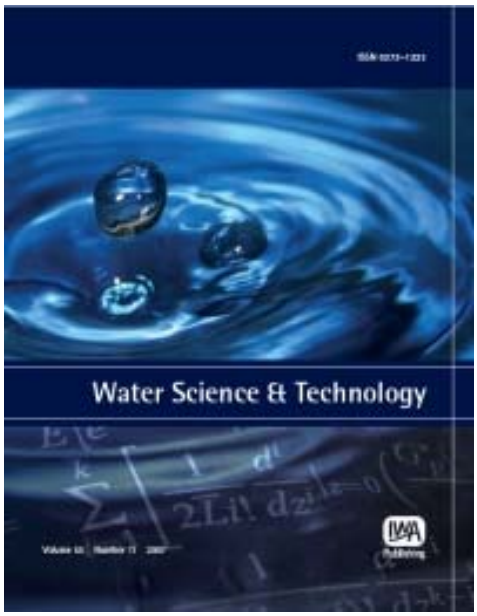

Comparative 16S rRNA gene surveys of granular sludge from three upflow anaerobic bioreactors treating purified terephthalic acid (PTA) wastewater

S. D. Perkins, N. B. Scalfone and L. T. Angenent

ABSTRACT

The microbial communities from three upflow anaerobic bioreactors treating purified terephthalic acid (PTA) wastewater were characterized with 16S ribosomal RNA gene sequencing surveys. Universal bacterial and archaeal primers were used to compare the bioreactor communities to each other. A total of 1,733 bacterial sequences and 383 archaeal sequences were characterized. The high number of *Syntrophus* spp. and *Pelotomaculum* spp. found within these reactors indicates efficient removal of benzoate and terephthalate. Under anaerobic conditions benzoate can be degraded through syntrophic associations between these bacteria and hydrogen-scavenging microbes, such as *Desulfovibrio* spp. and hydrogenotrophic methanogens, which remove H_2 to force the thermodynamically unfavourable reactions to take place. The authors did not observe a relatively high percentage of hydrogenotrophic methanogens with the archaeal gene survey because of a high acetate flux (acetate is a main component in PTA wastewater and is the main degradation product of terephthalate/benzoate fermentation), and because of the presence of *Desulfovibrio* spp. (a sulfate reducer that scavenges hydrogen). The high acetate flux also explains the high percentage of acetoclastic methanogens from the genus *Methanosaeta* among the archaeal sequences. A group of uncultured bacteria (OD1) may be involved in the degradation of *p*-toluate (4-methyl benzoate), which is a component of PTA wastewater.

Key words | anaerobic digestion, methanogens, *p*-toluate, PTA wastewater, 16S rRNA gene survey, terephthalate

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INTRODUCTION

Purified terephthalic (*p*-phthalic or 1,4-benzenedicarboxylic) acid (PTA) wastewater has been successfully treated in anaerobic digesters at chemical companies that produce polyethylene terephthalate to manufacture, for example, plastic bottles for the beverage industry (Qiu *et al*. 2004; Kleerebezem *et al*. 1999b, 2005). This wastewater contains terephthalate, acetate, benzoate, and *p*-toluate (4-methyl-benzoate) among other chemicals (Zhang *et al*. 2006). Even though these chemicals are not inhibiting the methanogenic biomass at the concentrations observed in PTA wastewater, terephthalate and *p*-toluate are not always degraded in anaerobic upflow bioreactors, while the biodegradation of acetate and benzoate is relatively easy (Kleerebezem *et al*. 2005). Terephthalate under anaerobic conditions is degraded at low rates after long lag periods, which can be accomplished in upflow bioreactors, such as upflow anaerobic sludge blanket (UASB) reactors and its offshoots, with granular biomass to sustain long residence times (Kleerebezem *et al*. 2005). But even after a sustainable period of terephthalate degradation, high concentrations of acetate and benzoate (itself the product of terephthalate degradation) can disrupt this (Kleerebezem *et al*. 1999b). These authors also discussed that the same bacterium may be degrading both terephthalate and benzoate. *p*-Toluate can be removed, albeit at even lower rates than terephthalate in methanogenic environments, which is why some have suggested the use of aerobic post-treatment to remove *p*-toluate (Zhang *et al*. 2006). In high-rate anaerobic bioreactors, such as UASB reactors or expanded sludge bed reactors (EGSBs), the high concentration of granular

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biomass and long cell residence times may overcome the slow removal rates of *p*-toluate. Here, we surveyed three granular samples from upflow anaerobic bioreactors treating PTA wastewater to characterize their microbial communities with nonculturing methods. We used Sanger sequencing to sequence near full-length sequences of 16S ribosomal RNA (rRNA) genes that were amplified with bacterial or archaeal specific PCR primers. Our goal was to compare the communities of the upflow bioreactors with different operating conditions and performance, especially in regard to the removal of *p*-toluate.

METHODS

Granular samples

We surveyed granular samples from two lab-scale UASB reactors (samples U1 and U2, received 17 June 2008); and from a full-scale expanded granular sludge bed (EGSB)-Biobed reactor (E1, received 5 August 2008). The UASB reactors had an internal diameter of 0.10 m with a height of 1.2 m (~9 L wet volume). U1 was operated with an upflow velocity of 1.9 m/h, a temperature of 38 °C, and an average pH of 7.2 during a three-month period before sampling. This reactor had been operating for a period of 15 months after inoculation with granular sludge from upflow anaerobic bioreactors located at a brewery (70% v/v) and a papermill (8%), and from U2 (22%). A synthetic PTA wastewater was fed to U1 at a loading rate of 42.6 g COD/L/day, a hydraulic retention time (HRT) of 14 h, and a soluble COD (sCOD) removal efficiency of 98.7%. U2 was operated with an upflow velocity of 1.7 m/h, a temperature of 38 °C, and an average pH of 7.2 during a three-month period. This reactor had been operating for a period of 21 months after inoculation with granular sludge from upflow anaerobic bioreactors at two breweries and a petrochemical company. A synthetic PTA wastewater was fed to U2 at a loading rate of 27.4 g COD/L/day, a HRT of 12 h, and a sCOD removal efficiency of 99.0%. The difference between U1 and U2 was the composition of the PTA wastewater, which mimicked two different processing plants. The high sCOD removal efficiencies show that almost all chemicals (including *p*-toluate) in this type of wastewater were completely removed under methanogenic conditions and converted to biogas, and that the intermediate volatile fatty acids were maintained at very low concentrations in the effluent. The full-scale E1 system is located at a petrochemical industrial site and consists of a tank with an internal diameter of 13 m and a height of 15 m (~2,000 m^3 wet volume). This reactor was started ~4 years prior to sampling with a sCOD removal efficiency of ~60%. This relatively low sCOD removal efficiency indicates that the most difficult to be removed chemicals, such as *p*-toluate, were not degraded in the anaerobic system.

DNA extraction and PCR amplification

Received samples were immediately stored at –80 °C until further processing. Granules were crushed using a sterile spatula (Corning 3003, Corning, NY) before 0.5 ml of sample was submitted to a bead-beating and phenol:chloroform extraction protocol. The 16S rRNA genes from bacteria were amplified with a 30-cycle PCR (initial 2 min denaturing step at 94 °C, followed by 30 cycles at 92 °C for 30 s 45 °C for 90 s, 72 °C for 90 s, and a final 72 °C extension for 15 min). The 50-μl solution contained 1.25 units of GoTaq (Promega Corp, Madison, WI), 0.4 pmol/μl forward and reverse primers (8F-5′ AGAGTTTGATCCTGGCTCAG-3′; 1391R-5′ GACGGGCGGTGWGTRCA-3′), 0.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.8 mg/mL BSA, and 2-μl of template. Archaeal 16S rRNA genes were amplified with a 30-cycle PCR (initial 2 min denaturing step at 94 °C, followed by 30 cycles at 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s, and a final 72 °C extension for 10 min). A similar PCR solution was used with different primers: 21F-5′ AAGGAGGTGATC CAGCC-3′; 1492R-5′ GGTTACCTTGTTACGACTT-3′. Control positive and negative reactions were included with each reaction set.

Cloning and sequence analysis

PCR products were cleaned and concentrated (Montage PCR cleaning kit, UFC7PCR50, Millipore, Billerica, MA) and sent to the Genome Sequencing Center, Washington University School of Medicine in St. Louis, for cloning, purification, and Sanger sequencing on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Three independent PCR amplicon materials were sequenced per sample for bacteria and one for Archaea. 16S rRNA gene sequences were edited and assembled into consensus sequences using PHRED and PHRAP aided by XplorSeq (Frank 2008). Bases with a PHRAP quality score <20 were trimmed from the dataset before being aligned using the NAST online tool (DeSantis *et al*. 2006b). Chimeras were detected and removed using Bellerophon (Huber *et al*. 2004). Nonchimeric sequences were compared to the

greengenes public database and imported into ARB (Ludwig *et al*. 2004). A pair-wise identity of 97% or greater was used to bin sequences into 'species'-level operational taxonomic units (OTUs). Distance matrices constructed in DOTUR were used to assess the number of OTUs and cluster them by pair-wise identity (percent ID) with a furthest-neighbour algorithm and a precision of 0.01 (Schloss & Handelsman 2005). Assignment of the majority of bacterial sequences to phyla was based on their position after parsimony insertion to the ARB dendrogram in the greengenes database (DeSantis *et al*. 2006a). Bacteria phyla classifications were double-checked and a percent ID was calculated for nonchimeric sequences with the Ribosomal Database Project II (RDP) (Cole *et al*. 2007). However, for archaeal sequences only RDP was used to classify to the family level. A phylogenetic tree model was found using jModeltest 0.1 and an Adaike Information Criterion to rank 88 candidate models with an objective scoring method (Posada 2008). The best-fit model was used to create a maximum likelihood phylogenetic tree with PAUP (Wilgenbusch & Swofford 2003). Finally, UniFrac was used to conduct a jackknife replication weighted and normalized test, replicated 100 times to compare the three reactor environments by clustering them based upon phylogenetic similarity between sample communities (Lozupone *et al*. 2006).

Nucleotide sequence accession numbers

Near full-length bacterial 16S rRNA gene sequences were deposited in GenBank under accession numbers GQ181242-GQ182974. Near full-length archaeal 16S rRNA gene sequences were deposited in GenBank under accession numbers GQ201538-GQ201920.

RESULTS AND DISCUSSION

Bacterial and archaeal sequences showed a diverse granular community

We obtained 1,733 near full-length nonchimeric bacterial 16S rRNA gene sequences for the three granular samples. The phylum Proteobacteria was the dominant bacterial taxonomic phyla with the class of Deltaproteobacteria dominating the sequences from all three reactors (Figure 1(a)). In addition, the phyla Firmicutes and Chloroflexi were abundant in all three reactors. Further analyses were performed to bin sequences, to assess diversity, to compare communities, and to classify 16S rRNA gene sequences at the species level. We found 538 unique OTUs for the 1,733 bacterial sequences (Figure 1(a)). Next, rarefaction curves showed that the community from E1 was more diverse

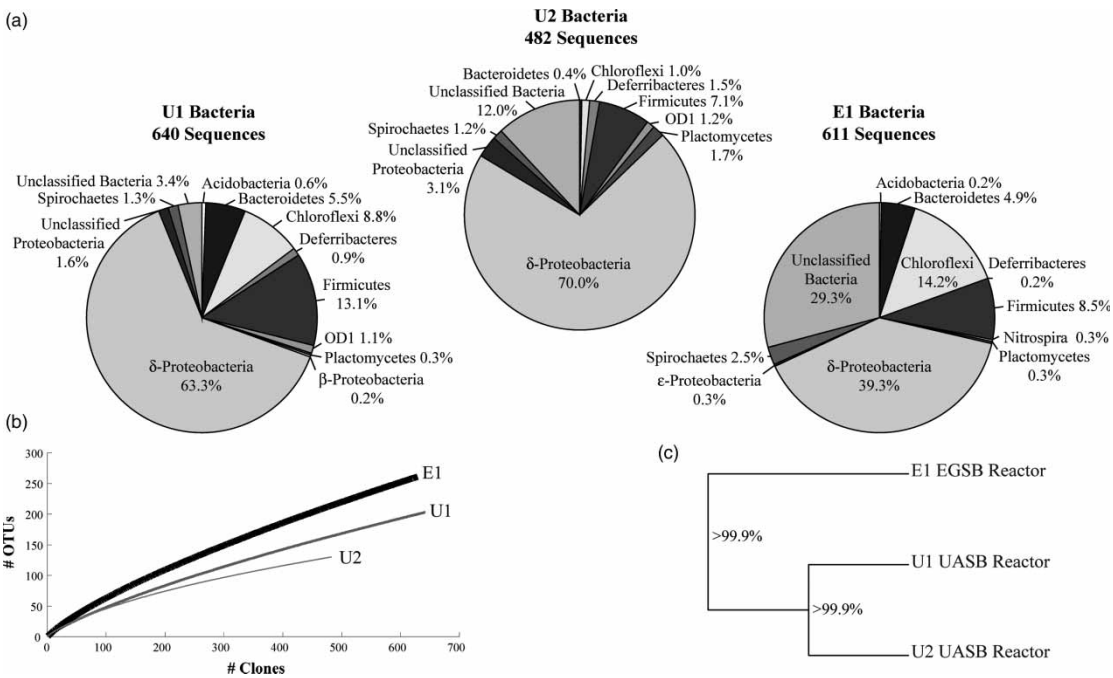

Figure 1 | Comparison of bacterial 16S rRNA gene sequences for each upflow anaerobic bioreactor (U1, U2, and E1): (a) Phylum or class distribution (proteobacteria was divided into the classes alpha-, beta-, gamma-, and deltaproteobacteria); (b) Rarefaction curves, which represent diversity based on the number of OTUs; and (c) Cluster analysis (weighted and normalized UniFrac). Percentages indicate bootstrap from 100 jack-knifed subsamples.

(steeper curve) than from U1 and U2 (Figure 1(b)), which makes sense because of the more complex nature of real PTA wastewater (E1) compared to synthetic wastewater (U1 and U2). All three curves also show that the granular biomass samples were not fully characterized (no asymptotes; Figure 1(b)), or, in other words, more sequencing would have resulted in additional unique OTUs. We used the UniFrac distance method to compare the three communities to each other by quantifying the taxonomical differences between samples, and found that U1 and U2 were more similar to each other than E1 (Figure 1(c)). This may have identified an effect of differences in reactor operating conditions (upflow velocity), in wastewater conditions (real versus synthetic PTA wastewater), and in reactor performance (complete versus incomplete removal of chemicals) between the UASB and EGSB reactors on the community structure. In almost all bacterial phyla, closely related OTUs were represented from all three reactors, which clearly shows a similar functioning community (data not shown).

We obtained 383 near full-length nonchimeric archaeal 16S rRNA gene sequences. In all three reactors, more than 90% of the archaeal sequences were from the family Methanosaetaceae, while the rest of the archaeal sequences were only from three other methanogenic families (Table 1). Wu *et al*. (2001) had found more than 80% of 72 archaeal sequences in granular biomass degrading terephthalate to be closely affiliated with *Methanosaeta* spp., which, therefore, verifies the relatively large percentage of this family in a similar type of reactor. In our study, maximum likelihood analysis (data not shown) showed that the majority of the members of the Methanosaetaceae family were closely related to a sequence from a cultured *Methanosaeta concilii*, which is an acetoclastic methanogen (Boone *et al*. 1993). Acetate is an important constituent of PTA wastewater and is also a product from benzoate degradation (3 mol of acetate per mol of benzoate), and this explains the abundance of these acetoclastic methanogens. It is well accepted that low acetate concentrations allow for slow-growing *Methanosaeta* spp. to have a competitive advantage over *Methanosarcina* spp. (Jetten *et al*. 1992). Thus, the abundance of Methanosaetaceae found within these anaerobic digesters is indicative of a low acetate concentration in the effluent.

Syntrophy between *Syntrophus* spp. and hydrogenotrophic methanogens or *Desulfovibrio* spp. explains benzoate degradation

Almost 46% (804/1733) of the bacterial sequences in this study were from *Syntrophus* spp. This genus (in the class of Deltaproteobacteria) is known to degrade fatty acids or aromatic acids with, for example, a hydrogenotrophic methanogen or *Desulfovibrio* spp. (a sulfate reducer that scavenges hydrogen) in a syntrophic partnership. Wu *et al*. (2001) had found 78% of 106 bacterial sequences in granular biomass degrading terephthalate to be of this class, including several *Syntrophus* spp.-related sequences. We performed maximum likelihood analysis within the phyla of Proteobacteria, and found that the majority of *Syntrophus* spp. sequences for U1 and U2 matched the pure culture strain *S. buswellii*, while for E1 the *Syntrophus* spp. sequences matched environmental sequences. Mountfort *et al*. (1984) isolated *S. buswellii* from anaerobic digester sludge and aquatic sediments; their work characterized *S. buswellii* as a benzoate catabolizer ($C_7H_5O_2^- + 7H_2O \leftrightarrow 3H_2 + HCO_3^- + 3CH_3COO^- + 3H^+$; $\Delta G^{\circ\prime} = +59.6$ kJ/mol (Kleerebezem *et al*. 1999a)). Anaerobic benzoate degradation is not thermodynamically favourable unless syntrophic hydrogenotrophic microbes within the community scavenge the hydrogen to keep the hydrogen partial pressure below 10^{-3}–10^{-5} bar (Auburger & Winter 1995). These authors further characterized the syntrophic relationship between *S. buswellii* and the hydrogenotrophs *Methanospirillum hungatei* and *Desulfovibrio* spp. using benzoate. Our

Table 1 | RDP classification of archaeal sequences from reactors U1, U2, and E1

Family	U1		U2		E1	
Methanobacteriaceae	4	2.8%	0	0.0%	1	0.5%
Methanomicrobiaceae	0	0.0%	3	7.9%	0	0.0%
Methanosaetaceae	134	93.1%	35	92.1%	195	97.0%
Methanosarcinaceae	6	4.2%	0	0.0%	5	2.5%
Total	144		38		201	

A total of 383 sequences were classified to the family level. For each sample the amount of sequences is given and its relative presence.

maximum likelihood distribution analyses matched *Desulfovibrio* spp. to cultured sequences for all three reactors (Figure 2), and *M. hungatei* was identified in reactor U2 (data not shown). Therefore, both *Desulfovibrio* spp. and the hydrogen-utilizing methanogens of the families Methanobacteriaceae, Methanomicrobiaceae, or Methanosarcinaceae were the likely synergistic partner of the Deltaproteobacteria in our study to explain benzoate removal.

Syntrophy between *Pelotomaculum* spp. and hydrogenotrophic methanogens explains terephthalate degradation

A study conducted by Qiu *et al*. (2004) enriched and isolated terephthalate-degrading microbes from anaerobic sludge from digesters treating PTA wastewater. One of the isolates (*Pelotomaculum* spp. in the Desulfotomaculum lineage I) was able to degrade terephthalate (and also benzoate) in

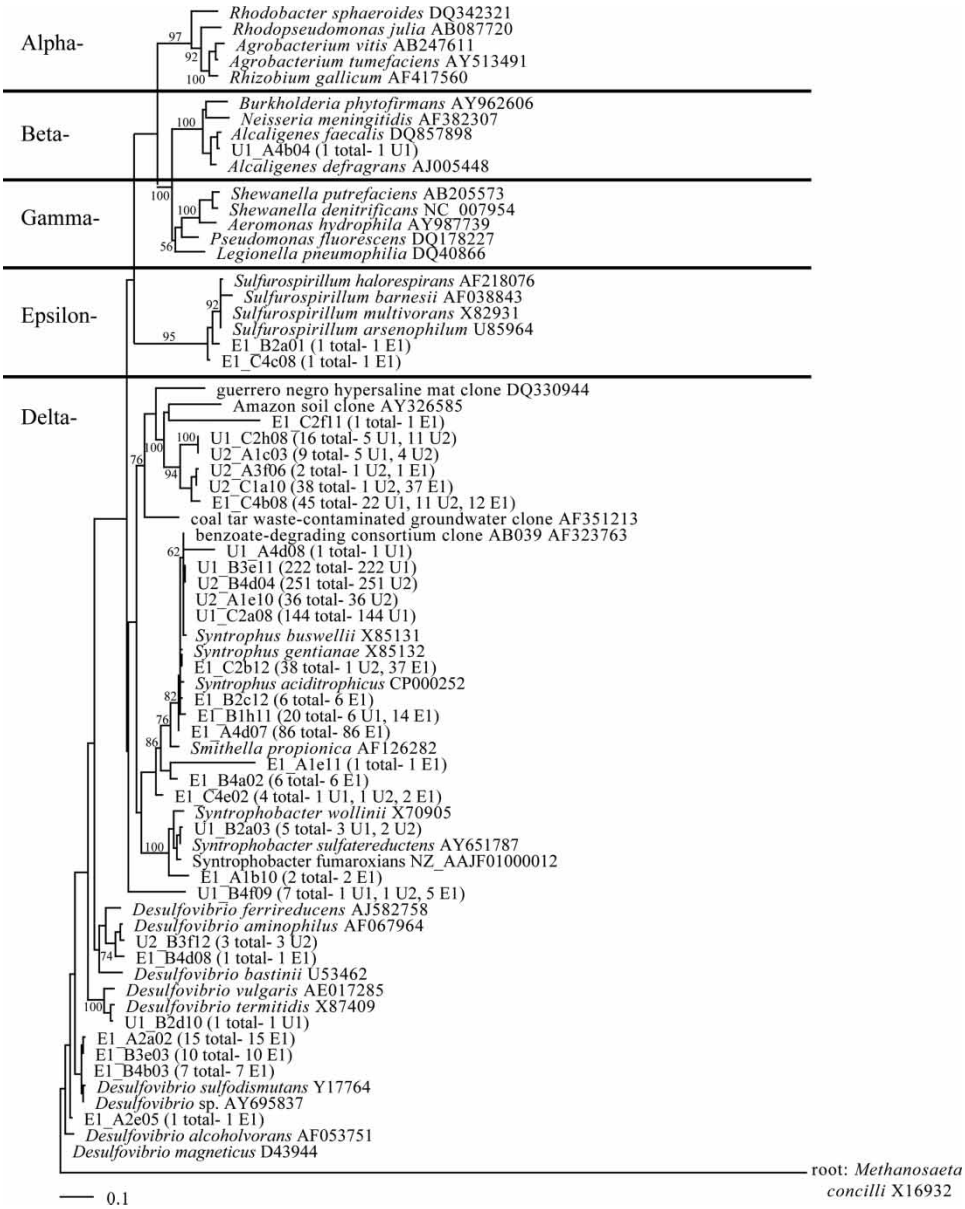

Figure 2 | Maximum likelihood phylogenetic distribution of select 16S rRNA gene sequences from our study and closely related pure-culture sequences from the phylum Proteobacteria. Reference sequences from the NCBI database are included with their accession numbers. Individual sequences that represent one OTU are labelled with U1, U2, and E1 identifiers followed by the sample label and the unique sequence identity. The number of sequences in the OTU found in this study are in parenthesis. The scale bar represents base changes per site.

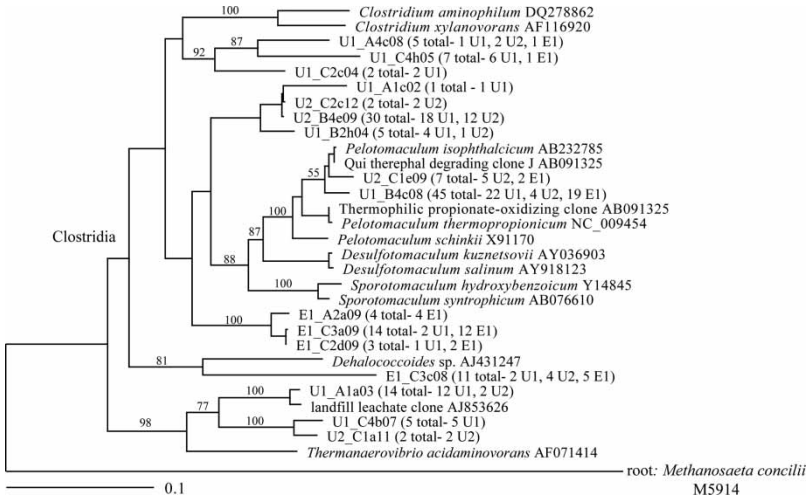

Figure 3 | Maximum likelihood phylogenetic distribution of select 16S rRNA gene sequences from our study and closely related pure-culture sequences from the phyla Firmicutes. Reference sequences from the NCBI database are included with their accession numbers. Individual sequences that represent one OTU are labelled with U1, U2, and E1 identifiers followed by the sample label and the unique sequence identity. The number of sequences in the OTU found in this study are in parenthesis. The scale bar represents base changes per site.

co-culture with the hydrogenotroph *M. hungatei*. Here, *Pelotomaculum* spp. were present in all reactor samples in relatively high numbers (Figure 3). Similarly to benzoate degradation, terephthalate removal ($C_8H_4O_4^- + 8H_2O \leftrightarrow 3H_2 + 2HCO_3^- + 3CH_3COO^- + 3H^+$; $\Delta G^{\circ\prime} = +38.9$ kJ/mol (Kleerebezem *et al.* 1999a)) also requires a syntrophic partner to remove thermodynamic constraints. The presence of hydrogenotrophic methanogens (including *M. hungatei* in U2) – the relatively low percentage of archaeal sequences (Table 1) does not exclude these methanogens as important partners – explains the syntrophic removal of terephthalate from all our reactors.

Other organisms found within the reactors play key roles in the overall community dynamics

The phylum Chloroflexi was the third most abundant phylum in the reactors (after Firmicutes and Proteobacteria), and has been recognized as an important component of mesophilic and thermophilic granular sludge. The filamentous web-like structures formed by these bacteria play an important role in maintaining the structural integrity of sludge granules (Yamada *et al.* 2005). It may, therefore, have an important function in these upflow bioreactors. As mentioned earlier, many OTUs in our study overlapped between the three reactors (Figures 2 and 3), and almost all phyla included OTUs from all three reactors. One distinct discrepancy to this was the phyla OD1 found in reactors U1 and U2, but not in E1. OD1 is an uncultured candidate division recently split from the uncultured candidate division OP11. Its members are often found within marine and aquifer sediments (Harris *et al.* 2004), but their function is unknown. The important difference between U1/U2 and E1 was that the former reactors were able to convert *p*-toluate, while the latter reactor missed this important characteristic. Therefore, members of OD1 may have been involved in *p*-toluate biodegradation. However, further time-resolved studies with highly parallel sequencing efforts would be necessary to prove this hypothesis. The enrichment or isolation of this group of bacteria may be very difficult under laboratory conditions.

CONCLUSIONS

The community structure of three upflow anaerobic bioreactors with granular biomass explains their bioconversion of PTA wastewater into methane. A very high level of syntrophic partnerships between benzoate- and terephthalic-degrading bacteria and hydrogenotrophic methanogens or *Desulfovibrio* spp. was found. The genera *Syntrophus* and *Pelotomaculum* were the likely degraders of benzoate and terephthalate. We have identified a possible group of uncultured bacteria that could be responsible for *p*-toluate degradation, but further work is necessary to explore this hypothesis. Finally, acetate was degraded by the acetoclastic methanogens in the genus *Methanosaeta*. The relatively high percentage of these methanogens

compared to hydrogenotrophic methanogens (>90%) seems to be a signature for PTA-wastewater-treating digesters because acetate is another important constituent of PTA wastewater and the main intermediate degradation product of benzoate and terephthalate.

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