Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system

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Received 6 February 1998; revised 27 February 1998; accepted 27 February 1998

Abstract

Methanogen populations in the rumen and in model rumen systems (operated over a 240-h period) were studied using the small subunit (SSU) rRNA phylogenetic framework for group-specific enumerations. Representatives of the family Methanobacteriaceae were the most abundant methanogen population in the rumen, accounting for 89.3% (± 1.02%) of total archaea in the rumen fluid and 99.2% (± 1.8%) in a protozoal fraction of rumen fluid. Their percentage of archaea in the model rumen systems declined from 84% (± 8.5%) to 54% (± 7.8%) after 48 h of operation, correlated with loss of protozoa from these systems. The Methanomicrobiales, encompassed by the families Methanomicrobiaceae, Methanocorpusculaceae, and Methanospirillaceae were the second most abundant population and accounted for 12.1% (± 2.1%) of total SSU rRNA in rumen fluid. Additionally this group was shown to be essentially free living, since only a negligible hybridization signal was detected with the ruminal protozoal fraction. This group constituted a more significant proportion of total archaea in whole rumen fluid, 12.1% (± 2.1%) and model rumen fluid containing no protozoa (26.3 ± 7.7%). In contrast, the Methanosarcinales, generally considered the second most abundant population of rumen methanogens, accounted for only 2.8% (± 0.3%) of total archaeal SSU rRNA in rumen fluid. © 1998 Published by Elsevier Science B.V.

Keywords: Methanogen; Rumen protozoan; Symbiosis; Phylogeny; rRNA

1. Introduction

Rumen methanogens participate in the terminal steps of the degradation of plant organic matter in the rumen primarily by the removal of hydrogen generated by fermentation. Hydrogen removal promotes the more complete oxidation of fermented substrates and greater energy recovery by the fermenting organisms [1, 2]. Methanogens are therefore thought to be integral components of the microbial food chain in the rumen. The predominant species of methanogens so far identified by cultural enumeration are Methanobrevibacter ruminantium and Methanosarcina barkeri [2, 3]. While techniques are available for culture-based enumeration of methanogens, a complication in some environments is their frequent and intimate association with protozoa. The methanogen–protozoa association is a common feature of many anaerobic systems, observed between
hydrogenotrophic methanogens and bovine ruminal ciliates [4], ovine ruminal ciliates [5], termite hindgut flagellated protozoa [6], cockroach gut ciliate Nycotherus ovalis [7], marine ciliate Metopus contortus [9], lakewater ciliate Metopus pachyurus [10] and Tri... sp. [11]. This association is proposed to be of selective value to both organisms since it provides the capacity for interspecies hydrogen transfer [12]. Rumen protozoa depend upon a hydrogen evolving fermentation that provides substrate for the methanogens. The protozoa, in turn, benefit from hydrogen removal, since hydrogen is inhibitory to their metabolism if not removed.

We earlier developed a set of small subunit ribosomal RNA (SSU rRNA) probes for the major groups of methanogens [13,14] and have used them to study the population structure of natural and engineered communities [15]. We are currently using these and other group-specific probes to study the community structure of artificial rumens, using the Hoover dual flow continuous culture fermenters as a model system [16,17]. A key feature of these systems is the loss of protozoa during long term system operation. Since the protozoa are hosts to many rumen methanogens, their loss from the model systems was hypothesized to alter the methanogen population structure. To evaluate this hypothesis, taxon specific probes for methanogens were used to quantify shifts in methanogen populations that accompanied loss of protozoa from the model rumens. This revealed a specific association between rumen protozoa and certain taxonomic groups of methanogens.

2. Methods

2.1. Collection of rumen fluid

Rumen fluid was removed from the rumen of a multiparous Holstein cow fed a total mixed diet [Alfalfa hay (600 g), dry shelled corn (329 g), soybean meal (58 g), dicalcium phosphate (8 g), trace mineral salt (4 g), vitamin A, D and E mix (1 g kg⁻¹)] twice daily for 14 days before sampling. The fluid was transferred to a prewarmed (38°C) container, filled to the top and covered to exclude air for transport to the laboratory.

2.2. Operation of model rumens

The original model rumen system (dual flow continuous culture system) and the modified version have been previously described [16,17]. This system was designed to simulate differential solid-liquid phase removal rates that occur in the rumen. Each vessel was supplied with feed (11 g) every 3 h [alfalfa hay (600 g), dry shelled corn (329 g), soybean meal (58 g), dicalcium phosphate (8 g), trace mineral salt (4 g), vitamin A, D and E mix (1 g kg⁻¹)] by an automatic feeding device. The pH was maintained at 6.25 by a pH sensor and infusion pumps delivering sodium hydroxide or hydrochloric acid. The temperature of the system was kept at 38°C using a thermocoupled probe to monitor temperature. Four model systems were operated for 240 h. Samples were removed for nucleic acid extraction at 0, 6, 12, 24, 48, 72, 96, 120, 168 and 240 h after inoculation by graduated pipette, placed in 2-ml screw-cap polypropylene tubes (Sarstedt, Inc., Newton, NC) containing 500 mg of zirconium beads, and immediately frozen on dry ice.

2.3. Preparation of the protozoal fraction

One liter of rumen fluid was transferred to a prewarmed (38°C) separative funnel previously flushed with nitrogen gas to maintain anaerobiosis. After 15 min of incubation, the protozoa had settled to form a milky layer at the bottom of the funnel. Excellent fractionation was confirmed by microscopic inspection and by hybridization (below). Aliquots of the protozoal fraction (0.5 ml) were sampled and immediately frozen in 2.0-ml screw-cap polypolyethylene tubes (Sarstedt, Inc., Newton, NC) containing 500 mg of zirconium beads on dry ice. Samples of rumen fluid were similarly frozen for subsequent analysis.

2.4. Extraction of nucleic acid

Total nucleic acids were recovered from the samples using a slight modification of a published procedure [18]. Mechanical disruption on a reciprocating shaker (Beadbeater, Biospec Products, Bartelsville, OK) with zirconium beads (0.1 mm) was employed to extract total nucleic acid from about 1 g of sample. Approximately 1 g beads was
used with each sample, together with 50 μl of 20% SDS (w/v), 500 μl of phenol equilibrated with 50 mM sodium acetate-10 mM EDTA buffer (pH 5.1). The samples were reciprocated for 3 min at room temperature and transferred to a 60°C waterbath for 10 min before a further 3-min period of reciprocation. Samples were extracted again with buffer-equilibrated phenol before extracting twice with phenol/chloroform (4:1) and twice with chloroform. Total nucleic acid was precipitated with ammonium acetate (2 M final concentration) and two volumes of ethanol. Concentrations of the recovered nucleic acid were measured spectrophotometrically, assuming that 1 mg of RNA per ml was equal to 20 optical density units at a wavelength of 260 nm.

2.5. Oligonucleotide probe hybridization

The RNAs extracted from samples and reference organisms were diluted and applied in triplicate to Magna Charge Nylon membranes as previously described [15]. Established hybridization conditions were used for three group-specific probes for methanogens, a universal probe (S-Univ-1392-a-A-15) and probes for each of the three domains, Archaea (S-D-Arch-0915-a-A-20), Bacteria (S-D-Bact-0338-a-A-18), and Eucarya (S-D-Euca-1379-a-A-16) [12,13]. The universal probe encompasses virtually all known life and was used to quantify total SSU rRNA. The methanogen probe S-F-Mbac-0310-a-A-22 encompasses the genera *Methanobacterium*, *Methanobrevibacter*, and *Methanosphaera*. These are representatives of the family Methanobacteriaceae within the order Methanobacteriales [14]. The orders Methanocorpusculaceae and Methanosarcinaceae were quantified using two probes, S-O-Mmic-1200-a-A-21 (Methanocorpusculaceae, Methanococcaceae, and Methanospirillaceae) and S-O-Msar-0860-a-A-21 (Methanosarcinaceae and Methanosaetaceae), respectively. The reference organisms used for each probe group were: *E. coli* (S-Univ-1392-a-A-15), *Saccharomyces cerevisiae* (S-D-Euca-1379-a-A-16), *Filrobacter saccharinisogens* S85 (S-D-Bact-0338-a-A-18), *Methanobacterium bryantii* DSM 862 (S-D-Arch-0915-a-A-20 and S-F-Mbac-0310-a-A-22), *Methanosarcina frisius* C16 (S-O-Msar-0860-a-A-21), *Methanogenium cariaci* DSM1947 (S-O-Mmic-1200-a-A-21). Bound probe was quantified using Storage Phosphor Screens and a PhosphorImager (Molecular Dynamics, Sunnyvale, CA [14]).

3. Results and discussion

The quantification of natural microbial populations is dependent on representative sampling and unbiased enumeration. Of these two requirements, unbiased enumeration is the more fundamental since representative sampling cannot be established without it. Although the utility of culture based techniques for enumeration is well established, they are not generally feasible for studies encompassing a wide variety of physiological types of differing abundance and including a large number of samples [18]. For example, in the absence of a selective medium, culture-based enumeration of low abundance populations is difficult [18]. Thus, a fundamental utility of molecular methods is the ability to establish general conditions for enumerating the full spectrum of microbial diversity. In this regard, key issues of nucleic acid extraction efficiency from different populations, and the stringency and precision of hybridization, have been established in previous studies [1,19]. Also, the phylogenetic framework provides an additional basis for evaluation of these data. A key point of validation is consistency between quantification using general and specific probes. For example, in this study domain probe summation (the total hybridization response from Archaea, Eucarya and Bacteria) is generally within 10% of total SSU rRNA measured using the universal probe. This internal consistency provides essential support for data interpretation.

3.1. Domain abundance

Domain representation is expressed as a percentage of the total SSU rRNA in each sample quantified using the universal probe (S-Univ-1392-a-A-15). As noted, we routinely determine domain summation for each sample as a key element of experimental validation. For example, we have observed that partial sample degradation results in greatly elevated domain summation as a consequence of the sensitivity of the universal probe target site to nuclease digestion [1]. In this study, the sum of the domain
probes was comparable to the universal probe quantification: 95.2 ± 11.6% for the protozoal fraction, 103.1 ± 2.1% for the rumen fluid samples and 96.3 ± 5.6% for the model rumen samples. This also demonstrated that fractionation of the protozoa was very effective: eucaryotic SSU rRNA in the protozoal fraction was 76.6 ± 9.6%, compared to 44.6 ± 2.3% for rumen fluid (Fig. 1). Bacterial SSU rRNA accounted for 17.2 ± 3.2% in the protozoal fraction, compared to 57.7 ± 3.2% in the rumen fluid (Fig. 1). Bacteria were not visible in this fraction using microscopic examination, suggesting that much of the bacterial signal represented bacteria ingested by, or associated with, protozoa.

The relative abundance of eucaryotic SSU rRNA in the model rumens rapidly declined following inoculation (Fig. 2), from 39.5 ± 6.3% at the start of the operation to 6.8 ± 1.1% after 48 h of operation. Microscopic observation has shown that this decline represents the loss of protozoa from the model systems (unpublished data). The residual eucaryotic signal may represent anaerobic rumen fungi, known to be another significant eucaryotic population in the rumen [20]. The proportion of bacterial SSU rRNA increased from 32.6 ± 6.3% to 90.5 ± 14.4% as the eucaryotic biomass was lost from the systems (Fig. 2).

3.2. Archaeal abundance

Archaeal SSU rRNA comprised a relatively small proportion of the total SSU rRNA in rumen fluid, the protozoal fraction and model rumen samples. Archaeal SSU rRNA accounted for 1.46 ± 0.31% of protozoal fraction SSU rRNA (Fig. 1), 3.12 ± 0.38% of the ruminal sample and 3.99 ± 1.72% of the model rumen samples. The protozoal archaeal fraction corresponds well to the previous estimate of the proportion of ciliate volume occupied by methanogens in some free-living anaerobic ciliates (1–2%) [21,22].

3.3. Summation of individual methanogen probe target groups

Since methanogens are the only Archaea that have been isolated from either the rumen or the large intestine of non-ruminants, we anticipated that archaeal abundance would correspond to the abundance of methanogens. Thus, the sum of the methanogen groups quantified with the individual methanogen probes should equal the value obtained by the archaeal probe. Fig. 3 shows the proportion of each group of methanogens in the protozoal fraction, rumen fluid, and the model rumen (72 h), expressed as a proportion of archaeal SSU rRNA. The average proportion of total archaeal rRNA in the protozoal fraction and rumen fluid samples accounted for by the three methanogen probes was 102 ± 1.9% and
107 ± 2.1%, respectively. In the model rumen samples, the summation was 105 ± 9.9%. This indicates that the diversity of Archaea in these samples was well circumscribed by these probes. However, we also note that a previous study of other gut content samples, most notably the cecum and lower GI tracts of goats, suggested the presence of an abundant archaeal population not encompassed by these probes [23].

3.4. Taxonomic composition of methanogens in rumen fluid and protozoal fraction

The Methanobacteriaceae was the most abundant family of methanogens in all the samples, comprising 99.2 ± 1.8% of the archaeal SSU rRNA in the protozoal fraction, 92.4 ± 1.8% in rumen fluid, and 72.5 ± 2.4% in the model rumen sample (72 h). The presence of the family Methanobacteriaceae in all three samples suggests that members of this family can exist either free living or associated with protozoa. Methanobrevibacter ruminantium, a member of the family Methanobacteriaceae, has been considered the most significant rumen methanogen, based on the frequency of cultural isolation [2,24]. Our results are consistent with these findings. Several methanogens in the family Methanobacteriaceae have been isolated from similar environments including additional strains of Methanobrevibacter ruminantium, Methanosphaera stadtmanae, Methanobacterium for-
virtually none was protozoal associated. This indicates that ruminal representatives were primarily free living in this study animal.

The association of the rumen protozoa with members of the Methanobacteriaceae is different from specificities observed in some other environments. The marine ciliate *Metopus contortus* together with *Trimyema compressum* and *Plagiopyla frontata* have been shown to form intracellular associations with close relatives of *Methanocorpusculum parvum* and *Methanocalicium marisnigri*, respectively, as their associated methanogens using rRNA-based approaches [9,27]. Culture-based techniques have been used to isolate *Methanoplanus* endosymbionts from *Metopus contortus* [28]. These species are representatives of the families Methanocorpusculaceae and Methanomicrobiaceae within the order Methanomicrobiales. In contrast, our results show that members of this order do not significantly associate with the rumen protozoa. Examples of Methanobacteriaceae association with protozoa are less numerous; ribosomal rDNA closely related to *Methanobacterium bryantii* has been isolated from the protozoon *Metopus palaeiformis* [27].

The rumen harbors a complex protozoan community, including flagellates, chytrids, and ciliates. The community of ciliates constitute the most conspicuous component of the rumen, the entodiniomorphs being the most diverse. Altogether 42 genera are recognized, common genera in the rumen of cattle are species of *Entodinium*, *Polyplastron* and *Ophryoscolex* [29]. Given this diversity it is surprising that the rumen protozoa show the same general specificity for methanogenic symbionts.

### 3.5. The composition of methanogen populations in the model rumen systems

The abundance of each population of methanogens expressed as a proportion of the total archaeal rRNA in individual model rumens is shown in Figs. 4-6. The composition of the methanogen populations in the inoculant (time 0) used for the model rumens was, as expected, very similar to the rumen fluid samples. However, after 6 h of operation, it was apparent that the methanogen populations had been perturbed. Over the 240 h period of operation, the relative abundance of Methanobacteriaceae declined from 84.2 ± 3.6% to 54.9 ± 3.95% of total archaea. This was most apparent in the first 48 h of operation, the period of most rapid loss of rumen protozoa, and was consistent with a protozoal association.

The relative abundance of a subset of the Methanomicrobiales increased from 9.6 ± 3.5% to 26.3 ± 7.7% (Fig. 5). A relative increase in other populations was not observed. Thus, this group may
have replaced those methanogens lost via protozoal displacement.

Methanosarcinales increased in abundance shortly after inoculation into the model systems, from $3.4 \pm 2.02\%$ to $11.9 \pm 5.13\%$. However, they returned to initial abundance after 72 h. Although there was much variation between the individual model systems during the first 48 h, they were remarkably consistent at later times. We suggest that their transient increase reflects the nutritional versatility of this group. *Methanosarcina barkerii* isolated from the rumen contents of both cows and sheep uses a variety of substrates including methanol and methyl amines [30]. Most *Methanosarcina* species utilize acetate as a carbon and energy source [31].

The use of molecular methods provided by comparative rDNA sequencing has greatly facilitated the accurate identification and classification of endosymbionts and their hosts. This in turn reveals important information about the diversity and evolution of symbioses. Of the four major lineages of methanogens (Methanobacteriales, Methanomicrobiales, Methanosarcinales, and Methanococcales), characterized endosymbionts are drawn from throughout the order Methanomicrobiales and from the family Methanobacteriaceae within the order Methanobacteriales. The results obtained in these studies suggested the importance of Methanobacteriaceae as symbionts of rumen protozoa. A more specific set of phylogenetic probes for the Methanobacteriaceae will be used in future studies to more fully resolve the character of these symbioses.

Syntrophic and competitive relationships are key determinants of microbial community structure in the rumen. The techniques used for this study could be equally valuable for evaluating various of these key microbial relationships. Since the nutrition of the host animal is largely determined by the products of the overall metabolism of this community (volatile fatty acids, bacterial protein and vitamins), the metabolic interactions that define the microbial community and its collective activities also determine host nutrition and production. We anticipate that information provided by the use of molecular techniques combined with established culture based enumerations and rumen fermentation characteristics will help resolve the contribution of microbial interactions to overall ruminal metabolism and host production.

**Acknowledgments**

This work was supported by a grant from the United States Department of Agriculture. The authors wish to thank the personnel at the University of Minnesota Dairy Research and Teaching Facility for care and feeding of the rumen fluid donor cow.

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