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Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems

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Abstract In order to obtain insight into the prokaryotic diversity and community in leachate sediment, a culture-independent DNA-based molecular phylogenetic approach was performed with archaeal and bacterial 16S rRNA gene clone libraries derived from leachate sediment of an aged landfill. A total of 59 archaeal and 283 bacterial rDNA phylotypes were identified in 425 archaeal and 375 bacterial analyzed clones. All archaeal clones distributed within two archaeal phyla of the Euryarchaeota and Crenarchaeota, and well-defined methanogen lineages, especially *Methanosaeta* spp., are the most numerically dominant species of the archaeal community. Phylogenetic analysis of the bacterial library revealed a variety of pollutant-degrading and bio-transforming microorganisms, including 18 distinct phyla. A substantial fraction of bacterial clones showed low levels of similarity with any previously documented sequences and thus might be taxonomically new. Chemical characteristics and phylogenetic inferences indicated that (1) ammonium-utilizing bacteria might form consortia to alleviate or avoid the negative influence of high ammonium concentration on other microorganisms, and (2) members of the Crenarchaeota found in the sediment might be involved in ammonium oxidation. This study is the first to report the composition of the microbial assemblages and phylogenetic characteristics of prokaryotic populations extant in leachate sediment. Additional work on microbial activity and contaminant biodegradation remains to be explored.

Keywords Bacterial diversity · Archaeal diversity · 16S rRNA gene · Clone library · Leachate sediment

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Introduction

Landfill is a physically, chemically, and biologically complex heterogeneous system, which takes the hydrological conditions, refuse composition and compaction, temperature, and moisture content along with the seasonal variations as its key characters and functions. Different types of microorganisms coexist and interact in these extremely complicated and variable ecosystems. The understanding of microbial populations participating in the degradation processes in municipal solid waste landfills is still limited. Microbial diversity in different depths of a municipal solid waste landfill (Sawamura et al. 2010), landfill leachate (Huang et al. 2004, 2005) and landfill cover soil (Wang et al. 2008), etc., has been revealed. *Clostridium*, methanogenic, and methanotrophic populations in landfills have been characterized using molecular methods (Uz et al. 2003). However, much of what is known or assumed about the microbe in landfills comes indirectly from studies of anaerobic reactors treating leachate (Calli et al. 2006). But, little attention is given to microbial populations of sediment in leachate collection ponds.

Microbial communities in sediments of different environments have been studied extensively (Humayoun et al. 2003; Zhang et al. 2007; Hunter et al. 2006; Dong et al. 2006), indicating that local microbial communities and functions were determined by their environments. Because the diversity of microbial communities in sediment is massive (Torsvik et al. 1996), it leads to complicated interactions between ecosystem stability and community development for sediment microbial communities. Sediments in extreme habitats, such as thermophilic sediment, cryophilic sediment, and contaminated sediment (Weber and Jorgensen 2002; Reed et al. 2009), attract much attention with their unique communities and metabolism, but not the sediment of leachate ponds. To our knowledge, few or no previous studies have examined community composition using genetic cloning/sequencing in leachate sediment of a considerably high concentration of ammonium.

Leachate collection ponds are used for the long-term storage of leachate before it flows to wastewater treatment systems. Contaminants such as organics, inorganics, heavy metals, and ammonium accumulate in leachate. Chemical oxygen demand (COD) in leachate vary from 140 to 152,000 mg l^{-1}, and ammonium–nitrogen varies from 50 to 2,200 mg l^{-1} (Christensen et al. 2001). More than 200 organic compounds have been identified in municipal landfill leachate (Slack et al. 2005). Sediments in leachate collection ponds are the ultimate reservoir for the numerous chemical contaminants which are contained in effluents originating from landfills and allow exchange of pore water with the overlying leachate. This habitat is characterized by immediate contact with a large quantity of leachate and an excess of electron donors (i.e., ammonium) but also by a shortage of electron acceptors. Numerous pollutants could restrain or affect microbial metabolism, leading to interactions in microbial and coexistence in this extreme habitat. Such drastically different physicochemical parameters are likely to support the microbial community with a composition very different from that in fine-grained sediments. Therefore, it is interesting to study microbial diversity, identify novel microorganisms, and understand their function in this ecosystem because of its importance in microbial ecology and waste management.

This study focused on microbial communities inhabiting the sediment of a leachate collection pond, a relatively stable environment in which the amount of substrate only delivered from landfill leachate. Due to negative influence caused by high concentration of ammonium and accumulation of toxic substances, novel microbial lineages, special metabolism, and community structure would be detected in this particular environment. The identities and physiological capabilities of prokaryotes in the environment are very poorly understood. In order to obtain insight into the types of prokaryotes in leachate sediment, a culture-independent DNA-based molecular phylogenetic approach was performed with archaeal and bacterial 16S rRNA gene clone libraries derived from three leachate sediment samples of an aged leachate collection pond. Additional insights from this study and other related analysis would improve understanding of the microbiology of waste decomposition in landfills and assigning specific microorganisms to particular biogeochemical processes.

Materials and methods

Site description and sediments sampling

Dongyang landfill is located in south of Dongyang (29°14′49″ N, 120°15′57″ E), Zhejiang Province, People's Republic of China. This landfill has a surface area of approximately 74,000 m^2, and a capacity of 3,000,000 m^3. It began operation in 1993 as a waste disposal site for municipal solid waste, with a daily loading rate of 400 t and a daily leachate generation of 100 m^3.The capacity of leachate collecting pond is about 8,000 m^3. This pond had been used to store leachate for 16 years; the depth of the leachate is more than 2 m, and the thickness of sediment is about 30 cm. Samples were collected in April 2009 from three different locations of the leachate collecting pond using a core sediment sampler. Sediment samples were mixtures of the 0–8-cm layers situated between leachate and deep sediment, and then split into two parts. One part was frozen at −20°C for DNA isolation; the other part was mixed evenly and stored at 4°C for analysis of chemical characteristics, such as moisture content, pH, NH_4^+, NO_3^-, and NO_2^-, within 24 h. Leachate samples just above the sediment were also collected to detect chemical parameters.

The pH (KCl) and nitrogen concentration of sediment were determined by shaking 5 g of moist sediment with 10 ml of 1 M KCl for 2 h (Zhang et al. 2007). Subsequently, samples were taken and centrifuged at 15,000×*g* for 10 min. The supernatant was sampled for further measurement of pH, nitrate, nitrite, and ammonium. Leachate samples were analyzed for pH, COD, nitrate, nitrite, and ammonium. All the analyses were conducted according to the standard methods (State Environmental Protection Administration of China 2002). pH, electrical conductivity (EC), and dissolved oxygen (DO) were measured by YSI 556MPS (YSI Inc., USA), and COD was analyzed by the standard digestion method. Nitrate, nitrite, and ammonium concentrations were determined by spectrophotometric method. Moisture content of the sediments was represented by the difference of weight before and after drying at 105°C for 24 h. Organic matter was analyzed by measuring the weight difference after combustion of the samples at 550°C for 2 h. Triplicates were carried out for every measurement.

DNA extraction, PCR amplification, and construction of 16S rRNA gene library

Total sediment DNA was extracted from approximately 500 mg sediment using a beating method (FastDNATM SPIN Kit for Soil; Bio101 Inc., USA) following the manufacturer's protocol. DNA extracts were stored at −20°C for further test.

Bacterial and archaeal 16S rRNA gene were amplified by PCR with DNA samples from triplicate sediment samples, using the combination of respective universal primer pairs 27f and 1492r for Bacteria (Lane 1991) and Arch21F and Arch958R for Archaea (Delong 1992). PCR was run in a Hybrid PCR Express thermal cycler (Bio-Rad, USA) in 0.2-ml tubes using 50-μl reaction volumes. The reaction mixture contained the following components: 10 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$ (Bacteria) or 2.5 mM $MgCl_2$ (Archaea), 0.2 mM of each dNTP, 0.4 μM of each primer, 0.25 U of *Taq* DNA polymerase (TaKaRa), 1 μl of template DNA (Bacteria), or 2 μl of template DNA (Archaea). Prior to amplification, DNA was denatured at 94°C for 5 min; 25 cycles (Bacteria) or 30 cycles (Archaea) of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min (Bacteria) or 45 s (Archaea), were then performed. A final extension was set at 72°C for 10 min. The expected size of the fragment amplified from the 16S rRNA gene was approximately 1,460 bp for Bacteria and 930 bp for Archaea. Amplified products were analyzed on 1.5% agarose gel running in TAE buffer, stained with SYBR Green I (Invitrogen Biotechnology Co., Ltd., USA) and UV illuminated. The amplicons were purified with AxyPrep kit (Axygen, USA) in accordance with the manufacturer's instructions and re-suspended in nuclease-free water. Purified bacterial and archaeal PCR products were ligated into a pMD19-T vector system (TaKaRa), and cloned into *Escherichia coli* DH5α competent cells. Positive clones were detected from overnight cultures by the appearance of white colonies in LB plates containing 40 μg ml^{-1} of X-Gal, 24 μg ml^{-1} of IPTG, and 100 μg ml^{-1} of ampicillin. White colonies were selected at random. Three independent sublibraries were created for each separated sediment sample and mixed to create the bacterial and archaeal 16S rRNA gene clone libraries.

Screening of rDNA clones and DNA sequencing

Preliminary screening was done by directly reamplifying recombinant clones with M13-47 and RV-M vector primer pair, and analyzed for plasmids containing inserts by 1.5% agarose gel electrophoresis. Amplified ribosomal DNA restriction analysis (ARDRA) of the positive clones were carried out by digesting the reamplified products with restriction enzymes Hha I and Msp I (TaKaRa), and the digested nucleotide sequences were electrophoresed in 3% TAE agarose gels. Clones were grouped according to ARDRA banding patters, and scanning image analyses were performed manually. Then unique phylotypes were identified. Sequencing was carried out by Invitrogen Corporation (USA) in China with an Applied Biosystems 3730xl DNA Analyzer.

Phylogenetic analysis and clone library analysis

All the sequences obtained in this work were checked for chimeras using CHIMERA-CHECK on line analysis program from Pintail (http://www.bioinformatics-toolkit.org/index.html). Four archaeal and 14 bacterial chimeras were determined in the archaeal and bacterial clone libraries, respectively. The chimeric sequences identified were not included in further phylogenetic analysis and clone library analysis. The non-chimeric sequences were submitted to the BLAST network service (http://www.ncbi.nlm.nih.gov) to determine approximate phylogenetic affiliations (Altschul et al. 1997). Multiple alignments of the sequences from this study and reference sequences were performed using CLUSTAL X (Thompson et al. 1997). An average of at least 1,480 and 930 nucleotides was included in the phylogenetic analysis of bacterial and archaeal clones, respectively. The phylogenetic trees were constructed based on neighbor-joining algorithm (Saitou and Nei 1987) in the MEGA4 computer software program (Tamura et al. 2007), using the Jukes–Cantor model (Jukes and Cantor 1969). The confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1,000 resamplings.

The rarefaction curves of the bacterial and archaeal 16S rRNA gene clone libraries were performed by Analytic Rarefaction Software, a web-based program written by Holland and Zaffos (http://www.uga.edu/strata/software/index.html). The diversity index was analyzed by species prediction and diversity estimation (SPADE [http://chao.stat.nthu.edu.tw/softwareCE.html]). Estimated sample coverage (Good 1953), species richness (Chao and Lee 1992), Shannon Index (Chao and Shen 2003), and Simpson Index were chosen to estimate the diversity of the libraries.

Nucleotide sequence accession numbers

The sequences of the leachate sediments archaeal 16S rRNA gene clones have been deposited in the GenBank database under accession numbers HQ141798 to HQ141856; and the bacterial sequences were assigned the accession numbers HQ183746 to HQ184028.

Results

Leachate and sediment sampling and characterization

Sediment samples in this study were the 0–8-cm layers situated between leachate and deep sediment, which was the inevitable way of material exchange in the leachate-sediment interface. The chemical parameters of the leachate and sediment samples, including pH, nitrate, nitrite, ammonium, etc., were measured and used to provide a preliminary indication of the leachate quality and microbial habitat (Table 1). Leachate and sediment samples from Dongyang landfill contain a high level of ammonium–nitrogen, 51.75 and 0.123 mM, respectively. The leachate and sediment were both alkaline, with high concentrations of ammonium. The ammonium–nitrogen in leachate and sediment would inhibit or affect microbial activity. Also detected was a high level of NO_3^- and NO_2^- in the leachate and sediment, which differed from fresh leachate in other landfills. The electrical conductivity of leachate reached a high level, implying plenty of anion and cation and high salinity. Extremely low dissolved oxygen implied anoxic environment in the leachate, so the sediment was possibly anoxic or anaerobic.

Rarefaction analysis and diversity index of 16S rRNA gene clone libraries

The compositions of the archaeal and bacterial communities in the leachate sediment were determined by 16S rRNA gene phylogenetic analysis of clone libraries derived from three separated sediment samples (independent repetitions of PCR and ligation reactions and transformations). A total of 425 recombinant archaeal clones and 375 recombinant bacterial clones were randomly selected, and their rDNA inserts were subjected to amplified ribosomal DNA restriction analysis by separate enzymatic digestions, resulting in 59 different phylotypes for Archaea and 283 different phylotypes for Bacteria. This indicated that compared to archaeal community, the bacterial community associated with the landfill leachate sediment was much more complicated.

Rarefaction curves and calculation of diversity indexes were both based on the ARDRA groups. Rarefaction analysis was conducted to determine if a sufficient number of clones from a library were screened to estimate diversity within the clone library sampled. Rarefaction analysis showed the number of archaeal clones sequenced had covered most of the diversity in the archaeal library (Fig. 1). In addition, the curve reached saturation for archaeal clones, and the coverage C of the archaeal library was 90.4%, which both indicated that a sufficient number of archaeal clones were sampled to represent the diversity of the archaeal library. In contrast, the curve did not reach saturation for the bacterial library. Additional sampling of the bacterial clones would be needed to reveal the full extent of the diversity. However, numerically dominant phylotypes were obtained.

The diversity indexes of archaeal and bacterial clone libraries were analyzed by SPADE (Table 2). Using abundance-based coverage estimator (ACE) to evaluate the species richness of the two libraries, the value of bacterial clone library was almost eight times more than that of Archaea. Furthermore, all statistical estimators, including rarefaction, species richness, Shannon Index, and Simpson Index entirely indicated that the diversity of Bacteria was more abundant than Archaea (Table 2).

Phylogenetic analysis of archaeal clone library based on the 16S rRNA gene

A total of 59 phylotypes affiliated with two phyla Euryarchaeota and Crenarchaeota were examined among

Table 1 General characteristics of the leachate and sediment samples

Parameter	Leachate	Sediment
Moisture content (%)	–	76.26±0.83
Organic matter (%)	–	4.09±0.07
pH (KCl)	8.56	8.06±0.06
NH_4^+–N (mM)	51.75±0.96	0.123±0.005
NO_3^-–N (μM)	2,732.71±25.93	9.06±0.24
NO_2^-–N (μM)	9.39±0.20	0.30±0.002
COD (mg l^{-1})	1,690±22	–
DO (mg l^{-1})	0.03	–
EC (μs/cm)	8,640	–

The meaning of the values in the table is mean±standard deviation.

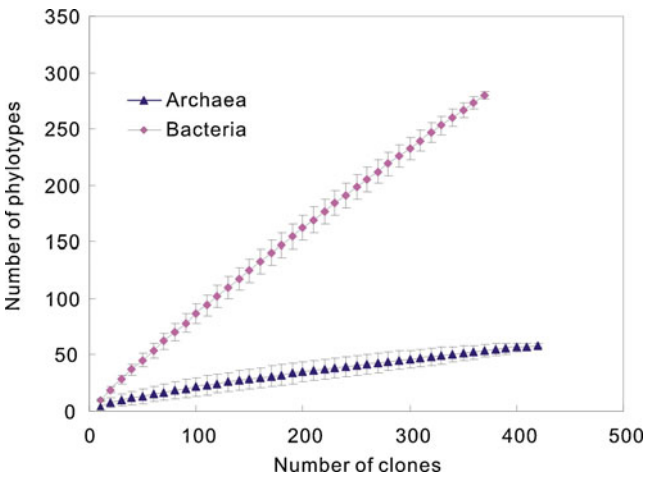


Fig. 1 Rarefaction curve for different phylotypes of archaeal and bacterial 16S rRNA gene clones. *Error bars* are standard deviations

Table 2 Diversity index of the archaeal and bacterial clone libraries

Sample	Diversity estimate								
	No. of clones	No. of phylotypes	C	Species richness		Shannon Index		Simpson Index	
				ACE	95% CIs	MLE	95% CIs	MLE	95% CIs
Archaea	425	59	0.904	209.7	(129.5, 381.4)	2.109	(1.934, 2.285)	3.402	(2.817, 3.988)
Bacteria	375	283	0.347	1,719.3	(1,121.0, 2,744.9)	5.386	(5.294, 5.478)	120.1	(119.6, 120.5)

C estimated sample coverage, *ACE* abundance-based coverage estimator, *CIs* confidence interval, *MLE* maximum likelihood estimator

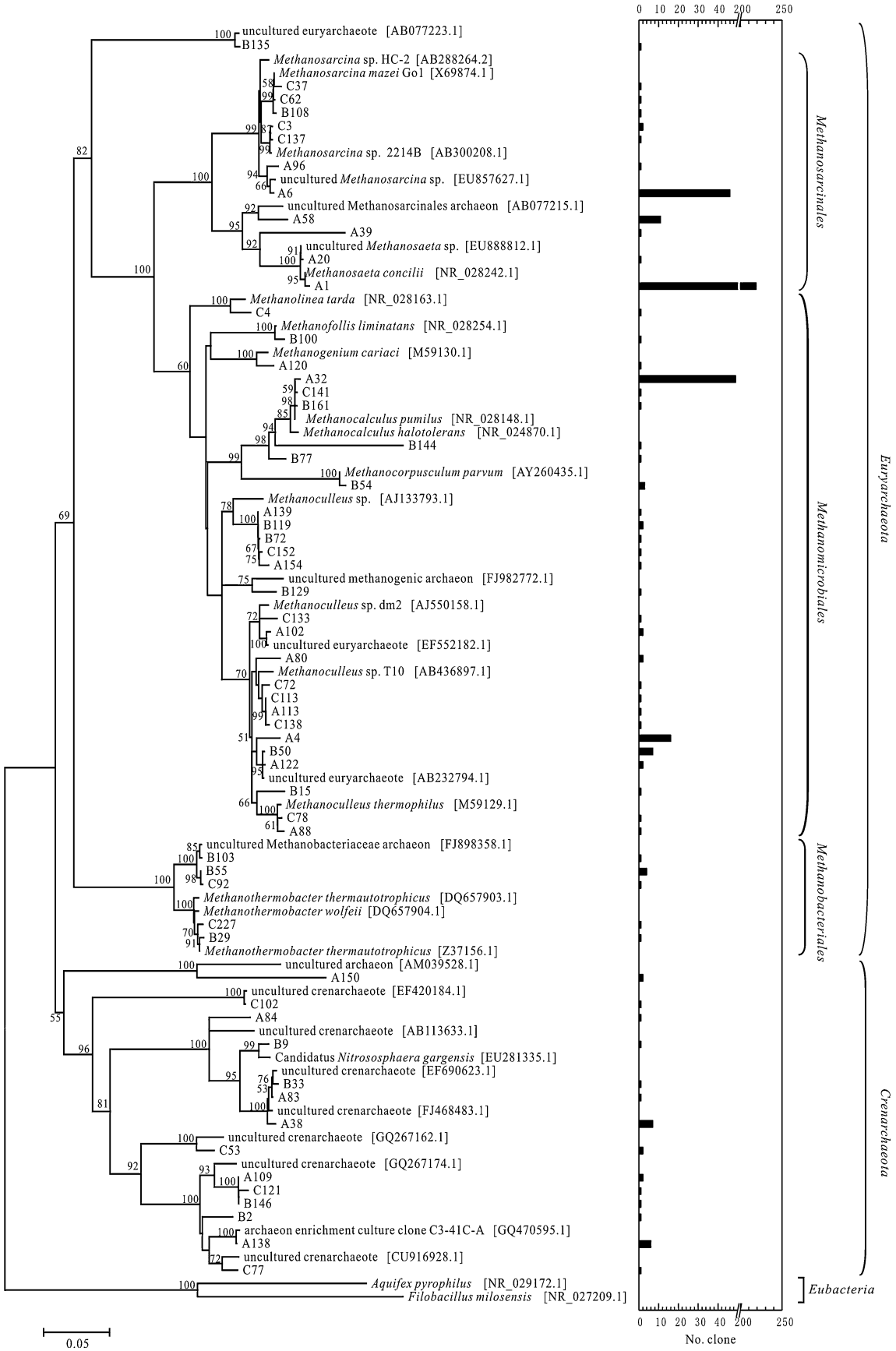
425 archaeal rDNA clones. Forty-five phylotypes out of 59 were identified as Euryarchaeota, representing 397 out of the total 425 clones (93.41%). The other 14 phylotypes, occurring less frequently, were affiliated with the phylum Crenarchaeota, representing the other 28 clones. Nearly all of the sediment archaeal sequences had relatively high level (>95%) of similarity with their closest counterparts in the public databases (Table 3), except four clones which revealed lower similarity to their corresponding closest relatives. The phylogenetic relationships of archaeal sequences were analyzed with 59 phylotypes and closely relative reference sequences obtained from the GenBank database (Fig. 2), and sequences from the three sublibraries have the prefixes "a," "b," and "c," respectively, in their designations.

The database search and phylogenetic analysis indicated that a great abundance of euryarchaeotic clones were related to cultured lineages. Forty-four euryarchaeotic phylotypes were affiliated with three of the six described orders of methanogen: 28 with Methanomicrobiales, 11 with Methanosarcinales, and 5 with Methanobacteriales. The remaining euryarchaeotic phylotype, affiliated with none of the known orders, could not be determined to a similar taxonomic level. All the methanogen-related phylotypes were phylogenetically associated with genera from *Methanoculleus*, *Methanocorpusculum*, *Methanocalculus*,

Table 3 Distribution of phylogenetic affiliation of clones from the archaeal and bacterial 16S rRNA gene libraries

Phylogenetic affiliation	No. of phylotypes	Proportion of phylotypes (%)	No. of clones	Proportion of clones (%)	Sequence similarity to its closest relatives (%)
Archaea	59		425		
Euryarchaeota	45	76.27	397	93.41	89–99
Crenarchaeota	14	23.73	28	6.59	94–99
Bacteria	283		375		
Firmicutes	70	24.73	81	21.60	88–99
Proteobacteria	66	23.32	72	19.20	90–99
β-Proteobacteria	22	7.77	24	6.40	96–99
α-Proteobacteria	19	6.71	21	5.60	93–99
γ-Proteobacteria	13	4.59	14	3.73	92–99
δ-Proteobacteria	11	3.89	12	3.20	90–99
ε-Proteobacteria	1	0.35	1	0.27	98
Chloroflexi	26	9.19	33	8.80	90–99
Actinobacteria	23	8.13	26	6.93	93–99
Bacteroidetes	20	7.07	25	6.67	90–99
OP8	8	2.83	36	9.60	91–99
Planctomycetes	7	2.47	9	2.40	91–99
Spirochaetes	6	2.12	8	2.13	88–97
Other lineages[a]	18	6.36	23	6.13	94–99
Unclassified	39	13.78	62	16.53	84–99

[a] Groups of 10 phyla related clones which accounted for less than 2% of the total bacterial clones. The 10 phyla are Fusobacteria, Synergistetes, Verrucomicrobia, Deinococcus-Thermus, Deferribacteres, Acidobacteria, candidate division TM7, candidate division WWE1, OP10, and OP11 clades


uncultured euryarchaeote [AB077223.1]
B135
Methanosarcina sp. HC-2 [AB288264.2]
Methanosarcina mazei Go1 [X69874.1]
C37
C62
B108
C3
C137
Methanosarcina sp. 2214B [AB300208.1]
A96
uncultured Methanosarcina sp. [EU857627.1]
A6
uncultured Methanosarcinales archaeon [AB077215.1]
A58
A39
uncultured Methanosaeta sp. [EU888812.1]
A20
Methanosaeta concilii [NR_028242.1]
A1
Methanolinea tarda [NR_028163.1]
C4
Methanofollis liminatans [NR_028254.1]
B100
Methanogenium cariaci [M59130.1]
A120
A32
C141
B161
Methanocalculus pumilus [NR_028148.1]
Methanocalculus halotolerans [NR_024870.1]
B144
B77
Methanocorpusculum parvum [AY260435.1]
B54
Methanoculleus sp. [AJ133793.1]
A139
B119
B72
C152
A154
uncultured methanogenic archaeon [FJ982772.1]
B129
Methanoculleus sp. dm2 [AJ550158.1]
C133
A102
uncultured euryarchaeote [EF552182.1]
A80
Methanoculleus sp. T10 [AB436897.1]
C72
C113
A113
C138
A4
B50
A122
uncultured euryarchaeote [AB232794.1]
B15
Methanoculleus thermophilus [M59129.1]
C78
A88
uncultured Methanobacteriaceae archaeon [FJ898358.1]
B103
B55
C92
Methanothermobacter thermautotrophicus [DQ657903.1]
Methanothermobacter wolfeii [DQ657904.1]
C227
B29
Methanothermobacter thermautotrophicus [Z37156.1]
uncultured archaeon [AM039528.1]
A150
uncultured crenarchaeote [EF420184.1]
C102
A84
uncultured crenarchaeote [AB113633.1]
B9
Candidatus Nitrososphaera gargensis [EU281335.1]
uncultured crenarchaeote [EF690623.1]
B33
A83
uncultured crenarchaeote [FJ468483.1]
A38
uncultured crenarchaeote [GQ267162.1]
C53
uncultured crenarchaeote [GQ267174.1]
A109
C121
B146
B2
archaeon enrichment culture clone C3-41C-A [GQ470595.1]
A138
uncultured crenarchaeote [CU916928.1]
C77
Aquifex pyrophilus [NR_029172.1]
Filobacillus milosensis [NR_027209.1]
0.05
0 10 20 30 40 200 250
No. clone
Methanosarcinales
Methanomicrobiales
Methanobacteriales
Euryarchaeota
Crenarchaeota
Eubacteria

◀ **Fig. 2** Phylogenetic relationships of partial 16S rRNA gene sequences from 59 archaeal clones from leachate sediment libraries (in *boldface*) and 36 sequences from GenBank databases. The prefixes "a," "b," and "c" in designations indicated sequences were from the three sublibraries. The sequences were aligned with ClustalX; distance matrices and phylogenetic trees were constructed by using the Jukes–Cantor and neighbor-joining algorithms, respectively. Division level groupings are indicated on the *right*. *Aquifex pyrophilus* and *Filobacillus milosensis* were used as outgroup. The *numbers at the nodes* are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications. Bootstrap values less than 50% are not shown. *Bar*, 0.05 change per sequence position. *Histograms* denote the number of clones in the archaeal library that were affiliated with each particular archaeal phylotype

Methanogenium, *Methanofollisa*, *Methanolinea*, *Methanosaeta*, *Methanosarcina*, and *Methanothermobacter*. The most numerically dominant phylotype A1 was closely related to *Methanosaeta concilii* (99.2% similarity) previously isolated from a mesophilic sewage digester (Eggen et al. 1990), comprising 51.5% of the total archaeal clones. The second most dominant phylotype A32, accounting for 11.5% of the total archaeal clones, was closely related to *Methanocalculus pumilus* (99.1% similarity) previously isolated from leachate (Mori et al. 2000).

All the crenarchaeotic clones could not be affiliated with any known divisions in Crenarchaeota. They were clustered with unclassified environmental clones, except that clone B9 was 98.3% identical to a moderately thermophilic ammonium-oxidizing crenarchaeote "Candidatus *Nitrososphaera gargensis*" (Hatzenpichler et al. 2008).

Phylogenetic analysis of bacterial clone library based on the 16S rRNA gene

Analysis of 283 phylotypes out of 375 total bacterial clones revealed much more diversity relative to the archaeal rDNA library. Two hundred and forty-four phylotypes out of 283 were affiliated with 18 distinct phyla, representing 313 out of the total 375 clones (83.47%). The other 39 phylotypes were irrelated with any known division, representing 62 clones (Table 3). It was determined that most of the sediment bacterial sequences had a relatively high level of similarity with their closest counterparts in public databases (Table 3). However, some clones displaying low levels of similarity (<90%) to any other reported rRNA gene sequences were retrieved. With few exceptions, more than half of the bacterial clones obtained were related to as yet uncultured bacterial lineages from various environments. The phylogenetic relationships of bacterial sequences were analyzed with 244 phylotypes and closely relative reference sequences obtained from the GenBank database (Fig. 3); sequences from the three sublibraries have the suffixes "a," "b," and "c," respectively, in their designations.

The most frequently detected phylum was Firmicutes, comprising 21.60% of the total bacterial clones. A separate distance-based neighbor-joining tree was constructed with the 70 Firmicutes-related phylotypes and reference sequences from GenBank database (Fig. 3a). The Firmicutes-related clones grouped into two classes, i.e., Bacilli and Clostridia. Of the 81 clones, 19.75% (16 of 81) clustered within class Bacilli order Bacillales. A majority of sequences collected from this class were closely affiliated with known species. Phylotypes 29b and 41b clustered with *Paenibacillus* sp. YT0039 and *Paenibacillus* sp. YT0073 within family Paenibacillaceae, which were previously characterized as high-CO_2 dependent strains (Ueda et al. 2008). Phylotype 30b was 97.0% identical with *Ammoniphilus oxalaticus*, an ammonium and oxalate-utilizing bacterium (Zaitsev et al. 1998). Three phylotypes 84a, 111b, and 44b grouped with an uncultured Firmicutes bacterium from a tar oil-impacted aquifer where hydrocarbon degradation depends mainly on sulfate reduction (Winderl et al. 2008). Phylotype 119b, 92.7% identical with a low GC Gram-positive bacterium strain AHT28 (HM046584), was in a clade distinct from the other lineages of Bacillales on the tree, as supported by strong bootstrap values.

The other 65 Firmicutes-related phylotypes were most closely affiliated with the class Clostridia and represented 17.33% of the total bacterial clones. Compared to the Bacillales-related phylotypes, the clostridial phylotypes were more diverse, representing multiple families and uncultivated lineages. Six phylotypes grouped within family Syntrophomonadaceae, whose ecologically important ability is to use stearate and other long-chain saturated fatty acids (Wu et al. 2007; Hatamoto et al. 2007). 60b and 163c were most closely affiliated with *Syntrophomonas palmitatica* and uncultured Thermoanaerobacteriaceae bacterium, respectively, which were usually a group of component in hydrogenotrophic methanogenic community (Erkel et al. 2005; Hatamoto et al. 2007). Phylotype 229c was clustered with an obligately anaerobic saccharolytic bacterium, *Alkalibacter saccharofermemtans* (Garnova et al. 2004).

A second distance-based neighbor-joining tree was constructed with 66 Proteobacteria-related phylotypes (Fig. 3b). The proteobacterial clones comprised 19.20% of the total bacterial clones and they grouped into five classes, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, and Epsilonproteobacteria (α-, β-, γ-, δ-, and ε-proteobacteria), which was the second most abundant division. As with the Bacillales-related phylotypes, the great mass of the proteobacterial phylotypes were closely related to classified species. Overall, the β- and α-phylotypes exhibited the greatest phylogenetic diversity of all proteobacterial lineages detected; the γ- and δ-phylotypes were less diverse. Phylogenetic analysis indicates all the phylotypes concerned with denitrification were detected within

phylum Proteobacteria. The only phylotype related to genus *Thiobacillus* is 79a, which clustered with an inorganic sulfur-oxidizing denitrifier *Thiobacillus denitrificans* (Kelly and Wood 2000). Phylotypes 68a and 52c clustered within the genus *Thauera* and were closely related to oxygen-containing monoterpenes and succinate degrading denitrifiers, *Thanuera terpenica* and *Thanuera* sp. R-25071, (Foss and Harder 1998; Heylen et al. 2006). Three phylotypes, represented by 169c, 86c, and 25c, branched into a clade with organic polymer utilizing denitrifiers, betaproteobacterium NOS8, *Acidovorax* sp. PD-10, and *Comamonas* sp. PG6-1 (Horiba et al. 2005; Khan et al. 2002, 2007). Two phylotypes 75a and 156c, related to Alphaproteobacteria, branched into a clade with a denitrifying bacterium capable of degrading halobenzoates, *Mesorhizobium* sp. 4FB11 (Song et al. 2000). Four phylotypes 74a, 43c, 2c, and 34c were most affiliated with *Steroidobacter denitrificans*, a steroidal hormone-degrading denitrifying bacterium (Fahrbach et al. 2008). The only phylotype 178c grouped with a denitrifier, *Thermomonas fusca* isolated from denitrification reactor with PCL as fixed bed (Mergaert et al. 2003), in a clade distinct from the other γ-proteobacteria on the tree, as supported by good bootstrap values (Fig. 3b).

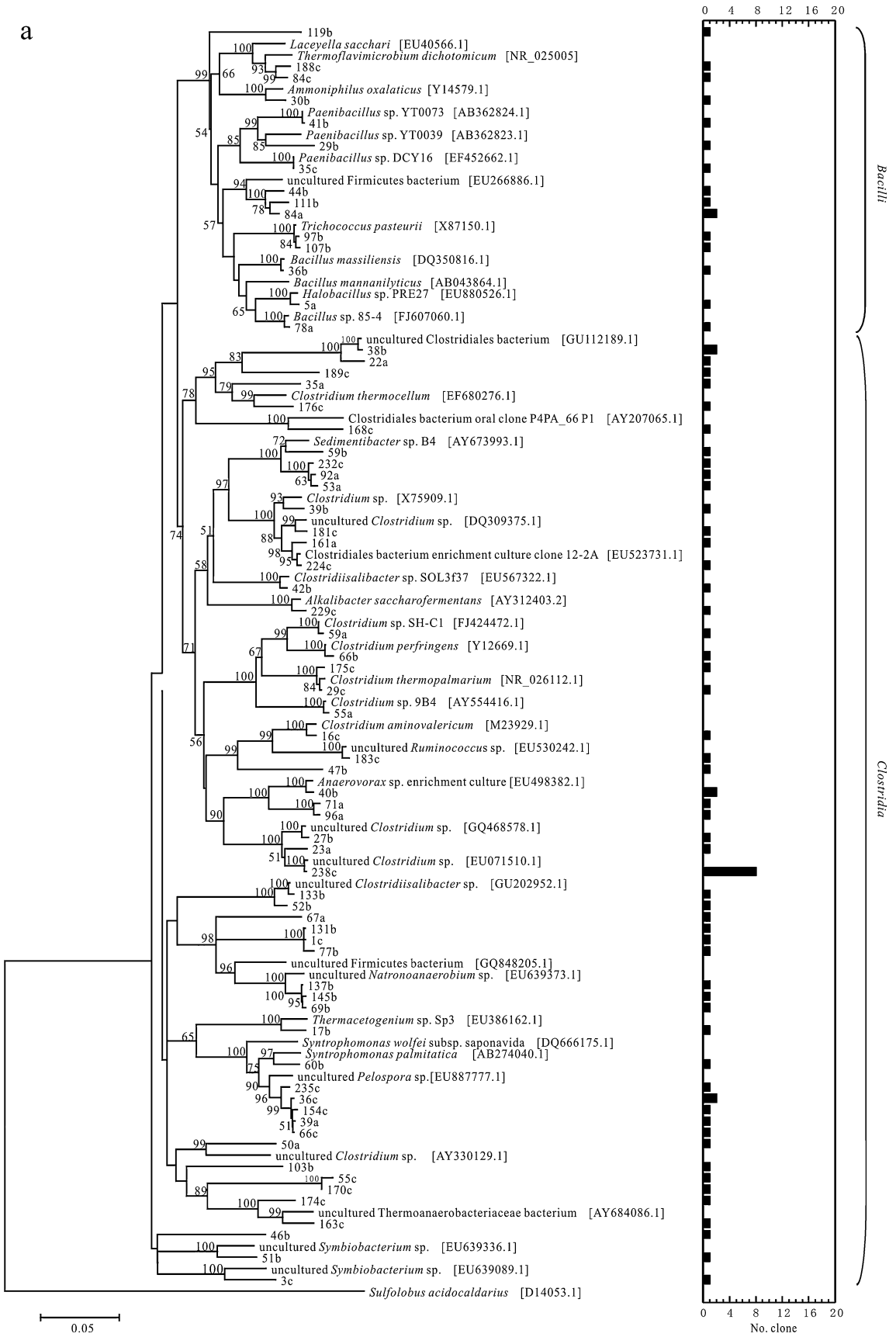
Two distance-based neighbor-joining trees were constructed with non-Proteobacteria and non-Firmicutes-related classifiable phylotypes and included predominatedly uncultured bacterial lineages (Fig. 3c, d). Twenty-six phylotypes were affiliated with phylum Chloroflexi, whereas they were all closely related to environmental clone sequences, except that 70c branched into a clade with *Bellilinea caldifistulae*, a strictly anaerobic filamentous bacterium isolated from methanogenic propionate-degrading consortia (Yamada et al. 2007). Twenty-three phylotypes, representing 26 bacterial clones grouped within phylum Actinobacteria. Of these, three phylotypes, each representing a single clone, were clustered with genus *Lucobacter*, whose strains were mostly isolated from chromium-contaminated environments (Morais et al. 2004). Most phylotypes affiliated with phylum Bacteroidetes, Planctomycetes, Spirochaetes, and candidate division OP8 clade were not closely related to any cultivated organisms. With the exception of phylotypes 73a and 46c, the other Planctomycetes-related phylotypes were <97% similar to any previously identified rRNA gene sequences. Although the phylum Planctomycetes has a single class, order, and family currently identified (Garrity et al. 2005), all the phylotypes are clustered apart from the cultured Planctomycetes and supported by good bootstrap values. Clones related to phyla Fusobacteria, Synergistetes, Verrucomicrobia, Deinococcus-Thermus, Deferribacteres, Acidobacteria, and candidate division TM7, WWE1, OP10, and OP11 clades were observed; however, these clones occurred infrequently ($n<6$), and they were assembled and expressed as "other lineage" in Table 3.

Fig. 3 **a** Phylogenetic relationships of Firmicutes-related phylotypes from bacterial 16S rRNA gene clone library. **b** Phylogenetic relationships of Proteobacteria-related phylotypes from bacterial 16S rRNA gene clone library. **c** Phylogenetic relationships of Chloroflexi-, Actinobacteria-, and Bacteroidetes-related phylotypes from bacterial 16S rRNA gene clone library. **d** Phylogenetic relationships of 39 phylotypes of the other 13 phyla from bacterial 16S rRNA gene clone library. The suffixes "a," "b," and "c" in designations indicated sequences were from the three sublibraries. The sequences were aligned with ClustalX; distance matrices and phylogenetic trees were constructed by using the Jukes–Cantor and neighbor-joining algorithms, respectively. Division level groupings are indicated on the *right*. *Sulfolobus acidocaldarius* is used as outgroup. GenBank accession numbers are in *brackets*. The *numbers at the nodes* are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications and only values greater than 50% are reported. The *scale bar* represents 0.05 substitutions per nucleotide position. *Histograms* denote the number of clones in the bacterial library that were affiliated with each particular bacterial phylotype

No similar taxonomic level could be determined for the remaining 39 bacterial phylotypes (Table 4). These phylotypes represented 62 clones and accounted for 16.53% of the total bacterial clones. However, some of them had low levels of similarity (<90%) with any previously documented sequences in the public databases, or it was difficult to infer their phylogenetic placements, and several of them were individually deep branched in the phylogenetic tree, suggesting that these isolates might be taxonomically new at the species.

Discussion

Phylogenetic composition of bacterial and archaeal communities in the landfill leachate treatment systems and the effluent leachate of a full-scale recirculating landfill have been studied using 16S rRNA gene (Calli et al. 2006; Huang et al. 2004). However, this study is the first description to report the microbial diversity and community structure of prokaryotic populations inhabiting aged leachate sediment based on 16S rRNA gene clone libraries. Leachate sediment contains various amounts of pollutants such as high concentration of ammonium–nitrogen which could affect microbial community composition in sediment. Therefore, microorganisms which tolerate high-concentration ammonium and potentially metabolize in particular ways would be enriched. However, less is known with regard to microbial community composition in leachate sediment. Investigations of the microbial community composition are important steps in understanding the role of bacterial and archaeal populations in biogeochemical processes in this special habitat. The microbial diversity of leachate sediment has just begun to be revealed, and


a
119b
Laceyella sacchari [EU40566.1]
Thermoflavimicrobium dichotomicum [NR_025005]
188c
84c
Ammoniphilus oxalaticus [Y14579.1]
30b
Paenibacillus sp. YT0073 [AB362824.1]
41b
Paenibacillus sp. YT0039 [AB362823.1]
29b
Paenibacillus sp. DCY16 [EF452662.1]
35c
uncultured Firmicutes bacterium [EU266886.1]
44b
111b
84a
Trichococcus pasteurii [X87150.1]
97b
107b
Bacillus massiliensis [DQ350816.1]
36b
Bacillus mannanilyticus [AB043864.1]
Halobacillus sp. PRE27 [EU880526.1]
5a
Bacillus sp. 85-4 [FJ607060.1]
78a
uncultured Clostridiales bacterium [GU112189.1]
38b
22a
189c
35a
Clostridium thermocellum [EF680276.1]
176c
Clostridiales bacterium oral clone P4PA_66 P1 [AY207065.1]
168c
Sedimentibacter sp. B4 [AY673993.1]
59b
232c
92a
53a
Clostridium sp. [X75909.1]
39b
uncultured Clostridium sp. [DQ309375.1]
181c
161a
Clostridiales bacterium enrichment culture clone 12-2A [EU523731.1]
224c
Clostridiisalibacter sp. SOL3f37 [EU567322.1]
42b
Alkalibacter saccharofermentans [AY312403.2]
229c
Clostridium sp. SH-C1 [FJ424472.1]
59a
Clostridium perfringens [Y12669.1]
66b
175c
Clostridium thermopalmarium [NR_026112.1]
29c
Clostridium sp. 9B4 [AY554416.1]
55a
Clostridium aminovalericum [M23929.1]
16c
uncultured Ruminococcus sp. [EU530242.1]
183c
47b
Anaerovorax sp. enrichment culture [EU498382.1]
40b
71a
96a
uncultured Clostridium sp. [GQ468578.1]
27b
23a
uncultured Clostridium sp. [EU071510.1]
238c
uncultured Clostridiisalibacter sp. [GU202952.1]
133b
52b
67a
131b
1c
77b
uncultured Firmicutes bacterium [GQ848205.1]
uncultured Natronoanaerobium sp. [EU639373.1]
137b
145b
69b
Thermacetogenium sp. Sp3 [EU386162.1]
17b
Syntrophomonas wolfei subsp. saponavida [DQ666175.1]
Syntrophomonas palmitatica [AB274040.1]
60b
uncultured Pelospora sp.[EU887777.1]
235c
36c
154c
39a
66c
50a
uncultured Clostridium sp. [AY330129.1]
103b
55c
170c
174c
uncultured Thermoanaerobacteriaceae bacterium [AY684086.1]
163c
46b
uncultured Symbiobacterium sp. [EU639336.1]
51b
uncultured Symbiobacterium sp. [EU639089.1]
3c
Sulfolobus acidocaldarius [D14053.1]
0.05
0 4 8 12 16 20
No. clone
Bacilli
Clostridia

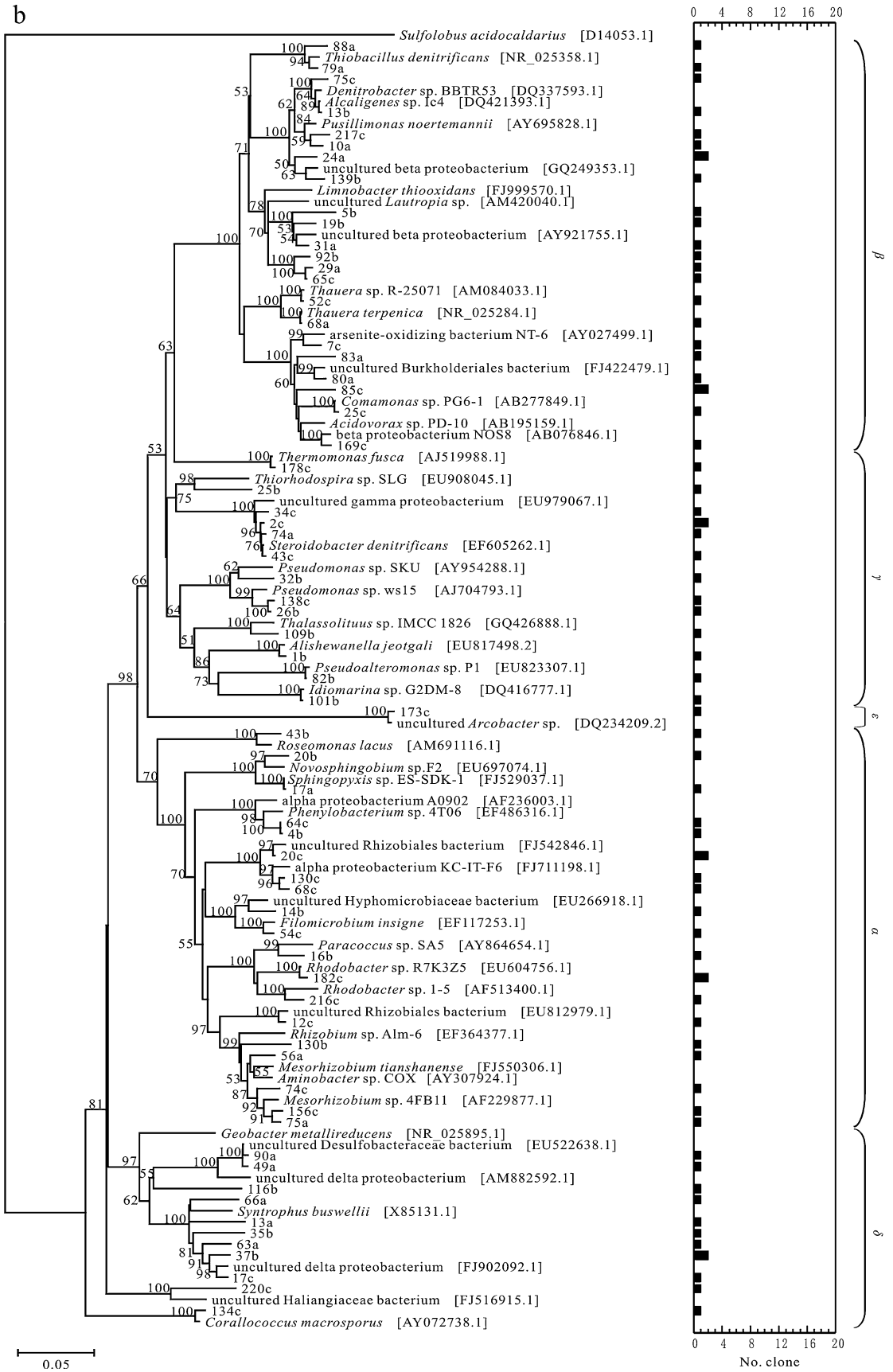


Fig. 3 (continued)

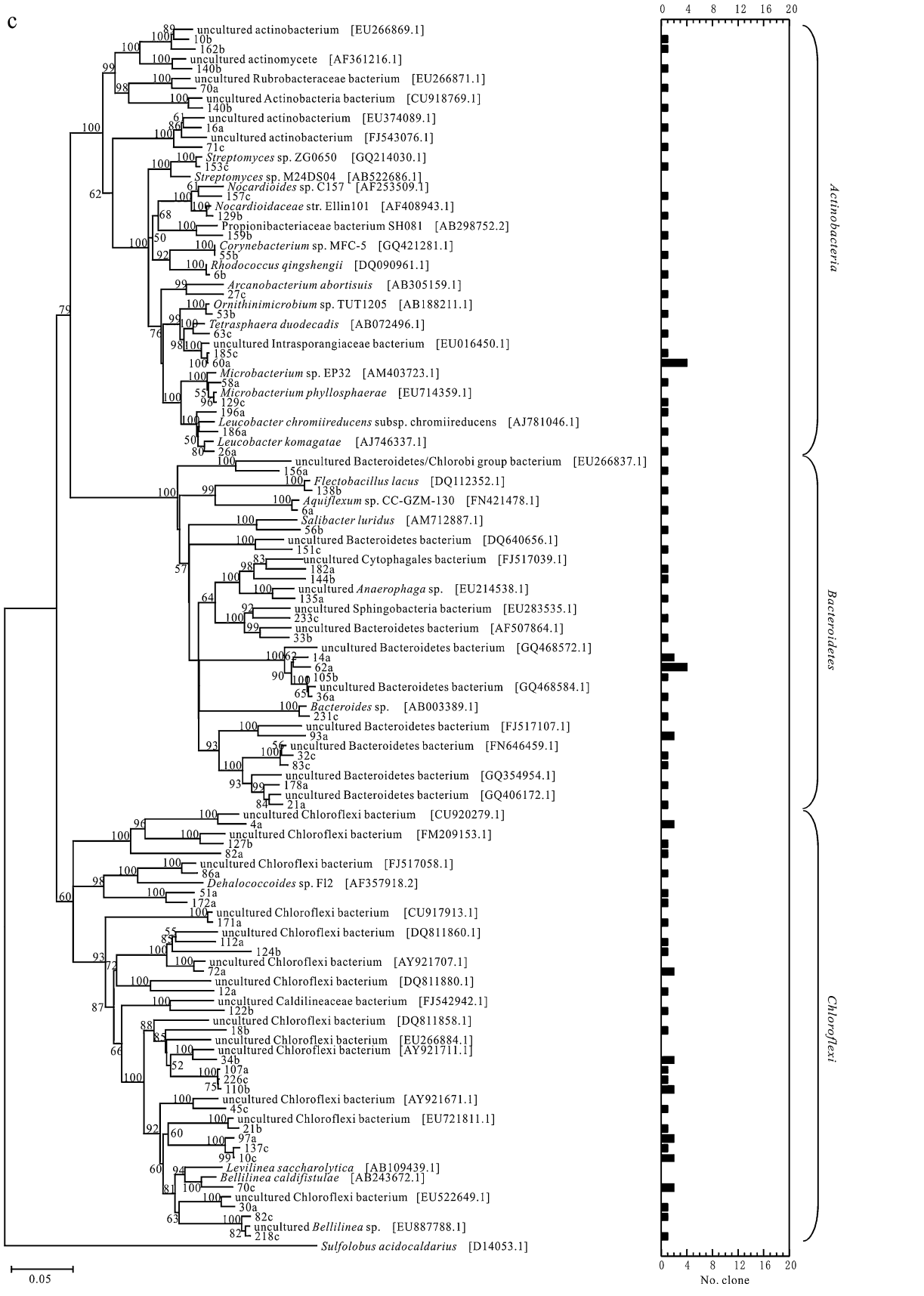


Fig. 3 (continued)

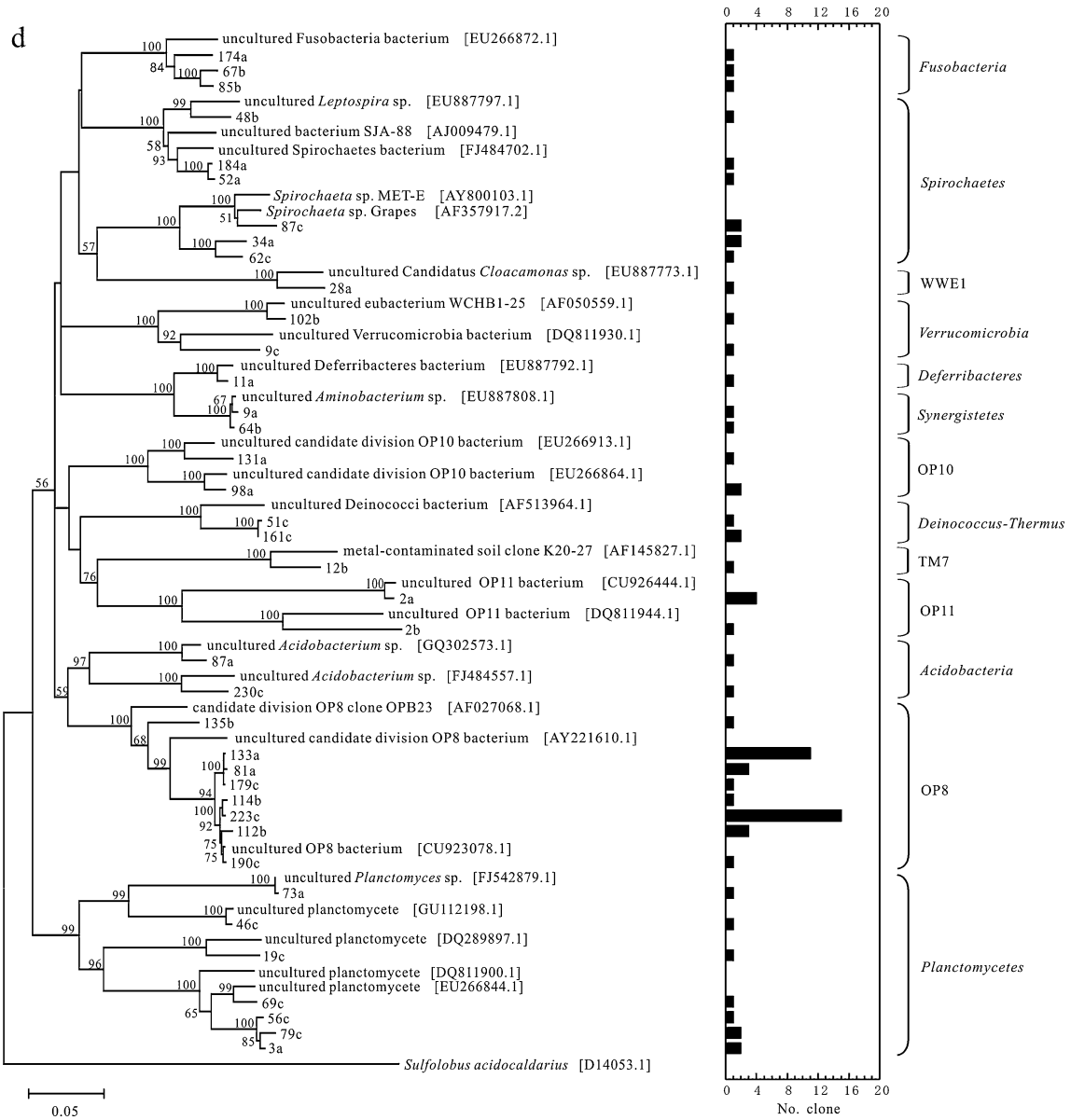


Fig. 3 (continued)

clonal analysis provides a solid sequence database for the development of metabolically active microbial groups in these poorly studied but biogeochemically significant ecosystems.

Archaeal community structure in the leachate sediment

Anaerobic decomposition of landfill solid waste generates significant amounts of greenhouse gas mainly comprising methane and carbon dioxide. Landfills have been implicated as the largest anthropogenic source of atmospheric methane in the world and as a significant contributor to global warming in greenhouse gas scenarios (Bogner et al. 1999). *M. concilii* was the most numerically dominant species of the archaeal community in leachate sediment, thus indicating that leachate collecting pond is one of the important sources for methane generation in landfills. *Methanosaeta* spp. are known to produce energy obligately through acetoclastic pathway (Boone et al. 1993). It is generally accepted that at a certain acetate concentration, long-sheathed rod *Methanosaeta* species with high surface to volume ratio have a competitive advantage over coccus-like *Methanosarcina* species (Jetten et al. 1992; Raskin et al. 1994). As has been reported previously that acetoclastic *Methanosaeta* species are prevalent in anaerobic upflow filter treating landfill leachate (Calli et al. 2006). Ribosomal DNA sequences highly homologous to *Methanosaeta* have also been retrieved as major clones from hydrocarbon-contaminated

Table 4 Representative unclassified phylotypes from bacterial 16S rRNA gene clone library

Representative phylotype	Closest relatives		
	Isolation source	Accession no.	% Identity
24c	Hypersaline microbial mat	EU245416.1	89.9
230c	Hypersaline microbial mat	EU245570.1	95.1
132b	River water	FJ230924.1	96.8
42c	River water	FJ230924.1	97.6
155c	Household biogas digester	EU407211.1	99.7
33a	Methane seep sediment	FJ264787.1	94.1
42a	Sawmill sink water column	FJ716342.1	94.0
28b	Hydrogen fermentor	GQ167173.1	93.1
101b	Subsurface water	DQ234647.1	90.7
40a	Anaerobic ammonium oxidation reactor	FJ710781.1	99.3
85a	Rice paddy soil	AB486816.1	97.2
100a	Deep coal seam groundwater	AB294309.1	89.8
77a	Human fecal sample from subject FA	EF400519.1	88.3
149b	Full or pilot scale municipal compost	FN667494.1	99.4
61a	Oil well	EU721821.1	95.4
870a	Hypersaline microbial mat	EU245165.1	95.4
95a	Oil-contaminated soil	EU735601.1	84.9
104b	Groundwater	AB179664.1	80.1
74b	Landfill leachate	AJ853552.1	99.6
91a	Soil	EU735751.1	93.3
136c	Evry municipal wastewater treatment plant	CT573834.1	96.8
100a	Marine sediment	GQ249498.1	96.3
48a	Harbor sediment	DQ395066.1	93.6
76a	Marine sediment	GQ249498.1	96.0
57b	Hypersaline sediment	EU592434.1	94.0
160b	Farm soil adjacent to a silage storage bunker	AY921783.1	97.7
131c	Mesophilic biogas digester treating pig manure	EU358733.1	96.3
38a	Mesophilic anaerobic digester at 35°C	EF559145.1	98.3
78c	Mesophilic anaerobic digester at 35°C	EF559145.1	99.5
133c	Low-temperature biodegraded Canadian oil reservoir	AY570587.1	99.5
170a	Mesophilic anaerobic digester which treats municipal wastewater sludge	CU922882.1	99.6
40c	Mesophilic anaerobic digester at 35°C	EF559154.1	99.7
69a	Mesophilic anaerobic digester which treats municipal wastewater sludge	CU924177.1	99.3
134b	Mesophilic biogas digester treating pig manure	EU358731.1	96.6
237c	Anoxic filter from a wastewater treatment plant treating RDX	EU334517.1	99.5
62b	Noxic filter from a wastewater treatment plant treating RDX	EU334517.1	98.8
195c	Mesophilic anaerobic digester which treats municipal wastewater sludge	CU921910.1	99.7
67c	Anaerobic ammonium oxidation (Anammox) bioreactor	GQ356151.1	88.0
181a	Siliciclastic sediment from Thalassia sea grass bed	EU488087.1	89.1

These phylotypes were listed as “unclassified” in Table 3

groundwater (Dojka et al. 1998) and hexadecane-degrading (Zengler et al. 1999) methanogenic consortia. However, we did not detect any Methanococcales-, Methanocellales-, and Methanopyrales-related clones in the archaeal library. Methanococcales- and Methanocellales-related clones are perhaps less abundant in the sediment. Representatives of the specific order of methanogen, Methanopyrales, are extremely thermophilic (Boone et al. 1993) and unlikely to exist in leachate sediment. Nevertheless, much more clear methanogen community

structure could be established though detailed characters of leachate sediment, such as soluble COD, acetate and volatile fatty acids, etc.

As strictly anaerobic microorganisms, methanogen thrive in leachate sediment, which suggests that leachate sediment provides a favorable anaerobic environment for various methanogen to grow. The Archaea represented by methanogen-related clones in this analysis produce methane gas as their end product of metabolism by utilizing a limited number of simple carbon compounds as substrates, via the hydrogenotrophic CO_2-reducing, methylotrophic, or acetoclastic pathways. For the conversion of complex organic substrates to methane, methanogen might be accompanied with fermentative and acetogenic bacteria. Thus, it is not unexpected to observe fermentative bacterial clones that are closely associated with macromolecular organic compounds degrading strains in this habitat. Burrell et al. (2004) have proved that *Clostridium* populations are responsible for cellulose degradation in methanogenic landfill leachate bioreactor. These fermentative bacteria hydrolyze and then ferment complex substrates to produce longer chain fatty acids, acetate, carbon dioxide, H_2, NH_4^+, and HS^- (McInerney and Bryant 1981). H_2-producing acetogens convert fatty acids, alcohols, and some aromatic and amino acids to H_2, carbon dioxide, and acetate needed by methanogen. This collection of different microbial species is referred to as a methanogenic consortium (Zinder 1993).

Ammonium in leachate is released from the waste mainly by decomposition of proteins. The only mechanism by which the ammonium concentration can decrease during refuse decomposition is leaching because no mechanism is present for its degradation under methanogenic conditions (Burton and Watson-Craik 1998). It implies that there is little nitrite and nitrate in fresh leachate, and this point has been substantiated by some studies (Vigneron et al. 2007; Laitinen et al. 2006). But, relatively high concentrations of nitrite and nitrate were detected in the leachate and sediment samples of this study. The detection of clone closely related to Candidatus *N. gargensis* not only suggests ammonium-oxidizing Archaea (AOA) might be present in the leachate sediment, but also could explain the appearances of nitrite and nitrate. Although leachate sediment provides an anaerobic environment, the concomitant presence of AOA with anammox bacteria and high abundance of AOA in deep soils suggest that AOA are involved in nitrification under very low oxygen levels and anoxic conditions (Coolen et al. 2007; Leininger et al. 2006). Moreover, leachate sediment could supply AOA with high level of CO_2 and ammonium as the carbon and energy sources. Thus, it is possible that AOA inhabit in leachate sediment and provide nitrite and nitrate which is needed for denitrification.

Bacterial community structure in the leachate sediment

Leachate and sediment sample from Dongyang landfill contain 51.75 and 0.123 mM ammonium–nitrogen, respectively. The high concentration of ammonium and amounts of pollutants would make a negative influence on microbial activity. For comparison, soil moisture ammonium concentrations in moderately nitrogen-polluted Dutch flood plains are only 0–100 μM (Lamers et al. 2006). High concentrations of ammonium–nitrogen in leachate usually inhibit microbial growth and activity of activated sludge and methanogen in mesophilic solid-substrate anaerobic digestion (PoggiVaraldo et al. 1997; Li and Zhao 1999). Consortia in leachate sediment, including ammonium-utilizing microorganisms, might consume surrounding ammonium to form a relatively low ammonium microenvironment and to alleviate or avoid negative influence on other microorganisms.

A part of the bacterial clones were affiliated with chemoheterotroph bacteria which take various complex and simple organic compounds as substrate and energy to grow and produce H_2, CO_2, methylated compounds, and acetate. Clostridial clones observed in leachate sediment could degrade cellulose to produce hydrogen and acetic acid. These populations would provide methanogen with substrates, including acetate, H_2/CO_2, and methylated compounds. Amounts of CO_2 generated by degrading of organic compounds provide comfortable conditions for high-CO_2 dependant strains as well. Phylogenetic analysis indicates that most denitrifying phylotypes in the leachate sediment are affiliated with chemoorganoheterotroph denitrifiers. They belong to Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Their closest counterparts (>98% similarities) could utilize halobenzoate, oxygen-containing monoterpenes, acetate, 3HB, steroidal hormone, and organic polymers as carbon source to reduce nitrate and nitrite to dinitrogen (Foss and Harder 1998; Fahrbach et al. 2008; Khan et al. 2002, 2007; Horiba et al. 2005; Song et al. 2000). Some phylotypes were identical to uncultured organisms found in varied denitrifying reactors.

Microbial populations in the landfill are capable of a variety of reactions depending upon the prevailing environmental conditions and the organism substrate specificity (Ragle et al. 1995). The amount of pollutants in the leachate sediment results in a complicated heterogeneous environment, in which different kinds of pollutants interrelated microorganisms would inhabit. Saccharolytic bacteria, benzoate-degrading bacteria, and proteolytic bacteria-related clones in bacterial library were also detected (Mountfort et al. 1984; Narihiro et al. 2004; Garnova et al. 2004). Other pollutant-degrading and biotransforming-related clones present in leachate sediment include arsenite-oxidizing bacterium (arsenite-oxidizing bacterium NT-6),

demulsifying bacterium (*Sphingopyxis* sp. ES-SDK-1 and *Pusillimonas* sp. ES-SD-3) (Huang et al. 2010), CO-oxidizing *Aminobacter* sp. COX (King 2003), carbendazim-degrading *Rhodococcus* sp. djl-6 (Xu et al. 2006), and uncultured organisms from oil-contaminated environments and chromium-contaminated environments (Morais et al. 2004, 2006).

Alkaline and high level of electrical conductivity is another characteristic of the leachate sediment. Some phylotypes affiliated with extreme halophilic and halotolerant organisms were detected, which are alkaliphilic and alkaline tolerant. Recently, anaerobic denitrifying methanotrophic bacteria have been reported (Ettwig et al. 2008). However, this kind of organism was not detected. The occurrence of diverse denitrifying populations and production of abundant methane suggests a highly favorable environment for anaerobic methane oxidation coupled to denitrification in leachate sediment. Nevertheless, collaborating molecular and chemical data will need to be obtained to substantiate our hypothesis.

Phylogenetic analysis also indicated that part of bacterial clones clustered tightly with other environmental clones from mesophilic anaerobic digesters, oil-contaminated areas, and denitrifying bioreactors. There were a degree of resemblances in the microbial populations between current study and leachate of a closed municipal solid waste landfill and a full-scale recirculating landfill (Huang et al. 2003, 2005). They had similar bacterial constituents, such as phylum Firmicutes, Proteobacteria, Bacteroidetes, etc., and similar metabolic type, methanogen. However, differences exist in their microbial populations as well. For example, most archaeal clones were closely related to acetoclastic *Methanosaeta* spp. in this study, but no sequences related to known *Methanosaeta* spp. were retrieved in a full-scale recirculating landfill. Specific bacterial group distributed in leachate sediment, such as phylum Chloroflexi, Candidate division OP8 and OP10. Distinct landfill circumstances and different physicochemical properties of leachate and sediment would result in discrepant microbial communities in leachate and leachate sediment. Otherwise, environment factors and pollutants would make strong effects on microbial communities in the leachate sediment.

Numerous pollutants and drastically different physicochemical characters make the leachate sediment a complicated heterogeneous environment. This study revealed the prokaryotic diversity and microbial populations within the leachate sediment of an aged municipal solid waste landfill. Archaeal community is dominated by methanogen, especially *M. concilii*. Methanogen accompanied with fermentative and acetogenic bacteria form a methanogenic consortium in the sediment and suggest that leachate collecting pond is one of the important sources for methane generation in the landfill. Bacterial community is dominated by clones affiliate with pollutant-degrading and biotransforming bacteria, such as denitrifiers and cellulose-degrading bacteria. As is often the case in environmental sequence analysis, the majority of bacterial phylotypes detected were not closely related to any cultivated representatives, and the sequences of several microbial groups have not been observed in past studies of landfill ecosystems. This environment could be a source of novel species belonging to new lineages with still unknown physiological characteristics.

The molecular phylogenetic approach of this study has given a first useful insight of the substantial diversity in the microbial community within the leachate sediment of a municipal solid waste landfill and could be used as a starting point for further studies. Further information on the microbial mediated contributions to carbon and nitrogen cycling and the activity of prokaryote with respect to their direct and indirect contributions to contaminant biodegradation remains to be explored.

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