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Effect of Biowaste Sludge Maturation on the Diversity of Thermophilic Bacteria and Archaea in an Anaerobic Reactor[▽]

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Prokaryotic diversity was investigated near the inlet and outlet of a plug-flow reactor. After analyzing 800 clones, 50 bacterial and 3 archaeal phylogenetic groups were defined. *Clostridia* (>92%) dominated among bacteria and *Methanoculleus* (>90%) among archaea. Significant changes in pH and volatile fatty acids did not invoke a major shift in the phylogenetic groups. We suggest that the environmental filter imposed by the saline conditions (20 g liter^{-1}) selected a stable community of halotolerant and halophilic prokaryotes.

The anaerobic digestion of organic wastes constitutes a major research focus due to the global needs for waste recycling and renewable energy production. Currently, the linkage between digester performance and the diversity and dynamics of anaerobic prokaryotes is still not fully understood (2). Bacterial diversity in anaerobic reactors has always been judged to be greater than archaeal diversity (9, 13, 30). This probably reflects the metabolic flexibility of bacteria and the range of available substrates in complex input materials. However, several recent discoveries pose the question as to whether archaeal diversity and physiological versatility are greater than currently thought: that is, the huge diversity of yet-to-be cultured archaea (4, 6), the detection of energy metabolisms not known previously in archaea (e.g., chemoorganotrophy [1]), and the unexpected predominance of archaeal groups among prokaryotes in unstressed environments, such as ammonia oxidizers in soils (19).

Several surveys have investigated the shifts in prokaryotic diversity occurring with waste maturation or under different reactor operating conditions. Some evidence demonstrates bacterial phylogenetic stability under constant operation conditions (18). Generally, however, the dominant bacterial communities are very dynamic, showing chaotic shifts even with stable reactor performance (9, 32). Hypothetically, this is due to the functional redundancy among diverse phylogenetic groups allowing oscillations of their populations with no effects on the reactor function (2). Archaeal communities are less dynamic than bacterial communities (32), their shifts being related to changes in reactor performance (6) and correlated with important process parameters such as volatile fatty acids (VFAs) (13, 16).

We aimed to analyze the change in prokaryotic diversity in a plug-flow reactor associated with the maturation of biowastes. In a previous study, stable bacterial and archaeal denaturing gradient gel electrophoresis patterns were found in the sludge collected close to the outlet over a year of unstable reactor performance (23). This temporal pattern contradicts the general idea of extremely dynamic bacterial communities proliferating in bioreactors. Here, we investigated the phylogenetic identity of the organisms in sludge samples collected near the inlet and outlet pipes after a period of stable operation and performance in terms of pH and biogas production.

Operation of the bioreactor and sampling of sludges. Samples were taken from the thermophilic (50 to 55°C) anaerobic plug-flow reactor located in Roppen (Tirol, Austria), which has a capacity of 750,000 liters. At the time of sampling (October 2006), the reactor had been operating stably for several years. That is, it had been fed daily with 20 to 25 Mg biowaste (i.e., source-separated organic household waste mixed with garden residues in a season-dependent ratio) with continuous radial stirring and a directional axial flow from the inlet to the outlet, with no backward flow possible, and a 3-week hydraulic retention time. The biogas production had been stable for several months, averaging 120 m^3 h^{-1}, with 62% CH_4 and 38% CO_2. Fresh and mature sludges were collected from two 1-m-depth sampling ports located on top of the reactor near the inlet and outlet pipes (ca. 30 m away from each other). Five 1-liter samples were collected per sampling port.

Physical and chemical sludge properties. Table 1 shows the physical and chemical parameters of fresh and mature sludges. The C and N contents of oven-dried (60°C) ground sludge samples were measured in an automated CNHS analyzer (TruSpec; LECO). VFAs were analyzed by high-pressure liquid chromatography, after injection of 20 μl of solution (i.e., supernatant after centrifuging 2 ml of 1:4 sludge-water suspensions) into a Shimadzu LC-20A *prominence* high-pressure liquid chromatograph. VFAs were separated with an Aminex HPX-87H column (300 by 7.8 mm) and detected photometrically (220 nm).

The fresh and mature sludges differed significantly in their main chemical properties (Table 1). The high content of organic substances in the fresh sludge resulted in the production of a large amount of short-chain fatty acids. This explains the significantly lower pH and higher electrical conductivity of the fresh sludge compared with the mature sludge. As expected, the recalcitrance of the organic matter in the biowastes increased together with the process of anaerobic digestion, as indicated by the decrease in both the C/N ratio and the levels of VFAs, which dropped from 22.5 mM in the fresh sludge to

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TABLE 1. Physical and chemical properties of the fresh and mature sludges

Parameter	Result for sludge[a]	
	Fresh	Mature
Total solids (%)	29.1 (1.0)	27.1 (1.8)
Volatile solids (%)	57.3 (2.2)	54.8 (2.2)
pH at 1:5 (wt/vol)	7.93 (0.05)	8.65 (0.03)*
Electrical conductivity at 1:10 wt/vol (mS cm^{-1})	3.97 (0.05)*	3.75 (0.10)
C (%)	30.07 (2.50)*	24.72 (1.50)
N (%)	2.00 (0.08)	1.92 (0.11)
C/N ratio	15.0 (0.5)*	12.9 (0.2)
Acetate (mM)	10.02 (1.01)*	1.01 (0.20)
Propionate (mM)	7.16 (0.73)	6.69 (0.49)
Butyrate (mM)	3.31 (0.40)*	0.06 (0.04)
i-Valeriate (mM)	1.03 (0.11)*	0.52 (0.10)
i-Butyrate (mM)	0.57 (0.03)*	0.28 (0.03)
Valeriate (mM)	0.37 (0.05)	0.35 (0.24)

[a] Standard deviations are given in parentheses ($n = 5$). Asterisks indicate significant differences at $P < 0.001$ after Student's *t* test or multivariate one-way analysis of variance (for VFAs) with SPSS 15.0.

8.9 mM in the mature sludge. All VFAs underwent a significant decrease during the digestion, except for propionate, which remained at high and constant levels, and valeriate, which was present at low levels in both sludges.

Biogas production from the fresh and mature sludges. Biogas production was measured in a batch incubation assay. Within 5 h of sampling, 2.5 g sludge was transferred to 250-ml Schott bottles flushed with 100% N_2 and incubated in the dark at 50°C with continuous stirring. The pressure within the test bottles was measured for 17 days using a digital manometric system (Sensomat; Aqualytic, Germany). Nonlinear regression analysis was used to estimate the kinetic parameters of biogas production using SigmaPlot v9.01.

The reduction in substrate concentration during the digestion invoked a decrease in the rate of biogas production. After 17 days of incubation, biogas production decreased from 19.2 ml biogas g^{-1} fresh sludge to 5.6 ml biogas g^{-1} mature sludge (wet weight). Both curves fitted well ($R^2 = 0.995$) to a first-order kinetic equation, and the rate constant in the fresh sludge was twofold that of the mature sludge (0.18 and 0.09 day^{-1}).

DNA extraction and PCR amplification. DNA was extracted from 0.25 g sludge within 5 h of sampling using the PowerSoil DNA isolation kit (MO BIO Laboratories). DNA was subjected to PCR amplification of the 16S rRNA genes with universal bacterial and archaeal primers (Table 2). PCR amplifications were performed in a Thermo Hybaid PCR Express Thermalcycler (Fisher Scientific) in 25-μl volumes, with each reaction mixture containing 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]) (Applied Biosystems), 200 μM each deoxynucleoside triphosphate (dNTP) (Genxpress, Austria), 0.2 μM each primer, 1× enhancer (Peqlab, Germany), 0.4 mg ml^{-1} bovine serum albumin, 10 mM tetramethylammonium chloride, 1.25 mM $MgCl_2$, 0.63 U AmpliTaq Gold DNA polymerase LD (Applied Biosystems), and sterile water. Two microliters of extracted DNA (diluted 1/10 for archaea and 1/50 for bacteria) was applied to the PCR mix. Bacterial DNA was amplified with an initial denaturation at 95°C for 5 min, 25 amplification cycles (45 s at 95°C, 45 s at 52°C, and 4 min at 72°C), and a final elongation at 72°C for 10 min. Archaeal DNA was amplified as described for bacteria, but amplification cycles consisted of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C. PCR products obtained for the five independent replicates were pooled and purified with the GenElute PCR clean-up kit (Sigma).

Clone library construction and analysis. A total of four clone libraries were constructed from the DNA extracted from the fresh (F) and mature (M) sludges. DNA amplified with both the bacterial (-B) and archaeal (-A) primers was sub-

TABLE 2. Primers used in this study

Target	Primer	Sequence (5′ → 3′)	*E. coli* position	Reference
Bacteria	8F	AGAGTTTGATYMTGGCTC	8–27	21
	1492R	GGYTACCTTGTTACGACTT	1492–1507	
Archaea	109F	ACKGCTCAGTAACACGT	109–125	12
	934R	GTGCTCCCCCGCCAATTCCT	915–934	
Methanoculleus[a]	298F	GGAGCAAGAGCCCGGAGT	298–315	10
	586R	CCAAGAGACTTAACAACCCA	586–606	
Methanothermobacter[a]	410F	CTCTTAACGGGGTGGCTTTT	410–440	10
	667R	CCCTGGGAGTACCTCCAGC	667–686	
Uncultured group[a]	195F	AAAACTCCGGTGCCTTAGGATT	195–230	10
	330R	CCCGTAGGGCCTGGACTCA	330–348	
Methanosaeta[a]	MS1b	CCGGCCGGATAAGTCTCTTGA	585–606	28
	SAE835R	GACAACGGTCGCACCGTGGCC	835–851	
Methanosarcina[a]	240F	CCTATCAGGTAGTAGTGGGTGTAAT	240–264	10
	589R	CCCGGAGGACTGACCAAA	589–606	

[a] Source DNA for preparing standards: clone 2FA36 (AM947508), *Methanoculleus bourgensis*; DSM 2970, *Methanothermobacter wolfeii*; clone 1FA28 (AM947509), uncultured archaea; DSM 2139, *Methanosaeta concilii*; DSM 800, *Methanosarcina barkeri*. All pure cultures from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

TABLE 3. Distribution of clones in the fresh and mature sludge bacterial clone libraries

Putative division	No. of clones (no. of phylotypes)		Closest cultured species or environmental clone (accession no.)[a]	% Sequence identity
	F-B	M-B		
Firmicutes				
Clostridiales	69 (9)	82 (15)	Clone TIBH10 (DQ887962.1)	90–99
	1 (1)	11 (3)	Clone MBA02 (AB114312.1)	98–99
	19 (6)	12 (5)	Clone GMB4D08 (DQ887933.1)	90–99
	0	4 (3)	Clone GMB4H01 (DQ887946.1)	88–97
	2 (1)	2 (2)	Clone MRE50b01 (AY684075.1)	92
	9 (2)	10 (4)	Clone GMB4C10 (DQ887942.1)	98
	2 (1)	7 (4)	Clone TIBA10 (DQ887956.1)	93–98
	11 (4)	4 (4)	*Carboxydocella ferrireducens* (EF092457.1)	83–87
	4 (3)	1 (1)	*Catabacter hongkongensis* (AY574991.1)	86–88
	9 (2)	6 (2)	*Tepidimicrobium quinonicus* (AY656718.1)	93–94
	6 (2)	6 (3)	*Clostridium cellulolyticum* (X71847.1)	91
	6 (1)	1 (1)	*Clostridium piliforme* (D14639.1)	86
	1 (1)	0	*Clostridium stercorarium* (AJ310082.1)	90
	0	2 (1)	*Clostridium ultunense* (Z69293.1)	94
	0	1 (1)	*Syntrophomonas erecta* (AY536889.1)	90
	0	1 (1)	*Syntrophomonas wolfei* (M26492.1)	94
	0	1 (1)	*Pelotomaculum isophthalicicum* (AB232785.1)	94
Halanaerobiales	40 (6)	30 (9)	*Halocella cellulosilytica* (X89072.1)	91–92
Bacillales	9 (3)	4 (4)	*Geobacillus caldoxylosilyticus* (AF067651.1)	82–87
Thermotogae	3 (2)	1 (1)	*Petrotoga mobilis* (Y15479.1)	77–91
Bacteroidetes	2 (2)	2 (2)	*Bacteroides uniformis* (AB050110.1)	84
	0	1 (1)	Clone GMB4C12 (DQ887931.1)	99

[a] Environmental clones are given for sequences not closely related to any cultured species.

jected to cloning with the TOPO TA cloning kit using One Shot TOP10 competent cells (Invitrogen). To analyze the clones for inserts, thermal cycling was performed in 20-μl volumes, with each reaction mixture containing 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.01% Tween 20], 200 μM each dNTP, 0.2 μM T3 (5′-ATTAACCCTCACTAAAGGGA) and T7 (5′-TAATAC GACTCACTATAGGG) primers, 0.45 U BioTherm DNA polymerase (GeneCraft, Germany), and sterile water. A small amount of the colony was directly added to the PCR mix. The initial denaturation (94°C, 5 min) was followed by 30 cycles of amplification (50 s at 94°C, 50 s at 50°C, and 2 min at 72°C) and a final extension (10 min at 72°C).

DNA from insert-containing clones was analyzed by restriction digestion analysis. Ten microliters of PCR product was digested in a 10-μl reaction mixture containing 0.5 U HaeIII, 1× Tango buffer (Fermentas, Germany), and sterile water. Products digested at 37°C for 3 h were separated in 2.5% agarose gels (1× Tris-borate-EDTA buffer, 50 V, 60 min) and stained (0.1% [vol/vol] ethidium bromide). Analysis of the restriction digestion patterns was carried out with GelCompar 3.1 (Applied Maths, Belgium). Ward's method was applied to calculate dendrograms, after which the phylotypes (i.e., clones with the same restriction digestion patterns) were defined.

Sequencing and phylogenetic analysis. DNA from at least one representative of each phylotype was amplified as described above. The DNA was purified with the NucleoSpin Extract II kit (Macherey-Nagel, Germany), and forward and reverse sequencing reactions were performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). PCR products were analyzed using a 3130 Genetic Analyzer (Applied Biosystems) at the BioSeq facilities of the University of Innsbruck, Innsbruck, Austria. Full-length sequences were NAST-aligned with Greengenes (7) and checked for anomalies using Bellephoron (15). The phylogenetic affiliation of error-free sequences was determined with the ARB software package (22). Neighbor-joining trees of nearly complete 16S rRNA gene bacterial and partial 16S rRNA gene archaeal sequences and their corresponding similarity matrices were generated by the neighbor-joining distance method. Operational taxonomic units (OTUs) were defined as phylotypes having 16S rRNA gene sequences with ≥97% identity. Bootstrap values were calculated based on 1,000 replications.

Quantitative RT-PCR. Sludge DNA was subjected to real-time PCR (RT-PCR) amplification with specific primers for several groups of methanogens (Table 2) as described in reference 10. Amplifications were conducted using the Quantimix Easy SYG kit (Biotools, Spain) in a Rotor-Gene 6000 (Corbett Life Sciences, Australia) in 20-μl volumes including 2 μl sludge DNA (1/70 diluted). Standard curves were constructed with PCR-amplified 16S rRNA genes using the source DNA and methanogen-specific primers in Table 2. Spiking experiments were performed to check for PCR efficiency reduction due to the presence of inhibitors in the sludge matrix. The amplification efficiency of the unspiked standard curves was similar to that of the standard curves spiked with sludge DNA (data not

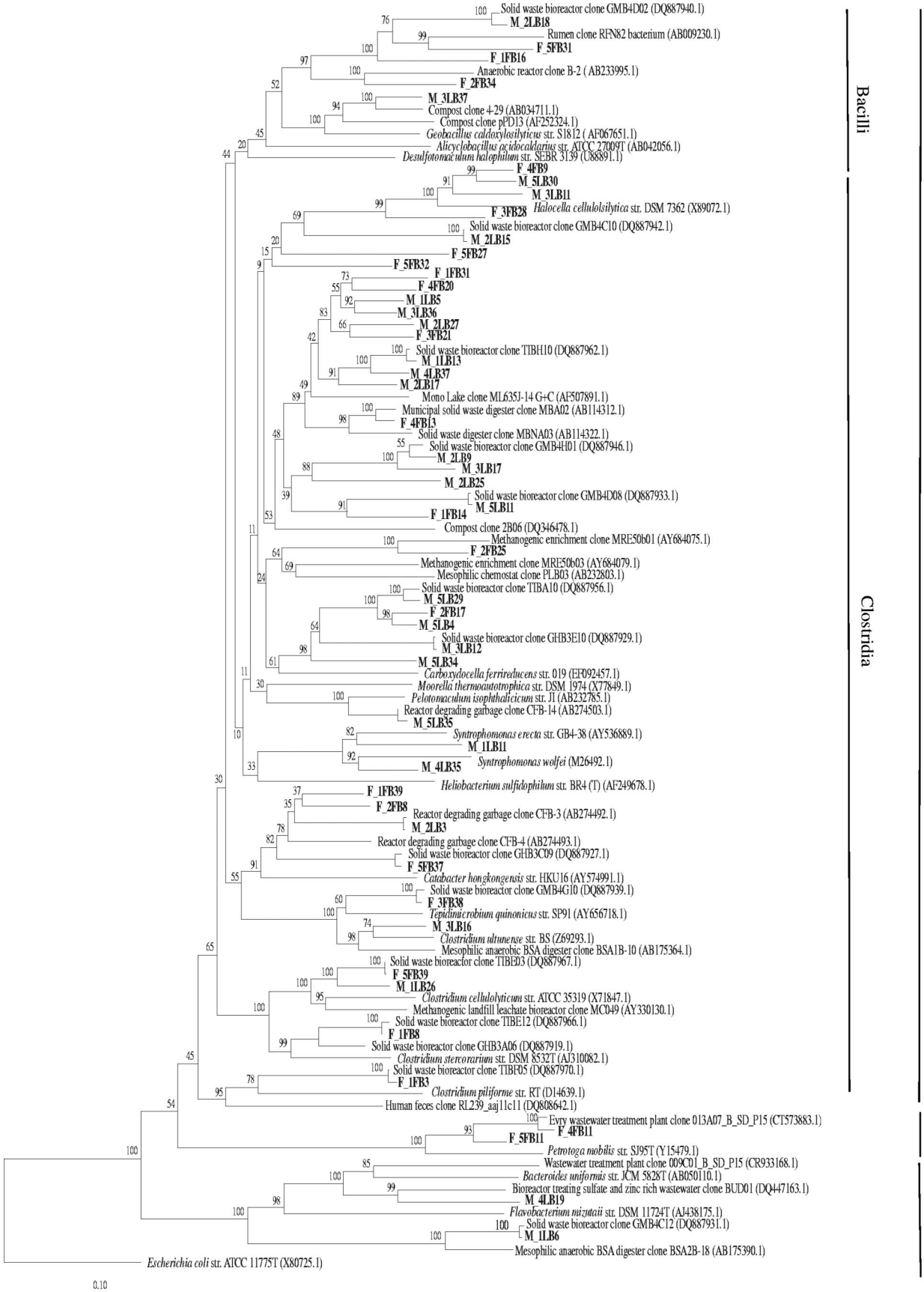

FIG. 1. Neighbor-joining tree of partial 16S rRNA gene sequences depicting the phylogenetic relationships of 16S rRNA gene bacterial clones from fresh (F) and mature (M) sludge DNA. Numbers at nodes represent bootstrap values based on 1,000 replications. *Escherichia coli* was used as the outgroup of the tree. Sequences from this study are shown in bold.

TABLE 4. Distribution of clones in the fresh and mature sludge archaeal clone libraries

Putative division	No. of clones (no. of phylotypes)		Closest cultured species or environmental clone (accession no.)[a]	% Sequence identity
	F-A	M-A		
Methanomicrobiales	177 (2)	201 (3)	*Methanoculleus bourgensis* (AY196674)	96–98
Methanobacteriales	1 (1)	0	*Methanothermobacter thermautotrophicus* (EF100758)	98
Uncertain	17 (1)	0	Clone GZK24 (AJ576219)	99

[a] Environmental clones are given for sequences not closely related to any cultured species.

shown). Thus, the effect of inhibitors in the sludge DNA was considered to be negligible.

Bacterial community structure. Bacterial clone libraries were constructed from the fresh and mature sludges (F-B and M-B), and 400 clones were screened. The number of phylotypes indicated a higher bacterial diversity in the mature sludge, with 46 phylotypes detected in F-B and 70 in M-B (Table 3). However, the numbers of OTUs were similar: 31 and 37 OTUs were defined in F-B and M-B, respectively, 18 of which were common to both libraries. *Firmicutes* (97.7%), *Bacteroidetes* (1.3%) and *Thermotogae* (1.0%) were the bacterial phyla detected in the bioreactor (Fig. 1). The same phyla have been reported to dominate a lab-scale thermophilic reactor fed with organic household waste (20).

The class *Clostridia* dominated both clone libraries (92.7% in F-B and 95.8% in M-B), a finding reported previously for cellulolytic environments (5). Among these, clones being most closely related to several groups of uncultured *Clostridia* were the most abundant (52.8% and 67.7% in F-B and M-B). Some of these uncultured *Clostridia* have been previously detected in thermophilic anaerobic bioreactors (8). The ecophysiological function of these organisms is not certain, but often *Clostridium* spp. show hydrolytic fermentative (5) and acetate-oxidizing activity (17). Also abundant in both clone libraries were *Clostridia* most closely related to *Halocella cellulosilytica* (20.7% and 15.9% in F-B and M-B), a halophilic cellulolytic organism (29). Therefore, halophilic and halotolerant bacteria able to thrive at the high salt concentrations prevailing in the fermenter (20 g liter^{-1}), particularly those of ammonium (2.7 g liter^{-1}), dominated the reactor ecosystem.

It can be concluded that bacterial diversity, based on the number of phylotypes in the clone libraries, increased with the maturation of wastes, but phylogenetically the dominant communities remained mostly unchanged during the process of anaerobic digestion. Presumably, the environmental filter imposed by the saline environment in the biowaste-degrading fermenter selected a phylogenetically stable community dominated by groups physiologically adapted to osmotic stress. Under such conditions, the significant changes detected in other chemical parameters, such as pH or VFAs, did not provoke a major shift in the phylogenetic groups of bacteria. The selective filter imposed by the saline environment would also explain the constant denaturing gradient gel electrophoresis patterns detected in the mature sludge of the same reactor over a whole year of unstable performance (23). Our results contradict previous reports describing bacterial populations as very dynamic under pH-changing conditions (13) as well as in stably performing reactors (9). However, in those studies, lab-scale reactors fed with synthetic wastewater containing glucose as the only carbon and energy source were used, eliminating the possible influence of ammonia on the dynamics.

Finally, a set of singletons appeared in the mature sludge (M-B) that were closely related to syntrophic bacteria, namely, *Clostridium ultunense* (26), *Syntrophomonas* sp. (24, 31), and *Pelotomaculum* sp. (25). No syntrophs were detected in the fresh sludge, whereas these constituted 2.5% of the bacterial

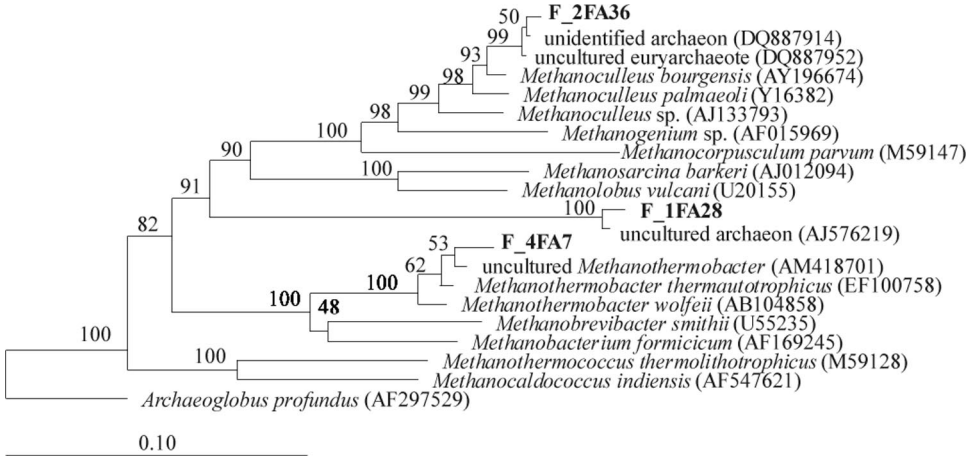

FIG. 2. Neighbor-joining tree of partial 16S rRNA gene sequences depicting the phylogenetic relationships of 16S rRNA gene archaeal clones from fresh (F) and mature (M) sludge DNA. Numbers at nodes represent bootstrap values based on 1,000 replications. *Archaeoglobus profundus* was used as the outgroup of the tree. Sequences from this study are shown in bold.

TABLE 5. 16S rRNA gene copy numbers for the groups of methanogens quantified by RT-PCR in the fresh and mature sludges

Target	16S rRNA gene copy no. (copies g^{-1} sludge) in[a]:	
	Fresh sludge	Mature sludge
Methanoculleus	3.9×10^8 (1.2×10^8)	9.0×10^8 (2.2×10^8)*
Uncultured group	5.7×10^6 (1.3×10^6)*	2.3×10^6 (4.3×10^5)
Methanothermobacter	1.6×10^6 (6.0×10^5)	1.4×10^6 (7.4×10^5)
Methanosaeta	ND	ND
Methanosarcina	ND	ND

[a] Standard deviations are given in parentheses ($n = 5$). Asterisks indicate significant differences at $P < 0.05$, after Student's t test with SPSS 15.0. ND, not detected (<600 gene copies g^{-1} sludge).

community in the mature sludge. Interestingly, no propionate-oxidizing bacteria were detected, and, indeed propionate concentrations did not decrease during the process; thus, propionate was presumably not degraded. It is possible that the anaerobic digestion period was too short to allow the proliferation of propionate degraders (5). Alternatively, high ammonia levels could have inhibited the growth of these syntrophs (3).

Archaeal community structure. Two archaeal clone libraries were constructed from the fresh and mature sludges (F-A and M-A). After screening 400 clones, four phylotypes were detected in F-A and three in M-A. These corresponded to three OTUs in F-A, of which only one persisted in M-A. This low archaeal diversity has been previously reported in thermophilic reactors (13, 30). Both clone libraries were dominated by organisms related to *Methanoculleus bourgensis* (Table 4 and Fig. 2), a hydrogenotrophic methanogen (11). Its frequency increased from 90.8% in F-A to 100% in M-A. In F-A, other clones appeared that were most closely related to clone GZK24, detected in the leachate of a municipal solid waste landfill (14), or to the hydrogenotroph *Methanothermobacter thermautotrophicus* (11). Therefore, there was a decrease in the archaeal clonal diversity following maturation of the biowastes, with a total disappearance of methanogens other than *Methanoculleus* sp.

RT-PCR confirmed the dominance of *Methanoculleus* (>98%) and the significant increase of its abundance with the maturation of wastes (Table 5). However, the group of uncultured archaeons and *Methanothermobacter* could be amplified from both sludge types. The lack of detection of these organisms in the clone library constructed from the mature sludge could be due to the low probability of sampling them randomly among all PCR-generated products because of their low abundance.

RT-PCR also confirmed the absence in the samples of the two genera of known acetotrophic methanogens (*Methanosarcina* and *Methanosaeta*). This indicates that the syntrophic oxidation of acetate was the only pathway for methanogenesis from biowastes. Acetate concentrations above 1 mM, as was the case here, should be enough to support an acetoclastic community of methanogens (13). On the other hand, VFAs in the reactor were not above the reported inhibitory concentrations for acetotrophic methanogens (27). Therefore, the most plausible explanation for the inhibition of the acetotrophic pathway is the high concentration of salts, particularly those of ammonium (2.7 g $liter^{-1}$) (27). Also the prevalence of *Methanoculleus* over other hydrogenotrophic methanogens might be related to its tolerance to high salt concentrations (27).

In conclusion, the prokaryotic phylogenetic groups in the anaerobic sludge of a thermophilic plug-flow reactor remained relatively unaltered despite changes in the environmental conditions concurrent with the maturation of the biowastes. We demonstrated that biogas production was based on syntrophic acetate oxidation by a community of organisms able to thrive at the high salt levels prevailing in the reactor. This environmental factor presumably determined the minor shift in the phylogenetic groups between the fresh and mature sludges, despite significant changes in pH and VFAs, which have been shown to be influential for anaerobic community dynamics in previous studies. Whether the saline conditions impose a selective filter that determines phylogenetic stability in anaerobic reactors should be further investigated.

Nucleotide sequence accession numbers. The EMBL Nucleotide Sequence Database accession numbers of the 16S rRNA gene sequences obtained in this study are AM947508 to AM947560.

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