Nitrogen and Oxygen Isotope Effects of Ammonia Oxidation by Thermophilic *Thaumarchaeota* from a Geothermal Water Stream

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ABSTRACT

Ammonia oxidation regulates the balance of reduced and oxidized nitrogen pools in nature. Although ammonia-oxidizing archaea have been recently recognized to often outnumber ammonia-oxidizing bacteria in various environments, the contribution of ammonia-oxidizing archaea is still uncertain due to difficulties in the *in situ* quantification of ammonia oxidation activity. Nitrogen and oxygen isotope ratios of nitrite ($\delta^{15}\text{N}_{\text{NO}_2^-}$ and $\delta^{18}\text{O}_{\text{NO}_2^-}$, respectively) are geochemical tracers for evaluating the sources and the *in situ* rate of nitrite turnover determined from the activities of nitrification and denitrification; however, the isotope ratios of nitrite from archaeal ammonia oxidation have been characterized only for a few marine species. We first report the isotope effects of ammonia oxidation at 70°C by thermophilic *Thaumarchaeota* populations composed almost entirely of “*Candidatus Nitrosocaldus*.” The nitrogen isotope effect of ammonia oxidation varied with ambient pH (25‰ to 32‰) and strongly suggests the oxidation of ammonia, not ammonium. The $\delta^{18}\text{O}$ value of nitrite produced from ammonia oxidation varied with the $\delta^{18}\text{O}$ value of water in the medium but was lower than the isotopic equilibrium value in water. Because experiments have shown that the half-life of abiotic oxygen isotope exchange between nitrite and water is longer than 33 h at 70°C and pH ≥ 6.6 , the rate of ammonia oxidation by thermophilic *Thaumarchaeota* could be estimated using $\delta^{18}\text{O}_{\text{NO}_2^-}$ in geothermal environments, where the biological nitrite turnover is likely faster than 33 h. This study extended the range of application of nitrite isotopes as a geochemical clock of the ammonia oxidation activity to high-temperature environments.

IMPORTANCE

Because ammonia oxidation is generally the rate-limiting step in nitrification that regulates the balance of reduced and oxidized nitrogen pools in nature, it is important to understand the biological and environmental factors underlying the regulation of the rate of ammonia oxidation. The discovery of ammonia-oxidizing archaea (AOA) in marine and terrestrial environments has transformed the concept that ammonia oxidation is operated only by bacterial species, suggesting that AOA play a significant role in the global nitrogen cycle. However, the archaeal contribution to ammonia oxidation in the global biosphere is not yet completely understood. This study successfully identified key factors controlling nitrogen and oxygen isotopic ratios of nitrite produced from thermophilic *Thaumarchaeota* and elucidated the applicability and its limit of nitrite isotopes as a geochemical clock of ammonia oxidation rate in nature. Oxygen isotope analysis in this study also provided new biochemical information on archaeal ammonia oxidation.

Ammonia oxidation is the first step in nitrification, where ammonia is converted to nitrate via nitrite (Fig. 1). Nitrate, the end product of nitrification, is removed from the biosphere by denitrification and anaerobic ammonium oxidation (anammox) as dinitrogen (N_2) or nitrous oxide (N_2O). Nitrification is therefore critical for balancing reduced and oxidized nitrogen pools in nature, which link the mineralization and removal processes of biologically available nitrogen. Because ammonia oxidation is generally the rate-limiting step in nitrification (1), it is important to understand the biological and environmental factors regulating the rate of ammonia oxidation in nature.

Since the historical isolation of nitrifying bacteria (2), it was believed for over a century that ammonia oxidation was conducted only by bacterial species belonging to the classes *Betaproteobacteria* and *Gammaproteobacteria*. The recent discovery of ammonia-oxidizing archaea (AOA) belonging to the phylum *Thaumarchaeota* in marine and terrestrial environments has revolutionized the concept (3–8). Molecular surveys suggest that the abundance of archaeal *amoA* genes, coding for the putative am-

monia monooxygenase (AMO), and transcripts exceeds those of bacterial counterparts in the ocean, ammonium-depleted soils, and terrestrial hot springs (9–13). It has become evident that some

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FIG 1 Schematic illustration of ammonia oxidation pathways in ammonia-oxidizing bacteria (A) and archaea (B) and associated isotope effects (based on reference 18). It is generally accepted that not ammonium but ammonia is the substrate for bacterial ammonia monooxygenase (AMO) (65), while the true substrate for archaeal AMO remains to be elucidated. Abbreviations: HAO, hydroxylamine dehydrogenase; NIR, nitrite reductase; NOR, nitric oxide reductase.

AOA members also produce N_2O , a greenhouse gas, as a by-product in ammonia oxidation (6, 14). These findings suggest that AOA play a significant role in the global nitrogen cycle. However, their contribution to ammonia oxidation in the global biosphere is yet to be fully understood.

The rate of ammonia oxidation (or nitrification) in nature has often been determined by incubation experiments with isotopically labeled tracers (^{15}N ammonium and ^{15}N nitrate). Such experiments provide key insights into the instantaneous rate of ammonia oxidation (or nitrification) but still have the following potential defects: (i) potential stimulation of nitrification by added tracers, (ii) underestimation of the ammonia oxidation rate by isotope dilution of nitrite arising from the concurrency of nitrification and denitrification, and (iii) potential change in microbial community composition during incubation. Experiments involving incubation with selective inhibitors of AOB or AOA activity (e.g., allylthiourea, 1-octyne, and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) could be useful to assess bacterial and archaeal contributions to total nitrification (15–17), but it is still uncertain to what extent the ammonia oxidation rates measured in the inhibition experiments represent bacterial and archaeal ammonia oxidation rates in nature, as the minimum concentrations of the inhibitors to stop ammonia oxidation activity in natural environments with complex physical and ecological characteristics (e.g., permeability and cell density in the local environment) are largely unknown (16, 18).

On the other hand, the natural abundances of nitrogen and

oxygen isotopes are alternative potential tools for calculating the *in situ* rates of ammonia oxidation and other nitrogen metabolic processes at an ecosystem scale (19). Both nitrogen and oxygen isotope ratios of inorganic nitrogen compounds (ammonia, nitrite, and nitrate) are altered by kinetic isotope effects during microbial metabolic transformations (20–23). The oxygen isotope ratio of nitrite ($\delta^{18}\text{O}_{\text{NO}_2^-}$) is also affected by abiotic isotope exchange with oxygen atoms in water, which pushes the $\delta^{18}\text{O}_{\text{NO}_2^-}$ value toward the isotopic equilibrium in a time scale comparable to that of biological nitrite turnover. Therefore, $\delta^{18}\text{O}_{\text{NO}_2^-}$ can be used to calculate the *in situ* rates of ammonia oxidation and other nitrite-involved metabolic processes (nitrite oxidation, denitrification, and anammox) if the relative fluxes of these processes are inferred from nitrogen isotopes, which are not affected by abiotic isotopic exchange in a time scale comparable to that of biological nitrite turnover. Another prerequisite for using the dual (nitrogen and oxygen) isotopic composition of nitrite as a geochemical clock of biological nitrite turnover is the knowledge of nitrogen and oxygen isotope effects during nitrite-producing and -consuming processes. The nitrogen and oxygen isotope effects of bacterial ammonia oxidation, nitrite oxidation, and denitrification have been determined for a range of microbial species (21–23). In contrast, the nitrogen and oxygen isotope effects of archaeal ammonia oxidation have been reported for only a few marine species (24). Given the potential differences between the ammonia oxidation pathways of AOA and ammonia-oxidizing bacteria (AOB) (Fig. 1) (25, 26), the nitrogen and oxygen isotope effects of archaeal ammonia oxidation may be distinguished from those of bacterial ammonia oxidation and should be determined for a range of archaeal species with different physiological properties and dwelling in different ecological niches to understand environmental and biological factors underlying the regulation of the magnitudes of the isotopic fractionations.

“*Candidatus Nitrosocaldus yellowstonii*,” present in geothermal environments, is the most deep-rooted AOA (5, 26). The evolutionary distance between the *amoA* gene sequences of *Nitrosocaldus* and marine AOA members and their physiological differences related to different growth temperatures may represent distinctive isotope effects and kinetics of ammonia oxidation by different AOA groups. Thermophilic ammonia oxidation also plays a significant role in the biomass production of chemolithotrophic microbial communities in geothermal environments. In previous studies, the thermodynamic calculation of potential chemolithotrophic catabolism, compositional and nitrogen isotopic properties of dissolved inorganic compounds, and microbial community composition strongly suggested that the ammonia oxidation by *Nitrosocaldus* members is the predominant energy metabolic process in the microbial mat communities of geothermal springs (12, 27). Given the widespread occurrence of the *amoA* genes of *Thaumarchaeota* in terrestrial hot springs (28, 29) and the potential metabolic versatility of various nitrogen compounds in thermophilic prokaryotes (30), the dual isotopic composition of nitrite could provide important insights into the contribution of AOA to the *in situ* kinetics and energetics of nitrogen metabolism and biomass production in global geothermal environments.

To evaluate if the nitrogen and oxygen isotope ratios of nitrite can be used to calculate the *in situ* kinetics of ammonia oxidation and other nitrogen metabolic processes in various environments with different temperature and pH conditions, we first report the

nitrogen and oxygen isotope effects of ammonia oxidation by thermophilic *Thaumarchaeota* populations composed almost entirely of “*Ca. Nitrosocaldus*.” Comparing the isotope effects of thermophilic *Thaumarchaeota* with those of mesophilic ammonia oxidizers, we discuss key factors that control the magnitudes of the isotopic fractionations and the applicability of the dual isotopic composition of nitrite as a clock of biological nitrite turnover in various environments. The rate of abiotic oxygen isotope exchange between nitrite and water was also measured at 70°C to estimate the minimum rate of biological nitrite turnover that can be measured using the $\delta^{18}\text{O}_{\text{NO}_2^-}$ value in geothermal environments.

MATERIALS AND METHODS

Microbial mat sampling. In June 2011, microbial mat samples attached to mineral grains were collected from an ammonium-enriched subsurface geothermal aquifer stream in Japan (31, 32). We focused on this stream because of the fact that previous investigations between 2001 and 2009 showed that uncultivated *Thaumarchaeota* members, which are closely related to “*Ca. Nitrosocaldus yellowstonii*,” dominated in the microbial mat communities through the entire stream, with a water temperature ranging from 55°C to 72°C (12, 33). Ammonia oxidation by the predominant *Thaumarchaeota* has been demonstrated by *in situ* biogeochemical and microbiological characterization of the stream (12).

To enrich thermophilic *Thaumarchaeota*, an intact mat sample with stream water was collected from one point of the stream and preserved at 20°C until cultivation (see the supplemental material for details). A mat sample for the analysis of the prokaryotic 16S rRNA gene community structure was collected from the same site using a sterilized plastic syringe, and in the field, the interstitial mat water was extracted from the mat sample through a 0.22- μm -pore-size polyethersulfone filter (Millex-GP syringe filter unit; Merck Millipore, Darmstadt, Germany). The temperature and pH of the stream water flowing over the microbial mat were measured *in situ* and were 65°C and 6.8, respectively.

Enrichment culture. To establish an ammonia-oxidizing enrichment culture, some mineral grains were incubated with the microbial mat under aerobic conditions at 70°C in a continuous-flow bioreactor (see Fig. S1 in the supplemental material). Based on the temperature range of the field, the enrichment was also tested at 50°C; however, ammonia-oxidizing enrichment did not successfully establish at this temperature. The bioreactor consists of a glass tube (with an inside volume of 41 ml), of which one-third of the space is filled with porous ceramics (Aero ring; Kyorin, Hyogo, Japan) and pumice (approximately 3 to 5 mm in diameter) as carrier materials for microbial cells. Both ends of the tube were sealed with butyl rubber stoppers and crimped with aluminum seals. A synthetic medium was supplied into the slightly inclined reactor by peristaltic pumps via a silicon tube and a needle. The synthetic medium then flowed downward, passed through the carriers by gravity, and, finally, drained from the reactor through a needle at a flow rate of 18 ml/h. The mean residence time of the medium in the reactor was 0.8 h. The headspace volume in the reactor was approximately 10 ml and was aerated by ventilation through a 21-gauge needle inserted into the reactor.

According to the chemical composition of the aquifer and interstitial waters at the sampling site, the synthetic medium was prepared with the following chemical composition: 3 mM NaCl, 0.6 mM KCl, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 500 μM Na_2SO_4 , 5 μM K_2HPO_4 , 200 μM NH_4Cl , 70 μM $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, and 2.5 mM Na_2SiO_3 . To 1,000 ml of this medium was added 1 ml of trace element solution. The trace element solution contained 30 mM FeCl_2 , 26.7 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6.4 mM CoCl_2 , 6.26 mM ZnCl_2 , 1.62 mM H_3BO_3 , 20 mM NiCl_2 , 0.1 mM AlCl_3 , 0.41 mM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.4 mM CuCl_2 . The synthetic medium was autoclaved, and NaHCO_3 was added to the medium at a final concentration of 0.01%; using HCl, the pH of the medium was adjusted to approximately 6.8 at room temperature.

Experiments on nitrogen and oxygen isotope effects during ammonia oxidation. The microbial community associated with the ceramics and pumice from the enrichment culture was sampled at 180 days of enrichment and incubated in a closed system (100-ml sterilized glass bottle) for up to 53.5 h with 40 ml of medium and 60 ml of air. The chemical composition of the medium was the same as that used for the enrichment culture, except for the NH_4Cl concentration (150 μM) and oxygen isotope ratio of water ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$). The water was ^{18}O labeled in various degrees ($\delta^{18}\text{O}_{\text{H}_2\text{O}} = -8$ [nonlabel], 41, and 116‰) by adding H_2^{18}O (97% ^{18}O ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to measure the oxygen isotope dependence of nitrite ($\delta^{18}\text{O}_{\text{NO}_2^-}$) on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. A total of 2.5 ml of the incubated solution was periodically sampled during the batch experiments, and the sampling interval typically ranged from 2.3 h to 4.5 h. Immediately after sampling of the batch cultures, the pH was measured using 0.2 ml of the solution sample, while the remaining sample was filtered through a 0.22- μm -pore-size polyethersulfone filter. Soon after the filtration, 0.1 to 1 ml of the solution was adjusted to pH 13 by adding 5 M NaOH solution, and it was stored at -20°C until analysis for the nitrogen and oxygen isotopes of nitrite ($\delta^{15}\text{N}_{\text{NO}_2^-}$ and $\delta^{18}\text{O}_{\text{NO}_2^-}$). The alkaline treatment was critical for preventing oxygen isotope exchange between nitrite and water during sample storage (28). The nitrite concentration and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of the filtered solution were measured within a day after the sampling. The remaining filtered solution was stored at -20°C until the measurement of the ΣNH_3 concentration. As a negative-control experiment, a portion of the enrichment culture was sterilized by autoclaving and incubated under the same conditions as described above.

The nitrogen isotope effect during ammonia oxidation under a higher ΣNH_3 concentration was also measured with the enrichment culture (135 days of enrichment) in the continuous-flow bioreactor. A synthetic medium as described under “Enrichment culture” above but with 14 mM ΣNH_3 ($\delta^{15}\text{N} = -5.8\text{‰} \pm 0.4\text{‰}$) was used as an influent solution at a flow rate of 18 ml/h.

Experiments with oxygen isotope exchange between nitrite and water. The rate of abiotic oxygen isotope exchange between nitrite and water was determined at a temperature of 70°C and a pH ranging from 5.4 to 8.0 (values at 70°C). Under each pH condition, a 50-ml polypropylene tube was filled with 30 ml of buffer solution already preheated to 70°C and incubated in an oven at 70°C immediately after addition of 150 μl of 6 mM NaNO_2 solution (at a final concentration of 30 μM). A buffer solution was prepared with ^{18}O -labeled water, CH_3COOH , and CH_3COONa or ^{18}O -labeled water, Tris buffer, and 0.1 N HCl to adjust the solution pH to 5.4, 6.5, or 8.0 at 70°C. NaNO_2 solutions with various $\delta^{18}\text{O}_{\text{NO}_2^-}$ values were prepared by dissolving NaNO_2 in water with various $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values at room temperature and a pH ranging from 6.5 to 7.0 for 25 days. Initial and time series measurements of nitrite concentration, $\delta^{18}\text{O}_{\text{NO}_2^-}$, and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ were performed by periodic sampling of the incubated solution (1.5 ml). Pretreatments of the sample solution until analyses were identical to those described under “Experiments on nitrogen and oxygen isotope effects during ammonia oxidation” above. The solution pH at 70°C was measured by a benchtop pH analyzer with an Ag/AgCl electrode coupled to a temperature compensation sensor (pH meter F-15; Horiba, Ltd., Kyoto, Japan).

Chemical and isotopic analysis. The ΣNH_3 concentration in sample solutions was determined by the indophenol blue method (34). The nitrite concentration was spectrophotometrically measured at 540 nm on reacting with sulfanilamide and *N*-1-naphthyl-ethylenediamine dihydrochloride (the detection limit was 0.3 μM and the reproducibility was better than 10%) (35). The nitrate concentration was measured using ion chromatography (IC-Pac A25S column; Thermo Fisher Scientific, MA) with UV detection at 220 nm (GL-7451; GL Science, Tokyo, Japan) (the detection limit was 0.1 μM and the reproducibility was better than 3%). The oxygen isotope ratio of water was determined by cavity ring-down spectroscopy (LWIA-24d; Los Gatos Research, Inc., CA). The precision achieved by the repeated analyses of water standards was typically better than 0.5‰ for $\delta^{18}\text{O}$.

The nitrogen and oxygen isotope ratios of nitrite were measured by gas chromatography coupled on-line with isotope ratio monitoring mass spectrometry (20/20; Sercon, Cheshire, United Kingdom) after chemical conversion to N_2O (12, 36). In-house $NaNO_2$ and KNO_2 isotopic standards that had been calibrated by Karen Casciotti and Matthew McIlvin at the Woods Hole Oceanographic Institution ($\delta^{15}N_{NO_2-} = 0.2\text{‰}$ to 68.4‰ and $\delta^{18}O_{NO_2-} = 1.1\text{‰}$ to 27.0‰) were analyzed in parallel to calibrate the measured $\delta^{15}N_{N_2O}$ and $\delta^{18}O_{N_2O}$ from $\delta^{15}N_{NO_2-}$ and $\delta^{18}O_{NO_2-}$, respectively. To compensate for the isotope effect of oxygen atom exchange between nitrite and water during chemical conversion to N_2O , the isotopic standards were dissolved in nitrite-free water with the same $\delta^{18}O_{H_2O}$ as present in the solution sample so that the $\delta^{18}O_{H_2O}$ during the chemical conversion of the isotopic standards was identical to that of the sample solution. The levels of precision achieved by the repeated analyses of the in-house standards were typically better than 0.4‰ for $\delta^{15}N$ and 0.5‰ for $\delta^{18}O$.

Isotope ratios are reported relative to air for nitrogen and relative to Vienna Standard Mean Ocean Water for oxygen.

Calculation. We defined the kinetic isotope effect as follows:

$$\varepsilon_k = (k_L/k_H - 1) \times 1,000 \quad (1)$$

where k_L is the first-order rate constant for the reaction of molecules containing lighter isotopes (e.g., ^{14}N and ^{16}O) and k_H is the rate constant for the reaction of molecules containing heavier isotopes (e.g., ^{15}N and ^{18}O).

The organism-level nitrogen isotope effect for ammonia oxidation ($^{15}\varepsilon_{k,\Sigma NH_3/NO_2-}$) was calculated using the Rayleigh accumulated product equation (37):

$$\delta^{15}N_{NO_2-,pro} = \delta^{15}N_{\Sigma NH_3,initial} + f \times \ln(f) \times (1-f)^{-1} \times ^{15}\varepsilon_{k,\Sigma NH_3/NO_2-} \quad (2)$$

where $\delta^{15}N_{NO_2-,pro}$ and $\delta^{15}N_{\Sigma NH_3,initial}$ are the nitrogen isotope ratios of nitrite produced in the experiments and ΣNH_3 initially present in medium, respectively, and f is the remaining fraction of ΣNH_3 ($f = [\Sigma NH_3]/[\Sigma NH_3]_{initial}$). Because all batch culture experiments were initiated with a small amount of nitrite carried over from the enrichment transfer (3 to $5 \mu M$), $\delta^{15}N_{NO_2-,pro}$ at any time since the initial time point was calculated from the measured $\delta^{15}N_{NO_2-}$ using the following isotope mass balance equation:

$$\delta^{15}N_{NO_2-,pro} = (\delta^{15}N_{NO_2-} \times [NO_2^-] - \delta^{15}N_{NO_2-,initial} \times [NO_2^-]_{initial}) / ([NO_2^-] - [NO_2^-]_{initial}) \quad (3)$$

Further, minor variations in $\delta^{15}N_{\Sigma NH_3,initial}$ should be expected between experiments due to the various mixing ratios of ΣNH_3 carried over from the enrichment transfer and NH_4Cl from freshly prepared medium. Therefore, to calculate $^{15}\varepsilon$ from equation 2, linear fitting was separately performed for each experiment using KaleidaGraph (Synergy Software, PA). Equation 2 can be approximated by the following equation in the continuous-culture experiment because the f value was greater than 0.99 and $f \times \ln(f) \times (1-f)^{-1} \approx -1$:

$$\delta^{15}N_{NO_2-,pro} = \delta^{15}N_{\Sigma NH_3,initial} - ^{15}\varepsilon_{k,\Sigma NH_3/NO_2-} \quad (4)$$

The calculation of the oxygen isotope effect for ammonia oxidation requires oxygen isotopic data for nitrite produced during batch culture experiments ($\delta^{18}O_{NO_2-,pro}$). The $\delta^{18}O$ value of nitrite produced in the intervals between the N^{th} and the $(N+1)^{th}$ sampling times was calculated using the following equation:

$$\delta^{18}O_{NO_2-,pro} = (\delta^{18}O_{NO_2-,N+1} \times [NO_2^-]_{N+1} - \delta^{18}O_{NO_2-,N} \times [NO_2^-]_N) / ([NO_2^-]_{N+1} - [NO_2^-]_N) \quad (5)$$

The rate of abiotic oxygen isotope exchange reaction between nitrite and water was calculated using the following equation (19):

$$\delta^{18}O_{NO_2-} = \delta^{18}O_{NO_2-,eq} + (\delta^{18}O_{NO_2-,initial} - \delta^{18}O_{NO_2-,eq}) \times \exp(-k \times t) \quad (6)$$

where $\delta^{18}O_{NO_2-,initial}$ and $\delta^{18}O_{NO_2-,eq}$ are the oxygen isotope ratios of

nitrite at the initial time point and at isotope equilibrium with water, respectively, k is the rate constant of the oxygen isotope exchange, and t is the reaction time. The half-life ($t_{1/2}$) of the oxygen isotope exchange (50% exchange) was expressed as follows:

$$t_{1/2} = (\log 2)/k \quad (7)$$

The equilibrium fractionation of oxygen isotopes between nitrite and water ($^{18}\varepsilon_{eq}$) was calculated as follows:

$$^{18}\varepsilon_{eq} = [(^{18}O/^{16}O)_{NO_2-} / (^{18}O/^{16}O)_{H_2O} - 1] \times 1,000 = \delta^{18}O_{NO_2-} - \delta^{18}O_{H_2O} \quad (8)$$

The ammonia/ammonium ratio in aqueous medium was calculated from the equilibrium constant K_a of the ammonium dissociation reaction [$NH_4^+ + OH^- \leftrightarrow NH_3(aq) + H_2O$]. The temperature (T [in Kelvin]) dependence of K_a was determined according to the method of Olofsson (38) as follows:

$$pK_a = 2,533/T - 0.5936 \times \ln T + 4.127 \quad (9)$$

The nitrogen isotopic equilibrium between ammonia and ammonium was calculated from the empirical relationship determined from the experimental temperature range from $23^\circ C$ to $70^\circ C$ (39):

$$^{15}\varepsilon_{eq}(NH_3(aq)/NH_4^+) = -25.94 \times 1,000/T + 42.25 \quad (10)$$

Analyses of 16S rRNA and *Nitrospira amoA* genes. DNA extraction from the microbial mat and bioreactor enrichment samples was performed using the ISOIL for Beads Beating kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. PCR amplification was performed using the Premix Ex Taq Hot Start version (TaKaRa Bio, Otsu, Japan), and the PCR mixture was prepared according to the manufacturer's instructions. For PCR amplification, the whole prokaryotic 16S rRNA gene-targeted primer pair 530F and 907R (40) was used, adhering to the PCR conditions previously described (40). PCR products were checked for size by electrophoresis on a 1.5% agarose gel using RedSafe stain (FroggaBio Inc., Toronto, Canada), purified using the MinElute gel extraction kit (Qiagen, Venlo, Netherlands), and quantified using a Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Life Technologies, Carlsbad, CA). To ligate Ion Torrent adapters, purified PCR products were subjected to end repair, ligation, and nick repair reactions using the Ion Plus fragment library kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The adapter-ligated PCR products were subjected to emulsion PCR using the Ion PGM Hi-Q sequencing kit (Life Technologies) and OneTouch and OneTouch ES instruments (Life Technologies) according to the manufacturer's protocol. Sequencing was performed using an Ion Torrent PGM (Life Technologies) and a 314 chip with the Ion PGM Hi-Q sequencing kit (Life Technologies). After sequencing, the shorter (<100 bp) and low-quality sequences were removed using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) with the following parameters: a Phred quality score of above 20 and a minimum length of 100 bp. Next, primer and the Ion Torrent adapter sequences were trimmed, and potential chimera sequences were removed using UCHIME software (41). The trimmed sequences were subjected to basic local alignment search tool (BLAST) analysis against a locally installed version of the ARB-SILVA database SSU Ref NR 199.1 (42, 43). The BLAST results were visualized using MEGAN software (44) with the following lowest-common-ancestor (LCA) parameters: minimum score, 50; maximum expected, 10^{-30} ; top percent, 10.0; minimum support percent, 0.0; minimum support, 1; LCA percent, 100.0; and minimum complexity, 0.0. To clarify the heterogeneity of *Thaumarchaeota* populations in the samples, all *Thaumarchaeota* sequences were subjected to a BLAST search against the reference thaumarchaeal 16S rRNA gene sequences used for Fig. S3 in the supplemental material. Detailed methods of archaeal 16S rRNA gene-based clone analysis and *Nitrospira amoA* gene PCR amplification are described in the supplemental material.

FIG 2 Prokaryotic 16S rRNA gene tag sequence compositions of the original microbial mat and enriched samples. In total, 19,901 and 14,973 sequences were identified in the original microbial mat (before incubation) and enriched samples (45 days after incubation). The “*Ca. Nitrosocaldus*” sp. within the phylum *Thaumarchaeota* is defined as the tag sequences exhibiting $\geq 97\%$ sequence similarity to the clonal sequence obtained in this study (see also Fig. S3 and S4 in the supplemental material). Taxonomic groups presenting $< 1.5\%$ of the total sequences were contained in the other phyla.

Accession number(s). The raw tag sequencing data obtained were deposited in the DDBJ Sequence Read Archive under accession number [DRA003792](https://www.ncbi.nlm.nih.gov/sra/DRA003792).

RESULTS

Microbial composition of the *in situ* mat community and enrichment culture. At the time of field sampling, the concentrations of ΣNH_3 , nitrite, and nitrate in the mat interstitial water were $91\ \mu\text{M}$, $78\ \mu\text{M}$, and $14\ \mu\text{M}$, whereas those in the geothermal stream water flowing over the microbial mat were $161\ \mu\text{M}$, $12\ \mu\text{M}$, and $17\ \mu\text{M}$, respectively. The chemical characteristics strongly suggest the production of nitrite from microbial ammonia oxidation within the mat community. An ammonia-oxidizing microbial community was developed and preserved in a continuous-flow bioreactor at 70°C during 791 days of operation under aerobic conditions. Based on the chemical characteristics of the influent and effluent solutions of the bioreactor, ammonia-consuming nitrite production was observed ($56\ \mu\text{M}$ ΣNH_3 consumption and $39\ \mu\text{M}$ nitrite production at 5 days of enrichment, as opposed to $89\ \mu\text{M}$ ΣNH_3 consumption and $119\ \mu\text{M}$ nitrite production at the time of batch culture experiments), whereas less nitrate was produced (up to $3\ \mu\text{M}$ [see Fig. S2 in the supplemental material]).

To investigate the shift in the microbial community during enrichment, 16S rRNA gene tag sequencing analysis was applied to the samples before and 45 days after enrichment (Fig. 2). After sequence processing, totals of 19,901 and 14,973 16S rRNA gene tag sequences were obtained from the original microbial mat and enriched samples, respectively. A compositional shift in the tag sequences became evident after enrichment culture. The predominant populations ($> 5\%$ of the total) in the original mat sample were *Thaumarchaeota*, OP1 (“*Candidatus* Acetothermus”), *Aquificae*, *Chloroflexi*, and *Proteobacteria*, whereas those in the enrichment sample were *Thaumarchaeota*, *Deinococcus-Thermus*, OD1, and *Chloroflexi*. After bioreactor cultivation, three groups exhibited an increase in relative sequence abundance: *Thaumar-*

chaeota (10.0% to 17.1%), *Deinococcus-Thermus* (2.5% to 34.8%), and OD1 (3.1% to 10.0%).

The 16S rRNA gene tag sequence data suggest that a heterogeneous *Thaumarchaeota* community was present in the samples. To clarify their composition and taxonomic positions, we constructed archaeal 16S rRNA gene-based clone libraries from the samples. We examined 160 archaeal clonal sequences (81 and 79 clones were obtained from the microbial mat and enrichment libraries, respectively). All the sequences were affiliated with a single phylotype within the phylum *Thaumarchaeota*. The closest cultured relative of the clonal sequence was “*Ca. Nitrosocaldus yellowstonii*” strain HL72, with a sequence similarity of 95.3% (see Fig. S3 in the supplemental material). The level of 16S rRNA gene sequence similarity suggests that our thaumarchaeon is distinct from strain HL72 but a member of “*Candidatus* Nitrosocaldus.” Using BLAST analysis with the reference sequences displayed in Fig. S3, including our clonal sequence, we checked whether “*Ca. Nitrosocaldus*” and the other *Thaumarchaeota* were present in the samples. Most thaumarchaeal tag sequences exhibited $\geq 97\%$ sequence similarity to the clonal sequence that we obtained (88.8% in the mat sample and 84.2% in the reactor enrichment sample) (see Fig. S4). This strongly indicates that most thaumarchaeal populations belong to “*Ca. Nitrosocaldus*” sp. The analysis also indicates that other members of *Thaumarchaeota* are in fact present (see Fig. S4), although they represent minor populations. These are closely related to the ThAOA clade, but detailed taxonomic classification of these sequences is not possible due to short sequence length.

AOB-affiliated sequences were detected in both samples, but they were very minor populations. *Nitrosococcus* (0.03% in the total) and *Nitrospira* (0.04%) sequences were found in the mat sample, and *Nitrospira* sequences (0.03%) were detected in the enrichment sample. The *Nitrospira* sequences were closely related to a recently discovered complete nitrifying “*Candidatus* Nitrospira inopinata” organism (ca. 98% sequence similarity). To in-

investigate whether *Nitrospira* had the potential to oxidize ammonia, we performed PCR with a “*Ca. Nitrospira inopinata*” *amoA* gene-specific primer set. No *amoA* genes could be amplified from either of the two samples. It remains to be seen whether *Nitrospira* has the ability to oxidize ammonia. If *Nitrospira* oxidized ammonia in the bioreactor, the effect on ammonia oxidation was very limited due to the presence of very minor populations. On the other hand, sequences related to nitrite-oxidizing bacteria were not found in either of the two samples, except for the *Nitrospira* sequences, although a small amount of nitrate was detected in the effluent of the bioreactor. Considering these facts, the *Nitrospira* organisms in the bioreactor may function as nitrite oxidizers.

The successful enrichment of the ammonia-oxidizing microbial community using the continuous-flow reactor was reproduced twice under the same conditions. In all bioreactor enrichments, “*Ca. Nitrosocaldus*” and *Deinococcus-Thermus* sequences were observed to increase in terms of relative abundance in the 16S rRNA gene phylotype composition compared with the compositions of the original microbial mat communities. “*Ca. Nitrosocaldus*” represented the potential ammonia-oxidizing population in the enrichment cultures (data not shown).

Compositional and isotopic characteristics of nitrogen compounds during ammonia oxidation. In the continuous-culture experiments with 14 mM ΣNH_3 ($\delta^{15}\text{N} = -5.8\text{‰}$), nitrite was produced in concentrations of 56 to 71 μM and with $\delta^{15}\text{N}$ values of $-37.8\text{‰} \pm 0.5\text{‰}$ ($n = 3$) when the mean residence time of the medium in the reactor was 0.8 h. The nitrate concentration in the effluent solution was 4 μM , whereas that in the influent solutions was 2 μM . In the negative-control experiment that supplied 14 mM ΣNH_3 to the sterilized culture sample, the nitrite and nitrate concentrations in the effluent solution were the same as those in the influent solution ($0.4 \pm 0.1 \mu\text{M}$ for nitrite and $0.5 \pm 0.3 \mu\text{M}$ for nitrate) when the mean residence time of the medium in the reactor was 0.8 h. Given the predominance of the “*Ca. Nitrosocaldus*”-like 16S rRNA gene phylotypes in the enrichment culture as shown by the tag sequencing and clonal analyses, these results suggest the occurrence of ammonia oxidation by “*Ca. Nitrosocaldus*” in the enrichment culture, although we cannot exclude the possibility of ammonia oxidation by the other *Thaumarchaeota* members that existed in minor populations in the culture. It is also speculated that the small increase in nitrate concentration in the effluent solution (compared with that in the influent solution) is derived from nitrite oxidation by *Nitrospira* spp., indicated as minor tag sequence components in the enrichment culture. The nitrogen isotope effect for ammonia oxidation was $32.0\text{‰} \pm 1.4\text{‰}$ (weighted mean $\pm 2\sigma$; $n = 3$) from equation 4 in the case of no nitrite oxidation. The calculated value of $^{15}\epsilon_{\text{k},\Sigma\text{NH}_3/\text{NO}_2^-}$ was $32.8\text{‰} \pm 2.2\text{‰}$, even if we considered a potential contribution of nitrite oxidation to $\delta^{15}\text{N}_{\text{NO}_2^-}$. The difference between the two calculation was negligible, because the fractions of nitrite potentially consumed were small ($<4\%$) and the associated isotopic alteration of $\delta^{15}\text{N}_{\text{NO}_2^-}$ was $<0.8\text{‰}$ ($^{15}\epsilon_{\text{k}} = -20\text{‰}$ for nitrite oxidation by thermophilic *Nitrospira* members) (12).

In all batch culture experiments, the nitrite concentration progressively increased with decreasing ΣNH_3 concentration (Fig. 3). From the early to the intermediate periods of the experimental time series, the sum of ΣNH_3 and nitrite concentrations was nearly equal to the initial concentration of ΣNH_3 (approximately 150 μM) when the ΣNH_3 concentration was higher than 10 μM . The nitrite concentration became 20 to 40 μM higher than the

FIG 3 Concentrations of inorganic nitrogen compounds during batch culture experiments. Identical colors indicate data obtained from the same batches.

initial concentration of ΣNH_3 when the ΣNH_3 concentration in the medium became below 10 μM . The nitrate concentration was quite constant (2 to 8 μM) throughout the experiments. In the negative-control experiment using the sterilized enrichment culture, the nitrite and nitrate concentrations were also quite constant (39 to 41 μM for NO_2^- and 2 to 6 μM for NO_3^-) in the presence of ΣNH_3 (130 μM). These results indicated that nearly stoichiometric oxidation of ΣNH_3 (in medium) to nitrite by “*Ca. Nitrosocaldus*” occurred when ammonia (ΣNH_3) was provided at a concentration higher than 10 μM . In contrast, when a low ΣNH_3 concentration ($<10 \mu\text{M}$) was provided, nitrite was likely to be produced not only by the oxidation of ΣNH_3 in the medium but also by the oxidation of the extra- and intracellular nitrogenous components via ΣNH_3 and/or other nitrogenous compounds (e.g., urea). The ΣNH_3 -depleted condition may energetically limit the biomass and function of ammonia-oxidizing microbial population, inducing biomass degradation on a community scale (e.g., stationary and death phases of the community). It is likely that little microbial nitrite ammonification occurred in the experiments because the ΣNH_3 -depleted condition is incompatible with active nitrite ammonification that leads to ΣNH_3 accumulation (see Fig. S5 in the supplemental material).

In all batch culture experiments, the $\delta^{15}\text{N}_{\text{NO}_2^-, \text{pro}}$ value increased with increasing nitrite concentration. When the ΣNH_3 concentration was higher than 10 μM , the $\delta^{15}\text{N}_{\text{NO}_2^-, \text{pro}}$ value was positively correlated with the value calculated by the following formula: $f/(1-f) \times \ln(f)$, where f is the fraction of initial substance remaining (Fig. 4). The positive correlation suggests that $\delta^{15}\text{N}_{\text{NO}_2^-, \text{pro}}$ is controlled by Rayleigh-type isotopic fractionation for ammonia oxidation in a closed system (equation 2) and that the potential isotope effect of nitrite ammonification on $\delta^{15}\text{N}_{\text{NO}_2^-, \text{pro}}$ is negligible (see Fig. S6 in the supplemental material). It is consistent with the above-mentioned inference that

FIG 4 Change in $\delta^{15}\text{N}_{\text{NO}_2^-}$ values during ammonia oxidation during batch culture experiments. The $\delta^{15}\text{N}_{\text{NO}_2^-}$ data on the plot were taken from the experimental periods when the ΣNH_3 concentration was higher than $10\ \mu\text{M}$.

ΣNH_3 in the medium was the sole nitrogen source of nitrite production at ΣNH_3 concentrations of $>10\ \mu\text{M}$. The nitrogen isotope effect for ammonia oxidation was $24.7\text{‰} \pm 2.1\text{‰}$ from equation 2 as the weighted mean of five experiments (Table 1).

The time series behavior of the $\delta^{18}\text{O}_{\text{NO}_2^-}$ value appeared to

TABLE 1 Nitrogen isotope effect during ammonia oxidation

Run ID	$^{15}\epsilon \pm 2\sigma$ (‰)	R	n	Reference
<i>“Ca. Nitrosocaldus”</i> ^{a,b} This study				
Batch culture experiments ($\Sigma\text{NH}_3 = 150\ \mu\text{M}$, temp = 70°C)				
Expt 1	26 ± 18	0.95	3	
Expt 2	23 ± 2	0.98	7	
Expt 3	22 ± 10	0.92	4	
Expt 4	27 ± 6	0.94	8	
Expt 5	26 ± 2	0.99	9	
Weighted mean ^c	24.7 ± 2.1			
Continuous-culture experiments ($\Sigma\text{NH}_3 = 14\ \text{mM}$, temp = 70°C)				
	32.0 ± 1.4		3	
<i>“Ca. Nitrosopelagicus brevis”</i> CN25 ^b 24				
<i>Nitrosomonas marina</i> ^d 46	14			
<i>Nitrosomonas</i> sp. strain C-113a ^d 46	19			
<i>Nitrospira tenuis</i> ^d 46	25			
<i>Nitrosomonas eutropha</i> ^d 46	33			
<i>Nitrosomonas europaea</i> ^d 46	38			
<i>Nitrosomonas europaea</i> ^d 37	35			
<i>Nitrosomonas europaea</i> ^d 20	26 ± 11 ^f			

^a The “*Ca. Nitrosocaldus*” obtained in this study is a mixed thaumarchaeal community. The detailed thaumarchaeal compositions are shown in Fig. S3 and S4 in the supplemental material.

^b Archaea.

^c The weighted mean of $^{15}\epsilon$ value is calculated as follows:
 $^{15}\epsilon = \sum_{i=1}^n w_i \times ^{15}\epsilon_i$, where $w_i = 1/(2\sigma_i)^2$

^d Bacteria.

^e 2 SD in 11 experiments.

^f 2 SD in nine experiments.

FIG 5 Time course behavior of the $\delta^{18}\text{O}_{\text{NO}_2^-}$ value during ammonia oxidation in batch culture experiments with water, of which the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values were 116‰ (A), 41‰ (B), and -8‰ (C). Error bars (1σ) were assigned to the $\delta^{18}\text{O}_{\text{NO}_2^-}$ values. Abbreviation: VSMOW, Vienna Standard Mean Ocean Water.

depend on the $\delta^{18}\text{O}$ value of water in the medium ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$). During the batch culture experiments with water in which $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was 116‰, $\delta^{18}\text{O}_{\text{NO}_2^-}$ increased from 5‰ to 90‰; however, the rate of increase in $\delta^{18}\text{O}_{\text{NO}_2^-}$ value was decreased with time (Fig. 5). The increase in the $\delta^{18}\text{O}_{\text{NO}_2^-}$ value was caused mainly by the mixing of two components: one is the carryover nitrite from the enrichment transfer ($\delta^{18}\text{O}_{\text{NO}_2^-} = 5\text{‰}$) and the other is the nitrite

TABLE 2 Oxygen isotope fractionation and exchange during ammonia oxidation at 70°C^a

Time (min)	$\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$		X_{AOA}	$^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$ (‰)	<i>n</i>
	Slope	<i>y</i> intercept			
0–195	0.65 ± 0.05	6.4 ± 3.5	0.31 ± 0.10	16 ± 11	6
195–411	0.79 ± 0.04	2.2 ± 2.5	0.57 ± 0.08	46 ± 14	5
411–574	0.73 ± 0.09	4.8 ± 4.3	0.46 ± 0.17	27 ± 18	5
574–778	0.74 ± 0.07	5.5 ± 3.6	0.49 ± 0.13	25 ± 16	4
778–1,044	0.71 ± 0.05	5.6 ± 3.2	0.42 ± 0.11	22 ± 12	4
Weighted mean ^b	0.73 ± 0.07	4.5 ± 1.5	0.46 ± 0.14	26 ± 15	

^a 2σ error.^b The weight of datum in each of the sampling period is defined as follows: $w_i = 1/(2\sigma_i)^2$.

produced during the experiment. The nitrite produced during each of the sampling periods showed relatively constant $\delta^{18}\text{O}$ values of 85‰ ± 12‰ (weighted mean ± 2σ; *n* = 7) for experiment 1 and 88‰ ± 6‰ (*n* = 7) for experiment 2, being lower than the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value in the medium (116‰) and the isotopic equilibrium value in water (128‰ [Fig. 5A]). Similarly, the nitrite produced in the experiments with medium water for which the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ was 41‰ or –8‰ showed constant $\delta^{18}\text{O}$ values of 33‰ ± 2‰ (*n* = 9; experiment 2 [Fig. 5B]) or 0‰ ± 2‰ (*n* = 7; experiment 1 [Fig. 5C]), respectively. Thus, the $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ values positively correlated with the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values in each sampling period, with a slope of 0.73 ± 0.07 and a *y*-intercept of 4.5 ± 1.5 (weighted mean ± 2σ [Table 2; Fig. 6]).

Abiotic oxygen isotope exchange between nitrite and water.

In all abiotic experiments at pHs 5.4, 6.5, and 8.0, the nitrite concentration did not change with time (30 μM). The oxygen isotope exchange rate systematically increased with decreasing pH. At pH 5.4, the $\delta^{18}\text{O}_{\text{NO}_2-}$ value converged with the value of 51.3‰ ± 0.6‰ (*n* = 3) after 1.6 h (Fig. 7). Thus, nitrite achieved an oxygen isotope equilibrium with water in ≤1.6 h at pH 5.4. In contrast,

the $\delta^{18}\text{O}_{\text{NO}_2-}$ value failed to reach isotope equilibrium for 72 h at pHs 6.5 and 8.0. From equation 6, the exponential fitting of the experimental results for $\delta^{18}\text{O}_{\text{NO}_2-}$ versus reaction time yielded rate constants of $(2.1 \pm 0.4) \times 10^{-2} \text{ h}^{-1}$ and $(9.6 \pm 9.6) \times 10^{-3} \text{ h}^{-1}$ at pHs 6.5 and 8.0, respectively (Table 3). The half-lives of the oxygen isotope exchange (50% exchange) were then estimated to be 32.6 ± 7.9 h and >33 h at pHs 6.5 and 8.0, respectively. At pH 6.5, the rate constant at 70°C was greater than that measured for nitrite in seawater at 25°C ($1.5 \times 10^{-2} \text{ h}^{-1}$) (19). As the rate constant at a given pH tends to increase with a rise in temperature, our results are consistent with those of a previous study (19).

The equilibrium fractionation of oxygen isotopes between nitrite and water ($^{18}\epsilon_{\text{eq}}$) was determined from the oxygen isotope compositions of nitrite and water at equilibrium. The equilibrium fractionation was 12.2‰ ± 0.5‰ (1σ) at 70°C from the experimental results at pH 5.4 ($\delta^{18}\text{O}_{\text{NO}_2-} = 51.3\text{‰} \pm 0.6\text{‰}$; $\delta^{18}\text{O}_{\text{H}_2\text{O}} = 39.0\text{‰} \pm 0.3\text{‰}$). This $^{18}\epsilon_{\text{eq}}$ value is consistent with the equilibrium values calculated from the exponential fitting of the experimental results for $\delta^{18}\text{O}_{\text{NO}_2-}$ versus reaction time at pHs 6.5 and 8.0 (10‰ ± 4‰ and 12‰ ± 29‰, respectively) and close to $^{18}\epsilon_{\text{eq}}$ measured for nitrite and seawater at 28°C (10.0‰ to

FIG 6 Correlation diagram between the $\delta^{18}\text{O}$ values of water and nitrite produced at five sampling periods (0 to 195 min, 195 to 411 min, 411 to 574 min, 574 to 778 min, and 778 to 1,044 min) of batch culture experiments.

FIG 7 Time course behavior of the $\delta^{18}\text{O}_{\text{NO}_2-}$ value in the abiotic oxygen isotope exchange experiments. Error bars (1σ) were assigned to the symbols.

TABLE 3 Rate of abiotic oxygen isotope exchange between nitrite and water

pH (70°C)	k (h ⁻¹)	$t_{1/2}$ (h)
8	$(9.6 \pm 9.6) \times 10^{-3}$	>33
6.5	$(2.1 \pm 0.4) \times 10^{-2}$	32.6 ± 7.9
5.4		<1.6

12.4‰) (19). Compared with the equilibrium fractionation of oxygen isotopes between carbonate ion and water, the $^{18}\epsilon_{\text{eq}}$ value between nitrite and water tends to decrease little with a rise in temperature (19, 45). Therefore, the difference in the $^{18}\epsilon_{\text{eq}}$ values at 28°C and 70°C may be small and comparable to the analytical uncertainty of $^{18}\epsilon_{\text{eq}}$ (2‰).

DISCUSSION

Nitrogen isotope effect during ammonia oxidation by thermophilic AOA. A series of gene analyses strongly suggest that the predominant ammonia oxidizer in the enrichment cultures was “*Ca. Nitrosocaldus*.” Nitrite production through bacterial ammonia oxidation and comammox (complete ammonia oxidation) was probably negligible in the enrichment cultures due to very minor populations of the corresponding microorganisms. It is unknown whether few *Thaumarchaeota* members other than “*Ca. Nitrosocaldus*” have the capability of ammonia oxidation. Collectively, the measured isotope effects for ammonia oxidation in this study mostly reflect the isotope effects by “*Ca. Nitrosocaldus*.” The nitrogen isotope effects for ammonia oxidation were $24.7\text{‰} \pm 2.1\text{‰}$ in the batch culture experiments (2σ ; $n = 5$) and $32.0\text{‰} \pm 1.4\text{‰}$ for the continuous-culture experiments (2σ ; $n = 3$). These $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values fall within the range of reported $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values for mesophilic ammonia-oxidizing microorganisms ($22\text{‰} \pm 5\text{‰}$ for AOA and 14‰ to 38‰ for AOB) (20, 24, 37, 46). Based on the observed variation in $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values for AOA, including thermophilic *Thaumarchaeota*, the difference in nitrogen isotope fractionation during ammonia oxidation between AOA and AOB is not apparent despite the potential different ammonia oxidation pathways between the two groups (25, 26).

Environmental and biological factors that may have led to the 7‰ difference in the $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ value between the batch culture and continuous-culture experiments include the effect of ambient pH on the kinetics of a rate-limiting step in ammonia oxidation. The ambient pH condition significantly affects the $\text{NH}_3/\text{NH}_4^+$ ratio and consequently the $\delta^{15}\text{N}$ value of the substrate for ammonia oxidation ($\delta^{15}\text{N}_{\text{NH}_3}$), whereas the overall isotope effect of the multistep process is controlled mainly by the isotope effect of the rate-limiting step (47). In our experiments, the mass balance of ΣNH_3 and nitrite suggests that intermediate products in the ammonia oxidation pathway do not significantly accumulate. The potential rate-limiting step in ammonia oxidation would be ammonia diffusion across the cell envelope or the AMO-catalyzed reaction.

It is generally considered that AOA have cell envelopes assembled by an array of a single type of protein (S-layer), and the active site of AMO is exposed to pseudoperiplasm, located between the S-layer and the cytoplasmic membrane (26, 48). The rate of ammonia diffusion (J_{NH_3}) across the S-layer of AOA is expressed using Fick's law:

$$J_{\text{NH}_3} = P_{\text{NH}_3} \times A \times \Delta C \quad (11)$$

where A is the surface area per cell (approximately $3 \mu\text{m}^2$ for “*Ca. Nitrosocaldus yellowstonii*”) (49) and ΔC is the difference in ammonia concentrations inside and outside the S-layer. Because the S-layer is more porous than the cytoplasmic membrane, the permeability of the S-layer for ammonia (P_{NH_3}) is assumed to be higher than that of the liposome, which is composed of glycerol dialkyl glycerol tetraether lipids ($P_{\text{NH}_3} = 2.4 \times 10^{-4} \pm 0.9 \times 10^{-4} \text{ m s}^{-1}$) (50), the predominant membrane lipids of “*Ca. Nitrosocaldus yellowstonii*” (5). The rate of ammonia diffusion in our experiments (with 10 to 6,000 μM NH_3) was then suggested to be higher than 10^1 to $10^4 \text{ fmol cell}^{-1} \text{ h}^{-1}$ when the ammonia content in the pseudoperiplasm was much lower than that in the medium. In contrast, the rate of cell-specific ammonia oxidation for “*Ca. Nitrosocaldus*” was 1 to 2 $\text{fmol cell}^{-1} \text{ h}^{-1}$ under optimal growth conditions (calculated from the data of de la Torre et al. [5]). These estimates suggest that the ammonia oxidation rate would be much lower than the ammonia diffusion rate, constrained, however, by the rate of the AMO-catalyzed reaction. By analogy with the nitrogen isotope effect of ammonia assimilation (51, 52), the $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values observed in this study would be described with the isotopic equilibrium between ammonia and ammonium ($^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3} = \delta^{15}\text{N}_{\Sigma\text{NH}_3} - \delta^{15}\text{N}_{\text{NH}_3}$) and the kinetic isotope effect associated with the AMO-catalyzed reaction ($^{15}\epsilon_{\text{amo}}$ [Fig. 1B]):

$$^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-} = ^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3} + ^{15}\epsilon_{\text{amo}} \quad (12)$$

According to equation 12, the 7‰ difference in $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ between the batch and continuous culture experiments would be caused by the different ambient pH conditions, leading to different $^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3}$ values. During the continuous-culture experiments (14 mM ΣNH_3), the pH of the medium was 8.0 ± 0.1 at 70°C. The fraction of NH_3 to ΣNH_3 was then calculated to be $43\% \pm 5\%$ (from equation 9), and the $^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3}$ value in the medium was calculated to be $19\text{‰} \pm 2\text{‰}$ using -34‰ for the isotopic equilibrium between ammonia and ammonium at 70°C (39). In contrast, the pH of the medium was 8.2 to 8.6 at 70°C during the batch culture experiments (20 to 150 μM ΣNH_3). The calculated fractions of NH_3 to ΣNH_3 and $^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3}$ were 55% to 75% and $12\text{‰} \pm 3\text{‰}$, respectively. Thus, the $^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3}$ value in the batch culture experiments was 7‰ smaller than that in the continuous-culture experiments, explaining the 7‰ difference in $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$.

Conclusively, both the batch culture and continuous-culture experiments revealed that the ϵ_{amo} value of thermophilic “*Ca. Nitrosocaldus*” could be 13‰ ($^{15}\epsilon_{\text{amo}} = ^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-} - ^{15}\Delta_{\Sigma\text{NH}_3 - \text{NH}_3} = 25\text{‰} - 12\text{‰}$ for the batch culture experiments; equation 12). However, the ϵ_{amo} values of the other microorganisms are currently uncertain for the following reasons: (i) AMO has never been purified and is often inactive in cell-free systems, and (ii) the $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values of the other microorganisms have been determined under low- NH_3 conditions.

Under such conditions, ammonia diffusion can become the rate-limiting step in the ammonia oxidation pathway, and the ammonium uptake via membrane-bound transporters and its isotope effect should be considered. For example, the reported $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ values of marine AOA ($22\text{‰} \pm 6\text{‰}$; $n = 24$) (24) are 20‰ smaller than the calculated value of $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ ($>44\text{‰}$ at 22°C and pH 8.0), if ammonia diffusion is much faster

TABLE 4 Oxygen isotope effects during ammonia oxidation by six different species of ammonia-oxidizing archaea and bacteria

Species	$\delta^{18}\text{O}_{\text{H}_2\text{O}} - \delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$		X_{AOA}	$^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$ (‰)	Habitat(s)	Reference
	Slope	y-intercept				
" <i>Ca. Nitrosocaldus</i> " ^{a,b}	0.73	4.5	0.46	26	Hot spring	This study
" <i>Ca. Nitrosopelagicus brevis</i> " CN25 ^b	0.54	3~5	0.08 ± 0.02	Not reported	Ocean	14
<i>Nitrosococcus oceanus</i> ^c	0.62	-1.9	0.25	38	Ocean	23
<i>Nitrosomonas</i> sp. strain C-113a ^c	0.61	5.3	0.23	18	Ocean	23
<i>Nitrosomonas europaea</i> ^c	0.56	3.5	0.11	19	Soil, freshwater	23
<i>Nitrospira briensis</i> ^c	0.5	-3.3	0.01	30		23

^a The "*Ca. Nitrosocaldus*" obtained in this study is a mixed thaumarchaeal community. The detailed thaumarchaeal compositions are shown in Fig. S3 and S4 in the supplemental material.

^b Archaea.

^c Bacteria.

than enzymatic ammonia oxidation and isotopically lighter ammonia is preferentially oxidized by AMO ($\epsilon_{\text{amo}} > 0\text{‰}$) (calculated from equations 9, equations 10, and equations 12). This suggests the active uptake and oxidation of ammonium by marine AOA. Although the *in vitro* measurement of ϵ_{amo} is necessary to fully understand the nitrogen isotope systematics of ammonia oxidation at an organism level, this study first and foremost highlights the isotopic fractionation effect of the AMO-catalyzed reaction.

Based on the findings described above, it is predicted that the nitrogen isotope effect for ammonia oxidation in geothermal environments would be highly controlled by ambient pH and temperature conditions. The ambient temperature and pH conditions of *Nitrosocaldus* population habitats are expected to be 57 to 80°C and 6.4 to 8.3, respectively, based on laboratory experiments and field observations (5, 12, 30, 53). Thus, the $^{15}\epsilon_{\text{k},\Sigma\text{NH}_3/\text{NO}_2-}$ values in geothermal environments are predicted to be 9‰ to 36‰ (from equations 9, 10, and 12) if we use the ϵ_{amo} value of 13‰. The predicted range of $^{15}\epsilon_{\text{k},\Sigma\text{NH}_3/\text{NO}_2-}$ values is consistent with the $^{15}\epsilon_{\text{k},\Sigma\text{NH}_3/\text{NO}_2-}$ values observed in the geothermal stream environment where the ammonia-oxidizing microbial mat community was developed (14‰ to 25‰) (12).

Oxygen isotopic exchange and fractionation during ammonia oxidation by thermophilic AOA. The relationship between $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ in the batch culture experiments provides valuable insight into the oxygen sources and enzyme level oxygen isotope effects of ammonia oxidation by thermophilic *Thaumarchaeota* because the potential isotope effect of nitrite ammonification on $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ was negligible (see Fig. S7 in the supplemental material). The slopes of linear regressions of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ versus $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ values were not 1 but approximately 0.7 (Fig. 6), suggesting that water is not the sole oxygen source of nitrite produced by "*Ca. Nitrosocaldus*." For example, AOB can incorporate one oxygen atom from dioxygen during the oxidation of ammonia to hydroxylamine and the second oxygen atom from water during the oxidation of hydroxylamine to nitrite (Fig. 1A) (54, 55). Given that hydroxylamine is a common intermediate in bacterial and archaeal ammonia oxidation (56), dioxygen would be the other oxygen source of nitrite produced by "*Ca. Nitrosocaldus*." This inference is consistent with the fact that the $\delta^{18}\text{O}_{\text{NO}_2}$ value has an intermediate property between the $\delta^{18}\text{O}_{\text{O}_2}$ (23.5‰) (57) and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values (Fig. 5).

Given the dual sources of oxygen atoms of nitrite, the following equation for bacterial ammonia oxidation (23) may be applied to explain the $\delta^{18}\text{O}$ value of nitrite produced from thermophilic archaeal ammonia oxidation:

$$\begin{aligned}\delta^{18}\text{O}_{\text{NO}_2-\text{pro}} &= [0.5(\delta^{18}\text{O}_{\text{O}_2} - ^{18}\epsilon_{\text{k},\text{O}_2}) + 0.5(\delta^{18}\text{O}_{\text{H}_2\text{O}} \\ &\quad - ^{18}\epsilon_{\text{k},\text{H}_2\text{O}})](1 - X_{\text{AOA}}) + (\delta^{18}\text{O}_{\text{H}_2\text{O}} + ^{18}\epsilon_{\text{eq}})(X_{\text{AOA}}) \\ &= 0.5(1 + X_{\text{AOA}})\delta^{18}\text{O}_{\text{H}_2\text{O}} + 0.5(1 - X_{\text{AOA}})(\delta^{18}\text{O}_{\text{O}_2} \\ &\quad - ^{18}\epsilon_{\text{k},\text{O}_2} - ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}) + (X_{\text{AOA}})^{18}\epsilon_{\text{eq}} \quad (13)\end{aligned}$$

where X_{AOA} is the fraction of nitrite oxygen atoms that have equilibrated with water catalyzed by AOA. The kinetic isotope effects for oxygen atom incorporation from dioxygen and water are represented as $^{18}\epsilon_{\text{k},\text{O}_2}$ and $^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$, respectively (Fig. 1). The abiotic oxygen isotope exchange experiments ensure that the fraction of abiotic oxygen isotope exchange between nitrite and water was limited during thermophilic archaeal ammonia oxidation (<0.03 at pHs 8.2 to 8.6 and 70°C; see "Abiotic oxygen isotope exchange between nitrite and water" above). The linear regression of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ versus $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ values thus yields the fractional exchange of nitrite oxygen atoms catalyzed by AOA (X_{AOA} ; calculated from the slope) and the overall kinetic isotope effect for oxygen atom incorporation ($^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$; calculated from the intercept) when a constant $\delta^{18}\text{O}_{\text{O}_2}$ value is assumed (23.5‰) (57). The assumption of a constant $\delta^{18}\text{O}_{\text{O}_2}$ is justified from the experimental design that only <5% of dioxygen in the gas phase of the medium would be consumed in the batch culture experiments.

The values for X_{AOA} and $^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$ did not change during the experiments for thermophilic archaeal ammonia oxidation and are likely to be independent of the growth state of "*Ca. Nitrosocaldus*" (Table 2). Our experiments suggest that the X_{AOA} value of thermophilic *Thaumarchaeota* was 0.46 ± 0.14 (2σ ; $n = 5$) and was higher than those of marine AOA (0.08) and several mesophilic AOB strains (0.01 to 0.25) (14, 23). A higher temperature would promote greater nitrite oxygen isotope exchange with intracellular water and cause a higher X_{AOA} in cases of thermophilic *Thaumarchaeota* than in cases of mesophilic ammonia-oxidizing microorganisms. In contrast, the value of $^{18}\epsilon_{\text{k},\text{O}_2} + ^{18}\epsilon_{\text{k},\text{H}_2\text{O}}$ for thermophilic *Thaumarchaeota* was suggested to be $26\text{‰} \pm 15\text{‰}$ (2σ ; $n = 5$) and was comparable to that of the mesophilic AOB (18‰ to 30‰ [Table 4]) despite the significantly different growth temperatures and ammonia oxidation pathways between the two groups.

An alternative explanation for the 70% dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (Fig. 6) is the involvement of nitric oxide (NO) in nitrite production. In contrast to bacterial ammonia oxidation, NO is suggested to be directly involved in archaeal ammonia oxidation (58) and is hypothesized to be a coreactant in the oxida-

tion of hydroxylamine by a proposed copper enzyme (Fig. 1B) (18). In the model of NO-dependent oxidation of hydroxylamine (18), nitrite is produced by the following enzymatic reaction: $\text{NH}_2\text{OH} + \text{NO} + 2\text{H}_2\text{O} \rightarrow 2\text{NO}_2^- + 7\text{e}^- + 7\text{H}^+$ (reaction 1), whereas NO is produced from nitrite reduction. It follows that 66% of oxygen atoms of nitrite originate from water, whereas 34% of oxygen atoms of nitrite originate from dioxygen if the oxygen-bearing compounds in reaction 1 (nitrite, hydroxylamine, NO, and water) reach oxygen isotope steady state and do not exchange oxygen atoms with each other. Additional oxygen atom exchange between nitrite and water (and possibly between NO and water) eventually set the dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (Fig. 1) and can explain the high dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ for thermophilic *Thaumarchaeota*. In contrast, to explain the 54% dependence of $\delta^{18}\text{O}_{\text{NO}_2}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ for marine AOA (“*Ca. Nitrosopelagicus brevis*” [14]) in the NO-dependent oxidation model, at least 84% of the oxygen atom of NO should originate from dioxygen (calculated from the oxygen isotope mass balance in reaction 1). Such NO with a high contribution of the O atom from dioxygen is not easily produced from the reduction of nitrite, of which only 46% of oxygen atoms originate from dioxygen. Therefore, even if NO-dependent oxidation of hydroxylamine is performed by some AOA members, this will not be the common step of archaeal ammonia oxidation. In the future, isotope-labeling experiments with $^{15}\text{N}^{18}\text{O}$ are necessary for AOA members of various lineages to clarify NO functions and the oxygen isotope effect during archaeal ammonia oxidation.

Application of nitrogen and oxygen isotopes of nitrite to ecological studies. The relationship between the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ values obtained is valuable for predicting the oxygen isotope ratio of nitrite produced in various geothermal environments. The $\delta^{18}\text{O}$ value of geothermal water depends on the origin of source water (meteoric water, seawater, or magmatic water) and geographic location (latitude or distance from shoreline), exhibiting a site-dependent variation ranging from -20‰ to 7‰ (59, 60). The $\delta^{18}\text{O}$ value of nitrite produced from thermophilic AOA such as “*Ca. Nitrosocaldus*” is thus expected to vary from -10‰ to 10‰ in natural environments, using the relationship of $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}} = (0.73 \pm 0.07) \times \delta^{18}\text{O}_{\text{H}_2\text{O}} + (4.5 \pm 1.5)$ determined in this study (Table 2). The calculated value of $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ was significantly lower than the $\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$ value at 70°C when the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value was higher than -16‰ , although the $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ value was indistinguishable from the $\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$ value in the case of a $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value of -17‰ to -20‰ (Fig. 8). Thus, ammonia oxidation by thermophilic AOA can be discerned from $\delta^{18}\text{O}_{\text{NO}_2-}$ in geothermal areas such as convergent plate boundaries in middle and low latitudes, of which the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value is generally higher than -16‰ (59, 61). In contrast, the $\delta^{18}\text{O}_{\text{NO}_2-}$ value may not be applicable as a geochemical indicator of ammonia oxidation in geothermal environments with $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values of -17‰ to -20‰ (e.g., Heart Lake Geyser Basin in the Yellowstone geothermal system) (62). It should be noted that the $\delta^{18}\text{O}_{\text{NO}_2-}$ value is also associated with microbial nitrite oxidation and/or denitrification (nitrate reduction to nitrite and nitrite reduction) because these microbiological processes can imprint the nonequilibrium oxygen isotope signature on nitrite (21, 22).

Combining the $\delta^{18}\text{O}_{\text{NO}_2-}$ value and kinetic data of abiotic oxygen isotope exchange between nitrite and water provides a basis for estimating nitrification and denitrification rates. This study

FIG 8 Relationship between $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ and $\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$ expected in geothermal environments. Error bars (2σ) were assigned to the symbols.

suggests that the half-life of abiotic oxygen isotope exchange between nitrite and geothermal water would be >33 h when the pH is greater than 6.6 at 70°C (see “Abiotic oxygen isotope exchange between nitrite and water” above). On the other hand, the biological nitrite turnover rate in geothermal environments would be variable in habitats where ammonia-oxidizing, nitrite-oxidizing, and denitrifying microorganisms are highly associated with environmental conditions such as temperature and pH. If the nitrite turnover rate is <33 h in a geothermal habitat of circumneutral pH, the $\delta^{18}\text{O}_{\text{NO}_2-}$ value would represent the signature that is different from the isotopic equilibration value ($\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$). In this case, the *in situ* rates of nitrification and denitrification may be quantified from the difference between $\delta^{18}\text{O}_{\text{NO}_2-}$ and $\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$ values. In addition to such geochemical signatures, molecular ecological quantification using specific functional genes and their transcripts will be applicable to deduce microbial community components operating ammonia oxidation, nitrite oxidation, and denitrification predominantly on an ecosystem scale (32, 63).

We finally evaluated the applicability of the dual isotopic composition of nitrite for estimating the *in situ* kinetics of nitrogen metabolism in other environments. This study suggests that the organism level nitrogen isotope effect for ammonia oxidation ($^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$) would vary with $^{15}\Delta_{\Sigma\text{NH}_3-\text{NH}_3}$ under ammonia-replete conditions (see “Nitrogen isotope effect during ammonia oxidation by thermophilic AOA” above). Because some soil environments are rich in ammonia, a variation of the species-specific $^{15}\epsilon_{\text{k}, \Sigma\text{NH}_3/\text{NO}_2-}$ value in such environments should be $<8\text{‰}$ (from equations 9, 10, and 12) even if assuming unusually high diurnal fluctuation of temperature (10°C to 30°C) and pH (5 to 8). This variation is much smaller than that assumed for calculating the *in situ* rates of nitrification and denitrification in the ocean (22‰) (24). Further, in middle- and low-latitude soil environ-

ments where the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value is often higher than -10‰ (64), the $\delta^{18}\text{O}_{\text{NO}_2-\text{pro}}$ values of mesophilic ammonia oxidizers are predicted to be significantly different from the $\delta^{18}\text{O}_{\text{NO}_2-\text{eq}}$ values (Table 4). Collectively, the *in situ* rates of biological nitrite turnover in ammonia-replete soils may be quantified more accurately than in the ocean, if nitrogen and oxygen isotopic compositions are characterized for nitrite produced from ammonia oxidizers that predominate in soils.

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