Physiological and Genotypic Characteristics of Nitrous Oxide (N₂O)-Emitting Pseudomonas Species Isolated from Dent Corn Andisol Farmland in Hokkaido, Japan

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(Received October 6, 2015—Accepted January 25, 2016—Published online April 22, 2016)

Dent corn Andisol at the Hokkaido University Shizunai Livestock Experimental Farm actively emits nitrous oxide (N₂O). In order to screen for culturable and active N₂O emitters with high N₂O emission potential, soft gel medium containing excess KNO₃ was inoculated with soil suspensions from farm soil samples collected at different land managements. Dominant bacterial colonies were searched for among 20 of the actively N₂O-emitting cultures from post-harvest soil and 19 from pre-tilled soil, and all isolates were subjected to the culture-based N₂O emission assay. Ten active N₂O-emitting bacteria, four from post-harvest soil and six from pre-tilled soil, out of 156 isolates were identified as genus Pseudomonas by 16S rRNA gene sequencing. These N₂O emitters showed clear responses to NO₃⁻ within a neutral pH range (5.5–6.7), and accelerated N₂O production with 1.5–15 mM sucrose supplementation, suggesting the production of N₂ during the denitrification process. However, the negative responses of 6 active N₂O emitters, 3 from post-harvest soil and 3 from pre-tilled soil, out of the 10 isolates in the acetylene-blocking assay suggest that these 6 N₂O emitters are incomplete denitrifiers that have lost their N₂O reductase (N₂OR) activity. Although the PCR assay for the denitrification-associated genes, narG and nirK/S, was positive in all 10 Pseudomonas isolates, those negative in the acetylene-blocking assay were nosZ-negative. Therefore, these results imply that the high N₂O emission potential of dent corn Andisol is partly attributed to saprophytic, nosZ gene-missing pseudomonad denitrifiers.

Key words: nitrous oxide (N₂O) emitter, Pseudomonas sp., nosZ-missing and nosZ-harboring denitrifiers, Andisol corn farm

Nitrous oxide (N₂O) is an active greenhouse gas that is responsible for more than 7% of global warming (21) and contributes to the depletion of the ozone layer (28). Global anthropogenic sources of N₂O include agriculture and industry, from which this gas is generated due to biomass burning, indirect emissions from reactive nitrogen leaching, runoff, and atmospheric deposition (12). The widespread use of nitrogenous fertilizers and manure in agricultural soil is a leading cause of N₂O emission (6, 22, 29, 32, 45). A recent study demonstrated that 60–70% of the annual anthropogenic N₂O yield was from agriculture (31), of which nearly 70% of N₂O gas emission was due to the processes of nitrification and denitrification by soil microorganisms in farm soil (15, 21).

Chemical fertilization using ammonia products, such as ammonium sulfate ([NH₄]₂SO₄) or urea (CO[NH₂]₂), often accelerates nitrification, leading to N₂O emission in upland farming soils (3, 47). Conversely, biological denitrification is another microbial process, otherwise known as nitrate respiration, in which NO₃⁻ or other oxygenated nitrogen compounds serve as electron acceptors for the degradation of organic substances under anaerobic conditions. In the process of denitrification, nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (N₂OR), encoded by the napA/narG, nirS/nirK, norB, and nosZ genes respectively, are relevant to N₂O emission from agricultural soils. N₂O in particular is actively released as the final reduced gas by some N₂OR-malfunctional, and often nosZ gene-missing, denitrifiers (26, 35).

The Shizunai Experimental Livestock Farm of Hokkaido University is a relatively active N₂O-emitting area in Japan (16). We established a reproducible N₂O emission assay system using gellan gum soft gel medium, and isolated and characterized culturable N₂O-emitting eubacteria from the farmland soils of reclaimed tropical peatland (9). In the Andisol farmland in Hokkaido, Paenibacillus sp. and a tentative Leptothrix sp., both of which showed relatively active N₂O emission, were directly isolated from a suspension of the thawing pasture topsoil. However, their N₂O-emitting potentials toward a suspension of the thawing soil itself were parts per thousand (38). We speculated that soil microbial communities in farmed corn soil in the summer and autumn are affected not only by plowing and fertilization, but also by the large mass of organic carbons provided by crop roots. Therefore, the saprophytic and facultatively anaerobic bacteria that emerged in sugarless soft gel medium were further examined for active N₂O emission, which resulted in the isolation of 10 bacteria that showed active N₂O emission potentials almost equivalent to the soil suspension.

In this study, we present the physiological and genetic characteristics of the N₂O-emitting bacteria isolated from pre-tilled and post-harvest vitric Andisol of a corn farm in Hokkaido, Japan.
Materials and Methods

Soil samples

Soil samples (approximately 10–50 g each) were collected from the Hokkaido University Shizunai Livestock Farm in Hokkaido, Japan (42°26’ N, 142°28’ E) after the corn harvest in November 2011 and the following spring before tilling and sowing in April 2012. With the untreated control as neither fertilizer nor manure (CC), 4 treatments had been applied to the corn field: fertilizer (05CF), manure (beef cattle manure with bedding litter) (05CM), and fertilizer and manure (05CFM), all of which were deposited from 2005 to the present date. The relatively newly converted grassland to corn farm (10CFM) had the following farm management history: grassland between 2004 and 2009 was tilled on November 30, 2009, and then converted to corn farm from 2010 to the present date, with fertilizer and manure being deposited from 2010. The fertilizer application rates were 104 kg ha⁻¹ N (80 kg ha⁻¹ ammonium-nitrogen and 24 kg ha⁻¹ urea), 144 kg ha⁻¹ P₂O₅, and 80 kg ha⁻¹ K₂O. Manure (beef cattle manure with bedding litter) application rates were 215 kg ha⁻¹ N, 397 kg ha⁻¹ P₂O₅, and 325 kg ha⁻¹ K₂O. At the same time, we collected soil samples from cultivated pasture plots that had been treated. Pasture samples were labeled as PC (control), PF (fertilizer), and PFM (fertilizer and manure). The fertilizer application rates were 66.1 kg ha⁻¹ N, 175 kg ha⁻¹ P₂O₅, and 100 kg ha⁻¹ K₂O. Manure application rates were 95.5 kg ha⁻¹ N, 74.8 kg ha⁻¹ P₂O₅, and 164.6 kg ha⁻¹ K₂O. Forty-eight soil samples were obtained from each plot at three points and two different depths, 5 cm (4–6 cm) and 15 cm (14–16 cm), in November 2011 and April 2012, respectively. Samples were obtained before tillage with a rotary cultivator and kept in zippered plastic bags at 4°C until used.

N₂O emission assay for soil suspensions under an alternative N source

In order to investigate the main causative microorganisms for N₂O production in soil in autumn (post-harvest) and spring (pre-tilled), two media for the culture-based N₂O emission assay were used alternatively. Since mineral N is the substrate for N₂O production, an excess concentration of (NH₄)₂SO₄ (500 mg L⁻¹ N, as 2.4 g L⁻¹) was added to Winogradsky’s medium solution separately with 0.01% (w/v) CaCO₃ (8, 38). The pH of the solution for the enrichment culture was adjusted to 5.0 with 1 M aqueous solution of H₂SO₄, followed by filtration through a polytetrafluoroethylene (PTFE) membrane (pore size, 0.45 μm) in order to remove insoluble mineral salts. Gellan gum powder 0.3% (w/v) was added as the gel matrix to the resulting solution. The mixture was heated at 117°C for 15 min, and mixed well as the mixture liquefied (7). After cooling to room temperature, 10.0 mL medium was poured into a 30-mL gas chromatographic vial (Nichiden-Rika Glass Co., Kobe, Japan), sealed with a butyl rubber plug and screw cap septum, and autoclaved at 121°C for 15 min. The headspace volume of the vial containing 10.0 mL medium was 22.6 mL (8). We further compensated the headspace volume as 22.5 mL due to considerations of the inoculant space volume as 22.5 mL due to considerations of the inoculant volume. The medium used in the preliminary culture-based N₂O emission assay to screen N₂O emitters (Figs. 1 and S1) did not contain any additional sugar, 15 mM sucrose was used for further incubations in order to analyze opti- mum pH and N₂O emission. After a 7-d incubation at 20°C in the dark, the headspace gas was analyzed with the GC.

Extraction of DNA from N₂O emitters and 16S rRNA gene amplification

Isolation of and screening for N₂O-emitting bacteria

Culture medium from which N₂O was actively produced during the N₂O emission assay was used as the source in the isolation of N₂O-emitting bacteria. A 100-μL aliquot of the suspension (medium diluted 10,000-fold with sterilized water) was inoculated into modified Winogradsky’s gellan gum plates (MWG, Winogradsky’s mineral mixture with 0.5% sucrose as the carbon source, 500 mg L⁻¹ N as the nitrogen source (3.6 g KNO₃ L⁻¹), pH 5.0 with H₂SO₄, and 2% gellan gum for the gel matrix). Plates were incubated at 20°C in the dark for 4 d. The dominant bacterial colonies apparent on the MWG plates were isolated and purified several times on MWG plates.

In order to examine N₂O emitters, two loops of the isolated bacterial colonies were inoculated on soft gel medium without supplementation with sugar and vortexed for 30 s. All vials were analyzed by ECD-gas chromatography after incubating at 20°C in the dark for 7 d, at which point the concentration of N₂O in the headspace gas reached its maximum level. When the headspace gas (a volume of 22.5 mL) contained 1 μL L⁻¹ of N₂O (1 ppmv, equivalent to 2.0 μg L⁻¹), the absolute amount of N₂O produced from the culture medium per vial was 45.2 ng. The absolute amount of N₂O in the headspace was simply divided by the incubation days, leading to N₂O emitted d⁻¹ from the 10-mL culture (as ng d⁻¹ or μg d⁻¹).

Optimal conditions for N₂O production

In order to evaluate the effects of a supplemented carbon source on N₂O emitters, a series of sucrose concentrations (0, 1.5, and 15 mM) were added to 10 mL of Winogradsky’s medium supplemented with 5 mg KNO₃ (N supplement). A. pseudomonas sp. was cultured on a shaker at 110 rpm in 50 mL of Winogradsky’s medium supplemented with 15 mM sucrose at 20°C for 24 h in the dark in order to obtain fresh inoculates for the N₂O emission assay. Inoculates were collected from 50 mL culture medium by centrifuging at 8,000 ×g at 4°C for 10 min, washed with Milli-Q water several times, and then re-suspended in sterilized water. The suspension of bacteria (10⁷ cells mL⁻¹) was added to Winogradsky’s medium (10 mL) in 30-mL gas chromatographic vials. The initial medium containing 1.5 mM sucrose was used for further incubations in order to analyze optimum pH and N₂O emission. After a 7-d incubation at 20°C in the dark, the headspace gas was analyzed with the GC.

The pH values of media before and after culturing were recorded in order to identify any marked changes in pH during the incubation period.
N2O-emitting Pseudomonas in fertilized corn farm Andisol

Adjustments in pH (3.5–7.6) were made with 1 M H2SO4 and 1 M KOH solutions before autoclaving. The optimal pH was measured using the portable pH meter Horiba F-22 (Horiba, Kyoto, Japan) connected to an Orion 8013BN glass electrode (Orion, Beverly, MA, USA). pH was measured in isolates from spring soil before tilling using the hand-held pH meter, the B-212

Fig. 1. N2O emission potential of soil microbial communities from corn farm Andisol, and bacteria isolated from culture media. N2O emission from soil suspensions incubated in gellan gum soft-gel media (pH 5.0) with 500 mg L⁻¹ NO3⁻ or 500 mg L⁻¹ NH4⁺ at 20°C for 7 d in the dark without sucrose supplementation. Soil samples were collected from farms (autumn 2011) for the preparation of inoculants. KNO3 was used as a substrate (A). N2O emission by bacteria isolated from culture media in A (B). Soil collected from the farms (spring 2012) were inoculated using KNO3 as the substrate (C). N2O emission by bacteria isolated from culture media in C (D). Note that the scales of the y-axes in C and D are 5-fold larger than those of the others. Samples marked with the symbol (▼) were further screened for N2O emitters. Blue-colored (▼) and red-colored (▼) symbols in C and D are nosZ-harboring and nosZ-missing denitrifiers respectively.
Twin Compact pH meter (Horiba). Four isolates (10CFM5-1B and 10CFM5-2D from post-harvest soil and 10CFM5-4A and 10CFM5-6A from pre-tilled soil) were used in this experiment.

Acetylene-blocking assay of N\textsubscript{2}O-emitting bacteria

In order to investigate the effects of acetylene on N\textsubscript{2}O emitters, N\textsubscript{2}O-emitting bacteria were inoculated into 10 mL soft gel medium in the same gas chromatographic culture vials as those used in the N\textsubscript{2}O emission assay, and pure acetylene gas (2.25 mL) was injected into the headspace (22.5 mL), giving a concentration of 10% acetylene (1). In order to allow excess gas to escape during the injection of acetylene, a sterile needle was inserted by penetrating the butyl rubber plug. Cultured vials with inoculates, but without an injection of acetylene gas were prepared as controls. Treated samples and controls were examined in triplicate. After an incubation for 5–7 d, the amount of N\textsubscript{2}O in the headspace gas was measured using ECD-gas chromatography (Shimadzu GC-14B equipped with Shimadzu ECD-2014) as described above.

Detection of the nosZ gene from N\textsubscript{2}O-emitting Pseudomonas spp. by PCR

In denitrifying pseudomonads, nosZ was detected by PCR, using the primer set nosZ-1111F (5’-STA CAA CWC GGA RAA SG-3’), nosZ-661F (5’-CGG CTG GGG GCT GAC CAA-3’), nosZ-1327R (5’-CTG RCT GTC GAD GAA CAG-3’), and nosZ-1773R (5’-ATR TCG ATC ARC TGB TCG TT-3’). The PCR conditions used were as follows: preheating at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. The reaction was completed at 72°C for 7 min. Sequencing of the PCR amplicons assignable as nosZ fragments was detected by agarose gel electrophoresis. If one bacterium gave an amplicon, its sequence determination followed by a homology search on the database (NCBI) was performed in order to confirm whether the bacterium possessed the nosZ gene in its genome.

Detection of narG and nirS genes from pseudomonad denitrifiers

A PCR assay was also performed for the narG and nirS genes, which encode the α-subunit of membrane-bound, respiratory nitrate reductase and nitrite reductase containing cytochrome-c\textsubscript{d}, respectively, in order to determine whether denitrification is linked to these reductoxidases. Regarding the specific detection of the narG gene, the conserved domain of the target enzyme was surveyed for the universal primer design. Amino acid sequences converted from the base sequence of the narG gene encoding the nitrate reductase α-subunit (NarG) were randomly collected from Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Actinobacteria, and some other minor phyla. Among the 50 eubacterial sequence data for N\textsubscript{2}O-R2, two conserved regions were selected in more than HYVQGKE (positions of amino acids at 567–573 for the nitrate reductase α-subunit of Pseudomonas fluorescens AK15 with accession no. U71398.1 (25) and DMPFPHI (positions at 792–798 for the same protein) for the design of the degenerated forward (5’-CAY TAY TGS GGS CAR G-3’) and reverse (5’-TGD ATR AAN GGR TGC A-3’) primers, respectively. A 600-bp amplicon (LC034274.2) was obtained from Pseudomonas sp. 05CF15-5C, and using this sequence, the secondary primers narG-2712F (5’-TGC GGC AAG GGC CAT GAG TAC-3’) and narG-2332R (5’-CTT CTT ACC AGG TGG CCG TCG-3’) for the shorter amplicon (approx. 200 bp) were further designed. Conversely, the partial nirS gene was amplified using a pair of known primers, nirScri3Af (5’-AAC GYS AAG GAR ACS GG-3’) and nirScri3Fd (5’-GAS TTC GCR TGS GTC T-3’) (40).

Phylogenetic analysis of N\textsubscript{2}O-emitting bacteria for the 16S rRNA gene

In the phylogenetic analysis of these 10 N\textsubscript{2}O-emitting Pseudomonas isolates, the 1456-bp sequences obtained for their 16S rRNA genes in the region from positions 74 to 1529 for Escherichia coli (accession no. J01859.1) were analyzed by the maximum composite likelihood estimation, along with 9 reference species selected from several groups of pseudomonads registered in the NCBI DNA database. This neighbor-joining tree method was conducted for the identification of pseudomonads at the species level using the computing tool for a phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA v. 6.06) software (39). Under an estimation of distances between all pairs of sequences with 1,000 bootstrap replicates, clustering of the complete denitrifiers and incomplete denitrifiers was performed and used for further determinations of their emergence in corn farm Andisol.

Taxonomic classification of the soil bacterial community using a 16S rRNA metagenomic analysis

A metagenomic analysis for the soil bacterial community structure was performed using the next-generation sequencing system Ion Torrent PGM (Thermo Fisher scientific, Waltham, MA USA) targeting the 16S rRNA gene. 16S rRNA metagenomic libraries were prepared following the instructions provided by the Ion 16S metagenomics kit (Life Technologies, CA, USA). Bacterial 16S rRNA gene regions were amplified by the Ion 16S metagenomics kit using the primer sets V2-4-8 and V3-6-7-9 on a 30-MU scale. PCR amplification was performed under the following conditions: an initial activation step was conducted at 95°C for 10 min, denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 20 s was repeated for 30 cycles, and the final extension was performed at 72°C for 7 min. The PCR sample amplified using V2-4-8 was mixed with an equal volume of the other sample amplified with V3-6-7-9, and the mixture thereof was subjected to purification with Agilent AMPure XP (Agilent Technologies, Santa Clara, CA, USA) according to the instructions provided. The PCR products thus purified were quantified by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

The DNA libraries of the 16S rRNA gene were prepared using the Ion Plus Fragment Library Kit (Life Technologies) and barcoded with the IonXpress Barcode Adapters 1-16 Kit (Life Technologies) in order to distinguish 7 samples of soil DNA with barcode numbers 1 to 7. Adapter-ligated and nick-repaired DNA chains were subjected to library amplification under the following conditions: 95°C for 5 min, 6 cycles at 95°C for 15 s, at 58°C for 15 s, and at 70°C for 1 min. The bioanalyzer with the Agilent High Sensitivity DNA kit (Agilent Technologies) was used to assess the quality of the libraries. Each DNA library from soil samples was diluted to 26 pM, subjected to emulsion PCR in order to amplify the sequencing template onto Ion Sphere Particles (ISPs) with the Ion OneTouch 2 system (Life Technologies), and enriched with the Ion OneTouch ES using the Ion PGM Template OT2 400 Kit (Life Technologies) according to the manufacturer’s protocol. Enriched ISPs were subjected to single-end sequencing on Ion Torrent PGM (Life Technologies) using the Ion PGM Sequencing 400 kit (Life Technologies) with Ion 316 Chip v2 (Life Technologies) for 850 flows. All sequences were processed for data analysis using Ion Torrent platform-specific pipeline software, Ion Reporter 5.0 16S Metagenomics Workflow (Life Technologies) automatically accessible to MicroSEQ 16S Reference Library v2013.1 (Thermo Fisher Science), and Greengenes v13.5 (The Greengenes Database Consortium, http://greengenes.secondgenome.com/). The number of copies needed for matching was set at 10.

Nucleotide sequence accession numbers

The sequences of the 16S rRNA genes and denitrification-related genes from N\textsubscript{2}O-emitting Pseudomonas isolates have been deposited in the GenBank/DDBJ databases. The accession numbers of the 16S rRNA genes from Pseudomonas isolates are AB856847–AB856850 (from post-harvest soil) and LC007966–LC007971 (from pre-tilled soil). LC047837–LC047840 are those of the partial nosZ gene, while LC047828–LC047836 and LC034243–LC034252 are those of the partial nirS and narG genes, respectively.
Results

N$_2$O-emitting capacities of soil microbial community members in post-harvest and pre-tilled corn farm Andisol

The N$_2$O-emitting capacities of culturable soil bacteria from post-harvest and pre-tilled corn farm Andisol were examined; the supernatant of each soil suspension was inoculated on 0.3% gellan gum soft gel medium in closed glass vials. Forty-eight and 44 soil samples obtained from post-harvest and pre-tilled farmland, respectively, were incubated in medium supplemented with an alternative mineral nitrogen source (NO$_3^-$ N or NH$_4^+$ N) in the absence of sucrose (Figs. 1 and S1). Some of the inoculants displayed particularly active N$_2$O emission, greater than 0.1 μg vial$^{-1}$ d$^{-1}$, in the headspace gas (equivalent to 15 ppmv N$_2$O in the headspace gas after 7 d) with the addition of NO$_3^-$ N. The most active Andisol suspension culture produced 2.0 μg vial$^{-1}$ d$^{-1}$ of N$_2$O (soil sample 10CFM15-5). All active cultures were from fertilized corn farm soil (10CFM, 05CFM, or 05CF) and not pasture soils (Fig. 1A–D).

N$_2$O emitters isolated from farm soils

Among the ten soft gel cultures of the post-harvest soil microbiota and nine cultures of the pre-tilled soil microbiota that showed relatively high active N$_2$O emission, the major emergent bacterial colonies of each culture, appearing as the 1st to 4th highest population size on a plate, were isolated and purified several times on MWG plates (Fig. 1A and B). We subsequently screened 76 microbial isolates from N$_2$O-producing cultures, and the N$_2$O emission potential of each bacterial isolate was determined. Among the 40 microbial community members obtained from the culture of post-harvest soil suspensions, four isolates exhibited N$_2$O-producing activity greater than 0.15 μg N$_2$O vial$^{-1}$ d$^{-1}$, and the most active N$_2$O emitter, isolate 10CMF5-1B, produced 0.38 μg N$_2$O vial$^{-1}$ d$^{-1}$ (Fig. 1C). Among the 36 microbes obtained from pre-tilled soil cultures, six isolates exhibited higher N$_2$O emission than those selected from post-harvest soil bacteria (more than 1.0 μg N$_2$O vial$^{-1}$ d$^{-1}$). The most active isolate 05CFM15-4A showed a performance of 1.9 μg N$_2$O vial$^{-1}$ d$^{-1}$ (Fig. 1D). All active N$_2$O-emitting bacteria screened from post-harvest and pre-tilled soil were identified as Gram-negative bacteria of the genus *Pseudomonas* (Table 1), which includes some well-known denitrifiers active as N$_2$O emitters (4, 27).

Identification of N$_2$O-emitting pseudomonads by a phylogenetic analysis of 16S rRNA gene sequences

The 16S rRNA gene sequences of the *Pseudomonas* denitrifiers, 10CMF5-1B, 10CMF5-2D, and 10CMF5-2A from post-harvest soil and 10CMF5-4D and 10CMF5-6A from pre-tilled soil formed a clade (group A). Group A was separated into two sub-groups, sub-group A1 for the five nosZ-missing pseudomonads and sub-group A2 for the two nosZ-harboring *Pseudomonas* denitrifiers 10CMF5-4A and 05CFM15-6B (Fig. 2). *Pseudomonas* sp. PAMC 26831 (accession no. KF011705), which had been isolated from subarctic Alaskan grassland soil by Park and Kim (24), showed the highest sequence homology to members in sub-group A1. The other nosZ-harboring denitrifiers *Pseudomonas* sp. 10CMF5-2B and 05CFM15-5C made another clade (group B) distinguishable from pseudomonads in group A. Group B consisted of the nosZ-missing denitrifier *Pseudomonas* sp. 05CFM15-6D from pre-tilled soil and some reference pseudomonads, including *P. fluorescens* (Fig. 2).

Fig. 2. Phylogenetic position of N$_2$O-emitting *Pseudomonas* species isolated from a corn farm Andisol suspension culture. The phylogenetic relationships among the N$_2$O-emitting *Pseudomonas* sp. obtained from chemically fertilized corn farmland soil were computed, along with 10 reference species selected from several groups of pseudomonads. This neighbor-joining tree was deduced based on the 16S rRNA gene sequences obtained of the 1456-bp region using the maximum composite likelihood method for the estimation of distances between all pairs of sequences simultaneously with 1,000 bootstrap replicates by the Kimura 2-parameter model. The scale bar represents 0.01 substitutions per nucleotide site. The same region of the sequence from *E. coli* (J01859.1) was used as the outgroup. nosZ-missing denitrifiers are shown by red characters and nosZ-harboring denitrifiers by blue characters.
Response of N$_2$O-emitting pseudomonads to supplemented sucrose in the production of N$_2$O

Since most denitrifiers are saprophytes and *Pseudomonas* bacteria are generally heterotrophic, the responses of N$_2$O-emitting pseudomonads to carbon source-rich conditions were investigated. The addition of sucrose as a carbon source to standard assay medium led to an increase in the production of N$_2$O by the N$_2$O emitters, *Pseudomonas* 10CMF5-1B and 10CMF5-2D (Fig. 3). N$_2$O production from culture medium without the addition of sugar was not significant (less than 2.0 μg N$_2$O vial$^{-1}$ d$^{-1}$) when test bacteria were cultured in sugarless medium, whereas N$_2$O production was approximately 20-fold higher in the presence of 1.5 mM sucrose, which produced 8.0 μg N$_2$O vial$^{-1}$ d$^{-1}$. N$_2$O emission from *Pseudomonas* spp. 10CMF5-1B cultured in 15 mM sucrose-supplemented medium was more than 4-fold higher than that incubated in 1.5 mM sucrose-supplemented medium, reaching 37 μg N$_2$O vial$^{-1}$ d$^{-1}$.

In contrast, two N$_2$O-emitting pseudomonads from pretilled soil (isolates 10CMF15-6A and 05CMF15-6D) also showed strong responses to 1.5 mM sucrose-supplemented medium; however, N$_2$O emission markedly decreased upon supplementation with a 10-fold higher concentration of sucrose (Fig. 3). The responses of these N$_2$O emitters to additional sucrose suggested that the carbon source is an important factor for active N$_2$O emission due to the active cell growth of the highly heterotrophic and denitrifying *Pseudomonas* spp. and active nitrate respiration. Among these N$_2$O emitters, *Pseudomonas* sp. 10CMF15-6A cultured in 1.5 mM sucrose-supplemented medium showed the most active production of N$_2$O (37 μg N$_2$O vial$^{-1}$ d$^{-1}$).

**Acetylene-blocking assay and PCR assay for the detection of N$_2$OR activity and/or the nosZ gene**

The addition of 10% acetylene did not result in any significant acceleration in the production of N$_2$O by the *Pseudomonas* isolates 10CMF5-1B, 10CMF5-2D, 10CMF15-4D, 10CMF15-6A, and 05CMF15-6D, suggesting that these isolates are incomplete denitrifiers that are almost missing N$_2$O-reducing activity to produce N$_2$ gas (Fig. 4). Conversely, *Pseudomonas* isolates 10CMF5-2B, 05CF15-5C, 05CF15-6B, and 05CMF5-4A demonstrated significantly higher (approximately 2-fold) N$_2$O emission in the presence of acetylene in the headspace (p<0.001), and, thus, these isolates were characterizable as complete denitrifiers that possess a functional nosZ gene.

In the PCR assay to detect the nosZ gene toward N$_2$O-emitting pseudomonads, four isolates, 10CMF5-2B, 05CF15-5C, 05CFM15-6D, and 05CMF5-6D, demonstrated nosZ gene amplification, confirming their denitrifying capacity.
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05CF15-6B, and 10CFM5-4A, gave amplicons of 850–900 bp (Fig. S2). All the PCR products sequenced were confirmed to be nosZ fragments by a homology search on the DNA database (NCBI). All positive pseudomonads in the nosZ-PCR assay also showed weak, but positive responses in the acetylene-blocking assay, proving that they are complete denitrifiers. Unlike the nosZ gene, the narG gene was detected from all N₂O-emittable Pseudomonas spp. reported in this study (Fig. S3).

Optimum pH for N₂O emission by Pseudomonas denitrifiers

The optimum pH of the Pseudomonas denitrifiers 10CFM5-1B (nosZ-missing denitrifier) and 10CFM5-2B (nosZ-harboring denitrifier) from post-harvest soil for N₂O production was measured in a series of pre-autoclaved media with a range of pH values (3.6–7.6). The pH values in medium slightly increased after autoclaving, but decreased after the incubation (38), while the pH values in medium remained nearly in the original region, even though it contained 1.5 mM sucrose (Fig. S4). Both of the N₂O emitters, nosZ-missing Pseudomonas 10CFM5-1B and nosZ-harboring Pseudomonas 10CFM5-2B, produced N₂O at an appropriate pH range after culturing (5.8–6.3 and 6.0–6.8, respectively). Pseudomonas sp. 10CFM5-1B showed a strong response at approximately pH 6.0 during the incubation, while nosZ-harboring 10CFM5-2B actively emitted N₂O in a range of 6.2–6.8.

We also investigated the optimum pH of two more Pseudomonas denitrifiers 10CFM5-4A (nosZ-harboring) and 10CFM5-15-6A (nosZ-missing) from pre-tilled soil for N₂O production at a range of pH values after the incubation (Fig. 5). The responses of nosZ-harboring 10CFM5-4A and nosZ-missing 10CFM5-15-6A to medium pH were both similar to the isolates from post-harvest soil.

nosZ-missing 10CFM5-1B and 10CFM5-6A both exhibited the ability to converge the medium pH at 6.0–6.1, in which medium increments in the final pH from 5.9 to 6.1 showed proportional increases in the production of N₂O and optimum pH for the active production of N₂O appeared to be at pH 6.1 or higher. Emission decreased gradually in the acidic region and was nearly zero at pH 5.1 or lower. Cell growth was very active in all media showing high turbidity. Similarly, an inverse relationship was observed between N₂O production...
and acidity in the pH range of 5.1–6.4 and 5.3–6.1, respectively, in two nosZ-harboring pseudomonads 10CFM5-2B and 10CFM5-4A. This was dissimilar to N₂O emitters that have adapted to medium-strongly acidic tropical peat soils (e.g., *Janthinobacterium* sp.) (9), but more similar to that of an active N₂O emitter isolated from the Andisol of an unfertilized pasture (tentative *Leptothrix* sp. of subclass *Betaproteobacteria*) (38).

**Detection of denitrification genes (narG, nirS, and nosZ) and each phylogenetic relationship among nosZ-harboring Pseudomonas**

In the PCR assay for the detection of the nosZ gene among N₂O-emitting pseudomonads, four isolates, 10CFM5-2B, 05CF15-5C, 05CF15-6B, and 10CFM5-4A, produced amplicons of 700–900 bp (Fig. S3). All pseudomonads with positive nosZ-PCR also showed positive responses to the acetylene-blocking assay, proving that they are denitrifiers with relatively low N₂O OR activity (Table 1).

Unlike the nosZ gene, the narG gene was detected in all pseudomonad denitrifiers using the PCR assay (Fig. S3). A phylogenetic analysis using the 204-bp sequence of their narG genes in the region from positions 2152 and 2355 for *P. fluorescens* AK15 (accession no. U71398.1) along with some reference species of pseudomonads showed phylogenetic relationships among the 10 N₂O-emitting *Pseudomonas* bacterial isolates. A dendrogram of the partial narG gene was similar to that obtained by the 16S rRNA gene analysis (Fig. 2). Furthermore, all isolates also harbored the nirS gene, except for *Pseudomonas* 10CFM5-2B, which possessed the nirK gene (data not shown). A phylogenetic analysis of the 366-bp sequence of the nirS gene in the region of positions 937 to 1316 for *P. stutzeri* (accession no. AAZ43111.1) showed a dendrogram that was very agreeable with that of the 16S rRNA gene-based phylogenetic tree (Fig. S5).

A phylogenetic analysis of these nosZ-harboring isolates and *P. stutzeri* A1501 as a reference bacterium showed that the partial sequences of the nirS and narG genes showed similar phylogenetic dendrograms to those drawn on the 1.5-kbp sequences of their 16S rRNA genes. In contrast, the partial sequences of the nosZ gene showed a clearly different phylogenetic pattern, in which two isolates 10CFM5-4A and 05CF15-6B of sub-group A2 were markedly closer to *P. stutzeri* than other isolates in group B (10CFM5-2B and 05CF15-5C) (Fig. 6).

**The soil bacterial community structure shown by the 16S metagenomic analysis**

Although soil sample storage at 4°C in a zippered plastic bag continued for more than 4 years, stored soil showed some characteristic bacterial compositions according to land management. Fertilized corn farm soil (10CF 15-5) that had shown active N₂O emission in culture medium had a more characteristic bacterial composition in the order level than unfertilized corn farm soil (CC 15-4) (Fig. S6); however, the order *Pseudomonadales* was minor and no *Pseudomonas* was detected. *Rhodocyclales* (class *Betaproteobacteria*), *Chromatiales* (class *Gammaproteobacteria*), *Nitrospirales* (phylum *Nitrospira*), *Myxococcales*, *Desulfuromonadales*, and *Desulfovibrionales* (class *Deltaproteobacteria*), and *Coribacteriales* and *Acidimicrobiales* (phylum *Actinobacteria*) were increased in fertilized corn farm soil. Bacterial community structures in pasture soils (unfertilized PC and fertilized PFM) were clearly distinguishable from corn farm soils. PFM soil contained characteristic bacteria of the orders *Rhizobiales* (class *Alphaproteobacteria*), *Berkholderiales* (class *Betaproteobacteria*), *Chromatiales* (class *Gammaproteobacteria*), and *Gaiellales* (phylum *Actinobacteria*), while the order *Methylophilales* (class *Betaproteobacteria*) was missing in pasture soil.

**Discussion**

Regarding the effects of low pH, many biogeochemical studies have reported that the N₂O/N₂ production ratio in denitrification is higher in acidic soil than in neutral and alkaline soil (18, 20, 36, 37), and ample evidence has been obtained in European farm soils (5). Active N₂O emission in acidic soil is due to incomplete denitrification led by the transient inhibition of N₂OR for the catalytic reduction of N₂O into N₂ (2, 10, 19, 30, 35). Consistent with these findings, our results suggest that one of the most important factors affecting N₂O production is the weakened or completely lost functionality of N₂OR necessary for complete denitrification in the microbial community. In nitrate respiration by N₂O activity-missing denitrifiers, N₂O becomes the

**Table 1.** N₂O-emitting bacteria isolated from the post-harvest and pre-tilled Andisol of a corn farm and their identification by means of 16S rRNA gene sequence homology.

<table>
<thead>
<tr>
<th>Isolated strain</th>
<th>Temporal identification</th>
<th>PCR for nosZ&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acetylene-blocking assay&lt;sup&gt;b&lt;/sup&gt;</th>
<th>16S rRNA gene (bp)</th>
<th>Accession no.</th>
<th>Type of denitrifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-harvest soil (sampled in 2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10CFM5-1B</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1518</td>
<td>AB856847</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>10CFM5-2D</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1518</td>
<td>AB856848</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>10CFM15-2A</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1517</td>
<td>AB856849</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>10CFM5-2B</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>+</td>
<td>1546</td>
<td>AB856850</td>
<td>nosZ-harboring</td>
</tr>
<tr>
<td>Pre-tilled soil (sampled in 2012)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05CF15-5C</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1518</td>
<td>LC007966</td>
<td>nosZ-harboring</td>
</tr>
<tr>
<td>05CF15-6B</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1518</td>
<td>LC007967</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>05CF15-6D</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1518</td>
<td>LC007968</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>10CFM5-4A</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1577</td>
<td>LC007969</td>
<td>nosZ-harboring</td>
</tr>
<tr>
<td>10CFM15-4D</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1572</td>
<td>LC007970</td>
<td>nosZ-missing</td>
</tr>
<tr>
<td>10CFM15-6A</td>
<td><em>Pseudomonas</em> sp.</td>
<td>–</td>
<td>–</td>
<td>1576</td>
<td>LC006671</td>
<td>nosZ-missing</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amplification with a combination of two forward primers (nosZ-661F and 1111F) and two reverse primers (nosZ-1527R and 1773R).

<sup>b</sup> Acetylene-blocking assay: incubation at 20°C for 7 d with the addition of 1.5 mM sucrose.

The source and identification of ten *Pseudomonas* N₂O emitters are summarized. In the PCR assay for the detection of the nosZ gene, the sequenced amplicon obtained was marked by (+) when it was the targeted gene region. In the acetylene-blocking assay, (−) indicated no significant difference between the treatment and control.
N2O-emitting Pseudomonas in fertilized corn farm Andisol

In the present study, N2O-emitting bacteria screened from culturable communities of Andisol showed such traits, and, hence, N2OR-inactive traits appear to be more important than the acidic pH of soil for active N2O emission from Andisol (Fig. 1).

In the phylogenetic analysis of genus Pseudomonas bacteria using the 1456-bp sequence of the 16S rRNA gene in the region of positions 74-1541 for E. coli (accession no. J01859.1), the five Pseudomonas grouped together in the same clade (Fig. 2) suggest that N2OR activity-missing pseudomonads widely inhabit Andisol corn farmland. The members of sub-group A1, five nosZ-missing denitrifiers 10CFM5-1B, 10CFM15-2A, 10CFM5-2D, 10CFM15-4D, and 10CFM15-6A, had a far phylogenetic relationship with common N2O-emitting Pseudomonas species often containing N2OR, such as Pseudomonas stutzeri (34) and Pseudomonas denitrificans (4, 33). Shina et al. (35) reported that nosZ-negative or positive is highly affected by soil types; however, our results suggest that the nosZ-missing or nosZ-harboring trait is strongly associated with genotypes.

A previous study demonstrated that repeated fertilization through the cropping seasons triggered active N2O emission from the volcanic Andisol of grassland at Nasu, Tochigi Prefecture, in central Japan. In this non-frozen region, only manure-input grassland actively emitted N2O in winter (23). In contrast, in the Shizunai Livestock Farm, Hokkaido Prefecture, the most active annual N2O efflux was observed in thawing, pre-tilled corn farm soil in early spring (16, 46). At the transition period (just at thawing) in early spring, NH4+ concentrations in corn farm soil temporally increased by approximately 10-fold that of frozen period, and this was followed by the rapid depletion of NH4+ and accumulation of NO3− soon after the thawing event (16). This marked accumulation of NO3− in thawing soils and the results of our culturing assay collectively suggest the importance of nitrification-coupled denitrification in active N2O emission. Conversely, nitrifier denitrification appears to contribute less to N2O emission because microaerobic conditions with the low availability of organic carbon in soils are required for this event (44).

The N2O-emitting abilities of the nosZ-missing denitrifiers Pseudomonas spp. 10CFM5-1B, 10CFM5-2D, 10CMF15-2A, 10CFM15-4D, 10CFM15-6A, and 05CFM15-6D were higher than those of the nosZ-harboring Pseudomonas spp. 10CFM5-2B, 05CF15-3C, 05CF15-6B, and 05CFM5-4A, particularly in the presence of an additional carbon source (Fig. 3). All the nosZ-missing denitrifiers of pseudomonads were present in post-harvest and pre-tilled fertilized soil that had been cultivating corn from 2010 (10CFM), while nosZ-harboring denitrifiers were particularly dominant in the pre-tilled spring soil of the corn farm that had been chemically fertilized since 2005 (05CF). The pseudomonads in group A showed the highest sequence homology to Pseudomonas sp. PAMC 26831 (accession no. KF011705), which had been isolated from subarctic Alaskan grassland soil by Park and Kim (24), and uncultured Pseudomonas sp. RF3-C12 (accession no. JN379403), reported as a catechin degrader in the rhizosphere of Rhododendron formosanum (42) (Fig. 2).
Pseudomonas N₂O emitters isolated from post-harvest and pre-tilled corn farm soil appeared to be root-associated because of their obvious saprophytic behaviors, which are highly responsive to supplemented sucrose (Fig. 3). Corn roots in post-harvest soil may enhance carbon and nitrogen turnover by soil microbes and soil ecosystems as reported previously by Henry et al. (11), and, accordingly, N₂O-emitting pseudomonads in pre-tilled farm soil may have survived winter on corn root residues as saprophyles. Furthermore, the neutrophilic properties of these pseudomonads for active N₂O emission at neutral regions may have been reasonable for rhizoplane bacteria because root surfaces generally maintain neutral pH even in acidic soils (11). In the crop-growing seasons, root-assOCIating, saprophytic pseudomonads may contribute to denitrification in fertilized Andisol corn fields. If denitriifiers were screened under the acetylene-blocking condition, we may have obtained highly N₂OR-active Pseudomonas denitrifiers as key bacteria in order to elucidate the mechanisms underlying passive activity-loss for N₂O. Unfortunately, such pseudomonads are mostly diminished from the soil community during long-term (more than 4 years) storage at 4°C (Fig. S6). The phylogenetic tree of N₂O-emitting Pseudomonas bacteria constructed using sequence variations in their partial narG and nirS genes sequences is consistent with the dendrogram constructed from the 16S rRNA gene sequences among the pseudomonads. However, the phylogenetic tree of the nosZ and 16S rRNA genes among nosZ-harbouring Pseudomonas showed very weak similarities (Fig. 6). Thus, the nosZ gene may be particularly unstable among denitrification-associated genes, and easyly undergoes the re-construction and deletion of gene regions, leading to the appearance of active N₂O emitters that have lost N₂OR activity. It has been hypothesized that nosZ is actively transferred by horizontal gene transmission because nosZ was previously found to be absent in some bacteria and archaea harboring nar and nir genes (48). An inspection of a wide range of 16S rRNA trees in the reliable nosZ data set was initially performed by Jones et al. (14), and they reported that nosZ genes in the denitrifiers of Proteobacteria were not of a monophyletic origin.

The results of the present study suggest that an important step in the emergence of N₂O-emitting bacteria in corn farm Andisol is decreased N₂O activity; however, these denitrifying Pseudomonas bacteria do not solely active N₂O emission in farmland soil. However, this speculation requires further evidence for the malfunctioning of N₂O OR or absent nosZ gene that progressively occurs in proteobacterial denitrifiers in corn farm Andisol, along with a metagenomic analysis on fresh soil from thawing and pre-tilled corn-cultivated farm soil in early spring. Not only the chemical and physical properties of vitric Andisol in the farm, but also its land management history, including cultivated crops as well as manure and chemical fertilizer input, may be key factors responsible for the appearance of such N₂O OR–emitting, heterotrophic denitrifiers that have lost N₂O OR activity. These N₂O emitters of Pseudomonas may be a useful bacterial tool to search for effective N₂O regulation, including delivery methods for functional nosZ genes. Hence, further studies on the relationship between N₂O emitters and vitric Andisol and corn roots in addition to their bias effects during trapping cultures in sugarless gellan gum medium are warranted.

Acknowledgements

This research was supported by Grant-in-Aids A (20248033 and 26252058 to YH) and B (26304042 to Y.H.). Scholarships to L.L. and Y.N. from the Chinese Scholarship Council (CSC 2011491196 and 20124910200 respectively) are also appreciated.

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