Revised ISMEJ-10-00866OA

Identification and Isolation of Active N_2O Reducers in Rice Paddy Soil

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ABSTRACT

Dissolved N_2O is occasionally detected in surface and ground water in rice paddy fields, while little or no N_2O is emitted to the atmosphere above these fields. This indicates the occurrence of N_2O reduction in rice paddy fields; however, identity of the N_2O reducers is largely unknown. In this study, we employed both culture-dependent and culture-independent approaches to identify N_2O reducers in rice paddy soil. In a soil microcosm, N_2O and succinate were added as the electron acceptor and donor, respectively, for N_2O reduction. For the stable isotope probing (SIP) experiment, ^{13}C-labeled succinate was used to identify succinate-assimilating microbes under N_2O-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 N_2O reductase gene were performed. For culture-dependent analysis, the microbes that elongated under N_2O-reducing conditions in the presence of cell division inhibitors were individually captured by a micromanipulator and transferred to a low-nutrient medium. The N_2O-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 N_2O-reducing conditions, in contrast to the control sample (soil incubated with only ^{13}C-succinate added). Results of the single-cell isolation technique also indicated that the majority of the N_2O-reducing strains belonged to the genera *Herbaspirillum*

(*Burkholderiales*) and *Azospirillum* (*Rhodospirillales*). In addition, *Herbaspirillum* strains reduced N_2O faster than *Azospirillum* strains. These results suggest that *Herbaspirillum* spp. may play an important role in N_2O reduction in rice paddy soils.

Key words: denitrification / *Herbaspirillum* / nitrous oxide / rice paddy soil / single-cell isolation / stable isotope probing

INTRODUCTION

Nitrous oxide (N_2O) is considered a major greenhouse gas and is a significant contributor to ozone layer destruction (Zumft and Kroneck, 2006). N_2O is mainly produced by denitrification, a microbial respiratory process in which nitrate/nitrite are reduced to gaseous forms (NO, N_2O, and N_2); however, other microbial processes, such as nitrification and dissimilatory nitrate reduction to ammonium (DNRA), can also produce N_2O (Conrad, 1996).

Agricultural fields are one of the main sources of N_2O emission (Philippot *et al.*, 2007; Minamikawa *et al*., 2010). In contrast to upland crop fields, little N_2O is emitted from rice paddy soils, even though paddy fields are known to have strong denitrification activity (Akiyama *et al*., 2006). Dissolved N_2O is occasionally detected in surface and ground water in rice paddy fields, while little or no N_2O is emitted to the atmosphere above these fields (Xiong *et al*., 2006; Minamikawa *et al*., 2010). This indicates that water-dissolved N_2O is possibly reduced by N_2O-reducing microorganisms in rice paddy fields.

N_2O can serve as an electron acceptor for microbial respiration. The standard reduction potential (E_0' at pH 7) of the reaction ($N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$) 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 N_2O. Although the reduction of N_2O to N_2 gas is part of denitrification, some denitrifiers do not have the ability to reduce N_2O (Tiedje, 1994).

Both N_2O-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 N_2O (Conrad, 1996). Therefore, it is difficult to use 16S rRNA gene sequences alone to identify N_2O reducers. Instead, the gene encoding N_2O reductase (*nosZ*) has been used to detect potential N_2O 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 N_2O 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 (e.g., ^{13}C, ^{15}N, ^{18}O) 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, ^{13}C-assimilating populations under denitrifying conditions were analyzed by DNA-based SIP analysis (Ginige *et al.*, 2004; Osaka *et al.*, 2006; Osaka *et al.*, 2008; Saito *et al.*, 2008). However, microbial populations responsible for N_2O reduction have not been examined to date.

Another approach to identifying such populations is to isolate and analyze N_2O 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 (e.g., 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 N_2O reduction rates, could also be measured (Tago *et al*., 2011).

Consequently, the objectives of the current study were (1) to identify ^{13}C-assimilating populations under N_2O-reducing conditions by SIP, (2) to isolate N_2O-reducing microorganisms from rice paddy soil by using the FSC isolation method, (3) to examine the N_2O 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 N_2O was used as an electron acceptor instead of nitrate. Succinate was used as an electron donor for N_2O reduction in this study. Because succinate is a member of TCA cycle and is considered as a non-fermentable carbon substrate, it can be used by various N_2O-reducers, but not by fermenting microbes. The optimum concentrations of electron acceptor and donor (N_2O and succinate, respectively) were determined by adding several combinations of N_2O (0%, 0.5%, 1%, 2%, 5%, and 20% in Ar base) and succinate (0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mg C per g soil), and were set at 5% N_2O and 0.1 mg succinate C per g soil. For SIP, ^{13}C-labeled succinate (Cambridge Isotope Laboratories, Andover, MA, USA) was used (0.1 mg [= 8.3 μmol] of ^{13}C per g soil). For FSC isolation, cell-division inhibitors (20 μg nalidixic acid, 10 μg pipemidic acid, 10 μg piromidic, and 10 μg cephalexin) (Joux and Lebaron, 1997) were added to the vial together with N_2O and succinate. The vial was then incubated under an Ar:N_2O (95:5) atmosphere and static conditions at 30°C for 24 h.

CO_2 and N_2O gases in the headspace of the vial were quantified by gas chromatography (GC) as described previously (Saito *et al*., 2008). When ^{13}C-labeled

succinate and ^{15}N-labeled N_2O (^{15}N, 99 atom. %; Cambridge Isotope Laboratories) were used, $^{44}CO_2$, $^{45}CO_2$, $^{44}N_2O$, $^{46}N_2O$, $^{28}N_2$, and $^{30}N_2$ 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). Fe^{2+} 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 N_2O and ^{13}C-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 N_2O and ^{12}C-succinate (sample 12SN), ^{13}C-succinate only (sample 13Su), and ^{12}C-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 ×*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 (PCA), 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 400–1000× 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 N_2O-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.

N_2O 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:^{15}N-labeled N_2O (95:5) gas. After incubation at 30°C for 1 week, the amounts of ^{15}N-labeled N_2 and N_2O 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 N_2O-reducing rate was also measured. Cells were grown in DNB-S medium in a vial with 5% nonlabeled N_2O 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:^{15}N-labeled N_2O (95:5) gas, and the vial was then incubated at 30°C. Amounts of ^{15}N-labeled N_2 and N_2O 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 from13SN 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 (Ishii *et al*., 2009b; Rich *et al*., 2003). 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 (rep-PCR) DNA fingerprinting was performed using the BOXA1R primer according to the protocol described by Rademaker et al. (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 digitalized 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 (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 N_2O disappeared within 24 h of incubation when <2% N_2O was added (data not shown). Since N_2O should always be present to minimize utilization of succinate by metal reducers, the concentration of N_2O should be >2%. Based on the Bunsen absorption coefficient and Henry's law, the concentration of the water-dissolved N_2O would be 1 mM when 5% N_2O 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 less than 0.1 mg succinate C did not significantly enhance N_2O reduction (Table S2). Addition of >0.1 mg succinate C significantly enhanced N_2O reduction (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% N_2O, all of the added succinate (0.1 mg C) was consumed within 24 h, while 32% of the added succinate remained unused in the absence of N_2O. Concentrations of Fe^{2+} 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% N_2O nor after anaerobic incubation without succinate addition (Table S3). These results suggest that succinate is likely used by N_2O reducers when N_2O is

present, but it can be used by metal reducers when N_2O 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 N_2O reduction.

Figure 1 shows time-course changes in N_2O, N_2, and CO_2 in the soil microcosms amended with the optimum concentrations of N_2O and succinate (5% and 0.1 mg C, respectively). The quantity of ^{15}N-labeled N_2 increased along with the decrease in ^{15}N-labeled N_2O in the microcosm, suggesting that N_2O was reduced to N_2. The N_2O decrease was larger than the amount of N_2 produced. This unbalanced N_2O mass may be attributed to N_2 fixation or other N_2O metabolism such as N_2O oxidation. The amount of ^{13}C-labeled CO_2 gradually increased and reached a plateau after 18 h, while nonlabeled CO_2 continued to increase after 24 h. About 10% of the added succinate (0.1 mg C = 8.3 μmol) was oxidized to CO_2. Because 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 N_2O reducers by SIP. SIP was performed to study succinate-assimilating populations under N_2O-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 (Fig. 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 (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 (Fig. 4A), many bands were from bacteria belonging to the order *Desulfuromonadales* (class *Deltaproteobacteria*) in the H fraction of the 13Su sample (Fig. 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* (Table S4).

In order to examine the community structure in the H fractions of the 13SN and 13Su samples in 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 (Fig. 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 (Fig. 4D).

Isolation of N_2O 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 N_2O-reducing conditions. No elongated cells were observed in the sample without cell-division inhibitors. After single-colony isolation and GC/MS analysis, 33 N_2O-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; Fig. 4E). 16S rRNA gene sequences of the isolated *Herbaspirillum* strains were >98% similar to the SIP clones obtained in this study (Fig. 5A). Strains related to the genera *Azospirillum* (seven strains) and *Burkholderia* (three strains) were the second and third most frequently obtained, respectively.

N_2O reductase gene. *nosZ* was detected in all N_2O-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* 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 due, in part, to the formation of chimeric sequences in SIP analysis.

N_2O reduction and denitrification activities. Based on the *nosZ* sequence information and rep-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 N_2O reduction activity. Since both an electron acceptor and an electron donor were abundantly present under the experimental conditions, the reaction ($N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$) followed zero-order kinetics. The N_2O 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 N_2O-reducing strains carried *nirS*, which encodes cytochrome cd_1 nitrite reductase, and were able to perform denitrification (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 N_2O reduction have not been well characterized. In the present study, we employed both culture-independent (SIP) and culture-dependent (FSC isolation) techniques to analyze N_2O reducers in rice paddy soil. Populations that assimilated succinate under N_2O-reducing conditions were examined by SIP analysis. The FSC isolation method was used to isolate microbes that were ready to grow under the same N_2O-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 N_2O reduction.

In the present study, succinate was used as an electron donor by the N_2O 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 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 N_2O (E_0' = +1355 mV). According to the thermodynamic theory (Thauer *et al*., 1977), N_2O is the preferred electron acceptor to Mn^{4+}, Fe^{3+}, and sulfate. Our results support this notion since the production of Fe^{2+} was suppressed by the addition of N_2O.

Succinate-assimilating populations under N_2O-reducing and non-reducing conditions were examined by SIP. Under non-N_2O-reducing conditions (sample 13Su), clones related to the genus *Geobacter* (order *Desulfuromonadales*) were most frequently (42%) obtained (Fig. 4D). Because production of Fe^{2+} 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*., 2010). In the present study, *Herbaspirillum* spp. and other *Burkholderiales* bacteria were also detected in the H fraction of the 13Su sample. Because 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 (N_2O-reducing conditions) (Fig. 4C). Involvement of these bacteria in N_2O reduction was also supported by culture-dependent FSC isolation (Fig. 4E). *Herbaspirillum* strains obtained by FSC isolation carried *nosZ* and reduced exogenous N_2O to N_2. The majority of the SIP *nosZ* clones were similar to *nosZ* of *Herbaspirillum* and other *Burkholderiales* N_2O reducers (Fig. 5). Similar *nosZ* clones have also been obtained from other paddy fields (e.g., 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 N_2O 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; Ishii *et al*., 2011), but the present study showed that they are also important players in N_2O reduction. Although some *Herbaspirillum* species (e.g., *Herbaspirillum seropedica*) can colonize rice roots and stems and fix atmospheric N_2 (Baldani *et al*., 1986; Elbeltagy *et al*., 2001), almost all strains obtained in this study did not show N_2-fixing ability (S. Ishii, unpublished data). In addition, 16S rRNA gene similarities between the *Herbaspiriilum* strains obtained in this study and other *Herbaspirillum* species were less than 97%. These results suggest that the N_2O-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 N_2O reduction. The N_2O reduction rates suggested that *Azispirillum* spp. reduced N_2O more slowly than *Herbaspirillum* spp. Although their *in situ* N_2O reduction rates are not known, these results indicated that the relative contribution of *Azospirillum* strains to N_2O reduction might be smaller than that of *Herbaspirillum* strains. Similar to other *Azospirillum* strains (e.g., *Azospirillum brasilense* and *Azospirillum* sp. B510; Isawa *et al*., 2010), our *Azospirillum* strains also showed N_2-fixing ability (S. Ishii, unpublished data). Relatively close phylogenetic relationship

between the *Azospirillum* strains obtained in this study and other *Azospirillum* strains (Fig. 5A) also suggested that they may be able to colonize plant roots and fix N_2.

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, since there are no annealing sites for the currently available PCR primers on the *nirK* sequence of *Azospirillum* sp. B510 (Ishii *et al*., 2011). However, it is also possible that these strains lack a nitrite reduction pathway since *nirK* of several *Azospirillum* strains is located on plasmids (Pothier *et al*., 2008; Kaneko *et al*., 2010).

In conclusion, our results suggest that most N_2O reducers are denitrifiers under the present study conditions, although some DNRA bacteria are known to reduce N_2O (Conrad, 1996). Among the N_2O reducers, *Burkholderiales* bacteria, especially those belonging to the genus *Herbaspirillum*, may play an important role in N_2O reduction in rice paddy soil. Because *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 (e.g., groundwater) while minimizing the emission of N_2O. Our study also identified several N_2O reducers lacking denitrification activity. These bacteria could be used to mitigate N_2O emission from agricultural fields while minimizing the loss of fertilizer N.

ACKNOWLEDGMENTS

We thank Daisuke Sasaki and Yasuo Igarashi at the University of Tokyo for their help with ultracentrifugation and fractionation. We also thank Hirofumi Shoun and Takayoshi Wakagi at the University of Tokyo, for allowing us to use their GC/MS instrument, and Takashi Tsuji and Yoshitaka Yoshimura at Tamagawa University for allowing us to use their micromanipulator.

This work was supported by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

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FIGURE LEGENDS

Figure 1. Time-course changes in $^{46}N_2O$ (–●–), $^{30}N_2$ (–■–), $^{45}CO_2$ (–○–), and $^{44}CO_2$ (–△–) in soil microcosms amended with $^{46}N_2O$ and ^{13}C-labeled succinate. Mean ± SE (n = 3) is shown. An arrow indicates the time when DNA was extracted (24 h).

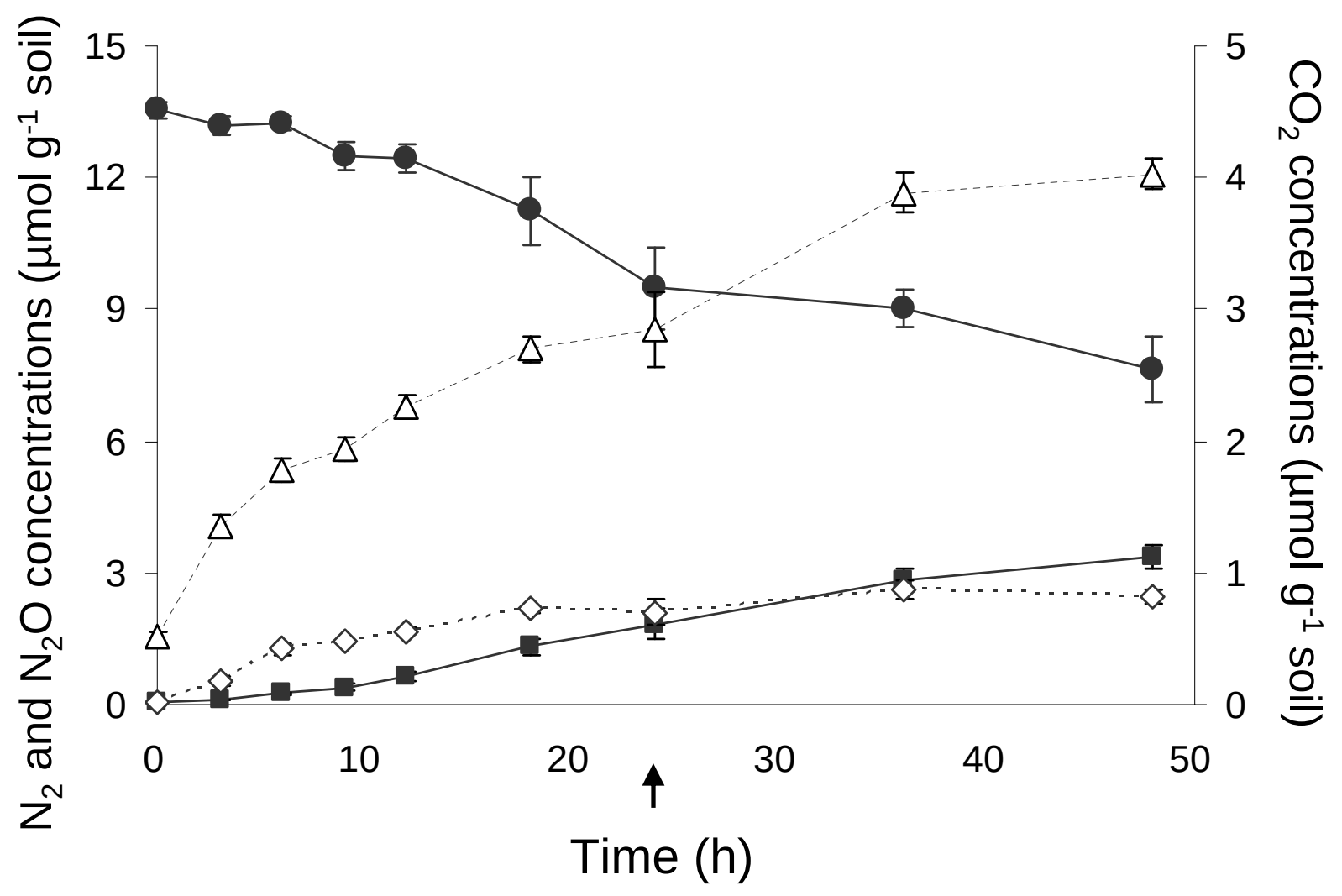
Figure 2. CsCl density gradient centrifugation of DNA extracted from soil. Buoyant densities of the light (L), heavy (H), and middle (M) density fractions were 1.70–1.715, 1.715–1.73, and 1.73–1.75 g cm^{-3}, respectively. Legend: 13SN sample (–■–), 13Su sample (–●–), 12SN sample (···□···), and 12Su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

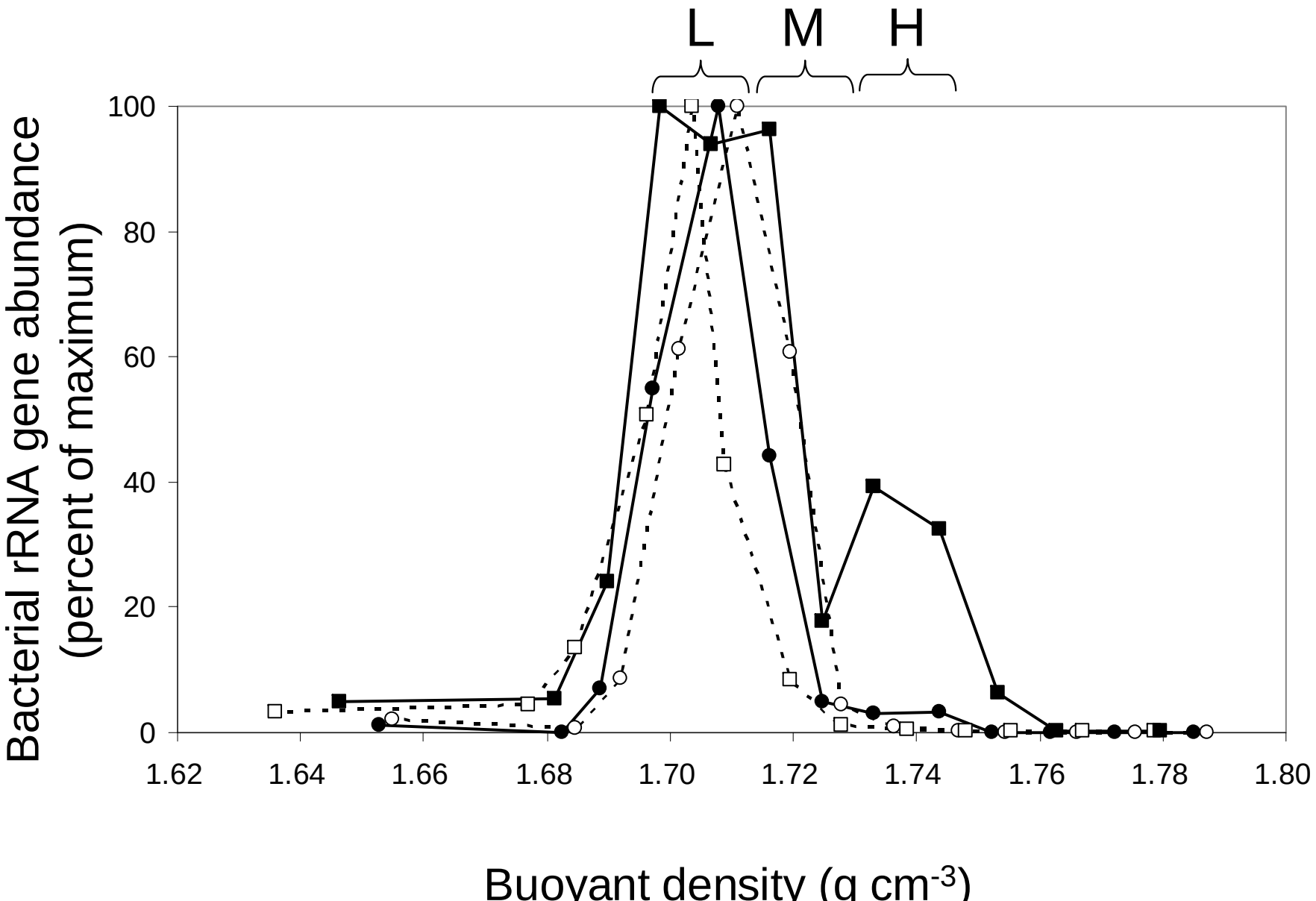
Figure 3. Community structure assessed by DGGE analysis. (A) DGGE banding profile from each fraction separated by CsCl density gradient centrifugation. Gel region shown is between 44% and 54% denaturant concentrations, as estimated by the DGGE marker II (Nippon Gene, Tokyo, Japan). L, M, and H correspond to the light, middle, and heavy DNA fractions as shown in Fig. 2. Bands specific to the H fractions of each sample (indicated by arrows) were excised, cloned, and sequenced (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.

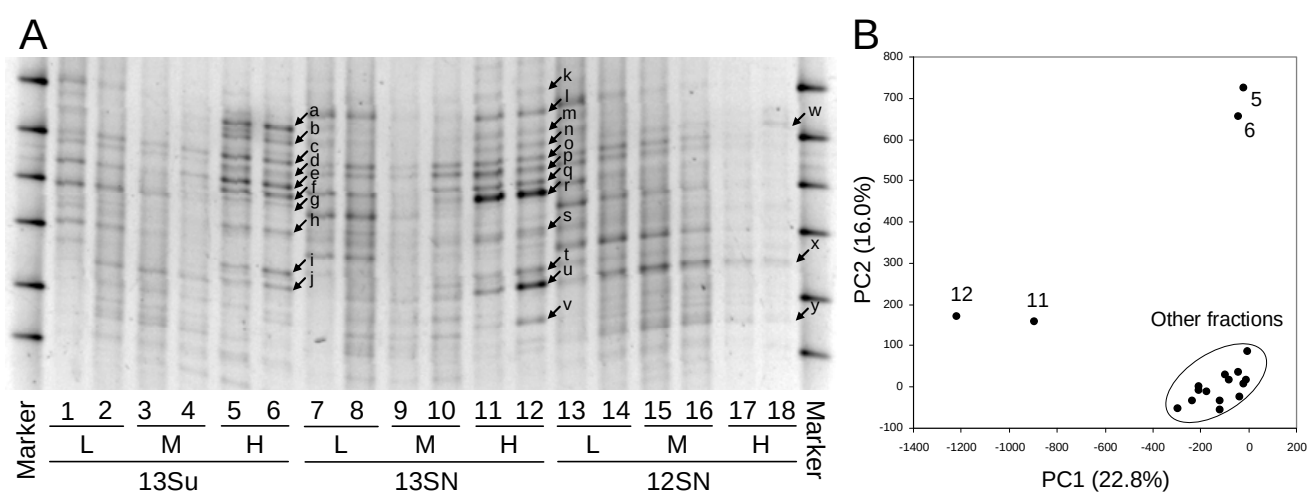
Figure 4. Taxonomic classification of the (A) DGGE band excised from the H fraction of the 13SN sample, (B) DGGE band excised from the H fraction of the 13Su sample, (C) clones obtained from the H fraction of the 13SN sample, (D) clones obtained from the H fraction of the 13Su sample, and (E) strains 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.* 180 bp) and clone library results (*ca*. 1450 bp), respectively. Relative intensities of the DGGE bands (see Table S2) correspond to the fraction of the assigned taxon.

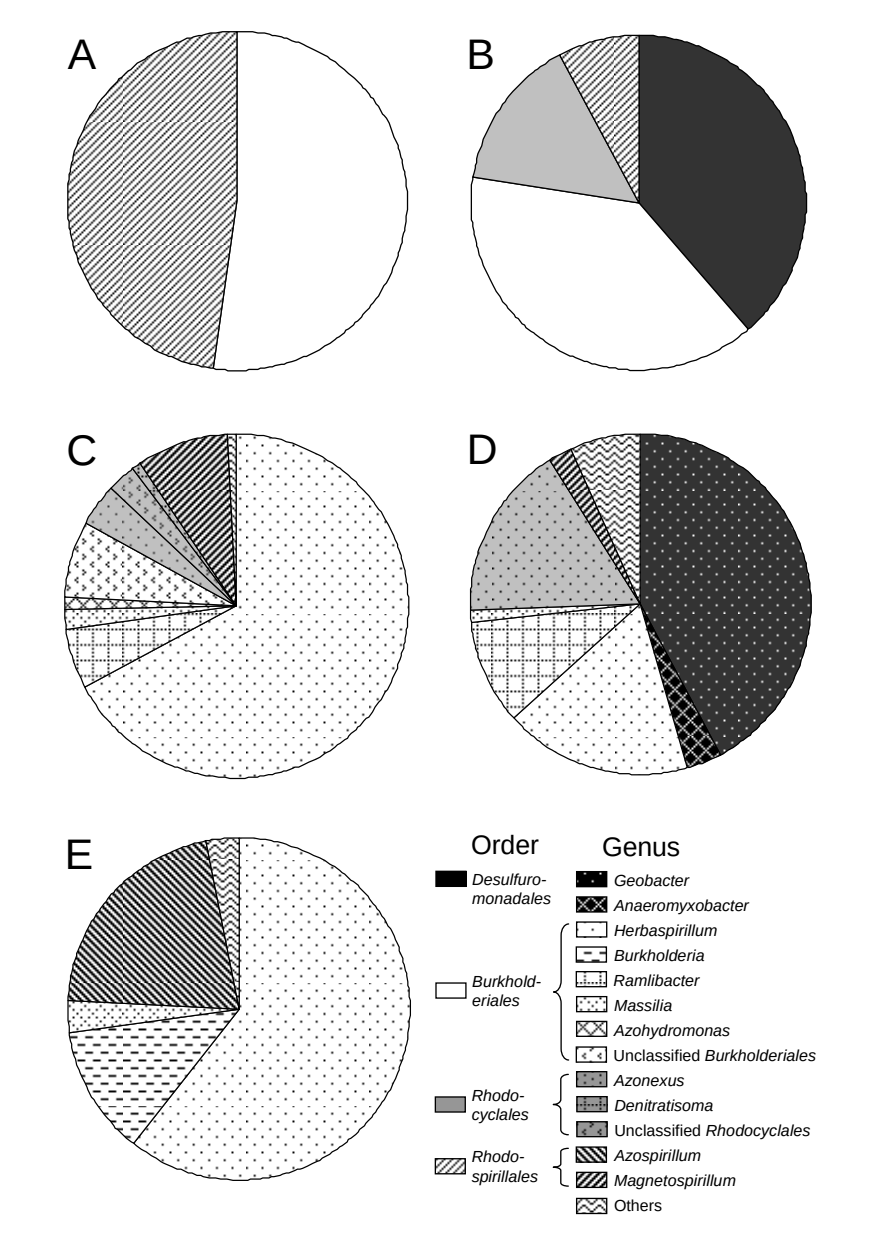
Figure 5. Phylogenetic relationships between SIP clones and FSC isolates. The phylogenetic trees were constructed based on (A) the 16S rRNA gene sequences and (B) deduced *nosZ* amino acid sequences, by using the maximum likelihood method. Clones obtained from the H fractions of the 13SN and 13Su samples 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.


15
12
9
6
3
0
5
4
3
2
1
0
0
10
20
30
40
50
Time (h)
N2 and N2O concentrations (µmol g-1 soil)
CO2 concentrations (µmol g-1 soil)


L
M
H
Bacterial rRNA gene abundance
(percent of maximum)
100
80
60
40
20
0
1.62
1.64
1.66
1.68
1.70
1.72
1.74
1.76
1.78
1.80
Buoyant density (g cm-3)


A
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
y
Marker
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Marker
L M H L M H L M H
13Su
13SN
12SN
B
PC2 (16.0%)
PC1 (22.8%)
800
700
600
500
400
300
200
100
0
-100
-1400 -1200 -1000 -800 -600 -400 -200 0 200
5
6
12
11
Other fractions


A
B
C
D
E
Order
Genus
Desulfuro-monadales
Geobacter
Anaeromyxobacter
Burkhold-eriales
Herbaspirillum
Burkholderia
Ramlibacter
Massilia
Azohydromonas
Unclassified Burkholderiales
Rhodo-cyclales
Azonexus
Denitratisoma
Unclassified Rhodocyclales
Rhodo-spirillales
Azospirillum
Magnetospirillum
Others

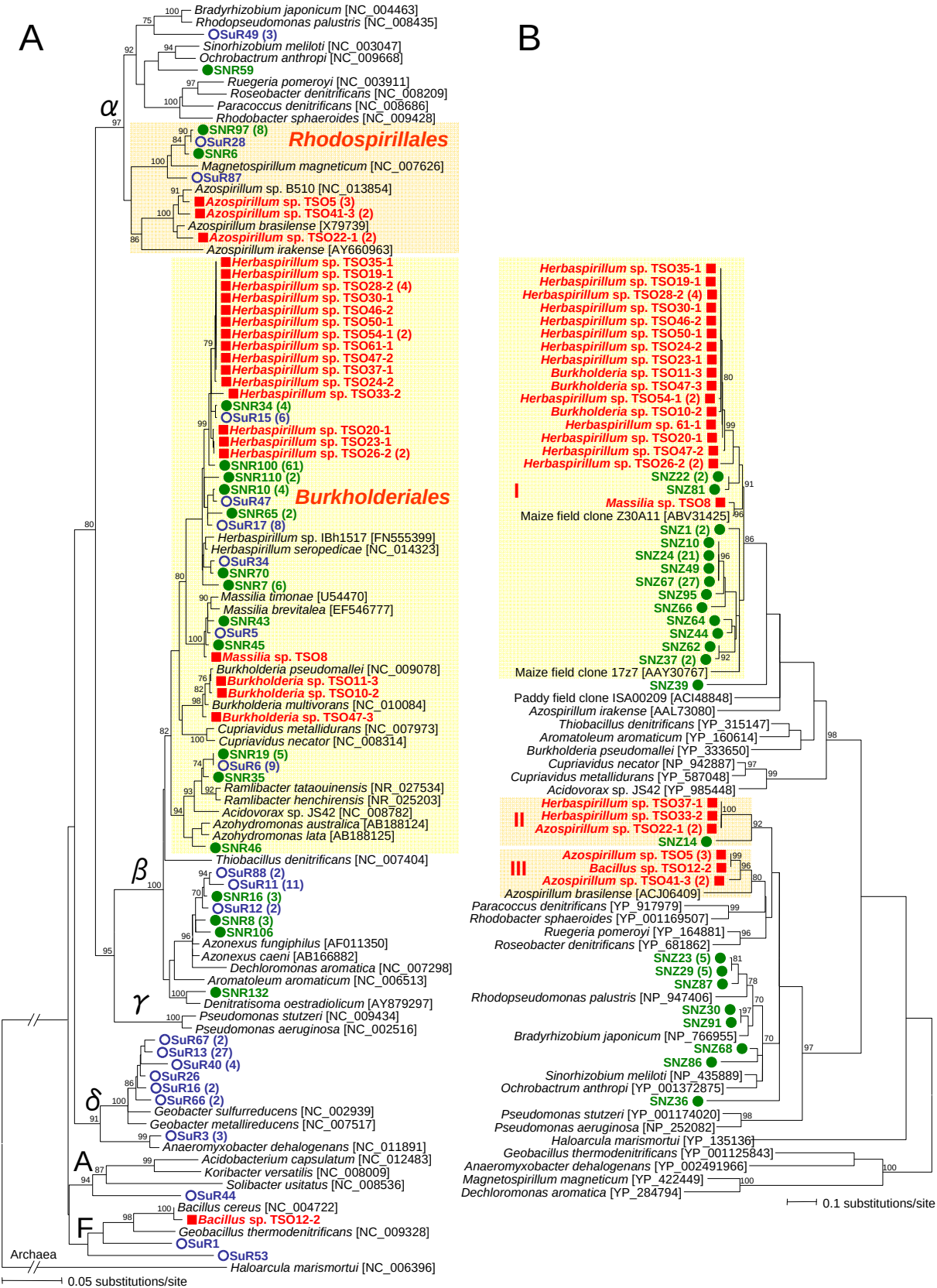

A
B
α
β
γ
δ
ε
ζ
Rhodospirillales
Burkholderiales
Bradyrhizobium japonicum [NC_004463]
Rhodopseudomonas palustris [NC_008435]
SuR49 (3)
Sinorhizobium meliloti [NC_003047]
Ochrobactrum anthropi [NC_009668]
SNR59
Ruegeria pomeroyi [NC_003911]
Roseobacter denitrificans [NC_008209]
Paracoccus denitrificans [NC_008686]
Rhodobacter sphaeroides [NC_009428]
SNR97 (8)
SuR28
SNR6
Magnetospirillum magneticum [NC_007626]
SuR87
Azospirillum sp. B510 [NC_013854]
Azospirillum sp. TSO5 (3)
Azospirillum sp. TSO41-3 (2)
Azospirillum brasilense [X79739]
Azospirillum sp. TSO22-1 (2)
Azospirillum irakense [AY660963]
Herbaspirillum sp. TSO35-1
Herbaspirillum sp. TSO19-1
Herbaspirillum sp. TSO28-2 (4)
Herbaspirillum sp. TSO30-1
Herbaspirillum sp. TSO46-2
Herbaspirillum sp. TSO50-1
Herbaspirillum sp. TSO54-1 (2)
Herbaspirillum sp. TSO61-1
Herbaspirillum sp. TSO47-2
Herbaspirillum sp. TSO37-1
Herbaspirillum sp. TSO24-2
Herbaspirillum sp. TSO33-2
SNR34 (4)
SuR15 (6)
Herbaspirillum sp. TSO20-1
Herbaspirillum sp. TSO23-1
Herbaspirillum sp. TSO26-2 (2)
SNR100 (61)
SNR110 (2)
SNR10 (4)
SuR47
SNR65 (2)
SuR17 (8)
Herbaspirillum sp. IBh1517 [FN555399]
Herbaspirillum seropedicae [NC_014323]
SuR34
SNR70
SNR7 (6)
Massilia timonae [U54470]
Massilia brevitalea [EF546777]
SNR43
SuR5
SNR45
Massilia sp. TSO8
Burkholderia pseudomallei [NC_009078]
Burkholderia sp. TSO11-3
Burkholderia sp. TSO10-2
Burkholderia multivorans [NC_010084]
Burkholderia sp. TSO47-3
Cupriavidus metallidurans [NC_007973]
Cupriavidus necator [NC_008314]
SNR19 (5)
SuR6 (9)
SNR35
Ramlibacter tataouinensis [NR_027534]
Ramlibacter henchirensis [NR_025203]
Acidovorax sp. JS42 [NC_008782]
Azohydromonas australica [AB188124]
Azohydromonas lata [AB188125]
SNR46
Thiobacillus denitrificans [NC_007404]
SuR88 (2)
SuR11 (11)
SNR16 (3)
SuR12 (2)
SNR8 (3)
SNR106
Azonexus fungiphilus [AF011350]
Azonexus caeni [AB166882]
Dechloromonas aromatica [NC_007298]
Aromatoleum aromaticum [NC_006513]
SNR132
Denitratisoma oestradiolicum [AY879297]
Pseudomonas stutzeri [NC_009434]
Pseudomonas aeruginosa [NC_002516]
SuR67 (2)
SuR13 (27)
SuR40 (4)
SuR26
SuR16 (2)
SuR66 (2)
Geobacter sulfurreducens [NC_002939]
Geobacter metallireducens [NC_007517]
SuR3 (3)
Anaeromyxobacter dehalogenans [NC_011891]
Acidobacterium capsulatum [NC_012483]
Koribacter versatilis [NC_008009]
Solibacter usitatus [NC_008536]
SuR44
Bacillus cereus [NC_004722]
Bacillus sp. TSO12-2
Geobacillus thermodenitrificans [NC_009328]
SuR1
SuR53
Haloarcula marismortui [NC_006396]
Archaea
0.05 substitutions/site
Herbaspirillum sp. TSO35-1
Herbaspirillum sp. TSO19-1
Herbaspirillum sp. TSO28-2 (4)
Herbaspirillum sp. TSO30-1
Herbaspirillum sp. TSO46-2
Herbaspirillum sp. TSO50-1
Herbaspirillum sp. TSO24-2
Herbaspirillum sp. TSO23-1
Burkholderia sp. TSO11-3
Burkholderia sp. TSO47-3
Herbaspirillum sp. TSO54-1 (2)
Burkholderia sp. TSO10-2
Herbaspirillum sp. 61-1
Herbaspirillum sp. TSO20-1
Herbaspirillum sp. TSO47-2
Herbaspirillum sp. TSO26-2 (2)
SNZ22 (2)
SNZ81
Massilia sp. TSO8
Maize field clone Z30A11 [ABV31425]
SNZ1 (2)
SNZ10
SNZ24 (21)
SNZ49
SNZ67 (27)
SNZ95
SNZ66
SNZ64
SNZ44
SNZ62
SNZ37 (2)
Maize field clone 17z7 [AAY30767]
SNZ39
Paddy field clone ISA00209 [ACI48848]
Azospirillum irakense [AAL73080]
Thiobacillus denitrificans [YP_315147]
Aromatoleum aromaticum [YP_160614]
Burkholderia pseudomallei [YP_333650]
Cupriavidus necator [NP_942887]
Cupriavidus metallidurans [YP_587048]
Acidovorax sp. JS42 [YP_985448]
Herbaspirillum sp. TSO37-1
Herbaspirillum sp. TSO33-2
Azospirillum sp. TSO22-1 (2)
SNZ14
Azospirillum sp. TSO5 (3)
Bacillus sp. TSO12-2
Azospirillum sp. TSO41-3 (2)
Azospirillum brasilense [ACJ06409]
Paracoccus denitrificans [YP_917979]
Rhodobacter sphaeroides [YP_001169507]
Ruegeria pomeroyi [YP_164881]
Roseobacter denitrificans [YP_681862]
SNZ23 (5)
SNZ29 (5)
SNZ87
Rhodopseudomonas palustris [NP_947406]
SNZ30
SNZ91
Bradyrhizobium japonicum [NP_766955]
SNZ68
SNZ86
Sinorhizobium meliloti [NP_435889]
Ochrobactrum anthropi [YP_001372875]
SNZ36
Pseudomonas stutzeri [YP_001174020]
Pseudomonas aeruginosa [NP_252082]
Haloarcula marismortui [YP_135136]
Geobacillus thermodenitrificans [YP_001125843]
Anaeromyxobacter dehalogenans [YP_002491966]
Magnetospirillum magneticum [YP_422449]
Dechloromonas aromatica [YP_284794]
0.1 substitutions/site

Table S1. Denitrification activity and the nucleotide sequence accession numbers for 16S rRNA gene, *nosZ*, and *nirS* of the strains obtained in this study.

Genus	Strain	Denitrification activity [%][a]	16S rRNA	*nosZ*	*nirS*
Azospirillum	TSO5	100	AB545618	AB545661	-[b]
	TSO7	100	AB545619	AB545687	-
	TSO9	90	AB545621	AB545688	-
	TSO22-1	55	AB545647	AB545697	AB545721
	TSO32-4	43	AB545633	AB545690	AB545710
	TSO35-2	30	AB545654	AB545698	-
	TSO41-3	10	AB545635	AB545691	-
Herbaspirillum	TSO19-1	39	AB545622	AB545662	AB545699
	TSO20-1	23	AB545623	AB545663	AB545700
	TSO23-1	30	AB545624	AB545664	AB545701
	TSO24-2	32	AB545648	AB545677	AB545722
	TSO26-2	41	AB545626	AB545666	AB545703
	TSO28-2	37	AB545628	AB545667	AB545705
	TSO29-2	48	AB545630	AB545669	AB545707
	TSO30-1	53	AB545649	AB545678	AB545723
	TSO32-1	31	AB545632	AB608727	AB545709
	TSO33-2	35	AB545652	AB545681	AB545726
	TSO35-1	40	AB545653	AB545682	AB545727
	TSO37-1	88	AB545634	AB545671	AB545711
	TSO45-3	41	AB545636	AB608728	AB545712
	TSO46-2	29	AB545637	AB545672	AB545713
	TSO47-2	33	AB545638	AB545673	AB545714
	TSO49-1	27	AB545655	AB545683	AB545728
	TSO50-1	34	AB545640	AB545674	AB545716
	TSO54-1	33	AB545641	AB545675	AB545717
	TSO56-1	56	AB545657	AB545685	AB545730
	TSO56-2	32	AB545658	AB545686	AB545731
	TSO61-1	47	AB545659	AB608729	AB545732
Burkholderia	TSO10-2	0	AB545644	AB545694	AB545720
	TSO11-3	0	AB545645	AB545695	-
	TSO47-3	0	AB545639	AB545692	AB545715
Massilia	TSO8	0	AB545620	AB545689	-
Bacillus	TSO12-2	3	AB545646	AB545696	-

[a]proportion of nitrate reduced to N_2O in the medium, as determined by acetylene block method (n=2)

[b]-, not detected by PCR

Table S2. Concentration of N_2O after 24-h incubation

Amount of succinate added (mg C g^{-1} soil)	N_2O conc. (%)[a]	
0.50	2.94 ± 0.70	A
0.25	3.55 ± 0.32	A
0.10	3.68 ± 0.25	AB
0.05	4.52 ± 0.05	BC
0.025	4.69 ± 0.07	C
0.01	4.58 ± 0.07	C
0	4.77 ± 0.01	C

[a]Means ± SE followed by the same letter are not significantly different ($P > 0.05$, $n = 3$)

Table S3. Concentration of Fe^{2+} in soil samples

Sample[a]	Additive	Fe^{2+} conc. (g kg^{-1} soil)[b]	
BI	-	2.22 ± 0.28	A
Su	Succinate only	3.52 ± 0.27	B
SN	Succinate + N_2O	2.92 ± 0.35	AB
N2O	N_2O only	2.43 ± 0.32	A

[a]BI, unincubated control; Su, soil sample incubated with 0.1 mg C succinate; SN, soil sample incubated with 0.1 mg C succinate and 5% N_2O; N2O, soil sample incubated with 5% N_2O.

[b]Means ± SE followed by the same letter are not statistically different ($P > 0.05$, $n = 3$)

Table S4. Taxonomic assignment of the 16S rRNA gene clones recovered from the DGGE bands shown in Fig. 3A. Relative intensity of each band is also shown.

Sample	Band ID	Accession number	Relative intensity (%)	Classifier results (bootstrap value %)					
				Order		Family		Genus	
13Su	a	AB608638	15.8	*Desulfuromonadales*	100%	*Geobacteraceae*	99%	*Geobacter*	99%
	b	AB608639	6.0	*Desulfuromonadales*	97%	*Geobacteraceae*	96%	*Geobacter*	96%
	c	AB608640	11.4	*Burkholderiales*	99%	*Oxalobacteraceae*	99%	*Massilia*	75%
	d	AB608641	4.8	*Burkholderiales*	100%	*Comamonadaceae*	99%	*Ramlibacter*	48%
	e	AB608642	13.9	*Desulfuromonadales*	98%	*Geobacteraceae*	85%	*Geobacter*	85%
	f	AB608643	11.4	*Burkholderiales*	98%	*Burkholderiaceae*	88%	*Cupriavidus*	54%
	g	AB608644	2.7	*Desulfuromonadales*	88%	*Geobacteraceae*	69%	*Geobacter*	69%
	h	AB608645	11.3	*Burkholderiales*	100%	*Comamonadaceae*	99%	*Ramlibacter*	51%
	i	AB608646	15.0	*Rhodocyclales*	100%	*Rhodocyclaceae*	100%	*Thauera*	43%
	j	AB608647	7.7	*Rhodospirillales*	100%	*Rhodospirillaceae*	100%	*Azospirillum*	100%
13SN	k	AB608648	1.7	*Burkholderiales*	96%	*Burkholderiaceae*	91%	*Ralstonia*	39%
	l	AB608649	6.7	*Burkholderiales*	98%	*Burkholderiaceae*	87%	*Cupriavidus*	58%
	m	AB608650	2.5	*Burkholderiales*	96%	*Oxalobacteraceae*	94%	*Massilia*	41%
	n	AB608651	2.8	*Burkholderiales*	99%	*Oxalobacteraceae*	97%	*Massilia*	41%
	o	AB608652	3.2	*Burkholderiales*	76%	*Burkholderiaceae*	80%	*Cupriavidus*	42%
	p	AB608653	4.1	*Burkholderiales*	100%	*Comamonadaceae*	100%	*Ramlibacter*	56%
	q	AB608654	6.1	*Rhodospirillales*	84%	*Rhodospirillaceae*	77%	*Magnetospirillum*	65%
	r	AB608655	25.2	*Burkholderiales*	92%	*Burkholderiaceae*	74%	*Cupriavidus*	31%
	s	AB608656	6.0	*Burkholderiales*	99%	*Comamonadaceae*	98%	*Ramlibacter*	53%
	t	AB608657	9.7	*Rhodospirillales*	94%	*Rhodospirillaceae*	94%	*Magnetospirillum*	91%
	u	AB608658	23.4	*Rhodospirillales*	91%	*Rhodospirillaceae*	87%	*Magnetospirillum*	84%
	v	AB608659	8.5	*Rhodospirillales*	74%	*Rhodospirillaceae*	72%	*Magnetospirillum*	30%
12SN	w	AB608660	29.6	*Bacillales*	100%	*Bacillaceae*	99%	*Bacillus*	77%
	x	AB608661	44.8	*Rhodospirillales*	96%	*Rhodospirillaceae*	92%	*Magnetospirillum*	85%
	y	AB608662	25.6	*Clostridiales*	80%	Incertae Sedis XVIII	79%	*Symbiobacterium*	79%