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Diversity and Abundance of Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea During Cattle Manure Composting

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Abstract Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) play important roles in nitrification in various environments. They may also be key communities for ammonia oxidation in composting systems, although few studies have discussed their presence. We investigated the relative diversity and abundance of AOB and AOA using cloning procedures, denaturing gradient gel electrophoresis analysis, and real-time PCR during several stages in the process of cattle manure composting. Our results revealed that the AOB community structure changed during the process. At the high-temperature stage (>60°C), a member of the *Nitrosomonas europaea/eutropha* cluster dominated while the uncultured *Nitrosomonas* spp. cluster appeared after the temperature decreased. Additionally, our analysis indicated that AOA sequences, which were classified into a soil/sediment cluster, were present after the temperature decreased during the composting process. At these stages, the number of the archaeal *amoA* gene copies (3.2 or 3.9×10^7 copies per gram freeze-dried compost) was significantly higher than that of bacterial *amoA* gene copies ($2.2–7.2\times10^6$ copies per gram freeze-dried compost). Our results suggest that both AOB and AOA are actively involved in nitrification of composting systems.

Introduction

Composting is one of the most effective techniques for treating organic solid waste, such as cattle manure [4, 14, 44]. Organic nitrogen (N) in fresh manure is decomposed by microorganisms and transformed into ammonium via ammonification [4]. Then, ammonium can be oxidized to nitrate through nitrification during the composting process [4, 25, 30, 46]. Compost products can be applied to agricultural lands as organic fertilizer, which contains large amounts of inorganic N that plants require for growth [12].

Nitrification consists of two reactions that involve the aerobic oxidation of ammonia by ammonia-oxidizing organisms to form nitrite [24], which is then transformed into nitrate by nitrite-oxidizing bacteria. Ammonia-oxidizing bacteria (AOB), initially considered the sole autotrophic ammonia oxidizers, have been detected in most aerobic environments, including freshwater [7, 18, 42], soil [20, 26, 37, 43], and wastewater treatment plants [13, 28, 35]. AOB may play an important role in nitrification during composting. In several composting materials, such as swine or chicken manure, several clusters are present, including the genera *Nitrosomonas* and *Nitrosospira* [25]. Another study also showed that both *Nitrosomonas* and *Nitrosospira* spp. were present throughout the composting of household waste by monitoring the community succession of AOB and ammonia emissions [21]. In the final product of cattle manure composting, AOB present were grouped into the *Nitrosomonas* lineage [20]. Other researchers have reported high-temperature-tolerant AOB that could be cultivated from composted cattle manure [41]. However, the AOB community was not well identified in the cattle manure composting process; indeed, there were few studies on AOB community structure using composting material [20, 30]. Additionally, the relationship between the AOB community and inorganic N should be investigated because of the influence of environmental factors, such as temperature, that dynamically change during the composting process.

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Recently, crenarchaeal autotrophic species that oxidize ammonia have been discovered [50]. Ammonia-oxidizing archaea (AOA) are also present in seawater [9, 11, 23, 48], estuarine sediments [3], soil [1, 6, 8, 17, 27, 33, 47], and wastewater treatment plants [36, 51]. They have also been detected in hot springs [16, 39, 52, 53] and have been cultivated at high temperatures that otherwise inhibit AOB growth [10]. Moreover, several studies on the ammonia-oxidizing community composition in soil have revealed that the copy number of the archaeal *amoA* gene can be higher than that of the bacterial *amoA* gene, suggesting that AOA, rather than AOB, are numerically superior in various soil environments [1, 17, 27, 33]. It was previously suggested that the composting process could permit the establishment of an AOA community, as well as the AOB community, due to the aerobic conditions and large amounts of ammonia produced during this process. Although there are only a few studies about the AOA community in composting systems, archaeal *amoA* gene was not detected from any stages of the composting process [30, 49].

Here, we identified both AOB and AOA communities in cattle manure compost using denaturing gradient gel electrophoresis (DGGE) analysis and cloning procedure. In addition, relative abundance of their genes was quantified using real-time PCR. Compost samples from four stages with different temperatures and chemical parameters were studied. The changes in AOB and AOA community structures were correlated with physical and chemical properties. Our results suggest that both AOB and AOA are actively involved in the ammonia oxidization process during cattle manure composting.

Materials and Methods

Compost Samples

Compost samples were collected from a field-scale facility owned by MIYAGI Agriculture Public Corporation (Miyagi, Japan) in January 2008. The facility consisted of a 70-m-long lane and storage pit. About 10,000 kg of beef cattle manure and bark were added to the lane and mixed daily using an automatic stirrer, with forced aeration for 25 days. Materials were then transferred to the pit and piled without aeration for about 35 days. Samples were collected at four stages: initial stage (day 0), high-temperature stage (day 4), end of the first treatment (day 25), and end of the second treatment (day 60). About 50 g of compost samples were collected at a depth of 30 cm from the surface of the compost. The compost temperature was also measured at this depth. Water content was measured with an infrared moisture meter (Kett Electric Laboratory, Tokyo, Japan). Chemical and physical parameters, such as pH, electrical conductivity, total ash, total N, and the carbon (C)/N ratio, were analyzed by the Institute of Livestock Industry's Environmental Technology group (Fukushima, Japan; Table 1). To measure the ammonium, nitrite ion, and nitrate ion concentrations, we suspended about 4 g of compost in 40 ml of ion-exchanged water and mixed for 30 min. After centrifugation, the supernatant was filtered using 0.45-μm cellulose acetate filters (Advantec MFS, Tokyo, Japan). The filtrate was stored at −85 °C until use, and was measured with ion chromatography systems ICS-1000 and ICS-2000 (Dionex, Sunnyvale, CA, USA). Chemical and physical properties of the samples are listed in Table 1.

DNA Extraction

All compost samples were freeze-dried using a freeze dryer (Tokyo Rikakikai, Tokyo, Japan) to maintain water content at the same low level (3–4%). Samples were then crushed and sieved through 0.5-mm pore filters. Total DNA was then extracted from 0.05 g of the freeze-dried compost using a PowerSoil™ DNA Isolation kit (MO Bio Labs, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was then dissolved in 80 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA) and stored at −20°C until PCR amplification.

PCR and DGGE Analysis

PCR was performed using Ex Taq™ (TaKaRa Bio, Shiga, Japan) with an iCycler (Bio-Rad, Hercules, CA, USA) and primer set for each community (Table 2). For amplifying the

Table 1 Changes in physical and chemical parameters during composting of cattle manure

Sampling time	Temperature (°C)	Water content (% WW)	Total ash (g/kg DW)	pH	EC (mS/cm)	Total N (%DW)	C/N ratio	NH_4^+ (gN/kg DW)	NO_2^- (gN/kg DW)	NO_3^- (gN/kg DW)
Day 0	14.0	79.1	10.3	7.8	4.3	1.4	32.6	5.820	ND	ND
Day 4	69.7	72.0	15.5	9.2	5.0	1.6	25.0	1.998	ND	0.016
Day 25	49.2	60.9	28.6	7.9	4.8	2.0	17.6	0.047	ND	0.049
Day 60	31.8	55.8	36.0	7.8	5.6	2.1	15.1	0.074	0.134	0.243

WW wet weight, *DW* dry weight, *ND* not detected

Table 2 PCR primers used in this study

Primer name	Sequence (5′–3′)	Targeted site	Use	Reference
amoA1F	GGGGTTTCTACTGGTGGT	Bacterial *amoA* gene	For cloning, PCR-DGGE and real-time PCR	40
amoA2R	CCCCTCKGSAAAGCCTTCTTC	Bacterial *amoA* gene	For cloning, PCR-DGGE and real-time PCR	40
Arch-amoAF	STAATGGTCTGGCTTAGACG	Archaeal *amoA* gene	For cloning and real-time PCR	11
Arch-amoAR	GCGGCCATCCATCTGTATGT	Archaeal *amoA* gene	For cloning and real-time PCR	11
AOA23F	ATGGTCTGGCTWAGACG	Archaeal *amoA* gene	For PCR-DGGE	47
AOA616R	GCCATCCATCTGTATGTCCA	Archaeal *amoA* gene	For PCR-DGGE	47
T7	TAATACGACTCACTATAGGG	Plasmid	For sequencing of clones	EMD Chemicals (WI, USA)
U19	GTTTTCCCAGTCACGACGT	Plasmid	For sequencing of clones	EMD Chemicals (WI, USA)

bacterial *amoA* gene, PCR was performed using a primer set amoA1F/amoA2R [40]. The forward primer contained GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) [32]. PCR products for AOA were applied directly to DGGE analysis since the primer set AOA23f/616r was used [47]. For AOB, PCR conditions were as follows: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 35 s. A final extension step at 72°C for 5 min was also performed. For AOA, the initial PCR conditions were as follows: initial denaturation at 95°C for 4 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 20 s, and extension at 72°C for 35 s. A final extension step at 72°C for 5 min was also performed. DGGE analysis was conducted with a D-code Multiple system (Bio-Rad) using an 8% polyacrylamide gel with a denaturing gradient of 30–70% for AOB and 20–60% for AOA. Electrophoresis was performed at 100 V for 8 h (for AOB) or at 120 V for 10 h (for AOA) at 60°C in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 10 mM EDTA-2Na·2H_2O). After staining gels with GelStar® Nucleic Acid Gel Stain (Lonza Rockland, Rockland, ME, USA) for 15 min, the DNA bands were excised and transferred to a 1.5-mL tube with 80 μL TE buffer. Part of each aliquot (1 μL) was used as the template for PCR to sequence DNA bands using primer sets without the GC-clamp. PCR products were then purified with ExoSAP®-IT (USB, Cleveland, OH, USA) following the manufacturer's instructions.

Cloning Procedure

PCR was performed using primer sets for each community (Table 2) and Ex Taq™ (TaKaRa Bio) for AOB and PrimeSTAR® (TaKaRa Bio) for AOA with an iCycler (Bio-Rad). For AOB, the PCR conditions were as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final extension step at 72°C for 5 min was also performed. For AOA, the PCR conditions were as follows: initial denaturation at 98°C for 1 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s, and extension at 72°C for 35 s. A final extension step at 72°C for 3 min was also performed. After all PCR products were purified using MagExtractor-PCR and Gel Clean Up (Toyobo, Osaka, Japan), the products were used for cloning with Novagen® Perfectly Blunt™ Cloning kits (EMD Chemicals, San Diego, CA, USA) according to the manufacturer's instructions. To amplify their vector sequences of clones, PCR was conducted using primer set T7/U19. PCR product sizes were confirmed by electrophoressis with 1.2% agrose gel. Then, PCR products with the correct size were purified with ExoSAP®-IT (USB) following the manufacturer's instructions.

Sequencing and Phylogenetic Analysis

The purified products were sequenced using the BigDye® Terminator Cycle Sequencing kit v.1.1 (Applied Biosystems, Carlsbad, CA, USA) with the appropriate primers listed in Table 2, according to the manufacturer's instructions. The products were analyzed using an ABI PRISM 3130xl Autosequencer (Applied Biosystems).

The sequences were then assembled and compared using BLAST from the DNA Databank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/index-e.html). After the sequences were aligned using ClustalW, a phylogenetic tree was constructed for the *amoA* sequences of each type of community using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1 [45]. Both phylogenetic trees were constructed using nucleic acid sequences obtained from DGGE bands and clones. Unrooted phylogenetic trees were generated using the neighbor-joining method and bootstrap tests were performed with 1,000 replicates. The *amoA* gene sequences obtained in this study are available in the DDBJ/EMBL/GenBank databases under accession numbers AB465013 to AB465033 (bacterial) and AB465037 to AB465040 and AB542178 (archaeal).

Real-Time PCR

The *amoA* gene copy numbers of AOB and AOA were determined using real-time PCR, as described previously [8]. Real-time PCR was conducted using primer sets listed in Table 2 with iQ™ SYBR® Green Supermix (Bio-Rad). Analysis was performed with a Chromo4^{TM} Four-Color Real-Time Detector added to a DNA Engine® cycler (Bio-Rad). To obtain a standard curve for each *amoA* gene product, PCR products amplified from activated sludge (for AOB) or a compost sample on day 60 (for AOA) were used after purification with MinElute® PCR purification kit (QIAGEN, Hilden, Germany). DNA concentration of both products were determined with a Nanodrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the copy numbers of *amoA* genes were calculated. The ranges of template copies used for generation of the standard curves were 1 to 1,000 copies for AOB and 7.8 to 7,818 copies for AOA, respectively. Standard curves from each run were analyzed with $r^2>0.99$ and gave amplification efficiency of about 87%. PCR reactions were analyzed in triplicate. The *t* test was performed using the SPSS® software 14.0J (SPSS, Tokyo, Japan) to evaluate the difference between archaeal and bacterial *amoA* gene copy numbers.

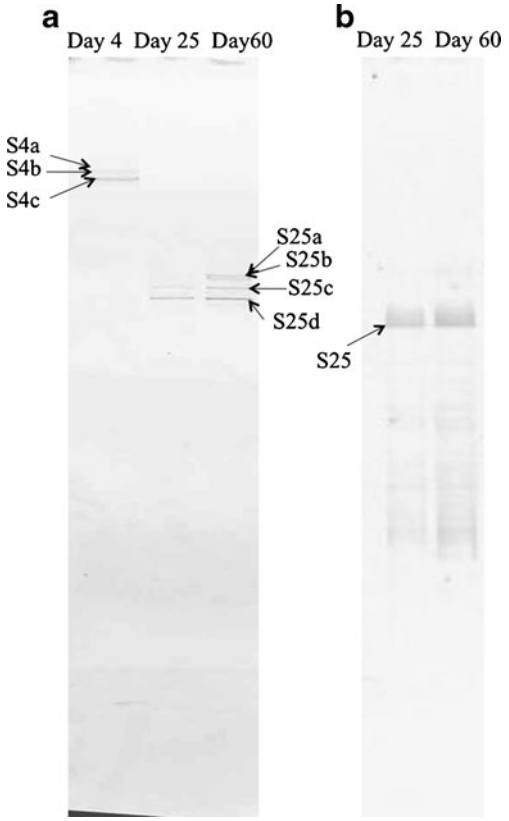

Figure 1 DGGE profiles of the compost samples for **a** AOB and **b** AOA communities. The numbered DNA bands were sequenced

Results and Discussion

Detection of AOB

PCR products for DGGE and cloning procedure were obtained only from three compost samples on days 4, 25, and 60. The DGGE profile consisted of a few DNA bands, depicting a rather simple AOB community structure (Fig. 1a). On day 4, all three sequences from the DGGE bands (S4a to S4c) were identical to a sequence originating from freshwater sediment (EU309909) [18] and were grouped into the *Nitrosomonas europaea/eutropha* cluster. The clone library analysis also revealed a dominance of *N. europaea*, and the clone sequences were the same as those of the bands from the DGGE fingerprint. This result was consistent with a previous report showing that only *N. europaea* dominated at temperatures over 60°C in compost from household wastes [21]. Additionally, the *N. europaea/eutropha* cluster has often been detected in wastewater treatment facilities [35], constructed wetlands [19], and soil-loaded swine manure [15], which are considered to be high-ammonium and -pH environments. A recent study reported that *Nitrosomonas europaea*-like *amoA* gene was detected at the surface layer of the compost pile made from cattle manure, suggesting that nitrification occurred by these AOB species [30]. Some AOB sequences obtained in this study were identical to their sequences. Our results confirmed that the *N. europaea/eutropha* cluster has a tolerance for high ammonia content as shown on day 4 [24] and the presence of a member of the *Nitrosomonas* cluster under high temperatures during cattle manure composting, as well as household waste composting [21].

The DGGE profiles from samples obtained on days 25 and 60 suggested that both samples had similar AOB communities and differed from the AOB community present in the sample from day 4. This change might be linked to a rapid decrease in ammonia concentration (from 1.998 to 0.047 gN/kg DW). All four sequenced DGGE bands of the sample from day 25 (S25a to S25d) were identical to an *amoA* gene sequence obtained from an uncultured *Nitrosomonas* spp. group (AB495028), which was also detected in cattle manure compost [30]. Eighteen of 31 sequenced clones (AB465020 to AB465022, AB465024, AB465026, and AB465028 to AB465030) had high identities (99% or 100%) when compared with the same sequence. Eleven clones (AB465023, AB465027, and AB465033) were grouped near this cluster but were more closely related to other sequences obtained from

activated sludge (GQ258534). Interestingly, this group differed from the *Nitrosomonas halophila* cluster, which is closest to cultured AOB (85% similarity). This result might be due to two potential reasons: (1) multiple gene copies or different strains of *N. halophila* or (2) a novel AOB species, because of greater than 80% identity with *amoA* genes [38]. In the former case, salt concentrations in composting material, which were revealed by an increase in electrical conductivity [29], as measured from 4.3 to 5.6 mS/cm, might be a potential factor for selection of dominant AOB species in compost. The members of *N. halophila*, which are known as alkali-tolerant organisms isolated from soda lakes [42], can tolerate up to 900 mM NaCl, whereas *N. europaea* does not have a salt requirement and can survive up to 400 mM NaCl [24]. The other case is that a novel AOB species could live in compost, and it was classified into the same cluster as another AOB sequence detected from activated sludge (Fig. 2). Compost-specific AOB might

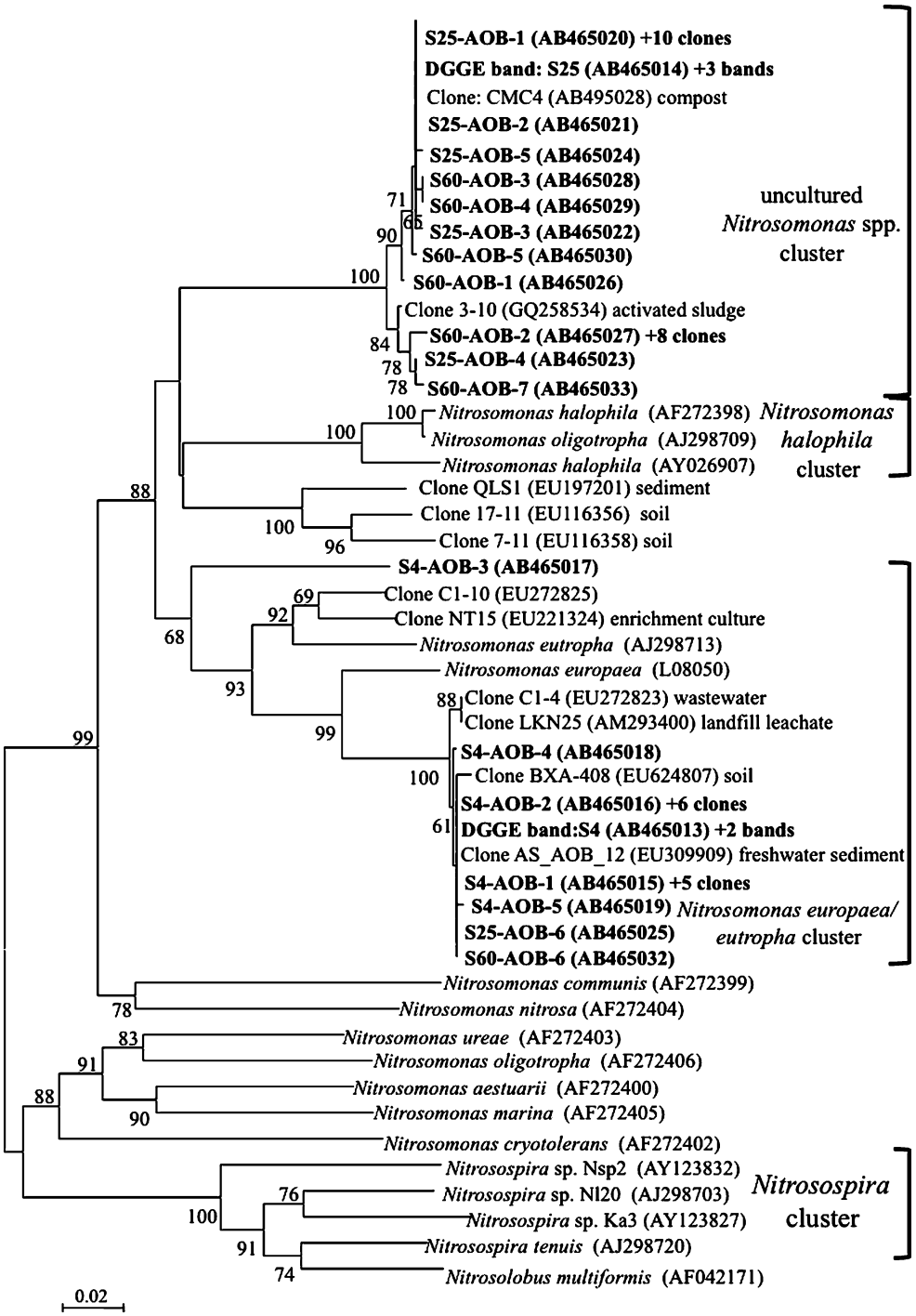

Figure 2 Phylogenetic tree of the bacterial *amoA* sequences obtained from composting materials. Boot-strap values (>60%) are indicated at the *branch points*. All sequences obtained from this study are given in *bold letters*. The *scale bar* represents 5% sequence divergence. Accession numbers are given in *parentheses*. *Numbers* following accession numbers indicate the number of bands or clones with same sequence

grow in the composting material with low ammonium instead of *N. europaea*, which is a weak competitor for ammonium [5]. Our results provide further evidence that *Nitrosomonas* spp. might act as an ammonia oxidizer in the composting of cattle manure, as well as final product [20, 30]. In soil environment, AOB community was changed by different fertilizer treatments and temperature [2]. We propose that the changes in community structure might also be caused by a shift in salt concentration, temperature, and ammonia content.

In this study, no PCR product was amplified from the sample taken on day 0. To our knowledge, the presence of AOB in cattle manure has not been investigated. Another study has shown very low numbers of AOB in fresh chicken manure, whereas large numbers of AOB were detected from chicken bed materials or swine manure that were stored for 1 week [25]. This indicates that the AOB community grew in manure that was stored at ambient temperature. AOB might not have been present in the fresh cattle manure used in our study, but rather grew after mixing with bark compost.

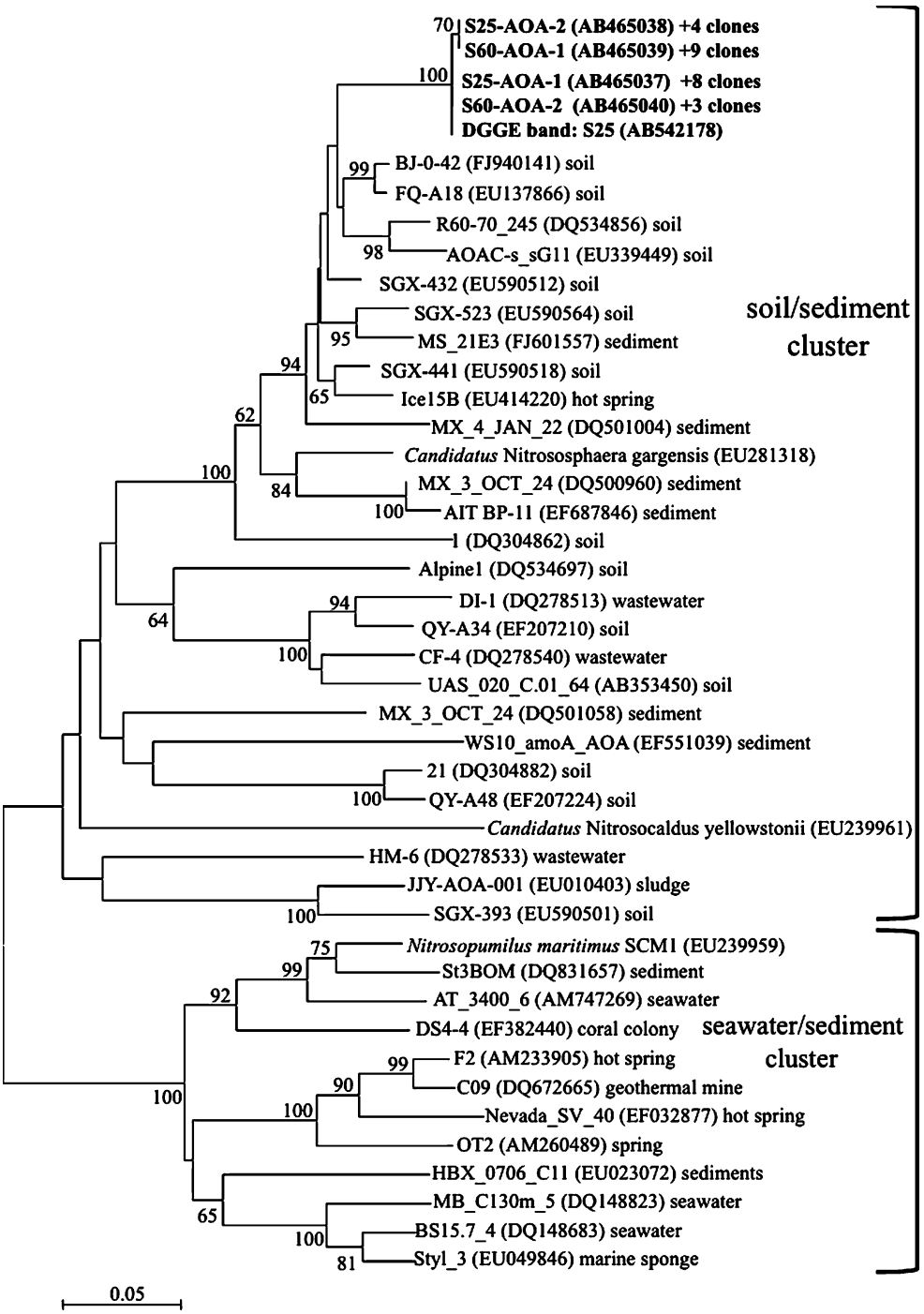

Figure 3 Phylogenetic tree of the archaeal *amoA* sequences obtained from composting materials. Boot-strap values (>60%) are indicated at the *branch points*. All sequences obtained from this study are given in *bold letters*. The *scale bar* represents 5% sequence divergence. Accession numbers are given in *parentheses*. *Numbers* following accession numbers indicate the number of bands or clones with same sequence

No members of the *Nitrosospira* cluster were detected in any of the samples, consistent with the report of only *Nitrosomonas* spp. being detected in cattle manure compost [20]. Another study also showed that *Nitrosospira* spp. dominated in compost made from green waste, bio-waste, and sewage sludge. Other studies have also reported the detection of both *Nitorosomonas* and *Nitrosospira* clusters in swine and chicken manure composts [25]. Compared with their data, cattle manure compost in this study has less N and ammonia content than swine or chicken manure compost [14, 25]. This difference in species dominance may be due to the chemical properties in original materials as described above [4]. However, further studies are needed to understand the shift in AOB community and the role of uncultured *Nitrosomonas* spp. in cattle manure compost.

Detection of AOA

PCR products for DGGE analysis and cloning procedure were amplified only from samples on days 25 and 60 with low ammonia content. Our results also showed that AOA could not be detected in the first 4 days of composting. Initial compost materials have lower ammonia levels than activated sludge, but AOA growth in compost might also be inhibited by ammonia. Previous reports concluded that significant inhibition of AOA growth occurred in hot springs with 3.08 mM ammonium [16]. Another survey of activated sludge with high ammonia content (about 1.0–3.0 mM) [51] also supported the assumption that the appearance or absence of the AOA community might be ammonia-dependent. Although the ammonia content in later stages of composting was higher than that of moderate soil studied in a previous report, the amount was similar to that in soil-inoculated swine manure [15]. Also, cultured AOA strain, *Candidatus* Nitrosopumilus martimus strain SCM1, survived with lower ammonium levels than cultured AOB strains [31]. Previous studies indicate that AOA can grow after ammonia reached the level observed in soil during the composting process. The DGGE profiles for both samples were identical, consisting of one strong band (Fig. 1b). Both DNA bands and all clones (12 clones per sample) showed relatively low identity (93%) to a soil AOA (FJ940141). These sequences were grouped into the soil/sediment cluster (Fig. 3). In this study, the AOA diversity was very low compared with that of other studies using soil samples, which had several clusters [6, 17]. Other studies indicate that the diversity is limited by high temperature or pH [33, 39]. Our results suggest that particular AOA species originating from surrounding environment such as soil accidently entered composting material and adopted the environmental changes during the composting process. For confirmation, a study on AOA community in soil environment around the facility is needed.

Determination of *amoA* Gene Copy Numbers with Real-Time PCR

Real-time PCR confirmed that AOB species survive at the high-temperature stage based on the high copy number of bacterial *amoA* genes detected (Fig. 4). Regarding the AOB community, our results confirmed a previous study that showed no PCR amplification from the initial 28 days of the process [49]. The number of archaeal *amoA* gene copies (3.2 or 3.9×10^7 copies per gram freeze-dried compost) was significantly higher than the number of bacterial *amoA* gene copies (2.2-7.2×10^6 copies per gram freeze-dried compost), which is consistent with results obtained from soil environments [3, 17]. The ratios of AOA:AOB gene copy numbers of samples taken from days 25 and 60 were 15 and 6, respectively. According to the melting curve analysis, specific products were obtained from day 4 to day 60 for AOB and days 25 and 60 for AOA, while no specific products were produced with samples from day 0 for both species and samples from day 4 for AOA.

Given that AOB on average have 2.5 *amoA* gene copies [34] and AOA have only one copy [18], the number of AOA cells may be slightly higher than that of AOB cells in the composting material. Higher archaeal *amoA* gene copy numbers have been reported in soil treated with mineral fertilizers and organic manure [17]. The compost might also provide ammonia oxidizers to soil and change their communities by supplying chemical nutrients affecting for growth of both organisms. Moreover, AOA communities are widespread in soil with various levels of pH (from acidic to neutral) or N content [1, 6, 8, 17, 27]. Our results suggest that a high number of AOA cells are present despite the alkaline conditions. However, the importance of AOB rather than AOA for ammonia oxidation in the agricultural

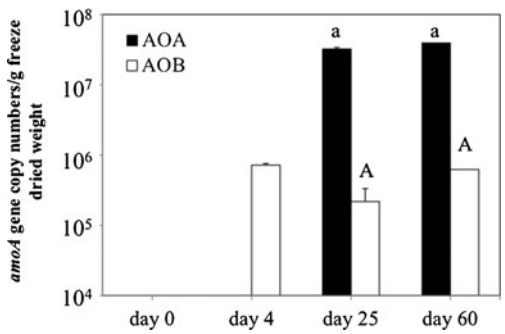

Figure 4 *AmoA* gene copy numbers for AOA and AOB. *Error bars* indicate standard deviation. Each sample was analyzed in triplicate. The sample without a bar indicates that gave non-specific PCR products. Different *letters* (a and A) indicate significant differences ($P<0.05$) determined by *t* test

soil has been reported [22]. To understand the role of AOA in ammonia oxidization during the composting process, further studies regarding potential ammonia oxidizing activities using mRNA as studied in soil [33] are needed with various types of cattle manure composts.

Conclusions

We demonstrated that not only specific AOB but also AOA communities exist in large amounts in cattle manure compost and may play important roles in ammonium oxidation during composting. The AOB community clearly develops rapidly within the first 4 days. These data indicate that AOB must be almost exclusively responsible for nitrification at high temperature, whereas, in the later stages with lower ammonia concentrations, dominant AOB species then changed with shifts in various environmental factors (e.g., salt, ammonium, and pH). Coincidentally, AOA were dominant after the temperature or ammonia concentration decreased. Our study indicates that AOA might contribute more significantly in the later stage of composting. The data also suggest that compost can be a source for bacterial and archaeal ammonia oxidizers in soil environments. The present study provides useful information for further research to understand nitrification in composting systems.

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