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Insights into the physiology of ammonia-oxidizing microorganisms

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Nitrification is the aerobic process of the nitrogen cycle that converts ammonia to nitrate and is facilitated by ammonia-oxidizing and nitrite-oxidizing microorganisms. Ammonia-oxidizers are unique chemolithotrophs that evolved specialized networks of electron carriers to generate proton motive force using ammonia as a sole energy source as well as mechanisms to tolerate cytotoxic intermediates of their metabolism. Cultivation and genome sequencing of ammonia-oxidizing bacteria (AOB), archaea (AOA), and comammox bacteria (i.e. COMplete AMMonia OXidizers) have revealed new enzymology, mechanisms to tolerate low pH and hypoxia, and mechanisms for production of the potent greenhouse gas, nitrous oxide. The role of ammonia-oxidizers in natural and engineered environments is of keen interest as they are essential to the nitrogen cycle, wastewater treatment, and flux of greenhouse gases to the atmosphere.

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Introduction

Nitrification is a microbial process that aerobically converts ammonia to nitrate, and joins nitrogen fixation and denitrification as primary functions of the global nitrogen cycle [1]. Nitrification is initiated by the oxidation of ammonia by specialized groups of bacteria 'AOB' and archaea 'AOA.' For nearly 125 years, ammonia-oxidizing microorganisms were thought to produce only nitrite from their metabolism, which was released as the substrate for nitrite-oxidizing bacteria to produce nitrate. In 2015, the first reports of 'comammox,' a bacterium that performs complete ammonia oxidation from ammonia to nitrate, were published [2,3]. This discovery has renewed interest in characterizing and discriminating the enzymology, regulation, and respective niches of AOB, AOA, and comammox in natural and engineered environments. This review primarily focuses on studies published from 2016 to 2018, covering the topics of isolation and cultivation of new ammonia-oxidizers, genomics and metabolic modeling, enzymology, and roles of ammonia-oxidizers in complex ecosystems. Because of the contribution of ammonia-oxidizers to the potent greenhouse gas, nitrous oxide (N_2O), insights into pathways and abiotic processes leading to N_2O are highlighted.

Cultivation of new ammonia-oxidizers

Our understanding of ammonia-oxidizers and the complexities of their physiology has been strongly facilitated by studying axenic cultures. The first isolation of an ammonia-oxidizer, *Nitrosomonas europaea*, was reported in 1890 by Sergei Winogradsky [4]. Since then, several strains of AOB representing two classes of Proteobacteria (Betaproteobacteria and Gammaproteobacteria), AOA in the subphylum Thaumarchaeota, and comammox bacteria in the Nitrospirae phylum have been brought into culture from numerous ecosystems including soils, freshwater, marine systems, estuaries, hot springs, hot water pipe biofilms, aquaria, sediments, wastewater treatment facilities, drinking water systems, and many others [5,6••]. Isolation of ammonia-oxidizers is not a trivial task as they resist losing the tight partnerships formed with microbes that detoxify their metabolic intermediates, protect them from oxidative stress [7], or perform reciprocal feeding functions, such as cyanate degradation to ammonia by associated nitrite-oxidizers [8]. Ammonia-oxidizers often have long generation times and are sensitive to environmental factors like substrate concentration, temperature, light, pH, and oxygen [9]. Ammonia-oxidizers are also highly sensitive to reactive oxygen species. The requirement of pyruvate in cultivation medium of AOA was initially thought to sustain mixotrophic metabolism, but was instead found to detoxify hydrogen peroxide [10•]. Because of the challenges with axenic cultivation, the majority of available genome sequences for AOB, AOA, and comammox bacteria are from metagenomic data or enrichment cultures rather than from isolates. Published reports of recently isolated ammonia-oxidizers into axenic culture are listed in Table 1.

Genomics and metabolic models

It has become commonplace to include a genome sequence with the report of a new microbial isolate

Table 1

Recently isolated ammonia-oxidizing microorganisms

solate	Year	Environment	Genome sequence	Ref.
Gammaproteobacteria (AOB)				
'*Candidatus* Nitrosoglobus terrae'	2017	Acidic soil	Yes	[45]
Nitrosococcus wardiae D1FHS	2016	Eutrophic marine sediment	No	[46]
Betaproteobacteria (AOB)				
Nitrosomonas sp. PY1	2017			
Nitrosomonas sp. NP1		Activated sludge	No	[47]
Nitrosomonas sp. SN1				
Nitrosomonas mobilis Ms1	2016	WWTP granules	Yes	[48]
Thaumarchaeota (AOA)				
'*Candidatus* Nitrosocaldus cavascurensis'	2018	Hot spring	Yes	[26]
'*Candidatus* Nitrosocaldus islandicus'	2018	Hot spring biofilm	Yes	[27]
Nitrosomarinus catalina SPOT01	2017	Temperate Pacific	Yes	[49]
Nitrosopumilus cabalaminigenes HCA1	2017	Tropical marine fish tank	No	[50]
Nitrosopumilus oxyclinae HCE1				
Nitrosopumilus ureiphilus PS0				
'*Candidatus* Nitrosocosmicus exaquare G61'	2017	WWTP	Yes	[51]
'*Candidatus* Nitrosopumilus sp. NF5'	2016	Adriatic Sea	Yes	[52]
'*Candidatus* Nitrosopumilus sp. D3C'				
'*Candidatus* Nitrosocosmicus franklandus'	2016	Neutral pH soil	No	[53]
Nitrospirae (Comammox)				
Nitrospira inopinata	2017	Hot water pipe biofilm	Yes	[6••]

Enrichment cultures, metagenomes, and unpublished strains are not included.

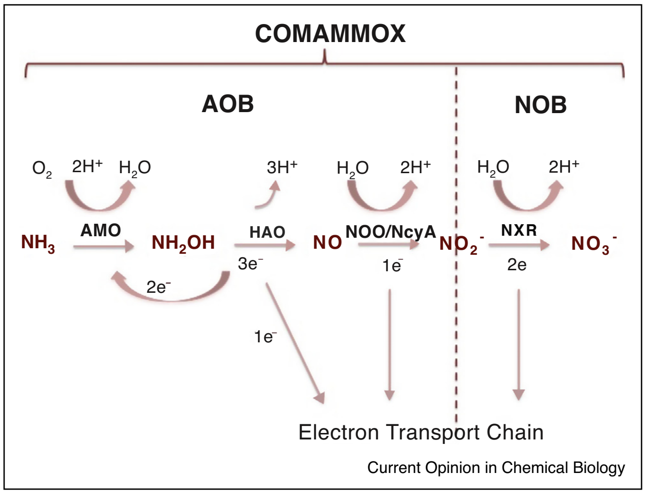
(Table 1). Access to genome sequence information enables characterization and comparison of interesting physiological, regulatory, and evolutionary features of microbes in addition to a platform for building predictive genome-scale metabolic models. Genomic inventory on its own assists with generating hypotheses for interesting physiologies, such as acid tolerance of the AOA isolate, '*Candiatus* Nitrosotalea devanaterra' [11] and related strains [12]. Comparison of four '*Ca.* Nitrosotalea' genome sequences with 23 other AOA genomes revealed 743 shared core proteins [12], which is fewer than the 860 shared core proteins reported in a prior AOA genome comparison [13]. All AOA genomes encode the 3-hydroxyproionate/4-hydroxybutyrate CO_2 fixation pathway, central carbon pathways, and enzymes for ammonia-oxidation. Unique genes in acid-tolerant '*Ca.* Nitrosotalea' genomes include a Na^+/solute symporter, metal transporters, and a chaperone specific for proline-rich proteins [12]. Soil AOA encode unique features such as envelope modifications for biofilm formation, polysaccharide production, and cell–cell adhesion [13]. A prior analysis of the '*Ca.* N. devanaterra' genome alone predicted 51 candidate genes conferring adaptations to low pH including high-affinity substrate transport, membrane impermeability, and a number of cation transporters [11]; however, the more robust comparative analysis found that all but 10 of these genes are in AOA genomes of non-acidophiles [12]. These analyses highlight the need to compare across as many genome sequences as possible to identify unique gene sets that confer specialized functions, but also suggests that gene inventory alone is an inaccurate predictor of specialized pathways and functions.

Genome-scale metabolic network modeling assembles layers of functional information (e.g. genomic, physiological, transcriptomic, proteomic, metabolomic) from a microorganism into a mathematical representation. When iteratively refined with experimental data, the model can accurately predict global metabolic responses of a microbe before confirmation with wet-lab experiments, thus extending functional information far beyond the genome sequence alone. Metabolic network models have been used to predict N_2O formation from nitric oxide (NO)-producing reactions in AOB consortia with nitrite-oxidizing bacteria [14,15] and to describe adaptation of *N. europaea* to anoxic-oxic transitions [16•]. The latter study found that exposure of *N. europaea* to repeated anoxic-oxic cycles resulted in a reduction of N_2O production and that long-term changes in flux of nitrogen oxide metabolites correlated to changes at the protein, rather than transcript, levels [16•]. The implication of this study is that N_2O emissions from AOB are likely overestimated in wastewater treatment operations that regularly utilize anoxic-oxic cycling. With rapidly increasing accessibility to genome sequences, 'omics, and physiological information on ammonia-oxidizing isolates, genome-scale metabolic network modeling is rapidly becoming a primary tool for resolving remaining questions on ammonia-oxidizer metabolism, whole genome regulation, and control of reactive metabolites, like NO and N_2O.

Enzymology of ammonia-oxidation

Ammonia oxidation is a highly specialized metabolism among prokaryotes due to the toxicity of its intermediates and products (i.e. hydroxylamine, NO and nitrite) and the requirement for specific electron carriers to deliver reductant to the quinone pool to generate proton motive force and ATP [1]. AOB, AOA, and comammox bacteria oxidize ammonia to hydroxylamine using conserved ammonia monooxygenase (AMO) enzymes [17,18]. All ammonia-oxidizers also oxidize hydroxylamine and produce nitrite, but the enzymology for this part of the pathway remains partially unresolved. In 2017, Caranto *et al.* demonstrated that the enzyme hydroxylamine dehydrogenase (HAO) of AOB oxidizes hydroxylamine to NO rather than nitrite [19••]. This finding overturned the decades-old model of direct oxidation of hydroxylamine to nitrite by HAO, and presented a new challenge to identify a nitric oxide oxidoreductase (NOO) (Figure 1). Thus far, two NOO candidates have been suggested for AOB: nitrosocyanin, enoded by *ncyA*, and reversely operating copper-containing nitrite reductase, encoded by *nirK* [20]. The *ncyA* gene is unique to AOB and is found in every AOB genome sequence thus far, except the oligotrophic isolate, *Nitrosomonas* sp. Is79 [21]. Proteomic analysis of three AOB species showed high expression of NcyA along with other enzymes in the ammonia-oxidation pathway, suggesting its role as an essential player [22]. Conversely, NirK expression in AOB is not constitutive, and is instead regulated by exposure to high ammonia [22], NO [23], and anoxia [16•]. NirK is also absent from some AOB genomes [24]. Furthermore, the *nirK* gene can be knocked-out in *N. europaea* with no ill effect on growth, validating its non-essential function at least in this model AOB strain [25].

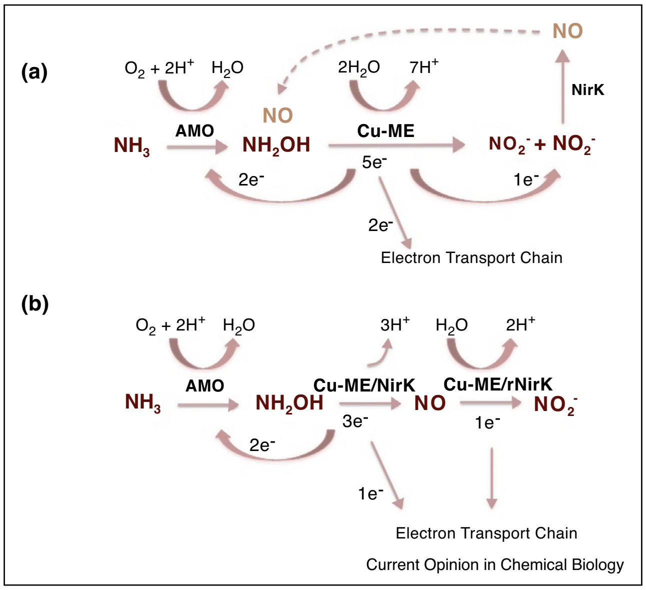
Figure 1



Pathways for energy conservation by ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and comammox bacteria. AMO = ammonia monooxygenase, HAO = hydroxylamine dehydrogenase, NOO = nitric oxide oxidoreductase, NXR = nitrite oxidoreductase. The NOO is proposed to be nitrosocyanin (NcyA) or an uncharacterized enzyme, with consideration to the lack of *ncyA* in the genome of *Nitrosomonas* sp. Is79.

The pathway is less clear for AOA as there are no heme-based enzymes encoded in AOA genomes; hence, the hydroxylamine-oxidizing and nitric-oxide oxidizing enzymes must be copper-based. NirK has been found in all AOA genomes except *Cenarcheaum symbiosum* and members of the *Nitrosocaldus* genus [13,26,27]. Yet unlike AOB, consistently high expression of *nirK* in metatranscriptome and transcriptome data sets suggests an essential function for several taxonomic clusters of AOA, especially those from marine environments [28]. NO is an essential metabolite in AOA that is produced and immediately consumed with tight control, whereas AOB tend to produce and release NO [29,30•]. Furthermore, ammonia-oxidation by AOA, but not AOB, is completely inhibited by the NO scavenger, PTIO, suggesting a requirement for free (i.e. able to be scavenged) NO in the AOA ammonia oxidation pathway [29,30•,31]. These observations led to the proposed pathway in Figure 2a, whereby hydroxylamine and NO act as co-substrates for an enzyme complex that produces two

Figure 2



Proposed energy conserving pathways for ammonia-oxidizing archaea (AOA). Panel **(a)**: After AMO oxidation of ammonia to hydroxylamine, a single enzyme complex co-oxidizes hydroxylamine and nitric oxide to two molecules of nitrite, with one molecule reduced to NO by the NirK nitrite reductase and returned back to the copper-based metalloenzyme complex (Cu-ME) as proposed by Kozlowski *et al.* [30•]. Panel **(b)**: The pathway is essentially the same as for AOB in Figure 1, in that two enzymes are proposed for the oxidation of hydroxylamine to nitrite: NirK either oxidizes hydroxylamine to NO, or oxidizes NO to nitrite (rNIR) in a reverse function, and a Cu-ME is either the hydroxylamine-oxidoreductase or NO-oxidoreductase depending on a yet-to-be determined role of NirK or other Cu-ME that can fulfill these functions. These models were proposed by Carini *et al.* from metagenome, transriptome, and metatranscriptome comparisons of marine AOA [28]. Neither model in Panel (b) accounts for the lack of *nirK* in genomes of *C. symbiosum* and *Nitrosocaldus* spp.

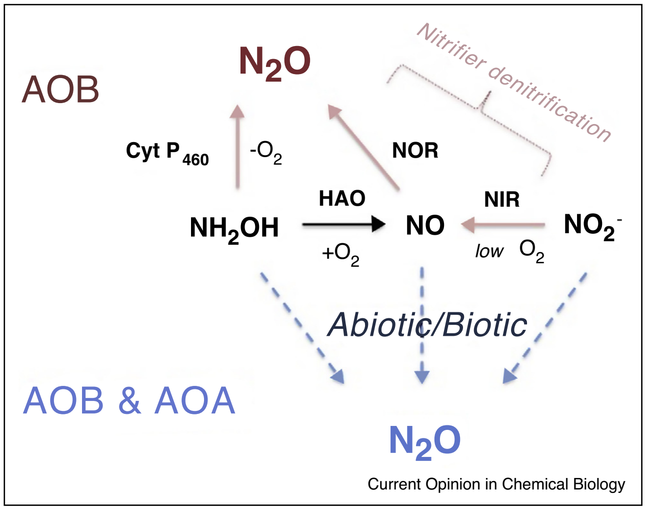
molecules of nitrite [30•]. In this model, NirK is an important enzyme for generating NO by reducing one of the two nitrite molecules back to NO to continue the process. As marine AOA co-express *nirK, amo* and several other genes within a coherent genomic module, two alternative models incorporate NirK as either a hydroxylamine-oxidizing or NO-oxidizing enzyme [28] (Figure 2b). Limited biochemical evidence suggests that NirK is not a suitable candidate for either of these reactions; purified NirK was shown to oxidize NO to nitrite at 1/100 the rate of the maximum forward reaction above pH 8 [32], and there is no evidence in the literature to support hydroxylamine as a native substrate for NirK. However, archaeal NirK enzymes have yet to be fully characterized and could potentially differ from their bacterial counterparts.

The physiology of comammox bacteria suggests that they are adapted to extremely oligotrophic environments [6••]. The isolate *Nitrospira inopinata* has a high affinity for ammonia, more in line with AOA than with AOB, but a much lower affinity for nitrite compared to canonical nitrite oxidizers [6••]. Core genes encoding enzyme complexes for ammonia oxidation, nitrite oxidation, urea hydrolysis, and carbon fixation via the rTCA cycle were identified in a comparison of several comammox bacterial genomes [33•], but like *Nitrosomonas* sp. Is79, comammox genomes lack *ncyA* (nitrosocyanin) but do encode *nirK* (nitrite reductase). Thus, as with AOB and AOA, the precise physiological role(s) of NirK and the identity of the NOO enzyme complex in comammox bacteria remain unresolved.

Ammonia-oxidizers in the environment and production of N_2O

Since the early 1980s, AOB have been recognized as significant producers of the greenhouse gas N_2O. Unabated fertilizer use, hypoxia in aquatic and coastal ecosystems subject to nitrate runoff, and mid-20th century wastewater treatment systems all stimulate N_2O production by resident AOB through an enzymatic process termed 'nitrifier denitrification' or from biotic and abiotic transformations of their metabolic intermediates (Figure 3). The majority of cultivated AOB encode and express NirK and nitric oxide reductase enzymes (NorB/Y), which in theory should reduce nitrite to NO and N_2O. However, only the NorB enzyme was found essential to nitrifier denitrification activity in a gene knock-out study of *N. europaea* [25]. Furthermore, oligotrophic strains that lack NorB cannot perform nitrifier denitrification [34] and the strain *Nitrosomonas communis* naturally lacks NirK, yet retains nitrifier denitrification activity [24,34]. These studies indicate that unknown nitrite reductases are yet to be discovered in the AOB and, again, the physiological role of NirK remains undefined. Other mechanisms of N_2O generation by AOB include the anaerobic oxidation of hydroxylamine by the enzyme cytochrome P_{460} [35] and abiotic reactions of metabolic intermediates — NO, hydroxylamine, and nitrite — with each other, with media components, and with metals [30•,36–39] (Figure 3).

Figure 3



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Pathways for N_2O production by AOB and AOA. Reactions with red arrows are catalyzed by enzymes and only by AOB. Nitrifier denitrification activity in AOB is stimulated at low O_2 concentrations to maintain intracellular redox balance and is not a respiratory pathway. Reactions with blue dashed arrows are abiotic, or biotic via partnering microorganisms, and occur for both AOB and AOA from intermediates produced during active ammonia oxidation.

Interestingly, AOA do not have the physiological capacity to perform nitrifier denitrification [30•]. Abiotic reactions of metabolic intermediates yield N_2O (Figure 3), which can be substantial where AOA are abundant and active, such as marine ecosystems [40]. Abiotic formation of N_2O from intermediates of AOA metabolism has also been confirmed in soils, and is favored under conditions of low ammonium supply [41,42]. These studies confirmed that production of N_2O by nitrifier denitrification in AOB is significantly greater than abiotic N_2O production by AOA, and is more favored in soils with high ammonium supply [42,43•]. Studies are underway to determine whether comammox bacteria contribute to N_2O emissions, but their strict oligotrophic lifestyle would indicate that they produce very little, if any [6••]. It should be noted, however, that elucidation of specific sources of N_2O is an ongoing methodological challenge, complicated by the lack of interlaboratory calibration of standards and instrumentation [44].

Conclusions

Our understanding of ammonia-oxidizing microorganisms has accelerated over the past few years through the discovery and cultivation of novel microbes, a rapid proliferation of genome sequence and functional ‘omics information, intensive biochemical work that both expanded functionality of known enzymes and challenged us to identify novel enzymes, and ecosystem studies that revealed interconnections of ammonia-oxidizers with each other and with partner microbes. We now recognize that both enzymology and abiotic interactions contribute to the generation of atmospheric N_2O by ammonia-oxidizers and that diverse mechanisms exist. We also recognize that NO is the pivotal intermediate in all ammonia-oxidation pathways. Remaining questions include the identification of NOO enzymes, the diverse roles of NirK and cupredoxins in production/consumption of NO, the capacity for comammox bacteria to produce N_2O (if at all), and genomic-regulatory features that enable specialized functions and niche preference among AOB, AOA, and comammox bacteria. Resolution of these questions will enable real-world mitigation for fertilizer overuse and N_2O production and harnessing of ammonia-oxidizers for industrial and environmental purposes. Many revelations still await as key aspects of these fascinating microorganisms and their unique metabolism remain unresolved.

Conflict of interest statement

Nothing declared.

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