Enrichment of a Novel Marine Ammonia-Oxidizing Archaeon Obtained from Sand of an Eelgrass Zone

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Ammonia-oxidizing archaea (AOA) are generally cultivated at ammonium concentrations of less than 2 mM. The physiology and abundance in the environment of AOA suggest an important role in the nitrogen cycle. We report here a novel marine ammonia-oxidizing crenarchaeote, strain NM25 belonged to ‘*Candidatus Nitrosopumilus*’, that was enriched from coastal sand of an eelgrass zone and grew in a medium containing 15 mM ammonium at 30°C. A phylogenetic analysis based on the 16S rRNA gene revealed this crenarchaeote was related to the ammonia-oxidizing archaeon ‘*Candidatus Nitrosopumilus maritimus*’ strain SCM1, with 98.5% identity. The ammonia monooxygenase subunit A (*amoA*) gene of strain NM25 was less closely related to that of known cultivable AOA (>95%) and environmental clones (>97%). This finding suggests the existence of AOA adapted to high ammonium-containing environments.

Key words: ammonia-oxidizing archaea, marine crenarchaeote, nitrifying archaea, enrichment culture, ‘*Nitrosopumilus*’

Ammonia-oxidizing bacteria (AOB) able to convert ammonium (NH₄⁺) to nitrite (NO₂⁻) have been considered solely responsible for the first and rate-limiting steps of nitrification in marine environments since the discovery of the marine ammonia-oxidizer *Nitrosococcus marina* (46). Ammonia monooxygenase catalyzes the conversion of ammonia (NH₃) to hydroxylamine (NH₂OH) and is a key enzyme for nitrification (6). Several ammonia-oxidizers belonging to the genera *Nitrospira* and *Nitrosomonas* within *Betaproteobacteria* (26, 30) and the genus *Nitrosococcus* within *Gammaproteobacteria* (2) have already been isolated from marine environments. However, microorganisms within *Crenarchaeota* Group 1.1a (Marine *Crenarchaeota* Group I) were recently found to be ammonia-oxidizers based on metagenomic studies of seawater (45) and the isolation of an archaeon, ‘*Candidatus Nitrosopumilus maritimus*’ strain SCM1, from a marine aquarium tank (25). *Crenarchaeota* Group 1.1a are often detected in marine environments (12, 16), and dominate in the deep sea (24). To elucidate the ecology of ammonia-oxidizing archaea (AOA) in marine systems, enrichment cultures of a nitrifying crenarchaeote were established from sea water (48) and estuarial sediment (31, 40). Moreover, recent molecular ecological studies based on the putative ammonia monooxygenase A subunit gene (*amoA*) have demonstrated the worldwide distribution of AOA in coastal, estuarial and deep-sea water and sediment, suggesting that AOA as well as AOB play an important role in the nitrogen cycle of a marine ecosystem (ex. 5, 7, 8, 15, 31, 33).

An archaeon, ‘*Candidatus Nitrosopumilus maritimus*’ SCM1, was sustainable with a low ammonium concentration

(less than 1 mM) in the medium (25), and able to utilize a very low concentration of ammonium (29). Similarly, enrichment cultures of marine and freshwater AOA (31, 40, 48), thermophilic ammonia-oxidizers, ‘*Candidatus Nitrosocaldus yellowstonii*’ (44) and ‘*Candidatus Nitrososphaera gargensis*’ (19), derived from terrestrial hot springs, were also maintained at a low concentration of ammonium. Additionally, putative crenarchaeotal *amoA* genes have been detected in low ammonium-containing environments such as sea-water, sediment and hot springs (<1 mM ammonium) (14). However, *amoA* genes were also found in bioreactors with ammonium concentrations ranging from 1.2 to 3.2 mM (36). As yet, there is a little information on the cultivation of AOA compared to AOB. Here we report the culture of a novel marine ammonia-oxidizing archaeon maintained at a high ammonium concentration.

Materials and Methods

Sample collection

Sand samples were collected about 0–5 cm below the seafloor by divers using sterile plastic tubes (50 mL) in the eelgrass zone in Tanoura Bay of Shimoda, Shizuoka, Japan, on 17 May, 2007. The temperature of the sea water was 18.5°C. The concentration of ammonium in pore water was 558 µM. It has been previously suggested that AOA were responsible for nitrification in the eelgrass zone as well as ammonia-oxidizing bacteria (AOB) (5). The samples were stored at 4°C and transferred to the laboratory that same day.

Cultivation of marine AOA

One gram of sample was diluted with 10 mL of modified ASW-medium (33). This suspension was thoroughly mixed and serially diluted (10-fold), and test tubes (inner diameter, 14 mm) were incubated at 10, 20 and 30°C in the dark without shaking. The medium contained (NH₄)₂SO₄ 1.0 g, NaCl 5.84 g, MgCl₂·6H₂O 10 g, Na₂SO₄

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3.91 g, CaCl₂ 1.1 g, KCl 0.66 g, NaHCO₃ 0.19 g, K₂HPO₄ 0.01 g, Fe(III)-EDTA 0.01 g, MOPS [3-(*N*-morpholino) propanesulfonic acid] 10 g, and 1 mL of bromothymol blue solution (bromothymol blue 0.01 g L⁻¹, Na₂HPO₄ 0.095 g L⁻¹, and KH₂PO₄ 0.005 g L⁻¹ in distilled water) in 1,000 mL of distilled water. The pH of the medium was adjusted to 8.0. The highest dilution at which nitrite production was observed was serially diluted (10-fold) with the medium and incubated. After this step was repeated four times, the following were added to one liter of the modified ASW-medium for a 30°C-incubation: 1 mL of a nonchelated trace element mixture (47), 1 mL of a selenite-tungstate solution (0.4 g L⁻¹ NaOH, 6 mg L⁻¹ Na₂SeO₃·5H₂O, 8 mg L⁻¹ Na₂WO₄·2H₂O), 1 mL of a vitamin mixture (40 mg L⁻¹ of 4-aminobenzoic acid, 10 mg L⁻¹ of D-biotin, 100 mg L⁻¹ of nicotinic acid, 50 mg L⁻¹ of calcium D-pantothenate and 150 mg L⁻¹ of pyridoxine dihydrochloride dissolved in 10 mM sodium phosphate buffer [pH 7.1]), 1 mL of a thiamin solution (100 mg L⁻¹ of thiamine chloride dihydrochloride dissolved in 25 mM sodium phosphate buffer [pH 3.4]) and 1 mL of vitamin B₁₂ solution (50 mg L⁻¹ of cyanocobalamin dissolved in distilled water). Cultures were routinely transferred (10% volume per transfer) to fresh medium in test tubes (inner diameter, 14 mm) when nitrite concentrations reached approximately 3 mM. Growth was monitored based on nitrite production, PCR-denaturing gradient gel electrophoresis (DGGE), and microscopy. The enrichment culture was sand-free and stable, resulting from repeated dilution for over two years.

For growth tests, test tubes (22 mm) containing 30 mL of modified ASW-medium were used. The concentration of ammonium was adjusted to 15 mM or 26 mM. To test the tolerance to ammonium in the enrichment cultures, test tubes (14 mm) with 10 mL of modified ASW-medium were used. The concentration of ammonium in the medium was adjusted to 0.8, 12, 26, 36, 47, 61 or 76 mM.

Chemical analyses

The concentration of nitrite in the culture medium was determined colorimetrically with Griess-Ilosvey reagent (20). The concentration of ammonium was determined colorimetrically by a modified indophenol reaction (23). The concentration of nitrate was measured with an ion-chromatography system-1000 attached to an UltiMate 3000 Variable Wavelength Detector (Dionex, Sunnyvale, CA).

Fluorescence in situ hybridization

For fluorescence *in situ* hybridization (FISH), cells were collected with 0.2-µm-pore-size polycarbonate GTBP membrane filters (Millipore, Billerica, MA, USA) and fixed in 2% paraformaldehyde. The fixed cell suspensions were spotted on precleaned, gelatin-coated microscopic slides. Dehydration and *in situ* hybridization were performed according to the procedure described by Amann *et al.* (4). The following 16S rRNA-targeted oligonucleotides were used: a Cy3-labeled CREN500nm probe for strain NM25 belonged to 'Nitrosopumilus' (Table 1), a Cy3-labeled ARCH915 probe for the domain *Archaea* (41) and an FITC-labeled EUB338 probe for the domain *Bacteria* (3). The CREN500nm probe was modified from CREN499R for the kingdom *Crenarchaeota* (9). Cell numbers of CREN500nm- or EUB338-stained microbes were determined by counting cells in more than 3 microscopic fields per sample, each of which contained more than 50 DAPI-stained cells. The specificity of the labeling was confirmed by a failure to hybridize *Pyrobaculum aerophilum* NBRC 100827^T and *Nitrosospora multififormis* ATCC 25196^T. For counting total cell numbers, the cells were fixed in 2% paraformaldehyde and stained with 4',6'-diamidino-2-phenylindole (DAPI). The cells were filtrated on 0.2-µm polycarbonate GTBP membrane filter. The total cell counts were estimated from 5 microscopic fields per sample.

16S rRNA and amoA sequences and phylogenetic analysis

Cells were collected by centrifugation. Nucleic acids were then

Table 1. Oligonucleotides used for FISH and the 16S rRNA sequences of target and nontarget species

Probe, target and nontarget organism(s), and environmental clones	Probe and target sequences ^a
CREN500Rnm	3' - CCC CAC CCG TTC AAG ACC - 5'
'Nitrosopumilus sp.' NM25	5' - GGG GUG GGC AAG UUC UGG - 3'
'Nitrosopumilus maritimus' SCM1	5' - GGG GUG GGC AAG UUC UGG - 3'
'Cenarchaeum symbiosum'	5' - GGG GUG GGC AAG UUC UGG - 3'
Marine fosmid DeepAnt-EC39	5' - GGG GUG GGC AAG UUC UGG - 3'
'Nitrososphaera gargensis'	5' - GGG GUG GGC AAG U-C UGG - 3'
'Nitrosocaldus yellowstonii'	5' - GGG GUG GGC AAG U-U UGG - 3'
<i>Pyrobaculum aerophilum</i> IM2	5' - GCG GGG GGC AAG U-C UGG - 3'
<i>Pyrodicticum occultum</i> PL-19	5' - GCG GGG GGC AAG U-C UGG - 3'
'Nanoarchaeum equitans' Kin4-M	5' - GCG GUG GGC AAG A-G GGG - 3'
EUB338	3' - TGA GGA TGC CCT CCG TCG - 5'
<i>Bacteria</i>	

^a Formamide concentration in hybridization buffer was 20%.

extracted with Lysing Matrix E (MP Biomedicals, Solon, OH, USA) and ISOPLANT II (Nippon Gene, Toyama, Japan). A near complete archaeal 16S rRNA gene was PCR amplified using archaeal-specific primers Ar7f (28) and Univ1492R (27) with the following protocol: 94°C for 5 min and then 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 9 min. For PCR-DGGE, DNA fragments encoding 16S rRNA genes were amplified using either primer sets ARC344F-GC and ARC915R for *Archaea* (11) or EUB341F-GC and 907R for *Bacteria* (32) with the protocol described above. Crenarchaeotal *amoA* was amplified using crenAMO_F and crenAMO_R (18), again with the protocol described above. The betaproteobacterial *amoA* gene was amplified using *amoA*-1F and *amoA*-2R (39). The gammaproteobacterial *amoA-amoB* gene was amplified using *amoA*-3F and *amoB*-4R (38). Amplified fragments were cloned with a TOPO TA PCR cloning kit (Invitrogen, Carlsbad, USA) and sequenced with the primers M13F, M13R, Ar7f, ARC344F, ARC344R, A571F (10), ARC915F, ARC915R, Ar1384r (28), Univ1492R, EUB341F and 907R as described previously (34). The nucleotide and amino acid sequences were manually aligned using the CLUSTAL W program in MEGA4 (42) and Clustal X version 1.83 (43). Phylogenetic analyses were performed with neighbor-joining, maximum-parsimony and maximum-likelihood using MEGA4 and MOLPHY (1).

Quantitative PCR

Crenarchaeotal and betaproteobacterial *amoA* genes in culture medium were quantified by real-time PCR in a LightCycler system 350S (Roche Diagnostics, Mannheim, Germany) with a SYBR Premix Ex Taq (Perfect Real Time) kit (Takara Bio, Otsu, Japan) using either crenAMO_F/crenAMO_R or *amoA*-1F/*amoA*-2R (5, 34). Cycling conditions were as follows: for both *amoA* genes, an initial denaturation step at 95°C for 30 sec and then 35 cycles at 95°C for 15 sec, 57°C for 15 sec and 72°C for 30 sec. Fluorescence intensity was measured at 81°C. Plasmids containing copies of *amoA* genes of the crenarchaeotal clone G35259 (5) or *N. multififormis* ATCC25196^T were used to make a standard curve.

Nucleotide sequence accession numbers

The 16S rRNA and *amoA* gene sequences were submitted to DDBJ/EMBL/GenBank and have been assigned the following accession numbers: AB546961–AB546965.

Results and Discussion

Establishment of enrichment cultures

An ammonia-oxidizing enrichment culture derived from coastal sand, that was sediment-free and stable, was first established at 30°C by repeated passage into modified ASW-medium containing ammonium sulfate (7.6 mM) and bicarbonate (2.3 mM) as a sole source of energy and carbon for more than two years. The crenarchaeotal *amoA* gene was successfully amplified from only the 30°C-incubated tubes after four rounds of serial dilution without any amplification of the bacterial *amoA* gene. However, the proportion of archaeal microbes among all prokaryotes was estimated at 0.02% in medium at the highest dilution tube where nitrite production was observed, using quantitative PCR based on 16S rRNA. The archaeal microbes were highly abundant after the two years. It was reported that AOA medium required vitamins and trace elements (25). In this study too, it was necessary to supplement the medium with trace elements and vitamins to maintain the enrichment after serial dilution. In addition, the enrichment of ‘*Candidatus Nitrosopumilus*’-like archaea was successful on the transfer of 10% inoculum into fresh medium in glass tubes instead of Erlenmeyer flasks. It seems that using a test tube for cultivation of AOA inhibited the growth of heterotrophic microbes due to a decrease in the amount of oxygen diffused into the medium. The enrichment culture was stable in test tubes (inner diameter, 14 mm) containing 7.6 mM ammonium sulfate, trace metals, and vitamins. Although filtrations was performed for isolation, a pure culture has not yet been established. It is possible that AOA is maintained by an unknown compound released from microorganisms in the enrichment culture.

Ammonium oxidation by crenarchaeote

Tiny rod-shaped or peanut-shaped cells (diameter of 0.2–0.4 μm and length of 0.4–0.9 μm) stained by the CREN500nm probe for NM25 belonged to ‘*Nitrosopumilus*’ were observed as the dominant population throughout incubation of the enrichment NM25 culture under the epifluorescence microscope (Fig. 1A and Fig. 1B). Bacterial cells remained in the culture (Fig. 1C). Peanut-shaped cells (<1 μm) were previously reported for an ammonia-oxidizing archaeon ‘*Candidatus Nitrosopumilus maritimus*’ SCM1 (25), a psychrophilic crenarchaeon ‘*Candidatus Cenarchaeum symbiosum*’ inhabiting a marine sponge (37) and marine planktonic archaeal cells (13). In addition, the ‘*Candidatus Nitrosopumilus*’-like cells were smaller than the cells of the marine AOB *Nitrosomonas aestuarii* (1.0–1.3 by 1.4–2.0 μm) and *Nitrosomonas marina* (0.7–0.9 by 1.4–2.3 μm) (26). Although NM25 belonged to ‘*Nitrosopumilus*’ possessed the region of the 16S rRNA gene sequence in which the ARCH915 probe for *Archaea* bound, the ‘*Candidatus Nitrosopumilus*’-like cells were not labeled with this probe. Similarly, cells of the nanosized hyperthermophilic archaeon ‘*Candidatus Nanoarchaeum equitans*’, measuring approximately 0.4 μm in diameter, were not labeled with the ARCH915 probe (21). However, after the CREN500nm probe was designed based on the ‘*Candidatus Nitrosopumilus*’ sequence, at the same position as the

Fig. 1. Epifluorescence microscopy of the NM25 belonged to ‘*Nitrosopumilus*’ enrichment culture after 16 days of incubation. A, Fluorescence micrograph of cells stained with DAPI. B, Fluorescence micrograph of cells after hybridization with the Cy3-labeled probe CREN500nm (‘*Nitrosopumilus*’ strain NM25). C, Fluorescence micrograph of cells after hybridization with the FITC-labeled probe EUB338 (*Bacteria*). Scale bars represent 5 μm .

515mcR probe for ‘*Candidatus Nanoarchaeum equitans*’ (Table 1), the ‘*Candidatus Nitrosopumilus*’-like cells showed bright fluorescence (Fig. 1B). It is, therefore, likely that the position from 500 to 519 is the accessible binding site of the small-subunit rRNA for the nanosized archaeon.

Growth of microbes in the enrichment culture containing 26 mM ammonium was associated with the consumption of ammonium accompanying the accumulation of nitrite (Fig. 2). Cell density ranged from 9.7×10^6 to 3.0×10^8 cells mL^{-1} , with a minimum generation time of 48 h. The maximal density of ‘*Candidatus Nitrosopumilus*’-like cells was approximately ten times higher than previously reported cell densities of isolated and enriched AOA (25, 44, 48). No nitrate was detected in the enrichment cultures.

FISH revealed that the ratio of cells stained by the

Fig. 2. Time-course of changes in ammonium and nitrite concentrations and concomitant cell growth in the enrichment culture of NM25 belonged to ‘*Nitrosopumilus*’ in test tubes (inner diameter 22 mm, $n=3$) at 30°C.

Fig. 3. Correlation of ammonia oxidation with growth of NM25 belonged to ‘*Nitrosopumilus*’. A, Time-course of changes in ammonium and nitrite concentrations in the enrichment culture in test tubes (inner diameter 22 mm, $n=3$) at 30°C. B, Change in the ratio of NM25 belonged to ‘*Nitrosopumilus*’ (CREN500nm) to *Bacteria* (EUB338) cell counts in the same enrichment culture as determined by FISH. The ratio was estimated by the following formula $\% = \text{CREN500nm} / (\text{CREN500nm} + \text{EUB338}) \times 100$. Error bars represent the standard deviation of the mean.

CREN500nm probe for NM25 belonged to ‘*Nitrosopumilus*’ increased gradually from 9- to 18-days’ incubation (reaching 89%) accompanying the increase in nitrite (Fig. 3). Of all the cells stained by DAPI ($n=1304$), $89.9 \pm 10.2\%$ ($n=3$) yielded a positive signal with the CREN500nm probe after 18-days’ incubation. The molar ratio of oxidation of ammonium to nitrite (nitrite ammonium⁻¹) was 0.8 after 18 days of incubation. It is assumed that 1.0 mmol of nitrite is formed per

Fig. 4. Denaturing gradient gel electrophoresis of archaeal and bacterial communities in the enrichment culture of NM25 belonged to ‘*Nitrosopumilus*’. 16S rRNA gene fragments were amplified with primer sets for the domain *Archaea* or *Bacteria*.

mmol of ammonium, according to the following equation for complete oxidation of ammonium: $\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+$. Indeed, 0.8 mmol of nitrite was formed per mmol of ammonium. This measured oxidation balance is similar to the above stoichiometry. It seems that part of the ammonium was utilized by archaea and bacteria. The ammonium oxidation rate of the ‘*Candidatus Nitrosopumilus*’-like cells in the enrichment culture ($0.19\text{--}0.43 \text{ fmol NH}_4^+ \text{ cell}^{-1} \text{ h}^{-1}$) was similar to the range of rates estimated for pure and enrichment cultures of known AOA, $0.08\text{--}0.59 \text{ fmol NH}_4^+ \text{ cell}^{-1} \text{ h}^{-1}$ (22).

The DGGE profile retrieved from the enrichment culture consisted of a single dominant archaeal 16S rDNA band (A1) during incubation (Fig. 4). The sequence of this band corresponded to that of NM25 belonged to ‘*Nitrosopumilus*’ (Fig. 5). The sequences of bacterial DGGE bands (B1, B2 and B3) were non-AOB (marine chemoorganotrophic bacteria *Neptuniibacter* and *Marinobacter* spp. and bacteria within the *Deltaproteobacteria*), indicating that marine bacteria utilized organic matter released by AOA. Moreover, quantitative PCR also revealed that the abundance of the crenarchaeotal *amoA* gene increased with the production of nitrite in the enrichment cultures (reaching 4.3×10^8 copies mL⁻¹). However, neither betaproteobacterial nor gammaproteobacterial *amoA* was detected in the enrichment cultures. Thus, these results demonstrated that ammonium was converted to nitrite by a single population of crenarchaeote NM25 belonged to ‘*Nitrosopumilus*’ in the enrichment culture.

Phylogenetic analysis of ‘*Candidatus Nitrosopumilus* sp.’ strain NM25

Analysis of the 16S rRNA gene revealed that NM25 belonged to *Crenarchaeota* Group 1.1a (Fig. 5). NM25 was most closely related to ‘*Candidatus Nitrosopumilus maritimus*’ SCM1 (25) and the environmental clone SAT 3E6

Fig. 5. Phylogenetic analysis of archaeal 16S rRNA gene sequences (1,352 bases) amplified from NM25 belonged to 'Nitrosopumilus'. The underline represents cultivable ammonia-oxidizing archaea. The tree was constructed with the neighbor-joining method. The scale bar represents an estimated sequence divergence of 5%. Neighbor-joining (left), maximum-parsimony (center) and maximum-likelihood (right) bootstrap values providing more than 50% support are indicated.

obtained from salt marsh sediment (35), with 98.5% and 99.4% identity, respectively. Analysis of the *amoA* gene revealed that NM25 belonged to the Marine cluster containing AOA enriched from estuarial sediment (31), the ammonia-oxidizing archaeon 'Candidatus Nitrosopumilus maritimus' SCM1 (25) and the psychrophilic crenarchaeon 'Candidatus Cenarchaeum symbiosum' (37), with sequence similarity of <95%, 94% and 74%, respectively (Fig. S1). In addition, the *amoA* gene sequence of NM25 was less closely related to that of known environmental clones (>97%). These results suggest that the ammonia-oxidizing archaeon NM25 belonged to 'Nitrosopumilus' was a novel species within *Crenarchaeota* Group 1.1a.

Ammonium tolerance

In general, AOA are known for their high sensitivity to their substrate ammonium (25, 29, 31, 40, 48). The known culturable AOA were maintained at less than 2 mM of ammonium independent of sampling sites (Table 2). However, the enrichment culture of NM25 belonged to 'Nitrosopumilus' was able to grow in the presence of 26 mM ammonium within the test tube (inner diameter 22 mm)

Fig. 6. Tolerance to ammonium of the NM25 enrichment culture in test tubes (inner diameter 14 mm, $n=3$) at 30°C. A, The nitrite concentration and cell density after 30 days of incubation. Error bars represent the standard deviation of the mean. B, The maximum production rate of nitrite per day is plotted against the ammonium concentration.

(Fig. 2). In addition, the optimal ammonium concentration for NM25 was approximately 15 mM in the test tube (14 mm) (Fig. 6). A concentration of more than 35 mM of ammonium resulted in an almost complete inhibition of growth.

Ecological observation

No *amoA* gene sequence closely related to that of the archaeon NM25 belonged to 'Nitrosopumilus' was obtained in sand of an eelgrass zone in a previous study (5), although NM25 was derived from the same sampling site. This result suggests that NM25 was a minor population in the *in situ* habitats of an eelgrass zone. The enrichment culture of archaeon NM25 was established at 15 mM ammonium. However, the concentrations of ammonium in sand samples

Table 2. Culture conditions that differentiate NM25 belonged to 'Nitrosopumilus' from other ammonia-oxidizing archaea

Isolate or enrichment culture	Ammonium (mM)	Temperature (°C)	Source of inoculum	Reference
'Nitrosopumilus sp.' NM25	15 ^a , 26 ^b	30	Coastal sand of an eelgrass zone	This study
'Nitrosopumilus maritimus' SCM1	0.5–1.0	28	Rocky substratum of a tropical marine tank	25
Enrichment cultures	0.5	22	Sediments of estuary	31
Enrichment cultures	0.15	25	Sea water	48
Enrichment cultures	2.0	20	Freshwater coastal sediments	40
'Nitrososphaera gargensis'	2.0	46	Microbial mat of terrestrial hot spring	19
'Nitrosocaldus yellowstonii'	0.5–1.0	65–72	Sediment of terrestrial hot spring	44

^a The value was obtained from the test tube (inner diameter 14 mm).

^b The value was obtained from the test tube (inner diameter 22 mm).

of the eelgrass zone were <1 mM (5). It would seem that the difference in ammonium levels led to the cultivation of a novel ammonia-oxidizing archaeon, which adapted to a higher concentration of ammonium than known AOA. On the other hand, putative crenarchaeotal *amoA* genes were usually obtained at less than 1 mM ammonium in nature (14). It is therefore likely that the growth of major members of AOA within sand of the eelgrass zone was inhibited by the higher concentration of ammonium in the culture medium.

Conclusion

We showed for the first time that a novel marine ammonium-oxidizing archaeon, NM25 belonged to 'Nitrosopumilus', within *Crenarchaeota* Group 1.1a, was successfully enriched at a high ammonium concentration from coastal sand of an eelgrass zone. This finding suggests that AOA distribute not only in low ammonia-containing environments but also in high ammonia-containing environments. A marine ammonia-oxidizing archaeon, '*Candidatus Nitrosopumilus maritimus*' SCM1, and a thermophilic ammonia-oxidizing archaeon, '*Candidatus Nitrosocaldus yellowstonii*', were reported to be obligate autotrophs (25, 44). On the other hand, a psychrophilic marine crenarchaeon, '*Candidatus Cenarchaeum symbiosum*', was suggested to be an obligate autotroph or a mixotroph utilizing both carbon dioxide and organic matters (17, 18). Further study is needed to determine whether the archaeon NM25 belonged to 'Nitrosopumilus' is an obligate autotroph or a mixotroph.

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