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Minireview

Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea

Takashi Narihiro[1] and Yuji Sekiguchi[2]*

[1]*International Patent Organism Depositary (IPOD) and* [2]*Bio-medical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan.*

Summary

For the identification and quantification of methanogenic archaea (methanogens) in environmental samples, various oligonucleotide probes/primers targeting phylogenetic markers of methanogens, such as 16S rRNA, 16S rRNA gene and the gene for the α-subunit of methyl coenzyme M reductase (*mcrA*), have been extensively developed and characterized experimentally. These oligonucleotides were designed to resolve different groups of methanogens at different taxonomic levels, and have been widely used as hybridization probes or polymerase chain reaction primers for membrane hybridization, fluorescence *in situ* hybridization, rRNA cleavage method, gene cloning, DNA microarray and quantitative polymerase chain reaction for studies in environmental and determinative microbiology. In this review, we present a comprehensive list of such oligonucleotide probes/primers, which enable us to determine methanogen populations in an environment quantitatively and hierarchically, with examples of the practical applications of the probes and primers.

Introduction

Methanogenic archaea (methanogens) are strictly anaerobic microorganisms producing methane as a result of their anaerobic respiration (Schink, 1997; Thauer, 1998). For methanogenesis, they can utilize a limited number of substrates such as carbon dioxide, acetate and methyl-group-containing compounds under anoxic conditions (Liu and Whitman, 2008). Most of the known methanogens are hydrogenotrophs reducing carbon dioxide to form methane; among them, formate is also often utilized as the electron donor instead of hydrogen. Some of the hydrogenotrophic methanogens can also utilize secondary alcohols such as 2-propanol as the electron donor. Acetate is an important intermediate substance in the anaerobic decomposition of organic matter, and is generally exclusively utilized by limited groups of methanogens to form methane under anoxic conditions, where external electron acceptors other than carbon dioxide are unavailable. Methyl-group-containing compounds, such as methanol and methylamines, are also utilized by some methanogens through disproportionation of methyl groups.

Methanogens are frequently found in anoxic environments, such as rice paddy fields (Iino *et al*., 2010; Sakai *et al*., 2010), wetlands (Cadillo-Quiroz *et al*., 2009; Bräuer *et al*., 2010), permafrost (Krivushin *et al*., 2010; Shcherbakova *et al*., 2010), landfills (Laloui-Carpentier *et al*., 2006), subsurfaces (Doerfert *et al*., 2009; Mochimaru *et al*., 2009) and ruminants (Frey *et al*., 2009), which are known to be the major sources of atmospheric methane. It has been estimated that the annual global emission of methane is 500–600 Tg, and atmospheric methane concentration has risen threefold over the past 200 years (Liu and Whitman, 2008). With the increased interests in global climate change and environmental issues, studies on the diversity and ecophysiological functions of methanogens in such environments have been extensively conducted using cultivation-dependent and cultivation-independent approaches (Liu and Whitman, 2008). In addition to such environments, methanogens play key roles in fields of anaerobic digestion technology, which is widely used as a means for treating municipal and industrial waste/wastewater containing high levels of organic compounds (Sekiguchi, 2006; Narihiro and Sekiguchi, 2007; Talbot *et al*., 2008; Tabatabaei *et al*., 2010). Methanogens are often critical components of such bioconversion systems, resulting in the recovery of

Received 6 July, 2010; accepted 12 November, 2010. *For correspondence. E-mail y.sekiguchi@aist.go.jp; Tel. (+81) 29 861 7866; Fax (+81) 29 861 6400.

gaseous methane from those wastes as reusable energy resource. To better manage the bioconversion systems and achieve a higher efficiency in removing organic compounds in wastes, methanogens in these systems have been extensively studied and the quantitative monitoring of such methanogenic populations in these systems has been conducted (Narihiro and Sekiguchi, 2007).

To explore the ecological significance of methanogens in these natural and engineered ecosystems, identification and quantification techniques for different methanogen groups are indispensable. For the purpose, analyses of membrane lipid (Weijers *et al*., 2004; Strapoc *et al*., 2008), autofluorescence (Neu *et al*., 2002; Tung *et al*., 2005; Mochimaru *et al*., 2007), activity measurement (Lehmann-Richter *et al*., 1999; Weijers *et al*., 2004) and immunoenzymatic profiling (Visser *et al*., 1991; Sorensen and Ahring, 1997) have been used. In addition to these methods, cultivation-independent, nucleic acid-based analysis by using oligonucleotide probe/primers, such as membrane hybridization, fluorescence *in situ* hybridization (FISH), gene cloning, quantitative polymerase chain reaction (qPCR), and cleavage method with ribonuclease H (RNase H) were most widely and frequently used as means to detect and quantify methanogens more specifically and accurately. In this review, we present a catalogue of previously developed oligonucleotide probes/primers targeting genes of methanogens. Particular emphasis is placed on the probes/primers for 16S rRNA, 16S rRNA gene and the gene for the α-subunit of methyl coenzyme M reductase (*mcrA*), which are generally used for the taxonomic classification of methanogens (Friedrich, 2005; Liu and Whitman, 2008).

Phylogeny of methanogens

All the methanogens isolated and characterized to date have been classified into the phylum *Euryarchaeota* of the domain *Archaea* (Garrity *et al*., 2007). They are assigned into 33 genera of the classes '*Methanomicrobia*', *Methanobacteria*, *Methanococci* and *Methanopyri* (Fig. 1, Table 1). The class '*Methanomicrobia*' is the most phylogenetically and physiologically diverse group of methanogens consisting of three orders (*Methanosarcinales*, *Methanomicrobiales* and *Methanocellales*); 23 genera belonging to seven families (Fig. 1, Table 1). Within the order *Methanosarcinales*, the genera *Methanosarcina* and *Methanosaeta* are known to play a key role in the conversion of acetate into methane in various anaerobic environments, and the rest are known to metabolize relatively broad ranges of substrates, such as hydrogen, methanol and methylamines (Garrity and Holt, 2001). Known members of the order *Methanomicrobiales* are all hydrogenotrophs, and some of them are often observed in anaerobic environments as important hydrogen scavengers (Liu and Whitman, 2008). Members of the class *Methanobacteria*, consisting of the families *Methanobacteriaceae* and *Methanothermaceae*, are recognized as important hydrogenotrophs that have also been widely found in anaerobic ecosystems (Garrity and Holt, 2001). *Methanobacteriaceae* comprises four genera, *Methanobacterium*, *Methanosphaera*, *Methanobrevibacter* and *Methanothermobacter*. The class *Methanococci* includes the families *Methanococcaceae* and *Methanocaldococcaceae*, which are widely distributed in natural ecosystems such as marine sediments and deep sea geothermal sediments (Liu and Whitman, 2008). The class *Methanopyri* consists of solely the genus *Methanopyrus*, a hyperthermophilic, hydrogenotrophic methanogen isolated from the deep-sea hydrothermal field (Takai *et al*., 2008).

The isolation and characterization of novel methanogens from various ecosystems are ongoing, and the descriptions of such methanogens have been carried out at an encouraging rate. Recently, hydrogenotrophic methanogens, which are novel at high taxonomic levels (*Methanocella paludicola* and *Methanocella arvoryzae*), have been isolated, and the novel order *Methanocellales* was proposed (Sakai *et al*., 2008; 2010). These methanogens have long been considered as the uncultivable methanogen group (Rice cluster I), and responsible for the major part of methanogenesis in rice paddy soil (Conrad *et al*., 2006). In addition, novel hydrogenotrophic methanogens associated with previously uncultivated phylogenetic groups of the order *Methanomicrobiales* (formerly known as E1/E2 or Fen cluster) were isolated from anaerobic bioreactors (Imachi *et al*., 2008; Yashiro *et al*., 2009) and wetlands (Cadillo-Quiroz *et al*., 2009; Bräuer *et al*., 2010). Novel strains of the genera *Methanofollis* (Imachi *et al*., 2009), *Methanolobus* (Doerfert *et al*., 2009; Mochimaru *et al*., 2009), *Methanospirillum* (Iino *et al*., 2010) and *Methanobacterium* (Krivushin *et al*., 2010; Shcherbakova *et al*., 2010) have also been reported recently.

Despite these efforts in cultivating as yet uncultivable methanogens present in environments, there are still a vast number of uncultivable archaeal taxa that may have similar metabolic functions as those of known methanogens. For example, 16S rRNA gene types assigned into the WSA2 (or ArcI) group were frequently retrieved from methanogenic waste/wastewater treatment systems (Sekiguchi and Kamagata, 2004; Chouari *et al*., 2005). The WSA2 group is considered to be an archaeal taxon at the class level with no cultured representatives (Hugenholtz, 2002). However, Chouari and colleagues have found that WSA2-related cells can be enriched using formate- or hydrogen-containing culture media, suggesting that they harbour methanogenic activity (Chouari *et al*., 2005). Another example similar to the Rice Cluster I group is Rice Cluster II (RC-II). Members of the RC-II group were also considered to be methanogens, because

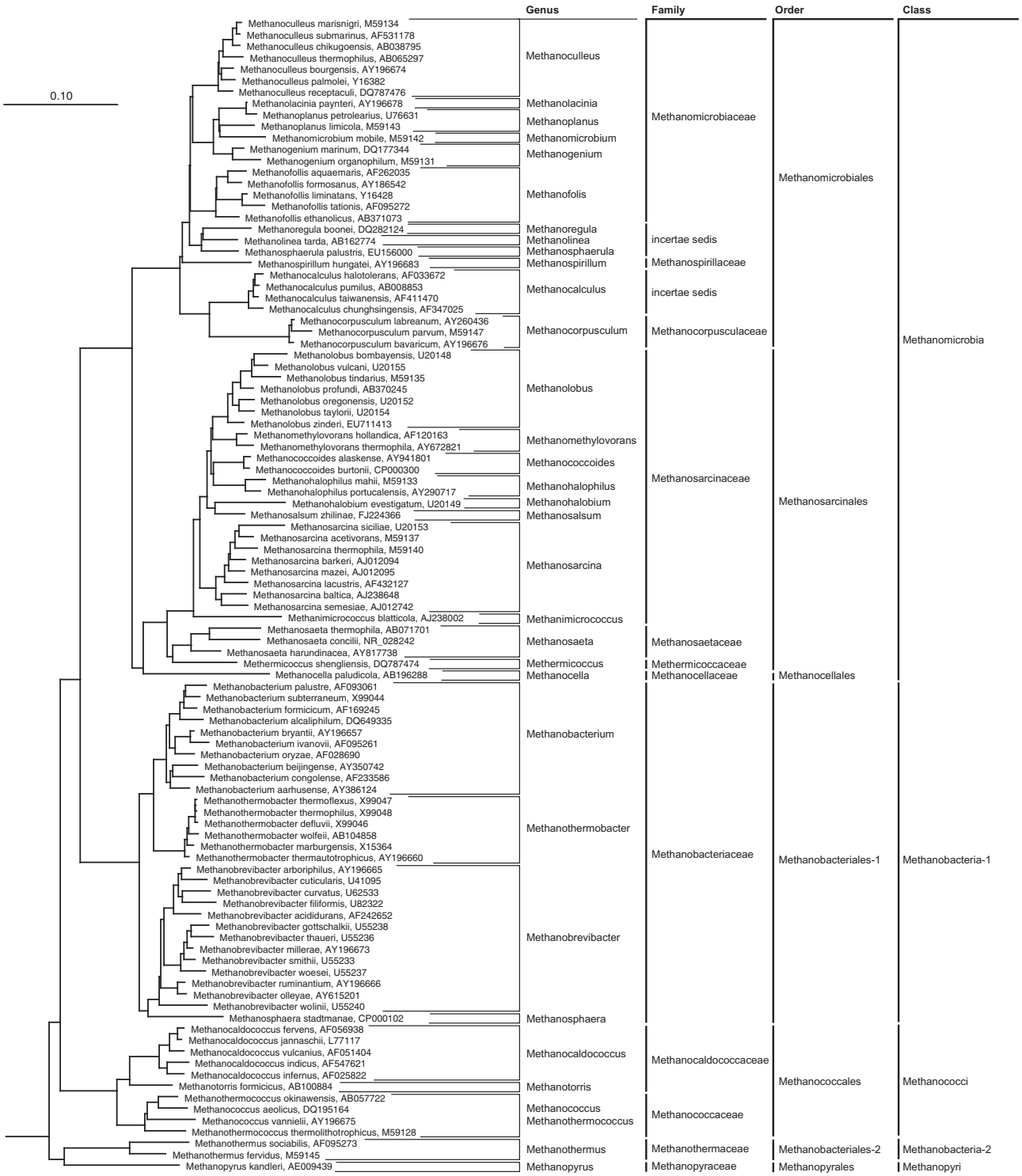

Fig. 1. Phylogeny and taxonomy of methanogens. The neighbour-joining tree was constructed on the basis of 16S rRNA gene sequences using the ARB package (Ludwig *et al*., 2004) with the data set (Yarza *et al*., 2008) provided from silva databases (http://silva.mpi-bremen.de/), showing representative species of methanogens that have been described to date.

the 16S rRNA gene clones affiliated with this group were frequently observed in methanogenic enrichment cultures containing ethanol as an electron donor, and because the RC-II group is a lineage within the phylogenetic radiation of the orders *Methanosarcinales* and *Methanomicrobiales* (Lehmann-Richter *et al*., 1999). As can be noted from these examples, there is no doubt that the actual biodiversity of methanogens will be much expanded in the

Table 1. Oligonucleotide probes and primers targeting the 16S rRNA gene of methanogens.

Target group	Probe name	Probe sequence (5′–3′)[a]	Application	Probe length (mer)	Reference
Most methanogens	Arch f2[b]	TTCYGGTTGATCCYGCCRGA	PCR (forward)	20	Skillman *et al.* (2004)
	Arch r1386	GCGGTGTGTGCAAGGAGC	PCR (reverse)	18	Skillman *et al.* (2004)
	A1f	TCYGKTTGATCCYGSCRGAG	PCR (forward), DGGE	20	Embley *et al.* (1992)
	A1100r	TGGGTCTCGCTCGTTG	PCR (forward), DGGE	16	Embley *et al.* (1992)
	Met83F	ACKGCTCAGTAACAC	PCR (forward)	15	Wright and Pimm (2003)
	Met86F	GCTCAGTAACACGTGG	PCR (forward)	16	Wright and Pimm (2003)
	Met448F	GGTGCCAGCCGCCGC	sequencing	15	Wright and Pimm (2003)
	Met1027F	GTCAGGCAACGAGCGAGACC	sequencing	20	Wright and Pimm (2003)
	Met1340R	CGGTGTGTGCAAGGAG	PCR (reverse)	16	Wright and Pimm (2003)
	109f	ACKGCTCAGTAACACGT	PCR (forward)	17	Grosskopf *et al.* (1998)
	146f	GGSATAACCYCGGGAAAC	PCR (forward)	18	Marchesi *et al.* (2001)
	1324r	GCGAGTTACAGCCCWCRA	PCR (reverse)	18	Marchesi *et al.* (2001)
	ARC344f	ACGGGGYGCAGCAGGCGCGA	PCR (forward), DGGE	20	Casamayor *et al.* (2001)
	25f	CYGGTYGATYCTGCCRG	PCR (forward)	17	Dojka *et al.* (1998)
	1391r	GACGGGCGGTGTGTRCA	PCR (reverse)	17	Barns *et al.* (1994)
	A24f	TCYGKTTGATCCYGSCRGA	PCR (forward), DGGE	19	Yu *et al.* (2008)
	A357f	CCCTACGGGGCGCAGCAG	PCR (forward), DGGE	18	Yu *et al.* (2008)
	A329r	TGTCTCAGGTTCCATCTCCG	PCR (reverse), DGGE	20	Yu *et al.* (2008)
	A348r	CCCCRTAGGGCCYGG	PCR (reverse), DGGE	15	Yu *et al.* (2008)
	A693r	GGATTACARGATTTC	PCR (reverse), DGGE	15	Yu *et al.* (2008)
	Met630F	GGATTAGATACCCSGGTAGT	qPCR (forward), DGGE	20	Hook *et al.* (2009)
	Met803R	GTTGARTCCAATTAAACCGCA	qPCR (reverse), DGGE	21	Hook *et al.* (2009)
	A1040f	GAGAGGWGGTGCATGGCC	PCR (forward), DGGE	18	Reysenbach and Pace (1995)
	ARC344	TCGCGCCTGCTGCICCCCGT	MH	20	Raskin *et al.* (1994b)
	ARC915	GTGCTCCCCCGCCAATTCCT	PCR (reverse), DGGE, MH, FISH	20	Raskin *et al.* (1994b)
	MER1	GGGCACGGGTCTCGCT	PCR (reverse)	16	Hales *et al.* (1996)
Class *Methanomicrobia*	1068R	ATGCTTCACAGTACGAAC	PCR (reverse)	18	Banning *et al.* (2005)
	CMSMM1068m	GGATGCTTCACAGTACGAAC	RNase H	20	Narihiro *et al.* (2009b)
Order *Methanocellales*					
Family *Methanocellaceae*					
Genus *Methanocella*	SANAE1136	GTGTACTCGCCCTCCTCG	FISH	18	Sakai *et al.* (2007)
Order *Methanomicrobiales*	MG1200	CGGATAATTCGGGGCATGCTG	MH, FISH	21	Raskin *et al.* (1994b)
	MG1200m	CCGGATAATTCGGGGCATGCTG	RNase H	22	Narihiro *et al.* (2009b)
	M(SA/MI)355	GTAAAGTTTTCGCGCCTG	MH	18	Ovreås *et al.* (1997)
	MMB282F	ATCGRTACGGGTTGTGGG	qPCR (forward)	18	Yu *et al.* (2005)
	MMB749F	TYCGACAGTGAGGRACGAAAGCTG	qPCR (probe)	24	Yu *et al.* (2005)
	MMB832R	CACCTAACGCRCATHGTTTAC	qPCR (reverse)	21	Yu *et al.* (2005)
Family *Methanomicrobiaceae*					
Genus *Methanoculleus*	298F	GGAGCAAGAGCCCGGAGT	qPCR (forward)	18	Franke-Whittle *et al.* (2009a)
	586R	CCAAGAGACTTAACAACCCA	qPCR (reverse)	20	Franke-Whittle *et al.* (2009a)
	F2SC668	TCCTACCCCCGAAGTACCCCTC	RNase H	22	Narihiro *et al.* (2009b)
	F2SC732	TCGAAGCCGTTCTGGTGAGGCG	RNase H	22	Narihiro *et al.* (2009b)
	AR934F	AGGAATTGGCGGGGGAGCAC	qPCR (forward)	20	Shigematsu *et al.* (2003)
	MCU1023TAQ	GAATGATTGCCGGGCTGAAGACTC	qPCR (probe)	24	Shigematsu *et al.* (2003)
	MG1200b	CCGGATAATTCGGGGCATGCTG	qPCR (reverse)	22	Shigematsu *et al.* (2003)
Species *M. thermophilus*	Mc412f	CTGGGTGTCTAAAACACACCCAA	qPCR (forward)	23	Hori *et al.* (2006)
	Mc578r	ATTGCCAGTATCTCTTAG	qPCR (reverse)	18	Hori *et al.* (2006)
	SMCUT1253	GCCTTTCGGCGTCGATACCC	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanofollis*	F3SC984	CATATCGCTGTCCTACCCGG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanogenium*	GMG1128	CGTTCCGGAGAACAAGCTAG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanomicrobium*	GMM829	CTCGTAGTTACAGGCACACC	FISH, RNase H	20	Yanagita *et al.* (2000)
Genus *Methanoplanus*					
Species *M. limicola*	SMPL623[c]	TTCTCTTAAACGCCTGCAGG	RNase H	20	Narihiro *et al.* (2009b)
Species *M. endosynbiosus*	SMPL623[c]	TTCTCTTAAACGCCTGCAGG	RNase H	20	Narihiro *et al.* (2009b)
Species *M. petrolearius*	SMPP1252[d]	CTTCTCAGTGTCGTTGCTCA	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanolacinia*	SMPP1252[d]	CTTCTCAGTGTCGTTGCTCA	RNase H	20	Narihiro *et al.* (2009b)
Family *Methanospirillaceae*					
Genus *Methanospirillum*	F7SC1260	TATCCTCACCTCTCGGTGTC	RNase H	20	Narihiro *et al.* (2009b)
	MSP1025TAQ	GAATGATAGTCGGGATGAAGACTCTA	qPCR (probe)	26	Tang *et al.* (2005)
Genus *Methanosphaerula*					
Genus *Methanolinea*	NOBI109f	ACTGCTCAGTAACACGT	qPCR (forward)	17	Imachi *et al.* (2008)
	NOBI633	GATTGCCAGTTTCTCCTG	qPCR (reverse), FISH	18	Imachi *et al.* (2008)
Family *Methanocorpusculaceae*					
Genus *Methanocorpusculum*	F6SC393[e]	GACAGGCACTCAGGGTTTCC	RNase H	20	Narihiro *et al.* (2009b)
	GMCP489	GCCCTGCCCTTTCTTCACAT	RNase H	20	Narihiro *et al.* (2009b)
Family incertae sedis					
Genus *Methanocalculus*	F6SC393[e]	GACAGGCACTCAGGGTTTCC	RNase H	20	Narihiro *et al.* (2009b)
	GMCL488	CCCCGCCCTTTCTCCTGGTG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanoregula*					
Species *M. boonei*	6A8 644	TCTTCCGGTCCCTAGCCTGCCA	FISH	22	Bräuer *et al.* (2006)
Species *M. formicica*	SMSP129	TATCCCCTTCCATAGGGTAGATT	FISH	23	Yashiro *et al.* (2009)
Order *Methanosarcinales*	MSMX860	GGCTCGCTTCACGGCTTCCCT	MH	21	Raskin *et al.* (1994b)
	MSSH859	TCGCTTCACGGCTTCCCT	FISH	18	Boetius *et al.* (2000)
	MSr r859	TCGCTTCACGGCTTCCCTG	PCR (reverse)	19	Skillman *et al.* (2004)
	MSMX860m	GCTCGCTTCACGGCTTCCCT	RNase H	20	Narihiro *et al.* (2009b)
	MSL812F	GTAAACGATRYTCGCTAGGT	qPCR (forward)	20	Yu *et al.* (2005)
	MSL860F	AGGGAAGCCGTGAAGCGARCC	qPCR (probe)	21	Yu *et al.* (2005)
	MSL1159R	GGTCCCCACAGWGTACC	qPCR (reverse)	17	Yu *et al.* (2005)

Table 1. *cont.*

Target group	Probe name	Probe sequence (5′–3′)[a]	Application	Probe length (mer)	Reference
Family *Methanosaetaceae*					
Genus *Methanosaeta*	MX825	TCGCACCGTGGCCGACACCTAGC	MH, FISH	23	Raskin *et al.* (1994b)
	MX825mix	TCGCACCGTGGCYGACACCTAGC	RNase H	23	Narihiro *et al.* (2009b)
	MX1361	ACGTATTCACCGCGTTCTGT	FISH	20	Crocetti *et al.* (2006)
	S-G-Msae-0332-a-A-22	TTAGGTCCGGGATGCXCCACGT	MH, FISH	22	Zheng and Raskin (2000)
	Mst702F	TAATC CTYGA RGGAC CACCA	qPCR (forward)	20	Yu *et al.* (2005)
	Mst753F	ACGGC AAGGG ACGAA AGCTA GG	qPCR (probe)	22	Yu *et al.* (2005)
	Mst862R	CCTAC GGCAC CRACM AC	qPCR (reverse)	17	Yu *et al.* (2005)
	MS1b	CCGGCCGGATAAGTCTCTTGA	qPCR (forward)	21	Shigematsu *et al.* (2003)
	SAE761TAQ	ACCAGAACGGACCTGACGGCAAGG	qPCR (probe)	24	Shigematsu *et al.* (2003)
	SAE835R	GACAACGGTCGCACCGTGGCC	qPCR (reverse)	21	Shigematsu *et al.* (2003)
	S-F-Msaet-0387-S-a-21	GATAAGGGRAYCTCGAGTGCY	qPCR (forward)	21	Sawayama *et al.* (2004)
	S-F-Msaet-0540-A-a-31	AGACCCAATAAHARCGGTTACCACTCGRGCC	qPCR (probe)	31	Sawayama *et al.* (2004)
	S-F-Msaet-0573-A-a-17	GGCCGRCTACAGACCCT	qPCR (reverse)	17	Sawayama *et al.* (2004)
Species *M. concilii*	Rotcl1	CTCCCGGCCTCGAGCCAGAC	FISH	20	Zepp Falz *et al.* (1999)
	MS1	CCGGATAAGTCTCTTGA	MH	17	Rocheleau *et al.* (1999)
	MS2	CTGAATGAGAGCGCTTTCTTT	MH	21	Rocheleau *et al.* (1999)
	MS5	GGCCACGGTGCGACCGTTGTCG	MH, FISH	22	Rocheleau *et al.* (1999)
	MMX1273	GGTTTTAGGAGATTCCCGTC	RNase H	20	Narihiro *et al.* (2009b)
	GTMS393m	ACCCAGCACTCGAGGTCCCC	RNase H	20	Narihiro *et al.* (2009b)
Species *M. thermophila*	Ms413f	CAGATGTGTAAAATACATCTGTT	qPCR (forward)	23	Hori *et al.* (2006)
	Ms578r	TCTGGCAGTATCCACCGA	qPCR (reverse)	18	Hori *et al.* (2006)
	TMX745	CCCTTGCCGTCGGATCCGTT	RNase H	20	Narihiro *et al.* (2009b)
Family *Methanosarcinaceae*	MS1414	CTCACCCATACCTCACTCGGG	MH, FISH	21	Raskin *et al.* (1994b)
	EelMS240[f]	CTATCAGGTTGTAGTGGG	FISH	18	Boetius *et al.* (2000)
	Msc380F	GAAACCGYGATAAGGGGA	qPCR (forward)	18	Yu *et al.* (2005)
	Msc492F	TTAGCAAGGGCCGGGCAA	qPCR (probe)	18	Yu *et al.* (2005)
	Msc828R	TAGCGARCATCGTTTACG	qPCR (reverse)	18	Yu *et al.* (2005)
	R15F[g]	GCTACACGCGGGCTACAATGA	qPCR (forward)	21	Zhang *et al.* (2008a)
	FMSC394	ATGCTGGCACTCGGTGTCCC	RNase H	20	Narihiro *et al.* (2009b)
	MS821m[h]	GCCATGCCTGACACCTAGCG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanimicrococcus*	GMIB1254	CACCTTTCGGTGTAGTTGCC	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanosarcina*	MS821	CGCCATGCCTGACACCTAGCGAGC	MH, FISH	24	Raskin *et al.* (1994b)
	SARCI551	GACCCAATAATCACGATCAC	FISH	20	Sorensen and Ahring (1997)
	SARCI645	TCCCGGTTCCAAGTCTGGC	FISH	19	Sorensen and Ahring (1997)
	MB1	TTTGGTCAGTCCTCCGG	MH	17	Rocheleau *et al.* (1999)
	MB3	CCAGACTTGGAACCG	MH	15	Rocheleau *et al.* (1999)
	MB4	TTTATGCGTAAAATGGATT	MH, FISH	19	Rocheleau *et al.* (1999)
	240F	CCTATCAGGTAGTAGTGGGTGTAAT	qPCR (forward)	25	Franke-Whittle *et al.* (2009a)
	589R	CCCGGAGGACTGACCAAA	qPCR (reverse)	18	Franke-Whittle *et al.* (2009a)
	MB1b	CGGTTTGGTCAGTCCTCCGG	qPCR (forward)	20	Shigematsu *et al.* (2003)
	SAR761TAQ	ACCAGAACGGGTTCGACGGTGAGG	qPCR (probe)	24	Shigematsu *et al.* (2003)
	SAR835R	AGACACGGTCGCGCCATGCCT	qPCR (reverse)	21	Shigematsu *et al.* (2003)
	S-G-Msar-0450-S-a-19	TAGCAAGGGCCGGGCAAGA	qPCR (forward)	19	Sawayama *et al.* (2006)
	S-P-Msar-0540-A-a-31	AGACCCAATAATCACGATCACCACTCGGGCC	qPCR (probe)	31	Sawayama *et al.* (2006)
	S-G-Msar-0589-S-a-20	ATCCCGGAGGACTGACCAAA	qPCR (reverse)	20	Sawayama *et al.* (2006)
Genus *Methanococcoides*	GMCO441	ACATGCCGTTTACACATGTG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanohalobium*	GMHB842	TCGGCACTAGGAACGGCCGT	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanohalophilus*	GMHP1258	CCGTCACTTTTCAGTGTAGG	RNase H	20	Narihiro *et al.* (2009b)
Genus *Methanolobus*	GMLB834	TGAAACGGTCGCACCGTCCCAG	RNase H	22	Narihiro *et al.* (2009b)
Species *M. psychrophilus*	R15F	GCTACACGCGGGCTACAATGA	qPCR (forward)	21	Zhang *et al.* (2008a)
	R15R	AATTTAGGTTCGAACACGGCATGAA	qPCR (reverse)	25	Zhang *et al.* (2008a)
Genus *Methanomethylovorans*					
Genus *Methanosalsum*	GMSS261	GTCGGCTAGCAGGTACCTTG	RNase H	20	Narihiro *et al.* (2009b)
Family *Methermicoccaceae*					
Genus *Methermicoccus*					
Class *Methanobacteria*					
Order *Methanobacteriales*	MB310	CTTGTCTCAGGTTCCATCTCCG	MH	22	Raskin *et al.* (1994b)
	MB311	ACCTTGTCTCAGGTTCCATCTCC	FISH	23	Crocetti *et al.* (2006)
	Mbac f331	CTTGTCTCAGGTTCCATCTC	PCR	20	Skillman *et al.* (2004)
	MB1174	TACCGTCGTCCACTCCTTCCTC	MH, FISH	22	Raskin *et al.* (1994b)
	MBT857F	CGWAGGGAAGCTGTTAAGT	qPCR (forward)	19	Yu *et al.* (2005)
	MBT929F	AGCACCACAACGCGTGGA	qPCR (probe)	18	Yu *et al.* (2005)
	MBT1196R	TACCGTCGTCCACTCCTT	qPCR (reverse)	18	Yu *et al.* (2005)
	1401R	KTTTGGGTGGYGTGACGGGC	PCR (reverse)	20	Banning *et al.* (2005)
Family *Methanobacteriaceae*	MB1175m	CCGTCGTCCACTCCTTCCTC	RNase H	20	Narihiro *et al.* (2009b)
	MEB859	AGGGAAGCTGTTAAGTCC	FISH	18	Boetius *et al.* (2000)
Genus *Methanobrevibacter*	fMbb1	CTCCGCAATGTGAGAAATCG	PCR	20	Skillman *et al.* (2004)
	GMB406	GCCATCCCGTTAAGAATGGC	RNase H	20	Narihiro *et al.* (2009b)
Species *M. ruminantium*	MBR1001	TCAGCCTGGTAATCATACA	FISH	19	Yanagita *et al.* (2000)
Species *M. smithii*	Forward	CCGGGTATCTAATCCGGTTC	qPCR (forward)	20	Armougom *et al.* (2009)
	Reverse	CTCCCAGGGTAGAGGTGAAA	qPCR (reverse)	20	Armougom *et al.* (2009)
	Probe	CCGTCAGAATCGTTCCAGTCAG	qPCR (probe)	22	Armougom *et al.* (2009)
Genus *Methanobacterium*	fMbium	CGTTCGTAGCCGGCYTGA	PCR	18	Skillman *et al.* (2004)
	GMBA755	TGGCTTTCGTTACTCACC	RNase H	18	Narihiro *et al.* (2009b)
	S-F-Mbac-0398-S-a-20	CCCAAGTGCCACTCTTAACG	qPCR (forward)	20	Sawayama *et al.* (2006)
	S-G-Mbac-0526-A-a-33	AAYGGCCACCACTTGAGCTGCC GGTGTTACCGC	qPCR (reverse)	33	Sawayama *et al.* (2006)
	S-G-Mbac-0578-A-a-22	AGACTTATCAARCCGGCTACGA	qPCR (probe)	22	Sawayama *et al.* (2006)
Genus *Methanosphaera*	GMSP838	CCGGAACAACTCGAGGCCAT	RNase H	20	Narihiro *et al.* (2009b)

Table 1. *cont.*

Target group	Probe name	Probe sequence (5′–3′)[a]	Application	Probe length (mer)	Reference
Genus *Methanothermobacter*	Mt392f	ACTCTTAACGGGGTGGCTTTT	qPCR (forward)	21	Hori *et al.* (2006)
	Mt578r	TCATGATAGTATCTCCAGC	qPCR (reverse)	19	Hori *et al.* (2006)
	410F	CTCTTAACGGGGTGGCTTTT	qPCR (forward)	20	Franke-Whittle *et al.* (2009a)
	667R	CCCTGGGAGTACCTCCAGC	qPCR (reverse)	19	Franke-Whittle *et al.* (2009a)
	GMTB541	AAAAGCGGCTACCACTTGAGCT	RNase H	22	Narihiro *et al.* (2009b)
	S-F-Mbac-0398-S-a-20	CCCAAGTGCCACTCTTAACG	qPCR (forward)	20	Sawayama *et al.* (2006)
	S-G-Mthb-0549-S-a-32	CGGACGCTTTAGGCCCAATAAAAGCGGCTACC	qPCR (probe)	32	Sawayama *et al.* (2006)
	S-G-Mthb-0589-A-a-25	GGGATTTCACCAGAGACTTATCAG	qPCR (reverse)	25	Sawayama *et al.* (2006)
Family *Methanothermaceae*					
Genus *Methanothermus*	FMTH1183	TACGGACCTACCGTCGCCCGCA	RNase H	22	Narihiro *et al.* (2009b)
Class *Methanococci*					
Order *Methanococcales*	M(CO/BA)377	CCCCCGTCGCACTTKCGTG	MH	19	Ovreås *et al.* (1997)
	Mcc r	WASTVGCAACATAGGGCACGG	PCR (reverse)	21	Skillman *et al.* (2004)
	MCC495F	TAAGGGCTGGGCAAGT	qPCR (forward)	16	Yu *et al.* (2005)
	MCC686F	TAGCGGTGRAATGYGTTGATCC	qPCR (probe)	22	Yu *et al.* (2005)
	MCC832R	CACCTAGTYCGCARAGTTTA	qPCR (reverse)	20	Yu *et al.* (2005)
	1202R	CCAGGRGATTCGGGGCATGC	PCR (reverse)	20	Banning *et al.* (2005)
Family	S-F-Mcc-1109-b-A-20	GCAACATGGGGCRCGGGTCT	MH	20	Nercessian *et al.* (2004)
Methanocaldococcaceae	MC504	GGCTGCTGGCACCGGACTTGCCCA	FISH	24	Crocetti *et al.* (2006)
	FMCMT1044[i]	GTCAACCTGGCCTTCATCCTGC	RNase H	22	Narihiro *et al.* (2009b)
Genus *Methanocaldococcus*					
Genus *Methanotorris*					
Family *Methanococcaceae*	MC1109	GCAACATAGGGCACGGGTCT	MH	20	Raskin *et al.* (1994b)
Genus *Methanococcus*	GMC728	ACCCGTTCCAGACAAGTGCCTT	RNase H	22	Narihiro *et al.* (2009b)
	GMC231	ACTACCTAATCGAGCGCAGTCC	RNase H	22	Narihiro *et al.* (2009b)
	GMC416	TTGATAAAAGCCCATGCTGTGC	RNase H	22	Narihiro *et al.* (2009b)
Genus *Methanothermococcus*	GMTL416	TAGAAAAGCCTACGCAGTGC	RNase H	20	Narihiro *et al.* (2009b)
Class *Methanopyri*					
Order *Methanopyrales*					
Family *Methanopyraceae*					
Genus *Methanopyrus*	FMCMT1044[i]	GTCAACCTGGCCTTCATCCTGC	RNase H	22	Narihiro *et al.* (2009b)
	S-G-Mp-0431-a-A-20	TTACACCCCGGTACAGCCGC	MH	20	Nercessian *et al.* (2004)
	GMPK1331	GGTTACTACCGATTCCACCTTC	RNase H	22	Narihiro *et al.* (2009b)

a. IUPAC Ambiguity Codes: Y = C or T, R = A or G, K = G or T, S = C or G, W = A or T, M = A or C, H = A or C or T, V = A or C or G
b. Arch f2 probe covers members of the orders *Methanomicrobiales, Methanosarcinales* and *Methanococcales.*
c. SMPL623 probe covers members of the *Methanoplanus limicola* and *M. endosynbiosus.*
d. SMPP1252 probe covers members of the *Methanoplanus petrolearius* and *Methanolacinia.*
e. F6SC393 probe covers members of the genera *Methanocorpusculum* and *Methanocalculus.*
f. EelMS240 probe targets for members of the genera *Methanolobus, Methanohalophilus, Methanococcoides* and *Methanomethylovorans.*
g. R15F probe covers members of the genera *Methanomethylovorans* and *Methanosarcina* and *Methanolobus psychrophilus.*
h. MS821m probe covers members of the genera *Methanimicrococcus* and *Methanosarcina.*
i. FMCMT1044 probe covers members of the family *Methanocaldococcaceae* and genus *Methanopyrus.*
MH, membrane hybridization.

future as the number of isolated and described methanogens continues to increase. However, in this review, we mainly focus on the quantitative monitoring tools for previously cultured methanogens.

Oligonucleotide probes/primers for 16S rRNA and its gene

16S rRNA and its gene are the most frequently used biomarkers for the determination of methanogenic populations in environments. 16S rRNA gene-targeted probes/primers frequently used for identifying methanogens are listed in Table 1. To entirely describe methanogenic populations in ecosystems of interest, 16S rRNA gene-targeted primer sets for a wide range of methanogen taxa, such as 146f/1324r (Marchesi *et al.*, 2001) and Met83F (Met86F)/Met1340R (Wright and Pimm, 2003), were developed. In addition, a number of oligonucleotide probes/primers for specifically and hierarchically detecting methanogens at different taxonomic levels were designed to resolve different methanogen populations in waste/wastewater treatment anaerobic sludges (Rocheleau *et al.*, 1999; Zheng and Raskin, 2000; Hori *et al.*, 2006; Ariesyady *et al.*, 2007; Franke-Whittle *et al.*, 2009a; Narihiro *et al.*, 2009a,b), the rumen (Yanagita *et al.*, 2000; Skillman *et al.*, 2004), subseafloor sediments (Boetius *et al.*, 2000; Nercessian *et al.*, 2004), sediments (Falz *et al.*, 1999), the human gut (Armougom *et al.*, 2009) and wetlands (Bräuer *et al.*, 2006; Zhang *et al.*, 2008a,b) (Table 1). Nowadays, almost all of the known culturable methanogens can be detected using these probes/primers at the class, order, family genus and even species levels; at the genus level, it should be noted that the probes/primers targeting for the genera *Methermicoccus*, *Methanomethylovorans*, *Methanocaldococcus* and *Methanotorris* are lacking.

Oligonucleotide probes/primers for *mcrA* gene

The 16S rRNA gene has been best used for the identification of methanogens in environments. However, because archaeal 16S rRNA genes other than those of methanogens can also often be detected using PCR

Table 2. Oligonucleotide PCR primers and probes targeting the *mcrA* gene.

Probe/primer name	Name	Direction/ Application	Probe sequence (5′–3′)	Probe length (mer)	Reference	Specificity
PCR primer						
Set 1	MCRf	Forward	TAYGAYCARATHTGGYT	17	Springer *et al.* (1995)	Most methanogens
	MCRr	Reverse	ACRTTCATNGCRTARTT	17		
Set 2	ME1	Forward	GCMATGCARATHGGWATGTC	20	Hales *et al.* (1996)	Most methanogens
	ME2	Reverse	TCATKGCRTAGTTDGGRTAGT	21		
Set 3	MLf	Forward	GGTGGTGTMGGATTCACACARTAYGCWACAGC	32	Luton *et al.* (2002)	Most methanogens
	MLr	Reverse	TTCATTGCRTAGTTWGGRTAGTT	23		
Set 4	ME1	Forward	GCMATGCARATHGGWATGTC	20	Hales *et al.* (1996)	Most methanogens
	ME2b	Reverse	TCCTGSAGGTCGWARCCGAAGAA	23	Shigematsu *et al.* (2004)	
Set 5	MrtA_for	Forward	AAACAATCAACCACGCACTC	20	Scanlan *et al.* (2008)	*Methanosphaera stadtmanae*
	MrtA_rev	Reverse	GTGAGCCCAATCGAAGGA	18		
Set 6	METH-f	Forward	RTRYTMTWYGACCARATMTG	20	Colwell *et al.* (2008)	Most methanogens
	METH-r	Reverse	YTGDGAWCCWCCRAAGTG	18		
Set 7	mlas	Forward	GGTGGTGTMGGDTTCACMCARTA	24	Steinberg and Regan (2008)	Most methanogens
	mcrA-rev	Reverse	CGTTCATBGCGTAGTTVGGRTAGT	24		
Set 8	ME3MF	Forward	ATGTCNGGTGGHGTMGGSTTYAC	23	Nunoura *et al.* (2008)	Most methanogens
	ME3MF-e	Forward	ATGAGCGGTGGTGTCGGTTTCAC	23		
	ME2r'	Reverse	TCATBGCRTAGTTDGGRTAGT	21		
Probe						
	ME3	Clone screening	GGTGGHGTMGGWTTCACACA	20	Hales *et al.* (1996)	Most of methanogens
	SAE716TAQ	TaqMan probe	AGGCCTTCCCCACTCTGCTTGAGGAT	26	Shigematsu *et al.* (2004)	Genus *Methanosaeta*
	SAR716TAQ	TaqMan probe	AGAAATTCCCAACAGCCCTTGAAGAC	26	Shigematsu *et al.* (2004)	Genus *Methanosarcina*
	MCU716TAQ	TaqMan probe	AGCAGTACCCGACCATGATGGAGGAC	26	Shigematsu *et al.* (2004)	Genus *Methanoculleus*
	mbac-mcrA	TaqMan probe	ARGCACCKAACAMCATGGACACWGT	25	Steinberg and Regan (2009)	Family *Methanobacteriaceae*
	mrtA	TaqMan probe	CCAACTCYCTCTCMATCAGRAGCG	24	Steinberg and Regan (2009)	Family *Methanobacteriaceae*
	mcp	TaqMan probe	AGCCGAAGAAACCAAGTCTGGACC	24	Steinberg and Regan (2009)	Family *Methanocorpusculaceae*
	msp	TaqMan probe	TGGTWCMACCAACTCACTCTCTGTC	25	Steinberg and Regan (2009)	Family *Methanospirillaceae*
	Fen	TaqMan probe	AAVCACGGYGGYMTCGGMAAG	21	Steinberg and Regan (2009)	Genus *Methanoregula*
	msa	TaqMan probe	CCTTGGCRAATCCKCCGWACTTG	23	Steinberg and Regan (2009)	Family *Methanosaetaceae*
	msar	TaqMan probe	TCTCTCWGGCTGGTAYCTCTCCATGTAC	28	Steinberg and Regan (2009)	Genus *Methanosarcina*
	McvME0	FISH	GGAAAAATTCGAAGAAGATC	20	Kubota *et al.* (2006)	*Methanococcus vannielii*
	McvME3r	FISH	TGTGTGAAACCTACGCCACC	20	Kubota *et al.* (2006)	*Methanococcus vannielii*
	McvME1r	FISH	GACATTCCAATCTGCATTGC	20	Kubota *et al.* (2006)	*Methanococcus vannielii*

The probes/primers listed here.

primer sets for a wide range of methanogen taxa, it has limitation in exclusively describing the population structure of methanogens. Therefore, there is a need to detect methanogens on the basis of functional genes that are found to be unique in methanogenesis. Such a functional gene frequently used is *mcrA*. Methyl coenzyme M reductase (mcr) is the terminal enzyme involved in methanogenesis, which reduces the methyl group bond of methyl coenzyme M with the release of methane (Friedrich, 2005). Because the α-subunit of *mcr* (*mcrA*) and its isoenzyme gene (*mrtA*) are highly conserved among methanogens, and that these genes are almost exclusively found in methanogens, *mcrA*/*mrtA*-based detection of methanogens has been used. The phylogeny of methanogens determined using *mcrA*/*mrtA* (or translated amino acid) sequences is in good accordance with those determined using 16S rRNA gene sequences (Friedrich, 2005). Previously reported, frequently used probes/primers for *mcrA*/*mrtA* are categorized into three primer sets, namely, MCR (Springer *et al.*, 1995), ME (Hales *et al.*, 1996) and ML (Luton *et al.*, 2002) (Table 2). The targeted regions of the forward primers of these sets are considerably different, whereas those of the reverse primers are almost the same. The MCR primer set was originally designed to determine the phylogeny of the family *Methanosarcinaceae* (Springer *et al.*, 1995). The ME primer set was designed to describe methanogenic populations in wetlands (Hales *et al.*, 1996), for which the difficulty in amplifying *mcrA*/*mrtA* relevant to *Methanosarcinaceae* and *Methanobacteriaceae* was pointed out later (Lueders *et al.*, 2001; Juottonen *et al.*, 2006). The ML primer set was developed on the basis of the *mcrA* sequences obtained from five orders, comprising *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales* and *Methanopyrales* (Luton *et al.*, 2002). Four other primer sets and probes for specific taxonomic groups have also been developed recently (Table 2).

Assessing the biodiversity of methanogens in complex communities by PCR detection and cloning of methanogen genes

Some of the noted primers for 16S rRNA and methyl coenzyme M reductase genes have often been used for the detection and identification by PCR to explore the diversity of methanogens in environmental samples (Table 3). For example, the 146f/1324r primer set for most of all the known methanogens was designed for the 16S rRNA gene clone analysis of deep sediment gas hydrate deposit, and the results showed that gene clones (phylotypes) affiliated with *Methanosarcina* and *Methanobrevibacter* predominated in the sediments (Marchesi *et al.*, 2001). Similarly, some of these primers shown in Table 1 have been used for PCR to profile methanogen popula-

Table 3. Examples of oligonucleotide primer sets for PCR-based analyses for methanogens.

Type of sample	Application	Target gene	Target group	Probe set (forward/reverse/probe)[a]	Reference
Anaerobic process	qPCR	16S rRNA	*Methanomicrobiales*	MMB282F/MMB832R/MMB749F	Yu *et al.* (2005)
			Methanosarcinales	MSL812F/MSL1159R/MSL860F	
			Methanobacteriales	MBT857F/MBT1196R/MBT929F	
			Methanococcales	MCC495F/MCC832R/MCC686F	
			Methanosarcinaceae	Msc380F/Msc828R/Msc492F	
			Methanosaeta	Mst702F/Mst862R/Mst753F	
	qPCR	*mcrA*	*Methanocorpusculaceae*	mlas/mcrA-rev/mcp	Steinberg and Regan (2009)
			Methanospirillaceae	mlas/mcrA-rev/msp	
			Methanosaetaceae	mlas/mcrA-rev/msa	
			Methanobacteriaceae	mlas/mcrA-rev/mbac-mcrA	
			Methanobacteriaceae	mlas/mcrA-rev/mrtA	
			Methanoregula	mlas/mcrA-rev/Fen	
			Methanosarcina	mlas/mcrA-rev/msar	
	qPCR	16S rRNA	*Methanoculleus*	298F/586R	Franke-Whittle *et al.* (2009a)
			Methanosarcina	240F/589R	
			Methanothermobacter	410F/667R	
	qPCR	16S rRNA	*Methanoculleus thermophilus*	Mc412f/Mc578r	Hori *et al.* (2006)
			Methanosaeta thermophila	Ms413f/Ms578r	
			Methanothermobacter	Mt392f/Mt578r	
	qPCR	16S rRNA	*Methanosaeta*	MS1b/SAE835R/SAE761TAQ	Shigematsu *et al.* (2003)
			Methanosarcina	MB1b/SAR835R/SAR761TAQ	
			Methanoculleus	AR934F/MG1200b/MCU1023TAQ	
	qPCR	*mcrA*	*Methanosaeta*	ME1/ME2b/SAE716TAQ	Shigematsu *et al.* (2004)
			Methanosarcina	ME1/ME2b/SAR716TAQ	
			Methanoculleus	ME1/ME2b/MCU716TAQ	
	qPCR	16S rRNA	*Methanosaeta*	S-F-Msaet-0387-S-a-21/ S-F-Msaet-0540-A-a-31/ S-F-Msaet-0573-A-a-17	Sawayama *et al.* (2004)
	qPCR	16S rRNA	*Methanosarcina*	S-G-Msar-0450-S-a-19/ S-P-Msar-0540-A-a-31/ S-G-Msar-0589-S-a-20	Sawayama *et al.* (2006)
			Methanobacterium	S-F-Mbac-0398-S-a-20/ S-G-Mbac-0526-A-a-33/ S-G-Mbac-0578-A-a-22	
			Methanothermobacter	S-F-Mbac-0398-S-a-20/ S-G-Mthb-0549-S-a-32/ S-G-Mthb-0589-A-a-25	
	qPCR	16S rRNA	*Methanospirillum*	AR934F/MG1200b/MSP1025TAQ	Tang *et al.* (2005)
	qPCR	16S rRNA	*Methanolinea*	NOBI109f/NOBI633	Imachi *et al.* (2008)
	PCR-cloning	16S rRNA	Most methanogens	109f/UNIV1492r[b]	Narihiro *et al.* (2009a)
	PCR-cloning	16S rRNA	Most methanogens	25f/1391r 25f/UNIV1492r[b] 109f/UNIV1492r[b]	Ariesyady *et al.* (2007)
Anaerobic process, wetland	PCR-cloning	*mcrA*	Most methanogens	mlas/mcrA-rev	Steinberg and Regan (2008)
Wetland	PCR-cloning	*mcrA*	Most methanogens	ME1/ME2	Hales *et al.* (1996)
	qPCR	16S rRNA	*Methanolobus psychrophilus*	R15F/R15R	Zhang *et al.* (2008a)
Rumen	qPCR, DGGE	16S rRNA	Most methanogens	Met630F/Met803R	Hook *et al.* (2009)
	PCR, DGGE	16S rRNA	Most methanogens	A357f/A693r A24f/A329r A24f/A348r	Yu *et al.* (2008)
	PCR-typing	16S rRNA	Most methanogens	Arch f2/Arch r1386	Skillman *et al.* (2004)
			Methanosarcinales	Arch f2/MSr r859	
			Methanobacteriales	Mbac f331/Arch r1386	
			Methanobacterium	fMbium/Arch r1386	
			Methanococcales	Arch f2/Mcc r	
			Methanobrevibacter	fMbb1/Arch r1386	
	qPCR	16S rRNA	*Methanobrevibacter smithii*	forward/reverse/probe	Armougom *et al.* (2009)
Gastrointestinal tract	PCR-cloning	*mcrA*	Most methanogens	MrtA_for/MrtA_rev	Scanlan *et al.* (2008)
Deep sea sediments	PCR-cloning	16S rRNA	Most methanogens	146f/1324r	Marchesi *et al.* (2001)
	qPCR	*mcrA*	Most methanogens	METH-f/METH-r	Colwell *et al.* (2008)
	qPCR	*mcrA*	Most methanogens	ME3MF and ME3MF-e/ME2r'	Nunoura *et al.* (2008)
Lake sediment	PCR-cloning	16S rRNA	*Methanomicrobia*	355F[c]/1068R	Banning *et al.* (2005)
			Methanobacteriales	109f/1401R	
			Methanococcales	344F[d]/1202R	
Sulfurous lake	PCR, DGGE	16S rRNA	Most methanogens	ARC344f/ARC915	Casamayor *et al.* (2001)
Landfill	PCR-cloning	*mcrA*	Most methanogens	MLf/MLr	Luton *et al.* (2002)
Ciliate endosymbiont	PCR, DGGE	16S rRNA	Most methanogens	A1f/A1100r	Embley *et al.* (1992)
Rice paddy soil	PCR, DGGE	16S rRNA	Most methanogens	109f/ARC915	Grosskopf *et al.* (1998)
Pure cultures	PCR-ribotyping	16S rRNA	Most methanogens	Met83F (or Met86F)/Met1340R	Wright and Pimm (2003)
	PCR-cloning	*mcrA*	Most methanogens	MCRf/MCRr	Springer *et al.* (1995)

a. The primer sequences were shown in Tables 1 and 2.
b. UNIV1492r reverse primer was originally referred from Lane (1991) as an universal primer.
c. 355F forward primer was originally referred as M(SA/MI)355 probe developed by Ovreås *et al.* (1997) as shown in Table 1.
d. 344F forward primer was originally referred as ARC344 probe developed by Raskin and colleagues (1994b) as shown in Table 1.

tions by denaturing gradient gel electrophoresis (DGGE) (e.g. (Casamayor *et al*., 2001; 2002; Yu *et al*., 2005; 2006; 2008). As examples, Wright and Pimm (2003) developed PCR and sequencing primers for the 16S rRNA gene of methanogens, and used them for the ribotyping of members of the classes '*Methanomicrobia*' and *Methanobacteria*. The detection of methanogens by PCR in lamb rumen samples was performed using methanogen-specific primers targeting different taxonomic levels (Skillman *et al*., 2004). Banning and colleagues (2005) designed novel reverse primers to provide specific amplification of the 16S rRNA genes of '*Methanomicrobia*' (*Methanomicrobiales* and *Methanosarcinales*), *Methanobacteriales* and *Methanococcales*, and successfully used them for the identification of methanogenic population structures in lake sediments.

Massive parallel sequencing of PCR-amplified 16S rRNA genes using next generation sequencers (such as the FLX pyrosequencers) allows us to obtain a huge number of community sequence tags (for example *c*. 10 000–100 000 16S pyrotags for each sample), which is more than any Sanger-based cloning study to date, and have been used for characterizing archaeal populations (including methanogens) in hydrothermal chimneys (Brazelton *et al*., 2010a,b). The methodological advancements of 16S rRNA gene pyrosequencing include higher resolution (more sequences) for gene-based community structure analysis, analysis of multiple related samples and use of metadata (Tringe and Hugenholtz, 2008). Because of these advancements, as well as recent development of analytical tools for massive sequence data such as QIIME (Caporaso *et al*., 2010), the method may be further used for characterizing diversity of methanogens in ecosystems.

Similarly, the primers for methyl coenzyme M reductase genes have often been used for PCR detection and identification to exclusively explore the diversity of methanogens in samples. For example, the MCR set was used to elucidate the diversity of methanogens in various environments with PCR-based cloning (Kemnitz *et al*., 2004; Dhillon *et al*., 2005; Alain *et al*., 2006) and T-RFLP analyses (Ramakrishnan *et al*., 2001; Kemnitz *et al*., 2004). Such cloning analyses were also conducted using the ME (Hales *et al*., 1996; Nercessian *et al*., 1999; Galand *et al*., 2002; 2005; Tatsuoka *et al*., 2004) and ML primer sets (Luton *et al*., 2002; Castro *et al*., 2004; Juottonen *et al*., 2005; Nercessian *et al*., 2005; Ufnar *et al*., 2007; Smith *et al*., 2008). Comparative studies using these three primer sets have indicated that the ML primer set is more efficient for retrieving phylogenetically diverse methanogens in the wetland than others (Juottonen *et al*., 2006; Jerman *et al*., 2009). Owing to this advantage, the ML set has been used extensively to determine the diversity of methanogens in various anaerobic ecosystems. In addition, it has been noted that these *mcrA*-targeted primer sets (especially ME-related primer set) were also used for the quantitative detection of anaerobic methanotrophic archaea (ANME) in methane seep sediments (Inagaki *et al*., 2004; Nunoura *et al*., 2006; 2008). This is due to the fact that anaerobic methane oxidation represented by the ANME group is considered to proceed with mcr-type enzymes (Thauer and Shima, 2008). Detailed information about the *mcrA*-based qPCR for ANMEs is described below.

Polymerase chain reaction-based molecular techniques, such as PCR-cloning, pyrosequencing, DGGE and T-RFLP are adequate to gain entire community composition and diversity of methanogens in ecosystems. Based on the frequency of retrieval of phylotypes in gene library (or relative intensity of DGGE or T-RF bands in electropherogram), relative abundance of phylotypes of interest can be inferred. However, it should be noted that entire microbial community structure analysis based on bulk cell lysis, DNA extraction, PCR and cloning are often suspect because of several biases involved in each of the steps (Dahllof, 2002). Therefore, one should be careful to discuss on the abundance of phylotypes in samples based solely on the data obtained by these methods. More reliable methods to carry out quantitative detection of different groups of methanogens in samples would be to use the following quantitative molecular techniques.

Identification and quantification of methanogens in complex communities by membrane hybridization method

Quantitative membrane hybridization of labelled DNA probes to community rRNAs has been applied to various environmental rRNAs for the quantitative detection of specific groups of microbes present in complex communities (Stahl *et al*., 1988; Raskin *et al*., 1994a). RNA-dependent community analysis is known to indicate the *in situ* activity of individual members in ecosystems, because of the reasons that RNA synthesis is known to reflect the *in situ* growth rates of organisms (Poulsen *et al*., 1993; Amann *et al*., 1995), and that the turnover of RNA is thought to be much higher than that of DNA. Therefore, rRNA-dependent molecular techniques like the present one provide precise information about the dynamic nature of individual microbes in systems. In 1994, Raskin and colleagues carried out the first leading studies on the development of eight oligonucleotide probes for the quantitative detection of methanogens in anaerobic wastewater treatment sludges (Stahl and Amann, 1991; Raskin *et al*., 1994a,b). In these studies, they established the group-specific oligonucleotide probes targeting *Methanomicrobiales* (probes MG1200 and MSMX860), *Methanobacteriaceae* (probes MB310 and MB1174) and *Methanococcales* (probe MC1109). Because of the importance of methane production from acetate in anaerobic bioreactors, specific probes

for aceticlastic methanogens, such as the members of *Methanosarcinaceae* (probes MS1414 and MS821) and *Methanosaeta* (probe MX825), were also developed.

These probes have been successfully applied to the quantification of methanogens in laboratory- and full-scale anaerobic bioreactors based on rRNA (Raskin *et al*., 1995; Griffin *et al*., 1998; Liu *et al*., 2002; McMahon *et al*., 2004; Zheng *et al*., 2006). Although membrane hybridization enables the sensitive quantification of individual species of rRNA molecules, this method requires several laborious experimental steps, often radioactively labelled DNA probes, and reference rRNA samples as external standards for each experiment. Thus, the method itself may be replaced by similar but much rapid and simpler methods, such as real-time RT-PCR and RNase H methods. However, the probes used for membrane hybridization experiments may be also used as probes/primers in other experiments shown below.

FISH for methanogens

Whole-cell FISH based on 16S rRNA is now commonly used to detect specific groups of microbes and to quantify populations of interest in environments by direct counting under a microscope (Amann *et al*., 1995). In addition, FISH is used for visualizing the spatial distribution of the population of interest in biofilms, such as those of methanogens in sludge granules in methanogenic wastewater treatment systems (Sekiguchi *et al*., 1999). Basically, the probes developed for membrane hybridization of methanogen 16S rRNAs or reverse primers for PCR amplification of methanogen 16S rRNA genes can directly be used as oligonucleotide probes for *in situ* hybridization studies, the probes previously designed by Raskin and colleagues (Raskin, *et al*., 1994b) have frequently be used for the purpose of FISH studies as well. These probes have been used for the quantitative detection of methanogens using the FISH technique in various anaerobic ecosystems, such as peat bog (e.g. Horn *et al*., 2003), aquifer (e.g. Kleikemper *et al*., 2005), landfills (e.g. Laloui-Carpentier *et al*., 2006) and anaerobic wastewater treatment processes (e.g. Sekiguchi *et al*., 1999; Plumb *et al*., 2001; Boonapatcharoen *et al*., 2007; Chen *et al*., 2009). Recently, the improvement of the specificity and sensitivity of the probes designed by Raskin and colleagues (1994b) has been reported. Crocetti and colleagues (2006) refined the experimental conditions of such probes for FISH analysis to accurately and sensitively detect methanogens.

In addition to the quantification, the probes (Table 1) have also been used for investigating the localization of methanogens in biofilms (sludge granules) [e.g. (Rocheleau *et al*., 1999; Sekiguchi *et al*., 1999; Plumb *et al*., 2001; Zheng *et al*., 2006; Vavilin *et al*., 2008; Chen *et al*., 2009)]. In anaerobic sludge granules, hydrogenotrophic methanogens are often juxtaposed with syntrophic substrate-degrading bacteria, such as syntrophic propionate-oxidizing bacteria such as members of the genera *Syntrophobacter* and *Pelotomaculum*; such close proximity between syntrophic bacteria and methanogens has been observed by FISH with confocal laser scanning microscopy (Harmsen *et al*., 1995; 1996; Sekiguchi *et al*., 1999; Imachi *et al*., 2000). Anaerobic ciliates often posses endosymbiotic methanogens within their cells, and the distribution of such methanogens in eukaryotic cells has been observed by the FISH method [e.g. (Embley *et al*., 1992; Shinzato *et al*., 2007)].

Although FISH is a powerful method for visualizing the cells of interest, there are some drawbacks in detecting cells; one of such problems is concerned with the penetration of oligonucleotide probes into the cells (Amann *et al*., 1995). For methanogens, FISH staining is often difficult for some *Methanobacterium* and *Methanobrevibacter* cells, for which oligonucleotide probes do not penetrate into their cells (Sekiguchi *et al*., 1999; Yanagita *et al*., 2000; Nakamura *et al*., 2006). To solve this problem, fixed cells were subjected to freeze-thaw cycles before hybridization, resulting in the improvement of probe penetration (Sekiguchi *et al*., 1999). Another way to solve this problem is the use of recombinant pseudomurein endoisopeptidase, which increases the permeability of oligonucleotide probes into cells, and allows a better visualization of methanogens in anaerobic granular sludge and the endosymbiotic methanogens in the anaerobic ciliate *Trimyema compressum* (Nakamura *et al*., 2006). An improved protocol of catalysed reporter deposition-FISH for methanogens with recombinant pseudomurein endoisopeptidase has also been reported, which can increase fluorescence signal intensity in FISH for detecting cells with a low rRNA content (Kubota *et al*., 2008).

Recently, *mcrA*-based *in situ* detection of methanogens has been performed using the two-pass tyramide signal amplification-FISH approach combined with locked nucleic acids (Kubota *et al*., 2006; Kawakami *et al*., 2010). These attempts were, at this point, only partially successful in detecting methanogen cells, because *mcrA* is generally present as a single copy gene on their chromosome, which results in a low sensitivity of detection.

qPCR

Quantitative PCR of 16S rRNA gene and *mcrA* has also been used to quantify the abundance of methanogens in recent years. Examples of qPCR primer and probe sets for different taxa of methanogens are listed in Table 3. For example, the primers Met630F/Met803R were developed for the SYBR green-based real-time qPCR for almost all the known methanogens in the rumen of the dairy cow (Hook *et al*., 2009). Yu and colleagues (2005) designed

TaqMan-based qPCR probes/primer sets (six sets in total) for each of the orders *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales* and *Methanococcales*, as well as the families *Methanosaetaceae* and *Methanosarcinaceae*. They applied a part of these sets to quantifying aceticlastic methanogens in methanogenic sludges for treating sewage sludges, cheese whey wastewater and synthetic wastewater, and revealed that the population of aceticlastic methanogens is affected by the acetate concentration in the wastewaters (Yu *et al*., 2006). qPCR detection using specific primers for particular groups of methanogens of interest, such as *Methanoculleus* (Shigematsu *et al*., 2003; Hori *et al*., 2006; Franke-Whittle *et al*., 2009a), *Methanolinea* (Imachi *et al*., 2008), *Methanospirillum* (Tang *et al*., 2005), *Methanosaeta* (Shigematsu *et al*., 2003; Sawayama *et al*., 2004; Hori *et al*., 2006), *Methanosarcina* (Shigematsu *et al*., 2003; Sawayama *et al*., 2006; Franke-Whittle *et al*., 2009a), *Methanolobus* (Zhang *et al*., 2008a,b), *Methanobrevibacter* (Armougom *et al*., 2009), *Methanobacterium* (Sawayama *et al*., 2006) and *Methanothermobacter* (Hori *et al*., 2006; Sawayama *et al*., 2006; Franke-Whittle *et al*., 2009a) have also been reported to date (Table 3).

For the qPCR detection of *mcrA*, the ME primer set was used for the quantification of methanogenic and methanotrophic populations in methane seep sediments (Inagaki *et al*., 2004; Nunoura *et al*., 2006). Afterwards, Nunoura and colleagues (2008) slightly modified the ME primer series, and showed that the mixture of the ME3MF and ME3MF-e forward primers and the ME2' reverse primer is most suitable for the qPCR detection of the methanogens and ANMEs in the environments. The results showed that a significant amount of methanogens and ANMEs was found in anaerobically digested sludge and methane seep sediments. The ML primer set was also used for the quantitation of methanogenic archaeal populations in the rumen (Denman *et al*., 2007) and human subgingival plaque (Vianna *et al*., 2008). Moreover, Steinberg and Regan (2008; 2009) developed the mlas/mcrA-rev primer set, which is a derivative of the ML primer set, for the clone library construction and qPCR analyses of methanogens in oligotrophic fen and anaerobic digester sludge. In addition, the genus-specific TaqMan probes for the *mcrA*-based quantitative detection of the *Methanosaeta*, *Methanosarcina* and *Methanoculleus* resident in acetate-fed chemostats, and the results showed that dilution rate is a key factor in the acetate bioconversion pathway (Shigematsu *et al*., 2004).

Quantitative PCR method provides sensitive, quantitative data of gene of interest with a sufficiently high dynamic range of quantification (Zhang and Fang, 2006). Therefore, in addition to the use of digital PCR (Ottesen *et al*., 2006), qPCR may be further used for quantitative monitoring of methanogen taxa of interests in complex microbial communities. However, it should be noted that the method is PCR-based and hence their data can be suspect because of biases involved in DNA extraction and primer/probe mismatches.

Assessing methanogen population by RNase H method

Although the above-mentioned quantitative methods such as membrane hybridization and qPCR are becoming general means to determine the abundance of the population of interest in a complex microbial community, there is a need to develop more simple and rapid techniques that meet the needs for real-time monitoring of the population of interest in a complex community. Recently, a simple and rapid quantification method, namely, the RNase H method, has been developed (Uyeno *et al*., 2004). This method is based on the sequence-specific cleavage of 16S rRNA with ribonuclease H (RNase H) and oligonucleotide (scissor) probes. RNAs from a complex community were first mixed with an oligonucleotide and subsequently digested with RNase H. Because RNase H specifically degrades the RNA strand of RNA : DNA hybrid heteroduplexes, the targeted rRNAs are cleaved at the hybridization site in a sequence-dependent manner and are consequently cut into two fragments. In contrast, non-targeted rRNAs remain intact under the same conditions. For the detection of cleaved rRNAs, the resulting RNA fragment patterns can be resolved by gel electrophoresis using RNA-staining dyes. The relative abundance of the targeted species of 16S rRNA fragments in total 16S rRNA can also be quantified by determining the signal intensity of individual 16S rRNA bands in an electropherogram (without the use of external standards). Because this method does not require an external RNA standard for each experiment, as is required in membrane hybridization, and because the present method is relatively easy to perform within a short time (i.e. within 2–3 h), this technique may provide direct, rapid and easy means of the quantitative detection of particular groups of anaerobes based on their rRNA, such as those of methanogens as well.

This method has been successfully applied to the quantification of active methanogens in anaerobic biological treatment processes (Uyeno *et al*., 2004; Sekiguchi *et al*., 2005; Narihiro *et al*., 2009b). In general, oligonucleotide probes used in FISH and membrane hybridization methods can directly be used as scissor probes in the RNase H method. Recently, a total of 40 probes, including newly designed and previously reported probes listed in Table 1, have been optimized for the specific quantification of methanogens at different taxonomic levels for use in the RNase H method and have been applied to quantitative and comprehensive detection of methanogens in various types of anaerobic biosystems (Narihiro *et al*.,

2009b). As a result, methanogen populations were identified at different taxonomic levels and were influenced by the process temperature and wastewater compositions. Because of the reasons that this method is based on rRNA and that the RNA (rRNA) level is often dependent on the *in situ* activity of individual cells as described above, this method may be used for real-time monitoring of active methanogens and other important bacteria in engineered ecosystems such as waste/wastewater treatment systems to better control such bioreactors.

Stable isotope probing (SIP)-based detection of active methanogen populations in environments

To identify metabolically active populations in environments, SIP of DNA (Radajewski *et al*., 2000) and RNA (Manefield *et al*., 2002) has been used in recent years. In principle, SIP technology is based on the incorporation of ^{13}C-labelled substrates into the nucleic acids. The separation of isotopically labelled (active) fractions from unlabeled (inactive) fractions is generally performed with density gradient centrifugation. The substrate-assimilated microorganisms in the labelled fractions are identified by a set of PCR-based molecular techniques such as gene cloning, DGGE and other methods. Therefore, for the purpose of identifying active methanogens that are responsible for particular metabolisms in environments, the probes/primers listed in Tables 1 and 2 can be used.

As examples, active methanogen populations involved in the syntrophic propionate oxidation in anoxic soil were analysed on the basis of rRNA-SIP, and it was found that the members of the genera *Methanobacterium*, *Methanosarcina* and *Methanocella* play a key role in scavenging hydrogen/formate/acetate in syntrophic association with propionate-oxidizing bacteria (Lueders *et al*., 2004). Conrad and coworkers have studied the detection of active methanogen populations using DNA-SIP combined with ^{13}C-labelled CO_2, and the results of T-RFLP profiling and phylogenetic analysis for clonal 16S rRNA gene fragments suggest that members of the RC-I group (*Methanocellales*) serve as important methanogens in rice paddy fields (Lu and Conrad, 2005; Lu *et al*., 2005). The active methanogenic populations in enrichment culture of municipal solid waste digester residues spiked with ^{13}C-labelled substrates (such as cellulose, glucose and sodium acetate) were determined by DNA-SIP followed by cloning analysis (Li *et al*., 2009).

Other methods and future perspectives

DNA microarray platform, like PhyloChip, is becoming an important tool for parallel detection of different community members of microbes in ecosystems. For high throughput and comprehensive detection of methanogens in parallel, ANAEROCHIP (Franke-Whittle *et al*., 2009b) and GeoChip (Wang *et al*., 2009) have been developed recently. The primers/probes summarized in this review may be integrated into such a platform for parallel and hierarchical detection of methanogens. These primer/probes for methanogens can also be used in novel PCR-based techniques, such as the hierarchical oligonucleotide primer extension method (Wu and Liu, 2007), which has recently been developed for quantitative, multiplex detection of targeted microbial genes among PCR-amplified genes. SIP technology has been noted as an important pretreatment step for functional microbial community analyses, such as Raman microscopy-FISH (Huang *et al*., 2007; 2009) and metagenomic approaches (Kalyuzhnaya *et al*., 2008; Sul *et al*., 2009). Moreover, recent advances in analytical chemistry, such as isotope ratio mass spectrometry (Penning *et al*., 2006; Vavilin *et al*., 2008) and secondary ion mass spectrometry (Orphan *et al*., 2001), hold great promise for the highly sensitive determination of targeted microbes. Thus, in addition to describing the diversity of methanogens in particular environments of interest on the basis of DNA and RNA, such function-related analyses of methanogens may become important in the fields of environmental, determinative and applied microbiology.

As described in this minireview, a vast number of probe/primers have been developed for describing and quantifying methanogen populations, covering most parts of the known culturable methanogens described so far. A variety of molecular methods have also been developed that are used in combination with the probe/primers. Because these molecular methods have their own advancements and drawbacks, researchers need to select appropriate combinations of methods and probe/primers depending on what the researchers need to know. For details, recent reviews may be helpful for the selection of molecular techniques to be used (Talbot *et al*., 2008; Tabatabaei *et al*., 2010). In molecular ecology, multiple approaches are best to gain a complete picture of methanogen populations in environments. Therefore, the use of appropriate (multiple) molecular techniques in combinations with other non-molecular based methods like membrane lipid, autofluorescence, activity measurement and immunoenzymatic profiling should be considered. It should also be noted that there are still a number of uncultivated methanogens in various environments, and that they should be further isolated and characterized in detail. Monitoring tools for such uncultured methanogens remain to be developed to further increase in the coverage of methanogens present in environments.

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