Microbial Ecology

Analysis of Methanogen Diversity in the Rumen Using Temporal Temperature Gradient Gel Electrophoresis: Identification of Uncultured Methanogens

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Abstract

A temporal temperature gradient gel electrophoresis (TTGE) method was developed to determine the diversity of methanogen populations in the rumen. Tests with amplicons from genomic DNA from 12 cultured methanogens showed single bands for all strains, with only two showing apparently comigrating bands. Fingerprints of methanogen populations were analyzed from DNA extracted from rumen contents from two cattle and four sheep grazing pasture. For one sheep, dilution cultures selective for methanogens were grown and the culturable methanogens in each successive dilution examined by TTGE. A total of 66 methanogen sequences were retrieved from bands in fingerprints and analyzed to reveal the presence of methanogens belonging to the *Methanobacteriales*, the *Methanosarcinales*, and to an uncultured archaeal lineage. Twenty-four sequences were most similar to *Methanobrevibacter ruminantium*, five to *Methanobrevibacter smithii*, four to *Methanosphaera stadtmanae*, and for three, the nearest match was *Methanimicrococcus blatticola*. The remaining 30 sequences did not cluster with sequences from cultured archaea, but when combined with published novel sequences from clone libraries formed a monophyletic lineage within the *Euryarchaeota*, which contained two previously unrecognized clusters. The TTGE bands from this lineage showed that the uncultured methanogens had significant population densities in each of the six rumen samples examined. In cultures of dilutions from one rumen sample, TTGE examination revealed these methanogens at a level of at least 10^5 g^{-1}. Band intensities from low-dilution cultures indicated that these methanogens were present at similar densities to *Methanobrevibacter ruminantium*-like methanogens, the sole culturable methanogens in high dilutions (10^6–10^{-10} g^{-1}). It is suggested that the uncultured methanogens together with *Methanobrevibacter* spp. may be the predominant methanogens in the rumen. The TTGE method presented in this article provides a new opportunity for characterizing methanogen populations in the rumen microbial ecosystem.

Introduction

Methane eructated from ruminants is a significant (6–10%) loss of dietary energy to the animal and is of interest to nutritionists wishing to increase productivity. This "ruminant" methane also has adverse environmental consequences because it is a significant contributor to global warming [1, 13]. As a result, the microbial populations involved in ruminal methanogenesis have become a focus of increasing scientific attention over recent years. In the rumen, methane is formed by methanogenic archaea (methanogens), an important part of the microbial ecosystem. Methanogens in the rumen have been identified by culture methods [11, 14, 20, 26], and most recently by a range of molecular techniques developed to obtain information on microbial populations in natural habitats. DNA hybridization studies using specific DNA probes have been used to identify methanogens in the rumen [17, 23, 27], and ruminal methanogen populations have been identified from clone libraries of archaeal 16S rRNA gene sequences [24, 25, 29, 33–35]. These molecular ecological techniques have revealed a much greater diversity of methanogens in the rumen than recognized from culture studies.

There is a need for nonculture techniques to quickly fingerprint methanogen populations in rumen samples. Such methods include denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel

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electrophoresis (TTGE). These provide information on the phylogenetic diversity in samples by separating polymerase chain reaction (PCR) products from 16S rRNA genes on the basis of G + C content. DGGE has been used to fingerprint very diverse eubacterial populations in microbial ecosystems including the rumen [16, and references therein], but in the case of PCR primers with broad specificities, a comprehensive sequencing strategy is difficult because of the complexity of the population profiles. The less-complex archaeal populations in the rumen are more suited to these techniques.

In the present study, we developed a TTGE methodology for fingerprinting methanogens using ruminal methanogens from our culture collection as indicators. This technique then was used to prepare fingerprints of methanogen populations both in rumen samples from grazing cattle and sheep, and in a dilution series of cultures grown from one of the samples. We report in this article an analysis of sequences in bands from TTGE profiles to provide a comprehensive picture of the ruminal methanogens in these animals.

Materials and Methods

Animals and Sample Collection. Rumen contents (approximately 500 mL) consisting of fluid and solids were collected from four sheep and two cows grazing together on a ryegrass/clover pasture in the spring of 2004. Samples were collected via rumen fistulae into screw-top glass jars previously flushed with O_2-free CO_2 and processed immediately. For each sample, 40 g was transferred into a Waring blender flushed with O_2-free CO_2, 160 mL of the basal RF30 medium of Joblin *et al.* [12] added, and the contents homogenized for 60 s with a pause after the first 15 s.

Dilution Cultures. A series of dilution cultures was prepared by decimal dilutions of the homogenized rumen contents from one sheep (sheep 64). Media preparation and dilution cultures were carried out using the methanogen enumeration method described by Joblin [14]. Cultures were pressurized with H_2/CO_2 (80:20), incubated with shaking (100 rpm) for 3 weeks at 39°C, and cells for DNA extraction were pelleted from cultures by centrifugation at 7000×*g* for 15 min.

Reference Methanogens. The following methanogens from our culture collection were grown and DNA extracted for use in DGGE and TTGE tests: *Methanobrevibacter ruminantium* strains DSM1093, CT, YLMI, and NT7; *Methanobacterium formicicum* BRM9, *Methanosarcina barkeri* CM1, *Methanococcus voltae* DSM1357, *Methanobacterium bryantii* DSM863, *Methanobrevibacter wolinii* ABM4, *Methanosphaera stadtmanae* DSM3091, *Methanobrevibacter smithii* strains DSM861, and SM9. The reference standard for TTGE fingerprints was created by combining equal quantities of PCR products from strains SM9, DSM3091, ABM4, DSM863, CM1, and NT7.

DNA Isolation. Genomic DNA was isolated from homogenized rumen samples, cultured dilutions, and pure cultures using a FastDNA kit and FastPrep instrument (QBiogene, France), with minor modifications from the manufacturer's protocol. Homogenized rumen contents (1 mL) or culture (1 mL) were measured into FastPrep tubes containing a ceramic bead and stored at −20°C overnight. The samples were thawed and centrifuged in a microfuge for 1 min, after which the supernatants were discarded and the pellets resuspended in 100 μl sterile saline EDTA (0.5M EDTA, pH 8.0; 0.15 M NaCl). One milliliter of lysis buffer (CLS TC) was added to each tube and the cells were mechanically disrupted using a FastPrep instrument at speed 5 for 10 s. The tubes were then centrifuged for 15 min at 14,000×*g* at 4°C. The supernatants (600 μl) were transferred to separate tubes and combined with equal volumes (600 μl) of a binding matrix. After 5 min incubation at room temperature, matrix-bound DNA was recovered by centrifugation in a microfuge for 1 min. For each tube the matrix was then washed twice in 500 μl of wash buffer (SEWS-M) before eluting the DNA from the matrix in 100 μl of elution buffer (DES). Eluted DNA was diluted 1/10 in molecular biology grade water.

Primer Selection and PCR. Three archaea-specific primers were selected from previous methanogen population diversity studies. Primers 0357aF [24, 31] and 0915aR [31] were used to amplify a ~559-bp product covering the V3 to V5 regions of the 16S rRNA gene. Primers 0915aF (complementary to 0915aR) and 1386R were used to amplify a ~491-bp product covering the V6–V8 regions, and primer 0357aF was used in combination with 1386R to produce a ~1029-bp product containing regions V3 to V8. For TTGE, a GC-clamp sequence was incorporated at the 5′ end of one of either primer in each pair (Table 1). This gave six possible primer combinations for testing.

PCR reactions were performed using Advantage II DNA polymerase (BD Clontech) and contained the following: 2.5 μl 10× buffer, 2.5 μl template DNA, primers 0915aFGC and 1386R (0.4 μM each), deoxyribonucleotide triphosphates (dNTP)s (200 μM each), 0.5 μl polymerase, and molecular biology grade water to make a final reaction volume of 25 μl. Touchdown PCR was used for DNA amplification: 95°C for 4 min; then 20 cycles of 95°C for 30 s, followed by 68°C for 30 s (decreasing 0.5°C each cycle) and 68°C for 30 s; then 10

Table 1. Primer sequences for TTGE

Primer	*Original primer name and source*	*Primer sequence*	*Associated primer*	*Amplicon size (bp)*	*Target region*
0357aF (forward)	Archf364 [24] 0357F [31]	CCCTACGGGGYGCAGCAGG	0915aR	~559	V3–V5
			1386R	~1029	V3–V8
0915aF (forward)	0915aR [31]	AGGAATTGGCGGGGGAGCAC	1386R	~491	V6–V8
0915aR(reverse)	0915aR [31]	GTGCTCCCCCGCCAATTCCT			
1386R (reverse)	Archr1386 [24]	GCGGTGTGTGCAAGGAGC			

GC clamp versions were constructed by the addition of the following to the 5′ end of primers:
5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG 3′

cycles of 95°C for 30 s, 58°C for 30 s and 68°C for 30 s; with a final elongation step of 68°C for 5 min.

Electrophoresis. TTGE was performed using a four-place TTGE system (CBS Scientific, USA). Gels for TTGE were made using 5% acrylamide:bisacrylamide (29:1), 1 × TAE buffer and 7.0 M urea. Polymerization was initiated using 120 µl 10% ammonium persulphate and 12 µl TEMED for 22 mL of gel solution. Electrophoresis was performed for 6.5 h at 150 V (7.5 V/cm), with a linear temperature increase from 59 to 67°C. Gels were stained using SYBR Gold (Molecular Probes, USA) in which 10 mL of stain (1 µl of 10,000× concentrate in 10 mL double-distilled water) was spread over the surface of the gel and incubated for 10 min at room temperature in the dark. Gels were washed in double-distilled water, visualized using UV trans-illumination and photographed using a Gel Logic 200 imaging system (Kodak, USA).

Analysis of Electrophoretic Patterns. TTGE fingerprints were analyzed with the BioNumerics software package (Applied Maths). After normalization, similarities between the fingerprints were analyzed from the presence and absence of different bands using the unweighted pair group method of arithmetic averages (UPGMA) with Dice binary coefficient.

Cloning, Sequencing, and Phylogenetic Analysis. For extraction of DNA from TTGE bands, bands were cut from the gel using a clean, sharp scalpel and transferred to a 1.5-mL microcentrifuge tube. DNA was eluted from polyacrylamide gel slices according to the method of Etokebe *et al.* [6]. Gel slices were each washed by incubation in 50 µl molecular biology grade water for 15 min at room temperature. The water was then removed and discarded. DNA was recovered by the addition of 50 µl of molecular biology grade water to each tube, which was then vortexed for 5 s, centrifuged for 1 min, and the supernatant transferred to a clean tube. The supernatant (5 µl) was included as template in a final PCR reaction volume of 20 µl using Platinum *Taq* DNA polymerase (Invitrogen, USA), and subjected to 25 PCR cycles (95°C for 30 s, 58°C for 30 s, 72°C for 30 s followed by a final extension for 10 min at 72°C). PCR reactions contained 5 µl template DNA (see above), 2 µl of 10× reaction buffer, 2.5 mM $MgCl_2$, dNTPs (200 µM each), primers 0915aF and 1386R (0.4 µM each), 0.1 µl *Taq* polymerase and molecular biology grade water to make a final volume of 20 µl. PCR products were cloned into a plasmid vector (pCR 2.1) and transformed into an *E. coli* TOP-10 cells using TOPO-TA cloning system (Invitrogen, USA). Plasmids containing inserts were sequenced using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.) to approximately 99% accuracy (PHRED 20). Sequences were assembled using Contig Express in the Vector NTI suite of programs (Version 7, Informax) and traces were visually inspected for sequencing accuracy. Sequences were analyzed using the Ribosomal Database Project Chimera Check program [3]. Phylogenetic analyses were performed using the ARB software package and its associated databases [18] after alignment and manual adjustment and after consideration of secondary structures. A distance matrix was subsequently calculated based on percentage nucleotide-identity values. A dendrogram was calculated using the Neighbor-joining method with Felsenstein correction [7]. Sequences were assigned to clusters based upon branching patterns within the dendrogram. TTGE-derived sequences have been deposited in EBI database and assigned accession numbers of AM261762–AM261810.

Results

TTGE Separation of Methanogen Amplicons. When combinations of the three archaea-specific PCR primers (Table 1) were used to amplify 16S rRNA genes from 12 methanogen strains, the most optimal separation was achieved for amplicons generated from primers 1386R and 0915aFGC. The forward primer was derived from primer 0915aF by the addition of the GC-clamp sequence at its 5′ end (Table 1). The amplicons of approximately 491 bp spanned the V6–V8 region and their separation is shown in Fig. 1. For some closely related strains, pronounced separation was achieved. TTGE bands from strains of *M. ruminantium* (lanes 1–4, Fig. 1) migrated at opposite ends of the gel to bands from *M. smithii* strains

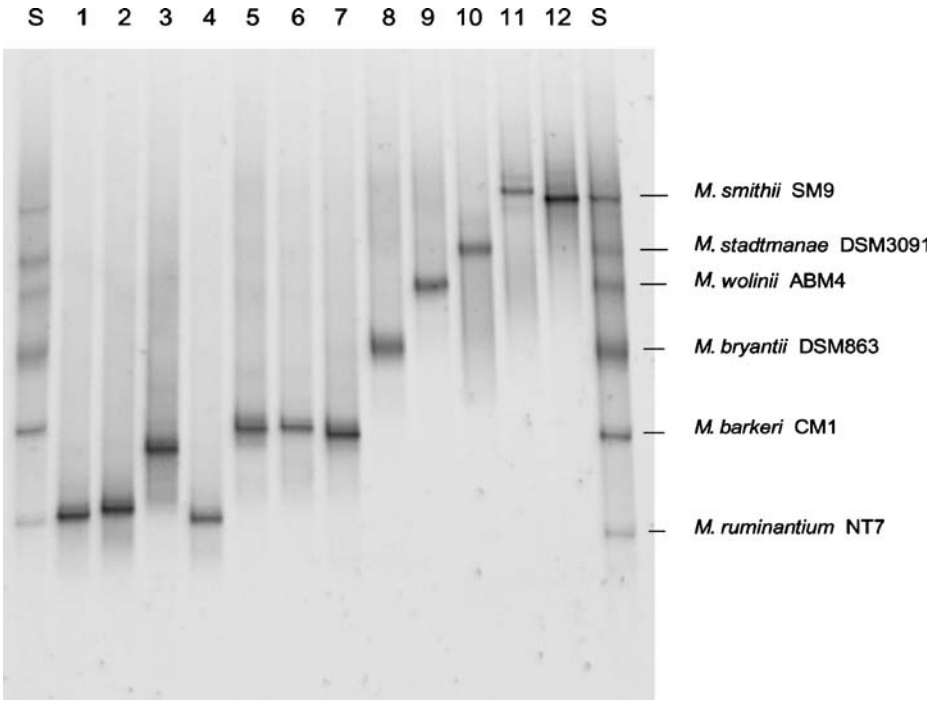

Figure 1. TTGE separation of the V6–V8 region of 16S rRNA genes amplified from pure cultures of methanogens using primers 0915aFGC and 1386R. TTGE separation of rDNA amplicons from pure methanogens. Lane 1, *Methanobrevibacter ruminantium* DSM 1093; lane 2, *M. ruminantium* CT; lane 3, *M. ruminantium* YLM-1; lane 4, *M. ruminantium*, NT7; lane 5, *Methanobacterium formicicum* BRM9; lane 6, *Methanosarcina barkeri* CM1; lane 7, *Methanococcus voltae* DSM1537; lane 8, *Methanobacterium bryantii* DSM863; lane 9, *Methanobrevibacter wolinii* ABM4; lane 10, *Methanosphaera stadtmanae* DSM3091; lane 11, *Methanobrevibacter smithii* DSM861; lane 12, *M. smithii*. SM9. S = standard mixture containing six methanogen amplicons as labeled.

(lanes 11–12). Conversely, in the case of amplicons from different genera (*M. formicicum*, *M. barkeri*, and *M. voltae*) bands migrated to similar positions (lanes 5–7, Fig. 1). A mixture of six amplicons in the same lane provided a reference standard (Figs. 1, 2, and 3).

TTGE Analysis of Methanogens in Rumen Samples. TTGE fingerprints of methanogens in rumen samples from two cattle and four sheep are given in Fig. 2. Each rumen sample showed a broad range of amplicons. Cluster analysis of band profiles did not reveal any relationship between methanogen fingerprint and host species (data not shown). In each fingerprint, a predominant group of bands was present between the equivalent migratory positions of the *M. wolinii* ABM4 and *M. bryantii* DSM863 standards. Other bands were present close to the equivalent migratory position of the *M. ruminantium* NT7 standard. The fingerprints from two of the animals (cow 37 and sheep 62) had additional bands above and below the position of the NT7 standard.

TTGE fingerprints for methanogens in each culture of a dilution series derived from rumen contents from sheep 64 are given in Fig. 3. This shows that banding patterns similar to those of the rumen samples were present in the low-dilution cultures ($<10^{-5}$). In subsequent dilutions there was a transition from a predominance of midregion bands to a predominance of bands migrating at the *M. ruminantium* NT7 region. In the culture from the 10^{-10} dilution, only bands in the NT7 region were detectable.

A total of 67 sequences were obtained from clones prepared from 30 selected bands (indicated by arrows in Figs. 2 and 3). Analysis of sequences using Chimeracheck [3] showed that one sequence (4-1) was a chimera so this was discarded. A tree showing inferred phylogenetic relatedness between TTGE-derived sequences and sequences from databases is given in Fig. 4. The TTGE origin (band number followed by clone number) of sequences in each of the clusters in the tree together with percentage similarities within each cluster obtained from similarity matrices (data not shown) are listed in Table 2. All 66 sequences grouped within the domain Archaea and, on the basis of branching within the tree, belonged to seven taxonomic groups (Fig. 4). Thirty-six of the sequences formed four taxonomic groups clustering with sequences from *M. ruminantium*, *M. smithii*, *M. stadtmanae*, and the *Methanosarcinales*, respectively. The remaining 30 sequences clustered with a small number of database sequences from uncultured archaea to form a monophyletic lineage consisting of three taxonomic groups (Fig. 4). These three groups did not cluster with 16S rDNA sequences from cultured species.

Methanobrevibacter ruminantium-like Sequences. This group consisted of 24 very similar (>97.2% identity) sequences, which were 97.9–100% identical to the sequence of *M. ruminantium* NT7. Several clone-library sequences obtained from rumen samples in other investigations also clustered with this group, but it is not practicable to show these in Fig. 4. The majority of *M. ruminantium*-like sequences (22 of 24) were obtained from a tight group of bands that migrated to a position equivalent to that of the NT7 standard in almost every lane

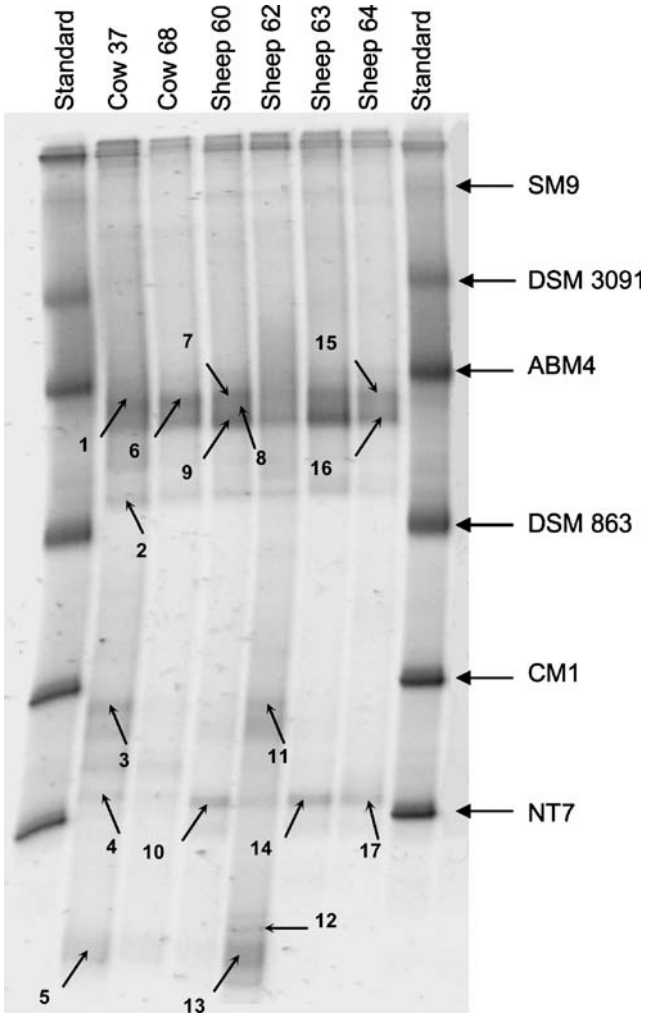

Figure 2. TTGE fingerprints of ruminal methanogens in two cattle and four sheep. The fragments are the ~491 bp V6–V8 regions of 16S rRNA genes amplified with primers 0915aFGC and 1386R. Numbered arrows indicate bands selected for reamplification and sequencing. Standard profiles consisted of amplicons from *M. smithii.* SM9, *M. stadtmanae* DSM3091, *M. wolinii* ABM4, *M. bryantii* DSM863, *M. barkeri* CM1, and *M. ruminantium* NT7.

examined (Figs. 2 and 3). The remaining two sequences were obtained from bands 9 and 26 (Figs. 2 and 4, respectively). Only one *M. ruminantium*-like sequence (4-2) was obtained from the bovine rumen, whereas seven were obtained from the ovine rumen (Fig. 2) and 16 from the dilution cultures from sheep 64 (Fig. 3).

Methanobrevibacter smithii-like Sequences. This group consisted of five very similar (>98.5% identity) sequences, which were 98.2–100% identical to the sequence of *M. smithii* SM9. Several clone-library sequences obtained from rumen samples in other investigations grouped with these, but are not shown in Fig. 4. Sequences in this group were obtained from five TTGE bands (Table 2) dispersed over the upper half of the gel. All these bands also yielded other sequence types suggesting a comigration of different sequence types. One *M. smithii*-like sequence (1-1) was obtained from the bovine rumen and the remaining four from bands in dilution cultures from sheep 64.

Methanosphaera-like Sequences. Four highly similar sequences were closely related (95.9–99.8% identity) to the sequence from *Methanosphaera stadtmanae* DSM3091. Of these, three (19-2, 21-1, and 21-2) were 99.6–100% identical to each other. Database sequences clustering with this *Methanosphaera*-like group included clone-library sequences from rumen samples [32] and sequences from tropical estuarine sediment [4] (not shown). The sequences within this group were all obtained from dilution cultures from one sheep (sheep 64).

Sequences Clustering Within the Methanosarcinales. Three sequences clustered with sequences from members of the *Methanosarcinales.* Sequences 2-1 and 23-3 were 99.6% identical to a clone-library sequence obtained from the rumen of a Japanese cow [29]. The most closely related (94.5–94.9%) cultivated methanogen was a strain of *Methanimicrococcus blatticola* isolated from the cockroach intestine [28]. The next closest (89.2–89.8%

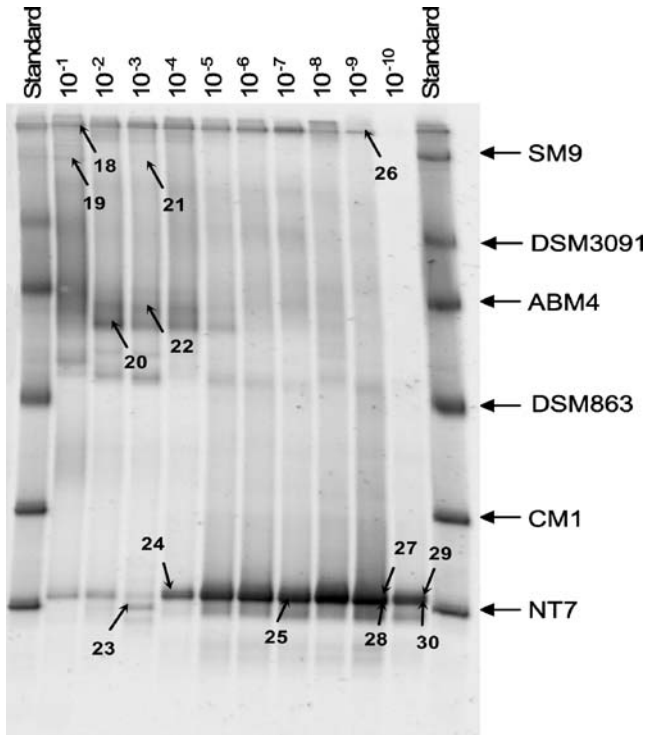

Figure 3. TTGE profiles for methanogens in cultured dilutions of rumen contents from sheep 64. The fragments are the ~491 bp V6–V8 regions of 16S rRNA genes amplified with primers 0915aFGC and 1386R. Numbered arrows indicate bands that were selected for reamplification and sequencing. Standards are the same as those in Fig. 2.

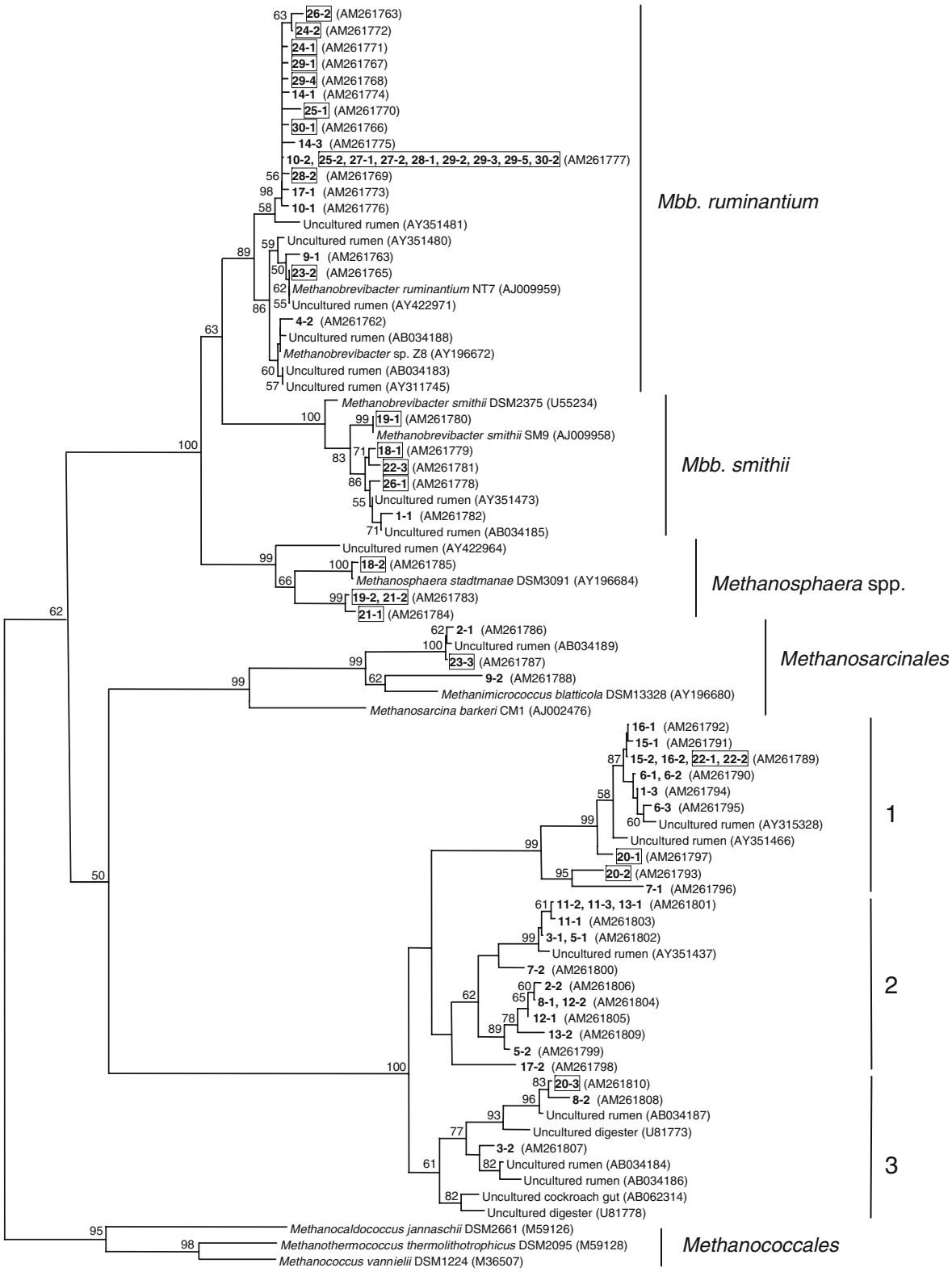
26-2 (AM261763)
24-2 (AM261772)
24-1 (AM261771)
29-1 (AM261767)
29-4 (AM261768)
14-1 (AM261774)
25-1 (AM261770)
30-1 (AM261766)
14-3 (AM261775)
10-2, 25-2, 27-1, 27-2, 28-1, 29-2, 29-3, 29-5, 30-2 (AM261777)
28-2 (AM261769)
17-1 (AM261773)
10-1 (AM261776)
Uncultured rumen (AY351481)
Uncultured rumen (AY351480)
9-1 (AM261763)
23-2 (AM261765)
Methanobrevibacter ruminantium NT7 (AJ009959)
Uncultured rumen (AY422971)
4-2 (AM261762)
Uncultured rumen (AB034188)
Methanobrevibacter sp. Z8 (AY196672)
Uncultured rumen (AB034183)
Uncultured rumen (AY311745)
Mbb. ruminantium
Methanobrevibacter smithii DSM2375 (U55234)
19-1 (AM261780)
Methanobrevibacter smithii SM9 (AJ009958)
18-1 (AM261779)
22-3 (AM261781)
26-1 (AM261778)
Uncultured rumen (AY351473)
1-1 (AM261782)
Uncultured rumen (AB034185)
Mbb. smithii
Uncultured rumen (AY422964)
18-2 (AM261785)
Methanosphaera stadtmanae DSM3091 (AY196684)
19-2, 21-2 (AM261783)
21-1 (AM261784)
Methanosphaera spp.
2-1 (AM261786)
Uncultured rumen (AB034189)
23-3 (AM261787)
9-2 (AM261788)
Methanimicrococcus blatticola DSM13328 (AY196680)
Methanosarcina barkeri CM1 (AJ002476)
Methanosarcinales
16-1 (AM261792)
15-1 (AM261791)
15-2, 16-2, 22-1, 22-2 (AM261789)
6-1, 6-2 (AM261790)
1-3 (AM261794)
6-3 (AM261795)
Uncultured rumen (AY315328)
Uncultured rumen (AY351466)
20-1 (AM261797)
20-2 (AM261793)
7-1 (AM261796)
1
11-2, 11-3, 13-1 (AM261801)
11-1 (AM261803)
3-1, 5-1 (AM261802)
Uncultured rumen (AY351437)
7-2 (AM261800)
2-2 (AM261806)
8-1, 12-2 (AM261804)
12-1 (AM261805)
13-2 (AM261809)
5-2 (AM261799)
17-2 (AM261798)
2
20-3 (AM261810)
8-2 (AM261808)
Uncultured rumen (AB034187)
Uncultured digester (U81773)
3-2 (AM261807)
Uncultured rumen (AB034184)
Uncultured rumen (AB034186)
Uncultured cockroach gut (AB062314)
Uncultured digester (U81778)
3
Methanocaldococcus jannaschii DSM2661 (M59126)
Methanothermococcus thermolithotrophicus DSM2095 (M59128)
Methanococcus vannielii DSM1224 (M36507)
Methanococcales
0.10

Table 2. Phylogenetic placement of sequences obtained from TTGE bands

Taxonomic group	*Number of sequences*	*Sequence similarity (%)*	*Sequence identifier*
M. ruminantium-like	24	>97.2	4-2, 9-1, 10-1, 10-2, 14-1, 14-3, 17-1, 23-2, 24-1, 24-2, 25-1, 25-2, 26-2, 27-1, 27-2, 28-1, 28-2, 29-1, 29-2, 29-3, 29-4, 29-5, 30-1, 30-2
M. smithii-like	5	>98.5	1-1, 18-1, 19-1, 22-3, 26-1
M. stadtmanae-like	4	>95.7	18-2, 19-2, 21-1, 21-2,
Methanosarcina-like	3	>93.4	2-1, 9-2, 23-3
Uncultured cluster 1	13	>94.3	1-3, 6-1, 6-2, 6-3, 7-1, 15-1, 15-2, 16-1, 16-2, 20-1, 20-2, 22-1, 22-2
Uncultured cluster 2	14	>95.4	2-2, 3-1, 5-1, 5-2, 7-2, 8-1, 11-1, 11-2, 11-3, 12-1, 12-2, 13-1, 13-2, 17-2
Uncultured cluster 3	3	>96.8	3-2, 8-2, 20-3

identical) was a sequence from a ruminal strain of *M. barkeri* [11]. Database sequences clustering with this group included some from rumen samples and one from the cockroach intestine [10]. These sequences were obtained from bands 29, 20, and 7, all of which also yielded comigrating sequences different from those of the *Methanosarcinales* (Table 2).

Sequences not Clustering with Known Methanogens. The 30 sequences that did not cluster with known methanogens formed a monophyletic group with three discrete taxa (clusters 1, 2, and 3) within the lineage (Fig. 4). Cluster 1 consisted of 13 sequences that share a minimum identity of 94.3% (Table 2). The only database sequences clustering with these 13 sequences were one from the ovine rumen [33] and one unpublished sequence (accession number AY315328) from the bovine rumen. All sequences in this cluster were obtained from bands that migrated to a position slightly below the *M. wolinii* ABM4 standard (Figs. 2 and 3). Sequences in this cluster were obtained from both ovine and bovine rumens and the dilution series.

Cluster 2 contained 14 sequences, which share a minimum of 95.5% identity (Table 2). The only database sequence grouping within this cluster was a clone-library sequence from the ovine rumen [33]. This was 95.8–99.6% similar to other sequences in cluster 2. Our cluster 2 sequences were obtained from both bovine and ovine rumen samples. None were obtained from the dilution series.

Cluster 3 contained three TTGE-derived sequences, which were 96.5–99.8% identical to each other. These sequences clustered with a group of database archaeal sequences including sequences from the bovine rumen [29], an anaerobic digester [9], and from the cockroach intestine [10]. The TTGE sequences in cluster 3 were obtained from the bovine and ovine rumens and from dilution cultures.

The level of sequence identity between clusters 1 and 2, 1 and 3, and 2 and 3 was 89–93%, 90–92%, and 92–97%, respectively. In comparison, the level of sequence identity between the *M. ruminantium*-like and *M. smithii*-like clusters in Fig. 4 was 92–93% (data not shown).

◀**Figure 4.** Neighbor-joining tree showing inferred phylogenetic relationships of TTGE-derived methanogen 16S rDNA sequences. Sequences from this study are labeled in bold, with band number and clone number with those derived from dilution cultures in boxes. Sequences from uncultured methanogens are clustered in 1, 2, and 3. Accessions numbers are in parentheses. The bootstrap values on nodes are % confidence levels from 1000 resamplings. The bar represents 10% sequence divergence.

Discussion

Denaturing gradient gel electrophoresis in its various forms has been used to investigate the ecology of microorganisms in many different environments, including the study of bacterial, fungal, and protozoal populations in the rumen [2, 15, 21]. In the case of methanogens, DGGE has been used to investigate methanogen populations in paddy fields [31], and TTGE has been applied to methanogen populations in a eutrophic lake [5]. To the best of our knowledge, the present study is the first application of a DGGE-based technique to populations of methanogenic archaea in the rumen.

PCR primers were chosen on the basis of their expected amplicons, which need to be sufficiently large for phylogenetic analysis yet sufficiently small for resolution by TGGE, and on their ability to amplify all known methanogens without degeneracy in primer sequences. These three criteria were met by primers 915aFGC and 1386R (Table 1) selected from published archaea-specific primers after alignment and comparisons with methanogen 16S rDNA database sequences. In tests with genomic DNA extracted from 12 methanogens, we were unable to adequately separate the resulting ~490-bp amplicons using conventional DGGE (data not shown). Instead, a TTGE method was developed to achieve better separation. The increased resolving power we observed for TTGE may be a result of the flexibility

offered by the temporal temperature gradient in comparison to the chemical denaturant gradient, which, at its limits, tended to result in diffuse bands rather than improved separation.

TTGE gave well-resolved bands for all the test strains, and discriminated between some strains of *M. ruminantium* and between the two strains of *M. smithii* tested. When the method was applied to amplicons obtained from rumen contents, the TTGE fingerprints of ruminal methanogens in two cows and in four sheep grazing ryegrass/clover pasture were surprisingly similar overall (Fig. 2). In the main, each fingerprint had a cluster of bands migrating adjacent to a *M. wolinii* reference and bands migrating near a *M. ruminantium* reference. The TTGE method developed in this study should enable the effects of diet and host on ruminal methanogen populations to be determined. In the case of grazing ruminants, our methanogen fingerprints from sheep and cows suggest that there may be no strong host-specificities for methanogens in the rumen.

Methanogenic archaea, together with several other archaeal lineages comprise the kingdom Euryarchaeota within the domain Archaea [22]. To date, five orders of methanogenic archaea, namely, Methanobacteriales, Methanomicrobiales, and Methanosarcinales, Methanococcales and Methanopyrales have been described [8]. Representatives of the first three orders have been isolated from the rumen [14; and references therein], and methanogens belonging to the Methanococcales have been detected in the rumen using a *Methanococcaceae*-specific hybridization probe [17]. In the present study, a wide range of representative bands were selected from TTGE profiles, but further information probably exists in unsequenced bands. About half of the sequences obtained from rumen samples and from serial dilution cultures were found to group with previously cultured ruminal methanogens. Of 33 sequences clustering within the Methanobacteriales, 24 grouped with *M. ruminantium*, five with *M. smithii*, and four with *M. stadtmanae*. Three sequences clustered within the Methanosarcinales.

The *M. ruminantium*-like sequences (Fig. 3) clustered with sequences obtained from the bovine rumen in Canada [32], sequences from both bovine and ovine rumen in Japan [29, 35], sequence types from the ovine rumen in Western Australia [33], and sequences from the bovine rumen in New Zealand [25]. The apparently high representation of *M. ruminantium*-like sequences in the present study (Fig. 4) is a result of the inclusion of sequences from *M. ruminantium*-like bands from dilution cultures (Fig. 3). *M. smithii*-like sequences, similar to the ones we have found, have been reported in all the above studies except for the Canadian one [32].

Our finding of *Methanosphaera*-like sequences is also consistent with results from previous examinations of methanogen 16S rDNA sequences from the rumen [25, 32, 33]. Of four *Methanosphaera*-like sequences, three were 95.9% similar and one 99.8% similar with that of *M. stadtmanae*. Of the three sequences clustering within the Methanosarcinales, two were similar to sequences previously found in methanogen clone libraries from the rumen [29, 32]. In this case, the closest identity (94.5–94.9%) of the three sequences with that of a cultured methanogen was with *Methanimicrococcus blatticola*, a methanogen found in the cockroach intestine [28], suggesting that these sequences may represent a new *Methanimicrococcus* species.

In the present study, 30 of the 66 sequences obtained from TTGE profiles did not cluster with established orders of methanogens. Twenty-two of these were nonidentical sequences demonstrating that this was not a chance selection of the same sequence type, and formed a monophyletic lineage containing three distinct clusters (Fig. 4). Members of the lineage were well represented in the rumens of all the animals tested. The lineage described in this study provides a framework for published ruminal sequences that do not cluster with recognized methanogens. From clone libraries prepared from the bovine rumen, Tajima *et al.* [33] identified a substantial number of novel archaeal sequences and concluded that these were likely to represent a new group of ruminal archaea. These, together with our sequences, sequences from an anaerobic digester, and a sequence from the cockroach gut grouped to form cluster 3 of the lineage. The finding of sequences from nonrumen environments in cluster 3 suggests that these types of methanogen may occur widely in the biosphere. From a clone library prepared from the ovine rumen, Wright *et al.* [33] reported two novel archaeal sequences and suggested that these might represent a new order. One of these sequences grouped within our cluster 1 and the other grouped within our cluster 2.

The finding of these sequences in all six ruminants in this study and in ruminants in previous studies suggests that the members of this lineage are normal rumen inhabitants. The microbes represented appear to be methanogens. Of the 66 sequences generated by our primer pair, all were related to cultured methanogens except for those in clusters 1, 2, and 3. Moreover, TTGE examination of cultured dilutions from a rumen sample showed that these microorganisms established on H_2/CO_2 (the most common substrate for ruminal methanogens) until overgrown by *M. ruminantium*-like methanogens. The relative abundance of sequences from the uncultured methanogens (Fig. 4) suggests that they may have significant population densities in the rumen. The branching in the phylogenetic tree (Fig. 4) suggests that the three clusters may each represent at least one species of methanogen.

It is somewhat surprising that there have been few previous reports on this archaeal lineage in the rumen.

This may be the result of cloning biases or of factors affecting primer specificities [30]. The apparent diversity of ruminal methanogens revealed from 16S rDNA clone libraries has been shown to depend on the choice of methanogen-specific PCR primers used to create the libraries [25, 29]. In the present study, the broad diversity revealed suggests that our primers are not strongly biased toward any particular sequence type. The TTGE profiles contained not only sequences from the three clusters, but also sequences of known ruminal methanogens *M. ruminantium*, *M. smithii*, *M. stadtmanae*, and *Methanosarcina* spp.

To obtain more information on methanogen diversity and population densities, the rumen sample from one sheep was diluted through methanogen-selective medium and cultured for 21 days. TTGE profiles from the low-dilution cultures ($<10^{-4}$) showed a range of methanogens including those in the uncultured lineage. Band intensities suggested that uncultured methanogens were present at densities equivalent to *M. ruminantium*-like methanogens (Fig. 3). At the highest dilutions, *M. ruminantium*-like methanogens were the sole culturable methanogens, indicating that they were present in the original rumen sample at about 10^{10} g^{-1}. This is in agreement with general findings that species of *Methanobrevibacter* spp. are predominant ruminal methanogens [19, 24]. It appears that the unidentified methanogens did not compete well with *M. ruminantium* in the culture system used.

This study has demonstrated the power of TTGE for analyzing methanogen populations in the rumen, and suggests that fingerprints generated by TTGE may offer a quick method for monitoring changes occurring within the populations. There is now a need to investigate the methanogens in the lineage revealed in this study, and in particular obtain cultures so that their physiology and ecological roles can be investigated.

After submission of this manuscript, a study of methanogen 16S rDNA sequences from the ovine rumen was published [34]. From a phylogenetic tree generated from 1300 bp sequences, these researchers found a grouping of novel uncultured methanogens, a finding in close agreement with the results in the present study. Our finding of a monophyletic lineage of uncultured methanogens is also in close agreement with their conclusion that these represent a new order of methanogens.

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