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Cultivation of Autotrophic Ammonia-Oxidizing Archaea from Marine Sediments in Coculture with Sulfur-Oxidizing Bacteria▽†

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The role of ammonia-oxidizing archaea (AOA) in nitrogen cycling in marine sediments remains poorly characterized. In this study, we enriched and characterized AOA from marine sediments. Group I.1a crenarchaea closely related to those identified in marine sediments and "*Candidatus* Nitrosopumilus maritimus" (99.1 and 94.9% 16S rRNA and *amoA* gene sequence identities to the latter, respectively) were substantially enriched by coculture with sulfur-oxidizing bacteria (SOB). The selective enrichment of AOA over ammonia-oxidizing bacteria (AOB) is likely due to the reduced oxygen levels caused by the rapid initial growth of SOB. After biweekly transfers for ca. 20 months, archaeal cells became the dominant prokaryotes (>80%), based on quantitative PCR and fluorescence *in situ* hybridization analysis. The increase of archaeal 16S rRNA gene copy numbers was coincident with the amount of ammonia oxidized, and expression of the archaeal *amoA* gene was observed during ammonia oxidation. Bacterial *amoA* genes were not detected in the enrichment culture. The affinities of these AOA to oxygen and ammonia were substantially higher than those of AOB. [^{13}C]bicarbonate incorporation and the presence and activation of genes of the 3-hydroxypropionate/4-hydroxybutyrate cycle indicated autotrophy during ammonia oxidation. In the enrichment culture, ammonium was oxidized to nitrite by the AOA and subsequently to nitrate by *Nitrospina*-like bacteria. Our experiments suggest that AOA may be important nitrifiers in low-oxygen environments, such as oxygen-minimum zones and marine sediments.

Archaea have long been known as extremophiles, since most cultivated archaeal strains were cultivated from extreme environments, such as acidic, hot, and high-salt environments. The view of archaea as extremophiles (i.e., acidophiles, thermophiles, and halophiles) has radically changed by the application of molecular technologies, including PCR in environmental microbiology. Using *Archaea*-specific PCR primers, novel archaeal 16S rRNA gene sequences were discovered in seawater (23, 27). Following these discoveries, an ever-increasing and unexpectedly high variety of archaeal 16S rRNA gene sequences has been reported from diverse "nonextreme" environments (67). This indicates that archaea are, like bacteria, ubiquitous in the biosphere rather than exclusively inhabiting specific extreme niches. *Archaea* are abundant in water columns of some oceanic provinces (33, 36) and deep-subsea floor sediments (11, 12, 48). Despite the increasing number of reports of the diversity and abundance of these nonextreme archaea by molecular ecological studies, their physiology and ecological roles have remained enigmatic.

Oxidation of ammonia, a trait long thought to be exclusive to the domain *Bacteria* (13), was recently suggested to be a trait of archaea of the crenarchaeal groups I.1a and I.1b, based on a metagenome analysis (79) and supported by the discovery of archaeal *amoA*-like genes in environmental shotgun sequencing studies of Sargasso Sea water (80) and genomic analysis of "*Candidatus* Cenarchaeum symbiosum," a symbiont of a marine sponge (30). Molecular ecological studies indicated that these ammonia-oxidizing archaea (AOA) are often predominant over ammonia-oxidizing bacteria (AOB) in ocean waters (9, 53, 87), soils (17, 47), and marine sediments (61). Critical evidence for autotrophic archaeal ammonia oxidation was obtained by the characterization of the first cultivated mesophilic crenarchaeon (group I.1a), "*Candidatus* Nitrosopumilus maritimus SCM1," from an aquarium (38), and a related archaeon from North Sea water (87) and subsequently by enrichment of thermophilic AOA (22, 31). Whole-genome-based phylogenetic studies recently indicated that the nonthermophilic crenarchaea, including the AOA, likely form a phylum separate from the *Crenarchaeota* and *Euryarchaeota* phyla (15, 16, 72). This proposed new phylum was called *Thaumarchaeota* (15).

Microorganisms in marine sediments contribute significantly to global biogeochemical cycles because of their abundance (85). Nitrification is essential to the nitrogen cycle in marine sediments and may be metabolically coupled with denitrification and anaerobic ammonium oxidation, resulting in the removal of nitrogen as molecular nitrogen and the generation of greenhouse gases, such as nitrous oxide (19, 75). Compared with studies on archaeal nitrification in the marine water column, only limited information on archaeal nitrification in marine sediments is available so far. Archaeal *amoA* genes have been retrieved from marine and coastal sediments (8, 26, 61), and the potentially important role of AOA in nitrification has

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been suggested based on the abundance of archaeal *amoA* genes relative to that of bacterial *amoA* genes in surface marine sediments from Donghae (South Korea) (61). Cultivation of AOA, although difficult (38), remains essential to estimating the metabolic potential of archaea in environments such as soils (47) and marine sediments (61). Here, we report the successful enrichment of AOA of crenarchaeal group I.1a from marine sediments by employing a coculture with sulfur-oxidizing bacteria (SOB) which was maintained for ca. 20 months with biweekly transfers. In this way, we were able to characterize AOA from marine sediments, providing a clue for the role of AOA in the nitrogen cycle of marine sediments.

MATERIALS AND METHODS

Collection of samples. Sediment samples (~100 g of sediment and 1 liter of seawater) were collected from Donghae (128°35′E, 38°20′N; depth, 650 m) and Svalbard (Arctic region, 16°28′E, 78°21′N; depth, 78 m) (referred to as the SJ and AR samples, respectively) in sterilized glass bottles. Approximately 10 ml of each well-mixed sediment slurry was transferred to a sterile conical tube and transported back to the laboratory at 4°C.

Cultivation of AOA and chemical analysis. Cultures were grown aerobically in natural seawater media, which contained autoclaved coastal seawater from Donghae (South Korea) supplemented with ammonium chloride (1 mM), sodium thiosulfate (0.1 mM to 1 mM), sodium bicarbonate (2.5 mM), potassium phosphate (0.1 mM), trace element mixture (1×) (86), and vitamin solution (1×) (86). The pH was adjusted to 8.2 by using 1 N NaOH or HCl. Marine sediment slurries (0.5 g) from Donghae, South Korea, and the Arctic sea of Svalbard were taken as inocula for 10-ml media in glass tubes sealed with butyl-rubber stoppers. The cultures were incubated at a static condition with daily intermittent inverting instead of continuous shaking. After oxidation of ammonia (typically after 2 weeks), 5% of the total culture volume was transferred to a fresh seawater medium at 25°C under dark conditions. Unless otherwise stated, we supplemented each starting batch culture with 1 mM ammonia and 0.1 mM thiosulfate as energy sources. The pH of the medium remained almost constant (8.0 to 8.2) during the culture cycle.

Determination of ammonia and thiosulfate was conducted colorimetrically (71, 81). The concentrations of nitrite and nitrate were determined by using an ion chromatograph (ICS-1500; Dionex, Sunnyvale, CA) with an OnGuard II Ag cartridge (Dionex) (51). Dissolved oxygen (DO) and pH were measured by using the 3-Star DO meter (Thermo, Beverly, MA) and S20 SevenEasy pH meter (Mettler Toledo, Switzerland), respectively. During oxidation of thiosulfate and ammonia, the oxygen concentration was decreased and maintained low. After depletion of both electron donors, the oxygen concentration was rebounded to original level. This was caused by a combination of the cease of oxygen uptake and the continued diffusion of oxygen from the headspace of the culture.

Determination of kinetics of ammonium oxidation and oxygen uptake. The kinetic study of the oxygen uptake and ammonia oxidation was performed with the AR culture as described in a previous study (14, 52) with appropriate modifications. For this study, we used the AR culture in which nitrite oxidizers were removed by the inhibitor of nitrite oxidizer, chlorate (10 μM) (7). Oxygen uptake was measured in an Oxygraph system (Hansatech Instruments Ltd., England) equipped with an S1 Clark-type polarographic oxygen electrode disc, 2-ml DW1 borosilicate glass reaction/sample vessel, magnetic stir bars, and supplied Oxygraph Plus software. Oxygen microsensors were polarized continuously for >3 h before use. All measurements were done in a recirculated water bath at 28°C. Activity measurements were carried out with late-exponential- or early-stationary-phase cells by monitoring ammonia oxidation and nitrite production in the AR culture. Aliquots (10 ml) were removed from cultures and immediately transferred to prewarmed 25-ml glass tubes in a 28°C water bath. Subsamples were then filled into the 2-ml DW1 electrode glass vessel assembled with the oxygen electrode disc and carefully sealed with adapted plunger assembly. Oxygen uptake was monitored continuously after an initial equilibration of at least 40 min. Ammonium was added as necessary from stock solution in medium by a Hamilton syringe. The apparent half-saturation constants (K_m) for oxygen and ammonia and the maximum velocity (V_{max}) were determined from plots of the oxygen uptake rate. For comparison, *Nitrosomonas europaea* strain C-31 grown under the same culture condition with AOA in parallel was used as a reference.

FISH and transmission electron microscope (TEM) analysis. Fluorescence *in situ* hybridization (FISH) was carried out on paraformaldehyde-fixed samples as described by Amann et al. (5). The cells from the 10-ml culture were harvested and resuspended in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate, pH 7.5), fixed by the addition of 3 volumes of cold paraformaldehyde solution (4% in PBS), and stored at 4°C for 16 h. The cells were harvested and washed with PBS solution 3 times to remove residual fixation solution and then concentrated 10-fold in PBS solution. The fixed cell suspension was mixed with the same volume of cold absolute ethanol, and the mixture was stored at −20°C. For the FISH analysis of *Bacteria* and *Archaea*, paraformaldehyde-fixed samples were triple hybridized with Cy3-labeled archaeon-specific probe (Arch915) (3) and 6-carboxyfluorescein (FAM)-labeled bacterium-specific probe (EUB338) (4). DAPI (4′,6-diamidino-2-phenylindole) was used for visualization of total cells. Samples were observed by use of a Nikon 80i instrument (Tokyo, Japan) with an oil immersion objective.

To enable transmission electron microscopy analyses of AOA from the enrichment cultures, the cells from the 10-ml culture were fixed in 2.5% paraformaldehyde–1.5% glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded ethanol series (70% to 100%), transferred to propylene oxide, and embedded in Epon-812 (TAAB, England) (50). Ultrathin sections generated with the UltraCut E (Leica, Austria) ultramicrotome were stained with uranyl acetate and lead citrate and examined under a CM 20 electron microscope (Philips, Netherlands) (65).

Genomic DNA and total RNA extractions. Genomic DNA and total RNA were extracted to determine the copy numbers of specific genes in genomic DNA and their expression levels. Cells were harvested from 50-ml culture by centrifugation (i) in the middle of the ammonia oxidation phase (i.e., when about 0.5 mM ammonia remained) and (ii) after 2 days of ammonia depletion and were immediately frozen and stored at −70°C until further analysis. DNA was isolated from frozen cells by using the Solgent genomic DNA prep kit (South Korea) according to the manufacturer's instructions. RNA was isolated from the frozen cells by using the RNeasy minikit (Qiagen, Germany), and cDNA was synthesized by the SuperScript First Strand synthesis system (Invitrogen, San Diego, CA) by following the manufacturer's instructions. Concentrations of DNA, RNA, and cDNA were determined by using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Construction of clone library and phylogenetic analysis of 16S rRNA, ITS, archaeal *amoA*, and carbon fixation genes. Bacterial and archaeal 16S rRNA, 16S-23S rRNA internal transcribed spacer (ITS), putative archaeal *amoA*, and key carbon fixation genes (*acc*, *mce*, and the 4-hydroxybutyryl–coenzyme A [CoA] dehydratase [4-BUDH] gene) of the 3-hydroxypropionate/4-hydroxybutyrate pathway were amplified by PCR using the primers listed in Table 1. The putative key carbon fixation gene sequences of the 3-hydroxypropionate/4-hydroxybutyrate pathway were selected from the publication of Berg et al. (10) for design of primers for PCR amplification. The conditions of the PCR for all the genes were 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, and then 7 min at 72°C. Clone libraries were constructed using PCR products which were purified by using the PCR purification system kit (Solgent, South Korea), ligated using the T&A cloning vector kit (Real Biotech Corporation, Taiwan), and thereafter transformed into *Escherichia coli* DH5α cells according to the manufacturer's instructions. Putative positive clones were transferred to a 96-well plate that contained Luria-Bertani broth (LB) supplemented with ampicillin (100 μg/ml). The clones were grown overnight at 37°C and then stored in LB-glycerol at −70°C before PCR screening and sequencing. Library clones were screened directly by PCR for the presence of inserts by using two M13 universal primers, M13F (5′-GTTTCCCAGTCACGAC-3′) and M13R (5′-TCACACAG GAAACAGCTATGAC-3′). The conditions for PCR screening were 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and then 5 min at 72°C. The positive clones from each library were randomly selected, and the PCR products were purified by using the PCR purification kit (Solgent, South Korea). PCR products were sequenced directly by using ABI Prism BigDye Terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA) and an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). For phylogenetic analysis, the gene sequences of related taxa were obtained from the GenBank database, and multiple alignments were performed using the Clustal X program (78). The phylogenetic trees were constructed by using the MEGA 3 computer software program (43).

Quantification of gene copy numbers by real-time PCR. All quantitative real-time PCR experiments were carried out using the MiniOpticon real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and built-in Opticon Monitor software version 3.1 (Bio-Rad Laboratories, Hercules, CA). For the amplification of all genes, the thermal cycling parameters were 15 min at 95°C

TABLE 1. Primers used for PCR amplification for library construction and real-time quantification

Target gene and corresponding primer pair(s)	Position	Sequence (5′–3′)	Application	Reference(s)
Archaea				
16S rRNA				
519F (DQ831586)[a]	519–533[c]	CAGCMGCCGCGGTAA	Quantification	61, 83
727R	712–727[c]	GCTTTCRTCCCTCACCGT		
arch20F	2–20[c]	TTCCGGTTGATCCYGCCRG	Clone library	23
arch958R	958–976[c]	TCCGGCGTTGAMTCCAATT		
ITS 51R (with arch20F)	51–71[c]	TCGCAGCTTRSCACGYCCTTC	Clone library	28
amoA				
amoAF (EF534487)[a]	217–237[b]	GCTCTAATTATGACAGTATAC	Quantification	61
amoAR	399–422[b]	AYCATGTTGAAYAATGGTAATGAC		
FamoAF	19–38[g]	STAATGGTCTGGCTTAGACG	Clone library	26
FamoAR	582–601[g]	ACATACAGATGGATGGCCGC		
4-Hydroxylbutyl-CoA dehydratase				
Budh-3F (FJ656518)[a]	3–25[b]	GCATCAAATACAAGCACATTACC	Quantification and clone library	This study
Budh-500R	500–525[b]	CATATTTGTCATCTGAATATTTGTA		
Acetyl-CoA carboxylase				
ACA-490F (FJ656668)[a]	498–517[b]	GCAAGTATWGGWCCTTCTGC	Quantification	This study
ACA-841R	842–868[b]	GATAATWTCTTTCATWTCATAWGGTTG		
ACA-385F	385–517[b]	AYTCWGGWGGTGCAMGAAT	Clone library	
ACA-1100R	1090–1109[b]	GAACCWGGCATGTTACCWGG		
Methylmalonyl-CoA epimerase[f]				
MMN-323F	323–348[b]	CGATATGCRTTTTGYTTTTGYCTWAT	Quantification and clone library	This study
MMN-1073R	1073–1092[b]	CAAAATGAYATHYTMAAAGA		
Bacteria				
16S rRNA				
Bac518F (FJ656473)[a]	518–534[c]	CCAGCAGCCGCGGTAAT	Quantification	6, 55
Bac786R	786–803[c]	CTACCAGGGTATCTAATC		
27F	8–27[c]	AGAGTTTGATCMTGGCTCAG	Clone library	
1492R	1510–1492[c]	TACGGYTACCTTGTTACGACTT		
227R (with 27F)	227–244[c]	ATGGTCCGCGAACTCATC	Quantification (*Nitrospina* clade)	70
NCC214NF (with 1492R)	214–231[c]	AGCTCGCACCGATGGATG	PCR (*Nitrococcus* clade)[f]	
NIT3_1035R (with 27F)	1035–1048[c]	CCTGCGCTCCATGCTCCG	PCR (*Nitrobacter* clade)[f]	54
amoA				
amoA1F (EF617305)[a]	332–349[d]	GGGGTTTCTACTGGTGGT	Quantification and PCR	66
amoA2R	820–822[d]	CCCCTCKGSAAAGCCTTCTTC		
A189F[f]	172–189[e]	GGNGACTGGGACTTCTGG	PCR	56
A682R	665–682[e]	GAASGCNGAGAAGAASGC		
amoA-amoB[f]				
amoA3F	295–310[e]	GGTGAGTGGGYTAACMG	PCR	64
amoB4R	30–44[e]	GCTAGCCACTTTCTGG		
Eukarya				
18S rRNA[f]				
82F	82–98[c]	GAAACTGCGAATGGCTC	PCR	60
1520R	1505–1520	CYGCAGGTTCACCTAC		

[a] Primer and reference gene sequence used for real-time PCR.
[b] Numbering of the positions for this gene is based on the gene of "*Ca*. Nitrosopumilus maritimus" SCM1 (NC 010085).
[c] Numbering of the positions is based on the 16S rRNA gene of *Escherichia coli* and 18S rRNA gene of *Gracilariopsis* sp. (M33639).
[d] Numbering of the positions is based on bacterial *amoA* of *Nitrosomonas europaea* (L08050).
[e] Numbering is based on bacterial *amoA* of *Nitrosococcus oceani* (AF047705).
[f] Target genes could not be amplified from our cultures using the primer set.
[g] Numbering of the positions for this gene is based on the gene of a metagenomic clone from the Sargasso Sea (AACY01435967).

and then 40 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C, with readings taken between each cycle. Standard curves were generated in each run by using standards of reference genes ranging from 0 to 10^9 gene copies per reaction and used to estimate gene abundance in the enrichment samples. Standard curves showed the relationship between a known copy number of genes and cycle threshold (C_T) values as previously described (61). Specificity of real-time PCRs was tested by analyzing melting curves, checking the sizes of reaction products by using gel electrophoresis, and sequencing of the reaction products. Information about the PCR amplification primers for each target gene is listed in the Table 1.

Bicarbonate incorporation analysis. In order to determine bicarbonate incorporation by AOA during ammonia oxidation, [^{13}C]bicarbonate (99% ^{13}C; Cambridge Isotope Laboratories, Andover, MA) (5 mM) was added after complete depletion of thiosulfate (0.1 mM or 1.0 mM thiosulfate) to exclude uptake of [^{13}C]bicarbonate by SOB but before initiation of archaeal growth in 10-ml media in Hungate tubes sealed with butyl-rubber stoppers. The ratio of the [^{13}C]bicarbonate concentration to the unlabeled bicarbonate concentration was determined by measuring bicarbonate concentrations before and after the spiking with [^{13}C]bicarbonate. A control culture was incubated under the same conditions without the addition of [^{13}C]bicarbonate.

Total cells were harvested by centrifugation before completion of ammonia oxidation and freeze dried for archaeal membrane lipid analysis as described by Wuchter et al. (88). The freeze-dried cells were ultrasonically extracted once with methanol and three times with dichloromethane. The extracts were combined, and the water was removed with a small pipette Na_2SO_4 column. To measure the ^{13}C incorporation in archaeal membrane lipids, biphytanes were released from the glycerol dialkyl glycerol tetraethers (GDGTs) present in the lipid extract by treating with HI-$LiAlH_4$ (68) and measured by gas chromatography (GC) and isotope-ratio-monitoring GC-mass spectrometry as described previously (87).

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database (accession numbers FJ656506 to FJ656704, FJ656473 to FJ656505, and FJ971108 to FJ971126).

RESULTS

Establishment of AOA enrichment cultures by using consortia of SOB. Previously, we showed the presence of AOA in marine sediments around South Korea (61). Attempts to enrich these AOA by using media containing ammonia as the sole energy source as previously reported by Könneke et al. (38), regardless of the presence of antibiotics, were not successful. Next, we tried to stimulate growth of the AOA in cocultures with (mostly autotrophic) bacteria, e.g., sulfur oxidizers, hydrogen oxidizers, methanotrophs, phototrophs, and lignin decomposers. Stimulation of ammonia oxidation by AOA was observed only in the coculture medium for SOB, which contained thiosulfate as a supplementary energy source in addition to ammonia. After successive biweekly transfers upon completion of ammonia oxidation for ca. 20 months, AOA became dominant members in the enrichment cultures. No eukaryotic rRNA genes could be amplified from any of the cultures. Using the SOB coculture conditions, we could enrich members of the crenarchaeal group I.1a AOA from the coastal sediments of Donghae, South Korea (SJ culture), and Svalbard in the Arctic region (AR culture).

Typically, in our SOB coculture enrichment cultures, oxidation of ammonia and coincident increase of archaeal 16S rRNA gene copy numbers initiated only after complete consumption of 0.1 mM thiosulfate by SOB with a concomitant drop (typically down to 10%) of the oxygen concentration (Fig. 1A). After consumption of 1 mM ammonia, AOA became predominant, as demonstrated by quantitative PCR experiments (81% of the total prokaryotic 16S rRNA gene copies) (Fig. 1B and Table 2) and FISH (>80% of DAPI-stained cells; Fig. 2A and B). The batch culture profile was stable and reproducible after only several transfers in ca. 3 months, and we obtained the batch culture profile shown in Fig. 1 late in the enrichment process (i.e., after 2 years).

Electron photomicrographs showed most AOA cells were slender rod-type cells (0.15 to 0.2 μm in width and 0.5 to 1.0 μm in length) (Fig. 2C and D) with a morphology similar to that reported for "*Ca*. Nitrosopumilus maritimus" (38). The enriched AOA were identified as crenarchaea closely related to those identified in marine sediments (61) and "*Ca.* Nitrosopumilus maritimus" (Fig. 3). Average identities of archaeal 16S rRNA gene sequences (approximately 900 bp in size) among clones of SJ and AR cultures were 99.3% ± 0.4%. The closest cultured relative of the SJ and AR AOA is "*Ca*. Nitrosopumilus maritimus," with 99.1% ± 0.4% 16S rRNA gene sequence identity.

Our experimental data indicated that archaea belonging to crenarchaeal group I.1a were the only ammonia oxidizers in our enrichment cultures. First, the increase of archaeal 16S rRNA gene copy numbers was coincident with the amount of ammonia oxidized (Fig. 4). Crenarchaea were the only microorganisms growing during the phase of oxidation of ammonia. 16S rRNA gene sequences related to crenarchaeal group I.1b or the pSL12 clade, potential other marine AOA (53, 61), were under the detection limit of the analysis of denaturing gradient gel electrophoresis (DGGE; data not shown) and clone libraries from archaeal 16S rRNA genes (Fig. 3). Second, the expression of the archaeal *amoA* gene was observed during oxidation of ammonia and decreased 2 to 3 orders magnitude after depletion of ammonia (Table 2). In the case of the AR enrichment culture, the archaeal *amoA* gene expression declined from 4.1×10^3 to 4.5×10^1 copies per ng RNA as ammonia became depleted. Third, beta- and gammaproteobacterial *amoA* genes could not be amplified by PCR from genomic DNA and cDNA generated from the enrichment cultures. Fourth, the addition of bacterial antibiotics (streptomycin or kanamycin) to the enrichment cultures during oxidation of ammonia caused the accumulation of nitrite (see Fig. S1 in the supplemental material), with concomitant AOA growth as previously observed for "*Ca*. Nitrosopumilus maritimus" (38). Furthermore, nitrification inhibitors (100 μM chlorite and 50 μM nitrapyrin) strongly inhibited archaeal ammonia oxidation and growth (data not shown), similar to what has been shown for the thermophilic AOA, "*Ca*. Nitrososphaera gargensis," in the presence of allylthiourea (50 μM) (31).

Due to the formation of aggregates, determination of growth rates of AOA based on FISH was inappropriate. Therefore, we used copy numbers of 16S rRNA genes to indirectly infer

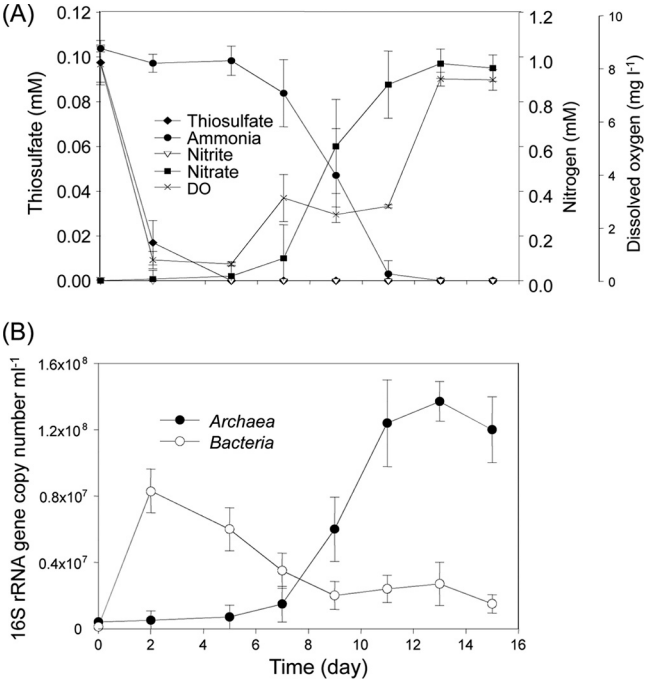

FIG. 1. Ammonium oxidation by AOA in coculture with SOB oxidizing thiosulfate in the SJ enrichment culture. (A) Concentrations of ammonia, nitrite, nitrate, thiosulfate, and dissolved oxygen. (B) *Bacteria* and *Archaea* gene copy numbers. Error bars represent the standard deviations of the means from triplicate experiments.

growth rates. Maximum growth rates (0.57 day^{-1} for SJ culture and 0.65 day^{-1} for AR culture) under our culture conditions (25°C, pH 8.2, 1 mM ammonia) were slightly lower than that of "*Ca.* Nitrosopumilus maritimus" (0.78 day^{-1}) (38). However, this rate is still higher than the growth rates of natural bacterioplanktonic communities (0.05 to 0.3 day^{-1}) (24). Incomplete consumption of ammonia was observed at ammonia concentrations of 4 mM (Fig. 4), and nitrification stopped at levels of >4 mM.

Growth of SOB oxidizing thiosulfate was essential for the AOA activity in our cultures. When we transferred the cultures to media without thiosulfate, only ca. 20% of ammonia was oxidized in 2 weeks, and the activity could not be resumed during further transfers (data not shown). Increasing the size of the inoculum or reducing oxygen tension did not affect this phenomenon. When reduced sulfur compounds other than thiosulfate (e.g., sulfite, tetrathionate, and elemental sulfur) were tested as substrates for SOB in our cocultures, sulfite and tetrathionate also stimulated growth of AOA, while elemental sulfur did not (data not shown). Thiosulfate at initial levels ranging from 0.1 to 2.0 mM led to optimal conditions maintaining activity of AOA. However, at a concentration of 5 mM thiosulfate, no AOA activity was evident (data not shown).

Kinetics of ammonia oxidation and oxygen uptake. The nitrification rate was determined from the batch growth experiment of AOA (Fig. 1). The specific oxidation rates of ammonia were 2.8 (SJ culture) and 3.0 (AR culture) fmol $cell^{-1}$ day^{-1}, which are slightly lower than those from the known reports for AOA enrichment cultures from the North Sea (4.3 fmol $cell^{-1}$ day^{-1}) (87) and "*Ca.* Nitrosopumilus maritimus" (4.0 fmol $cell^{-1}$ day^{-1}) (38).

Ammonia oxidation was severely inhibited by shaking during incubation, but the extent of thiosulfate oxidation was not affected (data not shown). This finding suggests that the SOB activity led to a reduction of the dissolved oxygen level below a certain level. Indeed, measured dissolved oxygen levels rapidly decreased to a low level (<3 mg $liter^{-1}$) before oxidation of ammonia commenced (Fig. 1A). This suggests that the AOA in our enrichment cultures are capable of dealing with low levels of oxygen, conditions typical for marine surface sediments.

Oxygen uptake coupled with ammonia oxidation was respirometrically measured for determination of affinities (via K_m values) for oxygen and ammonia in the AR culture. Kinetics of the ammonia oxidation and oxygen consumption by the AR culture followed Michaelis-Menten-type kinetics (see Fig. S2 in the supplemental material). Our data indicate that affinities of AOA to oxygen and ammonia in the AR culture were much greater than those of AOB and that the maximum rates of oxygen uptake and ammonia oxidation of AOA in the AR culture were much lower than those of AOB (Table 3). The ammonia affinities of AOB are broad, ranging from ca. 30 to 2,000 μM (40, 45, 63, 73). The affinities of AOA to oxygen and ammonia (and the maximum rates of oxygen uptake and ammonia oxidation) in the AR culture were similar to those of "*Ca*. Nitrosopumilus martimus" (52).

Carbon fixation of AOA enrichment cultures. We could not observe any significant effect of an increased concentration of bicarbonate on bacterial or archaeal growth in our culture, as demonstrated by quantification of the 16S rRNA gene copy

TABLE 2. Copy numbers of 16S rRNA, *amoA*, and carbon fixation genes in genomic DNA and cDNA during and after completion of ammonia oxidation

DNA	Culture	AOA growth phase[a]	Gene copy no.[f]								*amoA*/16S rRNA gene copy no. ratio
			Archaeal 16S rRNA gene	Bacterial 16S rRNA gene	Archaeal *amoA*	NOB (*Nitrospina*) 16S rRNA gene	Carbon fixation				
							4-BUDH[b]	ACC[c]	MCE[d]		
Genomic DNA	SJ	NH_3 oxidizing	1.5×10^6 (8.3×10^5)	3.9×10^5 (1.8×10^5)	2.9×10^6 (2.2×10^5)	2.1×10^3 (9.9×10^2)	1.4×10^6 (4.4×10^5)	2.1×10^5 (1.2×10^4)	ND	2.0×10^0
	AR	NH_3 depleted	8.3×10^5 (3.4×10^5)	2.0×10^5 (9.4×10^4)	3.3×10^6 (2.3×10^5)	5.5×10^2 (2.3×10^2)	1.9×10^6 (6.2×10^5)	1.7×10^5 (1.1×10^4)	ND	3.9×10^0
		NH_3 oxidizing	6.7×10^5 (7.4×10^5)	8.6×10^4 (9.2×10^4)	2.7×10^5 (1.4×10^5)	6.1×10^2 (1.9×10^2)	7.6×10^4 (5.9×10^4)	2.0×10^4 (7.9×10^3)	ND	0.4×10^0
		NH_3 depleted	1.1×10^6 (8.9×10^5)	1.7×10^5 (7.8×10^4)	3.1×10^6 (4.2×10^4)	1.0×10^3 (5.4×10^2)	2.3×10^6 (7.8×10^5)	1.4×10^5 (1.4×10^4)	ND	2.9×10^0
cDNA	SJ	NH_3 oxidizing	3.2×10^5 (2.1×10^5)	4.6×10^4 (2.4×10^4)	4.1×10^3 (1.3×10^3)	—[e]	4.8×10^0 (1.1×10^0)	2.9×10^0 (1.4×10^0)	—	1.3×10^{-2}
		NH_3 depleted	3.8×10^5 (2.8×10^5)	9.6×10^4 (3.0×10^4)	4.5×10^1 (3.6×10^0)	—	2.6×10^0 (2.2×10^0)	4.5×10^{-1} (4.5×10^{-1})	—	1.2×10^{-4}
	AR	NH_3 oxidizing	5.9×10^5 (3.3×10^5)	5.5×10^4 (3.3×10^4)	1.0×10^4 (1.5×10^3)	—	9.2×10^0 (4.2×10^0)	5.7×10^0 (3.1×10^0)	—	1.7×10^{-2}
		NH_3 depleted	4.5×10^5 (3.3×10^5)	1.5×10^5 (1.5×10^5)	4.4×10^1 (1.2×10^1)	—	9.4×10^0 (2.0×10^0)	7.4×10^{-1} (9.8×10^{-1})	—	9.8×10^{-5}

[a] Cells were collected at 7 days of culture (ammonia-oxidizing phase) and at 17 days (3 days after ammonia consumption) (ammonia-depleted phase).
[b] 4-BUDH, 4-hydroxybutyryl CoA dehydratase.
[c] ACC, acetyl-CoA carboxylase.
[d] MCE, methylmalonyl CoA epimerase; ND, not detected.
[e] —, not determined.
[f] For comparison, copy numbers of genomic DNA and cDNA were normalized based on ng of genomic DNA and total RNA, respectively. Numbers in parentheses indicate standard deviations from triplicate measurements.

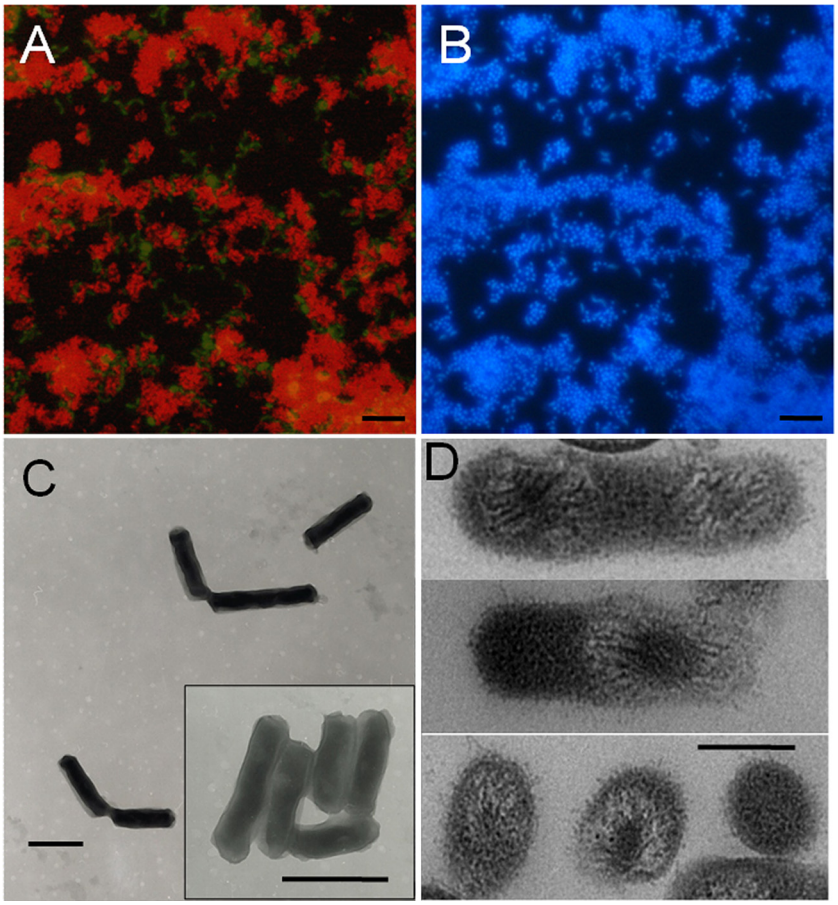

FIG. 2. Photomicrograph of archaeal cells of the SJ culture. For FISH analysis, archaeal cells were stained with Cy5-labeled probe (red, Arch915) and bacterial cells were stained with fluorescein-labeled probe (green, EUB 338) (A); total cells were stained with DAPI (blue) (B). The same position in the FISH image was taken for panels A and B. Scale bar, 5 μm. (C) Transmission electron micrograph of negative-stained cells. The inset shows a magnified view. Scale bar, 0.5 μm. (D) Transmission electron micrograph of thin-sectioned cells. Scale bar, 0.2 μm.

numbers (data not shown), suggesting that this concentration was not limiting. To investigate the pathway of carbon fixation of the enriched AOA, we performed labeling with [^{13}C]bicarbonate on the SJ and AR enrichment cultures. This resulted in the incorporation of a substantial amount of ^{13}C label into the characteristic crenarchaeal membrane lipids (20), providing convincing evidence for autotrophic growth of the AOA (Table 4). The degree of ^{13}C labeling of the archaeal lipids did not depend on the amount of thiosulfate added (Table 4).

The 3-hydroxypropionate/4-hydroxybutyrate cycle has been proposed as the autotrophic carbon fixation pathway in diverse crenarchaea, including mesophilic AOA, such as "*Ca*. Cenarchaeum symbiosum" (10, 30) and "*Ca*. Nitrosopumilus maritimus" (82). We were able to amplify two key genes involved in the 3-hydroxypropionate/4-hydroxybutyrate cycle in our enrichment cultures, i.e., those encoding 4-hydroxybutyryl–CoA dehydratase (4-BUDH) and acetyl-CoA carboxylase (ACC), which have 81 to 92% and 86 to 89% nucleotide sequence identities with these genes in "*Ca*. Nitrosopumilus maritimus," respectively (see Fig. S3 in the supplemental material). The expression levels of the two genes were significantly lower than that of *amoA* (Table 2). Unexpectedly, we were not able to amplify another key gene for the 3-hydroxypropionate/4-hydroxybutyrate cycle, that encoding methylmalonyl-CoA epimerase (MCE), although several combinations of the PCR primers specific for this cluster were tried (Table 1).

Microdiversity of AOA. Although rRNA gene sequences are widely used for identification of members of microbial communities, it is not always appropriate for a detailed assessment of the ecotype level microdiversity. We, therefore, also analyzed the archaeal 16S-23S ITS sequences (28). This experiment showed that AOA of SJ and AR enrichment cultures are not uniclonal (Fig. 5). However, most of the obtained sequences are closely related and constitute a distinct group ("Crena-MS," indicating those from marine sediments) together with the ITS sequence of "*Ca.* Nitrosopumilus maritimus" (with 93.7% ± 0.2% ITS sequence identity). This group is distinct from the ITS sequences of crenarchaea from the marine water column, namely, Crena-A (Antarctic water), -S1 and -S2 (surface water), and -D (deep water) (28). Notably, a few clones (6 out of 26) of the SJ culture were closely related to clones from water column samples (Crena-S1 and Crena-A) (Fig. 5).

The microdiversity of archaeal communities of the enrichment cultures was also reflected by the heterogeneity of archaeal *amoA* genes in the SJ and AR enrichment cultures (Fig. 6). Average

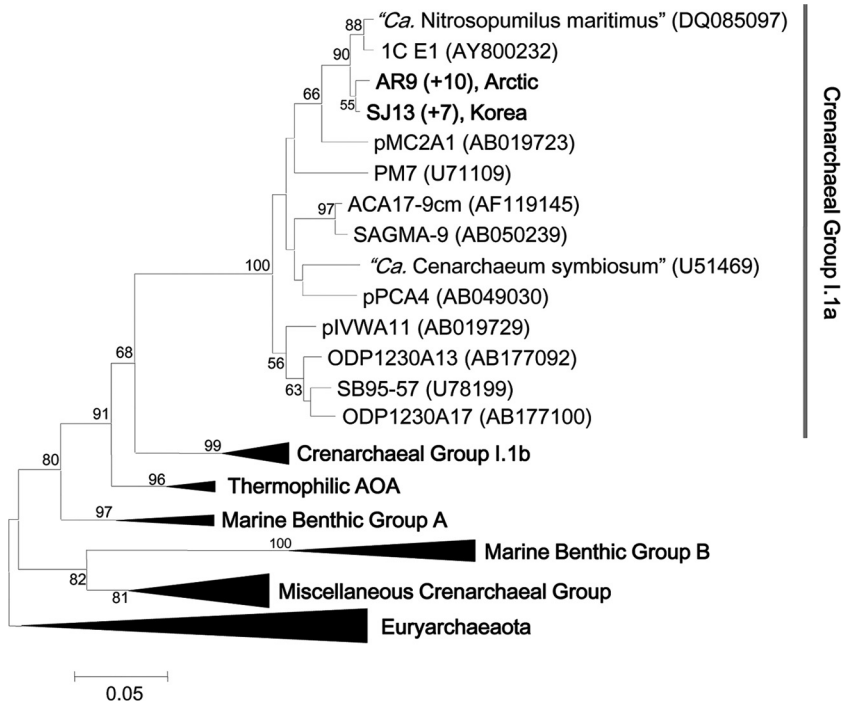

FIG. 3. A phylogenetic tree of archaeal 16S rRNA gene sequences (about 900 bp) obtained from the SJ and AR cultures. Branching patterns supported by >60% bootstrap values (1,000 iterations) by means of neighbor joining are denoted by their respective bootstrap values. Numbers in parentheses indicate the frequencies of redundant clones. The scale bar represents 5% estimated sequence divergence.

inter-*amoA* gene sequence similarities of the SJ and AR enrichment cultures were 97.5% ± 1.7% and 97.5% ± 2.5%, respectively. The SJ and AR enrichment cultures contained a major phylotype, represented by SJ-amoA-6 and AR-amoA-13, respectively (Fig. 6). All these *amoA* gene sequences are closely related to that of "*Ca*. Nitrosopumilus maritimus" and belong to the sediment/water column group.

Identity of sulfur- and nitrite-oxidizing bacteria in the enrichment cultures. We investigated the bacterial diversity of our cocultures, specifically to identify the main SOB and nitrite-oxidizing bacteria. Bacterial 16S rRNA gene sequence analysis of SJ and AR cultures showed that all recovered sequences belonged to the *Proteobacteria* (alpha [71 clones], gamma [42 clones], epsilon [11 clones], and delta [3 clones] subdivisions) (see Fig. S4 in the supplemental material). Our molecular analysis indicated that both facultative and obligatory chemoautotrophic SOB are major members in the SJ and AR cultures. Interestingly, bacterial communities between two cultures are rather different although the enriched AOA are similar. The SJ enrichment culture contains two major candidate SOB clusters. One is *Sulfurovum*-related sequences (11 clones) of *Epsilonproteobacteria. Sulfurovum* species are known as chemolithoautotrophic SOB (34). SOB falling in the chemolithoautotrophic *Epsilonproteobacteria* have been shown to be important primary producers in redoxcline ecosystems (29). The other major group (13 clones) is related to *Kordiimonas gwangyangensis* of the *Alphaproteobacteria* isolated from bay sediments. These bacteria are able to degrade polycyclic aromatic hydrocarbons (44). It is presently unknown if this group contains facultative SOB.

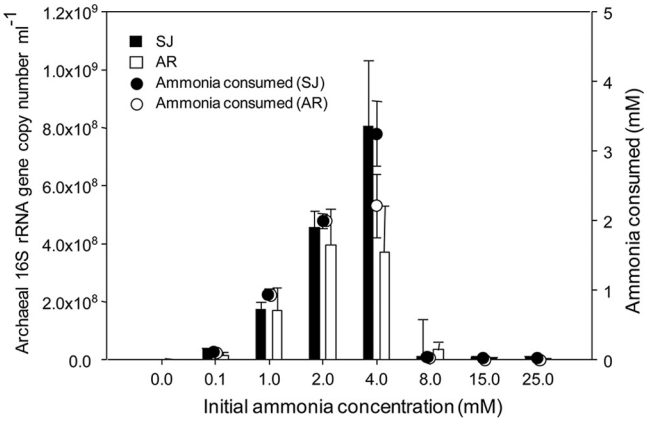

FIG. 4. Dependence of archaeon-specific 16S rRNA gene copy number on ammonia concentration in the SJ and AR enrichment cultures. Incomplete oxidation of ammonia was observed in 4 mM ammonia medium, and complete inhibition of ammonia oxidation was observed above 4 mM ammonia. Error bars represents the standard deviations of the means from triplicate experiments.

In contrast with the case for the SJ enrichment culture, the major presumed SOB in the AR enrichment culture (20 clones) belong to a unique cluster of *Roseobacter* clade, comprised of facultative thiosulfate oxidizers isolated from abyssal plains and continental slopes (shaded area in Fig. S4 in the supplemental material) (77). Other major clones of the AR enrichment culture are related to symbiotic *Proteobacteria* associated with toxic dinoflagellates (11 clones) (58) and *Oligobrachia mashikoii* (9 clones) (42). The endosymbionts of *Oli-*

TABLE 3. Kinetics of ammonia oxidation and oxygen uptake

Strain	Ammonia (NH_4^+)[a]		Oxygen		Reference
	K_m (μM)	V_{max} (μmol per mg protein per h)	K_m (μM)	V_{max} (μmol per mg protein per h)	
AOA					
AR enrichment culture	0.61 (0.02)[b]	14[c]	2.01 (0.45)[b]	11.4[c]	This study
"*Ca*. Nitrosopumilus martimus" SCM1	0.13 (0.04)[d]	24	3.90 (0.6)[d]	22.5	52
AOB					
Nitrosomonas europaea C-31 (ATCC 25978)	1,300	36	186	129	This study
Nitrosomonas europaea ATCC 19718	530	122	ND	ND	52
Activated sludge	59	ND[e]	15.6	ND	32

[a] The values are given for NH_4^+ and NH_3.
[b] The numbers in parentheses indicate the standard deviations from triplicate experiments.
[c] We assumed that protein content per cell of AOA in AR culture equals that of "*Ca*. Nitrosopumilus maritimus" (52).
[d] Data from a study by Martens-Habbena et al. (52).
[e] ND, not determined.

gobrachia mashikoii are suggested to provide organic carbon for their host by growing on reduced inorganic sulfur compounds.

The only group of bacteria which was found in both the SJ (8 clones) and AR (6 clones) enrichment cultures is closely related to *Alcanivorax venustensis* (falling into the *Gammaproteobacteria* taxon), which is an aliphatic-compound-degrading bacterium isolated from seawater (49).

During oxidation of ammonia, we could not observe nitrite accumulation at any time during the batch culture but observed a near-stoichiometric amount of nitrate accumulated in the medium (Fig. 1A). Addition of bacterial antibiotics (streptomycin or kanamycin) to the enrichment cultures during oxidation of ammonia caused accumulation of nitrite (see Fig. S1 in the supplemental material). This observation suggested that nitrite-oxidizing bacteria (NOB) oxidized the nitrite formed by the AOA immediately, but only one clone related to *Nitrospina* was detected from the bacterial 16S rRNA gene library of the SJ culture by using general bacterial primers. PCR amplification of the 16S rRNA gene of the group of marine NOB (i.e., *Nitrospina*, *Nitrospira*, and *Nitrococcus* species) resulted in detection of sequences related to *Nitrospina*, which comprised 0.5% (SJ enrichment culture) or 2.8% (AR enrichment culture) of the total bacterial 16S rRNA gene copies (Table 2). The level of 16S rRNA gene sequence similarity between *Nitrospina*-like clones and *Nitrospina gracilis* isolated from Atlantic Ocean was ca. 90% (see Fig. S4 in the supplemental material) (76). Mincer et al. (53) found that abundance and distribution of *Nitrospina* group of NOB coincided with those of marine crenarchaea at Station ALOHA and suggested potential metabolic interactions between these groups in the water column of the ocean. The similarity of the *Nitrospina*-like 16S rRNA gene sequences recovered from Station ALOHA (HF500_19A12; DQ989201) and those of our SJ and AR enrichment cultures was ca. 96% and supports this suggestion.

DISCUSSION

Factors leading to the selective enrichment of sedimentary AOA. We successfully enriched AOA from marine sediments (up to ca. 80% of all prokaryotic cells) by coculturing with SOB consortia which used thiosulfate as an electron donor. However, all our attempts to grow AOA without SOB consortia failed. In addition, we could not transfer and maintain growth of AOA in the presence of antibiotics (either as single components or as cocktails), even in the media containing spent culture media and/or whole-cell lysates of the SJ culture. Hence, the SOB consortia most probably played a key role in the growth of AOA in our enrichment cultures. Possibly, SOB provided some growth factors essential for AOA. Indeed, nitrifiers and SOB occupy similar niches at redox gradients of stratified sediments (89), and a tight association between crenarchaea and SOB was observed in a terrestrial cold sulfidic spring (37). However, our phylogenetic analysis of SOB 16S rRNA gene sequences in the two enrichment cultures indicates that the SOB communities in the two cultures are completely different.

Alternatively, the growth of SOB leads to conditions in the

TABLE 4. ^{13}C label incorporation into archaeal lipids during incubation in the presence of [^{13}C]bicarbonate[b]

Culture	Thiosulfate (mM)	Biphytane abundance (%)[a]				Biphytane ^{13}C atom (%)[a]				Avg ^{13}C in total lipids (%)
		I	II	III	IV	I	II	III	IV	
SJ	0.1	38	16	25	21	49	49	49	49	49
	1.0	45	14	23	18	46	46	46	46	46
AR	0.1	45	12	23	20	48	46	47	46	47
	1.0	37	15	25	23	46	46	46	46	46

[a] Acyclic, monocyclic, bicyclic, and tricyclic biphytanes are indicated by I, II, III, and IV, respectively (see reference 68, but note that the structure of the tricyclic biphytane has been corrected since then [see reference 20]).
[b] After 1 day of complete oxidation of thiosulfate, 5 mM [^{13}C]bicarbonate was added. Cells were harvested before ammonia was completely consumed.

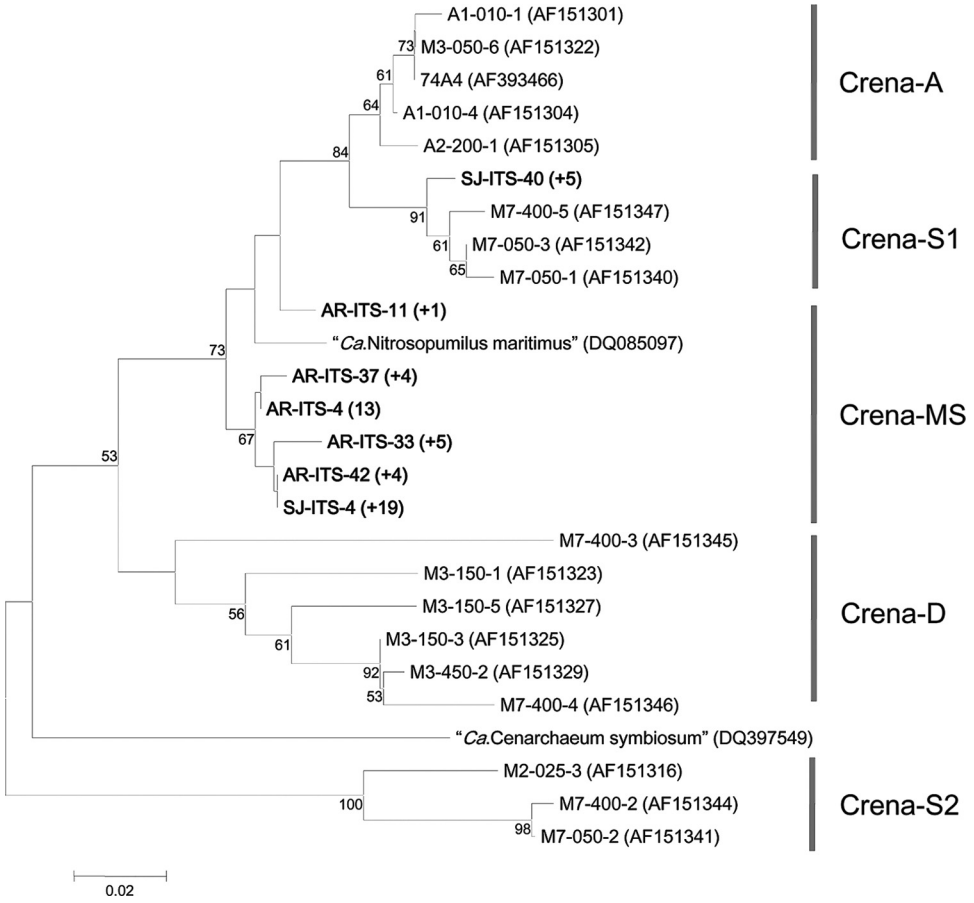

FIG. 5. Phylogenetic tree of internal transcribed spacer (ITS) sequences (140 bp) of AOA of the SJ and AR cultures. Sequences from marine group were used as references. The *p*-distances (complete-deletion parameter) were used to build a neighbor-joining tree, with bootstrap values (1,000 iterations) greater than 60% shown for the main nodes. The clusters Crena-A, -D, -S1, and -S2 were previously defined (28), and Crena-MS is defined here. Numbers in parentheses indicate the frequencies of redundant clones. The scale bar represents 2% estimated sequence divergence.

culture which positively affect growth of AOA. The SOB activity led to a substantial reduction of the dissolved oxygen level, i.e., from a level of 250 μM to ca. 30 μM (i.e., about 10% of the saturation level; Fig. 1A). At this relatively low oxygen concentration, the AOA performed nitrification at the maximum growth rate of ca. 0.6 day^{-1}. In our respirometer experiment, the maximum nitrification rate was observed only at ca. 20 μM (see Fig. S3 in the supplemental material). This is consistent with the results of Martens-Habbena et al. (52), who recently reported that "*Ca.* Nitrosopumilus maritimus" achieved maximum levels of oxygen uptake (and thus the maximum nitrification rate) at oxygen concentrations of only ca. 30 μM. These authors argued that the ability of AOA to perform nitrification at ammonium levels lower than the levels for AOB is perhaps a key factor of why AOA are able to compete with AOB, and even phytoplankton, in oligotrophic parts of the ocean. This may also hold for the oxygen concentration (25). The ability to perform nitrification at a relatively low oxygen concentration as observed here, and thereby an ability to outcompete AOB which have low affinity to oxygen (59, 84), would explain why AOA are found in relatively high abundances in suboxic environments, such as the oceanic oxygen minimum zones (9, 26, 69), the chemocline of the Black Sea (18, 46), and surface marine sediments (61).

Although the exact mechanism for successful enrichment of sedimentary AOA is not entirely clear yet, our enrichment method has enabled us to study the characteristics of sedimentary AOA, a potentially environmentally significant group of organisms in the sedimentary marine nitrogen cycle.

Carbon assimilation by sedimentary AOA. The carbon assimilation mechanism of marine AOA is still a subject to be studied. The possibility of heterotrophic growth of marine crenarchaea is supported by studies which showed that ^{14}C-labeled amino acids were incorporated into archaeal cells of marine crenarchaea (33, 57). In contrast, incorporation of [^{13}C]bicarbonate into the archaeal membrane lipids (88) and archaeal cells (33) and the natural radiocarbon content of archaeal lipids from the marine water column (35, 62) support a predominantly autotrophic physiology of marine crenarchaea. The isolation of "*Ca*. Nitrosopumilus maritimus" demonstrated the chemolithoautotrophic growth of marine AOA (38). Chemolithoautotrophic growth of our enrichment cultures of sedimentary AOA was clearly established by (i) the strong incorporation of [^{13}C]bicarbonate into the archaeal cell

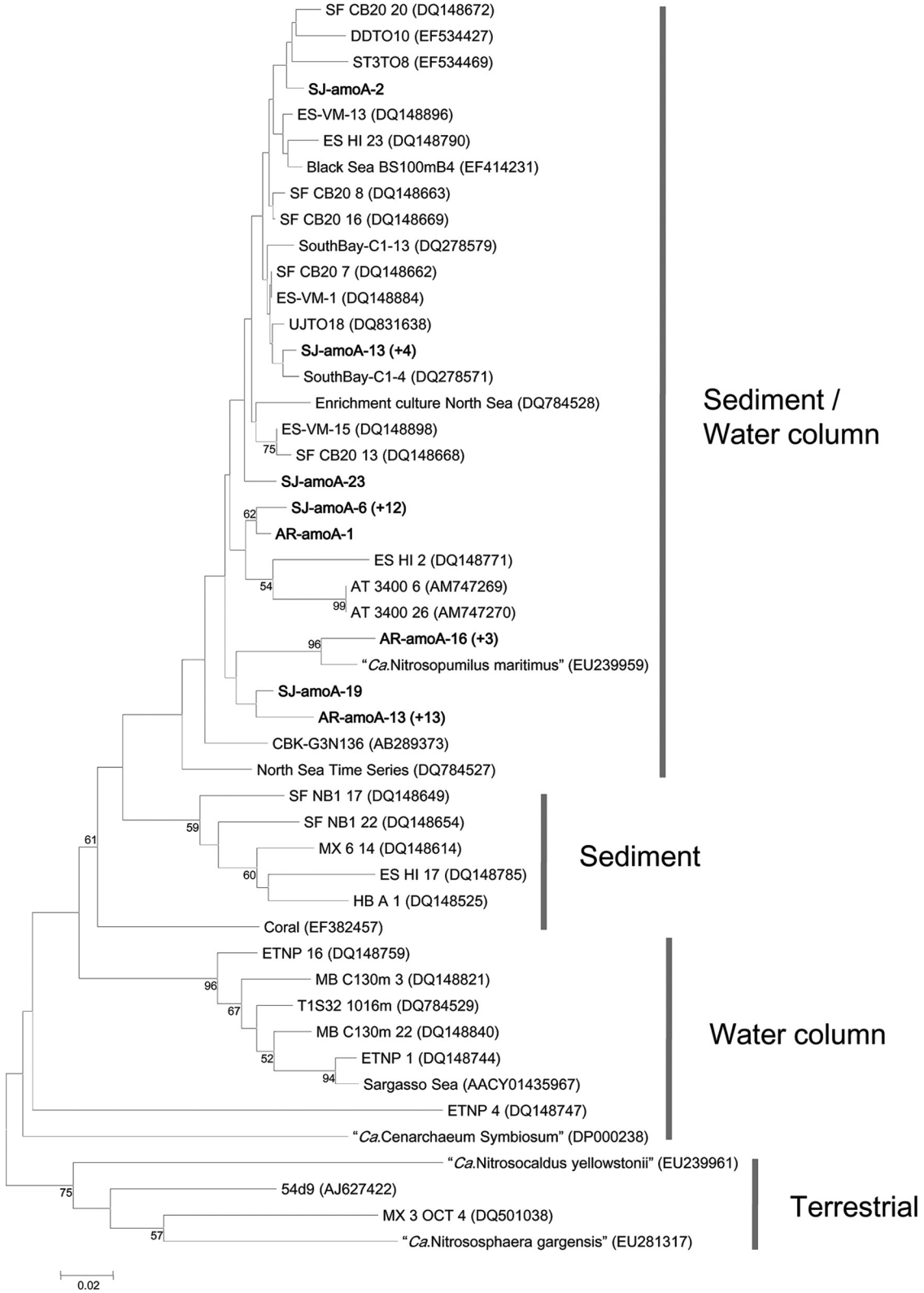

FIG. 6. A phylogenetic tree of archaeal *amoA* gene sequences (about 600 bp) obtained from the SJ and AR enrichment cultures. Branching patterns supported by more than 60% bootstrap values (1,000 iterations) by means of neighbor joining are indicated by their respective bootstrap values. Numbers in parentheses indicate the frequencies of redundant clones. Based on the origin of reference sequences, cluster groups are denoted at the right of the figure. The scale bar represents 2% estimated sequence divergence.

membrane lipids and (ii) the presence and activation of key genes of the carbon fixation pathway of crenarchaea (10). This suggests that sedimentary AOA might be important not only for the sedimentary N cycle but also for the sedimentary C cycle, as they fix inorganic carbon to produce organic cell material.

It has been questioned whether the marine archaeal community includes both autotrophs and heterotrophs or if it is a single population with a uniformly mixotrophic metabolism (33, 35). The ratio of the archaeal *amoA* gene copy number to the 16S rRNA gene copy number has been used as an indicator of the relative abundance of autotrophic archaea, for which ratios between 1 and 3 have been used to indicate that the crenarchaea all possess the ability to perform nitrification (2, 46, 87). In contrast, Agogue et al. (2) found that archaeal *amoA*/16S rRNA gene copy number ratios were well below 1 in the bathypelagic waters of the Atlantic Ocean, suggesting that a large part of the deep-water crenarchaea do not have the ability to perform nitrification. It should be noted, however, that a recent metagenomic study of deep-sea microbial communities indicated that the reported low archaeal *amoA*/16S rRNA gene copy number ratio for deep-sea crenarchaea may be artificial due to a mismatch of the primers used (39). The *amoA*/16S RNA gene copy number ratios of our sedimentary AOA enrichment cultures ranged between 2.9 and 3.9 (Table 2), suggesting that our enrichment cultures were mostly comprised of crenarchaea possessing the capacity to perform ammonia oxidation, despite the observed microdiversity of the archaeal populations. The archaeal *amoA*/16S rRNA gene copy number ratio was also used as an indicator of ecotype in different redox environments in the Black Sea (18, 46), where the observed ratios corresponding to oxygen-depleted environments are high compared to those corresponding to oxygen-saturated and anoxic environments. This ratio in our enrichment culture is much closer to those of the suboxic zone (1.2 to 2.8) than those of the oxic (0.3 to 0.7) and anoxic (0.01 to 0.04) zones, further supporting the idea that the sedimentary AOA that we enriched are adapted to grow at low dissolved oxygen concentrations.

Niche adaptation of AOA. The crenarchaea within marine sediments are typically composed of sequences belonging to the marine benthic group. However, group I.1a AOA have also been frequently reported in surface of marine sediments and are especially dominant in the marine water column (2, 25). Molecular phylogeny using the 16S rRNA gene typically reveals only small differences among group I.1a crenarchaea (for an example, see Fig. 5). A slightly different picture emerges, however, when molecular phylogeny of *amoA* gene sequences is used where separate clusters present in different environments are observed (1, 21, 26, 61). The *amoA* gene sequences of our enrichment cultures fall into the sediment/water column group. This group contains diverse environmental clones, which are retrieved from marine sediments, including Donghae, South Korea (ST3TO8; Fig. 6) (61), from which the SJ enrichment culture originated. In addition, this group contains environmental clones from the suboxic zone of Black Sea (BS100mB4; 100-m depth) (46), water from just above the halocline of a deep, anoxic brine lake (AT 3400 6) (89), and an AOA enrichment culture (MESO_11) and time series (CNS_07) from the North Sea (87).

Our enrichment cultures of sedimentary AOA are genetically quite similar to "*Ca*. Nitrosopumilus maritimus," suggesting similar physiological properties. However, an important difference between our sedimentary AOA cultures and "*Ca*. Nitrosopumilus maritimus" is their sensitivity toward ammonia. Incomplete ammonia oxidation was reported even at ca. 0.5 mM for "*Ca*. Nitrosopumilus maritimus" (38), whereas our enrichment cultures completely oxidized ammonia up to 2 mM and still performed nitrification at 4 mM ammonia (Fig. 4). This suggests that our enriched AOA are specialized in dealing with higher ammonium concentrations. A recent study of the thermophilic AOA "*Ca*. Nitrososphaera gargensis" showed that the cells could oxidize an ammonia concentration near 3 mM (31). However, this inhibitory concentration is still substantially lower than that for AOB, which are inhibited only at concentrations above 20 mM (sometimes even 600 mM) ammonia (41, 74). Indeed, marine surface sediments typically contain higher concentrations of ammonium than the marine water column does, and thus, our sedimentary AOA seem to have a physiology specifically adapted to their environmental niche, i.e., high ammonia concentrations under low-oxygen conditions. Considering these characteristics of AOA and dominance of AOA over AOB in marine sediments (61), we suggest that AOA play an important role in nitrification in marine sediments.

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REFERENCES

1. **Abell, G. C., A. T. Revill, C. Smith, A. P. Bissett, J. K. Volkman, and S. S. Robert.** 2010. Archaeal ammonia oxidizers and nirS-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary. ISME J. **4:**286–300.
2. **Agogué, H., M. Brink, J. Dinasquet, and G. J. Herndl.** 2008. Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic. Nature **456:**788–791.
3. **Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin.** 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. **62:**3557–3559.
4. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
5. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
6. **Baker, G. C., J. J. Smith, and D. A. Cowan.** 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods **55:**541–555.
7. **Belser, L. W., and E. L. Mays.** 1980. Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Appl. Environ. Microbiol. **39:**505–510.
8. **Beman, J. M., and C. A. Francis.** 2006. Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahia del Tobari, Mexico. Appl. Environ. Microbiol. **72:**7767–7777.
9. **Beman, J. M., B. N. Popp, and C. A. Francis.** 2008. Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California. ISME J. **2:**429–441.
10. **Berg, I. A., D. Kockelkorn, W. Buckel, and G. Fuchs.** 2007. A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Science **318:**1782–1786.
11. **Biddle, J. F., S. Fitz-Gibbon, S. C. Schuster, J. E. Brenchley, and C. H. House.** 2008. Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. Proc. Natl. Acad. Sci. U. S. A. **105:**10583–10588.

12. **Biddle, J. F., J. S. Lipp, M. A. Lever, K. G. Lloyd, K. B. Sorensen, R. Anderson, H. F. Fredricks, M. Elvert, T. J. Kelly, D. P. Schrag, M. L. Sogin, J. E. Brenchley, A. Teske, C. H. House, and K. U. Hinrichs.** 2006. Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru. Proc. Natl. Acad. Sci. U. S. A. **103:**3846–3851.
13. **Bock, E., and M. Wagner.** 2006. Oxidation of Inorganic nitrogen compounds as an energy source, p. 457–495. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes. Springer, New York, NY.
14. **Bodelier, P., J. Libochant, C. Blom, and H. Laanbroek.** 1996. Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl. Environ. Microbiol. **62:**4100–4107.
15. **Brochier-Armanet, C., B. Boussau, S. Gribaldo, and P. Forterre.** 2008. Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nat. Rev. Microbiol. **6:**245–252.
16. **Brochier-Armanet, C., S. Gribaldo, and P. Forterre.** 2008. A DNA topoisomerase IB in Thaumarchaeota testifies for the presence of this enzyme in the last common ancestor of Archaea and Eucarya. Biol. Direct. **3:**54.
17. **Chen, X. P., Y. G. Zhu, Y. Xia, J. P. Shen, and J. Z. He.** 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environ. Microbiol. **10:**1978–1987.
18. **Coolen, M. J., B. Abbas, J. van Bleijswijk, E. C. Hopmans, M. M. Kuypers, S. G. Wakeham, and J. S. Sinninghe Damste.** 2007. Putative ammonia-oxidizing Crenarchaeota in suboxic waters of the Black Sea: a basin-wide ecological study using 16S ribosomal and functional genes and membrane lipids. Environ. Microbiol. **9:**1001–1016.
19. **Dalsgaard, T., B. Thamdrup, and D. E. Canfield.** 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. Res. Microbiol. **156:**457–464.
20. **Damsté, J. S., S. Schouten, E. C. Hopmans, A. C. van Duin, and J. A. Geenevasen.** 2002. Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. J. Lipid Res. **43:**1641–1651.
21. **Dang, H., X. Zhang, J. Sun, T. Li, Z. Zhang, and G. Yang.** 2008. Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea. Microbiology **154:**2084–2095.
22. **de la Torre, J. R., C. B. Walker, A. E. Ingalls, M. Konneke, and D. A. Stahl.** 2008. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. Environ. Microbiol. **10:**810–818.
23. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. U. S. A. **89:**5685–5689.
24. **Ducklow, H.** 2000. Bacterial production and biomass in the oceans, p. 85–120. *In* D. L. Kirchman (ed.), Microbial ecology of the oceans. Wiley, New York, NY.
25. **Erguder, T. H., N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete.** 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol. Rev. **33:**855–869.
26. **Francis, C. A., K. J. Roberts, J. M. Beman, A. E. Santoro, and B. B. Oakley.** 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci. U. S. A. **102:**14683–14688.
27. **Fuhrman, J. A., K. McCallum, and A. A. Davis.** 1992. Novel major archaebacterial group from marine plankton. Nature **356:**148–149.
28. **García-Martínez, J., and F. Rodriguez-Valera.** 2000. Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine Archaea of group I. Mol. Ecol. **9:**935–948.
29. **Glaubitz, S., T. Lueders, W. R. Abraham, G. Jost, K. Jurgens, and M. Labrenz.** 2009. ^{13}C-isotope analyses reveal that chemolithoautotrophic Gamma- and Epsilonproteobacteria feed a microbial food web in a pelagic redoxcline of the central Baltic Sea. Environ. Microbiol. **11:**326–337.
30. **Hallam, S. J., T. J. Mincer, C. Schleper, C. M. Preston, K. Roberts, P. M. Richardson, and E. F. DeLong.** 2006. Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. PLoS Biol. **4:**e95.
31. **Hatzenpichler, R., E. V. Lebedeva, E. Spieck, K. Stoecker, A. Richter, H. Daims, and M. Wagner.** 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proc. Natl. Acad. Sci. U. S. A. **105:**2134–2139.
32. **Henze, M., W. Gujer, T. Mino, and M. van Loosdrecht.** 2000. Activated sludge models ASM1, ASM2, ASM2d and ASM3. IWA Publishing, London, United Kingdom.
33. **Herndl, G. J., T. Reinthaler, E. Teira, H. van Aken, C. Veth, A. Pernthaler, and J. Pernthaler.** 2005. Contribution of *Archaea* to total prokaryotic production in the deep Atlantic Ocean. Appl. Environ. Microbiol. **71:**2303–2309.
34. **Inagaki, F., K. Takai, K. H. Nealson, and K. Horikoshi.** 2004. Sulfurovum lithotrophicum gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the epsilon-Proteobacteria isolated from Okinawa Trough hydrothermal sediments. Int. J. Syst. Evol. Microbiol. **54:**1477–1482.
35. **Ingalls, A. E., S. R. Shah, R. L. Hansman, L. I. Aluwihare, G. M. Santos, E. R. Druffel, and A. Pearson.** 2006. Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon. Proc. Natl. Acad. Sci. U. S. A. **103:**6442–6447.
36. **Karner, M. B., E. F. DeLong, and D. M. Karl.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409:**507–510.
37. **Koch, M., C. Rudolph, C. Moissl, and R. Huber.** 2006. A cold-loving crenarchaeon is a substantial part of a novel microbial community in cold sulphidic marsh water. FEMS Microbiol. Ecol. **57:**55–66.
38. **Könneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl.** 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437:**543–546.
39. **Konstantinidis, K. T., J. Braff, D. M. Karl, and E. F. DeLong.** 2009. Comparative metagenomic analysis of a microbial community residing at a depth of 4,000 meters at station ALOHA in the North Pacific subtropical gyre. Appl. Environ. Microbiol. **75:**5345–5355.
40. **Koops, H.-P., and A. Pommerening-Röser.** 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. **37:**1–9.
41. **Koops, H. P., B. Bottcher, U. C. Moller, A. Pommerening-Roser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. and *Nitrosomonas halophila* sp. nov. J. Gen. Microbiol. **137:**1689–1699.
42. **Kubota, N., M. Kanemori, Y. Sasayama, M. Aida, and Y. Fukumori.** 2007. Identification of endosymbionts in Oligobrachia mashikoi (Siboglindiae, Annelida). Microbes Environ. **22:**136–144.
43. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinformatics **5:**150–163.
44. **Kwon, K. K., H. S. Lee, S. H. Yang, and S. J. Kim.** 2005. Kordiimonas gwangyangensis gen. nov., sp. nov., a marine bacterium isolated from marine sediments that forms a distinct phyletic lineage (Kordiimonadales ord. nov.) in the 'Alphaproteobacteria.' Int. J. Syst. Evol. Microbiol. **55:**2033–2037.
45. **Laanbroek, H. J., P. L. E. Bodelier, and S. Gerards.** 1994. Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* grown in mixed continuous cultures at different oxygen concentrations. Arch. Microbiol. **161:**156–162.
46. **Lam, P., M. M. Jensen, G. Lavik, D. F. McGinnis, B. Muller, C. J. Schubert, R. Amann, B. Thamdrup, and M. M. Kuypers.** 2007. Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. Proc. Natl. Acad. Sci. U. S. A. **104:**7104–7109.
47. **Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature **442:**806–809.
48. **Lipp, J. S., Y. Morono, F. Inagaki, and K. U. Hinrichs.** 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature **454:**991–994.
49. **Liu, C., and Z. Shao.** 2005. Alcanivorax dieselolei sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int. J. Syst. Evol. Microbiol. **55:**1181–1186.
50. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9:**409–414.
51. **Manning, D. A. C., and A. Bewsher.** 1997. Determination of anions in landfill leachates by ion chromatography. J. Chromatogr. A **770:**203–210.
52. **Martens-Habbena, W., P. M. Berube, H. Urakawa, J. R. de la Torre, and D. A. Stahl.** 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature **461:**976–979.
53. **Mincer, T. J., M. J. Church, L. T. Taylor, C. Preston, D. M. Karl, and E. F. DeLong.** 2007. Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. Environ. Microbiol. **9:**1162–1175.
54. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
55. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
56. **Nold, S. C., J. Zhou, A. H. Devol, and J. M. Tiedje.** 2000. Pacific Northwest marine sediments contain ammonia-oxidizing bacteria in the β subdivision of the *Proteobacteria*. Appl. Environ. Microbiol. **66:**4532–4535.
57. **Ouverney, C. C., and J. A. Fuhrman.** 2000. Marine planktonic archaea take up amino acids. Appl. Environ. Microbiol. **66:**4829–4833.
58. **Palacios, L., D. R. Arahal, B. Reguera, and I. Marin.** 2006. Hoeflea alexandrii sp. nov., isolated from the toxic dinoflagellate Alexandrium minutum AL1V. Int. J. Syst. Evol. Microbiol. **56:**1991–1995.
59. **Park, H. D., and D. R. Noguera.** 2007. Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. J. Appl. Microbiol. **102:**1401–1417.
60. **Park, S. J., B. J. Park, V. H. Pham, D. N. Yoon, S. K. Kim, and S. K. Rhee.** 2008. Microeukaryotic diversity in marine environments, an analysis of surface layer sediments from the East Sea. J. Microbiol. **46:**244–249.

61. **Park, S. J., B. J. Park, and S. K. Rhee.** 2008. Comparative analysis of archaeal 16S rRNA and amoA genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments. Extremophiles **12:**605–615.
62. **Pearson, A., A. P. McNichol, B. C. Benitez-Nelson, J. M. Hayes, and T. I. Eglinton.** 2001. Origins of lipid biomarkers in Santa Monica Basin surface sediment: a case study using compound-specific D14C analysis. Geochim. Cosmochim. Acta **65:**3123–3137.
63. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
64. **Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66:** 5368–5382.
65. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
66. **Rotthauwe, J.-H., K.-P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704–4712.
67. **Schleper, C., G. Jurgens, and M. Jonuscheit.** 2005. Genomic studies of uncultivated archaea. Nat. Rev. Microbiol. **3:**479–488.
68. **Schouten, S., M. J. L. Hoefs, M. P. Koopmans, H. J. Bosch, and J. S. Sinninghe Damsté.** 1998. Structural identification, occurrence and fate of archaeal ether-bound acyclic and cyclic biphytanes and corresponding diols in sediments. Org. Geochem. **29:**1305–1319.
69. **Sinninghe Damsté, J. S., W. I. Rijpstra, E. C. Hopmans, F. G. Prahl, S. G. Wakeham, and S. Schouten.** 2002. Distribution of membrane lipids of planktonic *Crenarchaeota* in the Arabian Sea. Appl. Environ. Microbiol. **68:**2997–3002.
70. **Siripong, S., J. J. Kelly, D. A. Stahl, and B. E. Rittmann.** 2006. Impact of prehybridization PCR amplification on microarray detection of nitrifying bacteria in wastewater treatment plant samples. Environ. Microbiol. **8:**1564–1574.
71. **Solorzano, L.** 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. **14:**799–801.
72. **Spang, A., R. Hatzenpichler, C. Brochier-Armanet, T. Rattei, P. Tischler, E. Spieck, W. Streit, D. A. Stahl, M. Wagner, and C. Schleper.** 2010. Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. Trends Microbiol. **18:**331–340.
73. **Stehr, G., B. Böttcher, P. Dittberner, G. Rath, and H. P. Koops.** 1995. The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol. Ecol. **17:**177–186.
74. **Suwa, Y., Y. Imamura, T. Suzuki, T. Tashiro, and Y. Urushigawa.** 1994. Ammonia-oxidizing bacteria with different sensitivities to $(NH_4)_2SO_4$ in activated sludges. Water Res. **28:**1523–1532.
75. **Tal, Y., J. E. Watts, and H. J. Schreier.** 2005. Anaerobic ammonia-oxidizing bacteria and related activity in Baltimore Inner Harbor sediment. Appl. Environ. Microbiol. **71:**1816–1821.
76. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
77. **Teske, A., T. Brinkhoff, G. Muyzer, D. P. Moser, J. Rethmeier, and H. W. Jannasch.** 2000. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. Appl. Environ. Microbiol. **66:**3125–3133.
78. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
79. **Treusch, A. H., S. Leininger, A. Kletzin, S. C. Schuster, H. P. Klenk, and C. Schleper.** 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ. Microbiol. **7:**1985–1995.
80. **Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y. H. Rogers, and H. O. Smith.** 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science **304:**66–74.
81. **Voroteliak, V., D. M. Cowley, and T. H. Florin.** 1993. Improved colorimetric determination of urinary thiosulfate to study intermediate sulfur metabolism in humans. Clin. Chem. **39:**2533–2534.
82. **Walker, C. B., J. R. de la Torre, M. G. Klotz, H. Urakawa, N. Pinel, D. J. Arp, C. Brochier-Armanet, P. S. Chain, P. P. Chan, A. Gollabgir, J. Hemp, M. Hugler, E. A. Karr, M. Konneke, M. Shin, T. J. Lawton, T. Lowe, W. Martens-Habbena, L. A. Sayavedra-Soto, D. Lang, S. M. Sievert, A. C. Rosenzweig, G. Manning, and D. A. Stahl.** 2010. Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proc. Natl. Acad. Sci. U. S. A. **107:** 8818–8823.
83. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
84. **Wells, G. F., H. D. Park, C. H. Yeung, B. Eggleston, C. A. Francis, and C. S. Criddle.** 2009. Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea. Environ. Microbiol. **11:**2310–2328.
85. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U. S. A. **95:**6578–6583.
86. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. Springer, Berlin, Germany.
87. **Wuchter, C., B. Abbas, M. J. Coolen, L. Herfort, J. van Bleijswijk, P. Timmers, M. Strous, E. Teira, G. J. Herndl, J. J. Middelburg, S. Schouten, and J. S. Sinninghe Damste.** 2006. Archaeal nitrification in the ocean. Proc. Natl. Acad. Sci. U. S. A. **103:**12317–12322.
88. **Wuchter, C., S. Schouten, H. T. Boschker, and J. S. Sinninghe Damste.** 2003. Bicarbonate uptake by marine Crenarchaeota. FEMS Microbiol. Lett. **219:** 203–207.
89. **Yakimov, M. M., V. La Cono, R. Denaro, G. D'Auria, F. Decembrini, K. N. Timmis, P. N. Golyshin, and L. Giuliano.** 2007. Primary producing prokaryotic communities of brine, interface and seawater above the halocline of deep anoxic lake L'Atalante, Eastern Mediterranean Sea. ISME J. **1:**743–755.