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PHYLOGENETIC DIVERSITY OF BACTERIAL POPULATION IN RUMEN

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A B S T R A C T

In order to investigate the microbial diversity in rumen, bacterial 16S rRNA genes were amplified from total microbial DNA isolated from rumen fluid. The amplificated genes were incorporated into pBluescript SK II vector and electroporated into *E.coli* JM109 cells. The transformants, recognized in hybridization experiments (colony blot) by oligonucleotide DNA probe specific for the *Cytophaga-Flavobacterium* subgroup of the *Cytophaga-Flexibacter-Bacteroides* phylum and *Prevotella-Bacteroides* specific probe, were subjected to the RFLP analysis. On the basis of the RFLP profiles the Jaccard's similarity coefficients were calculated and the dendrograms were created using the UPGMA method. The representative clones were chosen from the established groups of clones and the 16S rDNA was partially sequenced, compared to already known rRNA sequences in data banks and analysed using Clustal and Phylip phylogenetic packages. A large genetic variability of the rumen bacteria belonging to the *Cytophaga-Flexibacter-Bacteroides* phylum was established.

Key words: microbiology / bacteria / molecular genetic / rRNA / phylogeny / rumen

FILOGENETSKA RAZNOLIKOST BAKTERIJSKE POPULACIJE V VAMPU

I Z V L E Č E K

Za preučevanje mikrobne raznolikosti v vampu smo z reakcijo PCR pomnožili bakterijske gene za 16S rRNK (16S rDNK) iz skupne DNK, izolirane iz vampnega soka, in jih vgradili v vektor pBluescript SK II. Z elektroporacijo smo vektor vnesli v *E. coli* JM109. Z analizo profilov RFLP smo ugotavljali raznolikost med nukleotidnimi zaporedji 16S rDNK transformant, ki so reagirale s sondo, specifično za podskupino *Cytophaga-Flavobacterium* filogenetske skupine *Cytophaga-Flexibacter-Bacteroides* in s sondo, specifično za skupino *Prevotella-Bacteroides*. Iz profilov RFLP smo izračunali Jaccardov koeficient podobnosti in z metodo UPGMA naredili fenograme. Na podlagi le teh smo iz skupine BAC-PRE in CFB-CF izbrali reprezentativne klone in ugotovili delna nukleotidna zaporedja za 16S rDNK. Zaporedja smo primerjali z že znanimi zaporedji v bankah podatkov in jih analizirali s programi iz računalniških paketov Clustal in Phylip. Z analizo restrikcijskih profilov in z analizo nukleotidnih zaporedij 16S rDNK smo ugotovili veliko raznolikost med pripadniki skupine *Cytophaga-Flexibacter-Bacteroides* v vampu.

Ključne besede: mikrobiologija / bakterije / molekularna genetika / rRNK / filogenija / vamp

INTRODUCTION

Rumen has been intensively studied by conventional microbiological techniques and a great variability within it was established. High population density, wide diversity, and complexity of interactions between rumen bacteria are its characteristics. Rumen contains large number of bacteria (up to 10^{11} viable cells/ml) comprising at least 200 species. Actually it seems that only 30 or 40 bacterial species make out the bulk of the bacterial biomass, carrying out the major part of the microbial digestion (Krausse in Russel, 1996). Molecular biology methods developed and used in recent years enabled detailed studies of the complex microbial habitats such as soil (Holben *et al*., 1988, Liesack and Stackebrandt, 1992, Stackebrandt *et al*., 1993), hydrothermal vents (Stahl *et al*., 1985), and also competitive ecosystems such as rumen (Stahl *et al*., 1988, Odenyo *et al*., 1994, Forster *et al*., 1997a, Forster *et al*., 1997, Whitford *et al*., 1998). The amplification of 16S rDNA genes from environmental samples by PCR reaction has proven to be an attractive technique for characterizing members of complex microbial communities. The subsequent comparison of 16S rDNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria in the rumen (Amann *et al*., 1995).

MATERIAL AND METHODS

Extracted total DNA from a rumen sample (Ramšak and Avguštin, 1995) was used as a target for specific 16S rDNA amplification in a PCR reaction using conserved bacterial primers fD1 (5' ccgaattcgtcgacaacagagtttgatcctggctcag3') (*E. coli* numbering 7-26, Weisburg *et al*., 1991) and 1492 (5'aagcttgcggccgctacggytaccttgttacgactt3') (*E. coli* numbering 1492-1513, Lane, 1991). The 1492 primer was modified to contain a recognizing sequence for *Not I* restriction enzyme (underlined sequence). The amplified genes for 16S rDNA were cloned into pBluescript II SK+ (Stratagene, La Jolla) and electroporated into *E. coli* JM 109 (Promega). Analysis of transformed cells was performed by colony blot hybridization using a broad range CF319a oligonucleotide probe (5' tgg tcc gtg tct cag tac3') (Manz *et al*., 1996) and more group specific BacPre oligonucleotide probe (5' tcaccgttgccggcgtactc3') (Avguštin *et al*., 1994). Oligonucleotide probes were labelled with digoxigenin. All clones recognised by the CF319a and BacPre probes were further analysed by restriction analysis using *Alu I*, *Dde I*, *Hha I* and *Taq I* endonucleases.

On the basis of RFLP profiles of clones from the BacPre and CFB CF groups Jaccard's and simple match coefficients were calculated. Phenograms were made with UPGMA method (NTSYS pc 1.80 package, Applied Biostatistics Inc., Rohlf, 1994). Representative clones from the established groups of clones were subjected to sequence analysis using 27f forward, 519r and 907r reverse primer (Lane, 1991). Sequence fragments were assembled with the GCG program package (ICGEB, Trieste, Italy). Putative chimeric sequences were identified using the program Check Chimera (Maidak *et al*., 1996). The sequences were automatically aligned (multiple sequence alignment) by Clustal V (Higgins *et al*., 1991). Phylogenetic trees were developed using a neighbour-joining method (Saito and Nei, 1987) included in the Phylip package (Phylogeny Inference Package version 3.57c, Felsenstein, 1993). In order to statistically evaluate the dendrograms, bootstrapping (Felsenstein, 1985) was carried out with data resampled 2000 times. *E. coli* 16S rRNA sequence was used as outgroup (accession number E05133). Kimura 2-parameter method (Jin and Nei, 1990) was used for DNA distance corrections.

RESULTS AND DISCUSSION

28 (app. 4%) of 640 recovered transformants hybridised with probe CF319a, specific for *Cytophaga-Flavobacterium* subgroup of *Cytophaga-Flexibacter-Bacteroides* phylum (CFB CF), and 52 (app. 8%) of transformants hybridized with probe BacPre, recognizing members of *Bacteroides* and *Prevotella* genera from the CFB phylum. Strains *P. ruminicola* 23^T, *P. albensis* M384, *P. bryantii* B_I4, TF1-3 and TS1-5 and *P. brevis* Fc4 and Fc6 (Avguštin *et al*., 1997) were included in hybridizing experiment and restriction analysis as positive controls. A considerable number of 16S rDNA restriction profiles was observed when analysing the transformants belonging to the *Cythophaga-Flavobacterium* and *Prevotella-Bacteroides* cluster, respectively. Transformants recognized by *Cytophaga-Flavobacterium* cluster specific probe were assigned into 14 clearly distinct groups and transformants recognized by *Prevotella-Bacteroides* probe into as many as 25 distinct groups respectively (Avguštin *et al*., 1997).

On the basis of both phenograms, transformants were chosen for cycle sequencing experiments using three different sequencing primers. 54 sequences were obtained from BacPre group and 42 sequences were obtained from CFB CF group of transformants. Multiple sequence alignments were made with determined sequences and with added most-similar sequences found in the GenBank.

The first tree shows the phylogenetic relations between the sequences from BacPre group (this study), sequences of the type strains of the species: *P. ruminicola* (strain 23^T), *P. brevis* (strain $GA33^T$), *P. bryantii* (strain B_I4) and *P. albensis* (strain M384) (Avguštin *et al*., 1994, Avguštin *et al*.,1997), and 11 sequences from unknown rumen bacteria deposited in the GenBank (Figure 1). The latter sequences were retrieved from a similar study made in Japan (sequences designated RF and RC, until now still unpublished). Multiple sequence alignments were made using sequences covering the V4 and V5 regions of 16S rRNA gene (the length of the sequences was approximately 350 bp).

Sequences of BacPre transformants were not too closely related with the sequences from type strains of different species from the genus *Prevotella*. Almost all formed clusters were supported by high values of confidence (bootstrap analysis, data not shown). One cluster consisted only from sequences obtained in this study (194p, 68p, 262p, 62p, 509p and 353p) and was clearly separated from all other sequences. 16S rRNA sequence from strain TC2-24, designated as *P. ruminicola* like (Avguštin *et al*., 1997), and 9 other sequences (7 from this study and 2 from Japan) formed another separated cluster. This finding seems particularly interesting since it shows that samples from geographically distinct locations do contain highly related organisms. The TC2-24 strain was designated previously as *P.ruminicola* like although it was quite different to the "true" *P.ruminicola* strains, merely because no other similar isolate was available. This study shows, however, that TC2-24 related organisms do represent a distinct, ubiqitous and presumably also abundant group, well worth classifying as separate species.

Three other major clusters were also formed. The first contained *P.ruminicola* type strain sequence and related sequences, while the second cluster contained *P.brevis* and *P.albensis* type strain sequences as well as sequences from the *P.ruminicola* like strain 223/M2/7 and several sequences obtained from PCR clone libraries (2 from this study). Finally, sequence of one transformant, 268p, was phylogenetically quite separated from all other sequences.

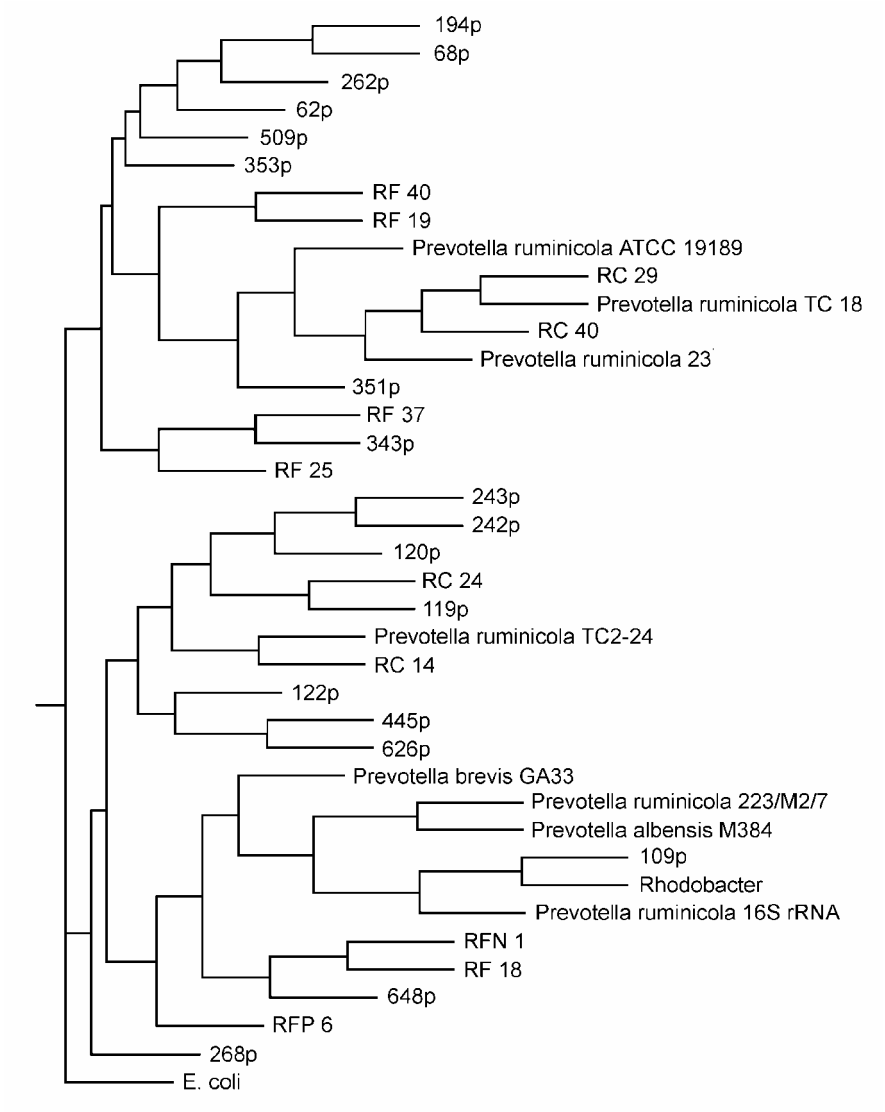

Figure 1. Unrooted phylogenetic tree constructed from sequences derived from region V4 and V5 16S rRNA genes.

Slika 1. Nerazvejeno filogenetsko drevo, narejeno iz nukleotidnih zaporedij iz regij V4 in V5 genov za 16S rRNK.

The second tree was generated including longer sequences (approx. 470 bp) from V1 and V2 regions of the 16S rRNA genes of the CFB CF and BacPre transformants and 62 sequences from phylogenetic subgroups *Bacteroides distasonis*, *B.fragilis*, *Prevotella* and from phylogenetic group *Sphingobacterium*. Sequences belonging to species *Selenomonas ruminicola*, *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens*, *Flavobacterium breve* and *Fibrobacter succinogenes* were also included. 8 sequences were derived from different strains of *P. ruminicola* isolated from the rumen, and 11 sequences from unidentified rumen bacteria.

The majority of the sequences derived from BacPre and CFB CF group transformants were clustered into one cluster comprising also sequences from *P. ruminicola* isolates and unidentified rumen bacteria derived from a Japanese study. The cluster was also supported with high confidence values (bootstrap method, data not shown). Only 4 sequences (73c, 550c, 648p, and 362p) were clustered separately, somewhat closer to sequences from *Prevotella* species inhabiting the human gastrointestinal tract.

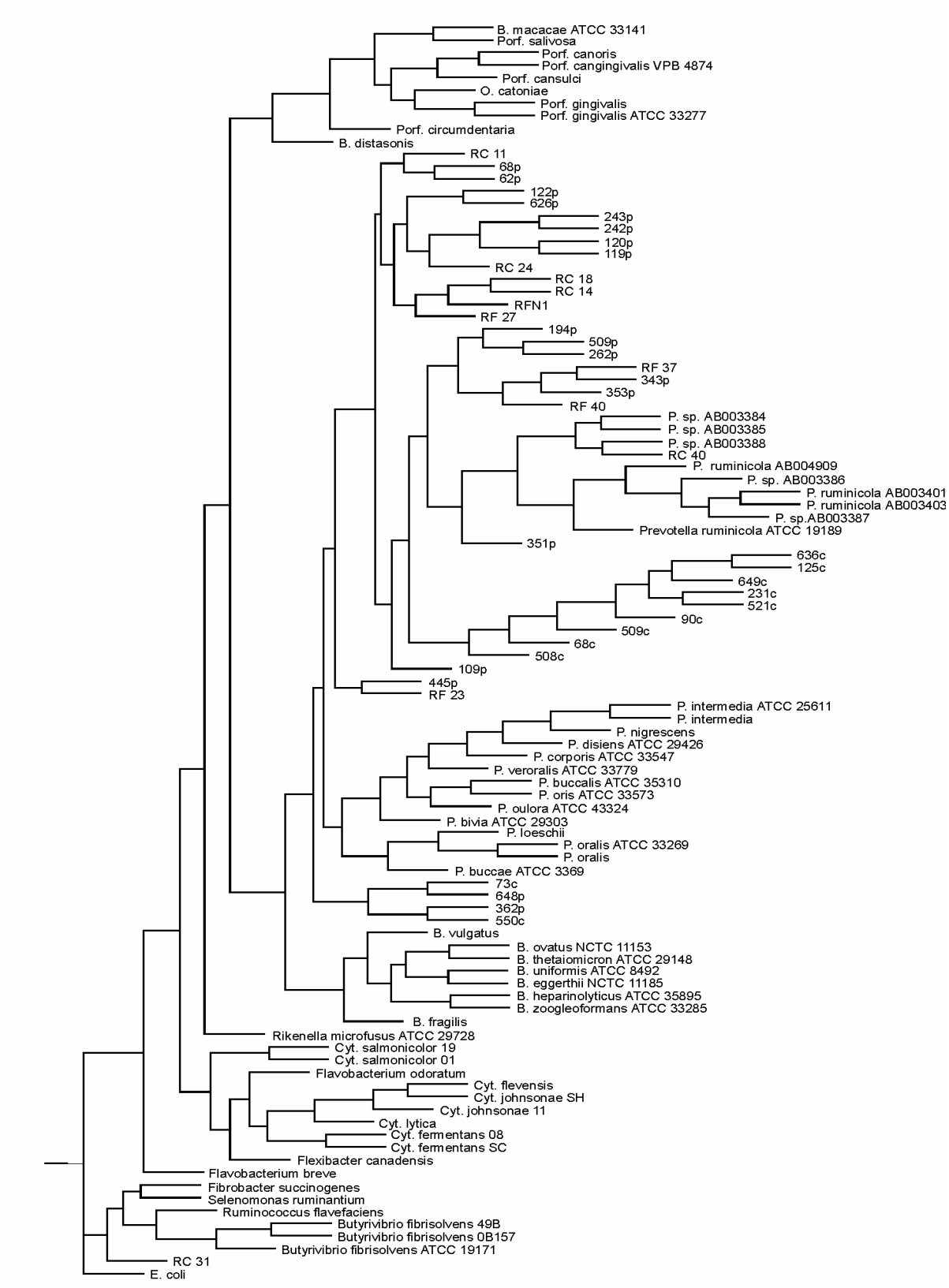

Figure 2. Unrooted phylogenetic tree constructed from sequences derived from region V1 and V2 16S rRNA genes.

Slika 2. Nerazvejeno filogenetsko drevo, narejeno iz nukleotidnih zaporedij iz regij V1 in V2 genov za 16S rRNK.

Clusters comprising sequences of *B. fragilis* and *B. distasonis* phylogenetic subgroups were phylogenetic homogenous and clearly separated from other sequences. Separated cluster was formed from sequences of the phylogenetic group *Cythophaga Flexibacter*. The phylogenetic arrangement of *B. fragilis*, *B. distasonis* and *Cytophaga* phylogenetic subgroups was checked with the phylogenetic tree in the RDP database, and the comparison showed the same clustering of described phylogenetic subgroups. The sequences from rumen bacteria *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens* 498, *B. fibrisolvens* OB157 and *B. fibrisolvens* ATCC 19171 formed a separated, phylogeneticaly most distinct cluster. Indeed, these sequences were clearly unrelated to the *Cytophaga-Flexibacter-Bacteroides* phylum.

The RFLP analysis of the 16S rDNA amplified from the total rumen DNA and data from sequencing analysis demonstrate clearly a great genotypic diversity of rumen microorganisms belonging to the *Cytophaga-Flexibacter-Bacteroides* phylum. The described study underlines the general underestimation of the microbial diversity in complex ecosystem such as rumen. Our results are in accordance with the recent findings about large and underestimated bacterial diversity in the rumen, established also on the basis of the ribosomal sequence analysis (Whitford *et al*., 1998) or RFLP data (Wood *et al*., 1998). Concentrating on only one phylogenetic group, it was possible to obtain a deeper insight into such important genera as *Prevotella* and *Bacteroides*. However, rumen Prevotellas comprise a cluster, distinct from the cluster comprising organisms inhabiting the human hindgut or oral cavity. It seems that rumen members of the genus *Prevotella* may well face yet another taxonomic rearrangement.

POVZETEK

Vamp je kompleksen in odprt ekosistem, gosto naseljen z raznoliko in kompetitivno mikrobno populacijo. Preiskovalci mikrobne raznolikosti v vampu, ki so temeljile na klasičnih tehnikah, so ocenili, da je v vampu več kot 200 bakterijskih vrst, od tega 22 prevladujočih (Krausse in Russel, 1996). Seveda so bile omenjene ocene precej ohlapne, povezane z zahtevo po izolaciji in gojenju teh organizmov "*in vitro*". Na osnovi analize nukleotidnih zaporedij iz genov za 16S rRNK (16S rDNK) lahko opišemo mikrobne združbe brez omejitev, na katere naletimo pri gojitvi in biokemični identifikaciji (Amann in sod., 1995).

Proučevali smo filogenetsko raznolikost bakterijske populacije v vampu z metodami molekularne biologije. Klonirano bakterijsko 16S rDNK smo uporabili v hibridizacijskih eksperimentih s sondo, specifično za skupino *Bacteroides-Prevotella* in s sondo, specifično za filogenetsko skupino *Cytophaga-Flavobacterium*. Nekatere od klonov smo izbrali za ciklično sekvenciranje in nukleotidna zaporedja nato uporabili za izdelavo filogenetskega drevesa z metodo združevanja najbližjega soseda. Restrikcijska analiza 16S rDNK, pomnožene iz celotne vampne DNK, in rezultati sekvenčne analize izražajo veliko genotipsko raznolikost med vampnimi mikroorganizmi, ki spadajo v filogenetsko skupino *Cytophaga-Flexibacter-Bacteroides* in poudarjajo veliko, a doslej podcenjeno mikrobno raznolikost v kompleksnem ekosistemu, kot je vamp. Naši rezultati so v skladu z novejšimi ugotovitvami nekaterih raziskovalcev, ki so opisali veliko bakterijsko raznolikost v vampu na osnovi nukleotidnih zaporedij (Whitford in sod., 1998) in polimorfizma restrikcijskih fragmentov (RFLP) (Wood in sod., 1998). Ugotovili smo, da večina nukleotidnih zaporedij, ugotovljenih v tej študiji, predstavlja dokaj sorodne vampne bakterije, ki pa se ločijo od *Prevotell* iz človekovega prebavnega trakta. Zanimivo je tudi opažanje, da so različni avtorji iz vzorcev, odvzetih na geografsko zelo oddaljenih lokacijah, pridobili klone z ribosomskimi geni zelo sorodnih bakterij.

REFERENCES

Amann, R. I./ Ludwig, W./ Schleifer, K-H. Phylogenetic identification and In Situ detection of individual microbial cells without cultivation. Microb. Rev., 59 (1995), 143-169.

Avguštin, G./ Wright, F./ Flint, H. Genetic diversity and phylogenetic relationships among strains of *Prevotella* (*Bacteroides*) *ruminicola* from the rumen. Int. J. Syst. Bacteriol., 44 (1994), 246-255.

Avguštin, G./ Wallace, J. R./ Flint, H. J. Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*. Int. J. Syst. Bacteriol., 47 (1997), 284-288.

Avguštin, G./ Ramšak, A./ Peterka, M./ Nekrep, F.V./ Flint, H.J. Evolutionary relationships and the diversity of the rumen bacteria belonging to the *Cytophaga-Flexibacter-Bacteroides* phylum. Reproduction, Nutrition and Development. Suppl. Evolution of the rumen microbial ecosystem., 1997, 27-28.

Felsenstein, J. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle 1993.

Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(1985), 783-791.

Forster, R. J./ Whitford, M. F./ Beard, C. E./ Gong, J. An investigation of microbial diversity in the rumen od dairy cattle using comparative sequence analysis of cloned 16S rRNA genes. Reproduction, Nutrition and Development. Suppl. Evolution of the rumen microbial ecosystem, (1997), 28-29.

Forster, R. J./ Gong, J./ Teather, R. M. Group-specific 16S rRNA hybridization probes for determinative and community structure studies of *Butyrivibrio fibrisolvens* in the rumen. Appl. Environ. Microbiol., 63 (1997), 1256-1260.

Higgins, D. G./ Bleasby, A. J./ Fuchs, R. Clustal V: improved software for multiple sequence alignment. CABIOS, 8(1991), 189-191.

Holben, W. E./ Jansson, J. K./ Chelm, B. K./ Tiedje, J. M. DNA probe methods for the detection of specific microorganism in the soil bacterial community. Appl. Environ. Microbiol., 54(1988), 703-711.

Jin, L./ Nei, M. Limitations of the evolutionary parsimony method of phylogenetic analysis. Molecular Biology and Evolution, 7(1990), 82-102.

Krausse, D. O./ Russel, J. B. How many ruminal bacteria are there? J. Dairy Sci., 79 (1996), 1467-1475.

Larsen, N./ Olsen, G.J./ Maidak, B.L./ McCaughey, M.J./ Overbeek, R./ Macke, T.J./ Marsh, T.L./ Woese, C.R. The ribosomal database project. Nucleic Acids Res., (1993)21, 3021.

Liesack, W./ Stackebrandt, E. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriology, 174(1992), 5072-5078.

Maidak, B. L./ Olsen, G. J./ Larsen, N./ Overbeek, R./ McCaughey, M. J./ Woese, C. R. The Ribosomal Database Project (RDP). Nucleic Acids Res., 24(1996), 82-85.

Manz, W./ Amann, R./ Ludwig, W./ Vancanneyt M./ Schleifer K-H. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology, 142(1996), 1097-1106.

Odenyo, A. A./ Mackie, R./ Stahl, D. A./ White, B. A. The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: development of probes for *Ruminococcus* species and evidence for bacteriocin production. Appl. Environm. Microbiol., (1994)60, 3688-3696.

Ramšak, A./ Avguštin, G. Izolacija nukleinskih kislin iz ekoloških vzorcev in *in vitro* pomnoževanje ribosomalnih genov: vamp. Zb.Biotehniške fak. Univ. v Ljubljani, Kmetijstvo (Zootehnika), 66(1995), 179-187.

Rohlf, J.F. NTSYS. Numerical taxonomy and multivariate analysis system. Exeter software, New York, 1994.

Saitou, N./ Nei, M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol.Biol. Evol., 4(1987), 406-425.

Stackebrandt, E./ Liesack, W./ Goebel, B. M. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J., 7(1993), 232-236.

Stahl, D. A./ Flesher, B./ Mansfield, H. R./ Montgomery, L. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol., 54(1988), 1079-1084.

Stahl, D. A./ Lane, D. J./ Olsen, G. J./ Pace, N. R. Characterization of a Yellowstone Hot Spring microbial commmunity by 5S rRNA Sequences. Appl. Environ. Microbiol., 49(1985), 1379-1384.

Weisburg, W. G./ Barns, S. M./ Pelletier, D. A./ Lane, D. J. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173(1991), 697-703.

Whitford, M. F./ Forster, R. J./ Beard, C. E./ Gong, J./ and Teather, R. M. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe, 4(1998), 153-163.

Woese, C. Bacterial evolution. Microbiol. Rev., (1987)51, 221.

Wood, J./ Scott, K. P./ Avguštin, G./ Newbold, C. J./ Flint, H. J. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Appl. Environ. Microbiol., 64(1998), 3683-3689.