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Pyrosequencing of *mcrA* and Archaeal 16S rRNA Genes Reveals Diversity and Substrate Preferences of Methanogen Communities in Anaerobic Digesters

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Methanogenic archaea play a key role in biogas-producing anaerobic digestion and yet remain poorly taxonomically characterized. This is in part due to the limitations of low-throughput Sanger sequencing of a single (16S rRNA) gene, which in the past may have undersampled methanogen diversity. In this study, archaeal communities from three sludge digesters in Hong Kong and one wastewater digester in China were examined using high-throughput pyrosequencing of the methyl coenzyme M reductase (*mcrA*) and 16S rRNA genes. *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* were detected in each digester, indicating that both hydrogenotrophic and acetoclastic methanogenesis was occurring. Two sludge digesters had similar community structures, likely due to their similar design and feedstock. Taxonomic classification of the *mcrA* genes suggested that these digesters were dominated by acetoclastic methanogens, particularly *Methanosarcinales*, while the other digesters were dominated by hydrogenotrophic *Methanomicrobiales*. The proposed euryarchaeotal order *Methanomassiliicoccales* and the uncultured WSA2 group were detected with the 16S rRNA gene, and potential *mcrA* genes for these groups were identified. 16S rRNA gene sequencing also recovered several crenarchaeotal groups potentially involved in the initial anaerobic digestion processes. Overall, the two genes produced different taxonomic profiles for the digesters, while greater methanogen richness was detected using the *mcrA* gene, supporting the use of this functional gene as a complement to the 16S rRNA gene to better assess methanogen diversity. A significant positive correlation was detected between methane production and the abundance of *mcrA* transcripts in digesters treating sludge and wastewater samples, supporting the *mcrA* gene as a biomarker for methane yield.

Methane from anaerobic digestion is an important source of renewable energy that can circumvent the problems of dwindling fossil fuels and of atmospheric carbon dioxide (CO_2) emissions due to fossil fuel combustion (1). Anaerobic digestion is becoming a key part of municipal wastewater treatment, as it allows recovery of energy (biogas) from waste streams to offset onsite energy consumption. The anaerobic digestion of heterogeneous organic substrates for methane production is a complex process involving four major sequential phases: hydrolysis, fermentation, acetogenesis, and methanogenesis (2). Methanogens are strictly anaerobic archaea that produce methane from a limited number of substrates, including hydrogen (H_2), acetate, and some C_1 compounds (2). Phylogenetically, methanogens belong to the *Euryarchaeota* with six established (*Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanocellales*, *Methanopyrales*, and *Methanosarcinales*) and one proposed (*Methanomassiliicoccales*) order(s) (3) and at least 31 genera (4). As well as being major functional components of anaerobic digester communities (5, 6), methanogens are found in other anoxic environments such as peatlands (7), landfills (8), rice paddy fields (9), and ruminant gut (10), all of which emit methane to the atmosphere. The diversity and abundance of methanogens in anaerobic digesters are critical to operating efficiency, since methanogenesis is usually the rate-limiting step (2). Evidence from the better-characterized bacterial component of digester communities suggests that a high level of functional redundancy despite variable taxonomic composition may be an important feature of these communities (11, 12). However, many of the major methanogen groups in anaerobic digesters remain unknown or poorly understood (5), and variation between digesters has been little examined.

Methanogen phylogeny can be determined by sequencing the 16S rRNA gene using archaea-specific (13) or methanogen-specific (14) primers, and/or the α subunit of the methyl coenzyme M reductase (*mcrA*) gene (14, 15). *mcrA* encodes the enzyme catalyzing the terminal step in methanogenesis and is ubiquitous among known methanogens (15). Methanogen phylogenies determined using the *mcrA* and 16S rRNA genes are largely congruent (8, 15), and both genes have been used to elucidate the diversity and phylogeny of methanogens in anaerobic digesters (14–17). Methanogens in mixed communities have traditionally been investigated either by cultivation-dependent methods (18, 19) or Sanger sequencing of 16S rRNA or *mcrA* gene clone libraries (14, 20). Thus far, pyrosequencing of the *mcrA* gene has only seen limited application, e.g., to examine methanogens in river sediments (21) and an algal fed anaerobic digester (22), and this method has been underutilized relative to the potential taxonomic

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TABLE 1 Operating conditions and performance of the digesters analyzed in this study

Parameter[a]	Treatment plant[b]			
	ST	SWH	YL	GZ
Salinity (ppt)	9.7	0	0	0
Daily methane production (m^3)	9,663	2,476	455	211
Daily vol processed (m^3)	1,910	453	163	700
Operating temp (°C)	32−37	34−37	33−35	27−28
pH[c]	6.6 ± 0.1	7.2 ± 0.1	6.5	7.2 ± 0.2
Retention time (days)	11	23	40	0.5
Removal of total solids (%)	27	47	62	
Removal of volatile solids (%)	24	11	12	
Removal of chemical oxygen demand (%)				87.6

[a] ppt, parts per thousand. Daily methane production measurements were obtained at ambient temperature and pressure.
[b] ST, SWH, and YL are located in Hong Kong and treat sludge from secondary treatment; GZ is in Guangzhou, China, and treats organic wastewater. The typical operating parameters are shown. Removal percentages are averages of 1 month of data before and after sampling as provided by the respective plants.
[c] Ranges are given for 1 month of data. For YL, only one pH measurement was taken by plant operators during the month.

diversity. An improved taxonomic understanding of the methanogens involved will aid the optimization of anaerobic digesters to increase methane yield and other industrially important parameters.

In the present study, we report the taxonomic diversity of methanogens in four full-scale anaerobic digesters by analyzing the *mcrA* and archaeal 16S rRNA genes with 454 pyrosequencing. Of the four digesters, three treat fresh or saline municipal sludge, and one treats industrial organic wastewater. Digesters with different operating conditions and input streams were selected to examine methanogen composition across a broad range of digester types. In addition, the correlation between *mcrA* transcription and methane production is experimentally assessed to evaluate the applicability of *mcrA* expression as a biomarker for methanogenesis.

MATERIALS AND METHODS

Sample collection and DNA extraction. Samples were collected during October and November 2011 from three full-scale municipal anaerobic digesters that treat sludge following secondary treatment, located at Sha Tin (ST), Shek Wu Hui (SWH), and Yuen Long (YL) in Hong Kong, and from one industrial anaerobic digester that treats organic wastewater from a beverage manufacturing company located in Guangzhou (GZ), China. Detailed descriptions of the four digesters, including operational parameters measured by the plant operators using standard methods (23), are provided in Table 1. Multiple sludge samples were collected from each digester while the system was in stable operation. Samples for molecular analysis were centrifuged at 6,200 × *g* for 10 min at 4°C and stored at −80°C until processing. Samples for cultivation experiments were kept at 35°C and used as inocula within 48 to 72 h. Genomic DNA (gDNA) was extracted as described previously (24). Briefly, two independently collected ~250-mg sludge samples from each digester were pooled and DNA extracted with a PowerSoil DNA extraction kit (MoBio Laboratories, CA). The final DNA concentration was ~100 ng/μl, and the A_{260}/A_{280} ratio was ~1.90.

PCR amplification and 454 pyrosequencing. gDNA was amplified with primers specific to the *mcrA* gene (8) and the archaeal 16S rRNA gene (25) according to the PCR protocols indicated in these references. To enable sample multiplexing during sequencing, barcodes were incorporated between the adapter and forward primer. Triplicate PCRs were performed for each sample, and the amplicons were pooled and purified. Equimolar concentrations were sequenced on a Roche 454 GS FLX Titanium platform (Roche, NJ) as described previously (24).

Sequence analysis. A total of 16,810 raw *mcrA* reads were generated, and were processed using the mothur pipeline (v1.32) with default parameters (26). Denoising was performed using the mothur command shhh.flows, an implementation of the PyroNoise algorithm (27) using the default parameters (maximum of 1,000 iterations; minimum change between iterations before stopping, 10^{-6}; cutoff, 0.01; sigma, 0.06; flow order, TACG). Chimeras were identified with the mothur command chimera.uchime, a wrapper for the UCHIME package (28), with default parameters, and likely chimeric sequences were removed. Low-quality reads that likely resulted from pyrosequencing errors (>2-bp difference from primer sequence in primer region; >1-bp difference from barcode sequence in barcode region; >8-bp homopolymer runs; <300-bp length) were removed from further analysis. Barcode and primer sequences were removed. The remaining reads were compared to the NCBI nonredundant (nr) database using BLAST to ensure the top hit (sequence similarity) was a *mcrA* gene. After quality control, 16,634 reads were retained with 3,388 in the GZ digester sample, 4,947 in ST, 3,717 in SWH, and 4,582 in YL. These reads were then clustered into operational taxonomic units at 97% sequence similarity (OTU_{mcrA}).

A custom database of *mcrA* sequences was constructed by downloading all *mcrA* sequences from the Functional Gene Repository v.7.3 (29). The sequences were checked against the NCBI nr database, and 939 sequences that attracted matches with taxonomic information from the domain to family levels were retained. The database was manually curated to ensure all major methanogen families were represented. The OTU_{mcrA} were taxonomically classified against this database using the Wang algorithm (30) implemented in mothur.

In order to account for differences in sequencing depth between samples, the read sets were normalized by randomly selecting 3,388 sequences (the number in the smallest sample, GZ) from each sample. Weighted UniFrac distances were calculated in mothur, and Principal Coordinates Analysis (PCoA) was performed with the cmdscale function in the R package vegan (31, 32). A Venn diagram was constructed using the R package VennDiagram (33) to evaluate the number of OTU_{mcrA} shared between the four digesters. To visualize the relationship between the most abundant OTU_{mcrA} and the differences between each digester, Pearson correlation coefficient was calculated between the rarefied abundances for the 20 most abundant OTU_{mcrA} (abundance averaged over the four digesters) and the PCoA axes using the add.spec.scores function from BiodiversityR (34). OTU_{mcrA} richness (Chao1 estimator), evenness (Pielou index, J'), and diversity (Shannon-Weaver index, H') were calculated in vegan. Rarefaction curves were generated using mothur. To investigate the detailed phylogenetic affiliations of the most abundant OTU_{mcrA}, representative sequences for the 20 most abundant OTU_{mcrA} (abundance averaged over the four digesters) and 28 additional sequences (selected representatives of each methanogen order and *mcrA* sequences from the NCBI nr database with high BLAST similarity to the abundant OTU_{mcrA}) were aligned using MUSCLE (35), and a neighbor-joining (NJ) tree with Jukes-Cantor correction constructed in MEGA v.5.2.2 (36). The tree was rooted to accurately represent the evolutionary relationship between methanogen groups and allow comparison with 16S rRNA-based phylogeny. The *Methanopyrus kandleri mcrA* gene was selected as the outgroup sequence as it is deeply branching; no nonmethanogen sequence could be used since only methanogens carry the *mcrA* gene (8).

Quality checks and sequence clustering were performed as described above via the mothur pipeline for the archaeal 16S rRNA gene sequences to form OTU_{16S}. OTU_{16S} were taxonomically classified against the Greengenes database (37) using the Wang algorithm as described above. In total, 8,446 high-quality archaeal 16S rRNA sequences were used for downstream analyses, with 1,159 in the GZ digester sample, 2,849 in ST, 2,682 in SWH, and 1,756 in YL. The subsampling depth for normalization was set

TABLE 2 Read counts, OTU counts, and alpha diversity indices for *mcrA* and 16S genes[a]

Treatment plant	*mcrA*					16S				
	No. of reads (no. rarefied)	No. of OTU (no. rarefied)	Chao1	*H′*	*J′*	No. of reads (no. rarefied)	No. of OTU (no. rarefied)	Chao1	*H′*	*J′*
GZ	3,388 (3,388)	119 (119)	371	1.2	0.36	1,159 (1,159)	85 (88)	194	2.0	0.59
ST	4,947 (3,388)	214 (167)	538	1.6	0.46	2,849 (1,159)	119 (58)	317	0.97	0.34
SWH	3,717 (3,388)	221 (215)	580	1.7	0.50	2,682 (1,159)	105 (67)	202	1.5	0.49
YL	4,582 (3,388)	210 (180)	592	1.2	0.38	1,756 (1,159)	138 (109)	272	2.1	0.63

[a] Alpha diversity indices were calculated from rarefied read sets. The numbers of reads refer to reads that passed quality checks.

at 1,159 reads. Calculation of diversity indices and weighted UniFrac distances, PCoA ordination, regression of shared OTU onto PCoA axes, and Venn diagram construction were performed as described above.

***mcrA* transcription.** Sludge samples collected from the GZ and SWH digesters were incubated with food waste as described previously (24). Briefly, 50 ml of sludge was incubated with 5 g of volatile solids/liter of food waste as a heterogeneous substrate and incubated at 35°C. Each experiment was run in duplicate, and control experiments without substrate were also prepared. Methane concentration was measured by a gas chromatograph equipped with a flame ionization detector (GC-2010 Plus; Shimadzu, Japan) and methane production rate was calculated by the difference in methane yield between time points. After a linear increase in methane concentration commenced, 1 ml of culture was periodically collected from each replicate, pooled, and centrifuged at 13,800 × *g* for 6 min at 4°C, and the cell pellet was stored at −80°C until processing.

Total RNA was extracted from the frozen cell pellet using the RNeasy minikit (Qiagen, CA) and residual DNA was removed with the Qiagen RNase-free DNase set. Total RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (NanoDrop Products, DE). A portion (2 μl) of total RNA was reverse transcribed to cDNA with random hexamers according to the manufacturer's protocol using the SuperScript III first-strand synthesis system (Invitrogen, CA). After reverse transcription (RT), *mcrA* transcript abundances were determined on a StepOnePlus quantitative PCR (qPCR) system (Applied Biosystems, CA) with 1× SYBR green PCR master mix (Applied Biosystems), 0.3 μM *mcrA*-F/*mcrA*-R primers (8), and 2 μl of cDNA template in a 25-μl reaction volume. Triplicate RT-qPCRs were performed for each sample along with a control reaction without reverse transcriptase.

Absolute quantification of *mcrA* transcripts was based on RNA standards transcribed *in vitro* using the MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's protocol with *mcrA* PCR product as the template. The synthesized RNA was treated with DNase to remove DNA contaminants and complete DNA removal was confirmed by triplicate RT-qPCRs without reverse transcriptase in which no fluorescence signal was detected after 40 cycles. Copy number was calculated from the size of the input PCR product and an average molecular mass of 340 Da per RNA nucleotide.

Accession number. Sequences obtained in the present study have been deposited in the NCBI Sequence Read Archive (SRA) (BioProject accession number PRJNA245382).

RESULTS

Characteristics of the anaerobic digesters. The four digesters examined in the present study are operated under different conditions and are geographically separated, with three (ST, SWH, and YL) located in Hong Kong and one (GZ) about 200 km to the north (details on digester operating conditions and performance provided in Table 1). Among the four digesters, only ST treats saline wastewater (9.7 ppt salinity). ST, SHW, and YL treat concentrated sludge following secondary treatment and operate at similar temperatures, but the processing capacity and volume of methane produced vary in the order ST > SHW > YL. The retention time of the three digesters also differs, with ST having the shortest (11 days) and YL the longest (40 days). In contrast to the other three digesters, GZ treats organic wastewater from industrial manufacturing and is small compared to other industrial wastewater systems (700 m^3/day) with a short retention time (12 h). GZ's operating temperature is ~5°C lower than the other digesters (Table 1).

Sequencing statistics and diversity estimates. After the low-quality reads were filtered out, a total of 16,634 *mcrA* and 8,446 16S rRNA gene reads were retained for downstream analyses (Table 2). Rarefaction curves for both OTU_{mcrA} and OTU_{16S} did not plateau in any sample, indicating additional sampling effort would be required to completely assess community diversity (see Fig. S1 in the supplemental material), and neither gene had a clear advantage in capturing overall diversity, with greater diversity captured by the 16S rRNA gene in samples GZ and YL but with greater diversity captured by the *mcrA* gene in samples SWH and ST. This was supported by the Shannon-Weaver diversity index (*H′*, Table 2). The archaeal (16S rRNA gene) and methanogenic (*mcrA*) communities were highly uneven, with fewer than six OTU_{mcrA} or five OTU_{16S} from any sample comprising more than 100 sequences (see Fig. S2 in the supplemental material).

Community composition of methanogens and archaea. Methanogens (OTU_{mcrA}) from the order *Methanomicrobiales* were dominant in the ST (63% of sequences) and GZ (79%) digesters, while *Methanosarcinales* dominated SWH (43%) and YL (52%) (Fig. 1). Each of the two *Methanobacteriales* families *Methanobacteriaceae* and *Methanothermaceae* was also detected, with the former comprising 40% of *mcrA* sequences in YL, 17% in SWH, 2.2% in GZ, and 1.5% in ST, and the latter found only in ST (0.040%). The majority of *Methanomicrobiales* OTU_{mcrA} were unclassified, although the families *Methanomicrobiaceae* (0.22 to 1.0%), *Methanoregulaceae* (0.020 to 2.7%), and *Methanospirillaceae* (GZ 0.12%, ST 16%, SWH 10%, YL 0.48%) were identified in all four digesters and *Methanocorpusculaceae* in ST (0.061%). The failure to classify most *Methanomicrobiales* to the family level may be due to the paucity of available *mcrA* sequences for candidate or recently described *Methanomicrobiales* genera (e.g., *Methanolinea* and *Methanoregula*) (38). Of the order *Methanosarcinales*, the family *Methanosaetaceae* was most abundant (GZ 8.3%, ST 1.0%, SWH 42%, YL 52%), though *Methanosarcinaceae* were also detected (0.27 to 2.3%). Finally, a small proportion of OTU_{mcrA} from family *Methanopyraceae* (order *Methanopyrales*) were detected in GZ (5.1%), SWH (0.027%), and YL (0.48%) (although the dominant *Methanopyrales* OTU_{mcrA} may be better classified as a member of the *Methanomassiliicoccales*; see *Discussion*). No OTU_{mcrA} were classified as members of the methanogenic orders *Methanococcales* or *Methanocellales*, despite there be-

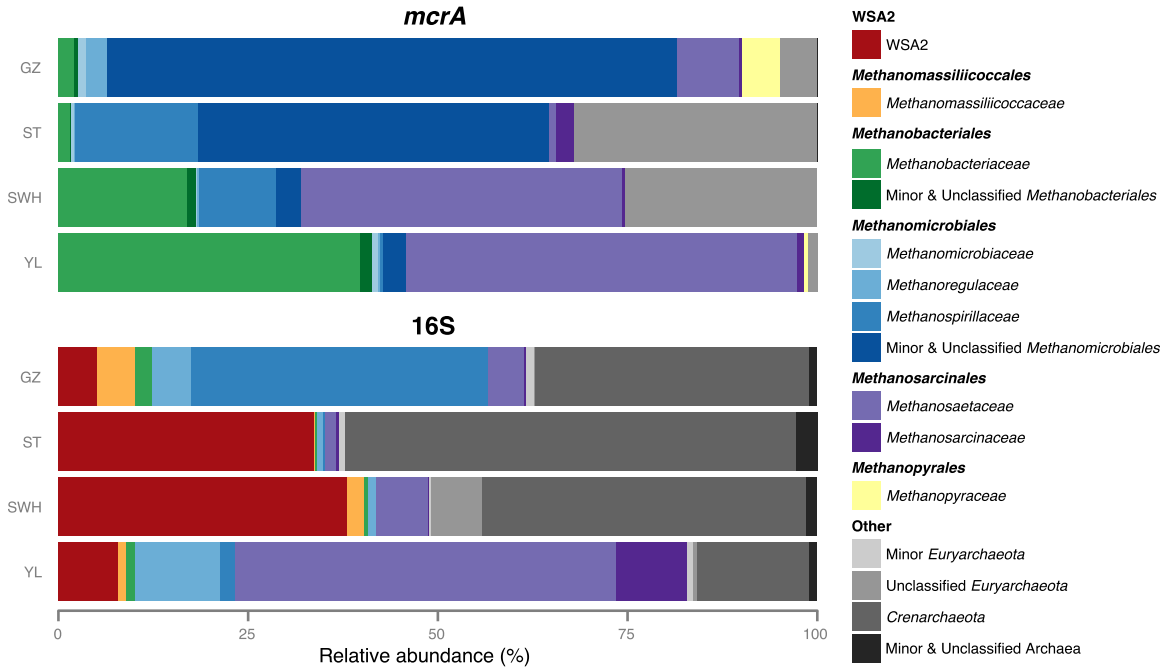

FIG 1 Taxonomic composition of methanogen (*mcrA*) and archaea (16S) communities. Selected nonmethanogen, low-abundance, and unclassified taxa (including OTU assigned by the Wang algorithm to poorly defined taxa) have been aggregated for clarity. Note that a large proportion of *Methanopyrales* OTU_{mcrA} may be better classified as *Methanomassiliicoccales*, and a large proportion of unclassified OTU_{mcrA} may represent the WSA2 group (see Discussion).

ing representative *mcrA* sequences for these orders in the reference database.

Overall, the OTU_{mcrA} profile of each digester could be characterized as either *Methanomicrobiales* dominated (GZ and ST) or *Methanosaetaceae* and *Methanobacteriaceae* dominated (SWH and YL), with each also containing a minority population of the nondominant order(s) as well as *Methanopyrales* and unclassified OTU_{mcrA}. However, these taxonomic similarities may break down at the OTU level (see "Comparison of digesters," below).

In the archaeal community, OTU_{16S} affiliated with both the *Crenarchaeota* and *Euryarchaeota* were detected in addition to some OTU_{16S} unclassifiable past the domain level (0.21 to 0.74%) (Fig. 1). A large proportion of OTU_{16S} (GZ 5.2%, ST 34%, SWH 38%, YL 7.9%) were assigned to the uncultured group WSA2, classified in the Greengenes v.13_5 taxonomy as a family of the order *Methanobacteriales*. WSA2 (sometimes named "ArcI" or "Arc I" after reference 39) has been previously detected at high abundance in anaerobic digesters and found to form a class-level monophyletic lineage within the *Euryarchaeota* distinct from the *Methanobacteriales* (16, 39), a phylogeny supported by a meta-analysis of anaerobic digester 16S rRNA gene sequences (5). Accordingly, we treat the WSA2 OTU_{16S} here as a class of the *Euryarchaeota* separate from the *Methanobacteriales*. Excluding WSA2 thus, very few *Methanobacteriales* OTU_{16S} were detected (GZ 2.2%, ST 0.35%, SWH 0.48%, YL 1.2%), with all from the family *Methanobacteriaceae* except for 0.057% of YL sequences assigned to candidate division MSBL1 (40).

Other methanogenic OTU_{16S} were of the order *Methanomicrobiales* (GZ 44%, ST 1.0%, SWH 1.2%, YL 13%), dominated in GZ by the *Methanospirillaceae* (39%) and YL by the *Methanoregulaceae* (11%); and the *Methanosarcinales* (GZ 4.9%, ST 1.9%, SWH 6.9%, YL 60%), dominated by the *Methanosaetaceae* (GZ 4.7%, ST 1.5%, SWH 6.8%, YL 50%). In ST only, a small population (0.035%) of *Methanococcales* was found, all of the family *Methanococcaceae*. No *Methanocellales* or *Methanopyrales* OTU_{16S} were detected, although there were representative sequences for both in the reference database.

Nonmethanogenic *Euryarchaeota* were also identified in all digesters, including OTU of the classes *Halobacteria* (0.070 to 0.86%), *Thermoplasmata* (0.06 to 0.49%), and miscellaneous unclassified sequences and candidate divisions (1.7 to 7.0%). Most of the remaining OTU_{16S} were classified to the *Crenarchaeota*, divided between the families *Nitrososphaeraceae* (0.21 to 11%), *Cenarchaeaceae* (GZ 0.26%, ST 59%, SWH 1.5%, YL 0.63%), class *Thermoprotei* (GZ 3.5%, ST 0.21%, SWH 35%, YL 2.8%), the uncultured Miscellaneous Crenarchaeotal Group (MCG) (GZ 22%, ST 0.35%, SWH 0.67%, YL 1.1%), and the Marine Benthic Group B (0 to 0.086%) with the remainder unclassified *Thaumarchaeota* (0.075 to 0.11%) (in the Greengenes v.13_5 taxonomy, the *Thaumarchaeota* are classified under the phylum *Crenarchaeota*).

Phylogeny of abundant methanogens. To investigate the detailed phylogenetic affiliation of the most abundant OTU_{mcrA}, the 20 most abundant OTU_{mcrA} (averaged across the four digesters) were aligned with 28 reference sequences and a NJ tree constructed (Fig. 2). Three OTU_{mcrA} clustered with reference *Methanobacteriales* sequences, six with *Methanomicrobiales*, four with *Methanosarcinales*, one with *Methanococcales*, and one with representatives of the proposed order *Methanomassiliicoccales* (3). The remaining five OTU_{mcrA} were placed in two clusters branching deeply within the *Methanomicrobia*. These OTU_{mcrA} included two highly abundant sequences from ST (OTU006) and SWH (OTU007) and, while clustering closely with a number of unclassified *mcrA* sequences from previous studies, were not affiliated with any cultured isolates.

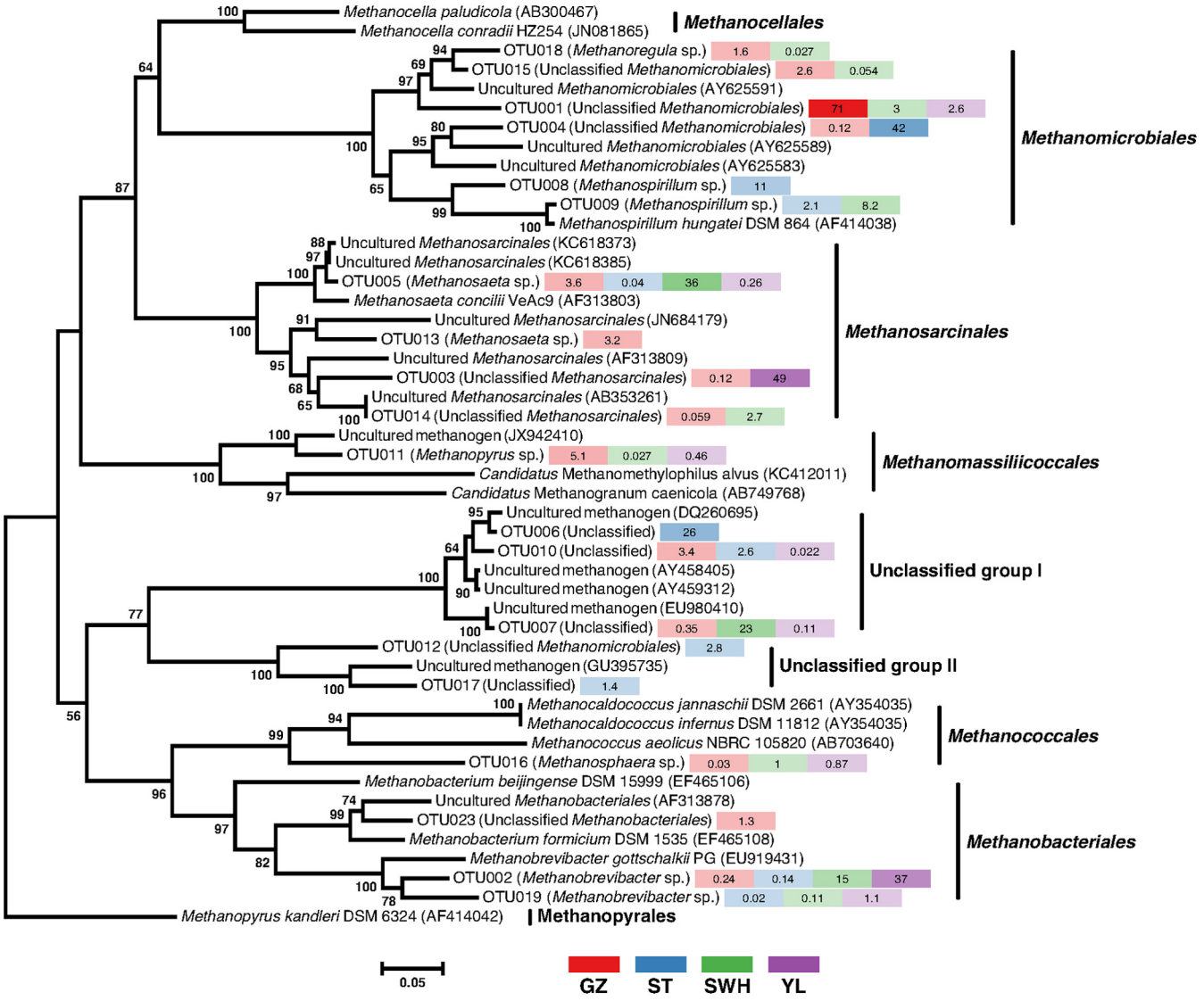

FIG 2 Neighbor-joining tree of 20 most abundant OTU$_{mcrA}$, with selected reference sequences. Relative abundances (%) for each digester are given to the right of each OTU$_{mcrA}$. Bootstrap values >50% (500 repetitions) are shown on nodes. The scale bar indicates sequence dissimilarity between nodes. For each OTU$_{mcrA}$, the Wang algorithm's taxonomic assignment (class or finer) is indicated in parentheses.

Consistent with the taxonomic classifications assigned by mothur, the most abundant OTU$_{mcrA}$ in GZ (OTU001) and ST (OTU004) were placed in the *Methanomicrobiales* cluster, while those in SWH (OTU005) and YL (OTU003) were associated with the *Methanosarcinales*. Only two of the most abundant OTU$_{mcrA}$, OTU005 (*Methanosarcinales*) and OTU002 (*Methanobacteriales*) were present in all four digesters, reflecting the overall low level of community overlap (see Fig. S3 in the supplemental material).

Comparison of digesters. The digesters' methanogen and archaeal communities were compared by PCoA ordination of the weighted UniFrac distances between samples. For both communities, the SWH and YL digesters were closely clustered and separated from the other two digesters (Fig. 3). Correlations between dominant OTU and the PCoA axes showed that the majority of dominant OTU were strongly associated with either a single digester or SWH+YL. Venn diagrams for both methanogenic and archaeal communities showed little overlap between the digesters, with 93% of OTU$_{mcrA}$ and 90% of OTU$_{16S}$ found only in a single digester (see Fig. S3 in the supplemental material).

Correlation between abundance of *mcrA* transcripts and methane production. The *mcrA* gene is a good candidate for a biomarker of methanogenesis rates in anaerobic digesters. Inocula from the GZ and SWH digesters were incubated on a food waste substrate to investigate the relationship between the methane production rate and transcription of the *mcrA* gene. Significant linear correlations ($R^2 > 0.87$) were found between the methane production rates and abundance of *mcrA* transcripts for both digester inocula (Fig. 4). A negligible amount of methane was produced from the control samples without substrate.

DISCUSSION

Methanogenesis is a major function of anaerobic digesters in wastewater treatment plants. However, the taxonomic identity of the methanogen groups involved and their community composi-

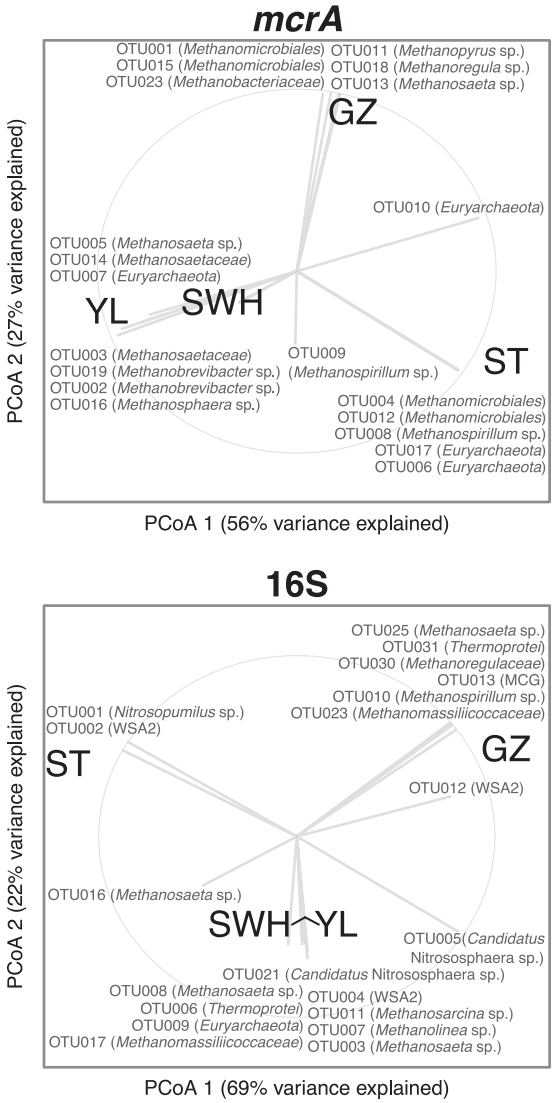

FIG 3 Principle coordinates analyses (PCoA) of methanogen (*mcrA*) and archaea (16S) communities. Vectors indicate correlation (ρ) between the abundances of the 20 most abundant OTU and the PCoA axes and should not be interpreted as identifying OTU that explain the variance between digesters. The light gray circle represents ρ = 1.

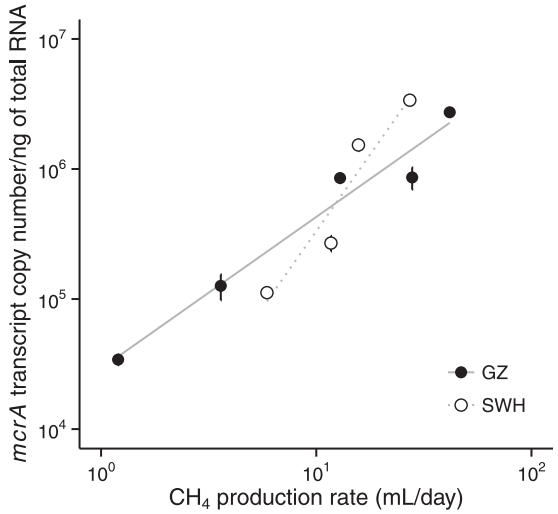

FIG 4 Relationship between *mcrA* transcription and the rate of methane (CH_4) production in anaerobic cultures inoculated with material from the GZ and SHX digesters. Each point represents one time point during the incubation. Error bars represent standard deviation for triplicate RT-qPCRs (vertical axis) and the 95% confidence intervals for CH_4 production (horizontal axis, smaller than the point in most cases).

tion under different operating conditions are still not well understood (5, 6). Although the 16S rRNA gene is a common target for community analysis, the *mcrA* gene has been used for the taxonomic classification of methanogens either independently (41, 42) or in concert with the 16S rRNA gene (14, 20). In the present study, pyrosequencing of both genes was applied to investigate the community composition of methanogens from four different anaerobic digesters.

Previous investigations mainly relied on Sanger sequencing of 16S rRNA gene clone libraries (16, 20, 41, 42), which can underestimate richness and diversity due to lack of sequencing depth. The development of high-throughput pyrosequencing has expanded our view of the diversity, abundance and structure of microbial communities in many environments (43). In the present study, 694 OTU_{mcrA} (from 16,634 reads) and 391 OTU_{16S} (8,446 reads) were recovered at the 97% sequence similarity level (Table 2), and rarefaction suggests richness was not sampled to exhaustion (see Fig. S1 in the supplemental material). A meta-analysis of archaeal 16S rRNA gene sequences from anaerobic digesters found that ~90% of 97% similar OTU were identified with <3,000 sequence reads and estimated through rarefaction a total richness of 327 OTU across all digesters studied (5). Although the greater sampling depth enabled by high-throughput sequencing may explain the higher observed richness here, it does not account for the higher estimated total richness. The higher OTU_{16S} richness in the present study may be due to the use of the hypervariable V1 region as sequencing target, resulting in a finer phylogenetic resolution compared to more conserved targets. However, OTU_{mcrA} richness was also high relative to comparable studies using the 97% sequence similarity threshold. For example, a study of 118 *mcrA* sequences from an anaerobic batch reactor identified 21 OTU_{mcrA} at an estimated 90% coverage of total richness (20), whereas a study of 123 *mcrA* clones from an anaerobic biogas reactor identified 28 OTU_{mcrA} at 89% estimated coverage (42). It is therefore possible that the digesters selected for the present study are more diverse than others previously reported on. Alternatively, previous clone library-based studies may have underestimated OTU richness due to low evenness combined with undersampling (44). In this investigation, Pielou's evenness J' was generally low (Table 2), an observation consistent with the rank-abundance curves (see Fig. S2 in the supplemental material), which suggests the digesters are dominated by a small number of abundant OTU. The majority of both OTU_{mcrA} and OTU_{16S} were represented by a single sequence (singleton OTU). An important caveat is that PCR and sequencing error can inflate richness estimates by generating spurious OTU. Appropriate denoising and quality control steps were applied (see Materials and Methods), and the OTU identity threshold used in the present study (97%) should be sufficient to compensate for typical 454 sequencing error (45). When OTU containing only one read across all samples (global singletons) are excluded, a conservative filter given that only one sample was

taken from each digester, 201 OTU_{mcrA} remain, still exceeding previous reports, although only 114 OTU_{16S} are retained. Despite this and the quality control steps taken, it is still possible that spurious OTU contributed in part to the richness observed in this study.

This study also provides an opportunity to compare the 16S rRNA and *mcrA* genes as markers for characterizing methanogen assemblages. Previous studies have found greater diversity using *mcrA* primers than archaeon-specific 16S rRNA gene primers at 97% sequence similarity (20) or by restriction fragment length polymorphism fingerprint (46), but similar diversity when sequence similarity thresholds are calibrated to taxonomic rank (14). In the present study, rarefaction did not suggest a systematic difference in the total richness revealed by the two genes at the 97% level, with more OTU_{16S} identified in samples GZ and YL but more OTU_{mcrA} in samples SWH and ST. This pattern was reflected by the Shannon-Weaver diversity index (Table 2), although the Chao1 index was consistently higher for OTU_{mcrA}, likely due to the higher sequencing depth. It is difficult to meaningfully compare OTU richness between the two genes since even at the same sequence similarity threshold they likely represent different levels of taxonomic resolution (14) and since the OTU_{16S} include non-methanogens. There is no widely used *mcrA* similarity threshold for delineating methanogen species, although studies generally agree that interspecies *mcrA* gene similarity is much lower than 16S rRNA gene similarity (14, 47).

The two gene targets also gave strikingly different pictures of the digesters' taxonomic compositions (Fig. 1). The same methanogen families were identified with both genes, except for the *Methanopyraceae* which was identified only by *mcrA* despite several representative sequences in the 16S rRNA gene reference database, the WSA2 group for which no *mcrA* sequence is available, and some minor (<3 sequences) families. However, there were large differences between the genes in the relative abundances of methanogen groups. Methanogens of the order *Methanobacteriales*, while abundant in the SWH and YL OTU_{mcrA}, were only a small fraction of the OTU_{16S}. Similarly, while the *mcrA*-based abundances suggested GZ and ST methanogens are dominated by *Methanomicrobiales* and SWH and YL by *Methanosarcinales*, in ST and SWH the 16S rRNA gene abundances suggested only small populations of these orders with the difference made up by WSA2 OTU. A known problem with some *mcrA*-targeting primers is that they also amplify the paralogous *mrtA* gene (encoding methyl coenzyme M reductase II) found in members of the *Methanobacteriales* and *Methanococcales*, increasing the observed proportions of these groups (8, 15). However, the observed differences are not explained by a systematic overrepresentation of the *Methanobacteriales*. Some *mcrA* primer sets are also known to exclude certain methanogen groups, with, e.g., the *mcrA3* set unable to detect members of the genus *Methanosaetaceae* (20, 46), although the primer set used in the present study does not appear to exclude any major methanogen groups (8). Differences in 16S rRNA (48) and *mcrA* (15) copy numbers between organisms may also have biased the taxonomic profiles. Overall, our results provide further support for the conclusion that a combination of the two genes is valuable in assessing the full spectrum of methanogen diversity (20).

While the digesters harbored distinct methanogen and archaeal communities, the SWH and YL digesters were most similar in overall taxonomic composition (Fig. 1 and 3). This reflects the similarity between the SWH and YL treatment plants, which treat secondary sludge from municipal sewage (unlike GZ) at low salinity (unlike ST), although at different scales (Table 1). SWH and YL shared more OTU from both communities than all other digester combinations except the GZ+YL methanogens (see Fig. S3 in the supplemental material). Overall, however, there was little overlap between the communities; among the most abundant OTU, the majority were strongly associated with either a single digester or SWH+YL (Fig. 3). This lack of overlap is probably not solely attributable to undersampling or low evenness, as even among the most abundant OTU in each community the overlap was low. It may reflect small differences in operating conditions and feedstock composition between the digesters. As a metagenomic study of the ST and SWH digesters found that they did not significantly differ in genomically encoded metabolic functions (49), it may also be due to stochastic occupation by closely related and functionally interchangeable taxa of the limited set of biochemical niches within the anaerobic digestion process. A high level of functional redundancy is commonly reported in anaerobic digester communities (12).

The NJ tree of abundant OTU_{mcrA} sequences produced class-level clades generally congruent with the accepted phylogeny of the *Methanomicrobia* (Fig. 2) and supported the Wang algorithm's taxonomic classifications with two exceptions. The first exception was OTU016, which clustered with the *Methanococcales* despite being classified to genus *Methanosphaera* by the Wang algorithm. Given the lack of closely related reference sequences and the deep branching of this OTU relative to the *Methanococcales* and the very low proportion of 16S rRNA gene sequences classified to the *Methanococcales*, this may be a misplacement of a *Methanobacteriales* sequence. The second exception was OTU011, most abundant in GZ (5.1%) but also present in SWH and YL (<0.5%), which had been classified to the genus *Methanopyrus* but did not have high sequence similarity with the *Methanopyrus kandleri* sequence subsequently used to root the tree. OTU011 was instead affiliated with the *mcrA* genes of "*Candidatus* Methanomethylophilus alvus" and "*Candidatus* Methanogranum caenicola," members of the proposed methanogen order *Methanomassiliicoccales* (3, 50, 51), as well as an unclassified reference sequence. Nine OTU_{16S} were classified by the Wang algorithm to the family *Methanomassiliicoccaceae*, with the highest collective abundance in GZ (GZ 4.9%, ST 0.18%, SWH 2.3%, and YL 1.0%), supporting the presence of *Methanomassiliicoccaceae* in the digesters and at highest abundance in GZ. "*Ca.* Methanomethylophilus alvus" is a putative obligate hydrogen-dependent methylotrophic methanogen (52) with the genomic potential to utilize a wide range of methylated compounds, a metabolic ability which may be common to the proposed order (3). Similarly, "*Ca.* Methanogranum caenicola" was isolated from anaerobic packed-bed reactor sludge and determined in culture to produce methane through hydrogen-dependent reduction of methanol (50). Notably, OTU011 was only identified at significant abundance in GZ (5.1%), which treats organic wastewater from industrial manufacturing, as opposed to the other three digesters (<0.5%) which treat concentrated sludge following secondary treatment of sewage. The high abundance of OTU011 in GZ may therefore reflect the presence of methanol or other methylated compounds in this industrial waste stream. Since OTU011 comprised almost all of the *mcrA* sequences assigned by the Wang algorithm to the *Methanopyrales* (the exception being a single YL sequence), reassigning this OTU

to the *Methanomassiliicoccaceae* is consistent with the lack of *Methanopyrales* OTU_{16S} and suggests the *Methanopyrales* are either present at extremely low abundance in, or absent from, the digesters.

The WSA2 group was a major component of the OTU_{16S} assemblages, particularly in ST and SWH (Fig. 1). Although classified in Greengenes as a member of the *Methanobacteriales*, it has been identified at high abundance in other anaerobic digesters and consistently found to form a class-level monophyletic lineage within the *Euryarchaeota* distinct from the *Methanobacteria* (5, 16, 39). Based on this phylogenetic placement, growth in culture on formate and H_2/CO_2 (39), and possible competition with *Methanosarcinales* for acetate (16), WSA2 group organisms are very likely methanogens. The *mcrA* tree constructed here contained two groups of deeply branching, abundant unclassified OTU_{mcrA} designated unclassified group (UG) I and UGII (Fig. 2). Two UGI OTU_{mcrA} were highly abundant in SWH (OTU007, 23%) and ST (OTU006, 26%), in similar proportions to those of WSA2 OTU_{16S} in those digesters (Fig. 1) and accounting for the majority (77%) of the unclassified *mcrA* sequences observed here. Although systematic biases due to PCR bias and copy number variation very likely influence the observed relative abundances, the similarity in proportions across the four digesters is consistent with the hypothesis that the genes originate from the same organism(s). It is thus plausible the UGI and possibly UGII *mcrA* sequences originate from the WSA2 group, for which no representative *mcrA* sequences yet exist. Future experiments, e.g., dual-probe fluorescence in situ hybridization or construction of a draft WSA2 genome from metagenomic sequences, will be useful to confirm this hypothesis.

Both *Crenarchaeota* and *Euryarchaeota* OTU_{16S} were detected in all digesters. ST *Crenarchaeota* consisted almost exclusively of a single OTU_{16S} classified to the genus *Nitrosopumilus*, the only species of which is the abundant marine archaeon *N. maritimus*, likely reflecting the widespread use of seawater for toilet flushing in the region served by the ST wastewater treatment plant. *Crenarchaeota* in the other three digesters were predominantly from the class *Thermoprotei*, common in anaerobic digesters (5), the ammonia-oxidizing family *Nitrososphaeraceae*, and the poorly characterized Miscellaneous Crenarchaeotal Group (MCG). Although *Crenarchaeota* are frequent components of anaerobic digester communities (5), their role in the digestion process is largely unknown, although they have been found to collocate with active *Methanosaeta* cells in a granular digester biofilm (53), suggesting some metabolic interaction and potentially syntrophy with methanogens.

Methanogens of the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* have previously been reported as abundant in anaerobic digesters treating cow manure (20, 41), wastewater algae (22), and municipal solid waste (54). *Methanobacteriales* and *Methanomicrobiales* produce methane by reduction of CO_2 with H_2 as an electron donor (hydrogenotrophic methanogenesis), while *Methanosarcinales* directly cleave acetate to methane and CO_2 (acetoclastic methanogenesis) (55). Stoichiometric modeling (56, 57) and measurements of natural methanogenic systems (58) have been used to predict acetate accounts for ~70% of methane production, and a meta-analysis of anaerobic digester studies found that the acetoclastic genus *Methanosaeta* accounted for 55% of archaea (although a large proportion of sequences were unclassifiable to genus) (5). However, several studies of biogas reactors have identified a dominance of hydrogenotrophic methanogens, though the relative proportions of *Methanobacteriales* and *Methanomicrobiales* vary (41, 42, 46). In the present study, *mcrA*-based taxonomic classification suggested the SWH and YL digesters had large or dominant populations of acetoclastic *Methanosarcinales*, while GZ and ST were dominated by hydrogenotrophic *Methanomicrobiales*. Within the *Methanosarcinales*, *Methanosaetaceae* far outnumbered *Methanosarcinaceae* in all digesters and with both genes (Fig. 1). *Methanosaetaceae* dominance is characteristic of digesters with low acetate concentrations and moderate retention times (20, 59, 60). Acetate was not detected in any of the sampled digesters (data not shown), consistent with the dominance of *Methanosaetaceae* and indicating that syntrophic acetoclastic methanogenesis in these reactors keeps pace with acetogenesis. A previous metagenomic study of the ST and SWH digesters similarly found the *Methanosaeta* to be dominant in all but one sample (49), suggesting this pattern is stable over time.

In addition to its use in determining methanogenic diversity, the *mcrA* gene is a potential biomarker of methane yield from methanogenesis. Using qPCR with broad-specificity *mcrA* primers, the *mcrA* gene copy number has been found to correlate with methanogen abundance in sludge from anaerobic digesters and in methane seep sediments (61) and with methane production rates in a biogas reactor (17). However, gene copy number is not a reliable predictor of metabolic activity and is unable to capture the real-time responses of methanogens to changing operating conditions. In the present study, we used RT-qPCR to measure *mcrA* transcription and demonstrated a significant linear correlation between *mcrA* transcript quantity and methane production rate in two digester microbial communities (Fig. 4). This result confirms that the physiological activity of methanogens in a complex system can be gauged by analyzing the expression of the *mcrA* gene, and the expression level can also be used to predict methane yield.

Anaerobic digestion is widely used in the treatment of wastewater sludge, and yet the microbial consortia performing this process are poorly understood. A thorough understanding of the methanogens and other archaea in biogas-producing reactors is essential to improving methane yield and other industrially important parameters. In the present study, pyrosequencing of the *mcrA* gene detected both hydrogenotrophic and acetoclastic methanogens in each digester and found an overall taxonomic composition quite different to that found with the 16S rRNA gene. The identification of the proposed order *Methanomassiliicoccales* and the uncultured but ubiquitous WSA2 lineage suggest these groups may play roles in the anaerobic digestion process and are important targets for future investigation. Finally, a significant positive correlation was identified between methane production rates and the abundance of *mcrA* transcripts in digesters despite very different methanogen compositions. Overall, our study confirmed the efficacy of the *mcrA* gene as a marker for both methanogen taxonomy and metabolic activity and also reinforced the value of using multiple genes to assess microbial diversity.

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