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Distinctive Archaebacterial Species Associated with Anaerobic Rumen Protozoan *Entodinium caudatum*

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ABSTRACT. The diversity of archaebacteria associated with anaerobic rumen protozoan *Entodinium caudatum* in long term *in vitro* culture was investigated by denaturing gradient gel electrophoresis (DGGE) analysis of hypervariable V3 region of archaebacterial 16S rRNA gene. PCR was accomplished directly from DNA extracted from a single protozoal cell and from total community genomic DNA and the obtained fingerprints were compared. The analysis indicated the presence of a solitary intensive band present in *Entodinium caudatum* single cell DNA, which had no counterparts in the profile from total DNA. The identity of archaebacterium represented by this band was determined by sequence analysis which showed that the sequence fell to the cluster of ciliate symbiotic methanogens identified recently by 16S gene library approach.

Abbreviation
DGGE denaturing gradient gel electrophoresis

Ruminal methane is considered a loss of dietary energy for ruminant livestock (Blaxter and Clapperton 1965). In the rumen, methane is formed by methanogenic archaea (methanogens), an important part of the microbial ecosystem. Methanogenesis itself is an important metabolic activity of the rumen microflora because ruminal methanogens contribute to elimination of reducing equivalents produced by fermentative hydrogen-producing bacteria, anaerobic fungi and ciliate protozoa (Šurín *et al*. 2006). It is suggested that the activity of ciliate protozoa is significantly related to the rumen methanogenesis. Indeed, the elimination of ciliate protozoa from the rumen may prevent 30–45 % of ruminal methane emissions (Jouany 1991) and a close relationship between ruminal methanogens and ciliate protozoa was proposed by several authors. Some of the reports provide direct evidence for association of methanogens with anaerobic protozoa (Vogels *et al*. 1980; Finlay e*t al*. 1994). According to these authors, the methanogens are found in both endo- and ectosymbiotic associations with protozoa and the key to the symbiotic relationship is H_2 transfer between protozoa and methanogens.

Several species of methanogens have been isolated from ruminants and identified by culture methods (Jarvis *et al.* 2000) and most recently by a range of molecular techniques developed to obtain information on microbial populations in natural habitats. Consistent with these results, *Methanobacteriaceae* was the best-represented methanogenic family both in the rumen fluid (92 % of *Archaea*) and in the protozoal fraction (99 %), suggesting that members of this family thrive both as free-living cells and in protozoal association (Sharp *et al.* 1998).

The aim of our study was to analyze the archaeal community associated with the prominent and the smallest anaerobic rumen ciliate *Entodinium caudatum* in long term *in vitro* culture. The culture-independent DGGE fingerprinting method was used for this purpose.

MATERIAL AND METHODS.

Organism and culture conditions. The rumen ciliate *E. caudatum* was isolated from sheep rumen fluid. Incubation of the whole protozoal culture (protozoa plus bacteria) was done according to Kišidayová (2000).

Single cell DNA isolation. A single protozoal cell of *E. caudatum* was picked under the microscope from an *in vitro* culture, washed twice in a drop of sterile water and put into 50 μL solution of 5 % Chelex-100 (*BioRad*, USA) in water. Proteinase K (*Merck*, Germany) was then added to the reaction mixture to a final concentration of 20 μg/mL. After proteinase treatment (30 min, 55 °C), DNA was released from

the cell by heating the sample for 5 min at 98 °C. After rapid cooling to 0 °C and centrifugation (3000 ***g***, 5 min) an aliquot (10 μL) of the supernatant was used directly for PCR amplification.

Total DNA isolation. Genomic DNA (archaeal and eubacterial) was extracted from 10 mL of *in vitro* culture of *E. caudatum* by a standard sodium dodecyl sulfate lysis and subsequent chloroform extractions according to Pospiech and Neumann (1995).

PCR amplification. Nearly full length archaeal 16S rDNA was amplified by PCR using the primers ArcF7 and ArcR1326 (van Hoek *et al*. 2000). The PCR with 35 thermal cycles was performed (45 s 94 °C, 45 s 50 °C, 1.5 min 72 °C) with an initial denaturation (5 min 94 °C) and a final extension (10 min 72 °C). Amplification products were visualized by electrophoresis in 0.8 % agarose gels (Maniatis *et al*. 1982) and further used as a template (10× diluted) to amplify the variable V3 region of archaeal 16S rDNA. PCR amplification of 16S rRNA V3 region was carried out by using the primers of Muyzer *et al*. (1993), 344F (GC) and S-*-Univ-0518-a-A-17. Amplification was done using the following steps: an initial denaturation step (5 min 94 °C) followed by 35 cycles of denaturation (45 s 94 °C), annealing (1 min 55 °C), extension (30 s 72 °C) and a final cycle step (10 min 72 °C). In both cases PCR amplification was done in Techne Progene thermal cycler (*Techne*, UK) in a 50 μL PCR mix containing either 250 ng of purified genomic DNA or 1 μL of 10× diluted PCR product from amplification of the full-length archaeal 16S rDNA of a single cell, 200 μmol/L of each dNTP, 1× reaction buffer, 1.25 U Platinum Taq DNA polymerase (*Invitrogen*, USA) and 25 pmol of each primer. PCR amplification products were visualized on 1 % (*V*/*W*) agarose gels prior to DGGE analysis.

DGGE conditions. DGGE was performed by using the *BioRad* DCode Universal Mutation Detection System according to manufacturer's guidelines. PCR products (40 μL) were loaded onto 8 % (*W*/*V*) polyacrylamide gels containing 35–60 % denaturant gradient (where 100 % denaturant contains 7 mol/L urea and 40 % formamide). Electrophoresis was performed in 1× TAE (40 mmol/L Tris, 20 mmol/L acetate, 1 mmol/L EDTA) buffer at a constant voltage of 50 V for 17 h at 60 °C. After the completion of electrophoresis, the gels were stained in an ethidium bromide (0.5 μg/mL) and documented with a MiniBis UV Vis documentation system (*Micro Photonics*, USA).

Cloning, sequencing and phylogenetic analysis. For extraction of DNA from DGGE gel, bands were cut from the gel using a clean scalpel and transferred to a microcentrifuge tube. DNA was recovered from polyacrylamide gel slices by the addition of 50 μL of water to each tube, which was then vortexed for 5 s and centrifuged for 1 min. The supernatant was transferred to a clean tube and used as template in a final PCR reaction. The primers used in this PCR were 344F (GC) and S-*-Univ-0518-a-A-17, as mentioned *above*. PCR products were cloned into the plasmid vector (pCR2.1) using a TOPO TA cloning kit (*Invitrogen*). Recombinant clones were selected and plasmid DNA extracted using a plasmid Miniprep kit (*Qiagen*, USA). Sequencing was performed on both strands and sequence obtained was used further in phylogenetic analyses.

The sequence of DGGE band was submitted to the *GenBank* nucleotide sequence database under the accession number EF657886.

RESULTS AND DISCUSSION

DGGE in its various forms has been used to investigate the ecology of microorganisms in different environments, including the study of bacterial and protozoal populations in the rumen (Kocherginskaya *et al*. 2001; Regensbogenova *et al*. 2004*a*). We used this method for determining the diversity of methanogenic archaea associated with anaerobic protozoan *E. caudatum* in long-term (2 years) *in-vitro* culture. The archaebacterial 16S rRNA gene of the methanogenic symbionts of the above anaerobic ciliate was amplified by PCR on DNA from single ciliates and from total community (protozoa and archaea) using archaea-specific primers. The amplicons of ≈180 bp spanned the hypervariable V3 region of 16S rDNA and were used for DGGE analysis (Fig. 1).

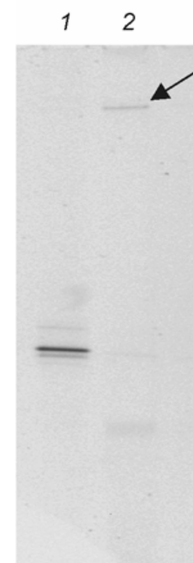

Fig. 1. DGGE separation of the V3 region of archaebacterial 16S rRNA genes amplified from total community DNA (lane *1*) and from single protozoal cell of *E. caudatum* (*2*) using primers 344F (GC) and S-*-Univ-0518-a-A-17; *arrow* indicates DGGE band used for further analysis.

Band profiles did not reveal any correlation between methanogen fingerprint of the total community DNA and of the single protozoal cell. The fingerprint of the single cell of *E. caudatum* showed one intensive DGGE band only. The identity of the organism represented by this solitary band was determined by sequencing the band excised from the gel, re-amplifying and cloning. Identical sequences were obtained for two randomly picked clones.

Phylogenetic affiliation of the obtained sequence was analyzed by its comparison with the *GeneBank* database and the phylogenetic tree was constructed (by using the Neighbor-joining method). A separated cluster was obtained of ciliate symbiotic methanogen with significant phylogenetic distance (Fig. 2). Sequence analysis showed that sequence obtained is closely related to the group of methanogenic *Archaea* identified earlier by 16S gene library approach showing very low similarity with the sequences in database (Regensbogenova *et al*. 2004*b*). The absence of the DGGE band representing identified methanogen in the total

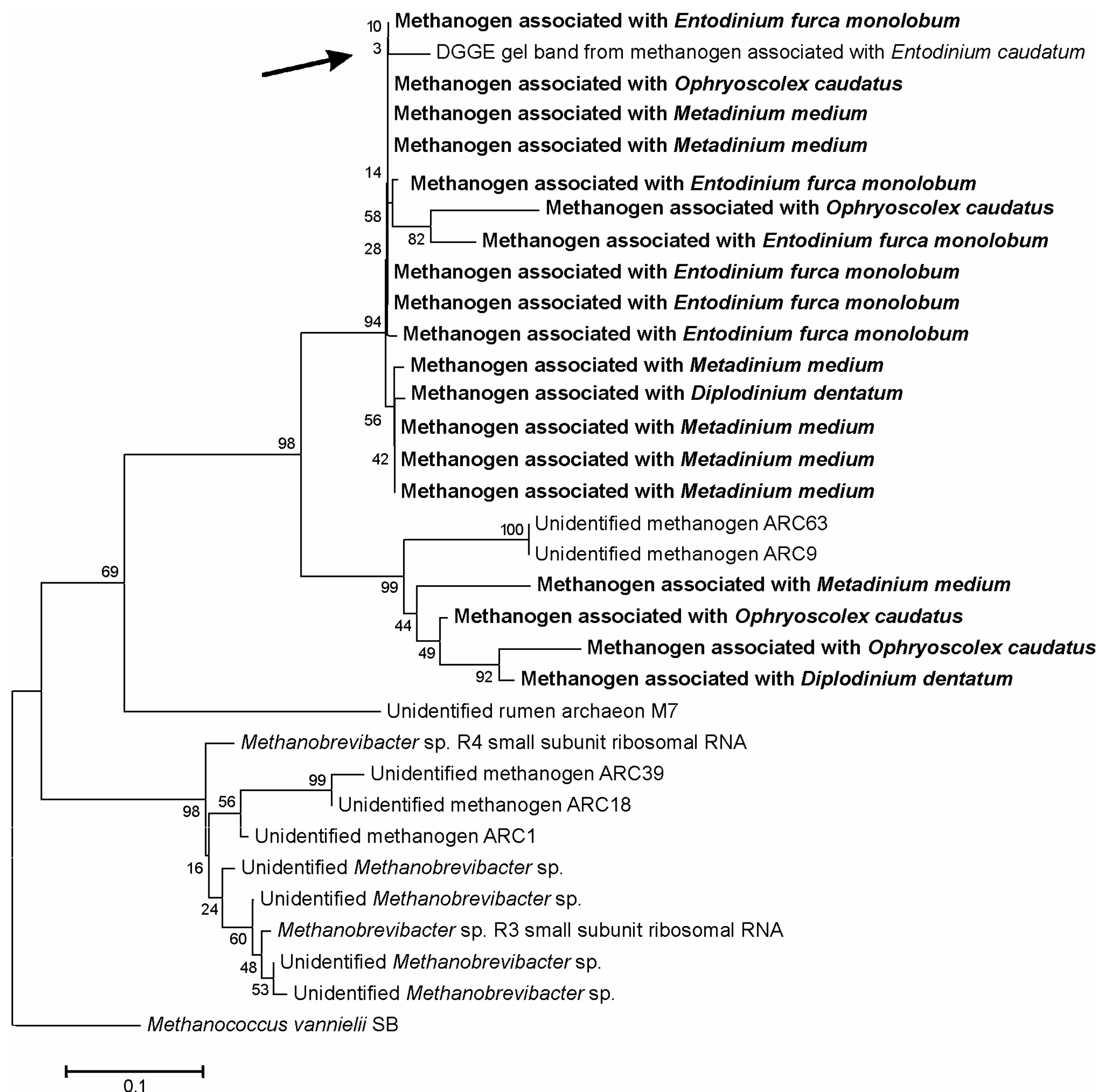

Fig. 2. Phylogenetic relationship of ruminal archaeal 16S rDNA sequence (indicated by *arrow*) retrieved from single protozoal cell of *E. caudatum*; protozoal archaesymbionts are shown in **bold**; *numbers at nodes* are bootstrap values (in %) that measure the reliability of the derived tree.

community DNA indicates that this methanogen does not occur in the free-living bacterial community in studied *in vitro* protozoal culture. Considering our results, this methanogen is phylogenetically distinct from known methanogens and probably represents the dominant methanogen endosymbiont specific to the rumen ciliate *Entodinium caudatum*.

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