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Phylogenic diversity analysis of rumen acetogens in adult sheep fed on conventional roughage diet

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ABSTRACT

The study was conducted to screen the Indian sheep for the presence as well as diversity of rumen acetogens. Rumen liquor samples from 3 adult female sheep (Mandya × Rambouillet) was collected through stomach tube. Genomic DNA was isolated and amplified using touchdown PCR protocol for partial *formyltetrahydrofolate synthetase (fhs)* gene sequence. Bioinformatic analysis of obtained sequences was done to construct phylogenic tree for molecular characterization and diversity analysis. PCR based amplification and further agarose gel electrophoresis revealed the presence of *fhs* functional gene in sheep rumen confirming the occurrence of acetogens in adult sheep fed on conventional roughage diet. However, we could not amplify the gene *acs* conserved with acetogens described by Australian workers. From the phylogenic tree it is clear that *act*1 group is distant from all other clones (*act*2 - *act*10) obtained in this study and represent a distinct uncultured acetogens genera. The *act*3 and *act*5 clones are also present in different clusters and are distant to *act*2, *act*10, *act*4 and *act*6 are closer to each other in the phylogenic tree but distant from *act*1. From the phylogeny tree it is clear that majority of the acetogens in these sheep are different from those acetogens reported elsewhere in the world. Thus, there is an urgent need to screen our livestock for the acetogens species/strains and to culture them for exploring the prospects of their use as alternate H_2 sink for methane mitigation from ruminants.

Key words: *fhs* gene, Molecular characterization, Phylogenic diversity, Rumen acetogens, Sheep

Methanogenesis is the major route in rumen for fermentative hydrogen utilization, however, in marsupials such as kangaroos and wallabies analogous gut fermentation is the alternate disposal route for hydrogen utilization where it converts into acetate through reductive acetogenesis (Malik *et al.* 2012). Reductive acetogens exist in sediments, wastewater treatment systems, soils, animal gut systems and are likely to natural microbiota in rumen (Joblin 1999). Thus, the augmentation of reductive acetogen microbes in rumen would not only ensure the reduction in methane emission (Morrison 2009) but also lead an energetic gain (Boccazzi and Patterson 2011). Till date no documented report is available about the diversity and existence of reductive acetogens in Indian ruminant and this study is the first attempt in this direction. Keeping these facts in mind, the study was undertaken to screen the Indian adult sheep for exploring the molecular diversity and existence of acetogens in rumen through functional gene analysis.

MATERIALS AND METHODS

Rumen liquor samples from 3 adult female sheep *Mandya × Rambouillet* (BW 26.7±2.4 kg) maintained at Institute's experimental livestock unit was collected using stomach tube as per Malik *et al.* (2014). These animals were maintained on *ragi* straw (*ad lib.*) and concentrate (500 g/d) based diet and fed as per standards (Ranjhan 1998). Rumen liquor samples were collected into 15 ml plastic tube after filtering through double layer of muslin cheese cloth and tubes were placed into ice box to avoid the degradation of genomic DNA. Genomic DNA was isolated from the rumen liquor samples as per Gagen *et al.* (2010) and thereafter, quantitation of genomic DNA was done using nanodrop spectrophotometer ND1000 at 260 nm. The integrity of genomic DNA was confirmed through 0.8% agarose gel electrophoresis. Partially conserved functional gene *formyltetrahydrofolate synthetase (fhs)* in acetogens was amplified using specific forward and reverse primer. For the amplification of *fhs* functional gene, FTHFS_f (5'-TTYACWGGHGAYTTCCATGC-3') and FTHFS_r (5'-GTATTGDGT YTTRGCCATACA-3') primers described by Leaphart and Lovell (2001) and Gagen *et al.* (2010) were used. Reaction volume of 25 µl comprising 1×PCR buffer, 0.25 mM $MgCl_2$, 250 µM of each DNTP's, 1µM forward primer, 1µM reverse primer, 1.5U of Dream *Taq* DNA polymerase and ~60 ng of template DNA and nuclease free water was prepared for PCR amplification. Amplification of *fhs* functional gene, a modified touchdown PCR protocol

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from the method of Leaphart and Lovell (2001); Matsui *et al.* (2008) was developed in laboratory. The details of modified touchdown PCR protocol are as follows: 94°C for 3 min followed by 94°C 30 sec, 56°C 30 sec (0.5°decrease/cycle; 8 cycles), 72°C for 60 sec and then 22 cycles with 94°C 30 sec; 52°C 30 sec and 72°C 60 sec and finally 72°C 7 min. PCR product was purified using GeneJET purification kit to get rid from unused primers, dNTP's, enzymes, salt etc. Quality of amplified product was confirmed on 1% agarose gel before processing the amplicons for transformation and cloning. PCR product was transformed and cloned into *E.coli* D5á and plasmid pGEM-T easy vector following the standard protocols. Positive clones were screened and picked from library and thereafter plasmid was isolated from *E. coli* using JeneJET plasmid miniprep kit following the manufacturer's instructions. The *fhs* gene insert was released from plasmid by restriction digestion with *Eco*R1 followed by confirmation of release through 1.5% agarose gel electrophoresis. Positive clones were sequenced with primers T_7 and SP_6 using a BigDye terminator according to the manufacturer's instructions. Sequences were determined by using ABI3730XL sequencer and sequence similarity to *fhs* was determined by BLASTX analysis. DNA sequences were translated before alignment using ExPASy online tool. Bioinformatic analysis of nucleotide sequences was done and Bootstrap consensus phylogenic tree was constructed using Parsimony for the diversity analysis of Indian sheep acetogens with other known acetogens. Phylogenetic analyses were conducted in MEGA4.

RESULTS AND DISCUSSION

Genomic DNA isolation and amplification of fhs gene: Good quality undegraded genomic DNA was obtained from the rumen liquor samples obtained from 3 adult sheep. Amplification of *acs*/*fhs* functional gene in the isolated genomic DNA was tried using the primers viz. ACS_f/ACS_r; ACSf1/ACSr1; FTHFS_f/FTHFS_r and fhs1f/fhs1r, however, finally FTHFS_f/FTHFS_r combination was selected for the amplification of partial *fhs* functional gene of rumen acetogens. On the amplification of genomic DNA using FTHFS_f/FTHFS_r primer single clear band was obtained from all the samples. A band of ~1.1Kb size was appeared from the amplicons as confirmed through agarose gel electrophoresis. We also tried ACS_f/ACS_r; ACSf1/ACSr1 primers combination in our lab to amplify the *acs* gene sequence from the isolated DNA samples obtained from sheet however, could not succeed to amplify the *acs* gene (an another gene conserved to reductive acetogens). On date only one report is available for the amplification of *acs* gene in rumen liquor samples of bovine rumen and tammar wallaby fore-stomach (Gagen *et al.* 2010). First report on the amplification of *formyltetrahydrofolate synthetase* (*fhs*) gene sequence is from Leaphart and Lovell (2001) who amplified this gene from the root of smooth cordgrass, *Spartina alterniflora* and from horse manure samples using FTHFS-f/r primer. Matsui *et al.* (2008); Gagen *et al.* (2010); Henderson *et al.* (2010) also amplified partial *fhs* gene sequences using same primers. Similarly, Otteson and Leadbetter (2011) amplified the *fhs* gene sequence in termite with same primers. Large abundance of *fhs* gene in sheep rumen fed on conventional roughage based diet was confirmed through the agarose gel electrophoresis.

Phylogenic diversity analysis: Bootstrap consensus phylogenic tree was constructed from *fhs* sequences using Maximum Parsimony method. For comparison, *Thermoplasma acidophilum* (*AL445067*) was used as out group reference while 16 others well known acetogens and sulfate reducing bacteria from bovine rumen or termite were used for group reference to compare the acetogens present in adult sheep. Layout of phylogenic tree is given in Fig. 1. Sixty clones were picked from the library and accommodated under *act*1 to *act*10 on the basis of their sequence along with other sequences of acetogens submitted to the Gene bank.

From the phylogenic tree it may be inferred that *act*1 group is distant to all other clones (*act*2 to *act*10) and represent a distinct uncultured acetogens genera. The *act*3 and *act*5 are also present in different clusters and are distant to *act*2, *act*7 and *act*9 are closer. Similarly, *act*10, *act*4 and *act*6 are closer to each other in the phylogenic tree but distant from *act*1. On comparison with other known rumen acetogens, it was found that *act*1 clone group had 100% similarity with *lcF*78 (HQ186617) uncultured and *lcF*108 (HQ186574) uncultured acetogens from gnotobiotic lambs in Australia. Similarly, *act*2 group of clones from Indian sheep also showed 100% similarity with *Blautia hydrogenotrophica* DSM 10507. Phylogenic diversity analysis envisaged that most of clone group except *act*1, *act*2 and *act*5 showed limited similarity with known acetogens like *Acetitomaculum ruminis, Eubacterium limosum, Acetobacterium woodii* and other uncultured acetogens. Clone group *act*5 showed 99% similarity with uncultured clone *fnb*11 (AB085423) and 72% resemblances to PastF6 uncultured. From the phylogeny tree it is clear that majority of the acetogens (>70%, Fig. 1) exist in Indian adult sheep sustaining on roughage diet are different from the cultured or uncultured rumen acetogens reported in sheep or cattle elsewhere in Australia, New Zealand and Japan. The deviation in acetogens population in Indian sheep from the reported acetogens may be attributed to entirely different feeding practices in the country and to geographical location also accountable for the variable rumen microbial diversity.

PCR based amplification of *fhs* functional gene revealed the presence of acetogens in adult sheep fed on conventional roughage diet. From the phylogenic analysis it may be concluded that majority of the acetogens in Indian sheep are different from those reported earlier and there is a need to screen our livestock for the acetogens and to culture them for exploring the prospects of their competitiveness to rumen methanogens from enteric methane mitigation point of view.

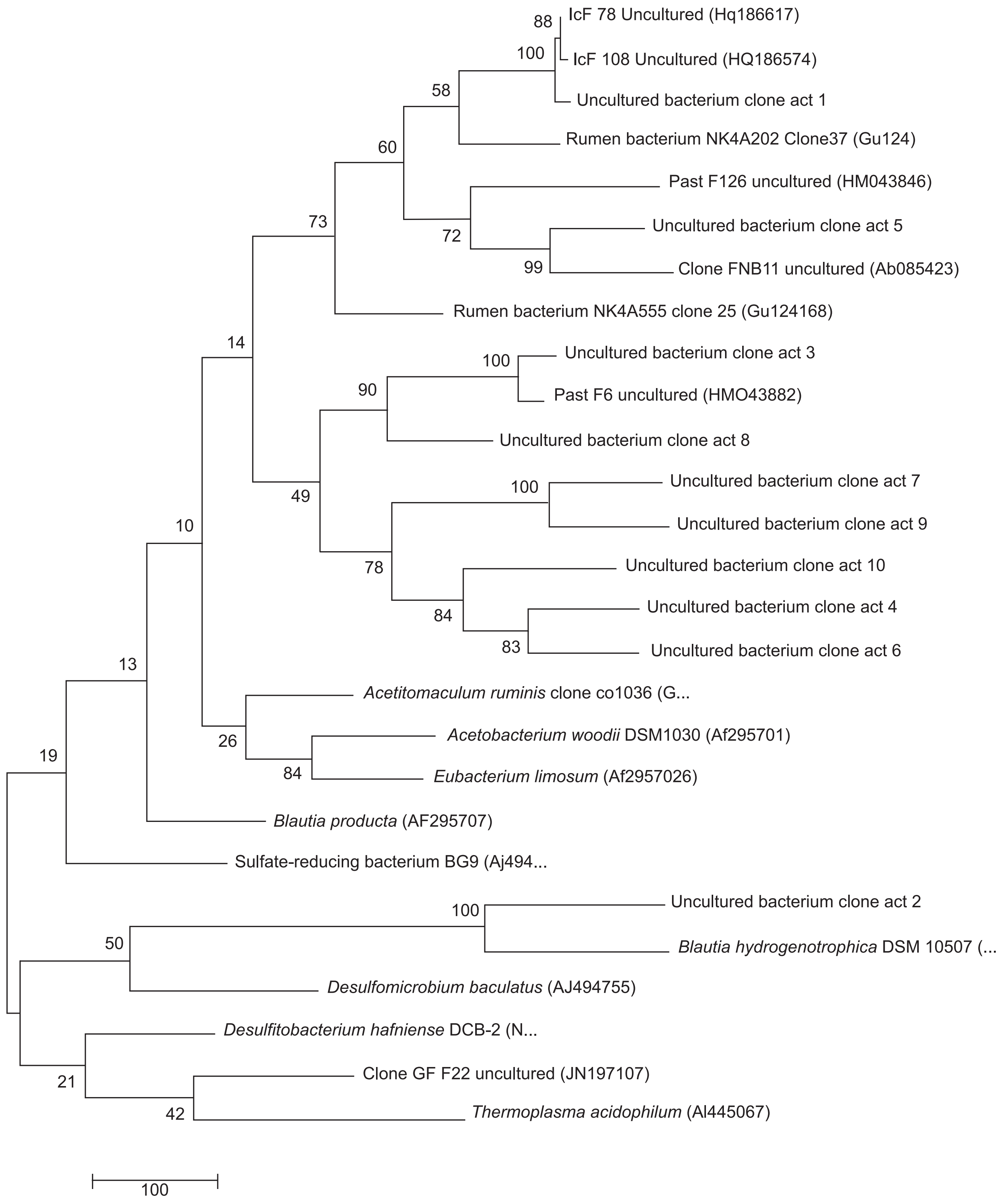

Fig. 1. Bootstrap consensus phylogenic tree using Parsimony. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3.

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