Physiology and Diversity of Ammonia-Oxidizing Archaea

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Abstract

The discovery of ammonia-oxidizing archaea (AOA), now generally recognized to exert primary control over ammonia oxidation in terrestrial, marine, and geothermal habitats, necessitates a reassessment of the nitrogen cycle. In particular, the unusually high affinity of marine and terrestrial AOA for ammonia indicates that this group may determine the oxidation state of nitrogen available to associated micro- and macrobiota, altering our current understanding of trophic interactions. Initial comparative genomics and physiological studies have revealed a novel, and as yet unresolved, primarily copper-based pathway for ammonia oxidation and respiration distinct from that of known ammonia-oxidizing bacteria and possibly relevant to the production of atmospherically active nitrogen oxides. Comparative studies also provide compelling evidence that the lineage of *Archaea* with which the AOA affiliate is sufficiently divergent to justify the creation of a novel phylum, the *Thaumarchaeota*.

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INTRODUCTION

Our understanding of the microbiological underpinnings of the global nitrogen cycle was significantly altered in both 1999 and 2005. The year 1999 marked the first formal description of an organism mediating anaerobic ammonia oxidation (72), and in 2005 the first description of an ammonia-oxidizing archaeon (AOA) was published (36). Anaerobic decomposition of ammonia to dinitrogen gas via the anammox reaction is now recognized to be a major, if not dominant, process in many natural systems such as the Black Sea and certain marine oxygen-minimum zones (38, 39). The more recent revision in our understanding of the nitrogen cycle followed the isolation of the first AOA, *Nitrosopumilus maritimus* [*nitrosus* (Latin): nitrous; *pumilus* (Latin): dwarf; *maritimus* (Latin): of the sea], a small marine organism closely related to an abundant population of planktonic marine archaea first identified in the 1990s by Furhman et al. (21) and DeLong (17) by molecular typing of 16S rRNA genes.

However, the biogeochemical significance of marine archaea, comprising 20–40% of marine bacterioplankton (34), had remained mysterious until data from marine metagenome sequencing reported an archaeal open-reading frame coding for a protein distantly related to monooxygenases of ammonia-oxidizing bacteria (AOB) and methanotrophs (77). In these bacteria the monooxygenase activates either ammonia or methane for further metabolic transformation by inserting one atom from molecular oxygen into the substrate, yielding hydroxylamine or methanol. A homologous gene was subsequently described in an archaeal fosmid clone derived from a soil metagenome study (75). And, more recently, a related but functionally distinct monooxygenase, functioning in the hydroxylation of butane, was described for a bacterial butane oxidizer (64). The presumptive annotation of the archaeal homologs served for speculation that some marine archaea may function as ammonia oxidizers (77).

Interestingly, the alternative possibility that these divergent monooxygenases from marine and soil archaea served an alternative catabolic function was surprisingly not formally considered. The bacterial particulate methane monooxygenase (pMMO) and bacterial ammonia monooxygenase (AMO) share an amino acid similarity of approximately 74% (64). In contrast, the putative archaeal AMO was only distantly related to the bacterial pMMO and AMO. Thus, a functional assignment

AOA: ammonia-oxidizing archaea

Monooxygenase: an enzyme that incorporates one atom of molecular oxygen into its substrate as a hydroxyl group

AOB: ammonia-oxidizing bacteria

MMO: methane monooxygenase

AMO: ammonia monooxygenase

to ammonia oxidation was based on only ~40% amino acid similarity. For perspective, the BmoA subunit of the recently described putative butane monooxygenase from a gram-positive bacterium (*Nocardioides* sp. strain CF8) is more closely related to proteobacterial PmoA and AmoA sequences (37–38% average amino acid identity) than to the putative archaeal AmoA sequence (20% average amino acid identity) (64). However, given the importance of ammonia oxidation in contributing to both positive and negative environmental impacts, and the possibility that a major player in the nitrogen cycle had been overlooked since the pioneering work of Sergei Winogradsky in the late nineteenth century (81), the metagenome annotation did engender some attention. Nonetheless, it was only the isolation of a representative microorganism that conclusively demonstrated the physiological capacity of ammonia oxidation and that fully caught the attention of oceanographers, limnologists, and soil scientists (36). Numerous studies and reviews published in the past five years now provide persuasive data in support of the general dominance of archaea in controlling the fate of ammonia in both soil and aquatic systems (42, 57, 76, 84).

AmoA, AmoB, AmoC: three protein subunits composing the ammonia monooxygenase

A BRIEF HISTORY OF DISCOVERY

The paths to discovery are rarely direct, and the backstories generally not published nor attributable to an individual effort. The discovery of AOA is no exception, and we relate events that led to the isolation of *N. maritimus*. Our initial indication of a novel group of ammonia oxidizers came from our studies in the late 1990s as part of a census of bacterial diversity in the nitrifying reactor systems used to treat water in the large saltwater aquaria at the Shedd Aquarium (Chicago, Illinois). PCR amplification of bacterial 16S rRNA genes from DNA recovered from the saltwater aquaria failed to yield sequences closely related to known bacterial ammonia oxidizers (J.L. Flax & D.A. Stahl, unpublished observations). Because PCR amplification has its associated biases, the results were put aside to be revisited.

A few years later, similar observations were made in a study of the microbiology of nitrogen processing in Plum Island Sound (Massachusetts) estuary sediments (36). Again, general and specific primers for bacterial ammonia oxidizers failed to amplify the expected sequence types from nitrifying enrichments developed in collaboration with investigators at the Woods Hole Oceanographic Institution. However, by expanding the PCR-based screen to encompass archaeal 16S rRNA gene sequences from a clade of marine archaea then known as marine Group 1 crenarchaeota, related sequences were found to be abundant in the estuary sediment enrichments and in the nitrifying filtration systems at the Shedd Aquarium (36). Those results stimulated a follow-up study of nitrifying filtration systems at the Seattle Aquarium, which also revealed high representation of the same sequence type (36). Thus, an archaeal population closely related to the abundant Group 1 crenarchaeota was clearly implicated in ammonia oxidation. Subsequent development of enrichment cultures using the saltwater aquaria material as inoculant, and a medium containing a much lower concentration of ammonia than typically used to enrich bacterial ammonia oxidizers, yielded an active ammonia-oxidizing culture highly enriched in an archaeal population affiliated with the Group 1 crenarchaeota. Following another year or so of painstaking end-point dilutions, combined with selection by size and antibiotic resistance, the first pure culture of an AOA, *N. maritimus* strain SCM1, was described (36).

In a research environment where metagenomics and extensive environmental gene sequence surveys are increasingly common, this story offers a counterpoint. An organism relevant to a major environmental process was tracked down using culture-independent methods to first associate the process (ammonia oxidation) with a phylotype (Group 1 crenarchaeota) as a prelude to investing the significant effort necessary for isolation in culture. Since the isolation of *N. maritimus* in 2005,

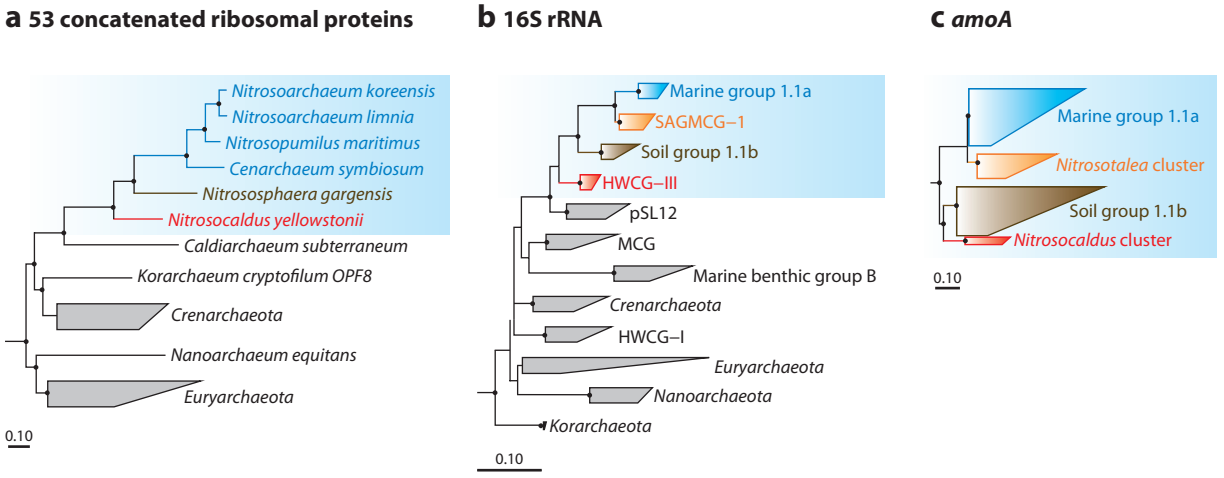


Figure 1

Phylogeny of ammonia-oxidizing archaea. (*a*) Maximum-likelihood analysis of 53 concatenated conserved ribosomal proteins constructed using 6,138 amino acid positions. Eukaryotic sequences were used as an outgroup. The tree was constructed using PhyML with an LG model with gamma correction (four site categories and using estimated proportion of invariable sites). (*b*) Maximum-likelihood tree of archaeal 16S ribosomal rRNA sequences, calculated using 594 positions and a Hasegawa-Kishino-Yano model with gamma correction (four site categories and using estimated proportion of invariable sites). Eukaryotic sequences were used as an outgroup. (*c*) Maximum-likelihood tree of archaeal *amoA* gene sequences, calculated using 1,265 positions and a Hasegawa-Kishino-Yano model with gamma correction (four site categories and using estimated proportion of invariable sites). Bacterial *amoA* and *pmoA* sequences were used as an outgroup. In all trees, nodes supported by bootstrap values greater than 80% (100 replicates) are indicated with a block dot. Blue background boxes indicate lineages belonging to the *Thaumarchaeota*. Abbreviations: SAGMCG-1, South African gold mine crenarchaeotic group 1; HWCG-I, hot water crenarchaeotic group I; HWCG-III, hot water crenarchaeotic group III; MCG, miscellaneous crenarchaeotic group.

the same general approach to enrichment and isolation has been used successfully to culture new AOA from soils and geothermal environments (5, 16, 24, 30, 41, 74).

PHYLOGENETIC DIVERSITY

Three major lineages of marine archaea were identified in the late 1990s, using 16S rRNA gene sequences as a proxy for resolving genetically distinct populations (phylotypes) (17, 21, 48). One lineage (Group 1) was proposed to be affiliated with the kingdom *Crenarchaeota* and the remaining two groups affiliated with the kingdom *Euryarchaeota*. Subsequent extensive 16S rRNA gene surveys of both soil and aquatic systems revealed that Group 1 archaea were widely distributed in moderate habitats. They were abundant throughout the marine water column (34), representing the dominant deep marine microbial population, and common in estuaries (3, 4, 14), sediments (26, 44, 65, 79), and soils (31). As additional sequences were compiled, additional divisions related to the Group 1 crenarchaeota were defined (**Figure 1**). The majority of soil and marine sequences could be assigned to two clades, defined as Group 1.1a (marine) and Group 1.1b (soil). Because no organism was available in culture, insights into the biogeochemical significance of these globally distributed and abundant microorganisms were initially inferred from in situ measurements of natural isotopic composition of diagnostic ether lipids (27, 55) and from incorporation of radiolabeled organic and inorganic substrates (25, 53, 82). These analyses suggested a significant inorganic carbon source of cellular carbon by the deep-water marine Group 1 population but also established a capacity to assimilate organic material. The physiological basis for these assimilation

properties remained unknown until the isolation of the AOA and the analysis of their genomes (23, 80).

THAUMARCHAEOTA: A NEW DIVISION WITHIN THE *ARCHAEA*

Since the description of *N. maritimus*, the phylogenetic diversity of AOA has been under constant revision as new organisms are described. The current census reveals an assemblage that spans tremendous depth within the *Archaea*, affiliated with marine and soil groups initially assigned to the *Crenarchaeota* on the basis of comparative 16S rRNA sequence comparisons (**Figure 1**), and more divergent clades derived from geothermal environments. Phylogenetic inference based on larger sets of phylogenetically informative genes (including concatenated ribosomal protein sequence alignments) now made available by complete genome sequences has provided support for the creation of a new division within the *Archaea*, the *Thaumarchaeota*, with which all characterized AOA affiliate (7, 71).

The current understanding of phylogenetic and physiological diversity within the *Thaumarchaeota* Group 1 marine and soil archaea is based primarily on 16S rRNA sequence diversity. The few cultures of AOA can be used to physiologically anchor some of the clades initially inferred from environmentally derived 16S rRNA sequences. However, because all described members of this new kingdom have been brought into culture owing to an ability to grow via ammonia oxidation, we are left with the unusual impression that ammonia oxidation is the defining characteristic of this newly defined division, as is methanogenesis a defining characteristic of the *Euryarchaeota*. The possibility that a deeply diverging group within the *Archaea* split from the main line following development of the capacity to grow by extracting energy and electrons from ammonia would raise fundamental questions concerning both the origins of the *Archaea* and the age of the contemporary nitrogen cycle. Deeply diverging clades within the *Archaea* are dominated by anaerobic physiology, and early development of respiratory systems using oxygen as a terminal electron acceptor would seem to require an early nonphototrophic source of O_2 (11).

Thaumarchaeota: a recently defined division (phylum) within the *Archaea* encompassing all known ammonia-oxidizing archaea, including organisms previously classified as Group 1 crenarchaeota

Crenarchaeol: a glycerol dialkyl glycerol tetraether containing four cyclopentane moieties plus an additional cyclohexane moiety, associated specifically with *Thaumarchaeota*

HABITAT RANGE

The other remarkable picture emerging from the coalescence of culture-independent and culture-dependent techniques is the extraordinary range of habitats occupied by the AOA, far exceeding that of cultured bacterial ammonia oxidizers. Abundance and diversity patterns have been inferred primarily from sequencing and quantification of the gene coding for the putative archaeal *amoA* (2, 20), a measure generally well correlated with the abundance of a unique glycerol dialkyl glycerol tetraether, crenarchaeol, first associated with marine Group 1 archaea and now recognized to be made by both mesophilic and thermophilic AOA (15, 16, 42, 54). AOA appear to be the dominant archaeal clade in soils (generally comprising 1–5% of all prokaryotes) (40, 51), are a dominant marine group (comprising 20–40% of all marine bacterioplankton) (12, 34), and appear to be a major ammonia-oxidizing population in geothermal habitats (16, 19, 60, 83). Thus, their habitat range far exceeds that of their known bacterial counterparts.

Those AOA now available in culture grow at temperatures as high as 74°C (*Nitrosocaldus yellowstonii*) (16) and at pH values as low as 4 (*Nitrosotalea devanaterra*) (41). The existence of thermophilic representatives of AOA was initially indicated by the recovery of the diagnostic glycerol dialkyl glycerol tetraether lipid (crenarchaeol) previously associated only with the marine Group 1.1a and by the amplification of the archaeal homolog of *amoA* from hot springs (54). The moderate thermophile *Nitrososphaera gargensis* [*nitrosus* (Latin): nitrous; *sphaera* (Latin): spherical;

gargensis (Latin): from Garga spring], enriched from a Siberian hot spring, has an optimum growth temperature of about 46°C (24).

Half-saturation constant (K_m or K_s): the concentration of a limiting nutrient supporting one-half the maximum growth rate of an organism

Continued surveys of geothermal habitats based on both nitrification rate measurements and selective amplification of the archaeal *amoA* and gene transcripts suggest their distribution includes hot springs with temperatures as high as 97°C and pH values as low as 2.5, but with greater diversity and abundance trending to springs with temperatures below 75°C (29, 60, 85). This broad habitat range is also reflected by the tremendous phylogenetic diversity of major archaeal lineages defined by 16S rRNA sequence types recognized to have ammonia-oxidizing affiliates and the corresponding diversity of putative archaeal *amoA* sequences (**Figure 1**).

PHYSIOLOGICAL PROPERTIES

Kinetics and Stoichiometry of Ammonia Oxidation

Initial physiological studies of *N. maritimus* strain SCM1, the only available marine isolate, have provided important insights into the basis for the ecological success of AOA. Studies of reaction stoichiometry using microrespirometry to measure oxygen and ammonia consumption relative to nitrite production showed that the overall stoichiometry of ammonia oxidation by *N. maritimus* is indistinguishable from that of AOB (46):

$$1\,NH_3 + 1.5\,O_2 \Rightarrow 1\,NO_2^- + H_2O + H^+.$$

Studies of ammonia oxidation kinetics subsequently demonstrated that this marine isolate is an extreme oligotroph, having an apparent half-saturation constant (K_m) for total ammonia (ammonium plus ammonia) of 132 nM (~3 nM NH_3 at near-neutral pH) (46). More than 50% of maximum activity could be elicited by the addition of 200 nM ammonium to resting cells. This is equivalent to the addition of one teaspoon of concentrated ammonium hydroxide to an Olympic size swimming pool. Furthermore, *N. maritimus* cells did not tolerate ammonia at concentrations significantly above 1 mM. Similar observations have been made with the moderately thermophilic AOA *N. gargensis* (24). In contrast, characterized AOB have K_m values that are more than 200-fold higher than the K_m value of *N. maritimus* (46). The extremely low apparent K_m and high maximum activity of *N. maritimus* contribute to a specific affinity for ammonia ($V_{max} \cdot K_m^{-1}$) of approximately 69,000 liter g [wet weight]$^{-1}$ h^{-1}. This is among the highest substrate affinity reported for any microorganism, not only for organic substrates but also for assimilation of ammonia by bacterial heterotrophs and marine phytoplankton for cellular growth (10, 46). As extreme oligophiles, the marine populations are plausibly responsible for maintaining oceanic concentrations of ammonia in the low nanomolar range, effectively outcompeting the bacterial ammonia oxidizers in accessing ammonia (46). In contrast to the very low ammonium concentrations required for growth, *N. maritimus* has an affinity for oxygen that is more typical of aerobic microorganisms (~4 μM) and is unable to grow anaerobically under culture conditions so far evaluated (D.A. Stahl & J.R. de la Torre, unpublished observations). Because AOA have been implicated in providing the nitrite used by anaerobic ammonia oxidizers in oxygen-minimum zones, such organisms may have significantly higher oxygen affinities than *N. maritimus*.

The soil AOA have been only more recently brought into culture and there is less direct physiological data available. *Nitrososphaera viennensis* [*nitrosus* (Latin): nitrous (nitrite producer); *sphaera* (Latin): spherically shaped; *viennensis* (Latin): from Vienna], isolated from garden soil in Vienna, Austria, tolerates higher concentrations of ammonia than does *N. maritimus*, demonstrating complete conversion of ammonia at initial ammonia concentrations as high as 3 mM (74). Growth can be initiated at higher concentrations (greater than 10 mM ammonia) but stopped when

nitrite originating from ammonia oxidation exceeds ~3 mM (74). Nitrite alone is not associated with growth inhibition, as growth can be initiated in fresh culture medium supplemented with 10-mM nitrite. Thus, an unknown metabolite or intermediate was invoked as inhibitory (74). These culture-based observations are generally consistent with in situ studies showing that AOA populations grow over a wide range of ammonia concentrations in contrast to AOB that require significantly higher ammonia concentrations to initiate growth (57, 78, 84). A more recently described AOA enriched from low pH soils, *Nitrosotalea devanaterra* [*nitrosus* (Latin): nitrous (nitrite producer); *talea* (Latin): slender rod; *devana* (Latin): Aberdeen; *terra* (Latin): soil], has an optimum pH between 4 and 5 (41). This marked the first description of an acidophilic ammonia oxidizer and provided a clear microbiological explanation for significant nitrification rates measured in acidic soils, even though no such capacity was known for AOB (6). The ability of *N. devanaterra* to grow at extremely low pH values is also suggestive that ammonium, rather than un-ionized ammonia, is the substrate for growth. One explanation for the failure of AOB to grow at pH values significantly below pH 7 is their requirement for the un-ionized form as growth substrate (1, 8). Because the concentration of the un-ionized ammonia form decreases 10-fold for every 1-unit reduction in pH, the AOB would become rapidly substrate limited as pH is lowered. For example, in a system containing a total concentration of ammonia/ammonium of 100 mg total N liter^{-1} (28°C), there is 7 mg NH_3-N liter^{-1} at pH 8 and only 0.0007 mg NH_3-N liter^{-1} at pH 4. A preference, or requirement, for ammonium may also be true of the marine populations, because at a half-saturation value of 100 nM for *N. maritimus*, the concentration of un-ionized ammonia near pH 7 is only approximately 1–3 nM. We therefore suspect that the AOA use a mechanism for the collection of ammonia for oxidation (catabolism) entirely different from that used for assimilation. If not so, a high-affinity catabolic pathway would impoverish an anabolic pathway used for cellular synthesis. These data point to a novel physiology and supporting biochemistry.

In soils, studies linking nitrification rates to the nitrifier abundance and activity (using molecular proxies such as *amoA* gene and transcript abundances) have suggested that high affinity for ammonia also appears to provide soil AOA with a competitive advantage over AOB. In general, AOB tend to dominate in systems receiving high direct additions of inorganic ammonia (28, 57), whereas systems sustained by mineralization (ammonification) of organic material select for AOA (18, 52). Thus, with representatives that function as extreme oligotrophs, these chemoautotrophic organisms presumably function as a key valve in nitrification by controlling the rate of ammonia oxidation, generally considered to be the rate-limiting step in nitrification. This has significant implications for the global nitrogen cycle and for trophic interactions in both terrestrial and marine environments. Nitrification serves a key function in the nitrogen cycle by providing the oxidized species of nitrogen (nitrite and nitrate) that are essential substrates for both denitrification and anaerobic ammonia oxidation, activities that serve to return fixed nitrogen to the relatively inert atmospheric form of molecular nitrogen. The recognition of archaeal groups adapted to extremely low ammonia concentrations, acidic pH, and high temperatures now points to a fully functional nitrogen cycle in a much greater range of habitats than previously recognized. In turn, if AOA effectively compete with phytoplankton, heterotrophs, and other autotrophs in the ocean, or with plants and other microorganisms in soils, this may mean that assimilation of ammonia is a minor pathway for the metabolism of ammonia released through mineralization of organic material. Thus, the AOA may be ammonia thieves and conceivably force other groups to invest reducing power in the reduction of nitrate/nitrite to ammonia for biosynthesis. Resolution of the major operative pathway, assimilation versus oxidation of ammonia released through mineralization, is then of great significance to developing a better understanding of microbial controls of nitrogen form and availability to associated micro- and macrobiota in both marine and terrestrial systems.

Evidence for Autotrophic, Mixotrophic, and Heterotrophic Growth

Available data suggest that the AOA are generally capable of fixing CO_2. In addition to the clear demonstration of autotrophic growth by *N. maritimus*, CO_2 fixation was previously inferred from environmental studies of the isotopic composition of signature lipids and microautoradiographic imaging of natural samples incubated with labeled organic or inorganic carbon (37, 53, 55). These data also suggested some ability for the incorporation of organic carbon (27). The latter is consistent with genome sequence annotation of *N. maritimus*, pointing to the presence of a number of transporters for organic molecules. Recent studies in our laboratories have demonstrated some stimulation of *N. maritimus* growth by the addition of central metabolism intermediates (e.g., pyruvate and α-ketoglutarate) (47). A larger set of test substrates (including amino acids and fatty acids) did not stimulate growth. *N. viennensis* is also capable of chemolithoautotrophic growth. Although growth was obligately coupled to ammonia oxidation, it could be significantly enhanced by including pyruvate in the growth medium (74).

In addition to a capacity for mixotrophic growth by the marine AOA, a recent publication from the Wagner and Head laboratories (50) suggests that some *Thaumarchaeota*, although expressing an *amoA* gene closely related to that of the Group 1.1b soil clade, lack the capacity to either fix CO_2 or oxidize ammonia. This study examined a population of presumptive AOA in a petroleum refinery wastewater treatment system. Initially identified by combined 16S rRNA gene and *amoA* sequence characterization, stimulation of the ammonia oxidizers in this reactor by ammonia addition failed to stimulate incorporation of radiolabeled CO_2 by the AOA, even though coresident AOB did incorporate label. Modeling studies suggested that the AOB enumerated in the same treatment system could account for all ammonia oxidation. Thus, although active transcription of the archaeal *amoA* was confirmed, there was no evidence of a contribution to ammonia oxidation. These authors suggested that, as has been recently reported for an organism harboring a butane monooxygenase closely related to bacterial AMO and pMMO (64), the AMO-like monooxygenase of this archaeal population functions in the biodegradation of organic material, a well-characterized function of hydroxylases of petroleum-degrading bacteria. These data further emphasize the difficulty in assigning function solely on the basis of homology, even when the gene codes for a closely related and functionally well-characterized enzyme. More generally, this raises the question of whether the abundance of *amoA* genes or gene transcripts in marine or terrestrial systems can or even should be directly associated with nitrification.

COMPARATIVE GENOMICS POINTS TO UNUSUAL BIOCHEMISTRY AND CELL BIOLOGY

The completion of the genome sequence (1.64 Mbp circular chromosome) of the first isolated AOA, *N. maritimus*, revealed three major deviations from the canonical bacterial system of ammonia oxidation and carbon fixation: (*a*) a role for copper (rather than iron) as the major redox active metal in electron transfer reactions, (*b*) the absence of any homolog to the bacterial oxidoreductase (hydroxylamine oxidoreductase, HAO) responsible for the oxidation of hydroxylamine to nitrite, and (*c*) a variant of the 3-hydroxypropionate/4-hydroxybutyrate cycle for CO_2 fixation (as opposed to fixation by the ribulose bisphosphate carboxylase/oxygenase of the Calvin-Bassham-Benson cycle employed by characterized bacterial ammonia oxidizers) (80). Thus, the current picture of carbon metabolism suggests that these representatives of the marine and soil AOA derive energy and electrons primarily from the oxidation of ammonia but can supplement carbon from CO_2 fixation using a limited set of simple compounds that feed directly into central metabolism. The combined

properties of having selective organic material uptake, CO_2 fixation by the 3-hydroxypropionate/4-hydroxybutyrate pathway, and the presence of a branched (incomplete) tricarboxylic acid (TCA) cycle now point to a relatively simple metabolic architecture of carbon assimilation and anabolism. Other distinctly novel features indicated by the genome sequences included an unusual system of cell division and the capacity to synthesize novel phosphonate compounds (80).

Phosphonate: organic compound containing a direct C-P bond

Nitroxyl (HNO): the one electron reduced and protonated congener of nitric oxide is a highly reactive nitrogen species implicated in mammalian cell signaling and as a possible intermediate in archaeal oxidation of ammonia

The Biochemistry of Archaeal Ammonia Oxidation

The most glaring problem presented by annotation of available genome sequences of AOA is the absence of the canonical bacterial pathway for ammonia oxidation (**Figure 2**). The available sequence information indicates that the AOA are missing all elements of the bacterial pathway other than genes coding for the presumptive AMO. *Nitrosopumilus* lacks a homolog of the bacterial HAO and the capacity for synthesis of *c*-type cytochromes (23, 80). Bacterial *c*-type cytochromes compose the redox-active centers of the HAO and mediate respiratory transfer of electrons from HAO to the terminal oxidase (**Figure 2**). These distinctive features had been previously suggested from the annotation of a metagenomic sequence assembled from a Group 1 symbiont of a marine sponge, *Cenarchaeum symbiosum* (23). Although the physiology of that uncultured archaeon is unknown, the general genome features are similar to that of *N. maritimus* and suggestive of a capacity for ammonia oxidation that may function in detoxification of sponge nitrogenous waste.

As yet there is no evidence that the product of ammonia oxidation by the archaeal AMO is hydroxylamine. An alternative pathway proposed by Klotz, Arp, and colleagues (80) suggested that nitroxyl (HNO) could be the product of the archaeal AMO (66) (**Figure 2**). Some clarification of the archaeal pathway for ammonia oxidation is anticipated to derive from genome comparisons of evolutionarily divergent species. Assuming a novel core pathway for ammonia oxidation is conserved among AOA (such as a novel Cu-based HAO), such features should be conserved across all lineages. *Nitrosocaldus yellowstonii* is now the most divergent representative of the AOA, distantly related to the marine and soil types, and as such a good candidate for such comparisons. As shown in **Figure 3**, the genes coding for the presumptive AMO remain the most diagnostic feature of an ammonia oxidation pathway common to all available genome sequences. However, initial comparative annotation of the *Nitrosocaldus* and *Nitrosopumilus* genomes has not yet served to further constrain the biochemistry of archaeal ammonia oxidation. In addition, only two small plastocyanin-like proteins are shared by all AOA. These redox-active copper proteins may participate in electron transfer from the unknown product of ammonia oxidation (e.g., hydroxylamine or nitroxyl) to a membrane-bound electron transfer chain (**Figure 2**).

Although the archaeal pathway for ammonia oxidation has not been resolved by comparative genomics, recent studies using nitric oxide (NO) sensitive microelectrodes are suggestive that NO may function in the biochemistry (47). Measurable amounts of NO are produced during ammonia oxidation. This has led to speculation that NO may be an intermediate or function as a redox shuttle, for instance, delivering electrons to the AMO (**Figure 2**). In contrast, the AOB draw electrons required by the monooxygenase from the membrane-associated quinone pool. Either the formation of nitroxyl as the first product of ammonia oxidation or the use of NO as an electron redox shuttle for hydroxylamine generation would eliminate the need to draw electrons directly from the quinone pool, either by obviating a requirement for reductant through formation of nitroxyl or by drawing electrons from a lower potential donor in the reduction of nitrite to NO (**Figure 2**). Equations 1 and 2 show possible recycling of an NO redox shuttle. The associated thermodynamic calculations assume that electrons for nitrite reduction originate from a donor species with an electrical potential (230 mV) approximately that of a c_1-type cytochrome and in

the known range of plastocyanins.

$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \quad 1.$$

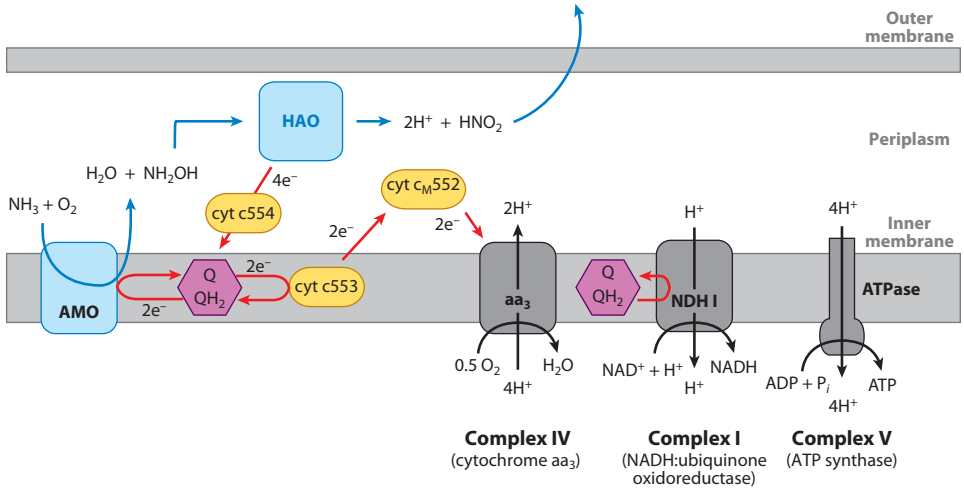
$$2NO + O_2 + NH_3 + H_2O \rightarrow NH_2OH + 2NO_2^- + 2H^+ \quad 2.$$

$$\text{Rx2}\ \Delta G^{o'} = -103.4 \frac{KJ}{mole\ NH_3}$$

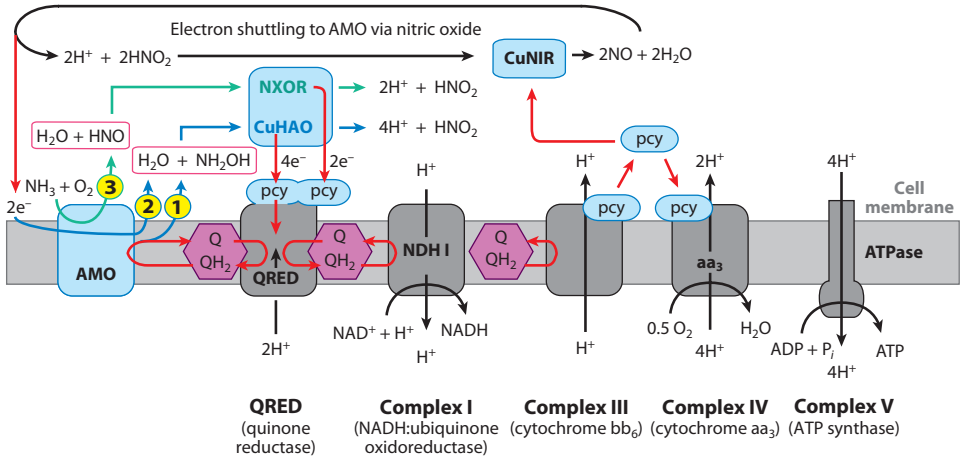
$$\text{Rx1}\ \Delta G^{o'} = -12.5 \frac{KJ}{mole\ NO_2^-}.$$

Because the formation of hydroxylamine as the immediate product of the presumptive AMO has not been demonstrated yet, the archaeal pathway for ammonia oxidation must be considered unresolved at this time.

a AOB (*Nitrosomonas europaea*)



b AOA (*Nitrosopumilus maritimus*)

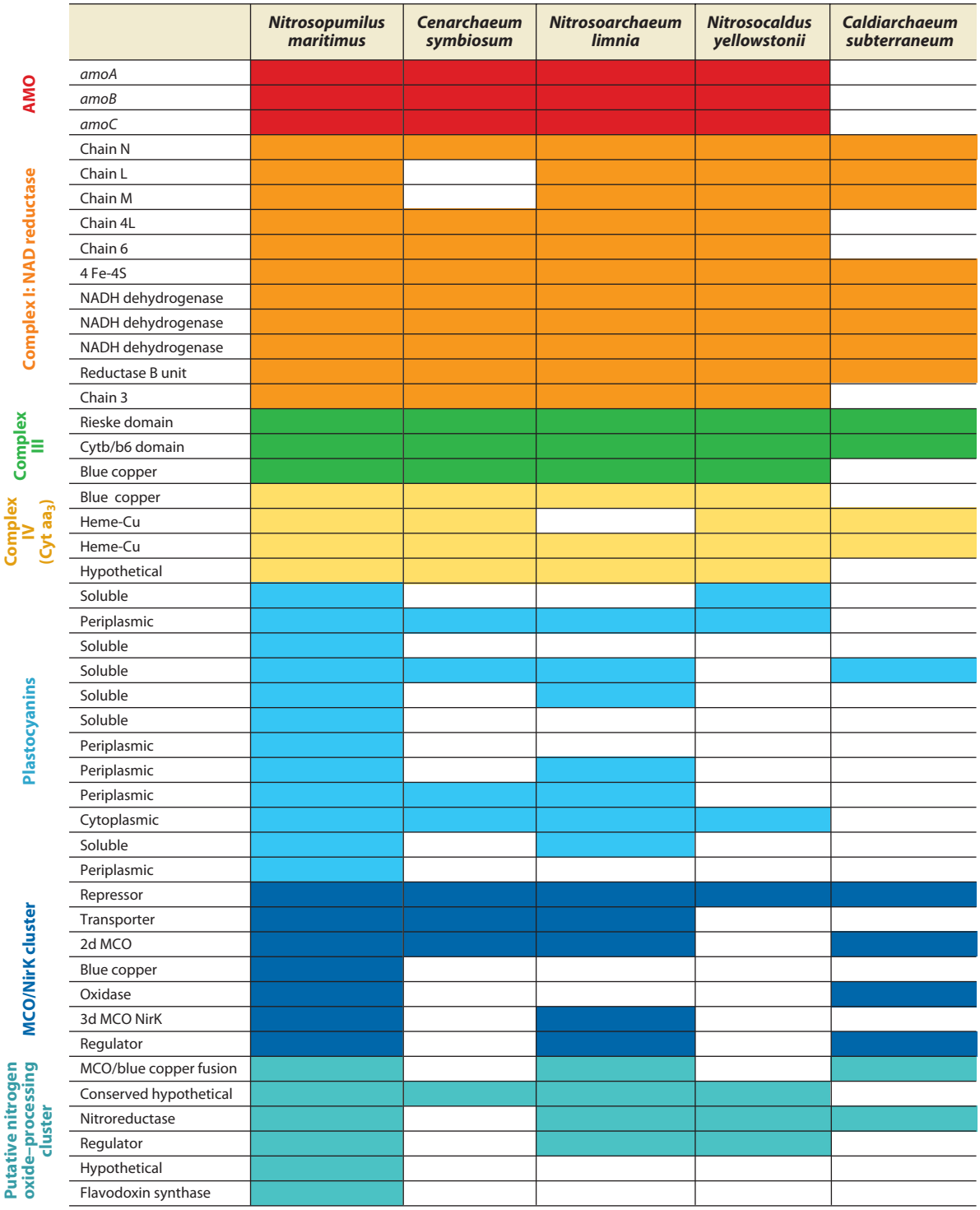


Cell Cycle and the Machinery of Cell Division

The AOA are distinctive in having genes coding for two alternative systems of cell division, the CdvABC-based and FtsZ-based division systems (43, 61). The recently described Cdv division system used by certain members of the *Crenarchaeota* is composed of three proteins, two of which (CdvB and CdvC) are homologs of the eukaryotic ESCRT-III–like sorting complex involved in vesicular sorting and cytokinesis (43, 62). The FtsZ-based division system, mediated by FtsZ protein filaments that form a constricting ring structure (45), is more widely distributed and is found in most major groups of bacteria and in the euryarchaeal and korarchaeotal branches of the *Archaea*. All the AOA genomes examined to date share the unusual characteristic of having genes diagnostic for both systems of cell division (5, 23, 35, 71, 80). Recent studies in collaboration with the Rolf Bernander group at the University of Uppsala used flow cytometry and immunofluorescence microscopy to examine the cell cycle and division system in *N. maritimus* (56). Fluorescence microscopy combined with cell staining using antibodies against the CdvA, CdvB, and CdvC proteins established that their expression was associated with cell division. Centrally positioned banding patterns of CdvA and CdvC were correlated with the presence of segregated nucleoids. Expression of two of three CdvB paralogs also correlated with segregated nucleoids, but neither formed distinct banding patterns. In contrast, the FtsZ protein was neither spatially nor temporally correlated with nucleoid segregation and strong FtsZ staining was observed in a majority of cells regardless of cell cycle state. Together, these results provided strong support for a Cdv system (ESCRT-III–like) in *N. maritimus*. As yet we can only speculate about possible function(s) of the FtsZ homolog. Hypothesized functions include a role in chromosome segregation or cell wall growth (9, 56).

Figure 2

Proposed respiratory pathways for ammonia oxidation in AOB and AOA. (*a*) Proposed pathway for ammonia oxidation in the AOB *Nitrosomonas europaea*. Ammonia is oxidized to NH_2OH by the membrane enzyme complex AMO. Subsequently, hydroxylamine is oxidized to nitrite in the periplasm by HAO. Four electrons from this oxidation are transferred to the quinone pool by cytochrome c554. Two electrons from the reduced quinone pool return to AMO and are required to initiate ammonia oxidation. The remaining two electrons enter the electron transport chain via cytochrome c553 and cytochrome c_M552 to generate the proton motive force necessary for ATP synthesis. (*b*) Proposed pathway for ammonia oxidation in the AOA *Nitrosopumilus maritimus*. Three alternative pathways are indicated in this speculative diagram. In pathways 1 and 2, the immediate product of ammonia oxidation by the archaeal AMO would be hydroxylamine. However, these two pathways differ in the origin of electrons required to initiate ammonia oxidation by the monooxygenase. Pathway 1 is of the bacterial type, in which electrons produced by the oxidation of hydroxylamine to nitrite by a presumed CuHAO are transferred to pcy electron carriers and then to the quinone pool by a membrane-associated QRED. Two electrons would be recycled to AMO and the remaining two electrons would be transferred to the electron transport chain. Pathway 2 speculates that NO, produced by the reduction of nitrite by a proposed CuNIR, is the source of electrons for AMO. The possibility that HNO is the product of the archaeal AMO is shown by pathway 3. This pathway would eliminate the requirement for electron recycling during the initial oxidation of ammonia. Subsequently, HNO would be oxidized to nitrite by a presumed NXOR. The two electrons extracted during this oxidation would be transferred to QRED and the electron transport chain as indicated above. Red arrows indicate electron flow. Blue shading denotes copper-containing proteins. Hexagons containing Q and QH_2 represent the oxidized and reduced quinone pool, respectively. Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NO, nitric oxide; HNO, nitroxyl; CuHAO, copper hydroxylamine oxidoreductase; CuNIR, copper-dependent nitrite reductase; NXOR, putative nitroxyl oxidoreductase; pcy, plastocyanins; NDH, NAD(P)H:quinone oxidoreductase; NH_2OH, hydroxylamine; QRED, quinone reductase. Figure adapted with permission from Reference 80.



Flow-cytometry-based analysis of the cell cycle also indicated a distinctive cell biology (56). The timing of the cell cycle of *N. maritimus* differs substantially from characterized hyperthermophilic crenarchaea, having a much longer prereplication phase (G_1) and a shorter postreplication phase (G_2, mitosis, and cell division). Replication of the small 1.64-Mbp genome required 15–18 h and tended to arrest if ammonia was depleted before replication was completed. We suspect slow replication and arrest may relate to adaptation to extreme nutrient limitation. Although ammonia is available only at generally low nanomolar concentrations in the open ocean, there is nonetheless a continuous supply of ammonia through mineralization of organic material. It is unlikely that oceanic populations of this organism ever experience complete ammonia depletion as occurs at the termination of growth in batch culture.

PEP: phosphoenolpyruvate

POTENTIAL EFFECTS ON ATMOSPHERIC CHEMISTRY

The ubiquitous and globally abundant AOA have recently been implicated as a direct or indirect source of the atmospherically reactive gasses methane (CH_4) and nitrous oxide (N_2O). The possibility that these organisms generate significant amounts of nitrous oxide was suspected by analogy with the activities of AOB known to produce N_2O in association with ammonia oxidation, or for some species to fully reduce nitrite to N_2O via a poorly characterized partial denitrification pathway (70). A recent study comparing the natural isotopic signature of bulk N_2O ($\delta^{15}N$ and $\delta^{18}O$ values) produced in cultures by AOA or AOB served to associate most oceanic production of N_2O with the AOA (63). Because the oceans are a source for as much as 30% of global N_2O inputs to the atmosphere, this observation provides additional impetus to resolve the biochemistry of archaeal ammonia oxidation.

An unsuspected association with atmospheric chemistry was the outcome of studies designed to identify phosphonate compounds predicted by the genome sequence to be synthesized by *N. maritimus*. Phosphonates are organic compounds containing a direct C-P bond and thus are distinct from the more common esterified form of phosphate. The family of biologically produced phosphonates includes antibiotics, modified extracellular polysaccharides, lipids, and phosphorus storage compounds (49). Although poorly characterized structurally, phosphonates comprise 20–30% of organic phosphorus in the oceans (13) and thus are an important source of phosphorus for organisms expressing the C-P lyase necessary to cleave the bond and release the phosphate (32, 58). A gene in the *N. maritimus* genome annotated as coding for phosphoenolpyruvate (PEP) mutase, the enzyme catalyzing the first step in a variety of pathways for phosphonate biosynthesis (conversion of PEP to phosphonopyruvate) (68, 69), was associated with a gene cluster implicated in production of both a novel phosphonate and extracellular polysaccharides. Collaborative studies between the Metcalf and van der Donk groups have since established the in vitro production of methylphosphonic acid by enzymes coded by genes in this cluster and confirmed the presence of methylphosphonate in whole-cell extracts by NMR (W.W. Metcalf, B.M. Griffin, R.M. Cicchillo,

Figure 3

Distribution of genes thought to play key roles in ammonia oxidation and energy generation in genome sequences of representative AOA and of the related archaeon "*Candidatus* Caldiarchaeum subterraneum" (of the proposed candidate phylum *Aigarchaeota*). Colored boxes indicate the presence of orthologs in each genome. Identification and clustering of orthologs were computed using OrthoMCL v1.4 and an E-value cutoff of 1E-10. Abbreviations: AOA, ammonia-oxidizing archaea; AMO, ammonia monooxygenase; MCO, multicopper oxidase; NAD+/NADH, nicotinamide adenine dinucleotide (oxidized and reduced forms, respectively).

J. Gao, S.C. Janga, H.A. Cooke, B.T. Circello, B.S. Evans, W. Martens-Habbena, D.A. Stahl & W.A. van der Donk, manuscript submitted).

Discovery of a biological source of methylphosphonate may provide a partial explanation for the long-standing ocean methane paradox (59, 67, 73). This paradox originates from the observation that the aerobic surface ocean is supersaturated in CH_4 with respect to the atmosphere, contributing as much 4% of the global methane budget (59). Because a contribution by the obligately anaerobic methanogens remains controversial, there has been no generally accepted explanation of origin. An intriguing hypothesis was put forward recently by Karl et al. (33), who suggested a new model in which methane would be produced when marine microorganisms use methylphosphonic acid as a source of phosphorus. The model was supported empirically by addition of commercially available methylphosphonate to seawater (33). The genes encoding C-P lyase are common among marine microorganisms, and those organisms were present in sufficient numbers to release methane from the added methylphosphonate and to use the released phosphate for cellular synthesis. The only significant difficulty with the model was the absence of a known biological source of methylphosphonate, which has now been provided. In addition to our discovery of the pathway in *N. maritimus*, a screen of available marine metagenome sequences for homologs of the *N. maritimus* diagnostic enzyme (methylphosphonate synthase) has shown the pathway to be present in other abundant marine clades, including representative *Prochlorococcus* and *Pelagibacter* species. Given the high abundance of these marine groups, they can produce the substantial amounts of methylphosphonate necessary to provide the missing link in the ocean methane paradox. However, apart from providing an explanation for an unknown methane source, there remains the equally significant question: What is the biological function of a phosphonate-modified outer cell wall?

FUTURE RESEARCH

These are exciting times in studies of the global nitrogen cycle. In a rapidly changing world that has more than doubled global inputs of fixed nitrogen into the biosphere since the pre-Industrial period (22), primarily through agricultural practice, it is imperative that the microbiological engines that drive the major biogeochemical cycles be more fully resolved. Ammonia-oxidizing microorganisms are essential to the proper functioning of the nitrogen cycle. As part of this critical nutrient cycle they produce the oxidized nitrogen species used by both anammox and denitrifying organisms to convert the generally biological available forms of nitrogen (ammonia, nitrate, nitrite) to the large and relatively inert atmospheric reservoir of molecular nitrogen. If the AOA do control rates of ammonia oxidation in most natural systems, as available data now indicate, it is necessary that their ecology, physiology, and underlying biochemistry are explored more fully. Such investigations are now being facilitated by the isolation of new AOA in culture and the power of comparative biology made possible by rapid genome sequencing. We suspect these studies will not only reveal novel biochemistry, but ultimately also offer a new understanding of the evolutionary origins of the *Archaea*.

SUMMARY POINTS

1. AOA are now thought to be the predominant ammonia-oxidizing population in most natural environments in which ammonia is present at very low concentrations. The naturally low concentration is attributable in part to the high affinity of the AOA for ammonia.

2. The biochemistry of archaeal ammonia oxidation is unique and as yet unresolved, sharing only genes distantly related to those coding for the AMO of characterized bacterial ammonia oxidizers.
3. Characterized AOA are chemolithoautotrophs; they use a variant of the 3-hydroxypropionate/4-hydroxybutyrate pathway for CO_2 fixation and are now believed to have only a limited capacity to assimilate different forms of fixed carbon for cellular synthesis.
4. Copper, as opposed to iron, appears to be the primary metal used in the respiratory redox chemistry of AOA.
5. NO, and possibly nitroxyl, may be important in the biochemistry of archaeal ammonia oxidation.
6. The AOA affiliate with a novel phylum, the *Thaumarchaeota*, recently recognized to constitute an early radiation within the *Archaea* on the basis of divergent features of genome sequence.
7. The AOA may contribute significantly to atmospherically active gases, including nitric and nitrous oxides and methane.
8. The habitat range of AOA, which includes hot springs and acidic soils, is significantly broader than that of characterized AOB.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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