Appl Microbiol Biotechnol (2012) 93:2181–2191
DOI 10.1007/s00253-011-3529-3

ENVIRONMENTAL BIOTECHNOLOGY

Molecular analysis of the biomass of a fluidized bed reactor treating synthetic vinasse at anaerobic and micro-aerobic conditions

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Received: 20 May 2011 / Revised: 22 July 2011 / Accepted: 3 August 2011 / Published online: 23 August 2011

Abstract The microbial communities (Bacteria and Archaea) established in an anaerobic fluidized bed reactor used to treat synthetic vinasse (betaine, glucose, acetate, propionate, and butyrate) were characterized by denaturing gradient gel electrophoresis (DGGE) and phylogenetic analysis. This study was focused on the competitive and syntrophic interactions between the different microbial groups at varying influent substrate to sulfate ratios of 8, 4, and 2 and anaerobic or micro-aerobic conditions. Acetogens detected along the anaerobic phases at substrate to sulfate ratios of 8 and 4 seemed to be mainly involved in the fermentation of glucose and betaine, but they were substituted by other sugar or betaine degraders after oxygen application. Typical fatty acid degraders that grow in syntrophy with methanogens were not detected during the entire reactor run. Likely, sugar and betaine degraders outnumbered them in the DGGE analysis. The detected sulfate-reducing bacteria (SRB) belonged to the hydrogen-utilizing *Desulfovibrio*. The introduction of oxygen led to the formation of elemental sulfur (S^0) and probably other sulfur compounds by sulfide-oxidizing bacteria (*γ-Proteobacteria*). It is likely that the sulfur intermediates produced from sulfide oxidation were used by SRB and other microorganisms as electron acceptors, as was supported by the detection of the sulfur respiring *Wolinella succinogenes*. Within the Archaea population, members of *Methanomethylovorans* and *Methanosaeta* were detected throughout the entire reactor operation. Hydrogenotrophic methanogens mainly belonging to the genus *Methanobacterium* were detected at the highest substrate to sulfate ratio but rapidly disappeared by increasing the sulfate concentration.

Keywords Sulfate-reducing bacteria · DGGE · Micro-aerobic · Sulfur-oxidizing bacteria · Methanogenesis

Electronic supplementary material The online version of this article (doi:10.1007/s00253-011-3529-3) contains supplementary material, which is available to authorized users.

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Introduction

Anaerobic treatment of sulfate-rich wastewaters such as vinasse, the aqueous by-product from the distillation of ethanol, is widely used as an effective and economical treatment option (de Menezes 1989; van Haandel and Catunda 1994). The anaerobic digestion of organic material in the presence of sulfate is a complex process in which different physiological microbial groups mineralize organic matter to ultimately CH_4, CO_2, and H_2S (Schink and Stams 2006). In this process, sulfate-reducing bacteria (SRB) are able to couple the oxidation of organic compounds and hydrogen to sulfate reduction producing H_2S. However,

SRB are physiologically versatile microorganisms that are also able to grow in the absence of sulfate in syntrophy with methanogens (Castro et al. 2000; Stams et al. 2005).

Metabolic interactions between the different physiological microbial groups involved in the anaerobic process are an important research aspect. In anaerobic bioreactors, SRB can compete with methanogens and acetogens for common substrates. Since SRB are more involved in the last stages of mineralization (Rivers-Singleton 1993), it is generally agreed they do not effectively compete with hydrolytic and primary fermentative bacteria (Colleran et al. 1995), even though some SRB have the ability to grow on sugars (Sass et al. 2002), amino acids (Stams et al. 1985), and other substrates.

Hydrogen constitutes an important intermediate for which different microbes compete. Thermodynamically, the outcome of the competition between SRB and methanogens is clear; methanogens are outcompeted by SRB. This is clearly supported by several works (O'Flaherty et al. 1998; Sousa et al. 2009). However, despite that acetogenesis is less favorable than sulfate reduction, the outcome of the competition between SRB and acetogens is not always clear. In fact, the co-existence of these two bacterial groups has been widely reported. In a bioreactor fed with H_2 and small quantities of CO_2, Weijma et al. (2002) observed that sulfate reduction was the main process, but acetogenesis occurred as well. Since some members of SRB found in that study use acetate as carbon source, Stams et al. (2005) proposed that SRB were dependent for growth on acetogens. In anaerobic digesters, where carbon sources are not a limiting factor, the co-existence of SRB and acetogens has been reported. The capacity of acetogens to grow heterotrophically on a wide range of organic compounds and their mixotrophic ability and versatility has been proposed to explain their prevalence (Zehnder and Stumm 1988).

Butyrate and propionate are important intermediates in the anaerobic degradation process. Propionate and butyrate oxidation are energetically very unfavorable reactions and syntrophic interaction with methanogens or SRB is needed to make these oxidations feasible (Schink and Stams 2006). Thermodynamically, SRB easily outcompete the syntrophic fatty-acid-degrading consortia, though the interactions between these microbial groups are quite complex. Stams et al. (2005) proposed that the concentration of sulfate determines the competition. As hydrogen-utilizing SRB seem to have higher affinity for sulfate than acetate-, propionate-, or butyrate-degrading SRB it is likely to assume that syntrophic sulfate-reducing cocultures are most important at sulfate-limiting conditions. In fact, *Syntrophobacter* species which are able to grow on propionate and sulfate prefer to grow syntrophically in coculture with *Desulfovibrio*.

Regarding to acetate, the outcome of the competition between SRB and methanogens is not clear. Results reported are contradictory, e.g., a predominance of SRB and acetoclastic methanogens has been shown (Gupta et al. 1994; Oude Elferink et al. 1995), but a predominance of methanogens also has been reported (Omil et al. 1996, O'Flaherty et al. 1998). Moreover, acetate-degrading SRB have only slightly better growth kinetic properties than *Methanosaeta* (Stams et al. 2005).

Besides the importance of the knowledge about the interactions between the microorganisms, sulfide production receives considerable attention in the anaerobic treatment of sulfate-rich wastewaters. Sulfide in the liquid can cause inhibition of the anaerobic organisms. The pH seems to be an important factor, since only the unionized form of sulfide is able to pass through the cell membrane (Speece 1983). Also, the different threshold of sulfide toxicity between the different bacterial groups (McCartney and Oleskiewicz 1991; O'Flaherty et al. 1998) and the position of the microorganisms in the process, e.g., in granular reactors, are important factors. Other problems associated to sulfide either in the effluent or in the biogas are unpleasant odors, corrosiveness, and toxicity. Hence, their formation has to be avoided. Driven by this prerequisite, introduction of limited quantities of oxygen/air to anaerobic bioreactors has been considered to lower the levels of sulfide. This technique involves the oxidation of sulfide by sulfur-oxidizing bacteria (SOB) under limiting oxygen conditions. Various studies have demonstrated that, under oxygen limitation, sulfide is mainly converted to elemental sulfur (S^0) and thiosulfate by SOB (van den Ende and van Gemerden 1993; van den Ende et al. 1997; van der Zee et al. 2007; Janssen et al. 1999). Therefore, the introduction of low oxygen concentrations in sulfate-rich wastewaters complicates the anaerobic degradation process since the sulfur intermediates produced by the SOB can be used by the SRB and other anaerobic bacteria (van den Ende et al. 1997).

Molecular tools, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), allow to get insight into the microorganisms involved in the anaerobic process and to elucidate the role of these microorganisms and the interactions between them. This technique was used to study the microbial communities and their temporal variation established in an anaerobic fluidized bed reactor supplied with synthetic vinasse under three different substrate to sulfate ratios and under anaerobic or micro-aerobic conditions.

Materials and methods

Granular activated carbon reactor operation

An anaerobic fluidized bed reactor (V=4 L) containing granular activated carbon (GAC) as support material (average particle diameter between 0.42 and 0.45 mm)

and inoculated with sludge from an industrial digester treating wastewater from sugar production was fed with synthetic vinasse consisting of betaine, acetate, butyrate, propionate, and glucose in the chemical oxygen demand (COD) ratio of 1.0:2.3:2.3:2.3:3.4 to a total COD in each operation phase. The synthetic influent was buffered with $NaHCO_3$, and micro- and macro-nutrients were added as described by Angelidaki et al. (2009). The pH in the reactor was not controlled, and it ranged between 7.0 and 7.3. The reactor was operated for approximately 700 days at 35±2°C with a hydraulic retention time of 2 days. The expansion of the bed reactor was maintained at ~30% by controlling the effluent recycle flow of the reactor (400). The upflow velocity was established at 13 mh^{-1}.

Three operation phases were imposed to the reactor based on different substrate to sulfate ratios of 8, 4, and 2. This was achieved by varying the COD and the sulfate concentration or only the sulfate concentration added to the reactor. Additionally, low concentrations of oxygen were introduced from the bottom into the reactor since phase 2 till the end of the experiment. Table 1 summarizes the details of the different operation phases applied during the experiment.

Chemical analysis

The performance of the fluidized bed reactor was routinely monitored by measuring pH, temperature, redox potential, total and soluble COD, total and volatile suspended solids (TSS, VSS), sulfate, sulfide, nitrite, nitrate, and total Kjeldahl nitrogen (TKN). Total and soluble COD, TSS, VSS, and TKN were determined according to standard methods (Clesceri et al. 1998). Biogas composition (O_2, N_2, CH_4, CO_2, H_2S) was determined by gas chromatography (GC) on a Hewlett Packard 6890 series II equipped with a TCD (thermal conductivity detector) and on a Varian CP3800 equipped with a TCD. Carrier gas was helium in both cases. Anions (sulfate and thiosulfate) were determined by high-performance liquid chromatography.

Sample collection, DNA isolation, and 16S rRNA gene amplification

The effect on the microbial community at the different substrate to sulfate ratios and the anaerobic or micro-aerobic operations was studied. GAC samples used for this purpose were taken in each operation phase from the mid-section of the reactor, immediately frozen at −20°C and stored until DNA extraction was performed. Biomass samples of the headspace walls of the reactor, the upper part of the headspace (the lid) of the reactor, and seed were collected too and stored at −20°C. Table 1 shows the details of sample collection. DNA was extracted with the Fast® DNA Spin Kit for Soil (MP Biomedicals, LLC) according to the manufacturer protocol, adjusting the speed and the time of shaking of samples in the Mini-Bead-beater apparatus and the time of the samples on a rotator to allow binding of DNA to a silica matrix (from 2 min to 1 h). DNA was stored at −20°C.

The V6–V8 regions of the bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the universal bacterial primers 968-F-GC and 1401-R (Sigma-Aldrich, St. Louis, MO, USA; Nübel et al. 1996). The V2–V3 regions of the archaeal 16S rRNA genes were amplified for DGGE using the primers A109(T)-F and 515-GC-R (Sigma-Aldrich, St. Louis, MO, USA) (Lane 1991; Muyzer et al. 1993; Grosskopf et al. 1998). The PCR mixture (50 μl) contained 1 μl of each primer (10 ng μl^{-1} each primer), 10 μl

Table 1 Different bioreactor conditions applied and details of sampling

Process	Operation phases	Sampling day	Periods in each phase	Sample name	O_2 flow rate (ml min^{-1})	OLR (kg m^{-3} d^{-1})	COD (mg l^{-1})	SO_4^{2-} (mg l^{-1})
		0	–	Seed	–	–	–	–
Anaerobic	Phase I COD/SO_4^{2-}=8	95	1	1	0	0.5	1,000	120
		184	2	2	0	1	2,000	240
		197	3	3	0	2	4,000	480
		280	4	4	0	3.1	6,000	720
	Phase II COD/SO_4^{2-}=4	349	5	5	0	3.1	6,000	1,440
		426		6				
Micro-aerobic	Phase II COD/SO_4^{2-}=4	502	6	7	0.26	3.1	6,000	1,440
		533		8				
	Phase III COD/SO_4^{2-}=2	645	7	9	0.52	3.1	6,000	2,880
		709		10				
		769	–	Headspace	–	–	–	–
		769	–	Lid	–	–	–	–

OLR organic loading rate, *COD* chemical oxygen demand

of Taq and GO PCR Mastermix (MP Biomedicals, LLC) containing *Taq* DNA polymerase, PCR reaction buffer and dNTPs (MP Biomedicals, LLC), 1 or 2 μl of DNA template, and Milli-Q water until the final volume of the PCR reaction. PCR was performed in an iCycler Thermal Cycler (Bio Rad Laboratories, Inc.) with the following thermo-cycling program for bacterial amplification—2 min of pre-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. For archaeal amplification, the thermo-cycling program differed only in the annealing step, which was 52°C for 40 s, and the elongation step, which was 90 s long. Size and yield of PCR products were estimated using a 2,000-bp DNA ladder, Hypperladder II (Bioline, USA Inc.) in 1.8% agarose gel (*w/v*) electrophoresis and SYBR Green I staining.

DGGE analysis

DGGE analysis of the amplicons was performed on 8% (*w/v*) polyacrilamide gels using urea/formamide denaturant gradients of 42–67% and 30–60% for bacterial and archaeal communities, respectively (Ben-Amor et al. 2005; Roest et al. 2005a). Electrophoresis was performed with a D-Code Universal Mutation Detection System (Bio Rad Laboratories, Inc.) in 0.5× TAE buffer at 60°C and 85 V for 16 h for bacterial amplicons and 60°C and 65 V for 18 h for archaeal amplicons. During the first 5 min of the electrophoresis, a voltage of 200 V was applied. The gels were stained with SYBR Green I nucleic acid gel stain (1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) for 1 h.

Sequencing and phylogenetic analysis

Individual bands were excised from the DGGE gel with a sterile blade, resuspended in 50 μl of ultrapure water, and maintained at 60°C for 1 h to allow DNA extraction from the gel. A volume of 5 μl of the supernatant was used for reamplification with the original primer sets. Some reamplified PCR products were run again on a DGGE to check their purity and mobility on the DGGE gel. Before sequencing, PCR products were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). The taxonomic hierarchy of the sequences was determined using the RDP classifier tool (Wang et al. 2007) with 80% confidence level. Sequences were also compared with sequences in GenBank by BLAST search tool at the NCBI (National Centre for Biotechnology Information) (McGinnis and Madden 2004). Alignment of the 16S rRNA sequences was performed by using the RDP INFERNAL aligner (Nawrocki et al. 2009) at the RDP website. Subsequently, the alignment was manually checked and corrected when necessary, and phylogenetic trees were constructed by 1,000-fold bootstrap analysis using neighbor-joining methods. Trees were edited using MEGA 3 (Kumar et al. 1994).

Sequences generated from this work are deposited at GenBank under accession numbers JF937202- JF937239.

Results

Reactor operation

The averaged reactor performance data over the operation periods are summarized in Table 2. COD removal efficiencies ranged between 90% and 97% during the two first operation phases, but a slight decrease in COD removal was observed for operation phase 3, being 86.7±9.3% (the COD contribution of sulfide was not considered). The mean sulfate removal efficiency was about 100% during the different reactor operation periods, except for operation phase 3 where the sulfate removal efficiency decreased to an average value of about 78%. Thiosulfate was not

Table 2 Mean values obtained for the different operation phases of the GAC bioreactor

Process	Operation phases	Periods in each phase	COD removal (%)	SO_4^{2-} removal (%)	Biogas composition (%)				
					O_2	CO_2	N_2	H_2S	CH_4
Anaerobic	Phase I $COD/SO_4^{2-}=8$	1	91.1±2.9	100.0±0.0	0.8±0.3	10.6±0.7	11.3±3.7	0.1±0.1	77.2±3.4
		2	90.4±0.0	–	0.3±0.1	28.1±3.8	2.0±2.6	0.4±0.1	69.3±1.5
		3	91.7±0.1	95.5±6.3	0.3±0.3	28.6±1.9	1.6±1.1	0.7±0.1	68.8±1.9
		4	92.9±3.7	96.8±4.2	0.1 ±0.1	29.7±4.2	1.0±0.4	1.2±0.8	67.9±4.1
	Phase II $COD/SO_4^{2-}=4$	5	95.0±2.8	99.7±0.5	0.1±0.2	30.2±5.3	1.0±0.8	2.5±0.5	66.2±5.3
Micro-aerobic	Phase II $COD/SO_4^{2-}=4$	6	96.8±2.2	95.3±4.9	4.1±2.9	38.3±3.0	1.2±0.7	0.7±0.9	55.7±2.0
	Phase III $COD/SO_4^{2-}=2$	7	86.7±9.3	78.3±13.1	4.3±2.4	41.8±3.8	2.7±1.2	1.0±1.4	50.2±5.3

detected during the entire anaerobic process operation, but concentrations of 0.5 to 2.5 mg l^{-1} were detected in the effluent during the micro-aerobic phase (data not shown).

During the anaerobic process (periods 1 to 5), methane yields of the GAC bioreactor constituted between 77% and 66% of the total biogas produced, but in the micro-aerobic phase, the methane levels in the biogas decreased noticeably (between 50% and 58%). The opposite was observed for CO_2. During the micro-aerobic phase, the gas contained between 38% and 42% CO_2. Sulfide levels in the biogas increased throughout the anaerobic process due to the increase in the organic loading rate at phase 1 and due to the increase sulfate concentrations in the influent from the operation phase 1 to the operation phase 2. During the micro-aerobic operation period, not all sulfide was removed from the biogas, but the sulfide levels decreased considerably. From the oxygen balance calculated, a considerably part of the oxygen introduced into the reactor was observed in the biogas (data not shown). Table 3 shows the sulfur balance, which was calculated taking into account the mean values of the influent and effluent sulfate and thiosulfate concentrations and the mean values of the sulfide detected in the biogas and in the liquid effluent. Under anaerobic conditions, the gap in the sulfur balance was low (1–9%) in contrast to the gap of 50–53% observed during the micro-aerobic phase. This was mainly due to the elemental sulfur accumulated at the headspace of the reactor (Fig. 1). This compound could not be quantified.

Bacterial DGGE dynamics and phylogenetic analysis

The shift in the predominant bacterial population due to different conditions along reactor operation was recorded by DGGE profiling of 16S rRNA gene fragments. Figure 2 illustrates the profile of the bacterial 16S rRNA gene DGGE. The phylogenetic trees of 16S rRNA gene sequences are presented in Figs. 3 and 4; Fig. 3 shows the phylogenetic tree constructed with the sequences belonging to the Firmicutes phylum, and Fig. 4 shows the phylogenetic tree constructed with the sequences belonging to the Proteobacteria and Actinobacteria phyla. Taxonomic assignments (based on the RDP classifier tool (80% confidence level)) of the sequenced bands, the closest relatives with each corresponding sequence similarity and the environment from which they were retrieved, as well as the presence or absence of the DGGE bands in each phase are listed in Supplement Table 1.

In general, the bacterial profile changed over the time course (Fig. 2). In most cases, these changes could be related with operation changes applied to the reactor. Using the RDP classifier with a bootstrap value of 80%, nine bands from the bacterial DGGE profile were assigned to the Firmicutes phylum, namely in the Clostridia class (52–100% confidence level). Four bands were affiliated with the Proteobacteria phylum (γ-Proteobacteria, δ-Proteobacteria, and ε-Proteobacteria) with confidence levels higher than 80%. Representatives of the Actinobacteria phylum (bands 18, 19, 20, and 21) were also found, namely in the Actinobacteria class (100% confidence level). According to the results from the RDP classifier, four out of the nine bands within the Firmicutes phylum were affiliated to different genera with bootstrap values of 80% or higher. Band 1 was assigned to *Anaerostipes*, band 6 to *Acetobacterium* and fragments 8 and 11 to *Sporomusa* and *Tissierella* genera, respectively. Within the *Proteobacteria*, sequences corresponding to fragments 15 and 17, and 24 were affiliated to *Desulfovibrio* and *Wolinella* genera, respectively (80% or higher confidence level). The 16S rRNA fragments 18, 20, and 21 within the *Actinobacteria* showed bootstrap confidence values which ranged between 73% and 85% with members of *Eggerthella* genus, while band 19 was assigned to the *Propionibacterium* genus (100% confidence level). In general, the results given by the RDP classifier tool showed consistency with the results obtained from the NCBI Blast search tool.

Archaeal DGGE dynamics and phylogenetic analysis

The shift in the predominant archaeal populations due to the different conditions along reactor operation was

Table 3 Bioreactor S-balance

Process	Operation phases	Periods in each phase	Influent mgS d^{-1}	Effluent mgS d^{-1}	Gap-S (%)
Anaerobic	Phase I COD/SO_4^{2-}=8	1	–	–	–
		2	126	123	2
		3	261	253	3
		4	485	478	1
	Phase II COD/SO_4^{2-}=4	5	688	630	9
Micro-aerobic	Phase II COD/SO_4^{2-}=4	6	714	334	53
	Phase III COD/SO_4^{2-}=2	7	1363	680	50

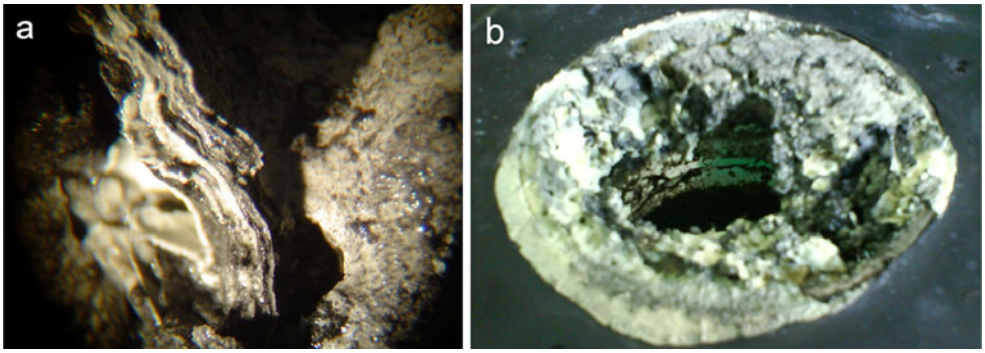

Fig. 1 **a** Observation of the elemental sulfur attached to the headspace under magnifying glass. **b** Elemental sulfur attached to the headspace of the reactor

recorded by DGGE profiling of 16S rRNA gene fragments (Fig. 5). The phylogenetic affiliation of 16S rRNA sequences is presented in Fig. 6. The closest groups of the archaeal DGGE bands determined through an NCBI GenBank similarity search, sequence similarity, taxonomic affiliation (RDP classifier), and the presence or absence of the DGGE bands in each phase are shown in Supplement Table 2. The 16S rRNA sequences were affiliated to a unique phylum, *Euryarchaeota* except two sequences (bands 2 and 4) which remained unclassified. The classified sequences were assigned to two classes, Methanomicrobia and Methanobacteria. Most of the bands belonged to the Methanomicrobia class and were assigned to three different genera, *Methanomethylovorans*, *Methanosaeta*, and *Methanosarcina* with confidence values higher than 90%. Fragments 19 and 13, in the *Methanobacteria* class, were affiliated to the *Methanobacterium* genus (100% confidence level).

With the archaeal primers, no DNA could be amplified from samples collected from the lid of the reactor. In general, DGGE banding patterns from archaeal communities showed a more homogenous population than the bacterial banding patterns, appearing to be more conserved over the time course (Fig. 5).

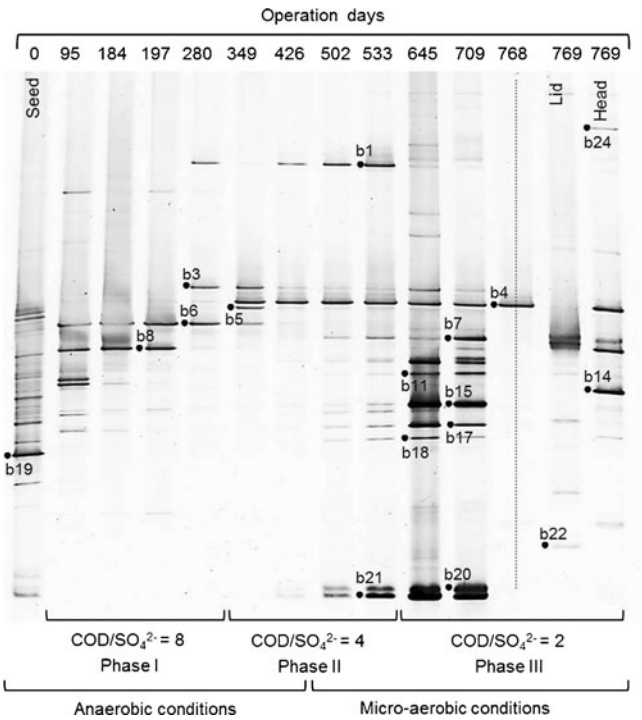

Fig. 2 Bacterial DGGE profiles of 16S rRNA amplicons from the GAC reactor. *Lane upper numbers* indicate the operation day on which each sample was collected. Sample on day 768 was not considered due to a bioreactor operation problem. The substrate to sulfate ratios and micro-aerobic or anaerobic conditions applied are indicated by keys in the lower part of the gel. DGGE bands are indicated with "*b*" letter and the corresponding *number* of each band

Discussion

This study evaluated the shift in the microbial community structure in an anaerobic fluidized bed reactor fed with betaine, glucose, propionate, butyrate, and acetate, after changing the substrate to sulfate ratios and switching from anaerobic to micro-aerobic conditions using the PCR–DGGE technique. The molecular analysis together with the results obtained by chemical analysis allowed for linking the population structure and composition with function and provides insight into the metabolic interactions of the microbial communities in the reactor. The PCR products from archaeal primers included some bacterial sequences as revealed by comparing with databases at NCBI. This was also reported before and was attributed to either mismatching by the archaeal primers or caused by a relatively high abundance of bacterial DNA in relation to archaea DNA in samples (Leclerc et al. 2004; Roest et al. 2005b). Carbon support samples collected at day 768 in the bacterial and archaeal DGGE profiles were not discussed due to a bioreactor operation problem (at day 715, the reactor lost support material). Although PCR-DGGE profiles produced using DNA templates may not completely reflect the abundance of target sequences in the reactor samples, results from this study revealed the presence of different phylogenetic groups that can be linked to the different conditions applied to the bioreactor.

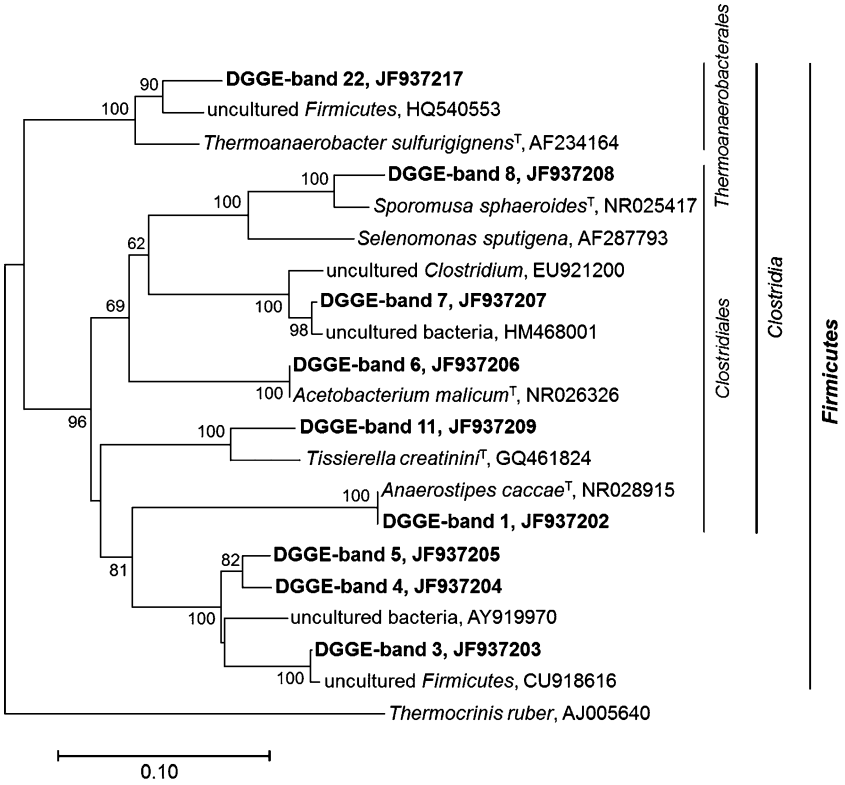

Fig. 3 Bacterial phylogenetic tree based on 16S rRNA sequences affiliated to the *Firmicutes* phylum and reference sequences. Accession numbers are indicated. Sequences obtained in this study are in *bold face*. *Numbers* on the nodes indicate bootstrap values of 50% and higher (1,000 replicates). The *scale bar* indicates 10% sequence difference. *Thermocrinis ruber* (AJ005640)) was used as outgroup

During the anaerobic conditions, DGGE results indicated the presence of acetogens related to *Acetobacterium*, *Sporomusa*, and *Anaerostipes* (bands 6, 8, 1, respectively). Acetogens are able to grow autotrophically with H_2, producing acetate, but based on thermodynamic considerations, it is unlikely that they effectively outcompeted SRB or methanogens for hydrogen. Rather, they can degrade organic substrates such as glucose or betaine and produce hydrogen. In fact, acetogens can convert a variety of substrates to acetate as the major end product (Diekert and

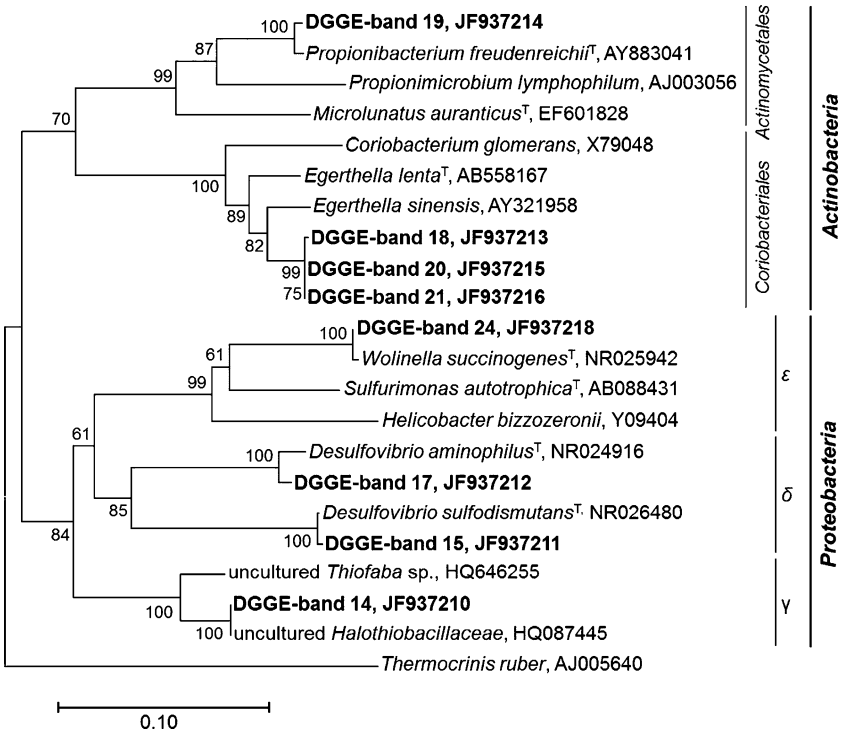

Fig. 4 Bacterial phylogenetic tree based on 16S rRNA sequences affiliated to the Proteobacteria and Actinobacteria phyla and reference sequences. Accession numbers are indicated. Sequences obtained in this study are in *bold face*. *Numbers* on the nodes indicate bootstrap values of 50% and higher (1,000 replicates). The *scale bar* indicates 10% sequence difference. *Thermocrinis ruber* (AJ005640)) was used as outgroup

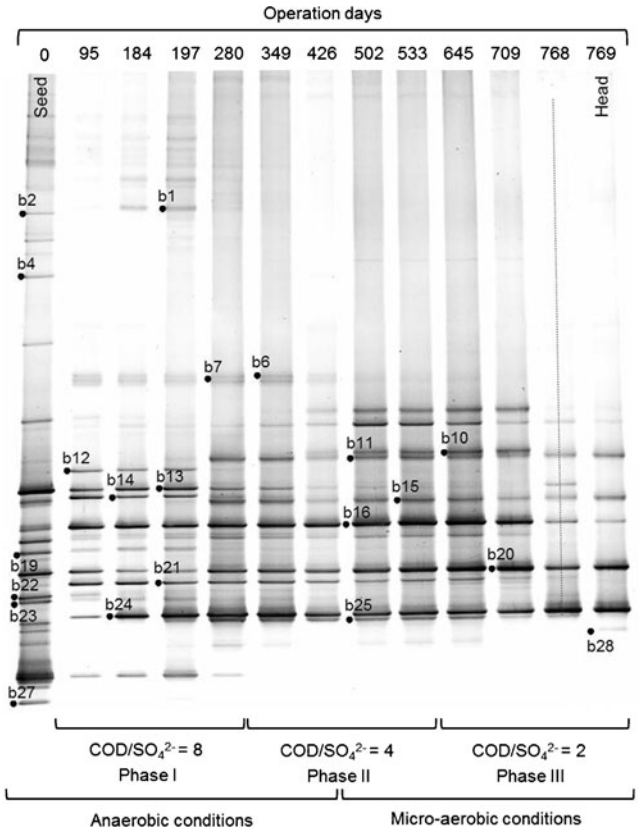

Fig. 5 Archaeal DGGE profiles of 16S rRNA amplicons from the GAC reactor. *Lane upper numbers* indicate the operation day on which each sample was collected. Sample on day 768 was not considered due to a bioreactor operation problem. Sample from the lid of the reactor was also not considered due to the lack of DNA amplification with the archaeal primers used. The substrate to sulfate ratios and micro-aerobic or anaerobic conditions applied are indicated by keys in the lower part of the gel. DGGE bands are indicated with "*b*" letter and the *corresponding number* of each band

Wohlfarth 1994). After oxygen introduction, DGGE bands belonging to acetogens almost disappeared, and they were substituted by other potential sugar or betaine degraders such as members of the Coriobacteriaceae family (fragments 18, 20, and 21) and the *Tissierella* genus (band 11). This shift might be related to a different oxygen tolerance or even to oxygen utilization.

Typical fatty-acid-degrading bacteria such as *Syntrophobacter*, and *Syntrophomonas* and *Syntrophus* which are important propionate- and butyrate-degrading bacteria, respectively (Stams et al. 2005), were not detected by the 16S rRNA gene analysis. In another study (Roest et al. 2005a) in which carbohydrates and short-chain fatty acids were degraded in an anaerobic reactor, fatty-acid degraders were also not detected as dominant microorganisms, but their presence could only be demonstrated by specific enrichment. From these observations, it is likely that sugar or betaine degraders outnumbered fatty-acid degraders in the DGGE analysis.

SRB detected in this work belonged to *Desulfovibrio* genus (fragments 15 and 17). During the anaerobic phase, sulfate was almost completely removed suggesting limiting sulfate concentrations for SRB. Hence, the intensity of the bands of *Desulfovibrio* was low. Based on the ability of *Desulfovibrio* to grow with hydrogen (Voordouw 1995) and their high affinity for sulfate (Laanbroek et al. 1984; O'Flaherty et al. 1998), they likely used hydrogen for sulfate reduction. The observed increase in the intensity of the bands of *Desulfovibrio* just after oxygen introduction could not be attributed to an increase in sulfate availabilty since the substrate to sulfate ratio was maintained. Since sulfate reducers can use many other electron acceptors for growth (Muyzer and Stams 2008), the most likely explanation is that *Desulfovibrio* used other sulfur compounds produced from sulfide oxidation, which results in higher cell yields than with sulfate (Colleran et al. 1995). Also, under micro-aerobic conditions, but at a substrate to sulfate ratio of 2, a further increase in the intensity of the bands of *Desulfovibrio* was observed. In this case, this could be due to the increase in sulfate availabilty, the presence of other electron acceptors, or both. In fact, not all sulfate in the feed was removed at this substrate to sulfate ratio. Likely, acetate was mainly consumed by methanogens, and a depletion of easily degradable substrates for SRB such as hydrogen could have occurred. No SRB are known to degrade fatty acids were detected in this work. Some members in this group are able to ferment fatty acids such as members of *Syntrophobacter* (Stams et al. 2005). In fact, it has been proposed that the concentration of sulfate determines whether the oxidation of butyrate or propionate is directly or indirectly coupled to sulfate reduction (Stams et al. 2005). Since sulfate seemed to be a limited factor for SRB that degrade fatty acids, it is likely that these compounds were degraded by acetogens in syntrophy with H_2-utilizing SRB.

The RDP classifier tool placed the band 14 within the γ-Proteobacteria class. This band was classified in the Halothiobacillaceae family with a low confidence value (38%). Furthermore, closest organisms in GeneBank showed some uncultured Halothiobacillaceae (HQ087445; 99% similarity) and uncultured *Thiofaba* (HQ646255) (95% similarity). Members in the γ-Proteobacteria such as *Halothiobacillus* and *Thiofaba* organisms are SOB, which agrees with the observed decrease in the biogas sulfide levels after oxygen addition. In this work, SOBs were only detected in the sulfur-containing biomass attached to the headspace of the reactor (Fig. 1), suggesting that the oxidation of sulfide occurred at this site. This could be related to the low oxygen solubility in water. Oxygen was introduced from the bottom into the reactor, and a considerably part of the oxygen was detected in the biogas. Similarly, Kobayashi et al. 2011 found that species related

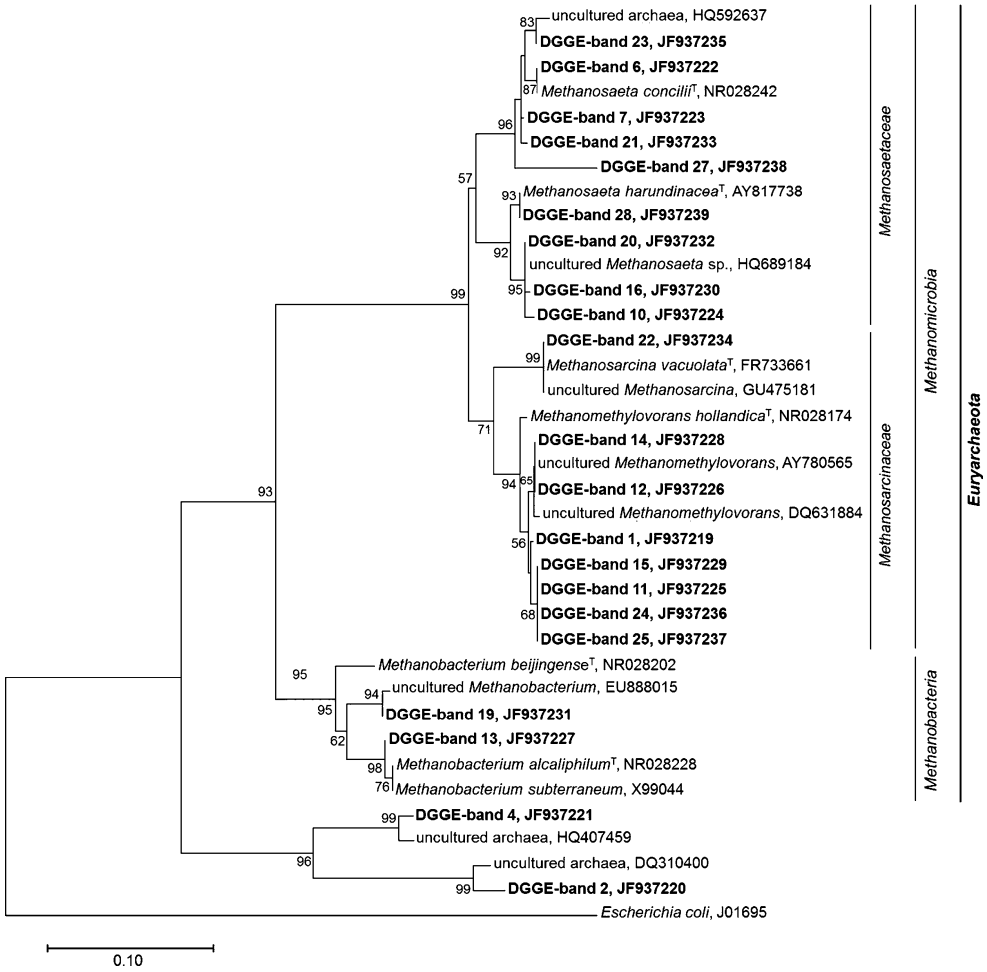

Fig. 6 Phylogenetic tree based on 16S rRNA sequences affiliated to the Archaea domain and reference sequences. Accession numbers are indicated. Sequences obtained in this study are in *bold face*. *Numbers* on the nodes indicate bootstrap values of 50% and higher (1,000 replicates). The *scale bar* indicates 10% sequence difference. *Escherichia coli* (J01695) was used as outgroup

to *Halothiobacillus* played a key role in the sulfide oxidation and sulfur production in the headspace of an anaerobic digester. The large quantity of elemental sulfur found suggests that this was the main compound formed from sulfide oxidation. However, the formation of thiosulfate cannot be ruled out since small amounts of this compound were detected in the effluent of the reactor during the micro-aerobic phase. Other studies have also been shown the oxidation of sulfide to $S_2O_3^-$ and S^0 by SOB under oxygen limitations (Van den Ende and van Gemerden 1993). Moreover, sulfur-respiring organisms affiliated to *Wolinella succinogenes* (fragment 24) were found at the headspace of the reactor. It is known that the intermediates formed by SOB, such as $S_2O_3^-$ and S^0 can be used again by SRB or other bacteria as electron aceptor. Even so, they may disproportionate these compounds (van den Ende et al. 1997). Hence, it is likely that members of the *Wolinella* genus used sulfur for growth-producing sulfide. Fragment 4 was assigned to the Firmicutes phylum. This band could not be classified at lower taxonomic levels; at the genus level, this band showed 18% confidence level with *Desulfitibacter*. Band 4 was present at high intensity in the headspace of the reactor and also in the carbon support samples during the micro-aerobic phase and the anaerobic phase at a substrate to sulfate ratio of 4. The presence of this band at the lowest substrate to sulfate ratios and in the headspace suggests that sulfur compounds could act as electron acceptors for them. Members of *Desulfitibacter* can grow with different sulfur compounds as electron acceptors using substrates such as betaine, formate, or pyruvate (Nielsen et al. 2006). The utilization of sulfur intermediates (produced from sulfide oxidation) by SRB or other sulfur metabolism microorganisms, indicates that part of these intermediates returned again to sulfide. Therefore, a positive feedback mechanism in which sulfur intermediates are converted to sulfide and vice versa is suggested. In this way, the increase CO_2 observed in the biogas during the micro-aerobic phases could be explained by an increased sulfidogenesis over methanogenesis due to the formation of sulfur intermediates. Linked with that, a slightly decrease in methane production was observed during the micro-aerobic phases, i.e., 56% and 50% at the substrate to sulfate ratios of 4 and 2, respectively. However, an inhibitory effect of oxygen on methanogens cannot be ruled out.

The archaea DGGE profiles indicated that hydrogen-utilizing methanogens in the *Methanobacterium* genus

(bands 19 and 13) were present at the higher substrate to sulfate ratio, suggesting that not all hydrogen produced in the reactor was degraded via sulfate reduction but also via hydrogenotrophic methanogenesis. At lower substrate to sulfate ratios, these bands rapidly disappeared, suggesting that they were outcompeted by the hydrogen-utilizing SRB. This agrees with previous findings (O'Flaherty et al. 1998; Sousa et al. 2009). Moreover, prominent bands related to acetoclastic methanogens within the *Methanosaeta* genus (bands 10, 16, 20, 21) and to methylotrophic methanogens within the *Methanomethylovorans* genus (fragments 11, 12, 14, 15, 24, 25) were detected during the entire reactor operation. This suggests that acetate was mainly consumed via methanogenesis. On the other hand, this also suggests that methane was mainly produced via acetoclastic or methylotrophic methanogenesis in the reactor. The decrease in methane yields at micro-aerobic conditions could be related to a detrimental effect of oxygen on methanogens, but this was not reflected in the archaea DGGE profiles. Likely, sulfidogenesis gained in importance over methanogenesis at micro-aerobic conditions.

Acknowledgments This research was supported by the Spanish Ministry of Education and Science (contract project CTQ2007-64324 and CONSOLIDER-CSD 2007–00055). The Regional Government of Castilla y Leon (Ref. VA038A07) is also gratefully acknowledged. The authors wish to thank to Lorena López and Irene Sánchez for helpful in sequence analysis.

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