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Long-Term Monensin Supplementation Does Not Significantly Affect the Quantity or Diversity of Methanogens in the Rumen of the Lactating Dairy Cow▽

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A long-term monensin supplementation trial involving lactating dairy cattle was conducted to determine the effect of monensin on the quantity and diversity of rumen methanogens in vivo. Fourteen cows were paired on the basis of days in milk and parity and allocated to one of two treatment groups, receiving (i) a control total mixed ration (TMR) or (ii) a TMR with 24 mg of monensin premix/kg of diet dry matter. Rumen fluid was obtained using an ororuminal probe on day −15 (baseline) and days 20, 90, and 180 following treatment. Throughout the 6-month experiment, the quantity of rumen methanogens was not significantly affected by monensin supplementation, as measured by quantitative real-time PCR. The diversity of the rumen methanogen population was investigated using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone gene libraries. DGGE analysis at each sampling point indicated that the molecular diversity of rumen methanogens from monensin-treated cattle was not significantly different from that of rumen methanogens from control cattle. 16S rRNA gene libraries were constructed from samples obtained from the rumen fluids of five cows, with a total of 166 clones examined. Eleven unique 16S rRNA sequences or phylotypes were identified, five of which have not been recognized previously. The majority of clones (98.2%) belonged to the genus *Methanobrevibacter*, with all libraries containing *Methanobrevibacter* strains M6 and SM9 and a novel phylotype, UG3322.2. Overall, long-term monensin supplementation was not found to significantly alter the quantity or diversity of methanogens in the rumens of lactating dairy cattle in the present study.

Rumen methanogens are involved in interspecies hydrogen transfer and the production of methane gas as an end product of fermentation (28). The accumulation of hydrogen as a waste product of rumen microbe fermentation has the ability to inhibit metabolism, and so the removal of hydrogen by methanogens is important to maintain normal rumen functioning (28). Methane produced by rumen methanogens, as well as being a potent greenhouse gas, is produced at a loss, ranging from 2 to 12%, of gross energy for the animal (13). For these reasons, the inhibition of methane is an important area of research in greenhouse gas mitigation and ruminant production systems.

Recent efforts have been directed at methane mitigation in the bovine rumen, and monensin treatment is one strategy that is being investigated due to the role of monensin as a carboxylic polyether ionophore capable of interfering with ion flux within prokaryotic cells through its action as an ion carrier (2). It is generally accepted that the impact of monensin on methane production is through its suppression of other rumen microorganisms that provide methanogens with substrates (2, 4, 31). In a recent in vivo study, the use of long-term monensin supplementation for lactating dairy cattle decreased ruminal methane production by 7% (18). Along with the information now available about the long-term success of monensin treatment as a methane reduction strategy, it is also important to know the mechanism of action in the rumen and, more specifically, whether or not monensin is altering the quantity and/or diversity of the methanogens in the rumen to accomplish this decrease in methane output.

The aim of the present study was to provide a molecular analysis of the effect of long-term monensin supplementation on the numbers and diversity of rumen methanogens in lactating dairy cattle in Ontario, Canada, who were fed a total mixed ration (TMR) for milking cows.

MATERIALS AND METHODS

Animals. The protocol for this study was approved by the University of Guelph Animal Care Committee. The animals and treatments have been described previously by Odongo et al. (18). Briefly, 14 lactating Holstein dairy cows housed in a tie-stall barn at Elora Dairy Research Centre (University of Guelph, Guelph, Canada) were paired based on days in milk (average ± standard error [SE] of 106.86 ± 13.27 days) and parity in a double-blind, color-coded experiment and assigned to one of two treatments. Cows had an average age of 3.14 ± 1.17 years at the trial commencement and an average body weight of 637.5 ± 78.8 kg throughout the trial. Cows in the treatment group received the TMR used at Elora Dairy at a 60:40 forage-to-concentrate ratio with 24 mg of Rumensin premix (Elanco Animal Health, Guelph, Canada)/kg of diet dry matter, with the monensin incorporated into a soy hull carrier. Cows in the control group received the TMR with soy hulls only. A listing of diet ingredients and chemical compositions can be found in a previous publication by Odongo et al. (18). Cows were milked at 0500 h and 1500 h and pair fed ad libitum once daily following the first milking, with allowance for 5 to 10% refusal, and the dry matter intake (DMI) was recorded daily. Cows were also given free access to water. On days when samples of rumen digesta were obtained, cows were fed following sampling at 0830 h.

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Rumen sampling. Rumen sampling was performed on four separate occasions for each pair of cows: (i) 15 days prior to the start of feeding with experimental diets (baseline; day −15) and (ii) 20 days, (iii) 90 days, and (iv) 180 days after the start of feeding with experimental diets (days 20, 90, and 180), with the experimental diets commencing on day 0. Samples of rumen digesta were collected using a weighted ororuminal probe at approximately 0800 h, and sampling equipment was washed between sample collections from different experimental animals. The first 100- to 200-ml sample of rumen contents per animal was discarded to limit contamination with saliva. Approximately 50 ml of rumen contents from the second sample was filtered through four layers of autoclaved gauze, and the resulting rumen fluid was immediately placed on ice. Samples were aliquoted into sterile 2.0-ml Cryovial tubes in a 1:1 dilution with 100% ethanol for storage. DNA extraction followed the glass milk protocol of Sundset et al. (29).

Sample coding. Samples from control cows (3030, 3158, 3241, 3322, 3340, 3350, and 3352) were coded 1 to 7, respectively, whereas samples from monensin-treated cows (3088, 3267, 3336, 3343, 3349, 3355, and 3364) were coded 8 to 14, respectively. This coding system was used throughout the analyses.

Quantitative real-time PCR. External standards were prepared using a mixture of pure cultures of *Methanobrevibacter ruminantium* $M1^T$ and *Methanobrevibacter smithii* PS^T as described by Christophersen (5) and diluting the culture samples to a range of 1.0×10^3 to 1.0×10^8 cells per standard. Each pure isolate was cultured, and cells were counted using a Coulter Counter (Multisizer TM3; Beckman). Methanogen real-time PCR primers Met630F (5′-GGA TTA GAT ACC CSG GTA GT-3′) (5) and Met803R (5′-GTT GAR TCC AAT TAA ACC GCA-3′) (5) were used to enumerate the methanogens represented in the extracted DNA from rumen samples by using the protocol of Christophersen (5). Amplification was performed using Bio-Rad's Icycler (Hercules) with a volume of 25 μl containing the following reagents: 12.5 μl of Platinum Sybr green quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 5 μM (each) PCR primers, and 5.0 μl of template DNA. Real-time amplification commenced with a hot start of 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Fluorescence was measured during extension by using an excitation wavelength of 470 nm and a detection wavelength of 530 nm. Melting-curve analysis occurred by monitoring fluorescence continuously between 60 and 95°C, with 10-s increments of 0.5°C. The Icycler software (version 3.5) automatically determined the threshold cycles, and PCR efficiency (ε) for each extract was determined using the equation $\varepsilon = 10^{-1/\text{slope}}$, based on a linear regression of the cycle number where fluorescence was detected (threshold cycle) versus the log dilution, as outlined by Liu and Saint (16). The *N* constant was calculated by the equation $N = N_0 \times \varepsilon^n$, where N_0 is the number of methanogens per the quantity of DNA initially present in the sample, ε is the PCR efficiency, and *n* is the cycle number (5). DNA amplification was performed in triplicate, and only sample dilutions that had a PCR efficiency between 1.8 and 2.1 were used to calculate the mean PCR efficiency for that sample. If the PCR efficiencies for all dilutions were outside the 1.8-to-2.1 range, then real-time PCR amplifications were repeated for the sample.

Statistical analysis. The investigators were blinded to the treatments until after statistical analysis was complete. The response of methanogens per gram (wet weight) was analyzed as a randomized complete block design with repeated measures, where pairs of animals were blocks and measurements were obtained on days 20, 90, and 180. Data were log transformed prior to analysis, and the baseline number of methanogens per gram (wet weight), the square of the baseline number of methanogens per gram (wet weight), and the DMI were used as covariates. Analysis was performed as a mixed procedure in the SAS program (version 9.1.3; SAS Institute, Inc., Cary, NC) by following the method described by Wang and Goonewardene (32).

Denaturing gradient gel electrophoresis (DGGE) and analysis. Methanogen 16S rRNA gene amplification was carried out using primers described by Christophersen (5), Met630F and Met803R, except that a 40-bp G-C clamp was added to the 5′ end of the forward primer. PCR amplification was performed with a 25-μl reaction volume containing 0.2 μl of ExTaq polymerase (TaKaRa Bio, Inc., Japan), 2.5 μl of buffer, 500 nM (each) primers, 3 μl of deoxynucleoside triphosphate mix, and 1 μl of template DNA. Amplification occurred using a Mastercycler gradient (Eppendorf AG, Hamburg, Germany) with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation (95°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 90 s), with a final extension at 72°C for 8.5 min (5). PCR products were run on a 2% agarose gel with ethidium bromide staining to confirm amplification.

According to the protocol of Christophersen (5), samples were loaded onto an 8% polyacrylamide gel with a 40 to 55% denaturing gradient, and aliquots of approximately 10 μl of a 1-kb Plus DNA ladder (Invitrogen, Carlsbad, CA) were added to both ends of the gel. Fifteen microliters of the PCR product was combined with 10 μl of 2× loading dye, and the mixture was loaded onto the DGGE gel. Each gel represented one sampling day (i.e., day −15, 20, 90, or 180), and gels were run at 59°C for 13 h at 75 V in 0.5× Tris-acetate-EDTA buffer (5). Gels were incubated in silver stain buffer A (10% ethanol, 0.5% acetic acid) for 20 min and then submerged in silver stain buffer B (0.15% $AgNO_3$, 0.05% formaldehyde) for 7 min. Gels were rinsed in deionized water for 10 s and incubated in a mixture of silver stain buffer C (1.5% NaOH) and 200 μl of formaldehyde for 40 min. Finally, gels were submerged in silver stain buffer D (10% ethanol, 5% acetic acid) for 5 min.

A total of 26 distinct bands on the DGGE gels across all four time points were numbered 1 through 26, and their presence or absence in each lane was recorded as 0 for absent or 1 for present. The matrix was input into RESTDIST (PHYLIP; version 3.66) (8) to obtain restriction distances for the analysis of a time effect. An additional restriction distance matrix was also made to analyze the treatment effect without data from day −15, when no treatment was applied. Both restriction matrices were analyzed in DOTUR (version 1.3) (22) for the number of unique operational taxonomic units (OTUs) and the Shannon diversity index (23).

Clone library construction and restriction fragment length polymorphism analysis. Based on the banding patterns observed in the DGGE analysis, four rumen samples that produced unique banding patterns (i.e., day 20 samples 4 and 5, day 90 sample 1, and day 180 sample 12) were selected and one rumen sample (day −15 sample 3) was selected to represent the standard banding pattern. These five samples (1, 3, 4, 5, and 12) were used to make clone libraries, labeled A to E, respectively. The amplification of the rumen methanogen DNA was performed using the PCR primers Met86F and Met1340R by following the protocol of Wright and Pimm (35).

Methanogen 16S rRNA gene clone libraries were constructed using a TOPO cloning kit and the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) with chemically competent *Escherichia coli* TOP10F′ according to the methods described by Wright et al. (39, 40).

The digestion of the 16S rRNA gene insert was performed using the restriction enzyme HaeIII according to the manufacturer specifications (Invitrogen), and the digest was separated on a 4% molecular screening agarose gel alongside a 1-kb Plus ladder at 100 V for 45 min and visualized by ethidium bromide staining under UV light. Initial identification of the phylotypes was made based on the comparison of the banding patterns with those published previously (35–38, 40). HaeIII cannot differentiate *Methanobrevibacter* sp. strains M6, SM9, and PS, so a subsequent digestion with Sau3AI was required to distinguish these samples. Clones that could not be identified based on restriction fragment length polymorphism were sequenced as described by Wright et al. (38). Furthermore, at least one representative clone from each identified phylotype was also selected for sequencing to confirm identification.

Sequence analysis. Sequences were checked for chimeras by using Bellerophon (11). GenBank's basic local alignment and search tool (BLAST) (1) was used to initially identify all sequences. All the clone library sequences were assembled in BioEdit (version 7.0.0; Ibis Therapeutics, Carlsbad, CA) and aligned with ClustalW (30). Distance data were generated using the Kimura two-parameter model (14) and analyzed using DOTUR (22) with 98% identity to determine OTUs and the Shannon diversity index (23) for each library, including the pooled library (3). Pielou's evenness index (*E*) (20) was also calculated to measure how equal the variations within a population were or how even the distribution of phylotypes within a population was by using the following equation: $E = H'/\ln S$, where H' equals the Shannon diversity index and S equals number of phylotypes present in a clone library.

Phylotypes were assigned a label with the prefix UG, followed by two numbers; the first number is the identification number of the experimental cow from which the phylotype was obtained, and the second is the unique phylotype number. For example, phylotype 2 isolated from the rumen fluid of cow 3322 was designated UG3322.2.

Phylogenetic analysis. Fifty-two 16S rRNA gene sequences, including 11 unique sequences obtained in the present study, were aligned using the Dedicated Comparative Sequence Editor program (7). Members of the *Crenarchaeota*, *Pyrolobus fumarii*, *Sulfolobus acidocaldarius*, and *Thermosphaera aggregans*, were selected as the out-group. A neighbor-joining (21) distance matrix tree was generated using the Kimura two-parameter model (14) and subjected to bootstrap resampling 1,000 times (9).

Nucleotide sequence accession numbers. 16S rRNA gene sequences obtained in the present study were deposited in GenBank with the accession numbers EU161638 to EU161642 and EU574621 to EU574626.

TABLE 1. Real-time PCR enumeration of methanogens in experimental rumen samples grouped by treatment

Day	Control group			Monensin-treated group		
	Cow ID[a] no.	PCR efficiency[b]	No. of methanogens per g (wet wt)	Cow ID[a] no.	PCR efficiency[b]	No. of methanogens per g (wet wt)
−15	1	1.9	1.66×10^7	8	2.0	9.64×10^7
20	1	2.1	1.81×10^7	8	1.8	1.25×10^7
90	1	2.0	1.45×10^9	8	1.9	2.05×10^8
180	1	2.0	2.56×10^6	8	1.9	7.16×10^8
−15	2	1.8	6.01×10^7	9	2.0	1.23×10^5
20	2	2.0	7.21×10^7	9	2.0	1.06×10^6
90	2	2.0	5.79×10^6	9	2.0	9.38×10^7
180	2	2.0	2.88×10^6	9	2.0	9.76×10^4
−15	3	2.0	5.13×10^6	10	2.0	3.80×10^7
20	3	1.8	1.55×10^8	10	1.9	2.64×10^6
90	3	1.8	1.66×10^8	10	2.1	7.40×10^6
180	3	1.9	6.38×10^5	10	2.0	2.10×10^6
−15	4	1.9	3.31×10^7	11	1.9	3.70×10^7
20	4	1.9	9.68×10^7	11	2.0	3.34×10^7
90	4	2.0	9.67×10^4	11	2.0	3.43×10^6
180	4	2.0	1.26×10^6	11	1.9	1.05×10^8
−15	5	2.1	7.93×10^4	12	1.9	2.62×10^8
20	5	2.1	2.18×10^5	12	2.0	1.58×10^5
90	5	1.9	7.19×10^8	12	2.0	8.20×10^5
180	5	1.9	3.88×10^6	12	1.9	2.64×10^7
−15	6	2.0	5.26×10^7	13	1.9	2.14×10^6
20	6	2.1	1.62×10^9	13	2.0	7.39×10^5
90	6	1.8	1.20×10^8	13	1.9	6.96×10^7
180	6	1.8	7.98×10^7	13	2.0	6.33×10^7
−15	7	1.9	5.49×10^7	14	1.9	4.89×10^6
20	7	1.9	3.10×10^7	14	1.9	4.07×10^6
90	7	2.0	7.00×10^5	14	1.8	7.21×10^7
180	7	1.8	1.32×10^8	14	2.0	3.78×10^5

[a] ID, identification.
[b] PCR efficiency, ε, was calculated by the equation $\varepsilon = 10^{-1/\text{slope}}$, based on linear regression of threshold cycle versus log dilution (16).

RESULTS AND DISCUSSION

The target used for both quantification and diversity analyses in the present study was the 16S rRNA gene segment representative of methanogenic archaea. The *mcrA* gene has been used previously to identify and enumerate methane-producing microbes (6, 10), and it appears that 16S rRNA and *mcrA* provide similar results (P. N. Evans, unpublished data), making them complementary targets. Studies conducted by Wright et al., with both unpublished and published results (36–38, 40), have examined more than 1,500 methanogen sequences by using the 16S rRNA gene target, identifying over 120 novel sequences and thereby illustrating the robustness of this method of detection.

Quantification of methanogens by real-time PCR. The correlation coefficient R^2 for the real-time PCR measurements was 0.999. The average PCR efficiency (ε) of the standards was determined to be 1.94. The N constant of 4.79×10^{11} was used to determine the number of cells per gram (wet weight) in dilutions of the experimental samples.

TABLE 2. Mean number of methanogens per gram (wet weight) and SE as determined by real-time PCR for each treatment

Day	Control group		Monensin-treated group	
	Mean	SE	Mean	SE
20	2.85×10^8	2.23×10^8	7.80×10^6	4.56×10^6
90	3.52×10^8	2.07×10^8	6.46×10^7	2.75×10^7
180	3.19×10^7	2.00×10^7	1.30×10^8	9.87×10^7

The N_0 and PCR efficiency were calculated for each dilution of the experimental samples. The number of methanogens per gram (wet weight) was determined for each of the sampling time periods (days −15, 20, 90, and 180) (Table 1). In the baseline measurements, independent of the treatment group, the number of methanogens per gram (wet weight) ranged from 7.93×10^4 to 2.62×10^8, with an average ± SE of $4.73 \times 10^7 \pm 1.82 \times 10^7$ methanogens/g (wet weight). The average and SE for each treatment comparison on days 20, 90, and 180 can be found in Table 2.

The numbers of methanogens per gram (wet weight) reported in the present study are higher than those found in a study based on classical microbial counts in the rumens of grazing cows by Jarvis et al. (12) (10^6 cells/ml) and a study of Svalbard reindeer in the high Arctic by Orpin et al. (19) (10^4 to 10^7 cells/ml). Classical microbial counts often involve culture-based techniques, which can select against difficult-to-cultivate methanogens and ultimately underestimate the size of the methanogen population. Culture-independent techniques, such as quantitative real-time PCR, are not affected by this bias, resulting in higher numbers of methanogens being detected. It is possible that quantitative real-time PCR may overestimate the number of methanogens present due to copy number differences, since the standards used are extracted DNA samples from a counted number of cells. If overestimation occurred, the number of methanogens would be inflated for both control and monensin-treated animals, which would have no effect on the outcome of the study. The numbers of methanogens per gram (wet weight) in the present study are in agreement with quantitative real-time PCR measurements by Sundset et al. (29) (3.17×10^9 cells/g [wet weight]) and by Denman et al. (6) (10^8 to 10^9 cells/ml), who enumerated methanogens by using the *mcrA* gene and different primers and standards. Estimates of methanogen populations have also been made using oligonucleotide probing of extracted rRNA from the rumen, such as in the studies by Lin et al. (15) and Sharp et al. (24), but the quantities are represented as proportions of the rRNA present instead of actual quantities of cells. Lin et al. (15) extracted rRNAs from steers, cows, sheep, and goats, with the methanogen populations estimated to correspond to a range of 1.6 to 2.4% of the total rRNA from bovine rumen samples, 0.6% of that from sheep rumen samples, and 2.3% of that from goat rumen samples. Sharp et al. (24) used a similar technique to estimate the methanogen population in bovine rumen fluid and found that 3.12% of the extracted rRNA hybridized with an archaeon-specific probe.

Statistical analysis of numbers of methanogens per gram (wet weight). When the baseline number and the square of the baseline number of methanogens per gram (wet weight) were used as a covariate, there was no significant effect of treatment

TABLE 3. OTUs and Shannon diversity indices for DGGE gels, calculated by DOTUR at the unique level

DGGE sample	OTU	Mean Shannon diversity index ± SE[a]
Day −15	2	0.26 ± 0.37
Day 20	4	0.76 ± 0.55
Day 90	2	0.26 ± 0.37
Day 180	4	0.76 ± 0.55
Control group[b]	8	1.76 ± 0.37
Monensin group[b]	5	1.36 ± 0.28

[a] The Shannon diversity index is a measure of the diversity within a sample and can be used to compare diversities between samples. DGGE gels with greater diversity have higher Shannon diversity indices.
[b] Samples from control and monensin-treated animals for days 20 to 180 were compiled to compare treatments during the entire experimental period.

($P = 0.67$), time ($P = 0.53$), or the treatment-time interaction ($P = 0.25$) on the number of methanogens per gram (wet weight). The differences from the baseline number of methanogens per gram (wet weight) ($P = 0.47$) and the square of the baseline number of methanogens per gram (wet weight) ($P = 0.45$) were also not significant.

The number of methanogens per gram (wet weight) was also analyzed using the DMI as a covariate. In this case as well, the effects of treatment ($P = 0.64$), time ($P = 0.53$), and the treatment-time interaction ($P = 0.20$) were not significant. The baseline DMI ($P = 0.13$) and the DMI ($P = 0.20$) did not significantly affect the number of methanogens per gram (wet weight). Throughout the experimental period, the DMIs, as measured in kilograms per day, did not significantly differ between monensin-treated and control animals (18).

The numbers of methanogens per gram (wet weight) in the rumens of monensin-treated cattle were lower than those in control cattle (Table 2). However, there was no significant treatment effect or time effect on the number of methanogens per gram (wet weight), indicating that if long-term monensin supplementation decreases the number of methanogens in the rumen, then we were unable to detect it here. The significant reductions in methane output, milk fat, and milk protein observed with monensin supplementation, compared to the levels for control animals, would suggest that monensin was having the anticipated effects on the treated animals (18). The current theory of the effect of monensin on rumen methanogens is that it acts indirectly to suppress methanogen activity by limiting substrate availability in the rumen through its inhibition of protozoa and gram-positive bacteria (4, 31). If this is the case, then it would explain why monensin-treated animals did not have a significant reduction in methanogens per gram (wet weight), although long-term monensin use has been shown to significantly reduce methane output, in terms of grams of methane per day, by 7% (18).

DGGE analysis. No treatment effect on the banding pattern at any time point was observed using DGGE. Banding patterns different from those in the other samples from the same day were observed for day −15 sample 12, day 20 samples 6 (control group), 4 (control group), and 5 (control group), day 90 sample 1 (control group), and day 180 samples 2 (control group), 8 (monensin-treated group), and 12 (monensin-treated group). Based upon the DGGE banding patterns, the degrees of diversity of the methanogens, as expressed by the Shannon diversity index (at the level of uniqueness), were not significantly different between treatment groups, although the diversity in control animals was greater numerically (Table 3).

Interestingly, the diversity tended to increase from day −15 to day 20, decreased back to baseline levels by day 90, and then increased again to day 20 levels by day 180. The same trend was seen for the OTUs as well. However, these changes were not statistically significant over time based upon DOTUR's Shannon diversity index at the unique level (Table 3), indicating that from the baseline to the end of the trial, the overall diversity of the methanogen community was largely maintained. There were no notable changes in feeding or animal handling at these time points, so this observation may represent normal fluctuations in methanogen diversity over time and seasons.

It is known that the level of resistance of methanogens to monensin varies (4, 31, 34), providing the possibility for some phylotypes to be repressed by monensin supplementation directly. Control animals were found to have 8 OTUs, and monensin-treated animals had 5 OTUs (Table 3), but this difference was not substantial enough to significantly alter the overall diversity of the methanogen population. Therefore, DGGE analysis does not rule out the possibility that some methanogen phylotypes may have been repressed by monensin supplementation, but the effect of the long-term supplementation in this study was not sufficiently inhibitory to significantly alter methanogen diversity.

TABLE 4. Methanogen 16S rRNA clones from the present study and their sequence similarities to validly described methanogens

16S rRNA phylotype	No. of clones[a]	Size (bp)	Most closely related valid taxon	% Sequence similarity
UG3322.1	66	1,262	*Methanobrevibacter smithii*	97.4
UG3322.2[b]	52	1,259	*Methanobrevibacter ruminantium*	96.7
UG3340.3	25	1,262	*Methanobrevibacter smithii*	97.9
UG3349.4	13	1,262	*Methanobrevibacter smithii*	100.0
UG3349.5[b]	2	1,262	*Methanobrevibacter smithii*	96.9
UG3030.6[b]	2	1,267	*Methanobrevibacter smithii*	94.7
UG3340.8	2	1,260	*Methanobrevibacter ruminantium*	99.6
UG3340.9[b]	1	1,262	*Methanobrevibacter thaueri*	98.9
UG3241.12[b]	1	1,263	*Methanosphaera stadtmanae*	93.5
UG3241.13	1	1,260	*Methanobacterium aarhusense*	95.2
UG3241.14	1	1,261	*Methanosphaera stadtmanae*	93.6

[a] A total of 166 clones were examined.
[b] Previously unidentified.

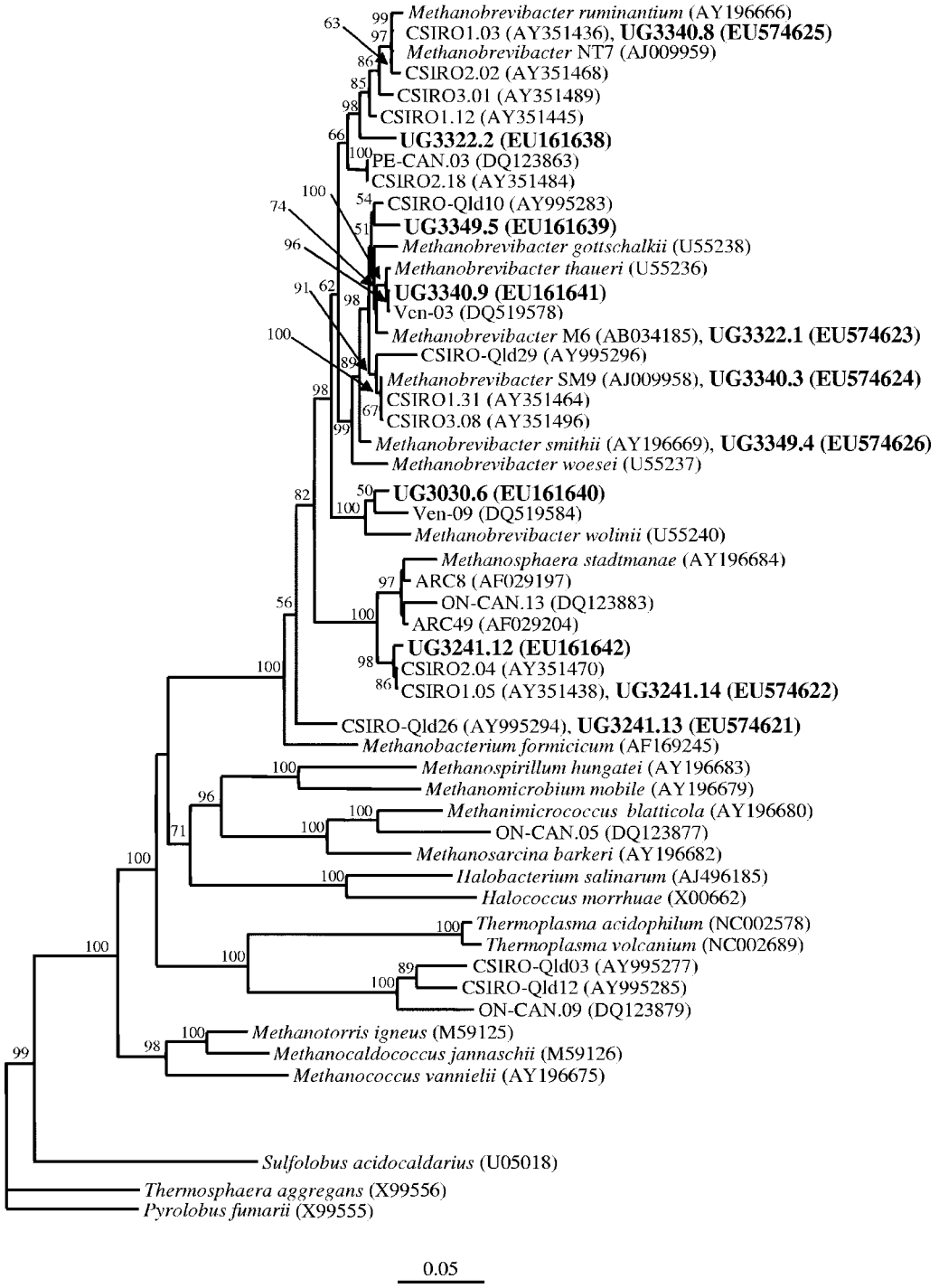

FIG. 1. Phylogenetic relationships among methanogenic archaea based on 16S rRNA gene evolutionary distances derived from the Kimura two-parameter model (14), constructed into a tree by the neighbor-joining method (21). The tree was subjected to bootstrap analysis (9) 1,000 times, and bootstrap values greater than 50% are shown on the nodes. GenBank accession numbers for all sequences are given in parentheses. Two or more taxa on the same branch show 100% sequence identity. The scale bar indicates 5 nucleotide substitutions per 100 positions.

Sequence analysis. Eleven different 16S rRNA gene sequences were obtained in the present study (Table 4; Fig. 1). Three chimeras were also identified and omitted from the analyses. Five previously unidentified 16S rRNA gene sequences (corresponding to phylotypes designated UG3322.2, UG3349.5, UG3030.6, UG3340.9, and UG3241.12) were found to have only 93.5 to 98.9% identity to sequences from validly described methanogens in GenBank (Fig. 1). The remaining six 16S rRNA gene sequences (corresponding to phylotypes designated UG3322.1, UG3340.3, UG3349.4, UG3340.8,

UG3241.13, and UG3241.14) had 100% identity to previously identified clones in GenBank (Fig. 1). Approximately 98.2% of all clones were related to taxa belonging to the genus *Methanobrevibacter* (Fig. 1). This finding is in agreement with those of previous studies showing that *Methanobrevibacter* is the predominant genus in the rumens of cattle and sheep (15, 24, 27, 33, 37, 38, 40).

Methanosphaera stadtmanae has been identified previously in the rumen by others (36, 38, 40), and in some cases, it has been found to be an important member of the methanogen community (17, 33), but this does not appear to be the case in the present study. *Methanobacterium* spp. have also been identified previously in the rumen, although *Methanobacterium* is not considered to be a prominent genus (12, 25, 27, 36). Phylotypes UG3322.1 and UG3340.3 have been identified previously in the rumens of cattle from Prince Edward Island, Canada (38), while UG3322.1, UG3340.3, and UG3349.4 have been found in the rumens of cattle from Ontario, Canada (38), and are predominant clones worldwide (17, 26, 36–38, 40). Phylotypes UG3340.8, UG3241.13, and UG3241.14 were all 100% identical to phylotypes found in the rumens of sheep in Australia (36, 40), and UG3241.13 had 100% identity to a phylotype recently found in the rumens of sheep from Venezuela (37) (Fig. 1). This finding suggests that these methanogens are ubiquitous in both sheep and cattle.

Following DOTUR analysis (22) of the clone libraries, at 98% identity (3), all libraries were found to have between 2 and 4 OTUs, with the pooled library having 8 OTUs. Based on the Shannon diversity indices, libraries A, B, C, D, and E were not significantly different from one another, with indices of 0.83 ± 0.20, 1.03 ± 0.27, 0.62 ± 0.22, 0.85 ± 0.22, and 0.75 ± 0.26, respectively, resulting in the libraries' being pooled. The pooled clone library of 166 clones contained 11 unique sequences and had a Shannon diversity index of 1.13 ± 0.15 at 98% identity (Table 4).

There have been two previous studies focused on investigating the diversity of methanogens in the rumens of cattle in Canada. The first study, by Whitford et al. (33), focused on midlactation dairy cattle fed a TMR, and a 16S rRNA gene clone library was constructed from sequences obtained the rumen fluids of five cows. Of the 41 sequences, 24 were found to cluster with *Methanobrevibacter ruminantium* sequences and 11 sequences clustered with *Methanosphaera stadtmanae* sequences (33). The diet was very similar to that in the present experiment, and *Methanobrevibacter* species were the predominant methanogens recovered, as in the present experiment.

The second study, by Wright et al. (38), investigated the diversity of rumen methanogens in feedlot cattle in Ontario (receiving a corn-based diet) and Prince Edward Island (receiving a potato-based diet). A clone library containing 127 clones from the Ontario cattle was constructed. Many of the sequences were found to be similar to those in the study by Whitford et al. (33), and almost 50% of the clones were *Methanobrevibacter ruminantium*-like, while *Methanosphaera stadtmanae*-like clones were also recovered, as in the present study. In contrast, approximately 50% of the 114 clones from the Prince Edward Island library clustered with uncultured archaea, with only 21% of the Prince Edward Island clones closely related to *Methanobrevibacter ruminantium*. Wright et al. (38) concluded that diet seems to affect the rumen methanogen diversity more than geographical location and that diet should be considered in comparing clone libraries from different experiments. This factor would explain the strong similarities between the clones in the present study and those found in the study by Whitford et al. (33) and the Ontario methanogen clone library of Wright et al. (38).

The present study has shown that the methanogen quantity and diversity in the rumen does not change with long-term monensin supplementation based on concurrent quantitative real-time PCR and DGGE analyses. Monensin has been shown to be a valid approach for methane mitigation in the rumen, and the present study suggests that monensin supplementation does not have a direct effect on any one group of methanogens, leaving rumen methanogen quantity and diversity unaltered. As a result, more microbiology-based studies are necessary to further identify the impact of monensin on the methanogens and on other microbial communities of the rumen. This study has also provided insight into the diversity and sizes of the methanogen populations of lactating dairy cattle in Ontario and highlighted the extent of diversity found within these populations, adding to data from previous national and international reports.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Chang, W. Miller, and D. I. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bergen, W. G., and D. B. Bates.** 1984. Ionophores: their effect on production efficiency and mode of action. J. Anim. Sci. **58:**1465–1483.
3. **Brenner, D. J., J. T. Staley, and N. R. Krieg.** 2001. Classification of procaryotic organisms and the concept of bacterial speciation, p. 27–31. *In* G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed. Springer, New York, NY.
4. **Chen, M., and M. J. Wolin.** 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. **38:**72–77.
5. **Christophersen, C. T.** 2007. Ph.D. thesis. University of Western Australia, Perth.
6. **Denman, S. E., N. W. Tomkins, and C. S. McSweeney.** 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol. Ecol. **62:**313–322.
7. **de Rijk, P., and R. de Wachter.** 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. Comput. Appl. Biosci. **9:**735–740.
8. **Felsenstein, J.** 2006. PHYLIP (phylogenetic inference package) documentation files, version 3.66. Department of Genome Sciences, University of Washington, Seattle.
9. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
10. **Galand, P. E., S. Saarnio, H. Fritze, and K. Yrjala.** 2002. Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. FEMS Microbiol. Ecol. **42:**441–449.
11. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317–2319.
12. **Jarvis, G. N., C. Strompl, D. M. Burgess, L. C. Skillman, E. R. Moore, and K. N. Joblin.** 2000. Isolation and identification of ruminal methanogens from grazing cattle. Curr. Microbiol. **40:**327–332.
13. **Johnson, K. A., and D. E. Johnson.** 1995. Methane emissions from cattle. J. Anim. Sci. **73:**2483–2492.

14. **Kimura, M.** 1980. A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
15. **Lin, C., L. Raskin, and D. A. Stahl.** 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. FEMS Microbiol. Ecol. **22:**281–294.
16. **Liu, W., and D. A. Saint.** 2002. Validation of a quantitative method for real time PCR kinetics. Biochem. Biophys. Res. Commun. **294:**347–353.
17. **Nicholson, M. J., P. N. Evans, and K. N. Joblin.** 2007. Analysis of methanogen diversity in the rumen using temporal temperature gradient gel electrophoresis: identification of uncultured methanogens. Microb. Ecol. **54:**141–150.
18. **Odongo, N. E., R. Bagg, G. Vessie, P. Dick, M. M. Or-Rashid, S. E. Hook, J. T. Gray, E. Kebreab, J. France, and B. W. McBride.** 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. J. Dairy Sci. **90:**1781–1788.
19. **Orpin, C. G., S. D. Mathieson, Y. Greenwood, and A. S. Blix.** 1985. Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). Appl. Environ. Microbiol. **50:**144–151.
20. **Pielou, E. C.** 1966. The measurement of diversity in different types of biological collections. J. Theor. Biol. **13:**131–144.
21. **Saito, N., and M. Nei.** 1987. The neighbour-joining method: a new method for constructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
22. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71:**1501–1506.
23. **Shannon, C. E., and W. Weaver.** 1949. The mathematical theory of communication. University of Illinois Press, Urbana.
24. **Sharp, R., C. J. Ziemer, M. D. Stern, and D. A. Stahl.** 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol. Ecol. **26:**71–78.
25. **Shin, E. C., B. R. Choi, W. J. Lim, S. Y. Hong, C. L. An, K. M. Cho, Y. K. Kim, J. M. An, J. M. Kang, S. S. Lee, H. Kim, and H. D. Yun.** 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. Anaerobe **10:**313–319.
26. **Skillman, L. C., P. N. Evans, C. Strompl, and K. N. Joblin.** 2006. 16S rDNA directed PCR primers and detection of methanogens in the bovine rumen. Lett. Appl. Microbiol. **42:**222–228.
27. **Skillman, L. C., P. N. Evans, G. E. Naylor, B. Morvan, G. N. Jarvis, and K. N. Joblin.** 2004. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe **10:**277–285.
28. **Stewart, C. S., H. J. Flint, and M. P. Bryant.** 1997. The rumen bacteria, p. 10–72. *In* P. N. Hobson and C. S. Stewart (ed.), The rumen microbial ecosystem. Chapman and Hall, London, United Kingdom.
29. **Sundset, M. A., J. E. Edwards, Y. F. Cheng, R. S. Senosiain, M. N. Fraile, K. S. Northwood, K. E. Praesteng, T. Glad, S. D. Mathiesen, and A.-D. G. Wright.** 8 July 2008. Molecular diversity of the rumen microbiome of Norwegian reindeer on natural summer pasture. Microb. Ecol. doi:10.1007/s00248-008-9414-7.
30. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
31. **Van Nevel, C. J., and D. I. Demeyer.** 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. **34:**251–257.
32. **Wang, Z., and L. A. Goonewardene.** 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. **84:**1–11.
33. **Whitford, M. F., R. M. Teather, and R. J. Forster.** 2001. Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol. **1:**5.
34. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 2006. The methanogenic bacteria, p. 165–207. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes, vol. 3. Springer, New York, NY.
35. **Wright, A.-D. G., and C. L. Pimm.** 2003. Improved strategy for presumptive identification of methanogens using 16S riboprinting. J. Microbiol. Methods **55:**337–349.
36. **Wright, A.-D. G., A. F. Toovey, and C. L. Pimm.** 2006. Molecular identification of methanogenic archaea from sheep in Queensland, Australia reveal more uncultured novel archaea. Anaerobe **12:**134–139.
37. **Wright, A.-D. G., X. Ma, and N. E. Obispo.** 2008. *Methanobrevibacter* phylotypes are the dominant methanogens in sheep from Venezuela. Microb. Ecol. **56:**390–394.
38. **Wright, A.-D. G., C. H. Auckland, and D. H. Lynn.** 2007. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. Appl. Environ. Microbiol. **73:**4206–4210.
39. **Wright, A.-D. G., K. Tajima, and R. I. Aminov.** 2005. 16S/18S ribosomal DNA clone library analysis, p. 163–174. *In* H. P. S. Makkar and C. S. McSweeney (ed.), Methods in gut microbial ecology for ruminants. Springer, Dordrecht, The Netherlands.
40. **Wright, A.-D. G., A. J. Williams, B. Winder, C. T. Christophersen, S. L. Rodgers, and K. D. Smith.** 2004. Molecular diversity of rumen methanogens from sheep in Western Australia. Appl. Environ. Microbiol. **70:**1263–1270.