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Changes in the actinomycetal communities during continuous thermophilic composting as revealed by denaturing gradient gel electrophoresis and quantitative PCR

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ABSTRACT

Actinomycetes degrade cellulose and solubilize lignin during composting. Changes in the diversity of the actinomycetal communities and the 16S rDNA copy numbers of actinomycetes were monitored by denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR), respectively, during continuous thermophilic composting (CTC) and traditional composting (TC). qPCR indicated that the copy numbers from the CTC samples were 25–80% higher than those from the TC samples during similar phases of active composting and they were lower than 3×10^9 gene copies/g (dry weight) in the mature compost from both runs. DGGE showed a more diverse actinomycetal community in the CTC than in TC, averaging 16 bands as compared to 12 bands, at the post peak temperature phase. The study suggested that temperatures higher than 50 °C in CTC benefited the growth of actinomycetes.

1. Introduction

Composting is a self-heating, aerobic, bio-decomposition process of organic waste that has advantages over other disposal strategies since it reduces waste volume by 40–50%, kills pathogens by the heat generated during the thermophilic phase and provides a final product that can be used as a soil conditioner or fertilizer (Larney et al., 2003; Wong et al., 1995). Continuous thermophilic composting (CTC), which was first applied to a 55-gal rotating drum composting system by Schulze (1962), was demonstrated to be more effective than traditional composting (TC) since it produced a more stable product and shortened the processing (Xiao et al., 2009a). However, only a few studies (Schulze, 1962; Suler and Finstein, 1977; Strom, 1985) have explored the degradation mechanism of organic materials during the CTC process. It is known that the decomposition of organic substrates in compost is mainly carried out by microorganisms and that actinomycetes, a group of Gram-positive mycelium-forming bacteria, are efficient in degrading macromolecules such as proteins and cellulose, and in solubilizing lignin (Tuomela et al., 2000). These bacteria tolerate higher temperatures and pH than most fungi and can survive as spores under adverse conditions (Cross, 1968). Actinomycetes thus are important agents of lignocellulose degradation, although their ability to degrade cellulose and lignin is not as high as that of fungi (Crawford, 1983; Godden et al., 1992). Actinomycetes are also important decomposers of other organic matter and of other bacteria during composting (Ryckeboer et al., 2003).

Temperatures in a CTC pile are higher than 50 °C throughout the whole composting process (Xiao et al., 2009a), and these conditions are likely to select for different microbial populations than those found during TC process. Strom (1985) studied the diversity of bacteria in thermophilic solid-waste composting using a cultivation method, and found that species diversity markedly decreased in laboratory composting at 60 °C and above and 10 of the 15 isolated taxa were included in genus *Bacillus*.

Since no study has been conducted to specially investigate the actinomycetes in CTC compost, we analyzed the composition of actinomycetal communities by denaturing gradient gel electrophoresis (DGGE), and determined the quantity of actinomycetes by quantitative polymerase chain reaction (qPCR).

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2. Methods

2.1. Composting

The source materials for composting, consisting mainly of kitchen waste, leaves, grass and small branches, were shredded to a size of 1–3 cm after collection from a municipal transfer station near the campus of Hunan University in Changsha, China, and separation from inorganic materials such as glass and inert components such as plastics. The moisture ratio of the materials was adjusted to about 60% by spreading them on the ground for drying in the open air for 12 h.

The composting experiment was designed as described previously (Xiao et al., 2009a) and included packing 20 kg (wet weight, about 28 L) of source materials into a bench-scale composting reactor (30 cm in diameter, 45 cm in depth). The reactors were placed in an incubator and sequentially operated under TC or CTC conditions (TC run A and CTC runs B-1, B-2, C-1 and C-2) for 60 days. In run A, the incubator temperature was maintained at 30 °C. The incubator temperature was maintained at 50 °C during the first 30 days in runs B-1 and B-2 while it was maintained at 40 °C on the first day and then raised to 50 °C during the remaining 29 days of thermophilic composting in runs C-1 and C-2. Air from a compressor was supplied at a flow rate of 0.25 L/(min kg (wet weight)) to maintain aerobic conditions throughout the experimental runs, and a higher flow rate was used to bring down the temperature to 60 °C in runs B-2 and C-2 when the compost pile temperature exceeded 60 °C. Each compost run was conducted in triplicate, and three subsamples of each sample were tested. The compost was turned manually every 24 h, and samples with a total weight of 50 g (wet weight) were collected daily at five random locations (10 g from each location) in each pile immediately after window turning and subjected to physicochemical properties analyses or stored at −20 °C for other uses.

C/N ratio, germination index (GI), specific oxygen uptake rate (SOUR), dissolved organic carbon (DOC) and dehydrogenase activity (DA) were monitored daily to determine the average composting cycle (Xiao et al., 2009a).

2.2. DNA extraction and purification

Total DNA was extracted from each 1-g compost sample (wet weight) with protease K and CTAB as described by Yang et al. (2007). After purification with a TIANquick Midi Purification Kit (TianGen, Beijing, China), the DNA was dissolved in 200 μL of EB buffer (DNA concentrations ranged from about 100 to 200 ng/μL), and 5 μL of DNA was used for agarose gel electrophoresis.

2.3. PCR amplification

Universal bacterial 16S rRNA gene forward primer GC341F (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′) (Muyzer et al., 1998; Xiao et al., 2009b) and actinomycetes-specific reverse primer Act704R (5′-TCT GCG CAT TTC ACC GCT AC-3′) (Jin et al., 1998) were used for amplification of the V3 and V4 regions of 16S rRNA genes (Chakravorty et al., 2007). Each 100 μL PCR reaction mixture contained about 300 ng of template DNA, 10 μL of 10× buffer with 15 mmol/L $MgCl_2$ (TianGen), 40 nmol each dNTP mixture (TianGen), 12 pmol each primer (Sangon, Shanghai, China), 5 U *Taq* DNA polymerase (TianGen), 20 μg of bovine serum albumin (BSA) V (Sangon) and 77.4 μL of sterilized Milli-Q water. PCR amplification on a MyCycler (Bio-Rad, Hercules, CA, USA) was run using the following cycling conditions: 4 min at 95 °C; 35 cycles with each cycle consisting of 30 s at 95 °C, 40 s at 56 °C, and 40 s at 72 °C; followed by a final 7-min extension at 72 °C. Amplification products were stored at −20 °C before further analysis.

About 5 μL of each PCR product (about 400 bp) was stained with 0.5 μL of 100× SYBR™ Green I, electrophoresed in a 1.5% (w/v) agarose gel and quantified by comparison with a DNA Marker V (TianGen) under the Gel Doc XR System (Bio-Rad). For the DGGE analysis, 50 μL of the PCR product was purified and concentrated to 30 μL (about 20 μg) with a purification kit (TianGen) following the manufacturer's instructions.

2.4. DGGE analysis

The DGGE was carried out by using a DCode™ Universal Detection System instrument and gradient former model 475 according to the manufacturer's instructions (Bio-Rad). The denaturant solution was prepared as described by Muyzer (Muyzer et al., 1998), and electrophoresis was performed in 8% acrylamide gels with a denaturing gradient of 35–70%. The 30 μL of purified PCR products were applied for each DGGE analysis. Gels were run in 1× TAE buffer at 60 °C for 14 h at 120 V. Gels were stained with 1× YBR™ Green I, unstained with Milli-Q water and visualized under UV light with the Gel Doc XR System (Bio-Rad). Bands were recognized by the program QuantityOne V4.63 (Bio-Rad), and some faint bands that could not been recognized by the program were identified manually under UV light.

2.5. Identification and phylogenetic analysis of DGGE bands

All recognized bands, even those co-migrating in different lanes, were excised from DGGE gels under UV light, and a bead beating method was applied to extract DNA from the gel slices (Muyzer et al., 1998). The purified DNA was amplified using primer pair GC341F/Act704R. After purification with a kit (TianGen Beijing, China), the products were ligated into the pGM-T easy vector (TianGen) and resulting plasmids were electroporated into competent *Escherichia coli* DH5α cells using a MicroPulser Electroporator (Bio-Rad). For each band, three different clones were selected for sequencing with the help of penbritin and blue-white spot screening (Aslanidis and de Jong, 1990). Plasmid DNA was obtained from transformant using alkali lysis method (Birnboim and Doly, 1979), and the insert was amplified using the primer pair GC341F/Act704R for re-DGGE to affirm that the insert was the targeted band. For each excised band, three clones were selected and sequenced. A few differences in the sequences of the three clones were detected and the sequences were aligned and a consensus sequence was established. Twenty distinct bands, designated as A to T in the DGGE profile, were identified from the SYBR™ Green I stained denaturing gradient gels and phylogenetic identity was determined by comparing the partial 16S rRNA gene sequences of the clones with sequences in GenBank using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was constructed with MegAlign in Lasergenge 7.0 (DNASTAR Inc., Madison, WI, USA) using the Clustal V method. Sequences with a similarity of more than 97% were considered to belong to the same species (McCaig et al., 1999).

2.6. qPCR analysis

Real-time PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad) to determine the 16S rRNA gene copy numbers of actinomycetes in the compost. Forward primer 517F (5′-CCA GCA GCC GCG GTA AT-3′), which was modified from a universal bacterial 16S rRNA gene reverse primer 517R (5′-ATT ACC GCG GCT GCT GG-3′) (Murray et al., 1996), and reverse primer Act704R were used for qPCR analysis, targeting the V3 region of 16S rRNA gene (Chakravorty et al., 2007). The amplifications were carried out in

a total volume of 25 μL. The qPCR mix contained 2 ng of compost microbial DNA, 4 pmol of each primer (517F and Act704R), 5 μg of BSA V, 10 μL of 2.5× RealMasterMix (TianGen) and 1.5 μL of 20× SYBR solution (TianGen). The two-step Amp+Melt protocol was as follows: (i) amplification step: denaturing at 95 °C for 4 min, 40 cycles of 30 s at 94 °C and 30 s at 55 °C, 1 min at 95 °C, 1 min at 55 °C, and (ii) melting curve analysis step: 81 cycles of 30s at 55 °C. The fluorescent signal was measured at the end of each annealing/extension step. A negative control without the corresponding template DNA was included in every qPCR assay for each primer and probe set. All experiments were done in triplicate. Data analysis was carried out with the iQ5 Optical System Software (version 1.0.1384.0 CR) (Bio-Rad). The parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. Before qPCR, primer pair 517F/Act704R was tested by PCR and determined not to produce non-specific product. 16S rRNA gene fragments of *Streptomyces griseus* were PCR amplified with primer pair of 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Mayhew et al., 2008) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) (Muyzer et al., 1998; Xiao et al., 2009b). The PCR products were cloned into the Target Clone™ vector (Toyobo, Osaka, Japan). The plasmids were extracted in the range of 10^8–10^9 copies/μL, serially diluted, and used as templates in qPCR for standard curves generation, as described by Lim et al. (2008).

2.7. Cluster analysis of DGGE bands

To monitor changes in the actinomycetal communities during composting, hierarchical cluster analysis with the SPSS version 16 (SPSS Inc., Chicago, IL, USA) was performed to analyze the DGGE pattern using the Ward method. Only band positions, not intensity were analyzed.

2.8. Nucleotide sequence accession numbers

The retrieved 20 distinct 16S rRNA gene sequences (bands A to T) of the 16S rRNA gene clones have been deposited orderly in the GenBank database under accession numbers GU188876–GU188857.

3. Results and discussions

3.1. Composting and sampling

Run B-1 showed the shortest average composting cycle of 14 days as determined by C/N ratio, GI, SOUR, DOC and DA, while runs A, B-2, C-1 and C-2 required 28, 16, 18 and 19 days, respectively. Therefore, two typical runs, designated as A and B from the TC run A and CTC runs B-1, respectively, were selected for sampling as similar change curves of each physicochemical index were presented in the same run series. The sample taken from the source materials was designated "0", the other samples were designated based on run and sampling time (e.g. "A1" indicates a sample taken from run A on day 1). Fourteen samples were selected from runs A and B (Table 1).

3.2. DGGE analysis

No obvious differences between the banding patterns of the triplicate samples were detected, and a representative gel images is shown in Fig. 1. The banding patterns suggest that a diverse community of actinomycetes were present in the source materials (lane 0 in Fig. 1a). Fewer bands appear to be present in the samples collected within the initial 8 and 5 days in runs A and B, respectively, and most of the bands were also observed in the source material sample. Therefore, the banding patterns for samples 0, A1, A3, A5, A8, B1, B3 and B5 were grouped into the same cluster II on the dendrogram (Fig. 1b). The apparent reduction in actinomycetes diversity during the initial composting phase could be due to the rapidly changing environmental factors inside these piles, especially temperature and pH (Table 1), which could be conducive to some, but not all initially present actinomycete.

The peak temperatures of 55 °C and 73 °C appeared around the day 5 in runs A and B (Table 1), respectively. After the peak temperature phase, the diversity of actinomycetes increased remarkably since more bands were identified from lanes A13, A20, B8, B10, B14 and B28 than from lanes A1, A3, A5, A8, B1, B3 and B5 (Fig. 1a). Nearly half of the sequenced bands (I, M, N, O, P, Q, R, S and T) originated from these samples. The banding patterns for the six samples were clustered together in cluster I on the dendrogram (Fig. 1b). Actinomycetal communities with apparently

Table 1
Summarize of the samples.

Sample name	Source	T[a]/°C[b]	pH[b]	TOC[c]/%[b]	TKN[d]/%[b]	C/N[b]	DOC[e]/(g/kg)[b]	SOUR[f]/(mg O_2/g VS/h)[b]
0	Source material	30.0	6.83	46.26	0.92	50.28	43.18	9.94
A1	Traditional compost run A	36.1	6.67	45.72	0.92	49.70	41.58	12.47
A3		43.9	6.22	42.78	0.91	47.01	38.26	16.87
A5		55.4	6.58	38.13	0.90	42.37	30.53	15.63
A8		47.9	6.91	30.06	0.87	34.55	17.82	11.64
A13		37.4	7.25	20.94	0.83	25.23	8.48	8.75
A20		33.0	7.21	18.45	0.81	22.78	6.21	1.94
A28		30.1	7.06	18.00	0.79	22.78	5.42	1.26
B1	Continuous thermophilic compost run B	52.1	6.46	44.25	0.92	48.10	42.11	16.47
B3		65.6	7.75	40.25	0.91	44.23	35.16	17.61
B5		69.6	8.09	34.07	0.89	38.28	26.76	15.33
B8		57.2	7.85	22.41	0.84	26.68	12.71	12.41
B10		52.1	7.75	19.07	0.82	23.26	7.33	8.63
B14		50.0	7.55	18.06	0.79	22.86	5.03	1.59
B28		50.0	7.21	18.01	0.78	23.09	4.98	1.25

[a] Temperature.
[b] Xiao et al., 2009a.
[c] Total organic carbon.
[d] Total Kjeldahl nitrogen.
[e] Dissolved organic carbon.
[f] Specific oxygen uptake rate.

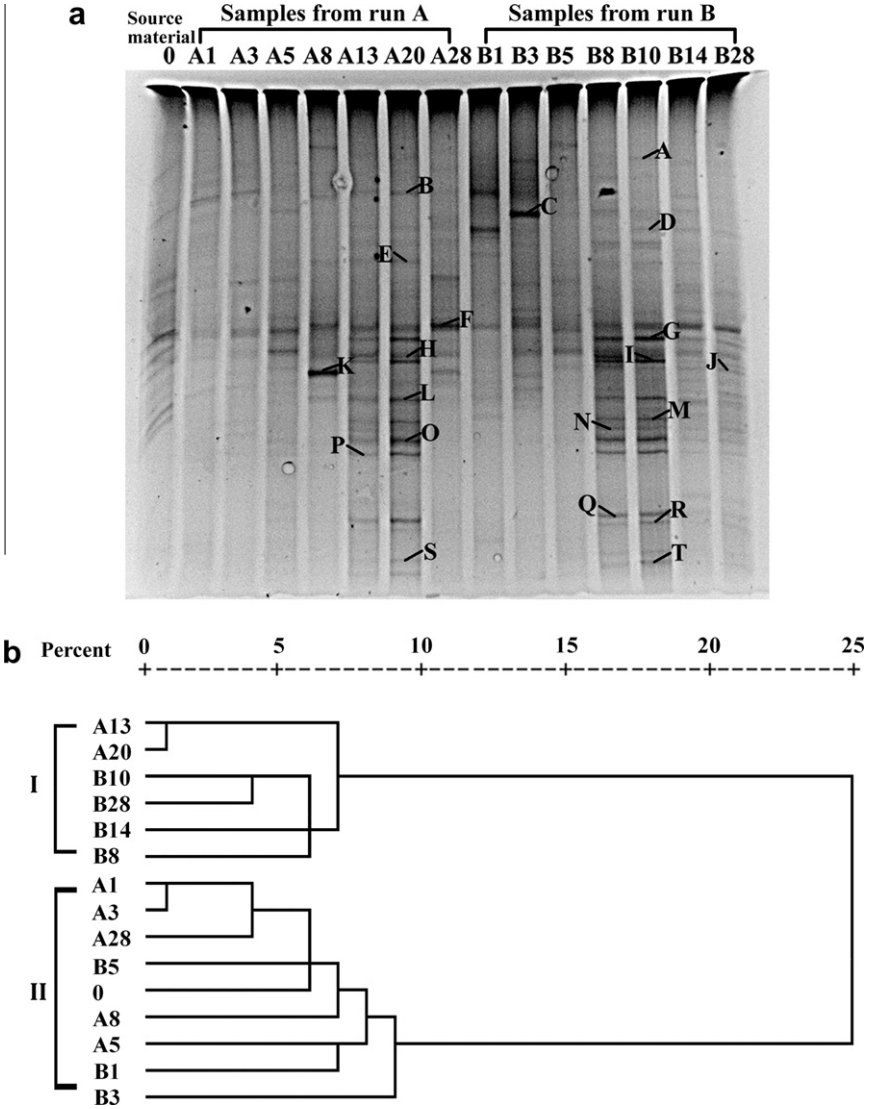

Fig. 1. (a) DGGE pattern of the amplified 16S rRNA gene fragments; (b) dendrogram of cluster analysis based on the position of bands on DGGE pattern.

similar composition were detected in A13, A20, B8, B10, B14 and B28, which formed in run B five days earlier than that in run A. The diverse actinomycetal community was still detected in mature compost (B14 and B28) while it was only detected from days 13–20 in run A. The results therefore indicated that the CTC compost had provided a much more suitable environment for the growth of actinomycetes.

3.3. Phylogenetic analysis

With the exception of the sequence corresponding to band A (97% identity to the sequence from *Laceyella sacchari*), the sequences from the DGGE bands shared a high degree of similarity (>97%) with those from microbes in the phylum *Actinobacteria* (Fig. 2). Band A was only identified in lanes B3 and B10 from the CTC run. *L. sacchari* is known to grow at 55 °C (Yoon et al., 2005) and be able to degrade cellulose (Yoon et al., 2005).

The sequences corresponding to bands F, M, O, P, Q and S were ⩾97% identical to sequences inform the genus *Streptomyces*, which usually are dominant actinomycetes in soils (Otsuka et al., 2008). Only band F was detected throughout the entire composting in both runs. The sequence of band F suggests that it originated from *Streptomyces violaceoruber*, a bacterium known to degrade cellulose (Fergus, 1960). Bands M, O, P, Q and S were only identified in six lanes in cluster I (Fig. 1b) which suggests that these strains were thermotolerant actinomycetes. Band H appears to have originated from an organism related to *Cellulosimicrobium cellulans*, a microorganism producing a wide variety of cell wall-degrading enzymes such as β-1,3-glucanase, protease and chitinase (Fleuri and Sato, 2008). Band B, with a sequence 97% identical to that of *Micrococcus luteus*, was detected during the entire active composting process, except in samples A5 and B1.

Band D, with a sequence 98% identical to that of *Amycolatopsis sacchari*, was detected in A13, A20, A28, B1, B10, B14 and B28. *A. sacchari* was reported to be a moderately thermophilic actinomycete whose growth occurs between pH 5 and 8 and between 20 and 45 °C but not at 55 °C (Goodfellow et al., 2001). Band J apparently originated from an obligate thermophile since the band was only detected in lanes A5, B8, B10, B14 and B28. Its sequence is 98% identical to that of *Amycolatopsis thermoflava*, an actinomycete growing at 45 °C but not at 10 °C (Chun et al., 1999). Bands G, R and T were mainly detected in samples from the post high-temperature phase (Fig. 1a) and their corresponding sequences were similar to those of species in the genus *Micromonospora*, which are decomposers of cellulose (El-Tarabily et al., 1996). Band C, a band detected in high temperature samples only, had 97% sequence

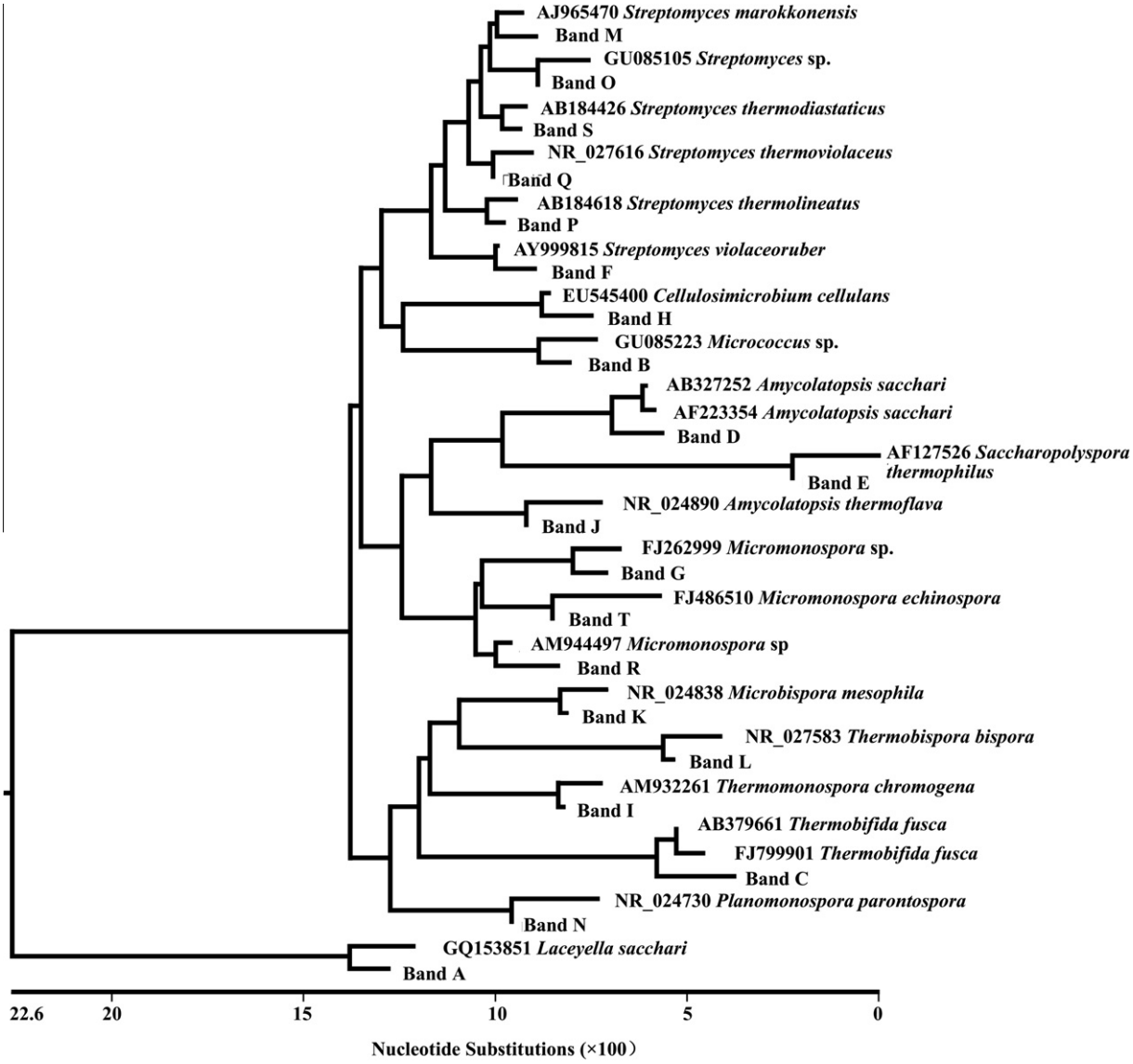

Fig. 2. Phylogenetic tree of the identified bands on DGGE pattern constructed by MegAlign in Lasergene 7.0 using the Clustal V method.

similarity to that from *Thermobifida fusca*, a producer of cellulases (Li et al., 2007).

The sequences corresponding to bands I, L and N were ⩾97% identical to those of *Thermomonospora chromogena*, *Thermobispora bispora* and *Planomonospora parontospora,* respectively. The organism represented by these bands are likely moderate thermophilic actinomycetes since the bands were only observed during the post peak temperature phase in runs A and B (Fig. 1a). Among these actinomycetes, *Thermomonospora chromogena* possesses the ability to degrade cellulose in compost (Ramírezy and Coha, 2003).

3.4. Quantitative analysis of actinomycetes

qPCR, which is a fast, reliable, sensitive and convenient method to enumerate various cultivable or uncultivable microorganisms, has recently been applied and optimized to quantify bacteria from various environments (Kindaichi et al., 2006; Nakamura et al., 2006). When applied to samples obtained from composts in runs A and B, the qPCR-based 16S rDNA copy numbers shown in Fig. 3 were obtained. It is not possible to equate 16S rDNA copy numbers

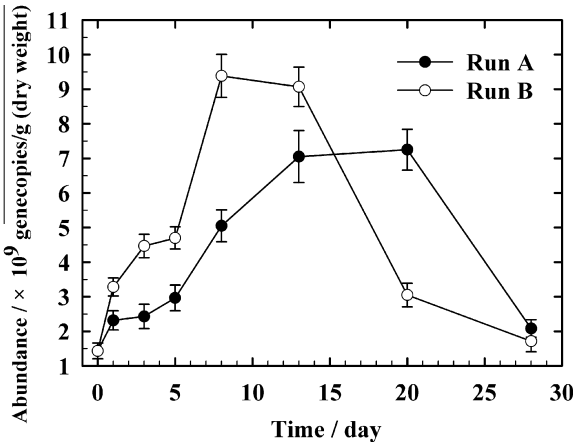

Fig. 3. qPCR-based determination of actinomycetal 16S rRNA gene copies in samples from the active composting phase in runs A and B. The temperature of incubator was maintained at 50 °C in run A throughout the entire composting, while it was maintained at 30 °C in run B.

with numbers of bacteria since different numbers of 16S rRNA genes are found in different bacteria (Farrelly et al., 1995). Similar change to the copy numbers were observed in runs A and B (Fig. 3). The numbers increased quickly on day 1 which might be ascribed to the rising temperature and high substrates availability in the piles (Table 1). The copy numbers from the CTC samples were 25 to 80% higher than those from the TC samples during similar phases of active composting, e.g. B3 vs A3, B8 vs A13 and B10 vs A20. Higher temperature and pH in the CTC runs might have accelerated the proliferation of actinomycetes, compared with the TC run.

Higher actinomycete-derived 16S rRNA gene copy numbers were detected in the two piles in the post peak temperature phase than in mature compost. The copy numbers from the mature CTC samples B14 and B28 were as low as those in A28, although a diverse actinomycetal community was detected by DGGE analysis in these samples.

4. Conclusion

DGGE and qPCR analyses indicated a higher diversity and larger numbers of actinomycetes in CTC run than in TC, which suggested that raising the temperatures to 50 °C or higher would benefit the growth of actinomycetes in the post peak temperature phase during composting. However, the activities of actinomycetes in degrading organic matters are still unclear, and future studies of enzymes such as cellulases derived from actinomycetes will contribute to further explanation of the function of actinomycetes in CTC.

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