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16S rDNA Analysis of Bacterial Diversity in Three Fractions of Cow Rumen

CHO, SOO JEONG[§], KYE MAN CHO[1], EUN CHULE SHIN[1], WOO JIN LIM[1], SU YOUNG HONG[1], BYOUNG ROCK CHOI[1], JUNG MI KANG[1], SUN MI LEE[1], YONG HEE KIM[1], HOON KIM[3], AND HAN DAE YUN[1,2]*

[1]*Division of Applied Life Science, Gyeongsang National University, Chinju 660-701, Republic of Korea*
[2]*Research Institute of Life Science, Gyeongsang National University, Chinju 660-701, Republic of Korea*
[3]*Department of Agricultural Chemistry, Sunchon National University, Sunchon 540-742, Republic of Korea*

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Abstract The bacterial diversity of the bovine rumen was examined using a PCR-based approach. 16S rDNA sequences were amplified and cloned from three fractions of rumen (solid, fluid, and epithelium) that are likely to represent different bacterial niches. A total of 113 clones were sequenced, and similarities to known 16S rDNA sequences were examined. About 47.8% of the sequences had 90–97% similarity to 16S rDNA database sequences. Furthermore, about 62.2% of the sequences were 98–100% similar to 16S rDNA database sequences. For the remaining 6.1%, the similarity was less than 90%. Phylogenetic analysis was also used to infer the makeup of the bacterial communities in the different rumen fractions. The *Cytophaga-Flexibacter-Bacteroides* group (CFB, 67.5%), low G+C Gram-positive bacteria (LGCGPB, 30%), and *Proteobacteria* (2.5%) were represented in the rumen fluid clone set; LGCGPB (75.7%), CFB (10.8%), *Proteobacteria* (5.4%), high G+C Gram-positive bacteria (HGCGPB, 5.4%), and *Spirochaetes* (2.7%) were represented in the rumen solid clone set; and the CFB group (94.4%) and LGCGPB (5.6%) were represented in the rumen epithelium clone set. These findings suggest that the rumen fluid, solid, and epithelium support different microbial populations that may play specific roles in rumen function.

Key words: Rumen, bacteria, molecular diversity, 16S rDNA, phylogeny

Previous studies designed to identify bacteria present in complex microbial communities such as the rumen have relied on traditional techniques, but have been hampered by the fastidious growth requirements of many bacteria and problems associated with the phenotypic criteria used to define microbial taxa of those culturable bacteria. Oligonucleotide probes targeting specific 16S rRNA sequences have been used to identify, quantify, and visualize ruminal bacterial populations [2, 7, 28, 32]. However, the probe sequences have been based on 16S rRNA sequences of organisms that successfully grow in culture collections.

The first use of comparative analysis of 16S rDNA sequences to examine the makeup of bacterial communities in the rumen was undertaken by Whitfort *et al.* [37]. Tajima *et al.* [33, 34] investigated the molecular diversity of rumen bacteria by PCR amplification and sequencing of 16S rDNA clone libraries prepared from the rumen fluid and solid of Holstein cows. The existence of distinct bacterial communities in the rumen was suggested by differences in the sequences found in the two different fractions of rumen content. Recently, Mitsumori *et al.* [23] detected *Proteobacteria*, including methanotrophs, in the rumen fluid and the rumen epithelium by PCR with methanotroph-specific primers. They concluded that these rumen fluid fractions and the rumen epithelium host different populations of *Proteobacteria* and suggested further that the rumen fluid and the epithelium generally support different microbial populations that would play specific roles in rumen function. A phylogenetic study, based on 16S ribosomal DNA sequences together with chemotaxonomic and genomic analyses, is one of the most useful methods for inferring the relationships between genera or between species belonging to a genus [9, 13, 36].

The goal of this study was to examine the phylogenetic diversity of the bacterial community in three putative niches of the bovine rumen (the rumen fluid, solid, and epithelium) by direct retrieval and analysis of 16S rDNA sequences in a culture-independent manner. To the best of our knowledge, this is the first characterization of rumen bacterial populations in three different fractions as rumen niches.

*Corresponding author
Phone: 82-55-751-5469; Fax: 82-55-747-0178;
E-mail: hdyun@nongae.gsnu.ac.kr

[§]Present Address; Laboratory of Microbial Functions, Bioprocess Engineering Division, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Escherichia coli DH5α and recombinant *E. coli* cells were cultured in Luria-Bertani (LB) broth containing ampicillin (50 μg/ml).

Sampling

Samples of rumen content were obtained from a closed herd at the Chinju National University (Chinju, Korea). The study used rumen-fistulous Korean cow (Hanwoo) with body weights of about 400 kg. The animal was fed twice daily with a mixed ration of rice hull and concentrated feed (purchased from Daehan Food, Ulsan, Korea) in 4:1 ratio. Three replicate samples of total rumen contents were collected for 3 days from the animal via the rumen fistula before the morning feeding. The samples were separated into rumen fluid for the first library construction, and feed particle fractions for the second library construction. To obtain these samples, the rumen contents were squeezed through two layers of cheesecloth, and the resulting rumen fluid fraction from the cow was pooled and sampled. The remaining feed particle sample from one cow was also pooled and subsampled. At the same time, microorganisms of three replicate samples were collected from the rumen epithelium of the dorsal sac by scraping with the spatula for 3 days, which were used for the third library construction.

Extraction of Total DNA

The collected rumen fluid (BF), rumen solid (BS), and rumen epithelium (BE) samples were centrifuged at 14,000 ×*g* for 5 min at 4°C [29, 30]. The obtained pellet (approximately 0.2 ml) was subjected to DNA extraction using the G-spin™ Genomic DNA Extraction Kit (Intron Biotechnology, Suwon, Korea), as recommended by the supplier. The extracted DNA was used as a template for PCR to amplify 16S rDNA.

PCR Amplifications

The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers, 5'-CGG-AGA-GTT-TGA-TCC-TGG-3'(#877, forward) and 5'-TAC-GGC-TAC-CTT-GTT-ACG-AC-3'(#878, reverse) [18]. Subsequently, rDNAs were amplified by PCR using the metagenomic DNA and Super-Therm DNA polymerase (JMR, Side Cup, Kent, U.K.). Based on the manufacturer's instruction, the PCR reaction mixture (50 μl) contained 1 μl of *Taq* polymerase (25 unit), 3 μl each of primers #877 and #878 (10 pmol), 5 μl of reaction buffer, 15 mM $MgCl_2$, 5 μl of 2 mM dNTP, 5 μl of template DNA, and 28 μl of sterile water. Fifteen cycles (denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec) were followed by a final incubation at 72°C for 10 min. The anticipated product of approximately 1.5 kb (Fig. 1) was isolated by agarose gel electrophoresis of the amplified mixture using a gel extraction kit (NucleoGen, Seoul, Korea).

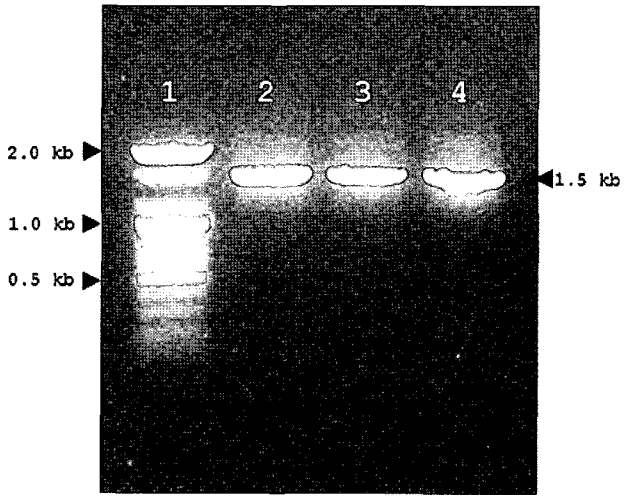

Fig. 1. Electrophoresis of PCR products when the primers #877F and #878R were used for the identification and detection of identified 16S rDNA.
Lane 1, 100 bp-ladder (Bioneer, Daejoen, Korea); lane 2, rumen fluid; lane 3, rumen solid; lane 4, rumen epithelium.

Cloning and Sequencing

PCR products were directly cloned into the pGEM®-T Easy vector (Promega, Midson, WI, U.S.A.), and recombinant colonies were randomly picked. Recombinant plasmids were extracted using the alkaline lysis miniprep method [5]. Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, U.S.A.). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing was done on full-length cloned PCR product. Assembly of the nucleotide sequences analysis was performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada).

Sequence Analysis

All reference sequences were obtained from the GenBank and RDP (Ribosomal Database Project) [22] databases. Sequences were analyzed using the CHECK_CHIMERA program [22] to identify and exclude sequences arising from chimeric rDNA clones. Similarity searches against database entries were carried out by online BLAST search [21]. Sequences were aligned using the multiple sequence alignment program, CLUSTAL W [35]. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using neighbor-

joining methods [27]. Bootstrap analysis was performed using data resampled 1,000 times using the DNAMAN analysis system.

Nucleotide Sequence Accession Numbers and Nomenclature

Nucleotide sequences have been deposited in the GenBank database under the accession numbers AY244879-AY244991. Clone names in the rumen fluid library begin with the letters BF (e.g. BF01), whereas the library with a BS prefix (e.g. BS01) represents the rumen solid, and a BE prefix represents (e.g. BE01) the rumen epithelium.

RESULTS

Detection and Cloning of Bacterial rDNA

Rumen samples were obtained from Korean cow. PCR amplification using total DNA prepared from rumen fluid (BF), rumen solid (BS), and rumen epithelium (BE) samples

Table 1. Similarity values of 16S rDNA sequences retrieved from the rumen fluid.

Clone (Accession No.)	Phylum	Nearest relative[d] (Accession No.)	Similarity (%)
BF01 (AY244879)	CFB[a]	URB[c] RFN29 (AB009187)	95
BF02 (AY244880)	LGCGPB[b]	URB 4C0d-2 (AB034016)	95
BF03 (AY244881)	CFB	URB RFN67 (AB009214)	97
BF04 (AY244882)	CFB	NA[c]	
BF05 (AY244883)	LGCGPB	URB 6C3d-34 (AB034084)	94
BF06 (AY244884)	CFB	NA	
BF07 (AY244885)	LGCGPB	URB RFN24 (AB009182)	**99**[f]
BF08 (AY244886)	CFB	NA	
BF09 (AY244887)	LGCGPB	URB RFN24 (AB009182)	99
BF10 (AY244888)	LGCGPB	URB RFN24 (AB009182)	99
BF11 (AY244889)	LGCGPB	URB RFN24 (AB009182)	99
BF12 (AY244890)	LGCGPB	URB 4C0d-2 (AB034016)	95
BF13 (AY244891)	LGCGPB	URB 4C0d-18 (AB034031)	97
BF14 (AY244892)	LGCGPB	URB RFN24 (AB009182)	99
BF15 (AY244893)	CFB	URB RF37 (AF001768)	99
BF16 (AY244894)	CFB	URB RFN64 (AB009211)	98
BF17 (AY244895)	CFB	URB RFN9 (AB009236)	98
BF18 (AY244896)	CFB	URB 12-12 (AF018458)	93
BF19 (AY244897)	CFB	URB RF19 (AF001751)	98
BF20 (AY244898)	CFB	URB 4C3d-5 (AB034103)	99
BF21 (AY244899)	CFB	URB RF37 (AF001768)	98
BF22 (AY244900)	CFB	URB 12-70 (AF018477)	98
BF23 (AY244901)	CFB	URB RFN29 (AF544206)	95
BF24 (AY244902)	CFB	NA	
BF25 (AY244903)	CFB	URB RFN79 (AB009226)	96
BF26 (AY244904)	CFB	URB RF31 (AF001763)	99
BF27 (AY244905)	CFB	URB JW7 (AF018440)	93
BF28 (AY244906)	CFB	URB 12-70 (AF018477)	98
BF29 (AY244907)	CFB	URB RC18 (AF001709)	96
BF30 (AY244908)	LGCGPB	Bacterium ASF500 (AF157051)	93
BF31 (AY244909)	CFB	URB RF19 (AF001751)	97
BF32 (AY244910)	LGCGPB	*Selenomonas ruminantium* (M62702)	98
BF33 (AY244911)	CFB	*Prevotella ruminicola* (AF218619)	94
BF34 (AY244912)	CFB	*Prevotella albensis* (AJ011683)	90
BF35 (AY244913)	Proteobacteria	*Stenotrophomonas maltophilia* (AJ293463)	98
BF36 (AY244914)	CFB	*Prevotella ruminicola* (AF218619)	91
BF37 (AY244915)	CFB	*Prevotella ruminicola* (AB003401)	99
BF38 (AY244916)	CFB	*Prevotella ruminicola* (AF218619)	95
BF39 (AY244917)	LGCGPB	*Ruminococcus flavefaciens* (X83430)	96
BF40 (AY244918)	CFB	*Prevotella* aff. *ruminicola* (AJ009933)	94

[a]CFB: *Cytophaga-Flavobacter-Bacteroides* group.

[b]LGCGPB: low G+C Gram-positive bacteria.

[c]NA: not available.

[d]Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence was given.

[e]URB: unidentified rumen bacterium.

[f]Database sequences with >97% similarity are shown in bold.

Table 2. Similarity values of 16S rDNA sequences retrieved from the rumen solid.

Clone (Accession No.)	Phylum	Nearest relative[e] (Accession No.)	Similarity (%)
BS01 (AY244955)	LGCGPB[b]	*S. ruminis* (X81137)	97
BS02 (AY244956)	LGCGPB	URB[f] RFN24 (AB009182)	**99**[g]
BS03 (AY244957)	LGCGPB	*S. ruminis* (X81137)	97
BS04 (AY244958)	LGCGPB	URB 3C0d-12 (AB034012)	94
BS05 (AY244959)	Spirochetes	NA[d]	
BS06 (AY244960)	LGCGPB	URB 4C0d-10 (AB034023)	97
BS07 (AY244961)	LGCGPB	URB 3C0d-12 (AB034012)	96
BS08 (AY244962)	LGCGPB	URB RFN24 (AB009182)	99
BS09 (AY244963)	CFB[a]	URB RC16 (AF001707)	94
BS10 (AY244964)	LGCGPB	URB RFN24 (AB009182)	98
BS11 (AY244965)	CFB	URB 12-80 (AB185735)	94
BS12 (AY244966)	CFB	NA	
BS13 (AY244967)	LGCGPB	*S. ruminis* (X81137)	95
BS14 (AY244968)	CFB	URB JW30 (AF018449)	97
BS15 (AY244969)	LGCGPB	URB RFN24 (AB009182)	99
BS16 (AY244970)	LGCGPB	URB RFN24 (AB009182)	99
BS17 (AY244971)	LGCGPB	URB 6C3d-34 (AB034084)	95
BS18 (AY244972)	LGCGPB	URB RFN24 (AB009182)	99
BS19 (AY244973)	LGCGPB	*S .ruminis* (X81137)	95
BS20 (AY244974)	LGCGPB	URB RFN24 (AB009182)	99
BS24 (AY244975)	LGCGPB	URB RFN24 (AB009182)	99
BS25 (AY244976)	LGCGPB	URB RFN24 (AB009182)	99
BS26 (AY244977)	LGCGPB	*Clostridium putrefaciens.* (AF127024)	99
BS27 (AY244978)	LGCGPB	*Clostridium putrefaciens* (AF127024)	99
BS28 (AY244979)	LGCGPB	*Carnobacterium* (AB083414)	99
BS29 (AY244980)	LGCGPB	*Clostridium algidicarnis* (AF127023)	99
BS30 (AY244981)	LGCGPB	*Clostridium putrefaciens* (Y18177)	99
BS31 (AY244982)	LGCGPB	*Clostridium putrefaciens* (Y18177)	99
BS32 (AY244983)	Proteobacteria	*Agrobacterium larrymoorei* (Z345545)	98
BS33 (AY244984)	LGCGPB	*Clostridium putrefaciens* (Y18177)	99
BS34 (AY244985)	HGCGPB[c]	*Olsenella uli* (AF292373)	92
BS35 (AY244986)	LGCGPB	*Clostridium algidicarnis* (AF127023)	99
BS36 (AY244987)	Proteobacteria	*Roseomonas genomospecies* 5 (AF533356)	98
BS37 (AY244988)	LGCGPB	*Clostridium algidicarnis* (AF127023)	99
BS38 (AY244989)	LGCGPB	*Clostridium putrefaciens* (Y18177)	99
BS39 (AY244990)	HGCGPB	*Rathayibacter rathayi* (D45062)	98
BS40 (AY244991)	LGCGPB	*Clostridium algidicarnis* (AF127023)	99

[a]CFB : *Cytophaga-Flavobacter-Bacteroides* group.
[b]LGCGPB: low G+C Gram-positive bacteria.
[c]HGCGPB: high G+C Gram-positive bacteria.
[d]NA: not available.
[e]Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence was given.
[f]URB: unidentified rumen bacterium.
[g]Database sequences with >97% similarity are shown in bold.

as template with bacterial-specific primers produced a single product of approximately 1.5 kb. The products from the three fractions were used to create three libraries. The product was isolated from an agarose gel, cloned into the pGEM®-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. A total of 167 clones was obtained, and 113 clones from the three libraries were sequenced (Tables 1, 2, and 3).

Similarity with Database Sequences

All clones were subjected to sequence analysis, followed by online homology searches using two databases; GenBank, which implements the BLAST algorithm [21], and the RDP database, which implements the SIMILARITY_RANK program [22] (Tables 1, 2, and 3). Although there are no exact 16S rDNA similarity limits for defining specific taxa such as genus and species, species definition in general requires sequence similarities greater than 98% [36]. Thus, if a sequence has greater than 98% similarity to a 16S rDNA of a known bacterium, it is considered to be a member of that species. In our libraries, only 19 sequences out of the 113 clones (16.8%) were identified as belonging to a particular genus and species. The following species were represented: *C. algidicarnis* (four clones), *C. putrefaciens*

Table 3. Similarity values of 16S rDNA sequences retrieved from the rumen epithelium.

Clone (Accession No.)	Phylum	Nearest relative[d] (Accession No.)	Similarity (%)
BE01 (AY244919)	CFB[a]	*Prevotella ruminicola* (AF218619)	92
BE03 (AY244920)	CFB	*Prevotella ruminicola* (AB003401)	**99**[f]
BE04 (AY244921)	CFB	URB[e] RFN26 (AB009184)	98
BE05 (AY244922)	CFB	URB 6C3d-25 (AB034102)	97
BE06 (AY244923)	CFB	URB RC18 (AF001709)	97
BE07 (AY244924)	CFB	URB 12-70 (AF018477)	98
BE08 (AY244925)	CFB	URB RFN78 (AB009225)	97
BE09 (AY244926)	CFB	URB RF20 (AF001752)	95
BE10 (AY244927)	CFB	URB 12-70 (AF018477)	98
BE11 (AY244928)	CFB	URB RF37 (AF001768)	99
BE12 (AY244929)	CFB	URB RF37 (AF001768)	96
BE13 (AY244930)	CFB	URB 12-70 (AF018477)	94
BE14 (AY244931)	CFB	URB RFN40 (AB009196)	90
BE15 (AY244932)	CFB	URB 6C3d-25 (AB034102)	93
BE16 (AY244933)	CFB	URB RFN67 (AF544208)	97
BE17 (AY244934)	CFB	URB 6C3d-16 (AB034100)	97
BE18 (AY244935)	CFB	URB RFN79 (AB009226)	96
BE19 (AY244936)	CFB	*Prevotella* aff. *ruminicola* (AJ009933)	93
BE20 (AY244937)	CFB	URB RFN79 (AB009226)	96
BE21 (AY244938)	CFB	URB RFN29 (AF544206)	95
BE22 (AY244939)	CFB	URB 12-70 (AF018477)	98
BE23 (AY244940)	CFB	*Prevotella* aff. *ruminicola* (AJ009933)	93
BE24 (AY244941)	CFB	NA[c]	
BE25 (AY244942)	CFB	URB RFN73 (AF544208)	95
BE26 (AY244943)	CFB	URB RFN41 (AB009197)	96
BE27 (AY244944)	CFB	URB 4C3d-1(AB034097)	99
BE28 (AY244945)	CFB	URB RF19 (AF001751)	96
BE29 (AY244946)	CFB	URB RFN79 (AB009226)	96
BE30 (AY244947)	CFB	URB 12-70 (AF018477)	98
BE32 (AY244948)	CFB	URB 12-70 (AF018477)	96
BE33 (AY244949)	LGCGPB[b]	URB RC23 (AF001713)	98
BE34 (AY244950)	CFB	URB 6C3d-16 (AB034100)	94
BE35 (AY244951)	CFB	URB RF25 (AF001757)	98
BE36 (AY244952)	CFB	URB RFN50 (AB009207)	98
BE37 (AY244953)	CFB	URB RFN29 (AB009187)	95
BE38 (AY244954)	LGCGPB	*Clostridium xylanovorans* (AF116920)	100

[a]CFB: *Cytophaga-Flavobacter-Bacteroides* group.

[b]LGCGPB: low G+C Gram-positive bacteria.

[c]NA: not available.

[d]Accession number of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence was given.

[e]URB: unidentified rumen bacterium.

[f]Database sequences with >97% similarity are shown.in bold.

(six clones), *A. larrymoorei* (one clone), *Carnobacterium* (one clone), *R. genomospecies* 5 (one clone), *C. xylanovorans* (one clone), *S. ruminantium* (one clone), *S. maltophilia* (one clone), *R. rathayi* (one clone), and *P. ruminicola* (two clones). Thirty-three additional clones (29.2%) were also 98% similar to sequences in the databases, but those sequences corresponded to uncultured rumen bacteria. About 47.8% of the sequences was 90–97% similar to database sequences, and the similarity was less than 90% for the remaining 6.1% (Tables 1, 2, and 3). Each library will be separately considered below.

The BF library was composed of 40 clones (Table 1). Seventeen of them could be assigned to operational taxonomic units (OTUs) represented in databases. However, only three clones had similarity with the cultured isolate of an OTU. BF32, BF35, and BF37 were similar to *Selenomonas ruminantium*, *Stenotrophomonas maltophilia*, and *Prevotella ruminicola*, respectively. The other 14 clones gave database matches with sequence entries corresponding to uncultured rumen bacteria. The remaining 23 sequences in this library were less than 98% similar to sequences in the database (Table 1).

The BS library was composed of 37 clones (Table 2). This library had more matches (98 to 100% similarity) with cultivated rumen isolates than the other two libraries (Tables 1 and 3). Fourteen sequences were defined as

belonging to specific OTUs: *C. putrefaciens* (six clones: BS26, BS27, BS30, BS31, BS33, and BS38), *C. algidicarnis* (four clones: BS29, BS35, BS37, and BS40), *R. rathayi* (BS39), *R. genomospecies* 5 (BS36), *A. larrymoorei* (BS32), and *Carnobacterium* (BS28). The other nine clones in the 98–100% similarity range were similar to sequence entries corresponding to uncultured rumen bacteria. The remaining 14 sequences were less than 98% similar to database sequences (Table 2).

The BE library was composed of 36 clones (Table 3). Two sequences belonged to the OTUs corresponding to *P. ruminicola* and *C. xylanovorans*. The other ten clones in the 98–100% similarity range gave matches with sequence entries corresponding to uncultured rumen bacteria. The remaining 24 sequences in this library were less than 98% similar to database sequences.

Phylogenetic Placement of Sequences

The result of phylogenetic analysis of the BF library is shown in Fig. 1A. More than half of the sequences (67%) were placed within the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum. One sequence in the *Proteobacteria* phylum (BF35) was related to the typical rumen isolate, *Stenotrophomonas maltophillia*. Four clones (BF04, BF06, BF08, and BF24) formed a novel separate group that did not include any sequences from the databases. Twenty clones clustered with the type strain of *P. ruminicola*. The sequence of F34 was associated with the type strain of *Prevotella albensis*. Within the LGCGPB (low G+C Gram-positive bacteria), two sequences were related to the typical rumen isolates, *Selenomonas ruminantium* (BF32) and *Ruminococcus flavefaciens* (BF39). Two clones, BF02 and BF12, were associated with the *Cyanobacteria* phylum.

The result of phylogenetic analysis of the BS library is shown in Fig. 1B. The majority of sequences were placed within the LGCGPB phylum. Two sequences (BS32 and BS36) fell to the *Proteobacteria* and the other one (BS05) to the *Spirochaetes*. However, unlike the BF library, there were no *Cyanobacteria*-related sequences. Two sequences (BS34 and BS39) were placed within the high G+C Gram-positive bacteria (HGCGPB). Within the *Proteobacteria* phylum, one sequence (BS32) was not related to the typical rumen isolates, but clustered with *A. larrymoorei*. Another clone (BS36) was associated with the type strain of *R. genomospecies* 5. Within the *Spirochaetes* phylum, one clone (BS05) formed a novel separate cluster. Within the HGCGPB phylum, one sequence (BS34) was associated with the type strain of *Olsenella uli*, and another clone (BS39) was related to *R. rathayi*. Twelve clones (BS01, BS02, BS03, BS08, BS10, BS13, BS15, BS16, BS19, BS20, BS24, and BS25) were related to the type strain of *Succiniclasticum ruminis*. Six clones (BS26, BS27, BS30, BS31, BS33, and BS38) and four clones (BS29, BS35, BS37 and BS40) were clustered with ruminal bacteria such as *C. putrefaciens* and *C. algidicarnis*, respectively. The BS28 was associated with the type strain of *Carnobacterium mobile*. Within the CFB phylum, three clones (BS09, BS11, and BS14) were clustered, and one clone (BS12) was apparently not related to any sequences in the database (Fig. 1B).

The result of phylogenetic analysis of the BE library is shown in Fig. 1C. The majority of sequences belonged to the CFB phylum, as was the case with the BF sequences. However, unlike the BF library, there were no *Proteobacteria* and *Cyanobacteria*-related sequences. One clone (BE24) was not related to any sequences in the database. Within the CFB phylum, some sequences were affiliated with a *Prevotella* cluster. The sequences were closely associated with the type strain of *P.* aff. *ruminicola* Tc2-24, *P. ruminicola* TC2-28, and *P. ruminicola*. Two clones, BE8 and BE19, were associated with the *Bacteroides* cluster. Seven sequences (BE16, BE18, BE20, BE25, BE26, BE29, and BE36) were placed in a *Porphyromonas* cluster. Within the LGCGPB phylum, one clone (BE38) was related to the type strain of *Clostridium xylanovorans*.

DISCUSSION

In the present study, we examined rumen bacterial composition by PCR-based analysis of bacterial 16S rDNA molecules. This culture-independent approach offers the possibility of characterizing microbial ecosystems independent of isolation, maintenance, and propagation of bacteria under laboratory conditions. However, PCR-based methodologies are subject to certain limitations [4, 6, 10, 12, 20, 39, 41, 42]. As discussed by Wintzingerode *et al.* [40], in PCR-based analysis, care should be taken in experimental procedures and in the interpretation of the results.

PCR-retrieved 16S rDNA libraries were made from the rumen fluid, solid, and epithelium. The number of hits (in the range of 97–100%) with database entries was substantially higher (Tables 1, 2 and 3); however, the majority of these hits, especially in the BF and BE libraries, were within *in vitro*-retrieved sequences, indicating the necessity of more studies using specific sequence signatures of new phylogroups in the rumen. The BS library had a high number of hits with cultured rumen isolates, compared with the BF and BE libraries (38% vs ~8%) (Fig. 2).

One striking characteristic of the PCR-retrieved sequences was that the majority of them showed little similarity with known ruminal isolates and could not be placed within a specific genus and species. Six sequences from the 113 clones closely resembled that of *C. putrefaciens*, and sequences of four clones were related to *C. algidicarnis*. Seven clones were related to *A. larrymoorei*, *Carnobacterium*, *R. genomospecies*, *C. xylanovorans*, *S. ruminantium*, *S.*

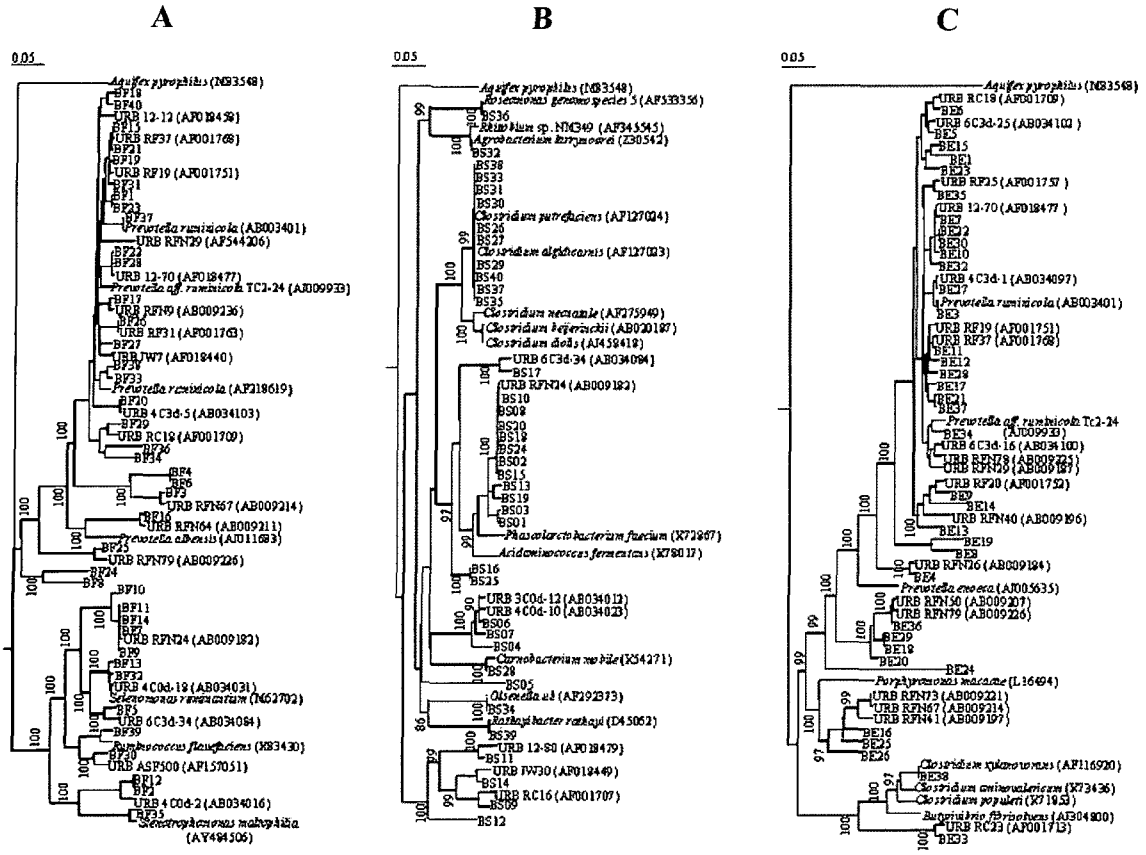

Fig. 2. Phylogenetic placement of 16S rDNA sequences from the rumen fluid (A), rumen solid (B), and rumen epithelium (C). Numbers above each node are confidence levels (%) generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only values of 60% or above are shown.

maltophilia, and *R. rathayi*. Two clones were related to *P. ruminicola*. The 47.8% of the sequences were similar with database sequences (90–97%), and the remaining 6.1% was less than 90%.

A second striking feature is that phylogenetic analysis of the retrieved sequences placed most of them into two phyla, LGCGPB (37%) and CFB (57%), with a small number belonging to the *Spirochaetes* (1%), *Proteobacteria* (3%), and HGCGPB (2%) phyla. The predominance of sequences located within the CFB and LGCGPB phyla accentuates the prominence and role of these groups in structure and function of the rumen bacterial community (Fig. 3).

A third feature is that the species distributions among the three rumen fractions were different. This suggests that the three fractions are likely to represent different microbial niches. For example, the solids fraction contained sequences related to *C. putrefaciens*, *C. algidicarnis*, *Carnobacterium*, *R. rarhayi*, and *A. larrymoorei* (sequences BS26, BS27, BS28, BS29, BS30, BS31, BS32, BS33, BS35, BS36, BS37, BS38, BS39, and BS40), but none of these were detected in the BF and BE libraries. This finding confirms previous observations from culture-based experiments [1, 19]. These researchers showed that a number of strains able to solubilize plant material can also effectively colonize plant cell wall material. Representatives of known bacteria were also found to be more abundant in the BS library. Two novel clones (BS5 and BS12) were found only in the rumen solids. Approximately half of the LGCGPB-related sequences were affiliated with the *Clostridium* group. Representatives of this group, such as *M. multiacida* and *S. ruminantium*, are phylogenetically close [27] and utilize a similarly wide range of substrates including lactate. The prevalence of clones from this lactate-utilizing group may explain why the residual lactate concentration in the rumen fluid during the experiment was very low: apparently, most of it was metabolized by this group of bacteria. *S. bovis* is one of the predominant bacteria under rumen acidosis condition [25, 26], but no related sequences were retrieved in the BS library. One sequence of *Spirochaetes* was identified to be not affiliated with any previously known ruminal spirochetes (Table 3).

The rumen fluid contained *S. maltophilia*, *S. ruminantium*, and *P. ruminicola* (sequences BF32, BF35, and BF37), whose related sequences were not detected in the BE and BS libraries: *S. ruminantium* is related to lactate-utilizing bacteria. The rumen epithelium library contained sequences

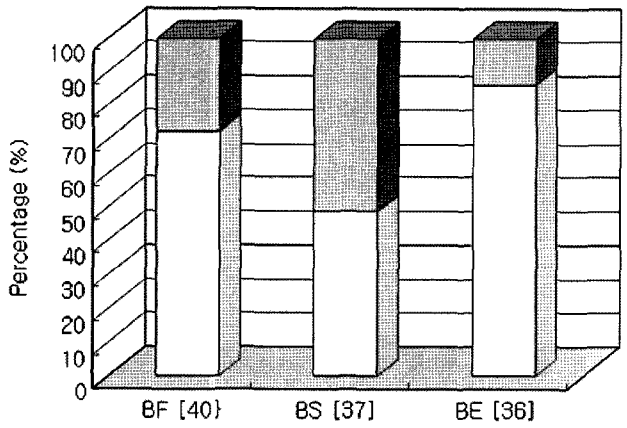

Fig. 3. Distribution of identified and unidentified rumen bacterial clones in the library BF, BS, and BE.
Numbers in square brackets give the total number of clones in that library. BF, rumen fluid; BS, rumen solid; BE, rumen epithelium. Numbers in square brackets give the total number of clones in that library. Uncultured rumen bacteria (open); cultured rumen bacteria (grey).

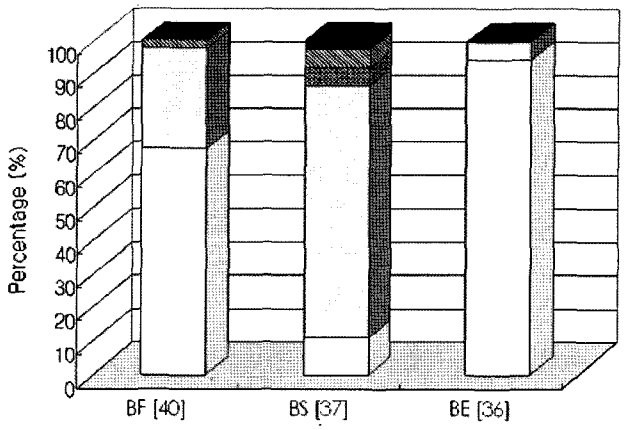

Fig. 4. Distribution of clones in each of the three libraries, BF, BE, and BS.
Numbers in square brackets give the total number of the corresponding clones in that library. Percentage of microcosm in each of the three libraries is shown. LGCGPB, low G+C Gram-positive bacteria (grey); HGCGPB, high G+C Gram-positive bacteria (squared); CFB, *Cytophaga-Flavobacter-Bacteroides* group (open); *Proteobacteria* (hatched); *Spirochetes* (black).

related to *C. xylanovorans* (BE38). No sequences related to this bacterium were detected in the BF and BS libraries. One clone (BE03) was related to *P. ruminicola* that was detected in the BF library. One novel clone (BE24) was found exclusively in the epithelium library. Sequences belonging to the CFB phylum were predominant in this library.

It is quite possible that one cattle used in the present study is not enough to unravel the diversity of the bacteria in rumen. In order to elucidate the exact population of bacteria in rumen contents, it would be necessary to compare the biodiversity of bacteria in rumen samples collected from various kinds of cow grown in the different places. Nevertheless, the present demonstrates the usefulness of 16S rDNA-specific primers to evaluate the biodiversity of bacterial communities in three fractions of the rumen, and indicates possible limitations of the approach that requires consideration. Furthermore, the scope of functional roles and the extent of bacterial diversity have yet to be understood, since most bacteria in the three fractions of the rumen remain unidentified. Considering that the PCR primer to amplify 16S rDNA used in this study was not enough to cover most phyla of bacteria in rumen, the use of a more specific set of phylogenetic probes for the bacteria and quantitative PCR techniques would help unravel the quantitative significance of the newly identified groups and the distribution of the identified bacteria clusters in rumen microbial communities.

GenBank (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) was utilized to search 16S rDNA related to rumen bacteria as a token set (rumen). A total of 523 rumen bacterial 16S rDNA fragments of more than 1.5 kb in sequence were extracted and analyzed. We compared the result of this experiment with that of the GenBank database. Interestingly, the frequency distribution of bacterial size classes in our rumen experiment was similar to that of the GenBank database (Fig. 4).

How many different ruminal bacteria are there? It is now commonly accepted that the cultured species of bacteria represent only a minor fraction of the diversity existing. The previously accepted estimate of 22 species of predominant ruminal bacteria is clearly underestimated, because many ruminal species have strains with little DNA or rRNA similarity [16]. At present, more than 60 species

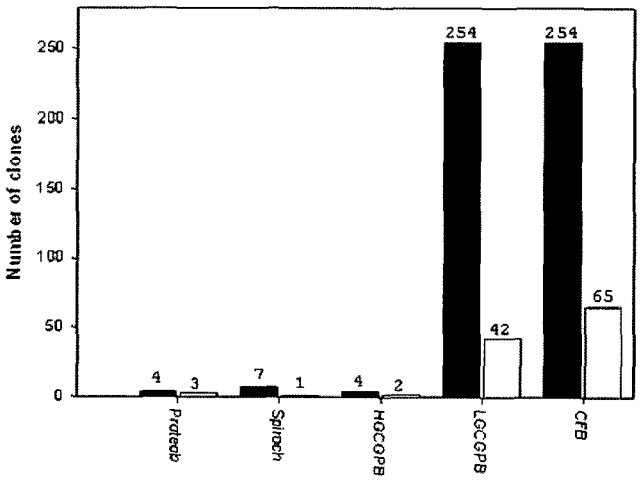

Fig. 5. Relative abundance of 113 clones obtained from this work and 523 clones containing more than 1.5 kb of rDNA sequence from the GenBank database.
Clones in GenBank were extracted using rumen as a token set at http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi. LGCGPB, low G+C Gram-positive bacteria; HGCGPB, high G+C Gram-positive bacteria; CFB, *Cytophaga-Flavobacter-Bacteroides* group; Proteob, *Probacteria*; Spiroch, *Spirochaetes*. This work (open); GenBank data (black).

of ruminal bacteria are stocked in the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and the Japan Collection of Microorganisms (JCM). A larger question is: how many different microbes are there in the rumen? The analysis of the rumen metagenome is complicated by the fact that the majority of rumen microorganisms have not been cultured (estimates range from 85 to 95%) [6, 7, 31, 38], and probably comprise upwards of 1,000 individual species of bacteria, fungi, and protozoa [16]. Excluding duplicated and nonruminal clones, less than 30% of clones corresponded to previously identified bacteria, whereas more than 70% of clones corresponded to unidentified isolates. Based on these numbers, we can estimate that there might be more than 300 different bacterial species in the rumen, if the 60 kinds of the identified bacterial species are considered to correspond to 20%.

Until recently, examining bacterial diversity was a tedious exercise, including cultivation, classification, and enumeration; however, recently developed approaches utilizing PCR technologies allow much more facile explorations of diversity, even of uncultured bacteria [8, 11, 14, 15, 24]. The resulting recent surge of research in molecular microbial ecology provides compelling evidence for the existence of large numbers of novel microorganisms. The rumen clearly contains a diverse mix. 16S rRNA clone libraries indicate that a vast reservoir of physiologies is present in the rumen [20, 31, 32]. Moreover, genomics technologies, including metagenomics, now provide rumen microbiologists with their best opportunity in both functional and comparative studies. This is an exciting prospect. Clearly, genomics will greatly accelerate the rate of information acquisition, and both novel and conventional methodologies and techniques must flourish, when we fully realize the potential of genomics [17]. Renewed interest in microbial physiology and in the isolation of "not-yet-cultured" or "unculturable" microbes is also necessary, if we are to fully exploit the opportunities provided by genomics.

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