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mcrA-Targeted Real-Time Quantitative PCR Method To Examine Methanogen Communities[▽]

Lisa M. Steinberg and John M. Regan*

Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, Pennsylvania 16802

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Methanogens are of great importance in carbon cycling and alternative energy production, but quantitation with culture-based methods is time-consuming and biased against methanogen groups that are difficult to cultivate in a laboratory. For these reasons, methanogens are typically studied through culture-independent molecular techniques. We developed a SYBR green I quantitative PCR (qPCR) assay to quantify total numbers of methyl coenzyme M reductase α-subunit (*mcrA*) genes. TaqMan probes were also designed to target nine different phylogenetic groups of methanogens in qPCR assays. Total *mcrA* and *mcrA* levels of different methanogen phylogenetic groups were determined from six samples: four samples from anaerobic digesters used to treat either primarily cow or pig manure and two aliquots from an acidic peat sample stored at 4°C or 20°C. Only members of the *Methanosaetaceae*, *Methanosarcina*, *Methanobacteriaceae*, and *Methanocorpusculaceae* and Fen cluster were detected in the environmental samples. The three samples obtained from cow manure digesters were dominated by members of the genus *Methanosarcina*, whereas the sample from the pig manure digester contained detectable levels of only members of the *Methanobacteriaceae*. The acidic peat samples were dominated by both *Methanosarcina* spp. and members of the Fen cluster. In two of the manure digester samples only one methanogen group was detected, but in both of the acidic peat samples and two of the manure digester samples, multiple methanogen groups were detected. The TaqMan qPCR assays were successfully able to determine the environmental abundance of different phylogenetic groups of methanogens, including several groups with few or no cultivated members.

Methanogens are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases, during organic matter degradation in anaerobic soils and sediment (8). Methanogens are widespread in anaerobic environments, including tundra (36), freshwater lake and wetland sediments (9, 12), estuarine and marine sediments (2), acidic peatlands (4, 14), rice field soil (10, 16), animal guts (41), landfills (30), and anaerobic digesters treating animal manure (1), food processing wastewater (27), and municipal wastewater and solid waste (37, 57). Methane produced in anaerobic digesters may be captured and used for energy production, thus offsetting some or all of the cost of operation and reducing the global warming potential of methane release to the atmosphere.

Methanogens are difficult to study through culture-based methods, and therefore many researchers have instead used culture-independent techniques to study methanogen populations. The 16S rRNA gene is the most widely used target for gene surveys, and a number of primers and probes have been developed to target methanogen groups (9, 11, 31, 36, 38, 40, 46, 48, 57). To eliminate potential problems with nonspecific amplification, some researchers have developed primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) (17, 30, 49). The Mcr is exclusive to the methanogens with the exception of the methane-oxidizing *Archaea* (18) and shows mostly congruent phylogeny to the 16S rRNA gene, allowing *mcrA* analysis to be used in conjunction with, or independently of, that of the 16S rRNA gene (3, 30, 49). A number of researchers have examined methanogen communities with *mcrA* and have found uncultured clades quite different in sequence from cultured methanogen representatives (9, 10, 12, 14, 17, 22, 28, 47).

Previous studies described methanogen communities by quantitation of different clades through the use of rRNA-targeted or rRNA gene-targeted probes with techniques such as dot blot hybridization (1, 27, 37, 38, 48) and fluorescent in situ hybridization (11, 40, 44, 57). Real-time quantitative PCR (qPCR) is an alternate technique capable of determining the copy number of a particular gene present in the DNA extracted from an environmental sample. Only a few studies have used qPCR to quantitatively examine different clades within methanogen communities, and most of these studies have exclusively targeted the 16S rRNA gene (19, 41, 42, 54–56). Far fewer researchers have used qPCR to quantify methanogen clades by targeting the *mcrA* (21, 34, 45), and these studies were limited to only a few phylogenetic groups.

In this paper we present a methodology for determining methanogen gene copy numbers through the use of qPCR targeting the *mcrA*. Methanogens were quantified in total using methanogen-specific primers in SYBR green assays and also as members of nine different phylogenetic groups using TaqMan probes targeting specific subsets of methanogens.

MATERIALS AND METHODS

Design of methanogen-specific probes. We previously constructed clone libraries for the *mcrA* and *mrtA* genes from two distinct methanogenic environments: sediment of an acidic transitional fen known as Bear Meadows Bog, and the primary digester of a municipal wastewater treatment plant treating combined

* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 865-9436. Fax: (814) 863-7304. E-mail: jregan@engr.psu.edu.

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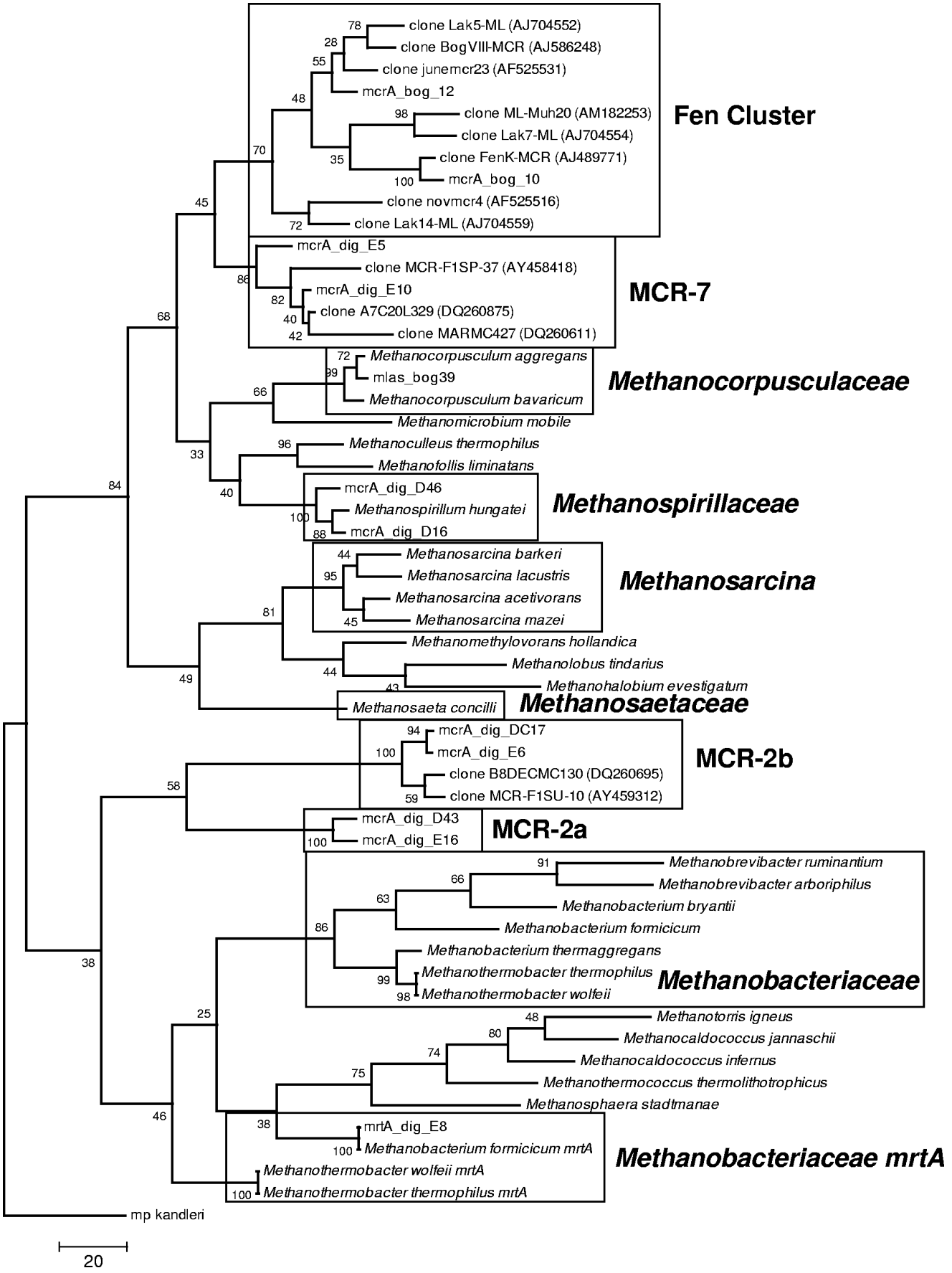
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mcrA_bog_12
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clone Lak7-ML (AJ704554)
clone FenK-MCR (AJ489771)
mcrA_bog_10
clone novmcr4 (AF525516)
clone Lak14-ML (AJ704559)
Fen Cluster
mcrA_dig_E5
clone MCR-F1SP-37 (AY458418)
mcrA_dig_E10
clone A7C20L329 (DQ260875)
clone MARMC427 (DQ260611)
MCR-7
Methanocorpusculum aggregans
mlas_bog39
Methanocorpusculum bavaricum
Methanomicrobium mobile
Methanocorpusculaceae
Methanoculleus thermophilus
Methanofollis liminatans
mcrA_dig_D46
Methanospirillum hungatei
mcrA_dig_D16
Methanospirillaceae
Methanosarcina barkeri
Methanosarcina lacustris
Methanosarcina acetivorans
Methanosarcina mazei
Methanosarcina
Methanomethylovorans hollandica
Methanolobus tindarius
Methanohalobium evestigatum
Methanosaeta concilli
Methanosaetaceae
mcrA_dig_DC17
mcrA_dig_E6
clone B8DECMC130 (DQ260695)
clone MCR-F1SU-10 (AY459312)
MCR-2b
mcrA_dig_D43
mcrA_dig_E16
MCR-2a
Methanobrevibacter ruminantium
Methanobrevibacter arboriphilus
Methanobacterium bryantii
Methanobacterium formicicum
Methanobacterium thermaggregans
Methanothermobacter thermophilus
Methanothermobacter wolfeii
Methanobacteriaceae
Methanotorris igneus
Methanocaldococcus jannaschii
Methanocaldococcus infernus
Methanothermococcus thermolithotrophicus
Methanosphaera stadtmanae
mrtA_dig_E8
Methanobacterium formicicum mrtA
Methanothermobacter wolfeii mrtA
Methanothermobacter thermophilus mrtA
Methanobacteriaceae mrtA
mp kandleri
20

TABLE 1. TaqMan probes designed for this study and used to quantify different methanogen groups from environmental DNA

Probe name	Target group	Probe sequence (5′–3′)[a]	Position[b]	Clone(s) used as standard(s) (GenBank accession no.)
mbac-mcrA	*Methanobacteriaceae mcrA*	ARGCACCKAACAMCATGGACACWGT	1154	*M. wolfeii* (AB300780), *M. arboriphilus* (AF 414035)
mrtA	*Methanobacteriaceae mrtA*	CCAACTCYCTCTCMATCAGRAGCG	1433	*M. thermoauto-trophicum* (NC 000916), mrtA_dig_E8 (EU980398)
mcp	*Methanocorpusculaceae*	AGCCGAAGAAACCAAGTCTGGACC	1319	mlas_bog39 (DQ680603)
msp	*Methanospirillaceae*	TGGTWCMACCAACTCACTCTCTGTC	1419	mcrA_dig_D16 (EU980421), mcrA_dig_D46 (EU980419)
MCR-7	Uncultured MCR-7 group	TGSCTTGACCTTRTCCWTCTCGYTS	1134	mcrA_dig_E5 (EU980422), mcrA_dig_E10 (EU980423)
MCR-2a	Uncultured MCR-2 group	CCACTCTACTGCCGGTATCAACG	1317	mcrA_dig_D43 (EU980402), mcrA_dig_E16 (EU980418)
MCR-2b	Uncultured MCR-2 group	ATGTATCTCTGCAGCAGCCGGTACA	1269	mcrA_dig_DC17 (EU980412), mcrA_dig_E6 (EU980407)
Fen	Uncultured Fen Cluster group	AAVCACGGYGGYMTCGGMAAG	1071	mcrA_bog_10 (EU980434), mcrA_bog_12 (EU980424)
msar	*Methanosarcina*	TCTCTCWGGCTGGTAYCTCTCCATGTAC	1272	*M. barkeri* (Y00158), *M. acetivorans* (NC 003552)
msa	*Methanosaetaceae*	CCTTGGCRAATCCKCCGWACTTG	1107	*M. concilli* (AF414037)

[a] R = A or G; K = G or T; M = A or C; W = A or T; Y = C or T; S = G or C; V = A, C, or G.
[b] The 5′ end of the sense strand, based on *M. thermoautotrophicum mcrA* delta H (U10036) numbering.

primary and secondary sludge (50). The alignment of these clone sequences along with sequences from cultured methanogens were used to design TaqMan probes for groups of methanogens dominant in both environments (Fig. 1; Table 1). Originally we attempted to design primers to differentiate these phylogenetic groups in a SYBR green-based qPCR, but we were unable to achieve the necessary specificity without the inclusion of the group-specific TaqMan probes. Targeted groups that were observed in the municipal wastewater sludge digester included members of the *Methanosaetaceae*, *Methanobacteriaceae mcrA* and the isoenzyme *mrtA*, *Methanospirillaceae*, MCR-7, and two subgroups of MCR-2 which were labeled MCR-2a and MCR-2b. Uncultured clades MCR-7 and MCR-2 derive from a naming scheme used by Castro et al. (9). Targeted groups observed in the clone library from the acidic fen include *Methanosarcina* spp., *Methanocorpusculaceae*, and the Fen cluster, which was named in a study by Galand et al. (14). Probes were designed for a melting temperature of 68 to 72°C, a G+C content of 40 to 60%, and a length of 23 to 30 bp. The melting temperatures of probes were calculated with Integrated DNA Technologies' Oligo Analyzer program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Probe specificity was checked with a BLAST search of GenBank sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed experimentally as described below. Probes were ordered from Biosearch Technologies (Novato, CA) and labeled with 6-carboxyfluorescein, Cal Fluor Orange, or Cal Fluor Red as the reporter dye and Black Hole 1 or Black Hole 2 as the quencher dye.

Collection of environmental DNA. Methanogen communities were analyzed from four anaerobic digesters and two incubations of acidic peat. Case studies for the design and operation of the digesters examined in this study are available through the Pennsylvania State University Department of Agricultural and Biological Engineering website (www.biogas.psu.edu). The Penn England dairy farm began operating a two-compartment reactor with a flexible cover in August 2006. Each compartment is heated to 37°C and mixed for approximately 1 hour twice daily. The design retention time in each compartment of the reactor is 20 days with 8 to 9% solids, and the system receives dairy manure, bedding, and milk parlor wash water. Bedding used in the dairy barns is composed entirely of the dried digested solids from the reactor. Grease trap waste from local restaurants is added to increase methane production. Brookside Farms began operating a plug flow reactor in April 2006 with a retention time of 30 to 33 days and 8 to 10% solids. The reactor is heated to 37°C and receives manure, sawdust bedding, and milk parlor wash water. Brewery waste and cheese whey are also added to the influent of the reactor. The Schrack dairy farm began operating a plug flow reactor in August 2006 with a retention time of 30 days and 10 to 12% solids. The reactor is heated to 37°C and receives manure, sawdust bedding, and milk parlor wash water. Crone Farms operates a plug flow digester with a retention time of 30 days and 8 to 10% solids. The reactor is heated to 37°C and treats only raw pig manure with no bedding or other amendments.

In addition to the anaerobic digesters, samples were also taken from acidic peat that had been stored for nearly 2 years at either room temperature (about 20°C) or 4°C. The peat incubations were included because, based on the previous cloning results, we expected that some of the groups targeted by these TaqMan probes would not be present in manure digesters but would be present in incubations of peat. The peat was collected in September 2006 from Bear Meadows Bog, an acidic transitional fen. Peat was transferred to sterile Pyrex medium bottles under aerobic conditions, and bottles were capped and stored with no amendments for nearly 2 years before withdrawing samples for DNA analysis. DNA from all samples was extracted using a PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. Contaminants carried over during DNA extraction inhibited PCR, but inhibition was eliminated by diluting extracts fivefold in PCR-grade water.

Preparation of templates for qPCR standard curves. Cultures of *Methanobacterium thermoautotrophicum*, *Methanobacterium wolfeii*, *Methanosarcina acetivorans*, and *Methanosarcina barkeri* MS were generously provided by the laboratory of Christopher House (Penn State University, University Park, PA). DNA from these methanogen cultures was extracted with a DNeasy DNA extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions for DNA extraction from gram-positive cells. The *mcrA* or *mrtA* gene was amplified with primers mlas and mcrA-rev by using a previously described method (50). PCR products were ligated into a pCR 2.1 vector and used to transform *Escherichia coli* Top10 cells according to the manufacturer's instructions (TA cloning kit; Invitrogen, Carlsbad, CA). Blue-white screening was used to identify transformants, and positive clones were grown overnight at 37°C in LB broth containing ampicillin. Plasmids were purified with a PrepEase plasmid purification kit (USB, Cleveland, OH) and quantified based on absorbance at 260 nm. To confirm the presence of the correct insert, plasmids were sequenced at the Nucleic Acid Facility at Penn State University. As we did not have methanogen

FIG. 1. Phylogenetic tree of representative *mcrA* sequences and standards used for real-time quantitative PCR. Boxes denote sequences targeted by the respective TaqMan probe listed in Table 1. The tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 1,000 trials. All positions containing gaps and missing data were eliminated from the data set. The scale bar represents the number of changes over the whole sequence. Classification of clusters is based on that reported by Castro et al. (8) for MCR-2, MCR-5, and MCR-7 and that of Juottonen et al. (24) for the Fen cluster.

TABLE 2. Reproducibility of the SYBR green I assay for total *mcrA* copies conducted for 18 standard curves in three separate qPCRs[a]

Plate no.	Slope	Efficiency (%)	*y* intercept	R^2
1	−3.416 ± 0.054	96.2 ± 2.1	38.78 ± 0.72	0.9954 ± 0.0037
2	−3.470 ± 0.097	94.2 ± 3.7	39.48 ± 0.49	0.9964 ± 0.0030
3	−3.464 ± 0.099	94.4 ± 3.9	40.24 ± 0.36	0.9974 ± 0.0069
Combined	−3.451 ± 0.079	94.9 ± 3.1	39.40 ± 0.69	0.9962 ± 0.0030

[a] Values are means ± standard deviations.

cultures representing *Methanospirillaceae*, *Methanocorpusculaceae*, or the Fen cluster, we used environmental clones previously obtained from a municipal sludge anaerobic digester and Bear Meadows Bog (50). Supercoiled plasmid DNA containing each of the 18 cloned fragments was used as templates for standard curves (Table 1). Plasmid DNA from each clone was diluted 10-fold in PCR-grade distilled water to create a dilution series ranging from 2.1×10^9 to approximately 2 copies per μl. From this original dilution series, it was determined experimentally that the consistent linear range for obtaining quantitative data was 2.1×10^7 to approximately 207 copies per μl, which translated to 4.15×10^7 to 415 copies per reaction mixture.

SYBR green I assays for total *mcrA* gene copies. Quantitation of total *mcrA* gene copies was performed with primers mlas and mcrA-rev (50) using the nonspecific fluorophore SYBR green I (Molecular Probes, Invitrogen). The full genomes from 19 methanogens submitted to GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) were examined for the presence of multiple copies of *mcrA*. All the genomes contained only one copy of *mcrA*, but members of the *Methanococcales* and *Methanobacteriales* also contained a copy of the gene for the isoenzyme Mrt. The primers mlas and mcrA-rev also amplify *mrtA*, so methanogen numbers inferred by gene copy numbers may be overestimated by as much as a factor of 2 when these two orders are prevalent.

The SYBR green qPCR conditions included 1× *Taq* polymerase buffer and 0.03 U/μl *Taq* (USB), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates containing uracil in place of thymidine, 0.01 U/μl of heat-labile uracil-DNA glycosylase, a 0.25 μM concentration of each primer, 0.3 M betaine, 250 μg/ml of bovine serum albumin, 10 nM fluorescein (Bio-Rad, Hercules, CA) as a reference dye, and a 75,000× dilution of SYBR green I dye (Molecular Probes). Quantitative PCR was run on a Bio-Rad iCycler with the following protocol: 20 min at 37°C to degrade contaminating PCR products, then 3.5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55° for 45 s, extension at 72°C for 30 s, and image capture at 83°C, followed by a final extension at 72°C for 7 min. Melt curve analysis to detect the presence of primer dimers was performed after the final extension by increasing the temperature from 50 to 95°C in 0.5°C increments every 10 s. Image capture at 83°C was necessary to exclude fluorescence from the amplification of primer dimers.

SYBR green qPCR was first performed in triplicate on a dilution series of each of 18 clones used as a standard for the group-specific methanogen TaqMan probes (Table 1). This was done to ensure similar amplification efficiencies when using these primers with all phylogenetic groups, as the primers used for the SYBR green assays were also used for the TaqMan probe assays. Three separate 96-well plates were necessary to quantify the dilution series for all clones. The means, standard deviations, and coefficients of variance were calculated for the dilution series within each plate and among all three plates. After testing the assay with these 18 standards, total *mcrA* copies from environmental DNA were determined in triplicate, with dilution series of clones containing the *M. thermoautotrophicum mrtA* and *Methanosaeta concilli mcrA* genes run in duplicate as controls.

TaqMan qPCR for *mcrA* copies of individual methanogen groups. TaqMan assays for *mcrA* copies from methanogen groups were verified before quantitation of environmental DNA. Each probe was checked for specificity in TaqMan assays by running all of the nontarget clone standards as negative controls. A dilution series of clones used as standards for each probe was run in three separate reactions to test the reproducibility of the TaqMan probe quantitation. The master mix for TaqMan qPCR was identical to that used for SYBR green I assays, including the primers, except that it contained 3.5 mM $MgCl_2$ instead of 2.5 mM $MgCl_2$ and 150 nM of TaqMan probe in place of SYBR green I and fluorescein dyes. Quantitative PCR was performed with the following protocol: 20 min at 37°C to degrade any contaminating product and 3.5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s and annealing/extension with image capture at 55° for 1 min. For quantitation of environmental DNA, one or two clones were used as standards for each TaqMan assay. One dilution series was constructed for each standard, and standards were run in duplicate while environmental samples were run in triplicate. The standard deviation of *mcrA* concentration for each environmental sample was determined from the standard deviation of the three threshold cycle values, which was then log transformed to gene copy number.

TABLE 3. Reproducibility of TaqMan assays targeting different methanogen groups from three standard curves from three separate qPCRs[a]

Probe	Slope	Efficiency (%)	*y* intercept	R^2
mbac-mcrA	−3.518 ± 0.141	92.4 ± 5.3	42.20 ± 0.89	0.9949 ± 0.0004
mrtA	−3.538 ± 0.064	91.7 ± 2.3	41.06 ± 0.32	0.9937 ± 0.0035
mcp	−3.487 ± 0.040	93.6 ± 1.5	42.75 ± 1.44	0.9912 ± 0.0041
msp	−3.570 ± 0.104	90.6 ± 3.7	41.66 ± 0.43	0.9930 ± 0.0040
MCR-7	−3.505 ± 0.040	92.9 ± 1.5	41.41 ± 0.26	0.9964 ± 0.0014
MCR-2a	−3.513 ± 0.077	92.6 ± 2.8	40.67 ± 0.97	0.9953 ± 0.0057
MCR-2b	−3.427 ± 0.355	95.8 ± 15.8	42.73 ± 1.47	0.9864 ± 0.0130
Fen	−3.345 ± 0.121	99.4 ± 5.2	41.24 ± 1.46	0.9930 ± 0.0043
msar	−3.477 ± 0.094	93.9 ± 3.6	43.64 ± 1.24	0.9953 ± 0.0034
msa	−3.481 ± 0.074	93.8 ± 2.8	42.45 ± 1.45	0.9977 ± 0.0016

[a] Values are means ± standard deviations.

RESULTS

Quantitative PCR assays. Standard curves for the SYBR green I qPCR were run with dilution series of all clones used as standards for methanogen group-specific TaqMan assays. A total of 18 curves were collected from three separate qPCR 96-well plates, and the means and standard deviations were calculated for assays within each plate and for all the data combined (Table 2). Similar results were obtained for each standard curve within and among plates, with an average slope of −3.451, average efficiency of 94.9%, average *y* intercept of 39.40, average R^2 of 0.9962, and a lower limit of detection of approximately 415 copies/reaction mixture. This limit of detection corresponds to approximately 2.6×10^4 gene copies/ml sludge, which takes into account the fivefold dilution of the original DNA extractions to eliminate inhibition. A slope of −3.32 represents 100% efficiency, or a doubling of the DNA products in each cycle of PCR.

TaqMan probes were tested in triplicate for specificity and reproducible amplicon detection with dilution series of clones used as standards for each probe (Table 1). Amplification and detection varied from probe to probe, with efficiencies ranging from 91 to 99% (Table 3). The lower limit of detection for each TaqMan assay was approximately 415 copies/reaction mixture, and similar *y* intercepts were obtained with each probe, suggesting all probes had about the same sensitivity for their respective targets. Quantitative PCR assays with the same probe were also similar for all three assays, which demonstrated the reproducibility of these methods. No negative controls or controls lacking template were amplified in any of the TaqMan qPCR assays, which confirmed the specificity of the probes to their targets. Several probes had two clones that were used as standards, and amplification of both clones was nearly identical for these probes (data not shown).

Quantitation of environmental DNA. DNA was extracted from six environmental samples, including four anaerobic digesters used to treat primarily animal waste and two incubations of an acidic peat maintained at either 20°C or 4°C for

nearly 2 years. Total *mcrA* was measured using a SYBR green I method, and *mcrA* from different methanogen groups was measured using the TaqMan probe method. Total *mcrA* was also calculated as the sum of the methanogen numbers obtained with each TaqMan probe. Total *mcrA* numbers obtained with both the SYBR green I and TaqMan probe methods were similar, although not identical (Table 4). The structure of the methanogen communities differed in the six environments both in terms of total *mcrA* numbers and in the numbers of different methanogen groups that were represented. All three cattle manure digesters were dominated by members of the *Methanosarcina* genus, whereas the two incubations of peat were dominated by both members of *Methanosarcina* and the Fen cluster. The Brookside digester sample showed detectable levels of only *Methanosarcina*, and the Crone digester sample showed only *Methanobacteriaceae*. The Penn England digester sample contained *Methanosaetacae*, *Methanosarcina*, *Methanobacteriaceae*, and *Methanocorpusculaceae*, and the Schrack digester sample contained *Methanosarcina* and *Methanocorpusculaceae*. The two peat incubations were the only environmental samples to contain members of the Fen cluster, but although both incubations were inoculated from the same sample, the methanogen communities were different in the two incubations. Besides members of the *Methanosarcina* genus and Fen cluster, the 20°C incubation mixture also contained *Methanosaetaceae*, whereas the 4°C incubation mixture contained members of *Methanobacteriaceae*.

TABLE 4. Total methanogen *mcrA* copies and *mcrA* from different methanogen groups from six different environments

Targeted group	Copies/ml of original sample[a]					
	Penn England	Brookside	Schrack	Crone	20°C incubation	4°C incubation
Methanosaetaceae	$7.07 \times 10^5 \pm 1.91 \times 10^5$	0	0	0	$3.45 \times 10^4 \pm 1.88 \times 10^4$	0
Methanosarcina	$3.20 \times 10^6 \pm 9.18 \times 10^5$	$1.54 \times 10^6 \pm 2.42 \times 10^5$	$4.49 \times 10^6 \pm 1.54 \times 10^6$	$1.40 \times 10^6 \pm 2.29 \times 10^6$	$1.24 \times 10^5 \pm 1.19 \times 10^5$	$1.27 \times 10^6 \pm 1.12 \times 10^5$
Methanobacteriaceae	$6.81 \times 10^5 \pm 3.96 \times 10^5$	0	0		0	$2.98 \times 10^6 \pm 1.50 \times 10^6$
Methanocorpusculaceae	$5.95 \times 10^5 \pm 9.78 \times 10^4$	0	$3.90 \times 10^5 \pm 5.23 \times 10^4$	0	0	0
Fen cluster	0	0	0	0	$6.36 \times 10^5 \pm 1.62 \times 10^5$	$6.94 \times 10^5 \pm 1.25 \times 10^5$
Total of TaqMan assays	$5.18 \times 10^6 \pm 1.60 \times 10^6$	$1.54 \times 10^6 \pm 2.42 \times 10^5$	$4.88 \times 10^6 \pm 1.59 \times 10^6$	$1.40 \times 10^6 \pm 2.29 \times 10^6$	$7.95 \times 10^5 \pm 3.00 \times 10^5$	$4.94 \times 10^6 \pm 1.74 \times 10^6$
SYBR green I	$2.53 \times 10^6 \pm 3.99 \times 10^5$	$1.04 \times 10^6 \pm 4.36 \times 10^5$	$3.13 \times 10^6 \pm 6.68 \times 10^5$	$3.63 \times 10^6 \pm 2.37 \times 10^5$	$3.55 \times 10^6 \pm 3.61 \times 10^5$	$3.95 \times 10^6 \pm 5.57 \times 10^5$

[a] Values represent copies per milliliter of original sample, which was digester effluent for the Penn England, Brookside, Schrack, and Crone environments and peat slurry for the 20°C and 4°C incubations. Only those methanogen groups detected in at least one environment are shown.

DISCUSSION

A number of studies have quantified methanogens belonging to different phylogenetic groups with techniques such as dot blot hybridization and fluorescent in situ hybridization (27, 38, 57), as well as qPCR (19, 41, 42, 54–56). Quantitation in these studies has targeted 16S rRNA or the rRNA gene, but studies by Springer et al. (49) and Bapteste et al. (3) suggest that the *mcrA* gene demonstrates the same phylogenetic relationships as the 16S rRNA gene between organisms. A major advantage to the use of *mcrA* as a target is that this gene is exclusive to methanogens, with the exception of the closely related anaerobic methane-oxidizing *Archaea*. To the best of our knowledge, the only other study besides ours that quantified phylogenetically different groups of methanogens by targeting *mcrA* is by Shigematsu et al., who designed TaqMan probes to target *Methanosaeta*, *Methanosarcina*, and *Methanoculleus* in acetate-fed anaerobic reactors (45). Our study is the first to quantify methanogens of other phylogenetic groups using *mcrA* as the target, including methanogens of the uncultured group MCR-2, and also members of the previously uncultured Fen cluster and MCR-7 groups.

Methanosarcina was the most commonly detected methanogen clade in the six samples and was the only methanogen group detected in the Brookside digester. Both *Methanosarcina* and *Methanosaetaceae* were detected in the Penn England digester sample and the acidic peat incubation at 20°C, and in both samples there were approximately four times more *Methanosarcina* than *Methanosaetaceae* detected. Although *Methanosarcina* and *Methanosaetaceae* are the only aceticlastic methanogens, *Methanosaetaceae* are obligately aceticlastic, whereas *Methanosarcina* are also able to produce methane

from H_2/CO_2 and methylated compounds (52). *Methanosaetaceae* have a lower K_s for acetate than *Methanosarcina*, but as cleavage of acetate yields the least energy for *Methanosarcina* among its metabolic options (52), members of these two genera may not always directly compete for acetate, and *Methanosarcina* has been reported to dominate the methanogenic community of anaerobic digesters even when acetate levels are low (26, 55). Although both *Methanosarcina* and *Methanosaetaceae* were detected in the 20°C acidic peat incubation, *Methanosarcina* was the only detected aceticlastic methanogen in the 4°C incubation (Table 4). At colder temperatures homoacetogenic bacteria, which produce acetate from H_2 and CO_2, outcompete hydrogenotrophic methanogens for H_2 (33, 43) and more methane is produced from acetate than H_2/CO_2 (36). Thus, in tundra, deep lake sediments, and other permanently cold environments methanogenesis proceeds predominantly from cleavage of acetate (32, 33, 43). It is possible that in the 20°C incubation, acetate was sufficiently low to allow *Methanosaetaceae* to outcompete *Methanosarcina*, but at 4°C homoacetogenesis may have led to the accumulation of acetate and dominance of the faster-growing *Methanosarcina*. Similar to our findings, some studies of permanently cold environments have found *Methanosarcina* to be the only aceticlastic methanogen present (23, 32).

Both *Methanocorpusculaceae* and *Methanobacteriaceae* are commonly detected in anaerobic digesters treating a variety of wastes. In this study, *Methanocorpusculaceae* was detected in the Penn England and Schrack digesters, while *Methanobacteriaceae* was also detected in the Penn England digester and was the only methanogen clade detected in the Crone digester. The genus *Methanobrevibacter*, within the *Methanobacteriaceae*, is often the dominant methanogen in animal guts (51, 53), so it is not surprising that this clade was the only methanogen group detected in a digester treating only pig waste. It is interesting that *Methanobacteriaceae* were the dominant methanogens in the 4°C peat incubation but were not detected in the 20°C peat incubation (Table 4). Although *Methanobacteriaceae* have been detected in northern wetlands and freshwater lake sediments which experience yearly cold seasons (20, 24, 32), they are rarely detected in permanently cold sediments, such as tundra and polar lakes. This observation, coupled with the fact that no psychrophilic members of the *Methanobacteriaceae* have been isolated, suggests that methanogens of this family do not compete well at cold temperatures. So, while it is not unusual that *Methanobacteriaceae* would be present in the original peat prior to incubation, it is unusual that they would become the predominant methanogen at 4°C but not at 20°C. Although *mcrA* of *Methanobacteriaceae* was detected, the isoenzyme *mrtA* of *Methanobacteriaceae* was not. The primers used in this study previously amplified both *mcrA* and *mrtA* from *Methanobacteriaceae* (50), so the failure to detect the *Methanobacteriaceae mrtA* is most likely due to a limitation in the coverage of the probe designed for this study. For quantitative analysis, a probe for *mcrA* of *Methanobacteriaceae* alone is sufficient. Originally, probes for both *mcrA* and *mrtA* of *Methanobacteriaceae* were designed to allow greater coverage of this diverse family.

The Fen cluster was detected in both of the acidic peat incubations but not in any of the digester samples. Bräuer et al. recently isolated and characterized a member of the Fen cluster, "*Candidatus* Methanoregula boonei," from an acidic peatland (6). This isolate was found to be hydrogenotrophic and acidophilic, with optimum growth at pH 5. The Fen cluster, which has also been referred to as MCR-5 (9) or E1 and E2 (4), commonly dominates acidic freshwater environments (7, 13). If members of this cluster exhibit maximal growth at acidic pH, then this may indicate why they were not detected in any of the circumneutral anaerobic digester environments.

Although the Penn England, Brookside, and Schrack dairy manure digesters treat similar waste, Penn England had the most diverse methanogen community. The Brookside, Schrack, and Crone digesters are all plug flow designs, whereas the Penn England reactor is a periodically mixed tank reactor. In addition, bedding used in the Penn England barns is composed of the dried, digested solids from the reactor, which return to the reactor when the barns are cleaned. If some methanogens present in the digested solids survive desiccation and oxygen exposure, they are eventually returned to a favorable environment in the reactor. Active methanogens have been obtained from oxic environments, including aerated activated sludge (15) and the A horizon of forest, savanna, and desert soils (35). This may essentially increase the retention time for both methanogens and other bacteria in this reactor and may allow a more diverse methanogen population to emerge.

As the detection limit for the TaqMan qPCR method was approximately 2.6×10^4 copies per ml of reactor effluent, minor methanogen groups may have existed in numbers lower than this in the environmental samples but could not be detected by this qPCR method. So, although only one methanogen clade was detected in the Brookside and Crone digesters, this does not mean that only one methanogen clade was present. In addition, a TaqMan probe was not designed to capture some members of the order *Methanomicrobiales*, including the genus *Methanoculleus*, which is commonly detected in anaerobic digesters (25, 39). We designed TaqMan probes to target methanogen clades present in clone libraries obtained from a peat bog and an anaerobic digester treating municipal wastewater sludge, and *Methanoculleus* was not detected in these libraries (50). The inability to detect minor community members may partially explain the difference between total *mcrA* numbers as detected by SYBR green I assays and the total *mcrA* calculated by adding the numbers obtained in TaqMan assays, although it is more likely that differences in these numbers are due to the errors of the methods themselves. Even though the two calculations of total *mcrA* do not perfectly agree, they are close (Table 4). Sufficiently large clone libraries may be able to detect minor community members that are present in numbers too low to be detected by qPCR, but PCR bias may render abundance of a sequence in a clone library an unreliable predictor for abundance of that organism in the actual sample. Quantitative PCR may overcome some of these biases and thus serve as a complementary molecular technique for determination of relative abundance of a sequence in an environment (22, 29). Other sources of bias, such as differences in cell lysis and DNA extraction efficiencies among different phylogenetic groups or environmental samples, still affect qPCR results, but the determination by qPCR of the relative numbers of microbes of different clades

provides a more in-depth examination of a microbial community than can be provided by clone libraries alone.

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