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# Phylogenetic identification of methanogens assimilating acetate-derived carbon in dairy and swine manures

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ABSTRACT

In order to develop approaches for reducing the carbon footprint of the swine and dairy industries, it is important first to identify the methanogenic communities that drive methane emissions from stored manure. In this study, the metabolically active methanogens in substrate-starved manure samples taken from two dairy and one swine manure storage tanks were identified using [$^{13}$C]-acetate and DNA stable-isotope probing (DNA-SIP). Molecular analysis of recovered genomic [$^{13}$C]-DNA revealed that two distinct clusters of unclassified methanogen populations affiliated with the *Methanoculleus* genus, and the populations affiliated with *Methanoculleus chikugoensis* assimilated acetate-derived carbon (acetate-C) in swine and dairy starved manure samples, respectively. Furthermore, carbon flow calculations indicated that these populations were the primary contributors to methane emissions during these anoxic SIP incubations. Comparative analysis of *mcrA* gene abundance (coding for a key enzyme of methanogenesis) for *Methanoculleus* spp. in fresh feces and a wider range of stored dairy or swine manure samples, by real-time quantitative PCR using newly designed specific primers, demonstrated that the abundance of this genus significantly increased during storage. The findings supported the involvement of these particular methanogen populations as methane emitters from swine and dairy manure storage tanks. The study revealed that the ability to assimilate acetate-C for growth in manure differed within the *Methanoculleus* genus.



## Introduction

Reducing the carbon footprint of food production is a major challenge faced by the agricultural sector. For the last two decades in both Canada (1990–2012) and the United States (1990–2011), annual agricultural greenhouse gas (GHG) emissions accounted for approximately 7% of total GHG emissions [10,11]. For the period 1990–2011, methane emissions from manure management increased by 65% in the United States, which was primarily due to increased use of liquid systems for manure storage on both swine and dairy farms [11]. For these livestock operations, manure is typically stored in outdoor tanks or lagoons prior to application onto agricultural soils for fertilization. In this context, methane is the primary GHG emitted and it represents a serious environmental problem [27].

Methane emissions from stored manure result from the activities of complex microbial communities that metabolize manure organic matter. Fermentative bacteria convert organic matter into volatile fatty acids (VFAs, including acetate), $H_2$, formate and $CO_2$. Acetate can be metabolized by acetoclastic methanogens and/or by acetate-oxidizing bacteria syntrophically associated with hydrogenotrophic methanogens that produce methane [34]. Identifying metabolically active methanogens is an important step toward understanding the mechanisms that contribute to

*Abbreviations:* Acetate-C, acetate-derived carbon; DNA-SIP, DNA stable-isotope probing; GHG, greenhouse gas; *mcrA*, alpha subunit of the methyl coenzyme M reductase; LH-mcrA, amplicon length heterogeneity of the mcrA gene; OTU, operational taxonomic unit; qPCR, quantitative PCR; VFA, volatile fatty acid.

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GHG emissions during manure management and for developing strategies that can mitigate methane emissions from livestock operations.

Typically, methanogenic archaeal communities present in animal feces or stored manure are investigated by examining the diversity of 16S rRNA or *mcrA* gene (coding for the alpha subunit of methyl coenzyme M reductase) sequences [41,42]. Although these studies have demonstrated the presence of methanogens, microbial community compositions can vary considerably. The methanogenic community of pig feces is dominated by *Methanobrevibacter* spp., *Methanosphaera* spp. and unidentified *Archaea* [23,41]. In contrast, the communities in stored swine manure consist mainly of *Methanobrevibacter* spp., *Methanogenium* spp., *Methanocorpusculum* spp., *Methanoculleus* spp. and unidentified *Archaea* in diverse proportions [4,29,36,42]. In the rumen of dairy cows, methanogenic communities largely consist of *Methanobrevibacter* spp. [7,20,46], and this also appears to be the case for dairy cow feces [13]. Comparison between methanogen communities in feces and stored manure indicates that they may change during storage [29], and that the methanogens indigenous to feces may not be those adapted to the environment of a manure storage tank, as has been concluded for the associated bacterial communities [43]. Therefore, there can be discrepancies between microorganisms measured as abundant in storage tanks versus those that are most active during methanogenesis.

Our previous study based on amplicon length heterogeneity of the *mcrA* gene (LH-mcrA) fingerprint analysis of methanogen communities in two swine and two dairy manure tanks indicated that community composition and structure (relative abundance of phylotypes) significantly varied from tank to tank [5]. The dynamics of the methanogenic community of manure samples from these two swine tanks was further investigated by clone library sequence analysis of the *mcrA* gene [4]. An enrichment of *Methanoculleus* spp. was observed in the community over time, indicating that, under anaerobic conditions simulating manure storage, this genus would be responsible for methane production. This is in contrast to other methanogens, such as *Methanocorpusculum* spp., that decreased in relative abundance for *in vitro* anaerobic incubation [4].

DNA stable-isotope probing (DNA-SIP) is a useful approach for identifying the microorganisms within a complex community that take part in specific assimilatory processes [26,30]. Recently, we conducted a DNA-SIP proof-of-principle experiment, with a single sample from a swine manure storage tank [3], to demonstrate that this experimental approach could be applied to identify active methanogens in such a carbon-rich ecosystem. *Methanoculleus* spp., although not representing the dominant genus in the initial sample, assimilated acetate-C and contributed to methanogenesis. However, evidence that this initial finding could be generalised to other swine manure storage tanks and also to those containing dairy manure was lacking.

Therefore, the present work aimed at identifying the *Archaea* involved in methanogenesis during anoxic incubations of representative swine and dairy manure samples taken from three additional farms (two dairy and one swine) using the DNA-SIP approach. *Methanoculleus* genus-specific real-time quantitative PCR (qPCR) primers were developed in this study and used to quantify its abundance in several animal feces and stored manure samples in order to assess its ability to persist and adapt to the environmental conditions in the tank.

## Materials and methods

### *Feces and manure sampling*

Manure samples stored outdoors in concrete tanks were obtained from a swine finishing operation (sample S1) and two commercial dairy farms (samples D1 and D2) located near Sherbrooke, Québec, Canada. The swine manure sample S1 was collected at a different farm than the one used in our preliminary work [3]. Details of the sampling procedure for manure samples have been described in a previous study [5]. Briefly, 1 kg manure samples were taken from the bottom of the outdoor storage tank of a swine farm (manure sample S1 from farm A) in April 2010, and two dairy farms (manure samples D1 and D2 from farms B and C, respectively) in June 2010. The manure in the tank for sample D1 had been mixed prior to sampling. From these primary (day 0) manure samples, four 0.5 mL subsamples were frozen on site in liquid nitrogen for subsequent DNA extraction, LH-mcrA fingerprinting, and qPCR quantification of *Methanoculleus* spp. The remainder of each manure sample was kept at ambient temperature during transport to the laboratory.

For studying the abundance of *Methanoculleus* spp. and *Methanosarcina* spp. across multiple samples reflecting a range of manure management stages, samples of fresh feces were collected from animals grown in the Dairy and Swine Research and Development Centre (Sherbrooke, Canada) and from manure storage tanks located on nine different commercial farms in Québec (farms A, B, C, D, H, I) and Ontario (farms E, F, G), Canada. Details regarding these samples are provided in the supplementary material (Table S1). Triplicate samples were taken from each storage tank. Following transport to the laboratory (*i.e.* always less than 24 h on ice), four aliquots of 0.5 mL were taken and frozen in liquid nitrogen prior to DNA extraction and purification for subsequent qPCR analyses.

### *Pre-incubation of manure samples S1, D1, and D2 for endogenous substrate starvation and subsequent DNA stable-isotope probing*

Duplicate 230 g subsamples of manure samples S1, D1 and D2 were pre-incubated in 500 mL sealed glass bottles with butyl rubber stoppers under anaerobic conditions in order to induce substrate (endogenous carbon) starvation, as previously described [4]. During the pre-incubation, biogas production rates were determined with a 2089 pressure gauge (Ashcroft Inc., Stratford, USA), and biogas compositions and volatile fatty acid (VFA) concentrations were determined by standard gas chromatography techniques (GC), as previously described [4]. On day 40 of the incubation and at starvation (*i.e.* when the VFA concentrations decreased to levels of less than 50 mg $L^{-1}$ and biogas production had decreased; on days 213, 132 and 173 for starved manure samples S1, D1 and D2, respectively), four 0.5 mL subsamples were removed from each incubated duplicate 230 g subsample. Then, the subsamples were immediately frozen in liquid nitrogen and stored at −80 °C for further qPCR (day 40 samples) and clone library analyses (starved samples).

Acetate was chosen as the substrate for DNA-SIP because it is the main precursor for methane production and it stimulates the growth of the majority of methanogens [15]. The starved manure samples were used for SIP incubations, as previously detailed [3]. Briefly, [$^{12}$C]- or [$^{13}$C]-acetate were added to each 45 mL subsample of S1, D1 and D2 manures to a final concentration of 6 g $L^{-1}$. Over a 35-day anaerobic incubation period at 25 °C, acetate degradation and biogas production were monitored by GC, and 0.5 mL aliquots of manure were sampled and immediately frozen at various time points. Then, DNA extraction and density fractionation followed by LH-mcrA fingerprinting and clone library analyses were carried out. Carbon flow associated with acetate metabolism and biogas production during SIP incubations were assessed as previously described [3].

### *DNA extraction, density fractionation and LH-mcrA fingerprinting*

In addition to extracting DNA from all farm manure samples (Table S1), DNA was extracted with a bead-beating method [31]