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Microbial Community in a Microbiological Additive and Composting Process

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ABSTRACT

There are many types of commercial microbiological additives (MAs), including feed additives, that are used for controlling odor and speed up composting for animal manure treatments. The detailed microbial composition for most MAs is not disclosed, and therefore the fate and the functions of MAs during animal manure treatments are uncertain. When MAs are used to improve the animal manure treatment process, it is essential to determine the functions and mechanisms of MAs. In addition, to monitor the structure of a microbial community and succession during treatment is an important issue for the understanding of the functions of MAs. This review summaries the effect of a commercial MA on the changes in chemical properties and microbial succession during the composting process, and describes the culture-dependent as well as culture-independent methods for monitoring the predominant microbial population during the treatment.

Keywords: compost, livestock waste, microbial diversity, microbial succession
Abbreviations: MA, microbiological additive; **PCR-DGGE**, polymerase chain reaction-denaturing gradient gel electrophoresis; **rRNA**, ribosomal RNA; **TS**, trypto soy

CONTENTS

INTRODUCTION

Livestock waste and wastewater are treated through the biological decomposition and stabilization of organic compounds under controlled conditions (Nakai 2001). These treatments are biological processes that are carried out by microorganisms, and therefore various microbial species play an important role in the decomposition of organic matter such as odourous and recalcitrant compounds (Tiquia and Michel 2002). Microbial composition at initial stage of the process may affect the rate of the organic compounds decomposition in latter stages and end products. Therefore, the microbial population and their activity in raw materials are important factors affecting waste and wastewater treatment processes.

There are numerous microbial-based additives that are commercially available (Dubois *et al*. 2004; Barrene *et al*. 2006; Wakase *et al*. 2008). In Japan, there are approximately 100 types of commercial microbiological additives (MAs) that are used for the acceleration of composting in waste and wastewater treatments (Sasaki *et al*. 2006). In North America, many microbial-based products are also commercially available (Dubois *et al*. 2004; Hill *et al*. 2007). These microbial-based products are aimed at the deodorization, decolorization and the removal of nitrogen compounds. For the laboratory conditions, the MAs contain the microbial consortia that are capable of nitrification, degradation of high concentration of volatile fatty acids and nitrogen compounds were experimentally inoculated into composts (Liao and Bundy 1994; Zhu 2000; Sasaki *et al*. 2005). Furthermore, humic-like substances including humic acid are known to be one of the major constituents of dark matter in soils, composts and wastewater, and microbial decolorization of humic-like substances or humic acid has also been attempted in recent studies (Yanagi *et al*. 2002; del Carmen *et al*. 2006; Wei *et al*. 2007). In the laboratory scale conditions, many of these microbial consortia or one of the microbial species has been confirmed to achieve the removal of nitrogen compounds, deodorization and decolorization. For the commercially available MAs, however, only a limited number of manufacturers reveal the identity of the microorganisms present in those MAs. Further, the fate and functions of the microbial population and the biochemical characteristics of most of those commercial additives during the waste treatment processes are not sufficiently clarified. It is necessary to determine the functions and mechanisms of the additives in order to improve waste treatment processes.

We have investigated the microbial communities in MAs and composts (Nakai *et al*. 2004; Sasaki *et al*. 2006; Wakase *et al*. 2008). In the present paper, we report and review the effects of the microbiological additive on the

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microbial population and succession of livestock waste-based composting. We also summarize the microbial community determined by both culture-dependent and -independent methods.

CHANGES IN TEMPERATURE AND CHEMICAL COMPONENTS DURING COMPOSTING PROCESS BY ADDING MA

Sasaki *et al*. (2006) reported that effects of a commercial MA on composting process. Briefly, the temperature attained with the MA-treated compost was higher than that attained with the control after the second turning (5-10°C). The MA-treated compost showed a rapid temperature increase at the beginning of composting and first turning. Chen *et al*. (2007) reported that inoculation of thermophilic microorganisms into compost resulted higher temperature than the control compost without inoculation during composting process. However, the higher temperature in the inoculated group was not always observed. When nitrite-oxidizing microorganisms were inoculated, no differences in temperature between the inoculated compost sample and control without inoculation were observed (Fukumoto *et al*. 2006).

Some of the objectives to improve the composting processes by adding MAs are considered to reduce odorous compounds, nitrogen compounds and recalcitrant compounds. For the removal of odorous compounds, many studies have attempted to clarify the correlation among MAs, raw materials and physical conditions (Goldstein *et al*. 1985; Bourque *et al*. 1987; Al-Kanani *et al.* 1992; Liao and Bundy 1994; Zhu 2000). The detailed results and perspectives have been summarized and discussed in the review by Zhu (2000). In order to remove the ammonia-nitrogen, one of the effective mechanisms is considered to be nitrification that is carried out by both of ammonia-oxidizing and nitrite-oxidizing microorganisms (Bothe *et al*. 2000; Geets *et al*. 2006; Peng and Zhu 2006; Ren *et al*. 2008). In recent studies, greenhouse gases have been also known to be emitted from composting (Hao *et al*. 2001, 2004). Fukumoto *et al*. (2006) reported that the inoculation of nitrite-oxidizing microorganisms after the thermophilic phase of composting reduced nitrous oxide emission from compost and simultaneously inhibit nitrate accumulation in compost. As summarized in a review by Larney and Hao (2007), nitrification is considered to be one of the most effective treatment for the removal of ammonia-nitrogen in compost. On the other hand, the assimilatory pathways of ammonia-nitrogen by adding MA were also suggested. Pramanik *et al*. (2007) reported that inoculation of nitrogen-fixing microorganisms (*Bacillus polymyxa*) into compost caused increase of total nitrogen contents compared with the other species of microorganisms. Sasaki *et al*. (2006) observed that inoculation of MA led to the decrease of ammonia gas emission from compost and nitrogen compounds including nitrite and nitrate accumulation tended to be increase, and they also suggested that ammonia might be metabolized by microbial assimilation. Tiquia and Tam (2000) reported that 30–35 days were required for the initiation of nitrification during the composting process. Sasaki *et al*. (2007) suggested that ammonia emission is prevented in composting due to the assimilation of ammonia nitrogen by the microorganisms during the composting process. Further, various species of prokaryotes are known to assimilate nitrate as the sole nitrogen source (Merrick and Edwards 1995). For the reduced emission of ammonia-nitrogen, several species of microorganisms that existed in the MA might be metabolized by assimilation as well as nitrification during the early stage of composting with MA treatment.

DETERMINATION OF MICROBIAL COMPOSITION IN MA

There are many commercial MAs that contain an undisclosed microbial composition. To determine the microbial

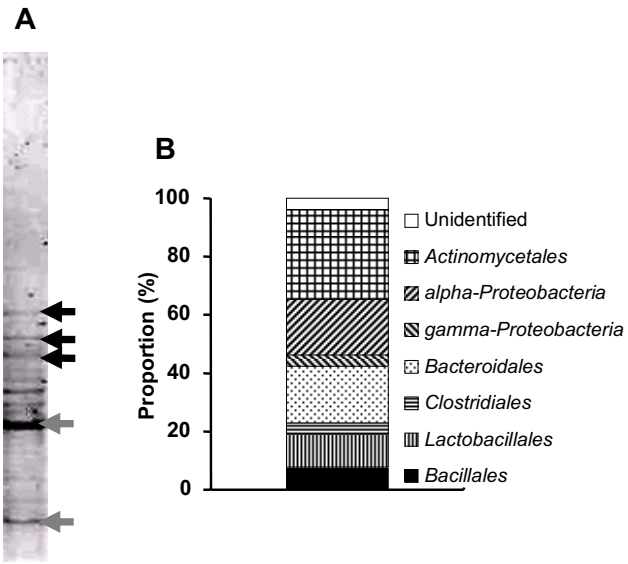

Fig. 1 DGGE bands profiles (A) and proportion of identified clones by the phylogenetic group of bacteria in libraries of 16S rRNA (B) of MA. The arrows in DGGE profiles indicate that the corresponding band was excised and sequenced. Reprinted from **Wakase S, Sasaki H, Ito K, Otawa K, Kitazume O, Nonaka J, Satoh M, Sasaki T, Nakai Y** (2008) Investigation of the microbial community in a microbiological additive used in a manure composting process. *Bioresource Technology* **99**, 2687-2693, with kind permission from Elsevier Ltd., ©2008.

community in complex microbial environments such as soils, composts and wastewater, many studies conducted to use culture-dependent methods as well as culture-independent methods (Muyzer *et al*. 1993; Ishii and Fukui 2001; Kisand and Wikner 2003; Zhou 2003; Dubois *et al*. 2004; Sasaki *et al*. 2005; Otawa *et al.* 2006; Takaku *et al*. 2006). These many studies revealed that the microbial species identified by both of culture-dependent and -independent methods were different, and suggested that the combined several methods including culture-dependent and -independent methods might be necessary for monitoring the microbial community. For instance, Wakase *et al*. (2008) used the MA that was composed of the genera *Alcaligenes*, *Bacillus*, *Clostridium*, *Enterococcus* and *Lactobacillus*, as disclosed by the manufacture, and they identified the cultivable thermotolerant microorganisms as *Bacillus*, *Paenibacillus* and *Clostridium* species. In addition, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis was carried out to determined bacterial community structure in the MA (**Fig. 1A**), and the identified microorganisms in MA (indicated by black arrows) were classified under the phylum *Bacteroidetes* and *Lactobacillus* species. Further, a 16S rRNA clone library in the extract from the MA was constructed, and the clone library method indicated that the bacteria belonging to the phyla *Actinobacteria*, α-*Proteobacteria* and *Bacteriodales* were the dominant microorganisms; PCR-DGGE demonstrated that these bacteria were additional microorganisms (**Fig. 1B**). The predominant clones by PCR-DGGE and the clone library method in the MA were identified as members of the phylum *Bacteroidetes* and *Lactobacillus* species, and none of the cultivable isolates coincided with the microorganisms identified by PCR-DGGE. These results indicate that the microorganisms obtained by the cultivation method were not the dominant microbial community in the MA. Therefore, to determine microbial composition in MA should be combined both of culture-dependent and -independent methods.

In the laboratory scale conditions, many studies used the MA contained microbial consortia or one of the microbial species which were isolated and characterized in preliminary experiments. One of the most widely used microorganisms for MA is thermotolerant microorganisms such as species of *Bacillus*, *Clostridium* and *Streptomyces* (Naka-

saki *et al*. 1998; Fang *et al*. 2001; Xi *et al*. 2005; Sasaki *et al*. 2006; Barrena *et al*. 2006; Chen *et al*. 2007; Pramanik *et al*. 2007; Tang *et al*. 2007b; Vargas-García *et al*. 2007). Almost of these MAs were considered to be effectiveness for degradation of soluble organic carbon, odorous compounds and nitrogen compounds during composting process, and several inoculants aimed to prevent a plant disease in final products. Further, keratinase-producing microorganisms were isolated and identified as species of *Bacillus*, *Flavobacterium*, *Streptomyces* and *Vibrio*, and these microorganisms were reported to be one of the candidates that were inoculated to compost contained poultry feathers, as a MA (Letourneau *et al*. 1998; Sangali and Brandelli 2000; Ichida *et al*. 2001; Riffel and Brandelli 2002; Werlang and Brandelli 2005). For the compost contained high level of cellulose and lignin contents, the mixtures of lignocellulolytic microorganisms were often used as a MA. In many cases, the lignocellulolytic microorganisms are composed of both eubacteria and fungi. Of these, several species of genus *Bacillus*, *Streptomyces* and class *Actinobacteria* as a eubacteria, and genus *Trichoderma* and so-called white-rot fungi as a fungus were confirmed to degrade lignocellulosic substances efficiently in complex microbial conditions (López *et al*. 2002; Lu *et al*. 2004; Vargas-García *et al*. 2005; Yu *et al*. 2007; Vargas-García *et al*. 2007).

CHANGES IN MICROBIAL COMMUNITY DURING COMPOSTING PROCESS BY ADDING MA

By adding MA, increase of total number of microorganisms in compost is often observed. In particular, total number of microorganisms in early stage of composting is markedly increased compared with that of untreated composts, and after the active composting phase total number of microorganisms did not show significant differences between both composts (Sasaki *et al*. 2006; Chen *et al*. 2007). However, the number of certain microorganisms that were considered to be derived from the MAs was confirmed to be increased during composting process in several studies. Briefly, addition of nitrifying microorganisms achieved to continuous increase of nitrite-oxidizing microorganisms during pig manure composting process (Fukumoto *et al*. 2006). Also, inoculation of the MA that was contained thermotolerant microorganisms led to increase of total number of thermotolerant microorganisms in the MA-treated compost (Barrena *et al*. 2006; Sasaki *et al*. 2006; Chen *et al*. 2007). Variations in succession and diversity in microbial communities during the thermophilic phase of composting have been reported (Fogarty and Tuovinen 1991; Tiquia 2005). Xi *et al.* (2005) reported that consumption of oxygen was gradually increased with increase of inoculants contained mixture of *Bacillus* species into compost. In the active phase of the composting process that were performed with inoculation of MA, the temperature rapidly increased and the nitrate concentration and ammonia emission decreased more than in the process without MA. The elevation of temperature and reduction of the nitrogen source in the composting process was generally considered to be due to the metabolization of both carbon and nitrogen sources by the microorganisms (Ryckeboer *et al*. 2003). These results suggest that the increase in the total number of microorganisms by the inoculation of MA actively metabolized the nitrogen to assimilate it and accelerate the temperature elevation of the compost; this does not occur during the process without MA. It also suggests that some functional microorganisms that actively metabolized the nitrogen might exist in MA under those conditions.

Changes in microbial succession during composting process are monitored by the cultivation methods as well as the DNA analysis. One of the conventional methods to analyze structure of microbial community is a PCR-DGGE method that can differentiate PCR products of identical lengths differing in sequence by even a single base (Muyzer *et al*. 1993). This method is being widely used for analysis of microbial succession during composting processes, and many recent studies confirmed that the predominant species

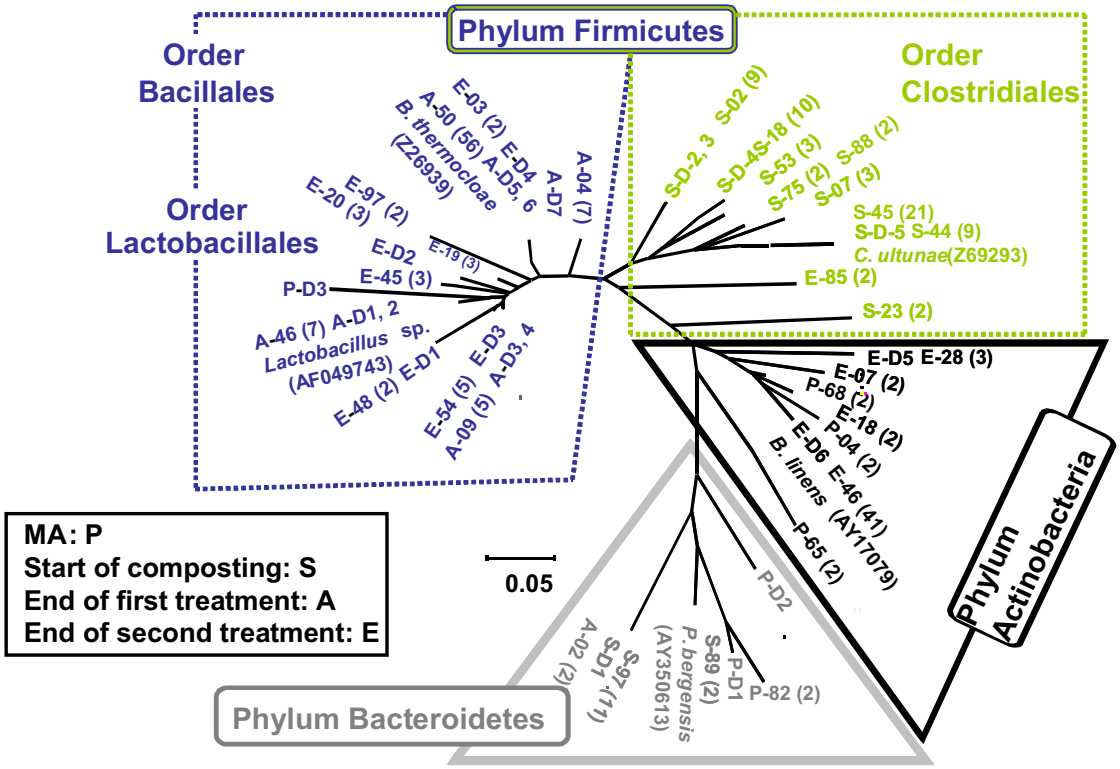

Fig. 2 Phylogenetic analysis of 16S rRNA sequence obtained from MA and compost samples. In the clone library results, only 2 more colonies are shown. Letters P, S, A and E indicate MA, start of composting, end of first treatment, and end of second treatment, respectively. Letter D indicates the DGGE result. When the letter D is replaced by a numeral, it represents the clone number, and a numeral in parenthesis indicates the number of clones. Reprinted from **Wakase S, Sasaki H, Ito K, Otawa K, Kitazume O, Nonaka J, Satoh M, Sasaki T, Nakai Y** (2008) Investigation of the microbial community in a microbiological additive used in a manure composting process. *Bioresource Technology* **99**, 2687-2693, with kind permission from Elsevier Ltd., ©2008.

changed during composting process by the fact in which PCR-DGGE band patterns of samples obtained from different treatment stages of the composting process differed from each other (Ishii and Fukui 2001; Sasaki *et al*. 2005; Takaku *et al*. 2006; Tang *et al*. 2007a; Poulsen *et al*. 2008; Wakase *et al*. 2008). Of these, Wakase *et al*. (2008) observed the microbial community structure in the course of chicken manure composting with adding MA by using PCR-DGGE method and identified the predominant microorganisms as *Clostridium* species at the start of composting (S), *Bacillus* species and an identified uncultured bacterium at the end of the first treatment (A), and *Bacillus* and *Corynebacterium* species at the end of the second treatment (E). Furthermore, Wakase *et al*. (2008) constructed the clone libraries and compared with identification results of the PCR-DGGE method. Phylogeny of the isolates obtained from both methods during the composting process is shown in **Fig. 2**. Of the organisms present in stage S, almost of clones were classified under the orders *Clostridiales* and *Bacteroidales*, respectively, in stage A, approximately 100 clones were classified under the order *Bacillales*, and in stage E, almost clones were classified under the orders *Actinobacterialles* and *Bacillales*, respectively. Although almost of the identified species by the clone library was agreement with that by the PCR-DGGE method, the clone library showed the presence of additional species than the PCR-DGGE analysis. PCR biases are known to give rise to erroneous DGGE profiles; however, PCR-DGGE analysis gave valuable information regarding the microbial community, which is additional to the results that were obtained from the cultivation method (Ishii and Fukui 2001; Janse *et al*. 2004). The resulting microorganisms following analyses by PCR-DGGE or the clone library method in the composting stages corresponded with the microorganisms demonstrated in the MA either by the cultivation method or by DNA analysis.

The phylogenetic relationship of the dominant species obtained in each treatment stage (**Fig. 2**) shows that the dominant species in the MA belonged to the phyla *Bacteroidetes* and *Actinobacteria*, those in the start of composting belonged to the phylum *Bacteroidetes* and order *Clostridiales*, those at the end of first treatment belonged to the order *Bacillales*, and those at the end of the second treatment belonged to the order *Bacillales* and phylum *Actinobacteria*. The phylogenetic analysis shows the change in dominant species from one systematic group to another during the treatment process.

CHANGES IN MICROBIAL COMMUNITY IN MA DURING CULTIVATION

Identification of microbial species that can actually dominate in MA-treated compost is essentially important issue in order to speculate and evaluate the function of MA. Although there are many types of commercial available MAs, only a few studies were conducted on analysis of microbial composition in commercial available MAs. One of the methods to clarify the predominant species and microbial composition in MA itself are considered to be the DNA analysis including clone library and PCR-DGGE method. Dubois *et al*. (2004) analyzed the commercial available MAs by using PCR-DGGE and microarray assay, and in particular microarray assay that can be performed with oligonucleotide probes was effectiveness for assessing the MAs. In addition, it was considered that there were methodological advantages compared with the PCR-based DNA analysis because of free from any bias introduced through DNA amplification (Dubois *et al*. 2004). The other methods to clarify the microbial species that can dominate in treatment process are considered to be combined both of cultivation method and DNA analysis. Wakase *et al*. (2008) reported that the commercial available MA itself was inoculated into trypto soy (TS) broth and cultivated together with monitoring by PCR-DGGE (**Fig. 3**). Band patterns changed as the incubation at 55°C proceeded, and dominant microorganisms belonged to

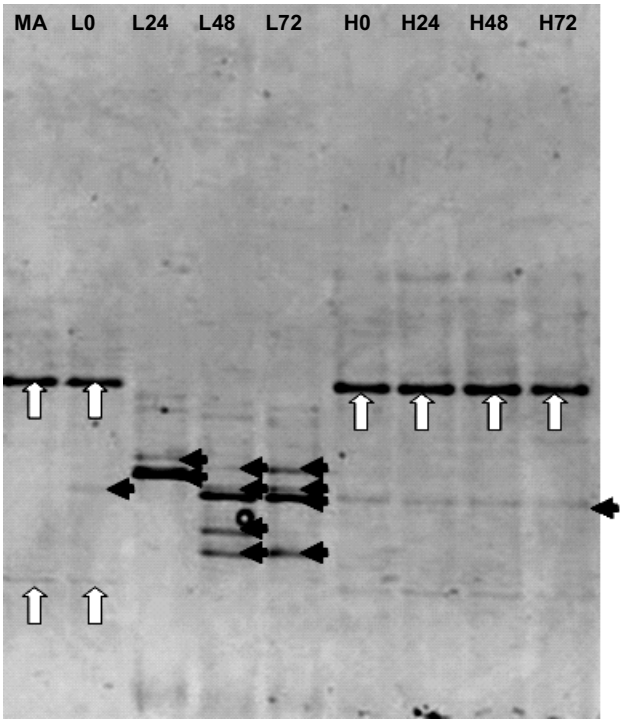

Fig. 3 DGGE profile of cultivation of MA at 55 and 72°C. White arrows indicate 18S rRNA, black arrows indicate 16S rRNA. L: Cultivation at 55°C; H: Cultivation at 72°C. Cultivation time write down by temperature. Reprinted from **Wakase S, Sasaki H, Ito K, Otawa K, Kitazume O, Nonaka J, Satoh M, Sasaki T, Nakai Y** (2008) Investigation of the microbial community in a microbiological additive used in a manure composting process. *Bioresource Technology* **99**, 2687-2693, with kind permission from Elsevier Ltd., ©2008.

Bacillus species and the order *Clostridiales*. Following incubation at 72°C, the dominant microorganism obtained was an uncultured bacterium of the phylum α-*Proteobacteria*. The results of these dominant microorganisms detected from cultures at 55 and 72°C incubation did not correspond with those detected from composting processes and the MA. Wakase *et al*. (2008) indicated that the MA contained a variety of microorganisms including thermophilic microorganisms, and also suggested that the population of these microorganisms did not become dominant in the composting process. The growth of the microorganisms in the MA might be suppressed by thermal conditions that exist during certain stages of composting. Nevertheless, the orders *Bacillales* and *Clostridiales* were mainly detected in DGGE gels of cultured MA at 55 and 72°C, suggesting that not all microorganisms were inactivated by the thermal conditions. Furthermore, the results with regard to these dominant microorganisms after cultivation did not correspond to the isolates obtained during the composting process. In this method, there might be several shortcomings. Briefly, liquid conditions and using a TS broth might stimulate the growth of specific microorganisms that were derived from MA. Therefore, the solid phase conditions, which are able to keep the homogeneity for the microbial analysis, should be developed for the evaluation of MA by culturing.

CONCLUDING REMARKS

DNA analyses, including 16S rRNA-based approaches, have been employed to analyze the diversity of environmental microorganisms (Zwart *et al*. 1998; Philips and Verstraete 2001; Herbert *et al*. 2002; Salles *et al*. 2002; Callia *et al*. 2006). PCR-DGGE and clone library analyses might have some biases; however, these techniques are useful in determining the dominant microbial population even in complex microbial systems such as composts (Gonzalez *et al*. 2003). The phylogenetic analysis revealed that several dominant microorganisms separated by PCR-DGGE did not

completely correspond with the isolates obtained from the clone library. A similar sequence of some isolates, which were separated by PCR-DGGE, were not obtained from the clone library method. Moreover, some clones that were determined by the clone library method were not detected by PCR-DGGE (Takaku *et al*. 2006; Wakase *et al*. 2008). Kisand and Wikner (2003) reported that the overlaps of species identified by PCR-DGGE and the clone library method, PCR-DGGE and the cultivation method, and the clone library method and isolation method were estimated at 9, 3, and 7%, respectively, and the overlap of species identified by these three methods together was estimated at only 1%. Torsvik *et al*. (1990) reported that less than 1% of the soil microorganisms were cultivable, and for composts, the percentage may be considerably lower. Therefore, any one of these approaches used alone might result in the identification of only a small part of the microbial community and a combination of these methods is required for monitoring the composting process.

Our previous studies revealed that when cattle manure compost was inoculated with MA, its temperature rapidly increased at the beginning of the process, and after the first turning, ammonia emission from the compost pile and nitrate production decreased more than in the composting process without the MA (Nakai *et al*. 2004; Sasaki *et al*. 2006). However, none of the dominant species detected in the MA were identified during the composting process by PCR-DGGE analysis (Wakase *et al*. 2008). These results may indicate that non-dominant species in the MA affected the complex conditions in the composting process. Microorganisms that are not dominant but have a few functions in the MA might function actively during the biodegradation of the manure and compost.

Although there are many types of commercial available MAs, the detailed microbial composition and function for most of MAs are not disclosed and elucidated. In fact, there is no evidence that most of commercial available MAs are effectiveness for compost, and also there are few reports dealt with a commercial MA. Thus, it is necessary to asses and evaluate commercial available MAs that are not disclosed their microbial composition with the unified methods. Simultaneously, the relationships of the predominant species in MA itself and the species that can dominate during composting process by adding MA should be clarified. Further, the interactions between maturities of composts and the MAs should be verified in future studies.

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