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

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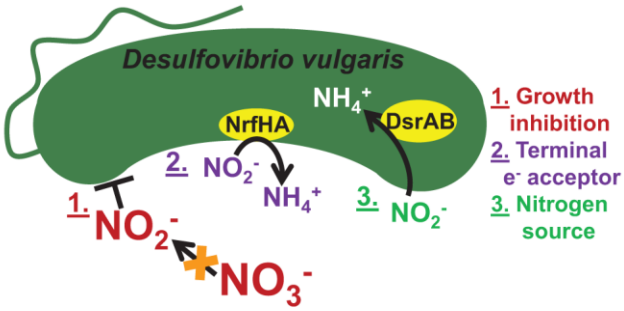


ABSTRACT

Sulfate-reducing microbes, such as *Desulfovibrio vulgaris* Hildenborough, cause "souring" of petroleum reservoirs through produced sulfide and precipitate heavy metals, either as sulfides or by alteration of the metal reduction state. Thus, inhibitors of these microbes, including nitrate and nitrite ions, are studied in order to limit their impact. Nitrite is a potent inhibitor of sulfate reducers and it has been suggested that nitrate does not inhibit these microbes directly but by reduction to nitrite which serves as the ultimate inhibitor. Here we provide evidence that nitrate inhibition of *D. vulgaris* can be independent of nitrite production. We also show that *D. vulgaris* can use nitrite as a nitrogen source or terminal electron acceptor for growth. Moreover, we report that use of nitrite as a terminal electron acceptor requires nitrite

reductase (*nrfA*) as a *D. vulgaris nrfA* mutant cannot respire nitrite but remains capable of utilizing nitrite as a nitrogen source. These results illuminate previously uncharacterized metabolic abilities of *D. vulgaris* that may allow niche expansion in low-sulfate environments. Understanding these abilities may lead to better control of sulfate-reducing bacteria in industrial settings and more accurate prediction of their interactions in the environment.

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.[1] Such souring leads to corrosion of pipes,[2, 3] to "plugging" of oil reservoirs by metal sulfides, and to health hazards for personnel working in the petroleum industry.[2] To prevent these problems, nitrate[4-6] and nitrite[2, 7] 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.[8] Survival in the presence of nitrate or nitrite may be especially beneficial in nitrate- and heavy metal-contaminated environmental sites[9] where sulfate reducers may help to immobilize the heavy metals by changing their redox state[10-12] or by precipitating them as insoluble sulfides.[13] Thus, studies have attempted to make the effects of nitrate[14-16] and nitrite[17, 18] 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.[14] In fact, nitrate and nitrite are sometimes considered to be mechanistically interchangeable in their effects on sulfate-reducing bacteria.[19] 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[14] or sodium nitrite[17] indicated few similarities between the stress responses.[14] Furthermore, we have recently identified a cluster of genes that, when mutated, conferred resistance to nitrate but not nitrite.[16, 20] 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.

MATERIALS AND METHODS

Strains and media

Strains and plasmids used in this study are listed in Table 1. We used the DvH parental strain JW710 that is deleted for *upp* for making marker exchange and markerless deletion strains.[21] Therefore, JW710 will be referred to as "wild-type" DvH in this study. Unless otherwise specified, DvH strains were grown in MO Basal Salts[22] 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.

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,[18] 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.

Plasmid and Strain Construction

Plasmids pMO4500, pMO4501, and pMO4505 were constructed and JW4500 marker-exchange deletion mutant was generated as previously described**.**[16] Primers are listed in Table S1. Construction of the markerless deletion, JW4502, was achieved as previously described[16] 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[16] 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.

Nitrite and Nitrate Determination

A scaled-down version of a previously described nitrite assay[23] 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. S1), and the instrument detection limit was 0.15 ± 0.05 µM. Nitrate determination was as described previously.[16]

Protein Determination

Whole cell protein concentrations were determined with the Bradford assay[24] 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 were confirmed with final whole cell protein measurements, and these values were well-correlated (Fig. S2).

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.[25] In brief, $MV^{+\cdot}$ stock was prepared in an anaerobic chamber by zinc reduction of MV^{2+} followed by filtration to remove the metal.[25] 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 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 (pDV1) Δ*upp*; 5-FU^r (Parent strain for markerless deletion mutants)	[21]
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	[21]
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	[26]
pMO9075	pMO719 containing P_{npt} for constitutive expression of complementation constructs; pBG1 stable SRB replicon; Sp^r	[27, 28]
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

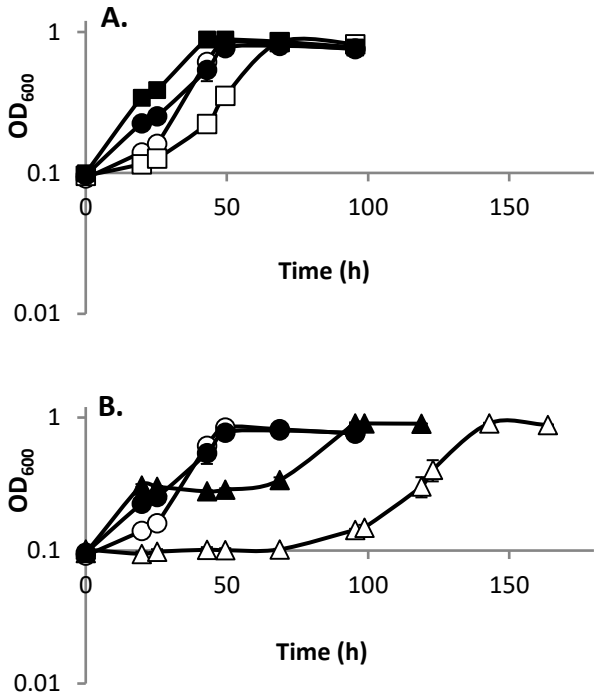
[a]Km, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript "r" or "s", resistance or sensitivity

[b] *nrfA* is DVU0625 of *D. vulgaris* Hildenborough

RESULTS

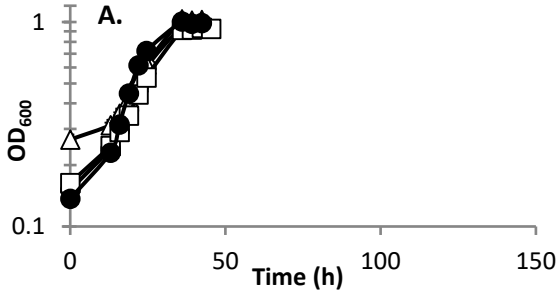
Since *in vitro* studies have reported that nitrite can bind to the dissimilatory sulfite reductase of DvH,[25] we first sought to investigate whether nitrite competitively inhibits the sulfite reductase *in vivo*. If so, we predicted that sulfite would relieve nitrite inhibition by outcompeting nitrite 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. 1A) and only partially relieved inhibition by 100 mM nitrate (Fig. 1B). 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.[22] 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,[29] stimulates growth of DvH (Fig 1). 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.

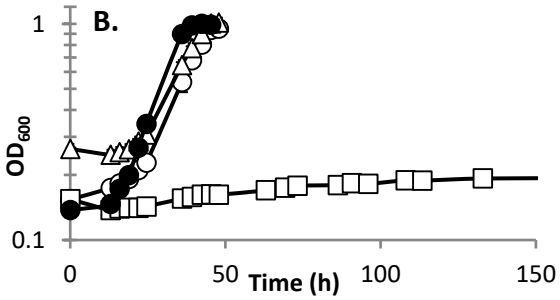
Figure 1 Growth of wild-type *D. vulgaris* Hildenborough (JW710) in lactate-sulfate medium with **(A)** nitrite or **(B)** nitrate plus sulfite. **(A)** Growth of DvH with no additions (○), 5 mM sulfite (●), 1 mM nitrite (□), or 5 mM sulfite plus 1 mM nitrite (■). **(B)** Growth of DvH with 100 mM nitrate (△), 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.[18, 30] 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. 2A) amended with 1 mM nitrite (Fig. 2B) or 100 mM nitrate (Fig. 2C). 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 2 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).





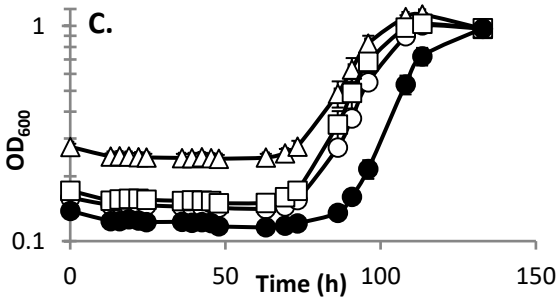


Table 2. Specific activity of nitrite reductase (NrfA) in strains of *D. vulgaris* Hildenborough

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

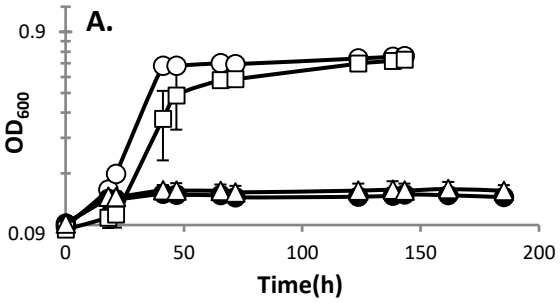
[a] Specific activity is reported in: μmoles $MV^{+\cdot}$ oxidized min^{-1} mg of total $protein^{-1}$. Activities were determined from three independent measurements with standard deviations shown.

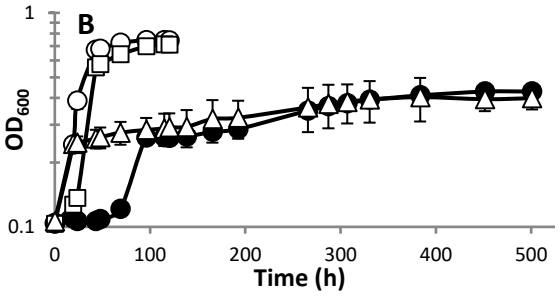
While the NrfA mutant with the empty vector was strongly inhibited in the presence of 1mM nitrite, the complemented mutant strain grew like the parental strain under these conditions (Fig. 2B). The successful complementation of the NrfA mutant, confirmed by enzyme assays (Table 2), is evidence that the P_{npt} promoter used for constitutive expression of *nrfA* provides robust expression of the complemented gene. In contrast to nitrite effects, the parental strain, deletion and complement grew similarly in the presence of 100 mM nitrate (Fig. 2C), 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.[16] Therefore, one interpretation of these results could be that nitrate inhibition is not mediated by the production of nitrite.

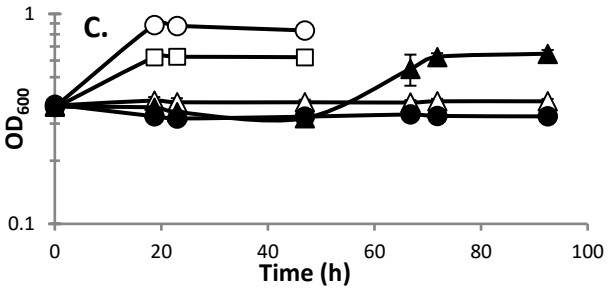
However, we considered the possibility that the nitrite accumulation from nitrate could be sufficiently low that its effect might be the same on the parent and mutant strains. It was previously reported that single colonies of a NrfA mutant of a DvH derivative had the same nitrite sensitivity as the parent strain, which was inhibited by concentrations above 40 μM nitrite.[18] 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,[31] it follows that DvH should be able to use subinhibitory concentrations of nitrite as a nitrogen source.

Figure 3 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.







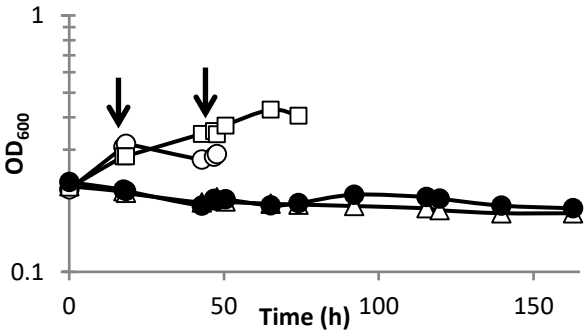
Further, we predicted that if small amounts of nitrite were produced when DvH is exposed to 100 mM nitrate, then nitrate could also be used by DvH as a sole nitrogen source. Ammonium is an energetically favorable nitrogen source for DvH, but strains containing the native plasmid pDV1 can also fix dinitrogen gas if sufficient energy is available.[32] 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. 3A). 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. 3B). 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. 3B) was expected, as this concentration has been shown to drastically increase the lag phase of DvH cultures.[14, 16, 33] 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. 3C). 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. 4). In contrast, as expected, 100 mM nitrate was not used as an electron acceptor (Fig. 4). This result prompted us to consider what enzyme was allowing this use of nitrite. Both the cytoplasmic sulfite reductase[25] and the periplasmic nitrite reductase[31] have been shown to mediate nitrite reduction, but previous attempts to demonstrate the use of nitrite as a terminal electron acceptor were unsuccessful.[31] To determine which enzyme, if either, might provide

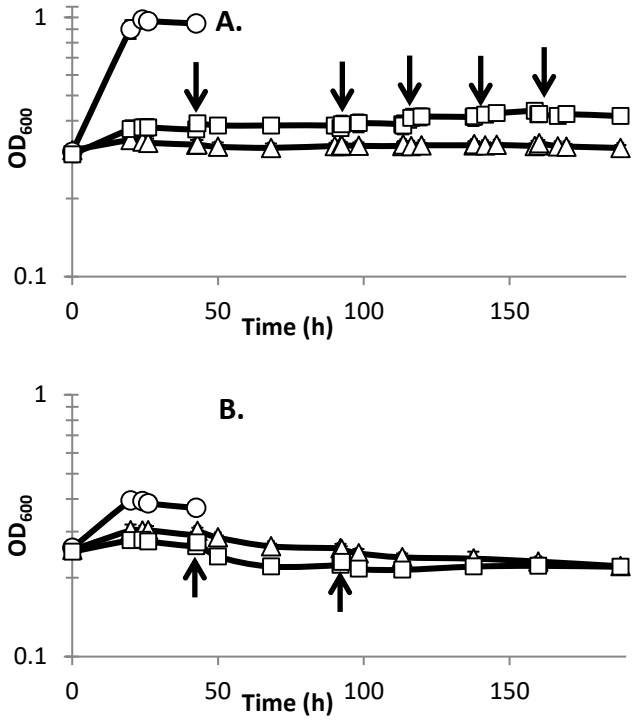
energy conservation, we tested the mutant deleted for *nrfA* to determine whether subinhibitory concentrations of nitrite could serve as a nitrogen source or an electron acceptor.

Figure 4. 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. 5A), it was unable to grow with nitrite as sole electron acceptor (Fig. 5B). 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.[18] The nitrite reduction capability is likely enabled by the sulfite reductase, DsrABD.[18] 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 5. 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).



DISCUSSION

The results reported here clarify fundamental interactions of DvH with nitrate and nitrite. We confirmed that, consistent with prior inferences,[14, 34] 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[16] 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/).[16, 20] This bacterium recovered from growth inhibition within 50 h in the presence of 150 mM nitrate in lactate-sulfate

medium.[16] Evidence has been presented that was consistent with nitrate inhibiting the sulfate reduction pathway, but not necessarily the sulfite reductase.[20]

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.[14] 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.[14] 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.[30, 35] 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.[30]

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.[17] In contrast, a gene encoding glutamine synthetase, which assimilates ammonium into amino acids,[36] was induced by the addition of 2.5 mM nitrite.[17] 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.[17] The reduction of nitrite by NrfA in *D. vulgaris* is particularly relevant to agricultural settings[37] 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[31] which has been cited[38, 39] as evidence of a lack of nitrite ammonification by this organism. However, in light of more current studies of nitrite toxicity,[17, 18] the high levels of nitrite used in the prior study[31] 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*,[38-42] 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[25] 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.[39, 41] Respiration of nitrite in DvH may therefore be very similar to the NrfHA-mediated nitrite respiration of the model nitrite reducer *Wolinella succinogenes*.[43] The coupling could be through menaquinone cycling with electrons from lactate dehydrogenase, which is apparently capable of delivering electrons to menaquinones.[44, 45]

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[2] 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[4] 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,[9] 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.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

405 **Table S1. Primers used for PCR amplification, Southern probe generation and sequencing**

Primer name	Primer sequence (5'-3')	Application
56HK-nrfA-up-47-F	GCCTTTTGCTGGCCTTTTGCTCACAT GCGTGGCGACTAT CTGTGCAA	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[16]]. Amplification of Southern probe for confirmation of DVU0625 deletion. forward
57HK-nrfA-up-52-R	GCGACAAGATATTCGGCACCAAGTA AGTTATTCATCGGCGACCTCTCTCGT G	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[16]]. Amplification of Southern probe for confirmation of DVU0625 deletion. reverse
58HK-nrfA-dn-46-F	GCGCCCCAGCTGGCAATTCCGGTTC CCGCTCTTTCG CAAAGGTATG	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[16]]. forward
59HK-nrfA-dn-46-R	GTCGAGGCATTTCTGTCCTGGCTGG CTTGCAGTACG CTCATGGGCT	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[16]]. 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	CAACGTTCGACG GTCGCAA	Sequencing primer to confirm downstream region of deletion cassette of pMO4500.
62HK-4500-4-upstrm-22-R	CCCATGAACTGG ACATGGCAGA	Sequencing primer to confirm upstream region of deletion cassette of pMO4500.
63HK-4500-4-dnstrm-20-F	ATGCAGGTGTGCGAGGTGTT	Sequencing primer to confirm downstream region of deletion cassette of pMO4500.

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 [16]. 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		

406

407 **Figure S1.** Standard curve for nitrite assay. Absorbance readings show averages of three replicates and
408 error bars show standard deviations.

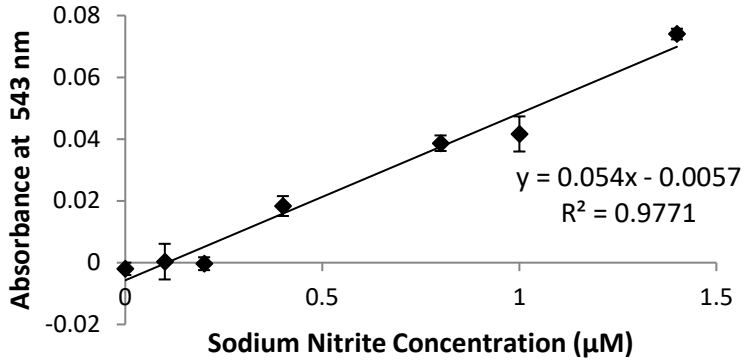
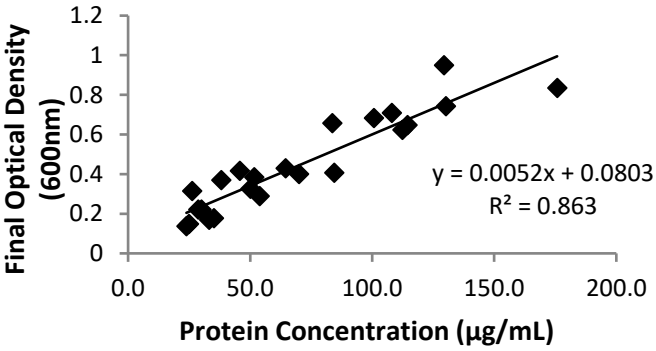


Figure S2. Final Optical Density of *D. vulgaris* Hildenborough cultures correlated with final whole cell proteins. Final average optical densities of cultures shown in Fig. 3-5 are plotted against the average final whole cell protein measurements for these same samples.



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