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MOLECULAR IDENTIFICATION OF METHANOGENIC ARCHAEA FROM SURTI BUFFALOES (*BUBALUS BUBALIS*), REVEALS MORE HYDROGENOTROPHIC METHANOGENS PHYLOTYPES

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ABSTRACT

Methane emissions from ruminant livestock are considered to be one of the more potent forms of greenhouses gases contributing to global warming. Many strategies to reduce emissions are targeting the methanogens that inhabit the rumen, but such an approach can only be successful if it targets all the major groups of ruminant methanogens. Therefore, a thorough knowledge of the diversity of these microbes in breeds of buffaloes, as well as in response to geographical location and different diets, is required. Therefore, molecular diversity of rumen methanogens in Surti buffaloes was investigated using 16S rRNA gene libraries prepared from pooled rumen contents from three Surti buffaloes. A total of 171 clones were identified revealing 23 different sequences (phylotypes). Of these 23 sequences, twelve sequences (12 OTUs, 83 clones) and 10 sequences (10 OTUs, 83 clones) were similar to methanogens belonging to the orders Methanomicrobiales and Methanobacteriales, and the remaining 1 phylotype (5 clones) were similar to *Methanosarcina barkeri*. These unique sequences clustered within a distinct and strongly supported phylogenetic group. Further studies and effective strategies can be made to inhibit the growth of Methanomicrobiales and Methanobacteriales phylotypes to reduce the methane emission from rumen and thus help in preventing global warming.

Key word: Methanomicrobiales, Methanobacteriales, Surti buffaloes, phylotypes, 16s DNA.

INTRODUCTION

Methanogens are members of the domain Archaea, and fall within the kingdom Euryarchaeota (48) Methanogens 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 (8). Methanogens are widespread in anaerobic environments, including tundra (30), freshwater lake and wetland sediments (7, 13), estuarine and marine sediments (2), acidic peatlands (3, 17), rice field soil (8, 16), animal guts (33), landfills (22), and anaerobic digesters treating animal manure (1,34), food processing wastewater (23), and municipal wastewater and solid waste (15, 53). Interest in methanogens

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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 (http://www.indiastat.com). 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% (11). 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 (35) and it is likely that major species of rumen methanogens are yet to be identified (31, 50). The most common species of methanogens isolated from the rumen are strains of *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina* (50, 18). 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 (16, 26, 29, 28, 32, 39, 37, 45, 15, 49). Methanogens are frequently found in association with protozoa (41, 21, 42, 43, 38). 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.

MATERIALS AND METHODS

Sampling and DNA extraction

The experiments were carried out on 3 adult Surti buffaloes, approximately three years of age and with a mean live weight of 201±18kg. They 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 0,2,4,6hrs post feeding (52,19). 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, 6 hrs x 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 samples. The total DNA mixture (pooled) was used as a template in PCR to amplify 16S r DNA.

PCR primers and amplification

The primers used were 1Af (5'- TCYGKTTGATCCY GSCRGAG-3') and 1100Ar (5'- TGGGTCTCGCTCGTTG-3'). (12).Subsequently 16S rDNA were amplified (1100bp) 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 approximately1.1 kb was purified using Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were cloned in PTZ57R/T vector (Fermentas, UK) as per the instructions of the manufacturer and transformed into *E. coli* DH alpha competent cells. 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 primers M13F (5'-GTAAAACGAC GGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') and nucleotide sequences of cloned genes 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.

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the Genbank/EMBL/DDBJ/RDP (4). Sequences (~600 bp) from the current study were trimmed (remove low-quality base calls from the start and end of DNA sequence) manually and analysed by the CHECK_CHIMERA program (27) 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 (25) and alignment was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html (47). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (14) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (40) with 95% confidence intervals to quantify the diversity of phylotypes and total of 23 OTUs were generated based on furthest-neighbor algorithm at cut offs of 10 % difference. The sampling effort of library was evaluated by calculating the percentage of coverage (C) according to the equation C= 1 - (n/N)X 100, where n is the number of sequences represented by a single clone (Tables 1) and N is the total number of clones analyzed in the library. Phylogenetic tree was constructed by the neighbour joining method using MEGA 4.0 (46). Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (46).The prefix meth was used to denote OTUs identified and nucleotide sequences have been deposited in the Genbank database under the accession numbers are depicted in Fig 1.

RESULTS AND DISCUSSION

One hundred seventy one 16S rDNA sequences were analyzed. On the basis of sequence similarity, all of the sequences were related to methanogens. 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 (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 (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 (15 OTUs), similarity value of 16S sequences that were ranged from 85% to 90% and members of this group are belonged *Methanomicrobium mobile*-like clones (Table 1). Boone *et al*. (6) considered that a sequence similarity of 98 % or less was evidence for separate species within the methanogens. Based on this, Methanomicrobials would be considered as *Methanomicrobium mobile*-like strains, however, as pointed out by Martinez-Murcia *et al*. (24), 16S sequence similarity values recommended to define a species provide a working definition that has been empirically derived, and values should not be treated as absolute or fixed. For example, 16S rRNA sequences from strains of *Methanobacterium thermoformicicum* and *M. thermoautotrophicum* used in this

study were greater than 98 % similar, yet these organisms are considered distinct species. Shin *et al*. (51) reported that 85% (89 of 104 clones) of the total clones from the bovine rumen belonged to the order Methanomicrobiales, with 61 clones resembling *Methanomicrobium mobile.* Similar results are also reported by (53) in Feedlot Cattle from Ontario and Prince Edward Island. Interestingly, *Methanomicrobium mobile* was not detected in sheep from Western Australia (51). Phylotypes within the Methanobacteriales represented 48.5% (83 clones) of total clones and spanned ten OTUs. Within this cluster, the cloned sequences 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 OTUs representing 2.9% of total clones were closely related to cultured species belonging to order Methanosarcinales.

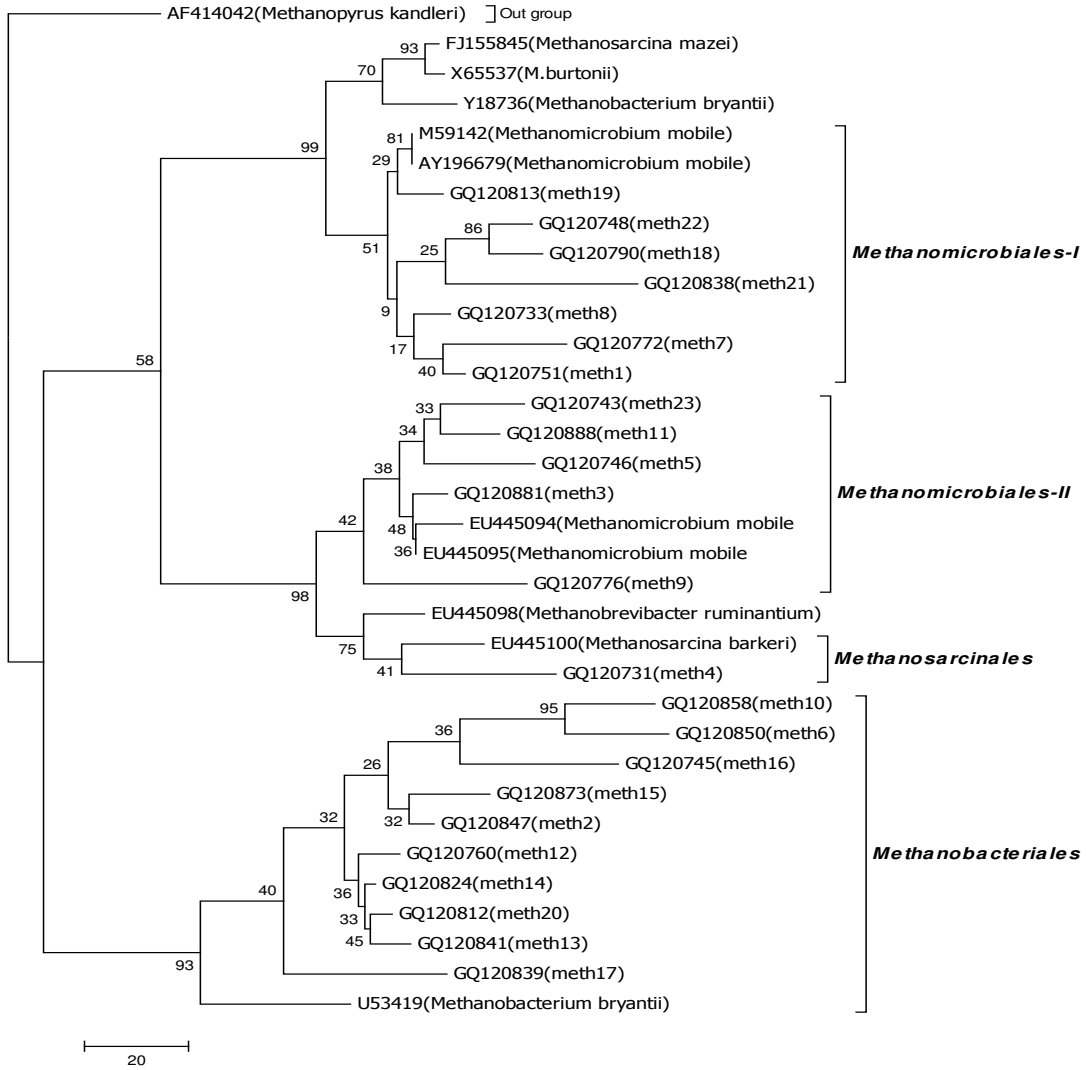

Figure 1. Phylogenetic relationships of partial 16S r DNA sequences of clones recovered from Surti rumen samples. The rooted tree was constructed as a maximum parsimony tree using close-neighbor interchange level 1 and bootstrapped with 1,000 trials, using the MEGA 4 tree building program. All positions 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.

Zinder (54) showed 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 H2/CO2 can multiply easily and are observed in high abundance in rumen [54]. 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 (44, 14). 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. The results of the present study corroborate with earlier observations where in the Surti rumen 16S r RNA library 97% clones belonged to the hydrogenotrophic methanogens, while the acetoclastic methanogens represented merely 3% of the total clone diversity (Table 1). Previous culture based studies have isolated methanogens of the genus *Methanomicrobium* and *Methanobacterium* from the bovine rumen (18), although *Methanobrevibacter* and *Methanosarcina* tend to be isolated at higher population levels (29). 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. The methanogen community of Surti buffalo should reflect the same trend as observed in a typical cattle rumen such as the greater abundance of hydrogenotrophic methanogens than the acetoclastic methanogens. The results of the present study corroborate with earlier observations (33) where in the fresh cow dung *mcrA* library 93.5% clones belonged to the hydrogenotrophic methanogens, while the acetoclastic methanogens represented merely 6.5% of the total clone diversity, in the 8-month-old dung, which included 80% of clones belonging to hydrogenotrophic methanogens, while acetoclastic methanogens constituted only 20% of the total clone diversity.

Table 1. Analysis of 16S rDNA phylotypes diversity retrieved from the rumen fluid of Surti buffaloes

Items	**16S r DNA libraries**
Library size [a] (N)	171
OTUs [b]	23
Single clone OTU [c] (n)	7
Good's coverage [d] (%)	96
Clone distribution	
(i) Methanomicrobiales [e]	12 OTU (83 clones)
(ii) Methanobacteriales [e]	10 OTU (83 clones)
(iii) *Methanosarcinales* [g]	
a. *Methanosarcina barkeri*	1 OTU (5 clones)
Total hydrogenotrophic methanogens (%)	97% (166 clones)
Total acetoclastic methanogens (%)	3% (5 clones)

[a] Number of clones analyzed from library
[b] OTUs based on 16S r DNA sequences
[c] OTUs containing only single clone
[d] The higher percentage coverage means more diversity is captured
[e]Hydrogenotrophic methanogens
[g]Acetoclastic methanogens

Although some feeding strategies reduce ruminal methane emissions, the amount of CH_4 produced during ruminal fermentation is dependent upon the nature of the substrate being fermented. In general, methanogenic potential of the ruminal microflora is greatest for the fermentation of structural carbohydrates compared to that of non-structural carbohydrates (5).The addition of fat or individual fatty acids to ruminal cultures decrease CH_4 production (34). 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. 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. 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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