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Succession and Phylogenetic Profile of Methanogenic Archaeal Communities during the Composting Process of Rice Straw Estimated by PCR-DGGE Analysis

Vita Ratri Cahyani[1], Kazuo Matsuya*, Susumu Asakawa, and Makoto Kimura

*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464–8601 Japan; and *Aichi-ken Anjo Research and Extension Center, Anjo, 446–0073 Japan*

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The succession and phylogenetic profile of methanogenic archaeal communities for rice straw (RS) in the composting process were studied by polymerase-chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) analysis followed by sequencing. Three groups of DGGE bands of methanogenic archaeal communities appeared successively in the present composting process. The first group of DGGE bands characterized the communities that were probably associated with soil contamination and survived during the early stage of composting. The second and the third groups of DGGE bands characterized the communities that proliferated and played a role in the anaerobic decomposition of RS during the middle and curing stages. Methanogenic archaeal communities were detected at every stage of rice straw composting except for one sampling time on day 14 at the end of the thermophilic stage. Among the 12 sequenced DGGE bands, 7 bands corresponded to Methanomicrobiales, 4 bands to novel uncultured euryarchaeota belonging to "Rice Cluster I," and one band to Methanosarcinales. Two DGGE bands which appeared from day 28 of the second temperature peak (48°C) to the end of composting corresponded to the thermophilic strain of *Methanoculleus thermophilus* CR-1. Six bands corresponded to the methanogens that originated from paddy field soils, which indicated that methanogens in RS compost were derived mainly from rice fields.

***Key Words*: compost, DGGE, methanogen, phylogeny, rice straw, succession.**

Although the effect of the application of rice straw (RS) compost on methane emission from rice fields was significantly less conspicuous than that of RS (Yagi and Minami 1990; Corton et al. 2000), methanogenic populations were higher in the rice field with RS compost application than in the field with RS application (Asakawa et al. 1998). RS compost not only contributes to methanogenesis in soil as substrate, but also it may be colonized by a considerable number of methanogenic archaea.

Methanogenic activity during the composting process was demonstrated by the presence of methane in the air evolving from various compost materials (Derikx et al. 1988, 1989; Lopez-Real and Baptista 1996; Eleazer et al. 1997; Hellmann et al. 1997; Miura 2000; Sommer and Moller 2000; Zeman et al. 2002). Furthermore, Hellmann et al. (1997) reported that the amount of ether lipids increased with elevated methane emission in the composting process of a mixture of municipal and yard waste, indicating the enhanced growth of methanogenic archaea. Asakawa et al. (1998) reported that the number of methanogens in RS compost was about 10^8 g^{-1} dry matter, based on the cultivation method. Derikx et al. (1989) also isolated a high number of thermophilic methanogenic archaea, "*Methanobacterium thermoautotrophicum*" (presently *Methanothermobacter thermoautotrophicus*), from the compost pile during the first stage of preparation of mushroom compost.

Although composting is considered to be an aerobic decomposition process (Finstein and Morris 1975), reports on the presence and activitiy of methanogenic archaea in the composting process, as described above, indicated that anaerobic microenvironments were developed in compost piles. This estimation was supported by the presence of bacteria that corresponded to strictly anaerobic *Spirochaeta zuelzerae* and clostridia in the

[1] Present address: Soil Biology Laboratory, Department / Study Program of Soil Science, Faculty of Agriculture, Sebelas Maret University, Jl. Ir Sutami No. 36A Kentingan Surakarta, Jawa Tengah 57126, Indonesia.

composting of RS (Cahyani et al. 2003).

Although the increase in methane emission from the RS field by RS compost application and the presence and activity of methanogens in various compost materials have been revealed, there is no information on the methanogenic archaeal communities during the composting process of RS. This phenomenon calls for a phylogenetic study on methanogenic archaea during the composting process of RS. Therefore, the present study aimed at elucidating the succession and phylogenetic profile of methanogenic archaeal communities during the composting process of RS by polymerase chain reaction (PCR)–denaturaing gradient gel electrophoresis (DGGE) analysis that was followed by sequencing.

MATERIALS AND METHODS

All the samples used in the present study were the same as those used in the three previous studies, in revealing the succession of biota during the composting process of RS by using PLFA analysis (Cahyani et al. 2002) and PCR-DGGE analysis followed by sequencing, for elucidating the succession of bacterial communities (Cahyani et al. 2003) and for eukaryotic communities (Cahyani et al., in press).

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

Setup of the compost pile and sampling method. Composting was performed based on a conventional method used in Japan with RS and ammonium sulfate ($[NH_4]_2SO_4$) as materials (Cahyani et al. 2002). The size of the compost pile was approximately 210 cm long, 180 cm wide, and 80 cm high. 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) maintained in a storage facility for compost material was collected 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 and purification. DNA extraction and purification were carried out according to the methods described in the previous study (Cahyani et al. 2003). During the composting process, five subsamples were collected at every sampling time. All the procedures were performed in duplicate for the RS materials samples and for a mixture of five subsamples of RS compost from each sampling time.

PCR-DGGE analysis. The methanogenic archaeal 16S rDNA was amplified with PCR using a set of two primers, 0357F with GC clamp (*Escherichia coli* position: 340–357, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCTACGGGGCGCAGCAG-3′, the underlined sequence corresponded to the GC clamp) and 0691R (*E. coli* position: 707–691, 5′-GGATTACARGATTTCAC-3′) (Watanabe et al. 2004) which were modified primers from the PARCH-340f forward primer (Øvreås et al. 1997) and 0690aR reverse primer (Achenbach and Woese 1995), respectively. PCR was performed in a total volume of 50 μL in a 200-μL microtube, which contained 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mM of dNTP mixture, 5 μL of 10× *Ex Taq* buffer (20 mM Mg^{2+}; TaKaRa, Tokyo, Japan), 0.5 μL of 5 units μL^{-1} *Ex Taq* DNA polymerase (TaKaRa), 1.3 μL of DNA template (about 15 ng) and 37.2 μL of milli-Q water. Cycle conditions for the amplification corresponded to the PCR program 1 as follows: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 8 min, with TaKaRa PCR Thermal Cycler Model TP 240 (TaKaRa). The PCR product was analyzed on 2% (w / v) agarose gels, as described previously (Cahyani et al. 2003). The DNA fragments of the PCR products were separated on a DGGE gel which was prepared according to the method of Muyzer et al. (1998) with a denaturing gradient from 25 to 65%. A mixture of PCR products from genomic DNAs of 13 methanogenic archaeal strains was also used for the positive control. The electrophoresis was conducted in an electrophoresis cell D-code™ System at 60°C and 100 V for 14 h. Visualization of the DGGE bands was performed as described previously (Cahyani et al. 2003). We carried out DGGE for the PCR products of a duplicated mixture of five subsamples and found exactly the same patterns for each duplication at each sampling time. Therefore, a single replicate for each sampling time is shown in Fig. 1.

Statistical analysis. To estimate the succession of methanogenic archaeal communities during the composting process of RS, the data obtained from the DGGE patterns, based on the band intensity and position, were subjected to cluster analysis and principal component analysis as described previously (Cahyani et al. 2003).

Direct sequencing. All the DGGE bands with different mobilities (2 or 3 replications) from different lanes were excised from the polyacrylamide gel. The DNA in the excised gel strip was eluted in 50 μL TE buffer at 4°C overnight. The eluted DNA was amplified with a set of two primers, 0357F with GC clamp and 0691R using the PCR program 2 as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of

denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 8 min. The PCR products were subjected to mobility check by comparing the position with the original DGGE pattern. As for the PCR products that matched the position with the original bands, the primer pair without GC clamp (0357F and 0691R) was applied to the same eluted DNA for the amplification of the template used in the subsequent sequencing with the PCR program 3 as follows: initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 8 min. Sequencing was performed with a 373S DNA Automated Sequencer (Applied Biosystems, Chiba, Japan) using the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions, by using two primers, 0365F (5′-GCAGCAGGCGCGAAA-3′) and 0691R (Watanabe et al. 2004).

Cloning and sequencing. Cloning was performed before sequencing for the bands which failed to be sequenced directly. DNA eluted from the excised DGGE band was amplified with a set of two primers, 0357F with GC clamp and 0691R using the PCR program 2, as described above. Cloning and transformation were conducted by using the pT7 Blue T-vector (Novagen, Darmstadt, Germany) and competent cells of *E. coli* XLI blue (TaKaRa), as described previously (Cahyani et al. 2003). PCR was performed for the formed colonies using a set of two primers, 0357F with GC clamp and 0691R, with the PCR program 2 as 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 conducted in order to select the correct clones which matched the target bands for sequencing. Plasmid extraction from the selected clones and sequencing were performed as described previously (Cahyani et al. 2003) and above. Among the sequenced bands, only two bands were cloned before sequencing and the others were successfully sequenced directly. We analyzed two clones (for each band) which showed the same mobility as the original excised band and we obtained the same sequences. By direct sequencing, the same sequences were also obtained for the excised bands with the same mobility from different lanes (at least two lanes).

Phylogenetic analysis. Sequences of the DGGE bands were compared to the archaeal 16S rDNA sequences obtained by the BLAST search from the database of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/E-mail/homology.html) to determine the closest relatives and phylogenetic affiliation. A phylogenetic tree was constructed for the obtained DGGE sequences and the sequences of closest relatives deposited at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) by the neighbor-joining method using the Clustal W program.

Nucleotide sequence accession number. The archaeal 16S rDNA partial sequences obtained in this study are available in the DDBJ database under the accession numbers AB159051–AB159062.

RESULTS AND DISCUSSION

DGGE band patterns of methanogenic archaeal communities during the composting process of RS

The succession of methanogenic archaeal communities in the composting process of RS was observed in the DGGE band patterns shown in Fig. 1. No band was detected in the sample on day 14 due to failure of PCR amplification. This indicated that there was no or a very small amount of 16S rDNA of methanogenic archaea in the sample on that day because bacterial 16S and eukaryotic 18S rDNAs were successfully amplified from the same DNA sample (Cahyani et al. 2003, in press) (see also the discussion below). A total of 12 different bands were observed in the DGGE image. The number of DGGE bands ranged from 6 to 9 for the RS materials and RS under the composting process (Fig. 2). The closest relatives associated with the respective DGGE bands and the phylogenetic relationships among them are presented in Fig. 3 and Table 1.

The number of total different bands observed in the DGGE patterns of the methanogenic archaeal communities was considerably lower than that of the bacterial

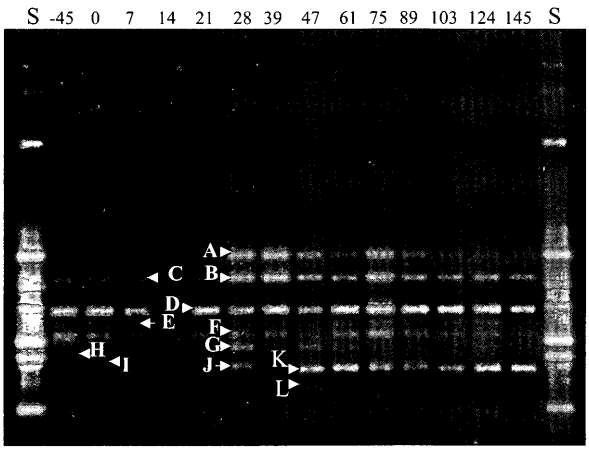

Fig. 1. DGGE pattern of the methanogenic archaeal communities associated with the composting process of rice straw. Numbers above the lane denote the composting time (d). S indicates the DGGE pattern of a mixture of PCR products from genomic DNAs of 13 methanogenic archaeal strains.

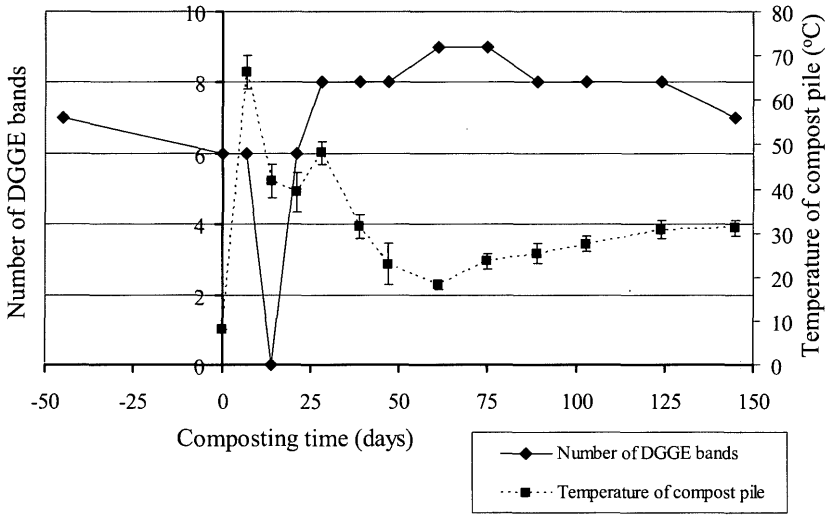

Fig. 2. Number of bands in the DGGE pattern of the methanogenic archaeal communities associated with the composting process of rice straw. The temperature curve is superimposed.

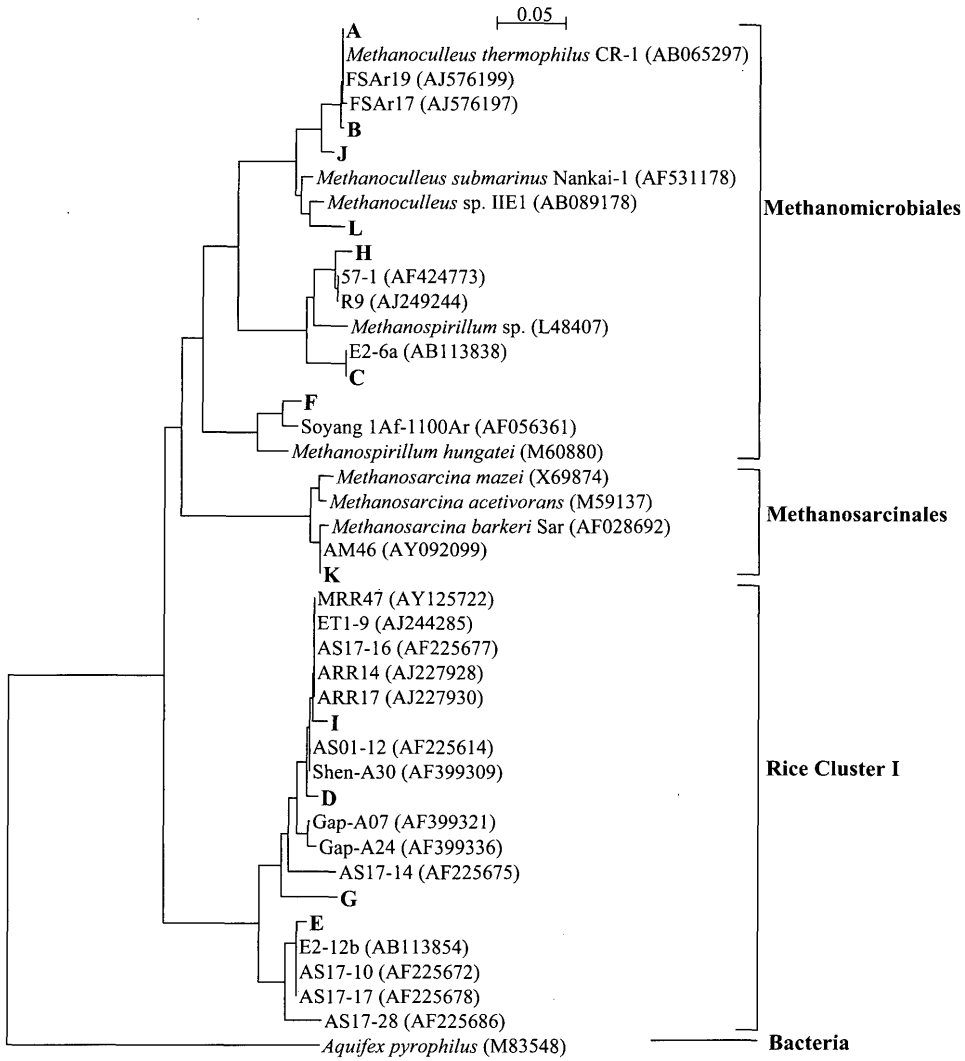

Fig. 3. Neighbor-joining tree showing the phylogenetic relationship among the DGGE bands characterizing the methanogenic archaeal communities associated with the composting process of rice straw and their closest relatives. The scale bars represent 5% estimated difference in nucleotide sequence position. *Aquifex pyrophilus* was used as the outgroup.

and eukaryotic communities (57 and 88, respectively) (Cahyani et al. 2003, in press). The present findings suggested that the methanogenic archaeal communities in RS during the composting process displayed a lower diversity than the bacterial and eukaryotic communities there.

Table 1. Closest relatives of the methanogenic archaea associated with the composting process of rice straw that were characterized by respective DGGE bands.

DGGE band	PCA analysis Eigen value	Seq bp	Closest relatives			Similarity (%)	Alignment	Source and reference
			Microorganisms	Phylogenetic affiliation	Accession number			
A	1st PC (+) 0.380	280	*Methanoculleus thermophilus* CR-1	Methanomicrobiales	AB065297	100	280 / 280	Rivard and Smith 1982
			Uncultured archaeon FSAr19	Methanomicrobiales	AJ576199	100	280 / 280	food soil of *Pachnoda ephippiata* larva (Egert et al. 2003)
B	1st PC (+) 0.419	280	*Methanoculleus thermophilus* CR-1	Methanomicrobiales	AB065297	99	279 / 280	Rivard and Smith 1982
			Uncultured archaeon FSAr19	Methanomicrobiales	AJ576199	99	279 / 280	food soil of *Pachnoda ephippiata* larva (Egert et al. 2003)
C	1st PC (−) −0.425	280	Uncultured archaeon E2-6a	Methanomicrobiales	AB113838	100	280 / 280	paddy field soil
D		284	Uncultured euryarchaeote Shen-A30	Rice cluster I	AF399309	98	281 / 284	rice field soil (Ramakrishnan et al. 2001)
			Uncultured archaeon AS01-12	Rice cluster I	AF225614	98	281 / 284	rice field soil (Lueders and Friedrich 2000)
E		284	Uncultured archaeon AS17-17	Rice cluster I	AF225678	99	282 / 284	rice field soil (Lueders and Friedrich 2000)
			Uncultured archaeon E2-12b	Rice cluster I	AB113854	99	282 / 284	paddy field soil
F		280	Uncultured archaeon Soyang 1Af-1100Ar	Methanomicrobiales	AF056361	97	264 / 272	sediment of Lake Soyang
G	2nd PC (+) 0.307	284	Uncultured euryarchaeote Gap-A24	Rice cluster I	AF399336	95	270 / 284	rice field soil (Ramakrishnan et al. 2001)
			Uncultured euryarchaeote Gap-A07	Rice cluster I	AF399321	95	270 / 284	rice field soil (Ramakrishnan et al. 2001)
H	2nd PC (+) 0.288	281	Uncultured methanogen R9	Methanomicrobiales	AJ249244	98	276 / 281	petroleum-contaminated aquifer
			Uncultured archaeon 57-1	Methanomicrobiales	AF424773	98	276 / 281	municipal wastewater sludge
I	1st PC (−) −0.412	284	Unidentified archaebacteria ARR17	Rice cluster I	AJ227930	99	281 / 283	rice root (Großkopf et al. 1998)
			Anaerobic methanogenic archaeon ET1-9	Rice cluster I	AJ244285	99	281 / 283	stable cellulose-degrading enrichment culture (Chin et al. 1999)
			Uncultured archaeon AS17-16	Rice cluster I	AF225677	99	281 / 283	rice field soil (Lueders and Friedrich 2000)
J	2nd PC (+) 0.642	280	Uncultured archaeon FSAr17	Methanomicrobiales	AJ576197	97	274 / 280	food soil of *Pachnoda ephippiata* larva (Egert et al. 2003)
K	2nd PC (−) −0.503	284	Uncultured archaeon AM46	Methanosarcinales	AY092099	100	284 / 284	anaerobic digester for pulp and paper mill solid waste
L	2nd PC (−) −0.278	280	*Methanoculleus submarinus* Nankai-1	Methanomicrobiales	AF531178	95	265 / 278	marine sediments
			Methanoculleus sp. IIE1	Methanomicrobiales	AB089178	94	264 / 278	paddy field soil

1st PC(+), positive large Eigen value in the first Principal Component; 1st PC(−), negative large Eigen value in the first Principal Component; 2nd PC(+), positive large Eigen value in the second Principal Component; 2nd PC(−), negative large Eigen value in the second Principal Component.

Statistical analysis of the DGGE patterns of the methanogenic archaeal communities in the initial RS materials and RS under the composting process included principal component analysis and cluster analysis. Principal component analysis showed that the total contribution percentages of the 1st and 2nd principal components were 56 and 22%, respectively. The DGGE bands with positive and negative large Eigen values in the 1st principal component were Bands B and A, and Bands C and I, respectively. On the other hand, the DGGE bands with positive and negative large Eigen values in the 2nd principal component were Bands J, G, and H, and Bands K and L, respectively. The distribution of the score plots of the DGGE bands of methanogenic archaeal communities during the composting process is shown in Fig. 4.

Cluster analysis identified three clusters for the DGGE patterns of the methanogenic archaeal communities in the initial RS materials and RS under the composting process (Fig. 5). The DGGE patterns were first divided into two clusters, the first cluster for the communities associated with the initial RS materials and the RS composts on days 7 and 21, and the second cluster for the communities associated with RS composts from day 28 onwards. The second cluster was further divided into two sub-clusters as follows: communities associated with RS composts on days 28 and 39, and RS composts from day 47 onwards. The domain of each cluster is shown in Fig. 4.

In our previous studies, the phospholipid fatty acid (PLFA) composition and the DGGE patterns of the bacterial and eukaryotic communities for the same samples were statistically divided exactly into the same four groups, namely those characterizing the communities associated with the pre-composting stage (initial RS materials on days −45 and 0), and thermophilic (RS composts on days 7 and 14), middle (RS composts on days 21, 28, 39, and 47), and curing stages of the compost (RS composts on days 61, 75, 89, 103, 124, and 145) (Cahyani et al. 2002, 2003, in press). In addition, the bacterial and eukaryotic members that characterized the respective stages corresponded to the dominant biomarker PLFAs for every stage. However, the present study revealed a different grouping of DGGE patterns for the methanogenic archaeal communities from the previous one. The difference in the grouping was considered to be firstly due to the lack or scarcity of thermophilic methanogens that were able to survive at the peak temperature (66°C, Fig. 2) in the RS composting process, which was suggested from the absence of amplification of 16S rDNA in the sample on day 14 (see also the discussion below). As a result, the communities were divided into two main clusters before and after the thermophilic stage. Secondly, the difference in grouping was considered to be due to the DGGE patterns of the methanogenic archaeal communities representing the anaerobic microbial succession in the composting process of RS, and those of the bacterial and eukaryotic communities representing the aerobic, anaerobic, and facultative anaerobic communities. In addition, it is

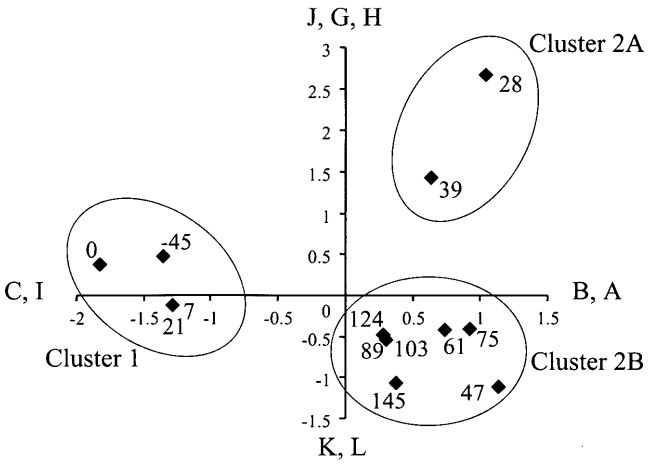

Fig. 4. Principal component analysis of the DGGE patterns of the methanogenic archaeal communities associated with the composting process of rice straw. Numbers next to the symbol denote the composting time (d).

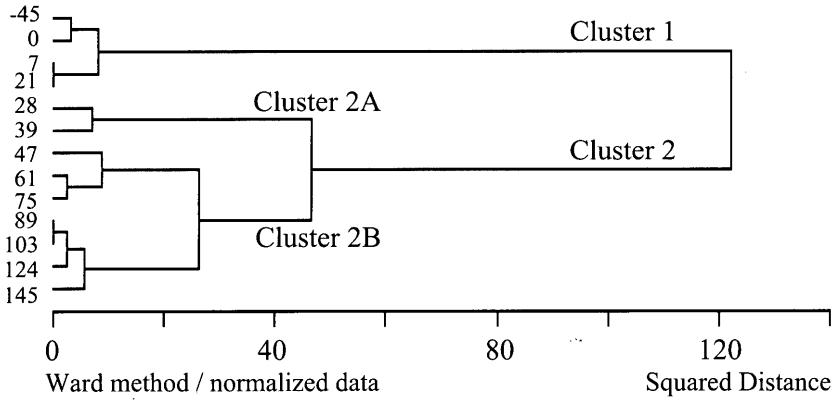

Fig. 5. Cluster analysis of the DGGE patterns of the methanogenic archaeal communities associated with the composting process of rice straw. Numbers next to the cluster denote the composting time (d).

important to note that the squared distance of cluster analysis for the DGGE pattern of methanogenic archaeal communities was very small. Therefore, the apparent difference observed in Fig. 5 was smaller than that for the bacterial and eukaryotic communities.

Succession and phylogenetic profile of methanogenic archaeal communities

Based on the DGGE image shown in Fig. 1 and the distribution of the score plots of the DGGE bands of the methanogenic archaeal communities in the initial RS materials and RS under the composting process depicted in Fig. 4, the grouping, succession, and phylogenetic profile of the methanogenic archaeal communities during the composting process of RS were summarized as follows:

First group of communities. The first group of methanogenic archaeal communities consisted of the communities associated with RS materials taken 45 d before composting (day −45) and on the day of compost piling (day 0), and RS composts on days 7 and 21. The DGGE patterns were nearly identical (Fig. 1). Then, there was no band in the lane of RS compost on day 14. A similar pattern to that of first three lanes appeared for the RS compost on day 21. The score plots of the DGGE bands of the first group communities were located on the middle-left side in Fig. 4. Bands C and I (negative large Eigen values in the 1st principal component) and Band H (positive large Eigen value in the 2nd principal component) characterized the first group of methanogenic archaeal communities. Their closest relatives were the members of Methanomicrobiales (Bands C and H) and Rice Cluster I (Band I) (Fig. 3 and Table 1). The DGGE patterns in the first group of communities consisted of 7 different bands (Fig. 1). Sequence analysis of those bands indicated that 4 bands corresponded to Methanomicrobiales and 3 bands to Rice Cluster I (Fig. 3 and Table 1).

The consistent appearance of the DGGE bands of the methanogenic archaeal communities in both RS materials indicated that the methanogenic archaeal communities in the air-dried RS materials were associated with soil contamination, because the proliferation of the methanogenic archaea on dry RS materials was unlikely. Compared with the DGGE bands in both RS materials, no additional band appeared in the RS compost on days 7 or 21, indicating that no thermophilic methanogens grew during the thermophilic stage (66°C, Fig. 2). Thus, the communities in the first group seemed to represent methanogenic archaea that were associated with soil contamination of RS materials and which survived during the early stage of composting. This finding was not in agreement with the observation of Derikx et al. (1989), who reported the existence of a methanogenic activity by thermophilic strains during the thermophilic stage in mushroom compost. They detected on the average 410 ppm of CH_4 in the air evolved above the compost pile at the end of the thermophilic stage (average temperature of 63°C). They also reported that compost samples taken at that time contained up to 2×10^8 thermophilic methanogenic archaea per gram dry matter and that the isolated methanogenic archaea were identified as strains of "*Methanobacterium thermoautotrophicum*" (presently *Methanothermobacter thermoautotrophicus*). The optimal temperature and the pH range for growth were reported to be 65–70°C and 6.0–8.8, respectively, for this species (Wasserfallen et al. 2000). Hellmann et al. (1997) also observed that the amount of ether lipids increased with the elevated CH_4 emission in the composting process of municipal and yard waste mixture, when the temperature was around 60°C.

As shown in Figs. 1 and 2 (the temperature curve is superimposed), the DGGE bands appeared on day 7 when the highest temperature of the compost pile was monitored, followed by the disappearance of the DGGE bands on day 14. These findings were attributed to the retrieval of methanogenic archaeal DNA on day 7, although the methanogenic archaeal communities were estimated to be sterilized at that time. Then, the DNA of dead methanogenic archaeal communities was decomposed by day 14. Turning of the compost pile that was performed immediately after the sampling of day 14 might have incorporated the methanogenic biomass from the outer part of the compost pile, where methanogenic archaea had avoided the lethal high temperature during the thermophilic stage, into the center, resulting in the detection of the same DGGE bands on day 21 as those on day 7.

Bands C, D, E, and I were closely related to the uncultured methanogens originating from paddy field environments, either from paddy field soil (Chin et al. 1999; Lueders and Friedrich 2000; Ramakrishnan et al. 2001; Watanabe et al. 2004) or rice roots (Großkopf et al. 1998). This finding also indicated that methanogenic archaeal communities in the RS compost were derived from rice fields.

Second and third groups of communities. The second and the third groups of methanogenic archaeal communities in the composting process of RS consisted of the communities associated with RS composts on days 28 and 39, and RS compost from day 47 onwards, respectively. As shown in Fig. 1, the DGGE patterns of the second and the third communities were slightly different from each other. The score plots of the DGGE bands of the second and the third communities were located on the right-side in Fig. 4, the second group in the 1st quadrant, and the third group in the 4th quadrant, respectively. Bands B and A (positive large

Eigen values in the 1st principal component) were considered to characterize the communities in these two groups which were placed in the same cluster (cluster 2). Then, different specific bands separated these two groups into the different 2A and 2B sub-clusters: Bands J and G characterized the second group and Bands K and L characterized the third group, respectively. These characteristic bands corresponded to the members of Methanomicrobiales (Bands A, B, J, and L), Rice Cluster I (Band G), and Methanosarcinales (Band K).

The DGGE patterns in the second and third communities consisted of 8 and 9 different bands, respectively (Fig. 1). Among the 8 different bands found in the second group, 5 bands corresponded to Methanomicrobiales and 3 bands to Rice Cluster I. Sequence analysis of 9 different bands detected in the third group indicated that 5 bands corresponded to Methanomicrobiales, 3 bands to Rice Cluster I, and 1 band to Methanosarcinales (Fig. 3 and Table 1). Among the sequenced bands, Bands A and B corresponded to *Methanoculleus thermophilus* CR-1, which was used as the positive control in the DGGE pattern analysis. Band A which displayed the same mobility as that of the DGGE band of this strain showed a 100% similarity, while Band B which migrated at a lower position showed a 99% similarity to the strain (Fig. 1 and Table 1).

The second and the third groups of communities seemed to represent the actual proliferation of methanogenic archaea which played a role in methane production during the composting process. The results indicated that favorable environmental conditions had developed from day 28 for the proliferation of these methanogens. It was interesting to note the appearance of two characteristic DGGE bands (Bands A and B) on day 28 which corresponded to *Methanoculleus thermophilus* CR-1 whose optimal temperature for growth was 55°C, when the second peak of compost temperature (48°C) was recorded. As the lower and upper limits of the temperature for growth of the strain were below 37°C and 65°C, respectively (Rivard and Smith 1982), the proliferation of the strain was expected to occur before the first peak temperature of 66°C. However, the pH at the early stage of RS composting was about 8.4 and was considered to be too high for the active growth of the strain (the optimal pH was 7.0 with an upper limit above 7.8) (Rivard and Smith 1982). This strain utilizes only formate, or hydrogen and carbon dioxide as growth substrates and for methane formation, and is not able to utilize ethanol, methanol, acetate, propionate, and pyruvate (Rivard and Smith 1982). The temperature did not appear to be the sole environmental factor which determined the composition of the subsets of microbial communities at a certain stage of composting.

The reason for the appearance of the characteristic Bands K and L from day 47 remains to be elucidated. This phenomenon suggested that the requirements of environmental conditions and substrates for optimum growth were complex and specific for each strain.

Common methanogenic archaea in the composting process. Three bands (Bands D, E, and F) always appeared from the RS materials until the end of composting except for the sample on day 14 (Fig. 1). Two bands corresponded to Rice Cluster I (Bands D and E), and one band to Methanomicrobiales (Band F) (Fig. 3 and Table 1). As the first group of methanogenic archaeal communities was considered to be associated with soil contamination and survived during the early stage of composting, the actual proliferation of these common methanogenic archaea was estimated to occur concurrently with the second and the third groups of communities as described above.

In addition to two common Bands D and E, Band G in the second group and Band L in the third group of methanogenic communities corresponded to methanogens found in paddy field soils (Lueders and Friedrich 2000; Ramakrishnan et al. 2001). These clones seemed also to be derived from rice field soils. The other bands were related to methanogenic archaea from diverse anaerobic environments, e.g. digester, aquifer or sludge, lake sediment, and guts of soil-feeding macroinvertebrates (Egert et al. 2003). Since the closest relatives corresponding to the respective DGGE bands were dominated by uncultured strains, further characteristics of those methanogens remained unclear.

Although it was reported in several studies that methane emission occurred during the composting process, evidence related to the methanogenic communities responsible for methanogenesis is still limited (Lopez-Real and Baptista 1996; Eleazer et al. 1997; Hellmann et al. 1997; Miura 2000; Sommer and Moller 2000; Zeman et al. 2002). Therefore, methanogenic archaeal communities that were identified in the present study, could provide important information for their control during the RS composting process.

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