Molecular and conventional analyses of microbial diversity in mesophilic and thermophilic upflow anaerobic sludge blanket granular sludges

Y. Sekiguchi*, **, Y. Kamagata**, A. Ohashi* and H. Harada*

*Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan
**Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

Abstract The microbial community structure of mesophilic (35°C) and thermophilic (55°C) methanogenic granular sludges was surveyed by using both cultivation-independent molecular approach and conventional cultivation technique in order to address the fundamental questions on the microbial populations, i.e. who are present, where they are located, and what they are doing there. To elucidate the microbial constituents within both sludges, we first constructed 16S ribosomal DNA clone libraries, and partial sequencing of the clones was conducted for phylogenetic analysis. In this experiment, we found a number of unidentifiable clones within the domain Bacteria as well as clones that were closely related with 16S rDNAs of cultured microbes. The unidentifiable clones accounted for approximately 60–70% of the total clones in both mesophilic and thermophilic libraries. 16S rRNA-targeted in situ hybridization combined with confocal laser scanning microscopy was subsequently employed to examine where the uncultured populations were located within sludge granules. Spatial organization of uncultured microbes was visualized in thin-sections of both types of granules using fluorescent oligonucleotide probes, which were designed based on the clone sequences of certain novel clusters. This resulted in the detection of two types of uncultured cells in specific locations inside the granules. Finally, the goal-directed conventional cultivation technique was employed to recover such uncultured anaerobes and uncover their physiology and functions. In this approach, a total of five new species of thermophilic microorganisms were isolated, including several types of syntrophs and a novel sugar-fermenting bacterium. In the previous molecular approaches, all of these isolates were suggested to be significant populations within thermophilic granular sludge, hence obtaining these isolates in pure culture decreased the fraction of unknown clones in the previous thermophilic clone library from 70% to 40%. In conclusion, these approaches successfully revealed biodiversity and spatial organization of microbes of interest in sludge granules, and enlarged the fundamental knowledge of microbial constituents functioning as significant populations in the UASB processes.

Keywords 16S rRNA gene; fluorescence in situ hybridization; granular sludge; syntroph

Introduction

Upflow anaerobic sludge blanket (UASB) process has been widely used over the past decades mainly for the treatment of medium- and high-strength organic wastewaters (Lettinga, 1995). Among a number of characteristic features of the UASB process, granulation of sludge is undoubtedly the most important and interesting phenomenon. Granulation forms dense spherical aggregates, in which several metabolic groups of microbes reside for complete mineralization of organic matters. In addition, the granule pellets involve close interactions among the microorganisms linked to the food web in anaerobic organic degradation. This unique trait of the UASB process has been an intriguing research topic for both engineers and microbiologists. Until recent years, microbial populations within such sludge granules have been intensively monitored mainly with conventional techniques such as methane-producing activity and most probable number (MPN)-counting. The internal architecture of granules has been also surveyed with traditional methods such as
scanning electron microscopy and the immunohistochemical technique. Nevertheless, only a fraction of the whole microbial community structure has been clarified so far; this is largely attributed to the limitations of the conventional techniques used to date. Recent cultivation-independent molecular approaches have been, however, overcoming some drawbacks of these techniques, and small subunit ribosome DNA (rDNA) and rRNA-based molecular techniques are becoming the most powerful tool for microbial community structure analysis. In recent years, these molecular techniques have been applied to methanogenic bioreactors, and several new findings have been reported (Sekiguchi et al., 2001). Since a large part of the microbial constituents in sludge granules still remains to be revealed, more detailed surveys on the community structure are needed using such emerging new techniques. In this study, we surveyed the microbial community structure of two types of methanogenic granular sludge, mesophilic (35°C) and thermophilic (55°C) granules that had been treating the same artificial wastewater by using molecular-based analyses in combination with conventional cultivation techniques.

Materials and methods

Operation of UASB reactors. Granules were collected from two lab-scale mesophilic (operated at 35°C) and thermophilic (55°C) UASB reactors (each 13L capacity) (Sekiguchi et al., 1998; Sekiguchi et al., 1999). Both reactors were fed with the synthetic substrate containing sucrose, acetate, propionate, and yeast extract (or peptone) (4.5: 2.25: 2.25: 1, chemical oxygen demand [COD]-ratio), over three years of operation.

16S rDNA clone library and determination of 16S rDNA of pure cultures. DNA extraction, PCR amplification, and cloning procedures for constructing 16S rDNA clone libraries were reported previously (Sekiguchi et al., 1998). 16S rDNA of isolates was amplified by PCR as described elsewhere (Sekiguchi et al., 2000). The PCR products were purified with a MicroSpin Column (Amersham Pharmacia Biotech), and were subjected to further analysis.

Sequencing and phylogenetic analysis. Sequences of representative rDNA clones as well as the 16S rDNA of pure cultures were determined by Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech) and an automated sequence analyzer (DSQ-1000L: Shimadzu). Phylogenetic analyses were constructed as described elsewhere (Imachi et al., 2000).

In situ hybridization. Fixation and sectioning of granules were done as described elsewhere (Sekiguchi et al., 1999). Whole cell in situ hybridization was performed based on the method as described elsewhere (Sekiguchi et al., 1998; Sekiguchi et al., 1999). 16S rRNA-targeted oligonucleotide probes used in this study are shown in Table 1. The stringency of hybridization was adjusted by adding formamide to the hybridization buffer (5% [v/v] for EUB338; 10% for SYB701; 15% for TGP690; 20% for GNSB633; 35% for ARC915). For double staining of the granule sections, Cy-5 and rhodamine-labeled probes were used simultaneously.

Media and cultivation. The culture medium used for enrichment and isolation of the target-ed microbes was prepared as described previously (Sekiguchi et al. 2000). All cultivations were carried out at 55°C in 50 ml serum vials containing 20 ml of medium (pH 7.2) under an atmosphere of N2:CO2 (80/20, vol/vol) without shaking.

Microscopy and analytical methods. Cells immobilized and hybridized on glass slides were viewed with a fluorescent microscope (Olympus BX50F), and the sections hybridized with
the probes were examined under a confocal laser scanning microscopy (OLYMPUS FLUOVIEW BX50). Scanning electron micrographs were obtained as described previously (Sekiguchi et al., 1999). Short chain fatty acids, alcohols, carbohydrates, and several gases (Methane, hydrogen, and carbon dioxide) were determined as described elsewhere (Sekiguchi et al., 2001).

Results and discussion

16S rDNA-cloning analysis of mesophilic and thermophilic granular sludge. Two laboratory-scale UASB reactors were operated over two years using well settleable granules (1 to 2 mm in diameter) adapted to the same wastewater but with different operational temperatures (Sekiguchi et al., 1998). In order to elucidate the microbial constituents in both sludges, 16S rDNA-cloning analysis was first performed (Sekiguchi et al., 1998). Of 115 mesophilic granule and 110 thermophilic granule clones sequenced, 19% and 22%, respectively, were phylogenetically affiliated with the domain Archaea, and the remainders 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 contrast, in the thermophilic clone library, Thermodesulfovibrio group (19%), the green non-sulfur bacteria (18%), and the low G+C subclass of the Gram-positive bacteria (18%) were the predominant clones. Both libraries contained a large number of unknown clones as functionally unknown organisms, such as clones relative to the green non-sulfur bacteria. Supposing that clones with more than 97% sequence similarity with those of cultured organisms can be grouped into the same species, 38% and 27% of total clones in mesophilic and thermophilic clone libraries, respectively, were thought to be cultivable, i.e. known, populations. This finding suggested that a large fraction of the microbial community in both sludges was comprised of diverse microbes that have yet to be cultured.

Internal architecture of mesophilic and thermophilic granular sludges. To examine whether the recovered clones were derived from the dominant populations in the two sludges, and to elucidate where those microbes were located inside the granules, 16S rRNA-targeted in situ hybridization combined with confocal laser scanning microscopy was employed (Sekiguchi et al., 1999). Spatial organization of microbes was visualized in thin-sections of the granules using fluorescent oligonucleotide probes specific to several phylogenetic groups of microbes. For specific detection of unknown bacteria that were detected in the 16S rDNA clone libraries, we employed the full cycle rRNA approach (Amann et al., 1995), in which oligonucleotide probes were designed based on the clone sequences. In this approach, we designed probes specific to clonal 16S rDNAs related with unidentified green non-sulfur bacteria (GNSB633 probe), and clones related with Syntrophobacter species (SYB701 probe) (Sekiguchi et al., 1999). The probe SYB701

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Target group</th>
<th>Probe sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Bacteria</td>
<td>GCTGCCCTCCCGTGGAGT</td>
<td>Amann et al., 1995</td>
</tr>
<tr>
<td>ARC915</td>
<td>Archaea</td>
<td>GTGCTCCCAGGCCAATTCCCT</td>
<td>Stahl and Amann, 1991</td>
</tr>
<tr>
<td>SYB701</td>
<td>clone MUG28</td>
<td>AAATGCGATTITCAATGCAC</td>
<td>Sekiguchi et al., 1999</td>
</tr>
<tr>
<td>GNSB633</td>
<td>clones in the green non-sulfur bacteria (strain UNI-1)</td>
<td>TAGCCCGCAGTCCTGAACG</td>
<td>Sekiguchi et al., 1999</td>
</tr>
<tr>
<td>TGP690</td>
<td>clone TUG16 (strain SI)</td>
<td>CTCAAGTCCCTCAGTTCAA</td>
<td>Imachi et al., 2000</td>
</tr>
</tbody>
</table>

Table 1 Fluorescently labeled oligonucleotide probes used in this study
hybridized with coccoid cells in the inner layer of the mesophilic granule sections; the morphology of the detected cells resembled those of the genus *Syntrophobacter*. In contrast, the probe GNSB633 detected filamentous cells in the outermost layers of the thermophilic sludge granule sections. Since almost all part of the thin-filamentous cells in the outermost layer of the granules seemed to be stained with GNSB633 probe, it was suggested that the thin filamentous microbes present on the surface of the thermophilic granules were uncultured bacteria which could be phylogenetically classified within the green non-sulfur bacteria. In thermophilic UASB reactors treating high strength wastewaters, it was frequently observed that long, thin filamentous microbes were predominant on the surface of the sludge granules (Uemura and Harada, 1993). This type of organism might be responsible for granulation of sludge and preservation of its structure, but phylogenetic, metabolic and functional information about this organism has not been clearly known so far. Our results fully demonstrated the advantages of the full cycle rRNA approach in use of the microbial community analysis of sludge granules, giving new insight into the uncultured populations that we have never known before.

**Conventional cultivation approach.** Despite the advantages of these molecular approaches, the physiological and functional information on microbial constituents, particularly unidentifiable populations, has yet to be uncovered because the molecular phylogeny based on clone sequences implies only indirect information on the functions of each clone (microbe). In contrast, pure cultures of microbe can provide plenty of information on such characteristics. Thus, we employed a cultivation-based, goal-directed approach to recover the microbes of significant populations; i.e. the microbes that were detected as dominant populations in the previous molecular-based approach. In this attempt, we focused on the thermophilic granular sludge as inoculum, and conventional batch culture was used to enrich and isolate the microbes of interest with various organic substrates as energy and carbon source. All cultivations were performed at 55°C anaerobically, and grown cells in the cultures were monitored by either in situ hybridization with probes designed above or 16S rDNA-based cloning analysis to check whether the grown cultures contained the targeted cells. Through this approach, a total of five new species of thermophilic microorganisms that were detected in the molecular analyses, together with a number of bacteria that were not found in our clone libraries, were finally isolated.

One of the isolates from the thermophilic granular sludge is an anaerobic, thermophilic, syntrophic fatty acid-oxidizing bacterium designated strain TGB-C1 (Sekiguchi *et al.*, 2000). A primary enrichment was performed using 20 mM butyrate as substrate, and the culture was further enriched by successive transfers. To identify the bacterial populations in the highly enriched culture, we then performed rDNA-cloning analysis. Ten bacterial 16S rDNA clones were randomly selected, and the clones were subsequently sequenced. In this analysis, we found that almost all of the clones had the same sequence as the clone TUG15, which was found in the previous thermophilic clone library (one clone in a total of 110 clones). After several attempts to isolate the butyrate-oxidizer, we obtained the bacterium in pure culture with crotonate as substrate.

A syntrophic propionate-oxidizing bacterium designated strain SI was also isolated (Imachi *et al.*, 2000). This strain was primarily enriched with propionate (20 mM) as the sole substrate. We enriched and characterized the enrichment culture as similar to the butyrate-oxidizing syntroph. 16S rDNA-cloning analysis for a highly enrichment culture showed that a large fraction of the bacterial clones was shown to be derived from the microbes that were affiliated with the genus *Desulfotomaculum*. According to the previous 16S rDNA-cloning analysis of the thermophilic sludge, one clone (TUG16) in a total of 110 clones was found to be the same sequence as these clones. Based on the clone
sequence, we designed an oligonucleotide probe to specifically detect the microbes representing the clones. The designed probe (TGP690) was then used to check the cells grown on various organic substrates from the enrichment culture. After several attempts, we isolated the propionate-oxidizing syntroph (Figure 1A).

Thermophilic lactate-degrading syntrophs, designated strain TGE-P1 and TGL-LS1, were isolated in pure culture and co-culture with M. thermautotrophicus. Primary enrichment was made with lactate (20 mM) as sole substrate, and successive transfer was carried out to further purify the lactate-degraders. We performed rDNA-cloning analysis for highly enriched cultures, showing that major clones were derived from two types of 16S rDNA sequences; both of which were closely related to the members of the genus Thermodesulfovibrio. In addition, both sequences were detected in the previous cloning analysis as one of the major clones (they accounted for 19% of the total thermophilic clones). After several attempts, we obtained two strains using the medium supplemented with lactate and sulfate as the substrates. In pure-culture, they could oxidize lactate and hydrogen with sulfate reduction, and could ferment pyruvate. In syntrophic co-culture, the strains oxidized only lactate in the absence of sulfate. The members of Thermodesulfovibrio have been recognized as thermophilic sulfate-reducing microbes, and no syntrophic growth has been reported so far. It is strongly suggested that Thermodesulfovibrio-type cells were one of the significant populations within thermophilic granular sludge, and are likely to function as syntrophic lactate-degraders since the wastewater contained only a small amount of sulfate in our reactor experiment.

Finally, we tried to cultivate the uncultured filamentous cells that were detected by the previous full cycle rRNA approach (Sekiguchi et al., 1999; Sekiguchi et al., 2001). For selective isolation of the filaments, the cells grown on different substrates were screened by using in situ hybridization with GNSB633 probe. After several attempts to isolate the filamentous bacteria for a long time, we finally obtained GNSB633-positive, anaerobic, filamentous cells using sucrose (20 mM) and yeast extract (0.01%) as the substrates (Sekiguchi et al., 2001). The isolate, designated strain UNI-1, was a Gram-negative, very thin-filamentous, non-motile bacterium (Figure 1B). In the presence of yeast extract, they fermented several carbohydrates, and produced carbon dioxide, acetate, and hydrogen as the end product. Growth of the cells in pure culture was weak and very slow. However, co-culture of the strain with hydrogenotrophic methanogens significantly enhanced the growth. According to the previous 16S rDNA-cloning analysis, the strain contains the identical sequence with an unidentifiable clone TUG8 and was very closely related to clones.

![Figure 1 Phase contrast micrographs of strains isolated in the goal-directed cultivation approach. A: strain SI, a thermophilic syntrophic propionate-oxidizing bacterium grown on pyruvate. B: strain UNI-1, a sugar-fermenting thermophile belonging to the group green non-sulfur bacteria subdivision I](https://iwaponline.com/wst/article-pdf/45/10/19/424733/19.pdf)
TUG9 and TUG10 in green non-sulfur bacteria group (these clones accounted for 18% of the total thermophilic clones). This finding together with the previous in situ hybridization analyses suggested that this type of organism was widely distributed in the thermophilic granular sludge as one of the major populations.

In the previous molecular approaches, all of these isolated strains were suggested to be significant populations within thermophilic granular sludge. By obtaining these isolates in pure culture, the fraction of unknown clones in the previous thermophilic clone library decreased from 70% to 40% (Figure 2). As the number of such cultured representatives continues to expand, we will be able to further understand the microbial community structure of methanogenic granular sludges.

**Conclusions**

The molecular-based microbial community structure analyses revealed biodiversity and detailed spatial organization of microbes of interest in sludge granules, and conventional cultivation techniques together with molecular techniques could enlarge our fundamental knowledge of the functions of significant populations in the UASB processes. For further elucidating the fundamental knowledge, detailed molecular surveys for microbial communities in anaerobic sludges, in particular population changes over time, and continued challenges to cultivate relevant but almost always recalcitrant anaerobes are needed. Those approaches will also bridge the gaps between engineer (reactor operation) and microbiologist (culture-based study) and lead to an interactive communication between them for improving reactor performance.

**References**


