RESEARCH LETTER

Effects of nitrite on ammonia-oxidizing activity and gene regulation in three ammonia-oxidizing bacteria

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Abstract

Nitrite is the highly toxic end product of ammonia oxidation that accumulates in the absence of a nitrite-consuming process and is inhibitory to nitrifying and other bacteria. The effects of nitrite on ammonia oxidation rates and regulation of a common gene set were compared in three ammonia-oxidizing bacteria (AOB) to determine whether responses to this toxic metabolite were uniform. Mid-exponential-phase cells of *Nitrosomonas europaea* ATCC 19718, *Nitrosospira multiformis* ATCC 25196, and *Nitrosomonas eutropha* C-91 were incubated for 6 h in mineral medium supplemented with 0, 10, or 20 mM $NaNO_2$. The rates of ammonia oxidation (nitrite production) decreased significantly only in $NaNO_2$-supplemented incubations of *N. eutropha*; no significant effect on the rates was observed for *N. europaea* or *N. multiformis*. The levels of *norB* (nitric oxide reductases), *cytL* (cytochrome P460), and *cytS* (cytochrome *c*′-β) mRNA were unaffected by nitrite in all strains. The levels of *nirK* (nitrite reductase) mRNA increased only in *N. europaea* in response to nitrite (10 and 20 mM). Nitrite (20 mM) significantly reduced the mRNA levels of *amoA* (ammonia monooxygenase) in *N. multiformis* and *norS* (nitric oxide reductase) in the two *Nitrosomonas* spp. Differences in response to nitrite indicated nonuniform adaptive and regulatory strategies of AOB, even between closely related species.

Introduction

Nitrification, the oxidative conversion of ammonium to nitrate via nitrite, is carried out by chemolithoautotrophic ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively); ammonia-oxidizing thaumarchaea also convert ammonia to nitrite. Environments like wastewater treatment systems (van Dongen *et al.*, 2001) and axenic cultures of AOB (Stein & Arp, 1998) can accumulate very high concentrations of nitrite, often in the range of 25–30 mM. Yet, the physiological mechanisms that AOB use to adapt to and resist high nitrite concentrations have not been broadly investigated and are limited to a single AOB strain, *Nitrosomonas europaea* ATCC 19718, and enrichment cultures (Tan *et al.*, 2008). These studies show that nitrite and free nitrous acid have toxic effects on AOB (Tan *et al.*, 2008) and specifically and irreversibly inactivate ammonia monooxygenase enzymes of *N. europaea* (Stein & Arp, 1998).

In *N. europaea*, the gene cluster, *ncgABC-nirK*, which encodes a copper-containing nitrite reductase and three functionally related proteins (Beaumont *et al.*, 2004a, 2005), is under direct regulation by nitrite via a NsrR repressor protein (Beaumont *et al.*, 2004a). No other genes in *N. europaea* have been identified as part of a nitrite regulon, although *norB*, encoding nitric oxide reductase, was shown to be more highly expressed in batch cultures of *N. europaea* in the presence of supplemental nitrite (Yu & Chandran, 2010). Furthermore, both *nirK* and *norB* genes were found to be essential for the anaerobic growth of *N. europaea* in which nitrite acts as the terminal electron acceptor (Schmidt *et al.*, 2004). The irreversible inactivation of ammonia monooxygenase enzymes by nitrite in *N. europaea* was found to be under post-translational, but not transcriptional control (Stein & Arp, 1998).

The present study investigated the effect of moderately high nitrite concentrations on three genome-sequenced AOB strains: *N. europaea* ATCC 19718, the long-standing

model strain that provided foundational knowledge of AOB physiology, biochemistry, and genetics (Chain *et al.*, 2003); *Nitrosomonas eutropha* strain C-91, a close taxonomic relative of *N. europaea* that is apparently restricted to environments with very high ammonium loads like wastewater treatment plants (Stein *et al.*, 2007); and *Nitrosospira multiformis* strain ATCC 25196, a representative of the most common AOB genus found in soils (Norton *et al.*, 2008). The effects of nitrite on the ability of these three AOB to further convert ammonia to nitrite and on the expression of a common gene set were compared to determine whether the strains had similar or different responses to this toxic end product of their metabolism. Uniform responses would indicate that prior studies of nitrite effects on *N. europaea* could be universalized to other AOB strains. Different responses would indicate that each strain has evolved its own set of genetic and physiological adaptations to high-nitrite environments that must be explored independently.

Materials and methods

Bacterial cultures

Nitrosospira multiformis strain ATCC 25196, *N. europaea* strain ATCC 19718, and *N. eutropha* strain C-91 were grown in a mineral medium containing per liter: 10 mM $(NH_4)_2SO_4$, 0.4 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM KCl, 0.05% Phenol red, 1 mL of trace element solution (per liter distilled water: 11.5 mM Na_2-EDTA, 10 mM $FeCl_2 \cdot 4H_2O$, 0.5 mM $MnCl_2 \cdot 2H_2O$, 0.1 mM $NiCl_2 \cdot 6H_2O$, 0.1 mM $CoCl_2 \cdot 6H_2O$, 0.1 mM $CuCl_2 \cdot 2H_2O$, 0.5 mM $ZnCl_2$, 0.1 mM $Na_2MoO_4 \cdot 2H_2O$, 1 mM H_3BO_3), and 15 mM HEPES buffer pH 7.5. The pH was maintained at *c.* 7.5 using 5% sodium bicarbonate, added daily following 48 h of growth. *Nitrosomonas europaea* and *N. eutropha* were also grown in the same medium buffered with 43 mM phosphate (per liter: 5.47 g KH_2PO_4 and 0.47 g NaH_2PO_4, pH 8) in place of HEPES. *Nitrosospira multiformis* was incapable of consistent growth in phosphate-buffered medium. Cultures were grown with shaking (180 r.p.m.) at 28 °C in the dark.

The maximum doubling times were similar at 24 h (± 1.90) for *N. europaea*, 20.6 h (± 1.73) for *N. eutropha*, and 22.1 h (± 1.71) for *N. multiformis* (Supporting Information, Fig. S1). *Nitrosospira multiformis* cultures produced half the cell numbers, but biomass equivalent to that of *Nitrosomonas* cultures. All cultures produced 13–15 mM nitrite by the late exponential phase. The maximum doubling times were significantly shorter at 7.1 (± 0.68) and 9.2 (± 1.38) h for *N. europaea* and *N. eutropha*, respectively, when grown in phosphate- instead of HEPES-buffered medium (Fig. S1) and produced *c.* 18 mM nitrite (± 0.04) by the late exponential phase.

Experimental incubations

Cells were harvested in the mid-exponential growth phase as determined by the levels of nitrite accumulation (*c.* 10 mM ± 0.76 for *N. multiformis* and *c.* 13 mM ± 0.23 for *Nitrosomonas* cultures). Cells were collected by centrifugation (15 000 ***g***, 10 min), washed three times in HEPES buffer (15 mM, pH 7.5) or sodium phosphate buffer (50 mM NaH_2PO_4, 2 mM $MgSO_4$, pH 8) for HEPES or phosphate-grown cells, respectively, and resuspended in 10 mL of a fresh medium to a concentration of 10^9 cells mL^{-1} as determined by a Petroff–Hausser counting chamber and phase-contrast light microscopy. The medium was amended with 0, 10, or 20 mM $NaNO_2$. Flasks were incubated with shaking (180 r.p.m.) at 28 °C in the dark. Samples (2 mL) were taken at $t = 0$, 0.5, 2, 4, and 6 h and cells were collected by centrifugation (21 000 ***g***, 2 min). The supernatant was used for pH and nitrite measurements (Hageman & Hucklesby, 1971), and cell pellets were immediately treated with 500 μL RNAprotect (Qiagen, Valencia, CA) for storage at − 80 °C. Three to seven replicates of each incubation condition using batches of cells grown on separate days were compared.

Dot-blot hybridization

Cross-comparisons of nucleotide and predicted protein sequences were performed using genome sequences and BLAST functions available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). GenBank accession numbers for genome sequences are *N. europaea*, AL954747; *N. eutropha*, CP000450; and *N. multiformis*, CP000103.

Nucleotide sequences of each targeted gene from each strain were used to design specific primers using PRIMER3 INPUT 0.4.0 software (Rozen & Skaletsky, 2000) (Table S1). PCR reactions included standard reagents and concentrations for Taq polymerase (Sambrook & Russell, 2001) and isolated genomic DNA (AquaPure Genomic DNA Isolation Kit, Bio-Rad, Hercules, CA) as a template. Amplification conditions for all primer sets were: 95 °C for 5 min; 30 cycles of 95 °C for 40 s, 55 °C for 40 s and 72 °C for 50 s; and 72 °C for 7 min (iCycler, Bio-Rad). Single PCR products of appropriate size were verified by agarose gel and purified (QIAquick PCR Purification kit, Qiagen). Purified PCR products were labeled (Prime-a-Gene, Promega, Madison, WI) with [α-^{32}P]-dCTP (3000 Ci $mmol^{-1}$; Perkin-Elmer, Waltham, MA) and random hexamers. The dynamic range of detection for each probe was tested with 0.25–3 μg mRNA from control incubations of each culture. The r^2 values for the slope of hybridization intensity vs. microgram of RNA concentration were between 0.92 and 1.00 for all probes and all strains (data not shown).

Total RNA was extracted from cell pellets using the Aurum Total RNA Mini kit as per the manufacturer's instructions (Bio-Rad). Nucleic acid concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE). Two micrograms of total RNA from each sample were blotted onto Zeta-Probe GT nylon membranes (Bio-Rad) using a Minifold filtration manifold (Schleicher & Schuell, Keene, NH). RNA from exponential-phase cells harvested directly from culture (and not resuspended into a fresh medium) was blotted onto the same membrane as RNA from cells subjected to short-term incubations to ensure comparability of the hybridization signals. Membranes were dried overnight and UV-cross-linked (FB-UVXL-1000, Fisher Scientific, Pittsburgh, PA). Prehybridization, hybridization, and washing of membranes were performed according to the manufacturer's instructions (Bio-Rad) at 30 °C. To allow reprobing, membranes were stripped of radioactivity by washing twice in a 0.1 × SSC/0.5% SDS solution at 95–100 °C for 20 min. Hybridization intensity was analyzed using a TYPHOON PHOSPHORIMAGER and IMAGEQUANT software (Amersham, Piscataway, NJ).

Data analysis

Background from nonspecific binding of the probes to the membrane was subtracted from the hybridization signals. The relative hybridization intensity was normalized by dividing the gene-specific signals by 16S rRNA gene probes. The fold difference in the levels of mRNA for each gene, time point, and organism resulting from $NaNO_2$ exposure was determined by dividing hybridization intensities from dot blots of RNA extracted from $NaNO_2$ amended to those from unamended incubations. A twofold change in transcript level was considered a significant effect of nitrite on gene expression. Student's *t*-tests ($P < 0.05$) were performed to verify significant differences between treatments for both activity and gene expression levels.

Results

Effects of $NaNO_2$ on the rates of ammonia oxidation to nitrite

Short-term (6 h) incubations were used to determine the effects of $NaNO_2$ on the ability of the AOB to oxidize ammonia to nitrite. Concentrations of $NaNO_2$ similar to that applied in previous studies of nitrite effects on *N. europaea* were used (Stein & Arp, 1998; Beaumont *et al.*, 2004a, b). The final pH was significantly higher in $NaNO_2$ amended than in unamended incubations for all three AOB, indicating less acidification and thus reduced rates of ammonia oxidation (Table 1). However, among the three AOB, only *N. eutropha* showed significantly slower rates of and less net nitrite production when incubated in the $NaNO_2$-amended medium, although this strain also had the fastest maximum nitrite production rate among the three strains (Table 1). Similar results were observed for *N. eutropha* and *N. europaea* cells incubated in phosphate-buffered, rather than HEPES-buffered, medium (data not shown). Thus, among the three AOB, the ammonia-oxidizing activity of *N. eutropha* was the most negatively affected by the presence of high nitrite concentrations.

Effect of nitrite on levels of select gene expression

Genes selected for this study included those with demonstrated involvement in the ammonia oxidation and/or the nitrite reduction pathways of *N. europaea* (Klotz & Stein, 2011). The genes were *amoA*, encoding the α-subunit of ammonia monooxygenase; *nirK*, encoding copper-containing nitrite reductase; *norB* and *norS*, both encoding cytochrome *c*-dependent nitric oxide reductases; *cytS*, encoding cytochrome *c*′-β; and *cytL*, encoding cytochrome P460. NirK and NorB have demonstrated activity in reducing nitrite to nitrous oxide via nitric oxide in *N. europaea* (Beaumont *et al.*, 2002, 2004b; Schmidt *et al.*, 2004). The *norS* gene has been identified only in AOB and a few other bacteria (Stein *et al.*, 2007; Norton *et al.*, 2008) and encodes a nitric oxide reductase with high similarity to NorB (J. Hemp, pers. commun.). Cytochrome *c*′-β has a putative function in nitrogen oxide detoxification, while the evolutionarily related cytochrome P460 was shown to oxidize hydroxylamine to nitrite in *N. europaea* (Elmore *et al.*, 2007). Comparisons of similarity between nucleotide and translated protein sequences of genes in *N. eutropha* and *N. multiformis* to orthologues in *N. europaea* are shown in Table 2. *Nitrosospira multiformis* lacks cytochrome P460, and as it belongs to a different genus, there was less sequence similarity between *N. multiformis* and *N. europaea* than between the two *Nitrosomonas* strains for all genes.

Incubations supplemented with $NaNO_2$ only caused significant changes in the expression levels of three of the six functional genes examined. No significant change was detected in the levels of *norB*, *cytL*, or *cytS* mRNA of any AOB, suggesting no regulation of these genes by nitrite (data not shown). The levels of *amoA* mRNA of *N. multiformis* were significantly reduced in incubations supplemented with 20 mM $NaNO_2$, but not with 10 mM $NaNO_2$ (Fig. 1). Similarly, the levels of *norS* mRNA of *N. europaea* and *N. eutropha* were also significantly reduced in incubations supplemented with 20 mM $NaNO_2$, but not 10 mM $NaNO_2$ (Fig. 2). The levels of *nirK* mRNA were only significantly increased in *N. europaea* with either 10 or 20 mM $NaNO_2$, although the increase was short lived (Fig. 3). Similar trends in gene expression were observed for *N. europaea* and *N.*

Table 1. pH and nitrite production measurements for incubations of AOB strains in HEPES-buffered medium with or without $NaNO_2$ supplementation

	pH at 6 h			Net NO_2^- (mM) produced at 6 h			Maximum rate of NO_2^- production ($mM\ h^{-1}$)		
Organism	No amendment	10 mM NO_2^-	20 mM NO_2^-	No amendment	10 mM NO_2^-	20 mM NO_2^-	No amendment	10 mM NO_2^-	20 mM NO_2^-
N. europaea	5.80 (0.03)[a]	**5.95** (**0.06**)[c]	**6.04** (**0.07**)[e]	4.55 (0.17)[g]	3.98 (0.42)[h]	3.33 (0.92)[i]	1.83 (0.14)[j]	1.80 (0.10)[l]	1.57 (0.21)[n]
N. eutropha C91	5.57 (0.04)[b]	**5.72** (**0.02**)[d]	**5.81** (**0.02**)[f]	4.84 (0.07)[g]	**4.61** (**0.08**)[h]	**4.43** (**0.14**)[i]	2.26 (0.02)[k]	**2.15** (**0.03**)[m]	**2.03** (**0.02**)[o]
N. multiformis	5.57 (0.12)[ab]	**5.90** (**0.07**)[c]	**6.07** (**0.06**)[e]	4.49 (0.19)[g]	4.34 (0.43)[h]	4.89 (0.53)[i]	1.62 (0.33)[j]	1.80 (0.50)[lm]	1.50 (0.57)[no]

Values in parentheses indicate the SE ($n = 3$–7). Bold values indicate a significant difference between measurements for the same organism incubated in medium with $NaNO_2$ supplementation relative to medium without added $NaNO_2$. Superscript letters indicate measurements within the same statistical grouping for an individual treatment among the three AOB. Significant differences were determined using Student's *t*-test at $P < 0.05$. Initial pH: 7.58 ± 0.02 for all incubations. The rates of NO_2^- production were measured over the maximum linear range for each incubation.

Table 2. Similarity of nucleotide and predicted protein sequences of genes in *Nitrosomonas eutropha* C91 and *Nitrosospira multiformis* compared with related sequences in *Nitrosomonas europaea*

	% Sequence identity relative to similar sequences in *Nitrosomonas europaea* (nucleotide/translated protein sequence)	
Gene/translated product	*Nitrosomonas eutropha* C91	*Nitrosospira multiformis*
amoA/ammonia monooxygenase subunit A*	88/97	84/84
nirK/nitrite reductase†	79/82	NS/32
norB/nitric oxide reductase	82/90	NS/41
norS/nitric oxide reductase‡	82/91	NS/51
cytS/cytochrome *c*′-β	81/82	NS/32
cytL/cytochrome P460	82/87	Not present
16S rRNA gene	97	92

*Two and three copies of nearly identical *amoA* genes reside in the two *Nitrosomonas* spp. and in *N. multiformis*, respectively; thus, the percent identity for the multiple copies is the same when comparing between species.

†*nirK* genes are part of a four-member operon in the two *Nitrosomonas* spp. and is a singlet in *N. multiformis* (Cantera & Stein, 2007a).

‡*norS* genes are annotated as cytochrome *c* oxidase subunit II due to conservation among the heme-copper oxidase superfamily. Biochemical evidence shows NorS to be a functional nitric oxide reductase (J. Hemp, pers. commun.).

NS, no significant sequence similarity at a cut-off value of $1e^{-05}$.

eutropha grown in phosphate-buffered medium, although *norS* mRNA levels decreased less than twofold relative to the no nitrite control (data not shown). No significant differences were found in the hybridization intensities of mRNA extracted from cells immediately harvested from culture vs. those taken at $t = 0$ from the short-term incubations, indicating no immediate effects from resuspending cells into a fresh medium with or without $NaNO_2$ amendment (data not shown).

Discussion

The nonuniformity of the physiological and transcriptional responses of these three AOB to relatively high nitrite concentrations demonstrates that each strain, even those as closely related as *N. europaea* and *N. eutropha*, has a different ability and mechanism to tolerate the major end product of their metabolism. Therefore, the effects of nitrite on *N. europaea* found in this and prior studies cannot be universalized to other AOB.

Previous studies of *N. europaea* have shown that the expression of *amoA* is regulated primarily by the availability of NH_3 (Sayavedra-Soto *et al.*, 1996) and O_2 (Yu & Chandran, 2010). However, exponential-phase *N. europaea* showed decreased *amoA* mRNA levels when grown in batch cultures supplemented with nitrite (Yu & Chandran, 2010), although this particular study involved a longer time course and supplementation of media with nitrite before inoculating cells for growth experiments, likely exposing them to a higher overall nitrite load than in the present study. In the present study, there was no acute effect of nitrite on *amoA* mRNA levels in either *Nitrosomonas* strain, only in *N. multiformis* (Fig. 1). The decrease in *amoA* mRNA did not translate to a significant decrease in the nitrite production rate of *N. multiformis* (Table 1). Similarly, the unchanged *amoA* mRNA levels in *N. eutropha* did not correlate with its decreased nitrite production rate. Thus, the expression of *amoA* did not correlate to ammonia-oxidizing activity in any of the AOB, at least in these short-term incubations. These observations indicate that caution must be exercised

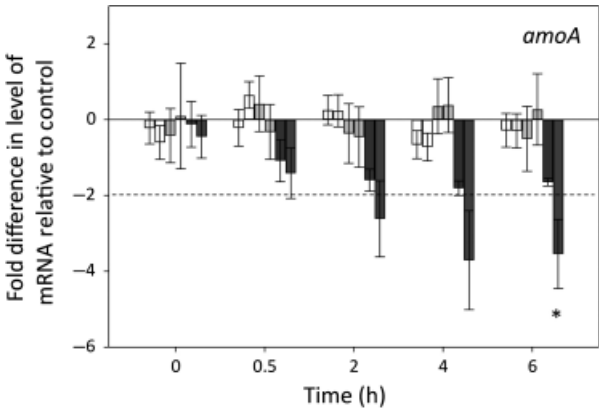

Fig. 1. Fold difference in *amoA* mRNA levels over a 6-h time course for incubations of *Nitrosomonas europaea* ATCC 19713 (white bars), *Nitrosomonas eutropha* (light gray bars), and *Nitrosospira multiformis* (dark gray bars) supplemented with 10 mM (first bar of each couple) or 20 mM (second bar of each couple) $NaNO_2$ relative to a control culture with no nitrite supplementation. *Significant difference in the level of mRNA between the 10 mM and the 20 mM $NaNO_2$ condition. Error bars represent the SE for three to seven replicate experiments with cells from different cultures grown on different days.

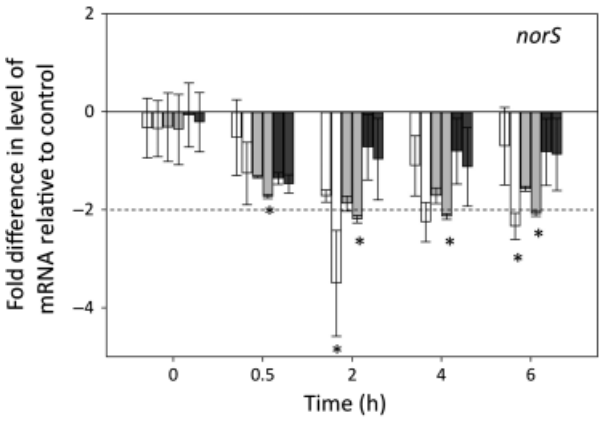

Fig. 2. Fold difference in *norS* mRNA levels over a 6-h time course. Bars, error bars, and asterisks are the same as those reported for Fig. 1.

when using absolute *amoA* gene expression as a proxy for acute rates of ammonia-oxidizing activity.

Of the two genes encoding nitric oxide reductase, *norB* and *norS*, only the levels of *norS* mRNA in the two *Nitrosomonas* spp. were significantly decreased in incubations with nitrite supplementation (Fig. 2). A prior study showed upregulation of *norS* in NirK-deficient *N. europaea* under conditions where hydroxylamine conversion to nitrous oxide was highly favored (Cho *et al.*, 2006; Cantera & Stein, 2007b). Thus, the decrease of *norS* mRNA levels could have been in response to less favorable conditions for ammonia oxidation, and hence hydroxylamine formation,

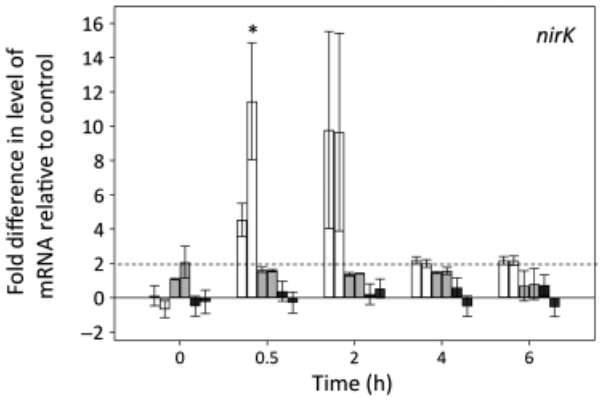

Fig. 3. Fold difference in *nirK* mRNA levels over a 6-h time course. Bars, error bars, and asterisks are the same as those reported for Fig. 1.

if NorS indeed plays a specific role in reducing nitric oxide arising from incomplete hydroxylamine oxidation to nitrite.

Studies in *N. europaea* have linked the expression of *nirK* and *norB* genes with the reduction of nitrite to nitrous oxide via nitric oxide (Beaumont *et al.*, 2002, 2004b; Schmidt *et al.*, 2004). Similarly, the ability of *Nitrosospira* spp. to produce nitrous oxide has been suggested to involve orthologous genes (Shaw *et al.*, 2006; Garbeva *et al.*, 2007), although a direct linkage between this activity and *nirK* or *norB* expression has not yet been demonstrated in any *Nitrosospira* spp. The present study showed no effect of nitrite on the expression of either *nirK* (Fig. 2) or *norB* (data not shown) in *N. multiformis*, which is understandable at the molecular level as neither gene has a recognizable nitrite-responsive regulatory protein-binding motif in its promoter region (Norton *et al.*, 2008). The more surprising result was the lack of increased *nirK* mRNA levels in *N. eutropha* from exposure to nitrite (Fig. 2) as the *ncgABC-nirK* operon, promoter-proximal NsrR-binding motif, and NsrR repressor share high sequence identity between *N. europaea* and *N. eutropha* (Cantera & Stein, 2007a; Stein *et al.*, 2007). Together, the data suggest that while the expression of the NirK enzyme is vital to nitrite reduction (Schmidt *et al.*, 2004) and tolerance (Beaumont *et al.*, 2005; Cantera & Stein, 2007b) in *N. europaea*, it may play a lesser role in *N. eutropha* and *N. multiformis* or is constitutively expressed to perform these functions.

mRNA levels of the three remaining genes, *norB*, *cytL* (encoding cytochrome P460), and *cytS* (encoding cytochrome c'-β) were not affected by nitrite in any of the AOB, suggesting constitutive expression in the presence of this toxic metabolite (data not shown). In *N. europaea*, it was suggested that *norB* is constitutively expressed during aerobic metabolism (Beaumont *et al.*, 2004b), but is induced during anaerobic metabolism (Beyer *et al.*, 2009) and during growth in the presence of $NaNO_2$ (Yu & Chandran, 2010). We were unable to confirm induction of *norB* expression by

$NaNO_2$, but did indicate a constant presence of *norB* mRNA (i.e. 0.03–0.08% of the 16S rRNA gene pool) for all three AOB in all incubations.

Although the present study examined only a small subset of shared genetic inventory among three AOB strains, the data revealed that the regulation of these genes was not predictable based on sequence or regulatory motif similarities. This observation was particularly surprising for the *nirK* genes of the two *Nitrosomonas* strains. Thus, nitrite and probably other metabolites of AOB are certain to have physiological and genetic effects that vary from strain to strain. This variability must be recognized when building predictive models of how environmental factors, like transiently high nitrite loads, affect AOB physiology, gene expression, and nitrification rates.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth curves of AOB cultivated in HEPES- (a) and phosphate- (b) buffered medium; *Nitrosomonas europaea* (squares), *Nitrosomonas eutropha* (circles), and *Nitrosospira multiformis* (triangles).

Table S1. Genes and PCR primers used in this study.

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