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Temporal shifts in diversity and quantity of *nirS* and *nirK* in a rice paddy field soil

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ABSTRACT

Denitrification is an important part of the nitrogen cycle in the environment, and diverse bacteria, archaea, and fungi are known to have denitrifying ability. Rice paddy field soils have been known to have strong denitrifying activity, but the microbes responsible for denitrification in rice paddy field soils are not well known. Present study analyzed the diversity and quantity of the nitrite reductase genes (*nirS* and *nirK*) in a rice paddy field soil, sampled four times in one rice-growing season. Clone library analyses suggested that the denitrifier community composition varied over sampling time. Although many clones were distantly related to the known NirS or NirK, some clones were related to the NirS from Burkholderiales and Rhodocyclales bacteria, and some were related to the NirK from Rhizobiales bacteria. These denitrifiers may play an important role in denitrification in the rice paddy field soil. The quantitative PCR results showed that *nirK* was more abundant than *nirS* in all soil samples, but the *nirK*/*nirS* ratio decreased after water logging. These results suggest that both diversity and quantity changed over time in the rice paddy field soil, in response to the soil condition.

1. Introduction

Denitrification, a microbial redox process in which nitrogen oxides (NO_3^- and NO_2^-) are reduced stepwise to gaseous end products (NO, N_2O, and N_2) (Zumft, 1997), is an important step in the environmental nitrogen cycle (Philippot et al., 2007; Hayatsu et al., 2008). In agricultural fields, denitrification can cause nitrogen loss from soils as well as emission of N_2O gas, which may contribute to global warming and destruction of the ozone layer (Hofstra and Bouwman, 2005; Philippot et al., 2007). Atmospheric concentration of N_2O has been increasing annually, and agriculture is a major source of this greenhouse gas (Philippot et al., 2007). Among agricultural soils, rice (*Oryza sativa*) paddy fields are one of the areas where denitrification occurs actively (Liesack et al., 2000; Hofstra and Bouwman, 2005). However, emission of N_2O was much lower in rice paddy fields than upland crop fields (Nishimura et al., 2004; Akiyama et al., 2006). This phenomenon is most likely due to complete denitrification with N_2 as the end product (Nishimura et al., 2004). However, denitrifiers in rice paddy field soils are not well characterized.

Many types of bacteria (Gamble et al., 1977; Bothe et al., 2000; Liesack et al., 2000), as well as some archaea (Philippot, 2002; Hayatsu et al., 2008) and fungi (Shoun et al., 1992; Hayatsu et al., 2008) have been reported to possess denitrifying ability. When assessing diversity of denitrifiers in soil, culture-independent approach has been frequently used because many soil microbes are difficult to be cultivated. Since many phylogenetically diverse microbes have the ability to denitrify, it is difficult to identify denitrifiers by using specific primers or probes based on the 16S rRNA gene (16S rDNA) (Philippot et al., 2007). To analyze denitrifier community, the cytochrome cd_1-containing nitrite reductase gene (*nirS*) and the Cu-dependent nitrite reductase gene (*nirK*) are frequently used as gene markers, since only denitrifiers harbor these genes (Braker et al., 2000). Furthermore, genetic heterogeneity within the *nirS* or *nirK* allows us to examine diversity of denitrifiers (Braker et al., 2000). Specific primer sets for these genes have been evaluated (Throbäck et al., 2004), and a variety of environmental samples such as meadow (Bremer et al., 2007), glacier foreland (Kandeler et al., 2006), forest soil (Priemé et al., 2002; Katuyama et al., 2008), and arable soil (Throbäck et al., 2004; Wolsing and Priemé, 2004) have been studied using these genes. In addition, quantity of these genes can be measured by using real-time PCR approach (Kandeler et al., 2006). Therefore, we selected *nirS* and *nirK* as gene markers to study denitrifier community in the rice paddy field soil.

Recently, Saito et al. (2008) applied stable isotope probing (SIP) to study the active denitrifying population in a rice paddy soil using ^{13}C-succinate as an electron donor and a carbon source for

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denitrifiers. Based on the clone library analyses targeting 16S rDNA, *nirS*, and *nirK*, they concluded that Burkholderiales and Rhodocyclales bacteria, and novel betaproteobacteria closely related to the Rhodocyclales bacteria may contribute to denitrification in the microcosm containing a rice paddy field soil. However, their SIP approach is applicable only to the laboratory microcosm since ^{13}C-succinate should be assimilated under anaerobic condition, which is difficult to control under field conditions. Therefore, denitrifying populations in rice paddy field soils is still not well known.

By using real-time PCR and clone library analysis targeting *nirS* and *nirK*, we expected to clarify the temporal shift in denitrifier communities in a rice paddy field soil. In general, it is known that the redox potential of rice paddy soils decreases drastically in 10–14 days after water logging, around when denitrification activity was most significant (Takai and Uehara, 1973). Water-logged condition is one of the unique characteristics of rice paddy soils, in which various biogeochemical processes occur as redox potential decreases (Takai and Uehara, 1973; Kikuchi et al., 2007). We hypothesized that denitrifier population changed by time, and denitrifiers identified in the previous SIP experiment might be observed in the field soil.

Consequently, the objectives of this study were (1) to investigate the diversities of *nirS* and *nirK* in a rice paddy field soil, (2) to monitor diversity and quantity of the two genes over time during a rice-growing season, and (3) to compare results obtained from the field samples with those obtained by SIP study (Saito et al., 2008). Although *nirS* and *nirK* diversities have been analyzed in various environments including field agricultural soils, this is the first report elucidating temporal dynamics of *nirS* and *nirK* diversities in a rice paddy field soil.

2. Materials and methods

2.1. Field soil samples

Soil samples were collected from the rice paddy field (3000 m^2) in the Field Production Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo (Nishitokyo, Tokyo, Japan; 35° 44′ N, 139° 32′ E). The soil was derived from volcanic ash (Andisol), rich in humic substances, and classified into Clay Loam (35.3% sand, 30.3% silt, and 24.3% clay). The chemical and physical properties of the soil were described elsewhere (Saito et al., 2008). 60 kg ha^{-1} of the chemical fertilizer (12% N–NH_4^+, 18% P–phosphate, 16% K, 3% Mg, 0.4% Mn, and 0.2% B) was homogeneously applied and incorporated into the soil on May 16, and rice (*O. sativa*) nursery plants were transplanted on May 26, 2006. Field soil sampling was conducted on May 18 (just before paddy water logging and fertilizer application), June 2 (two weeks after water logging), June 19, and July 27, 2006. In general, denitrification activity in the rice paddy field soils was at its peak two weeks after water logging (Takai and Uehara, 1973). Samplings at 1 and 2 months after water logging (June 19 and July 27, respectively) were performed to monitor denitrifier community under the succession of soil redox potential (i.e., decreasing soil Eh). On each occasion, subsamples (approximately 50 g) were collected from top 5-cm surface soil by using sterile soil probe from five points; each located approximately 5 m apart. The five subsamples were then mixed well and stored cool (~4 °C) until processed (usually within 2 h) for DNA extraction. DNA was extracted in triplicate from each soil sample.

2.2. DNA extraction and PCR

DNA was extracted and purified from each 1 g soil sample using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan). The extracted DNA was eluted in 100 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). Purified DNA was diluted 50-folds to reduce the influence of the PCR inhibitors such as humic acids, and used for PCR targeting *nirS* and *nirK*. For amplification of *nirS*, the primer pair cd3aF (Michotey et al., 2000) and R3cd (Throbäck et al., 2004) was used, while another primer pair F1aCu and R3Cu (Hallin and Lindgren, 1999) was used for *nirK*-targeting PCR. It was reported that these primer pairs have relatively high specificity and broad host coverage (Throbäck et al., 2004). The PCR reaction mixture (20 μl) contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w v^{-1}) gelatin, 0.2 mM dNTPs, 1 mM each of the forward and reverse primers, 10 μg of BSA, 2 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 1 μl soil DNA. PCR was performed in a thermal cycler, the GeneAmp PCR System 9700 (Applied Biosystems) under the condition described by Throbäck et al. (2004). After the reaction, 2 μl of the PCR products were separated by electrophoresis using a 1.5% agarose gel, and visualized by ethidium bromide staining to check for the presence of amplified DNA. In addition to the *nirS*- and *nirK*-targeting PCR, 16S rDNA was also amplified by using 357F and 520R primers, and denaturing gradient gel electrophoresis (DGGE) was performed as previously described (Otsuka et al., 2008), to compare community structures among replicate samples.

2.3. Cloning and sequencing

The *nirS* and *nirK* PCR products were passed through MicroSpin S-400 HR Columns (GE Healthcare Biosciences, Piscataway, NJ, USA) to remove the remaining dNTPs and primers. Since community structure assessed by DGGE analysis was similar among triplicate samples (data not shown), the purified PCR products from three subsamples were mixed in equal volume and then cloned with the pGEM-T Easy System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cloned insert DNA was amplified by PCR with M13 vector primers M3 (5′- GTA AAA CGA CGG CCA GT -3′) and RV-P (5′- GGA AAC AGC TAT GAC CAT G -3′), purified as mentioned above, and subjected to DNA sequencing. Bidirectional sequence reactions were performed with a DTCS Quick Start Kit for Dye Terminator Cycle Sequence (Beckman Coulter, Fullerton, CA, USA) and Genetic Analysis System CEQ 8000 (Beckman) with primer M3 or RV-P.

2.4. Real-time PCR

Real-time PCR targeting *nirS* and *nirK* was performed in duplicates to quantify abundance of each gene in the soil samples. To minimize the influence of the PCR inhibitors, the extracted DNA samples were further purified by using Power Soil DNA Purification Kit (MoBio Laboratories, Carlsbad, CA, USA), and diluted 10-folds. Primer pairs modified-cd3aF and -R3cd (Kandeler et al., 2006) were used for *nirS* amplification, and *nirK*876F and *nirK*1040R (Henry et al., 2004) were used for *nirK* amplification. The reaction mixture (20 μl) contained 10 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.2 mM each of the forward and reverse primers, 8 μg of BSA, and 1 μl of the purified DNA template. Amplification was performed in a StepOne Real-Time PCR System (Applied Biosystems) using SYBR Green as a detection system under the same conditions described by Henry et al. (2004). Amplification of the correct-size products was verified by dissociation curve analysis and agarose gel electrophoresis. Analysis of variance (ANOVA) was performed by using R program ver. 2.8.1 (http://www.r-project.org) to examine significant differences ($\alpha = 0.05$) in *nirS* and *nirK* gene copy numbers among samples.