The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester

Yueqin Tang a, Toru Shigematsu a,*, Ikbal b, Shigeru Morimura a, Kenji Kida a,c

a Department of Materials and Life Science, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto City, Kumamoto 860-8555, Japan
b Agency for the Assessment and Application of Technology (BPPT), Jalan MH Thamrin no. 8, Jakarta 10340, Indonesia
c Department of Applied Chemistry and Biochemistry, Faculty of Engineering, Kumamoto University, 2-39-1 Kurokami, Kumamoto City, Kumamoto 860-8555, Japan

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Abstract

We demonstrated previously that micro-aeration allows construction of an effective thermophilic methane-fermentation system for treatment of municipal solid waste (MSW) without production of H2S. In the present study, we compared the microbial communities in a thermophilic MSW digester without aeration and with micro-aeration by fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), phylogenetic analysis of libraries of 16S rRNA gene clones and quantitative real-time PCR. Moreover, we studied the activity of sulfate-reducing bacteria (SRB) by analysis of the transcription of the gene for dissimilatory sulfite reductase (dsr). Experiments using FISH revealed that microorganisms belonging to the domain Bacteria dominated in the digester both without aeration and with micro-aeration. Phylogenetic analysis based on 16S rRNA gene and analysis of bacteria by DGGE did not reveal any obvious difference within the microbial communities under the two aeration conditions, and bacteria affiliated with the phylum Firmicutes were dominant. In Archaea, the population of Methanosarcina decreased while the population of Methanoculleus increased as a result of micro-aerations as revealed by the analysis of 16S rRNA gene clones and quantitative real-time PCR. Reverse transcription and PCR (RT–PCR) demonstrated the transcription of dsrA not only in the absence of aeration but also in the presence of micro-aeration, even under conditions where no H2S was detected in the biogas. In conclusion, micro-aeration has no obvious effects on the phylogenetic diversity of microorganisms. Furthermore, the activity of SRBs in the digester was not repressed even though the concentration of H2S in the biogas was very low under the micro-aeration conditions.

Keywords: Methane fermentation; Hydrogen sulfide; Sulfate-reducing bacteria; Phylogenetic analysis; Municipal solid waste

1. Introduction

The amount of municipal solid waste (MSW) is increasing every year but landfill and incinerator capacities are not increasing [1]. Therefore, alternative systems for treatment of MSW are of great interest. Anaerobic digestion of MSW using a methane-fermentation process appears to have considerable potential utility. Anaerobic digestion involves many classes of microorganisms and several intermediate reactions, which catalyze the mineralization of organic components to carbon dioxide, methane and water via hydrolysis, acidogenesis and methanogenesis [2]. In most anaerobic systems, methanogenic archaea and
sulfate-reducing bacteria (SRB) are mainly responsible for consumption of hydrogen gas, which is generated during hydrolysis and acidogenesis. Methane is the main and most desirable end product and is generated by methanogens. However, hydrogen sulfide (H₂S), generated by SRB with the consumption of H₂, has an inhibitory effect on methanogenesis. The concentration of sulfide in a digester must be controlled to eliminate its toxic effect on microbes, in particular in large-scale treatment systems with high water levels.

The analysis of 16S rRNA can be used in studies of complex microbial communities, such as those found in methane-fermentation systems, eliminating the need to culture microorganisms [32]. Methods based on certain functional genes can also be useful. For example, the gene for methyl-coenzyme M reductase (mcr) and the gene for dissimilatory sulfite reductase (dsr) have been used for the specific detection and phylogenetic analysis of methanogens and SRBs, respectively [4,5].

In a previous study, we showed that MSW could be treated effectively by methane fermentation with exogenously supplied trace metals and that micro-aeration could effectively repress the production of H₂S without any effects on the efficiency of digestion and methanogenic activity [6]. In the present study, to evaluate the effects of micro-aeration on microbial diversity, we examined microbial communities in an MSW digester without aeration and with micro-aeration by fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), phylogenetic analysis and quantitative real-time PCR. We also monitored the activity of SRB in terms of the transcription of the dsr gene. There are several earlier reports of studies of the digestion of MSW [7,8], but, to our knowledge, there have been no studies of the effects of micro-aeration on microbial diversity during such digestion.

2. Materials and methods

2.1. Apparatus, feedstock and operation of the anaerobic digester

A synthetic preparation of MSW (Table 1) with a total concentration of solid of 10% (w/v) was used in this study. A gas circulation-type digester with a working volume of 5 l was operated at 53°C. For operation under micro-aeration conditions, air was introduced continuously into the digester at a rate of approximately 7.5% (v/v) of the biogas-generation rate. The digester was operated for more than 1.5 months under each condition to reach a steady state in each case. The digester and its operation were described in detail in a previous paper [6].

<table>
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<tr>
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<td>Carrot</td>
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<td>Chinese noodle</td>
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</table>

<table>
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<th>14</th>
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<tr>
<td>Fish (with bone)</td>
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</tr>
<tr>
<td>Egg</td>
<td>4</td>
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</table>

2.2. Autofluorescence

Coenzyme F₄2₀ is found in all CO₂/H₂ grown methanogens and it can be used as an indicator for the identification of hydrogenotrophic methanogens because of its high level in these cells and its intense fluorescence. Moreover, it is localized predominantly at the inner surface of the cytoplasmic membrane and, thus, the profiles of hydrogenotrophic methanogens can also visualized by fluorescence microscopy. We used a fluorescence microscope (BX60; Olympus Optical Co. Ltd., Tokyo, Japan) for our analyses.

2.3. Fluorescent in situ hybridization

We examined cells by FISH as described by Amann [3] using two domain-specific probes: EUB338 (S-D-Bact-0338-a-A-18) [9], which had been labeled with rhodamine; and ARC915 (S-D-Arch-0915-a-A-20) [10], which had been labeled with fluorescein isothiocyanate (FITC). For the detection of SRB, we used FITC-labeled SRB385 [11] as the probe. The probes were synthesized by Hokkaido System Science Co. Ltd. (Sapporo, Japan). For each analysis, we used 50 μl of culture broth from the digester.

2.4. Extraction of DNA and RNA

A 30 ml aliquot of culture from the digester was centrifuged at 8000 × g for 10 min at 4°C and the resulting pellet was washed with phosphate-buffered
saline (PBS). DNA and total RNA were extracted from the pellet as described by Griffiths et al. [12] with a multi-beads shocker (Yasui Kikai Co. Ltd., Osaka, Japan). For extraction of RNA, the washed pellet was frozen in liquid nitrogen for 20 min before beating with 0.5 g of glass beads (diameter, 0.1 mm; Sigma-Aldrich). The extracted RNA was purified with an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany).

2.5. Amplification by PCR and cloning

We used the primer sets Ar28F (5'-TGGTTGATCCTCTGCACAGGG-3') and 1490R (5'-GGTTACCTGTGTTACAGCTT-3'), Eu27F (5'-AGAGTATGGTACCTGGGCATAG-3') and 1490R, and 530F (5'-GTGCCCAGC(A/C)GCCCAGG-3') and 1490R to amplify 16S rRNA gene from the total-community DNA, targeting *Archaea*, *Bacteria* and total prokaryotes, respectively. We used primers, DSR1F (5'-AC(C/G)GACGAGGAGG-3') and DSR4R (5'-GTGTAG-GTGTACCAGCAGCA-3') [5] to amplify the dsr gene from extracted DNA, targeting almost all SRB. The thermal profile used for amplification of rRNA gene was as follows: incubation at 95°C for 9 min; and then 35 cycles of incubation at 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. We used the same thermal profile for amplification of the dsr gene with one exception: the annealing temperature was 54°C. Amplified DNAs were cloned directly into the pT7Blue T-vector (Novagen Inc., Madison, WI, USA) using DNA Ligation Kit version 2 (Takara, Kyoto, Japan). Libraries of clones were constructed using *Escherichia coli* JM109 or DH5α competent cells (Takara) according to the manufacturer’s instructions.

2.6. Sequencing and phylogenetic analysis

We prepared cloned 16S rRNA genes and dsr genes from randomly selected recombinants and used them as templates for sequencing with a Beckman CEQ8000 sequencer and a CEQ DTCS-Quick Start Kit (Beckman Coulter Inc., Fullerton, CA) in accordance with the manufacturer’s instructions. We checked all sequences manually for chimeric artifacts using the CHIMERA-CHECK program version 2.7 in the Ribosomal Database Project II (RDP-II) [13]. We searched for similar sequences with the BLASTN program [14]. Multiple alignments were generated with the Clustal X program, version 1.8 [15] and phylogenetic trees were constructed with MEGA program version 2.1 [16] on the basis of evolutionary distances that were calculated by the Neighbor-Joining method [17] with Kimura’s two-parameter model [18]. We performed bootstrap resampling analysis [19] for 500 replicates to estimate degrees of confidence in tree topologies. Identical sequences were recognized by analysis of phylogenetic trees and manual comparisons, in which sequences that were more than 99% similar were defined as identical, and these sequences were used for further phylogenetic analysis as an operational taxonomic unit (OTU). Eight libraries of clones were constructed with total-community DNA that had been extracted from the digester with and without micro-aeration. After operation without aeration, we constructed the following libraries: an archaeal rRNA gene library (OTUs, MANA01 and 02); a bacterial rRNA gene library (OTUs, MBNA01–08); and prokaryotic universal rRNA gene (OTUs, MUNA01–08) and dsr gene (OTUs, MSNA01 and 02) libraries. After operation of the digester with micro-aeration, we constructed the following four libraries: an archaeal rRNA gene library (OTUs, MAA01–08); a bacterial rRNA gene library (OTUs, MBA01–09); and prokaryotic universal rRNA gene (OTUs, MUA01–09) and dsr gene (OTUs, MSA01) libraries. The GeneBank/EMBL/DDBJ accession numbers for the sequences of OTUs MAA01–MSNA02 are AB114301–AB114347.

2.7. Denaturing gradient gel electrophoresis

The V3 regions of genes for 16S rRNA from the DNA extracted from the digester were amplified by PCR and the amplified products were used for analysis by DGGE. We performed PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA), a total volume of 100 μl in 0.2 ml reaction tubes and GeneAmp PCR system 2400 (Applied Biosystems). The primer sets and GC clamp used were the same as those described by Øvreås et al. [20]. For amplification of bacterial rRNA gene, we used PRBA338F and PRUN518R as primers. The thermal profile was as follows: incubation at 95°C for 9 min; and then 30 cycles of incubation at 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. Archaeal rRNA gene was obtained by nested PCR with a thermal profile similar to that used for bacterial rRNA gene, with the following exceptions: there were 35 cycles of amplification and the annealing temperature was only 53.5°C. For nested PCR, we used PRA46F and PREA1100R primers to produce fragments of rRNA gene, and then we used these fragments as template for the second PCR with primers PARCH340F and PARCH519R. We performed DGGE with a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). The amplified fragments of rRNA gene were loaded onto a 10% (w/v) polyacrylamide gel in 0.5 × TAE (pH 8.0). Each gel was prepared with a denaturing gradient that ranged from 30% to 60%. Electrophoresis was performed at 60°C, first for 10 min at 25 V and then for 8 h at 130 V.
2.8. Real-time quantitative PCR

For detection and quantification of methanogens by real-time quantitative PCR, we used the TaqMan fluorogenic PCR system (Applied Biosystems, Foster City, CA) and the same as reported previously [21]. The primer/probe sets for the genera Methanoseta, Methanosarcina and Methanocalculus were MS1b/SAE835R/SAR761TAQ, MB1b/SAR835R/SAR761TAQ and AR934F/MG1200b/MCU1023TAQ, respectively.

2.9. Reverse transcription–PCR

We examined the transcription of the dsr gene by reverse transcription and PCR (RT–PCR). We performed two-step RNA PCR using a GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol with primer DSR3FR (5′-GCTGCTAC(G/A)CAGTCATT-3′) for the RT reaction. We used primers DSR3FRExt (5′-GGACCGCTGCTACCGAGCTCATTCCGAGCAG-3′) and DSR1FExt (5′-CGCTATTCAGACCTGCCGGAAGATTTCTC-3′) for amplification of approximately 440 bp of the dsrA gene. These primers were based on the cloned sequences of dsr in MSNA and MSA libraries. Reverse transcription was allowed to proceed at 45°C for 20 min. The thermal profile for amplification of the cDNA was as follows: incubation at 95°C for 10 min; and then 35 cycles of incubation at 95°C for 1 min, 64°C for 1 min and 72°C for 2 min. The products of RT–PCR were purified on a Microspin S-400 HR Columns (Amersham Biosciences, Piscataway, NJ) and then sequenced.

3. Results

3.1. Operation of the digester

A laboratory-scale system for the thermophilic anaerobic digestion of MSW was constructed. Synthetic MSW was fed into the system continuously at a total solid (TS) loading rate of 6 g/l/day and a hydraulic retention time (HRT) of 15 days.

Under steady-state conditions, the efficiency of volatile suspended solid (VSS) digestion was approximately 80% under both non-aeration and micro-aeration conditions (Table 2). The concentrations of total organic carbon (TOC) and of volatile fatty acids in the digester, the biogas-generation rate and the concentration of methane in the biogas were also almost identical under the two sets of conditions (Table 2). Only the concentration of H2S in the biogas was different, being approximately 700 ppm under non-aeration conditions and less than 5 ppm under micro-aeration conditions.

The concentrations of sulfate ions in the digester were approximately 1250 and 1330 mg/l under non-aeration and micro-aeration conditions, respectively. There were no obvious differences between results obtained under the two aeration conditions, although there was a slightly lower concentration of sulfate ions under non-aerobic conditions. The biogas that contained H2S at a concentration of 700 ppm was mixed with air to a final concentration of 7.5% (v/v) and introduced into 5 l of water. The water turned turbid and a light yellow precipitate, which was probably sulfur was formed (data not shown).

3.2. Fluorescence microscopy

We used FISH and examination of the autofluorescence of F420 to monitor cells in the digester under non-aeration and micro-aeration conditions. FISH using the probes EUB338 and ARC915 revealed that cells in the domain Bacteria were predominant in the digester under non-aeration and micro-aeration conditions (Figs. 1A, A0, B and B0). Most bacterial cells were long, short or curved rods under both sets of conditions. The number of cells that hybridized to the SRB385 probe was very low under both aeration conditions (the yellow cells in Figs. 1C0 and D0), suggesting that the number of SRBs in the digester that were related to known cultured SRBs was very low.

The number of cells in the domain Archaea was low under both aeration conditions. Most of the archaeal cells were irregular rods or cocci (Figs. 1A0 and B0). Cells with autofluorescent F420 have similar morphotypes to the cells in Fig. 1A0 and B0 (Figs. 2A0, A0, B and B0), suggesting that hydrogenotrophic methanogens were predominant in the digester under both sets of aeration conditions.

When we looked for aceticlastic methanogens, we found Methanosarcina-like aggregates with F420

Table 2
Parameters in MSW digester under no-aeration and micro-aeration steady-state conditions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TOC (mg/l)</th>
<th>VFA (mg/l)</th>
<th>VSS digestion efficiency (%)</th>
<th>H2S concentration in biogas (ppm)</th>
<th>Methane concentration in biogas (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No aeration</td>
<td>~1600</td>
<td>~400</td>
<td>~83</td>
<td>~680</td>
<td>~50%</td>
</tr>
<tr>
<td>Micro-aeration</td>
<td>~1500</td>
<td>~100</td>
<td>~84</td>
<td>&lt;5</td>
<td>~50%</td>
</tr>
</tbody>
</table>
autofluorescence under non-aeration conditions only (Figs. 2C and C'), and we found no Methanosaeta-like filamentous cells at all.

3.3. General phylogenetic analysis

We prepared six 16S rRNA gene libraries. After growth without aeration, we constructed archaeal (MANA), bacterial (MBNA) and prokaryotic universal (MUNA) libraries. After growth under micro-aeration conditions, we constructed archaeal (MAA), bacterial (MBA) and prokaryotic universal (MUA) libraries. All the clones in the archaeal libraries (MANA and MAA) were affiliated with the phylum Euryarchaeota (Table 3). All the clones in the bacterial libraries (MBNA and MBA) were
affiliated with the phylum *Firmicutes*. All the clones in the prokaryotic universal libraries (MUNA and MUA) were affiliated with the domain *Bacteria*, indicating that bacterial cells were dominant in our reactor. Most of the clones in the MUNA library and all the clones in the MUA library were affiliated with the phylum *Firmicutes*. Only two OTUs (two clones) in the MUNA library were affiliated with the phylum *Thermotogae*.

### 3.4. Domain Archaea

In the domain *Archaea*, there were two OTUs (15 clones) in the MANA library, constructed from cards.
that had grown in the digester without aeration, and eight OTUs (19 clones) in the MAA library, constructed from cells that had grown under micro-aeration conditions. All of these clones were assigned to the phylum *Euryarchaeota* (Fig. 3). In the MANA library, one OTU (MANA01, seven clones) was closely related to *Methanosarcina thermophila* with 99% sequence similarity. The other OTU (MANA02, eight clones) was closely related to *Methanoculleus palmolei* with 97% similarity. In the MAA library, all OTUs (MAA01–08, 19 clones) were closely related to *Methanoculleus palmolei* with 96–98% similarity. These OTUs were widely distributed in the genus *Methanoculleus*. No clones that were closely related to the genus *Methanosarcina* were detected in the MAA library.

### 3.5. Domain Bacteria

#### 3.5.1. The phylum Firmicutes (low G+C Gram-positive bacteria)

In the domain *Bacteria*, we obtained eight OTUs (22 clones), nine OTUs (18 clones), eight OTUs (16 clones) and nine OTUs (18 clones) in the MBNA, MBA, MUNA and MUA libraries, respectively (Table 3). The dominant phylum in the domain *Bacteria* was *Firmicutes* with 14 OTUs (36 of a total of 38 bacterial clones) and 18 OTUs (36 of a total of 36 bacterial clones) under non-aeration and micro-aeration conditions, respectively. We analyzed the phylogenetic relationships among these 32 OTUs in the phylum *Firmicutes* (Fig. 4). There were no apparent differences among the phylogenetic positions of the clones between the four rRNA gene

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**Fig. 3.** Distance matrix tree showing the genetic relationships among the clones affiliated with the order *Methanosarcinales* and the order *Methanomicrobiales*. The tree was constructed by the Neighbor-Joining method [17] using partial sequences of 16S rRNA gene. MSWNA and MSWA refer to clones derived from growth under non-aeration and micro-aeration conditions, respectively. Numbers of clones with identical sequences are shown in parentheses. The bar represents two substitutions per 100 nucleotide positions. Bootstrap probabilities [19] are indicated at branch nodes. The DDBJ/EMBL/GeneBank accession numbers for reference strains and clones obtained in this study are shown in parentheses in this and other figures. The tree was rooted using *Methanobacterium bryantii* as the outgroup.
Fig. 4. Distance matrix tree showing the phylogenetic relationships among the clones affiliated with the phylum *Firmicutes* (low G+C Gram-positive bacteria). The tree was constructed by the Neighbor-Joining method [17] using partial sequences of 16S rRNA gene. The bar represents two substitutions per 100 nucleotide positions. Numbers in parentheses and numbers at branch nodes are defined in the legend of Fig. 3. The tree was rooted using *Arthrobacter globiformis* as the outgroup.
libraries. This result indicated that no obvious bias had been introduced by the choice of primer sets for the bacterial and prokaryotic universal libraries and that there was no apparent difference between non-aeration and micro-aeration conditions. Most clones in the phylum *Firmicutes* (seven OTUs in MBNA, 21 clones; seven OTUs in MBA, 15 clones; five OTUs in MUNA, 13 clones; seven OTUs in MUA, 16 clones) formed a cluster (MSW cluster 1 in Fig. 4) with the uncultured clone ML635J-38 that was obtained from Mono Lake [22]. MSW cluster 1 was closely related to *Clostridium* cluster III [23] and the genus *Desulfotomaculum*. No sequences of rDNAs from cultured strains were related to MSW cluster 1. One OTU, obtained under non-aeration conditions (MUNA02, one clone), was assigned to *Clostridium* cluster VIII [23] and was closely related to the genus *Syntrophomonas*. Another OTU obtained under non-aeration conditions (MBNA01, one clone) was assigned to *Clostridium* cluster XII [23] and was closely related to *Clostridium ultunense* [24]. Four OTUs obtained under micro-aeration conditions (MBA05, MBA09, MUA05 and MUA08; five clones) were assigned to *Clostridium* cluster III.

### 3.5.2. The phylum Thermotogae

Two OTUs obtained under non-aeration conditions (MUNA04 and MUNA06, two clones) were assigned to the phylum *Thermotogae* and were closely related to *Coprothermobacter proteolyticus* [25] with 98% sequence similarity (Fig. 5). In the MUA library obtained under micro-aeration conditions, one clone, which was considered to be a chimeric artifact, had a partial sequence with a significant similarity to the rRNA gene of *Coprothermobacter proteolyticus* (data not shown). These results suggest that a small but significant number of relatives of *Coprothermobacter* were present in the digester under both aeration conditions.

### 3.6. Analysis of community structure by DGGE

We analyzed the DNA derived from cells in the MSW reactor under non-aeration and micro-aeration conditions by DGGE. We amplified fragments of approximately 200 bp in the V3 region of archaeal and bacterial 16S rRNA gene separately and subjected them to DGGE. We detected at least six and five bands after DGGE of the amplified archaeal rRNA genes derived from cells grown in the MSW digester under non-aeration and micro-aeration conditions, respectively (bands A1–A9 in lanes 1 and 2 in Fig. 6). Among these bands, A1, A2 and A3 were common to both aeration conditions. Band A3, which was the dominant band under non-aeration condition, was correlated with clone MANA01 that was affiliated with the genus *Methanosarcina*. Band A4, which was also predominant under non-aeration condition, was correlated with clone MANA02 that was affiliated with the genus *Methano- culleus*. Bands A7, A8 and A9, which appeared only under micro-aeration conditions, were correlated with clones MANA01, and MAA03–MAA08, respectively, all of which were affiliated with the genus *Methanoculleus*. These results supported the results of the analysis of rRNA gene clones, namely, that clones of rRNA gene that were affiliated with *Methanoculleus* were distributed within the genus. Most of the clones of archaeal rRNA gene obtained in the analysis of libraries of clones were detected as bands after DGGE.

We detected at least 13 bands in the analysis of amplified bacterial rRNA genes derived from cells grown in the MSW digester under both non-aeration conditions.

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**Fig. 5.** Distance matrix tree showing the phylogenetic relationships among the clones affiliated with the phylum *Thermotogae*. The tree was constructed by the Neighbor-Joining method [17] using partial sequences of 16S rRNA gene. The bar represents five substitutions per 100 nucleotide positions. Numbers in parentheses and numbers at branch nodes are defined in the legend of Fig. 3. The tree was rooted using *Methanosarcina thermophila* as the outgroup.
and micro-aeration conditions (bands B1–B13 in lanes 3 and 4 in Fig. 6). Most of these bands were common to both aeration conditions. Two bands (bands 9 and 11) were correlated with DGGE bands obtained from bacterial rRNA gene clones. Band B9 was correlated with MBA01, MBA07, MBA09, MBNA08 and MBNA06. Band B11 was correlated with MBA02, MBA06, MBNA01, MBNA02, MBNA03 and MBNA04.

3.7. Real-time quantitative PCR

We performed real-time quantitative PCR to estimate the numbers of methanogens in various taxonomic groups. We used three sets of primer and TaqMan probe, namely, MS1b, SAE835R and SAE761TAQ; MB1b, SAR835R and SAR761TAQ; and AR934F, MG1200b and MCU1023TAQ [21], to quantify the 16S rRNA gene in the genera *Methanosaeta*, *Methanosarcina* and *Methanoculleus*, respectively, and we analyzed 500 ng of DNA derived from cells grown in the MSW digester under non-aeration and micro-aeration conditions. No rRNA gene affiliated with the genus *Methanosaeta* was detected under both aeration conditions (Table 4). This result was consistent with the results of our analysis of clone libraries. The number of rRNA genes affiliated with the genus *Methanosarcina* derived from cells grown in the digester under micro-aeration conditions was approximately half that of rRNA genes obtained under non-aeration conditions. However, the number of rRNA genes affiliated with the genus *Methanoculleus* obtained from cells grown in the digester under micro-aeration conditions was approximately 200 times larger than that obtained under non-aeration conditions.

3.8. Diversity and sulfate-reducing activity of sulfate-reducing bacteria in the MSW digester

To evaluate the diversity of SRBs in the digester, we constructed libraries of genes of dissimilatory sulfite reductase (dsrAB) from DNA derived from cells grown in the MSW digester. This gene is strongly conserved in SRB [5]. Two OTUs (MSNA01, five clones; MSNA02, three clones) and one OTU (MSA01, eight clones) were obtained from DNA from cells grown under non-aeration and micro-aeration conditions, respectively. The nucleotide sequences of OTUs MSNA01 and MSA01 were almost identical (similarity, 99.8%). We compared the deduced amino acid sequences of the dsrA proteins of the three OTUs with those of other known dsrAs (Fig. 7). All three dsrAs derived from the MSW digester were affiliated with the dsrAs of Gram-positive

![Fig. 6. Results of DGGE for fragments of 16S rRNA gene from Archaea (lanes 1 and 2) and Bacteria (lanes 3 and 4) in the MSW digester. Lanes 1 and 3 show results for non-aeration conditions and lanes 2 and 4 show the results for micro-aeration conditions. Bands are numbered as indicated.]

### Table 4
Quantitation of 16S rRNA genes in methanogens by analysis of DNA from culture broth taken from the MSW digester

<table>
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<th>Primer/probe set</th>
<th>Target organism</th>
<th>No. of copies of 16S rRNA gene per 500 ng of total DNA</th>
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</thead>
<tbody>
<tr>
<td>MS1b/SAE835R/SAE761TAQ</td>
<td><em>Methanosaeta</em></td>
<td>N.D. (^a)</td>
</tr>
<tr>
<td>MB1b/SAR835R/SAR761TAQ</td>
<td><em>Methanosarcina</em></td>
<td>7.83 × 10⁷</td>
</tr>
<tr>
<td>AR934F/MG1200b/MCU1023TAQ</td>
<td><em>Methanoculleus</em></td>
<td>6.71 × 10³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Micro-aeration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.72 × 10⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.34 × 10⁶</td>
</tr>
</tbody>
</table>

\(^a\) N.D., not detected.
spore-forming SRBs. The phylogenetic relationships among \( \text{dsrB} \) of our OTUs and known \( \text{dsrB} \)s were essentially identical to those of the various \( \text{dsrA} \)s (data not shown).

3.9. Repression of the production of \( \text{H}_2\text{S} \) by micro-aeration

The difference in the concentration of \( \text{H}_2\text{S} \) in the biogas between the two aeration conditions might have been caused by differences in levels of expression of relevant genes in the SRBs in the digester. We evaluated the transcription of \( \text{dsr} \) genes under non-aeration conditions and micro-aeration conditions by RT–PCR.

Under both aeration conditions, fragments of 440bp were amplified from the total RNA derived from cells grown in the digester (Fig. 8). We cloned and sequenced these fragments and found that the nucleotide sequences obtained under both aeration conditions were identical to MSA01 and MSNA01 (data not shown). These observations indicated that transcription of the \( \text{dsr} \) gene was not repressed by micro-aeration.

4. Discussion

The micro-aeration technique has proved useful for constructing an effective thermophilic methane-fermentation system for processing MSW without the production of \( \text{H}_2\text{S} \) [6]. In the present study, we characterized the structure of the microbial communities in the thermophilic MSW digester without aeration and with micro-aeration. Our analysis of the community structure by FISH revealed that microorganisms in the domain \textit{Bacteria} were dominant in the digester under non-aeration and micro-aeration conditions. Analysis of bacteria by DGGE failed to reveal any major difference between microbial communities under the two aeration conditions. Phylogenetic analysis based on 16S rRNA gene showed that microorganisms affiliated with the phylum \textit{Firmicutes} were dominant, independent of the aeration conditions. Within the \textit{Firmicutes}, most of the rRNA gene clones that we obtained formed a cluster (Fig. 4) and they were significantly different from cultured bacterial strains. Micro-aeration did not cause a dramatic shift in the structure of the microbial community in the MSW digester. The predominant
microorganisms in this cluster probably play a major role in the decomposition of the complex organic matter in MSW but the actual metabolic functions of individual cells in the digester remain to be clarified. Some clones were closely related to Clostridium thermocellum, which has been shown to digest cellulose [26], and to Coprothermobacter proteolyticus, which has been shown to digest proteins [25].

In the domain Archaea, we detected clones that were closely related to the genera Methanoculleus and Methanosarcina. Methanoculleus has been reported to be a hydrogenotrophic methanogen [27], and Methanosarcina can utilize both acetate and H2 plus CO2 [27]. In general, in a methane-fermentation process, approximately 70% of the methane generated is derived from acetate [2]. Thus, in our MSW digester, Methanoculleus and Methanosarcina cells probably produced methane from H2 plus CO2 and from acetate, respectively, under non-aeration conditions. The size of the population of Methanosarcina was reduced by micro-aeration, as revealed by the analysis of the library of 16S rRNA genes clones and by quantitative real-time PCR, although a slight discrepancy was observed between the results of these two analytical methods. Conversely, the size of population of Methanoculleus was increased by micro-aeration. These findings suggest that Methanosarcina might be more sensitive to air than Methanoculleus and, moreover, that a significant portion of the acetate produced by acetogens is converted to methane by syntrophic acetate-oxidizing bacteria [28] and hydrogenotrophic methanogens under micro-aeration conditions. The genus Methanosaeta, which consists of aceticlastic methanogens and has been detected in a number of methane-fermentation reactors [27], was not detected in our MSW digester under either of the aeration conditions used in this study. Petersen and Ahring [28] reported that, in a thermophilic anaerobic reactor that contained acetate, the population of Methanosaeta was so small that this genus could not be detected. In our MSW digester, as well as in the reactor of Petersen et al., it is unlikely that Methanosaeta played an important role in methanogenesis.

In our analysis of clonal dsrA sequences, we detected two OTUs of dsr (MSNA01 and MSNA02) under non-aeration conditions and only one OTU (MSA01) under micro-aeration conditions. A single phylogenetic type of dsr (MSNA01 and MSA01) was detected irrespective of the aeration conditions. This result suggests that there was one phylogenetic type of SRB in the digester throughout our experiment even though it is possible that some bias was introduced during amplification by PCR.

The OTUs MSNA01 and MSA01 were not closely related to the dsr genes of any genus of pure cultured SRBs. However, the OTUs were clearly related to dsr genes in the genera Desulfotomaculum, Desulfitobacterium and Desulfoспорisimus. These genera reportedly belong to the phylum Firmicutes. Our analysis of 16S rDNA libraries indicated that rDNA clones affiliated with the phylum Firmicutes were dominant irrespective of the aeration conditions. Thus, some of the bacteria that were detected as rRNA gene clones and were affiliated with Firmicutes might correspond to the dominant SRB, which has a dsr gene of the phylogenetic type, in the digester.

Amplification by RT–PCR revealed that transcription of the phylogenetic type of dsrA gene (OTUs MSNA01 and MSA01) occurred not only under non-aeration conditions but also under micro-aeration conditions, even when a minimal amount of H2S was detected in the biogas. This finding suggests that SRBs in the digester might have produced H2S even under micro-aeration conditions and that the H2S produced was subsequently oxidized by air. This hypothesis is supported by our finding that, when biogas containing H2S at 700 ppm was mixed with air to a final concentration of 7.5% (v/v), it formed a pale yellow precipitate in water.

Fuselier et al. reported that sulfide could be oxidized to sulfate and/or sulfite by some SRBs, with oxygen as the final acceptor of electron [29–31]. Therefore, it is possible that, in our MSW digester, the H2S that had been produced was oxidized to sulfite and/or sulfate by microorganisms under micro-aeration conditions. It might be possible to elucidate the mechanism of repression of the production of CH2S during micro-aeration if we could determine the sulfur balance in our MSW digester.

5. Conclusions

The effect of micro-aeration on the microbial communities in the digester was investigated in this study by molecular biological techniques. Microorganisms belonging to the domain Bacteria dominated in the digester both without aeration and with micro-aeration. No obvious difference was shown within the microbial communities under the two aeration conditions, and bacteria affiliated with the phylum Firmicutes were dominant. In Archaea, the population of Methanosarcina decreased while the population of Methanoculleus increased as a result of micro-aerations. The transcription of dsrA had happened not only in the absence of aeration but also in the presence of micro-aeration. In conclusion, micro-aeration has not shown obvious effects on the microbial diversity in the MSW digester and, the activity of SRBs in the digester was not repressed under the micro-aeration conditions.

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References


