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MINIREVIEW

Diversity, Physiology, and Niche Differentiation of Ammonia-Oxidizing Archaea

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Nitrification, the aerobic oxidation of ammonia to nitrate via nitrite, has been suggested to have been a central part of the global biogeochemical nitrogen cycle since the oxygenation of Earth. The cultivation of several ammonia-oxidizing archaea (AOA) as well as the discovery that archaeal ammonia monooxygenase (*amo*)-like gene sequences are nearly ubiquitously distributed in the environment and outnumber their bacterial counterparts in many habitats fundamentally revised our understanding of nitrification. Surprising insights into the physiological distinctiveness of AOA are mirrored by the recognition of the phylogenetic uniqueness of these microbes, which fall within a novel archaeal phylum now known as *Thaumarchaeota*. The relative importance of AOA in nitrification, compared to ammonia-oxidizing bacteria (AOB), is still under debate. This minireview provides a synopsis of our current knowledge of the diversity and physiology of AOA, the factors controlling their ecology, and their role in carbon cycling as well as their potential involvement in the production of the greenhouse gas nitrous oxide. It emphasizes the importance of activity-based analyses in AOA studies and formulates priorities for future research.

The discovery of archaeal ammonia oxidizers has radically challenged our bacteriocentric view of nitrification and stands as an example of the fascinating complexity of microbes involved in biogeochemical cycling. For over a century, ammonia-oxidizing bacteria (AOB) have been known to catalyze the first step of nitrification, the aerobic oxidation of ammonia (NH_3) to nitrite (NO_2^-). Their metabolic hallmark, ammonia monooxygenase (AMO), the enzyme catalyzing the initial oxidation of NH_3, has become one of the most widely used molecular markers in environmental microbiology. The first indication of the involvement of archaea in ammonia oxidation (AO) came when *amo*-like genes associated with archaeal scaffolds were discovered in marine surface waters (137) and soil (118, 132).

DIVERSITY OF AMMONIA-OXIDIZING ARCHAEA

Shortly after these insights from metagenomics, the isolation of the first ammonia-oxidizing archaeon (AOA), *Nitrosopumilus maritimus* SMC1 of the until then enigmatic group I.1a archaea, was reported (65). Members of this lineage are ubiquitously distributed in open ocean and coastal waters and have been demonstrated to represent 20% to 30% of marine microbes (48, 59, 82, 144). Within the past few years, additional *N. maritimus* strains have been obtained in enrichment cultures (99, 144). Furthermore, the uncultivated marine sponge symbiont "*Candidatus* Cenarchaeum symbiosum" was shown to encode genes essential for the oxidation of NH_3 and thus became regarded as an AOA (44, 45, 107). However, this organism has not yet been shown to catalyze the oxidation of NH_3 and, until further data are available, should be considered an *amoA*-encoding archaeon (AEA; 25). Later, the group I.1a AOA "*Ca.* Nitrosoarchaeum limnia" SFB1 was enriched from a low-salinity sediment and its genome was nearly completely reconstructed via a combination of metagenomics and single-cell sequencing (15). The genomes of "*Ca.* Nitrosoarchaeum koreensis" and "*Ca.* Nitrosopumilus salaria" were obtained from enrichment cultures from agricultural soil and estuary sediment, respectively (58, 60, 88). Very recently, novel (as-yet-unnamed) AOA species were enriched from freshwater sediment (36), expanding our knowledge about the environmental distribution of this archaeal lineage. Besides these group I.1a archaea, two thermophilic AOA species, "*Ca.* Nitrososphaera gargensis" (46) and "*Ca.* Nitrosocaldus yellowstonii" (29), have been described. While the former was the first characterized member of group I.1b archaea, the latter represents a deep-branching lineage (thermophilic AOA [ThAOA] group; formerly hot water crenarchaeotal group III [HWCG-III]) with wide distribution in high-temperature habitats.

One of the milestones of research on mesophilic archaea was the isolation of the first representative of soil-inhabiting AOA, *Nitrososphaera viennensis* EN76 (131). This group I.1b archaeon could serve as a model organism for future studies, most importantly because it represents a population of archaea exhibiting global distribution in soils (42, 61, 72, 103). Until 2011, archaeal ammonia oxidizers capable of growth at low pH had not been cultivated. Thus, the discovery of the first obligately acidophilic AOA "*Ca.* Nitrosotalea devanaterra" of the group I.1a-associated lineage is of particular importance because acidic soils (pH < 5.5) can exhibit high nitrification rates and comprise ~30% of the ice-free terrestrial surface (see reference 71 and references therein).

Table S1 in the supplemental material lists important features of characterized AOA species, and their phylogeny as determined on the basis of AmoA sequences is shown in Fig. S1 in the supplemental material. Besides the known lineages of AOA (group I.1a, group I.1a-associated, group I.1b, ThAOA), sequence data suggest that more, as-yet-unidentified *amoA*-encoding and potentially ammonia-oxidizing groups might exist (84, 102, 103, 108).

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THAUMARCHAEOTA: BEYOND AMMONIA OXIDATION

In 2008, analysis of the "*Ca.* Cenarchaeum symbiosum" genome led to the proposal of a novel phylum of archaea, *Thaumarchaeota*, which was later confirmed when more genome sequences became available (19, 124). The idea of the uniqueness of *Thaumarchaeota* is supported by the phylum's distinct phylogenetic position, a characteristic set of archaeal marker genes, and specific insertion and deletion sites in certain tRNA synthetases and proteins engaged in ribosomal functioning and cell division, as well as by the presence of a phylum-specific membrane lipid, thaumarchaeol (see references 19, 102, and 124 and references therein).

Presently, chemolithoautotrophic growth on NH_3 is the only physiology known for members of the *Thaumarchaeota*. It is, however, highly probable that their true metabolic diversity has as yet escaped our attention. Recently, it was reported that in some wastewater treatment plant sludges (wwtps), thaumarchaeotes that express putative *amo* genes (for their phylogenetic context, see Fig. S1 in the supplemental material) were highly enriched. Outnumbering AOB up to 10,000-fold, their cell numbers were 100 to 1,000 times higher than could be sustained by autotrophic AO alone. In highly dynamic systems such as wwtps, these high cell numbers can be explained only by active growth, suggesting the use of unknown heterotrophic substrates to conserve energy (92). This observation serves as an important reminder that for complex samples, the detection and enumeration of gene sequences are insufficient to propose a physiology. In addition, the discovery of giant thaumarchaeotes that may be involved in sulfur cycling in mangrove swamps demonstrated our lack of knowledge on the biology of members of this phylum (91). Despite its close relatedness to *N. maritimus* on the 16S rRNA sequence level (>97.7% identity), *Giganthauma* exhibits remarkable differences from this planktonic, sub-micrometer-sized AOA. If these data can be confirmed and extended in future experiments, this newly discovered microbe has the potential to shed new light on this still largely enigmatic archaeal phylum (91).

PHYSIOLOGY

AMO. A key enzyme in the biochemistry of AOA and AOB is ammonia monooxygenase (AMO). AMO is a member of the AMO/pMMO/pBMO/pXMO enzyme group, a diverse family of copper-containing membrane-associated monoxygenases (CuMMOs) that engage in the oxidation of ammonia (AMO), methane (pMMO), butane (pBMO), and possibly propane and ethane, as well as other as-yet-unidentified substrates (pXMO) (see reference 129 and references therein). CuMMOs are heterotrimers and in bacteria are commonly genetically encoded in the subunit order *CAB*, while in AOA the arrangement of these genes differs between different lineages (6, 15, 60, 124, 140). In ammonia oxidizers, the genes *amoA*, *amoB*, and *amoC* encode the three subunits of AMO. Whereas multiple, nearly identical *amoCAB* copies occur in AOB (3), no AOA has yet been found to carry more than a single copy of *amoA* or *amoB*, while two *amoC* copies are regularly found (15, 45, 140). Based on data obtained from the soluble fraction of its homologue PmoB, AmoB is thought to harbor the active site of AMO (4, 74). In AOB, AmoC is thought to exhibit chaperonic activity with respect to the other subunits to assist in their integration into the membrane (62) and has been shown to be involved in recovery from NH_3 starvation (13, 14). Unfortunately, AMO has so far defied all attempts for functional isolation and structural analyses. Thus, an unambiguous assignment of the roles of individual Amo proteins cannot yet be drawn.

With the exception of fosmid 19c08, all currently known genomes or genome fragments of AEA/AOA encode a conserved hypothetical protein directly upstream of *amoA* (6). Interestingly, a recent metatranscriptomic study demonstrated that homologues of this gene are among the most highly expressed mRNAs in estuarine bacterioplankton, amouting to ~70% and ~296% of *amoA* and *amoB* transcript numbers, respectively (49). It was proposed that this gene may be associated with the Amo proteins and referred to as "*amoX*" (6). In the same publication, the first evidence for the potential transposon-linked mobilization of archaeal *amo* genes was presented, which might also provide an explanation for the disconnection of *amoA* and "*amoX*" in fosmid 19c08 (6).

Proposed pathways. In AOB, the membrane-associated AMO catalyzes the aerobic oxidation of NH_3 to hydroxylamine (NH_2OH) which is subsequently oxidized to NO_2^- by the periplasmic hydroxylamine oxidoreductase (HAO) (16). The downstream electron (e^-) flow has not yet been fully resolved (see references 64 and 123 and references therein). Under standard conditions (pH 7.0, 25°C), ~0.7% of the total pool of ammonia plus ammonium (NH_4^+) is available as NH_3. While it is generally accepted that NH_3 and not NH_4^+ is the substrate for bacterial AMO (128), archaeal AMO has never been tested in that regard. It is unclear whether archaeal AMO catalyzes the same reaction as its bacterial counterpart, because no *hao* homologue, enzymes for the detoxification of NH_2OH, or cytochrome *c* has been found in any AOA genome (see, e.g., references 123 and 140; for a list of sequenced AOA, see Table S1 in the supplemental material). In addition, the observation that archaeal AmoB and bacterial AmoB have significantly different predicted structures might indicate a different function of this protein in AOA (140). Thus, either an unidentified enzyme substitutes for HAO in AOA or the archaeal AMO reaction yields a different product (15, 45, 60, 124, 140). It was suggested that nitroxyl hydride (HNO) may be generated by a monooxygenase reaction of the archaeal AMO. Subsequently, HNO could be oxidized to NO_2^- via a nitroxyl oxidoreductase (NxOR) (119, 140). The activation of O_2 for the monooxygenase reaction could also be achieved by nitric oxide (NO), the reaction product of nitrite reductase (NIR), which would result in the production of N_2 gas (119). It was reported that archaeal *nirK* (encoding copper-dependent NIR) genes are expressed under aerobic conditions (see, e.g., references 5, 49, 58, 78, and 132), suggesting a different function of these enzymes in AOA compared to their bacterial counterparts. *nirK* homologues are present in all published AOA genomes but are absent in the genome of "*Ca.* Cenarchaeum symbiosum." Thus, if this microbe is indeed an AOA, then the model described above might not be fully valid.

In all of the discussed physiological pathways, two of the released e^- participate in the generation of a proton motive force and lead to the conservation of energy via an ATPase. For both AOA and AOB, the exact amount of synthesized ATP per mole of NH_3 is unknown, because assessment of the number of e^- available for energy conservation is dependent on the growth stage and the extent of reverse e^- flow, as well as other factors, and on the fact that their e^- transport process is yet not fully understood (16, 140). However, the lack of cytochrome *c* proteins and the existence of numerous genes encoding copper-containing proteins (multicopper oxidases and plastocyanin-like domain proteins) in AOA suggest a different e^- transport mechanism (15, 45, 140) from that of the highly iron-heme-dependent AOB (38, 39). A copper-based biochemistry would help to explain the ecological

success of marine AOA (compared to AOB), because dissolved copper concentrations are generally an order of magnitude higher than those of iron in seawater (see reference 134 and references therein).

Specificity. Bacterial AMO does not exhibit high substrate specificity: methane (CH_4) and carbon monoxide (CO) as well as some aliphatic and aromatic compounds may be oxidized and act as competitive inhibitors of AMO (see references 51 and 102 and references therein) (109). These substrates, however, cannot be used for energy conservation and do not support the growth of AOB (16, 51). This low specificity is regarded as a reflection of the evolutionary history of AMO as a member of the CuMMO enzyme family (63). Given their substrate promiscuity, it has been hypothesized that not the type of CuMMO but the downstream biochemical machinery defines an organism's substrate specificity (129). The substrate spectrum of archaeal CuMMOs remains largely unknown, and growth of *N. maritimus* SCM1 on CH_4 or other organic or inorganic e^- donors has not been observed (134). However, given the substrate promiscuity of known CuMMOs as well as the high abundance of AEA in the environment, the existence of other (potentially energy-yielding) substrates of archaeal CuMMOs seems plausible.

Inhibitors. In contrast to AOB data (7, 16, 51), only limited information on potential inhibitors is available for AOA. While the metal chelator allylthiourea is observed to lead to a decrease, but not complete inhibition, of metabolic activity (46, 58, 61, 90), acetylene has been demonstrated to cause a total suppression of AO in cultivated AOA (71, 131) as well as *in situ* (56, 96, 97). Contrasting observations have been reported for dicyandiamide (30, 31, 58, 61, 151), and nitrapyrin has been successfully tested only on "*Ca.* Nitrosoarchaeum koreensis" and "*Ca.* Nitrososphaera sp." JG1 (58, 61). Use of the antibiotic sulfadiazine offers the ability to differentiate between AOA- and AOB-catalyzed AO via the preferential inhibition of bacteria (117). In addition, both AOA (36, 83) and AOB (43, 52) exhibit species- and wavelength-specific photoinhibition, which in AOB is attributed to photooxidation damage of AMO (50, 53). Different light sensitivities of AOA and AOB support the idea of a potential mechanistic difference between their respective versions of AMO (36, 83).

EMISSION OF NITROUS OXIDE

As a result of classical and nitrifier denitrification—the reduction of NO_2^-/NO_3^- in several steps to N_2—nitrous oxide (N_2O) is produced and partially escapes into the gas phase (18, 64). With its long atmospheric lifetime, N_2O has a greenhouse warming potential 310 times higher than carbon dioxide (CO_2) and is responsible for 5% to 7% of the observed greenhouse effect, making it the third most important greenhouse gas (after CO_2 and CH_4) (18, 55). Due to its reaction with atomic oxygen, N_2O also has detrimental effects on Earth's ozone (O_3) layer (110). Together with canonical denitrifiers, AOB (64, 75) and, potentially, AOA (58, 76, 90, 115, 116) are suspected to be responsible for ~70% of the global N_2O emissions (55).

Potential sources. In AOB, two sources of this gas can be distinguished. Small amounts are produced via the chemical decomposition of NH_2OH to NO and N_2O (18). In addition, under O_2-limiting conditions, AOB readily use NO_2^- as a terminal e^- acceptor, leading to the formation of nitrogen-containing gases via the activity of NIR and nitric oxide reductase (NOR) enzymes (18). For AOA, the enzymatic production of N_2O has yet not been demonstrated, and observed N_2O emissions may be due to spontaneous chemical reactions of metabolic intermediates. A NOR enzyme has not yet been identified in any fully sequenced AOA (for a list, see Table S1 in the supplemental material), and NH_2OH is currently not regarded as an intermediate in their AO pathway. While the reactions leading to the formation of N_2O apparently differ between AOA and AOB (76), ^{18}O labeling studies suggest the same ratio of oxygen sources in NO_2^- (one atom originating from H_2O and one from O_2) (115). Moreover, the site preference for $^{15}N_{N_2O}$ produced by enrichments (115) and pure cultures (76) of AOA is consistent with the values for AOB cultures seen under AO conditions. This suggests that in AOA (at least under the tested conditions), N_2O originates from an intermediate in the AO process and not AOB-like nitrifier denitrification (76). Assuming that HNO is indeed an intermediate in the AOA AO pathway, it might be the source of archaeal N_2O emission (M. G. Klotz, personal communication). In water, HNO molecules dimerize to form hyponitrous acid ($H_2N_2O_2$), which is subsequently dehydrated to N_2O—a process first proposed for denitrifying bacteria (see references 85 and 153 and references therein).

Yield. While the amount of N_2O emitted from an enrichment culture containing "*Ca.* Nitrosoarchaeum koreensis" is considerably lower than that seen with *Nitrosomonas europaea* (58), N_2O production rates up to five times higher were reported for *N. maritimus* SCM1 versus *Nitrosococcus oceani* and *Nitrosomonas marina* (76). An explanation for this apparently conflicting result may be an overestimation of rates resulting from the use of high-density cultures that may not be representative of the environment (76). Other explanations may be that soil and marine ammonia oxidizers differ in their (relative) responses to low O_2 conditions or the potential presence of N_2O-removing microbes in the enrichment culture (58), leading to an underestimation of the rates. In *N. maritimus* SCM1 cultures, N_2O emission inversely correlates with the O_2 concentration, an observation that is in accordance with the idea that the gross amount of N_2O generated in analyzed marine oxygen minimum zones (OMZ) potentially originates from group I.1a archaea (76).

NICHE DIFFERENTIATION

The high numbers of AEA/AOA in many natural and man-made systems raise questions on the exact nature of their cellular and biochemical adaptations that lead to this wide distribution, as well as on their relative significance in nitrification. This section discusses the most important factors known to shape the ecology of AOA compared to AOB. While it gives examples of the niche differentiation of specific AOA populations, this discussion is not intended to present an exhaustive list of habitats in which *amoA*-like gene sequences have been detected, and it emphasizes the importance of activity-based analyses in environmental studies.

Oligotrophy. One of the principal factors controlling the relative distribution of AOA versus AOB is the substrate concentration. In contrast to several analyzed AOB, *N. maritimus* SCM1 has been demonstrated to exhibit exceptionally high affinities (K_m = 133 nM total NH_4^+) and low (≤10 nM) thresholds with respect to its substrate (79, 80). Most importantly, these values correlate well with the *in situ* kinetics of the oligotrophic open oceans that have been shown to contain large amounts of AEA/AOA (see reference 79 and references therein). If most marine *N. maritimus*-related populations were indeed AOA, they would be responsible for the bulk of AO observed in these habitats, which would call for a

reinvestigation of current biogeochemical models (79). A preference for low NH_3 levels had initially been reported for "*Ca.* Nitrososphaera gargensis" (46) and was recently also demonstrated for "*Ca.* Nitrosotalea devanaterra" (71). In addition, three sedimentary/freshwater AOA enrichments were shown to extend their lag phases with increasing concentrations of NH_3 (36; see also Table S1 in the supplemental material). This is in contrast to the soil AOA *N. viennensis* EN76 and "*Ca.* Nitrosoarchaeum koreensis," which were found to be adapted to considerably higher substrate concentrations (58, 131). While the inhibitory NH_4^+ concentrations (10 to 20 mM) of these AOA (see Table S1 in the supplemental material) are comparable to that of the most oligotrophic AOB known (21.4 mM for *N. oligotropha* JL21), they are still low compared to the highest NH_4^+ tolerance of AOB (50 to 1,000 mM) (66). A preference of AEA/AOA for low NH_4^+ concentrations has also been reported from several environmental studies, especially for soils (see, e.g., references 30, 106, and 138). This might be explained by either higher affinities or higher densities of archaeal AMO or transporters for NH_3/NH_4^+ compared to AOB (134). However, it is still unclear what the exact localization of the catalytic site of AMO is (i.e., whether it faces the periplasm or cytoplasm), whether the mentioned transporters are used for the accumulation of NH_3, or whether archaeal AO is transporter dependent (134). Interestingly, reads assigned to group I.1a archaeal NH_3 transporters and permeases are among the most commonly detected genes and transcripts in marine samples (49, 120, 125, 133).

Cell (ultra)structure. For *N. maritimus* SCM1, the presence of a single genome copy per cell as well as a very low rate of replication (15 to 18 h for a 1.65-Mb genome) supports the idea of the organism's adaptation to oligotrophic habitats (101). According to cryoelectron tomography data, *N. maritimus* SCM1 cells in exponential growth harbor ~1,000 ribosomes per ~0.023-μm^3 cell volume (134). Data on the stability of mRNAs, AMO, and ribosomal proteins of AOA are lacking but could be essential in understanding the ecological adaptations of AOA compared to AOB, which are known for the exceptionally high *in vivo* stabilities of their *amo* mRNA and proteins as well as the high level of their ribosomal contents (16, 66). While energetically expensive, consistently high numbers of ribosomes offer organisms the ability to respond quickly to changing environmental conditions, for example, fluctuating NH_3 levels. The observations described above are consistent with the hypothesis that most archaea, in contrast to bacteria, are highly adapted to energy-stressed environments (135). The lower membrane permeability of AOA cells, a direct consequence of their preference of tetraether lipids, has been proposed to result in a reduction in ion cycling and, thus, lower levels of maintenance energy relative to AOB (135).

Furthermore, most AOA have cell volumes that are 10 to 100 times smaller than those of known AOB (for cell sizes and references, see Table S1 in the supplemental material). This has profound implications for their per-cell AO rates, which for *N. maritimus* SCM1 were reported to be ~10-fold lower (0.53 fmol NH_3 $cell^{-1}$ h^{-1} at highest activity) than those of AOB (see references 79 and 134 and references therein). Thus, the high relative abundance of AOA compared to AOB in many environments does not *per se* implicate a major importance in net nitrification, necessitating the use of activity-correlated analyses that can differentiate between their individual contributions.

NH_3 source. Besides the substrate concentration, the form in which NH_3 is supplied also governs niche adaptation. Due to the low nitrogen uptake efficiency of crops and leaching of NO_2^-/NO_3^-, ~70% of fertilizer nitrogen is lost to the atmosphere or washed out from soils before assimilation into biomass can occur (see reference 21 and references therein). The preference of AOA versus AOB for different NH_3 sources thus could have important consequences for agricultural fertilization strategies that aim to minimize nitrogen loss and maximize crop production. In all studies that reported growth of soil AOA, NH_3 originated from mineralized organic material (31, 73, 96, 117, 150). On the other hand, when NH_4^+ fertilizer or urea (NH_2CONH_2) was provided as the source of substrate, AOB usually strongly outcompeted AOA (see, e.g., references 30 and 56). Also, the total nitrogen and organic carbon content of a range of different soils has been shown to negatively correlate with the species richness of putative AOA (103).

pH. The observation of the preference of AOA for low NH_3 concentrations is in accordance with studies that reported high numbers and activities of AEA/AOA in acidic soils (41, 42, 77, 93). A recent study demonstrated that, among seven physicochemical parameters measured (pH; carbon, nitrogen, and organic matter content; C:N ratio; soil moisture; and vegetation), the pH value was the major factor governing AEA community structure (42). Low-pH conditions decrease the availability of NH_3 while increasing the toxicity of NO and N_2O as well as gaseous nitrogen dioxide (NO_2) (26, 27). While some AOB populations are adapted to coping with low pH, cultured representatives show no or only very limited activity at pH < 6.5 (see references 41, 67, and 93 and references therein). Many but not all species of AOB (105) and some AEA/AOA (45, 131, 133, 147) encode ureases, enzymes that catalyze the conversion of urea to CO_2 and NH_3. The reaction products can then be used as sources of carbon and energy, respectively, or potentially to regulate the pH in the vicinity of the cell. Compared to AOB, AEA/AOA are more transcriptionally active in acidic soils and both microbial groups harbor phylotypes that are specifically adapted to low-pH conditions (42, 93, 103). In a study targeting *amoA*-like gene diversity in a wide range of globally distributed soils, several lineages within group I.1b and the I.1a-associated group clearly exhibited adaptation to certain pH regimens, and these results were coherent at the global, regional, and local level of sampling sites. Most prominently, a strong correlation of the two sublineages of group I.1a-associated *amoA*-like sequences with acidic soils (pH < 5) was found (42). These findings are in accordance with the observation that in some acidic soils (pH 3.75 and 5.4) in which AOB could not be detected, AOA closely related to *Nitrosotalea* and *Nitrososphaera* grew when urea was provided as a substrate (77). Archaeal *amoA*-like gene abundance and diversity directly increase with soil pH (42, 103), and so far only one obligately acidophilic AOA has been described (71). However, the wide distribution of the *Nitrososphaera* cluster and other *amoA* lineages in acidic soils suggests the existence of other low-pH-adapted AOA (42, 103, 145).

Differential activities of soil AOA populations. AEA/AOB *amoA* ratios increase with soil depth (31, 47, 56, 72, 93), and specific AEA phylotypes exist in different soil horizons, suggestive of populational adaptations to such microenvironments (72, 94). Only rarely, however, were such studies combined with activity tests (see, e.g., references 56, 106, 145, and 150), and available data are partially conflicting, which makes it hard to judge the relative importance of AOA in soil nitrification (aside from acidic habi-

tats). A preferred tool in comparative soil studies has been stable isotope labeling (SIP) of nucleic acids. Using RNA SIP, it was demonstrated that, although AOA incorporate $^{13}CO_2$-derived carbon into their *amoA* and carbon fixation gene transcripts (i.e., are metabolically active), their growth (i.e., replication of DNA) cannot be detected via DNA SIP (56, 106). This is in contrast to other studies that reported growth of AOA even after relatively short incubation times (4 compared to up to 12 weeks in other studies) (145, 150). Irrespective of their high numerical dominance in most soil habitats (42, 72, 103), only a limited number of studies reported the activity of *Nitrosotalea*- and *Nitrososphaera*-related archaea (77, 145). In contrast, several investigations observed the preferential activity and growth of the low-abundance group I.1a AOA (96, 130, 138, 150). These results are a warning that we still do not appreciate the functional diversity of the numerically dominant group of soil archaea (i.e., group I.1b). In addition, many soil AEA actually may not (always) be directly involved in AO because of either a potential for mixotrophic growth (see next chapter) or additional or different functions of their CuMMOs.

Elevated temperatures. Specific soil-inhabiting AEA subpopulations (mostly group I.1a related), but not AOB, increase expression of their *amoA*-like genes and probably nitrify upon incubation at >30°C (96, 130). Due to the limited number of studies, we do not yet know whether this differential temperature response is a general trend for soil ammonia oxidizer communities. Interestingly, however, AOB have so far not been detected in environments experiencing constant temperatures of >40°C. Archaeal *amoA*-like genes (33, 111, 141, 149) and transcripts (57, 149), on the other hand, have been retrieved from geothermal habitats at up to 97°C. Many retrieved sequences are highly similar to those of *amoA* genes of the cultured thermophilic AOA "*Ca.* Nitrosocaldus yellowstonii" and "*Ca.* Nitrososphaera gargensis" (29, 46), but several other species still await characterization (57, 111, 149). Thermodynamic calculations of potential chemolithotrophic reactions in two geothermal springs demonstrated that AO is among the highest energy-yielding physiologies under such conditions (34). Consistently, *in situ* measurements of AO in several hot springs have indicated the essential role of heat-adapted AOA in these systems (33, 111). Recent data support the idea of a thermophilic ancestor of *Thaumarchaeota* and a geothermal origin of archaeal AO (20, 29, 40, 46, 95). According to this hypothesis, today's nearly ubiquitous mesophilic AEA/AOA are the result of secondary adaptations to the lower temperatures of terrestrial and marine systems (20, 29, 46).

Zonation with water depth. Many studies have addressed the distribution of *amoA*-like gene sequences in the marine water column, but only a limited number have characterized the *in situ* activity of AOA (see, e.g., references 2, 9, 114, 125, and 147). Soon after the discovery of AOA, it was recognized that archaeal *amoA*-like gene numbers correlate with NO_2^-/NO_3^- maxima in the oceans (see, e.g., references 9, 24, and 84). Besides the widely distributed *N. maritimus*-like sequences, many sequences that have been obtained from marine samples fall within two phylogenetically distinct clusters. Group A or "shallow" genes and transcripts are primarily derived from the shallow, euphotic zone (<200 m depth), while group B or "deep clade" sequences represent deep water (>200 m depth) ecotypes (see, e.g., references 9, 23, 84, 87, and 114). A similar zonation has been observed for genes encoding putative thaumarchaeotal carbon-fixing 4-hydroxybutyryl-coenzyme A (CoA) dehydratase (Hcd) and acetyl-CoA carboxylase (AccA/PccB), as well as *nirK* and urease enzymes (78, 147). In the photic zone, such patterns could be due to either differential photoinhibition or variable success of AOA populations in competition with phytoplankton for NH_3 (9, 84). While these ideas have not been directly tested so far, it was recently reported that the sensitivity to light for three AOA species was greater than that for AOB (36, 83). While most studies recorded a strong correlation of AO with 16S rRNA and *amoA*-like gene sequences of "shallow" group A (9, 23), trends are not always clear for deep-water thaumarchaeotes, partly due to the presence and activity of AOB in the same waters (2, 114). In contrast to most other reports, two studies found stark discrepancies between thaumarchaeotal 16S rRNA and *amoA*-like gene copy numbers (2, 28), but these conflicting results are at least partly due to primer biases (114, 147).

Oxygen deprivation. An intriguing feature of some marine AOA is their apparent preference for regions of low (<10 μM) levels of dissolved O_2, where AO might be coupled to anaerobic ammonium oxidation (anammox) and/or denitrification (9, 69, 70, 86, 104, 125). It was thus suggested that AOA and AOB together provide 30% to 40% of the NO_2^- required by anammox in the OMZ of the Black Sea (69) and off Peru (70), but no indication for such coupling was found in the Arabian Sea (104). These results largely coincide with observations of pure and enrichment cultures which demonstrate a much higher affinity of *N. maritimus* SMC1 to O_2 (79, 80) and likely also of other group I.1a AOA (58, 99) compared to AOB. In addition, three AOA enrichments that had been obtained from freshwater sediment were recently demonstrated to be active under conditions of reduced O_2 concentrations (0.5% to 2% O_2 in the headspace). The observation that other microbes, such as *Escherichia coli*, are able to grow aerobically at O_2 concentrations ≤ 3 nM (126) raises the possibility that microbes with other physiologies could also be adapted to such minute O_2 concentrations. It must be kept in mind that the nitrification rates observed in OMZ (20 to 150 nmol liter^{-1} day^{-1}) are well within the range of the prevalent O_2 concentrations (69, 70) and that the O_2 levels required to sustain such rates are below the detection limit of currently used in-field O_2 sensors (~1 μM). In either case, AOA are expected to have evolved molecular adaptations to cope with periods of O_2 deprivation, as known for AOB performing microaerophilic nitrification (37).

Investigations of the adaptivity and tolerance of AOA with respect to low-O_2 regimens are of utmost importance, given the high abundance of archaeal *amoA*-like gene sequences in other habitats such as sediments, seasonally oxygen-deficient water zones, or certain agricultural soils (see, e.g., references 1, 10, 22, 68, 86, and 99). Unfortunately, the contribution of AOA to the *in situ* nitrification as well as N_2O emission rates in these systems is largely unknown.

Other factors. Additional variables that might be involved in shaping the ecology of AEA/AOA have been brought into discussion, but data are yet not conclusive. Some potential factors are the levels of sulfide, phosphate, salinity, soil moisture, and others (see references 17, 35, and 148 and references therein).

CARBON SOURCES

Autotrophy. Although both AOA and AOB are usually regarded as autotrophic organisms, important differences exist in how they fix inorganic carbon and use organic carbon. While AOB rely on the Calvin-Benson-Bassham (Calvin) cycle for carbon fixation

(3), a modified version of the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle seems to operate in AOA (11, 15, 99, 131, 140). In addition, for *N. maritimus* SCM1 and "*Ca.* Cenarchaeum symbiosum" as well as for marine planktonic thaumarchaeotes, a reverse tricarboxylic acid (rTCA) cycle for carbon fixation has been suggested (45, 81, 140). The preference for different carbon-fixing pathways has important consequences for the ecological adaptation of microbes, as recently reviewed (11). Most importantly, in the 3HP/4HB cycle, bicarbonate (HCO_3^-) is the fixed carbon species, while the Calvin cycle fixes CO_2. This is of high importance, considering that at neutral and slightly alkaline pH, as found in marine waters, HCO_3^- is the predominant carbon species. In addition, a complete or even rudimentary 3HP/4HB cycle provides the organism the capability to coassimilate many different organic compounds, including fermentation products. For marine archaea, the ability to use 3HP, an intermediate in the metabolism of the ubiquitously distributed osmoprotectant dimethylsulfoniopropionate (DMSP), may be ecologically more important (11). It raises the question of whether these microbes are directly involved in the breakdown of DMSP or its products, e.g., acrylate, and whether this could potentially account for the observed heterotrophy and mixotrophy of marine thaumarchaeotal populations (2, 54, 136).

Mixotrophy? A potential for mixotrophic growth has been reported from analyses of the genomes of *N. maritimus* SCM1 and "*Ca.* Cenarchaeum symbiosum" (44, 45, 140), but these hypotheses have not yet been experimentally supported. In contrast, the addition of organic substances (yeast extract, peptone, and acetate) has been shown to inhibit AO by *N. maritimus* SCM1 (65) and "*Ca.* Nitrosocaldus yellowstonii" (29). However, the tested compounds may not be representative of the substrates present in their respective niches. *N. maritimus* SCM1 and "*Ca.* Cenarchaeum symbiosum" harbor transporters for different amino acids, di- and oligopeptides, glycerol, and sulfonates or taurine, making these compounds ideal candidates for future experiments (44, 45, 140). A metatranscriptomic study did not report any expression of these transporters in coastal bacterioplankton, while transcripts assigned to the 3HP/4HB and tricarboxylic acid cycle were detected (49). However, the genomic analyses described above are consistent with studies that demonstrate the genetic potential (133) and metabolic ability of marine thaumarchaeotal populations to use amino acids (48, 98) or other organic matter (2, 54, 136). Interestingly, cultures of the soil AOA *N. viennensis* EN76 exhibit cell yields that are 12 times higher for growth under mixotrophic (pyruvate) rather than purely autotrophic conditions (131). However, less than 10% of cellular carbon seems to be derived from this substrate under the tested conditions (131). While key components of the 3HP/4HB cycle could be identified in the draft genome of *N. viennensis* EN76, no indications of its dependence on pyruvate compared to that of *N. maritimus* SCM1 could be found (131). Very recently, SIP experiments demonstrated the incorporation of ^{13}C from labeled rice callus into the DNA of both AOA and AOB (142). While potential cross-feeding—via respiration of labeled substrates by other microbes and subsequent fixation of exhaled $^{13}CO_2$—cannot be ruled out completely, the results of this study correspond to those of other reports of root-colonizing group I.1b thaumarchaeotes (121, 122, 146).

OUTLOOK

PCR-based studies. Based on the pairwise comparison of thaumarchaeotal 16S rRNA and *amoA* genes from metagenomic fragments and cultured AEA/AOA, archaeal *amoA*-like sequences deposited in public databases as of June 2010 represented ~113 to 120 AEA/AOA species (i.e., *amoA* identities < 85%; 103). This range of values might be a conservative estimate, given that the primers that have most frequently been used in past research do not effectively replicate the full diversity of the *amoA*-like genes now known (103). Primer sets which together target the whole range of archaeal *amoA*-like sequence diversity were recently published (32, 103, 130), and their use in future studies is recommended (primers are listed in Table S2 in the supplemental material). In the future, PCR-based analyses of AOA populations are expected to be expanded to genes encoding enzymes involved in pathways other than AO. Potential candidates include *hcd* and *accA*/*pccB* (11, 97) as well as *nirK* (5, 78, 132). Homologues of these genes are present in all genome-sequenced AOA (*nirK* is, however, absent from the genome of the AEA "*Ca.* Cenarchaeum symbiosum"), and their expression in enrichment cultures as well as in natural populations has recently been shown (see, e.g., references 5, 49, 58, 78, and 106). Another potential target is the *uvrBAC* cluster, proposed to encode an enzyme involved in DNA excision repair, which can be found in all published thaumarchaeotal genomes. The single unknown is "*Ca.* Nitrosoarchaeum limnia," the genome of which harbors a partial *uvrAB* cluster at a contig end (6).

Correlation with activity. While PCR-driven studies are expected to keep on being an essential part in AOA research, as discussed in this review, the detection—or even demonstration of expression—of *amo*-like genes is an insufficient basis for the proposal that the respective organism is indeed an obligate autotrophic ammonia oxidizer (92). Thus, experiments need to be carefully designed and combined with adequate controls (e.g., inhibitors) as well as tests of activity, most importantly, isotopic labeling techniques (targeting the level of single cells, DNA, RNA, lipids, etc.). With the successful cultivation of several species (see Table S1 in the supplemental material) as well as the more widespread application of combined "meta-omics" approaches—concomitantly targeting the genomic, transcriptomic, proteomic, metabolic, and/or geochemical level—and single-cell-resolving techniques within the last years, we are now able to directly approach fundamental problems in the AOA field.

Some open questions. Currently, the most urgent issue is that of the biochemistry of archaeal AO and its implications for the niche separation from AOB. The determination of the substrate range of AOA as well as their involvement in the emission of N_2O is expected to have profound consequences for our understanding of the impact of archaea on biogeochemical cycles. Substitution experiments performed with isotopically labeled nitrogen compounds as well as inhibition tests scavenging potential reaction intermediates should enable us to decipher AOA physiology. In addition, the use of meta-analyses in the comparative study of several AOA in parallel promises to yield important information on the underlying protein machinery and regulatory factors of AO. Similar analyses could also help us to shed light on potential additional functions of archaeal AMO.

It is surprising that, despite their importance for humans and the high diversity of archaeal *amoA*-like gene sequences detected

in these habitats (see, e.g., references 100, 112, 113, 143, and 152), wwtps as well as ground- and freshwater systems have only very rarely been the focus of AOA-targeted enrichment and activity studies, with notable recent exceptions (36, 92). Thus, the available data make it hard to judge the contribution of AOA to nitrogen cycling in these habitats. However, the study of these environments is of particular importance not only for our understanding of nitrogen cycling but also for the optimization of detoxification and removal of municipal and industrial waste. Furthermore, the potential *in situ* nitrifying activity of sediment-dwelling thaumarchaeotes deserves more attention, considering the large inputs of anthropogenic nitrogen into many freshwater and estuary systems and the high abundance of AEA in the marine habitats (see, e.g., references 8, 10, 12, 89, and 99).

Another topic currently insufficiently studied is the relationship of AOA with nitrite-oxidizing bacteria (NOB). The mutual dependence of AOB and NOB is a typical textbook example of the metabolic coupling of two functional guilds of microbes. Hence, our lack of knowledge of a potential codependence of AOA and NOB is highly unfortunate. Besides the demonstration of a spatial-temporal co-occurrence of these microbes in marine and soil habitats (84, 114, 145), a deeper understanding of their relationship is so far lacking.

In our search for novel AOA, we should try to more directly address the biology of lineages with assumed ammonia-oxidizing activity, e.g., the pSL12, I.1c, and ALOHA groups (77, 84, 102, 127). This could be achieved by a combination of direct single-cell sorting from environmental samples with (meta)genomic sequencing to determine the genetic potential of these groups. After this screening phase, environmental samples could be incubated in the presence of stable or radioactively labeled substrates. They could then be subjected to activity tests using either molecular tools such as differently targeted SIP techniques or single-cell-resolving methods such as microautoradiography, Raman spectroscopy, or dynamic secondary-ion mass spectrometry (see reference 139 and references therein). Finally, given the potential physiological flexibility of some members of the *Thaumarchaeota* (2, 54, 56, 91, 92, 98, 136), we should extend our studies on these microbes beyond the single metabolism of ammonia oxidation.

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