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ORIGINAL ARTICLE

Isolation and characterization of methanogens from rumen of Murrah buffalo

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Abstract Methanogens were isolated from the rumen of Murrah buffaloes (*Bubalus bubalis*). These isolates (BRM-1, -2 and -3) were found to utilize CO_2+H_2 mixture, formate and acetate as substrate, but failed to grow on ethanol and methanol. Their physiological analysis showed that they could tolerate NaCl and bile salts up to 1.0% but 2.0% bile salt inhibited their growth. Based on 16S rRNA/*mcrA* gene sequence analysis, the isolates showed their phylogenetic relation with genus *Methanobrevibacter* and *Methanomicrobium*. BRM-1 and -3 showed 100% similarity with *Methanobrevibacter smithii*, while BRM-2 showed 100% similarity with *Methanomicrobium mobile.* The *mcrA* protein-based phylogeny also showed similar results to the *mcrA* gene, suggesting no apparent difference in the phylogeny between DNA and amino acid sequences of these isolates.

Keywords Methanogens · Rumen · Buffalo · 16S rRNA · *mcrA*

Introduction

Methanogens belong to domain *Archaea* and are characterized by their ability to produce methane under highly anoxic conditions (Guo et al. 2005). In rumen, methane emission accounts for the loss of 2–15% of ingested energy (Moss et al. 2000). Therefore, reduction of methane emissions could be an important area for ensuring the sustainability of ruminant-based agriculture production. Methane is a normal product of rumen fermentation, representing a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. During the oxidation of sugars via the EMP pathway, NAD^+ is reduced to NADH, which has to be reoxidised to NAD^+ to allow fermentation to continue. Under the anaerobic conditions prevailing in the rumen, where electron transfer to acceptors other than oxygen must regenerate NAD^+, the major sink is the reduction of carbon dioxide to methane (although the sink include sulfate, nitrate and fumarate). Because methanogens present in the mixed microbial ecosystem use hydrogen, it does not accumulate in the rumen. Indeed, even traces of hydrogen in the rumen inhibit hydrogenase activity and limit the oxidation of sugar when alternative pathways for disposal are absent (McAllister and Newbold 2008). Two methods utilized for disposal of reducing equivalents are production of more reduced volatile fatty acids and the production of hydrogen by membrane-bound hydrogenases. However, these hydrogenases have an acute sensitivity to an increased partial pressure of hydrogen. Therefore, the role of methanogens in the rumen is to scavenge and keep the partial pressure low enough for the hydrogenases to function (Russell 2002). In order to identify key methanogens involved in methanogenesis and to have a better understanding of their interaction with other microorganisms, they have been isolated from a wide range of habitats (Luton et al. 2002; Chaudhary and Sirohi 2009; Kumar et al. 2009). To our knowledge, there is no published information available on methanogens isolated from domesticated ruminants in India. Hence, the present study was aimed to isolate and characterize methanogens form rumen of Murrah buffalo.

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Materials and methods

Enrichment of cultures

Rumen liquor was collected before feeding from fistulated Murrah buffaloes maintained at cattle yard, NDRI, Karnal, on a standard diet (concentrate:roughage ratio= 40:60). BY media (Joblin 2005) was used for the enrichment and isolation of methanogens. Initially, a mixture of CO_2 and H_2 (20:80), sodium formate (0.5% w/v) and their combinations were used as substrates. Pre-reduced BY media was dispensed in oxygen-free CO_2 flushed serum bottles of 125 mL volume and autoclaved. After autoclaving, filter sterilized vitamin solution (1%), and antibiotics [streptomycin and penicillin, 0.1 mg/mL; vancomycin, 0.2 mg/mL and nystatin, 200 U/mL (HiMedia)], 1% reducing solution (containing 2.5 g each of L-cysteine.HCl and sodium sulfite in 205 mL of distilled water; pH 10.0) were added just before inoculation. Rumen liquor sample was inoculated (5%) using pre-reduced syringe and the bottles were then incubated at 39±1°C for 90 days in the dark (Sowers 1995).

Screening for methane production

After incubation, 5 mL of gas was withdrawn from the head space of the incubated serum bottle using a gas-tight syringe (Hamilton, USA) and analyzed for methane using 'Gas Chromatograph' (Nucon 5700, India) equipped with a flame-ionization detector and stainless steel column packed with Poropak–Q (80/100 mesh range and 2 m×1/8″×2 mm SS). The injector temperature was 40°C, while for the detector and column, the temperature was 50°C. The flow rate of N_2 (carrier gas) and H_2 was 30 mL/min and air was 300 mL/min. The standard gas (Spantech Calibration Gas, Surrey, England) used for methane estimation was composed of 50% each of methane and carbon dioxide. The peak of methane was identified on the basis of retention time of standard and the response factor obtained was used to calculate percent of methane in the sample. The methane produced from the substrate was also corrected using blank values. The volume of methane (mL) produced was calculated as follows:

$$\text{Methane production (mL)} = \text{Total gas produced (mL)} \times \text{Percent methane in sample}$$

Isolation of methanogens

Roll tubes (Hungate 1969) were prepared from methane positive serum bottles using 1.0 mL inoculum from 10^{-6} dilution. After incubation for 4 weeks at 39±1°C purity of cultures and cell morphology was confirmed microscopically. Their physiological characteristics like ability to utilize 50 mM sodium formate, sodium acetate, methanol and ethanol as growth substrates were also evaluated using discrete colonies. Growth was determined by measuring methane and optical density at 660 nm (OD_{660}) with a Jenway spectrophotometer (Rea et al. 2007). For checking the sensitivity of cultures to NaCl and bile salt, cultures were inoculated at 10% in BY medium containing different concentrations of NaCl (0.25, 0.50 and 1.0%) and bile salt (0.5, 1.0 and 2.0% oxbile). The isolates were allowed to grow at 39±1°C for 21 days. Growth was considered positive after estimation of methane and optical density.

Molecular characterization

DNA was extracted and purified by using QIAamp® DNA stool kit (Qiagen). The PCR amplification was performed using 16S rRNA based (Met86F and Met1340R) and *mcrA* gene based primers (Sigma). The reaction mixture (25 μL) was comprised of 10X Taq buffer F, $MgCl_2$ (25 mM), dNTPs (10 mM each), primers (20 pmol/μL each) and nuclease-free water. The steps and conditions of thermal cycling for Met 86F/1340R targeting 16S rRNA and *mcrA* gene were according to Wright et al. (2004) and Luton et al. (2002), respectively. The amplified PCR products were sequenced (Xcelris Genomic Centre, Ahmedabad). BLAST search was performed with the sequences obtained to find out the homology with published methanogen sequences in GenBank database.

Phylogenetic analysis

Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Ten additional 16S rRNA sequences [*Methanobrevibacter millerae* (AY196673.1); *Methanobrevibacter* sp. 1Y (DQ135988.1); *Methanobrevibacter smithii* (U55234.1); *Methanobrevibacter smithii* (AY196667.1); *Methanomicrobium mobile* (M59142.1); *Methanomicrobium* sp. (X99139.1); *Methanobrevibacter gottschalkii* (U55238); *Methanobrevibacter smithii* (AY196668.1); *Methanobrevibacter smithii* (AY196669.1); *Methanomicrobium mobile* (AY196679.1)}/ four *mcrA* gene sequences {*Methanobrevibacter smithii* (DQ251046.1); *Methanobrevibacter* sp. WBY1 (EU919429.1); *Methanobrevibacter gottschalkii* strain PG (EU919431.1); *Methanomicrobium mobile* DSM (AF414044.1)] representing methanogens were included in phylogenetic analyses. Phylogeny was further confirmed by *mcrA* protein sequences [*Methanobrevibacter smithii* (ABB77886); *Methanobrevibacter* sp. WBY1

(EU919429); *Methanobrevibacter gottschalkii* strain PG (ACK56066); *Methanomicrobium mobile* strain DSM 1539 (AAl29293)].

Results and discussion

Growth and methane emission were checked after enrichment and incubation for 30 days, but no methane emission was observed, suggesting the need to extend the incubation period (Skillman et al. 2004). After 90 days of incubation, different types of colonies were developed and, based on colony morphology, three (named as BRM-1, -2 and -3) were selected for further analysis. The colony size of BRM-1, -2 and -3 that increased with increasing incubation period were in the range of 0.2–0.7 mm in diameter (Table 1). The colony size of methanogens in range of 0.5–1.0 mm is well documented by Ma et al. (2005).

Microscopic observation revealed that cells of BRM-1 and -3 stained Gram-positive, whereas BRM-2 cells stained Gram-negative. Both BRM-2 and -3 utilized sodium formate and CO_2+H_2 as substrate. The above results are in agreement with Joblin (2005). After physiological characterization, it was found that all the isolates could metabolize formate and acetate as carbon as well as energy source, but not methanol and ethanol (Table 2). Formate utilization by *Methanobrevibacter* and *Methanomicrobium* as carbon and energy source is in agreement with Bryant (1974). Although the use of acetate as carbon source is well reported in *Methanomicrobium* and *Methanobrevibacter* (Tanner and Wolfe 1988; Rea et al. 2007), its utilization as energy source has not been found elsewhere in the literature. Our results indicate that, in the absence of an electron donor in the growth medium, these electrons must have come from acetate for the reduction of carbon dioxide available in

Table 1 Colony and cell morphology of rumen methanogens

Isolate	Colony morphology	Gram reaction	Substrates
BRM-1	Creamish white; 0.3–0.5 mm diameter; regular margin	Gram-positive cocci	Sodium formate and CO_2+H_2
BRM-2	Light brown; 0.2–0.4 mm diameter, entire margin	Gram-negative thin rods, slightly curved	Sodium formate and CO_2+H_2
BRM-3	Brown; 0.2–0.7 mm diameter; regular margin	Gram-positive cocci	Sodium formate and CO_2+H_2

Table 2 - Effect of different substrates, NaCl and oxbile on the growth of rumen methanogens

Isolate	Substrate					NaCl (%)				Oxbile (%)			
	CO_2+H_2 (20:80)	Sodium formate (50 mM)	Sodium acetate (50 mM)	Methanol (50 mM)	Ethanol (50 mM)	0.0	0.25	0.50	1.00	0.0	0.50	1.00	2.00
Optical density at 660 nm													
BRM-1	0.23±0.11	0.15±0.01	0.11±0.01	0.09±0.01	0.03±0.00	0.22±0.13	0.21±0.11	0.21±0.01	0.21±0.11	0.24±0.12	0.13±0.01	0.10±0.02	0.01±0.01
BRM-2	0.18±0.09	0.13±0.03	0.10±0.01	0.02±0.00	0.01±0.00	0.19±0.21	0.23±0.03	0.21±0.01	0.18±0.10	0.19±0.01	0.17±0.01	0.11±0.01	0.06±0.01
BRM-3	0.21±0.02	0.12±0.01	0.10±0.02	0.01±0.00	0.02±0.00	0.22±0.02	0.22±0.01	0.23±0.02	0.23±0.01	0.22±0.02	0.12±0.01	0.13±0.01	0.04±0.01
Methane produced in µmol/mL head space													
BRM-1	14.07±0.09	5.34±0.09	2.87±0.00	0.04±0.02	0.06±0.02	15.33±0.09	14.34±0.09	13.87±0.03	14.04±0.02	12.34±0.03	7.67±0.01	7.73±0.02	0.04±0.01
BRM-2	10.90±0.12	4.63±0.02	2.36±0.10	0.02±0.01	Absent	13.03±0.12	14.63±0.02	13.36±0.10	11.02±0.01	11.90±0.10	9.33±0.12	8.41±0.10	0.02±0.01
BRM-3	11.59±0.01	3.55±0.01	1.80±0.02	0.0±0.04	0.04±0.33	13.66±0.12	12.55±0.02	14.80±0.01	14.76±0.02	12.16±0.04	12.41±0.10	10.80±0.02	Absent

the headspace. The amount of methane produced (Table 2) with acetate was less in comparison to formate, suggesting that the growth was not coupled to methane production and that the cells might be generating methane for maintenance rather than growth (Sowers and Noll 1995). The difference in growth pattern with different substrate utilization (i.e., CO_2+H_2; formate and acetate) might be due to the free energy available for methanogenesis, which is greater for hydrogen followed by formate (Muller 1993). In contrast, no growth was observed when methanol and ethanol were used as substrate. Although some rumen isolates (i.e., *Methanosarcina barkeri* and *Methanobacterium ruminantium*) have been reported to utilize methanol and ethanol (Smith and Hungate 1958), but no growth was seen with our isolates.

Results of growth at different NaCl concentration (Table 2) showed that all the isolates could survive at 0.25, 0.5 and 1.0% NaCl concentration. The results obtained are in accordance with Rea et al. (2007), who reported that different *Methanobrevibacter* strains and other species, i.e., *Methanobrevibacter gottschalkii* HO^T, *Methanobrevibacter thaueri* CW^T, and *Methanobrevibacter smithii* PS^T, could tolerate 0.45 M (2.6%) NaCl. In addition, no difference was observed in growth pattern at different NaCl concentrations compared to control. Hence, it can be concluded that NaCl at 0.25–1.0% neither supported nor inhibited the growth of these isolates. Our results are also in agreement with Thakker and Ranade (2002), who observed the growth of *Methanosarcina* spp. in presence of 3.0% NaCl, with optimum growth at 0.5% (w/v) NaCl. They also reported that isolate produced methane at low salt (0.06%) concentration, whereas methanogenesis was completely inhibited at NaCl higher than 3.0% (w/v). Lomans et al. (1999) has also reported that *Methanomethylovorans hollandica* strain DMS1T could grow at NaCl concentrations of 0–40 mM. The salt tolerance range of the strain was 0–300 mM, similar to our study.

The bile sensitivity results (Table 2) showed that the presence of bile salts in the medium suppressed the growth of all the isolates and almost complete inhibition was observed at 2.0% of bile salts. From Table 2, it is well evident that growth was observed in 0, 0.5 and 1.0% bile, though there were variations in growth pattern at these concentrations. Medium without bile supported the growth, whereas 0.5 and 1.0% bile limited the growth of BRM-1, -2 and -3. Similar results were obtained by Rea et al. (2007), showing that *Methanobrevibacter* strains (i.e., AK-87, OCP and ZA-10^T and KM1H5-1P^T) and other species (i.e. *Methanobrevibacter gottschalkii* HO^T, and *Methanobrevibacter smithii* PS^T) were sensitive to bile (2.0%) except *Methanobrevibacter ruminantium* M1^T and *Methanobrevibacter thaueri* CW^T. The results obtained by Savant et al. (2002) are also in accordance with our study, stating that *Methanobrevibacter acididurans* was sensitive to bile (2.0%).

PCR based on 16S rRNA gave an amplicon size of 1,260 bp (Fig. 1a), thus confirming that BRM-1, -2 and -3 belongs to rumen methanogenic *Archaea*. The identification was also confirmed by *mcrA* gene-based amplification that resulted in product size of 460–490 bp (Fig. 1b).

When analyzed by BLAST and at RDP II, the sequence of BRM-1 and -3 showed a high degree of similarity to many *Methanobrevibacter* sequences, including many partial sequences, uncharacterized species and clones from environmental samples. For subsequent analyses, only those sequences with more than 1,000 bases (16S rRNA) and 400 bases (*mcrA*), excluding uncultured organisms were compared and phylogenetic tree was drawn.

No major differences were observed between DNA and amino acid sequences or between the different algorithms used (Fig. 2a–c). The phylogentic tree with 16S rRNA (Fig. 2a), *mcrA* gene (Fig. 2b) and protein sequence (Fig. 2c) showed two major clusters: cluster 1 and 2, which were entirely different from each other.

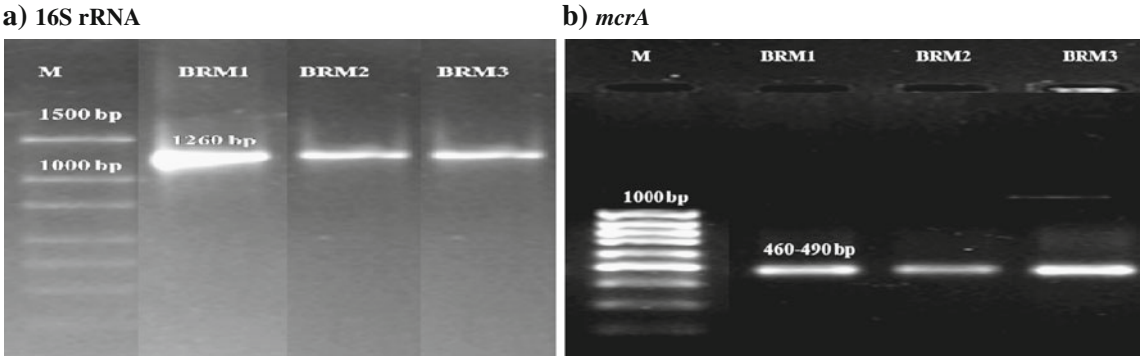

Fig. 1 Agarose gel electrophoresis of PCR products

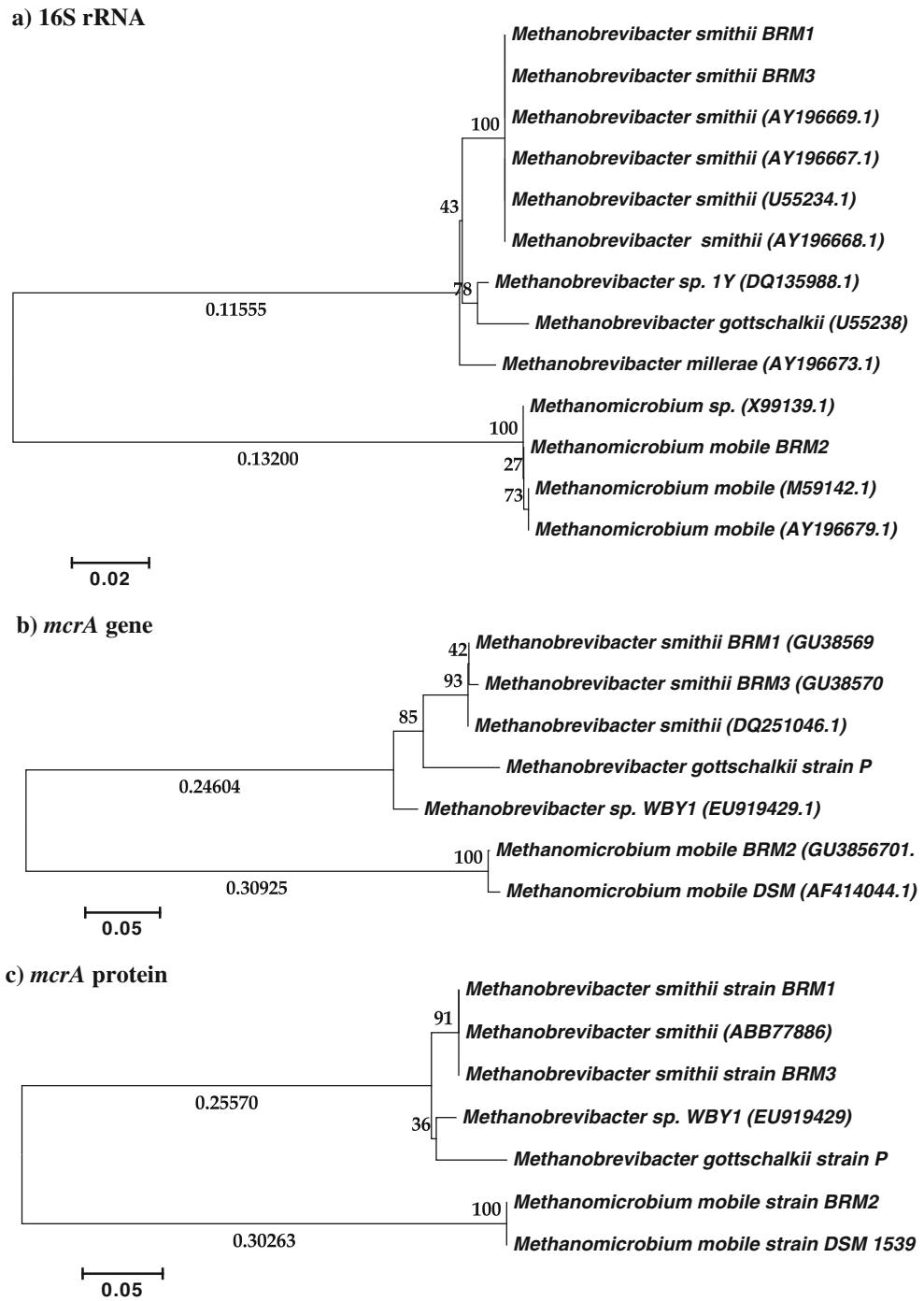

Fig. 2 Phylogenetic trees of cultured rumen methanogens based on 16S rRNA, *mcrA* gene and *mcrA* protein sequences**.** The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown above the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). **a** The optimal tree with the sum of branch length=0.28939019 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. There were a total of 1,018 positions in the final dataset. **b** The optimal tree with the sum of branch length= 0.68617123 is shown. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 358 positions in the final dataset. **c** The optimal tree with the sum of branch length=0.63459853 is shown. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. There were a total of 128 positions in the final dataset.

Cluster 1 contained 3 subclusters; with BRM-1 and -3 in sub cluster 1 that showed 100% similarity with *Methanobrevibacter smithii* ATCC 35061 and *Methanobrevibacter smithii* (Accession No. DQ251046.1). BRM-2 was present in major cluster 2 and shared 100% similarity with *Methanomicrobium mobile*, whereas subcluster 3 includes other species of *Methanobrevibacter*. All the trees showed similar phylogenetic results and thus are in accordance with Luton et al. (2002) who stated that *mcrA* gene sequence can be used as an alternative to 16S rRNA-based sequences.

Conclusion

Methanogens isolated from Murrah buffalo showed different patterns of substrate utilization and salt tolerance. The 16S rRNA- and *mcrA* gene-based analysis showed them to be *Methanobrevibacter smithii* and *Methanomicrobium mobile.* Although *Methanobrevibacter* spp. in ruminants has been reported from Australia and Canada, this appears to be the first report of *Methanobrevibacter* isolation from Murrah buffaloes, suggesting their presence in the Indian subcontinent.

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