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Growth of Ammonia-Oxidizing Archaea and Bacteria in Cattle Manure Compost under Various Temperatures and Ammonia Concentrations

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Abstract A recent study showed that ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) coexist in the process of cattle manure composting. To investigate their physiological characteristics, liquid cultures seeded with fermenting cattle manure compost were incubated at various temperatures (37°C, 46°C, or 60°C) and ammonium concentrations (0.5, 1, 4, or 10 mM NH_4^+-N). The growth rates of the AOB and AOA were monitored using real-time polymerase chain reaction analysis targeting the bacterial and archaeal ammonia monooxygenase subunit A genes. AOB grew at 37°C and 4 or 10 mM NH_4^+-N, whereas AOA grew at 46°C and 10 mM NH_4^+-N. Incubation with allylthiourea indicated that the AOB and AOA grew by oxidizing ammonia. Denaturing gradient gel electrophoresis and subsequent sequencing analyses revealed that a bacterium related to *Nitrosomonas halophila* and an archaeon related to *Candidatus* Nitrososphaera gargensis were the predominant AOB and AOA, respectively, in the seed compost and in cultures after incubation. This is the first report to demonstrate that the predominant AOA in cattle manure compost can grow and can probably oxidize ammonia under moderately thermophilic conditions.

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Introduction

Composting is a technique that is widely used to degrade organic solid waste, such as animal manure [1, 2]. It enhances the effectiveness and handleability of organic waste as fertilizer. Animal manure compost is rich in nitrogen (N) and its application to agricultural land supplies inorganic N, which plants require for growth [3]. In the composting process, organic N in fresh manure is decomposed by microorganisms and transformed into ammonium [4]. The ammonium can subsequently be oxidized to nitrate, the principal N source for most plants, through nitrification [1, 5, 6].

Nitrification is a two-step process that involves the oxidation of ammonia to nitrite and then to nitrate. Ammonia oxidation is an important aspect of the nitrogen cycle in the composting process. Jarvis et al. [7] suggested that ammonia oxidation reduced ammonia emissions in the high-temperature phase of composting, which caused a loss in fertilizer N [8], odor, the acidification of rain and the environment [9], and the eutrophication of aquatic environments and forest ecosystems [10]. Ammonia oxidation, however, increases the risk that N elements are converted via denitrification to nitrous oxide (N_2O) [5], which is a dominant greenhouse gas. N_2O can also be emitted during ammonia oxidation at low oxygen partial pressures [10]. Thus, a better understanding of the microbiology of ammonia oxidizers in the composting process is required to reduce these gas emissions.

Given the importance of ammonia oxidizers in N dynamics, several studies have examined the community composition of ammonia oxidizers in compost. Bacterial archaeal ammonia monooxygenase subunit A genes (*amoA*) gene sequences encoding the subunit of ammonia monooxygenase (AMO), a key functional enzyme in ammonia oxidation, have been detected in fully fermented composts made from various materials, such as swine or chicken manure; these bacteria belong to the genera *Nitrosospira* and *Nitrosomonas* [4]. Another study also showed that both *Nitrosomonas* and *Nitrosospira* spp. were present throughout the composting of household waste [6]. In the final product of cattle manure composting, the ammonia-oxidizing bacteria (AOB) present were grouped into the *Nitrosomonas* lineage [11]. Furthermore, significantly more copies of the archaeal than the bacterial *amoA* gene were detected in cattle manure compost, and more ammonia-oxidizing archaea (AOA) were detected when composting was conducted under thermophilic conditions [12].

Although some information about the composition of ammonia oxidizers in compost is available, we lack data on the physiological characteristics of ammonia oxidizers in composts. In particular, suitable growth temperature and ammonium concentration are important because these factors vary widely during the composting process. Generally, the temperature in compost rises immediately to more than 60°C during the first few days then returns gradually to ambient temperatures over several months. The ammonium concentration in cattle manure compost is at least 100 mM in the early phase of composting and decreases to less than 10 mM after thermophilic conditions. Therefore, this study examined the suitable growth temperature and ammonium concentration of ammonia oxidizers in compost. Inorganic liquid cultures seeded with compost were incubated at various temperatures and ammonium concentrations, and the archaeal and bacterial *amoA* gene copy numbers and AOA and AOB communities before and after incubation were monitored. Furthermore, to investigate whether the putative ammonia oxidizers actually oxidized ammonia, growth tests were conducted in the presence of an ammonia oxidation inhibitor.

Materials and Methods

Seed Composts

Compost samples were collected from a field-scale facility owned by Tohoku University (Miyagi, Japan) in September 2009. The facility consisted of an 80-m long lane and storage pit. About 1,000 kg of dairy cattle manure and sawdust were added to the lane and mixed daily using an automatic stirrer with forced aeration for about 1 month. The material was then transferred to a pit and piled without aeration for about 1 month. For a five-replicate experiment, five samples of compost were taken from five locations within the same pile. These locations were less than 30 cm from the surface of the pile and had temperatures of 38–49°C.

Cultivation of Ammonia Oxidizers

First, 3 g of the compost samples were suspended in 30 mL of ion-exchanged water and 0.1 mL of these suspensions was inoculated into 5 mL of inorganic liquid medium. The medium was prepared as described by Krümmel and Harms [13] and contained the following chemicals (/L distilled water): 49.3 mg $MgSO_4$ $7H_2O$, 147 mg $CaCl_2$ $2H_2O$, 136.1 mg KH_2PO4, 74.4 mg KCl, various quantities of NH_4Cl, and 5.0 g $CaCO_3$. The pH was adjusted to 7.8. To investigate suitable growth temperatures and ammonium concentrations, the liquid cultures were incubated at various temperatures (37°C, 46°C, or 60°C) and ammonium concentrations (0.5, 1, 4, or 10 mM NH_4^+-N) for 2 weeks. Five samples were cultured under each condition, resulting in a total of 60 cultures.

Nitrite and Nitrate Concentrations

To estimate the ammonia oxidation activity in each culture, nitrite and nitrate concentrations were measured before and after the 2-week culturing period using ion chromatography (ICS-1000, ICS-2000; Dionex, Osaka, Japan) [14].

Quantification of *amoA* Gene Copies

DNA from the culture samples was extracted using a Power Soil Extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was then subjected to real-time polymerase chain reaction (PCR) analysis to determine the bacterial and archaeal *amoA* gene copy numbers as described previously [15]. Real-time PCR was conducted with SYBR Premix Ex *Taq* II (TaKaRa Bio, Tokyo, Japan) using primer sets amoA1F(5′-GGG GTT TCT ACT GGT GGT-3′)/amoA2R(5′-CCC CTC KGS AAA GCC TTC TTC-3′) [16] for bacterial *amoA* genes and AOA23F(5′-ATG GTC TGG CTW AGA CG-3′)/AOA616R(5′-GCC ATC CAT CTG TAT GTC CA-3′) [17] for archaeal *amoA* genes. The analyses were performed using a Chromo4™ Four-Color Real-Time Detector added to a DNA Engine® cycler (Bio-Rad, Hercules, CA, USA). To obtain a standard curve for each *amoA* gene product, the PCR products amplified from cattle manure compost were used after purification with a MinElute® PCR purification kit (Qiagen, Hilden, Germany). The DNA concentrations of the products

were determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the copy numbers of the *amoA* genes were calculated. A range of 10–1,000,000 template copies was used to generate the standard curves. Standard curves from each run were analyzed, with $r^2>0.99$. A *t* test was performed using the Excel analytical toolbox (Microsoft, Redmond, WA, USA) to evaluate the difference between *amoA* gene copy numbers before and after incubation.

Growth Test with an Ammonia Oxidation Inhibitor

To investigate the relationship between *amoA* gene copy number and the occurrence of ammonia oxidation, changes in *amoA* gene copy numbers were monitored after adding allylthiourea (ATU) to the medium to obtain a final concentration of 100 μM. ATU is a specific ammonia oxidation inhibitor that chelates copper on active AMO sites, but has no reported effect on the oxidation of substrates, except NH_4^+ [18–20].

Culturing and analytical procedures were as described above. Incubation temperatures were 37°C and 46°C, and the ammonium concentration was 10 mM NH_4^+-N. The cultures were performed in triplicate.

AOB and AOA Communities

The AOB and AOA communities in a seed compost sample and five replicate cultures after incubation were analyzed using specific PCR amplification followed by denaturing gradient gel electrophoresis (DGGE) and sequencing of the *amoA* gene, as described by Yamamoto et al. [12], except that the samples were not freeze-dried before DNA extraction. Briefly, DNA extracted from a sample was amplified with PCR using Ex *Taq*™ (TaKaRa Bio) in an iCycler (Bio-Rad), using the primer set amoA1F/amoA2R [16] for AOB community analysis and AOA23F/AOA616R [21] for AOA community analysis. The forward primer amoA1F contained GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) [22]. DGGE was performed using 8% polyacrylamide gels with a denaturing gradient of 25–60% for AOB community analysis and 6% polyacrylamide gels with a denaturing gradient of 20–60% for AOA community analysis. After staining the gels with ethidium bromide for 10 min and washing for 5 min with distilled water, the DNA bands were excised and transferred to 1.5-mL tubes containing 100 μL TE buffer. Part of each aliquot (1 μL) was used as the template for PCR to sequence the DNA bands using primer sets without the GC clamp. The PCR products were then purified with ExoSAP®-IT (USB, Cleveland, OH, USA) following the manufacturer's instructions. The purified products were sequenced using a BigDye® Terminator Cycle Sequencing kit (v. 1.1; Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The products were analyzed using an ABI PRISM 3130xl autosequencer (Applied Biosystems). The analyzed sequences were compared with sequences registered in the database of the DNA Databank of Japan using the Basic Local Alignment Search Tool system (http://blast.ddbj.nig.ac.jp/top-j.html).

Results and Discussion

Ammonia Oxidation Activity

At 37°C, large amounts of nitrite and nitrate accumulated in the 4.0 and 10 mM NH_4^+-N cultures (Fig. 1). Accumulations of nitrite and nitrate were also observed at 46°C,

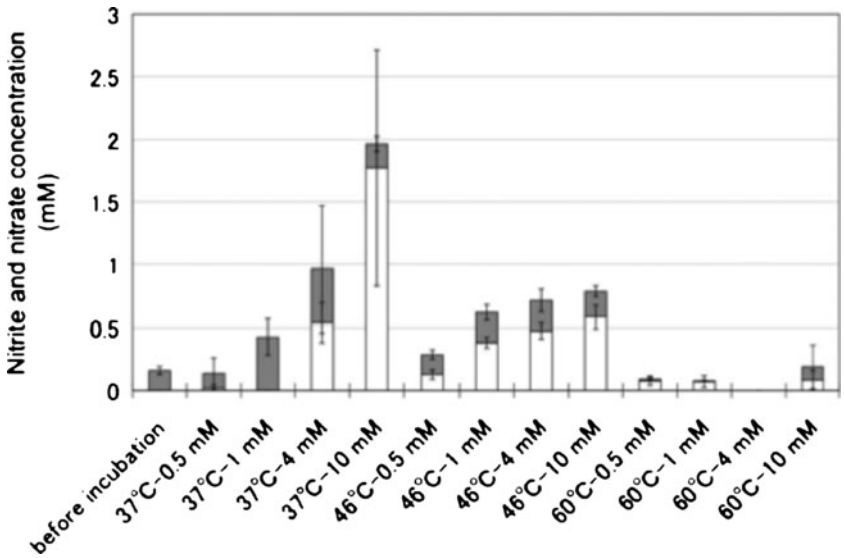

Figure 1 Nitrite (*white bars*) and nitrate (*gray bars*) concentrations in compost-seeded cultures after incubation at different temperatures and ammonia concentrations. The *bar on the far left* shows the nitrate concentration before incubation. *Error bars* indicate standard deviations

suggesting the existence of moderately thermophilic ammonia oxidizers. At 60°C, little nitrite or nitrate accumulated. Generally, composting temperatures rise immediately to more than 60°C during the first few days, then return gradually to ambient temperatures over several months. Temperature also varied at different locations within the compost pile [5]. These results suggest that ammonia oxidation occurred not only under mesophilic conditions, but also under moderately thermophilic conditions during composting.

Bacterial and Archaeal *amoA* Gene Copy Numbers

Although the archaeal *amoA* copy numbers after incubation were similar to those before incubation, the bacterial *amoA* copy numbers increased significantly at 37°C and 4 and 10 mM NH_4^+-N (Fig. 2). Conversely, the archaeal *amoA* copy numbers increased significantly at 46°C and 10 mM NH_4^+-N, indicating that AOA in the compost grew under moderately thermophilic conditions. The bacterial *amoA* gene copy numbers decreased in the 46°C cultures, in which the AOB cells likely suffered serious heat stress. At 60°C, both the bacterial and archaeal *amoA* gene copy numbers decreased at all ammonium concentrations. Nitrate and nitrite concentrations (Fig. 1) and *amoA* copy numbers (Fig. 2) indicated that the growth temperature limit of the ammonia oxidizer in the compost was lower than 60°C.

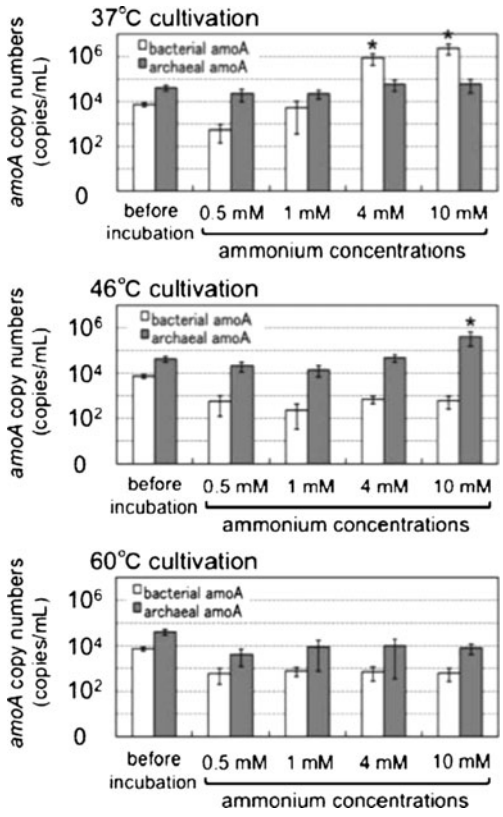

Figure 2 Bacterial and archaeal *amoA* copy numbers in cultures after incubation. The *bar on the far left* shows the *amoA* copy number before incubation. *Error bars* indicate standard deviations. *Asterisks* indicate *amoA* copy numbers that are significantly higher than before incubation ($p<0.05$)

Growth Test with an Ammonia Oxidation Inhibitor

The effects of ATU on the increase in bacterial *amoA* gene copy numbers in cultures at 37°C and 10 mM NH_4^+-N, and on the increase in archaeal *amoA* gene copy numbers in cultures at 46°C and 10 mM NH_4^+-N, were investigated. No nitrite or nitrate accumulated in the cultures containing ATU, indicating that ammonia oxidation was strongly inhibited (Fig. 3). Bacterial and archaeal *amoA* gene numbers did not increase in cultures with ATU, but did increase significantly in the positive controls lacking ATU (Table 1). ATU has been reported to selectively inhibit ammonia oxidizers at a concentration of 86 μM [23] and our results suggest that the growth of AOB and AOA depends on ammonia oxidation. Yamamoto et al. [12] reported more AOA than AOB in 25- and 60-day-old compost, but could not determine whether the AOA was oxidizing ammonia. In this study, AOA that grew at 46°C oxidized ammonia. During the composting process, AOB oxidized ammonia under mesophilic conditions, and AOA oxidized ammonia under thermophilic conditions.

AOB and AOA Communities

Several lanes of the DGGE profile for the AOB communities were blank (46°C, 0.5 and 1 mM NH_4^+-N; 60°C, 0.5, 1, 4, and 10 mM NH_4^+-N) because no amplification of bacterial *amoA* genes was observed in the DNA samples extracted from these cultures (Fig. 4). These amplification failures likely resulted from low bacterial *amoA* gene copy numbers in these cultures (Fig. 2) and the reduction in amplification efficiency caused by the attached GC clamp [24].

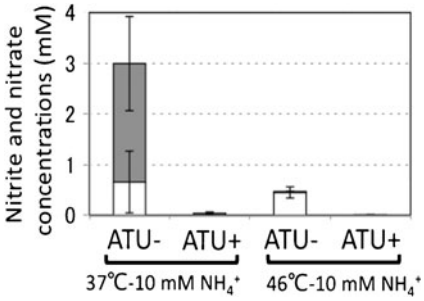

Figure 3 The effect of adding ATU on ammonia-oxidizing activity. Nitrite (*white bars*) and nitrate (*gray bars*) concentrations in cultures after incubation are shown. *ATU−* positive controls that lacked ATU. *ATU+* cultures to which 100 μM of ATU was added

Table 1 Effect of ATU on bacterial and archaeal *amoA* gene copy numbers

	ATU*	Before incubation	After incubation
Bacterial *amoA* copy number at 37°C-10 mM	−	$3.1 \times 10^4 \pm 1.3 \times 10^4$	$2.1 \times 10^6 \pm 6.1 \times 10^5$*
	+		$2.0 \times 10^4 \pm 7.3 \times 10^3$
Archacal *AmoA* copy number At 46°C-10 mM	−	$4.8 \times 10^4 \pm 1.3 \times 10^4$	$4.1 \times 10^5 \pm 6.1 \times 10^4$*
	+		$4.5 \times 10^4 \pm 1.8 \times 10^4$

The *amoA* copy numbers in cultures before and after incubation with and without ATU are shown

(−) positive controls that lacked ATU, (+) indicates cultures to which 100 μM of ATU was added

*$p<0.05$, significant differences against the value of before incubation

The band patterns, ignoring very weak bands, showed that the predominant AOB and AOA in seed compost and in cultures after incubation were each a single species. The predominant species did not change in any culture, including those in which the bacterial and archaeal *amoA* copy numbers increased significantly, indicating that the predominant AOB and AOA in the seed composts grew in the inorganic medium under the respective suitable conditions.

All four bacterial *amoA* sequences from the DGGE bands were identical to sequences originating from cattle manure compost from other facilities (AB495028, AB465020, AB465027, and AB465033) [5, 12], and were similar to the *amoA* sequence of *Nitrosomonas halophila* (87% identity, AY026907) [25]. In addition, all four archaea *amoA* sequences from the DGGE bands were identical to sequences originating from cattle manure compost from another facility (AB465038 and AB465039) [12] and were similar to the *amoA* sequence of *Candidatus* Nitrososphaera gargensis (87% identity, EU281318) [26].

Figures 2 and 4 show that the copy numbers of the *Candidatus* Nitrososphaera gargensis-like *amoA* sequence increased in the cultures at 46°C, whereas the copy numbers of the *N. halophila*-like *amoA* sequence increased in the cultures at 37°C. These results demonstrate that archaea related to *Candidatus* Nitrososphaera gargensis originating from cattle manure compost can grow under moderately thermophilic conditions. *Candidatus* Nitrososphaera gargensis has been found in a hot spring and can grow at 46°C [26]. Growth under moderately thermophilic conditions may be a common characteristic of this related group of archaea. Although Shimaya et al. [17] reported that ammonia oxidation occurred in liquid cultures at 50°C inoculated with cattle manure compost, the ammonia oxidizers in the cultures were not identified. Yamamoto et al. [12] also reported a greater archaeal than bacterial *amoA* gene copy number on day 25 at a temperature of 49.2°C. Our results also show that archaea related to *Candidatus* Nitrososphaera gargensis could grow at 46°C, whereas bacteria related to *N. halophila* could not. In addition, ATU

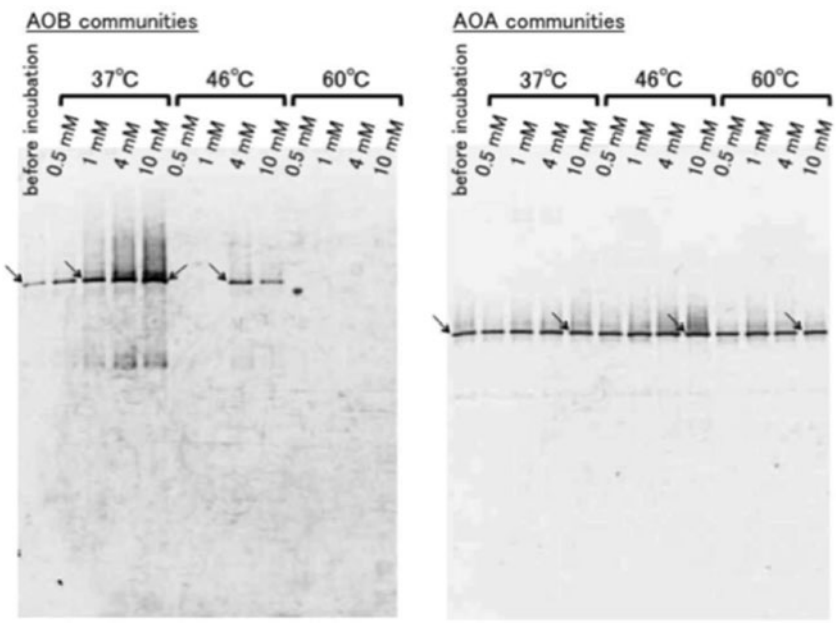

Figure 4 DGGE profiles for AOB and AOA communities in the cultures after incubation at various temperatures and ammonia concentrations. The lanes on the far left show the profiles before incubation. The bands indicated by *arrows* were sequenced

experiments showed that the archaeon related to *Candidatus* Nitrososphaera gargensis oxidized ammonia at 46°C. These results and previous reports suggest that AOA can oxidize ammonia under moderately thermophilic conditions during the composting process, and that the archaeon related to *Candidatus* Nitrososphaera gargensis may play an important role in this oxidization. Further studies of in situ ammonia-oxidizing activity, such as mRNA assays targeting *amoA*, are needed to clarify the role of AOA in ammonia oxidization during composting.

The archaeon related to *Candidatus* Nitrososphaera gargensis grew at 10 mM NH_4^+-N. Hatzenpichler et al. [10] reported that *Candidatus* Nitrososphaera gargensis isolated from a hot spring was partially inhibited by 3.08 mM NH_4^+-N at pH 7.8. However, the archaeon related to *Candidatus* Nitrososphaera gargensis that was present in our compost was not inhibited by 10 mM NH_4^+-N, indicating that it has greater ammonia tolerance than *Candidatus* Nitrososphaera gargensis isolated from a hot spring. Although the ammonium concentrations in compost generally decrease during the composting process, around 5 mM of ammonium remained in cattle manure compost matured for over 2 months. The AOA detected in this study were adapted to the ammonium concentrations in mature compost.

During composting, ammonia volatilization was higher under thermophilic conditions than under mesophilic conditions. In this study, AOA detected in the compost could oxidize ammonia under moderately thermophilic conditions. In a future study, we plan to produce an enriched AOA culture and to augment the thermophilic condition of the compost to decrease ammonia volatilization.

Conclusions

This study demonstrated that a bacterium related to *N. halophila* and an archaeon related to *Candidatus* Nitrososphaera gargensis, which dominated the AOB and AOA in the compost, respectively, were mesophilic and moderately thermophilic organisms, respectively. Neither of these organisms was inhibited by 10 mM NH_4^+-N at pH 7.8 and both adapted to the ammonium concentrations in mature compost. These results support the important contribution of AOA to ammonia oxidation during cattle manure composting under moderately thermophilic conditions.

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