N$_2$O Reduction by *Gemmatimonas aurantiaca* and Potential Involvement of *Gemmatimonadetes* Bacteria in N$_2$O Reduction in Agricultural Soils

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Agricultural soil is the primary N$_2$O sink limiting the emission of N$_2$O gas into the atmosphere. Although *Gemmatimonadetes* bacteria are abundant in agricultural soils, limited information is currently available on N$_2$O reduction by *Gemmatimonadetes* bacteria. Therefore, the effects of pH and temperature on N$_2$O reduction activities and affinity constants for N$_2$O reduction were examined by performing batch experiments using an isolate of *Gemmatimonadetes* bacteria, *Gemmatimonas aurantiaca* (NBRC100505$^T$). N. *aurantiaca* reduced N$_2$O at pH 5–9 and 4–50°C, with the highest activity being observed at pH 7 and 30°C. The affinity constant of *G. aurantiaca* cells for N$_2$O was 4.4 μM. The abundance and diversity of the *Gemmatimonadetes* 16S rRNA gene and nosZ encoding nitrous oxide reductase in agricultural soil samples were also investigated by quantitative PCR (qPCR) and amplicon sequencing analyses. Four N$_2$O-reducing agricultural soil samples were assessed, and the copy numbers of the *Gemmatimonadetes* 16S rRNA gene (clades G1 and G3), nosZ DNA, and nosZ mRNA were 8.62–9.65×10$^8$, 5.35–7.15×10$^8$, and 2.23–4.31×10$^9$ copies (g dry soil)$^{-1}$, respectively. The abundance of the nosZ mRNA of *Gemmatimonadetes* bacteria and OTU91, OUT332, and OTU122 correlated with the N$_2$O reduction rates of the soil samples tested, suggesting N$_2$O reduction by *Gemmatimonadetes* bacteria. *Gemmatimonadetes* 16S rRNA gene reads affiliated with OTU4572 and OTU3759 were predominant among the soil samples examined, and these *Gemmatimonadetes* OTUs have been identified in various types of soil samples.

Key words: Nitrous oxide (N$_2$O) reduction, nitrous oxide reductase nosZ, agricultural soil, *Gemmatimonadetes*, *Gemmatimonas aurantiaca*

N$_2$O gas is a notorious greenhouse gas because of its strong global warming potential (265-fold greater than that of carbon dioxide) and persistence in the atmosphere (ca. 114 years) (Ravishankara et al., 2009; Montzka et al., 2011; IPCC, 2014). N$_2$O gas also contributes to the loss of stratospheric ozone and has been recognized as the dominant ozone-depleting substance (Ravishankara et al., 2009; Montzka et al., 2011). Terrestrial soils greatly contribute to N$_2$O emissions (i.e., 6–7 ton g$^{-1}$ year$^{-1}$, corresponding to ca. 60% of total N$_2$O emissions) (Butterbach-Bahl et al., 2013; Cui et al., 2013; Tian et al., 2020), and N$_2$O is discharged from soils as a net result of soil N$_2$O production and consumption (Holtan-Hartwig et al., 2000). The application of nitrogenous fertilizers to agricultural soils is a common practice, but markedly increases N$_2$O emissions from agricultural soils (Seitzinger et al., 2000; Liu and Greaver, 2009; Bouwman et al., 2013). More than 50% of the annual consumption of nitrogenous fertilizers is currently derived from urea-based fertilizer consumption (Gilbert et al., 2014), and urea is hydrolyzed to ammonia and carbon dioxide by ureolytic microorganisms (Oshiki et al., 2018). The ammonia produced is subsequently oxidized to nitrite and/or nitrate by nitrification, followed by the reduction of nitrite and nitrate to nitrogen gas by denitrification and/or anammox processes (Isobe and Ohte, 2014; Oshiki et al., 2016). N$_2$O gas is produced biotically and abiotically by these nitrification and denitrification processes (Ishii et al., 2011). Regarding the consumption of N$_2$O, the biological reduction of N$_2$O is the only reaction that acts as a N$_2$O sink. This N$_2$O reduction reaction is catalyzed by the multicopper enzyme, nitrous oxide reductase (NosZ), which catalyzes the reduction of two electrons of N$_2$O to produce N$_2$ (Richardson et al., 2009; Simon and Klotz, 2013). The abundance and diversity of nosZ in soils have received a great deal of attention as a functional gene marker of N$_2$O reducers. nosZ has been found in various bacterial and archaeal genomes and classified into two phylogenetically distinct clades, nosZ clades I and II, based on sequence homology (Sanford et al., 2012; Jones et al., 2013). The abundance and diversity of nosZ clade I have been investigated in various types of soils, whereas those of nosZ clade II were largely overlooked until 2012 (Sanford et al., 2012). nosZ clade II was overlooked because the previ-
ously published oligonucleotide primer set (i.e., the nosZ1F and nosZ1R primers) (Henry et al., 2006) utilized for the PCR amplification of environmental nosZ sequences did not cover the sequence divergence of nosZ clade II. Recent molecular analyses, including quantitative PCR (qPCR), an amplicon sequencing analysis of nosZ clade II, and a metagenomic analysis of soil DNA, revealed the abundant distribution of nosZ clade II in various types of soils, similar to nosZ clade I (Sanford et al., 2012; Jones et al., 2013; Jones et al., 2014; Orellana et al., 2014; Domeignoz-Horta et al., 2015; Samad et al., 2016; Juhanson et al., 2017). In addition to their phylogenetic differences, N₂O reducers carrying nosZ clade II showed an affinity for N₂O that was up to two orders of magnitude higher than those carrying nosZ clade I (Yoon et al., 2016; Suenaga et al., 2018), suggesting their significant contribution to N₂O mitigation from soils because the concentration of N₂O is generally low in soils (i.e., typically less than 1 μM) (Schreiber et al., 2012). A linear regression analysis (Domeignoz-Horta et al., 2015; 2018; Samad et al., 2016) and structural equation modeling and network analysis (Jones et al., 2014) of the gene abundance and diversity of nosZ clade II in terrestrial soils indicated the greater contribution of N₂O reducers carrying nosZ clade II to the soil N₂O sink capacity than those carrying nosZ clade I.

The phylum Gemmatimonadetes is currently recognized as one of the nine dominant soil phyla (Janssen, 2006) because the Gemmatimonadetes 16S rRNA gene has been frequently and abundantly detected in various terrestrial environments (Janssen, 2006; DeBruyn et al., 2011). The phylum Gemmatimonadetes contains phylogenetically diverse bacterial members that have been classified into at least five sublineages (Hanada and Sekiguchi, 2014), and the following isolates have been described: Gemmatimonas aurantiaca (Zhang et al., 2003), G. phototrophica (Zeng et al., 2017), G. groenlandica (Zeng et al., 2021), Gemmatirosa kalamazoonensis (DeBruyn et al., 2013), Roseisolibacter agrii (Pascual et al., 2018), and Longimicrobium terrae (Pascual et al., 2016). G. aurantiaca and Gt. kalamazoonensis carry nosZ clade II (accession numbers AP009153.1 and CP007128.1, respectively), and N₂O reduction by G. aurantiaca was recently demonstrated in a culture-dependent manner (Park et al., 2017; Chee-Sanford et al., 2019). Although G. aurantiaca has been characterized as an obligate aerobic heterotroph (Zhang et al., 2003), this bacterium reduced N₂O under not only aerobic, but also microaerobic and anoxic conditions when partially oxic conditions were present (Chee-Sanford et al., 2019). G. aurantiaca cells transcribed nosZ when they reduced N₂O (Park et al., 2017), suggesting that the abundance of Gemmatimonadetes nosZ mRNA correlates with that of a metabolically-active Gemmatimonadetes bacterial population. The metabolic capability of N₂O reduction by G. aurantiaca and the widespread distribution of Gemmatimonadetes bacteria in various types of soils including agricultural soils (Jones et al., 2014; Orellana et al., 2014; Samad et al., 2016) led us to expect the contribution of Gemmatimonadetes bacteria to N₂O mitigation from soils. However, the physiological characteristics of G. aurantiaca for N₂O reduction have not yet been examined in detail, and limited information is currently available on the involvement of Gemmatimonadetes bacteria in N₂O reduction in soils. Physiological pH and temperature ranges and affinity constants are key physiological information for obtaining a more detailed understanding of microbial activities in natural and man-made ecosystems (Oshiki et al., 2016).

Therefore, the present study investigated 1) the physiological characteristics of G. aurantiaca associated with N₂O reduction, and 2) the involvement of Gemmatimonadetes bacteria in N₂O reduction in agricultural soils. The effects of pH and temperature on N₂O reduction activities and affinity constants for N₂O reduction by G. aurantiaca were examined by performing batch incubations and assessing N₂O reduction activities. The involvement of Gemmatimonadetes bacteria in N₂O reduction in agricultural soils was then analyzed by a soil incubation experiment in which the relationship between the N₂O reduction rates of soils and the abundance of Gemmatimonadetes bacteria was investigated. Agricultural soil samples were incubated with the addition of ¹⁵NO₃⁻ to evaluate N₂O reduction rates, and the abundance and diversity of the Gemmatimonadetes 16S rRNA gene and nosZ in the soil samples tested were evaluated by qPCR and amplicon sequencing analyses. The above DNA-based analyses potentially detect metabolically-inactive Gemmatimonadetes bacteria; therefore, qPCR and amplicon sequencing analyses of Gemmatimonadetes nosZ mRNA were also conducted, and the relationship between N₂O reduction rates and the abundance of nosZ mRNA was investigated. This is the first study to show a correlation between N₂O reduction rates and the abundance of Gemmatimonadetes nosZ mRNA in agricultural soils, and the potential involvement of Gemmatimonadetes bacteria in N₂O reduction in soil is discussed.

Materials and Methods

Bacterial culture and incubation conditions

G. aurantiaca (NBRC100505) cells were cultivated aerobically at 30°C with shaking at 90 rpm. NBRC822 medium (L-glutamic acid, 0.5 g; peptone [BD Difco, Becton Dickinson and company], 0.5 g; yeast extract [BD Difco], 0.5 g; sodium glutamate, 0.5 g; KH₂PO₄, 0.44 g; [NH₄]₂SO₄, 0.1 g; MgSO₄·7H₂O, 0.1 g; pH 7.0) was used for cultivation. Stationary-phase cells were harvested by centrifugation (13,420×g, 10 min), washed, and resuspended in fresh NBRC822 medium. The cell suspension was subjected to the following activity tests.

Effects of pH and temperature on the N₂O reduction activity of G. aurantiaca

G. aurantiaca cells were incubated at pH 5 to 10 and at 4 to 80°C, and the consumption of N₂O was examined. Three milliliters of NBRC822 medium was dispensed into 7.7-mL serum glass vials (Nichiden-Rika Glass), which were then sealed with butyl rubber stoppers and aluminum caps. The pH of NBRC822 medium was adjusted in the range of pH 5 to 10 by adding the following pH buffer at a final concentration of 20 mM: 2-morpholinoethanesulfonic acid, monohydrate (MES) for pH 5 to 6.5, 2-[4-(2-hydroxyethyl)-1-piperaziny]ethanesulfonic acid (HEPES) for pH 7 to 7.5, and N-[Tris(hydroxymethyl)methyl]glycine (Tricine) for pH 8 to 10. After purging the liquid phase with argon gas for 3 min, the headspace was replaced with pure He gas (>99.99995%). One hundred microliters of the G. aurantiaca cell
suspending and N₂O gas (GL Science) were injected into the vials at a final concentration of 0.1–0.5 mg protein mL⁻¹ and 180 nmol (N vial)⁻¹, respectively, using a gas tight syringe. The vials were incubated at 4 to 80°C in the dark, and changes in N₂O concentrations in the headspace over time were examined.

Partialoxic conditions were essential for initiating N₂O reduction by *G. aurantiaca*, which occurred after the depletion of O₂ (Park et al., 2017). Although O₂ was not added externally to the above vials, certain amounts of oxygen were available in the vials due to 1) the incomplete removal of O₂ (e.g., 3 min of an argon gas purge) and 2) the carryover of O₂ from the inoculum, which were adequate to initiate N₂O reduction by *G. aurantiaca*.

**Affinity of *G. aurantiaca* cells for N₂O**

The value of *K₅* for N₂O was calculated based on the N₂O consumption rate assessed using a N₂O-specific microsensor (N₂O-MR) and micro-respirometric system (Unisense) as previously described (Suenaga et al., 2018). Briefly, *G. aurantiaca* cells suspended in NBR822 medium (pH 7) were dispensed into a 3.0-mL closed chamber (Unisense) and stirred with a dedicated stirrer bar at 300 rpm. N₂O-saturated NBR822 medium (27–24 mM N₂O at 20–25°C) was added using a Hamilton syringe to reach a final N₂O concentration of 30 μM. The chamber was incubated at 30°C, and the N₂O concentration in the liquid phase was continuously monitored using SensorTrace Suite ver.2.8.0 (Unisense). N₂O concentration profiles were smoothed in Sigma Plot 13.0 to remove high frequency noise. The value of *K₅* was assessed by fitting N₂O concentrations and instantaneous consumption rates to the Michaelis-Menten equation using the solver function in Microsoft Excel. N₂O-MR was calibrated prior to incubations using aqueous N₂O solution as described in the manual provided by the supplier.

**N₂O reduction by agricultural soils**

Agricultural soils were incubated with the addition of ¹⁵⁴⁰N₃O, and the production of ¹⁵⁴⁰N₂O and ¹⁵⁴⁰N₂ gas was examined (Fukushi et al., 1984; Shan et al., 2016). Agricultural soils (designated Soils A, B, C, and D) were collected at four sites in Nagaoka city, Niigata, Japan (Table S1). Surface layers (0 to 5 cm) were collected from the inoculum, which were incubated in the dark at 30°C in quadruplicate. After an incubation period of 69 h, incubated soils were collected from the vials using a sterile spatula and subjected to total DNA and RNA extraction.

**Total DNA and RNA extractions**

Total DNA and RNA extractions were performed using a Power Soil DNA Isolation and RNA PowerSoil Total RNA Isolation Kit (Qiagen Japan), respectively, according to the manufacturer's protocols. Extracted RNA was transcribed to cDNA using a random 6mer primer and Prime Script RT Reagent Kit (TaKaRa Bio) (Kobayashi et al., 2017).

**qPCR assay**

The copy numbers of the 1) prokaryotic and 2) Gemmatimonadetes 16S rRNA gene (clades G1 and G3), 3) Gemmatimonadetes nosZ DNA, and 4) Gemmatimonadetes nosZ mRNA (i.e., synthesized cDNA) were assessed using the MiniOpticon Real-Time PCR System (Bio-Rad, Hercules). The reaction mixture (20 μL) contained the KAPA SYBR FAST qPCR master mix (Kapen Genetics) (10 μL), 0.8 μL of each forward and reverse primer (10 μM), and 1.6 μL of extracted DNA or the synthesized cDNA template. The oligonucleotide primers used for PCR amplification were as follows: 515F (5′-GTGCCAGCM GCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAA T-3′) for the prokaryotic 16S rRNA gene (Caporaso et al., 2011), GIG3-673F (5′-GAATGCGTAGAGATCC-3′) and 907r (5′-CCG TCAATTCMTTTRAGTTT-3′) for the Gemmatimonadetes 16S rRNA gene affiliated to clades G1 and G3, which were previously described by DeBruyn et al., (2011), and nosZ-123-145-F (5′-AA CAAAGAACASSAAGAYCG-3′) and nosZ-481-499-R (5′-ATRTC CCAARTCTGTYT-3′) for Gemmatimonadetes nosZ (the present study). Cycling conditions were as follows: 95°C for 30 s; 40 cycles at 95°C for 5 s and 55°C for 10 s; and 65°C to 95°C in increments of 0.5°C for the melting curve analysis. Negative controls (i.e., distilled water and an RNA template that was not reverse transcribed) were subjected to qPCR in parallel, and no amplicon was obtained from these negative controls. The genomic DNA of *G. aurantiaca* with a single copy of the 16S rRNA gene and nosZ was used as a standard for quantification. DNA concentrations were measured using the Qubit dsDNA BR assay kit and Qubit 3.0 fluorospectrometer (Thermo Fisher Scientific). Genomic DNA was serially diluted with distilled water to concentrations of 10⁴ to 10⁶ copies μL⁻¹.

The nosZ-123-145-F and nosZ-481-499-R primers were newly designed in the present study. We attempted to amplify the partial sequences of clade II nosZ with the nosZ-II-F and nosZ-II-R primers (Jones et al., 2013). However, no specific amplicon was obtained by PCR amplification using nosZ-II-F and nosZ-II-R primers from the DNA and cDNA samples prepared from the soil samples tested, even after the optimization of PCR conditions (i.e., Taq polymerase and the addition of DMSO and betaine) and cycling parameters (annealing temperature and extension time). A similar phenomenon was previously reported (Samad et al., 2016); therefore, we designed a new set of oligonucleotide primers for the specific detection of Gemmatimonadetes nosZ. The nosZ-123-145-F and nosZ-481-499-R primers were designed by performing a blastn search using the *G. aurantiaca* nosZ sequence as a query sequence against the *nr* database (NCBI, accessed on December 2016). The top 500 nosZ sequence hits were aligned using MUSCLE software under default conditions (18 iterations) (Edgar, 2004), and the conserved regions suitable for PCR primer design were manually examined. The coverage of the designed primers was examined by aligning Gemmatimonadetes nosZ sequences and the designed nosZ primer sequences, and by counting the numbers of primer-template mismatches.

**Amplification sequencing analysis of the 16S rRNA gene and nosZ**

The prokaryotic 16S rRNA gene and Gemmatimonadetes nosZ were amplified by PCR using the above oligonucleotide primers containing Illumina tag sequences at the 5' end of the forward and reverse primers (5′-TGGTCCTGACGCTCACTATGGCTATCTCGAGCAAGATGTGTTAT AGAGACAG-3′ and 5′-GTCTCTGGTGTCGTTAGAGATGTAT CAAGAACCSAAGGAYCG-3′, (2016)). The PCR mixture had a volume of 20 μL and contained 2 μL of extracted DNA or synthesized cDNA, oligonucleotide primers (1 μM each), dNTPs (200 μM), 2% (v/v) DMSO, 1×PCR buffer, and ExTaq polymerase (0.025 U μL⁻¹). Thermal cycling conditions were as follows: 35 cycles at 98°C for 10 s, followed by 55°C for 30 s, then 72°C for 30 s; and 72°C for 10 min. PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics). Purified PCR products were tagged with a sample-unique index and Illumina adapter sequences at their 5’ ends (Nextera XT Index Kit v2; Illumina) by PCR. The PCR reaction mixture (20 μL) contained 1×KAPA HiFi HS ReadyMix (Nippon Genetics), 1 μL of each forward and reverse primer (10 μM), and 2 μL of the recovered PCR products. PCR was run under the following cycling conditions: 95°C for 3 min, 10 cycles of 95°C for 20 s, 65°C for 15 s, and 72°C for 1 min; and 72°C for 5 min. After agarose gel electrophoresis, PCR products were excised from the gel and purified using the Agencourt AMPure XP Kit (Beckman Coulter). The tagged amplicons were pooled and sequenced on an Illumina MiSeq platform in a 250-bp paired-end sequencing reaction using the v2 reagent kit (Illumina).
Bioinformatics

The generated 16S rRNA gene and nosZ sequence reads were processed for the removal of adapter sequences using cutadapt and for quality trimming using Trimmomatic v0.33 (Bolger et al., 2014). Reads containing <50 bp or those associated with an average Phred-like quality score <30 were removed. Paired-end sequence reads were assembled in the paired-end assembler of the Illumina sequence software package (PANDAseq) (Masella et al., 2012). The nosZ reads obtained were subjected to a blastn search (threshold e-value; 10^-10) against the 22,647 nosZ sequences downloaded from the fungene database (http://fungene.cme.msu.edu/) to remove non-nosZ sequences. Regarding 16S rRNA, assembled sequence reads with ≥97% sequence identity were grouped into OTUs by UCLUST (Edgar, 2004). The phylogenetic affiliations of the OTUs were identified by a blastn search against reference sequences in Greengenes database version 13.5 (DeSantis et al., 2006) and in the nr database (NCBI). Regarding nosZ, sequence reads with ≥80% sequence identity were grouped into OTUs as previously reported (Palmer et al., 2009), and their phylogenetic affiliation was examined by a blastn search against the nr database. Putative chimeric sequences were removed using UCHIME (Edgar et al., 2011). Alpha diversity indices (observed species, Chao1, Good’s coverage, and Simpson’s index) were calculated in QIIME (Callahan et al., 2011). Chao1 was computed at sampling depths of 6,500 and 2,700 reads for 16S rRNA and nosZ, respectively. The alignment of nucleic acid and protein sequences was performed using MUSCLE software (Edgar, 2004) with 18 iterations, and a phylogenetic tree was constructed in MEGA 7.0.26 (Kumar et al., 2016) using the maximum likelihood method (Jones-Taylor-Thornton model).

Chemical analysis

14-14N2O, 15-15N2O, and 15-15N2O concentrations were measured by gas chromatography-mass spectrometry (GS/MS) as previously described (Isobe et al., 2011; Yoshinaga et al., 2011). Ten microliters of the headspace gas was collected using a 100-μL gas-tight glass syringe and immediately injected into the gas chromatograph GCMS-QP 2010 SE (Shimadzu) equipped with a fused silica capillary column (Agilent Technologies). Peaks at m/z = 30, 44, and 46, corresponding to 15-15N2, 14-14N2O, and 15-15N2O were monitored, and concentrations were calculated using standard curves prepared using the standard 14-14N2O gas for both 14-14N2O and 15-15N2O (Shimakyu) and the 15-15N2 gas (Cambridge Isotope Laboratories).

Biomass concentrations were measured by the Lowry method using the DC-protein assay kit (Bio-Rad) as previously reported (Oshiki et al., 2011). Bovine serum albumin was used to prepare calibration curves.

Correlation analysis between N2O reduction rates and physicochemical parameters or the abundance of the Gemmatimonadetes 16S rRNA gene or nosZ

A linear regression analysis was performed using Microsoft Excel 16.57 to assess the coefficient of determination (R2) between N2O reduction rates (16.6, 5.0, 24.3, and 9.5 nmol·N·[dry soil]⁻¹·h⁻¹) in Soils A, B, C, and D, respectively, see below) and physicochemical parameters (Table S1) or the copy numbers of the prokaryotic 16S rRNA gene, Gemmatimonadetes nosZ DNA, and Gemmatimonadetes nosZ mRNA (copies [g dry soil]⁻¹) (Fig. 4). The copy numbers of Gemmatimonadetes nosZ mRNA, Gemmatimonadetes nosZ mRNA and Gemmatimonadetes nosZ mRNA were calculated by multiplying the relative abundance of each OTU (%) by the copy numbers of Gemmatimonadetes nosZ mRNA.

Nucleotide sequence accession number

Raw sequence data obtained in the amplicon sequencing analysis were deposited in the DDBJ nucleotide sequence database under accession number DRA006974. The sequence reads of each OTU are available under accession numbers LC390430 to LC401807 and IADF01000001 to IADF01000317 for the 16S rRNA gene and nosZ, respectively.

Results

pH and temperature ranges and affinity for N2O reduction by G. aurantiaca

G. aurantiaca cells were incubated under different pH (pH 5 to 10) and temperature (4 to 80°C) conditions, and the activities of N2O consumption were examined. As shown in Fig. 1a and b, G. aurantiaca cells consumed N2O at pH 5–9 and 4–50°C, with the highest activity being observed at pH 7 and 30°C. The affinity of G. aurantiaca cells for N2O was examined by continuously measuring N2O concentrations using N2O-MR. The relationship between N2O reduction rates and N2O concentrations is shown in Fig. 2, and the apparent affinity constant for N2O (K) was 4.4 μM.
N$_2$O uptake (µM h$^{-1}$)

N$_2$O concentration (µM)

$R^2 = 0.96$

Fig. 2. Affinity constant of Gemmatimonas aurantiaca for N$_2$O reduction. G. aurantiaca cells were cultivated with the addition of 30 µM N$_2$O, and N$_2$O consumption was monitored using a N$_2$O microsensor. Circle symbols correspond to the data set obtained by N$_2$O microsensor measurements, and the red line indicates a fitted Michaelis-Menten curve. The coefficient of determination ($R^2$) was calculated at a range of 0.1 to 18 µM N$_2$O.

N$_2$O reduction activities and abundance of Gemmatimonadetes in soil

Four agricultural soil samples collected in Nagaoka city, Niigata, Japan were incubated with the addition of $^{15}$NO$_3^-$. All soil samples reduced $^{15}$NO$_3$ and produced $^{15}$-15N$_2$O and $^{15}$-15N$_2$ (Fig. 3). N$_2$O production was more prominent than N$_2$ production during the early phase of the incubation (up to 24 to 69 h), and N$_2$O concentrations then stabilized or decreased. In contrast, $^{15}$-15N$_2$ concentrations continuously increased during the incubation, except for Soil C, which showed a decreased after an incubation for 93 h. $^{15}$-15N$_2$O reduction rates were calculated as a slope of $^{15}$-15N$_2$O concentrations during 0 to 93 h of the incubation, and were 16.6, 5.0, 24.3, and 9.5 nmol N (g dry soil)$^{-1}$ h$^{-1}$ for Soil A, B, C, and D, respectively. These rates were similar or higher than those of Chinese paddy soils (2.37 to 8.31 nmol N g$^{-1}$ h$^{-1}$) (Shan et al., 2016).

The abundance and diversity of the Gemmatimonadetes 16S rRNA gene and nosZ (DNA and mRNA) were examined using the above 4 soil samples collected after an
incubation for 69 h. The copy numbers of the prokaryotic and Gemmatimonadetes (clades G1 and G3) 16S rRNA genes were 1.19–16.7×10^10 and 8.62–9.65×10^8 copies (g dry soil)^{-1}, respectively (Fig. 4). The new oligonucleotide primers nosZ-123-145-F and nosZ-481-499-R were designed to assess the copy number of Gemmatimonadetes nosZ, and the sequence coverage of the designed primers is shown in Fig. S1. The copy numbers of Gemmatimonadetes nosZ DNA and mRNA (i.e., cDNA) were 5.35–7.15×10^8 and 2.23–4.31×10^9 copies (g dry soil)^{-1}, respectively (Fig. 4). PCR amplicons of the prokaryotic 16S rRNA gene and Gemmatimonadetes nosZ mRNA were subjected to an amplicon sequencing analysis. Overall, 6,572 to 12,252 sequence reads of the 16S rRNA gene were obtained from each soil sample and then clustered based on ≥97% sequence identity into 2,982 to 4,021 OTUs (Table S2a). Gemmatimonadetes 16S rRNA reads accounted for 4.7 to 8.9% of the total reads (Fig. S2), and the phylogeny of Gemmatimonadetes 16S rRNA reads is shown in Fig. 5.

Regarding Gemmatimonadetes nosZ mRNA, 2,711 to 6,328 sequence reads were obtained (Table S2b). Gemmatimonadetes nosZ reads were screened from the total reads by a phylogenetic analysis, and more than 72% of the total reads were affiliated with the Gemmatimonadetes nosZ clades; i.e., 74, 86, 72, and 76% of nosZ reads obtained from Soil A, B, C, and D, respectively, were affiliated with a putative Gemmatimonadetes nosZ clade. Gemmatimonadetes nosZ reads were clustered (≥80% sequence identity) (Palmer et al., 2009) into 90 Gemmatimonadetes nosZ OTUs. The phylogeny of the 44 major OTUs is shown in Fig. 6, and no OTU shared ≥80% sequence similarity with G. aurantiaca nosZ.

The relationships between N₂O reduction rates and physicochemical parameters (Table S1) or the abundance of the Gemmatimonadetes 16S rRNA gene (clades G1 and G3) and nosZ were examined using a linear regression analysis. As shown in Table 1, the abundance of the nosZ mRNA of Gemmatimonadetes bacteria, OTU91, OTU332, and OTU122 strongly correlated (R²; >0.84) with the N₂O reduction rates of the soil samples tested.

**Discussion**

The relative abundance of the Gemmatimonadetes 16S rRNA gene in the soils examined in the present study ranged between 0.5 and 7.8% of the prokaryotic 16S rRNA copy number and between 4.7 and 8.9% of the total 16S rRNA gene amplicon reads, indicating that Gemmatimonadetes bacteria were the dominant soil bacteria. The abundance of Gemmatimonadetes 16S rRNA gene copy numbers to prokaryotic 16S rRNA gene copy numbers assessed by qPCR was generally lower than the relative abundance estimated by the amplicon sequencing analysis. The G1G3-673F primer used for qPCR of the Gemmatimonadetes 16S rRNA gene targeted the 16S rRNA gene sequences of Gemmatimonadetes clades G1 and G3, but not G2 (DeBruyn et al., 2011), which may have resulted in an underestimation of the copy numbers of the Gemmatimonadetes 16S rRNA gene in the soils examined. On the other hand, although
**Fig. 5.** Phylogeny and abundance of *Gemmatimonadetes* 16S rRNA sequences detected in agricultural soil samples. 16S rRNA gene reads obtained by amplicon sequencing were clustered into species-level operational taxonomic units (OTUs) with ≥97% sequence identity, and a phylogenetic tree was constructed using the maximum likelihood method with the Jones-Taylor-Thornton model and the 16S rRNA of *Escherichia coli* (accession number BA000007.2) 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 5% sequence divergence.
putative Gemmatimonadetes nosZ clade

Fig. 6. Phylogeny and abundance of 44 most abundant operational taxonomic units (OTUs) of Gemmatimonadetes nosZ mRNA. nosZ reads were clustered into species-level OTUs with ≥80% sequence identity (Palmer et al., 2009), and a phylogenetic tree was constructed using the maximum likelihood method with the Jones-Taylor-Thornton model and Robigintadella biformata nosZ (accession number; CP001712) as an outgroup. Branching points that support a probability >80% in bootstrap analyses (based on 500 replicates) are shown as filled circles. The phylogenetic positions of Gemmatimonadetes aurantiaca and Gemmatirosa kalamazoonesis nosZ in the Gemmatimonadetes nosZ clade tentatively proposed in the present study are shown in red. The scale bar represents 10% sequence divergence. The OTUs highlighted with a star symbol (i.e., OTU91, OTU139, and OTU122) are the major OTUs (>1% relative abundance in a soil sample) showing a strong correlation with soil N$_2$O reduction rates (Table 1).
the nosZ-123-145-F and nosZ-481-499-R primers covered >92% of the Gemmatimonadetes nosZ sequences, the amplification sequencing analysis of Gemmatimonadetes nosZ mRNA revealed that between 14 and 28% of the total nosZ reads were not assigned to the putative Gemmatimonadetes nosZ clade. This result suggests that the copy numbers of Gemmatimonadetes nosZ mRNA (and potentially nosZ DNA) were overestimated by 28%. Therefore, caution is needed when comparing the copy numbers of the Gemmatimonadetes 16S rRNA gene and nosZ.

Previous DNA-based PCR and sequencing analyses of nosZ clade II revealed metabolically inactive populations (i.e., dead and dormant cells) in soil. G. aurantiaca and other N₂O reducers transcribed nosZ mRNA when they reduced N₂O (Henderson et al., 2010; Mania et al., 2016; Park et al., 2017), and, thus, an mRNA-based analysis of Gemmatimonadetes nosZ was herein performed to identify N₂O-reducing Gemmatimonadetes bacterial populations. Gemmatimonadetes nosZ mRNA was successfully detected in N₂O-reducing soils (Fig. 4 and 6), and correlations were observed between N₂O reduction rates and the abundance of Gemmatimonadetes nosZ mRNA (R²=0.91). On the other hand, a weak correlation was noted between N₂O reduction rates and the abundance of Gemmatimonadetes nosZ DNA (R²=0.36), which may have been due to the detection of a metabolically inactive Gemmatimonadetes population. Additionally, the abundance of the Gemmatimonadetes 16S rRNA gene (clades G1 and G3) did not show a correlation (R²=0.02). nosZ has been found in particular Gemmatimonadetes genomes and is not commonly conserved among this bacterial phylum, which may have contributed to the lack of a correlation between N₂O reduction rates and the abundance of the Gemmatimonadetes 16S rRNA gene. The correlations observed between the N₂O reduction rates of the soil samples tested and the abundance of the nosZ mRNA of Gemmatimonadetes bacteria, OTU91, OTU332, and OTU122 (Table 1) indicated the involvement of Gemmatimonadetes bacteria in N₂O reduction in these soil samples. This is the first study to show a correlation between N₂O reduction rates and the abundance of Gemmatimonadetes nosZ mRNA in soil. These correlations were examined using 4 agricultural soils in the present study, and, thus, more detailed studies are warranted using larger numbers and various types of soil samples. Furthermore, the soil samples examined in the present study contained phylogenetically diverse microorganisms other than Gemmatimonadetes bacteria (Fig. 2), and their contribution to N₂O reduction and other nitrogen transformation reactions in soils currently remains unclear. A metatranscriptomic analysis other than target-specific qPCR is a powerful tool for investigating microbial nitrogen transformation reactions in soil ecosystems (Masuda et al., 2017).

To gain further insights into N₂O reduction by Gemmatimonadetes bacteria in soil, the effects of pH and temperature as well as affinity for N₂O reduction were examined using G. aurantiaca. The influence of pH and temperature on the N₂O reduction activities of Gemmatimonadetes bacteria have not yet been examined; nevertheless, this physiological information is key for understanding their involvement in N₂O reduction in soils. pH and temperature conditions markedly affected the N₂O reduction activities of G. aurantiaca in the present study; relative activity decreased by 50±28% at pH 6.5 (Fig. 1). G. aurantiaca reduced N₂O to N₂ at pH 5–9 and 4–50°C, and agricultural soils showed these pH and temperature ranges; i.e., pH 5.4–8.1 (Holtan-Hartwig et al., 2000; Domeignoz-Horta et al., 2015) and 3.6–25.8°C (Takata et al., 2011). The Ks value for N₂O (i.e., 4.4 μM) was in the range of those previously reported in cultures of denitrifiers and bacteria catalyzing dissimilatory nitrite reduction to ammonium (0.324–100 μM) (Betlach and Tiedje, 1981; Conrad, 1996; Yoon et al., 2016; Park et al., 2017; Suenaga et al., 2018) and using bulk agricultural soils (0.1–5.8 μM) (Holtan-Hartwig et al., 2000). N₂O concentrations in agricultural soils were generally less than 1 μM, but increased up to 400 μM (Schreiber et al., 2012 and references therein) with nitrogenous fertilizer treatment, which was higher than the Ks value of G. aurantiaca. Collectively, these findings indicate that soil management practices, including pH and temperature control and nitrogenous fertilizer treatment, have a significant impact on N₂O reduction by Gemmatimonadetes bacteria (and likely N₂O emissions from soil). Although the aforementioned physiological characteristics of G. aurantiaca support our hypothesis that Gemmatimonadetes bacteria participated in N₂O reduction in the soils examined, it is important to note that the G. aurantiaca 16S rRNA gene and nosZ mRNA reads were not detected in the soil samples examined (Fig. 5 and 6); therefore, the physiological characteristics of soil-inhabiting Gemmatimonadetes bacteria need to be investigated in future studies. The 16S rRNA gene reads affiliated with OTU4572 and OTU3759 were commonly and abundantly found in the soils examined, and also from various types of European, American, and Asian soils as assessed by

| pH | Water content | TC | TN | P | NO₂⁻ | NO₃⁻ | NH₄⁺ |
|----|---------------|----|----|---|------|------|------|
| 0.09 | 0.57 | 0.58 | 0.62 | 0.03 | 0.42 | 0.41 | 0.41 |

Table 1. Relationships between N₂O reduction rates and physicochemical parameters or the abundance of the Gemmatimonadetes 16S rRNA gene and nosZ in soil samples. The table shows R² values between the N₂O reduction rates of the soil samples examined and (upper) physicochemical parameters or (bottom) the abundance of the Gemmatimonadetes nosZ mRNA OTUs; the major OTUs (>1% of relative abundance in a soil sample, Fig. 6) with R² values >0.8 are shown.

| Prokaryotic | Gemmatimonadetes |
|------------|------------------|
| 16S rRNA | nosZ DNA | nosZ mRNA | OTU91 nosZ mRNA | OTU332 nosZ mRNA | OTU122 nosZ mRNA |
| gene      | gene            |            |                |                 |                  |
| 0.11      | 0.02 | 0.36 | 0.91 | 0.97 | 0.84 | 0.92 |
a blastn search against the nr database (Table S3a and b). Further studies are warranted to examine the physiological characteristics of the Gemmatimonadetes bacteria affiliated with OTU4572 and OTU3759.

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