Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system

Jer-Horng Wu,¹ Wen-Tso Liu,² I-Cheng Tseng³ and Sheng-Shung Cheng¹

Author for correspondence: Wen-Tso Liu. Tel: +886 3422 7151 ext. 4683. Fax: +886 3426 9401.
e-mail: liuwt@cc.ncu.edu.tw

¹ Department of Environmental Engineering, National Cheng Kung University, Tainan, Taiwan

² Graduate Institute of Environmental Engineering, National Central University, Chungli 32054, Taiwan

³ Department of Biology, National Cheng Kung University, Tainan, Taiwan

The microbial composition and spatial distribution in a terephthalate-degrading anaerobic granular sludge system were characterized using molecular techniques. 16S rDNA clone library and sequence analysis revealed that 78.5% of 106 bacterial clones belonged to the δ subclass of the class *Proteobacteria*; the remaining clones were assigned to the green non-sulfur bacteria (7.5%), *Synergistes* (0.9%) and unidentified divisions (13.1%). Most of the bacterial clones in the δ -*Proteobacteria* formed a novel group containing no known bacterial isolates. For the domain *Archaea*, 81.7% and 18.3% of 72 archaeal clones were affiliated with *Methanosaeta* and *Methanospirillum*, respectively. Spatial localization of microbial populations inside granules was determined by transmission electron microscopy and fluorescent *in situ* hybridization with oligonucleotide probes targeting the novel δ -proteobacterial group, the acetoclastic *Methanosaeta*, and the hydrogenotrophic *Methanospirillum* and members of *Methanobacteriaceae*. The novel group included at least two different populations with identical rod-shape morphology, which made up more than 87% of the total bacterial cells, and were closely associated with methanogenic populations to form a nonlayered granular structure. This novel group was presumed to be the primary bacterial population involved in the terephthalate degradation in the methanogenic granular consortium.

Keywords: denaturing gradient gel electrophoresis, clone library, 16S rDNA, *in situ* hybridization, UASB

INTRODUCTION

Terephthalic acid (1,4-benzenedicarboxylic acid) and its isomers are the major components used in the production of polyester fibres, films, adhesives, coatings and plastic bottles (Franck & Stadelhofer, 1988). In Taiwan, approximately $2.8 \times 10^4 \text{ m}^3 \text{ d}^{-1}$ of wastewater is generated during terephthalate acid manufacturing processes, and terephthalate was identified as a major component (~25–30% of total chemical oxygen demand) in the wastewater, followed by 4-methylbenzoate, benzoate and acetate (Cheng *et al.*, 1997).

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization; GNS, green non-sulfur; OTU, operational taxonomic unit; TEM, transmission electron microscopy; UASB, upflow anaerobic sludge bed.

The GenBank accession numbers for the sequences obtained in this work are AF229774–AF229793.

Wastewater containing terephthalic acid isomers is typically treated by aerobic biological treatment systems (Lau, 1977), but anaerobic treatment processes, which require less energy and nutrients than traditional aerobic processes, have become attractive alternatives (Lettinga, 1995).

- (1) $\text{Terephthalate}^{2-} + 8\text{H}_2\text{O} \rightarrow \text{acetate}^- + 3\text{H}^+ + 2\text{HCO}_3^- + 3\text{H}_2$ ($\Delta G_0' = +38.9 \text{ kJ mol}^{-1}$)
- (2) $\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$ ($\Delta G_0' = -135.6 \text{ kJ mol}^{-1}$)
- (3) $\text{Acetate}^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{CH}_4$ ($\Delta G_0' = -31.0 \text{ kJ mol}^{-1}$)

Recently, the upflow anaerobic sludge bed (UASB) reactor was successfully demonstrated to treat terephthalate-containing wastewater (Cheng *et al.*, 1997; Kleerebezem *et al.*, 1997). In this system, microbial populations aggregate as granules or as individual

micro-ecosystems. Each micro-ecosystem is composed of acetogenic bacteria which degrade complex organic compounds to a mixture of acetate, hydrogen and formate, and methanogenic *Archaea*, which continuously mineralize the intermediates to methane and carbon dioxide. These populations interact syntrophically, because the fermentative step from terephthalate to acetate is energetically unfavourable ($\Delta G'_0 > 0$) (equation 1) and requires the methanogenesis step (equations 2 and/or 3) as a coupling reaction to proceed (Theile & Zeikus, 1988). Kleerebezem *et al.* (1999a, b) proposed that in a methanogenic consortium, terephthalate was degraded via decarboxylation to the transient intermediate benzoyl-CoA, and then to acetate and hydrogen, which were further mineralized to methane and carbon dioxide. They suggested that the degradation of terephthalate to acetate and hydrogen was performed by a fermentative population, and the methanogenesis step was performed by acetoclastic and hydrogenotrophic methanogens. However, due to the difficulty of isolating syntrophic bacteria (Schink, 1997), the proposed metabolic pathway remains to be verified. Furthermore, the diversity and phylogenetic position of the fermentative population responsible for the initial terephthalate degradation have not been identified.

The advent of molecular techniques has made it feasible to study the microbial community of environmental ecosystems without cultivation (Amann *et al.*, 1995). 16S rDNA-based methods employing denaturing gradient gel electrophoresis (DGGE) (Muyzer & Smalla, 1998; Nielsen *et al.*, 1999) and molecular cloning (Barns *et al.*, 1994; Godon *et al.*, 1997; Sekiguchi *et al.*, 1998) can provide a rapid estimate of the microbial diversity and composition of a community. In addition, fluorescent *in situ* hybridization (FISH) with oligonucleotide probes targeting the 16S rRNA of micro-organisms can be used to evaluate the abundance and distribution of specific phylogenetic groups in their natural micro-habitats (Amann *et al.*, 1992). These molecular techniques have been successfully applied to identify syntrophic propionate-oxidizing bacteria (Harmsen *et al.*, 1996), and to localize methanogens in anaerobic granular sludge systems (Rocheleau *et al.*, 1999; Sekiguchi *et al.*, 1999). In this study, a molecular approach was used in combination with electron microscopy to characterize the microbial consortia in a laboratory-scale terephthalate-degrading UASB reactor.

METHODS

Granular sludge reactor. A 20 litre UASB reactor was inoculated with anaerobic sludge from a full-scale UASB reactor treating terephthalic-acid wastewater (Pereboom *et al.*, 1994), and was fed with synthetic terephthalate wastewater (pH 7.0–7.4) containing terephthalate (0.4–2.7 g l⁻¹), NH₄Cl (0.2–0.5 g l⁻¹), trace metals (2–3 ml l⁻¹) and FeCl₂ (0.5 ml l⁻¹) (Owen *et al.*, 1979). The reactor was maintained under mesophilic conditions by circulating water at 35 °C through the outer water jacket of the double-layer Plexiglas reactor. After 2 years' operation, the terephthalate-degrading granular sludge was used as an inoculum to inoculate a 12.5 litre UASB reactor treating the same synthetic terephthalate wastewater.

The concentration of terephthalate fed into the 12.5 litre reactor was 0.5 g l⁻¹ initially and was gradually increased to 3.0 g l⁻¹ at day 200. The hydraulic retention time was controlled at 20–24 h. The removal efficiency of terephthalate was monitored during the operation. After a further 1 year of operation, the granular sludge was taken from the 12.5 litre UASB reactor for microbial community analysis.

Anaerobic batch experiment. A 305 ml serum bottle was charged with 250 ml disrupted granular sludge (final volatile suspended solids, ~300 mg l⁻¹), oxygen-free medium and 3 mM terephthalate. The oxygen-free medium (pH 7.2) consisted of vitamins, trace metals and 50 mM carbonate buffer (Owen *et al.*, 1979). During the anaerobic experiment at 37 °C for 20 d, 1 ml liquid sample was withdrawn every 1–4 d from the serum bottle with a syringe, and filtered through a 0.45 µm disposable filter. Volatile fatty acid concentrations were analysed using a gas chromatograph equipped with a flame-ionization detector (Shimadzu 8A) and a Thermo-3000 (Shincarbon 60/80 mesh)-packed glass column. The temperatures at injection, oven and detector ports were controlled at 180, 150 and 180 °C, respectively. Aromatic compounds (terephthalate and benzoate) were determined by HPLC using a Hitachi L-3000 instrument equipped with a photodiode array detector and a separation column (LiChroCART 250-4 RP-18e, Merck). Mixed solvents, which were used as the mobile solutions in HPLC, were acetonitrile and water containing 1% (v/v) acetic acid. Final gaseous products (methane and hydrogen) were analysed by GC with a thermal conductivity detector (China GC 8900). The 2 m stainless column was packed with Haysep Q (60/80 mesh) and installed inside a 60 °C oven. Pure N₂ was used as the carrier gas at a constant flow rate of 10 ml min⁻¹.

Transmission electron microscopy (TEM). Sludge granules were first fixed in 0.1 M phosphate buffer solution (PBS) containing 2.5% (v/v) glutaraldehyde and 4% (w/v) para-formaldehyde for at least 12 h at 4 °C and washed three times with 0.1 M PBS. The granules were subsequently fixed in 0.1 M PBS containing 1% (w/v) osmium tetroxide for 2 h at 4 °C and washed three times with 0.1 M PBS. The samples were dehydrated with a series of ethanol washes (50, 75, 85, 95 and 100%), and exposed to acetone for 15 min twice. The dehydrated sample was gradually washed in a series of acetone solutions containing 50% (v/v) and then 75% (v/v) Spurr's resin at room temperature with gentle shaking (12 h each wash). The mixture was decanted and replaced with 100% Spurr's resin, followed by incubation for 24 h at room temperature. The specimens were embedded in fresh resin and cured at 70 °C for 14 h. A diamond knife was used to produce 60–70 nm ultra-thin sections. The granule section was retrieved onto an uncoated 300-mesh copper grid (Ted Pella). The grid was stained in 50% ethanol solution containing 2% (w/v) uranyl acetate for 20 min, and then in 0.4% (w/v) lead citrate solution for 5 min and air-dried. The sectioned samples were analysed with a transmission electron microscope (JEOL Jem 1200EX).

DNA extraction and microbial community analysis. Granular sludge samples were homogenized with a tissue grinder and suspended in 750 µl lysis buffer (100 mM Tris/HCl, 100 mM EDTA and 0.75 M sucrose, pH 8.0). Following cell lysis, phenol/chloroform extraction and ethanol precipitation procedures (Liu *et al.*, 1997), total DNA from the sludge sample was obtained.

16S rDNA clone libraries for the domains *Bacteria* and *Archaea* were constructed to reveal the microbial community structure in the granular sludge. Initially, the 16S rDNAs from members of the domains *Bacteria* and *Archaea* were PCR-

amplified with two frequently used primer sets, eu11F/eu1512R (Bond *et al.*, 1995) and A23F/A1392R (Barns *et al.*, 1994), respectively. The PCR reaction was performed in 1 × PCR buffer (Gibco-BRL) containing 200 µM (each) of deoxynucleoside triphosphates, 1.5 mM MgCl₂, 0.1 µM (each) of primers and 2.5 U *Taq* polymerase (Gibco-BRL) in a final volume of 100 µl, using a PCR Express programmable thermal cycler (Hybaid). The thermal cycling programs used were described previously (Barns *et al.*, 1994; Bond *et al.*, 1995). After PCR amplification, PCR products were cloned into the pPCR-Script AmpSK(+) vector using a commercial cloning kit (Stratagene) according to the manufacturer's instructions.

For rapid clone screening, white colonies containing 16S rDNA inserts of the correct size (~1.5 kb) in vectors were identified using PCR amplification with vector-specific primers (M13F and M13R). Products of the correct length (~1.6 kb) were diluted and used as the DNA template in a subsequent DGGE-PCR reaction. The DGGE primers used for the amplification of 16S rDNA from members of the domain *Bacteria* were U968F-gc (Heuer *et al.*, 1997) and U1392R (Ferris *et al.*, 1996). The primers for the amplification of 16S rDNA from members of the domain *Archaea* were A934F (5'-AGGAATTGGCGGGGAGCA-3') and 1390R-gc (5'-CGCCCGGGGCGCGCCCGGGCGGGGCGGGGCGGGGCGGGGCGGTGTGTGCAA-3'), which were designed in this study (underlined sequences are the gc-clamp region). The forward primer perfectly matched at least 91.2% of the archaeal 16S rRNA gene sequences in the Ribosomal Database Project (RDP) database (Maidak *et al.*, 1997). The reverse primer matched more than 85% of all sequences in the current RDP database at one-mismatch specificity. The thermal program used for amplification of bacterial 16S rRNA genes was described previously (Nielsen *et al.*, 1999). For members of the domain *Archaea*, a touch-down thermal program was used. An initial denaturation step (95 °C, 3 min), followed by five cycles of touch-down amplification [denaturation (95 °C, 45 s), annealing (68 °C, 45 s with 1 °C decrease each cycle to 63 °C) and extension (72 °C, 2 min)], 23 cycles of amplification [denaturation (95 °C, 45 s), annealing (63 °C, 45 s) and extension (72 °C, 2 min)], and a final extension (72 °C, 3 min) was used. Nested DGGE-PCR products were verified by electrophoresis in a 0.8% agarose gel, and were screened by DGGE using a gel with a denaturant gradient from 40% to 60%.

DGGE was performed using the DCode system (Bio-Rad) at 200 V, 60 °C for 3.5 h, as previously described (Nielsen *et al.*, 1999). The separated DNA fragments were visualized by silver staining (Riesner *et al.*, 1989) and photographed using an image-capture system equipped with a Kodak DC-120 digital camera. Clones that produced PCR products which migrated to different positions in the DGGE gel were identified and sequenced with a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems). Sequencing was performed with an ABI DNA Sequencer model 377 (Applied Biosystems) at Mission Biotech (Taipei, Taiwan). In addition to the amplification primers, bacterial primer 926R (Amann *et al.*, 1992) and archaeal primer 934R (Amann *et al.*, 1995) were used as primers in the sequencing reaction.

Phylogenetic analysis and probe design. Approximate phylogenetic affiliations of the inferred 16S rRNA gene sequences obtained were initially compared to the GenBank database using the NCBI BLAST program. Sequences closely related (sequence identity >90%) to the clone sequences were retrieved and imported into the sequence alignment program of ARB (provided by Oliver Strunk & Wolfgang Ludwig, Technical University of Munich, Munich, Germany) for

further phylogenetic analysis. The 16S rRNA gene sequences obtained in this study and the most closely related sequences from the GenBank database were aligned using the sequence alignment editor in ARB and then manually corrected. Phylogenetic trees were built from these aligned sequences using the neighbour-joining method provided in the ARB program. For distance correction, the algorithm of Jukes & Cantor (1969) was used. Alignment positions at which less than 50% of the sequences in the entire data set shared the same residues were excluded from the calculations. 16S rRNA-targeted oligonucleotide probes were designed from the cloned sequences using the probe design function in ARB. Probe specificity was checked using the Check Probe analysis in the RDP (Maidak *et al.*, 1997). Fluorescently labelled probes were synthesized by Operon Technologies (Alameda, CA).

Fixation, sectioning and FISH of sludge granules. Sludge granules were gently washed three times with 0.1 M PBS, fixed in 0.1 M PBS containing 4% (w/v) paraformaldehyde at 4 °C overnight, and washed three times with 0.1 M PBS. To improve the penetration efficiency of oligonucleotide probes, the granule was subjected to at least five cycles of freeze-and-thaw (-80 °C to +60 °C) after fixation (Sekiguchi *et al.*, 1999). The fixed sample was then dehydrated in a 50% ethanol solution at 4 °C overnight, and washed with a series of ethanol/water solutions (50, 80 and 96%, 3 min each), then with an ethanol/xylene mixture (50:50, v/v), and finally with 100% xylene. The xylene solution was gradually replaced with an equal volume of paraffin/xylene mixture with the paraffin content varying from 25%, 50%, 75% to 100% at 62 °C (12 h for each replacement). The granule-embedded paraffin block was cooled and sectioned into 2–3 µm slices with a rotary microtome (Microm type HM310). The centre sections of sludge granules were collected and floated on 0.8% (v/v) formaldehyde solution on the surface of a poly-L-lysine-coated microscope slide at 40 °C, and air-dried. The paraffin in the granule sections was removed by washing twice with 100% xylene for 40 min and then the sections were washed twice more with 100% ethanol for 40 min. The slides with granule sections were air-dried at room temperature.

In situ hybridization was conducted according to protocols previously described (Nielsen *et al.*, 1999; Sekiguchi *et al.*, 1999). Initially, 10 µl hybridization buffer (0.9 M NaCl, 1% SDS, 100 mM Tris/HCl, pH 7.2) containing 50 ng of each labelled probe (see Table 1) was added to each well on the slide and hybridized at 45 °C for at least 12 h. The hybridization stringency was controlled by adding different amounts of formamide to the hybridization buffer (10% for MG1200; 15% for EUB338; 20% for MX825 and delta-TA1; 30% for delta402; and 35% for ARC915, MB1174 and delta-TA2). For washing, the slide was briefly rinsed with double-distilled water and incubated in the same hybridization solution without the addition of probes for 30 min at 48 °C. The slide was briefly rinsed with milliQ water, and air-dried prior to examination using an epifluorescence microscope equipped with a cooled CCD camera (Quntax; Photometrics). When multiple probes were used, the probes with higher-stringency hybridization conditions were applied first, followed by probes with lower-stringency hybridization conditions.

The optimal hybridization stringency for a newly designed probe was empirically obtained by simultaneously comparing the hybridization signal between non-target reference strains and granular sections under different formamide concentrations from 0 to 45%. *Saccharopolyspora erythraea* (ATCC 11635), used as the closest reference strain, has three and more than three mismatched nucleotides with probes TA1 and TA2, respectively. In addition, *Desulfovibrio* sp. strain TSL8-2 from

Table 1. Oligonucleotide probes

Probe	OPD name*	Specificity	Sequence (5'-3')†	Fluorescent label(s)	Reference
EUB338	S-D-Bact-0338-a-A-18	Bacteria	GCTGCCTCCCGTAGGAGT	Cy3, Cy5	Amann <i>et al.</i> (1992)
ARC915	S-D-Arch-0915-a-A-20	Archaea	GTGCTCCCGCCCAATTCCT	FITC, Cy3	Stahl & Amann (1991)
MG1200	S-O-Mmic-1200-a-A-21	Methanomicrobiales	CGGATAATTGGGGCATGCTG	Cy3	Raskin <i>et al.</i> (1994)
MX825	S-F-Msac-0825-a-A-23	Methanosaetaceae	TCGCACCGTGGCCGACACCTAGC	Cy5	Raskin <i>et al.</i> (1994)
MB1174	S-F-Mbac-1174-a-A-22	Methanobacteriaceae	TACCGTCGTCCACTCCTTCCTC	Cy3	Raskin <i>et al.</i> (1994)
delta402	S-Sc-dProt-0402-a-A-18	δ -Proteobacteria	CGGGCTCGCTGCGTCAGG	Cy3	Amann <i>et al.</i> (1995)
delta-TA1	S-S-dProt-01031-a-A-18	Group TA (Fig. 3b)	TCCAAGYTCCCCTTACGG	Cy5	This study
delta-TA2	S-S-dProt-0100-a-A-19	TA11/TA12	TGTTCCATCGGGTACTCG	Cy3	This study

* OPD, oligonucleotide probe database (Alm *et al.*, 1996).

† Y = T or C.

the δ -Proteobacteria was used for optimizing the probe specificity of TA1 and TA2. When quantitative FISH was performed, at least 5–10 microscopic fields were analysed for the hybridization of individual probes using the quantification function provided in the image-analysis software, V-for-Window (Photometrics). In some cases, total cells targeted by a probe were expressed as the percentage of total cells targeted by a bacterial-specific or an archaeal-specific probe. Table 1 lists the specificity, sequences, target sites and fluorescent labels of the oligonucleotide probes used in this study.

RESULTS AND DISCUSSION

Reactor performance

Fig. 1(a) shows the overall removal efficiency of terephthalate in a 12.5 litre UASB reactor during the course of 300 d operation. The terephthalate loading was gradually increased from 500 mg l⁻¹ to 3000 mg l⁻¹ at day 200. The removal efficiencies of terephthalate were consistently above 95% after day 120. In addition, anaerobic batch experiments were carried out to determine the degradation of terephthalate. Fig. 1(b) indicates that 3 mM terephthalate was completely converted into methane with no fermentative intermediates accumulated throughout the experiment. The rates of terephthalate degradation and methane production were similar to those previously reported (Kleerebezem *et al.*, 1999a).

Microbial community as revealed by electron microscopy

TEM observations (Fig. 2a) revealed at least three different predominant morphotypes in the microbial consortium, including bamboo-shaped cells (0.6–0.8 × 1.5–2 µm) (arrow 1), fat rods (0.8 × 0.6 µm) (arrow 2), and short rods (0.3 × 0.8–1.5 µm) with an opaque background (arrow 3). The bamboo-shaped cells with electron-opaque bodies and a sheath-like structure strongly resembled acetoclastic *Methanosaeta*-like organisms (Whitman *et al.*, 1992), and formed dense clusters throughout the granule. The fat rods with a rough cell boundary usually occurred singly or in pairs. Such morphology has been previously observed for cells of anaerobic benzoate degraders (Mountfort *et al.*, 1984) and these cells contained electron-dense regions

Fig. 1. (a) Overall reactor performance of a 12.5 litre UASB reactor degrading terephthalate-acid wastewater (—, terephthalate influent concentration; ○, terephthalate effluent concentration; ■, removal efficiency). (b) Degradation of terephthalate under an anaerobic batch experiment (●, terephthalate concentration; ○, methane production).

(Fig. 2b, arrow 4). These fat rods were closely surrounded by electron-opaque short rods that were possibly *Methanobrevibacter*- or *Methanobacterium*-like organisms (Grotenhuis *et al.*, 1991; Wu *et al.*, 1996) from the family *Methanobacteriaceae*, which utilize hydrogen or formate (Whitman *et al.*, 1992). In addition to the predominant morphotypes, *Methanospirillum*-like organisms were occasionally observed during the TEM examination (data not shown).

Fig. 2. Transmission electron micrographs of various predominant micro-organisms observed in a terephthalate-degrading granule section at a low magnification (a) and a high magnification (b).

Microbial community structure

Microbial diversity in the syntrophic consortium was determined by using a 16S rDNA clone library approach. In total, 72 and 106 16S rDNA clones were obtained for the archaeal and bacterial populations, respectively. The clones that contained different 16S rDNA inserts were screened by DGGE, which differentiated identical-length DNA fragments based on their electrophoretic migration in a denaturing gradient gel. This method was suggested to be an effective means to screen a large number of 16S rDNAs with a high degree of sequence similarity (Muyzer & Smalla, 1998). A clone with a unique electrophoretic position on the DGGE gel was defined as an operational taxonomic unit (OTU). However, it is possible that two different DNA fragments could migrate to the same position and decrease the clone screening resolution. The phylogenetic affiliation of each 16S rDNA clone was further revealed after sequence analysis and comparison to the GenBank database. Based on the above approach, 5 and 15 different DGGE band positions or OTUs were found among the archaeal and bacterial 16S rDNA clones, respectively, suggesting the presence of at least this many archaeal and bacterial populations in the granular consortium.

The phylogenetic analysis indicated that archaeal OTUs found in the syntrophic granule were all close relatives of methanogens in the *Euryarchaeota* (Fig. 3a). Three OTUs (TA1, TA4 and TA5), which accounted for 81.7% of the total clones, were closely affiliated with *Methanosaeta concilii* (formerly known as *Methanotherix soehngenii*) (99% sequence identity). The other two OTUs (TA2 and TA3) were closely related to the genera *Methanospirillum* and *Methanogenium*, in the

family *Methanospirillaceae* and the order *Methanomicrobiales*, respectively. Both acetoclastic *Methanosaeta* spp. and hydrogenotrophic methanogens (*Methanospirillum*, *Methanobacterium* and *Methanobrevibacter*) are frequently found in anaerobic sludge granules (Sekiguchi *et al.*, 1998; Grotenhuis *et al.*, 1991), and the former was suggested to be important for sludge granulation (Grotenhuis *et al.*, 1992). The TEM results also support the clone library finding that *Methanosaeta*-like organisms were the most predominant archaea. In contrast, methanogens putatively from the family *Methanobacteriaceae* were detected by TEM, but no archaeal clone related to this family was found.

In the domain *Bacteria*, most clones were affiliated with the δ -subclass of the *Proteobacteria* (10 OTUs, 78.5% of the total clones), the green non-sulfur (GNS) bacteria (1 OTU, 7.5%) and *Synergistes* (1 OTU, 0.9%) (Fig. 3b). For the 10 OTUs found in the δ -*Proteobacteria*, two (TA7 and TA20) were closely affiliated with bacterial genera (*Syntrophus* and *Smithella*) that form syntrophic relationships with methanogens to degrade aromatic compounds such as benzoate (Mountfort *et al.*, 1984). The other eight OTUs (66.8% of the clones of *Bacteria*) appeared to form a novel cluster with no close affiliation to any known bacterial isolate. Several environmental clones obtained from environments contaminated with aromatic compounds or solvent (Dojka *et al.*, 1998; Wintzingerode *et al.*, 1999) were closely affiliated within this novel bacterial group (Fig. 3b).

In the GNS division, OTU TA17 (7.5% of the total clones of *Bacteria*) is closely associated with subdivision I (Hugenholtz *et al.*, 1998). Since this subdivision is currently composed of only environmental 16S rDNA clones obtained from various ecosystems, members of

Fig. 3. Phylogenetic affiliations of the 16S rDNA retrieved from the cloning analysis of domain Archaea (a) and domain Bacteria (b). The phylogenetic trees were constructed based on the neighbour-joining method with bootstrapping. The archaeal and bacterial trees were rooted with the 16S rRNA gene sequences of *Methanobrevibacter smithii* and *Aquifex pyrophilus*, respectively. The scale bars correspond to 10 nucleotide substitutions per 100 nucleotide positions. Bootstrap values that were greater than 45% are shown at each node. The abundance of clones found in the domain Archaea or Bacteria is indicated at the end of each clone in parentheses.

this subdivision were suggested to play an important role in the environment (Hugenholtz *et al.*, 1998). Sekiguchi *et al.* (1998) retrieved several 16S rRNA gene sequences within this subdivision from mesophilic and thermophilic UASB systems treating sucrose, acetate and propionate. Their results based on *in situ* hybridization with a specific probe targeting this subdivision further revealed that members of this bacterial group are filamentous, and presumably were responsible for degradation of carbohydrates (Sekiguchi *et al.*, 1999). Taking this together with the observation of the GNS-I 16S rRNA gene sequences in the terephthalate-degrading

UASB system, it may be suggested that members of this group are important in the degradation of substrate under methanogenic conditions. The exact metabolic traits of the GNS-I in UASB systems warrant further studies.

In the *Synergistes* division, one OTU (TA19) was found, but it only accounted for 0.9% of the total bacterial clones. Clone TA19 was closely related to an environmental clone, vandinHA73, obtained from an anaerobic digester (Godon *et al.*, 1997), and formed a cluster that was deeply branched and phylogenetically distant from

Fig. 4. FISH of granule sections for the UASB reactor degrading terephthalate. (a) Topographical view of a granule section that was simultaneously hybridized with a bacterial-domain probe (EUB338) labelled with Cy3 (red) and an archaeal-domain probe (ARC915) labelled with FITC (green) (bar, 100 μ m). (b) FISH of a granule section with the MX825 probe (Cy5, green) for *Methanosaeta* and the MG1200 probe (Cy3, red) for *Methanomicrobiales* (bar, 100 μ m). (c) FISH of a granule section with the MX825 probe (Cy5, green) for *Methanosaeta* and the MB1174 probe (Cy3, red) for *Methanobacteriaceae* (bar, 20 μ m). (d) FISH of a granule section with the EUB338 probe (Cy3, red) and the delta-TA1 probe (Cy5, green), which targeted the novel δ -proteobacterial group found in the sludge granule (bar, 20 μ m). (e) The diversity of the novel δ -proteobacterial group as revealed by sequential FISH with the delta-TA1 probe (Cy5, green) and the delta-TA2 probe (Cy3, red) targeting the OTUs TA11 and TA12 (bar, 20 μ m).

the *Synergistes* genus. Furthermore, three OTUs (TA6, TA8 and TA18), accounting for 13.1% of the total bacterial clones, were found to have a low degree of similarity to any known 16S rRNA sequences in the database. Very few environmental 16S rDNA clones were found to affiliate with OTUs TA19, TA6, TA8 and TA18. As a result, no conclusions could be drawn on the metabolic and physiological traits of microbial populations represented by these OTUs.

Microbial topography as revealed by FISH

The distribution of archaeal and bacterial populations in the granular consortium was determined using *in situ* hybridization with *Archaea* (ARC915)- and *Bacteria* (EUB338)-specific probes, respectively. As with the TEM

observations (Fig. 2), most of the archaeal (green) and bacterial (red) cells observed were randomly distributed and of equal abundance (bacterial cells = $52.9 \pm 5.4\%$) in the sludge granule (Fig. 4a). In some areas where bacterial cells and archaeal cells were closely associated, yellow signals were observed. This is due to the inability of epifluorescence microscopy to differentiate signals from close but different focal planes in a granular thin section thicker than 1–2 μ m. Microcolonies of archaeal and bacterial cells were observed to be denser at the outer region than at the inner region of the granule. A fraction of the granule at the inner region was composed of void areas or micro-channels, which have been suggested to facilitate diffusive transport of substrates, nutrients and by-products within the granule (Wu *et al.*, 1991). This so-called nonlayered architecture observed

in the terephthalate-degrading granule was similar to that observed in granular sludges that degraded propionate (Grotenhuis *et al.*, 1991) or glutamate (Fang *et al.*, 1994a) and hydrolysed proteins (Fang *et al.*, 1994b). The formation of a nonlayered granular structure was suggested to be highly dependent on the degradation mechanism, which usually includes an acetogenesis step followed by a methanogenesis step (Fang *et al.*, 1995). Because the acetogenesis step is often reported as being rate-limiting (Fang *et al.*, 1995; Rocheleau *et al.*, 1999), a slow but steady flux of intermediates (i.e. acetate and hydrogen) into the granule can be provided. A terephthalate-degrading granule with a relatively small size (~ 0.6 mm) could easily provide constant flux of terephthalate, degradation intermediates, nutrients and final gaseous products into and out of the granule, leading to the formation of a nonlayered structure.

Distribution of methanogens

The predominance of acetoclastic *Methanosaeta* and hydrogenotrophic *Methanospirillum* observed in the cloning analysis was further confirmed using combined whole-cell hybridization with two probes specific to the genus *Methanosaeta* (MX825 probe) and the order *Methanomicrobiales* (MG1200 probe), which includes the genera *Methanogenium* and *Methanospirillum*. Fig. 4(b) shows that members of both types of methanogens were present at a significant level, and randomly distributed throughout the entire granule section. Quantitative FISH analysis showed that the acetoclastic *Methanosaeta* ($67 \pm 6.7\%$ of total hybridized cells) was more abundant than the hydrogenotrophic methanogens (i.e. *Methanomicrobiales*). This finding correlated with the cloning result that *Methanosaeta*-related clones were the most predominant archaeal population ($> 70\%$ of total *Archaea* clones).

The cloning results did not reveal any sequences from the family *Methanobacteriaceae*, but cells morphologically resembling *Methanobacteriaceae* were observed using TEM. This discrepancy was addressed by using *in situ* hybridization with probes targeting the *Methanobacteriaceae* (probe MB1174) and *Methanosaeta* (probe MX825). Fig. 4(c) shows that a significant fraction of cells hybridized with the MB1174 probe (red), and the cells were closely clustered with cells that hybridized with the *Methanosaeta*-specific probe (green). The inability to retrieve 16S rRNA gene sequences from *Methanobacteriaceae* was likely due to the bias associated with total community DNA extraction (Sekiguchi *et al.*, 1998), PCR amplification (Suzuki & Giovannoni, 1996) and 16S rDNA cloning (Amann *et al.*, 1995).

Syntrophic bacterial populations

The 16S rDNA cloning analysis revealed that the novel group found in the δ -*Proteobacteria* was the predominant group (Fig. 3b). Thus, the abundance and localization of this novel bacterial group in the granular consortium were further determined by FISH. Two 16S rRNA-targeted oligonucleotide probes (Table 1) were

designed to encompass this novel bacterial group at different levels of specificity as shown in Fig. 3(b). Probe delta-TA1, a 'group-specific' probe, targeted the novel δ -proteobacterial group TA. It had at least three mismatches with the 16S rRNA gene sequence of any known bacterium (e.g. *S. erythraea*) outside the novel group, and more than three mismatches with other bacterial sequences in the δ -*Proteobacteria*. Probe delta-TA2 was a 'subgroup-specific' probe that targeted the most abundant OTUs, TA11/TA12 (27.3% of the total bacterial clones), within the novel bacterial group. This probe had one mismatch with TA11, three mismatches with TA7, TA10, TA14 and TA16 within the novel group, and more than four mismatches with any known bacterial species from the δ -*Proteobacteria*. The specificity of these two probes was tested under different hybridization conditions, and the optimal hybridization condition showed no reaction to the non-target organisms, *S. erythraea* and *Desulfovibrio* sp.

Fig. 4(d) shows the combined FISH of the granular consortium with a bacterial-domain probe (EUB338) and a group-specific probe (delta-TA1). At least $87 \pm 7.6\%$ of the clustered bacterial cells hybridized with the probe delta-TA1 and showed as yellow coloured as a result of overlapping red (EUB338) and green (delta-TA1) probes. The red cells represent bacterial populations outside the novel bacterial group. Unlike cells belonging to the novel bacterial group, these red cells did not form dense microcolonies, but were randomly scattered throughout the sludge granule. The predominance of the novel group TA was further confirmed by the combined use of a probe (delta402) specific for the δ -*Proteobacteria* and probe delta-TA1 (data not shown). This finding closely agreed with the 16S rDNA cloning analysis result.

The population diversity within the novel δ -proteobacterial group TA was further determined by the combined use of probes delta-TA1 and delta-TA2. At least two different populations with similar rod-shaped morphology were observed (Fig. 4e). Based on the hybridization signal, the microbial population targeted by probe TA2 made up a significant proportion ($72 \pm 9.6\%$) of the novel bacterial group. This result also verified that the optimal hybridization specificity was obtained for probes delta-TA1 and delta-TA2. Thus, the novel δ -proteobacterial group should include at least two or more than two members, considering that at least 10 different 16S rRNA gene sequences have been found within this novel group from various environments (Dojka *et al.*, 1998; Wintzingerode *et al.*, 1999).

Conclusions

In summary, a not-yet-cultured, rod-shaped novel group in the δ -*Proteobacteria* was presumed to be the primary population responsible for degrading terephthalate to acetate and hydrogen; it formed a close spatial association with different methanogenic populations (i.e. *Methanosaeta*, *Methanospirillum* and *Methanobacteriaceae*) that convert acetate, hydrogen and carbon

dioxide into methane. Enrichment culture and characterization of the terephthalate-degrading consortium are being undertaken to further confirm the role of the novel δ -proteobacterial group in terephthalate degradation.

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