

# Methanogenic Archaeal Census of Ruminal Microbiomes

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**Abstract** The objective of the study was to undertake a phylogenetic diversity census of ruminal archaea based on a meta-analysis of 16S rRNA gene sequences that were publicly available in the Ribosomal Database Project. A total of 8,416 sequences were retrieved from the Ribosomal Database Project (release 11, update 5) and included in the construction of a taxonomy tree. Species-level operational taxonomic units (OTUs) were analyzed at a 97% sequence similarity by using the QIIME program. Of the 8,416 sequences, 8,412 were classified into one of three phyla; however, the remaining four sequences could not be classified into a known phylum. The Euryarchaeota phylum was predominant and accounted for 99.8% of the archaeal sequences examined. Among the Euryarchaeota, 65.4% were assigned to *Methanobrevibacter*, followed by *Methanosphaera* (10.4%), *Methanomassillicoccus* (10.4%), *Methanomicrobium* (7.9%), *Methanobacterium* (1.9%), *Methanimicrococcus* (0.5%), *Methanosarcina* (0.1%), and *Methanoculleus* (0.1%). The 7,544 sequences that had been trimmed to the V2 and V3 regions clustered into 493 OTUs. Only 17 of those 493 OTUs were dominant groups and accounted for more than 1% of the 7,544 sequences. These results can help guide future research into the dominant ruminal methanogens that significantly contribute to methane emissions from ruminants, research that may lead to the development of anti-methanogenic compounds that inhibit these methanogens regardless of diet or animal species.

**요약** 본 연구의 목적은 Ribosomal Database Project에서 공적으로 활용 가능한 16S rRNA 유전자 시퀀스들의 메타분석을 통해 반추위 고세균의 계통발생 다양성을 조사하는 것이다. 총 8,416개의 시퀀스가 Ribosomal Database Project(출시버전 11, 업데이트 5)로부터 회수되었고, taxonomy tree를 구축하는데 사용되었다. Species 수준의 OTUs가 97% sequence 유사성 기준으로 QIIME 프로그램을 사용하여 분석되었다. 총 8,416개의 시퀀스 중에서 8,412개의 시퀀스는 총 3개의 문으로 분류되었고, 나머지 4개의 시퀀스는 어떤 알려진 문으로 분류되지 못했다. *Euryarchaeota*는 가장 우점하는 문으로, 전체 고세균 시퀀스의 99.8%를 차지하였다. 이 중에서 차례로 *Methanobrevibacter*가 65.4%, *Methanosphaera*가 10.4%, *Methanomassillicoccus*가 10.4%, *Methanomicrobium*가 7.9%, *Methanobacterium*가 1.9%, *Methanimicrococcus*가 0.5%, *Methanosarcina*가 0.1%, *Methanoculleus*가 0.1%를 차지하였다. V2와 V3 영역으로 자른 7,544개의 시퀀스는 493개의 OTUs로 분류되었다. 총 493 OTUs 중에서 단지 17개만 우점하였고, 총 7,544 시퀀스 중 1% 이상을 차지하였다. 본 연구는 반추동물로부터 메탄발생에 크게 기여하는 반추위 우점 메탄생성균 분석에 대한 향후 연구를 인도하는데 도움을 주고, 사료나 가축 품종이 달라져도 이러한 메탄생성균을 억제하는 메탄저감제를 개발하는데 도움을 줄 것이다.

**Keywords** : 16S rRNA Gene, Meta-Analysis, Phylogenetic Diversity, Ribosomal Database Project, Ruminal Microbiome, Methanogenic Archaea

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## 1. Introduction

Ruminal archaea are mostly composed of methane-producing methanogens that contribute potent greenhouse gases. Ruminal methanogens belong to the phylum *Euryarchaeota*, where *Methanobrevibacter* is the most dominant genus and accounts for about 50% of the total ruminal archaeal sequences [1,2]. Although considerable effort has been devoted to isolating ruminal methanogens, only seven methanogen species have been cultured due to the limited number of isolation techniques [3,4].

Since an archaeal 16S rRNA gene was first used as a phylogenetic marker, ruminal microbial communities have been investigated using various culture-independent methods, such as the construction of clone libraries, denaturing gradient gel electrophoresis (DGGE), quantitative real-time polymerase chain reaction (PCR), phylogenetic microarrays, and next-generation sequencing [5-10]. Small 16S rRNA gene sequence libraries constructed using Sanger sequencing technology have been catalogued for the Ribosomal Database Project (RDP). Kim et al. [1] examined ruminal archaeal census data using a meta-analysis of ruminal 16S rRNA gene sequences deposited in the RDP database. However, this study used only 3,516 sequences of rumen origin with outdated versions of their taxonomic classifiers.

The community composition of ruminal methanogens is influenced by different breeds, ages, diets, geographic regions, and seasons [1]. Therefore, ruminal methanogenic taxa contributing to greenhouse gas emissions varies with the factors described above. This means that anti-methanogenic compounds developed in one study can exhibit inconsistent efficacy in other studies conducted in different breeds, ages, diets, geographic regions, and seasons.

Because individual studies have mostly focused on specific methanogenic archaeal ecosystems in

the rumen, the research conducted to date may bias our knowledge of the structure of ruminal methanogenic archaea. Therefore, a collective view of ruminal archaeal communities is necessary to reduce this bias and understand dominant ruminal methanogenic taxa irrespective of different breeds, ages, diets, geographic regions, and seasons. In our study, we performed a meta-analysis to construct a collective view of the structure of ruminal methanogenic archaea using the updated 16S rRNA gene libraries with a new taxonomic classification system.

## 2. Materials and Methods

All 16S rRNA gene sequences from the ruminal archaea were retrieved from the RDP release 11 (Update 5) as described previously [11,12]. Only high-quality sequences were selected using the 'Quality' option in the RDP database. Both 'rumen' and 'ruminal' were used as search terms. In addition, archaeal 16S rRNA gene sequences of rumen origin were manually added from American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and Japan Collection of Microorganisms (JCM), as described previously [1]. Sequences that did not originate from ruminal archaea were manually excluded. We constructed taxonomic trees with Bergey's taxonomy using the ARB program [13], as described previously [1].

The retrieved archaeal sequences were trimmed to the V2 and V3 regions to calculate operational taxonomic units (OTUs) at 97% sequence similarity using the QIIME software package 1.9.0 [14]. Sequences including the V2 and V3 regions were selected for OTU clustering. From a rarefaction curve, the asymptote that indicates the maximum number of OTUs was predicted using the nonlinear model procedure

of SAS (V9.2, SAS Institute Inc., Cary, NC) as described previously [1].

### 3. Results and Discussion

#### 3.1 Data summary

We retrieved a total of 8,416 archaeal 16S rRNA gene sequences of rumen origin from the RDP (Release 11, Update 5). These 8,416 sequences were obtained from 103 studies, including 53 unpublished studies based on Sanger sequencing technology, where 8,412 sequences were classified to three phyla (Fig. 1). The remaining four sequences could not be classified into a known phylum and was assigned to unclassified Archaea. *Euryarchaeota* was the first predominant phylum and accounted for 8,402 of the 8,412 sequences, whereas phyla *Crenarchaeota* and *Thaumarchaeota* were represented by only one and 9 sequences, respectively.

The trimmed V2 and V3 regions were obtained from 7,544 of the 8,402 *Euryarchaeota* sequences. These 7,544 sequences were clustered into 493 OTUs, where 17 OTUs (OTUs #1-17) were dominant groups and accounted for approximately 1% of the 7,544 sequences (Table 1). Species corresponding to these 17 OTUs may be predominant ruminal methanogens, irrespective of factors affecting ruminal archaeal diversity. Development of anti-methanogenic compounds inhibiting these 17 putative methanogen species may greatly contribute to mitigating methane emissions from ruminant animals irrespective of different breeds, ages, diets, geographic regions, and seasons. Of the 493 OTUs, 370 were represented by only one sequence, which suggests that these species may be minor methanogens and not commonly found in the rumen. Since these 370 putative methanogen species are not dominant, development of anti-methanogenic compounds for these methanogens may not be effective to reduce methane emissions from ruminant animals.

#### 3.2 Methanogenic archaeal taxonomy

Ruminal methanogens consisted of 12 known genera and were represented by 96.8% of all the 8,416 archaeal sequences (Fig. 1). *Methanobrevibacter* and *Methanosphaera* were the first and the second dominant genera represented by 5,505 and 877 sequences (65.4% and 10.4% of all sequences), respectively. *Methanomassilicoccus* (873 sequences) belonging to order *Thermoplasmatales* was represented by 873 sequences (10.4% of all sequences), followed by *Methanomicrobium* (666 sequences), *Methanobacterium* (163 sequences), *Methanimicrococcus* (42 sequences), *Methanosarcina* (11 sequences), *Methanoculleus* (five sequences), *Methanotherix* (one sequence), *Methanolinea* (one sequence), *Methanopyrus* (one sequence) and *Methanocorpusculum* (one sequence). Because *Methanotherix*, *Methanolinea*, *Methanopyrus* and *Methanocorpusculum* were represented by only one sequence, these four methanogen genera may not be residents in the rumen. Since *Methanobrevibacter* is the most abundant genus irrespective of different breeds, ages, diets, geographic regions and seasons, more efforts are needed to elucidate the mechanisms involved in methanogenesis by *Methanobrevibacter* species and develop novel anti-methanogenic compounds showing lasting efficacy in future studies.

We were unable to assign a total of 256 sequences to a known genus (Fig. 1). The most dominant group was unclassified *Methanobacteriaceae* (224 sequences); it was followed by unclassified *Euryarchaeota* (28 sequences) and unclassified *Thermoplasmatales* (4 sequences). These unclassified taxa also may greatly contribute to methane emissions from ruminant animals and will need to be evaluated if these unclassified taxa can be used as target rumen methanogens for methane mitigation in future studies.

### 3.3 Cultured methanogens

Janssen and Kirs [3] reported that the following seven methanogens have been isolated from the rumen: *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanomicrobium mobile*, and *Methanoculleus olentangyi*. *Methanosarcina barkeri* has also been isolated from the rumen [15]. Recently, *Methanobrevibacter boviskoreani* has been isolated from the rumen of native Korean cattle [16]. In addition, several unpublished studies in the RDP database indicate that *Methanobacterium beijingense*, *Methanoculleus marisnigri*, *Methanoculleus bourgensis*, and *Methanosarcina mazei* have been isolated from the rumen of goats, Indian crossbred cattle, Holstein cattle, and Korean Hanwoo cattle, respectively. *Thermoplasmatales archaeon* BRNA1 was an isolate assigned to unclassified *Euryarchaeota* and has been described as a pyrrolysine-dependent methanogen (unpublished study). However, the RDP database showed that sequences recovered from cultured methanogens accounted for only 0.7% of the total ruminal archaeal sequences.

Since the present meta-analysis showed *Methanobrevibacter*, *Methanosphaera* and *Methanomassillicoccus* were dominant genera, more efforts are required to isolate and characterize novel methanogen species belonging to these three genera. This will help better understand the mechanisms involved in methanogenesis by these three genera and develop effective strategies to mitigate methane emissions from ruminant animals.

### 3.4 Methanogenic archaeal diversity

Of the 493 OTUs, 17 were dominant groups, accounting for more than 1% of the trimmed 7,544 sequences (Table 1). Similarly, of the 493

OTUs, 18 were represented by at least one sequence recovered from methanogen isolates, whereas six OTUs were dominant groups, accounting for more than 0.7% of the total sequences.

The first dominant genus, *Methanobrevibacter*, was represented by nine (OTU #1-3, 6, 7, 11, 14-16) of the 17 dominant OTU groups (Table 1). OTU #1 was represented by 1,514 of the 7,544 sequences and included an isolate sequence recovered from *Methanobrevibacter ruminantium*. OTU #3 and #11 were represented by 992 and 123 sequences that included *Methanobrevibacter millerae* and *Methanobrevibacter boviskoreani* isolate sequences, respectively. These three OTUs were represented by sequences recovered from the rumens of various ruminant animals such as cattle [17-21], goats [22], sheep [23,24], sika deer [25], reindeer [26,27], and yak [28]. It seems that *M. ruminantium*, *M. millerae*, and *M. boviskoreani* are dominant methanogen species that are responsible for substantial methane emission irrespective of diet and animal species. OTU #2 and #6 were represented by 1,462 and 217 sequences, respectively, and included *Methanobrevibacter* isolate sequences that were unnamed at the species level. Because these two OTUs were represented by sequences recovered from various ruminal animals, as described in the studies listed above, *Methanobrevibacter* species corresponding to these two OTUs may be commonly found in the rumen, irrespective of diet and animal species. The remaining four OTUs (OTU #7 and #14-16) were dominant groups but did not include any sequences recovered from *Methanobrevibacter* isolates. Methanogen species corresponding to these four OTUs will need to be isolated and characterized in future studies.

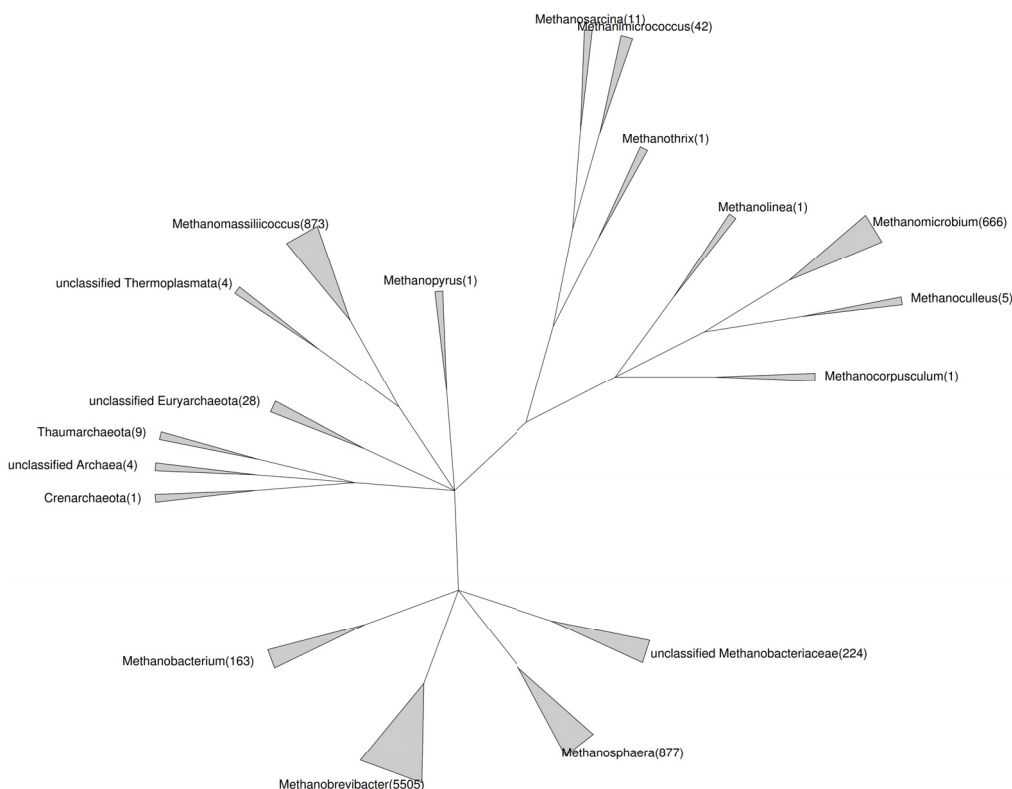


Fig. 1. A taxonomic tree showing rumen archaea. A total of 8,416 sequences of ruminal archaea were retrieved from the RDP Release 11 (Update 5). Information on the number of sequences was indicated in parentheses.

The second dominant genus, *Methanosphaera*, was represented by two (OTU #4, 8) of the 17 dominant OTU groups but had no sequence recovered from *Methanosphaera* isolates. OTU #4 was represented by sequences recovered from the rumen of cattle [18-21], muskoxen (unpublished study), sheep [29,30], goats [31], and buffalo (unpublished study), whereas OTU #8 was represented by sequences recovered from the rumen of cattle [20], sika deer [25], reindeer [26], sheep [23], goats (unpublished study), and muskoxen (unpublished study). *Methanosphaera* species corresponding to these two species may contribute large quantities of methane emissions, irrespective of diet and animal species, and will need to be isolated and characterized to develop

novel anti-methanogenic compounds in future studies.

The third dominant genus, *Methanomicrobium*, was represented by one (OTU #5) of the 17 dominant OTU groups and included an isolate sequence recovered from *Methanomicrobium mobile*. OTU #5 was represented by sequences recovered from the rumen of cattle [21, 28, 32, 33], reindeer [26], yak [28], and muskoxen (unpublished study). Notably, *M. mobile* may also greatly contribute to ruminal methane emissions. *Methanobacterium* was represented by one (OTU #9) of the 17 dominant OTU groups but included no isolate sequence recovered from *Methanobacterium*.

Table 1. OTU groups of ruminal archaea

OTU ID	Taxonomy	No. of sequences	No. of isolate sequences
1	<i>Methanobrevibacter</i>	1,514	14
2	<i>Methanobrevibacter</i>	1,462	7
3	<i>Methanobrevibacter</i>	992	2
4	<i>Methanosphaera</i>	406	
5	<i>Methanomicrobium</i>	378	1
6	<i>Methanobrevibacter</i>	217	1
7	<i>Methanobrevibacter</i>	210	
8	<i>Methanosphaera</i>	176	
9	<i>Methanobacterium</i>	161	
10	<i>Methanomassillicoccus</i>	148	
11	<i>Methanobrevibacter</i>	123	5
12	<i>Methanomassillicoccus</i>	102	
13	<i>Methanomassillicoccus</i>	93	
14	<i>Methanobrevibacter</i>	93	
15	<i>Methanobrevibacter</i>	90	
16	<i>Methanobrevibacter</i>	83	
17	<i>Methanomassillicoccus</i>	78	
22	<i>Methanobrevibacter</i>	47	1
63	<i>Methanosarcina</i>	4	4
73	<i>Methanobacterium</i>	3	3
75	<i>Methanoculleus</i>	3	2
113	<i>Methanobrevibacter</i>	2	1
124	<i>Methanobacterium</i>	1	1
129	<i>Methanobacterium</i>	1	1
132	<i>Methanosarcina</i>	1	1
135	<i>Methanoculleus</i>	1	1
139	<i>Methanoculleus</i>	1	1
375	<i>Methanomassillicoccus</i>	1	1
409	<i>Methanobacterium</i>	1	1

The remaining four dominant OTUs (OTU #10, 12, 13, and 17) were assigned to the genus *Methanomassillicoccus* but were not represented by any isolate sequences. These OTUs were also represented by sequences recovered from the rumen of various animal species, indicating that species corresponding to these four OTUs may play an important role in ruminal methane emissions, irrespective of diet and animal species. Additional efforts to isolate and characterize species corresponding to these four OTUs are necessary.

Development of novel anti-methanogenic compounds that inhibit methanogen species belonging to these dominant OTUs may greatly contribute to reducing methane emissions from ruminant animals irrespective of diet and animal species.

Of the total 493 OTUs, 12 were not dominant OTU groups but included a sequence (or sequences) recovered from methanogen isolates. These 12 OTUs were assigned to *Methanobrevibacter* (OTU #22, 113), *Methanobacterium* (OTU #73, 124, 129, 409), *Methanoculleus* (OTU #75, 135, 139), *Methanosarcina* (OTU #63, 132), and *Methanomassillicoccus* (OTU #375).

OTU #113 was represented by one *Methanobrevibacter ruminantium* sequence, indicating that this *M. ruminantium* strain is phylogenetically different from a *M. ruminantium* strain corresponding to the first dominant OTU #1 and may play a minor role in ruminal methane emissions. OTU #73, #124, and #129 were represented by a sequence recovered from *Methanobacterium formicicum*, *Methanobacterium bryantii*, and *Methanobacterium beijingense*, respectively. It seems that these three *Methanobacterium* species play a minor role in ruminal methane emission. OTU #75 and #139 were represented by a sequence recovered from *Methanoculleus bourgensis* and *Methanoculleus marisnigri*, whereas OTU #63 and #132 were represented by a sequence recovered from *Methanosarcina barkeri* and *Methanosarcina mazei*. These four species may also play a minor role in ruminal methane emission and may not be effective as target methanogens for methane mitigation.

The maximum number of OTUs estimated from the rarefaction curve was 957, indicating that 52% of ruminal archaeal diversity has been sampled. Therefore, novel methanogenic archaea will need to be identified in future studies.

#### 4. Conclusion

In conclusion, our study has provided an overview of methanogenic archaea using a meta-analysis of individual studies using Sanger sequencing technology. A collective view of

ruminal archaeal communities using a meta-analysis has helped to reduce biased knowledge concerning the structure of ruminal methanogenic archaea. The information on dominant methanogenic taxa with reduced bias may contribute to developing anti-methanogenic compounds exhibiting consistent efficacy irrespective of different breeds, ages, diets, geographic regions, and seasons. Only 0.7% of the total sequences were recovered from cultured methanogens, indicating that additional efforts to isolate new ruminal methanogens are warranted to achieve sustainable methane reduction from ruminant animals.

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