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The Phylogenetic Affiliation of an Uncultured Population of Ammonia-Oxidizing Bacteria Harboring Environmental Sequences of *amoA* Cluster-3

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We investigated the phylogenetic diversity of ammonia-oxidizing bacteria (AOB) in Yellow Sea continental shelf sediment by the cloning and sequencing of PCR-amplified *amoA* and 16S rRNA genes. Phylogenetic analysis of the *amoA*-related clones revealed that the diversity of AOB was extremely low at the study site. The majority (92.7%) of *amoA* clones obtained belonged to a single cluster, environmental *amoA* cluster-3, the taxonomic position of which was previously unknown. Phylogenetic analysis on AOB-specific 16S rRNA gene sequences also demonstrated a very low diversity. All of the cloned 16S rRNA gene sequences comprised a single phylotype that belonged to the members of uncultured *Nitrosospira* cluster-1, suggesting that AOB belonging to the uncultured *Nitrosospira* cluster-1 could carry *amoA* sequences of environmental *amoA* cluster-3.

Keywords: Ammonia-oxidizing bacteria, *amoA*, phylogeny

Nitrification, the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and ultimately nitrate (NO_3^-) by chemolithoautotrophic microorganisms, plays a crucial role in the global nitrogen (N) cycle and provides a link between ammonification (the mineralization of organic nitrogen) and denitrification (the loss of fixed nitrogen). Because the low energy yield from ammonia oxidation leads to low biomass yield, the cultivation of ammonia-oxidizing bacteria (AOB) takes several months [18]. Until recently, only members of the genera *Nitrosococcus*, *Nitrosospira*, and *Nitrosomonas* of the phylum Proteobacteria were known as cultured AOB [6, 28]. Therefore, the study of community structure and evolutionary history of AOB relies mainly on molecular methods targeting an AOB-specific 16S rRNA gene or *amoA* sequences encoding the first subunit of ammonia monooxygenase [20].

According to previous studies based on 16S rRNA gene sequences, β-proteobacterial AOB comprise nine clusters [23, 27]. Clusters 0~4 have been shown to belong to *Nitrosospira*; the remaining clusters (5~8) belong to *Nitrosomonas*. In particular, most sequences that belong to *Nitrosospira* cluster-1 and *Nitrosomonas* cluster-5 are found in marine environments, and no cultured representatives have been discovered for these two clusters. The results of phylogenetic analyses of AOB using *amoA* and 16S rRNA gene sequences seem similar, but not identical [23]. Generally, environmental clones of the *amoA* sequences are divided into three clusters [30]. Although analysis of *amoA* sequences can provide great resolution of genetic differences in natural AOB populations [22, 23], *amoA* alone cannot determine the phylogenetic position of AOB, and the phylogenetic position of uncultured AOB harboring environmental *amoA* sequences remains unclear.

In this study, we investigated the community structure and diversity of AOB in Yellow Sea continental shelf sediment using *amoA* and 16S rRNA gene sequences. We observed a very simple AOB community structure at the study site. Interestingly, the site harbored only previously uncultured AOB with a limited number of phylotypes of *amoA* and 16S rRNA gene sequences. This provided us circumstantial information to speculate the taxonomic position of the uncultured AOB that carry environmental *amoA* sequences belonging to cluster-3, the taxonomic position of which was previously unknown.

Sampling Site and Molecular Phylogenetic Analysis

The sampling site was located in the southeastern Yellow Sea (125°30'E and 33°30'N), west of Je-Ju Island, where a shield volcano formed during the middle Pleistocene epoch through deep and shallow extrusions of alkaline basaltic magma. A sediment core sample (depth, ca. 80 m) was

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collected using a gravity corer. After removing the seawater from the top of the core, the sediment sample was stored at <0°C until analysis. A detailed description of the sample was presented by Jeong *et al*. [14].

DNA was extracted from the sediment sample using a Power Soil DNA isolation kit (MoBio, Solana Beach, CA, USA) and subjected to amplification of the 16S rRNA gene using PCR with primers NitA and NitB (NitA, 5'-CTT AAG TGG GGA ATA ACG CAT CG-3', and NitB, 5'-TTA CGT GTG AAG CCC TAC CCA-3', corresponding to positions 137~159 and 1214~1234 of *E. coli* 16S rRNA gene) specific for β-proteobacterial AOB [31]. To amplify the *amoA* gene from the community DNA, primers A189F (5'-GGN GAC TGG GAC TTC TGG-3') and A692R (5'-GAA SGC NGA GAA GAA SGC-3') [12] were used. The 16S rRNA and *amoA* gene amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valentia, CA, USA), and were cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). To analyze the 16S rRNA gene clones, sequences were checked for possible chimeric origins using MALLARD software [1]. A few potentially suspicious sequences were excluded from subsequent analyses, and NCBI-BLAST and RDP Sequence Match were used to find closely related sequences. Cloned and reference sequences were aligned using CLUSTALW [29] and subjected to phylogenetic reconstruction using MEGA software [16]. The evolutionary distances were calculated according to Kimura's two-parameter model [15]. For *amoA* clones, *amoA* nucleotide sequences as well as deduced amino acid sequences were used for phylogenetic analysis. The similarity scores for the amino acid sequences were calculated based on the PAM matrix [26]. Phylogenetic trees were inferred using the neighbor-joining algorithm, and the tree topology was statistically evaluated by 1,000 bootstrap resamplings.

Ammonia Monooxygenase Gene (*amoA*) Sequences

A total of 41 sequences were obtained with the *amoA* specific primers. The similarities between the cloned sequences were very high (97.6±0.04%), and the BLAST search revealed that only three sequences in the public database were close relatives of our cloned sequences. Clone *amoA*-SW21 was the closest relative (similarity, 97.4±0.30%) of the majority (90.2%) of our cloned *amoA* sequences. The remaining *amoA* clones were closely related (similarity, ~94.9%) to clones SAG-sed1 and *amoA*-Ts11, which were recovered from a marine sponge in a previous study [3]. The cloned *amoA* sequences were subjected to phylogenetic analysis with *amoA* sequences of cultured AOB and environmental *amoA* sequences. All *amoA* clones obtained in this study belonged to environmental *amoA* cluster-3 (Fig. 1) and were closely related to the *amoA* of *Nitrosomonas*. However, the phylogenetic tree constructed using the deduced amino acid sequences translated *in silico* from *amoA* sequences showed that our deduced amino acid sequences were more similar to AmoA of *Nitrosospira* than of *Nitrosomonas* (Fig. 2), whereas the bootstrap value marginally supported their monophyly.

Although many previous studies have discovered environmental *amoA* sequences belonging to the cluster-3, the phylogenetic position of bacteria harboring the cluster-3 *amoA* remains uncertain. A previous study suggested that the *amoA* cluster-3 might be *Nitrosospira-amoA* or *Nitrosospira*-sister clade *amoA* [5, 8, 13]. However, Urakawa *et al.* [30] argued that this cluster belonged to neither *Nitrosospira-amoA* nor *Nitrosomonas-amoA*. Although the *amoA* sequence data alone are not enough to determine the taxonomic identity of bacteria carrying cluster-3 *amoA* sequences, our observation that all *amoA* sequences obtained from this study site formed a single phylotype allowed us to speculate the phylogenetic affiliation of AOB carrying cluster-3 *amoA.* To be specific, we questioned whether AOB 16S rRNA gene clones were observed as a single phylotype, like the *amoA* clones.

16S rRNA Gene Sequences of Ammonia-Oxidizing Bacteria

We investigated the community structure of AOB using 16S rRNA gene sequences. The 16S rRNA gene clone library of AOB was constructed from PCR amplicons using the AOB-specific primers. A total of 71 clones were obtained, three of which were revealed as chimeric sequences using the MALLARD program. The remaining 68 clones were phylogenetically analyzed with the formerly published 16S rDNA sequences of cultured and environmental AOB [10, 30]. Through a BLAST search, clone SS1-B-03-51, recovered previously from Arctic Sea sediment, was found to be the closest relative (similarity, 99.0±0.20%) of 64.7% of the clones. Clones P0X3b1H04 and P0X4b2G08 were closely related to the remaining 4 and 20 clones with 98.9±0.30% and 99.0±0.20% similarity, respectively.

The phylogenetic tree showed that all of the 16S rRNA gene clones belonged to *Nitrosospira*-like cluster-1 (Fig. 3). Similar to the results of the analysis based on the *amoA* clones, the 16S rRNA gene clones formed a restricted cluster with high intragroup similarity (99.4%). This indicates that the environmental *amoA* sequences of cluster-3 might be carried by β-proteobacterial ammonia-oxidizing bacteria of *Nitrosospira*-like cluster-1. A similar idea that environmental *amoA* cluster-3 might correspond to *Nitrosospira*-like cluster-1 has been suggested previously by O'Mullan and Ward [22]. Their study found insufficient evidence to prove this, but here we present circumstantial information to support this idea in that we obtained a single 16S rRNA phylotype and a single *amoA* phylotype, representing an AOB population predominating in this continental shelf sediment.

Although environmental *amoA* cluster-3 (*Nitrosospira* cluster-1) is known as a widespread and dominant sequence

Fig. 1. Phylogenetic relationships between *amoA* sequences of cultured ammonia-oxidizing bacteria and clones obtained from Yellow sea continental shelf sediment (marked with closed circles; GenBank accession numbers JF416324–JF416364).

The cluster designations were adopted and modified from those of Urakawa *et al*. [30]. The phylogenetic distances were calculated using the Kimura two-parameter model, and the tree was constructed using the neighbor-joining algorithm. The numbers at the nodes indicate the bootstrap score (as a percentage) and are shown for frequencies at or above the threshold of 50%. The scale bar represents the expected number of substitutions per nucleotide position.

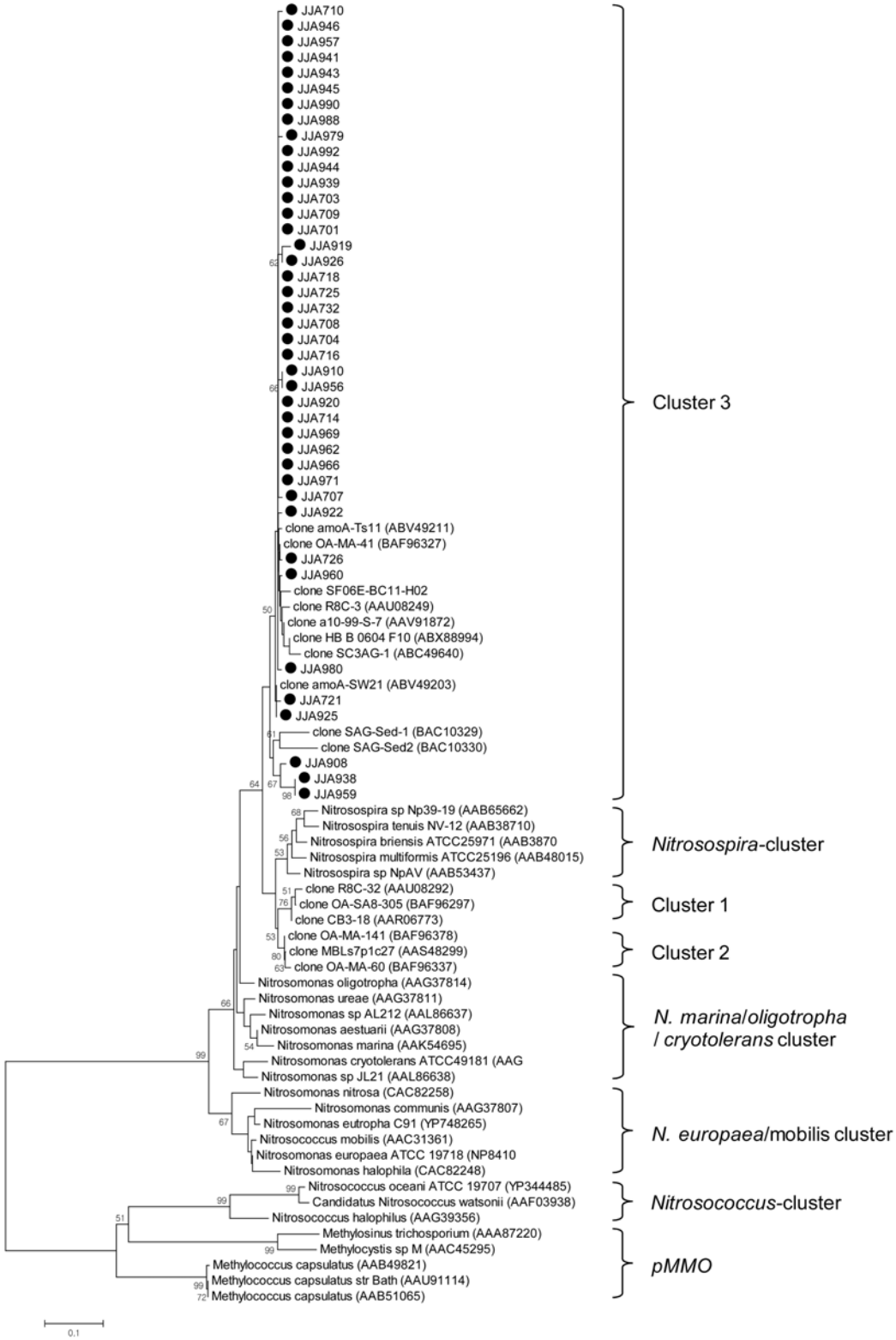

Fig. 2. Phylogenetic relationships between deduced amino acid sequences of cloned *amoA* sequences from Yellow Sea continental shelf sediment (marked with closed circles) and AmoA amino acid sequences of cultured ammonia-oxidizing bacteria.
The cluster designations were adopted and modified from those of Urakawa *et al.* [30]. The phylogenetic distances were calculated using the PAM matrix, and the tree was constructed using the neighbor-joining algorithm. The numbers at the nodes indicate the bootstrap score (as a percentage) and are shown for frequencies at or above the threshold of 50%. The scale bar represents the expected number of substitutions per amino acid position.

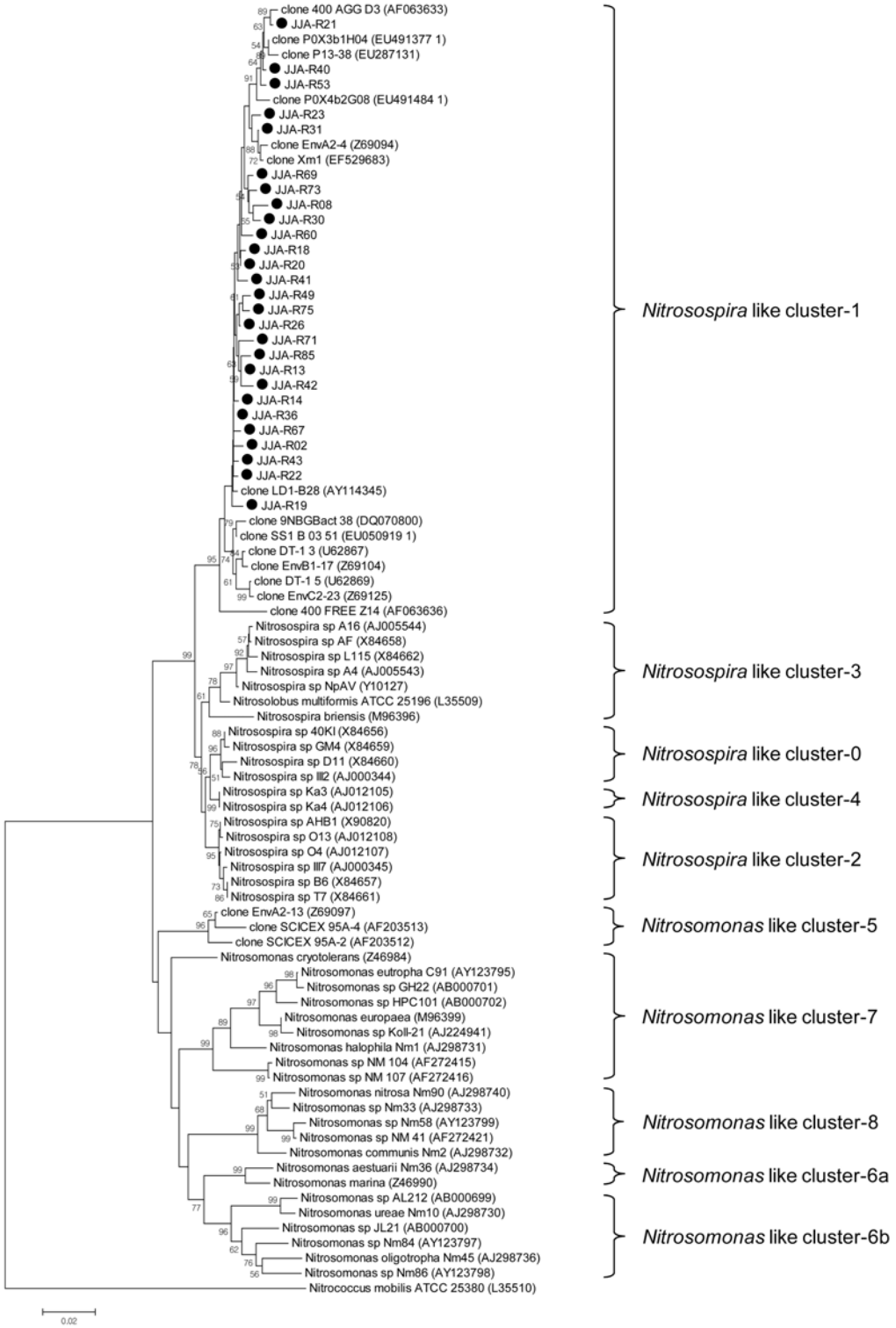

Fig. 3. The phylogenetic position of 16S rRNA gene sequences of ammonia oxidizing bacteria obtained from Yellow Sea continental shelf sediment (marked with closed circles; GenBank accession numbers JF416365–JF416432).

Representative sequences are presented for each group of identical sequences. Database sequences for cultured ammonia-oxidizing bacteria and environmental sequences are derived from Purkhold *et al.* [23], and cluster designation originated from Stephen *et al.* [27]. The phylogenetic distances were calculated using the Kimura two-parameter model, and the tree was constructed using the neighbor-joining algorithm. The numbers at the nodes indicate the bootstrap score (as a percentage) and are shown for frequencies at or above the threshold of 50%. The scale bar represents the expected number of substitutions per nucleotide position.

type of AOB in marine environments [2, 4, 5, 10, 11, 19, 22], no cultured representative has been described for this cluster. Previous studies have found that AOB corresponding to this cluster might tolerate salinity [4, 8, 11, 21], suggesting adaptation of possible survival characteristics to high salinity environments [11]. Additionally, this adaptation is reflected by high evolutionary distances that are as long as independent lineages (*e.g.*, *Nitrosomonas cryotolerans* and *Nitrosomonas marina* lineages) [24]. The reason for the low diversity of AOB in the Yellow Sea continental shelf sediment was unclear, although several environmental factors, such as ammonia concentration, temperature, and salinity, have been suggested as factors that control the diversity and community structure of AOB. The low diversity of AOB in our study site was consistent with previous studies of marine sediments of the South Atlantic Bight and Huntington Beach in the USA, where only two phylotypes were recovered [13, 25]. The texture of these sediments was identical to those of our sediment samples, but other information on physicochemical properties was unavailable for further comparison. Specifically, in marine environments, salinity could be considered the key factor influencing AOB diversity and community structure by affecting their NH_4^+ adsorption and growth rate [7, 17]. A previous study found that the diversity of AOB in three sediment samples from an estuary at different salinity levels was lower at the higher salinity [5]. The salinity level was suggested to shift AOB community structure; *Nitrosospira*-like AOB usually dominate in high salinity environments, and *Nitrosomonas*-like AOB in low salinity environments [5, 8, 9, 21]. However, the Yellow Sea is mixoeuhaline, and its salinity (*ca*. 33 psu) is slightly higher than other marine environments. Recently, an investigation of AOB in aquariums provided evidence that low temperature might lead to a low diversity of AOB [30]. However, the temperature of the Yellow Sea continental shelf varies greatly, between 15°C and 28°C during a year (http://kodc.nfrdi.re.kr/); this proposed relationship between temperature and low AOB diversity is also unlikely. To further elucidate the causes leading to lower AOB diversity in marine sediments and its consequences, comprehensive studies focusing on the relationships between environmental parameters (*e.g.*, sediment porosity and oxygen availability) as well as other organisms contributing to the marine N-cycle (*e.g.*, denitrifying bacteria) are important and timely.

Concluding Remarks

The community structure of AOB in our Yellow Sea continental shelf sediment demonstrated extremely low diversity, with only one phylotype representing both 16S rRNA gene and *amoA* clone libraries. This low diversity allowed us to obtain circumstantial evidences supporting the assertion that the sequence of environmental *amoA* cluster-3 could be carried by ammonia-oxidizing bacteria belonging to *Nitrosospira* cluster-1. The wide distribution of this cluster in marine environments and its possible salinity tolerance indicate that *Nitrosospira* cluster-1 might perform an important role in the marine N-cycle. However, the molecular data obtained in this study suggest only possible characteristics of *Nitrosospira*-like cluster-1. Hence, to obtain direct and convincing evidences of genotypic and phenotypic characteristics, pure or enriched cultures analysis and metagenomic studies are essential.

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