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# Culture-Independent Analysis of Microbial Succession During Composting of Swine Slurry and Mushroom Cultural Wastes

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**Bacterial diversity and the composition of individual communities during the composting process of swine and mushroom cultural wastes in a field-scale composter (Hazaka system) were examined using a PCR-based approach. The composting process was divided into six stages based on recorded temperature changes. Phylogenetic analysis of eighty 16S rRNA sequences from uncultured composting bacterial groups revealed the presence of representatives from three divisions, including plant pathogenic bacteria, high-molecule-degrading bacteria and spore-forming bacteria. The plant pathogen *A. tumefaciens* gradually decreased in abundance during the composting process and eventually disappeared during the thermophilic and cooling stage. A bacterium homologous to *Bacillus humi* first appeared at the early thermophilic stage and was established at the intermediate thermophilic, post-thermophilic, and cooling stages. It was not possible to isolate the *B. humi* during any of the stages using general culture techniques.**

**Keywords:** Field-scale composter, swine slurry and mushroom cultural wastes, uncultivable bacterium, bacterial diversity, 16S rRNA gene

Composting is an aerobic process by which organic materials are degraded through the activities of successive groups of microorganisms [2, 10]. Application of the final composted products includes such things as the restoration of construction sites, gardening, fertilizers, soils, and indigenous plant pathogen- suppressive potting mixes. The positive effects of composts on arable soil have been reported by many researchers [3, 24].

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A typical composting process is driven by dynamic changes in microbial communities under aerobic, moist, and self-heating conditions. The increase in temperature is a consequence of the biological activity and growth of thermophilic organisms during the initial phase of composting; lactic acid bacteria, Gram-negative bacteria, yeasts, and fungi are characteristic of this stage [12, 16, 23, 28]. In the subsequent degradation stage, thermophilic organisms take over, and many low G+C Gram-positive bacteria (LGCGPB) and high G+C Gram-positive bacteria (HGCGPB) have been isolated, and nonthermotolerant microbes are inhibited during this stage [5, 16, 34]. The final cooling and maturing period is characterized by the growth and development of a new mesophilic community, including *Bacteroidetes* and HGCGPB such as *Arthrobacter* sp. [23, 31, 33].

Animal waste composts (swine and cattle wastes) have been used as a nutrient source in crop production [9]. However, composts may also contain substances harmful to the environment such as pathogens, bioaerosols, heavy metals, and toxic organics. The pathogens may be viruses, bacteria, fungi, protozoa, nematodes, and helminthes. Pathogens are commonly present in sewage sludge, household waste, yard waste, and animal waste [17, 19], all of which are commonly composted. However, composting can be an efficient method for the destruction of such pathogens [11]. Swine waste alone cannot be subjected to composting because it requires other materials to support the process. Likewise, the large amount of mushroom cultural wastes (MCWs) generated by mushroom industries faces the same problems as that of swine waste while composting. To overcome these problems, both of the materials are mixed for composting. In addition, environmentally acceptable spent mushroom compost (SMC) management solutions combined with the development of new value-added products are of primary importance for this agro-industrial sector [1, 25].

Several cultivation-independent techniques including analyses of phospholipids fatty acids [7] and quinolines [32], oligonucleotide microarrays [14], and the analyses of rDNA genes encoding for the small ribosomal subunit DNA (16S rRNA in bacteria) [2, 10, 13, 27, 30, 31] have dramatically increased knowledge about the contribution of different microorganisms to various compost production phases. There is a general agreement among those in the field that the identification and classification of microorganisms should be performed according to phylogenetic analysis based on rDNA [26].

Currently, no systematic approach has been employed to elucidate the stability and/or change in the culture-independent bacterial community within the Hazaka system. In this study, we investigated the bacterial diversity of a field-scale composter using this system. Composting can be completed within less than 4 weeks, which is considered very efficient for the treatment of organic waste. In this context, we studied the bacterial diversity during composting of swine waste and MCW samples taken from five sites based on the temperature at the site. We also applied a traditional culture-dependent method in order to identify and isolate representative thermophilic bacteria.

## MATERIALS AND METHODS

### Sampling

Sampling is based on the different ranges/variation of temperature in the composting process. Five samples were collected from each stage at Yonghyeon Nonghyup Compost Factory [Yonghyeon-myeon, Sacheon, Korea; Hazaka system; 40 m (length) × 6 m (width) × 1.5 m (depth)]. Composting materials were mixed with swine and MCW (8:2) and stacked for a month. After 2 weeks, it was processed *via* machinery agitation and ventilation until composted fully. Five replicate samples at six different spots were collected from a depth of 10 to 20 cm from the surface. Samples were classified into six groups upon the change of temperature that occurred at the sampling site; S0 site (0 days), S1 site (1st days), S2 site (3rd day), S3 site (7th day), S4 site (10th day), S5 site (14th day) (Fig. 1A).

### Isolation of Thermophilic Bacteria

Ten g of each compost sample and 90 ml of 0.85% (w/v) NaCl were placed into a flask (500 ml). The mixture was homogenized in a shaker at 170 rpm for 30 min to disperse the bacteria and then was serially diluted to a factor of $10^{-8}$. Bacteria were isolated on nutrient agar (NA; Difco, U.S.A.), tryptic soy agar (TSA; Difco, U.S.A.), and plate count agar (PCA; Difco, U.S.A.) and were incubated at 60°C for 48 h. The selected colonies were picked on TSA.

### Extraction of Total DNA

The collected six composting samples and isolated thermophilic bacteria were centrifuged at 14,000 ×*g* for 5 min at 4°C. The obtained pellet (approximately 0.3 ml) was subjected to total DNA extraction using the G-spin Genomic DNA Extraction Kit (Intron Biotechnology, Suwon, Korea), as recommended by the supplier. The total DNA of six composting samples was purified with a gel purification kit (Intron Biotechnology, Suwon, Korea). The efficiency of DNA extraction varied from 0.75 μg to 1.0 μg according to a 260/280 ratio observed in a UV spectrophotometer (GeneQuant II; Pharmacia Biotech., Cambridge, U.K.). The extracted DNA was used as a template for PCR to amplify 16S rRNA.

### PCR Amplifications

The PCR primers used to amplify 16S rRNA fragments were the bacteria-specific primers, 5'-CGG AGA GTT TGA TCC TGG-3' (#877F, forward) and 5'-TAC GGC TAC CTT GTT ACG AC-3' (#878R, reverse) [8]. Subsequently, rDNAs were amplified by PCR using the metagenomic DNA and Super-Therm DNA polymerase (JMR; Side Cup, Kent, U.K.). Based on the manufacturer's instruction, the PCR reaction mixture (50 μl) contained 1 μl of *Taq* polymerase (25 unit), 3 μl each of primers 1BR and 2BR (10 pmol), 5 μl of reaction buffer, 15 mM $MgCl_2$, 5 μl of 2 mM dNTP, 5 μl of template DNA, and 28 μl of sterile water. Thirty cycles (denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec) were followed by a final extension at 72°C for 10 min. The anticipated product of approximately 1.5 kb was isolated by agarose gel electrophoresis. The bacterial 16S rRNA gene amplicons were purified with a PCR Purification Kit (Intron Biotechnology, Suwon, Korea).

### 16S rRNA Library

Amplified bacterial 16S rRNA genes were cloned in *E. coli* DH5α with the pGEM-T easy vector (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. Recombinant clones were randomly picked and recombinant plasmids were extracted using the Plasmid Purification Kit (Intron Biotechnology, Suwon, Korea). Purified plasmids were checked for correct insert size *via* standard vector-targeted PCR and gel electrophoresis.

### DNA Sequencing and Sequence Analysis

Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye Terminator/Primer Cycle Sequencing Kit (Perkin-Elmer Corp., Norwalk, CN, U.S.A.). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing was done on full-length cloned PCR product. All reference sequences were obtained from the GenBank and RDP (Ribosomal Database Project) [20] databases. Sequences were analyzed using the CHECK CHIMERA program [20] to identify and exclude sequences arising from chimeric rDNA clones. The 16S rRNA similarity sequences searches were performed using the BLASTN as well as PSI-BLAST tools in the NCBI Web site [21]. Sequences were aligned using the multiple sequence alignment program, CLUSTAL W [36]. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using neighbor-joining methods [29]. Bootstrap analysis was performed using data resample of 1,000 times with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada).

### Nucleotide Sequence Accession Numbers and Nomenclature

Nucleotide sequences have been deposited in the GenBank database under the accession numbers DQ345454 to DQ346645. In the first library (mixes materials, 0), clone names begin with the letters 0B (*e.g.*, 0B01). In the second library (about the 1st day), clones names begin with 1B (*e.g.*, 1B01). In the third library (about the 3rd day), clone name begin with letters 2B (*e.g.*, 2B01). In the fourth library

(about the 7th day), clones begin with letters 3B (*e.g.*, 3B01). In the fifth library (about the 10th day), clone names begin with letters 4B (*e.g.*, 4B01). In the sixth library (about the 14th day), clone names begin with letters 5B (*e.g.*, 5B01).

## Results

### Temperature Changes During the Composting Process

The composting process was characterized by an early period of self-heating due to rapid microbial metabolism. Based on the pattern of temperature changes, the following six stages of composting were identified: the preliminary stage as materials decomposed (organic and inorganic degrading molecules, 43±5°C, materials stocking period), mesophilic (43±5°C, 24±5 h), early thermophilic (57±5°C, 72±5 h), intermediate thermophilic (73±5°C, 96±5 h), post-thermophilic (65±5°C, 72±5 h), and cooling stages (40±5°C, 96±5 h). The organic composting materials were mixed and initially processed to 43±5°C at the preliminary stage (0 day, S0 site) and the mesophilic stage (1 day, S1 site). The temperature of the compost rapidly reached the early thermophilic levels (57±5°C, S2 site) by day 3. Thereafter, the temperature increased to 73±5°C at the intermediate thermophilic stage (7 day, S3 site). This stage corresponded to hot compost levels. Temperatures of the post-thermophilic (10 day, S4 site) and cooling stages (14 day, S5 site) decreased to 65±5°C and 40±5°C, respectively (Fig. 1).

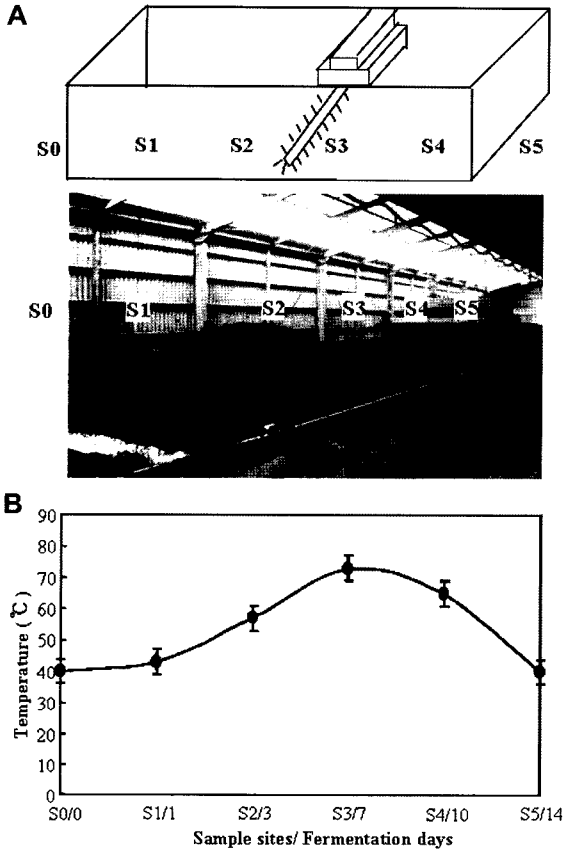


**Fig. 1. A.** Sampling sites during the composting process of swine and mushroom cultural wastes (MCW) in a field-scale composter. **B.** Change of temperature during the composting process of swine and MCW in a field-scale composter.
S0, preliminary phase as decomposing materials (0 day, 43±5°C, materials stocking period); S1, mesophilic phase (1st day, 43±5°C, 24±5 h); S2, early thermophilic phase (3rd day, 57±5°C, 72±5 h); S3, intermediate thermophilic phase (7th day, 73±5°C, 96±5 h); S4, post-thermophilic phase (10th day, 65±5°C, 72±5 h); S5, cooling-down or maturation phase (14th day, 40±5°C, 96±5 h).

### Similarity of Isolates with Database Sequences

About 163 sequences from 240 clones in our libraries (67.1%) were identified as *Bacillus humi* (98 clones) and the remaining clones were identified as *Agrobacterium tumefaciens* (18 clones), *Bacillus thermocloaceae* (16 clones), uncultured bacteria (7 clones), low G+C Gram-positive bacteria (4 clones), *Paenibacillus* sp. LMG 20245 (4 clones), *Lactobacillus* sp. 121B (3 clones), *Sporosarcina* sp. (3 clones), *Oligella ureolytica* (2 clones), *Bacillus* sp. KSM-P358 (3 clones), *Bacillus* sp. SSCS22-2 (1 clone), *Bacillus* sp. 112442 JS2 (1 clone), and *Bacillus* sp. MSP06G (1 clone). The percentage homologies of different clones with the database are described in Table 1.

During the preliminary and mesophilic stages, sequences were classified into 16 and 17 operational taxonomic units (OTUs) according to the 16S rRNA sequence (Table 1). Twenty clones shared similarity with the cultured isolate of an OTU at the preliminary stage (0B library). 0BII, 0BIII, 0BIV, 0BX (0B18), 0BXI, 0BXIII, and 0BXIV (0B30, 0B32, and 0B37) were similar to *A. tumefaciens, B. thermocloaceae, Bacillus* sp., *Lactobacillus* sp., and *Sporosarcina* sp., respectively. The other four clones shared sequence identity with database entries corresponding to uncultured bacteria. During the mesophilic stage (1B library), only four sequences were defined as belonging to the OTUs *A. tumefaciens* (1BVII) and *Oligella ureolytica* (1BX) (Table 1).

Clones were distributed into 23, 12, 6, and 4 OTUs at the early thermophilic, intermediate thermophilic, post-thermophilic, and cooling stages, respectively (Table 1). Thirteen clones shared similarity with the cultured isolate of an OTU at the early thermophilic stage (2B library). 2BXII, 2BXIII, 2BXIV, and 2BXVIII were similar to *B. humi, B. thermocloaceae, Bacillus* sp., and *Paenibacillus* sp., respectively. The other four clones matched sequence entries corresponding to uncultured bacteria. At the intermediate mesophilic stage (3B library), 33 sequences were defined as belonging to specific OTUs including *B. humi* (3BII), *B. thermocloaceae* (3BIII), *Bacillus* sp. (3BIV and 3BV), and *Paenibacillus* sp. (3BIX). Thirty-eight sequences belonged to the OTUs corresponding to *B. humi, B. thermocloaceae, Bacillus* sp., and *Paenibacillus* sp. in the post-thermophilic stage (4B library). Finally, at

**Table 1.** Similarity values of 16S rRNA sequences retrieved from the clones of each library.

| Group | Clone | Phylum | Nearest relative[a] | Accession No. | Similarity (%) |
|---|---|---|---|---|---|
| **0B (0 D)** | | | | | |
| 0BI | 0B01 | Bacteroidetes | *Sphingobacterium* sp. MG2 | AY556417 | 91 |
| 0BII | 0B02, 0B04, 0B06, 0B08, 0B09, 0B17, 0B19, 0B24, 0B25, 0B27, 0B28, 0B29, 0B34, 0B35, 0B39, 0B40 | Proteobacteria | *Agrobacterium tumefaciens* | AE009348 | **98–100**[b] |
| 0BIII | 0B07 | Firmicutes | *Anaerococcus octavius* | Y07841 | 94 |
| 0BIV | 0B036 | Firmicutes | *Bacillus fordii* | AY443039 | 96 |
| 0BV | 0B38 | Firmicutes | *Bacillus hackensackii* | AY148129 | 95 |
| 0BVI | 0B03 | Firmicutes | *Bacillus lentus* | AB021189 | 95 |
| 0BVII | 0B20 | Firmicutes | *Bacillus thermocloaceae* | Z26939 | **99** |
| 0BVIII | 0B14, 0B18 | Firmicutes | *Bacillus* sp. SSCS22-2 | AB21189 | 95–**99** |
| 0BIX | 0B15 | Firmicutes | *Bacillus* sp. 11244JS2 | AF071857 | **99** |
| 0BX | 0B11, 0B12 | Firmicutes | *Enterococcus casseliflavus* | AF039903 | 96 |
| 0BXI | 0B21, 0B22, 0B31 | Firmicutes | *Lactobacillus* sp. 121B | AF305930 | **99** |
| 0BXII | 0B26, 0B30, 0B32, 0B37 | Firmicutes | *Sporosarcina* sp. F73 | DQ73393 | 97–**98** |
| 0BXIII | 0B05 | Firmicutes | LGCGPB[c] M52 | AB116130 | **99** |
| 0BXIV | 0B10, 0B16, 0B33 | Firmicutes | LGCGPB T134 | AB116139 | **99–100** |
| 0BXV | 0B13, 0B23 | Firmicutes | UFB[d] | DQ129529 | 96 |
| **1B (1 D)** | | | | | |
| 1BI | 1B03, 1B12 | Bacteroidetes | Bacteroidetes endosymbiont | AY753171 | 89–90 |
| 1BII | 1B02, 1B19, 1B22, 1B26, 1B34, 1B37, 1B39, 1B40 | Bacteroidetes | *Sphingobacterium* sp. MG2 | AJ556417 | 91 |
| 1BIII | 1B05, 1B06, 1B09, 1B15, 1B18, 1B32 | Bacteroidetes | UBB[e] | AJ318191 | 91 |
| 1BIV | 1B21, 1B24 | Bacteroidetes | UBB | AJ853590 | 92–93 |
| 1BV | 1B10, 1B27 | Bacteroidetes | UBB | AJ582209 | 96 |
| 1BVI | 1B23 | Bacteroidetes | UBB | AJ223454 | 92 |
| 1BVII | 1B04, 1B33 | Proteobacteria | *Agrobacterium tumefaciens* | AE009348 | **99** |
| 1BVIII | 1B08, 1B14, 1B25, 1B29, 1B35, 1B38 | Proteobacteria | *Alcaligenes* sp. R-21939 | AJ786800 | 93 |
| 1BIX | 1B01 | Proteobacteria | *Bacterium* sp. rM17 | AB021352 | 93 |
| 1BX | 1B31, 1B36 | Proteobacteria | *Oligella ureolytica* | AJ251912 | **99** |
| 1BXI | 1B17, 1B20 | Proteobacteria | *Pusillimonas noertemannii* | AY695828 | 95 |
| 1BXII | 1B11 | Proteobacteria | UPB[f] | AJ853534 | **98** |
| 1BXIII | 1B16 | Proteobacteria | UPB | AY770946 | 96 |
| 1BXIV | 1B28 | Proteobacteria | UPB | AY676483 | 97 |
| 1BXV | 1B13, 1B30 | Firmicutes | *Halanaerobium salsuginis* | L22890 | 88 |
| 1BXVI | 1B07 | Firmicutes | *Thermaerobacter subterraneus* | AF343566 | 89 |
| **2B (3 D)** | | | | | |
| 2BI | 2B08 | Bacteroidetes | Bacteroidetes endosymbiont | AY753171 | 89 |
| 2BII | 2B07 | Bacteroidetes | Bacteroidet bacterium | AJ565431 | 91 |
| 2BIII | 2B24 | Bacteroidetes | *Flexibacter tractuosus* | AB078071 | 91 |
| 2BIV | 2B03, 2B12, 2B15, 2B34 | Bacteroidetes | UBB[e] | AJ318191 | 91 |
| 2BV | 2B09 | Bacteroidetes | UBB | AY838493 | 91 |
| 2BVI | 2B01 | Bacteroidetes | UBB | DQ103637 | 92 |
| 2BVII | 2B22 | Proteobacteria | *Amaricoccus veronensis* | U88043 | 93 |
| 2BVIII | 2B10, 2B18 | Proteobacteria | *Pusillimonas noertemannii* | AY695828 | 95 |
| 2BIX | 2B37 | Proteobacteria | *Rhizobium* sp. TANU14 | AJ971481 | 94 |
| 2BX | 2B13 | Proteobacteria | *Rhodovibrio* sp. 2Mbl | AY987846 | 91 |
| 2BXI | 2B02, 2B14, 2B21, 2B23 | Proteobacteria | UPB[d] | AJ853534 | **98–99** |
| 2BXII | 2B28, 2B33, 2B38 | Firmicutes | *Bacillus humi* | AJ627209 | **98** |

**Table 1.** Continued.

| Group | Clone | Phylum | Nearest relative[a] | Accession No. | Similarity (%) |
|---|---|---|---|---|---|
| 2BXIII | 2B04, 2B05, 2B11, 2B17, 2B20, 2B25, 2B26, 2B30 | Firmicutes | *Bacillus thermocloaceae* | Z26939 | **99** |
| 2BXIV | 2B32 | Firmicutes | *Bacillus* sp. KSM-P358 | AB073167 | **98** |
| 2BXV | 2B39 | Firmicutes | *Paenibacillus pabuli* | AB073191 | 95 |
| 2BXVI | 2B27 | Firmicutes | *Paenibacillus timonensis* | AY323610 | 94 |
| 2BXVII | 2B29 | Firmicutes | *Paenibacillus* sp. 19508 | AJ315076 | 93 |
| 2BXVIII | 2B35 | Firmicutes | *Paenibacillus* sp. LMG20245 | AJ316315 | **99** |
| 2BXIX | 2B40 | Firmicutes | *Virgibacillus picturae* | AJ276808 | 95 |
| 2BXX | 2B06, 2B16 | Firmicutes | UFB[e] | AB114316 | 90 |
| 2BXXI | 2B14 | Firmicutes | UFB | AF252323 | 96 |
| 2BXXII | 2B31 | Firmicutes | UFB | AY559417 | 95 |
| 2BXXIII | 2B38 | Firmicutes | UFCB[f] | AB034713 | 96 |
| **3B (7 D)** | | | | | |
| 3BI | 3B26 | Proteobacteria | *Rhizobium* sp. TANU14 | AJ971481 | 94 |
| 3BII | 3B01–3B06, 3B08–3B10, 3B12, 3B14, 3B15, 3B17–3B20, 3B23, 3B25, 3B27, 3B30, 3B31, 3B33–3B36, 3B38, 3B40 (28 clones) | Firmicutes | *Bacillus humi* | AJ627209 | **98–99** |
| 3BIII | 3B11 | Firmicutes | *Bacillus thermocloaceae* | Z26939 | **99** |
| 3BIV | 3B39 | Firmicutes | *Bacillus* sp. KSM-P358 | AB073167 | **98** |
| 3BV | 3B21 | Firmicutes | *Bacillus* sp. MSP06G | AB084065 | **98** |
| 3BVI | 3B32 | Firmicutes | *Paenibacillus pabuli* | AB073191 | 95 |
| 3BVII | 3B29 | Firmicutes | *Paenibacillus* timonensis | AY323610 | 94 |
| 3BVIII | 3B07 | Firmicutes | *Paenibacillus* sp. 19508 | AJ315076 | 93 |
| 3BIX | 3B16, 3B28 | Firmicutes | *Paenibacillus* sp. LMG20245 | AJ316315 | **98–99** |
| 3BX | 3B13 | Firmicutes | *Virgibacillus picturae* | AJ276808 | 95 |
| 3BXI | 3B22 | Firmicutes | UFB | AY559417 | 95 |
| 3BXII | 3B37 | Firmicutes | UFCB | AB034713 | 96 |
| **4B (10 D)** | | | | | |
| 4BI | 4B02–4B06, 4B08–4B15, 4B17, 4B–4B23, 4B25–4B36, 4B38–4B40 (34 clones) | Firmicutes | *Bacillus humi* | AJ627209 | **98–99** |
| 4BII | 4B18 | Firmicutes | *Bacillus thermocloaceae* | Z26939 | **99** |
| 4BIII | 4B01, 4B2 | Firmicutes | *Bacillus* sp. KSM-P358 | AB073167 | **98** |
| 4BIV | 4B07 | Firmicutes | *Paenibacillus* sp. LMG20245 | AJ316315 | **99** |
| 4BV | 4B16 | Firmicutes | UFB | DQ129330 | **99** |
| 4BVI | 4B37 | Firmicutes | UFCB | AB034713 | 96 |
| **5B (14 D)** | | | | | |
| 5BI | 5B02–5B06, 5B09–5B15, 5B17, 5B18–5B21, 5B23–5B25, 5B27–5B34, 5B36-5B40 (33 clones) | Firmicutes | *Bacillus humi* | AJ627209 | 94–**99** |
| 5BII | 5B07, 5B08, 5B16, 5B26, 5B35 | Firmicutes | *Bacillus sp.* KSM-P358 | AB073167 | **98–99** |
| 5BIII | 5B01 | Firmicutes | UFB | DQ129330 | **98** |
| 5BIV | 5B22 | Firmicutes | UFCB | AB034713 | 96 |

[a]Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.
[b]Database sequences with >97% similarity are shown in bold.
[c]UBB: Uncultured bacteroidetes bacterium.
[d]UPB: Uncultured proteobacteria bacterium.
[e]UFB: Uncultured firmicutes bacterium.
[f]UFCB: Uncultured firmicutes composting bacterium.

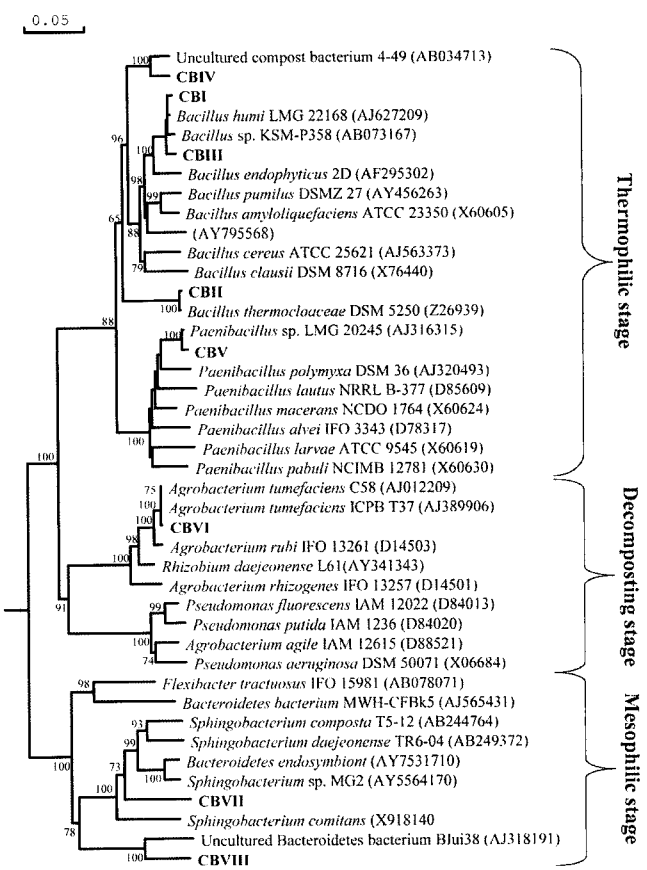


**Fig. 2.** Phylogenetic relationships of major group of composting bacteria and other related bacteria based on 16S rRNA sequences.
The number above each node is confidence levels (%) generated from 1,000 bootrap trees. CBI, group I (AJ627209); CBII, group II (Z26939); CBIII, group III (AB073167); CBIV, group IV (AB034713); CBV, group V (AJ316315); CBVI, group VI (AJ38990); CBVII, group VII (AY5564170); CBVIII, group VIII (AJ318191).

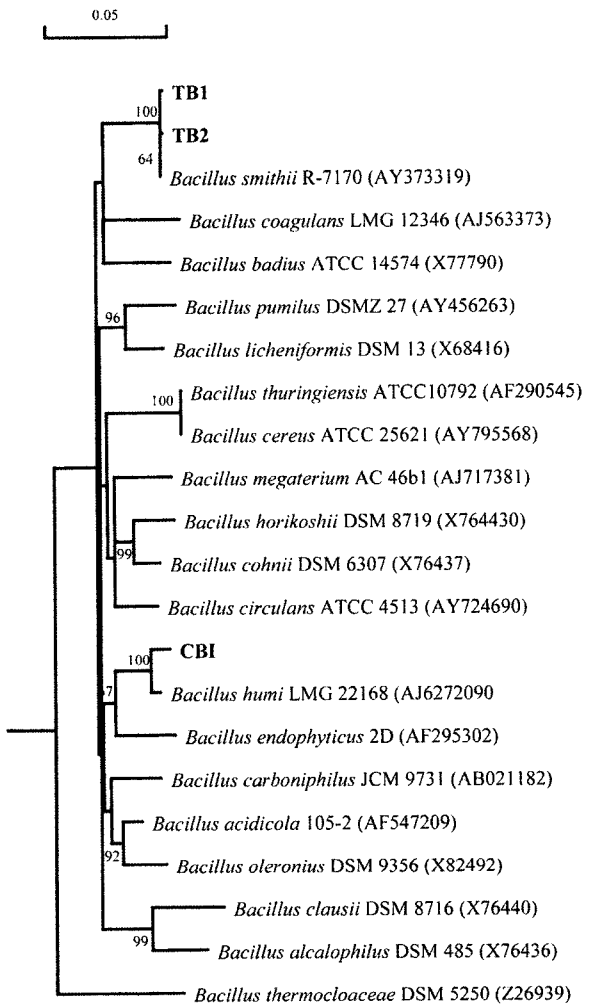


**Fig. 3.** Phylogenetic relationships of CBI, TB, and TB2 and other related *Bacillus* based on 16S rRNA sequences.
The number above each node is confidence levels (%) generated from 1,000 bootrap trees. CBI, group I (AJ627209); TB1 and TB2, isolated thermophilic bacteria.

the cooling stage (5B library), four of the clones could be assigned to OTUs represented in the database. Thirty-seven clones had similarity with the cultured isolate of an OTU. 5BI and 5BII were similar to *B. humi* and *Bacillus* sp., respectively (Table 1).

### Phylogenetic Analysis of 16S rRNA Sequences

The results of phylogenetic analysis from the major group of uncultured composting bacteria are shown in Fig. 2. Of the major groups of uncultured composting bacteria analyzed, 80 clones were from the preliminary stage as decomposing materials, mesophilic, thermophilic, and cooling stages. Sequences recovered from the preliminary and mesophilic stages belonged to mesophilic bacterial species, and thermophilic and cooling stages contained sequences belonging to thermophilic bacterial species (Fig. 2).

Sequences isolated during the preliminary stage revealed that the bacterial species present were related to the plant pathogenic bacterial species *A. tumefaciens*. Two *Sphingobacterium* sp. MG2 and uncultured bacteroidetes bacteria (UBB) species were present during the mesophilic stage. At the early thermophilic, intermediate thermophilic, post-thermophilic, and cooling stages, the sequences were related to the spore-forming bacterial species *B. thermocloaceae*, *B. humi*, *Bacillus* sp. KSM-P358, and *Paenibacillus* sp. LMG20245 (Fig. 2).

The thermophilic bacteria incubated at 60°C in plates selected were the specific form and high community of four classes of bacteria. We obtained four culturable thermophilic bacteria, all of which we sequenced, and found that these were *B. pumilus*, *B. licheniformis*, *B. cereus*, and *B. smithii*. Specifically, two strains (TB1 and TB2) of thermophilic bacteria were isolated, and their optimal temperatures were 55°C and 65°C, respectively. Strain TB1 was faster-growing and showed a wider range of growth temperatures than

strain TB2 (data not shown). A phylogenetic tree constructed from these sequences shows that strains TB1 and TB2 were within evolutionary radiation, which encompasses the genus *Bacillus* and occupies a distinct phylogenetic position within this genus. The level of similarity of the 16S rRNA sequences between strains TB1, TB2, and the *Bacillus* species ranges from 90.4% to 99.9%, and from 90.3% to 99.9%, respectively. The highest 16S rRNA sequence similarity (99.9%) was observed between strain TB1, TB2, and *Bacillus smithii* R-7170 (AY373319). This phylogenetic study clearly establishes that strains TB1 and TB2 are closely related to *Bacillus* sp. and *Bacillus smithii* R-7170 (AY373319). However, the lowest level of 16S rRNA similarity (94.7%) was observed between strain TB1, TB2, and *Bacillus humi* LMG 22168 (AJ272090). Likewise, the lowest 16S rRNA sequence similarity (94.3%) was between the uncultured composting bacteria CBI group and *Bacillus smithii* R-7170 (AY373319) (Fig. 3).

### Transformation of the Major Group of Composting Bacteria

The predominant composting bacteria varied according to different composting stages: *A. tumefaciens* (CBVI, AE009348) at the preliminary stage (16 clones), *Sphingobacterium* sp. MG2 (CBVII, AJ556417) at the mesophilic stage (8 clones), *B. thermocloaceae* (CBII, Z26939) at the early thermophilic stage (8 clones), and *B. humi* (CBI, AJ6272090) at the intermediate thermophilic stage (28 clones), post-thermophilic stage (34 clones), and cooling stage (33 clones) (Fig. 4).

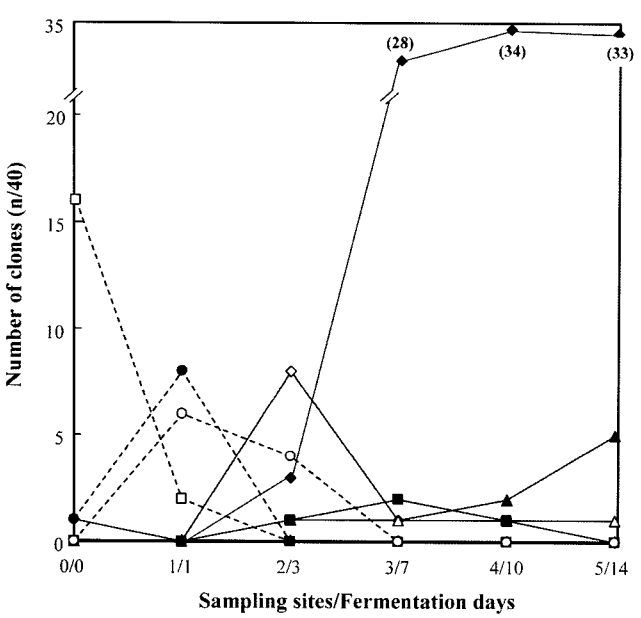


**Fig. 4.** Change of the major group of composting during composting period with temperature.
The line and dotted line represent the mesophilic bacteria and thermophilic bacteria, respectively. Symbols: ◆, group I (AJ627209 ); ◇, group II (Z26939); ▲, group III (AB073167); △, group IV (AB034713); ■, group V (AJ316315); □, group VI (AJ38990); ●, group VII (AY5564170); ○, group VIII (AJ318191).

The majority of the composting bacteria can be placed among the thermophilic/spore-forming bacteria at the early thermophilic, intermediate thermophilic, post-thermophilic, and cooling stages. At the early thermophilic stage, the majority of the composting bacteria were related to *B. thermocloaceae* (CBII), *B. humi* (CBI), *Bacillus* sp. KSM-P358 (CBIII, AB073167), *Paenibacillus* sp. LMG20245 (CBV, AJ316315), and UBB (CBVIII), and amounted to three, one, four, and one clones, respectively. *Bacillus humi* first appeared at this stage. The majority of the composting bacteria in the intermediate thermophilic stage could be placed within *B. humi* (CBI). *B. thermocloaceae* (CBII), *Bacillus* sp. KSM-958 (CBIII), and *Paenibacillus* sp. LMG20245 (CBV) each accounted for one, one, and two clones, respectively. At the post thermophilic stage, the majority of the composting bacteria belonged to *B. humi* (CBI). *B. thermocloaceae* (CBII), *Bacillus* sp. KSM-958 (CBIII), and *Paenibacillus* sp. LMG20245 (CBV) accounted for one, two, and one clone, respectively. Finally, the predominant composting bacteria in the cooling stage were located within *B. humi* (CBI). *Bacillus* sp. KSM-P358 (CBIII) accounted for five clones (Fig. 4).

In this study, an attempt was made to isolate the CBI clone (homologous to *B. humi*) from the thermophilic stage. Instead, the thermophilic bacteria strains TB1 and TB2 were isolated from this sample. Nevertheless, the lowest level of 16S rRNA similarity (94.7%) was observed between those and *B. humi* LMG 22168 (AJ272090) (Fig. 3). This result suggests that homologous *B. humi* (CBI) cannot be isolated by the current culture techniques.

## Discussion

During the composting process, physical characteristics such as temperature, pH, nutrient availability, and moisture content indicate the extent of decomposition [35]. The period of high temperatures most likely ensured sanitization of the material, but at the same time, rather high temperatures probably depressed the decomposition rate. Therefore, total N, C, and C:N ratio were influenced by differences in the components of the initial compost material. The C contents varied from 521 and 531 g/kg; N contents between 20 and 28.5 g/kg; C:N ratios between 18 and 25.8 (data not shown). The stability of compost is connected to the microbial activities during composting. During composting, the degradable organic matter and nitrogenous compounds in pig manure are broken down by microorganisms [9]. Towards the end of composting, no further decomposition is taking place as C and N became stabilized. Thus, it has been suggested that in addition to temperature, the availability

of specific substrates are key factors in the selection of microbial communities [27, 31].

Samples were collected during the composting process based on the temperature of the material (Fig. 1). 16S rRNA was amplified by PCR, and six libraries were constructed. Among the 240 sequences obtained from the samples, only 163 clones were considered OTUs, and these were distributed into the different groups in each sample based on similarity with a match from the database. Since the majority of our sequences had similarity values that were too low to classify into any given taxa with a reasonable degree of confidence, phylogenetic analysis was performed to clarify their taxonomic position. It has been suggested that phylogenetic clustering of bacterial groups, rather than a specific similarity value, should be used as a guide for defining bacterial taxa. Phylogenetic analysis based on 16S rRNA sequences showed that the sequences of clones isolated in this study belonged to three major phylogenetic groups including *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. At the preliminary stage, the composting bacteria could be divided into the phyla *Firmicutes*, *Bacteriodetes*, and *Proteobacteria*. The majority of the composting bacteria in this stage belonged to the *Firmicutes* (57.5%) and *Proteobacteria* (40.0%) phyla. The *Bacteriodetes* phylum accounted for 2.5%. The majority of the composting bacteria were assigned to *A. tumefaciens* (CBVI, 40.0%). *Agrobacterium tumefaciens* appeared only in the early stage and the mesophilic stage and disappeared in the thermophilic phase. The period of high temperatures most likely ensured sanitization of the material. Thus, pathogenic microorganisms die during compost processing [6]. Our samples were stocked for one month and this could be the reason that

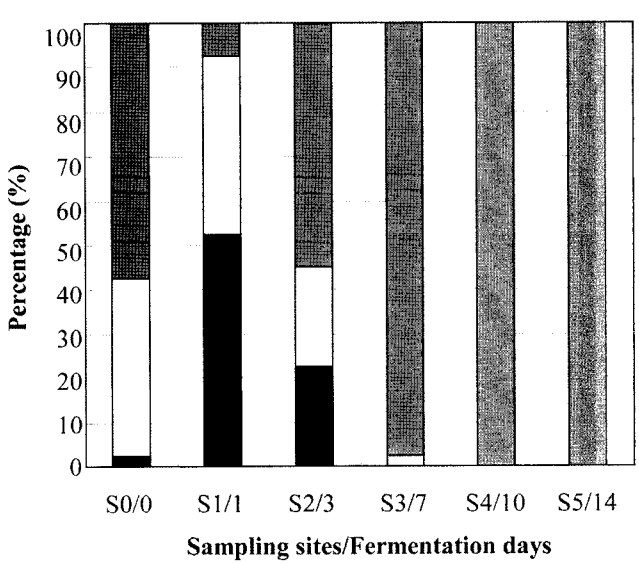


**Fig. 5.** Population dynamics of dominant species during composting process of swine and MCW in a field-scale composter.
Percentage of microcosm in each of the six libraries is shown. *Bacteroidetes* (black); *Proteobacteria* (open); *Firmicutes* (gray).

many of the animal pathogens could have not been detected during the composting process or some minor population may have missed the PCR amplification. At the mesophilic stage, the majority of composting bacteria were placed within the *Bacteriodetes* (52.5%) and *Proteobacteria* (40.0%) phyla. The *Firmicutes* phylum accounted for 7.5% (Fig. 5). The phylum *Bacteroidetes* contains a wide variety of bacteria known for their utilization of macromolecules such as proteins, starch, cellulose, chitin, and lignin, and members of this phylum have been previously detected by molecular methods in various types of compost [22]. In the previous study, many *Clostridium* sp. were reported in swine manure compost, but our result did not detect any stages related to the *Clostridium* group. PCR-based methodologies are subject to certain limitations such as the PCR amplification error and formation of chimera [37]. In addition, Lee and Kim [18] suggested that PCR amplification cannot be strictly co-related with the ratio of target DNA to total DNA. As a result, some minor population may have been missed in our present study. Recently, Ntougias *et al.* [25] reported that bacterial diversity in SMC is greatly affected by the origin of the initial material, its thermal pasteurization treatment, and the potential for unintended colonization of the mushroom substrate during the cultivation process. This result suggested that mainly thermotolerant bacteria existing in the initial materials are expected to survive, and then during conditioning they could rapidly colonize the substrate. Thus, the nature of microorganisms present in the MCW is of interest since this material has a great recycling potential and valorization potential.

The composting bacteria were divided into three phyla (*Firmicutes*, *Bacteriodetes*, and *Proteobacteria*) at the early thermophilic stage, two phyla (*Firmicutes* and *Proteobacteria*) at the intermediate thermophilic stage, and only one phylum (*Firmicutes*) at the post-thermophilic and cooling stages. More than half (55.5%) of the composting bacteria were placed within the *Firmicutes* phylum at the early thermophilic stage. The *Bacteriodetes* and *Proteobacteria* phyla accounted for 22.5% of the samples. The majority of the composting bacteria in the intermediate thermophilic stage belonged to the *Firmicutes* phylum (97.5%), whereas the *Proteobacteria* phylum amounted to only 2.5%. At the post-thermophilic and cooling stages, all composting bacteria were placed within the *Firmicutes* phylum (Fig. 4). Previous culture-dependent and culture-independent composting studies have detected the phyla *Firmicutes* and *Actinobacteria*. However, we did not obtain any samples related to the phylum *Actinobacteria*. The phylum *Firmicutes* typically includes spore-forming bacteria, which explains their presence in compost because of their abundance in the initial substrates and to their thermotolerance, allowing them to be active in or at least survive the hot composting stage [2, 10, 13, 16, 31]. Most *Firmicutes* detected in this study were closely related to members of the *Bacillus* and *Paenibacillus*

groups. It has been previously reported that diverse bacterial species are present in hot compost [5, 10, 27], and an increase in respiratory activity occurs at temperatures above 60°C [4]. These observations support our finding that the *Bacillus* group from *Firmicutes* is generally present at the hot composting stage, even in the case of systems containing different organic wastes.

In this study, we found that the most predominant clones were from the thermophilic stage and belonged to clone CBI (homologous to *B. humi*, a thermophilic bacterium). These clones were dominant throughout the entire thermophilic stage, and persisted into the cooling stage. Furthermore, we isolated the thermophilic bacterium *Bacillus smithii* R-7170 (Fig. 3) from the same compost sample, and as expected, we did not detect any other bacteria similar to *B. humi*. Recently, *B. humi* was isolated in soil from the Drentse Agriculture Research area in The Netherlands. *B. humi* produces ellipsoidal or spherical endospores that appear in subterminal or terminal positions and can swell the sporangia. Optimum growth occurs at approximately 30°C [15]. Here, we showed that one of our CBI clones shared sequence similarity with the bacterium *B. humi*, indicating that it belongs to *B. humi* and was a metagenomic, uncultivable bacterium.

This study revealed that culture-independent methods can be successfully applied to an environment processing sample, such as swine and MCW mixed composting. The data presented in this study should provide a useful frame of reference for further studies of population dynamics during the composting process of poultry and mushroom wastes in field-scale composters, and for control of the composting process. It may also be possible to continue the composting of swine and MCW by recycling the produced compost, in which the *B. humi* is highly concentrated, as a starter for the following treatment.

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## References

1. Adams, J. D. W. and L. E. Frostick. 2008. Investigating microbial activities in compost using mushroom (*Agaricus bisporus*) cultivation as an experimental system. *Bioresour. Technol.* **99:** 1097–1102.
2. Alfreider, A., S. Peters, C. C. Tebbe, A. Rangger, and H. Insam. 2002. Microbial community dynamics during composting of organic matter as determined by 16S ribosomal DNA analysis. *Compost Sci. Util.* **10:** 303–312.
3. Bailey, K. L. and L. G. Lazarovits. 2003. Suppressing soil-borne diseases with residue management and organic amendments. *Soil Tillage Res.* **72:** 169–180.
4. Beffa, T., M. Blanc, P. F. Lyon, G. Vogt, M. Marchiani, J. L. Fischer, and M. Aragno. 1996. Isolation of *Thermus* strains from hot composts (60 to 80°C). *Appl. Environ. Microbiol.* **62:** 1723–1727.
5. Blanc, M., L. Marilley, T. Beffa, and M. Aragno. 1999. Thermophilic bacterial communities in hot composts as revealed by most probable number counts and molecular (16S rRNA) methods. *FEMS Microbiol. Ecol.* **28:** 141–149.
6. Bollen, G. J. 1985. The fate of plant pathogens during composting of crop residues, pp. 282–290. *In* J. K. R. Gasser (ed.), *Composting of Agricultural and Other Wastes*. Elsevier Applied Science, London.
7. Cahyani, V. R., A. Watanabe, K. Matsuya, S. Asakawa, and M. Kimura. 2002. Succession of microbiota estimated by phospholipids fatty acid analysis and changes in organic constituents during the composting process of rice straw. *Soil Sci. Plant Nutr.* **48:** 735–743.
8. Cho, S. J., K. M. Cho, E. C. Shin, W. J. Lim, S. Y. Hong, B. R. Choi, *et al.* 2006. 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. *J. Microbiol. Biotechnol.* **16:** 92–101.
9. Dambreville, C., S. Hallet, C. Nguyen, T. Morvan, J. C. Germon, and L. Philippot. 2006. Structure and activity of the denitrifying community in a maize-cropped field fertilized with composted swine manure or ammonium nitrate. *FEMS Microbiol. Ecol.* **56:** 119–131.
10. Dees, P. M. and W. C. Ghiorse. 2001. Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolated and extracted DNA. *FEMS Microbiol. Ecol.* **35:** 207–216.
11. Dumontet, S., H. Dinel, and S. B. Baloda. 1999. Pathogen reduction in sewage sludge by composting and other biological treatments: A review. *Biol. Agr. Hort.* **16:** 409–430.
12. Epstein, E. 1997. Microbiology, pp. 53–76. *In* E. Epstein (ed.), *The Science of Composting*. CRC Press, Washingtion, DC.
13. Fracchia, L., A. B. Dohrmann, M. G. Martinotti, and C. C. Tebbe. 2006. Bacterial diversity in a finished compost and vermicompost: Differences revealed by cultivation-independent analyses of PCR-amplified 16S rRNA genes. *Appl. Microbiol. Biotechnol.* **71:** 942–952.
14. Franke-Whittle, I. H., S. H. Klammer, and H. Insam. 2005. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. *J. Microbiol. Methods* **62:** 37–56.
15. Heyrman, J., M. Rodríguez-Díaz, J. Devos, A. Felske, N. A. Logan, and P. De Vos. 2005. *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov and *Bacillus humi* sp. nov., isolated from soil. *Int. J. Syst. Evol. Microbiol.* **55:** 111–117.
16. Juteau, P., D. Tremblay, R. Villemur, J. G. Bisaillon, and R. Beaudet. 2005. Analysis of the bacterial community inhabiting

an aerobic thermophilic sequencing batch reactor (AT-SBR) treating swine waste. *Appl. Microbiol. Biotechnol.* **66:** 115–122.
17. Larney, F. J. and X. Hao. 2007. A review of composting as a management alternative for beef cattle feedlot manure in southern Alberta, Canada. *Bioresour. Technol.* **98:** 3221–3227.
18. Lee, H. J. and J. Kim. 2000. Multiplex PCR-based detection and identification of *Leuconostoc* species. *FEMS Microbiol. Lett.* **193:** 243–247.
19. Lemunier, M., C. Francou, S. Rousseaux, R. Houot, P. Dantigny, P. Piveteau, and J. Guzzo. 2005. Long-term survival of pathogenic and sanitation indicator bacteria in experimental biowaste composts. *Appl. Environ. Microbiol.* **71:** 5779–5786.
20. Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Jr. Parker, P. R. Saxman, J. M. Stredwick, *et al.* 2000. The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.* **28:** 173–174.
21. McGinnis, S. and T. L. Madden. 2004. BLAST: At the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res.* **32:** 20–25.
22. Michel, F. C., T. J. Jr. Marsh, and C. A. Reddy. 2002. Bacterial community structure during yard trimmings composting, pp. 25–42. *In* H. Insam, S. Riddech, and S. Klammer (eds.), *Microbiology of Composting*. Springer, Berlin, Heidelberg, NY.
23. Miller, F. C. 1993. Composting as a process based on the control of ecologically selective factors., pp. 515–539. *In* F. Blaine and J. Metting (eds.), *Soil Microbial Ecology – Applications in Agricultural and Environmental Management*. Marcel Dekker, NY.
24. Nakasaki, K., S. Hiraolka, and H. Nagata. 1998. A new operation for producing disease-suppressive compost from grass clippings. *Appl. Environ. Microbiol.* **64:** 4015–4020.
25. Ntougias, S., G. I. Zervakis, N. Kavroulakis, C. Ehaliotis, and K. K. Papdopoulou. 2004. Bacterial diversity in spent mushroom compost assessed by amplified rDNA restriction analysis and sequencing of cultivated isolates. *System. Appl. Microbiol.* **27:** 7416–754.
26. Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen. 1986. The analysis of natural microbial population by ribosomal RNA sequences. *Adv. Microb. Ecol.* **9:** 1–55.
27. Peters, S., S. Koschinsky, F. Schwieger, and C. C. Tebbe. 2000. Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. *Appl. Environ. Microbiol.* **66:** 930–936.
28. Ryckeboer, J., J. Mergaert, K. Vaes, S. Klammer, D. De Clercq, J. Coosemans, H. Insam, and J. Swings. 2003. A survey of bacteria and fungi occurring during composting and self-heating processes. *Ann. Microbiol.* **53:** 349–410.
29. Saito, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
30. Shuang, J. L., C. H. Liu, S. Q. An, Y. Xing, G. Q. Zheng, and Y. F. Shen. 2006. Some universal characteristics of intertidal bacterial diversity as revealed by 16S rRNA gene-based PCR clone analysis. *J. Microbiol. Biotechnol.* **16:** 1882–1889.
31. Takaku, H., S. Kodaira, A. Kimoto, M. Nashimoto, and M. Takagi. 2006. Microbial communities in the garbage composting with rice hull as an amendment revealed by culture-dependent and -independent approaches. *J. Biosci. Bioeng.* **101:** 42–50.
32. Tang, J. C., T. Kanamori, Y. Inoue, T. Yasuta, S. Yoshida, and A. Katayama. 2004. Changes in the microbial community structure during thermophilic composting of manure as detected by the quinine profile method. *Process Biochem.* **39:** 1999–2006.
33. Ten, L. N., G. M. Liu, W. T. Kim, Z. Aslam, and S. T. Lee. 2006. *Sphingobacterium composti* sp. nov., a novel DNase-producing bacterium isolated from compost. *J. Microbiol. Biotechnol.* **16:** 1728–1733.
34. Tiquia, S. M., J. M. Ichida, H. M. Keener, D. L. Elwell, E. H. Jr. Burtt, and F. C. Jr. Michel. 2005. Bacterial community profiles on feathers during composting as determined by terminal restriction fragment length polymorphism analysis of 16S rDNA genes. *Appl. Microbiol. Biotechnol.* **67:** 412–419.
35. Tiquia, S. M. and N. F. Y. Tam. 2000. Co-composting of spent swine litter and sludge with forced aeration. *Bioresour. Technol.* **72:** 1–7.
36. Tompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.
37. Wintzingerode, F., U. B. Göbel, and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21:** 213–229.