UC Berkeley

UC Berkeley Previously Published Works

Title

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

Permalink

<https://escholarship.org/uc/item/0wn7s5qs>

Journal

Environmental science & technology, 49(2)

ISSN

0013-936X

Authors

Korte, Hannah L
Saini, Avneesh
Trotter, Valentine V
et al.

Publication Date

2015

DOI

10.1021/es504484m

Peer reviewed

1 Independence of nitrate and nitrite inhibition of *Desulfovibrio vulgaris* Hildenborough 2 and use of nitrite as a substrate for growth

3 Hannah L. Korte,^{1,2} Avneesh Saini,^{2,3} Valentine V. Trotter,^{2,3} Gareth P. Butland,^{2,3} Adam P. Arkin,^{2,4} and
4 Judy D. Wall*,^{1,2}

5 ¹Department of Biochemistry, University of Missouri, Columbia, MO, USA

6 ²Ecosystems and Networks Integrated with Genes and Molecular Assemblies, Berkeley, CA, USA

7 ³Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

8 ⁴Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

9 *Corresponding author: wallj@missouri.edu; 573-882-8726

10 TOC/Abstract ART:

11

12 ABSTRACT

13 Sulfate-reducing microbes, such as *Desulfovibrio vulgaris* Hildenborough, cause “souring” of
14 petroleum reservoirs through produced sulfide and precipitate heavy metals, either as sulfides or by
15 alteration of the metal reduction state. Thus, inhibitors of these microbes, including nitrate and nitrite ions,
16 are studied in order to limit their impact. Nitrite is a potent inhibitor of sulfate reducers and it has been
17 suggested that nitrate does not inhibit these microbes directly but by reduction to nitrite which serves as the
18 ultimate inhibitor. Here we provide evidence that nitrate inhibition of *D. vulgaris* can be independent of
19 nitrite production. We also show that *D. vulgaris* can use nitrite as a nitrogen source or terminal electron
20 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, nitrate⁴⁻⁶ 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 sites⁹ where sulfate reducers may help to immobilize the heavy metals by changing their redox state¹⁰⁻¹² or by precipitating them as insoluble sulfides.¹³ Thus, studies have attempted to make the effects of nitrate¹⁴⁻¹⁶ 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 nitrate¹⁴ or sodium nitrite¹⁷ 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 Salts²² 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 25°C with an atmosphere of approximately 95% N₂ and 5% H₂.

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 34°C incubator for growth. A Genesys 20 spectrophotometer (Thermo Scientific,

Waltham, 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 described¹⁶ 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 constructs¹⁶ 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 assay²³ 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 R² 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

96 **Protein Determination**

97 Whole cell protein concentrations were determined with the Bradford assay²⁴ with bovine serum
98 albumin as the standard. Absorbance at 595 nm was measured with a Genesys 20 spectrophotometer. Final
99 optical densities for the growth kinetics studies shown in Figures 3-5 were confirmed with final whole cell
100 protein measurements, and these values were well-correlated (Fig. S2).

101 **Enzymatic Studies**

102 Nitrite-dependent oxidation of the chemically reduced radical cation methyl viologen ($MV^{+•}$) was
103 used as a marker for nitrite reductase (NrfA) activity. To prepare cell-free extract for activity assays, *D.*
104 *vulgaris* strains JW710 and JW4502, containing *nrfA* expression plasmid (pMO4501) or empty vector
105 (pMO9075), were grown in MOYLS4 liquid medium [supplemented with 0.2% (wt/vol) yeast extract
106 instead of 0.1% (wt/vol)] except that prior to inoculation the medium was reduced with Na_2S at a final
107 concentration of 1 mM. Cells were routinely cultured in the presence of spectinomycin (100 μ g/mL) with a
108 10% (vol/vol) inoculum and harvested by centrifugation from 1-L early stationary phase cultures. Cell
109 pellets were lysed at room temperature in an anaerobic chamber (Coy Laboratory Products, Inc.;
110 atmosphere of approximately 96% N_2 and 4% H_2) via resuspension in 2 mL of B-PER (Thermo Scientific)
111 containing 200 μ L of 10X protease inhibitor (Pierce Protease Inhibitor tablets, Product #8825) and 1 μ L of
112 Benzonase nuclease (Sigma) and 2 μ L of lysozyme (Sigma, 50 mg/mL) for 15 min. Cell lysate was cleared
113 by centrifugation for 15 min at 8,000 x g. Specific activity of NrfA was assayed in cell-free extract by
114 monitoring the decrease in absorbance at 578 nm of reduced $MV^{+•}$ used as electron source for the enzyme
115 during turnover. Nitrite reductase activity was assayed essentially as reported previously, with minor
116 modifications.²⁵ In brief, $MV^{+•}$ stock was prepared in an anaerobic chamber by zinc reduction of MV^{2+}
117 followed by filtration to remove the metal.²⁵ The assay was continuously monitored using a temperature-
118 controlled (set to 30°C) HP diode array spectrophotometer (Agilent Technologies) inside an anaerobic
119 chamber. All reagents were prepared with anoxic buffers and 3-ml open-top cuvettes were used for assays.
120 A standard nitrite reductase assay contained 2 mL activity assay buffer (50 mM Tris [pH 8.0], 0.1 M NaCl),

121 to which 12.5 mM reduced MV^{+} was added to give a starting OD_{578} of ~2 OD units. Sodium
122 diethyldithiocarbamate (10 μ M) was then added to the assay mixture to inhibit the non-enzymatic reduction
123 of nitrite by MV^{+} . OD readings were then allowed to stabilize for 30 seconds. A small volume of the cell-
124 free extract to be assayed (2-10 μ L) was added to the cuvette and any changes in OD_{578} monitored for 30
125 seconds. No baseline oxidation of MV^{+} was observed. The reaction was initiated by adding 12.5 mM of
126 sodium nitrite. One unit of specific activity is defined as the amount of MV^{+} oxidized (μ mol) over time
127 normalized to the amount of cell free protein extract used (μ mol min⁻¹ mg⁻¹ of total protein; extinction
128 coefficient, 9.8 mM⁻¹ cm⁻¹). Specific activities were determined for three independent experiments. No
129 oxidation of MV^{+} was observed for control assays containing only sodium nitrite and no cell-free extract.
130 Hydrogenases did not interfere by oxidizing reduced MV^{+} during the assay. Apparently the presence of
131 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
<i>Escherichia coli</i>		
α-Select (Silver Efficiency)	F ⁻ <i>deoR endA1 recA1 relA1 gyrA96 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 phoA Δ(lacZYA-argF)U169 Φ80lacZΔM15 λ⁻</i>	Bioline
<i>Desulfovibrio vulgaris</i>		
ATCC 29579	Wild-type <i>D. vulgaris</i> Hildenborough (pDV1); 5-FU ^s	ATCC
JW710	WT (pDV1) Δ <i>upp</i> ; 5-FU ^r (Parent strain for markerless deletion mutants)	²¹
JW4500	JW710 Δ <i>nrfA</i> ^b ::(<i>npt upp</i>); Km ^r , 5-FU ^s	This study
JW4502	JW710 Δ <i>nrfA</i> ; 5-FU ^r	This study
Plasmids		
pCR [®] 4-TOPO [®]	Cloning vector, Ap ^r , Km ^r , pUC <i>ori</i> .	Invitrogen Life Technologies
pCR [®] 8/GW/TOPO [®]	Cloning vector, Sp ^r , pUC <i>ori</i>	Invitrogen Life Technologies
pMO719	pCR [®] 8/GW/TOPO [®] containing SRB replicon (pBG1); Sp ^r ; source of Sp ^r , pUC <i>ori</i> fragment for marker exchange and markerless deletion suicide plasmid construction	²¹
pMO746	<i>upp</i> in artificial operon with <i>npt</i> and linked to Ap ^r -pUC <i>ori</i> from pCR [®] 4-TOPO [®] , P _{<i>npt-npt-upp</i>} ; Km ^r ; source of Kan ^r , <i>upp</i> fragment for marker exchange and markerless deletion suicide plasmid construction	²⁶
pMO9075	pMO719 containing P _{<i>npt</i>} for constitutive expression of complementation constructs; pBG1 stable SRB replicon; Sp ^r	^{27, 28}
pMO4500	Sp ^r and pUC <i>ori</i> from pMO719 plus upstream and downstream DNA regions from DVU0625 (<i>nrfA</i>) flanking the artificial operon of P _{<i>npt-npt-upp</i>} from pMO746; for marker exchange deletion mutagenesis; Sp ^r and Km ^r	This study
pMO4505	Sp ^r and pUC <i>ori</i> from pMO719 plus upstream and downstream DNA regions from DVU0625 (<i>nrfA</i>); for markerless deletion mutagenesis	This study
pMO4501	pMO9075 with DVU0625 (<i>nrfA</i>) constitutively expressed from P _{<i>npt</i>}	This study

^aKm, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript “r” or “s”, resistance or sensitivity

^b *nrfA* is DVU0625 of *D. vulgaris* Hildenborough

155 RESULTS

156 Since *in vitro* studies have reported that nitrite can bind to the dissimilatory sulfite reductase of
157 DvH,²⁵ we first sought to investigate whether nitrite competitively inhibits the sulfite reductase *in vivo*. If
158 so, we predicted that sulfite would relieve nitrite inhibition by outcompeting nitrite for the enzyme. If
159 nitrate inhibition were mediated by nitrite production, nitrate inhibition would also be relieved by sulfite.
160 Growth kinetics were determined for wild-type DvH inhibited by either 1 mM sodium nitrite or 100 mM
161 sodium nitrate in lactate-sulfate medium. Addition of 5 mM sulfite to DvH completely relieved inhibition
162 by 1 mM nitrite (Fig. 1A) and only partially relieved inhibition by 100 mM nitrate (Fig. 1B). Furthermore,
163 thiosulfate addition was similar to sulfite addition in its effect on nitrite or nitrate inhibition of DvH growth
164 (data not shown), since thiosulfate is reduced to sulfite before further reduction.²² Stocks of 100 mM
165 nitrate were assayed for nitrite content and found to contain less than 1 μ M, a concentration not inhibitory
166 to these bacteria (data not shown). Any inhibition caused by nitrite would therefore have required its
167 production from the 100 mM nitrate by the bacteria. Although we inferred a possible connection between
168 nitrate and nitrite inhibition through these results, we noted that low concentrations of sulfite, an electron
169 acceptor that does not require activation by ATP,²⁹ stimulates growth of DvH (Fig 1). Therefore, we
170 reasoned that sulfite might relieve nitrate and nitrite inhibition by the general effects of sulfite stimulation
171 and not by outcompeting nitrite for the sulfite reductase. Thus, whether nitrate was reduced to nitrite,
172 which then acted as the ultimate inhibitor of DvH growth, remained inconclusive.

173

174

175

176

177

178

179

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

187

188

189

190 To explore the inhibitory mechanisms of nitrate and nitrite further, a markerless, in-frame deletion
 191 of the gene encoding NrfA, the catalytic subunit of the periplasmic nitrite reductase, NrfHA, was
 192 constructed. The NrfHA enzyme is known to provide DvH protection against inhibition by nitrite.^{18, 30} We
 193 predicted that if nitrite were produced when DvH was exposed to 100 mM nitrate, then a NrfA mutant
 194 should be more sensitive to both nitrite and nitrate than the parental strain. Growth of this NrfA mutant was
 195 compared to that of the wild-type in lactate-sulfate medium (Fig. 2A) amended with 1 mM nitrite (Fig. 2B)
 196 or 100 mM nitrate (Fig. 2C). The mutant was also complemented with a constitutively expressed copy of
 197 the *nrfA* gene to confirm the absence of polar effects in the deletion mutant.

198 **Figure 2** Growth of *D. vulgaris* Hildenborough wild-type vs. $\Delta nrfA$ mutant in lactate-sulfate medium with
 199 inhibitory nitrogen species. (A) no additions, (B) 1 mM sodium nitrite, or (C) 100 mM sodium nitrate.
 200 Growth of the parental strain with empty vector [JW710(pMO9075)]($\circ$), parental strain with *nrfA*
 201 overexpression plasmid [JW710(pMO4501)](Δ), $\Delta nrfA$ mutant with empty vector [JW4502(pMO9075)]($\square$)
 202 and $\Delta nrfA$ mutant with *nrfA* complement plasmid [JW4502(pMO4501)]($\bullet$). A 28% (vol/vol) subculture
 203 from the initial 4 mL culture was made and a 9% (vol/vol) inoculum from this subculture was used for
 204 growth kinetic studies. Optical density readings show averages of three samples, and error bars show
 205 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 + <i>nrfA</i> complement	4.70 ± 0.49
JW4502(pMO9075); JW710 $\Delta nrfA$ + empty vector	0.13 ± 0.00
JW4502(pMO4501); JW710 $\Delta nrfA$ + <i>nrfA</i> complement	4.74 ± 0.75

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

215 While the NrfA mutant with the empty vector was strongly inhibited in the presence of 1mM nitrite, the
216 complemented mutant strain grew like the parental strain under these conditions (Fig. 2B). The successful
217 complementation of the NrfA mutant, confirmed by enzyme assays (Table 2), is evidence that the P_{npt}
218 promoter used for constitutive expression of *nrfA* provides robust expression of the complemented gene. In
219 contrast to nitrite effects, the parental strain, deletion and complement grew similarly in the presence of 100
220 mM nitrate (Fig. 2C), showing no effect of NrfA on the nitrate inhibition. It was expected that if nitrite
221 were produced from the 100 mM nitrate, it might be present in the NrfA mutant during the lag/inhibition
222 phase because the mutant had a decreased ability to reduce nitrite compared with the parental strain. The
223 nitrite concentration was therefore measured in wild type (empty vector) and NrfA mutant (empty vector)
224 cultures grown 70 h in the presence of 100 mM nitrate and was less than $15 \pm 5 \mu\text{M}$ (the limit of our
225 detection for diluted cultures) in both sets of cultures. A preliminary report of these nitrite measurements
226 was previously made and was consistent with the absence of measurable nitrate consumption by DvH
227 cultures grown in the presence of 100 mM nitrate.¹⁶ Therefore, one interpretation of these results could be
228 that nitrate inhibition is not mediated by the production of nitrite.

229 However, we considered the possibility that the nitrite accumulation from nitrate could be
230 sufficiently low that its effect might be the same on the parent and mutant strains. It was previously
231 reported that single colonies of a NrfA mutant of a DvH derivative had the same nitrite sensitivity as the
232 parent strain, which was inhibited by concentrations above $40 \mu\text{M}$ nitrite.¹⁸ We reasoned that if nitrate-
233 inhibited cells produced only micromolar concentrations of nitrite, then the NrfA mutant might not be
234 expected to have increased sensitivity to nitrate compared with the parent strain. However, such
235 concentrations of nitrite might be sufficient and therefore detectable for other metabolic roles such as a
236 nitrogen source or terminal electron acceptor.

237 Any conversion of nitrate to assimilable nitrogen in the form of ammonium requires intermediate
238 production of nitrite. Given that the NrfHA enzyme is known to be able to convert nitrite to ammonium,³¹
239 it follows that DvH should be able to use subinhibitory concentrations of nitrite as a nitrogen source.

240 **Figure 3** Growth of wild-type *D. vulgaris* Hildenborough with various nitrogen sources in lactate-sulfate
 241 lacking ammonium. **(A)** Low nitrate concentration, **(B)** high nitrate concentration and **(C)** high nitrate with
 242 nitrite. **(A)** Nitrogen additions were 1.5 mM NH_4Cl ($\circ$), 1.5 mM NaNO_3 ($\bullet$), 1.5 mM NaNO_2 ($\square$), or no
 243 additions ($\triangle$). A 6.4% (vol/vol) subculture from the initial 5 mL culture was made into NH_4 -free medium
 244 and a 6.4% (vol/vol) inoculum from this subculture was used for growth kinetic studies. **(B)** Nitrogen
 245 additions were 1.5 mM NH_4Cl ($\circ$), 100 mM NaNO_3 ($\bullet$), 1.5 mM NaNO_2 ($\square$), or no additions ($\triangle$). A 33%
 246 (vol/vol) subculture from the initial 5 mL culture was made into NH_4 -free medium and a 6.4% (vol/vol)
 247 inoculum from this subculture was used for growth kinetic studies. **(C)** Nitrogen additions were 5 mM
 248 NH_4Cl ($\circ$), 100 mM NaNO_3 ($\bullet$), 1 mM NaNO_2 ($\square$), 100 mM NaNO_3 plus 1 mM NaNO_2 ($\blacktriangle$), or no
 249 additions ($\triangle$). A 13% (vol/vol) subculture from the initial 5 mL culture was made into NH_4 -free medium
 250 and a 28% (vol/vol) inoculum from this subculture was used for growth kinetic studies. Optical density
 251 readings show averages of three or more samples and error bars show standard deviations (often within
 252 symbols). All tubes were degassed with argon.

256 Further, we predicted that if small amounts of nitrite were produced when DvH is exposed to 100 mM
 257 nitrate, then nitrate could also be used by DvH as a sole nitrogen source. Ammonium is an energetically
 258 favorable nitrogen source for DvH, but strains containing the native plasmid pDV1 can also fix dinitrogen
 259 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 reductase²⁵ and the periplasmic nitrite reductase³¹ 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

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

286 **Figure 4.** Growth of wild-type *D. vulgaris* Hildenborough in lactate medium with sulfite, nitrite, or nitrate
 287 as sole electron acceptor. Initial additions were 3 mM NaSO₃ (○), 100 mM NaNO₃ (●), 3 mM NaNO₂ (□),
 288 or no additions (△). Where indicated by arrows, additions of approximately 3.7 mM NaNO₂ were made to
 289 5 mL cultures that had started with 3 mM nitrite. To cultures with no additions or 100 mM nitrate, the
 290 same volume of deionized water was added at these times. No additions were made to cultures with 3 mM
 291 sulfite. A 33% (vol/vol) subculture from the initial 5 mL culture was made into lactate-sulfate medium and
 292 a 19% (vol/vol) inoculum from this subculture was used for growth kinetic studies. Optical density
 293 readings show averages of three samples, and error bars show standard deviations (often within symbols).

294

295

296 We found that while the NrfA mutant could use nitrite as a nitrogen source (Fig. 5A), it was unable to grow
 297 with nitrite as sole electron acceptor (Fig. 5B). The ability of the NrfA mutant to reduce very low
 298 concentrations of nitrite is consistent with the reported observations of nitrite reduction by a NrfA mutant in
 299 a DvH strain lacking the native plasmid.¹⁸ The nitrite reduction capability is likely enabled by the sulfite
 300 reductase, DsrABD.¹⁸ However, the inability of the NrfA mutant to use nitrite as a terminal electron
 301 acceptor may indicate that the NrfHA enzyme is responsible for energy conservation or that the increased
 302 sensitivity of the mutant to added nitrite prevented an observable nitrite-dependent growth.

303

304

305

306

307

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

320 DISCUSSION

321 The results reported here clarify fundamental interactions of DvH with nitrate and nitrite. We
 322 confirmed that, consistent with prior inferences,^{14, 34} nitrate inhibition of pure cultures of DvH is not
 323 mediated by the production of nitrite under the conditions tested. This means that predictions of bacterial
 324 responses to nitrate and nitrite stress in the environment should consider these ions as separate inhibitors
 325 and not as a single entity. Indeed, our prior work suggested unique inhibitory mechanisms¹⁶ for nitrate and
 326 nitrite. Separate inhibitory mechanisms were also exhibited by the model sulfate reducer *Desulfovibrio*
 327 *alaskensis* G20, which lacks an annotated nitrite reductase (<http://microbesonline.org/>).^{16, 20} This bacterium
 328 recovered from growth inhibition within 50 h in the presence of 150 mM nitrate in lactate-sulfate

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

331 The ability of *D. alaskensis* to grow in the presence of high nitrate is, therefore, analogous to the
332 nitrate resistance of the NrfA mutant of DvH presented in this work. The results presented here are
333 surprising in light of previous reports that, in the presence of nitrate, the nitrite reductase *nrfA* gene of DvH
334 is overexpressed.¹⁴ Increased transcription of the *nrfA* gene in the presence of high nitrate contributed to
335 the prior assumption that nitrate inhibition was mediated by nitrite production.¹⁴ In the present study, nitrite
336 was undetected by assays or by production of a nitrogen source used to support growth of DvH
337 monocultures in the presence of 100 mM nitrate.

338 In a new isolate of *Desulfovibrio desulfuricans* capable of growth by nitrate ammonification,
339 Dalsgaard and Bak (1994) reported that nitrate reduction was dramatically inhibited by the presence of
340 quite low concentrations of sulfide. The presence of sulfide carried over from inoculating cultures in our
341 experiments could potentially inhibit conversion of nitrate to nitrite by DvH. Additional studies would be
342 needed to determine if DvH can convert nitrate to nitrite under sulfide-free conditions. However, the
343 results presented here are relevant since bacteria that are known to produce nitrite from nitrate are often
344 present in environments where sulfate-reducing bacteria are found.^{30, 35} DvH may respond to nitrate as a
345 signal that nitrite may also be present and prepare DvH for detoxification of nitrite produced by nearby
346 nitrate-reducing bacteria.³⁰

347 In addition, increased transcription of *nrfA* genes may enable use of nitrite as an environmental
348 nitrogen source. Production of ammonium from nitrite could be especially helpful in an environment low
349 in reduced nitrogen substrates. DvH may have specific response mechanisms to assist in this process, as
350 the addition of 2.5 mM nitrite to mid-log-phase DvH cells has been reported to cause down-regulation of
351 genes involved in amino acid transport and catabolism.¹⁷ In contrast, a gene encoding glutamine
352 synthetase, which assimilates ammonium into amino acids,³⁶ was induced by the addition of 2.5 mM
353 nitrite.¹⁷ In light of the present study, these prior results indicate that DvH sensed an excess of ammonium

354 availability and was able to slow down costly transport pathways in favor of nitrogen assimilation by
355 glutamine synthetase.¹⁷ The reduction of nitrite by NrfA in *D. vulgaris* is particularly relevant to
356 agricultural settings³⁷ because dissimilatory nitrite reduction to ammonium promotes nitrogen retention,
357 rather than loss (as N₂ or N₂O), in soil.

358 Shown in this work is a previously unknown role for nitrite in DvH metabolism, its use as a terminal
359 electron acceptor supporting growth. In soils or fresh water environments low in sulfate, this capacity
360 could allow niche expansion of DvH. Our report that DvH can respire nitrite refutes a previous report³¹
361 which has been cited^{38, 39} as evidence of a lack of nitrite ammonification by this organism. However, in
362 light of more current studies of nitrite toxicity,^{17, 18} the high levels of nitrite used in the prior study³¹ would
363 be expected to completely inhibit DvH, compromising the test for nitrite respiration. The current study
364 indicates that nitrite can indeed be used by *D. vulgaris* as an electron acceptor when supplied at
365 subinhibitory concentrations. This metabolic ability may be especially useful to investigators studying the
366 essential components of sulfate reduction, because nitrite could be used as an alternative electron acceptor
367 for mutant strains unable to grow with sulfate as an electron acceptor.

368 Use of nitrite as electron acceptor brings up the question of which enzyme is allowing this growth.
369 The results reported here, as well as a wealth of *in vitro* studies of the NrfHA “model” enzyme from *D.*
370 *vulgaris*,³⁸⁻⁴² indicate that it is likely the NrfHA enzyme complex, rather than the sulfite reductase, that
371 allows *D. vulgaris* to use nitrite as an electron acceptor. While the sulfite reductase can reduce nitrite, its
372 high affinity for nitrite and a low turnover number for nitrite reduction²⁵ may inhibit its ability to use nitrite
373 efficiently as a terminal electron acceptor. Furthermore, there is strong evidence that NrfHA accepts
374 electrons from the menaquinone pool.^{39, 41} Respiration of nitrite in DvH may therefore be very similar to
375 the NrfHA-mediated nitrite respiration of the model nitrite reducer *Wolinella succinogenes*.⁴³ The coupling
376 could be through menaquinone cycling with electrons from lactate dehydrogenase, which is apparently
377 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 industry² 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 recover⁴ 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.

ACKNOWLEDGEMENTS—This material by ENIGMA- Ecosystems and Networks Integrated with Genes and Molecular Assemblies (<http://enigma.lbl.gov>), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological & Environmental Research under contract number DE-AC02-05CH11231. We thank Paul D. Adams, LBNL SFA Laboratory Research Manager for ENIGMA, for his leadership and guidance.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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

Primer name	Primer sequence (5'-3')	Application
56HK-nrfA-up-47-F	<u>GCCTTTTGCTGGCCTTTTGCTCACAT</u> 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 <i>ori</i> fragment [SpecRpUC-R ¹⁶]. Amplification of Southern probe for confirmation of DVU0625 deletion. forward
57HK-nrfA-up-52-R	<u>GCGACAAGATATTCGGCACCAAGTA</u> <u>AGTTATTCATCGGCGACCTCTCTCGT</u> 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 , <i>upp</i> fragment [UppCterm ¹⁶]. Amplification of Southern probe for confirmation of DVU0625 deletion. reverse
58HK-nrfA-dn-46-F	<u>GCGCCCCAGCTGGCAATTCCGGTTC</u> 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 , <i>upp</i> fragment [KanPromNterm ¹⁶]. forward
59HK-nrfA-dn-46-R	<u>GTCGAGGCATTTCTGTCCTGGCTGG</u> 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 <i>ori</i> 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	<u>AGGTTGGGAAGCCCTGCAATGCAGT</u> <u>CCCAGGAGGTACCATATGAATAACC</u> AGAAGACGTTCAAGGGGT	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	<u>GATCGTGATCCCCTGCGCCATCAGA</u> <u>TCCTTGCTACTGCTTGGCGGAGACC</u> A	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	<u>CATACCTTTGCGAAAGAGCGGGAAT</u> TATTCATCGGCGACCTCTCTCGTG	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	<u>CACGAGAGAGGTCGCCGATGAATAA</u> TTCCCGCTCTTTCGCAAAGGTATG	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.

References

1. Ligthelm, D. J.; de Boer, R. B.; Brint, J. F.; Schulte, W. M. Reservoir souring. An analytical model for H₂S generation and transportation in an oil reservoir owing to bacterial activity. In *Offshore Eur. 91 - Proc.*, Society of Petroleum Engineers of AIME: Aberdeen, Scotland, 1991; pp 369-378.
2. Voordouw, G.; Buziak, B.; Lin, S.; Grigoryan, A.; Kaster, K. M.; Jenneman, G. E.; Arensdorf, J. J. In *Use of nitrate or nitrite for the management of the sulfur cycle in oil and gas fields*, SPE Internat. Symp. Oilfield Chem., Houston, TX, 2007; Society of Petroleum Engineers: Houston, TX, 2007; pp 485-491.
3. Zhu, X. Y.; Modi, H.; Kilbane II, J. J. Efficacy and risks of nitrate application for the mitigation of SRB-induced corrosion. In *CORROSION*, NACE International: San Diego, California, 2006; pp 065241-065244.

4. Voordouw, G.; Grigoryan, A. A.; Lambo, A.; Lin, S.; Park, H. S.; Jack, T. R.; Coombe, D.; Clay, B.; Zhang, F.; Ertmoed, R.; Miner, K.; Arensdorf, J. J. Sulfide remediation by pulsed injection of nitrate into a low temperature Canadian heavy oil reservoir. *Environ. Sci. Technol.* **2009**, *43*, (24), 9512-9518.
5. Grigoryan, A.; Lambo, A.; Lin, S.; Cornish, S. L.; Jack, T. R.; Voordouw, G. Souring remediation by field-wide nitrate injection in an Alberta oil field. *J. Canadian Petroleum Technol.* **2009**, *48*, (5), 58-61.
6. Callbeck, C. M.; Dong, X.; Chatterjee, I.; Agrawal, A.; Caffrey, S. M.; Sensen, C. W.; Voordouw, G. Microbial community succession in a bioreactor modeling a souring low-temperature oil reservoir subjected to nitrate injection. *Appl. Microbiol. Biotechnol.* **2011**, *91*, (3), 799-810.
7. Kaster, K. M.; Grigoriyan, A.; Jenneman, G.; Voordouw, G. Effect of nitrate and nitrite on sulfide production by two thermophilic, sulfate-reducing enrichments from an oil field in the North Sea. *Appl. Microbiol. Biotechnol.* **2007**, *75*, (1), 195-203.
8. Callbeck, M. C.; Agrawal, A.; Voordouw, G. Acetate production from oil under sulfate-reducing conditions in bioreactors injected with sulfate and nitrate. *Appl. Environ. Microbiol.* **2013**, *79*, (16), 5059-5068.
9. Green, S. J.; Prakash, O.; Jasrotia, P.; Overholt, W. A.; Cardenas, E.; Hubbard, D.; Tiedje, J. M.; Watson, D. B.; Schadt, C. W.; Brooks, S. C.; Kostka, J. E. Denitrifying bacteria from the genus *Rhodanobacter* dominate bacterial communities in the highly contaminated subsurface of a nuclear legacy waste site. *Appl. Environ. Microbiol.* **2012**, *78*, (4), 1039-47.
10. Lovley, D. R.; Roden, E. E.; Phillips, E. J. P.; Woodward, J. C. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geol.* **1993**, *113*, (1-2), 41-53.
11. Lloyd, J. R.; Ridley, J.; Khizniak, T.; Lyalikova, N. N.; Macaskie, L. E. Reduction of technetium by *Desulfovibrio desulfuricans*: biocatalyst characterization and use in a flowthrough bioreactor. *Appl. Environ. Microbiol.* **1999**, *65*, (6), 2691-2696.
12. Chardin, B.; Dolla, A.; Chaspoul, F.; Fardeau, M.; Gallice, P.; Bruschi, M. Bioremediation of chromate: thermodynamic analysis of the effects of Cr(VI) on sulfate-reducing bacteria. *Appl. Microbiol. Biotechnol.* **2003**, *60*, (3), 352-360.
13. Jalali, K.; Baldwin, S. A. The role of sulphate reducing bacteria in copper removal from aqueous sulphate solutions. *Water Res.* **2000**, *34*, (3), 797-806.
14. He, Q.; He, Z.; Joyner, D. C.; Joachimiak, M.; Price, M. N.; Yang, Z. K.; Yen, H. C. B.; Hemme, C. L.; Chen, W.; Fields, M. M.; Stahl, D. A.; Keasling, J. D.; Keller, M.; Arkin, A. P.; Hazen, T. C.; Wall, J. D.; Zhou, J. Impact of elevated nitrate on sulfate-reducing bacteria: a comparative study of *Desulfovibrio vulgaris*. *ISME J.* **2010**, *4*, (11), 1386-1397.
15. Redding, A. M.; Mukhopadhyay, A.; Joyner, D. C.; Hazen, T. C.; Keasling, J. D. Study of nitrate stress in *Desulfovibrio vulgaris* Hildenborough using iTRAQ proteomics. *Brief. Funct. Genomic. Proteomic.* **2006**, *5*, (2), 133-143.
16. Korte, H. L.; Fels, S. R.; Christensen, G. A.; Price, M. N.; Kuehl, J. V.; Zane, G. M.; Deutschbauer, A. M.; Arkin, A. P.; Wall, J. D. Genetic basis for nitrate resistance in *Desulfovibrio* strains. *Front. Microbiol.* **2014**, *5*, (153).
17. He, Q.; Huang, K. H.; He, Z.; Alm, E. J.; Fields, M. W.; Hazen, T. C.; Arkin, A. P.; Wall, J. D.; Zhou, J. Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Appl. Environ. Microbiol.* **2006**, *72*, (6), 4370-4381.
18. Haveman, S. A.; Greene, E. A.; Stilwell, C. P.; Voordouw, J. K.; Voordouw, G. Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *J. Bacteriol.* **2004**, *186*, (23), 7944-7950.
19. Gieg, L. M.; Jack, T. R.; Foght, J. M. Biological souring and mitigation in oil reservoirs. *Appl. Microbiol. Biotechnol.* **2011**, *92*, (2), 263-282.
20. Carlson, H. K.; Kuehl, J. V.; Hazra, A. B.; Justice, N. B.; Stoeva, M. K.; Sczesnak, A.; Mullan, M. R.; Iavarone, A. T.; Engelbrektson, A.; Price, M. N.; Deutschbauer, A. M.; Arkin, A. P.; Coates, J. D. Mechanisms of direct inhibition of the respiratory sulfate-reduction pathway by (per)chlorate and nitrate. *ISME J.* **2014**.

- 481 21. Keller, K. L.; Bender, K. S.; Wall, J. D. Development of a markerless genetic exchange system for
482 *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation
483 efficiency. *Appl. Environ. Microbiol.* **2009**, 75, (24), 7682-7691.
- 484 22. Zane, G. M.; Bill Yen, H. C.; Wall, J. D. Effect of the deletion of *qmoABC* and the promoter-distal
485 gene encoding a hypothetical protein on sulfate reduction in *Desulfovibrio vulgaris* Hildenborough. *Appl.*
486 *Environ. Microbiol.* **2010**, 76, (16), 5500-5509.
- 487 23. American Public Health Association *Standard methods for the examination of water and waste*
488 *water, 18th ed.*; American Water Works Association and Water Pollution Control Federation: Washington,
489 DC, 1992.
- 490 24. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of
491 protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, 72, 248-54.
- 492 25. Wolfe, B. M.; Lui, S. M.; Cowan, J. A. Desulfovibridin, a multimeric-dissimilatory sulfite reductase
493 from *Desulfovibrio vulgaris* (Hildenborough). *Eur. J. Biochem.* **1994**, 223, (1), 79-89.
- 494 26. Parks, J. M.; Johs, A.; Podar, M.; Bridou, R.; Hurt Jr, R. A.; Smith, S. D.; Tomanicek, S. J.; Qian,
495 Y.; Brown, S. D.; Brandt, C. C.; Palumbo, A. V.; Smith, J. C.; Wall, J. D.; Elias, D. A.; Liang, L. The
496 genetic basis for bacterial mercury methylation. *Science* **2013**, 339, (6125), 1332-1335.
- 497 27. Keller, K. L.; Wall, J. D.; Chhabra, S. Methods for engineering sulfate reducing bacteria of the
498 genus *Desulfovibrio*. *Methods Enzymol.* **2011**, 497, 503-517.
- 499 28. Keller, K. L.; Rapp-Giles, B. J.; Semkiw, E. S.; Porat, I.; Brown, S. D.; Wall, J. D. New model for
500 electron flow for sulfate reduction in *Desulfovibrio alaskensis* G20. *Appl. Environ. Microbiol.* **2014**, 80,
501 (3), 855-868.
- 502 29. Postgate, J. R. *The Sulfate-Reducing Bacteria, 2nd ed.*; Cambridge University Press: Cambridge,
503 U.K., 1984.
- 504 30. Greene, E. A.; Hubert, C.; Nemati, M.; Jenneman, G. E.; Voordouw, G. Nitrite reductase activity of
505 sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria.
506 *Environ. Microbiol.* **2003**, 5, (7), 607-617.
- 507 31. Pereira, I. A. C.; LeGall, J.; Xavier, A. V.; Teixeira, M. Characterization of a heme c nitrite
508 reductase from a non-ammonifying microorganism, *Desulfovibrio vulgaris* Hildenborough. *Biochim.*
509 *Biophys. Acta - Protein Struct. Mol. Enzymol.* **2000**, 1481, (1), 119-130.
- 510 32. Heidelberg, J. F.; Seshadri, R.; Haveman, S. A.; Hemme, C. L.; Paulsen, I. T.; Kolonay, J. F.; Eisen,
511 J. A.; Ward, N.; Methe, B.; Brinkac, L. M.; Daugherty, S. C.; Deboy, R. T.; Dodson, R. J.; Durkin, A. S.;
512 Madupu, R.; Nelson, W. C.; Sullivan, S. A.; Fouts, D.; Haft, D. H.; Selengut, J.; Peterson, J. D.; Davidsen,
513 T. M.; Zafar, N.; Zhou, L.; Radune, D.; Dimitrov, G.; Hance, M.; Tran, K.; Khouri, H.; Gill, J.; Utterback,
514 T. R.; Feldblyum, T. V.; Wall, J. D.; Voordouw, G.; Fraser, C. M. The genome sequence of the anaerobic,
515 sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat. Biotech.* **2004**, 22, (5), 554-559.
- 516 33. Elias, D. A.; Mukhopadhyay, A.; Joachimiak, M. P.; Drury, E. C.; Redding, A. M.; Yen, H. C. B.;
517 Fields, M. W.; Hazen, T. C.; Arkin, A. P.; Keasling, J. D.; Wall, J. D. Expression profiling of hypothetical
518 genes in *Desulfovibrio vulgaris* leads to improved functional annotation. *Nucleic Acids Res.* **2009**, 37, (9),
519 2926-2939.
- 520 34. Wall, J. D.; Bill Yen, H. C.; Drury, E. C. Evaluation of stress response in sulphate-reducing bacteria
521 through genome analysis. In *Sulphate-reducing bacteria: environmental and engineered systems*; Barton, L.
522 L.; Hamilton, W. A., Eds.; Cambridge University Press: New York, 2007; pp 141-165.
- 523 35. Haveman, S. A.; Greene, E. A.; Voordouw, G. Gene expression analysis of the mechanism of
524 inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrate-reducing, sulfide-oxidizing bacteria. *Environ.*
525 *Microbiol.* **2005**, 7, (9), 1461-1465.
- 526 36. Merrick, M. J.; Edwards, R. A. Nitrogen control in bacteria. *Microbiological Reviews* **1995**, 59, (4),
527 604-22.
- 528 37. Welsh, A.; Chee-Sanford, J.; Connor, L.; Löffler, F.; Sanford, R. Refined NrfA phylogeny improves
529 PCR-based *nrfA* gene detection. *Appl. Environ. Microbiol.* **2014**, 80, (7), 2110-2119.

- 530 38. Martins, G.; Rodrigues, L.; Cunha, F. M.; Matos, D.; Hildebrandt, P.; Murgida, D. H.; Pereira, I. A.;
531 Todorovic, S. Substrate binding to a nitrite reductase induces a spin transition. *J. Phys. Chem. B.* **2010**, *114*,
532 (16), 5563-6.
- 533 39. Rodrigues, M. L.; Oliveira, T. F.; Pereira, I. A.; Archer, M. X-ray structure of the membrane-bound
534 cytochrome c quinol dehydrogenase NrfH reveals novel haem coordination. *EMBO J.* **2006**, *25*, (24), 5951-
535 60.
- 536 40. Rodrigues, M. L.; Oliveira, T.; Matias, P. M.; Martins, I. C.; Valente, F. M.; Pereira, I. A.; Archer,
537 M. Crystallization and preliminary structure determination of the membrane-bound complex cytochrome c
538 nitrite reductase from *Desulfovibrio vulgaris* Hildenborough. *Acta Crystallogr. Sec.t F Struct. Bio.l Cryst.*
539 *Commun.* **2006**, *62*, (Pt 6), 565-8.
- 540 41. Rodrigues, M. L.; Scott, K. A.; Sansom, M. S.; Pereira, I. A.; Archer, M. Quinol oxidation by c-type
541 cytochromes: structural characterization of the menaquinol binding site of NrfHA. *J. Mol. Biol.* **2008**, *381*,
542 (2), 341-50.
- 543 42. Todorovic, S.; Rodrigues, M. L.; Matos, D.; Pereira, I. A. C. Redox properties of lysine- and
544 methionine-coordinated hemes ensure downhill electron transfer in NrfH2A4 nitrite reductase. *J. Phys.*
545 *Chem. B.* **2012**, *116*, (19), 5637-5643.
- 546 43. Simon, J. Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol.*
547 *Rev.* **2002**, *26*, (3), 285-309.
- 548 44. Reed, D. W.; Hartzell, P. L. The *Archaeoglobus fulgidus* D-lactate dehydrogenase is a Zn(2+)
549 flavoprotein. *J. Bacteriol.* **1999**, *181*, (24), 7580-7.
- 550 45. Keller, K. L.; Wall, J. D. Genetics and molecular biology of the electron flow for sulfate respiration
551 in *Desulfovibrio*. *Front. Microbiol.* **2011**, *2*, (135).
552
553