

Limited Genetic Variability in *Megasphaera elsdenii* Strains

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ABSTRACT. Levels of phenotypic and genotypic diversity among seven *Megasphaera elsdenii* strains recovered from rumen contents of cattle, sheep and lambs were determined by a combination of antibiotic-resistance analysis and PCR fingerprint techniques targeted both to the ribosomal RNA operon (ARDRA, RISA) and the whole genome (ERIC-PCR, RAPD-PCR). Despite exhibiting different antibiotic resistance profiles, the tested strains represent genetically nearly identical isolates. Close genetic relatedness was found among *M. elsdenii* isolates that originated from vastly different habitats worldwide, as revealed by the comparison of 16S rDNA sequences.

The rumen of herbivores is one of the most extensively and best studied microbial ecosystems containing a diverse and competitive population of anaerobic bacteria, fungi, protozoa and even bacteriophages. In the past 50 years, all major representatives of rumen bacterial microflora have been identified and characterized morphologically and nutritionally. However, recent phylogenetic analyses using modern methods of bacterial classification based on comparison of 16S rRNA genes have indicated that the diversity of rumen bacteria has been greatly underestimated (Krause and Russel 1996). At present, various approaches at molecular level are used for the analysis of ruminal bacterial inter- and intraspecies variability. Unfortunately, genetic studies have been initiated only with some predominant rumen species such as *Selenomonas ruminantium* (Zatkovič *et al.* 2000) or rumen bacteria of CFB phylum (Ramsak *et al.* 2000) and there is scarce information on metabolic and genetic diversity at the intraspecies level of the less abundant ruminal bacteria, including *Megasphaera elsdenii*.

M. elsdenii (formerly *Peptostreptococcus elsdenii*) is a Gram-negative, obligately anaerobic, non-motile coccus that ferments soluble sugars and lactate. It is found mainly in the rumens of young animals (cattle, sheep) and in animals receiving high grain rations in which the fermentation of lactate assumes particular importance (Stewart and Bryant 1988). It can also be found in intestinal contents of humans and pigs (Giesecke *et al.* 1970; Marounek *et al.* 1989). *M. elsdenii* is considered to be the predominant lactate-utilizing species, fermenting up to 97 % of ruminal lactate (Counotte *et al.* 1981). In addition to fermenting lactate, it is thought to play a major role in the production of branched-chain volatile fatty acids in the rumen (Allison 1978).

Previous studies described basic metabolic characteristics (Forsberg 1978), metabolism of lactate (Sanseverino *et al.* 1989) and role of this microorganism in rumen ecosystem. To date, information about genetic analyses of *M. elsdenii* is scarce (Piknová *et al.* 2004a). The aim of our work was to investigate the intraspecies genetic variability of seven *M. elsdenii* isolates found in rumen contents of Slovak cattle, sheep and lambs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. elsdenii* strains were isolates from the rumen contents of lamb (3J, 5J, 7J), calf (2T, 5T) and sheep (4O, 5O), and were previously characterized (Piknová *et al.* 2004b) in our laboratory according to their ability to utilize saccharides, analysis of fermentation end products and Gram staining (Holdeman *et al.* 1973). All cultivations were done anaerobically (Piknová *et al.* 2004b).

Antibiotic sensitivity of the bacterial strains toward different antibiotics was studied using commercial Sensi-La disks (*Lachema*, Czechia). The following antimicrobial compounds (in µg or U per disk) were examined: chloramphenicol (30), tetracycline (30), erythromycin (10), oxacillin (10), polymyxin B (100 U), colistin (10), kanamycin (30), penicillin (10 U), gentamicin (10), ampicillin (10), neomycin (30) and streptomycin (30). According to the manufacturer's protocol, the growth-inhibition zone around the antibiotic disk was measured and the response was classified as resistant, variable or sensitive. Antibiotic susceptibility and/or resistance profiles were assessed by using the Kirby–Bauer disk diffusion method.

Polymerase chain reaction. Total (genomic) DNA was purified according to Pospiech and Neumann (1995). PCR amplification was done in 50 µL reaction mixture (Piknová *et al.* 2004c). Universal eubacterial primers fD1 and rP2 (Weisburg *et al.* 1991) were used for amplification of 16S rRNA gene. The primers IGSFor (5'-TGG GGT GAA GTC GTA ACA AGG TA-3') and IGSRev (5'-TAC TTA GAT GTT TCA GTT CC-3') (Gürtler and Stanisich 1996) were used for the study of 16S–23S spacer-region polymorphism (RISA). ERIC (enterobacterial repetitive intergenic consensus)-PCR analysis was done according to Zatkovič *et al.* (2000) using ERIC1R and ERIC2 primer pair. RAPD-PCR (random amplified polymorphic DNA) analysis was done with OPD-01, OPD-12, OPD-15, OPD-17 and OPD-20 primers, obtained from *Qiagen Operon* (Germany). Cycling conditions were as follows: 16S rRNA gene and RISA – 35 cycles (93 °C 30 s, 40 °C 45 s, 72 °C 2 min); RAPD – 42 cycles (94 °C 1 min, 36 °C 1 min, 72 °C 2 min). Restriction analysis of amplified 16S rDNA (ARDRA) was done with restriction endonucleases *Nla*III, *Mse*I, *Sau*3AI, *Hae*III or *Taq*I (*New England Biolabs*, USA) according to the manufacturer's instructions.

Sequence analysis and comparisons. The amplified 16S rDNA sequence was cloned into pKRX vector using the TA cloning procedure and sequenced by dideoxy termination method in *MWG Biotech DNA Sequencing Laboratory* (Germany). Homology searches were done using the BLAST search algorithm (Altschul *et al.* 1990). The sequences were aligned by Clustal W program, DNA distances were calculated with the DNAdist program and phylogenetic tree was generated using a neighbor-joining method implemented in BioEdit software package (Hall 1999).

The partial *M. elsdenii* 5T 16S rDNA sequence was deposited in the *GenBank* database under accession no. DQ146765.

RESULTS AND DISCUSSION

Levels of phenotypic and genotypic diversity among seven *M. elsdenii* strains recovered from rumen contents of Slovak cattle, sheep and lambs were determined by a combination of antibiotic resistance analysis and PCR fingerprint techniques targeted both to the ribosomal RNA operon – ARDRA (amplified ribosomal DNA restriction analysis), RISA – and the whole genome (ERIC-PCR, RAPD-PCR).

Antibiotic sensitivity patterns. All seven strains tested were sensitive to chloramphenicol, polymyxin B, colistin and ampicillin. However, the strains exhibited substantial differences in resistance to 8 other antimicrobials including tetracycline, erythromycin, oxacillin, kanamycin, penicillin, gentamicin, neomycin, and streptomycin (*not shown*).

Variability at rRNA operon level. The universal eubacterial primers were used for amplification of nearly full-length 16S rRNA gene producing the specific band with a size of ≈1500 bp. ARDRA was carried out on the amplified DNA of each sample with the tetranucleotide restriction enzymes *Hae*III, *Nla*III, *Mse*I, *Sau*3AI or *Taq*I. Restriction profiles (Fig. 1A) were identical, indicating a low discriminatory power of ARDRA analysis for *M. elsdenii* strains. Similar results were obtained with RISA analysis. The study of 16S–23S spacer-region polymorphism was shown to be a valuable tool for differentiation of bacteria at the species or even at the strain level (Garcia-Martinez *et al.* 1999). However, PCR amplification of 16S–23S intergenic spacer region resulted in the production of only two types of RISA profiles (*not shown*), indicating thus the limited genetic diversity of *M. elsdenii* strains.

Fingerprinting of M. elsdenii genomic DNA. Since 16S rDNA sequences are highly conserved among the species and useful especially to distinguish between distantly related organisms, two whole genome fingerprinting techniques were used for distinguishing the *M. elsdenii* strains. As the ERIC sequences were used to characterize different Gram-negative and -positive bacterial strains, it was quite surprising that the PCR fingerprinting of *M. elsdenii* strains resulted in identical ERIC-PCR patterns (*not shown*). Similarly, limited variability was observed using RAPD DNA analysis (Fig. 1B).

16S rRNA sequence comparisons. The fingerprint data presented here showed striking genetic similarity among the *M. elsdenii* strains. On the basis of ARDRA, RISA, ERIC-PCR and RAPD fingerprint patterns, it could be assumed that all tested *M. elsdenii* strains, coming from three different herbivorous animals, represent nearly identical isolates. The phylogenetic analyses showed that the 16S rDNA sequence of *M. elsdenii* strain 5T is identical or nearly identical (differing in less than 5 nucleotides) to sequences of

M. elsdenii isolates that originate from vastly different habitats, e.g., from swine colon (strain 7-11), dairy cow rumen in USA (strain YJ-4), Australia (strain La03), and South Africa (strain ATCC 25940).

One could speculate that low genetic intraspecies variability reflects a nutrition strategy of *M. elsdenii*. This coccus is a well-known mutualist organism whose ecological niche lies at the intersection of various metabolic pathways of other rumen bacteria. It is a saccharide (glucose, fructose, mannitol; Stewart and Bryant 1988)-fermenting ruminal bacterium that can ferment amino acids (especially L-threonine and L-serine; Wallace 1986) but in the rumen its primary niche is lactate utilization. Due to a relatively invariable spectrum of substrates, comprising especially intermediates of various biochemical pathways, *M. elsdenii* may not be significantly affected by dietary changes and forced to adapt quickly to different energy

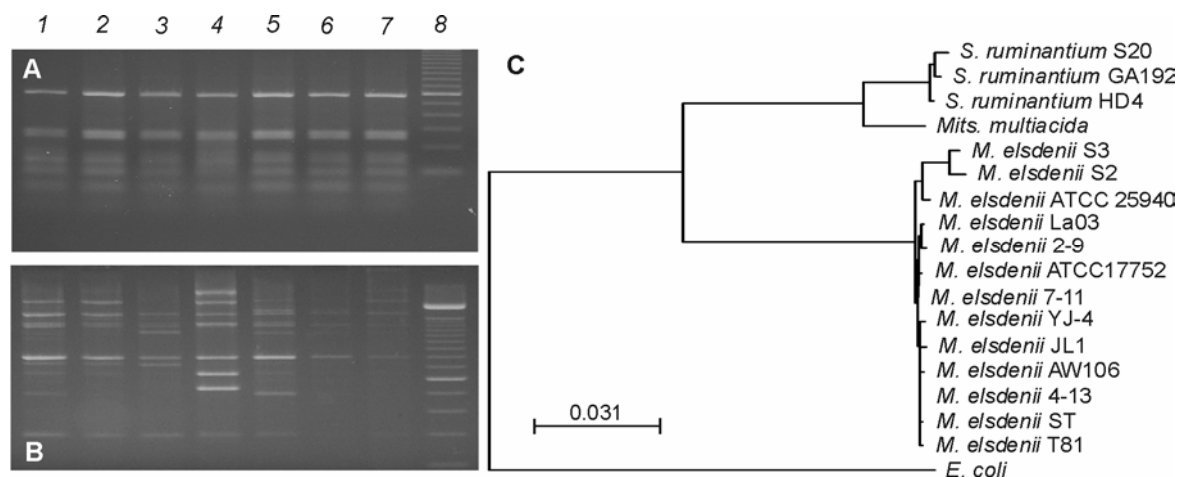


Fig. 1. Genetic relatedness of *M. elsdenii* strains. **A:** ARDRA patterns obtained after restriction of the amplified 16S rRNA genes with *Mse*I restriction endonuclease; **B:** RAPD profiles amplified from genomic DNA of *M. elsdenii* strains by using primer OPD-01. Strains: 1 – 40; 2 – 50; 3 – 2T; 4 – 5T; 5 – 3J; 6 – 5J; 7 – 7J; 8 – molar-mass standards (100-bp DNA ladder; *Invitrogen*, USA). **C:** phylogenetic tree inferred from 16S rRNA sequences showing the phylogenetic placement of *M. elsdenii* strain 5T; phylogenetic relationships were constructed by using neighbor-joining method; operational taxonomic units were obtained from the *GenBank* and RDP databases.

supplies compared to, e.g., the extremely genetically variable plant polysaccharide degraders (Ramsak *et al.* 2000; Kopečný *et al.* 2001). Alternatively, low genetic variability could indicate that *M. elsdenii* strains may have diverged recently from an ancestor strain and the phenotypic diversity observed is due to epigenetic factors, such as mobile gene elements (plasmids, transposons or bacteriophages), gene rearrangements and mutations that easily promote transfer of genes between phylogenetically homologous populations contributing to the genetic diversity.

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