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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

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We describe an approach for determining the genetic composition of *Bacteroides* and *Prevotella* populations in gut contents based on selective amplification of 16S rRNA gene sequences (rDNA) followed by cleavage of the amplified material with restriction enzymes. The relative contributions of different ribotypes to total *Bacteroides* and *Prevotella* 16S rDNA are estimated after end labelling of one of the PCR primers, and the contribution of *Bacteroides* and *Prevotella* sequences to total eubacterial 16S rDNA is estimated by measuring the binding of oligonucleotide probes to amplified DNA. *Bacteroides* and *Prevotella* 16S rDNA accounted for between 12 and 62% of total eubacterial 16S rDNA in samples of ruminal contents from six sheep and a cow. Ribotypes 4, 5, 6, and 7, which include most cultivated rumen *Prevotella* strains, together accounted for between 20 and 86% of the total amplified *Bacteroides* and *Prevotella* rDNA in these samples. The most abundant *Bacteroides* or *Prevotella* ribotype in four animals, however, was ribotype 8, for which there is only one known cultured isolate, while ribotypes 1 and 2, which include many colonic *Bacteroides* spp., were the most abundant in two animals. This indicates that some abundant *Bacteroides* and *Prevotella* groups in the rumen are underrepresented among cultured rumen *Prevotella* isolates. The approach described here provides a rapid, convenient, and widely applicable method for comparing the genotypic composition of bacterial populations in gut samples.

Methods for enumerating gut bacteria that are based on cultivation, isolation, and biochemical testing are generally laborious and do not guarantee recovery of the less easily cultivated species. This is a particular problem for obligately anaerobic bacteria, which make up the great majority of organisms present in densely populated gut habitats such as the rumen and hind gut (13, 31). For this reason, there has been increasing interest in the rapid enumeration of microbial groupings by analysis of nucleic acids extracted from gut samples. Probing of extracted RNA with radiolabelled or fluorescently labelled oligonucleotide probes has been used in several studies (6, 14, 20, 30) but relies on developing panels of probes for different groups from available sequence data. Sequencing of random PCR-amplified 16S rRNA gene (rDNA) clones has provided valuable information on total eubacterial diversity for human fecal microflora (37). However, more rapid approaches to the study of diversity that allow the examination of large numbers of samples are required, and a semiquantitative PCR detection approach based on serial dilution has been reported for some of the predominant gut anaerobes (35). The approach we take here is to perform selective PCR amplification of 16S rRNA genes from the gram-negative anaerobic genera *Bacteroides* and *Prevotella* by using DNA extracted from gut samples and then to estimate the genotypic composition of samples from restriction enzyme cleavage patterns (restriction fragment length polymorphism [RFLP]) of the amplified DNA (PCR-RFLP). 16S rDNA PCR-RFLP approaches have proved valuable for typing isolated bacterial strains (see, e.g., references 10 and 15) and assessing the diversity of cloned, amplified 16S rDNA sequences from bacteria at hydrothermal vents (23), but they do not appear to have been applied previously to sequences directly amplified from mixed gut communities.

Members of the *Bacteroides-Cytophaga-Flexibacter* phylum (25, 38) are often reported to be among the most numerous culturable microbes present in the rumen and hind gut, where they play important roles in the breakdown of protein and carbohydrate and, in some cases, act as opportunistic pathogens (28). Rumen *Prevotella* spp. form a diverse group that is distinct from the human hind-gut *Bacteroides* spp. based on 16S rRNA sequencing and other criteria (3, 18, 29). The single species recognized formerly, *Prevotella ruminicola*, contained considerable variation, and its recently proposed reclassification into four species, *P. ruminicola*, *P. bryantii*, *P. brevis*, and *P. albensis* (4), is followed here.

MATERIALS AND METHODS

Bacteria. The origins of the *Prevotella* spp. have been described previously (3, 19). *Bacteroides uniformis* 1004 was obtained from A. Salyers, University of Illinois; *B. vulgatus* 10583, *B. ovatus* 11153, and *B. levii* 11028 were obtained from the National Collection of Type Cultures, Aberdeen, United Kingdom; *B. vulgatus* 1447 was from the DSM collection, Braunschweig, Germany. Bacteria were grown anaerobically (8) at 38°C in M2GSC medium (22) under O_2-free CO_2.

Animals and diets. DNA was extracted from samples of rumen fluid removed from cannulated animals (one cow and six sheep). Unless otherwise stated, the samples were obtained 2 h after the morning feed and the microbial DNA was immediately extracted. Diet 1 consisted of 500 g of grass hay, 299.5 g of barley, 100 g of molasses, 91 g of white fishmeal, and 9.1 g of mineral-vitamin mixture per kg (cow, 4 to 5 kg, once daily; sheep 1, 0.7 kg, twice daily). Diet 2 consisted of 300 g of grass hay and 150 g of grass nut (sheep 2 and 3, fed twice daily). Sheep 4 was a defaunated animal that received diet 1 (1.4 kg, once daily). Diet 3 consisted of 400 g of bruised barley, 100 g of hay, twice daily, and diet 4 consisted of 200 g of bruised barley and 300 g of hay, twice daily (sheep 5 and 6).

DNA extraction. DNA was extracted from isolated strains as described previously (2, 3). DNA was extracted from rumen and fecal samples by a modification of the method of Stahl et al. (30). A sterile 2-ml screw-cap Eppendorf tube was

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half filled with sterile zirconium beads, 0.1 mm in diameter, and 1 ml of sample was added so that the tube was filled completely. The sample was beaten with a mini bead beater (Biospec Products) for 30 s and then chilled on ice for at least 1 min. This procedure was carried out six times, and the sample was then added immediately to an equal volume of 1:1 (vol/vol) phenol-chloroform and vortexed. Further extractions were performed until the aqueous phase no longer appeared cloudy. Nucleic acids were recovered from the aqueous phase by ethanol precipitation and resuspended in a suitable volume of sterile distilled H_2O (dH_2O).

Humic material had to be removed from the DNA extracted from rumen fluid and feces prior to PCR amplification. This was achieved by passing the DNA through an Elutip-d column as specified by the manufacturer (Schleicher and Schuell, Dassel, Germany). The DNA was then precipitated in 2 volumes of ethanol and resuspended in sterile dH_2O. This procedure had to be performed at least twice to obtain DNA of a quality suitable for amplification.

Oligonucleotide primers and probes. The universal eubacterial primers fD1 (5′-AGAGTTTGATCCTGGCTCAG, positions 7 to 26 in the *Escherichia coli* 16S rRNA gene [7]) and rP2 (ACGGCTACCTTGTTACGACTT, positions 1513 to 1494) are those used in reference 36. The Uni16S primer (ACGGGCGGTG TGTACAAGGCC, positions 1383 to 1402) is that used in reference 30. The *Bacteroides*- and *Prevotella*-specific primer BacPre (GAGTACGCCGGCAACG GTGA, positions 887 to 907) its reverse complement rBacPre (TCACCGTTG CCGGCGTACTC), and the *P. ruminicola* 23-specific probe (ATCTTGAGTG AGTTCGATGTTGG, positions 650–673) are those used in reference 3. For end labelling of primers or probes, 100 ng of the oligonucleotide was diluted to a final volume of 16 μl with sterile dH_2O, incubated at 70°C for 1 min, and immediately placed on ice. T4 polynucleotide kinase buffer, 50 μCi of [γ-^{32}P]ATP, and 10 U of T4 polynucleotide kinase were added in a final volume of 25 μl, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by heating to 70°C for 10 min. Unincorporated ^{32}P was removed by passing the mixture through Chroma spin-10 columns (Clontech) as specified by the manufacturer.

PCR amplification of ruminal 16S rDNA and PCR-RFLP analysis. Approximately 200 to 250 ng of chromosomal DNA was amplified with a Techne PHC-3 thermal cycler in a 100-μl reaction mix containing 0.04 mM each deoxynucleoside triphosphate, 20 pmol of each primer, and 1× reaction buffer, 0.5 U of *Taq* polymerase. Reaction conditions for the amplification with the forward fD1 primer and the reverse rBacPre primer involved an initial cycle of 94°C for 5 min, 60°C for 2 min, and 72°C for 2 min, followed by 29 cycles of 94°C for 2 min, 60°C for 30 s, and 72°C for 2 min, with a final cycle step at 72°C for 10 min. Amplification with the universal primers, fD1 and rP2, was performed under the same conditions, except that the annealing temperature was 57°C.

For PCR-RFLP analysis, PCR products were digested to completion with the appropriate enzyme and analyzed by electrophoresis in either 1.5% agarose or 3% MetaPhor agarose (Flowgen) gels. Radioactive bands resulting from 5′-end labelling of the rBacPre primer were analyzed with a Packard InstantImager after the gel was dried.

Some additional sequencing of 16S rDNA amplified from isolated *Prevotella* strains was undertaken with an ABI373 automated sequencer to extend the previous partial-sequence information.

Estimation of *Bacteroides* and *Prevotella* DNA by hybridization. PCR products were transferred to positively charged nylon membranes (Boehringer Mannheim) by Southern blotting. After transfer, the DNA was fixed to the membrane by UV cross-linking at 120 mJ. The membranes were prehybridized for 3 to 4 h at 65°C in 0.2 volume of 20× Denhardt's solution (0.2 mg of bovine serum albumin, 0.2 mg of Ficoll, and 0.2 mg of polyvinylpyrrolidone in 10 ml of sterile dH_2O)–0.2 volume of 1% herring sperm DNA–0.2 volume of 25× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.06 volume of 5% sodium dodecyl sulfate (SDS)–0.34 volume of sterile dH_2O. This solution was boiled for 2 to 3 min and then chilled on ice for 2 to 3 min before being added to the membrane. Labelled oligonucleotide (100 ng) was then added, and the membrane was incubated overnight at 54°C. Hybridized membranes were washed twice with 2× SSC–0.1% SDS and twice with 0.1× SSC–0.1% SDS, all for 15 min at 54°C. The membranes were then sealed in a bag and placed in a Packard InstantImager or exposed to X-ray film.

Nucleotide sequence accession number. The sequence for *P. bryantii* B_14 is available as accession no. AJ00647.

RESULTS

Restriction enzyme profiles of 16S rDNA sequences amplified with a PCR primer combination specific for *Bacteroides* and *Prevotella* spp. The aim of this work was to derive information on the relative abundance in the community of different *Bacteroides* and *Prevotella* ribotypes from restriction enzyme cleavage of 16S rDNA sequences amplified from gut samples. A universal eubacterial primer, fD1 (36), and rBacPre, the reverse complement of a primer specific for *Prevotella* spp. and *Bacteroides* spp. (3), were used to amplify a 900-bp portion of the 16S rRNA gene. The recognition spectrum of the rBacPre oligonucleotide was established by using the Checkprobe program, which confirmed a 100% match for all 26 species of *Prevotella* and *Bacteroides* listed in the Ribosomal database (17), except for *B. levii* and *B. splanchnicus*, which showed a 90% match. Seven species not belonging to either of these genera (two *Flectobacillus*, *Flexibacter*, *Runella*, two *Cytophaga*, and *Thermonema*) were also recognized, but none of these have been found in rumen contents.

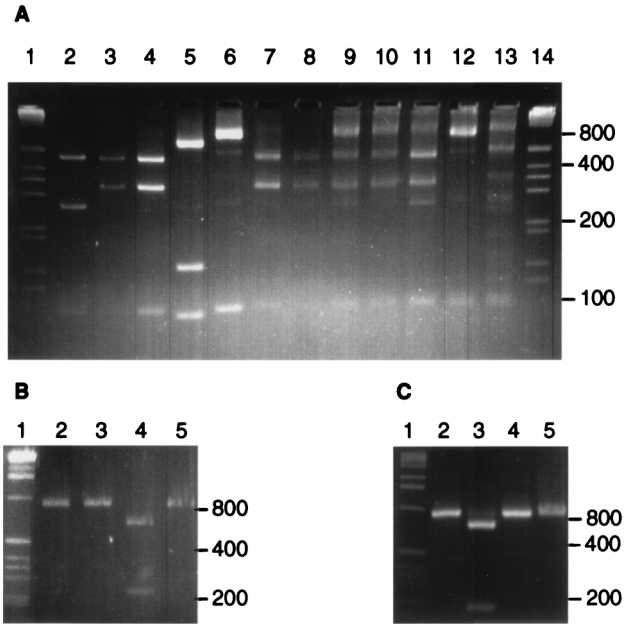

FIG. 1. Restriction enzyme cleavage of PCR amplification products from 16S rDNA. Cleavage of amplified sequences from isolated strains *P. bryantii* B_14 (lane 2), *P. ruminicola* 23 (lane 3), *P. brevis* GA33 (lane 4), *P. albensis* M384 (lane 5), and *B. vulgatus* 1447 (lane 6) by *Hha*I (A), *Aat*II (B), and *Stu*I (C) are shown. Lanes 7 to 13 show *Hha*I-cut amplification products from rumen samples derived from a cow (lane 7) and from sheep 1 to 4 (lanes 8 to 11) and human and porcine fecal samples (lanes 12 and 13, respectively). Size markers (1-kb ladder [Gibco BRL]) are shown in lanes 1 and 14.

The rBacPre-plus-fD1 primer combination was used to amplify 16S rDNA sequences from isolated strains, and restriction enzyme cleavage patterns were analyzed for the enzymes *Hha*I, *Aat*II, and *Stu*I, which were predicted from computer analysis to discriminate between *Prevotella* species (Fig. 1). Combining the results obtained with the three enzymes, it was possible to define 11 ribotypes for the 26 rumen *Prevotella* and 6 *Bacteroides* strains studied (Table 1). It should be noted that certain species of human colonic or oral origin, not studied here, are predicted to belong to additional ribotypes that were not detected in this work (Table 1, footnote *b*).

Analysis of 16S rDNA sequences amplified from rumen samples. DNA suitable for PCR amplification with the rBacPre-plus-fD1 primer combination was extracted from rumen samples as described in Materials and Methods. When the amplified products were cleaved with *Hha*I, *Aat*II, or *Stu*I, most of the products of restriction enzyme cleavage correlated with bands obtained for the isolated strains (Fig. 1). The relatively simple banding patterns obtained and the ability to correlate these bands with ribotypes of isolated strains are consistent with highly specific amplification by the rBacPre-plus-fD1 primer pair. The 323-bp band obtained after *Hha*I cleavage, predicted for ribotypes 4 and 6, was shown to hybridize with a signature oligonucleotide probe designed to recognize strains related to *P. ruminicola* 23, which belongs to ribotype 4. No hybridization was obtained for the same probe when the am-

TABLE 1. Restriction fragment patterns obtained from rumen *Prevotella* isolates and from colonic *Bacteroides* spp. after cleavage of an 896- to 898-bp region of 16S rDNA amplified with the rBacPre-plus-fD1 primer set

Bacterium	Ribotype	Enzyme	Observed band size or database prediction size (bp)[a]	Database prediction[b]
Bacteroides levii 11028	1	*Hha*I	**897**	
		*Stu*I	**897**	
		*Aat*II	**897**	
Bacteroides vulgatus 1447	2	*Hha*I	**794–799**, 101–104	*B. forsythus*, *B. maccacae*, *B. distasonis*,[d] *Porphyromonas salivosa*, *Porphyromonas oulera*
Bacteroides vulgatus 10583		*Stu*I	**892–900**	
Bacteroides uniformis 1004		*Aat*II	**892–900**	
Bacteroides ovatus 11153				
Bacteroides thetaiotaomicron 5482	3	*Hha*I	**519**, 275, 102–104	*B. fragilis*
		*Stu*I	**896–898**	
		*Aat*II	**896–898**	
Prevotella ruminicola 23	4	*Hha*I	472, **323**, 102	
Prevotella ruminicola 118B[c]		*Stu*I	736, **161**	
Prevotella ruminicola TC18[c]		*Aat*II	**897**	
Prevotella ruminicola TC35[c]				
Prevotella ruminicola TC44[c]				
Prevotella ruminicola TC27[c]				
Prevotella ruminicola TS1-2[c]				
Prevotella ruminicola TF1-2[c]				
Prevotella bryantii $B_1$4	5	*Hha*I	472, **266**, 102, 57	*P. oralis*,[d] *P. disiens*[d]
Prevotella bryantii TC1-1[c]		*Stu*I	**897**	
Prevotella bryantii 92/2[c]		*Aat*II	897	
Prevotella ruminicola TF1-5[c]				
Prevotella ruminicola TF1-10[c]				
Prevotella brevis GA33	6	*Hha*I	472, **323**, 102	
Prevotella brevis FC2[c]		*Stu*I	**897**	
Prevotella brevis FC4[c]		*Aat*II	661, **237**	
Prevotella brevis FC6[c]				
Prevotella ruminicola-like TC2-24[c]				
Prevotella albensis M384[c]	7	*Hha*I	529, **158**, 108, 102	*P. veroralis*, *P. melaninogenica*
Prevotella albensis 52/3[c]		*Stu*I	**897**	
Prevotella albensis 79/1[c]		*Aat*II	**897**	
Prevotella ruminicola TC2-3[c]	8	*Hha*I	470, **325**, 100	
		*Stu*I	**900**	
		*Aat*II	**900**	
Prevotella brevis-like TS2-7[c]	9	*Hha*I	**520**, 170, 100	
		*Stu*I	**900**	
		*Aat*II	**900**	
Prevotella ruminicola-like TF2-5[c]	10	*Hha*I	470, **325**, 100	
		*Stu*I	740 **160**	
		*Aat*II	660 **240**	
Prevotella ruminicola-like 223/M2/7[c]	11	*Hha*I	510, **160**, 110, 100, 20	
		*Stu*I	**900**	
		*Aat*I	**900**	

[a] The rBacPre terminal fragment is indicated in boldface type.
[b] Additional species predicted to give the same ribotype with respect to the rBacPre terminal fragment; other species predicted to belong to additional ribotypes (not shown) include *B. eggerthii*, *B. heparinolytica*, *B. zoogleoformans*, *P. oris*, *P. buccae*, *P. bivia*, *P. buccalis*, *P. loeschii*, *P. intermedia*, *P. denticola*, *P. corporis*, *P. nigrescens*, and *Porphyromonas gingivalis*.
[c] Full 16S rDNA sequence not available; either approximate fragment sizes or the fragment sizes for sequenced representatives of the same ribotype are shown.
[d] Predicted to show the same rBacPre terminal fragment, but differing in other *Hha*I digest products.

plified DNA was cut with *Taq*I, which is known to cut within the target site for the *P. ruminicola* 23 probe (results not shown).

As a test for bias in amplification, DNA was extracted from mixtures containing different proportions of *P. ruminicola* 23 and *P. bryantii* $B_1$4 cells and amplified with the rBacPre-plus-fD1 primer set. No evidence of bias was found, since the intensity of diagnostic bands for each strain reflected the relative contributions of the input cells (Fig. 2). The same result was obtained when purified DNA from the two strains was mixed in different proportions and subjected to amplification (results not shown).

Estimating the relative abundance of different *Bacteroides* and *Prevotella* rDNA ribotypes. PCR amplifications in which the rBacPre primer was end labelled with [γ-^{32}P]dATP were next performed. This simplifies the banding pattern, since only one terminal fragment is labelled, and also allowed the proportional contributions of particular labelled bands to the total radioactivity present in the amplified PCR product to be estimated by using a Packard β scanner (Fig. 3). Since only one ^{32}P

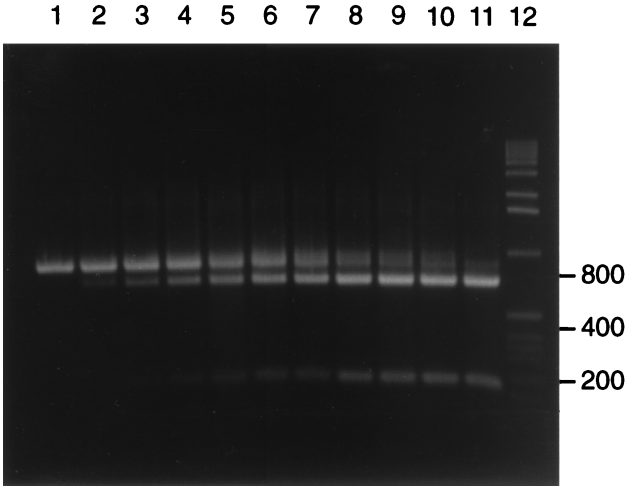

FIG. 2. *Stu*I digests of PCR amplification products obtained from mixtures of *P. ruminicola* 23 and *P. bryantii* B_14 cells, using the rBacPre-plus-fD1 primer combination. Lanes: 1 B_14 DNA only; 11, 23 DNA only; 2 to 10 contained B_14 and 23 cells in the ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, respectively.

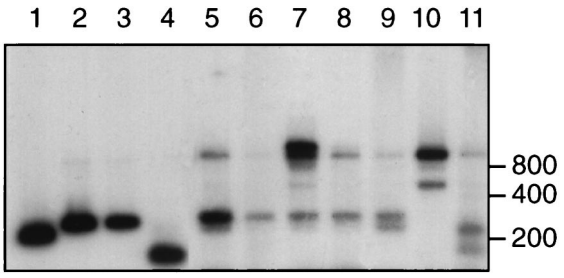

FIG. 3. Detection of ^{32}P-labelled fragments derived from digestion of 16S rDNA sequences amplified with rBacPre and end-labelled fD1 primer. Lanes: 1 to 4 PCR-amplified fragments from *P. bryantii* B_14, *P. ruminicola* 23, *P. brevis* GA33, and *P. albensis* M384, respectively, cut with *Hha*I; 5 to 9, *Hha*I-cut amplification products from rumen samples derived from a cow (lane 5) and from sheep 1 to 4 (lanes 6 to 9); 10 and 11, human and porcine fecal samples. Material in lane 7 was incompletely digested in this gel.

atom is present per fragment, detection is independent of fragment size. The sizes of the labelled restriction fragments were predicted by computer analysis for all of the *Bacteroides* and *Prevotella* spp. that gave an exact match with the rBacPre primer (Table 1). This confirmed that the ribotypes that include *P. ruminicola* 23 and *P. brevis* GA33 (ribotypes 4 and 6, respectively) do not include any other known organisms that give a PCR product with the rBacPre-plus-fD1 primer combination. Ribotypes 5 and 7 are predicted to include some other *Prevotella* spp. in addition to *P. bryantii* B_14 and *P. albensis* M384 (Table 1). The end-labelling approach could not distinguish between ribotypes 7 and 11 or between ribotypes 3 and 9, and these pairs are treated together here, as are ribotypes 1 and 2.

The relative abundance of the six most common ribotypes in rumen samples is shown in Table 2 for four sheep and one cow and in Table 3 for two further sheep. Ribotypes 4, 5, 6, and 7 plus 11, which include the best-defined rumen *Prevotella* species, together accounted for between 20 and 86% of the total amplified material from these animals. Up to 47% was due to ribotype 8, for which only one cultured rumen isolate (*P. ruminicola* TC2-3) is currently available. Ribotype 8 may represent a genetically divergent group that is underrepresented because its members are difficult to culture, and the functional properties of this group are largely unknown. Surprisingly, between 10 and 56% of ruminal material was due to representatives of ribotypes 1 plus 2, which include *Bacteroides* and *Porphyromonas* spp. Other recent studies have found evidence for *Bacteroides*-related organisms in rumen contents (5, 12).

To examine the stability of the rumen community with respect to *Bacteroides* and *Prevotella* ribotypes, rumen samples were taken from two sheep before and after a change in diet (Table 3). The results reveal a considerable difference initially in the strain profiles of the two animals. Apart from a consistent increase in ribotypes 1 plus 2, the effects of the dietary shift were quite different in the two animals. A likely explanation for this is that the two sheep harbored functionally distinct strains belonging to the same ribotypes.

A human fecal sample gave a low proportion (<10%) of bands characteristic of *Prevotella* ribotypes and a high proportion (90%) of bands of ribotypes 1 plus 2, corresponding to *Bacteroides* spp. A fecal sample from a pig gave significant proportions of ribotypes 1 plus 2, 5, and 7 plus 11, which include *Bacteroides* spp., *P. bryantii* B_14, and *P. albensis* M384, but no detectable material closely related to ribotypes 4 and 6, which include *P. ruminicola* 23 and *P. brevis* GA33, respectively (Table 2). *Prevotella* strains apparently related to ruminal isolates have been isolated from the large intestinal contents of pigs (27).

Contribution of *Bacteroides* and *Prevotella* 16S rDNA sequences to total eubacterial 16S rDNA. To estimate the amount

TABLE 2. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in amplified 16S rDNA sequences from gut samples

Sample and source	% of *Prevotella* and *Bacteroides* 16S rDNA present as ribotype:						% of *Prevotella* and *Bacteroides* in total eubacterial 16S rDNA
	1 + 2	4	5	6	7 + 11	8	
Rumen, cow	15.6	13.1	16.0	8.5	4.1	42.7	36.5
Rumen, sheep							
1	15.8	5.1	24.1	12.5	7.9	34.5	12.4
2	56.2	0.9	9.9	7.6	1.9	24.4	22.6
3	52.3	2.7	14.5	7.7	1.9	20.9	18.8
4	25.1	3.7	27.0	3.0	3.0	38.2	19.2
Feces, human	89.8	0.0	3.5	1.3	0.0	5.3	5.8
Feces, pig	39.7	0.0	27.7	0.0	13.2	19.4	2.4

TABLE 3. Changes in *Bacteroides* and *Prevotella* ribotypes with diet and sampling time in rumen liquor from two sheep

Sample and source	Sampling time[a]	Diet[b]	% of *Prevotella* and *Bacteroides* 16S rDNA present as ribotype:						% of *Prevotella* and *Bacteroides* in total eubacterial 16S rDNA
			1 + 2	4	5	6	7 + 11	8	
Rumen, sheep 5	Pre	3	12.7	15.0	8.4	5.4	14.7	43.7	62.0
	Post	3	14.1	11.4	9.0	3.3	15.4	46.7	45.0
	Pre	4	42.2	7.3	21.6	0.0	0.0	28.7	54.0
	Post	4	42.7	14.8	21.5	0.0	10.2	10.8	42.0
Rumen, sheep 6	Pre	3	10.8	4.5	49.5	29.3	6.5	0.0	45.0
	Post	3	12.3	3.0	55.4	26.2	1.6	1.5	49.0
	Pre	4	30.3	7.9	12.2	1.7	22.8	20.1	57.0
	Post	4	44.1	3.2	7.7	2.6	21.3	21.4	60.0

[a] Pre, samples taken immediately before feeding; Post, samples taken 2 h after feeding.
[b] The two animals were first fed diet 3 and then switched to diet 4 (see the text) and sampled again 28 days later.

of *Bacteroides* and *Prevotella* DNA relative to total eubacterial DNA, two universal eubacterial primers, fD1 and rP2 (36), were used to amplify most of the 16S rRNA gene. Amplified material was transferred to filters by Southern blotting and probed with a general eubacterial oligonucleotide, Uni16S (30), or with the *Bacteroides*- and *Prevotella*-specific oligonucleotide BacPre. The approximate proportion of *Bacteroides* and *Prevotella* 16S rDNA, shown in Tables 2 and 3, was calculated from the relative binding of these two probes to material amplified from gut samples and from pure cultures, correcting for any differences in probe-specific activity or hybridization kinetics (Fig. 4). *Bacteroides* and *Prevotella* sequences were estimated to account for between 12 and 62% of total eubacterial 16S rDNA in the rumen samples examined here (Tables 2 and 3).

Combining the estimates of the relative abundance of *Prevotella* and *Bacteroides* ribotypes with the estimated contribution of *Prevotella* and *Bacteroides* sequences to total eubacterial rDNA allows calculation of the contributions of individual *Prevotella* ribotypes. For example, the greatest abundance for ribotypes 4, 5, 6, and 7 plus 11 was 9, 27, 13, and 13% respectively, as percentages of total eubacterial 16S rDNA in the rumen samples studied here.

DISCUSSION

The four ribotypes that include the major rumen *Prevotella* species identified previously by culture approaches were present as a significant proportion of *Bacteroides* and *Prevotella* 16S rDNA sequences in all seven ruminant animals examined here and accounted for 20 to 86% of *Bacteroides* and *Prevotella* rDNA or 4 to 43% of the total eubacterial rDNA. At present, the largest single group of cultured rumen *Prevotella* strains (9) is probably represented by ribotype 4. Among *Prevotella* isolates from silage-fed cattle studied by van Gylswyk (33), more than 50% were *P. ruminicola* belonging to ribotype 4 (3). On the other hand, isolations of strains showing dipeptidyl aminopeptidase I (DAPI) activity (thought to be characteristic of rumen *Prevotella* strains) from sheep fed similar diets and held at the same site as those studied here (19) yielded mainly *P. bryantii*, *P. brevis*, or *P. albensis*. The present observation that ribotypes 5 and 6 were more abundant than ribotype 4 in sheep rumen samples is therefore consistent with the results of previous isolation studies. On the other hand, the most abundant *Bacteroides* and *Prevotella* ribotypes in six of the seven animals (ribotypes 8 and 1 plus 2) are represented by very few cultured strains of rumen origin. Recent investigations through random sequencing of amplified 16S rDNA from the rumen have indicated a greater diversity of *Bacteroides* and *Prevotella* spp. than previously recognized (5, 12). It appears, therefore, that certain groupings may be underrepresented among cultured strains because of difficulties in their recovery through cultivation. Studies of other ecosystems have revealed large discrepancies between viable and direct microscopic microbial counts (1), although there are reasons to expect that discrepancies would be smaller for gut ecosystems in which a certain growth rate is required to prevent washout from the system. The viable count from the rumen was previously found to vary between 14 and 75% of the total direct count for cattle fed two different diets, depending on the diet and the time after feeding (16). These discrepancies may reflect a failure to recover the full range of rumen microbial diversity, as well as changes in the viability of known organisms (21).

It is possible that certain *Bacteroides* and *Prevotella* strains are overrepresented in amplified 16S rDNA due to PCR bias (32, 34), or differential extractability of nucleic acids, but there was little evidence of this in the control experiments reported

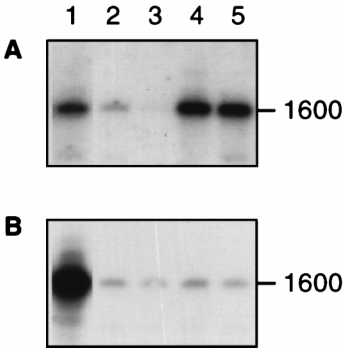

FIG. 4. Estimation of the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA sequences. Amplified sequences were transferred onto a filter by Southern blotting and probed with either the Uni16S eubacterial probe (A) or the BacPre probe (B). Lanes: 1, amplified DNA from *P. ruminicola* 23 control; 2 to 5, DNA from four different sheep rumen samples. To obtain the data shown in the final columns in Tables 2 and 3, radioactivity was estimated for each band by using a Packard beta scanner. The proportion of eubacterial 16S rDNA sequences due to *Bacteroides* and *Prevotella* was estimated as $(a_e/a_b) \times (b_b/b_e)$ where a_e and b_e are the counts obtained for the control and unknown cultures, respectively, with the universal eubacterial probe uni16S, and a_b and b_b are the corresponding counts obtained with the BacPre probe.

here. PCR bias was detected by Wilson and Blitchington (37), who obtained slightly different estimates of relative sequence abundance after 35 cycles compared with 9 cycles of PCR in amplifications of rDNA sequences from human fecal material, although the amplified region was larger than in the present study. In addition, the number of rRNA operons can vary among different bacteria (11, 24, 26), and it is not known how much variation occurs between strains of *Bacteroides* and *Prevotella*. In general, such biases may prove less of a problem when comparisons are being made, as here, within a phylogenetic grouping than among dissimilar groupings.

The approach described here offers a simple, rapid, and convenient method for obtaining information on the population structure of bacteria present in gut ecosystems. In future, more convenient quantification should be possible, for example by using fluorescently labelled rather than radioactively labelled primers for PCR. Although it cannot be assumed that ribotype frequencies correspond precisely to the abundance of different genotypes in the sample, for reasons discussed above, they can nevertheless provide important indicators of population changes between samples. This simple profiling approach therefore appears ideally suited for testing hypotheses to explain in vivo population dynamics and interanimal variability of important components of gut microbial communities. For the *Bacteroides* and *Prevotella* group, it should prove directly applicable to other anaerobic systems such as the human and animal hind gut.

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