J Ind Microbiol Biotechnol (2008) 35:1345–1352
DOI 10.1007/s10295-008-0417-7

ORIGINAL PAPER

Use of order-specific primers to investigate the methanogenic diversity in acetate enrichment system

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Received: 24 March 2008 / Accepted: 29 July 2008 / Published online: 20 August 2008

Abstract The applicability of order-specific primers in minimizing the possible underestimation of microbial diversity was evaluated via denaturing gradient gel electrophoresis (DGGE) analysis of a lab-scale anaerobic digester. Initially, a population analysis with real-time quantitative PCR demonstrated the existence of three methanogenic orders—*Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales*—throughout the reaction period. DGGE analyses with three pairs of order-specific primers yielded eight operational taxonomic units (OTUs), whereas DGGE analysis with two independent Archaea-specific primers identified only five. Moreover, the order-specific primers amplified at least one OTU affiliated with each order, whereas no members of *Methanobacteriales* or *Methanomicrobiales* were identified with Archaea-specific primers in most samples. These findings provide evidence that order-specific analysis can detect the diversity of methanogens in greater detail than conventional Archaea-specific analysis.

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Keywords Diversity of methanogens · Taxonomic level · Order-specific primers · Real-time quantitative PCR · Denaturing gradient gel electrophoresis

Introduction

Anaerobic digestion is an effective way to treat organic wastes with the production of methane, a valuable energy source [31, 37]. Anaerobic digestion involves a series of symbiotic reactions that can be grouped into acidogenesis and methanogenesis [16]. Because methanogenesis is usually the rate-limiting step in the overall process, the appropriate control of the methanogenic phase has been a key factor in the successful operation of anaerobic processes [26, 35]. Therefore, methanogens, the sole mediators of methanogenesis, have received particular attention in engineered environments [12, 15, 18, 21]. Nonetheless, because our knowledge of the involved microbial ecosystems is limited, field-scale anaerobic digesters have been empirically designed and operated to avoid failures, possibly resulting in unnecessarily large digester volumes and insufficient treatment [7, 14].

Culture-independent molecular techniques, particularly those based on 16S rRNA gene PCR fragments, are powerful tools with which to investigate microbial communities in various environments [6, 9]. Denaturing gradient gel electrophoresis (DGGE), one of the most frequently used of these methods, has been widely applied to anaerobic digestion processes [21, 27, 33]. This technique is effective in detecting microbial community shifts and in identifying the phylogenetic affiliations of microbial populations in mixed culture systems [32]. However, the PCR amplification of a DNA mixture from diverse organisms is a competitive reaction among different templates, and thus

a numerically minor population (ca. <1% of the total target group) is generally not detected by PCR-based techniques, including DGGE [9, 19]. This means that a primer set targeting a higher taxonomic level has a greater likelihood of missing a small population than does a set targeting a lower taxon when environmental DNA samples are analyzed. Such limitations possibly result in the underestimation of microbial diversity [9, 17]. Logically, the higher the target taxonomic level, the greater the possible error will be. Nevertheless, many studies have used PCR primers targeting extremely high taxonomic levels, such as the prokaryotic kingdom (kingdom Monera), the Archaea domain, or the Bacteria domain, without considering this issue. Although some effort has recently been made to compare or modify such universal primers to better describe microbial diversity [3, 6], little attention has been paid to their detection threshold.

In this study, we assessed the nesting of methanogenic species in terms of their diversity, employing an anaerobic bioreactor treating synthetic acetate wastewater, by comparing the DGGE profiles analyzed with several primer sets targeting different taxonomic levels. For this purpose, five primer sets targeting the total archaeal population or distinct methanogenic orders were used for the DGGE analyses of reactor DNA samples. One of the five methanogenic orders, *Methanopyrales*, was not considered because its members are unlikely to occur in engineered environments because their growth temperature is extremely high (>80 °C) [15]. In parallel with the DGGE analysis, we quantified the occurrence of each methanogenic order using real-time quantitative PCR (Q-PCR). This study demonstrates that methanogenic populations are better detected with order-specific analysis than with domain-specific analysis, based on the total numbers of operational taxonomic units (OTUs) detected with DGGE. To the best of our knowledge, this is the first study to apply the nesting concept to the DGGE analysis of methanogenic community structures.

Materials and methods

Bioreactor operation

A completely stirred anaerobic digester with a working volume of 6 L was used to cultivate the anaerobes. Synthetic wastewater containing acetate (12 g/L) and nutrients was used as the substrate. The concentrations of the nutrients were modified from a previous study [1], as follows (mg/L): NH_4Cl 2440, KH_2PO_4 750, $K_2HPO_4 \cdot 3H_2O$ 1450, NaCl 600, KCl 370, $MgSO_4 \cdot 7H_2O$ 247, citric acid·H_2O 210, nitrilotriacetic acid 40, $FeCl_3 \cdot 6H_2O$ 4.9, $CuCl_2 \cdot 2H_2O$ 4.3, $CaCl_2 \cdot 2H_2O$ 2.0, $CoCl_2 \cdot 6H_2O$ 1.5, $NiCl_2 \cdot 6H_2O$ 1.2, $MnCl_2 \cdot 4H_2O$ 0.9, $ZnCl_2$ 0.9, $Na_2SeO_3 \cdot 5H_2O$ 0.6, H_3BO_3 0.17, and $Na_2MoO_4 \cdot 2H_2O$ 0.1. The digester was initially operated in batch mode with an anaerobic seed inoculum (2% v/v) from a stable local municipal wastewater treatment plant. The reactor was further operated as a continuously stirred tank reactor (CSTR) with a hydraulic retention time of 15 days. Steady state was determined when the acetate concentration, the soluble chemical oxygen demand, the volatile suspended solids concentration, and the methane production rate did not change significantly. The temperature was held at 35 °C and the pH was maintained at 6.8 with 6.0 N NaOH and 3 N HCl.

Figure 1 shows the overall process performance of the anaerobic bioreactor. The initial batch operation was terminated at day 27, when no residual acetate or methane production was detectable. A steady state was observed after day 103. Acetate was converted to methane at the steady state, when the residual acetate concentration was <7 mg/L and the methane yield was 263 mL CH_4/g acetate reduced [equivalent to 246 mL CH_4/g chemical oxygen demand (COD) reduced]. Samples were collected at four sampling points (at the start and the end of the batch, at an intermediate point, and at steady state) for further microbial analysis.

Extraction of DNA

Total DNA was extracted using a fully automated nucleic acid extractor (Magtration System 6GC, Precision System Science, Chiba, Japan) using magnetic bead technology [16, 24]. An aliquot (1 mL) of the sample was centrifuged at 14,000×*g* for 10 min and the supernatant was decanted. The pellet was then washed with 1 mL of deionized distilled water (DDW) and centrifuged again in the same manner, to ensure the maximal removal of residual medium. The supernatant was carefully removed and the pellet was resuspended in 100 μL of DDW. The genomic DNA in

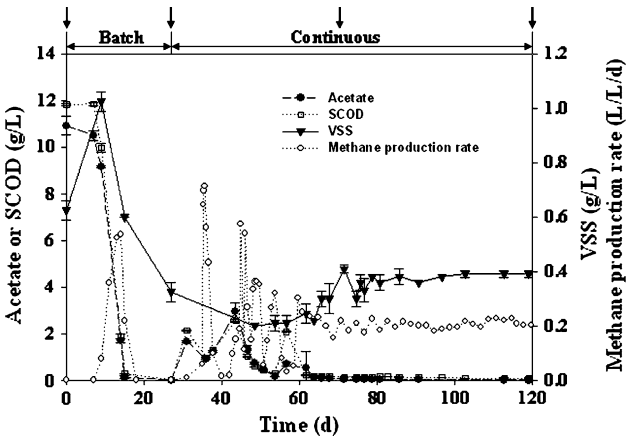

Fig. 1 Changes in acetate, SCOD, and VSS concentrations and methane production rate of the anaerobic digester. *Arrows* indicate DNA sampling points

the suspension was immediately extracted using an automated nucleic acid extractor with a Magtration Genomic DNA Purification Kit (Precision System Science). The purified DNA was eluted with 100 μL of Tris—Cl buffer (pH 8.0) and stored at −20 °C until use. All DNAs were extracted in duplicate.

Q-PCR analysis

Primer and probe sets targeting the orders *Methanobacteriales* (MBT-set), *Methanococcales* (MCC-set), *Methanomicrobiales* (MMB-set), or *Methanosarcinales* (MSL-set) have been described previously [37]. The nucleotide sequences of the TaqMan probes were as follows: MBT929F, 5′-AGCACCACAACGCGTGGA-3′; MCC686F, 5′-TAGCGGTGRAATGYGTTGATCC-3′; MMB749F, 5′-TYCGACAGTGAGGRACGAAAGCTG-3′; MSL860F, 5′-AGGGAAGCCGTGAAGCGARCC-3′. Q-PCR was performed using the LightCycler 480 (Roche Diagnostics, Mannheim, Germany). The 20 μL Q-PCR mixture was prepared using the LightCycler 480 Probes Master kit (Roche Diagnostics): 10 μL of 2 × reaction solution, 1 μL of each primer (final concentration 500 nM), 1 μL of the TaqMan probe (final concentration 100 nM), 2 μL of template DNA, and 5 μL of PCR-grade water. The two-step amplification protocol was as follows: initial denaturation for 10 min at 94 °C followed by 45 cycles of 10 s at 94 °C and combined annealing and extension for 30 s at 60 °C (62 °C for the MMB set). The fluorescent signal was measured at the end of each annealing/extension step. Duplicate DNA samples were analyzed at each point.

Standard curves were generated for the methanogen strains, as described previously [20, 35]. Nearly full-length 16S rRNA gene fragments of *Methanobacterium formicicum* M.o.H. (DSM 863), *Methanomicrobium mobile* BP (DSM 1539), and *Methanosarcina barkeri* MS (DSM 800) were amplified with PCR. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and designated pGEM-MF, pGEM-MM, and pGEM-MB, respectively. The plasmids were extracted in the range of 10^8–10^9 copies/μL, serially diluted, and used as templates in Q-PCR with the corresponding sets (i.e., pGEM-MF for the MBT-set, pGEM-MM for the MMB-set, and pGEM-MB for the MSL-set).

PCR–DGGE and band identification

The 16S rRNA gene primers used for DGGE analysis included two distinct Archaea primers and three methanogen-order-specific primers (Table 1) [25, 37]. A 40-bp GC-clamp was added to the 5′ end of either the forward or reverse primer to stabilize the melting behavior of the PCR fragments [22, 34]. A PCR premix (AccuPower HF PCR PreMix, Bioneer, Daejon, Korea) was used with 1 μL of each primer (final concentration 500 nM), 1–5 μL of template DNA, and the appropriate amount of DDW for a final volume of 20 μL. Touch-down PCR was conducted in a thermal cycler (PTC-100, MJ Research Inc., Watertown, MA) using the following protocol: initial denaturation at 94 °C for 10 min; 20 cycles of denaturation at 94 °C, annealing at 65–55 °C (reducing the temperature by 0.5 °C per cycle) for 30 s, and extension at 72 °C for 30 s; an additional 25 cycles of 94, 55, and 72 °C for 30 s each; and a final extension at 72 °C for 7 min. All PCRs were run in duplicate and mixed to minimize PCR bias.

The PCR products were directly loaded onto 8% (w/v) acrylamide gels containing denaturant gradients: 20–70% for PCR products amplified with the ARC and PARCH primers, 40–60% for the MBT and MMB primers, and 30–60% for the MSL primers [100% denaturant was 7 M urea with 40% (v/v) formamide]. DGGE (DCode system, Bio-Rad, Hercules, CA) was run at 150 V for 7 h in 1 × TAE buffer. The gels were stained with ethidium bromide and the visible bands were excised and eluted into 40 μL of sterile DDW. The eluted solution was further amplified with PCR using the corresponding primers without the GC-clamp. The PCR products were purified from a 1% agarose gel and cloned into the pGEM-T Easy vector (Promega). The cloned 16S rRNA gene fragments were sequenced with a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) using the T7 primer. The sequencing results were compared with the reference sequences in the GenBank database using the BLAST program. The nucleotide sequences reported in this paper have been deposited under GenBank accession numbers EU586058–EU586108.

Analytical methods

The COD and suspended solids were measured according to the procedures in Standard Methods [8]. A gas chromatograph (6890 Plus, Agilent, Palo Alto, CA), equipped with an Innowax capillary column (Agilent) and a flame ionization detector, was used to quantify acetic acid. Another identical gas chromatograph, equipped with an HP-5 capillary column (Agilent) and a thermal conductivity detector, was used to analyze the composition of the biogas.

Results

Q-PCR analysis

The methanogen population dynamics in the bioreactor were estimated at the order level (Fig. 2). The 16S rRNA

Table 1 Primers used in PCR for DGGE analysis

Target group	Name	Sequence[a]	Amplicon size	Reference
Archaea	PARCH340f-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG CCCTACGGGGYGCASCAG	152	[25]
	PARCH519r	TTACCGCGGCKGCTG		
	ARC787F-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG ATTAGATACCCSBGTAGTCC	271	[37]
	ARC1059R	GCCATGCACCWCCTCT		
Methanobacteriales	MBT857F	CGWAGGGAAGCTGTTAAGT	342	[37]
	MBT1196R-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCGCGCCCG TACCGTCGTCCACTCCTT		
Methanomicrobiales	MMB282F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG ATCGRTACGGGTTGTGGG	506	[37]
	MMB832R	CACCTAACGCRCATHGTTTAC		
Methanosarcinales	MSL812F	GTAAACGATRYTCGCTAGGT	354	[37]
	MSL1159R-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG GGTCCCCACAGWGTACC		

[a] Underlined sequences are 40-bp GC-clamps attached to the 5′ ends of the reported primers

gene copy number of *Methanosarcinales* increased from 1.4×10^7 copies/mL to 1.3×10^9 copies/mL during the batch period. *Methanosarcinales* had become the most abundant methanogenic order after the batch period, when the 16S rRNA gene of this group contributed more than 99% of the total methanogenic 16S rRNA gene population. Conversely, the *Methanomicrobiales* gene copy number decreased continuously from 1.5×10^7 copies/mL (48% of the total) on day 0 to 1.5×10^6 copies/mL (0.5% of the total) on day 119. *Methanobacteriales* continued to decline and constituted the smallest proportion of methanogens during the reaction period. The *Methanococcales* group was not detected. The standard curves for the three sets of organisms detected were nearly linear (correlation coefficients > 0.998) in a template concentration range of at least six orders of magnitude (data not shown).

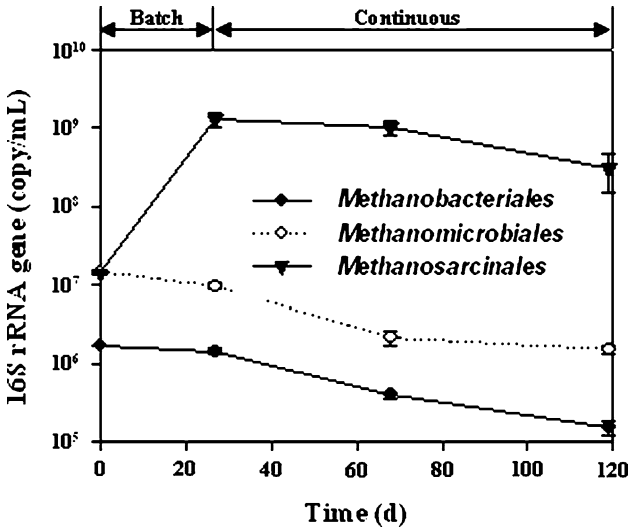

Fig. 2 Methanogen population dynamics in the anaerobic digester. Four methanogenic orders were examined using Q-PCR. The order Methanococcales was not detected

PCR–DGGE and band identification

PCR–DGGE and subsequent band analysis were conducted to identify the methanogenic species present in the digester (Fig. 3). Archaeal universal primers are commonly used for methanogenic diversity studies [10, 27, 33] because methanogens are classified exclusively within the domain Archaea [11] and most of the Archaea in anaerobic digesters are likely to be methanogens [37]. Therefore, a set of representative Archaea-specific primers was used to determine the total methanogenic community [25]. Accordingly, PARCH340f-GC and PARCH519r (PARCH primers) (Table 1) were used as the primers for PCR in the Archaea DGGE analysis. Five bands (P1–5) from the DGGE profile were excised for subsequent sequencing analysis (Fig. 3a). The 16S rRNA gene fragments from P1 to 5 were grouped into three OTUs, as shown in Table 2. The three OTUs clustered into either *Methanomicrobiales* (P1) or *Methanosarcinales* (P2–5); no OTU belonging to the order *Methanobacteriales* was identified. Moreover, P1 was only detected on day 0. These findings are inconsistent with the Q-PCR results, which showed that the *Methanobacteriales* and *Methanomicrobiales* groups were present throughout the reaction period.

Although the PARCH primers have been successfully used to describe several archaeal communities [13, 25], PCR–DGGE with the PARCH primers did not fully

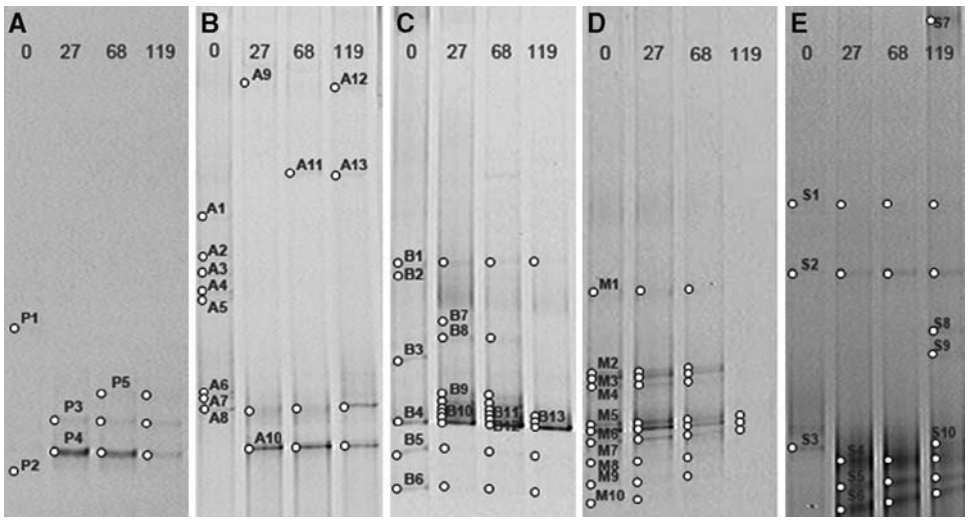

Fig. 3 DGGE profiles of the PCR products amplified with Archaea- or order-specific primers: **a** PARCH primers, **b** ARC primers, **c** MBT primers, **d** MMB primers, and **e** MSL primers. Lane numbers (*0*, *27*, *68*, and *119*) indicate the sampling day in the reactor process

detect the methanogenic OTUs in this reactor. Therefore, a second PCR–DGGE was designed to link the Q-PCR and DGGE results. Primers ARC787F and ARC1059R are specific to the domain Archaea [37], and when combined with an internal TaqMan probe, can be used in nested analysis with the order-specific primer and probe sets used in this study (data not shown) [36]. Therefore, we used these Archaea-specific primers with a GC-clamp attached to the 5′ end of the forward primer (ARC primers) in a second PCR–DGGE analysis (Table 1). Thirteen distinct bands were analyzed from this DGGE profile (Fig. 3b). Among these bands, four OTUs were identified, as described in Table 2. However, repetitive discrepancies were observed. No member of the order *Methanobacteriales* was detected and only *Methanosarcinales*-affiliated OTUs were found at day 27 or later.

We hypothesized that the relative, not absolute, abundance of *Methanobacteriales* and *Methanomicrobiales* was the main constraint in their detectability in PCR–DGGE with the PARCH or ARC primers. In this context, primers must target narrower taxa to successfully amplify these groups. Consequently, a set of PCR–DGGEs was conducted with the order-specific primers that were used in the Q-PCR analysis (Fig. 3c–e). A GC-clamp was added to the 5′ end of either the forward or the reverse primer of each primer set, and the resulting primers were designated MBT (*Methanobacteriales*-specific), MMB (*Methanomicrobiales*-specific), and MSL (*Methanosarcinales*-specific) primers (Table 1). DGGE profiles of the PCR products amplified with the MBT, MMB, or MSL primers produced two, three, and three OTUs, respectively (Table 2). At least one OTU affiliated to each order was detected in the four DNA samples that were examined.

Discussion

Acetate is regarded as the precursor for more than 70% of methane formation in many anaerobic digestion processes [31, 35]. A group of methanogens, clustered in the order *Methanosarcinales*, utilize acetate directly [11, 38] and are present in most anaerobic environments [10, 17, 30]. Among these aceticlastic methanogens, the family *Methanosarcinaceae* has a high growth rate with a relatively low acetate affinity, whereas the family *Methanosaetaceae* has a higher substrate affinity but a lower growth rate [35]. *Methanosarcinales* became the abundant group according to Q-PCR after day 27, indicating that aceticlastic methanogenesis was the major pathway of acetate degradation (Fig. 2). Three OTUs were identified within this order (Table 2). *Methanosaeta concilii*-like bands were detected throughout the reactor period, with relatively higher intensities on days 0 and 119 (Fig. 3a, b, e). Another OTU belonging to the *Methanosaetaceae* family, *Methanosaeta harundinacea*-related OTU, was visualized only on day 119. However, *Methanosarcinaceae*-affiliated bands (*Methanosarcina mazei*-like) appeared mainly after day 27 with strong band intensity. These observations are consistent with the general kinetic characteristics of the two families because the seed inoculum and the steady-state reactor experienced low-acetate conditions, whereas the high acetate concentration in the batch period (between days 0 and 27) was responsible for the outgrowth of the *Methanosarcina mazei*-like species.

Acetate is anaerobically converted to methane and carbon dioxide either via its direct utilization by aceticlastic methanogens or via acetate oxidation by syntrophic microbial consortia [5]. Although the aceticlastic pathway is often preferred, acetate oxidation is accelerated under high-temperature and/or low-acetate conditions [5].

Table 2 Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands

Band(s)		Nearest sequence	Accession no.	Percentage similarity
Archaea DGGE	Order DGGE			
P1		Uncultured archaeon clone 4LOC8	AY835818	99
P2	S1,3,7,10	*Methanosaeta concilii*	X16932	98–100
A6–8,12				
P3–5	S2,4–6,8–9	*Methanosarcina mazei*	AY196685	98–100
A9–11				
A1–5	M1–8	Uncultured *Methanomicrobiales* LF-ProM-C	AB236107	98–100
A13		*Methanosaeta harundinacea*	AY970347	99
	M9	Uncultured archeon CBs-a1D	DQ301907	98
	M10	*Methanospirillum hungatei* JF-1	AY196683	92
	B1–2,4,6–13	*Methanobacterium formicicum*	AY196659	97–99
	B3	*Methanosphaera stadtmanae*	AY196684	99
	B5	*Methanobrevibacter smithii* PS	AY196669	98

Each sequence was compared with the GenBank database

Recently, the syntrophism within mesophilic acetate oxidation has been investigated, showing that hydrogen-utilizing methanogens can be involved in this process by eliminating the end products of their metabolic partners [23, 28]. Organisms that belong to the orders *Methanobacteriales* or *Methanomicrobiales* are hydrogenotrophic methanogens, which do not utilize acetate directly [11, 38]. Thus, the *Methanobacteriales*- or *Methanomicrobiales*-affiliated OTUs that were identified in the digester (Table 2) may have utilized the hydrogen and carbon dioxide that originated from acetate.

With a CSTR, assumptions are often made that the reactor is homogenized and that the effluent is identical to the reactor matrix [29]. Thus, a nongrowing microorganism can be deduced according to the following equation:

$$X = X_0 \exp(-Dt)$$

where X is the microbial concentration at present, X_0 is the initial microbial concentration, D is the dilution rate, and t is the time between the initial reaction and the present. Based on this equation and with day 27 as the initial time, the 16S rRNA gene concentrations for *Methanobacteriales* and *Methanomicrobiales* at day 119 can be predicted to be 3.0×10^3 copies/mL and 2.1×10^4 copies/mL, respectively, compared with the experimental values of 1.5×10^5 copies/mL and 1.5×10^6 copies/mL (Fig. 2). The discrepancies between the predicted and measured values suggest that the two groups of hydrogenotrophic methanogens had grown in this anaerobic digester.

Overall, five OTUs were observed in PCR–DGGE with the PARCH and ARC primers, whereas eight OTUs were identified with the order-specific primers. Only three OTUs, *Methanosaeta concilii*-like, *Methanosarcina mazei*-like, and clone LF-ProM-C-related, were found in both analyses. They represented relatively intense bands, including bands M2–7 and S4-6, in the order-specific PCR–DGGEs (Fig. 3d, e). This observation, together with the fact that the sum of the *Methanosarcinales* and *Methanomicrobiales* 16S rRNA genes always predominated (Fig. 2), indirectly supports the proposition that the three OTUs were the abundant methanogenic species present in the bioreactor.

Five OTUs were only identified with the order-specific PCR–DGGEs (Table 2). These OTUs were grouped in the orders *Methanobacteriales* and *Methanomicrobiales*. Because the two orders constituted relatively less-abundant populations (<1% of the total) after day 27, the competitive aspects of the PCR may have limited the detection of members of these groups in the Archaea-specific PCR–DGGEs [9]. In particular, no *Methanobacteriales*-affiliated bands were recovered with the Archaea-specific PCR–DGGEs (Fig. 3a, b), even when the proportion of this order had reached 5.5% at day 0. Therefore, we conclude that order-specific PCR–DGGE can be used to determine methanogenic populations when it is difficult to identify members of a relatively low-abundance subgroup with Archaea-specific PCR–DGGE. It should be mentioned that a previous study used three group-specific primers to construct methanogenic clone libraries, but the advantages of the lower-taxon analyses could not be emphasized [4]. The concept of nesting can also be applied to the detection of microorganisms when the group of interest (a higher taxon) is clearly divided into several lower taxa, such as the β-proteobacterial ammonia-oxidizing bacteria [20].

In contrast, two OTUs, closely related to clone 4LOC8 or *Methanosaeta harundinacea*, were only observed in the PCR–DGGEs with Archaea-specific primers (Table 2). Because the two OTUs were successfully visualized in the PARCH or ARC DGGE profiles (Fig. 3a, b), they must have

Table 3 Comparison of the order-specific primer sequences with the nearest sequences of P1 and A13 bands

Primer and nearest sequence	Nucleotide sequences (5′-3′)																	
MMB282F	A	T	C	G	R	T	A	C	G	G	G	T	T	G	T	G	G	G
Clone 4LOC8	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
MSL1159R	G	G	T	A	C	W	C	T	G	T	G	G	G	G	A	C	C	
M. harundinacea	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	

Each matching sequence was represented by a dot

been present at concentrations above the detection limit in the corresponding DNA samples. However, they were not recovered as visible bands in the order-specific PCR–DGGEs, where in principle, there should have been fewer PCR competitors compared with those in the Archaea-specific PCR–DGGEs. One possible explanation for this inconsistency is that the order-specific primers contained mismatches relative to the 16S rRNA genes of these OTUs. Because the two OTUs were only partially sequenced, the 16S rRNA gene sequences of the nearest neighbors, shown in Table 2, were instead matched to the corresponding order-specific primers. As a result, clone 4LOC8 (AY835818) had three mismatches with the MMB282F primer and *M. harundinacea* (AY970347) had one mismatch with the MSL1159R primer (Table 3). The high sequence similarities between the two OTUs and the reference sequences suggest that the methanogenic OTUs had the same mismatches with the order-specific primers. Although primer–template mismatches can be tolerated in PCR [2], inefficient amplification is likely to occur from a quantitative point of view [20]. The use of order-specific primers that allow such false negative results can underestimate species richness, although at present these problems are inevitable because the database is still growing [3].

Acknowledgments This work was supported in part by the Korea Ministry of Education (MOE) through the BK-21 program and by the Korea Science and Engineering Foundation (KOSEF) through the Advanced Environmental Biotechnology Research Center (R11-2003-006) at POSTECH. This research was also supported by New and Renewable Energy R&D program (2006-N-BI02-P-09) under the Korea Ministry of Commerce, Industry, and Energy (MOCIE).

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