The ISME Journal (2009) 3, 522–535

www.nature.com/ismej

ORIGINAL ARTICLE

Quantitative detection of culturable methanogenic archaea abundance in anaerobic treatment systems using the sequence-specific rRNA cleavage method

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A method based on sequence-specific cleavage of rRNA with ribonuclease H was used to detect almost all known cultivable methanogens in anaerobic biological treatment systems. To do so, a total of 40 scissor probes in different phylogeny specificities were designed or modified from previous studies, optimized for their specificities under digestion conditions with 32 methanogenic reference strains, and then applied to detect methanogens in sludge samples taken from 6 different anaerobic treatment processes. Among these processes, known aceticlastic and hydrogenotrophic groups of methanogens from the families *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriaceae*, *Methanothermaceae* and *Methanocaldococcaceae* could be successfully detected and identified down to the genus level. Within the aceticlastic methanogens, the abundances of mesophilic *Methanosaeta* accounted for 5.7–48.5% of the total archaeal populations in mesophilic anaerobic processes, and those of *Methanosarcina* represented 41.7% of the total archaeal populations in thermophilic processes. For hydrogenotrophic methanogens, members of the *Methanomicrobiales*, *Methanobrevibacter* and *Methanobacterium* were detected in mesophilic processes (1.2–17.2%), whereas those of *Methanothermobacter*, *Methanothermaceae* and *Methanocaldococcaceae* were detected in thermophilic process (2.0–4.8%). Overall results suggested that those hierarchical scissor probes developed could be effective for rapid and possibly on-site monitoring of targeted methanogens in different microbial environments.
The ISME Journal (2009) **3,** 522–535; doi:10.1038/ismej.2009.4; published online 12 February 2009
Subject Category: microbial population and community ecology
Keywords: anaerobic wastewater treatment process; methanogen; RNase H method

Introduction

Methane-producing archaea (methanogens) are known to proliferate in natural anaerobic ecosystems (Brauer *et al.*, 2006; Mehta and Baross, 2006; Miyata *et al.*, 2007; Sakai *et al.*, 2007; Nunoura *et al.*, 2008) and anaerobic biological systems (Sekiguchi and Kamagata, 2004), where external electron accepters (for example, oxygen, sulfate or ferric iron) other than carbon dioxide are relatively limited. These methanogens closely interact with anaerobic syntrophs (that is, fermentative heterotrophs and proton-reducing bacteria) by converting important intermediates such as hydrogen, formate and acetate, which are derived from the breakdown of complex organic matter, to methane and carbon dioxide (Schink, 1997; Hattori, 2008). Based on 16S rRNA sequence as the phylogenetic marker, methanogens include mainly members of the phylum *Euryarchaeota* of the domain *Archaea* (Garrity *et al.*, 2007) and can be assigned into at least 29 genera from the classes

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Received 24 September 2008; revised 12 December 2008; accepted 21 December 2008; published online 12 February 2009

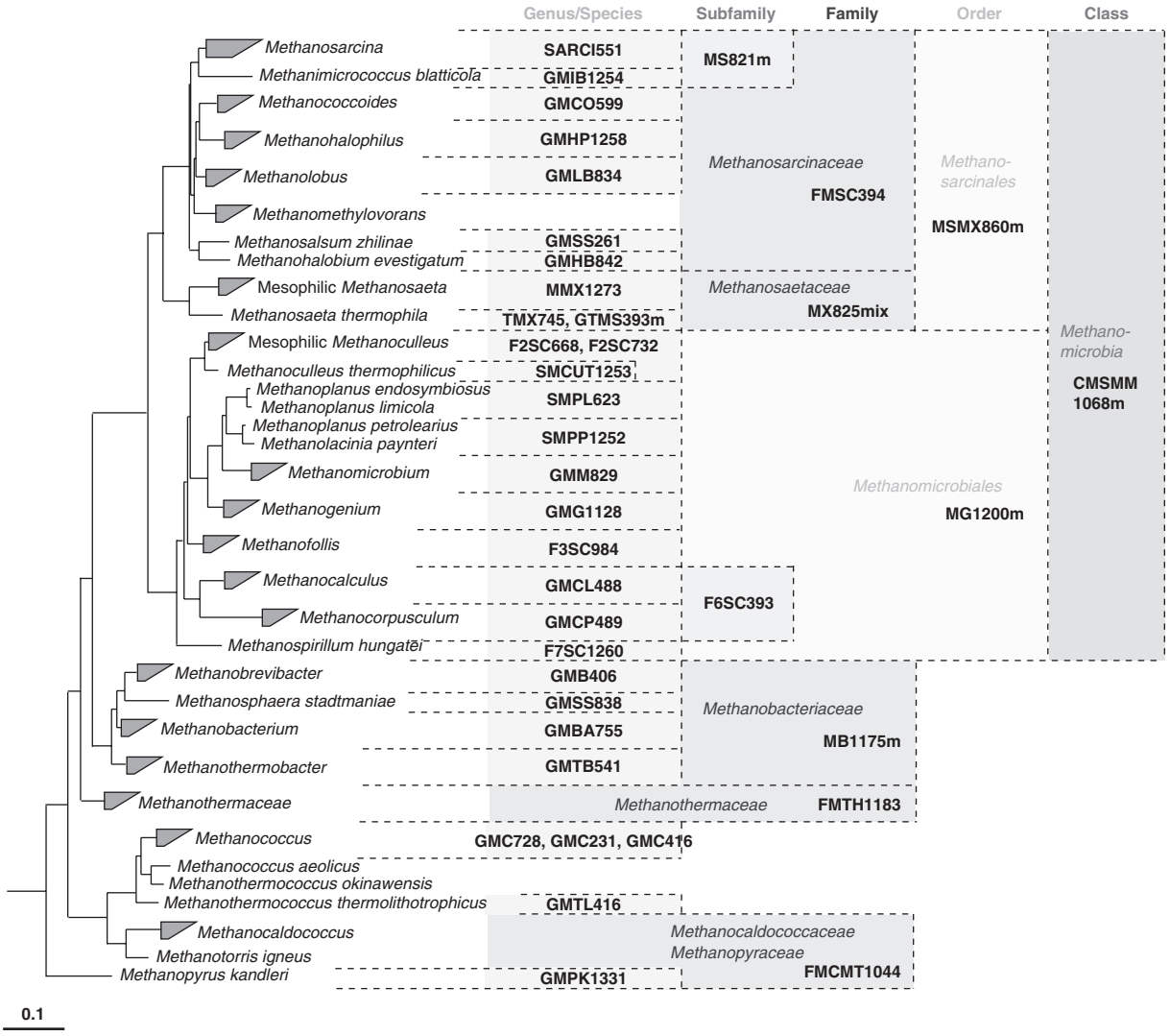


Figure 1 Phylogenetic tree of previously known methanogens based on neighbor-joining analysis of partial 16S rRNA gene sequences. Coverages of oligonucleotide scissor probes are shown in dotted brackets. *Desulfurococcus mobilis* was used as an outgroup. The bar indicates 10% base substitution.

Methanomicrobia, *Methanobacteria*, *Methanococci* and *Methanopyri* within the phylum *Euryarchaeota* (Figure 1).

Due to the ecological importance of methanogens in the carbon cycle, several rRNA-based molecular tools have been developed to quantify these methanogens in environments. Membrane hybridization technique, together with the use of a comprehensive set of rRNA-targeted oligonucleotide probes, can detect most methanogens at different levels of specificity (that is, order and genus) in various anaerobic processes (Raskin *et al*., 1994a, b; Zheng and Raskin, 2000; McMahon *et al*., 2004). These rRNA-targeted oligonucleotide probes are further used in fluorescence *in situ* hybridization (FISH) analysis (Crocetti *et al*., 2006; Nakamura *et al*., 2006; Zheng *et al*., 2006) and PCR-based methods (Banning *et al*., 2005; Hori *et al*., 2006; Yu *et al*., 2006) to quantify the abundance of different methanogens in environments. Though providing quantitative measurement of different methanogens in environments, the detection specificity of these molecular methods is rather limited to the order or family level, or to functionally important methanogens in environments, and their operations tend to be laborious and time consuming for on-site detection in the biological systems. Thus, there is a need for a more rapid and simpler method to detect the abundance of different target organisms and link their abundance to process performance.

Recently, a simple method based on the reaction of ribonuclease H (RNase H) on probe–rRNA duplex has been developed for the quantification of specific SSU rRNA in microbial environments (Uyeno *et al*., 2004). In this method, oligonucleotide probes (scissor probes) are used to bind onto targeted SSU rRNAs and form RNA–DNA duplexes. By digesting these RNA–DNA duplexes with RNase H, the

fractions of the digested duplexes can be separated by size and quantified using electrophoresis. This method has been successfully applied to quantify targeted microbial populations in different environments such as termite gut (Noda *et al.*, 2005), biological treatment processes (Uyeno *et al.*, 2004; Sekiguchi *et al.*, 2005), daily cattle rumen (Uyeno *et al.*, 2007), cow feces (Uyeno *et al.*, 2004) and human feces (Uyeno *et al.*, 2008). In this study, this method is further extended to quantify different methanogens in anaerobic treatment processes through the use of a comprehensive set of 40 16S rRNA-based scissor probes targeting almost all known methanogens at different phylogeny specificities. To improve the efficiency of cleavage reaction, thermostable RNase H was used instead of the *E coli* RNase H used previously. After optimizing their conditions in the RNase H digestion against reference strains, these probes were used to quantify the rRNA contents of different methanogens in samples taken from six different anaerobic biological treatment systems. The results provided good insights into the relationships between the traits of the anaerobic processes and the constituents of methanogenic archaea.

Materials and methods

Microorganisms and sludge samples

In total, 32 methanogen cultures purchased from Japan Collection of Microorganisms (JCM, Wako, Japan) or Deutshe Sammlung von Mikroorganismen

Table 1 Microorganisms used in this study

Microorganisms	*Strain no.*[a]
Domain *Archaea*	
Order *Methanosarcinales*	
Methanosarcina barkeri DSM800	1
Methanosarcina thermophila DSM2905	2
Methanimicrococcus blatticola DSM13328	3
Methanococcoides methylutens DSM2657	4
Methanohalophilus mahii DSM5219	5
Methanolobus tindarius DSM2278	6
Methanosalsum zhilinae DSM4017	7
Methanohalobium evestigatum DSM3721	8
Methanosaeta concilii DSM3671	9
Methanosaeta thermophila DSM6194	10
Order *Methanomicrobiales*	
Methanoculleus bourgensis DSM3045	11
Methanoculleus thermophilus DSM2373	12
Methanoplanus limicola DSM2279	13
Methanolacinia paynteri DSM2545	14
Methanomicrobium mobile DSM1539	15
Methanogenium cariaci DSM1497	16
Methanofollis tationis DSM2702	17
Methanocalculus halotolerans DSM14092	18
Methanocorpusculum parvum DSM3823	19
Methanospirillum hungatei DSM864	20
Order *Methanobacteriales*	
Methanobrevibacter arboriphilus DSM2462	21
Methanobrevibacter ruminantium DSM1093	22
Methanosphaera stadtmanae DSM3091	23
Methanobacterium bryantii DSM863	24
Methanobacterium formicicum DSM1535	25
Methanothermobacter thermautotrophicus DSM1053	26
Methanothermus fervidus DSM2088	27
Order *Methanococcales*	
Methanococcus vannielii DSM1224	28
Methanothermococcus thermolithotrophicus DSM2095	29
Methanocaldococcus jannaschii DSM2661	30
Methanotorris igneus DSM5666	31
Order *Methanopyrales*	
Methanopyrus kandleri DSM6324	32
Domain *Bacteria*	
Phylum *Proteobacteria*	
Escherichia coli DSM5717	33
Thiothrix disciformis DSM 14473	34
Stenotrophomonas maltophilia DSM50170	35
Syntrophobacter wolinii DSM2805M	36
Desulfovibrio vulgaris subsp. *vulgaris* DSM644	37
Phylum *Gemmatimonadetes*	
Gemmatimonas aurantiaca DSM 14586	38
Phylum *Actinobacteria*	
Microlunatus phosphovorus DSM10555	39
Phylum *Firmicutes*	
Syntrophomonas wolfei subsp. *wolfei* DSM2245A	40
Desulfotomaculum nigrificans DSM574	41
Clostridium acetobutyricum DSM792	42
Desulfitobacterium hafniese DSM10664	43
Bacillus subtilis DSM10	44
Phylum *Bacteroidetes*	
Prevotella bryantii DSM 11371	45
Bacteroides fragilis DSM 2151	46
Phylum *Fibrobacteria*	
Fibrobacter succinogenes ATCC 19169	47
Phylum *Spirohaetes*	
Treponema bryantii DSM 1788	48
Phylum *Chloroflexi*	
Chloroflexus aurantiacus DSM635	49
Anaerolinea thermophila DSM14523	50
Phylum *Nitrospirae*	
Thermodesulfovibrio yellowstonii DSM11347	51
Phylum *Thermodesulfobacteria*	
Thermodesulfobacterium commune DSM2178	52
Phylum *Deinococcus-Thermus*	
Deinobacter grandis DSM3963	53
Domain *Eucarya*	
Saccharomyces cerevisiae	54

[a]Numbers for each strain are used for abbreviation in Table 3.

Table 2 Specifications of the methane fermentation processes

Reactor No.	*Wastewater type*	*Reactor type*	*Volume (m^3)*	*Temperature (°C)*	*COD loading rate ($kg\,COD\,m^{-3}$ per day)*	*COD removal (%)*	*VFA concentration in influent ($mg\,COD\,l^{-1}$)*	
							Acetate	*Propionate*
1	Sugar processing	UASB	380	35–40	10	87	410	260
2	Amino-acid processing	UASB	65	35–40	3.7	55	430	110
3	Clear-liquor processing	UASB	2.5	50–55	60	89	3000	ND
4	Clear-liquor processing	UASB	2	37	36	90	2600	ND
5	Sewage wastewater	EGSB	0.071	23	2.0	36	16	10
6	Sewage wastewater	Digester	170	30	0.038	93	NT	NT

Abbreviations: COD, chemical oxygen demand; EGSB, expanded granular sludge blanket; ND, not detected; NT, not tested; UASB, upflow anaerobic sludge blanket; VFA, volatile fatty acid.

und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) were used as the reference organisms (Table 1). Anaerobic sludge samples were taken from six different treatment processes including a mesophilic (35–40 °C) full-scale upflow anaerobic sludge blanket (UASB) reactor treating wastewater from a food-processing plant (no. 1 and 2), a thermophilic (50–55 °C) pilot-scale UASB reactor treating wastewater from a alcohol-producing plant (no. 3), a mesophilic (37 °C) laboratory-scale UASB reactor treating wastewater from an alcohol-producing plant (reactor no. 4), a mesophilic (23 °C) laboratory-scale expanded granular sludge blanket (EGSB) reactor treating municipal sewage (no. 5) and a mesophilic (30 °C) full-scale anaerobic digester treating municipal sewage (no. 6). Analytical procedures for chemical oxygen demand (COD) and volatile fatty acid (VFA) concentrations were described previously (Harada *et al.*, 1996). Reactors 1, 3, 4 and 6 exhibited good COD removal performance (>87%), but reactors 2 and 5 had low COD removal rate (Table 2). In addition, reactor 1 was an UASB process, which often showed sludge-bulking phenomenon caused by uncultured anaerobic bacteria (Yamada *et al.*, 2007). Sludge samples taken were immediately subjected to RNA extraction or stored at −80 °C. Specifications and operational conditions of all reactors were summarized in Table 2.

Probe design

rRNA-targeted oligonucleotide probes (Table 3) were newly designed or modified from probes previously reported for methanogens. These probes were evaluated *in silico* using the ARB software package (Ludwig *et al.*, 2004). All the oligonucleotide probes were purchased from Tsukuba Oligo Service Co. Ltd (Tsukuba, Japan). The probe specificities were tested against those reference methanogen strains. The 16S rRNAs of all the reference methanogens were *in vitro* transcribed using T7 RiboMAX Express Large Scale RNA Production System (Promega Corp., Madison, WI, USA) as previously described (Uyeno *et al.*, 2004).

RNA extraction and sequence-specific cleavage of rRNA with RNase H

The extraction and quantification of rRNA from sludge samples were performed as described previously (Uyeno *et al.*, 2004). Briefly, pellets of cells (or samples; approximately 0.1–0.2 g (wet weight)) were harvested in conical 2.2-ml screw-cap tubes; each tube contained 1 g of baked glass beads (0.1 mm in diameter) and 1 ml of pH 5.1 buffer (10 mM EDTA, 50 mM sodium acetate, pH 5.1). The remaining volume in the tubes was filled with phenol equilibrated with pH 5.1 buffer. The tubes were then subjected to mechanical disruption for 1 min on a bead-beating device (FastPrep machine; Bio101, Holbrook, NY, USA). To purify the RNA molecules, additional extractions were performed with pH 5.1 buffer-equilibrated phenol, pH 5.1 buffer-equilibrated phenol/chloroform/isoamyl alcohol and chloroform, and the purified RNA were recovered by ethanol precipitation. After DNase treatment, shorter RNA fragments of approximately 500 or less bases (nt) were removed with a purification column (MicroSpin column S-400; GE Healthcare, Uppsala, Sweden).

For RNase H reaction, the hybridization mixture contained 1 μl of RNA template (approximately $1\,\mu g\,\mu l^{-1}$), 1 μl of each scissor probe solution ($10\,pmol\,\mu l^{-1}$), 2.5 μl of 10 × hybridization buffer (250 mM Tris-HCl, 10 mM EDTA, 250 mM NaCl), and a given amount of formamide (pH 7.5; Uyeno *et al.*, 2004). It was heated at 95 °C for 1 min to denature RNA molecules, and then incubated at 50 °C for 1 min. Cleavage reaction was initiated by adding 5 μl of 10 × enzyme mixture (200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 125 mM NaCl, 10 mM dithiothreitol, $300\,\mu g\,\mu l^{-1}$ bovine serum albumin, $5\,U\,\mu l^{-1}$ RNase H), and immediately incubated at 50 °C for 15 min. To terminate the digestion reaction, 25 μl of 3 × stop solution (30 mM EDTA, 0.9 M sodium acetate (pH 7.0)) was added to the mixture. The RNA mixture was then deproteinized by washing with acid phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The supernatant was collected by

Table 3 Oligonucleotide scissor probes for quantitative detection of methanogens

Probe name	*Target group*	*Probe sequence (5′–3′)*	*Probe length (mer)*	*Target site (*E. coli *position)*[a]	*16S rRNA used*[b]		*Optimal cleavage condition*		*Reference*
					Target organism	*Nontarget organism*	*Formamide (%)*	*Cleavage coefficient*	
ARC915	Domain *Archaea*	GTGCTCCCCCGCCAATTCCT	20	912–929	1, 2, 9, 10, 15, 20, 24, 26, 28, 32	33, 54	70	1	Stahl *et al.* (1988)
CMSMM1068m	Class *Methanomicrobia*	GGATGCTTCACAGTACGAAC	20	1068–1087	1–20	22, 24	35	1	Banning *et al.* (2005)
MSMX860m	Order *Methanosarcinales*	GCTCGCTTCACGGCTTCCCT	20	860–879	1–10	11, 16	45	1	Raskin *et al.* (1994b)
MG1200m	Order *Methanomicrobiales*	CCGGATAATTCGGGGCATGCTG	22	1200–1221	11–20	1, 9	40	0.96	Raskin *et al.* (1994b)
FMSC394	Family *Methanosarcinaceae*	ATGCTGGCACTCGGTGTCCC	20	394–417	1,2,4,5,7	13, 20	50	0.99	This study
MX825mix[c]	Family *Methanosaetaceae*	TCGCACCGTGGCYGACACCTAGC	23	825–845	9	4, 6	60	1	Raskin *et al.* (1994b); Crocetti *et al.* (2006)
MB1175m	Family *Methanobacteriaceae*	CCGTCGTCCACTCCTTCCTC	20	1175–1194	21–26	9, 17	60	1	Raskin *et al.* (1994b)
FMTH1183	Family *Methanothermus*	TACGGACCTACCGTCGCCCGCA	22	1183–1204	27	1, 4, 10, 32	65	0.97	This study
FMCMT1044	Family *Methanocaldococcaceae*, Family *Methanopyraceae*	GTCAACCTGGCCTTCATCCTGC	22	1044–1044	30–32	5, 28	50	0.89	This study
MS821m	Subfamily *Methanosarcinales*	GCCATGCCTGACACCTAGCG	20	824–841	1–3	5	55	0.99	Raskin *et al.* (1994b)
F2SC668	Subfamily *Methanomicrobiales* SC-2	TCCTACCCCCGAAGTACCCCTC	22	668–690	11, 12	9, 13	70	0.99	This study
F2SC732	Subfamily *Methanomicrobiales* SC-2	TCGAAGCCGTTCTGGTGAGGCG	22	732–753	11, 12	1, 9	50	0.99	This study
F3SC984	Subfamily *Methanomicrobiales* SC-3	CATATCGCTGTCCTACCCGG	20	984–1044	17	14	55	1	This study
F6SC393	Subfamily *Methanomicrobiales* SC-6	GACAGGCACTCAGGGTTTCC	20	393–420	18, 19	11	50	0.99	This study
F7SC1260	Subfamily *Methanomicrobiales* SC-7	TATCCTCACCTCTCGGTGTC	20	1260–1279	20	18	50	1	This study
SARCI551	Genus *Methanosarcina*	GACCCAATAATCACGATCAC	20	551–570	1, 2	7, 8, 9	35	0.93	Sorensen *et al.* (1997)
GMIB1254	Genus *Methanomicrococcus*	CACCTTTCGGTGTAGTTGCC	20	1254–1273	3	11, 19	45	0.97	This study
GMHB842	Genus *Methanohalobium*	TCGGCACTAGGAACGGCCGT	20	842–859	8	7	45	0.96	This study
GMSS261	Genus *Methanosalsum*	GTCGGCTAGCAGGTACCTTG	20	261–280	7	4	45	0.98	This study
GMCO441	Genus *Methanococcoides*	ACATGCCGTTTACACATGTG	20	441–492	4	5	40	0.81	This study
GMLB834	Genus *Methanolobus*	TGAAACGGTCGCACCGTCCCAG	22	834–851	6	5	70	0.84	This study
GMHP1258	Genus *Methanohalophilus*	CCGTCACTTTTCAGTGTAGG	20	1258–1277	5	24	30	0.96	This study
GMM829	Genus *Methanomicrobium*	CTCGTAGTTACAGGCACACC	20	829–845	15	13	40	1	Yanagita *et al.* (2000)
GMG1128	Genus *Methanogenium*	CGTTCCGGAGAACAAGCTAG	20	1128–1139	16	5, 13, 15	35	0.99	This study
GMCP489	Genus *Methanocorpusculum*	GCCCTGCCCTTTCTTCACAT	20	489–507	19	14, 16	60	1	This study
GMCL488	Genus *Methanocalculus*	CCCCGCCCTTTCTCCTGGTG	20	488–506	18	11, 17	60	0.99	This study
GMB406	Genus *Methanobrevibacter*	GCCATCCCGTTAAGAATGGC	20	406–436	21, 22	24, 25, 26	45	0.98	This study
GMBA755	Genus *Methanobacterium*	TGGCTTTCGTTACTCACC	18	755–772	24, 25	26, 27, 28	25	0.98	This study
GMSP838	Genus *Methanosphaera*	CCGGAACAACTCGAGGCCAT	20	838–853	23	24	45	1	This study
GMTB541	Genus *Methanothermobacter*	AAAAGCGGCTACCACTTGAGCT	22	541–562	26	21, 23, 25	55	0.90	This study
GMC728	Genus *Methanococcus*	ACCCGTTCCAGACAAGTGCCTT	22	728–749	28	29, 31	55	0.98	This study
GMC231	Genus *Methanococcus*	ACTACCTAATCGAGCGCAGTCC	22	231-252	28	31	30	0.83	This study
GMC416	Genus *Methanococcus*	TTGATAAAAGCCCATGCTGTGC	22	416–445	28	29	35	0.85	This study
GMTL416	Genus *Methanothermococcus*	TAGAAAAGCCTACGCAGTGC	20	416–443	29	31	30	0.96	This study
GMPK1331	Genus *Methanopyrus*	GGTTACTACCGATTCCACCTTC	22	1331–1352	32	29	35	0.99	This study
GTMS393m	Thermophilic *Methanosaeta* group	ACCCAGCACTCGAGGTCCCC	20	393–416	10	9	65	1	Zheng and Raskin (2000)
TMX745	Thermophilic *Methanosaeta* group	CCCTTGCCGTCGGATCCGTT	20	743-762	10	4, 9	65	0.97	This study
MMX1273	Mesophilic *Methanosaeta* group	GGTTTTAGGAGATTCCCGTC	20	1273–1292	9	7, 10	45	1	This study
SMCUT1253	*Methanoculleus thermophilus*	GCCTTTCGGCGTCGATACCC	20	1253–1272	12	16, 22	59	0.76	This study
SMPL623	*Methanoplanus limicola* *Methanoplanus endosynbiosus*	TTCTCTTAAACGCCTGCAGG	20	623–641	13	19	20	0.99	This study
SMPP1252	*Methanoplanus petrolearius* *Methanolacinia paynteri*	CTTCTCAGTGTCGTTGCTCA	20	1252–1273	14	13	40	1	This study
EUB338	Most of the domain *Bacteria*	GCTGCCTCCCGTAGGAGT	18	338–355	33-49, 51-53	9, 54	65	0.97	Amann *et al.* (1990)
EUB338III	phylum *Verrucomicrobia* class *Anaerolineae*	GCTGCCACCCGTAGGTGT	18	338–355	50	10	65	0.97	Daims *et al.* (1999)

[a]Position of 16S rRNA was referred as *Escherichia coli* numbering system designed based on Brosius *et al.* (1978).
[b]Abbreviated strain name are shown in Supplementary Table 1.
[c]MX825mix probe consists of equal parts of MX825 (Raskin *et al.*, 1994a, b) and MX825c (Crocetti *et al.* (2006)) probe.

centrifugation, and the rRNA was precipitated through ethanol wash and dissolved in 4 μl of diethylpyrocarbonate-treated water. Furthermore, the efficiency and optimal conditions of thermostable RNase H from *Thermus thermophilus* (TOYOBO, Osaka, Japan) in the sequence-specific cleavage reaction was evaluated by using a perfect matched probe (327-18, 5′-TGTCTCAGTTCCAGTGTG-3′; Uyeno *et al.*, 2004) and single-base mismatched probe (327-18M9a, 5′-TGTCTCAGTACCAGTGTG-3′; Uyeno *et al.*, 2004) targeting *E. coli* whole RNA.

The quantity and integrity of intact and digested 16S rRNA fragments were evaluated using the Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) with RNA 6000 Nano kit (Agilent) according to the manufacturer's instructions. The percentage of digested 16S rRNA was calculated using the following equation: percentage of digested 16S rRNA in the total 16S rRNAs $= (a+b)/(a+b+c) \times 100$, where a and b are the respective peak areas of cleaved 16S rRNAs derived from the cleavage, and c is the peak area of the intact (that is, uncleaved) 16S rRNA. The percentage was converted to the 16S rRNA population of the target group in total 16S rRNAs by the following calculation: (the 16S rRNA population of the target group) = (the percentage of cleavage 16S rRNA)/(cleavage coefficient of the scissor probe). Each cleavage experiment was performed in duplicate.

Construction of archaeal 16S rRNA genes clone library and phylogeny analysis

Community DNA of sludge samples was extracted as described previously (Yamada *et al.*, 2005). For clone library construction, archaeal 16S rRNA gene fragments were PCR amplified from the extracted DNA using forward primer ARC109f (Grosskopf *et al.*, 1998) and reverse primer 1492r (Lane, 1991; Weisburg *et al.*, 1991) under the following thermal program: preheating (95 °C, 9 min), 20 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 30 s) and extension (72 °C, 2 min), and a final postextension (72 °C, 10 min). PCR amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and cloned using a TA cloning kit (Novagen, Madison, WI, USA). Cloned 16S rRNA genes were sequenced with a Quick start kit (Beckman Coulter, Fullerton, CA, USA) and analyzed with a CEQ 2000XL automated sequence analyzer (Beckman Coulter).

Distance matrix trees based on 16S rRNA gene sequences of more than 1000 nucleotides were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the ARB software package. Insertions of shorter length sequences were performed with the parsimony insertion tool of the ARB program package. The topology of the trees was evaluated by the bootstrap analysis (1000 replicates; Felsenstein, 1985) with the PAUP* 4.0 program package (Swofford, 2002). For 16S rRNA gene sequences found in the libraries examined, sequences exhibiting more than 97% similarities were grouped into one phylotype, and the representative sequence of each phylotype was used as an operational taxonomic unit (OTU).

The 16S rRNA gene sequences obtained here were deposited under DDBJ/EMBL/GenBank accession numbers AB266892 to AB266895 and AB266917 to AB266919.

Results

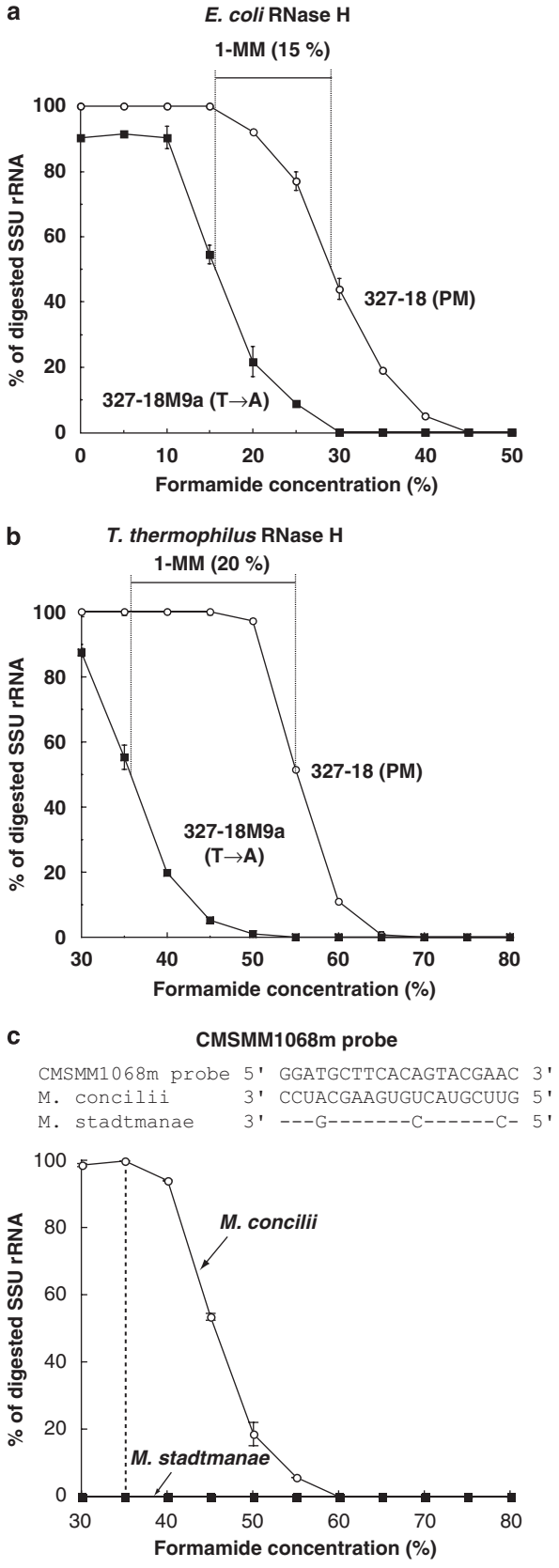
Development of hierarchical group-specific probes

To determine the abundance of methanogens using RNase H analysis, 40 16S rRNA-targeted oligonucleotide probes at different phylogeny specificities of class, order, family, genus and species were developed (Figure 1). Among them, 31 probes were newly designed with at least two or more mismatches (MMs) against nontargeted sequences, and 9 were adapted or modified from previously studies by shifting target position or by changing probe length to increase the specificity and probe binding affinity of RNA cleavage. To detect aceticlastic methanogens, probe SARCI551 (for the genus *Methanosarcina*) and probe MX825mix (for the family *Methanosaetaceae*) were used (Figure 1). Probe MX825mix has complementary sequence to 16S rRNA of *Methanosaeta thermophila*, a thermophilic aceticlastic methanogen, but, possibly due to the effect of the tertiary structure of 16S rRNA of *M. thermophila* on the probe accessibility during hybridization/digestion reaction, this probe showed a low cleavage coefficient of 0.26 with synthesized *M. thermophila* 16S rRNA. To better estimate the abundance of thermophilic *Methanosaeta* group, probes TMX745 and GTMS393m (Zheng and Raskin, 2000) were designed. To detect mesophilic members of the *Methanosaetaceae*, probe MMX1273 was used. Furthermore, four probes (CMSMM1068m, MSMX860m, FMSC394 and MS821m) with broader specificities were used to target aceticlastic methanogens together with hydrogenotrophic methanogens. The remaining probes mainly targeted the hydrogenotrophic methanogens, including example *Methanoculleus thermophilicus* (SMCUT1253), *Methanothermobacter* (GMTB541), *Methanothermaceae* (FMTH1183), *Methanothermococcus thermolithotrophicus* (GMTL416), *Methanocaldococcus*, *Methanotorris* and *Methanopyrus* (FMCMT1044). Table 3 provides detailed information on the nucleotide sequence, and targeted position of individual probes.

Optimization of RNase H reaction with thermostable RNase H

Figure 2 shows the improvement of RNAse cleavage reaction. The use of thermostable RNase H to improve single-base mismatch discrimination was evaluated by using two *E. coli*-targeted probes with

perfect-matched or single-mismatched sequences against targeted *E. coli* 16S rRNA sequence. Results showed that the single-base mismatch discrimination at 50% of cleavage efficiency was improved from a 15% disparity of formamide concentration with RNase H (Figure 2a) to a 20% disparity with thermostable RNase H (Figure 2b), suggesting that the use of thermostable RNase H for cleavage could increase the optimum formamide concentration for each probe (that is, probes can hybridize under a more stringent condition). Therefore, thermostable RNase H was used in this study hereafter for the determination and optimization of the specificities of all the probes used in the RNase H reaction. For example, CMSMM1068m probe could completely cleave the 16S rRNA of *Methanosaeta concilii* 16S rRNA (cleavage coefficient = 1.0) at a formamide concentration of 35%, and did not cleave the 16S rRNA of *Methanosphaera stadtmanae* at a formamide concentration between 30% and 80% (Figure 2c) or related nontarget 16S RNAs at 35% formamide concentration. Thus, an optimum formamide concentration of 35% for probe CMSMM1068m was chosen. The dissociation curves obtained for three other probes MG1200m, MX825mix and F2SC668 were shown in Supplementary Figure 1. Using the same criteria described above, the specificities of all probes were evaluated, and their optimum formamide concentrations and their cleavage coefficients determined (Table 3).



Quantitative detection of microbial populations in anaerobic digestion processes

Among those six different sludge samples determined, the relative abundances of 16S rRNA from members of the domains *Bacteria* and *Archaea* were shown in Table 4. It ranged from 26% to 74% for *Bacteria* and 28.1–78.9% for *Archaea*. The total abundance of bacterial and archaeal populations as determined by probes EUB338, EUB338III and ARC915 for all sludge samples varied from 68% to 108%. The highest bacterial population was observed with reactor 1 (74%), where sludge bulking was frequently reported. Reactor 1 also had the lowest abundance of archaeal populations. For reactors 2 and 5, showing low COD removal rates, the abundances of the domain *Bacteria* were relatively low, <30% of the total SSU rRNA.

Figure 2 Dissociation curves of scissor probes under different formamide conditions. Percentages of digested 16S rRNA in the total 16S rRNAs were calculated based on the peak area of electropherograms of 16S rRNA fragments. All experiments were run in duplicate and error bar showed standard deviations. Effect of single-base mismatches between oligonucleotides and *E. coli* 16S rRNA on the rRNA scission reaction with RNase H from *Escherichia coli* (**a**) and with thermostable RNase H from *Thermus thermophilus* (**b**). The one-base mismatch discrimination at 50% of cleavage efficiency was indicated in the figures. (**c**) Dissociation curves of probe CMSMM1068m for the class *Methanomicrobia*, and the vertical dotted lines show the optimum formamide concentration.

Table 4 Comparative microbial community analysis of anaerobic processes by using sequence-specific cleavage of SSU rRNA

Target group	*Probe name*	*Target SSU rRNA population (%) at reactor no.*						*No. of clones retrieved from reactor no.*[a]	
		1	*2*	*3*	*4*	*5*	*6*	*1*	*2*
Domain *Bacteria*	EUB338	70.2 ± 2.6	23.8 ± 4.6	50.9 ± 3.7	31.5 ± 1.2	29.4 ± 2.8	42.0 ± 0.2		
	EUB338III	3.3 ± 0.5	2.3 ± 0.3	ND[b]	2.2 ± 1.1	ND	9.3 ± 0.3		
Domain *Archaea*	ARC915	28.1 ± 0.9	42.0 ± 3.2	57.0 ± 1.4	70.9 ± 0.3	78.9 ± 0.9	33.9 ± 3.5	49 (100)	75 (100)
Class *Methanomicrobia*	CMSMM1068m	19.4 ± 1.9	33.1 ± 2.4	38.4 ± 0.4	59.3 ± 1.6	69.2 ± 0.2	22.3 ± 2.0	25 (51)	53 (70.1)
Order *Methanosarcinales*	MSMX860m	15.4 ± 2.1	32.8 ± 1.0	39.2 ± 1.3	55.8 ± 1.4	69.0 ± 0.5	15.6 ± 1.0	25 (51)	53 (70.1)
Family *Methanosarcinaceae*	FMSC394m	ND	ND	39.9 ± 0.2	ND	ND	ND	0 (0)	0 (0)
Subfamily *Methanosarcinaceae*	MS821m	ND	ND	40.1 ± 5.2	ND	ND	ND	0 (0)	0 (0)
Genus *Methanosarcina*	SARCI551	ND	ND	41.7 ± 3.1	ND	ND	ND	0 (0)	0 (0)
Family *Methanosaetaceae*	MX825mix	9.2 ± 0.2	33.5 ± 0.9	ND	54.8 ± 1.2	64.4 ± 0.0	16.6 ± 0.7	25 (51)	53 (70.1)
Mesophilic *Methanosaeta* group	MMX1273m	5.7 ± 1.7	23.9 ± 0.9	ND	48.5 ± 0.8	41.8 ± 3.4	12.1 ± 0.6	12 (24.5)	53 (70.1)
Order *Methanomicrobiales*	MG1200m	2.9 ± 0.3	17.2 ± 0.6	ND	5.0 ± 0.1	ND	10.3 ± 1.5	9 (18.4)	11 (14.7)
Methanoplanus limicola	SMPL623	ND	ND	ND	2.0 ± 0.1	ND	ND	0 (0)	0 (0)
Family *Methanobacteriaceae*	MB1175m	2.0 ± 0.0	ND	1.8 ± 0.0	12.7 ± 0.3	10.3 ± 2.0	ND	15 (30.6)	11 (14.7)
Genus *Methanobrevibacter*	GMB406	1.2 ± 0.6	ND	ND	ND	ND	ND	0 (0)	0 (0)
Genus *Methanobacterium*	GMBA755	ND	ND	ND	12.7 ± 0.5	9.8 ± 0.5	ND	15 (30.6)	11 (14.7)
Genus *Methanothermobacter*	GMTB541	ND	ND	2.0 ± 0.0	ND	ND	ND	0 (0)	0 (0)
Family *Methanothermaceae*	FMTH1183	ND	ND	4.7 ± 0.4	ND	ND	ND	0 (0)	0 (0)
Family *Methanocaldococcaceae*	FMCMT1044	ND	ND	4.8 ± 0.6	ND	ND	ND	0 (0)	0 (0)

[a]The number in parentheses shows the percentage of respective clones in the total clones examined.
[b]Not detected (below detection limit (<1% of the total SSU rRNA)).

Table 4 further lists the abundance of methanogens at different phylogeny specificities within those six anaerobic sludge systems. It was observed that only 15 out of those 40 probes gave positive cleavage reactions with the extracted rRNA. Within the class *Methanomicrobia* (targeted by CMSMM1068m probe), members of the order *Methanosarcinales* (MSMX860m) were detected in all the reactors, and members of the order *Methanomicrobiales* (MG1200m) were detected only in four mesophilic processes (reactors 1, 2, 4 and 6). Within the order *Methanosarcinales*, members of the family *Methanosaetaceae* (MX825mix), especially mesophilic *Methanosaeta* (MMX1273), were detected as a major aceticlastic methanogen in the mesophilic processes (reactors 1, 2, 4, 5 and 6). However, it was noted that only 61–89% of the archaeal populations in the family *Methanosaetaceae* (MX825mix) were detected by probe MMX1273, suggesting other yet-to-be-defined methanogens in the family *Methanosaetaceae* in anaerobic treatment processes. It was further noted that high abundance of the genus *Methanosarcina* (SARCI551) in the subfamily *Methanosarcinaceae* (MS821m) was detected only in reactor 3 operated under thermophilic conditions.

Within the order *Methanobacteriales*, members of the family *Methanobacteriaceae* (MB1175m) were found in both mesophilic and thermophilic processes. They were mainly represented by methanogens in the genus *Methanobrevibacter* (GMB406) in reactor 1, the genus *Methanothermobacter* (GMTB541) in reactor 3 or the genus *Methanobacterium* (GMBA755) in reactors 4 and 5. For members of the families *Methanothermaceae* and *Methanocaldococcaceae*, they represented hyperthermophilic hydrogenotrophic methanogens, and were detected only in reactor 3 operated under thermophilic conditions.

It was further observed in reactors 4 and 5 that the summation of the 16S rRNA abundances estimated by the probes targeting all lower rank taxa in the domain *Archaea* closely accounted for the archaeal populations determined by probe ARC915, 101–102%. In contrast, for samples taken from reactors 1, 2, 3 and 6, the archaeal populations detected by lower rank probes only accounted for 66–87% of ARC915 detectable archaeal populations. The observations suggested that other methanogenic and/or nonmethanogenic archaea that could not be detected by those probes developed in this study were present in these processes.

16S rRNA gene clone library

To validate the results of RNase H method, we subsequently constructed archaeal 16S rRNA gene clone library for reactors 1 and 2. The relative abundances of 16S rRNA gene clones were shown in Table 4, and a neighbor-joining phylogeny tree comprising of these clones were shown in Figure 3. For aceticlastic methanogens, clones closely related to *Methanosaeta harundinacea* (clone SwA12, 27% of total clones) and *M. concilii* (clone DtA103, 68% of total clones) were predominant in reactors 1 and 2, respectively. For hydrogenotrophic methanogens, clones related to uncultured clones were frequently observed in both reactors. For example, clones SwA77 and DtA4 were closely related to uncultured anaerobic sludge clone (AY426480), and estimated to be 31% and 6.7%, respectively.

The clone library results in general closely agreed with the rRNA cleavage results. The *Methanosaetaceae* populations, accounting for 51–70% of the total clones retrieved, were similar to 33–80% of the archaeal rRNA as determined by RNase H method using a *Methanosaetaceae*-specific probe (MX825mix). There were however differences in the populations between two approaches, especially in the detection of *Methanobacteriaceae* and *Methanomicrobiales* population. We could not detect cleaved rRNA signals from *Methanobacterium* (GMBA775) using RNase H method, but observed that 15–31% of the total 16S rRNA clones were assigned to this genus. In addition, *Methanomicrobiales* members in reactor 2 accounted for 41% of the archaeal population based on RNase H method, but 16S rRNA gene clone library showed a lower abundance (14.7%).

Discussion

This study has successfully demonstrated the use of sequence-specific cleavage method to quantitatively detect the rRNA level of different methanogenic populations in anaerobic digestion processes. In comparison to the currently widely used molecular tools such as FISH, quantitative PCR and membrane hybridization which are relatively laborious and time consuming, the RNase H method can rapidly detect targeting microorganisms at different phylogeny levels based on the abundance of rRNA. This method can detect more than 20 different methanogens at different taxonomic level within 3–4 h. The results show that bacterial and archaeal populations represented 26.1–73.5% and 28.1–78.9%, respectively, of total rRNA extracted from those six anaerobic treatment processes. These observations are in close agreement with the results obtained on microbial community analysis of anaerobic wastewater treatment processes through the uses of membrane hybridization (Raskin *et al.*, 1994a; Zheng and Raskin, 2000) and FISH (Sekiguchi *et al.*, 1998), and from our preliminary RNase H-based analyses (Uyeno *et al.*, 2004; Sekiguchi *et al.*, 2005).

Using the probe set developed, we could detect most of the methanogens in those anaerobic treatment systems down to the genus level. For aceticlastic methanogens, members of the family *Methanosaetaceae*, especially the mesophilic

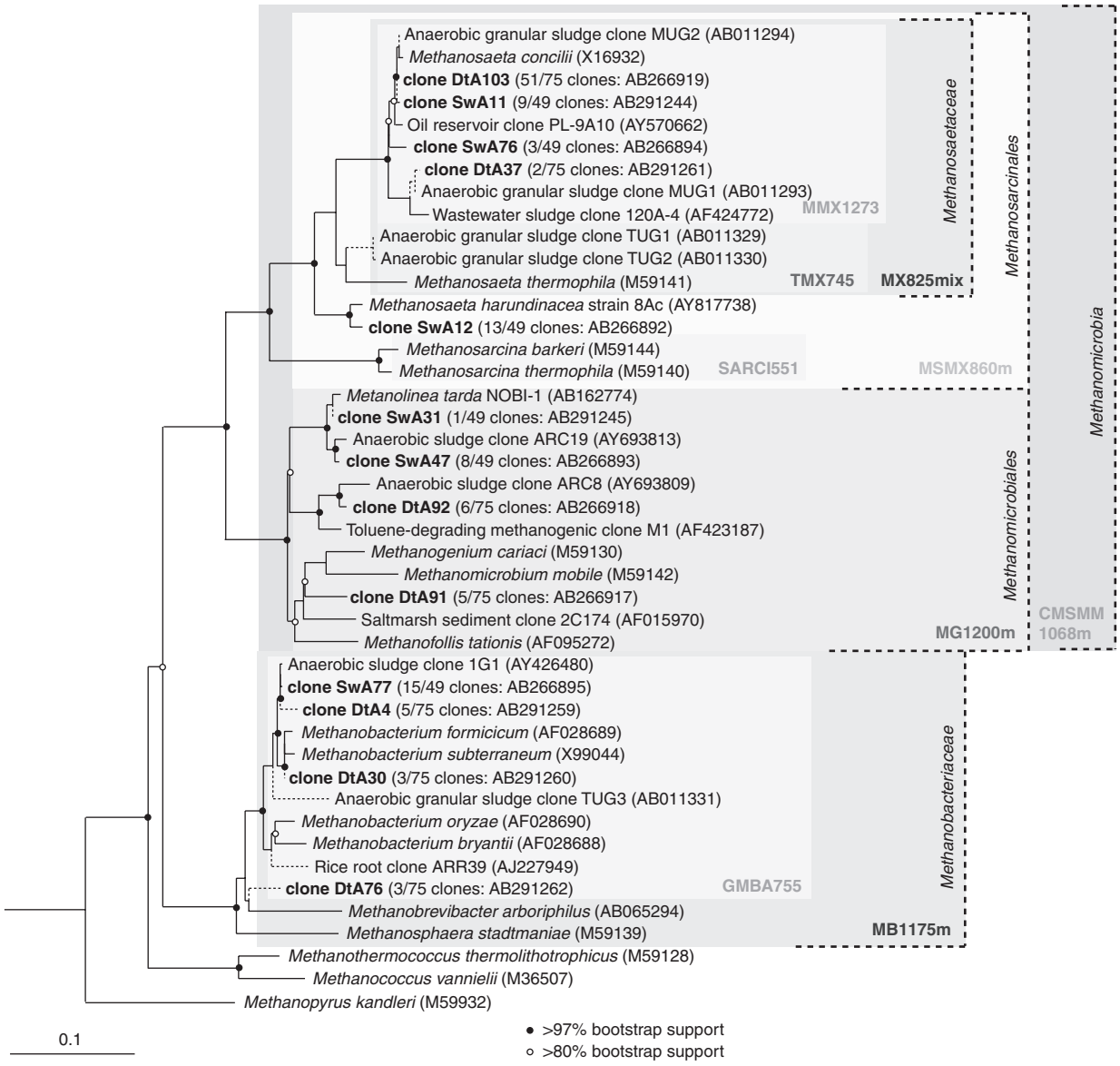


Figure 3 Distance matrix tree of archaeal 16S rRNA gene clones retrieved from the reactors 1 and 2 based on neighbor-joining analysis. The sequence of *Desulfurococcus mobilis* was used as an outgroup to root the tree. The bar indicates 10% base substitution. Distance matrix tree was initially constructed with 16S rRNA gene sequences with more than 1000 nucleotides. Shorter length sequences (<1000 nt) were inserted into the tree with the parsimony insertion tool of the ARB program (dashed line). Boldface indicates the clones obtained in this study, and those labeled with SwA and DtA were obtained from the reactor 1 and 2, respectively. The symbols at nodes indicate bootstrap values with 1000 resampling analysis.

Methanosaeta group were observed to be predominant, independent on their reactor types and wastewater composition, accounting for 20–68% of the archaeal population in the reactors operated under mesophilic conditions (reactors 1, 2, 4, 5, and 6; Table 4). This observation was supported by the 16S rRNA gene clone library results, revealing that 51% and 70% of archaeal 16S rRNA gene clones were affiliated to the family *Methanosaetaceae* in the reactors 1 and 2, respectively (Table 4), and by previous studies using molecular-based approaches (Raskin *et al.*, 1994a; Sekiguchi *et al.*, 1998; Zheng and Raskin, 2000; Mchugh *et al.*, 2003; McMahon *et al.*, 2004; Yu *et al.*, 2006). In contrast, in reactor 3 operated at relatively high temperatures (50–55 °C), members of the genus *Methanosarcina* were predominant. This difference in the dominance of different aceticlastic methanogens is obviously operational temperature dependent (mesophilic vs thermophilic). The kinetics of acetate utilization could also be relevant as *Methanosarcina* spp. can outcompete *Methanosaeta* spp. under high acetate concentrations (Min and Zinder, 1989). In reactor 3, acetate concentration of influent was relatively high (3000 mg-COD l^{-1}).

It was further noted that members of the family *Methanosaetaceae* could not be fully covered by probes MMX1273 and TMX745 (Table 4). This

suggested the presence of other, possibly novel, methanogens that are not covered by those probes developed in this study. For example, probe MMX1273 could not target *M. harundinacea* (single MM in targeted 16S rRNA sequence) and related clones (Figure 3; accession no. AB266892), which were recently isolated or obtained from UASB process treating beer-processing wastewater (Ma *et al.*, 2006). Hence, further improvement of scissor probes for *Methanosaetaceae* group is necessary to better quantify their abundance in anaerobic environments.

For hydrogenotrophic and other substrate-oxidizing methanogens (for example, formate and alcohols), their abundances could be clearly differentiated among those reactors studied. In mesophilic reactors 1, 2, 4 and 6, the presence of *Methanomicrobiales*-related methanogens (MG1200m) was observed (Table 4), but their detailed phylogenetic affiliations within this family could not be properly identified using subfamily-, genus- and species-specific probes (Table 3), because major members of the order *Methanomicrobiales* in reactors 1 and 2 were related to uncultured environmental clones and a novel methanogen (*Methanoline tarda*) isolated and characterized very recently (Imachi *et al.*, 2008) (Figure 3). Thus, additional scissor probes are needed to account for the presence of these yet-to-be-identified taxa.

Hydrogenotrophic methanogens from the order *Methanobacteriaceae* (MB1175 m) were also detected in four of the six reactors (that is, 1, 3, 4 and 5; Table 4). In reactor 1, approximately half of the detectable methanogens was affiliated with the genus *Methanobrevibacter* commonly observed in mesophilic methanogenic bioprocesses treating several types of wastewater (Grotenhuis *et al.*, 1991; Wu *et al.*, 1991; Harmsen *et al.*, 1996; Zellner *et al.*, 1997). It was reported that the relative abundances of *Methanobrevibacter*-related methanogens represented $<1.5\%$ of total microbial populations during the start-up operation of a UASB process (Zheng *et al.*, 2006). However, 16S rRNA gene clones related to this taxon were seldom retrieved from anaerobic treatment processes (Ariesyady *et al.*, 2007) in this study (Figure 3). This suggested that members of the genus *Methanobrevibacter* can participate in hydrogenotrophic methanogenesis even they are present at lower abundance. In thermophilic reactor 3, members of *Methanothermobacter* were detected at low abundance (3.5% of the total archaeal RNA). They were reported as the dominant populations in thermophilic anaerobic processes (Sekiguchi *et al.*, 1998; Chen *et al.*, 2008) and could play an important role in secondary fermentation with thermophilic syntrophic bacterium *Pelotomaculum thermopropionicum* (Imachi *et al.*, 2000). Likewise, high abundant of the *Methanobacterium* (12–18% of total archaeal RNA) were observed in mesophilic reactors 4 and 5. In reactors 1 and 2, members of *Methanobacterium* spp. (GMBA775) were not detected by RNase H method, however, numerous numbers of 16S rRNA gene clones from this taxon were obtained. This discrepancy indicated that *Methanobacterium*-related species were not as active as other hydrogenotrophic methanogens in the reactors.

We further detected high abundance of hyperthermophilic methanogens (the families *Methanothermaceae* and *Methanocaldococcaceae*) in thermophilic UASB reactor 3, and a fraction of *Methanoplanus* group in the mesophilic reactor 4. To our knowledge, no study has observed these methanogens in anaerobic wastewater treatment processes (Sekiguchi and Kamagata, 2004). This observation demonstrated the ability of the RNase H method to discover new insights into the diversity of methanogenic archaea in anaerobic wastewater treatment process. Further studies using cultivation- and molecular-based approaches are needed to determine the *in-situ* ecophysiological traits of these hyperthermophilic methanogens in anaerobic processes.

We further noted that the abundances of the domain *Bacteria* were relatively low (below 30% of the total SSU rRNA) in reactors 2 and 5 (Table 4) with low COD removal rates (Table 2). The decrease in the primary and secondary fermentative bacteria could likely lead to unstable operation or poor treatment efficiency with these processes. On the other hand, a high proportion of the domain *Bacteria* was observed in reactor 1 treating sugar-processing wastewater (Table 4). We previously reported that an anaerobic filamentous bacterium assigned to uncultured clone cluster KSB3 could trigger sludge bulking by populating on the outermost layer of the granules and may be involved in primary fermentation of carbohydrates in this process (Yamada *et al.*, 2007). Our preliminary results based on the RNase H method indicate that the population of the KSB3-related organism is more than 30% of total RNA in this process, and unexpected outgrowth of this microorganism seems to be involved in anaerobic sludge bulking phenomenon (T Yamada *et al.*, unpublished data). Thus, further studies using scissor probes for members of the domain *Bacteria,* which play an important role in primary and secondary fermentation steps and in-process failure, will be required to determine the microbial function of anaerobic ecosystems.

Lastly, we occasionally observed that the total abundance of those methanogens detected by lower rank probes at genus level could not fully account for the total abundance of methanogens detected at higher taxonomic levels. The possibility that the presence of other archaeal populations or methanogens that were not covered by those probes developed in this study was confirmed by the construction of archaeal 16S rRNA gene clone libraries for the sludge samples taken from reactors 1 and 2. Approximately 51% and 21% of the clones analyzed for reactors 1 and 2, respectively, were

closely related to uncultured archaeal 16S rRNA gene clones that were not targeted by our probe set (Figure 3). In addition to these uncultured methanogen-like groups, it is known that clones assigned to the candidate taxon WSA2 of the *Euryarchaeota* and the subphylum C2 of the *Crenarchaeota* have been retrieved from some methanogenic sludges in abundance (Chouari *et al.*, 2005; Collins *et al.*, 2005), although the archaeal rRNA gene cloning analysis for the sludges in this study showed no detection of such phylotypes. Thus there is a further need to develop additional scissor probes to cover these uncultured *Archaea* to better determine the abundance of the archaeal populations in natural and engineered environments in the future. This may be done by designing scissor probes based on rRNA gene sequences of uncultured archaeal phylotypes that are frequently retrieved from such ecosystems.

Conclusions

The single-base mismatch discrimination of sequence-specific cleavage method was improved with the use of thermostable RNase H, and successfully demonstrated to rapidly quantify methanogens in anaerobic bioprocesses. The overall results revealed that the dynamics of culturable aceticlastic and hydrogenotrophic methanogens at different taxonomic levels in anaerobic treatment processes are closely related to the process temperature and COD source. As the present probe set was mainly developed for known methanogen isolates, more probes should be designed and included in the RNase H method to fully detect certain yet-to-be-cultured methanogens in anaerobic processes. By further incorporating the quantitative detection of the domain *Bacteria*, including primary and secondary fermenting bacteria, with methanogens, RNase H method can allow to better manage process operation and achieve better removal efficiency in a long run.

Acknowledgements

This study was supported by the Project 'Development of Technologies for Analyzing and Controlling the Mechanism of Biodegrading and Processing', which was ensured to the New Energy and Industrial Technology Development Organization (NEDO). This work was also supported by the Global Environment Research Fund (RF-076) of the Ministry of the Environment, Japan.

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