Nitril ammonification by Nautilia profundicola AmH: experimental evidence consistent with a free hydroxylamine intermediate

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INTRODUCTION

Nitril ammonification is a major fixed nitrogen pool in the global N-cycle (Falkowski and Godfrey, 2008; Klotz and Stein, 2008). Nitril ammonification can be reduced via demitrification to nitrous oxide or dinitrogen gas, whereby it is lost from terrestrial and aquatic ecosystems, or via ammonification to ammonium that can be retained (Brandes et al., 2007; Klotz and Stein, 2008, 2010). In both pathways, nitril ammonification is reduced to nitril ammoniation. In heterotrophic demitrifiers and ammonia oxidizers, nitrite is further reduced by NO-forming nitrite reductases: NirS or NirK. Nitrite reductase module (reverse-HURM) pathway. Instead of a classical ammonia-forming nitrite reductase that performs a 6 electron-transfer process, the pathway is thought to employ two catalytic redox modules operating in sequence: the reverse-HURM reducing nitril ammoniation followed by a hydroxylamine reductase that converts hydroxylamine to ammonium. Experiments were performed on Nautilia profundicola strain AmH, whose genome sequence led to the reverse-HURM pathway proposal. N. profundicola produced ammonium from nitrate, which was assimilated into biomass. Furthermore, genes encoding the catalysts of the reverse-HURM pathway were preferentially expressed during growth of N. profundicola on nitril ammoniation as an electron acceptor relative to cultures grown on polysulfide as an electron acceptor. Finally, nitril ammoniation grown cells of N. profundicola were able to rapidly and stoichiometrically convert high concentrations of hydroxylamine to ammonium in resting cell assays. These experiments are consistent with the reverse-HURM pathway and a free hydroxylamine intermediate, but could not definitively exclude direct nitrite reduction to ammonium by the reverse-HURM with hydroxylamine as an off-pathway product. N. profundicola and related organisms are models for a new pathway of nitril ammonification that may have global impact due to the wide distribution of these organisms in hypoxic environments and symbiotic or pathogenic associations with animal hosts.

Keywords: hydroxylamine, hydroxylamine oxidoreductase, nitrite, Epsilonproteobacteria, nitril ammonification
NAP complex in *Epsilonproteobacteria* usually lacks a NapC protein (Kern and Simon, 2009; Klotz and Stein, 2010; Simon and Klotz, 2013). Based on genome-informed metabolic reconstruction (Campbell et al., 2009), we recently proposed a novel pathway for nitrate assimilation (Figure 1) whereby nitrate is reduced to hydroxylamine by a hydroxylamine:ubiquinone redox module (HURM, i.e., a quinone-dependent hydroxylamine dehydrogenase), which we term the reverse-HURM pathway. In *N. profundicola*, the HURM is thought to consist of a NapC/NrfH-related cytochrome cM552 (cycB, NAMH_0559) that mediates electron transfer between the quinone pool and a periplasmic hydroxylamine oxidoreductase (HAO, NAMH_1280). HURM, operating in the forward direction and consisting of hydroxylamine dehydrogenase (EC:1.7.2.6; HaoA3) connected to a cytochrome c protein electron shuttle (c554 and/or cM552 encoded by cycA and cycB, respectively), links the oxidation of hydroxylamine to the quinone pool in aerobic ammonia-oxidizing bacteria (Klotz and Stein, 2008; Simon and Klotz, 2013). In anaerobic ammonia-oxidizing (anammox) bacteria, the redox module consists of hydrazine oxidoreductase and an as yet unidentified cytochrome c (Jetten et al., 2009; Simon and Klotz, 2013). In the reverse-HURM pathway, hydroxylamine generated from nitrite in the periplasm is transported into the cytoplasm and reduced to ammonium via a hydroxylamine reductase, also known as the hybrid cluster protein (Har/Hcp). Thus, the proposed electron accepting reactions of the reverse-HURM pathway are:

| Reaction | Enzyme |
|----------|--------|
| (1)      | 2H⁺ + NO⁻³ + 2e⁻ → NO₂⁻ + H₂O  | Nitrate reductase |
| (2)      | 5H⁺ + NO₂⁻ + 4e⁻ → NH₂OH + H₂O | HURM |
| (3)      | 3H⁺ + NH₂OH + 2e⁻ → NH₄⁺ + H₂O | Hydroxylamine reductase |
| (4)      | 10H⁺ + NO⁻³ + 8e⁻ → NH₄⁺ + 3H₂O | Sum |

![FIGURE 1](image-url)  
*FIGURE 1* | The reverse-HURM pathway in *N. profundicola*. Individual steps are noted with capital letters in the figure. Numbers in the depicted proteins refer to locus tags from the AmH genome (i.e., 1280 = NAMH_1280 = HaoA). (A) Nitrate reduction by respiratory periplasmic nitrate reductase (NapABGH, 0556-53), (B) HURM (Hydroxylamine:Ubiquinone Redox Module) comprised of a reversely operating hydroxylamine oxidoreductase (HAO, 1280) functioning as an ocataheme nitrite reductase, that produces hydroxylamine utilizing electrons donated from a tetra-heme cytochrome c in the NapC/NrfH/O₅₅₂ family (CycB, 0559). (C) Transport of hydroxylamine via ammonia transporters related to AmtB. (D) Reduction of hydroxylamine to ammonium by a hybrid cluster protein/hydroxylamine reductase (Hcp/Har, 1034) utilizing reducing power from a predicted Fe-S containing protein (1302) whose electron donor (X) is currently unknown. Assimilation of ammonium into biomass occurs via glutamine synthetase and glutamine:2-oxoglutarate aminotransferase (GS-GOGAT).
The genomes of *N. profundicola*, *C. concisus, C. curvis, C. fetus, and Caminibacter mediatlanticus* each contain genes encoding the enzymes of the reverse-HURM pathway while the genomes of other *Epsilonproteobacteria* encode homologues of the classical NO-forming NirS/NirK and ammonium-forming assimilatory siroheme NirA, or respiratory pentaheme NrfA nitrite reductases [Table 1, (Kern and Simon, 2009)]. We hypothesize that the reverse-HURM pathway improves the survival and/or dispersal of *N. profundicola* and *Campylobacter* spp. in ammonium-deficient host or non-host associated environments. However, for this hypothesis to be true, the reverse-HURM pathway must provide ammonium for biosynthesis and/or facilitate energy conservation in these organisms.

**RESULTS**

**ENERGETICS OF GROWTH IN *N. profundicola***

*N. profundicola* utilizes both formate and hydrogen as electron donors for energy metabolism and is typically grown on polysulfide as the electron acceptor (Campbell et al., 2001; Smith et al., 2008). Calculations under standard conditions (1 M of all species, 23°C, pH = 7) indicate that when nitrate is reduced by the reactions of the reverse-HURM pathway, the Gibbs free energy (ΔG°) is substantially greater than that available from the reduction of polysulfide (Table 2). For example, the ΔG° per mole of formate oxidized with nitrate is 7.4-fold increased over polysulfide (−237 kJ mol⁻¹ vs. −32 kJ mol⁻¹). From this calculation, one can predict that the growth of *N. profundicola* should be stimulated in cultures with nitrate as the electron acceptor relative to polysulfide.

**GROWTH OF *N. profundicola* WITH NITRATE**

Prior characterization and growth of *N. profundicola* utilized media with polysulfide as a combined sulfur source and electron acceptor (Campbell et al., 2001, 2009; Smith et al., 2008). To further clarify the role of nitrate in the energy metabolism of *N. profundicola*, strain AmH was grown with sulfide as the sole sulfur source so that nitrate was the sole electron acceptor (Figure 2). The electron donor was a mixture of hydrogen and formate in a medium with no other sources of sulfur or nitrogen (Campbell et al., 2001). Growth rates with nitrate as potential electron acceptor and nitrogen source and sulfide (sulfur source) or polysulfide (sulfur source and potential electron acceptor) were more than double the growth rates observed in cultures using ammonium as the sole nitrogen source and polysulfide as an electron acceptor and sulfurre source. As expected, negative control (no electron acceptor) cultures provided with ammonium as the nitrogen source and sulfide as the sulfur source were unable to grow.

**NITROGEN BALANCE IN *N. profundicola* CULTURES**

To confirm that the level of ammonium produced by *N. profundicola* was in excess of biosynthetic needs, we calculated the biosynthetic nitrogen demand as follows. Assuming a C:N ratio of 32:6.4 for exponentially growing bacterioplankton (Vrede et al., 2002), 288 fg of C µm⁻³ for exponentially growing *Escherichia coli* (Loferer-Krossbacher et al., 1998), and a cell volume of 0.021 µm³ calculated from the reported dimensions of *N. profundicola* (Smith et al., 2008), the production of 1 × 10⁸ cells ml⁻¹ requires ~120 ng ml⁻¹ of N (~9 µM), a figure that would not change regardless of the N-source utilized. *N. profundicola* did not utilize a significant amount of the ammonium provided during growth with polysulfide as the electron acceptor (Figure 3, open circles), which agrees with the low N requirement calculated to produce the observed cell densities. The ammonium plus sulfide culture did not grow (Figure 2) and also did not detectably consume ammonium (Figure 3, closed circles). In contrast, the amount of ammonium produced by cells grown with 5 mM nitrate was 4.6 mM with polysulfide and 5.2 mM with sulfide (Figure 3, open and closed squares, respectively). If the reverse-HURM pathway in the *Epsilonproteobacteria* primarily serves to produce ammonium for biosynthesis, we would expect low levels of exogenous ammonium to strongly repress HURM pathway gene expression and nitrate reduction activity. However, the stoichiometric conversion of nitrate to ammonium in excess

![Table 1](https://www.frontiersin.org) | Nitrogen metabolism gene inventories in genome-sequenced Epsilonproteobacteria.
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|   | napA/nasA | nirA | nrfA | haoA | cycB/napC | NAMH_1302 | nirS | norB | norE | norZ | nosZ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NAUTILIALES | | | | | | | | | | | |
| *N. profundicola* | + | − | − | + | + | + | + | − | − | − | − |
| *C. mediatlanticus* TB-2 | + | + | NF | + | + | + | + | NF | NF | NF |
| CAMPYLOBACTERIALES | | | | | | | | | | | |
| *C. fetus* 82-40 | + | − | + | + | + | + | + | − | − | − | − |
| *C. concisus* 13826 | + | − | − | + | + | + | + | − | norZ | − | − |
| *C. curvis* 525.92 | + | − | − | + | + | + | + | − | norZ | − | − |
| *A. butzleri* | + | + | + | − | − | − | + | + | + | + | + |
| *W. succinogenes* | + | − | + | − | − | − | − | + | + | + | + |
| *S. denitrificans* | + | + | − | − | − | − | − | + | + | + | + |

Components of the proposed reverse-HURM pathway are noted in bold text in the shaded columns.

+, present in complete genome sequence; −, absent in complete genome sequence; NF, absent in draft genome sequence with multiple contigs.

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Table 2 | Calculated free energies of reaction under standard conditions (1 M all species, 25°C and pH = 7) for electron accepting half reactions of nitrogen compounds and polysulfide and coupled reactions with formate or hydrogen as the electron donor.

| HALF REACTIONS                                                                 | \( E^0 \) (V) | \( \Delta G^0 \) (kJ mol\(^{-1}\)) |
|--------------------------------------------------------------------------------|---------------|----------------------------------|
| \( 2H^+ + NO_3^- + 2e^- \rightarrow NO_2^- + H_2O \)                          | +0.873        | −169                             |
| \( 5H^+ + NO_2^- + 4e^- \rightarrow NH_2OH + H_2O \)                          | +0.270        | −104                             |
| \( 3H^+ + NH_2OH + 2e^- \rightarrow NH_4^+ + H_2O \)                          | +1.780        | −344                             |
| \( S_{3-8}^- + H^+ + 2e^- \rightarrow S_{2-7}^{2-} + HS^- \)                 | −0.267        | +52                              |

| COUPLED REACTIONS                                                             | \( \Delta G^0 \) (kJ mol\(^{-1}\)) | per H2 or CH2O2 |
|--------------------------------------------------------------------------------|----------------------------------|----------------|
| H\(_2\) oxidation                                                             |                                  |                |
| \( 4H_2 + NO_3^- + 2H^+ \rightarrow NH_4^+ + 3 H_2O \)                       | −937                            | −234           |
| \( 3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2 H_2O \)                       | −688                            | −229           |
| \( H_2 + S_{3-8}^{2-} \rightarrow H^+ + S_{2-7}^{2-} + HS^- \)              | −28                             | −28            |
| Formate oxidation                                                             |                                  |                |
| \( 4CH_2O_2 + NO_3^- + 2H^+ \rightarrow 4CO_2 + NH_4^+ + 3 H_2O \)           | −946                            | −237           |
| \( 3CH_2O_2 + NO_2^- + 2H^+ \rightarrow 3CO_2 + NH_4^+ + 2 H_2O \)           | −695                            | −232           |
| \( CH_2O_2 + S_{3-8}^{2-} \rightarrow CO_2 + S_{2-7}^{2-} + H^+ + HS^- \)   | −32                             | −32            |

of the predicted N requirement and observed ammonium consumption in the ammonium + polysulfide cultures is consistent with the hypothesis that nitrate is utilized as a terminal electron acceptor by *N. profundicola* and that nitrate ammonification is not subject to repression by ammonium.

**15N-NO\(_3^\) INCORPORATION INTO BIOMASS**

The potential for nitrate assimilation was assessed by examining the incorporation of 15N-labeled ammonium or nitrate into *N. profundicola* biomass during growth with all above combinations of electron donors and potential acceptors. *N. profundicola* grown on 20 Atom% 15N-labeled ammonium with polysulfide produced biomass enriched to ∼10 Atom% 15N after one round of batch culturing (Figure 4), whereas the unlabeled control was not enriched in 15N. Cultures grown with sulfide and 20 Atom% 15N-labeled nitrate produced biomass enriched to 16 Atom% 15N (Figure 4). Nitrate-grown biomass contained approximately 5-fold more N than ammonium-grown biomass (data not shown), which may explain the more extensive enrichment of 15N in the nitrate-grown samples.

**TRANSCRIPTION OF KEY PATHWAY GENES**

Transcript abundance for genes that encode enzymes of the proposed reverse-HURM pathway in *N. profundicola* was determined by quantitative real-time PCR in batch cultures grown with ammonium or nitrate. All components of the proposed pathway displayed a strong increase in transcript abundance in
nitrate grown cultures (Figure 5). mRNAs encoding a nitrate reductase subunit (napA) were 4.6-fold \( (P < 0.05, \text{two-tailed heteroscedastic } t\text{-test}) \) more abundant in nitrate + sulfide grown cells relative to ammonium + polysulfide grown cells. Transcripts of both components of the HURM, haoA and cycB/napC, were increased by 8.5- and 7.1-fold \( (P < 0.05 \text{ for both}) \), respectively. Consistent with our prediction that hydroxylamine produced in the periplasm by the HURM would be transported through major facilitator protein channels, mRNAs encoding both homologs of the ammonium/methylammonium transporter \( \text{amtB} \) were increased by 4.5-fold \( (\text{amtB}-1, \text{NAMH}_0397, \ P < 0.08) \) and 10.3-fold \( (\text{amtB}-2, \text{NAMH}_0215, \ P > 0.10) \). Hydroxylamine is a powerful mutagen that must be detoxified as it arrives in the cytoplasm. We propose that this is accomplished by NADH-dependent Har/Hcp based on studies of the \( hcp \) gene in the nitrate assimilation gene cluster of \( \text{Rhodobacter capsulatus} \) \( \text{E1F1} \) where it is required for the reduction and assimilation of hydroxylamine produced by a cytoplasmic assimilatory nitrate reductase (Cabello et al., 2004; Pino et al., 2006). Consistent with this proposal, the largest change in transcript abundance was seen for \( \text{har} \), which increased by 11.7-fold, but this change was not significant \( (P > 0.10) \) due to the high variability in independent measurements of \( \text{har} \) transcript abundance. Originally, we had proposed that the electron donor to Har was an NADH dehydrogenase encoded by \( \text{NAMH}_0542 \) (Campbell et al., 2009). However, we now believe a better candidate for this function is \( \text{NAMH}_1302 \), a predicted 4Fe-4S cluster protein, based on conservation in other \( \text{Epsilonproteobacteria} \) (Table 1). Consistent with this prediction, transcripts of \( \text{NAMH}_1302 \) were 4.6-fold more abundant in nitrate grown cells relative to ammonium, but the change was not significant \( (P > 0.10) \).

To test for potential ammonium repression of pathway expression, ammonium was added to nitrate + sulfide grown cultures and the mRNA abundance quantified for the same genes. In general, ammonium addition decreased mRNA abundances of the reverse-HURM pathway encoding genes from 1.2- to 3.6-fold (Figure 5, gray bars), though this effect was only significant \( (P < 0.10) \) for \( \text{haoA} \) and \( \text{napC/cycB} \) transcripts that encode the HURM subunits. Interestingly, the expression of \( \text{napA} \) decreased the least (1.2-fold), indicating the need for nitrate respiration in these cells. In no case, did the addition of ammonium reduce transcript levels to those seen in cultures grown in ammonium + polysulfide.

**EVIDENCE FOR A HYDROXYLAMINE INTERMEDIATE**

Direct measurements of hydroxylamine in filtrates of \( \text{N. profundicola} \) cultures grown with nitrate found low, but consistently detectable levels of hydroxylamine \( (5.7 \pm 3.5 \mu M, SD, \ n = 3) \). This low level is consistent with an intermediate whose production and consumption rates are closely matched.

To determine if hydroxylamine could be metabolized by \( \text{N. profundicola} \), washed suspensions of nitrate-grown cells were incubated in nitrate-free growth medium with hydrogen as the sole electron donor. Hydroxylamine was added as a potential electron acceptor and the concentrations of both hydroxylamine and ammonium were followed over time. The results of this experiment indicate that nitrate-grown cells of \( \text{N. profundicola} \) rapidly and completely convert hydroxylamine to ammonium (Figure 6). The specific rate of hydroxylamine conversion in this experiment was \( 28.6 \mu M \text{ min}^{-1} \text{ } 10^8 \text{ cells}^{-1} \). The ammonium production rate from nitrate calculated for exponential growth in Figure 3 \((8-24 \text{ h})\) is \( \sim 3 \mu M \text{ min}^{-1} \text{ } (10^8 \text{ cells})^{-1} \). Thus, the measured hydroxylamine conversion rate is far in
The data presented here are consistent with a new pathway for nitrite reduction to ammonium by *N. profundicola*. The concentrations of hydroxylamine (closed circles) and ammonium (open circles) were followed after the addition of 5 mM hydroxylamine to a resting cell suspension. The points are the mean values from three replicates ± the standard deviation.

excess of the ammonium production rate observed during growth of *N. profundicola* with nitrate. A similarly tight coupling between hydroxylamine production and removal was recently reported for the chemolithotrophic ammonia-oxidizing archaeon, *Nitrosopumilus maritimus*, where hydroxylamine oxidation is an essential catabolic step (Vajrala et al., 2013).

**DISCUSSION**

The data presented here are consistent with a new pathway for nitrate reduction in organisms that lack classical NO-forming (NirS or NirK) or ammonium-forming (NirB/NirA) nitrite reductases with *N. profundicola* strain AmH as a useful model system. A reverse-HURM pathway has been proposed whereby nitrite is reduced to hydroxylamine in the periplasm and subsequently reduced to ammonium in the cytoplasm (Campbell et al., 2009). The original pathway has been slightly modified here to include a more likely candidate for the proximal electron donor to Har/Hcp, a predicted 4Fe-4S containing protein encoded by NAMH_1302 (*Figure 1*). Homologs of NAMH_1302 are conserved in *C. concisus* (GenBank accession YP_001466207), *C. curvus* (YP_001408944), *C. fetus* (YP_892678), and *C. mediatlanticus* (ZP_01871213) that possess all other genes for the reverse-HURM pathway. In addition, NAMH_1302 displayed an increase in transcript abundance in *N. profundicola* cells grown on nitrate relative to ammonium.

While the production of hydroxylamine from nitrate and nitrite both via chemical (Rollefson and Oldershaw, 1932) and biological routes (Lindsey and Rhines, 1932) has long been known and a [HaoA] complex was shown to reduce nitrite to hydroxylamine when supplied with reductant (Koston et al., 2010), the ratio of hydroxylamine vs. ammonium production from nitrite in the reverse-HURM pathway is not yet clear. The uptake of extracellular hydroxylamine by bacteria is known; exogenously supplied hydroxylamine accumulates readily in the anammoxosome of *Kuenenia stuttgartiensis* (Lindsay et al., 2001; Schmidt et al., 2004), a compartment that can be reached only by crossing both the cell and anammoxosome membranes. The proposed uptake of the highly mutagenic hydroxylamine necessitates protection against DNA lesions, which may partially explain the extensive complement of DNA repair systems identified in *N. profundicola* (Campbell et al., 2009). Hydroxylamine has also been proposed as an intermediate of ammonium production from nitrite in plants and algae as ferredoxin-dependent nitrite reductase displays high reactivity and specificity for this substrate (Kuznetsova et al., 2004; Hirasawa et al., 2010).

Unlike the quinone reductase function of HURM in ammonia-oxidizers (Klotz and Stein, 2008; Simon and Klotz, 2013), we propose that HURM in *N. profundicola* acts, similar to NrfAH, as a periplasmic quinol oxidase system that shuttles electrons from the quinol pool to reduce nitrite to hydroxylamine. It is likely that the reverse-HURM-pathway evolved from a common ancestor of the NrfAH and ONR ammonification module in anammox bacteria. We hypothesize that this occurred before HURM—including a covalently bound trimeric HaoA complex capable of hydroxylamine disproportionation—was used as a catalytic module in both anaerobic and aerobic ammonia-oxidizing bacteria (Jetten et al., 2009; Klotz and Stein, 2010; Campbell et al., 2011; Kern et al., 2011; Simon and Klotz, 2013). A similar reversal of function from reducing to oxidizing activity effected by covalent bond-directed complex formation has been discussed for the NO-reducing cytochrome c’-beta (cysT) and the NO-oxidizing cytochrome P460 (cysL) (Elmore et al., 2007). Genes encoding the NrfAH and reverse-HURM modules coexist in only one bacterial genome sequence, *C. fetus* 82-40 (*Table 1*). It was shown recently that octaheme cytochrome c hydroxylamine dehydrogenase evolved as a member of a multi-heme cytochrome c protein superfamily with functions in the nitrogen and sulfur cycles and that cytochrome c552 (CycB), NrfH, and NapC are members of another cytochrome c superfamily (Bergmann et al., 2005; Klotz and Stein, 2008; Kern et al., 2011; Simon and Klotz, 2013). It is also known that NAP complexes in *Epsilonproteobacteria* generally do not contain NapC subunit proteins (Klotz et al., 2006; Sievert et al., 2008; Kern and Simon, 2009; Klotz and Stein, 2010; Simon and Klotz, 2013). The proposed reverse function of a [HaoA] complex (CycB/NapC) complex as a nitrite reductase—quinol oxidase complex is feasible given the presence of both transcripts and the absence of the critical tyrosine protein ligand in *N. profundicola* HaoA (Campbell et al., 2009), which separate the N-oxide reducing NrfA and ONR from the N-oxide oxidizing HAO complexes (Bergmann et al., 2005; Klotz and Stein, 2008; Kern et al., 2011; Simon and Klotz, 2013).

The 15N assimilation data established that *N. profundicola* incorporates nitrate-nitrogen into biomass during growth when it is provided as the sole nitrogen source. However, the vast excess production of ammonium from nitrate over the calculated demands for biosynthesis leads us to conclude that the primary function of nitrate reduction to ammonium by *N. profundicola* is that of an efficient respiratory electron acceptor.
This conclusion is supported by the increased growth rates and yields in *N. profundicola* cultures with nitrate as the sole electron acceptor. Furthermore, the gene expression data agree with our proposal (Campbell et al., 2009) that this occurs via the concerted function of Nap, HURM ([HaoA]3 and cF532) and Har/Hcp as a novel N-assimilation and energy conservation pathway: the reverse-HURM pathway. Finally, the data indicate that *N. profundicola* has the ability to rapidly reduce hydroxylamine to ammonium, consistent with the proposal of free hydroxylamine as an intermediate in the reverse-HURM pathway. The high rate of hydroxylamine uptake observed likely explains why it is not observed consistently during growth of cultures on nitrate. Preliminary measurements of ammonium production from nitrate or nitrite in resting cells are similar to the $3 \mu$M min$^{-1}$ (10$^8$ cells)$^{-1}$ rate calculated from cultures, ~10-fold lower than the observed hydroxylamine consumption rate. Given these rates, hydroxylamine should be consumed immediately after production in cultures growing on nitrate. As hydroxylamine is mutagenic, we hypothesize that this reflects previous selective pressure to maintain low hydroxylamine levels during growth.

While labeling of biomass has been attempted with $^{15}$N-NH$_2$OH in batch *N. profundicola* cultures, it has not yet been successful (data not shown). Given that hydroxylamine in batch culture must be supplied at low concentrations relative to the unlabeled nitrate to avoid toxicity and that the vast majority of N from hydroxylamine should be converted to ammonium (>90% based on Figure 3), this result is not surprising. Short-term labeling experiments with much higher concentrations of labeled hydroxylamine as the sole N-source may definitively establish this point.

In conclusion, the data presented here are consistent with a model where *N. profundicola* reduces nitrate via a free hydroxylamine intermediate. These experiments do not yet completely exclude the possibility that the reverse-HURM activity proposed in *N. profundicola* functions as an ammonium-forming nitrite reductase that transfers 6 electrons without releasing an N-oxide intermediate. Unpublished results indicate that a few point mutations in the *nrfA* gene may render pentaheme cytochrome c nitrite reductase into a leaky enzyme that releases N oxide intermediates and fails to convert nitrite stoichiometrically into ammonium (Jörg Simon, pers. communi.). Given the evolutionary relationship between *nrfA* and *haoA* (Bergmann et al., 2005; Simon and Klotz, 2013), the reverse-HURM module in *N. profundicola* might naturally function as an ammonium and hydroxylamine-producing enzyme complex whereas extant NrfA operates “leak-free” as an ammonium-producing nitrite reductase. Further experiments using inhibitors of Har/Hcp with nitrate or nitrite as a substrate in whole cells and/or biochemical studies of the reverse-HURM complex to determine the *in vitro* product should provide additional direct tests of this hypothesis. Irrespective of the specific mechanism, the extensive production of ammonium far in excess of biosynthetic needs by *N. profundicola* suggests that it and related Epsilonproteobacteria may serve an ecological role as a source of ammonium in nitrate rich, ammonium deprived, hypoxic environments where they are commonly found.

### MATERIALS AND METHODS

#### FREE ENERGY CALCULATIONS

Gibbs free energies (Table 2) were calculated from standard redox potentials of the half reactions by the Nernst equation.
CULTURING AND NITROGEN COMPOUND ANALYSIS

N. profundicola strain AmH (ATCC BAA-1463) was cultured and cells counted as previously described (Smith et al., 2008; Campbell et al., 2009). Ammonium in culture supernatants was quantified after derivatization with dansyl chloride and separation by HPLC with fluorescence detection (Shakila et al., 2001). Nitrate was quantified by HPLC using UV/Vis detection. Hydroxylamine concentrations were determined in culture filtrates (0.2 µm) as described by Frear and Burrell (1955).

15N LABELING PROCEDURES

Stock solutions of 20 Atom% 15N-nitrate and 15N-ammonium were prepared by mixing >99 Atom% and natural abundance salts to prepare medium at a final concentration of 5 mM nitrate or ammonium as needed. Cells were harvested from 15N labeled cultures by centrifugation and the cell pellet washed three times with nanopure water to remove salts. The cell suspension was dried in tin capsules which were sent to the University of North Carolina to Martin G. Klotz.

HYDROXYLAMINE UPTAKE

N. profundicola was grown on H2 and formate as electron donors and nitrate as the sole electron acceptor to late exponential phase. Cells were harvested by centrifugation. All transfers and cell washes were performed in a Coy Laboratories anaerobic chamber. Cells were resuspended with nitrate-free growth medium to a final concentration of ∼105 cells ml−1 and incubated at the 45°C for 2 h under an atmosphere of 80% H2 + 20% CO2 prior to the addition of hydroxylamine (5 mM final concentration) from an anaerobically prepared stock solution. Aliquots of the cell suspension were filtered and assayed for hydroxylamine as described above and for ammonium by HPLC after derivatization with diethyl ethoxymethylenemalonate (Gómez-Alonso et al., 2007).

AUTHOR CONTRIBUTIONS

Thomas E. Hanson, Barbara J. Campbell, and Martin G. Klotz collaboratively designed experiments and interpreted results, Thomas E. Hanson performed all nitrogen compound analyses and prepared samples for mass spectrometry, Barbara J. Campbell grew all cultures and performed quantitative PCR, Katie M. Kalis performed hydroxylamine uptake experiments, Mark A. Campbell identified NAMH_1302 as a relevant gene. Thomas E. Hanson drafted the paper, which was subsequently revised by Thomas E. Hanson, Barbara J. Campbell, and Martin G. Klotz.

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