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ORIGINAL PAPER

Molecular characterization of mesophilic and thermophilic sulfate reducing microbial communities in expanded granular sludge bed (EGSB) reactors

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Abstract The microbial communities established in mesophilic and thermophilic expanded granular sludge bed reactors operated with sulfate as the electron acceptor were analyzed using 16S rRNA targeted molecular methods, including denaturing gradient gel electrophoresis, cloning, and phylogenetic analysis. Bacterial and archaeal communities were examined over 450 days of operation treating ethanol (thermophilic reactor) or ethanol and later a simulated semiconductor manufacturing wastewater containing citrate, isopropanol, and polyethylene glycol 300 (mesophilic reactor), with and without the addition of copper(II). Analysis, of PCR-amplified 16S rRNA gene fragments using denaturing gradient gel electrophoresis revealed a defined shift in microbial diversity in both reactors following a change in substrate composition (mesophilic reactor) and in temperature of operation from 30°C to 55°C (thermophilic reactor). The addition of copper(II) to the influent of both reactors did not noticeably affect the composition of the bacterial or archaeal communities, which is in agreement with the very low soluble copper concentrations (3–310 μg l^{-1}) present in the reactor contents as a consequence of extensive precipitation of copper with biogenic sulfides. Furthermore, clone library analysis confirmed the phylogenetic diversity of sulfate-reducing consortia in mesophilic and thermophilic sulfidogenic reactors operated with simple substrates.

Keywords Anaerobic wastewater treatment · Copper · Ethanol · Methanogens · Sulfate reducing bacteria · DGGE · 16S rRNA gene clone library

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Introduction

Sulfate-reducing bacteria (SRB) have long been studied as an integral part of the global sulfur cycle, which comprises important instances of electron exchange in oceans, sediments, and water systems. SRB, as well as sulfate-reducing archaea, are able to use sulfate as a terminal electron acceptor for growth using an organic compound or hydrogen as an electron donor. SRB are a polyphyletic group as they are commonly found in the class of *δ-Proteobacteria*, the low G+C Gram positive class *Clostridia*, the genus *Thermodesulfovibrio* within *Nitrospira*, and the genus *Thermodesulfobacterium* (Stahl et al. 2002).

The ability of dissimilatory sulfate reduction also occurs in a branch of hyperthermophilic archaea including several *Archaeoglobus* species (Burggraf et al. 1990; Stetter et al. 1993).

Recent studies have shown the potential of sulfate reducing bioreactors for the removal of heavy metals from wastewater (Lens et al. 1998). Removal of heavy metals by SRB is mainly due to the formation of highly insoluble precipitates with biogenic sulfide, including entrapment of precipitated sulfide minerals by biolfilm exopolymers. The solubility product of most metal sulfides is extremely low (Stumm and Morgan 1996) and, therefore, this technology can reduce metal concentrations in the wastewater to very low concentrations (in the ppb range). Studies of mesophilic SRB applied to metal decontamination have increased in recent years, but existing applications of these environmental biotechnologies at the practical scale are still limited. Technological application of thermophilic SRB bacteria for metal removal has not yet been described. Thermophilic bioreactors could potentially accommodate hotter process waters resulting from closed-cycle manufacturing.

An interesting application of sulfate reducing bioreactors is in the treatment of the high flows of copper-rich wastewaters generated from semiconductor manufacturing. Sulfidogenic bioreactors could also facilitate the removal of organic compounds in these effluent streams. Anaerobic microorganisms can effectively remove a variety of organic compounds often found in semiconductor manufacturing effluents, including citric acid, polyethylene glycol (average M_n 300), isopropanol, and oxalic acid using sulfate as the main electron acceptor (Hollingsworth et al. 2005).

Despite considerable interest shown by microbiologists and environmental engineers, the present understanding of the microbial diversity in sulfate reducing bioreactors is incomplete, especially in thermophilic processes. The aim of this study was to analyze the microbial communities established in a mesophilic reactor (MR) and a thermophilic reactor (TR) operated at 30°C and 55°C, respectively, and supplied with sulfate as the main electron acceptor and ethanol as carbon source and electron donating substrate. A second objective was to evaluate the impact on the microbiota present in the reactor biofilms resulting from the addition of copper(II) to the feed of TR, and from the shift in TR from ethanol to a simulated semiconductor manufacturing wastewater containing a mixture of isopropanol, citrate and polyethylene glycol (PEG, Mn = 300) with and without copper(II). A complementary set of methods targeting 16S rRNA and the encoding gene were used, including denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments, restriction fragment length polymorphism (RFLP) fingerprinting, cloning and sequencing. Phylogenetic analysis of partial sequences of clone libraries was performed to reveal the identity of the predominant groups of bacteria and archaea in the sulfidogenic biofilms, and to gain insights into the transformation processes occurring in the reactor biofilms.

Materials and methods

EGSB bioreactor operation, wastewater composition, and sample fixation

Two laboratory scale expanded granular sludge blanket (EGSB) bioreactors (V = 2.9 l) were inoculated with granular sludge from an industrial sulfidogenic reactor fed ethanol (10 g volatile suspended solids (VSS) l^{-1}). The sludge was adapted to a wastewater containing ethanol (493–1,480 mg l^{-1}) and sulfate (1,800–5,320 mg l^{-1}) for 46 days prior to inoculation. A mesophilic reactor (MR) at 30°C and a thermophilic reactor (TR) at 55°C were operated for approximately 450 days with non-limiting concentrations of sulfate, at a hydraulic retention time of 8 h. The influent of MR was mixed with recycled effluent in a fluidized-bed crystallization reactor (V = 0.4 l) placed upstream from the bioreactor. The crystallizer was supplied with 150 grams of acid-washed quartz sand (50–70 mesh) to promote heterogeneous nucleation of copper minerals. The effluent recirculation in the bioreactor was maintained at a recycle ratio of 15, in order to maintain fluidization of the sand in the crystallizer as well as maintain sludge bed fluidization in the bioreactor. The TR operated as a stand-alone reactor and heavy metal precipitation by biogenic sulfides occurred within the reactor. Both reactors were initially fed with 1,480 mg l^{-1} ethanol and 5,320 mg l^{-1} sulfate. At day 199, the electron donor

in the MR was switched from ethanol to a simulated semiconductor manufacturing wastewater containing 425 mg l^{-1} isopropanol, 1,370 mg l^{-1} citric acid, and 615 mg l^{-1} polyethylene glycol (average M_n approx. 300). Divalent copper (Cu(II)) was added to the influent of MR and TR at an average concentration of 4.2 ± 0.4 mg l^{-1} on day 374 and further increased to 24.6 ± 0.1 mg l^{-1} on day 407. The concentration of Cu(II) in the influent of the MR was increased further to 66.4 ± 0.4 mg l^{-1} on day 430. Cu(II) was added to the medium as $CuCl_2{\cdot}2H_2O$.

The basal mineral medium contained (in mg l^{-1}): NH_4Cl (280), $NaHCO_3$ (4,000), KCl (270), K_2HPO_4 (169), $CaCl_2 \cdot 2H_2O$ (10), $MgCl_2 \cdot 6H_2O$ (150), yeast extract (20), and 1 ml l^{-1} of trace element solution (Hollingsworth et al. 2005). The initial pH of the reactor medium ranged between 7.7 and 8.0, resulting in effluent pH values from 7.4 to 8.0.

Sludge samples were taken from both bioreactors periodically and immediately frozen at –80 °C and stored until DNA extraction was performed. The MR was sampled for clone library analysis on day 280, 197 and 447, and for DGGE analysis on days 8, 16, 22, 30, 62, 84, 140, 197, 280, 367, 424, and 447. The TR was sampled for clone library analysis on day 62, 367 and 447, and for DGGE analysis on days 8, 16, 22, 30, 62, 84, 104, 140, 197, 367, 424, and 447.

DNA and RNA extraction and 16S rRNA gene amplification

DNA was extracted from frozen sludge samples, and RNA was extracted immediately from fresh sludge samples (only day 447 samples) and reverse transcribed as described previously (Roest et al. 2005a). Purified DNA and cDNA samples were stored at −20°C. The V6–V8 regions of the bacterial 16S rRNA genes were amplified for DGGE using universal bacterial primers 968GC-f and 1401r (MWG Biotech, Ebersberg, Germany; Eurogentec, Seraing, Belgium) (Nubel et al. 1996). The V2-V3 regions of archaeal 16S rRNA genes were amplified for DGGE using the primers A109(T)f and 515GC-r (Sigma-Aldrich, St. Louis, MO, USA) (Grosskopf et al. 1998; Lane 1991; Muyzer et al. 1993). Reactions of 50 µl consisted of 1.25 U *Taq* DNA polymerase, 1× PCR reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, and 1 µl template DNA. The thermal sequence used for bacterial amplification was 2 min of initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and elongation at 72°C for 1 min, with a final 5 min elongation at 72°C. The thermal sequence used for archaeal amplification differed only in the annealing step, which was 52°C for 40 s, and the elongation step, which was 90 s long.

Cloning and RFLP analysis

Bacterial and archaeal 16S rRNA gene clone library analysis was performed with total DNA extracted from the biofilm samples, with the exception of the clone libraries of samples obtained on day 447 which used cDNA. The bacterial primers 968f (no GC clamp) and 1401r and the archaeal primers A109(T)-f and 515r (no GC clamp) were used in PCR amplification, as described above. Amplified 16S rRNA gene products were purified and cloned as described previously (Roest et al. 2005b). Cell lysates were screened using RFLP and restricting with MspI, CfoI, and AluI (Promega Corp., Madison, WI, USA). Restriction fragments were analyzed on 12% Poly(NAT)® gels (Elchrom Scientific, Cham, Switzerland). One or two representative clones were sequenced for each unique restriction pattern.

Sequencing and phylogenetic analysis

Clones were sequenced commercially using T7-Sp6 primers, and primer residues were removed from recovered sequences before alignment. Clone libraries ranged in size with 57 clones in the MR280 library, 87 in MR447, 89 in TR062, 89 in TR447, 15 in MRA197, 37 inMRA447, 43 in TRA367, and 35 in TRA447. 16S rRNA sequence similarity searches were performed in the GenBank database of NCBI (*http://www.ncbi.nlm.nih.gov*) using the Basic Local Alignment Search Tool (BLAST) algorithm (McGinnis and Madden 2004). Subsequently, sequences were aligned using the FastAligner automated alignment tool of the ARB software package and further checked and manually aligned for secondary structure (Ludwig et al. 2004). Distance trees were constructed in ARB using the neighbor-joining method, as previously described, and with Felsenstein correlations and outgroup, as indicated (Egert et al. 2003; Felsenstein 1985). Operational Taxonomic Units (OTUs) were defined for sequences that shared

greater than 97% similar as determined from the distance method in ARB.

Sequences obtained in this study were deposited in Genbank under accession numbers EF512353–EF512459.

DGGE analysis

DGGE was performed using gradients of 35–55% and 30–50% for bacterial and archaeal amplicons, respectively (Ben-Amor et al. 2005; Roest et al. 2005b). Gels were stained using silver nitrate, scanned at 400 dpi and analyzed with BioNumerics, v. 4.0 software package (Applied Maths, Sint-Martens-Latem, Belgium) as previously described to calculate similarity indices and moving window correlations using the Pearson product-movement correlation (Konstantinov et al. 2006).

Analytical methods

Sulfate and citrate were determined by ion chromatography with suppressed conductivity detection, and volatile fatty acids (acetic, propionic and butyric acids), ethanol, isopropanol and acetone by gas chromatography with flame ionization detection (Hollingsworth et al. 2005). The concentration of PEG was determined by subtracting the theoretical chemical oxygen demand (COD) contributions of other measured chemical species from the overall COD measurements. Sulfide was analyzed colorimetrically (Trüper and Schlegel 1964). Copper was determined by inductively coupled plasma–mass spectroscopy (Karri et al. 2006). COD (colorimetric micro-method) and VSS content were determined according to standard methods (APHA 1998).

Results and discussion

Reactor operation

Details of MR and TR samples used for clone libraries, and average reactor performance data over the 14 days prior to sample collection are summarized in Table 1. Both bioreactors were operated under non-sulfate limiting conditions to favor the dominance of SRB over methanogens. This approach was successful, and the electron flow in both bioreactors was almost exclusively directed towards sulfate reduction, with methanogenesis accounting for 7.9% or less of the influent COD. Average substrate removal by the MR exceeded 95% during the various periods of operation with ethanol alone or with a mixture of citric acid, isopropanol (IPA) and polyethylene glycol (PEG, M_n 300), with the exception of IPA which was partially degraded to acetone. This finding is in agreement with studies reporting the ability of a number of sulfate-reducing bacteria (Janssen and Schink 1995b; Platen et al. 1990) and methanogenic archaea (Garcia 1990) to oxidize IPA to acetone. IPA (Davies and Stephenson 1941; Ensign

Table 1 Details of MR and TR samples used for clone libraries, and average reactor performance data over the 14 days prior to sample collection

Reactor	Day	Clone library ID		Reactor conditions[a]	% Total COD flux[b]		
		Bacterial	Archaeal		Sulfide	Methane	Acetate
MR	8	–	–	Ethanol + Sulfate	79.4 ± 6.7	2.4 ± 1.3	1.7 ± 3.1
	197	–	MRA197	Ethanol + Sulfate	88.2 ± 6.4	7.9 ± 1.4	5.3 ± 3.0
	280	MR280	–	IPA/PEG/CA + Sulfate	60.7 ± 2.7	3.8 ± 0.4	3.3 ± 0.2
	447	MR447	MRA447	IPA/PEG/CA + Sulfate	60.1 ± 3.5	0.5 ± 0.3	0.5 ± 0.3
TR	8	–	–	Ethanol + Sulfate	13.1 ± 4.2	2.0 ± 1.6	74.3 ± 20.1
	62	TR062	–	Ethanol + Sulfate	7.9 ± 5.9	0.9 ± 0.5	62.6 ± 18.3
	367	–	TRA367	Ethanol + Sulfate	43.5 ± 5.3	3.4 ± 1.2	43.6 ± 8.3
	447	TR447	TRA447	Ethanol + Sulfate + Cu(II)	30.0 ± 5.1	0.4 ± 0.2	41.7 ± 3.7

[a] IPA = isopropanol; PEG = polyethylene glycol (M_n 300); CA = citrate. See Materials and methods for concentrations of ethanol, sulfate, IPA, PEG, and CA. When indicated, copper(II) concentration is 24.6 mg l^{-1}

[b] Reactor performance data are averaged over the 14 days prior to the collection of each sample. For day 8, data are averaged over the 8 days prior to sample collection

et al. 1998; Widdel 1986) and acetone (Ensign et al. 1998; Platen et al. 1990; Platen and Schink 1987, 1989) can serve as growth-supporting substrates for a number of diverse anaerobic microorganisms. The factors responsible for the poor removal of both compounds by the microbiota in the sulfate-reducing bioreactor are unclear. Inhibition of IPA and acetone degraders due to the high sulfide concentrations prevailing in the bioreactor (729–809 mg $S^{2-}l^{-1}$) is a plausible explanation. Manganese limitation due to formation of insoluble MnS with biogenic sulfide might have contributed to accumulation of acetone in the MR. Bacterial acetone decarboxylase has recently been shown to be a manganese-dependent metalloenzyme (Boyd et al. 2004).

Similarly to the MR, the microbial community in the TR was found to utilize ethanol readily. However, acetate, the major intermediate of ethanol oxidation, was not degraded during the initial 62 days of TR operation and only partly degraded (approx. 33–35% removal) thereafter. The lack of further acetate consumption might be related to sulfide toxicity, as SRB and methanogens that utilize acetate are known to be very sensitive to sulfide inhibition (Lens et al. 1998; O'Flaherty and Colleran 1999). Severe inhibition of sulfate reduction with acetate was observed in thermophilic experiments (55 °C) at total sulfide concentrations ranging from 33 to 450 mg l^{-1} (Visser et al. 1993), depending on the pH. These concentrations are in the range of those prevailing in the TR (112–686 mg l^{-1}).

Although the feed of the MR and TR systems were supplied with elevated copper(II) levels, the concentration of soluble copper in the reactor contents was very low, 18–162 μg l^{-1} in MR and 3–310 μg l^{-1} in TR, due to the effective precipitation of this metal by biogenic sulfides. These concentrations are several orders of magnitude lower compared to 50% inhibitory values reported for copper(II) which generally range from a few ppm to as much as 100 mg l^{-1} (Karri et al. 2006; Utgikar et al. 2001).

The inoculum of the MR and TR systems consisted of sulfate-reducing sludge in the form of granular biofilms. The morphology of the sludge in both reactors remained chiefly granular throughout the whole experiment, although the size of the granules in TR decreased upon increasing the temperature to 55°C.

Bacterial clone libraries

Clone library analysis was used to assess the diversity of microbial communities in the different reactors, and to identify predominant populations detected by DGGE fingerprinting. In total, 13 OTUs were identified for MR280 (labeled MR-1 through MR-13), 16 for MR447 (MR-14 through MR-29), 35 for TR062 (TR-1 through TR-35), and 12 for TR447 (TR-36 through TR-47). The phylogenetic affiliations of the 16S rRNA gene clones and OTUs are listed in Table 2. Phylogenetic trees were constructed based on 16S rRNA sequences from the four bacterial clone libraries and reference sequences (Figs. 1–3).

The phylogenies of the sequences retrieved from the day-447 samples differed greatly from earlier samples both in the diversity of species and range of phylogeny represented within each clone library. For the MR bacterial community, a marked difference, even at the class level, was observed between MR280 and MR447. The MR280 OTUs were found to be most closely related to species within the *Firmicutes* division (93% of the clones), namely in the *Bacilli* and *Clostridia* classes, and *δ-Proteobacteria* (7% of the clones). None of the 10 OTUs within the *Firmicutes* division were closely related to species known to reduce sulfate. Conversely, in MR447, all clones were affiliated with the *δ-Proteobacteria* subdivision, and the closest relatives of 15 of the 16 OTUs were well known sulfate-reducing organisms, obviously more closely reflecting the operational conditions. Similarly, in the TR clone libraries, significantly more OTUs were closely related to species capable of sulfate reduction in the TR447 library derived from RNA (89%), than the TR062 library (46%). These dissimilarities could be partly due to the fact that sludge RNA was used as the template for the day 447 clone libraries rather than DNA, resulting in a better reflection of the metabolically active fraction in the reactor microbiota. The difference, therefore, between the identity of the most active organisms and the numerically most abundant organisms in this reactor community appears to be substantial. This was also confirmed by DGGE analysis, revealing distinct profiles generated from sludge RNA as compared to DNA-derived fingerprints (Fig. 7).

The dominant clone in the MR280 library was closely related to *Trichococcus flocculiformis*

Table 2 Distribution of the 16S rRNA gene bacterial clones and archaeal OTUs obtained from the mesophilic and thermophilic reactors

Phylogenetic group	Mesophilic reactor				Thermophilic reactor			
	Clone libraries				Clone libraries			
	MR280		MR447		TR062		TR447	
	OTUs	% Clones (n = 57)	OTUs	% Clones (n = 87)	OTUs	% Clones (n = 89)	OTUs	% Clones (n = 89)
Firmicutes								
Bacilli	5	70.2	–	–	–	–	–	–
Clostridia > *Clostridiales*	5	22.7	–	–	15	46.1	–	–
Clostridia > *Thermoanaerobacteriales*	–	–	–	–	10	41.5	–	–
Proteobacteria								
δ-Proteobacteria	3	7.1	16	100	3	3.4	6	88.7
γ-Proteobacteria	–	–	–	–	–	–	5	7.9
Chloroflexi > *Unclassified Chloroflexi*	–	–	–	–	3	3.4	–	–
Spirochaetes > *Spirochaetes*	–	–	–	–	1	1.1	1	3.4
Nitrospirae > *Nitrospirales*	–	–	–	–	3	4.5	–	–
Total	13	100	16	100	35	100	12	100

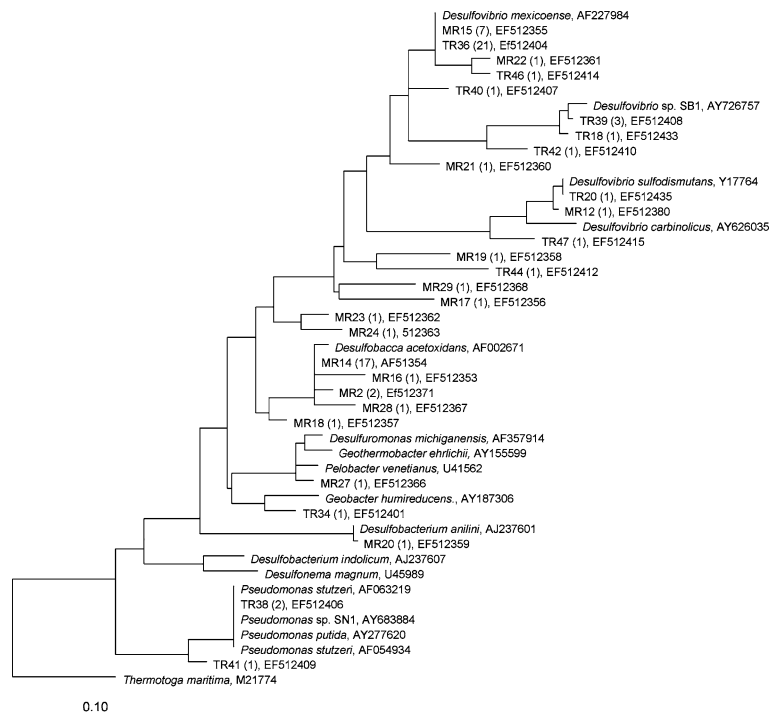

Fig. 1 Phylogenetic tree of 16S rRNA sequences affiliated with the *Proteobacteria* division. For tree construction, neighbor joining distance matrix method in the ARB software package was used based on *E. coli* positions 969 to 1373 with the ARB filter for *Bacteria*. *Thermotoga maritima* (M21774) was used as outgroup to root the tree. GenBank accession numbers are provided. Total numbers of clones comprising individual OTUs are given in parentheses. The bar indicates 10% sequence difference. Tree topology was confirmed by application of alternative tree building algorithms as implemented in the ARB software package

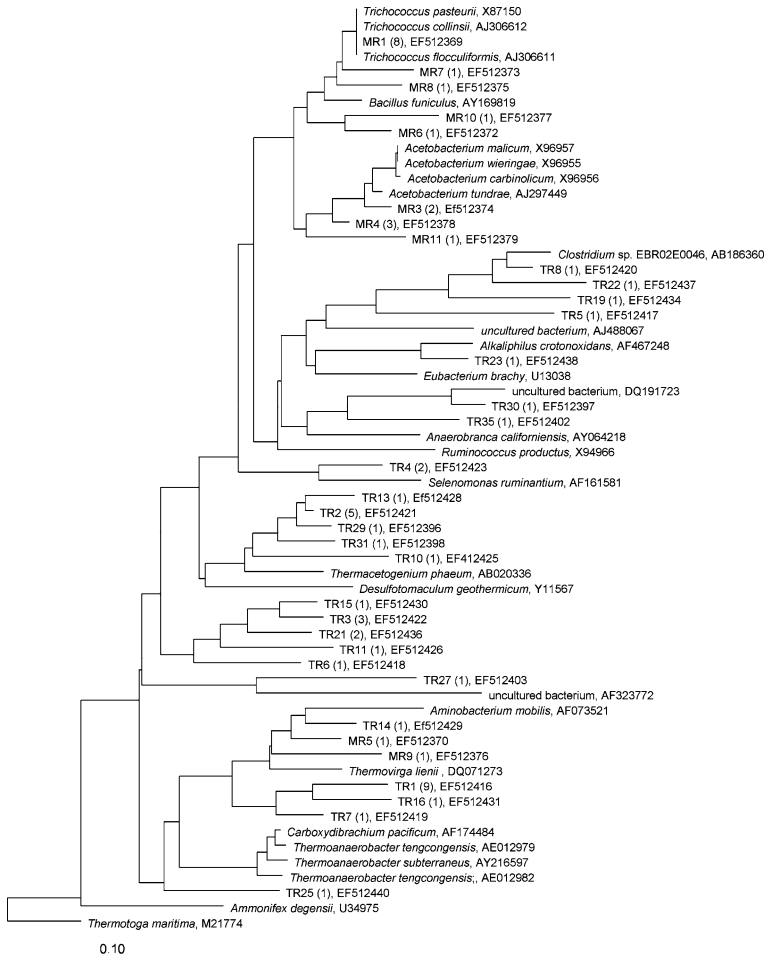

Fig. 2 Phylogenetic tree of 16S rRNA sequences affiliated with the *Firmicutes* division. Tree construction followed that described in the caption for Fig. 1 except that positions 985–1373 in combination with the ARB filter for *Firmicutes* was used. Details of tree (bar definition, notation, outgroup) are the same as Fig. 1

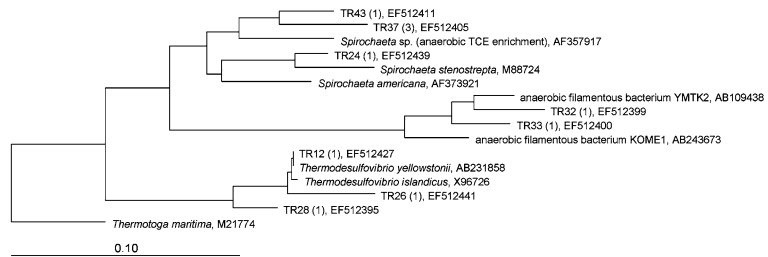

Fig. 3 Phylogenetic tree of 16S rRNA sequences affiliated with the *Chloroflexi*, *Nitrospira*, and *Spirochaeta* divisions. Tree construction followed that described in the caption for Fig. 1. Details of tree (bar definition, notation, outgroup) as Fig. 1

(AJ306611), a fermentative organism in the low G-C Gram positive bacteria, originally isolated from anoxic digestor sludge (Scheff et al. 1984). *T. flocculiformis* can grow with citrate (Liu et al. 2002) and might have contributed to the effective removal of this substrate in the MR. Within the class *Clostridia*, three clone OTUs were found in the genus *Acetobacterium*, which closest cultured relatives were *A. malicum* (X96957), *A. wieringae* (X96955) and *A. tundrae* (AJ297449). These acetogenic

microorganisms might be implicated in the removal of PEG. A number of *Acetobacterium* species, including *A. malicum*, can grow at the expense of glycol ethers which are metabolized to acetate (Schuppert and Schink 1990; Tanaka and Pfennig 1988). Anaerobic fermentation of polymeric PEG compounds has been described for an *Acetobacterium* sp. (Schramm and Schink 1991) and for a few other fermenting bacteria such as *Pelobacter venetianus* (Schink and Stieb 1983), *Bacteroides* sp. (Dwyer and Tiedje 1986), and a propionate-forming fermenting bacterium (Wagener and Schink 1988). *Thermovirga lienii* (DQ071273) was the closest cultured relative of the other two clone OTUs affiliated with the *Clostridia*. *Th. lienii* is a moderately thermophilic bacterium with a fermentative type of metabolism, which can reduce elemental sulfur to sulfide (Dahle and Birkeland 2006). Sequences of sulfate reducers such as *Desulfococcus biacutus* and *Desulfobacterium cetonicum* which are able to oxidize acetone to CO_2 (Janssen and Schink 1995a, b; Platen et al. 1990) or other microorganisms in those genera were not detected in the clone libraries, which is in agreement with the observed accumulation of acetone in the MR.

Within the class *Proteobacteria*, clones were only found in the delta (*δ)* sub-division. *Desulfovibrio mexicanus* (AF227984) and *Desulfobacca acetoxidans* (AF002671) were the closest cultured relatives of the most abundant clones in the *δ-Proteobacteria* cluster of the MR280 and MR447 libraries. *D. mexicanus*, a SRB isolated from a Mexican upflow anaerobic granular sludge blanket (UASB) digester treating cheese factory wastewater, can oxidize ethanol to acetate (Hernandez-Eugenio et al. 2000). *D. acetoxidans* is a sulfate-reducer that can utilize acetate as the sole electron donor, but it is unable to use ethanol or propanol (Oude-Elferink et al. 1998). Acetate was only detected at very low concentrations in the effluent of the MR (Table 1), which is in agreement with phylogenetic data confirming the presence of acetate-utilizing microorganisms. *D. acetoxidans*-like populations were the most abundant relative to the total SRB population in several lab-scale and full-scale sulfidogenic bioreactors that received ethanol as energy source (Dar et al. 2007). The abundance of *D. acetoxidans* in many sulfate-reducing reactors has been attributed to the higher specific growth rate of this sulfate-reducer (μ_{max} = 0.013–0.017 h^{-1}) compared to acetate-degrading methanogenic Archaea, like *Methanosaeta* spp. (μ_{max} = 0.003–0.012 h^{-1}), which often dominate in methanogenic bioreactors (Oude-Elferink et al. 1998). Four additional OTUs were found in the *δ-Proteobacteria*, three of them most closely related to the sulfate reducers, *Desulfonema magnum* (U45989), *Desulfobacterium anilini* (AJ237601) and *Desulfovibrio sulfodismutants* (Y17764), and the other to *Geobacter ehrlichii* (AY155599). Some of these microorganisms have the ability to completely oxidize organic electron donors. *D. magnum* can oxidize acetate completely to CO_2, but it is unable to use ethanol or propanol (Fukui et al. 1999). *G. ehrlichii* is a thermophile that grows with organic acids as electron donors and Fe(III) or nitrate as electron acceptor (Kashefi et al. 2003).

The thermophilic clone libraries were the most diverse of the study with respect to number of unique OTUs (Table 2). This finding underscores the possibility of maintaining complex microbial communities on a simple substrate as ethanol, as was previously observed in a mesophilic ethanol-utilizing sulfate-reducing fluidized-bed reactor (Kaksonen et al. 2004). The TR062 library contained clones most closely related to species in the *Chloroflexi*, *Firmicutes*, *Nitrospira*, *Proteobacteria*, and *Spirochaetes* divisions. Despite the wide range of diversity, the majority of clones (88%) were found within the *Firmicutes*, nearly evenly split between the orders *Clostridiales* (47%) and *Thermoanaerobacteriales* (41%). In the TR447 clone library, all OTUs fell within the classes of *δ*- and *γ-Proteobacteria*. A majority of clones, 89%, were found solely within the *Desulfovibrio* genus, with the remaining OTUs being most closely related to various strains of *Pseudomonas* and *Spirochaeta* species.

The three dominant clones in the TR062 library affiliated with the *Firmicutes* and they were most closely related to thermophilic microorganisms, including the sulfate reducer, *Desulfotomaculum geothermicum* (Y11567), the synthrophic acetate-degrader *Thermacetogenium phaeum* (AB0200336), and the fermentative bacterium, *Thermovirga lienii* (DQ071273). *D. geothermicum* (Daumas et al. 1988) and *Th. phaeum* (Hattori et al. 2000) can grow acetogenically on several alcohols, including ethanol, the only electron donor supplied to the TR. *Th. lienii* can utilize proteinous substrates, some single amino

acids and a limited number of organic acids, but not sugars, fatty acids or alcohols (Dahle and Birkeland 2006). The decay rates of thermophilic bacterial are high, and microorganisms related to *Th. lienii* in the TR might have thrived on products from cell decay. Three of the OTUs were most closely related to the thermophilic, fermentative bacteria, *Thermoanaerobacter tengcongensis* and *Thermoanaerobacter subterraneus*, both species which have been proposed to be reassigned a novel genus and species, *Caldanaerobacter subterraneus* (Fardeau et al. 2004). Within the *Nitrospirae* division, three OTUs were most closely related to *Thermodesulfovibrio yellowstonii* (AB231858), a thermophilic sulfate reducer that does not grow on acetate or ethanol (Henry et al. 1994).

Within the class *Proteobacteria*, 3 OTUs in the TR062 library and 2 OTUs in the TR447 library affiliated with the *Desulfovibrio* cluster, the closest cultured relative of these OTUs being *D. carbinolicus* (AY626035), *D. mexicanus* (AF227984), and *D. sulfodismutans* (Y17764). These three species can oxidize ethanol to acetate using sulfate as electron acceptor (Bak and Pfennig 1987; Hernandez-Eugenio et al. 2000; Nanninga and Gottschal 1987). Five OTUs in the TR447 library were affiliated to *Pseudomonas* species, whereas three other OTUs clustered with *Sprirochaeta americana* (AF373921), a mesophilic haloalkaliphile (Hoover et al. 2003). Three OTUs in the TR067 library were most closely related to the *Chloroflexi* division (green non-sulfur bacteria) with environmental clones isolated from methanogenic UASB sludge granules (Yamada et al. 2005). Isolates belonging to the class-level taxon '*Anaerolineae*' of the bacterial division *Chloroflexi* have been found to be widely distributed in mesophilic and thermophilic anaerobic granular sludge and their physiological function has been hypothesized to include carbohydrate degradation and cellular matter degradation (Yamada et al. 2005, 2006).

Acetate accumulated in the effluent throughout the operation of the thermophilic reactor (Table 1). This seems contrary to the phylogenetic results that identified *Th. phaeum*, a synthrophic acetate-oxidizer, as the closest relative of 28% of the TR062 clones. The bacterium can grow on acetate with concomitant reduction of sulfate, and it can oxidize acetate in co-culture with a thermophilic hydrogenotrophic methanogens to form methane (Hattori et al. 2000).

Archaea clone libraries

Eight OTUs were retrieved for the archaeal clone library MRA197 (labeled MRA-1 through MRA-8), five for MRA447 (MRA-9 through MRA-13), eleven for TRA367 (TRA-1 through TRA-11), and seven for TRA447 (TRA-12 through TRA-18). The PCR products from archaeal primers included some bacterial sequences as revealed through cloning and sequencing and were determined to be a result of either mispriming of the archaeal primers or due to the high relative abundance of bacterial genomic DNA in relation to archaea in the samples (Leclerc et al. 2004; Roest et al. 2005a). The closest cultured species of the archaeal clones of both reactors were determined through an NCBI GenBank similarity search (Table 3). A phylogenetic tree was created based on 16S rRNA gene sequences (Fig. 4).

Table 3 Distribution of the 16S rRNA gene archaeal clones and archaeal OTUs obtained from the mesophilic and thermophilic reactors

Phylogenetic group	Mesophilic reactor				Thermophilic reactor			
	Clone libraries				Clone libraries			
Euryarchaeota	MRA197		MRA447		TRA367		TRA447	
	OTUs	% Clones (n = 15)	OTUs	% Clones (n = 37)	OTUs	% Clones (n = 43)	OTUs	% Clones (n = 35)
Methanobacteria	1	26.6	2	18.9	6	58.2	2	37.1
Methanomicrobia	6	66.7	3	51.4	2	9.3	5	54.3
Uncultured *Archaea*	1	6.7			3	16.3	–	–
Bacterial Sequences[a]	–	–	–	29.7	–	16.3	–	8.6
Total	8	100	5	100	11	100	7	100

[a] Bacterial sequences found in the archaeal libraries were not assigned OTUs, but still considered in population statistics

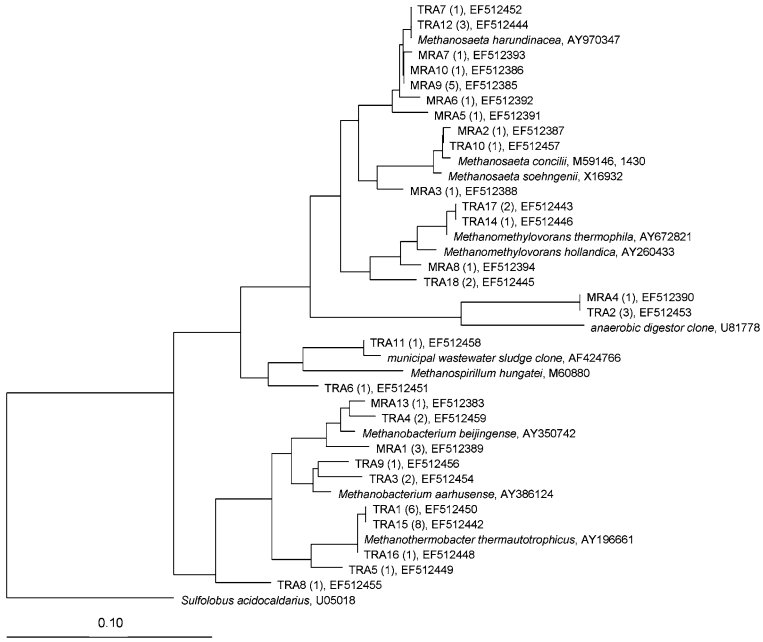

Fig. 4 Phylogenetic tree of 16S rRNA sequences affiliated with the *Archaea* kingdom. For tree construction, neighbor joining distance matrix method in the ARB software package was used based on *E. coli* positions 117–399) regions of the archaeal OTUs with the ARB filter for *Archaea*. *Sulfolobus acidocaldarius* (U05018) was used as outgroup to root the tree. All sequences are given with their corresponding GenBank accession number. The bar indicates 10% sequence difference

In the mesophilic libraries, most of the closest cultured relatives were mesophilic archaea, falling mainly within the *Methanomicrobia* and *Methanobacteria* classes. The dominant clones were most closely related to *Methanobacterium beijingense* (AY350742), *Methanosaeta harundinacea* (AY970347), and *Methanosaeta concilii* (M59146). *M. beijingense*, a methanogen recently isolated from an anaerobic digester, can utilize H_2/CO_2 or formate to form methane, but not acetate (Ma et al. 2005). In contrast, *M. harundinacea*, a microorganism isolated from a UASB reactor treating brewery wastewater (Ma et al. 2006), and *M. concilii*, a methanogen commonly found in anaerobic reactors (Ekiel et al. 1985; Patel and Sprott 1990), are obligate acetoclastic methanogens. In spite of the presence of excess sulfate, methane formation was detected in the MR throughout the experiment, albeit at low rates. Substrate utilization by methanogens decreased from approximately 7.9% of the total COD on day 197 to almost negligible values at the end of the experiment (Table 1). IPA might have provided an additional growth substrate for some methanogens. Several species in the genus *Methanobacterium* have been reported to degrade IPA to acetone (Joulian et al. 2000).

In the thermophilic libraries, clone OTUs were found in the *Methanomicrobia* and *Methanobacteria* classes. In addition, three OTUs in the TR367 library were most closely related to sequences of uncultured archaeons retrieved from methanogenic bioreactor sludge and anaerobic sediments (Chauhan and Ogram 2006). The dominant clones in the thermophilic libraries were *M. harundinacea* (AY970347), *Methanothermobacter thermautotrophicus* (AY196661), a microorganism originally isolated from sewage sludge (Zeikus and Wolfe 1972), and *Methanomethylovorans thermophila* (AY672821), first isolated from a methanol-fed thermophilic UASB reactor (Jiang et al. 2005). Three OTUs in the TR367 library were most similar to *M. beijingense* (AY350742), whereas two other OTUs in the same library were related to *Methanobacterium aarhusense* (AY386124) and *M. concilii* (M59146), respectively, and one OTU in the TR197 library was affiliated with *Methanomethylovorans hollandica* (AY260433), originally isolated from a freshwater pond (Lomans et al. 1999). *M. thermoautotrophicus* and other archaea in the *Methanobacteriales* are generally hydrogenotrophic, using H_2 to reduce CO_2 to CH_4. Some members of this order can use formate, CO, or

secondary alcohols as electron donors for CO_2 reduction, but no acetate (Garcia 1990). *M. thermophila* and *M. hollandica* are thermophilic, obligately methylotrophic, methanogenic archaeons that do not use H_2/CO_2 or acetate as a substrate (Jiang et al. 2005; Lomans et al. 1999). The archaeal diversity observed in the thermophilic reactor is somewhat surprising since methane production was marginal (Table 1). Some of the sequences retrieved might correspond to resting cells already present in the granular inoculum.

DGGE analysis and DGGE band identification

DNA extracted from sludge samples from both reactors was analyzed by 16S rRNA-targeted DGGE fingerprinting of the bacteria and archaea communities, revealing overall higher diversity of bacterial profiles when compared to archaeal communities (Figs. 5, 6). To allow for the identification of significant changes in the profiles during reactor operation, moving window correlation was applied, for which similarity indices were calculated for

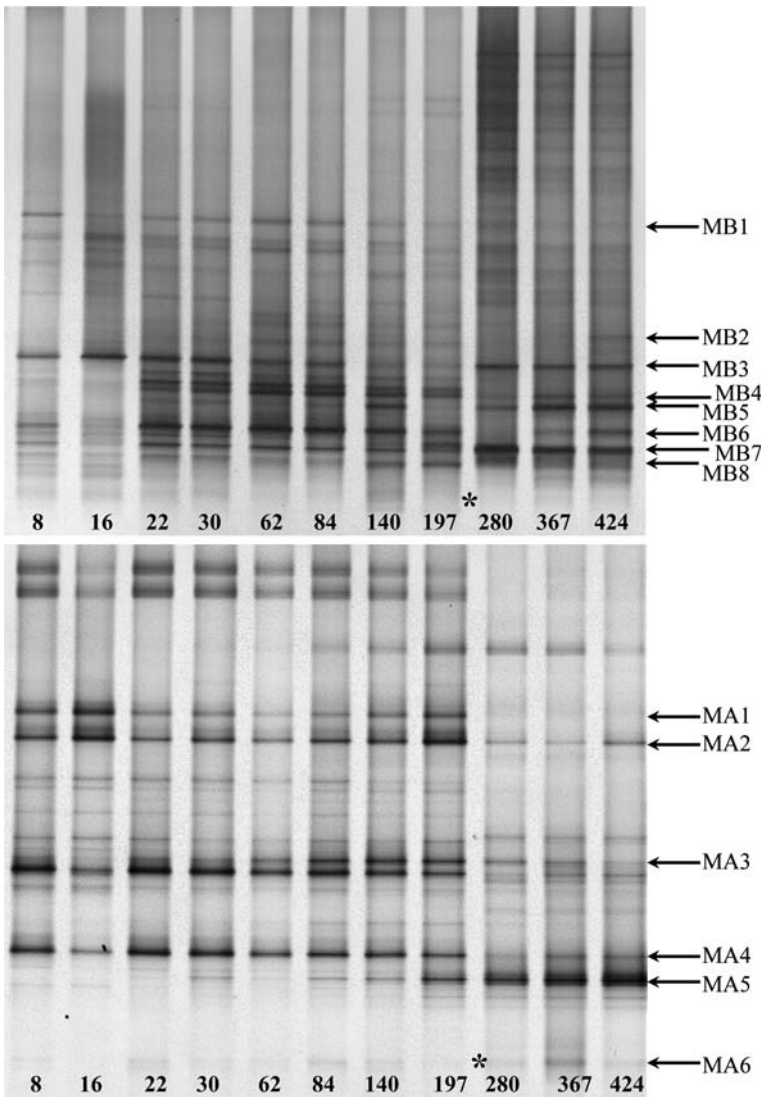

Fig 5 DGGE profiles of 16S rRNA amplicons from the mesophilic reactor (MR). Bacterial DGGE profiles (Top panel) and archaeal DGGE profiles (Lower panel) with lane numbers indicating the day each sample was collected. The asterisk (*) indicates when substrate change occurred. Identified DGGE bands are labeled to the right, see text for details

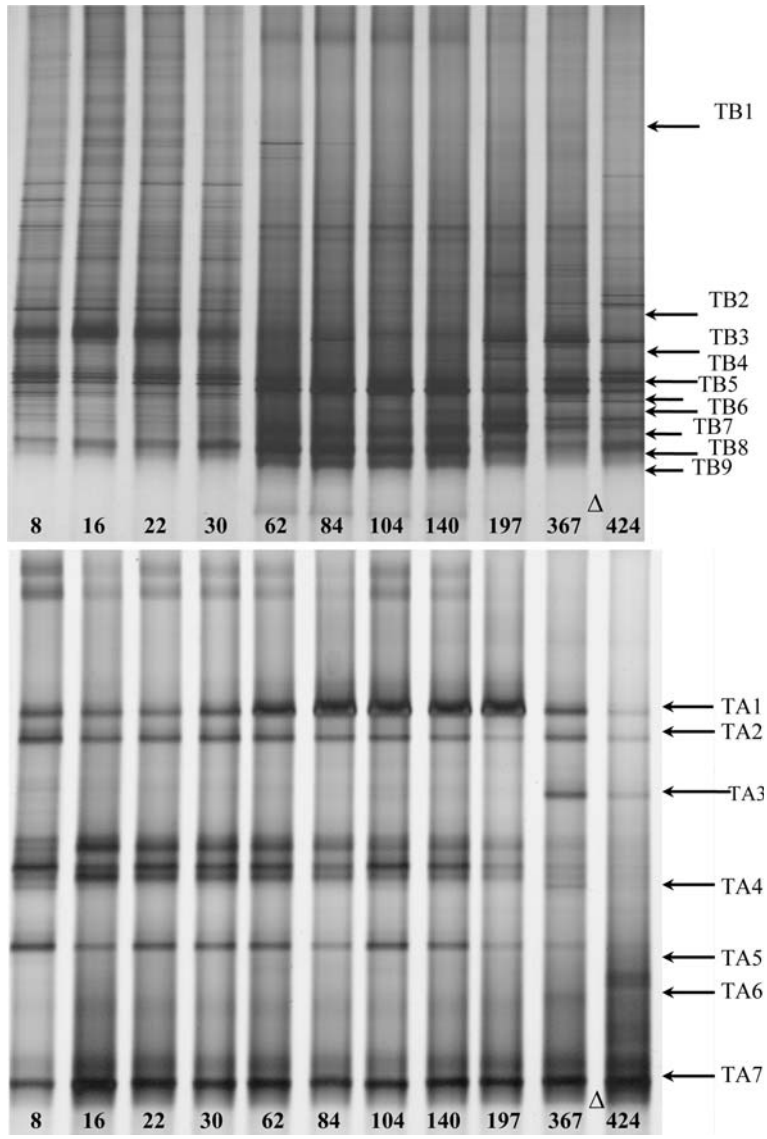

Fig. 6 DGGE profiles of 16S rRNA amplicons from the thermophilic reactor (TR). Bacterial DGGE profiles (Top panel) and archaeal DGGE profiles (Lower panel) with lane numbers indicating the day each sample was collected. The asterisk (*) indicates when Cu(II) was added to the influent at concentrations ranging 4.2–24.6 mg l^{-1}. Identified DGGE bands are labeled to the right, see text for details

each pair of successive time points and plotted according to which sample pair was analyzed (Fig. 7). The archaeal profiles appear to be more conserved through the time course with fewer bands appearing and more bands maintaining intensity throughout.

Apparent shifts in bacterial and archaeal communities could in most cases be directly related to operational changes in the reactor. As expected, the change from ethanol to a synthetic semiconductor wastewater containing citrate, IPA and PEG in the MR resulted in a noticeable shift in the bacterial DGGE profile as the consortium adjusted to consume the new substrates (Figs. 5, 7). The very low concentrations of soluble copper in the MR liquid contents (18–162 μg l^{-1}) are unlikely to have played a role in altering the structure of the microbial community in MR. In the TR, the bacterial community appeared to undergo a significant change between days 30 and 62, while the archaea did not

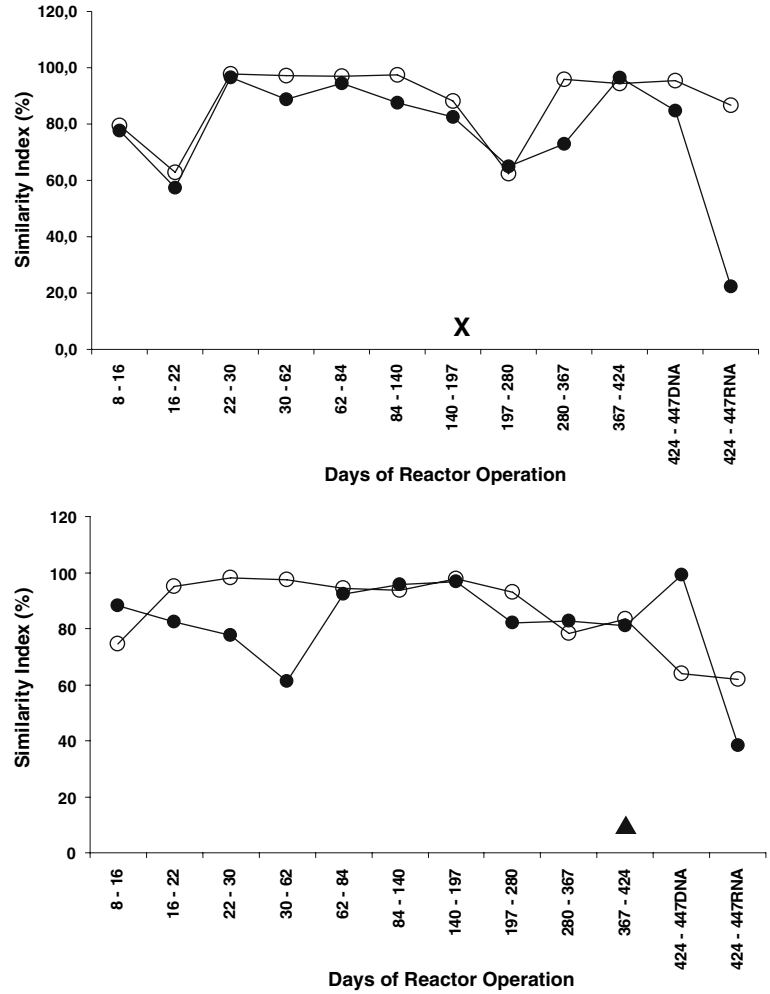

Fig. 7 Moving window correlation of bacterial and archaeal diversity in the sulfidogenic mesophilic (MR) and thermophilic reactor (TR). Diversity of DGGE profiles assessed as similarity index of successive sampling points based on Pearson product-moment correlation. The samples which are compared at each point are indicated on the x-axis. For the MR (Top panel) and TR (Lower panel), bacterial DGGE patterns (●) and archaeal DGGE patterns (◯) are shown. The symbol (**X**) indicates when substrate change occurred in the MR and the triangle (▲) indicates when Cu(II) was added to the influent of the TR at concentrations ranging 4.2 to 24.6 mg l^{-1}. A similarity index of 100% indicates profiles with identical densitometric curves

seem affected at this point (Fig. 6). This shift could not be related to an apparent change in reactor performance. The moving window correlation of similarity indices indicated stable bacterial and archaeal profiles between days 367 and 424 samples in the TR, suggesting that Cu(II) did not trigger dramatic changes in the microbial community structure (Fig. 7). This finding is not surprising since the Cu(II) amendment (up to 24.6 mg l^{-1}) precipitated with biogenic sulfide, resulting in very low concentrations of soluble Cu(II) in the liquid phase (3–310 μg l^{-1}). To further assess, whether the large differences observed between clone libraries prepared from sludge DNA and RNA, respectively, were indeed related to differences between most abundant and most active populations, DGGE analysis was performed for samples taken from both reactors on day 447. While DNA-derived profiles were highly similar to those taken earlier during reactor operation, this was not the case for the RNA fingerprints (Fig. 7), reinforcing the notion that RNA-based analyses can be used to identify the most active, rather than the most abundant microorganisms (Felske et al. 1998; Wagner 1994).

Representative clones were analyzed using DGGE to identify the phylogenetic affiliation of the predominant populations observed with the original banding patterns. The banding of individual clones was evaluated along with sequencing information to assign OTUs to their position on the original banding patterns (Table 4). The closest cultured relative of each OTU is shown along with class-level phylogeny associations. Numerous bands of bacterial profiles could not be associated with any of the clone sequences retrieved, particularly in TR.

The analysis of the bacterial and archaeal banding patterns of both reactors revealed that several OTUs often migrated in a single band. Conversely, some OTUs most closely related to the same species frequently migrated into multiple bands. This occurred in the bacterial and archaeal populations of both reactors (Table 4). This outcome is justified since OTUs are less than 97% similar by definition. In some cases, these similar OTUs are present in bands that both increase and decrease in intensity through the time course of the reactors, complicating assessment of the

Table 4 Association of OTUs with DGGE banding patterns

Band	OTU label	Affiliation(s) of OTUs closest related cultured species	Taxonomy class[a]
MB1	MR-20	*Desulfobacterium anilini*	DP
MB2	MR-12, 19	*Desulfovibrio mexicanus*, *Desulfovibrio sulfodismutans*	DP
MB3	MR-4, 8	*Acetobacterium malicum*, *Trichococcus flocculiformis*	C, B
MB4	MR-17, 27	*Desulfobacca acetoxidans*, *Geothermobacter ehrlichii*	DP
MB5	MR-14, 16, 18, 23, 24, 25	*D. acetoxidans*, *D. mexicanus*	DP
MB6	MR-1, 6, 15, 21	*T. flocculiformis*, *D. mexicanus*	B, DP
MB7	MR-1	*T. flocculiformis,*	B
MB8	MR-3, 9, 11	*Acetobacterium tundrae*, *Acetobacterium wieringae*, *Thermovirga lienii*	C
MA1	MRA-4	Unidentified *Archaea* species	–
MA2	MRA-13	*Methanobacterium beijingense* 8–2	MB
MA3	MRA-4	Unidentified *Archaea* species	–
MA4	MRA-2	*Methanosaeta concilii*	MM
MA5	MRA-5, 7, 9	*Methanosaeta harundinacea*	MM
MA6	MRA-9, 10, 11	*M. harundinacea*	MM
TB1	TR-38, 43	*Pseudomonas* sp. SN1, *Pseudomonas sp. DVS6a*	GP
TB2	TR-37	*Spirochaeta americana* ASpG	S
TB3	TR-4, 41	*Selenomonas ruminantium*, *Pseudomonas* sp. JQR2–5	C, GP
TB4	TR-36, 42, 44, 46	*D. mexicanus*, *Pseudomonas* sp. SN1	DP, GP
TB5	TR-6, 21	*Desulfotomaculum geothermicum*	C
TB6	TR-39	*Desulfovibrio gigas*	DP
TB7	TR-12, 25, 26, 28	*Thermodesulfovibrio yellowstonii*, *Thermoanaerobacter tengcongensis MB4*	C, N
TB8	TR-1, 2, 10, 40	*T. lienii, Thermacetogenium phaeum*, *D. mexicanus*	C, DP
TB9	TR-3	*Thermoanaerobacter subterraneus*	C
TA1	TRA-2, 3, 9, 16	*Methanobacterium aarhusense*, *Methanothermobacter thermoautotrophicus* GC-1	MB
TA2	TRA-4	*M. beijingense* 8-2	MB
TA3	TRA-8, 11, 17, 18	*M. aarhusense*, *Methanomethylovorans thermophilia*	MB, MM
TA4	TRA-14	*M. thermophilia*	MM
TA5	TRA-7, 10	*M. concilii*, *M. harundinacea*	MM
TA6	TRA-6	Unidentified *Archaea* species	–
TA7	TRA-1, 5, 15	*M. thermoautotrophicus* GC-1	MB

[a] Abbreviations for taxonomical classes within Bacteria and Archaea kingdoms: B – Bacilli, C – Clostridia, DP – δ-Proteobacteria, GP – γ-Proteobacteria, N – Nitrospira, S – Spirochaetes, MB – Methanobacteria, MM – Methanomicrobia

impact of particular phylogenetic groups on reactor performance. These findings demonstrate the inherent difficulties in DGGE analysis, and they indicate the need of clone library and sequencing analysis to understand DGGE banding patterns.

The bands that were associated with the identified clones provide insight into the changing community structure and, for the majority of cases, also confirm the relative abundance of OTUs based on clone library analysis. In the MR, for example, OTUs related to *D. acetoxidans* corresponded to the most prominent band at day 447, MB5, and also represent 77% of all clones in the MR447 library. On the other hand, the bands at position MB3 and MB8, related to *A. malicum*-like *s*equences, and to *A. tundrae* and *A. wieringae*-like sequences, respectively, increased in intensity after day 197, suggesting that the shift to the influent containing citrate, IPA and PEG, lead to an increase in the relative abundance of the *Acetobacterium* community. The increasing intensity of band MB6 at day 424 most probably corresponds with the increasing abundance of *D. mexicanus* related OTUs in the clone library MR447.

Similar correlations between relative OTU abundance in clone libraries and DGGE were observed for the archaeal community. As an example, the OTU MR-9/10 was found to contain over 50% of clones related to the methanogen, *M. harundinacea*, corresponding to the prominent band MA5 observed in the archaeal DGGE profiles of samples collected from the mesophilic reactor after 197 days of operation. Similarly, the dominant species in the TR archaeal clone libraries, *M. thermautotrophicus* GC-1, corresponded to the intense TA7 band.

This study confirms the biodiversity of mesophilic and thermophilic microbial communities maintained in anaerobic bioreactors fed with simple electron donors such as ethanol or with a mixture of citrate, isopropanol and polyethylene glycol (M_n 300). Comparisons of RNA- and DNA-targeted analyses revealed that the molecular diversity of the metabolically active microbial fraction appeared to vary significantly from the overall diversity both in dominant phylogenetic associations and number of unique clones in bacterial and archaeal libraries.

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References

Bak F, Pfennig N (1987) Chemolithotrophic growth of *Desulfovibrio sulfodismutans* sp. nov. by disproportionation of inorganic sulfur-compounds. Arch Microbiol 147:184–189

Ben-Amor K, Heilig H, Smidt H et al (2005) Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. Appl Environ Microbiol 71:4679–4689

Boyd JM, Ellsworth H, Ensign SA (2004) Bacterial acetone carboxylase is a manganese-dependent metalloenzyme. J Biol Chem 279:46644–46651

Burggraf S, Jannasch HW, Nicolaus B et al (1990) *Archaeoglobus profundus* sp. nov, represents a new species within the sulfate-reducing archaebacteria. Syst Appl Microbiol 13:24–28

Chauhan A, Ogram A (2006) Fatty acid-oxidizing consortia along a nutrient gradient in the Florida Everglades. Appl Environ Microbiol 72:2400–2406

Clesceri LS, Greenberg AE, Eaton AD (eds) (1998) Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association, Washington, DC

Dahle H, Birkeland NK (2006) *Thermovirga lienii* gen. nov., sp nov., a novel moderately thermophilic, anaerobic, amino-acid-degrading bacterium isolated from a North Sea oil well. Int J Syst Evol Microbiol 56:1539–1545

Dar SA, Yao L, Van Dongen U et al (2007) Diversity and activity of sulfate reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and *dsr*B genes as molecular markers. Appl Environ Microbiol 73:594–604

Daumas S, Cordruwisch R, Garcia JL (1988) *Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H2 from geothermal ground-water. Anton Leeuwen 54: 165–178

Davies R, Stephenson M (1941) Studies on the acetone-butyl-alcohol fermentation. 1. Nutritional and other factors involved in the preparation of active suspensions of *Clostridium acetobutylicum* (Weizmann). Biochem J 35:1320–1331

Dwyer D, Tiedje J (1986) Metabolism of polyethylene glycol by two anaerobic bacteria, *Desulfovibrio desulfuricans* and a *Bacteroides* sp. Appl Environ Microbiol 52: 852–856

Egert M, Wagner B, Lemke T, Brune A et al (2003) Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (*Coleoptera: Scarabaeidae*). Appl Environ Microbiol 69:6659–6668

Ekiel I, Sprott GD, Patel GB (1985) Acetate and CO_2 assimilation by *Methanothrix concilii*. J Bacteriol 162:905–908

Ensign SA, Small FJ, Allen JR, Sluis MK (1998) New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. Arch Microbiol 169:179–187

Fardeau ML, Salinas MB, L'Haridon S et al (2004) Isolation from oil reservoirs of novel thermophilic anaerobes phylogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus, Thermoanaerobacter yonseiensis*, *Thermoanaerobacter tengcongensis* and *Carboxydibrachium pacificum* to *Caldanaerobacter subterraneus* gen. nov., sp nov., comb. nov. as four novel subspecies. Int J Syst Evol Microbiol 54:467–474

Felsenstein J (1985) Confidence-limits on phylogenies – an approach using the bootstrap. Evolution 39:783–791

Felske A, Wolterink A, Van Lis R, Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl Environ Microbiol 64:871–879

Fukui M, Teske A, Assmus B et al (1999) Physiology, phylogenetic relationships, and ecology of filamentous sulfate-reducing bacteria (genus *Desulfonema*). Arch Microbiol 172:193–203

Garcia JL (1990) Taxonomy and ecology of methanogens. Fems Microbiol Rev 87:297–308

Grosskopf R, Janssen PH, Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol 64:960–969

Hattori S, Kamagata Y, Hanada S et al (2000) *Thermacetogenium phaeum* gen. nov., sp nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int J Syst Evol Microbiol 50:1601–1609

Henry EA, Devereux R, Maki JS et al (1994) Characterization of a new thermophilic sulfate-reducing bacterium – *Thermodesulfovibrio yellowstonii*, gen. nov. and sp. nov. – its phylogenetic relationship to *Thermodesulfobacterium commune* and their origins deep within the bacterial domain. Arch Microbiol 161:62–69

Hernandez-Eugenio G, Fardeau ML, Patel BKC et al (2000) *Desulfovibrio mexicanus* sp nov., a sulfate-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor treating cheese wastewaters. Anaerobe 6:305–312

Hollingsworth J, Sierra-Alvarez R, Zhou M et al (2005) Anaerobic biodegradability and methanogenic toxicity of key constituents in copper chemical mechanical planarization effluents of the semiconductor industry. Chemosphere 59:1219–1228

Hoover RB, Pikuta EV, Bej AK et al (2003) *Spirochaeta americana* sp nov., a new haloalkallphilic, obligately anaerobic spirochaete isolated from soda Mono Lake in California. Int J Syst Evol Microbiol 53:815–821

Janssen PH, Schink B (1995a) Catabolic and anabolic enzyme-activities and energetics of acetone metabolism of the sulfate-reducing bacterium *Desulfococcus biacutus*. J Bacteriol 177:277–282

Janssen PH, Schink B. 1995b. Metabolic pathways and energetics of the acetone-oxidizing, sulfate-reducing bacterium, *Desulfobacterium cetonicum*. Arch Microbiol 163:188–194

Jiang B, Parshina SN, van Doesburg W et al (2005) *Methanomethylovorans thermophila* sp nov., a thermophilic, methylotrophic methanogen from an anaerobic reactor fed with methanol. Int J Syst Evol Microbiol 55:2465–2470

Joulian C, Patel BKC, Ollivier B et al (2000) *Methanobacterium oryzae* sp nov., a novel methanogenic rod isolated from a Philippines ricefield. Int J Syst Evol Microbiol 50:525–528

Kaksonen AH, Plumb JJ, Franzmann PD et al (2004) Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. Fems Microbiol Ecol 47:279–289

Karri S, Sierra-Alvarez R, Field JA (2006) Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. Chemosphere 62:121–127

Kashefi k, Holmes DE, Baross JA et al (2003) Thermophily in the *Geobacteraceae*: *Geothermobacter ehrlichii* gen. nov., sp. nov., a novel thermophilic member of the *Geobacteraceae* from the ''Bag City'' hydrothermal vent. Appl Environ Microbiol 69:2985–2993

Konstantinov SR, Awati AA, Williams BA et al (2006) Post-natal development of the porcine microbiota composition and activities. Environ Microbiol 8:1191–1199

Lane D (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley & Sons Ltd, Chichester, UK, pp 115–175

Leclerc M, Delgenes JP, Godon JJ (2004) Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. Environ Microbiol 6:809–819

Lens PNL, Visser A, Janssen AJH et al (1998) Biotechnological treatment of sulfate-rich wastewaters. Crit Rev Environ Sci Technol 28:41–88

Liu JR, Tanner RS, Schumann P et al (2002) Emended description of the genus *Trichococcus*, description of *Trichococcus collinsii* sp nov., and reclassification of *Lactosphaera pasteurii* as *Trichococcus pasteurii* comb. nov and of *Ruminococcus palustris* as *Trichococcus palustris* comb. nov in the low-G+C Gram-positive bacteria. Int J Syst Evol Microbiol 52:1113–1126

Lomans BP, Maas R, Luderer R, den Camp H et al (1999) Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. Appl Environ Microbiol 65:3641–3650

Ludwig W, Strunk O, Westram R et al (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Ma K, Liu XL, Dong XZ (2005) *Methanobacterium beijingense* sp nov., a. novel methanogen isolated from anaerobic digesters. Int J Syst Evol Microbiol 55:325–329

Ma K, Liu XL, Dong XZ (2006) *Methanosaeta harundinacea* sp nov., a novel acetate-scavenging methanogen isolated

from a UASB reactor. Int J Syst Evol Microbiol 56: 127–131

McGinnis S, Madden TL (2004) BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32:W20–W25

Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal RNA. Appl Environ Microbiol 59:695–700

Nanninga HJ, Gottschal JC (1987) Properties of *Desulfovibrio carbinolicus* sp. nov. and other sulfate-reducing bacteria isolated from an anaerobic-purification plant. Appl Environ Microbiol 53:802–809

Nubel U, Engelen B, Felske A et al (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol 178:5636–5643

O'Flaherty V, Colleran E (1999) Effect of sulphate addition on volatile fatty acid and ethanol degradation in an anaerobic hybrid reactor. I: process disturbance and remediation. Bioresource Technol 68:101–107

Oude-Elferink S, Luppens SBI, Marcelis CLM et al (1998) Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge. Appl Environ Microbiol 64:2301–2303

Patel GB, Sprott GD (1990) *Methanosaeta concilii* gen. nov, sp. nov (*Methanothrix concilii*) and *Methanosaeta thermoacetophila* nom. rev, comb. nov. Int J Syst Bacteriol 40:79–82

Platen H, Temmes A, Schink B (1990) Anaerobic dgradation of acetone by *Desulfococcus biacutus* spec. nov. Arch Microbiol 154:355–361

Platen H, Schink B (1987) Methanogenic degradation of acetone by an enrichment culture. Arch Microbiol 149:136–141

Platen H, Schink B (1989) Anaerobic degradation of acetone and higher ketones by newly isolated denitrifying bacteria. J Gen Microbiol 135:883–891

Roest K, Altinbas M, Paulo PL et al (2005a) Enrichment and detection of microorganisms involved in direct and indirect methanogenesis from methanol in an anaerobic thermophilic bioreactor. Microb Ecol 50:440–446

Roest K, Heilig H, Smidt H et al (2005b) Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. Syst Appl Microbiol 28:175–185

Scheff G, Salcher O, Lingens F (1984) *Trichococcus flocculiformis* gen nov. sp. nov. a new Gram-positive filamentous bacterium isolated from bulking sludge. Appl Microbiol Biotechnol 19:114–119

Schink B, Stieb M (1983) Fermentative degradation of polyethylene glycol by a strictly anaerobic, gram-negative, non-sporeforming bacterium, *Pelobacter venetianus* sp. nov. Appl Environ Microbiol 45:1905–1913

Schramm E, Schink B (1991) Ether-cleaving enzyme and diol dehydratase involved in anaerobic polyethylene glycol degradation by a new *Acetobacterium* sp. Biodegradation 2:71–79

Schuppert B, Schink B (1990) Fermentation of methoxyacetate to glycolate and acetate by newly isolated strains of *Acetobacterium* sp. Arch Microbiol 153:200–204

Stahl DA, Fishbain S, Klein M et al (2002) Origins and diversification of sulfate-respiring microorganisms. Antonie Leeuwen 81:189–195

Stetter KO, Huber R, Blochl E et al (1993) yperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature 365:743–745

Stumm W, Morgan JJ (1996) Aquatic chemistry: chemical equilibria and rates in natural waters, 3rd edn. John Wiley & Sons, Inc, New York, USA

Tanaka K, Pfennig N (1988) Fermentation of 2-methoxyethanol by *Acetobacterium malicum* sp. nov. and *Pelobacter venetianus*. Arch Microbiol 149:181–187

Trüper HG, Schlegel HG (1964) Sulphur metabolism in *thiorhodaceae*: quantitative measurements on growing cells of *Chromatium okenii*. Antonie Leeuwen 30:225–238

Utgikar VP, Chen BY, Chaudhary N, Tabak HH, Haines JR, Govind R (2001) *Environ*. Toxicol Chem 20:2662–2669

Visser A, Nozhevnikova AN, Lettinga G (1993) Sulfide inhibition of methanogenic activity at various pH levels at 55°C. J Chem Technol Biotechnol 57:9–13

Wagener S, Schink B (1988) Fermentative degradation of nonionic surfactants and polyethylene-glycol by enrichment cultures and by pure cultures of homoacetogenic and propionate-forming bacteria. Appl Environ Microbiol 54:561–565

Wagner R (1994) The regulation of ribosomal RNA synthesis and bacterial cell growth. Arch Microbiol 161:100–109

Widdel F (1986) Growth of methanogenic bacteria in pure culture with 2-propanol and other alcohols as hydrogen donors. Appl Environ Microbiol 51:1056–1062

Yamada T, Sekiguchi Y, Imachi H et al (2005) Diversity, localization, and physiological properties of filamentous microbes belonging to *Chloroflexi* subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl Environ Microbiol 71:7493–7503

Yamada T, Sekiguchi Y, Hanada S et al (2006) *Anaerolinea thermolimosa* sp nov., *Levilinea saccharolytica* gen. nov., sp nov and *Leptolinea tardivitalis* gen. nov., so. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov and *Caldilineae* classis nov in the bacterial phylum *Chloroflexi*. Int J Sys Evol Microbiol 56:1331–1340

Zeikus JG, Wolfe RS (1972) *Methanobacterium thermoautotrophicus* sp n, an anaerobic, autotrophic, extreme thermophile. J Bacteriol 109:707–713