ORIGINAL ARTICLE

Identification and isolation of active N₂O reducers in rice paddy soil

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Dissolved N₂O is occasionally detected in surface and ground water in rice paddy fields, whereas little or no N₂O is emitted to the atmosphere above these fields. This indicates the occurrence of N₂O reduction in rice paddy fields; however, identity of the N₂O reducers is largely unknown. In this study, we employed both culture-dependent and culture-independent approaches to identify N₂O reducers in rice paddy soil. In a soil microcosm, N₂O and succinate were added as the electron acceptor and donor, respectively, for N₂O reduction. For the stable isotope probing (SIP) experiment, ¹³C-labeled succinate was used to identify succinate-assimilating microbes under N₂O-reducing conditions. DNA was extracted 24 h after incubation, and heavy and light DNA fractions were separated by density gradient ultracentrifugation. Denaturing gradient gel electrophoresis and clone library analysis targeting the 16S rRNA and the N₂O reductase gene were performed. For culture-dependent analysis, the microbes that elongated under N₂O-reducing conditions in the presence of cell-division inhibitors were individually captured by a micromanipulator and transferred to a low-nutrient medium. The N₂O-reducing ability of these strains was examined by gas chromatography/mass spectrometry. Results of the SIP analysis suggested that *Burkholderiales* and *Rhodospirillales* bacteria dominated the population under N₂O-reducing conditions, in contrast to the control sample (soil incubated with only ¹³C-succinate). Results of the single-cell isolation technique also indicated that the majority of the N₂O-reducing strains belonged to the genera *Herbaspirillum* (*Burkholderiales*) and *Azospirillum* (*Rhodospirillales*). In addition, *Herbaspirillum* strains reduced N₂O faster than *Azospirillum* strains. These results suggest that *Herbaspirillum* spp. may have an important role in N₂O reduction in rice paddy soils.

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Introduction

Nitrous oxide (N₂O) is considered as a major greenhouse gas and is a significant contributor to ozone layer destruction (Zumft and Kroneck, 2006). N₂O is mainly produced by denitrification, a microbial respiratory process in which nitrate/nitrite is reduced to gaseous forms (NO, N₂O and N₂); however, other microbial processes, such as nitrification and dissimilatory nitrate reduction to ammonium (DNRA), can also produce N₂O (Conrad, 1996).

Agricultural fields are one of the main sources of N₂O emission (Philippot *et al.*, 2007; Minamikawa *et al.*, 2010). In contrast to upland crop fields, little

N₂O is emitted from rice paddy soils, even though paddy fields are known to have strong denitrification activity (Akiyama *et al.*, 2006). Dissolved N₂O is occasionally detected in surface and ground water in rice paddy fields, whereas little or no N₂O is emitted to the atmosphere above these fields (Xiong *et al.*, 2006; Minamikawa *et al.*, 2010). This indicates that water-dissolved N₂O is possibly reduced by N₂O-reducing microorganisms in rice paddy fields.

N₂O can serve as an electron acceptor for microbial respiration. The standard reduction potential (E_0' at pH 7) of the reaction (N₂O + 2H⁺ + 2e⁻ → N₂ + H₂O) is 1.35 V with $\Delta G_0'$ of -339.5 kJ mol⁻¹ (Zumft and Kroneck, 2006). Phylogenetically diverse bacteria and archaea have the ability to reduce N₂O. Although the reduction of N₂O to N₂ gas is part of denitrification, some denitrifiers do not have the ability to reduce N₂O (Tiedje, 1994). Both N₂O-reducing strains and non-reducing strains may be present within the same species (Sameshima-Saito *et al.*, 2006). In addition, some DNRA bacteria have the ability to reduce N₂O (Conrad, 1996).

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Therefore, it is difficult to use 16S rRNA gene sequences alone to identify N₂O reducers. Instead, the gene encoding N₂O reductase (*nosZ*) has been used to detect potential N₂O reducers in various environments (Rich *et al.*, 2003). Although *nosZ* phylogeny is generally in agreement with 16S rRNA gene phylogeny, horizontal gene transfer may have occurred among closely related microorganisms (Dandie *et al.*, 2007; Jones *et al.*, 2008) and we therefore cannot identify N₂O reducers on the basis of *nosZ* sequence information alone.

One approach to link microbial identity to a specific function is stable isotope probing (SIP) of nucleic acids (Radajewski *et al.*, 2000; Gutierrez-Zamora and Manefield, 2010). In the SIP approach, microbes that have incorporated heavy stable isotopes (for example, ¹³C, ¹⁵N, ¹⁸O) into their DNA (or RNA) can be identified by analyzing the heavy DNA fractions separated by density gradient ultracentrifugation. Using the SIP approach, we can analyze the 16S rRNA and functional gene diversities of microbial populations involved in specific functions. Previously, ¹³C-assimilating populations under denitrifying conditions were analyzed by DNA-based SIP analysis (Ginige *et al.*, 2004; Osaka *et al.*, 2006, 2008; Saito *et al.*, 2008). However, microbial populations responsible for N₂O reduction have not been examined to date.

Another approach to identifying such populations is to isolate and analyze N₂O reducers that are active and dominant in the environment. We previously developed a single-cell isolation technique to obtain actively growing microorganisms from environmental samples and designated it the functional single-cell (FSC) isolation method (Ashida *et al.*, 2010). In this method, individual cells growing in response to certain conditions (for example, denitrification-inducing conditions) are elongated or enlarged, and can be individually captured with a micromanipulator. Single-cell isolation techniques provide an environment without resource competition, thereby allowing microbes, including slow-growing microorganisms, to multiply without interference from fast-growing ones (Ishii *et al.*, 2010a). The FSC isolation method allowed us to obtain denitrifiers that were shown to be active and dominant by culture-independent analyses (Ishii *et al.*, 2011). By analyzing the isolated strains, we were able to directly link the 16S rRNA gene and functional gene phylogenies. In addition, various cell properties, such as denitrification and N₂O reduction rates, could also be measured (Tago *et al.*, 2011).

Consequently, the objectives of the current study were (1) to identify ¹³C-assimilating populations under N₂O-reducing conditions by SIP, (2) to isolate N₂O-reducing microorganisms from rice paddy soil by using the FSC isolation method, (3) to examine the N₂O reduction rates of the isolated strains and (4) to compare the results obtained by SIP and the FSC isolation method.

Materials and methods

Soil microcosm

Soil samples were collected from rice paddy fields at the Institute for Sustainable Agro-Ecosystem Services, The University of Tokyo, Nishitokyo City, Tokyo, Japan (Saito *et al.*, 2008). A soil microcosm setup was established based on the previous reports (Saito *et al.*, 2008; Ishii *et al.*, 2009b), except N₂O was used as an electron acceptor instead of nitrate. Succinate was used as an electron donor for N₂O reduction in this study. As succinate is a member of the TCA cycle and is considered as a non-fermentable carbon substrate, it can be used by various N₂O reducers, but not by fermenting microbes. The optimum concentrations of electron acceptor and donor (N₂O and succinate, respectively) were determined by adding several combinations of N₂O (0%, 0.5%, 1%, 2%, 5% and 20% in Ar base) and succinate (0 mg, 0.01 mg, 0.025 mg, 0.05 mg, 0.1 mg, 0.25 mg and 0.5 mg C per g soil), and were set at 5% N₂O and 0.1 mg succinate C per g soil. For SIP, ¹³C-labeled succinate (Cambridge Isotope Laboratories, Andover, MA, USA) was used (0.1 mg (= 8.3 μmol) of ¹³C per g soil). For FSC isolation, cell-division inhibitors (20 μg nalidixic acid, 10 μg pipemidic acid, 10 μg piromidic and 10 μg cephalixin) (Joux and Lebaron, 1997) were added to the vial together with N₂O and succinate. The vial was then incubated under an Ar:N₂O (95:5) atmosphere and static conditions at 30 °C for 24 h.

CO₂ and N₂O gases in the headspace of the vial were quantified by gas chromatography (GC) as described previously (Saito *et al.*, 2008). When ¹³C-labeled succinate and ¹⁵N-labeled N₂O (¹⁵N, 99 atom. %; Cambridge Isotope Laboratories) were used, ⁴⁴CO₂, ⁴⁵CO₂, ⁴⁴N₂O, ⁴⁶N₂O, ²⁸N₂ and ³⁰N₂ were separately quantified by GC–mass spectrometry (GC/MS) using the gas chromatograph/mass spectrometer QP5050 (Shimadzu, Kyoto, Japan) as described elsewhere (Miyahara *et al.*, 2010). Succinate was extracted from soil with 5 ml water and quantified by high-performance liquid chromatography as described previously (Saito *et al.*, 2008). Fe²⁺ was anaerobically extracted from soil with 1 M ammonium acetate solution (pH 3) and quantified colorimetrically as described previously (Ishii *et al.*, 2009b).

SIP

DNA was extracted from the soil microcosms (*n* = 10) amended with N₂O and ¹³C-succinate (sample 13SN) using ISOIL for bead beating (Nippon Gene, Tokyo, Japan). As controls, DNA was also extracted from the soil microcosms (*n* = 10) amended with N₂O and ¹²C-succinate (sample 12SN), ¹³C-succinate only (sample 13Su), and ¹²C-succinate only (sample 12Su). Community structures among the replicate samples were analyzed and compared by PCR–denaturing gradient gel

electrophoresis (DGGE) targeting 16S rRNA gene as described previously (Ishii *et al.*, 2010b). After confirming that the DGGE profiles looked similar among the replicate samples, purified DNA from 10 replicate samples was pooled to ensure a sufficient amount of DNA for ultracentrifugation. Cesium chloride density gradient ultracentrifugation was performed as described by Neufeld *et al.* (2007) at an average of $177\,000 \times g$ (53 200 rpm) using a P100VT rotor (Hitachi Koki, Tokyo, Japan). After 40 h centrifugation, gradients of density-resolved DNA were fractionated and purified as described elsewhere (Neufeld *et al.*, 2007). The copy number of the 16S rRNA gene in each fraction was determined by quantitative PCR as described previously (Fierer *et al.*, 2005; Ishii *et al.*, 2009b). Community structures among the DNA fractions were analyzed by the 16S rRNA gene-based PCR–DGGE and principal component analysis as described previously (Ishii *et al.*, 2009a) and clone library analysis as described below.

Single-cell isolation

Metabolically active cells were stained with 5-carboxyfluorescein diacetate acetoxymethyl ester as described previously (Ashida *et al.*, 2010). Fluorescing cells were observed under a fluorescent microscope (Diaphot 300, Nikon, Tokyo, Japan) with $400\text{--}1000\times$ magnification.

Single cells were isolated using a micromanipulator (MTA-31, Daiwa Union, Iida, Nagano, Japan) equipped with a microinjector (UJI-A, Daiwa Union) as described previously (Ashida *et al.*, 2010). After a single cell was captured in the capillary of the micromanipulation system, the tip of the capillary was soaked in 70% ethanol for 30 s to disinfect its outside. The captured cell was then ejected into a test tube containing 100-fold diluted nutrient broth (Hashimoto *et al.*, 2009) supplemented with 4.4 mM succinate (DNB–S medium) and incubated at 30 °C under N₂O-reducing conditions for 2 weeks. To obtain purified isolates, the cultures in the DNB–S media were streaked onto DNB–S agar and incubated at 30 °C for 2 weeks.

N₂O reduction and denitrification activities of the strains

Each strain was inoculated into 5 ml of DNB–S medium in a 10 ml glass serum vial, and the headspace air was replaced with Ar:¹⁵N-labeled N₂O (95:5) gas. After incubation at 30 °C for 1 week, the amounts of ¹⁵N-labeled N₂ and N₂O were measured by GC/MS as described above. Denitrification activities of the strains were also measured in duplicate (two vials for each strain) by the acetylene block method (Tiedje, 1994) described previously (Ishii *et al.*, 2011; Tago *et al.*, 2011).

For selected strains, the N₂O-reducing rate was also measured. Cells were grown in DNB–S medium in a vial with 5% non-labeled N₂O gas in Ar base.

After 1-week incubation, cells were harvested and inoculated, in triplicate (three vials for each strain), into fresh, 5 ml of DNB–S medium at 10^5 cells ml^{−1}. The headspace air was replaced with Ar:¹⁵N-labeled N₂O (95:5) gas, and the vial was then incubated at 30 °C. Amounts of ¹⁵N-labeled N₂ and N₂O were measured at 3, 6, 9, 12, 18 and 24 h after inoculation by GC/MS as described above.

PCR, cloning and sequencing

For culture-independent clone library analysis of the microbial community in the heavy fractions from 13SN and 13Su samples, the 16S rRNA gene and *nosZ* were PCR amplified using primers m-27F and m-1492R (Tyson *et al.*, 2004) and *nosZ*-F-1181 and *nosZ*-R-1880 (Rich *et al.*, 2003), respectively. PCR was performed using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) under conditions described elsewhere (Rich *et al.*, 2003; Ishii *et al.*, 2009b). After removing excess primers and dNTP by using a Wizard DNA Cleanup system (Promega, Madison, WI, USA), PCR products were cloned into a pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* JM109 high-efficiency competent cells (Promega) according to the manufacturer's instructions. DNA inserts from randomly selected clones were amplified by PCR with vector primers T7-1 and SP6, and sequenced as described previously (Saito *et al.*, 2008).

For isolated strains, DNA was extracted from cells as described previously (Ashida *et al.*, 2010). PCR was performed to amplify the 16S rRNA gene and *nosZ* as described above. In addition, the nitrite reductase gene (*nirK* or *nirS*) was amplified using primers F1aCu and R3Cu (Throbäck *et al.*, 2004) or cd3aF and R3cd (Throbäck *et al.*, 2004), respectively, as described previously (Yoshida *et al.*, 2010). PCR products were purified using a Wizard DNA Cleanup system (Promega) and directly sequenced as described previously (Ashida *et al.*, 2010).

DNA fingerprinting analysis

Repetitive element palindromic-PCR DNA fingerprinting was performed using the BOXA1R primer according to the protocol described by Rademaker *et al.* (2008) to examine the relatedness of the strains (Ishii and Sadowsky, 2009). The amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel at 80 V for 8 h, and the image was visualized under UV light. The image was digitized and analyzed as described previously (Ishii *et al.*, 2009a). Strains with >80% DNA fingerprint similarity were considered identical.

Phylogenetic analysis

The nucleotide sequences were trimmed and assembled as described previously (Ishii *et al.*, 2009b; Ashida *et al.*, 2010). Taxonomic assignment of the

clones or strains was performed based on their 16S rRNA gene sequences by using the Ribosomal Database Project classifier program (Wang *et al.*, 2007) with 80% as the bootstrap cutoff. Operational taxonomic units were determined at 97% nucleotide sequence similarity by using MOTHUR program (Schloss *et al.*, 2009). The nucleotide or deduced amino acid sequences from multiple strains were aligned with reference sequences obtained from the DDBJ/EMBL/GenBank databases. A phylogenetic tree was constructed based on the maximum likelihood method by using MEGA version 5 (Tamura *et al.*, 2007).

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA gene and *nosZ* from the isolated strains were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB545618–AB545660 and AB545661–AB545698, respectively (Supplementary Table S1). The nucleotide sequences of the 16S rRNA gene and *nosZ* from the culture-independent analysis were also in the databases under the accession numbers AB608638–AB608703 and AB608704–AB608729, respectively.

Results

Evaluation of the soil microcosm

Based on the preliminary experiments, all of the added N₂O disappeared within 24 h of incubation when <2% N₂O was added (data not shown). As N₂O should always be present to minimize utilization of succinate by metal reducers, the concentration of N₂O should be >2%. Based on the Bunsen absorption coefficient and Henry's law, the concentration of the water-dissolved N₂O would be 1 mM when 5% N₂O was added to a 10 ml vial containing 1 g soil submerged in 1 ml water. This concentration is 10- to 100-fold less than the N level found in the rice paddy field right after the fertilizer application (Saito *et al.*, 2008).

Preliminary experiments also showed that the addition of <0.1 mg succinate C did not significantly enhance N₂O reduction (Supplementary Table S2). Addition of >0.1 mg succinate C significantly enhanced N₂O reduction (Supplementary Table S2), but 33% and 58% of the added succinate remained unused when 0.25 and 0.5 mg succinate C was added, respectively. In the presence of 5% N₂O, all of the added succinate (0.1 mg C) was consumed within 24 h, whereas 32% of the added succinate remained unused in the absence of N₂O. Concentrations of Fe²⁺ in the soil significantly increased ($P < 0.05$) after 24 h anaerobic incubation with 0.1 mg succinate C, but not after anaerobic incubation with 0.1 mg succinate C and 5% N₂O nor after anaerobic incubation without succinate addition (Supplementary Table S3). These results suggest

Figure 1 Time-course changes in ⁴⁶N₂O (●), ³⁰N₂ (■), ⁴⁵CO₂ (○) and ⁴⁴CO₂ (△) in soil microcosms amended with ⁴⁶N₂O and ¹³C-labeled succinate. Mean ± s.e. (n = 3) is shown. An arrow indicates the time when DNA was extracted (24 h).

that succinate is likely used by N₂O reducers when N₂O is present, but it can be used by metal reducers when N₂O is absent. Based on these results, we considered 0.1 mg succinate C per gram soil to be sufficient and the minimum required for enhancing N₂O reduction.

Figure 1 shows time-course changes in N₂O, N₂ and CO₂ in the soil microcosms amended with the optimum concentrations of N₂O and succinate (5% and 0.1 mg C, respectively). The quantity of ¹⁵N-labeled N₂ increased along with the decrease in ¹⁵N-labeled N₂O in the microcosm, suggesting that N₂O was reduced to N₂. The N₂O decrease was larger than the amount of N₂ produced. This unbalanced N₂O mass may be attributed to N₂ fixation or other N₂O metabolism such as N₂O oxidation. The amount of ¹³C-labeled CO₂ gradually increased and reached a plateau after 18 h, whereas non-labeled CO₂ continued to increase after 24 h. About 10% of the added succinate (0.1 mg C = 8.3 μmol) was oxidized to CO₂. As all of the added succinate was consumed within 24 h, the remaining *ca.* 90% of the added succinate was assumed to be used as a C source by actively growing microbes.

Identification of N₂O reducers by SIP

SIP was performed to study succinate-assimilating populations under N₂O-reducing and non-reducing conditions. Figure 2 shows the relative amount of the 16S rRNA gene in DNA fractions separated by CsCl density gradient ultracentrifugation. All four samples had peaks in the light DNA fractions (L fraction) with buoyant densities of 1.70–1.715 g cm⁻³. An additional peak was also observed in the 13SN sample in the heavy DNA fraction (H fraction) with buoyant densities of 1.73–1.75 g cm⁻³. A small amount of DNA was also seen in the H fraction from the 13Su sample.

PCR-DGGE analysis showed that the community structure differed between the H and L fractions

within a sample (Figure 3). The community structure also differed among the H fractions originating from the 13SN, 13Su and 12SN samples. Bands specific to each fraction were excised and sequenced (Supplementary Table S2). While most bands originated from bacteria belonging to the orders *Burkholderiales* (class *Betaproteobacteria*) and *Rhodospirillales* (class *Alphaproteobacteria*) in the H fraction of the 13SN sample (Figure 4a), many bands were from bacteria belonging to the order *Desulfuromonadales* (class *Deltaproteobacteria*) in the H fraction of the 13Su sample (Figure 4b). Bands appearing in the H fraction of the 12SN sample were similar to that of the 16S rRNA gene sequence of bacteria belonging to the orders *Bacillales* and *Clostridiales* (phylum *Firmicutes*) and the order *Rhodospirillales* (Supplementary Table S4).

In order to examine the community structure in the H fractions of the 13SN and 13Su samples in

Figure 2 Cesium chloride density gradient centrifugation of DNA extracted from soil. Buoyant densities of the light (L), middle (M) and heavy (H) density fractions were 1.70–1.715, 1.715–1.73 and 1.73–1.75 g cm⁻³, respectively. 13SN sample (■), 13Su sample (●), 12SN sample (□) and 12Su sample (○).

detail, we performed clone library analysis based on the near-full length 16S rRNA gene. Similar to the PCR–DGGE results, most clones were related to the orders *Burkholderiales* and *Rhodospirillales* in the H fraction of the 13SN sample (Figure 4c). Among these, clones closely related to the genus *Herbaspirillum* (order *Burkholderiales*) were most frequently obtained. In contrast, clones related to the genus *Geobacter* (order *Desulfuromonadales*) dominated the H fraction of the 13Su sample (Figure 4d).

Isolation of N₂O reducers

In addition to the culture-independent analyses, culture-based analysis was also performed in this study. During FSC isolation, 61 elongated single cells were captured from the soil microcosm incubated under N₂O-reducing conditions. No elongated cells were observed in the sample without cell-division inhibitors. After single-colony isolation and GC/MS analysis, 33 N₂O-reducing strains were obtained.

Similar to the results obtained by clone library analysis, strains closely related to the genus *Herbaspirillum* were most frequently obtained (20 strains; Figure 4e). 16S rRNA gene sequences of the isolated *Herbaspirillum* strains were >98% similar to the SIP clones obtained in this study (Figure 5a). Strains related to the genera *Azospirillum* (seven strains) and *Burkholderia* (three strains) were the second and third most frequently obtained, respectively.

N₂O reductase gene

nosZ was detected in all N₂O-reducing strains. Diverse *nosZ* sequences were also obtained from the clone library constructed based on the H fraction of the 13SN sample. Figure 5b shows the phylogenetic tree constructed based on the *nosZ*

Figure 3 Community structure assessed by DGGE analysis. (a) DGGE banding profile from each fraction separated by CsCl density gradient centrifugation. Gel region shown is between 44% and 54% denaturant concentrations, as estimated by the DGGE marker II (Nippon Gene). L, M and H correspond to the light, middle and heavy DNA fractions as shown in Figure 2. Bands specific to the H fractions of each sample (indicated by arrows) were excised, cloned and sequenced (Supplementary Table S2). (b) Principal component analysis plot based on the DGGE profile. The normalized location and intensity of each DGGE band were used (Ishii *et al.*, 2009a). The numbers in the plot correspond to the lanes in panel a. The percentages in parentheses are the percentages of variation explained by the components.

sequences obtained in this study. With some exceptions, similar *nosZ* sequences were obtained from phylogenetically closely related strains. For example, *nosZ* sequences of most *Burkholderiales* bacteria (*Burkholderia* spp., *Herbaspirillum* spp. and *Massilia* spp.) were clustered together (cluster I). The *nosZ* sequences of some *Herbaspirillum* strains were distantly related to these sequences and were more closely related to the *nosZ* of *Azospirillum* spp. (cluster II).

Figure 5b also shows the relatedness between *nosZ* sequences obtained from SIP and FSC analyses. From the H fraction of the 13SN sample, *nosZ* sequences in cluster I were most frequently obtained (78%), and these sequences were >76% similar to those of *Burkholderiales*. We did not find 100% match in the *nosZ* sequences between isolated

strains and SIP clones. This may be due, in part, to the formation of chimeric sequences in SIP analysis.

N₂O reduction and denitrification activities

Based on the *nosZ* sequence information and repetitive element palindromic-PCR DNA fingerprinting, three *Herbaspirillum* strains (TSO23-1, TSO35-1 and TSO37-1), three *Azospirillum* strains (TSO5, TSO22-1 and TSO41-3) and two *Burkholderia* strains (TSO10-2 and TSO47-3) were selected for measurement of N₂O reduction activity. As both an electron acceptor and an electron donor were abundantly present under the experimental conditions, the reaction ($\text{N}_2\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2 + \text{H}_2\text{O}$) followed zero-order kinetics. The N₂O reduction rates of *Herbaspirillum* spp., *Burkholderia* spp. and *Azospirillum* spp. were 1.72 ± 0.13 , 1.39 ± 0.24 and 0.65 ± 0.06 pmol h⁻¹ cell⁻¹, respectively; these differed by genus ($P < 0.05$) but not by *nosZ* cluster (cluster I vs II + III).

The majority (76%) of the N₂O-reducing strains carried *nirS*, which encodes cytochrome *cd*₁ nitrite reductase, and were able to perform denitrification (Supplementary Table S1). No strains were detected with *nirK*, which encodes copper-containing nitrite reductase. We could not amplify the nitrite reductase gene from the denitrifying *Azospirillum* strains TSO5, TSO7, TSO9 and TSO35-2. *Azospirillum* sp. strain TSO41-3, *Burkholderia* sp. strain TSO11-3, and *Massilia* and *Bacillus* strains did not show denitrification activity and nitrite reductase genes were not detected.

Discussion

Although denitrifying and nitrate-reducing communities have been studied in various environments including rice paddy soils (Philippot *et al.*, 2007; Ishii *et al.*, 2009b), microbial communities responsible for N₂O reduction have not been well characterized. In the present study, we employed both culture-independent (SIP) and culture-dependent (FSC isolation) techniques to analyze N₂O reducers in rice paddy soil. Populations that assimilated succinate under N₂O-reducing conditions were examined by SIP analysis. The FSC isolation method was used to isolate microbes that were ready to grow under the same N₂O-reducing conditions used for the SIP analysis. Combined analysis of the results obtained by SIP and FSC isolation allowed us to assess the phylogeny, function and physiology of the microbes responsible for N₂O reduction.

In the present study, succinate was used as an electron donor by the N₂O reducers. Previous studies have shown that anaerobic incubation of soil with nitrate and succinate greatly enhances denitrification activity (Saito *et al.*, 2008; Ishii *et al.*, 2009b). Under such conditions, succinate could be

Figure 4 Taxonomic classification of the (a) DGGE band excised from the H fraction of the 13SN sample, (b) DGGE band excised from the H fraction of the 13Su sample, (c) clones obtained from the H fraction of the 13SN sample, (d) clones obtained from the H fraction of the 13Su sample and (e) strains obtained by the FSC isolation method. Taxonomic assignment was performed using the Ribosomal Database Project classifier program (Wang *et al.*, 2007) at the order and genus level for the DGGE results (ca. 180bp) and clone library results (ca. 1450bp), respectively. Relative intensities of the DGGE bands (see Supplementary Table S2) correspond to the fraction of the assigned taxon.

used by various denitrifiers, whereas there would be little utilization of succinate for other functions, such as fermentation, DNRA, and metal and sulfate reduction (Saito *et al.*, 2008; Ishii *et al.*, 2009b). Oxidation of succinate ($E_0' = +33$ mV) can also be coupled with reduction of N₂O ($E_0' = +1355$ mV). According to the thermodynamic theory (Thauer *et al.*, 1977), N₂O is the preferred electron acceptor to Mn⁴⁺, Fe³⁺ and sulfate. Our results support this notion as the production of Fe²⁺ was suppressed by the addition of N₂O.

Succinate-assimilating populations under N₂O-reducing and non-reducing conditions were examined by SIP. Under non-N₂O-reducing conditions (sample 13Su), clones related to the genus *Geobacter* (order *Desulfuromonadales*) were most frequently (42%) obtained (Figure 4d). As production of Fe²⁺ was observed in the 13Su sample, bacteria identified in the 13Su clone library may be involved in metal reduction with succinate as an electron donor. The *Geobacter* species is well known for its capacity to reduce metals (Lovley *et al.*, 2004). Similar to our study, RNA-based SIP analysis has revealed that *Geobacter*, *Anaeromyxobacter*, and a novel *Betaproteobacteria* closely related to the order *Rhodocyclales* were acetate-assimilating iron reducers in Italian rice paddy soil (Hori *et al.*, 2009). In the present study, *Herbaspirillum* spp. and other *Burkholderiales* bacteria were also detected in the H fraction of the 13Su sample. As these bacteria were not detected in the control sample (H fraction of the 12SN sample), they are most likely enriched under succinate-assimilating and metal-reducing conditions. Similar to our study, clones related to *Herbaspirillum* have been frequently obtained in a sediment sample (collected at Oak Ridge, TN, USA) incubated without nitrate (Li and Krumholz, 2008) and in a sediment sample (collected at Hanford, WA, USA) incubated with organic acids (Lee *et al.*, 2010).

In contrast to the results obtained from the 13Su sample, clones related to *Herbaspirillum* spp. and other *Burkholderiales* bacteria dominated the population in the clone library constructed from the H fraction of the 13SN sample (N₂O-reducing conditions) (Figure 4c). Involvement of these bacteria in N₂O reduction was also supported by culture-dependent FSC isolation (Figure 4e). *Herbaspirillum* strains obtained by FSC isolation carried *nosZ* and reduced exogenous N₂O to N₂. The majority of the SIP *nosZ* clones were similar to *nosZ* of *Herbaspirillum* and other *Burkholderiales* N₂O reducers

(Figure 5). Similar *nosZ* clones have also been obtained from other paddy fields (for example, GenBank Accession No. ACI48848) and maize rhizospheric soils (Mounier *et al.*, 2004; Dambreville *et al.*, 2006; Henry *et al.*, 2008). Considering the general agreement between the 16S rRNA gene and *nosZ* phylogenies (Jones *et al.*, 2008; Palmer *et al.*, 2009), these results suggested that *Herbaspirillum* and other *Burkholderiales* bacteria may be important players in N₂O reduction, not only in rice paddy soils but also in other environments. *Herbaspirillum* strains were previously shown to be involved in nitrate reduction of rice paddy soil (Ishii *et al.*, 2009b, 2011), but the present study showed that they are also important players in N₂O reduction. Although some *Herbaspirillum* species (for example, *Herbaspirillum seropedica*) can colonize rice roots and stems and fix atmospheric N₂ (Baldani *et al.*, 1986; Elbertagy *et al.*, 2001), almost all strains obtained in this study did not show N₂-fixing ability (S. Ishii, unpublished data). In addition, 16S rRNA gene similarities between the *Herbaspirillum* strains obtained in this study and other *Herbaspirillum* species were <97%. These results suggest that the N₂O-reducing *Herbaspirillum* strains obtained in this study may constitute a new species.

The SIP and FSC isolation results also suggested that *Azospirillum* spp. and other *Rhodospirillales* bacteria were responsible for N₂O reduction. The N₂O reduction rates suggested that *Azospirillum* spp. reduced N₂O more slowly than *Herbaspirillum* spp. Although their *in situ* N₂O reduction rates are not known, these results indicated that the relative contribution of *Azospirillum* strains to N₂O reduction might be smaller than that of *Herbaspirillum* strains. Similar to other *Azospirillum* strains (for example, *Azospirillum brasilense* and *Azospirillum* sp. B510; Isawa *et al.*, 2010), our *Azospirillum* strains also showed N₂-fixing ability (S. Ishii, unpublished data). Relatively close phylogenetic relationship between the *Azospirillum* strains obtained in this study and other *Azospirillum* strains (Figure 5a) also suggested that they may be able to colonize plant roots and fix N₂.

Some *Azospirillum*, *Burkholderia*, *Massilia* and *Bacillus* strains did not have a detectable nitrite reductase gene and did not show denitrification ability. The lack of detection of a nitrite reductase gene may be attributed to the primers used in this study, as there are no annealing sites for the currently available PCR primers on the *nirK*

Figure 5 Phylogenetic relationships between SIP clones and FSC isolates. The phylogenetic trees were constructed based on (a) the 16S rRNA gene sequences and (b) deduced *nosZ* amino acid sequences, by using the maximum likelihood method. Clones obtained from the H fractions of the 13SN and 13Su samples are shown in green closed circle and blue open circle, respectively; strains obtained by the FSC isolation method are shown in red square. Taxonomic assignment of the strains obtained by the FSC isolation method was performed using the Ribosomal Database Project classifier program (Wang *et al.*, 2007). The numbers in parentheses are the numbers of clones in the operational taxonomic units (for SIP) or the number of strains that have the identical DNA fingerprinting patterns as the representatives (for FSC isolation). The accession numbers of the reference strains in the DDBJ/EMBL/GenBank databases are indicated in brackets. The bootstrap values (>70%) from 500 replicates are indicated next to the branches.

sequence of *Azospirillum* sp. B510 (Ishii *et al.*, 2011). However, it is also possible that these strains lack a nitrite reduction pathway as *nirK* of several *Azospirillum* strains is located on plasmids (Pothier *et al.*, 2008; Kaneko *et al.*, 2010).

In conclusion, our results suggest that most N₂O reducers are denitrifiers under the present study conditions, although some DNRA bacteria are known to reduce N₂O (Conrad, 1996). Among the N₂O reducers, *Burkholderiales* bacteria, especially those belonging to the genus *Herbaspirillum*, may have an important role in N₂O reduction in rice paddy soil. As *Herbaspirillum* bacteria are potential key players in nitrate reduction (Ishii *et al.*, 2009b), these bacteria can be used for the removal of contaminated nitrate from environments (for example, groundwater) while minimizing the emission of N₂O. Our study also identified several N₂O reducers lacking denitrification activity. These bacteria could be used to mitigate N₂O emission from agricultural fields while minimizing the loss of fertilizer N.

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