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Bacterial Communities Developing during Composting Processes in Animal Manure Treatment Facilities*

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ABSTRACT : We analyzed succession of the bacterial communities during composting of animal manure in three individual facilities. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) targeting for the bacterial 16S rRNA gene were used to clarify the changes of bacterial community throughout each composting process. Our study revealed that the bacterial community structures differed during the composting process. The bacterial community in composting of facility A showed little change throughout the process. In the compost sample from facility B, its community had a small shift as the temperature increased. In compost from facility C, the temperature dynamically changed; it was shown that various bacterial communities appeared and disappeared as follows: in the initial phase, the members of phylum Bacteroidetes dominated; in the thermophilic phase, some bacteria belonging to phylum Firmicutes increased; towards the end, the community structure consisted of three phyla, Bacteroidetes, Firmicutes, and Proteobacteria. This study provides some information about the bacterial community actually present in field-scale composting with animal manure. (**Key Words :** 16S rRNA Gene, Animal Manure, Bacteria, Composting, PCR-DGGE)

INTRODUCTION

Composting is one of the most successful methods for treating organic waste such as animal manure (Harada, 1992). Livestock manure accounts for a large part of the total waste generated and can cause environmental problems (e.g., air, water, and soil pollution) (Hall, 1999); but using composting treatment can reduce these problems (Taiganides, 1977) and apply to agricultural soil as nitrogen fertilizer (Kaku et al., 2004). We focus on changes in microbial community throughout composting process because it is known that microorganisms play important roles in the process of composting by degrading easily decomposable organic matter in the raw material (Haga, 1999; Insam et al., 2002). To follow microbial community whose structure and diversity change dramatically during composting, researchers have been used a variety of culture-based or unculture-based techniques.

Denaturing gradient gel electrophoresis (DGGE) is a useful uncultured method because it can separate DNA fragments amplified by polymerase chain reaction (PCR) according to the differences in base-pair sequences and visualize the bacterial community as a band fingerprint (Muyzer et al., 1993). By using DGGE, some studies observed the changes in bacterial community during composting when synthetic food waste is the raw material (Ishii et al., 2000; Dees and Ghiorse, 2001). Green et al. (2004) analyzed the community in finished compost, which were produced using cow manure with windrow method. However, there are only a few researches that have studied microbial community composition in field-scale composting with animal manure (Sasaki et al., 2009). It is essential to analyze the microbial community in animal manure compost in order to obtain data that will be useful to improve composting in a livestock farm. Indeed, many results obtained by studying microbial communities in compost from other materials (e.g., dog food, food waste, and plant waste) may not always be extrapolated to the communities found in animal manure compost.

In this study, we analyzed the structure of bacterial communities during composting of animal manure in three individual facilities using PCR-DGGE analysis in order to get their succession in composting of animal manure.

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Table 1. Compost samples collected in three facilities

Facility	Raw materials	Aeration	Turnover	Sample No.	Composting step	Temperature (°C)
A	Dairy cattle manure			1	Initial stage	38.1
	Beef cattle manure	No	No	2	Intermediate stage	30.6
				3	End stage	39.1
B	Cattle manure		Yes	1	Initial stage	18.6
	Swine manure	Yes	Twice/week	2	Intermediate stage	28.0
	Sawdust and rice chaff			3	End stage	50.0
C		No	No	1	Initial stage	21.6
	Beef cattle manure			2	Starting of second treatment	60.1
	Sawdust	Yes	Yes	3	Highest temperature phase	67.1
	Rice chaff		Once/day	4	Decreasing temperature phase	52.4
				5	End of second treatment	38.4
		No	No	6	End stage	32.0

MATERIALS AND METHODS

Compost samples

Compost samples were collected from 3 different field-scale composting facilities (Table 1) in December 2004. All the samples were collected from a depth of 20 cm from the top of the compost pile, and the temperature was measured. The steps of the composting process and the temperatures of the collected samples are shown in Table 1.

Composting facility A was the flat- bed type (approximately 6 m×80 m). The moisture content of the raw materials was not adjusted before starting the composting process. In addition, the materials were piled without turning or aeration during winter. Samples were collected at the initial (sample A1), intermediate (A2), and end (A3) stages of the composting process.

Composting facility B had a length of 25 m. The materials were mixed twice every week and aerated from the bottom of the pile. Samples were collected at the initial (B1), intermediate (B2), and end (B3) stages of composting.

Composting facility C had 2 treatment steps. In the first step of treatment, rice chaff was mixed to the beef cow manure and sawdust in order to control the moisture content. Then, materials were piled for 2 weeks without aeration and transferred to the second treatment system, which had a length of approximately 90 m and had appurtenances for aeration. After treatment, compost was piled again for about 2 months. Samples were collected at the following time points: beginning of the first treatment (C1), beginning of the second treatment (C2), the highest-temperature phase (C3), the decreasing-temperature phase (C4), end of the second treatment (C5), and piling (C6).

DNA extraction

DNA was extracted from compost by the methods of Sasaki et al. (2009) modified in this study. One g of each compost sample was added to 10 ml of 10 mM sodium tripolyphosphate and mixed for 10 min. The mixture was then centrifuged at 690×g for 5 min, and the supernatant was then centrifuged at 17,360×g for 2 min to obtain the microorganisms as a pellet. DNA in the sample was completely extracted from the pellet using a MagExtractor-Genome (TOYOBO CO., LTD., Osaka, Japan). Freeze-thawed method (5 times a set of freezing at -85°C for 5 min and thawing with tap water for 3 min) was added in order to destroy bacterial cells efficiently. Finally, the DNA was dissolved in 80 μl of TE buffer (10 mM tris-HCl, 1 mM EDTA) and stored at 4°C till PCR amplification was performed.

PCR amplification

PCR was performed using primer sets 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACC GCGGCTGCTGG-3') targeting the V3 region of bacterial 16S rRNA genes (Muyzer et al., 1993). For DGGE, the forward primer had a GC-rich sequence at its 5' end described by Myzer et al. (1993). The PCR mixture consisted of 1× Ex Taq™ buffer, 0.20 mM of dNTP mixture, 0.25 μM of each primer, 1.25 U of Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), and 1 μl of the sample. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s. A final extension step at 72°C for 5 min was also performed. PCR products were electrophoresed on a 1.2% agarose gel. The agarose gel was stained with ethidium bromide to determine the PCR product size.

DGGE analysis

DGGE analysis was performed with a Dcode Multiple System (Bio-Rad Laboratories Inc., Hercules, CA, USA) by using the following protocol: 35 μl of the PCR products were loaded onto an 8% polyacrylamide gel with a denaturant gradient 30 to 70%. Electrophoresis was performed at 130 V for 5 h at 60°C in 1×TAE buffer (40 mM Tris, 40 mM acetic acid, and 10 mM EDTA $2Na.2H_2O$). The gel was then stained with ethidium bromide for 5 min, visualized with Printgraph (ATTO Corporation, Tokyo,

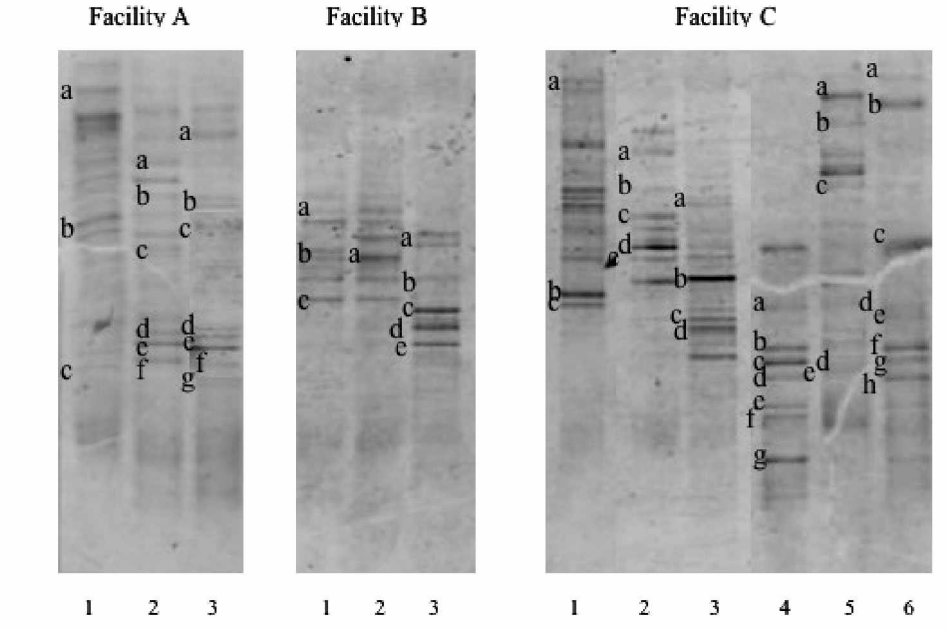

Figure 1. DGGE profile of the samples in the composting processes. The number expressed below DGGE profiles corresponds to each sample. Each letter indicated the bands were sequenced.

Japan), and photographed using a CCD video camera module (ATTO Corporation, Tokyo, Japan). The DNA were excised from the DGGE gels using sterile razor blades and transferred to a 1.5 ml tube with 80 μl of TE buffer. The tubes were then stored overnight at 4°C. One microliter of each aliquot was used as a template for PCR and DGGE to confirm that each PCR product formed a single band. The single DNA fragments obtained were amplified using primer sets 341F/518R. The PCR products were purified using MagExtractor-PCR & Gel Clean Up (TOYOBO CO., LTD., Osaka, Japan) following manufacturer's instructions, and 25 μl of sterile milliQ water (Millipore Corporation, Billerica, MA, USA) was added.

To compare DGGE profiles between samples, cluster analysis based on band position and intensity was used. Profiles were processed with ImageJ 1.41 (http://rsb.info.nih.gov/ij/) and calculated Pearson's product-moment correlation coefficient. Samples were then clustered by using unweighted pair group method with arithmetic averages.

DNA sequencing

Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems, Foster City, CA, USA) using the primers 341F and 518R in accordance with the manufacturer's instructions. The reaction products were precipitated and dried for 10 min. The products were analyzed using an ABI PRISM 310 Autosequencer (Applied Biosystems, Foster City, CA, USA).

The sequences obtained were assembled and compared with 16S rRNA gene sequences available in the database using the BLAST WWW system present in the DNA Databank of Japan. The sequences obtained were deposited in GenBank under accession numbers AB442088 to AB442144.

RESULTS AND DISCUSSION

DGGE profiles from all compost samples are shown in Figure 1. The bands represented by letters were excised and subsequently sequenced. Table 2 shows information of analyzed sequences from all samples.

Three samples (A1, A2, and A3) in facility A were collected at low temperatures up to 40°C (Table 1). The DGGE band pattern of A1 was differed from A2 and A3 by cluster analysis (data not shown). However, Table 2 shows that all sequences obtained from three samples are grouped to either class Bacteroidetes or class Firmicutes and that most sequences belong to the members of class Clostridia. These organisms are known to be anaerobic organisms living in soil, sediments, rumen, feces etc. (Wiegel et al., 2006). In fact, the bacteria grouped in class Clostridia are often found in cow rumen fluid (Whitford et al., 1998) and cow manure (Ozutsumi et al., 2005). Because the composting materials in facility A were not aerated or turned and the moisture content was not controlled, anaerobic conditions might have been created. We believe that anaerobes such as members of class Clostridia present in animal manure survived in the compost material and were responsible for incomplete degradation of organic compounds.

Table 2. Characterization of sequences obtained from DGGE profiles

Band		Closet relatives (accession number)	Similarity (%)	Phylum or class
A1	a	Uncultured bacterium clone AFYEL_aaj68b03 (EU465103)	97	Flavobacteria
	b	Uncultured bacterium clone SU49(OTU-26) (EU862889)	98	Clostridia
	c	Uncultured bacterium clone 2G1-27 (EU160210)	100	Clostridia
A2	a	Uncultured bacterium clone SU49(OTU-26) (EU862889)	98	Clostridia
	b	Uncultured compost bacterium isolate 2b (AY489028)	99	Unclassfied Bacteroidetes
	c	Uncultured bacterium clone:31L (AB206781)	100	Unclassfied Bacteroidetes
	d	Uncultured bacterium clone E70 (FJ205837)	100	Clostridia
	e	Uncultured bacterium clone A0-061006-e1 (EU814595)	100	Clostridia
	f	Uncultured bacterium clone BL47 (EU586252)	100	Firmicutes
A3	a	Uncultured compost bacterium clone 2B01 (DQ346473)	100	Flavobacteria
	b	Uncultured bacterium clone BL42 (EU586247)	100	Clostridia
	c	Uncultured bacterium clone BL24 (EU586229)	100	Clostridia
	d	Uncultured bacterium isolate: DGGE band 19 (AB364404)	98	Flavobacteria
	e	Uncultured bacterium clone C55_D6_H_B_E04 (EF559026)	100	Clostridia
	f	Uncultured bacterium clone E70 (FJ205837)	99	Clostridia
	g	Uncultured bacterium clone A0-061006-e1 (EU814595)	100	Clostridia
B1	a	Uncultured bacterium clone MABRDTU33 (FJ529986)	98	Unclassfied Bacteroidetes
	b	Uncultured bacterium isolate PCR-DGGE gel band T8 (AY332599)	98	Chlorobi
	c	Uncultured bacterium clone C09 (EU136279)	91	Proteobacteria
B2	a	Uncultured bacterium clone S2-48 (EF491364)	99	Proteobacteria
B3	a	Uncultured bacterium clone: TP16_K (AB290375)	91	Clostridia
	b	Uncultured bacterium clone C55_D6_H_B_E04 (EF559026)	100	Clostridia
	c	Uncultured bacterium clone: clone-43 (AB375719)	100	Clostridia
	d	Uncultured bacterium clone A0-061006-e1 (EU814595)	100	Clostridia
	e	Uncultured bacterium clone BL47 (EU586252)	100	Clostridia
C1	a	Uncultured bacterium clone AFYEL_aaj68b03 (EU465103)	97	Flavobacteria
	b	Bacterium LZX27 (DQ359949)	96	Unclassfied Bacteroidetes
	c	Bacterium LZX27 (DQ359949)	98	Unclassfied Bacteroidetes
C2	a	Uncultured bacterium clone Fin_CL-100621_OTU-32 (EU808411)	98	Flavobacteria
	b	Uncultured bacterium clone EV818CFSSAHH37 (DQ336988)	100	Unclassfied Bacteroidetes
	c	Uncultured bacterium clone SMG152 (AM930317)	99	Unclassfied Bacteroidetes
	d	Uncultured bacterium isolate: DGGE clone AB14-28 (AB474790)	99	Unclassfied Bacteroidetes
	e	*Bacillus sp.* 50LAy-1 (AB375754)	100	Bacilli
C3	a	*Ureibacillus sp.* S22-47 (AM932280)	100	Bacilli
	b	*B. thermocloacae* (Z26939)	100	Bacilli
	c	Uncultured bacterium clone M55_D21_H_B_C10 (EF586062)	98	Bacilli
	d	Uncultured bacterium clone A0-061006-e1 (EU814595)	100	Clostridia
C4	a	Uncultured bacterium clone M55_D21_H_B_C10 (EF586062)	100	Bacilli
	b	Uncultured bacterium isolate DGGE gel band M76_4 (EU921820)	97	Clostridia
	c	Uncultured bacterium clone biogas-DM1-b48 (DQ419667)	100	Clostridia
	d	Uncultured compost bacterium clone: SWC13 (AB438006)	100	Clostridia
	e	Uncultured compost bacterium clone 1B07 (DQ346486)	100	Clostridia
	f	Uncultured compost bacterium clone: SWC13 (AB438006)	95	Clostridia
	g	Uncultured bacterium clone SMG94 (AM930302)	99	Clostridia
C5	a	Uncultured Bacteroidetes bacterium clone Blui38 (AJ318191)	100	Unclassfied Bacteroidetes
	b	Uncultured bacterium isolate PCR-DGGE gel band S1-2 (AY332579)	97	Unclassfied Bacteroidetes
	c	Uncultured bacterium clone P4T_047 (EF552043)	100	Proteobacteria
	d	Uncultured compost bacterium clone 2B06 (DQ346478)	99	Clostridia
	e	Uncultured bacterium clone NGD65 (EF614088)	94	Chloroflexi
C6	a	Uncultured bacterium clone ATB-KS-1411 (EF686944)	100	Bacteroides
	b	Uncultured bacterium clone E37 (FJ205818)	100	Bacteroides
	c	Uncultured compost bacterium clone 2B01 (DQ346473)	100	Flavobacteria
	d	Uncultured bacterium clone M55_D21_H_B_C10 (EF586062)	100	Bacilli
	e	Uncultured bacterium clone A0-061006-e1 (EU814595)	100	Clostridia
	f	Uncultured compost bacterium clone 2B06 (DQ346478)	99	Clostridia
	g	Uncultured compost bacterium clone: SWC22 (AB438015)	100	Clostridia
	h	Uncultured bacterium clone biogas-DM1-b48 (DQ419667)	100	Clostridia

DGGE band patterns of samples B1 and B2, which were at ambient temperature (B1: 18.6°C and B2: 28.0°C), were similar by cluster analysis (data not shown). In both samples, 2 bands (B1b and B1c) were related to delta-subclass proteobacteria and 1 band (B1c) was related to gamma-subclass proteobacteria although few reports have shown that Proteobacteria are present in the initial stage of composting. Many studies revealed that members of *Bacillus* spp. dominate the initial stages (Peters et al., 1999; Ishii et al., 2000; Dees and Ghiorse, 2001; Schloss et al., 2003). These might be because the composting materials were different, e.g., livestock manure, food waste, and industrial waste.

However, the DGGE pattern of sample B3 was significantly different from those of B1 and B2 and new bands (3a, 3c, 3d and 3e) appeared. Additionally, when the compost temperature increased to 50°C, there was a shift in the band pattern, suggesting that thermophilic bacteria dominate in this stage. In addition, we could not detect any sequences belonging to *Bacillus spp.* in facility B. Because the lanes for mixing compost material in facility B were shorter (approximately 25 m) than that in the other facilities, compost temperature could not increase beyond 50°C and high temperature could not be maintained for a long time. The operational characteristics of facility B prevented the dominance of thermophilic aerobes such as *Bacillus spp.* even though the temperature of the compost material was high.

In facility C, temperature changed dynamically (between 21.6 and 61.7°C) during composting (Table 1). In the DGGE profile, a number of bands appeared and disappeared during the course of composting. This suggests that specific bacterial communities, which could adapt to the conditions in the composting material, were established in each stage of composting. It also implies that the succession of bacterial communities occurred in a short time.

In sample C1, only class Bacteroidetes-related sequences were obtained (Table 2). The results suggest that the bacteria showing family Flavobacteriaceae-related sequences might play an important role in initial phase of composting from cattle manure.

In samples C2 to C4, composting material was maintained at high temperature (>50°C), and their bacterial communities were different compared to that in sample C1. In sample C2 and C3, most bands corresponded to sequences grouped to the class Bacillales (Table 2). Our data was followed by some studies that Bacillus-type spp. were mainly detected at thermophilic stage of composting (Ishii et al., 2000; Pedoro et al., 2001). The appearance of these bacteria suggests that organic compounds had begun to degrade aerobically by them under thermophilic conditions.

In sample C4, the temperature had begun to decrease and many sequences grouped to class Clostridia were detected (C4b to C4g). Thus, the bacterial community from sample C4 was not the same as that which was present in composts of animal manure. This observation supported the idea that bacterial community structure changed significantly through the high-temperature stage of composting in facility C. Considering that *Clostridium spp.* have the ability to convert organic compounds to sugar, acids, and alcohols (Wiegel et al., 2002), the community related to class Clostridia found in sample C4 played a significant role in compost maturation. Therefore, we speculate that after oxygen is consumed by aerobes, a new anaerobe community, which differs from the fecal bacterial community, might start growing and degrade organic compounds in composts.

During the maturation stage, sequences obtained from samples C5 and C6 were grouped to several phyla, that is, phyla Bacteroidetes, Proteobacteria, and Firmicutes following a decrease in compost temperature. Phylum Bacteroidetes-related sequences (C5a, C5b, C6a, C6b, and C6c) as well as phylum Firmicutes-like sequences (C5d, C6d, C6e, C6f, C6g, and C6h) were detected in samples C5 and C6. Green et al. (2004) reported the presence of DNA sequences close to phylum Bacteroidetes in the compost product made from daily manure and sawdust or straw. Takaku et al. (2006) also found that the members of phylum Bacteroidetes dominated in the maturing stage of garbage composting. A Firmicutes bacterium has also been detected in compost products (Ishii et al., 2000; Pedoro et al., 2001). This phylum contains several organisms that can utilize protein and starch. Our results indicate that the bacteria belonging to Bacteroidetes and Firmicutes have important roles in degrading organic materials in the last stage of composting. In addition, some bands with same sequence were detected from several samples (C3d and C6e, C4a and C6d, and C4c and C6h) and grouped within phylum Firmicutes. The members of phylum Firmicutes particularly could contribute the degradation of composting materials.

Differences in analysis procedures such as the DNA extraction method or the PCR primer sets used may influence the differences in DGGE profiles. Arbeli and Fuentes (2007) found that the differences in DNA extraction and purification methods affected DNA yield and the quality of DNA. In addition, DGGE profile cannot give us quantitative data of microbial property as described by Ishii et al. (2000) and Pedoro et al. (2001). For a better understanding of the microbial community in composts, detailed data of microbial communities should be obtained using the most effective procedures though qualitative analysis such as real-time PCR or fluorescence *in situ* hybridization. Moreover, it is necessary to detect and characterize specific bacteria called functional bacteria, e.g.,

ammonia-oxidizing bacteria (Kowalchuk et al., 1999) and sulfur-oxidizing bacteria (Asano et al., 2007).

In conclusion, bacterial community of compost from facility A showed little change throughout the process. In compost sample from facility B, there is a small shift in their community structure as its temperature increased. Compost sample from facility C, which was recorded a considerable temperature change, showed clear variations in the bacterial community structure throughout its composting process. These results must be confirmed by further research because we did not study the reproducibility of our results. However, this study provides a part of information about bacterial community actually present in field-scale compost with animal manure

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