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ORIGINAL ARTICLE

Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota

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Chemolithotrophic ammonia-oxidizing bacteria and Thaumarchaeota are central players in the global nitrogen cycle. Obligate ammonia chemolithotrophy has been characterized for bacteria; however, large gaps remain in the Thaumarchaeotal pathway. Using batch growth experiments and instantaneous microrespirometry measurements of resting biomass, we show that the terrestrial Thaumarchaeon *Nitrososphaera viennensis* EN76^T exhibits tight control over production and consumption of nitric oxide (NO) during ammonia catabolism, unlike the ammonia-oxidizing bacterium *Nitrosospira multiformis* ATCC 25196^T. In particular, pulses of hydroxylamine into a microelectrode chamber as the sole substrate for *N. viennensis* resulted in iterative production and consumption of NO followed by conversion of hydroxylamine to nitrite. In support of these observations, oxidation of ammonia in growing cultures of *N. viennensis*, but not of *N. multiformis*, was inhibited by the NO-scavenger PTIO. When based on the marginal nitrous oxide (N_2O) levels detected in cell-free media controls, the higher levels produced by *N. multiformis* were explained by enzyme activity, whereas N_2O in *N. viennensis* cultures was attributed to abiotic reactions of released N-oxide intermediates with media components. Our results are conceptualized in a pathway for ammonia-dependent chemolithotrophy in Thaumarchaea, which identifies NO as an essential intermediate in the pathway and implements known biochemistry to be executed by a proposed but still elusive copper enzyme. Taken together, this work identifies differences in ammonia-dependent chemolithotrophy between bacteria and the Thaumarchaeota, advances a central catabolic role of NO only in the Thaumarchaeotal pathway and reveals stark differences in how the two microbial cohorts contribute to N_2O emissions.

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Introduction

Ammonia-oxidizing archaea, in the phylum Thaumarchaeota, and ammonia-oxidizing bacteria are abundant and diverse microorganisms that control the oxidation of ammonia (NH_3) to nitrite (NO_2^-) in the global biogeochemical nitrogen cycle. Through many decades of research, the biochemical pathway for chemolithotrophic growth of ammonia-oxidizing bacteria has been principally elucidated (Sayavedra-Soto and Arp, 2011); however, this pathway has yet to be characterized in the more recently discovered thaumarchaeotal ammonia-oxidizers. This lesser understanding is largely due to the difficulty of growing reliable and sufficient biomass from pure cultures for performing physiological experiments, thus making identification of the genetic inventory that supports chemolithotrophic growth of the thaumarchaeotal ammonia-oxidizers a challenge. In contrast, the pathways for the autotrophic assimilation of carbon have been identified in both cohorts (Arp *et al*., 2007; Könneke *et al*., 2014).

Previous experiments with the marine isolate *Nitrosopumilus maritimus* SCM1 indicated that ammonia oxidation is dependent on the activity of the ammonia monooxygenase enzyme and (an) unknown enzyme(s) that convert(s) hydroxylamine (NH_2OH) to NO_2^- and provide electrons for energy

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conservation (Vajrala *et al.*, 2012). In ammonia-oxidizing bacteria, this second step is performed by hydroxylamine dehydrogenase (EC 1.7.2.6); however, no homologues of hydroxylamine dehydrogenase-encoding genes have been identified in genome sequences obtained from any pure or enrichment culture of Thaumarchaea (Walker *et al.*, 2010; Kim *et al.*, 2011; Tourna *et al.*, 2011; Spang *et al.*, 2012). In addition to NH_2OH, there is also evidence that nitric oxide (NO) plays an important role in the Thaumarchaeotal but not in the bacterial ammonia oxidation pathway (Shen *et al.*, 2013; Martens-Habbena *et al.*, 2015). Martens-Habbena *et al.* (2015) demonstrated that NO accumulated in *N. maritimus* SCM1 cultures during active oxidation of NH_4Cl in a closed microrespirometry chamber, and was released at higher levels under saturating versus non-saturating availability of NH_4Cl. Exposure to increasing concentrations of an NO-scavenging compound over a 24 h period resulted in decreased levels of nitrite production in batch cultures of ammonia-oxidizing Thaumarchaea, but not bacteria (Martens-Habbena *et al.*, 2015). The authors concluded that NO was either released as a free intermediate during ammonia oxidation by *N. maritimus*, or it could serve a functional role as an electron delivery mechanism to ammonia monooxygenase, an idea that has been proposed previously (Schleper and Nicol, 2010).

Although the detection of nitrous oxide (N_2O) has been reported for both enrichments and pure cultures of Thaumarchaea engaged in ammonia oxidation (Santoro *et al.*, 2011; Loscher *et al.*, 2012; Jung *et al.*, 2014; Stieglmeier *et al.*, 2014b), the isotope data reported by Stieglmeier *et al.* (2014b) revealed that ammonia-oxidizing Thaumarchaea cannot enzymatically reduce NO_2^- to N_2O via NO in the pathway known as 'nitrifier denitrification'. Several publications have suggested that ammonia-oxidizing Thaumarchaea are a major source of N_2O to the environment based on their relative abundance in oxic environments, the isotopic signature of the detected N_2O, and that the authors failed to detect known bacterial denitrification genes and pertinent activities (Santoro *et al.*, 2011; Loscher *et al.*, 2012; Jung *et al.*, 2014). Yet, control experiments to verify or falsify chemical formation of N_2O facilitated by interaction of Thaumarchaeotal metabolites with components of the cultivation or incubation media or assay solutions remain absent from the literature. It should be noted that interactions of ammonia oxidation intermediates with iron, manganese, and organic compounds could generate substantial amounts of N_2O under environmentally relevant conditions (Zhu-Barker *et al.*, 2015).

The present study addresses critical ecophysiological questions about how two different cohorts of microorganisms, simultaneously involved in the biogeochemical nitrogen cycle through ammonia-oxidation, vary in their contributions, particularly to production of nitrous oxide. This study also furthers the observation of NO as an intermediate for ammonia chemolithotrophy in the terrestrial Thaumarchaeon *Nitrososphaera viennensis* strain EN76^T (Stieglmeier *et al.*, 2014a) by examining its complete profile of NO production and consumption during substrate oxidation at oxic conditions and the transition into an extended period of anoxia. In contrast to the above referenced studies of 'N_2O production' by ammonia-oxidizing Thaumarchaea, our results do not support any scenario in which *N. viennensis* enzymatically reduces NO to N_2O through a denitrification pathway. Instead, the results support that N_2O was formed abiotically from NO by interaction with media components or with debris in killed cell controls. We further demonstrated that NO is an active and necessary intermediate during the oxidation of NH_2OH to NO_2^- in ammonia-oxidizing Thaumarchaea rather than participating directly in the oxidation of NH_3 to NH_2OH as suggested previously (Schleper and Nicol, 2010). Based on these results, a new pathway for obligate ammonia-dependent chemolithotrophy for ammonia-oxidizing Thaumarchaea is proposed that implicates a novel copper enzyme to perform a biochemistry known to occur in ammonia-oxidizing bacteria facilitated by heme-containing cytochrome *c*.

Materials and methods

Strains and cultivation

N. viennensis strain EN76^T was maintained at 37 °C in 50 ml freshwater medium (FWM) supplemented with 2 mM NH_4Cl, 0.5 mM sodium pyruvate and 50 μg/ml carbenicillin and buffered with HEPES (Tourna *et al.*, 2011; Stieglmeier *et al.*, 2014a) and inoculated at 4% v/v. Cultures were grown in Wheaton bottles (150 ml) sealed with caps inlaid with grey butyl rubber stoppers. *Nitrosospira multiformis* ATCC 25196^T was maintained at 28 °C in 100 ml HEPES-buffered HK medium (HKM) (Krümmel and Harms, 1982) containing 3 mM ammonium and phenol red as pH indicator (pH of 7.5–8) and inoculated at 5% v/v into 250 ml Wheaton bottles. The pH of *N. multiformis* cultures was maintained with regular additions of 10% $NaHCO_3$.

Growth experiments with NO-scavenger PTIO

For monitoring activity in the presence of an NO-scavenging compound, *N. viennensis* was cultivated in 20 ml FWM. *N. multiformis* was cultivated in 20 ml phosphate-buffered mineral medium (Skinner and Walker, 1961) amended with 1 mM NH_4Cl and pH was adjusted regularly with 5% Na_2CO_3. In early to mid-exponential phase of growth, 150 μM of 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; Sigma-Aldrich, Vienna, Austria), a chemical that scavenges NO (Goldstein *et al.*, 2003)

was injected into the cultures. Ammonium consumption and nitrite production were measured over a period of 6–8 days using standard colorimetric assays (Clesceri *et al*., 1998) and N_2O was measured via GC (AGILENT 6890N, Vienna, Austria; injector: 120 °C, detector: 350 °C, oven: 35 °C, carrier gas: N_2) in connection with an automatic sample-injection system (DANI HSS 86.50, Head-space-Sampler, Sprockhövel, Germany). Detailed sampling and sample preparation has been described previously (Stieglmeier *et al*., 2014a).

Instantaneous measurement of NO and N_2O during oxidation of ammonia

In preparation for experiments measuring instantaneous O_2 consumption and either NO or N_2O production, *N. viennensis* was inoculated at 4% v/v into 2 l of HEPES-buffered FWM and *N. multiformis* was inoculated at 5% v/v into 250 ml HKM. Cells were harvested at late exponential phase (*N. viennensis*, 1–1.5 mM NO_2^-; *N. multiformis*, 2–2.5 mM NO_2^-) by filtration on Supor200 0.2 μm filters (Pall, Ann Arbor, WI, USA) and rinsed three times with substrate-free media (*N. viennensis*, FWM; *N. multiformis*, HKM). Washed cells (*N. viennensis*, *ca*. 1×10^{11} total cells; *N. multiformis*, *ca*. 1×10^{10} total cells) were resuspended into 10 ml of substrate-free growth medium for each strain in a 10 ml two-port microrespiratory (MR) chamber with fitted injection lids (Unisense, Aarhus, Denmark). Cell concentrations for microrespirometry experiments were chosen on the basis of comparable oxygen consumption rates between the two strains. O_2 concentration was measured using an OX-MR 500 μm tip diameter MR oxygen electrode (Unisense), N_2O concentration was measured using an N_2O-500 N_2O minisensor electrode with 500 μm tip diameter (Unisense), and NO was measured using an ami-600 NO sensor with 600 μm tip diameter (Innovative Instruments Inc., Tampa, FL, USA). The availability of O_2 in the MR chamber, a closed system, corresponded to either *ca*. 207 μM O_2 (FWM) or *ca*. 243 μM O_2 (HKM) respectively, based on equilibrium O_2 concentration at operating temperatures and medium salinities. For microrespirometry experiments involving ammonia oxidation, cells were provided 2 mM NH_4Cl. The microrespirometry chamber was maintained at 37 °C and 28 °C for measurements with *N. vienennsis* and *N. multiformis* cells, respectively, reflecting their optimal growth temperatures.

Instantaneous measurement of NO from N. viennensis *during oxidation of NH_2OH*

For experiments measuring the oxidation of NH_2OH (99.999% purity, Sigma-Aldrich, St Louis, MO, USA), *N. viennensis* was provided with multiple additions of 200 μM NH_2OH (based on chamber volume) to maintain a steady rate of O_2 consumption. NO production was measured until O_2 was undetectable in the chamber. Samples were taken post-experiment for NO_2^- measurements.

Instantaneous ammonia and hydroxylamine oxidation by N. viennensis *in the presence of PTIO*

Microrespirometry experiments with the NO-scavenger PTIO were performed with *N. viennensis* cells harvested as described above; cells were incubated with 200 μM PTIO in the dark with shaking at 37 °C for 1 h prior to adding the cells to a 2 ml 1-port MR chamber at 37 °C for the measurement of NH_4^+- and NH_2OH-dependent O_2 consumption (Supplementary Figure S1). Confirmation of the NO-scavenging activity of PTIO was confirmed chemically by addition of 1 μl PAPA NONOate ((Z)-1-[N-(3-aminopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate; Cayman Chemical, Ann Arbor, MI, USA; half-life of 15 min at 37 °C liberating 2 moles of NO per mole of parent compound) to FWM in the 2 ml MR chamber with the NO sensor at 37 °C. Once the rate of NO release from PAPA NONOate slowed, 200 μM PTIO was added to the chamber and NO disappearance was immediately measured. After ~7 min of NO-chelation by PTIO, another 1 μl of PAPA NONOate was added but NO levels remained below detection levels (Supplementary Figure S2).

Instantaneous measurement of N_2O from media and killed-cell controls

To measure the abiotic production of N_2O from either FWM or HKM, 10 ml of cell-free media was added to the 10 ml MR chamber. The NO-donor MAHMA NONOate (6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine, NOC-9; Cayman Chemical) was added to the MR chamber in increasing additions of 20–100 μl, which is equivalent to the release of *ca*. 1.1–5.5 μM NO, or in a single addition of 100 μl (*ca*. 5.5 μM NO). The half-life of MAHMA NONOate at pH 7.4 is 1 min and 3 min at 37 °C and 22–25 °C, respectively. N_2O production was measured using the N_2O microelectrode during the decay of 1 mol MAHMA NONOate into 2 moles NO in either FWM or HKM. Experiments were performed at 37 °C and 28 °C for FWM and HKM, respectively, after sparging to *ca*. 0–3% O_2 saturation with N_2 (Praxair) as determined by O_2 electrode. Chemical controls to confirm that FWM alone did not react with NH_2OH to form measureable NO involved addition of 200, 400 and 600 μM NH_2OH to sparged (*ca*. 0–3% O_2) FWM or FWM+200 μM NO_2^- to reflect maximum concentration available once cells depleted MR chamber O_2 (Supplementary Figures S3a and b). Control experiments with heat killed *N. viennensis* (*ca*. 1×10^{11} cells) were performed in sparged FWM+$NaNO_2$ (200 μM) with measurement of NO and N_2O upon addition of 200 μM NH_2OH (Supplementary Figure S4) to determine whether NH_2OH interacts with cellular debris.

Results

Effects of the NO-scavenger PTIO on N_2O levels measured in cultures of N. viennensis *and* N. multiformis

To investigate the role of NO in chemolithotrophic oxidation of ammonia to nitrite and generation of N_2O in these two organisms, batch cultures of *N. viennensis* or *N. multiformis* were grown to mid-log phase, at which point PTIO (150 µM) was added (Figure 1). Addition of PTIO resulted in the immediate saturation of N_2O levels in either culture as would be expected in the absence or decreasing levels of NO intermediates. However, in cultures of *N. viennensis*, PTIO also caused an inhibition of both ammonium consumption and nitrite production (Figure 1a), whereas in cultures of *N. multiformis,* ammonia oxidation and nitrite production continued at the same rate as before PTIO addition (Figure 1b). These results indicated that NO is an essential, dynamic, intermediate in the process of ammonia oxidation to nitrite and thus ammonia-dependent chemolithotrophy for *N. viennensis*, but not for *N. multiformis*.

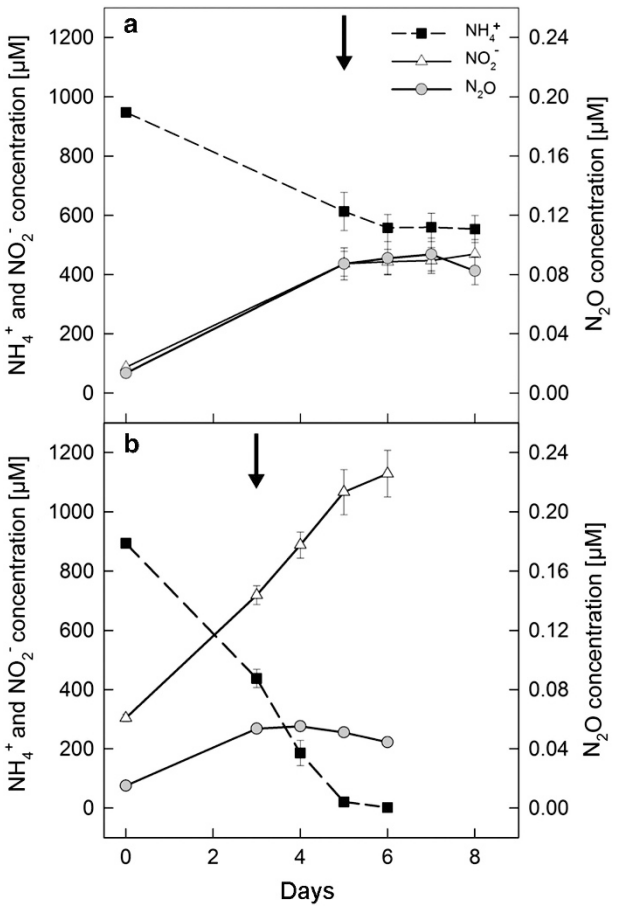

Figure 1 Inhibition of *N. viennensis* (**a**) and *N. multiformis* (**b**) with the NO-scavenger PTIO. Ammonium consumption (black squares, dotted line) and nitrite production (white triangles, solid line) as well as N_2O production (gray circles, solid line) are plotted. The black arrow indicates the time point of PTIO addition (150 µgml^{-1}) to the cultures. Mean values of five-fold replicated experiments with standard deviations are shown.

Dynamics of NO and N_2O production during and following ammonia oxidation

The effect of PTIO on growing cultures of *N. viennensis* and *N. multiformis* indicated different requirements for NO during ammonia oxidation. Using a MR chamber, the dynamics of NO production and consumption were measured during and after ammonia oxidation (2 mM NH_4Cl) by *N. viennensis* or *N. multiformis* as determined by O_2 consumption profiles (Figures 2a and c). To achieve an equivalent rate of O_2 consumption for rate comparison, 10 times more *N. viennensis* than *N. multiformis* cells were required in the MR chamber. Whereas *N. multiformis* showed a linear rate of O_2 consumption during ammonia oxidation (Figure 2c), the initial rate of O_2 consumption by *N. viennensis* was quite rapid, followed by a slower, linear rate (Figures 2a and b). *N. viennensis* produced a maximum of ca. 1.41 nM of NO per 1×10^{10} cells ($n = 4$) at the beginning of substrate oxidation, concomitant with the initial rapid rate of O_2 consumption. The NO was immediately re-consumed as the cells achieved the slower, linear rate of O_2 consumption (Figure 2a). After *ca.* 3 min from the point at which O_2 became undetectable, *N. viennensis* cells began to release NO reaching a maximum of *ca.* 1.39 nM per $1x10^{10}$ cells ($n = 4$). None of the NO released by *N. viennensis* following O_2 depletion was re-consumed. In contrast, *N. multiformis* produced a maximum of ca. 92.15 nM NO per 1×10^{10} cells ($n = 4$) and re-consumption of NO began once *ca.* 50% of the available O_2 was consumed (Figure 2c).

Levels of N_2O were measured during and following ammonia oxidation; however, no N_2O was detectable during ammonia oxidation by cells of either microbe (Figures 2b and d). Assays including *N. viennensis* cells contained measurable N_2O levels increasing at a non-linear rate after *ca.* 5 min following depletion of O_2, yielding an average maximum at *ca.* 40 min of 0.19 µM per 1×10^{10} cells (Figure 2b; $n = 4$). In contrast, assays including *N. multiformis* cells contained measurable N_2O levels immediately upon O_2 depletion increasing at a linear rate to an average maximum of 5.6 µM per 1×10^{10} cells at *ca.* 40 min (Figure 2d; $n = 4$).

Dynamics of NO production and consumption during NH_2OH oxidation by N. viennensis *EN76*^{T}

Although the experiments described above indicated that *N. viennensis* cultures produce and consume NO during ammonia oxidation to nitrite, it was not clear whether NO acted as an intermediate in the ammonia- or hydroxylamine-oxidizing step of the pathway. Therefore, we examined production and consumption of NO by *N. viennensis* cells when fed with NH_2OH instead of ammonium. NH_2OH was added in 200 µM pulses to the MR chamber containing *N. viennensis* cells to support linear O_2 consumption until all of the available O_2 was

consumed (Figure 3). Each subsequent addition of equal aliquots of NH_2OH led to the production of *ca.* 5 nM NO per 1×10^{10} cells ($n = 3$), followed by an immediate re-consumption of NO and O_2 until the next addition of NH_2OH (Figure 3). NO_2^- accumulated to *ca.* 206 μM ($n = 3$) in the culture medium, which matched the *ca.* 207 μM O_2 consumed during the time course of the experiment. Importantly, free conversion of NH_2OH to NO in the absence of cells was stochastic and insignificant (Supplementary Figure S3a). Addition of NH_2OH to FWM containing 200 μM $NaNO_2$ resulted in the production of *ca.* 4 nM NO but only once a concentration of 1.2 mM NH_2OH was reached in the MR chamber (Supplementary Figure S3b).

In an effort to demonstrate the requirement of NO by *N. viennensis* for the oxidation of either NH_3 or NH_2OH, washed *N. viennensis* cells were incubated with 200 μM PTIO for 1 h prior to measurement of NH_4^+- or NH_2OH-dependent O_2 consumption. The presence of PTIO did not prevent substrate-dependent O_2 consumption of either substrate by *N. viennensis* cells (Supplementary Figure S1), although PTIO was able to effectively scavenge NO in cell-free FWM containing the NO-donating compound, PAPA-NONOate (Supplementary Figure S2).

Detection of abiotic N_2O in growth media without viable cells

Inspired by the observed differences in N_2O production profiles between cultures of *N. viennensis* and *N. multiformis*, we performed abiotic experiments in the MR chamber using cell-free FWM or HKM and the NO-donating compound, MAHMA NONOate. Addition of MAHMA NONOate to FWM released *ca.* 5.5 μM NO, 70% of which was converted to N_2O (Figure 4a). In contrast, addition of an equal aliquot of MAHMA NONOate to HKM resulted in only a 20% conversion of the released NO to N_2O (Figure 4c). Continuous addition of MAHMA NONOate to produce 1.1–5.5 μM NO in FWM or HKM resulted in a sustained high-efficiency conversion of released NO to N_2O only in FWM, but not

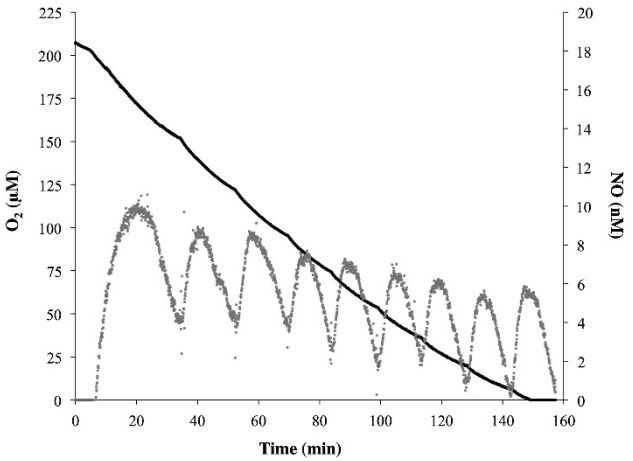

Figure 3 Instantaneous measurements of O_2 consumption (black line) and NO production (grey dots) from 200 μM pulses of NH_2OH in liquid phase incubations of *N. viennensis* in the absence of NH_4^+. Plot is a single representative of replicable experiments ($n = 4$).

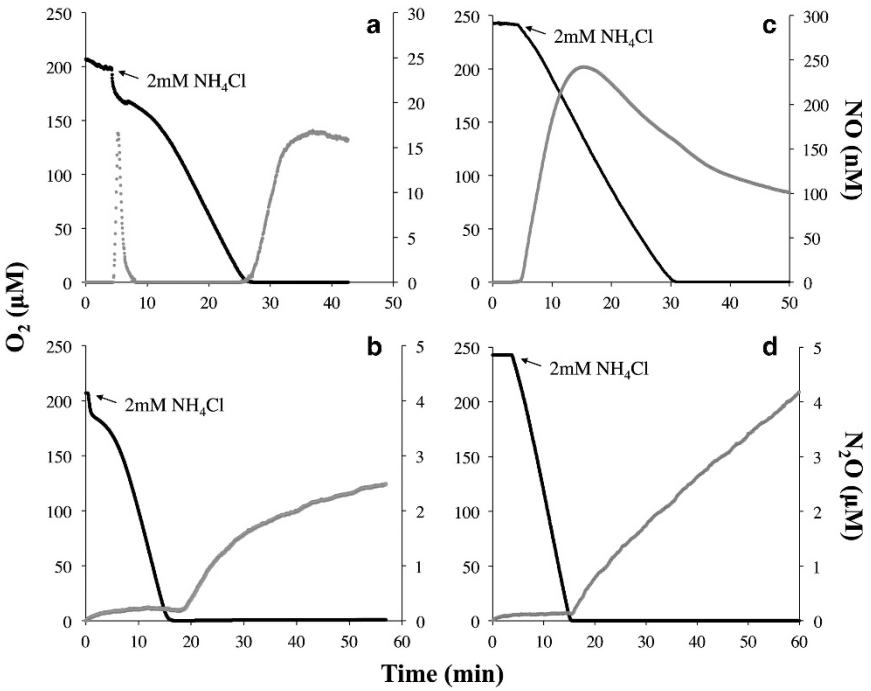

Figure 2 Instantaneous measurements of O_2 (black line), NO (**a** and **c**) and N_2O (**b** and **d**) (gray dots) after addition of 2 mM NH_4Cl in liquid phase suspensions of *N. viennensis* (**a** and **b**) and *N. multiformis* (**c** and **d**) cells. Panels are single representative measurements of reproducible results ($n = 4$). Note that y-axes for NO are on different scales for *N. viennensis* versus *N. multiformis*. *N. viennensis* cell concentration was 10^{11} cells per ml, whereas *N. multiformis* cell concentration was 10^{10} cells per ml to achieve equivalent rates of O_2 consumption by the two strains.

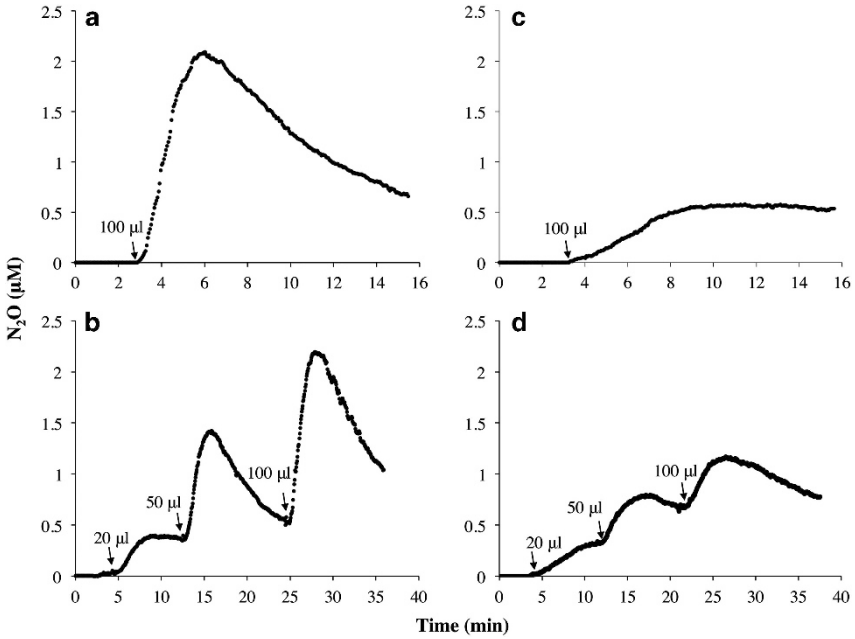

Figure 4 Abiotic production of N_2O from the NO-donor MAHMA NONOate in either FWM (**a** and **b**) or HKM (**c** and **d**). Panels are single representative measurements of reproducible results ($n = 3$). The addition of varying concentrations of MAHMA NONOate is indicated by arrows.

HKM (Figures 4b and d). Reactivity of NH_2OH in FWM+heat-killed *N. viennensis* cells was also explored (Supplementary Figure S4). When NH_2OH was introduced into the MR chamber containing heat-killed cells, accumulation of NO reached *ca.* 110 nM NO over 5 min (Supplementary Figure S4a). After 30 min, N_2O accumulated to levels of *ca.* 90 µM, demonstrating that NH_2OH was eventually converted to N_2O in the absence of physiologically active cells (Supplementary Figure S4b).

Discussion

The NO-scavenger, PTIO, stops ammonia-dependent chemolithotrophy of N. viennensis

The measured cessation of ammonium consumption and nitrite production upon PTIO addition to growing cultures of *N. viennensis* demonstrates the requirement of free NO for ammonia chemolithotrophy that was not observed for *N. multiformis*. These results confirm prior growth experiments with enrichment cultures (Jung *et al.*, 2014) and reported effects on nitrite production and activity by ammonia-oxidizing Thaumarchaea and bacteria incubated with PTIO (Shen *et al.*, 2013; Martens-Habbena *et al.*, 2015). For both *N. viennensis* and *N. multiformis*, PTIO addition abolished N_2O production, suggesting that the presence of the enzyme-generated free NO intermediate is required for formation of N_2O production by both strains, regardless of whether NO is reduced biotically by enzyme activity or abiotically.

NO is produced and immediately consumed during active ammonia oxidation by N. viennensis *EN76*^{T}

The initial, rapid production of NO followed by its equally rapid consumption during ammonia-dependent O_2 consumption by *N. viennensis* differed from results in similar experiments with *N. maritimus* SCM1 (Martens-Habbena *et al.*, 2015). In this prior study, *N. maritimus* SCM1 produced NO at a steady-state level prior to its consumption once NH_4^+ was depleted or its partial consumption at saturating concentrations of NH_4^+. A major difference in the two profiles observed for both cultures was that O_2 levels remained quite high in assays with *N. maritimus* SCM1 such that complete consumption of NO was not observed as a function of time and O_2 consumption as observed for *N. viennensis* EN76^T. Even so, experiments with both *N. viennensis* and *N. maritimus* confirm that NO is being produced and consumed during ammonia oxidation. In addition, the present experiments demonstrate that NO is being released at the onset of anoxia. A likely fate of released NO at anoxia was its conversion to N_2O, because 1000 times more N_2O than NO was measured once the microrespirometry chamber reached anoxia, suggesting rapid conversion of released NO to N_2O (Figures 2a and c). Another contributor to N_2O levels measured in anoxic assays with *N. viennensis* cells could be the reactivity of cell components with released NH_2OH as heat-killed cells showed a rapid conversion of exogenous NH_2OH to measureable NO and N_2O (Supplementary figure S4).

NO dynamics during ammonium-dependent O_2 consumption by *N. multiformis* showed a vastly different profile compared to that of either *N. viennensis*

or *N. maritimus,* revealing *ca.* 10 times more NO released from the cells, some of which was slowly re-consumed during ammonia oxidation and through anoxia. The comparison of NOx profiles in microrespirometry measurements with cells of *N. multiformis* and the ammonia-oxidizing Thaumarchaea reveals an intriguing difference in how NOx is metabolized during ammonia oxidation by bacteria and Thaumarchaea, which requires further investigation. Unlike *N. viennensis*, N_2O production by *N. multiformis* during anoxia was linear and 10 times more N_2O was produced per number of cells. This is a confirmatory evidence that ammonia-oxidizing bacteria, but not *N. viennensis*, are capable of producing N_2O enzymatically via nitrifier denitrification (Stieglmeier *et al.*, 2014b).

NO is produced and consumed during NH_2OH oxidation to NO_2^- in N. viennensis

The rapid production and consumption of NO during NH_2OH oxidation by *N. viennensis* along with the stoichiometric production of NO_2^- with O_2 consumption suggest that NO is directly participating in the dehydrogenation of NH_2OH. Recent models have postulated that NO is involved in providing reductant to ammonia monooxygenase (Schleper and Nicol, 2010; Stahl and de la Torre, 2012); however, if this were the case then the rapid production/consumption cycle of NO during NH_2OH oxidation would not be observed. Experiments to demonstrate the role of NO in NH_2OH oxidation by pre-incubating washed cells with PTIO with the goal to observe quenching of NH_3 or NH_2OH-dependent O_2 consumption were inconclusive. It is possible that PTIO is only effective during active growth of *N. viennensis* (which was observed; Figure 1), or perhaps PTIO was ineffective at chelating rapidly cycling NO at the high cell densities used in the MR chamber.

N_2O in N. viennensis *cultures originates from the abiotic reaction of biotic N-oxide intermediates with medium or cellular components*

Previous studies measuring N_2O in pure and enrichments cultures of ammonia-oxidizing Thaumarchaea suggested an enzymatic origin of measured N_2O (Santoro *et al.,* 2011; Loscher *et al.,* 2012; Jung *et al.,* 2014); however, control experiments to test for abiotic reduction of NO to N_2O, including by medium components, were not performed. We observed a high rate of NO reduction to N_2O in FWM both in the presence of an NO-donating molecule and in the presence of NH_2OH plus heat-killed cells. Based on the difference in metal content of both media, we propose that reduction of NO to N_2O in FWM is facilitated by iron, which is present in FWM at a relatively high final concentration of 7.5 uMl^{-1} in the form of FeNaEDTA but absent from HKM. Under anoxic conditions, in which the metal components of the medium are reduced, the Fe(II) and reduced trace metals act as chemical catalysts for NO reduction to N_2O. This 'chemodenitrification' process has been implicated by hypothesis in contributing to abiotic N_2O production in reduced environments where Fe(II) is abundant (Samarkin *et al.,* 2010; Kampschreur *et al.,* 2011; Jones *et al.,* 2015).

Proposed pathway for ammonia chemolithotrophy in ammonia-oxidizing Thaumarchaea in which NO facilitates NH_2OH oxidation

Our revised model of ammonia-dependent chemolithotrophy of the Thaumarchaeota places NO as a necessary co-reactant for the oxidation of NH_2OH to NO_2^- (Figure 5a). This NO-dependent dehydrogenation of NH_2OH to NO_2^- is not based on novel chemistry because ammonia-oxidizing bacteria, and others such as aerobic methane-oxidizing bacteria, utilize the heme-containing cytochrome P460 enzyme to facilitate this reaction (Figure 5b; Simon and Klotz, 2013). Instead, the central reaction in the Thaumarchaeotal nitrification pathway is based on a proposed novel copper enzyme capable of performing known P460 activity. This model achieves the proper substrate stoichiometry and reductant flow. In addition, the modelled rapid cycling of NO (and, concomitantly electrons) to support NH_2OH oxidation would logically preclude any enzymology for NO reduction to N_2O. In agreement with this requirement, none of the sequenced genomes of ammonia-oxidizing Thaumarchaeota revealed the presence of canonical and alternate inventory for NO reduction to N_2O. Nitrite reductase (*nirK*) is encoded in the genomes of all published sequences of ammonia-oxidizing Thaumarchaea (Bartossek *et al.*, 2010, 2012) and *nirK* transcripts have been detected at very high steady-state levels in environmental metatranscriptomes (Hollibaugh *et al.*, 2011; Radax *et al.*, 2012), which makes this enzyme the most parsimonious source of the NO needed to support ammonia-dependent chemolithotrophy. The proposal that NO_2^- reduction and not NH_2OH oxidation is the more likely source of the NO required for the oxidation of NH_2OH to NO_2^- is supported by the following logic and reasoning:

(1) A two-step oxidation of NH_2OH to NO_2^- via a NO intermediate would require the operation of two enzyme complexes that feed extracted electrons (3+1) via two redox shuttles to two quinone-reactive enzymes. In addition to requiring additional unknown inventory, such a pathway would also not generate enough electrons needed to provide for effective linear electron flow ($4-2-1=1$). In contrast, the proposed one-step model provides for effective linear electron flow ($5-2-1=2$; Figure 5). The bioenergetics contrast stands in the context that an observed active NirK activity would draw one electron per reduced NO_2^- in both scenarios in addition to that the two-step model would include two linearly connected sources of NO production in a genomic background not encoding identifiable NO detoxification inventory and a scenario

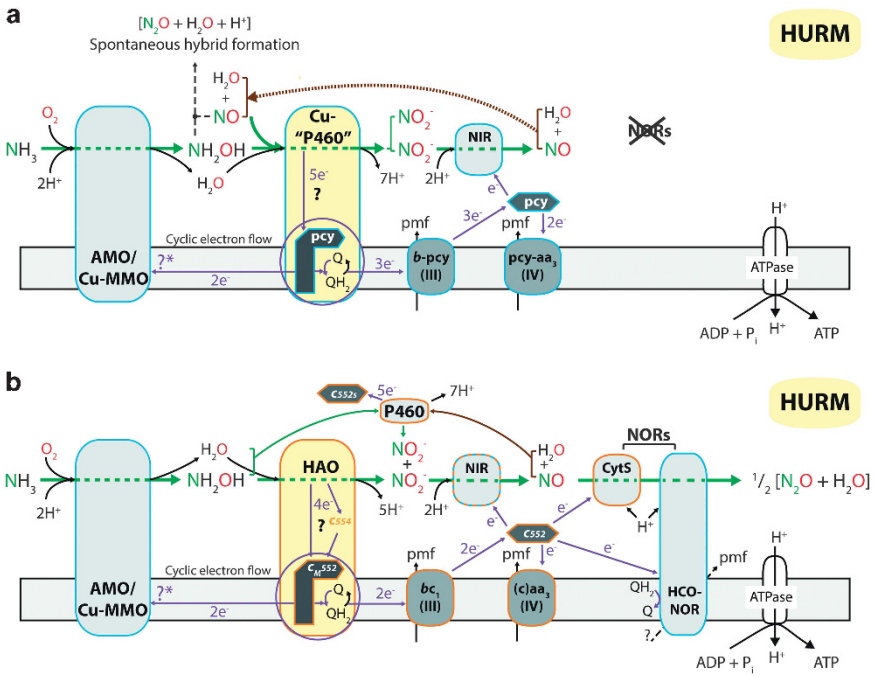

Figure 5 Proposed pathway for ammonia-dependent chemolithotrophy in the ammonia-oxidizing Thaumarchaea (**a**) compared with known pathways of N-oxide transformation in ammonia-oxidizing bacteria (**b**). The model presents a central role of NO in the oxidation of NH_2OH, and its contribution to hybrid formation of N_2O as proposed by Stieglmeier *et al.* (2014b). Due to the lack of heme proteins including HAO and quinone-reactive proteins such as c_M552 (CycB), redox processes in ammonia-oxidizing archaea are likely mediated by Cu protein complexes (Walker *et al.*, 2010; Stahl and de la Torre, 2012). The present literature suggests that NH_3 is monooxygenated to NH_2OH by ammonia monooxygenase (AMO) and that NH_2OH is dehydrogenated to NO_2^- by activities of a number of unknown enzymes (Walker *et al.*, 2010; Stahl and de la Torre, 2012; Vajrala *et al.*, 2012). Based on existing chemistry facilitated by heme proteins in ammonia-oxidizing bacteria (**b**), the model in (**a**) proposes that the oxidation of NH_2OH to NO_2^- and subsequent extraction of five electrons results from a reaction of NH_2OH with NO and H_2O facilitated by a novel Cu-containing enzyme. This could be one of the multi-copper oxidases encoded in all genomes of ammonia-oxidizing Thaumarchaeota (Bartossek *et al.*, 2010, 2012; Walker *et al.*, 2010). NO is provided by the Cu-containing NirK, which enzymatically reduces one NO_2^- per NH_3 oxidized to NO. A fraction of the enzyme-produced NO and NH_2OH could react to form N_2O by hybrid formation. The figure was adapted from Simon and Klotz (2013). AMO/Cu-MMO, ammonia monooxygenase; *c*552, cytochrome *c* redox carrier; CytS: cytochrome *c*'-beta (see Simon and Klotz, 2013, and references therein); HAO, hydroxylamine dehydrogenase; HCO, heme-copper oxidase; HURM, hydroxylamine:ubiquinone redox module (see Simon and Klotz, 2013, and references therein); NirK, Cu-containing NO-forming nitrite reductase; NOR, nitric oxide reductase; P460, tetraheme cytochrome *c* protein P460 (CytL; see Simon and Klotz, 2013, and references therein); pcy, plastocyanin; pmf, proton-motive force; Q/QH_2, quinone/quinol pool.

that should not lead to stoichiometric conversion of $N\text{-}NH_3$ to $N\text{-}NO_2^-$.

(2) Isotopic measurements of $^{15}N_2O$ produced by *N. viennensis* suggest a 'hybrid signature' in that one N atom would originate from NH_3 (contributed as NH_2OH) and one atom would originate from NO_2^- (contributed as NO) (Stieglmeier *et al.*, 2014b). This finding also contradicts a two-step model and supports the model shown in Figure 5.

The proposed one-step model is parsimonious in that it requires the innovation of only one enzyme in ammonia-oxidizing Thaumarchaea. Based on existing knowledge, this novel enzyme is copper-based and facilitates known redox chemistry in context with known enzyme complexes such as the NH_2OH-producing ammonia monooxygenase, NO-producing NirK, plastocyanin redox carriers and a quinone-reactive membrane protein, all of which are copper proteins and have been identified in all sequenced genomes of ammonia-oxidizing Thaumarchaea (Walker *et al.*, 2010; Bartossek *et al.*, 2012; Stahl and de la Torre, 2012).

We propose that the model of catabolic electron flow presented here (Figure 5a) applies to all obligate chemolithotrophic ammonia-oxidizing Thaumarchaea because it is based on and supported by results from above referenced experiments with marine ammonia-oxidizing Thaumarchaea including *N. maritimus* SCM1 and experiments with terrestrial ammonia-oxidizing Thaumarchaeota including the data presented here for *N. viennensis* $EN76^T$.

Conclusion

The present study establishes that both ammonia-oxidizing Thaumarchaea and bacteria contribute to the production of N_2O, although the mechanisms by which they do so are distinct. Whereas the ammonia-oxidizing bacteria produce N_2O enzymatically through nitrifier denitrification, the ammonia-oxidizing Thaumarchaea release intermediates (NO and/or NH_2OH), which are then reduced non-enzymatically to N_2O in anoxic microenvironments

(Zhu-Barker *et al*., 2015). Due to the relatively high abundance and activity of Thaumarchaea across terrestrial, freshwater, and marine environments (Zhang *et al*., 2010; Pratscher *et al*., 2011; French *et al*., 2012; Berg *et al*., 2015) and their established tolerance of low ammonium and oxygen environments (Martens-Habbena *et al*., 2009), their contributions to NOx emissions is likely of high global significance (Babbin *et al*., 2015). For instance, marine Thaumarchaea may be essential in providing a substantial concentration of NO to denitrifying microorganisms within oxygen minimum zones, and in return, the denitrifiers could provide organic carbon to the Thaumarchaeota to establish a nitrifying-denitrifying consortium (Karner *et al*., 2001; Beman *et al*., 2012; Ganesh *et al*., 2015). The present study also supports that both ammonia-oxidizing Thaumarchaea and bacterial ammonia-oxidizers likely contribute to chemodenitrification in terrestrial environments through the release and subsequent transformation of metabolites (NH_2OH, NO and NO_2^-) either abiotically or via denitrifying consortia (Jones *et al*., 2015), which dominate in less oligotrophic environments. The elucidation of NO as an essential pathway intermediate and released metabolite of the ammonia-oxidizing Thaumarchaea in the absence of a nitrifier denitrificaiton pathway will allow refinement of the relative contributions of ammonia-oxidizing microorganisms to global N_2O production.

Conflict of Interest

The authors declare no conflict of interest.

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