The ISME Journal (2010), 1–12

www.nature.com/ismej

ORIGINAL ARTICLE

Impact of elevated nitrate on sulfate-reducing bacteria: a comparative Study of *Desulfovibrio vulgaris*

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Sulfate-reducing bacteria have been extensively studied for their potential in heavy-metal bioremediation. However, the occurrence of elevated nitrate in contaminated environments has been shown to inhibit sulfate reduction activity. Although the inhibition has been suggested to result from the competition with nitrate-reducing bacteria, the possibility of direct inhibition of sulfate reducers by elevated nitrate needs to be explored. Using *Desulfovibrio vulgaris* as a model sulfate-reducing bacterium, functional genomics analysis reveals that osmotic stress contributed to growth inhibition by nitrate as shown by the upregulation of the glycine/betaine transporter genes and the relief of nitrate inhibition by osmoprotectants. The observation that significant growth inhibition was effected by 70 mM $NaNO_3$ but not by 70 mM NaCl suggests the presence of inhibitory mechanisms in addition to osmotic stress. The differential expression of genes characteristic of nitrite stress responses, such as the hybrid cluster protein gene, under nitrate stress condition further indicates that nitrate stress response by *D. vulgaris* was linked to components of both osmotic and nitrite stress responses. The involvement of the oxidative stress response pathway, however, might be the result of a more general stress response. Given the low similarities between the response profiles to nitrate and other stresses, less-defined stress response pathways could also be important in nitrate stress, which might involve the shift in energy metabolism. The involvement of nitrite stress response upon exposure to nitrate may provide detoxification mechanisms for nitrite, which is inhibitory to sulfate-reducing bacteria, produced by microbial nitrate reduction as a metabolic intermediate and may enhance the survival of sulfate-reducing bacteria in environments with elevated nitrate level.

The ISME Journal advance online publication, 6 May 2010; doi:10.1038/ismej.2010.59
Subject Category: integrated genomics and post-genomics approaches in microbial ecology
Keywords: *Desulfovibrio*; nitrate; stress response; sulfate-reducing bacteria; functional genomics; systems biology

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Received 5 February 2010; revised 26 March 2010; accepted 26 March 2010

Introduction

Exploitation of microbially mediated reduction of redox-sensitive metals has been proposed as a promising strategy to remediate metal-contaminated subsurface environments *in situ* (Valls and de Lorenzo, 2002; Wall and Krumholz, 2006). Owing

to this ability to reduce and accumulate heavy metals and radionuclides (Jones *et al.*, 1976; Lovley *et al.*, 1993a; Chardin *et al.*, 2002), sulfate-reducing bacteria (SRBs) have drawn particular attention for potential applications in heavy metal immobilization. It has been well documented that SRBs can reductively precipitate redox metals through enzymatic pathways (Lovley and Phillips, 1992; Abdelouas *et al.*, 1998) or can simply precipitate metals as metallic sulfides. Enzymatic reduction of soluble metal oxyanions to insoluble forms has been specifically shown for*Desulfovibrio* spp. (Lovley *et al.*, 1993b; Lloyd *et al.*, 1999; Payne *et al.*, 2002), which are the model SRBs most extensively studied for their bioremediation capacity. More importantly, SRB populations are also found to be significant members of microbial communities involved in such metal reduction and are ubiquitous even in extreme environments (Chang *et al.*, 2001; Gillan *et al.*, 2005; Bagwell *et al.*, 2006; Fields *et al.*, 2006). Therefore, stimulation of SRB activities has been considered as a useful approach for the immobilization of heavy metals and radionuclides (Landa, 2005; Lloyd and Renshaw, 2005).

To exploit SRB effectively for the remediation of heavy metal and radionuclide contaminated sites, it is important to understand the microbial responses to the adverse environmental factors commonly encountered in these subsurface environments. One such factor is the high nitrate concentration of many contaminated sites at the US nuclear weapon complexes managed by the Department of Energy (Riley and Zachara, 1992; NABIR, 2003). The presence of nitrate may pose a specific stress to SRB as nitrate has been observed to suppress sulfate reduction activity *in situ* (Jenneman *et al.*, 1986; Davidova *et al.*, 2001). Thus, it is important to examine the responses of sulfate-reducing microorganisms in metabolic and regulatory pathways following nitrate exposure to understand their defense mechanisms. Furthermore, as nitrate is a broadly available electron acceptor readily used by a large number of microorganisms in natural environments, nitrate reduction as an ecologically more competitive process could have major impacts on the survival and persistence of SRB in microbial communities, as well as on the functions of SRB in nitrate-impacted environments. Therefore, insights into the mechanisms of the ecological adaptability of SRB in nitrate-impacted environments would facilitate the development of strategies to monitor and predict the performance of these microorganisms in bioremediation (Hazen and Stahl, 2006).

In this report, we used *Desulfovibrio vulgaris* Hildenborough as a model organism to investigate the inhibition of sulfate reduction by nitrate as compared with other related stress conditions. Our results from physiological analyses indicate the presence of inhibitory mechanisms in addition to the expected osmotic stress responses. Subsequent functional studies revealed that nitrate stress response by *D. vulgaris* was linked to components of both osmotic and nitrite stress responses.

Materials and methods

High-throughput monitoring of cell growth with various stressor concentrations

The growth response of *D. vulgaris* cells to various concentrations of sodium nitrate ($NaNO_3$) or sodium chloride (NaCl) was monitored using the Phenotype MicroArray platform (Biolog Inc., Hayward, CA, USA). Culture handling and instrument operation were carried out following a previously described procedure (Borglin *et al.*, 2009). The OmniLog instrument was calibrated against *D. vulgaris* cell densities as measured by a spectrophotometer at OD_{600} and direct cell counts. All were comparable at 95% confidence interval (CI) for the exponential growth phase. Specifically, OL(Omnilog) readings were converted to cell density (cells ml^{-1}) using the following experimentally determined linear expression ($r^2 = 0.933$, $n = 37$): cell density $= 2.34 \times 10^7 \cdot \mathrm{OL} + 34.3$.

Impacts of osmoprotectants on growth responses to nitrate stress

Glycine betaine was selected as the osmoprotectant to test the presence of potential osmotic stress responses when *D. vulgaris* was exposed to high nitrate levels as this osmolyte had been shown to protect *D. vulgaris* from salt stress (Mukhopadhyay *et al.*, 2006). Initially, glycine betaine was added into the Yen45 defined medium (Bender *et al.*, 2007) to a final concentration of 2 mM, together with additional $NaNO_3$ at 100 or 200 mM. This medium has fewer precipitates that interfere with optical density determinations than does the LS4D medium (Mukhopadhyay *et al.*, 2006). Controls without glycine betaine and those without either glycine betaine or nitrate were also compared. Following a 2% (v/v) inoculation with a late-log phase culture ($OD_{600} \sim 0.8$–0.9), growth was monitored by optical density measurements at 600 nm.

Biomass production for microarray and proteomics analysis

Cultures for biomass production were initiated with 10% (v/v) inocula from stocks of *D. vulgaris* frozen at −80 °C (fully grown cells in LS4D with 10% (v/v) glycerol) into LS4D medium as previously described (Mukhopadhyay *et al.*, 2006). All production cultures were grown in triplicate (three control cultures and three stressed cultures). When the production cultures reached an OD_{600} of 0.3, 50 ml was taken from each replicate culture as the T0 samples. Once the T0 samples were taken, degassed $NaNO_3$ solution was immediately added to the three treatment cultures to a final concentration of 105 mM (6500 p.p.m. nitrate, which was shown to inhibit the

growth rate of the log-phase cultures by approximately 50%), and an equivalent volume of sterile, distilled, degassed water was added to each control culture. Culture samples of 50 ml were collected from each culture at 30, 60, 120 and 240 min post-addition while cells were still in exponential growth phase. To minimize mRNA or protein changes during sample collection and processing, cell samples were rapidly chilled and pelleted using a previously described method (Mukhopadhyay *et al.*, 2006). The final pellet was flash-frozen in liquid nitrogen and stored at −80 °C for microarray analysis.

The same procedure was followed to collect the biomass for proteomics analysis, with the exception that sampling from the production cultures was conducted at only two time points. Briefly, when the production cultures reached an OD_{600} of ca. 0.3, 100 ml of sample was taken from each triplicate culture as the T0 samples. Following nitrate (105 mM) addition, 100 ml each from the three control cultures and 100 ml each from the three stressed cultures were collected at 240 min post-exposure as the T1 sample. Culture samples from the triplicate treatment or control cultures at each time point were subsequently pooled to provide adequate biomass for protein extraction. Cell mass from the four pooled culture samples, T1 and T0 for the treatment and control, respectively, were harvested using the same procedure as described above for microarray analysis. The final pellet was flash-frozen in liquid nitrogen and stored at −80 °C until proteomics analysis.

Microarray transcriptomic analysis

A previously described whole-genome oligonucleotide DNA microarray (He *et al.*, 2006), covering more than 98.6% of the annotated protein-coding sequences of the *D. vulgaris* genome, was used for global transcriptional analysis of nitrate stress response. The accuracy of the microarrays in global transcriptional profiling has been extensively tested and validated in previous studies on stress response pathways in *D. vulgaris* (Clark *et al.*, 2006; He *et al.*, 2006). All microarray procedures including the extraction and labeling of nucleic acids, microarray hybridization and washing, and data analysis were performed using previously published protocols (He *et al.*, 2006). Total RNA extraction, purification and labeling were performed independently on each cell sample using previously described protocols (He *et al.*, 2006; Butler *et al.*, 2007). Each replicate sample consisted of cells from 300-ml cultures. Labeling of cDNA targets from purified total RNA was carried out using the reverse transcriptase reaction with random hexamer priming, and the fluorophore Cy5-dUTP (Amersham Biosciences, Piscataway, NJ, USA). Genomic DNA was extracted from *D. vulgaris* cultures at stationary phase and labeled with the fluorophore Cy3-dUTP (Amersham Biosciences). To hybridize a single glass slide, the Cy5-dUTP-labeled cDNA targets obtained from stressed or non-stressed control cultures were mixed with the Cy3-dUTP-labeled genomic DNA. After washing and drying, the microarray slides were scanned using the ScanArray Express microarray analysis system (Perkin Elmer, Fremont, CA, USA). The fluorescent intensity of both Cy5 and Cy3 fluorophores was analyzed with ImaGene software version 6.0 (Biodiscovery, Marina Del Rey, CA, USA). Log ratios of differential gene expression between treatment and control cultures were determined using previously described data processing and analysis methods, and statistical significance was assessed using standard *Z*-scores (Chhabra *et al.*, 2006). Pairwise correlation coefficients between any two transcriptional profiles were computed with the centered Pearson's correlation using the entire transcriptional expression profiles obtained by the *D. vulgaris* microarray. Color heat map representations comparing gene expression under various growth conditions were generated using the software JColorGrid (Joachimiak *et al.*, 2006). The microarray results were deposited at the GEO database with the accession number GSE20079.

Three-dimensional nano LC-MS/MS proteomics analysis of nitrate-stressed biomass

Total protein extracted and treated from the control and stressed samples was used for fractionation by three-dimensional liquid chromatography, followed by tandem mass spectrometry analysis to determine the protein identities, as described previously (Wei *et al.*, 2005). The relative abundance of proteins in each sample was estimated on the basis of the hypothesis that the more abundant a peptide ion is in a mixture, the more likely it is that the peptide ion is sampled during the course of a tandem mass spectrometry experiment (Wolters *et al.*, 2001; Liu *et al.*, 2004). Accordingly, the total numbers of qualified spectral counts represented the relative abundance of each protein under a specific condition. To identify proteins for which there were significant changes under certain conditions, the statistical 'local-pooled-error' test (Jain *et al.*, 2003) was used. Only protein changers with a *P*-value of less than 0.05 were considered to be significant.

Results

Growth inhibition of D. vulgaris *by nitrate*

The inhibitory effect of nitrate was evaluated by monitoring the growth of *D. vulgaris* in the presence of various concentrations of sodium nitrate. Although a slow-growth phase (with no detectable growth) of approximately 20 h was observed in control cultures without nitrate addition, an extended phase of slow growth followed by normal growth was observed with increasing concentrations of nitrate in the culture medium, indicative of

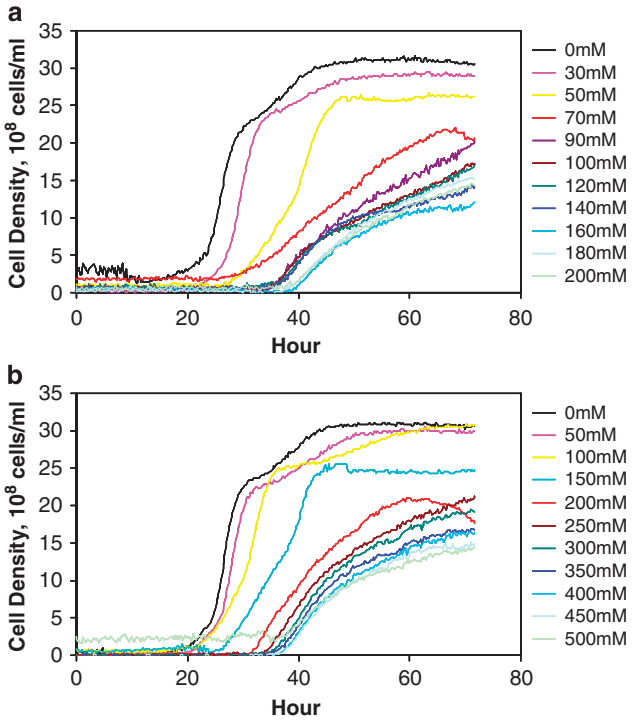

Figure 1 Growth response of *Desulfovibrio vulgaris* to varying concentrations of (**a**) $NaNO_3$ or (**b**) NaCl.

a moderate inhibitory effect (Figure 1a). A more severe inhibition pattern, characterized by a sharp decrease in growth rate accompanied by an increasingly longer slow-growth phase, was apparent when the nitrate concentration reached 70 mM, as indicated by the reduced slope of the growth curve (Figure 1a).

As sodium nitrate is an ionic solute, high concentrations of nitrate are expected to result in osmotic stress as a non-specific inhibitory mechanism. To identify any inhibitory effects specific to nitrate, a comparison was made between the growth responses of *D. vulgaris* to sodium nitrate versus sodium chloride, which are known to cause osmotic stress. In sharp contrast to the 70 mM sodium nitrate addition needed for growth inhibition, a significant decrease in the growth rate of *D. vulgaris* was observed only when 200 mM sodium chloride was added into the LS4D medium (Figure 1b). These results suggest that sodium nitrate inhibition resulted from at least some interactions specific to nitrate and not simply from a salt-induced osmotic effect.

Global transcriptomic analysis of nitrate stress

To understand the mechanisms of nitrate inhibition and the potential response pathways used by *D. vulgaris* cells to alleviate nitrate stress, microarray experiments were carried out to compare global gene expression profiles between nitrate-stressed *D. vulgaris* cultures and control cultures without nitrate exposure. *D. vulgaris* cells were challenged by a nitrate level of 105 mM, which was effective in inhibiting, but not eliminating, cell growth in log-phase cultures.

Changes in the gene expression profile were observed at 30 min following nitrate exposure and peaked at 120 min, with 298 genes being differentially expressed, either up or down, greater than twofold (Supplementary Figure S1). A similar number of genes (288) remained differentially regulated at 240 min. It is noted that the number of genes with reduced expression level considerably exceeded the number of genes with increased expression at 30, 60 and 120 min, consistent with the inhibitory effect of nitrate observed in the growth study (Figure 1). As the number of down-regulated genes peaked at 120 min, the number of up-regulated genes, however, continued to rise throughout the duration of the experiment, which is indicative of an active response to nitrate treatment following the initial inhibition.

Effects of osmoprotectants on growth inhibition by nitrate

Given the presence of osmotic stress at high nitrate concentrations, indications of osmotic stress response following nitrate exposure were examined. Indeed, transcriptional profiling showed an increase in the gene expression of the periplasmic-binding protein of the glycine/betaine/proline ABC transporter (DVU2297; $\log_2 R = 1.6$ at 240 min), although not the putative permease or ATP binding protein. As glycine betaine is a known osmoprotectant (Cayley and Record, 2003) and has been shown to relieve osmotic stress in *D. vulgaris* (Mukhopadhyay *et al.*, 2006), the up-regulation of this gene supports the expected overlap between osmotic stress and nitrate stress. To further confirm that nitrate inhibition is associated with osmotic stress, growth was monitored following the addition of glycine betaine as an osmoprotectant into *D. vulgaris* cultures in nitrate-supplemented defined medium (Figure 2).

Similar to stress-inducing concentrations of NaCl, elevated $NaNO_3$ concentrations resulted in a prolonged lag phase and significantly reduced the final cell density. The addition of glycine betaine led to the complete recovery of the final cell density in *D. vulgaris* cultures exposed to 100 mM $NaNO_3$, but provided only a 16% reduction of the lag phase (Figure 2a), which is in contrast to the near-complete reversal of growth inhibition by glycine betaine in NaCl stress (Mukhopadhyay *et al.*, 2006). The inability of glycine betaine to relieve nitrate stress entirely indicates that osmotic stress does not account for all the inhibition of cellular activities by nitrate stress.

In contrast, growth inhibition by 200 mM $NaNO_3$ was more significantly relieved by the inclusion of glycine betaine in the medium, with the lag phase shortened from approximately 300 to 100 h

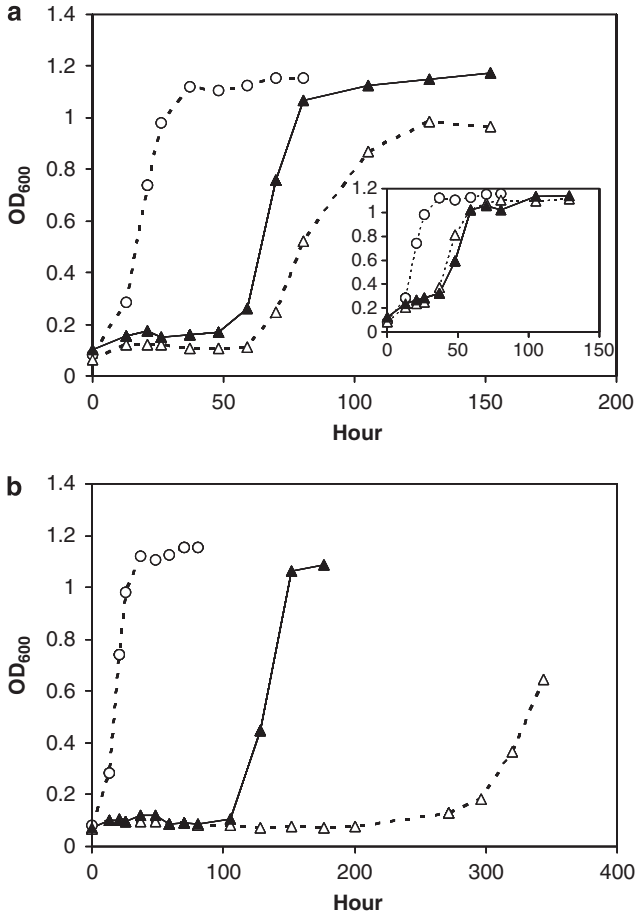

Figure 2 Impact of glycine betaine on the growth of *D. vulgaris* exposed to 100 mM (**a**) and 200 mM (**b**) sodium nitrate. The inset in panel a shows the growth of *D. vulgaris* when 2 mM sodium nitrite was in place of sodium nitrate. *D. vulgaris* cultures were inoculated to a defined medium (Control, open circles), medium supplemented with sodium nitrate / nitrite only (open triangles), or medium supplemented with sodium nitrate / nitrite plus 2 mM glycine betaine as an osmoprotectant (closed triangles). Note different time scales on graphs. Results were typical of three experiments.

(Figure 2b). This observation was likely the result of the increasing importance of osmotic stress with higher levels of nitrate. Nonetheless, only partial relief of nitrate stress was provided by the addition of osmoprotectant, further suggesting the presence of additional sources of growth inhibition that were specific to nitrate stress, but not osmotic stress.

Genes involved in methyl/SAM metabolism

In nitrate-stressed *D. vulgaris,* a group of genes involved in the methyl metabolism were among those with the greatest increases in expression (Supplementary Table S1), including *metF* (DVU0997), *metE* (DVU3371) and *ahcY* (DVU0607). All these genes have functions in the metabolism of methionine and regeneration of *S*-adenosylmethionine (SAM), a major methyl donor in various cellular processes (Wang and Frey, 2007). A careful examination of the genes up-regulated under nitrate stress further revealed the increased expression of the gene encoding another key enzyme in SAM biosynthesis, *S*-adenosylmethionine synthetase MetK (DVU2449; $\log_2 R = 1.7$). From the co-expression patterns of all these genes (Supplementary Figure S2), scattered across the genome, we infer the presence of a regulatory mechanism that might be involved in the increased turnover of SAM.

Interestingly, the enzyme activating the pyruvate formate-lyase (DVU2825), which was also among the most up-regulated genes under nitrate stress (Supplementary Table S1), has been shown to require the methyl donor SAM in other bacteria (Chase and Rabinowitz, 1968; Knappe and Schmitt, 1976), providing a potential link between energy metabolism and methyl/SAM metabolism (Supplementary Figure S2).

Genes involved in energy metabolism

Nitrate does not support the growth of *D. vulgaris* as an electron acceptor or nitrogen source (Haveman *et al.*, 2004, 2005). However, in many other anaerobes nitrate metabolism is directly linked to energy metabolism via multiple redox reactions (Moura *et al.*, 1997). Thus, the involvement of genes in energy metabolism was investigated when elevated nitrate constituted a stress condition. Transcriptional analysis indicated that a small number of genes with functions in energy metabolism were among those highly up-regulated under nitrate stress, such as the genes related to the catabolism of pyruvate as a key metabolic intermediate: a pyruvate formate-lyase (DVU2824) and its activating enzyme (DVU2825) (Supplementary Table S1). These two genes form an operon with two other genes encoding a tripartite ATP-independent periplasmic (TRAP) dicarboxylate transporter (DVU2822–2825). In addition, a formate dehydrogenase gene cluster (DVU0586–0588) had increased expression under nitrate stress (data not shown). The composite of these differentially expressed genes appears to be consistent with an increased flow of reducing equivalent cycling through formate as a metabolic intermediate, as suggested under certain growth conditions (He *et al.*, 2006; Pereira *et al.*, 2008).

The gene encoding the hybrid cluster protein (DVU2543), which was suggested to be involved in the response to reactive nitrogen species generated in nitrate metabolism in other microorganisms (van den Berg *et al.*, 2000; Wolfe *et al.*, 2002), was also up-regulated ($\log_2 R = 1.8$). It is noted that this gene was among the most highly up-regulated ($\log_2 R = 6.4$) under nitrite stress (Haveman *et al.*, 2004; He *et al.*, 2006). The iron–sulfur cluster-binding protein, predicted to be encoded promoter distal in the same operon (DVU2544), was also increased in expression ($\log_2 R = 1.9$), representing a shared response to nitrate and nitrite stress (Table 1). Nonetheless, the differential expression of the hybrid

Table 1 Comparison of the gene expression of selected gene groups in response to $NaNO_3$, $NaNO_2$ and NaCl in *D. vulgaris*[a]

Gene ID	*Log₂ ratio of transcriptional response*[b]			*TIGR Annotation*
	$NaNO_3$[c]	*$NaNO_2$*[c]	*NaCl*[c]	
Methyl metabolism				
DVU0606	**2.5**	**2.0**	−1.0	Regulator/methyltransferase, UbiE/COQ5 family
DVU0607	**2.7**	**2.4**	1.1	Adenosylhomocysteinase, AhcY
DVU0997	**2.9**	**2.2**	0.6	5,10-Methylenetetrahydrofolate reductase, MetF
DVU2449	**1.7**	**2.1**	−1.3	*S*-Adenosylmethionine synthetase, MetK
DVU3371	**2.7**	**3.8**	−1.4	5-Methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase, MetE
Carbon metabolism				
DVU2822	**3.4**	0.9	**1.5**	TRAP dicarboxylate family transporter
DVU2823	**1.8**	0.4	1.9	TRAP dicarboxylate transporter family protein
DVU2824	**2.5**	0.5	0.9	Formate acetyltransferase
DVU2825	**2.9**	0.6	0.7	Pyruvate formate-lyase 1 activating enzyme
DVU0586	**2.5**	0.5	**−1.4**	Hypothetical protein
DVU0587	**1.5**	0.7	**−1.8**	Formate dehydrogenase, alpha subunit, selenocysteine-containing
DVU0588	**1.6**	0.3	**−1.3**	Formate dehydrogenase, beta subunit, putative
DVU1569	0.8	**1.1**	0.4	Pyruvate ferredoxin oxidoreductase, alpha subunit PorA
DVU1570	-0.5	**1.3**	−1.2	Pyruvate ferredoxin oxidoreductase, beta subunit PorB
Nitrogen metabolism				
DVU2543	**1.8**	**5.7**	**−1.2**	Hybrid cluster protein
DVU2544	**1.9**	**6.2**	0.5	Iron-sulfur cluster-binding protein
DVU0624	0.3	**4.4**	**−1.3**	NapC/NirT cytochrome *c* family protein
DVU0625	0.7	**4.1**	**−1.3**	Cytochrome *c* nitrite reductase, catalytic subunit NrfA

Abbreviation: TRAP, tripartite ATP-independent periplasmic.
[a]Gene expression profiles following $NaNO_3$ (105 mM), $NaNO_2$ (2.5 mM) and NaCl (250 mM) treatment in *D. vulgaris* were obtained from this study, He *et al*., 2006, and Mukhopadhyay *et al*., 2006, respectively.
[b]Expression ratios represent the highest levels of differential gene expression throughout the time course (240 min) of the stress exposure. Expression levels were obtained at the same time points from both the treatment and control cultures for the calculation of the expression changes resulting from the stressor. Positive Log_2 (Expression Ratio) values denote increases in expression level and negative values indicate decreases in expression level. Values with $Z > 2$ are shown in boldface type.
[c]The treatment cultures received addition of $NaNO_3$, $NaNO_2$ or NaCl, and the control cultures received none.

Table 2 Effect of nitrate exposure on the transcriptional responses of *Desulfovibrio vulgaris* genes in the predicted PerR regulon[a]

Gene ID	*Description*	*Log₂ (Expression ratio)*[b]			
		30 min	*60 min*	*120 min*	*240 min*
DVU0772	Hypothetical protein	0.1	0.7	**2.0**	**2.4**
DVU2247	Alkyl hydroperoxide reductase C, *ahpC*	0.2	0.5	0.7	**1.6**
DVU2318	Rubrerythrin, putative, *rbr2*	0.4	0.6	0.9	**2.2**
DVU3093	Rubredoxin-like protein, *rdl*	−0.2	0.0	0.8	**1.2**
DVU3094	Rubrerythrin, *rbr*	−0.2	−0.1	−0.3	0.7
DVU3095	Peroxide-responsive regulator PerR	−0.5	0.0	0.8	**1.4**

[a]Predicted PerR regulon from Rodionov *et al*., 2004.
[b]Expression ratios represent the levels of gene expression at various time points following the addition of 105 mM nitrate into cultures compared with controls without nitrate addition. Expression levels were obtained at the same time points from both the treatment and control cultures for the calculation of the expression changes resulting from the stressor. Positive Log_2 (Expression Ratio) values denote increases in expression level and negative values indicate decreases in expression level. Values with $Z > 2$ are shown in boldface type.

cluster protein operon was much weaker in response to nitrate than in response to nitrite. No significant changes in gene expression were observed in other known genes participating in nitrogen metabolism.

Another highly up-regulated gene with annotated functions in energy metabolism encodes a putative rubrerythrin (DVU2318) (Supplementary Table S1), which is predicted to be under the regulation of the peroxide-responsive regulator (PerR) (Rodionov *et al*., 2004). A survey of the gene expression profile indicated that all genes in the predicted PerR regulon had increased expression to various extents under nitrate stress (Table 2). However, comparison of gene expression profiles found that the PerR regulon was consistently up-regulated throughout different stress conditions (Chhabra *et al*., 2006; He *et al*., 2006; Mukhopadhyay *et al*., 2006, 2007), indicating that the increased expression of the PerR regulon was likely a part of the general stress response.

Proteomics analysis of nitrate stress response

Liquid chromatography–mass spectrometry proteomics analysis of the stress response to nitrate in the wild-type strain was performed to complement transcriptional analysis. Ribosomal proteins were among the most downregulated, consistent with transcriptional analysis and growth inhibition observed with exposure to elevated nitrate (Table 3). Proteomics results also confirmed the up-regulation of the glycine/betaine/proline ABC transporter (DVU2297) and MetE (DVU3371), which is a key enzyme in the methyl/SAM metabolic pathway (Supplementary Figure S2). A *phi* coefficient of correlation of 0.6 was achieved for genes/proteins with significant changes in both transcriptional and proteomics analyses, which is indicative of the good agreement on the direction of regulation at both the messenger RNA and protein levels. This is also largely consistent with previous comparisons between transcriptional and proteomics profiles in *D. vulgaris* (Chhabra *et al.*, 2006; Mukhopadhyay *et al.*, 2006), confirming the validity of the microarray technique and subsequent transcriptional analysis for making regulatory event inferences in *D. vulgaris*.

Comparison of nitrate stress response with other stress conditions

The above analyses show that nitrate stress shared with two related stresses, NaCl stress and nitrite stress, similar patterns of gene expression in a number of genes, including the glycine/betaine/proline ABC transporter (DVU2297) and hybrid cluster protein (DVU2543) genes. To further determine the potential correlations in gene expression between nitrate, nitrite and NaCl stress responses in *D. vulgaris*, all genes with significant changes in expression were identified at 30 min following stress exposure, which corresponds to the earliest post-stress time point and likely represents the most direct stress responses in the early phase of the

Table 3 Comparison of transcript abundance with corresponding protein levels following nitrate exposure in *D. vulgaris*[a]

Gene ID	*Description*	*Log₂(Expression Ratio)*[b]	
		mRNA[c]	*Protein*[d]
DVU0470	Tryptophan synthase, beta subunit, *trpB-2*	$+1.1^{60}$	+0.8
DVU0764	DNA-binding protein HU, *hup-2*	−1.1	−1.3
DVU0777	ATP synthase, F_1 alpha subunit, *atpA*	-1.1^{120}	−0.4
DVU0873	Translation elongation factor Ts, *tst*	−1.4	+1.1
DVU1077	Inner membrane protein, 60 kDa, *yidC*	-1.3^{120}	−1.0
DVU1089	Alanyl-tRNA synthetase, *alaS*	−1.3	+1.4
DVU1295	Sulfate adenylyltransferase, *sat*	+1.4	+0.4
DVU1300	Translation elongation factor G, *fusA-1*	−1.3	+0.5
DVU1303	Ribosomal protein L3, *rplC*	−1.7	−0.7
DVU1306	Ribosomal protein L2, *rplB*	−1.5	−1.0
DVU1308	Ribosomal protein L22, *rplV*	−1.7	−0.9
DVU1317	Ribosomal protein S8, *rpsH*	−1.3	−0.9
DVU1326	Ribosomal protein S13, *rpsM*	−1.2	−1.3
DVU1434	Hypothetical protein	−1.1	+2.0
DVU1443	Flagellar hook protein FlgE, *flgE*	-1.4^{120}	−2.4
DVU1575	Ribose-phosphate pyrophosphokinase, *prsA*	−1.4	+1.5
DVU1636	Inorganic pyrophosphatase, manganese-dependent, *ppaC*	+1.2	+0.7
DVU1896	Ribosomal protein S20, *rpsT*	−2.1	−1.3
DVU2105	Hypothetical protein	-1.6^{30}	−0.7
DVU2108	MTH1175-like domain family protein	$+1.1^{30}$	+0.6
DVU2215	RNA-binding protein	-1.4^{60}	+0.8
DVU2289	Hydrogenase, CooX subunit, putative, *b2488*	-1.1^{120}	−2.0
DVU2297	Glycine/betaine/L-proline ABC transporter, periplasmic-binding protein	+1.6	+0.6
DVU2347	Acetylornithine aminotransferase, *argD*	+1.4	+0.8
DVU2364	Aminotransferase, classes I and II	-1.2^{120}	−0.8
DVU2927	Ribosomal protein L7/L12, rplL	-1.5^{120}	+0.9
DVU3228	Chemotaxis protein CheY, *cheY-3*	$+1.0^{120}$	+1.3
DVU3371	5-Methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, *metE*	+2.7	+0.7

[a]Genes with significant changes at both the mRNA and protein levels (absolute value of $Z > 2$) subsequent to nitrate treatment were selected for comparison.
[b]Expression ratios represent the levels of expression following the addition of 105 mM nitrate into cultures compared with controls without nitrate addition. Expression levels were obtained at the same time points from both the treatment and control cultures for the calculation of the expression changes resulting from the stressor. Log_2(Expression Ratio) values greater than 0 denote increases in expression level and values less than 0 indicate decreases in expression level.
[c]Transcript abundance was determined from cDNA microarray analysis. Transcriptional expression ratios (log_2R) are shown for genes with $Z > 2$ (absolute value) at 240 min following nitrate treatment unless otherwise indicated. For genes with $Z < 2$ (absolute value) at 240 min, data are shown for a different time point (min; appears as an italic superscript).
[d]Protein abundance was determined from MS-MS proteomics analysis of protein extracts from cell samples subjected to 240-min of nitrate treatment. Protein abundance ratios (log_2R) are shown for genes with $Z > 2$ (absolute value) at 240 min following nitrate addition.

stress experiments. Genes with changes in expression at later post-stress time points, however, might not necessarily be representative of nitrate-specific responses. Instead, these genes could be involved in general stress response subsequent to the nitrate-specific primary responses, such as the genes characteristic of the general oxidative stress responses in the PerR regulon discussed above, which were significantly upregulated at later time points during the experimental period (Table 2).

It is revealed that there were variable numbers of differentially expressed genes in response to each stress, from 40 in nitrate stress and 60 in salt stress (Mukhopadhyay *et al.*, 2006), to 261 in nitrite stress (He *et al.*, 2006) (Supplementary Figure S3). The numbers of differentially expressed genes in common between these experiments were very few, with ten between nitrite and NaCl, six between nitrate and nitrite, one between nitrate and NaCl, and none among all three stress responses. Thus, when considering common genes with significant change in expression at the time point when the stress response was most expected (30 min), it is evident that there was little similarity between these stress responses.

To further examine the presence of stress response pathways common between nitrate stress and various other stress conditions, analyses of gene expression overlap proportions and correlations were performed across all pairs of time points in eight stress responses, including nitrate (this study), nitrite (He *et al.*, 2006), NaCl and KCl (Mukhopadhyay *et al.*, 2006), heat shock (Chhabra *et al.*, 2006), low oxygen (1000 p.p.m.) (Mukhopadhyay *et al.*, 2007), high oxygen (air) (Mukhopadhyay *et al.*, 2007) and alkaline stress (Stolyar *et al.*, 2007). The highest values for gene overlap proportions (Figure 3) and gene expression correlations (Supplementary Figure S4) were observed between time points of the same stress response, as expected. Considering comparisons across different stress responses at 30 min post-stress, the two salt stresses NaCl and KCl showed the largest gene expression overlap proportions among all experimental pairs (excluding comparisons of time points from the same experiment) (Figure 3) and the highest correlation of 0.71 at 30 min (Supplementary Figure S4). The heat shock and high oxygen (air) stress exhibited the second largest overlap proportion and a correlation of 0.51 at 30 min (Figure 3 and Supplementary Figure S4), indicative of the similarities in stress response. It is evident that stress pairs showing the most gene expression overlap and highest gene expression correlations did so at all time points following the stress treatment. In contrast, the comparisons between nitrate, nitrite and NaCl showed minimal gene overlap proportions and gene expression correlations (Figure 3 and Supplementary Figure S4). For example, the gene expression correlation at 30 min for nitrate and nitrite was 0.08, for nitrate and NaCl 0.11, and for nitrite and NaCl 0.19. Slightly better gene expression overlap proportions were observed between the last nitrate stress time point at 240 min and selected nitrite stress time

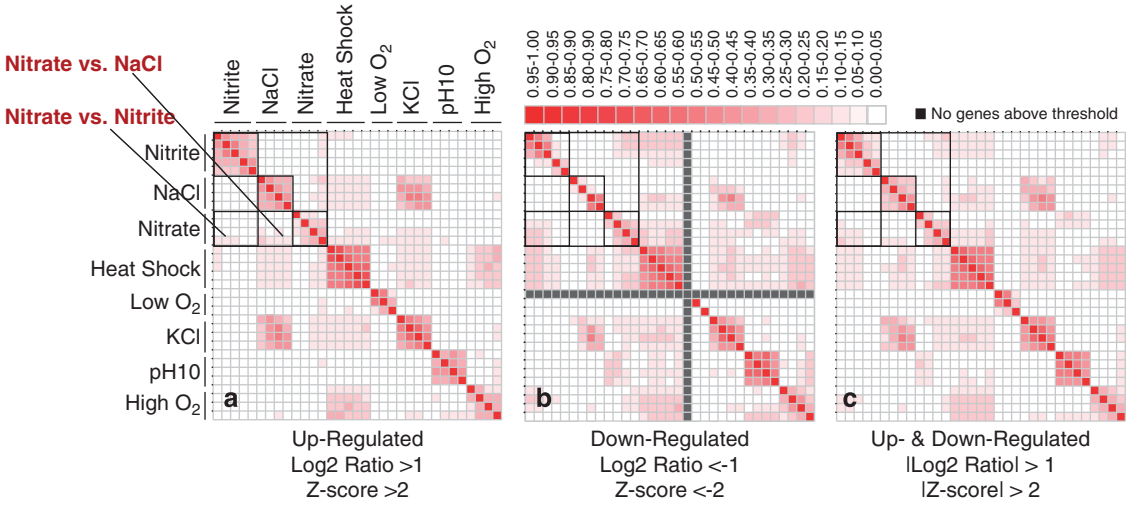

Figure 3 Gene expression overlap proportions between experimental time points of eight different stress conditions for *D. vulgaris*, including nitrate, nitrite, NaCl, KCl, heat shock, low O_2 (1000 ppm), high O_2 (air) and alkaline (pH 10) stress. Shown are overlap proportions for genes in three categories: (**a**) up-regulated; (**b**) down-regulated; and (**c**) both up- and down-regulated. The gene expression overlap proportion between the two transcriptional profiles was computed as the number of genes above the threshold, i.e. Z-score and $\log_2$Ratio, common between a pair of transcriptional profiles normalized by the root of the product of the number of genes above threshold in each transcriptional profile. The nitrate, nitrite and NaCl pairwise comparisons are in the top left corner of the heatmaps and are outlined in the black frame. Each square represents the gene expression overlap proportion of one pairwise comparison between two experiment time points. Time point increments for each stress condition progress left to right horizontally and top to bottom vertically from 30 min to 240 min post stress. Solid black squares indicate cases where one or both of the experimental time points being compared had no genes with significant change in expression. The bright red squares along the diagonal correspond to the greater proportions of common genes between time points within the same experiment.

points. Similarly, the last nitrate stress time point had low overlap of gene expression with several NaCl time points. Since the last time point in the nitrate stress may not represent a primary response to this stress but secondary effects, these low similarity measures do not support similarity between the nitrate, nitrite and NaCl responses.

Discussion

Nitrate is a common co-contaminant in the subsurface environments impacted by radionuclides and heavy metals (Brooks, 2001). Nitrate inhibition of metal-reducing microbial populations, such as the SRB, hinders bioremediation efforts exploiting these microbial biocatalysts (Abdelouas *et al.*, 1998; Finneran *et al.*, 2002; Istok *et al.*, 2004; Nyman *et al.*, 2006). However, the persistence of sulfate-reducing bacteria at contaminated sites with high nitrate levels suggested the presence of potential resistance mechanisms (Gu *et al.*, 2005; Bagwell *et al.*, 2006; Fields *et al.*, 2006), which were explored in this study using physiological and genomics approaches.

Growth inhibition by nitrate in the form of osmotic stress was demonstrated by the up-regulation of the glycine/betaine transporter genes and the relief of nitrate inhibition by osmoprotectant (Figure 2). However, osmotic stress response is not likely the only pathway contributing to the inhibitory effect of nitrate, given the minimal similarity in the transcriptional profiles between nitrate stress and NaCl stress (Figure 3 and Supplementary Figure S4). Indeed, the finding that *D. vulgaris* cells were significantly more sensitive to $NaNO_3$ than NaCl (Figure 1) indicates the involvement of inhibitory mechanisms in addition to the osmotic stress resulting from the elevated nitrate concentrations. Presumably, the more severe growth inhibition under nitrate stress (Figure 1a) could be attributed to the presence of the nitrate ions, as compared with chloride ions.

In contrast to Cl^-, in some bacteria, the nitrate ion is redox active and can serve as a terminal electron acceptor in energy metabolism or as a source of nitrogen for biosynthesis, both requiring the reduction of nitrate coupled with electron transfer. However, nitrate-dependent growth of *D. vulgaris* has not been observed, which is consistent with the absence of nitrate reductase genes in the sequenced genome of *D. vulgaris* (Moura *et al.*, 1997; Haveman *et al.*, 2004; Heidelberg *et al.*, 2004). Thus, it is unlikely that copious amounts of nitrogenous intermediates would be generated as toxic intermediates from nitrate reduction in *D. vulgaris*. It is suggested, however, that small amounts of nitrite, and subsequently other reactive nitrogen species, could be produced from non-specific reduction of nitrate by low potential reductases in *D. vulgaris* cells, such as the multiheme *c*-type cytochromes (Wall *et al.*, 2007). Owing to the specificity of nitrite toxicity to sulfate reduction (Greene *et al.*, 2003; Haveman *et al.*, 2004), nitrite derived from nitrate could represent a major stress condition for *D. vulgaris*. It appears that the significant up-regulation of the hybrid cluster protein genes (DVU2543–2544) upon nitrate exposure (Table 1), which resembled a similar response pattern specific to nitrite stress (Greene *et al.*, 2003; Haveman *et al.*, 2004; He *et al.*, 2006), would support the suggestion that nitrite stress is a result of nitrate exposure.

The initiation of nitrite stress responses upon exposure to nitrate in *D. vulgaris* could be of particular ecological significance in the persistence of SRB in environments with elevated levels of nitrate, which has been shown to effectively inhibit SRB populations in the environment (Jenneman *et al.*, 1986; Davidova *et al.*, 2001). More importantly, the nitrate inhibition of SRB is shown to be caused by nitrite, a key intermediate during microbial nitrate reduction (Greene *et al.*, 2003; Voordouw *et al.*, 2009). Thus, given the known toxicity of nitrite to SRB (Haveman *et al.*, 2004; He *et al.*, 2006), a potential impact of nitrate stress on SRB in the natural environment could be the subsequent occurrence of nitrite stress with the onset of microbial nitrate reduction. Even though nitrate cannot serve as an electron acceptor for *D. vulgaris*, nitrate is a common electron acceptor readily used by many other microorganisms. As a result, the presence of nitrate in the environment would rapidly result in the production of nitrite by microbial nitrate reduction. The initiation of stress responses for nitrite detoxification upon nitrate exposure would therefore, prepare the SRB population for the imminent inhibitory effects of nitrite produced from nitrate reduction and provide a physiological advantage to the survival of SRB in the environment.

Aside from components of salt stress and nitrite stress, involvement of oxidative stress response was also implicated during nitrate stress in *D. vulgaris*, with the up-regulation of the genes in the PerR regulon (Table 2), which is known to be responsive to oxidative stress (Rodionov *et al.*, 2004). However, examination of the responses of *D. vulgaris* to other stress conditions reveals that the up-regulation of the Per-R regulon takes place not only under oxygen stress (Mukhopadhyay *et al.*, 2007) but also under nitrite (He *et al.*, 2006), salt (Mukhopadhyay *et al.*, 2006) and heat (Chhabra *et al.*, 2006) stress, suggesting that the response of the PerR regulon may not be specifically linked to nitrate. The same non-specific response could also be suggested for several other genes responsive to nitrate stress. For example, the gene for the phage shock protein A (DVU2988) was up-regulated in nitrate stress (Supplementary Table S1). However, this gene was also up-regulated under conditions of salt (Mukhopadhyay *et al.*, 2006), heat (Chhabra *et al.*, 2006) and oxygen (Mukhopadhyay *et al.*, 2007) stress, suggesting that this response was not directly related to nitrate stress, rather a form of general stress response.

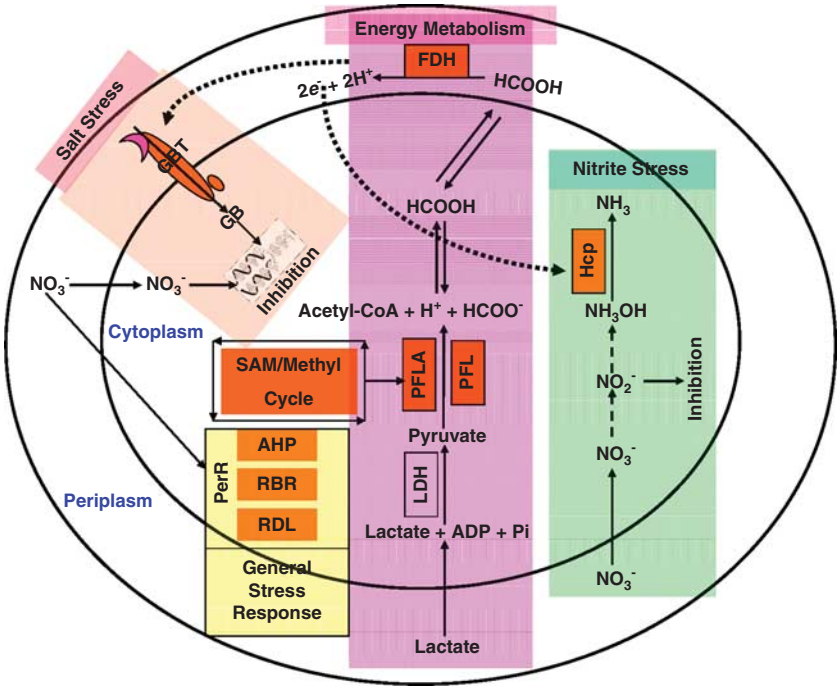

Figure 4 Conceptual model of responses to nitrate stress by *D. vulgaris* with linkages to both salt and nitrite stress in addition to shifts in energy metabolism. Colored symbols designate up-regulation. GBT: glycine/betaine ABC transporter; Hcp: hybrid cluster protein; PFL: pyruvate formate-lyase; PFLA: pyruvate formate-lyase activating enzyme; FDH: formate dehydrogenase; LDH: lactate dehydrogenase; AHP: alkyl hydroperoxide reductase; RBR: Rubrerythrin; RDL: Rubredoxin-like protein; SAM/Methyl cycle: *S*-adenosyl methionine cycle (details described in Supplementary Figure S2).

In contrast, the energetic consequences of nitrate stress could be considered as potential mechanisms contributing to the inhibition of *D. vulgaris* by nitrate. Indeed, our results indicate that a number of genes with functions in energy metabolism were among those that were highly up-regulated under nitrate stress, such as an operon consisting of genes related to the catabolism of pyruvate as a key metabolic intermediate, a pyruvate formatelyase (DVU2824) and its activating enzyme (DVU2825), as well as another operon encoding a formate dehydrogenase (DVU0586-0588) (Table 1). These regulatory events implicate a shift in energy metabolism to the increased flow of reducing equivalents through formate as a metabolic intermediate during nitrate stress. Notably, the activation of the pyruvate formate-lyase (DVU2824), a key enzyme in the generation of formate from the central metabolite pyruvate, has been shown to require the methyl-donor SAM in other bacteria (Chase and Rabinowitz, 1968; Knappe and Schmitt, 1976). The increased flow of reducing equivalent during nitrate exposure could potentially be used by *D. vulgaris* as a mechanism to meet the demand for an increased electron flow by redox processes, such as the detoxification of nitrite (He *et al.*, 2006) generated from the reduction of nitrate by other microbial populations, thus providing a physiological advantage to the survival of these microorganisms in natural environments with high nitrate levels. Interestingly, genes involved in methyl/SAM metabolism were among those that were the most responsive to nitrate stress in *D. vulgaris* (Supplementary Table S1), thus linking the methyl/SAM metabolic pathway to the shift in energy metabolism (Supplementary Figure S2). However, a definitive relationship between the shift in energy metabolism and nitrate inhibition could not be established, given the limited scope of this study. Future work should be focused on elucidation of the roles of energy metabolism in nitrate stress.

Therefore, the response to nitrate stress by *D. vulgaris* was shown to be linked to the components of both osmotic and nitrite stress responses (Figure 4), which is illustrated by the up-regulation of the glycine/betaine transporter genes known to relieve salt stress (Mukhopadhyay *et al.*, 2006) and the hybrid cluster protein gene identified specifically in nitrite stress (He *et al.*, 2006). Nitrate stress also had an impact on energy metabolism by way of increased expression of the SAM/methyl cycle, along with the up-regulation of the pyruvate formate-lyase, resulting in a shift to the increased flow of reducing equivalents through formate as a metabolic intermediate. The increased flux of formate is likely processed by the periplasmic formate dehydrogenase, which was expressed at a higher level during nitrate stress. The involvement of the oxidative stress response pathway, however, might be the result of a more general stress response. Given the low similarities between the response profiles to nitrate and other stresses, less defined

stress response pathways could also be important in nitrate stress, which might involve the shift in energy metabolism.

Acknowledgements

This work was a part of the Environmental Stress Pathway Project (ESPP) of the Virtual Institute for Microbial Stress and Survival (http://vimss.lbl.gov) supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics: GTL Program, through contract DE-AC02-05CH11231 with the Lawrence Berkeley National Laboratory.

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