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Molecular identification and dynamics of microbial communities in reactor treating organic household waste

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Abstract The prokaryotic diversity associated with organic household waste (OHW), leachate (start-up inoculum), and mesophilic anaerobic digestion processes in the degradation of OHW for 44 and 90 days was investigated using a culture-independent approach. Bacterial and archaeal 16S rRNA and *mcr*A gene clone libraries were constructed from community DNA preparations. Bacterial clones were affiliated with 13 phyla, of which *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were represented in all libraries, whereas *Actinobacteria*, *Thermotogae*, *Lentisphaerae*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Synergistetes*, *Spirochaetes*, *Deferribacteres*, and *Deinococcus-Thermus* were exclusively identified in a single library. Within the *Archaea* domain, the *Euryarchaeota* phylum was the only one represented. Corresponding sequences were associated with the following orders of hydrogenotrophic methanogens: *Methanomicrobiales* (*Methanoculleus* genus) and *Methanobacteriales* (*Methanosphaera* and *Methanobacterium* genera). One archaeal clone was not affiliated with any order and may represent a novel taxon. Diversity indices showed greater diversity of *Bacteria* when compared to methanogenic *Archaea*.

Keywords 16S rRNA gene · *mcr*A gene · Clone library · Anaerobic reactor · Organic household waste · Leachate

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Introduction

Millions of tons of household solid waste (HSW) are produced daily worldwide, and this has become a serious environmental, social, and public health problem for urban communities due to a lack of available areas for its disposal. Thus, one of the greatest challenges of contemporary society is to reduce and treat HSW.

Currently, HSW treatment has gained relevance due to an increased interest in renewable energy sources, as it combines the removal of organic pollutants and energy conservation in the form of biogas production (Lastella et al. 2002). Anaerobic digestion has been extensively and successfully applied to organic waste treatment over the past decades (Abegglen et al. 2008; Fernández et al. 2008). It takes place in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and is the consequence of a series of metabolic interactions among bacterial and archaeal groups. The first group of microorganisms secretes enzymes which hydrolyze polymers to monomers, such as glucose, amino acids, lactate, and ethanol. These are subsequently converted by acidogenic and acetogenic bacteria to H_2 and volatile fatty acids. Finally, methanogenic *Archaea* convert H_2, formate, and acetate to CH_4 and CO_2.

Biogas produced during methanogenesis is a natural source of energy. It can be collected and used to generate heat energy and electricity, reducing the consumption of fossil fuels, and commercialized as carbon credits in accordance with the Kyoto protocol (Yadvika et al. 2004).

The anaerobic process is very fragile and during digestion; it is necessary to maintain the balance among different microbial populations. The use of molecular tools to biomonitor digesters helps to know the microorganisms present during the process which later can be useful improving the efficacy of organic matter degradation, methane production, and stability of the process (Talbot et al. 2007).

The use of both 16S rRNA genes and functional gene clone libraries is a culture-independent approach that has been applied to detect the diversity of prokaryotes from different environmental samples. Previous culture-independent analyses of anaerobic reactors have been based on phylogenetic markers like the 16S rRNA and *mcrA* genes (Sasaki et al. 2006, 2007; Krause et al. 2008; Rastogi et al. 2008). The *mcrA* gene encodes a subunit of methyl-coenzyme M reductase, a key enzyme in the methane production pathway (Banning et al. 2005). However, little information on the dynamics of prokaryote communities from organic household waste treated in anaerobic reactors is available. In this study, the bacterial and archaeal diversity of organic household waste (OHW), leachate (LC, start-up inoculum), and OHW at two stages of treatment in a mesophilic anaerobic reactor was investigated by analyzing sequences of 16S rRNA genes and *mcr*A functional gene clone libraries, thus extending our understanding of the involvement of these microorganisms in the operation of anaerobic reactors.

Materials and methods

Reactor conditions and chemical analysis

Two continuous, 20 L reactors, with an internal diameter of 30 cm and a height of 40 cm, were built at the DEFLOR LTDA Company (Contagem, state of Minas Gerais, Brazil) to treat OHW. The reactors were operated for 90 days at 35°C (mesophilic), and pH was maintained around 6.5 to 7.5 by neutralizing with sodium carbonate. The first reactor was fed with 40% (w/v) OHW (fruit and meal leftovers, cut into pieces) only at the beginning of the treatment, and 60% (v/v) of leachate (used as start-up inoculum), collected from an OHW anaerobic reactor after 90 days of treatment, which had been previously inoculated with anaerobic sludge collected from the anaerobic reactor UASB of the Arrudas wastewater treatment station (Contagem, Minas Gerais state, Brazil). These conditions were similar to

those of the first reactor previously described. The volume of mass (OHW+LC) in the reactor was 16 L, and pH was adjusted early on to 7.5. Prokaryote community structure was analyzed in the LC, OHW, and OHW treated for 44 and 90 days (OT44 and OT90, respectively). OHW+LC and OT90 samples were decanted and analyzed for pH, total solids (TS), fixed solids (FS), and volatile solids (VS). The measured cumulative production of biogas was investigated after treatment. Methane and carbon dioxide content was measured by gas chromatography.

DNA extraction

DNA was extracted from approximately 10 g (wet weight) of LC, OHW, OT44, and OT90 residues using the MegaPrep Power Max ™ Soil KIT (Mo Bio Laboratories) according to the manufacturer's instructions. This procedure is effective for removing polymerase chain reaction (PCR) inhibitors. Total DNA from samples was quantified by absorbance at 260 nm using a NanoDrop Spectrophotometer (NanoDrop Technologies). DNA purity was assessed with the A260/A280 and A260/A230 ratios. Isolated DNA was stored at −20°C until further processing.

PCR amplification and clone library construction

Bacterial and archaeal 16S rDNA and *mcrA* genes fragments were amplified by touchdown PCR. Reactions were carried out with the bacterial-targeted primer set 8F (5′-AGAGTTT GATYMTGGCTCAG-3′) and 907R (5′-CCGTCAAT TCMTTTRAGT-3′; Lane 1991); and archaea-targeted primer set Arc21F (5′-CCGGTTGATCCYGCCGGA-3′) and Arc958R (5′-YCCGGCGTTGAMTCCAATT-3′; Delong 1992). To amplify the *mcrA* gene fragments from methanogenic *Archaea*, we used primer set mcrAF (5′-GGTGGT GTMGGATTCAC-3′) and mcrAR (5′-TTCATTCGRTA GTTWGGRTAGTT-3′; Luton et al. 2002). Polymerase chain reaction mixtures (20 μL) consisted of 10 μL of Hot Star Taq Master Mix KIT® 24 (Quiagen, CA 2005), 0.5 μM of each primer, and 20 ng of DNA. PCR was conducted with a Mini-cyclerTM PTC-100 (MJ Research Inc., Waltham, MA) with an initial cycle of 5 min at 94°C, followed by 18 cycles of denaturation at 94°C for 1 min, annealing beginning at 57°C and ending at 49°C for 1 min, and extension at 72°C for 3 min. Annealing temperature was lowered by 1°C every two cycles until it reached 49°C; 12 additional cycles were carried out at the last annealing temperature. The cycles were finished with an elongation step of 10 min at 72°C. Gene amplification was achieved after 30 thermal cycles for bacterial and archaeal 16S rRNA genes, whereas amplification of the *mcrA* gene was achieved using a two-round approach. Thus, a 1-μL aliquot of the first round PCR

product was subjected to a second round of PCR. Amplicons were visualized on 1% agarose gel stained with ethidium bromide and purified directly with the QIAquick Gel extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

For cloning, the purified amplicons were cloned into the PCR 4-TOPO vector from a TOPO TA Cloning Kit (Invitrogen, EUA) as per the manufacturer's instructions and transformed into electro competent *Escherichia coli* DH5α. At least 800 positive clones from each clone library were transferred to 96-well plates with Luria–Bertani medium containing ampicillin (100 µg mL^{-1}) and 20% glycerol, incubated overnight at 37°C and stored at −80°C. Plasmids containing 16S rDNA and *mcrA* inserts were extracted, using a standard alkaline lysis method (Ausubel et al. 1990), and the inserts were subsequently PCR-amplified with vector-specific primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′).

16S rRNA, *mcrA* gene sequencing, and phylogenetic analysis

Sequencing was performed in a MegaBace 1.000 capillary sequencer (Amersham Biosciences) using standard protocols with a DYEnamic ET dye terminator kit (Amersham Biosciences). The sequences (800 bp for bacterial and archaeal 16S rDNA) were compared against sequences held in the Ribosomal Database Project (RDP) using Library Compare and against sequences held in GenBank using BLASTN to search for similar homologous sequences of 16S rRNA gene partial sequences in clone libraries. The *mcrA* gene (490 bp) was compared only to GenBank sequences. To accomplish this, the 16S rRNA gene sequences were base-called, checked for quality, aligned, and analyzed with Phred v.0.020425, Phrap v.0.990319 and Consed 12.0. RDP's CHECK-CHIMERA program (Maidak et al. 2001) was used to detect and omit chimeric DNAs. Phylogenetic relationships were inferred by MEGA 3.1 (Kumar et al. 2004) using the neighbor-joining method (Saitou and Nei 1987) and Kimura's 2-P model of sequence evolution. DOTUR (Schloss and Handelsman 2005) was used to assign sequences to operational taxonomic units (OTUs). Sequences were grouped into an OTU at distance values of 0.03 (≥97% sequence similarity), which correspond to species level (Stackebrandt and Goebel 1994). Nucleotide sequences generated were deposited in the GenBank database under accession numbers FJ98280–FJ982838, FJ982839–FJ982863, FJ982864–FJ982879, and FJ945735–FJ945748 for 16S rRNA gene sequences from LC, OHW, OT44, and OT90 *Bacteria*, respectively. Accession numbers FJ982799 and FJ982800 correspond to 16S rRNA gene sequences from LC *Archaea*, and FJ982880–FJ982898, FJ982899–FJ982904, and FJ982905–

FJ982912 correspond to *mcrA* gene sequences from LC, OHW, and OT44, respectively.

Estimation of bacterial diversity and clone library similarity

The DOTUR program was applied to calculate rarefaction, richness, and diversity, as well to estimate the representation of the OTUs and to characterize the bacterial and archaeal diversity in the samples. The diversity indices included nonparametric richness estimators abundance-based coverage estimator (ACE) and Chao1 (Chao 1984), Shannon diversity index (1/*D*), and Shannon–Weaver diversity index. Rarefaction curves with 95% confidence intervals were constructed by comparing the number of clones in each 16S rRNA and *mcrA* gene library. The coverage (*C*) of each clone library, a measure of captured diversity, was calculated as: $C=1-(n/N)$, where *n* is the number of different OTU types from a clone library that were encountered only once (Henriksen 2004), and *N* is the total sequenced number of clones in the library (Good 1953).

Phylogenetic diversity in the bacterial libraries was compared using LIBSHUFF (Singleton et al. 2001; Hughes and Bohannan 2004) and S-LIBSHUFF (Schloss et al. 2004). These programs use Good's coverage formula (Good 1953) to generate homologous and heterologous coverage curves (C_X and C_{XY}, respectively) for two 16S rDNA clone libraries (*X* and *Y*) at a similarity level or evolutive distance (*D*). To determine whether coverage curves $C_X(D)$ and $C_{XY}(D)$ were significantly different, the Crámer-von Mises (Pettitt 1982) statistic test was used to determine the distance between the two curves. Curves were considered significantly different if $P<0.05$.

Results

Characterization of the samples and biogas production

Total solids, volatile solids, fixed solids, and pH of the samples OHW+LC and OT90, before and after treatment in the mesophilic anaerobic reactor, are shown in Table 1. Biogas yield in the reactor reached 570 L over 90 days (Fig. 1). Methane and carbon dioxide content in the produced biogas at the end of the process were 37.5% and 42.5%, respectively.

PCR amplification and clone library analysis

To determine the composition of the bacterial and archaeal communities, clone libraries were constructed from PCR-amplified 16S rRNA gene (bacterial and archaeal) and methanogenic *Archaea mcrA* gene fragments retrieved from the four samples. The archaeal 16S rRNA gene yielded a

Table 1 Physical and chemical characteristics of the organic household waste in the anaerobic reactor

Measurement (%)	Sample	
	LC+OHW	OT90
TS	21.6	14.72
VS	78.7	54.31
FS	21.3	45.69
pH	7.0	6.9

TS total solids, *VS* volatile solids, *FS* fixed solids, *LC+OHW* leachate used as start-up inoculum plus the pre-treatment organic household waste, *OT90* OHW treated for 90 days

PCR product only for DNA from LC and *Halobacterium salinarum* ATCC 19700 (positive control); consequently, archaeal clone libraries could not be constructed for the samples from OHW, OT44, and OT90. Clone libraries were constructed where PCR amplification was successful. However, no OT90 clone library was obtained using the *mcrA* gene partial amplicon, despite four attempts to do so. Therefore, eight clone libraries from *Bacteria* (OHW, LC, OT44, and OT90), *Archaea* (LC), and methanogenic *Archaea* (OHW, LC, and OT44) were generated. The number of clones obtained in each library ranged from 800 to 2,000. A total of 601 clones from the eight aforementioned libraries were randomly picked and sequenced. Twenty-seven chimeric sequences were excluded from further analyses (Table 2).

Bacterial 16S rDNA clone library diversity

Phylogenetic analysis of the nucleotide sequences revealed a significant degree of bacterial diversity in the four clone libraries (Figs. 2, 3, 4, and 5). The LC library was most diverse, with few or no OTUs in common with the other libraries (Table 2, Figs. 2 and 6, and Electronic supplementary material, Table S 1). The bacterial libraries contained 93
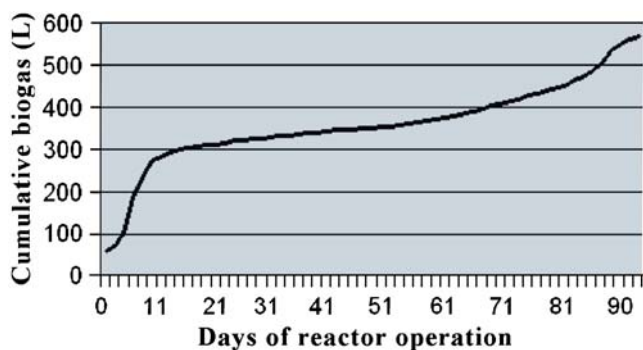

Fig. 1 Temporal variation of greenhouse gases production in an anaerobic reactor having organic household waste as substrate and leachate as inoculum

distinct OTUs, which were affiliated to 13 different phyla. Most of the sequences had high identity to those of uncultured organisms obtained from various environments (Electronic supplementary material, Table S 1). OTUS from four libraries were affiliated with *Firmicutes* (47%), *Proteobacteria* (31%), and *Bacteroidetes* (11%), followed by *Actinobacteria*, *Thermotogae*, *Lentisphaerae*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Synergistetes*, *Spirochaetes*, *Deferribacteres*, and *Deinococcus-Thermus* detected at low frequency and exclusively in a single library (Fig. 6 and Electronic supplementary material, Table S 1).

The 44 OTUs affiliated with *Firmicutes* were represented by classes *Clostridia* and *Bacilli* in all libraries. Among the identified genera, namely *Sporanaerobacter*, *Thermacetogenium*, *Clostridium*, *Leuconostoc*, *Bacillus*, and *Lactobacillus*, the latter was most abundant (Figs. 2, 3, 4, and 5 and Electronic supplementary material, Table S 1).

All classes of *Proteobacteria* were present. The predominant γ-proteobacterial OTUs from a variety of environments were reported, as well as some that are closely related to cultured representatives of the genera *Providentia*, *Klebsiella*, *Schineria*, *Escherichia*, and *Enterobacter*. Other proteobacterial classes (α, β, δ, and ε) were represented by a small number of sequences (Figs. 2, 3, 4, and 5 and Electronic supplementary material, Table S 1).

The *Bacteroidetes* phylum was represented by ten OTUs, two of which belong to the genera *Empedobacter* and *Flavobacterium*. OTUs affiliated with the phyla *Actinobacteria* (two OTUs), *Thermotogae*, *Spirochaetes*, *Lentisphaerae*, *Acidobacteria*, *Chloroflexi*, *Deferribacteres*, *Cyanobacteria*, *Synergistetes*, and *Deinococcus-Thermus* (one OTU each) were all detected at low frequency (Figs. 2, 3, 4, and 5 and Electronic supplementary material, Table S 1).

Archaeal clone libraries diversity

Phylogenetic analysis of methanogenic archaeal (*mcrA* gene) and archaeal (16S rRNA gene) libraries (Figs. 7 and 8) revealed 33 and two distinct OTUs, respectively, that were assigned to the phylum *Euryarchaeota* (Fig. 9). Two hydrogenotrophic methanogenic orders, *Methanomicrobiales* and *Methanobacteriales*, are phylogenetically associated with this phylum. Most of the sequences had high identity to those of uncultured organisms obtained from biogas plants (Electronic supplementary material, Table S 2).

Twenty-one out of 33 OTUS from the methanogenic archaeal libraries were affiliated to *Methanomicrobiales*, and all of the sequences were closely related to uncultured representatives of the genus *Methanoculleus*, whereas the OT44-6 OTU was affiliated with the species *Methanoculleus bourgensis*. *Methanobacteriales* was represented by 11 OTUs closely related to uncultured representatives with sequences similar to the genera *Methanosphaera* and

Table 2 Sequence diversity and library coverage estimates

LC leachate, *OHW* organic household waste, *OT44* OHW treated for 44 days, *OT90* OHW treated for 90 days

[a] Chimeric sequences were included in the total number of clones, but then discarded from phylogenetic analysis.

Parameters	Bacteria				Archaea	mcrA		
	LC	OHW	OT44	OT90	LC	LC	OHW	OT44
Clones[a]	171	79	83	54	26	96	55	37
Chimera	13	4	4	6	0	0	0	0
OTUs	38	25	16	4	2	19	6	8
Shannon index diversity	3.036	2.450	2.140	2.06	0.92	2.25	1.03	1.27
Simpson index diversity	0.063	0.102	0.117	0.116	0.16	0.11	0.38	0.26
Chao1 estimator	73	33	19	15	8	19	9	9
ACE estimator	95	47	22	16	10	20	10	10
Good's coverage (%)	85.5	83	92.5	91	96	94	96	90

Methanobacterium. Two OTUs (LC8 and LC12) were closely related to the species *Methanosphaera stadtmanae* (Fig. 7 and Electronic supplementary material, Table S 2).

The A-LC2 OTU from the LC archaeal library was related to an uncultured archaeal clone (EU591668, no accession), with 91% identity to *Methanofollis formosanus*, as inferred by BLAST analysis. However, it was not grouped with the clade of *M. formosanus* by phylogenetic tree analysis and was not related to any of the five orders of methanogenic *Archaea* (Fig. 8). Thus, this archaeon may potentially be classified as a new order. Another OTU (A-LC1) identified in this library was closely related to an uncultured *Euryarchaeote* clone with 99% identity to *M. bourgensis* (Fig. 8 and Electronic supplementary material, Table S 2).

Species richness and diversity

Rarefaction curves were obtained by plotting the number of OTUs observed against the number of clones sequenced for each of the eight libraries (supplementaryElectronic supplementary material, Fig. S 3A, B). A decrease in the rate of OTU detection was observed on every curve, indicating that the majority of diversity in every library might have been detected. This result was further supported by calculating the coverage of the libraries, which was >83% (Table 2).

The Shannon–Weaver and Simpson diversity indices, ACE, and Chao1 estimator values obtained for the eight libraries showed that the *mcrA* and bacterial 16S rRNA gene libraries from the LC sample had the highest diversity among the libraries (Table 2).

Comparison among the bacterial clone libraries

We applied the LIBSHUFF algorithm to assess whether observed differences in the LC and OHW bacterial community composition were statistically significant (supplementary Electronic supplementary material, Fig. S 4A, B). Pairwise comparisons by LIBSHUFF revealed that the bacterial community composition of the OHW and LC libraries differed significantly (OHW $P=0.005$ and LC $P=0.002$ for each combination). More information on the differences between clone libraries was obtained by examining the distribution of $(C_X - C_{XY})^2$ as a function of evolutionary distance (D). The coverage curves for representative pairs of clone libraries clearly showed major differences at low levels of genetic distance ($D<0.2$). LIBSHUFF analysis also indicated that the LC library was characterized by a deeper divergence than the OHW library (Electronic supplementary material, Fig. S 4A, B).

Analysis using S-LIBSHUFF (Electronic supplementary material, Table S 5) to compare all bacterial libraries also showed a significant difference between the LC and OHW libraries ($P<0.003$), in accordance with the LIBSHUFF analysis, whereas the remaining matchings were not significantly different from each other ($P>0.08$).

Discussion

Work by several authors has revealed that the culture-independent approach provides insight into microbial community structure. In the present study, phylogenetic analysis of 16S rDNA and *mcrA* sequences was used to compare the microbial diversity of LC, OHW, and organic household waste treated at different stages in a mesophilic anaerobic reactor, detecting great bacterial diversity. To the authors' knowledge, this is the first phylogenetic evaluation of OHW and its degradation in an anaerobic reactor operating for 90 days.

Previous studies about microbial diversity in organic household waste anaerobic reactors have been reported (Tang et al. 2004; Levén et al. 2007; Weiss et al. 2008; Xian Qu et al. 2009). In our samples, the bacteria were associated to 13 phyla (Fig. 6). In other studies, using denaturing gradient gel electrophoresis (Weiss et al. 2008) and clone libraries construction followed by sequencing (Levén et al. 2007), the bacteria were associated with four and nine phyla, respectively. *Lentisphaerae*, *Acidobacteria*,

Fig. 2 Neighbor-joining tree deduced from bacterial 16S rRNA gene sequences of clone library of the LC sample. The *numbers at the nodes* indicate percentages of occurrence in 1,000 bootstrapped trees. *Halobacterium salinarum* (AJ496185) was used as an outgroup
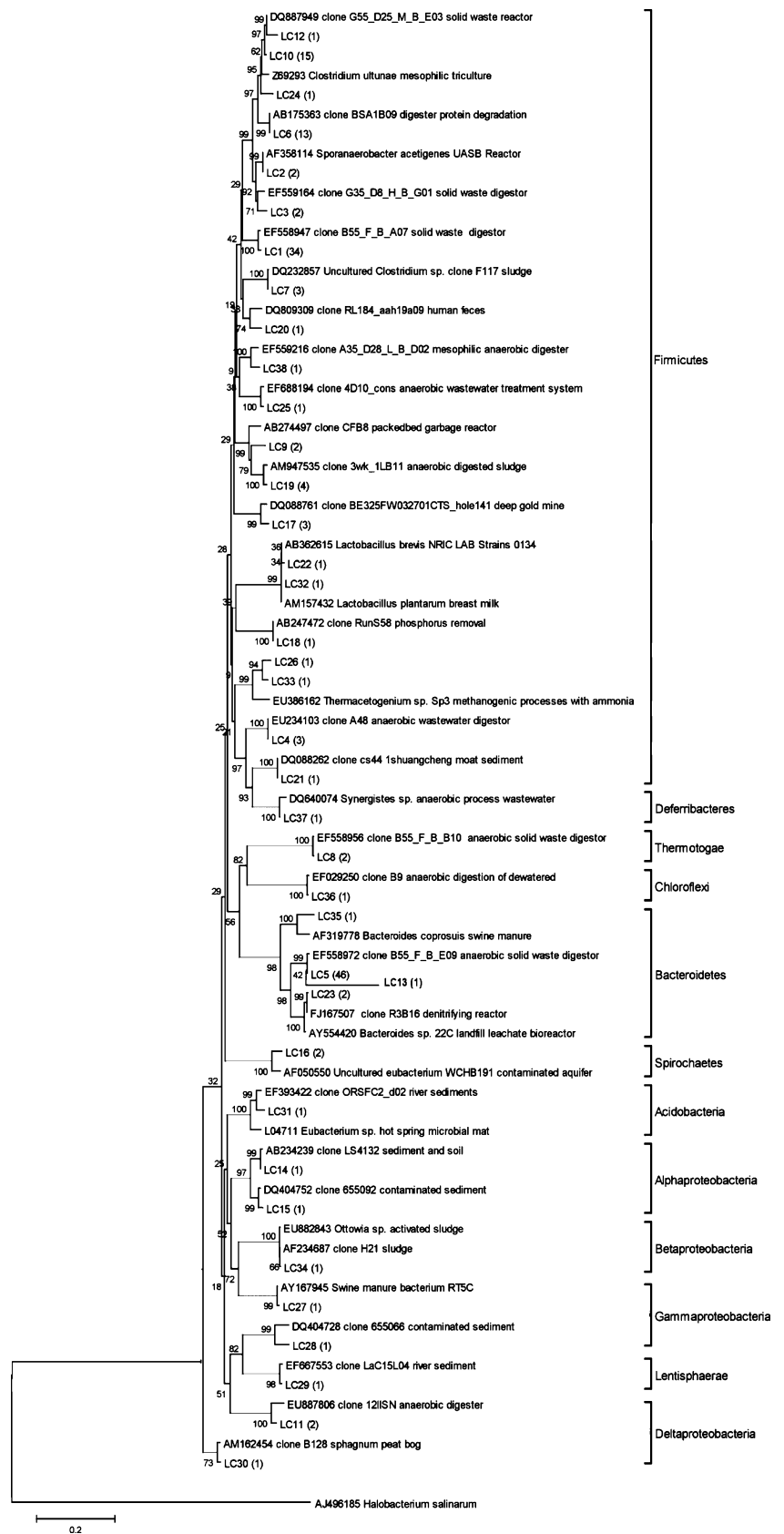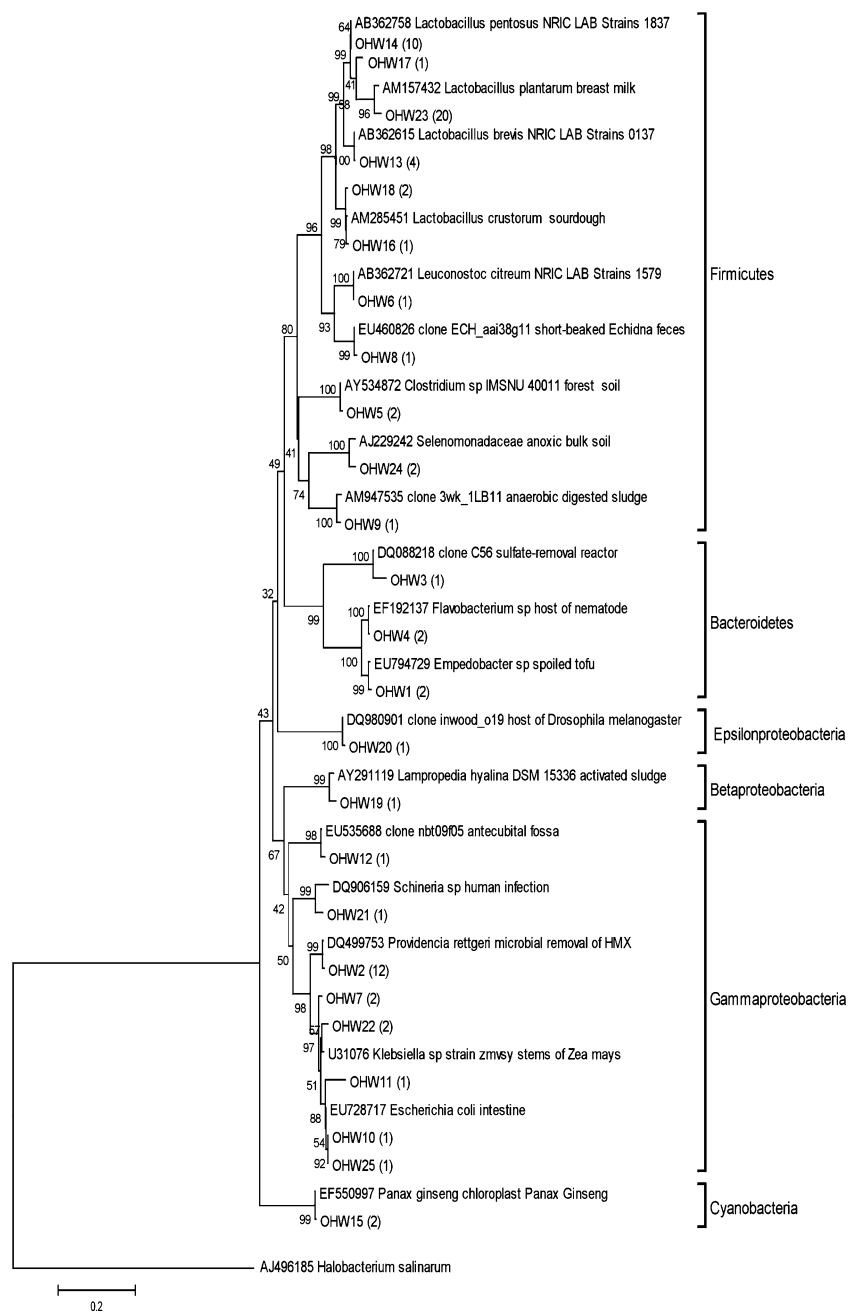
Fig. 3 Neighbor-joining tree deduced from bacterial 16S rRNA gene sequences of clone library of the OHW sample. The *numbers at the nodes* indicate percentages of occurrence in 1,000 boot-strapped trees. *H. salinarum* (AJ496185) was used as an outgroup
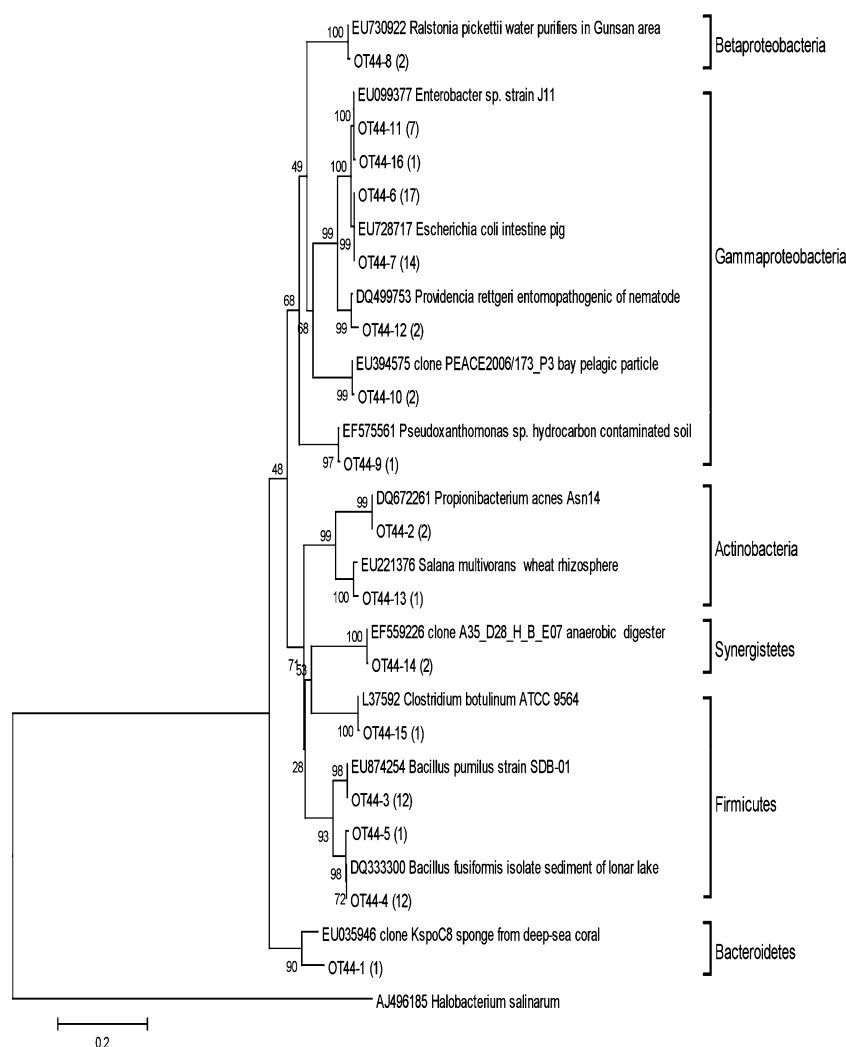

Deferribacteres, and *Deinococcus-Thermus* were detected in our analysis but were not observed in the analyses of Levén et al. (2007). In addition to these phyla, *Proteobacteria*, *Bacteriodetes*, *Chloroflexi*, *Cyanobacteria*, and *Spirochaetes* found in our study were not detected by Weiss et al. (2008). In contrast, the candidate divisions of OD1 and OP9 were detected only by Levén et al. (2007). On the other hand, *Methanosaeta* and *Methanosarcina*, which are common in anaerobic reactors (Levén et al. 2007; Weiss et al. 2008; Xian Qu et al. 2009), were not identified in our archaeal and methanogenic archaeal libraries (Fig. 7 and 8). The direct analysis of 16S rRNA and *mcrA* genes through a

PCR-based approach can potentially produce biased diversity estimations and underestimate the genetic diversity present in a given habitat (Moyer et al. 1994). Obviously, some factors such as the choice of primers and the proportion of 16S rRNA and *mcrA* gene sequences in a library may produce results that do not necessarily correspond to the actual proportion of these sequences in a natural community, but the qualitative complexity of a microbial community can be assessed (Suzuki et al. 1998).

In the bacterial 16S rDNA clone libraries, *Firmicutes* predominated in LC, OHW, and OT90 (Figs. 2, 3, and 5), whereas *Proteobacteria* occurred most frequently in the

Fig. 4 Neighbor-joining tree deduced from bacterial 16S rRNA gene sequences of clone library of the OT44 sample. The *numbers at the nodes* indicate percentages of occurrence in 1,000 bootstrapped trees. *H. salinarum* (AJ496185) was used as an outgroup

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        100 ┌ EU730922 Ralstonia pickettii water purifiers in Gunsan area    ] Betaproteobacteria
            └ OT44-8 (2)
                 100 ┌ EU099377 Enterobacter sp. strain J11
    49               └ OT44-11 (7)
                 100 ┌ OT44-16 (1)
                     ┌ OT44-6 (17)
                  99 ┌ EU728717 Escherichia coli intestine pig
                  99 └ OT44-7 (14)
    68               ┌ DQ499753 Providencia rettgeri entomopathogenic of nematode
    68            99 └ OT44-12 (2)                                             ] Gammaproteobacteria
                     ┌ EU394575 clone PEACE2006/173_P3 bay pelagic particle
                  99 └ OT44-10 (2)
                     ┌ EF575561 Pseudoxanthomonas sp. hydrocarbon contaminated soil
                  97 └ OT44-9 (1)
    48            99 ┌ DQ672261 Propionibacterium acnes Asn14
                  99 └ OT44-2 (2)                                              ] Actinobacteria
                     ┌ EU221376 Salana multivorans  wheat rhizosphere
                 100 └ OT44-13 (1)
              100 ┌ EF559226 clone A35_D28_H_B_E07 anaerobic  digester        ] Synergistetes
    75 50        └ OT44-14 (2)
                     ┌ L37592 Clostridium botulinum ATCC 9564
                 100 └ OT44-15 (1)
    28         98 ┌ EU874254 Bacillus pumilus strain SDB-01
                  └ OT44-3 (12)                                               ] Firmicutes
               93 ┌ OT44-5 (1)
               98 ┌ DQ333300 Bacillus fusiformis isolate sediment of lonar lake
               72 └ OT44-4 (12)
                  ┌ EU035946 clone KspoC8 sponge from deep-sea coral          ] Bacteroidetes
               90 └ OT44-1 (1)
    └ AJ496185 Halobacterium salinarum
    ├───┤ 0.2
```

OT44 library (Fig. 4). This result could be compared to previous works (Lozada et al. 2004; Tang et al. 2004; Figuerola and Erijman 2007) which suggested that *Firmicutes* and *Proteobacteria* could be the most dominant groups in anaerobic reactors. Classes *Clostridia* and *Bacilli* were the most abundant in our samples (Figs. 2, 3, 4, and 5 and Electronic supplementary material, Table S 1). *Bacilli* are Gram-positive lactic acid bacteria, tolerant to low pH, which convert carbohydrates into organic acids, mainly lactic acid (Filya 2003). *Clostridia* ferment carbohydrates and cellulose, hydrolyze proteins, and grow in association with some methanogenic *Archaea* (Wiegel et al. 2005; Krause et al. 2008). Although most OTUs of *Firmicutes* and *Proteobacteria* from LC, OHW, and OT90 clone libraries are distinct, representatives of these phyla take part in the same stages of organic matter anaerobic digestion (hydrolysis and fermentation).

Proteobacteria includes several bacterial species with diverse phenotypes, lifestyles, and trophic capabilities (Kersters et al. 2003). γ-*Proteobacteria* predominated among the different proteobacterial classes (Figs. 2, 3, 4, and 5). It is worth noting that some members of the β-*Proteobacteria* that are known to be responsible for fixing nitrogen and oxidizing ammonia produced in large quantities in anaerobic reactors (Bond et al. 1995) were found in small numbers in our samples (Figs. 2, 3, and 4).

Bacteroidetes, which are typically strictly anaerobic degraders, are ubiquitous in a variety of environments and have been observed in wastewater reactors (Figuerola and Erijman 2007) and an agricultural biogas reactor (Krause et al. 2008). *Deinococcus-Thermus*, which was detected in the OT90 sample (Fig. 5), is known to be able to reduce compounds such as iron and manganese, and low concentrations of these metals are required for methanogenic *Archaea* to produce methane (Slobodkin 2005; Lovley 1991). The phylum *Thermotogae*, which was identified in the LC library (Fig. 2) and encompasses carbohydrate-degrading thermophilic species, was also detected at very low frequency in the mesophilic temperature reactor by Levén et al (2007). Other phyla were specific to a single

Fig. 5 Neighbor-joining tree deduced from bacterial 16S rRNA gene sequences of clone library of the OT90 sample. The *numbers at the nodes* indicate percentages of occurrence in 1,000 bootstrapped trees. *H. salinarum* (AJ496185) was used as an outgroup
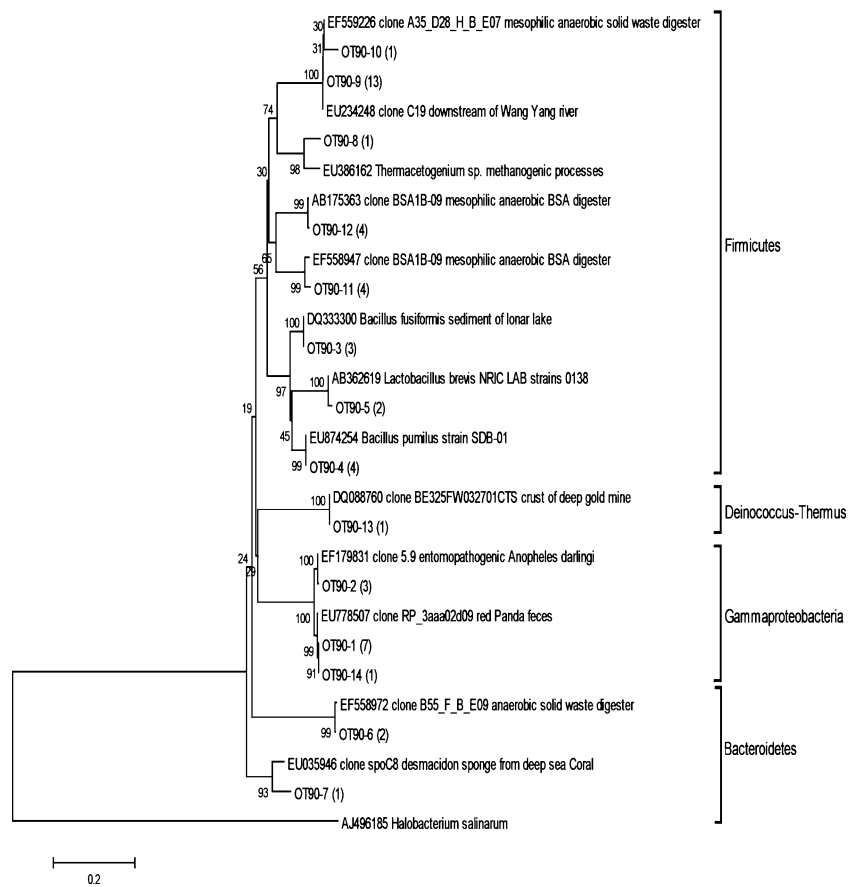

Fig. 6 Phylogenetic distribution of bacterial 16S rDNA sequences generated from LC (**a**), OHW (**b**), OT44 (**c**), and OT90 (**d**) samples
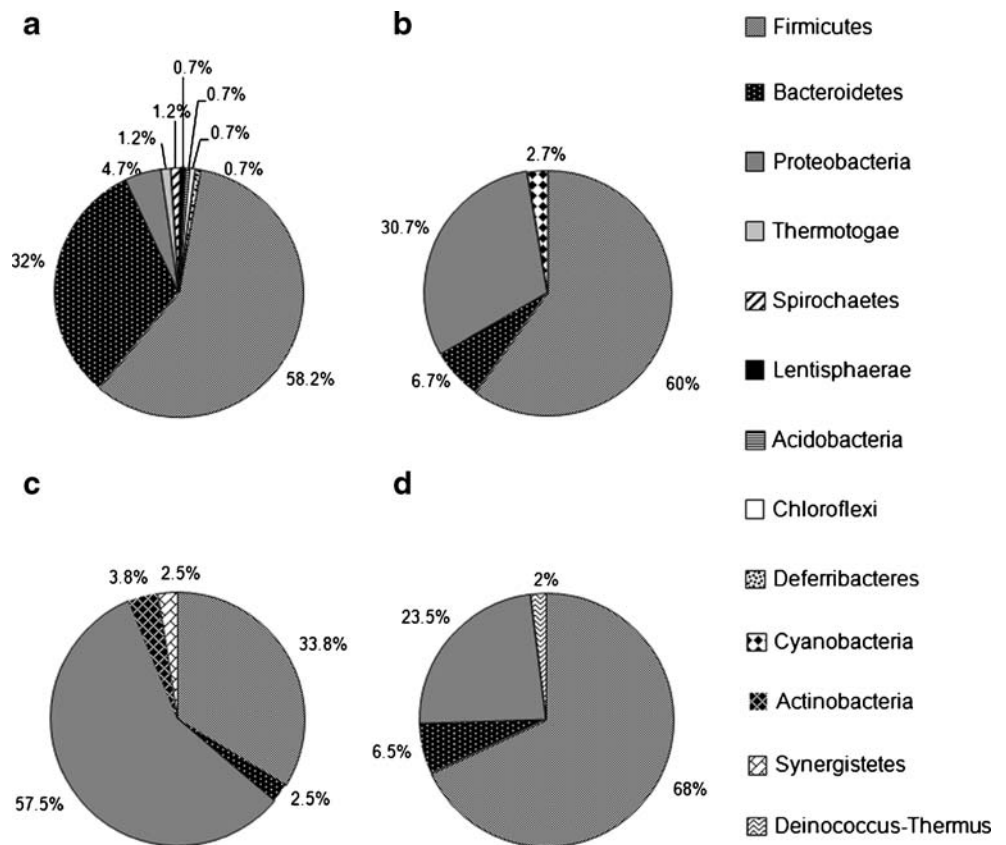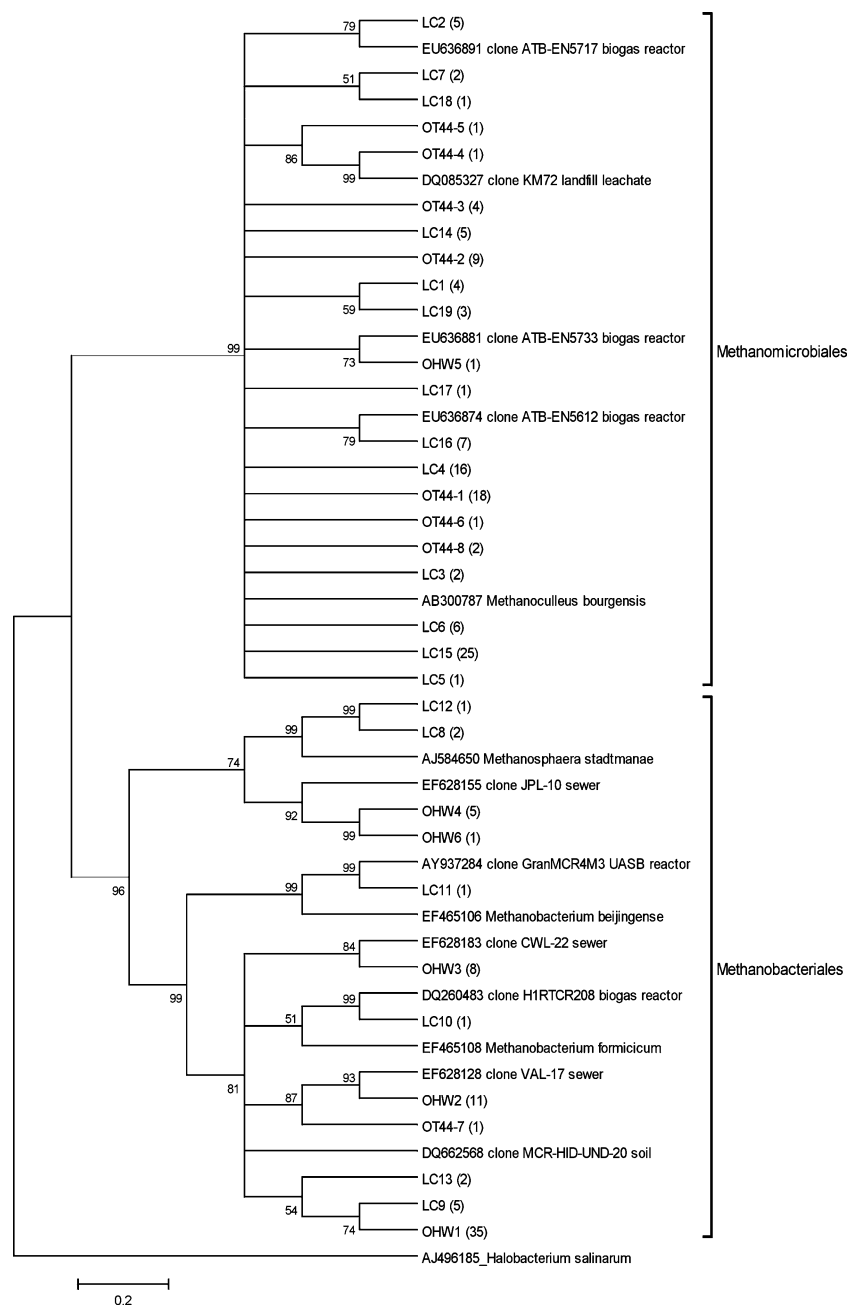
Fig. 7 Neighbor-joining tree deduced from methanogenic *Archaea mcrA* gene sequences of clone libraries of the LC, OHW, and OT44 samples. The *numbers at the nodes* indicate percentages of occurrence in 1,000 bootstrapped trees. *H. salinarum* (AJ496185) was used as an outgroup

library, such as *Cyanobacteria* (Fig. 3), *Deferribacteres*, *Spirochaete*, and *Chloroflexi* (Fig. 2), which were also identified in a household waste reactor by Levén et al. (2007). *Lentisphaera* and *Acidobacteria* (Fig. 2), which are found in acidic environments, have been identified in wastewater anaerobic reactors (Figuerola and Erijman 2007).

Phylogenetic analysis revealed greater diversity within the *mcrA* library than in the 16S rDNA library and showed that the archaeal clones were closely related to the hydrogenotrophic methanogens (Figs. 7 and 8). The differences in diversity within the mcrA and 16S rDNA libraries may be caused by an inadequately designed 16S rDNA primer

set for detection of some species, although the archaeal 16S rDNA primer set used is potentially able to amplify representatives from all archaeal groups. In a balanced anaerobic reactor, the majority of methanogenic *Archaea* present are acetotrophic methanogenic A*rchaea*, mainly *Methanosaeta* sp., and not hydrogenotrophic methanogenic *Archaea*, which are usually observed only in anaerobic reactors in an imbalanced condition. However, hydrogenotrophic methanogens appear to be important during the initial phase of waste treatment in anaerobic reactors (Montero et al. 2007). Identification only of this group was also reported in anaerobic reactors treating industrial

Fig. 8 Neighbor-joining tree deduced from archaeal 16S rRNA gene sequences of clone library of the LC sample. The *numbers at the nodes* indicate percentages of occurrence in 1,000 bootstrapped trees. *Methanopyrus kandleri* (U57340) was used as an outgroup
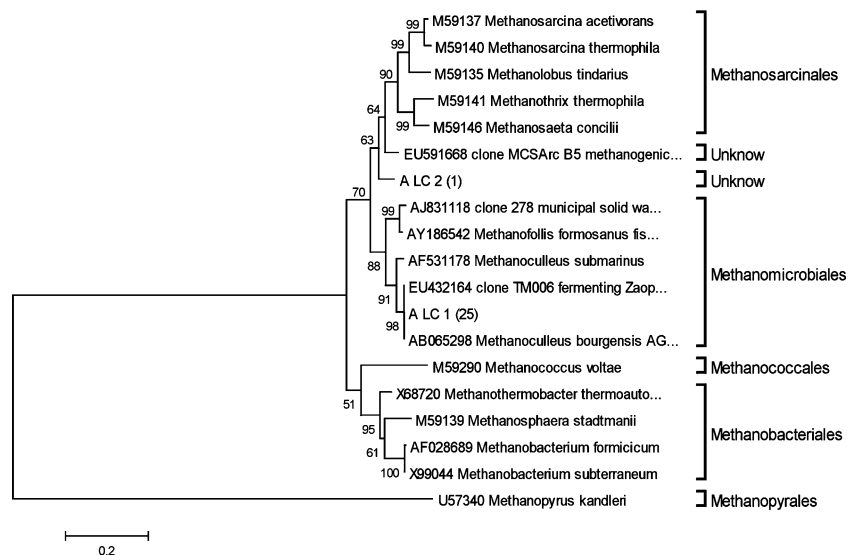
waste under imbalanced conditions (Schnurer et al. 1999). This result can probably be explained by an inhibition of aceticlastic methanogens due to high accumulation of ammonium (Karakashev et al. 2005) and volatile fatty acids from hydrolysis of long-chain fatty acids and subsequent production of methane by a less sensitive hydrogenotrophic mechanism (Schnurer et al. 1999; Lalman and Bagley 2001). According to Speece (1983), aceticlastic and hydrogenotrophic methanogenic *Archaea* contribute with 70% and 30%, respectively, of methane production in industrial wastewater

Fig. 9 Phylogenetic distribution of archaeal sequences generated from *mcrA* gene LC library (**a**), 16S rRNA gene archaeal LC library (**b**), *mcrA* gene OHW library (**c**), *mcrA* gene OT44 library (**d**)

treatment. However, Angenent et al. (2002) reported that the major route of methane production is through a syntrophic relationship between acetate-oxidizing bacteria and hydrogenotrophic methanogens. Some members of the genera *Thermacetogenium* and *Clostridium*, which were identified in our libraries, are syntrophic bacteria and oxidize acetate in co-culture with hydrogenotrophic methanogens, as observed by Schnuërer et al. (1996) and Hattori et al. (2001).

In this study, the identification of *Methanobacterium*, *Methanosphaera*, and *Methanoculleus*, which were detected in great number in our libraries (Figs. 7 and 8), agrees with other community analyses in anaerobic reactors (Leclerc et al. 2004; Tang et al. 2004), suggesting that these microorganisms were responsible for hydrogenotrophic methanogenesis in such systems. The absence of acetotrophic methanogens could probably be caused by acid (acetate, propionate, and butyrate) and ammonium concentrations in the reactor that were above the threshold level for acetotrophic methanogen growth. Furthermore, the genera *Methanoculleus*, *Methanosphaera*, and *Methanobacterium* might compete for acetate with acetotrophic methanogens, given that hydrogenotrophic methanogens require acetate as their sole energy source for biomass production, despite not catabolizing acetate into methane (Boone et al. 2001).

The diversity indices (Table 2) indicated a high diversity level in the bacterial and methanogenic archaeal communities represented by the LC library and are in accordance with the rarefaction curves (Electronic supplementary material, Fig. S 3A, B). The results suggest that the number of analyzed clones encompassed the majority of the bacterial and archaeal diversity present in every sample. The S-LIBSHUFF and LIBSHUFF analyses showed a deeper divergence between the OHW and LC libraries. Unlike these results, according to the S-LIBSHUFF comparisons, these
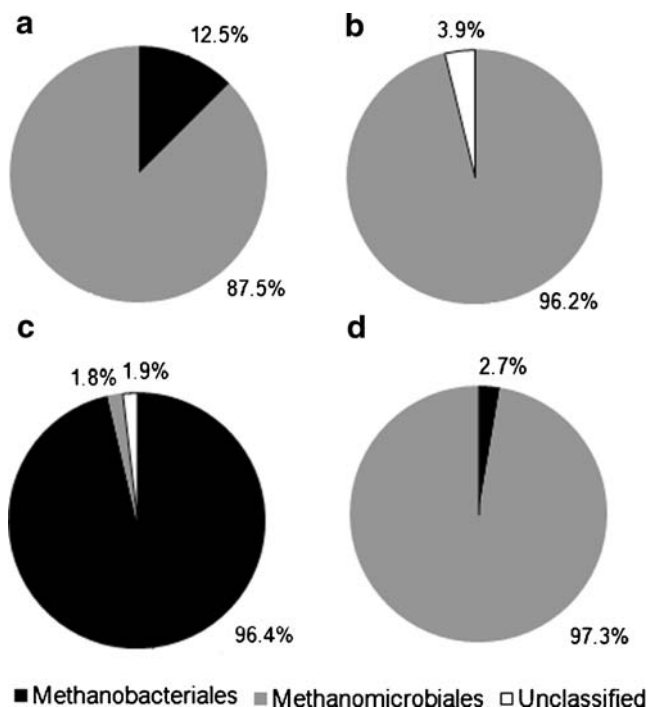
libraries did not show significant *P* values for the OT44 and OT90 samples nor between libraries OT44 and OT90, since the bacterial communities of the OT44 and OT90 samples were obtained from LC and OHW initial samples (Electronic supplementary material, Fig. S4A, B and Table S 5).

The microbial community changed during anaerobic digestion, and the results suggest that the production of methane was carried out mainly using CO_2 as electron acceptor and hydrogen as electron donor via the hydrogenotrophic pathway. The fact that aceticlastic methanogens could not be identified, the low abundance of methanogens, the low percentage of methane in the biogas produced, and the low organic matter degradation in the anaerobic reactor treating OHW suggest that this reactor could have been under imbalanced conditions, which might have been caused by its being fed only at treatment start-up, as occurs in batch reactors.

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