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Methanogen diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis

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ABSTRACT

The methanogenic communities in buffalo rumen were characterized using a culture-independent approach of a pooled sample of rumen fluid from three adult Surti buffaloes. Buffalo rumen is likely to include species of various methanogens, so 16S rDNA sequences were amplified and cloned from the sample. A total of 171 clones were sequenced to examine 16S rDNA sequence similarity. About 52.63% sequences (90 clones) had ⩾90% similarity, whereas, 46.78% of the sequences (81 clones) were 75–89% similar to 16S rDNA database sequences, respectively. Phylogenetic analyses were also used to infer the makeup of methanogenic communities in the rumen of Surti buffalo. As a result, we distinguished 23 operational taxonomic units (OTUs) based on unique 16S rDNA sequences: 12 OTUs (52.17%) affiliated to Methanomicrobiales order, 10 OTUs (43.47%) of the order Methanobacteriales and one OTU (4.34%) of *Methanosarcina barkeri* like clone, respectively. In addition, the population of Methanomicrobiales and Methabacteriales orders were also observed, accounting 4% and 2.17% of total archea. This study has revealed the largest assortment of hydrogenotrophic methanogens phylotypes ever identified from rumen of Surti buffaloes.

1. Introduction

Methanogens are members of the domain Archaea, and fall within the kingdom Euryarchaeota (Woese et al., 1990) and are integral to carbon cycling, catalyzing the production of methane and carbon dioxide, both potent greenhouse gases, during organic matter degradation in anaerobic soils and sediment (Chin et al., 2004). Methanogens are widespread in anaerobic environments, including tundra (Raskin et al., 1994), freshwater lake and wetland sediments (Castro et al., 2004; Earl et al., 2003), estuarine and marine sediments (Banning et al., 2005), acidic peatlands (Basiliko et al., 2003; Galand et al., 2002), rice field soil (Chin et al., 2004), animal guts (Rastogi et al., 2008), landfills (Luton et al., 2002), and anaerobic digesters treating animal manure (Angenent et al., 2002; Soliva et al., 2004), food processing wastewater (Liu et al., 2002), and municipal wastewater and solid waste (Zheng and Raskin, 2000). Interest in methanogens from ruminants has resulted from the role of methane in global warming and from the fact that enteric methane emission is a major source of greenhouse gas in agriculture (US Energy Information Administration). Currently, India possesses the world's largest livestock population of 485 million, which accounts for 13% of the global livestock population (Intergovernmental Panel on Climate Change 2001). It has 57% of the world's buffalo and 16% of the cattle population. Contribution of methane emission in India by buffalo is 42% (Chhabra et al., 2009). Reducing enteric methane emissions has been identified as one way of lowering global methane emissions. However, the effectiveness of any strategy that will reduce greenhouse gas emissions and also increase production or nutritional efficiency will likely depend upon having an understanding of the numbers and/or distribution of methanogen species among ruminant livestock. Several species of methanogens have been isolated from ruminants, but few have been consistently found in high numbers (Stewart et al., 1997) and it is likely that major species of rumen methanogens are yet to be identified (Rocheleau et al., 1999; Wright et al., 2004). The most common species of methanogens isolated from the rumen are strains of *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina* (Wright et al., 2004; Jarvis et al., 2000). Methanogens are difficult to study through culture-based methods, and therefore many researchers have instead used culture-independent techniques to study methanogen populations. The 16S rRNA gene is the most widely used target for gene surveys, and a number of primers and probes have been developed to target methanogen groups (Purdy et al., 2003;

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Shin et al., 2004; Tatsuoka et al., 2004; Saengkerdsub et al., 2007). Methanogens are frequently found in association with protozoa (Tajima et al., 2001; Sizova et al., 2003). To date, relatively little is known of the dominant methanogens in ruminants, particularly Surti buffaloes in western India. This paper uses comparative sequence analysis of cloned 16S rRNA genes (rDNA) amplified from total DNA extracted from rumen fluid to analyse the dominant methanogens present in the rumen of Surti buffalo.

2. Materials and methods

2.1. Sampling and DNA extraction

The experiments were carried out on three adult Surti buffaloes, approximately three years of age and with a mean live weight of 201 ± 18 kg, which were reared at the Department of Animal Nutrition, College of Veterinary Science and A.H., Anand. The permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. All the animals were maintained under uniform feeding regime (Indian Council of Agricultural Research, 1998) for minimum 21 days. The diet comprised of green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*) and compound concentrate mixture (20% CP, 65% TDN). The animals were offered 10 kg green, ad-lib dry grass and 2.5 kg of concentrate mixture daily. Approximately 500 ml of rumen fluid was collected *via* a stomach tube located in the mid part of the rumen and connected to a vacuum pump at 0, 2, 4 and 6 h post feeding (Yuangklang et al., 2005; Khampa et al., 2006). About 100 ml rumen fluid was passed through four layers of cheese cloth to remove particulate matter. Remaining rumen fluid was stored at −80 °C for further study. Total DNA (0, 2, 4 and 6 h × 3 animals) was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA) and finally pooled the all DNA extracts. The total DNA mixture (pooled) was used as a template in PCR to amplify 16S rDNA.

2.2. PCR primers and amplification

The primers used were 1Af (5′-TCYGKTTGATCCYGSCRGAG-3′) and 1100Ar (5′-TGGGTCTCGCTCGTTG-3′) (Embley et al., 1992). Subsequently 16S rDNA were amplified (1100 bp) by PCR using the metagenomic DNA and Master mix (Fermentas, UK). A total of 25 μl of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, 12.5 μl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) and PCR conditions were adjusted in laboratory. The anticipated product of approximately 1.1 kb was was purified using Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the manufacturer's instructions.

2.3. Cloning and sequencing

The purified PCR products were cloned in pTZ57R T/A vector (Fermentas, UK) as per the instructions of the manufacturer and transformed into *Escherichia coli* DH-5α competent cell. Ampicillin- and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)-amended LB agar was used for blue–white screening of transformants. The recombinant plasmids then were extracted by the Qiagen mini-prep plasmids extraction kit (QIAGEN, CA). Plasmid inserts were amplified with M13 universal primer [F-5′-GTAAAACGACGGCCAG-3′ and R-5′-CAGGAAACAGCTATGAC-3′] and nucleotide sequences of cloned fragments were determined by sequencing with M13F/M13R primer in ABI Prism 310 Genetic analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) at Animal Biotechnology laboratory, AAU, Anand, Gujarat, India.

2.4. Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the Genbank/EMBL/DDBJ/RDP (Benson et al., 2007). Sequences (∼500 bp) from the current study were trimmed (remove low-quality base calls from the start and end of DNA sequence) manually and analyzed by the CHECK_CHIMERA program (Maidak et al., 2001) to remove any chimeric rDNA clone. The similarity searches for sequences were carried out by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) (Madden et al., 1996) and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Thompson et al., 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (Felsenstein, 1985) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (Schloss and Handelsman, 2005) with 95% confidence intervals to quantitate the diversity of phylotypes and total of 23 OTUs were generated based on furthest-neighbor algorithm at cut offs of 10% difference. Phylogenetic tree was constructed by the neighbor joining method using MEGA 4.0 (Tamura et al., 2007). The evolutionary distance was computed using the maximum composite likelihood method (Tamura et al., 2004). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (Felsenstein, 1985). The prefix meth was used to denote identified OTU and nucleotide sequences have been deposited in the Genbank database under the accession numbers are depicted in Fig. 1.

2.5. Real time-PCR analysis

Plasmid DNA containing the total archea, Methanomicrobiales, and Methanobacteriales gene sequence, used as the standard DNA in real-time PCR, was obtained by PCR cloning with the specific primer sets (Yu et al., 2004). After the confirmation of a single band of the correct size with respective pair of primers on an agarose gel, the PCR products were excised from the gel. The PCR products were purified using the Qiagen gel Purification Kit (Qiagen, CA), and then ligated into pTZR57 T/A cloning vector (Fermetas, UK). The ligated products were transformed to competent *E. coli* DH5α cells by heat shock. Plasmids were purified from positive clones using a QIAprep spin miniprep kit (Qiagen, USA), and the plasmids containing the correct insert were screened out by PCR amplification with respective primer sets. The concentration of the plasmid was determined with a Nanodrop spectrophotometer and copy number of each standard plasmid was calculated using formula: Copy No./μl = Concentration of plasmids (gm/μl) × 6.022×10^{23}/length of recombinant plasmid (bp) × 660, (660 = Molecular weight of nucleotide base, 6.022×10^{23} = Avogadro's number). Tenfold dilution series ranging from 10^9 to 10 copies were prepared for each target. Real-time PCR was performed with ABI system (ABI 7500). The Qiagen 2X SYBR Green master mix (2X) was used for PCR reaction. The optimal amplification conditions for each primer set were obtained with 10 pmol each primer with the combination of annealing temperature and extension time as described by Yu et al. (2004). The tenfold dilution series of the standard plasmid for the respective target was run along with the corresponding samples in duplicate. The copy numbers of 16S rRNA genes of targeted methanogens per ml rumen fluid were calculated using the following equation: $(QM \times C \times DV)/(S \times V)$, where QM was the quantitative mean of

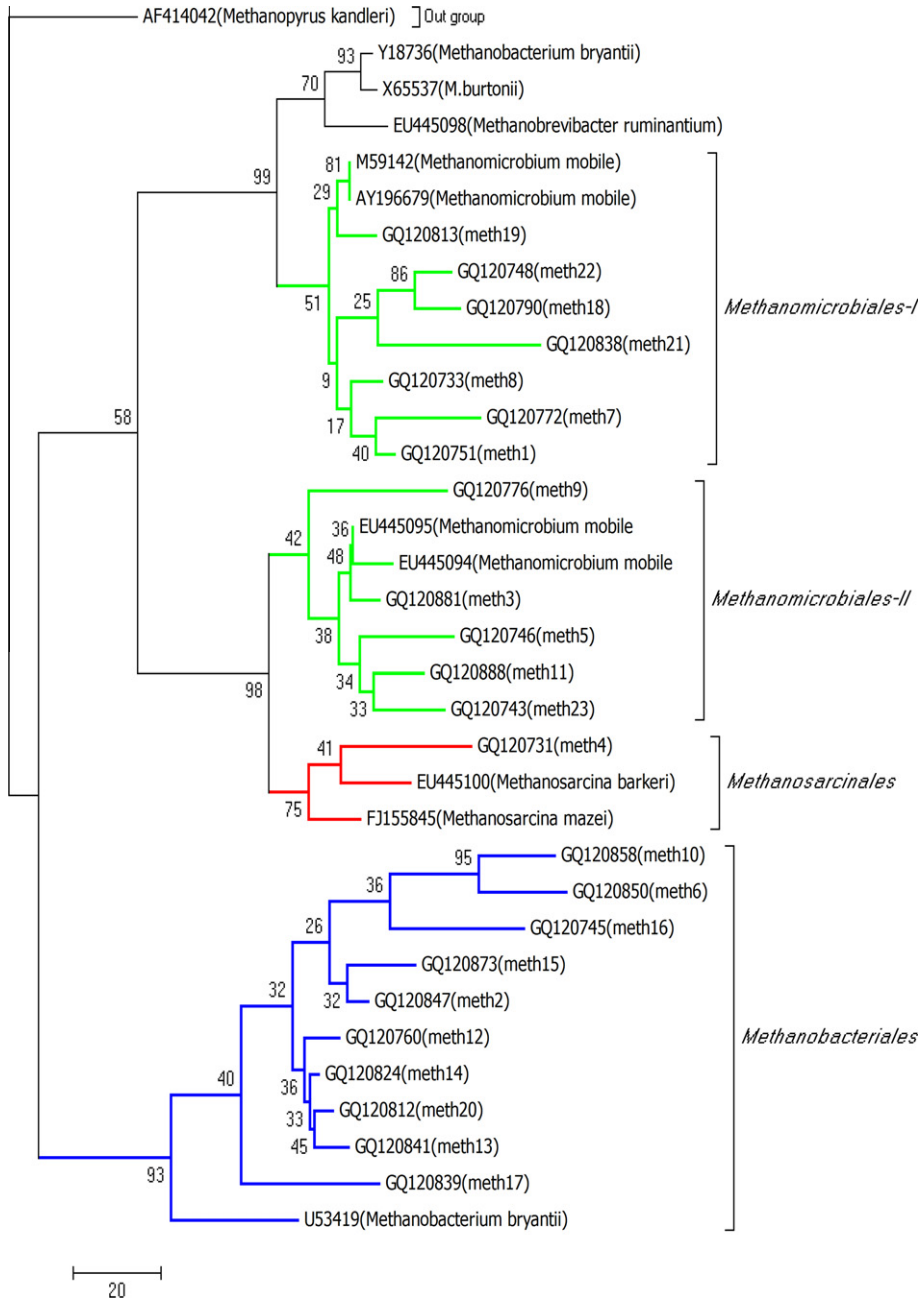

Fig. 1. Phylogenetic relationships of partial 16S rDNA sequences of clones recovered from Surti rumen samples. The rooted tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 1000 trials, using the MEGA 4 tree building program. All positions containing gaps and missing data were eliminated from the data set.The *Methanopyrus kanderi* (AF414042) are used as the out-group for rooting the tree. The scale bar represents the number of changes over the whole sequence.

the copy number *C* was the DNA concentration of each sample, DV was dilution volume of extracted DNA, *S* was the DNA amount (ng) subjected to analysis and *V* was rumen fluid volume subjected to DNA extraction. In the reaction, nearly perfect linear regressions (r^2 = 0.9930–0.9995 and slope (−3.2 to −4) were obtained between threshold cycle and quantities of standard for all targets and data generated from the reaction were used for further analysis.

3. Results

One hundred seventy one 16S rDNA sequences were analyzed and on the basis of sequence similarity, all of the sequences were related to methanogens. In our library, about 52.63% sequences (90 clones) had >90% similarity to the 16S rDNA database sequences. Furthermore, about 46.78% of the sequences (81 clones) were 75–89% similar to 16S rDNA database sequences (Table 1). The sample preparation technique, centrifugation before DNA extraction, allowed us to preferentially examine methanogens isolated from the fluid fraction of rumen contents. Two distinct clusters were generated by Maximum Parsimony method analysis of sequences (Fig. 1). The first largest cluster contained 12 OTUs (52.17% of total OTUs; 83 clones) grouped with order Methanomicrobials forming two distinct subclusters that were supported by high bootstrap values (Fig. 1). Subcluster Methanomicrobials I consisted of 51 clones (7 OTUs) identical or nearly identical sequences

Table 1
Similarity values of operational taxonomic units (OTUs) based on 16S rDNA sequences of 171 clones retrieved from the rumen fluid of Indian Surti buffalo.

OTU	No. of clone	Nearest valid relative	Accession no.	Similarity (%)
Meth1	37	*Methanomicrobium mobile*	M59142	94
Meth 2	29	*Methanomicrobium mobile*	AY196679	88
Meth 3	24	*Methanomicrobium* sp.	X99139	90
Meth 4	5	Uncultured *Methanobrevibacter* sp.	FJ938102	89
Meth 5	1	Uncultured methanogenic archaeon	EU794805	76
Meth 6	3	Uncultured Methanobacteriales archaeon	DQ402018	93
Meth 7	4	*Methanomicrobium mobile*	AY196679	85
Meth 8	2	*Methanomicrobium mobile*	AY196679	89
Meth 9	2	*Methanomicrobium mobile*	AY196679	86
Meth 10	1	Uncultured Methanobacteriales archaeon	DQ402018	75
Meth 11	3	*Methanomicrobium mobile*	AY196679	86
Meth 12	22	Uncultured rumen archaeon	FJ586387	88
Meth 13	1	*Methanomicrobium mobile*	AY196679	91
Meth 14	21	Uncultured archaeon clone	EU487521	96
Meth 15	2	*Methanomicrobium mobile*	AY196679	87
Meth 16	2	*Methanomicrobium mobile*	AY196679	90
Meth 17	1	N/A	–	–
Meth 18	4	*Methanomicrobium mobile*	AY196679	85
Meth 19	1	Uncultured compost archaeon	DQ365171	91
Meth 20	1	Uncultured archaeon clone	EU487521	90
Meth 21	1	Uncultured rumen archaeon	FJ586416	84
Meth 22	2	*Methanomicrobium mobile*	AY196679	86
Meth 23	2	*Methanomicrobium mobile*	AY196679	85

N/A: not available.

(similarity values ranged from 85 to 96%) that were similar to *Methanomicrobium mobile*-like clones. The second subcluster, Methanomicrobials II, formed a deeper branch consisting of 32 clones (5 OTUs), similarity value of 16S sequences that were ranged from 85% to 90% and members of this group are belonged *M. mobile*-like clones (Table 2). Phylotypes within the Methanobacteriales represented 48.5% (83 clones) of total clones which spanned 10 OTUs (43.47%). Within this cluster, the cloned sequences also formed two subclusters. It should be noted that the significance of the subclusters is not supported by high bootstrap values (Fig. 1). Although, the rDNA sequences may represent species of *Methanobacterium bryantii*. A total of 1 OTU (4.34%) representing 2.9% of total clones were closely related to cultured species belonging to order Methanosarcinales. Total archaea was detected 6.0×10^7 copies/ml of ruminal fluid. The number of 16S rRNA gene copies of *Methanomicrobiales* (2.4×10^6 copies/ml of ruminal fluid) and *Methanobacteriales* (1.3×10^6 copies/ml of ruminal fluid) were also observed, accounting 4.0% and 2.17% of total arcahea, respectively.

4. Discussion and conclusion

Ruminal methane is formed by the action of methanogenic archaea typified by hydrogenotrophic methanogens, which is present in ruminants fed upon a wide variety of diets worldwide. The results of the present study show that Surti rumen 16S rRNA library consist about 97% clones belonged to the hydrogenotrophic methanogens (Methanomicrobials and Methanobacterials), while the acetoclastic methanogens represented merely 3% of the total clone diversity. Earlier phylogenetic studies based on the 16S rRNA and *mcrA* genes also revealed that majority of the sequences retrieved from bovine rumens and cattle dung were affiliated to hydrogenotrophic methanogens belonging to Methanomicrobiales and Methanobacteriales (Tatsuoka et al., 2004; Rastogi et al., 2008). The methanogen community of buffaloes rumen should reflect the same trend as observed in a typical cattle rumen such as the greater abundance of hydrogenotrophic methanogens than the acetoclastic methanogens.

Similarly, Shin et al., (2004) reported that 85% (89 of 104 clones) of the total clones from the bovine rumen belonged to the order Methanomicrobiales, with 61 clones resembling *M. mobile*. Interestingly, *M. mobile* was not detected in sheep from Western Australia (Wright et al., 2004). While, *Methanobrevibacter*-like clone could not detect in the present study. This may be due to differences in sample preparation, animal diet or geographic region.

Zinder (1993) has observed that in a typical cattle rumen, the approximate steady-state amounts of volatile fatty acids (VFA) are 63% acetate, 21% propionate, and 16% butyrate and other higher fatty acids. VFA are generally absorbed by the rumen epithelium and subsequently converted to animal proteins, and therefore not available for utilization as a carbon source by acetoclastic methanogens (Methanosarcinales) residing in rumen. Therefore, acetoclastic methanogens make up only a small percentage of total methanogen community in cattle rumen. The only carbon source available in plenty for methanogens is H_2/CO_2, thus hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) that are capable of using H_2/CO_2 can multiply easily and are observed in high abundance in rumen (Zinder, 1993). Similarly, Denman et al. (2007) reported that the population of *Methanobacteriales* order was the major hydrogenotrophic methanogens in rumen of Brahman-crossbred (*Bos indicus*) steers. The results of the present study also corroborate with earlier observations of Rastogi et al. (2008), where in the fresh cow dung *mcrA* library 93.5% clones belonged to the hydrogenotrophic methanogens, while the acetoclastic methanogens (Methanosarcinales) represented merely 6.5% of the total clone diversity, in the 8-month-old dung, which

Table 2
Analysis of diversity of 16S rDNA phylotypes retrieved from the rumen of Indian Surti buffalo.

Taxon	No. of operational taxonomic units (OTUs) and % of total OTUs	No. of clones
Methanomicrobiales	12 OTUs (52.17%)	83
(a) Methanomicrobials I	7 OTUs	51
(b) Methanomicrobials II	5 OTUs	32
Methanobacteriales	10 OTUs (43.47%)	83
Methanosarcinales(*Methanosarcina barkeri*)	1 OTU (4.34%)	5
Total	23 OTUs	171

included 80% of clones belonging to hydrogenotrophic methanogens, while acetoclastic methanogens constituted only 20% of the total clone diversity.

Genome sequences would provide new insights into the lifestyle and cellular processes of this important rumen hydrogenotrophic methanogens (Methanomicrobiales and Methanobacteriales) under control feeding regime. It would also define vaccine and chemogenomic targets for broad inhibition of rumen methanogens and represents a significant contribution to worldwide efforts to mitigate ruminant methane emissions and reduce production of anthropogenic greenhouse gases.

Over all more studies are needed on the effects of diets composition and animal species on the diversity of methanogens and enteric methane emission in the rumen. This study has revealed the largest assortment of hydrogenotrophic methanogens phylotypes ever identified from rumen of Surti buffaloes and the need to better understand the factors influencing methanogen diversity with methane emission. Further studies are needed to examine methanogen diversity in goat, sheep and dairy cattle located in the Gujarat state as well as other state of India. Such studies would significantly enhance our knowledge and ability to use novel methods to manipulate the rumen methanogen populations to reduce methane production from ruminant animals. Reducing enteric methane emissions is likely to be one of the key mitigation strategies for the reduction of greenhouse gas emissions in the agricultural sector.

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