Original research article

Analysis of rhizosphere bacteria of rice cultivated in Andosol lowland and upland fields using molecular biological methods

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Abstract: Bacteria in the rhizosphere influence plant growth and interact with plant roots. Microscopy and culture method have been used for studies of microorganisms of the rhizosphere, but these methods are insufficient for evaluation because most rhizosphere bacteria are viable but non-culturable (VBNC). Bacteria in the rhizosphere of rice cultivated in Andosol lowland and upland fields were analyzed in this study using PCR-DGGE and FISH, in combination with modified pretreatments. Results show that the two methods with the pretreatments, more than conventional methods, provided a rapid and simple analysis of rhizosphere bacteria. The 16S rDNA band pattern of bacteria in the rhizosphere obtained using PCR-DGGE indicated different species composition of bacterial community in the two ecosystems and greater diversity of bacteria in the rhizosphere in upland field. Sequencing of major 16S rDNA bands identified Bacterium A35 and *Clostridium bifermentans* as dominant bacteria in the rhizosphere of rice in lowland fields and *Klebsiella planticola* and *Bacillus fusiformis* in upland fields. Furthermore, FISH observation indicated the predominance of gram-positive low GC bacteria in both rhizospheres and a higher proportion of *Clostridium* spp. in lowland fields, which is consistent with results of PCR-DGGE analysis. The results suggest that the bacteria in the rice rhizosphere can be changed depending on aerobic and anaerobic conditions of fields. It is expected to apply the PCR-DGGE and FISH to agricultural field experiments as reliable methods to evaluate the rhizosphere bacteria.

Keywords: 16S rDNA, bacteria, FISH, PCR-DGGE, rhizosphere, rice (*Oryza sativa* L.).

Abbreviations: PCR-DGGE, polymerase chain reaction with denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization

Introduction

Plants take up water and nutrients from soil through plant roots. Plant roots, especially their apex including the root cap, exudes mucigel (Jenny and Grossenbacher 1963) which mainly includes polysaccharides with amino and organic acids (Rovira 1969, Eastman and Peterson 1985) to provide a carbohydrate source to soil microorganisms (Jimenez et al. 2003). For that reason, diverse and large populations of microorganisms are present in the rhizosphere: the soil that is circumjacent to the root. Bacteria in the rhizosphere influence plant growth because they affect soil chemical properties and interact with plant roots, where the influence can be beneficial, neutral, or deleterious (Russell 1981, Morita 2000, Sakai et al. 2004). Understanding rhizosphere microorganisms is important for their effective use for low-input sustainable agriculture (LISA) and phytoremediation (Siciliano and Germida 1998, Doi et al.2004, 2006). Microorganisms in the rhizosphere are known to compose the complex of the microbial community, which might be changeable depending on plant root exudates (Rovira et al. 1974, Kimura 1994, Watt et al 2006b, 2006c).

Conventionally, microscopic examination and/or culture method such as dilution-plate culture have been used to study soil microorganisms (Hattori 1976). Microscopic examination, however, necessitates

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proficient knowledge and experience, and culture method take several days until the results are obtained (Ohishi et al. 2001). In addition, it has recently been suggested that conventional methods are insufficient to examine environmental microorganisms for the following reasons (Doi et al. 2006, Tani et al. 2006). Microscopic examination cannot distinguish active and inactive microorganisms. Moreover, most environmental microorganisms are viable but non-culturable (VBNC) microorganisms that cannot be detected as colonies in nutritional cultivation (Colwell et al. 2004). Reportedly, culture method can detect only 1% of soil environmental bacteria as they are (Amann et al. 1995).

For those reasons, to examine microorganisms, a rapid and accurate method is required. Recently, methods in molecular biology are rapidly developing to meet the needs of medical and environmental sciences. Especially, databases of base sequences of DNA in 16S subunit of ribosomes (16S rDNA), which consist of ca. 1500 base genes existing in any bacterial species, have been enriched to render them effective for comparison of their biodiversity from evolutionary viewpoints (Okabe 2005), e.g., in aquatic environmental microbiology (Colwell et al. 2004).

Moreover, the combination of polymerase chain reaction with denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescent *in situ* hybridization (FISH) are anticipated as new simple and prompt molecule biological methods using 16S and/or 23S rDNA/RNA to analyze eubacteria. DGGE imposes a parallel gradient of denaturing conditions caused by urea and formamide along a polyacrylamide gel to migrate PCR amplicons of double-stranded DNA (Muyzer and Smalla 1998, Thies 2006). Because native double stranded DNA migrates faster than partially denatured DNA, and because the sequence affects duplex stability, the sequence of a fragment determines the point in the gradient gel at which the DNA fragment forms the band. The resulting gel yields a ladder of bands in each lane, in which each band indicates the presence of a species and the whole band pattern obtained by DGGE reflects the species composition of the eubacteria community in the sample. In the field of the environment microbiology, PCR-DGGE is applied to distinguish bacteria species and to estimate the proportion of respective species among all bacteria (Fujita et al. 2006). Another technique, FISH, uses an oligonucleotide probe conjugated with a fluorescent molecule. The probe for detection of a bacterium is designed to bind to complementary sequences in the 16S and/or 23S rRNA within bacterial cells. Because metabolically active cells contain numerous rRNA, concentration of the fluorescently labeled probe is high inside the cells, causing them to fluoresce under UV light (Thies, 2006). For environmental microbiology, FISH has facilitated direct observation of target bacteria as individuals (Delong 1989, Frischer et al. 2000). Recently, PCR-DGGE and FISH have come to be applied to analyses of microbial community structure in studies for bioremediation of oil-contaminated water (Syutsudo 2005) and for the biomass energy industry, which uses hydrogen and methane fermentation (Ohba et al. 2006).

Although application of PCR-DGGE and FISH to study bacteria in the rhizosphere is anticipated (Thies 2007), the use of these methods has remained limited because of the difficulty in isolation of the target DNA/RNA fragments from soil in comparison to materials in environmental sciences (e.g., water and sludge). In fact, DNA extraction is inhibited by humus and clay minerals from soil. Humus contaminates DNA extraction; in addition, DNA is adsorbed by clay minerals, which might influence PCR-DGGE analyses (Hoshino and Hasebe 2004). Moreover, autofluorescence of soil particles disturbs the fluorescent observation used for FISH (Doi et al. 2001). Accordingly, modification of pretreatments is necessary for application of PCR-DGGE and FISH to study rhizosphere microorganisms. For the present study, we sought to improve the treatments of DNA/RNA extractions from soil to apply PCR-DGGE and FISH and compare the rhizosphere bacteria of rice that were grown in flooded lowland and upland fields of Andosol soil.

Materials and Methods

Rice cultivation and rhizosphere soil sampling

Rice plants (*Oryza sativa* L. cv. Nipponbare) were conventionally cultivated in a lowland paddy field and an upland field at the Field Production Science Center of the University of Tokyo in Nishitokyo, Japan (35°43′N, 139°32′E) during April–October 2002. The soil was Andosol, a volcanic ash soil named Kanto loam. The topsoil layer of ca. 0.35 m was a dark, humic silty loam; the subsoil layer (below 0.35 m) was a red-brown silty clay loam in both lowland and upland fields (Yamagishi et al., 2003). Two dry spells of longer than 10 days occurred in August (Kato et al., 2006).

In the lowland field, lowland rice seedlings were transplanted on 24 May with spacing of 300 mm × 150 mm (22.2 hills m^{-2}). The rice straw of the former year’s production was cut and plowed into the soil after harvesting in October 2001. Nitrogen (6 g m^{-2}), phosphate (10 g m^{-2}), and potassium (5 g m^{-2}) fertilizers were applied as basal dressing; nitrogen (1.5 g m^{-2}) fertilizer was applied as topdressing at 20 July. Heading of 50% of panicles of rice in the lowland field

occurred on 25 August. The lowland field was flooded until the heading stage. In the upland field, rice was seeded directly by drill seeding (24 plants m^{-2}) on 30 April. Nitrogen (6 g m^{-2}), phosphate (9 g m^{-2}), potassium (8 g m^{-2}) fertilizers and compost (500 g m^{-2}) were applied as a basal dressing, and nitrogen (3 g m^{-2}) fertilizer was applied on 19 June as a topdressing. Heading of 50% of panicles of rice in the upland field occurred on 1 September. The rice was cultivated in a rain-fed upland condition without irrigation.

The soil, with roots around hills, was taken with a shovel as far as approximately 0.15 m from the hill with approximately 0.2 m depth at the ripening stage. Precipitation during the growing period was slight in that year. Therefore, soil moisture in the top 0.12 m was only 15–20% (v/v) in the upland rice field at sampling. The water table was ca. 50 mm below the soil surface and the soil Eh was ca. -150 mV at 100-mm depth in the lowland field.

Rhizosphere soil is separable from roots by rapid manual washing of roots (Nakamoto, 1999). We improved that method using an ultrasonic washer for stable treatment to separate rhizosphere soil from roots and non-rhizosphere soils in phosphate buffered saline (PBS). Non-rhizosphere soil was removed using 20-s ultrasonic treatment for upland rice and 30 s for lowland rice; then soil particles adhering to the roots were separated from the roots using 3 min ultrasonic treatment. The soil was collected by centrifugation and was then designated as rhizosphere soil. After washing in PBS and collection by centrifugation, each sample of rhizosphere soil was fixed using 4%-paraformic aldehyde and kept at 4°C in a refrigerator (Nielsen et al. 1999).

Analysis of rhizosphere soil bacteria using methods of molecular biology

An outline of the molecular biological methods used in this study is presented in Fig. 1. After DNA extraction, PCR-DGGE was performed for microbial analysis of bacterial populations by 16S rDNA segments; the domain bacteria were identified using sequential gene analysis of the 16S rDNA (Syutsudo 2005). In addition, fixed rhizosphere eubacteria were labeled using FISH for observation using fluorescent microscopy. Details of PCR-DGGE and FISH protocols are described below.

PCR-DGGE

For PCR-DGGE, DNA was extracted from each sample using bead-beating: 0.5 g of soil samples was added to a 2 mL tube, then disrupted at 55,000 rpm for 30 s using a bead shocker (Micro smash; TOMY). Suspension of the extracted DNA was clarified by centrifugation at 15,000 rpm for 3 min. The washed DNA samples contained some humus and protein. For that reason, they were purified further using a spin column (GFX ™ kit; Amersham Pharmacia Biotech Co. Ltd.) (Ritchie et al. 2000). This purified DNA was used as the PCR template.

The oligonucleotide with forward primer 357F-GC, 5'-cct acg gga ggc agc ag -3' (*Escherichia coli* positions 341–357), which was attached to an oligonucleotide GC clamp (cgc ccg ccg cgc gcg gcg ggg gcg ggg gca cgg ggg g) at the 5'-terminus, and the oligonucleotide reverse primer 518R, 5'-att acc gcg gct gct gg -3' (*E. coli* positions 518–534), which encoded the V_3 region of the 16S ribosome DNA (Muyzer et al.1993), were used for PCR. The PCR was conducted as an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min and annealing at 53°C for 1 min and primer extension at 72°C for 2 min (Kurisu et al., 2000). The amplification of the 16S rDNA by PCR was verified by electrophoresis in 2% agarose gel prior to DGGE. The PCR products were examined after DGGE (D-Code system; Bio-Rad Laboratories) on a polyacrylamide gel with a 20–50% denaturation gradient under a 120 V field for 60°C four hours (Clayton et al. 2001). After DGGE, the polyacrylamide gel was stained (SYBER Green I; Molecular Probes Inc.) in a dark room for 20 min. Then the 16SrDNA profile was verified and photographed. Two samples each from the lowland (L1, L2) and upland (U1, U2) fields were analyzed using PCR-DGGE. The two samples were taken at two sites 1-2 m away from each other in each of lowland and upland fields. After intensely stained DGGE bands were excised from the gels, the 16SrDNAs were purified and amplified using a QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany). Purified PCR 16SrDNAs were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and DNA sequencer (ABI Prism 3100; Applied Biosystems). Dominant bacteria were identified using data from the DNA data bank of Japan (DDBJ) and a basic local alignment search tool (BLAST) (Ishii et al. 2000). The sequences obtained from the DGGE bands were sent to the DNA database for a homology search using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

FISH

Microorganisms in the rhizosphere soil samples were separated using Percoll-density gradient solution centrifugation. For that process, 500 μL dilution of Percoll (specific gravity 1.2) was added to the 1 mL soil sample fixed using 4%-paraformic aldehyde in a 2 mL tube, followed by centrifugation at 13,000 rpm

for 10 min. The supernatant was transferred to a new 2-mL tube for cleaning with 1 mL PBS.

The following 16S and 23S rRNA-targeted oligonucleotide probes were used for labeling soil bacteria (Table 1): EUB338 probe (5'-gctgcctcccgtaggagt-3') (Amann et al., 1990) for all eubacteria; LowGC probe (5'-tgtagcccargcata-3') (Meier et al., 1999) for the low guanine + cytosine content gram-positive bacteria group (low-GC bacteria)(Macrae et al., 2001); HiGC probe (5'-tat agt tac cac cgc gt-3') for the high DNA guanine + cytosine content gram-positive bacteria group (high-GC bacteria) (Roller et al., 1994); Pseudo probe (5'-act gca tcc aaa act ggc aa -3') for *Pseudomonas* spp. (Duteau et al., 1998); and Clost I probe (5'-ttc ttc cta atc tct acg ca -3') for *Clostridium* spp. (Kusel et al., 1999).

A Pseudo-probe was applied because *Pseudomonas* spp. sometimes exist in large quantities in the rhizosphere (Lochhead and Chase 1943) and because *Pseudomonas* spp. have been recognized as plant-growth promoting rhizosphere bacteria (PGPR; Kloepper et al.1980). Gram stain in a pre-experiment indicated the presence of numerous gram-positive bacteria in both lowland and upland soil samples. Therefore, LowGC and HiGC probes were applied. In addition, Clost I probe was applied to detect *Clostridium* spp., which belong among low-GC bacteria because *Clostridium* spp. were reported as major gram-positive soil bacteria in lowland and upland fields (Singleton et al.1996). Gram-positive bacteria such as *Clostridium* spp. form endospores (Sneath 1986). Therefore, cell permeability for oligonucleotide probe is inferior (Ohnishi et al. 2003). For that reason, we performed 15-min pretreatment using lysozyme to lysis peptideglucan to improve cell permeability.

The protocol described by Amann et al. (1995) for detection of marine bacteria was applied to the in-situ hybridization procedure. First, a 10-μL fixed sample was mounted on slide glass using a micropipettor. It was them prehybridized in 5×SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent, and formamide for 15-min at 45°C (Bach et al. 2001). After removal of the prehybridization solution, 10 μL hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate) was applied to the sample. A couple of 5 ng of fluorescence EUB338 and a specific probe (e.g. LowGC) were applied to the buffer. Hybridization was carried out at 46°C for 3 h. The stringency of hybridization was adjusted by adding formamide to the hybridization buffer 30 % (vol/vol) (Sekiguchi et al. 1999). Non-binded probes were rinsed out by incubation with the hybridization buffer at 48°C for 20 min.

To enumerate all microorganisms in the samples, the cells were stained with 4', 6'-diamidino-2-phenylindole (DAPI) (Yamaguchi et al.2004) at the final concentration of 5 μg μL^{-1}. Soil bacterial cells immobilized and hybridized on glass slides were observed using a fluorescence microscope (BX60; Olympus Optical Co. Ltd.). The percentage of the EUB338-labeled area in the view was measured using computer image analysis (Scion-Image); that percentage was used as an index of the relative amount of active bacteria. The degree of dominance

Table 1. 16S and 23S rRNA oligonucleotide probes used for hybridization.

Probe	Target site*	Specificity	Probe sequence (5'→3')	Reference
EUB338	338 16S rRNA	Eubacteria	gct gcc tcc cgt agg agt	Amann et al.,1990
LowGC	344 16S rRNA	Low GC content gram positive bacteria group	tgt agc cca rgc ata	Meier et al.,1999
HiGC	1901 23S rRNA	High GC content gram positive bacteria group, Actinobacteria group	tat agt tac cac cgc gt	Roller et al.,1994
Pseudo	1467 23S rRNA	*Pseudomonas* spp.	att tca gcc tac cac ctt aa	DuTeau et al.,1998
Clost I	696 16S rRNA	*Clostridium* spp.**	ttc ttc cta atc tct acg ca	Kusel et al.,1999

* *Escherichia coli* numbering (Brosius et al., 1981).
*******Clostridium* spp. belong to low GC content gram positive bacteria group.

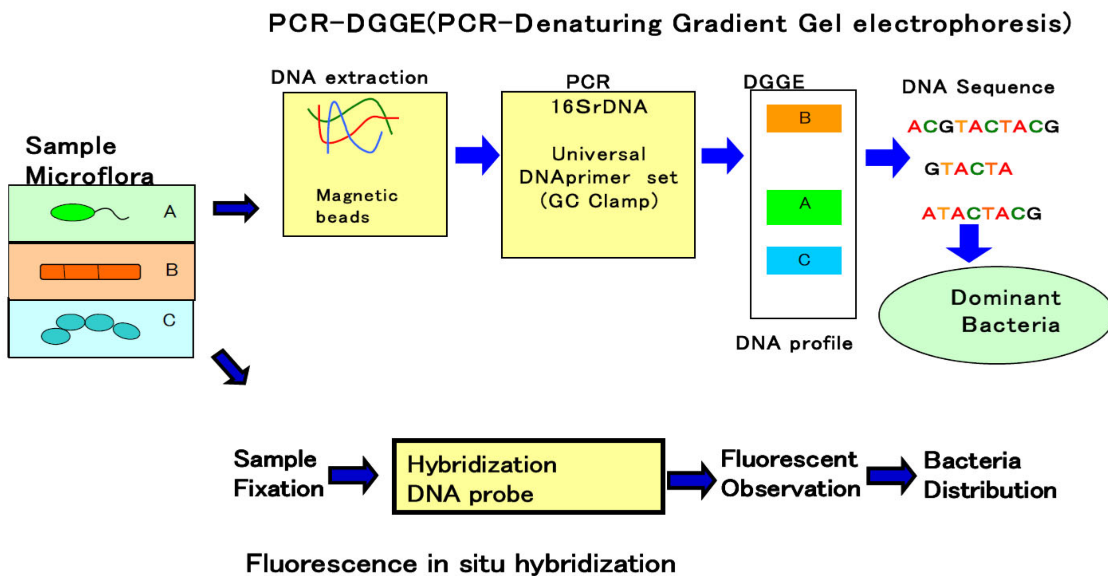

Fig. 1. Procedure of molecular biological methods for analysis of rhizosphere bacteria in the present study.

of bacteria revealed using LowGC, HiGC, ClostI and Pseudo was evaluated through comparison of individual-probe-labeled areas with the EUB338-labeled area in the microscopic view.

Culture method examination

Culture method was conducted to evaluate the microorganism populations. Extracts from fresh rhizosphere soil samples were cultivated on nutritional agars and DNB (for oligotrophic bacteria): DNB is meat extract medium diluted 100 times, which is suitable for growth of the environment microorganisms (Hattori 1976). It comprises the following: 0.1 g meat extract, 0.1 g peptone, 0.05 g NaCl, 1 L H_2O. All cultivations were carried out at 37°C in a Petri dish containing 20 mL of medium pH 7.2 in the incubator. The colonies were counted after 24-hour incubation at 37°C to calculate the population.

Results and Discussion

PCR-DGGE

Using agarose-gel electrophoresis, PCR amplification of the target sequence in 16S rDNA from extracted soil microbial DNA was confirmed. Bead-beating and spin column pretreatments extracted and purified the DNA sufficiently.

Band patterns of 16S rDNA in the PCR-DGGE profiles from the lowland and upland fields differed. Rhizosphere microorganisms of the upland field (U1, U2 in Fig. 2) exhibited more DNA bands than those of the lowland field (L1, L2 in Fig. 2). Moreover, unique DNA bands were obtained that were respectively specific to lowland and upland fields (A–D in Fig. 2). Different DNA band patterns by PCR-DGGE suggest different compositions of microorganisms (Ikenega et al. 2002). The band patterns obtained in this study suggest that different species of microorganisms exist in the respective rice rhizospheres of lowland and upland fields. In addition, band patterns of 16S rDNA in the lowland fields differed among samples, even among those from the same plot (L1 and L2 in Fig. 2). The difference indicates that microorganisms in the rhizosphere could differ depending on the parts of a whole root system (for example, young or aged roots) and/or the variation of microenvironments in soil.

Sequential analyses indicated that unique bands A and B from the lowland field and C, D from the upland field (Fig. 2) were derived respectively from the bacterial species Bacterium A35 (A), *Clostridium bifermentans* (B), *Klebsiella planticola* (C), and *Bacillus fusiformis* (D). The unique species in each of upland and lowland field detected using PCR-DGGE and DNA sequencing are consistent with knowledge from previous reports related to the four bacterial groups when considering soil conditions in lowland and upland fields. Namely, members of the genus *Clostridium* are gram-positive strictly anaerobic motile rods found in soil, water, and the intestinal tracts of humans and other animals, all of which are anaerobic spore formers in soil (Black 1993). On the other hand, the PCR-DGGE result indicated that *K. planticola* and *B. fusiformis* were dominant bacteria species of the rice rhizosphere in upland fields. In fact, *K. planticola* are gram-positive rods found in soil and plants (Singleton et al., 1996). *Bacillus* are gram-positive, aerobic or facultative aerobic en-

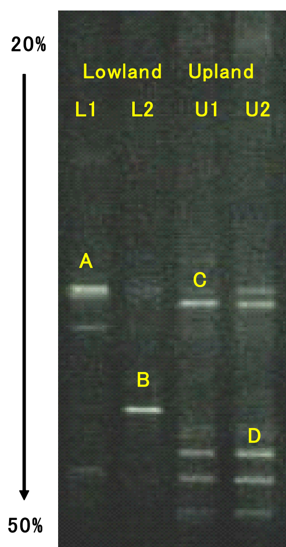

Fig. 2. 16S rDNA PCR-DGGE profile. Polyacrylamide gel had a 20–50% denaturation gradient. A–D indicate the bands of unique dominant bacteria in the respective samples.

dospore-forming, chemoorganotrophic bacilli with meretricious flagella; they are ubiquitous in soil. In addition, they are resistant to desiccation and ultraviolet light (Singleton et al., 1996, Stetzenbach et al., 2004). *Bacillus* bacteria are distinguishable by the location of their spores and by growth temperatures (Black, 1993). Detection of anaerobic *Clostridium bifermentans* and aerobic *Bacillus fusiformis* is supported also by the fact that lowland soil is in a reductive environment and upland soil is an aerobic and dry environment.

FISH

Figure 3 shows fluorescent photographs of rhizosphere microorganisms separated from soil samples. Separation of bacteria from soil particles using Percoll-density gradient solution as a pretreatment improved the fluorescent microscopy. This pretreatment obviated auto fluorescent analysis of soil particles, which disturbs the detection of hybridized bacteria (compare Fig. 3a and Fig. 3c). Watt et al. (2006 a, b, c) succeeded to observe bacteria on the root surfaces (epidermis) by FISH. They used limited wave length (633 mm) of confocal laser microscope in combination with specific dye (Cy5, Cy5.5) for labeling DNA probes to avoid autofluorescence of soil and roots (Watt et al. 2006a). The pretreatment using Percoll solution in the present study enabled the use of various fluorescent dyes (e.g., FITC, Cy3) and fluorescent microscopes for FISH analysis on the bacteria in the rhizosphere soil.

Areas labeled by EUB338 and LowGC in the fluorescent view were larger in upland rice samples

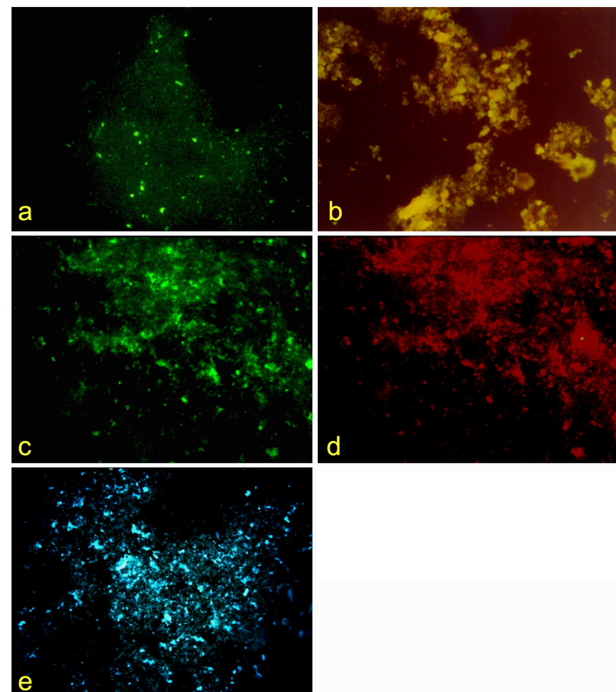

Fig. 3. Fluorescent photographs of rhizosphere bacteria labeled using FISH. a, Whole eubacteria hybridized using EUB338-FITC probe (lowland field). b, Whole eubacteria hybridized using EUB338-FITC probe without pretreatment by Percoll solution. Autofluorescence of soil particles disturbed the observation of FITC labels of eubacteria (lowland field). c, Whole eubacteria hybridized using EUB338-FITC probe (upland field). d, gram-positive low GC bacteria hybridized using LowGC-Cye3 probe (upland field). e, 4’, 6’-diamidino-2-phenylindole (DAPI) staining of whole microorganisms (upland field).

than those in lowland rice samples, suggesting larger populations of eubacteria and gram-positive bacteria with low GC in the upland rice rhizosphere. Moreover, because the distribution of EUB338 and LowGC almost overlapped, gram-positive low-GC bacteria were dominant among the eubacteria in both upland and lowland fields. In our previous study on garbage, *Bacillus* spp. and *Clostridium* spp. in garbage were labeled by FISH using the LowGC probe (Doi et al. 2006). The results of sequencing of the PCR-DGGE dominant bands in the present study also support the dominance of these two groups in the rice rhizosphere. The two groups are rod-shaped bacilli. Numerous rod-shaped bacilli were detected through microcopy of DAPI-stained samples of both lowland and upland rice.

Figure 4 shows the ratio of individual probe-labeled areas to EUB338-labeled area as an index of dominance of individual groups of bacteria in rhizosphere. The low-GC bacteria were dominant in both fields. *Clostridium* spp. detected by Clost I probe accounted for many of the low-GC bacteria in the lowland field, whereas *Clostridium* spp. was

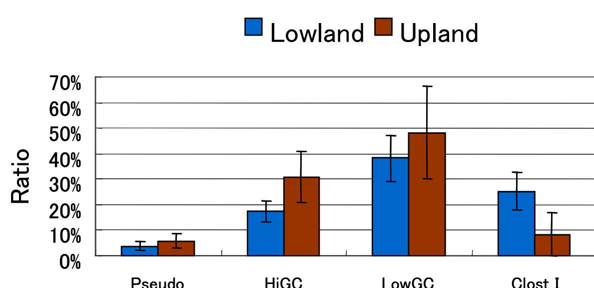

Fig. 4. The ratio of the labeled area by individual probes to the area labeled by EUB338, as a relative index of dominance of the individual bacterial groups among the whole eubacteria community of the rice rhizosphere. Pseudo, *Pseudomonas* spp.; HiGC, high DNA G + C content gram positive bacteria group (Actinobacteria group); LowCG, low DNA G + C content gram positive bacteria group; Clost I, *Clostridium* sp. *Clostridium* spp. belongs to the low GC content gram positive bacteria group. The vertical error bars indicate standard errors.

present only to a minor degree in the upland field, which supports the result of PCR-DGGE and sequencing. Another group of gram-positive bacteria, high GC, showed non-negligible status in rhizosphere flora, particularly in the upland field. The ratio of one gram-negative bacterium, *Pseudomonas* spp., was very low in both the lowland and upland fields.

Culture method examination

In the result of culture method examination, colonies were approximately 1.5 times more numerous in upland samples (9.6×10^6 CFU g^{-1} rhizosphere soil) than in lowland samples (6.6×10^6 CFU).

It should be considered that aerobic conditions during incubation might have suppressed the growth of anaerobic bacteria collected from lowland rhizosphere soil. However, this result of the culture method examination is consistent with the result of FISH, which suggested a larger population of rhizosphere eubacteria in the upland field than in the lowland field.

Conclusions

This study of microorganisms using two new molecular biological methods with modified pretreatments showed that rice grown in an upland field holds a larger population and diversity of rhizosphere bacteria than rice grown in a lowland field. The gram-positive low-GC bacteria were dominant in both fields; the species identified using PCR-DGGE and sequencing respectively reflected the soil water conditions of upland and lowland fields. More studies are required to generalize the results of this study as the difference between rice rhizospheres of upland and lowland fields; comparisons between the two ecosystems at different growth stages of rice and for different rice cultivars and managements are desirable. However, results of this study, which were consistent with the result of culture method and former knowledge, indicate the reliability of the molecular biological methods with adequate pretreatments in the study of rhizosphere microorganisms in agricultural fields. In addition, the methods are more rapid than incubation examination, and require no highly proficient knowledge such as that of conventional microscopic observation to identify the species. Further improvement of fluorescence image processing and combination with some biochemical method such as Quinone profile method (Fujie et al. 1998, Fujita et al. 2004) might facilitate sufficient quantitative analyses of biomass for the study of rhizosphere icroorganisms.

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References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA 1990 Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56: 1919-1925.

Amann RI, Ludwig W, Scheifer KH 1995 Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial Rev. 59: 143-169.

Bach HJ, Hartmann A, Schloter M, Munch JC 2001 PCR primers and functional probes for amplification and detection of bacterial genes for extracellular peptidases in single strains and in soil. J. Microbiol. Methods 44: 173-182.

Black JG, 1993 Microbiology: principles and applications. 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, NJ, USA, pp. 80-83, pp. 248-251.

Brosius J, Dul TJ, Sleeter DD, Noller HF 1981 Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. 148: 107-127.

Clayton SJ, Clegg CD, Murrary PJ, Gregory PJ 2001 Impact of defoliation on bacteria community structure in the rhizosphere. Root Res.(Extra issue 1): 408-409.

Colwell RR, Grimes DJ. 2000 Nonculturable Microorganisms in the Environment. 2000 ASM Press, Washington DC,USA.

Delong EF 1989 Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. Science 243: 1360-1363.

Doi T, Abe J, Zhang B, Morita S 2001 Application of

fluorescent *in situ* hybridization(FISH) for observation of rhizosphere microorganisms. Root Res.(Extra issue 1): 430-431.

Doi T, Matsumoto H, Ohshita N, Takemoto Y, Shinada T 2006 Microflora analysis on hydrogen fermentation of feedstock wastes. J. Water Waste 48: 784-790. (In Japanese)

DuTeau NM, Rogers JD, Bartholomay CT, Reardon KF 1998 Species-specific oligonucleotides for enumeration of *Pseudomonas putida* F1, *Burkholderia* sp. strain JS150, and *Bacillus subtilis* ATCC 7003 in biodegradation experiments. Appl. Environ. Microbiol. 64: 4994-4999.

Eastman A , Peterson RL 1985 Root apex structure in *Regenellidium diphyllum* (Marsileaceae). Bot. Gaz. 146: 44-45.

Frischer M, Danforth J, Healy M, Saunders F 2000 Whole-cell versus total RNA extraction for analysis of microbial community structure with 16S rRNA-targeted oligonucleotide probes in salt marsh sediments. Appl. Environ. Microbiol. 66: 3037-3043.

Fujie K, Hu H, Tanaka H, Urano, K, Saito K, Katayama A 1998 Analysis of respiratory quinines in soil for characterization of micro biota. Soil Sci. Plant Nutr. 44: 393-404.

Fujita M, Ike M 2006 Bio-environmental engineering. CMC Press, Tokyo, pp. 89-99. (in Japanese)

Fujita M, Furumai A, Nakajima F 2004 Quinone profile analysis of activated sludge during start-up phase in anaerobic/oxic sequencing batch reactor. J. Jpn. Soc. Water Environ. 27: 189-194. (in Japanese with English abstract)

Hattori T 1976 Plate count of bacteria in soil on a diluted nutrient broth as a culture medium. Rep. Inst. Agr. Res. Tohoku Univ. 27: 23-30.

Hoshino YT, Matsumoto N 2004 An improved DNA extraction method using skim milk from soils that strongly absorb DNA. Microbes Environ.19: 13-19.

Igarashi Y, 2001 Utilization of microbial communities for recycling of organic resources. J. Brew. Soc. Jpn. 96: 374-378.

Ikenaga M, Asakawa S, Muraoka Y, Kimura M 2004 Community structure of Eukaryotes in rice roots estimated by PCR-DGGE pattern and sequence analyses. Soil Sci. Plant Nutr. 50: 583-589.

Ishii K, Takii, S Fukunaga S, Aoki K 2000 Characterization by denaturing gradient gel electrophoresis of bacterial communities in deep groundwater at the Kamaishi Mine. Japan. J. Gen. Appl. Microbial. 46: 85-93.

Jenny H, Grossenbacher K 1963 Root-soil boundary zone as seen in the electron microscope. Proc. Soc. Soil Sci. Am. 27: 273-277.

Jimenez MB, Flores SA, Zapata EV, Campos EP, Bouquelet S, Zenteno E 2003 Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant Soil 249: 271-277.

Kato Y, Kamoshita A, Yamagishi J, Abe J 2006 Growth of three rice (*Oryza sativa* L.) cultivars under upland conditions with different levels of water supply. 1. Nitrogen content and dry matter production. Plant Prod. Sci. 9: 422-434.

Kimura M 1994 Rhizosphere microorganisms. In: Morita S, Abe J Eds., Root Handbook. JSRR, Tokyo, pp. 173-174. (In Japanese Title translated by authors).

Klopper JW, Leong J, Teintze M, Schroth MN 1980 Enhanced plant growth by siderophores produced by plant growth -promoting rhizobacteria. Nature 286: 885-886.

Kurisu F, Satoh H, Mino T, Matsuo T 2000 Microbial community analysis of thermophilic contact oxidation process by using PCR-DGGE method. Proceedings of the International Symposium of the COE project on Establishment and Evaluation of Advanced Water Treatment Technology Systems Using Function of Complex Microbial Community (Tokyo, 2000). pp 60-69.

Kusel K, Pinkart H, Drake L, Devereux R 1999 Acetogenic and sulfate-reducing-bacteria inhabiting the rhizosphere and deep cortex cells of the sea grass *Halodule wrightii*. Appl. Environ. Microbiol.65: 5117-5123.

Lochhead AG, Chase,FE 1943 Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. Soil Sci. 55: 185-195.

Macrae A, Lucon C, Rimmer D, Donnell A 2001 Sampling DNA from rhizosphere of *Brassica napus* to investigate rhizosphere community structure. Plant Soil 233: 223-230.

Meier H, Amann R, Ludwig W, Schleifer KH 1999 Oligonucleotide probe for in situ detection of a major group of gram-positive bacteria with low DNA G+C content system. Appl. Microbiol. 22: 186-195.

Morita S 2000 Root growth and root system development. University of Tokyo Press, Tokyo, pp. 13-21. (in Japanese)

Muyzer G, de Waal EC, Uitterlinden AG 1993 Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59: 695-700.

Muyzer G, Smalla K 1998 Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73: 127-141.

Nakamoto T 2004 Rhizosphere research in agro-ecosystem. In: AE-Bio Ed., Experiments in agricultural and environmental biology. Asakura Shoten, Tokyo, pp. 150-159. (in Japanese)

Nielsen PH, Andreasen K, Lee N, Wagner M 1999 Use of microautoradiography and fluorescent for characterization of microbial activity in activity in activation sludge. Wat. Sci. Tech. 39: 1-9.

Ohba M, LiY, Noike T 2006 Analysis of microbial community in two-phase circulating process for hydrogen and methane fermentation, J. Jpn. Soc. Water Environ. 29: 399-406. (in Japanese with English abstract)

Ohnishi A, Fujimoto N, Suzuki M 2003 Development of technique that detect to thermophilic bacteria in compost applied the FISH method. Environ. Conserv. Eng. 32: 976-985. (in Japanese with English abstract)

Ohnishi A, Fujimoto N, Suzuki M 2004 Analysis of initial microflora in garbage-composting process by cultivation and molecular biological approaches. Environ. Conserv. Eng. 33: 620-630. (in Japanese with English abstract)

Okabe S 2005 Molecular approaches to understanding the structure and function of complex microbial communities. J. Jpn. Soc. Water Environ. 28: 460-465. (in Japanese with English abstract)

Pallud C, Viallard V, Balandreau J, Normand P, Grundmann G 2001 Combined use of a specific probe and PCAT medium to study *Burkholderia* in soil. J. Microbiol. Methods. 47: 25-34.

Plumb JJ, Bell J, Stuckey DC 2001 Microbial populations associated with treatment of an industrial dye effluent in an anaerobic baffled reactor. Appl. Environ. Microbiol. 67: 3226-3235.

Ritchie N, Schutter M, Dick R, Myrold D 2000 Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Appl. Environ. Microbiol. 66: 1668-1675.

Roller C, Wagner M, Amann R, Ludwig W, Schleifer KH 1994 *In situ* probing of gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. Microbiology 140: 2849-2858.

Rovira A D 1969 Plant root exudates. Bot. Rev. 35: 35-57.

Rovira AD, Davey CB 1974 Biology of the rhizosphere. In: Carson EW Ed., The plant root and its environment. University Press of Virginia, Charlottesville, VA, USA, pp. 153-204.

Russell RS 1981 Plant root systems: their function and interaction with the soil. pp.151-187.

Sakai M, Matsuka A, Komura T, Kanazawa S 2004 Application of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant root. J. Microbiol. Methods. 59: 81-89.

Sekiguchi Y, Ohshi A, Harada H 1999 Fluorescence in situ hybridization using 16Sr RNA-targeted oligonucleotidies reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. 65: 1280-1288.

Siciliano SD, Germida JJ 1998 Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. Environ. Rev. 6: 65-79.

Singleton P, Sainsbury D 1996 Dictionary of microbiology and molecular biology. John Wiley & Sons, New York.

Sneath PHA 1986 Endospore-forming gram-positive rods and cocci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG Eds., Bergey's manual of systematic bacteriology, vol 2. Williams & Wilkins, Baltimore, MD, USA. pp. 1104-1139.

Syutsubo K 2005 Microbial Community Structure analysis of crude oil-degrading consortia. J. Jpn. Soc. Water Environ. 28: 466-469. (in Japanese with English abstract)

Tani K, Yamaguchi N, Nasu M 1997 Rapid detection of specific microbial cells by fluorescent staining techniques. J. Jpn. Toxicol. Environ. Health. 43: 145-154. (in Japanese with English abstract)

Thies JF 2007 Molecular methods for studying soil ecology. In: Paul EA Ed., Soil microbiology, ecology and biochemistry. 3rd ed. Elsevier, Oxford. pp. 85-118.

Watt M, Hugenholtz P, White R, Vinall K 2006a Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence *in situ* hybridization (FISH). Environ. Microbiol. 8: 871-884.

Watt M, Kirkegaard JA, Passioura J 2006b Rhizosphere biology and crop productivity– a review. Aust. J. Soil Res. 44: 299-317.

Watt M, Silk W, Passioura J 2006c Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. Ann. Bot. 97: 839-855.

Yamagishi J, Nakamoto T, Richner W 2003 Stability and variability of wheat and maize biomass in a small field managed under two contrasting tillage systems over 3 years. Field Crops Res.81: 95-108.

Yamaguchi N, Kenzaka T, Nasu M 1997 Rapid in situ enumeration of physiologically active bacteria in river waters using fluorescent probes. Microbes Environ. 12: 1-8.

Zelles L, Bai QY, Beck T, Beese F 1992 Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biol. Biochem. 24: 317-323.

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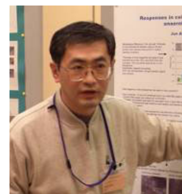

Dr. Jun Abe studies root morphology of crop plants from the eco-physiological viewpoints for the improvement of stress tolerance. His body has shown secondary thickening like a dicot root by middle-age spread in these several years.

Prof. Shigenori Morita is an expert of developmental morphology of rice and cereal plants, and the founder of the Japanese Society for Root Research (JSRR). He recently takes a leadership role in the use of whole-crop rice for bioethanol production in Japan.