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Recommended Citation
Morris, R. L.; Tale, Vaibhav Panjabrao; Mathai, P. P.; Zitomer, Daniel; and Maki, James, "*mcrA* Gene Abundance Correlates with Hydrogenotrophic Methane Production Rates in Full-scale Anaerobic Waste Treatment Systems" (2016). *Biological Sciences Faculty Research and Publications*. 506.
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Mcra Gene Abundance Correlates with Hydrogenotrophic Methane Production Rates in Full-Scale Anaerobic Waste Treatment Systems

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Abstract

Anaerobic treatment is a sustainable and economical technology for waste stabilization and production of methane as a renewable energy. However, the process is under-utilized due to operational challenges. Organic overload or toxicants can stress the microbial community that performs waste degradation, resulting in system failure. In addition, not all methanogenic microbial communities are equally capable of consistent, maximum biogas production. Opinion varies as to which parameters should be used to monitor the fitness of digester biomass. No standard molecular tools are currently in use to monitor and compare full-scale operations. It was hypothesized that determining the number of gene copies of *mcrA*, a methanogen-specific gene, would positively correlate with specific methanogenic activity (SMA) rates from biomass samples from six full-scale anaerobic digester systems. Positive correlations were observed between *mcrA* gene copy numbers and methane production rates against H_2 : CO_2 and propionate (R^2 = 0·67–0·70, P < 0·05) but not acetate (R^2 = 0·49, P > 0·05). Results from this study indicate that *mcrA* gene targeted qPCR can be used as an alternate tool to monitor and compare certain methanogen communities in anaerobic digesters.

Significance and Impact of the Study

Using quantitative PCR (qPCR), we demonstrate that the abundance of *mcrA*, a gene specific to methane producing archaea, correlated with specific methanogenic activity (SMA) measurements when H_2 and CO_2, or propionate were provided as substrates. However, *mcrA* abundance did not correlate with SMA with acetate. SMA values are often used as a fitness indicator of anaerobic biomass. Results from qPCR can be obtained within a day while SMA analysis requires days to weeks to complete. Therefore, qPCR for *mcrA* abundance is a sensitive and fast method to compare and monitor the fitness of certain anaerobic biomass. As a monitoring tool, qPCR of *mcrA* will help anaerobic digester operators optimize treatment and encourage more widespread use of this valuable technology.

Introduction

Anaerobic waste treatment is often a sustainable, economical process in which the degradation of organic compounds in industrial and municipal wastes result in the biological production of a renewable energy source: methane. However, anaerobic bioprocesses may be under-utilized due to operational challenges, such as difficult start-up and transient periods of poor operation (Speece **1996**). Prudent use of monitoring and control has been used or suggested to help prevent failure or encourage faster start-up and recovery of stressed digesters (Castellano *et al*. **2007**; Schauer-Gimenez *et al*. **2010**). Activity testing, such as specific methanogenic activity (SMA) assays, has been used to determine maximum methane production rates of anaerobic biomass given specific substrates (Coates *et al*. **1996**, **2005**). Unfortunately, days to weeks are typically required to obtain activity results, limiting applicability for real-time optimization.

Digester microbial communities are complex, with multiple trophic levels in which different groups of organisms carry out waste degradation in a series of steps (Schink **1997**; Fernandez *et al*. **1999**; White **2000**; Leclerc *et al*. **2004**; Liu and Whitman **2008**; Rivière *et al*. **2009**). The methanogens are especially important as the last link in the food chain, performing the final step in the degradation of organic waste to methane (McCarty and Smith **1986**). Additionally, their function is closely tied to that of the syntrophic fatty-acid degrading bacteria that metabolize these compounds and depend upon hydrogen removal by methanogens (Schink **1997**; Conrad and Klose **1999**). Furthermore, methanogenesis is often considered to be the rate-limiting step in anaerobic treatment of many wastes (Liu and Whitman **2008**). Therefore, methanogens are critical for digester stability. Monitoring this specific group of organisms may provide an important link between digester function and microbial community structure.

Methanogens possess an operon that encodes the methyl coenzyme M reductase (MCR). The presence and transcription of the gene that encodes the alpha subunit of MCR (*mcrA*) has been used to detect methanogen presence and activity in the environment (Springer *et al*. **1995**; Luton *et al*. **2002**; Juottonen *et al*. **2008**; Steinberg and Regan **2009**; Kampmann *et al*. **2012**; Zeleke *et al*. **2013**; Bocher *et al*. **2015**). Previously, we showed that *mcrA* gene copy numbers, but not transcript numbers, positively correlated with SMA values in laboratory methanogenic enrichments (Morris *et al*. **2014**). An advantage of qPCR of *mcrA* over SMA analysis is that relevant data can be obtained in a much shorter period of time.

This study was performed to test the hypothesis that the *mcrA* gene copy numbers positively correlate to SMA values determined for industrial and municipal anaerobic biomass. Herein, we report the comparison of *mcrA* gene abundance to SMA values from biomass collected from six full-scale anaerobic treatment plants.

Results and discussion

Methanogen community fingerprint with denaturing gradient gel electrophoresis (DGGE)

Biomass from the six different full-scale digesters (Table **1**) was examined for the presence and structure of the methanogen community by fingerprinting using the *mcrA* gene and DGGE. The results revealed a unique methanogen community within each full-scale digester (Fig. **1**a). A dendogram constructed from the densitometric data from the gel confirmed that the communities were different even when the substrates were similar (Fig. **1**b). For example, the biomass from both MSS and MBR, which were municipal anaerobic digesters, appeared in different clades (Fig. **1**b).

Table 1. Description of anaerobic waste treatment systems and operating conditions. Results provided by operators in response to a questionnaire

Digester	Substrate	Operating temperature (°C)	pH	Biogas production	Organic loading rate	Hydraulic retention time
CB	Brewery waste	26·7–29·4	6·5–8·0	351·3 l kg^{-1} COD removed	NP	4·4 h
KI	Milk-derived food additive waste	35·6–36·7	7·0	19 369 SCM day^{-1}	NP	7–10 days

CF	Dairy cow manure waste	NP	NP	NP	NP	NP
JBS	Beef slaughter waste	36·1	7·0	130 SCM day^{-1}	1·6 kg COD m^{-3} day^{-1}	3–4 days
MBR	Municipal waste	36·2	7·1	6884 SCM day^{-1}	0·80 kg VS m^{-3} day^{-1}	53 days
MSS	Municipal waste	35·8	7·2	17 238 SCM day^{-1}	0·32 kg VS m^{-3} day^{-1}	47 days

NP = not provided by operator; COD = chemical oxygen demand; SCM = standard cubic meter; VS = volatile solids.

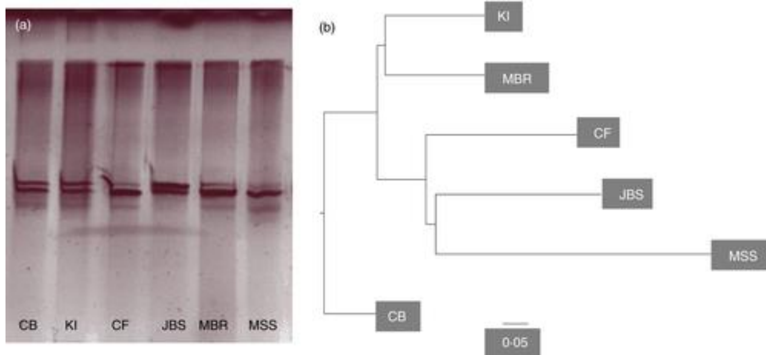

Figure 1. Community fingerprint analysis using denaturing gradient gel electrophoresis (DGGE) of *mcrA* from full-scale industrial and municipal anaerobic digesters**.** (a) DGGE fingerprint of *mcrA* genes present in full-scale biomass samples. Lanes are labelled with sample names. See Table 1 for more information regarding each sample. (b) Dendrogram showing relationships between the methanogen communities in the full-scale digesters based on optical density data from the DGGE gel.

Quantitative PCR (qPCR) of *mcrA*

All qPCR analyses were performed in one run, and critical parameters were as follows: slope −3·662, y-intercept 5·465, correlation coefficient 0·933, efficiency 87·5%, C_t of the no-template control 26·45. Total *mcrA* copy number per ng DNA (Fig. **2**a) and per g of wet pellet biomass (Fig. **2**b) was calculated from qPCR results. When the results were normalized to g of pellet as opposed to ng of DNA the digester with the highest *mcrA* copy number switched from biomass JBS to biomass CB (Fig. **2**). This normalization with pellet biomass was performed because it was thought to be more similar to the volatile suspended solids (VSS) measurement used to normalize the SMA values.

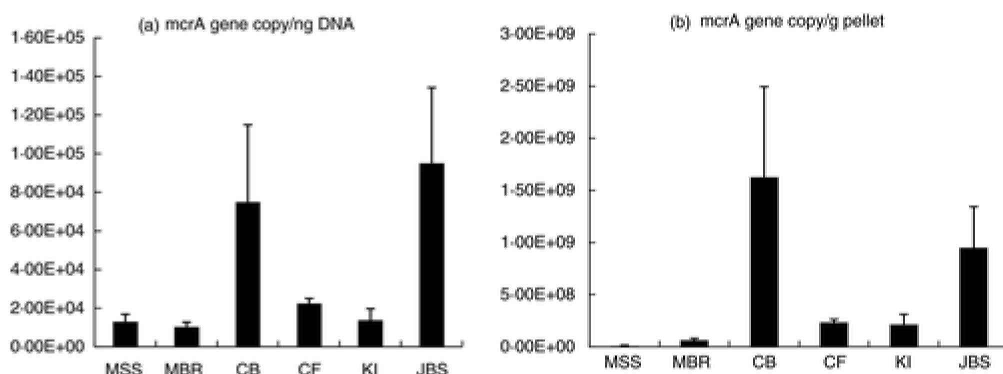

Figure 2. Results of qPCR using industrial and municipal anaerobic digester biomass. Each bar represents results from three technical replicates. Error bars show standard deviation from the mean. (a) *mcrA* gene copies ng^{-1} DNA. (b) *mcrA* gene copies g^{-1} pellet of centrifuged biomass with the supernatant removed. Note that the *y*-axis numeration is different between a and b.

SMA assays

SMA assays were used to compare the biomass samples' maximum specific ability to produce methane given a particular substrate (Table **2**) (Coates *et al*. **1996**, **2005**). Biomass sample CB had the highest SMA values for all three substrates tested (Table **2**), biomass KI had the second highest activity against propionate and acetate, followed by biomass CF. However, biomasses MSS, MBR and JBS had higher activities against $H_2 : CO_2$ than KI and CF.

Table 2. Anaerobic waste treatment systems from which biomass was collected and their specific methanogenic activity (SMA) assay results

Digester	**Substrate**	**SMA against calcium propionate**		**SMA against calcium acetate**		**SMA against $H_2 : CO_2$**	
		(in ml CH_4 g^{-1} VSS^{-h})					
		Average	**Cv (%)a**	**Average**	**Cv (%)**	**Average**	**Cv (%)**
CB	Brewery waste	14·9	6·7	21·7	2·5	31·4	0·0
KI	Milk-derived food additive waste	3·6	6·3	11·0	6·3	8·8	3·7
CF	Dairy cow manure waste	1·1	97·0	10·1	9·4	8·6	16·0
JBS	Beef slaughter waste	0·8	29·6	4·7	48·5	12·1	0·0
MBR	Municipal waste	0·0	0·0	5·6	66·3	10·2	26·0
MSS	Municipal waste	0·0	0·0	6·6	97·4	15·0	17·0

[a] Cv (%) is the coefficient of variation for the triplicates from each sample.

Methanogen abundance and methane production rates

Digester biomass qPCR results normalized to g of wet pellet correlated well with corresponding SMA against $H_2 : CO_2$ (R^2 = 0·67, *P* = 0·046; Fig. **3**a) and propionate (R^2 = 0·70, *P* = 0·038; Fig. **3**b) but not with acetate (R^2 = 0·49, *P* = 0·12; Fig. **3**c). These data obtained from SMA assays and qPCR performed on full-scale digester samples confirmed findings from a previous study that demonstrated a relationship between methane production rates and methanogen abundance in hydrogen enrichment cultures (Morris *et al*. **2014**), although the correlation in this study was not as strong as found in the previous

study. This finding is likely due to the nature of the biomass from the laboratory maintained enrichment cultures when compared to the industrial and municipal biomass.

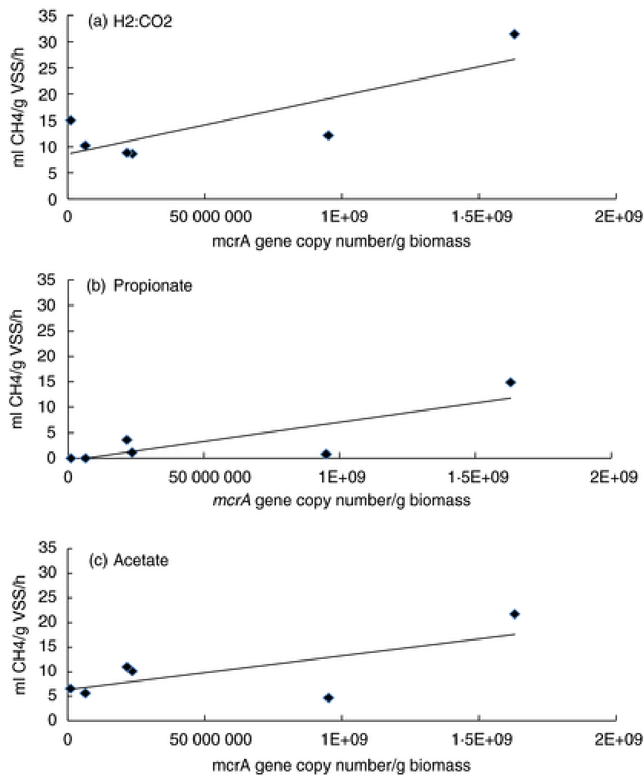

Figure 3. Correlation between specific methanogenic activity (SMA) assays and *mcrA* gene copy number per unit biomass from the six industrial and municipal digesters sampled. (a) SMA and H_2 : CO_2. (b) SMA and propionate. (c) SMA and acetate.

The association between SMA against acetate in this study was not significant (Fig. **3**c). This latter finding likely resulted from the fact that several groups of micro-organisms in digesters, in addition to acetoclastic methanogens, are able to utilize acetate, and that the relative abundance of these organisms could vary among the biomass samples tested.

The relationship between SMA and *mcrA* copy number was not dependent on the structure of a particular methanogen community or its composition (Fig. **1**). This agreed with the results of the previous study which compared clone libraries to SMA values (Morris *et al*. **2014**).

Taken together, the results of this study support the hypothesis that *mcrA* copy number can be used to monitor and compare methanogen communities in some anaerobic digesters. Useful methods of monitoring systems must be sensitive and fast (Castellano *et al*. **2007**; Molina *et al*. **2009**). The ability of this methodology to produce results within a day that correlate well with SMA assays (which may take days to weeks to complete) satisfies both of these criteria, suggesting that quantifying methanogens may be a very useful technique for comparing biomass from different sources.

Although methanogenesis is often proposed to be the rate-limiting step in anaerobic digestion, the actual metabolic process that limits the rate of methane production may depend upon substrate composition (Liu and Whitman **2008**). While monitoring micro-organisms at all trophic levels of the

anaerobic food chain could provide valuable diagnostic information, this study shows a direct link between methanogen abundance and methane production rates in certain situations.

Materials and methods

Sample collection

Anaerobic biomass was collected from six industrial/mu-nicipal full-scale anaerobic systems that varied in substrate in the state of Wisconsin, USA (Table **1**). Biomass samples CB and JBS were taken from an upflow anaerobic sludge blanket reactor and an anaerobic contact process, respectively, whereas the remaining biomass samples were from continuously-stirred tank reactors with no recycle. Biomass samples were collected in DNase-free centrifuge bottles, placed on dry ice for transport, and then stored at −80°C until processing (24–48 h).

SMA assays

Methanogenic activity assays were conducted in triplicate under anaerobic conditions in 160-ml serum bottles with 25 ml (<3 g VSS, l^{-1}) of biomass. The VSS concentration was determined according to Standard Methods (American Public Health Association *et al*. **1998**) at the beginning and the end of activity tests and the average of the two values was employed for specific activity calculations. SMA for $H_2 : CO_2$ was determined by the method of Coates *et al*. (**1996**). Serum bottles were sparged with gas (4 : 1 v/v $H_2 : CO_2$) and sealed with solid Balch-type butyl rubber stoppers. Immediately thereafter, 100 ml of the $H_2 : CO_2$ gas blend at ambient pressure and temperature was injected through the stopper using a syringe and a needle. Acetate and propionate SMAs were determined as described by Sorensen and Ahring (**1993**). Assays were supplied with 3 g l^{-1} propionate or 10 g l^{-1} acetate, each as the calcium salt, whereas the control assays were not supplied with substrate (Sorenson and Ahring **1993**; Zitomer *et al*. **2008**). All the propionate and acetate assays were then sparged with gas (7 : 3 v/v $N_2 : CO_2$) to establish anaerobic conditions and sealed as stated above. Immediately after the addition of substrate to the test assays, all bottles were incubated at 35°C and shaken at 150 rev min^{-1}. Bottle head-space volume was measured at ambient pressure (approx. 1 atm) for 30 days by inserting the needle of a glass syringe with wetted barrel. The syringe content was re-injected into the serum bottle after volume measurement. Headspace methane content was analysed by gas chromatography (Agilent Technologies, Santa Clara, CA). Methane produced by the control assays accounted for endogenous decay, and was subtracted from methane produced by the test assays. Finally, maximum methane production rate (ml CH_4 g^{-1} VSS-h^{-1}) was determined as described elsewhere (Owen *et al*. **1979**).

DNA extraction

Frozen biomass samples were thawed at room temperature. All samples were centrifuged at 10 000 ***g*** for 10 min at 4°C (Avanti J-25, Beckman Coulter, Brea, CA). The supernatant was discarded, and DNA was extracted from the pellet using a combination of treatments from the RNA PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory Kit (MOBIO, Carlsbad, CA) according to manufacturer's instructions. DNA was then purified using the PowerClean™ DNA Clean-up Kit (MOBIO). DNA integrity was examined by using a 1·5% (w/v, Tris-acetate-EDTA buffer, Sambrook and Russell **2001**) agarose gel. DNA concentration of purified extracts was determined spectrophotometrically (Nanodrop ND-1000, Thermo-Scientific, Wilmington, DE).

Quantitative PCR (qPCR)

qPCR was performed according to the recommendations by Smith *et al*. (**2006**) and Smith and Osborn (**2009**) except for the standard curve, and according to MIQE guidelines (Bustin *et al*. **2009**) which were applicable to environmental samples. qPCR standards were created using pooled *mcrA* DNA clones from anaerobic biomass samples as described previously (Morris *et al*. **2014**). Quantification was performed using the primers designed by Luton *et al*. (**2002**): mcrF 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′ and mcrR 5′-TTCATTGCRTAGTTWGGRTAGTT-3′, as described previously (Vianna *et al*. **2006**; Goffredi *et al*. **2008**; Freitag and Prosser **2009**; Freitag *et al*. **2010**; Morris *et al*. **2014**). The final qPCR mix per 25 μl reaction was as follows: 1× iQ™ SYBR®-Green Supermix reaction buffer containing dNTPs, iTaq DNA polymerase and 3 mmol l^{-1} $MgCl_2$ (Bio-Rad, Hercules, CA); 750 nmol l^{-1} mcrF and mcrR; and template DNA (0·3–1 ng). Each qPCR run included a no-template control. Quantification was performed on the MyIQ™ Single-Color Real-Time PCR Detection System (Bio-Rad) using the following programme: initial denaturation at 95°C (10 min), 35 cycles of 95°C (30 s), 58·5°C (1 min), and 72°C (3 min), with a final extension of 7 min at 72°C. The amplification programme was followed by a denaturation curve programme (80 cycles 10 s in length starting at 55°C and increasing in 0·5°C increments) to check for product specificity. Products from optimization runs were also examined for specificity using 1·5% agarose gels as described above. Starting quantity amounts and threshold cycle values were calculated using the Myiq™ optical system software ver. 1.0 (Bio-Rad). Normalization 'per g of wet pellet' was also calculated using the g of biomass pellet after centrifugation and supernatant removal. Results were normalized to this parameter to account for differences in total solids among the biomass samples.

Statistical analysis of qPCR and SMA results

Linear correlation of the SMA data with the qPCR results normalized to ng of DNA and g of biomass pellet, respectively, was performed using *R* to calculate R^2 and *P*-values (Team **2008**). Values were plotted with a trend line for visual analysis.

Denaturing gradient gel electrophoresis

DNA extracts from the full-scale digester biomass samples were PCR amplified with the *mcrA* specific primers described above with the exception that the forward primer was modified to include a GC clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) (Muyzer *et al*. **1993**; Luton *et al*. **2002**). The final component concentrations per 50 μl PCR reaction were as follows: 100 nmol l^{-1} each primer, 0·2 mmol l^{-1} dNTPs, 1× Colorless GoTaq Reaction Buffer (Promega, Madison, WI) which contained 1·5 mmol l^{-1} $MgCl_2$ and 1·2U goTaq Polymerase (Promega). Template concentrations were approx. 100 ng per reaction tube. The PCR conditions were as follows: initial denaturation at 95°C (5 min), 35 cycles of 95°C (1 min), 58°C (1 min), and 72°C (3 min), with a final extension of 10 min at 72°C. The programme included a slow ramp in temperature (0·1°C s^{-1}) between the annealing and extension steps of the first five cycles of the protocol as recommended by Luton *et al*. (**2002**). The amplicon size was confirmed using a 1% agarose gel as described above. Forty µl of PCR product was then used for DGGE in a 1 mm thick 8% polyacrylamide gel (37·5 : 1 acrylamide to bis-acrylamide) with 40–70% denaturant gradient (urea and formamide). Electrophoresis at 100V for 15 h was performed using the DCode™ Universal Mutation Detection System (Bio-Rad). The DGGE gel

was stained with 1% SYBR Gold Nucleic Acid Stain (Invitrogen, Grand Island, NY) for 30 min and visualized using the GelDoc™-It Imaging System (UVP, Upland, CA).

A dendogram representing the relationships between DGGE patterns of full-scale biomass samples was constructed using optical density data collected using labworks™ software (Lablogics, Inc., Mission Viejo, CA). Pearson's correlation coefficient (*r*) was calculated using the densitometric data for each pair of samples. A distance matrix representing the relationships among the densitometric data was calculated using 1-*r* values. An unweighted pair group method with arithmetic mean (UPGMA) tree was plotted using the distance matrix and the PHYLIP software package (Felsenstein **2005**).

Acknowledgements

We thank the owners and operators of the full-scale industrial and municipal anaerobic digesters that were sampled for providing us with biomass and operating information, and Mike Dollhopf of the Water Quality Center, Marquette University, for all his assistance during this investigation. Support for this work was provided by WE Energies and by Marquette University.

Conflict of Interest

There is no conflict of interest for any of the authors of this manuscript.

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