

Out of the blue: Planktonic *Crenarchaea* and the ocean's nitrogen cycle

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Over the past two decades, many new microbial lineages have been discovered in the course of cultivation-independent molecular phylogenetic environmental surveys (13). New sequencing technologies have extended our appreciation of the depth and breadth of microbial diversity even further. While recognition of the vast extent of microbial diversity has been exhilarating, it has also raised large questions about our current limited understanding of the nature, activities and ecological significance of extant microbial life on Earth. What are the specific biological properties of all these previously unrecognized microbial groups? Are these new microbes significantly different from commonly cultivated laboratory cultivars? How do newly recognized microbial species impact their surrounding environment, and what specific environmental processes are they involved in? Characterizing the specific biochemical attributes, physiological properties, and ecological function of newly recognized microbial life is an critical area for Biology to develop in the coming century. Our current understanding of marine *Crenarchaea* provides a good example of the flow path from initial recognition of a new microbial lineage, to a deeper appreciation of its biological properties and ecological significance.

Molecular phylogenetic surveys in natural habitats have revealed major, previously uncultivated microbial taxa in almost every habitat imaginable (13). This microbial census-taking has shown for example that members of the domain *Archaea*, once considered to thrive only in extreme environments, are actually widely distributed in soils, lakes, sediments and the world's oceans. Evidence for planktonic *Crenarchaea* was initially reported based on a handful of archaeal rRNA clones recovered from deep Pacific Ocean waters (5), and in an independent, quantitative survey of several coastal habitats (4). Subsequent studies have verified the ubiquity and abundance of these marine *Archaea* (7). Surprisingly, the *Archaea* that were discovered in cold aerobic marine plankton communities most closely related to

high-temperature-adapted *Archaea* from hot springs. We now know that marine *Crenarchaea* can comprise 20% or more of total cell numbers below the photic zone in coastal or open ocean waters. They are also present in considerable numbers in Antarctic or Arctic circumpolar regions, in near surface and deep equatorial waters, in specific association with marine invertebrates, and within oxygen minimum zone regions. The best available evidence now also suggests that ancestors of planktonic *Crenarchaea* once lived in high temperature habitats, later adapting to the cold, oxygenated environment of contemporary ocean waters.

Many studies have now shown that planktonic *Crenarchaea* are globally abundant, what biological niche do they occupy? In her thesis work Ann Pearson, used ^{14}C found in oceanic organic matter and CO_2 as a tracer to track crenarchaeal lipid biosynthesis precursors, and showed that deep-water marine *Crenarchaea* use dissolved CO_2 as their main carbon source – that is, they are autotrophs (14). Subsequent studies using ^{13}C labeled bicarbonate provided further support for crenarchaeal autotrophy (17). But if marine *Crenarchaea* are indeed chemoautotrophs, what is their energy source? Definitive demonstration of marine crenarchaeal chemoautotrophy came from the first isolation of crenarchaeal pure cultures, and demonstration of their growth on NH_4 as the sole energy source, and CO_2 as the sole carbon source (8). Genome analyses and metagenomic surveys (6, 16) provide further evidence that crenarchaeal ammonia oxidation may be a widespread in the marine environment, and potentially more important for marine ammonia oxidation (nitrification) than what was previously supposed to be a solely bacterial process.

Outstanding questions remain concerning the biological properties, activities and ecological significance of planktonic *Crenarchaea*. Are deep-sea *Crenarchaea* predominantly autotrophic, heterotrophic, or mixotrophic? Based on discrepancies between quantitative PCR counts of crenarchaeal rRNA and ammonia monooxygenase (*amoA*) genes, some authors have suggested that deep-sea *Archaea* are heterotrophic, not chemoautotrophic, since they appear to lack the *amoA* gene (1). Other work suggests however that most planktonic *Crenarchaea* actually do possess *amoA* genes, casting

doubt on this line of reasoning (3, 9, 12). What role do planktonic *Crenarchaea* play in oxygen minimum zone communities? Although it has been proposed that planktonic *Crenarchaea* may function anaerobically (15), current evidence seems to indicate that they are primarily active in oxygenated regions as aerobic nitrifiers (10). Given their environmental ubiquity and function, how might ammonia oxidizing *Crenarchaea* compete or interact with other bacterial nitrifiers? Interesting biochemical and environmental clues are now providing new insight into potential *in situ* interactions between marine *Crenarchaea* and other ammonia-oxidizing and nitrite-oxidizing bacteria (11, 12). Finally, the phylogenetic position of marine planktonic *Crenarchaea* and their relatives has been a matter of some debate, since they are phylogenetically positioned on a long branch rooted deep at the base of the archaeal lineage. Based on concatenated ribosomal protein and other gene phylogenies, some authors now propose a tripartite division of phyla within the Domain *Archaea*: the *Crenarchaea*, the *Euryarchaea*, and a newly proposed phylum, the *Thaumarchaeota*, that would encompass marine planktonic *Crenarchaea* and their close relatives (2). Regardless of the specific taxonomic designator, be it *Crenarchaea* or *Thaumarchaeota*, it seems clear that this ubiquitous archaeal lineage is ecologically significant in many different environmental and biogeochemical contexts, and likely to reveal more surprises as its biological properties and variability become more well characterized.

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The ecophysiology of marine ammonia-oxidizing archaea

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Since the discovery of nitrification by Sergey Winogradsky more than a hundred years ago it was thought that the aerobic oxidation ammonia to nitrite is exclusively catalysed by two distinct groups of Bacteria. The isolation of a chemolithoautotrophic ammonia-oxidizing crenarchaeon revealed first direct evidences for nitrification within the domain Archaea. In addition, the cultivation of the first mesophilic crenarchaeote *Candidatus Nitrosopumilus maritimus* further provided an organismic link between an abundant phylogenetic group, the marine group 1.1a crenarchaeotes, and the marine nitrogen cycles. Subsequent molecular surveys using mainly archaeal ammonia-moonxygenase subunit A genes demonstrated that ammonia-oxidising archaea (AOA) form a quite diverse microorganism group and may even represent the predominant ammonia oxidisers in certain marine and terrestrial habitats. Both, archaeal and bacterial ammonia oxidisers share the same chemolithoautotrophic lifestyle by gaining energy via aerobic ammonia oxidation to nitrite and fixing inorganic carbon as sole carbon source. However, recent genomic, biochemical and physiological studies on *N. maritimus* revealed differences between biochemical pathways operating in AOA and in AOB. For instance, all known AOB use the Calvin-cycle for carbon assimilation while the 3-hydroxypropionate/4-hydroxybutyrate-pathway was found to operate in AOA. The lack of genes coding for an AOB hydroxylamine oxidoreductase, a key enzyme in AOB, in the genome of *N. maritimus* points to a different mechanism for ammonia oxidation. The novel pathways found in *N. maritimus*, serving as model organism for a marine archaeal ammonia oxidizer, exhibit physiological properties that enable AOA to successfully outcompete their bacterial counterparts in ammonia-poor, oligotrophic environments. Furthermore, the cultivation of novel mesophilic and thermophilic AOAs from natural habitats extended the temperature limits of nitrification and allows us to proof hypothesised mechanisms in AOA.

Ammonia oxidizing bacteria and archaea distribution patterns and dynamics in estuaries and oceans

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Ammonia-oxidizing bacteria (AOB) have been the model group for investigation of functional diversity since the beginning of the molecular microbial ecology revolution in the 1990's. Their narrow phylogenetic distribution and highly specialized physiological capabilities implied a strong link for both rRNA genes and signature functional genes with ecological function. Our understanding of nitrification, its role in the N and C cycles and its environmental regulation, have all been challenged by the recent discovery that ammonia oxidation in the ocean and terrestrial environments is dominated by ammonia-oxidizing archaea (AOA) rather than AOB. AOB and AOA appear to share the first enzymatic step in the ammonia oxidation pathway, which is encoded by the ammonia monooxygenase (*amo*) gene. Based on the published diversity of *amoA* gene sequences, it appears that both groups contain a similar breadth of diversity. Although not as diverse as some functional genes (e.g., *nirS* in denitrifiers), the diversity of *amoA* in AOB and AOA implies the maintenance of high diversity even within an apparently narrow ecophysiological niche.

Unlike the open ocean where AOA clearly dominate, the relative abundance of AOA and AOB varies among different estuarine systems. In most systems, salinity is a very important variable in determining the distribution and community composition of AOB, and several studies have documented a decrease in both abundance and diversity of AOB with increasing salinity along an estuarine gradient. Although fewer data on AOA are available, they appear to follow the opposite trends; AOA are least important in the low salinity region of estuaries. Exceptions have been reported, however, and it is clear that other variables must be important in determining the distribution and activity of both AOB and AOA.

The great importance of estuaries in modulating N fluxes between land and sea has motivated our research on the functional diversity of AOB and AOA in Chesapeake Bay. In order to obtain high throughput high resolution analysis of community composition, we developed functional gene microarrays, which contain archetype oligonucleotide probes representing all published *amoA* sequences for either AOA or AOB (separate arrays). The archetype probes are designed using an iterative algorithm such that each probe hybridizes with all sequences of $\geq 87\%$ identity. The 70-mer oligos represent the fragment of the gene that contains the highest diversity across all known homologous genes. Hybridization patterns on the arrays are interpreted in terms of relative abundance of each archetype in DNA or RNA extracted from environmental samples. Using an internal standard present in each feature on the array, the hybridization data yield relative fluorescence ratios (RFR) that may be quantitatively compared between probes across arrays.

Three stations representing the oligohaline, mesohaline and euryhaline regions of Chesapeake Bay were sampled 11 times over the course of four years from the surface

sediments and at three depths in the water column. DNA extracts were labeled and hybridized to the AOB *amoA* array in triplicate. Diversity of AOB increased with depth in the water column and was greatest in the sediments. The strongest hybridization signals were derived from archetype probes representing estuarine *amoA* sequences. Probes representing marine archetypes yielded significant signals only from the deep water and sediments of the euryhaline station.

Using a combination of non-weighted discrimination analysis and principal components analysis, the RFR data were analyzed to identify temporal patterns among the archetypes. For the deep water location in the upper estuary (oligohaline) where AOB dominate, we detected repeating seasonal patterns among groups of archetypes over multiple years. The 26 archetypes clustered into three groups, and each had a distinct seasonal pattern with one group most important in April, one in August and one in October. Each pattern repeated consistently across the four years, suggesting each cluster of archetypes represents AOB with characteristics favored by the seasonally varying conditions. PCA identified the same clusters of archetypes as covarying and identified correlations with different environmental variables for each cluster. For example, the two estuarine archetypes that had the strongest signals overall belonged to different clusters with different temporal patterns. One of them was positively correlated with nitrite concentration and negatively with total dissolved nitrogen while the other dominant archetype (and its cluster of probes with a similar temporal pattern) had the opposite correlations.

The surface water at the same station also showed seasonal patterns among archetype clusters. The most interesting pattern was a one time event associated with Hurricane Isabel. The hurricane flooded the Chesapeake Bay region with rain and runoff when it made landfall in September 2003. Our sample from the subsequent October had an unusual community composition, dominated by probes representing sequences associated with oligotrophic environments. This was consistent with the anomalous nutrient and salinity levels measured in this sample as a result of dilution by the hurricane floodwaters. After the hurricane event, the community composition returned to the pattern observed in previous years.

In addition to the relative quantification of the microarrays, we used quantitative-PCR to evaluate the distributions of AOB and AOA in several marine environments, from Chesapeake Bay to the Sargasso Sea, the Eastern Tropical South Pacific (ETSP) and the Arabian Sea. AOA dominate all of the oceanic environments numerically. Among the AOA, striking biogeographical patterns were observed in hybridization patterns with the *amoA* AOA microarray. For example, archetypes representing sequences found only in clone libraries from deep low oxygen waters were found in the ETSP and Arabian Sea, but not in the Sargasso Sea, consistent with the biogeochemistry of these regions. Although sequences from clone libraries tend to show geographical clustering, we detected “soil” archetypes in several of the marine environments.

Using quantitative PCR and functional gene microarrays representing all known phylotypes of either group, we have documented spatial and temporal patterns in abundance and assemblage composition of AOB and AOA. These patterns suggest niche separation within the AOB and AOA on the basis of parameters such as substrate and oxygen concentration. In the open ocean, however, it is not clear which environmental

parameters might be important in influencing community composition, even though marine AOA assemblages appear to show distinct biogeographical distributions. AOA are consistently found in oxygen minimum zones, which might suggest they have some anaerobic metabolic capabilities. Their role, if any, in producing nitrous oxide, formerly attributed to AOB metabolism, remains unknown, along with the physiological characteristics of AOA from most natural environments. How the AOA and AOB partition the environment, whether they compete and how they vary in time in the ocean are critical research questions. It's clear that much remains to be learned about both AOB and AOA, and the role of nitrification in the ocean's C and N cycles.

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Session #3 Nitrification

Title: "Regulation of Aerobic Ammonia-Oxidizing Metabolism"

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Aerobic ammonia oxidation is an essential process in the global nitrogen cycle as it creates N-oxide intermediates that, when left unchecked by assimilation, nitrite oxidation, denitrification or anaerobic ammonia oxidation (anammox), can have dire effects on ecosystem quality. For instance, biogenic emissions of nitric and nitrous oxides contribute to global warming and destruction of the stratospheric ozone layer (Stein & Yung, 2003) while nitrate-rich run-off causes eutrophication of surface waters. The release of nitrogen oxides is a consequence of N-overloading, favoring the loss of N-oxide intermediates from overlapping processes of the N-cycle rather than returning fixed-N back to the atmospheric dinitrogen pool. We have now reached a point where the anthropogenic contributions of fixed-N to the world's ecosystems exceeds that of all natural sources (Röckstrom, et al., 2009). This massive imbalance of the N-cycle and release of N-oxides has been taken as a sure sign that we are leaving the Holocene epoch altogether for the Anthropocene.

For the past 120 years, our understanding of how aerobic ammonia oxidation is regulated has relied almost exclusively on a single model organism, *Nitrosomonas europaea* that is far from representative of natural ammonia-oxidizer diversity. Only recently have other model systems emerged to encompass a broader range of ammonia-oxidizer diversity. Through metagenomic studies it was discovered that a group of mesophilic ammonia-oxidizing Crenarchaea are numerically dominant in many soil (Leininger, et al., 2006) and marine ecosystems (Massana, et al., 1997). An obligate ammonia-oxidizing marine Crenarchaeon, *Nitrosopumilus maritimus*, has been brought into pure culture (Könneke, et al., 2005). In addition, the complete genome sequence of *N. maritimus* (Walker, et al., 2010) and several ammonia-oxidizing bacteria (Chain, et al., 2003, Klotz, et al., 2006, Norton, et al., 2008, Stein, et al., 2007) are available such that we can dissect, reconstruct and compare metabolic pathways.

Obligatory ammonia oxidation is possible through the combination of two functional modules – one for the oxidation of ammonia to a reactive N-oxide intermediate and one for oxidizing the N-oxide intermediate to nitrite. Ammonia-oxidizing bacteria and *N. maritimus* encode and express ammonia monooxygenase (AMO), although the product of ammonia oxidation by AMO is likely different between bacteria and archaea. Ammonia-oxidizing bacteria produce hydroxylamine, whereas a likely intermediate of archaeal ammonia oxidation is nitroxyl (Walker, et al., 2010). This hypothesis is supported by the lack of homologous genes for hydroxylamine oxidoreductase (*haoAB*) and cytochrome *c* proteins in the *N. maritimus* genome. Ammonia-oxidizing bacteria express the cytochrome *c*-rich hydroxylamine oxidoreductase along with its electron carrying partners to connect hydroxylamine oxidation to the quinone pool for energy generation, reverse electron flow, and ammonia oxidation (Klotz & Stein, 2008). In contrast, *N. maritimus* likely uses a novel nitroxyl hydrolase (or a novel non-iron hydroxylamine oxidoreductase) to produce nitrite and metabolically useful electrons that are conveyed to the quinol pool via copper-containing plastocyanin carriers. Even among the ammonia-oxidizing bacteria and archaea, genes encoding ammonia monooxygenase are regulated by diverse cues and AMO is regulated posttranslationally (Berube, et al., 2007, El Sheikh & Klotz, 2008, Stein, et al., 2000, Treusch, 2005). Differential regulation of AMO, and thus the ammonia-oxidation process, is a consequence of niche differentiation among the obligate ammonia-oxidizers.

Thus far, all characterized ammonia-oxidizing bacteria and several of the *amo*-encoding Crenarchaea also encode copper-containing nitrite reductase (NirK). In *N. europaea*, NirK is used as a redox-balancing enzyme under conditions of high nitrite and low oxygen (Cantera &

Stein, 2007) and can also be used as a terminal reductase for anoxic growth (Schmidt, et al., 2004). However, NirK enzymes are extremely diverse in structure and genomic context in ammonia-oxidizing bacteria and crenarchaea, and some crenarchaea that encode ammonia monooxygenase lack *nirK* homologues. One possibility for NirK function in *N. maritimus* is to reduce nitrite to nitric oxide which could then act as an alternate oxidant for AMO under low oxygen tension.

The hydroxylamine oxidoreductase (HAO) of the ammonia-oxidizing bacteria shares ancestry with pentaheme and octaheme nitrite reductases and the HAO from anammox bacteria (Klotz, et al., 2008). Furthermore, several gammaproteobacterial methanotrophs maintain functional HAO to rid themselves of hydroxylamine formed from the oxidation of ammonia via particulate methane monooxygenase enzymes (pMMO) (Campbell, et al., 2010, Nyerges & Stein, 2009, Poret-Peterson, et al., 2008). AMO and pMMO also share a common ancestry (Klotz & Norton, 1998, Norton, et al., 2002). Thus, ammonia-oxidizing methanotrophs constitute a population that does not use the process obligatorily for cell growth. These “facultative” ammonia-oxidizers are ecologically significant as they likely account for a significant fraction of ammonia conversion to nitrogen oxides, including nitrous oxide, in N-impacted environments.

One of the biggest differences between the ammonia-oxidizing bacteria (obligate and facultative) and the ammonia-oxidizing archaea is their contribution to nitrous oxide emissions. Nitrous oxide is produced by two functionally distinct pathways in ammonia-oxidizing bacteria: the oxidation of hydroxylamine and the reduction of nitrite (Wrage, et al., 2001). As ammonia-oxidizing archaea apparently lack HAO enzymes, the hydroxylamine-linked pathway for nitrous oxide production is likely missing. Furthermore, ammonia-oxidizing archaea are favored in environments with exceedingly low levels of ammonia (Martens-Habbena, et al., 2009) such that leakage N-oxide intermediates would be virtually undetectable. In contrast, ammonia-oxidizing bacteria are highly competitive with the ammonia-oxidizing archaea as ammonia levels climb (Erguder, et al., 2009). Therefore, the increase in fixed-N to the world’s ecosystems is favoring the activity and growth of bacteria that contribute to nitrous oxide flux while decreasing the biological methane sink.

Based on the differential presence and regulation of *amo*, *hao*, and *nirK* genes and the connection between gene expression and physiological function, we now have a more complete understanding of how diverse ammonia-oxidizing prokaryotes correspond to differences in nitrifying activities and the release of nitrogen oxides to the surrounding environment.

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