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Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries

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Abstract

Molecular diversity of rumen bacteria was analyzed by PCR amplification and sequencing of 16S rDNA clone libraries prepared from the rumen content of Holstein cows fasted for 16 h. A total of 84 clones, containing almost full size 16S rDNA sequences (about 1.5 kb long), were completely sequenced and subjected to an on line similarity search. Four sequences from the 84 clones closely resembled that of *Butyrivibrio fibrisolvens* and one clone was found to be related to *Treponema bryantii*. For 38% of the sequences, the similarity with database sequences was in the range of 90%–98% and for the remaining 56% the similarity was less than 90%. The bacterial community structure was also revealed by phylogenetic placement of sequences in relation to different fractions of rumen content. In the library from the rumen fluid, the sequences were affiliated with the following major phyla: low G+C Gram-positive bacteria (52.4%), *Cytophaga-Flexibacter-Bacteroides* (38.1%), *Proteobacteria* (4.7%) and *Spirochaetes* (2.4%). 2.4% had an uncertain affiliation. The vast majority of sequences from the rumen solids were found to be related to low G+C Gram-positive bacteria (71.4%) and the remaining sequences were placed within the *Cytophaga-Flexibacter-Bacteroides* (26.2%) and *Spirochaetes* (2.4%) phyla.

Keywords: Rumen contents; 16S rDNA library; Bacterial diversity; Phylogenetic analysis

1. Introduction

The rumen ecosystem is populated by a highly diverse collection of obligately anaerobic microorganisms, including fungi, protozoa, bacteria and archaea. The anaerobic bacterial component of the system has been a subject of intensive studies over the past 40 years, beginning with the recognition of the fact that large numbers of bacteria are present in the rumen [1,2]. Since then, numerous studies describing the isolation and identification of a wide variety of aerobic, facultative and anaerobic bacterial strains from ruminants of different ages, health conditions, geographical locations, seasons and diets have been published (reviewed in [1–5]). However, considering that only a small fraction of the total

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microbial diversity in natural ecosystems can be recovered by cultural methods [6], it is not clear whether these descriptions accurately reflect bacterial species distribution in the rumen.

Recently, with the introduction of methods for direct retrieval and sequence analysis of some target genes, mainly those of ribosomal RNAs, it has become possible to evaluate the genetic diversity and phylogenetic relationships of microorganisms in different ecosystems without cultivation. Early studies of this type revealed the existence of unknown species [7,8]. A similar situation may exist in the bacterial diversity of the rumen ecosystem since, as in other microbial ecosystems, the direct microscopic count of rumen bacteria substantially exceeds the cultivable count. Most of the bacteria in natural ecosystems probably enter the 'viable, but non-recoverable' physiological state which complicates the isolation and phenotypic characterization and curtails the actual number of species. Also, isolation techniques may not always reproduce in vivo conditions, especially those of highly specialized or symbiotic ecosystems, such as the syntropic relation between *Desulfovibrio* and a butyrate oxidizing bacterium from bovine rumen [9]. Another example of this kind is a large rumen bacterium, *Quinella ovalis*, which has never been obtained in axenic cultures and its taxonomic position has been established on the basis of 16S rDNA sequence analysis [10]. Analyses of target genes amplified directly from the rumen-extracted DNA may eventually help to describe the bacterial composition independent of isolating, maintaining and propagating bacteria under laboratory conditions. The recent works [11,12] have demonstrated successful application of in vitro-based techniques to analysis of rumen microbiota.

Our present work also is an attempt to describe the bacterial diversity in the rumen by direct retrieval and analysis of 16S rDNA sequences in a culture-independent manner.

2. Materials and methods

2.1. Sampling

Samples of rumen content were obtained from a closed herd at the National Institute of Animal Industry (Ibaraki, Japan). The animals were rumen-fistulated Holstein dry cows, fed a mixed ration (alfalfa-timothy hay and concentrate in a 4:1 ratio) twice a day. The corn and barley-based (43%) concentrate, Select 16, was purchased from Zen-Raku-Ren (Tokyo, Japan). The exact composition of the concentrate is available upon request. Since the rumen is an open system, some bacterial isolates may be transient and do not occupy a secure ecological niche. For that reason an 16 h interval was allowed before sampling to avoid the background influx of bacteria with feed. Representative samples of total rumen contents (250 g) were collected from two animals via the ruminal fistula before the morning feeding. The samples on ice were immediately transferred into an anaerobic box under a nitrogen:carbon dioxide:hydrogen (80:10:10) atmosphere and were separated into the rumen fluid (used for the first library construction) and feed particles (used for the second library construction) fractions. For this, the rumen contents were squeezed through two layers of cheesecloth and the resulting rumen fluid fractions from two animals were pooled, subsampled and frozen at −80°C. The remaining feed particle samples from two animals were also pooled, subsampled and stored at −80°C in tightly closed 50 ml Falcon tubes.

2.2. Total DNA extraction

Two methods of DNA extraction were tested in our experiments. The first method was based on *N*-lauroyl sarcosine/proteinase K lysis and the second method was the modified freezing/thawing protocol designed for a broad range of cell types [13]. In the first protocol, the rumen fluid or feed particles of the rumen content were thawed on ice and the concentrated buffer components were added to a final concentration of 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 2% *N*-lauroyl sarcosine and 2 mg ml^{-1} proteinase K. This suspension was gently mixed and incubated at 65°C for 45 min with agitation. After extraction with an equal volume of buffer-equilibrated phenol-chloroform-isoamyl alcohol (50:49:1) solution, nucleic acids were precipitated by the addition of 1/10 volume of 5 M NaCl and 1 volume of isopropanol, washed several times with 70% ethanol, dried and dissolved in TE buffer

(pH 8.0) containing DNase-free RNase (100 μg ml^{-1}). The second protocol included the physical treatment of the rumen fluid or the feed particles of the rumen content in the same buffer by five cycles of freezing at −80°C for 60 min, followed by heating in a water bath at 65°C for 30 min. The total DNA was subsequently isolated as in the first protocol. In the preliminary experiments, the quality and representativeness of two model libraries based on different DNA isolation techniques was tested. The library which was constructed using the DNA templates according to the second extraction protocol represented a broader range of molecular diversity. At the same time, the library based on DNA templates isolated by the first extraction method was biased to 'easy to lyse' Gram-negative bacteria and showed a considerably lower extent of genetic diversity (data not shown). We also tested the second DNA isolation protocol with subsequent PCR reactions (see Section 2.3) using pure cultures of *Prevotella ruminicola*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Streptococcus bovis*, *Ruminobacter amylophilus*, *Ruminococcus albus* and *R. flavefaciens*. No preferential amplification of a particular template was detected among these strains. Therefore, only data based on the second DNA isolation protocol are presented in this report.

2.3. PCR procedures

The primers used for the PCR amplification of the almost full length 16S rDNA sequences were 27f and 1544r [14]. Several sources of thermostable DNA-polymerases for PCR were tested for their fidelity. Enzymes from two PCR kits, Expand™ High Fidelity PCR System (Boehringer Mannheim, Germany) and TaKaRa Ex Taq™ (Takara Shuzo, Japan) were found to be less prone to misincorporation errors. Since the latter kit provided a higher efficiency of PCR products cloning (∼80%) into the TA Cloning® Kit (Invitrogen, USA), it was chosen for further library construction experiments. Because the standard number of PCR cycles may introduce biases during retrieval of sequences from complex gastrointestinal systems [15], we reduced the number of amplification cycles to 10 and performed the amplification as follows: initial denaturation at 95°C for 3 min, followed by 10 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 60°C and 1.5 min of elongation at 72°C with a final extension at 72°C for 7 min using a PE480 thermal cycler (Perkin Elmer, USA). A typical PCR mixture contained 300 nM of each primer, 1 μg of purified template DNA, 1×Ex Taq reaction buffer, 200 mM of each deoxynucleoside triphosphate and 2.5 U of Ex Taq DNA-polymerase, adjusted to a total volume of 50 μl. The PCR products were separated by electrophoresis in 1.5% H14 agarose gels (Takara) and stained with ethidium bromide. The products were excized from the gel and placed into 1.5 ml microtubes. The gel fragments were crushed and 500 μl of buffer-equilibrated phenol was added. The samples were treated with three cycles of freezing at −80°C for 20 min, followed by melting at room temperature for 20 min. After that, the samples were centrifuged at $10\,000\times g$ for 20 min. The aqueous phase was collected and PCR fragments were recovered by ethanol precipitation.

2.4. Cloning and sequencing

PCR products were cloned into the TA Cloning Kit (Invitrogen, USA) and the transformants were randomly picked up. The recombinant plasmids were extracted by using the alkaline lysis miniprep method [16]. Cycle sequencing was performed with the SequiTherm EXCEL™ Long-Read DNA Sequencing (Epicentre Technologies, USA) and Thermo Sequenase™ (Amersham, UK) kits. The sequencing reaction products were read on a LI-COR M4000L automated DNA sequencer (LI-COR, USA).

2.5. Sequence analysis

All reference sequences were obtained from the GenBank and RDP (Ribosomal Database Project) [17] databases. Our sequences were analyzed by the CHECK_CHIMERA program [17] to remove chimeric rDNA clones. Sequences were aligned using the multiple sequence alignment program CLUSTAL W version 1.6 [18]. Gaps and regions of alignments for which homology of residues could not be reasonably assumed were excluded from the phylogenetic analysis. The two parameter model of Kimura [19] was used for the construction of neighbor joining

trees [20]. The statistical significance of the tree branches was evaluated by bootstrap analysis [21] involving the construction of 1000 trees from re-sampled data.

2.6. Nomenclature

For the first library (rumen fluid), clone names begin with the letters RF or RFP (e.g. RF1 or RFP4). For the second library (rumen solids), the prefixes are RC or RCP (e.g. RC1 or RCP10).

2.7. Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank and DDBJ nucleotide sequence databases under accession numbers AF001692-AF001743, AF001745-AF001747 and AF001749-1776.

3. Results

3.1. Similarity with database sequences

A total of 84 sequences from two libraries (rumen fluid and feed particles) were subjected to sequence analysis with subsequent on line homology searches using two databases, Genbank which implements the BLAST algorithm [22] and the RDP database which implements the SIMILARITY_RANK program [17] (Table 1, Table 2). Although there are no exact 16S rDNA similarity limits for defining specific taxa such as genus and species, in general, species definition requires sequence similarities greater than 98%. Only five sequences from the 84 clones in our libraries (6%) can be identified as belonging to *Butyrivibrio fibrisolvens* (four clones) and *T. bryantii* (one clone). About 38% of the sequences have a similarity level with database sequences in the range of 90–98% and for the remaining 56%, the similarity was less than 90% (Table 1, Table 2). Since for a majority of our sequences the similarity value was too low to allocate them with a reasonable degree of confidence to the corresponding taxa, phylogenetic analysis was performed to clarify their taxonomic position. It has been suggested that phylogenetic clustering of bacterial groups, rather than a specific similarity value, should be used as a guide for defining bacterial taxa [23].

3.2. Phylogenetic placement of sequences from rumen fluid

The results of phylogenetic analysis are shown in Fig. 1. In this library, the majority of sequences were placed within the low G+C Gram-positive bacteria (LGCGPB) and *Cytophaga-Flexibacter-Bacteroides* (CFB) phyla. Two sequences fell to the *Proteobacteria* and the other two to the *Spirochaetes*. Two sequences in the *Proteobacteria* phylum (RF32 and RF33) were not related to the typical rumen isolates but clustered with *Cowdria ruminantium*, a representative of the alpha subclass of this phylum. Within the *Spirochaetes* phylum, clone RF13 was clustered with the sequence of a rumen bacterium, *T. bryantii*. However, the clustering of the second clone, RFP12, within the phylum was doubtful since it was deeply branched in the phylogenetic tree and the bootstrap confidence level was only 85.2% (Fig. 1).

Five clones within the CFB phylum (RF2, RF14, RF15, RF26 and RF36) formed a novel separate cluster which was not affiliated with any sequence data (Fig. 1). The stability of this cluster was verified by bootstrap analysis with a confidence level of 100%. Another sequence, RF17, was deeply branched from that of *Bacteroides distasonis*, but this species itself does not fulfil sensu stricto definition and, on the basis of the 16S rRNA data, has been placed in the porphyromonas cluster [24].Only one clone, RF25, clustered with the type strain of *P. ruminicola*, the other nine RF sequences were clearly divided from this cluster (Fig. 1). This is in agreement with the earlier observations on phenotypic and genetic diversity among the cultivated ruminal prevotellas [25,26].

More than half of our sequences (52.4%) were phylogenetically placed within the LGCGPB phylum (Fig. 1), suggesting that this numerical significance may be a consequence of their functional importance in symbiotic relationship with the host animal. One clone (RFP7) was associated with a high confidence with a rumen bacterium *S. ruminantium*. Together with another sequence, RF4, they fell into cluster IX [27] of the phylum. The stability of clustering of four clones (RF3, RF8, RF9 and RF39) with the

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

Clone	Phylum	Nearest relative	Similarity (%)
RF1	LGCGPB[a]	*Butyrivibrio crossotus*	90
RF2	CFB[b]	NA[c]	
RF3	LGCGPB	NA	
RF4	LGCGPB	NA	
RF5	LGCGPB	*Eubacterium halii*	93
RF6	LGCGPB	NA	
RF7	LGCGPB	*Termitobacter aceticus*	90
RF8	LGCGPB	NA	
RF9	*Mycoplasmas*	NA	
RF10	LGCGPB	NA	
RF13	Spirochaetes	*Treponema bryantii*	98
RF14	CFB	NA	
RF15	CFB	NA	
RF17	CFB	NA	
RF18	CFB	*Prevotella ruminicola*	92
RF19	CFB	*Prevotella ruminicola*	95
RF20	CFB	*Prevotella ruminicola*	92
RF21	LGCGPB	NA	
RF22	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO 2394)	95
RF23	LGCGPB	*Eubacterium halii*	94
RF24	CFB	NA	
RF25	CFB	*Prevotella ruminicola*	93
RF26	CFB	NA	
RF27	CFB	*Prevotella ruminicola*	92
RF28	LGCGPB	NA	
RF29	LGCGPB	NA	
RF30	LGCGPB	NA	
RF31	CFB	NA	
RF32	*Proteobacteria*	NA	
RF33	*Proteobacteria*	NA	
RF34	LGCGPB	NA	
RF35	LGCGPB	NA	
RF36	CFB	NA	
RF37	CFB	*Prevotella ruminicola*	94
RF38	LGCGPB	*Clostridium celerecrescens*	90
RF39	*Mycoplasmas*	NA	
RF40	CFB	*Prevotella ruminicola*	95
RFP4	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO 2435)	96
RFP6	CFB	*Prevotella ruminicola*	91
RFP7	LGCGPB	*Selenomonas ruminantium*	94
RFP12	NA		
RFP18	LGCGPB	*Butyrivibrio fibrisolvens* (ATCC 19171)	93

[a]LGCGPB, low G+C Gram-positive bacteria.
[b]CFB, the *Cytophaga-Flavobacter-Bacteroides* group.
[c]NA, not available.

ruminal mycoplasmas was verified by bootstrap with a confidence level of 91.3% (Fig. 1). Two clones (RF21 and RF35) were placed in cluster IX and four clones (RF6, RF10, RF30 and RF34) in cluster I (Fig. 1). Interestingly, the rumen fluid sequences formed a separate entity from that of the feed particles in cluster I. Three clones (RF7, RF28 and RF29) belonged to cluster III of LGCGPB. No sequences were associated with cluster IV which includes extensively studied ruminal bacteria such as

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

Clone	Phylum	Nearest relative	Similarity (%)
RC1	Spirochaetes	*Treponema bryantii*	97
RC2	CFB[a]	NA[b]	
RC3	LGCGPB[c]	*Butyrivibrio fibrisolvens* (Bu 43)	99
RC4	LGCGPB	NA	
RC5	LGCGPB	NA	
RC6	LGCGPB	NA	
RC7	LGCGPB	NA	
RC9	CFB	NA	
RC10	CFB	NA	
RC11	CFB	NA	
RC13	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO2223)	98
RC14	CFB	*Prevotella ruminicola*	91
RC15	LGCGPB	NA	
RC16	CFB	NA	
RC17	CFB	NA	
RC18	CFB	*Prevotella ruminicola*	91
RC20	LGCGPB	NA	
RC21	LGCGPB	*Clostridium xylanolyticum*	90
RC22	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO 2398)	99
RC23	LGCGPB	NA	
RC24	CFB	*Prevotella ruminicola*	92
RC25	LGCGPB	NA	
RC26	LGCGPB	NA	
RC27	LGCGPB	*Eubacterium ventriosum*	90
RC28	LGCGPB	*Clostridium celerecrescens*	90
RC29	CFB	*Prevotella ruminicola*	95
RC30	LGCGPB	NA	
RC31	LGCGPB	NA	
RC32	LGCGPB	*Butyrivibrio crossotus*	90
RC34	LGCGPB	NA	
RC35	LGCGPB	NA	
RC36	LGCGPB	NA	
RC37	LGCGPB	*Ruminococcus flavefaciens*	96
RC38	LGCGPB	NA	
RC39	LGCGPB	*Butyrivibrio crossotus*	91
RC40	CFB	*Prevotella ruminicola*	97
RCP1	LGCGPB	NA	
RCP5	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO 2398)	98
RCP6	LGCGPB	*Ruminococcus flavefaciens*	95
RCP10	LGCGPB	NA	
RCP17	LGCGPB	*Butyrivibrio fibrisolvens* (NCDO 2398)	93
RCP19	CFB	*Prevotella ruminicola*	96

[a]CFB, the *Cytophaga-Flavobacter-Bacteroides* group.
[b]NA, not available.
[c]LGCGPB, low G+C Gram-positive bacteria.

R. albus and *R. flavefaciens* (Fig. 1). Two clones, RFP4 and RFP18, were clustered with *B. fibrisolvens* lineage 2 [28] and the other two clones (RF1 and RF38) were related to *B. fibrisolvens* lineage 1. One clone, RF22, was associated with *B. fibrisolvens* lineage 3 which includes a cultivable rumen isolate *Pseudobutyrivibrio ruminis* (Fig. 1). Two other clones, RF5 and RF23, were phylogenetically placed within cluster XIVa of LGCGPB as well, but their

further taxonomic definition cannot be reliably assumed (Fig. 1).

3.3. Phylogenetic placement of sequences from rumen solids

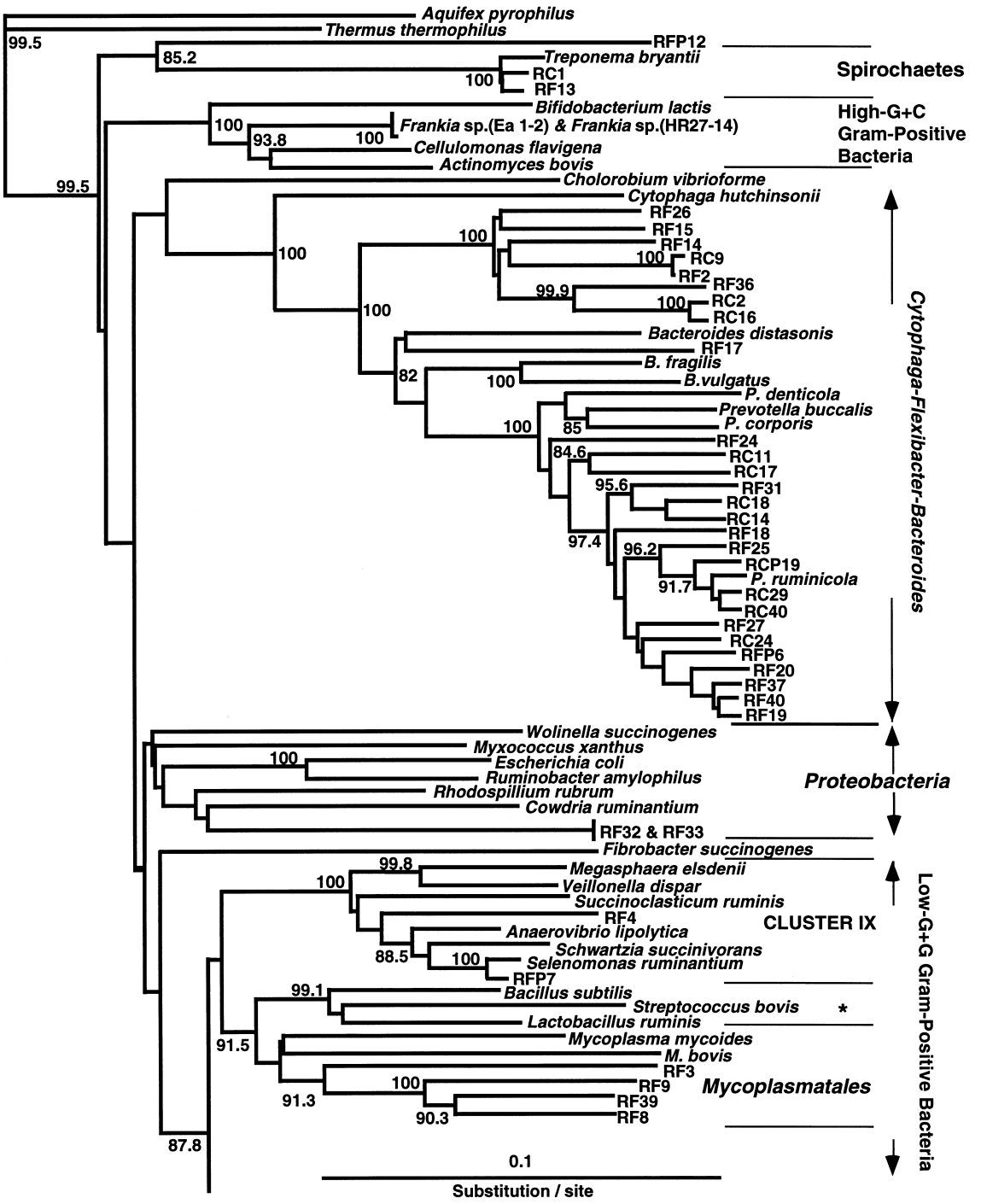
The majority of sequences in this library belonged to the LGCGPB and CFB phyla, but the number of clones phylogenetically placed within the former was higher than in the library from the rumen fluid fraction (71.4% versus 52.4%). Similar to the rumen fluid library, there was one sequence (RC1) related to *T. bryantii*. (Fig. 1). But, unlike the rumen fluid library, there were no *Proteobacteria*-related sequences.

Within the CFB phylum, three sequences (RC2, RC9 and RC16) were affiliated with a novel cluster encountered during the first library analysis (Fig. 1). This clustering supports the independent taxonomic entity of this group and necessitates further isolation/identification of their culturable representatives. Three clones (RCP19, RC29 and RC40) were closely associated with the type strain of *P. ruminicola* while the remaining five clones (RC11, RC14, RC17, RC18 and RC24) were scattered within the heterogeneous ruminal prevotella cluster together with the rumen fluid sequences (Fig. 1).

Within the LGCGPB phylum, no sequences related to clusters IX and XI as well as to the ruminal mycoplasmas could be detected in this library (Fig. 1). Also, in cluster I, three sequences from the feed particles library (RC5, RC6 and RC30) formed an independent entity, separated from the rumen fluid sequences with a 100% bootstrap confidence (Fig. 1). Clones from the solid fraction were clearly enriched by the representatives of cluster IV which were absent in the liquid fraction of rumen content. Two clones (RCP6 and RC37) were clustered with *R. flavefaciens* and one clone, RC23, with *Eubacterium siraeum* (Fig. 1). Clones from cluster III, which were weakly associated with *Termitobacter aceticus* in the rumen fluid library, formed two groups in the library from feed particles. One clone (RC15) was affiliated with *T. aceticus* as in the first library, but the other three (RC4, RC31 and RC38) were assembled in a separate entity (Fig. 1). Clones from this library were certainly enriched by the representatives of cluster XIVa (19 clones from a total of 42 in the library). In particular, the existence of two new groups (represented by (i) RC26 and RC35 and (ii) RC7, RCP10 and RC20 sequences), having no close cultivable relatives or matches with the rumen fluid sequences, was verified in several calculations with a confidence level of 100% (Fig. 1). Additionally, four sequences (RC25, RC27, RC28 and RC34), also with no matches to any sequence from the rumen fluid library, were found to be weakly related to *R. productus* and *Clostridium clostridiiforme* (Fig. 1). Association of our sequences with the established classification scheme of *B. fibrisolvens* [28] was as follows: (i) four clones (RC3, RCP5, RCP17 and RC22) were located within lineage 2, (ii) two clones (RC32 and RC39) within lineage 1, (iii) one clone, RC13, related to lineage 3 (Fig. 1). The remaining three clones within cluster XIVa (RC10, RC21 and RC36), together with two clones from the rumen fluid library (RF5 and RF23), formed a novel phylogenetic group only distantly related to *C. aminovalericum* (Fig. 1).

4. Discussion

Together with two recent works by other authors [11,12], our study was an attempt to characterize the rumen bacterial composition by PCR-based analysis of bacterial 16S rDNA molecules. The culture-independent approach offers the possibility of characterization of microbial ecosystems independent of isolating, maintaining and propagating bacteria under laboratory conditions. However, PCR-based methodologies are also not free from certain limitations [15,29,30]. As has been pointed out by Wintzingerode et al. [30], in PCR-based analysis, care should be taken in experimental procedures and in the interpretation of results. In the present study, a number of preliminary experiments were accomplished to minimize the possible biases of PCR-based analysis. First, two protocols of total DNA isolation (see Section 2) were tested for biases introduced during the DNA release step. The modified protocol of Barns et al. [13], designed for a broad range of cell types, allowed us to recover a broader range of bacterial molecular diversity (data not shown). Second, preliminary experiments with pure cultures of *P. ruminicola*, *F. succinogenes*, *S. ruminantium*, *M. elsdenii*, *S. bovis*, *R. amylophilus*, *R. albus* and *R. flavefaciens*



*Bacillales and Lactobacillales

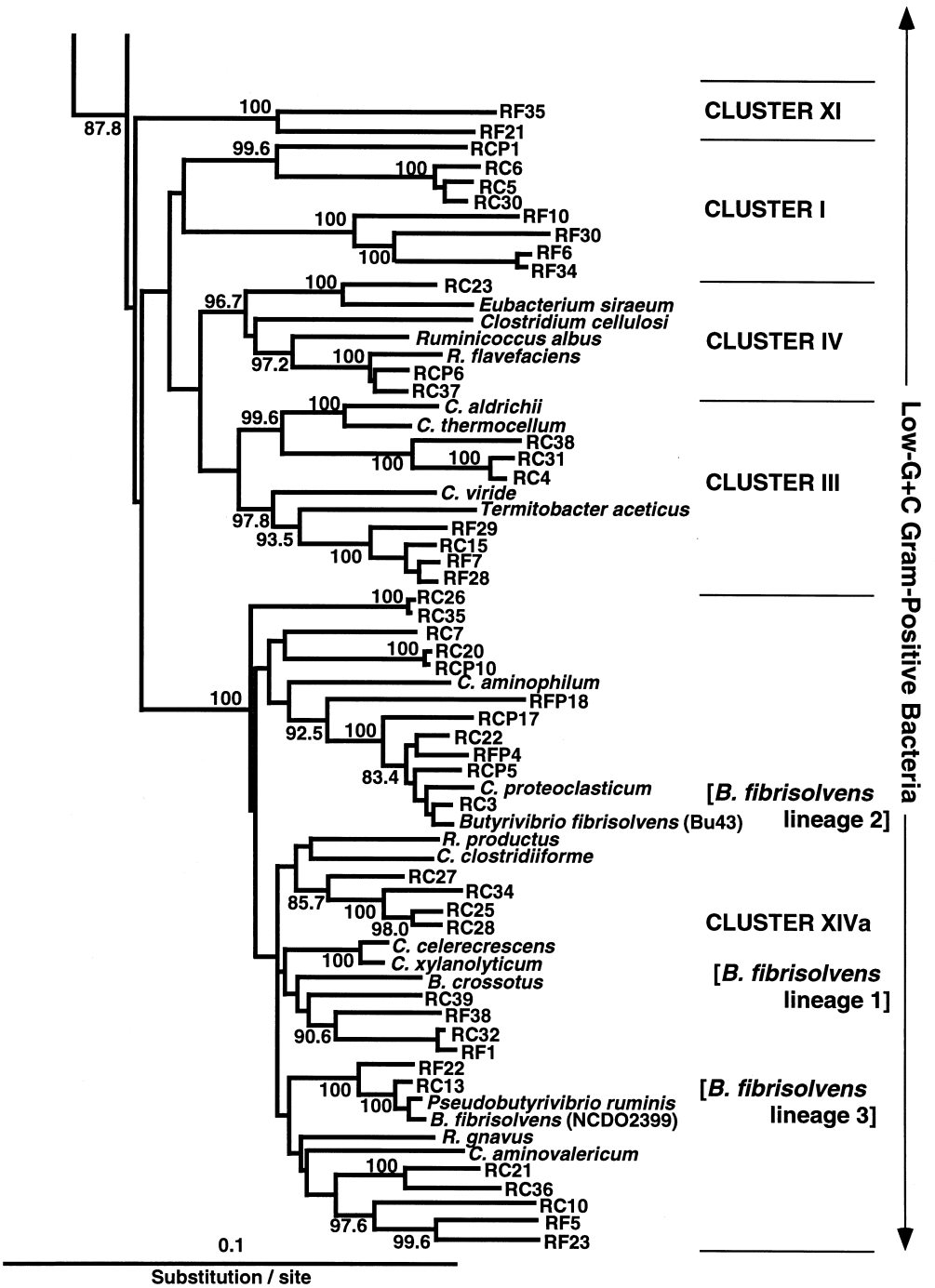


Fig. 1. Phylogenetic placement of 16S rDNA sequences from the rumen fluid (begin with RF or RFP followed by a clone number) and rumen solids (begin with RC or RCP followed by a clone number) fractions. The *Aquifex pyrophilus* sequence is used as the outgroup for rooting the tree. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees [21]. The scale bar is in fixed nucleotide substitutions per sequence position.

showed no preferential amplification of particular 16S rDNA sequences (data not shown). To reduce further the distorting effects of PCR, the polymerase with a proof reading activity (Takara) was chosen and the number of cycles was reduced to 10.

The first noticeable characteristic of the PCR-retrieved sequences was that the majority of them bear little similarity with the described ruminal isolates and cannot be placed within specific taxa such as genus and species. Four sequences from the 84 clones closely resembled that of *B. fibrisolvens* and one clone was found to be related to *T. bryantii*. For 38% of the sequences the similarity with database sequences was in the range of 90%–98% and for the remaining 56% the similarity was less than 90%. Second, the only sequence encountered twice during the analysis was that of an unidentified proteobacterium (RF32 and RF33), other sequences were unique. This even bacterial species distribution in the rumen dictates the necessity of further analytical efforts in order to describe the full range of their diversity. Third, phylogenetic analysis of the retrieved sequences placed them essentially into the two phyla, LGCGPB (61.9%) and CFB (32.1%), with a small number of clones belonging to the *Spirochaetes* (3.6%) and *Proteobacteria* (2.4%) phyla. Predominance of sequences located within the LGCGPB phylum accentuates the prominence and role of this group in structure and function of the rumen bacterial community. The majority of highly hydrolytic and acetogenic cultivable bacteria, such as ruminococci, butyrivibrios and clostridia, belong to this phylum as well. Fourth, the molecular retrieval approach with subsequent phylogenetic analysis allowed us to reveal new taxonomic entities for which no cultivable representatives are known. The most prominent example of this kind is a group of ruminal bacteroides (RF26, RF15, RF14, RC9, RF2, RF36, RC2 and RC16) located within the CFB phylum (Fig. 1). These observations suggest the need of purposeful isolation methodology based on screening of cultivable rumen isolates using probes or primers designed on the basis of PCR-retrieved libraries.

The species distribution in different fractions of rumen content confirms some earlier observations established in culture-based experiments [31,32]. These researchers showed that a number of strains able to solubilize plant material can also effectively colonize plant cell wall material. The solids fraction in our study contained sequences related to *R. flavefaciens* and *E. siraeum* (cluster IV, sequences RCP6, RC27 and RC37), while such sequences were not detected in the rumen fluid fraction. Representatives of cluster XIVa were also more abundant in the library from the rumen solids. Moreover, two novel groups within the cluster (RC26 and RC35 and RC7, RCP10 and RC20) were formed exclusively by sequences from the solid material of the rumen. The rumen fluids, in its turn, contained *S. ruminantium*-, *Mycoplasma*- and *Proteobacteria*-related sequences that were not detected in the rumen solids (food particle-associated) library. Ruminal prevotellas were equally represented in both fractions of the rumen content and the large number of CFBs in the fluid fraction was due to the elevated number of sequences (RF2, RF14, RF15, RF26 and RF36) forming a cluster with no close cultivable relatives. Despite the well-documented association of *F. succinogenes* with the fibrous material [32], corresponding sequences were not found in library 2 (as well as in library 1). Although we experimentally addressed the possibilities of poor lysis of this organism, of lower competitiveness of its DNA template in PCR, of less efficient cloning of its 16S molecules into *Escherichia coli* and of the copy number effect of its rRNA operons, it seems that none of these factors could account for the absence of the corresponding sequences in our libraries. Since the full range of bacterial diversity in our libraries was not completely covered, some bacteria with a ratio of less than 1.2% may have escaped detection.

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