HAL
open science

Identification of Methanoculleus spp. as active methanogens during anoxic incubations of swine manure storage tank samples

Maialen Barret, Nathalie Gagnon, Martin L. Kalmokoff, Edward Topp, Yris Verasteguy, Stephen P. J. Brooks, Fernando Matias, Josh D. Neufeld, Guylaine Talbot

To cite this version:

Maialen Barret, Nathalie Gagnon, Martin L. Kalmokoff, Edward Topp, Yris Verasteguy, et al.. Identification of Methanoculleus spp. as active methanogens during anoxic incubations of swine manure storage tank samples. Applied and Environmental Microbiology, 2013, vol. 79, pp. 424-433. 10.1128/AEM.02268-12 . hal-00917867

HAL Id: hal-00917867

https://hal.science/hal-00917867v1

Submitted on 12 Dec 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Open Archive TOULOUSE Archive Ouverte (OATAO)
OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in : http://oatao.univ-toulouse.fr/
Eprints ID : 10456

To link to this article : DOI:10.1128/AEM.02268-12
URL : http://dx.doi.org/10.1128/AEM.02268-12

To cite this version : Barret, Maialen and Gagnon, Nathalie and Kalmokoff, Martin L. and Topp, Edward and Verasteguy, Yris and Brooks, Stephen P. J. and Matias, Fernando and Neufeld, Josh D. and Talbot, Guylaine. *Identification of Methanoculleus spp. as active methanogens during anoxic incubations of swine manure storage tank samples.* (2013) Applied and Environmental Microbiology, vol. 79 (n° 2). pp. 424-433. ISSN 0099-2240

Any correspondance concerning this service should be sent to the repository administrator: staff-oatao@listes-diff.inp-toulouse.fr

Identification of *Methanoculleus* spp. as Active Methanogens during Anoxic Incubations of Swine Manure Storage Tank Samples

Maialen Barret,[a] Nathalie Gagnon,[a] Martin L. Kalmokoff,[b] Edward Topp,[c] Yris Verastegui,[d] Stephen P. J. Brooks,[e] Fernando Matias,[e] Josh D. Neufeld,[d] Guylaine Talbot[a]

Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada, Sherbrooke, Quebec, Canada[a]; Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada[b]; Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada[c]; Department of Biology, University of Waterloo, Waterloo, Ontario, Canada[d]; Health Canada, Bureau of Nutritional Sciences, Health Products and Foods Branch, Banting Research Centre, Ottawa, Ontario, Canada[e]

Methane emissions represent a major environmental concern associated with manure management in the livestock industry. A more thorough understanding of how microbial communities function in manure storage tanks is a prerequisite for mitigating methane emissions. Identifying the microorganisms that are metabolically active is an important first step. Methanogenic archaea are major contributors to methanogenesis in stored swine manure, and we investigated active methanogenic populations by DNA stable isotope probing (DNA-SIP). Following a preincubation of manure samples under anoxic conditions to induce substrate starvation, [U-^{13}C]acetate was added as a labeled substrate. Fingerprint analysis of density-fractionated DNA, using length-heterogeneity analysis of PCR-amplified *mcrA* genes (encoding the alpha subunit of methyl coenzyme M reductase), showed that the incorporation of ^{13}C into DNA was detectable at *in situ* acetate concentrations (~7 g/liter). Fingerprints of DNA retrieved from heavy fractions of the ^{13}C treatment were primarily enriched in a 483-bp amplicon and, to a lesser extent, in a 481-bp amplicon. Analyses based on clone libraries of the *mcrA* and 16S rRNA genes revealed that both of these heavy DNA amplicons corresponded to *Methanoculleus* spp. Our results demonstrate that uncultivated methanogenic archaea related to *Methanoculleus* spp. were major contributors to acetate-C assimilation during the anoxic incubation of swine manure storage tank samples. Carbon assimilation and dissimilation rate estimations suggested that *Methanoculleus* spp. were also major contributors to methane emissions and that the hydrogenotrophic pathway predominated during methanogenesis.

In 2008, greenhouse gas (GHG) emissions from the agricultural sector accounted for 8.5% of the total GHG emissions in Canada, of which 12% originated from manure management (1). In livestock buildings, manure is first collected in gutters located below the animals and then transferred to an outdoor tank, where it is stored prior to being spread on agricultural fields. While strategies to treat manure using a variety of physical and biological processes are available, these approaches are not generally adapted to regional requirements and in most cases remain unaffordable for individual farms (2). Although a passive ambient alternative is the installation of an air-tight cover on these storage tanks, allowing for anaerobic digestion (3), this practice has not been widely adopted. Emissions of GHGs represent one of the major environmental problems associated with the open storage of manure. Park et al. (4) demonstrated that N_2O emissions from such facilities were insignificant, and methane was a primary GHG emitted from the manure tanks.

The conversion of manure organic matter into methane results from the activity of complex anaerobic consortia consisting of fermentative bacteria together with methanogenic archaea. Hydrolytic, acidogenic, and acetogenic bacteria ferment the organic matter producing volatile fatty acids (VFAs), including acetate and formate, as well as H_2 and CO_2. The direct conversion of H_2 (or formate) and CO_2 to methane is catalyzed by hydrogenotrophic methanogens. Related to the current study, the conversion of acetate to methane and CO_2 can be performed through two alternative pathways. The first pathway is a cleavage of the acetate molecule into methane and CO_2, which is catalyzed by acetoclastic methanogens. The second pathway relies on a syntrophic association where acetate-oxidizing bacteria first convert acetate into H_2 and CO_2, which are then used by the hydrogenotrophic methanogens to produce methane (5). In both pathways, the methanogenic archaea play a key role as terminal oxidizers of the community.

Previous cultivation-independent studies have identified *Methanobrevibacter*, *Methanogenium*, and *Methanocorpusculum* spp. as the dominant archaea associated with stored pig manure (6–8). However, manure storage tanks are similar to fed-batch bioreactors fed with communities indigenous to fresh manure, which are not necessarily active in the environmental conditions of storage tanks. The relative abundance of microorganisms may more closely reflect those associated with fresh manure rather than the community adapted to storage substrates and environmental conditions. On this basis, it is important to identify those microorganisms which are most active in methanogenesis rather than most abundant. Previously, we identified phylotypes related to *Methanoculleus* which became enriched during *in vitro* incubation of samples from swine manure storage tanks, supporting their involvement in methanogenesis (9). The DNA stable isotope probing (DNA-SIP) method (10, 11) is appropriate for identifying microorganisms that are actively involved in specific assimilatory processes under conditions that approximate those found in an

Address correspondence to Guylaine Talbot, guylaine.talbot@agr.gc.ca.

environment of interest. This cultivation-independent approach relies on the incorporation of an isotopically heavy substrate into the nucleic acids of metabolically active organisms in complex microbial communities, providing information on microbial networks participating in a wide range of metabolic functions, including methanogenesis in soils (12), sediments (13), and sludges (14) and from different substrates (15, 16).

The objective of this study was to identify the methanogenic archaea responsible for methane emissions during anoxic incubations of swine manure storage tank samples using DNA-SIP with [U-^{13}C]acetate as the labeled substrate. Identifying active manure storage tank methanogens represents an important first step to understanding the contribution of methanogenic archaea to GHG emissions.

MATERIALS AND METHODS

Manure sampling. A swine finishing farm located near Sherbrooke, Québec, Canada, was selected for the study. In April 2010, a 1-liter manure sample was collected from the bottom of the outdoor storage tank with a total manure depth of 3.5 m. The sampling apparatus consisted of a 3.6-m-long aluminum rod connected to a container with a retractable lid which was submerged and opened at the bottom of the tank. From the primary manure sample, four 0.5-ml aliquots were removed and immediately frozen in liquid nitrogen for molecular biology analyses. The remaining sample was maintained at an ambient temperature during transport from the farm to the laboratory.

Anoxic incubation for *in situ* substrate starvation. Within 4 h of collection, duplicate 230-g subsamples were transferred into 500-ml incubation bottles. The remainder (~500 ml) was frozen at −20°C and stored for subsequent physical and chemical analyses. The incubation bottles were flushed with nitrogen and then sealed with butyl rubber stoppers and aluminum crimp seals. These bottles were incubated at 25°C in a temperature-regulated chamber to mimic the summer temperature in Canadian storage tanks (17). At least once a week, biogas volume was measured with a 2089 pressure gauge (Ashcroft Inc.) and then released. An 8-ml sample was collected from the gaseous phase of one of the replicate bottles to determine the CH_4, CO_2, H_2S, and N_2 content with a Hach Carle 04131-C gas chromatograph (Loveland, Co.). Following sampling of the headspace gas, the bottles were shaken manually to homogenize the contents, and 0.5 ml was removed to determine volatile fatty acid (VFA) concentrations using an Autosystem gas chromatograph (PerkinElmer, Woodbridge, Canada). On day 78, when the levels of VFAs had fallen below 50 mg/liter and biogas production had decreased, indicating substrate starvation, four 0.5-ml subsamples were removed from one of the duplicate bottles, immediately frozen in liquid nitrogen, and stored at −80°C prior to DNA extraction. The remaining manure was used for the SIP incubations.

Stable-isotope probing incubations. The starved manure was the only sample used for SIP experiments. It was mixed in a 1:1 ratio with deionized water, and 45-ml subsamples were transferred into four 100-ml serum bottles. These manipulations were carried out quickly to minimize contact with air, and the bottles were immediately flushed with nitrogen and then sealed with butyl rubber stoppers and aluminum crimp seals. Stock solutions of [^{12}C]acetate and [U-^{13}C]acetate (99% ^{13}C; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) were prepared at 10 and 100 g/liter in sterile deionized water. Approximately 3 ml of the appropriate stock solution was added to reach 0.75 g/liter [U-^{13}C]acetate in bottle A, 0.75 g/liter [^{12}C]acetate in bottle B, 6.5 g/liter [U-^{13}C]acetate in bottle C, and 6.5 g/liter [^{12}C]acetate in bottle D. On days 0, 1, 3, 6, 10, 13, and 20, biogas volume was measured with a 2089 pressure gauge (Ashcroft Inc.), and excess pressure was released. Following each biogas measurement, the bottles were shaken manually to homogenize the contents, and a 0.5-ml sample was removed to determine VFA concentrations. In addition, on days 6 and 15, four 0.5-ml aliquots were removed and immediately frozen in liquid nitrogen for molecular analyses. At the end of the incubation, an 8-ml sample was collected from the gaseous phase of each bottle to analyze biogas composition as described previously (9).

Carbon flow calculation. The carbon flow (r_C, in mmol$_C$ kg$_{VSS}^{-1}$ day^{-1}; VSS stands for volatile suspended solids) associated with biogas production was assessed with the equation $r_C = (r_{CO2} + r_{CH4})/(V_{mol}[VSS])$, where r_{CO2} and r_{CH4} are the production rates of CO_2 and CH_4 (ml kg$_{manure}^{-1}$ day^{-1}), respectively, V_{mol} is the molar volume of an ideal gas at 25°C (24.5 liters/mol), and [VSS] is the concentration of volatile suspended solids in manure (kg$_{VSS}$/kg$_{manure}$). This calculation neglects the equilibrium of CO_2 between liquid and gas phases.

The carbon flow associated with acetate metabolism (r_C, in mmol$_C$ kg$_{VSS}^{-1}$ day^{-1}) was assessed with the equation $r_C = 2r_{Ac}/(M_{Ac}[VSS])$, where r_{Ac} is the consumption rate of acetate (mg kg$_{manure}^{-1}$ day^{-1}) and M_{Ac} is the molecular weight of acetate (g/mol).

DNA extraction and fractionation. From the frozen subsamples taken at days 0 and 78 of starvation incubation and at days 6 and 15 of SIP incubation, DNA was extracted as previously described (18), with minor modifications (19). Density fractionation of DNA was performed by ultracentrifugation on a cesium chloride gradient as specified by Neufeld et al. (10). Approximately 6 μg of DNA was loaded in each gradient prior to centrifugation, and dropwise collections of 420-μl fractions were taken from the bottom of the ultracentrifugation tube by pumping water into the top of the tube with a constant-flow syringe pump (420 μl/min; Braintree Scientific). The fraction densities were measured with an AR200 refractometer (Reichert) and ranged from 1.69 to 1.75 g/ml, with a median density of 1.72 g/ml.

Fingerprinting of archaea. To generate fingerprints of methanogenic archaeal communities, we used length heterogeneity (LH)-*mcrA* as detailed in Gagnon et al. (20), which is a PCR method based on natural length variations of the gene coding for the alpha subunit of the methyl-coenzyme M reductase involved in methane formation by methanogenic archaea. This method uses universal primers (mcrAfornew, 5′-GGT GTM GGD TTC ACH CAR TAY GC-3′; mcrArevnew, 5′-6-FAM-TTC ATN GCR TAG TTH GGR TAG TT-3′) to amplify the *mcrA* gene, which is then analyzed using capillary electrophoresis. PCR mixtures consisted of 1× *Taq* buffer (Bioshop Canada Inc., Burlington, Ontario, Canada), 1.5 mM $MgCl_2$ (Bioshop Canada Inc.), 0.5 μM each primer (Applied Biosystems Canada), 0.1 mM deoxynucleotide triphosphate (Bioshop Canada Inc.), DNA from manure (100 ng), and 0.625 U of *Taq* polymerase (Bioshop Canada Inc.) in a final volume of 25 μl. DNA denaturation was performed at 94°C for 2 min, followed by 28 cycles at 94°C for 60 s, annealing at 55°C for 60 s, elongation at 72°C for 60 s, and then a final extension step at 72°C for 10 min in an Eppendorf gradient thermal cycler (Fisher Scientific Ltd.). Amplifications were performed in duplicate. A 1-μl aliquot of appropriately diluted LH-*mcrA* amplification products was mixed with 0.06 μl of GeneScan 500 LIZ size standard (Applied Biosystems Canada) and 12.3 μl of Hi-Di formamide (Applied Biosystems Canada). Electrophoresis was performed with POP-4 polymer (Applied Biosystems Canada) on an ABI Prism 310 47-cm capillary DNA sequencer (Applied Biosystems Canada) for 40 min. Length analysis between 300 and 500 bp and determination of peak height were done using the GeneScan analysis software (Applied Biosystems Canada). Five distinct peaks were detected: 464-, 467-, 481-, 483-, and 485-bp amplicons. The relative abundance of amplicons was based on peak height. The corrected abundance was calculated from the dilution factor prior to electrophoresis and the volume of DNA extract used for LH-*mcrA*.

Cloning and sequencing. Clone libraries of the *mcrA* and 16S rRNA genes were constructed from the total DNA extract from day 0 of SIP incubation (control library) and from the pooled high-density fractions (1.717 and 1.722 g/ml) of DNA extract from day 6 (HD-2 library) and day 15 (HD-5 library) of [U-^{13}C]acetate incubation at the 6.5 g/liter initial concentration. PCR amplification of *mcrA* and 16S rRNA genes was performed using the same conditions as those for LH-*mcrA*, except that the final volume was 50 μl. Archaeal 16S rRNA genes were amplified with

TABLE 1 Physicochemical characteristics of the freshly sampled manure

Sample content or parameter	Result
TS[a] (g/liter)	113 ± 5
TSS[a] (g/liter)	111 ± 2
VS[a] (g/liter)	87 ± 4
VSS[a] (g/liter)	83 ± 3
TKN (g/liter)	5.1
NH3-N (g/liter)	3.6
pH	7.2
Acetate (g/liter)	7.43
Propionate (g/liter)	1.80
Isobutyrate (g/liter)	0.40
Butyrate (g/liter)	0.66
Isovalerate (g/liter)	0.50
Valerate (g/liter)	0.16
Caproate (g/liter)	0.06

[a] Data are averages and standard deviations calculated from three measurements.

Arch109f (5′-ACKGCTCAGTAACACGT-3′) and the universal 1492r primer (5′-ACGGYTACCTTGTTACGACTT-3′) (21). PCR products were used for library construction and sequence analysis as described by Barret et al. (9). Good's estimator of coverage of the libraries was calculated using the formula $[1 - (n_1/N)] \times 100$, where n_1 is the number of singletons and N the number of individuals.

Construction of phylogenetic trees. Sequences of the partial *mcrA* and 16S rRNA genes were initially aligned using MUSCLE (22). Aligned sequences were imported into the ARB program (23), compared using a similarity matrix, and assigned to consensus groups. Nearest relatives were obtained from NCBI following BLAST comparison of consensus sequences. Also included within the alignment were *mcrA* and 16S rRNA genes from the whole genomic sequences of various methanogens. All sequences were realigned using MUSCLE. The phylogenetic tree was generated with the PHYLO_WIN program (24) using the nearest neighbor algorithm and a Jukes-Cantor correction (25) with pairwise gap removal. In order to statistically evaluate the tree, bootstrap values were calculated using data resampled 1,000 times (26).

Physical and chemical analyses. The frozen primary manure sample (approximately 500 ml) was thawed and then homogenized using a PT10/35 Polytron (Binkman Instruments, Rexdale, Canada). The sample was analyzed for total solids (TS), total suspended solids (TSS), volatile solids (VS), VSS, total Kjeldhal nitrogen (TKN), ammoniacal nitrogen (NH_3-N), and pH as described previously (9).

Nucleotide sequence accession numbers. Sequences from clone libraries have been deposited in GenBank under accession numbers JX110124 to JX110159.

RESULTS

The physicochemical characteristics of the initial pig manure sampled for this study are provided in Table 1. Briefly, this manure contained 113 ± 5 g/liter of total solids and the ammonium content was 3.6 g/liter. Total VFA concentrations reached 11.0 g/liter and consisted of 67% acetic acid. Overall, these data are consistent with those of previous reports on stored swine manure, where acetate typically represents the most abundant intermediate of organic matter degradation (7, 27).

Based on LH-*mcrA* analysis (20), the methanogenic archaeal community within the manure storage tank was dominated by a single major amplicon of 485 bp (Fig. 1A). This peak represented 71% relative abundance and was followed by minor peaks at 467, 465, and 481 bp (11, 7, and 5%, respectively). In this study (Table 2) and in previous ones (9, 20), we identified the 485-bp amplicon as representative of members belonging to the *Methanomicrobiales* order (*Methanogenium*, *Methanospirillum*, and *Methanocorpusculum* spp.), the 467-bp amplicon corresponds to an uncultivated eukaryote, the 465-bp amplicon is associated with a *Methanobrevibacter* sp. sequence, and the 481-bp amplicon is associated with *Methanosarcina* spp. and *Methanoculleus* spp. In terms of the abundant genera, the archaeal community in this storage tank was similar to previously characterized communities (6–8).

From the beginning of the anoxic incubation for *in situ* substrate starvation until day 49, biogas was produced at a constant rate of 235 ± 17 ml kg_{manure}^{-1} day^{-1} (Fig. 2) and consisted of 73% ± 10% methane (averages and standard deviations of the two replicate bottles). During the first 10 days, acetate and propionate concentrations increased, suggesting that both acidogenic and acetogenic bacteria were producing these intermediates more rapidly than syntrophic bacteria and methanogenic archaea consumed them. Following this initial phase, acetate and propionate concentrations decreased and reached levels lower than 100 mg/liter by day 49 (Fig. 2), reflecting the ability to use acetate in the subsequent SIP incubation. Following day 49, the biogas production rate decreased and stabilized at the residual rate of 79 ± 7 ml kg_{manure}^{-1} day^{-1} until day 78 (averages and standard deviations from the two replicate bottles), when the anoxic incubation for *in situ* substrate starvation was terminated. This residual rate represented the endogenic activity of manure.

On day 78, the archaeal community was still dominated by the 485-bp amplicon, whereas the relative abundance of the 465- and 467-bp amplicons had decreased substantially (Fig. 1B). A 483-bp amplicon emerged and encompassed ~30% of LH-*mcrA* relative abundance (Fig. 1B). This amplicon aligned with operational tax-

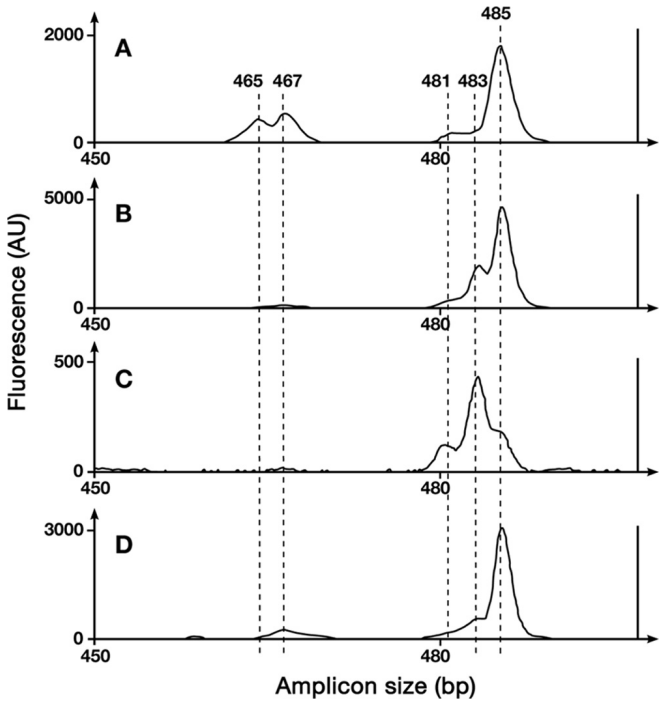

FIG 1 LH-*mcrA* fingerprints of methanogen populations in manure at the sampling day (A), manure at the end of the starvation period (day 0 of SIP incubation) (B), and high-density (C) and low-density (D) fractions at the end of [U-^{13}C]acetate incubation with 5 $mmol_C/g_{VSS}$ at day 15.

TABLE 2 LH-*mcrA* amplicon length of phylotypes and their abundance in *mcrA* gene clone libraries

Phylotype[a]	n^b	Amplicon length (bp)		Abundance in library[e] (%)		
		Seq[c]	LH-*mcrA*[d]	Control (n = 46)	HD-2 (n = 46)	HD-5 (n = 46)
Unidentified cluster						
LB14H05	4	470	466.4	0	2	7
LB11A06	3	470	465.7	4	0	2
LB11B05	3	470	466.4	4	2	0
LB11B04	1	470	466.7	2	0	0
LB11C05	1	470	464.9	2	0	0
Total				13	4	9
Methanosarcinaceae						
LB11A10	4	483	480.8	9	0	0
Methanoculleus						
LB11A01	48	488	482.5	22	48	35
LB17C08	14	488	480.7	0	17	13
LB11E10	7	488	482.7	2	9	4
LB11C09	6	488	483.7	2	2	9
LB14B01	4	488	484.1	0	4	4
LB14B12	2	488	480.8	0	4	0
LB14C10	1	488	483.8	0	2	0
LB14D12	1	488	481.6	0	2	0
LB17D12	1	488	482.6	0	0	2
Total				26	89	67
Methanocorpusculum						
LB11A05	6	488	484.8	11	0	2
LB11D08	2	488	485.7	2	0	2
Total				13	0	4
Methanogenium						
LB11B08	15	488	485.0	22	2	9
LB17D10	11	488	485.1	15	4	4
LB11F10	1	488	484.9	2	0	0
Total				39	7	13
Methanospirillum						
LB17B03	2	488	485.3	0	0	4
LB17G02	1	488	485.0	0	0	2
Total				0	0	6

[a] Clone libraries were sequenced starting with the control library (LB11) and ending with the HD-5 library (LB17). Clone designations begin with LB11 if they were first found in library LB11.
[b] Number of times that the phylotype was encountered across the three libraries.
[c] Theoretical amplicon length from sequence alignment with the newly designed *mcrA* primers.
[d] Experimental amplicon length determined by the LH-*mcrA* method.
[e] Control, control sample, day 0 of SIP incubation; HD-2, high-density DNA fraction of [U-^{13}C]acetate incubation at day 6; HD-5, high-density DNA fraction of [U-^{13}C]acetate incubation at day 15.

onomic units (OTUs) related to *Methanoculleus* (control library) (Table 2; also see Fig. 5A). Thus, the LH-*mcrA* patterns combined with clone library analysis indicated that uncultivated methanogenic archaea related to *Methanoculleus* were major contributors of methanogenesis that use endogenous substrates in this manure.

The kinetics of [U-^{13}C]acetate and [^{12}C]acetate degradation during the SIP incubations are shown in Fig. 3. Typically, incorporation of 0.005 to 0.5 mmol$_C$ per g soil or sediment is sufficient for DNA labeling (10). In subsample bottles A and B, 0.75 g/liter acetate, which is equivalent to ~0.6 mmol$_C$/g$_{VSS}$, was well within the range of concentrations typically used in ^{13}C-labeling experiments. However, this value is 10-fold lower than the acetate concentration within the ecological niche of a manure storage tank. Therefore, in subsample bottles C and D, a final concentration of 6.5 g/liter acetate was added and is equivalent to 5 mmol$_C$/g$_{VSS}$, which is more representative of the substrate concentration found in the stored manure. Acetate was depleted by 95% on days 10 and 13 at the lower and higher acetate concentrations, respectively, resulting in ^{13}C fluxes of 74 and 384 mmol$_C$ kg$_{VSS}^{-1}$ day^{-1}, respectively. The biogas production rates during acetate utilization represented C dissimilation rates of 50 ± 4 and 142 ± 1 mmol$_C$ kg$_{VSS}^{-1}$ day^{-1}, respectively (Table 3). When acetate concentrations became lower than 50 mg/liter, the C dissimilation rate associated with the manure endogenic activity in the four SIP incubations was 25 ± 2 mmol$_C$ kg$_{VSS}^{-1}$ day^{-1}. The percentage of dissimilated carbon from exogenous acetate was similar in the four SIP incubations, at 32% ± 2% (Table 3), and was in the range of that found in similar incubations (28, 29). The proportion of methane in the biogas generated over the incubation period reached 80, 79, 89, and 90% in bottles A, B, C, and D, respectively, indicating that methanogenesis occurred during the SIP incubations. In an attempt to maximize the ^{13}C labeling of DNA but avoid cross-feeding, manure samples from day 6 of the incubation at the lower acetate concentration (corresponding to 80% of acetate consumption) and from days 6 and 15 of the incubation at the higher acetate concentration (corresponding to 40 and 100% of acetate consumption, respectively) were chosen for further analyses. In these samples, the amount of acetate metabolized by the manure community reached 0.4, 2, and 5 mmol$_C$/g$_{VSS}$, respectively. From the percentage of acetate-C recovered in biogas (Table 3) and by neglecting the fraction of ^{13}C remaining in liquid phase, the percentage of ^{13}C assimilated by biomass could be estimated at 68, 70, and 70%, respectively. DNA from these samples was extracted, density fractionated, and profiled by LH-*mcrA* analysis.

In the sample from incubation with maximum ^{13}C utilization (5 mmol$_C$/g$_{VSS}$), LH-*mcrA* fingerprints from high-density fractions clearly differed from those of low-density fractions. The 483-bp amplicon had the most abundant peak in profiles from the heavy DNA fraction (1.721 g/ml) (Fig. 1C), whereas its relative abundance decreased in the light DNA fraction (1.708 g/ml)

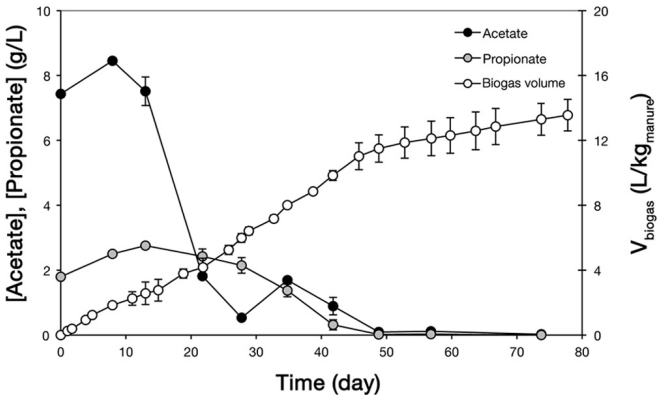

FIG 2 Dynamics of acetate concentration (black), propionate concentration (gray), and biogas production (white) during the incubation of manure for *in situ* substrate starvation.

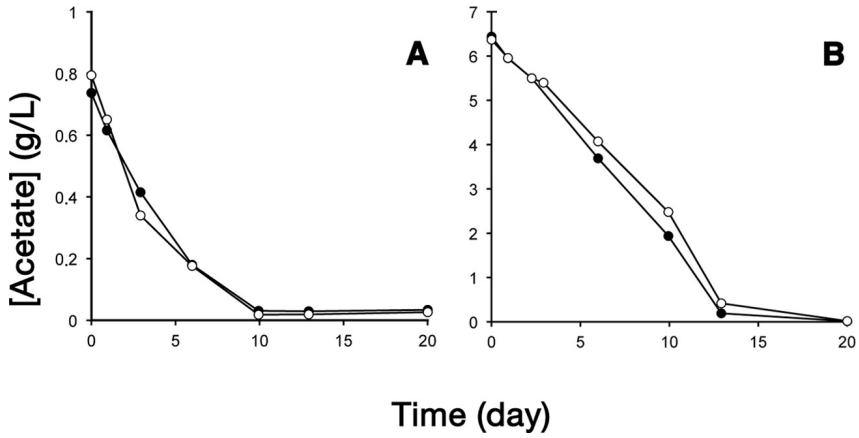

FIG 3 Kinetics of [U-^{13}C]acetate (black) and [^{12}C]acetate (white) consumption during the SIP incubation with 0.75 g/liter (A) and 6.5 g/liter (B) initial acetate concentration.

(Fig. 1D). The 485-bp amplicon was even more dominant in the light fraction than at day 0 of SIP incubation (Fig. 1B and D). These observations provide good evidence of DNA labeling with U-^{13}C-labeled substrate.

To better characterize the abundance of specific LH-*mcrA* amplicons associated with labeled DNA fractions, the corrected abundance of each of the five LH-*mcrA* amplicons was calculated in each density fraction and at the three levels of metabolized acetate in ^{13}C SIP incubations as well as in ^{12}C control incubations. Abundance results for the 481-, 483-, and 485-bp LH-*mcrA* amplicons are presented in Fig. 4. At 0.4 $mmol_C/g_{VSS}$ of metabolized acetate, these three amplicons were found in light DNA fractions only in both the ^{13}C incubation and the ^{12}C control. For SIP incubations at 2 $mmol_C/g_{VSS}$ of metabolized acetate, the 481- and 483-bp LH-*mcrA* amplicons were both associated with high-density fractions from [U-^{13}C]acetate incubations. For incubations at 5 $mmol_C/g_{VSS}$ of metabolized acetate, the abundance of the 481- and 483-bp amplicons increased in high-density fractions and surpassed the abundance of those same peaks in the low-density fractions. The ^{12}C controls demonstrated that the shift of the 481- and 483-bp amplicons to heavier fractions was conditional on the addition of [U-^{13}C]acetate. Interestingly, the absolute abundance of the 483-bp amplicon was four times higher than the abundance of the 481-bp amplicon, suggesting the higher growth and ^{13}C incorporation of the 483-bp-related archaea. In all SIP incubations, the 485-bp amplicon was only associated with light DNA fractions regardless of the amount of metabolized acetate (Fig. 4). This was also true for the 464- and 467-bp amplicons (data not shown). Taken together, these results indicate that two LH-*mcrA* phylotypes of methanogenic archaea responded in the SIP incubations by assimilating acetate during anoxic incubation of this swine manure sample.

TABLE 3 Carbon flow rates and dissimilation yields calculated from acetate utilization and biogas production rates during the starvation and SIP incubations

Incubation[a]	Rate of exogenous acetate utilization ($mmol_C\ kg_{VSS}^{-1}\ day^{-1}$)	Biogas production rate ($mmol_C\ kg_{VSS}^{-1}\ day^{-1}$)		Exogenous C dissimilated (%)
		Total[b]	Endogenous[c]	
A	74	47	24	32
B	83	52	23	35
C	384	142	25	30
D	381	143	27	30

[a] Incubations with 0.75 g/liter (A) or 6.5 g/liter (C) of [U-^{13}C]acetate and with 0.75 g/liter (B) or 6.5 g/liter (D) of [U-^{12}C]acetate.
[b] Biogas production rate resulting from exogenous acetate and endogenous substrates calculated during the utilization of exogenous acetate.
[c] Biogas production rate resulting from endogenous substrates calculated after the starvation of exogenous acetate.

In order to characterize the phylogeny and diversity of the archaeal phylotypes involved in acetate assimilation, clone libraries of *mcrA* and 16S rRNA genes were constructed from the total DNA extracts of the day 0 sample, the bulk SIP incubation sample (control library), and from the high-density fractions of the 2 $mmol_C/g_{VSS}$ (HD-2 library) and the 5 $mmol_C/g_{VSS}$ (HD-5 library) [U-^{13}C]acetate SIP samples. Overall, seven phylogenetic lineages were associated with these libraries, with gene sequences aligning with *Methanoculleus* spp., *Methanocorpusculum* spp., *Methanogenium* spp., *Methanospirillum* spp., *Methanosphaera* spp., *Methanosarcina* spp., and a lineage containing no known cultivated representatives. Phylotypes related to *Methanocorpusculum* spp. only occurred in *mcrA* gene clone libraries (Table 2 and Fig. 5A). The single clone related to *Methanosphaera* spp. was found only in the 16S rRNA gene clone control library (Table 4 and Fig. 5B). The Good's estimator of coverage was 87, 87, and 89% in control, HD-2, and HD-5 libraries of the *mcrA* gene, respectively, and 79, 95, and 92% in that of the 16S rRNA gene.

In *mcrA* gene clone libraries, the only phylogenetic group which had a significantly higher relative abundance in both HD-2 and HD-5 libraries than in the control library consisted of OTUs related to the *Methanoculleus* genus (Table 2 and Fig. 5A). Two *Methanoculleus* phylotypes not closely related to previously isolated species exhibited large increases in the high-density DNA fractions. The first, LB11A01, increased in relative abundance from 22% in the control library to 48 and 35% in HD-2 and HD-5 libraries, respectively. Because LB11A01 had an apparent LH-*mcrA* length of 482.5 bp (Table 2), its strong increase supported the emergence of the 483-bp amplicon in LH-*mcrA* fingerprints of high-density DNA fractions (Fig. 1C). The second phylotype, LB17C08, although not detectable in the control library, encompassed 17 and 13% of sequence abundance in HD-2 and HD-5

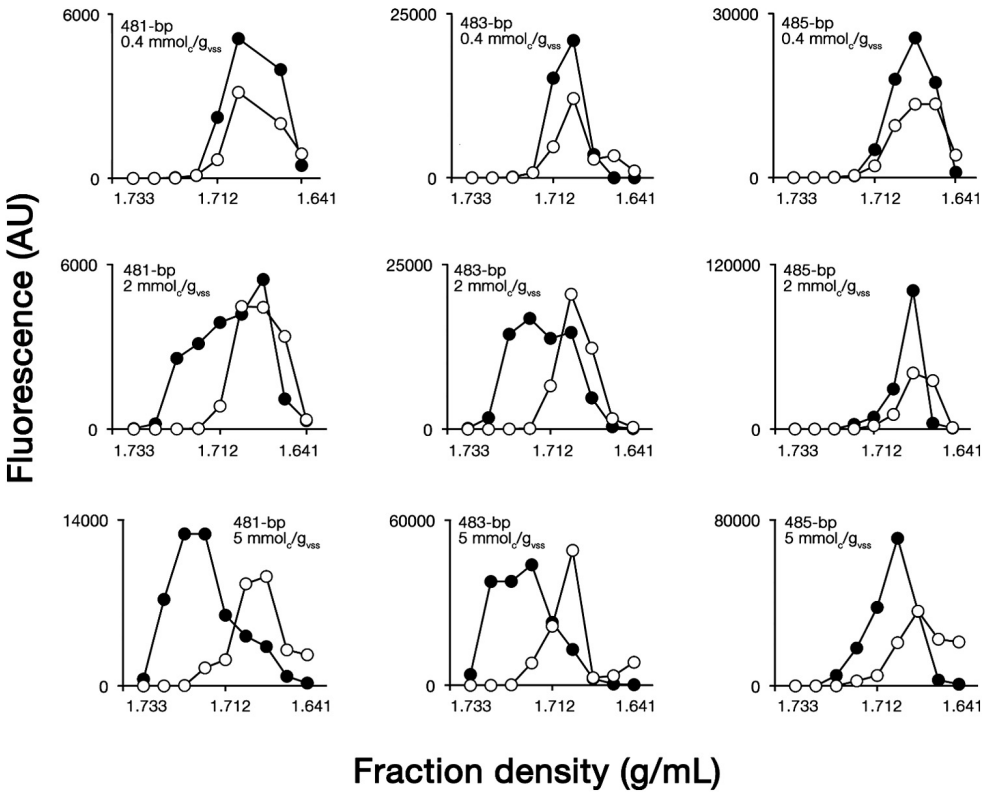

FIG 4 Abundance of 481-, 483-, and 485-bp amplicons in LH-*mcrA* fingerprints across the density fractions following the metabolization of 0.4, 2, and 5 $mmol_C/g_{VSS}$ in [U-^{13}C]acetate (black) and [^{12}C]acetate (white) incubation.

libraries, respectively. The increased abundance of this 481-bp LH-*mcrA* phylotype in the high-density DNA fraction library also correlated with the higher proportion of a 481-bp amplicon peak in LH-*mcrA* fingerprints after SIP incubation (Fig. 1B and C). Thus, the LH-*mcrA* patterns coupled with *mcrA* gene clone library analysis support that the methanogenic archaea which mainly assimilated ^{13}C from acetate during SIP incubation were related to *Methanoculleus* spp. The analysis of 16S rRNA gene clone libraries corroborated this finding, because the phylogenetic group of OTUs related to *Methanoculleus* spp. was also the only one which had a much higher relative abundance in HD-2 and HD-5 libraries than in the control library (Table 4 and Fig. 5B).

Moreover, the OTUs related to *Methanospirillum* spp. exhibited an increased abundance between control and HD-5 libraries (but not the HD-2 library): from 0 to 4% in *mcrA* gene clone libraries (Table 2) and from 3 to 18% in 16S rRNA gene clone libraries (Table 4). However, the abundance of the 485-bp LH-*mcrA* amplicon from the high-density DNA fractions at day 15 of SIP incubation was not increased compared to the one from the DNA at day 0 (Fig. 1B and C). The predominance, at day 0 of SIP incubation, of *Methanogenium* and *Methanocorpusculum* spp. (Tables 2 and 4, control library) at the same LH-*mcrA* amplicon size (485 bp) likely masked this increase.

DISCUSSION

The analysis of *mcrA* and 16S rRNA clone libraries labeled with 2 $mmol_C/g_{VSS}^{-1}$ of [U-^{13}C]acetate revealed that OTUs related to *Methanoculleus* spp. were enriched in high-density DNA fractions. Although some representatives of other genera may have assimilated small amounts of ^{13}C that did not allow for SIP detection, our results indicate that *Methanoculleus* spp. were the methanogenic archaea that mainly assimilated ^{13}C from acetate. Most species within this genus use H_2 and CO_2 as catabolic substrates and acetate as a carbon source for growth (30–32). Four possible assimilation/dissimilation schemes could be consistent with the assimilation of ^{13}C from acetate by *Methanoculleus* spp. during SIP incubation: (i) *Methanoculleus* spp. assimilated ^{13}C from acetate and catabolized acetate-derived H_2 and CO_2, (ii) *Methanoculleus* spp. assimilated ^{13}C from acetate and catabolized endogenous H_2 and CO_2, (iii) other hydrogenotrophs catabolized H_2 and CO_2 derived from [U-^{13}C]acetate and used an endogenous C source, and (iv) acetoclastic methanogens used [U-^{13}C]acetate as an electron source and endogenic substrates as the carbon source. However, the carbon flow associated with the endogenic activity of manure represented 18% of the total biogas production during acetate utilization in SIP incubations (Table 3), indicating that 82% of C dissimilation resulted from the degradation of exogenous acetate in SIP incubation. Furthermore, the C dissimilation during acetate utilization represented the typical yield of 32% ± 2% of metabolized acetate-C, suggesting that no major additional endogenic source of C was assimilated. The estimation of C flow in assimilatory and dissimilatory processes therefore suggests that not only was acetate assimilated by *Methanoculleus* spp. but it also provided the major part of the catabolic substrate to *Methanoculleus* spp. The *Methanoculleus* spp. that were enriched in high-density DNA fractions thus represented major contributors to methanogenesis during SIP incubation. Because known *Methanoculleus* spp. are hydrogenotrophs (30, 31, 33), this finding sug-

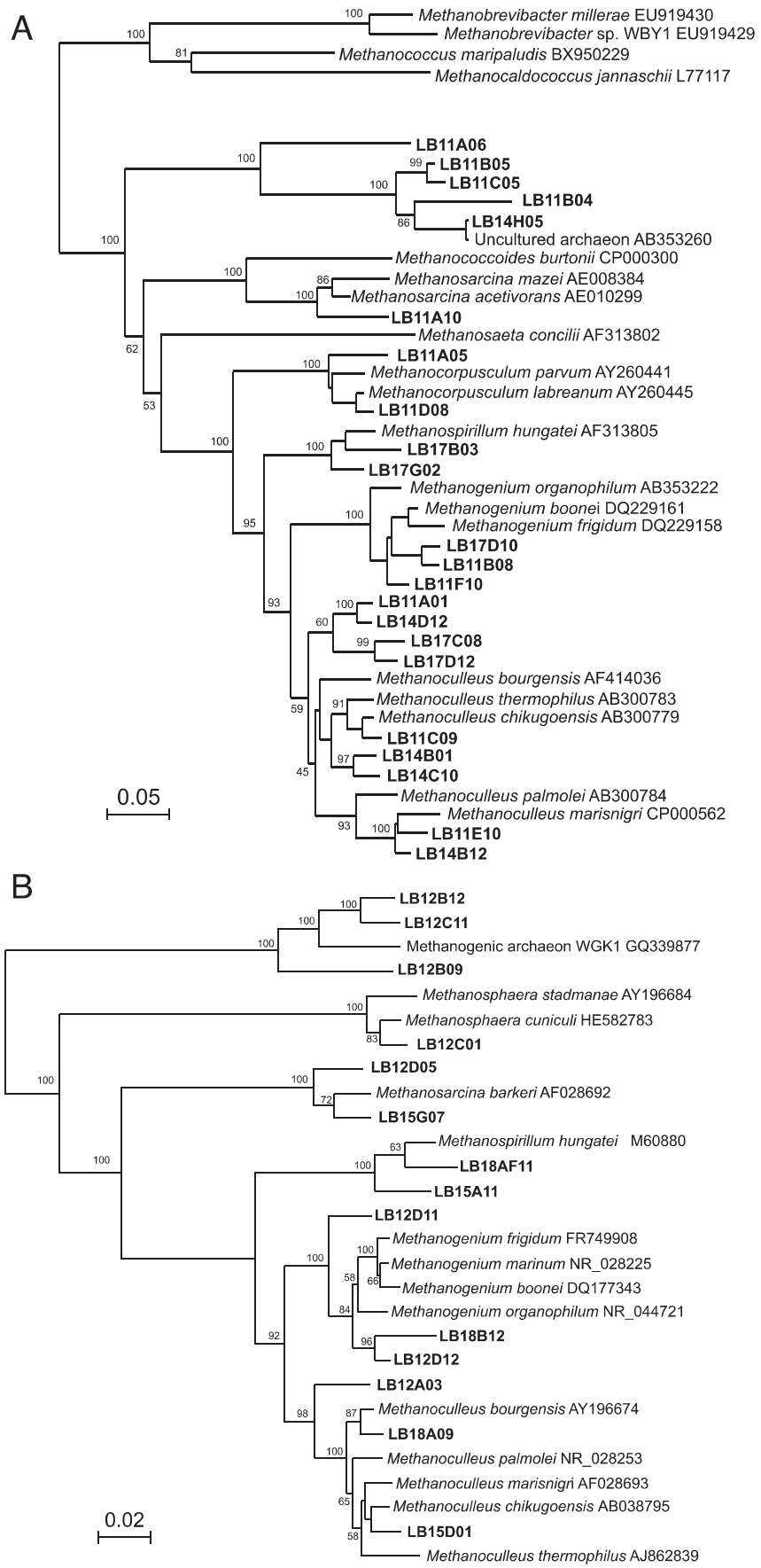

FIG 5 Phylogenetic tree derived from the alignment of DNA clones of *mcrA* (A) and 16S rRNA (B) with previously reported sequences. The legend bar represents a 5% sequence divergence. The tree was generated by neighbor joining with bootstrap values for 1,000 permutations indicated at the branch points. Clones are indicated in boldface.

TABLE 4 Analysis of phylotype abundance in 16S rRNA gene clone libraries

Phylotype	n^a	Abundance in library[b] (%)		
		Control (n = 39)	HD-2 (n = 39)	HD-5 (n = 39)
Unidentified cluster				
LB12B09	2	3	0	3
LB12B12	2	5	0	0
LB12C11	1	3	0	0
Total		10	0	3
Methanosphaera				
LB12C01	1	3	0	0
Methanosarcina				
LB12D05	1	3	0	0
LB15G07	1	0	3	0
Total		3	3	0
Methanoculleus				
LB18A09	50	23	54	51
LB15D01	9	3	21	0
LB12A03	2	3	0	3
Total		28	74	54
Methanogenium				
LB12D12	31	46	15	18
LB18B12	3	3	0	5
LB12D11	3	5	0	3
Total		54	15	26
Methanospirillum				
LB18F11	10	3	5	18
LB15A11	1	0	3	0
Total		3	8	18

[a] Number of times that the phylotype was encountered across the three libraries.
[b] Control, control sample, day 0 of SIP incubation; HD-2, high-density DNA fraction of [U-^{13}C]acetate incubation at day 6; HD-5, high-density DNA fraction of [U-^{13}C]acetate incubation at day 15.

gests that syntrophic bacteria oxidized acetate into H_2 and CO_2 during SIP incubation (34). Syntrophic association of acetate-oxidizing bacteria with hydrogenotrophic methanogens has been reported in the natural environment and engineered systems (34–37). However, the enrichment of bacterial phylotypes in high-density DNA fractions could not be detected by LH-PCR fingerprinting using primers that target the bacterial 16S rRNA gene (data not shown), probably because of the higher richness of bacterial communities than archaeal communities (9) and low abundance of syntrophic bacteria.

Comparisons of clone libraries suggested that *Methanospirillum* spp. were also active but only during the final phase of SIP incubation (days 6 to 15). The lower acetate concentration between 6 and 15 days of incubation (Fig. 3) may have affected the competition between methanogens due to the interspecies differences in terms of sensitivity to inhibition by acetate and affinity for acetate as a carbon source among the methanogens. In addition, the concentrations of H_2 and CO_2 also might have changed over the incubation period, which could affect the competition between methanogens due to the interspecies differences in terms of affinity for these methanogenesis substrates. The specific growth rates of *Methanoculleus* and *Methanospirillum* spp. are similar (32, 38). In contrast, the minimal threshold for hydrogen metabolism reported for *Methanoculleus submarinus* (39) is lower than that reported for *Methanospirillum hungatei* (40): 0.1 and 3.0 Pa, respectively. Due to interspecies differences in terms of threshold and affinity for hydrogen, the variations of H_2 concentration could have affected the competition between methanogens over the incubation period.

However, the results obtained over the initial 1 to 6 days are likely a better representation of the metabolically active methanogen community in storage conditions, as the acetate concentration better reflects that found *in situ* and short SIP incubation time avoids any cross-feeding interferences (41).

Although 0.4 $mmol_C/g_{VSS}$ of metabolized acetate is usually adequate for SIP experiments in terrestrial and aquatic environments (10), our results showed that it was insufficient to label the DNA from high-biomass manure communities. One possible reason is that the assimilation of the ^{12}C-labeled substrate background associated with the endogenic activity of this system was comparable to the assimilation rate of [U-^{13}C]acetate. The assimilation rate of ^{12}C-labeled substrate background can hardly be estimated. Nonetheless, the dissimilatory rate of ^{12}C-labeled substrate background estimated when the exogenous acetate was starved represented 24 $mmol_C\ kg_{VSS}^{-1}\ day^{-1}$, i.e., approximately half of the total dissimilatory rate during the utilization of exogenous acetate. These estimates indicate that the assimilated U-^{13}C-labeled substrate was diluted by background organic carbon by a 2-fold factor. This dilution may have resulted in an undetectable increase in DNA density, which represents a well-identified limitation of DNA-SIP (11, 41).

Two weaknesses are attributable to the experimental design of this study. The first weakness concerns the preincubation prior to SIP incubations. As discussed previously, the increase of DNA density was not detected when the ratio between the dissimilation rates of ^{13}C from exogenous acetate and ^{12}C from background was 1:1. The dissimilation rate from exogenous acetate degradation during SIP incubation at 6.5 g/liter represented 117 $mmol_C\ kg_{VSS}^{-1}\ day^{-1}$, although the biogas production rate at the beginning of the preincubation of the manure sample was equivalent to 116 $mmol_C\ kg_{VSS}^{-1}\ day^{-1}$. The SIP incubation of freshly sampled manure therefore would have resulted in a 1:1 ratio between the assimilation rates of exogenous acetate and endogenous substrates, i.e., a likely undetectable increase of DNA density. This estimate underlines the necessity of a preliminary starvation step for this swine manure, although this preliminary incubation did influence the structure of the archaeal community. A similar starvation approach has also been used for other substrate-rich communities (42). The second caveat that should be stated concerns the single sample used for SIP incubations. Previously, we reported that methanogenic activity at 25°C in samples from the bottom of this manure storage tank were more than 2-fold higher than those found in samples taken at shallow depths (9). Studying the bottom communities is thus of higher importance. Furthermore, the bacterial and archaeal communities in two samples taken from the bottom of the storage tank were very similar on the sampling day and after long-term anoxic incubations (9). On this basis, the single sample used here for DNA-SIP is likely a reasonable representation of the starved manure communities from this storage tank.

Theoretically, the U-^{13}C-labeled substrate selected for this experiment can be metabolized by methanogenic consortia through

both the hydrogenotrophic and acetoclastic pathways. However, our identification of *Methanoculleus* spp. as major contributors to methanogenesis together with the fact that species within this genus utilize H_2 and CO_2 and formate as electron sources (30–32) suggests that under these test conditions the hydrogenotrophic pathway predominated. The important question is whether this also holds true *in situ*. Several factors control acetoclastic versus hydrogenotrophic methanogenesis and competition between hydrogenotrophs. Inhibitor concentrations represent one factor. The hydrogenotrophic pathway was shown to be dominant at the high ammonia (43) and acetate (44) concentrations that are typical of swine manure, with the hydrogen-utilizing methanogens being less sensitive to inhibition by these compounds. The second factor is hydrogen availability, which is thought to represent a limiting factor for hydrogenotrophic methanogens (45). In the swine manure storage tank, the hydrogen availability may be different from that in SIP incubations with acetate. Indeed, the fermentation of other manure substrates represents an additional source of hydrogen, and hydrogen transfer within the bulk and during the gas phase is different, because storage tanks are mixed more seldomly (only prior to land spreading), the partial pressure of hydrogen at the surface of the storage tank is probably lower, and the manure height is higher than that in laboratory incubations. The final factor is temperature. A switch from the acetoclastic to hydrogenotrophic pathway was observed in rice soil when the temperature of incubation was increased from 25 to 50°C (12). Our laboratory incubations were carried out at 25°C, whereas the temperature in this storage tank had an annual mean of 10°C with peaks at approximately 25°C (data not shown). The storage conditions might not favor the hydrogenotrophic versus acetoclastic pathway to the same extent as the experimental conditions during laboratory incubations did. The storage conditions might also favor other hydrogenotrophic genera over *Methanoculleus* spp. that have different hydrogen affinities, growth rates, and temperature optima. More research is needed to verify if, *in situ*, hydrogenotrophic methanogenesis predominates over the acetoclastic pathway and if *Methanoculleus* spp. are in fact major contributors to *in situ* methanogenesis.

While *Methanoculleus* spp. represented major contributors to methanogenesis during SIP incubations, they were not numerically dominant methanogens in the storage tank (9). The potential discrepancy between the most abundant microorganisms in the swine manure community compared to those which may represent the most active methanogens as suggested by our SIP incubations could be related to several factors. First, the fed-batch operation of storage tanks implies that the stored manure is fed with communities indigenous to fresh manure. These communities, adapted to the ecological niche of the pig digestive tract, are not necessarily active when their environmental conditions change to those of the storage tank (e.g., different substrate availability, inhibitor concentrations, pH, and temperature). Second, the low temperatures associated with this storage tank (annual mean of 10°C with peaks at 25°C; data not shown) may not allow sufficient growth to enrich the metabolically active microorganisms *in situ*. Both factors imply that the major contributors to *in situ* methanogenesis are not abundant in storage tank communities.

Several recent studies have found that the hydrogenotrophic pathway and the *Methanoculleus* genus were predominant in reactors fed with liquid swine manure (46, 47) or with manures mixed with other agricultural wastes (48, 49). Not only are *Methanoculleus* spp. active in controlled and engineered systems, these archaea also may be important contributors to methanogenesis within the manure storage tank studied here. More research is needed to investigate the phylogenetic variability of active methanogens between different manure storage tanks, taking into account the spatial and temporal variations of microbial communities and environmental conditions in storage tanks. This first SIP study of swine manure storage tank samples provides new information that will help improve the assessment and modeling of methane emissions during manure storage.

ACKNOWLEDGMENTS

This work was financially supported by Agriculture and Agri-Food Canada under the Sustainable Agriculture Environmental Systems (SAGES) research program. J.D.N. was supported by Discovery and Strategic Project Grants from the National Sciences and Engineering Research Council of Canada (NSERC).

REFERENCES

1. **Blain D, Liang C, MacDonald D.** 2010. National inventory report 1990–2008: greenhouse gas sources and sinks in Canada, p 153–167. *In* Agriculture, part 1, chapter 6. Environnement Canada—Greenhouse Gas Division, Gatineau, Canada.
2. **Martinez J, Dabert P, Barrington S, Burton C.** 2009. Livestock waste treatment systems for environmental quality, food safety, and sustainability. Bioresource Technol. **100**:5527–5536.
3. **Nohra JA, Barrington S, Frigon JC, Guiot SR.** 2003. In storage psychrophilic anaerobic digestion of swine slurry. Resource Conserv. Recyc. **38**: 23–37.
4. **Park KH, Thompson AG, Marinier M, Clark K, Wagner-Riddle C.** 2006. Greenhouse gas emissions from stored liquid swine manure in a cold climate. Atmos. Environ. **40**:618–627.
5. **Schink B, Stams A.** 2006. Syntrophism among prokaryotes, p 309–335. *In* Dworkin EA (ed), The prokaryotes. Springer, New York, NY.
6. **Peu P, Brugère H, Pourcher AM, Kérourédan M, Godon JJ, Delgenès JP, Dabert P.** 2006. Dynamics of a pig slurry microbial community during anaerobic storage and management. Appl. Environ. Microbiol. **72**:3578–3585.
7. **Snell-Castro R, Godon JJ, Delgenès JP, Dabert P.** 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiol. Ecol. **52**:229–242.
8. **Whitehead TR, Cotta MA.** 1999. Phylogenetic diversity of methanogenic archaea in swine waste storage pits. FEMS Microbiol. Lett. **179**:223–226.
9. **Barret M, Gagnon N, Morissette B, Topp E, Kalmokoff M, Brooks S, Matias F, Massé D, Masse L, Talbot G.** 2012. Methanoculleus spp. as a biomarker of methanogenic activity in swine manure storage tanks. FEMS Microbiol. Ecol. **80**:427–440.
10. **Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell CJ.** 2007. DNA stable-isotope probing. Nat. Protoc. **2**:860–866.
11. **Radajewski S, Ineson P, Parekh NR, Murrell JC.** 2000. Stable-isotope probing as a tool in microbial ecology. Nature **403**:646–649.
12. **Rui J, Qiu Q, Lu Y.** 2011. Syntrophic acetate oxidation under thermophilic methanogenic condition in Chinese paddy field soil. FEMS Microbiol. Ecol. **77**:264–273.
13. **Schwarz JIK, Lueders T, Eckert W, Conrad R.** 2007. Identification of acetate-utilizing Bacteria and Archaea in methanogenic profundal sediments of Lake Kinneret (Israel) by stable isotope probing of rRNA. Environ. Microbiol. **9**:223–237.
14. **Hatamoto M, Imachi H, Ohashi A, Harada H.** 2007. Identification and cultivation of anaerobic, syntrophic long-chain fatty acid-degrading microbes from mesophilic and thermophilic methanogenic sludges. Appl. Environ. Microbiol. **73**:1332–1340.
15. **Lu Y, Conrad R.** 2005. In situ stable isotope probing of methanogenic Archaea in the rice rhizosphere. Science **309**:1088–1090.
16. **Lu Y, Lueders T, Friedrich MW, Conrad R.** 2005. Detecting active methanogenic populations on rice roots using stable isotope probing. Environ. Microbiol. **7**:326–336.
17. **Massé DI, Masse L, Claveau S, Benchaar C, Thomas O.** 2008. Methane emissions from manure storages. Trans. ASABE **51**:1775–1781.

18. **Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ.** 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. **66**:5488–5491.
19. **Roy CS, Talbot G, Topp E, Beaulieu C, Palin MF, Massé DI.** 2009. Bacterial community dynamics in an anaerobic plug-flow type bioreactor treating swine manure. Water Res. **43**:21–32.
20. **Gagnon N, Barret M, Topp E, Kalmokoff M, Massé D, Masse L, Talbot G.** 2011. Novel fingerprinting technology to assess the diversity of methanogens. FEMS Microbiol. Lett. **325**:115–122.
21. **Lane DJ.** 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt EG (ed), Nucleic acid techniques in bacterial systematics. Wiley, Chichester, United Kingdom.
22. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32**:1792–1797.
23. **Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar A, Buchner A, Lai T, Steppi S, Jacob G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßbmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32**:1363–1371.
24. **Galtier N, Gouy M, Gautier C.** 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. **12**:543–548.
25. **Jukes TH, Cantor SR.** 1969. Evolution of protein molecules, p 81–132. *In* Munro HN (ed), Mammalian protein metabolism. Academic Press, New York, NY.
26. **Felsenstein J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.
27. **Massé DI, Croteau F, Patni NK, Masse L.** 2003. Methane emissions from dairy cow and swine manure slurries stored at 10°C and 15°C. Can. Biosyst. Eng. **45**:6.1–6.6.
28. **Hori T, Noll M, Igarashi Y, Friedrich MW, Conrad R.** 2007. Identification of acetate-assimilating microorganisms under methanogenic conditions in anoxic rice field soil by comparative stable isotope probing of RNA. Appl. Environ. Microbiol. **73**:101–109.
29. **Liu F, Conrad R.** 2010. Thermoanaerobacteriaceae oxidize acetate in methanogenic rice field soil at 50°C. Environ. Microbiol. **12**:2341–2354.
30. **Dianou D, Miyaki T, Asakawa S, Morii H, Nagaoka K, Oyaizu H, Matsumoto S.** 2001. Methanoculleus chikugoensis sp. nov., a novel methanogenic archaeon isolated from paddy field soil in Japan, and DNA-DNA hybridization among Methanoculleus species. Int. J. Syst. Evol. Microbiol. **51**:1663–1669.
31. **Mikucki JA, Liu Y, Delwiche M, Colwell FS, Boone DR.** 2003. Isolation of a methanogen from deep marine sediments that contain methane hydrates, and description of Methanoculleus submarinus sp. nov. Appl. Environ. Microbiol. **69**:3311–3316.
32. **Zellner G, Messner P, Winter J, Stackebrandt E.** 1998. Methanoculleus palmolei sp. nov., an irregularly coccoid methanogen from an anaerobic digester treating wastewater of a palm oil plant in North Sumatra, Indonesia. Int. J. Syst. Bacteriol. **48**:1111–1117.
33. **Cheng L, Qiu T-L, Li X, Wang W-D, Deng Y, Yin X-B, Zhang H.** 2008. Isolation and characterization of Methanoculleus receptaculi sp. nov. from Shengli oil field, China. FEMS Microbiol. Lett. **285**:65–71.
34. **Schnürer A, Zellner G, Svensson BH.** 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol. Ecol. **29**:249–261.
35. **Nüsslein B, Chin K-J, Eckert W, Conrad R.** 2001. Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). Environ. Microbiol. **3**:460–470.
36. **Peterson S, Ahring B.** 1991. Acetate oxidation in a thermophilic anaerobic sewage-sludge digestor: the importance of non-aceticlastic methanogenesis from acetate. FEMS Microbiol. Ecol. **86**:149–158.
37. **Shigematsu T, Era S, Mizuno Y, Ninomiya K, Kamegawa Y, Morimura S, Kida K.** 2006. Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. Appl. Microbiol. Biotechnol. **72**:401–415.
38. **Koster IW, Koomen E.** 1988. Ammonia inhibition of the maximum growth rate (μ_m) of hydrogenotrophic methanogens at various pH-levels and temperatures. Appl. Microbiol. Biotechnol. **28**:500–505.
39. **Chong SC, Liu Y, Cummins M, Valentine DL, Boone DR.** 2002. Methanogenium marinum sp. nov., a H_2-using methanogen from Skan Bay, Alaska, and kinetics of H_2 utilization. Antonie Van Leeuwenhoek **81**:263–270.
40. **Zinder S.** 1993. Physiological ecology of methanogens, p 128–206. *In* Ferry JG (ed), Methanogenesis. Chapman & Hall, New York, NY.
41. **Neufeld JD, Dumont MG, Vohra J, Murrell JC.** 2007. Methodological considerations for the use of stable isotope probing in microbial ecology. Microb. Ecol. **53**:435–442.
42. **Li T, Mazéas L, Sghir A, Leblon G, Bouchez T.** 2009. Insights into networks of functional microbes catalysing methanization of cellulose under mesophilic conditions. Environ. Microbiol. **11**:889–904.
43. **Angenent LT, Sung S, Raskin L.** 2002. Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Water Res. **36**:4648–4654.
44. **Hao LP, Lü F, He PJ, Li L, Shao LM.** 2011. Predominant contribution of syntrophic acetate oxidation to thermophilic methane formation at high acetate concentrations. Environ. Sci. Technol. **45**:508–513.
45. **Bagi Z, Nács Bálint B, Horváth L, Dobó K, Perei KR, Rákhely G, Kovács KL.** 2007. Biotechnological intensification of biogas production. Appl. Microbiol. Biotechnol. **76**:473–482.
46. **Patil SS, Kumar MS, Ball AS.** 2010. Microbial community dynamics in anaerobic bioreactors and algal tanks treating piggery wastewater. Appl. Microbiol. Biotechnol. **87**:353–363.
47. **Zhu C, Zhang J, Tang Y, Zhengkai X, Song R.** 2011. Diversity of methanogenic archaea in a biogas reactor fed with swine feces as the mono-substrate by mcrA analysis. Microbiol. Res. **166**:27–35.
48. **Jaenicke S, Ander C, Bekel T, Bisdorf R, Dröge M, Gartemann KH, Jünemann Kaiser O, Krause L, Tille F, Zakrzewski M, Pühler A, Schlüter A, Goesmann A.** 2011. Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing. PLoS One **6**:e14519. doi:10.1371/journal.pone.0014519.
49. **Nettmann E, Bergmann I, Pramschüfer S, Mundt K, Plogsties V, Herrmann C, Klocke M.** 2010. Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. Appl. Environ. Microbiol. **76**:2540–2548.