NITRATE AND NITRITE GROWTH INHIBITION OF *DESULFOVIBRIO* STRAINS

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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To my Guardian Angel, a faithful friend through many trials

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NITRATE AND NITRITE GROWTH INHIBITION OF *DESULFOVIBRIO* STRAINS

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ABSTRACT

Sulfate-reducing bacteria (SRB) can perform desirable functions such as immobilization of environmental heavy metals, but they also cause oil "souring" because of their sulfide end product. Growth of SRB can be controlled by the inhibitory effects of nitrate and nitrite. However, prior studies have suggested that nitrate does not directly inhibit SRB. Rather, it was thought that nitrate is converted to the more toxic nitrite, which serves as the ultimate inhibitor. Here we tested whether nitrate can inhibit SRB by a mechanism other than through nitrite inhibition, and therefore whether responses of SRB to these different inhibitors might also be different. We measured growth kinetics and the fitness of thousands of mutants of the model SRB *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio alaskensis* G20 in lactate-sulfate plus nitrate. We found that mutations in homologous gene clusters (DVU0251-DVU0245/Dde_0597-Dde_0605) and in the Rex transcriptional regulator (DVU0916/Dde_2702) of these SRB confer resistance to nitrate. The same mutations did not confer nitrite resistance, and no nitrate consumption was observed. We also found that *D. vulgaris* can use subinhibitory concentrations of nitrite, but not nitrate, as a nitrogen source or terminal electron acceptor for growth. Since nitrate did not support growth of *D. vulgaris* as a nitrogen source, we infer that significant nitrite is not generated from the nitrate. These results show that nitrate inhibition of SRB can be independent of nitrite production. Furthermore, they reveal previously uncharacterized metabolic abilities which may allow niche expansion of *D. vulgaris* in low-sulfate environments containing nitrogen oxides. These insights into the interactions of SRB with nitrate and nitrite may lead to better control of SRB in industrial settings and better prediction of their interactions in the environment

CHAPTER 1

INTRODUCTION

I. Significance of sulfate-reducing bacteria (SRB)

Sulfate-reducing bacteria (SRB) are environmentally and industrially significant microorganisms that use sulfate as a terminal electron acceptor in anaerobic respiration. These obligate anaerobes produce sulfide as the end product of dissimilatory sulfate reduction, their cell respiration process (Postgate, 1984). Sulfide is toxic to most organisms (Caffrey and Voordouw, 2010), including humans (Voordouw et al., 2007), and the production of sulfide by SRB is considered to be the chief cause of oil souring in the petroleum industry (Ligthelm et al., 1991; Sunde et al., 1993). In addition to these negative roles, however, SRB can have positive effects in the environment. They are essential players in global nutrient cycling, especially carbon and sulfur cycling (Postgate, 1984). Furthermore, because of their ability to precipitate heavy metals, they have been exploited in studies of heavy metal bioremediation (Jiang and Fan, 2008; Martins et al., 2009). SRB precipitate heavy metals both by reduction (Lovley et al., 1993a; Lovley et al., 1993b; Lloyd et al., 1999; Chardin et al., 2003) and by formation of insoluble metal sulfides (Jalali and Baldwin, 2000). The metabolism of SRB, therefore, is studied to understand better how to minimize their detrimental economic effects and maximize their positive metabolic traits.

I.A. Introduction to sulfate reduction by SRB

In the central respiratory metabolism of SRB, sulfate is reduced to sulfide by multiple enzymes (Postgate, 1984). These enzymes are represented in Figure 1.1. A variety of electron donors are known to be used by the SRB (Peck, 1993). However, many studies of the model SRB *Desulfovibrio vulgaris* Hildenborough have been conducted with lactate as electron donor and sulfate as electron acceptor because of the environmental relevance of these substrates (He et al., 2006; Redding et al., 2006; Mukhopadhyay et al., 2007; He et al., 2010a; Zhou et al., 2013). This is the reason for the predominant use of lactate-sulfate media in the studies described in this work.

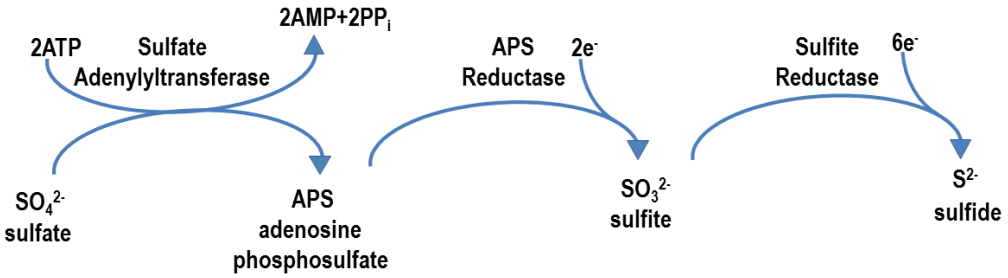

Figure 1.1. Dissimilatory Sulfate Reduction. Sulfate is activated by sulfate adenylyltransferase to adenosine phosphosulfate (APS), and this process consumes 2 ATP equivalents (Peck, 1993). APS reductase reduces APS with two electrons to form sulfite, which is then reduced by sulfite reductase with six electrons to form sulfide (Peck, 1993). The reduction of APS and sulfite allows for generation of a proton gradient which energetically offsets the initial input of two ATP molecules and also allows enough energy for growth (Thauer et al., 2007).

I.B. Nitrate and nitrite as inhibitors of SRB

Growth by sulfate reduction can be inhibited by many chemicals. Among such inhibitors are nitrate and nitrite ions, although several sulfate-reducing bacteria are known to be capable of using nitrate and nitrite in dissimilatory nitrate reduction (Moura et al., 2007). Probably the best-studied (Moura et al., 2007) of these bacteria is *Desulfovibrio desulfuricans* ATCC 27774, which has both nitrate- and nitrite-reduction enzymes. However, there are many sulfate-reducing bacteria, such as *Desulfovibrio vulgaris* Hildenborough (Haveman et al., 2004), that can reduce nitrite but apparently not nitrate (Moura et al., 1997). In fact, the presence of a nitrite reductase serves as a resistance factor for sulfate-reducing bacteria in the presence of nitrite (Greene et al., 2003). Studies have indicated that neither nitrate (Mitchell et al., 1986; Seitz and Cypionka, 1986) nor nitrite (Pereira et al., 2000) could be used by *D. vulgaris* as a terminal electron acceptor in energy conservation, which suggested that the role of the nitrite reductase is entirely for detoxification of nitrite. Nitrite is much more inhibitory to sulfate-reducing bacteria than is nitrate (Haveman et al., 2004; He et al., 2006; He et al., 2010a). For example, millimolar levels of nitrite can completely inhibit *D. vulgaris* Hildenborough growth (Haveman et al., 2004); whereas, the same bacterium can recover from inhibition by ca.100 mM nitrate (Redding et al., 2006; He et al., 2010a). The strong inhibition by nitrite is thought to result from competition of nitrite with sulfite for binding to the dissimilatory sulfite reductase (Fig. 1.1). Both *in vitro* biochemical analysis of enzyme activities (Wolfe et al., 1994) and *in vivo* genetic studies (Haveman et al., 2004) support this hypothesis. Such enzyme inhibition has been suggested to cause a decrease in bacterial viability in *Desulfovibrio* cultures incubated in human saliva containing both

nitrate and nitrite together (Mitsui et al., 2013). However, for some of the study participants, *Desulfovibrio* viability was greater in saliva samples with higher concentrations of nitrate and nitrite (Mitsui et al., 2013). This suggests that those bacteria were partially resistant to nitrate and nitrite inhibition. Since *Desulfovibrio* have been found in the human oral cavity (Willis et al., 1999) and have been associated with periodontitis (Loubinoux et al., 2002) and ulcerative colitis (Rowan et al., 2010), such resistance may have important medical implications. There is much debate about whether dietary nitrates and nitrites are harmful or beneficial for human health (Hord et al., 2009). Therefore, the interactions of bacteria with these ingested ions will be an increasingly more important field of study as more is understood about these interactions.

The inhibition of SRB by nitrate and nitrite has been used industrially to control the growth and sulfide production of sulfate-reducing bacteria. For example, nitrate was used as early as 1929 to control sulfide odors produced by SRB in industrial sewage (Fales, 1929). In 1986, it was suggested that nitrate could be used in microbially enhanced oil recovery to reduce the "souring" of oil that is caused by production of sulfide (Jenneman et al., 1986). Such souring leads to corrosion of pipes (Zhu et al., 2006; Voordouw et al., 2007) as well as to "plugging" of oil reservoirs by metal sulfides (Voordouw et al., 2007). This is a major economic problem because SRB are widespread in oil reservoirs, including low-temperature (22.6°C)(Gao et al., 2013), mesothermic (30°C)(Voordouw et al., 2009) and thermic (80°C)(Gittel et al., 2009) reservoirs. Even if bacterial growth is limited by high temperatures in the oil field (80°C), sulfide can be produced in surface processing facilities where the temperature is lower (Agrawal et al., 2014). Furthermore, SRB tend to be enriched by produced water reinjection, a process

used to increase the output of oil reservoirs (Gieg et al., 2011). In this process, water is used to "push" oil toward production wells in reservoirs that have lost pressure over their production life (Gieg et al., 2011). Seawater, which has a high sulfate concentration and therefore promotes the growth of SRB, is usually used in this process (Gieg et al., 2011). In order to alleviate the negative effects of sulfide production, nitrate has been successfully used in oil wells to control the activity of sulfate-reducing bacteria (Jenneman et al., 1999; Voordouw et al., 2009).

The inhibition of sulfide production in oil wells by nitrate is thought to have a number of different causes. One of these is thought to be production of nitrite by nitrate-reducing bacteria (Greene et al., 2003), which are also indigenous to oil wells (Jenneman et al., 1999; Gao et al., 2013). Nitrite even at low concentrations is strongly inhibitory to SRB (Greene et al., 2003; Haveman et al., 2004; Haveman et al., 2005; Greene et al., 2006), and therefore its production limits the growth and subsequent sulfide production of SRB. However, nitrate-reducing bacteria are also thought to inhibit sulfide production by at least two additional mechanisms. The first is competition of organotrophic nitrate-reducing bacteria with SRB for electron donors (Grigoryan and Voordouw, 2008). These donors may include lactate (Hubert and Voordouw, 2007; Hubert et al., 2009) or a mixture of volatile fatty acids such as acetate, propionate, and butyrate (Grigoryan et al., 2008). Volatile fatty acids are more likely to be present as the electron donors in oil fields, compared with lactate (Grigoryan et al., 2008). Since the organotrophic nitrate-reducing bacteria use the same electron donors as the sulfate reducers, they are thought to inhibit growth, and therefore sulfide production, of the SRB (Grigoryan and Voordouw, 2008). Another kind of nitrate-reducing bacteria, the nitrate-reducing, sulfide-oxidizing

bacteria, can limit accumulation of sulfide by oxidizing sulfide back to sulfate (Greene et al., 2003) or sulfur (Gadekar et al., 2006). This directly reduces the sulfide concentration in the oil well. However, both kinds of nitrate-reducing bacteria, sulfide-oxidizing and organotrophic, also produce the toxic nitrite from nitrate.

What is less well understood is whether, under these circumstances, nitrate itself directly inhibits the bacteria. The concentrations of nitrate used to treat oil wells or to treat bioreactors meant to mimic oil wells have often been low concentrations. In these cases they are concentrations of nitrate which are not inhibitory to monocultures of the model sulfate-reducing bacterium *D. vulgaris* Hildenborough. For example, 10 mM nitrate does not noticeably inhibit *D. vulgaris* (Haveman et al., 2004) or, apparently, several other sulfate-reducing bacteria (Greene et al., 2003). In one study, 10 mM nitrate was the maximum nitrate concentration used in a bioreactor testing competition of SRB with nitrate-reducing bacteria for volatile fatty acids (Grigoryan et al., 2008). Therefore, the inhibitory effect of nitrate observed was not likely due to direct nitrate inhibition in this case. In an oil field study in Alberta, Canada, only 2.4 mM nitrate was added to the injection water used to force the production of oil (Grigoryan et al., 2009). In this case, its effect was also not likely a result of direct inhibition by nitrate. However, in a more complex field study, both continuous injection of 2.4 mM nitrate and a pulsed injection of nitrate were used (Voordouw et al., 2009). The pulses lasted one hour per week at one point of injection and the pulses produced a peak concentration of 760 mM nitrate. This pulsed strategy was found to be more effective in sulfide (souring) control than continuous injection. The reason for this increased effectiveness was proposed to be the disruption of different "zones" of bacteria that had become established as a result of

nitrate addition. The zones of bacteria were thought to allow growth of SRB, and, therefore, the disruption of these zones allowed souring control to resume (Voordouw et al., 2009). Since 100 mM nitrate is known to delay growth of *D. vulgaris*, it is reasonable to suggest that the 760 mM nitrate may also have had direct inhibitory effects on SRB in the affected oil field. Nitrate at a concentration of 100 mM has also been used in a study of the inhibition of sulfide production in a bioreactor with oil from a Canadian oil field (Callbeck et al., 2013). The authors found that, in the bioreactor, sulfide production was completely inhibited during pulses of 100 mM nitrate. However, after each pulse, sulfide production resumed. This resumption of sulfide production indicated that the SRB were resistant, or became resistant, to the high nitrate as well as to the nitrite produced by nitrate-reducing bacteria in the bioreactor community (Callbeck et al., 2013). Taken together, these data suggest that further study into the mechanisms of SRB inhibition by and resistance to nitrate and nitrite would assist efforts to control souring in oil fields.

The inhibition of sulfate-reducing bacteria by nitrate can also be detrimental at times. For example, in the treatment of greenhouse wastewater, the removal of both nitrate and sulfate is desirable. Therefore, inhibition of sulfate reduction by nitrate reduction might not be beneficial under these circumstances (Gruyer et al., 2013). In addition, nitrate addition to SRB can increase, rather than decrease, microbiologically influenced corrosion (Dall' Agnol et al., 2014). However, it has been suggested that this increased corrosion in nitrate-treated fields was a result of incomplete oxidation of sulfide to corrosive sulfur (Agrawal et al., 2014). Sulfur formation might therefore be avoided by the use of a higher nitrate-to-sulfide ratio (Agrawal et al., 2014). This would be similar to the approach of increasing the oxygen-to-sulfide ratio during wastewater

treatment to encourage complete microbial oxidation of sulfide to sulfate, rather than incomplete oxidation to sulfur (Buisman et al., 1990). In contrast, it is possible that the nitrate-to-sulfide ratio could be intentionally limited (Agrawal et al., 2014) so that sulfur formation, sedimentation, and removal would be possible (Buisman et al., 1990). Adjustment of the concentration of oxygen in one wastewater treatment study allowed for the near-complete removal of both sulfate and nitrate contamination from the wastewater, with approximately 70% recovery of elemental sulfur (Xu et al., 2014). Such successful simultaneous nitrate reduction and sulfate reduction requires a balance of the inhibitory effects of nitrite and sulfide on the bacteria in such systems. A better understanding of the interactions of nitrate and sulfate with the bacteria present would allow for better predictions of the response of such systems to perturbations.

Furthermore, the detrimental effect of nitrate and nitrite on sulfate reduction may limit bioremediation of heavy metals (He et al., 2010a). Sulfate-reducing bacteria are capable of sequestering heavy metals by reduction and by sulfide precipitation, and therefore are used in studies of bioremediative strategies (Lovley et al., 1993a; Lovley et al., 1993b; Lloyd et al., 1999; Chardin et al., 2003; Jiang and Fan, 2008; Martins et al., 2009). Sites co-contaminated with nitrate and heavy metals include former nuclear weapons production sites currently managed by the U.S. Department of Energy (Riley and Zachara, 1992; Venkatramanan et al., 2013). In such sites, nitrate concentrations can exceed 100 mM (Green et al., 2012), and denitrifying bacteria, which can produce nitrite from nitrate, are known to be present (Venkatramanan et al., 2013). In such sites, therefore, production of nitrite from nitrate may limit growth of sulfate-reducing bacteria. However, the conversion of nitrate to nitrite can be inhibited if the environment lacks

molybdenum (Glass et al., 2012), since molybdenum is a cofactor for nitrate reductase (Ringel et al., 2013). Low molybdenum levels are indeed found in high-nitrate environments, and may explain the lack of disappearance of nitrate in those environments (Michael Adams, unpublished data, https://www.orau.gov/gsp2014/abstracts/adams_paul_08.pdf). Currently, it is not known whether nitrate at high concentrations (e.g. 100 mM) can inhibit SRB directly, without production of small amounts of nitrite (He et al., 2010a). If nitrate inhibits directly, the existence of mechanisms of nitrate inhibition and resistance not previously known is implicated (Redding et al., 2006; He et al., 2010a). Such mechanisms may be particularly important in low-molybdenum, high-nitrate environments in which conversion of nitrate to nitrite is not occurring. It is therefore desirable to distinguish between the effects of nitrate and nitrite on SRB. Studies of model SRB monocultures allow for separation of the effects of nitrate alone from the effects of nitrite (Haveman et al., 2004) produced by nitrate-reducing bacteria (Greene et al., 2003).

I.C. *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio alaskensis* G20 as model SRB for genetic studies

For monoculture studies of nitrate and nitrite inhibition of SRB, good genetic models are desirable. Strains of *Desulfovibrio,* a genus found at the uranium-contaminated bioremediation test zone at the US Department of Energy Oak Ridge site (Leigh et al., 2014), have been chosen as model SRB. They are good model organisms because of their relatively rapid growth rate, variety of electron donors and acceptors used, and ability to grow on agar plates (Wall, 1993). The latter is a result of improved anaerobic culture techniques (Wall, 1993) to accommodate the oxygen sensitivity of

these bacteria (Postgate, 1984; Johnston et al., 2009; Yurkiw et al., 2012). Although the first successful marker exchange mutagenesis in a sulfate-reducing bacterium was reported in 1991 (Rousset et al., 1991) for *Desulfovibrio fructosovorans*, development of genetic systems for SRB has been challenging. Reasons for this include their abundant restriction endonucleases, which can destroy non-native DNA introduced into the cell for genetic manipulation (Wall, 1993), and their resistance to several antibiotics (Postgate, 1984; Wall, 1993), which limits the number of antibiotic resistance markers that can be used to make multiple gene deletions in one strain. Despite these limitations, deletions have also been made in *Desulfovibrio gigas* (Broco et al., 2005; Rodrigues et al., 2006c) and *D. vulgaris* Hildenborough. The most advanced deletion system in a *Desulfovibrio* strain is the markerless deletion strategy developed for *D. vulgaris* Hildenborough (Keller et al., 2009). In addition, cloning in *Desulfovibrio* is possible because of the construction of various shuttle vectors (Rousset et al., 1998) based on the cryptic plasmid pBG1 isolated from *D. alaskensis* G20 parent (Wall et al., 1993). Therefore, constitutive expression of a cloned gene has been employed for mutant complementation (Zane et al., 2010) and gene overexpression (Korte et al., 2014) in *D. vulgaris*. *D. vulgaris*, therefore, because of the possibility of in-depth deletion and complementation studies, is an excellent choice for monoculture studies of nitrate and nitrite inhibition. In addition, high-throughput genetic profiling methods have been developed for both *D. vulgaris* and *D. alaskensis* G20.

D. vulgaris Hildenborough and *D. alaskensis* G20 (formerly *Desulfovibrio desulfuricans* G20), both have completely sequenced genomes (Heidelberg et al., 2004; Hauser et al., 2011). Because of this, high-throughput methods based upon genome

sequencing are possible. Transposon mutant libraries, which enable high-throughput phenotypic screening, have been generated for *D. alaskensis* (Price et al., 2013), and *D. vulgaris* (Grant Zane, Tom Juba, Adam Deutschbauer, personal communication). A high-throughput gene fitness profiling method based on deep sequencing has also been developed for *D. vulgaris* (Fels et al., 2013). This method is similar to the HITS (High-throughput Insertion Tracking by deep Sequencing) method developed in *Haemophilus influenzae* (Gawronski et al., 2009), the Tn-seq method developed in *Streptococcus pneumoniae* (van Opijnen et al., 2009), and the TraDIS (Transposon Directed Insertion-site Sequencing) method developed in *Salmonella enterica* serovar Typhi (Langridge et al., 2009). However, the method developed for *D. vulgaris*, TnLE-seq (Transposon Liquid Enrichment sequencing), is especially well-adapted to slow-growing, oxygen-sensitive bacteria that have low electroporation efficiency (Fels et al., 2013). Because of these advances, *D. vulgaris* Hildenborough and *D. alaskensis* G20 can be used for high-throughput genetic analysis of nitrate and nitrite inhibition of sulfate-reducing bacteria.

II. Study of nitrate inhibition and resistance of *D. vulgaris* Hildenborough

II.A. Current literature on nitrate vs. nitrite inhibition and limitations

Studies of nitrate stress in pure cultures of *D. vulgaris* have consisted largely of mRNA transcript analyses, proteomic analyses, and comparison of transcript abundance to that of other stress responses (Redding et al., 2006; He et al., 2010a). Such analyses have the potential to detect and monitor changes in the metabolism of bacteria in contaminated environments (Steinberg et al., 2008). Monitoring of those changes may assist in the development of more effective bioremediation strategies. However, for understanding

the underlying mechanisms involved in *D. vulgaris* stress responses, neither transcription changes nor proteomics data are sufficient (Torres-García et al., 2009). Current proteomics data for nitrate stress in *D. vulgaris* provide an incomplete picture of the proteome. In addition, there seems to be a poor correlation between the expression of transcripts and the expression of proteins in *D. vulgaris* in response to nitrate stress (Redding et al., 2006; He et al., 2010a). For example, He et al. (He et al., 2010a) reported 28 genes for which the mRNA and protein levels were both significantly changed in nitrate stress conditions. However, for 7 of these 28 genes, the mRNA was significantly down-regulated while the protein abundance was significantly up-regulated (He et al., 2010a). This poor correlation may be a result of meaningful regulatory mechanisms (Lu et al., 2007), but it is difficult to interpret. Furthermore, it is now known that bacterial transcript abundance frequently does not correlate well with gene fitness (Price et al., 2013). In fact, it was found that nearly a quarter of the genes of the metal-reducing bacterium *Shewanella oneidensis* MR-1 have a significantly detrimental effect on the fitness of this organism in some conditions (Price et al., 2013). This may be because these genes are often highly expressed in conditions in which they are detrimental (Price et al., 2013). The same authors also found that gene fitness and RNA levels were poorly correlated in the ethanol-producing bacterium *Zymomonas mobilis* ZM4 (Price et al., 2013). Furthermore, whereas *Escherichia coli* has careful control of biosynthetic pathway genes, *Z. mobilis* ZM4 and *D. alaskensis* G20 do not have such control (Price et al., 2013). Similarly, for the model yeast *Saccharomyces cerevisiae*, functional profiling with a knockout library revealed that the level of expression of a gene is a poor indicator of the fitness of its corresponding mutant in the same growth condition

(Giaever et al., 2002). Taken together, these results highlight the inadequacy of gene expression data for determining the contribution of individual genes to the ability of a bacterium to survive and grow in a particular condition. Therefore, alternate methods, such as high-throughput gene fitness profiling (Giaever et al., 2002; Deutschbauer et al., 2011) and follow-up studies with individual mutants (Deutschbauer et al., 2011), are preferred for inferring the relative importance of genes for fitness, as measured by growth and biomass accumulation, in a particular condition.

II.B. Genetic approaches for further study of nitrate vs. nitrite inhibition

Mutant analysis is the gold standard of phenotypic analysis, as the construction of the Keio collection, a library of 3,985 nonessential genes in the model bacterium *E. coli,* demonstrates (Baba et al., 2006). The original description of this collection (Baba et al., 2006) has been cited over 1,300 times. Such a collection allows for high-throughput parallel screening of mutants in a variety of conditions (Maynard et al., 2010), which can lead to understanding molecular mechanisms of bacterial responses to stress. An even more efficient high-throughput method, fitness profiling of pooled mutants, can provide similar information. For example, fitness profiling has been used in *E. coli* to study ethanol tolerance (Goodarzi et al., 2010). In that study, the authors found that the mechanisms of adaptation to ethanol inferred from the results of fitness profiling were consistent with the adaptations they observed in laboratory-evolved ethanol-tolerant *E. coli* strains. Therefore, fitness profiling was an effective tool in elucidating the molecular mechanisms of ethanol tolerance (Goodarzi et al., 2010). Transposon mutant fitness profiling can be also be used to probe the molecular mechanisms of the nitrate stress

response of *D. vulgaris*. However, analysis in nitrate-stress conditions alone is not sufficient to elucidate the mechanisms involved in nitrate inhibition of *D. vulgaris*.

Previous studies have proposed that nitrate inhibition of *D. vulgaris* is mediated by the production of small amounts of nitrite from non-specific reduction of nitrate (Wall et al., 2007). The reports of microarray studies of nitrate and nitrite stress in *D. vulgaris* indicate that the expression of at least 21 genes is significantly changed in the same direction at one or more time points in both treatments. Of these, 9 are coordinately up-regulated and 12 are coordinately down-regulated for at least one time point (He et al., 2006; He et al., 2010a). It is not known whether these statistically significant changes are biologically significant and, if so, whether they represent general stress responses or responses specific to nitrate stress. Purified desulfoviridin (DsrAB), the dissimilatory sulfite reductase of *D. vulgaris*, reduces nitrite *in vitro* (Wolfe et al., 1994). DsrAB has high affinity (K_m = 0.028 mM) and a low turnover number (k_{cat} = 0.038 mol $\cdot$ $s^{-1}\cdot$ mol $heme^{-1}$) for nitrite reduction compared with sulfite reduction (K_m = 0.06 mM; k_{cat} = 0.31 mol $\cdot$ $s^{-1}\cdot$ mol $heme^{-1}$)(Wolfe et al., 1994). These observations were interpreted to mean that nitrite competitively inhibits DsrAB (Greene et al., 2003). Furthermore, comparison of the growth of sulfate-reducing strains with and without a nitrite reductase (NrfA) showed that the presence of an active NrfA is associated with the ability to reduce sulfate in the presence of nitrite (Greene et al., 2003). Inhibition of DsrAB by nitrite is therefore thought to be at least partially overcome by the up-regulated expression of NrfHA, the periplasmic nitrite reductase of *D. vulgaris*, in the presence of nitrite (Haveman et al., 2004; He et al., 2006). The expression of this enzyme is also up-regulated in the presence of nitrate (He et al., 2010a), which suggests that nitrite might be produced in

pure cultures of *D. vulgaris* stressed by nitrate. Therefore, any fitness profiling of *D. vulgaris* to probe nitrate stress must include nitrite stress as a control. It is also necessary to control for osmotic stress responses. It is not surprising that when 105 mM sodium nitrate was added to the medium, the resulting expression profile had some overlap with the expression profile of *D. vulgaris* cultures with 250 mM sodium chloride added to the medium (He et al., 2010a). It was proposed (He et al., 2010a) that nitrate stress responses are a combination of nitrite stress responses and osmotic stress responses. However, the same authors also proposed, given the rather minimal overlap of these expression profiles with the nitrate profile, that unique stress responses were also involved. In contrast to the often confusing results of expression profiling, "fitness profiling" can effectively reveal the contribution of each bacterial gene to the ability of the cell to survive and grow in a particular condition (Deutschbauer et al., 2011; Deutschbauer et al., 2014). It is a high-throughput mutant screen. Because of this, fitness profiling is a rapid tool to elucidate differences in the responses of *D. vulgaris* to the presence of nitrate, nitrite, and osmotic stresses. The results of fitness profiling can then be followed up and confirmed with in-depth analysis of isolated mutants.

Analysis of individual mutants is necessary because of the potential interfering effects of pooling mutants. It is possible, due to the exchange of metabolic products in a population of different mutants, that a single mutant might have different growth phenotypes in pooled versus isolated growth conditions (Pierce et al., 2007). Furthermore, transposon mutant phenotypes are sometimes different from deletion mutant phenotypes. This is because a transposon insertion may not fully inactivate the gene it interrupts, or it may disrupt the expression of downstream genes in a polar effect

(Baba et al., 2006). It is therefore ideal to make an in-frame deletion of the gene for mutant studies. Complementation of the deleted gene on a stable plasmid (Zane et al., 2010) or integrated into the genome itself (Parks et al., 2013) is then undertaken to restore the original phenotype to the bacterium. Restoration of the parental phenotype confirms that the phenotype of the mutant was specific to the inactivation of the gene of interest. Mutant and complemented strains can then be grown individually and subjected to appropriate biochemical analyses. These analyses allow for in-depth knowledge that supports and confirms the hypotheses that can be generated by fitness profiling. In this way, mutant analysis and fitness profiling of this kind can work together to elucidate molecular mechanisms (Deutschbauer et al., 2011), such as those of nitrate and nitrite inhibition and resistance.

CHAPTER 2

GENETIC BASIS FOR NITRATE RESISTANCE IN *Desulfovibrio* STRAINS

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Genetic basis for nitrate resistance in *Desulfovibrio* strains

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Author contributions: H.L.K., S.R.F. and J.D.W. designed the experiments. S.R.F. conducted the TnLE-seq experiments and analyzed them. G.A.C. constructed the Δ*rex* mutant and showed its impairment on thiosulfate. M.N.P. and A.M.D. generated and analyzed the G20 fitness profiling data. J.V.K. constructed the G20 transposon mutant library. G.M.Z. constructed the DvH transposon mutant library, confirmed and provided the individual transposon mutants tested. A.P.A. and J.D.W. supervised the project. H.L.K, M.N.P., and J.D.W. interpreted the data and wrote the manuscript.

I. Introduction

Sulfate-reducing bacteria are environmentally and industrially significant microorganisms that use sulfate as a terminal electron acceptor in anaerobic respiration. These anaerobes produce sulfide as the end product of sulfate respiration (Postgate, 1984). Sulfide is toxic to most organisms (Caffrey and Voordouw, 2010), and its production causes oil souring in the petroleum industry (Ligthelm et al., 1991; Sunde et al., 1993). Despite the undesirable features of this metabolic end product, SRB have been exploited in studies of heavy metal bioremediation (Jiang and Fan, 2008; Martins et al., 2009) because of the ability of sulfide to form insoluble complexes with heavy metals (Jalali and Baldwin, 2000). SRB also precipitate heavy metals by directly changing the metal redox state to a less soluble form (Lovley et al., 1993a; Lovley et al., 1993b; Lloyd et al., 1999; Chardin et al., 2003). The metabolism of SRB is studied, therefore, to understand how to minimize the detrimental economic effects of these bacteria and to maximize their positive metabolic traits.

Such traits have been studied extensively in *Desulfovibrio vulgaris* Hildenborough (DvH), a model SRB with a sequenced genome (Heidelberg et al., 2004). DvH has been examined under a variety of stress conditions, including elevated nitrite (He et al., 2006; Bender et al., 2007) or nitrate concentrations (Redding et al., 2006; He et al., 2010a), heat shock (Chhabra et al., 2006), high salt (Mukhopadhyay et al., 2006; He et al., 2010b), oxygen (Mukhopadhyay et al., 2007), or electron donor depletion (Clark et al., 2006). The data obtained help in the prediction of responses of SRB in heavy metal-

contaminated sites, which also contain many chemicals that inhibit these bacteria. For example, nitrate concentrations can be greater than 100 mM at US nuclear weapon complexes overseen by the Department of Energy (Green et al., 2012), and these waste sites are also contaminated with heavy metals (Riley and Zachara, 1992). High nitrate inhibits the growth of DvH (He et al., 2010a). Although some SRB can also use nitrate as a terminal electron acceptor (McCready et al., 1983), nitrate is successfully used by the petroleum industry to control the growth of SRB and the oil souring that their sulfide production causes (Sunde and Torsvik, 2005). The mechanism of nitrate inhibition of SRB is still unclear. In the environment, at least part of the inhibition by nitrate is indirect: nitrate-reducing, sulfide-oxidizing bacteria produce nitrite that is toxic to SRB at much lower concentrations than is nitrate (Haveman et al., 2005; He et al., 2006). Furthermore, in oil wells, heterotrophic nitrate-reducing bacteria can compete with sulfate-reducing bacteria for volatile fatty acid electron donors, further reducing the production of sulfide (Grigoryan et al., 2008). However, nitrate is also inhibitory to DvH in the absence of nitrate-reducing bacteria (Redding et al., 2006; He et al., 2010a).

It has been suggested that this pure culture nitrate inhibition is also a result of nitrite stress, since DvH itself may produce small amounts of nitrite from non-specific reduction of nitrate (Wall et al., 2007). In addition, high concentrations of nitrate could potentially induce a nonspecific osmotic shock response in the bacteria (Wall et al., 2007). However, microarray data reveal few common gene expression changes among nitrate, nitrite, and sodium chloride stress conditions (He et al., 2010a). He et al. suggested that unique nitrate stress responses might account for these discrepancies (He et al., 2010a). Understanding the mechanism of nitrate inhibition of DvH and the genes involved in the

nitrate stress response should facilitate the prediction and monitoring of the effectiveness of bioremediation strategies that employ SRB (Hazen and Stahl, 2006).

Past studies of the mechanisms of nitrate stress responses in DvH have relied primarily on transcript analyses (He et al., 2010a) and protein determination (Redding et al., 2006) techniques. However, mutant analysis is a more reliable method of determining gene essentiality in a particular stress condition (Price et al., 2013). Fitness profiling of many mutants en masse is a high-throughput approach complementary to classical genetic techniques that has allowed rapid annotation of genes (Deutschbauer et al., 2011). In this study, we used random transposon mutant fitness profiling in two completely sequenced (Heidelberg et al., 2004; Hauser et al., 2011) model SRB, *Desulfovibrio alaskensis* G20 ("G20," formerly called *Desulfovibrio desulfuricans* G20) and DvH, to probe the molecular mechanisms of their nitrate stress responses. Because 58% of G20 genes (1954/3371) are also present in DvH, which has 3503 genes (http://www.microbesonline.org/), we predicted that the strains would have similar nitrate stress responses. Therefore, pools of DvH and G20 transposon mutants with mutations saturating the non-essential genes under permissive growth conditions were subjected to high concentrations of nitrate. Illumina sequencing or microarrays were used to locate the transposons in mutants surviving the nitrate exposure and, by comparison with mutants not exposed to stress, to identify genes essential for survival in high nitrate. Generally, those mutants lost from the stress treatment represent genes whose functions are needed for stress survival. From the fitness profiling reported here, surprisingly we identified mutants with dramatically increased fitness in nitrate stress conditions that we further analyzed in pure cultures. However, the same mutations did not confer resistance

to nitrite. These results confirmed the predicted existence of unique nitrate-resistance mechanisms (He et al., 2010a) and suggested that environmental models of nitrate inhibition require expansion.

II. Materials and Methods

II.A. Strains and media

The strains used in this study are listed in Table 2.1. All DvH and G20 strains were grown in defined MOLS4 medium [MO Basal Salts (Zane et al., 2010) with 60 mM sodium lactate and 30 mM sodium sulfate]. The medium used to grow DvH cultures was reduced with 1.2 mM sodium thioglycolate; whereas, the medium for G20 was reduced with 0.38 mM titanium citrate. DvH and G20 manipulations, including setup of growth kinetic studies, were done at about 25°C in an anaerobic growth chamber (Coy Laboratory Products, Inc., Grass Lake, MI) with an atmosphere of approximately 95% N_2 and 5% H_2. Optical densities (600 nm) were determined with a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA).

Table 2.1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Source and/or reference
E. coli		
α-Select (Silver Efficiency)	F$^-$ *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA-*arg*F)U169 Φ80*lac*ZΔM15 λ$^-$	Bioline
D. alaskensis		
G20	Spontaneously nalidixic acid-resistant derivative of *Desulfovibrio desulfuricans* G100A lacking the endogenous cryptic 2.3-kb plasmid, pBG1	(Wall et al., 1993)
D. vulgaris		
ATCC 29579	Wild-type (WT) *D. vulgaris* Hildenborough (pDV1); 5-FU^s (Parent for GZ strains)	ATCC
JW710	WT Δ*upp* (pDV1); 5-FU^r (used as "WT" control for DvH growth kinetics in this study; parent strain for deletions)	(Keller et al., 2009)
JW3311	JW710 ΔDVU0916::(*npt upp*); Km^r; 5-FU^s (Δ*rex* marker exchange)	This study
GZ9685	DVU0245-773::Tn*5*-RL27; insertion at bp 773/1110 for the gene; Km^r	Wall Laboratory
GZ12997	DVU0246-111::Tn*5*-RL27; insertion at bp 111/2235 for the gene; Km^r	Wall Laboratory
GZ2640	DVU0247-211::Tn*5*-RL27; insertion at bp 211/360 for the gene; Km^r	Wall Laboratory
GZ12015	DVU0250-427::Tn*5*-RL27; insertion at bp 427/588 for the gene; Km^r	Wall Laboratory
GZ10694	DVU0251-80::Tn*5*-RL27;insertion at bp 80/963 for the gene; Km^r	Wall Laboratory
GZ2179	Genome position 658487::Tn*5*-RL27; insertion at intergenic region 327 bp upstream of VIMSS209534, DVU0590; Km^r (Control strain for transposon mutant growth kinetics)	Wall Laboratory
Plasmids		
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); Sp^r; source of Sp^r and pUC *ori* fragment for marker exchange suicide plasmid construction	(Keller et al., 2009)
pMO746	*upp* in artificial operon with *npt* and Ap^r-pUC *ori*; P_{npt}-*npt-upp*; Km^r; 5-FU^s; for marker exchange suicide plasmid construction	(Parks et al., 2013)
pMO9075	pMO719 containing P_{npt} for constitutive expression of complementation constructs; pBG1 stable SRB replicon; Sp^r	(Keller et al., 2011; Keller et al., 2014)
pMO3311	Sp^r and pUC *ori* from pMO719 plus 1630 bp upstream and 1590 bp downstream DNA regions from DVU0916 (*rex)* flanking the artificial operon of P_{npt}-*npt-upp* from pMO746; for marker exchange deletion mutagenesis; Sp^r and Km^r	This study
pMO3313	pMO9075 with DVU0916 (*rex*) constitutively expressed from P_{npt}	This study
pRL27	Tn*5*-RL27 (Km^r-*ori*R6 K) delivery vector; for transposon mutagenesis of DvH strains	(Larsen et al., 2002)

[a]Km, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript "r" or "s", resistance or sensitivity

II.B. Growth kinetics

Growth kinetic studies with wild-type G20 were carried out as described below for the G20 fitness profiling, with the following exceptions: No kanamycin was used with the wild-type cells, and each wild-type G20 freezer stock was pelleted to reduce carryover of glycerol used as the cryoprotectant before inoculation of starter cultures. For all DvH growth kinetics, 5mL MOLS4 cultures (with 1.2 mM sodium thioglycolate) were started by inoculation with the pelleted cells from a freezer stock. Geneticin (G418) sulfate (400 µg/mL) or spectinomycin dihydrochloride pentahydrate (100 µg/mL) were added to DvH cultures where indicated. Each condition tested was prepared as 14.5 mL of inoculated culture plus 1 mL deionized water (for "no additions" controls) or inhibitory salts (sodium nitrate, sodium nitrite) dissolved in deionized water. Aliquots of 5.1 mL from this 15.5 mL culture were grown as triplicates in 27-mL anaerobic tubes, each capped with a butyl rubber stopper and grown at 34°C. All G20 and DvH inocula were grown to late exponential or stationary phase. 100 mM sodium nitrate was used for experiments with DvH, compared with 150 mM for G20 experiments, due to the greater sensitivity of DvH to nitrate. With a few exceptions, all growth kinetics experiments were repeated at least twice with triplicates in each experiment. Triplicate growth experiments for the DVU0250 transposon mutant and intergenic control transposon mutant experiments were done once. Triplicates of the Δ*rex* mutant in the presence of nitrite were also grown once.

II.C. G20 transposon mutant fitness studies

Fitness data were collected with two pools of G20 transposon insertion mutants (4,069 and 4,056 mutants, respectively) that are described in more detail elsewhere (Kuehl et al., 2014). Briefly, 1174 strains are present in both pools, leaving 6951 strains that are present only once in the complete library. 498 genes are represented only once in the library. 571 genes are represented twice in the library; that is, either a single strain is present in each of the pools or two different strains with a transposon insertion in the same gene are present in the library. 1,341 genes are represented in the library three or more times. A total of 2410 unique genes and 212 unique intergenic regions are represented. Thus, about 71% of G20 genes are represented in the library, providing excellent coverage of nonessential genes. Transposon insertions were mapped to the genome by a two-step arbitrary PCR as described previously (Oh et al., 2010). Each mutant has a "TagModule" that contains two different variable segments, an "uptag" and a "downtag"(Oh et al., 2010). Within each pool, each strain has a unique TagModule, so that the abundance of the TagModule is a proxy for the abundance of that strain. Only the uptags are amplified from the “uptag pool,” Pool 1 (4,069 strains) and only the downtags are amplified from the “downtag pool” Pool 2 (4,056 strains). Amplified tags from both pools can be hybridized to the same array because only one tag (up or down) from a TagModule has been shown to be necessary for accurate quantification of strain abundance and there is no overlap of tags in the two pools (Oh et al., 2010; Deutschbauer et al., 2011). Each pool was grown overnight to late logarithmic phase (OD_{600} about 0.87) in about 10 mL MOLS4 medium amended with titanium citrate (0.38 mM) as

reductant and kanamycin (800 µg/mL). 750 µL of each pool was added to 15 mL MOLS4 + 1650 µL sterile MO Basal salts (Zane et al., 2010) or salt (sodium nitrate, sodium chloride, etc.) dissolved in MO Basal Salts. Each amended medium plus mutants (17.4 mL) was aliquoted into three 27-mL anaerobic tubes, about 5.8 mL per tube, each capped with a butyl rubber stopper and grown at 34°C. When the cultures had reached stationary phase (OD_{600} >1), 0.5 mL from each control or stress condition was pelleted and processed as described previously; that is, genomic DNA was extracted (Deutschbauer et al., 2011), and the uptags and downtags were PCR amplified, hybridized to an Affymetrix 16K TAG4 array, and scanned (Pierce et al., 2007). The number of doublings of the population was estimated by using the doubling in OD_{600} to approximate doubling of the cell population.

Fitness data for G20 were analyzed as described for similar experiments with *Shewanella oneidensis* MR-1 (Deutschbauer et al., 2011), with slight modifications (Price et al., 2013). Briefly, strain fitness = $\log_2$(END/START), where those values ("END" and "START") are averages of the gene location-specific uptag and downtag $\log_2$ intensities. Mutant strains with low START values were excluded, leaving measurements for 3726 strains in Pool 1 and 3865 strains in Pool 2. Strain fitness was normalized across the genome so that the median was 0; this was done separately for the two pools. Since a gene could be mutated at different sites, gene fitness was calculated as the average fitness of strains with mutations in a particular gene. Gene fitness was normalized to remove the effect of chromosomal position on gene fitness and to set the mode of fitness values to zero, as previously described (Price et al., 2013). One difference from the previously described protocol (Price et al., 2013) was that only one

Affymetrix chip was used per experiment; up- and downtags were hybridized to the same array. The z scores were computed as described previously with control experiments for G20 (Price et al., 2013). The results of the "MOLS4 no stress" condition were similar to the LS4D controls described previously (Price et al., 2013), including similarly "sick" auxotrophs that would be expected in a defined medium when compared with lactate-sulfate medium containing yeast extract. Two quality metrics were used for each experiment. Strain correlation (Table 2.2) is the correlation of the strain fitness values for the same strains between the two pools. Operon correlation (Table 2.2) is the correlation of gene fitness values between adjacent genes predicted (http://www.microbesonline.org/) to be in the same operon. The low quality metrics for the sodium nitrate and potassium nitrate conditions (Table 2.2) reflect the predominance of a few strains in the culture; essentially only the data for these few strains is biologically meaningful, while the majority of strains did not have the opportunity to grow at all before the culture reached stationary phase. The complete data from these experiments are available at http://www.microbesonline.org/.

Table 2.2. Quality metrics for *D. alaskensis* G20 fitness profiling experiments

Experiment	Strain Correlation[a]	Operon Correlation[b]
No stress Control	0.650	0.416
150 mM Sodium Nitrate	0.311	0.377
150 mM Potassium Nitrate	0.317	0.393
150 mM Sodium Chloride	0.843	0.490
150 mM Potassium Chloride	0.907	0.493
0.25 mM Sodium Nitrite	0.659	0.409

[a]The correlation of the strain fitness values for the strains present in both pools: 1091 strains measured

[b]The correlation of gene fitness values between adjacent genes predicted (http://www.microbesonline.org/) to be in the same operon

II.D. DvH TnLE-seq fitness studies

This nitrate resistance study was one of five multiplexed TnLE-seq pools that were part of a protocol described elsewhere (Fels et al., 2013). One advantage of this method is that individual mutants do not need to be isolated and confirmed, as in a catalogued library. They also do not need to be frozen en masse and recovered from the freezer. Rather, hundreds of thousands of unique transposon mutations are created by conjugation at the beginning of each experiment. The only experimental difference between this study and those published was the addition of 100 mM nitrate to the MOLS4 defined medium for growth of the mutant pool. As expected given the strong stress of 100 mM nitrate, this pool was delayed by about 92 h in reaching an OD_{600} of 0.4 compared with only about 40 h in the defined MOLS4 medium without nitrate. However, the nitrate pool was harvested and the fitness values were determined as previously described (Fels et al., 2013). The total number of cells in the final 500 mL culture (1×10^{11} cells) was determined by plating for individual colony-forming units, as previously described (Fels et al., 2013). The number of doublings of the culture was estimated by assuming that only the genes with $\log_2$ fitness scores >0 (38 genes) contributed significantly to the final population. Therefore, the number of unique insertions in these genes (1904) was considered to be number of cells in the starting pool. The complete data from these experiments are available at http://desulfovibriomaps.biochem.missouri.edu/fitness/.

II.E. Plasmid and strain construction

Genomic DNA from DvH was isolated with the Wizard® Genomic DNA Purification Kit (Promega, USA). Plasmids were isolated from both *E. coli* and DvH with the GeneJET Plasmid Miniprep Kit (Fermentas, Thermo Scientific, Glen Burnie, MD). All primers

were obtained from Integrated DNA Technologies (Coralville, IA). The pMO3311 and pMO3313 plasmids were constructed by Sequence- and Ligation-Independent Cloning (SLIC) (Li and Elledge, 2007). PCR products from template plasmids were agarose gel-purified to reduce transformation of the parent plasmid. All products were cleaned with a Wizard® SV Gel and PCR Clean-up System (Promega, USA) before the SLIC procedure. The plasmids were constructed by amplification of DNA regions (Table 2.3) with Herculase II polymerase (Agilent cat# 600675), as previously described for a similar procedure (Parks et al., 2013). DNA products were transformed into Silver Efficiency α-Select *E.coli* cells (Bioline) and plated on solidified LC medium (Zane et al., 2010). Electroporation procedures were similar to those previously described (Keller et al., 2011) with electroporation parameters 1500 V, 250 Ω, and 25 μF. Cells recovered overnight after electroporation were plated on MOYLS4 with 1.2 mM thioglycolate as reductant and about 0.2% (wt/vol) yeast extract. Sequence confirmation of the mutagenic cassette and the complementing gene was performed at the University of Missouri DNA Core Facility (http://www.biotech.missouri.edu/dnacore/).

II.F. Nitrate determination

A scaled-down version of a previously described colorimetric method (Cataldo et al., 1975) was used to determine nitrate concentrations. Briefly, 200 μL of salicylic acid solution (1g salicylic acid dissolved in 20 mL of approximately 98% [vol/vol] sulfuric acid) was added to each 25 μL sample that had been diluted 25-fold in deionized water. This was mixed and incubated 20 min at room temperature and then 4.75 mL of 2M NaOH was added to each tube. Absorbance at 410 nm was measured with a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA). R^2 for a standard curve was

> 0.99, instrument detection limit 0.1 ± 0.1 mM.

II.G. Protein determinations

Whole cell protein concentrations were determined with the Bradford assay (Bradford, 1976) with bovine serum albumin as the standard. Absorbance at 595 nm was measured with a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA).

Table 2.3. Primers used for PCR amplification, Southern probe generation and sequencing in *D. vulgaris*

Primer name	Primer sequence (5'-3')	Application
DVU0916-1	GCCTTTTGCTGGCCTTTTGCTCACATGATGCTGAGAAGTTCGGTCCGAAG	For amplification of DVU0916 upstream region from gDNA with DVU0916-2 primer to make pMO3311. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment (SpecRpUC-R). Amplification of Southern probe for confirmation of DVU0916 deletion. forward
DVU0916-2	GCGACAAGATATTCGGCACCAAGTAAGCGTTCGTTAACTTCACTTTTTGCAATGCAC	For amplification of DVU0916 upstream from gDNA with DVU0916-1 primer to make pMO3311. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment (UppCterm). Amplification of Southern probe for confirmation of DVU0916 deletion. reverse
DVU0916-3	GCGCCCCAGCTGGCAATTCCGGCTGGAGCGTGAACGCCTCC	For amplification of DVU0916 downstream from gDNA with DVU0916-4 to make pMO3311. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment (KanPromNterm). forward
DVU0916-4	GTCGAGGCATTTCTGTCCTGGCTGGGATTTCATGGGCCCCGATGTATTGG	For amplification of DVU0916 downstream region from gDNA with DVU0916-3 primer to make pMO3311. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment (SpecRpUC-F). reverse
SpecRpUC-F	CCAGCCAGGACAGAAATGCCTCG	For amplification of Sp^r and pUC *ori* from pMO719 to make pMO3311. Used as overhang for SLIC. forward
SpecRpUC-R	ATGTGAGCAAAAGGC	For amplification of Sp^r and pUC *ori* from

	CAGCAAAAGGC	pMO719 to make pMO3311. Used as overhang for SLIC. reverse
KanPromNterm	CCGGAATTGCCAGCTGGG	For amplification of Km^r from pMO719 to to make pMO3311. Used as overhang for SLIC. forward
UppCTerm	CTTACTTGGTGCCGAATATCTTGTCGC	For amplification of Km^r from pMO719 to to make pMO3311. Used as overhang for SLIC. reverse
SpecRpUC-up	GGGAAACGCCTGGTATCTTTATAGTCCT	For colony PCR, screen of and sequencing upstream region of pMO3311 deletion cassette. forward
pMO719XbaI-Dn	TGGGTTCGTGCCTTCATCCG	For colony PCR, screen of and sequencing downstream region of pMO3311 deletion cassette; also for sequencing complementation constructs. Sequencing primer to confirm inserts into pMO9075 for complementation of DVU0916 (pMO3313). reverse
Kan-int-Fwd-rev-comp	CTCATCCTGTCTCTTGATCAGATCT	For sequencing downstream region of pMO3311 deletion cassette. forward
DvH-Upp gene Cterm-out	GCTGAAGCGCATCGTGGACAA	For sequencing upstream region of pMO3311 deletion cassette. reverse
pBG1-2199-F	GCTGAAAGCGAGAAGAGCGCAC	Sequencing primer to confirm inserts into pMO9075 for complementation of DVU0916 (pMO3313).
DVU0916-UP-int-F	CCTACGGCCAACGTCAACACCAAC	Sequencing primer to confirm upstream region of deletion cassette of pMO3311. forward
DVU0916-UP-int-R	GTTGGTGTTGACGTTGGCCGTAGG	Sequencing primer to confirm upstream region of deletion cassette of pMO3311. reverse
DVU0916-DWN-int-F	GGATAGCGTGACATTCCCGGACGTG	Sequencing primer to confirm downstream region of deletion cassette of pMO3311. forward
DVU0916-DWN-int-R	CACGTCCGGGAATGTCACGCTATCC	Sequencing primer to confirm downstream region of deletion cassette of pMO3311. reverse
SLIC-DVU0916-comp-F	AGGTTGGGAAGCCCTGCAATGCAGTCCCAGGAGGTACCAT ATGACCAACATCAAAAGCGAACACATCC	For amplification of DVU0916 to make pMO3313 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. forward
SLIC-DVU0916-	GATCGTGATCCCCTGCGCCATCAGATCCTT	For amplification of DVU0916 to make pMO3313 complementation construct.

comp-R	GCTATTTGTTGCGCGAGAACGTGATGTT	Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. reverse
pMO9075-SLIC-F	CAAGGATCTGATGGCGCAGGG	For amplification of pMO9075 fragment for SLIC to make pMO3313 complementation construct. forward
pMO9075-SLIC-R3	CTGGGACTGCATTGCAGGGCTTCCCAACCT	For amplification of pMO9075 fragment for SLIC to make pMO3313 complementation construct. reverse

III. Results

III.A. Response of DvH to nitrate exposure

It has been reported (Elias et al., 2009; He et al., 2010a) that DvH cells can grow rapidly and abundantly after a long lag phase in high nitrate concentrations. It was unclear, however, whether this rapid growth was due to elimination of the toxic nitrate, some modification of cell metabolism allowing adaptation to the continued presence of nitrate, or outgrowth of preexisting nitrate-resistant mutants. Furthermore, it was not known whether the cells that grew in nitrate had a growth advantage over naïve cells when subcultured into a fresh medium amended with nitrate. To test this, JW710 (Table 2.1), the parent for making marker exchange and markerless deletion strains (Keller et al., 2009), was used. JW710 will therefore be referred to as the wild-type control for all DvH growth kinetics in this study. This wild-type control was grown in lactate-sulfate medium amended with 100 mM nitrate (Fig. 2.1A).

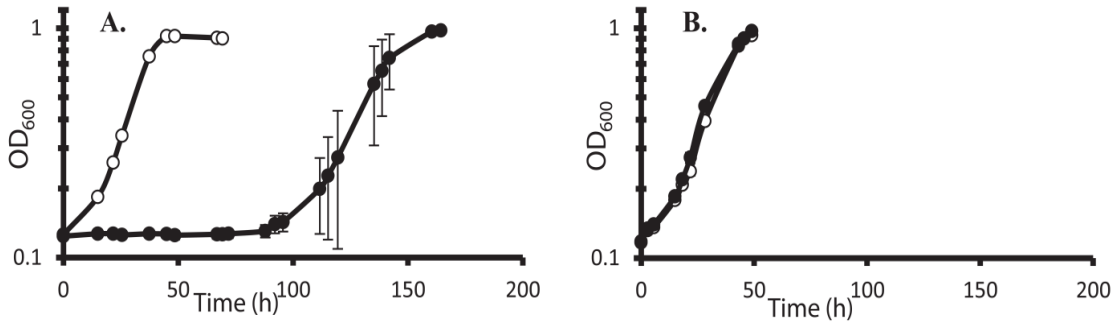

Figure 2.1 Growth and subculture of wild-type *D. vulgaris* Hildenborough (JW710) in lactate-sulfate medium with inhibitory nitrogen species. (A): Growth of DvH with no additions (○) or 100 mM sodium nitrate (●) **(B)**: Growth of DvH subcultured from original 100 mM nitrate culture shown in panel A; no additions (○), 100 mM sodium nitrate (●). Approximately 6-6.5% (vol/vol) inocula were used for the original culture and subcultures. Readings reflect averages of three samples, and errors bars show standard deviations.

It was determined that, at the end of growth in the presence of 100 mM nitrate, no gross consumption of nitrate was detected (Table 2.4). The persistence of the nitrate suggested that the ability of DvH to grow in the presence of 100 mM nitrate was due to adaptation to nitrate or outgrowth of spontaneous mutants, rather than a detoxification of nitrate itself. To further confirm this lack of nitrate metabolism, nitrite was measured (American Public Health Association, 1992) during the lag/inhibition phase for JW710 cells exposed to 100 mM nitrate and the nitrite concentration was below 15 ± 5 µM (data not shown). This nitrite concentration is below the concentration (40 µM) reported to inhibit plated single colonies of *D. vulgaris* (Haveman et al., 2004). Thus, secondary production of nitrite is not likely the cause of nitrate sensitivity. To begin to test whether

the nitrate adaptation was due to a reversible gene regulation or to successful growth of spontaneous mutants in the culture, nitrate-adapted strains were subcultured back into medium with or without nitrate. We found that nitrate-stressed cultures grew without a prolonged lag and maintained nitrate resistance when subcultured into fresh medium with 100 mM nitrate (Fig. 2.1B). This resistance continued over the course of three subcultures (data not shown). Further, even nitrate-resistant cultures that were subcultured into medium lacking nitrate retained nitrate resistance when subcultured back into 100 mM nitrate (data not shown). As with the original exposure to nitrate, no gross consumption of nitrate was observed over the course of the subcultures (Table 2.4). We suggest that spontaneous mutations in the culture lead to increased nitrate resistance of some cells which then predominate in the population.

Table 2.4. Nitrate concentrations from stationary phase cultures of *D. vulgaris* Hildenborough grown in MOLS4 medium amended as indicated

Subculture[a]	Inoculum History	Amendment	$[NO_3^-]$[b] in mM
0-A[c]	Lactate/SO_4^{2-}	none	not measured
0-B[c]	Lactate/SO_4^{2-}	100 mM NO_3^-	101 ± 3
1-A[d]	From 0-B	none	8 ± 1
1-B[d]	From 0-B	100 mM NO_3^-	102 ± 3
2-A	From 1-B	none	6 ± 2
2-B	From 1-B	100 mM NO_3^-	99 ± 1
3-A	From 2-A	none	not detected
3-B	From 2-A	100 mM NO_3^-	101 ± 7
3-C	From 2-B	none	8 ± 2
3-D	From 2-B	100 mM NO_3^-	103 ± 5

[a]Inocula were 6.5% (vol/vol)
[b]Concentrations determined from triplicate determinations with standard deviations shown
[c]Growth curves in Fig. 2.1A
[d]Growth curves in Fig. 2.1B

III.B. Fitness profiling with G20

To test what mutations might be causing this nitrate resistance, we employed transposon mutant fitness profiling. A catalogued transposon mutant library, which enables high-

throughput phenotypic screening, had been generated in G20 prior to that produced in DvH (Price et al., 2013). Each mutant strain in the library is identified by two unique DNA barcode sequences or "tags," the "up" tag and the "down" tag (Oh et al., 2010; Deutschbauer et al., 2011). Strain abundance is measured by reading the abundance of the barcodes through fluorescence in microarrays made to detect the barcodes (Pierce et al., 2006; Pierce et al., 2007).

We predicted that comparison of fitness profiles of nitrite- and nitrate-stressed G20 cells would reveal mutants with responses unique to nitrate. That is, fitness profiling in the two conditions would allow us to see which mutants differentially increased in relative abundance during a pooled growth competition and which decreased. The fitness of a particular strain is calculated as $\log_2$ of the ratio of the relative abundance of the strain after growth competition to the relative abundance of the strain before growth competition. Therefore, if the relative abundance of a particular strain in the pool remained the same before and after stress, its fitness would be equal to zero (Oh et al., 2010):

$$\text{Fitness of a G20 mutant} = \log_2 \left(\frac{\text{barcode microarray signal from cells after stress}}{\text{barcode microarray signal from cells prior to stress}} \right)$$

If a strain decreased in relative abundance after the stress condition because it was outcompeted or unable to cope with the stress, it would have negative (<0) fitness. If its relative abundance increased, it would have positive (>0) fitness. For the pools, the fitness calculated for a particular gene, referred to as the "mean log ratio," is expressed as $\log_2$ of the average fitness of strains with a mutation in that particular gene.

The G20 pools were grown in lactate-sulfate medium amended with 150 mM $NaNO_3$, 150 mM KNO_3, 150 mM NaCl, 150 mM KCl, or 0.25 mM $NaNO_2$. NaCl and KCl

conditions were osmotic controls; KNO_3 vs. KCl allowed a control for anion specificity. Concentrations of 150 mM nitrate and 0.25 mM nitrite were chosen because these concentrations severely but not completely inhibited wild-type G20 (Fig. 2.2A and B). Since a long lag phase had been observed before exponential growth of nitrate-stressed cultures of both DvH and G20 in lactate-sulfate conditions (Fig. 2.1A and 2.2B), it was reasonable to hypothesize that spontaneous mutants in wild-type cultures were selected in the population after the lag.

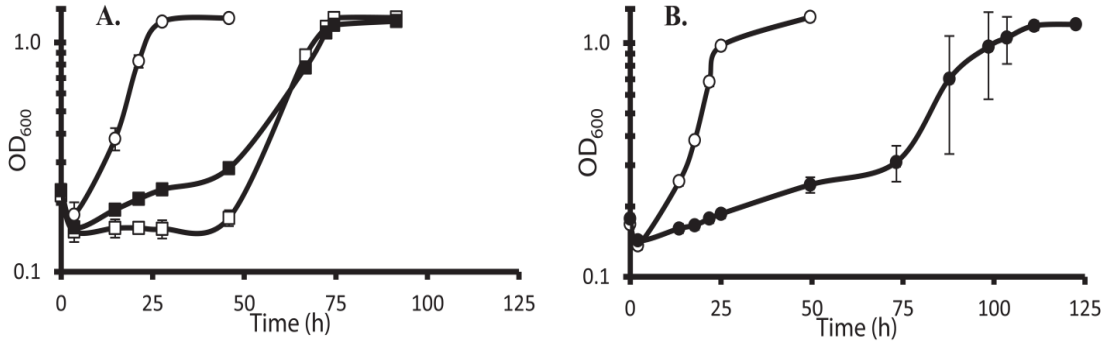

Figure 2.2 Growth of wild-type *D. alaskensis* G20 in lactate-sulfate medium with inhibitory nitrogen species. (A): Growth of G20 with no additions (○), 150 mM sodium nitrate (□), or 150 potassium nitrate (■). **(B)**: Growth of G20 with no additions (○) or 0.25 mM sodium nitrite (●). Approximately 4.5% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars show standard deviations.

Therefore, this hypothesis was confirmed when we found that, in the transposon mutant pools, several mutant strains predominated in cultures growing in the presence of nitrate (Tables 2.5 and 2.6). That is, transposon insertion conferred a growth advantage, and therefore a high fitness, on these particular strains in the nitrate stress condition. The top ten genes interrupted in the strains that grew abundantly in sodium nitrate had fitness values (mean log ratios) greater than 2 in that condition, but fitness values less than 0.25

in sodium chloride, potassium chloride, sodium nitrite, and in the absence of stress (Table 2.6). In contrast, interruption of those same genes was advantageous in both sodium nitrate and potassium nitrate (Table 2.6). Growth of the mutants in both salts of nitrate supports the specificity of the nitrate anion as the driver for selection of these resistant mutants. The interrupted gene conferring the highest fitness in sodium nitrate was Dde_2702 (Table 2.5), a gene annotated as encoding Rex, a redox-sensing regulatory protein (Ravcheev et al., 2012). Particularly surprising was the high fitness conferred by mutation of a cluster of poorly annotated genes, Dde_0597 through Dde_0605, hereafter called the "nitrate cluster." Both the *rex* gene and the nitrate cluster (Table 2.5) have homologs in DvH. Because of these homologies, including shared synteny of the nitrate cluster in G20 (Fig. 2.3), it seemed reasonable that mutations of the homologs in DvH would confer similar nitrate-resistant phenotypes.

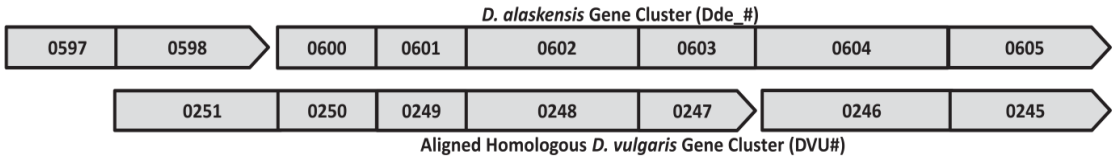

Figure 2.3 *Desulfovibrio* nitrate resistance gene cluster. Operon predictions were from http://microbesonline.org/; boxes represent predicted genes, arrows indicate direction of transcription, and contiguous boxes ending in an arrow represent predicted operons.

Table 2.5. *Desulfovibrio* genes interrupted in strains with high fitness in lactate-sulfate conditions amended with sodium nitrate

G20 gene (Dde)	fitness[a]	DvH homolog (DVU)	fitness[b]	Annotations
2702	4.23	0916	3.81	AT-rich DNA binding protein (COG2344); Transcriptional repressor, redox-sensing, Rex (IPR022876)
0597	2.25	no homolog	No data	Uncharacterized protein conserved in archaea (COG2043); Protein of unknown function DUF169 (IPR003748)
0598	3.01	0251	11.44	Transmembrane protein TauE like (IPR002781); predicted permease (COG0730); sulfite exporter TauE/SafE (pfam01925)
0600	2.88	0250	-5.82	Conserved hypothetical protein
0601	3.51	0249	3.86	PtxB, putative (http://microbesonline.org/); ABC-type phosphate/phosphonate transport system, periplasmic component (COG3221); outer membrane-associated homodimer (Walian et al., 2012)
0602	2.34	0248 (pseudo-gene)	1.43	Signal transduction histidine kinase (COG5002); PAS fold (IPR013767); ATPase-like, ATP-binding domain (IPR003594); HAMP linker domain (IPR003660); PAC motif (IPR001610)
0603	3.08	0247	9.14	Signal transduction response regulator, receiver domain (IPR001789); CheY-like superfamily (IPR011006); ntrX (http://microbesonline.org/)
0604	3.41	0246	2.18	Pyruvate phosphate dikinase, PEP/pyruvate-binding (IPR002192); PEP-utilizing enzyme, mobile domain (IPR008279); ATP-grasp fold, subdomain 1 (IPR013815); ATP-grasp fold, subdomain 2 (IPR013816)
0605	2.08	0245	-6.04	Protein-tyrosine/Dual-specificity phosphatase (IPR000387)

[a] $\log_2\left(\frac{\text{barcode microarray signal from cells after stress}}{\text{barcode microarray signal from cells prior to stress}}\right)$; fitness of stationary-phase G20 cultures grown for about 3.3 doublings (about 63 hours) in lactate-sulfate medium amended with 150 mM sodium nitrate

[b] $\log_2\left[\left(\frac{\#\text{ insertions in gene}}{\text{length of gene}}\right) / \left(\frac{\#\text{ insertions in }\mathbf{all}\text{ genes}}{\text{length of }\mathbf{all}\text{ genes}}\right)\right]$; fitness determined from mid-log phase DvH cultures grown for about 25.5 doublings (92 hours) in lactate-sulfate medium amended with 100 mM nitrate

Table 2.6. *D. alaskensis* G20 fitness profiling results in lactate-sulfate medium with top ten fitness scores in sodium nitrate compared with other amendments

		No Stress		150 mM Sodium Nitrate		150 mM Potassium Nitrate		150 mM Sodium Chloride		150 mM Potassium Chloride		0.25 mM Sodium Nitrite	
Gene (Dde)	Annotation	Mean Log_2 Ratio[a]	Z Score	**Mean Log_2 Ratio**	Z Score	**Mean Log_2 Ratio**	Z Score	Mean Log_2 Ratio	Z Score	Mean Log_2 Ratio	Z Score	Mean Log_2 Ratio	Z Score
2702	Rex, DNA-binding protein	-2.05	-3.07	**4.23**	3.33	**4.51**	3.42	-1.57	-3.63	-1.43	-3.88	-1.63	-3.05
0601	phosphate/ phosphonate transport system, putative	-0.04	-0.11	**3.51**	3.86	**3.53**	3.66	-0.10	-1.39	-0.05	-1.15	0.06	0.42
0604	PEP/pyruvate binding domain protein	-0.11	-1.08	**3.41**	3.80	**3.42**	3.80	0.01	0.20	0.07	0.81	-0.02	-0.04
0603	RR receiver domain-containing	0.12	0.41	**3.08**	2.84	**3.10**	3.12	0.06	0.95	0.03	0.37	0.08	0.46
0598	DUF	0.12	1.04	**3.01**	3.49	**3.03**	3.80	0.07	0.88	0.01	0.16	0.03	0.35
0600	CHyp	-0.11	-0.52	**2.88**	2.91	**2.87**	2.90	0.05	0.45	-0.01	-0.09	-0.17	-0.91
1268	Na+/proline symporter	-0.01	-0.04	**2.42**	2.71	**2.36**	2.67	0.14	1.05	0.05	0.65	0.24	1.24
0602	Sensory box sensor HK/RR, putative	0.21	0.92	**2.34**	1.91	**2.94**	2.66	0.07	0.88	0.08	1.03	0.03	0.29
0597	DUF	0.07	0.41	**2.25**	3.76	**2.38**	3.76	-0.03	-0.28	0.00	0.12	0.05	0.68
0605	Dual specificity phosphatase, catalytic domain	0.03	0.31	**2.08**	2.79	**2.23**	2.98	0.03	0.55	0.00	0.06	0.01	0.15

[a] Mean Log_2 Ratio = Gene Fitness Score = average of individual mutant fitness scores for mutations of that gene.

Individual mutant fitness score = $\log_2 \left(\frac{\text{barcode microarray signal from target mutant cells after stress}}{\text{barcode microarray signal from target mutant cells prior to stress}} \right)$. Signals used to calculate fitness scores were determined by microarray hybridization of barcode tags and normalized as described in the methods.

III.C. Fitness profiling with DvH

In order to test the hypothesis that both G20 and DvH used the same mechanisms for nitrate resistance, we had the opportunity to employ a different high-throughput fitness profiling method, Transposon Liquid Enrichment sequencing (TnLE-seq). This method (Fels et al., 2013) is based on deep sequencing of random transposon mutations to query DvH. TnLE-seq is a modification of the HITS (High-throughput Insertion Tracking by deep Sequencing) (Gawronski et al., 2009), Tn-seq (van Opijnen et al., 2009), and TraDIS (Transposon Directed Insertion-site Sequencing) (Langridge et al., 2009) methods. However, the TnLE-seq method developed for DvH is especially well-adapted to oxygen-sensitive bacteria that have low electroporation efficiency (Fels et al., 2013). The mutated culture is grown in control vs. stress conditions, and deep sequencing then determines the abundance and location of mutations at the end of growth. Because of the differences in methods, the calculation of fitness is also different from that of the mutant library experiment (Fels et al., 2013). The fitness value shown below is in $\log_2$R format, for easier comparison with the G20 pools:

$$\text{Fitness of a DvH gene} = \log_2 \left[\left(\frac{\#\text{ insertions in gene}}{\text{length of gene}} \right) / \left(\frac{\#\text{ insertions in } \mathbf{all} \text{ genes}}{\text{length of } \mathbf{all} \text{ genes}} \right) \right]$$

As previously described (Fels et al., 2013), fitness was calculated from insertions only in the 5-85% region of the coding sequence of genes, as such insertions are more likely to impair the function of gene products. As with the G20 pool described above, negative fitness indicates a fitness defect, whereas positive fitness indicates that the mutation confers a fitness advantage in that particular condition. For nitrate stress, the transposon mutants were grown in lactate-sulfate medium amended with 100 mM sodium

nitrate. As expected, the results showed that mutations in a predicted *rex* gene annotated as encoding a transcriptional regulator (DVU0916) as well as mutations in homologs of the G20 "nitrate cluster" (DVU0251, DVU0249, DVU0247, and DVU0246) conferred fitness values among the ten highest values (Tables 2.5 and 2.7). In fact, mutation of DVU0251 led to the highest fitness value, 11.44, or 2780-fold. Essentially, there was a "jackpot effect" in which a small percentage of mutants predominated in the population, a consistent result between the DvH and G20 fitness experiments. Despite these consistencies, in-depth, individual mutant analysis was necessary to confirm and elucidate the results of high-throughput fitness profiling (Deutschbauer et al., 2011).

Table 2.7. Selected results of fitness profiling of *D. vulgaris* Hildenborough--top ten fitness scores in sodium nitrate compared with MOLS4

Fitness	Gene annotation in *D. vulgaris*	fitness score[a]	fitness score
gene		MOLS4	MOLS4 + 100 mM NO_3
DVU0251	membrane protein, putative (TIGR)	2.05[b]	11.44
DVU0247	response regulator (TIGR), ntrX	1.23[b]	9.14
DVUA0023	ABC transporter, permease protein, putative (TIGR), atoC	0.18	6.10
DVU0249	conserved hypothetical protein (TIGR)	0.26	3.86
DVU0916	AT-rich DNA-binding protein (TIGR)	-2.86	3.81
DVU0123	membrane protein, putative (TIGR)	0.80	3.01
DVU2515	HD domain protein (TIGR)	-0.09	2.95
DVU0540	sensor histidine kinase (TIGR)	0.75	2.77
DVU1999	sulfate transporter family protein (TIGR)	-0.80	2.20
DVU0246	pyruvate phosphate dikinase, PEP/pyruvate binding domain protein (TIGR)	0.36	2.18

[a] $\text{Fitness score of a DvH gene} = \log_2 \left[\left(\frac{\#\text{ insertions in gene}}{\text{length of gene}}\right) / \left(\frac{\#\text{ insertions in } \mathbf{all} \text{ genes}}{\text{length of } \mathbf{all} \text{ genes}}\right)\right]$

[b] These values are artificially inflated because of "barcode bleed" (Kircher et al., 2012), an artifact of having both of these pools on the same Illumina™ HiSeq lane (Fels et al., 2013). A similar analysis of WT *D. vulgaris* in MOYLS4 (MOLS4 with 0.1% wt/vol yeast extract), unaffected by barcode bleed, resulted in fitness of 1.10 for DVU0251 and 1.75 for DVU0247 (Fels et al., 2013). As these genes are not predicted to be involved in amino acid biosynthesis, it is expected that their fitness values in MOLS4 should be similar to this original MOYLS4 fitness experiment.

III.D. Confirmation of fitness profiling with individual mutants

DvH was chosen for confirmation studies because a catalogued transposon mutant library of DvH was also available, in-frame deletion mutants can be made with greater facility (Keller et al., 2009), and complementation of mutants is readily accomplished. For an initial confirmation of the physiological relevance of the "nitrate cluster" to nitrate resistance, we determined growth kinetics of five DvH isolated mutants with transposon insertions in genes in that cluster (Fig. 2.4A-E). The control strain used had a transposon at an intergenic position 327 base pairs upstream of a gene encoding a "random"

hypothetical protein, DVU0590, and, therefore, was not predicted to be involved in nitrate stress responses. We found that the mutants with transposon insertions in DVU0246, DVU0247, DVU0250 and DVU0251 grew with indistinguishable kinetics with or without 100 mM nitrate. In contrast, 100 mM nitrate inhibited the control and the DVU0245 transposon mutants (Fig. 2.4A-F). Inhibition of the DVU0245 mutant in high nitrate is consistent with the low fitness of the DVU0245 mutant in nitrate (fitness -6.04, Table 2.5) and consistent with the inhibition of a deletion mutant of DVU0245 (data not shown). None of the mutants grew better than the control strain in 1 mM sodium nitrite (Fig. 2.4A-F). We interpret these results to mean that the growth advantage of these mutants is specific to nitrate and not simply an advantage in the presence of inhibitory nitrogen species.

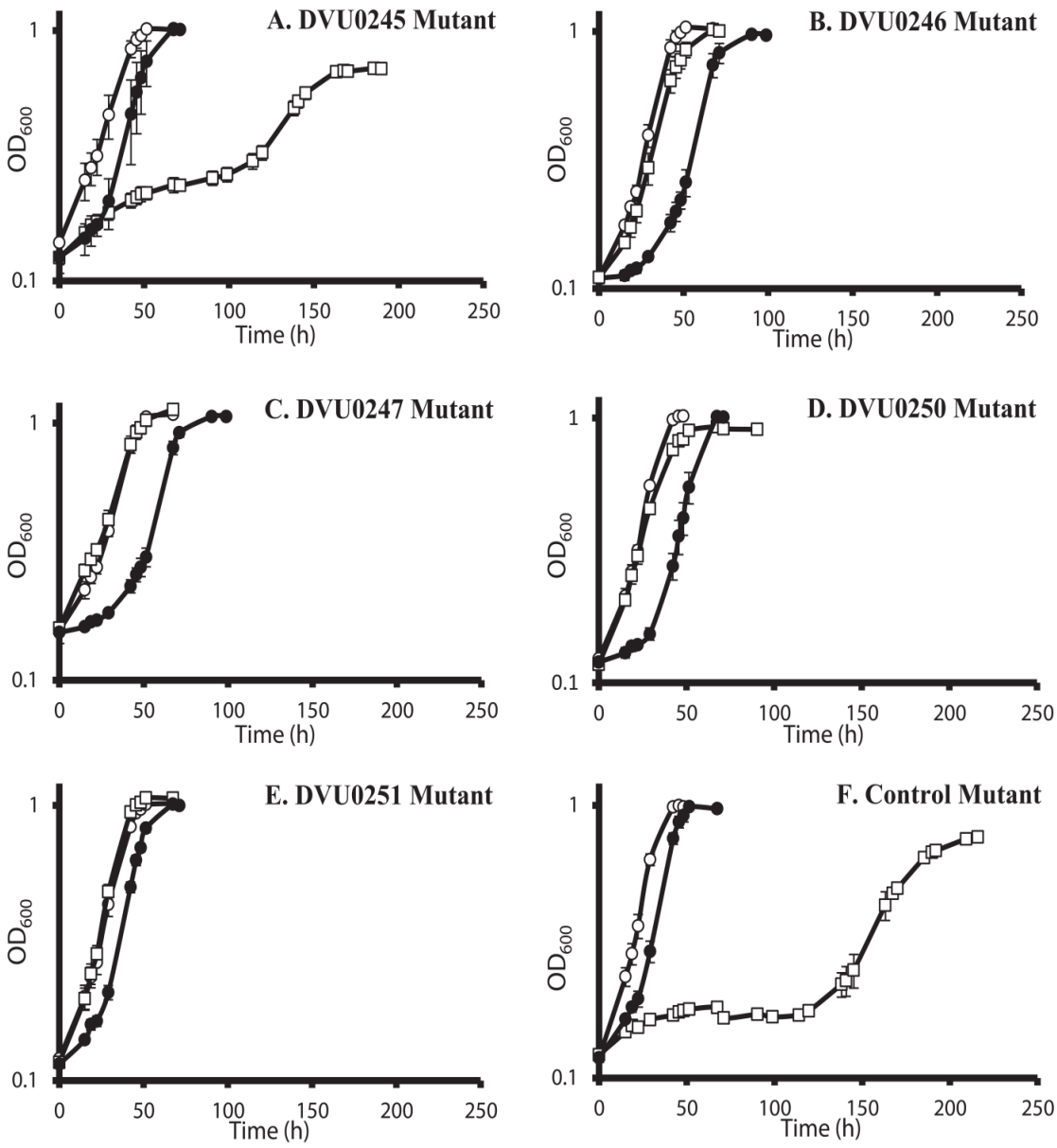

Figure 2.4 Growth of *D. vulgaris* Hildenborough transposon mutants in lactate-sulfate medium with inhibitory nitrogen species. Growth of the DVU0245 **(A)**, DVU0246 **(B)**, DVU0247 **(C)**, DVU0250 **(D)**, DVU0251 **(E)** and intergenic transposon control **(F)** mutants in the presence of no additions (○), 100 mM sodium nitrate (□), or 1 mM sodium nitrite (●). Approximately 5.3% (vol/vol) inocula were used. Optical density readings reflect averages of three samples, and errors bars show standard deviations.

The results of the G20 and DvH fitness studies indicated similar responses of homologous genes. In the G20 results, the predicted *rex* mutant had the highest fitness (Dde_2702). The fitness score for the DvH *rex* mutant was also in the top ten fitness scores, along with four DvH "nitrate cluster" (DVU0251, DVU0247, DVU0249, DVU0246) mutants (Tables 2.5 and 2.7). However, both the DVU0245 and the DVU0250 mutants had low fitness in nitrate, whereas mutants of their G20 homologs had high fitness in nitrate (Table 2.5). The nitrate resistance of the pooled versus the isolated DvH mutants was also not entirely consistent. The rapid growth of the isolated DVU0250 mutant (Fig. 2.4D) in the presence of nitrate was unexpected because of the low TnLE-seq fitness conferred in the same condition by mutation of DVU0250 (Table 2.5). In addition, preliminary growth kinetic data (not shown) suggest that the transposon mutant of DVU0248, which is annotated as a pseudogene in DvH, has little or no growth advantage over the control strain in the presence of 100 mM nitrate. In contrast, in the pooled experiment the DVU0248 mutations conferred positive fitness in nitrate (Table 2.5). While more data will be needed either to confirm or to change the annotation of DVU0248 as a pseudogene, we suggest that similar nitrate-resistance mechanisms are operating in G20 and DvH. The discrepancies between pooled and individual mutant studies confirm the need for follow-up studies of high-throughput experiments.

Such follow-up was pursued with gene deletion and complementation of the DvH gene encoding the predicted transcription regulator Rex. Interruption of the *rex* gene conferred the highest fitness in G20 but not in DvH (Table 2.5). We found that a deletion of DvH *rex* (DVU0916) had a clear advantage over the JW710 parent strain in lactate-

sulfate medium with 100 mM added nitrate (Fig. 2.5A and B). Like the "nitrate cluster" transposon mutants described above (Fig. 2.4A-F), the *rex* mutant is not demonstrably resistant to nitrite (Fig. 2.5A and B). Interestingly, when the mutant was complemented with *rex* expressed from a constitutive promoter, the phenotype in the presence of 100 mM nitrate was different from either parent or mutant phenotypes (Fig. 2.5C). In contrast, the parent strain with *rex* overexpressed appeared to be at least as sensitive to nitrate as the parent strain (Fig. 2.5C). The unique phenotype of the complemented mutant may result from some of the bacterial population losing the plasmid containing the complemented *rex* gene, in spite of antibiotic selection. While spectinomycin selects for plasmid retention, nitrate should select for plasmid loss in a Δ*rex* strain grown in high nitrate. Cells containing the plasmid may produce enough of the antibiotic-modifying enzyme to confer sufficient resistance to allow other cells to survive without containing the plasmid. If this is the case, then the Δ*rex* cells containing the plasmid should grow slowly while those which have lost the plasmid should grow more rapidly in the presence of 100 mM nitrate. The result would be a population growth rate in-between that seen for wild-type vs. Δ*rex* strains. Indeed, the phenotype of the complemented Δ*rex* strain exhibits this growth property (Fig. 2.5C). Finally, nitrate concentrations in the cultures with empty vector or *rex* complementing plasmids (Fig. 2.5C and D) were measured colorimetrically at the end of growth. As with the wild-type cultures described above, gross consumption of nitrate was not detected for any of these strains (data not shown). This is evidence of genuine nitrate resistance in these strains. Taken together, these growth and gene fitness data support transcriptomic predictions that nitrate stress responses involve mechanisms independent of nitrite stress responses (He et al., 2010a).

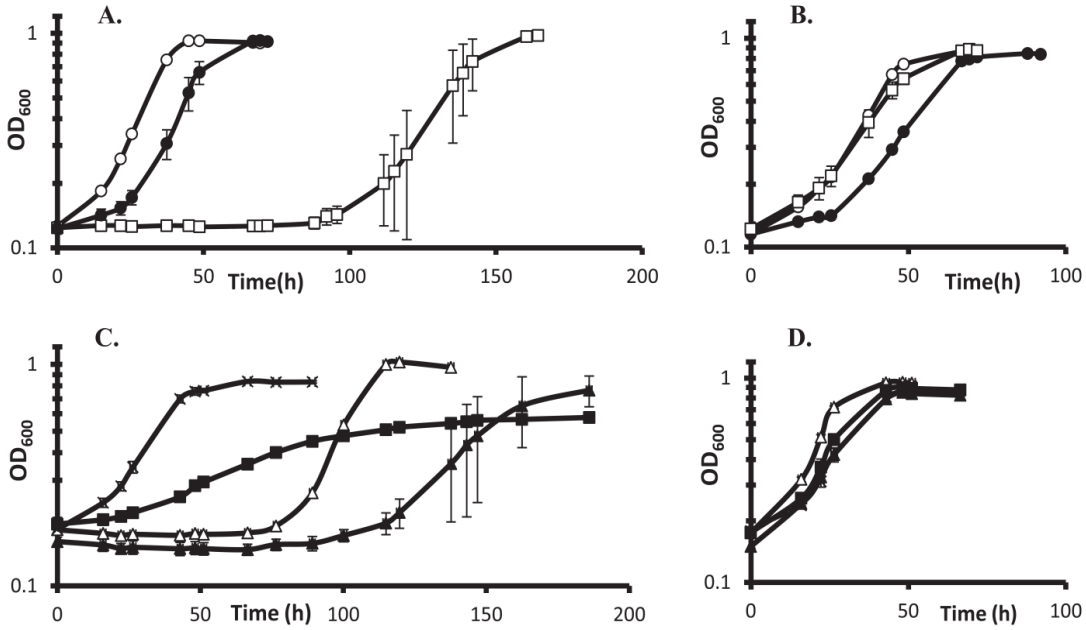

Figure 2.5 Growth of DvH wild-type vs. *Δrex* mutant in lactate-sulfate medium with inhibitory nitrogen species. (A) and **(B)**: Growth of the "wild-type" parental strain JW710 **(A)** vs. *Δrex* **(B)** mutant in the presence of no additions (○), 100 mM sodium nitrate (□), or 1 mM sodium nitrite (●). Approximately 6% (vol/vol) inocula were used. **(C)** and **(D)** show four strains grown with 100 mM nitrate **(C)** or no additions **(D)**. Wild-type with empty vector [JW710(pMO9075)],(△); wild-type with *rex* overexpression plasmid [JW710(pMO3313)], (▲); *Δrex* strain with empty vector [JW3311(pMO9075)], (x); *Δrex* strain with *rex* complement plasmid [JW3311(pMO3313)], (■). Approximately 7.5% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.

IV. Discussion

Whereas previous reports (Bender et al., 2007; Elias et al., 2009) have revealed mutations that led to increased sensitivity to both nitrate and nitrite, here we report the unexpected discovery of DvH mutants with increased resistance to nitrate but not nitrite. The data presented confirm that the *rex* deletion and the "nitrate cluster" transposon mutants, the top candidates from fitness profiling, confer resistance to nitrate in DvH. Such resistance also developed in the non-mutagenized DvH parental strain after subculture from 100 mM nitrate (Fig. 2.1B), likely as a result of the outgrowth of preexisting spontaneous nitrate-resistant mutants. The lack of nitrate metabolism of DvH is consistent with a report that 10 mM nitrate did not noticeably inhibit DvH (Haveman et al., 2004). Furthermore, DvH has been shown to reduce nitrite (Haveman et al., 2004), but not nitrate.

The ability of mutations in a subset of non-essential genes to confer nitrate resistance may in part account for the recently reported fluctuating sulfide levels produced by sulfate-reducing bacteria in a bioreactor inoculated with oil from a Canadian oil field (Callbeck et al., 2013). In this bioreactor, sulfide production was completely inhibited during pulses of 100 mM nitrate. However, after each pulse, sulfide production resumed, indicating that some sulfate-reducing bacteria persisted in the presence of the nitrate (Callbeck et al., 2013). The results of the work presented here suggest that persistence of SRB in nitrate-treated oil reservoirs may be the result of mutant resistance. Even if total oil-well nitrate concentrations reach low millimolar levels, the initial concentration of nitrate near the injection site will be much higher than this. For example, the peak nitrate concentration in one study of pulsed nitrate injection was reported as 760 mM

(Voordouw et al., 2009). Resistance to nitrate in the presence of a mixed culture is consistent with preliminary fitness profiling data from G20 grown in coculture with the nitrate reducer *Pseudomonas stutzeri* RCH2 in the presence of 100 mM nitrate. Under these mixed-culture conditions, the "nitrate cluster" mutants and the *rex* mutant gained a fitness advantage (A. Deutschbauer, unpublished data) very similar to that observed in pools of G20 mutants alone in the presence of 150 mM nitrate (Table 2.5).

These fitness studies bring clarity to questions that neither transcriptomic nor proteomic data could answer. While "omics" studies can assist detection and monitoring of changes in the metabolism of bacteria in contaminated environments (Steinberg et al., 2008), they are not sufficient (Torres-García et al., 2009) for elucidating underlying inhibitory mechanisms. In fact, there are poor correlations between the expression of transcripts and the expression of proteins in DvH in response to nitrate stress (Redding et al., 2006; He et al., 2010a). It has been reported (He et al., 2010a) that there were 28 genes for which the mRNA and protein levels were both significantly changed in nitrate stress conditions. However, for 7 of these 28 genes, the mRNA was significantly downregulated while the protein was significantly upregulated (He et al., 2010a). Although this poor correlation may be a result of meaningful regulatory mechanisms (Lu et al., 2007), transcript abundance is difficult to interpret and does not always correlate well with gene fitness (Price et al., 2013). For example, there is an upward trend of expression of the "nitrate cluster" genes in both nitrate (He et al., 2010a) and nitrite (He et al., 2006) stress conditions (http://microbesonline.org/). Because mutation of the nitrate cluster genes confers a growth advantage in high nitrate, increased expression of these genes should be detrimental to growth of DvH in high nitrate. This is consistent

with the recent deduction (Price et al., 2013) that, counterintuitively, detrimental bacterial genes are often not downregulated. This apparent suboptimal regulation of the nitrate cluster genes in the presence of high concentrations of nitrate likely contributes to the explanation of why their interruption confers such a strong growth advantage.

The roles of the genes of the nitrate cluster in nitrate sensitivity are not immediately obvious from their annotation (Table 2.5). The native functions of the nitrate cluster genes are not likely involved with nitrate, since neither DvH (Seitz and Cypionka, 1986; Pereira et al., 2000) nor G20 (J. Wall, unpublished data) have been shown to use nitrate for energy conservation. One hypothesis we proposed was that they allow nonspecific nitrate transport by a leaky thiosulfate transporter. The G20 mutants in this cluster were mildly sick when grown with 10 mM thiosulfate as a terminal electron acceptor (A. Deutschbauer, unpublished data), which suggested a possible role for these genes in thiosulfate uptake. However, preliminary growth kinetic data indicated that the DVU0251 mutant can grow rapidly and abundantly in lactate/thiosulfate and pyruvate/thiosulfate (G. Christensen, unpublished data), suggesting that thiosulfate uptake is not compromised in this mutant. However, preliminary data also show that a Δ*rex* DvH strain grows more slowly than the parent strain with 30 mM thiosulfate as a terminal electron acceptor (Christensen et al., in preparation). Therefore, we suggest that mutation of the *rex* gene might relieve nitrate inhibition by barring entry of nitrate into the cell. Further study will be needed to explore the native functions of the nitrate cluster genes.

Several additional genes outside of the "nitrate cluster" appear to have high fitness values during nitrate stress, indicating that their absence may improve growth. Follow-up

studies with individual mutants will be necessary to confirm these predictions. The results from this study clearly indicate that DvH and G20 have common nitrate resistance mechanisms that should be considered in environmental modeling.

CHAPTER 3

INDEPENDENCE OF NITRATE AND NITRITE INHIBITION OF *Desulfovibrio vulgaris* HILDENBOROUGH AND USE OF NITRITE AS A SUBSTRATE FOR GROWTH

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Independence of nitrate and nitrite inhibition of *Desulfovibrio vulgaris* Hildenborough and use of nitrite as a substrate for growth

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Author contributions: H.L.K. and J.D.W. designed the experiments. H.L.K. constructed the new bacterial strains and plasmids and conducted and analyzed all growth kinetic studies. V.V.T. prepared the cells for enzymatic studies. A. S. conducted the enzymatic studies and analyzed the enzyme data. A.P.A., G.P.B., and J.D.W. supervised the project. H.L.K, G.P.B, and J.D.W. interpreted the data and wrote the manuscript.

I. Introduction

Much study has been conducted to determine molecular mechanisms of inhibition of sulfide production in the petroleum industry because the production of sulfide "sours" the oil (Ligthelm et al., 1991). Such souring leads to corrosion of pipes (Zhu et al., 2006; Voordouw et al., 2007), to "plugging" of oil reservoirs by metal sulfides, and to health hazards for personnel working in the petroleum industry (Voordouw et al., 2007). To prevent these problems, nitrate (Grigoryan et al., 2009; Voordouw et al., 2009; Callbeck et al., 2011) and nitrite (Kaster et al., 2007; Voordouw et al., 2007) have been used in oil wells and bioreactor models of oil wells to limit sulfide production by sulfate-reducing bacteria. However, sulfate reducers have the ability to survive inhibition by nitrate and nitrite (Callbeck et al., 2013). Survival in the presence of nitrate or nitrite may be especially beneficial in nitrate- and heavy metal-contaminated environmental sites (Green et al., 2012) where sulfate reducers may help to immobilize the heavy metals by changing their redox state (Lovley et al., 1993a; Lloyd et al., 1999; Chardin et al., 2003) or by precipitating them as insoluble sulfides (Jalali and Baldwin, 2000). Thus, studies have attempted to make the effects of nitrate (Redding et al., 2006; He et al., 2010a; Korte et al., 2014) and nitrite (Haveman et al., 2004; He et al., 2006) on sulfate reducers more predictable. Previous studies have proposed that nitrate inhibition of the model sulfate reducer *Desulfovibrio vulgaris* Hildenborough (DvH) is mediated by the production of small amounts of nitrite from nonspecific reduction of nitrate (He et al., 2010a). In fact, nitrate and nitrite are sometimes considered to be mechanistically interchangeable in their effects on sulfate-reducing bacteria (Gieg et al., 2011). However, the evidence for the conversion of nitrate to nitrite in monocultures of *D. vulgaris* is unclear. Annotation of

the DvH genome indicates a lack of a functional nitrate reductase (http://www.microbesonline.org/). Transcript analyses of DvH stressed with sodium nitrate (He et al., 2010a) or sodium nitrite (He et al., 2006) indicated few similarities between the stress responses (He et al., 2010a). Furthermore, we have recently identified a cluster of genes that, when mutated, conferred resistance to nitrate but not nitrite (Carlson et al., 2014; Korte et al., 2014). Together these studies strongly suggest that nitrate inhibition of monocultures of DvH may be entirely independent of nitrite production. To explore this question, we analyzed growth characteristics of a nitrite reductase (NrfA) mutant of DvH. In addition, we tested the use of subinhibitory levels of nitrite or nitrate as either a nitrogen source or terminal electron acceptor by DvH. Here we provide evidence that nitrate inhibition of pure cultures of DvH can be independent of the production of nitrite. We further show that nitrite can be used by DvH as either a nitrogen source or as an electron acceptor. The clarification of these interactions of DvH with oxidized nitrogen species will allow for more accurate predictions of the role sulfate-reducing bacteria in environmental settings.

II. Materials and Methods

II.A. Strains and media

Strains and plasmids used in this study are listed in Table 3.1. We used the DvH parental strain JW710 that is deleted for *upp* for making marker exchange and markerless deletion strains (Keller et al., 2009). Therefore, JW710 will be referred to as "wild-type" DvH in this chapter. Unless otherwise specified, DvH strains were grown in MO Basal Salts (Zane et al., 2010) plus 60 mM sodium lactate, 30 mM sodium sulfate and, as reductant, 1.2 mM sodium thioglycolate (MOLS4 medium). MOLS4 medium

supplemented with 0.1% (wt/vol) yeast extract is referred to as MOYLS4. Cultures were started in an anaerobic growth chamber (Coy Laboratory Products, Inc., Grass Lake, MI) at about 25ºC with an atmosphere of approximately 95% N_2 and 5% H_2.

II.B. Growth kinetics

DvH cultures were started by inoculation of 5 mL MOLS4 (or 4 mL, for the strains with plasmids) with pelleted cells from 1-2 mL freezer stocks. These are referred to as "initial cultures" in this study. These stocks contained cells either in late exponential phase of growth or in stationary phase and frozen in growth medium plus approximately 10% (vol/vol) glycerol. To all plasmid-containing cultures, spectinomycin dihydrochloride pentahydrate (100 µg/mL) was added. Cultures for growth kinetics were set up as 5-mL triplicates in 27-mL anaerobic Balch tubes. Tubes were sealed with butyl rubber stoppers and transferred to a 34ºC incubator for growth. A Genesys 20 spectrophotometer (Thermo Scientific, Walthman, MA) was used to determine optical densities (600 nm). It is important to note that nitrite sensitivity of DvH, as previously indicated (Haveman et al., 2004), is highly dependent upon cell concentration. This is the reason for the use of relatively high concentrations of DvH inocula in these studies. Additions to cultures were made from stocks (sodium nitrate, sodium nitrite, sodium sulfite, ammonium chloride) prepared in deionized water. Where indicated, tubes were degassed with argon to eliminate dinitrogen as a substrate for nitrogen fixation.

II.C. Plasmid and strain construction

Plasmids pMO4500, pMO4501, and pMO4505 were constructed and JW4500 marker-exchange deletion mutant was generated as previously described (Korte et al., 2014). Primers are listed in Table 3.1. Construction of the markerless deletion, JW4502,

was achieved as previously described (Korte et al., 2014) except that after an approximately 24-h recovery of cells transformed with pMO4501, 40 µg of 5-fluorouracil/mL was added to the plating medium to select for 5-FU^r caused by loss of the *upp* gene. For stable plasmid introduction, electroporation was used as previously described for introduction of deletion constructs (Korte et al., 2014) and cells recovered overnight after electroporation were plated on MOYLS4 containing spectinomycin (100 µg/mL). The plasmids pMO9075 or pMO4501 isolated from JW710 were used in electroporation of JW4502 (NrfA mutant), and the recovered cells were plated on MOYLS4 with increased sodium thioglycolate, ca. 1.8 mM.

II.D. Nitrate and nitrite determination

A scaled-down version of a previously described nitrite assay (American Public Health Association, 1992) was used. Briefly, standards were prepared in 5 mL deionized water. Culture samples were diluted 100-fold into a total volume of 5 mL deionized water. Color Reagent (200 µL of 8.5% [vol/vol] phosphoric acid, 1% [wt/vol] sulfanilamide, 0.1% [wt/vol] N-(1-naphthyl)-ethylenediamine dihydrochloride) was added to each 5-mL diluted sample. Samples were mixed thoroughly and incubated 10 min at room temperature. Absorbance was read at 543 nm with a Genesys 20 spectrophotometer. The R^2 value for standard curves was >0.96 (Fig. 3.1), and the instrument detection limit was 0.15 ± 0.05 µM. Nitrate determination was as described previously (Korte et al., 2014).

II.E. Protein determination

Whole cell protein concentrations were determined with the Bradford assay (Bradford, 1976) with bovine serum albumin as the standard. Absorbance at 595 nm was measured with a Genesys 20 spectrophotometer. Final optical densities for the growth

kinetics studies shown in Figures 3.5-3.7 were confirmed with final whole cell protein measurements, and these values were well-correlated (Fig. 3.2).

II.F. Enzymatic studies

Nitrite-dependent oxidation of the chemically reduced radical cation methyl viologen ($MV^{+\cdot}$) was used as a marker for nitrite reductase (NrfA) activity. To prepare cell-free extract for activity assays, *D. vulgaris* strains JW710 and JW4502, containing *nrfA* expression plasmid (pMO4501) or empty vector (pMO9075), were grown in MOYLS4 liquid medium [supplemented with 0.2% (wt/vol) yeast extract instead of 0.1% (wt/vol)] except that prior to inoculation the medium was reduced with Na_2S at a final concentration of 1 mM. Cells were routinely cultured in the presence of spectinomycin (100 µg/mL) with a 10% (vol/vol) inoculum and harvested by centrifugation from 1-L early stationary phase cultures. Cell pellets were lysed at room temperature in an anaerobic chamber (Coy Laboratory Products, Inc.; atmosphere of approximately 96% N_2 and 4% H_2) via resuspension in 2 mL of B-PER (Thermo Scientific) containing 200 µL of 10X protease inhibitor (Pierce Protease Inhibitor tablets, Product #8825) and 1 µL of Benzonase nuclease (Sigma) and 2 µL of lysozyme (Sigma, 50 mg/mL) for 15 min. Cell lysate was cleared by centrifugation for 15 min at 8,000 x *g*. Specific activity of NrfA was assayed in cell-free extract by monitoring the decrease in absorbance at 578 nm of reduced $MV^{+\cdot}$ used as electron source for the enzyme during turnover. Nitrite reductase activity was assayed essentially as reported previously, with minor modifications(Wolfe et al., 1994). In brief, $MV^{+\cdot}$ stock was prepared in an anaerobic chamber by zinc reduction of MV^{2+} followed by filtration to remove the metal (Wolfe et al., 1994). The assay was continuously monitored using a temperature-

controlled (set to 30°C) HP diode array spectrophotometer (Agilent Technologies) inside an anaerobic chamber. All reagents were prepared with anoxic buffers and 3-ml open-top cuvettes were used for assays. A standard nitrite reductase assay contained 2 mL activity assay buffer (50 mM Tris [pH 8.0], 0.1 M NaCl), to which 12.5 mM reduced $MV^{+\cdot}$ was added to give a starting OD_{578} of ~2 OD units. Sodium diethyldithiocarbamate (10µM) was then added to the assay mixture to inhibit the non-enzymatic reduction of nitrite by $MV^{+\cdot}$. OD readings were then allowed to stabilize for 30 seconds. A small volume of the cell-free extract to be assayed (2-10µL) was added to the cuvette and any changes in OD_{578} monitored for 30 seconds. No baseline oxidation of $MV^{+\cdot}$ was observed. The reaction was initiated by adding 12.5 mM of sodium nitrite. One unit of specific activity is defined as the amount of $MV^{+\cdot}$ oxidized (µmol) over time normalized to the amount of cell free protein extract used (µmol min^{-1} mg^{-1} of total protein; extinction coefficient, 9.8 mM^{-1} cm^{-1}). Specific activities were determined for three independent experiments. No oxidation of $MV^{+\cdot}$ was observed for control assays containing only sodium nitrite and no cell-free extract. Hydrogenases did not interfere by oxidizing reduced MV^{+} during the assay. Apparently the presence of hydrogen in the headspace of assay cuvettes ensured that hydrogen production would be inhibited.

Table 3.1 Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Source and/or reference
Escherichia coli		
α-Select (Silver Efficiency)	F$^-$ *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA-*arg*F)U169 Φ80*lacZ*ΔM15 λ$^-$	Bioline
Desulfovibrio vulgaris		
ATCC 29579	Wild-type *D. vulgaris* Hildenborough (pDV1); 5-FU^s	ATCC
JW710	WT (pDV1) Δ*upp*; 5-FU^r (Parent strain for markerless deletion mutants)	(Keller et al., 2009)
JW4500	JW710 Δ*nrfA*[b] ::(*npt upp*); Km^r, 5-FU^s	This study
JW4502	JW710 Δ*nrfA*; 5-FU^r	This study
Plasmids		
pCR®4-TOPO®	Cloning vector, Ap^r, Km^r, pUC *ori*.	Invitrogen Life Technologies
pCR®8/GW/ TOPO®	Cloning vector, Sp^r, pUC *ori*	Invitrogen Life Technologies
pMO719	pCR®8/GW/TOPO® containing SRB replicon (pBG1); Sp^r; source of Sp^r, pUC *ori* fragment for marker exchange and markerless deletion suicide plasmid construction	(Keller et al., 2009)
pMO746	*upp* in artificial operon with *npt* and linked to Ap^r-pUC *ori* from pCR®4-TOPO®, P_{npt}-*npt*-*upp*; Km^r; source of Kan^r, *upp* fragment for marker exchange and markerless deletion suicide plasmid construction	(Parks et al., 2013)
pMO9075	pMO719 containing P_{npt} for constitutive expression of complementation constructs; pBG1 stable SRB replicon; Sp^r	(Keller et al., 2011; Keller et al., 2014)
pMO4500	Sp^r and pUC *ori* from pMO719 plus upstream and downstream DNA regions from DVU0625 (*nrfA)* flanking the artificial operon of P_{npt}-*npt*-*upp* from pMO746; for marker exchange deletion mutagenesis; Sp^r and Km^r	This study
pMO4505	Sp^r and pUC *ori* from pMO719 plus upstream and downstream DNA regions from DVU0625 (*nrfA)*; for markerless deletion mutagenesis	This study
pMO4501	pMO9075 with DVU0625 (*nrfA*) constitutively expressed from P_{npt}	This study

[a]Km, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript "r" or "s", resistance or sensitivity
[b] *nrfA* is DVU0625 of *D. vulgaris* Hildenborough

Table 3.2 Primers used for PCR amplification, Southern probe generation and sequencing

Primer name	Primer sequence (5'-3')	Application
56HK-nrfA-up-47-F	GCCTTTTGCTGGCCTTTTGCTCACATGCGTGGCGACTATCTGTGCAA	For amplification of DVU0625 upstream region from gDNA with 57HK-nrfA-up-52-R primer to make pMO4500. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment [SpecRpUC-R(Korte et al., 2014)]. Amplification of Southern probe for confirmation of DVU0625 deletion. forward
57HK-nrfA-up-52-R	GCGACAAGATATTCGGCACCAAGTAAGTTATTCATCGGCGACCTCTCTCGTG	For amplification of DVU0625 upstream from gDNA with 56HK-nrfA-up-47-F primer to make pMO4500. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment [UppCterm(Korte et al., 2014)]. Amplification of Southern probe for confirmation of DVU0625 deletion. reverse
58HK-nrfA-dn-46-F	GCGCCCCAGCTGGCAATTCCGGTTCCCGCTCTTTCGCAAAGGTATG	For amplification of DVU0625 downstream from gDNA with 59HK-nrfA-dn-46-R to make pMO4500. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment [KanPromNterm(Korte et al., 2014)]. forward
59HK-nrfA-dn-46-R	GTCGAGGCATTTCTGTCCTGGCTGGCTTGCAGTACGCTCATGGGCT	For amplification of DVU0625 downstream region from gDNA with 58HK-nrfA-dn-46-F primer to make pMO4500. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment [SpecRpUC-F(Korte et al., 2014)]. reverse
60HK-4500-4-upstrm-23-F	CGCACAATCTGTTGGCAAAGCTA	Sequencing primer to confirm upstream region of deletion cassette of pMO4500.
61HK-4500-4-dnstrm-19-R	CAACGTTCGACGGTCGCAA	Sequencing primer to confirm downstream region of deletion cassette of pMO4500.
62HK-4500-4-upstrm-22-R	CCCATGAACTGGACATGGCAGA	Sequencing primer to confirm upstream region of deletion cassette of pMO4500.
63HK-4500-4-dnstrm-	ATGCAGGTGTGCGAGGTGTT	Sequencing primer to confirm downstream region of deletion cassette of pMO4500.

20-F		
66HK-nrfA-SLIC-69-F	AGGTTGGGAAGCCCTGCAATGCAGTCCCAGGAGGTACCATATGAATAACCAGAAGACGTTCAAGGGGTT	For amplification of DVU0625 to make pMO4501 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. forward
67HK-nrfA-SLIC-51-R	GATCGTGATCCCCTGCGCCATCAGATCCTTGCTACTGCTTGGCGGAGACCA	For amplification of DVU0625 to make pMO4501 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. reverse
72HK-pMO4501-2871-R	GATACATGTCGGCAGGGTCGAAA	Sequencing primer to confirm pMO4501 complementation construct. reverse
73HK-pMO4501-2848-F	GTTTCGACCCTGCCGACATGTAT	Sequence primer to confirm pMO4501 complementation construct. forward
90HK-nrfA-MLD-upR-49	CATACCTTTGCGAAAGAGCGGGAATTATTCATCGGCGACCTCTCTCGTG	For amplification of DVU0625 upstream region from gDNA with 56HK-nrfA-up-47-F primer to make pMO4505. Underlined portion used as overhang for SLIC with DVU0625 downstream region. reverse
91HK-nrfA-MLD-dnF-49	CACGAGAGAGGTCGCCGATGAATAATTCCCGCTCTTTCGCAAAGGTATG	For amplification of DVU0625 downstream from gDNA with 59HK-nrfA-dn-46-R to make pMO4505. Underlined portion used as overhang for SLIC with DVU0625 downstream region. forward
Other primers for making and confirming these and similar plasmids have been previously described (Korte et al., 2014). These include SpecRpUC-R, KanPromNterm, UppCTerm, SpecRpUC-F, SpecRpUC-up, pMO719XbaI-Dn, Kan-int-Fwd-rev-comp, DvH-Upp gene Cterm-out, pBG1-2199-F, pMO9075-SLIC-F, and pMO9075-SLIC-R3		

Figure 3.1. Standard curve for nitrite assay. Absorbance readings show averages of three replicates and error bars show standard deviations.

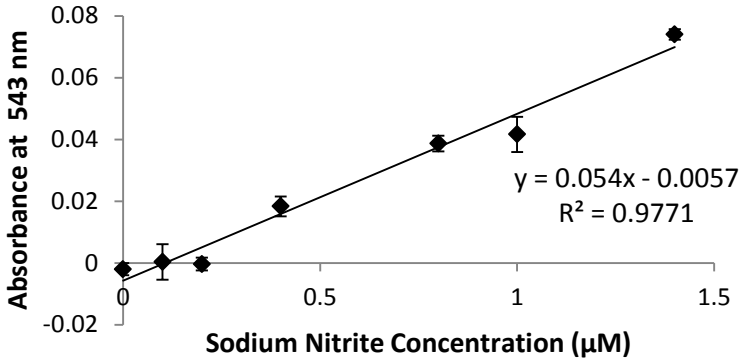
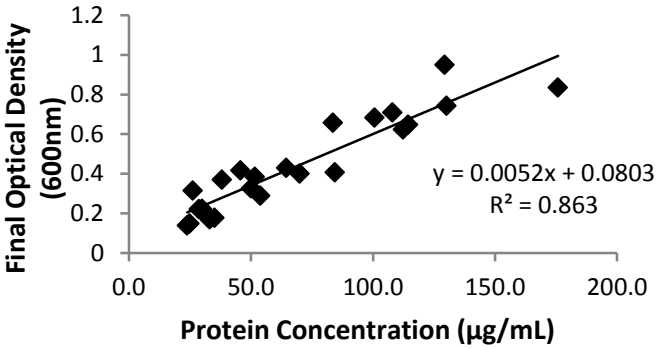


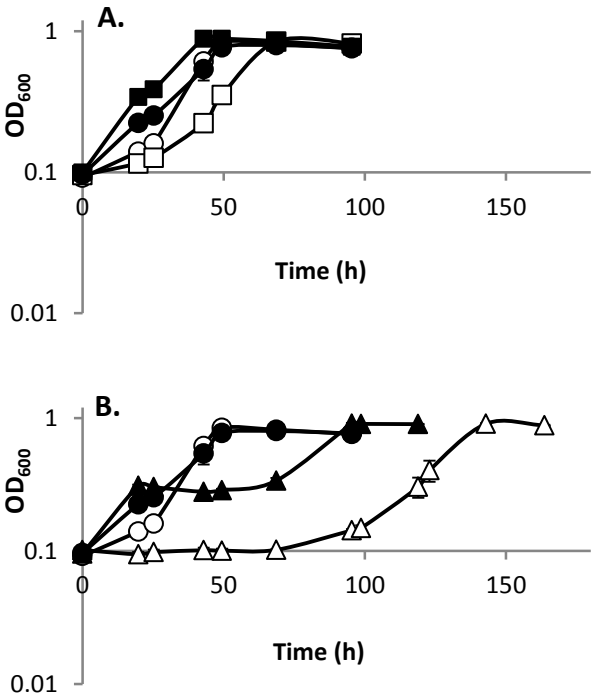
Figure 3.2. Final Optical Density of *D. vulgaris* Hildenborough cultures correlated with final whole cell proteins. Final average optical densities of cultures shown in Fig. 3.5-3.7 are plotted against the average final whole cell protein measurements for these same samples.



III. Results

Since *in vitro* studies have reported that nitrite can bind to the dissimilatory sulfite reductase of DvH (Wolfe et al., 1994), we first sought to investigate whether nitrite competitively inhibits the sulfite reductase *in vivo*. If so, we predicted that sulfite would relieve nitrite inhibition by outcompeting nitrite for the enzyme. If nitrate inhibition were mediated by nitrite production, nitrate inhibition would also be relieved by sulfite. Growth kinetics were determined for wild-type DvH inhibited by either 1 mM sodium nitrite or 100 mM sodium nitrate in lactate-sulfate medium. Addition of 5 mM sulfite to DvH completely relieved inhibition by 1 mM nitrite (Fig. 3.3A) and only partially relieved inhibition by 100 mM nitrate (Fig. 3.3B). Furthermore, thiosulfate addition was similar to sulfite addition in its effect on nitrite or nitrate inhibition of DvH growth (data not shown), since thiosulfate is reduced to sulfite before further reduction (Zane et al., 2010). Stocks of 100 mM nitrate were assayed for nitrite content and found to contain less than 1 μM, a concentration not inhibitory to these bacteria (data not shown). Any inhibition caused by nitrite would therefore have required its production from the 100 mM nitrate by the bacteria. Although we inferred a possible connection between nitrate and nitrite inhibition through these results, we noted that low concentrations of sulfite, an electron acceptor that does not require activation by ATP (Postgate, 1984), stimulates growth of DvH (Fig. 3.3). Therefore, we reasoned that sulfite might relieve nitrate and nitrite inhibition by the general effects of sulfite stimulation and not by outcompeting nitrite for the sulfite reductase. Thus, whether nitrate was reduced to nitrite, which then acted as the ultimate inhibitor of DvH growth, remained inconclusive.

Figure 3.3 Growth of wild-type *D. vulgaris* Hildenborough (JW710) in lactate-sulfate medium with (A) nitrite or (B) nitrate plus sulfite. (A) Growth of DvH with no additions (○), 5 mM sulfite (●), 1 mM nitrite (□), or 5 mM sulfite plus 1 mM nitrite (■). **(B)** Growth of DvH with 100 mM nitrate (△), or 5 mM sulfite plus 100 mM nitrate (▲). Curves of no additions (○) and 5 mM sulfite (●) are redrawn for comparison. Approximately 4.7% (vol/vol) inocula were used. Optical density readings show averages of three samples, and error bars show standard deviations (often within symbols).



To explore the inhibitory mechanisms of nitrate and nitrite further, a markerless, in-frame deletion of the gene encoding NrfA, the catalytic subunit of the periplasmic nitrite reductase, NrfHA, was constructed. The NrfHA enzyme is known to provide DvH protection against inhibition by nitrite (Greene et al., 2003; Haveman et al., 2004). We predicted that if nitrite were produced when DvH was exposed to 100 mM nitrate, then a NrfA mutant should be more sensitive to both nitrite and nitrate than the parental strain. Growth of this NrfA mutant was compared to that of the wild-type in lactate-sulfate medium (Fig. 3.4A) amended with 1 mM nitrite (Fig. 3.4B) or 100 mM nitrate (Fig. 3.4C). The mutant was also complemented with a constitutively expressed copy of the *nrfA* gene to confirm the absence of polar effects in the deletion mutant.

Figure 3.4 Growth of *D. vulgaris* Hildenborough wild-type vs. Δ*nrfA* mutant in lactate-sulfate medium with inhibitory nitrogen species. **(A)** no additions, **(B)** 1 mM sodium nitrite, or **(C)** 100 mM sodium nitrate. Growth of the parental strain with empty vector [JW710(pMO9075)](○), parental strain with *nrfA* overexpression plasmid [JW710(pMO4501)](△), *ΔnrfA* mutant with empty vector [JW4502(pMO9075)](□) and *ΔnrfA* mutant with *nrfA* complement plasmid [JW4502(pMO4501)](●). A 28% (vol/vol) subculture from the initial 4 mL culture was made and a 9% (vol/vol) inoculum from this subculture was used for growth kinetic studies. Optical density readings show averages of three samples, and error bars show standard deviations (often within symbols).

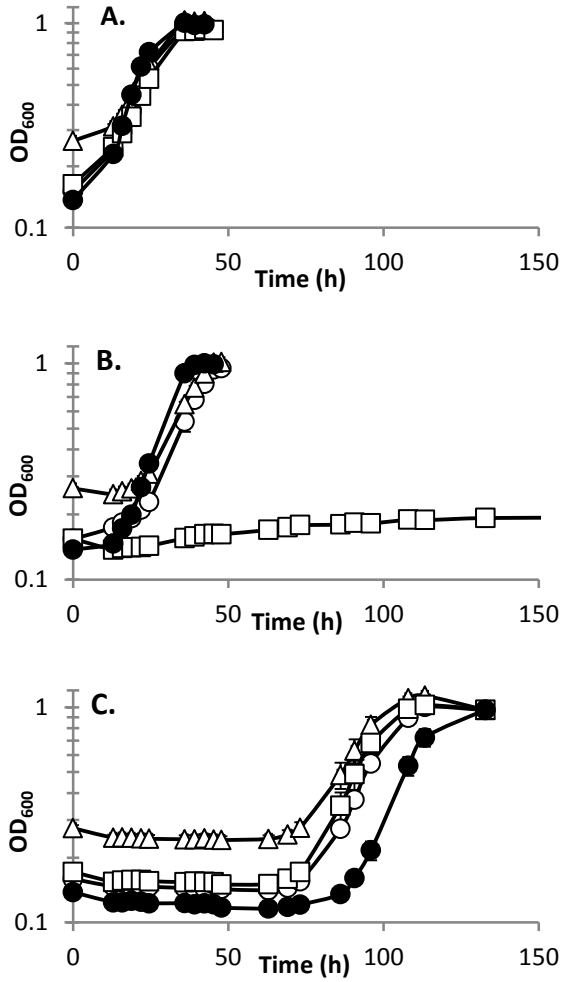


Table 3.3. Specific activity of nitrite reductase (NrfHA) in strains of *D. vulgaris* Hildenborough

Strain	**Specific Activity**[a]
JW710(pMO9075); Wild-type + empty vector	1.58 ± 0.07
JW710(pMO4501); Wild-type + *nrfA* complement	4.70 ± 0.49
JW4502(pMO9075); JW710 Δ*nrfA* + empty vector	0.13 ± 0.00
JW4502(pMO4501); JW710 Δ*nrfA* + *nrfA* complement	4.74 ± 0.75

[a] Specific activity is reported in: μmoles $MV^{+\cdot}$ oxidized min^{-1} mg of total $protein^{-1}$. Activities were determined from three independent measurements with standard deviations shown.

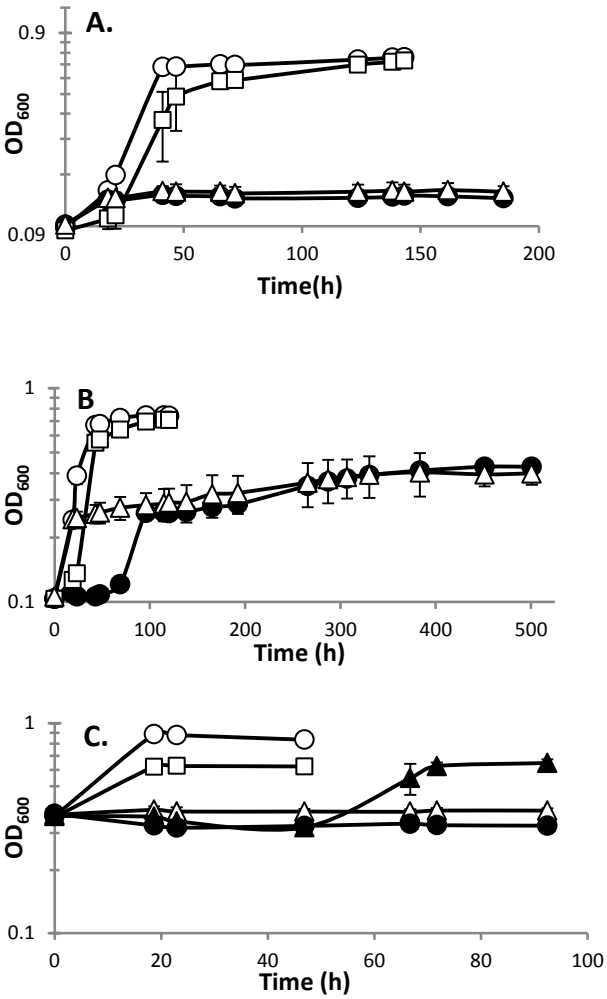
While the NrfA mutant with the empty vector was strongly inhibited in the presence of 1mM nitrite, the complemented mutant strain grew like the parental strain under these conditions (Fig. 3.4B). The successful complementation of the NrfA mutant, confirmed by enzyme assays (Table 3.3), is evidence that the P_{npt} promoter used for constitutive expression of *nrfA* provides robust expression of the complemented gene and that deletion of *nrfA* did not disrupt the function of the *nrfH* gene. In contrast to nitrite effects, the parental strain, deletion and complement grew similarly in the presence of 100 mM nitrate (Fig. 3.4C), showing no effect of NrfA on the nitrate inhibition. It was expected that if nitrite were produced from the 100 mM nitrate, it might be present in the NrfA mutant during the lag/inhibition phase because the mutant had a decreased ability to reduce nitrite compared with the parental strain. The nitrite concentration was therefore measured in wild type (empty vector) and NrfA mutant (empty vector) cultures grown 70 h in the presence of 100 mM nitrate and was less than 15 ± 5 μM (the limit of our detection for diluted cultures) in both sets of cultures. A preliminary report of these nitrite measurements was previously made and was consistent with the absence of

measurable nitrate consumption by DvH cultures grown in the presence of 100 mM nitrate (Korte et al., 2014). Therefore, one interpretation of these results could be that nitrate inhibition is not mediated by the production of nitrite.

However, we considered the possibility that the nitrite accumulation from nitrate could be sufficiently low that its effect might be the same on the parent and mutant strains. It was previously reported that single colonies of a NrfA mutant of a DvH derivative had the same nitrite sensitivity as the parent strain, which was inhibited by concentrations above 40 µM nitrite (Haveman et al., 2004). We reasoned that if nitrate-inhibited cells produced only micromolar concentrations of nitrite, then the NrfA mutant might not be expected to have increased sensitivity to nitrate compared with the parent strain. However, such concentrations of nitrite might be sufficient and therefore detectable for other metabolic roles such as a nitrogen source or terminal electron acceptor.

Any conversion of nitrate to assimilable nitrogen in the form of ammonium requires intermediate production of nitrite. Given that the NrfHA enzyme is known to be able to convert nitrite to ammonium (Pereira et al., 2000), it follows that DvH should be able to use subinhibitory concentrations of nitrite as a nitrogen source.

Figure 3.5 Growth of wild-type *D. vulgaris* Hildenborough with various nitrogen sources in lactate-sulfate lacking ammonium. (A) Low nitrate concentration, **(B)** high nitrate concentration and **(C)** high nitrate with nitrite. **(A)** Nitrogen additions were 1.5 mM NH_4Cl (○), 1.5 mM $NaNO_3$ (●), 1.5 mM $NaNO_2$ (□), or no additions (△). A 6.4% (vol/vol) subculture from the initial 5 mL culture was made into NH_4-free medium and a 6.4% (vol/vol) inoculum from this subculture was used for growth kinetic studies. **(B)** Nitrogen additions were 1.5 mM NH_4Cl (○), 100 mM $NaNO_3$ (●), 1.5 mM $NaNO_2$ (□), or no additions (△). A 33% (vol/vol) subculture from the initial 5 mL culture was made into NH_4-free medium and a 6.4% (vol/vol) inoculum from this subculture was used for growth kinetic studies. **(C)** Nitrogen additions were 5 mM NH_4Cl (○), 100 mM $NaNO_3$ (●), 1 mM $NaNO_2$ (□), 100 mM $NaNO_3$ plus 1 mM $NaNO_2$ (▲), or no additions (△). A 13% (vol/vol) subculture from the initial 5 mL culture was made into NH_4-free medium and a 28% (vol/vol) inoculum from this subculture was used for growth kinetic studies. Optical density readings show averages of three or more samples and error bars show standard deviations (often within symbols). All tubes were degassed with argon.



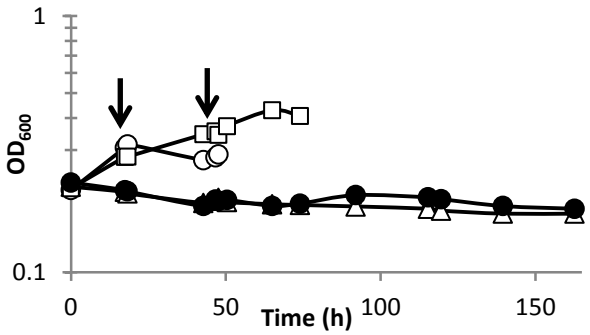
Further, we predicted that if small amounts of nitrite were produced when DvH is exposed to 100 mM nitrate, then nitrate could also be used by DvH as a sole nitrogen source. Ammonium is an energetically favorable nitrogen source for DvH, but strains containing the native plasmid pDV1 can also fix dinitrogen gas if sufficient energy is available (Heidelberg et al., 2004). Thus, nitrogen assimilation was tested in cultures with an argon headspace, to avoid the possibility of confounding the results by nitrogen fixation. Nitrogen assimilation was first tested in nitrogen-starved cells with additions of 1.5 mM sodium nitrate, 1.5 mM sodium nitrite, 1.5 mM ammonium chloride, or no added

nitrogen. While both ammonium and nitrite served as excellent nitrogen sources, cultures with nitrate added did not grow any more than the negative control (Fig. 3.5A). These results confirmed that 1.5 mM nitrite, but not 1.5 mM nitrate, could be used as a nitrogen source under these conditions. In addition, DvH was also unable to use 100 mM nitrate as a nitrogen source (Fig. 3.5B). This is consistent with the lack of a measurable loss of nitrate in a culture of DvH incubated for 500 h in the presence of 100 mM nitrate as sole nitrogen source (data not shown). The characteristic delay in growth of the culture with 100 mM nitrate (Fig. 3.5B) was expected, as this concentration has been shown to drastically increase the lag phase of DvH cultures (Elias et al., 2009; He et al., 2010a; Korte et al., 2014). Given that any conversion of nitrate to ammonium requires intermediate production of nitrite, these data strongly suggest that no nitrite was produced by DvH in the presence of 1.5 mM or 100 mM nitrate under the conditions tested. However, we considered the possibility that high nitrate levels might inhibit the use of nitrite as a nitrogen source. Therefore, we also showed that 1 mM nitrite could be used as a nitrogen source even in the presence of 100 mM nitrate (Fig. 3.5C). Taken together, these data confirm that nitrate inhibition of DvH under these conditions is not mediated by the production of nitrite.

The successful use of subinhibitory levels of nitrite as a nitrogen source caused us to revisit the question of whether nitrite could be used by *D. vulgaris* as a terminal electron acceptor. When subinhibitory concentrations of nitrite were added incrementally to DvH provided lactate and no other electron acceptor, nitrite reduction supported growth with lactate (Fig. 3.6). In contrast, as expected, 100 mM nitrate was not used as an electron acceptor (Fig. 3.6). This result prompted us to consider what enzyme was

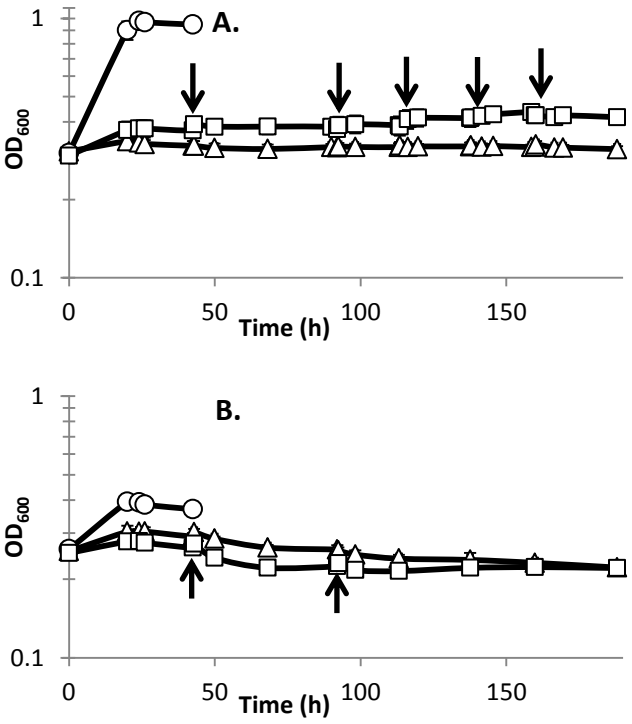
allowing this use of nitrite. Both the cytoplasmic sulfite reductase (Wolfe et al., 1994) and the periplasmic nitrite reductase (Pereira et al., 2000) have been shown to mediate nitrite reduction, but previous attempts to demonstrate the use of nitrite as a terminal electron acceptor were unsuccessful (Pereira et al., 2000). To determine which enzyme, if either, might provide energy conservation, we tested the mutant deleted for *nrfA* to determine whether subinhibitory concentrations of nitrite could serve as a nitrogen source or an electron acceptor.

Figure 3.6 Growth of wild-type *D. vulgaris* Hildenborough in lactate medium with sulfite, nitrite, or nitrate as sole electron acceptor. Initial additions were 3 mM $NaSO_3$ (○), 100 mM $NaNO_3$ (●), 3 mM $NaNO_2$ (□), or no additions (△). Where indicated by arrows, additions of approximately 3.7 mM $NaNO_2$ were made to 5 mL cultures that had started with 3 mM nitrite. To cultures with no additions or 100 mM nitrate, the same volume of deionized water was added at these times. No additions were made to cultures with 3 mM sulfite. A 33% (vol/vol) subculture from the initial 5 mL culture was made into lactate-sulfate medium and a 19% (vol/vol) inoculum from this subculture was used for growth kinetic studies. Optical density readings show averages of three samples, and error bars show standard deviations (often within symbols).



We found that while the NrfA mutant could use nitrite as a nitrogen source (Fig. 3.7A), it was unable to grow with nitrite as sole electron acceptor (Fig. 3.7B). The ability of the NrfA mutant to reduce very low concentrations of nitrite is consistent with the reported observations of nitrite reduction by a NrfA mutant in a DvH strain lacking the native plasmid (Haveman et al., 2004). The nitrite reduction capability is likely enabled by the sulfite reductase, DsrABD (Haveman et al., 2004). However, the inability of the NrfA mutant to use nitrite as a terminal electron acceptor may indicate that the NrfHA enzyme is responsible for energy conservation or that the increased sensitivity of the mutant to added nitrite prevented an observable nitrite-dependent growth.

Figure 3.7 Growth of Δ*nrfA* mutant with nitrite as sole nitrogen source or electron acceptor. **(A)** Nitrogen sources in NH_4-free lactate-sulfate medium were 5 mM NH_4Cl (○), 0.25 mM $NaNO_2$ (□), or no additions (△). **(B)** Potential electron acceptors in lactate medium lacking sulfate were 3 mM $NaSO_3$ (○), 0.25 mM $NaNO_2$ (□), or no additions (△). Where indicated by arrows, additions of approximately 0.2 mM $NaNO_2$ were made to 5 mL cultures initially containing nitrite. To cultures with no additions, the same volume of deionized water was added at these times. No additions were made to the cultures with either NH_4Cl or sulfite. An approximately 9% (vol/vol) subculture from the initial 5 mL culture was made into NH_4-free lactate-sulfate medium and a 21% inoculum from this culture was used for growth kinetic studies for (A) or (B). All tubes were degassed with argon. Optical density readings show averages of three or more replicates and error bars show standard deviations (often within symbols).



IV. Discussion

The results reported here clarify fundamental interactions of DvH with nitrate and nitrite. We confirmed that, consistent with prior inferences (Wall et al., 2007; He et al., 2010a), nitrate inhibition of pure cultures of DvH is not mediated by the production of nitrite under the conditions tested. This means that predictions of bacterial responses to nitrate and nitrite stress in the environment should consider these ions as separate inhibitors and not as a single entity. Indeed, our prior work suggested unique inhibitory mechanisms (Korte et al., 2014) for nitrate and nitrite. Separate inhibitory mechanisms were also exhibited by the model sulfate reducer *Desulfovibrio alaskensis* G20, which lacks an annotated nitrite reductase (http://microbesonline.org/)(Carlson et al., 2014; Korte et al., 2014). This bacterium recovered from growth inhibition within 50 h in the presence of 150 mM nitrate in lactate-sulfate medium (Korte et al., 2014). Evidence has been presented that was consistent with nitrate inhibiting the sulfate reduction pathway, but not necessarily the sulfite reductase (Carlson et al., 2014).

The ability of *D. alaskensis* to grow in the presence of high nitrate is, therefore, analogous to the nitrate resistance of the NrfA mutant of DvH presented in this work. The results presented here are surprising in light of previous reports that, in the presence of nitrate, the nitrite reductase *nrfA* gene of DvH is overexpressed (He et al., 2010a). Increased transcription of the *nrfA* gene in the presence of high nitrate contributed to the prior assumption that nitrate inhibition was mediated by nitrite production (He et al., 2010a). In the present study, nitrite was undetected by assays or by production of a nitrogen source used to support growth of DvH monocultures in the presence of 100 mM nitrate.

In a new isolate of *Desulfovibrio desulfuricans* capable of growth by nitrate ammonification, Dalsgaard and Bak (1994) reported that nitrate reduction was dramatically inhibited by the presence of quite low concentrations of sulfide. The presence of sulfide carried over from inoculating cultures in our experiments could potentially inhibit conversion of nitrate to nitrite by DvH. Additional studies would be needed to determine if DvH can convert nitrate to nitrite under sulfide-free conditions. However, the results presented here are relevant since bacteria that are known to produce nitrite from nitrate are often present in environments where sulfate-reducing bacteria are found (Greene et al., 2003; Haveman et al., 2005). DvH may respond to nitrate as a signal that nitrite may also be present and prepare DvH for detoxification of nitrite produced by nearby nitrate-reducing bacteria (Greene et al., 2003).

In addition, increased transcription of *nrfA* genes may enable use of nitrite as an environmental nitrogen source. Production of ammonium from nitrite could be especially helpful in an environment low in reduced nitrogen substrates. DvH may have specific response mechanisms to assist in this process, as the addition of 2.5 mM nitrite to mid-log-phase DvH cells has been reported to cause down-regulation of genes involved in amino acid transport and catabolism (He et al., 2006). In contrast, a gene encoding glutamine synthetase, which assimilates ammonium into amino acids (Merrick and Edwards, 1995), was induced by the addition of 2.5 mM nitrite (He et al., 2006). In light of the present study, these prior results indicate that DvH sensed an excess of ammonium availability and was able to slow down costly transport pathways in favor of nitrogen assimilation by glutamine synthetase (He et al., 2006). The reduction of nitrite by NrfA in *D. vulgaris* is particularly relevant to agricultural settings (Welsh et al., 2014) because

dissimilatory nitrite reduction to ammonium promotes nitrogen retention, rather than loss (as N_2 or N_2O), in soil.

Shown in this work is a previously unknown role for nitrite in DvH metabolism, its use as a terminal electron acceptor supporting growth. In soils or fresh water environments low in sulfate, this capacity could allow niche expansion of DvH. Our report that DvH can respire nitrite refutes a previous report (Pereira et al., 2000) which has been cited (Rodrigues et al., 2006b; Martins et al., 2010) as evidence of a lack of nitrite ammonification by this organism. However, in light of more current studies of nitrite toxicity (Haveman et al., 2004; He et al., 2006), the high levels of nitrite used in the prior study (Pereira et al., 2000) would be expected to completely inhibit DvH**,** compromising the test for nitrite respiration. The current study indicates that nitrite can indeed be used by *D. vulgaris* as an electron acceptor when supplied at subinhibitory concentrations. This metabolic ability may be especially useful to investigators studying the essential components of sulfate reduction, because nitrite could be used as an alternative electron acceptor for mutant strains unable to grow with sulfate as an electron acceptor.

Use of nitrite as electron acceptor brings up the question of which enzyme is allowing this growth. The results reported here, as well as a wealth of *in vitro* studies of the NrfHA "model" enzyme from *D. vulgaris* (Rodrigues et al., 2006a; Rodrigues et al., 2006b; Rodrigues et al., 2008; Martins et al., 2010; Todorovic et al., 2012), indicate that it is likely the NrfHA enzyme complex, rather than the sulfite reductase, that allows *D. vulgaris* to use nitrite as an electron acceptor. While the sulfite reductase can reduce nitrite, its high affinity for nitrite and a low turnover number for nitrite reduction (Wolfe

et al., 1994) may inhibit its ability to use nitrite efficiently as a terminal electron acceptor. Furthermore, there is strong evidence that NrfHA accepts electrons from the menaquinone pool (Rodrigues et al., 2006b; Rodrigues et al., 2008). Respiration of nitrite in DvH may therefore be very similar to the NrfHA-mediated nitrite respiration of the model nitrite reducer *Wolinella succinogenes* (Simon, 2002). The coupling could be through menaquinone cycling with electrons from lactate dehydrogenase, which is apparently capable of delivering electrons to menaquinones (Reed and Hartzell, 1999; Keller and Wall, 2011).

The ability of the NrfA mutant to grow with nitrite as sole nitrogen source provides evidence that reduction of nitrite by the sulfite reductase produces sufficient ammonium for growth. Importantly, the ammonification could allow sulfate-reducing bacteria, regardless of whether they contain a NrfHA enzyme complex, to use subinhibitory levels of nitrite in the environment as a nitrogen source. Thus low levels of environmental nitrite may directly impact the petroleum industry (Voordouw et al., 2007) or any other situation in which nitrite or nitrate is used to inhibit the growth and sulfide production of sulfate-reducing bacteria. The uses of nitrite beneficial to the sulfate reducers may contribute to their ability to recover (Voordouw et al., 2009) from inhibition. Furthermore, the clarification of the relationship between nitrate and nitrite inhibition of these bacteria should allow for better predictions of the activity of sulfate reducers in a variety of environments. For example, in former nuclear weapons production sites in which there are persistent high levels of nitrate (Green et al., 2012), nitrate may inhibit sulfate reducers entirely independently of nitrite production. In

conclusion, the results presented here should improve the predictability of models that include environmental activities of the sulfate-reducing bacteria.

CHAPTER 4

A PROPOSED FUNCTION FOR THE NITRATE GENE CLUSTER IN *Desulfovibrio vulgaris* HILDENBOROUGH

The nitrate ion is a common, regulated contaminant in the environment (Thorburn et al., 2003; Wick et al., 2012). Environmental nitrate levels are usually much lower than those found at former nuclear weapons production sites like the Oak Ridge Field Research Center (Green et al., 2012). The Maximum Contaminant Level Goal (MCLG) for nitrate in the United States is 10 ppm (mg/L) nitrate-N, which is equivalent to 45 ppm nitrate or 726 µM nitrate (http://water.epa.gov/drink/contaminants/basicinformation/nitrate.cfm, http://www.ext.colostate.edu/pubs/crops/00517.pdf). A study of nitrate concentrations in California groundwater revealed that about 6% of the wells sampled had nitrate concentrations above the MCLG (Kent and Landon, 2013). As of 2010, the maximum level allowed in groundwater in Europe was 50 ppm, or about 800 µM nitrate (http://ec.europa.eu/environment/pubs/pdf/factsheets/nitrates.pdf). Although in some areas of Europe the groundwater nitrate concentration was reported to be greater than 800 µM, widespread measurements suggest that in most of Europe the groundwater concentration was less than 400 µM in 2010 (http://ec.europa.eu/environment/pubs/pdf/factsheets/nitrates.pdf). These data were supported by a study of nitrate contamination of groundwater in Austria in 2012, in which the maximum nitrate level measured was just under the 50 ppm limit (Wick et al.,

2012). In 2003, the levels of nitrate in groundwater were similar in intensive agricultural areas of Australia (Thorburn et al., 2003). Thus a small number, 3%, of sampled wells had nitrate concentrations above the desired upper limit (Thorburn et al., 2003). In the ocean, the euphotic zone (sunlight zone) was reported to have a mean nitrate concentration of 7 µM, whereas the aphotic zone (no sunlight) had a mean nitrate concentration of 31 µM (Gruber, 2008). Thus, it is reasonable to expect that sulfate-reducing bacteria in the environment have not been selected to tolerate concentrations of nitrate in the millimolar range. It is no surprise, therefore, that 100 mM nitrate constitutes a severe stress for the model sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough (DvH).

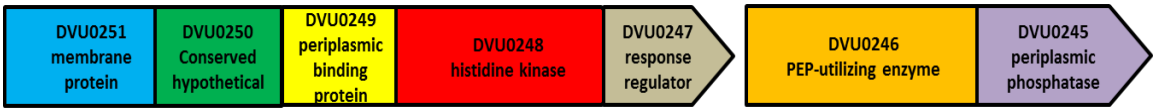


Figure 4.1 *D. vulgaris* Hildenborough "Nitrate Cluster." Mutations in these genes confer resistance to 100 mM nitrate. Operon predictions are from http://www.microbesonline.org/; boxes represent predicted genes, arrows indicate direction of transcription, and contiguous boxes ending in an arrow represent predicted operons.

A cluster of genes has been found which, upon mutation, confers resistance to high nitrate (100 mM or more) in DvH (DVU0251-DVU0245, Fig. 4.1) and *D. alaskensis* G20 (Dde_0597-Dde_0605) (Korte et al., 2014). The assumption is made here that the increased nitrate resistance of the "nitrate cluster" mutants was a result of the inactivation of each individual gene, rather than a result of polar mutations disrupting the function of

downstream genes (Korte et al., 2014). Taking into account sequence-based annotations of these genes, a model of the putative function of this cluster is presented here. For simplicity, the model presented here is for DvH. It is likely that "nitrate cluster" homologs in other closely-related sulfate-reducing bacteria, such as *D. alaskensis*, have a similar function. While only a few *Desulfovibrio* strains appear to have shared synteny for all of the seven "nitrate cluster" genes described here, many sulfate-reducing strains have homologs of one or more of these genes (Fig. 4.2).

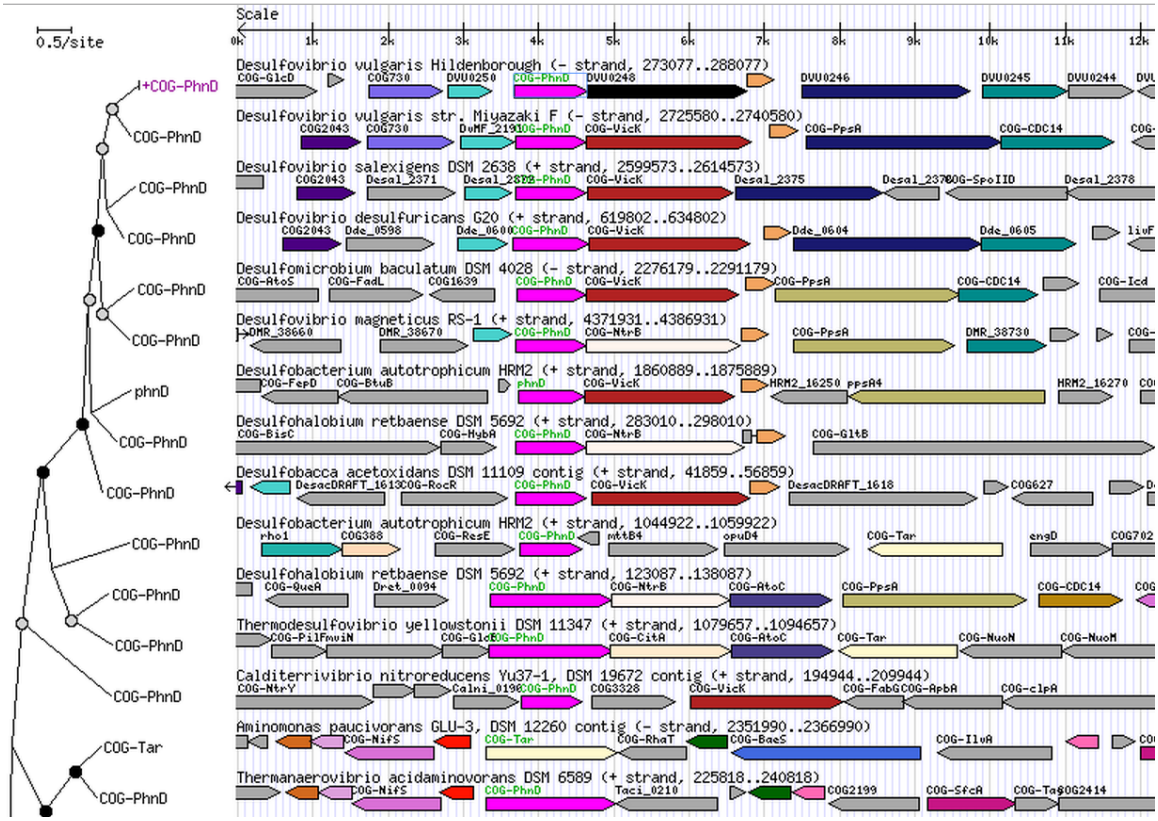


Figure 4.2 Gene tree for DVU0249 (COG-PhnD) and comparison of nitrate cluster homologs (http://www.microbesonline.org/, 11-4-14). Boxes represent predicted genes, arrows indicate direction of transcription, and boxes with the same color (in different rows) represent homologs.

Presented here is the hypothesis that this cluster encodes a group of proteins which work together to detect low levels of extracellular phosphate and to import phosphate ions when low phosphate levels are detected (Fig. 4.3). According to this model, high nitrate levels disrupt phosphate binding but also cause incorrect signaling such that a "low phosphate" signal is detected by these proteins. That signal then leads to increased uptake of phosphate and, inadvertently, uptake of nitrate ions (Fig. 4.3). This nitrate uptake is an energy sink which then slows or halts the growth of the bacteria. Therefore, interruption of these genes and subsequent disruption of the function of the proteins prevents aberrant use of the energy resources of the cell and allows robust growth in the presence of 100 mM nitrate. The following is a detailed look at each of the proteins encoded by this gene cluster and an analysis of the plausibility of this hypothesis. The proteins represented in the model (Fig. 4.3) are color-coded to correspond to the genes that encode them (Fig. 4.1).

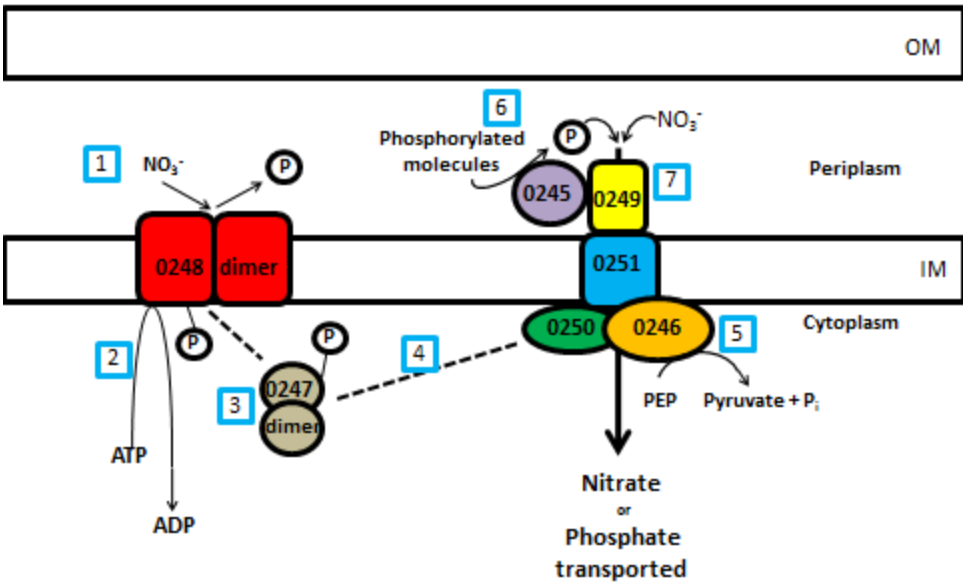


Figure 4.3 Model of the native function of *D. vulgaris* Hildenborough "nitrate cluster" genes and their interaction with nitrate. **1)** The protein encoded by DVU0248 senses phosphate, a negative regulator for this histidine kinase and its subsequent signaling. Nitrate disrupts this binding but transmits an "empty" signal (no phosphate bound) which initiates signaling. **2)** ATP is used for phosphorylation of the histidine kinase. **3)** The histidine kinase in turn phosphorylates the response regulator (DVU0247) dimer. **4)** The response regulator binds to the protein encoded by DVU0250 and activates the phosphate transporter. **5)** The enzyme encoded by DVU0246 uses PEP to power phosphate (or nitrate) transport. **6)** The periplasmic phosphatase (DVU0245) frees phosphate from phosphorylated molecules so that phosphate can bind to the periplasmic phosphate-binding protein (DVU0249). **7)** Phosphate (or nitrate) bound to the binding protein can then be transported through the transmembrane protein (DVU0251) and into the cell.

DVU#	Domain Name for best hit(s)	Domain Accession #	Domain Description	E value for domain hit	Length of protein (aa)	Amino acid range of domain within protein
0251	TauE	pfam01925	Sulfite exporter TauE/SafE; family formerly known as DUF81	7.94e-14	320	61-306
0250			Conserved hypothetical in sulfate-reducing bacteria—no putative conserved domains			
0249	PBP2_PhnD_like	cd01071	Substrate binding domain of phosphonate uptake system-like, a member of the type 2 periplasmic-binding fold superfamily (includes PhnD-like proteins that may bind phosphate, phosphonate, or hypophosphate)	1.52e-73	323	53-307
0248	PAS	cd00130	PAS domain	1.91e-09	702	333-434
	HisKA	cd00082	Histidine Kinase A (dimerization/phosphoacceptor) domain	4.22e-12		450-513
	HATPase_c	cd00075	Histidine kinase-like ATPases	2.31e-18		564-659
	PRK11360 (multi-domains)	PRK11360	sensory histidine kinase AtoS; Provisional	3.21e-60		289-660
0247	REC	cd00156	Signal receiver domain; examples are CheY, OmpR, NtrC, and PhoB; contains conserved aspartic acid residue for phosphorylation	2.04e-27	119	5-117
	AtoC (multi-domains)	COG2204	Response regulator	1.35e-27		1-117
0246	PPDK_N	pfam01326	Pyruvate phosphate dikinase, PEP/pyruvate binding domain	1.47e-72	744	8-337
	COG3848	COG3848	Phosphohistidine swiveling domain	4.10e-09		355-455
	PEP_synth (multi-domains)	TIGR01418	phosphoenolpyruvate synthase	4.64e-58		2-465
0245	DSPc	cd00127	Dual specificity phosphatases (DSP); Ser/Thr and Tyr protein phosphatases	3.47e-17	369	34-158

Table 4.1. Annotations of Nitrate Cluster genes. Blastp, 10-23-14, searching database "Reference proteins (refseq_protein)" with the Blastp (protein-protein BLAST) algorithm and selecting Conserved Domains for domain information (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)

More work will be necessary to clarify whether DVU0248 is a pseudogene or a true gene (Korte et al., 2014). The hypothesis that DVU0248 encodes a functional protein is supported by the fact that the mutation of its *D. alaskensis* homolog, Dde_0602, confers a strong fitness advantage in 150 mM nitrate (Korte et al., 2014). DVU0248 putatively encodes a 702-amino acid protein that is homologous to a histidine kinase and has putative PAS, dimerization/phosphoacceptor, and ATPase domains (Table 4.1). PAS domains, present in all three domains of life (Pellequer et al., 1998), are involved in sensing and signal transduction (Pellequer et al., 1999). For example, PAS domains are found in proteins such as ion channels and sensors of light, oxygen, and small ligands (Pellequer et al., 1999). Histidine kinases generally form dimers (Wolanin et al., 2002), so it is likely that the protein product of DVU0248 dimerizes in the process of signal transduction. DVU0248 may therefore be a sensor for phosphate ions which is turned "off" when high phosphate is present in the periplasmic space (phosphate is bound to the receptor). When extracellular concentrations of nitrate are high, the nitrate ion displaces phosphate as the ligand for the receptor (Step 1 in Fig. 4.3). When it does this, the binding of nitrate does not have the same effect on the protein's conformation that phosphate has. Therefore, nitrate binding causes the transmission of an "empty" (no phosphate bound, low extracellular phosphate) signal instead of the "phosphate bound" (high extracellular phosphate) signal. This "empty" signal then leads to transfer of a phosphate from ATP to the histidine kinase dimer (Steps 2 in Fig. 4.3). This signal can then be propagated to a response regulator (Step 3 in Fig. 4.3).

The 119 amino acids of DVU0247 share homology with Rec domains, or signal-reception domains of two-component regulatory systems (Table 4.1). Two Rec domains

form a homodimer and this homodimer is phosphorylated by a histidine kinase, as in the PhoR/PhoB phosphate regulatory system of *E. coli* (Hsieh and Wanner, 2010). The phosphoacceptor of the Rec receiver domain is an aspartic acid residue (Stock et al., 1990) which is conserved in DVU0247 (Table 4.1). Interestingly, the response regulator in a bacterial two-component regulatory system is usually covalently linked to an effector domain, such as a DNA-binding domain (Pao and Saier, 1995). The protein encoded by DVU0247 has no predicted effector domain, which suggests that the signal may be transmitted noncovalently to an effector (Pao and Saier, 1995). This opens up the possibility that DNA transcription is not the downstream target of this response regulator. According to the model presented here, the protein encoded by DVU0247 dimerizes and is phosphorylated by the histidine kinase encoded by DVU0248 (Step 3 in Fig. 4.3). The response regulator can then interact with a phosphate uptake system to compensate for the "low" levels of phosphate detected (Step 4 in Fig. 4.3).

DVU0250 encodes a 195-amino acid conserved hypothetical protein with no annotated domains (Table 4.1). Although automatic annotation is unavailable for DVU0250, the interruption of this gene has been shown to confer resistance to 100 mM nitrate (Korte et al., 2014). The location of this gene in the "nitrate cluster" therefore suggests that the gene encoded by DVU0250 interacts with the other nitrate cluster proteins. According to the model presented here, then, the protein encoded by DVU0250 is a part of the phosphate transport complex which receives the signal from the response regulator encoded by DVU0247. That is, the protein encoded by DVU0250 acts as a novel "effector domain" which connects the "low phosphate" signal with the uptake response (Step 4 in Fig. 4.3).

The 744-amino acid protein encoded by DVU0246 is a putative phosphoenolpyruvate-utilizing enzyme with homology to pyruvate-phosphate dikinase or phosphoenolpyruvate synthase (Table 4.1). Pyruvate phosphate dikinase (E.D. 2.7.9.1), or PPDK, catalyzes the following reversible reaction:

ATP + pyruvate + phosphate ⇔ AMP + phosphoenolpyruvate + diphosphate

(http://enzyme.expasy.org/EC/2.7.9.1)(Evans and Harland, 1968). In contrast, phosphoenolpyruvate synthase (Table 4.1) catalyzes the following reaction:

ATP + pyruvate + H_2O ⇔ AMP + phosphoenolpyruvate + phosphate

(http://enzyme.expasy.org/EC/2.7.9.2). Enzyme I of the PEP: sugar phosphotransferase system (PTS) of *E. coli* also shares homology with PPDK (Herzberg et al., 1996). Enzyme I of the PTS system is phosphorylated by PEP (Teplyakov et al., 2006), and this system in which a series of proteins is sequentially phosphorylated controls sugar transport in *E. coli* (Deutscher et al., 2006). It is therefore possible that DVU0246 is a PEP-utilizing enzyme that catalyzes neither of the above reactions. Therefore, in the model shown here, DVU0246 encodes a protein that uses PEP as an energy source for the phosphate-uptake complex (Step 5 in Fig. 4.3). DVU0246 also has a 279-amino acid C-terminal region with unpredicted function in the "best hit" domain model (Table 4.1). This region may be able to interact with other proteins. Furthermore, the 279-amino acid C-terminal region of this protein may allow it to bind to the other proteins of the complex (Fig. 4.3).

DVU0245 encodes a 369-amino acid protein with a domain homologous to dual-specificity Ser/Thr and Tyr protein phosphatases (Table 4.1). However, this domain only comprises about half the protein; the 211-amino acid C-terminal region of the protein has

no predicted function in the model of the "best hit" domain (Table 4.1). This region may be able to interact with other proteins. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/, for Gram-negative bacteria, sensitive cutoff: 0.34) software predicts that the protein encoded by DVU0245 has a signal peptide with a potential cleavage site between amino acids 17 and 18 (Petersen et al., 2011). Therefore, it is reasonable to suggest that the protein encoded by DVU0245 is located in the periplasm. In the model shown here, this protein scavenges phosphate by cleaving it from phosphorylated molecules, for example from nucleic acids released by neighboring lysed bacterial cells (Step 6 in Fig. 4.3). In this way, this enzyme would resemble the *E. coli* periplasmic alkaline phosphatase PhoA, the production of which increases upon phosphate starvation (Wanner and Latterell, 1980). Furthermore, the 211-amino acid C-terminal region of this protein may associate with the phosphate-transporting complex (Fig. 4.3) and contribute to the function of the complex.

The 323-amino acid protein encoded by DVU0249 is predicted to be the periplasmic substrate-binding component of a phosphate/phosphonate ABC transport uptake system (Table 4.1), known as PhnD in *E. coli* (Alicea et al., 2011). This particular kind of binding protein binds its ligand in the cleft between two lobes (Alicea et al., 2011), like a venus fly trap (Felder et al., 1999). A periplasmic localization for this protein is supported by the report that it is associated with the outer membrane and is a putative lipoprotein (Walian et al., 2012). Furthermore, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/, for Gram-negative bacteria, default cutoff: 0.45) software predicts that the protein encoded by DVU0249 has a signal peptide with a potential cleavage site between amino acids 30 and 31 (Petersen et al., 2011). In the

model shown here, DVU0249 encodes a phosphate-binding periplasmic protein that allows for phosphate recognition by the phosphate-uptake complex (Step 7 in Fig. 4.3). Furthermore, it is hypothesized here that nitrate can also bind to this protein (Fig. 4.3) and thus be transported (aberrantly) into the cell.

DVU0251 encodes a 320-amino acid predicted permease (Table 4.1). More specifically, it has a conserved domain of a sulfite exporter in the TauE superfamily (Table 4.1), formerly known as DUF81 (Weinitschke et al., 2007). This transmembrane, ion-transport annotation suggests that this protein could be involved with transport of other ions, such as phosphate or nitrate. In the model presented here, DVU0251 encodes the transmembrane portion of the phosphate-uptake complex that also aberrantly transports nitrate (Step 7 of Fig. 4.3).

Further evidence for this proposed mechanism is that high phosphate levels are known to suppress the activity of the high-affinity phosphate transport (Pst) complex in *E. coli* (Hsieh and Wanner, 2010). This is thought to be a negative regulatory process in which the transporter is active when P_i is not bound to the sensor (Hsieh and Wanner, 2010). *D. vulgaris* has genes annotated as phosphate transporters (Table 4.2), and functional redundancy is common with *D. vulgaris* genes (http://www.microbesonline.org/). It is therefore reasonable to suggest that the nitrate cluster, whose deletion does not impair bacterial growth under the control conditions tested, encodes a redundant phosphate transport mechanism.

Gene name	DVU #	Annotated function
pstA-2	2479	phosphate ABC transporter, permease protein, putative
pstB-1	1084	phosphate ABC transporter, ATP-binding protein
pstB-2	2664	phosphate ABC transporter, ATP-binding protein
pstC-2	2478	phosphate ABC transporter, permease protein, putative
pstS	2477	phosphate ABC transporter, periplasmic phosphate-binding protein, putative
pstS	2667	phosphate ABC transporter, periplasmic phosphate-binding protein
phoU	1085	phosphate transport system protein PhoU

Table 4.2 *D. vulgaris* homologs of the *E. coli Pst* operon (*pstS*, *pstC*, *pstA*, *pstB* and *phoU*) (Ferreira and Spira, 2008). Gene annotations from http://www.microbesonline.org/, 11-4-14.

The proposed connection between these proteins would mean that inactivation of the genes in the "nitrate cluster" leads to inactivation of this entire process. According to the hypothesis presented here, this inactivation saves the bacterial cell the signaling energy of ATP hydrolysis, and, more significantly, the energy provided by PEP for phosphate or nitrate transport (Fig. 4.3). Inactivation of any one of several of the genes in this cluster (DVU0251, 0250, 0249, 0247, 0246) confers nitrate resistance to DvH (Korte et al., 2014). More studies will be needed to confirm whether DVU0248 interruption confers a nitrate-resistant phenotype to DvH as the interruption of its homolog does in *D. alaskensis* (Korte et al., 2014). It is possible that the protein encoded by DVU0248 interacts with more than one response regulator and that this "crosstalk" is important for optimal cell function (Wang et al., 2009). This could account for an ambiguous phenotype for the DVU0248 mutant in nitrate-stress conditions. In addition, the DVU0245 mutant appears to be partially resistant to 100 mM nitrate (Korte et al., 2014) but also hindered in overall growth in these conditions (Appendix 1). This unique

phenotype amongst the "nitrate cluster" genes may be related to the putative phosphate-scavenging function of this putative phosphatase. That is, interruption of most of the genes in the cluster is not likely to affect other functions in the cell, except perhaps the putative histidine kinase as mentioned above. This is because there are other annotated phosphate uptake mechanisms in DvH (Table 4.2), and their function should not be impaired by the disruption of this transporter. However, deletion of the phosphatase may in fact hinder the uptake of phosphate by all phosphate transporters in the presence of high nitrate. This would explain why most of the "nitrate cluster" mutants essentially "ignore" 100 mM nitrate: phosphate uptake is sufficient to allow normal growth, and no excess energy is being expended for aberrant transport. The DVU0245 mutant has a shorter lag phase than the wild-type grown in the presence of 100 mM nitrate, presumably because no excess energy is being expended in aberrant transport (Appendix 1). However, its growth rate and growth extent is not as robust as the other nitrate cluster mutants possibly due to a decreased ability to get phosphate from the medium (Appendix 1).

There is some evidence that at least DVU0249 is co-regulated with genes involved with the transport and metabolism of inorganic ions and carbohydrates (Serdar Turkarslan, personal communication). The carbohydrate connection may be related to the involvement of PEP in the proposed transport process. One of the potential regulators for DVU0249 is oxygen (Serdar Turkarslan, personal communication). Hydrogen peroxide stress leads to at least some down-regulation of most of the genes in the nitrate cluster (DVU0251, 0250, 0249, 0247, 0246, 0245). No expression data are available for DVU0248, since it is currently annotated as a pseudogene. DVU0249 had greater than 5-

fold down-regulation (http://www.microbesonline.org/) in the presence of 1 mM hydrogen peroxide at 2 hours versus a sterile water control at the same time point (Zhou et al., 2010). There was also up-regulation of these six genes (http://www.microbesonline.org/) in the presence of 105 mM nitrate at four h after addition compared with the zero time point of nitrate addition (He et al., 2010a). Again, the most pronounced expression change was with DVU0249, which had about 3.5-fold decreased expression compared with the control. Interestingly, these six genes were also up-regulated (http://www.microbesonline.org/) in the presence of 2.5 mM nitrite at 60 min compared with the no additions control at the same time point (He et al., 2006). At this time point, the expression of DVU0250, the biggest changer, was about 2.5-fold higher in the presence of 2.5 mM nitrite (http://www.microbesonline.org/) than its expression in the control condition at the same time point (He et al., 2006). These expression results may indicate changes in phosphate metabolism in the presence of oxidative stresses. Currently, there are no *D. vulgaris* or *D. alaskensis* expression data available for low- or high-phosphate conditions. These would be interesting conditions to test in light of this hypothesis. Although the DVU0247 response regulator likely has no interaction with DNA, there may be another regulator that allows for increased expression of the nitrate cluster genes in the presence of limiting phosphate.

Additional experiments to test the native function of these genes should include tests of the growth rate of "nitrate cluster" mutants in limiting phosphate or high phosphate. Based on the model presented here, it is reasonable to predict that nitrate cluster mutants, particularly the DVU0245 phosphatase mutant, will be hindered in their growth rate and growth extent compared with wild-type *D. vulgaris* cells in phosphate-

limited conditions. The DVU0245 mutant should be particularly hindered because of a reduced ability to scavenge phosphate from molecules released by lysed cells in the culture medium. With increased added phosphate, it is reasonable to predict that wild-type cultures would have increased nitrate resistance. Additionally, the levels of expression of these genes in the above-mentioned conditions would illuminate whether they are regulated by phosphate levels.

Further testing of the proposed model should include testing whether nitrate is indeed transported into the cells. If so, it is reasonable to predict that the nitrate cluster mutants will have reduced nitrate uptake compared with wild-type DvH. Furthermore, the possibility remains that this system may sense and transport anions other than phosphate, such as sulfite. The hypothesis of phosphate transport is presented here as one possible function of the proteins encoded by the nitrate cluster genes. Future studies of these genes will shed light on not only the function of DvH, but also of closely related organisms, such as *D. alaskensis*, which have homologs of these genes. In addition, these studies may lead to a greater understanding of hypothetical genes in other organisms.

CHAPTER 5

CONCLUSION

In this study we explored the roles of nitrate and nitrite as inhibitors of sulfate-reducing bacteria (SRB). In petroleum production sites, amendments of nitrate and nitrite are used to prevent SRB production of sulfide that causes souring of oil wells (Voordouw et al., 2007). SRB, which can precipitate heavy metals, (Lovley, Roden et al. 1993; Lovley, Widman et al. 1993; Lloyd, Ridley et al. 1999; Chardin, Dolla et al. 2003), are also present at former nuclear weapons production sites that are contaminated with both heavy metals and high levels of nitrate (Green et al., 2012). Therefore, the presence of nitrate at such sites is predicted to inhibit the ability of the SRB to precipitate the heavy metals. A better understanding of nitrate stress responses in the model SRB, *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio alaskensis* G20, will strengthen predictions of environmental outcomes of nitrate application.

To study these responses, we employed fitness profiling and follow-up gene deletion analyses. The nitrite stress response was compared to the nitrate stress response by comparing fitness profiles of pooled *D. alaskensis* mutants grown under these different conditions. We predicted that if the nitrate stress response is mediated by nitrite stress, mutants should have similar fitness under both conditions. However, based on prior transcriptomic and proteomic analyses of nitrate and nitrite stress (He et al., 2006; Redding et al., 2006; He et al., 2010a), we predicted that we would find unique nitrate-stress responses. We predicted that mutants that grew better than the average strain—less

sensitive mutants—would have a mutation in a gene that facilitates nitrate inhibition. Likewise, we predicted that more sensitive mutants have a deletion in a gene that protects the cell from nitrate stress. We found that a small group of *D. alaskensis* genes, when mutated, conferred resistance to nitrate that allowed them to predominate in the population of mutants. Mutations in homologs of these genes in *D. vulgaris* also conferred nitrate resistance in a high-throughput TnLE-seq fitness profiling study. In the presence of nitrate, then, the nitrate-sensitive strains had little opportunity to grow before the resources in the medium were consumed. These results led to a number of follow-up studies, including gene deletion and complementation in *D. vulgaris*, in order to probe the mechanisms of nitrate resistance. We found that individual mutants impaired in the function of the genes indicated by fitness profiling had strong nitrate-resistant phenotypes. Furthermore, with a number of genetic and biochemical analyses, we found that nitrate inhibition of *D. vulgaris* can be independent of nitrite production. This finding refutes prior inferences about the mechanism of nitrate inhibition of *D. vulgaris* (He et al., 2010a; Gieg et al., 2011). Furthermore, *D. vulgaris* can use subinhibitory levels of nitrite as either a nitrogen source or a terminal electron acceptor. This finding refutes the results of a prior test for the use of nitrite as an electron acceptor (Pereira et al., 2000). However, the results of that prior test were likely compromised by the use of inhibitory levels of nitrite (Pereira et al., 2000).

The clarification that nitrate and nitrite inhibition can be independent allows for better predictions of the interactions of these molecules with SRB in environmental and industrial settings. For example, a couple of questions highlight the importance of this distinction. In a particular environmental setting, how will SRB respond and grow if high

nitrate is present but nitrite is absent? Conversely, how will SRB survive and grow in an environment in which nitrite is present but nitrate is absent? Clearly, if nitrate and nitrite are essentially the "same inhibitor," the answers to these questions should be the same. They would activate a nitrite-resistance response, which includes high expression of the nitrite reductase (Haveman et al., 2004; He et al., 2006). If the concentration of nitrite present was sufficiently low, they would be able to recover from the inhibition by reducing the nitrite (Greene et al., 2003; Haveman et al., 2004). However, we know from the results presented in this work that this picture is incomplete. In the case of high nitrate with nitrite absent, this is possible in molybdenum-limited environments. Molybdenum is an essential cofactor of nitrate reductases, and therefore its absence precludes the conversion of nitrate to nitrite (Glass et al., 2012). Even though nitrate-reducing microorganisms and SRB are often collocated (Greene et al., 2003), the nitrate reducers will not produce nitrite without sufficient molybdenum (Glass et al., 2012). In fact, low molybdenum levels appear to be limiting nitrate reduction at the Oak Ridge field research center (Michael Adams, unpublished data, https://www.orau.gov/gsp2014/abstracts/adams_paul_08.pdf). This site is contaminated with heavy metals and nitrate plumes of greater than 100 mM nitrate (Green et al., 2012).

In light of the data presented in this work, it is reasonable to predict that, in the absence of other inhibitors, spontaneous nitrate-resistant mutants of SRB will be selected for growth in environments high in nitrate but lacking nitrite. Preliminary data from re-sequencing of the genome of *D. vulgaris* cultures grown in 100 mM nitrate suggests that spontaneous nitrate-resistant mutants do not necessarily have mutations (*e.g.* single-nucleotide polymorphisms) in the *rex* gene (DVU0916), in the "nitrate cluster" genes

(DVU0251-0245), or in the promoter regions of these genes. This is not surprising since spontaneous mutations have the potential to improve the function of genes and not simply inactivate genes. In contrast, transposon mutants disrupt the function of genes. The nitrate-resistant mutants found in this study shed light on possible mechanisms of nitrate resistance. However, if resistance is a result of more efficient energy conservation, then it would be expected that many different spontaneous mutations—some perhaps improving cell function—could lead to a nitrate-resistant phenotype.

In contrast to high-nitrate, low-nitrite environments, the petroleum industry can readily create an environment in which nitrite but not nitrate is added for the purpose of decreasing the sulfide production in oil wells (Voordouw et al., 2007). In such an environment, high concentrations of nitrite would be expected to completely inhibit SRB. Subinhibitory concentrations of nitrite, however, would be used by the bacteria as a nitrogen source or terminal electron acceptor. The ability for SRB to use nitrite as a growth substrate may explain in part why these bacteria can recover from inhibition by nitrate or nitrite in oil wells (Voordouw et al., 2009).

More work will be necessary to understand the mechanisms by which nitrate-resistant mutations confer their resistance, as well as what functions of the cell nitrate inhibits. Nonetheless, this study contributes substantially to our ability to understand and predict the effects of nitrate on sulfate reducers in agricultural settings and heavy metal-contaminated environmental sites. Furthermore, it will be helpful for those who use nitrate or nitrite in industrial settings for the limiting of SRB growth and sulfide production.

Additional Publication

H.L. Korte and J.D. Wall (2011). Cloning vector pDSK519, complete sequence. *NCBI GenBank*, ACCESSION JQ173098

APPENDIX 1

INTERACTIONS OF *DESULFOVIBRIO VULGARIS* HILDENBOROUGH MUTANTS WITH NITRATE AND NITRITE

Acknowledgement of contributions: H.L. K. conducted all growth kinetic experiments; prepared plasmids pMO4503, pMO4504, and pMO4506; and generated strain JW4501. Grant M. Zane constructed strain JW9171and all *Desulfovibrio vulgaris* transposon mutants presented here. Valentine V.Trotter prepared the cells for enzymatic studies. Avneesh Saini conducted the enzymatic studies and analyzed the enzyme data.

I. Introduction

Sulfate-reducing bacteria (SRB) can precipitate heavy metals by changing their redox state (Lovley et al., 1993a; Lovley et al., 1993b; Lloyd et al., 1999; Chardin et al., 2003) and by sulfide precipitation of the heavy metals (Jalali and Baldwin, 2000). Therefore SRB have been explored as a sustainable tool for heavy metal bioremediation (Jiang and Fan, 2008; Martins et al., 2009). However, co-contamination of heavy metal-contaminated sites with compounds that inhibit sulfate-reducing bacteria, such as nitrate (Green et al., 2012) may limit the effectiveness of bioremediation. Therefore, nitrate (He and Zhou, 2010; Korte et al., 2014) and nitrite (He et al., 2006) stress responses have been studied in the model SRB *Desulfovibrio vulgaris* Hildenborough (DvH). It was discovered that nitrate and nitrite have unique inhibitory mechanisms (Korte et al., 2014)

(Korte et al., in review, Chapter 3). However, while it is well accepted that nitrite inhibits the dissimilatory sulfite reductase (Haveman et al., 2004), the mechanisms by which nitrate inhibits SRB are still unknown. The preliminary results presented here address whether nitrate-resistant mutants are also resistant to high levels of sodium chloride; whether the DVU0245 "nitrate cluster" gene has a role in nitrate sensitivity; whether nitrate resistance is related to ion transport; and whether nitrate inhibits the sulfate reduction pathway.

Table A1.1. Strains and plasmids used in this study

Strain or plasmid	**Genotype or relevant characteristics[a]**	**Source and/or reference**
Escherichia coli		
α-Select (Silver Efficiency)	F$^-$ *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA-*arg*F)U169 Φ80*lac*ZΔM15 λ$^-$	Bioline
Desulfovibrio vulgaris		
ATCC 29579	Wild-type *D. vulgaris* Hildenborough (pDV1); 5-FU^s	ATCC
JW710	WT Δ*upp* (pDV1); 5-FU^r (Parent strain for markerless deletion mutants)	(Keller et al., 2009)
JW4501	JW710 ΔDVU0245::(*npt upp*); Km^r; 5-FU^s	This study
JW4502	JW710 Δ*nrfA* (DVU0625); 5-FU^r	Korte et al., in review, Chapter 3
JW9021	WT Δ*qmoABCD*, Km^r	(Zane et al., 2010)
JW9171	JW710 ΔDVU1999; 5-FU^r	Zane et al., in preparation
JW9320	Mutation in "IR 1&2" of Rex repressor binding site upstream of *sat* (DVU1295)	(Christensen et al., 2014)
JW3311	JW710 ΔDVU0916::(*npt upp*); Km^r; 5-FU^s (Δ*rex* marker exchange)	(Korte et al., 2014)
JW3319	JW710 ΔDVU0916 (Δ*rex*); 5-FU^r	Christensen et al., in preparation
GZ9685	DVU0245-773::Tn*5*-RL27; insertion at bp 773 of the predicted 1110 bp of the protein coding sequence; Km^r	Wall Laboratory

GZ12997	DVU0246-111::Tn*5*-RL27; insertion at bp 111 of the predicted 2235 bp of the protein coding sequence; Km^r	Wall Laboratory
GZ2640	DVU0247-211::Tn*5*-RL27; insertion at bp 211 of the predicted 360 bp of the protein coding sequence; Km^r	Wall Laboratory
GZ12015	DVU0250-427::Tn*5*-RL27; insertion at bp 427 of the predicted 588 bp of the protein coding sequence; Km^r	Wall Laboratory
GZ10694	DVU0251-80::Tn*5*-RL27; insertion at bp 80 of the predicted 963 bp of the protein coding sequence; Km^r	Wall Laboratory
GZ2179	Genome position 658487::Tn*5*-RL27; insertion at intergenic region 327 bp upstream of DVU0590; Km^r (Control strain for growth kinetics of transposon mutants)	Wall Laboratory
Plasmids		
pCR®4-TOPO®	Cloning vector, Ap^r, Km^r, pUC *ori*.	Invitrogen Life Technologies
pCR®8/GW/TOPO®	Cloning vector, Sp^r, pUC *ori*	Invitrogen Life Technologies
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); Sp^r; source of Sp^r,pUC *ori* fragment for marker exchange and markerless deletion suicide plasmid construction	(Keller et al., 2009)
pMO746	*upp* in artificial operon with *npt* and linked to Ap^r-pUC *ori* from pCR®4-TOPO®, P_{npt}-*npt-upp*; Km^r; source of Kan^r, *upp* fragment for marker exchange and markerless deletion suicide plasmid construction	(Parks et al., 2013)
pMO3313	pMO9075 with DVU0916 (*rex*) constitutively expressed from P_{npt}	(Korte et al., 2014)
pMO4503	Sp^r and pUC *ori* from pMO719 plus upstream (1089 bp plus 15 bp of the DVU0245 sequence, including the ATG predicted start codon—1104 bp total) and downstream (1016 bp plus the predicted TGA stop codon—1019 bp total) DNA regions from DVU0245 flanking the artifical operon of P_{npt}-*npt-upp* from pMO746; for marker exchange deletion mutagenesis; Sp^r and Km^r	This study
pMO4506	pMO9075 with DVU0245 constitutively expressed from P_{npt}	This study
pMO4504	pMO9075 with DVU0402-0404 (*DsrABD*) constitutively expressed from P_{npt}	This study
pMO9117	*qmoABCD* (DVU0848-0851) constitutively expression from P_{npt}; Sp^r	(Zane et al., 2010)

[a]Km, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript "r" or "s", resistance or sensitivity

II. Materials and methods

II.A. Strains and media

The strains used in this study are listed in Table A1.1. DvH strains were grown in defined MO Basal Salts (Zane et al., 2010) with either 60 mM sodium lactate/30 mM sodium sulfate (MOLS4), 60 mM sodium lactate/20 mM sodium sulfite (MOLS3), or MOLS4 with 0.1% (wt/vol) yeast extract (MOYLS4), unless otherwise indicated. DvH media were reduced with 1.2 mM sodium thioglycolate, unless otherwise indicated. DvH manipulations were done at about 25°C in an anaerobic growth chamber (Coy Laboratory Products, Inc., Grass Lake, MI) with an atmosphere of approximately 95% N_2 and 5% H_2. Optical densities (600 nm) were determined with a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA).

II.B. Growth kinetics

DvH cells removed from a 1-2 mL freezer stock by centrifugation were grown in 4 or 5 mL of MOLS4 or MOLS3 (for studies with MOLS3 medium). Most of the 10% (vol/vol) glycerol used as a cryoprotectant was removed by this centrifugation step. For growth of all plasmid-containing cultures, spectinomycin dihydrochloride pentahydrate (100 µg/mL) was added to starter cultures and to the media for growth kinetic studies. geneticin (G418) sulfate (400 µg/mL) was added to starter cultures of JW4501 but not to the media for growth kinetic studies. Intermediate subculture volumes varied with the experiment and strain. To determine growth rates and extents of growth, triplicate cultures were grown in 5 mL medium in 27-mL anaerobic tubes, each capped with a butyl rubber stopper and incubated at 34°C. Growth kinetic data reported in this chapter are preliminary and will need to be repeated for confirmation.

II.C. Plasmid and strain construction

Plasmids pMO4503, pMO4504, and pMO4506 were constructed as previously described (Korte et al., 2014). Primers are found in Table A1.2. Generation of the marker exchange deletion of DVU0245, strain JW4501, was performed as previously described (Korte et al., 2014). Strains with stable plasmids were constructed according to the same protocol used to make deletion strains (Korte et al., 2014). Modifications to the methods used to construct plasmids for increased protein production were as follows: JW710 (wild-type) cells were electroporated with pMO4504 (*DsrABD*). After recovery, the potential transformants were plated on a MO basal salts (Zane et al., 2010) medium with 30 mM sodium lactate, 30 mM sodium pyruvate, 30 mM sodium sulfate, 10 mM sodium sulfite, 1.8 mM sodium thioglycolate, 0.2% (wt/vol) yeast extract and spectinomycin (100 µg/mL). The pMO4504 plasmid re-isolated from a transformant of JW710 was then transformed into JW4502 (NrfA mutant) by electroporation and plated on MOYLS4 with spectinomycin and about 1.8 mM thioglycolate.

JW4501 (DVU0245 deletion strain) electroporated with pMO4506 (DVU0245 complement) was plated on MOYLS4 with spectinomycin (100 µg/mL) and geneticin (G418) sulfate (400 µg/mL). The pMO4506 plasmid re-isolated from JW4501 was used to electroporate JW710, and transformants were plated on MOYLS4 with spectinomycin only.

II.D. Enzymatic studies

Biochemical studies of NrfA enzyme activity were conducted as previously described (Korte et al., in review, Chapter 3).

Table A1.2. Primers used for PCR amplification, Southern probe generation, and sequencing in *D. vulgaris*

Primer name	Primer sequence (5'-3')	Application
76HK-DVU0245-up-F-21	GCCTTTTGCTGGCCTTTTGCTCACAT CGC ATG AAG GCG TCG TGT TTG	For amplification of DVU0245 upstream region from gDNA with 77HK primer to make pMO4503. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment (SpecRpUC-R). Amplification of Southern probe for confirmation of DVU0245 deletion. forward
77HK-DVU0245-up-R-20	GCGACAAGATATTCGGCACCAAGTAAG GCG CAG AAA GGT CAT GGG TG	For amplification of DVU0245 upstream from gDNA with 76HK primer to make pMO4503. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment (UppCterm). Amplification of Southern probe for confirmation of DVU0245 deletion. reverse
78HK-DVU0245-dn-F-20	GCGCCCCAGCTGGCAATTCCGG TGA CCA AGG CGC AGA CAC TG	For amplification of DVU0245 downstream from gDNA with 79HK primer to make pMO4503. Underlined portion used as overhang for SLIC with Km^r, *upp* fragment (KanPromNterm). forward
79HK-DVU0245-dn-R-20	GTCGAGGCATTTCTGTCCTGGCTGG CCG CGA TGC CTT CGA CTT CT	For amplification of DVU0245 downstream region from gDNA with 78HK primer to make pMO4503. Underlined portion used as overhang for SLIC with Sp^r,pUC *ori* fragment (SpecRpUC-F). reverse
SpecRpUC-F	CCAGCCAGGACAGAAATGCCTCG	For amplification of Sp^r and pUC *ori* from pMO719 to make pMO4503. Used as overhang for SLIC. forward
SpecRpUC-R	ATGTGAGCAAAAGGCCAGCAAAAGGC	For amplification of Sp^r and pUC *ori* from pMO719 to make pMO4503. Used as overhang for SLIC. reverse
KanPromNterm	CCGGAATTGCCAGCTGGG	For amplification of Km^r from pMO719 to to make pMO4503. Used as overhang for SLIC. forward
UppCTerm	CTTACTTGGTGCCGAATATCTTGTCGC	For amplification of Km^r from pMO719 to to make pMO4503. Used as overhang for SLIC. reverse
SpecRpUC-up	GGGAAACGCCTGGTATCTTTATAGTCCT	For colony PCR, screen of and sequencing upstream region of pMO4503 deletion cassette. forward
pMO719XbaI-Dn	TGGGTTCGTGCCTTCATCCG	For colony PCR, screen of and sequencing downstream region of pMO4503 deletion cassette; also for sequencing complementation constructs. Sequencing primer to confirm inserts into pMO9075 for complementation/overexpression. reverse
Kan-int-Fwd-rev-comp	CTCATCCTGTCTCTTGATCAGATCT	For sequencing downstream region of pMO4503 deletion cassette. forward
DvH-Upp gene Cterm-out	GCTGAAGCGCATCGTGGACAA	For sequencing upstream region of pMO4503 deletion cassette. reverse
Topo-8-R	AGACACGGGCCAGAGCTG	Sequencing primer to confirm inserts into pMO9075 for complementation/overexpression.

pBG1-2199-F	GCTGAAAGCGAGAAGA GCGCAC	Sequencing primer to confirm inserts into pMO9075 for complementation/overexpression.
84HK-pMO4503-1386-F-21	GGT ACT CGA ACA GTC CGG CTT	Sequencing primer to confirm upstream region of deletion cassette of pMO4503. forward
85HK-pMO4503-977-R-21	GTC GTC GAG GTT CAG CAC GTA	Sequencing primer to confirm upstream region of deletion cassette of pMO4503. reverse
86HK-pMO4503-4000-F-21	CGT GCA GAT GGC TCG ACA GA	Sequencing primer to confirm downstream region of deletion cassette of pMO4503. forward
87HK-pMO4503-3510-R-21	GGA ACG TAG CGA TGC GCA AAG	Sequencing primer to confirm downstream region of deletion cassette of pMO4503. reverse
108HK-0245-SLIC-F-59	AGGTTGGGAAGCCCTG CAATGCAGTCCCAGGA GGTACCAT ATG ACC TTT CTG CGC GCC C	For amplification of DVU0245 to make pMO4506 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. forward
109HK-0245-SLIC-R-50	GATCGTGATCCCCTGCG CCATCAGATCCTTG TCA GGG CTT GCC GGT GTT C	For amplification of DVU0245 to make pMO4506 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. reverse
pMO9075-SLIC-F	CAAGGATCTGATGGCG CAGGG	For amplification of pMO9075 fragment for SLIC to make complementation constructs. forward
pMO9075-SLIC-R3	CTGGGACTGCATTGCA GGGCTTCCCAACCT	For amplification of pMO9075 fragment for SLIC to make complementation constructs. reverse
88HK-DsrABD-F-62	AGGTTGGGAAGCCCTG CAATGCAGTCCCAGGA GGTACCAT ATG GCG AAA CAT GCA ACT CCC A	For amplification of DVU0402-0404 (*DsrABD*) to make pMO4504 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. forward
89HK-DsrABD-R-51	GATCGTGATCCCCTGCG CCATCAGATCCTTG CTA GTC TTC GTG CTC GGC CG	For amplification of DVU0402-0404 (*DsrABD*) to make pMO4504 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. reverse
92HK-pMO4504-2890-F-22	CTG GGG CAA GTT CGA CAT CGA A	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. forward
93HK-pMO4504-4181-R-25	GTT CGT TCT TGA TGG CGA TGG TGT T	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. reverse
94HK-pMO4504-3739-F-22	TAG CCT CAA GGC CCT GAA GGA A	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. forward
95HK-pMO4504-3276-R-22	GGA GCG ATT TCG GTC ACT TCG A	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. reverse
96HK-pMO4504-4441-F-23	CAT CGG TTG GGA ACG CTT CTT CT	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. forward
97HK-pMO4504-2426-R-25	GCA GGT AGT CGG CAG AGT AGT ACT T	Sequencing primer to confirm DVU0402-0404 (*DsrABD*) insert of pMO4505. reverse

III. Preliminary results and discussion

III.A. Sodium chloride stress studies with nitrate-resistant mutants

In order to understand nitrate resistance mechanisms, further characterization of the growth of known nitrate-resistant mutants of DvH was initiated (Korte et al., 2014). These strains were grown with additions of 250 mM sodium chloride because this additional sodium concentration was known to inhibit wild-type DvH grown under similar conditions (Mukhopadhyay et al., 2006). It would therefore be possible to determine if the nitrate-resistance mutants were more resistant to osmotic stress than the control strain. Since these mutants were dramatically more resistant to nitrate than the control strain (Korte et al., 2014), if much of the resistance observed resulted from production of an osmolyte, their osmotic stress resistance would be increased relative to that of the control strain. Therefore, a NaCl concentration that might reveal a growth advantage of the nitrate-resistant mutants was chosen. The results showed that none of the mutants was particularly resistant to NaCl compared with a nitrate-sensitive control strain (Fig. A1.1 A-F). In contrast, the Δ*rex* mutant did have noticeable resistance to high levels of sodium chloride (Fig. A1.2 A and B), and this might account for some of the resistance of this mutant to sodium nitrate (Korte et al., 2014). The Δ*rex* mutant with empty complementation vector also appeared to be slightly more resistant than the parental strain (Fig. A1.2 C and D). However, the complemented Rex mutant, unexpectedly, was also resistant to sodium chloride (Fig. A1.2 C and D). This complemented strain did not grow exactly like the parental strain in 100 mM nitrate, either (Korte et al., 2014), and I suggest that partial loss of the plasmid (Korte et al., 2014) may lead to these unusual results. Further investigation will be required to better

characterize the complemented Rex mutant. It has been shown previously that relieving osmotic stress does not fully relieve nitrate stress (He et al., 2010a) and that the Rex mutant did not predominate in mutant libraries with sodium chloride or potassium chloride added (Korte et al., 2014). Thus, as previously concluded (Korte et al., 2014), factors unique to the nitrate ion are likely responsible for the nitrate resistance of the Rex mutant as well as the DvH "nitrate cluster" mutants (DVU0251-DVU0245).

Figure A1.1 Growth of *D. vulgaris* Hildenborough transposon mutants in lactate-sulfate medium with additional sodium chloride. Growth of the DVU0245 **(A)**, DVU0246 **(B)**, DVU0247 **(C)**, DVU0250 **(D)**, DVU0251 **(E)** and intergenic transposon control **(F)** mutants in the presence of no additions (○), or with 250 mM additional sodium chloride (△). Approximately 5.3% (vol/vol) inocula were used. Optical density readings reflect averages of three samples, and errors bars show standard deviations. Controls from the experiment shown have been previously reported (Korte et al., 2014).

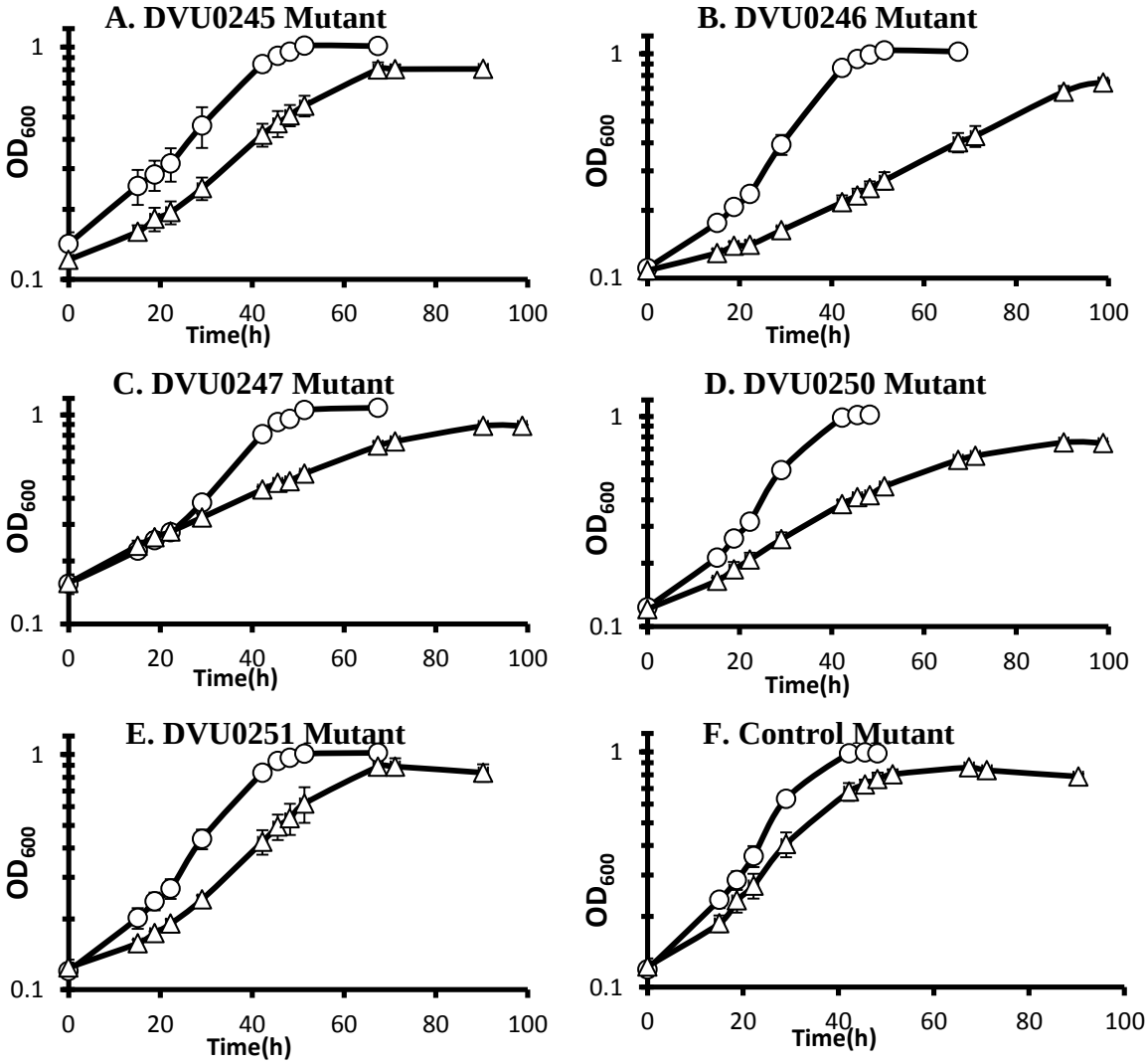
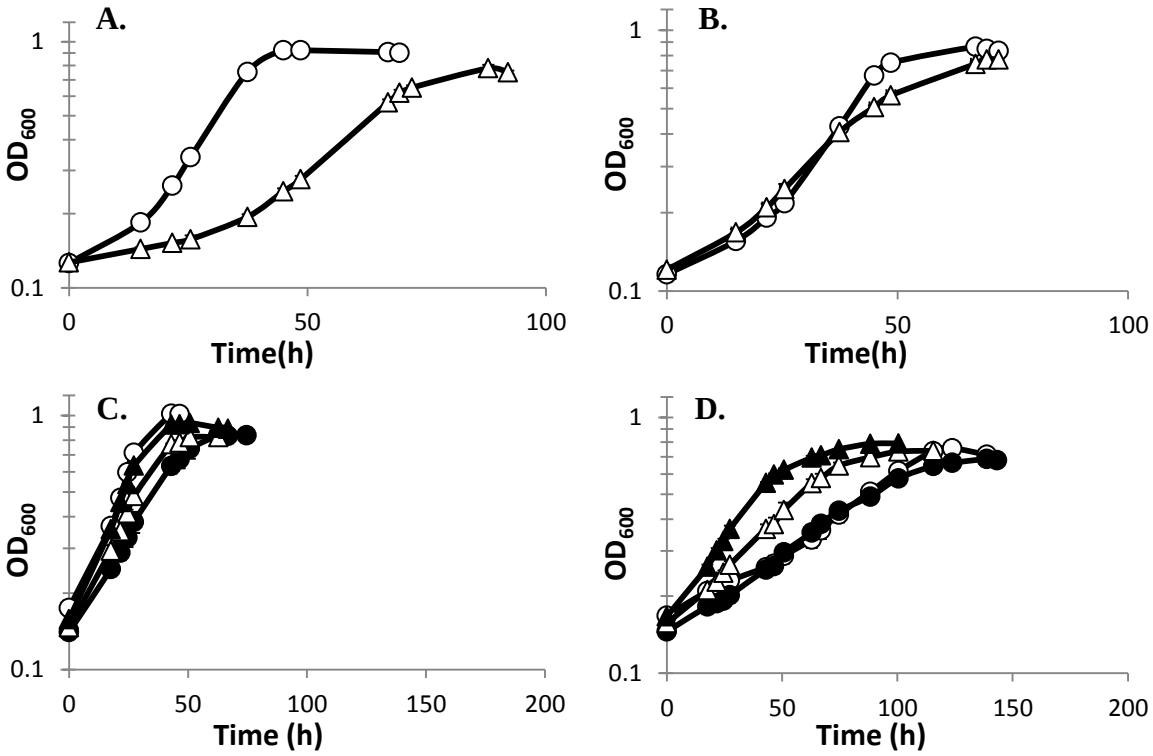


Figure A1.2 Growth of parental *D. vulgaris* Hildenborough vs. *Δrex* mutant in lactate-sulfate medium with additional sodium chloride. (A) and (B): Growth of the parental strain JW710 **(A)** vs. *Δrex* **(B)** mutant in the presence of no additions (○) or with 250 mM additional sodium chloride (△). Approximately 6% (vol/vol) inocula were used. Controls from the experiment shown in **(A)** and **(B)** have been previously reported (Korte et al., 2014). **(C)** and **(D)** show four strains grown with no additions **(C)** or with 250 mM additional sodium chloride **(D)**. Parent with empty vector [JW710(pMO9075)],(○); Parent with *rex* overexpression plasmid [JW710(pMO3313)], (●); *Δrex* strain with empty vector [JW3311(pMO9075)], (△); *Δrex* strain with *rex* complement plasmid [JW3311(pMO3313)], (▲). Approximately 8.2% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.



III.B. DVU0245 "nitrate cluster" deletion mutant studies

The mechanisms of inhibition and resistance may be complicated for the mutant of one the genes in the nitrate cluster, DVU0245. The G20 mutant with a transposon interruption of Dde_0605, the homolog of DVU0245, was resistant to nitrate in pooled growth of the transposon mutant pool (Korte et al., 2014). However, the DvH mutant of DVU0245 did not have similar resistance in pooled growth (Korte et al., 2014). An in-frame deletion of DVU0245 was made to further investigate this discrepancy. This mutant was grown in a lactate-sulfate medium supplemented with 100 mM nitrate, 250 mM sodium chloride, or 1 mM sodium nitrite. It was found that while nitrite and sodium chloride sensitivity of the parent and mutant strains were very similar, the DVU0245 mutant had an unusual phenotype in the presence of 100 mM nitrate. It had a shorter lag phase than the parental strain but grew more slowly and to a lower final optical density (Fig. A1.3 A and B). To clarify these results, complemented strains were grown. While the parental strain and DVU0245 mutant with empty vector grew similarly to the strains without plasmids (Fig.A1.3 B and Fig. A1.4 A and B) in the presence of 100 mM nitrate, the strains with the DVU0245 complementation/overexpression vector grew with entirely different kinetics than either parent or mutant strain (Fig. A1.4 A and B). These strains had faster growth in 100 mM nitrate but a lower final optical density (OD_{600}) than the parental strain with empty vector (Fig. A1.4 A and B). Because DVU0245 is expressed from a multi-copy number plasmid from a constitutive [*aph*(3')-II*p*,(Zane et al., 2010)] promoter, the gene is likely to be more highly expressed in these strains than it is in the parent strain (Korte et al., in review, Chapter 3). Overexpression of DVU0245 in both mutant and parent strains is likely the reason for their unexpected phenotypes, but the

similarity of those phenotypes indicates that complementation was successful. If, as the arrangement of the nitrate cluster (Korte et al., 2014) suggests, DVU0245 interacts with the other proteins produced from this cluster, its overexpression may lead to abnormal protein associations. Such associations may interfere with the function of the other proteins produced from the nitrate cluster. Since mutation of those genes confers nitrate resistance, disruption of the function of the proteins produced from those genes would be expected to increase nitrate resistance. More study will be needed to understand the function of the DVU0245 gene and the reason for the unusual phenotypes it demonstrates in the presence of 100 mM nitrate.

Figure A1.3 Growth of parental *D. vulgaris* Hildenborough vs. ΔDVU0245 mutant in lactate-sulfate medium with additions of sodium nitrate, sodium nitrite, or sodium chloride. (A) Growth of the parent with 1 mM nitrite (○), parent with an additional 250 mM sodium chloride (●), DVU0245 mutant (JW4501) with 1 mM nitrite (△), and DVU0245 mutant with an additional 250 mM sodium chloride (▲). **(B)** Growth of the parental strain JW710 with no additions (○), parent with 100 mM nitrate (●), DVU0245 mutant (JW4501) with no additions (△), and DVU0245 mutant with 100 mM nitrate (▲). Approximately 7% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.

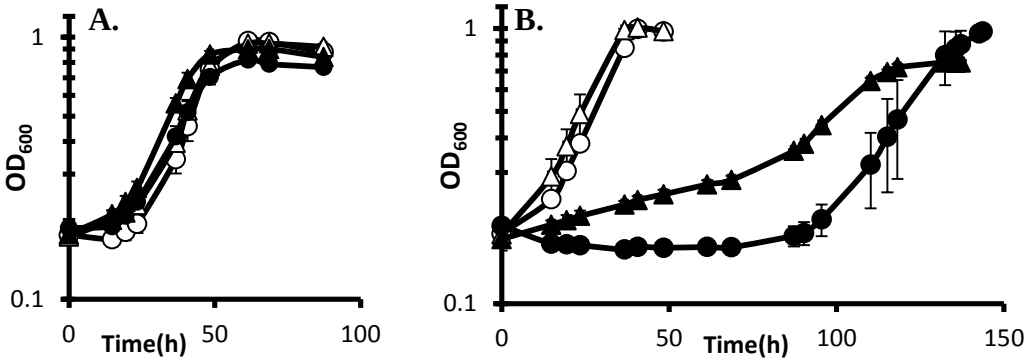
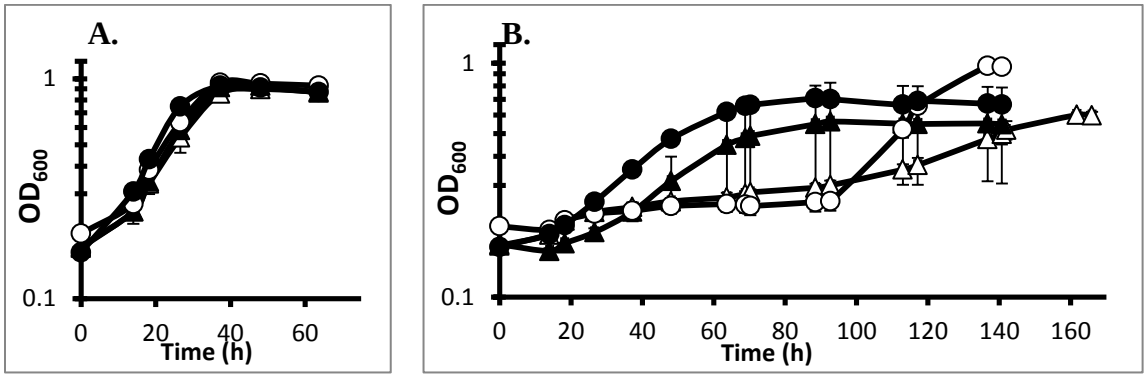


Figure A1.4 Growth of parental *D. vulgaris* Hildenborough vs. complemented ΔDVU0245 mutant in lactate-sulfate medium with sodium nitrate addition. (A) and (B) Growth of parental strain with empty vector [JW710(pMO9075)],(○); parent with DVU0245 overexpression plasmid [JW710(pMO4506)], (●);ΔDVU0245 strain with empty vector [JW4501(pMO9075)], (△);ΔDVU0245 strain with DVU0245 complement plasmid [JW4501(pMO4506)] (▲); with no additions **(A)** or 100 mM nitrate **(B).** Approximately 8.5% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.

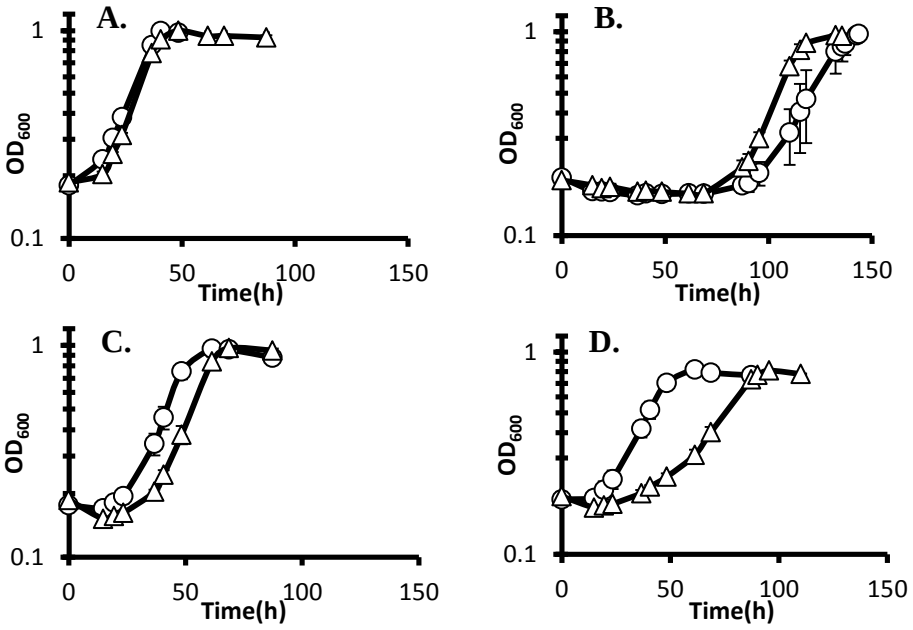


III.C. Additional follow-up of high-throughput nitrate studies

The discovery of a cluster of mutants resistant to nitrate (Korte et al., 2014) prompted us to investigate whether other candidate mutants from high-throughput fitness profiling had nitrate-resistant phenotypes when grown as individual mutants. In high-throughput gene fitness profiling by TnLE-seq (Fels et al., 2013) with DvH grown in the presence of 100 mM nitrate, a mutant containing a transposon in a putative sulfate permease gene (DVU1999) exhibited the ninth-highest gene fitness score (Korte et al., 2014) of all the mutants in that condition. This was interpreted to mean that this mutation

conferred an increase in growth kinetics relative to the average of all other non-essential genes. However, a mutant with an in-frame deletion of this gene had only a slight advantage over the parental strain in the presence of 100 mM nitrate (Fig. A1.5 A and B), coming out of lag phase only about ten hours faster than the parental strain. Such an advantage may have been sufficient to explain its outgrowth in the high-throughput study (Korte et al., 2014).

Figure A1.5 Growth of parental *D. vulgaris* Hildenborough vs. sulfate permease mutant (ΔDVU1999, JW9171) in lactate-sulfate medium with additions of sodium nitrate, sodium nitrite, or sodium chloride. (A), (B), (C) and (D) Growth of the parental strain JW710 (○) or sulfate permease mutant (△) with no additions (A), 100 mM nitrate (B), 1 mM nitrite (C), or an additional 250 mM sodium chloride (D). Approximately 7% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.



However, it is worth noting that the DVU0246 "nitrate cluster" transposon mutant, which had lower fitness than the DVU1999 transposon mutant in the high-throughput experiment, had much more pronounced nitrate resistance when isolated (Korte et al., 2014). Interestingly, the ΔDVU1999 mutant may have had a slight disadvantage in the presence of nitrite (Fig. A1.5 C) and had a clear disadvantage compared with JW710 in the presence of an additional 250 mM sodium chloride (Fig. A1.5 D). Its disadvantage in nitrite may be an experimental artifact, since the ΔDVU1999 mutant did have a slightly longer lag than the parental strain in the "no additions" control (Fig. A1.5 A). However, the dramatic disadvantage of this strain in sodium chloride (Fig. A1.5 D) may explain why its resistance to nitrate is less than the resistance of the "nitrate cluster" mutants (Korte et al., 2014), since osmotic stress responses are thought to be a component of nitrate stress responses (He et al., 2010a). One hypothesis consistent with the data is that the putative sulfate permease mutant has decreased transport of nitrate, and therefore slightly decreased nitrate sensitivity. Nitrate transport should have no benefit to *D. vulgaris*, since it cannot use nitrate as a substrate for growth under the conditions tested here (Korte et al., in review, Chapter 3). Thus, any nonspecific transport of nitrate should cause energy loss from the cells. Elimination of this aberrant transport would lead to more efficient growth in the presence of nitrate. Further, any disrupted transport might adversely affect osmotic stress responses. However, since the mechanism of nitrate inhibition is as yet unknown, it is not clear whether nitrate is taken into the cells. These hypotheses will require further testing. Among the tests that would be informative is growth of a triple putative sulfate permease deletion mutant (JW9201) in the presence of

100 mM nitrate. If this triple mutant has increased nitrate resistance compared with the single, putative sulfate permease deletion, that result would be consistent with a decreased ability to transport nitrate into cells.

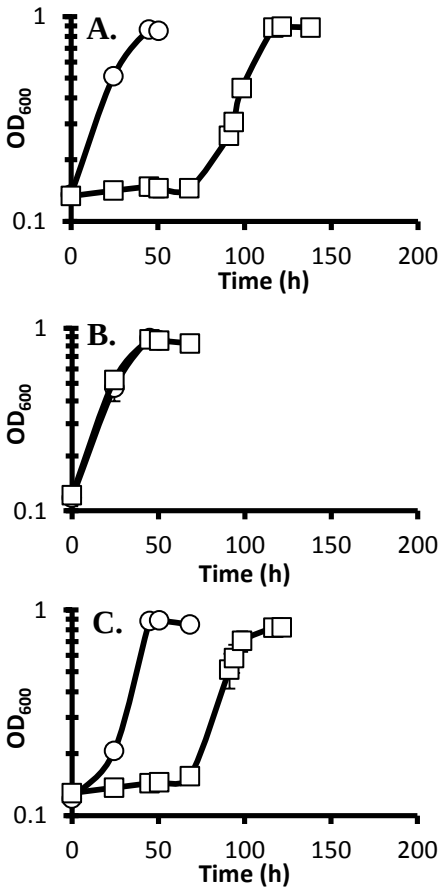
Additional high-throughput data that support this transport hypothesis include the report that a mutant with an insertion in a native plasmid gene (DVUA0023) encoding a putative ABC transporter permease (http://www.microbesonline.org/) had the third-highest nitrate resistance in a DvH fitness profiling study (Korte et al., 2014). These results prompted us to test whether loss of the native plasmid could confer nitrate resistance, as plasmid loss would then be a mechanism by which nitrate resistance could arise in a population of wild-type DvH. It was found that a strain lacking the native plasmid (JW801) did not exhibit increased resistance to nitrate (data not shown). Further studies of a DVUA0023 mutant will be necessary to confirm whether the loss of this gene alone indeed confers nitrate resistance.

III.D. Nitrate resistance and the sulfate-reduction pathway

One regulator gene with a clear connection to nitrate, the gene encoding the Rex regulator, was identified in our prior studies as conferring very high nitrate resistance when inactivated (Korte et al., 2014). Because the Rex protein, encoded by DVU0916, represses the sulfate adenylyl transferase (*sat*) gene (Christensen et al., 2014), a critical gene for sulfate reduction (Postgate, 1984), we explored whether the overexpression of *sat* resulted in increased resistance to nitrate. Overexpression was used instead of deletion because the genes that are essential for the process of sulfate reduction are essential for respiration in lactate-sulfate conditions.

A strain with mutations in the Rex binding site in the promoter of *sat* that overexpressed the *sat* gene (Christensen et al., 2014) was tested for resistance to nitrate. It had no advantage in nitrate stress conditions, especially compared with the Rex mutant (Fig. A1.6 A-C). Therefore, overexpression of the *sat* gene alone is not the mechanism by which the Rex mutant resists nitrate stress.

Figure A1.6 Growth of parental *D. vulgaris* Hildenborough vs. Δ*rex* mutant or mutant of Rex regulator binding site of sulfate adenylyltransferase (*sat*) in lactate-sulfate medium with addition of sodium nitrate. (A), (B), and (C) Growth of parental strain JW710 (A), Rex markerless deletion mutant (JW3319) (B), or mutant of Rex binding site of *sat* in which *sat* is overexpressed (JW9320) (C) with no additions (○) or 100 mM nitrate (□). Approximately 7% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.



Since nitrite is thought to inhibit the dissimilatory sulfite reductase (DsrAB) (Haveman et al., 2004) competitively, overexpression of this enzyme was attempted to determine whether such a construct would have greater resistance to nitrite. It has been shown that the sulfite reductase can slowly reduce nitrite *in vitro* (Wolfe et al., 1994), and therefore that nitrite has the potential to interfere with normal substrate binding (Greene et al., 2003). It was found that DsrABD overexpression relieved neither nitrite nor nitrate inhibition in the JW710 parental strain (Fig. A1.7 A). Furthermore, overexpression of this gene did not confer greater nitrate or nitrite resistance to a NrfA deletion mutant (Fig. A1.7 B). The strains with DsrABD overexpressed were tested for nitrite reductase activity and found to have no more nitrite reductase activity than the empty-vector control (Table A1.3). Confirmation of overexpression of Dsr in these strains has not been completed. Thus, it is possible that no functional Dsr enzyme was produced from the overexpressed genes. The results shown here (Fig. A1.7 A and B and Table A1.3), may reflect a lack of successful overexpression of DsrABD. It is also possible that the DsrD, which is not well-characterized, has an inhibitory function in the Dsr enzyme complex. Further, a lack of available electrons for reduction may explain the inability of the overexpressed genes to confer a beneficial phenotype in these stress conditions. Further work will be needed to determine whether Dsr activity has an influence on the nitrate- or nitrite-stress responses.

Figure A1.7 Growth of parental *D. vulgaris* Hildenborough vs. Δ*nrfA* nitrite reductase mutant with DsrABD overexpression in lactate-sulfate medium with additions of sodium nitrate or sodium nitrite. Growth of parental strain JW710 (A) or Δ*nrfA* markerless deletion mutant JW4502 (B) with empty vector (pMO9075) and no additions,(○); empty vector and 100 mM nitrate (△); empty vector and 1 mM nitrite (□); DsrABD overexpression plasmid (pMO4504) and no additions (●); DsrABD overexpression plasmid and 100 mM nitrate (▲); or DsrABD overexpression and 1 mM nitrite (■). Approximately 8.5% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.

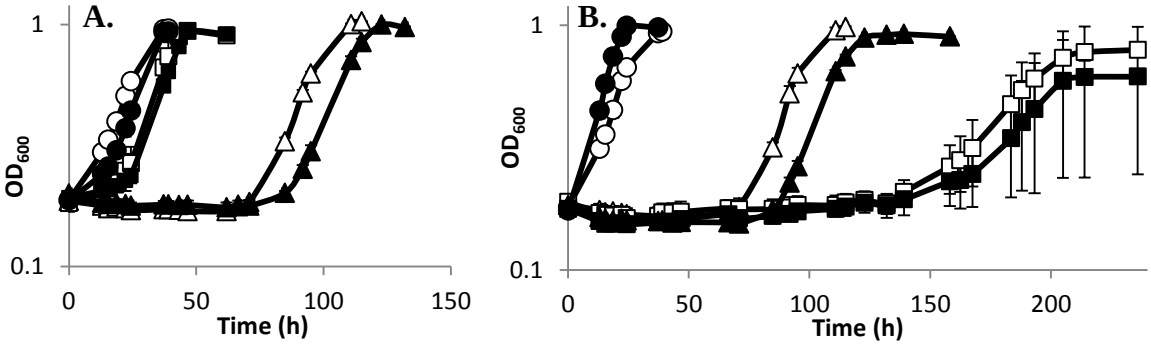


Table A1.3. Specific activity of nitrite reductase (NrfA) in strains of *D. vulgaris* Hildenborough

Strain	Specific Activity[a]
JW710+pMO9075 (Parent + empty vector)	1.58 ± 0.07
JW710+pMO4504 (Parent + DsrABD)	1.62 ± 0.19
JW4502+pMO9075 (Δ*nrfA* Mutant + empty vector)	0.13 ± 0.00
JW4502+pMO4504 (Δ*nrfA* Mutant + DsrABD)	0.12 ± 0.01

[a] Specific activity is reported in μmoles $MV^{+\cdot}$ oxidized min^{-1} mg of total $protein^{-1}$.

Activities were determined from three independent measurements with standard deviations shown.

For further investigation of the sulfate reduction pathway, the role of the proteins encoded by the *qmoABCD* genes in nitrate and nitrite stress responses was investigated. *qmoABCD* encode a quinone-interacting membrane-bound oxidoreductase that is essential for sulfate reduction and is thought to transfer electrons to the dissimilatory adenosine-5′- phosphosulfate reductase (APS reductase) in DvH (Zane et al., 2010; Ramos et al., 2012). A *qmoABCD* mutant and the complemented mutant (Zane et al., 2010) were grown in the presence of high nitrate. However, because the *qmoABCD* mutant is unable to grow with sulfate as a terminal electron acceptor (Zane et al., 2010), sulfite was used as the terminal electron acceptor. Under these conditions, neither nitrate nor nitrate was particularly inhibitory to any of the strains tested (Fig. A1.8 A-C). While this does, as previously noted (Korte et al., 2014), suggest a possible connection between nitrate and nitrite inhibition, it may also be the result of nonspecific stimulation of growth by sulfite. Therefore, the effect of *qmoABCD* overexpression on nitrate resistance of *D. vulgaris* grown in lactate-sulfate conditions was tested. The results indicated that overexpression of *qmoABCD* provides no growth advantage to DvH in the presence of 1 mM nitrite or 100 mM nitrate (Fig. A1.9 A and B). Therefore, I conclude that the proteins encoded by *qmoABCD* are unlikely to be directly involved in either nitrate or nitrite stress responses.

Figure A1.8 Growth of wild-type *D. vulgaris* Hildenborough vs. Δ*qmoABCD* mutant with *qmoABCD* overexpression/complementation in lactate-sulfite (MOLS3) medium with additions of sodium nitrate or sodium nitrite. (A), (B), and (C) Growth of wild-type with empty vector (pMO9075) (○), wild-type with *qmoABCD* overexpression plasmid (pMO9117) (●), Δ*qmoABCD* mutant with empty vector [JW9021 (pMO9075)](△), or Δ*qmoABCD* mutant with *qmoABCD* complementation [JW9021 (pMO9117)] (▲) with no additions **(A)**, 100 mM nitrate **(B)**, or 1 mM nitrite **(C)**. Approximately 5.5% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.

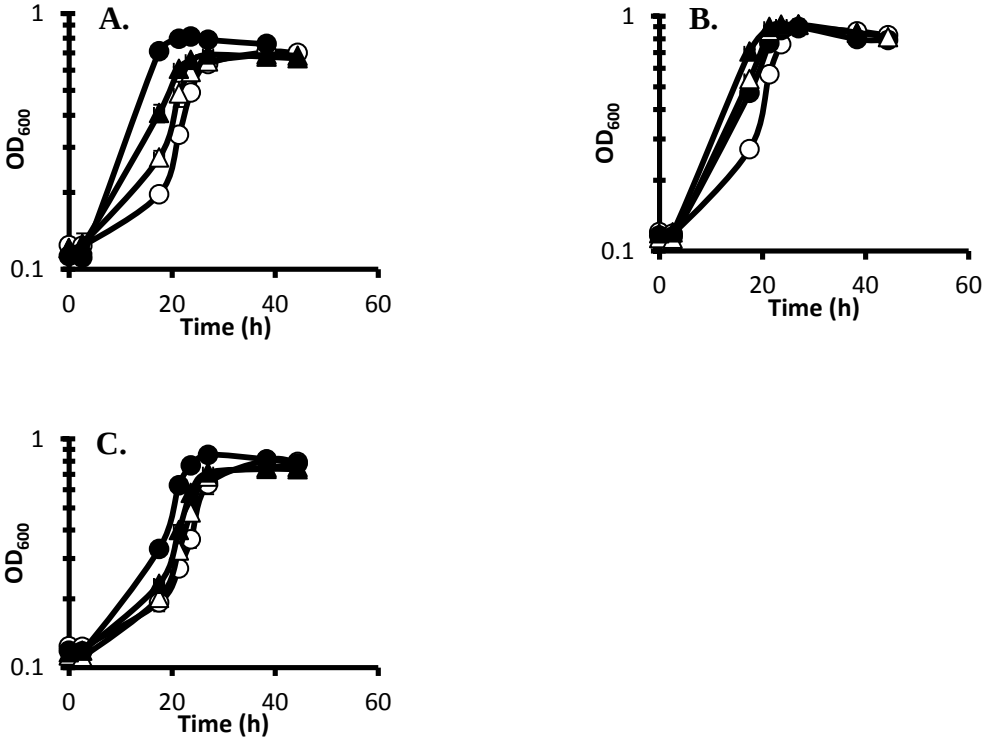
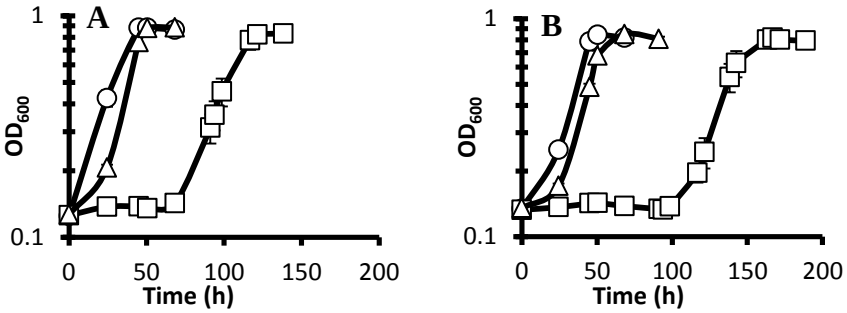


Figure A1.9 Growth of wild-type *D. vulgaris* Hildenborough with *qmoABCD* overexpression in lactate-sulfate medium with additions of sodium nitrite or sodium nitrate. (A) and (B) Growth of wild-type with empty vector (pMO9075) **(A)** or *qmoABCD* overexpression plasmid (pMO9117) **(B)** with no additions (○), 1 mM nitrite (△), or 100 mM nitrate (□). Approximately 7% (vol/vol) inocula were used. Readings reflect averages of three samples, and errors bars, which were often within the symbols, show standard deviations.



Taken together, these data addressing the connection between nitrate and the sulfate reduction pathway suggest that this pathway may not be the inhibitory target of nitrate. In contrast, it is well established that nitrite inhibits this pathway, specifically the dissimilatory sulfite reductase (Wolfe et al., 1994; Haveman et al., 2004). The data presented here therefore provide further evidence that nitrate and nitrite inhibition involve separate mechanisms (Korte et al., in review, Chapter 3). More data, however, will be necessary to provide definitive answers to the question of how nitrate inhibits sulfate-reducing bacteria.

IV. Discussion and future directions

The results shown here support the conclusion that not all nitrate-resistant mutants are also resistant to high levels of sodium chloride (IIIA); the DVU0245 "nitrate cluster" gene likely has a unique role in nitrate sensitivity (IIIB); nitrate resistance may be related to ion transport (IIIC); and that nitrate likely does not inhibit the sulfite reductase pathway (IIID). However, more will be required to elucidate the mechanism(s) of nitrate inhibition. For example, it is unclear why nitrate inhibits growth in lactate-sulfate medium but does not inhibit growth in lactate-sulfite medium. Sulfite is an energetically more favorable electron acceptor than sulfate (Thauer et al., 2007), and this energetic difference points to a mechanism by which nitrate may drain the energy resources of the cell. For example, nitrate may target the proton gradient across the cytoplasmic membrane or the cycling of electrons and protons. One component of these pathways, formate, was proposed to be an important intermediate when DvH was stressed with nitrate (He et al., 2010a). Preliminary data showed that a triple formate dehydrogenase markerless deletion mutant (JW2127) came out of lag phase about 30% faster than the parental strain in 100 mM nitrate (data not shown). This observation supports a connection between nitrate resistance and formate metabolism, although more studies will be needed to confirm these results and elucidate this connection. The results presented here do not exclude the possibility that nitrate inhibits sulfate reduction. A recent study of *D. alaskensis* G20 provides evidence that nitrate does inhibit the sulfate reduction pathway (Carlson et al., 2014), but more work will be needed to confirm whether this is the case.

Another mechanistic question which remains is how DvH can use nitrite as a

terminal electron acceptor. Studies have clearly reported that the NrfHA enzyme in *D. vulgaris* can reduce nitrite to ammonia with the quinone pool (menaquinol) as the electron donor (Rodrigues et al., 2006a; Rodrigues et al., 2006b; Rodrigues et al., 2008; Martins et al., 2010; Todorovic et al., 2012). Our current knowledge of electron flow in *D. vulgaris* and *D. alaskensis* indicates that electrons from lactate can directly enter the quinone pool through lactate dehydrogenase (Keller and Wall, 2011; Keller et al., 2014). However, when pyruvate is the electron donor, the electrons must travel through a transmembrane complex, out into the periplasm, through cytochrome c_3, and then back into the quinone pool through the Qrc complex (Keller et al., 2014). It is thought that electron confurcation may then allow electrons from the quinone pool to "merge" with electrons from ferredoxin (which is reduced in the further oxidation of pyruvate to acetate, inside the cell) for reduction of sulfate (Keller et al., 2014). However, if it is true that NrfHA only receives electrons from the quinone pool, then cytochrome c_3 mutants of *D. vulgaris* would be expected to grow with reduced efficiency (compared with the wild-type strain) with either lactate or pyruvate as an electron donor and nitrite as electron acceptor. Presumably no electrons from ferredoxin could reach nitrite as the final electron acceptor without the mediation of cytochrome c_3. This is in contrast to sulfate as electron acceptor, where lactate oxidation is not impaired in the c_3 mutant (Keller et al., 2014). Furthermore, a mutant lacking the Qrc complex, which is thought to mediate the transfer of electrons from cytochrome c_3 to the Qmo complex (Keller et al., 2014), should not be able to reduce electrons from pyruvate. These predictions are summarized in Table A1.4.

Table A1.4. Proposed tests to determine whether electrons flow through the quinone pool to NrfHA

Growth condition	Predicted result (reasoning)
Wild-type DvH with pyruvate/nitrite	Growth (electrons can get to quinone pool through cytochrome c_3)
Cyto c_3 mutant with pyruvate/nitrite	No growth (electrons cannot get to quinone pool because they would need to travel to periplasm/through cyto c_3 and Qrc to do so)
Cyto c_3 mutant with lactate/nitrite	Partial growth (not as good as wild-type, because electrons from pyruvate cannot reach the quinone pool; whereas, two electrons from lactate can reach that pool)
Qrc mutant with pyruvate/nitrite	No growth (electrons cannot get to quinone pool because they would need to travel to periplasm/through cyto c_3 and Qrc to do so)
Qrc mutant with lactate/nitrite	Partial growth (not as good as wild-type, because electrons from pyruvate cannot reach the quinone pool; whereas, two electrons from lactate can enter that pool)

In *Wolinella succinogenes*, formate and hydrogen have been shown to serve as electron donors for nitrite reduction (Simon, 2002). The oxidation of these substrates is coupled to proton production in the cytoplasm by the hydrogenase or formate dehydrogenase, and proton "pumping" also occurs through quinone cycling upon oxidation (Simon, 2002). Proposed studies to probe whether formate or hydrogen are oxidized to reduce nitrite are summarized, along with predicted results, in Table A1.5.

Table A1.5. Proposed tests to determine whether formate and/or hydrogen are necessary for DvH use of nitrite as electron acceptor

Growth condition	Predicted result (reasoning)
Wild-type DvH with formate/acetate/nitrite	Growth (electrons from formate are accessible to the NrfHA complex)
Wild-type DvH with hydrogen/acetate/nitrite	Growth (electrons from hydrogen are available to the NrfHA complex)
Formate dehydrogenase mutant with lactate/nitrite or pyruvate/nitrite	Impaired growth* compared with wild-type DvH (inability to get electrons from formate to NrfHA)
Hydrogenase mutants with lactate/nitrite or pyruvate/nitrite	Impaired growth* compared with wild-type DvH (inability to get electrons from hydrogen to NrfHA)

*Formate dehydrogenases and hydrogenases may compensate for one another in the reduction of nitrite. Thus, it may be necessary to make a deletion of all critical hydrogenases and formate dehydrogenases in order to produce a null phenotype under these conditions.

QmoABCD and Sat overexpression were conducted in part because of data from *D. alaskensis* G20. A Rex (Dde_2702) mutant of *D. alaskensis* was shown to have higher expression of Sat and Qmo genes in lactate-sulfate minimal medium than wild-type *D. alaskensis* (Kuehl et al., 2014). While the results reported here suggest that increased expression of Sat, DsrABD, or QmoABCD does not account for the nitrate resistance of the DvH Rex mutant, more data will be needed to confirm this.

It remains unclear why deletion of Rex would confer such a dramatic advantage in high nitrate. One possible reason is to allow the use of nitrite as a substrate for growth in the presence of high nitrate. To test this, the Rex mutant could be grown with nitrite as the sole nitrogen source in the presence of 100 mM nitrate. If the response to nitrate is as it is in lactate-sulfate conditions (Korte et al., 2014), then the mutant should be able to immediately use nitrite as an electron acceptor, without a lag (Korte et al., in review,

Chapter 3). Furthermore, the Rex mutant should be able to grow with nitrite as an electron acceptor in the presence of high nitrate. Preliminary data indicate that high nitrate compromises the use of nitrite as electron acceptor in wild-type *D. vulgaris* cultures (Korte et al., in review, Chapter 3). A Rex deletion might be able to overcome this limitation, perhaps by a shift in its electron distribution in the cell. In conclusion, additional studies will be needed to determine the mechanisms of nitrate inhibition and resistance, as well as the mechanisms by which nitrite is used as an electron acceptor.

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VITA

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