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Comparative Analysis of Denitrifying Activities of *Hyphomicrobium nitrativorans*, *Hyphomicrobium denitrificans*, and *Hyphomicrobium zavarzinii*

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***Hyphomicrobium* spp. are commonly identified as major players in denitrification systems supplied with methanol as a carbon source. However, denitrifying *Hyphomicrobium* species are poorly characterized, and very few studies have provided information on the genetic and physiological aspects of denitrification in pure cultures of these bacteria. This is a comparative study of three denitrifying *Hyphomicrobium* species, *H. denitrificans* ATCC 51888, *H. zavarzinii* ZV622, and a newly described species, *H. nitrativorans* NL23, which was isolated from a denitrification system treating seawater. Whole-genome sequence analyses revealed that although they share numerous orthologous genes, these three species differ greatly in their nitrate reductases, with gene clusters encoding a periplasmic nitrate reductase (Nap) in *H. nitrativorans*, a membrane-bound nitrate reductase (Nar) in *H. denitrificans*, and one Nap and two Nar enzymes in *H. zavarzinii*. Concurrently with these differences observed at the genetic level, important differences in the denitrification capacities of these *Hyphomicrobium* species were determined. *H. nitrativorans* grew and denitrified at higher nitrate and NaCl concentrations than did the two other species, without significant nitrite accumulation. Significant increases in the relative gene expression levels of the nitrate (*napA*) and nitrite (*nirK*) reductase genes were also noted for *H. nitrativorans* at higher nitrate and NaCl concentrations. Oxygen was also found to be a strong regulator of denitrification gene expression in both *H. nitrativorans* and *H. zavarzinii*, although individual genes responded differently in these two species. Taken together, the results presented in this study highlight the potential of *H. nitrativorans* as an efficient and adaptable bacterium that is able to perform complete denitrification under various conditions.**

Hyphomicrobium spp. are restricted facultative methylotrophs that reproduce by budding at the tip of a polar prostheca. They are ubiquitous in water and soil but can also be found in sewage treatment plants (1). Some strains have been characterized by their denitrification capacities (2–5). They have often been identified as major players in denitrification systems supplemented with methanol (4, 6–8), and their presence has been associated with high denitrification rates (9, 10).

Denitrification takes place in bacterial cells where N-oxides and/or N-oxyanions serve as the terminal electron acceptor instead of oxygen (O_2) for energy production when oxygen depletion occurs, leading to the production of gaseous nitrogen (N_2) (11). Four sequential reactions are essential for the reduction of nitrate to gaseous nitrogen via nitrite, nitric oxide, and nitrous oxide intermediates, and each of these reactions is catalyzed by different enzymes, namely, nitrate reductases (Nar and Nap), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (12–14).

Previous studies using DNA hybridization with probes targeting genes encoding the catalytic subunit of denitrification reductases (*narG*, *nirK*, *nirS*, and *nosZ*) detected denitrification genes in several members of the genus *Hyphomicrobium*, including *H. denitrificans* and *H. zavarzinii* (2, 3). *H. denitrificans* (5) is commonly found in soils, freshwater environments, and activated sludge. This bacterium has the capacity to denitrify (2), and its nitrite and nitrous oxide reductases were previously characterized (15–18). *H. zavarzinii* is a soil bacterium that has been studied mainly for the biotechnological potential of the exceptional formaldehyde dehydrogenase found in strain ZV580 (19, 20). Recently, we described a new denitrifying species of the genus *Hyphomicrobium*, *H. nitrativorans* NL23^T (21, 22). This bacterium was isolated from a methanol-fed denitrification system treating seawater at the Montreal Biodome in Canada. *H. nitrativorans* was one of the most abundant bacterial species in the denitrifying biofilm, along with *Methylophaga nitratireducenticrescens* JAM1^T, which was also isolated from this biofilm and which can reduce nitrate into nitrite (23, 24). Given that pure cultures of *H. nitrativorans* grow poorly in seawater (21), we believe that *M. nitratireducenticrescens* JAM1 and *H. nitrativorans* NL23 work together in syntrophy in the biofilm to achieve the complete denitrification pathway.

Despite the presence of denitrifying *Hyphomicrobium* spp. in many environments or denitrification processes, genetic and physiological information regarding their denitrification capacities is poorly documented. This could be related to the fact that *Hyphomicrobium* spp. can be hard to culture, especially under denitrifying conditions. In this study, three denitrifying *Hyphomi-*

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crobium species, *H. nitrativorans*, *H. denitrificans*, and *H. zavarzinii*, were compared at the genetic and physiological levels. Because *H. nitrativorans* was isolated from a marine environment, we focused on the effect of salt on denitrification. Among the major differences found between their genomes was the type of nitrate reductase that they encode. Our results showed that *H. nitrativorans* sustained better denitrifying activity than the other two species and had a better tolerance to higher nitrate and NaCl concentrations. The effect of variations in the nitrate and NaCl concentrations on the relative expression levels of the nitrate and nitrite reductase genes was also assessed.

MATERIALS AND METHODS

Genome sequence analyses. *Hyphomicrobium nitrativorans* strain NL23^T (ATCC BAA-2476), *Hyphomicrobium denitrificans* strain X^T (ATCC 51888), and *Hyphomicrobium zavarzinii* strain ZV622^T (ATCC 27496) were used in this study. The general characteristics of the three bacterial species are presented in Table S1 in the supplemental material. Publicly available genome sequences of *H. nitrativorans* NL23 (GenBank accession number NC_022997) (22), *H. denitrificans* ATCC 51888 (GenBank accession number NC_014313) (25), and *H. zavarzinii* ATCC 27496 (GenBank accession numbers KB911255 to KB911269 for 15 scaffolds) were retrieved from GenBank, imported into RAST (Rapid Annotation Using Subsystem Technology) (26) for annotation, and processed in SEED viewer (27) to identify denitrification gene clusters and associated regulatory genes. Orthologous genes were determined with the "sequence-based comparison" tool in SEED viewer and refined by hand. Briefly, the deduced amino acid sequences for all the annotated genes of one genome were compared to those of the other two genomes by BLAST (automated process in SEED viewer). This comparison was performed three times with a different genome set as the reference each time. Only genes that resulted from reciprocal analysis were considered orthologous. The three genomes were also annotated by the Integrated Microbial Genomes (IMG) comparative analysis system of the Joint Genome Institute (http://img.jgi.doe.gov/) (28). The majority of the differences in annotations between RAST and IMG were in genes encoding hypothetical proteins with unknown functions. However, both annotation systems provided similar results for orthologous genes. The results were derived from RAST because supplemental options were not available in IMG. A genome comparison of the three species was further performed with MAUVE (29). The average nucleotide identity (ANI) was determined for each pair of species (http://enve-omics.ce.gatech.edu/ani/). Deduced amino acid sequences derived from the *napA*, *narG*, *nirK*, *cnorB*, and *nosZ* genes that were retrieved from the genomes of the three *Hyphomicrobium* species, as well as sequences of their closest relatives and representative sequences of the *Proteobacteria*, were aligned by using ClustalW, and phylogenetic trees were constructed by using the neighbor-joining algorithm (30) with the Poisson correction method in MEGA version 6.06 (31).

Bacterial strains and growth conditions. Bacteria were cultured in modified *Hyphomicrobium* medium 337a (32) [composition (per liter), 1.3 g KH_2PO_4, 1.13 g Na_2HPO_4, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 3.09 mg $CaCl_2 \cdot 2H_2O$, 2.0 mg $FeSO_4 \cdot 7H_2O$, 1.0 mg $Na_2MoO_4 \cdot 2H_2O$, 0.88 mg $MnSO_4 \cdot 4H_2O$, and 10 μg $CuCl_2 \cdot 2H_2O$ (pH 7.5)] at 30°C. The NaCl concentrations were variable depending on the assay and are specified below. When required, nitrate was added to the growth medium as sodium nitrate ($NaNO_3$) at a concentration of 14.3 mM (200 mg NO_3-N liter^{-1}, unless otherwise specified). Nitrate was added to the precultures for all of the assays conducted under denitrifying conditions (unless otherwise specified). Methanol was added to the growth medium as a carbon source at a concentration of 0.3% (vol/vol) prior to inoculation. Vitamin B_{12} was also added at a concentration of 0.1 mg liter^{-1} prior to inoculation to stimulate bacterial growth on solid medium and in liquid medium to start cultures from bacterial colonies. The inoculum was made of fresh aerobic liquid cultures. Bacterial cells were pelleted, dispersed in a 0.85% NaCl solution, and homogenized prior to inoculation. The initial optical density of the cultures was adjusted to reach an optical density at 600 nm (OD_{600}) close to 0.1. In order to assess the capacity of the three *Hyphomicrobium* species to achieve complete denitrification, the N_2O concentrations in the headspace of cultures incubated with and without 10% (vol/vol) acetylene, an inhibitor of the nitrous oxide reductase, were compared (33).

Aerobic cultures used for RNA extraction were incubated with constant shaking (250 rpm) in 200-ml Erlenmeyer flasks containing 30 ml of growth medium. For cultures under denitrifying conditions, 30 ml of growth medium supplemented with nitrate was dispensed into 70-ml serum vials. The vials were sealed with rubber stoppers and metal seals, flushed for 10 min with N_2 to remove oxygen from the headspace, and autoclaved. Substrate addition, inoculation, and sampling of these vials were performed through the rubber stopper using a syringe and a needle. All cultures were incubated in the dark. Bacterial growth was monitored by spectrophotometry (OD_{600}). The nitrate and nitrite concentrations in the cultures were determined by ion chromatography using an 850 Professional IC instrument (Metrohm, Herisau, Switzerland) with a Metrosep A Supp 5 analytical column (250 by 4.0 mm), while N_2O was analyzed by injecting 250 μl of the headspace into an Agilent 6890 gas chromatograph equipped with a microelectron capture detector.

RNA extraction. RNA was extracted from 15 to 30 ml of bacterial culture in the mid-exponential growth phase. Bacterial cells were centrifuged, transferred into 2-ml tubes containing 500 mg of 0.1-mm zirconia-silica beads (Biospec Products, Bartlesville, OK, USA), and dispersed in 700 μl of a solution containing precooled TPM buffer (50 mM Tris-HCl [pH 7.0], 1.7% [wt/vol] polyvinylpyrrolidone K25, 20 mM $MgCl_2$), 35 μl of 20% SDS, and 500 μl of phenol. The samples were flash frozen with liquid nitrogen and stored at −80°C until RNA extraction was performed. After thawing, RNA was extracted by bead beating twice with a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA), with the power set at 6.5 for 45 s. The tubes were then centrifuged at 20,000 × *g* for 5 min at 4°C, and the upper phase was transferred into a new tube. The bead beating and centrifugation steps were repeated following the addition of 700 μl of PBB buffer (5 mM Tris-HCl [pH 7.0], 5 mM Na_2 EDTA, 1% [wt/vol] SDS, 6% [vol/vol] water-saturated phenol) to the tubes containing the bacterial cells. Both upper phases were further extracted with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and chloroform-isoamyl alcohol (24:1 [vol/vol]). For each extraction step, centrifugation was performed at 20,000 × *g* for 5 min at 4°C. RNA was recovered by precipitation with a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at −20°C overnight. After centrifugation at 20,000 × *g* for 45 min at 4°C, the supernatant was discarded, and the pellets were washed with 70% ethanol (13,000 × *g* for 10 min at 4°C), dried at room temperature for 15 min, dissolved in diethyl pyrocarbonate (DEPC)-treated water, and treated with Turbo DNase (Ambion; Life Technologies Inc., Burlington, ON, Canada) for 30 min to remove all contaminating DNA. Finally, RNA extracts were purified by using the RNeasy MinElute Cleanup kit according to the manufacturer's instructions (Qiagen, Toronto, ON, Canada). RNA integrity was verified by gel electrophoresis, and RNA quantification was performed by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The absence of DNA was confirmed by endpoint PCR analysis using 16S rRNA gene universal primers.

Reverse transcription and quantitative PCR assays. cDNAs were generated from RNA by using hexameric primers and the reverse transcription system from Promega (Madison, WI, USA) with 1 μg of extracted RNA, according to the manufacturer's protocols. Real-time quantitative PCR (qPCR) assays were performed with PerfeCTa SYBR green SuperMix ROX (Quanta; VWR International, Ville Mont-Royal, QC, Canada). The final volume of all of the reaction mixtures was 20 μl, and the amplification mix was composed of 10 μl of PerfeCTa SYBR green SuperMix, 0.4 μl of each primer (40 pmol), 4.2 μl of RNA-free water, and 5 μl of cDNA (50 ng). All of the reactions were performed with a Corbett

TABLE 1 General characteristics of the genomes of the three *Hyphomicrobium* species[a]

Characteristic	Value for species		
	H. nitrativorans	*H. zavarzinii*	*H. denitrificans*
Size (bp)	3,653,837	4,651,795	3,638,969
GC content (%)	63.9	63.7	60.8
No. of scaffolds	1	15	1
No. of genes	3,599	4,399	3,702
No. of CDSs	3,548	4,348	3,653
rRNA operons	3	3	3
tRNA	48	48	46
No. of CDSs with predicted function	2,182	2,764	2,293
No. of CDSs without predicted function	1,366	1,584	1,360

[a] Sequence analyses were performed in RAST. CDSs, coding DNA sequences.

Rotor-Gene 6000 real-time PCR machine (Qiagen). PCR started with an initial denaturation step for 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The fluorescence signal was acquired at the end of the 72°C step and at the end of a 15-s reading step at 80°C that was added to the end of each cycle to avoid fluorescence from primer dimers. Primers were designed with Primer3web version 4.0.0 (34, 35) to target the *napA*, *narG*, *nirK*, *cnorB*, and *nosZ* denitrification genes and the *rpoB* and *dnaG* reference genes of the three *Hyphomicrobium* species (see Table S2 in the supplemental material). Each of the primer sets was tested for specificity and efficiency of amplification by qPCR with serial dilutions of purified PCR fragments (efficiencies of between 0.92 and 1.05).

Calculations and statistical analyses. Growth and denitrification rates were obtained by calculating the slope of the linear portion of the growth and denitrification curves, respectively. Relative gene expression was calculated according to the $\Delta\Delta C_T$ method (36). For each calculation, only one housekeeping gene was used as a control. For each *Hyphomicrobium* species, the control gene was chosen from among the two genes tested (*rpoB* and *dnaG*) by using RefFinder (http://www.leonxie.com/referencegene.php), an online tool that allows the selection of the appropriate control gene based on the lowest variability according to geNorm (37), Normfinder (38), BestKeeper (39), and the comparative $\Delta\Delta C_T$ method (40). Statistical analyses were performed with R (version 2.9.0; R Foundation for Statistical Computing). The effects of nitrate and NaCl concentrations on growth and denitrification rates were tested by using factorial analysis of variance (ANOVA). Prior to factorial ANOVA, normality was tested by using the "shapiro.test" function. When necessary, data were log transformed to meet parametric ANOVA assumptions. ANOVA and *post hoc* Tukey honestly significant difference (HSD) tests were then carried out by using the "aov" and "TukeyHSD" functions, respectively. If significant interactions were detected between the two factors, ANOVA was performed on a subset of data that were generated for each factor. Normality was tested again on each data subset prior to ANOVA. The statistical significance of the $\Delta\Delta C_T$ values was tested by Student's *t* test (two tailed, heteroscedastic).

RESULTS

Genome comparison. The general characteristics of the three genomes are presented in Tables 1 and 2 and Fig. 1. A total of 1,961 orthologous genes are shared among the three species (Fig. 1), with 2,150 orthologous genes shared between *H. nitrativorans* and *H. denitrificans*, 2,687 shared between *H. nitrativorans* and *H. zavarzinii*, and 2,355 shared between *H. denitrificans* and *H. zavarzinii* (Table 2). The number of unique genes (found in one species but not in the other two) constitutes between 16.3 and 25.7% of the total genes, with the highest number of unique genes being found in *H. denitrificans* (Fig. 1). However, a large number (>75%) of these unique genes are annotated as hypothetical proteins with unknown functions (HP). When the genomes were compared for the average deduced amino acid sequence identity between orthologous genes and for the average nucleotide identity (ANI) between whole-genome sequences, *H. nitrativorans* and *H. zavarzinii* showed a higher level of identity with each other than with *H. denitrificans* (Table 2). These results are consistent with the results of phylogenetic analysis of the 16S rRNA gene sequences (21).

Putative unique pathways were found in each species or in two out of three species (see Table S3 in the supplemental material). A long cluster of genes associated with photosynthesis is present in *H. zavarzinii*, although there is no indication in the literature of photosynthesis-related activities in this bacterial species. The urea decomposition pathways differ among the three species. Urea is degraded into CO_2 and NH_3 by urease in *H. denitrificans* and by urea carboxylase and allophanate hydrolase in *H. zavarzinii*. No urea decomposition pathway was found in *H. nitrativorans*. Genes encoding the Na^+/H^+ multisubunit antiporter were found in *H. nitrativorans*, and NhaA-type and NhaD-type Na^+/H^+ antiporter genes were found in the other 2 species. *H. nitrativorans* and *H. zavarzinii* carry genes encoding methylamine dehydrogenase and associated proteins. These genes were not found in *H. denitrificans*, which is surprising because this bacterium can utilize monomethylamine, dimethylamine, and trimethylamine as sole carbon sources (5). Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated proteins were found in *H. nitrativorans*, whereas only CRISPR-associated proteins were found in *H. zavarzinii*. However, the draft genome showed an ~3,000-bp gap flanking the chromosomal region of the genome, suggesting a repeated region that could not be assembled properly.

Genes associated with the denitrification pathway. All three *Hyphomicrobium* species studied here possess gene clusters encoding the full set of enzymes necessary to achieve complete denitrification, although they differ greatly in their nitrate reductases (Table 3). The genome of *H. nitrativorans* contains a gene cluster encoding the periplasmic nitrate reductase Nap, the genome of *H. denitrificans* contains a gene cluster encoding the membrane-

TABLE 2 Comparative analysis of the genomes of the *Hyphomicrobium* species

Species	*H. nitrativorans*			*H. zavarzinii*		
	No. of orthologs[a]	Ortholog identity (%)[b]	ANI (%)	No. of orthologs[a]	Ortholog identity (%)[b]	ANI (%)
H. zavarzinii	2,687	73.90	82.50			
H. denitrificans	2,150	60.60	80.80	2,355	61.80	81.00

[a] Number of orthologous genes in common between the respective species.
[b] Average percent deduced amino acid sequence identity between orthologous genes.

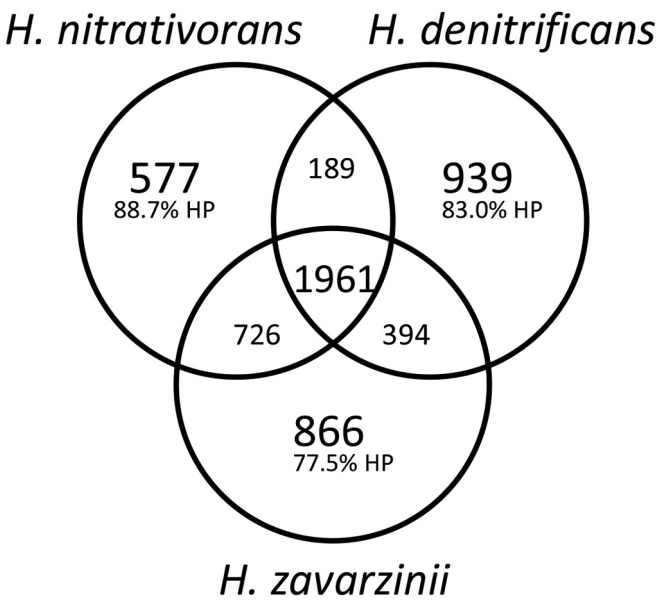

FIG 1 Venn diagram illustrating the number of orthologs found across the three species. Numbers in the nonsharing zones represent unique genes with no homology to sequences in the other two species. Percentages of unique genes that were identified as encoding a HP (hypothetical protein with unknown function) are illustrated. In the shared zones, only genes that resulted from reciprocal analysis (e.g., *H. nitrativorans* versus *H. denitrificans* and *H. denitrificans* versus *H. nitrativorans*) were considered.

bound nitrate reductase Nar, and the genome of *H. zavarzinii* contains gene clusters encoding one Nap and two Nar enzymes. The three species also have a *nirK* gene encoding the copper-containing nitrite reductase, along with the *nirV* gene encoding an accessory protein in *H. nitrativorans* and *H. zavarzinii*. Gene clusters encoding the nitric oxide (Nor) and nitrous oxide (Nos) reductases and with similar gene organizations were also detected in the three genomes. The only exception is *norE*, which is located upstream in a direction opposite that of *cnorCBQD* in *H. denitrificans* (Table 3). Genes associated with nitrate transport were detected upstream of the *nar* gene clusters in *H. denitrificans* and *H. zavarzinii*. Two distinct genes encoding NarK transporters, a NarK1-type symporter and a NarK2-type nitrate/nitrite antiporter, are located upstream of the *nar-2* gene cluster in *H. zavarzinii*. One gene encoding a fused NarK1-NarK2-type transporter (here named *narK12f*) (41) is located upstream of the *nar-1* gene cluster in *H. zavarzinii* and the *nar* gene cluster in *H. denitrificans*.

Genes commonly associated with the regulation of denitrification gene expression, such as *nnrS* and *nrrR*, were detected in the three genomes. Most of these genes belong to the CRP (cyclic AMP receptor protein)/FNR (fumarate and nitrate reductase regulation) family; some of them are located close to the denitrification gene clusters (Table 3). The *narXL* two-component transcription regulatory system was found in *H. denitrificans* and *H. zavarzinii*. However, it is not located in the vicinity of the denitrification gene clusters in either species.

Phylogenetic analyses were performed on the deduced amino acid sequences of genes encoding the catalytic subunits of the different reductases (NapA, NarG, NirK, cNorB, and NosZ) found in the three *Hyphomicrobium* species. All these reductases are phylogenetically related to known denitrification reductases found in other *Alphaproteobacteria*, with sequence identities generally above 80%, except for NarG2 and NosZ of *H. zavarzinii* (maximum of 69% identity in both cases) (see Fig. S1 in the supplemental material).

Effect of nitrate and NaCl on bacterial growth and denitrification activity. The three *Hyphomicrobium* species cultivated under aerobic conditions with or without the addition of 14.3 mM $NaNO_3$ showed similar growth curves. No nitrate reduction or nitrite production was detected under these conditions (data not shown). When incubated under denitrifying conditions at the same nitrate concentration, the three *Hyphomicrobium* species had the capacity to grow and to completely eliminate nitrate without nitrite accumulation, although growth was much slower than that under aerobic conditions (Fig. 2). The addition of acetylene led to the accumulation of N_2O in the headspace of the vials, while no (*H. nitrativorans*) or less (*H. denitrificans* and *H. zavarzinii*) N_2O accumulation was observed in cultures incubated without

TABLE 3 Gene clusters associated with the dissimilatory pathway of denitrification

Reductase	Associated gene cluster(s) (locus tags)		
	H. nitrativorans (GenBank accession no. CP006912)	*H. zavarzinii* (GenBank accession no. NZ_ARTG01000000)	*H. denitrificans* (GenBank accession no. CP002083)
Nar	NP	*narK12f-narGHJI* (*nar-1*) (F812RS0121405 to F812RS0121425) *narK1-narK2-narGHJI* (*nar-2*) (F812RS24645, F812RS24650, F812RS0118690 to F812RS0118710)	*narK12f-narGHJI* (Hden0925 to Hden0929)
Nap	*napEFDABC-napGH* (W91113865 to W91113890, W91102520 to W91102525)	*napDABC-napGH* (F812RS0114960 to F812RS0114975, F812RS0108875 to F812RS0108880)	NP[a]
Nir	*nirK-nirV-nnrR* (W91113050 to W91113060)	*nirK-nirV-nnrR* (F812RS0115595 to F812RS0115605)	*nirK-nnrS* (Hden0590 and Hden0591)
Nor	*nnrS-cnorCBQDE* (W91107415 to W91107440)	*cnorCBQDE* (F812RS0108785 to F812RS0108805)	*cnorE-cnorCBQD* (Hden0580 to Hden0584)
Nos	*nosRZDFYLX* (W91108030 to W91108060)	*nnrS-nosRZDFYLX* (F812RS24275 to F812RS24285, F812RS0116365 to F812RS0116390)	*nosRZDFYLX* (Hden1877 to Hden1883)

[a] NP, not present.

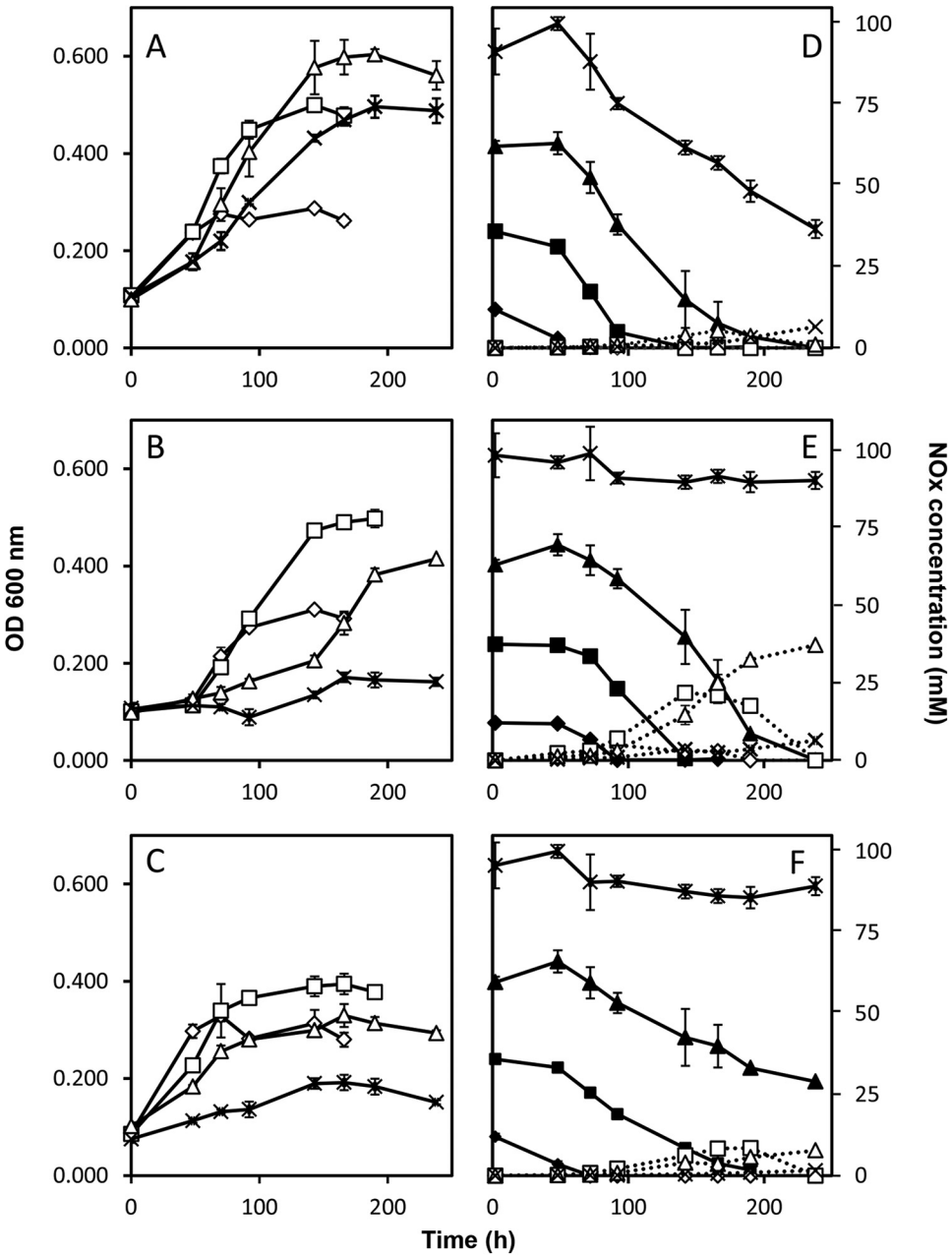

FIG 2 Effect of nitrate concentration on growth and denitrification. *H. nitrativorans* (A and D), *H. denitrificans* (B and E), and *H. zavarzinii* (C and F) were cultured in triplicate under denitrifying conditions with an initial concentration of 14.3 mM (diamonds), 42.8 mM (squares), 71.4 mM (triangles), or 107 mM (crosses) $NaNO_3$. At regular intervals, growth (A to C) and nitrate and nitrite concentrations (D to F) were measured. In panels D to F, full lines indicate nitrate concentrations, and dotted lines indicate nitrite (NOx) concentrations. Assays were performed with an NaCl concentration of 0.5% in the growth medium. The standard deviations of the means for each time point are presented.

acetylene (Fig. 3), indicating that the three species can achieve complete denitrification.

The effect of increasing nitrate concentrations on the growth and denitrification kinetics of the three *Hyphomicrobium* species was assessed under denitrifying conditions, with the $NaNO_3$ concentration ranging from 14.3 to 107 mM (200 to 1,500 mg NO_3-N/liter) (Fig. 2). *H. nitrativorans* had the capacity to grow (Fig. 2A) and denitrify without significant nitrite accumulation (Fig. 2D) at all of the concentrations of $NaNO_3$ tested, with optimal growth at 71.4 mM $NaNO_3$. In contrast, growth and denitrification by *H. denitrificans* and *H. zavarzinii* were almost completely inhibited at 107 mM $NaNO_3$ (Fig. 2B, C, E, and F). For *H. denitrificans* and *H. zavarzinii*, optimal growth and denitrification were observed at 42.8 mM $NaNO_3$, although higher levels of nitrite were constantly detected in cultures of *H. denitrificans*. While these higher levels of nitrite were transient at initial concentrations of 14.3 and 42.8 mM $NaNO_3$ in *H. denitrificans*, nitrite accumulation leading to incomplete denitrification was detected at 71.4 mM $NaNO_3$ (Fig. 2E).

Because *H. nitrativorans* was isolated from a marine environ-

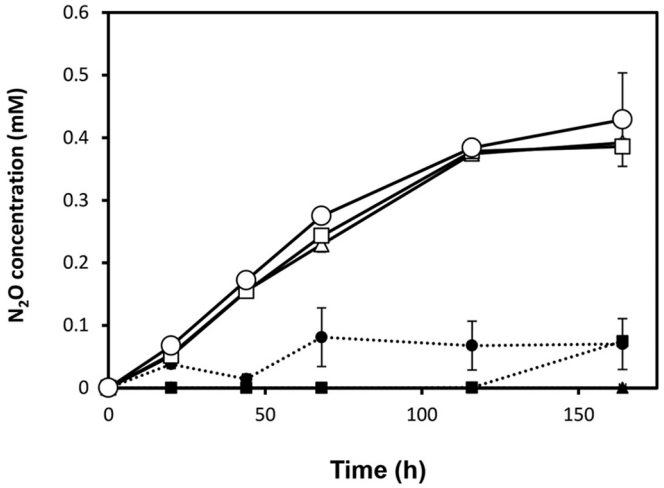

FIG 3 Effect of acetylene on the production of N_2O. *H. nitrativorans* (triangles), *H. denitrificans* (squares), and *H. zavarzinii* (circles) were cultured in triplicate under denitrifying conditions with (full lines) or without (dotted lines) acetylene at an initial concentration of 14.3 mM $NaNO_3$. At regular intervals, the nitrous oxide concentration in the headspace was measured.

ment, tolerance to increasing NaCl concentrations ranging from 0 to 2% NaCl was assessed under denitrifying conditions for the three species. Growth of and denitrification by *H. nitrativorans* were detected at all of the NaCl concentrations tested, without nitrite accumulation, although a concentration of 2% led to slower growth and denitrification (Fig. 4A and D). Increasing NaCl concentrations ranging from 0 to 1% led to delayed growth and denitrification in *H. denitrificans* (Fig. 4B and E) and slower growth and denitrification in *H. zavarzinii* (Fig. 4C and F). A transient accumulation of nitrite was also observed in *H. denitrificans* cultures. At 2% NaCl, these two species showed poor or no growth and no denitrifying activity.

To further evaluate whether the interaction between nitrate and NaCl could lead to significant differences in growth and denitrification rates in the three *Hyphomicrobium* species, we carried out a factorial experiment using two different concentrations of each of the two factors in question. Growth and denitrification were assessed at 14.3 and 71.4 mM $NaNO_3$ and at 0 and 0.5% NaCl (Table 4). The results showed that the three species responded differently to variations of nitrate and NaCl levels. While no significant interactions between the nitrate and NaCl concentrations were detected for *H. nitrativorans* and *H. denitrificans*, significant interactions between these two factors were detected for the growth rate and the denitrification rate in *H. zavarzinii* (Table 4). Single-factor analysis of the data subsets showed that growth and denitrification rates in *H. zavarzinii* were negatively affected by higher nitrate and NaCl concentrations, with the lowest rates being detected at 71.4 mM $NaNO_3$ and 0.5% NaCl (Tables 5 and 6). In *H. nitrativorans*, while nitrate and NaCl had no significant effect on the growth rate, a significant effect of both factors on the denitrification rate was detected, with higher values being observed at higher nitrate and NaCl concentrations (Tables 5 and 6). In contrast, neither of the two factors had a significant effect on the denitrification rate in *H. denitrificans* (Table 6), but the nitrate concentration had a significant effect on the growth rate, with a considerably lower value at 71.4 mM $NaNO_3$ than at 14.3 mM $NaNO_3$ (Table 5).

Effect of nitrate, NaCl, and oxygen on relative gene expression levels of the nitrate and nitrite reductase genes. Our results showed the influence of nitrate and NaCl on the growth and denitrification of the three *Hyphomicrobium* species. The effects of these parameters were also assessed at the nitrate and nitrite reductase gene expression levels. We focused our analysis on these genes because the three species differ greatly in their nitrate reductases; *H. denitrificans* also harbors a very distinct nitrite reductase, while NO and N_2O reductases found in the three species are more similar.

The three *Hyphomicrobium* species were cultured under the following denitrifying conditions: (i) 14.3 mM $NaNO_3$ and 0% NaCl, (ii) 71.4 mM $NaNO_3$ and 0% NaCl, (iii) 14.3 mM $NaNO_3$ and 0.5% NaCl, and (iv) 71.4 mM $NaNO_3$ and 0.5% NaCl. Relative expression levels of the *napA*, *narG*, and *nirK* genes were measured during the mid-exponential growth phase, with cultures grown in 14.3 mM $NaNO_3$ and 0% NaCl as the reference culture (Fig. 5A to C). In *H. nitrativorans*, higher nitrate and NaCl concentrations led to a small (1.4- to 3.4-fold) but significant increase in the relative gene expression levels of *napA* and *nirK* (Fig. 5A). In *H. denitrificans*, a significant effect was observed exclusively for relative gene expression levels of *nirK* in cultures grown with 71.4 mM $NaNO_3$ and 0% NaCl (5.8-fold increase) (Fig. 5B). In *H. zavarzinii*, the nitrate and NaCl concentrations affected the three nitrate reductase genes to different degrees. The relative gene expression level of *napA* did not change under any of the conditions tested. Both factors had a negative effect on *narG1* gene expression but a positive effect on *narG2* gene expression (Fig. 5C). Similar to *narG1*, the relative gene expression level of *nirK* in *H. zavarzinii* was negatively affected by higher nitrate and NaCl concentrations.

Oxygen is also known to have a major effect on denitrification and the denitrification reductases. The three *Hyphomicrobium* species were cultured under aerobic conditions, under aerobic conditions with 14.3 mM $NaNO_3$, or under denitrifying conditions with 14.3 mM $NaNO_3$. The relative expression levels of the *napA*, *narG*, and *nirK* genes were measured as described above, with the culture grown aerobic conditions set as the reference culture (Fig. 5D and E). In *H. nitrativorans*, the addition of nitrate to aerobic cultures led to small but significant increases in the relative gene expression levels of *napA* and *nirK* (1.8- and 3.2-fold, respectively), while incubation under denitrifying conditions led to much higher relative gene expression levels for both genes (7.1- and 35.1-fold, respectively) (Fig. 5D). In *H. denitrificans*, the only difference observed was for the relative gene expression level of *narG*, which was significantly higher in the presence of nitrate under aerobic conditions (Fig. 5E). In *H. zavarzinii*, none of the genes tested was significantly affected by the addition of nitrate to aerobic cultures (Fig. 5F). However, the incubation of this bacterial species under denitrifying conditions led to a strong increase in the relative gene expression level of *narG1* (36.4-fold), a slight but significant increase in the level of *narG2* (1.8-fold), and a significant decrease in the level of *napA* (−8.3-fold) (Fig. 5F).

DISCUSSION

H. nitrativorans, *H. denitrificans*, and *H. zavarzinii* possess genes encoding the full set of enzymes required to perform complete denitrification, and they were able to achieve this process. Under the culture conditions tested, *H. nitrativorans* was found to be the most efficient of the three *Hyphomicrobium* species evaluated. Compared to the two other species, it has the capacity to denitrify

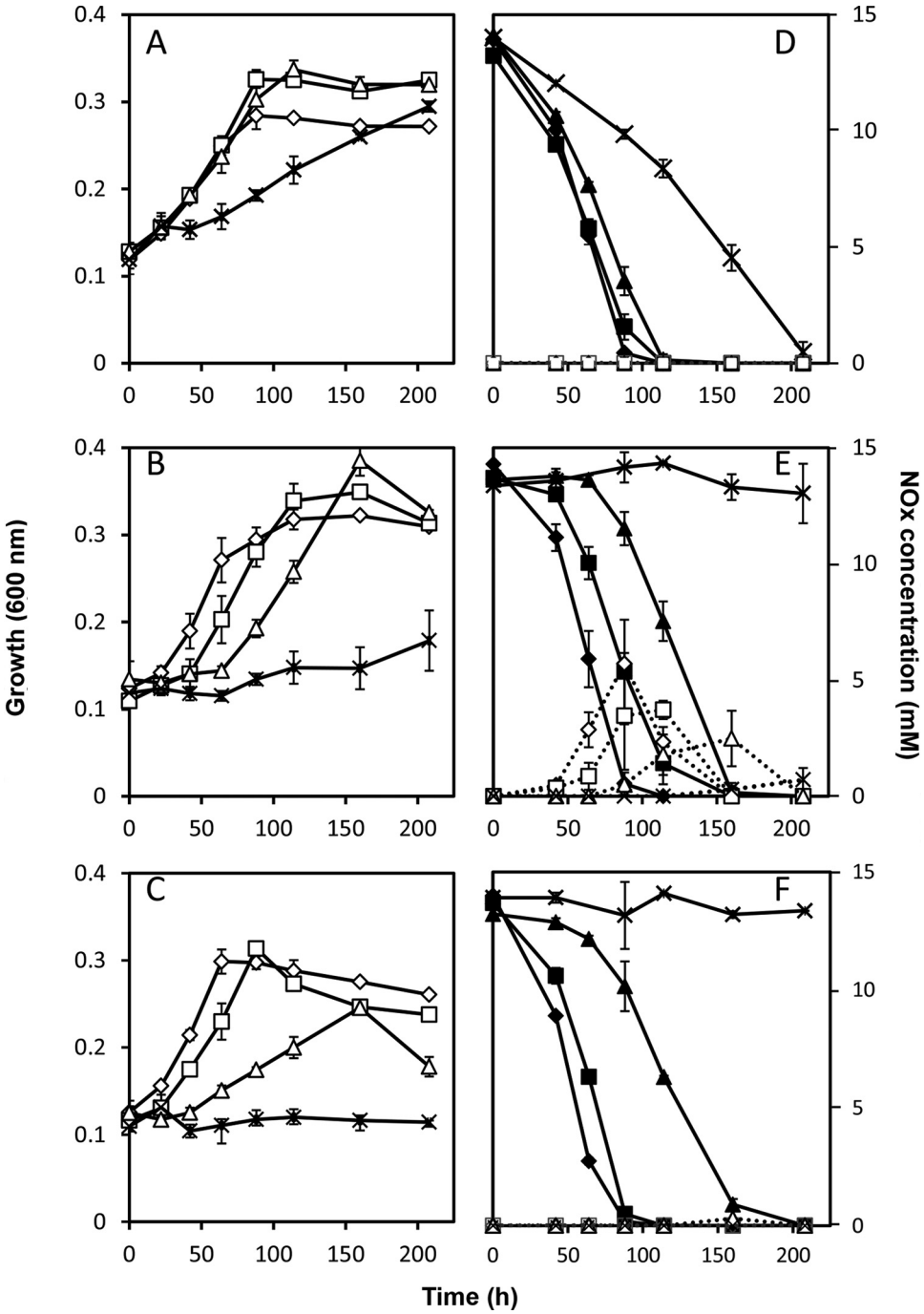

FIG 4 Effect of NaCl concentration on growth and denitrification. *H. nitrativorans* (A and D), *H. denitrificans* (B and E), and *H. zavarzinii* (C and F) were cultured in triplicate under denitrifying conditions with 0% (diamonds), 0.5% (squares), 1% (triangles), and 2% (crosses) NaCl. At regular intervals, growth (A to C) and nitrate and nitrite (NOx) concentrations (D to F) were measured. In panels D to F, full lines indicate nitrate concentrations, and dotted lines indicate nitrite concentrations. Standard deviations of the means for each time point are presented.

at high nitrate concentrations, without nitrite accumulation. Consistent with this observation, an increase in the nitrate concentration in the growth medium induced significant increases in *narG* and *nirK* relative gene expression levels in *H. nitrativorans*. In contrast, an increase of the initial nitrate concentration in the growth medium led to nitrite accumulation in *H. denitrificans* cultures despite a significant increase in the *nirK* relative gene expression level at a higher nitrate concentration. Nitrate inhibition of nitrite reduction was previously reported for several bacteria (42–44) and has been associated with a phenomenon of competition between nitrate and nitrite reductases for electron donors. These results suggest that in *H. denitrificans*, the nitrate reductase might have a higher affinity for electrons than the nitrite reductase. *H. denitrificans* is characterized by its unique nitrite re-

TABLE 4 Factorial ANOVA of the effects of nitrate and NaCl concentrations on growth and denitrification rates[a]

	P value					
	H. nitrativorans		*H. denitrificans*		*H. zavarzinii*	
Source of variation	Growth rate	Denitrification rate	Growth rate	Denitrification rate	Growth rate	Denitrification rate
Nitrate	NS	0.0041	0.0042	NS	<0.001	<0.001
NaCl	NS	0.0178	NS	NS	<0.001	<0.001
Nitrate + NaCl	NS	NS	NS	NS	0.0145	0.0087

[a] The nitrate concentrations tested were 14.3 and 71.4 mM $NaNO_3$; the NaCl concentrations tested were 0 and 0.5%. *P* values of <0.05 are considered statistically significant. NS, not significant.

ductase, which is clearly distinct from the nitrite reductases found in other *Hyphomicrobium* species. It is characterized by the presence of two type I Cu sites, as opposed to the single type I Cu site found in the nitrite reductases of most bacteria (16, 17, 45). The role of this second type I Cu site has not been elucidated yet. Currently, the role of this distinct nitrite reductase in the denitrifying capacity of *H. denitrificans* is unknown, but it might be linked to the nitrite accumulation observed in *H. denitrificans* cultures. Growth of *H. denitrificans* and *H. zavarzinii* was totally inhibited at the highest nitrate concentration tested. Because nitrate was supplied as $NaNO_3$, it is possible that this growth inhibition was exacerbated by the input of sodium in the growth medium, as previously observed for *Shewanella oneidensis* cultures (46).

As expected, *H. nitrativorans* also demonstrated better tolerance to NaCl than the two other *Hyphomicrobium* species tested and showed small but significant increases in the *napA* and *nirK* relative gene expression levels when NaCl was added to the growth medium. More surprisingly, *H. nitrativorans* showed better tolerance to NaCl when cultured under denitrifying conditions than previously reported for aerobic growth. Indeed, while total growth inhibition at NaCl concentrations of >1% was previously reported for *H. nitrativorans* under aerobic conditions (21), our results demonstrate growth and complete denitrification by this bacterium at NaCl concentrations of up to 2%. The mechanisms of this enhanced tolerance to NaCl could be associated with the multisubunit Na^+/H^+ antiporter. A gene cluster encoding this type of antiporter was found in the *H. nitrativorans* genome, whereas genes encoding an NhaA-type Na^+/H^+ antiporter were found in the genomes of the two other species. The multisubunit Na^+/H^+ antiporter has been associated with high Na^+ extrusion activity in halotolerant *Staphylococcus aureus* (47). Despite having better NaCl tolerance than the other *Hyphomicrobium* species, *H. nitrativorans* remains quite sensitive to NaCl compared to other bacteria isolated from denitrifying biofilms, such as *Methylophaga nitratireducenticrescens* JAM1, which can tolerate NaCl concentrations of up to 8% (24). We hypothesize that *H. nitrativorans* benefits from the osmoprotection of other bacteria, such as *M. nitratireducenticrescens* JAM1, to thrive in the artificial seawater of the Biodome aquarium (NaCl concentration of 2.4%). This hypothesis is currently being studied in our laboratory.

The detailed comparison of the *H. nitrativorans*, *H. denitrificans*, and *H. zavarzinii* genomes has highlighted the facts that a large number of orthologous genes are shared among these three species and that very few genes with known functions are unique to a single species. From this perspective, the fact that the three species differ greatly in their nitrate reductases is noteworthy, and it can be hypothesized that the differences observed in their denitrification capacities reflect these variations observed at the genetic level. Indeed, while *H. nitrativorans* and *H. denitrificans* are characterized by the presence of a single Nap or Nar gene cluster, respectively, one Nap and two Nar gene clusters have been identified in the genome of *H. zavarzinii*. Such an occurrence of multiple operons encoding nitrate reductases has previously been reported for other microorganisms. One well-characterized example is *E. coli* K-12, which also expresses two different cytoplasmic membrane nitrate reductases (NRA and NRZ), encoded by *narGHJI* and *narZYWV*, respectively, and one periplasmic nitrate reductase, encoded by *napFDAGHBC* (48–50). Gene deletions in *E. coli* K-12 indicate that Nap can support anaerobic growth when it is the only nitrate reductase available, although NRA is clearly the main enzyme responsible for nitrate reduction when all enzymes are present (48).

While Nap in *E. coli* is expressed under anaerobic conditions (50) and is specifically involved in nitrate reduction at low nitrate concentrations (48, 51), different functions have been proposed for the Nap system in other bacteria. In *Paracoccus* spp., in which both the Nar and Nap systems are present, Nar is expressed under anaerobic conditions, while Nap is expressed during aerobic

TABLE 5 Effect of nitrate and NaCl concentrations on growth rates of three *Hyphomicrobium* species[a]

	Growth rate (OD units h^{-1})								
	H. nitrativorans			*H. denitrificans*			*H. zavarzinii*		
$NaNO_3$ concn (mM)	0% NaCl	0.5% NaCl	Mean	0% NaCl	0.5% NaCl	Mean	0% NaCl	0.5% NaCl	Mean
14.3	0.00206	0.00249	0.00228	0.00325	0.00277	0.00301a	0.00357Aa	0.00287Ba	NA
71.4	0.00220	0.00225	0.00223	0.00234	0.00228	0.00231b	0.00291Ab	0.00138Bb	NA
Mean	0.00213	0.00237		0.00280	0.00253		NA	NA	

[a] Uppercase letters indicate significant differences between columns according to Tukey's test; lowercase letters indicate significant differences between rows according to Tukey's test. NA, not applicable.

TABLE 6 Effect of nitrate and NaCl concentrations on denitrification rates of three *Hyphomicrobium* species[a]

	Denitrification rate (mM h^{-1})								
	H. nitrativorans			*H. denitrificans*			*H. zavarzinii*		
$NaNO_3$ concn (mM)	0% NaCl	0.5% NaCl	Mean	0% NaCl	0.5% NaCl	Mean	0% NaCl	0.5% NaCl	Mean
14.3	0.121	0.174	0.148b	0.126	0.114	0.120	0.280Aa	0.220Ba	NA
71.4	0.186	0.204	0.196a	0.117	0.140	0.129	0.166Ab	0.123Bb	NA
Mean	0.154A	0.189A		0.122	0.127		NA	NA	

[a] Uppercase letters indicate significant differences between columns according to Tukey's test; lowercase letters indicate significant differences between rows according to Tukey's test. NA, not applicable.

growth, in the presence or absence of nitrate (13, 52, 53). This expression pattern is similar to our results for *H. zavarzinii*, with growth under denitrifying conditions leading to the repression of *napA* and the overexpression of *narG1* and, to a lesser extent, *narG2*. In *Paracoccus* spp., Nap is responsible for aerobic nitrate reduction, and it has been suggested that this activity might help dissipate excess reductant, which is further supported by the fact that Nap is induced by growth on highly reduced carbon sub-

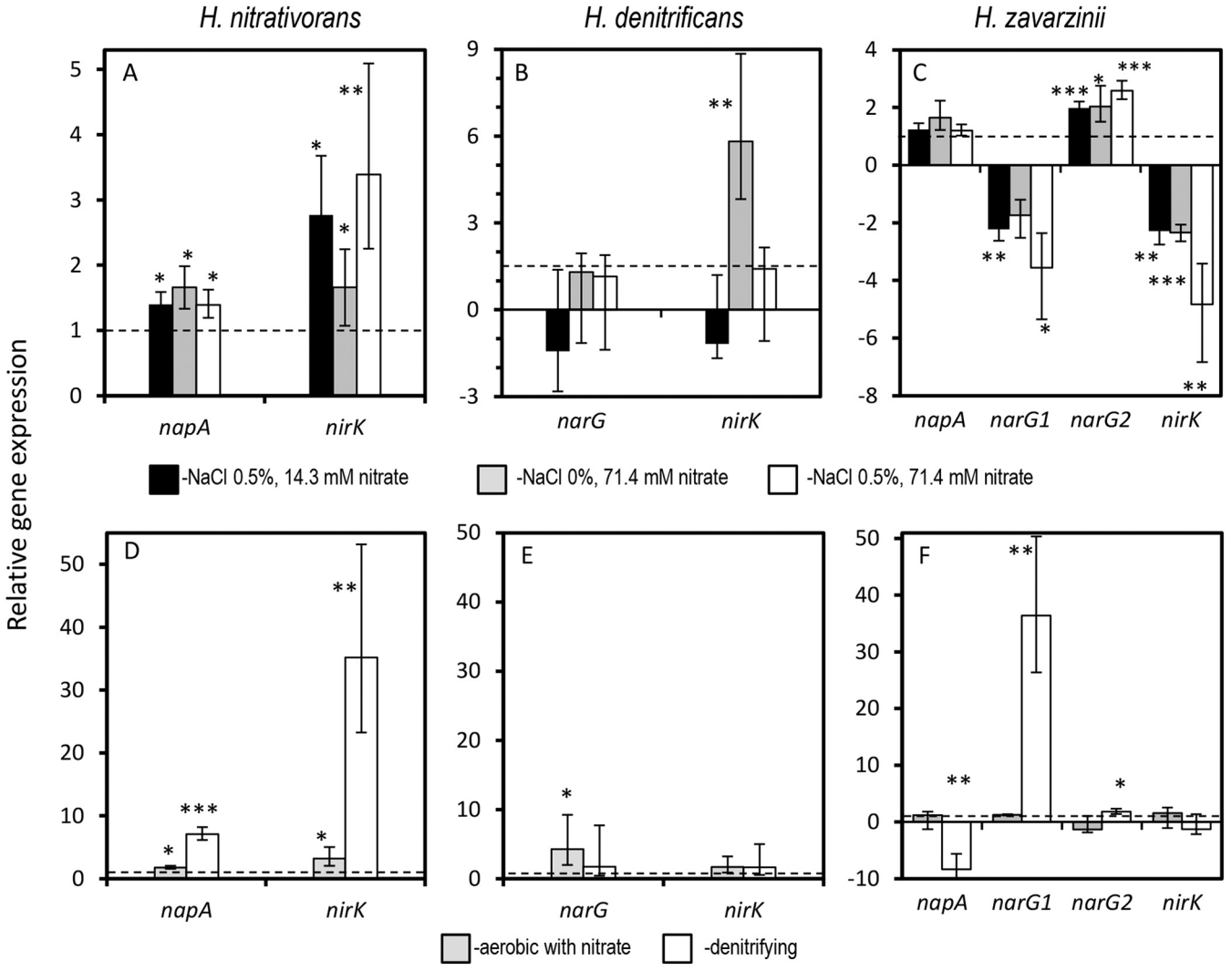

FIG 5 Relative expression levels of the nitrate and nitrite reductase genes in the three species cultured under different conditions. (A to C) Effects of nitrate and NaCl concentrations. The three species were cultured in triplicate under denitrifying conditions with 14.3 mM $NaNO_3$ and 0% NaCl (reference cultures are represented by the dashed line set at 1 in each panel), with 71.4 mM $NaNO_3$ and 0% NaCl, with 14.3 mM $NaNO_3$ and 0.5% NaCl, or with 71.4 mM $NaNO_3$ and 0.5% NaCl. (D and E) Effects of oxygen. The three species were cultured in triplicate under aerobic conditions (reference cultures are represented by the dashed line set at 1 in each panel), aerobic conditions with 14.3 mM $NaNO_3$, or denitrifying conditions with 14.3 mM $NaNO_3$. Aerobic precultures for this assay were prepared without adding nitrate to the growth medium. ***, $P < 0.001$; **, $0.01 > P > 0.001$; *, $0.05 > P > 0.01$.

strates (13, 52). Despite higher levels of Nap expression under aerobic conditions than under denitrifying conditions, we did not detect significant nitrate reduction in aerobic liquid cultures of *H. zavarzinii*. Similarly, the expression level of the Nap-α operon in *Rhodobacter sphaeroides* 2.4.3 was higher in aerobic liquid cultures than in anaerobic cultures, without any detectable nitrate reduction (54). However, aerobic reduction of nitrate into nitrite occurred when *R. sphaeroides* 2.4.3 was cultivated on solid medium, indicating a role of Nap-α in redox homeostasis specific for these culture conditions. Preliminary assays performed in our laboratory indicated that this is not the case for *H. zavarzinii*, but more extensive work will have to be performed to rule out this hypothesis.

While it is not uncommon to find multiple nitrate reductases in a single bacterium, the presence of multiple nitrate reductases of the same type has been reported only rarely in previous studies. *Rhodobacter sphaeroides* 2.4.3 possesses two Nap operons, one, identified as Nap-α, being similar to Nap operons found in other *Alphaproteobacteria* (*napKEFDABC*) and the other, identified as Nap-β, being similar to the Nap operons found in *Gammaproteobacteria* (*napFDAGHBC*) such as *Shewanella* spp. and *E. coli* (54). These two distinct Nap isoforms have also been reported for several strains of *Shewanella* (55, 56). Recently, we also described the new bacterial species *Methylophaga nitratireducenticrescens* JAM1 (*Gammaproteobacteria*), which expresses two distinct Nar nitrate reductases (Nar1 and Nar2) encoded by two *narGHJI* operons (23, 24, 57). For this bacterium, phylogenetic analyses revealed that Nar1 is more closely related to the Nar system of *Betaproteobacteria*, whereas Nar2 is more closely related to the Nar system found in other *Gammaproteobacteria*, suggesting that the Nar1 system was acquired by horizontal gene transfer (23). In *H. zavarzinii*, while *narG1* is closely related to *narG* sequences found in other *Alphaproteobacteria*, *narG2* is only distantly related to any known *narG* sequence. The expression patterns of these two genes were also slightly different, with a much stronger effect of anaerobic conditions on the expression of *narG1* than on the expression of *narG2*. Further work, including the production of gene knockout mutants for the different nitrate reductases in *H. zavarzinii*, is necessary to completely elucidate the physiological roles played by these enzymes.

Although the *napA* gene product in *H. nitrativorans* is more related to Nap-α than to Nap-β of *Rhodobacter sphaeroides* 2.4.3, a distinct *napA* expression pattern was observed. In *H. nitrativorans*, *napA* gene expression levels were significantly higher in the presence of nitrate and under denitrifying conditions than in the absence of nitrate and under aerobic conditions. Similar results have been reported for *Bradyrhizobium japonicum*, which also possesses a single periplasmic nitrate reductase of the α isoform (58). Although results from studies on *Paracoccus pantotrophus* and *Rhodobacter sphaeroides* point toward a specific role of the Nap-α isoform in aerobic denitrification and redox balancing, both the α and β isoforms have been associated with anaerobic nitrate respiration in *Shewanella* spp. (55, 56). It is important to note that the highest denitrification efficiencies were observed for a *Shewanella* strain lacking the *nap*-β operon (55) and for a *nap*-β mutant *Shewanella* strain (56), indicating that the α isoform plays a major role in anaerobic respiration in this bacterial genus. Taken together, these results further illustrate that there is no clear association between the isoform and the physiological role of Nap and that other factors, such as the presence of distinct enzymes with similar functions, might also play an important role.

Denitrifying conditions were shown to increase the relative gene expression level of *nirK* in *H. nitrativorans*. However, such an effect was not observed for *H. denitrificans* and *H. zavarzinii*. No previous studies assessed quantitatively the expression of *nirK* under different culture conditions in *Hyphomicrobium* spp. Microaerobic or anaerobic conditions are known to regulate *nirK* expression in many bacterial species (59–61). In *Rhodobacter sphaeroides* 2.4.3, *nirK* expression is regulated by NnrR, a transcriptional activator of the CRP/FNR family (60, 62). Gene annotation of the *H. denitrificans* genome did not reveal sequences closely related to *nnrR*, which could explain the absence of regulation of *nirK* expression. The lack of an effect of denitrifying conditions on *H. zavarzinii* is surprising, as *nnrR* was found downstream of *nirK*. In fact, *H. zavarzinii* and *H. nitrativorans* have the same gene arrangement in the *nirK* locus (Table 3), and both showed a potential NnrR binding site (data not shown). Further work will be required in order to elucidate this phenomenon.

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REFERENCES

1. **Gliesche CG, Fesefeldt A, Hirsch P.** 2005. Genus *Hyphomicrobium* Stutzer and Hartleb 1898, 76^{AL}, p 476–494. *In* Staley JT, Bryant MP, Pfennig N, Holt JG (ed), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, MD.
2. **Fesefeldt A, Kloos K, Bothe H, Lemmer H, Gliesche CG.** 1998. Distribution of denitrification and nitrogen fixation genes in *Hyphomicrobium* spp. and other budding bacteria. Can J Microbiol **44:**181–186. http://dx.doi.org/10.1139/w97-139.
3. **Kloos K, Fesefeldt A, Gliesche CG, Bothe H.** 1995. DNA-probing indicates the occurrence of denitrification and nitrogen fixation genes in *Hyphomicrobium*. Distribution of denitrifying and nitrogen fixing isolates of *Hyphomicrobium* in a sewage treatment plant. FEMS Microbiol Ecol **18:**205–213. http://dx.doi.org/10.1111/j.1574-6941.1995.tb00177.x.
4. **Timmermans P, Van Haute A.** 1983. Denitrification with methanol: fundamental study of the growth and denitrification capacity of *Hyphomicrobium* sp. Water Res **17:**1249–1255. http://dx.doi.org/10.1016/0043-1354(83)90249-X.
5. **Urakami T, Sasaki J, Suzuki K-I, Komagata K.** 1995. Characterization and description of *Hyphomicrobium denitrificans* sp. nov. Int J Syst Bacteriol **45:**528–532. http://dx.doi.org/10.1099/00207713-45-3-528.
6. **Harder W, Attwood MM.** 1978. Biology, physiology and biochemistry of *Hyphomicrobia*. Adv Microb Physiol **17:**303–359. http://dx.doi.org/10.1016/S0065-2911(08)60060-0.
7. **Baytshtok V, Lu H, Park H, Kim S, Yu R, Chandran K.** 2009. Impact of varying electron donors on the molecular microbial ecology and biokinetics of methylotrophic denitrifying bacteria. Biotechnol Bioeng **102:**1527–1536. http://dx.doi.org/10.1002/bit.22213.
8. **Satoh H, Yamakawa T, Kindaichi T, Ito T, Okabe S.** 2006. Community structures and activities of nitrifying and denitrifying bacteria in industrial wastewater-treating biofilms. Biotechnol Bioeng **94:**762–772. http://dx.doi.org/10.1002/bit.20894.
9. **Isaka K, Kimura Y, Osaka T, Tsuneda S.** 2012. High-rate denitrification using polyethylene glycol gel carriers entrapping heterotrophic denitrifying bacteria. Water Res **46:**4941–4948. http://dx.doi.org/10.1016/j.watres.2012.05.050.
10. **Baytshtok V, Kim S, Yu R, Park H, Chandran K.** 2008. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. Water Sci Technol **58**(2):359–365. http://dx.doi.org/10.2166/wst.2008.391.

11. **Knowles R.** 1982. Denitrification. Microbiol Rev **46:**43–70.
12. **Philippot L.** 2002. Denitrifying genes in bacterial and archaeal genomes. Biochim Biophys Acta **1577:**355–376. http://dx.doi.org/10.1016/S0167-4781(02)00420-7.
13. **Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ.** 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell Mol Life Sci **58:**165–178. http://dx.doi.org/10.1007/PL00000845.
14. **Kraft B, Strous M, Tegetmeyer HE.** 2011. Microbial nitrate respiration—genes, enzymes and environmental distribution. J Biotechnol **155:**104–117. http://dx.doi.org/10.1016/j.jbiotec.2010.12.025.
15. **Deligeer, Fukunaga R, Kataoka K, Yamaguchi K, Kobayashi K, Tagawa S, Suzuki S.** 2002. Spectroscopic and functional characterization of Cu-containing nitrite reductase from *Hyphomicrobium denitrificans* A3151. J Inorg Biochem **91:**132–138. http://dx.doi.org/10.1016/S0162-0134(02)00442-7.
16. **Yamaguchi K, Kataoka K, Kobayashi M, Itoh K, Fukui A, Suzuki S.** 2004. Characterization of two type 1 Cu sites of *Hyphomicrobium denitrificans* nitrite reductase: a new class of copper-containing nitrite reductases. Biochemistry **43:**14180–14188. http://dx.doi.org/10.1021/bi0492657.
17. **Nojiri M, Xie Y, Inoue T, Yamamoto T, Matsumura H, Kataoka K, Deligeer, Yamaguchi K, Kai Y, Suzuki S.** 2007. Structure and function of a hexameric copper-containing nitrite reductase. Proc Natl Acad Sci U S A **104:**4315–4320. http://dx.doi.org/10.1073/pnas.0609195104.
18. **Yamaguchi K, Kawamura A, Ogawa H, Suzuki S.** 2003. Characterization of nitrous oxide reductase from a methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans* A3151. J Biochem **134:**853–858. http://dx.doi.org/10.1093/jb/mvg211.
19. **Jérôme V, Hermann M, Hilbrig F, Freitag R.** 2007. Development of a fed-batch process for the production of a dye-linked formaldehyde dehydrogenase in *Hyphomicrobium zavarzinii* ZV 580. Appl Microbiol Biotechnol **77:**779–788. http://dx.doi.org/10.1007/s00253-007-1218-z.
20. **Klein CR, Kesseler FP, Perrei C, Frank J, Duine JA, Schwartz AC.** 1994. A novel dye-linked formaldehyde dehydrogenase with some properties indicating the presence of a protein-bound redox-active quinone cofactor. Biochem J **301:**289–295.
21. **Martineau C, Villeneuve C, Mauffrey F, Villemur R.** 2013. *Hyphomicrobium nitrativorans* sp. nov., isolated from the biofilm of a methanol-fed denitrification system treating seawater at the Montreal Biodome (Canada). Int J Syst Evol Microbiol **63:**3777–3781. http://dx.doi.org/10.1099/ijs.0.048124-0.
22. **Martineau C, Villeneuve C, Mauffrey F, Villemur R.** 2014. Complete genome sequence of *Hyphomicrobium nitrativorans* strain NL23, a denitrifying bacterium isolated from biofilm of a methanol-fed denitrification system treating seawater at the Montreal Biodome. Genome Announc **2**(1):e01165-13 http://dx.doi.org/10.1128/genomeA.01165-13.
23. **Auclair J, Lepine F, Parent S, Villemur R.** 2010. Dissimilatory reduction of nitrate in seawater by a *Methylophaga* strain containing two highly divergent *narG* sequences. ISME J **4:**1302–1313. http://dx.doi.org/10.1038/ismej.2010.47.
24. **Villeneuve C, Martineau C, Mauffrey F, Villemur R.** 2013. *Methylophaga nitratireducenticrescens* sp. nov. and *Methylophaga frappieri* sp. nov., isolated from the biofilm of the methanol-fed denitrification system treating the seawater at the Montreal Biodome. Int J Syst Evol Microbiol **63:**2216–2222. http://dx.doi.org/10.1099/ijs.0.044545-0.
25. **Brown PJB, Kysela DT, Buechlein A, Hemmerich C, Brun YV.** 2011. Genome sequences of eight morphologically diverse alphaproteobacteria. J Bacteriol **193:**4567–4568. http://dx.doi.org/10.1128/JB.05453-11.
26. **Aziz R, Bartels D, Best A, DeJongh M, Disz T, Edwards R, Formsma K, Gerdes S, Glass E, Kubal M, Meyer F, Olsen G, Olson R, Osterman A, Overbeek R, McNeil L, Paarmann D, Paczian T, Parrello B, Pusch G, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics **9:**75. http://dx.doi.org/10.1186/1471-2164-9-75.
27. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the Rapid Annotation of Microbial Genomes Using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206–D214. http://dx.doi.org/10.1093/nar/gkt1226.
28. **Markowitz VM, Chen I-MA, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides NC.** 2014. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res **42:**D560–D567. http://dx.doi.org/10.1093/nar/gkt963.
29. **Darling AE, Mau B, Perna NT.** 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One **5:**e11147 http://dx.doi.org/10.1371/journal.pone.0011147.
30. **Saitou N, Nei M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406–425.
31. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725–2729. http://dx.doi.org/10.1093/molbev/mst197.
32. **Atlas RM.** 2010. Handbook of microbiological media, 4th ed, vol 1, p 769–868. CRC Press, Boca Raton, FL.
33. **Yoshinari T, Knowles R.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem Biophys Res Commun **69:**705–710. http://dx.doi.org/10.1016/0006-291X(76)90932-3.
34. **Rozen S, Skaletsky H.** 1999. Primer3 on the WWW for general users and for biologist programmers, p 365–386. *In* Misener S, Krawetz S (ed), Bioinformatics methods and protocols, vol 132. Humana Press, Totowa, NJ.
35. **Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM.** 2007. Primer3Plus, an enhanced Web interface to Primer3. Nucleic Acids Res **35:**W71–W74. http://dx.doi.org/10.1093/nar/gkm306.
36. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
37. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:**RESEARCH0034. http://dx.doi.org/10.1186/gb-2002-3-7-research0034.
38. **Andersen CL, Jensen JL, Orntoft TF.** 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res **64:**5245–5250. http://dx.doi.org/10.1158/0008-5472.CAN-04-0496.
39. **Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP.** 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett **26:**509–515. http://dx.doi.org/10.1023/B:BILE.0000019559.84305.47.
40. **Silver N, Best S, Jiang J, Thein SL.** 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol **7:**33. http://dx.doi.org/10.1186/1471-2199-7-33.
41. **Goddard AD, Moir JWB, Richardson DJ, Ferguson SJ.** 2008. Interdependence of two NarK domains in a fused nitrate/nitrite transporter. Mol Microbiol **70:**667–681. http://dx.doi.org/10.1111/j.1365-2958.2008.06436.x.
42. **Polcyn W, Luciński R.** 2003. Aerobic and anaerobic nitrate and nitrite reduction in free-living cells of *Bradyrhizobium* sp. (Lupinus). FEMS Microbiol Lett **226:**331–337. http://dx.doi.org/10.1016/S0378-1097(03)00620-7.
43. **Almeida JS, Reis MAM, Carrondo MJT.** 1995. Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens*. Biotechnol Bioeng **46:**476–484. http://dx.doi.org/10.1002/bit.260460512.
44. **Kornaros M, Zafiri C, Lyberatos G.** 1996. Kinetics of denitrification by *Pseudomonas denitrificans* under growth conditions limited by carbon and/or nitrate or nitrite. Water Environ Res **68:**934–945. http://dx.doi.org/10.2175/106143096X127947.
45. **Ellis MJ, Grossmann JG, Eady RR, Hasnain SS.** 2007. Genomic analysis reveals widespread occurrence of new classes of copper nitrite reductases. J Biol Inorg Chem **12:**1119–1127. http://dx.doi.org/10.1007/s00775-007-0282-2.
46. **Zhang H, Fu H, Wang J, Sun L, Jiang Y, Zhang L, Gao H.** 2013. Impacts of nitrate and nitrite on physiology of *Shewanella oneidensis*. PLoS One **8:**e62629. http://dx.doi.org/10.1371/journal.pone.0062629.
47. **Hiramatsu T, Kodama K, Kuroda T, Mizushima T, Tsuchiya T.** 1998. A putative multisubunit Na^+/H^+ antiporter from *Staphylococcus aureus*. J Bacteriol **180:**6642–6648.
48. **Stewart V, Lu Y, Darwin AJ.** 2002. Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by *Escherichia coli* K-12. J Bacteriol **184:**1314–1323. http://dx.doi.org/10.1128/JB.184.5.1314-1323.2002.
49. **Potter LC, Millington P, Griffiths L, Thomas GH, Cole JA.** 1999.

Competition between *Escherichia coli* strains expressing either a periplasmic or a membrane-bound nitrate reductase: does Nap confer a selective advantage during nitrate-limited growth? Biochem J **344:**77–84. http://dx.doi.org/10.1042/0264-6021:3440077.

50. **Darwin AJ, Ziegelhoffer EC, Kiley PJ, Stewart V.** 1998. Fnr, NarP, and NarL regulation of *Escherichia coli* K-12 *napF* (periplasmic nitrate reductase) operon transcription *in vitro*. J Bacteriol **180:**4192–4198.
51. **Wang H, Tseng C-P, Gunsalus RP.** 1999. The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. J Bacteriol **181:**5303–5308.
52. **Bell LC, Richardson DJ, Ferguson SJ.** 1990. Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: the periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett **265:**85–87. http://dx.doi.org/10.1016/0014-5793(90)80889-Q.
53. **Sears HJ, Sawers G, Berks BC, Ferguson SJ, Richardson DJ.** 2000. Control of periplasmic nitrate reductase gene expression (*napEDABC*) from *Paracoccus pantotrophus* in response to oxygen and carbon substrates. Microbiology **146:**2977–2985.
54. **Hartsock A, Shapleigh JP.** 2011. Physiological roles for two periplasmic nitrate reductases in *Rhodobacter sphaeroides* 2.4.3 (ATCC 17025). J Bacteriol **193:**6483–6489. http://dx.doi.org/10.1128/JB.05324-11.
55. **Simpson PJL, Richardson DJ, Codd R.** 2010. The periplasmic nitrate reductase in *Shewanella*: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB. Microbiology **156:**302–312. http://dx.doi.org/10.1099/mic.0.034421-0.
56. **Chen Y, Wang F, Xu J, Mehmood MA, Xiao X.** 2011. Physiological and evolutionary studies of NAP systems in *Shewanella piezotolerans* WP3. ISME J **5:**843–855. http://dx.doi.org/10.1038/ismej.2010.182.
57. **Villeneuve C, Martineau C, Mauffrey F, Villemur R.** 2012. Complete genome sequences of *Methylophaga* sp. strain JAM1 and *Methylophaga* sp. strain JAM7. J Bacteriol **194:**4126–4127. http://dx.doi.org/10.1128/JB.00726-12.
58. **Delgado MJ, Bonnard N, Tresierra-Ayala A, Bedmar EJ, Müller P.** 2003. The *Bradyrhizobium japonicum napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. Microbiology **149:**3395–3403. http://dx.doi.org/10.1099/mic.0.26620-0.
59. **Baek SH, Shapleigh JP.** 2005. Expression of nitrite and nitric oxide reductases in free-living and plant-associated *Agrobacterium tumefaciens* C58 cells. Appl Environ Microbiol **71:**4427–4436. http://dx.doi.org/10.1128/AEM.71.8.4427-4436.2005.
60. **Tosques IE, Kwiatkowski AV, Shi J, Shapleigh JP.** 1997. Characterization and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. J Bacteriol **179:**1090–1095.
61. **Velasco L, Mesa S, Delgado MJ, Bedmar EJ.** 2001. Characterization of the *nirK* gene encoding the respiratory, Cu-containing nitrite reductase of *Bradyrhizobium japonicum*. Biochim Biophys Acta **1521:**130–134. http://dx.doi.org/10.1016/S0167-4781(01)00279-2.
62. **Laratta WP, Shapleigh JP.** 2003. Site-directed mutagenesis of NnrR: a transcriptional regulator of nitrite and nitric oxide reductase in *Rhodobacter sphaeroides*. FEMS Microbiol Lett **229:**173–178. http://dx.doi.org/10.1016/S0378-1097(03)00821-8.