

Archaeal nitrification in the ocean

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Edited by Carl R. Woese, University of Illinois at Urbana–Champaign, Urbana, IL, and approved June 6, 2006 (received for review January 30, 2006)

Marine Crenarchaeota are the most abundant single group of prokaryotes in the ocean, but their physiology and role in marine biogeochemical cycles are unknown. Recently, a member of this clade was isolated from a sea aquarium and shown to be capable of nitrification, tentatively suggesting that Crenarchaeota may play a role in the oceanic nitrogen cycle. We enriched a crenarchaeote from North Sea water and showed that its abundance, and not that of bacteria, correlates with ammonium oxidation to nitrite. A time series study in the North Sea revealed that the abundance of the gene encoding for the archaeal ammonia monooxygenase alpha subunit (*amoA*) is correlated with a decline in ammonium concentrations and with the abundance of Crenarchaeota. Remarkably, the archaeal *amoA* abundance was 1–2 orders of magnitude higher than those of bacterial nitrifiers, which are commonly thought to mediate the oxidation of ammonium to nitrite in marine environments. Analysis of Atlantic waters of the upper 1,000 m, where most of the ammonium regeneration and oxidation takes place, showed that crenarchaeotal *amoA* copy numbers are also 1–3 orders of magnitude higher than those of bacterial *amoA*. Our data thus suggest a major role for Archaea in oceanic nitrification.

Crenarchaeota | enrichment culture | North Sea | North Atlantic Ocean | nitrifying bacteria

Archaea constitute one of the three domains of life next to the Eukaryotes and Bacteria. Until a decade ago, Archaea were thought to mainly consist of organisms thriving in extreme environments such as sulfidic hot springs, salt brines, and anoxic environments. With the advent of molecular biological techniques, it became clear that Archaea are actually widespread and occur in diverse environments such as oceans, lakes, and soils (1–4). However, these nonextremophilic Archaea are not closely related to cultured relatives, and thus not much is known about their physiology and role in biogeochemical cycling.

Planktonic Archaea in the ocean comprise both Crenarchaeota and Euryarchaeota, of which the former appears to be the most abundant (1, 5). Marine Crenarchaeota are typically relatively more abundant in deep neritic waters and in the meso- and bathypelagic zones of the ocean (1, 5–7) and are thought to account for $\approx 20\%$ of all prokaryotic cells in the global ocean (1). The metabolism of these planktonic Crenarchaeota is a subject of current debate. *In situ* labeling (8) and microautoradiography (7) experiments showed that marine Crenarchaeota can use dissolved inorganic carbon as carbon source but are also able to take up amino acids (7, 9), suggesting a heterotrophic lifestyle. Recently, a crenarchaeote, *Candidatus Nitrosopumilus maritimus*, was isolated from a sea aquarium and shown to be autotrophic and able to oxidize ammonium to nitrite (10). Positive correlations between the abundance of Crenarchaeota and nitrite were observed in the Arabian Sea (11) and the Santa Barbara Channel time series (12) and with particulate organic nitrogen in Arctic waters (13). Furthermore, a diverse set of putative archaeal ammonia monooxygenase encoding genes (*amoA*) were recently reported from shallow (<300 m) marine waters and sediments (14) and was found in fosmid sequences of *Cenarchaeum symbiosum* (15). These findings hint that Crenarchaeota may be involved in the marine nitrogen cycle, possibly as

nitrifiers, but the relevance of crenarchaeotal nitrification for the marine nitrogen cycle is unknown. Until now it has been assumed that marine nitrification is mainly performed by two different groups of bacteria belonging to the β - and γ -proteobacteria (16).

Here, we provide experimental evidence that Crenarchaeota present in seawater are capable of aerobic ammonium oxidation (nitrification) by enrichment of a nitrifying crenarchaeote from coastal waters. We also show by quantitative analysis of both archaeal and bacterial *amoA* in coastal and open ocean waters that marine Crenarchaeota are likely important players of the present-day marine nitrogen cycle.

Results and Discussion

Enrichment Culture of a Nitrifying Crenarchaeote. We enriched a member of the Crenarchaeota from North Sea waters. The experimental setup was originally designed to examine the temperature adaptation of marine crenarchaeotal membrane lipids (see ref. 17 for details). During this experiment, coastal North Sea water was incubated in the dark for 6 months at 25°C in an 850-liter mesocosm tank without addition of nutrients. A substantial increase in archaeal membrane lipids was observed, which coincided with an almost complete consumption of ammonium (17). This initial experiment tentatively suggested a link between North Sea Crenarchaeota and the oxidation of ammonium. To further investigate this possibility, water from this mesocosm tank was incubated in the dark at 22°C and 25°C, and inorganic nutrients, including ammonium, were added while pH was kept constant at 8.2, the regular pH of surface seawater. The abundance of Crenarchaeota was monitored with catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) (18) and revealed a substantial enrichment of crenarchaeotal cells at both temperatures (Fig. 1 *Lower*; see also Fig. 4 and Table 2, which are published as supporting information on the PNAS web site). After a lag phase, crenarchaeotal abundance increased with a doubling time of 2 days to 4–5 $\times 10^6$ cells per ml in the incubation series and comprised at that time up to 40–70% of DAPI-stainable cells. Bacterial abundance increased during the first 3 days of incubation and then remained at <5% of DAPI-stainable cells during the whole incubation period (Fig. 1 *Lower*). Generally, 20–30% of the DAPI-stained cells are found to be composed of dead cell material (19), suggesting that our enrichment culture was dominated by Crenarchaeota at day 11 (Fig. 1 *Lower*). In fact, molecular analyses selective for Archaea revealed

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: AOB, ammonia-oxidizing bacteria; CARD-FISH, catalyzed reporter deposition–FISH; DGGE, denaturing gradient gel electrophoresis; QPCR, quantitative PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ659389–DQ659410 (archaeal 16S rDNA) and DQ784527–DQ784538 (*amoA*)].

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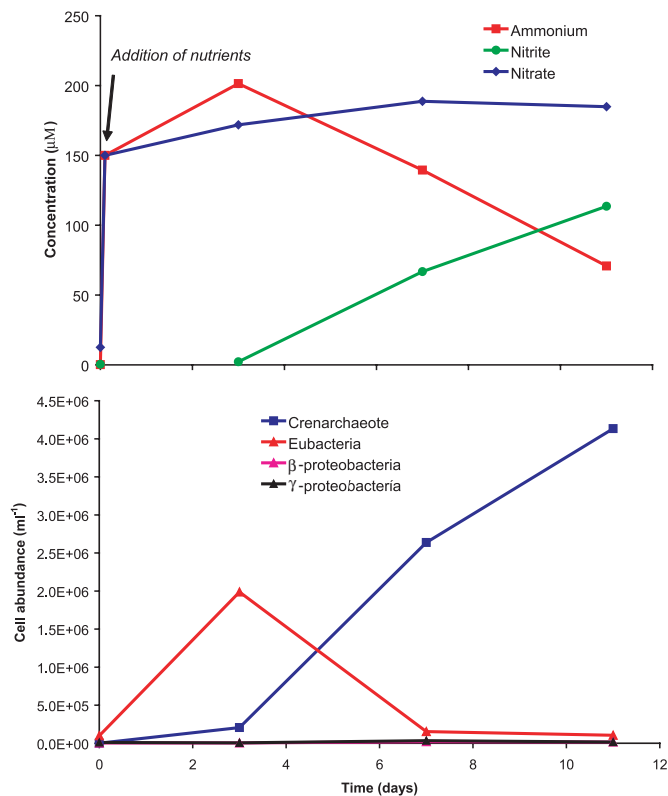


Fig. 1. Archaeal nitrification in an enrichment culture of a crenarchaeote from the North Sea. (Upper) Nutrient concentrations (micromolar) in the course of the experiment. (Lower) Crenarchaeotal, eubacterial, and β - and γ -proteobacterial cell numbers (cells per milliliter) as determined by CARD-FISH (18). Aged seawater from a large mesocosm experiment (see text and *Materials and Methods*) with added inorganic nutrients was incubated at two different temperatures in the dark. The data shown are those obtained at 25°C. The crenarchaeotal population in our enrichment culture was shown to consist of a single species, phylogenetically closely related to North Sea Crenarchaeota (Figs. 3–5).

that the incubated waters were dominated by a single member of the crenarchaeotal phylogenetic cluster 1a (6) (see Fig. 5, which is published as supporting information on the PNAS web site) with 99% sequence identity over the nearly complete 16S rRNA gene (1,324 bp) compared with the nitrifying crenarchaeote, *Candidatus "N. maritimus"* (10). When ammonium levels dropped, nitrite concentrations increased concomitantly with the increase in crenarchaeotal abundance (Fig. 1 Upper), similar to what was previously observed in the large mesocosm tank (see above) (17). Importantly, the abundance of β - and γ -proteobacteria, which include the known nitrifying bacteria, remained low (<1% of DAPI-stained cells) throughout the incubation. If all of the detected Proteobacteria had been nitrifiers and all of the nitrification activity had been mediated by these nitrifiers, the activity per cell would have been >100 fmol of NH_3 cell⁻¹·day⁻¹, substantially more than ever reported. However, if Crenarchaeota were responsible for nitrification, the ammonium conversion rates were between 2 and 4 fmol of NH_3 cell⁻¹·day⁻¹ at 22° and 25°C, respectively. These values are well within the ranges reported for bacterial aerobic ammonium oxidation (16) and compare very well with the ≈ 4 fmol of NH_3 cell⁻¹·day⁻¹ for *Candidatus "N. maritimus"* (estimated from figure 3 in ref. 10).

Further evidence for ammonia oxidation by the enriched marine crenarchaeote was provided by the identification of a single *amoA*. Its sequence is closely related (91% nucleotide and 98% amino acid sequence identities) to the *amoA* obtained from the archaeon *Candidatus "N. maritimus"* (10) and to archaeal *amoA* recovered

from the Sargasso Sea (4, 20) (90% nucleotide and 95% amino acid sequence identities; see Fig. 2) and only distantly related to known bacterial *amoA* sequences. Quantification of the abundance of the *amoA* and the marine crenarchaeotal 16S rRNA gene at day 7 in the enrichment culture yielded a relative ratio of 0.9:1, suggesting that the enriched crenarchaeote possesses a single copy of *amoA*. Thus, the distribution and coincidence of single phylotypes of *amoA* and marine crenarchaeotal 16S rRNA gene in relation to the observed changes in nutrient concentrations strongly suggest that the enriched marine crenarchaeote was involved in nitrification. Our result is in agreement with recent findings regarding an ammonium oxidizing crenarchaeote isolated from an aquarium (10) and the presence and expression of *amoA* in soil Crenarchaeota (21). Importantly, our findings suggest that this metabolism may be widespread among Crenarchaeota thriving in marine waters.

Importance of Archaeal Nitrification in an Ocean Margin System. The ecological significance of the observed crenarchaeotal nitrification was investigated in the coastal waters of the North Sea from which the marine crenarchaeote was enriched. PCR amplification of archaeal 16S rRNA gene by using a general archaeal primer followed by phylogenetic analysis of sequenced denaturing gradient gel electrophoresis (DGGE) fragments revealed that Crenarchaeota dominated the archaeal community from late fall to early spring. The recovered 16S rRNA crenarchaeotal gene sequences from the North Sea during the crenarchaeotal winter bloom were closely related to each other and to the enriched crenarchaeote with sequence similarities of >96%, and all belonged to the Group I.1a (6) of the Crenarchaeota (Fig. 5). The abundance of Crenarchaeota varied coincidentally with inorganic nitrogen species in North Sea waters: When ammonium levels dropped from 12.7 to 8.5 μM and nitrite levels were rising from 0.8 to 2.5 μM between November and December, crenarchaeotal abundance increased by 2 orders of magnitude (see Fig. 3 Middle). In December, abundances of Crenarchaeota decreased considerably, for reasons presently unclear, whereas ammonium levels remained relatively constant at ≈ 9 μM . In early January, crenarchaeotal abundance again increased substantially by 1 order of magnitude, coinciding with a decrease in ammonia levels from 10 to 3 μM . In contrast to the crenarchaeotal cell abundance, the cell abundance of β - and γ -proteobacteria as determined by CARD-FISH remained high but invariant throughout December to March with $4 \pm 1 \times 10^5$ cells per ml and did not show a distinct elevation in abundance when ammonium concentrations dropped.

Analyses of *amoA* genes of the North Sea time series yielded one dominant archaeal *amoA* gene. This gene was closely related to that of *Candidatus "N. maritimus"* (92% nucleotide and 97% amino acid identity) (Fig. 2). Importantly, quantification of the archaeal *amoA* copy numbers by quantitative PCR (QPCR) showed a strong positive linear correlation with both crenarchaeotal cell counts by CARD-FISH ($r^2 = 0.81$, $n = 9$) and crenarchaeotal 16S rRNA gene abundance ($r^2 = 0.94$, $n = 39$). The slopes of these correlation lines are 2.5 and 2.8, respectively, suggesting that each crenarchaeotal cell possessed two or three copies of *amoA*. This amount is significantly more than the single copy of *amoA* per cell in the enriched crenarchaeote. This finding may reflect either inaccuracies in our QPCR approach or variability in *amoA* copies within Crenarchaeota similar to ammonia-oxidizing bacteria (AOB) (22). Compared with archaeal *amoA*, β -proteobacterial *amoA* were present in equal or slightly higher numbers only during late spring and summer, while *amoA* of γ -proteobacteria could not be detected at all with our primer set. However, from late fall to early spring, during times of ammonium consumption, bacterial *amoA* was 1–2 orders of magnitude less abundant than Crenarchaeota-derived *amoA* (Fig. 3 Bottom). A recent study of ammonium-oxidizing β -proteobacteria in the North Sea reported the same seasonal abundance pattern (23). Thus, our results strongly suggest that the dominant crenar-

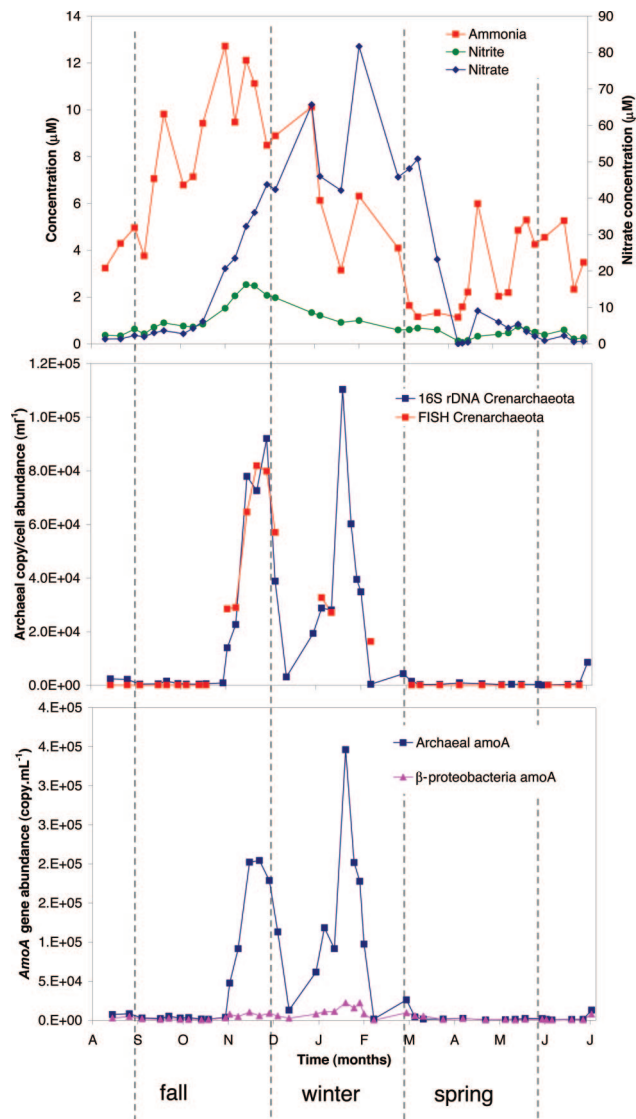


Fig. 3. Crenarchaeotal abundance in the North Sea between August 2002 and July 2003 as a response to changing nutrient concentrations. (*Top*) Nutrient concentrations (micromolar). (*Middle*) Cell abundances (cells per milliliter) of Crenarchaeota as determined by CARD-FISH (18) and abundances of 16S rDNA copies of Crenarchaeota as determined by QPCR (see *Materials and Methods*). (*Bottom*) Abundances of archaeal and β -proteobacterial *amoA* copy numbers as determined by QPCR. The sharp increases in crenarchaeotal cell numbers and archaeal *amoA* copy numbers in November and January co-occur with the transformation of ammonia to nitrate. In contrast, a far less pronounced increase in *amoA* copy numbers of β -proteobacterial ammonia oxidizers was observed in this period ($\approx 1.0 \times 10^4$) compared with the rest of the year ($\approx 2.3 \times 10^3$).

ocean waters by primary producers, ammonium regeneration from decomposing descending particulate organic nitrogen, subsequent oxidation of ammonium to nitrite by members of the *Nitrosomas/Nitrosospira* and *Nitrosococcus* groups, and oxidation of nitrite to nitrate by nitrite oxidizers such as *Nitrobacter* sp. (26). However, no molecular ecological study has so far revealed large numbers of known nitrifying bacteria in marine waters (16), whereas marine Crenarchaeota do constitute ≈ 20 –30% of the total prokaryotic community (1).

Analysis of cell numbers of Crenarchaeota in the upper 1,000 m of the North Atlantic as determined by CARD-FISH (7) and the abundance of archaeal *amoA* (Table 1) revealed a ratio of 1.2 ± 1.3

copies per cell, which is in between the ratios observed in the North Sea time series and the enriched crenarchaeote. The recovered *amoA* sequences fall into two phylogenetic clusters, including the one containing the North Sea enrichment culture *amoA* sequence and that of *Candidatus* “*N. maritimus*” (Fig. 2). Quantification of bacterial *amoA* gene in the same set of water samples (Table 1) revealed that the *amoA* gene abundance of β -proteobacteria was lower by 1–3 orders of magnitude compared with archaeal *amoA*, whereas *amoA* gene abundance of γ -proteobacteria was below our detection limit. These combined results suggest that Crenarchaeota in the mesopelagic layer of the open ocean are also involved in nitrification and may play a more important role than bacterial nitrifiers.

Our data can be combined with literature data to give a rough estimate of the global importance of archaeal nitrification. Mineralization in the meso- and bathypelagic zones of the ocean [2.2×10^{15} mol·C·yr $^{-1}$ (27)] releases $\approx 3.3 \times 10^{14}$ mol·N·yr $^{-1}$, assuming Redfield stoichiometry (28). If all of the generated ammonium would be oxidized by Crenarchaeota fixing one carbon atom for every ≈ 10 nitrogen molecules oxidized (29), then one would expect an archaeal inorganic carbon fixation rate of $\approx 3.3 \times 10^{13}$ mol·C·yr $^{-1}$. This estimate is consistent with the estimated rate of global inorganic carbon fixation in the dark ocean by Archaea of 6.6×10^{13} mol·C·yr $^{-1}$, of which $\approx 4.5 \times 10^{13}$ mol·C·yr $^{-1}$ may be taken up by Crenarchaeota (7), assuming that Euryarchaeota and Crenarchaeota are growing at equal rates. Archaeal nitrification thus may be an important process in the biogeochemical cycling of nitrogen in the ocean, although it remains uncertain whether all pelagic Crenarchaeota are nitrifiers. These data, together with the recently established importance of *Planctomycetes* in denitrification (30, 31) and unicellular cyanobacteria in dinitrogen fixation (32, 33), show the important role of hitherto unrecognized prokaryotes in the oceanic biogeochemical cycling of nitrogen.

Materials and Methods

Incubation Experiment Setup. Coastal North Sea water was kept in the dark at 25°C in an 850-liter mesocosm tank without addition of nutrients for 6 months (17). After these 6 months, batch cultures with aged mesocosm water were incubated in 20 liters of Clearboy tanks (Nalge Nunc, Rochester, NY) at 22° and 25°C in the dark. Nutrients were added before the incubation at concentrations of 150 μ M NaNO $_3$, 150 μ M NH $_4$ Cl, 25 μ M NaH $_2$ PO $_4$, and 2,666 μ M NaHCO $_3$ together with a sterile mix of 12.5 mg/liter yeast, 5 mg/liter peptone extract, vitamins, and trace elements. The pH was regularly adjusted to 8.2 by adding sterile 0.1 M NaOH or HCl, and salinity was maintained at 27 by addition of distilled water. The 20-liter tanks were continuously stirred and left open throughout the experiment, allowing constant gas exchange with the air. Samples for nutrient analysis, CARD-FISH, and DNA were taken every 3–4 days.

Coastal North Sea Time Series. The sampling site is situated at the western entrance of the North Sea into the Wadden Sea at the Island Texel (53°00'25"N, 4°78'27"E). Water samples for DNA and CARD-FISH were taken biweekly from August 2002 to July 2003.

CARD-FISH Analyses. Fifteen-milliliter water samples were fixed with formaldehyde (final concentration 4%) and stored at 4°C for at least 4 h. Thereafter, the samples were filtered onto 0.2- μ m polycarbonate filters (25-mm filter diameter; Millipore, Billerica, MA) with 0.45- μ m cellulose nitrate filters (Millipore) as supporting filters and stored frozen at -20° C until further analysis. Total picoplankton were enumerated after DAPI staining (34), and Bacteria and Archaea were enumerated by CARD-FISH (18) under the epifluorescence microscope. The oligonucleotide probes Eub338, BET42, and GAM42 were used for enumeration of Bacteria (35), β - and γ -proteobacteria (23), respectively, and specific probes were applied for the marine Crenarchaeota Group I,

clearly differed from that in the Atlantic Ocean (data not shown).

DGGE fragments were excised from the gels and subsequently sequenced for phylogenetic comparison with reference sequences from GenBank (39) by using the ARB software package (40). Archaeal 16S rDNA and *amoA* sequences obtained in this study have been deposited in GenBank under accession nos. DQ659389–DQ659410 and DQ784527–DQ784538, respectively.

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We thank Bouwe Kuipers, Govert van Noort, Harry Witte, and Karel Bakker (Royal NIOZ) for analytical assistance; Jesus Maria Arrieta for collecting the samples for molecular analyses in the North Atlantic during the TRANSAT cruises; Dr. Karen Casciotti (Woods Hole Oceanographic Institution, Woods Hole, MA) for supplying the *Nitrosococcus* and *Nitrosomonas* cultures; and Dr. G. Jurgens for the aligned ARB database. This work was supported in part by grants from the Netherlands Organisation for Scientific Research (NWO) (to J.S.S.D., G.J.H., and M.J.L.C.).