Analysis of Ammonia Monoxygenase and Archaeal 16S rRNA Gene Fragments in Nitrifying Acid-Sulfate Soil Microcosms

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The present study describes the occurrence of a unique archaeal ammonia monoxygenase alpha subunit (amoA) gene in nitrifying acid-sulfate soil microcosms at pH 3.5. The soil was collected from an abandoned paddie field in Thailand. Microcosms were incubated in the dark at 30°C for 372 days with the following three treatments: addition of ammonium sulfate solution once a month (I) or once a week (II), and addition of only sterilized water (III). A quantitative PCR analysis revealed an increase in abundance of the archaeal amoA gene in microcosms soils in which nitrate concentrations increased after incubation. A phylogenetic analysis indicated a predominance of the novel gene, and a predominance of a betaproteobacterial amoA gene affiliated with the genus Nitrosospira. A 16S rRNA gene-based PCR assay revealed that crenarchaeotic Group I.1d was predominant among the Crenarchaeota in microcosms. These results suggest the presence of ammonia-oxidizing archaea corresponding to the unique amoA lineage in nitrifying acid-sulfate soil microcosms at pH 3.5.

Key words: ammonia-oxidizing archaea, acid-sulfate soil, nitrification, low pH, ammonia-oxidizing bacteria

Non-thermophilic crenarchaeotic 16S rRNA genes can be detected in the soil of terrestrial ecosystems (30, 42). The phylogenetic groups II.b and II.c within the Crenarchaeota seem to be predominant in soils (18, 21, 32, 34, 35, 37). An environmental metagenomic study has suggested that ammonia-oxidizing archaea (AOA) within Group II.b exist in soil (50). Furthermore, molecular ecological approaches based on the archaeal ammonia oxygenase alpha subunit (amoA) gene have revealed a predominance of AOA among ammonia-oxidizing prokaryotes in several types of soils, suggesting that AOA may play an important role in nitrification within terrestrial ecosystems (2, 17, 25, 26). Recently, it has been demonstrated that archaeal amoA gene expression in soil was much higher than bacterial amoA expression at a low pH, but decreased as the pH increased from 4.9 to 7.5 (33). However, relatively little is known about AOA in acid soils, such as acid-sulfate soil with a pH of 3.5.

Nitrification is a two-step process, consisting of the conversion of ammonia to nitrate (NO3−) and the subsequent conversion of nitrite to nitrate (NO2−). The two processes are carried out by ammonia-oxidizing and nitrite-oxidizing microorganisms, respectively. The optimal pH range for the growth of isolated autotrophic ammonia-oxidizing bacteria (AOB) in a liquid culture is 7.0 to 8.5 (4). Nitrification, however, has occurred in acid soils (pH<4.5) of tea plantations (16, 54), forests (28, 36, 49) and heaths (8). In laboratory cultures, nitrification at low pH has been observed with aggregated AOB (4, 9, 13) and in the presence of an acid-tolerant nitrite-oxidizing bacterium (10). In addition, it has been reported that ammonia was oxidized by AOB at pH 4.0 through hydrolysis of urea (7). Molecular ecological approaches based on the 16S rRNA gene have revealed a predominance of Nitrosospira cluster 2 among the betaproteobacterial AOB in acid soil maintained at pH 4.2 (44, 45), and in acid forest soil with a pH of 2.7–4.3 (24). Moreover, it has been suggested that nitrification in acid soils might be not only due to autotrophic ammonia oxidizers but also due to heterotrophic ammonia oxidizers (3, 46).

The estimated worldwide extent of acid sulfate soils is approximately 12 million hectares (52), of which more than 5 million hectares are in Southeast Asia (51). In Thailand, the utilization of such areas for agriculture creates a high risk of sulfatic acidification (20). Soils of peat swamps in Southeast Asia have developed on marine clay sediments deposited during periods of high sea level and contain high concentrations of pyrite (FeS2). Accordingly, the pH value of acid sulfate soils drops below 4, and sometimes even below 3, due to the oxidation of pyrite by air (11). In such acid sulfate soils where only acid-tolerant plants are able to grow, little is so far known about the microbial community that contributes to nitrification.

In this paper, we report a unique archaeal amoA gene in nitrifying acid-sulfate soil microcosms with a pH of 3.5. We measured the concentrations of ammonium, nitrite and nitrate, and quantified the abundance of the crenarchaeotic and betaproteobacterial amoA genes in microcosms. Furthermore, we characterized the microbial community structure of AOA and AOB based on those amoA genes, and investigated the archaeal community structure based on 16S rRNA genes in microcosms to identify the predominant Crenarchaeota in nitrifying acid-sulfate soil microcosms.

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The total concentration of NH₄⁺ adding sterilized distilled water to replace loss through evaporation was retained at 40–50% by weighing pots three times a week and enrichment of AOA and AOB. The water content of microcosms included the following three treatments: addition of 5 mL of 40 mM ammonium sulfate once a week (two pots, ASA-M1-1 and ASA-M1-2), addition of 5 mL of 40 mM ammonium sulfate once a month (two pots, ASA-W1-1 and ASA-W1-2), addition of 5 mL of sterilized distilled water (one pot, SDW). The ammonium sulfate solution was added to soils with different frequencies to enhance the enrichment of AOA and AOB. The water content of microcosms was retained at 40–50% by weighing pots three times a week and adding sterilized distilled water to replace loss through evaporation. The total concentration of NH₄⁺ added to soil during the incubation for microcosms ASA-M1 and ASA-W1 was at about 230 μg [wet soil g⁻¹] and 1000 μg [wet soil g⁻¹], respectively. Given that the rate of conversion of NH₄⁺ into NO₂⁻ by AOA is assumed to be lower than that by AOB (15, 22) and that NO₂⁻ is absorbed by soil, the NH₄⁺ concentrations (about 3 mM in pore water) used in this study were considered suitable for the enrichment of AOA in acid-sulfate soil microcosms. Microcosms were incubated at 30°C in a dark room for 372 days. After incubation, soils were placed in a sterile plastic tube and stored at −80°C until further use. After measurement of the pH of incubated soil, concentrations of nitrite and nitrate were determined as described below.

Chemical analyses
Soil pH prior to and after incubation was determined with a 1:2.5 ratio of soil (wet weight) and deionized water (volume) using a twin pH meter (B-212, Horiba, Kyoto, Japan). After filtration of the water containing soil, we measured concentrations of nitrite and nitrate with an ion chromatography system-1000 attached to an UltiMate 3000 Variable Wavelength Detector (Dionex, Sunnyvale, CA, USA). The quantitative limits of NO₂⁻ and NO₃⁻ concentrations were 0.6 and 0.8 μg [wet soil g⁻¹], respectively. After the shaking of 0.5 g of soil mixed with 5 mL of ammonium extraction solution (1 M KCl and 0.01 M HCl) for 30 min, the concentration of ammonium in soil was determined colorimetrically by a modified indophenol reaction (19). The quantitative limit of the NH₄⁺ concentration was 1.8 μg [wet soil g⁻¹].

Amplification of amoA and 16S rRNA genes and cloning
Nucleic acids were extracted from 0.5 g of soil with an ISOIL for Beads Beating Kit (Nippon Gene, Toyama, Japan) following the manufacturer’s instructions, after vigorous stirring of the tube containing the lysis solution and beads for 45 s and incubation of the tube at 65°C for 1 h. Nucleic acids extracted in 20 μL of Tris-EDTA buffer were stored at −20°C until PCR. Partial amoA and archaeal 16S rRNA gene fragments were amplified with Takara Taq EX Tag DNA polymerase (Takara Bio, Otsu, Japan). The total volume of PCR mixture was 50 μL including 5 μL of 10× EX Taq buffer, 250 μM each of deoxynucleoside triphosphate, 50 pmol each of primer, and 1.25 U of EX Taq DNA polymerase. The nucleotide primers were amoA19F (5′-ATGGTCTCGGCTWAGACG-3′) (25) and amo643R (5′-TCCCATCTTGACGCGGCCATCAA-3′) (50) for the crenarchaeotic amoA gene, amoA-1F (5′-GGGGTTTCTACGTGTTG-3′) and amoA-2R (5′-GCCCTCGSAAAGCCTTCTT-3′) for the betaproteobacterial amoA gene (41), amoA-3F (5′-GCTGAAGTGGYTAACMG-3′) and amoA-4R (5′-GCTAGGCTTGCCGTTG-3′) for the gammaproteobacterial amoA-amoB gene (38), and ARC344F (5′-ACCGGGGGYGAGCAGGCGCGG-3′) and ARC915r (5′-GTGCCTCCCCGCACATCT-3′) for the archaeal 16S rRNA gene (40). Amplification was carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA, USA) and a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The following protocol was used for archael amoA: an initial denaturation step at 94°C for 5 min and then 35 cycles at 94°C for 30 s, 51°C for 30 s and 72°C for 45 s, with a final 5-min extension at 72°C. For betaproteobacterial amoA, there was an initial denaturation step at 94°C for 5 min and then 35 cycles at 94°C for 30 s, 57°C for 1 min and 72°C for 45 s, with a final extension at 72°C for 5 min. When no specific band was observed, re-amplification of the PCR products with 35 cycles was performed with 2 μL of the amplicons as template DNA to obtain betaproteobacterial amoA fragments. For gammaproteobacterial amoA, there was an initial denaturation step at 94°C for 5 min and then 35 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 45 s, with a final extension at 72°C for 2.25 min. For the archaeal 16S rDNA gene, an initial denaturation step at 94°C for 5 min was followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 40 s, with a final extension at 72°C for 10 min. After electrophoresis, PCR products were visualized on an agarose gel stained with ethidium bromide, and extracted with a QIAGEN II Gel Extraction Kit (Qiagen, Valencia, CA, USA). Amplified gene fragments were cloned into the vector pCR2.1-TOPO with a TOPO TA PCR cloning kit (Invitrogen, Carlsbad, CA, USA). White colonies were grown overnight at 37°C on fresh LB broth agar plates with kanamycin. The inserts were amplified by direct PCR from a single colony with the vector-specific primers M13F and M13R. After PCR products were electrophoresed again in an agarose gel to check the length of the bands, amplicons were purified with a QIAquick PCR purification kit (Qiagen) prior to sequencing.

Fluorescent quantitative PCR
Archael and betaproteobacterial amoA genes in soils were quantified by real-time PCR in a LightCycler system 350S (Roche Diagnostics, Mannheim, Germany) with SYBR Green I as a fluorescent dye using the primers amoA19F/amo643R and amoA-1F/amoA-2R. Each reaction was performed in a 20-μL volume containing 2 μL of template DNA solution, 0.2 μL μL⁻¹ of bovine serum albumin, 0.2 μL of each primer, and 10 μL of SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio). Cycling conditions were as follows: for archael amoA, an initial denaturation step at 95°C for 30 s and then 40–45 cycles at 95°C for 15 s, 55°C for 15 s and 72°C for 1 min. The fluorescence intensity was measured at 72°C. For the betaproteobacterial amoA gene, an initial denaturation step at 95°C for 30 s was followed by 45 cycles of 95°C for 15 s, 57°C for 15 s and 72°C for 30 s. The fluorescence intensity was measured at 72°C. After each run, the amplicon was visualized on an agarose gel to confirm specific product bands of the expected size as described before. Standardization of amoA templates was performed with a dilution series (3.8×10⁻⁸–7.5×10⁻⁹ copies μL⁻¹) of a plasmid (pCR2.1-TOPO, Invitrogen) containing the amoA clone of clone ASA_G32432 obtained in this study and generated with primers amoA19F/ amo643R, and with a dilution series (1.7×10⁻⁸–1.7×10⁻¹ copies μL⁻¹) of a plasmid containing the amoA of Nitrosospira multiformis ATCC25196 generated with primers amoA-1F/amoA-2R, respectively.

Sequencing and phylogenetic analysis
M13F/M13R PCR products were sequenced with a BigDye v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and vector primers M13F and M13R on a Model 3130xl DNA sequencer (Applied Biosystems). Similarities of amoA and 16S rRNA gene sequences were investigated in the databases of the National Center for Biotechnology Information and the DNA Data Bank of Japan using BLAST (5) and FASTA programs (27). Sequence similarity was determined with MEGA4 software (47).
Operational taxonomic units (OTUs) were defined as groups of sequences which differed by ≤3% from the nucleotide sequences. Deduced amino acid sequences for environmental amoA genes were determined from 604 bp of the AOA and 452 bp of the betaproteobacterial AOB nucleotide sequence. The nucleotide and amino acid sequences were manually aligned using the CLUSTAL W program in MEGA4 and Clustal X version 1.83 (48). Phylogenetic inferences were calculated from confidently aligned regions of homologous proteins and nucleic acid sequences using MEGA4 and the MOLPHY v2.3h3 package (1). Neighbor-joining and maximum parsimony bootstrap values were calculated from 1,000 resampled data sets using MEGA4. The maximum-likelihood bootstrap value was calculated from 100 resampled data sets using MOLPHY.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey tests were used to determine significant differences.

Nucleotide sequence accession numbers

The amoA and 16S rRNA gene sequences were submitted to DDBJ/EMBL/GenBank and have been assigned the following accession numbers: AB427052, AB427053, AB427055, AB457631 to AB457633 (amoA genes) and AB427062 to AB427064, AB427077 to AB427084 (16S rRNA genes).

Results and Discussion

Ammonium, nitrite, nitrate concentrations and pH of soil microcosms

The concentration of NH$_4^+$ in PFS prior to incubation was 2.9±2.2 (µg [wet soil g]$^{-1}$). After a 372-day incubation, the concentration of NH$_4^+$ in soil of microcosms ASA-M1-1, ASA-M1-2, ASA-W1-1, ASA-W1-2, and SDW was 22.1±16.4, 5.9±0.7, 12.6±21.8, 2.7±0.6, and 2.8±0.4 (µg [wet soil g]$^{-1}$), respectively. NO$_3^-$ was detected in ASA-M1-1 and SDW at 1.6±0.9 and 1.7±0.3 (µg [wet soil g]$^{-1}$) after the incubation, respectively. A difference in NO$_3^-$ concentrations of soil among the microcosms was observed after the 372-day incubation (Fig. 1). The NO$_3^-$ concentrations of ASA-M1-1, ASA-M1-2, and ASA-W1-1 increased significantly after the incubation ($P$=0.0001), and those of ASA-W1-2 and SDW also increased, though not significantly. The pH of the soil, at pH 3.3–3.5, was not significantly changed after the incubation. These results therefore demonstrate that nitrification occurred in the acid-sulfate soil microcosms at a pH of 3.3–3.5 during incubation.

Quantitative analysis of the amoA gene

The abundance of archaeal amoA increased after incubation in the microcosms ASA-M1-1, ASA-M1-2 and ASA-W1-1 (Fig. 2), although not significantly ($P$=0.051, 0.339 and 0.094 for ASA-M1-1, ASA-M1-2 and ASA-W1-1, respectively). The abundance of archaeal amoA in the microcosm SDW increased significantly after incubation ($P$<0.0001). The abundance of archaeal amoA was ten times higher than that of betaproteobacterial amoA for ASA-M1-1 and ASA-M1-2. The archaeal amoA gene copy numbers (1.3×10$^6$ copies [gram of wet soil]$^{-1}$) in this study were lower than those in previously reported soils, e.g., fertilized pH 3.7–6.0 soils in China (4×10$^6$–9×10$^7$ copies [gram of dry soil]$^{-1}$) (17), pH 5.5–7.3 soils in Europe (7×10$^6$–1×10$^7$ copies [gram of dry soil]$^{-1}$) (25), semiarid soils in USA (10$^7$–10$^8$ copies [gram of dry soil]$^{-1}$) (2), and grassland soil in France (1×10$^6$–6×10$^6$ copies [gram of dry soil]$^{-1}$) (26). In contrast, an increase in abundance of the betaproteobacterial amoA gene was observed after incubation in the microcosms ASA-W1-1 (N=3, $P$=0.042) and ASA-W1-2 (N=1). The abundance of archaeal amoA and NO$_3^-$ concentration in soil showed a positive correlation ($r$=0.739), although it was not significant ($P$=0.09). In contrast, no correlation was observed between the abundance of the betaproteobacterial amoA gene and NO$_3^-$ concentration ($r$=−0.283, $P$=0.65). This result is attributed to the disappearance of NO$_3^-$ with the production of N$_2$ and N$_2$O from NO$_3^-$ through abiological reaction at low pH (53) and the production of N$_2$O through AOB in acid soil (6, 29).

Phylogenetic analysis of archaeal amoA

We sequenced 11, 12, 9 and 6 clones, and obtained 1, 1, 1 and 1 OTU from the ASA-M1-1, ASA-W1-1, ASA-W1-2

![Fig. 1](image1.png)

**Fig. 1.** Concentration of nitrate in acid-sulfate soil microcosms after a 372-day incubation. PFS, paddy field soil prior to incubation; microcosms ASA-M1-1 and ASA-M1-2, treated with ammonium sulfate once a month; ASA-W1-1 and ASA-W1-2, treated with ammonium sulfate once a week; SDW, treated with only distilled water. Means of three replicates are presented with standard errors. Values with different letters differ significantly with $P$<0.05.

![Fig. 2](image2.png)

**Fig. 2.** Abundance of archaeal and betaproteobacterial amoA genes in acid-sulfate soil microcosms after a 372-day incubation. Means of three replicates are presented with standard errors. Means of two replicates (betaproteobacterial amoA genes of microcosms ASA-M1-1, ASA-M1-2 and ASA-W1-2) are presented without standard errors. ND indicates a value below the quantitative limit for archaeal or betaproteobacterial amoA genes.
Unique Archaeal amoA Gene

and SDW libraries, respectively. The sequence similarity among these clones was 99.2–100%. The novel archaeal amoA clones in ‘cluster ASS’ represented a separate but associated lineage with several amoA clones retrieved from fertilized soil in China (17), estuary sediment from the US (12), and acid soil (pH 4.5) from the US (14) in ‘clusters I and II’ (Fig. 3). Based on the FASTA program, the designated clones ASA-M1-1_G32432, ASA-W1-1_G32955, ASA-W1-2_G32968 and SDW_G32449 were found to be less closely related to environmental clones (>83% nucleotide similarity). These results clearly indicated the low diversity of AOA in the soil microcosms, suggesting the presence of AOA corresponding to the unique amoA lineage in nitrifying acid-sulfate soil microcosms at pH 3.5.

The diversity of archaeal amoA was lower for acid-sulfate soil than for other soils previously reported (12, 17). Recent studies of lower pH soils (pH 4.5–5.1) indicated that frequently detected archaeal amoA clones such as AOAB_sA01 and AOAC-u_sF08 fell into ‘clusters I and II’ (14, 33) (Fig. 3). It has been reported that the change in a predominantly crenarchaeotic community structure from Crenarchaeota Group I.1b to Group I.1c was due to the influence of soil maturation (lower pH and higher in nutrients) (14, 31). It is therefore likely that the low diversity of archaeal amoA in the incubated PFS (pH 3.3–3.5) is attributable to differences in soil pH as well as nutrient and/or soil water content.

Phylogenetic analysis of bacterial amoA

We sequenced 25 and 7 clones, and obtained 1 and 1 OTU from the ASA-W1-1 and SDW libraries, respectively. The sequence similarity among those clones was 97.8–100%. All betaproteobacterial amoA clones belonged to the Nitrosospira (39) (Fig. 4). The designated clones ASA-W1-1_G37487 and SDW_G37346 were closely related to Nitro-
sospira multiformis ATCC25196’ (similarity values, 99.8% and 99.3%, respectively) and corresponded to *Nitrosospira* cluster 3 based on the 16S rRNA gene-phylogenetic tree. The phylogenetic analyses of bacterial *amoA* genes similarly showed a low level of diversity and domination by beta-proteobacterial AOB within *Nitrosospira* cluster 3 that were most common in fertilized agricultural soils (17, 23). This result was not consistent with a previous study that detected several phylotypes within *Nitrosospira* cluster 3 in pH 3.7–5.8 fertilized soils (17) or with others that detected *Nitrosospira* cluster 2 as frequent phylotypes in acid soils (24, 44, 45).

**Phylogenetic analysis of archaeal 16S rRNA gene**

We sequenced 24 and 20 clones obtaining 6 and 4 OTU from the ASA-M1-1 and SDW libraries, respectively. The frequency of crenarchaeotic clones among all archaeal clones was 50% for ASA-M1-1 and 15% for SDW. Most of the crenarchaeotic 16S rRNA gene sequences from these two microcosms were affiliated with Group I.1d (21) (Fig. 5). The clonal frequency of Group I.1d among all crenarchaeotic clones was 83% for ASA-M1-1, and 100% for SDW. This finding was in disagreement with previous studies that detected crenarchaeot Group I.1b and Group I.1c as frequent phylotypes in terrestrial soil environments (18, 21, 32, 35, 37). It has been reported that a large number of *Crenarchaeota* of Group I.1b could be grown in an enrichment culture with tomato root extracts (43). In addition, it has been speculated that Group I.1c *Crenarchaeota* might be heterotrophic and involved in the nitrification of the upper layer of acidic forest soil in Germany (21). However, it is unclear what role the *Crenarchaeota* of Group I.1d play in acid-sulfate soil, or whether crenarchaeotic microorganisms corresponding to the novel phylogenetic *amoA* lineage are affiliated with Group I.1d. Hence, it is essential to conduct a further study in order to clarify whether or not they contribute to nitrification in the acid-sulfate soil of Thailand. In contrast, most of the euryarchaeotic 16S rRNA gene sequences were related to environmental clones retrieved from the soil of a temperate acidic forest in Germany, with 96–98% nucleotide similarity (21). Further study is needed to clarify the function of *Euryarchaeota* in acid-sulfate soil.

**Conclusion**

The present analysis of archaeal *amoA* gene sequences indicated an increase of a notable AOA corresponding to the unique *amoA* lineage in nitrifying acid-sulfate soil microcosms at pH 3.5. Furthermore, analysis of the 16S rRNA gene revealed the predominance of crenarchaeotic Group I.1d in nitrifying acid-sulfate soil microcosms.

**Acknowledgements**

We wish to thank M. Nakamura and the staff of the General Research Institute (Nihon University) for their support in the sequencing, D.-J. Kang and P. Vijarnso for their help with the field work, and K. Mori and T. Tanaka for assisting in the phylogenetic analysis. This study was supported by a Grant-in-aid for the 21st Century COE Program, the Open Research Center Projects, Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Nihon University Individual Research Grant for T.N. (2008).

**References**


