

Regulation of Nitrite Stress Response in *Desulfovibrio vulgaris* Hildenborough, a Model Sulfate-Reducing Bacterium

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ABSTRACT

Sulfate-reducing bacteria (SRB) are sensitive to low concentrations of nitrite, and nitrite has been used to control SRB-related biofouling in oil fields. *Desulfovibrio vulgaris* Hildenborough, a model SRB, carries a cytochrome *c*-type nitrite reductase (*nrhA*) that confers resistance to low concentrations of nitrite. The regulation of this nitrite reductase has not been directly examined to date. In this study, we show that DVU0621 (NrfR), a sigma54-dependent two-component system response regulator, is the positive regulator for this operon. NrfR activates the expression of the *nrhA* operon in response to nitrite stress. We also show that *nrhR* is needed for fitness at low cell densities in the presence of nitrite because inactivation of *nrhR* affects the rate of nitrite reduction. We also predict and validate the binding sites for NrfR upstream of the *nrhA* operon using purified NrfR in gel shift assays. We discuss possible roles for NrfR in regulating nitrate reductase genes in nitrate-utilizing *Desulfovibrio* spp.

IMPORTANCE

The NrfA nitrite reductase is prevalent across several bacterial phyla and required for dissimilatory nitrite reduction. However, regulation of the *nrhA* gene has been studied in only a few nitrate-utilizing bacteria. Here, we show that in *D. vulgaris*, a bacterium that does not respire nitrate, the expression of *nrhA* is induced by NrfR upon nitrite stress. This is the first report of regulation of *nrhA* by a sigma54-dependent two-component system. Our study increases our knowledge of nitrite stress responses and possibly of the regulation of nitrate reduction in SRB.

Sulfate-reducing bacteria (SRB) are important members of syntrophic anaerobic microbial communities. While SRB are useful in remediation of contaminated groundwater by reduction of toxic heavy metals (1), they are also a major problem in offshore oil industries, where they cause biofouling due to corrosive sulfide production (2). Additions of nitrate and nitrite have been used to control SRB growth and the resulting biofouling sulfide (3, 4). Nitrite is more effective for inhibition of SRB than nitrate (3), and most SRB are sensitive to low concentrations of nitrite (5, 6). Nitrite is toxic because it inhibits sulfite reduction by competing for the sulfite reductase enzyme (7, 8). Also, the reaction of nitrite with sulfide to form polysulfide results in the release of reactive nitrogen species (9).

The sensitivity to nitrite varies among SRB. Some SRB, such as *Desulfovibrio alaskensis* G20, lack any means for reducing the nitrite and are highly sensitive to small amounts of nitrite (10, 11). However, other SRB can reduce nitrite via a cytochrome *c*-type nitrite reductase, NrfA, and are able to tolerate low millimolar amounts of nitrite (7, 12). NrfA-carrying SRB also can utilize nitrite as the sole terminal electron acceptor (TEA), provided the nitrite is at subinhibitory concentrations (12–14). Some nitrite-reducing SRB also have the ability to utilize nitrate as the TEA via dissimilatory nitrate/nitrite reduction (15).

NrfA is a cytochrome *c* nitrite reductase that catalyzes a six-electron reduction of nitrite to ammonium, thus carrying out dissimilatory nitrite reduction (16) in contrast to the copper-containing or cytochrome *cd*₁ type of nitrite reductases that are involved in denitrification and catalyze the conversion of nitrite to nitric oxide (NO) (17). NrfA is widely present across the *Gamma*-, *Delta*-, and *Epsilonproteobacteria* (16) and in other phyla, such as *Firmicutes* and *Bacteroidetes* (18, 19), and has been well studied in

nitrate-respiring organisms, such as *Desulfovibrio desulfuricans* (20), *Escherichia coli* (21), and *Wolinella succinogenes* (22). The *nrhA* gene is associated with either *nrhH*, as seen in *Delta*- and *Epsilonproteobacteria*, or *nrhBCD*, as seen in the *Gammaproteobacteria*, and these accessory genes help in the transfer of electrons to NrfA (23). NrfA is also of particular interest in pathogenic organisms since it has also been shown to reduce NO and is one of the defenses used against nitrosative stress (24, 25).

The regulation of *nrhA* has been studied in only a few systems but in none for SRB, where this metabolism has specific importance. The induction of *nrhA* in response to nitrate and nitrite has been documented in *E. coli* via the two-component systems NarQP and NarXL (26) and in *Shewanella oneidensis* via NarQP (27). Fumarate and nitrate reductase regulator (FNR)-dependent activation in response to anaerobic conditions was seen in *E. coli*

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(26) and *Haemophilus influenzae* (28). NarQP- and FNR-dependent regulation of *nrfA* has been predicted for a number of *Gammaproteobacteria* (29). Cyclic-AMP receptor protein (Crp)-dependent transcription was also seen in *S. oneidensis* (27). Regulation by NO-sensitive transcription regulators was seen in *E. coli* (30) and has been suggested for *W. succinogenes* (31).

The SRB *Desulfovibrio vulgaris* Hildenborough does not respire nitrate and can use only nitrite as a TEA at low concentrations (12). Its NrfA has been proposed to function primarily as a detoxifying system to remove nitrite produced transiently by other community members, such as nitrate-respiring bacteria (7). The NrfHA complex has been purified from *D. vulgaris* and crystallized (32). The nitrite reductase activity appears to be constitutively present, even in the absence of nitrite (11, 33). Tiling array data also showed high expression of *nrfHA* genes in the absence of nitrite (34). However, microarray gene expression analysis revealed that the *nrfHA* genes have increased transcript abundance (6- to 12-fold) in the presence of nitrite (7, 35). Further, the *nrfHA* genes were determined to be the potential target for a sigma54-dependent two-component system, DVU0621-DVU0622 (NrfSR), in a system-wide *in vitro* microarray-based DNA-affinity-purified (DAP) chip assay that examined several response regulators in *D. vulgaris* (36).

Here, we demonstrate *in vivo* that NrfR is the physiologically relevant nitrite-responsive activator that induces transcription of the *nrfHA* operon in the presence of nitrite. While the constitutive expression of *nrfHA* is sufficient to overcome nitrite stress at high cell densities, NrfR strongly contributes to fitness for nitrite stress at low cell densities.

MATERIALS AND METHODS

***D. vulgaris* growth conditions.** *D. vulgaris* was grown in defined medium containing 8 mM MgCl₂, 20 mM NH₄Cl, 2.2 mM K₂PO₄, 0.6 mM CaCl₂, 30 mM Tris, 1 ml/liter of Thauer's vitamins (37), 12.5 ml/liter of trace element solution (38), and 640 µl/liter of resazurin (0.1%) and supplemented with 50 mM Na₂SO₄ and 60 mM sodium lactate (LS4D medium). The pH of the medium was adjusted to 7.2 with 1 N HCl. Cultures were grown at 30°C in an anaerobic growth chamber (Coy Laboratory Products, Grass Lake, MI). For transposon mutants, the medium was supplemented with the antibiotic G418 (400 µg/ml) (Sigma-Aldrich, St. Louis, MO), and for complemented strains, the medium was supplemented with the antibiotic spectinomycin (100 µg/ml).

Construction of GZ2270 (*nrfR*::mini-Tn5). The *nrfR* mutant was available from a library of transposon mutants (<http://desulfovibriomaps.biochem.missouri.edu/mutants/>). A description of the library is in preparation. The library has been used in other studies (39–41).

Complementation of GZ2270 (*nrfR*::mini-Tn5). Complementation of GZ2270 was accomplished by constructing a plasmid (pMO9381) that is nonreplicative in *D. vulgaris*. pMO9381 contains the pUC origin of replication, a gene conferring spectinomycin resistance, and the region from 1,352 bases upstream of *nrfR* (DVU0621) to the stop codon of *nrfR*. The plasmid was constructed by amplifying the regions of interest with the DNA polymerase Herculase II (Agilent Technologies, Cedar Creek, TX) and assembled using the sequence- and ligation-independent cloning (SLIC) method (42). The cloned region from *D. vulgaris* in pMO9381 was sequenced to ensure that no mutations were introduced in the complemented strain. GZ2270 was electroporated with pMO9381, and transformants were selected on MOYLS4 (43) plates containing spectinomycin. Next, to ensure that a complete, restored copy of *nrfR* followed the gene immediately upstream (DVU0622, *nrfS*) such that *nrfR* is expressed in a native context, several isolates were PCR screened to determine the location of the integration of pMO9381. An isolate that passed the screening criteria (JW9382) was used in this study. Primers were obtained from

Integrated DNA Technologies (Coralville, IA), and their sequences are listed in Table S1 in the supplemental material.

Effect of nitrite on growth. The *D. vulgaris* wild-type (WT), GZ2270 (*nrfR*::mini-Tn5), and JW9382 strains were inoculated at 5% (vol/vol) into 400 µl LS4D (4 replicates each) with and without 1 mM sodium nitrite and grown in honeycomb well plates (Growth Curves USA, Piscataway, NJ). Growth was measured with a Bioscreen C instrument (Growth Curves USA) housed within the anaerobic glove box at 30°C.

Nitrite determination. The *D. vulgaris* WT, GZ2270, and JW9382 strains were grown overnight in MOYLS4. Each strain was subcultured to an optical density at 600 nm (OD₆₀₀) of 0.04 in four tubes of LS4D (20 ml medium), two with and two without 1 mM sodium nitrite. At time zero and at time intervals of ~12 h, cultures were monitored for growth by measuring the OD₆₀₀ spectrophotometrically, and 400-µl aliquots were taken for nitrite analysis. The aliquots were centrifuged at 16,000 × g for 5 min at 4°C, and the supernatants (diluted 10-fold with water where necessary) were assayed for nitrite with a Griess reagent assay kit (Life Technologies, Grand Island, NY). The assay was set up in a 96-well microtiter plate, and absorbance at 580 nm was measured in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were determined with a sodium nitrite standard curve.

Nitrite stress and biomass production. The WT, GZ2270, and JW9382 strains were each grown in six 10-ml LS4D cultures (in 15-ml tubes) until the mid-log phase (OD₆₀₀ of 0.3). For each strain, sodium nitrite was added to three tubes to a final concentration of 2.5 mM, and equal amounts of sterile anoxic water were added to the remaining three tubes (control). After incubation for 60 min, 1.5-ml culture aliquots were centrifuged at 16,000 × g for 5 min at 4°C. The cell pellets were immediately frozen in liquid nitrogen and stored at –80°C until ready for RNA extraction.

Nitrate stress and NO stress. The WT, GZ2270, and JW9382 strains were each grown in nine 10-ml LS4D cultures (in 15-ml tubes) until the mid-log phase (OD₆₀₀ of 0.3). For each strain, three of the tubes received 100 mM sodium nitrate, three tubes received 10 µM NO donor compound S-nitrosoglutathione (GSNO), and the last three tubes received no additions. After incubation for 60 min, 3-ml culture aliquots were mixed with 6 ml of Bacteria Protect reagent (Qiagen, Valencia, CA) and stored at room temperature for 1 to 3 days until RNA extraction.

RNA extraction. Cells stored in Bacteria Protect reagent were pelleted by centrifugation at 9,000 × g for 15 min at 4°C. RNA was extracted from the cell pellets with an RNeasy minikit (Qiagen, Valencia, CA) after being lysed with lysozyme (1 mg/ml), according to the manufacturer's instructions. DNA contamination was removed by treatment with 1 µl of Turbo DNA-free DNase (Life Technologies, Grand Island, NY) at 37°C for 30 min. The DNase was inactivated with the inactivation reagent provided with the kit. RNA was quantified spectrophotometrically on a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Wilmington, DE). The quality was assessed by inspecting the 16S and 23S rRNA bands after electrophoresis on a 1% (wt/vol) agarose gel.

qRT-PCR. RNA (500 ng) was reverse transcribed with iScript reverse transcription mix (Bio-Rad, Hercules, CA). The resulting cDNA was diluted 10-fold, and 2 µl was used as the template for reverse transcriptase quantitative PCRs (qRT-PCRs). qRT-PCRs (10-µl volume) were set up with SsoAdvanced universal SYBR green supermix (Bio-Rad) and analyzed on a CFX96 real-time PCR instrument (Bio-Rad). Amplification efficiencies for the primer sets were determined by the standard curve method and were between 90 and 110%. Fold changes in expression were calculated with the CFX96 software that is based on qBase+ (44). Values were normalized to those of four reference genes: the sigma70 gene *rpoD* (DVU1788), the ribosomal protein gene *rplS* (DVU0835), the Clp protease subunit *clpP* (DVU1335), and a cysteinyl tRNA synthetase gene, *cysS* (DVU1579). Reference gene stability values, or *M* values (44), for the chosen reference genes were <0.5. The reference genes were chosen based on the available nitrite stress microarray data (35). Primers were designed with Primer3 software (45) and obtained from Integrated DNA Technol-

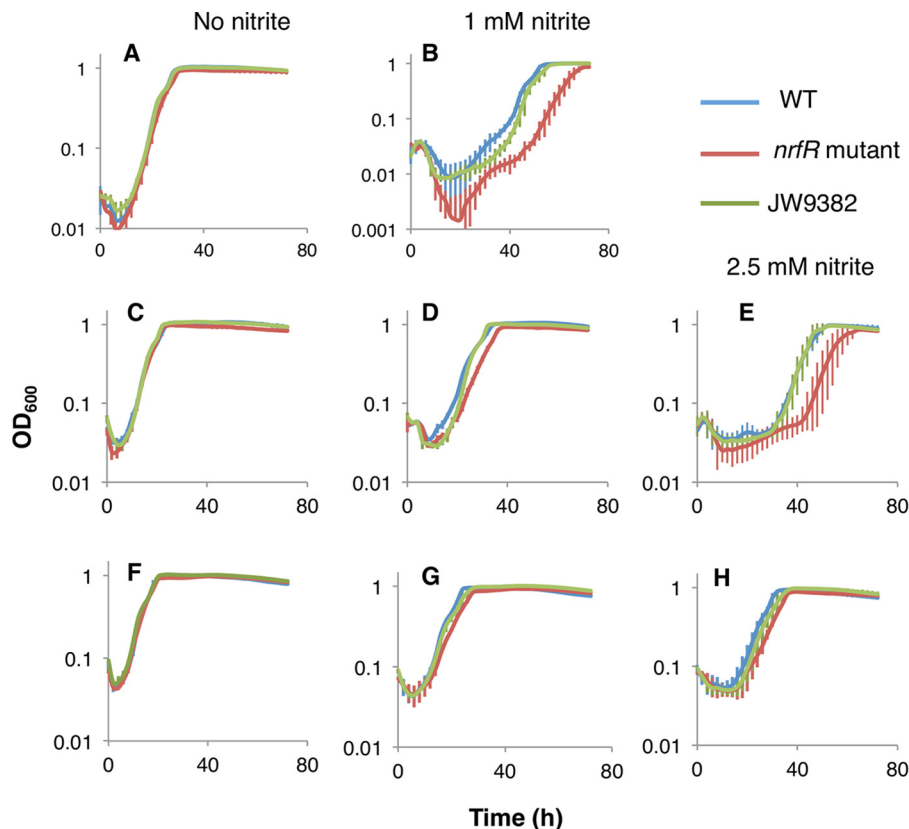


FIG 1 NrfR is needed for fitness during nitrite stress at low cell densities. The WT, *nrfR* mutant, and JW9382 strains were grown on LS4D with no nitrite addition (A, C, F) or with the addition of sodium nitrite at 1 mM (B, D, G) or 2.5 mM (E, H). Cells were inoculated from overnight cultures to a starting OD₆₀₀ of 0.02 (A, B), 0.05 (C to E), or 0.08 (F to H). Growth measurements were made in a Bioscreen growth analysis system. The data are averages from four biological replicates.

ogies (San Diego, CA). Sequences are provided in Table S1 in the supplemental material. Raw data and calculations are provided in Table S2 (for nitrite stress), Table S3 (for NO stress), and Table S4 (for nitrate stress) in the supplemental material.

Prediction of binding sites. For prediction of the NrfR binding motif, the RegPredict Web server was used (46). Sets of upstream sequences (bp −400 to +50 relative to the predicted translational start site) were searched for a common sequence motif by the Discover Profiles tool of RegPredict. The upstream sequences of a set of *nrfH* (DVU0624) orthologs from eight genomes containing *nrfR* genes were selected for identification of the NrfR binding motif. Binding sites were identified by a comparative genomics approach implemented on the RegPredict Web server as described previously (39).

Electrophoretic mobility shift assay. DNA substrates (25 bp) to test binding site motif predictions were prepared by annealing biotinylated oligonucleotides with their unlabeled complementary strands as described previously (36, 47). NrfR (~56-kDa) protein was purified with a C-terminal V5 epitope and a 6×His tag as described previously (36). NrfR protein was mixed with 100 fmol of biotinylated DNA substrate in 10 mM Tris HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and 1 μg/ml poly(dI-dC) in a total reaction volume of 20 μl and incubated at room temperature for 20 min. Electrophoresis, blotting, and chemiluminescent detection were performed as described previously (36). Final imaging of the blot was done using the FluorChem Q system (Protein Simple, Santa Clara, CA).

RESULTS

NrfR is required for an optimal response to nitrite stress. We tested whether a putative NrfR-null mutant of *D. vulgaris* exhibits

a growth defect in response to nitrite addition. The GZ2270 *nrfR*::mini-Tn5 mutant strain was generated by insertion of a transposon at nucleotide 208 of the 1,398 nucleotides predicted for the gene DVU0621. On LS4D, the wild type and the *nrfR* mutant grew similarly, but with slightly reduced yields for the mutant (Fig. 1A). Wild-type *D. vulgaris* grew with a long lag phase on LS4D with 1 mM nitrite (Fig. 1A and B), but the growth rates and final cell yields remained similar to those observed in the absence of nitrite (Fig. 1A and B), suggesting that growth resumes normally once the nitrite is reduced. This is consistent with the results of previous studies showing that sulfate reducers, such as *D. vulgaris* isolates that carry nitrite reductase genes, are only transiently inhibited by nitrite (6). We observed that the response to nitrite stress was highly dependent on cell densities, as was previously reported (7). At higher cell densities, WT *D. vulgaris* was able to grow in the presence of 2.5 mM and 5 mM nitrite (see Fig. S1 in the supplemental material).

When inoculated into LS4D with 1 mM sodium nitrite at a low starting OD₆₀₀ of 0.02, the *nrfR* mutant showed a longer lag than the WT, although the growth rates and yields were similar (Fig. 1B). At a higher starting OD₆₀₀ of 0.05, the fitness defect of the *nrfR* mutant was observed strongly at a nitrite concentration of 2.5 mM rather than 1 mM (Fig. 1C to E). When the cells were inoculated at even higher cell densities (OD₆₀₀ of 0.08), the *nrfR* mutant grew almost as well as the wild type (Fig. 1F to H).

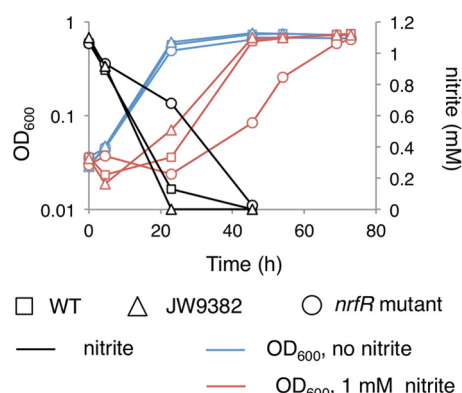


FIG 2 The *nrfR* mutant reduces nitrite slower than the WT. The WT, *nrfR* mutant, and JW9382 strains were grown on LS4D with and without 1 mM sodium nitrite. Growth was measured by monitoring the OD₆₀₀ spectrophotometrically. Nitrite consumption was monitored for the cultures with nitrite addition. The data are averages from two biological replicates.

To confirm that the growth impairment in the presence of nitrite at low cell densities was due to the absence of the *nrfR* gene, we complemented the mutant by inserting a chromosomal copy of the gene with its native promoter (strain JW9382). JW9382 behaved similarly to the WT with respect to growth on LS4D with or without nitrite additions (Fig. 1).

We also measured nitrite levels during growth of the WT and the *nrfR* mutant on LS4D with 1 mM nitrite. We observed that in the mutant, nitrite attenuation occurred at a slower rate than in the WT or JW9382 (Fig. 2). For all three strains, the cells enter the exponential phase of growth only after the nitrite was attenuated below detection levels (<1 μ M) (Fig. 2). Our results suggest not only that NrfR is needed for an optimal and faster response to nitrite stress but also that the NrfHA levels in the absence of *nrfR* are sufficient to eventually overcome the nitrite stress. This is consistent with earlier reports on the constitutive expression of nitrite reductase (11, 33, 34).

NrfR activates transcription of *nrfH* under nitrite stress.

Next, we examined gene expression by qRT-PCR analysis of the nitrite reductase *nrfH* gene under nitrite stress. The WT, *nrfR* mutant, and JW9382 strains were subjected to nitrite stress with 2.5 mM sodium nitrite at the mid-log phase (see Materials and Methods) for 60 min. The *nrfH* gene was upregulated after nitrite exposure in the WT and JW9382 (~4-fold) but not in the mutant (Fig. 3), indicating that NrfR activates the transcription of *nrfH*A in the presence of nitrite. The *nrfR* mutant showed ~4-fold downregulation of *nrfH* in the presence of nitrite, and we propose an explanation for this observation in Discussion.

The *nrfH* gene is not induced under nitrate or NO stress. Because SRB can occur in environments where they may be exposed to nitrite as a result of the activity of nitrate-reducing bacteria and also because *nrfH*A-carrying bacteria often have the ability to reduce nitrate, we tested the effect of sodium nitrate on *nrfH* gene expression. *D. vulgaris* cannot reduce nitrate, and growth is moderately inhibited by an unknown mechanism at 100 mM sodium nitrate (10, 48). The WT, *nrfR* mutant, and JW9382 strains were exposed to 100 mM sodium nitrate at the mid-log phase for 1 h. We observed that the expression levels of the reference genes of choice, *rplS* and *rpoD*, were strongly decreased (~5- to 10-fold) upon the addition of nitrate (Fig. 4A). Previous transcriptomic

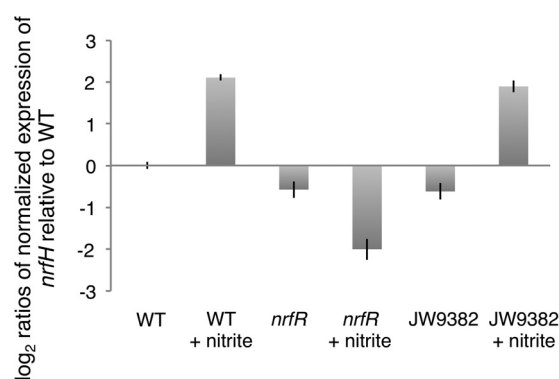


FIG 3 NrfR activates transcription of *nrfH* in response to nitrite. The WT, *nrfR* mutant, and JW9382 strains were subjected to nitrite stress at the mid-log phase with 2.5 mM sodium nitrite for 1 h. The qRT-PCR plot shows log₂ ratios of normalized expression of the *nrfH* gene relative to the WT without nitrite stress. Values were normalized to four reference genes. The data are averages from three biological replicates. The error bars represent standard deviations.

analysis of nitrate stress (48) also showed downregulation of several candidate reference genes. Therefore, we normalized the expression changes to that of total RNA. We observed no effect on *nrfH* transcription in the WT and JW9382 strains with nitrate addition. However, we did observe decreased transcript abundance (5-fold) for *nrfH* in the *nrfR* mutant in the presence of nitrate ($P = 0.002$, with respect to that in the WT) (Fig. 4A).

We then examined if *nrfH* is induced upon NO stress, since the NrfA nitrite reductase in some bacteria can reduce NO and plays a role in NO stress (24, 25). The NO donor *S*-nitrosoglutathione (GSNO) was added at 10 μ M to mid-log-phase cells. This concentration was previously shown to cause moderate inhibition of growth in *D. vulgaris* (41). Addition of GSNO did not affect *nrfH* transcription in the WT, the *nrfR* mutant, or the JW9382 strain (Fig. 4B). To confirm that the cells were experiencing NO stress, we examined expression of one of the hybrid cluster protein-encoding genes, *hcp2*, that has been shown previously to be induced by NO stress (41). We observed that the *hcp2* gene was strongly induced in all three strains in the presence of GSNO (Fig. 4B).

NrfR binds to predicted binding sites upstream of *nrfH*A. We used comparative genomics to predict the binding site motif for NrfR. We observed a 16-bp consensus sequence, GTC(A/T)(G/T)N TTTTCTG(A/T)N, in the upstream regions of orthologs of *nrfH*A in 11 genomes (Fig. 5A; see also Table S5 in the supplemental material). In *D. vulgaris*, the promoter region of *nrfH*A has two such predicted binding sites at -322 and -302 upstream of the start codon. Purified NrfR shifted this predicted motif in a gel shift assay (Fig. 5B). When substitutions were made in the conserved palindromic part of the motif, the shift was abolished (Fig. 5B).

Conservation of *nrfR* and *nrfH*A genes across *Desulfovibrio* spp. Since constitutive nitrite reductase activity has been observed in *D. vulgaris* (11, 33), *Desulfovibrio gigas* (49), and *D. desulfuricans* (50), we examined how conserved the *nrfR* gene was relative to the nitrite reductase *nrfH*A genes. The *nrfH*A genes are present in a cluster of species closely related to *D. vulgaris* Hildenborough (Fig. 6). These include *D. vulgaris* Miyazaki, *Desulfovibrio* sp. strain A2, *Desulfovibrio terminidis*, and species that are host associated, such as *D. desulfuricans* as well as the related *Bilophila* spp. These species also share the *nrfR* gene. *Lawsonia intracellularis* is a non-sulfate-reducing obligate intracellular pathogen that clusters

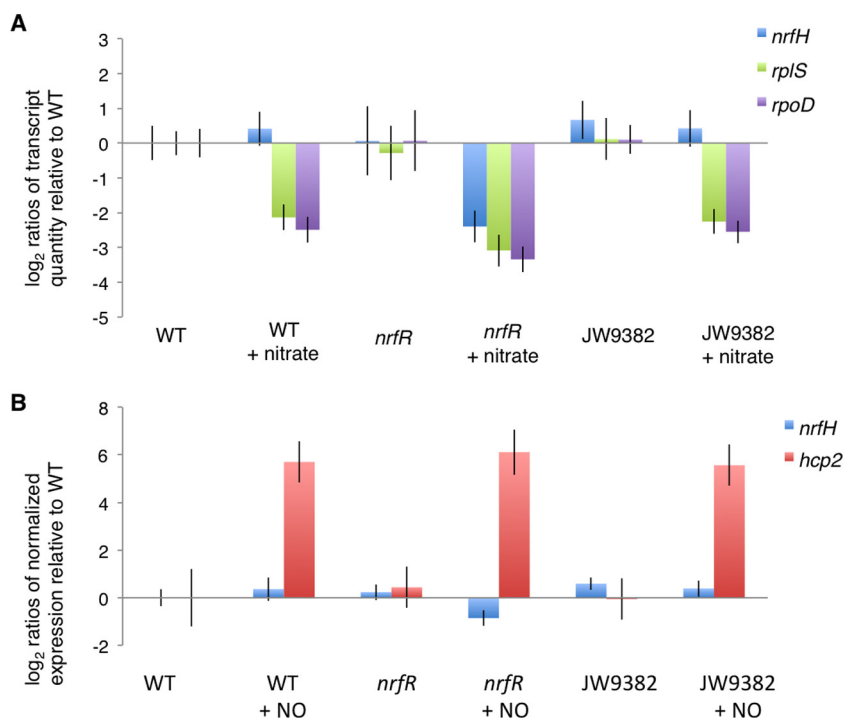


FIG 4 The *nrfH* gene is not induced in response to nitrate or NO stress. (A) The WT, *nrfR* mutant, and JW9382 strains were subjected to nitrate stress at the mid-log phase with 100 mM sodium nitrate for 1 h. The qRT-PCR plot shows log₂ ratios of *nrfH*, *rplS*, and *rpoD* transcripts relative to those in the WT without nitrate stress. Values were normalized to total RNA. The data are averages from two independent experiments with three biological replicates each. The error bars represent standard deviations. (B) The WT, *nrfR* mutant, and JW9382 strains were subjected to NO stress at the mid-log phase with 10 μ M GSNO for 1 h. The qRT-PCR plot shows log₂ ratios of normalized expression of *nrfH* and *hcp2* genes relative to that in the WT without NO stress. Values were normalized to the reference genes *rplS* and *rpoD*. The data are averages from six biological replicates in two independent experiments. The error bars represent standard deviations.

within the *Desulfovibrionaceae* family and is the only species within this cluster that is missing the *nrfHA* and *nrfR* genes. The *nrfR* gene is always associated with a sensor hybrid histidine kinase, NrfS. Seven other *Desulfovibrio* species that are scattered across the phylogenetic tree also carry the *nrfHA* genes but not the *nrfR* gene (Fig. 6).

Since nitrite reductase *nrfHA* genes are typically found in species that can utilize nitrate as a terminal electron acceptor, we also examined which of the *Desulfovibrio* species had the ability to grow on nitrate. Interestingly, we observed that the ability to use nitrate as a terminal electron acceptor was limited to a few species within the same cluster that also carries the *nrfR* gene. The exceptions were *D. vulgaris* Miyazaki and *D. vulgaris* Hildenborough. *Desulfovibrio cuneatus* has been reported to not grow on nitrate (51); however, the sequenced genome shows nitrate reductase genes orthologous to that seen in *D. desulfuricans*. Additionally, the *nrfR* gene in *D. cuneatus* is located upstream of the *nap* genes rather than the *nrfHA* genes. In all sequenced nitrate-utilizing *Desulfovibrio* species, NrfR binding sites were also found upstream of the nitrate reductase-encoding *nap* operon (see Table S5 in the supplemental material). sigma54-dependent promoters were also predicted for the *nap* operons in most of the above-named species (see Table S5).

Desulfovibrio piezophilus is an interesting case where the *nrfHA* genes are absent but the *nrfSR* genes are present upstream of a two-gene operon with a cytochrome *c* subunit and an octaheme oxidoreductase gene that has been hypothesized to function as a hydroxylamine oxidase or an octaheme nitrite reductase (52).

Three species (*D. vulgaris* Miyazaki, *D. termitidis*, and *Desulfovibrio* sp. A2) have two copies of *nrfHA* (see Table S6 in the supplemental material), with one copy located next to the *nrfSR* genes and the second copy located elsewhere in the genome. In all three cases, binding sites were found only upstream of the *nrfHA* copy that was adjacent to the *nrfSR* operon (see Table S5).

A search of 14 other available sequenced genomes belonging to the *Desulfovibrionales* family revealed that 9 of these genomes encoded the *nrfHA* genes; however, none of them had an NrfR ortholog (see Table S6 in the supplemental material). These genomes also did not encode homologs of the *nap* gene.

DISCUSSION

NrfA from *D. vulgaris* Hildenborough has been well studied and is one of the few proteins to be examined from a non-nitrate-reducing species. Earlier studies showed that the nitrite reductase has high basal expression (11, 33, 34) and was also induced further (7, 35), but no studies have explored the regulation of these genes. Here, we have shown that NrfR is the activator that induces transcription of *nrfHA* in response to nitrite exposure. This induction of *nrfHA* expression is important for optimal growth during nitrite stress at low cell densities (Fig. 1). At higher cell densities, the constitutive expression of *nrfHA* is sufficient to overcome nitrite stress, and an *nrfR* mutant does not have an obvious fitness defect.

NrfR is the first example of a sigma54-dependent regulator that regulates expression of an *nrfA*-type nitrite reductase. A predicted sigma54-dependent promoter has been identified at -196 bp from ATG of *nrfHA* (36). However, a high-confidence transcrip-

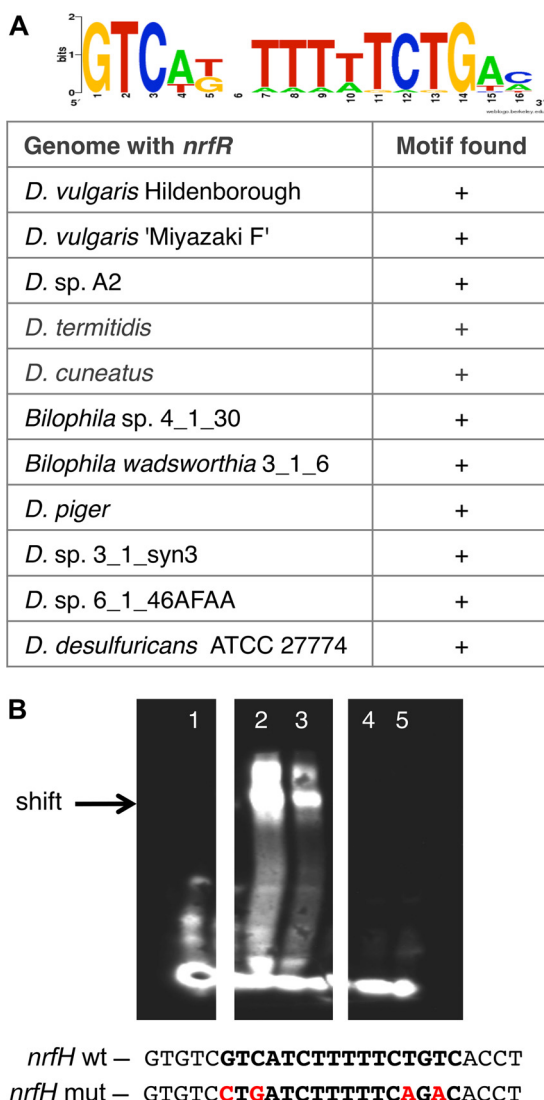


FIG 5 Purified NrfR binds to the predicted binding site. (A) A consensus-binding site motif for NrfR was predicted using the upstream regions of *nrfHA* orthologs in *Desulfovibrio* and *Bilophila* genomes (see the sequences in Table S5 in the supplemental material). The motif image was generated using WebLogo (53). (B) Electrophoretic mobility shift assay with purified NrfR and the predicted binding site (100 fmol). Lane 1, *nrfH* wild-type (wt) DNA only; lanes 2 to 3, *nrfH* WT DNA with 25 and 10 pmol of NrfR, respectively; lanes 4 to 5, *nrfH* mutant (mut) DNA with 25 and 10 pmol of NrfR, respectively. The sequences for *nrfH* WT and mutant DNA substrates are shown below the gel. The bases in red indicate substitutions made to conserved bases in the *nrfH* mutant DNA.

tion start site (TSS) was identified only at bp -98 from ATG (34). Since *nrfHA* has high expression even in the absence of nitrite, and the TSS was determined with cells grown in the absence of nitrite, it is possible that the constitutive expression of *nrfHA* occurs from another promoter located at -98 . A sigma70 promoter motif, however, was not identified at this TSS (34). The downregulation of *nrfHA* observed in the *nrfR* mutant under nitrite stress may be a result of this constitutive transcription being shut down in the presence of nitrite. In support of this hypothesis, of the two sigma70 genes that we tested as possible reference genes, we observed downregulation of *rpoH* (DVU1584) upon nitrite stress

(not shown). We hypothesize that under nitrite stress, transcription of *nrfHA* occurs from an alternate TSS adjacent to the sigma54-dependent promoter.

In *E. coli*, the *nrfA* gene is under complex regulation by two homologous two-component systems in response to both nitrate and nitrite, by the FNR in response to anaerobic conditions, and by the nitric oxide-sensitive repressor NsrR (26, 30). The regulation of *nrfHA* in *D. vulgaris* is likely to be much simpler, as NrfA performs a primarily nitrite-detoxifying function and the presence of nitrite does not signify a change in anaerobic conditions.

Among the *Desulfovibrio* spp., the *nrfHA* genes are present in several species; however, *nrfSR* is limited to a small number of species that are closely related to *D. vulgaris* Hildenborough. Interestingly, and unlike *D. vulgaris*, most strains that have *nrfR* also have the ability to grow on nitrate as a terminal electron acceptor. It is possible that *D. vulgaris* strains lost the *nap* genes or alternatively that *D. vulgaris* acquired the *nrfSR* and *nrfHA* genes through horizontal gene transfer. Since the nitrate-reducing *Desulfovibrio* species also have predicted NrfR binding sites upstream of the *nap* operon, it is probable that in these species NrfR responds to both nitrate and nitrite and that NrfR regulates both the *nrfHA* genes and the *nap* gene. Our data (Fig. 4A) as well as previous literature reports (7, 48) suggest that *D. vulgaris nrfHA* is not induced by nitrate. However, nitrate stress decreases the abundance of sigma70 genes, including *rpoH* (48) and *rpoD* (48; this study), and a decrease in the background expression of *nrfH* (from its constitutive promoter) may be expected. Since we observed this decrease only in the *nrfR* mutant, it is possible that in the WT (and JW9382), NrfR is activated by nitrate and induces *nrfHA* from its sigma54 promoter, such that a net increase or decrease in transcript abundance is not observed.

Nitrite stress may be associated with NO stress, as the formation of NO from nitrite is possible. The *D. desulfuricans* NrfA nitrite reductase has been shown to reduce nitric oxide at rates similar to those of nitrite (54). In *W. succinogenes*, NrfA is the primary defense against both oxidative and nitrosative stresses (25), and the *nrfA* gene is predicted to be regulated by the nitric oxide-sensitive regulator NssR (31). However, as we have shown in this paper, *D. vulgaris nrfH* was not induced in response to NO stress.

NrfS, the associated histidine kinase for NrfR, may be sensing nitrite and possibly nitrate but not NO. NrfS and its orthologs are predicted to be large hybrid histidine kinases (~ 800 amino acids [aa]) with a receiver and an Hpt domain at the C-terminal end, suggesting a multistep phosphorelay system that may provide a more fine-tuned response. In contrast, the sensor kinases NarX and NarQ of the nitrate/nitrite-responsive two-component systems of *E. coli* (and of other *Gammaproteobacteria*) are smaller nonhybrid kinases.

Since NrfR is a transcriptional activator for the *nrfHA* genes, it is interesting that *nrfHA* also has a relatively high constitutive expression in *D. vulgaris*. Constitutive expression of *nrfA* has also been observed in *D. desulfuricans* ATCC 27774, where nitrite or nitrate only slightly increased the transcript levels of *nrfA*, whereas the *nap* genes were strongly induced in the presence of nitrate (13). Studies with the *D. desulfuricans* strain Essex 6 found constitutive nitrite reductase activity that was induced further by nitrate but not nitrite and nitrate reductase activity that was induced only by nitrite or nitrate (50). Since nitrite is far more toxic than nitrate, being toxic at levels as low as 5 mM for both *D. vulgaris* (7, 35; this

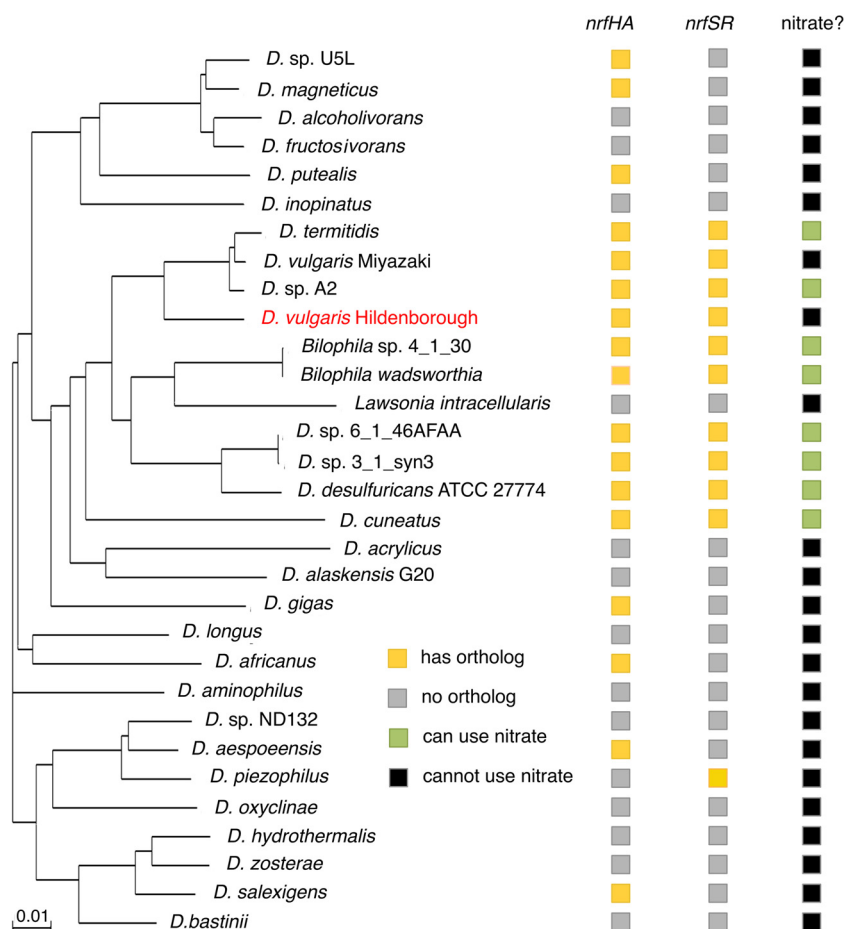


FIG 6 Conservation of *nrfR* and *nrfHA* genes and the ability to utilize nitrate as a terminal electron acceptor across sequenced *Desulfovibrio* species. The phylogenetic tree was constructed using the Distance Tree tool on the Integrated Microbial Genomes website (<https://img.jgi.doe.gov>). Orthologs for *nrfHA* and *nrfR* were determined using the Integrated Microbial Genomes (IMG) BLAST function with *D. vulgaris* Hildenborough genes as the query against the selected *Desulfovibrio* and related species. The data for nitrate-utilizing ability are based on either published literature reports or on the presence of orthologs of *D. desulfuricans* ATCC 27774 nitrate reductase *nap* genes (for details, see Table S6 in the supplemental material). *D. piger*, which the IMG Distance Tree tool left out, is closely related to *D. desulfuricans* ATCC 27774 and carries both *nrfHA* and *nrfR* but does not utilize nitrate.

study) and *D. desulfuricans* (13), constitutive expression of NrfHA ensures an immediate response to the presence of nitrite that may be released by other nitrate-reducing bacteria in these environments (6).

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