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Bacterial community composition and abundance in leachate of semi-aerobic and anaerobic landfills

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Abstract

The abundance and phylogenetic composition of bacterial community in leachate of semi-aerobic and anaerobic landfill were compared through real-time polymerase chain reaction and denaturing gradient gel electrophoresis. In semi-aerobic landfill scenario, the bacterial 16S rRNA copy numbers in leachate had no significant reduction from initial stage to stable period. In the scenario of anaerobic landfill, the largest bacterial 16S rRNA gene copy number was found in leachate at initial stage, but it reduced significantly at stable period. Moreover, methane-oxidizing bacteria population in stable period was lower than that in initial period in both two landfill processes. However, semi-aerobic landfill leachate had more methanotrophic bacteria populations than that in the anaerobic one. Furthermore, according to the sequences and phylogenetic analysis, obvious difference could be detected in bacterial community composition in different scenarios. Proteobacteria and bacteroidetes took up a dominantly higher proportion in semi-aerobic landfill leachate. To summarize up, different landfill methods and its landfill ages had crucial impacts on bacterial abundance and composition in leachate of semi-aerobic and anaerobic landfills.

Key words: real-time polymerase chain reaction; denaturing gradient gel electrophoresis; bacterial 16S rRNA gene; methane-oxidizing bacteria; phylogenetic analysis

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Introduction

Sanitary landfill is one of the dominant methods for municipal solid waste disposal in China (about 81% in 2007). However, gas emission released into atmosphere and leachate leakage discharged into groundwater from anaerobic landfills might cause serious environmental problems (Hudgins and March, 1998) and might last for decades (Kruempelbeck and Ehrig, 1999).

Aerobic landfill technology has been evaluated over the last few years in rapid stabilization, waste detoxification, and methane gas, volatile organic compounds and odor substances emissions reduction, as well as leachate quality improvement (Purcell, 2000a, 2000b; Read et al., 2001; Kim, 2005). But the high-cost and power-consumption limited the application of aerobic landfill.

The concept of semi-aerobic landfill was presented in the 1960s. In a semi-aerobic landfill site, ambient air was pumped into landfill bodies non-full leachate collecting pipes, which was motivated by the temperature differences between the inside and outside of the waste stacks. As a result, some aerobic and anaerobic zones existed in landfill waste layers (Shimaoka et al., 1997). Semi-aerobic landfill process had advantages as follows: (1) low-cost and energy-saving (compared with aerobic landfills); (2) low concentration of organic pollutants in leachate, such as COD_{Cr}, BOD_5 and NH_4^+-N; and (3) rapid garbage decomposition and stabilization because of large bacterial quantities and activities in aerobic landfill zones. Therefore, creating aerobic condition in solid waste layer in landfill site is crucial to accelerate garbage stabilization (Shimaoka et al., 1997).

Microorganisms play an important role in waste degradation and stabilization in landfill site. At past, microbiology of anaerobic digestion ecosystems was investigated mainly by traditional culture-based methods (Archer and Kirsop, 1990). Recently, 16S rRNA sequencing technology had revealed a higher microbial diversity in various anaerobic digesters (Godon et al., 1997; Sekiguchi et al., 1998; van Dyke and McCarthy, 2002; Wu et al., 2001). Pourcher et al. (2001) analyzed cellulosehydrolysis

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bacterial using traditional bacterial culture and 16S rDNA molecular biology. Huang et al. (2002) investigated the community and diversity of archaebacteria in leachate with different landfill processes in Guangzhou, China, and their results showed the big differences between two landfills types. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) approaches had been widely used to analyze the bacterial community structure (Lin et al., 2005; Shen et al., 2008; Uchida et al., 2009).

To date, few data of molecular microbiology in full-scale municipal landfills were reported, lacking of related studies focused on identification of members from the whole bacterial domain in the semi-aerobic landfills. In particular, none research had compared the effects of semi-aerobic and anaerobic landfill processes on leachate bacterial composition before, which is an important approach to reveal the different microbial mechanisms.

Some small cities in China, had bulit many semi-aerobic landfill sites, such as Weifang in Shandong Province, Mengzi in Yunnan Province, Langfang and Zhuozhou in Hebei Province. However, there was no investigation about bacterial community composition and abundance in semi-aerobic landfills.

In this article, molecular biological techniques of real-time and cloning analysis RCR-DGGE of 16S rRNA were applied to describe community composition and abundance of bacteria in leachate of both semi-aerobic and anaerobic landfills. The differences of bacterial community composition and abundance in semi-aerobic and anaerobic landfills were also presented.

1 Materials and methods

1.1 Sampling

Leachate samples used in semi-aerobic process were collected from Zhouzhou municipal waste semi-aerobic landfill site, located in Hebei Province, China. The semi-aerobic landfill site is 142 acres and had a total landfill capacity of 1.3 million ton with a daily processing capacity of 250 ton and 15 years of service time. Leachate samples used in anaerobic process were collected from Asu Wei municipal waste sanitary landfill site in northern of Beijing, China, which is 4300 acres, and had a total landfill capacity of 1.2×10^6 m^3, with processing capacity of 2000 ton per day and service time 17 years.

Because the stabilization times of semi-aerobic and anaerobic landfill sites were not synchronized, leachate samples only in the initial and stable stage were compared. According to pre-research results, the stabilization time of yielded leachate is 2–3 year for semi-aerobic landfills, and about 8–10 year for anaerobic landfills. Therefore, four samples were selected in this study: (1) a 6-month old initial leachate in semi-aerobic landfill (Z_A); (2) a 2-year old leachate in semi-aerobic landfill (Z_B); (3) a 6-month initial leachate in anaerobic landfill (Y_A); and (4) a 8-year old leachate in semi-aerobic landfill (Y_B). All leachate samples were obtained from the leachate collection pipes in August, 2009. Because every landfill area (representing a landfill stage) had a leachate outlet for sampling, three sub-samples from a leachate outlet were mixed. After sampling the samples were put into cool storage immediately. Samples used for physical and chemical analyses were kept at 4°C, while the ones for molecular biological experiments were stored at –20°C. Physical and chemical analyses of leachate samples were performed following the procedures described by Greenberg et al. (1992).

1.2 Total DNA extraction

Each leachate samples of 50 mL were centrifuged at 14,000 $\times g$ for 10 min to concentrate microbial cell. Supernatants were discarded so that the final volume of each solution containing microbial cells was less than 5 mL (Huang et al., 2004). Then total DNA was extracted using FastDNA SPIN kit for soil (MP, USA) according to the manufacturer's introduction. All treatments were performed in triplicates.

1.3 Quantity analysis of bacteria and methanotrophs by real-time PCR

Abundances of total bacteria were determined using real-time PCR assay by targeting 16S rRNA gene. Real-time PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad, USA). Amplification was performed in 25-μL reaction mixtures by using SYBR® Premix Ex Taq as described by the suppliers (Takara Bio, Otsu, Shiga, Japan). Quantification of bacterial 16S rRNA genes using primers 954f and 1369r was carried out with iQTM Supermix (Bio-Rad, USA). Primer pair A189/mb661 (Zheng et al., 2008) was applied for quantification of pmoA gene with SYBR(R) Premix Ex TaqTM (TaKaRa) (Table 1).

From standard curve for bacteria and methanotrophic bacteria (MOB) real-time PCR (Fig. 1), it can be seen that 16S rRNA and pmoA gene copies number had a good linear relation with threshold cycle with correlation

Table 1 Primer and PCR condition used for the real-time PCR

Target group	Primer	Sequence (5′–3′)	Length of amplicon	Thermal profile	Reference
Bacteria	954f 1369r	GCACAAGCGGTGGA-GCATGTGGGCCCGG-GAACGTATTCACCG	456	94°C for 2 min followed by 40 cycles of 30 sec at 94°C, 30 sec at 63°C, 1 min at 72°C, plate read at 83°C	Yu and Morrison, 2004
MOB	A189 mb661	GGNGACTGGGACTT-CTGGCCGGMGCAAC-GTCYTTACC	508	95°C for 2 min followed by 36 cycles of 1 min at 94°C, 1 min at 60°C, plate read at 83°C	Kolb et al., 2003; Zheng et al., 2008

MOB: methanotrophic bacteria.

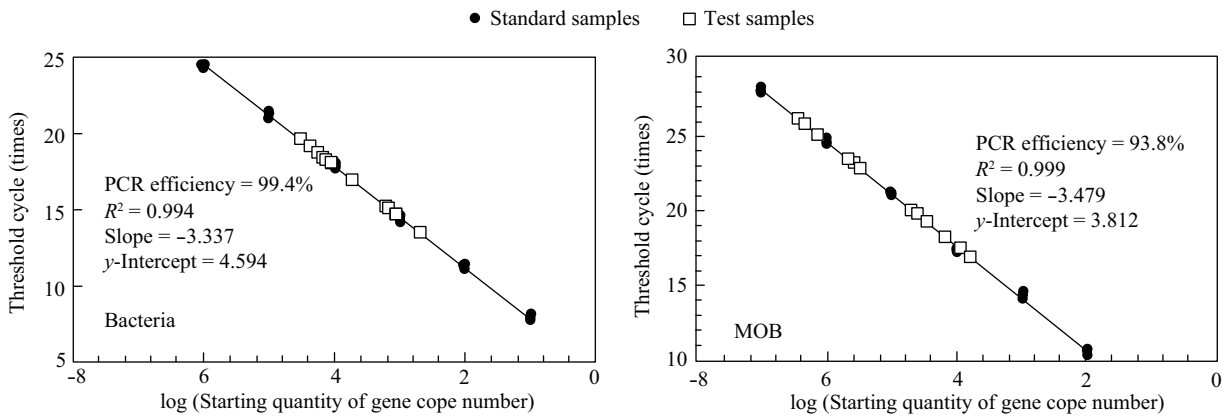

Fig. 1 Standard curve for real-time PCR.

coefficient 0.994 and 0.999, respectively.

1.4 PCR-DGGE analysis on microbial community structure

The 16S rRNA gene was PCR amplified through the extracted DNA as template and universal bacterial primers 954f (GCACAAGCGGTGGAGCATGTGG) with a GC clamp and 1369r (GCCCGGGAACGTATTCACCG) (Yu and Morrison, 2004). The PCR conditions were described as follows. Briefly, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L total dNTPs, 0.4 μmol/L of each primer, 2.5 U Taq polymerase, 1.0 μg/μL bovine serum albumin (BSA) and 1–10 ng of template DNA (Yu and Morrison, 2004) were used. A touchdown thermal cycle strategy included an initial preheating step for 5 min treatment at 94°C for melting double-stranded DNA. Then, a touch-down procedure (consisting of 30 sec at 94°C, annealing for 30 sec at temperature decreased from 61 to 56°C during the first 10 cycles, and ending with an extension step at 72°C for 1 min) followed the additional 25 cycles (consisting of 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C), and then a final extension for 10 min at 72°C were performed.

The obtained PCR products were loaded on 6% (*W*/*V*) acrylamide/bisacrylamide (37.5:1, *W*/*W*) gels containing a 35%–60% linear gradient of formamide and urea (100% denaturing solution contained 40% (*V*/*V*) formamide and 7 mol/L urea). The electrophoresis was run for 6 hr at 120 V and at a constant temperature of 60°C (Ge et al., 2008), using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA). The gels were stained with SYBR gold nucleic acidgel stain (1:10,000; Invitrogen-Molecular Probes, USA) for 30 min, scanned by the Gel Documentation System (Syngene, USA) and analyzed by the software Quantity One (Bio-Rad Laboratories).

1.5 Cloning, sequencing, and phylogenetic tree construction

DGGE gel strips of some distinguished bands among all treatments were excised and reamplified following previous PCR conditions. These PCR products were cloned by pGEM-T Easy Vector (Promega). Clones that contained correct inserted fragments were selected and sequenced by ABI PRISM 3730 DNA analyzer (BGI, Beijing, China). Sequences gained were manually calibrated readings and corrected if necessary, edited and aligned according to BioEdit version 4.8.5. These partial sequences of approximate 456 bp were aligned to 16S rRNA gene sequences obtained from the National Center for Biotechnology Information (NCBI) database based on the BLAST version 2.2.16 searching program. The most similar and representative GenBank sequences to the clones were extracted from the GenBank. Phylogenetic analyses were conducted under MEGA version 4.0 and the neighbor-joining trees were implemented by p-distance with 1000 replicates to produce bootstrap values.

1.6 Nucleotide sequence accession numbers

The (GU451117) 16S rRNA gene sequences were submitted to the GenBank database under accession numbers (GU4511162).

2 Results and discussion

2.1 Basic chemical properties of the different landfill leachates

Basic chemical properties of different landfill leachates are shown in Table 2. Significant differences ($P < 0.05$) in leachate pH value are observed among the four samples (Z_A, Z_B, Y_A, Y_B). Y_B had the highest pH value, while pH

Table 2 Basic chemical characteristics of test landfill leachate

Sample	pH	TS (mg/L)	VS (mg/L)	COD_{Cr} (mg/L)	TN (mg/L)	NH_4^+-N (mg/L)
Z_A	6.70 ± 0.05 cd	17,681 ± 1037 c	9613 ± 307 b	28,500 ± 1800 b	2067 ± 210 b	1022 ± 57 b
Z_B	6.99 ± 0.11 b	12,422 ± 1128 d	5467 ± 287 c	17,500 ± 1277 c	1276 ± 119 c	304 ± 45 c
Y_A	6.61 ± 0.14 d	27,210 ± 8990 b	14,142 ± 370 a	43,500 ± 3576 a	3987 ± 284 a	2187 ± 147 a
Y_B	8.10 ± 0.06 a	4013 ± 200 a	1857 ± 77 d	5500 ± 879 d	524 ± 83 d	256 ± 62 c

TS: total solids; VS: volatile solids; COD_{Cr}: chemical oxygen demand; TN: total nitrogen;
The different letters indicate significant differences between treatments at $P < 0.05$.

values of Z_A, Z_B and Y_A were at the same level. Because of unstable state in landfill site, the pH values of Z_A, Z_B and Y_A all indicated weakly acidic or neutral, while Y_B revealed obviously alkaline due to garbage was under stabilization after 8-year landfill disposal. Significant differences were found in total solid (TS) content, volatile solid (VS), COD_{Cr}, TN and NH_4^+-N among the four treatments ($P < 0.05$) with the same sequence: $Y_A > Z_A > Z_B > Y_B$. The comprehensive analysis on the indexes of the four waste landfill treatments revealed that leachate qualities were obviously impacted by different landfill technologies. Moreover, Y_B and Z_B reached relative stabilization state, and Z_A and Y_A kept initial unstable status.

2.2 Bacterial and methanotrophic abundance based on 16S rRNA gene and pmoA gene analysis

Quantification results of bacterial 16S rRNA gene copy numbers from leachate in semi-aerobic and anaerobic landfill sites are presented in Fig. 2a. The bacterial 16S rRNA gene copy numbers in the four treatments were Y_A (1.69×10^{10}) > Z_A (4.46×10^9) > Z_B (9.53×10^8) > Y_B (7.41×10^8). From the landfill-time-based point of view, Z_A and Z_B had no big difference, but obvious differences were found between Y_A and Y_B. Because semi-aerobic landfill was a half-open system all along landfill period, bacterial numbers had relatively less decreased. However, bacterial 16S rRNA copy numbers in stabilized leachate decreased dramatically compared with initial one, due to the aerobic-anaerobic conversion and organic matter declining in anaerobic process.

As shown in Fig. 2b, pmoA gene copy numbers of MOB in four samples (Z_A, Z_B, Y_A and Y_B) were 1.39×10^8, 3.06×10^7, 2.88×10^6 and 5.20×10^5 copies/mL respectively. MOB populations in stabilized leachate were one order of magnitude lower than that of initial ones in both semi-aerobic and anaerobic landfills scenarios. Compared Z_A to Y_A and Z_B to Y_B in same stage, the pmoA gene copy numbers in semi-aerobic landfill leachate were about 48 and 59 times higher than anaerobic ones respectively. The data demonstrated that leachate in semi-aerobic landfills had higher pmoA gene copy numbers than that in anaerobic ones. High ratios of MOB to total bacteria were disclosed in the semi-aerobic landfills, which provided a solid evidence that semi-aerobic landfills could reduce methane gas production and emission effectively (Liu et al., 2005).

As an influencing factor of bacterial abundance, Scheutz and Kjeldsen (2004) proposed the optimum pH value for bacterial abundance was in the range of 6.5 to 7.5. In this study, pH value of Z_A, Z_B and Y_B showed closed to neutral, while Y_B was alkaline, which was related to the obvious differences of the 16S rRNA gene and methanotrophic pmoA gene copies in two pH value types.

Besides the impact caused by pH, oxygen and methane contents were also the crucial factors affecting bacterial abundance, especially for MOB. Although MOB population was just a small fraction of the total bacterial population, it had close relation to methane gas emission reduction. In addition, the small fraction of MOB population changed sensitively in response to landfill oxygen condition, and varied significantly in abundance with different landfill processes (anaerobic or semi-aerobic landfills).

VS and COD_{Cr} values indicated the content of organic matters in leachate samples, which were also the factors to control bacteria abundance. Generally, the more the organic matter, the more bacterial aboudance. As shown in Fig. 2a, sequences of the bacterial 16S rRNA gene copies were the same with the VS and COD_{Cr} values in four treatments ($Y_A > Z_A > Z_B > Y_B$, Table 1). Due to the dominant impact caused by oxygen and methane conditions and other factors, while the same sequences were not found in methanotrophic pmoA gene copies.

2.3 DGGE profile of 16S rRNA gene fragments

Bacterial diversity characteristics of the four leachate samples using DGGE bands pattern data are shown in Table 3. Diversity indices were useful as a first approach to estimate the diversity of microbial communities. The higher Shannon idea (H) value, the greater the diversity of microbial community was. Diversity index consisted of two components: (1) the total numbers of species present or species richness and (2) the distribution of the number of individuals among those different species, called species evenness (Boon et al., 2002).

For the four leachate samples, the sequence of genotypic richness (S) value were $Z_A > Z_B > Y_A > Y_B$. Z_A and Z_B had higher numbers of bands, which were significant

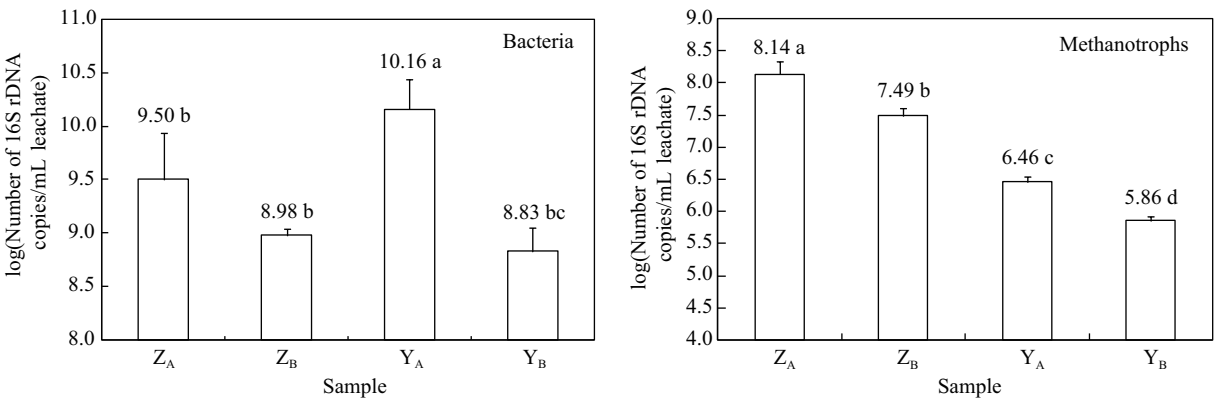

Fig. 2 Quantification of bacterial 16S rRNA (a) and methanotrophic pmoA gene (b) copy numbers from leachate in semi-aerobic and anaerobic landfill sites. Different letters above the bars indicated significant differences among samples at $P < 0.05$.

Table 3 Bacterial diversity characteristics of the four leachate samples using DGGE bands pattern data

Sample	Genotypic richness (*S*)	Shannon index (*H*)	Evenness index (*J*)
Z_A	33 ± 4 a	3.48 ± 0.15 a	0.993 ± 0.008 a
Z_B	31 ± 2 a	3.37 ± 0.09 a	0.995 ± 0.002 a
Y_A	22 ± 3 b	3.06 ± 0.17 b	0.993 ± 0.008 a
Y_B	17 ± 2 b	2.83 ± 0.19 b	0.995 ± 0.053 a

Different letters indicate significant differences between treatments at $P < 0.05$.

higher than Y_A and Y_B. No obvious differences of *S* were found between Z_A and Z_B, Y_A and Y_B. The *H* value of the four treatments revealed the similar regulations as $Z_A > Z_B > Y_A > Y_B$. Evenness index (*J*) of the four treatments had no significant difference. In general, *S* and *H* index showed that the bacterial diversities of two leachate samples in semi-aerobic landfills were significantly higher than that in anaerobic landfill sites.

DGGE profiles of PCR-amplified 16S rRNA gene segments from DNA extracted from the four leachate samples in semi-aerobic and anaerobic landfills with a dendrogram generated by cluster analysis comparison of DGGE patterns are shown in Fig. 3. The DGGE band patterns between replicates within each treatment were similar, but the DGGE band patterns between the four treatments had alterative differences. Dendrogram generated by cluster analysis of the four detected DGGE bands and their pixel intensities showed that there were slight differences between the replicates within Z_A, Z_B and Y_A due to close cluster characteristics (Fig. 3b), but Y_B showed big difference with others. Z_A and Z_B had a higher homology of 65%, and Y_A clustered together in another subcluster with Z_A and Z_B having the homology of 59%, but Y_B had only homology of 45% with the subcluster of Z_A, Z_B and Y_A, and showed obvious distinguish ability.

2.4 Phylogenetic analysis of 16S rRNA gene clones

Evolutionary distance tree of the sequence of 16S rRNA genes amplified from excised DGGE bands of the four leachate samples are shown in Fig. 4. Some distinct differences in the quantity of bands and diversity indices were found in the four samples, indicating that the landfill leachate bacterial community varied clearly according to different landfill years and landfill processes, and a total of 35 different bands in the DGGE gel were excised for further cloning analysis (Fig. 3a).

Three clones in a band were selected randomly for sequencing. By aligning with GenBank database, 46 different sequences were obtained, which were all identified as 16S rRNA gene sequences. Especially, some bands were found containing two or three sequences, such as bands 6, 9, 14, 17, 19, 21, 22, 25 and 30, which were further named as bands 6-1, 6-2, 9-1, 9-2. These sequences were classified into six groups: *Proteobacteria* (*Epsilonproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *DeltaProteobacteria*), *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Thermotoge* and unclassified bacteria. The results showed that 16 of 46 sequences (up to 34.8%) were classified as *Proteobacteria*, which was the dominant taxonomic group, followed by *Spirochaetes* (about 23.9%), and *Firmicutes* (about 23.9%), *Bacteroidetes* (about 8.7%), *Thermotoge* (about 6.5%), unclassified bacteria (about 2.2%). Except *Thermotoge*, all remaining bacterial groups were commonly detected in landfill leachate samples as reported previously (Röling et al., 2001; Huang et al., 2004). However, most of the bacterial sequence types were uncultured species, indicating that majority of bacterial

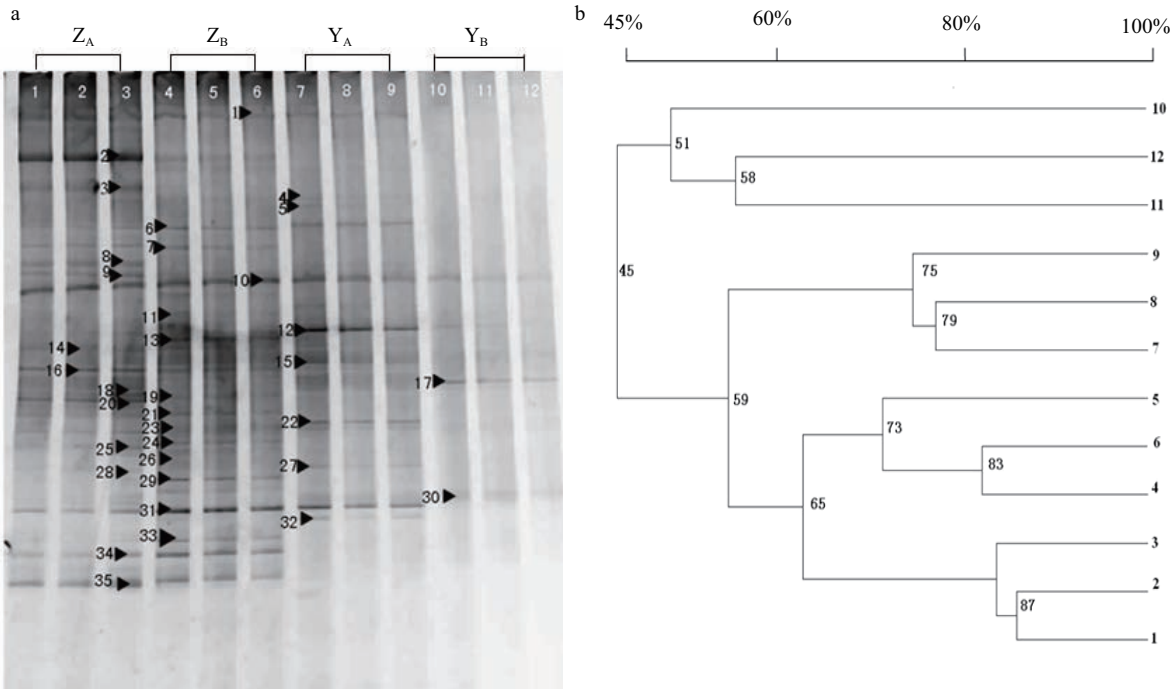

Fig. 3 DGGE profiles of PCR-amplified 16S rRNA gene segments from DNA extracted from the four leachate samples in semi-aerobic and anaerobic landfills (a), with a dendrogram generated by UPGMA cluster analysis comparison of DGGE patterns (b). The small black triangles and numbers on each lane indicated the bands were excised for further analysis.

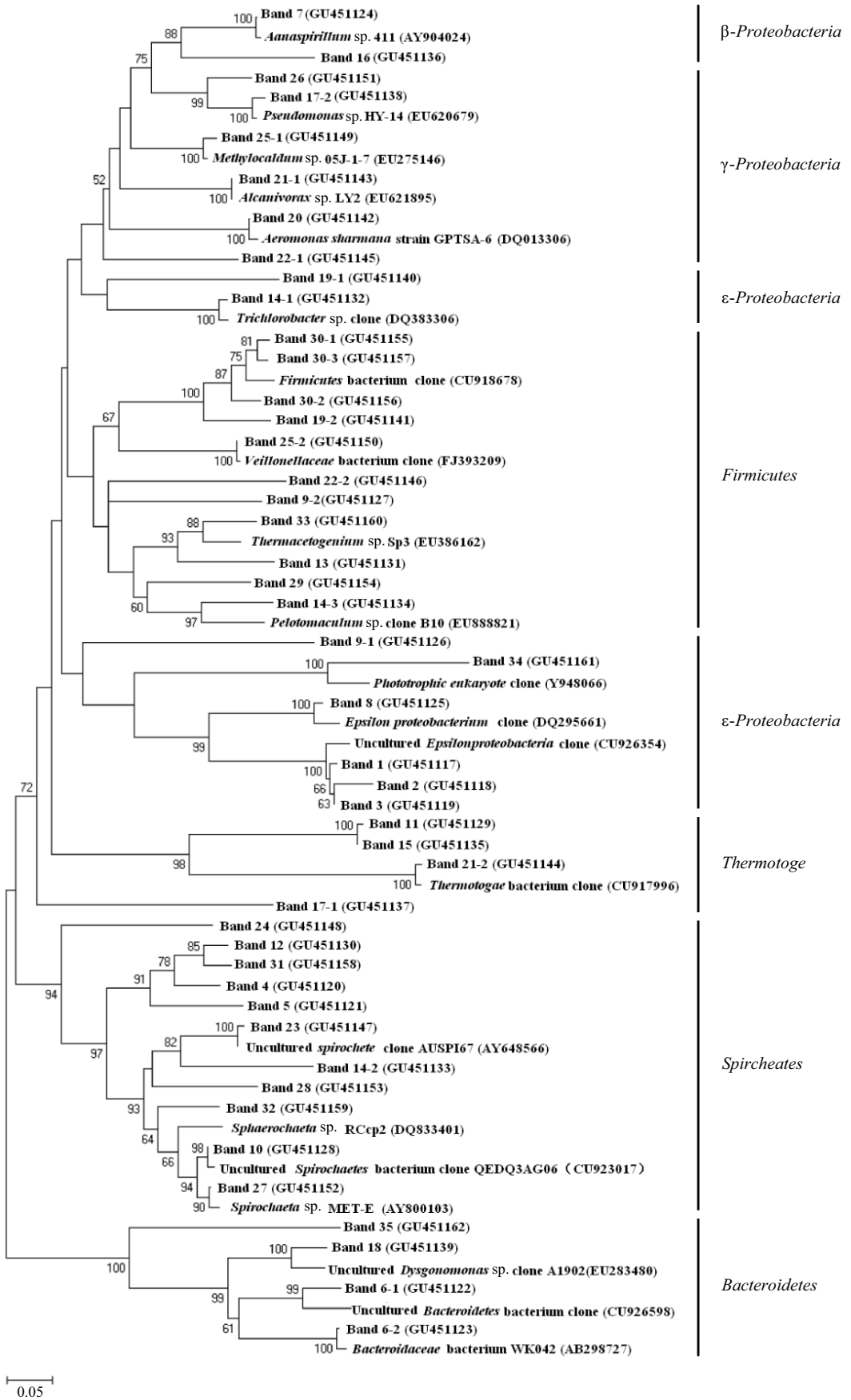

Fig. 4 Evolutionary distance tree of the derived amino acid sequence of 16S rRNA genes amplified from excised DGGE bands of the four leachate samples. Bootstrap values (> 50%) were indicated at branch points. The scale bar represented 5% estimated sequence divergence.

species associated within the landfill leachate remained to be identified.

In sample Z_A, bands belonged to *Proteobacteria* and *Bacteroidetes* were about 45%, these phyla were generally important contributors to biogeochemical processes or organic matter degradation (Barns et al., 1999; Spring et al., 2000) and had been usually found in landfill leachate environments (Huang et al., 2002). Comparing with Z_A, these two bacterial phylas in Y_A sample were relatively fewer, which revealed that garbage degradation and stabilization process in semi-aerobic landfills were promoted at landfill initial period because of the important role of *Proteobacteria* and *Bacteroidetes* for biogeochemical processes.

Compared Z_A with Z_B, high similarities of composition were detected, which because the semi-aerobic landfill was a half-open system during the landfill period, and the bacterial species were relatively not sensitive to landfill environmental changes. But a significantly composition difference between Y_A and Y_B was identified, and *Proteobacteria* and *Bacteroidetes* in Y_B were significantly lower than that in Y_A. In addition, the dominant groups in Y_B were S*pirochaetes*, which also was a general group usually found in leachate environments.

Aligning with GenBank database, band 25-1 presented in Z_A and Z_B treatments had been identified as *Methylocaldum* sp. 05J-I-7, which belonged to *Methanotrophic*. In detail, *Methanotrophic* were gram-negative bacteria that utilized methane as their sole source of carbon and energy, and played an important role in methane oxidation and methane emission reduction. Combined the quantification data of *Methanotrophic* in semi-aerobic leachate, the hypothesis that methane could be oxidized by *Methanotrophic* in the aerobic zone of semi-aerobic landfills had been strongly proved.

3 Conclusions and suggestions

In semi-aerobic landfill scenario, the bacterial 16S rRNA copy numbers of the leachate had no significant reduction from initial to stable period, but the significant reduction were found in aerobic landfill. Moreover, more MOB populations existed in semi-aerobic landfill leachate than that in the anaerobic scenario.

Bacterial diversities of two leachate samples in semi-aerobic landfills were significantly higher than that in anaerobic landfill ones. In addition, bacterial communities in semi-aerobic landfills leachate and initial anaerobic leachate had a relatively higher homology.

Bacterial communities in the tests can be classified into: *Proteobacteria*, *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Thermotoge* and unclassified bacteria. Howerever, the semi-aerobic leachates had dominantly higher proportions of *Proteobacteria* and *Bacteroidetes*. In addition, methane could be oxidized by *Methanotrophics* in the aerobic zones of semi-aerobic landfills body because the high number of MOB was founded in this area.

To accelerate landfill stabilization and reduction the methane emission, the suggestions were: (1) to design the high-efficient air pipe system which make sure the sufficient aerobic area in landfill bodies; (2) to recycle the leachate and control the environment condition for promoting organic matter degradation and methane oxidation bacteria.

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