Comparison of Nitrogen Oxide Metabolism among Diverse Ammonia-Oxidizing Bacteria

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Ammonia-oxidizing bacteria (AOB) have well-characterized genes that encode and express nitrite reductases (NIR) and nitric oxide reductases (NOR). However, the connection between presence or absence of these and other genes for nitrogen transformations with the physiological production of nitric oxide (NO) and nitrous oxide (N₂O) has not been tested across AOB isolated from various trophic states, with diverse phylogeny, and with closed genomes. It is therefore unclear if genomic content for nitrogen oxide metabolism is predictive of net N₂O production. Instantaneous microrespirometry experiments were utilized to measure NO and N₂O emitted by AOB during active oxidation of ammonia (NH₃) or hydroxylamine (NH₂OH) and through a period of anoxia. This data was used in concert with genomic content and phylogeny to assess whether taxonomic factors were predictive of nitrogen oxide metabolism. Results showed that two oligotrophic AOB strains lacking annotated NOR-encoding genes released large quantities of NO and produced N₂O abiologically at the onset of anoxia following NH₃-oxidation. Furthermore, high concentrations of N₂O were measured during active O₂-dependent NH₂OH oxidation by the two oligotrophic AOB in contrast to non-oligotrophic strains that only produced N₂O at the onset of anoxia. Therefore, complete nitrifier denitrification did not occur in the two oligotrophic strains, but did occur in meso- and eutrophic strains, even in Nitrosomonas communis Nm2 that lacks an annotated NIR-encoding gene. Regardless of mechanism, all AOB strains produced measureable N₂O under tested conditions. This work further confirms that AOB require NOR activity to enzymatically reduce NO to N₂O in the nitrifier denitrification pathway, and also that abiotic reactions play an important role in N₂O formation, in oligotrophic AOB lacking NOR activity.

Keywords: nitrogen oxides, nitrifier denitrification, ammonia-oxidizers, Nitrosomonas, Nitrosospira, nitrous oxide, nitric oxide, chemodenitrification

INTRODUCTION

Chemolithotrophic ammonia-oxidizing bacteria (AOB) are important players in the global biogeochemical nitrogen cycle and perform the first step in nitrification; the oxidation of ammonia (NH₃) to nitrite (NO₂⁻). AOB are abundant in a vast array of environments including soils, marine and fresh-water, and wastewater treatment plants (Klotz et al., 2006; Norton et al., 2008; Jia and Conrad, 2009; Ke et al., 2015) and are implicated in production of nitrous oxide (N₂O) through...
enzymatic (Stein, 2011; Kozlowski et al., 2014) and abiotic processes (Jones et al., 2015; Zhu-Barker et al., 2015). AOB have the potential to utilize NO$_3^-$ as an alternate terminal electron acceptor through the process of nitrifier denitrification (Stein, 2011) resulting in net production of N$_2$O (Stein and Yung, 2003; Kool et al., 2011; Zhu et al., 2013). N$_2$O has been measured from pure cultures of AOB from both the Nitrosomonas (Poth and Focht, 1985; Kozlowski et al., 2014) and Nitrosospira (Dundee and Hopkins, 2001; Wrage et al., 2004; Shaw et al., 2006) genera. However, studies on the enzymology and pathways of N$_2$O production by AOB have mostly focused on *N. europaea* ATCC 19718 (Beaumont et al., 2002, 2004; Cantera and Stein, 2007; Yu and Chandran, 2010; Yu et al., 2010; Kozlowski et al., 2014) leaving open the possibility that not all AOB strains share equivalent pathways and regulatory mechanisms.

The nitrifier denitrification pathway includes a nitrite reductase (NIR) to reduce NO$_3^-$ to nitric oxide (NO) and nitric oxide reductase (NOR) to reduce NO to N$_2$O. All closed AOB genomes, with the exception of *N. communis* Nm2 (Kozlowski et al., 2016b), have genes encoding the copper-containing NirK (Prosser et al., 2014). Furthermore, all AOB encode NOR genes (norB and/or norY) with the exception of *Nitrosomonas* sp. Is79A3 (Bollmann et al., 2013) and *N. ureae* Nm10 (Kozlowski et al., 2016a). Both *Nitrosomonas* sp. Is79A3 and *N. ureae* Nm10 are considered oligotrophic, growing optimally in medium containing 1–5 mM ammonium (Prosser et al., 2014). In contrast, *N. communis* Nm2 is considered eutrophic and prefers higher concentrations of 10–50 mM ammonium (Prosser et al., 2014).

Previous studies on the model organism *N. europaea*, a eutrophic strain, showed that both hydroxylamine (NH$_2$OH) oxidation and NO$_2^-$ reduction can lead to significant emission of N$_2$O (Cantera and Stein, 2007; Kozlowski et al., 2014). Previous work also revealed that NorB, but not NirK, is required for production of N$_2$O by *N. europaea* (Kozlowski et al., 2014). This observation, in addition to the lack of annotated NIR or NOR genes in some closed AOB genomes, has brought into question whether all AOB can even perform nitrifier denitrification and emit N$_2$O under similar conditions as *N. europaea*. There is also a question of whether uncharacterized NIR and/or NOR enzymes are expressed in AOB that can contribute to the process. The production and metabolism of NO and its role in N$_2$O emission is another understudied aspect of nitrogen oxide metabolism in AOB; *N. multiformis* ATCC 25196 was recently found to emit large quantities of NO during active NH$_3$-oxidation (Kozlowski et al., 2016c).

Due to the lack of comparative information on nitrogen oxide metabolism in AOB, five strains representing different phylogenies and trophic states and with closed genomes were selected for this study. Our main objectives were to: (i) compare NO and N$_2$O production profiles of the five strains during NH$_3$ and NH$_2$OH oxidation and over a period of anoxia when nitrifier denitrification is most active in *N. europaea*, and (ii) determine whether gene content, trophic state, and/or phylogeny of these diverse AOB were predictive of their capacity to metabolize and/or emit NO or N$_2$O.

### MATERIALS AND METHODS

#### Strains and Cultivation

AOB strains included *N. europaea* ATCC 19718T, *N. communis* strain Nm2T, *Nitrosomonas* sp. Is79A3, *N. ureae* Nm10T, and *N. multiformis* ATCC 25196T. All strains have closed genomes and grow under similar cultivation conditions to allow for proper comparisons across phylogenotypes and trophic status. Furthermore, an AOB strain was selected from each cluster in the *Betaproteobacteria* with a cultured representative, 3, 6, 7, and 8 (based on 16S rRNA phylogeny; Norton, 2011), with the exception of the newly cultured cluster 0 *N. lacus* sp. nov. as its genome is not yet closed (Garcia et al., 2013; Urakawa et al., 2014).

AOB cultures were grown and maintained in Wheaton bottles (250 mL) sealed with caps inlayed with butyl rubber stoppers at 28°C in 100 mL HEPES-buffered HK medium (Krümmel and Harms, 1982) and phenol red as pH indicator (pH of 7.5–8) with either 5 mM (NH$_4$)$_2$SO$_4$ for the meso- and eutrophic strains (*N. europaea*, *N. communis*, and *N. multiformis*), or 2.5 mM (NH$_4$)$_2$SO$_4$ for the oligotrophic strains (*Nitrosomonas* sp. Is79A3 and *N. ureae*; Prosser et al., 2014). All cultures were transferred (5% v/v inoculum) when ca. 80% of the NH$_3$ substrate was consumed as determined by NO$_3^-$ concentration (Bollmann et al., 2011). The pH of all cultures was adjusted as needed with 10% NaHCO$_3$.

#### Phylogenetic and Genome Analysis of AOB

PhyloPhlAn (Segata et al., 2013) was used to generate and analyze the genome-wide phylogeny of AOB. Genomes of 14 AOB were obtained from the National Center for Biotechnology Information. All of the predicted protein-coding sequences for each genome were exported into PhyloPhlAn to identify and align 400 broadly conserved protein sequences between all of the input genomes. PhyML 3.0 (Guindon et al., 2010) was used to construct a maximum likelihood phylogeny using the *Gammaproteobacteria* as the root and node support was calculated using 500 bootstrap replicates.

#### Microrespirometry Experiments

Instantaneous microrespirometry (MR) experiments of AOB were described in detail elsewhere (Kozlowski et al., 2016c). Briefly, MR experiments were performed at 28°C in a 10 mL 2-port injection lid glass chamber (Unisense, Aarhus, Denmark). For instantaneous experiments all strains were grown to late-log phase (7–8 mM NO$_3^-$), filtered on Supor® 200 0.2 µm filters ( Pall, Ann Arbor WI), and rinsed three times with NH$_4$-free HK media (Krümmel and Harms, 1982). Ca. 1 × 10$^{10}$ total cells were used per experiment for all strains as determined by direct cell count by phase-contrast light microscopy. All cells for instantaneous MR measurements were in a planktonic state, re-suspended in NH$_3$-free HK medium and provided either 2 mM NH$_4$Cl as substrate or pulses of 250 µM or 100 µM NH$_3$OH·HCl (final chamber concentration; 99.999% purity, Sigma–Aldrich, St Louis, MO).
Louis, MO, USA). Previous testing revealed that all strains could tolerate up to 250 $\mu$M NH$_3$OH (final chamber concentration) with the exception of *N. communis* which was unable to tolerate more than 100 $\mu$M NH$_3$OH (final chamber concentration) per injection (data not shown). Chamber O$_2$ was determined by an O$_2$ electrode (OX-MR 500 $\mu$m tip diameter MR oxygen electrode; Unisense, Aarhus, Denmark), and NO was measured using an am-600 NO sensor with 600 $\mu$m tip diameter (Innovative Instruments Inc., Tampa, FL, USA). The availability of O$_2$ in the MR chamber, a closed system, was ca. 243 $\mu$M O$_2$ based on equilibrium O$_2$ concentration at operating temperatures and medium salinities for experiments performed without N$_2$-sparged medium.

### Chemical Controls

Chemical controls were performed to determine the production of N$_2$O from reactivity of NH$_3$OH with media + NO$_2$, or from killed-cells (1 × 10$^{10}$ total cells) with media + NH$_3$OH. Chemical controls used N$_2$-sparged medium (to achieve 0–3% O$_2$ saturation in liquid phase) containing 250 $\mu$M NaNO$_2$ and then adding 250 $\mu$M NH$_3$OH (final chamber concentration) to reflect conditions in the chamber when testing for NO$_2$ consumption by AOB as an alternate terminal electron acceptor with NH$_3$OH as the electron donor. Cells for control experiments were heat-killed by boiling for 30 min. The heat-killed cell controls involved addition of 250 $\mu$M NH$_3$OH to the MR-chamber containing N$_2$-sparged media with 1 × 10$^{10}$ total heat-killed cells of each AOB strain. N$_2$O was measured as described above.

### RESULTS AND DISCUSSION

#### Phylogeny and Comparative Gene Inventory of AOB

A whole-genome analysis utilizing PhylopheLAN showed that each of the 5 Betaproteobacteria AOB chosen for physiological analysis in the present study separated into individual clades (Figure 1). The separation of each AOB into a unique branch, using 400 core protein markers from available complete genome sequences to form a high-resolution tree, shows a clearer and greater separation than currently available 16S rRNA or amoA single gene sequence phylogenies (Norton, 2011). The results of this multiple-marker, genome-wide, comparison highlight a need to reevaluate and perhaps reclassify some members of *Nitrosomonas* into different genera.

Comparison of inventory involved in central ammonia-oxidizing metabolism and NOx production revealed differences across the 5 strains (Table 1). In agreement with previous analyses of AMO gene clusters in betaproteobacterial AOB (Klotz and Stein, 2011) all AOB of the current study contain 1–2 copies of the *amoCABED* cluster encoding ammonia-monoxygenase (Table 1). All strains encoded at least one monocistronic copy of the *amoC* gene with the exception of *N. communis* (Table 1), a feature shared in common with the gammaproteobacterial AOB (Klotz et al., 2006; Arp et al., 2007; Campbell et al., 2011). The singleton AmoC is proposed to participate in cellular recovery from stressors such as elevated temperatures and starvation by stabilizing the AMO complex in the membrane of *N. europaea* (Berube and Stahl, 2012). Also, every strain encoded at least one copy of the *amoD* gene in tandem with *amoE*, a common feature of betaproteobacterial AOB still needing biochemical characterization (Klotz and Stein, 2011). It is also common for betaproteobacterial AOB to encode 2–3 complete or incomplete (lacking *cycB*) copies of the haoAB-cycAB cluster (Arp et al., 2007). However, *N. ureae* represents the first sequenced AOB to harbor 4 complete copies of the Hydroxylamine dehydrogenase (HAO) gene cluster (Table 1). Knockouts of one or two haoA gene copies from *N. europaea* did not result in a significant phenotype (Hommes et al., 2002), suggesting that the multiple copies are isofunctional. However, knockouts of individual amoA or amoB gene copies in *N. europaea* did result in different phenotypes, suggesting that operons encoding AMO are differentially regulated (Stein et al., 2000). For *N. ureae* and perhaps *Nitrosomonas* sp. AL212 (Suwa et al., 2011), additional gene clusters encoding AMO and HAO could be a strategy to thrive in oligotrophic environments to gain maximum reductant from available substrate; however, further studies are required to validate whether all of the gene copies are expressed, isofunctional, and/or differentially regulated. As with *N. europaea* and *N. eutropha*, one copy of the HAO gene cluster in *N. communis* lacks cycB (Table 1), encoding cytochrome Cm552 (Arp et al., 2007). All strains, with the exception of *Nitrosomonas* sp. Is79A3 (Bollmann et al., 2013), encode the AOB-specific red copper protein nitrosocyanin (Table 1) proposed to be involved in the NH$_3$-oxidation pathway as a redox sensitive electron carrier (Arciero et al., 2002; Sayavedra-Soto and Arp, 2011).

Analysis of NIR and NOR genes revealed that *N. communis* is the only sequenced and closed AOB genome without a copper-containing nitrite reductase (*nirK*; Kozlowski et al., 2016b) (Table 1). This is interesting as *nirK* is present in all published genomes of ammonia-oxidizing Thaumarchaeota (AOA; Bartossek et al., 2010), is highly expressed in metatranscriptomes (Hollibaugh et al., 2011; Radax et al., 2012), and is important for efficient substrate oxidation in *N. europaea* (Cantera and Stein, 2007; Kozlowski et al., 2014). Of the 5 strains, only *N. europaea* contains the operonic *nirK* and NO-responsive *nrsR* transcriptional regulator (Chain et al., 2003; Table 1), features shared by the closely related *N. eutropha* C-91 strain (Stein et al., 2007) (Figure 1). All the *Nitrosomonas* strains, but not *N. multiformis*, encode the NO-responsive NrsR transcriptional regulator. Two strains, *Nitrosomonas* sp. Is79A3 and *N. ureae*, both within the Cluster 6 AOB, lack annotated operons for cytochrome c nitric oxide reductases (Bollmann et al., 2013; Kozlowski et al., 2016a) (Table 1). The genome of the closely related *Nitrosomonas* sp. AL212 (Figure 1) does encode *norCQBD* but lacks genes for the other NOR frequently found in AOB genomes, *norSY-senC-orfJ* (Suwa et al., 2011). We hypothesize that environments with low substrate availability do not experience oversaturation of NH$_3$ and thus preclude accumulation of N-oxides such as NH$_3$OH and NO (Hooper and Terry, 1979). Thus, NORs may not be not required by some oligotrophic AOB as nitrosative stress should be minimal.
However, testing of strains such as *Nitrosomonas AL212*, an oligotrophic, NOR-encoding strain, must be accomplished to determine whether trophic state or gene content is more predictive of nitrifier denitrification activity. *N. multiformis* does not have an annotated cytochrome P460 (cytL) whereas *N. communis* has two copies (*Table 1*), a feature shared with *Nitrosomonas* sp. AL212 (Suwa et al., 2011). Cytochrome P460 has a proposed role in detoxification of NOx through the simultaneous oxidation of NH2OH and NO to NO−2 (Elmore et al., 2007; Stein, 2011) and may be important for alleviating nitrosative stress in AOB lacking NORs. All 5 genomes also contain sequences for cytochrome c′ beta, potentially having NOR activity (Elmore et al., 2007; Stein, 2011). Future work with focus on the transcription and activities of cytochromes P460 and c′ beta under conditions of nitrosative stress would better clarify the role of both enzymes as substitutes for lack of annotated NORs.

### Comparison of Instantaneous NOx Production from AOB during Oxidation of NH3 or NH2OH

Measurement of NO or N2O production during oxidation of NH3 or NH2OH were compared among the 5 strains and revealed that all AOB produce measureable quantities of NO during active oxidation of NH3 (*Figures 2A,C,E,G,I*). Although each AOB had a unique and dynamic NO production profile, making comparative rate calculations impractical, all strains produced >50 nM NO (per 1 × 10^18 total cells) prior to anoxia in the MR chamber. *N. europaea* produced the least amount of NO compared to the other strains during active oxidation and prior to anoxia (*Figure 2A; Supplementary Table S1*). As reported previously (Kozlowski et al., 2016c) *N. multiformis* began re-consuming NO once ca. 50% O2 was left in the MR-chamber (*Figure 2I*) and both *N. europaea* and *N. communis* re-consumed a small amount of NO following anoxia in the MR-chamber (*Figures 2A,C*). Interestingly, either immediately upon O2 depletion in the case of *Nitrosomonas* sp. Is79A3 (*Figure 2E*) or ca. 5 min. post-anoxia for *N. ureae*, these two strains released massive quantities of NO outside the limit for measurement by the ami-600 NO microsensor (*Figures 2E,G*). Unlike the other AOB strains, neither *Nitrosomonas* sp. Is79A3 nor *N. ureae* re-consumed NO during active NH3-oxidation or following anoxia in the MR-chamber.

Measurement of NO during active substrate oxidation has so far only been studied in pure cultures of *N. europaea* (Kester et al., 1997; Yu and Chandran, 2010; Yu et al., 2010) and *N. multiformis* (Kozlowski et al., 2016c), both of which have annotated nirK, norB, and norY genes (*Table 1*). It is known, however, that the thaumarchaeotal ammonia-oxidizers (AOA) also produce NO during NH3-oxidation (Martens-Habbema et al., 2015; Kozlowski et al., 2016c); however, they retain very tight control over its production and consumption (Kozlowski et al., 2016c). There are significant similarities in NO profiles of the AOA *Nitrososphaera viennensis* and the oligotrophic AOB of the present study in that once O2 was depleted in the MR chamber substantial quantities of NO were released (*Figures 2E,G; Kozlowski et al., 2016c*). This similarity between the AOA and the oligotrophic AOB, both lineages with a low *Km* and high affinity for ammonium (Martens-Habbema et al., 2009; Stahl and de la Torre, 2012;
et al., 2014). This was tested in the present study by measurement of NO release and abiotic media-dependent conversion to N₂O. Importantly, the N. europaea denitrification pathway, in the case of AOA (Kozlowski et al., 2016c), or perhaps N. multiformis (Kozlowski et al., 2014). This suggests that the lack of annotated NOR precludes a complete nitrifier-denitrification pathway in ammonia-oxidizers.

Following O₂ depletion and in the presence of NO₂⁻ some AOB can perform nitrifier denitrification (Stein, 2011; Kozlowski et al., 2014). This was tested in the present study by measurement of N₂O during active NH₃- or NH₂OH-oxidation and through a period of anoxia (Figures 2 and 3). It should be noted that the Kₘ for the copper-containing nitrite reductase, NirK, has not been tested for AOB and therefore it is not known whether ca. 162 or 243 µM NO₂⁻ following NH₃ or NH₂OH oxidation, respectively, in the chamber is at saturation for NirK.

| Strain                  | Nitrosomonas europaea ATCC 19718 | Nitrosomonas communis Nm2 | Nitrosomonas sp. Is79A3 | Nitrosomonas ureae Nm10 | Nitrosospira multiformis ATCC25196 |
|------------------------|----------------------------------|--------------------------|-------------------------|------------------------|----------------------------------|
| Ammonia monooxygenase (AMO) | amoCABED NE2064-59              | amoCABED AWW31_01090-70  | amoCABED NII79A3_0471-75 | amoCABED ATY38_01315-295 | amoCABED Nmul_A2326-22           |
|                        | NE0945-40 amoC NE1411           | AWW31_05385-65           | NII79A3_2086-82 amoCAB | ATY38_07250-70 amoCAB  | amoCABED Nmul_A0177              |
|                        |                                 |                          | NII79A3_1079-81 amoC   | ATY38_13760-50 amoCE  | amoC Nmul_A2467                 |
|                        |                                 |                          | NII79A3_1233           | ATY38_06315-10 amoC   |                                  |
|                        |                                 |                          | NII79A3_1595           | ATY38_09265           |                                  |
| Hydroxylamine dehydrogenase (HADO) | haoAB-cycAB NE0062-59 | haoAB-cycAB AWW31_16290-75 | haoAB-cycAB NII79A3_0822-25 | haoAB-cycAB ATY38_00640-55 | haoAB-cycAB Nmul_A1082-85       |
|                        | NE2239-36 haoAB-cycA NE2044-42 | haoAB-cycA AWW31_18275-65 | NII79A3_2942-39        | ATY38_10080-95 ATY38_15220-06 | Nmul_A2662-59                   |
| Nitrosocyanin           | NE0143                          | AWW31_00185              | Not Present            | ATY38_00645           | Nmul_A1601                       |
| Nitrile reductase (NirK) | nccABC-nirK NE0024              | Not Present               | nK NII79A3_2335        | nK ATY38_00959         | Nmul_A1998                       |
| Cytochrome c nitric oxide reductases | norCBQD NE2003-06 norSY-senC-orf1 NE0668-86 | norCBQD AWW31_09555-70 norSY-senC-orf1 AWW31_05895-910 | Not Present | Not Present | norCBQD Nmul_A1256-43 norSY-senC-orf1 Nmul_A2667-64 |
| Cytochrome c⁺ beta (cytS) | NE0824                          | AWW31_17525              | NII79A3_0363           | ATY38_05410           | Nmul_A2484                       |
| Cytochrome P460 (cytL)  | NE0011                          | AWW31_02040              | AWW31_00880           | ATY38_00655           | Not Present                       |
| NO-responsive transcriptional regulator (NirR) | NE0926                         | Not Present               | Not Present            | Not Present           | Not Present                       |
| NO-responsive transcriptional regulator (NsrR) | NE1722                         | AWW31_04320              | AWW31_06015           | AWW31_34312 ATY38_04220 | Not Present                       |

Locus tags from the sequenced and publicly accessible genomes are presented for each annotated gene and gene cluster.

Prosser et al., 2014), could be explained by a lack of NOR genes to combat high intracellular NO experienced during anoxia either due to release of NO directly from the NH₃-oxidation pathway, in the case of AOA (Kozlowski et al., 2016c), or perhaps from NO₂⁻ reduction in the case of the AOB (Stein, 2011). Importantly, the N₂O measured from N. viennensis following NH₃-oxidation and over an extended period of anoxia was a result of NO release and abiotic media-dependent conversion to N₂O (Kozlowski et al., 2016c). Also, in the nitrifier-denitrification pathway of N. europaea, it should be noted that NorB is required for NO₂⁻ reduction to N₂O (Kozlowski et al., 2014). This suggests that the lack of annotated NOR precludes a complete nitrifier-denitrification pathway in ammonia-oxidizers.

Following O₂ depletion and in the presence of NO₂⁻ some AOB can perform nitrifier denitrification (Stein, 2011; Kozlowski et al., 2014). This was tested in the present study by measurement of N₂O in the MR-chamber (Figures 2B,D,F,H,J). A greater delay of ca. 3 min in measurable N₂O was seen from traces with both N. communis (Figure 2D) and N. ureae (Figure 2H). The lowest concentrations and slowest rates of N₂O came from N. communis and N. multiformis (Figures 2D,J, Supplementary Table S1). N. europaea N₂O production in the MR-chamber began immediately following O₂-depletion and was produced at a rate of 0.47 µM N₂O per 10¹⁰ cells per minute (Figure 2B, Supplementary Table S1). As with NO production,
FIGURE 2 | Instantaneous measurement of O$_2$ consumption and NO or N$_2$O during oxidation of 2 mM NH$_4$Cl. Nitrosomonas europaea (A,B), N. communis (C,D), Nitrosomonas sp. Is79A3 (E,F), N. creati (G,H), Nitrosospira multiformis (I,J). Panels are single representative measurements of reproducible results (n = 3). Note differences in scale of x-axis for traces of NO production during NH$_3$-oxidation.
both *Nitrosomonas* sp. Is79A3 and *N. ureae* had similar N$_2$O traces with similarly fast rates for N$_2$O production following anoxia (Figures 2F,H; Supplementary Table S1).

With NH$_2$OH as substrate, the majority of N$_2$O in the MR-chamber from *N. europaea* (Figure 3A), *N. communis* (Figure 3B), and *N. multiformis* (Figure 3E) was produced in a linear fashion directly following anoxia suggesting enzymatic reduction of available NO$_3^-$ to N$_2$O and thus nitrifier denitrification. However, in the case of both *Nitrosomonas* sp. Is79A3 (Figure 3C) and *N. ureae* (Figure 3D) the majority of N$_2$O was measured during active NH$_2$OH-oxidation with production in both traces slowing upon complete O$_2$-depletion. Furthermore, the quantity of N$_2$O measured from both *Nitrosomonas* sp. Is79A3 and *N. ureae* during active NH$_2$OH-oxidation was much greater overall than that produced from any other AOB, suggesting a greater overall release of NO, or other reactive intermediates, during this process (Law et al., 2012) (Figure 3).

It is interesting that *N. communis*, the only AOB lacking NirK, had very weak non-linear N$_2$O production from NH$_3$, yet strong linear production when NH$_2$OH was provided (Figures 2D and 3B). The linearity of N$_2$O formation with NH$_2$OH as substrate suggests that there is an enzymatic pathway for N$_2$O formation under anoxic conditions, but this pathway is not active when NH$_3$ is provided as substrate. This observation provides insight into the function of unidentified enzymology that links direct NH$_2$OH oxidation to N$_2$O production in *N. communis* that requires further investigation. Similarly, an *N. europaea* NirK deficient mutant was also able to reduce NO$_2^-$ to N$_2$O (Cantera and Stein, 2007; Kozlowski et al., 2014), further supporting the presence of alternate, as yet unidentified, NIRs in AOB.

Contribution of AOB to Abiotic N$_2$O

The N$_2$O profiles of both *Nitrosomonas* sp. Is79A3 and *N. ureae* post-anoxia (Figures 2F,H) are congruent with a rapid and abundant release of NO (Figures 2E,G) being abiotically reduced to N$_2$O, a characteristic trait observed in the AOA *N. viennensis* (Kozlowski et al., 2016c). Also in support of an abiotic origin of N$_2$O for both *Nitrosomonas* sp. Is79 and *N. ureae* in comparison to the other AOB strains (Figure 3) is the observation that the majority of N$_2$O was measured during active oxidation of NH$_2$OH. Accumulation of NH$_2$OH can lead to NO and N$_2$O production at the active site of the HAO (Hooper and Terry, 1979; Stein, 2011). A high enough concentration of NO will react with components of the HK medium to form N$_2$O as well (Kozlowski et al., 2016c). Interestingly, the lack of NirK did not cause significant production of N$_2$O during active NH$_2$OH-oxidation by *N. communis*, as shown previously for NirK-deficient *N. europaea* (Cantera and Stein, 2007), suggesting a different configuration of the ammonia-oxidation pathway among AOB that lack NirK.

In previous control experiments the intermediate NH$_2$OH reacted with heat-killed cell moieties of the AOA, *N. viennensis* EN76, to produce abiological N$_2$O (Kozlowski et al., 2016c). In the present study, abiotic and heat-killed cell controls were performed to demonstrate if NH$_2$OH could react with either media components or heat-killed cells to produce N$_2$O in the absence of active cellular functioning (Supplementary Figure S1). NH$_4^+$/free HK medium + NaNO$_2$ or with heat-killed AOB and addition of 250 µM NH$_2$OH showed that medium + NaNO$_2$ or medium with heat-killed *N. europaea*, *N. communis*, and *N. multiformis* + NH$_2$OH did not facilitate significant measureable N$_2$O (Supplementary Figure S1). However, heat-killed cells of both *Nitrosomonas* sp. Is79A3 and *N. ureae* both produced measureable N$_2$O following addition of 250 µM NH$_2$OH. The reactivity of cellular moieties with NH$_2$OH is further evidence of similarities among these oligotrophic AOB and the AOA as heat-killed controls of
N. viennensis cells showed similar reactivity with NH$_2$OH in growth medium (Kozlowski et al., 2016c). Taken altogether, the data support that N. ureae cells similarly to that of the AOA, N. viennensis, likely due to their massive release of NO at anoxia and also the reactivity of their cellular moieties with NH$_2$OH and other medium constituents.

CONCLUSION

The present study highlights many new findings in the comparative phylogeny and nitrogen oxide metabolism of betaproteobacterial AOB. First, the data support the previous study of N. europaea that a cytochrome c-dependent NOR is required for nitrifier denitrification activity (Kozlowski et al., 2014). Second, the release of NO by the two oligotrophic strains in Cluster 6 of the AOB likely contributes to abiotic N$_2$O production (chemo-denitrification), especially under environmental conditions that facilitate NO or NH$_2$OH release (Jones et al., 2015; Zhu-Barker et al., 2015). This observation is congruent with the physiology of the oligotrophic AOA that lack NOR (Kozlowski et al., 2016c). Third, this study showcases the utility of comparative physiological studies on pure cultures of ammonia-oxidizers to characterize the diversity of mechanisms for NOx production and ultimately for N$_2$O release to the environment.

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AUTHOR CONTRIBUTIONS

JK and LS conceived the project; JK designed and performed all experiments, KK performed a PhyloPhAn analysis and created the phylogenetic tree; JK, KK, and LS analyzed the data, JK and LS wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

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