Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis

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The microbial diversity of two types of methanogenic granular sludge, mesophilic (35 °C) and thermophilic (55 °C), which had been treating sucrose/propionate/acetate-based artificial wastewater were compared. 16S rDNA clone libraries were constructed by PCR with a prokaryote-specific primer set, and partial sequencing of the clonal 16S rDNAs was conducted for phylogenetic analysis. Of 115 mesophilic granule and 110 thermophilic granule clones sequenced, 19 and 22 %, respectively, were phylogenetically affiliated with the domain *Archaea*, and the remainder in each case were assigned to the domain *Bacteria*. Within the domain *Archaea*, the 16S rDNA clones in both libraries showed relatively close relationships with those of methanogens. Within the *Bacteria*, a major group represented in the mesophilic clone library was the delta subclass of the *Proteobacteria* (27 %), in which high degrees of relatedness were observed between the clonal 16S rDNA sequences and those of previously identified syntrophic bacteria and sulfate-reducing bacteria. In contrast, in the thermophilic clone library, the *Thermodesulfovibrio* group (19 %), the green non-sulfur bacteria (18 %) and the low G+C subclass of the Gram-positive bacteria (18 %) were predominant. A significant difference between the two libraries was that no clone affiliated with the *Proteobacteria* was detected in the thermophilic clone library, whereas the *Proteobacteria* was the most predominant group in the mesophilic clones. Thirty-six and 24 different sequences were found in the mesophilic and thermophilic clones, respectively, suggesting that the microbial diversity of the thermophilic granule was lower than that of the mesophilic granule.

Keywords: 16S rDNA, granular sludge, thermophilic, upflow anaerobic sludge blanket (UASB) reactor

INTRODUCTION

Granulation of sludge is the most characteristic phenomenon of upflow anaerobic sludge blanket (UASB) reactors, in which micro-organisms form dense aggregates as a result of their self-immobilization property. In these reactors, one pellet of granular sludge is a functional unit which comprises all of the different

trophic groups of micro-organisms necessary for anaerobic degradation of organic compounds (Lettinga, 1995). In encompassing various competitive and syntrophic associations between micro-organisms, the consortium forms a quite unique microbial ecosystem within several millimetres of an aggregate (Grotenhuis *et al.*, 1991; Harmsen, 1996; Macario *et al.*, 1991; MacLeod *et al.*, 1990; Stams *et al.*, 1989). In the last decade, several micro-organisms in granular sludges have been isolated and characterized (Harmsen, 1996; Stams *et al.*, 1992; Wu *et al.*, 1992). However, cultivation-dependent methods have limitations in elucidating diversity in the microbial ecosystem (Ward *et al.*, 1990), and thus many of the component species may

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; MUR, mesophilic UASB reactor; OTU, operational taxonomic unit; TUR, thermophilic UASB reactor; UASB, upflow anaerobic sludge blanket.

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remain to be identified. In particular, the microbial community in thermophilic anaerobic processes is poorly characterized.

In recent years, the thermophilic anaerobic digestion process has become an attractive alternative for medium- and high-strength wastewater treatment, because it is capable of accommodating a very high loading rate at reasonable treatment efficiency. However, the thermophilic processes appear to be more sensitive to environmental changes than the mesophilic process (van Lier, 1996), and formation of thermophilic granular sludge is much more difficult than the mesophilic equivalent (Schmidt & Ahring, 1995; Wiegant, 1986). Consequently, full-scale thermophilic UASB processes are not in routine operation and further studies on the microbial composition of the thermophilic anaerobic community are required to establish stable and high-performance thermophilic processes.

Here, we characterize the microbial communities of two types of methanogenic sludge, mesophilic (35 °C) and thermophilic (55 °C) granules, based on 16S rRNA gene analysis.

METHODS

Operation of UASB reactors. Granules were sampled from two types of laboratory-scale UASB reactor (13 l capacity), i.e. mesophilic (MUR) (35 °C) and thermophilic (TUR) (55 °C). Both reactors had been fed with artificial wastewaters of similar composition comprising mainly sucrose, acetate, propionate and peptone or yeast extract [4.5:2.25:2.25:1, chemical oxygen demand (COD)-base] over 2 years operation. The wastewater for MUR comprised (mg l⁻¹): sucrose, 800; sodium acetate, 600; propionate, 300; peptone, 200; NH₄Cl, 110; Na₂SO₄, 120; KH₂PO₄, 150; KCl, 300; CaCl₂·2H₂O, 150; MgCl₂·2H₂O, 400; NaHCO₃, 2000; trace elements as indicated below. The wastewater for TUR comprised (mg l⁻¹): sucrose, 1600; sodium acetate, 1200; propionate, 600; yeast extract, 200; NH₄Cl, 222; Na₂SO₄, 233; KH₂PO₄, 11; KCl, 16; CaCl₂·2H₂O, 5; MgCl₂·6H₂O, 33; NaHCO₃, 2000; trace elements. The trace elements contained in both wastewaters were (mg l⁻¹): FeCl₂·4H₂O, 3.93; CoCl₂·6H₂O, 0.17; ZnCl₂, 0.07; H₃BO₃, 0.06; MnCl₂·4H₂O, 0.50; NiCl₂·6H₂O, 0.04; CuCl₂·2H₂O, 0.03; and NaMoO₄·2H₂O, 0.03. The influent for the reactors, consequently, had a strength of 2000 mg COD l⁻¹ for MUR, 4000 mg COD l⁻¹ for TUR (volumetric loading rate for MUR is 8 kg COD m⁻³ d⁻¹ and for TUR is 23 kg COD m⁻³ d⁻¹; hydraulic retention time for MUR is 6 h and for TUR is 4.2 h), respectively. The pH of the influent was approximately neutral. The seed sludges were mesophilically digested anaerobic sludge (for MUR) and thermophilically digested anaerobic sludge (for TUR).

DNA extraction. Granules were collected directly from two different sites of each UASB reactor which had been operated for over 2 years, immediately frozen in liquid nitrogen and stored at -20 °C. Before DNA extraction, frozen granules were slowly thawed, washed gently with phosphate buffer (10 mM, pH 7.2), and then three to five granules were homogenized in extraction buffer (10 mM Tris/HCl, pH 7.5, 50 mM EDTA, 0.5 M NaCl). The DNA extraction strategy was based on the method of Tsai & Olson (1991) with some modifications (Ohkuma & Kudo, 1996). Briefly, after 5 s sonication, the mixture was incubated in the presence of

lysozyme (final concentration 5 mg ml⁻¹) for 40 min at 37 °C. At the end of the incubation, proteinase K was added to a final concentration of 2 mg ml⁻¹ and incubated at 37 °C for another 40 min with occasional agitation. SDS was added to give a final concentration of 1% (w/v) and the mixture was agitated gently. The mixture was frozen in liquid nitrogen followed by thawing in a 65 °C water bath. This step was repeated three times to release a sufficient amount of nucleic acids. For purification of the mixture, an equal volume of phenol (saturated with 10 mM Tris/HCl, pH 8.0) was added followed by extraction with phenol/chloroform (1:1). Nucleic acids were recovered by addition of an equal volume of 2-propanol and centrifugation for 20 min at 12000 g, 4 °C. The pellets were rinsed with 70% ethanol and resuspended in 50 µl TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). RNA in the suspension was digested with RNase A (final concentration 50 µg ml⁻¹) for 30 min at 37 °C. Nucleic acids were precipitated with 2.5 vol. 100% ethanol and 0.25 vol. sodium acetate (3 M, pH 5.2). Extracted DNA was rinsed with 70% ethanol and dissolved in TE. Bulk DNA preparations extracted from the granules at two different sites of each reactor were combined for further analysis.

PCR amplification and cloning. Amplification of 16S rRNA genes from the purified DNA preparations was carried out by PCR with *Taq* polymerase (Perkin Elmer) according to the manufacturer's instructions (200 ng template DNA, 1 × *Taq* polymerase buffer, 2.5 units *Taq* polymerase, 200 µM dNTPs and 100 pmol of each primer in a 100 µl reaction volume). The PCR primers used in the amplification were universal primer 530F (5'-GTGCCAGCMGCCGCGG-3'; M represents A or C, 514–529 *Escherichia coli* position) and prokaryote-specific primer 1490R (5'-GGTTACCTTGTTACGACTT-3'; 1491–1509 *E. coli* position) (Lane, 1991; Weisburg *et al.*, 1991). The reaction conditions were as follows: initial denaturation at 95 °C for 9 min, followed by 15 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. PCR products corresponding to the expected size of amplified rDNA (1.0 kb) were purified with a GeneClean II kit (Bio 101). To reduce the possible bias caused by PCR amplification, rDNA was amplified in triplicate tubes and these were combined for the next cloning step. The rDNA fragments were cloned into plasmids using the TA cloning kit (Novagen).

Sequencing and phylogenetic analysis. Clonal rDNAs were prepared from randomly selected recombinants and used as template for sequencing. Sequencing was conducted with the 530F primer and PRISM Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and an automated sequence analyser (model 377; Applied Biosystems). rDNA sequences with a range of about 500–600 bases were obtained for all clones. All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997) and compared with similar sequences of the reference organisms by BLAST search (Altschul *et al.*, 1990). Sequence data were aligned with the CLUSTAL W package (Thompson *et al.*, 1994) and corrected by manual inspection. Phylogenetic trees were constructed by the neighbour-joining method (Saito & Nei, 1987) with the MEGA package (Kumar *et al.*, 1993). Bootstrap resampling analysis (Felsenstein, 1985) for 100 replicates was performed to estimate the confidence of tree topologies. Identical sequences were recognized by phylogenetic tree analysis and manual comparison, in which completely (100%) similar sequences were defined as identical, and used for further phylogenetic analysis as an operational taxonomic unit (OTU). The OTUs were designated MUG1–MUG36 for the MUR granule clones and TUG1–TUG24 for the TUR clones.

In situ hybridization. Fresh granule samples were homogenized, fixed with 4% paraformaldehyde in PBS (0.13 M NaCl in 10 mM Na₂HPO₄, pH 7.2), and left for 4 h. The samples were dispersed with sonication and immobilized on glass slides coated with Vectabond (Vector Laboratories). For hybridizations, we used the following rhodamine-labelled oligonucleotide probes complementary to specific regions of 16S rRNA: (i) EUB338, specific for the domain *Bacteria* (Amann *et al.*, 1990) and (ii) ARC915, specific for the domain *Archaea* (Stahl & Amann, 1991). Hybridizations were performed at 46 °C for 3 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS) containing each labelled probe (5 ng µl⁻¹). Hybridization stringency was adjusted by adding formamide to the hybridization buffer (5% for EUB338 and 35% for ARC915). After hybridization, the slides were washed at 48 °C for 20 min with washing buffer containing the same components of the hybridization buffer except the probes. To enumerate the total cell number in the samples, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 5 µg ml⁻¹. Over 1000 DAPI-stained cells were counted to determine the ratio of the probe-labelled cells to the total cells.

RESULTS

UASB reactor performance

Two laboratory-scale UASB reactors were operated over 2 years and formed well-settling granules adapted at different temperatures but with similar composition of substrate. Mesophilic and thermophilic granular sludges with a mean diameter of 1–2 mm were obtained after continuous operation of 3 and 7 months, respectively. COD removal of the reactors was 90–97%, and almost all of the COD removed (approximately 95%) was converted to methane in soluble and gaseous form. Since influent wastewater contained low concen-

trations of sulfate (81 mg l⁻¹ for MUR, 157 mg l⁻¹ for TUR), only a small amount of COD degradation appeared to be attributed to sulfate reduction (approx. 3% of the total COD removed).

PCR amplification

PCR amplification of 16S rRNA genes was conducted with prokaryote-specific primers, resulting in amplification of the domains *Archaea* and *Bacteria* simultaneously. To avoid involving the bias caused by template annealing in PCR amplification (Suzuki & Giovannoni, 1996), the thermal cycling in PCR was decreased to 15 cycles. Only a single band corresponding to the expected size of amplified DNA (approximately 1.0 kb) was observed for both granule DNA preparations on agarose gel electrophoresis. This fraction was used for the construction of 16S rDNA clone libraries for the granules.

Overall phylogenetic analysis

We prepared 115 and 110 clones of 16S rDNA libraries for the MUR and the TUR granules, respectively. Among the clones analysed, 37 different sequences (OTUs) were found in the MUR granule library and 24 OTUs in the TUR granule library. Large-scale phylogenetic tree analysis of these OTUs was performed to affiliate the clonal sequences to hitherto-determined groups (Maidak *et al.*, 1997; Woese, 1987; Woese *et al.*, 1990). From this analysis, 22 (19% of the total clones) and 24 (22%) 16S rDNA clone sequences from the MUR and TUR granule libraries were affiliated with the domain *Archaea* and 92 (80%) and 86 (78%) clones

Table 1. Distribution of 16S rDNA clones detected in the MUR and TUR granules

Group	MUR library			TUR library		
	No. of OTUs	No. of MUR clones	Percentage of total clones	No. of OTUs	No. of TUR clones	Percentage of total clones
<i>Archaea</i>						
<i>Euryarchaeota</i>	3	22	19	3	24	22
<i>Bacteria</i>						
Green non-sulfur bacteria	4	16	14	5	20	18
<i>Thermodesulfovibrio</i> group	2	6	5	2	21	19
<i>Synergistes</i>	1	1	1	1	6	5
Gram-positive bacteria						
High G + C subclass	2	5	4	—	—	—
Low G + C subclass	4	8	7	9	17	16
<i>Planctomyces</i> and relatives	5	11	10	3	14	13
<i>Cytophaga/Flexibacter/Bacteroides</i>	2	9	8	—	—	—
Spirochaetes and relatives	3	3	3	—	—	—
<i>Proteobacteria</i>						
Delta subclass	9	31	27	—	—	—
Green sulfur bacteria	1	2	2	1	8	7

Fig. 1. Phylogenetic tree of the clones among the class *Euryarchaeota* of the domain *Archaea*, based on neighbour-joining analysis (Saito & Nei, 1987) of partial 16S rDNA sequences. MUG represents the mesophilic granule clones and TUG represents the thermophilic clones. Numbers in parentheses indicate the frequency of appearance of the completely matched sequences (clones) in the total clones (number of identical clones/number of the total clones). Numbers at nodes represent bootstrap values (Felsenstein, 1985) for the nodes (100 times resampling analysis). *Desulfurococcus mobilis* is used as the outgroup.

Fig. 2. Phylogenetic tree of the clones among the *Thermodesulfobivibrio* group, the green non-sulfur bacteria and *Synergistes* of the domain *Bacteria*, based on neighbour-joining analysis of partial 16S rDNA sequences. *Synergistes* is included in the paraphyletic assemblage in the RDP phylogenetic list (Maidak *et al.*, 1997), but this taxon does not form a clade with that in this analysis. Numbers in parentheses and numbers at nodes are the same as in Fig. 1. *Aquifex pyrophilus* is used as the outgroup.

were assigned to the domain *Bacteria*, respectively. Only one clonal sequence in the MUR granule library could be recognized as a chimeric artifact.

We also performed *in situ* hybridization counts for both sludges using *Archaea* (ARC915)- and *Bacteria* (EUB338)-specific probes (Stahl & Amann, 1991; Amann *et al.*, 1990). By this approach, it was found that *Archaea* cells in the MUR and TUR granules accounted for approximately 37 and 42%, respectively, of the total

DAPI counts (data not shown). *Bacteria* cells accounted for 45 and 43% of the DAPI counts, respectively.

The major groups in the MUR library were the delta subclass of the *Proteobacteria* (27% of the total clones), the *Euryarchaeota* (19%) and the green non-sulfur bacteria (14%). There were no clonal sequences belonging to the other subclasses of the *Proteobacteria*. The major groups in the TUR granule library were the *Euryarchaeota* (22%), the *Thermodesulfobivibrio* group

Fig. 3. Phylogenetic tree of the clones among the class Gram-positive bacteria of the domain *Bacteria* based on neighbour-joining analysis of partial 16S rDNA sequences. Numbers in parentheses and numbers at nodes are the same as in Fig. 1. *Bacillus subtilis* is represented as the outgroup of the tree among the high G+C subclass (a) and *Arthrobacter globiformis* is used as the outgroup of the tree among the low G+C subclass (b).

(called 'paraphyletic assemblage' in the RDP; Maidak *et al.*, 1997) (19%), the green non-sulfur bacteria (18%) and the low G+C subclass of the Gram-positive bacteria (18%) (Maidak *et al.*, 1997) (Table 1). A significant difference between the two libraries was that a large number of 16S rDNA sequences belonging to the class *Proteobacteria* were detected in the MUR granule clones, whereas, interestingly, there was no clonal sequence affiliated with the *Proteobacteria* in the TUR granule library. Moreover, the MUR clones were

assigned to more diverse phylogenetic groups than the TUR clones.

Archaea domain

In the domain *Archaea*, we obtained three OTUs in each library (Fig. 1). All of these clones were assigned to the class *Euryarchaeota*, and all clones were close relatives of methanogens. Twenty completely identical sequences apparently belonging to *Methanosaeta concilii* (Patel &

Fig. 4. Phylogenetic tree of the clones among the class *Planctomyces* and relatives of the domain *Bacteria* derived by neighbour-joining analysis of partial 16S rDNA sequences. Numbers in parentheses and numbers at nodes are the same as in Fig. 1. *E. coli* is used as the outgroup.

Sprott, 1990) were detected in the MUR clones (MUG2). One clone showing 98.5% similarity to the 16S rDNA of *Methanosaeta concilii* (MUG1), and one clone affiliated with the group *Methanobacteriales* (MUG3) were also detected in the MUR clones. In the TUR granule library, the most dominant sequence affiliated with the *Archaea* was closely related to *Methanosaeta thermophila* ('*Methanothrix thermophila*') (Kamagata *et al.*, 1992) with 97.2–97.5% sequence similarity (TUG1 and TUG2, 20 clones). In addition, one OTU was recovered in the *Methanobacteriales* (TUG3, four clones), *Methanobacterium thermoformicum* being the closest organism to the clones (96.7% similarity).

Bacteria domain

(i) Green non-sulfur bacteria and relatives. We observed a number of clones affiliated with deeply branched groups in the domain *Bacteria*, the class green non-sulfur bacteria and the *Thermodesulfovibrio* group, at high frequencies in both MUR and TUR granule libraries (Fig. 2). Within the green non-sulfur bacteria, four and five OTUs, respectively, were detected from the MUR and TUR granule libraries at high frequencies (Table 1). Many of these clones (e.g. MUG6, TUG6) were related to the 16S rDNA of unidentified eubacterium RB41 detected from sediment of a Carolina bay by 16S rRNA gene analysis (Wise *et al.*, 1997), with sequence similarities of 87–93%. Some OTUs detected from each library were closely related to each other, although the adapted temperatures of the granules were different; e.g. MUG9 and TUG9 had 99.2% similarity. Within the *Thermodesulfovibrio* group, two OTUs were observed in each library (TUG4, 5 and MUG4, 5). The two OTUs (TUG4, 5) of the TUR clones had high degrees of relatedness to *Thermodesulfovibrio yellowstonii* (Henry *et al.*, 1994) with 96.8–98.0% sequence similarity. In addition, one OTU affiliated with the *Synergistes* clade was found in each library (MUG10, TUG11).

(ii) Gram-positive bacteria. In the class Gram-positive bacteria, six and nine OTUs were found in the MUR and TUR granule libraries, respectively (Fig. 3). In the high G + C subclass, we observed only two OTUs from the

MUR clones. These clones were closely related to *Propionibacterium* species (Cummins & Johnson, 1991). In the low G + C subclass, four and nine OTUs were detected in the MUR and TUR clones, respectively. Two OTUs (MUG13, TUG12) detected from both libraries were closely related to *Streptococcus bovis* (Hardie & Whaley, 1991) with 93.1–93.6% similarity. One OTU (MUG15) from the MUR clones was a close relative of *Clostridium acetobutylicum* (Hippe *et al.*, 1991) with 95.0% similarity. In the TUR library, one OTU (TUG15) was detected as a close relative of *Thermosyntropho lipolytica* (Svetlitsnyi *et al.*, 1996) (93.2% similarity), which is a thermophilic syntrophic fatty-acid-oxidizing species, and two OTUs were found as closely related sequences to propionate-oxidizing spore formers A and B (TUG16, 17; 95.9–96.1% similarity), which have been enriched as Gram-positive mesophilic, syntrophic propionate-oxidizing species (Harmsen, 1996). The remaining OTUs formed a clade with the low G + C subclass, but there was no closely related relevant organism in the databases.

(iii) Planctomyces and relatives. Within the *Planctomyces* and relatives, including the *Planctomyces*, *Chlamydia* and *Verrucomicrobiales* subclasses (Maidak *et al.*, 1997), five and three OTUs were found in the MUR and TUR libraries, respectively (Fig. 4). Within the *Planctomyces* subclass, we could retrieve several clones from both libraries, one of which (TUG21) from the TUR library could be a relative of the genus *Pirellula*. However, the remaining clones were phylogenetically distant from any reference organisms and were deeply branched within the group with relatively low bootstrap values. Within the *Chlamydia* and *Verrucomicrobiales* subclasses, one OTU was found in the MUR clones in both groups. However, there was no close relevant organism previously cultivated, and the bootstrap values of these clades were also quite low, suggesting that the affiliation of the OTUs cited above cannot be determined.

(iv) Cytophaga and relatives, and spirochaetes and relatives. In the class *Cytophaga/Flexibacter/Bacteroides*, we observed two OTUs only from the MUR library (MUG22, 23), to which the closest organism

Fig. 5. Phylogenetic tree of the clones among the class *Cytophaga/Flexibacter/Bacteroides*, the spirochaetes and relatives and the green sulfur bacteria of the domain *Bacteria* derived by neighbour-joining analysis of partial 16S rDNA sequences. Numbers in parentheses and numbers at nodes are the same as in Fig. 1. *E. coli* is used as the outgroup.

Fig. 6. Phylogenetic tree of the clones among the delta subclass of the *Proteobacteria*. Numbers in parentheses and numbers at nodes are the same as in Fig. 1. *E. coli* is used as the outgroup.

was *Cytophaga fermentans* (Bachmann, 1955) with 87.4–87.5% sequence similarity (Fig. 5). Nine clones were affiliated, and this fraction was the fourth predominant class in the MUR library. Within the class spirochaetes and relatives, three OTUs related to *Leptospira* species were found in the MUR clones (MUG25, 26, 27). Two OTUs (MUG 24, TUG 24) were affiliated with the class green sulfur bacteria with a 92% bootstrap value, but a relatively large evolutionary distance was estimated from hitherto-determined green sulfur bacterial species.

(v) *Proteobacteria*: delta subclass. Within the class *Proteobacteria*, we were able to find a number of clones only in the MUR library (Fig. 6). All clone sequences in the *Proteobacteria* were located in the delta subclass. In this clade, all of the clones were relatively close to the reference organisms in the databases. Some of these sequences were closely related to sulfate-reducing organisms, e.g. *Desulfovibrio* species (MUG34, 99.3% similarity), *Desulfobulbus propionicus* (Widdel & Pfennig, 1982) (MUG36, 98.5% similarity) and *Desulfobulbus elongatus* (Samain *et al.*, 1984) (MUG35, 97.4% simi-

larity). One OTU was closely related to *Syntrophobacter* species (MUG28, 95.2% with strain MPOB; Stams *et al.*, 1993), with a relatively high number of clones (six clones). Three OTUs (MUG29, 30, 31) assigned to a strain SYN7 clade, which was enriched from a UASB granular sludge as a mesophilic, syntrophic propionate-oxidizing strain (Harmsen, 1996; Harmsen *et al.*, 1996), were detected at high frequency (19 clones). Two OTUs (MUG32, MUG33) were recovered in the delta subclass, but there was no close reference sequence in the databases.

DISCUSSION

The microbial constituents of mesophilic and thermophilic methanogenic granular sludges were studied by 16S rDNA analysis. Granulation of both sludges occurred using wastewaters that contained very similar mixtures of defined substrates (sucrose; acetate; propionate; peptone or yeast extract) over 2 years operation. Although the seed sludge for each reactor was different, both performed high COD removal with methane formation at different temperatures, suggesting that both granular sludges contained micro-organisms necessary for complete mineralization of the substrates at different temperatures, i.e. both granules had the same function for COD removal.

For the construction of the 16S rDNA clone libraries, we used a primer set that could amplify members of the domains *Archaea* and *Bacteria* simultaneously for PCR. To minimize bias introduced in the analysis (Farrelly *et al.*, 1995; Suzuki & Giovannoni, 1996), we used the following strategies: (1) bulk community DNAs were extracted from the granules taken from two different sites of the reactor and combined for use; (2) 16S rDNAs were amplified by PCR in triplicate PCR tubes containing the extracted bulk community DNA, then they were combined for cloning; (3) 15 PCR cycles were employed to minimize biases in the PCR step (Suzuki & Giovannoni, 1996; Wilson & Blitchington, 1996). Although it is impossible to remove all possible biases that might occur during DNA extraction, PCR amplification and cloning, all experimental conditions in these steps for the mesophilic and thermophilic granules were identical, hence the difference in the composition of 16S rDNA sequence clones between the two granules could reflect the difference in microbial diversity *in situ*.

From this analysis, 22 (19% of the total clones) and 24 (22%) clonal 16S rDNA sequences from the mesophilic and thermophilic granule clone libraries, respectively, were affiliated with the domain *Archaea*, and the remaining clones were assigned to the domain *Bacteria*. However, direct counts by *in situ* hybridization with an *Archaea*-specific probe (ARC915) (Stahl & Amann, 1991) showed that the *Archaea* cells in the mesophilic and thermophilic granules comprised approximately 37 and 42% of the total cell number, respectively. The difference in the values between the cloning method and

in situ hybridization enumeration might be due to the low efficiency of the lysis of hydrogenotrophic methanogens residing in both granules, although the cell number cannot be compared directly with the number of corresponding 16S rDNA sequences which were obtained. Epifluorescence microscopy revealed that a number of F₄₂₀-autofluorescent curved rods morphologically similar to *Methanobacterium* were present. By using a pure culture of *Methanobacterium thermoautotrophicum*, it was found that the cells were not susceptible to lysis under the same conditions that we employed for the lysis of the granules (data not shown). The DNA extraction method employed here is milder than mechanical methods such as bead beating but we were concerned that vigorous extraction methods could lead to fragmentation of DNA strands due to shearing, which could have resulted in amplification of chimeric DNAs in the PCR step (Liesack *et al.*, 1991). To avoid generating chimeric DNAs, we used a mild extraction method, which, however, could result in lysis bias. In fact, we could obtain only four clonal sequences close to the *Methanobacteriales* of the 110 sequences from the thermophilic granules, and only one close sequence of 115 sequences from the mesophilic granules. This suggests that the cloning strategy may have resulted in a limited estimate of diversity by the failure to obtain DNA from certain community members.

The mesophilic granule contained at least 36 different 16S rDNA sequences within the microbial architecture and the thermophilic granule possessed at least 24 different sequences. From the cumulative number of different sequences plotted against the number of clones, we could estimate the difference in microbial diversity between the two granules (Fig. 7). We depicted approximate lines for the cumulative number of sequences versus the number of clones by calculating a simple parabola equation, $y = x/(ax + b)$, where x = number of clones, y = cumulative number of different sequences and a , b = coefficients, and estimated the possible total number of different sequences from this equation, in which y becomes $1/a$ if x was infinity. According to the calculations, the possible total number of sequences was estimated to be approximately 53 for the mesophilic granule and approximately 30 for the thermophilic granule. From this estimation, it is suggested that the microbial content in the mesophilic granules was almost twice as diverse as that of the thermophilic granule, although both of the granules had been exposed to similar substrates over 2 years operation.

In terms of microbial community structure, the difference between the two granules was clear. Major groups represented in the mesophilic clone library were the delta subclass of the *Proteobacteria* (27%) and the *Euryarchaeota* (possibly methanogens, 19%). In contrast, in the thermophilic clone library, the *Euryarchaeota* (possibly methanogens, 22%), the *Thermodesulfobivrio* group (19%), the green non-sulfur bacteria (18%) and the low G + C subclass of the Gram-positive bacteria (18%) were predominant groups. No thermophilic clone was recovered in the *Proteobacteria*,

Fig. 7. Estimation of microbial diversities in the mesophilic and thermophilic granular sludges. The numbers of cumulative different 16S rDNA sequences against the numbers of clones obtained were plotted for both of the sludges. Prediction of cumulative lines was performed by using a parabola equation, $y = x/(ax + b)$, where y = cumulative number of different sequences, x = number of clones made and a , b = coefficients.

whereas this class was the most predominant group in the mesophilic clone library (Table 1).

With regard to the relationship between substrate composition and expected trophic groups in the reactors, both may contain the following major trophic groups: sugar (sucrose)-fermenting microbes, acetate-utilizing microbes (acetoclastic methanogens), propionate-oxidizing microbes, microbes oxidizing other fermentation products (such as alcohols and fatty acids) and hydrogen (formate)-utilizing microbes (hydrogenotrophic methanogens). Although we should carefully avoid overinterpretation of data based solely on clonal sequences, we could find several clones closely related to the known trophic groups cited above, such as clones close to *Methanosaeta concilii* (Patel & Sprott, 1990) and *Methanosaeta thermophila* (Kamagata *et al.*, 1992) ('*Methanothrix thermophila*'), acetoclastic methane-producing methanogens, *Methanobacterium thermoformicum* (Zhilina & Ilarionov, 1985), a hydrogen-utilizing methanogen, *Syntrophobacter* (Boone & Bryant, 1980) performing syntrophic propionate oxidation, an anaerobic syntrophic propionate-oxidizing species (SYN7) enriched from an UASB granule (Harmsen, 1996; Harmsen *et al.*, 1996), some sulfate-reducers and clostridia. However, the remaining clones, possibly affiliated with the green non-sulfur bacteria, the spirochaetes and relatives, *Synergistes*, and the *Planctomyces* and relatives, in both libraries were distinct from known organisms. Hence the functions of the microbes represented by these sequences are unknown.

Recently Godon *et al.* (1997) reported on 16S-rRNA-based molecular microbial diversity within anaerobic

sludge used to treat wine distillation wastewater. They reported a large number of clones related to the green non-sulfur bacteria, the spirochaetes and relatives, *Synergistes*, and the *Planctomyces* and relatives as functionally unknown sequences. These types of sequences were also abundant in our clone libraries although our feed substrate was well-defined and much simpler, suggesting that the organisms represented by these sequences are certainly functioning as fundamental components in the anaerobic degradation of organic compounds.

Our study revealed the microbial composition and diversity of the mesophilic and thermophilic granules. Most importantly, the data suggest a large difference in microbial community structures between the two granules, and that the thermophilic granule has lower microbial diversity than the mesophilic granule. These findings could explain why thermophilic anaerobic processes are more susceptible to environmental changes than mesophilic processes (van Lier, 1996). Such elasticity in the mesophilic granule could be supported by the quite diverse microbes which contain multiple genera and species with the same metabolic functions. As the database continues to expand, we will be able to further elucidate the community structures of anaerobic sludge granules.

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