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Molecular Diversity of Rumen Methanogens from Sheep in Western Australia

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The molecular diversity of rumen methanogens in sheep in Australia was investigated by using individual 16S rRNA gene libraries prepared from the rumen contents obtained from six merino sheep grazing pasture (326 clones), six sheep fed an oaten hay-based diet (275 clones), and five sheep fed a lucerne hay-based diet (132 clones). A total of 733 clones were examined, and the analysis revealed 65 phylotypes whose sequences (1,260 bp) were similar to those of cultivated methanogens belonging to the order *Methanobacteriales*. Pasture-grazed sheep had more methanogen diversity than sheep fed either the oaten hay or lucerne hay diet. *Methanobrevibacter* strains SM9, M6, and NT7 accounted for over 90% of the total number of clones identified. M6 was more prevalent in grazing sheep, and SM9, despite being found in 16 of the 17 sheep, was more prevalent in sheep fed the lucerne-based diet. Five new species were identified. Two of these species exhibited very little sequence similarity to any cultivated methanogens and were found eight times in two of the six sheep that were grazing pasture. These unique sequences appear to represent a novel group of rumen archaea that are atypical for the rumen environment.

The rumen is a unique environment and is home to billions of microbes, including bacteria, methanogenic archaea, protozoa, and fungi. These different microbes form a complex community of organisms that interact with one another and play an important role in the digestion of feed and the supply of energy to the host in the form of volatile fatty acids and microbial protein. In the past decade, there has been an increasing amount of interest in the rumen methanogenic archaea. This has primarily resulted from the role of these organisms in global warming due to the production of methane by domesticated livestock.

In Australia, ruminant livestock are the single largest source of agricultural greenhouse gas emissions and alone account for at least 12% of Australian's total anthropomorphic national emissions (3). In New Zealand, enteric emissions are responsible for approximately 60% of that country's total greenhouse gas emissions (27). Approximately 95.5% of the methane emitted by ruminants is produced in the rumen (3), and the associated loss of energy for the ruminant has been estimated to be between 2 and 15% of the gross energy intake (18, 25, 40).

Methane production is influenced by feed intake and the digestibility of the dry matter in the feed that is consumed. The effects of diet on changes in the diversity and numbers of a wide range of bacterial species in the rumen are known (20, 30, 36, 37, 45), but there is little information available concerning the composition of the methanogen population and their numbers of methanogens with regard to diet. Therefore, it is necessary to understand the diversity of methanogens in the rumen.

In the past, methanogens from the digestive tracts of domesticated ruminants were identified by classical microbiological techniques (46). However, because of the fastidious growth requirements of rumen methanogens, it is reasonable to expect that non-culture-dependent molecular methods would reveal greater molecular diversity than is apparent with the techniques currently used to isolate, enumerate, or characterize methanogens in rumen digesta (4, 22, 31, 36). The use of molecular techniques has already revealed the enormous methanogen diversity and putative novel species in anaerobic treatment systems (6, 13, 34, 49), landfills, soils and rice fields (17, 21, 23, 28, 29, 42), wetlands and peat bogs (12, 14, 26), termites (5, 35), and the rumen (38, 39, 44, 50).

The Australian sheep industry is almost exclusively dependent upon pasture; some supplementary feeding occurs mainly for survival during seasonal feed shortages (15). This is especially true in southwestern Australia, which has a Mediterranean type of climate with wet mild winters and dry hot summers. In the present study, we constructed 17 individual 16S rRNA gene libraries from rumen fluid from 17 sheep to determine the molecular diversity of methanogens in the ovine rumen in Western Australia and to compare the compositions of the methanogen populations in pasture-grazed sheep with the compositions of the methanogen populations in sheep fed forage-based diets.

MATERIALS AND METHODS

Source of animals and diet. The first six 16S rRNA gene libraries were constructed from rumen fluid samples taken in August 2000 (experiment 1). Six merino wethers that were 12 to 15 months old were randomly selected from a

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TABLE 1. 16S rRNA clones from individual libraries obtained in experiment 1

16S rRNA phylotype	No. of clones[a]	Size (bp)	GenBank accession no.	Nearest taxon	% Sequence similary
CSIRO1.01	4	1,260	AY351434	*Methanobrevibacter* sp. strain NT7	97.7
CSIRO1.02	6	1,260	AY351435	*Methanobrevibacter* sp. strain NT7	99.5
CSIRO1.03	12	1,260	AY351436	*Methanobrevibacter ruminantium*	99.6
CSIRO1.04	5	1,256	AY351437	*Methanotorris igneus*	70.1
CSIRO1.05	6	1,261	AY351438	*Methanosphaera stadtmanae*	93.6
CSIRO1.06	17	1,262	AY351439	*Methanobrevibacter thaueri*	98.9
CSIRO1.07	24	1,262	AY351440	*Methanobrevibacter* sp. strain M6	99.4
CSIRO1.08	17	1,262	AY351441	*Methanobrevibacter* sp. strain M6	99.2
CSIRO1.09	26	1,262	AY351442	*Methanobrevibacter* sp. strain M6	99.4
CSIRO1.10	1	1,261	AY351443	*Methanobrevibacter* sp. strain M6	89.8
CSIRO1.11	9	1,261	AY351444	*Methanobrevibacter* sp. strain NT7	98.4
CSIRO1.12	3	1,259	AY351445	*Methanobrevibacter* sp. strain NT7	98.2
CSIRO1.13	2	1,262	AY351446	*Methanobrevibacter* sp. strain NT7	97.3
CSIRO1.14	1	1,262	AY351447	*Methanobrevibacter* sp. strain NT7	97.2
CSIRO1.15	2	1,262	AY351448	*Methanobrevibacter* sp. strain NT7	96.3
CSIRO1.16	10	1,269	AY351449	*Methanobrevibacter* sp. strain M6	98.6
CSIRO1.17	4	1,260	AY351450	*Methanobrevibacter thaueri*	97.2
CSIRO1.18	10	1,262	AY351451	*Methanobrevibacter* sp. strain SM9	98.2
CSIRO1.19	16	1,263	AY351452	*Methanobrevibacter* sp. strain M6	98.7
CSIRO1.20	19	1,261	AY351453	*Methanobrevibacter* sp. strain M6	98.7
CSIRO1.21	4	1,261	AY351454	*Methanobrevibacter* sp. strain M6	98.5
CSIRO1.22	2	1,262	AY351455	*Methanobrevibacter* sp. strain M6	99.0
CSIRO1.23	5	1,260	AY351456	*Methanobrevibacter* sp. strain SM9	99.0
CSIRO1.24	14	1,262	AY351457	*Methanobrevibacter* sp. strain SM9	98.8
CSIRO1.25	6	1,262	AY351458	*Methanobrevibacter* sp. strain SM9	99.0
CSIRO1.26	15	1,262	AY351459	*Methanobrevibacter* sp. strain SM9	99.1
CSIRO1.27	10	1,261	AY351460	*Methanobrevibacter* sp. strain SM9	99.0
CSIRO1.28	26	1,260	AY351461	*Methanobrevibacter* sp. strain SM9	99.4
CSIRO1.29	12	1,262	AY351462	*Methanobrevibacter* sp. strain SM9	99.4
CSIRO1.30	22	1,262	AY351463	*Methanobrevibacter* sp. strain SM9	99.5
CSIRO1.31	6	1,260	AY351464	*Methanobrevibacter* sp. strain SM9	100
CSIRO1.32	7	1,260	AY351465	*Methanobrevibacter* sp. strain SM9	99.7
CSIRO1.33	3	1,257	AY351466	*Methanotorris igneus*	71.7

[a] A total of 326 clones were examined.

flock of 90 sheep that were maintained under normal grazing conditions at CSIRO Yalanbee Research Station (Baker's Hill, Western Australia).

Six more libraries of cloned 16S rRNA genes were constructed from rumen fluid samples taken in November 2000 (experiment 2). Six Merino wethers that were 9 to 12 months old were randomly selected from a flock of 160 sheep that were housed in individual pens at the animal house facilities at CSIRO Livestock Industries (Perth, Australia). Each pen was provided with a feed bucket and a drinking nozzle. The sheep were offered 1.6 kg of formulated feed once a day, a ration estimated to maintain body weight. The dry matter content of the formulated feed was 67% oaten hay, 17.7% lupin grain, 10% barley, 3.0% canola meal, 2% SIROMIN (43), and 0.3% urea. Intake requirements were estimated by using the GrazFeed nutritional management software (11) and were calculated by using the specifications of the ration described above. Sheep were individually offered 1.6 kg at 90 to 95% voluntary intake and had free access to water at all times.

The final five 16S rRNA gene libraries were constructed from rumen fluid samples taken in June 2001 (experiment 3). Five mature Merino wethers were randomly selected from a flock of 10 sheep that were housed in individual pens at the large-animal facilities at The University of Western Australia (Perth, Australia). Feed buckets and drinking nozzles were at the front of each pen. Initially, the sheep were offered a mixture of 1.0 kg of pellets and 500 g of wheaten chaff once a day to familiarize them to the pellets. Then the sheep were offered 1.5 kg of pellets once a day to maintain body weight. The dry matter content of the pellets was 68% lucerne hay, 20% lupin grain, 10% molasses, and 2% minerals. Feed residue was removed after 3 h to train the sheep to eat within a 3-h period. The sheep had free access to water at all times.

Collection of rumen fluid samples. In experiments 1 and 2, 50 ml of rumen fluid was collected from each animal by using stomach tube attached to a vacuum pump. Each sheep in experiment 3 was fitted with rumen fistula, and 50-ml rumen fluid samples were taken through the fistula by inertial pumping by using a Perspex tube. Rumen fluid was strained through cheesecloth before it was divided into aliquots and placed into vials for storage. For each animal, fresh rumen fluid samples were stored in two 2-ml Eppendorf tubes on ice to prevent further microbial activity. The remaining rumen fluid was either fixed with an equal volume of 100% ethanol or immediately frozen and stored at −20°C.

DNA extraction and manipulation. A procedure for extraction of DNA from rumen fluid with cetyltrimethylammonium bromide was carried out as described previously (48). Methanogen-specific forward and reverse primers Met86F and Met1340R (47) were used for PCR amplification with an MJ Research PTC-100 thermal cycler (MJ Research, Inc., Watertown, Mass.) by using the following stringent parameters: 3-min hot start at 94°C and then 30 s of denaturation at 94°C, 30 s of primer annealing at 58°C, and 90 s of primer extension at 72°C. On the 40th and last cycle, the primer extension step was extended for 10 min.

Cloning of the 1.2-kb PCR product was performed by using a TOPO TA cloning kit (Invitrogen, GeneWorks, Adelaide, South Australia, Australia) with ampicillin (Sigma, Castle Hill, New South Wales, Australia) and blue-white screening (33), and positive clones were randomly selected and purified with a GenElute plasmid miniprep kit (Sigma). The plasmid insert was then reamplified by PCR by using the parameters and primers described above. The final PCR product was then digested initially with restriction enzyme *Hae*III (16, 47) used according to the manufacturer's specifications. Digested DNA was separated on a 4% molecular screening agarose (Roche Diagnostics) gel that was electrophoresed at 100 V for at least 2 h. Gels were visualized and annotated by using the Bio-Rad Gel Doc 2000 gel documentation system.

Restriction fragment length polymorphisms were grouped according to their riboprint patterns and were compared to a riboprint database for identification (47). In some cases, when two or more strains had the same *Hae*III riboprint, an additional digestion with another restriction enzyme was required to further differentiate closely related strains (47). All riboprints not identified in the riboprint database were considered to be novel and were sequenced along with 5 to 10 clones from each riboprint for confirmation. Sequencing was performed in both directions with an ABI Prism 373 automated DNA sequencer (Applied

TABLE 2. 16S rRNA clones from individual libraries obtained in experiment 2

16S rRNA phylotype	No. of clones[a]	Size (bp)	GenBank accession no.	Nearest taxon	% Sequence similary
CSIRO2.01	1	1,260	AY351467	*Methanobrevibacter* sp. strain NT7	99.5
CSIRO2.02	1	1,262	AY351468	*Methanobrevibacter* sp. strain NT7	99.4
CSIRO2.03	1	1,261	AY351469	*Methanobrevibacter acididurans*	94.8
CSIRO2.04	6	1,261	AY351470	*Methanosphaera stadtmanae*	93.4
CSIRO2.05	17	1,262	AY351471	*Methanobrevibacter thaueri*	98.9
CSIRO2.06	19	1,262	AY351472	*Methanobrevibacter* sp. strain M6	99.1
CSIRO2.07	24	1,262	AY351473	*Methanobrevibacter* sp. strain M6	99.0
CSIRO2.08	76	1,262	AY351474	*Methanobrevibacter* sp. strain SM9	97.9
CSIRO2.09	1	1,263	AY351475	*Methanobrevibacter* sp. strain M6	97.0
CSIRO2.10	16	1,259	AY351476	*Methanobrevibacter* sp. strain SM9	98.5
CSIRO2.11	1	1,262	AY351477	*Methanobrevibacter* sp. strain M6	99.1
CSIRO2.12	4	1,262	AY351478	*Methanobrevibacter* sp. strain NT7	97.1
CSIRO2.13	41	1,260	AY351479	*Methanobrevibacter* sp. strain NT7	99.7
CSIRO2.14	2	1,260	AY351480	*Methanobrevibacter* sp. strain NT7	99.4
CSIRO2.15	1	1,260	AY351481	*Methanobrevibacter* sp. strain NT7	98.8
CSIRO2.16	1	1,262	AY351482	*Methanobrevibacter* sp. strain NT7	96.8
CSIRO2.17	1	1,261	AY351483	*Methanobrevibacter* sp. strain NT7	96.7
CSIRO2.18	1	1,261	AY351484	*Methanobrevibacter* sp. strain NT7	96.2
CSIRO2.19	2	1,262	AY351485	*Methanobrevibacter* sp. strain SM9	99.6
CSIRO2.20	48	1,260	AY351486	*Methanobrevibacter* sp. strain SM9	99.8
CSIRO2.21	4	1,260	AY351487	*Methanobrevibacter* sp. strain SM9	99.9
CSIRO2.22	1	1,262	AY351488	*Methanobrevibacter* sp. strain SM9	98.9
CSIRO2.23	6	1,260	AY351464	*Methanobrevibacter* sp. strain SM9	100

[a] A total of 275 clones were examined.

Biosystems Inc., Foster City, Calif.) by using Big Dye terminator and *Taq*FS with two forward and two reverse internal universal 16S rRNA gene primers (47). Methanogen sequences were confirmed by using the Basic Local Alignment Search Tool (BLAST) (1) in GenBank.

Phylotypes were designated by using the prefix CSIRO followed by two sets of numbers; the first number indicates the experiment, and the second number indicates the unique phylotype (e.g., phylotype 17 from experiment 2 is CSIRO2.17).

Phylogenetic analysis. For this study, 32 methanogen archaeal 16S rRNA gene sequences from GenBank were globally aligned. The nucleotide sequences in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Methanobacterium curvum*, AF276958; *Methanobacterium formicicum*, AY196659; *Methanobacterium ivanovii*, AF095261; *Methanobrevibacter* sp. strain M6, AB034185; *Methanobrevibacter* sp. strain MB-9, AB017514; *Methanobrevibacter* sp. strain NT7, AJ009959; *Methanobrevibacter* sp. strain SM9, AJ009958; *Methanobrevibacter acididurans*, AF242652; *Methanobrevibacter arboriphilicus*, AY196665; *Methanobrevibacter curvatus*, U62533; *Methanobrevibacter cuticularis*, U41095; *Methanobrevibacter filiformis*, U82322; *Methanobrevibacter gottschalkii*, U55238; *Methanobrevibacter ruminantium*, AY196666; *Methanobrevibacter smithii*, AY196669; *Methanobrevibacter thaueri*, U55236; *Methanobrevibacter woesei*, U55237; *Methanobrevibacter wolinii*, U55240; *Methanocaldococcus jannaschii*, M59126; *Methanococcus vannielii*, AY196675; *Methanoculleus bourgensis*, AY196674; *Methanomicrobium mobile*, AY196679; *Methanomicrococcus blatticola*, AY196680; *Methanosaeta thermoacetophila*, M59141; *Methanosarcina barkeri*, AY196682; *Methanosphaera stadtmanae*, AY196684; *Methanospirillum hungatei*, AY196683; *Methanothermobacter wolfei*, X89406; *Methanothermococcus thermolithotrophicus*, M59128; *Methanothermus fervidus*, M59145; *Methanothermus sociabilis*, AF095273; and *Methanotorris igneus*, M59125.

Sequences were globally aligned by using the Dedicated Comparative Sequence Editor (DCSE) program (7). The sequence alignment was further refined by eye. Marine and deep-sea hydrothermal vent methanogens belonging to the order *Methanococcales* were used as the outgroup (24, 41) for the *Methanobacteriales* and *Methanomicrobiales*. PHYLIP (version 3.51C) (10) was used to calculate the sequence similarities and evolutionary distances between pairs of nucleotide sequences by using the Kimura two-parameter model (19). A distance matrix tree was then constructed by using the neighbor-joining method (32) and was bootstrap resampled (9) 1,000 times.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers AY351434 to AY351500.

RESULTS

Sequence analysis of 16S rRNA gene libraries. In the first experiment, a total of 326 clones were randomly selected from individual 16S rRNA gene libraries from six sheep grazing pasture. Riboprint analysis and preliminary sequence examination of these 326 clones revealed 33 unique phylotypes (Table 1). Eleven of these phylotypes represented by 33 clones were at least 98.2 to 100% similar to *Methanobrevibacter* sp. strain SM9 obtained from a ovine rumen in New Zealand (G. N. Jarvis, unpublished data), nine phylotypes (119 clones) were 98.5 to 99% similar to *Methanobrevibacter* sp. strain M6 obtained from a bovine rumen in Japan (38), and seven phylotypes (27 clones) were 96.3 to 99.5% similar to *Methanobrevibacter* sp. strain NT7obtained from a cervid rumen in New Zealand (Jarvis, unpublished). Two phylotypes (21 clones) were 97.2 to 98.9% similar to *Methanobrevibacter thaueri*, two phylotypes (8 clones) were 70.1% similar to *Methanotorris igneus*, one phylotype (12 clones) was 99.6% similar to *Methanobrevibacter ruminantium*, and one phylotype (6 clones) was 93.6% similar to *Methanosphaera stadtmanae*.

In the second experiment, a total of 275 clones were randomly selected from individual 16S rRNA gene libraries obtained from six sheep fed an oaten hay-based diet. These 275 clones belonged to 23 phylotypes (Table 2); seven of the phylotypes (153 clones) were 97.9 to 100% similar to *Methanobrevibacter* sp. strain SM9, nine phylotypes (53 clones) were 96.2 to 99.7% similar to *Methanobrevibacter* sp. strain NT7, and four phylotypes (45 clones) were 97 to 99.1% similar to *Methanobrevibacter* sp. strain M6. In addition, one phylotype (17 clones) was 98.9% similar to *Methanobrevibacter thaueri*, one phylotype (1 clone) was 94.8% similar to *Methanobrevibacter acididurans*, and one phylotype (6 clones) was 93.4% similar to *Methanosphaera stadtmanae*.

TABLE 3. 16S rRNA clones from individual libraries obtained in experiment 3

16S rRNA phylotype	No. of clones[a]	Size (bp)	GenBank accession no.	Nearest taxon	% Sequence similary
CSIRO3.01	1	1,260	AY351489	*Methanobrevibacter* sp. strain NT7	98.3
CSIRO3.02	1	1,261	AY351490	*Methanobrevibacter acididurans*	94.8
CSIRO3.03	1	1,262	AY351491	*Methanobrevibacter* sp. strain SM9	99.6
CSIRO3.04	2	1,260	AY351492	*Methanobrevibacter* sp. strain NT7	99.7
CSIRO3.05	1	1,260	AY351493	*Methanobrevibacter* sp. strain SM9	99.8
CSIRO3.06	1	1,260	AY351494	*Methanobrevibacter* sp. strain SM9	99.8
CSIRO3.07	1	1,262	AY351495	*Methanobrevibacter* sp. strain NT7	97.2
CSIRO3.08	1	1,260	AY351496	*Methanobrevibacter* sp. strain SM9	99.8
CSIRO3.09	1	983	AY351497	*Methanobrevibacter* sp. strain SM9	99.5
CSIRO3.10	2	1,260	AY351498	*Methanobrevibacter* sp. strain SM9	99.4
CSIRO3.11	4	1,262	AY351499	*Methanobrevibacter* sp. strain M6	100
CSIRO3.12	3	1,262	AY351500	*Methanobrevibacter* sp. strain SM9	99.4
CSIRO3.13	113	1,260	AY351464	*Methanobrevibacter* sp. strain SM9	100

[a] A total of 132 clones were examined.

In the third experiment, 132 clones were randomly selected from individual libraries from five sheep fed a lucerne hay-based diet, and these clones belonged to 13 phylotypes (Table 3). Eight phylotypes (123 clones) were 99.4 to 100% similar to *Methanobrevibacter* sp. strain SM9, three phylotypes (4 clones) were 97.2 to 99.7% similar to *Methanobrevibacter* sp. strain NT7, one phylotype (4 clones) was 100% similar to *Methanobrevibacter* sp. strain M6, and the remaining phylotype (1 clone) was 94.8% similar to *Methanobrevibacter acididurans*.

In total, 733 clones from 17 sheep in Western Australia were examined between August 2000 and June 2001. Among these 733 clones, 65 unique phylotypes were identified, and 20 phylotypes were represented by a single clone. Phylotypes CSIRO1.31, CSIRO2.23, and CSIRO3.13 exhibited 100% sequence similarity to SM9 and therefore were considered one phylotype. Likewise, CSIRO2.03 exhibited 100% sequence similarity to CSIRO3.02, and CSIRO1.05 was 100% similar to CSIRO2.04.

Phylogenetic analysis. Bootstrap data from the neighbor-joining tree (Fig. 1) supported the conclusion that the orders *Methanobacteriales* and *Methanomicrobiales* are monophyletic groups 80 and 94% of the time, respectively. The branching pattern within the order *Methanobacteriales* was consistent with division of this taxon into five genera, *Methanothermus*, *Methanothermobacter*, *Methanobacterium*, *Methanosphaera*, and *Methanobrevibacter*. The five genera formed monophyletic assemblages, and 62 of the 65 unique phylotypes clustered with the *Methanobrevibacter* clade. Of these 62 phylotypes, 41 represented by 615 clones clustered on a branch with *Methanobrevibacter thaueri* and *Methanobrevibacter* strains SM9 and M6. Twenty phylotypes (96 clones) clustered on a second branch with *Methanobrevibacter ruminantium*, *Methanobrevibacter* strain NT7, and unknown methanogen sequences from a bovine rumen obtained in Canada (45), while the other two phylotypes (two clones) grouped on a third branch with *Methanobrevibacter acididurans*. Two of the remaining four phylotypes (12 clones) grouped on a branch with *Methanosphaera stadtmanae* and unknown methanogen sequences from Canadian cattle (45), and the other two phylotypes (eight clones) clustered with uncultured archaeal sequences from swine waste (8) and a wine aerobic digestor (13) and with a novel group of archaea that are not affiliated with known methanogens and were found in sheep in Japan (38).

DISCUSSION

Assessing the diversity and structure of natural microbial communities has been a long-standing challenge in microbial ecology. The ribosomal DNA clones generated in this study exhibited high degrees of sequence similarity to seven methanogens, *Methanobrevibacter* strains SM9, M6, and NT7, *Methanobrevibacter thaueri*, *Methanobrevibacter ruminantium*, *Methanobrevibacter acididurans*, and *Methanosphaera stadtmanae*. Similarly, in a study performed by Shinzato et al. (35), *Methanobrevibacter*-like clones were the most abundant methanogens in the lower termite *Reticulitermes speratus*.

The clones resembling SM9, M6, and NT7 were the three major rumen methanogens in the 17 sheep from Western Australia, and SM9 and M6 were the only methanogens to be identified by 100% sequence comparison. On an individual-animal basis, SM9-like clones were identified as the dominant methanogen in 11 of the 17 sheep studied and were present in all but one animal (Table 4). Overall, the percentage of SM9-like clones was lowest in sheep grazing pasture and highest in sheep fed the lucerne hay-based diet. M6-like clones were found in all six animals grazing pasture. In contrast, M6-like clones were found in only three of the six sheep fed the oaten hay-based diet and in just two of the five sheep fed the lucerne hay-based diet. Despite the large variation in the number of clones (6 to 105 clones) obtained from the 17 sheep, the percentage of M6-like clones was 2 to 12 times higher in the pasture-grazed sheep than in the other sheep.

NT7-like clones were found in only one of the six pasture-grazed sheep, compared with five of the six sheep fed the oaten hay-based diet and three of the five sheep fed the lucerne hay-based diet. Clones putatively identified as *Methanosphaera stadtmanae* and *Methanobrevibacter thaueri* were found in only 3 of the 17 sheep, but they were not found together in the same sheep. Interestingly, neither *Methanosphaera stadtmanae*-like clones nor *Methanobrevibacter thaueri*-like clones were found in sheep fed the lucerne hay-based diet, and *Methanobrevibacter ruminantium*-like clones were found in only one pas-

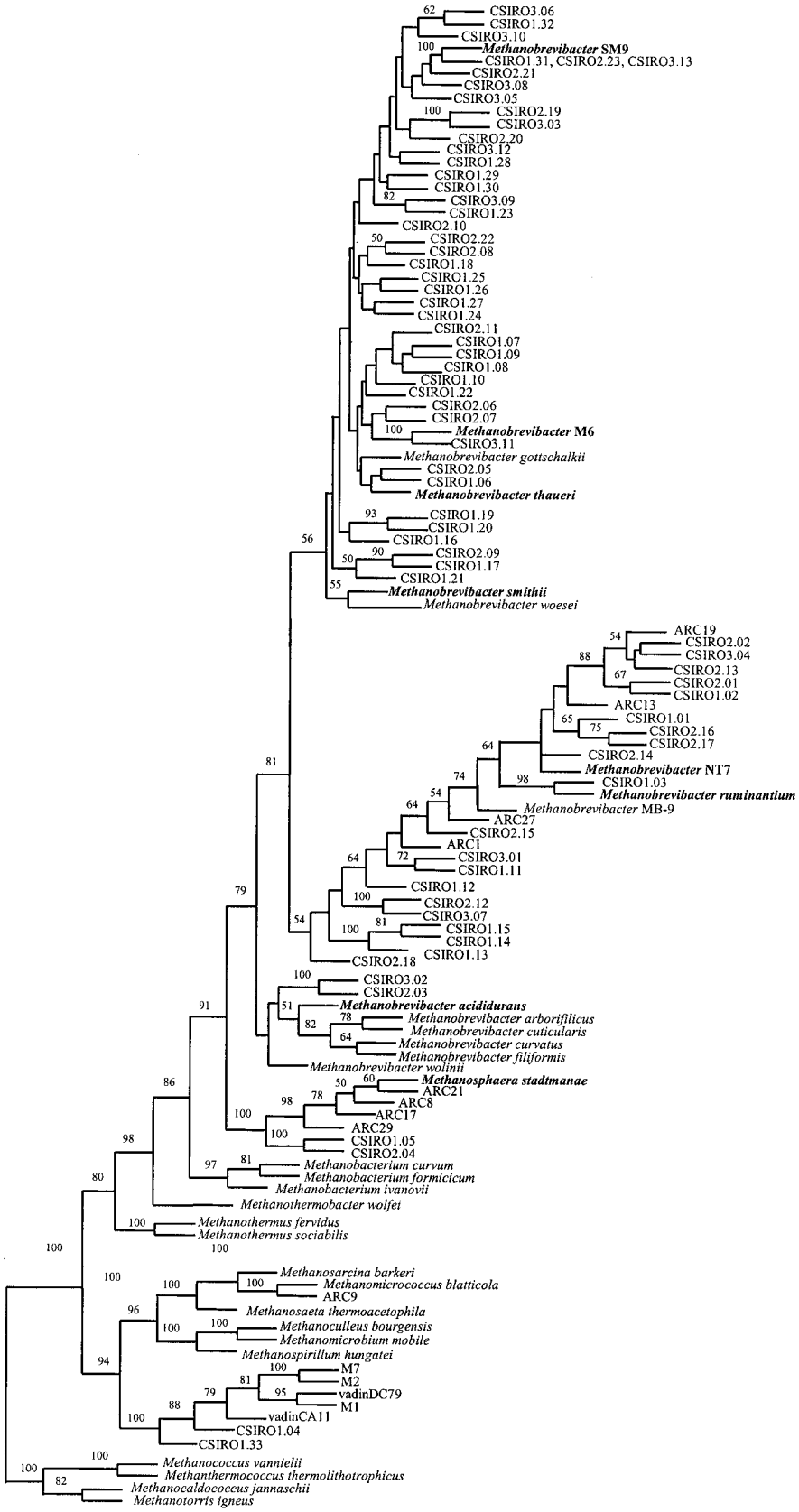

FIG. 1. Phylogenetic relationships of the methanogens derived from 16S rRNA gene evolutionary distances produced by the Kimura two-parameter correction model (19) and constructed by using the neighbor-joining method (32). The consensus trees for 1,000 bootstrap resamplings of the data set were computed independently, and the bootstrap resampling values for 16S rRNA gene sequences (expressed as percentages) are indicated at the nodes. Bootstrap values less than 50% are not indicated. No significance should be placed on the lengths of the branches connecting the species.

TABLE 4. Distribution of putatively identified 16S rRNA clones from individual sheep in three experiments

16S rRNA library	*Methanobrevibacter* sp. strain SM9		*Methanobrevibacter* sp. strain M6		*Methanobrevibacter* sp. strain NT7		*Methanobrevibacter thaueri*		*Methanobrevibacter ruminantium*		*Methanosphaera stadtmanae*		New species		*Methanobrevibacter acididurans*		Total no. of clones
	No. of clones	%	No. of clones	%	No. of clones	%	No. of clones	%	No. of clones	%	No. of clones	%	No. of clones	%	No. of clones	%	
Expt 1																	
Animal 1	43	41.0	18	17.1	27	25.7	12	11.4	0	NA[a]	0	NA	5	4.8	0	NA	105
Animal 2	25	56.8	19	43.2	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	44
Animal 3	38	63.3	10	16.7	0	NA	0	NA	12	20.0	0	NA	0	NA	0	NA	60
Animal 4	17	34.0	33	66.0	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	50
Animal 5	10	20.8	29	60.4	0	NA	9	18.8	0	NA	0	NA	0	NA	0	NA	48
Animal 6	0	NA	10	52.6	0	NA	0	NA	0	NA	6	31.6	3	15.8	0	NA	19
Subtotal[b]	133	40.8	119	36.5	27	8.3	21	6.4	12	3.7	6	1.8	8	2.5	0	NA	326
Expt 2																	
Animal 1	7	17.9	19	48.7	11	28.2	0	NA	0	NA	1	2.6	0	NA	1	2.6	39
Animal 2	49	100	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	49
Animal 3	15	21.7	25	36.3	12	17.4	17	24.6	0	NA	0	NA	0	NA	0	NA	69
Animal 4	58	96.6	1	1.7	1	1.7	0	NA	0	NA	0	NA	0	NA	0	NA	60
Animal 5	6	60.0	0	NA	4	40.0	0	NA	0	NA	0	NA	0	NA	0	NA	10
Animal 6	18	37.5	0	NA	25	52.1	0	NA	0	NA	5	10.4	0	NA	0	NA	48
Subtotal[b]	153	55.6	45	16.4	53	19.3	17	6.2	0	NA	6	2.2	0	NA	1	0.4	275
Expt 3																	
Animal 1	33	97.1	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	1	2.9	34
Animal 2	17	94.4	0	NA	1	5.6	0	NA	0	NA	0	NA	0	NA	0	NA	18
Animal 3	62	98.4	0	NA	1	1.6	0	NA	0	NA	0	NA	0	NA	0	NA	63
Animal 4	8	72.7	1	9.1	2	18.2	0	NA	0	NA	0	NA	0	NA	0	NA	11
Animal 5	3	50.0	3	50.0	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	6
Subtotal[b]	123	93.2	4	3.0	4	3.0	0	NA	0	NA	0	NA	0	NA	1	0.8	132
Total[c]	409	55.8	168	22.9	84	11.4	38	5.2	12	1.6	12	1.6	8	1.2	2	0.3	733

[a] NA, not applicable.
[b] Number of strains and percentage of methanogens for all clones generated in an experiment.
[c] Number of strains and percentage of methanogens for all clones examined in the three experiments.

ture-fed sheep. Clones of *Methanobrevibacter acididurans* were found in only two sheep, one fed the oaten hay-based diet and the other fed the lucerne hay-based diet.

Despite examining only five or six animals fed each of the three different diets, we did have the advantage that a total of 17 ribosomal DNA clone libraries were constructed, one for each sheep, instead of one clone library constructed from pooled samples for each diet. Hence, we examined a very large number of clones (n = 733) in this study compared to the numbers of clones examined in other studies (15 to 100 clones). In addition, our methanogen-specific primers and methodology were also validated as similar ribosomal DNA clones were identified from bovine and cervid rumens in New Zealand (Jarvis, unpublished) and bovine rumens in Japan and Canada (38, 45). It also appears that methanogen molecular diversity was greater in sheep grazing pasture than in sheep maintained on either the oaten hay- or lucerne hay-based diet (Table 4). In a recent study, Kocherginskaya et al. (20) analyzed the rumen bacterial diversity in steers and found that corn-fed animals also had more diverse bacterial populations than hay-fed animals. However, it is important to acknowledge that other factors, such as the sheep genotype, could have had an effect on the microbial diversity in both studies. Additional information from more animals fed similar and different diets would be desirable to test these hypotheses.

In a comprehensive review of phylogenetic identification and in situ detection of noncultivated microbial cells, Amann et al. (2) suggested that new 16S rRNA gene sequences with similarity values less than 95% should be regarded as new species. This is certainly true for most recognized methanogen species and suggests that sequences from CSIRO1.05 (100% similar to CSIRO2.04), CSIRO1.10, and CSIRO2.03 (100% similar to CSIRO3.02) should be considered representatives of three new species. The sequences from CSIRO1.15 and CSIRO2.18 may also be representatives of a new species (96.2% similar to NT7) once cultivated isolates are established and characterized.

The other two novel archaeal sequences discovered in this study, CSIRO1.04 and CSIRO1.33, were unique for the rumen environment. In a recent study to assess the molecular diversity of rumen archaea in Holstein dry cows in Japan, Tajima et al. (38) also found three novel sequences, M1, M2, and M7, that were unique for the rumen environment, but they concluded that they were likely from transient microbiota contaminating the animal feed, probably scavenging in an ecological niche in the rumen. In contrast, our two unique sequences were encountered eight times in two of the six sheep that were grazing pasture.

CSIRO1.04 and CSIRO1.33 were not encountered in the 11 sheep that were fed conserved forage. The sequence of CSIRO1.04 was only 88.5% similar to the sequence of CSIRO1.33, and these sequences were only 88.9 to 92.2% similar to the M1, M2, and M3 sequences (38). The other members of this unique clade include vadinCA11 and vadinDC79, both obtained from a wine anaerobic digestor in France (13), and WCHD302, obtained from an aquifer contaminated with hydrocarbons and chlorinated solvents (8). In a recent study characterizing the diversity and structure of archaea in the effluent leachate from a recirculating landfill, two new partial 16S rRNA gene sequences (500 bp) were also found to cluster with this unique group (17). Interestingly, the genetic distance between this novel clade and its nearest sister group (28%) was greater than the genetic distance between the orders *Methanobacteriales* and *Methanomicrobiales*. This suggests that a new order of methanogens should be created for this group once cultivars are isolated to validate the proposal. However, in the absence of cultivated isolates the biology and ecology of this novel group in the rumen remain unclear (17).

We believe that this study provided useful insight into the diversity of methanogens in sheep in Western Australia. However, more studies are needed to assess the molecular diversity of rumen methanogens in both sheep and cattle in this Mediterranean climate at different times of the year and when animals may have to be given supplementary feed during the dry summer months. Moreover, new studies are needed to examine methanogen diversity in sheep and cattle located in the temperate and tropical areas of Australia. Hopefully, additional information will increase our understanding of the effect of different diets and environments on methanogen diversity and numbers in ruminant livestock.

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