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Succession and Phylogenetic Composition of Bacterial Communities Responsible for the Composting Process of Rice Straw Estimated by PCR-DGGE Analysis

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PCR-DGGE analysis followed by sequencing of 16S rDNA was applied to reveal the succession and the phylogenetic composition of the bacterial communities responsible for the composting process of rice straw (RS). RS under the composting process exhibited more complex DGGE band patterns with more numerous bands and more diversification in intensity than the initial RS materials. The DGGE patterns of the bacterial communities in the initial RS materials and RS under the composting process were statistically divided into four groups, namely those characterizing the communities associated with the pre-composting stage, and thermophilic, middle, and curing stages of the compost, which corresponded exactly to the same grouping obtained from the PLFA pattern analysis for the same samples (Cahyani et al. 2002: *Soil Sci. Plant Nutr.*, 48, 735–743). Different bacterial members characterized the respective stages as follows: α-Proteobacteria for the initial RS materials, thermophilic *Bacillus* spp. and actinomycetes at the thermophilic stage, and *Cytophaga* and clostridial members at the middle and curing stages, respectively. In addition, mesophilic *Bacillus* members were always present throughout the composting process. Temperature and substrates available to bacteria seemed mainly to determine the composition of the bacterial members at the respective stages.

***Key Words*: bacteria, compost, DGGE, phylogenetic composition, succession.**

Traditional fertilizer, compost, contributes not only to the supply of plant nutrients, but also to the stimulation of the activity of soil microbiota, and furthermore it plays a role in disease control and acts as a soil conditioner (Inoko 1984; Guidi et al. 1988; De Ceuster and Hoitink 1999; Leita et al. 1999; Stewart et al. 2000; Crecchio et al. 2001).

The quality of compost is estimated based on the composition of the microbiota, and chemical and physical properties. Several studies indicated that the presence of certain microorganisms exerts a distinctive effect on the increase of the quality of compost or acceleration of the composting process (Nakasaki and Uehara 1996; Requena et al. 1996; Kuo-Shu et al. 1998; Badr EL-Din et al. 2000).

Application of rice straw (RS) compost to rice fields for sustainable rice production is a common practice in the Asian countries. However, the microbiota responsible for the composting process of RS have not been fully elucidated. More detailed information about the microbial succession and microbial composition throughout the composting process of RS is important to evaluate the degree of composting and to improve the quality of the compost end product.

By using phospholipid fatty acid (PLFA) analysis, the succession of the community structure of the microbiota responsible for the composting process of RS was revealed in the previous study (Cahyani et al. 2002). Based on the PLFA composition, fungi and Gram-negative bacteria were estimated to predominate in the initial RS materials. At the thermophilic stage (the first 2 weeks), Gram-positive bacteria and actinomycetes were dominant, and other Gram-positive bacteria became dominant during the subsequent, middle stage. Finally at the curing stage, the proportion of Gram-negative bacteria and eukaryotes increased, indicating the co-contribution of Gram-positive and -negative bacteria and fungi in the decomposition process at this stage. The results suggested that PLFA analysis was a suitable method for detecting rapid changes in different subsets of the microbial communities during the composting process of RS, as indicated in composting studies for various

materials (Hellmann et al. 1997; Herrmann and Shann 1997; Carpenter-Boggs et al. 1998; Klamer and Bååth 1998; Lei and VanderGheynst 2000).

Although information about the microbial communities obtained from PLFA analysis covered the whole microbiota including prokaryotes and eukaryotes, it had remained limited to higher taxa / groups. Therefore, further studies should be carried out to reveal the microbial succession at the genus or species level. The use of the genetic fingerprinting technique was expected to be suitable for this purpose, as reported in earlier studies on the composting process based on molecular DNA / RNA characterization for cultured isolates (Beffa et al. 1996a, b; Song et al. 2001), analysis of directly extracted DNA (Blanc et al. 1999; Ishii et al. 2000; Pedro et al. 2001; Haruta et al. 2002a, b), and both methods (Peters et al. 2000; Dees and Ghiorse 2001; Ueno et al. 2001). However, to our knowledge, no report on the use of these methods for the composting process of RS has been published yet.

The purpose of this study was to analyze the succession of community structure and the phylogenetic composition of the bacteria responsible for the composting process of RS by applying the PCR-DGGE method to extracted DNA, followed by sequencing. This is the first report on the succession of community structure of bacteria involved in the composting process of RS, by confirming the validity of two culture-independent techniques, PLFA analysis and molecular (16S rDNA) methods.

MATERIALS AND METHODS

All the samples used in the present study were the same as those used in the previous study (Cahyani et al. 2002).

Experimental site. Composting of RS was conducted in a storehouse at Aichi-ken Anjo Research and Extension Center, Central Japan (latitude 34°8′N, longitude 137°5′E).

Setup of the compost pile and sampling method. The composting process was performed based on a conventional method used in Japan (Cahyani et al. 2002). Briefly, RS materials were cut, moistened, then piled on the floor in the storehouse and covered with plastic sheets. At the time of the first turning, 2 weeks after the onset of composting, ammonium sulfate ($(NH_4)_2SO_4$) was applied at the rate of 10 kg Mg^{-1} of air-dried RS. The compost pile was turned once every month afterwards. Water was added every time that turning was performed. At each sampling time, five subsamples were collected randomly at a depth of about 30 cm, where the measured temperature was not different from that of the center. The samples were then stored at a temperature of −20°C.

Dried RS (compost material) stored in a storehouse was also sampled twice, 45 d before the onset of composting and on the day of composting. Samples were taken 12 times during the 145-d period of composting.

DNA extraction. DNA extraction was performed according to the procedure described by Weber et al. (2001) with small modifications. Each sample of compost RS was a mixture of the representative portions of 5 subsamples. The frozen samples were lyophilized overnight and homogenized using a mortar and a pestle under liquid nitrogen. About 125 mg of the sample was put in a sterile 2-mL Eppendorf tube and suspended in 1 mL of DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide (CTAB)), and shaken vigorously. Freezing at −196°C for 20 min in liquid nitrogen, and thawing at 65°C for 30 min in the water bath were repeated 3 times for the suspension. Afterwards, 0.1 mL of 50 mg mL^{-1} lysozyme was added and incubated for 2 h at 37°C, followed by the addition of 13.4 μL of 10 mg mL^{-1} proteinase K and 226.6 μL of 10% sodium dodecyl sulfate (SDS) and a further incubation for 1 h at 37°C. Then, the nucleic acids were extracted with 600 μL of phenol-chloroform-isoamylalcohol (PCI) (25 : 24 : 1, v / v / v) and centrifuged at 15,000 rpm for 5 min. The supernatant phase was transferred to a new Eppendorf tube and again extracted with 1 mL of PCI and centrifuged at 15,000 rpm for 5 min. The upper phase of the solution was then transferred to a new Eppendorf tube, to which 1 mL of chloroform-isoamylalcohol (24 : 1, v / v) was added, and centrifuged at 15,000 rpm at 5 min. After the transfer of the upper phase of the solution to another new Eppendorf tube, the DNA contained in this phase was precipitated with 1 mL of isopropanol at room temperature for 90 min and centrifuged at 15,000 rpm for 20 min. The supernatant was discarded and the pellet of crude DNA was washed with 70% cold ethanol, and thereafter with 100% cold ethanol. After drying on a heat block at 37°C, the DNA was dissolved in 200 μL of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and stored at 4°C for immediate use or at −20°C for long storage.

DNA purification. DNA purification was performed according to the method of Jackson et al. (1997) by using Sephadex G-200. Spin column was constructed by boring a sterilized 1.5 mL Eppendorf tube and plugging with glass wool (5 mm height from the bottom). The column was placed on a new 1.5 mL Eppendorf tube. Then, the column was packed with 500 μL of Sephadex G-200 and centrifuged at 3,700 rpm for 5 min. During the centrifugation, the liquid trapped in the gel matrix was drained into the lower 1.5 mL Eppendorf

tube. Repacking and centrifugation were continued until the column contained the gel matrix at a height of approximately 2.5 cm. The column was washed with 100 μL of TE buffer and centrifuged at 3,700 rpm for 10 min to remove the excess buffer. Afterwards, the lower tube was replaced by a new one. DNA extract (100 μL) of each sample was loaded onto each column, followed by centrifugation at 3,700 rpm for 5 min. The column was then loaded with 100 μL TE buffer and centrifuged again at 3,700 rpm for 5 min. Washing with TE buffer was repeated one more time, until the eluted solution reached a total volume of 300 μL. The DNA solution in the lower tube was then precipitated with 2 volumes of 100% cold ethanol and 1 / 10 volume of 3 M sodium acetate (pH 5.2) by incubation on ice for 15 min and centrifuged at 15,000 rpm for 15 min. The supernatant was discarded, and 1 mL of 70% cold ethanol was added to the tube and centrifuged again at 15,000 rpm for 5 min. After removal of the supernatant and drying up on a heat block at 37°C, the purified pellet DNA was dissolved in 100 μL of TE buffer.

PCR amplification. The 16S rDNA was amplified with PCR using the eubacterial set of two primers, 357f-GC clamp (*Escherichia coli* position: 341-357, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′, the underlined sequence corresponded to the GC clamp), and 517r (5′-ATTACCGCGGCTGCTGG-3′, *E. coli* position: 517-534) (Muyzer et al. 1993). PCR was performed in a total volume of 50 μL in 200-μL microtubes, which contained 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mM dNTP mixture, 5 μL of 10X *Ex Taq* buffer (20 mM Mg^{2+}; TaKaRa, Tokyo, Japan), 0.25 μL of *Ex Taq* DNA polymerase (TaKaRa), 1 μL of DNA template (about 15 ng) and 37.75 μL of milli-Q water. Cycle conditions for the amplification were as follows: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 8 min with TaKaRa PCR Thermal Cycles Model TP 240 (TaKaRa). The PCR product was analyzed on 2% (w / v) agarose gels containing 2% of 50 × TAE buffer (40 mM Tris-acetate, 2 mM EDTA) using ethidium bromide (10 mg mL^{-1}) staining. Electrophoresis of agarose gels was run with 1 × TAE buffer in a Mini Gel Electrophoretic System (Advance, Tokyo, Japan) at 100 V for 20 min. The gel was photographed under UV light.

DGGE analysis. The PCR products were separated on a polyacrylamide gel with a denaturing gradient from 30% (8% (w / v) acrylamide / bisacrylamide (37.5 : 1), 12% formamide, and 2.1 M urea) to 70% (8% (w / v) acrylamide / bisacrylamide (37.5 : 1), 28% formamide, and 4.9 M urea). The electrophoresis was performed in an electrophoresis cell D-code™ System (Bio-Rad Laboratories, Hercules, CA, USA) with 1 × TAE buffer at 60°C and 100 V for 14 h (Muyzer et al. 1998). Visualization of the bands was achieved by staining with SYBR™ Green I nucleic acid gel stain (BMA, Rockland, ME, USA) for 30 min and photography under UV light.

Statistical analysis. To estimate the succession of bacterial communities during the composting process of RS, the data obtained from the DGGE patterns, based on band intensity and position, were analyzed by cluster analysis and principal component analysis (PCA). Principal component analysis was performed using EXCEL STATISTICS 1997 for Windows (SRI, Tokyo, Japan). Correlation matrix was used in the analysis. Cluster analysis was performed according to the Blackbox program (Aoki 1996). Ward method was used in the analysis.

Direct sequencing. The selected DGGE bands were excised from the DGGE gels and put in 1.5 mL Eppendorf tubes. One hundred μL of TE buffer was added to the tube, and kept at 4°C overnight to diffuse DNA from the gel strip. One μL of eluted DNA was used as a template to amplify the DNA of the excised DGGE band. The primer set and PCR program were same as those described above. The resulting PCR products were checked by DGGE for comparison with the original DGGE pattern of the excised band. The PCR products that matched the position with the original bands were sequenced with the Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kit (Amersham, Tokyo, Japan) according to the instructions, by using the set of two primers, 357f (no GC clamp) and 517r, with the 373S DNA sequencer (ABI, Urayasu, Japan).

Cloning and sequencing. Although DNA fragments from the major bands in the DGGE gel were readily isolated and sequenced by direct sequencing, in the present study, several bands failed to be sequenced by the same method. Cloning was performed for these bands before sequencing. DNA extracted from the DGGE bands was reamplified with the same primers and same PCR program as those described above. The PCR product was cloned into pT7 Blue T-vector (Novagen, Darmstadt, Germany). The plasmids were transformed into competent cells of *Escherichia coli* XLI blue (TaKaRa) and were inoculated onto LB agar plate medium containing ampicillin sodium (50 μg mL^{-1}) together with 70 μL X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 mg mL^{-1}) and 8 μL IPTG (0.1 g mL^{-1}), and incubated at 37°C overnight. From each plate, about 25 white colonies were subcultured to the new LB agar plate medium for pure culturing of each colony. PCR was performed for the formed colonies using the set of two primers, 357f-GC clamp

and 517r, with the same program as that described above. The mobility of the resulting fragments was checked by DGGE and compared with the original pattern of the excised DGGE band. This cloning step was performed in order to select the correct clones which matched the target bands for sequencing. The plasmids from the selected clones were extracted with a QIAprep Spin Miniprep kit (Qiagen, Tokyo, Japan) and used as templates for sequencing with the set of M13 primer RV (5′-GTTTTCCCAGTCACGAC-3′) and M13 primer M4 (5′-CAGGAAACAGCTACGAC-3′), as described above. We excised the bands with the same mobility on DGGE gels from at least two lanes, and obtained the same sequence.

Phylogenetic analysis. Sequences of the DGGE bands were compared with the 16S rDNA sequences obtained with the BLAST search from the database of the DNA Data Bank of Japan (http//:www.ddbj.nig.ac.jp/E-mail/homology.html).

Nucleotide sequence accession number. The 16S rDNA partial sequences obtained in this study are available in the DDBJ database under the accession numbers AB103480–AB103504.

RESULTS

Succession of bacterial communities during the composting process of RS

Based on the temperature changes of the compost pile and PLFA analysis, the composting process could be divided into four stages as follows: pre-composting stage (the initial RS materials), thermophilic stage, middle stage, and curing stage of composting (Cahyani et al. 2002).

The succession of the bacterial communities responsible for the composting process of RS was visualized in the DGGE band patterns as shown in Fig. 1. The DGGE

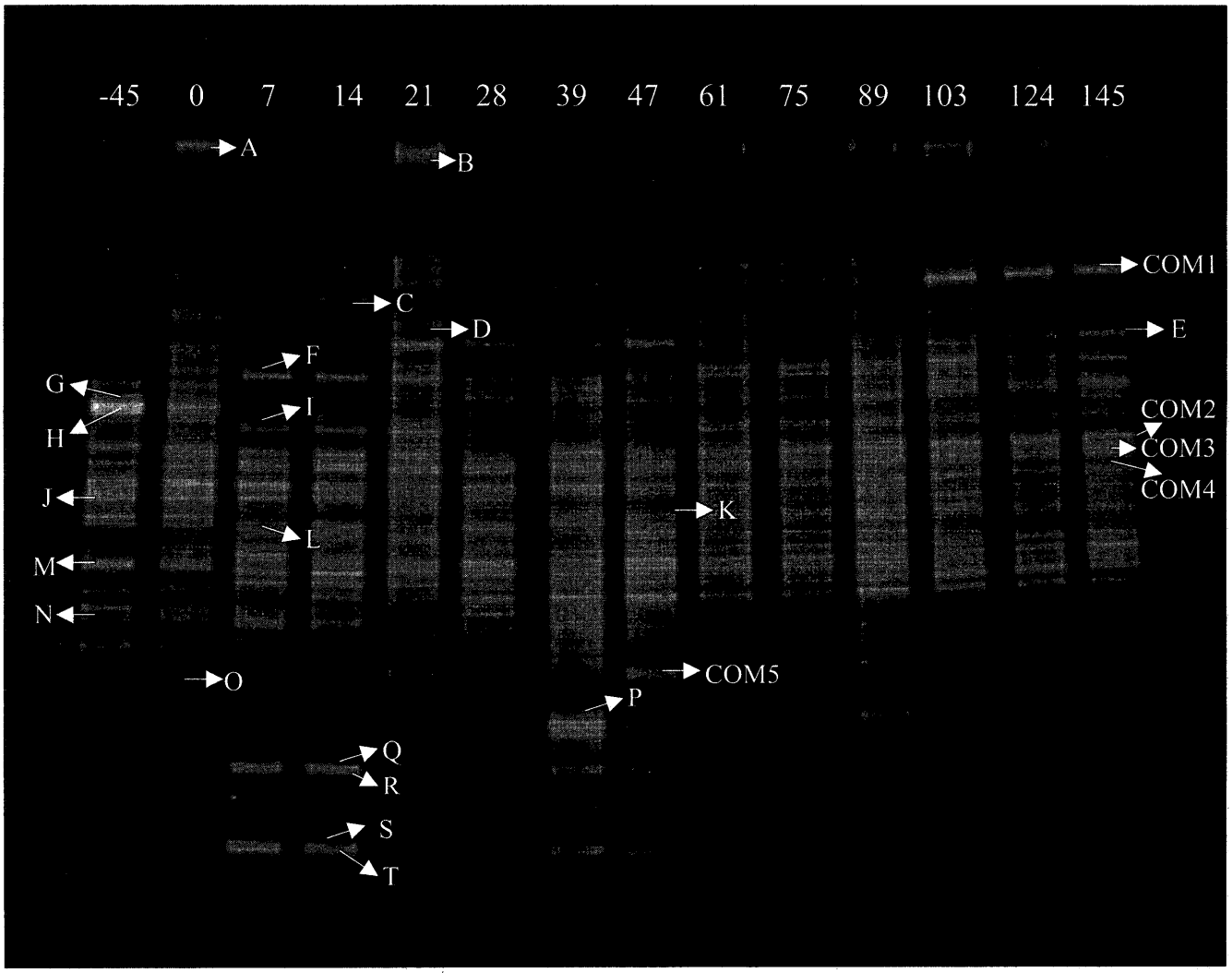

Fig. 1. DGGE pattern of the bacterial communities associated with the composting process of rice straw. Numbers above the lane denote the composting time (d).

pattern of the bacterial communities in the RS materials which were taken 45 d before composting and on day 0 of composting was similar, but the pattern changed drastically at the thermophilic stage (the first two weeks) of composting. At the middle stage (day 21 to day 47) of composting, the DGGE again showed a different pattern. At the curing stage, the DGGE pattern was relatively stable in terms of number and intensity of the respective bands.

The number of DGGE bands increased with the onset of composting, peaked at the middle stage on day 39, and then decreased to become relatively constant at the curing stage (Fig. 2). The number of DGGE bands ranged from 21 to 26 for the RS materials (45 d before composting and on day 0), from 32 to 33 during the thermophilic stage (day 7 to day 14), from 35 to 42 during the middle stage (day 21 to day 47), and from 28 to 34 during the curing stage (day 61 to day 145), respectively.

As shown in Fig. 1, specific bands appeared at each stage of the composting process. Bands G, J, N, and O, for example, showed a high intensity in the rice straw materials only. Bands Q, R, S, and T appeared with a high intensity from the thermophilic stage and the intensity decreased afterwards. Bands B, D, and E appeared from the middle stage to the end of composting. In addition, several common bands such as bands COM1, COM2, COM3, COM4, and COM5 always appeared throughout the composting period.

Statistical analysis of the DGGE patterns of the bacterial communities in the RS under the composting process was performed by principal component analysis and cluster analysis. Principal component analysis showed that the total contribution percentages of the 1st and 2nd principal components were 26.9% and 24.1%, respectively. The DGGE bands with positively and negatively large Eigen values in the 1st principal component were R, S, T, Q, L, F, I, and C, and M, A, and H, respectively. On the other hand, the DGGE bands with positively and negatively large Eigen values in the 2nd principal component were B, K, P, E, and D, and J, O, G, N, M, and H, respectively (Fig. 3).

The score plots of the DGGE bands of the initial RS materials were located on the lower-left side (the 3rd quadrant), and then the score plots shifted anti-clockwise as follows: the thermophilic stage in the 4th quadrant, the middle stage in the 1st quadrant, and the curing stage in the 2nd quadrant, respectively. Based on the distribution of the score plots of the RS samples under the composting process illustrated in Fig. 3 and the specific existence of the respective bands at each stage and their absence at the other stages (Fig. 1), the bacterial communities were considered to be characterized by bands J, O, G, N, M, H, and A for the initial RS materials, bands Q, R, S, T, L, F, I, and C for RS at the thermophilic stage, and bands B, K, P, E, and D at the middle and curing stages of composting, respectively.

Cluster analysis identified four clusters for the DGGE patterns of the bacterial communities in the RS under the composting process (data not shown). The DGGE patterns were divided first into three clusters: the first cluster for the initial RS materials, the second cluster for RS at the thermophilic stage of the composting process, and the third cluster for RS from the middle stage of composting onwards. The third cluster was further divided into two sub-clusters as follows: RS compost at the middle and the curing stages during the composting process. Domains A to D shown in Fig. 3 corresponded to the respective clusters and sub-clusters obtained in the cluster analysis.

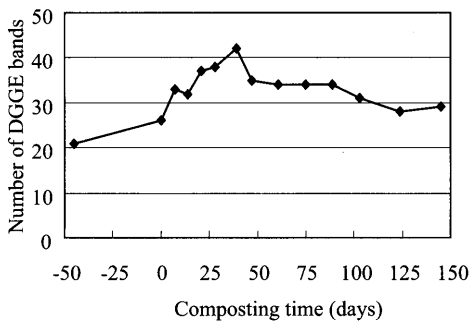

Fig. 2. Number of bands in the DGGE pattern of the bacterial communities associated with the composting process of rice straw.

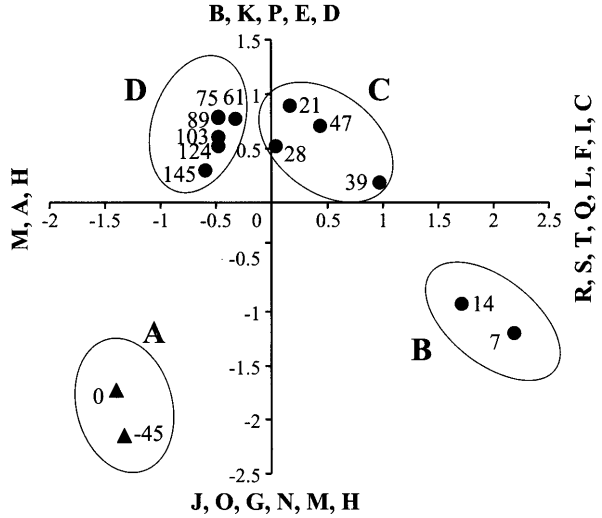

Fig. 3. Principal component analysis of the DGGE patterns of the bacterial communities associated with the composting process of rice straw. A: rice straw materials at the pre-composting stage, B: rice straw compost at the thermophilic stage, C: rice straw compost at the middle stage, D: rice straw compost at the curing stage. Numbers next to the symbol denote the composting time (d).

Table 1. Closest relatives of the bacteria with the characteristic DGGE bands detected by principal component analysis.

	DGGE band	Eigen value	Seq bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment	Source and reference
First PC (+)	R##	0.236	136	Streptosporangiaceae str.KACC-20141	Actinomycetes	AF345861	100	136/136	Mushroom composts (Song et al. 2001)
	S##	0.228	136	Streptosporangiaceae str.KACC-20141	Actinomycetes	AF345862	99	128/129	Mushroom composts (Song et al. 2001)
	T##	0.228	136	Streptosporangiaceae str.KACC-20141	Actinomycetes	AF345863	99	128/129	Mushroom composts (Song et al. 2001)
	Q##	0.227	136	Streptosporangiaceae str.KACC-20141	Actinomycetes	AF345864	100	136/136	Mushroom composts (Song et al. 2001)
	L##	0.219	160	*Bacillus* sp. S13	Bacillales	AF403022	97	156/160	Swine waste (Gagne et al. 2001)
				Ureibacillus thermosphaericus	Bacillales	AF515485	97	156/160	Compost
				Bacillus sp.B10EXG	Bacillales	AF537293	97	156/160	Waste compost
	F##	0.217	160	*B. thermosphaericus*	Bacillales	X90640	98	157/160	Air (Anderson et al. 1995); compost of garbage (Haruta et al. 2002b)
				Urebacillus thermosphaericus strain S11	Bacillales	AF403021	98	157/160	Swine waste (Gagne et al. 2001)
				Uncultured compost bacterium 5-11	Bacteria	AB034718	98	157/160	
	I##	0.213	155	*Oxalophagus oxalicus*	Bacillales	Y14581	90	138/152	Zaitsev et al. (1998)
	C##	0.207	160	*B. thermosphaericus*	Bacillales	X90640	98	157/160	Air (Anderson et al. 1995); compost of garbage (Haruta et al. 2002b)
				Urebacillus thermosphaericus strain S11	Bacillales	AF403021	98	157/160	Swine waste (Gagne et al. 2001)
				Uncultured compost bacterium 5-11	Bacteria	AB034718	98	157/160	
First PC (-)	M#	-0.213	160	*Pantoea ananatis* strain LMG 20103	γ-Proteobacteria	AF364847	99	159/160	Sproer et al. (1999)
				Pantoea sp. 'Cibuni-media-isolate-74')	γ-Proteobacteria	AF229453	99	159/160	Hot springs (Baker et al. 2001); Sproer et al. (1999)
	A#	-0.178	158	*Sphingobacterium multivorum*	Sphingobacteria	AB020205	90	143/158	Compost of food scraps (Ishii et al. 2000)
				Uncultured yard trimming compost bacteriu	Bacteria	AY095391	90	143/158	
	H#	-0.146	135	*Allorhizobium undicola*	α-Proteobacteria	Y17047	97	132/135	
				Uncultured bacterium clone EV12	α-Proteobacteria	AF379026	97	132/135	
				Rhizobium sp. strain DUS470	α-Proteobacteria	Y11084	97	132/135	
Second PC (+)	B###	0.242	155	*Cytophaga* sp. SA1	Cytophaga	AF414444	96	150/155	Paper mill slime
	K###	0.217	137	Uncultured sludge bacterium A4b	Bacteria	AF234686	90	124/137	Sludge
	P###	0.217	135	*Roseiflexus castenholzii*	Green nonsulfur bacteria	AB041226	92	125/135	Hanada et al. (2002)
	E###	0.215	127	*C. polysaccharolyticum* (DSM-1801)	Clostridia	X77839	96	118/122	
				Clostridium populeti	Clostridia	X71853	96	118/122	
				Clostridium sp. XB90	Clostridia	AJ229234	96	118/122	Anoxic rice paddy soil (Chin et al. 1999; Hengstman et al 1999)
	D###	0.205	135	*Eubacterium xylanophilum*	Clostridia	L34628	100	123/123	
				Clostridium herbivorans	Clostridia	L34418	100	123/123	Pig intestine
				Uncultured bacterium clone p-2409-55G5	Bacteria	AF371677	100	123/123	Pig intestine
Second PC (-)	J#	-0.22	135	*Agrobacterium tumefaciens*	α-Proteobacteria	AY043382	100	135/135	
				Uncultured soil bacterium clone 1315-2	α-Proteobacteria	AF423220	100	135/135	Soil
				Rhizobium sp. strain-113	α-Proteobacteria	D14512	100	135/135	
				Pseudomonas sp. G-179	α-Proteobacteria	AF109171	100	135/135	
				Agrobacterium larrymoorei	α-Proteobacteria	Z30542	100	135/135	
	O#	-0.217	160	Uncultured bacterium clone BCM1-37B	Bacteria	AY102881	100	160/160	Subtropical freshwater marsh
	G#	-0.217	136	*Oryza sativa* complete chloroplast genome	Chloroplast	X15901	99	136/137	*Oryza sativa* (Hiratsuka et al. 1989)
	N#	-0.213	140	*Aureobacterium resistens*	Actinomycetes	Y14699	100	140/140	
				Microbacterium aurum	Actinomycetes	D21340	100	140/140	
	M#	-0.201	160	see above (First PC (-))					
	H#	-0.191	135	see above (First PC (-))					
Common band	COM1		161	*Spirochaeta zuelzerae*	Spirochetes	M88725	96	156/161	Paster et al. (1991)
	COM2		160	*Bacillus firmus*	Bacillales	D16268	98	158/160	Suzuki and Yamasato (1994)
				Bacillus lentus	Bacillales	D16272	98	158/160	Suzuki and Yamasato (1994)
	COM3		161	*Bacillus* sp. PE4	Bacillales	AF500320	97	157/161	Soil
				Uncultured bacterium clone F5 824-08	Bacteria	AB059467	96	156/161	Sludge compost (Ueno et al. 2001)
	COM4		160	*Paenibacillus kribbensis*	Bacillales	AF391123	96	155/160	
				Paenibacillus amylolyticus	Bacillales	D85396	96	154/160	Shida et al. (1997)
	COM5		136	Uncultured Chloroflexaceae group bacteriu	Green nonsulfur bacteria	AJ309635	92	120/130	Nübel et al. (2001)

PC, Principal component; (+), positive value; (-), negative value; #, ##, ###, specific for rice straw materials, rice straw compost at the thermophilic stage, and at the middle and curing stages, respectively

Phylogenetic position of characteristic DGGE bands

Closest relatives of the DGGE bands with positively and negatively large Eigen values in the 1st and 2nd principal components are listed in Table 1. Closest relatives of the common bands found throughout the period of the composting process and of the specific bands at the respective stages of composting are also shown in the Table (see footnote in the Table).

As indicated in Table 1, most of the DGGE bands with positively and negatively large Eigen values in the 1st principal component corresponded to Gram-positive and Gram-negative bacteria, respectively. As for the 2nd principal component, most of the bands with positively and negatively large Eigen values also corresponded to Gram-positive and Gram-negative bacteria, respectively. Most of the common DGGE bands corresponded to Gram-positive bacteria.

RS materials used. As mentioned before, bands J, O, G, N, M, H, and A were considered to be specific to the initial RS materials. Band G was probably derived from the chloroplast of RS. Most of these specific bands (bands J, M, H, and A) corresponded to Gram-negative bacteria: division Proteobacteria for bands J, M, and H, and Sphingobacteria (member of CFB group) for band A. Band N was the only specific band that corresponded to Gram-positive bacteria (actinomycetes).

Thermophilic stage. Bands Q, R, S, T, L, F, I, and C were specific at the thermophilic stage. They appeared with a high intensity from the thermophilic stage and then decreased in intensity at the following stage. All of their closest relatives were found to correspond to Gram-positive bacteria. Bands Q, R, S, and T were closely related to the family Streptosporangiaceae of the thermophilic actinomycetes. Bands L, F, and C corresponded to the thermophilic bacteria belonging to the order Bacillales. Band I also corresponded to the order Bacillales but was related to mesophilic bacteria.

Middle and curing stages. Bands B, K, P, E, and D were specific at the middle and curing stages. The difference in intensity of the DGGE bands with positively and negatively large Eigen values in the 1st principal component enabled to separate the phase after the thermophilic stage into the middle and curing stages. At the middle stage, especially on day 39 and 47, the bands with the positively large Eigen values in the 1st principal component (bands Q, R, S, and T) reappeared or showed an increase in intensity, which tended to put this stage on the upper-right side (the 1st quadrant) in Fig. 3. Then, at the curing stage, the decrease in intensity of bands Q, R, S, and T and the reappearance of bands M and A (negatively large Eigen values in the 1st principal component) tended to put the curing stage on the upper-left side (the 2nd quadrant) in Fig. 3.

Bands B and P corresponded to Gram-negative *Cytophaga* (member of CFB group) and green nonsulfur bacteria, respectively. Bands D and E were derived from anaerobic bacteria belonging to Gram-positive bacteria (clostridia).

Common bands. Band COM1 which corresponded to *Spirochaeta zuelzerae* (Spirochetes) within Gram-negative bacteria showed an increase in intensity at the curing stage, especially from day 103 to the end of composting. Three of the common bands, COM2, COM3, and COM4 corresponded to the order Bacillales, Gram-positive bacteria. A sole common band (band COM5) was related to uncultured bacteria of the Chloroflexaceae group (member of green nonsulfur bacteria).

DISCUSSION

Succession of bacterial communities during the composting process of RS. The structural and numerical changes in the DGGE pattern from the initial RS materials to the end product of composting reflected the changes in the diversity and composition of the bacterial communities, which were probably influenced by the changes in environmental factors such as temperature, pH, and substrates (organic constituents of compost) (Cahyani et al. 2002). Compared with the materials before composting, RS under the composting process exhibited more complex band patterns with more numerous bands and more diversification in intensity, which suggested that the diversity of the bacteria responsible for the composting process of RS was higher and their physiological status was more complex than that of the initial RS materials.

Cluster analysis and principal component analysis of the DGGE patterns of the bacterial communities in the initial RS materials and RS under the composting process enabled to divide them into four groups as follows: those characterizing the communities associated with the pre-composting stage, and thermophilic, middle, and curing stages of the compost. Exactly the same grouping was obtained from cluster analysis and principal component analyses of the PLFA composition for the same samples (Cahyani et al. 2002).

As the community structure of the microbiota revealed by PLFA analysis included not only bacteria but also eukaryotes (Zelles 1999), the correspondence with PLFA analysis indicated that bacteria contributed predominantly to the microbial communities during the composting process of RS, although the proportion of biomarkers of eukaryotes was relatively high in the materials before composting and RS compost at the curing stage (Cahyani et al. 2002). Phylogenetic composition of fungal and other eukaryotic microbial communi-

ties remains to be elucidated during these periods.

Bacteria responsible for composting process of RS.

Common bacteria throughout the composting process: Three out of five common bacteria in the bacterial communities from the initial RS materials and RS under the composting process belonged to Bacillales. As both RS samples (45 d before composting and on day 0) were nearly air-dried, the DNA of the *Bacillus* members was considered to have been extracted mainly from spore forms. Therefore, the procedure used in the present experiment seemed to have extracted the DNA from bacteria under both active and resting / spore states. All the closest relatives affiliated to this order have been characterized as mesophilic bacilli (Slepecky and Hemphill 1992; Suzuki and Yamasato 1994; Shida et al. 1997). *Bacillus* spp. are spore formers, resistant to dry as well as thermal conditions, and also saccharolytic in general (Slepecky and Hemphill 1992). These characteristics of *Bacillus* spp. seemed to have resulted in their ubiquitous presence in the current study. In several studies on the composting process, the presence of *Bacillus* spp. which were found both at the mesophilic and thermophilic stages of composting (Strom 1985a, b; Peters et al. 2000) was also reported.

One of the common bacteria was related to *Spirochaeta zuelzerae*, an obligate anaerobic species of the genus *Spirochaeta* (division of Spirochetes) (Paster et al. 1991; Canale-Parola 1992). *Spirochaeta* are saccharolytic and they grow abundantly when the energy source available to them is cellobiose, a major product of cellulose depolymerization (Canale-Parola 1992). Two groups of saccharolytic bacteria (Bacillales and *Spirochaeta*) were considered to be involved in the composting of RS.

Specific bacteria in the initial RS materials: Specific bands in the bacterial communities in the initial RS materials were assigned to mainly Gram-negative bacteria (bands J, M, A, and H), which was in agreement with the results of PLFA analysis (Cahyani et al. 2002). The proportion of branched-chain PLFAs (biomarkers of Gram-positive bacteria; 5.8 to 6.5%) was smaller than that of mono-unsaturated PLFAs (biomarkers of Gram-negative bacteria; 20.0 to 28.7%).

In the present study, band G was derived from the chloroplast of *Oryza sativa* (Hiratsuka et al. 1989). Chelius and Triplett (2001) also recognized the amplification of the DNA from the mitochondria and chloroplast of *Zea mays* L. using the set of primers for eubacteria. As shown in Fig. 1, band G appeared with a strong intensity in the RS materials taken 45 d before the onset of piling and the intensity decreased in the RS materials taken on day 0 of composting. The band disappeared in the first week of the composting process, indicating the rapid decomposition of rice 16S rDNA within the first week, as observed for PLFAs derived from rice within 3 d under submerged soil incubation (Kimura et al. 2001).

Band A was related to *Sphingobacterium multivorum*, which was characterized as an aerobic, mesophilic, chemoorganotrophic bacterium (Holmes 1992). *S. multivorum* was found at the late stages (cooling-maturing stages) during the composting of food scraps, but its existence was not examined at the initial stage, because of the failure of PCR amplification of the sample on day 0 (Ishii et al. 2000). In the present study, band A appeared in the initial RS materials and at the middle and curing stages of composting (Fig. 1).

Bands J and H were related to the group of rhizobia, and band M to the genus *Pantoea* which contains aerobic / facultative anaerobic, mesophilic bacteria (Brenner 1992; Elkan and Bunn 1992; Kerr 1992; Sproer et al. 1999; Baker et al. 2001). Their coexistence with living rice plant may be related to the plant associative or pathogenic characters of some of the members within these groups, but further investigations should be carried out.

Among the specific bands in the initial RS materials, band N was the only band which was derived from Gram-positive bacteria. This band was closely related to the genera *Aureobacterium / Microbacterium* of actinomycetes which were characterized as aerobic, mesophilic / thermoduric bacteria (Collins and Bradbury 1992). Their role and activity during the storage period of RS materials remain to be elucidated.

Specific bacteria at the thermophilic stage: As shown in Table 1, almost all the closest relatives of the specific bands at the thermophilic stage were also found in the composting environments, such as mushroom compost (bands R, S, T, and Q), swine waste (bands L, F, and C), and compost from garbage (bands F and C). Two thermophilic strains isolated from the same source of swine waste, *Bacillus* sp. S13 (Accession No. AF403022) and *Ureibacillus thermosphaericus* strain S11 (Accession No. AF403021) (Gagne et al. 2001) were detected in the present composting process as the closest relatives of the bacteria characterized by band L and bands F and C, respectively.

All the specific bacteria at the thermophilic stage were estimated to belong to *Bacillus* and actinomycetes as their closest relatives (Table 1). Simultaneous increase in the proportion of branched-chain PLFAs (biomarkers of Gram-positive bacteria) and 10 Me-PLFAs (biomarkers of actinomycetes) at this stage also supported this assumption (Cahyani et al. 2002).

In contrast to the mesophilic *Bacillus* strains found as common bacteria throughout the composting process, the closest relatives of *Bacillus* at this stage that predominated were thermophilic strains (Anderson et al. 1995; Gagne et al. 2001; Haruta et al. 2002b), with the

sole exception of band I which was related to mesophilic *Oxalophagus oxalicus* (Zaitsev et al. 1998). Irrespective of the types of materials or the composting process, in many studies using molecular 16S rDNA / RNA methods, it was also reported that thermophilic *Bacillus* predominated at the thermophilic stage of composting as follows: the composting processes of food wastes (Ishii et al. 2000; Dees and Ghiorse 2001), family kitchen garbage (Haruta et al. 2002b), a mixture of RS, sugar cane dregs, and chicken / pig / cattle feces (Haruta et al. 2002a), mushrooms (Peters et al. 2000), a mixture of grass, kitchen and garden wastes, and shredded wood (Blanc et al. 1999), agricultural and industrial wastes (grass, bread, meat, and tofu) (Pedro et al. 2001), and sludge (Ueno et al. 2001). Based on the culture method, Strom (1985b) also reported that out of 652 randomly picked colonies of thermophilic bacteria in the composting of dried food scraps and shredded newspaper, 87% of the isolates were identified as *Bacillus* spp.

All the closest relatives of actinomycetes found at this stage were identified as strains of the family Streptosporangiaceae. These strains were characterized as thermophilic actinomycetes which had been isolated from mushroom compost (Song et al. 2001). An interesting finding was that these actinomycetes were still present when the temperature of the compost pile decreased until the end of the composting process (Fig. 1).

In the present study, whitening of the compost pile was observed at the thermophilic stage. Fergus (1964) indicated that this phenomenon was due to the growth of thermophilic actinomycetes. In earlier studies, the proliferation of thermophilic actinomycetes at the thermophilic stage of composting, either based on culture methods (Waksman et al. 1939; Fergus 1964) or molecular 16S rDNA methods (Peters et al. 2000; Dees and Ghiorse 2001) was also reported.

The thermophilic bacteria at the thermophilic stage may play a role in the decomposition of the hemicellulose and cellulose fractions in the composting of RS, as described previously (Cahyani et al. 2002). Nakasaki et al. (1985) suggested that two groups of microorganisms contributed to composting at the thermophilic stage by attributing the CO_2 evolution rate to the activity of thermophilic bacteria at the initial thermophilic stage and to the activity of thermophilic actinomycetes at the late thermophilic stage. Stutzenberger et al. (1970) and Waksman et al. (1939) reported that thermophilic actinomycetes may possibly display a cellulolytic activity.

Specific bacteria at the middle and curing stages: Specific bands were estimated to correspond to *Cytophaga*, green nonsulfur bacteria, and clostridia at the middle and curing stages. As all the members of Cytophagales are strict aerobes except for the Capnocytophagas (Reichenbach 1992) and clostridia which are strict anaerobes, the high moisture content at these stages in the present composting process (80 to 85%) seemed to have provided both aerobic and anaerobic conditions in the composting pile.

The members of *Cytophaga* are cellulose decomposers (Reichenbach 1992), and the clostridia estimated were affiliated to the group of mesophilic strains that enable to ferment various carbohydrates, including polymers like cellulose and xylan (Hippe et al. 1992). Therefore, both of them seemed to contribute to cellulose decomposition at these stages. Cahyani et al. (2002) revealed that the cellulose fraction was mainly decomposed at a relatively constant rate during the composting process of the same RS materials as those used in the present experiment.

Band E corresponded to the closest relative of the strain that was isolated from anoxic paddy soil (*Clostridia* sp. XB90 with Accession No. AJ229234) (Chin et al. 1999; Hengstmann et al. 1999). Many authors reported that clostridia predominated in the bacterial communities during RS decomposition in anoxic paddy soil (Chin et al. 1998, 1999; Hengstmann et al. 1999; Weber et al. 2001). Several clones from incubated RS in anaerobic soil slurries were also distinctly related to this anoxic paddy soil isolate XB90 (Weber et al. 2001). Therefore, contamination of RS materials with soil may have resulted in the decomposition of RS cellulose by the clone related to the soil clostridia in the present study.

A member of green nonsulfur-bacteria *Roseiflexus castenholzii* was detected as the closest relative of the bacteria characterized by band P at these stages. This strain was phylogenetically related to *Chloroflexus* spp. (Nübel et al. 2001). Thus, bacteria characterized by band P displayed the same affiliation as that of the uncultured *Chloroflexus* strain detected as a common bacterium in the present study (band COM5). These bacteria that contain bacteriochlorophyll and are metabolically versatile bacteria are capable of anoxygenic photoheterotrophy and aerobic chemoheterotrophy (Pierson and Castenholz 1992; Nübel et al. 2001; Hanada et al. 2002). Their contribution to the composting process has not been elucidated yet.

In conclusion, the composting process of RS was divided into four stages as follows: pre-composting, thermophilic, middle, and curing stages, and different bacterial members characterized the respective stages, namely α-Proteobacteria for the initial RS materials, thermophilic *Bacillus* spp. and actinomycetes at the thermophilic stage, and *Cytophaga* and clostridial members at the middle and curing stages, respectively. In addition, mesophilic *Bacillus* members were always present throughout the composting process. Temperature and substrates available to the bacteria seemed mainly to

determine the composition of the bacterial members at the respective stages and further investigations should be carried out to elucidate their role and activity during the composting process.

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