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Short-Term Effect of Elevated Temperature on the Abundance and Diversity of Bacterial and Archaeal *amoA* Genes in Antarctic Soils S

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Global warming will have far-reaching effects on our ecosystem. However, its effects on Antarctic soils have been poorly explored. To assess the effects of warming on microbial abundance and community composition, we sampled Antarctic soils from the King George Island in the Antarctic Peninsula and incubated these soils at elevated temperatures of 5°C and 8°C for 14 days. The reduction in total organic carbon and increase in soil respiration were attributed to the increased proliferation of Bacteria, Fungi, and Archaea. Interestingly, bacterial ammonia monooxygenase (*amoA*) genes were predominant over archaeal *amoA*, unlike in many other environments reported previously. Phylogenetic analyses of bacterial and archaeal *amoA* communities *via* clone libraries revealed that the diversity of *amoA* genes in Antarctic ammonia-oxidizing prokaryotic communities were temperature-insensitive. Interestingly, our data also showed that the *amoA* of Antarctic ammonia-oxidizing bacteria (AOB) communities differed from previously described *amoA* sequences of cultured isolates and clone library sequences, suggesting the presence of novel Antarctic-specific AOB communities. Denitrification-related genes were significantly reduced under warming conditions, whereas the abundance of *amoA* and *nifH* increased. Barcoded pyrosequencing of the bacterial 16S rRNA gene revealed that Proteobacteria, Acidobacteria, and Actinobacteria were the major phyla in Antarctic soils and the effect of short-term warming on the bacterial community was not apparent.

Keywords: Soil, Antarctica, nitrification, denitrification, ammonia-oxidizing bacteria, ammonia-oxidizing archaea

Introduction

Antarctica has one of the most extreme environments on Earth. Most regions of Antarctica are covered with ice and permafrost throughout the year; only 0.4% of the region is ice free [8]. Antarctica has unfavorable environmental conditions due to large variations in temperature [1] and chemical gradients [31] and intensive solar radiations including UV B [35]. The extreme and unfavorable conditions of the Antarctic environment limit biological activities. Remarkably, despite these unfavorable environmental factors, diverse microbial communities thrive in this region [34]. However, these harsh climatic conditions hamper microbial activity in Antarctica, as indicated by a respiration rate of 0.1 ± 0.08 μmol CO_2 m^{-2} s^{-1}, which is lower than the respiration rate reported for other harsh environments such as desert soils (0.6 μmol CO_2 m^{-2} s^{-1}) and soil from the Arctic tundra (1.6 μmol CO_2 m^{-2} s^{-1}) [13].

The low temperature is a key factor governing the Antarctic environment. However, the average temperature of Antarctica has increased continuously at the rate of 0.1°C per decade over the past 50 years [37]. To determine the effects of the warming climate, a good understanding of the current microbial diversity and how the microbial activity changes in response to the warmer temperatures is important. Previously, we investigated the microbial community and nitrogen cycle of Antarctic soils under increased temperature and nitrogen-added conditions [18]. The data

from our quantitative real-time PCR studies suggested that each microbial domain (Bacteria, Fungi, and Archaea) and the nitrogen cycle (nitrogen fixation, nitrification, and denitrification) respond differently to the environmental changes. We also previously examined soil enzyme activity as a representative indicator of microbial activities. In that previous study, we evaluated the activities of several enzymes, including phosphomonoesterase, dehydrogenase, β-glucosidase, urease, and arylsulfatase, and carried out fluorescein diacetate hydrolysis assays [14]. We observed that the change in soil enzyme activities in response to environmental factors such as change in nutritional input, hydrocarbon contamination, and temperature change varied for different enzymes. These results indicated that climate change in Antarctica can have an unpredictable effect on the diversity and activity of the microbial community in the region.

Interestingly, our previous study revealed that bacterial ammonia monooxygenase (*amoA*) genes were more abundant than archaeal *amoA* in the Antarctic soil [18], which was in contrast to the findings of other studies on the abundance of *amoA* in pristine and agricultural soils [24], estuarine soils [7], hot spring sediments [16], wastewater treatment plants [20], and marine ecosystems [4, 43]. Only a few reports have associated predominant bacterial *amoA* genes to increased salinity and C:N ratio as the determinant environmental factors. However, these environmental conditions have not been studied in the Antarctic soil.

In this study, we assessed the effect of warming on the microbial abundance and community compositions in the soils of the King George Island, Antarctic Peninsula. The abundance of a nitrogen-cycling community and the community composition of ammonia-oxidizing prokaryotes were of special interest because of the predominance of bacterial *amoA* over archaeal *amoA*. Barcoded pyrosequencing of the bacterial 16S rRNA gene was used to determine the composition of the bacterial community in Antarctic soils and the responses of these communities to warming.

Materials and Methods

Soil Sampling and Microcosm Design

The Korea Polar Research Institute (KOPRI) provided soil samples. All soil samples were collected from topsoil (<10 cm depth) and were subjected to storage below –80°C until analysis. The Antarctic soils were sampled from the King George Island, Antarctic Peninsula, where the King Sejong Station (Korean Polar Research Facility) is located. A composite soil sample was prepared from 5 sampling sites (62° 13′ 48.6S, 58° 46′ 24.6W; 62° 13′ 46.7S, 58° 47′ 00.9W; 62° 13′ 34.2S, 58° 46′ 00.3W; 62° 13′ 37.0S, 58° 46′ 13.1W; 62° 13′ 40.1S, 58° 44′ 45.3W) (Supplementary Fig. S1). The effects of warming were tested at 5°C and 8°C on a microcosm scale. We designated the samples as follows: Control (intact Antarctic soil, before incubation), 5C (incubated at 5°C), and 8C (incubated at 8°C). The design of the soil microcosm is depicted in Supplementary Fig. S2. A bottomless bottle contained 200 g of a composite soil sample on a 3 cm sand layer. To maintain a soil microcosm with constant water content, sterile water was continuously supplied to the Antarctic soils through the bottom sand layer. The sand and water were autoclaved to prevent the inoculation of microbial cells. CO_2-free air was supplied to the headspace of the microcosm at a flow rate of 10 ml/min. Soil respiration was determined by measuring CO_2 from soil with a LI-840A CO_2 analyzer (Li-COR, USA). Soil characteristics such as pH, electroconductivity (EC), total organic carbon (TOC), total nitrogen (TN), inorganic nitrogen, ammonium cation, nitrate, and nitrite were measured at day 0 for intact soil and after 14 days for incubated soils.

Soil DNA Extraction and Quantitative Real-Time PCR (qPCR)

Microbial communities were quantitatively evaluated *via* qPCR by using an iCycler iQ real-time PCR detection system (Bio-Rad, USA). Primers used in this study are listed in Supplementary Table S1. Soil DNA extraction, qPCR methods, and PCR primer design were carried out as described previously [18]. Soil DNA was isolated from 400 mg of duplicate soil samples by using a NucleoSpin soil kit (Macherey-Nagel, Germany) in accordance with the manufacturer's instructions. The PCR mixture contained 100 ng of soil DNA, 5 pmol of primers, and 12.5 µl of 2× SYBR Green iCycler iQ mixture (Bio-Rad, US) in a final volume of 25 µl. PCR was performed at 94°C for 90 sec, followed by 40 cycles of 94°C for 23 sec, 60°C for 23 sec, and 72°C for 23 sec. PCR specificity was verified by measuring the melting curves. qPCR data were analyzed using the iCycler iQ software (version 3.0). For qPCR standard curves, the reference genes were amplified and cloned into pGEM-T easy vector (Promega, USA) and transformed into *Escherichia coli* Top10. Cloned plasmids were isolated and digested to produce linearized template DNA. Standard curves were calculated from PCRs carried out using a known concentration of template DNA. Four independent experiments were performed and average values with standard deviation are presented.

Construction of AOA and AOB Clone Libraries and Phylogenetic Analysis

Archaeal and bacterial *amoA* genes were amplified by PCR using amoA-1F/amoA-2R and Arch-amoAF/Arch-amoAR primers, respectively [18]. The PCR conditions for bacterial *amoA* genes were as follows: 1 min 30 sec at 94°C, and 35 cycles consisting of 45 sec at 94°C, 45 sec at 60°C, and 45 sec at 72°C. The final extension step was carried out at 72°C for 5 min. The following PCR protocol was used for archaeal *amoA* gene: 4 min at 94°C, and 40 cycles consisting of 1 min at 94°C, 1 min at 53°C, and 1 min 30 sec at 72°C. The final extension step was carried out at 72°C for

20 min. The PCR products were cloned into pGEM-T easy vector (Promega, USA) and transformed into *E. coli* Top10 competent cells. Clone libraries were analyzed *via* RFLP analysis. PCR products amplified with T7 (5′-TAATACGACTACTCACTATAGGG-3′) and SP6 (5′-TACGATTTAGGTGACACTATAG-3′) primers were digested with *Msp*I and *Hae*III at 37°C for 1 h. The clones showing different RFLP patterns were sequenced. Sequence alignment and phylogenetic analysis were performed using MEGA 5 [38]. The clone libraries of *amoA* nucleotide sequences were deposited in GenBank (KC894758-KC894777).

Barcoded Pyrosequencing of Bacterial 16S rRNA Gene and Phylogenetic Analysis

Hypervariable regions (V1–V3) of a 16S rRNA gene were amplified using the V1-27F (5′-adaptor 1-TCAG-AC-GAGTTT GATCMTGGCTCAG-3′) and V3-541R (5′-adaptor 2-TCAG-barcode-AC-WTTACCGCGGCTGCTGG-3′) primers. The sequences of adaptor 1 and adaptor 2 were 5′-CCTATCCCCTGTGTGCCTTGGCAGTC-3′ and 5′-CCATCTCATCCCTGCGTGTCTCCGAC-3′, respectively [19]. PCR was performed with composite DNA samples, prepared by pooling equal amounts of PCR products from each sample. Pyrosequencing was performed by Macrogen (Korea) by using a 454 GS-FLX titanium system (Roche, Germany). The pyrosequencing data were processed and analyzed using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/) [9]. Sequences were checked for chimerism by using the Chimera Check program available at the Greengene website (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) [11]. All clones that displayed chimeric profiles were discarded from further analysis.

Statistical Analysis

The effect of warming on gene abundance was statistically tested using analysis of variation (ANOVA) at the 5% significance level. Microsoft Excel 2010 was used for the analyses.

Results

Soil Characteristics

The characteristics of the Antarctic soil are summarized in Supplementary Table S2. In this study, we examined the soil under the following conditions: intact Antarctic soil, prior to incubation (hereafter designated "Control"), soil incubated at 5°C (designated "5C"), and soil incubated at 8°C (designated "8C"). We observed that soil pH was 6.95 ± 0.06 in Control and was not significantly changed in 5C and 8C (6.91 ± 0.05 and 6.89 ± 0.02 in 5C and 8C, respectively). Before incubation, the Control group contained 20,466.32 ± 478.68 mg/kg of TOC. Following incubations, TOC was reduced to 18,679.25 ± 336.21 mg/kg and 11,403.29 ± 199.29 mg/kg in 5C and 8C, which were 91.3% and 55.7% of the initial TOC, respectively. Because the sum of the residual TOC and carbon equivalent of CO_2 was close to the initial TOC, the possibility exists that the reduced TOC contents were largely emitted as CO_2. Preliminary experiments confirmed that the sterilized sand layer and water below the Antarctic soil did not produce CO_2. TN was found to be unaffected by the elevated temperature. Most of the inorganic nitrogen content was accounted for as NH_4^+. Furthermore, nitrate and nitrite anions were present at negligible amounts.

Microbial Abundance in the Antarctic Soil

We reasoned that the reduction of TOC and CO_2 emissions were closely related to microbial activity. Therefore, we questioned if an increase in microbial abundance accompanied the utilization of TOC. Microbial abundance was determined from the copy number of a bacterial 16S rRNA gene, an archaeal 16S rRNA gene, and a fungal ITS, by using a qPCR-based method (Fig. 1). The most abundant microbial domain in the intact Antarctic soil was Bacteria ($5.80 \pm 1.45 \times 10^6$ copies per 100 ng of soil DNA), followed by Archaea ($3.16 \pm 0.52 \times 10^2$ copies per 100 ng of soil DNA), and Fungi ($2.07 \pm 0.24 \times 10^2$ copies per 100 ng of soil DNA). The abundance of the three domains increased after warming. Bacteria, Archaea, and Fungi increased 3.62-, 2.25-, and 2.42-fold in 5C and 10.49-, 16.81-, and 9.73-fold in 8C, respectively. We found that Bacteria was predominant in all samples ($2.10 \pm 0.40 \times 10^7$ and $6.08 \pm 0.38 \times 10^7$ copies per 100 ng of DNA in 5C and 8C, respectively). However, Fungi outnumbered the archaeal community in 8C, suggesting that Fungi may benefit from the warming more than Archaea. These results show that an overall increase of the microbial community in the Antarctic soil may result from warming and may prompt increased utilization of organic carbon and emission of CO_2.

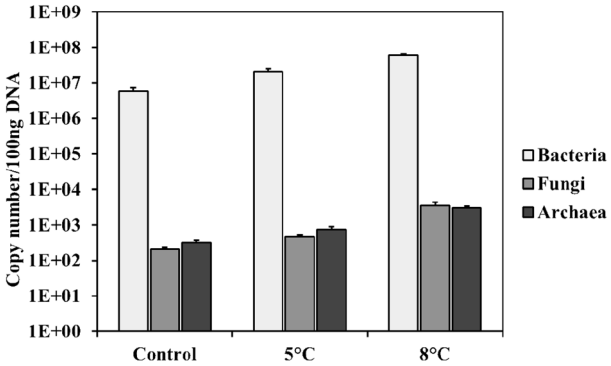

Fig. 1. The copy numbers of the bacterial 16S rRNA gene, archaeal 16S rRNA gene, and fungal ITS determined from Control, 5C, and 8C samples.

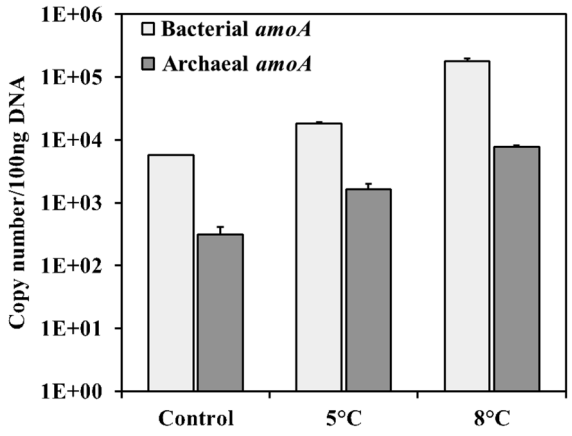

Fig. 2. The abundance of bacterial and archaeal *amoA*.

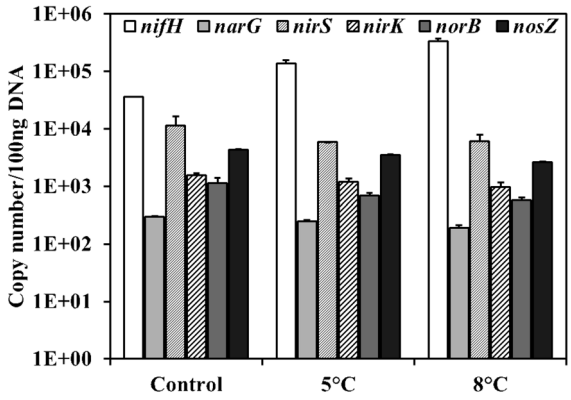

Fig. 3. The abundance of *nifH*, *narG*, *nirS*, *nirK*, *norB*, and *nosZ*.

Abundance of Bacterial and Archaeal *amoA* Genes

Several genera of β- and γ-Proteobacteria have long been regarded as exclusive participants in ammonia oxidation. Metagenomic studies conducted in open oceans and soils revealed the presence of archaeal *amoABC* genes that encode ammonia monooxygenase [40]. A number of recent reports have provided evidence that archaea are actively involved in ammonia oxidation [28, 45]. Therefore, we investigated the abundance of bacterial and archaeal *amoA* to identify the effect of warming on ammonia oxidation in Antarctic soil. Interestingly, we identified the predominance of bacterial *amoA* over archaeal *amoA* in all samples (Fig. 2). The copy number of bacterial *amoA* was $5.78 \pm 0.00 \times 10^3$, $1.84 \pm 0.08 \times 10^4$, and $1.80 \pm 0.16 \times 10^5$ copies per 100 ng of DNA in Control, 5C, and 8C, respectively. The copy number of archaeal *amoA* was found to be $3.15 \pm 0.99 \times 10^2$, $1.63 \pm 0.39 \times 10^3$, and $7.74 \pm 0.31 \times 10^3$ copies per 100 ng of DNA in Control, 5C, and 8C, respectively. Therefore, bacterial *amoA* outnumbered archaeal *amoA* by 18.39-, 11.28-, and 23.29-fold in copies per 100 ng of DNA. Increased temperature appeared to assist ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) to proliferate and resulted in 3.18- and 31.17-fold increases of bacterial *amoA* and 5.19- and 24.61-fold increases of archaeal *amoA* in 5C and 8C, respectively. Thus, the ratios of ammonia oxidizers to the whole community did not change significantly in the 5C and 8C. The copy number of archaeal *amoA* gene exceeded that of archaeal 16S rRNA gene in 5C and 8C samples. This seemed to be due to the different number of 16S rRNA genes and *amoA* per microbial genomes. Moreover, we could not exclude the possibility that the presence of extracellular DNA affected the measurement of gene abundance.

Abundance of Nitrogen Fixation- and Denitrification-Related Genes

Vegetation is practically absent in the Antarctic region; thus, a nitrogen-fixing microbial community would be essential for supplying nitrogen to the Antarctic soil. To determine the abundance of a nitrogen-fixing microbial community, we determined the copy number of the *nifH* gene, which encodes for a nitrogenase. We found that the initial copy number of *nifH* was $3.65 \pm 0.00 \times 10^4$ copies per 100 ng of DNA, and this value increased 3.81- and 9.31-fold in 5C and 8C, respectively. A denitrifying community was quantitatively assessed by measuring the copy numbers of *narG*, *nirS*, *nirK*, *norB*, and *nosZ*, which encode a nitrate reductase, a cytochrome *c* nitrite reductase, a copper-containing nitrite reductase, a nitric oxide reductase, and a nitrous oxide reductase, respectively. Notably, the abundance of the *nosZ* gene was the result from four sets of primers (Fig. 3). This strategy was used because conventional *nosZ* primers were based on Proteobacteria-*nosZ*, whereas *nosZ* is present, albeit with considerable sequence divergence, in diverse taxons such as Firmicutes and Bacteroidetes. We developed three sets of primers to detect *nosZ* from Firmicutes and Bacteroidetes in our recent study, and these primers were used in the analysis [20].

In many environments, the abundance of genes involved in later denitrification steps such as nitric oxide and nitrous oxide reduction is less than that of the genes involved in nitrate and nitrite reduction [18]. Microbial cells do not often harbor the genes for a complete denitrification pathway. However, our results showed that there is not a truncated denitrification process. The copy numbers of *narG*, *nirS*, *nirK*, *norB*, and *nosZ* decreased to 65%, 54%, 62%, 51%, and 61% of the initial copy numbers in 8C, respectively. Considering the increased abundance of the

bacterial community (bacterial 16S rRNA gene; Fig. 1), reduction of the denitrification genes is apparent. The percentages of denitrification genes to bacterial 16S rRNA gene were all less than 0.01% following the warming treatment.

Statistical Analysis of the Effect of Warming on Gene Abundance

ANOVA results determined that the abundance of all genes showed statistically significant changes following warming, except for *nirS* and *norB* (Supplementary Table S3). The *nirS* gene encodes a cytochrome *c* nitrite reductase and is the predominant nitrite reductase, over a copper-containing nitrite reductase (*nirK*), in this soil sample (Fig. 3). Our previous study conducted on Antarctic soil also showed that *nirS* was insensitive to warming. However, we noted that *norB* was found to be less abundant under warmed conditions than in our previous studies [18].

Phylogeny of Bacterial and Archaeal *amoA*

Phylogenetic analysis of a bacterial *amoA* clone library (n = 113) indicated that most bacterial *amoA* genes were classified into two clusters (Fig. 4A). Forty-four clones (including sequenced clones and clones with identical RFLP patterns) were closely related to the *Nitrosospira* lineage and we named this group as Antarctic soil cluster 1. Interestingly, 57 clones (including sequenced clones and clones with identical RFLP patterns) were not associated with either the *Nitrosospira* or *Nitrosomonas* lineages and were therefore included in Antarctic soil cluster 2. Manual

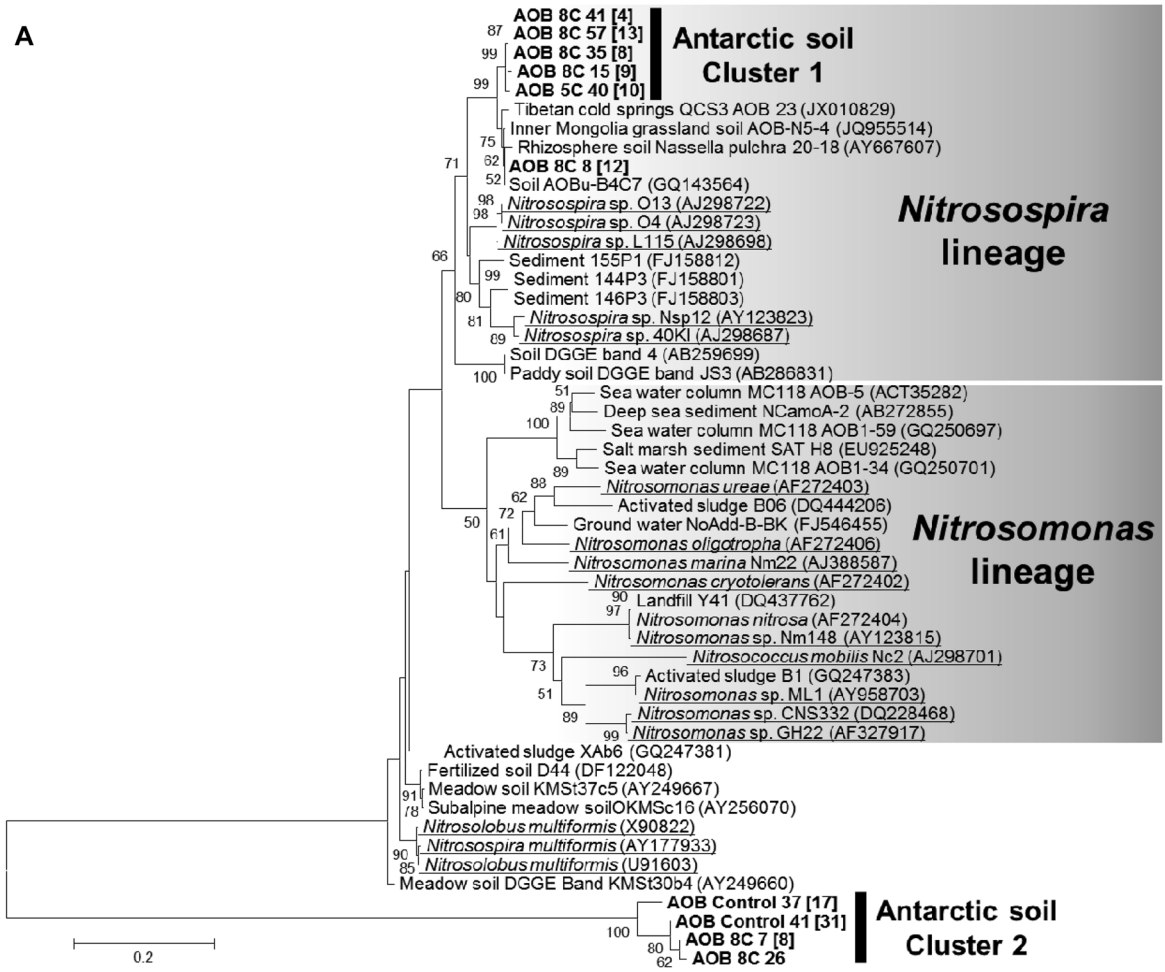

Fig. 4. Phylogenetic analysis (neighbor-joining) of the (**A**) bacterial and (**B**) archaeal *amoA* clone library.

Nucleotide sequences of ~600 and 440 bp were used for bacterial and archaeal *amoA* analysis, respectively. Sequences derived from this study are in boldface. The number of the identical RFLP patterns is in brackets. Sequences from culturable isolates are underlined. GenBank accession numbers are in parentheses.

curating ascertained the high quality of our sequencing results. Phylogenetic analysis with the closest BLAST match also confirmed the separation of Antarctic soil cluster 2 from the previously described AOB *amoA* sequences. Therefore, we suggest the presence of a previously unreported AOB community in Antarctic soils based on the results of our sequence analysis. A phylogenetic tree showed that the clone libraries from Control, 5C, and 8C were not distinguished from one another and implied that warming did not enrich for a specific group of AOB. Phylogenetic analysis of archaeal *amoA* clone library (n = 133) sequences showed that all clones were closely associated with the *Nitrososphaera* cluster (synonymously Group I.1b). However, AOA *amoA* clone library sequences were still grouped into distinguished nodes, and we assigned these sequences to AOA *amoA* Antarctic soil clusters 1, 2, and 3

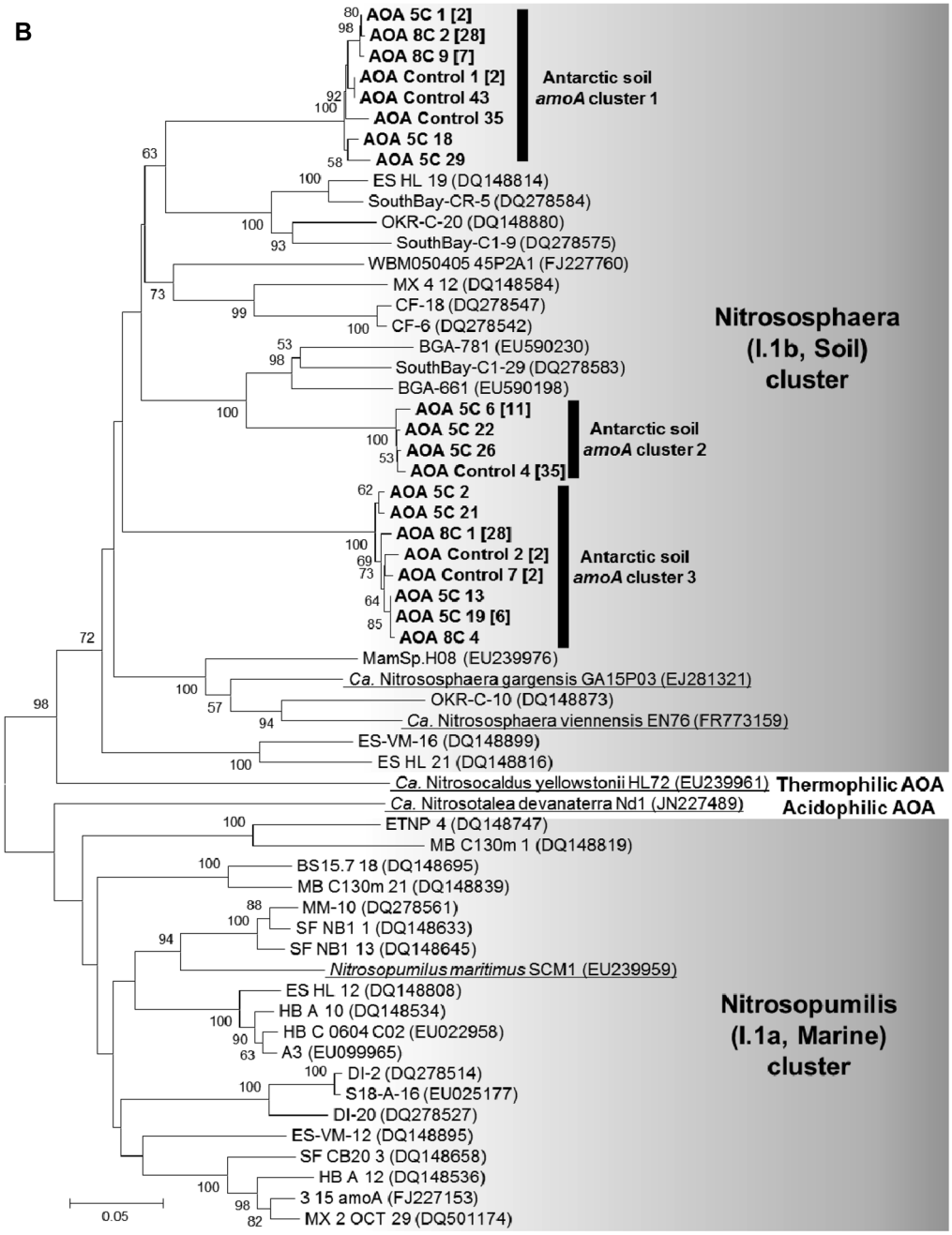

Fig. 4. Continued.

Table 1. Summary of the barcoded pyrosequencing data sets and statistical analysis.

Sample	No. of reads	No. of high quality reads	OTUs	Shannon-Weaver index (H′)[a]	Chao1	Evenness	No. of phyla	No. of genera
Control	16,626	10,617	1,146	5.73	1,596.25	0.81	18	170
5C	15,709	10,217	1,194	5.61	1,674.28	0.79	17	161
8C	10,884	7,110	910	5.61	1,380.10	0.82	16	142

OTUs: operational taxonomic units.

(Fig. 4B). Our results were consistent with the findings of a number of previous reports that thaumarchaeotal group I.1b was predominant over I.1a. [3, 15, 29].

Pyrosequencing of Bacteria 16S rRNA Gene

The bacterial community in the Antarctic soil was further investigated using barcoded pyrosequencing of the 16S rRNA gene. The results of pyrosequencing are summarized in Table 1. The Initial Processor of RDP pyrosequencing pipeline trimmed short and low-quality sequences and produced 10,617, 10,217, and 7,110 high-quality reads from 16,626, 15,709, and 10,884 raw sequences of Control, 5C, and 8C, respectively. The numbers of operational taxonomic units (OTUs) were 1,146, 1,194, and 910 from Control, 5C, and 8C, respectively. The Greengene database identified that 81-83% of sequencing reads from three samples had <97% sequence similarity with the previously known bacterial 16S rRNA genes. A large portion of poor taxonomic understanding has suggested the presence of novel species in Antarctic soils. Diversity indices such as Shannon-Weaver, Chao1, and Evenness indicated that the diversity of the bacterial community was slightly decreased in 8C. Moreover, the phyla, genera, and rarefaction curves also confirmed the reduction in the bacterial diversity of 8C (Fig. 5). The increased copy number of bacterial 16S rRNA gene (Fig. 1) and the decreased bacterial diversity in 8C suggest that a portion of the bacterial community was accustomed to the extreme environment of Antarctica. Therefore, warming could be detrimental to this psychrophilic community. The most abundant phylum was Proteobacteria, followed by Actinobacteria, Acidobacteria, and Bacteroidetes. These four phyla accounted for >70% of the whole bacterial community in all samples (data not shown). The predominant genera were *Gemmatimonas*, GP7, GP4, and GP16, and *Spartiobacteria*. Remarkably, besides *Gemmatimonas*, many dominant genera are poorly classified in terms of taxonomy. AOB such as *Nitrosospira* only comprised <1% of the whole community in all samples. *Nitrosomonas* and *Nitrosococcus* were not identified in our results. A small portion of *Nitrosospira* in the pyrosequencing results were consistent with the ratio of bacterial *amoA* gene to bacterial 16S rRNA gene, which was determined *via* qPCR analysis (Fig. 1).

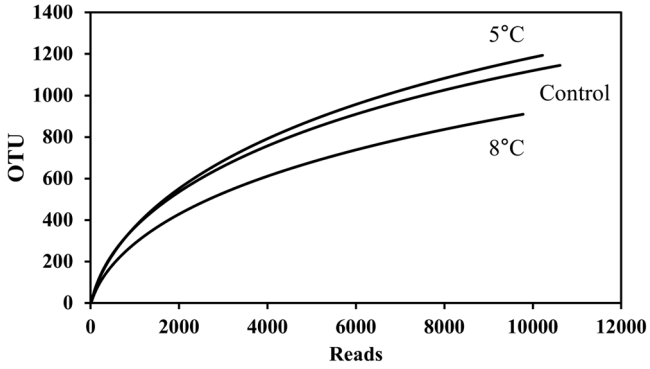

Fig. 5. Rarefaction curves of Control, 5C, and 8C derived from barcoded pyrosequencing results.

Discussion

Decomposition of organic matter in Antarctic soil is hindered by the extreme environmental conditions [6, 10]. Global climate change is expected to increase the temperature in the Antarctic terrestrial environment and subsequently alleviate current unfavorable environmental conditions. We determined the increased soil respiration of the Antarctic soil at warming conditions and found that the carbon equivalent of CO_2 was almost identical to the reduced amount of TOC. Proliferation of the microbial community (Fig. 1) seemed to be responsible for this CO_2 emission from soil TOC, which might impose a positive feedback on the warming climate. Our previous study indicated that the elevated temperature could also affect microbial soil enzyme activity and result in changes to biogeochemical cycles [14]. The nitrogen biogeochemical cycle is of particular interest to many researchers because it is related to agricultural practice, bioremediation, destruction of the ozone layer, and global warming [12, 33, 35, 36]. Therefore, changes in the nitrogen biogeochemical cycle that are induced by warming could affect the function of the

ecosystem at a global scale. Many studies have reported a greater abundance of archaeal over bacterial ammonia oxidizers in diverse environments [20, 21, 24, 39]. Remarkably, however, the predominance of bacterial *amoA* over archaeal *amoA* was found in this study. In our previous study conducted with soil from a relative proximity (around King Sejong Station), we also detected the predominance of bacterial *amoA*. These results are interesting because only limited information is available regarding the predominance of bacterial *amoA*. Zhang *et al.* [45] and Bernhard *et al.* [5] have reported that AOB outnumbered AOA in an estuarine environment and suggested that salinity was an important factor for the AOB abundance. However, it should be noted that conflicting results have been reported about bacterial *amoA* abundance resulting from an estuarine environment [5, 17, 23, 25, 26]. Ammonia-oxidizing communities of several wetlands comprised mainly AOB rather than AOA. However, a consensus result among the community compositions has not been established [42]. Currently, a determinant environmental factor shaping the predominance of bacterial *amoA* over archaeal *amoA* has not been suggested.

Phylogenetic analysis of a bacterial *amoA* clone library showed that Antarctic soil cluster 1 belonged to a *Nitrosospira* lineage. *Nitrosospira* species were associated with low ammonium environments [22, 32]. Supplementary Table S2 indicates that only a small amount of ammonium anion was detected from our tested soils and it was not affected by warming conditions. This environmental condition may be a presumable cause of the predominance of the *Nitrosospira* lineage. Interestingly, Antarctic soil cluster 2 was not associated with previously known *amoA* sequences. The best hits of a BLASTN search of 37 AOB Control, 41 AOB Control, 7 AOB 8C, and 26 AOB 8C clones were a fertilized soil clone D44, meadow soil clone KMSt37c5, subalpine meadow soil clone OKMSc16, and meadow soil DGGE band clone KMSt30b4, respectively. However, a neighbor-joining phylogenetic tree (Fig. 4A) showed that these best hits of BLASTN were grouped with *Nitrosolobus* sequences and that Antarctic soil cluster 2 was still separated. This result implies the presence of a novel AOB lineage in Antarctic soils.

Phylogenic analysis of archaeal *amoA* genes was recently reported by Pester *et al.* [30]. Phylogenetic analysis with all available *amoA* sequences and *amoA*-based deep sequencing revealed five clusters of AOA; namely, *Nitrosopumilus* cluster (I.1a or Marine cluster), *Nitrosotalea* cluster (SAGMGC-1 cluster), *Nitrosocaldus* cluster, *Nitrososphaera* cluster (I.1b or Soil cluster), and *Nitrososphaera* sister cluster. All archaeal *amoA* clone library sequences were associated with the *Nitrososphaera* cluster. Neighbor-joining phylogenetic tree (Fig. 4B) showed that our clone library sequences formed separated branches from previously known sequences. Hence, we designated them as Antarctic soil clusters 1, 2, and 3. Along with the phylogeny of the bacterial *amoA* gene, an ammonia-oxidizing prokaryotic community in the Antarctic soil will need to be investigated in detail.

Barcoded pyrosequencing analysis of the bacterial 16S rRNA gene determined that the predominant bacterial phylum was Proteobacteria followed by Actinobacteria, Acidobacteria, and Bacteroidetes. Our bacterial community analysis data were in accordance with those of the previous bacterial community analysis conducted in the Antarctic Peninsula (where King George Island is located). This analysis found the soil to contain Proteobacteria, Acidobacteria, and Actinobacteria as major bacterial phyla [44]. The Antarctic Peninsula is known to have a relatively warmer and wetter climate than the drier regions such as McMurdo Dry Valley [8]. Different environmental factors might affect the bacterial community composition, because Actinobacteria, Acidobacteria, and Bacteroidetes were dominant bacterial phyla in Antarctic cold desert mineral soils and low-productivity soils [1, 2, 27, 34]. The Antarctic soil bacterial communities tested in this study were not sensitive to the elevated temperature under the current experimental conditions. One possible explanation is that site factors (*e.g.*, soil parent material, moisture regime, TOC, TN, and total phosphorus) are the main determinants of the composition of a bacterial community. Long-term warming experiments in Arctic tundra soils have demonstrated that the site factors were the main determinants of community structure [41]. Furthermore, the compositions of the bacterial community in the Ross Sea regions were also discriminated by multivariate statistics based on soil properties [1]. Interestingly, pyrosequencing of the 16S rRNA gene did not determine previously known AOB such as *Nitrosospira* and *Nitrosomonas*, whereas detection and construction of a bacterial *amoA* clone library was successful. It may be explained by the phylogenetic tree of the AOB clone library showing that a large portion of the clone library contained phylogenetically distinguished bacterial *amoA* sequences. The RDP pyrosequencing pipeline system may not determine the 16S rRNA genes of these putative Antarctic-specific AOB as previously known AOB.

In conclusion, our data indicate that a short-term warming effect on the Antarctic soil environment can promote the proliferation of all three microbial domains. As a result, the functioning of the soil ecosystem may be altered in an unexpected way, as shown by the changes in

the abundance of the nitrogen biogeochemical cycle-related genes. The abundance of taxonomically vague genera and the Antarctic *amoA* clusters in the phylogenetic tree collectively suggest that further study of the Antarctic microbial community is required. To predict the response of the microbial community and the ecological impact of these responses in the warming climate, comprehensive research needs to be conducted.

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