Original research article

Study on rhizosphere bacterial community in lowland rice grown with organic fertilizers by using PCR-denaturing gradient gel electrophoresis

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Received on March 14, 2010; Accepted on December 7, 2010

Abstract: For the promotion of environment-friendly agriculture, use of organic fertilizers and green materials is increasingly attempted in rice farming. Although effects of organic fertilizers on soil bacteria in the rhizosphere can differ from those in non-rhizosphere soil, microbiological studies that specifically address the rice rhizosphere still limited. This study was undertaken to investigate the impact of organic fertilizers on soil bacteria communities through comparison of rhizosphere soils and bulk soils. Effects of soil types and seasonal change were also analyzed. Rice plants (*Oryza sativa* L. cv. Nipponbare) were cultivated in a lowland paddy field of Andosol soil. Applications of compost and rice bran in combination with chemical fertilizer were compared with control soil (chemical fertilizer only). Soil 16S rDNA extracted from rhizosphere soil collected using ultrasonic treatment of rice roots and from bulk soils were analyzed using PCR-denaturing gradient gel electrophoresis (DGGE). Principal component analysis based on PCR-DGGE profiles revealed clear differences in the community structures of soil bacteria between rhizosphere and bulk soils. Furthermore, rhizosphere bacterial community structures of compost and rice bran treatments were plainly different from that of control, and changed with the seasons. The organic fertilizers showed pronounced effects on bacterial communities until mid-summer, but small effects in autumn. Results of this study suggest that the rhizosphere microorganisms in paddy fields can be modified through organic fertilizer management. Moreover, effects of organic fertilizer application, soil type, and phenology on soil bacteria appear depending on interaction with the rice rhizosphere effects in paddy fields.

Keywords: bacterial community structure, compost, paddy field, rhizosphere effect, rice bran, soil reduction

Introduction

Rice plants are mainly grown in lowland paddy fields, where soil is characterized by remarkable soil reduction because of flooding (Kimura et al. 1983, Liesack et al. 2000). The community structure of microorganism in submerged paddy soil differs from that in upland soil because of the anaerobic environment; facultative and strict anaerobic fungi, bacteria (e.g., *Clostridium* spp., *Streptococcus* spp., *Staphylococcus* spp.), and archaea (e.g. methanogenic archaea) are often dominant in submerged paddy soil (Takeda and Furusaka 1970, Fetzer et al. 1993, Großkopf et al. 1998, Liesack et al. 2000, Weber et al. 2001).

The rhizosphere is surrounding the plant root surface and being affected by plant root activities. Plant roots secrete mucigel (Jenny and Grossen-bacher 1963) to supply carbohydrate sources to soil microorganisms (Jimenez et al. 2003). Rice roots also provide polysaccharides, amino acids and organic acids (Kimura 1977, 1983), as do upland plants (Rovira 1969). As a result, the community structures of soil microorganisms in the rice rhizosphere are expected to differ greatly from that in bulk soil (non-rhizosphere soil) of the same paddy field. Agar culture tests from pot experiments suggest that rice roots can show specific rhizosphere effects on both aerobic and anaerobic soil microbes (Kimura 1979, 1983). On the other hand, rhizosphere microorganisms, bacteria in particular, strongly influence plant growth

Doi T, Abe J, Shiotsu F, Morita S 2011 Study on rhizosphere bacterial community in lowland rice grown with organic fertilizers by using PCR-denaturing gradient gel electrophoresis. Plant Root 5:5-16. doi:10.3117/plantroot.5.5

through enhancement of nutrient uptake after decomposition of soil organic matters, competition for nutrients, and soil-borne diseases (Russell 1981, Matsuguchi 1986). Therefore, microbiological studies particularly addressing the rhizosphere are necessary to understand the rice and soil microbe interrelationship in a paddy field. We developed a method to separate rhizosphere soil that adheres to soil surface from non-rhizosphere soil with using an ultrasonic washer (Doi et al. 2007). This method enables us to study features of rhizosphere soil exactly. Furthermore, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is a method to compare different bacterial communities including 'viable but non-culturable' (VBNC) bacteria (Muyzer 1993). Difference in DNA sequence among bacterial species in a soil sample results in different DNA bands in PCR-DGGE. Thus, bacterial communities of different soil samples can be compared − which soil sample has more number of bacterial species; how many common bacterial species the soil samples have. Although sequencing of DNA bands detected by PCR-DGGE cannot identify bacteria species in details due to short length of DNA sequences amplified, it would be an effective tool for approximate evaluations − difference/similarity between rhizosphere and bulk soils, seasonal changes, etc.

Soil in farmers' paddy fields often contains rich organic matter such as old stubble, senescent roots, rice straw, and manure; various bacteria in soil contribute to decompose soil organic matter. Application of organic materials to soil and water in paddy fields can affect the bacterial community strongly. The total population of soil bacteria in paddy fields is increased by application of compost and rice straw (Kogawa et al. 1984). Kimura et al. (2002, 2006) reported a significant difference in the community structure of microbiota in flooded water among three different fertilizer managements: no fertilizer, chemical fertilizer, and compost. However, impacts of organic matter application on rhizosphere effects remain unknown, although the effects of organic material on bacterial communities in rhizosphere and bulk soils might differ.

Green materials are applied in rice farming for environment-friendly agriculture. Application of rice bran onto the surface of flooding water is a recent technique that is spreading among rice farmers in Japan. Rice bran is a by-product of the rice milling process of converting brown rice to white rice. Rice bran is effective not only as an organic fertilizer but as a green material for weed control. Decomposition of rice bran in the surface water of paddy fields suppresses the germination and growth of paddy weeds by enhancement of both hypoxia in water and reduction in soil, and by release of inhibitory organic acids such as isobutyric acid and acetic acid (Sousa et al. 2002, Ueno and Suzuki 2005). The components of rice bran are different from those of straw compost. Rice bran includes less than 10% fiber, and contains ca. 40% carbohydrates, 20% lipid and 10% protein/amino acids (Namba and Shibata 1985). Although such specific components may cause different effects on soil microorganisms, the impact of rice bran application on the community structure of soil bacteria in submerged paddy soil remains unknown.

In this study, we investigated the effects of organic matter application on the community structure of soil bacteria in a rice paddy field in relation to rhizosphere effects. Similarity and difference of the bacterial communities were evaluated in the rhizosphere and bulk (non-rhizosphere) soils applied with chemical fertilizer, rice straw compost (a traditional organic fertilizer), and rice bran (a recently used organic fertilizer) using PCR-DGGE method. Soil type of the paddy field examined in this study was Andosol. Since alluvion clay soil is more popular than Andosol in Japan, we also examined if the soil types may differ the effects of organic matters on soil bacterial community in a pot experiment.

Materials and Methods

Field experiment

The field experiment was performed in a lowland paddy field at the Field Production Science Center of the University of Tokyo in Nishitokyo, Japan (35°43′N, 139°32′E). The soil was volcanic ash soil (so-called Kanto loam) which the topsoil layer of ca. 0.35 m was a dark, humic silty loam (Andosol) and the subsoil layer (0.35 m and below) was a red-brown silty clay loam (Yamagishi et al. 2003). Three fertilizer treatments include “compost” (chemical fertilizer and compost; “M” in the figures), “rice bran” (chemical fertilizer and rice bran (*nuka* in Japanese); “N” in the figures), and “chemical fertilizer” (chemical fertilizer only; “F” in the figures). The three treatments with two replications (total six plots) were arranged by randomized design; individual plots were 3 m × 3m segmented by PVC corrugated plate (0.2 m deep in soil and 0.2 m high); intervals between plots were 1 m wide. On June 4, 2008, 28-day-old seedlings of rice (*Oryza sativa* L. cv. Nipponbare) were transplanted manually. The planting distance was 300 mm × 150 mm (planting density 22.2 hills m^{-2}); two seedlings were planted in each hill. A row was kept empty in each plot for the collection of bulk soil.

All the plots were fertilized with chemical fertilizer by basal dressing (N, 10; P_2O_5, 15; K_2O, 13 g m^{-2}) and top dressing (N: 0.5 g m^{-2}; August 8). Mixed chemical fertilizer (Kumiai Hukugo A907, Katakura

Chikkarin Co., Ltd; consisting of ammonium sulfate, calcium superphosphate and potassium chloride) was used for basal dressing and ammonium sulfate for top dressing, respectively. In addition, the compost (wheat straw compost with horse manure; C/N ratio 35) was applied into the soil at rate of 375 g m^{-2} (N, 5; P_2O_5, 3; K_2O, 8 g m^{-2}) in compost treatment. Rice bran (carbon, 45%; nitrogen, 2.5%) was disseminated to the surface of paddy field water at a rate of 45.5 g m^{-2} immediately after transplanting and 22.7 g m^{-2} in mid-August, respectively, in rice bran treatment. All plots were flooded (ca. 50–100 mm) during June–September 26; panicles emerged late August and matured in early October. Three rice hills were taken in each plot to measure plant height and shoot dry weight and to collect rhizosphere soil once a month during July−November (July 8, August 11, September 12, October 10 and November 8). A metallic cylinder (150 mm diameter) was used to sample the crown of rice with a root system in the top soil layer of Andosol, followed by ultrasonic treatment to collect rhizosphere soil. Bulk soil was collected from the topsoil layer in the empty row (300 mm distant from the neighboring rice rows) in each plot on the same days as the plant sampling.

As chemical properties of paddy soil during the experiment, redox potential (Eh) and pH of soil were monitored once a month during July− November. Soil Eh was measured using a compact Eh meter (PRN-41; Fujiwara Scientific Company Co. Ltd.). Platinum Eh electrodes were inserted approximately 0.1 m deep into the soil ca. 0.1 m away from a rice hill (rooting zone Eh) and in the non-planting row (bulk soil Eh).

Pot experiment

A pot experiment was conducted at the greenhouse in the Field Production Science Center of the University of Tokyo during July–October in 2008. Soils of two different types, Andosol and Arakida soil, were used. Arakida soil is an alluvial clay soil with water-retentive and argilliferous features (Oda and Yukimoto 1975, Ohnishi and Ishizaki 2008). In the pot experiment, three plots treated with “compost” (M), “rice bran” (N), and “chemical fertilizer” (F) corresponding to the field experiment. Wagner pots (1/50 m^2 and 150 mm deep; ca. 3 L soil) were filled with soils that had been mixed with chemical fertilizer: N, 0.6; P_2O_5, 0.9; K_2O, 0.8 g per pot by mixed fertilizer (Kumiai Hukugo A907; see "*Field experiment*" for details). Later, the same compost as that used in the field experiment was applied to the soil (8 g per pot) in the compost treatment (M), and rice bran was broadcast to the soil surface (15 g per pot) in the rice bran treatment (N). Thirty-day-old seedlings of rice (*Oryza sativa* L. cv. Nipponbare) were transplanted to the pots (two seedlings per pot). Another set of pots that contained soils that were similarly fertilized and treated were prepared for the collection of bulk soil, where rice was not planted, but soil was managed identically. The pots of same soil type and treatment were placed in a large plastic pool (length 1.2 m, wide 0.8 m) and flooded ca. 30−50 mm above the soil surface until the end of September. The plastic pools were rotated in the greenhouse once a month to unify the conditions among the soils and treatments. Light and temperature were not controlled during the experiment. Nipponbare is a photosensitive variety. Therefore, the flowering date was similar to that in the field experiment (late August). Soils were sampled from both the rhizosphere and bulk soil once a month (July 18, August 15, September 16, October 13) for analysis of bacterial communities. Namely, rhizosphere soil of whole root system of three rice hills (i.e. three planted pots) and bulk soil of three non-planted pots were collected every month.

Collection of rhizosphere soil

Rhizosphere soil had been collected by rapid manual washing of roots (Nakamoto 1999). We improved the method using an ultrasonic washer and phosphate buffered saline (PBS) for stable treatment to separate rhizosphere soil from roots and non-rhizosphere soils. Non-rhizosphere soil was removed using 3 min ultrasonic treatment; then soil particles adhering to the roots (i.e. rhizosphere soil) were separated from the roots using another ultrasonic treatment for 3 min, with subsequent collection by centrifugation (Doi et al. 2007). Bulk soil samples were taken from the non-planting rows of respective plots in the field experiment and the non-planting pots in the pot experiment. Soil samples were used for PCR-DGGE analysis and anaerobic culture examination.

Analysis of soil bacterial community structure using PCR-DGGE

DNA of bacteria was extracted from the soil for PCR-DGGE analysis. Sample soil was fixed preliminarily using 4%-paraformic aldehyde and kept at 4°C in a refrigerator for preventing phenotypical change (Nielsen et al. 1999). Skimmed milk was added to soil samples to prevent adsorption of extracted bacterial DNA by soil particles, as recommended by Takada-Hoshino and Matsumoto (2004). The DNA was extracted from each sample using a kit for PCR-DGGE (ISOIL for Beads Beating; Nippon Gene Co. Ltd.); 0.5 g of soil was added to a 2 mL tube, then disrupted at 5,500 rpm for 30 s using a bead shocker (Micro Smash; Tomy Seiko Co. Ltd.). The

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. They were purified using a spin column (GFX TM kit; Amersham Pharmacia Biotech Inc.) (Ritchie et al. 2000). Such purified DNA was used as the PCR template for analysis of the bacterial community structure. 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. Subsequently, PCR was conducted with 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. 1999). The PCR products were electrophoresed in 2% agarose gel to verify that the 16S rDNA had been amplified. The PCR products were analyzed using DGGE (D-Code system; Bio-Rad Laboratories Inc.) on a polyacrylamide gel with a 30–60% denaturation gradient of urea–formamide (Kurisu et al. 1999) under a 120 V field for 60°C 4 h (Clayton et al. 2001). The polyacrylamide gel was stained (SYBER Green I; Molecular Probes Inc.) in a dark room for 20 min; the 16S rDNA profile was verified and photographed. After intensely stained DGGE bands were excised from the gels, the 16S rDNAs were purified and amplified using a QIA quick PCR Purification Kit (Qiagen Inc., Hilden, Germany). The purified PCR 16S rDNAs were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and DNA sequencer (ABI Prism 3100; Applied Biosystems).

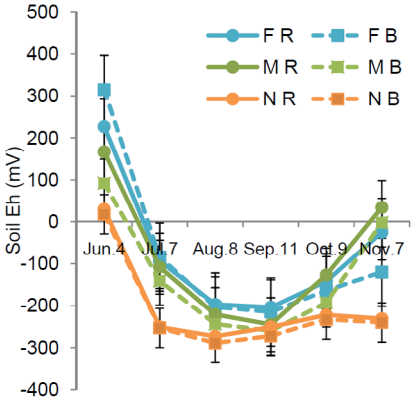

Fig. 1. Redox potential (Eh) of rooting zone and bulk soil in the field experiment: FR, chemical fertilizer/rooting zone; FB, chemical fertilizer/bulk soil; MR, compost/rooting zone; MB, compost/bulk soil NR, rice bran/rooting zone; NB, rice bran/bulk soil. The vertical bars indicate standard errors.

Detected 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 (www.ncbi.nlm.nih.gov/BLAST/).

The community structure of soil bacteria was examined concerning possible factors (rhizosphere vs. bulk, fertilizer managements, and seasonality) using principal component (PC) analysis based on the 16Sr DNA bands patterns detected by DGGE. A correlation matrix of the detection ratio of respective bands (detected ratio among the replications; minimum=0 and maximum=1) was used for PC analysis. A computer program (Excel Tokei; Social Information Services Ltd.) was used for computing statistics.

Anaerobic culture examination

Anaerobic agar culture was conducted to evaluate the bacterial populations in the field experiment using AnaeroPack-Anaero (Mitsubishi Gas Chemical Co. Inc.). Suspension solution of fresh rhizosphere and bulk soil (1 g soil in 10 mL sterilized water) was diluted 10^3–10^5 folds for cultivation on DNB agar (for oligotrophic bacteria); dilute nutrient broth (DNB) is a meat extract medium containing 0.1 g meat extract, 0.1 g peptone, 0.05 g NaCl and 1.0 L H_2O, diluted 100-fold. DNB agar is suitable for growth of environmental microorganisms (Hattori 1976). All cultivations were conducted in a petri dish containing 20 mL of medium pH 7.2 pouched in AnaeroPack. The colonies were counted after 24 h incubation at 37°C, and mean colony forming units(CFUs) were calculated among three sets of 10^4–10^5 dilution dishes for individual soil and treatments, because most 10^3 dilution dish overfilled before 24 h.

Results

Field experiment

Plant growth and soil properties

Plant height was increased by compost and rice bran application (ca. 15%) until mid-August (before heading), but the final plant height in none of the three treatments was significantly different from any other treatment. As for the shoot dry weight, no significant difference among treatments were detected with the limited number of plants examined (three plants × two replications).

Soil Eh was positive immediately after transplanting in June; it became negative in July followed by a gradual decrease indicating soil reduction with time under submerged conditions (Fig. 1). After the

drainage, soil Eh rose gradually. Compost application slightly lowered the Eh of bulk soil in comparison with the control during the early stage of rice growth. Rice bran application lowered the soil Eh immediately, and kept it lower than those of other two treatments throughout the experiment.

Soil pH was around 6.1 through 6.7 without showing significant difference among the treatments and during the experiments.

Anaerobic culture examination

The count of viable anaerobic bacteria of the rhizosphere soil was remarkably larger than that of bulk soil (Table 1). The bacterial count of rhizosphere soil was the highest in July; it decreased thereafter in the control. That in the compost and rice bran treatments increased from July to reach the maximum value in August, but it decreased thereafter. Consequently, the bacterial count in August is much larger in the compost and rice bran treatments than that in the control. The ratio of bacterial counts in the rhizosphere divided by that in the bulk soil (R/B in Table 1) indicates a rhizosphere effect on the bacterial population (Katznelson 1946, Kimura 1983). The rhizosphere effect enhances population increase when the R/B value is higher than 1. The R/B values in this experiment were mostly four or larger except those in post harvest season (November), which indicates much more bacteria in the rhizosphere than in bulk soil.

PCR-DGGE and DNA sequence

Different 16Sr DNA band patterns were obtained among soil samples by PCR-DGGE (Fig. 2). The detected 16Sr DNA bands were more numerous in the rhizosphere soil than in the bulk soil (Table 2). Moreover, the number of detected 16Sr DNA bands was affected significantly by fertilizer managements and season (i.e., sampling month) in rhizosphere soils (Table 3); more 16Sr DNA bands in the rhizosphere were detected in the organic fertilization treatments (compost and rice bran) than in the control (chemical fertilizer) (Table 2). In bulk soils, however, the effect of fertilizer managements or season was not significant (Table 3). Unique DNA bands of organic materials before application to the paddy field, which

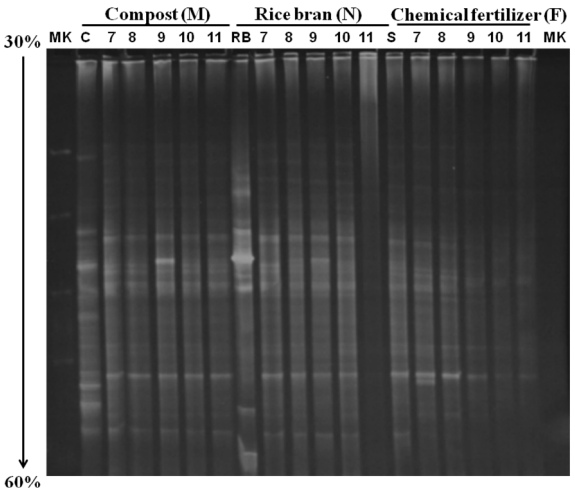

Fig. 2. 16S rDNA PCR-DGGE profiles of rhizosphere bacteria on a polyacrylamide gel with a 30–60% denaturation gradient of urea–formamide (first replication in field experiment). MK: molecular markers (Marker I, Nippon Gene Co. Ltd); C: compost material; RB: rice bran material; S: original soil; 7–11: rhizoshere soils taken from each treatment in July, August, September, October and November, respectively. The DNA bands were numbered according to the position from top to the bottom of the gel for the analysis of principal component (Fig. 3, Table 4).

Table 1. Viable anaerobic bacteria count ($\times 10^5$ CFU mL^{-1})

Treatment		Jul. 7	Aug. 8	Sep. 11	Oct. 9	Nov. 7
Chemical fertilizer (control)	R	262 ± 101	144 ± 30	151 ± 61	93 ± 31	92 ± 30
	B	29 ± 3	34 ± 13	20 ± 10	29 ± 54	15 ± 25
	R/B	9	4	8	3	6
Compost	R	226 ± 32	336 ± 70	207 ± 53	212 ± 34	102 ± 34
	B	45 ± 7	64 ± 11	9 ± 3	35 ± 18	41 ± 20
	R/B	5	5	23	6	2
Rice bran	R	199 ± 44	407 ± 138	303 ± 65	111 ± 7	32 ± 12
	B	26 ± 6	82 ± 33	17 ± 3	22 ± 1	37 ± 19
	R/B	8	5	18	5	1

R, rhizosphere soil; B, bulk soil; R/B, ratio of rhizosphere soil to bulk soil.

were not detected in the original soil before cultivation or in the other two treatments, were found in the compost material (four bands) and rice bran (three bands). But the unique DNA bands were not detected from soils of the compost/rice bran treatments during the rice cultivation, suggesting that unique bacteria provided from the organic materials did not survive in soil. The principal component analysis of the 16Sr DNA band patterns clearly indicated differences of bacterial community structure among the soil samples, although the contribution ratios of major principal components (PC1, 19%; PC2, 16%; PC3, 13%) were not so large. Figure 2a, which presents the combination of PC1 and PC2, shows different groups in rhizosphere and bulk soils (solid lines in Fig. 3a). In both rhizosphere soils and bulk soils, application of organic materials increased PC1 values in comparison with the controls (dotted lines in Fig. 3a). The effect of organic materials on PC1 was more apparent in the rhizosphere soil than in the bulk soil, but it disappeared in November (arrow in Fig. 3a). The PC3 indicated seasonal change in the community structure of soil bacteria; the PC3 decreased with time in most treatments (Fig. 3b).

The DNA bands that are closely related to the individual principal components are presented in Table 4. Taxonomic names could be estimated for eight of the bands through verification of the sequencing results with BLAST data. The bacterial species in *Glycomyces*, Nocardiopsaceae, and Chlorobi were detected in rhizosphere soils in the compost and rice bran treatments, and were rarely found in bulk soils and late stages of rice growth (October and November); they positively related to PC1 as a result. A species of Veillonellaceae was detected only in rhizosphere soils and related to PC2.

Pot experiment

Application of rice bran enhanced gleization in Arakida soil; the soil turned gray. Although the rice plants were shorter in the rice bran plot than in other two plots in both soils until August 5, no significant difference was found in the final plant height among the fertilizer treatments and soils (data not shown). Principal component analysis of the DGGE pattern showed a difference in the bacterial community structure between Andosol and Arakida soil and

Table 2. Number of DNA bands in the PCR-DGGE profile of field experiment samples

Treatment		Jul. 7	Aug. 8	Sep. 11	Oct. 9	Nov. 7
Chemical Fertilizer (control)	Rhizosphere	8.5	7.0	5.5	4.0	3.5
	Bulk	5.0	7.0	6.0	5.5	5.0
Compost	Rhizosphere	10.0	9.0	10.0	7.0	3.5
	Bulk	7.5	5.5	7.5	6.0	5.0
Rice bran	Rhizosphere	9.5	9.5	9.0	9.0	0.5
	Bulk	5.0	5.5	5.0	4.5	3.0

For the significance of the individual factors, see Table 3.

Table 3. Significance of factors on the number of DNA bands detected in PCR-DGGE profile

ANOVA target	Rhizosphere effect (R)[a]	Fertilizer managements (F)[b]	Sampling month (S)	R×F [c]	R×S [d]	F×S [e]
All soil samples	0.015	0.142	<0.001	0.143	0.070	0.612
Rhizosphere soils	–	0.045	0.001	–	–	0.252
Bulk soils	–	0.350	0.738	–	–	0.987

The figures indicate *p* values calculated by ANOVA. The original data are shown in Table 2.
a. Rhizosphere soil vs. bulk soil.
b. Control, compost, and rice bran.
c. Interaction between rhizosphere effect and fertilizer managements.
d. Interaction between rhizosphere effect and sampling month.
e. Interaction between fertilizer managements and sampling month.

among fertilizer treatments in rhizosphere soils, although such differences were not clear in bulk soils (Fig. 4). The contribution ratios of major principal components were the following: PC1, 16%; PC2, 11%; PC3, 7%; and PC4, 6%.

According to PC1 and PC2, the bulk soils of both soil types had similar bacterial communities, although rhizosphere samples showed large PC2 scores in

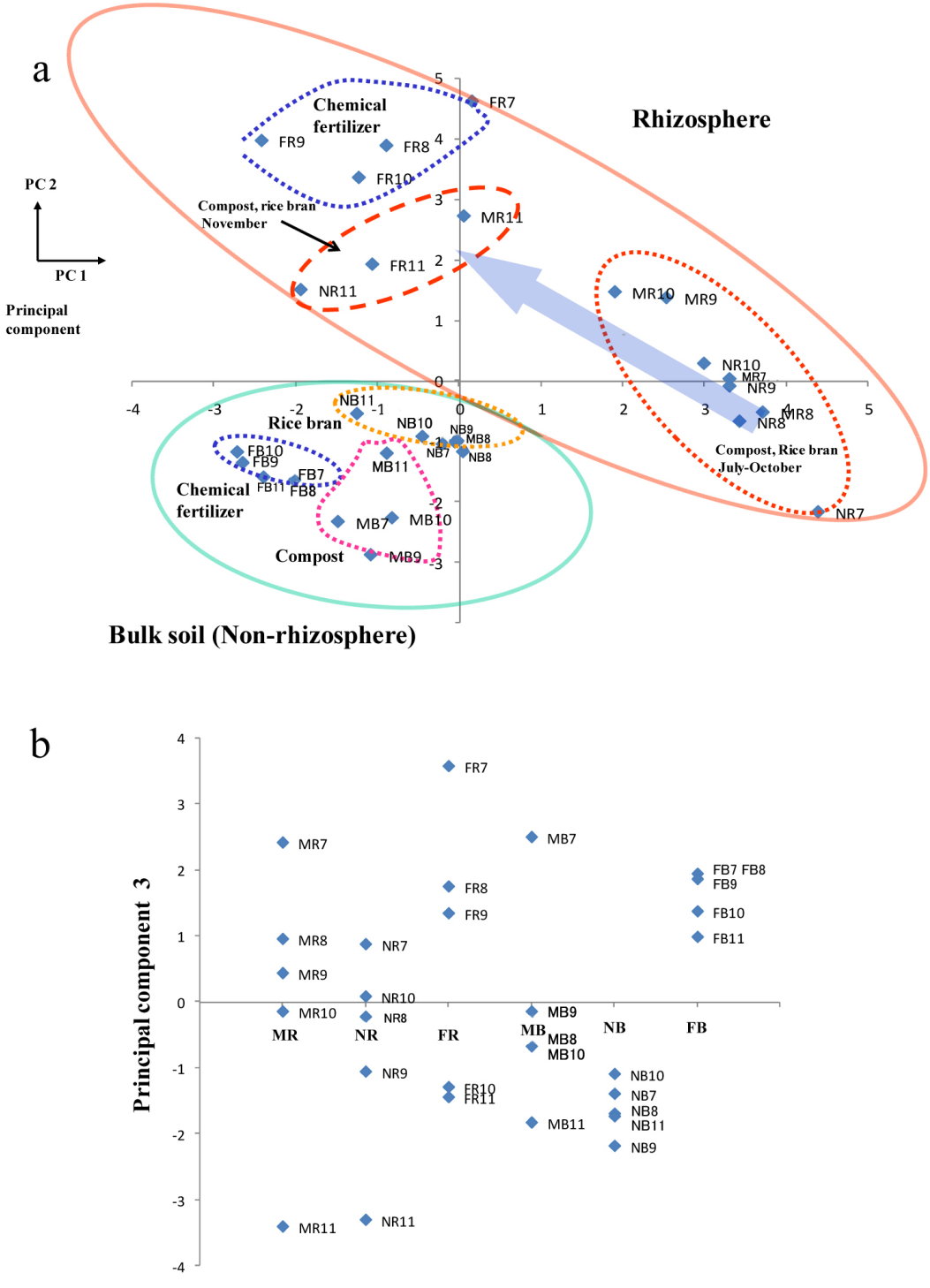

Fig. 3. Analysis of principal component (PC) based on bacterial DNA band patterns obtained using PCR-DGGE (field experiment). Similarity among the soil samples was analyzed using PC1 and PC2 (a) and PC3 only (b). MR, compost/rhizosphere; MB, compost/bulk soil; NR, rice bran/rhizosphere; NB, rice bran/bulk soil; FR, chemical fertilizer/rhizosphere; FB, chemical fertilizer/bulk soil. Number of plots (7–11) shows the month (July–November). The arrow in the figure shows the seasonal change in organic fertilizer treatments (M and N). For example, MR7 indicates the rhizosphere soil taken from compost treatment in July.

Andosol and large PC1 scores in Arakida soil (grouping by solid lines in Fig. 4a). The PC1 scores also featured bacterial communication structures of each fertilizer treatment in the rhizosphere (grouping by dotted lines in Fig. 4a). In Andosol, similarly to the field experiment, the rhizosphere soil with rice bran treatment showed particularly high PC1 sore in July. In Arakida soil, effect of compost to PC1 was much clearer than that of rice bran. In addition, the combination of PC1 and PC3 classified bacterial communities in the rhizosphere of Arakida soil among the chemical fertilizer, compost, and rice bran treatments (Fig. 4b). Furthermore, the fertilizer treatments in rhizosphere of Andosol soil were clearly classifiable by PC4 (Fig. 4c).

Discussion

The community structure of soil bacteria was largely influenced by rhizosphere effects, types of fertilizers and seasonal changes. The community structure of soil bacteria in the rhizosphere was significantly

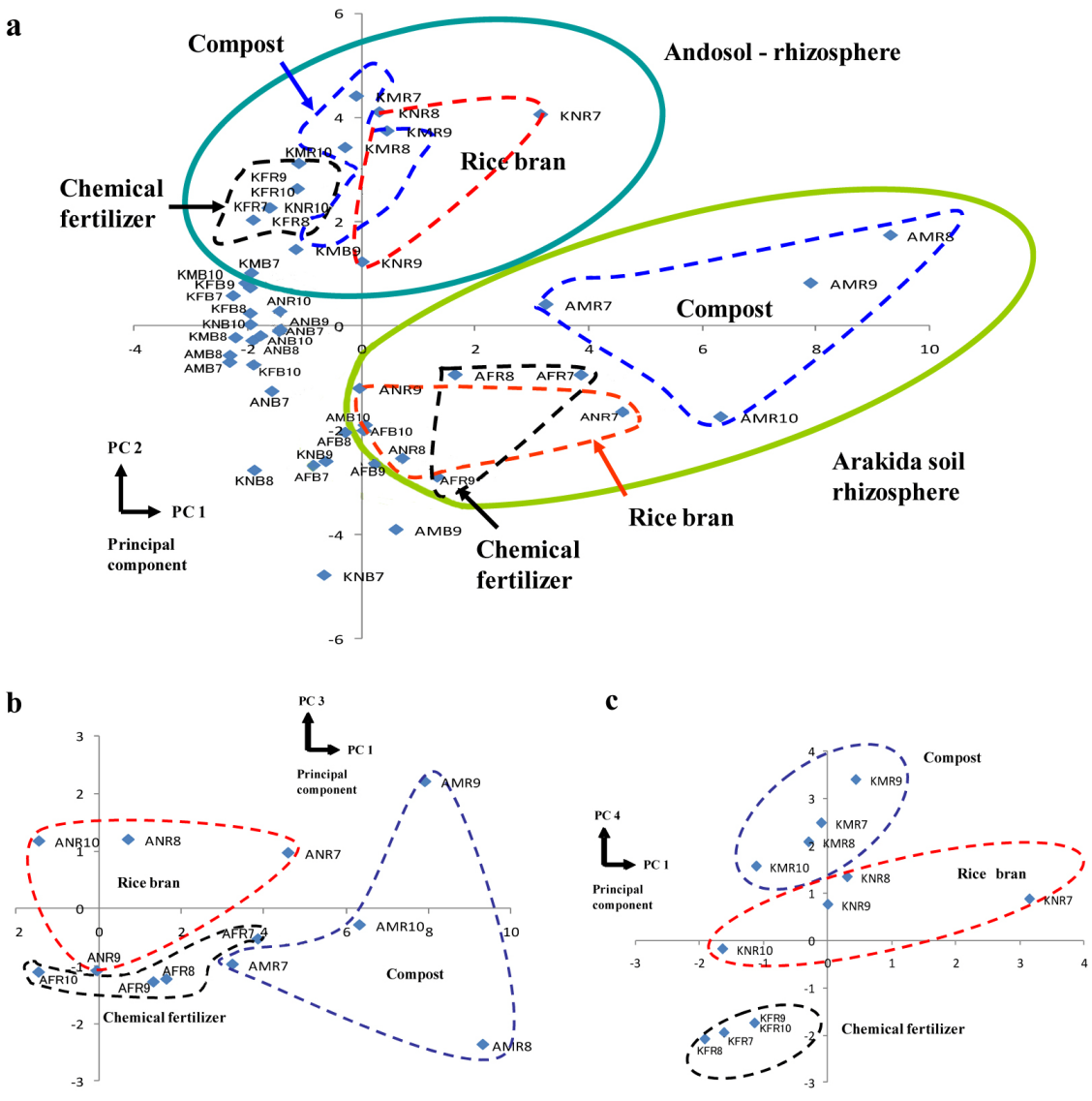

Fig. 4. Principal component (PC) analysis based on bacterial DNA patterns obtained using PCR-DGGE (pot experiment). Similarity among the soil samples was analyzed using PC1 in combination with PC2 (a), PC3 (b), and PC4 (c). In b and c, plots for rhizosphere of Arakida soil (b) and Andosol soil (c) are shown, respectively. A, Arakida soil; K, Andosol; MR, compost/rhizosphere; MB, compost/bulk soil; NR, rice bran/rhizosphere; NB, rice bran/bulk soil; FR, chemical fertilizer/rhizosphere; FB, chemical fertilizer/bulk soil. The number of each plot (7–10) shows the month (July–October). For example, AMR7 indicates rhizosphere soil taken from Arakida soil with compost treatment in July.

Table 4. Major DNA bands related to each principal component (PC1−PC3)[a]

Principal component	Positive		Negative	
	Band no.[b]	Component loading	Band no.	Component loading
PC1	19 (*Glycomyces* spp.)	0.847	5	-0.466
	30 (Nocardiopsaceae)	0.784	31	-0.333
	20 (Chlorobi)	0.779	17	-0.285
	14	0.690		
	8	0.648		
	1	0.551		
	11	0.550		
PC2	18 (Veillonellaceae)	0.731	31	-0.771
	29	0.602	8	-0.608
	17	0.547	9	-0.510
	15	0.450	24 (Methylocystaceae)	-0.481
	13	0.425	23 (Chloroflexi)	-0.443
PC3	3	0.776	10 (Chlorobi)	-0.503
	5	0.646	2	-0.429
	29	0.546	21 (Rhizobiales)	-0.429
	23 (Chloroflexi)	0.513	26	-0.429
	25	0.500	27	-0.429

a. DNA bands that contribute dominantly to each principal component were listed based on component loading.
b. Consecutive numbers were assigned to the DNA bands detected by DGGE in the sequence from the top to the bottom of the gel. The parenthetical references are taxonomic names estimated by verification of sequencing results using BLAST data.

different from that in bulk soil (Figs. 2a and 3a) with large population (Table 1). The rhizosphere provides a unique soil environment that differs from that of bulk soil. The rhizosphere is rich with organic components such as saccharides and organic acids exuded from roots, and soil pH is changed by the exhaust and absorption of ions by roots (Rovira 1969, Neumann and Römheld 1999, Uren et al. 2000, Bertin et al. 2003). Because rice roots also provide organic materials to rhizosphere soil through exuded chemicals and released border cells, rhizosphere soil has higher populations of both aerobic and anaerobic bacteria (Kimura et al. 1977, 1979, 1983). The degree of rhizosphere effects depends on the physiological activity of plant roots. Marschner et al. (2004) reported a difference of rhizosphere bacteria between young and matured parts of maize roots, possibly because of different root exudation. Aged and matured roots might cause small rhizosphere effects. Kimura et al. (1983) also pointed out that oxygen release can differ between young and aged roots of rice. In fact, the rhizosphere effects on bacterial biomass and diversity (Table 1, 2) were less or no significant in October and November in this study, when the rice plants had matured.

Moreover, effects of other factors such as fertilizer treatments, soil types and seasonal change were much more readily apparent in rhizosphere than in bulk soil. For example, population (Table 1) and community structure of soil bacteria (Figs. 3 and 4) were greatly changed by organic fertilizers in the rhizosphere, although the effects of organic fertilizers were not so large in bulk soils. The organic fertilizers (straw compost and rice bran for instance) provide carbon and nutrients to soil bacteria. There might also be synergy effects between organic fertilizer and root exudates. On the other hand, long-term utilization of straw compost showed no clear effects on the bacterial community in the rhizosphere of maize, barley, and soybean in an Andosol upland field of the same university farm (Doi et al. 2009). The great difference between lowland and upland fields is the soil oxygen condition. Oxygen release from rice roots in flooded lowland paddy field (Armstrong and Boatman 1967, Revsbech et al. 1999, Kirk 2003, Mano and Omori 2007) might affect the decomposition and utilization of organic materials and thereby alter the rhizosphere bacterial community. Using molecular biological methods, effects of long-term application of organic manure and/or compost on bacterial community of upland fields have been reported recently (Toyota and Kuninaga 2006, Chu et al. 2007, Li et al. 2008). This study showed remarkable effects of organic fertilizers on bacterial communities in the short term, probably because they particularly affect the rhizosphere soil adhering to roots collected by ultrasonic treatment and because of the anaerobic conditions in paddy field.

Although compost and rice bran had unique bacteria before application, the bacteria seemed to disappear in the paddy soil. The organic materials probably played role not as seed bacteria but to feed

substrates to soil originating bacterial species, which were not dominant in soil with poor organic materials. The decomposed straw compost and rice bran can be used vigorously as a carbon and nutrient source by bacteria in the rhizosphere; the bacterial population reached its maximum level in hot mid-summer (August) when decomposition should have proceeded actively, whereas bacterial population was decreased from July to August in the chemical fertilizer treatment (Table 1). Furthermore, the bacterial community structure of organic fertilizer treatments differed remarkably from that in chemical fertilizer treatment in summer (Fig. 3a). It gradually came to resemble that of chemical fertilizer treatment (arrow in Fig. 3a), and eventually became almost identical in late autumn (November), when degradable organic substrates in compost and rice bran were presumably consumed. We predicted different effects on soil bacteria between compost and rice bran because of their different organic components. But bacterial community structure analyzed using PCR-DGGE did not show clear difference between the two organic treatments in the field experiment (Table 2, Fig. 3), except that rice bran treatment in July, in which soil Eh was lowered quickly, showed specific PC scores (NR7 in Fig.3a). Some difference was detected between the two organic treatments in the pot experiment, but the difference between rhizosphere and bulk soils was much clearer (Fig.3a). Amount and/or decomposing rate, rather than the chemical components, of organic materials may affects on rhizosphere bacteria.

The organic materials may affect soil microorganisms not only as the substrates but also as a caution of soil anaerobic condition. Application of organic fertilizer enhances soil reduction in paddy soil as soil bacteria consume dissolved oxygen in water to decompose organic matter. In this study, the field soil Eh was lowered by application of organic fertilizers, in particular rice bran (Fig. 1). Ueno and Suzuki studied the mechanism of weed control by the broadcasting of rice bran in paddy fields (Ueno and Suzuki 2005). They reported rapid soil reduction with sharp increase of isobutyric acid in flooding water as the decomposed product. Similar rapid soil reduction observed by the rice bran treatment in June and July (Fig. 1) may have caused the change in bacterial community indicated by the specific PC scores (NR7 in Fig.3a). Although the details of bacterial community were not clarified well in this study, because 16Sr DNA sequence obtained from PCR-DGGE bands were not long sufficiently for identification of bacterial species, a group of bacteria detected in rhizosphere soil treated with organic materials was Chlorobi, which increased PC1 score (Table 4). Chlorobi is known as green sulfurbacteria mainly found in anoxic aquatic environment and able to carry out anaerobic photosynthesis withdrawing electrons from hydrogen sulfide (Gupta lab 2006). Detection of Chlorobi suggests the possibility that application of organic materials altered bacterial community through soil reduction in this experiment.

In addition to management practices, soil types may strongly influence the soil-bacteria community structure (Marschner et al. 2004, Doi et al. 2009). In the pot experiment described in this report, the bacterial communities of bulk soils were not clearly different between Andosol and Arakida soils (Fig. 4). On the other hand, large differences were found in bacterial community of rhizosphere soils between the two types of soil. Furthermore, the specific influence of fertilizer treatments in the individual soils was readily apparent only in rhizosphere soils. In Andosol, both the compost and rice bran affected rhizosphere bacterial community (in particular, rice bran effect in July) in the field and pot experiment, whereas effect of compost was more obvious than rice bran in the Arakida soil rhizosphere (Fig. 3, 4).

The caution and mechanism of the fact that rhizosphere bacterial community responds to organic matters and soil types much clearly than bulk soil are important theme of further studies. A recent trial of metabolome analysis for rice root exudates has demonstrated much larger diversity of organic compounds than expected before (Suzuki et al. 2008). Metabolome analysis has also shown that the amounts and components of root exudates can be affected by abiotic and biotic conditions of growing media (Paterson et al. 2000, Suzuki et al. 2008). Differences in nutritional and physical properties of soil caused by application of organic fertilizers and by types of soils can modify root exudates through changes of the physiological and metabolic conditions of rice plants. Therefore, application of organic fertilizers and soil types may indirectly but strongly affect bacterial communities in the rhizosphere. This study showed that the influence of fertilizer management and soil type on soil bacteria in a paddy field should be studied from the perspective of interaction with rhizosphere effects.

Acknowledgments

We thank Kenichiro Ichikawa, Ryuichi Soga, Keisuke Yatsuda, and Noboru Washizu (Institute for Sustainable Agro-ecosystem Services, Graduate School of Agricultural and Life Sciences, The University of Tokyo) for their managements of the paddy field.

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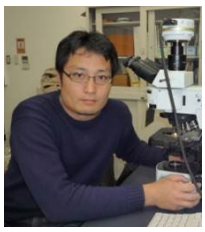

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