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FEMS Microbiology Letters 200 (2001) 67–72

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens

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Received 10 March 2001; received in revised form 19 April 2001; accepted 19 April 2001

First published online 11 May 2001

Abstract

Molecular diversity of rumen archaea was analyzed by PCR amplification and sequencing of two 16S rRNA clone libraries prepared from the bovine rumen fluid using two different archaea-specific primer sets. The first library of 19 clones which was generated with primers D30 and D33, produced essentially two groups of sequences, one affiliated with *Methanomicrobium mobile* (21% of clones) and the other – with the uncultured archaeal sequences from anaerobic digester, which are distantly associated with *Thermoplasma* (79% of clones). The second library of 25 clones, which was generated with primers 0025e Forward and 1492 Reverse, produced a higher degree of diversity: in addition to the previous two groups, with the *M. mobile*- (56%) and *Thermoplasma*-associated sequences (20%), four clones (16%) were identified as *Methanobrevibacter* spp. The remaining two sequences were associated with unidentified archaeal sequences from the rumen and swine waste. Phylogenetic placement of eight almost complete 16S rRNA sequences revealed the existence of a novel cluster of the rumen *Euryarchaeota*, which is not affiliated with the known methanogenic archaea. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Rumen; Molecular diversity; Archaea

1. Introduction

The symbiotic rumen ecosystem consists of mostly obligate anaerobic microorganisms including fungi, protozoa, bacteria and archaea. Molecular diversity of the bacterial part of the system, which is mainly responsible for the plant fiber breakdown process, has been intensively studied during the recent several years [1–4]. The extreme bacterial molecular diversity uncovered in these investigations reflects the complex metabolic network in which the rumen bacteria are involved. However, molecular diversity of other components of the system has received relatively little attention. The archaeal component of the ecosystem, which is thought to be represented exclusively by methanogens, is implicated in the removal of hydrogen through the synthesis and emission of methane thus completing the anaerobic fermentation [5]. Cultivation-based analyses of this component have identified *Methanobrevibacter* and *Methanosarcina* as predominant methanogenic genera in the rumen [5,6]. Also based on cultivation approach, earlier works have indicated that symbiotic relationships and close association exist between the rumen methanogens and protozoa [7,8]. These observations have been later confirmed with the use of an archaea-specific fluorescent probe [9]. Dot-blot hybridization analyses of the bovine rumen fluid and its protozoal fraction with taxon-specific 16S rRNA probes have implied that the protozoa-associated population consists mainly of the family Methanobacteriaceae and accounts for about 90% of all rumen methanogen population while the rest of it is free-living and represented by the Methanomicrobiales [10]. FISH-based enumerations have suggested that 54% of the total methanogens in the sheep rumen is *Methanomicrobium mobile* [11]. Taking into the consideration the fastidious

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0378-1097/01/$20.00
PII: S0378-1097(01)00201-4

growth requirements of ruminal archaea, it is reasonable to expect that PCR-retrieved 16S rDNA libraries would produce greater molecular diversity than the already cultivated archaea. However, a limited sequence information obtained from PCR-generated libraries from the total rumen fluid or protozoal fraction yielded sequences, which were similar to the already cultivated rumen archaea belonging to the *Methanomicrobium* and *Methanobrevibacter* genera [11,12]. In the present study, we attempted to recover the wider range of the rumen archaeal molecular diversity using two different sets of archaeal primers, which have not been used before in rumen microbiology studies. Indeed, we were able to recover several 16S rRNA sequences not clustering with known methanogens.

2. Materials and methods

2.1. Sample collection

Samples were collected as described previously [1]. Briefly, rumen contents were obtained from a closed herd at the National Institute of Animal Industry (Tsukuba, Japan). The animals were the rumen-fistulated Holstein dry cows fed a mixed ration (alfalfa-timothy hay and concentrate in a 4:1 ratio) twice a day. The corn and barley-based concentrate, Select 16, was purchased from Zen-Raku-Ren (Tokyo, Japan). To avoid the background influx of contaminating microorganisms with feed, the 18-h fasting interval was included and the samples were taken just before the morning feeding. The representative total rumen contents (250 g) were collected from two animals via the ruminal fistula. The rumen contents were squeezed through two layers of cheesecloth, and the resulting rumen fluid fractions from two animals were pooled, subsampled and frozen at −80°C in tightly closed 50-ml Falcon tubes.

2.2. Total DNA extraction

The rumen fluid was thawed on ice and the concentrated buffer components were added to final concentration of 100 mM Tris–HCl (pH 8.0), 50 mM EDTA (pH 8.0), 2% *N*-lauroyl sarcosine and 2 mg/ml proteinase K. This sample was treated by five cycles of freezing at −80°C for 30 min followed by heating in a water bath at 65°C for 30 min for DNA extraction. After extraction with equal volume of buffer-equilibrated phenol–chloroform–isoamyl alcohol solution, nucleic acids were precipitated by isopropanol, washed several times in 70% ethanol, dried, and dissolved in TE buffer (pH 8.0) containing DNase-free RNase (100 μg/ml).

2.3. PCR procedures

The first primer pair used for PCR amplification of archaeal 16S rRNA genes was D30 (ATTCCGGTTGATCCTGC) and D33 (TCGCGCCTGCGCCCCGT) [13]. The second set consisted of 0025e Forward (CTGGTTGATCCTGCCAG) and 1492 Reverse (GGTTACCTTGTTACGACTT) [14]. PCR was performed with TaKaRa *ExTaq* PCR kit (TaKaRa, Kyoto, Japan). The PCR reaction was conducted in a PE480 thermal cycler (Perkin Elmer Japan, Tokyo). The amplification conditions were: one cycle at 95°C for 3 min for initial denaturation, then 20 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min. A typical PCR mixture contained 300 nM of each primers, 0.35 μg of purified template DNA, 1×*ExTaq* reaction buffer, 200 μM of each deoxynucleoside triphosphate, and 2.5 U of *ExTaq* DNA polymerase, adjusted to a total volume of 50 μl. The PCR products were separated by electrophoresis in 1.0% agarose gels and stained with ethidium bromide. The products were excised from the gel and were purified with GenElute agarose spin column (Sigma, St. Louis, MO, USA). PCR fragments were recovered by ethanol precipitation.

2.4. Cloning and sequencing

PCR products were cloned with the TA-Cloning kit (Invitrogen, San Diego, CA, USA) and the transformants were randomly picked up. The recombinant plasmids were then extracted by the alkaline lysis miniprep procedure [15]. Cycle sequencing was performed with a ThermoSequenase kit purchased from Amersham (Tokyo branch, Japan). The sequencing reaction products were read on a M4000L automated DNA sequencer (LI-COR, Lincoln, USA).

2.5. Sequence and secondary structure analyses

All reference sequences were obtained from the GenBank and RDP (Ribosomal Database Project) [16]. Our sequences were analyzed by the CHECK_CHIMERA program [16] in RDP to remove chimeric rDNA clones. Similarity search against database entries was done using online BLAST search [17]. Sequence alignment and phylogenetic analysis was performed with the multiple sequence alignment software CLUSTAL W ver 1.74, [18]. Phylogenetic tree was constructed by the neighbor-joining method [19]. The tree was evaluated using the bootstrap test based on 1000 resamplings [20]. The secondly structure analysis and drawing was done according to [21].

2.6. Nucleotide sequence accession numbers

In the present study, a total of 44 archaeal clones were partially sequenced. Almost complete sequences were obtained for eight of them and these data were submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers AB304182 to AB304189.

3. Results

3.1. Sequence analysis of archaeal 16S rRNA clone libraries

In the first library, which was generated with D30 and D33 primers [13], 21% of clones were closely related to a known methanogen, *M. mobile* (Table 1). The remaining 79% of clones were related to uncultured archaeal sequences from anaerobic digester [22]. The second library of 25 clones, which was generated with primers 0025e Forward and 1492 Reverse [14], produced a higher degree of diversity: in addition to the previous two groups, with the *M. mobile*- (56%) and anaerobic digester archaea-associated sequences (20%), four clones (16%) were identified as *Methanobrevibacter* spp. The remaining two sequences were associated with unidentified archaeal sequences from the rumen (GenBank accession number AF029210) and swine waste [23]. Based on these preliminary sequence similarity data, we sequenced to the completion eight clones from library 2. These eight sequences covered the range of diversity discovered in our two libraries: one *M. mobile*-related sequence cHole9, three sequences (c9566, c61715, and cM16) similar to *Methanobrevibacter* sp., three sequences similar to uncultured archaea from anaerobic digester (cM1, cM2, and cM7), and one sequence resembling an uncultured archaeon from the rumen (c6172).

3.2. Phylogenetic analysis of archaeal 16S rDNAs

The results of phylogenetic placement of these eight sequences are shown in Fig. 1. The clones c9566 and c61715 were related to *Methanobrevibacter ruminantium*. The clone cM6 was also clustered within the *Methanobrevibacter* genus, however, there were no closely related reference sequences from cultivated isolates to define its taxonomic position more precisely. The clone c6172 was closely related with an unidentified archaeal sequence ARC63 from the bovine rumen (GenBank accession number AF029210) but again, with the closest cultivable relatives among the methanogens. The clone cHole9 was identified as *M. mobile*. Three clones, cM1, cM2 and cM7 were very atypical (Fig. 1). These clones clustered with PCR-generated archaeal clones from the anaerobic digester [22]. This group within the unclassified *Euryarchaeota* has very little similarity to any cultivated methanogen and only distantly relates to the thermoacidophilic scavengers, *Thermoplasma acidophilum* and *Picrophilus oshimae* (Fig. 1).

Generally, archaeal primers are less specific and some archaeal libraries may contain a high percentage of bacterial clones [22]. To exclude this possibility, we performed the secondary structure and sequence signature analyses of one of the representatives of this group, clone cM2.

3.3. Secondary structure and sequence signatures of cM2

16S rDNA secondary structure of cM2 was drawn to confirm the validity of the sequence and to determine the sequence position according to the *Escherichia coli* numbering (data not shown). Comparison of sequence signature distinguishing among the Bacteria, Archaea, and Eucarya domains was made as described in the work of Woese [24]. The sequence signatures of cM2 were analyzed in 43 positions and, with a few exceptions, its sequence signature is continuous and corresponds to the Archaea domain (Table 2). From these two analyses, the sequence of cM2 is a truly archaeal and, based on analysis for chimera formation (see Section 2), it is not chimeric.

Table 1
Similarity values of archaeal 16S rDNA sequences retrieved from rumen fluid

Clones	Nearest relative	Similarity	Library	Sequence length (bp)
c0601, c0610	unidentified archaeon clone vadinCA11	92	1	334
c0602, c0603, c0607	unidentified archaeon clone vadinCA11	94	1	337–338
c0608, c0612	unidentified archaeon clone vadinDC79	93	1	338
c0611	unidentified archaeon clone vadinCA11	95	1	336
c0701, c0704, c0705	*M. mobile*	99	1	329–337
c0702, c0703, c0706, c0708, c0709, c0710, c0711	unidentified archaeon clone vadinCA11	90	1	336–400
c0707	*M. mobile*	98	1	337
cHole1, cHole8, cHole9, c61711, c61717, c61720, cMB1, cMB2, cMB3, cMB4	*M. mobile*	99	2	480–1435
cHole4	unidentified archaeon clone vadinDC79	96	2	502
cHole5, cHole7, cMB5, c61713	*M. mobile*	98	2	397–756
cMB6, c9566, c61715	*Methanobrevibacter* sp. NT7	99	2	742–1439
cHole10	unidentified archaeon clone vadinCA11	93	2	817
cM1	unidentified archaeon clone vadinDC79	95	2	1430
cM2	unidentified archaeon clone vadinDC79	85	2	1437
cM6	*Methanobrevibacter* sp. SM9	98	2	1442
cM7	unidentified archaeon clone vadinDC79	94	2	1434
c6172	unidentified methanogen ARC63	96	2	1440
c61710	uncultured archaeon Ar28	93	2	851

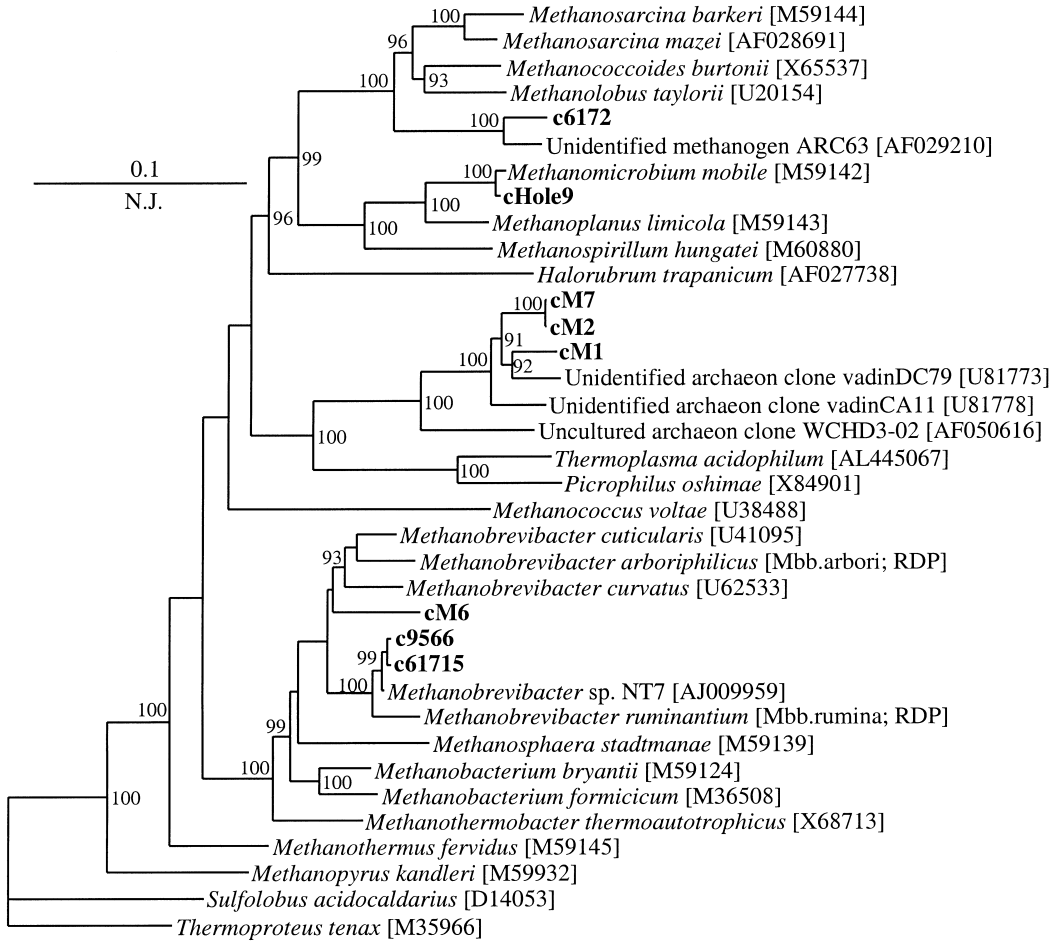

Fig. 1. Phylogenetic placement of archaeal 16S rDNA sequences. The database sequences have the GenBank and RDP accession numbers in brackets; our sequences are shown in bold. The numbers around the nodes are the confidence levels (%) generated from 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position.

4. Discussion

Cultivation-based studies of rumen archaea yielded five species, *Methanobacterium formicicum*, *M. ruminantium*, *Methanosarcina barkeri*, *Methanosarcina mazei*, and *M. mobile*, all of them methanogens [5,6,25]. This places the rumen archaea into essentially one functional group, which converts hydrogen produced by fermentative bacteria, fungi, and protozoa into methane. Unlike the 16S rRNA diversity of rumen bacteria, which has been studied by PCR retrieval and sequence analysis, the corresponding archaeal libraries have produced a limited range of molecular diversity [11,12]. These sequences were similar to the already cultivated rumen archaea and belonged to the known methanogens from the *Methanomicrobium* and *Methanobrevibacter* genera. 16S rRNA sequences of *Archaea* offer restricted opportunities for designing universal archaeal primers and the biases in archaeal libraries are evident such as cross-amplification and heavy contamination by bacterial sequences [22]. Perhaps the primers used for construction of the rumen archaeal libraries in two previous works [11,12] produced sequences biased toward the methanogenic populations, thus masking the real diversity. For construction of our libraries, we used two different sets of archaeal primers and they also exhibited different composition. However, the second library certainly displayed a broader range of diversity in comparison with the first library and the two libraries described earlier.

In addition to the known four clusters of methanogens, our libraries also uncovered a novel group of rumen archaeal sequences, which are rather atypical for this system. Because of this, we performed several additional verifications of these sequences to confirm that they are not the artefacts resulting from the PCR-based rRNA analysis [26] or contaminants coming with the animal feed. This group, which is exemplified by almost complete sequences of clones cM1, cM2, and cM7 (Fig. 1), was clustered with the uncultured clones from an anaerobic digester (vadinDC79 and vadinCA11) [22] and an aquifer contaminated with hydrocarbons and chlorinated solvents (clone WCHD3-02) [27]. This group has no closely related cultivable isolates and only distantly relates to the thermoacidophilic archaea *T. acidophilum* and *P. oshimae* [28,29].

Table 2
Comparison of domain-specific sequence signatures[a]

Position[b]	Bac[c]	Archaea	Eucar[d]	cM2	Position	Bac	Archaea	Eucar	cM2
9	G	C	C	C	756	C	G	A	G
10	A	Y	U	U	912	C	U	U	U
24	U	R	A	A	923	A	G	A	G
25	C	G	G	G	930	C	A	G	A
33	A	Y	A	C	931	C	G	G	G
53	R	C	C	A	952	U	C	C	C
113	G	C	C	C	962	C	G	U	G
338	A	G	A	G	966	G	U	U	U
339	C	G	C	G	973	G	C	G	C
350	G	Y	G	C	975	A	G	G	G
358	Y	G	G	U	1060	U	C	C	C
361	R	C	C	C	1086	U	C	Y	U
367	U	C	U	U	1087	G	C	U	C
377	G	C	Y	G	1109	C	A	A	A
386	C	G	R	C	1110	A	G	G	G
393	A	G	A	G	1197	A	G	G	G
403	C	A	A	A	1229	A	G	G	G
508	U	C	A	A	1381	U	C	C	C
523	A	C	A	C	1386	G	C	C	C
551	U	R	U	G	1387	G	U	C	U
585	G	C	U	C	1393	U	C	U	C
716	A	C	Y	C					

[a]Based on Woese [24]; Y, pyrimidine; R, purine.
[b]*E. coli* numbering.
[c]Bac, Bacteria.
[d]Eucar, Eucarya.

Recently, however, the archaeal sequences clustering within the order *Thermoplasma* have been amplified from the hindgut of the lower termite, *Reticulitermes speratus* [30]. Because of the differences in the regions determined we were not able to incorporate these sequences in our phylogenetic analysis. Nevertheless, the ruminal and termite *Thermoplasma* sequences demonstrated close relatedness within the common 498-bp region (94% similarity). Together with our data, this suggests that certain archaea within the order *Thermoplasma* may be neutrophilic and mesophilic while the typical representatives for which the metabolic information is available, *T. acidophilum* and *P. oshimae*, are the thermoacidophilic scavengers utilizing products of decomposition of other organisms [29,31]. Intuitively, with a very high microbial biomass turnover rate, the rumen may offer plenty of opportunities for scavengers to thrive. In the absence of cultivated isolates, however, the metabolic function of this group in the rumen and termite gut remains unclear. There might be also the possibility that these atypical sequences were from the transient microbiota contaminating the animal feed. To avoid this, we allowed the 18-h fasting period before sampling. Because of a very high turnover rate in the rumen, it is unlikely that such contaminating microbiota would reach the levels comparable, for example, with the methanogenic archaea *M. mobile* and *Methanobrevibacter* sp. Second, the most probable source of archaeal sequences, which could contaminate the feed, may originate from soil (especially the plant component of diet). We tested our sequences against the available archaeal libraries from soil [32] but did not detect any close similarity with these sequences. Therefore, we conclude that these archaea, which are not affiliated with the known methanogens or animal feed, perhaps may secure a scavenging ecological niche in the rumen.

Acknowledgements

The Rumen Microbiology Research Team was supported by grants from the National Institute of Animal Industry, Ministry for Agriculture, Forestry and Fisheries.

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