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Structure of the Archaeal Community of the Rumen$^\nabla$

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Members of the domain *Archaea* contribute about 0.3 to 3.3% of the microbial small subunit (16S and 18S) rRNA in the rumen (22, 39, 60). Archaea have a range of different metabolisms and are found in many habitats (6), but those known to exist in the rumen are strictly anaerobic methanogens. Yanagita et al. (59) observed that 2.8 to 4.0% of ruminal microorganisms displayed autofluorescence characteristic of F_{420}, a methanogen cofactor, able to be seen under UV illumination during microscopy. Taken together with the small subunit rRNA abundance data, this suggests that a large part of the archaeal population is made up of methanogens. Most species of methanogens can grow using H_2 and often formate as their energy sources and use the electrons derived from H_2 (or formate) to reduce CO_2 to CH_4. Some species can grow with methyl groups, oxidizing some to CO_2 to produce electrons that are used to reduce further methyl groups to methane. A few species can grow with acetate, effectively dissimilating acetate to CH_4 and CO_2. However, acetate is not metabolized to CH_4 to any significant extent in the rumen (13). This is probably because the rate of passage of rumen contents through the rumen is greater than the growth rate of acetate-utilizing methanogens (53).

In a normally functioning rumen, proteins and polymeric carbohydrates, which usually make up the largest part of the incoming feed, are fermented by a mixed microbial community to volatile fatty acids (VFAs), NH_4^+, CO_2, and H_2. The hydrogen is metabolized by the methanogens. The VFAs are taken up by the animal across the rumen wall and serve as major carbon and energy sources for the ruminant. A part of the VFAs, undigested feed components, and microbial cells leave the rumen and enter the rest of the animal's digestive tract. The central role of H_2 in the rumen fermentation (12) means that, although methanogenic archaea make up only a small part of the rumen microbial biomass, they play an important role in rumen function and animal nutrition. Efficient H_2 removal leads to a nutritionally more favorable pattern of VFA formation and to an increased rate of fermentation by eliminating the inhibitory effect of H_2 on the microbial fermentation (26, 53).

The rumen can be simplistically described as an open system with discontinuous solid (feed) and liquid (saliva and drinking water) inputs and multiple fractions that have different turnover rates (53). The methanogens in the rumen are found free in the rumen fluid, attached to particulate material and rumen protozoa, associated as endosymbionts within rumen protozoa, and attached to the rumen epithelium. The methanogens associated with these different fractions can be expected to have different growth rates since they will be removed from the rumen at different rates. In addition, the animal itself and the feed also influence the rate of passage of digesta through the rumen system (25). These different habitats may allow niche division among the methanogens and may explain some of the observed phylogenetic diversity of rumen archaea.

Cultured methanogens from the rumen. Methanogens have been classified into 28 genera and 113 species (11), but many more species can be expected to occur in nature (6). Surprisingly few methanogens have been isolated from the rumen. Those that have been cultured are assigned to only seven species. These are *Methanobacterium formicicum* (33), *Methanobacterium bryantii* (16), *Methanobrevibacter ruminantium* (43), *Methanobrevibacter millerae* (36), *Methanobrevibacter olleyae* (36), *Methanomicrobium mobile* (35), and *Methanoculleus olentangyi* (16). *Methanosarcina* spp. have also been cultured from the rumen (1, 34) but are not normally a major part of the archaeal community. In addition, *Methanobrevibacter smithii* (16) has been reported as being isolated from the rumen, but this is likely to be a strain more closely related to *M. millerae* (M. Kirs and P. H. Janssen, unpublished data).

Cultivation-based studies usually fail to uncover the full extent of microbial diversity. This is because some species are more readily culturable than others and because the size of any single cultivation-based survey is usually too small to give good insight into the community structure (15). The random isolation of methanogens in small-scale cultivation-based studies is useful for obtaining isolates for detailed investigation and also indicates the presence of a species. However, the abundance of different methanogens in different systems cannot be assessed comprehensively using data from the cultivation-based studies that have been made over the past 50 years.

Cultivation-independent surveys of ruminal methanogens. Surveys of methanogens and total archaea in the rumen have been made by using PCR to amplify the 16S rRNA genes of archaea, followed by a cloning step in *Escherichia coli* to separate the different gene variants within the mixed amplicon. An assessment of the dominant groups of archaea in the rumen can be made by comparative sequence analysis and phylogenetic placement of the 16S rRNA genes recovered in such surveys. In some of the studies, not all of the 16S rRNA genes

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TABLE 1. Numbers of clones and sequences in the studies summarized in this synthesis

Study subject and authors (reference no.)	No. of data sets	No. of sequences		No. of clones
		>870 nt	<800 nt	
Archaea in total rumen contents				
Shin et al. (40)	1	104	0	104
Skillman et al. (42)	1	10	0	10
Tajima et al. (48)	1	8	0	23
Whitford et al. (52)	1	41	0	41
Wright et al. (54)	3	43[a]	0	405
Wright et al. (55)	1	26	0	78
Wright et al. (56)	2	28	0	241
Wright et al. (57)	1	14	0	104
Yanagita et al. (59)	1	0	20	20
Protozoan-associated archaea				
Chagan et al. (3)	1	0	8	91
Irbis and Ushida (14)	1	0	14	2,398
Ohene-Adjei et al. (32)	1	0	139	139
Regensbogenova et al. (37)	1	11	9	20
Tokura et al. (50)	1	0	6	69

[a] An additional 26 sequences, representing 328 clones, were not included because they were withdrawn from GenBank.

were sequenced, but, instead, a method of screening, such as restriction fragment analysis, was used to group similar clones before determining the sequences of representative clones. In other studies, not all 16S rRNA gene sequences were deposited in publicly accessible databases. However, a number of publications provide enough detail to allow an accurate assessment of the number of occurrences of each sequence type and thus make estimating the abundance of different archaeal groups possible. Comparison of all sequences with a reference data set of almost full-length 16S rRNA gene sequences allows phylogenetic placement of all sequences with some degree of confidence.

The studies analyzed in this synthesis have been separated into two major types: studies of total rumen archaea and studies of protozoan-associated archaea (Table 1). Nine studies surveyed total rumen archaea, including free-living cells, particle-associated cells, and cells associated with other rumen microbes. Two of these studies (54, 56) reported multiple, distinctly different libraries, so that the nine studies were divided into 12 data sets containing a combined 294 useful sequences that represented 1,026 cloned 16S rRNA genes. Five other studies investigated protozoan-associated archaea. These formed five data sets containing a total of 187 sequences that represented 2,717 cloned 16S rRNA genes. 16S rRNA gene sequences were downloaded from GenBank databases (2) into a global data set curated using ARB (23). In addition, 138 16S rRNA gene sequences from a wide range of cultured archaea, largely methanogens, were included as reference sequences. These reference sequences were all >1,300 nucleotides (nt) long. Evolutionary analyses of these sequences resulted in construction of a dendrogram (Fig. 1), which allowed final assignment of all sequences and associated clones to clades of rumen archaea (Table 2).

The 14 studies summarized in this review were carried out by different research groups, with samples from a number of ruminant species and breeds fed different diets in various coun-

tries. Different samples were collected, and these were not always treated in the same way. The 16S rRNA genes were amplified using different primer sets, and so divergent amplification biases may have been introduced. Therefore, the studies are not strictly comparable, but taken together, the global data set does offer valuable insight into the identity of the dominant groups of rumen archaea.

Abundant archaea in the rumen. Based on the analysis of the global data set, the majority (92.3%) of rumen archaea detected in total rumen contents can be placed in three genus-level groups (Table 2). These are *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%), and a large group of uncultured rumen archaea labeled here as rumen cluster C, or RCC (15.8%). *Methanobrevibacter* spp. have been considered the dominant methanogens in the rumen (28, 57), and this synthesis supports this hypothesis.

Within the genus *Methanobrevibacter*, the cloned sequences fall into two major clades. One clade, defined by the species *Methanobrevibacter gottschalkii*, *Methanobrevibacter thaueri*, and *M. millerae*, contains the larger part of the *Methanobrevibacter*-related clones (a mean of 33.6% of rumen archaea). This group is designated the *M. gottschalkii* clade. The other major clade, defined by *M. ruminantium* and *M. olleyae*, contains 27.3% of rumen archaea, and is designated the *M. ruminantium* clade. Members of these two clades were found in nearly all of the data sets (Table 2). *Methanobrevibacter* spp. also appear to be early colonizers of the developing rumen (41). Members of other *Methanobrevibacter* spp., including *M. smithii* and *Methanobrevibacter wolinii*, appear to be rare.

Members of other groups of methanogens, including *Methanimicrococcus* spp., *Methanosphaera* spp., and *Methanobacterium* spp., occurred in fewer data sets or at lower abundances (Table 2). In addition, two groups of uncultured archaea, designated here the Qld26 group, and a clade of *Crenarchaeota* were also detected. The Qld26 group contains the 16S rRNA gene sequences CSIRO-Qld26 and Ven-04 (55, 57) and nine sequences, mainly associated with large particles in the rumen, detected by Shin et al. (40). The crenarchaeotes were detected rumen in only one study (40), and detection in other samples will be required to show that they were not transients. The physiologies of members of these two groups are not known.

The variation in the sequence composition within the major clades is great enough in some groups to result in almost continual gradation of sequence types with little evidence of subgroups. This is especially apparent within the *M. gottschalkii* clade. Some of the variation may be due to amplification and sequencing errors, and some may reflect true genetic diversity among strains of ruminal *Methanobrevibacter* spp. This gradation of 16S rRNA sequence types means that further division based on the available gene sequence evidence is not possible. There is a similar lack of differentiation of some of the other clades into discernable subgroups (e.g., the *Methanomicrobium* clade, the *Methanosphaera* clade, the Qld26 clade, the *Methanimicrococcus* clade, and most of the *M. ruminantium* clade). The RCC clade, in contrast, does display a considerable variation in sequence types that fall into a number of distinct lineages and may, therefore, represent a number of distinct species and perhaps multiple genera, with greater than 3% difference in sequence between members of different subgroups.
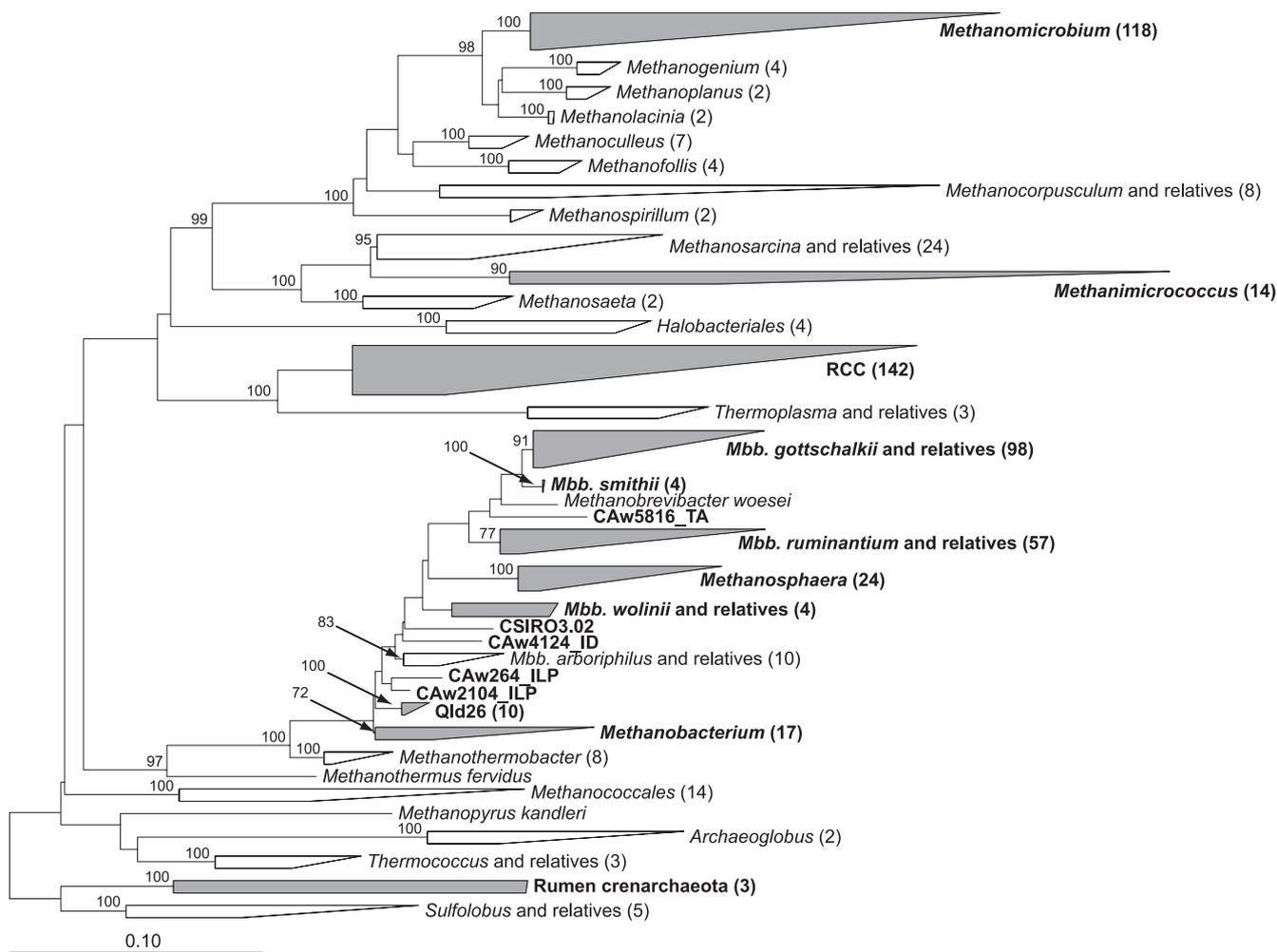
FIG. 1. Phylogenetic dendrogram of total rumen and rumen protozoan-associated archaea and selected reference sequences. Sequences of >870 nt long (138 reference sequences and 271 cloned sequences) were aligned using the EDIT4 primary sequence editor in ARB (23), and the alignments were checked and corrected to produce a master alignment. All positions were considered for the subsequent analyses. Phylogenetic dendrograms of just the 138 reference sequences and of the reference sequences plus 271 cloned sequences of >870 nt were generated in ARB using the Jukes-Cantor distance correction (17) and the neighbor-joining algorithm (38). These dendrograms were rooted with eight sequences from the *Crenarchaeota*. These alignments were exported from ARB, and 1,000 bootstrap data sets were generated from each in PAUP (46) using Juke-Cantor distances before 1,000 dendrograms were generated using neighbor joining. Consensus dendrograms were then generated in PAUP. These two consensus dendrograms, with bootstrap values, were used to define the clades referred to in this review. Key bootstrap values from the analysis of the larger data set have been added to the phylogenetic dendrogram illustrated. Sequences of <800 nt long (Table 1) were then inserted into the dendrogram of 271 cloned sequences of >870 nt plus 138 reference sequences using the parsimony insertion tool in ARB. For simplicity, clades are shown as shaded parallelograms, with the total number of sequences used in the dendrogram construction shown in parentheses. Sequences and clades containing sequences that originate from PCR-mediated surveys listed in Table 1 are indicated in boldface text. The scale bar indicates 0.10 inferred nucleotide substitutions per position. *Mbb, Methanobrevibacter*.

A difference of greater than 3% in the 16S rRNA gene sequences of prokaryotes is often used as an indicator of species-level separation of two strains (45). If the difference is less than 3%, it is not possible to use the 16S rRNA gene sequence comparison to determine whether strains belong to the same or different species. Dighe et al. (8) suggested that 16S rRNA gene sequence differences of >2% correlated with DNA-DNA hybridization similarities of <70% within the genus *Methanobrevibacter* and so could be used to separate sequences into species. This still relies on the definition of a species as being separated from its relatives by DNA-DNA hybridization values of <70%. In both of the major *Methanobrevibacter* clades discussed above, the named species have 16S rRNA gene se-

quence differences of <3%, and the decision to describe multiple species was based largely on differences in the genomes as determined by DNA-DNA hybridization, as well as a limited number of phenotypic differences (29, 36). Whether these can truly be regarded as different species is a matter of interpretation of the meaning of DNA-DNA hybridization results. This means that there may be a large number (>20) of rumen methanogen species within the genus *Methanobrevibacter* or only a very small number (approximately four). Detailed genome analysis may help reveal which interpretation is correct, although in the end the analysis will depend largely on the definition of a prokaryotic species, especially if the genomes also form a gradation of genotypes (20). It is more significant

TABLE 2. Assignment of clones to different clades of *Archaea* and the number of positive data sets for each clade[a]

Clade	Detection in total rumen contents			Detection in protozoan-associated archaea		
	Mean (%)	Maximum (%)	No. of data sets (n = 12)	Mean (%)	Maximum (%)	No. of data sets (n = 5)
Methanomicrobium	14.9	85.6	4	20.1	80.0	3
Methanimicrococcus	2.4	14.6	3	4.0	20.0	1
M. gottschalkii	33.6	81.2	10	42.9	98.6	3
M. smithii	0.1	0.8	1	0	ND[c]	0
M. ruminantium	27.3	60.0	10	12.5	62.6	1
M. wolinii	0.1	1.0	1	2.6	12.8	1
Other *Methanobrevibacter*[b]	0.5	5.6	1	0.6	2.9	1
Methanosphaera	3.7	26.8	5	1.9	4.0	4
Methanobacterium	0.2	2.9	1	0.4	1.1	2
RCC	15.8	80.8	5	15.1	74.1	2
Qld26	1.3	8.7	3	0	ND	0
Crenarchaeota	0.2	2.9	1	0	ND	0

[a] The clades are illustrated in Fig. 1. *n*, number of data sets (see Table 1).
[b] Not a phylogenetically coherent clade.
[c] ND, not detected in any data set.

to determine how many functionally different groups there are within these clades and what the biochemical and ecological differences are.

Comparison with other methods. RNA-targeted DNA probes have been used to analyze the archaeal community of the rumen using hybridization against extracted RNA (22, 39) or against RNA-containing cells using fluorescence in situ hybridization (FISH) (44, 58). In common with the clone library analyses, these studies found that members of family *Methanobacteriaceae* (which includes *Methanobrevibacter* spp., *Methanobacterium* spp., and *Methanosphaera* spp.) were the dominant members of the rumen archaeal community (30 to 99% of archaea). Members of the order *Methanomicrobiales* (which includes *Methanomicrobium* spp.) were less abundant (0 to 54%), and members of the order *Methanosarcinales* (which includes *Methanimicrococcus*) were rare (2 to 3%). Interestingly, significant populations of members of the family *Methanococcaceae* (8 to 44%) were detected using probes, but this group is wholly absent in gene library-based surveys (Table 2). This suggests that the probes used were not entirely specific or that significant biases occur in library generation. This remains to be resolved. However, members of the order *Methanococcales* have not been cultured from the rumen (16).

Temporal temperature gradient gel electrophoretic separation of 16S rRNA genes amplified by PCR from total rumen contents of pasture-fed sheep and cows, followed by sequencing prominent bands, confirmed the abundance of members of the *M. ruminantium*, *M. gottschalkii*, and RCC clades (31). Members of the *Methanimicrococcus* clade were also detected in the samples, and after a cultivation-based step, the presence of members of the *Methanosphaera* clade was also verified. These abundances agree with the global data set. No members of *Methanococcales* were detected.

Tatsuoka et al. (49) and Denman et al. (7) surveyed the diversity of the methyl-coenzyme M reductase (*mcrA*) gene in the rumen of cattle. This enzyme and its gene are good markers for the presence of methanogens (10). In both studies of the rumen, *mcrA* genes from *Methanobrevibacter* spp. dominated the libraries generated using primers that targeted most *mcrA* sequence types. Denman et al. (7) also detected a group

of *mcrA* sequences belonging to an unidentified group of archaea also detected in landfill by Luton et al. (24). It is not known if these are derived from one of the uncultured groups of archaea detected in the 16S rRNA libraries. No *mcrA* genes clearly assignable to the *Methanococcales* were detected in the two studies on the rumen (7, 49).

The methanogens that have been cultured from the rumen fall into the major clades detected in the global data set, with the exception of *Methanosarcina* spp. (1, 34) and *Methanoculleus olentangyi* (16). The isolation of a microorganism from an environment indicates its presence at the time of sampling, but unless abundance is quantified, it is not possible to make statements regarding the numerical significance the organism has in the community from which it was cultured. The 16S rRNA gene-based approach can be expected to detect mainly large populations. At present, the interpretation must be that *Methanosarcina* spp. and *Methanoculleus* spp. have been found in samples from the rumen but do not appear to be numerically significant parts of the rumen archaeal community. Methodological biases against their molecular detection could also result in their absence from libraries of PCR-amplified 16S rRNA genes, but the range of different primers used in the different studies suggests that primer bias is unlikely.

Archaeal community structure. The community composition uncovered in the different studies varied. This may be attributable to the ruminant host or the diet or to the DNA extraction methods and PCR primers used. Two of the studies were performed using the same DNA extraction methods and the same PCR primers (54, 55). Both assessed archaeal diversity in the rumen of sheep. The rumen archaea of sheep held at the CSIRO Yalanbee Research Station in Western Australia were dominated by members of the *M. gottschalkii* (75.3%) and *M. ruminantium* (19.5%) clades. In contrast, sheep in Queensland, Australia, had archaeal populations dominated by members of RCC clade (80.8%) and had only few members of the *M. gottschalkii* clade (9.0%), no detectable members of the *M. ruminantium* clade, and some members of the *Methanomicrobium* clade (7.7%). The differences in community composition could have been caused by differences in diet, environment, health, animal genotype, and animal age (27).

Shin et al. (40) and Tajima et al. (48) reported the dominance of *Methanomicrobium* spp. among the total rumen archaea of cows (85.6% and 60.9%, respectively) and found that *Methanobrevibacter* spp. were not detected (40) or not the dominant clade (17.4%) (48). In contrast, Whitford et al. (52) found that *Methanosphaera* spp. (26.8%) and *Methanimicrococcus* spp. (14.6%) were abundant in the cows they studied but that *Methanobrevibacter* spp. were the dominant archaea (58.5%). Wright et al. (56) found that *Methanobrevibacter* spp. (50.0 to 51.9%) and members of RCC (37.8 to 50.0%) dominated in feedlot cattle in two geographic locations in Canada, while sheep in Australia and Venezuela shared very similar archaeal communities (57). Whether these and other differences and similarities have a methodological basis or are host based or are controlled by diet or animal management choices still remains to be elucidated.

A more detailed analysis of data reported by Wright et al. (54) reveals that diet may have an effect on the composition of the methanogen community. Three groups of sheep at the same site were fed three different diets: pasture, oaten hay based, and lucerne hay based. The abundance of different clades of *Archaea* varied in these three cohorts ($P = 6.8 \times 10^{-6}$; χ^2-test). Diet is known to affect the composition of the bacterial community in the rumen (19, 47), but bacteria can be expected to interact more intimately with the diet since they will be using mainly feed components as their energy substrates. The majority of rumen archaea probably use hydrogen and possibly formate as their energy sources, which are formed during the primary fermentation of feed by the rumen bacteria, fungi, and protozoa. Diet can be expected to have an effect on methanogens due to changes in pH, which can affect methanogen activity (21), and through the presence of toxic compounds that may affect methanogens directly. Feed degradability will affect the rate of passage of digesta (25) and so may select for different species.

Abundant archaea associated with protozoa. Rumen protozoa are known to harbor ecto- and endosymbiotic methanogens, based on characteristic fluorescence of F_{350} and F_{420} and FISH using archaea-specific oligonucleotide probes (9, 51). Based on clone library analyses, the majority (93.8%) of protozoan-associated archaea can be placed in the same three genus-level groups found to dominate the total rumen archaea, the genera *Methanobrevibacter* and *Methanomicrobium* and the RCC clade (Table 2). Other genus-level groupings appear to be much less abundant. There were notable variations in the abundance of different groups of protozoan-associated archaea in the five different studies. Members of the *M. ruminantium* clade were abundant (62.6%) in the study reported by Chagan et al. (3) while *Methanomicrobium* spp. dominated in the study of Regensbogenova et al. (37), and members of the RCC clade dominated in the study of Ohene-Adjei et al. (32). Sequences known to have originated from protozoa are well interspersed among those from total rumen samples among the *Methanomicrobium*, *M. gottschalkii*, and RCC clades. There are not enough sequences to determine if this is also true for the *M. ruminantium* clade. It is not clear that there are different protozoan-associated and free-living lineages within these clades. All of the studies on rumen-associated archaea are strongly biased because these studies were not made on the total rumen protozoa but on selected protozoa. The abundance of the

individual protozoan species and the number of archaeal cells in or on each cell are not taken into account. The aim of these studies was not to describe total rumen protozoan-associated archaea, and so the interpretations made here must be regarded as a very tentative first analysis of abundant protozoan-associated rumen archaea.

The limited amount of information available does not support the idea that there are significant populations of uniquely protozoan-associated archaea in the rumen. Christophersen et al. (4) suggested that in some individual animals, the free-living and protozoan-associated archaea appeared to belong to the same species but that in other individuals they did not. Elimination of protozoa from the rumen of sheep resulted in changes in the archaeal community (30), but it was not clear if this was due to the disappearance of uniquely protozoan-associated methanogens or due to broader changes in rumen function as a result of defaunation that, in turn, affected the total archaeal community structure. The true significance of different clades of protozoan-associated archaea could be determined by careful analysis of the two groups in fractionated rumen samples and by using rRNA targeted FISH methods to observe the localization of cells of different archaeal groups in total rumen samples.

Roles and cooccurrence in the rumen. Of the 11 clades of *Archaea* detected (Table 2), 9 consist of hydrogen-utilizing methanogens. These are members of the genera *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, *Methanosphaera*, and *Methanimicrococcus*. It is not clear how these manage to coexist in the rumen, but there does appear to be some difference in the abundance of the different groups in different studies, suggesting a host or feed influence. This interpretation assumes no methodological biases. The rumen archaea may also interact with different hydrogen-producing organisms, for example, with protozoa, bacteria, or fungi.

The physiology of members of the Qld26 clade is not known, but based on their close relationship with *Methanobrevibacter* spp., *Methanobacterium* spp., and *Methanosphaera* spp., they are likely to be hydrogen-utilizing methanogens. The physiology of members of the RCC clade and the *Crenarchaeota* in rumen is not known. The RCC clade corresponds to group C of Kemnitz et al. (18), and relatives have been detected in a number of digestive tract and other anaerobic environments. Uncultured relatives falling into closely allied clades have been detected in soil, on plant roots, in hydrothermal and deep-sea sediments, and in aquifers (18). The RCC clade has been labeled as a group of methanogens (31), but there is little evidence for this. Isolation of members of these groups in pure culture or demonstration of their physiology using molecular ecological techniques is required to define the role of these archaea in the rumen.

Limiting the activity of rumen methanogens in domesticated ruminants may result in gains in animal productivity if the rates and patterns of feed fermentation are not adversely affected. Almost 40 years ago, Czerkawski (5) reviewed the ideas and research in this already active field. Interest in inhibiting rumen methanogens has recently been renewed (26) due to concerns about the amounts of methane generated from domesticated ruminants. This ruminant-derived methane accounts for about one-quarter of all anthropogenic methane emissions and is implicated in human-induced global climate change

(58). Knowledge of the ruminal methanogen community is an important part of developing strategies to mitigate rumen methane production. Assuming that the limited data available together constitute a good sample of global ruminant archaeal diversity, it can be concluded that only a few major groups of methanogens need to be targeted by antimethanogen agents in the first instance. It is not clear if elimination of these major groups will allow the less abundant members of other groups to take over the vacated niche. This will depend on the factors that limit or allow the different species to coexist in the rumen. These less abundant archaea may occupy specialist niches, but they may also be relegated to a minor part of the total community through competition. The limited diversity also means that strategies may be widely applicable across different countries. It is not clear whether other groups of methanogens are dominant in less-well-studied ruminant animals or if different feeds will allow otherwise minor groups of methanogens to dominate.

The few studies reported to date and summarized here give an indication of the global rumen archaeal community. This review is meant to act as a call for the development of more information rather than as a definitive summary. Many questions remain to be answered, and some have been briefly raised here. Fifty years after the initial isolation of methanogens from the rumen (1, 33, 43), the census of rumen archaea is beginning to show what groups are present, but this is not yet complete. New questions, such as how the groups apparently coexist and what determines the abundance of the different species, now need to be answered.

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