Genomic and Physiological Analysis of Nitrogen Oxide Metabolism in Ammonia-Oxidizers

By

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Abstract

Ammonia oxidizers come from two different domains of life, the Archaea and Bacteria, and control a vital step in the global biogeochemical Nitrogen cycle; the conversion of ammonia to nitrite. They are abundant in a wide range of environments including marine and freshwaters, terrestrial soils, and wastewater treatment plants. This group of organisms has also been implicated in the production of the nitrous oxide, a potent greenhouse gas. Nitrous oxide has been measured from ammonia oxidizing bacteria and archaea in pure and enrichment culture but the pathways, including enzymology, intermediates, and physiological conditions for nitrous oxide production are not well understood and therefore it is not possible to accurately model contributions of this group of microorganisms to global nitrous oxide emissions. The issue has been exacerbated by lack of available and closed genomes of ammonia oxidizers, physiological analyses of nitrous oxide production on pure cultures under environmentally relevant conditions, and chemical controls to elucidate differences in biological versus abiotic contributions to nitrous oxide production. Necessary studies to fill in the gaps in knowledge hindering the field were done by utilizing various approaches to studying ammonia oxidizer physiology. Physiological experiments included growth and resting cell assays along with headspace gas measurements, and instantaneous measurements of nitric oxide and nitrous oxide production during oxidation of native energy generating substrates. All studies were complemented with a genome-inferred approach to see if genomic inventory could explain physiological results. In conclusion, the present body of work addresses the above overarching problems in the field of Nitrification by (1) determining the pathways, including enzymology, involved in nitrous oxide production by the model ammonia-oxidizing bacterium *Nitrosomonas europaea* ATCC 19718, (2) showing that genomic inventory and phylogeny of ammonia oxidizing bacteria do not predict contributions to

nitrous oxide production, and (3) identifying key pathways and intermediates that explain differences in ammonia oxidation and nitrous oxide production between ammonia oxidizing bacteria and archaea.

Preface

This thesis consists of a compilation of individual research papers that are presently published or in preparation. The objectives of each individual paper and/or chapter and the relationship between each body of work are described in the opening chapter as well as in the text of the introduction and discussion of each manuscript or thesis chapter. Chapter 5 describes the major findings of the presented research and future directions suggested for the field. Appendix A and B consist of supplemental information to the body of work, especially research discussed in Chapter 3. The individual published research papers presented in this dissertation are:

Chapter 2: Kozlowski JA, Price J, Stein LY. (2014). Revision of N2O-Producing Pathways in the Ammonia-Oxidizing Bacterium Nitrosomonas europaea ATCC 19718. Appl Environ Microbiol 80: 4930–4935.

Chapter 4: Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY. (2016). Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. ISME J. Epub ahead of print February 16 2016.

Additional co-authored research papers in which the dissertation author was the primary author that are integral to the present thesis are included in appendices:

Appendix A: Kozlowski JA, Kits KD, Stein LY. (2016). Genome Sequence of Nitrosomonas communis Strain Nm2, a Mesophilic Ammonia-Oxidizing Bacterium Isolated from Mediterranean Soil. Genome Announc 4: e01541–15–2.

Appendix B: Kozlowski JA, Kits KD, Stein LY. (2016). Complete Genome Sequence of Nitrosomonas ureae Strain Nm10, an Oligotrophic Group 6a Nitrosomonad. Genome Announc 4: 1–2.

Additional co-authored publications that are not included in the dissertation are:

Campbell MA, Nyerges G, Kozlowski JA, Poret-Peterson AT, Stein LY, Klotz MG. (2011). Model of the molecular basis for hydroxylamine oxidation and nitrous oxide production in methanotrophic bacteria. FEMS Microbiol Lett. 322:82-89.

French E, Kozlowski JA, Mukherjee M, Bullerjahn G, Bollmann A. (2012). Ecophysiological Characterization of Ammonia-Oxidizing Archaea and Bacteria from Freshwater. Appl Environ Microbiol 78:5773-5780

Urakawa H, Garcia JC, Nielsen JL, Le VQ, Kozlowski JA, Stein LY, Lim CK, Pommerening-Roser A, Martens-Habbena W, Stahl DA, Klotz MG. (2014). Nitrosospira lacus sp. nov., a psychrotolerant ammonia-oxidizing bacterium from sandy lake sediment. IJSEM 65:242-250

Although the papers in chapter 2 and 4 as well as appendix A and B were co-authored, the first author on all papers (dissertation author) was involved in all aspects of the research encompassing the data collection, data analysis, and writing of manuscripts. Dr. Lisa Stein provided guidance on research directions, interpretation of data, and editing of manuscripts for all work presented in this thesis. In Chapter 2 Jennifer Price was involved in the original creation of the Nitrosomonas europaea ATCC 19718 nirK::Kan and norB::Gen knockout mutants. Work

done for the completion and publication of Chapter 4 was a joint effort between three research groups. The lab of Dr. Christa Schleper at the University of Vienna along with Dr. Michaela Stieglmeier provided the Nitrososphaera viennensis EN76 culture utilized for comparative physiological analysis in Chapter 4. Also, these researchers provided data on growth experiments with the NO-scavenger PTIO. Dr. Martin Klotz provided an evolutionary biology perspective and helped to formulate a model for the proposed pathway of thaumarchaeotal ammonia-oxidation which clarified data collecting in Chapter 4. In Appendix A and B K. Dimitri Kits assisted in the assembly of contigs obtained through PacBio sequencing as well as closure of the two genomes reported in these appendices. All co-authors have reviewed and approved final manuscripts before submission to journals.

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List of Symbols, Nomenclature, and Abbreviations

AMO: ammonia monooxygenase

AOA: ammonia-oxidizing Thaumarchaeota

AOB: ammonia-oxidizing bacteria

ATP: adenosine triphosphate

b_{c1}: respiratory complex III

$(c)_{aa3}$: respiratory complex IV

C_{554}: cytochrome C_{554}

C_{m552}: membrane cytochrome Cm_{552}

CH_4N_2O: urea

CO_2: carbon dioxide

Cu(II): copper oxide

cytL: cytochrome P460

cytS: cytochrome *c*' beta

dam^-/dcm^-: methyltransferase deficient chemically competent *Escherichia coli*

e^-: electron

Fe(II): iron oxide, ferrous iron

FWM: freshwater medium

Gen: Gentamycin

GHG: greenhouse gas

HAO: hydroxylamine dehydrogenase

HKM: Krümmel and Harms medium

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Kan: kanamycin

MAHMA NONOate (6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine, NOC-9

Mn(IV): manganese oxide

MR: Microrespirometry

MSM: mineral salts medium

N: Nitrogen

N-cycle: Nitrogen cycle

N-fixation: Nitrogen fixation

N-oxide/NOx: nitrogen oxide

N_2: Inert nitrogen/dinitrogen

N_2N_4: Hydrazine

N_2O: Nitrous Oxide

NADH: nicotinamide adenine dinucleotide

$NaNO_2$: sodium nitrite

NH_2OH: Hydroxylamine

NH_3/NH_4^+: ammonia/ammonium

NH_4Cl: ammonium chloride

NIR: nitrite reductase

NirK: copper-containing nitrite reductase

NO: nitric oxide

NO_2^-: nitrite

NO_3^-: nitrate

NOH: reactive nitrogen species

NOR: nitric oxide reductase

NorB: cytochrome pool oxidizing nitric oxide reductase

NorY: quinone oxidizing nitric oxide reductase

P460: cytochrome P460

PAPA NONOate: (Z)-1-[N-(3-aminopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate

pcy: plastocyanin

PTIO: 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide

Q/QH_2: quinone/quinol pool

REF: reverse electron flow

RuBisCo: ribulose bisphosphate carboxylase

TEA: terminal electron acceptor

WWTP: wastewater treatment plant

X-Gal: lactose analog/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: Introduction

1.1 The Global Biogeochemical Nitrogen Cycle

Nitrogen is the fourth most abundant element in all life. In the atmosphere it exists in an inert form as N_2 and is made bioavailable solely through microbial activities. The microbial nitrogen cycle (N-cycle) includes many interconnected processes of oxidation and reduction beginning with the conversion of atmospheric nitrogen from its inert form (N_2), through the microbial process of nitrogen fixation, to make bioavailable ammonia (NH_3) (Figure 1.1, 1) (Stein and Klotz, 2016). While nitrogen species are assimilated through NH_3 by all life to form the amino acid building blocks of cells (Figure 1.1, 8), the more oxidized nitrogen intermediates can also be used in a dissimilatory manner as terminal electron acceptors (Stein and Klotz, 2016). Microbial activities of the N-cycle beyond N-fixation include conversion of nitrogen in the reduced form of ammonia (NH_3) to very oxidized forms such as nitrate (NO_3^-) in the aerobic side of the cycle known as nitrification (Figure 1.1, 2 and 4) (Nitrification, 2011), and from reduction of NO_3^- back to either nitrous oxide (N_2O) or N_2 through anaerobic processes of denitrification (Figure 1.1, 5-7) (Zumft, 1997; Stein, 2011a; 2011b; Stein and Klotz, 2016). However, processes within the nitrogen cycle are highly interconnected between the aerobic and anaerobic reactions or by passage of intermediates between microbial metabolisms and also through abiotic conversion stimulated by release of reactive nitrogen species such as nitric oxide (NO) or hydroxylamine (NH_2OH) by candidate nitrogen cycling microorganisms in environments with favorable conditions such as acidic soils or with high reactive metal content (Kampschreur *et al.*, 2011; Zhu-Barker *et al.*, 2015; Jones *et al.*, 2015). Although intermediates of the nitrogen cycle are well known, the full extent of connectivity between microbial physiologies facilitating their conversion are still not fully elucidated. This makes the continued

study into the microbial physiology and enzymology of the nitrogen cycle complex but crucial to our understanding of global cycling of this essential building block of life.

1.1.1 The Anthropocene and Eutrophication

Industrial practices over the last *ca*. 100 years have reshaped the biogeochemical cycles on our planet leading to a new age in geological time, the Anthropocene (Waters *et al.*, 2016). This new epoch is defined by human activities directly changing the way that nutrients cycle and accumulate on earth. In particular, the amount of soil nitrogen has doubled in the past century due largely to fertilizer use (Waters *et al.*, 2016). The global biogeochemical nitrogen cycle is in a very delicate balance of interconverting nitrogen species between microorganisms and the environment making it highly sensitive to the input of nitrogen stemming from anthropogenic practices. An unbalancing in the global nitrogen budget arose mainly through increased inputs of bioavailable nitrogen, in the form of NH_3, through the man-made agriculturally relevant Haber-Bosch process. This process involves industrial formation of NH_3 for use in crop fertilization. This process is much more efficient than the microbial fixation of N_2 to NH_3 that previously existed as the only way of making bioavailable nitrogen. While extensive use of NH_3-based fertilizer to increase crop yields has led to exponential growth in food availability (Kissel, 2014) as well as population growth, it has also created detrimental and lasting consequences for the nitrogen cycle and beyond that the global climate. The vast increase in bioavailable nitrogen as $N\text{-}NH_3$ has led to eutrophication in our global water systems including the oceans now visible as eutrophication (Canfield *et al.*, 2010). The consequences of this include, but are not limited to, the formation of anoxic zones within which the nitrogen cycle is pushed towards dissimilatory anaerobic respirations that can terminate in N_2O production (Canfield *et al.*, 2010; Lam and Kuypers, 2011)

1.1.2 Nitrous Oxide

N_2O is considered the most detrimental of any studied nitrogen intermediate that originates from the microbial N-cycle. This greenhouse gas (GHG) has 300 times more global-warming potential than carbon dioxide (CO_2) (Stein and Yung, 2003) and, unlike CO_2, has only recently started to be included in many of the policy-making GHG budgets put forth by world governments. When the N-cycle is considered in microbial vs. anthropogenic contributions to N_2O, the *ca.* 16 million tonnes of N-N_2O released each year can be divided into 10-12 million tonnes arising from natural sources such as abiotic reactions and microbial metabolism occurring in the global soil and water systems and another 6-8 million tonnes can be attributed to human activities (Reay, 2015). The further increases in anthropogenic practices leading to nitrogen saturation and an unbalancing of the nitrogen cycle only further confounds this as we push the planet, and microbial-mediated N-transformation to the maximum.

N_2O can arise from a number of microbial-mediated and abiotic processes of the nitrogen cycle and is implicated in both nitrification and denitrification (Figure 1.1, 2-6) therefore leaving no side of the N-cycle free from potential for N_2O off-gassing. A microbial N-cycle cohort in which the processes that are aerobic (nitrification) and anaerobic (denitrification) in nature are bridged, through performing reactions of both simultaneously, are the nitrifiers.

1.2 Nitrification and the Microbial Nitrogen Cycle

Nitrogen is required by all life to form amino-acids and therefore all organisms have mechanisms for the assimilation of nitrogen as NH_3/NH_4^+ (Figure 1.1, 8). There exist microorganisms, however, that utilize nitrogen in its various forms as a sole source of energy, reducing equivalents, and nitrogen. This group is known as the chemolithotrophic nitrifiers and

they are capable of facilitating many of the N-species interconversions within the microbial N-cycle focused on in Figure 1.1.

The first step in making N bioavailable to nitrifiers and all other life is N-fixation to NH_3 (Figure 1.1, 1), a process performed by either free-living or symbiotic Bacterial or Archaeal diazotrophs that utilize nitrogenase enzymes for fixation of atmospheric inert N_2 (Dixon and Kahn, 2004). While N-fixation is performed in a wide range of microorganisms with diverse physiological capabilities, including aerobic and anaerobic metabolisms, the nitrogenase enzymes are highly sensitive to O_2 therefore making the specific process of N-fixation a non-aerobic one (Dixon and Kahn, 2004). Once nitrogen is fixed into NH_3 it can be assimilated to form amino-acid building blocks (Figure 1.1, 8) or oxidized to NO_2^-, through the intermediate hydroxylamine (NH_2OH), by aerobic nitrifying chemolithotrophic Gamma- and Betaproteobacteria (Arp *et al.*, 2007; Stein and Klotz, 2016) or Thaumarchaeota (Stahl and la Torre, 2012; Offre *et al.*, 2013; Vajrala *et al.*, 2013) in the first step of nitrification (Figure 1.1, 2). The oxidation of NH_3 in anaerobic environments is performed by the anaerobic ammonia-oxidizers (Anammox) of the phylum Planctomycetes (Figure 1.1, 3) (Kuenen, 2008). The second step of nitrification (Figure 1.1, 4) is performed by aerobic nitrite-oxidizing bacteria (NOB) that exist within many phyla including Proteobacteria and Nitrospirae and live by oxidizing NO_2^- to NO_3^- (Teske *et al.*, 1994; Stein and Klotz, 2016). The very recently identified comammox bacteria perform complete nitrification from NH_3 to NO_3^- (Figure 1.1, 2 and 4) and so far only include members of the phylum Nitrospirae (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Many processes inevitably cycle back to NH_3 and thus the first step of nitrification, either from N-fixation, from the breakdown of organic matter by saprophytes through ammonification, through breakdown of waste urea, or through human deposition of synthetic NH_3-based fertilizers (Reay,

2015). This high concentration of N in the form of NH_3 being constantly available and increasing, mainly due to agricultural practices, with time puts the ammonia-oxidizers at the center of interest for studies on the global impacts of N-loading on the biogeochemical N-cycle. Reviews on the effects of terrestrial N-loading globally all point to exponential N-loss as gaseous nitrogen, in the form of N_2O, over N-uptake by plants or soil retention of nitrogen as we increase deposition of nitrogen fertilizers (Niu *et al.*, 2016). This is worrisome as with increased N-availability and low O_2, conditions often seen in agricultural soils, the proteobacterial ammonia-oxidizers have been shown to increase activities of N-oxide production (Figure 1.1, 5 and 6) through a process known as nitrifier denitrification (Stein, 2011a).

Nitrification, nitrifier denitrification, and other interconversion of reduced forms of nitrogen to more oxidized forms within the microbial nitrogen cycle is also performed by certain heterotrophic microorganisms, methane-oxidizers (methanotrophs), and fungi (Stein, 2011a), however for the purposes and scope of this thesis work the main focus of pathways, N-conversions, and analyses of the N-cycle will be on the chemolithotrophic proteobacterial and thaumarchaeotal nitrifiers.

1.2.1 Relevant Industrial Processes Reliant on Nitrifiers

Microbial ammonia-oxidation is central to many of the industries we thrive on. One such example is the turnover of nitrogen within wastewater treatment plants (WWTP) globally (Okabe *et al.*, 2011). Large quantities of organic matter are sent through these systems and converted to their nitrogenous backbones of NH_3, NO_2^-, or NO_3^- through microbiologically facilitated breakdown. Nitrification is at the centre of this breakdown as a large portion of waste comes in the form of urea (CH_4N_2O) which contains two molecules of NH_3. WWTPs harbor a large population of ammonia-oxidizers involved in conversion of NH_3 to either NO_2^- or N_2 (Figure 1.1,

2 and 3). Under ideal processing conditions the complete turnover of nitrogen occurs when NO_2^- is then oxidized to NO_3^- by nitrite-oxidizing bacteria (Figure 1.1, 4) and then in anaerobic stages of the process is reduced through dissimilatory reduction to N_2 gas and released back to the atmosphere. While ideal conditions are always the hope of such a process there can be partial denitrification by some ammonia-oxidizers in which the NO_2^- is instead reduced in a dissimilatory fashion terminating in production of N_2O. This process, which will be examined in-depth in the next section, is referred to as "nitrifier denitrification" (Stein, 2011a; 2011b).

Agricultural settings are also a focus of the study of nitrification due to the importance of fixed nitrogen as NH_3 for crops. In agricultural soils nitrification can be detrimental because the NH_3 made available for plants might instead be oxidized to NO_2^- or NO_3^- and reduced to N_2O through the aforementioned nitrifier denitrification pathway by ammonia-oxidizers in soils. This competition for fixed nitrogen has led to implementation of a number of agricultural practices to bypass the issues including increased use of ammonia-based fertilizers, made available through the aforementioned Haber-Bosch process, and use of inhibitors on ammonia-oxidizers in soils. What is more concerning still is that although many reactions in nitrification are aerobic utilizing O_2 as a terminal electron acceptor for respiration, dissimilatory reduction of the intermediate NO_2^- as an alternate TEA to produce N_2O is a known reaction within some nitrifying microorganisms (Stein, 2011a; 2011b).

1.3 Chemolithotrophic Ammonia-Oxidation by the Proteobacterial and Thaumarchaeotal Nitrifiers

1.3.1 The AOB: ammonia-oxidizing proteobacteria

Nitrification in aerobic and microaerophilic environments is performed mainly by ammonia-oxidizing Proteobacteria (AOB) or Thaumarchaeota (AOA). Ammonia-oxidizers are involved in N-transformation in most environments, including marine and freshwater, terrestrial, and wastewater systems, through nitrification (Arp *et al.*, 2011; Stahl and la Torre, 2012; Prosser *et al.*, 2014). The majority of studies on ammonia-oxidizer physiology and metabolism have focused on the AOB as the model organism *Nitrosomonas europaea* ATCC 19718 has been in culture for over half a century (Meiklejohn, 1950; Chain *et al.*, 2003), whereas it was only in the past 10 years that the ammonia-oxidizing Thaumarchaeota were discovered (Könneke *et al.*, 2005). The AOB are Gram-negative Proteobacteria found in both the gamma-; harboring the *Nitrosococcus* genus of AOB, and betaproteobacterial class including the *Nitrosomonadaceae* group of AOB that encompasses both the *Nitrosomonas* and *Nitrosospira* genera (Arp *et al.*, 2007; Prosser *et al.*, 2014). Classification of AOB phylogenetically is based on 16S rDNA or *amoA*, the catalytic subunit of the ammonia-monooxygenase (AMO) enzyme, and has largely focused on betaproteobacterial AOB (Kowalchuk *et al.*, 1997; Purkhold *et al.*, 2000; Kowalchuk and Stephen, 2001; Koops *et al.*, 2006; Prosser *et al.*, 2014). Phylogenetic analysis of betaproteobacterial AOB 16S rDNA has led to the categorization of isolates into 11 clusters (7 clusters within the *Nitrosomonas* genus and 4 within the *Nitrosospira* genus) (Koops *et al.*, 2006; Norton, 2011; Prosser *et al.*, 2014), however, prior to this thesis, only 5 pure cultures of betaproteobacterial AOB were available with sequenced, closed, and published genomes able to be utilized in genome-inferred physiological analyses (Table 1.1) (Chain *et al.*, 2003; Stein *et al.*, 2007; Arp *et al.*, 2007; Norton *et al.*, 2008; Suwa *et al.*, 2011; Bollmann *et al.*, 2013). The majority of physiological and genomic analyses have also been performed on betaproteobacterial

AOB, isolated from freshwater or terrestrial environments, and therefore will constitute the majority of discussion and analysis in this thesis.

AOB genomes are relatively small, *ca*. 3-4 Mb, in comparison to other Proteobacteria genomes (Table 1.1) (Arp *et al.*, 2007) as they live a restricted lifestyle being chemolithoautotrophs acquiring all energy and reductant from oxidation of NH_3 and carbon mainly from fixation of carbon dioxide (CO_2) via the Calvin cycle (Arp *et al.*, 2007). However, one study suggested potential for facultative chemolithoorganotrophy by *N. europaea* utilizing either fructose or pyruvate as a sole carbon source (Hommes *et al.*, 2003) and recently the *N. europaea* sucrose synthase was crystalized (Wu *et al.*, 2015) suggesting potential of this AOB to make and use sugars as another source of carbon. How AOB overcome difficulties in gaining enough ATP to fix CO_2 into cellular biomass from NH_3 alone has been partially elucidated through the study of the biochemistry of ammonia-oxidation in the model AOB *N. europaea* ATCC 19718 as discussed in the following section and references therein.

1.3.2 Biochemistry of ammonia-oxidation in AOB

The oxidation of NH_3 to NO_2^- has a change in oxidation state of 6 electrons (e-) (NH_3 oxidation state -3; NO_2^- oxidation state +3) providing very little energy for proton motive force (PMF) generation or formation of cellular biomass. This alone can begin to explain the slow doubling-time commonly observed for AOB of *ca.* 8 hours. This also lends to the explanation of why many AOB retain multiple copies of the genes with products involved in oxidation of NH_3, ammonia monooxygenase (AMO), and extraction of e^- from NH_2OH, hydroxylamine dehydrogenase (HAO) (Table 1.1) (Arp *et al.*, 2007).

The pathway of ammonia-oxidation in AOB, based mostly on biochemistry and physiology of *N. europaea*, including intermediates and proven or proposed e^- flow is shown in Figure 1.2. The oxidation of NH_3 to the intermediate NH_2OH ($NH_3 + 2e^- + O_2 \rightarrow NH_2OH$) is facilitated by the AMO in an energy and O_2 consuming step (Whittaker *et al.*, 2000). NH_2OH is then oxidized to NO_2^- ($NH_2OH + H_2O \rightarrow NO_2^-$) by HAO and $4e^-$ are extracted which then are passed either from HAO to the membrane cytochrome C_{m552} or through the cytochrome pool ($C_{554} \rightarrow C_{m552}$) to the ubiquinone pool where $2e^-$ must past back to AMO to continue the monooxygenation of NH_3 and $2e^-$ pass on to complex III to make PMF (Whittaker *et al.*, 2000; Klotz and Stein, 2008). AOB genome analysis and biochemistry have revealed that cytochromes C_{554} and C_{m552} are present in a highly conserved gene cluster with HAO (Bergmann *et al.*, 2005) and facilitate the transfer of e^- between HAO and either the ubiquinone pool or other e^- acceptors available within the periplasm depending on the redox state of the cell (Whittaker *et al.*, 2000; Klotz and Stein, 2008; Stein, 2011b). It has been proposed that a cytochrome P460, present in all sequenced AOB genomes with the exception of *Nitrosospira multiformis* ATCC 25196 (Table 1.1) (Arp *et al.*, 2007; Norton *et al.*, 2008), can also facilitate NO_2^- production from NH_2OH and NO (Klotz and Stein, 2008; Simon and Klotz, 2013). To make NADH for cellular reductant and biomass the AOB utilize reverse electron flow in which complex III (bc_1) must pass electrons up the redox gradient to NADH dehydrogenase, proposed to reversely act in AOB (Whittaker *et al.*, 2000), to produce NADH from NAD^+. This also acts as an e^--sink along with AMO taking e^- away from the ubiquinone pool and thus away from PMF and ATP generation. The NH_3-oxidation pathway has largely heme-iron based enzymes whereas the AMO and terminal oxidase are copper-based enzymes (Klotz and Stein, 2008). Metal bioavailability throughout Earth's geological record gives some insight into the evolution of the AOB, including that AMO and

terminal oxidases were gained later in the evolution of the current NH_3-oxidation pathway, likely when nitrogen and copper became more bioavailable upon oxidation of the oceans (Klotz and Stein, 2008).

With increasing substrate concentration, as may be observed in a wastewater treatment facility or agricultural soils, the AOB require strategies for avoiding a build up of e^- and therefore a bottleneck affect within the electron transport chain (ETC) (Stein, 2011b). In particular, a slow down in the passage of e^- along the ETC between complexes III and IV (Figure 1.2) can result in slower oxidation of NH_2OH by HAO which is problematic due not only to the toxic and mutagenic side affects of high intracellular NH_2OH concentrations, but also due to the tendency of NH_2OH, a highly reactive intermediate, to break down into a number of other NOx intermediates including N_2O (Hooper and Terry, 1979; Cantera and Stein, 2007b; Pacheco *et al.*, 2011). To retain forward e^- transport along the ETC the AOB can funnel reductant towards alternative terminal electron acceptors (TEA's) such as NO_2^- or NO using nitrite or nitric oxide reductases (NIR and NOR) (Figure 1.2) found in most sequenced AOB genomes (Table 1.1) (Arp *et al.*, 2007; Cantera and Stein, 2007a; Garbeva *et al.*, 2007; Stein, 2011b;) in a process known as nitrifier denitrification.

1.3.3. N_2O Production from AOB

The discovery of denitrification inventory in aerobic ammonia-oxidizers changed the way we view the N-cycle as not only do the AOB bridge the aerobic/anaerobic pathways of N-transformation (Figure 1.1) but also exist as another potential source of N_2O release in many environments (Stein, 2011b). N_2O has been measured in the headspace gas of pure cultures of AOB in both the *Nitrosomonas* and *Nitrosospira* genera (Dundee and Hopkins, 2001; Wrage *et al.*, 2004; Shaw *et al.*, 2006; Cantera and Stein, 2007b). Genes for periplasmic copper-containing

NO_2^- reductase (*nirK*) have so far been found in all sequenced and annotated AOB genomes (Arp *et al.*, 2007; Norton *et al.*, 2008; Suwa *et al.*, 2011; Stein, 2011a; Bollmann *et al.*, 2013), whereas genes for membrane-bound NO-reductases including *norB* and *norY*, with the capacity to take on e^- from either the quinone (NorY) or cytochrome pools (NorB) (Figure 1.2) and reduce NO to N_2O, are found in all available AOB genomes with the exception of *Nitrosomonas* sp. Is79A3 that lacks annotated *norB* or *norY* genes (Bollmann *et al.*, 2013). Importantly, no sequenced AOB have nitrous oxide reductases (NOS) meaning that pathways of NOx production in AOB terminate in release of either NO or N_2O.

While the presence of denitrification inventory and measurement of headspace N_2O in pure cultures of AOB suggested enzymatic pathways for N_2O production, direct testing of this activity has only been done in *N. europaea* leading to the identification of two distinct pathways for N_2O production; the hydroxylamine oxidation pathway, favored under high atmospheric O_2 tensions and the nitrifier denitrification pathway, favored under low O_2 tensions (Figure 1.3) (Cantera and Stein, 2007b; Stein, 2011a; 2011b). To understand contributions of AOB to N_2O in the environment the physiological conditions, enzymes, and pathways of N_2O production need to be elucidated. It was previously suggested that both NirK and NorB could act to alleviate the e^- flow bottle neck in the ETC (Figure 1.2) caused by either high substrate concentration or low O_2 tension by placing more e^- on alternate TEA's such as NO_2^- and NO , however the conditions under which both enzymes are active and where in the N_2O production pathways they might participate in is not fully understood. To tackle this obstacle in our understanding of AOB-mediated N_2O production, studies on mutants of *N. europaea* strains deficient in either NirK or NorB activity were done. Studies with NirK-deficient *N. europaea* showed slower oxidation of NH_3 and build up of NH_2OH under atmospheric O_2 tension (*ca.* 21% O_2) (Schmidt, 2004;

Cantera and Stein, 2007b) suggesting NirK could play a role in efficient aerobic substrate oxidation possibly by HAO passing e^- through the cytochrome pool to NirK (Figure 1.2). Under low O_2 tension (*ca.* <5% O_2) results were misleading though as lack of NirK did not stop N_2O production from NO_2^- reduction through nitrifier denitrification when cultures were grown in batch (Beaumont *et al.*, 2002; Cantera and Stein, 2007b), whereas during chemostat growth NirK-deficient *N. europaea* was unable to reduce NO_2^-. Work on NorB-deficient *N. europaea* suggested an important role in NO reduction in the cell but not under atmospheric O_2 tensions (Beaumont *et al.*, 2004), conditions seen during N_2O production from the hydroxylamine oxidation pathway (Figure 1.2). However, NorY has been implicated in this pathway (Figure 1.2). An essential piece still missing in the study of AOB physiology is elucidation of the role of both NirK and NorB in substrate oxidation, growth, and N_2O production, particularly across O_2 tensions the AOB may see in terrestrial soils or WWTP for instance, starting with the model AOB *N. europaea*, so that pathways and contributions to N_2O production in other AOB isolates can be more accurately predicted.

While it is an important first step to characterize N_2O production in *N. europaea*, with use of a sequenced and closed genome as a guide for nitrogen oxide (N-oxide) metabolic pathways, other AOB isolated from diverse environments, in terms of substrate availability, are also available for genome-inferred studies of N-oxide production. Of particular interest for environmental studies are the contributions AOB can make to the accumulation of N-oxides in the environment. Modelling AOB contributions to NO and N_2O production have been made for terrestrial (Zhu *et al.*, 2013) and WWTP systems, however, use of pure cultures with sequenced and closed genomes, beyond *N. europaea*, have not been attempted. Without available data on measurements of NO or N_2O from more AOB with annotated and closed genomes during

oxidation of native substrates, we cannot confirm whether the presence or absence of certain NOx inventory such as nitrite or nitric oxide reductases are a predictive factor in physiological capability for NOx release. This is troublesome also, because gene markers such as *nirK* and *norB* are often utilized as environmental markers for nitrifier denitrification even though evidence supports NirK having a function in nitrification/substrate oxidation as opposed to denitrification activities (Schmidt, 2004; Cantera and Stein, 2007b) and the fact that AOB have alternate and hypothetical nitric oxide reductases beyond NorB.

1.3.4 The AOA: ammonia-oxidizing Thaumarchaeota

NH_3 is oxidized in aerobic environments by chemolithotrophic ammonia-oxidizing bacteria and archaea as a sole source of energy, reductant, and nitrogen (Figure 1.1). The archaeal ammonia-oxidizers are found in terrestrial, marine, and geothermal environments and fall within the Thaumarchaeota phylum (Offre *et al.*, 2013; Könneke *et al.*, 2014). Ammonia-oxidation by members of the domain Archaea is a relatively recent finding with the first isolate only *ca.* 10 years ago. Work on AOA suggests they are the dominant nitrifiers in marine (Martens-Habbena *et al.*, 2009) and certain terrestrial systems such as acid soils. The AOA have a higher Km for ammonium (NH_4^+) than the AOB (Martens-Habbena *et al.*, 2009), thought to aid in the highly oligotrophic lifestyle of surviving in the open ocean, and are often very sensitive to higher concentrations of NH_4^+ or NO_2^- as well. Like the AOB, the AOA are autotrophic and fix CO_2 to make cellular biomass. Genes for autotrophic CO_2 fixation via the hydroxypropionate/hydroxybutyrate cycle have been identified in all sequenced AOA (Walker *et al.*, 2010; Könneke *et al.*, 2014) and recent work on the most well-studied and first isolated AOA *Nitrosopumilus maritimus* SCM1 show use of a modified cycle utilizing the most energy efficient anabolic conversions possible, further showing adaptations of the AOA to oligotrophy.

Although AOA can live as obligate autotrophs they have enhanced growth with addition of organic carbon such as α-ketoglutarate or pyruvate. Given the difficulties of cultivating and isolating such tenuous oligotrophs there are very few pure cultures of AOA with sequenced and annotated genomes (Walker *et al.*, 2010; Tourna *et al.*, 2011) that are not still considered enrichments or "*Candidatus*" status.

The pathways and enzymology for extracting energy from NH_3 and utilizing it for cellular processes and PMF generation involving oxidation of NH_3 to NO_2^- are well-studied for the bacterial ammonia-oxidizers, however large gaps still exist in our understanding of the pathways in the thaumarchaeotal ammonia-oxidizers (AOA). Thaumarchaeotal ammonia-oxidizers do contain a copper AMO and grow by oxidation of NH_3 to NO_2^- with the same stoichiometry as in AOB (Figure 1.2) (Martens-Habbena *et al.*, 2009). Also, NH_2OH is the proposed intermediate of NH_3 oxidation to NO_2^- (Vajrala *et al.*, 2013) but the enzymology of this reaction is unclear as no enriched or pure culture of AOA has been found with an annotated HAO (Walker *et al.*, 2010; Tourna *et al.*, 2011; Kim *et al.*, 2012; Spang *et al.*, 2012) leaving it unclear how they are extracting electrons from NH_3. A further issue in resolving the AOA pathway for NH_3-oxidation is the fact that genomes of pure or enriched AOA lack annotated bacterial *c*-type cytochromes that make up the active center of bacterial HAO or the e^- transferring cytochrome pool in AOB. This has therefore led to the proposal of a novel copper-based enzymology for the pathway in the thaumarchaeotal ammonia-oxidizers; based on genomic data it has been suggested that copper-based plastocyanins take the place of iron based *c*-type cytochromes (Stahl and la Torre, 2012), but again no physiological data or biochemistry is available yet to support this claim. Vastly less is understood about electron flow and energy conservation in the AOA because obtaining AOA

in pure culture and at high concentration for studies on proteins have proven to be a great challenge in the field.

AMO copy from bacteria and thaumarchaeota is often used as a parameter for judging which group has a greater contribution to N-transformation in different environments, however there are very few physiological studies that directly compare bacterial and thaumarchaeotal ammonia-oxidizers (Stahl and la Torre, 2012; Vajrala *et al.*, 2013; Stieglmeier *et al.*, 2014). Of very high interest since the discovery of the AOA has been their potential to reduce NO_2^- as an alternate TEA and thus produce N_2O through pathways similar to the AOB such as nitrifier denitrification.

1.3.5 N_2O production from AOA

Another copper-based enzyme found in all sequenced AOA isolates is the copper-containing nitrite reductase NirK. This finding led many to question whether the AOA, like the AOB, could contribute to global N_2O production, especially given the finding that AOA are often in 10-fold higher abundance than AOB in many environments (Stahl and la Torre, 2012). Detection of N_2O from enrichment or pure cultures of AOA during active NH_3 oxidation furthered this claim even though annotated NORs were not found in any sequenced AOA genomes (Stahl and la Torre, 2012). Studies on the genome and physiology of *N. maritimus* suggested that NO could play some role in the NH_3-oxidation pathway as either an electron shuttle (reductant) or oxidant (Stahl and la Torre, 2012; Shen *et al.*, 2013; Martens-Habbena *et al.*, 2015) as opposed to being part of the dissimilatory reduction of NO_2^- as an alternate TEA. Work on the isotopic signature of N_2O produced from a pure culture of the terrestrial AOA *Nitrososphaera viennensis* EN76 showed a hybrid signature of the N_2O, suggesting production

resulting from interaction of intermediates in the NH_3-oxidation pathway and not solely from NO_2^- reduction (Stieglmeier *et al.*, 2014).

While there are a handful of pure cultures of AOA available with sequenced and annotated genomes, the ability to perform studies on the biochemistry of the NH_3-oxidation pathway or N_2O production are still far from simple. It is known that AOB seem to largely contribute to nitrification and N_2O production, at least in agricultural soils (Jia and Conrad, 2009; Hink *et al.*, 2016), but there still exists a need for understanding the pathway and intermediates of NH_3-oxidation and N_2O production by AOA to fully understand their role in the global N-cycle and N-oxide budget.

1.3.6 Abiotic N_2O Production from Nitrification

While it is vital to perform genome-directed physiological analyses of nitrifiers to understand how genomic content is linked to production of nitrogen oxides such as NO and N_2O, it is also important to keep in mind how products of nitrification can interact with the environment and lead to abiotic formation of N_2O. It is known that both AOB and AOA produce NO_2^- as a product of NH_3-oxidation, that NH_2OH is the likely intermediate in both NH_3-oxidation pathways, and that NO is an intermediate of either NH_3-oxidation or nitrifier denitrification (Stein, 2011b; Martens-Habbena *et al.*, 2015). There exist a number of abiotic environmental factors that have the thermodynamic potential to interact with these nitrogen oxide intermediates released by ammonia-oxidizers and produce N_2O. Chemo-denitrification of NO_2^- to N_2O in soils is proposed to be facilitated by high concentrations of reduced iron or reduced copper in the form of Fe(II) or Cu(II), increased organics, anoxia, or increased pH and the oxidation of NH_2OH to N_2O can be facilitated by Fe(III), Mn(IV), or increased salinity (Zhu *et al.*, 2013; Jones *et al.*, 2015; Zhu-Barker *et al.*, 2015; Wang *et al.*, 2016). In particular Fe(II)

has been shown, under the right conditions, to contribute to abiotic N_2O production from NO_2^- in hypersaline Antarctic ponds (Samarkin *et al.*, 2010) and WWTP's (Kampschreur *et al.*, 2011; Mampaey *et al.*, 2013) where both NO_2^- and reduced iron can be found in high concentration. The study of abiotic conversions of nitrogen oxides released by nitrifiers and other microorganisms involved in the nitrogen cycle brings about the importance of taking into account the organism, genomic inventory, physiology, and environment when studying potential contributions to nitrogen oxide release.

1.4 Conclusions: Thesis Objectives

The overarching goal of this thesis work is to use a genome-inferred approach towards the study of ammonia-oxidation and nitrogen oxide production in ammonia oxidizers. It is hypothesized that **(1)** genomic inventory and phylogeny are not accurate predictors of contributions of AOB to NOx production and **(2)** the ammonia-oxidizing Bacteria and Thaumarchaeota have different pathways and mechanisms for NH_3-oxidation and N_2O production. To address these issues in the field the below research objectives were followed:

1. Elucidate of the pathways of N_2O production in the model AOB *Nitrosomonas europaea* ATCC 19718:

a. To understanding the role of NirK and NorB in nitrogen oxide production from the model and most well-studied AOB *N. europaea*:

b. To provide a basis for the study of NOx production across other isolated and sequenced AOB

2. Understand how AOB isolated from different environments with sequenced genomes containing differences in annotated NOx inventory contribute to NOx production:

a. To add to the number of available, sequence, and closed terrestrial AOB genomes to allow more thorough comparative analyses

b. To show if AOB phylogeny or genes for N-transformation and NOx production are predictive of NOx metabolism

3. Elucidate the pathway of ammonia oxidation and N_2O production in Thaumarchaeota:

a. To propose the mechanisms by which the Thaumarchaeota oxide ammonia and produce N_2O

b. To model the contributions of Thaumarchaeota to N-transformation and NOx production vs. AOB in the environment

1.5 Tables and Figures

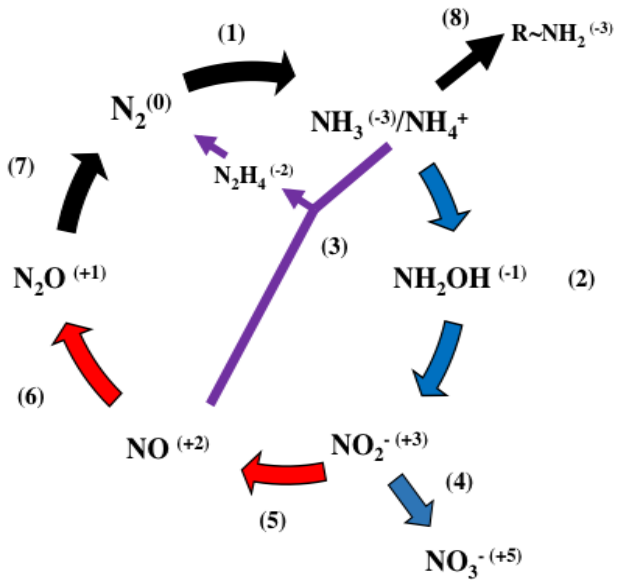

Figure 1.1 Interconversions of N-species facilitated by nitrifiers.

Processes shown are Nitrogen fixation (1), aerobic (2) and anaerobic (3) ammonia oxidation, Nitrite oxidation (4), nitrifier denitrification (5,6), denitrification to N_2 (7), and nitrogen assimilation through ammonia (8). Blue arrows indicate aerobic processes performed by nitrifiers, red and purple arrows indicate anaerobic processes performed by nitrifiers, black arrows indicate other N-transformations directly connected to processes performed by nitrifiers (7,1) or by all life (8). Redox states of N-species are in parentheses. Figure adapted from (Stein and Klotz, 2016).

Table 1.1 Terrestrial and freshwater (non-marine) AOB genomes that are sequenced, have closed genomes, and are available for comparative analysis of annotated inventory for ammonia-oxidation and nitrifier denitrification.

	Strain	Genome size (Mb)	# Predicted proteins :: # with putative function	AMO (*amoCAB*)	HAO (*haoAB*)	NirK	NORs	Cytochrome P460 (CytL)	CytS	RuBisCo type
cluster 7 AOB	*Nitrosomonas europaea* ATCC 19718	2.81	2,630::1,863	2	3	*ncgABC-nirK*	*norCBQD* *norSY-senC-orf1*	Present	Present	Form 1A
cluster 7 AOB	*Nitrosomonas eutropha* C-91	2.78	2,695::2,001	2	3	*ncgABC-nirK*	*norCBQD* *norSY-senC-orf1*	Present	Present	Form 1A
cluster 6 AOB	*Nitrosomonas* sp. Al212	3.18	3,238::2,536	3	3	*nirK*	*norCBQD*	Present	Present	Form 1A/1C
cluster 6 AOB	*Nitrosomonas* sp. Is79A3	3.78	3,597::2,866	3	3	*nirK*	Not present	Present	Present	Form 1A/1C
cluster 3 AOB	*Nitrosospira multiformis* ATCC 25196	3.23	2,885::2,026	2	3	*nirK*	*norCBQD* *norSY-senC-orf1*	Not present	Present	Form 1C

**State of the field previous to current thesis work.* Proteins reported are as follows; AMO: ammonia-monooxygenase, HAO: hydroxylamine dehydrogenase, NirK: copper-containing nitrite reductase, NORs: nitric oxide reductase (NorB, NorY, CytS (cytochrome c' beta)), CytL: cytochrome P460; RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase).

Phylogeny/cluster based on 16S (Norton, 2011). Figure adapted from information and tables found within (Norton, 2011; Prosser *et al*., 2014).

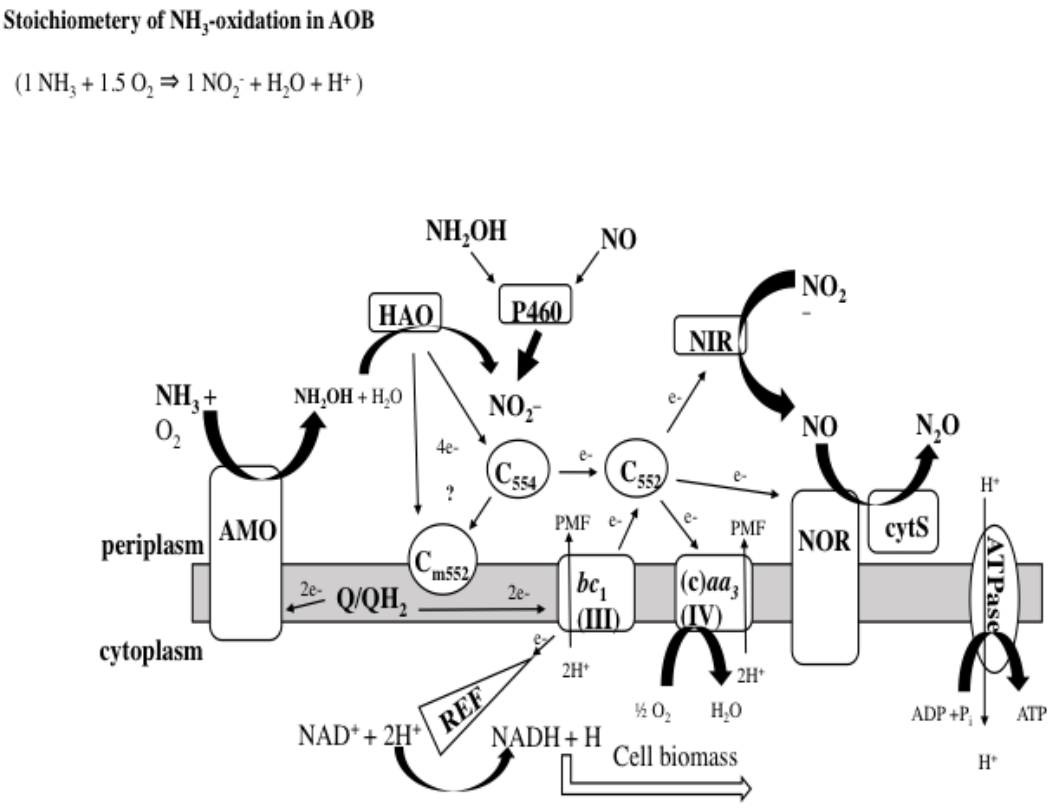

Figure 1.2 Intermediates, enzymology, and passage of electrons during ammonia-oxidation and N_2O production by AOB.

Enzymes not reported previously to this figure include bc1: cytochrome bc1 (complex III); Cm/552: membrane or periplasmic cytochrome C_{552}; C_{554}: cytochrome C_{554}; (c)aa_3: cytochrome (c)aa_3; NIR: nitrite reductases (copper-based or unidentified); PMF: proton motive force; REF: reverse electron flow; Q/QH_2: ubiquinone/the ubiquinone pool. Figure adapted from Whittaker et al., 2000; Klotz and Stein, 2008; Stein, 2011b.

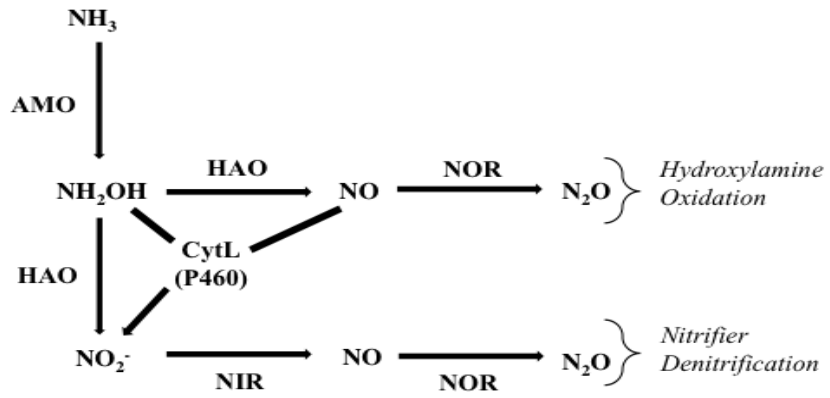

Figure 1.3 Proposed pathways and intermediates of N-oxide (NO and N_2O) production by AOB based on analysis of *Nitrosomonas europaea* ATCC 19718*

*Enzymology reported is as follows; AMO: ammonia-monooxygenase, HAO: hydroxylamine dehydrogenase, cu-P460: copper based P460, NIR (nitrite reductase; NirK or alternative NIR), NOR (nitric oxide reductase; NorB, NorY, cytochrome *c*'beta). Pathways adapted from (Cantera, 2007; Stein, 2011a; 2011b).

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CHAPTER 2: Revision of N_2O-Producing Pathways in the Ammonia-oxidizing bacterium *Nitrosomonas europaea* ATCC 19718

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2.1 Abstract

Nitrite reductase (NirK) and nitric oxide reductase (NorB) have long been thought to play an essential role in nitrous oxide (N_2O) production by ammonia-oxidizing bacteria. However, essential gaps remain in our understanding of how and when NirK and NorB are active and functional, putting into question their precise roles in N_2O production by ammonia-oxidizers.

The growth phenotypes of *Nitrosomonas europaea* ATCC 19718 wild type and mutant strains deficient in expression of NirK, NorB, and both gene products were compared under atmospheric and reduced O_2 tensions. Anoxic resting-cell assays and instantaneous nitrite (NO_2^-) reduction experiments were done to assess the ability of the wild type and mutant *N. europaea* strains to produce N_2O through the nitrifier denitrification pathway. Results confirmed the role of NirK for efficient substrate oxidation and growth of *N. europaea* and showed that NorB is involved in N_2O production during growth at both atmospheric and reduced O_2 tensions. Anoxic resting-cell assays and measurements of instantaneous NO_2^- reduction using hydrazine as an electron donor revealed that an alternate nitrite reductase to NirK is present and active. These experiments also clearly demonstrated that NorB was the sole nitric oxide reductase for nitrifier denitrification. The results of this study expand the enzymology for nitrogen metabolism and N_2O production by *N. europaea* and will be useful to interpret pathways in other ammonia-oxidizers that lack NirK and/or NorB genes.

2.2 Introduction

Ammonia-oxidizing bacteria (AOB) are obligate chemolithotrophs that oxidize ammonia (NH_3), through the intermediate hydroxylamine (NH_2OH), to nitrite (NO_2^-) as their primary energy metabolism. During ammonia oxidation AOB produce gaseous nitrogen oxides including nitrous oxide (N_2O), a greenhouse gas (GHG) with over 300 times the global warming potential of CO_2 (Stein and Yung, 2003), across a wide range of substrate and oxygen concentrations (Poth and Focht, 1985; Dundee and Hopkins, 2001; Shaw *et al.*, 2006). Genes that encode nitrogen oxide reductases, including a periplasmic copper-containing nitrite reductase (*nirK*) and a membrane-bound nitric oxide reductase (*norB*), are present in many closed AOB genome sequences (Arp *et al.*, 2007) including that of *Nitrosomonas europaea* strain ATCC 19718

(Chain *et al.*, 2003) - the model organism for this study. Previous work has denoted two N_2O-producing pathways in *N. europaea*, the pathway of hydroxylamine oxidation and the pathway of nitrifier denitrification. Generally, hydroxylamine oxidation is favored at atmospheric O_2 tension (Cho *et al.*, 2006; Law *et al.*, 2012) and nitrifier denitrification is favored at low O_2 tension (Poth and Focht, 1985; Schmidt *et al.*, 2004; Cantera and Stein, 2007b). Although previous work has been done to describe the roles NirK and NorB may play in electron flow during substrate oxidation and NO_2^- reduction to N_2O (Hooper, 1968; Whittaker *et al.*, 2000) many questions remain about the functionality of these gene products, particularly under reduced O_2 tension where nitrifier denitrification becomes environmentally relevant (Goreau *et al.*, 1980; Stein, 2011). Furthermore, screening by low-stringency Southern blotting and PCR to identify DNA sequences with similarity to *nirK* revealed no hybridization signals from genomic DNA of *Nitrosococcus mobilis* Nc2, *Nitrosomonas cryotolerans* Nm55, or *Nitrosomonas communis* Nm2 (Cantera and Stein, 2007a). In addition, the genome of the recently sequence *Nitrosomonas sp*. Is79 showed no homologues to the *norCBQD* gene cluster (Bollmann *et al.*, 2013). These observations suggest that NirK and NorB are either non-essential to the ammmonia-oxidizer lifestyle or that alternate mechanisms of reducing nitrogen oxides are present in AOB that lack these particular nitrogen oxide reductases.

Previous work on a NirK-deficient strain of *N. europaea* grown at atmospheric O_2 tension showed NirK activity to be important in tolerance of the bacteria to NO_2^- (Beaumont *et al.*, 2002) as well as for their efficient oxidation of NH_3 and NH_2OH (Schmidt *et al.*, 2004; Cantera and Stein, 2007b). Work on a NorB-deficient strain of *N. europaea* suggested that NorB is important for reduction of nitric oxide (NO) but not for net N_2O production under atmospheric O_2 tension (Beaumont *et al.*, 2004). However, previous studies present conflicting evidence regarding

whether NorB is essential for efficient oxidation of NH_3 and NH_2OH (Beaumont *et al.*, 2004; Schmidt *et al.*, 2004). Conflicting results are also present in work on NirK-deficient *N. europaea*, particularly the role of NirK in pathways of N_2O production. When grown in a chemostat, NirK-deficient *N. europaea* cells were unable to reduce NO_2^- as an alternate terminal electron acceptor (Schmidt *et al.*, 2004), versus in batch growth where, at reduced O_2 tension, there was no difference in the ability of NirK-deficient cells to reduce NO_2^- to N_2O when compared to wild type *N. europaea* (Cantera and Stein, 2007b).

The functional roles of NirK and NorB in growth, substrate oxidation, and N_2O production of *N. europaea* across a range of O_2 tensions have not been fully elucidated. Here, we compared the phenotypes of *N. europaea* wild type, NirK-deficient, NorB-deficient, and NirK- plus NorB-deficient strains to solidify our understanding of the enzymology for N_2O production as a function of variable O_2 levels and to determine the necessity of NirK and NorB for growth, substrate oxidation, and NO_2^- reduction to N_2O.

2.3 Materials and Methods

2.3.1 Bacterial Strains

Wild type *Nitrosomonas europaea* ATCC 19718 was used as the native strain for this study. The *nirK*::Kan strain of *N. europaea* was created in a previous study (Beaumont *et al.*, 2002) and was received as a gift from Dr. H.J.E. Beaumont. Confirmation of the *nirK*::Kan strain was done by PCR using primers nir10f: 5'- GGG CGA CAT ACC CAA GAG TG- 3'; nir10r: 5'- CAA GCC TAT GGG GGT TTA TAG- 3'; and nir26r: 5'- GTC ATA GCT GTT TCC TGT GTG AAA TT -3' as described previously (Beaumont *et al.*, 2002).

Creation of *norB*::Gen and *nirK*::Kan *norB*::Gen *N. europaea* strains followed methodology described elsewhere (Sayavedra-Soto and Stein, 2011). Briefly, the *norB*::Gen strain was generated by amplifying the *norB* gene from *N. europaea* ATCC 19718 genomic DNA using primers Ne_2004F: 5'-ACC CAG AAG CTT GCT TAC CC- 3' and Ne_2004R: 5'-TGT TCG GTG ACG ATG ACA CT- 3'. The amplified fragment was purified and ligated into the pGEM-T vector (Promega, Madison WI). The ligation mixture was transformed into *dam*$^-$/ *dcm*$^-$ competent *E. coli* cells (New England BioLabs Inc., Ipswich, MA) and transformants were selected via blue-white screening on LB agar plates containing 0.5 mM IPTG, 80 μg/mL X-Gal, and 100 μg/mL ampicillin. Plasmids from positive recombinants were purified using Wizard® *Plus* SV Minipreps DNA Purification System kit (Promega Corp., Madison, WI) and digested with the *KpnI* restriction enzyme (New England BioLabs Inc., Ipswich MA). The digest was run on a 0.8% agarose gel and linearized vector was gel-purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI). The gentamicin-resistance cassette from the pUGM vector was digested with *KpnI* and gel purified (QIAquick Gel Extraction Kit,QIAGEN, Venlo NL). The purified gentamicin cassette was then ligated into the previously *KpnI*-digested pGEM-T vector to disrupt the *norB* gene at nucleotide position 699/1347. Ligation mixture was transformed into *E. coli* JM109 cells and positive transformants were selected on LB plates containing 100 μg/mL ampicillin and 10 μg/mL gentamycin. Positive recombinants were verified by PCR and Sanger sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City USA). The plasmid with the correct construct was electroporated into prepared *N. europaea* cells (Sayavedra-Soto and Stein, 2011) using an *E. coli* Pulser™ Transformation Apparatus (BioRad Laboratories, Hercules, CA). Electroporated cells were inoculated into mineral salts medium (MSM) (Hyman and Arp, 1992) without

antibiotics and incubated at 28°C without shaking. After 24 h, 5 μg/ml gentamicin was added and cultures were monitored until turbidity was evident and approximately 10 mM NO_2^- was produced. Cell culture (1 ml) was then inoculated onto nitrocellulose membranes overlying agar-solidified mineral medium to select single recombinant colonies as described (Sayavedra-Soto and Stein, 2011). Cultures were PCR screened to confirm the location and orientation of the gentamicin resistance cassette within the *norB* gene that had recombined in the chromosome using primers Ne_2004F (as reported above), GenF: 5' TGC CTC GGG CAT CCA AGC AG -3' and GenR: GAG AGC GCC AAC AAC CGC TTC T-3'. The methods for creation of the *nirK*::Kan *norB*::Gen strain were identical to generation of the *norB*::Gen strain except that the *nirK*::Kan strain of *N. europaea* (Beaumont *et al.*, 2002) was used as the recipient instead of wild-type *N. europaea* and 5 μg/mL Gentamicin plus 30 μg/mL Kanamycin was added to the MSM of electroporated cells after 24 h incubation as described above.

All *N. europaea* strains were grown in 500 mL Erlenmeyer flasks with 250 mL of MSM containing 25 mM $(NH_4)_2SO_4$ (Hyman and Arp, 1992). Cultures were incubated at 30°C in the dark with shaking. Inoculation of fresh medium used 1% volume of culture in stationary phase, which was determined by NO_2^- concentration (Bollmann *et al.*, 2011). Concentrations of NO_2^- were determined using a standard curve from 1 mM-20 mM $NaNO_2$, and stationary phase was achieved at 15 mM NO_2^-.

2.3.2 Growth Experiments

Wild type and mutant *N. europaea* cultures (1 mL) were inoculated into MSM (100 mL) in Wheaton bottles (250 mL) sealed with caps inlaid with butyl rubber stoppers. Cultures were initiated at atmospheric (ca. 22%) or hypoxic (ca. 5%) levels of O_2. Hypoxia was achieved by aseptically sparging the bottles with nitrogen gas and injecting pure O_2 into the headspace. Final

headspace O_2 levels were confirmed by gas-chromatography (GC-TCD; Shimadzu; molecular sieve column, Alltech, DeerWeld IL). O_2 was measured again at the experimental endpoint (72 h) to determine the amount consumed. N_2O was measured in the gas headspace at 24, 48, and 72 h by GC-TCD (Hayesep Q column). Headspace concentrations of O_2 and N_2O in the cultures were determined by comparison to standard curves using pure gases (Sigma-Aldrich). Total cell counts were done at 0, 24, 48, and 72 h using a Petroff-Hauser counting chamber and contrast light microscopy. NO_2^- concentrations were determined at 0, 24, 48, and 72 h by colorimetric assay as described above. NH_2OH concentration was measured during growth between 0 h and 72 h in increments of 6 h using a colorimetric assay (Frear and Burrell, 1955). Statistical differences between measured values among the *N. europaea* strains and experimental conditions were evaluated using Student's *t* test at $P < 0.05$.

2.3.3 Resting-cell assays

The wild type and mutant strains of *N. europaea* were grown to stationary phase as described above. For each experiment, culture (1 mL) was transferred to a 12 mL vial sealed with a rubber stopper and aluminum crimp seal. The vial was sparged with nitrogen gas to anoxia. Electron donor (ascorbic acid; 1mM), and electron shuttle (phenazine methosulfate; 0.1mM) were added to the culture via Hamilton syringe. The vial was left to sit at 30°C in the dark for 72 h to allow adequate time for reduction of NO_2^- and accumulation of N_2O. Headspace N_2O concentration was measured at 0 and 72 h as described above. To confirm consistent anoxia, O_2 was measured at 0 and 72 h as described above.

2.3.4 MR measurements

In preparation for instantaneous O_2 consumption and NO_2^- reduction experiments, the wild type and mutant strains of *N. europaea* were grown in 250 mL Wheaton bottles in 100 mL MSM to stationary phase. Cells were harvested by filtration on Supor®200 0.2μm filters (Pall, Ann Arbor WI) and rinsed three times with sodium phosphate buffer (50 mM NaH_2PO_4, 2 mM $MgCl_2$; pH 8) to wash away remaining NO_2^- produced during growth. Ca. $5x10^{10}$ total cells were re-suspended into 10 mL sodium phosphate buffer in a 10 mL two-port microrespiratory (MR) chamber with fitted injection lids (Unisense, Aarhus DK). O_2 concentration was measuring using an OX-MR 500 μm tip diameter MR oxygen electrode (Unisense, Aarhus DK) and N_2O concentration was measuring using an N_2O-500 N_2O minisensor electrode with 500 μm tip diameter (Unisense, Aarhus DK). Hydrazine (N_2H_4) was added to the chamber as an electron donor for NO_2^- reduction at the beginning of each experiment at a concentration of 250 μM and again at a concentration of 125 μM after the cells had consumed more than half of the available O_2. Once the cells had consumed all available O_2 they were left to sit for 5-10 min under anoxia. Absence of N_2O production confirmed that no endogenous NO_2^- was present, after which 2 mM $NaNO_2$ was added to the chamber through the injection port. Instantaneous NO_2^- reduction to N_2O was measured for approximately 10 minutes.

2.4 Results

2.4.1 Growth phenotype of *N. europaea* strains

N. europaea wild type and mutant strains were grown under atmospheric (ca. 22%) and reduced (ca. 5%) O_2 tensions to evaluate and compare the phenotypes of strains deficient in NirK, NorB, or both gene products. Growth experiments beginning at atmospheric O_2 tension revealed that loss of NirK expression had a significant effect on doubling time with the double mutant (*nirK*::Kan *norB*::Gen) strain having a significantly slower doubling time with respect to

the other three strains, and the *nirK*::Kan strain having a significantly slower doubling time relative to the wild type (Figure 2.1; Table 2.1). The total amounts of NO_2^- produced by both the *nirK*::Kan and double mutant strain were significantly less than that of the wild type. In contrast, the *norB*::Gen strain showed no significant difference in doubling time or NO_2^- production relative to the wild type under atmospheric O_2 tension (Figure 2.1; Table 2.1)

Growth experiments beginning at reduced O_2 tension revealed no significant differences in doubling time (Table 2.1) or the period of logarithmic growth among the four strains (Figure 2.1, Table 2.1). The double mutant showed a significant accumulation of NO_2^- in comparison to all the other strains (Table 2.1). The *norB*::Gen strain showed no differences compared to the wild type (Table 2.1). Although NH_2OH was assayed from all of the cultures under all O_2 tensions, the assay was unable to detect significant differences over time or between strains (data not shown). Together, the results suggest that regardless of initial O_2 levels, NorB played no significant role in the growth phenotype of *N. europaea*; however, NirK was essential for efficient growth and nitrite production especially during growth initiated at atmospheric O_2 tension.

2.4.2 Effects of NirK and NorB absence on N_2O production by *N. europaea* under variable O_2 tensions

To evaluate the roles of NirK and NorB in the pathways of N_2O production for *N. europaea*, N_2O concentration in the gas headspace was measured during growth experiments initiated at both atmospheric and reduced O_2 tension and in resting cell assays in the absence of O_2. In confirmation of prior studies, N_2O in the headspace of the *nirK*::Kan strain at the endpoint (72 h) of growth in cultures initiated with atmospheric O_2 was approximately 15 times that of the wild type strain (Figure 2.2) (Beaumont *et al.*, 2002; Cantera and Stein, 2007b). However, the

resulting N_2O measured after hypoxic growth and in the anoxic resting cell assay revealed no difference between wild type and *nirK*::Kan strains of *N. europaea.* In contrast, the *norB*::Gen strain produced ca. 20% less N_2O in comparison to wild type following growth under atmospheric O_2, and approximately 70% less N_2O following growth under reduced O_2 (Figure 2.2). No N_2O was detected in the anoxic resting-cell assay of *norB*::Gen cells. The double mutant produced a similar amount of N_2O to the *norB*::Gen strain when grown under atmospheric O_2, but was unable to produce measurable N_2O after 72 h when grown under hypoxia or in the anoxic resting-cell assay.

2.4.3 Instantaneous O_2 consumption and N_2O production by wild-type and mutant *N. europaea* strains

The anoxic resting-cell assays revealed that *N. europaea* lacking NorB expression did not produce a measureable quantity of N_2O in the gas headspace from the reduction of available NO_2^- (15mM) in the medium. Therefore, instantaneous NO_2^- reduction experiments were conducted to confirm whether NorB is essential to NO_2^- reduction to N_2O by *N. europaea* and whether an alternative nitrite reductase to NirK was operating in the *nirK*::Kan strain to produce the same amount of N_2O as observed in the wild type (Figure 2.2).

Instantaneous NO_2^- reduction experiments were conducted in microelectrode chambers with the use of a non-nitrite forming intercellular electron donor, N_2H_4, and microelectrodes for O_2 and N_2O. The cells were allowed to consume all of the O_2 in the microelectrode chamber via oxidation of N_2H_4, after which $NaNO_2^-$ was added. Both the wild type and the *nirK*::Kan strains produced approximately 8 μmol N_2O per L per min, confirming the presence of an alternate nitrite reductase activity in *nirK*::Kan cells (Figure 2.3, A and B). Both the *norB*::Gen and double mutant strains showed only background levels of N_2O production from electrode drift upon the

addition of $NaNO_2$, confirming that activity of NorB is essential to this process (Figure 2.3, C and D).

2.5 Discussion

2.5.1 Function of NirK and NorB in efficient substrate oxidation and growth of *N. europaea* under variable O_2 tensions

The slowed doubling times and reduced production of NO_2^- by the *nirK*::Kan and double mutant strains of *N. europaea* when grown at atmospheric O_2 tension confirm previous reports of the requirement of NirK for efficient oxidation of NH_3 to NO_2^- during batch (Beaumont *et al.*, 2002; Cantera and Stein, 2007b) and chemostat cultivation (Schmidt *et al.*, 2004). Slowed substrate oxidation, and therefore diminished growth rate under atmospheric O_2 tension in the NirK-deficient strain of *N. europaea* was previously suggested to be caused by interruption of electron flow from NH_2OH to NO_2^- due to the inability of HAO to pass electrons on to NirK through cytochrome *c* electron carriers (Cantera and Stein, 2007b). A diminished ability of NirK-deficient *N. europaea* to oxidize exogenous NH_2OH during growth strengthens the hypothesis that NirK functions aerobically to facilitate efficient substrate oxidation (Cantera and Stein, 2007b). Furthermore, extensive accumulation of N_2O in the gas headspace of *nirK*::Kan cultures during growth under atmospheric O_2 tension validates previous measurements from growth of this strain in batch (Beaumont *et al.*, 2002; Cantera and Stein, 2007b) and chemostat (Schmidt *et al.*, 2004) cultures. One explanation for this result is that under conditions of high substrate throughput, NH_2OH accumulation inside *nirK*:Kan cells is chemically transformed into an unstable nitrosyl radical (NOH) that rapidly degrades to N_2O (Law *et al.*, 2012); however, our NH_2OH assay was not able to directly measure NH_2OH accumulation in this strain. Another possibility is that in the presence of high NH_2OH concentrations the HAO enzyme produces NO

due to incomplete oxidation of NH_2OH, which is then enzymatically reduced to N_2O (Stein, 2011). Our data suggest that both chemical transformation of NOH and enzymatic reduction of NO via NorB occur to produce N_2O from NH_2OH. During growth under atmospheric O_2 tension, the 20% reduction in N_2O produced by the *norB*::Gen strain could be accounted for by the lack of NorB activity, with the remaining N_2O being produced from NOH degradation (Figure 2.2). In the double mutant strain, growth and net NO_2^- production was likely slowed mostly by the lack of NirK in speeding substrate oxidation but also from the lack of NorB in preventing toxic accumulation of NO. Future work comparing NO accumulation between the wild type and mutant strains of *N. europaea* under variable O_2 tension would assist in validating this hypothesis.

Unlike the two strains deficient in NirK expression, the growth rate of *N. europaea* lacking NorB expression alone was not significantly impaired, which is in agreement with data from a previous study (Beaumont *et al.*, 2004)*;* however, contrary to this same study, N_2O production by *norB*::Gen cells was significantly less than that of the wild type (Figure 2.2). Schmidt *et al.* (2004) showed that NorB-deficient *N. europaea* had a significantly slower growth rate and yield in comparison to wild type, a similar N_2O production profile to the NirK-deficient strain, and significantly larger amounts of NH_2OH released to the growth medium relative to the wild type, suggesting similar inefficiency of substrate oxidation by both NirK- and NorB-deficient strains. Under the growth conditions of the present study, however, the results by Schmidt *et al*. *(*2004) were not validated. Rather, our results suggest that the absence of NorB expression alone in *N. europaea* had no effect on growth or substrate oxidation rates or on NH_2OH accumulation, but did result in diminished N_2O production in comparison to the wild type.

2.5.2 NorB, but not NirK, is required for anoxic reduction of NO_2^- to N_2O in *N. europaea*

The inability of *N. europaea* strains lacking NorB expression to make measurable N_2O in anoxic resting cells assays (Figure 2.2) and instantaneous NO_2^- reduction assays (Figure 2.3) pointed to NorB as the essential nitric oxide reductase involved in NO reduction in the absence of O_2. The significant accumulation of NO_2^- only during hypoxic growth of the double mutant strain (Table 2.1) could be explained by chemical decay of highly reactive NO that may accumulate from activity of the alternative nitrite reductase working in the absence of both NirK and NorB enzymes. However, the lack of similar results in the *norB*::Gen strain suggests that the activity of NirK in the absence of NorB has an effect on nitrogen oxide metabolism that is substantially different from the alternative NO_2^--reductase. Thus, exploration of alternative nitrogen oxide reductases active in *N. europaea* with and without expression of NirK and/or NorB will be helpful to elucidate the enzymology behind these phenotypes.

2.5.3 Amended pathways of N_2O production in *N. europaea* and other AOB

The most important finding of this study is the demonstration that NirK is not essential to the nitrifier denitrification pathway of *N. europaea* as has been assumed for many years in the literature. The similar headspace N_2O levels produced by both wild type and *nirK*::Kan strains of *N. europaea* during growth under reduced O_2 tension, in anoxic resting cell assays, and in anoxic instantaneous nitrite reduction experiments all revealed that an alternate nitrite reductase to NirK is active in the production of N_2O by *N. europaea* ATCC 19178 (Figure 2.4). It was previously suggested that an alternate nitrite reductase may be active in *N. europaea* (Cantera and Stein, 2007b); however, no other known homologues to nitrite reductase genes have been identified in its closed genome (Chain *et al.*, 2003). One possible candidate for an alternate nitrite reductase in AOB is C-terminal truncated HAO (HaoA') (Poret-Peterson *et al.*, 2008). Evolutionary

reconstructions showed that HAO evolved from an octaheme cytochrome *c* nitrite reductase (Klotz and Stein, 2008) and gene expression of HaoA' in the methanotrophic strain, *Methylococcus capsulatus* strain Bath, was induced in the presence of ammonia (Poret-Peterson *et al.*, 2008). *M. capsulatus* strain Bath can reduce NO_2^- to N_2O in the presence of NH_3 and NO_2^- (Campbell *et al.*, 2011) even though homologues to either *nirK* or *nirS* NO-forming nitrite reductases are absent from the closed genome sequence (Ward *et al.*, 2004). Although *nirK* genes have been found in the genomes of most AOB (Cantera and Stein, 2007a) and ammonia oxidizing archaea (Bartossek *et al.*, 2010; Hatzenpichler, 2012) and *nirK* has long been used as a marker for denitrification activity in the field of microbial ecology, the present study shows that at least in *Nitrosomonas europaea* ATCC 19718, *nirK* is not a marker for denitrification, but rather should be considered a marker for ammonia oxidation.

In addition to an alternate nitrite reductase, our results also demonstrate that NorB activity plays a role in the hydroxylamine oxidation pathway of N_2O production by *N. europaea* ATCC 19718 (Figure 2.4). While it is possible that chemical degradation of NOH could have accounted for the N_2O produced by the *norB*::Gen and double mutant *N. europaea* strains grown under atmospheric O_2 tension, a complete transcriptome of the *nirK*::Kan strain showed increased expression of genes for *norSY* (originally annotated as *coxAB*$_2$), an alternative nitric oxide reductase, in comparison to the wild type strain (Cho *et al.*, 2006). Also, *Nitrosomonas eutropha* C91 grown under continuous cultivation for three months in the presence of nitrogen dioxide (NO_2) gas showed increased expression of NorY protein (Kartal *et al.*, 2012). These observations suggest that NorY nitric oxide reductase could perhaps also contribute to N_2O production by the *norB*::Gen and the double mutant strains of *N. europaea* grown at atmospheric O_2 tension (Figure 2.2); however, this hypothesis remains to be validated.

Our results showing the inability of *norB*::Gen and the double mutant strains of *N. europaea* to reduce NO_2^- to N_2O in instantaneous NO_2^- reduction experiments (Figure 2.3, C and D), even with a readily available source of electrons, demonstrates that NorB is the sole enzyme involved in N_2O production through the nitrifier denitrification pathway (Figure 2.4). While these results are in agreement with those of Schmidt et al. (Schmidt *et al*, 2004; Cantera and Stein, 2007b), discrepancy remains regarding the role of NirK in this pathway.

2.6 Conclusions

This study is unique in its comparison of phenotypes of *N. europaea* lacking expression of NirK, NorB, and both enzymes together. Furthermore, our assays allowed comparison of phenotypes under O_2 initially present at atmospheric, hypoxic, and anoxic levels, each of which has a different effect on N_2O production by the two characterized pathways in *N. europaea* ATCC 19718 (Poth and Focht, 1985; Dundee and Hopkins, 2001; Stein and Yung, 2003; Shaw *et al.*, 2006; Stein, 2011). The main conclusions from this study are that: (i) NirK, but not NorB, plays an essential role in efficient substrate oxidation and growth under atmospheric O_2 tension; (ii) an alternate nitrite reductase to NirK is active in *N. europaea* under both hypoxic and anoxic conditions; (iii) NorB, along with chemical decay of NOH and/or other NOR enzymes, is active in *N. europaea* during growth under atmospheric O_2 tension; and (iv) NorB is the only nitric oxide reductase active in the nitrifier denitrification pathway. These results suggest that AOB have diverse enzymology beyond NirK and NorB leading to N_2O production that remains to be characterized.

2.7 Tables and Figures

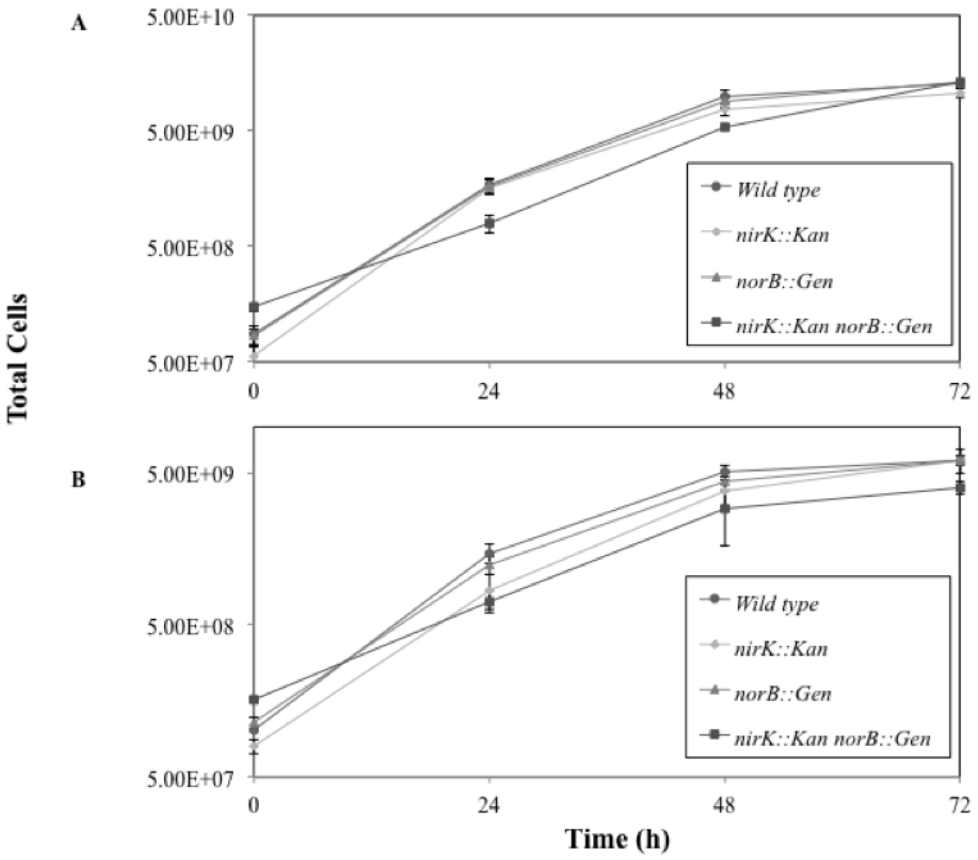

Figure 2. 1 Growth curves for *N. europaea* strains initiated at 22% O_2 (A) and 4.6% O_2 (B). Data represent mean values ± SEs (*ca.* 22% O_2, n=8, ca. 4.6% O_2, *n*=6).

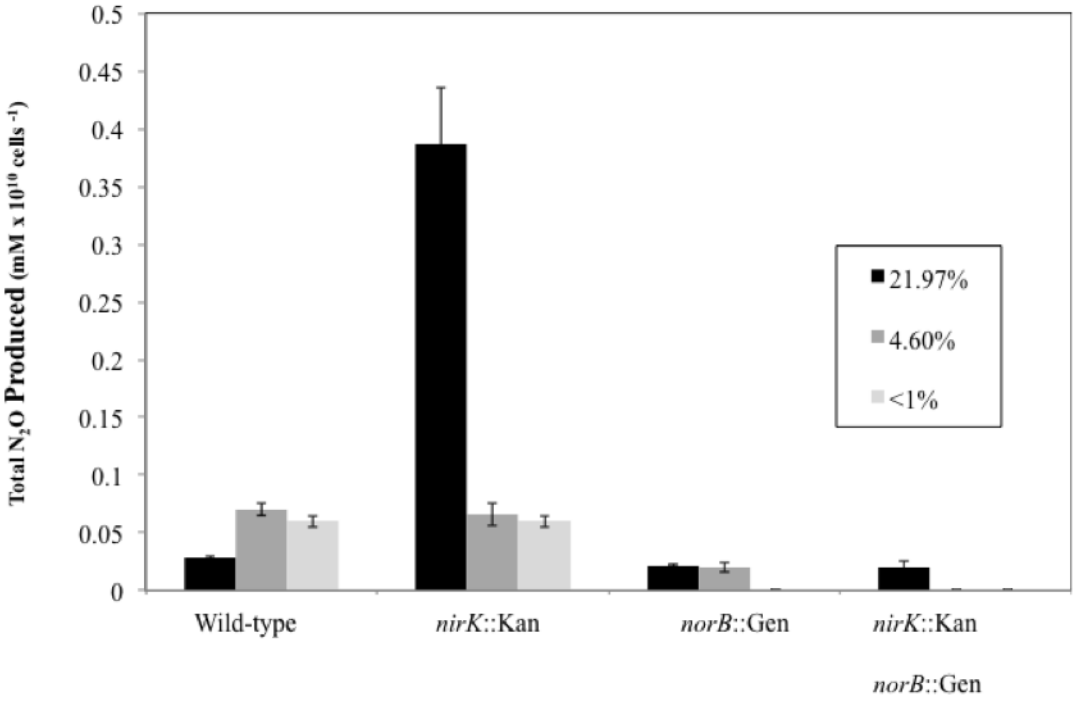

Figure 2. 2 Total N_2O produced by all strains after 72 h of growth at high (*n*=8) and low(*n*=6) oxygen. N_2O profiles under anoxia (*n*=8) were collected during resting cell-assays. Data are presented as means ± SEs.

Table 2. 1 Double time, total nitrite production, and percent remaining headspace O_2 for wild-type and mutant strains of *N. europaea* ATCC 19718 cultivated under atmospheric and reduced O_2 tensions*

	Value at indicated oxygen tension					
	Doubling time (h)		Total NO_2^--N produced ($mM \times 10^{10}$ $cells^{-1}$)		Remaining O_2 in headspace (%)	
Organism description	22%	4.6%	22%	4.6%	22%	4.6%
Wild type	6.5 d (0.6)	8.7 d (0.3)	7.3 bd (0.02)	4.2 d (0.4)	6.7 d (9.2e−5)	1.0 (6.7e−4)
nirK::Kan	6.7 d (0.2)	9.3 d (0.8)	5.8 a (0.01)	4.1 d (0.8)	6.8 d (1.8e−3)	1.2 d (2.4e−3)
norB::Gen	7.1 d (0.3)	9.2 d (0.2)	6.6 d (0.02)	4.3 d (0.4)	6.7 d (1.7e−3)	1.2 (2.4e−3)
nirK::Kan *norB*::Gen	9.6 abc (0.4)	14.3 abc (1.9)	5.6 ac (0.04)	8.0 abc (1.1)	8.0 abc (1.8e−3)	0.8 b (1.1e−4)

*Doubling times were calculated over the 0- to 48 h period of exponential growth. Total NO_2^- produced and remaining O_2 in the headspace were determined at 72 h for all cultures. Averages

and SEs (in parentheses) were calculated from 8 and 6 replicated experiments for cultures grown under 22 and 4.6% O_2, respectively. Significant differences ($P < 0.05$) are denoted by different letters as follows: "a," strain vs wild-type; "b," strain vs. *nirK*::Kan strain; "c," strain vs. *norB*::Gen strain; "d," strain vs *nirK*::Kan *norB*::Gen strain.

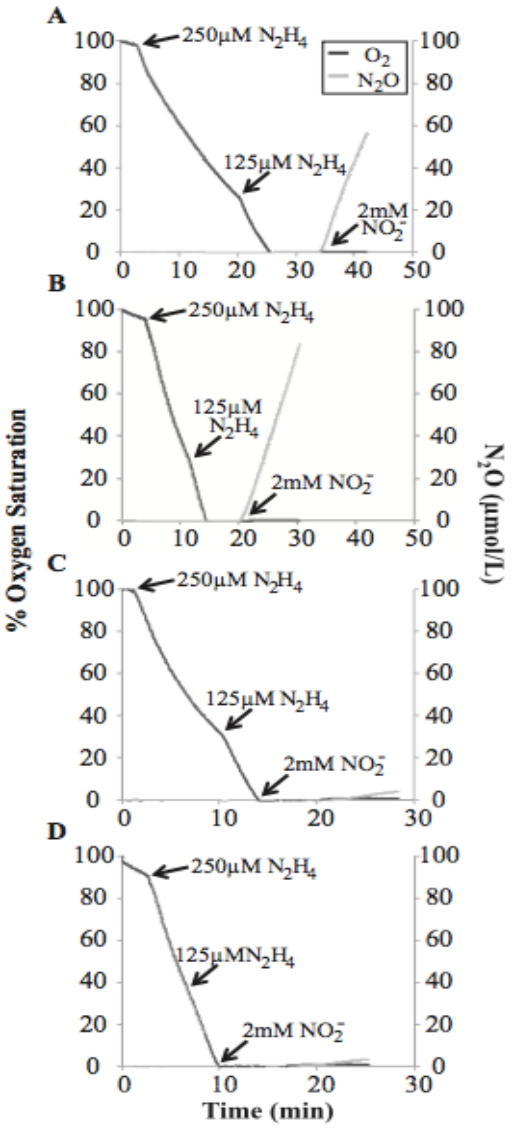

Figure 2. 3 Instantaneous oxygen consumption and nitrite reduction by wild-type (A), *nirK*::Kan (B), *norB*::Gen (C), and *nirK*::Kan *norB*::Gen (D) strains of *N. europaea*. Data are single representatives of reproducible results.

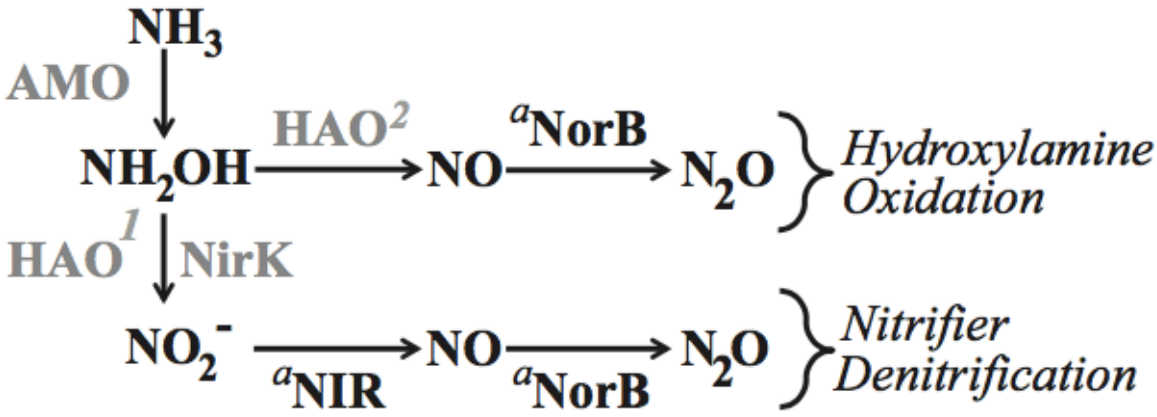

Figure 2. 4 Amended pathways of N_2O production by *Nitrosomonas europaea* ATCC 19718. AMO, ammonia monooxygenase; HAO, hydroxylamine dehydrogenase; NirK, nitrite reductase; NorB, nitric oxide reductase; NIR, unidentified alternate nitrite reductase. The role of enzymes in grey were characterized in previous studies as follows: AMO (Hyman and Wood, 1983), HAO^1 (Hooper *et al.*, 1978), HAO^2 (Pachecho *et al.*, 2011), NirK (Cantera and Stein, 2007). [a](Current Study).

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CHAPTER 3: Genome-wide Phylogeny and Inventory Are Not Predictive of NOx Metabolism in Ammonia-Oxidizing Bacteria

3.1 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 (NH_3) to nitrite (NO_2^-). 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 the global nitrous oxide (N_2O) budget through enzymatic (Stein, 2011; Kozlowski *et al*., 2014) and abiotic processes (Jones *et al.*, 2015; Zhu-Barker *et al.*, 2015). It is known that AOB have the potential to utilize NO_2^- as an alternate terminal electron acceptor (TEA) through the process of nitrifier denitrification (Stein, 2011) resulting in production of the potent greenhouse gas (GHG) nitrous oxide (N_2O) (Stein and Yung, 2003; Kool *et al.*, 2011; Zhu *et al.*, 2013) which 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 production of N_2O from AOB, specifically enzymology and intermediates of the process, have mostly focused on the model AOB *Nitrosomonas 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 share equivalent pathways and regulatory mechanisms.

Bacterial denitrification pathways generally include a nitrite reductase (NIR) to reduce NO_2^- to nitric oxide (NO) and then a nitric oxide reductase (NOR) to reduce NO to N_2O. All closed AOB genomes, with the exception of the newly sequenced and closed *Nitrosomonas communis* Nm2 (Kozlowski *et al.*, 2016b), have annotated genes for copper-containing *nirK* (Prosser *et al.*, 2014) and all have annotated NORs (*norB* and/or *norY*) with the exception of

Nitrosomonas sp. Is79A3 (Bollmann *et al.*, 2013) and *Nitrosomonas ureae* Nm10 (Kozlowski *et al.*, 2016a). Previous studies on *Nitrosomonas europaea* N_2O production pathways showed that both hydroxylamine (NH_2OH) oxidation and NO_2^- reduction can lead to significant emission of N_2O (Cantera and Stein, 2007; Kozlowski *et al.*, 2014). This work also revealed that NorB, but not NirK, is required for enzymatic NO_2^- reduction to N_2O through the nitrifier denitrification pathway (Kozlowski *et al.*, 2014). It was previously thought that AOB required both *nirK* and *norB* genes to produce N_2O, however with the aforementioned findings in *N. europaea* and the fact that there now exists closed AOB genomes that do not encode NIRs or NORs, it is unclear whether unknown genomic inventory exists that contributes to NO and N_2O production or even if all AOB can perform nitrifier denitrification. It was also recently discovered that *Nitrosospira multiformis* ATCC 25196 releases large quantities of NO during active NH_3-oxidation (Kozlowski *et al.*, 2016c) highlighting another yet unstudied aspect of nitrogen oxide metabolism in AOB.

The main objectives of the present study were to: 1) compare NO and N_2O production by several genome-sequenced AOB strains representing different phylogenetic clusters during NH_3 and NH_2OH oxidation, and 2) determine whether gene content and/or phylogeny were predictive of nitrogen oxide (NOx) metabolism by AOB.

3.2 Materials and Methods

3.2.1 Strains and Cultivation

AOB strains used in the present study include *Nitrosomonas europaea* ATCC 19718^T, *Nitrosomonas communis* strain Nm2, *Nitrosomonas* sp. Is79A3, *Nitrosomonas ureae* strain Nm10, and *Nitrosospira multiformis* ATCC 25196^T. Strains were chosen based on their

phylotype, availability of sequenced, annotated, and closed genomes, and their ability to grow under similar lab conditions. Also in the present study, an AOB strain is present from each cluster with cultured representatives; Cluster 3, 6, 7, and 8 (based on 16S; Norton, 2011) with the exception of the newly cultured and sequenced cluster 0 *Nitrosospira lacus* sp. nov. as the genome is not yet closed (Garcia *et al.*, 2013; Urakawa *et al*., 2014). All ammonia-oxidizers 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 $(NH_4)_2SO_4$ (*N. europaea*, *N. communis*, and *N. multiformis*) or 2.5 mM $(NH_4)_2SO_4$ (*N.* sp. Is79A3 and *N. ureae*). All cultures were transferred (5% v/v inoculum) when ca. 80% of the NH_3 substrate was consumed as determined by NO_2^- concentration (Bollmann *et al.*, 2011). The pH of all cultures was adjusted as needed with 10% $NaHCO_3$.

3.2.2 Phylogenetic and Genome Analysis of Published AOB

PhyloPhlAn (Segata *et al.*, 2013) was used to analyze phylogeny of AOB in the present study. Genomes of 14 ammonia-oxidizing bacteria were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/). All of the predicted protein coding sequences for each genome were exported and PhyloPhlAn (Segata *et al.*, 2013) was used 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.

3.2.3 Microrespirometry Experiments

Instantaneous microrespirometry (MR) experiments of AOB are 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_2^-), filtered on Supor®200 0.2 μm filters (Pall, Ann Arbor WI), and rinsed three times with substrate-free HK media (Krümmel and Harms, 1982). Ca. $1x10^{10}$ total cells were used per experiment across all strains. Cells were re-suspended for instantaneous MR experimentation in substrate-free HK medium and provided either 2 mM NH_4Cl as substrate or pulses of 250 μM or 100 μM NH_2OH-HCl (final chamber concentration; 99.999% purity, Sigma-Aldrich, St Louis, MO, USA). Previous experiments showing the maximum NH_2OH concentrations tolerated per organism revealed all strains could tolerate up to 250 μM NH_2OH (final chamber concentration) with the exception of *N. communis* which was unable to tolerate more than 100 μM NH_2OH (final chamber concentration) per injection (data not shown). Chamber O_2 was determined by an O_2 electrode (OX-MR 500 μm tip diameter MR oxygen electrode; Unisense, Aarhus DK), N_2O concentration was measured using an N_2O-500 N_2O minisensor electrode with 500 μm tip diameter (Unisense, Aarhus DK), and NO was measured using an ami-600 NO sensor with 600 μm tip diameter (Innovative Instruments Inc., Tampa FL). The availability of O_2 in the MR chamber, a closed system, was ca. 243 μM O_2 based on equilibrium O_2 concentration at operating temperatures and medium salinities for experiments performed without sparged medium.

3.2.4 Chemical Controls

Chemical controls were performed to determine the reactivity of NH_2OH with media + NO_2^- or heat-killed cells ($1x10^{10}$ total cells) with media + NH_2OH to form N_2O. Chemical

controls used N_2 sparged medium (to achieve 0-3% O_2 saturation in liquid phase) containing 250 μM $NaNO_2^-$ and then adding 250 μM NH_2OH (final chamber concentration) to reflect conditions in the chamber when AOB are utilizing NO_2^- as an alternate terminal electron acceptor with NH_2OH as electron donor. Heat-killed cell controls involved addition of 250 μM NH_2OH to the MR-chamber containing N_2-sparged media with $1x10^{10}$ total heat-killed cells of each AOB strain. N_2O was measured as described above.

3.3 Results and Discussion

3.3.1 Phylogeny and comparative gene inventory of AOB

A whole-genome analysis utilizing PhyloPhlAn showed that each of the 5 AOB chosen for physiological analysis in the present study separated into 5 clades (Figure 3.1). 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 highlights a need to reevaluate and perhaps reclassify 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 3.1). 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-monooxygenase (Table 3.1). All strains encoded at least one monocistronic copy of the *amoC* gene with the exception of *N. communis* (Table 3.1), a feature shared in common with the gammaproteobacerial 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). 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 HAO cluster (Table 3.1). Knockouts of one or two *haoA* gene copies from *N. europaea* did not result in a significant phenotype (Hommes *et al*., 2002). However, 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 highly oligotrophic environments to gain maximum reductant from available substrate. As with *N. europaea* and *N. eutropha*, one copy of the HAO gene cluster in *N. communis* lacks *cycB* (Table 3.1), encoding cytochrome C_m552 (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 3.1) proposed to be involved in the NH_3-oxidation pathway as a redox sensitive electron carrier (Arciero *et al.*, 2002; Arp *et al.*, 2007).

Analysis of NIR and NOR genes revealed that *N. communis* is the only sequenced and closed AOB genome without an annotated nitrite reductase (Kozlowski *et al*, 2016b) (Table 3.1). 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) . Only *N. europaea* contains the operonic *nirK* and NO-responsive *nsrR* transcriptional regulator (Chain *et al*., 2003; Table 3.1), features shared by the closely related *N. eutropha* C-91 strain (Stein *et al.*, 2007) (Figure 3.1). All the *Nitrosomonas strains*, but not *Nitrosospira multiformis*, encode the NO-responsive NnrS

transcriptional regulator. Two strains, *Nitrosomonas* sp. Is79A3 and *N. ureae*, within the Group 6 AOB (based on 16S rRNA; Norton, 2011), lack annotated operons for cytochrome *c* nitric oxide reductases (Bollmann *et al.*, 2013; Kozlowski *et al*, 2016a) (Table 3.1). The genome of the closely related *Nitrosomonas* sp. AL212 (Figure 3.1) does encode *norCBQD* but lacks genes for the other NOR frequently found in AOB genomes, *norSY-senC-orf1* (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_2OH and NO (Hooper and Terry, 1979). Thus, NORs are not required by oligotrophic AOB as nitrosative stress should be uncommon. *N. multiformis* does not have an annotated cytochrome P460 (*cytL*) whereas *N. communis* has two copies (Table 3.1), 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 NH_2OH and NO to NO_2^- (Elmore *et al.*, 2007; Stein, 2011) and may be important for alleviating nitrosative stress in AOB lacking NORs. All 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 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.

3.3.2 Instantaneous NOx production from AOB during oxidation of NH_3 or NH_2OH

Measurement of NO or N_2O production during oxidation of NH_3 or NH_2OH were compared among the 5 strains. Measurements revealed that all AOB produce measureable quantities of NO during active oxidation of NH3 (Figure 3.2A, C, E, G, and I). Although each AOB had a unique and dynamic NO production profile, making comparative rate calculations impractical, all strains produced >50 nM NO (per $1x10^{10}$ 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 3.2A; Table 3.2). As reported previously (Kozlowski *et al.*, 2016c) *N. multiformis* began re-consuming NO once *ca.* 50% O_2 was left in the MR-chamber (Figure 3.2I) and both *N. europaea* and *N. communis* re-consumed a small amount of NO following anoxia in the MR-chamber (Figure 3.2A and B). Interestingly either upon O_2 depletion, in the case of *Nitrosomonas* sp. Is79A3 (Figure 3.2E), 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 (Figure 3.2E and G). Unlike the other AOB, neither strain re-consumed NO during active NH_3-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 *et al.*, 2010; Yu and Chandran, 2010) and *N. multiformis* (Kozlowski *et al.*, 2016c), both of which have annotated *nirK*, *norB*, *and norY* genes (Table 3.1). It is known, however, that the thaumarchaeotal ammonia-oxidizers (AOA) also produce NO during NH_3-oxidation (Martens-Habbena *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 *N. viennensis* and the oligotrophic AOB of the present study, *Nitrosomonas* sp. Is79A3 and *N. ureae*. Once O_2 was depleted in the MR chamber (Figure 3.2E and G; Kozlowski *et al*., 2016c) measureable quantities of NO were released. This similarity between the AOA, considered to be oligotrophs with a high Km for ammonium (Martens-Habbena *et al.*, 2009; Stahl and la Torre, 2012), and the oligotrophic AOB could be explained by a lack of annotated NORs to combat high intracellular NO experienced following anoxia either due to release of NO directly from the NH_3-oxidation pathway, in the case of AOA (Kozlowski *et al.*, 2016c), or perhaps from NO_2^- reduction in the

case of the AOB (Stein, 2011). At least in the nitrifier denitrification pathway of *N. europaea*, NorB is required for NO_2^- reduction to N_2O (Kozlowski *et al.*, 2014).

Following O_2 depletion and in the presence of NO_2^- some AOB can perform nitrifier denitrification (Stein, 2011; Kozlowski *et al*., 2014) which was tested in the present study by measurement of N_2O during active NH_3- or NH_2OH-oxidation and through a period of anoxia (Figure 3.2 and 3.3). 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.* 243 μM NO_2^- in the chamber is at saturation of NirK following oxidation of NH_3 and NH_2OH.

N_2O was measured in the MR-chamber from all strains following NH_3-oxidation and upon anoxia when NH_3 was substrate (Figure 3.2B, D, F, H, and J). A greater delay of *ca.* 3 min in measureable N_2O was seen from traces with both *N. communis* (Figure 3.2D) and *N. ureae* (Figure 3.2H). The lowest concentrations of N_2O were measured from *N. communis* and *N. multiformis* (Figure 3.2D and J, Table 3.2). N_2O was measureable in the MR-chamber of *N. europaea* immediately following O_2-depletion and was produced at a rate of 0.47 μM N_2O per 10^{10} cells $^{-1}$ per min. and reaching *ca.* 7 μM 15 min. into anoxia (Figure 3.2B). As with NO production, both *Nitrosomonas* sp. Is79A3 and *N. ureae* had similar N_2O traces (Figure 3.2F and H) with similarly fast rates for N_2O measured in the MR-chamber following anoxia (Figure 3.2F and H; Table 3.2).

With NH_2OH as substrate, the majority of N_2O measured in the MR-chamber from *N. europaea* (Figure 3.3A), *N. communis* (Figure 3.3B), and *N. multiformis* (Figure 3.3E) was produced in a linear fashion directly following anoxia suggesting enzymatic reduction of available NO_2^- to N_2O. However, in the case of both *Nitrosomonas* sp. Is79A3 (Figure 3.3C) and *N. ureae* (Figure 3.3D) the majority of N_2O was measured during active NH_2OH-oxidation with

production in both traces slowing upon complete O_2-depletion. Also, the quantity of N_2O measured from both *Nitrosomonas* sp. Is79A3 and *N. ureae* during active NH_2OH-oxidation was much greater overall than that produced from any other AOB (Table 3.2).

It is interesting that *N. communis*, the only AOB lacking NirK, had very weak non-linear N_2O production from NH_3, yet strong linear production when NH_2OH was provided as substrate (Figure 3.2D and Figure 3.3B). The linearity of N_2O formation with NH_2OH as substrate suggests that there is an enzymatic pathway for N_2O formation under anoxic conditions, but this pathway is not active when NH_3 was provided as substrate. This observation provides insight into the function of unidentified enzymology in *N. communis* that requires further investigation. Similarly, an *N. europaea nirK*::Kan mutant was able to reduce NO_2^- to N_2O (Cantera and Stein, 2007; Kozlowski *et al*., 2014) suggesting alternate NIRs in AOB.

3.3.3 Contributions of AOB to abiotic N_2O

The NO and N_2O production profiles of *Nitrosomonas* sp. Is79 and *N. ureae* suggest that the N_2O is produced abiotically. The N_2O profiles of both AOB following anoxia (Figure 3.2F and H) are congruent with a rapid and abundant release of NO (Figure 3.2E and F) that can be abiologically transformed to N_2O, a characteristic trait observed from the AOA *N. viennensis* (Kozlowski *et al.*, 2016c). Also in support of an abiotic origin of N_2O for both *Nitrosomonas* sp. Is79 and *N. ureae* in comparison to the other strains (Figure 3.3) is the fact that the majority of N_2O was measured during active oxidation of the NH_2OH. Accumulation of NH_2OH can lead to NO and N_2O production at the 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_2O as well (Kozlowski *et al.*, 2016c). Interestingly, the lack of NirK did not appear to cause significant production of N_2O during active NH_2OH-oxidatation by *N. communis*, as shown

previously for NirK-deficient *N. europaea* (Cantera and Stein, 2007; Kozlowski *et al*., 2014), suggesting a different configuration of the ammonia-oxidation pathway between these two AOB.

The intermediate NH_2OH was shown to react with heat-killed cell moieties of the AOA *Nitrososphaera viennensis* EN76 and produce abiological N_2O (Kozlowski *et al.*, 2016c). In the present study, abiotic and heat-killed cell controls were performed to demonstrate if NH_2OH could react with either media components or heat-killed cells to produce N_2O in the absence of active cellular functioning (Figure 3.4). NH_4^+-free HK medium + $NaNO_2^-$ or with heat-killed AOB and addition of 250 μM NH_2OH showed that medium + $NaNO_2^-$ or medium with heat-killed *N. europaea*, *N. communis*, and *N. multiformis* + NH_2OH did not facilitate significant measureable N_2O (Figure 3.4). However, heat-killed cells of both *Nitrosomonas* sp. Is79A3 and *N. ureae* both produced measureable N_2O following addition of 250 μM NH_2OH. The reactivity of cellular moieties with NH_2OH is further evidence of similarities among oligotrophic AOB and the AOA as heat-killed cell controls of *N. viennensis* showed similar reactivity (Kozlowski *et al*., 2016c).

3.4 Conclusions

The present study highlights many new and significant findings in the fields of AOB phylogeny and their NOx metabolism. It is now clear that phylogeny and gene content are not predictors of whether AOB contribute to enzymatic NOx production, a finding already proposed for classical denitrifers (Roco *et al.*, 2016). Also, as has already been shown for AOA (Kozlowski *et al.*, 2016c), the release of NO is a likely contributor to abiological N_2O production (chemo-denitrification) especially under certain environmental conditions where NOx intermediates (i.e. NO or NH_2OH) are released (Zhu-Barker *et al.*, 2015; Jones *et al.*, 2015). This

work also showcases the necessity of physiological studies on pure cultures of ammonia-oxidizers to examine mechanisms of NOx production, rather than relying solely on genomic content, before hypotheses can be made about their relative contributions to N_2O emissions from specific ecosystems.

3.5 Tables and Figure

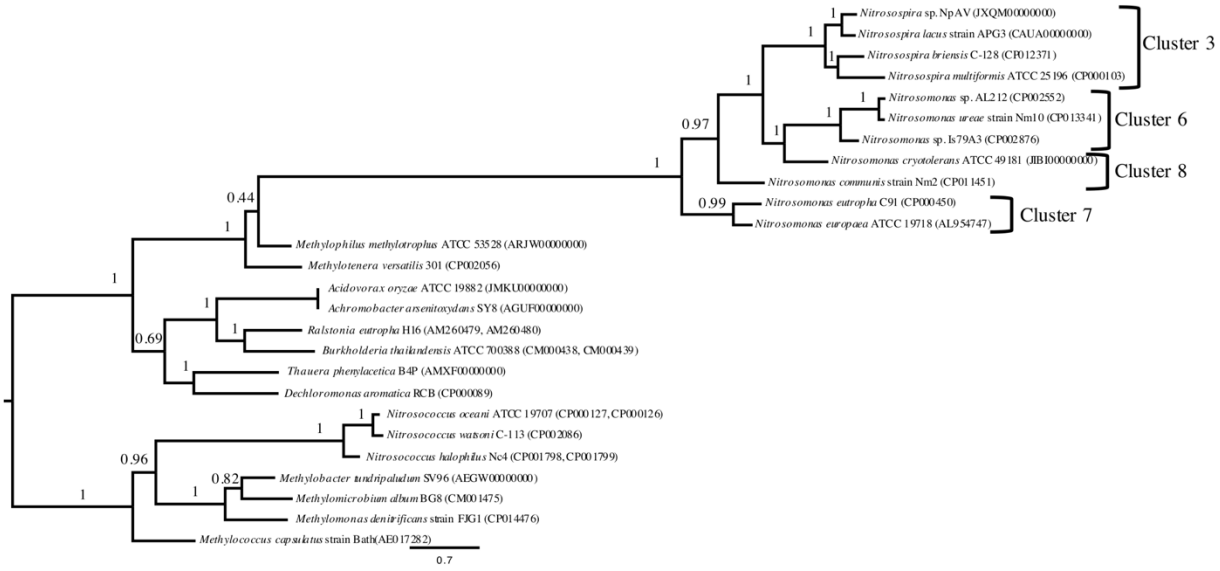

Figure 3. 1 Rooted maximum likelihood phylogeny of 14 publically available genomes of ammonia oxidizing bacteria based on 400 broadly conserved amino-acid sequences.

PhyloPhlAn (Segata *et al.*, 2013) was used to identify and align the amino-acid sequences in all input genomes. The tree was constructed using PhyML 3.0 (Guindon *et al.*, 2010) with the *Gammaproteobacteria* as the root. Bootstrap values (500 replicates) are denoted above the branches and branch lengths correspond to sequence differences as indicated by the scale bar at the bottom.

Table 3. 1 Annotated inventory with implications in ammonia-oxidation or N-oxide metabolism. Gene annotations from complete genomes of all AOB utilized in the present study.

Strain	*Nitrosomonas europaea* ATCC 19718	*Nitrosomonas communis* Nm2	*Nitrosomonas* sp. Is79A3	*Nitrosomonas ureae* Nm10	*Nitrosospira multiformis* ATCC 25196
Ammonia monooxygenase (*AMO*)	*amoCABED* (2) NE2064-59 NE0945-40 *amoC* (1) NE1411	*amoCABED* (2) AWW31_01090-70 AWW31_05385-65	*amoCABED* (2) Nit79A3_0471-75 Nit79A3_2886-82 *amoCAB* (1) Nit79A3_1079-81 *amoC* (2) Nit79A3_1233 Nit79A3_1595	*amoCABED* (2) ATY38_01315-295 ATY38_07250-70 *amoCAB* (1) ATY38_13760-50 amoCE (1) ATY38_06315-10 *amoC* (1) ATY38_09265	*amoCABED* (1) Nmul_A2326-22 *amoCAB* (1) Nmul_A0798-800 *amoC* (2) Nmul_A0177 Nmul_A2467
Hydroxylamine dehydrogenase (*HAO*)	*haoAB-cycAB* (2) NE0962-59 NE2339-36 *haoAB-cycA* (1) NE2044-42	*haoAB-cycAB* (2) AAW31_01285-70 AAW31_16290-75 *haoAB-cycA* (1) AAW31_18275-65	*haoAB-cycAB* (3) Nit79A3_0807-10 Nit79A3_0822-25 Nit79A3_2942-39	*haoAB-cycAB* (4) ATY38_00070-55 ATY38_06640-55 ATY38_10080-95 ATY38_15220-05	*haoAB-cycAB* (3) Nmul_A0805-02 Nmul_A1082-85 Nmul_A2662-59
Nitrosocyanin	Present NE0143	Present AAW31_00185	Not Present	Present ATY38_00645	Present Nmul_A1601
Nitrite reductase (NirK)	*ncgABC-nirK* NE0924	Not present	*nirK* Nit79A3_2335	*nirK* ATY38_00595	*nirK* Nmul_A1998
Cytochrome *c* nitric oxide reductases	*norCBQD* NE2003-06 *norSY-senC-orf1* NE0683-86	*norCBQD* AAW31_10555-70 *norSY-senC-orf1* AAW31_05895-910	Not Present	Not Present	*norCBQD* Nmul_A1256-43 *norSY-senC-orf1* Nmul_A2667-64
Cytochrome *c*' beta (*cytS*)	Present NE0824	Present AAW31_17525	Present Nit79A3_0363	Present ATY38_05410	Present Nmul_A2484
Cytochrome P460 (*cytL*)	Present NE0011	Present AAW31_02040 AAW31_00880	Present Nit79A3_1628	Present ATY38_00655	Not Present
NO-responsive transcriptional regulator (NsrR)	Present NE0926	Not Present	Not Present	Not Present	Not Present
NO-responsive transcriptional regulator (NnrS)	Present NE1722	Present AAW31_04320 AAW31_06015	Present Nit79A3_3412	Present ATY38_04220	Not Present

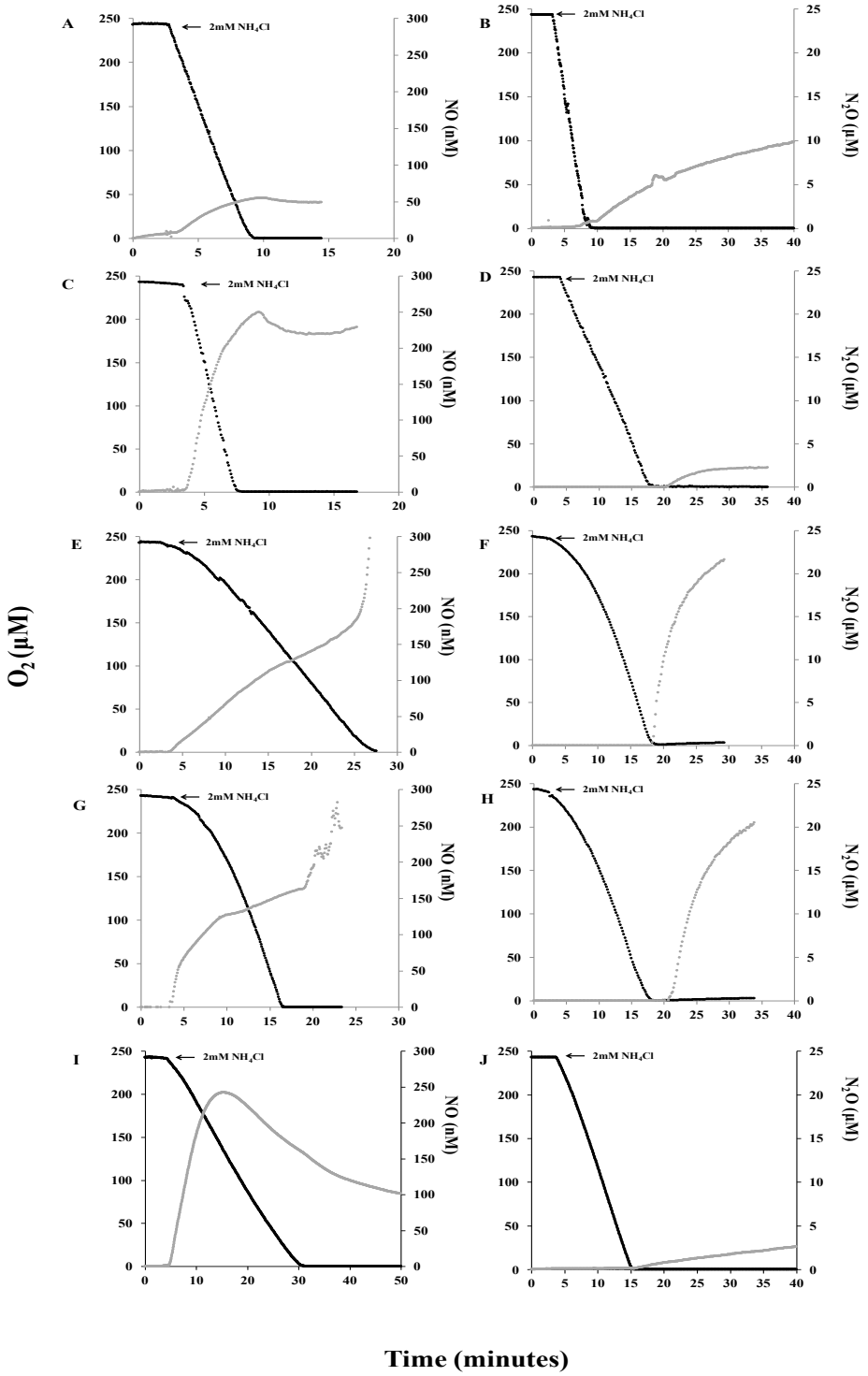

Figure 3. 2 Instantaneous measurement of O_2 consumption (black dots) and NO or N_2O production (gray dots) during oxidation of 2mM NH_4Cl by *Nitrosomonas europaea* (A and B), *Nitrosomonas communis* (C and D), *Nitrosomonas* sp. Is79A3 (E and F), *Nitrosomonas ureae* (G

and H) or *Nitrosospira multiformis* (I and J). Panels are single representative measurements of reproducible results (n=3)*

*Note differences in scale of X axis (time) for traces of NO production during NH_3-oxidation

Table 3. 2 Maximum NO produced during NH_3-oxidation prior to anoxia and rate of N_2O produced following NH_3-oxidation in anoxia. Data represent averages of replicate experiments (n=3) with standard error in parentheses.

	Max. NO produced during NH_3-ox. prior to anoxia (nM NO x 10^{10} cells $^{-1}$)	**Rate of N_2O production during anoxia following NH_3-ox. (µM N_2O/ 10^{10} cells $^{-1}$ /min.)**
***Nitrosomonas europaea* ATCC 19718**	65.81 (3.67)	0.47 (0.08)
***Nitrosomonas communis* Nm2**	218.38 (10.01)	0.22 (0.09)
***Nitrosomonas* sp. Is79A3**	215.30 (7.12)	4.69 (0.84)
***Nitrosomonas ureae* Nm10**	146.92 (17.97)	4.35 (1.15)
***Nitrosospira multiformis* ATCC 25196**	92.15 (17.95)	0.09 (0.04)

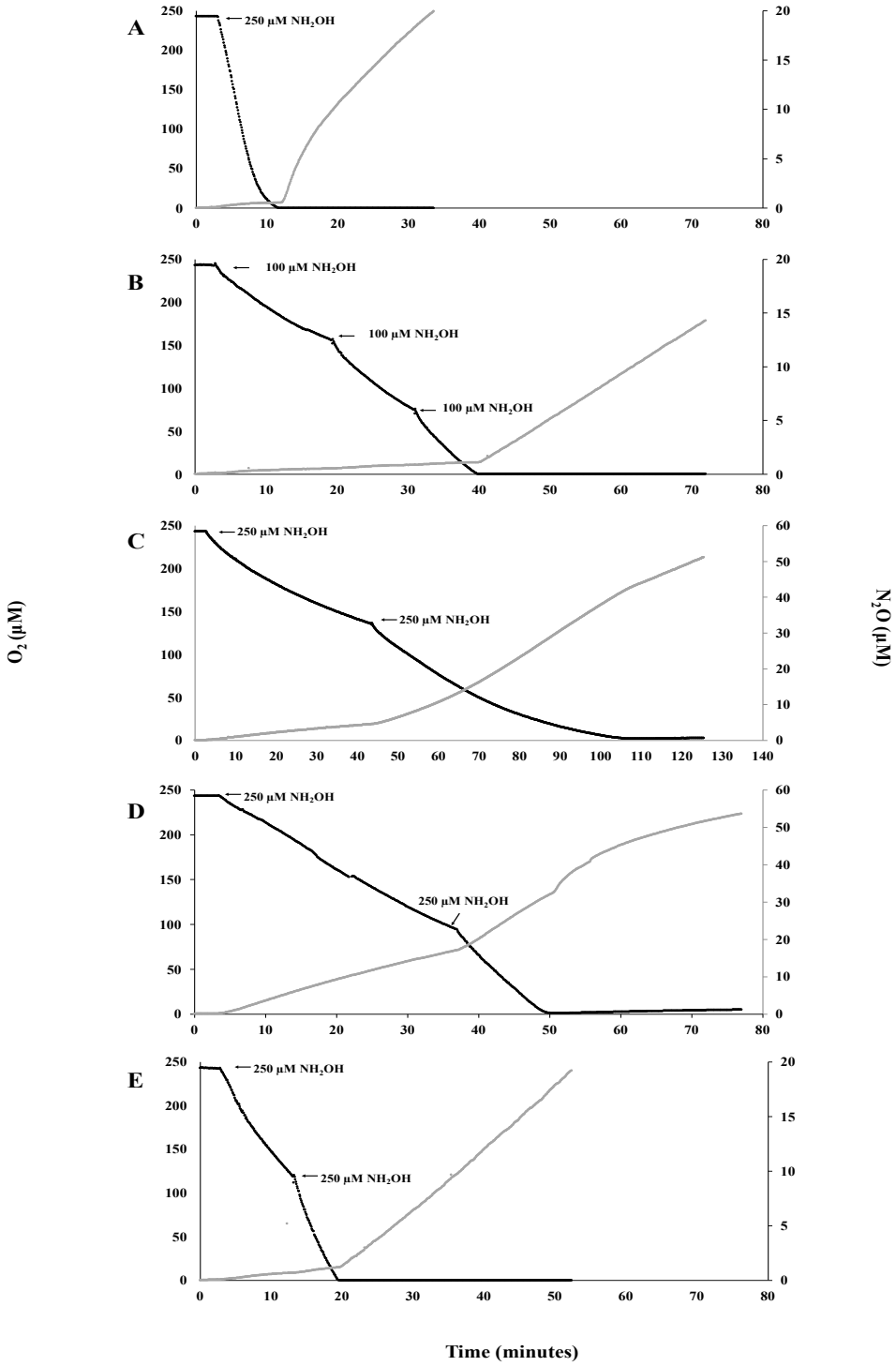

Figure 3. 3 Instantaneous measurement of O_2 consumption (black dots) and N_2O production (gray dots) during oxidation of NH_2OH by *Nitrosomonas europaea* (A), *Nitrosomonas communis* (B), *Nitrosomonas* sp. Is79A3 (C)* *Nitrosomonas ureae* (D)**, or *Nitrosospira multiformis* (E)

*Note the x-axis for *Nitrosomonas* sp. Is79A3 differs from all other traces

***Nitrosomonas* sp. Is79A3 and *N. ureae* have different y-axis for N_2O production

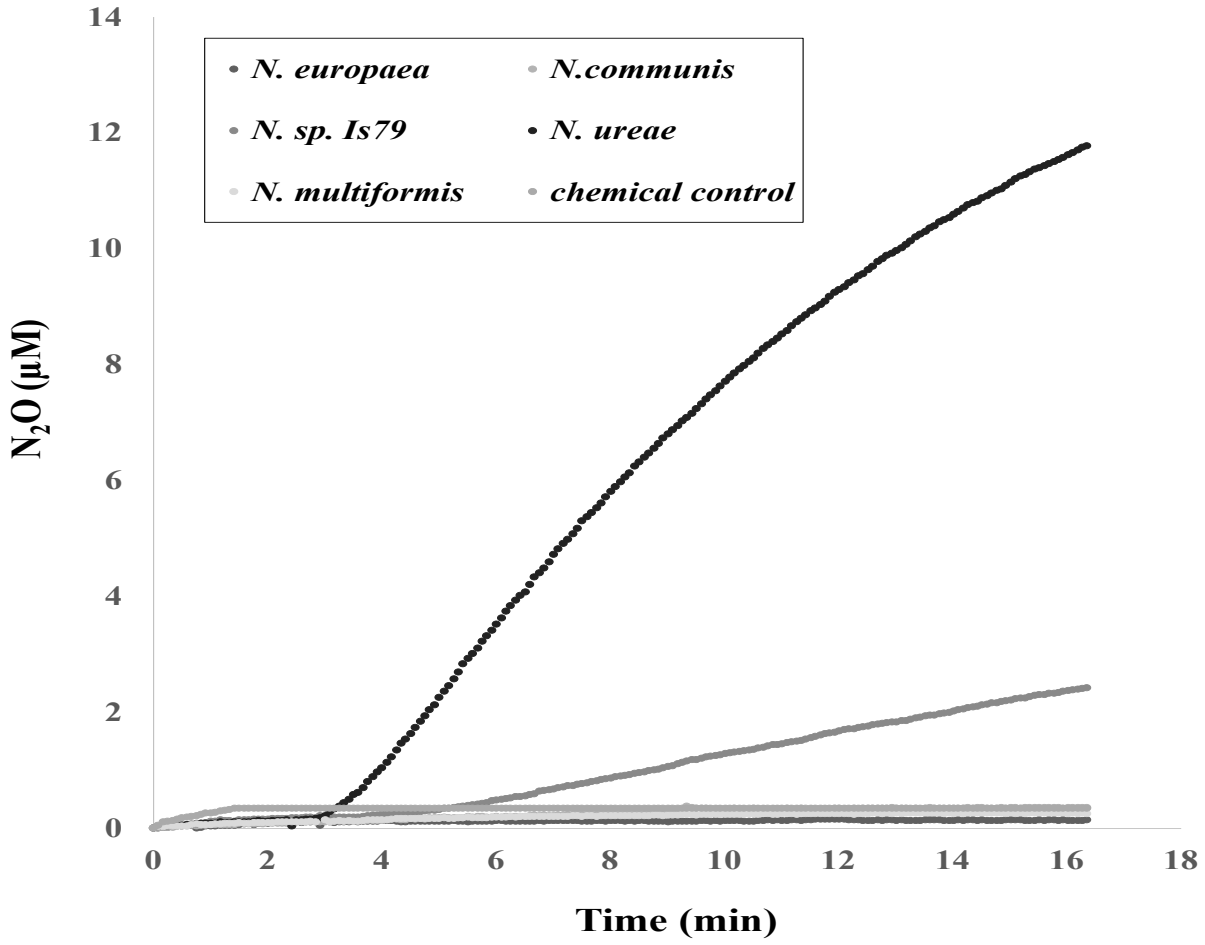

Figure 3. 4 Measurement of N_2O from either HK medium + 250 µM $NaNO_2^-$ or HK medium + heat-killed cells with addition of 250 uM NH_2OH.

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CHAPTER 4: Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota

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4.1 Abstract

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.

4.2 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 (AMO) enzyme and (an) unknown enzyme(s) that convert(s) hydroxylamine (NH_2OH) to NO_2^- and provide electrons for energy conservation (Vajrala *et al.*, 2013). In ammonia-oxidizing bacteria, this second step is performed by hydroxylamine dehydrogenase (HAO; EC 1.7.2.6); however, no homologues of HAO-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*., 2014). Martens-Habbena *et al.* (2014) 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.* 2014). 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 AMO, 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 interaction 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*.

4.3 Materials and Methods

4.3.1 Strains and cultivation

Nitrososphaera 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 50µg/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% $NaHCO_3$.

4.3.2. 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), 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).

4.3.3 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*, 1mM-1.5mM NO_2^-; *N. multiformis*, 2-2.5mM NO_2^-) by

filtration on Supor®200 0.2 μm filters (Pall, Ann Arbor WI) and rinsed three times with substrate-free media (*N. viennensis*, FWM; *N. multiformis*, HKM). Washed cells (*N. viennensis*, ca. $1x10^{11}$ total cells; *N. multiformis*, ca. $1x10^{10}$ total cells) were re-suspended 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 DK). 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, Aarhus DK), N_2O concentration was measured using an N_2O-500 N_2O minisensor electrode with 500 μm tip diameter (Unisense, Aarhus DK), and NO was measured using an ami-600 NO sensor with 600 μm tip diameter (Innovative Instruments Inc., Tampa FL). 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.

4.3.4 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), *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.

4.3.5 Instantaneous NH_3 and NH_2OH 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 (Figure 4.6). 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; half-life of 15 minutes 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 approximately 7 minutes of NO-chelation by PTIO, another 1 μL of PAPA NONOate was added but NO levels remained below detection levels (Figure 4.7).

4.3.6 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, Ann Arbor MI) 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 (Figure 4.8, A and B). Control experiments with heated killed *N. viennensis* (ca. $1x10^{11}$ cells) were performed in sparged FWM + $NaNO_2$ (200 μM) with measurement of NO and N_2O upon addition of 200 μM NH_2OH (Figure 4.9, A and B) to determine whether NH_2OH interacts with cellular debris.

4.4 Results

4.4.1 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 4.1). 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 4.1A) whereas in cultures of *N. multiformis,* ammonia oxidation and nitrite production continued at the same rate as before PTIO addition (Figure 4.1B). 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*.

4.4.2 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 microrespiratory (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 4.2A 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 4.2C), the initial rate of O_2 consumption by *N. viennensis* was quite rapid, followed by a slower, linear rate (Figures 4.2A and B). *N. viennensis* produced a maximum of ca. 1.41 nM of NO per $1x10^{10}$ 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 4.2A). After ca. 3 minutes 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 $1x10^{10}$ cells (n=4) and re-consumption of NO began once ca. 50% of the available O_2 was consumed (Figure 4.2C).

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 4.2B 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 $1x10^{10}$ cells (Figure 4.2B; 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 $1x10^{10}$ cells at ca. 40 min (Figure 4.2D; n=4).

4.4.3 Dynamics of NO production and consumption during NH_2OH oxidation by *N. viennensis* EN76T

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 4.3). Each subsequent addition of equal aliquots of NH_2OH led to the production of ca. 5 nM NO per $1x10^{10}$ cells (n=3), followed by an immediate re-consumption of NO and O_2 until the next addition of NH_2OH (Figure 4.3). 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 (Figure 4.8A). 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 (Figure 4.8B).

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 (Figure 4.6), although PTIO was able to effectively scavenge NO in cell-free FWM containing the NO-donating compound, PAPA-NONOate (Figure 4.7).

4.4.4 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 4.4A). 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 4.4C). 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 HKM (Figures 4.4B and D). Reactivity of NH_2OH in FWM + heat-killed *N. viennensis* cells was also explored (Figure 4.9). When NH_2OH was introduced into the MR chamber containing heat-killed cells, accumulation of NO reached ca. 110 nM NO over 5 min (Figure 4.9A). 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 (Figure 4.9B).

4.5 Discussion

4.5.1 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.*, 2014). 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.

4.5.2 NO is produced and immediately consumed during active ammonia oxidation by *N. viennensis* EN76T

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 *Nitrosopumilus maritimus* SCM1 (Martens-Habbena *et al.*, 2014). 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 4.2A 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 (Figure 4.9).

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 confirmatory evidence that ammonia-oxidizing bacteria, but not *N. viennensis*, are capable of producing N_2O enzymatically via nitrifier denitrification (Stieglmeier *et al.*, 2014b).

4.5.3 NO is produced and consumed during active 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 AMO (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 4.1), or perhaps PTIO was ineffective at chelating rapidly cycling NO at the high cell densities used in the MR chamber.

4.5.4 N_2O in *N. viennensis* cultures originates form the abiotic reactions 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 uM/L 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) .

4.5.5 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 4.5A). 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 4.5B; 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; Bartossek *et al*., 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 4.5). 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 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 4.5.

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 AMO, 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 4.5A) 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 *Nitrosopumilus maritimus* SCM1 and experiments with terrestrial ammonia-oxidizing *Thaumarchaeota* including the data presented here for *N. viennensis* $EN76^T$.

4.6 Conclusions

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 (OMZ), and in return, the denitrifiers could provide organic carbon to the *Thaumarchaeota* to establish a nitrifying-denitrifying consortium (Karner *et al.*, 2001; Ganesh *et al*., 2015; Beman *et al.* 2012). 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.

4.7 Tables and Figures

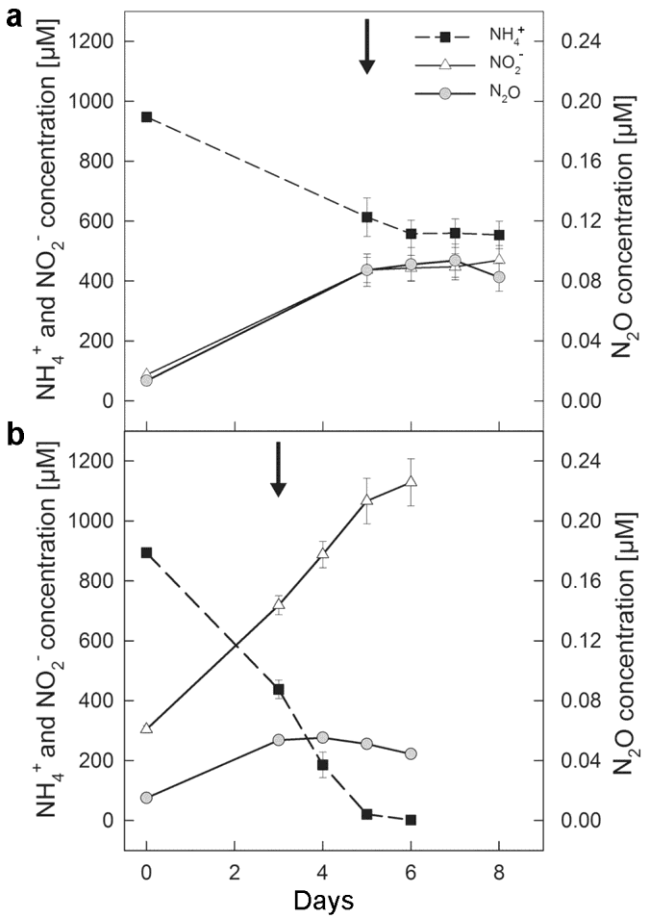

Figure 4. 1 Inhibition of *N. viennensis* (A) and *N. multiformis* (B) with the NO-scavenger PTIO. Ammonium consumption (black squares, dotted line) and nitrite production (white triangles, solid line) as well as N_2O production (gray circles, solid line) are plotted*

*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.

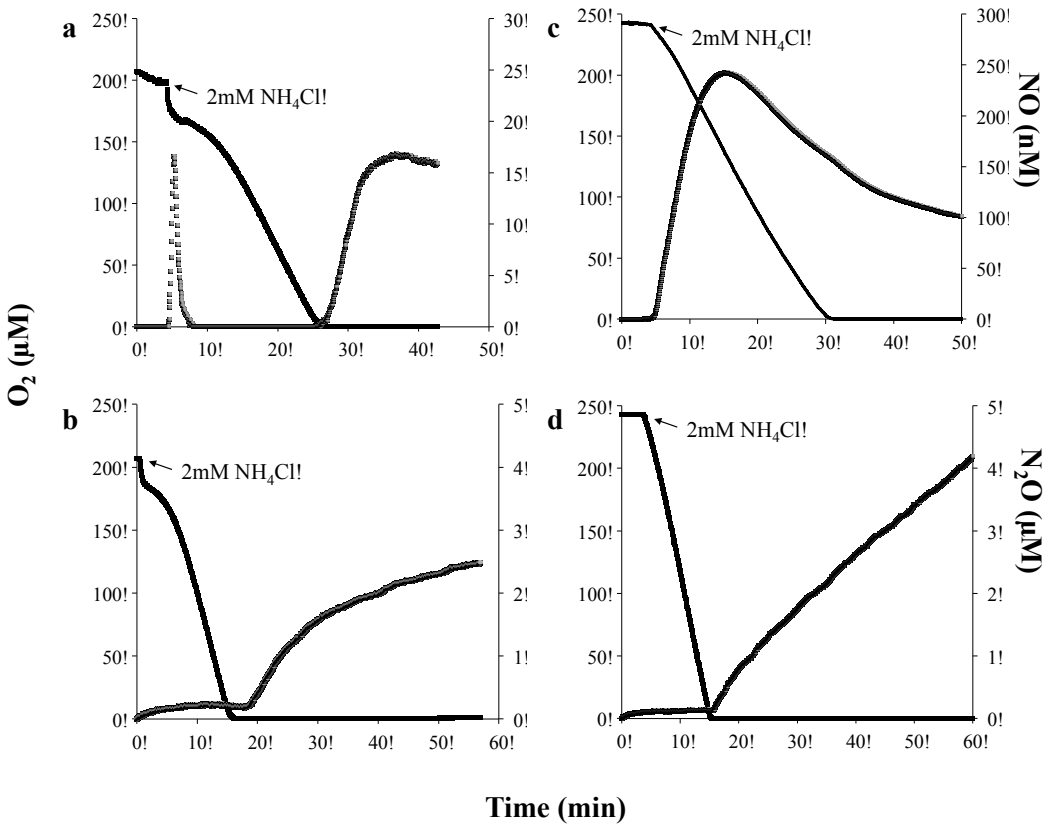

Figure 4. 2 Instantaneous measurements of O_2 (black line), NO (A and C) and N_2O (B and D) (gray dots) after addition of 2 mM NH_4Cl in liquid phase suspensions of *N. viennensis* (A and B) and *N. multiformis* (C and D) cells. Panels are single representative measurements of reproducible results (n=4). Note that y-axes for NO are on different scales for *N. viennensis* vs. *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.

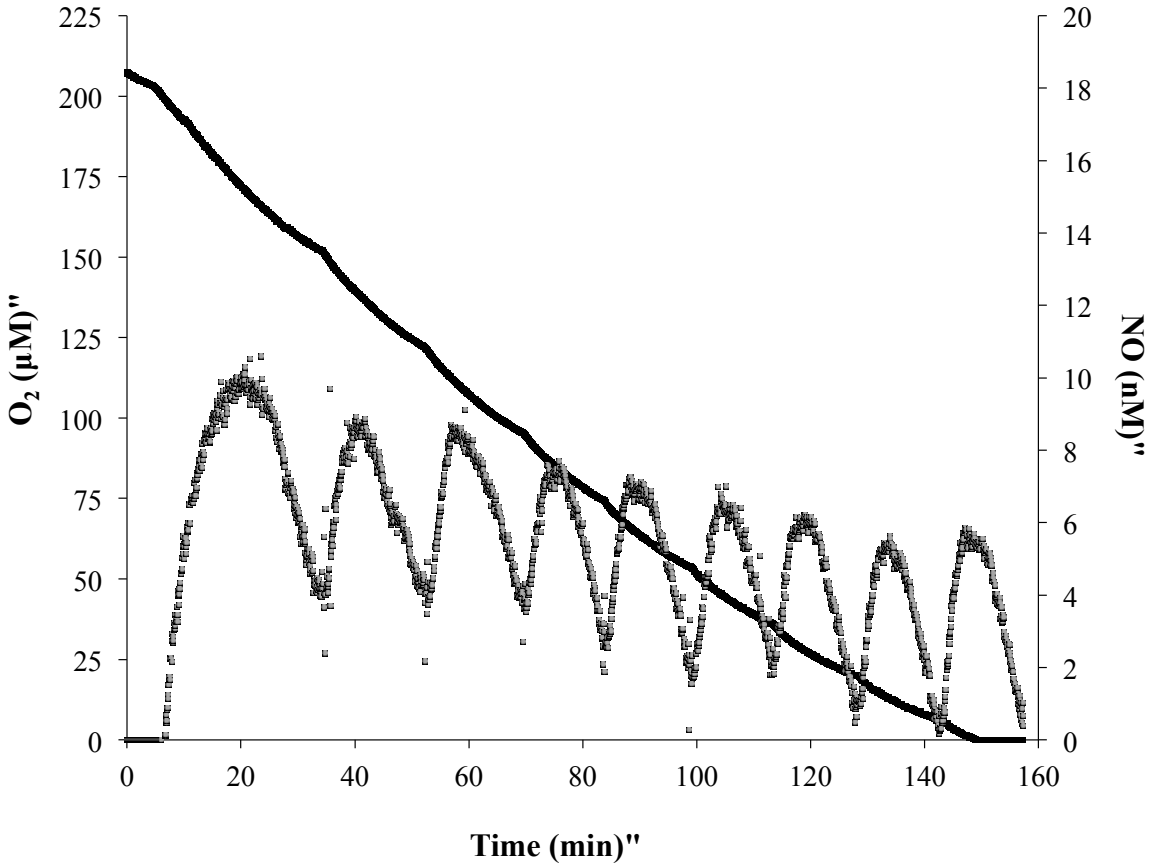

Figure 4. 3 Instantaneous measurements of O_2 consumption (black line) and NO production (grey dots) from 200 µM pulses of NH_2OH in liquid phase incubations of *N. viennensis* in the absence of NH_4^+. Plot is a single representative of replicable experiments (n=4).

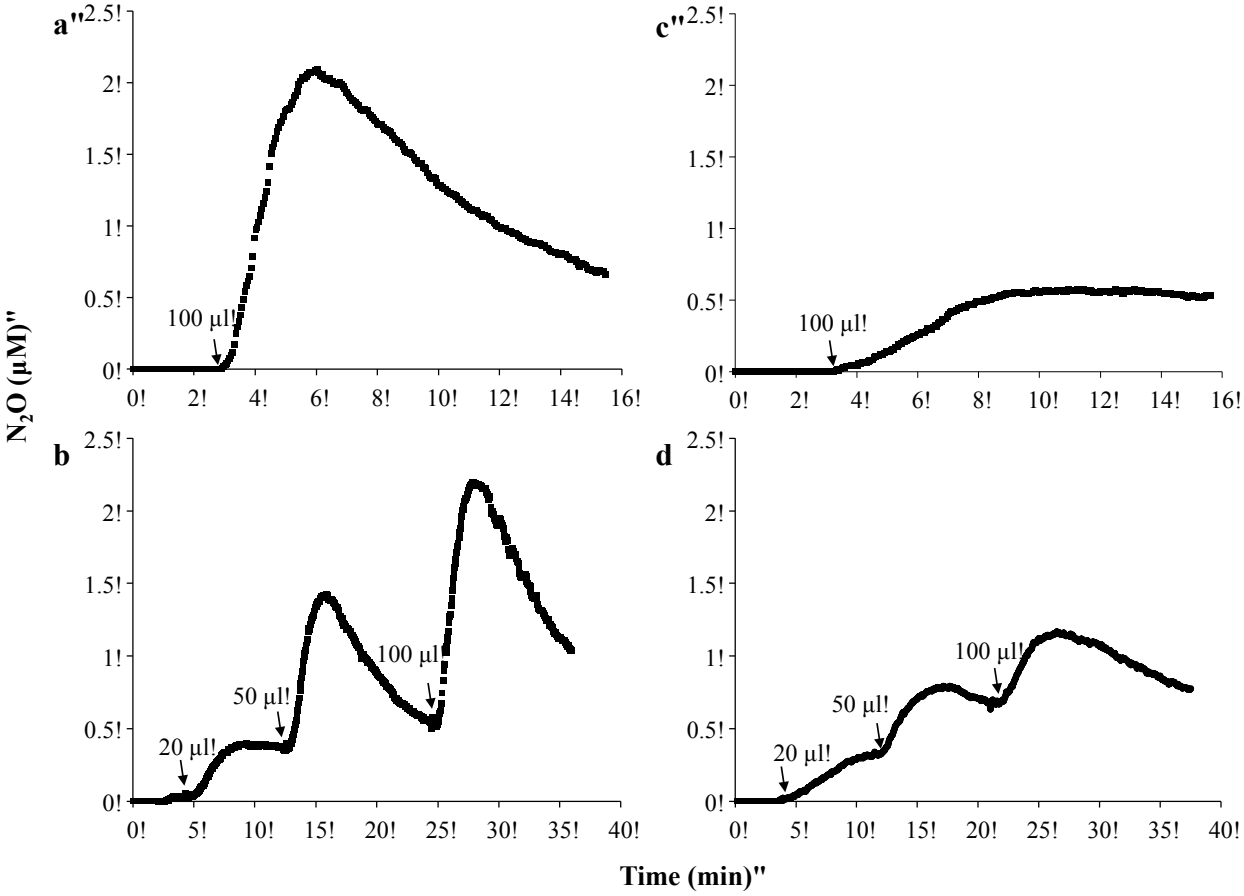

Figure 4. 4 Abiotic production of N_2O from the NO-donor MAHMA-NONOate in either FWM (A and B) or HKM (C and D). Panels are single representative measurements of reproducible results (n=3). The addition of varying concentrations of MAHMA-NONOate is indicated by arrows.

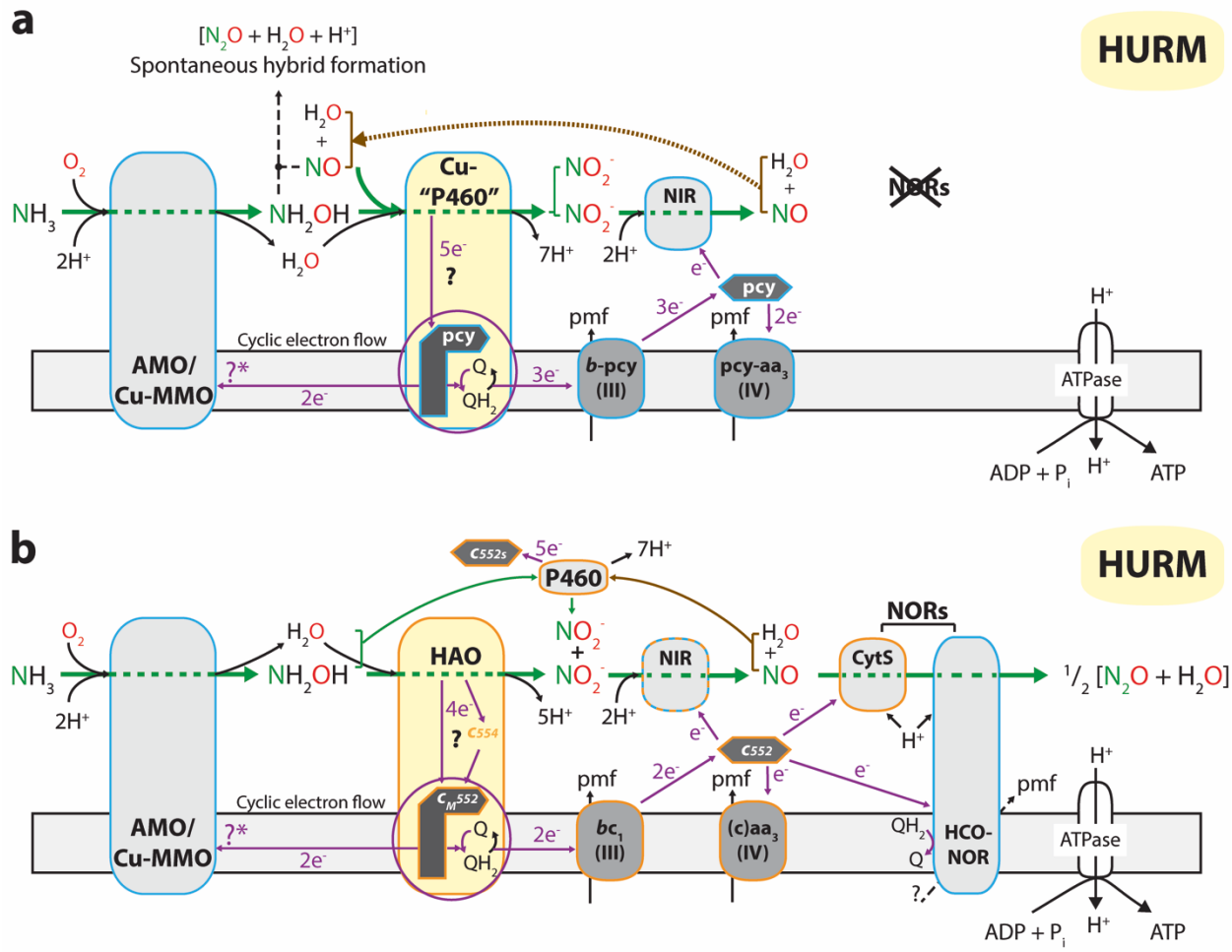

Figure 4. 5 Proposed pathway for ammonia-dependent chemolithotrophy in the ammonia-oxidizing thaumarchaea (Panel A) compared with known pathways of N-oxide transformation in ammonia-oxidizing bacteria (Panel B)*

*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 AMO and that NH_2OH is dehydrogenated to NO_2^- by activities of a number of unknown enzymes (Stahl and de la Torre, 2012; Vajrala *et al.*, 2012; Walker *et al.*, 2010). Based on existing chemistry facilitated by heme proteins in ammonia-oxidizing bacteria (Panel B), the model in Panel 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 (MCOs) 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). *c*552: cytochrome *c* redox carrier; Cu-MMO: ammonia monooxygenase (AMO); 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.

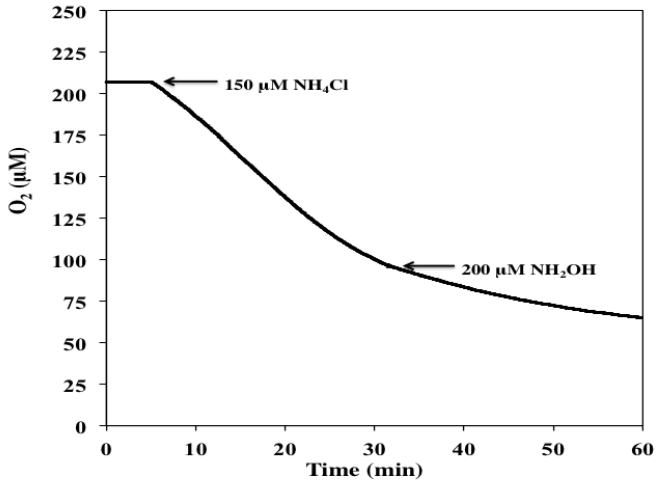

Figure 4. 6 Oxidation of NH_4Cl and NH_2OH by *N. viennensis* cells after 1h incubation with 200 µM PTIO.

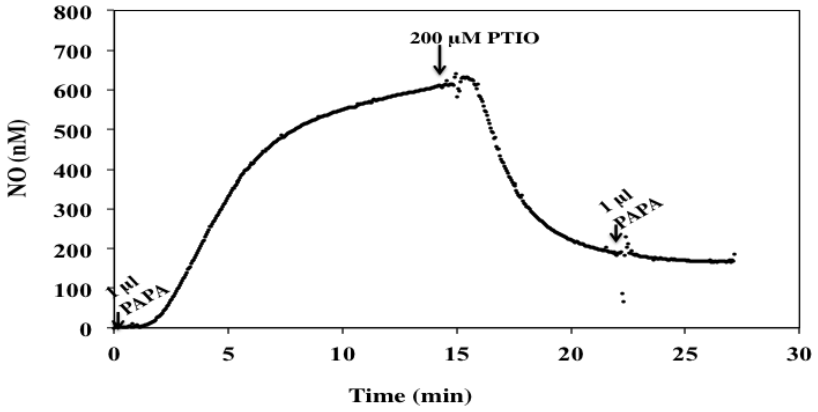

Figure 4. 7 Abiotic control confirming quenching of NO donated by PAPA-NONOate by the NO chelator PTIO.

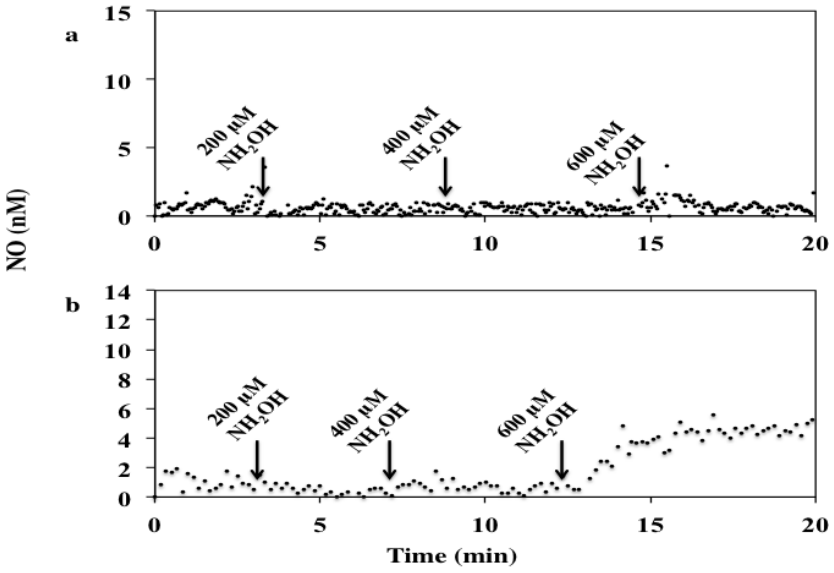

Figure 4. 8 Measurement of NO during addition of NH_2OH to FWM (A) or FWM amended with 200 µM NO_2^- (B). FWM was sparged with N_2 (0-3% O_2 saturation; determined by O_2 electrode) previous to NH_2OH addition.

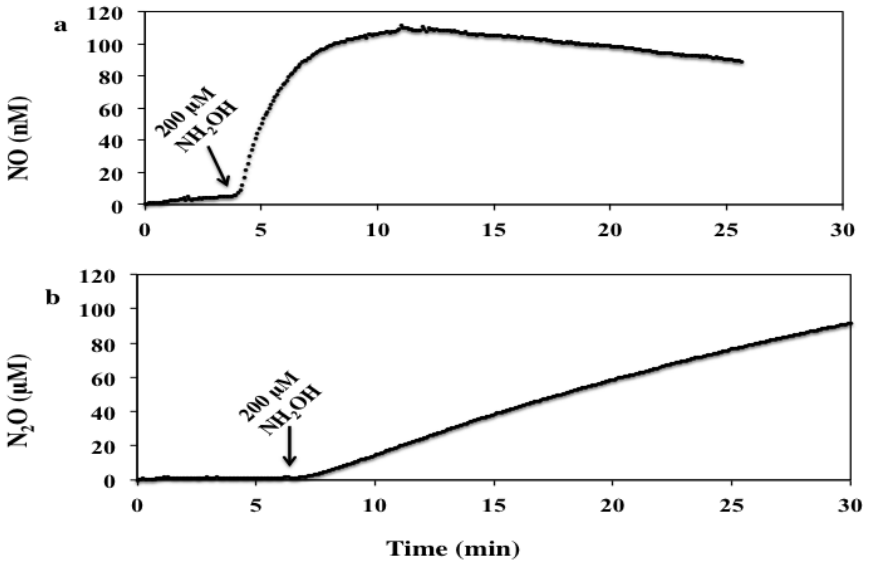

Figure 4. 9 Measurement of NO (A) and N_2O (B) from FWM + killed cells (1 x10^{11} total cells) amended with 200 µM NO_2^- upon addition of 200 µM NH_2OH. FWM was sparged with N_2 (0-3% O2 saturation; determined by O2 electrode) previous to NH_2OH addition.

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CHAPTER 5: General Conclusions and Future Research Directions

5.1 Overview

The purpose of this thesis was to form a comprehensive understanding of the nitrogen oxide metabolism of ammonia-oxidizers. The approach utilized included physiology, genomics, and phylogeny with specific focus on pathways and metabolism for production of nitrogen oxides under environmentally relevant conditions. The present thesis accomplishing this by 1) utilizing a genome-inferred physiological approach to elucidate pathways and enzymology of N_2O production in the model AOB *Nitrosomonas europaea* ATCC 19718, 2) applying this knowledge to the broader study of NOx metabolism of AOB isolated from various trophic states and with closed genomes, and 3) by comparative analysis of pathways for NH_3-oxidation and N_2O production by ammonia-oxidizing bacteria and Thaumarchaeota. The achievement of these goals supported the hypotheses that genomic inventory and phylogeny are not accurate predictors of contributions of AOB to NOx production and that ammonia-oxidizing Bacteria and Thaumarchaeota have different pathways and intermediates for NH_3-oxidation but nonetheless both contribute to global N_2O pools.

5.2 Summary and General Conclusions

Nitrifiers have been studied in lab culture for over 100 years and the model organism for the study of proteobacterial ammonia-oxidation, *Nitrosomonas europaea* ATCC 19718 has been in pure culture for over half a century (Meiklejohn, 1950). This lends an explanation not only to the abundance of AOB in diverse environments but also the great importance of the continued study of how this group of microorganisms controls important interconversions of nitrogen in both the oxidative and reductive sides of the biogeochemical nitrogen cycle.

When AOB were shown to possess genes with implications in denitrification through reduction of NO_2^- to N_2O, in particular copper-containing nitrite reductase *nirK* and nitric oxide reductase *norB* (Stein, 2011b), and gas head-space N_2O was measured in pure cultures (Dundee and Hopkins, 2001; Wrage *et al.*, 2004; Shaw *et al.*, 2006; Cantera and Stein, 2007; Stein, 2011a) the study of the pathways and conditions under which AOB were producing this potent greenhouse gas became a top priority in the field of Nitrification. The work of elucidating pathways of N_2O production originally focused on *N. europaea* as it was the first AOB able to be genetically manipulated to study the role of NirK and NorB in the process (Beaumont *et al.*, 2002; 2004; Schmidt, 2004; Cantera and Stein, 2007). Although previous work suggested NirK played a greater role in nitrification, as opposed to nitrifier denitrification, through efficient NH_3-oxidation and prevention of intracellular NH_2OH buildup (Cantera and Stein, 2007), it remained unclear if it also functioned as the nitrite reductase under low O_2 conditions when N_2O was produced through the nitrifier denitrification pathways (Figure 1.3). Also, the functional role of NorB in substrate oxidation and N_2O production pathways, particularly across a range of O_2 concentrations, remained incomplete. Chapter 2 clarifies these outstanding questions in the study of N_2O production pathways in *N. europaea* through use of knockout mutants deficient in *nirK*, *norB*, or both genes in comparison to wild-type phenotype at a range of O_2 tensions and with various technical approaches including batch growth, resting cell assays, headspace gas analysis, and instantaneous measurement of N_2O. The resulting work confirmed that for *N. europaea* NirK does enable efficient substrate oxidation, there exist (an) alternate nitrite reductase(s) involved in NO_2^- reduction through the nitrifier denitrification pathway (Figure 1.3), and that NorB is required for NO_2^- reduction to N_2O even though other annotated nitric oxide reductases exist in the *N. europaea* genome (Chain *et al.*, 2003).

The work and resultant publication of chapter 2 (Kozlowski *et al.*, 2014) solidified a need to further explore the NOx metabolism of other AOB available in pure culture and with sequenced genomes. Chapter 3 characterized the NOx metabolism of AOB isolated from different trophic states, with phylogenetic diversity (whole genome, Tables 3.1; 16S rRNA, (Norton, 2011)), and with differences in genes for nitrogen transformation or NOx metabolism (Table 3.1; Appendix A and B). This chapter expanded the study of NOx metabolism beyond NirK and NorB taking into account multiple aspects of the AOB to explain differences observed. Importantly, this study of AOB NOx metabolism warrants many new considerations in modeling production of N_2O from environments in which the AOB are prevalent. In particular, AOB genomic content and phylogeny are not suitable markers for contributions to N_2O production. Regardless of annotated genes or phylogeny, all AOB studied have significant NOx metabolisms and capacity to contribute to N_2O production under environmentally relevant conditions during substrate oxidation and at a range of O_2 tensions. Another major implication of the work in Chapter 3 is the potential for abiotic contributions of AOB to N_2O production. Most studies of nitrification and N_2O production have sought to model the role of AOB with parameters of gene content and headspace gas analyses. Now, with the availability of instantaneous measurement of NOx metabolites, NO and N_2O, as utilized for the majority of data collection in Chapter 3, it is clear that AOB do not need complete or functional pathways for reduction of NO_2^- to N_2O to be implicated in production of this detrimental and potent GHG. Especially for the oligotrophic AOB tested, having fewer annotated genes implicated in nitric oxide reduction, *Nitrosomonas* sp. Is79A3 and *N. ureae* both showed significant NO release with the onset of O_2 depletion following NH_3-oxidation and high concentrations of N_2O measured during but not following NH_2OH oxidation, suggesting abiological reactions. Substrate availability and transient anoxia

are both common parameters the AOB experience in environments from which they are identified and isolated including agricultural soils and WWTPs. This suggests that the NOx contributions of AOB does not necessarily require genes or pathways for these microorganisms to make considerable contributions to global N_2O levels via abiotic reactions.

In comparison to the AOB the discovery and isolation of the thaumarchaeotal ammonia-oxidizers is a relatively recent finding (Könneke *et al.*, 2005). Given the shared lifestyle of the bacterial and thaumarchaeotal ammonia-oxidizers for utilizing NH_3 as a sole source of reductant and nitrogen, it only made sense that comparative analyses of the AOA and AOB would become a major interest in the field. However, from the standpoint of physiological comparison this proved to be difficult. The biochemistry of the NH_3-oxidizer lifestyle has been studied in AOB for many decades leading to extensive knowledge and data-driven hypotheses about their central metabolic pathway of NH_3-oxidation (Sayavedra-Soto and Arp, 2011), the same is not true for the AOA (Stahl and la Torre, 2012). Major similarities between the AOA and AOB include the presence of ammonia-monooxygenase (AMO) in genomes of microorganisms from both domains (Stahl and la Torre, 2012), NH_2OH as the intermediate of NH_3-oxidation to NO_2^- (Whittaker *et al.*, 2000; Vajrala *et al.*, 2013), and presence of genes for the copper-containing nitrite reductase *nirK* in genomes or metagenomes of both (Stahl and la Torre, 2012; Bartossek *et al.*, 2010; Arp *et al.*, 2007; Bollmann *et al.*, 2013; Suwa *et al.*, 2011; Kozlowski, Kits and Stein, 2016a) with the exception of *Nitrosomonas communis* Nm2 (Kozlowski, Kits and Stein, 2016b). This information along with measurement of N_2O from pure and enrichment cultures of AOA (Santoro *et al.*, 2011; Löscher *et al.*, 2012; Jung *et al.*, 2014; Stieglmeier *et al.*, 2014) led to the suggestion that AOA may also perform nitrifier denitrification similarly to AOB. Claims that a nitrifer denitrification pathway exists in AOA remained problematic though as no AOA exists

with annotated nitric oxide reductases (Stahl and la Torre, 2012), there is wide support for NirK to be involved in efficient NH_3-oxidation and not denitrification by AOB, and most importantly the complete pathway of NH_3-oxidation including all intermediates and enzymology was not elucidated for the AOA. Chapter 4 tackled these long standing issues with the comparative study of AOA and AOB by physiological comparison of two widely abundant terrestrial ammonia-oxidizers; the AOA *Nitrososphaera viennensis* EN76 (Tourna *et al.*, 2011) and the AOB *Nitrosospira multiformis* ATCC 25196 (Norton *et al.*, 2008). Direct comparison using activity as the equalizing parameter highlighted the first major difference between the two groups of ammonia-oxidizers; ten times the number of AOA cells were needed for comparative activity to the AOB. Also, Chapter 4 showcased the striking difference for NO-metabolism between both ammonia-oxidizers. Previous work on the AOA either using NO-chelators or measurement of NO directly supported the claim that NO is somehow central to the NH_3-oxidation pathway of the AOA (Shen *et al.*, 2013; Martens-Habbena *et al.*, 2015; Stahl and la Torre, 2012), however, no work has conclusively answered where in the pathways it might be important or if it acts as an oxidant or reductant. The role of NO as an intermediate in the NH_3-oxidation pathway of AOA as well as an explanation of N_2O measurements from AOA in pure and enrichment culture are answered with the work and resultant publication of Chapter 4 (Kozlowski *et al.*, 2016). Comparative analysis of instantaneous production of nitrogen oxides NO and N_2O during and following NH_3-oxidation by *N. viennensis* and *N. multiformis*, abiotic media controls, and the NO-metabolism of *N. viennensis* during oxidation of the intermediate NH_2OH all facilitated the conceptualization of a model describing the NH_3-oxidation pathway of AOA as well as the mechanism behind N_2O production. Major findings included that NO is co-oxidized with NH_2OH in the intermediate step of NH_3-oxidation by AOA to conserve reductant and that AOA

release large quantities of NO when O_2 is no longer available for substrate oxidation leading to abiotic production of N_2O as facilitated by reduced metals in the growth medium. This data explained the futility of AOA to ever harbor annotated dissimilatory nitric oxide reductases as NO is an essential intermediate. Also it showed why N_2O could be measured in the headspace of AOA in pure or enrichment culture and have a hybrid signature, as seen previously from *N. viennensis* (Stieglmeier *et al.*, 2014). Additionally, the importance of abiotic transformations of NO introduced a new aspect to N_2O production by ammonia-oxidizing microorganisms. Finally, on a per cell basis the work demonstrated the importance of comparative physiology over use of gene markers alone as tools for forming hypotheses about the activity of microorganisms in the environment, especially when considering the study of nitrogen oxide metabolism.

5.3 Future directions in Nitrification

The present body of work answered many existing questions in the field of nitrification and greatly furthers our understanding of ammonia-oxidizers and their metabolism for nitrogen oxides. Looking forward, there exist many interesting and important directions for future work in the field including:

1. **Production of N_2O during steady state growth of AOB**. Most work involved in characterizing nitrogen oxide production from AOB focused on batch growth or instantaneous measurements of N_2O. Chapter 2 and 3 give some insights into the presence/absence of genes with implications in NOx metabolism and how they are involved in the process, however it is hypothesized that steady state growth under variable O_2 tensions could be used to identify the conditions under which these genes are

upregulated. Transcriptomic analysis could provide insight into when the AOB begin the passage of reductant to dissimilatory NO_2^- reduction instead of to O_2 reduction and PMF generation.

2. **Genome-wide comparative analysis of the *Nitrosomonas* genus.** With the addition of two new and closed *Nitrosomonas* genomes (Appendix A and B) and the first phylogenetic tree of AOB made with available sequenced genomes (Chapter 3), a reconsideration of the genera is required. Whole-genome phylogeny showcases that many AOB have a unique grouping and further analysis is proposed to lead to updated descriptions of new genera within the betaproteobacterial AOB. In further support of this, more sequenced and closed AOB genomes should be made available to strengthen genome-wide analysis of the AOB.
3. **Elucidation of AOB alternate nitrite reductase(s).** Work on AOB N_2O production pathways in Chapter 2 and 3 showed that *N. europaea* and *N. communis* likely have alternate nitrite reductases active in the nitrifier denitrification pathway. Work to identify (this) novel enzyme(s), possibly through the creation of an in-gel assay for nitrite reduction or other proteomic work, could identify further genes of AOB NOx metabolism.
4. **Identification of the AOA copper-containing cytochrome *c* P460-like NH_2OH and NO co-oxidase.** Chapter 4 showcases the unique and tight control AOA have over NO, specifically so it can be co-oxidized with NH_2OH to form NO_2^-. Identification of this enzyme with novel function in the NH_3-oxidation pathway of AOA would allow for use of the gene in activity and abundance studies and be a stronger marker than AMO for thaumarchaeotal N-transformation in many environments. It is hypothesized that in

proteomics studies this enzyme performing the intermediate step of NH_2OH oxidation will be found in very high abundance.

5. **Addition of NO-release as a physiological marker for nitrifier contributions to N_2O production.** This thesis has on many occasions highlighted that ammonia-oxidizers don't require the capacity to reduce NO to N_2O to be involved in overall N_2O production. The metabolism for NO release and environmental parameters known to facilitate abiotic NO reduction should all be taken into account in future studies of nitrogen oxide production by ammonia-oxidizers.

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Appendix A

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AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements

Genome Sequence of *Nitrosomonas communis* Strain Nm2, a Mesophilic Ammonia-Oxidizing Bacterium Isolated from Mediterranean Soil

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The complete genome sequence of *Nitrosomonas communis* strain Nm2, a mesophilic betaproteobacterial ammonia oxidizer isolated from Mediterranean soils in Corfu, Greece, is reported here. This is the first genome to describe a cluster 8 *Nitrosomonas* species and represents an ammonia-oxidizing bacterium commonly found in terrestrial ecosystems.

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The isolation and study of ammonia-oxidizing bacteria (AOB) are essential to furthering our understanding of their role in nitrogen cycling across diverse environments, and particularly their contribution to nitrate pollution and nitrous oxide (N_2O) emissions. The AOB *Nitrosomonas communis* Nm2 was first isolated from Corfu, Greece (1), and is a mesophilic aerobic betaproteobacterium belonging to *Nitrosomonas* cluster 8 (2).

The genome of *N. communis* was sequenced at the University of Washington, WA, using the PacBio RSII platform; 150,292 raw reads resulted in 110,554 quality-filtered trimmed reads yielding 429.2 Mb, with a mean genome-wide coverage of 96×. The filtered reads were assembled at the University of Alberta, Alberta, Canada, using HGAP version 2.3 (3), resulting in a 1-contig scaffold. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (4). The genome is 4.07 Mbp, with a mean G+C content of 44.73%, and it contains 3,189 predicted protein-coding genes. The genome includes 41 tRNA genes and a single copy of the 16S-23S-5S rRNA operon. Gene prediction analysis and comparative genomics were performed with IMG (5). The closest neighbor of *N. communis* was *Nitrosomonas* sp. Is79 (6), with an average nucleotide identity (ANI) (7) of 72.02%.

N. communis oxidizes ammonia to nitrite as a sole source of energy and reductant. The genome contains 2 operons for ammonia monooxygenase (*amoCAB*), one of which is followed by genes for copper transport and resistance proteins, *copCD* (8, 9). Three operons for hydroxylamine dehydrogenase (*haoAB-cycAB*) were found, one of which lacked a copy of *cycB*, a feature found in both *Nitrosomonas europaea* and *Nitrosomonas eutropha* (8, 9). A single copy of the AOB-specific red-copper protein nitrosocyanin (10) was also found. Although genes for a complete urease are present in some *Nitrosospira* genomes (11, 12), *N. communis* encodes only the urea carboxylase (EC 6.3.4.6) and putative allophanate hydrolase (EC 3.5.1.54), similar to inventory found in the oligotrophic *Nitrosomonas* sp. Is79 (6, 13). Genes for carbon fixation, including two copies of RubisCO-encoding genes, were identified with similarity to those found in *Nitrosomonas* sp. Is79 (6). AOB living in terrestrial ecosystems can experience high nitrogen loads and varied oxygen tensions, conditions connected with increased production of nitrous oxide. Inventory for nitrifier denitrification was identified in *N. communis*; two nitric oxide reductases (*norCBQD* and *norSY-senC-orf1*), two copies of the NO-responsive regulator *NnrS*, cytochrome P460 (*cytL*), and cytochrome *c'*-beta (14). Although the majority of AOB genomes contain *nirK* (15), the gene for the copper-containing nitrite reductase, no homologues were found in *N. communis* for either *nirK* or the cytochrome cd_1 *nirS* nitrite reductases; however, a recent study showed that NirK is not required for nitrifier denitrification in *N. europaea* (16).

Genes for nickel-iron-containing, NAD-reducing, hydrogen dehydrogenase were identified, with the exception of *hoxH* (11). For iron acquisition and storage, *N. communis* contains one set of genes for a *Streptococcus*-like ferric iron ABC transporter (8), three copies of TonB-associated ferric siderophore transporters (17), genes for siderophore synthesis and export, and three copies of bacterioferritin and associated genes for intracellular iron storage.

Nucleotide sequence accession number. The genome sequence has been deposited in GenBank under the accession number CP011451. The version described in this paper is the first version.

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Appendix B

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Complete Genome Sequence of *Nitrosomonas ureae* Strain Nm10, an Oligotrophic Group 6a Nitrosomonad

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The complete genome of *Nitrosomonas ureae* strain Nm10, a mesophilic betaproteobacterial ammonia oxidizer isolated from Mediterranean soils in Sardinia, Italy, is reported here. This genome represents a cluster 6a nitrosomonad.

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The isolation and genome sequencing of ammonia-oxidizing bacteria (AOB) remain vital to our understanding of the potential roles these organisms play in the global nitrogen cycle. To complement physiological studies on AOB, complete-genome sequences provide insight into how inventory relates to metabolic capacity and environmental niche. The AOB *Nitrosomonas ureae* Nm10 was first isolated from soils in Sardinia, Italy (1), and is an oligotrophic aerobic betaproteobacterium belonging to *Nitrosomonas* cluster 6a (2).

The genome of *N. ureae* was sequenced at the University of Washington, WA, using the PacBio RSII platform; 300,584 raw reads resulted in 166,852 quality-filtered trimmed reads yielding 1,340 Mb, with a mean genome-wide coverage of 311×. The filtered reads were assembled at the University of Alberta, Alberta, Canada, using HGAP version 2.3 (3), and resulted in a 1-contig scaffold. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (4). The genome is 3.3 Mbp, with a mean G+C content of 44.5% and 2,897 predicted protein-coding genes. The genome includes 40 tRNA genes and a single copy of the 16S-23S-5S rRNA operon. Gene prediction analysis and comparative genomics were performed with IMG (5). The closest neighbor of *N. ureae* is *Nitrosomonas* sp. strain AL212 (6), with an average nucleotide identity (ANI) (7) of 93.18%.

N. ureae oxidizes ammonia to nitrite as a sole source of energy and reductant. The genome contains 3 operons for ammonia monooxygenase (*amoCAB*), two of which are followed by the *orf4* and *orf5* genes that are often found in β-AOB (8). Two orphan *amoC* genes were also identified, along with a single copy of the AOB-specific red-copper protein nitrosocyanin (9). It is important to note that this is the first report of an AOB containing four complete operons for hydroxylamine dehydrogenase (*haoAB-cycAB*), as betaproteobacterial AOB usually contain 2 or 3 copies, and one copy often lacks the *cycB* gene (8).

N. ureae can utilize urea as an alternate nitrogen source (1) and contains both urea carboxylase (EC 6.3.4.6) and a putative allophanate hydrolase (EC 3.5.1.54) genes (10), as well as genes for a complete urease found in some *Nitrosospira* genomes (11). Carbon fixation genes, including two copies of form I RubisCO-encoding genes, were identified with similarity to those of *Nitrosomonas* sp. strain Is79 (12).

Terrestrial AOB can contribute to nitrogen-oxide release, including the production of nitric and nitrous oxide through nitrifier denitrification (13, 14). The genes in *N. ureae* that are implicated in this process include a copper-containing nitrite reductase (*nirK*), NO-responsive regulator *NnrS*, cytochrome P460 (*cytL*), and cytochrome *c*′ beta (*cytS*). Interestingly, no homologues for nitric oxide reductases were found in the genome, a featured shared by the closely related 6a AOB *Nitrosomonas* sp. Is79 (12).

The *N. ureae* genes for iron acquisition and storage include one copy of the ferric uptake regulation protein (FUR) (15), a *Streptococcus*-like ferric iron ABC transporter (16), two copies of TonB-associated ferric siderophore transporters (17), and two copies of bacterioferritin genes. Two copies of cyanophycin synthetase genes, utilized for nitrogen storage (18), were also identified.

Nucleotide sequence accession numbers. The genome sequence has been deposited in GenBank under the accession no. CP013341. The version described in this paper is the first version, CP013341.1.

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