

Phylogenetic Comparison of the Methanogenic Communities from an Acidic, Oligotrophic Fen and an Anaerobic Digester Treating Municipal Wastewater Sludge^{∇†}

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Methanogens play a critical role in the decomposition of organics under anaerobic conditions. The methanogenic consortia in saturated wetland soils are often subjected to large temperature fluctuations and acidic conditions, imposing a selective pressure for psychro- and acidotolerant community members; however, methanogenic communities in engineered digesters are frequently maintained within a narrow range of mesophilic and circumneutral conditions to retain system stability. To investigate the hypothesis that these two disparate environments have distinct methanogenic communities, the methanogens in an oligotrophic acidic fen and a mesophilic anaerobic digester treating municipal wastewater sludge were characterized by creating clone libraries for the 16S rRNA and methyl coenzyme M reductase alpha subunit (*mcrA*) genes. A quantitative framework was developed to assess the differences between these two communities by calculating the average sequence similarity for 16S rRNA genes and *mcrA* within a genus and family using sequences of isolated and characterized methanogens within the approved methanogen taxonomy. The average sequence similarities for 16S rRNA genes within a genus and family were 96.0 and 93.5%, respectively, and the average sequence similarities for *mcrA* within a genus and family were 88.9 and 79%, respectively. The clone libraries of the bog and digester environments showed no overlap at the species level and almost no overlap at the family level. Both libraries were dominated by clones related to uncultured methanogen groups within the *Methanomicrobiales*, although members of the *Methanosarcinales* and *Methanobacteriales* were also found in both libraries. Diversity indices for the 16S rRNA gene library of the bog and both *mcrA* libraries were similar, but these indices indicated much lower diversity in the 16S digester library than in the other three libraries.

Methanogens are an integral part of carbon cycling on the planet, anaerobically catalyzing the production of methane primarily through carbon dioxide reduction with hydrogen or the conversion of methylated compounds such as acetate (64). These *Archaea* are essential community members in anaerobic digesters, which may be used to treat a variety of domestic, agricultural, and industrial wastes with concomitant energy production from the combustion of the methane-containing biogas. Anaerobic digestion is typically operated at circumneutral pH and mesophilic (30°C to 37°C) or thermophilic (55°C to 65°C) temperatures, with process stability decreasing dramatically outside of this range of conditions (5, 34, 38, 39). In these systems, methanogens are considered to be the most sensitive members of the methanogenic consortium to nonoptimal conditions (1, 21, 29), yet methanogenesis is known to proceed in both cold and acidic natural environments such as tundra (28, 63), *Sphagnum*-dominated peatlands (35, 49), and lake sediments (48, 55). Presumably, this difference in psychro- and acidotolerance can be attributed, in part, to differences in the methanogen communities, and a knowledge of these differences might lend insights into community-based strategies to

increase digester stability with reduced chemical and energy inputs necessary to maintain narrow operating conditions.

Methanogens are difficult to isolate or culture under laboratory conditions, so communities are often examined through culture-independent techniques such as the amplification and sequencing of target DNA from environmental samples. The most widely used target for molecular analysis is the 16S rRNA genes, and a number of primers and probes have been developed specifically for methanogens or groups of methanogens (10, 44, 51, 52, 53, 57, 59, 65). 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*) (26, 43, 60). Mcr catalyzes the last step of methanogenesis and is conserved among all methanogens. Phylogenetic inference with *mcrA* sequences is similar to that obtained with 16S rRNA gene sequences, suggesting no lateral transfer (3, 43, 60). Moreover, Mcr is absent in all nonmethanogens, with the exception of the anaerobic methane-oxidizing *Archaea*, which are closely related to the methanogens (27). Due to the fact that methanogens may be examined exclusively from other bacteria present in an environment, *mcrA* has been increasingly used for phylogenetic analysis coupled with, or independent of, 16S rRNA genes.

The increasing use of molecular-based community analysis has led to the identification of phylogenetic clusters of methanogens that are quite divergent in sequence from those of isolated, phenotypically described methanogens. Ecological indices such as the Shannon-Wiener or Simpson's indices and

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species accumulation curves, which are used to determine the extent of sampling of an environment, have been adapted for use in microbial gene surveys (30). The use of these ecological indices requires that genes or gene fragments be organized into operational taxonomic units (OTUs) representing a species or strain. Boone et al. (6) previously suggested a 16S rRNA gene sequence similarity of less than 98% as evidence for a separate methanogen species, which is slightly more restrictive than the minimum 97% sequence similarity suggested previously by Stackebrandt and Goebel (61) for sequences to be considered from the same species. Although these studies provide a basis for determining if gene sequences represent new species, little information is available about sequence similarities used to determine new taxonomic levels of methanogens above the species level. In addition, although many researchers use *mcrA* sequences alone or coupled with 16S rRNA gene sequences to examine methanogenic communities, little attention has been given to appropriate sequence similarity limits for *mcrA* in establishing a separate species or genus.

The hypothesis of this research is that the methanogen community of a mesophilic anaerobic digester is distinct from that of an acidic peat bog. The methanogen communities from these two environments were explored by creating 16S rRNA gene and *mcrA* clone libraries. To characterize the extent of community differences between these libraries, in silico analysis was performed on existing *mcrA* and 16S rRNA gene sequences for phenotypically characterized methanogens to describe the range of sequence similarity within currently accepted taxonomic levels.

MATERIALS AND METHODS

Site characteristics and sampling. Bear Meadows Bog is an acidic, boreal peatland situated approximately 15 miles southeast of State College, PA. It is classified as a transitional bog, and vegetations consist predominantly of *Sphagnum* mosses, sedge grasses, and highbush blueberry, with peat approximately 1 m thick. The bog pH varies from 3.8 to 5.0, and bog waters contain little alkalinity, sulfate, or iron (J. F. Biddle, C. Turich, S. Brantley, and M. A. Bruns, presented at the American Geophysical Union, San Francisco, CA, 6 to 10 December 2002). Porewater methane reaches a maximum of 5,500 ppm at 30 cm below the water-sediment interface. Methane emitted from the bog has an isotopic signature of -60% , suggesting a biogenic origin (Biddle et al., presented at the American Geophysical Union, San Francisco, CA, 6 to 10 December 2002). Temperatures in the region range from approximately -15°C to 35°C and precipitation are fairly evenly spaced throughout the year, with an average of 98 cm of rain and 115 cm of snow (www.nws.noaa.gov).

Cores were taken from two locations at Bear Meadows Bog covered with both *Sphagnum* mosses and sedges. Each core was collected in a series of three 6-in.-long Teflon sleeves (Ben Meadows Company, Janesville, WI) for a total of 1.5 feet extracted at each location. Immediately upon collection, the cores were placed into a sealed container with a GasPak (BBL Inc., Franklin Labs, NJ) to create an anaerobic environment (H_2/CO_2) for transport.

Anaerobic digester sludge was collected from the State College Wastewater Treatment Plant. The plant operates two fixed-cover primary digesters, which receive primary sludge and thickened secondary sludge and are constantly mixed and heated to 37°C . The pH is kept near neutral by adjusting the organic loading rate, and the solid retention time is 25 days. Collected sludge was transported anaerobically in a sealed container with a GasPak.

Construction of 16S rRNA gene and *mcrA* clone libraries. Clone libraries were constructed for both methanogen-specific 16S rRNA genes and *mcrA* from the Bear Meadows Bog and anaerobic digester samples. DNA was extracted using a PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA). For the bog peat cores, DNA extractions were performed on sediment taken from the central portions of the top and bottom of each of the three 6-in. core segments for a total of six DNA extractions from the bog peat. All of the DNA extractions from the bog peat were pooled before performing PCR for library construction.

A fragment of the 16S rRNA genes was amplified with A21f, an *Archaea*-

specific forward primer (16), and Eury498 (8), a reverse primer developed from a *Euryarchaeota*-specific probe. PCR conditions were based on those reported previously by Girguis et al. (23). The *mcrA* sequences were amplified with three different forward primers, ME1 (26), ML-f (43), and mlas (5'-GGTGGTGTMGGDTTCACMCARTA-3'), which is a truncated version of forward primer ML-f, designed for a better melting temperature match with the reverse primer, a lower 3' stability to improve specificity, and two additional degeneracies to improve coverage (31). The ML primer set is reported to have wide coverage of methanogen groups, detecting members of the *Methanosarcinaceae*, *Methanosarcinaceae*, *Methanobacteriales* (both *mcrA* and *mrtA*), *Methanococcales*, and the uncultured groups fen cluster, rice cluster I, and MCR-2 (17, 18, 32, 43, 58). The ME primer set also captures a wide range of methanogens (2, 19, 20) and has been reported to capture methanogen groups related to the fen cluster not detected by the ML primer set, although the ME primers are reported to have difficulty amplifying members of the *Methanosarcinaceae* (33). We thought that by using both forward primers ML and ME, we would capture a greater representation of the methanogen diversities at these sites. All three forward primers were paired with the same reverse primer, *mcrA*-rev (5'-CGTTCATBGC GTA GTTVGGRTAGT-3'), which is a consensus sequence of three previously reported reverse primers for *mcrA* (26, 43, 60). Reaction conditions were the same for all three *mcrA*-targeted primer sets and included an initial denaturation step at 95°C for 3 min, followed by five cycles of denaturation at 95°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 30 s, with a ramp rate of $0.1^{\circ}\text{C}/\text{s}$ from the annealing to the extension temperature. These initial five cycles were followed with 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s, followed by a final extension step at 72°C for 10 min.

PCR products were ligated into vector pCR 2.1 using a TA cloning kit, and the ligation products were used to transform *Escherichia coli* Top10 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Ampicillin- and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)-amended LB agar was used for blue-white screening of transformants, which were subject to whole-cell PCR directly to amplify the plasmid insert. PCR products were sent to the Nucleic Acid Facility at Penn State University for sequencing, and sequencing electropherograms were examined for accuracy using Sequence Scanner v1.0 (Applied Biosystems, Inc., Foster City, CA).

Determination of sequence similarity cutoffs for methanogen taxonomic levels. 16S rRNA and *mcrA* gene sequences from isolated methanogens were analyzed to determine how to group clone sequences from the libraries into appropriate OTUs. A current list of methanogens with recognized taxonomic standing is maintained by the Subcommittee on Taxonomy of Methanogens of the International Committee on Systematics of Prokaryotes (<http://www.the-ictp.org/taxa/methanogenslist.htm>). Using this accepted taxonomic structure, corresponding 16S rRNA gene and *mcrA* sequences for these methanogens were obtained from GenBank, when available. Sequences were eliminated from the analysis if they were too short or contained several undetermined bases ($>2\%$ of the sequence). Sequences belonging to *Methanocaldococcus* sp. were eliminated from the analysis, as the placement of this group is still under study by the International Committee on Systematics of Prokaryotes. Sequences for *Methanospirillum hungatei* and *Methanopyrus kandleri* were also eliminated from the analysis, as they each represent the only sequence for their genus and family. Sequences were truncated from bp 114 to 1435 (*E. coli* numbering) for 16S rRNA genes and bp 1014 to 1423 (*Methanobacterium thermoautotrophicum* numbering) for *mcrA*. These truncations were necessary to ensure that all sequences were of the same length prior to the analysis. The sequences were aligned with ClustalW (<http://www.ebi.ac.uk/clustalw/>), and Mega3 (36) was used to create distance matrices based on the Jukes-Cantor algorithm. Sequences were grouped together by taxonomic level, and Mega3 was used to calculate the mean sequence similarities within each taxonomic unit and between taxonomic units. For instance, within the genus *Methanobacterium*, the average sequence similarity was calculated based on comparisons of each species to every other species in that genus. To calculate the percent similarity within the family *Methanobacteriaceae*, the mean similarity was calculated based on the similarities between each species in this family and every other species in a separate genus of this family.

Phylogenetic and diversity analyses of bog and digester communities. The program DOTUR (54) was used to group clone sequences into OTUs based upon decreasing levels of similarity using the furthest-neighbor algorithm, which states that any two sequences in a group defined by a percent similarity are at least that similar to one another. The input files for DOTUR were distance matrices generated by Mega3 using a Jukes-Cantor algorithm. EstimateS (version 7.5; R. K. Colwell [<http://purl.oclc.org/estimates/>]) was used to calculate Coleman rarefaction curves, Shannon-Wiener and Simpson indices, and Chao1 and abundance-based coverage estimator (ACE) values. For the 16S rRNA gene

TABLE 1. Sequence percent similarity values within the methanogens for 16S rRNA genes^a

Family	Mean % similarity	Genus	No. of isolates in analysis	Range of % similarity (mean)	GenBank accession no.
<i>Methanobacteriaceae</i>	92.8	<i>Methanobacterium</i>	11	92.1–99.8 (95.4)	M36508, AY386124, DQ649335, AY350742, M59124, AF233586, AF095261, AF028690, AF093061, DQ649330, AF095264
		<i>Methanobrevibacter</i>	9	88.2–97.1 (94.0)	AY196666, AF242652, AB065294, U62533, U41095, U82322, U55239, U55237, U55240
		<i>Methanosphaera</i>	1	NA	M59139
		<i>Methanothermobacter</i>	5	98.6–99.9 (99.3)	X68720, X99046, X99047, X99048, AB104858,
<i>Methanococcaceae</i>	93.4	<i>Methanococcus</i>	4	91.4–95.9 (93.5)	M36507, U39016, U38486, U38461,
		<i>Methanothermococcus</i>	2	(95.0)	M59128, AB057722,
<i>Methanocaldococcaceae</i>	95.6	<i>Methanocaldococcus</i>	5	95.7–98.8 (96.8)	M59126, AF056938, AF547621, AF025822, AF051404
		<i>Methanotorris</i>	1	NA	AB100884
<i>Methanomicrobiaceae</i>	92.7	<i>Methanomicrobium</i>	1	NA	M59142
		<i>Methanoculleus</i>	6	95.7–99.9 (97.3)	AB065298, AB038795, M59134, Y16382, AB065297, AF531178
		<i>Methanofollis</i>	3	95.7–99.7 (97.3)	AF095272, AY186542, Y16428,
		<i>Methanogenium</i>	3	90.0–98.4 (93.2)	M59130, AJ862839, DQ177345
		<i>Methanolacinia</i>	1	NA	AY196678
<i>Methanoplanaceae</i>	94.4	<i>Methanoplanus</i>	2	(94.4)	M59143, U76631,
<i>Methanocorpusculaceae</i>	99.9	<i>Methanocorpusculum</i>			
<i>Methanosarcinaceae</i>	92.9	<i>Methanosarcina</i>	4	99.7–100.0 (99.9)	AY260435, AF095266, AY260436, AF095268
		<i>Methanococcoides</i>	8	93.7–99.1 (96.6)	AY196682, AE010299, AY663809, DQ058823, AB065296, AJ012742, U89773, M59140
		<i>Methanohalobium</i>	2	(97.6)	M59127, X65537
		<i>Methanohalophilus</i>	1	NA	U20149
		<i>Methanolobus</i>	1	NA	M59133
		<i>Methanomethylovorans</i>	4	96.6–98.2 (97.4)	U20148, U20152, U20154, U20155,
		<i>Methanimicrococcus</i>	1	NA	AF120163
<i>Methanosaetaceae</i>	92.5	<i>Methanosaeta</i>	2	92.5	M59146, AB071701
Mean % similarity	94.3			96.0	
With multiple genera	93.5				

^a Ranges and means were calculated with distance matrices created with the Jukes-Cantor algorithm. NA, not applicable.

libraries, statistics were conducted for OTUs of 98 and 96% similarity, respectively, representing similarity within a methanogen species suggested previously by Boone et al. (6) and similarity within a methanogen genus as determined by this study. For the *mcrA* libraries, statistics were conducted for OTUs of 89% similarity, representing the average *mcrA* sequence similarity of methanogens within a genus, as there is no standard *mcrA* divergence for determining a new methanogen species. For each run, 100 randomizations were made with sample replacement. To construct phylogenetic trees, clones were grouped with DOTUR by family-level associations as determined in this study. One representative sequence was chosen from each clone group, and these clone sequences were aligned with ClustalW, and phylogenetic trees were created in Mega3 using the maximum parsimony method with clone-neighbor interchange search level 1 and bootstrapped with 100 trials. Trees for both the 16S rRNA genes and *mcrA* were rooted with *Methanopyrus kandleri* as the outgroup.

Nucleotide sequence accession numbers. Sequences derived from the analysis were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) under accession numbers DQ680352 to DQ680670 and DQ781023 to DQ781063.

RESULTS

Sequence similarities for methanogen taxonomic levels. The mean percent similarities of methanogen 16S rRNA gene se-

quences were calculated for 15 genera containing at least two sequences and eight families representing four orders of methanogens (Table 1). Results obtained using a Kimura two-parameter algorithm (data not shown) were nearly identical to those obtained with the Jukes-Cantor algorithm. The average sequence similarity of species within a genus was 96.0% with a range of 88.2 to 100%. The average sequence similarity within a family was 94.3% when considering all methanogen families and was 93.5% for families containing at least two genera. The mean percent similarities of *mcrA* sequences were calculated for 15 genera and eight families of methanogens (Table 2). The average percent similarity of *mcrA* sequences within a genus was 88.9%, which ranged from 69.3 to 100%. The average similarity overall within a family was 83.5% and was 79.0% for families containing at least two genera.

Collector's curves and diversity indices of bog and digester communities. A total of 97 methanogen 16S rRNA gene sequences were obtained, 47 from the bog sediment and 50 from

TABLE 2. Sequence percent similarity values within the methanogens for *mcrA*^a

Family	Mean % similarity	Genus	No. of isolates in analysis	Range of % similarity (mean)	GenBank accession no.
<i>Methanobacteriaceae</i>	71.7	<i>Methanobacterium</i>	4	69.3–75.8 (73.1)	AF414050, AY386125, AF313806, AY289750
		<i>Methanobrevibacter</i>	4	77.2–90.0 (82.6)	AF414046, AF414035, DQ251045, DQ251046
		<i>Methanosphaera</i>	1	NA	AF414047
		<i>Methanothermobacter</i>	4	97.2–100.0 (98.6)	U10036, AY303950, AY289752, AY289748
<i>Methanothermaceae</i>	98.0	<i>Methanothermus</i>	2	(98.0)	J03375, AY289747
<i>Methanococcaceae</i>	83.2	<i>Methanococcus</i>	4	76.0–92.7 (83.3)	M16893, AY354034, BX957223, X07793
		<i>Methanothermococcus</i>	2	85.9	AF414048, AY354033
<i>Methanocaldococcaceae</i>	81.4	<i>Methanocaldococcus</i>	2	82.5	AF414040, AY354035
		<i>Methanotorris</i>	1	NA	AF414039
<i>Methanomicrobiaceae</i>	79.5	<i>Methanomicrobium</i>	1	NA	AF414044
		<i>Methanoculleus</i>	5	89.3–100.0 (92.1)	AF414036, AB288270, NZAASI01000002, AF313804, DQ229156
		<i>Methanofollis</i>	1	NA	AF414041
		<i>Methanogenium</i>	4	82.8–93.8 (86.9)	DQ229157, DQ229158, DQ229159, DQ229160
<i>Methanocorpusculaceae</i>	94.9	<i>Methanocorpusculum</i>	3	93.8–97.1 (94.9)	AY260445, AF414049, AY260441
<i>Methanosarcinaceae</i>	79.4	<i>Methanosarcina</i>	7	88.3–99.3 (91.8)	Y10058, AE010299, AY260443, AF414043, U22248, U22250, U22251
		<i>Methanococcoides</i>	2	96.0	U22235, U22234
		<i>Methanohalobium</i>	1	NA	U22236
		<i>Methanohalophilus</i>	3	95.0–99.0 (96.7)	U22237, U22259, U22239
		<i>Methanolobus</i>	5	87.4–96.3 (91.6)	U22244, U22257, U22242, U22243, U22245
		<i>Methanomethylovorans</i>	1	NA	AY260442
		<i>Methanosalsum</i>	1	NA	U22252
<i>Methanosaetaceae</i>	79.6	<i>Methanosaeta</i>	2	79.6	AF313802, CP000477
Mean % similarity	83.5			88.9	
With multiple genera	79.0				

^a Ranges and means were calculated using distance matrices created with the Jukes-Cantor algorithm. NA, not applicable.

the digester sludge. A total of 259 *mcrA* sequences were obtained, 105 from the bog sediment and 154 from the digester sludge. For the 105 *mcrA* sequences derived from the bog, 35, 38, and 32 sequences were obtained with ME, ML, and mlas as the forward primer, respectively. For the 154 *mcrA* sequences obtained from the digester sludge, 21, 72, and 61 sequences were obtained with ME, ML, and mlas as the forward primers, respectively.

The 16S rRNA genes and *mcrA* libraries were analyzed by constructing species accumulation curves and calculating diversity indices. Minimum sequence similarities of 98 and 96% were used to group 16S rRNA gene sequences into OTUs, as these values represented the suggested sequence similarity within a methanogen species (6) and the calculated average sequence similarity of species within a genus from this study (Tables 1 and 2). Sequences for *mcrA* generated from all three forward primers were analyzed together, but sequences identified as the isoenzyme *mrtA* were excluded from this analysis. Only members of the *Methanobacteriales* and the *Methanococcales* possess the *Mrt* operon (21), so these sequences were excluded to avoid a biased overrepresentation of species from these orders in the library. At a sequence similarity of 98%, the 16S rRNA gene libraries were grouped into 26 and 16 clades

for the bog and digester, respectively. At a sequence similarity of 96%, the 16S rRNA gene libraries were grouped into 19 and 7 clades for the bog and digester, respectively. At a sequence similarity of 89%, *mcrA* sequences were grouped into 24 clades each for the bog and digester libraries.

Based on these clone groupings, Coleman rarefaction curves, which represent how extensively the genetic diversity at a site has been sampled, were constructed for both environments and gene targets (Fig. 1). The diversity indices Chao1, ACE, Shannon-Wiener index (H'), and Simpson's index (D) were calculated using EstimateS (Table 3). The values for Chao1 and ACE represent the expected number of OTUs present in an environment if sampling were complete (30). The Shannon-Wiener index (H') and Simpson's index are measures of species richness and the evenness of distribution of species within a community, and both increase with increasing genetic diversity at a site. Comparing the 16S rRNA gene libraries, diversity was lower and dominance was greater in the digester than in the bog library at both the 96 and 98% similarity cutoffs for OTU groupings (Table 3). As expected, for both 16S rRNA gene libraries, the rarefaction curves show a flattening with a cutoff of 96% versus 98%, but the change in curve shape was much more dramatic for the 16S rRNA gene library (Fig. 1).

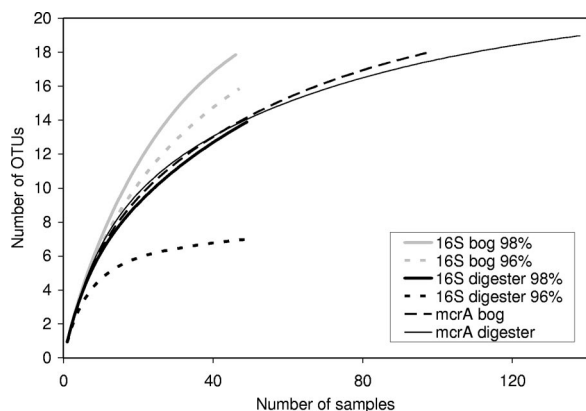


FIG. 1. Coleman rarefaction curves of 16S rRNA and *mcrA* gene clone libraries obtained from the acidic peatland and anaerobic digester environments. The 16S rRNA gene sequences were grouped by 98 or 96% similarity and *mcrA* sequences were grouped by 89% similarity using the program DOTUR. Rarefaction curves were generated using the program EstimateS at 100 randomizations made with sample replacement.

When the *mcrA* libraries from the bog and digester were compared, there was no difference in diversity or dominance indices (Table 3). The diversity indices for the *mcrA* libraries were estimated for a genus-level grouping and were compared with the 16S rRNA gene libraries at a genus-level grouping of 96% similarity. There was no difference in diversity when the 16S rRNA gene library was compared to the *mcrA* libraries of the bog and digester, but the 16S rRNA gene library did show less diversity than the other three libraries. Ecological indicators often underestimate the true richness of a community at low sample sizes (30), and as the 16S rRNA genes libraries were much smaller than the *mcrA* libraries, this may have contributed to the lower richness values for the 16S rRNA gene digester library.

Phylogeny of bog and digester communities. Sequences in the libraries were grouped at the family level using DOTUR, with minimum sequence similarity values of 93.5 and 79% for the 16S rRNA and *mcrA* genes, respectively. One representative sequence from each group was chosen for incorporation in the phylogenetic trees and was identified by BLAST analysis (see the supplemental material). The 16S rRNA gene sequences grouped into 15 clades for the bog and 7 clades for the digester, with no incidence of clones from both environments being present in the same clade (Fig. 2). For the 16S rRNA gene libraries, nearly all of the clones from Bear Meadows Bog

closely grouped with clones from acidic freshwater environments, including two central New York State peatlands (4, 9) and a freshwater lake located downstream of an acidic *Sphagnum* bog (12). The 16S rRNA gene digester sequences were related to clones obtained from a variety of environments, including full-scale anaerobic digesters (GenBank accession numbers DQ386720, DQ399803, and AB266913; see Table S1 in the supplemental material), lotus field soil (GenBank accession number AB236107), and part of the Florida Everglades impacted by agricultural runoff (13).

The *mcrA* sequences grouped into 12 clades for the bog and 14 clades for the digester, with two clades containing representative sequences from both the bog and digester libraries (Fig. 3). Trends are seen in the *mcrA* libraries that are similar to those seen with the 16S rRNA gene libraries, with most of the bog sequences being most closely related to clones from oligotrophic freshwater lakes and peatlands (17, 18, 19, 32) and the majority of the digester sequences being most closely related to clones from anaerobic digesters (GenBank accession numbers AY937276, AY937277, AY937285, DQ260701, DQ260681, DQ260578, and DQ262263; see Table S2 in the supplemental material) and pasture and rice paddy soil (22, 56).

Both the bog and digester libraries were dominated by clones that were related to uncultured methanogen groups. The majority of clones from the bog environment were members of the fen cluster, a deeply branching clade of the *Methanomicrobiales* that was first identified by Galand et al. (20) but has also been labeled by other researchers as MCR-5 and E1 and E2 (4, 10). Three groups of uncultured methanogens were represented exclusively within the bog clone libraries. These include rice clusters I and II, first described in Großkopf et al. (25), and two sequences that are related (89% similarity) to a branch within cluster MCR-5 (Fig. 3). Members of this cluster are related to members of the *Methanomicrobiales* isolated from an oligotrophic area of the Florida Everglades (10). The majority of the digester 16S rRNA gene clones grouped with sequences from cluster I, isolated from a variety of bioreactors and described previously by McHugh et al. (45), and with sequences from a nutrient-impacted area of the Florida Everglades that are grouped in *Methanomicrobiales* clusters E1 and E2, as described previously by Cadillo-Quiroz et al. (9). Most of the *mcrA* digester clones group with cluster MCR-7, which was identified previously by Castro et al. (10) during a molecular survey of oligotrophic and nutrient-impacted areas of the Florida Everglades. A number of digester clones grouped into cluster MCR-2, a cluster also defined in the study described

TABLE 3. Diversity indices for bog and digester 16S rRNA gene and *mcrA* libraries using species-level groupings

Library (% similarity)	Mean diversity index			
	Chao1 (95% confidence interval)	ACE ± SD	Shannon-Wiener index (<i>H'</i>) ± SD	Simpson's diversity index (1/ <i>D</i>) ± SD
16S bog (98)	28.02 (20.16, 65.43)	30.2 ± 8.96	2.51 ± 0.16	10.99 ± 3.15
16S bog (96)	20.66 (15.32, 54.5)	19.07 ± 4.58	2.28 ± 0.14	8.44 ± 1.9
16S digester (98)	17.33 (12.72, 48.36)	17.19 ± 4.78	2.05 ± 0.15	6.85 ± 1.18
16S digester (96)	10.73 (9.3, 24.78)	10.74 ± 1.86	1.84 ± 0.12	5.59 ± 0.81
<i>mcrA</i> bog	25.83 (21.69, 52.1)	24.27 ± 3.07	2.65 ± 0.09	11.93 ± 1.71
<i>mcrA</i> digester	26.06 (21.17, 57.1)	24.81 ± 4.3	2.5 ± 0.08	9.94 ± 0.93

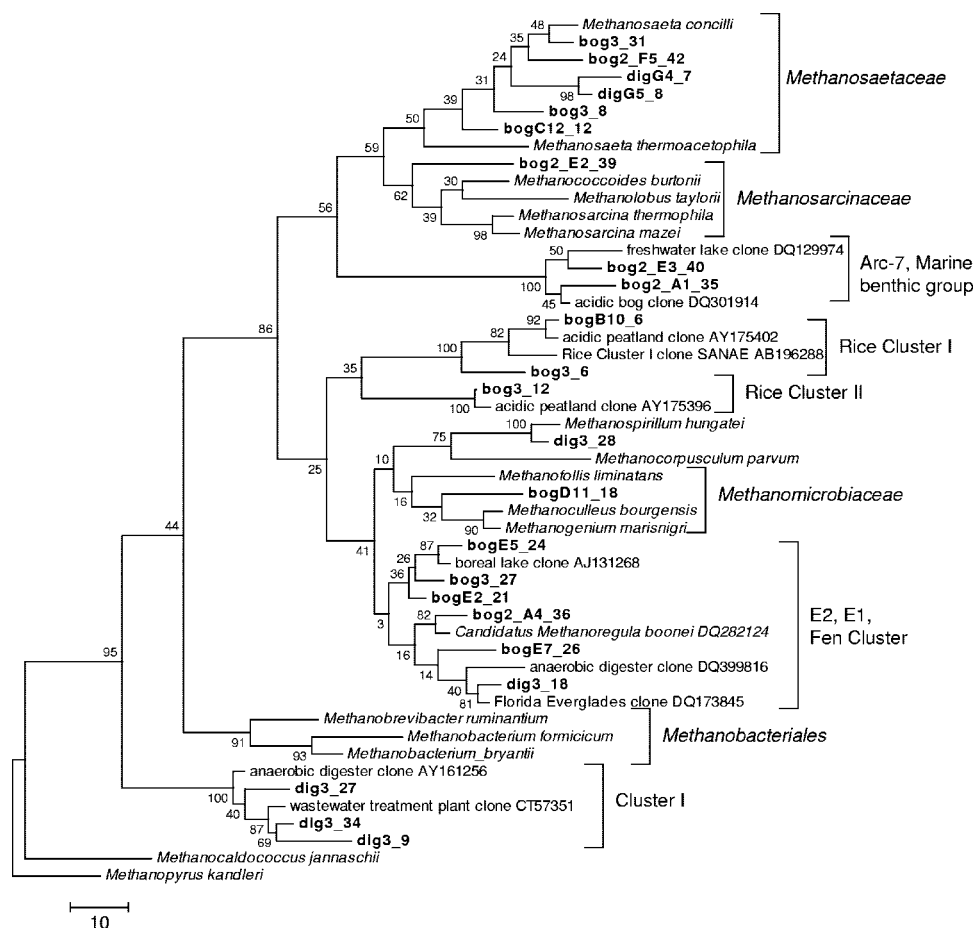


FIG. 2. Phylogenetic tree for representative 16S rRNA gene sequences (shown in Table S1 in the supplemental material) obtained from an acidic peatland (clones labeled “bog”) and an anaerobic digester treating municipal wastewater sludge (clones labeled “dig”). The tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 100 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. The classification of clusters is based on data described previously by Vetriani et al. (62) for the marine benthic group, which was also named Arc-7 by Castro et al. (10); Großkopf et al. (25) for rice clusters I and II; Cadillo-Quiroz et al. (9) for E1 and E2, also named fen cluster by Juottonen et al. (32); and McHugh et al. for cluster I (45).

previously by Castro et al. (10). In that study, all sequences belonging to MCR-2 and MCR-7 were found only in the digester environment.

Although the majority of clones obtained in this study were closely related to other uncultured clone sequences, a number of clone sequences from both libraries were closely related to cultured methanogen species. The bog environment included clones related to *Methanocorpusculum parvum* (GenBank accession no. AF414045) and *mrtA* of *Methanothermobacter thermophilus* (accession no. AY289753), and several clones from the digester environment were closely related to *Methanospirillum hungatei* strain JF-1 (accession no. NC007796) and *Methanobrevibacter ruminantium mcrA* (accession no. AF414406).

DISCUSSION

The methanogens of the bog and digester environments were found to be very different from one another, and although this result was expected, the degree of difference in comparing sequences from both environments was surprising. There was

nearly no overlap in either 16S rRNA or *mcrA* gene sequences even at a level of similarity representing the average similarity within a methanogen family. Few studies have compared methanogens in natural wetlands to those found in constructed environments, and the present study is the first to directly compare methanogen communities in a naturally occurring wetland and a constructed anaerobic reactor. In addition to containing very different methanogen sequences, both the bog and digester libraries were dominated by clones belonging to uncultured environmental groups. The majority of clones from both the 16S rRNA gene and *mcrA* libraries of the bog environment were related to the fen cluster, a deeply branching clade within the *Methanomicrobiales*. The fen cluster may no longer be considered an uncultured group, as Brauer et al. (7) recently isolated and described a member of this group, “*Candidatus Methanoregula boonei*,” which was found to be acid tolerant and hydrogenotrophic. The Fen cluster has been identified in a number of other peatland environments, many of which are acidic, oligotrophic bogs. The majority of clones from the 16S rRNA gene library of the digester environment

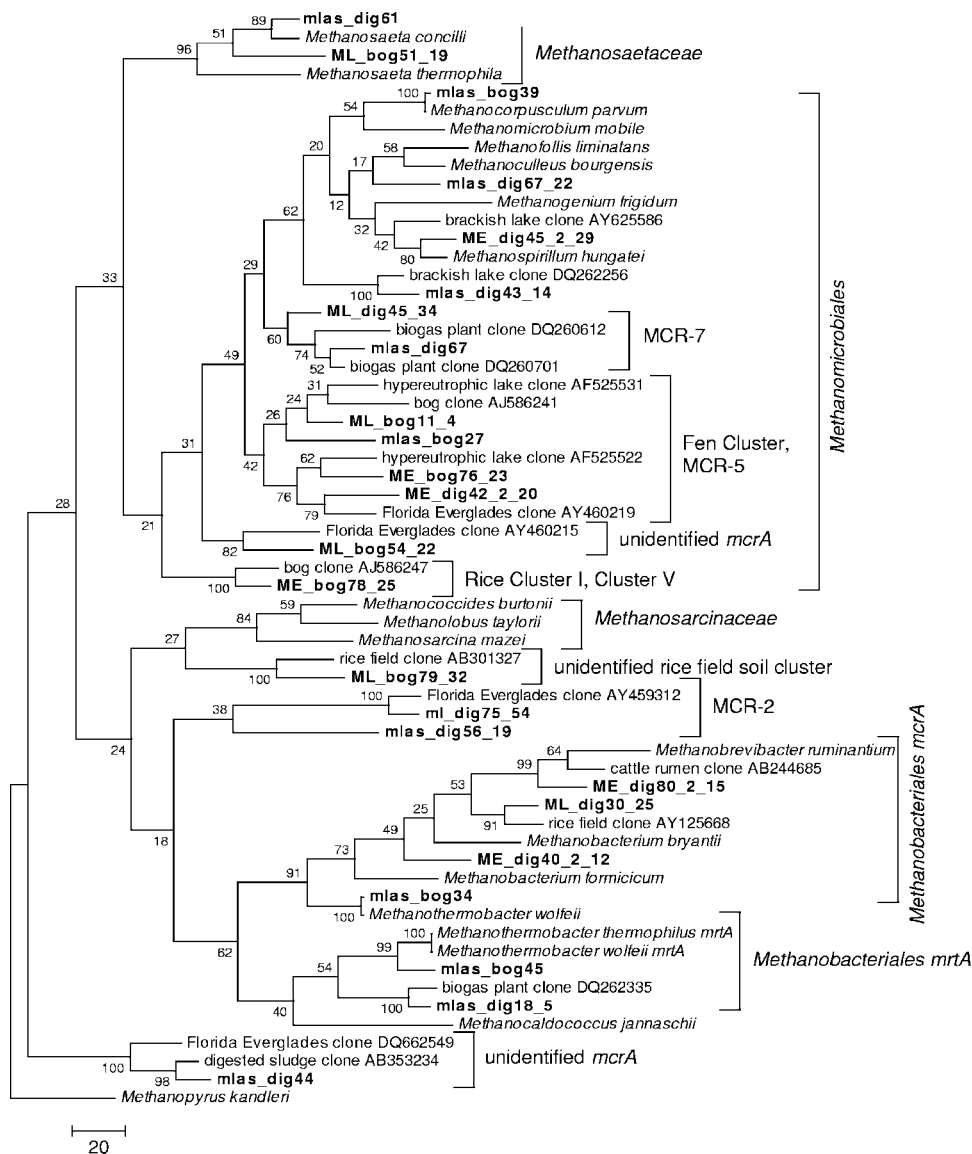


FIG. 3. Phylogenetic tree for representative *mcrA* sequences (shown in Table S2 in the supplemental material) obtained from an acidic peatland (bog) and an anaerobic digester treating municipal wastewater sludge (dig). The tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 100 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. The classification of clusters is based on data described previously by Castro et al. (10) for MCR-2, MCR-5, and MCR-7; Juottonen et al. (32) for the fen cluster; Großkopf et al. (27) for rice cluster I, also named cluster V by Galand et al. (20); and Lueders et al. (41) for the unidentified rice field soil cluster.

grouped with cluster I, members of which have been found in a variety of anaerobic digesters (14, 45, 50). The majority of clones in the *mcrA* library from the digester grouped with clade MCR-7, which has been found in a wide range of environments including oligotrophic and nutrient-impacted areas of the Florida Everglades (10), biogas plants (GenBank accession no. DQ260701, DQ260681, DQ260578, and DQ262263; see Table S2 in the supplemental material), and brackish lake sediment (2). Cluster I is related to the *Methanobacteriales*, and MCR-7 is found within the *Methanomicrobiales*, so it is likely that members of cluster I and MCR-7 are hydrogenotrophic. This suggests that both environments are dominated by hydrogenotrophic methanogens, although acetotrophic methanogen

sequences closely related to *Methanosaeta* were also found in both environments. Members of the *Methanosaeta*, which are strictly acetotrophic, appear to be ubiquitous and have been isolated from a number of different environments including freshwater marshes and lakes (10–12), acidic peatlands (4, 18, 32), rice field soil (41), and anaerobic digesters treating municipal solid waste and sewage sludge (24, 46), dairy wastewater (40), or industrial wastewater (15). Despite the abundance of clones representing hydrogenotrophic methanogens in all libraries, it should be noted that sequence abundance in a clone library does not necessarily relate to the abundance of the organism in the environment.

The analyses of sequences from accepted methanogen

taxonomy performed in this study illustrate the difficulty in gene fragment-based surveys of environmental microorganisms. Springer et al. (60) found that the average *mcrA* sequence distance between species was approximately three times greater than the distance of the 16S rRNA gene sequences. This correlates well to the difference between the average sequence distances found for the 16S rRNA gene and *mcrA* in this study. Despite this fact, the range of similarity within a methanogen genus varied from 88.2 to 99.9% for the 16S rRNA genes and from 69.3 to 100.0% for *mcrA*. Currently, the definition of species applied to methanogen 16S rRNA gene clone libraries varies from researcher to researcher, and there is no species definition applied to *mcrA* libraries. Ecological richness estimators can be used for microbial communities only if strict OTU definitions are consistently applied (30). The consistent application of an OTU definition also makes it possible to compare diversity measurements among studies. The goal for determining average sequence similarity within genera and families of methanogens in this study was primarily to calculate richness estimators and characterize differences between the two methanogen communities that we studied, but these data were also used to relate *mcrA* sequence disparity to phylogenetic distance.

The clone libraries for ME, ML, and mlas as the forward primers were compared to detect any difference in *mcrA* sequence recovery among the three forward primers. In general, a clone group sharing at least 80% similarity was detected by all three primers. Clone groups with only one or two clones were not detected by all three primers, but this is likely a limitation of the number of clones sampled in the different libraries. With both primers ML and mlas, clone groups with more than one clone were obtained solely with that primer. This was most dramatic for forward primer mlas, which had four groups of unique clones. These results suggest that forward primers ML and mlas may capture gene sequences not captured by forward primer ME. The ME primer set has been reported to not detect sequences from *Methanosaeta* sp. or sequences of the isoenzyme *mrtA* (2), a finding mimicked in the current study.

A few authors looked at primer bias in obtaining *mcrA* sequences from the natural environment. Lueders et al. (44) found that a cluster of *mcrA* sequences, which was named "unidentified rice field soil *mcrA*" by those authors, could be detected with the MCR, but not the ME, primer pair. Nercessian et al. (47) amplified *mcrA* sequences from deep-sea hydrothermal vents with ML and ME primer sets and recovered different sequences in both libraries, with only some overlap in clones (47). Juottonen et al. (33) examined potential bias in the three *mcrA* primer sets in obtaining clones from drained peatland sediment. Similar clone sequences were obtained with all sets, but four minor OTUs were not detected by all sets, although those authors could not rule out the number of clones sequenced as being responsible for these differences in coverage. The dominant OTUs obtained with each primer set differed, which in turn affected ecological diversity measurements of the clone libraries, namely, the Shannon-Wiener and Simpson's dominance indices (33). Results from these studies suggest that more comprehensive libraries of *mcrA* diversity may be obtained by using two or more primer sets. A limitation in comparing the results of these studies is that different PCR

parameters, notably the annealing temperature, were used in each study. Lueders and Friedrich previously demonstrated that the numbers and types of clones obtained in an *mcrA* library generated with the ML primer set was determined by the choice of annealing temperature (42), so both the choice of primer set and PCR conditions will affect the clones obtained in a library.

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