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Methyl Coenzyme M Reductase (*mcrA*) Gene Based Phylogenetic Analysis of Methanogens Population in Murrah Buffaloes (*Bubalus bubalis*)

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The aim of the present study was to decipher the diversity of methanogens in rumen of Murrah buffaloes so that effective strategies can be made in order to mitigate methane emission from these methanogens. In the present study diversity of rumen methanogens in Murrah buffaloes (*Bubalus bubalis*) from North India was evaluated by using *mcr-A* gene library obtained from the pooled PCR product from four animals and by using MEGA4 software. A total of 104 clones were examined, revealing 26 different *mcr-A* gene sequences or phylotypes. Of the 26 phylotypes, 16 (64 of 104 clones) were less than 97% similar to any of the cultured strain of methanogens. Seven clone sequences were clustered with *Methanomicrobium mobile* and three clone sequences were clustered with *Methanobrevibacter gottschalkii* during the phylogenetic analysis. Uncultured group of methanogens comes out to be the major component of the methanogens community structure in Murrah buffaloes. Methanomicrobium phylotype comes out to be major phylotype among cultured methanogens followed by Methanobrevibacter phylotype. These results help in making effective strategies to check the growth of dominant methanogenic communities in the rumen of this animal which in turn help in the reduction of methane emission in the environment and ultimately helps us in fighting with the problem of global warming.

***Keywords*:** *mcr-A* gene, methanogens, Murrah buffaloes, phylogenetics

Methanogens with other microorganisms in rumen play an important role in the digestion of feed and in the supply of energy in the form of microbial proteins and volatile fatty acids. During the accomplishment of this task a considerable amount of methane is also produced by the methanogens which is released in to the environment by these animals. It has been estimated that the worldwide production of methane from ruminants is around 86 million tonnes from rumens and a further 18 million tonnes from manure (Snell-Castro *et al*., 2005). In 2009, Indian livestock methane-emission was 11.75 million metric tons – higher than the 9 million metric tons estimated in 1994 (Steinfeld *et al*., 2006). So keeping in view the role of methane in global warming it is very important to keep a check on methane emission by ruminants. The first step in this direction is to have the knowledge of methanogenic communities present in the rumen of buffalo as they are fed on roughage based diet which is different from the diets of ruminants of other countries. Information is available on the changes in diversity of methanogens and wide range of bacterial species present in the rumen with respect to dietary modifications (Thauer, 1998; Tajima *et al*., 1999, 2000; Ramsak *et al*., 2000; Singh, 2009). Advancement of molecular techniques allow us to study the diversity of methanogens present in the rumen of ruminants more precisely (Wright *et al*., 2004, 2006; Denman *et al*., 2007). Most of the research on rumen methanogenic archaea diversity has been done using the 16S rRNA gene library approach. In this study we used Methyl coenzyme reductase (*mcr-A*) gene based approach to study the diversity of methanogens present in the rumen of Murrah buffaloes. *mcr* gene, catalyses the reduction of a methyl group bound to coenzyme-M, with the concomitant release of methane. This enzyme complex is thought to be unique to, and ubiquitous in, methanogens (Tajima *et al*., 2001), making it a suitable tool for their specific detection. The *mcr-A* form is thought to be present in all methanogens, whilst the presence of *mcr-II* form has only been demonstrated in members of the orders *Methanobacteriales* and *Methanococcales* (Kocherginskaya *et al*., 2001). In the present paper an effort has been made to describe the whole diversity of methanogens present in Murrah buffaloes by using *mcr-A* gene specific primers.

Material and Methods

Animals and diet

Four adult male Murrah Buffaloes, approximately 12 months old with an average body weight of 650 kg, were randomly selected to collect rumen liquor. All animals were individually accommodated in individual rooms located at National Dairy Research Institute, Karnal (29°41'0"N / 76°59'0"E), India. Each animal house was provided with a feed bucket and drinking nozzle with adequate amount of water at all times. Animals were individually fed once a day, eight weeks before collection of rumen contents. The buffaloes were fed twice a day on a standard diet (Concentrate: roughage=50:50). Rumen liquor samples from the buffaloes were obtained prior to the first

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morning feed. Rumen liquor samples were obtained after manual mixing of rumen contents.

Sample collection

Rumen liquor obtained was squeezed through four layers of cheese cloth and immediately processed for DNA isolation (Wright *et al*., 2004).

DNA extraction, PCR amplification, cloning, and sequencing

DNA was isolated from 1 ml of rumen liquor by using Genomic DNA Purification kit (Fermentas, USA) (Cat. No. K0512) according to the manufacturer's instructions. Amplification of genomic DNA was car-

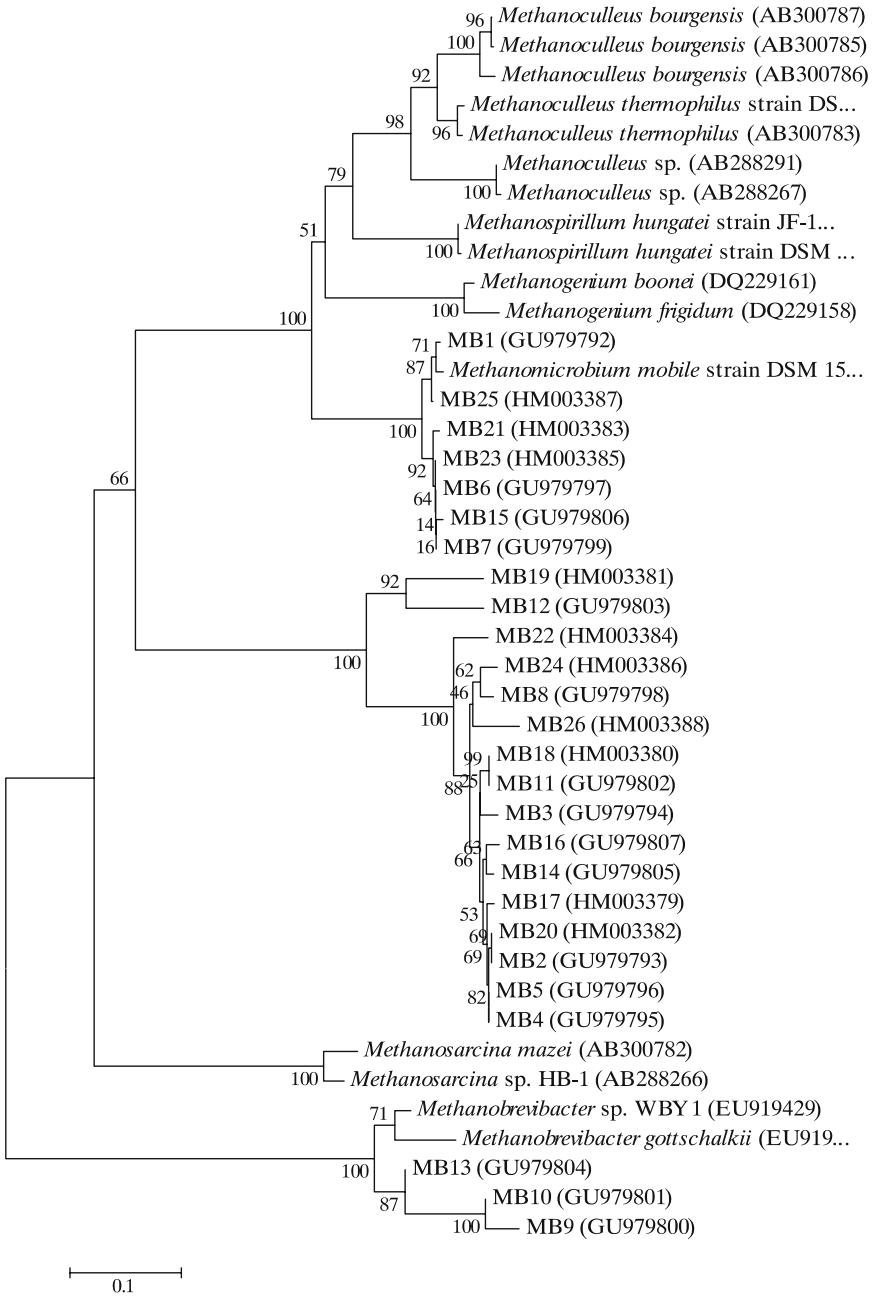

Fig. 1. Evolutionary relationships of 42 taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length=2.40959812 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+ 3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 388 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

ried out by using *mcr-A* gene specific primers (Luton *et al*., 2002) set in a Bio-Rad Thermal Cycler. PCR steps include 95°C for 5 min, 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1.5 min, and a final extension step of 10 min at 72°C. A *mcr-A* gene library was constructed from the pooled PCR products obtained from each animal. Cloning of the 470 bp PCR product, selection of positive clones on the basis of blue white screening, and re-amplification of the PCR products were done by the standard protocols. All the positive clones obtained were sent for sequencing at Xcelris Labs Limited (Ahmedabad, India).

Sequence analysis

Raw sequences obtained after sequencing were BLAST analysed to search for the sequence identity between other methanogen sequences available in the GenBank database. Then these sequences were aligned by using CLUSTAL W in order to remove any similar sequences.

Phylogenetic analysis

The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using MEGA software and the tree was evaluated using the bootstrap test based on 500 re-samplings of the dataset (Felsenstein, 1985).

Nomenclature

The prefix MB (*mcr-A* gene clone of Buffalo) was used to denote clones identified. All the *mcr-A* gene sequences have been deposited in GenBank under the accession numbers GU979792-GU979807 and HM003379-HM003388.

Reference sequences used in the study

Methanoculleus sp. (AB288291), *Methanoculleus* sp. (AB288267), *Methanoculleus thermophilus* strain DSM 2624 (AF313804), *Methanoculleus thermophilus* (AB300783), *Methanoculleus bourgensis* (AB300786), *Methanoculleus bourgensis* (AB300787), *Methanoculleus bourgensis* (AB300785), *Methanospirillum hungatei* strain JF-1 (AF313805), *Methanospirillum hungatei* strain DSM 864 (AF414038), *Methanogenium boonei* (DQ229161), *Methanogenium frigidum* (DQ229158), *Methanosarcina mazei* (AB300782), *Methanosarcina* sp. HB-1(AB288266), *Methanobrevibacter* sp. WBY1 (EU919429), *Methanobrevibacter gottschalkii* (EU919431), *Methanomicrobium mobile* strain DSM 1539 (AF414044), *Methanobrevibacter smithii* strain 4R_4_F02 (GU385700).

Results and Discussion

A total of 104 clones were examined from the pooled PCR products from four purebred Murrah buffaloes from India. After phylogenetic analysis all clones were clustered in three different groups. The largest number of clones (64 out of 104) constituting about 61.5% of total number of clones showed less than 97% sequence similarity with any cultured strain of methanogens and forms a cluster of uncultured novel methanogens. Earlier studies also supports our results in which the major groups are affiliated with uncultured group of methanogens (Tajima *et al*., 2001; Whitford *et al*., 2001; Wright *et al*., 2004, 2006; Castro *et al*., 2005).The second largest number of clones (28 out of 104) which constitutes about 26.9% of total number of clones clustered with *Methanomicrobium mobile* group of methanogens. These results are in agreement with one of our previous studies in which we reported that *Methanomicrobium* phylotype might be the most dominant component of the known strain of methanogens population in Murrah buffaloes (Chaudhary and Sirohi, 2009). Third and last cluster (12 out of 104) of clones clustered with *Methanobrevibacter gottschalkii* group of methanogens and constituting about 11.5% of the total no. of clones. The 104 clones examined in the present study revealed 26 different sequences or phylotypes. BLAST and CLUSTAL W alignment analyses of sequences from individual animals also revealed 26 different sequences with a range of 2-8 sequences per animal. Phylogenetic analysis (Fig. 1) revealed that ten phylotypes (64 clones) were less than 97% similar to any of the cultured strain of methanogens, whereas seven clone sequences were clustered with *M. mobile* and three clone sequences were clustered with *M. gottschalkii* during the phylogenetic analysis. Like our previous study in Murrah buffaloes which was carried out on the basis of 16S rDNA, we are not able to find any of our clone sequences 100% similar to any of the methanogens strain reported earlier in India or in any other part of the world (Chaudhary *et al*., 2009). This might be due to difference in the geographical location or feeding habits of ruminant livestock in India and other parts of the world. From this study we concluded that uncultured group of methanogens remains the largest group of methanogens followed by the group that resembles with *M. mobile* and *M. gottschalkii* group of methanogens in Murrah buffaloes as revealed by *mcr-A* gene based phylogenetic analysis. Earlier study carried out in surti buffaloes demonstrates the dominance of methanobacteriales group (Singh *et al*., 2010). But in our study after uncultured group *M. mobile* group comes out to be the major group. By these results we conclude that *mcr-A* gene acts as an essential and diagnostic gene of methanogenic pathway. However identification of rumen associated *mcr-A* group describes a functional genomic link between methanogenic and putative reverse methanogenic archaea (Singh *et al*., 2010).

To our knowledge this was the first study using *mcr-A* gene to decipher the diversity of methanogens in rumen of Murrah buffaloes. Furthermore extensive studies are required in order to find out the exact diversity of rumen methanogens in ruminant livestock.

Conclusion

Results of this study suggest that after uncultured group of methanogens, *Methanomicrobium* and *Methanobrevibacter* phylotypes respresents major group of methanogens with in the rumen of Murrah buffaloes. Effective strategies should be made in order to check the growth of these major group of methanogens, so that the methane emission from the ruminants should be minimized.

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