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Identification of Novel *Betaproteobacteria* in a Succinate-Assimilating Population in Denitrifying Rice Paddy Soil by Using Stable Isotope Probing

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Rice paddy soil has been shown to have strong denitrifying activity. However, the microbial populations responsible for the denitrification have not been well characterized. In this study, we employed Stable Isotope Probing (SIP) to study succinate-assimilating denitrifiers in soil microcosms amended with nitrate and ^{13}C-succinate. Microbial populations represented in ^{12}C- and ^{13}C-DNA fractions were different based on denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rRNA gene fragment. A nearly full-length 16S rRNA gene was also amplified, cloned, and sequenced from ^{13}C-DNA fraction. Both PCR-DGGE and clone library analyses revealed that *Burkholderiales* and *Rhodocyclales* dominated the succinate-assimilating population in denitrifying soil after 24 h incubation. Among these, novel *Betaproteobacteria*, possibly within the order *Rhodocyclales*, represented 43% of the clones obtained. Nitrite reductase genes, *nirS* and *nirK*, were also amplified and cloned from the ^{13}C-DNA fraction. While most *nirK* clones in this study were similar to the *nirK* sequences from *Rhizobiales*, a majority of the *nirS* clones were similar to the *nirS* sequences from *Burkholderiales* and *Rhodocyclales*, consistent with the 16S rRNA gene analysis. These groups of bacteria, including the novel *Betaproteobacteria*, may play an important role in denitrification in rice paddy soil.

Key words: denitrification, succinate-assimilating population, stable isotope probing, rice paddy soil, *Rhodocyclales*

Denitrification is a respiratory reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to gaseous products (NO, N_2O, and N_2), and has an important role in the nitrogen cycle[14,40,44]. Denitrification can cause a loss of nitrogen from agricultural soils as well as the emission of nitric oxide (NO) and nitrous oxide (N_2O), which may contribute to global warming and destruction of the ozone layer[32]. Denitrification process is also used to remove nitrogen compounds from wastewater[28,41].

Denitrification in soil is generally more active under anaerobic (e.g. waterlogged rice field) than aerobic (e.g. well-drained agricultural soil) conditions[1,40]. Long-term gas monitoring revealed that the emission of N_2O gas from waterlogged rice paddy fields is low compared to upland crop systems[1,26,27], probably due to high levels of N_2-generating denitrification activity in waterlogged soils. Priemé *et al.*[30] reported that the denitrifying community was more diverse in marsh soil than upland forest soil. Diversity of denitrifying community may influence ecosystem function in terms of N_2O production[5,17]. However, the microbial populations responsible for denitrification in rice field soils are not well characterized.

Denitrification ability has been found in phylogenetically diverse bacteria, as well as in some fungi and *Archaea*[4,14,29,36,40]. Therefore, the detection of denitrifiers based on ribosomal RNA genes is not a suitable approach unless a specific functional group is screened. Stable isotope probing (SIP) has been successfully applied to the selection of metabolically active microbial populations in environments[10,24,25,31]. To investigate active denitrifiers in activated sludge, the SIP approach was used with ^{13}C-labeled acetate and methanol[11,28]. Some denitrifying bacteria can assimilate methanol, but not others[28]; therefore, other substrates may be suitable when targeting broader range of denitrifiers. Heylen *et al.*[16] compared several different carbon compounds as a source of culture media for denitrifiers, and reported that ethanol and succinate were more suitable than glycerol, pyruvate, methanol, and glucose. Sato *et al.*[34,35] used 39 carbon compounds to investigate the impacts of different C sources on denitrification in waterlogged soils, and concluded that some types of carboxyl acids such as succinate and fumarate accelerated denitrification but not fermentation and dissimilatory reduction of NO_3^- to NH_4^+. On many occasions, succinate is produced as a product of fermentation. Under anaerobic conditions in the presence of nitrate, succinate can be preferentially used by denitrifiers as an electron donor and C source since denitrification has a higher $-\Delta G^\circ$ value than Mn^{4+}/Fe^{3+}/sulfate reductions[43]. Also, succinate is part of the TCA cycle, and therefore, can be used by broad groups of denitrifying bacteria if nitrate is present. These low-molecular weight organic acids are naturally abundant in soils, especially in waterlogged rice paddy fields[19,20]. Therefore, use of [^{13}C]succinate is a reasonable choice for a SIP experiment to screen the broad range of denitrifiers in waterlogged rice paddy soil. Since denitrification is a dissimilatory process, addition of [^{15}N]NO_3^- may not produce heavy DNA of denitrifiers, and therefore, was not used in this study.

Another approach to the study of denitrifying communities

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is to employ functional genes. Several functional genes have been used to study the diversity of denitrifiers, including copper-containing nitrite reductase gene (*nirK*) and cytocrome cd_1-containing nitrite reductase gene (*nirS*)[2,3,30,39,42]. These nitrite reductases are functionally equivalent and catalyze the reduction of nitrite to NO, a key step for denitrification[2]. The *nirS* and *nirK* genes are genetically heterogeneous, and often used to measure the diversity of denitrifiers[2,30]. However, heterogeneity in *nirS* and *nirK* does not necessarily reflect taxonomic diversity of denitrifiers. While dendrograms based on the *nirS* gene were reported to mostly reflect the 16S rRNA gene phylogeny at the family or genus level, *nirK* sequence analyses were incompatible with 16S rRNA gene phylogeny[15,29]. Horizontal transfer of *nir* genes may have occurred and contributed to the incongruence between *nir* and 16S rRNA gene phylogeny[8,15]. Use of SIP-enriched DNA enables one to compare 16S rRNA and *nir* gene diversities in the same metabolically active populations, assuming the substrate was used only by denitrifiers.

Consequently, the objectives of this study were to 1) examine the impacts of succinate and nitrate additions on denitrification in waterlogged soil, 2) use the SIP technique to investigate the active succinate-assimilating microbial community in waterlogged soil under denitrifying conditions, and 3) characterize the genetic diversity of *nirK* and *nirS* in the succinate-assimilating denitrifying community.

Materials and Methods

Soil properties

Rice paddy soil used in this study was collected from the Field Production Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo (Nishi-Tokyo, Tokyo, Japan) on 8 September 2004. Rice (*Oryza sativa*) was annually planted in this field with the addition of 100 kg N-NH_4 of the chemical fertilizer (12% N-NH_4^+, 18% P-phosphate, 16% K, 3% Mg, 0.4% Mn, and 0.2% B) per ha per year, of which 60% was applied at the beginning of the rice planting. The soil sample was air-dried, sieved through a 2-mm mesh, and stored in the dark. Properties of the soil were: 5.03% (w/w) organic matter, 14.6 mg N-NH_4^+ kg^{-1} soil, 52.3 mg N-NO_3^- kg^{-1} soil, pH(H_2O)=6.5, and pH(KCl)=5.7. The soil was derived from volcanic ash (Andisol), rich in humic substances, and classified as Clay Loam (39.3% sand, 33.7% silt, and 27% clay).

Soil microcosm

A soil microcosm was established to study denitrifying activity in waterlogged paddy soil. Microbes in 1 g air-dried soil were revived by incubating in a 20-ml serum bottle (Nichiden-Rika Glass, Kobe, Japan) with 3 ml H_2O at 30°C for 1 week (preincubation). This preincubation step was previously shown to deplete available NO_3^-, lower the Eh to around –0.2 V, and increase colony numbers of general and anaerobic microbes[37]. Depletion of available NO_3^- was also confirmed in this study by using HPLC as described below.

After preincubation, 2 ml of the upper soil-free water was removed, and optimum concentrations of nitrate and succinate, each dissolved in 100 µl sterile water, were added as electron acceptor and donor, respectively, for denitrifiers. The optimum concentrations were determined by adding several combinations of nitrate (0, 0.05, 0.1, 0.2, 0.5, and 1 mg N-NO_3^-) and succinate (0, 0.15, 0.3, 0.5, and 1 mg C-succinate) per g soil, and were set at 0.1 mg N-NO_3^- and 0.5 mg C-succinate per g soil. For stable isotope probing (SIP), ^{13}C-labeled succinate (Cambridge Isotope Laboratories, Andover, MA, USA) was used. Each bottle was tightly closed with a butyl rubber septum and an aluminum cap, and air-phase was replaced by Ar-C_2H_2 (90:10) gas at 0.1 MPa. The bottles were then incubated at 30°C for up to 48 h. Soil denitrification activity was monitored by N_2O production by the acetylene (C_2H_2) block method[40]. Three bottles were sacrificed every 6 h for GC/HPLC analyses.

GC/HPLC

The production of N_2O and CO_2 in soil microcosms was measured by gas chromatography (GC). Prior to the GC analysis, the bottles were vigorously shaken for 1 min to diffuse gases remaining in the soil to the headspace. A gas sample (0.5 ml) from each bottle was injected into a gas chromatograph GC14B (Shimadzu, Kyoto, Japan) equipped with a Porapack Q column (Shimadzu) and a thermal conductivity detector GC-TCD. The temperature of the injector, detector, and column were 50°C, and the flow rate of the He carrier gas was 45 ml/min. Chromatograms were analyzed by using C-R8A ChromatoPak (Shimadzu). The Bunsen absorption coefficient was used to estimate the quantity of water-dissolved N_2O[40].

Concentrations of nitrate and succinate were monitored by high performance liquid chromatography (HPLC). Nitrate and succinate were extracted from 1 g soil by vigorously shaking with 4 ml H_2O for 1 min. The soil solution was passed through a 0.2-µm-pore membrane filter (Sartorius Biotech, Göttingen, Germany). For the succinate measurement, the soil solution was further purified through a Maxi-Clean IC-Chelate cation exchange filter (Alltech, Deerfield, IL, USA). This additional filtering can remove cations that may interfere with the HPLC analysis, although some succinate may also be lost during the process. Nitrate was detected by using a Shimadzu HPLC system equipped with a IC-Pak Anion 4.6×50 mm column (Waters, Milford, MA, USA) and a conductivity detector CDD-6A (Shimadzu). The flow rate of the carrier (sodium borate/gluconate eluent) was 1.2 ml/min, and the column temperature was 40°C. Nitrite can be also detected and quantified by this method. Succinate was detected by using HPLC with an IC-Pak Ion Exclusion 78×50 mm column (Waters, Milford, MA, USA), UV spectrophotometric detector SDP-6A (Shimadzu), and refractive index detector RID-6A (Shimadzu). The flow rate of the carrier (0.2 M phosphate eluent, pH 2.5) was 1.0 ml/min, and the column temperature was 40°C. Other low molecular weight organic acids such as fumarate can be also detected by this method. Chromatograms were analyzed by using Chromato-PRO ver. 2.0.2 (Runtime Instruments, Sagamihara, Japan).

DNA isolation and ultracentrifugation

DNA was extracted from the soil microcosms (n=10) amended with nitrate and [^{13}C]succinate after 24 h incubation by using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan). The purified DNA (n=10) was pooled so as to have an amount sufficient for ultracentrifugation. As external references for the ultracentrifugation, [^{13}C]DNA and [^{12}C]DNA were extracted from *Escherichia coli* strain JM109 grown aerobically in M9 minimum medium[33] supplemented with 0.2% (w/v) pure ^{13}C- and ^{12}C-labeled succinate, respectively. Four ultracentrifuge tubes were prepared, each containing [^{12}C]DNA, [^{13}C]DNA, and a mixture of [^{12}C] and [^{13}C] DNA from *E. coli*, and DNA extracted from the soil microcosms. Ultracentrifugation was performed as described by Neufeld *et al.*[25] at an average of 158,000×*g* (41,900 rpm) using a RPV45T rotor (Hitachi Koki, Tokyo, Japan). After 66 h centrifugation, ^{12}C- and ^{13}C-labeled DNA bands were clearly separated (approx. 22 mm). DNA samples were taken from the [^{12}C]DNA fraction, the [^{13}C]DNA fraction, and the fraction in between the [^{12}C] and [^{13}C]DNA bands, by piercing the ultracentrifuge tubes with a 22-gauge needle. DNA from each fraction was purified using standard methods[25,33].

PCR conditions

For the DGGE analysis, partial bacterial 16S rRNA gene (V6,7,8 region) was amplified from the [^{12}C]DNA fraction, [^{13}C]DNA frac-

tion, and the fraction in between the [^{12}C] and [^{13}C]DNA bands, by using the primers EUBf933-GC-clamp and EUBr1387 as described by Iwamoto *et al.*[18]. For the clone library analysis, a nearly full-length of the 16S rRNA gene was amplified using the primers 27F and 1525R[21] from the [^{13}C]DNA fraction. Nitrite reductase genes, *nirS* and *nirK*, were also amplified from the [^{13}C]DNA fraction with cd3aF[22] and R3cd[39] primers and F1aCu and R3Cu primers[12], respectively.

The reaction mixture (50 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 µM each primer, 0.2 mM each dNTP, 0.5 µg/µl bovine serum albumin (Wako, Osaka, Japan), 0.001% (w/v) gelatin, 2 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 1 µl of DNA template. PCRs were performed using GeneAmp PCR system 9700 (Applied Biosystems) and the following conditions: initial annealing at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 57°C (55°C for Arch21F/Arch958R and 27F/1525R primers) for 30 s, and 72°C for 30 s (60 s for Arch21F/Arch958R primers and 90 s for 27F/1525R primers). After a final extension at 72°C for 7 min, the PCR mixtures were held at 4°C. The sizes of final PCR products were confirmed by agarose gel electrophoresis. PCR products amplified with the EUBf933-GC-clamp and EUBr1387 primers were used for the DGGE analysis, while the other products were used for cloning and sequencing as described below.

DGGE analysis

Denaturing gradient gel electrophoresis (DGGE)[18,23] was performed to compare bacterial community structures of the [^{12}C]DNA, [^{13}C]DNA, and middle fractions of the DNA extracted from the soil microcosm. Approximately 750 ng of the PCR products (100 ng for *E. coli* controls) were loaded onto a 6.5% (w/v) polyacrylamid gel in 1×TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA[pH 8]). The gel contained a gradient of denaturant (urea and formamide) ranging from 35 to 65%. The electrophoresis was performed at a constant temperature of 60°C for 16 h at 70 V, and the band patterns were visualized by staining with 0.01% (v/v) SYBR Gold (Molecular Probes, Eugene, Canada) for 30 min followed by UV transillumination. DNA bands relatively unique to the [^{13}C]DNA fraction were excised with a razor blade, and frozen at –80°C with 100 µl H_2O. The DNA was eluted through two freeze-thaw cycles (–80°C for 1 h and 37°C for 1 h) followed by centrifugation at 10,000×*g* for 5 min. Supernatant (1 µl) was used as a template for PCR with EUBf933 and EUBr1387 primers[18] as described above. The amplified products were cloned, and three clones per band were sequenced as described below.

Cloning and sequencing

PCR products were purified by using Wizard DNA Cleanup system (Promega, Madison, WI, USA) or MicroSpin S-400 HR Columns (GE Healthcare Biosciences, Piscataway, NJ, USA) to remove remaining dNTPs and primers. The purified PCR products were cloned into pGEM-T Easy vector (Promega), and transformed into *E. coli* JM109 high efficiency competent cells (Promega) according to the manufacturer's instructions. The plasmids with the insert were extracted by using a Qiagen Plasmid Mini Kit (Qiagen, Valencia, CA, USA), and used as a template for sequencing reactions. Occasionally, cloned insert DNA was amplified by PCR with vector primers T7-1 (5'-AATACGACTCACTATAG-3') and SP6 (5'-GATTTAGGTGACACTATAG-3'), purified as mentioned above, and directly sequenced. The sequencing reaction was performed with the DTCS Quick Start Kit for Dye Terminator Cycle Sequence (Beckman Coulter, Fullerton, CA, USA) and Genetic Analysis System CEQ 8000 (Beckman Coulter) with T7-1 or SP6 primers. For 16S rRNA gene sequencing, additional internal primers 341F[23], 519R[21], 926F[21], or 1115R[21] were used for sequencing nearly-full length of 16S rRNA gene. Three clones per DGGE band and around 50 clones per library were sequenced.

Phylogenetic analysis

DNA sequence chromatograms were edited and analyzed by using Finch TV ver. 1.4 (http://www.geospiza.com/finchtv/). Primer sequences were removed, and bidirectional sequences were aligned by using CLUSTALW ver. 1.83 (http://align.genome.jp/) to obtain consensus sequence for each clone. For functional gene (*nirS* and *nirK*) sequences, obtained consensus nucleotide sequences were translated to amino acid sequences by using TranSeq (http://www.ebi.ac.uk/emboss/transeq/). A sequence homology search was done by using BLASTN and BLASTX algorithms (http://www.ncbi.nlm.nih.gov/blast/). Reference sequences were retrieved from NCBI RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). The nucleotide or deduced amino acid sequences from multiple clones and reference were aligned with CLUSTALW. Obtained sequence alignments were confirmed manually by using GeneDoc ver. 2.6.002 (http://www.nrbsc.org/gfx/genedoc/index.html) and SeaView ver. 2.2 (http://pbil.univ-lyon1.fr/software/seaview.html). Aligned sequences were used to generate neighbor-joining trees with bootstrap values (n=1000) by using CLUSTALW provided with the SeaView program package.

Rarefaction calculation was done by using Analytic Rarefaction ver. 1.3 (http://www.uga.edu/~strata/software/Software.html). Operational taxonomic units (OTUs) were assigned using a 3% cut-off with the 16S rRNA gene sequence data. For rarefaction analyses with *nirS* and *nirK* genes, nucleotide sequences providing a unique amino acid sequence were considered to be one OTU.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rRNA, *nirS*, and *nirK* genes, and the DGGE bands determined in this study were deposited under accession numbers AB378583–AB378599, AB378600–AB378625, AB379559–AB379577, and AB430742–AB430748, respectively.

Results and Discussion

Evaluation of the soil microcosm

When a constant level of succinate (e.g. 0.5 mg C-succinate) was present, the addition of greater than 0.1 mg N-NO_3 per g soil did not increase the emission of N_2O after 24 h incubation at 30°C; however, the accumulation of nitrite and/or nitrate was observed (data not shown). This indicates that 0.1 mg N-NO_3^- is nearly the maximum amount of nitrate that can be used for N_2O production in 24 h. When a constant level of nitrate (0.1 mg N-NO_3^- per g soil) was incorporated into soil, N_2O production increased with a greater amount of succinate addition up to 0.3 mg C-succinate (data not shown), indicating that succinate was used as an electron donor for denitrification. The addition of greater than 0.3 mg C-succinate per g soil did not increase N_2O or CO_2 production (data not shown) similar to the previous report[34]. Based on these results, 0.1 mg N-NO_3^- and 0.5 mg C-succinate per g soil were set as the optimal concentrations for this study. Since soil organic matter can be also used as a C source for denitrifiers, 0.5 mg instead of 0.3 mg C-succinate was used to increase the incorporation of ^{13}C into microbial genomes. The N concentration of 0.1 mg N-NO_3^- per g soil is about twice as much as initially found in soil (see Materials and Methods), and approx. 10- to 100-fold less than the N level in the chemical fertilizer applied at the beginning of rice planting. Therefore, the concentration of nitrate added to soil in this study can be naturally observed.

Fig. 1A shows the time-course change in nitrate, succi-

nate, N_2O, and CO_2 quantities in the soil amended with the optimum concentrations of nitrate and succinate. Nitrous oxide emission increased greatly after 6 h and reached a plateau after 24 h. Similar trends were observed over multiple runs (data not shown), suggesting that this microcosm is reproducible in terms of substrate/product quantities. Nitrate and succinate concentrations decreased as N_2O and CO_2 emissions increased (Fig. 1A), indicating that these substrates were converted into the gaseous forms. Production of fumarate was observed and peaked at 12 h with an average of 12 μg C-fumarate, but decreased thereafter (data not shown).

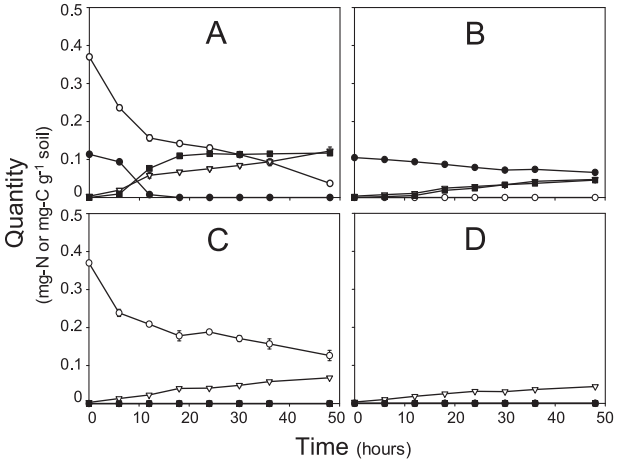

Fig. 1. Time-course change in nitrate (–●–), succinate (–○–), N_2O (–■–), and CO_2 (–▽–) quantities in the soil microcosms amended with A) 0.1 mg N-nitrate and 0.5 mg C-succinate; B) 0.1 mg N-nitrate only; C) 0.5 mg C-succinate only; and D) 200 μl sterile water (negative control). Mean±SE (n=3) is shown. Some succinate was lost during the extraction from soil, and therefore, only around 0.4 mg C-succinate was detected at 0 h (Fig. 1A and 1C).

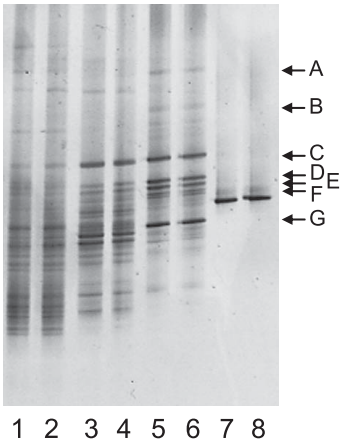

Fig. 2. PCR-DGGE 16S rRNA gene banding profiles of the ^{12}C-DNA fraction, ^{13}C-DNA fraction, and the fraction in between the ^{12}C- and ^{13}C-DNA bands separated by ultracentrifugation. DNA was extracted from soil microcosms after 24 h incubation with 0.1 mg N-nitrate and 0.5 mg ^{13}C-succinate. Lanes: 1–2, ^{12}C-DNA fraction; 3–4, the middle fraction beween ^{12}C and ^{13}C-DNA; 5–6, ^{13}C-DNA fraction; 7, ^{12}C-DNA from *E. coli*; and 8, ^{13}C-DNA from *E. coli*. Seven bands (A–G) relatively unique to the ^{13}C fraction were excised from Lane 6 of the gel, and three clones per band were sequenced. DNA sequences from each DGGE band were compared to the 16S rRNA genes in our clone library shown in Fig. 4.

This supports the oxidation of succinate to fumarate and eventually to CO_2. Based on these results, we chose 24 h incubation of the soil microcosm to study the succinate-assimilating community under denitrifying conditions.

Figure 1B, 1C, and 1D show the time-course change in nitrate, succinate, N_2O, and CO_2 quantities in the soil amended with 0.1 mg N-NO_3^- only, 0.5 mg C-succinate only, and 200 μl water (negative control), respectively. Production of N_2O was observed when nitrate only was added to soil (Fig. 1B), suggesting that soil organic matter can be used as an electron donor for denitrifiers. The concentration of nitrate gradually decreased as N_2O emission increased, indicating that nitrate was mostly converted to N_2O, and immobilization and other nitrate metabolism (e.g. dissimilatory nitrate reduction to ammonium) are minor. Succinate can be used when nitrate is absent (Fig. 1C) probably because Mn^{4+}/Fe^{3+}/sulfate reducers used it as an electron donor, but the rate of succinate utilization was lower than when nitrate is present. Fumarate was gradually accumulated with a greatest quantity at 24 h with an average of 48 μg C-fumarate, but decreased thereafter (data not shown). In the presence of nitrate, denitrification may account for the majority of anaerobic respiration based on the thermodynamic theory[43]. This implies that the majority of succinate is likely to be used by denitrifiers when nitrate is present.

Succinate-assimilating denitrifying population based on 16S rRNA gene analysis

Stable Isotope Probing was performed to study the [^{13}C]succinate-assimilating population in the soil microcosm under denitrifying conditions. Community structures representing [^{12}C]DNA, [^{13}C]DNA, and the middle fractions separated by CsCl-gradient ultracentrifuge were compared by PCR-DGGE analysis. The DGGE patterns shown in Fig. 2 indicate several distinct bands in the ^{13}C fractions. Similar DGGE patterns were observed in the ^{13}C-DNA fractions obtained over multiple SIP-labeling and ultracentrifugation runs (data not shown), indicating that the soil microcosms used in this study was reproducible in terms of metabolically-active bacterial population structure. Seven bands (~400 bp) relatively unique to the [^{13}C]DNA fractions were excised and three clones per band were sequenced. Most of

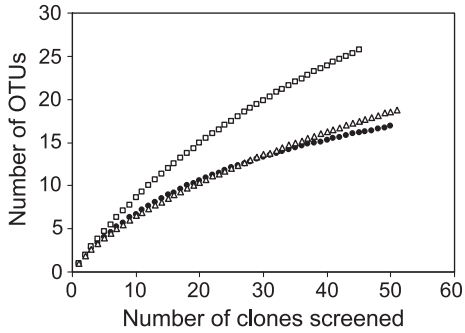

Fig. 3. Rarefaction curves indicating the diversity of the 16S rRNA (●), *nirS* (□), and *nirK* (△) genes cloned in this study. Operational taxonomic units (OTUs) were assigned using a 3% cut-off with 16S rRNA gene sequence data. For rarefaction analyses with *nirS* and *nirK*, nucleotide sequences providing a single amino acid sequence were considered to be one OTU.

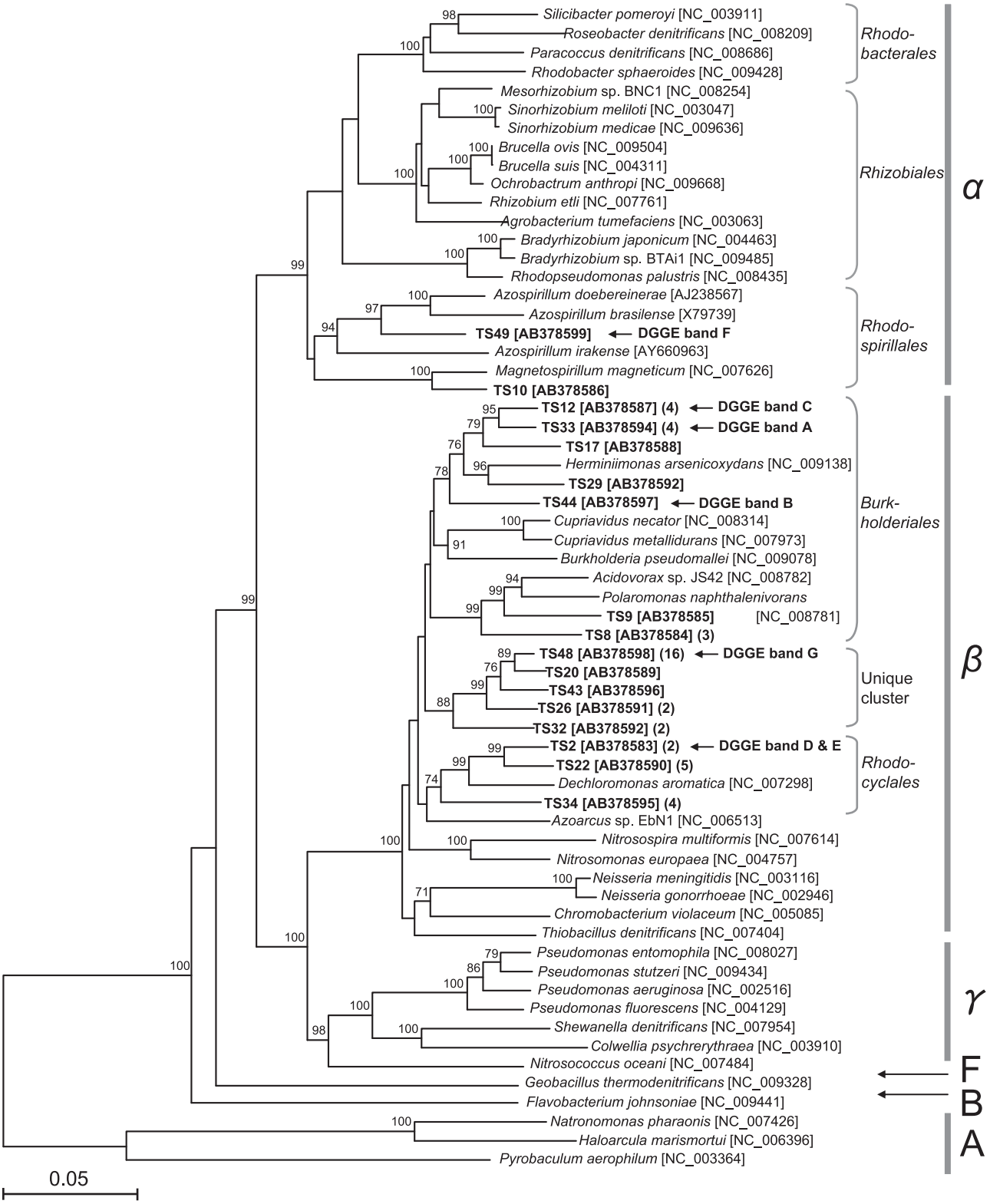

Fig. 4. Neighbor-joining phylogenetic tree based on the nearly full-length of 16S rRNA gene. Bootstrap values (%) were generated from 1000 replicates, and only values >70% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Clones obtained in this study are shown in bold with accession numbers. Clones with sequence similarities of >97% were considered to be the same OTU. Numbers in parentheses represent the total number of clones in the OTU. Legend: α, *Alphaproteobacteria*; β, *Betaproteobacteria*; γ, *Gammaproteobacteria*; F, *Firmicutes*, B, *Bacteroidetes*, and A, *Archaea*.

them belonged to *Burkholderiales* and *Rhodocyclales* within *Betaproteobacteria*, while one band belonged to *Rhodospirillales* within *Alphaproteobacteria*. Some DGGE bands (e.g. band C) were also observed in the middle fraction between ^{12}C- and ^{13}C-DNA (lane 3 and 4 in Fig. 2), probably due to insufficient incorporation of ^{13}C into DNA and/or

broad distribution of these DNA groups in the CsCl gradients. The PCR primers used for the DGGE analysis are specific to the universally conserved bacterial 16S rRNA gene, and do not amplify the archaeal 16S rRNA gene[18]. Since some *Archaea* have denitrifying ability[4,29], we investigated whether *Archaea* could be detected in the [^{13}C]DNA fraction by using PCR with *Archaea*-specific primers Arch21F and Arch 958R[7]. The amplification of the expected-size products were found only from [^{12}C]DNA fraction (data not shown), indicating that *Archaea* were present in the soil examined, but did not assimilate ^{13}C-succinate under the conditions we used.

To further characterize the community structure representing the [^{13}C]DNA fraction, a clone library analysis was employed with nearly full-length of 16S rRNA gene sequences. The rarefaction curve analysis shows that diversity based on 16S rRNA gene was not saturated with the number of clones in this study (n=51) (Fig. 3). We recognize that the clone library approach is not absolutely quantitative, but several clones were frequently observed (e.g. TS48 clone sequences, n=16). Similar to the DGGE analysis, the phylogenetic tree shows that the clone sequences obtained in this study were affiliated within *Alphaproteobacteria* and *Betaproteobacteria* (Fig. 4). DNA sequences recovered from the DGGE bands relatively unique to the ^{13}C-DNA fraction showed high similarity (>97%) to the 16S rRNA gene sequences obtained in the clone library analysis (Fig. 4). Most clones were clustered within the orders *Burkholderiales* (31%), *Rhodocyclales* (22%), and *Rhodospirillales* (4%). Twenty two clones (43%) including TS48 were distantly clustered from known reference sequences. The highest similarity to clone TS48 was with the 16S rRNA gene from recently proposed denitrifying bacterium *Denitratisoma oestradiolicum* JCM12830^{T} (AY879297; 96% sequence similarity)[9], and from nitrogen-fixing isolates obtained from wild rice (AY235684, AY235685, AY235687, AY235688; 96% sequence similarity). The ribosomal RNA gene from *Sterolibacterium denitrificans* ATCC

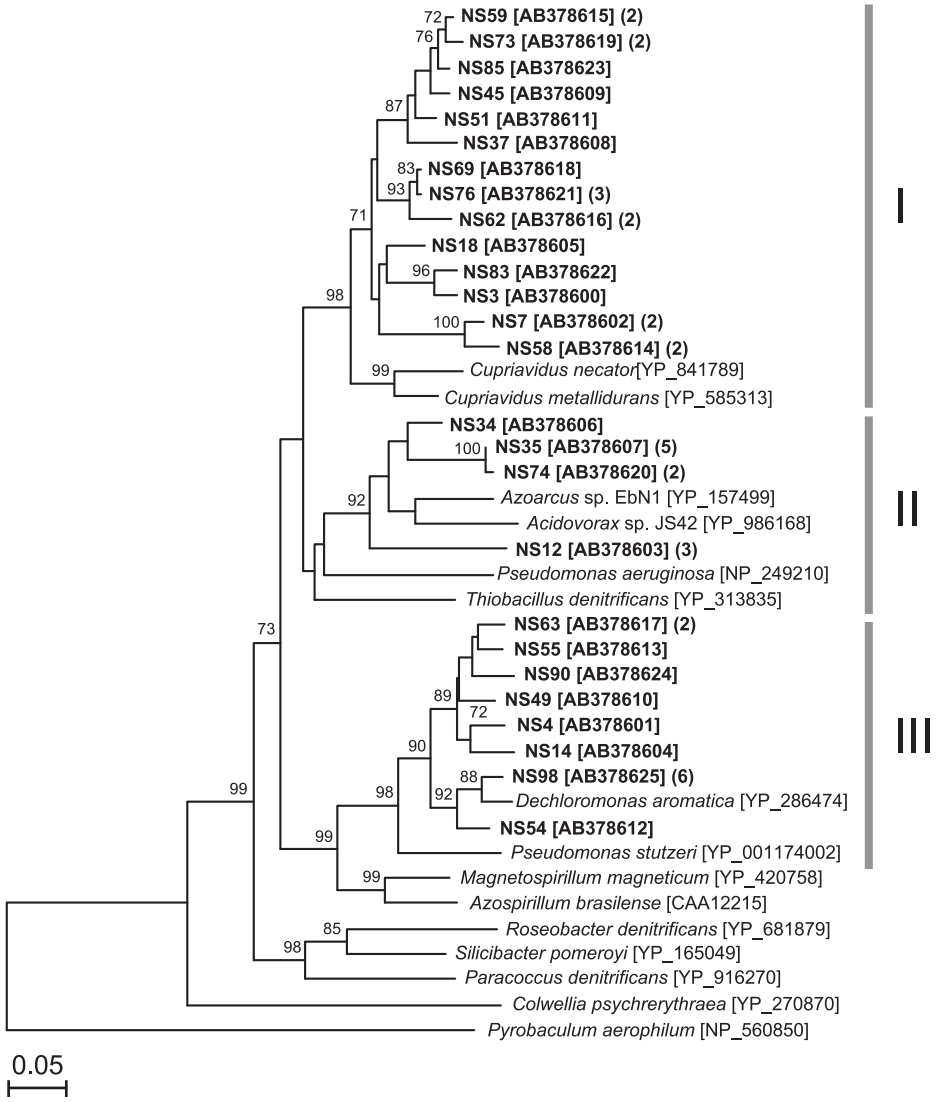

Fig. 5. Neighbor-joining tree based on deduced NirS amino acid sequences (137–142 aa). Bootstrap values (%) were generated from 1000 replicates, and only values >70% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Clones obtained in this study are shown in bold with accession numbers. Clones with identical amino acid sequences were considered to be the same OTU. Numbers in parentheses represent the total number of clones in the OTU.

BAA-354^T (AJ306683, 93% similarity)[38] and denitrifying bacterium strain 72Chol (Y09967, 94% similarity)[13], also had relatively high levels of similarity to TS48 clone. These strains are all affiliated to *Rhodocyclacea* (*Rhodocyclales*), suggesting that the members of the unique cluster (clone TS48 group) may also belong to *Rhodocyclales*. Furthermore, *Denitratisoma oestradiolicum*, *Sterolibacterium denitrificans*, and the strain 72Chol can anaerobically degrade aromatic compounds such as steroid and cholesterol by using nitrate as the electron acceptor[9,13,38] indicating that they, and probably the clone TS48 group, may have a selective advantage in the environment where aromatic compounds such as humic substances are abundant.

It is important to note that the assimilation of ^{13}C-succinate may not be restricted to denitrifying bacteria, although thermodynamic theory predicts that denitrification is more favorable than other succinate oxidizing processes such as Mn^{4+}/Fe^{3+}/sulfate reductions and nitrate respiration. However, *Burkholderiales* and *Rhodocyclales* have been reported to be dominant in denitrifying communities. For example, Osaka *et al.*[28] identified *Alphaproteobacteria* and *Betaproteobacteria* (especially *Burkholderiales*, *Rhodocyclales*, and *Rhodobacterales*) as dominant acetate-assimilating bacteria in activated sludge under nitrate-reducing conditions by using SIP analysis, although they also found clones belonging to other orders such as *Gammaproteobacteria* and *Epsilonproteobac-*

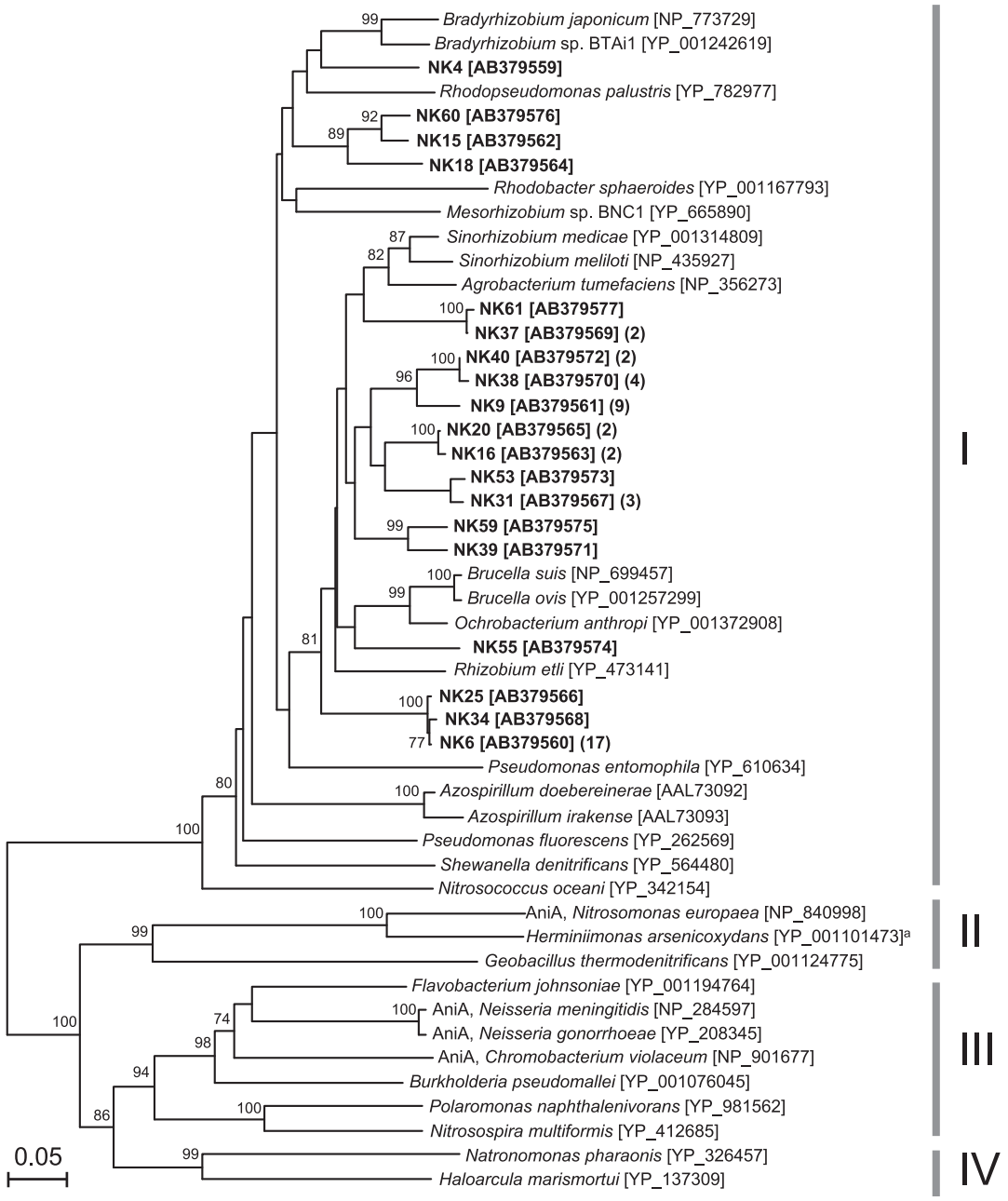

Fig. 6. Neighbor-joining tree based on deduced NirK amino acid sequences (145 aa). Bootstrap values (%) were generated from 1000 replicates, and only values >70% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Clones obtained in this study are shown in bold with accession numbers. Clones with identical amino acid sequences were considered to be the same OTU. Numbers in parentheses represent the total number of clones in the OTU.

teria. A culture-based approach also identified *Alphaproteobacteria* and *Betaproteobacteria* (especially *Rhodocyclaceae* members) as the most abundant (87%) denitrifiers in activated sludge[16]. These and other reports (e.g. Wagner and Loy[41]) suggest that the order *Rhodocyclales* potentially dominates in the denitrification in wastewater treatment systems. In rice paddy soil, members of *Rhodocyclales*, including the novel group identified in this study, may also play an important role in denitrification. In contrast, however, *Pseudomonas* (*Gammaproteobacteria*) and *Bacillus* were identified as the major denitrifiers in upland agricultural and turfgrass soils by using a culture-based approach with rich media[6,42]. Well-drained soils such as those of wheat fields and golf greens may have different denitrifying populations from wastewater and paddy soil, although these contradictory results may be also due, in part, to the incubation bias caused by the rich media.

Functional gene diversity

The diversity of the nitrite reductase genes (*nirS* and *nirK*) was examined in succinate-assimilating bacterial populations by using a clone library analysis. Rarefaction curves show that NirS was more diverse than NirK in this study (Fig. 3). A dendrogram based on deduced NirS amino acid sequences (137–142 aa) shows a high degree of genetic heterogeneity, although several clusters could be identified (Fig. 5). Archaea NirS from *Pyrobaculum aerophilum* (NP_560850) served as an outgroup in this dendrogram. Forty six NirS clone sequences obtained in this study were affiliated into three clusters (I, II, and III) in the dendrogram based on bootstrap values and sequence identity. The NirS clones in Cluster I (n=21) were related to NirS from *Cupriavidus* strains (YP_841789 and YP_585313). Relatedness between these NirS clones and the *Cupriavidus* NirS was supported by a relatively high bootstrap value (98%) and sequence identity (78–86%). The NirS clones in Cluster III (n=14) were related to NirS from *Dechloromonas aromatica* (YP_286474) at 84–95% identity. These clone sequences were also similar to NirS from *Dechloromonas*, *Thauera*, and other members of *Rhodocyclaceae* (e.g. CAJ76752, AAL86926, AAL86942). Many of the NirS sequences from marsh soil and activated sludge samples also showed high levels of identity to NirS from *Rhodocyclaceae*[28,30]. In contrast to the Clusters I and III, Cluster II consists of several reference strains from different orders. The majority of the bootstrap values in this cluster are also low, and therefore, identification of the NirS clones in this cluster could not be determined.

Similar to the previous report[29], the dendrogram based on deduced NirK amino acid sequences (145 aa) shows four main clusters, each containing taxonomically-diverse reference strains (Fig. 6). Sequences of AniA, a copper-containing nitrite reductase believed to be associated with the outer membrane of some bacteria, were also included in this dendrogram since NirK and AniA both catalyze the same reaction ($NO_2^- \rightarrow NO$) and they share two conserved copper-binding domains. All NirK clones in this study were related to NirK from *Rhizobiales* at a relatively high sequence identity (76–84%) similar to the previous report[3]. It is important to note, however, that the previously-designed PCR primers for *nirK*, including the ones used in this study, do not show homology to AniA and other NirK from Clusters II, III, and IV.

Comparison between 16S rRNA phylogeny and nitrite reductase phylogeny

Previous studies have reported that the NirS phylogeny was consistent with the 16S rRNA gene phylogeny with a few exceptions (e.g. *Pseudomonas stutzeri* in Cluster III in Fig. 5)[15,29]. This suggests that the NirS clones in Cluster I and III in Fig. 5 may originate from members within *Burkholderiales* and *Rodocyclales*, respectively. These members of bacteria were also frequently detected in the 16S rRNA gene clone library study shown in Fig. 4.

In contrast to NirS nitrite reductase, the NirK phylogeny was not necessarily in compatible with the 16S rRNA gene phylogeny[15,29]. Although Fig. 6 shows that many clones were related to NirK from *Rhizobiales*, they may not originate from members of this group. In fact, the 16S rRNA gene clone library approach did not detect *Rhizobiales*. Heylen *et al.*[15] examined *nirK* gene diversity among denitrifying strains obtained from activated sludge, and found that taxonomically-unrelated groups such as *Acidovorax*, *Pseudomonas*, *Paracoccus*, and *Ochrobactrum* (*Rhizobiales*), harbored very similar *nirK* sequences (AY078249–AY078253). When included, these NirK sequences were affiliated within Cluster I in Figure 6 (data not shown), suggesting that the NirK clones from Cluster I may originate from a diverse group of bacteria. More *nirK* sequence information from cultured bacteria is needed to further estimate the potential hosts of these NirK clones.

Conclusions

In this study, we proved that the SIP approach was useful to study the succinate-assimilating population in rice paddy soil under denitrifying conditions. Both 16S rRNA gene and *nirS* sequence analyses suggest that *Burkholderiales* and *Rhodocyclales* dominate the succinate-assimilating population in rice paddy soil. In particular, we identified a novel group, possibly within the order *Rhodocyclales*. Further research is necessary to identify if these members indeed have an ability to denitrify.

A potential limitation of the SIP-approach to the study of denitrifying populations is the detection of non-target functional groups: i.e. labeled succinate can be assimilated by other functional groups such as Mn^{4+}/Fe^{3+}/sulfate reducers. Recent improvements in SIP experiments[10,25] and the use of appropriate controls may overcome some problems.

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