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ORIGINAL ARTICLE

Isotopic signatures of N_2O produced by ammonia-oxidizing archaea from soils

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N_2O gas is involved in global warming and ozone depletion. The major sources of N_2O are soil microbial processes. Anthropogenic inputs into the nitrogen cycle have exacerbated these microbial processes, including nitrification. Ammonia-oxidizing archaea (AOA) are major members of the pool of soil ammonia-oxidizing microorganisms. This study investigated the isotopic signatures of N_2O produced by soil AOA and associated N_2O production processes. All five AOA strains (I.1a, I.1a-associated and I.1b clades of *Thaumarchaeota*) from soil produced N_2O and their yields were comparable to those of ammonia-oxidizing bacteria (AOB). The levels of site preference (SP), $\delta^{15}N^{bulk}$ and $\delta^{18}O$ -N_2O of soil AOA strains were 13–30%, −13 to −35% and 22–36%, respectively, and strains MY1–3 and other soil AOA strains had distinct isotopic signatures. A ^{15}N-NH_4^+-labeling experiment indicated that N_2O originated from two different production pathways (that is, ammonia oxidation and nitrifier denitrification), which suggests that the isotopic signatures of N_2O from AOA may be attributable to the relative contributions of these two processes. The highest N_2O production yield and lowest site preference of acidophilic strain CS may be related to enhanced nitrifier denitrification for detoxifying nitrite. Previously, it was not possible to detect N_2O from soil AOA because of similarities between its isotopic signatures and those from AOB. Given the predominance of AOA over AOB in most soils, a significant proportion of the total N_2O emissions from soil nitrification may be attributable to AOA.

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Introduction

Nitrous oxide (N_2O) is a long-lived greenhouse gas, which is also involved in ozone depletion. The atmospheric abundance of N_2O is approximately one-thousandth of CO_2 but its radiative efficiency (Wm^{-2} ppb^{-1}) is 216 times greater than that of CO_2 (Rahn and Wahlen, 1997). Soils are major sources of N_2O (Conrad, 1996) and are believed to contribute approximately 65% of the total N_2O emitted to the atmosphere (Seitzinger *et al.*, 2000). The atmospheric concentrations of N_2O have been increasing from pre-industrial levels of 0.27 ppmv to current concentrations approaching 0.32 ppmv (Conrad, 1996). Nitrogen deposition and nitrogen fertilization (Nevison and Holland, 1997) due to agricultural expansion and intensification have contributed to the conspicuous post-industrial increase in atmospheric N_2O loading via increased soil nitrogen availability.

The pathways of biological N_2O production include partial dissimilatory nitrate or nitrite reduction (denitrification), nitrifier denitrification (Wrage *et al.*, 2001), ammonia (hydroxylamine) oxidation and NOx detoxification (also known as the 'nitrosative stress' pathway) (Hendriks *et al.*, 2000). Nitrification-related pathways (nitrifier denitrification and ammonia oxidation) mediated by ammonia-oxidizing bacteria (AOB) are known to be a major source of N_2O production from terrestrial environments (Gödde and Conrad, 1999). The first N_2O-yielding route in AOB is related to the activity of hydroxylamine oxidoreductase (HAO), which mediates the conversion of hydroxylamine to nitrite, the second step in ammonia oxidation. The second N_2O-yielding route is through a denitrification pathway in AOB, the so-called nitrifier denitrification,

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where nitrite is reduced to NO and N_2O (Wrage *et al*., 2001; Arp and Stein, 2003), as in the classical heterotrophic denitrification pathway.

Isotopologue analysis of N_2O has many advantages over inhibitor- and tracer-based methods for the determination of production pathways. In addition to $\delta^{15}N^{bulk}$-N_2O and $\delta^{18}O$-N_2O, Yoshida and Toyoda (2000) suggested that analyses of the intramolecular distributions of ^{15}N in N_2O ($N^{\beta}=N^{\alpha}=O$) (isotopomers), which are often expressed as the site preference (SP) ($\delta^{15}N^{\alpha}$–$\delta^{15}N^{\beta}$), may provide critical information that could help identify the precise sources and sinks of this greenhouse gas. Site preference has emerged as a potential conservative tracer for microbial N_2O production because 1) it is independent of the isotopologue composition of the substrates and 2) it does not exhibit changes during the course of production. Distinct site preference values for N_2O production due to bacterial denitrification, including nitrifier denitrification, relative to hydroxylamine oxidation and fungal denitrification provide a fundamental basis that facilitates the resolution of production pathways in the natural environment (Sutka *et al*., 2006, 2008).

Strains of soil ammonia-oxidizing archaea (AOA) in the clade *Thaumarchaeota*, which were cultivated in laboratories, were shown to be capable of growing as autotrophic ammonia oxidizers (Jung *et al*., 2011; Lehtovirta-Morley *et al*., 2011; Kim *et al*., 2012). The thaumarchaeotal I.1a, I.1a-associated and I.1b clades are frequently recognized as the dominant ammonia-oxidizing organisms in terrestrial environments, possibly because of their high affinity for NH_3 (Jung *et al*., 2011; Kim *et al*., 2012). Thus, studies on the isotopic signatures of N_2O and the pathways of N_2O production by AOA are crucial for tracking the sources of N_2O emitted from soils and estimating the soil N_2O budget. AOA are highly fastidious and hence the ecophysiological characterization of AOA using cultured representatives is largely hindered. Recently, an AOA strain enriched from the ocean was shown to produce N_2O with distinct isotopic signatures (Santoro *et al*., 2011). N_2O production by AOA enriched from agricultural soils has been reported (Jung *et al*., 2011; Kim *et al*., 2012), but there has been no isotopic analysis of N_2O produced by soil AOA. The aim of this study was to investigate the isotopomeric signatures of N_2O produced by AOA strains enriched from soils and their pathways of N_2O production. This study may provide insights into the N_2O production mechanisms of AOA, thereby facilitating the tracking of archaeal sources of N_2O in terrestrial environments.

Materials and methods

Cultivation of AOA

Soil AOA strains (Table 1) were enriched from various soils. The enrichment culture procedure was basically the same as that described by Jung *et al*. (2011) and Kim *et al*. (2012), and various soil sources were used as inocula (see the Supplementary Materials and Methods). Initial enrichment cultures were set up in allylthiourea (ATU) (20 μM) and chlorate (50 μM) to selectively inhibit the growth of AOB and nitrite oxidizing bacteria (NOB), respectively. The enriched AOA cultures contained heterotrophic bacteria, and

Table 1 Ammonia-oxidizing microorganisms used in this study and their N_2O production

Strain	*Isolation site*	*Growth temperature (°C)*	*Growth pH*	*Taxonomic group*[a]	*Cell abundance (ml^{-1})*[b,c]	*Cell yield after ammonia oxidation (cells ml^{-1} μM^{-1})*	*N_2O production*[d]		
							N_2O (N_2O-N; μmol)[c]	*NO_2^- (NO_2^--N; μmol)*[c]	*N_2O yield (N_2O-N/NO_2^--N; %)*
AOA[e]									
MY1	Agricultural soil	25	6.5	I.1a	1.2×10^8 (1.0×10^7)	1.1×10^5	0.0555 (0.003)	51.21 (3.31)	0.11
MY2	Agricultural soil	25	6.5	I.1a	1.4×10^8 (1.3×10^7)	1.3×10^5	0.0551 (0.002)	50.83 (2.89)	0.11
MY3	Tar-contaminated soil	30	7.0	I.1b	9.3×10^7 (4.4×10^6)	8.8×10^4	0.0411 (0.003)	50.45 (3.73)	0.08
JG1	Agricultural soil	37	6.5	I.1b	1.8×10^8 (1.3×10^7)	1.7×10^5	0.4191 (0.023)	51.14 (4.01)	0.82
AR	Marine sediment	25	7.5	I.1a	1.3×10^8 (1.1×10^7)	1.2×10^5	0.0458 (0.004)	50.02 (3.89)	0.09
CS	Acid mine	25	5.0	I.1a-associated	5.1×10^7 (2.8×10^6)	4.8×10^5	0.1783 (0.009)	4.16 (0.21)	4.28
AOB									
N. europaea ATCC 19718	Soil	25	7.5	Beta-proteobacteria	9.7×10^7 (4.2×10^6)	9.2×10^4	0.1675 (0.011)	50.22 (4.12)	0.33

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria.
[a]The classification of thaumarchaeotal groups is based on Ochsenreiter *et al*. (2003).
[b]Cell counting was performed using real-time quantitative PCR after the depletion of 1 mM ammonia, except with strain CS. Strain CS was analyzed after the oxidation of 0.1 mM ammonia.
[c]The numbers in parentheses indicate the standard deviation for triplicate experiments.
[d]The yield of N_2O was determined after complete ammonia oxidation.
[e]MY1 (Jung *et al*., 2011), JG1 (Kim *et al*., 2012) and AR (Park *et al*., 2010) strains were described previously. MY2, MY3 and CS strains were obtained in this study.

attempts were made to further enrich AOA by successive culture in artificial freshwater medium (AFM) containing a mixture of ampicillin and kanamycin (each at 50 μg ml^{-1}). The composition of the culture media is described in the Supplementary Materials and Methods. After ca. 3 years (strains MY1, MY2 and JG1) and 2 years (strains MY3 and CS) of tri-weekly transfer, the cultures were serially diluted (10-fold) to extinction, and the highest dilution showing nitrifying activity was selected for isolating a single archaeal strain.

Soil AOA strains and a reference strain of AOB, *Nitrosomonas europaea* ATCC 19718, were incubated in an AFM. A marine AOA strain AR used as a reference strain in this study was incubated in an artificial seawater medium (ASM), which was modified from the study by Jung *et al.* (2013). For the N_2O studies, cultures of AOA and a strain of *N. europaea* were incubated with 50 ml of AFM or ASM in 120 ml serum bottles sealed with a butyl rubber stopper (Bellco Glass, Vineland, NJ, USA). All of the cultures were incubated in the dark at their optimum growth temperatures (Table 1) with unmodified ambient air in the head space. Sampling for gas and chemical analysis and supplementation with chemicals were conducted using syringes. The resistance of the butyl rubber stopper to the leakage of N_2O gas for 1 month was confirmed using standard gas. The cultures were routinely supplemented with 1 mM ammonium chloride as the sole energy source. After the ammonia oxidation was completed (in ca three weeks), 5% of the total culture was routinely transferred to fresh medium. The maximum ammonia oxidized by strain CS was ca 0.1 mM and hence strain CS was transferred when no further oxidation of ammonia occurred. The ammonia and nitrite concentrations were determined using an ion chromatograph (ICS-1500, Dionex, Sunnyvale, CA, USA) with an OnGuard II Ag Cartridge (Dionex) and colorimetrically, respectively (Solorzano, 1969). The N_2O yield after the completion of ammonia oxidation was determined on the basis of the nitrite produced from the same cultures. Cell growth was determined as described in the Supplementary Materials and Methods.

Isotopic signature of N_2O

N_2O was analyzed after the ammonia was depleted by oxidation. The ammonia level was monitored by measuring ammonia in the media and was depleted in 3 weeks. Strain CS was analyzed after the oxidation of 0.1 mM ammonium chloride. The headspaces of the 120 ml serum bottles were used for the isotopic N_2O analysis. The dual isotope and isotopomer signatures of N_2O were analyzed, that is, $\delta^{18}O$ of N_2O ($\delta^{18}O$-N_2O), average $\delta^{15}N$ ($\delta^{15}N^{bulk}$) and $\delta^{15}N$ from the central N-position ($\delta^{15}N^{\alpha}$), after cryofocusing by isotope ratio mass spectrometry (IRMS) using a Delta V IRMS (Thermo-Fisher, Bremen, Germany), which allowed simultaneous detection of m/z 44, 45 and 46 (Well and Flessa, 2008). The IRMS was connected to a gas chromatograph (GC) (Trace GC Ultra, Thermo-Fisher, Bremen, Germany) and a modified Precon (Thermo-Fisher) equipped with an autosampler (model Combi-PAL, CTC-Analytics, Zwingen, Switzerland) (Well and Flessa, 2008). The ^{15}N SP was determined as follows: $\delta^{15}N^{\beta} = 2 \times \delta^{15}N^{bulk} — \delta^{15}N^{\alpha}$. The dual isotope and isotopomer ratios of a sample (R_{sample}) were expressed as the ‰ deviation from $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of the reference standard materials (R_{std}), atmospheric N_2 and standard mean ocean water (SMOW):

$$\delta X = (R_{sample}/R_{std} - 1) \times 1000$$

where $X = {}^{15}N^{bulk}$, $^{15}N^{\alpha}$, $^{15}N^{\beta}$ or ^{18}O. The typical analytical precision levels were 0.2, 0.4 and 0.3‰ for $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$, respectively.

Isotopic signatures of H_2O, NO_2^-, and NH_4^+

The $\delta^{18}O$ values of the medium water were analyzed using a laser spectrometer (model L 1115-I, Picarro, Santa Clara, CA, USA), which was suitable for analyzing water as a liquid or vapor. To determine $\delta^{15}N$ and $\delta^{18}O$ values for NO_2^- in the media, the denitrifier method was used to transform NO_2^- quantitatively to N_2O (Casciotti *et al.*, 2002). The subsequent isotope analysis was conducted as described above. The $\delta^{15}N$ value of NH_4^+ and NO_2^- was measured by combustion of the pure salts (ammonium chloride and sodium nitrite, Sigma-Aldrich) using an elemental analyzer coupled to an IRMS (DeltaPlus, Thermo-Finnigan, Bremen, Germany).

N_2O tracer experiment

The N_2O tracer experiment was performed using the same culture conditions used for the isotope signature analysis. However, 0.5 mM 99 atm% $^{15}NH_4Cl$ (Cambridge Isotope laboratories, Tewksbury, MA, USA) was used as the sole electron donor in the presence of excess unlabeled nitrite (2 mM $NaNO_2$), which was added as a background to determine the N_2O fractions derived from preexisting nitrite via nitrifier denitrification. The same cultures used in the trace experiment with unlabeled substrates (0.5 mM unlabeled ammonium chloride with 2 mM unlabeled nitrite) were also used for comparative SP analysis. The amount of N_2O gas in the headspace was measured using a GC with a quadrupole mass spectrometer (QMS) (6890A (G1540A)/5973N, Agilent, Santa Clara, CA, USA). The quadrupole mass spectrometer recorded the relative quantitative intensity of ions according to their mass-to-charge ratios (*m/e*). From ^{15}N-labeled NH_4^+ and unlabeled NO_2^-, there were three possible combinations of N isotopes for N_2O: $^{14,14}N_2O$ (*m/e* 44), $^{14,15}N_2O$ (*m/e* 45) and $^{15,15}N_2O$ (*m/e* 46). The N_2O ion peaks produced by electron impact ionization

were measured in these three *m/e* positions and their background in the mass spectrometer was subtracted. Gas samples were taken from the headspace of culture bottles using a 5 ml gas-tight syringe and injected via a gas sampling valve using a 1 ml sample loop in the GC. The GC was fitted with a 4 m stainless steel column packed with Porapak Q (80/100 mesh, Restek, Bellefonte, PA, USA) to separate N_2O and CO_2 from the sample gas. The oven was isothermal at 50 °C and the N_2O ion peaks were measured three times for each sample. We used six certified reference gas mixtures of N_2O in nitrogen, that is, 0.198, 0.331, 0.550, 0.798, 12.0, and 94.9 N_2O μmol mol^{-1}, to calibrate the GC/QMS. These reference gas mixtures were prepared gravimetrically by the Korea Research Institute for Standards and Science and verified by international comparisons, that is, CCQM-K68 in 2010 (Lee *et al.*, 2011).

Effect of PTIO on nitrification

To evaluate the crucial role of NO as an intermediate in nitrification and N_2O production by soil AOA, the effect of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxyl (100 μM, PTIO), a NO scavenger (Amano and Noda, 1995; Ellis *et al.*, 2001), was tested on the growth and ammonia oxidation activities of AOA. The PTIO experiment was performed using the same growth conditions as those used for the isotopic signature studies. Cell growth and ammonia oxidation were determined during and after incubation.

Results and discussion

AOA strains

The soil AOA strains used in this study are shown in Table 1. An AOA strain (AR) from a marine sediment (Park *et al.*, 2010) and an AOB strain were also included as references. The phylogenetic analysis performed using archaeal 16S rRNA and *amoA* genes as described in Supplementary Materials and Methods indicated that I.1a (MY1, MY2 and AR), I.1a-associated (CS) and I.1b (MY3 and JG1) clades within *Thaumarchaeota* were included (Figure 1). The phylogenetic information regarding enriched AOA strains (based on 16S rRNA and *amoA* gene sequences) is described in the Supplementary Results.

All five soil AOA cultures used in this study grew on minimal medium, with ammonia as the sole electron donor. Each AOA culture was highly

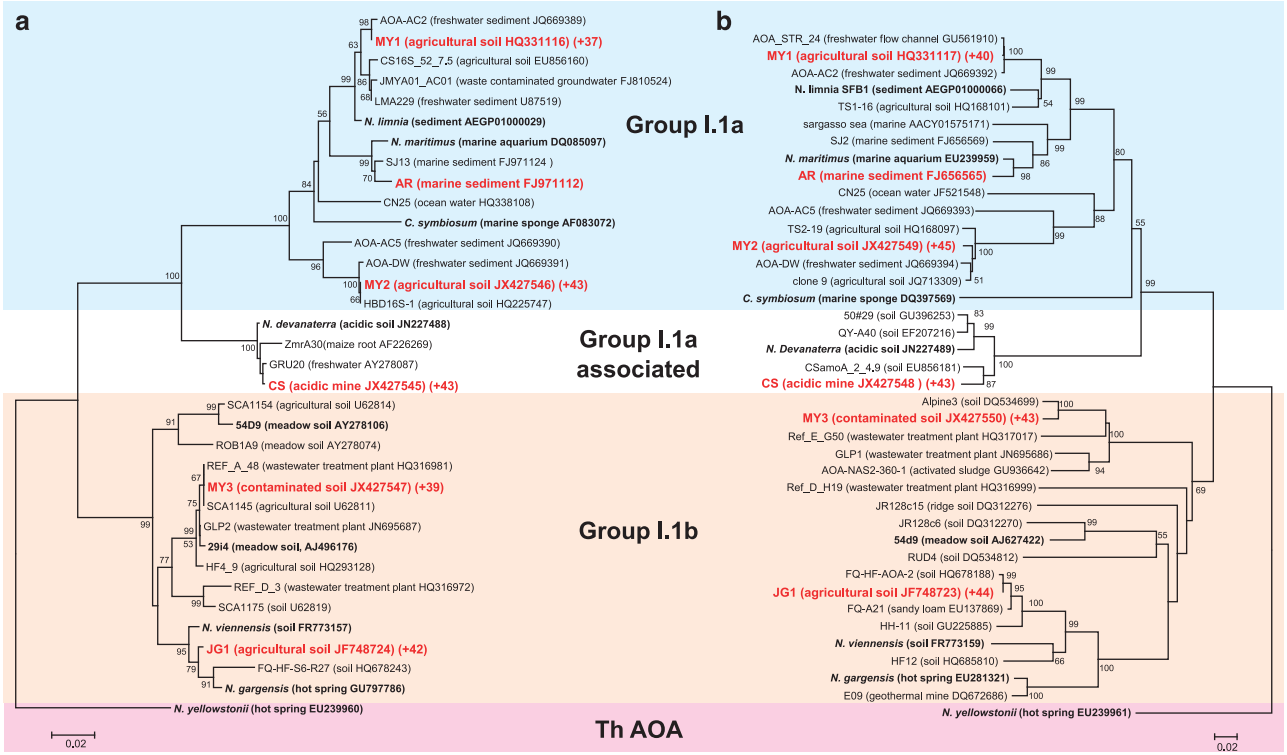

Figure 1 Comparative phylogenetic analysis of (**a**) 16S rRNA and (**b**) *amoA* gene sequences from *Thaumarchaeota* strains used in this study. The classification of thaumarchaeotal groups is based on Ochsenreiter *et al.* (2003). Branching patterns supported by bootstrap values (1000 iterations) >50% according to the neighbor-joining method are denoted by their bootstrap values. Strains indicated in red were used in this study. Strains marked in bold are reference strains or fosmid clones of *Thaumarchaeota*. The origins of each clone or strain are indicated in parentheses with their accession numbers. The number of 16S rRNA and *amoA* gene sequences used for checking the purity of AOA cultures is indicated in the second set of parentheses.

enriched and contained a single archaeal strain, as revealed by a single band corresponding to that of archaeal 16S rRNA gene sequence in a denaturing gradient gel electrophoresis. As described previously, clone libraries derived from the enrichment cultures confirmed that only one unique sequence (>99.7% identity within the error rate expected for PCR amplification (von Wintzingerode *et al.*, 1997; Könneke *et al.*, 2005)) for the archaeal 16S rRNA and *amoA* genes, respectively, was identified in each culture (Jung *et al.*, 2011; Kim *et al.*, 2012). The number of clones (>37 per library) that were sequenced and analyzed is shown in Figure 1. FISH with domain-specific probes and real-time quantitative PCR analysis confirmed that the archaeal abundance was >90% of the minor bacterial cells co-cultured. In this study, AOB were selectively inhibited at 20 μM ATU as reported previously (Bedard and Knowles, 1989; Ginestet *et al.*, 1998; Jung *et al.*, 2011). As reported by Santoro *et al.* (2011) and Shen *et al.* (2013), the AOA used in this study were not inhibited at 20 μM ATU (apart from strain CS, which was partially inhibited at 20 μM ATU, as previously reported in Lehtovirta-Morley *et al.* (2013)). The absence of AOB contamination was confirmed by the inability to amplify the bacterial 16S rRNA gene from betaproteobacterial AOB using specific nested PCR primers, and the *amoA* genes from gamma and betaproteobacterial AOB using specific PCR primers (Supplementary Table S1). The PCR results were also supported by the analysis of bacterial 16S rRNA gene clone libraries (Supplementary Table S2). Only the archaeal growth was tightly coupled to ammonia oxidation, as shown in other studies (Jung *et al.*, 2011; Kim *et al.*, 2012). These results suggested that ammonia-oxidizing archaea were solely responsible for the observed ammonia oxidation.

N_2O production by AOA

We observed N_2O production in all AOA cultures where ammonia oxidation occurred (Table 1). The inocula (early stationary phase cultures) had initial cell densities of ca 1×10^6 to 5×10^6 16S rRNA gene copies ml^{-1} for all AOA cultures and *N. europaea*. The cultures of AOA had stationary phase cell densities of ca 5×10^7 to 2×10^8 16S rRNA gene copies ml^{-1} after the oxidation of 1 mM ammonia, except strain CS (Table 1). The N_2O production by the AOA strains ranged from 0.041 to 0.419 μmol (N_2O-N) after the exhaustion of 1 mM ammonia. The yields (N_2O/NO_2^- produced) from AOA ranged from 0.11 to 4.28%, which were comparable to those from *N. europaea* (Table 1) and other AOB (Colliver and Stephenson, 2000). The total amount and production yield of N_2O from strains JG1 and CS were at the upper end of the range. The highest N_2O production yield from the acidophilic strain CS might have been related to its high sensitivity to nitrite toxicity (Supplementary Figure S1). Indeed, ca 0.1 mM nitrite was the maximum tolerable concentration for the CS cultures, as found with *Nitrosotalea devanaterra* (Lehtovirta-Morley *et al.*, 2011).

The archaeal strains were not pure isolates, but the involvement of minor contaminant (denitrifying) bacteria with N_2O production could be rejected based on the following experimental evidence. (1) N_2O production and ammonia oxidation were tightly coupled and N_2O production ceased after ammonia oxidation was completed, as found using strain MY1 (Jung *et al.*, 2011) and JG1 (Kim *et al.*, 2012) (see Supplementary Figure S2). This indicated that the contribution of bacterial denitrification to N_2O production from the nitrite and organic debris produced by AOA was negligible. (2) Chlorite (50 μM; ClO_2^-), an ammonia oxidation inhibitor that does not inhibit bacterial denitrification, completely inhibited N_2O production in the presence of 1 mM nitrite (Jung *et al.*, 2011; Kim *et al.*, 2012). Even when yeast extract (10 mg l^{-1}) was spiked in the medium containing chlorite, N_2O production was still not observed in the presence of 1 mM nitrite (data not shown). In this condition, the only bacterial cell growth (up to 10^8 cells ml^{-1}) that occurred corresponded to the final bacterial cell concentrations in the ammonia-oxidizing enrichment culture. (3) Heterotrophic bacterial strains with a denitrification capability (*Pseudomonas* sp. from culture JG1; *Acinetobacter* sp. from culture MY1; *Rhodococcus* sp. from culture MY3) were isolated from the ammonia-oxidizing cultures and cultivated with 10 mg l^{-1} yeast extract and 0.5 mM nitrite using the same medium and incubation conditions. The amount of N_2O produced after 2 weeks was negligible and was close to the atmospheric background level (0.003–0.005 N_2O-N μmol) (Supplementary Figure S3). These results showed clearly that the cultivation conditions were sufficiently oxic that N_2O production from bacterial denitrification was negligible.

Isotopic signatures of N_2O

In addition to bulk isotopic information ($\delta^{15}N$-N_2O and $\delta^{18}O$-N_2O), the N_2O molecule contains position-dependent isotopic information from nitrogen ($\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$). SP, the position-dependent isotope value of N_2O, is an isotopic signature that is commonly used to interpret the source of N_2O production (Toyoda and Yoshida, 1999; Sutka *et al.*, 2003; Ostrom and Ostrom, 2011). Studies using pure AOB cultures have indicated that a positive SP (30–38‰) is consistent with a N_2O source from hydroxylamine oxidation (ammonia oxidation), whereas nitrifier denitrification (and denitrification) is thought to produce N_2O with a SP value near or below zero (–10 to 0‰) (Frame and Casciotti, 2010; Sutka *et al.*, 2006). The N_2O emissions from various soils have positive (0–35‰) SP signatures (Pérez *et al.*, 2001; Park *et al.*, 2011, 2012; Toyoda *et al.*, 2011), which indicates that ammonia oxidation is an

important source of soil N_2O. The SP values of soil AOA strains (MY1-3 and JG1) were distinct and they ranged from 20 to –30‰, except strain CS (Table 2). The SP values of strain AR and *N. europaea* were at the upper end of the range. Intriguingly, the SP value of strain CS was 13‰, which was much lower than other ammonia-oxidizing microorganisms. Nitrite toxicity increased the nitrifier denitrification activity required for detoxification, which might have been attributable to the low SP and the high N_2O production by strain CS (Tables 1 and 2). A future SP analysis of AOB at acidic pH might be useful to verify whether the low SP value can be attributed to the increase in nitrifier denitrification required for nitrite detoxification under acidic conditions. The results of the present study indicated that SP analysis might not be a definitive method for distinguishing archaeal and bacterial nitrification sources of N_2O production.

The $\delta^{15}N^{bulk}$-N_2O values for all AOA strains were –13 to –17‰, except strain CS (–35.54‰), whereas the value for *N. europaea* was –19.88‰ (Table 2). The end-member mixing model was not applicable to the CS culture because of the partial oxidation of ammonia and hence the $^{15}N^{bulk}$-N_2O produced by strain CS did not reflect the isotopic shifts of NH_4^+. The ranges of $\delta^{15}N^{bulk}$-N_2O from soil AOA were similar to those (–10 to –50‰) from agricultural soils and forest soils (Kim and Craig, 1993; Rahn and Wahlen, 2000; Pérez *et al.*, 2001; Rock *et al.*, 2007). Previous studies found that the $\delta^{15}N^{bulk}$-N_2O values of marine AOB (*Nitrosomonas marina* C-113) were about –10‰ with 20% O_2 concentration (Frame and Casciotti, 2010), whereas the $\delta^{15}N^{bulk}$-N_2O values of *N. europaea* (ATCC 19718) in ammonia-oxidizing and nitrite-reducing conditions were –46.5‰ and –34.0‰, respectively (Sutka *et al.*, 2003, 2006). Surprisingly, the $\delta^{15}N^{bulk}$-N_2O values of a marine AOA strain CN25 were positive (8.7‰) (Santoro *et al.*, 2011), which differed significantly from the ideal isotope fractionation effect and from those of the soil AOA strains. These values were similar to those from surface oceans and the troposphere (5‰ to 10‰) (Kim and Craig, 1993; Rahn and Wahlen, 2000; Popp *et al.*, 2002). Strain AR was also from a marine environment but its $\delta^{15}N^{bulk}$ value (–12.91‰) was similar to those of the soil AOA strains and marine AOB (Frame and Casciotti, 2010). The 'apparent' (observable) isotope effect calculated from the data obtained by Santoro *et al.* (2012) would be $\varepsilon_{NH_4N_2O} = \delta^{15}N^{bulk}\text{-}N_2O - \delta^{15}N\text{-}NH_4 = 8.7 - 2.5 = 6.2‰$. As the intrinsic isotope effects of N_2O formation are always negative, that is, $\delta^{15}N$ of product is depleted when compared with $\delta^{15}N$ of substrate, our value of $\varepsilon_{NH_4N_2O} = \delta^{15}N^{bulk}\text{-}N_2O - \delta^{15}N\text{-}NH_4 = -17 - (-1) = -16‰$ is much closer to the expected value. The deviation of the apparent isotope effect from the intrinsic isotope effect (ideal Rayleigh behavior) could be caused by variations in the N_2O yield of nitrification over time. For example, if (1) the N_2O yield is higher at the end when the residual substrate (NH_4^+) is already substantially $\delta^{15}N$-enriched, or if (2) clumps of cells in the culture media create local areas of $\delta^{15}N$-enriched NH_4^+, the apparent isotope effect could be reversed (that is, positive).

Different N_2O production pathways incorporate elemental O from isotopically distinct sources

Table 2 Isotopic characteristics of N_2O produced during ammonia oxidation by soil AOA strains

Strain	$\delta^{18}O\text{-}N_2O$ (‰)	$\delta^{15}N^{bulk}\text{-}N_2O$ (‰)	$\delta^{15}N^{\alpha}$ (‰)	$\delta^{15}N^{\beta}$ (‰)	*SP* (‰)
AOA					
MY1	30.91 (2.06)	–13.53 (2.12)	–1.55 (3.63)	–25.42 (1.68)	23.87 (3.75)
MY2	29.26 (2.47)	–16.96 (1.81)	–6.04 (3.44)	–27.80 (1.33)	21.76 (3.75)
MY3	33.56 (3.27)	–16.49 (2.18)	–3.89 (4.15)	–28.98 (1.40)	25.09 (4.39)
JG1	21.59 (0.34)	–15.32 (0.16)	–5.48 (0.28)	–25.11 (0.03)	19.62 (0.24)
AR	36.11 (0.67)	–12.91 (1.50)	2.07 (1.33)	–27.77 (1.70)	29.83 (0.67)
CS[a]	22.44 (0.72)	–35.54 (0.89)	–28.97 (1.48)	–42.07 (0.32)	13.10 (1.20)
AOB					
N. europaea ATCC 19718	26.58 (0.18)	–19.88 (0.39)	–5.37 (0.58)	–34.31 (0.50)	28.94 (1.01)
Addition of 2 mM nitrite					
MY1	22.22 (3.23)	–17.67 (0.79)	–11.48 (3.10)	–23.86 (2.29)	12.38 (5.22)
N. europaea ATCC 19718	21.85 (0.49)	–27.39 (0.39)	–19.57 (1.37)	–35.14 (0.58)	15.57 (1.95)

Abbreviations:AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; ATCC, American Type Culture Collection; SP, site preference.
All experiments used air-equilibrated media with an assumed $\delta^{18}O\text{-}O_2$ of ca 23.5‰ versus VSMOW, and the initial 1 mM ammonia concentration was oxidized completely during each experiment. The $\delta^{15}N$ values are reported as ‰ versus AIR, while the $\delta^{18}O$ values are reported as ‰ versus VSMOW. The isotope values for N_2O are the isotopic composition of the N_2O produced after correcting for background material. $\delta^{18}O\text{-}H_2O$ was –7.97 (0.06)‰. $\delta^{15}N\text{-}NH_4^+$ was –1.20 (0.10)‰. The $\delta^{15}N\text{-}NO_2^-$ value of the nitrite added in the high nitrite background was –2.7 (0.3)‰. The standard deviation values were calculated from replicate experiments (refer to Supplementary Table S3 for the raw data) and are shown in parentheses.
[a]Only 10% of the ammonia (0.1 mM) was oxidized in this condition, possibly because of high sensitivity to the accumulated nitrite. Thus, the end-member mixing model did not explain the CS cultures (the $\delta^{15}N^{bulk}$-N_2O and $\delta^{18}O$-N_2O values produced by strain CS reflect isotopic shifts in substrates, i.e., Rayleigh effects (Mariotti *et al.*, 1981)).

(for example, H_2O or O_2) and they have unique fractionation factors (Ostrom *et al.*, 2000). The $\delta^{18}O$-N_2O values of surface oceans and the troposphere were 20–40‰, whereas those of agricultural soils and forest soils were 0–60‰ (Kim and Craig, 1993; Pérez *et al.*, 2001; Rock *et al.*, 2007). The $\delta^{18}O$-N_2O values of the AOA strains were 29–34‰ for MY1, 2, and 3, ca 22‰ for CS and JG1 and 36‰ for AR. The $\delta^{18}O$-N_2O value of *N. europaea* was 27‰. The oxygen isotope branching effects that affect $\delta^{18}O$ enrichment from oxygen sources during N_2O production may also be applicable to soil AOA strains as suggested for AOB and marine AOA (Frame and Casciotti, 2010; Santoro *et al.*, 2011). The $\delta^{18}O$-N_2O signature was not significantly different from those of AOB and hence the ratio of oxygen atom sources (H_2O to O_2) incorporated into nitrite (1:1) appears to hold for AOA and AOB. The $\delta^{18}O$-N_2O values of marine AOA (strain CN25) were also similar to those of soil AOA. The shift in $\delta^{18}O$-N_2O reflected changes in the proportion of N_2O derived from two different pathways, that is, ammonia oxidation and nitrifier denitrification; hence, the covariance of SP and $\delta^{18}O$-N_2O was observed in AOB. The covariance of SP and $\delta^{18}O$-N_2O for soil AOA may be inferred from the plot of SP versus $\delta^{18}O$-N_2O, including other ammonia-oxidizing AOA and AOB, as shown in Figure 2. Although the proportion of nitrifier denitrification varied between replicates (Supplementary Table 3 and Supplementary Figure S4), there was a distinct covariance between SP and $\delta^{18}O$-N_2O for each strain in the incubation conditions used in this study. This indicated that combined isotopic signatures can be used to improve the process identification, whereas individual signatures had limitations when trying to identify sources (Snider *et al.*, 2012).

There were similar covariance results from N_2O reduction to N_2 during denitrification (Jinuntuya-Nortman *et al.*, 2008; Well and Flessa, 2009). The SP/$\delta^{18}O$-N_2O slope of N_2O emitted from soil AOA (approx. 1) (Figure 2) was within the slope range reported for residual N_2O after partial reduction to N_2 (Well *et al.*, 2012). This similarity might further complicate the distinction of N_2O from nitrification and denitrification in environments.

^{15}N-labeling experiments

The mixing of two different N_2O production pathways by AOB (that is, ammonia oxidation and nitrifier denitrification) results in different SP values, which are well established using various approaches. The SP signatures of N_2O can be used to calculate the magnitude of each pathway's contribution to the total N_2O output based on its SP signature for AOB (Sutka *et al.*, 2003; Sutka *et al.*, 2006).

The mass of N_2O produced during the oxidation of ^{15}N-labeled NH_4^+ (0.5 mM) in the presence of excess unlabeled NO_2^- (2 mM) was determined to trace the sources of N_2O. The tracer experiment with

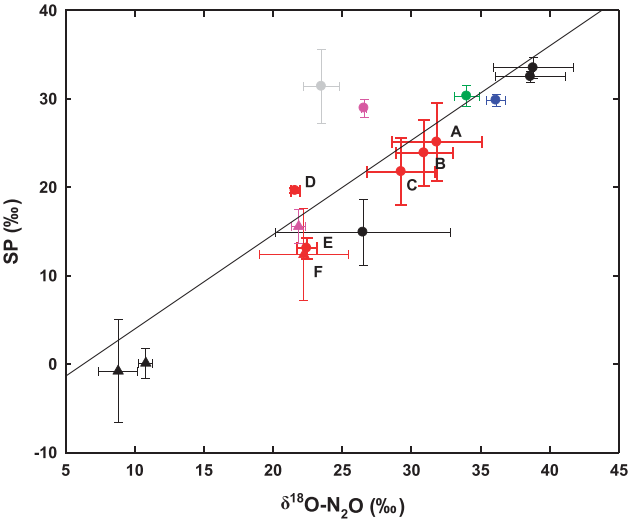

Figure 2 Covariance of SP and $\delta^{18}O$-N_2O. Symbols shown in red (soil AOA), blue (marine AOA strain AR), and pink (*N. europaea*) correspond to the data from this study (circles indicate values from ammonia-oxidizing cultures without nitrite addition; triangles indicate values from ammonia-oxidizing cultures with 2 mM nitrite): A, MY3; B, MY1; C, MY2; D, JG1; E, CS; F, MY1 with 2 mM nitrite. The green circle indicates marine AOA strain CN25 (Santoro *et al.*, 2011). The black circles and triangles indicate *N. europaea* under hydroxylamine-oxidizing and denitrifying conditions, respectively, whereas the gray circle indicates *N. europaea* under ammonia-oxidizing conditions (Sutka *et al.*, 2003; Sutka *et al.*, 2006). The error bars are based on replicate experiments. The raw data used in this plot are presented in Supplementary Table S3.

N. europaea (Ritchie and Nicholas, 1972) suggested that the dimerization of ^{15}NOH, derived from $^{15}NH_2OH$, and ^{14}NOH, derived from nitrite, might subsequently lead to the formation of $^{14,15}N_2O$. Three possible combinations of the N isotopes in N_2O were also possible for soil AOA via two different processes: $^{14,14}N_2O$ (nitrifier denitrification, *m/e* 44), $^{14,15}N_2O$ (mixing of nitrifier denitrification and ammonia oxidation, *m/e* 45) and $^{15,15}N_2O$ (ammonia oxidation, *m/e* 46) (Figure 3a). Strain CS could not be included in this experiment because of its high sensitivity to NO_2^-. The N_2O mass ratios varied, depending on the strains (Figure 3b). N_2O produced by the mixed process was dominant in JG1 and *N. europaea*, where the three different mass ratios of N_2O (*m/e* 46:45:44) from JG1 and *N. europaea* were 20.4%:71.2%:8.4% and 28.8%:62.8%:8.4%, respectively. Most of the N_2O produced by strains MY1, MY2, MY3 and AR had *m/e* values of 45 and 44, which indicated that N_2O derived from the mixed process and nitrifier denitrification were co-dominant (up to 90%) (Figure 3b). The proportions of mixed and pure ammonia-derived N_2O were not correlated with the SP values shown in Table 2. For example, SP was not high in strain JG1 despite the high proportion of mixed and pure ammonia-derived N_2O. The mixing process might be a key step that contributes to the high SP of ammonia-derived N_2O. The contribution made by

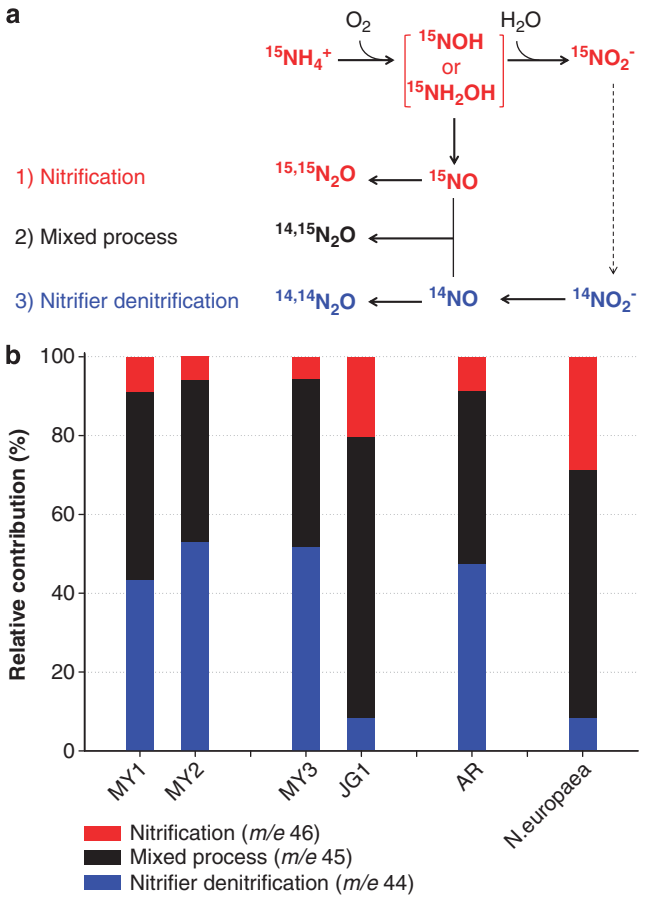

Figure 3 (**a**) Proposed biochemical processes during archaeal nitrification, showing the pathways leading to N_2O production in the tracer experiment. Two different NO sources permitted three possible combinations for N_2O production. HNO (nitroxyl) or hydroxylamine has been proposed as an intermediate during the oxidation of ammonia to nitrite (Walker *et al.*, 2010). (**b**) The composition of labeled N_2O produced during the tracer experiment. Soil AOA strains were incubated in the presence of 0.5 mM ^{15}N-labeled NH_4^+ and 2 mM nitrite.

nitrifier denitrification to N_2O production might be overestimated due to the high background (2 mM) nitrite level. In the present study, we observed an increased N_2O yield and reduced SP values in strain MY1 and in *N. europaea* in the presence of 2 mM nitrite (Figure 2, Table 2, and Supplementary Table S3), which indicated enhanced nitrifier denitrification; similar findings were reported for AOB (Frame and Casciotti, 2010). Therefore, the contribution made by the direct conversion of ^{15}N-labeled NH_4^+ to N_2O production, that is, via ammonia oxidation (*m/e* 46) and the mixed process (*m/e* 45), might be more significant in the absence of background nitrite. Thus, the tracer experiment was not applicable for determining the exact contributions made by the different processes in the isotopomer study. Furthermore, the fraction of N_2O produced via nitrifier denitrification by AOA in soil might be much lower because of the presence of nitrite-oxidizing bacteria. This study indicated that the isotopic signatures of N_2O produced by AOA might have been due to combinations of two different N_2O production processes (see Figure 3), similar to AOB (Ritchie and Nicholas, 1972). In a similar experiment using marine AOA with ^{15}N-labeled NH_4^+ addition, at least some of the N_2O was shown to be produced directly from the ^{15}N-enriched NH_3 pool without passing through the nitrite pool (Santoro *et al.*, 2011).

Effect of PTIO on nitrification

Bacterial ammonia oxidation is traditionally defined as the oxidation of ammonia to nitrite via hydroxylamine as an intermediate. In addition to hydroxylamine, the reactive intermediate nitroxyl (nitroxyl hydride; HNO) has been proposed as a key intermediate during archaeal ammonia oxidation (Walker *et al.*, 2010). The present study showed that archaeal ammonia oxidation was very sensitive to PTIO, a scavenger of free radical nitric oxide (NO) and nitroxyl (Amano and Noda, 1995; Ellis *et al.*, 2001; Samuni *et al.*, 2010), and neither ammonia oxidation nor archaeal growth was observed at 100 μM PTIO (Supplementary Figure S5, and Yan *et al.* (2012)). In addition, the inhibitory effects of 100 μM PTIO on AOB were negligible (Supplementary Figure S5 and Shen *et al.* (2013)). Recently, Vajrala *et al.* (2013) demonstrated hydroxylamine-induced oxygen consumption and ATP production by the marine AOA strain, *Nitrosopumilus maritimus*. In this case, NO was suggested to act as an electron shuttling factor in the same way as the quinone moiety in AOB. High expression of *nirK* gene was observed in strain MY1 during ammonia oxidation (Jung *et al.*, 2011). A more plausible explanation may be that the inhibitory effect of PTIO is related to NO-based electron shuttling in the hydroxylamine pathway rather than to the production of nitroxyl intermediates. Our results suggest that the ammonia oxidation mechanism in AOA and the N_2O production mechanism in AOA via ammonia oxidation might be distinct from those of AOB. By contrast, the isotopic signatures of $\delta^{15}N$-N_2O and $\delta^{18}O$-N_2O, the SP, and the results of the labeled tracer experiment did not differ greatly from those of the AOB strains.

Conclusions

Soil is the most important source of global N_2O emissions, and a significant but unknown fraction of the soil N_2O is produced by nitrification. The recent discovery of AOA in soils implies that it is essential to revise the current assumption that AOB are the sole sources of N_2O from soil nitrification. The present study showed that the isotopic signatures (SP, $\delta^{15}N^{bulk}$-N_2O, and $\delta^{18}O$-N_2O) of soil AOA strains were similar to those of AOB strains and also similar to those from agricultural soils and forest soils. Thus, the activities and roles of soil AOA during

N_2O emission from soil have been hidden in previous isotopic studies of N_2O. The current study, based on soil AOA cultures, indicates that a significant amount of N_2O could be produced from archaeal nitrification. However, there is controversy regarding AOA's contribution to autotrophic nitrification in terrestrial systems, despite their numerical dominance over AOB (Schleper, 2010). Global climate change and the upturn in atmospheric N_2O imply that it is imperative to incorporate archaeal N_2O production processes into global N_2O models to accurately predict the response of the N_2O flux in future soils. Selective inhibitors of nitrification, such as ATU for AOB and PTIO for AOA, could provide an important tool for differentiating the activity of soil AOA from that of AOB with respect to N_2O emissions. These results may provide fundamental information for facilitating future studies on the N_2O production pathway in AOA and the tracking of N_2O emission sources in soils.

Conflict of Interest

The authors declare no conflict of interest.

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