Short Communication

Nitric Oxide Production from Nitrite Reduction and Hydroxylamine Oxidation by Copper-containing Dissimilatory Nitrite Reductase (NirK) from the Aerobic Ammonia-oxidizing Archaea, *Nitrososphaera viennensis*

SHUN KOBAYASHI¹, DAISUKE HIRA², KEITARO YOSHIDA³, MASANORI TOYOFUKU³, YOSUKE SHIDA⁴, WATARU OGASAWARA⁴, TAKASHI YAMAGUCHI⁵, NOBUO AKAI¹, and MAAMORU OSHIKI*¹

¹Department of Civil Engineering, National Institute of Technology, Nagaoka College, Nagaoka, Japan; ²Department of Applied Life Science, Faculty of Biotechnology and Life Science, Sojo University, Ikeda, Kumamoto, Japan; ³Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan; ⁴Department of Bioengineering, Nagasaki University of Technology, Nagasaki, Niigata, Japan; and ⁵Department of Science of Technology Innovation, Nagasaki University of Technology, Nagasaki, Japan

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Aerobic ammonia-oxidizing archaea (AOA) play a crucial role in the global nitrogen cycle by oxidizing ammonia to nitrite, and nitric oxide (NO) is a key intermediate in AOA for sustaining aerobic ammonia oxidation activity. We herein heterologously expressed the NO-forming, copper-containing, dissimilatory nitrite reductase (NirK) from *Nitrososphaera viennensis* and investigated its enzymatic properties. The recombinant protein catalyzed the reduction of 15NO2⁻ to 15NO, the oxidation of hydroxylamine (15NH₂OH) to 15NO, and the production of 14-15N₂O from 15NH₂OH and 14NO₂⁻. To the best of our knowledge, the present study is the first to document the enzymatic properties of AOA NirK.

Key words: nitrite reduction, hydroxylamine oxidation, nitrous oxide production, ammonia oxidizing archaea, *Nitrososphaera viennensis*

Aerobic ammonia oxidation, a rate-limiting step of nitrification, drives the global nitrogen cycle (24, 40), which involves aerobic ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively) and complete ammonia oxidizers (comammox) (9, 44). Of these, AOA primarily contribute to aerobic ammonia oxidation in natural environments including soil and open ocean (19, 31, 46). AOA are affiliated with the phylum *Thaumarchaeota*, which includes phylogenetically and physiologically diverse members (6) and the soil-inhabiting archaeon *Nitrososphaera viennensis* (41). The biochemistry of aerobic ammonia oxidation by AOA has received a great deal of interest because ammonia oxidation to nitrite (NO₂⁻) proceeds in a different manner to that of AOB. AOA oxidize ammonia to hydroxylamine by ammonia monoxygenase (Amo) as well as AOB (43), while hydroxylamine is further oxidized to NO₂⁻ by an unidentified enzyme (17). All known AOA genomes lack the gene encoding hydroxylamine dehydrogenase (Hao), and the involvement of a copper-protein complex has been proposed (40, 45). In parallel with the oxidation of ammonia to NO₂⁻, AOA produce nitric oxide (NO) (22). NO is a key intermediate in AOA cells because this highly reactive molecule is essential for sustaining aerobic ammonia oxidation activity (17, 33, 36, 47). To date, the following 2 pathways have been reported as a source of prokaryotic NO formation: NO₂⁻ reduction to NO by copper-containing and cytochrome cd₁-type dissimilatory nitrite reductases (NirK and NirS, respectively) (38) and NH₂OH oxidation to NO by hydroxylamine oxidoreductase (Hao) (4, 21). Although neither nirS nor hao are found in AOA genomes (6), AOA commonly possess nirK, which is transcribed and expressed during aerobic ammonia oxidation (8, 15, 20, 37). These findings suggest that NirK is involved in NO formation in AOA cells. However, NO₂⁻ reduction to NO by AOA NirK has never been demonstrated.

Bacterial NirK have been characterized as homotrimeric enzymes, and each subunit has 2 Cu-binding sites (Type 1 and 2 Cu-binding sites). Type 1 Cu-binding sites receive an electron from an electron donor, such as type 1 Cu proteins (single-domain cupredoxins) and/or cytochrome c, and the electron is then further transferred to a type 2 Cu-binding site that is the catalytic center of NirK (14, 25). Bacterial NirK have been classified into 2 phylogenetically distinct groups (class 1 and class 2 groups) based on sequence similarities, and the NirK of the class 1 group contains linker loop and tower loop regions in the amino acid sequence (3). AOA NirK, including *Ns. viennensis* NirK, are affiliated with a distinct clade of bacterial class 1 and 2 groups (Fig. 1A). Lund et al. (20) reported that AOA NirK may be further classified into several phylogenetic clades showing specific geographic distributions. *Ns. viennensis* NirK has amino acid residues consistent with those of type 1 and 2 Cu-binding sites (His₁₀₆, His₁₄₀, and His₁₃₆ for type 1 Cu-binding sites and His₁₀₁, Cys₁₄₁, His₁₅₂, and Met₁₅₇ for type 2 Cu-binding sites) as well as the linker and tower loop regions, whereas the C terminus has unusual extensions of ~26 residues (Fig. 1B). These phylogenetic affiliations of and structural variations in *Ns. viennensis* NirK raise concerns regarding its enzymatic properties, such as specific enzymatic activity, affinity for NO₂⁻, and products of NO₂⁻ reduction.

Based on its unique sequence and lack of biochemical information, the purpose of the present study was to charac-
Prior to the present study, we aimed to isolate *Ns. viennensis* NirK from a batch culture of *Ns. viennensis* as a native enzyme. However, the activity of aerobic ammonia oxidation often disappeared when we scaled up the cultures (data not shown). Additionally, a slow growth rate ($\mu_{\text{max}} = 0.024 \text{ h}^{-1}$) (41) and low biomass concentration in the culture (ca. $10^7~10^8$ cells mL$^{-1}$) further precluded the preparation of the biomass required for protein purification. Since the biomass was difficult to obtain, we decided to use the N. oceani strain (WP 002812635.1) as a model system for our studies.

Fig. 1. Phylogeny (A) and sequence alignments (B) of prokaryotic NirK. A) A phylogenetic tree of prokaryotic NirK was constructed by the maximum likelihood method with the Jones-Taylor-Thornton model using the protein sequence of multicopper oxidase type 3 of *Nitrososphaera viennensis* (accession number; AIC14243.1) as an outgroup. Branching points that support a probability >80% in bootstrap analyses (based on 500 replicates) are shown as filled circles. The scale bar represents 10% sequence divergence. Sequence accession numbers are indicated in parentheses. B) Protein sequence alignment of *nirK*. NirK sequences were aligned using ClustalW software. Circles and triangles correspond to the amino acid residues of type 1 and 2 Cu-binding sites, respectively. Linker, Tower loop (3), and C-terminal extension regions are underlined. Abbreviations of microorganisms are as follows: *Nitrosomonas europaea* is *N. europaea*, *A. xylosoxidans* is *Achromobacter xylosoxidans*, *A. faecalis* is *Alcaligenes faecalis*, *N. gonorrhoeae* is *Neisseria gonorrhoeae*, *S. thermophilus* is *Sphaerobacter thermophilus*, and *Ns. viennensis* is *Nitrososphaera viennensis*.
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Regarding the expression of the recombinant protein in E. coli for PCR. The constructed plasmid was subjected to Sanger –1 ampicillin. in Luria-Bertani media containing 100 ng μL

Cells (JCM19564) was used as the DNA template Ns.

Sites of NdeI and HindIII in the forward and reverse primer (5’- CACCGGAGG-3’) oligonucleotide primers. The restriction nirK Ns. viennensis gene located in the genome properties. The, and investigate its enzymatic

Escherichia coli recombinant protein was dialyzed against buffer containing 20 mM Tris HCl (pH 8), 300 mM NaCl, and 0.5 mM CuSO4 at 4°C for 57 h. The protein solution was dialyzed again using the above Tris buffer without CuSO4 at 4°C for 6 h. The dialyzed recombinant protein was concentrated using a Vivaspin column (MWCO; 30 kDa) (GE Healthcare Japan, Tokyo, Japan). The recombinant protein was loaded onto a gel-filtration HiLoad 16/600 Superdex 200 pg column (GE Healthcare) to assess the molecular mass of the recombinant protein, which was 105±1.3 kDa (Fig. 2B). Since the deduced molecular mass of Ns. viennensis NirK was 39.7 kDa, the molecular mass obtained by gel filtration indicated that the recombinant protein forms a homotrimeric structure, similar to canonical NirK.

NirK have been characterized as metalloproteins showing a blue or green color spectrum, and exhibit absorption peaks at approximately 450 and/or 600 nm (3). Bacterial NirK, which belong to the class 1 group, often show a maximum absorption peak at approximately 450 nm, although an exception (Achromobacter xylosoxidans NirK) that shows a peak at 593 nm has been previously reported (16). The purified recombinant protein was pale blue in color, and showed an absorption peak at 590 nm (Fig. 2C). This feature indicated that Ns. viennensis NirK is affiliated with the subgroup of NirK showing a blue color spectrum. The blue or green color spectrum of NirK is derived from a copper atom in the type 1 Cu-binding site (14), while the type 2 Cu-binding site does not contribute to the UV or visible spectrum. The type 2 Cu-binding site shows a characteristic electron spin resonance (ESR) spectrum (7, 16); therefore, an ESR analysis was performed using a JES-FA200 spectrometer (JEOL, Tokyo, Japan) to test for the presence of the type 2 Cu-binding site in the recombinant protein. An axial type 2 Cu signal (g∥=2.24, A∥=18.31 mT, and g⊥=2.06) was found in the ESR measurement (Fig. 2D), indicating that the recombinant protein has a type 2 Cu-binding site coordinating with a copper atom. Additionally, we assessed the copper content of the recombinant protein by inducibly coupled plasma mass spectrometry (ICP-MS). The copper content was found to be 2.9 atoms per subunit of the recombinant protein, indicating that Cu was fully incorporated into the recombinant protein. Overall, the recombinant protein shared the structural and spectroscopic features of class 1 and 2 bacterial NirK, which is consistent with sequencing information.

The kinetics of NO2– reduction were examined by anoxically incubating the recombinant protein at 25°C and pH 6.5 with 15NO2– and artificial electron donors as previously described (7). All of the buffers and stock solutions were prepared anoxically as previously described (27). Two milliliters of reaction buffer (20 mM phosphate buffer, 0.1 to 1.6 mM Na15NO2, 0.5 mM benzyl viologen (BV), and 0.24 mM sodium dithionite) was dispensed into a 1-cm sealable quartz cuvette and placed in an anaerobic chamber in which the O2 concentration was maintained at lower than 1 ppm. BV was used as an artificial electron donor because it has been employed to examine the kinetics of the NO2– reduction of bacterial NirK (7, 13). The cuvette was set in a UV-VIS spectrometer UV-2700 (Shimadzu, Kyoto, Japan), and the initial absorbance of the prepared reaction mixture at a wavelength of 550 nm was approximately 2.0. The reaction was initiated by adding the recombinant protein (50 μL containing 250 μg of protein) using a gastight syringe, and the oxidation rate of reduced BV (molecular extinction coefficient, 10.4 mM1 cm1) (13) was monitored at 550 nm.
The recombinant protein reduced NO$_2^-$ by oxidizing BV, whereas no significant BV oxidation was found in the cuvette without the recombinant protein. The turnover number and $K_m$ value for NO$_2^-$ reduction by the recombinant protein were 3.1 s$^{-1}$ and 287 μM, respectively (Table 1), and the turnover number and affinity constant were markedly lower and higher, respectively, than those of other canonical NirK proteins, including those from AOB. The product of NO$_2^-$ reduction by the recombinant protein was examined using phenazine methosulfate (PMS) as the electron donor instead of BV.
When BV was used as the electron donor, NO$_2^-$ was reduced to NO, and further reduced to ammonia (approx. 60% of consumed $^{15}$NO$_2^-$) as observed in a previous study in which the NO$_2^-$ reduction activity of A. xylosoxidans NirK was examined using methyl viologen (MV) as the electron donor (1). BV and MV have low redox potentials (–350 and –440 mV, respectively) (23), resulting in the reduction of NO to NH$_3$; therefore, PMS with a higher redox potential (+80 mV) was used in the present study. The recombinant protein was incubated as described above in a 1.8-mL gas-tight vial with the addition of 0.5 mM PMS and 5 mM ascorbic acid instead of BV and dithionite, and the production of $^{15}$N-labeled gaseous compounds (i.e., N$_2$, NO, and N$_2$O) in the headspace was examined by gas chromatography mass spectrometry (GC/MS) as previously described (27). The diluted gases of $^{15}$-$^{15}$N$_2$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA), $^{14}$NO, and $^{14}$-$^{14}$N$_2$O (Cambridge Isotope Laboratories) were also analyzed to prepare standard curves for quantification. The recombinant protein reduced $^{15}$NO$_2^-$ with the oxidation of PMS, and 38 and 48% of consumed $^{15}$NO$_2^-$ were converted to $^{15}$NO and $^{15}$-$^{15}$N$_2$O, respectively. This is direct evidence to show that the recombinant protein is a NO-forming nitrite reductase. We found that the production of $^{15}$-$^{15}$N$_2$O was equal to the production of $^{15}$NO, which likely results from the reduction of $^{15}$NO$_2^-$ to H$^{15}$NO (i.e., NO$_2^- + 2e^- + 3H^+ \rightarrow$ HNO $+ H_2O$) and the chemical formation of $^{15}$-$^{15}$N$_2$O from the formed H$^{15}$NO (i.e., 2HNO $\rightarrow$ N$_2$O $+ H_2O$) (35), as previously observed for a sulfide-linked nitrite reductase (34).

Aside from NO$_2^-$ reduction, NH$_2$OH oxidation was also investigated using the recombinant protein because NH$_2$OH is produced as an intermediate during aerobic ammonia oxidation by AOA. The kinetics of NH$_2$OH oxidation were investigated by aerobically incubating the recombinant protein (245 μg mL$^{-1}$) at 30°C and pH 7.5 with 0.5 mM NH$_2$OH, with dissolved oxygen being available as an oxidant. The reaction was initiated by the addition of NH$_2$OH solution, and the concentration of NH$_2$OH was assessed colorimetrically (5). The concentration of H$_2$O$_2$, which may be produced by the oxidase activity of NirK (12), was also evaluated colorimetrically using horseradish peroxidase (Wako, Osaka, Japan) and 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Dojindo, Kumamoto, Japan) (2). As shown in Fig. S2, the recombinant protein oxidized NH$_2$OH with the production of H$_2$O$_2$. No NH$_2$OH oxidation or H$_2$O$_2$ production was observed when the incubation was repeated without the addition of the recombinant protein. The values for the turnover number and affinity constant for NH$_2$OH oxidation were 0.039 s$^{-1}$ and 97 μM (Table 1), respectively, and the value for the turnover number was two orders of magnitude lower than that observed for NO$_2^-$ reduction; therefore, the recombinant protein catalyzed NO$_2^-$ reduction more efficiently. The addition of cytochrome c from equine heart (1 mg mL$^{-1}$) or BV (0.5 mM) did not result in an increase in the reaction rate or affinity for NH$_2$OH oxidation. The product of NO$_2^-$ reduction by the recombinant protein was examined in a $^{15}$NH$_2$OH tracer experiment (29). The recombinant protein was incubated in a 1.8-mL gas-tight vial with the addition of 0.5 mM $^{15}$NH$_2$OH (Cambridge Isotope Laboratories) instead of $^{14}$NH$_2$OH. After a 2-h incubation, the concentrations of the $^{15}$N-labeled gaseous products were assessed by GC/MS. The recombinant protein oxidized $^{15}$NH$_2$OH and produced $^{15}$NO, $^{15}$-$^{15}$N$_2$O, and $^{15}$-$^{15}$N$_2$ gases quantitatively (Fig. 3), whereas the production of NO$_2^-$ and NH$_3$ was not detectable (detection limits: 50 and 100 μM, respectively). The oxidation of NH$_2$OH to NO has been

### Table 1. Enzymatic properties of archaeal and bacterial copper-containing nitrite reductase (NirK). ND; not determined.

| Organisms            | MW* (kDa) | Cu content† (atom per subunit) | Absorption (nm) | Activity‡ | Turnover (s$^{-1}$) | $K_m$ (μM) | Reference |
|----------------------|-----------|-------------------------------|-----------------|-----------|--------------------|------------|-----------|
| **Archaeal NirK**    |           |                               |                 |           |                    |            |           |
| *Nitrososphaera viennensis* | 1,715±1.5 | 2.9                           | 590             | 3.1       | 0.039              | 287        | This study |
| **Bacterial NirK (NO$_2^-$ reduction)** |           |                               |                 |           |                    |            |           |
| *Nitrosomonas europaea* | 96        | ND                            | 450, 597        | 288       | ND                 |            | 18        |
| *Nitrosococcus oceani* | 114       | 1.67                          | 455, 575        | 1,600     | 52                 |            | 16        |
| *Achromobacter xylosoxidans* | 110       | 1.99                          | 595             | 172       | 35                 |            | 14, 32    |
| *Candidatus Jettenia caeni* | 101       | ND                            | 449, 598        | 319       | 250                |            | ?         |

* Molecular weight (MW) of a trimeric NirK. The MW of Ca. Jettenia caeni NirK was calculated from amino acid sequences without a signal peptide sequence.  † Copper contents previously assessed by chemical analyses were shown. ‡ The following electron donors were used to evaluate the turn-over number of NO$_2^-$ reduction: methyl viologen for *N. europaea* and *Nc. oceani*, paeaoxazurine for *A. xylosoxidans*, and benzyl viologen for *Ns. viennensis* and Ca. Jettenia caeni NirK.
described in bacterial Hao (21); however, to the best of our knowledge, this is the first description of NH$_2$OH oxidation by NirK. We also observed $^{15}$N$_2$O production from $^{15}$NH$_2$OH oxidation, which likely resulted from the oxidation of $^{15}$NH$_2$OH to H$^{15}$NO and abiotic coupling of H$^{15}$NO, as previously described. Notably, $^{15}$N$_2$ was the major product of $^{15}$NH$_2$OH oxidation by the recombinant protein. Hydroxylamine disproportionation (30) may not be responsible for $^{15}$N$_2$ production because NH$_3$ production was not detectable in the liquid phase. The molecular mechanisms underlying the oxidation of $^{15}$NH$_2$OH to $^{15}$N$_2$ by the recombinant protein warrant further studies.

We repeated the above incubation with the addition of NH$_2$OH and NO$_3^-$ because both compounds are available in AOA cells during aerobic ammonia oxidation. Therefore, the above incubation was repeated with the addition of $^{15}$NH$_2$OH and $^{14}$NO$_2^-$ (each 0.5 mM) or $^{14}$NH$_2$OH and $^{15}$NO$_2^-$ (Cambridge Isotope Laboratories) (each 0.5 mM). In both cases, $^{14,15}$N$_2$O was the major product (Fig. 3), indicating that the recombinant protein produces N$_2$O by oxidizing NH$_2$OH using NO$_2^-$ as an electron acceptor. N$_2$O production by the denitrifier NirK from NH$_2$OH and NO$_3^-$ has been previously described (10), and the N-nitrosation reaction is involved in N$_2$O production (39). Notably, *Ns. viennensis* cells produce N$_2$O when they are incubated aerobically with NH$_3$ and NO$_2^-$ (42), although the *Ns. viennensis* genome lacks the gene encoding nitric oxide reductase (nor) that is involved in N$_2$O production from nitrifier-denitrification. Stiegler and *et al.* (42) suggested the involvement of *Ns. viennensis* NirK in the production of N$_2$O in an *Ns. viennensis* culture, and our results support this hypothesis. Although the catalytic efficiency of *Ns. viennensis* NirK for NH$_2$OH oxidation was markedly lower than that of NO$_2^-$ reduction (Table 1), *Ns. viennensis* NirK may act as an NH$_2$OH oxidase in *Ns. viennensis* cells and produce N$_2$O under oxic growth conditions. Aside from $^{14,15}$N$_2$O production, the production of $^{15}$NO and $^{15}$N$_2$O was also observed when the recombinant protein was incubated with $^{15}$NH$_2$OH and $^{15}$NO$_2^-$ (Fig. 3).

Although the recombinant protein catalyzes NO$_2^-$ reduction and NH$_2$OH oxidation, the catalytic efficiency of both reactions was low, as shown in Table 1. AOA nirK transcripts are abundant in the transcriptome (8, 11, 20, 37), suggesting the abundance of nirK genes in the *Nitrososphaera viennensis* genome. Our results reveal the core genome and recombinant proteins, and our study provides basic information that furthers our understanding of the biochemistry of AOA.

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### References

1. Abraham, Z.H.L., D.J. Lowe, and B.E. Smith. 1993. Purification and characterization of the dissimilatory nitrite reductase from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (N.C.I.M.B. 11015): Evidence for the presence of both type 1 and type 2 copper centres. Biochem. J. 296:587–593.

2. Bos, E.S., A.A. van der Doelen, N. van Rooy, and A.H.W.M. Schuurs. 1981. 3,3',5,5'-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J. Immunomassay 2:187–204.

3. Boulanger, M.J., and M.E. Murphy. 2002. Crystal structure of the soluble domain of the major anaerobically induced outer membrane protein (AniA) from pathogenic *Neisseria*: a new class of copper-containing nitrite reductases. J. Mol. Biol. 315:1111–1127.

4. Caranton, J.D., and K.M. Lancaster. 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proc. Natl. Acad. Sci. U.S.A. 114:8217–8222.

5. Fehr, D.S., and R.C. Burrell. 1955. Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27:1664–1665.

6. Hatzenpichler, R. 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Appl. Environ. Microbiol. 78:7501–7510.

7. Hirai, D., H. Toh, C.T. Mignita, H. Okubo, T. Nishiyama, M. Hattori, K. Furukawa, and T. Fujii. 2012. Anaamox organism KSU-1 expresses a NirK-type copper-containing nitrite reductase instead of a NirS-type complex with cytochrome cd. FEBS Lett. 586:1658–1663.

8. Hollibaugh, J.T., S. Gifford, S. Sharma, N. Bano, and M.A. Moran. 2011. Metatranscriptomic analysis of ammonia-oxidizing organisms in an estuarine bacterioplankton assemblage. ISME J. 5:866–878.

9. Isobe, K., and N. Ohto. 2014. Ecological perspectives on microbes involved in N-cycling. Microbes Environ. 29:4–16.

10. Iwasaki, H., and T. Matsubara. 1972. A nitrite reductase from *Achromobacter cycloclast*. J. Biochem. 71:645–652.

11. Jung, M.Y., S.J. Park, D. Min, J.S. Kim, W.L.C. Rijpstra, J.S. Sinninghe Damsté, G.J. Kim, E.L. Madsen, and S.K. Rhee. 2011. Enrichment and characterization of an autotrophic ammonia-oxidizing archaeon of mesophilic Crenarchaeotal group I, a from an agricultural soil. Appl. Environ. Microbiol. 77:8635–8647.

12. Kakutani, T., H. Watanabe, K. Arima, and T. Beppu. 1981. A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis* strain S-6. J. Biochem. 89:463–472.

13. Kataoka, K., H. Furusawa, K. Takagi, K. Yamaguchi, and S. Suzuki. 2000. Functional analysis of conserved aspartate and histidine residues located around the type 2 copper site of copper-containing nitrite reductase. J. Biochem. 127:345–350.

14. Kataoka, K., K. Yamaguchi, M. Kobayashi, T. Mori, N. Bokui, and S. Suzuki. 2004. Structure-based engineering of *Alcaligenes xylosoxidans* copper-containing nitrite reductase enhances intermolecular electron transfer reaction with pseudoazurin. J. Biol. Chem. 279:53374–53378.

15. Kerou, M., P. Offre, L. Valledor, S.S. Abby, M. Melcher, M. Nagler, W. Weckwerth, and C. Schleper. 2016. Proteomics and comparative genomics of *Nitrosophaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers. Proc. Natl. Acad. Sci. U.S.A. 113:E7937–E7946.
16. Kobayashi, K., K. Yoshimatsu, and T. Fujiwara. 2012. Expression, and molecular and enzymatic characterization of Cu-containing nitrite reductase from a marine ammonia-oxidizing gammaproteobacterium, *Nitrosococcus oceanii*. Microbes Environ. 27:407–412.

17. Kozlowski, J.A., M. Stieglmeier, C. Schleper, M.G. Klotz, and L.Y. Stein. 2016. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. ISME J. 10:1836–1845.

18. Lawton, T.J., K.E. Bowen, L.A. Sayavedra-Soto, D.J. Arb, and A.C. Rosenzweig. 2013. Characterization of a nitrite reductase involved in nitrifier denitrification. J. Biol. Chem. 288:25575–25583.

19. Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G.W. Nicol, J.J. Prosser, S.C. Schuster, and C. Schleper. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809.

20. Lund, M.B., J.M. Smith, and C.A. Francis. 2012. Diversity, abundance and expression of nitrite reductase (nirK)-like genes in marine thaumarchaea. ISME J. 6:1966–1977.

21. Maalcke, W.J., A. Dietl, S.J. Marritt, J.N. Butt, M.S.M. Jetten, J.T. Keltjens, T.R.M. Barends, and B. Kartal. 2014. Structural basis of biological NO generation by octaheme oxidoreductases. J. Biol. Chem. 289:1228–1242.

22. Martens-Habbena, W., W. Qin, R.E. Horak, et al. 2015. Expression of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. Environ. Microbiol. 17:2261–2274.

23. Nagashima, K.V.P. 2009. Redox titration for electron transfer proteins. Low Temp. Sci. 67:545–550 (in Japanese).

24. Nelson, M.B., A.C. Martiny, and J.B.H. Martiny. 2016. Global biogeography of microbial nitrogen-cycling traits in soil. Proc. Natl. Acad. Sci. U.S.A. 113:8033–8040.

25. Nojiri, M., H. Koteishi, T. Barends, and B. Kartal. 2014. Structural basis of nitrite reductase from *Alcaligenes* sp. F122. Methods Enzymol. 486:447–463.

26. Oshiki, M., M. Ali, K. Shinya-Hata, H. Satoh, and S. Okabe. 2013. Cultivation of planktonic anaerobic ammonium oxidation (anammox) bacteria by using membrane bioreactor. Microbes Environ. 28:436–443.

27. Oshiki, M., S. Ishii, K. Yoshida, N. Fujii, M. Ishiguro, H. Satoh, and S. Okabe. 2013. Nitrate-dependent ferrous iron oxidation by anaerobic ammonium oxidation (anammox) bacteria. Appl. Environ. Microbiol. 79:4087–4093.

28. Oshiki, M., R. Takagi, M. Hatamoto, T. Yamaguchi, and N. Araki. 2016. High-cell-density cultivation of *Nitrosomonas europaea* in a membrane bioreactor for performing protein purification and characterization studies. J. Gen. Appl. Microbiol. 62:330–333.

29. Oshiki, M., M. Ali, K. Shinya-Hata, H. Satoh, and S. Okabe. 2016. Hydroxylamine-dependent anaerobic ammonium oxidation (anammox) by *Candidatus Brocadia sinica*. Environ. Microbiol. 18:3133–3143.

30. Pacheco, A.A., J. McGarry, J. Kostera, and A. Corona. 2011. Techniques for investigating hydroxylamine disproportionation by hydroxylamine oxidoreductases. Methods Enzymol. 486:447–463.

31. Prosser, J.I., and G.W. Nicol. 2008. Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. Environ. Microbiol. 10:2931–2941.

32. Prudêncio, M., R.R. Eady, and G. Sawers. 1999. The blue copper-containing nitrite reductase from *Alcaligenes xylosoxidans* cloning of the nirA gene and characterization of the recombinant enzyme. J. Bacteriol. 181:2323–2329.

33. Sauder, L.A., A.A. Ross, and J.D. Neufeld. 2016. Nitric oxide scavengers differentially inhibit ammonia oxidation in ammonia-oxidizing archaea and bacteria. FEMS Microbiol. Lett. 363:fnw052.

34. Sawhney, V., and D.J.D. Nicholas. 1978. Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: purification and properties. J. Gen. Microbiol. 106:119–128.

35. Shafrinovich, V., and S.V. Lymar. 2002. Nitroxyl and its anion in aqueous solutions: Spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. Proc. Natl. Acad. Sci. U.S.A. 99:7340–7345.

36. Shen, T., M. Stieglmeier, M. Dai, T. Urich, and C. Schleper. 2013. Responses of the terrestrial ammonia-oxidizing archaeon *Ca. Nitrosopumilus maritimus* to the ammonia-oxidizing bacterium *Nitrosospira maritima* as a model system. Environ. Microbiol. 15:3146–3158.