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Methanogenic profiles by denaturing gradient gel electrophoresis using order-specific primers in anaerobic sludge digestion

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Abstract In the present study, the diversity of methanogenic populations was monitored for 25 days, together with the process data for an anaerobic batch reactor treating waste-activated sludge. To understand this microbial diversity and dynamics, 16S rRNA-gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting was conducted at two different taxonomic levels: the domain and order levels. The DGGE profiles of the domain Archaea and the three orders *Methanosarcinales*, *Methanomicrobiales*, and *Methanobacteriales* were comparatively analyzed after each DGGE band was sequenced to enable identification. The DGGE profiles of the three orders showed methanogens belonging to each order that were not detected in the DGGE profile of the Archaea. This discrepancy may have resulted from PCR bias or differences in the abundances of the three microbial orders in the anaerobic bioreactor. In conclusion, to fully understand the detailed methanogenic diversity and dynamics in an anaerobic bioreactor, it is necessary to conduct DGGE analysis with 16S rRNA gene primers that target lower taxonomic groups.

Keywords Anaerobic batch system · Archaea · Denaturing gradient gel electrophoresis (DGGE) · Methanogens · *Methanosarcinales* · *Methanomicrobiales* · *Methanobacteriales* · 16S rRNA gene primer

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Introduction

The anaerobic digestion process is a biological treatment method that is widely used to treat wastewater containing highly concentrated organic compounds. This approach has a number of important advantages, not only in the removal of organic pollutants from wastewater but also in the production of renewable energy as methane gas. The overall process involves three phases that each involves different microbial activities: hydrolysis, acidogenesis, and methanogenesis. In the anaerobic digestion of waste-activated sludge, understanding the microbial communities that stabilize the pollutional load of the sludge can clarify the behavior of the key microbes involved.

DGGE analysis is one of the most widely used fingerprinting techniques, enabling the identification of community members by the recovery and sequencing of amplification products (Muyzer et al. 1993; Curtis and Craine 1998). This genetic fingerprinting approach is particularly useful for detailed comparisons of microbial communities from different environments or in following changes in community structures over time, where the stability and performance of the methanogenesis process are strongly dependent upon complex microbial interactions (Muyzer et al. 1993; Liu et al. 2002; Sousa et al. 2007). Until now, many studies have revealed the relationships of bacterial populations at a defined taxonomic level; however, few investigations have compared microbial population structures at the domain and ordinal levels.

The aim of this study was to monitor and identify the methanogenic communities in an anaerobic digestion process treating waste-activated sludge, using DGGE profiles constructed with 16S rRNA gene primers targeting different phylogenetic groups. The diversity of the methanogenic microbial populations and their changes were

investigated relative to the changes in organic acid concentrations and methane gas production. A comparative analysis of the DGGE profiles constructed with domain-level and order-level 16S rRNA gene primers revealed differences between the DGGE profiles constructed with PCR primers targeting different taxonomic levels. The DGGE approach with primers specific for lower taxonomic levels (i.e., order-directed primers) gave more detailed information about the methanogenic populations in this digestion system.

Materials and methods

Reactor operation

The anaerobic batch reactor, with a working volume of 6 l, was operated at the mesophilic temperature (35°C) and pH 7.5 for 25 days. Waste-activated sludge was used as the substrate. The chemical oxygen demand (COD) and volatile solid (VS) concentrations of the substrate were 15.0 g/l and 10.6 g/l, respectively. The batch reactor was inoculated with 2% (*v*/*v*) anaerobic sludge with 17.4 g VS/l. The waste-activated sludge and seed inoculum used in this study were collected from a local municipal wastewater-treatment plant.

Process data analysis

For data analysis, a 24-h sampling interval was required. A gas chromatograph model 6890 Plus (Agilent, Palo Alto, CA) equipped with an Innowax capillary column and a flame ionization detector was used to determine the concentrations of C_2–C_6 volatile fatty acids (VFAs). Another 6890 Plus gas chromatograph (Agilent), with an HP-5 capillary column and a thermal conductivity detector, was used to analyze the gas composition of the biogas. The COD and solid concentrations were measured according to the procedures in Standard Methods (APHA-AWWA-WEF 2005). All analyses were duplicated, and the results are given as mean values.

DNA extraction and amplification

DNA was extracted from the anaerobic bioreactor at each sampling time using an automated nucleic acid extractor Magtration System 6GC (PSS, Chiba, Japan). To remove the DNA and debris derived from dead cells and residual wastewater materials from the waste-activated sludge, a 1-ml sample from the anaerobic bioreactor was centrifuged for 10 min, after which the supernatant was decanted and the pellet resuspended in 1 ml of deionized distilled water. The resulting suspension was dissolved by vortexing for 3 min and then centrifuged again in the same manner. The centrifugation and resuspension steps were repeated three times before DNA extraction with the automated nucleic acid extractor.

To construct the DGGE profiles, 16S rRNA gene primers (Table 1) for the Archaea or for specific orders of methanogens were used to amplify the extracted DNA using the touchdown polymerase chain reaction (PCR) method. We used the universal 16S rRNA gene primers for the Archaea (ARC) and specific primers for the orders *Methanomicrobiales* (MMB), *Methanosarcinales* (MSL), and *Methanobacteriales* (MBT) in the DGGE analysis. In the DGGE experiments, the 16S rRNA genes in each DNA sample were amplified with the primers specific for the target group. A 40-bp GC clamp was added to the 5′ end of the forward or reverse primer for each target group. The ARC787F and ARC1059R universal primers were used to amplify a 273-base pair (bp) fragment from microorganisms of the domain Archaea (Yu et al. 2005). The order-

Table 1 Primer sets for the domain Archaea and the three orders of methanogens analyzed in this study with DGGE

Primer	Target	Sequence(5′→3′)	Expected size (bp)	Reference
GC-ARC787F ARC1059R	Archaea	CGCCCGCCGCGCCCCGCGCCCGT CCCGCCGCCCCCGCCCGATTAGATAC CCSBGTAGTCC GCCATGCACCWCCTCT	273	Yu et al. 2005
MSL812F GC-MSL1159R	*Methanosarcinales*	GTAAACGATRYTCGCTAGGT CGCCCGCCGCGCC CCGCGCCCGTCCCGCCGCCCCCGCCCG GGTCCCCACAGWGTACC	354	Yu et al. 2005
GC-MMB282F MMB832R	*Methanomicrobiales*	CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGATCGRTACGGGTT GTGGG CACCTAACGCRCATHGTTTAC	506	Yu et al. 2005
MBT857F GC-MBT1196R	*Methanobacteriales*	CGWAGGGAAGCTGTTAAGT CGCCCGCCGCGC CCCGCGCCCGTCCCGCCGCCC CCGCCCGTACCGTCGTCCACTCCTT	343	Yu et al. 2005

A 40-bp GC clamp underlined was attached to the 5′ end of the forward or reverse primer of each target group

level primer pairs MMB282F and MMB832R, MSL812F and MSL1159R, and MBT857F and MBT1196R were used to amplify 506-bp, 354-bp, and 343-bp fragments, respectively. To amplify the target DNA, a touchdown PCR method was used with the following conditions: initial denaturation at 94°C for 10 min, followed by 20 cycles of denaturation at 94°C for 1 min; annealing at a temperature that decreased by 0.5°C every cycle from 65°C to the 'touchdown' at 55°C, remaining at each temperature for 1 min; and chain extension at 72°C for 1 min. This was followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Thus, the PCR was performed in a total of 40 cycles. A final extension step was performed at 72°C for 3 min.

DGGE analysis and DNA sequencing

The PCR products were loaded onto 8% polyacrylamide gels containing a range of different denaturant concentrations (100% denaturant was a mixture of 7 M urea and 40% [*v*/*v*] formamide). The ARC PCR products were applied to a gradient of 40–60% denaturant. The denaturant gradients used to separate the amplified MMB, MSL, and MBT PCR products were 30–60%, 35–65%, and 40–60%, respectively. Each DGGE was performed for 7 h at 150 V in 1×TAE electrophoresis buffer with the D-Code system (BioRad, Hercules, CA). Following electrophoresis, the gel was stained with ethidium bromide solution for 20 min, rinsed for 20 min in deionized water (DW), and photographed under UV transillumination. The visible DGGE bands in each DGGE profile were excised directly from the gels with a sterile blade, mixed with 40 μl of DW, and incubated overnight at 4°C. Each band was thus eluted into solution and 2 μl was used as the template in a reamplification reaction using the specific target primers. The PCR products were purified on 1% low melting point agarose gel. The final products, the partial 16S rRNA sequences amplified with the ARC, MMB, MSL, or MBT primers, were cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany). The 16S rRNA gene inserts were sequenced with a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Database homology searches for these sequences were performed using the BLAST program in the National Center for Biotechnology Information (NCBI) database.

Results

Performance of the anaerobic bioreactor

The batch process was monitored from the beginning to the point at which all the organic acids were degraded and methane gas was no longer being produced, a period of 25 days. Figure 1 shows the production and consumption of VFAs (C_2–C_6) and the production of methane gas. From these data for VFAs and methane gas, we inferred a close association between the production of methane gas and the degradation of organic acids. The concentration of total VFAs reached up to 1,182 mg/l and acetate was the major fermentation product, at a maximum concentration of 662 mg/l at day 4.8. The maximum propionate concentration was measured as 314 mg/l at day 12. Propionate was not totally consumed after day 20 while acetate and butyrate have been completely degraded at day 16. The acetate and propionate concentrations accounted for 70% of the total C_2–C_6 VFA concentration. The acetate concentration gradually decreased after 4.8 days of incubation. In this anaerobic bioreactor, we measured the methane gas of 2l produced during the first 9 days, and the methane gas of 5l that had formed from the 10th to 25th day. Although methane gas production commenced on day 2, it increased markedly from day 9 of the operation period.

PCR-based DGGE analysis of the domain Archaea

In Fig. 2, the ARC DGGE profiles show that the microbial community structure changed during the methanogenesis process. The ARC DGGE bands 1, 2, 7, 8, and 9 were observed in the waste-activated sludge and the anaerobic sludge. These bands were closely related to the methanogenic species *Methanosaeta concilii*, which is an acetate-utilizing methanogen (Rocheleau et al. 1999). Band 3 was observed in the anaerobic sludge and was closely related to *Methanocalculus pumilus*, a mesophilic hydrogenotrophic methanogen (Mori et al. 2000). This species uses H_2/CO_2 and formate as energy sources, and its optimal temperature for growth is 35°C and its optimal pH is 7. Band 10 was

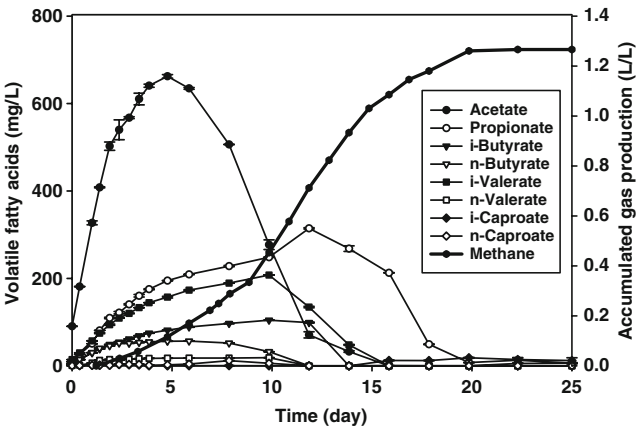

Fig. 1 Methane production according to the formation and degradation of volatile fatty acids in an anaerobic bioreactor treating waste-activated sludge

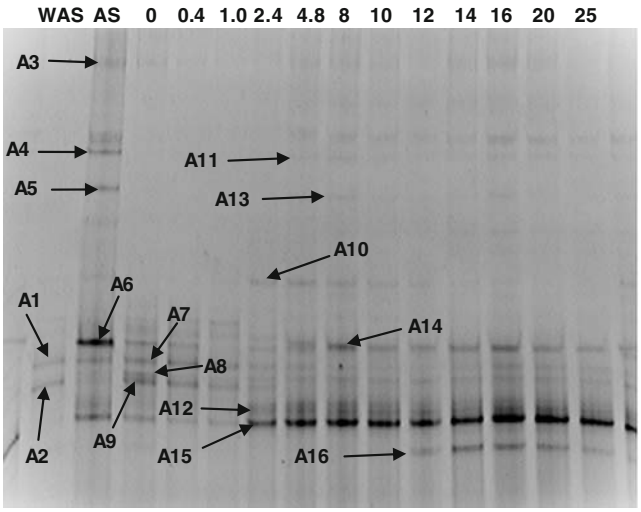

Fig. 2 Archaea DGGE banding profiles from an anaerobic batch system (*WAS* waste-activated sludge, *AS* anaerobic sludge; *lane labels* at the *top* show the sampling time [days] from start-up of the bioreactor; *numbers* on the DGGE gel indicate the bands excised for sequencing)

detected in the anaerobic bioreactor from day 2.4 and was closely related to *Methanocorpusculum bavaricum*.

Methane is produced from H_2/CO_2, formate, 2-propanol/CO_2, and 2-butanol/CO_2 by *M. bavaricum* (Zellner et al. 1989). Optimal growth of *M. bavaricum* occurs in the mesophilic temperature range and at a pH of around 7 (Zellner et al. 1989). ARC DGGE bands 4 and 6 were detected in the anaerobic sludge and another band pattern involving bands 11 and 13 was observed from day 4.8. These four bands were closely related to *Methanogenium marinum*, which utilizes H_2/CO_2 in methane production (Chong et al. 2002). Bands 5, 12, 14, and 15 in the ARC DGGE profile appeared as distinct bands from day 2.4 to the end of the operation, and showed 98% nucleotide similarity to the 16S rRNA gene of *Methanoplanus petrolearius*.

This species maintained a largely constant intensity until the end of the observation period, and is known to use H_2/CO_2 or propanol for methane production (Ollivier et al. 1977). It can be inferred that some of the methane gas observed in the overall process period was produced by the activity of *Methanoplanus petrolearius* because of this continuously dominant band pattern in the ARC DGGE profile. The other dominant ARC DGGE band, band 16, which was observed from day 12, corresponded to *Methanosarcina mazei*. This species converts acetate to methane gas during growth (Deppenmeier et al. 2002), and can also utilize other organics as substrates. Therefore, a proportion of the methane gas yield of the later period of the overall process is attributable to *Methanosarcina mazei*, as it was observed from day 12 and utilizes various organic acids.

Order-specific PCR-based DGGE analysis

Analysis of the ARC DGGE bands showed that all the sequenced bands clustered within the orders MMB and MSL. To confirm the ARC DGGE profiles and undertake a detailed analysis of this interesting microbial structure, we performed order-level DGGE experiments with group-specific primers targeting the 16S rRNA genes of the orders MMB and MSL. The ARC DGGE profiles and the order-specific DGGE profiles of MMB and MSL were compared after the identification of each DGGE band by sequencing.

To demonstrate more directly the involvement of the methanogenic populations in this anaerobic bioreactor during the treatment of waste-activated sludge, the extracted genomic DNA was PCR-amplified with order-specific 16S rRNA gene primers, followed by DGGE analysis. Group-specific primers targeting the 16S rRNA gene were used to detect methanogens in the orders MSL, MMB, and MBT (Yu et al. 2005).

Figure 3 illustrates the DGGE profiles of the two orders MSL and MMB in this anaerobic bioreactor. MSL DGGE bands 1, 5, and 6 were observed in the waste-activated sludge and the anaerobic sludge. These bands were all the same methanogen, *Methanolobus oregonensis*, which is a methanol-, methylamine-, and sometimes methyl-sulfide-utilizing methanogen (Kendall and Boone 2006; Liu et al. 1990). This species was not identified in the ARC DGGE profiles but was newly detected in the microbial community by MSL DGGE. Two other bands, 2 and 7, were also observed in the waste-activated sludge and anaerobic sludge. These bands corresponded to *Methanosaeta concilii* and showed a similar band pattern to that of *Methanosaeta concilii* in the Archaea DGGE profile. MSL DGGE bands 3 and 4, detected in the anaerobic sludge, were identified as *Methanosaeta harundinacea*, an acetate-scavenging methanogen (Ma et al. 2006). The other dominant bands (bands 8, 9, 10, and 11) in the MSL DGGE profiles were species similar to that of band 16, *Methanosarcina mazei*, detected in the later operational periods in the ARC DGGE profile.

In the MMB DGGE profiles, four species of the order *Methanomicrobiales* corresponded to methanogenic species found in the ARC DGGE profiles. The most dominant band in the MMB DGGE profile, band 8, was identified as *Methanoplanus petrolearius*, which maintained a fairly thick band pattern until the end of the overall operation. MMB DGGE band 6 was closely related to *Methanocalculus pumilus* and bands 2 and 3 were closely related to *Methanocorpusculum bavaricum*. The MMB DGGE bands 1, 4, 5, and 7 were closely related to *Methanogenium marinum*. In the MMB DGGE profiles, three microbial strains differed from the methanogenic bacteria identified in the ARC DGGE profiles. MMB DGGE band 10 was

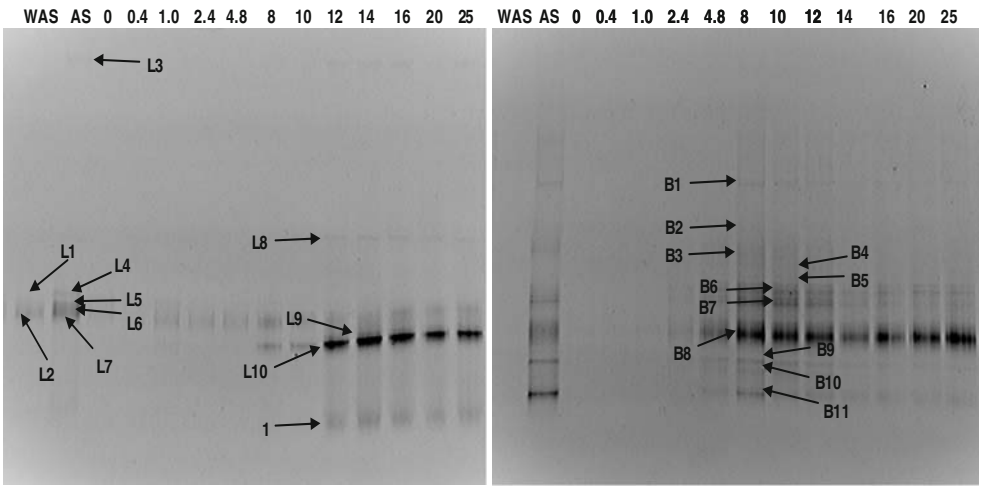

Fig. 3 Methanogenic bacterial community structures in an anaerobic batch system monitored using order-specific DGGE (left, *Methanosarcinales* DGGE profiles; right, *Methanomicrobiales* DGGE profiles)

closely related to *Methanofollis liminatans*, a methanogen that uses H_2/CO_2, formate, 2-propanol/CO_2, and 2-butanol/CO_2 as substrates for both growth and methanogenesis (Zellner et al. 1999).

Two other MMB DGGE bands, bands 9 and 11, corresponded to as yet uncultured microorganisms. From the DGGE profiles, we were able to discover the specific methanogens belonging to MSL and MMB unidentified by analysis of ARC DGGE profiles. *Methanolobus oregonensis* and *Methanosaeta harundinacea*, found in the MSL DGGE profiles, and *Methanofollis liminatans*, detected in the MMB DGGE profiles, are methanogens detected only in the order-level DGGE profiles. The ARC DGGE profiles showed no species of the order MBT, although this order is known to occur extensively, together with MSL and MMB, in methanogenic populations in anaerobic systems used to treat various environmental samples (Boone et al. 1993, Raskin et al. 1994).

Figure 4 shows the DGGE profiles of the order MBT in this anaerobic bioreactor. Five species belonging to the order MBT were identified by sequence analysis of the MBT DGGE bands and these bands maintained fairly constant intensities until the end of the observation period. Four bands in the MBT DGGE profiles (bands 1, 2, 3, and 9) were closely related *Methanobacterium subterraneum*, a methanogen that utilizes H_2/CO_2 or formate as substrates for growth and methanogenesis (Kotelnikova et al. 1998). Among the dominant bands of the MBT DGGE profiles, bands 4 and 5 were identified as *Methanobrevibacter smithii* and *Methanobrevibacter arboriphilus*, respectively, both belonging to the genus *Methanobrevibacter* of the order *Methanobacteriales*, which are methanogens that utilize H_2/CO_2 (Kotelnikova et al. 1998). MBT DGGE bands 6 and 7 were identified as *Methanosphaera stadtmanae*, which is a human intestinal archaeon (Fricke et al. 2006). This methanogen only generates methane by the reduction of methanol with H_2 and is dependent on acetate as a carbon source. MBT DGGE band 8 was closely related to *Methanobacterium oryzae*, a methanogen that uses H_2 and CO_2 or formate for growth and methanogenesis (Joulian et al. 2000).

In these DGGE profiles, we were able to monitor the dynamics of the microbial populations in an anaerobic bioreactor. The ARC DGGE primer set was similarly specific for each of the orders MMB, MSL, and MBT (Yu et al. 2005), but the ARC DGGE profiles generated with the ARC universal primers detected only the two orders MMB and MSL, and not MBT, in this bioreactor (Table 2). However, the MBT DGGE profile of the same samples had a distinct band pattern, as shown in Fig. 4. All the DGGE analysis data indicate that a proportion of the microbial

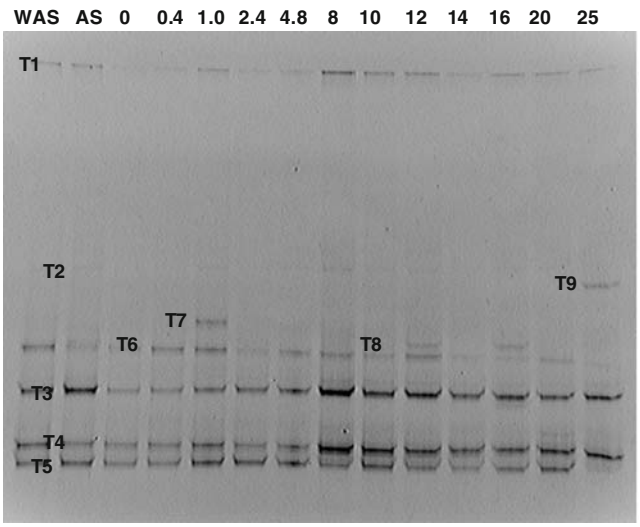

Fig. 4 Order *Methanobacteriales* DGGE banding profiles from an anaerobic batch system

Table 2 Identification of amplified 16S rRNA gene sequences excised from the DGGE gels shown in Figs. 2, 3, and 4

ARC DGGE band	MSL DGGE band	MMB DGGE band	MBT DGGE band	Highest similarity	GenBank accession number	Homology (%)
1,2,7,8,9	2,7			*Methanosaeta concilli*	X16932	269/271 (99%)
16	8,9,10,11			*Methanosarcina mazei*	AY196685	267/271 (98%)
3		6		*Methanocalculus pumilus*	AB008853	271/271 (100%)
10		2,3		*Methanocorpusculum bavaricum*	AY196676	266/272 (97%)
4,6,11,13		1,4,5,7		*Methanogenium marinum*	DQ177345	265/271 (97%)
5,12,14,15		8		*Methanoplanus petrolearius*	AY196681	266/271 (98%)
	1,5,6			*Methanolobus oregonensis*	U20152	345/354 (97%)
	3,4			*Methanosaeta harundinacea*	AY970347	344/354 (97%)
		9		Uncultured archaeon clone *F9T20L415*	DQ262579	503/506 (99%)
		10		*Methanofollis liminatans*	Y16428	503/506 (99%)
		11		Uncultured archaeon clone *OuO-39*	AJ556537	503/506 (99%)
			1,2,3,9	*Methanobacterium subterraneum*	DQ649330	333/339 (98%)
			6,7	*Methanosphaera stadtmanae*	AY196684	336/339 (99%)
			4	*Methanobrevibacter smithii*	AY196669	340/341 (100%)
			5	*Methanobrevibacter arborihilus*	AY196664	338/344 (98%)
			8	*Methanobacterium oryzae*	AF028690	335/342 (97%)

The numbers show the bands whose sequences were identified with the BLAST program in the National Center for Biotechnology Information (NCBI) database.
ARC, MSL, MMB, and MBT denote the domain Archaea and the three orders *Methanosarcinales*, *Methanomicrobiales*, and *Methanobacteriales*, respectively.

population producing methane gas could be detected with the ARC primers, but other methanogens involved in the process were not detected with these primers. It was therefore necessary to conduct DGGE experiments with order-level primers to detect the full complement of methanogens.

Discussion

Methanosaeta concilii-like species was faintly observed from the seed and substrate to the last days of incubation (Fig. 3). *M. mazei*-related bands (L8–11) were first observed at day 8 (Fig. 3) and this point coincided with the reduction of acetate concentration (Fig. 1). *M. concilii* and *M. mazei* belong to the families Methanosaetaceae and Methanosarcinaceae, respectively, under the order Methanosarcinales. If Methanosarcinaceae and Methanosaetaceae use acetate as their substrate in the same environment, we would anticipate that Methanosarcinaceae would predominate at higher acetate concentration, and Methanosaetaceae would predominate at lower acetate concentration (Yu et al. 2006). Accordingly, it was supposed that *M. mazei* preferentially participated in methane production using acetate because acetate level was as high as 662 mg/l, which would be favorable to Methanosarcinaceae (Yu et al. 2006). It is possible to suggest that *M. mazei* also utilized acetate derived from longer-chain fatty acids because further accumulation of acetate was not observed from the 8th to the 20th day when C_3–C_6 acids concentrations decreased (Fig. 1).

Hydrogenotrophic methanogens, including MMB and MBT, can utilize H_2 and CO_2 derived during the degradation of C_3–C_6 acids by nonmethanogenic organisms (Batstone et al. 2003; Stams et al. 1998). After the 8th day, butyrate and valerate concentrations decreased and methane production rate increased from 0.05 $l\,l^{-1}$ day^{-1} to 0.11 $l\,l^{-1}$ day^{-1} (Fig. 1). Unlike the other acids, propionate concentration decreased after day 12. The *M. subterraneum*-related (T3) and the *M. smithii*-related (T4) bands were observed with constant patterns from the beginning of incubation in MBT DGGE profiles, while the *M. petrolearius*-related band (B8) was observed from the 8th to the 25th day with distinct intensity in MMB DGGE profile (Fig. 3). Therefore, it is likely that *M. petrolearius* together with some species of MBT would participate as the consumer of H_2 and CO_2 derived from propionate, butyrate, and valerate catabolism.

In this work, DGGE of 16S rRNA gene fragments was used to analyze the microbial diversity in an anaerobic

batch treatment of waste-activated sludge. The DGGE profiles of the three orders showed methanogens belonging to each order that were not detected in the DGGE profile of the domain level. It was therefore necessary to use primers that target a lower taxonomic level, such as the MBT primers, to observe the more detailed microbial interactions that occurred during the operation.

It is important to note that different suites of methanogens were identified in the DGGE profiles of the whole domain Archaea and in the profiles of the three orders. Because the ARC primers used in this study have similar specificity for methanogens belonging to the three orders MMB, MSL, and MBT (Yu et al. 2005), the differences between the ARC DGGE profiles and the MMB, MSL, and MBT DGGE profiles may have resulted from two environmental factors. First, this discrepancy could be attributable to PCR bias. Research into PCR bias has shown that errors can occur during template annealing in the amplification of mixed samples (Suzuki and Giovannoni. 1996; Becker et al. 2000). Although the ARC primers used in this study have been shown to specifically amplify various orders of methanogens (MMB, MSL, and MBT) (Yu et al. 2005), it is difficult to ensure that these ARC primers always display unbiased specificity in the PCR annealing step in mixed samples of methanogen DNA extracted from a particular environmental sample.

Second, this discrepancy might be attributable to a quantitative problem involving the disproportionate occurrence of different methanogens in any sample. Thus, species of the order MBT may constitute a minor proportion of microbes in the overall process, whereas species of the orders MMB and MSL may constitute a major proportion of the microorganisms in the reaction. If members of the order MBT occur in smaller numbers than those of the other two orders, species of the order MBT could be detected by MBT-specific primer but not by ARC primers, which theoretically detect all species in the domain Archaea.

Although we can understand the general reaction between methanogens and their substrates using higher-taxonomic-level (domain Archaea) 16S rRNA gene primers, it is necessary to conduct DGGE experiments with 16S rRNA gene primers that target lower taxonomic levels to confirm the full and detailed microbial activity and dynamics occurring in a bioreactor.

These data provide insight into the diversity and distribution of functionally important methanogens in anaerobic sludge digestion.

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