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Analysis of the Structure of the Bacterial Community in the Livestock Manure-based Composting Process*

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ABSTRACT : We investigated the structure of bacterial communities present in livestock manure-based composting processes and evaluated the bacterial succession during the composting processes. Compost samples were derived separately from swine manure, dairy manure and sewage sludge. The structure of the bacterial community was analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) using universal eubacterial primers. The genus *Bacillus* and related genera were mainly detected following the thermophilic composting phase of swine and dairy manure composts, and the members of the phylum *Bacteroidetes* were mainly detected in the cattle manure waste-based and sewage sludge compost. We recovered and sequenced limited number of the bands; however, the PCR-DGGE analysis showed that predominant diversities during the composting processes were markedly changed. Although PCR-DGGE analysis revealed the presence of different phyla in the early stages of composting, the members of the phylum *Firmicutes* and *Bacteroidetes* were observed to be one of the predominant phyla after the thermophilic phase. (**Key Words :** Bacterial Community, Compost, Denaturing Gradient Gel Electrophoresis, Manure)

INTRODUCTION

In livestock manure treatment systems, various species of bacteria play an important role in the decomposition of organic matter (Chynoweth et al., 1999; Tiquia and Michel, 2002; Hanajima et al., 2004). By the analyzing microbial quinones, Hu et al. (2001) observed that microbial diversity changed markedly during the activated sludge process. Ishii et al. (2000) reported that the bacterial population and community structure became complex as the composting process proceeded into a laboratory-scale garbage compost. In order to efficiently carry out the process of livestock manure treatment, monitoring the structure of the bacterial community is an important issue. Although the bacterial diversity has been analyzed previously based on culture methods, many uncultured bacteria are known to exist in complex environmental conditions. Thus, methods that are based on gene analysis and do not depend on culture methods have been developed for the analysis of bacterial diversity. Among these methods, the most widely used technique is the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method that can differentiate PCR products of identical lengths differing in sequence by even a single base (Muyzer et al., 1993). In this method, banding patterns reveal the genetic diversity in a complex bacterial community. Therefore, this conventional method may further contribute to the understanding of the complex nature of the bacterial community in livestock manure treatment processes.

In this study, we investigated the bacterial succession in four different livestock manure-based composting processes and determined the predominant species in the bacterial community using PCR-DGGE.

MATERIALS AND METHODS

Compost samples

Sampling was carried out at four different composting facilities, and the compost samples were collected at each of the four treatment stages (Table 1). In all facilities, composting was conducted in four stages. In facility S (about 250 m^3 capacity), the raw material consisted of swine manure mixed with mature compost. The composting stages proceeded in each 20 days. In facility P (about 130 m^3 capacity), the raw material consisted of swine manure,

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Table 1. Characteristics of livestock manure-based compost samples used in this study

Facility	Source	Sample	Treatment stage	Temperature (°C)
S	Swine manure compost	Ss	Starting point of the lag phase	70
		St	Thermophilic phase	72
		Sa	Active composting phase	33
		Se	End of the composting phase	56
P	Swine manure compost	Ps	Starting point of the lag phase	16
		Pt	Thermophilic phase	58
		Pa	Active composting phase	46
		Pe	End of the composting phase	26
C	Dairy manure compost	Cs	Starting point of the lag phase	12
		Ct	Thermophilic phase	54
		Ca	Active composting phase	64
		Ce	End of the composting phase	27
A	Sewage sludge compost	As	Starting point of the lag phase	15
		At	Thermophilic phase	80
		Aa	Active composting phase	63
		Ae	End of the composting phase	28

chaff and mature compost. In both these facilities, an open type scoop mixing composter system with a floor-based air supply system was used. In facility C (about 9 m^3 capacity), a composter of the same type as that used in facilities S and P was used; however, this composter was not equipped with an air supply system. The raw material consisted of dairy manure, chaff and mature compost. The composting stages proceeded in each 25 days. Facilities S, P, and C used mature composts that were produced in their respective facilities. In facility A (about 6 m^3 capacity), the composter was a semi-closed type cubic-shaped composter with a floor-based air supply system and an air outflow system in the center of the composter (Sasaki et al., 2005). The raw materials used in facility A included sewage sludge and mature compost. The mature compost that was prepared from dairy cattle manure wastes containing bedding materials was obtained from the Iwate Agricultural Research Center. The composting stages proceeded in each 7 days. Compost samples were collected as follows: approximately 1 kg of compost sample was collected from four different points and then mixed well. A portion of this mixed sample was then used for analysis. The compost samples were collected from the four different treatment stages and subsequently transported at 4°C and maintained at this temperature until they were used.

PCR-DGGE analysis

The compost samples were dissolved in 10 mM sodium tripolyphosphate (1:20 w/v) by shaking for 15 min. Sediments including bacterial cells were harvested by centrifugation (10,000 g, 5 min), and total DNA was extracted by MagExtractor-Genome (Toyobo, Osaka, Japan; Boom et al., 1990).

Portions of the 16S rDNA V3 region were amplified from the extracted DNA samples using primer sets that target eubacteria (Muyzer et al., 1993). Both the DGGE primers GC-341F and 518R were used for direct amplification from the DNA samples. PCR primer sequences were as follows: GC-341F, 5′-CGCCCG CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG-3′; and 518R, 5′-ATTACCG CGGCTGCTGG-3′. PCR was performed with a Model iCycler (Bio-Rad, CA, USA). The PCR mixture contained 10×PCR amplification buffer, 25 mM $MgCl_2$, 2 mM dNTP, 25 μM of each primer, 1.25 U *Taq* DNA polymerase (AmpliTaq Gold, Applied Biosystems, CA, USA), and 1 μl of template DNA in 50 μl of PCR reaction mixture. Amplification was performed at 94°C for 10 min, and touchdown PCR was performed as follows: after denaturation at 94°C for 1 min, the annealing temperature was initially set at 65°C and was later decreased by 1°C after each 2 cycles until it reached 55°C. Primer extension was performed at 72°C for 2 min. The above reaction was performed for 20 cycles, followed by 15 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. A final extension step was performed for 10 min at 72°C.

DGGE analyses were performed with the method of Muyzer et al. (1993) using a DCode multiple system (Bio-Rad) by the following conditions. Polyacrylamide gels (8%) (acrylamide:bisacrylamide, 37.5:1) were prepared using denaturing gradients ranging from 40% to 60% for separating 16S rDNA fragments. The denaturant (100%) contained 7 M urea and 40% formamide. Electrophoresis was performed at 60°C for 16 h at 50 V. Following electrophoresis, the gel was stained for 10 min with ethidium bromide. The gels were scanned with Printgraph (ATTO, Tokyo, Japan) and visualized on a CCD video camera module (ATTO). Subsequently, the dominant bands were excised, and the slices were suspended overnight in 50 μl TE to elute the DNA from the gels. The purities of separated DNA were confirmed by repeating DGGE analysis.

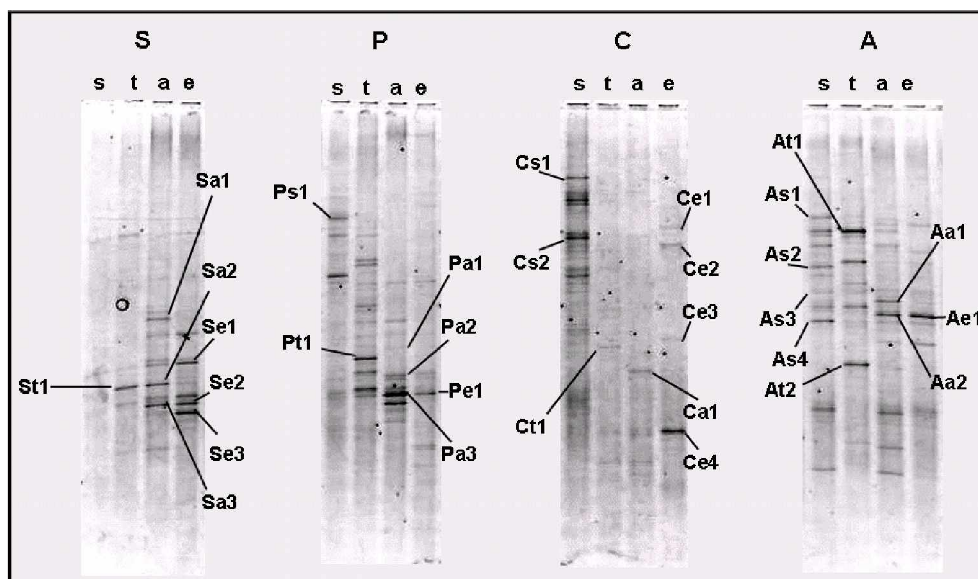

Figure 1. The analysis of four compost communities by PCR-DGGE. Sampling was carried out at four different composting facilities, Facility S, Facility P, Facility C, and Facility A. Samples were obtained from each of the facilities at starting point of the composting phase (phase s), the thermophilic phase (phase t), the secondary composting phase (phase a) and the end of the secondary composting phase (phase e).

Sequence analysis

The DNA isolated from DGGE gels were reamplified using the primer pair 341F-518R. The sequence of primer 341F was as follows: 5′-TACGGGAGGCAGCAG-3′. Briefly, amplification was performed as follows: denaturation at 94°C for 30 s, annealing at 57.5°C for 20 s and extension at 72°C for 30 s. Amplification was performed for 35 cycles, and finally, extension was performed at 72°C for 10 min. The amplified PCR products were purified by a MagExtractor-PCR&Gel Clean up (Toyobo). The sequencing reactions were carried out using a BigDye Terminator cycle sequencing kit (Applied Biosystems). The products of the sequencing reaction were analyzed with a ABI 310 autosequencer (Applied Biosystems). The closet matches of all the 16S rDNA sequences were identified through BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Multiple alignment analysis, distance matrix calculation and construction of a phylogenetic tree were carried out using the ClustalW program (Thompson et al., 1989). A phylogenetic tree was generated using the neighbor-joining algorithm (Saitou and Nei, 1987) and the Tree View program (Page, 1996) for the PCR amplified 16S rDNA bacterial sequences.

RESULTS

The structures of the bacterial community in the four different composts were determined by using PCR-DGGE (Figure 1). As expected, the banding pattern changed during each composting process.

Table 2 shows the identification results of the separated bands on the basis of percent similarity to the 16S rDNA sequence. In the gel of sample obtained from facility S (Figure 1), all the separated bands showed a similarity to the phylum *Firmicutes* (*Bacillus* spp., Halophilic bacterium and *Lentibacillus salicampi*). In the gels of sample obtained from facilities P and C (Table 2), the separated bands showed similarity to the phyla *α-Proteobacteria* (*Sphingomonas* sp.), *Firmicutes* (*Acholeplasma axanthum* and *Bacillus* spp.) and *Bacteroidetes* (*Flavobacterium* spp.). Most of the separated bands in the gel of facility A showed the presence of bacteria similar to the phylum *Bacteroidetes* (*Flavobacterium* spp. and *Tenacibaculum maritimum*), while other bands demonstrated a similarity to the phylum *γ-Proteobacteria* (*Pseudomonas halodenitrificans* and *Psychrobacter glacincola*). Figure 2 shows the phylogenetic relationships of the 16S rDNA sequences recovered from the DGGE gels. Bacteria belonging to the phylum *Firmicutes* and the phylum *Bacteroidetes* were determined to be the predominant species following the thermophilic phase in all the four compost samples (Figure 2).

DISCUSSION

The genus *Lentibacillus* that was detected in the facility S is phylogenetically closely related to the genus *Bacillus* (Yoon et al., 2001); therefore, the genus *Bacillus* and related genera are the dominant species following the thermophilic composting phase for the swine and dairy manure compost. The genus *Bacillus* has been known to be widely distributed in various compost raw materials such as garden, domestic

Table 2. Identification of separated bands on the basis of percent similarity to 16S rDNA sequence

Band number	Sample	Treatment stage	Identification	Accession No.	Similarity %	Phylum
St1	Swine manure compost	Thermophilic phase	*Bacillus thermocloacae*	Z26939	99	*Firmicutes*
Sa1		Active composting phase	*Bacillus licheniformis*	AY536574	98	*Firmicutes*
Sa2		Active composting phase	*Bacillus thermocloacae*	Z26939	99	*Firmicutes*
Sa3		Active composting phase	Halophilic bacterium	AB015022	94	*Firmicutes*
Se1		End of the composting phase	*Bacillus subtilis*	AY647297	97	*Firmicutes*
Se2		End of the composting phase	*Bacillus* sp.	AY690690	93	*Firmicutes*
Se3		End of the composting phase	*Lentibacillus salicampi*	AY057394	92	*Firmicutes*
Ps1	Swine manure compost	Starting point of the lag phase	*Sphingomonas* sp	AB066230	100	*α-Proteobacteria*
Pt1		Thermophilic phase	*Bacillus* sp	AY422984	95	*Firmicutes*
Pa1		Active composting phase	*Flavobacterium* sp.	AB164690	91	*Bacteroidetes*
Pa2		Active composting phase	*Flavobacterium* sp.	AY154889	100	*Bacteroidetes*
Pa3		Active composting phase	*Bacillus* sp	AB084065	99	*Firmicutes*
Pe1		End of the composting phase	*Bacillus* sp.	AB084065	99	*Firmicutes*
Cs1	Dairy manure compost	Starting point of the lag phase	*Acholeplasma axanthum*	U04657	90	*Firmicutes*
Cs2		Starting point of the lag phase	*Polaribacter* sp.	AF493676	94	*Bacteroidetes*
Ct1		Thermophilic phase	*Bacillus* sp	AB094472	98	*Firmicutes*
Ca1		Active composting phase	*Bacillus thermocloacae*	AY536574	100	*Firmicutes*
Ce1		End of the composting phase	*Bacillus licheniformis*	AY536574	98	*Firmicutes*
Ce2		End of the composting phase	*Bacillus subtilis*	AY647297	97	*Firmicutes*
Ce3		End of the composting phase	*Flavobacterium mizutaii*	AJ438175	91	*Bacteroidetes*
Ce4		End of the composting phase	*Flavobacterium* sp.	AF386740	89	*Bacteroidetes*
As1	Sewage sludge compost	Starting point of the lag phase	*Pseudomonas halodenitrificans*	X90867	100	*γ-Proteobacteria*
As2		Starting point of the lag phase	*Psychrobacter glacincola*	AJ430830	100	*γ-Proteobacteria*
As3		Starting point of the lag phase	*Flavobacterium* sp.	AY599661	94	*Bacteroidetes*
As4		Starting point of the lag phase	Rhizosphere soil bacterium	AJ252682	96	ND
At1		Thermophilic phase	*Flavobacterium odoratum*	D14019	96	*Bacteroidetes*
At2		Thermophilic phase	*Flavobacterium mizutaii*	AJ438175	94	*Bacteroidetes*
Aa1		Active composting phase	*Flavobacterium* sp.	AY599661	94	*Bacteroidetes*
Aa2		Active composting phase	*Tenacibaculum maritimum*	AB078057	93	*Bacteroidetes*
Ae1		End of the composting phase	*Tenacibaculum maritimum*	AB078057	93	*Bacteroidetes*

ND = Not determined.

and food wastes (Dees and Ghiorse 2001; Zhang et al., 2002). Strom (1985) reported that 87% of cultivated isolates from solid waste compost were identified as members of the genus *Bacillus*. In addition, these species have been known to degrade recalcitrant polymers and assimilate nitrogen compounds (Potter et al., 2001; Sasaki et al., 2007). Thus, a close relationship may exist between the dominance of the genus *Bacillus* in the bacterial community.

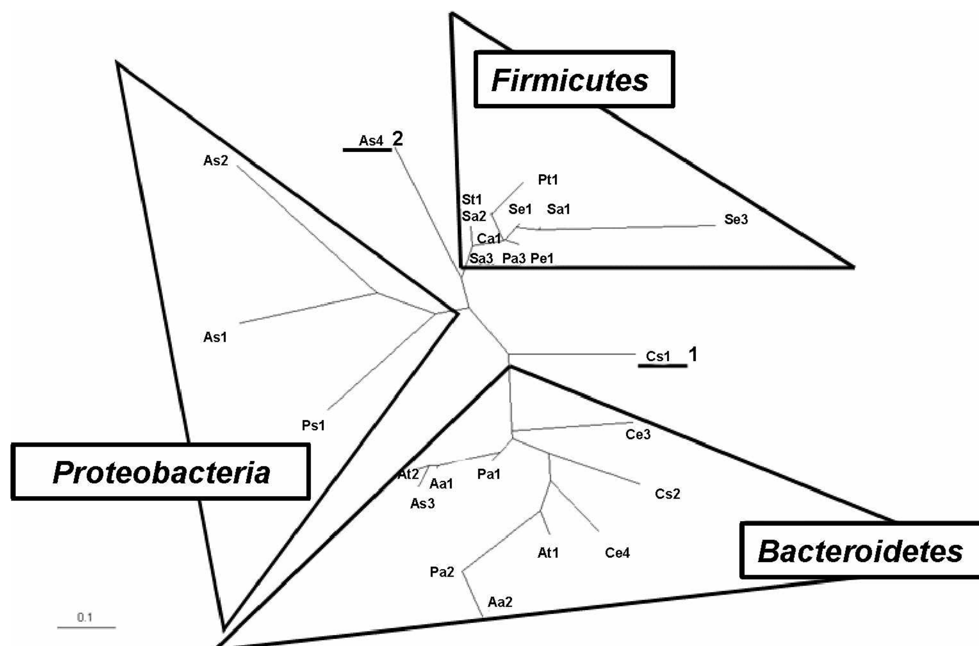

Figure 2. Phylogenetic analysis of the PCR amplified 16S rDNA bacterial sequences. The neighbor-joining method was used to construct a phylogenetic tree using the ClustalW program. 1: Cs1 was belonged to the phylum *Firmicutes*. 2: Phylogenetic group of As4 could not be determined based on the partial 16S rDNA sequence.

The separated bands in almost all the compost samples after the thermophilic phase indicated a similarity to the genus *Flavobacterium*. The phylum *Bacteroidetes* was identified as the predominant species in the samples from facilities P, C and A (Table 2). These species belonged to the phylum *Cytophaga-Flavobacter-Bacteroidetes* (CFB, Gherna and Woese 1992; Paster et al., 1994; Denger et al., 2002). Members of the CFB phylum have been reported to be distributed in the soil and water environments and are also known to utilize macromolecular compounds such as proteins, cellulose and chitin (Honschopp et al., 1996; Manz et al., 1996; Kenzaka et al., 1998; Battin et al., 2001). Green et al. (2004) reported that members of the phylum *Bacteroidetes* were determined to be the predominant bacteria in sawdust and straw-amended cow manure compost. Weber et al. (2001) reported that in the microbial community that degrades rice straw, 5% of the total microorganisms belonged to the CFB phylum. Furthermore, several species belonging to the CFB phylum were detected in the composting samples in which the microbiological additive (MA) was used (Wakase et al., 2008), and this MA was mainly composed of the mixture of winery solid residue, leaves and stem of corn and rice straw (Sasaki et al., 2006). The CFB phylum was detected only in the compost sample containing chaff and mixing materials such as bedding materials. Therefore, the presence of the members of the CFB phylum might be related to the degradation of fiber compounds that are derived from plants in the composting processes.

In the present study, we observed that members of the genus *Bacillus* and those belonging to the phylum *Bacteroidetes* were mainly detected after the thermophilic phase although bacteria belonging to different phyla were also detected during the early stage of composting. The PCR-DGGE method is widely used in environmental studies (White et al., 1999; Hong and Chen, 2007). However it has been reported that the banding patterns of PCR-DGGE were biased by methods of DNA extraction and PCR protocol (Eichner et al., 1999; Rolleke et al., 1999). It has been reported that changes in the annealing temperature and the amplification cycles of PCR protocol influenced the banding patterns of the DGGE gels (Polz et al., 1998; Ishii and Fukui, 2001). In this study, only a small number of bands appeared in several lanes (Ss, Ct and Ca) compared with the banding patterns of each treatment. Although the structures of microbial communities were considered to be remarkably changed after thermophilic stages during composting process, the results might be influenced by the conditions of amplification of the bacterial DNA. It might be necessary to use the additional methods such as culture depending method and the other DNA based methods including quantitative analysis in order to improve the monitoring of the succession of the microbial community. Comparison of these methods should be clarified in future study.

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