Genetic Diversity and Phylogenetic Relationships among Strains of *Prevotella* (*Bacteroides*) *ruminicola* from the Rumen

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A high degree of genetic diversity among 29 strains of *Prevotella* (*Bacteroides*) *ruminicola* from the rumen was revealed by comparing restriction fragment length polymorphisms in 16S rRNA genes, sodium dodecyl sulfate-polyacrylamide gel profiles of total-cell proteins, and G+C contents of chromosomal DNAs. In order to obtain information on phylogenetic relationships, the sequences of a 389-bp region of the 16S rRNA gene, including variable regions 4 and 5, were compared for 10 strains. These 10 strains formed a single group when their sequences were compared with 16S ribosomal DNA sequences from other species, including *Bacteroides* spp. from the human colon. On the other hand, the great genetic distances between many *P. ruminicola* strains, including *P. ruminicola* subsp. *brevis* B₁₄ and GA33 and *P. ruminicola* 23^T (T = type strain), support the hypothesis that these organisms should be reclassified into new species. We identified signature oligonucleotides based on 16S ribosomal DNA sequences that distinguished strains related to strains 23^T, B₁₄, GA33, and M384, as well as an oligonucleotide that specifically recognized all but one of the *Bacteroides* and *Prevotella* strains tested. On the basis of the priming activities of these signature oligonucleotides in PCR reactions and on other criteria, we concluded that 12 of the original 29 strains were related to strain 23^T, 4 were related to strain B₁₄, and 4 were related to strain GA33. While there are clear grounds for subdividing the species *P. ruminicola* on the basis of genotypic differences, it is appropriate to delay formal reclassification until further work on the phenotypic differentiation of the new groups is completed.

Prevotella (*Bacteroides*) *ruminicola* has long been recognized as one of the most numerous species inhabiting the rumen (5, 20) and can also account for a high proportion of the hind gut microflora of nonruminants, including pigs and humans (18, 30, 31). In a recent study, Van Gylswyk (43) found that as many as 60% of bacterial isolates from rumina of silage-fed cows belonged to this species. The new genus *Prevotella* was recently created (37) to distinguish certain former *Bacteroides* species, including *Bacteroides ruminicola*, *Bacteroides melaninogenicus*, and *Bacteroides oralis*, from the "true" *Bacteroides* species more closely related to *Bacteroides fragilis*. *P. ruminicola* has generally been regarded as a "widely adapted" species. One potential role of this organism is in the degradation of plant cell wall polysaccharides, such as hemicellulose (7, 47) and pectin (8). *P. ruminicola* strains lack a true cellulase system, are not able to degrade crystalline cellulose, and do not cause extensive solubilization of plant cell wall material in pure cultures. On the other hand, there is evidence that these organisms contribute to plant cell wall degradation by acting synergistically with cellulolytic bacteria (28). In addition, *P. ruminicola* strains can generally utilize cellodextrins (32), starch (6), and a range of soluble sugars. One of their most significant roles, however, may be in protein and peptide breakdown (26, 44, 45).

It has been shown that *P. ruminicola* strains exhibit a high degree of genetic divergence. Mannarelli et al. (23) found that the DNA G+C contents of 14 strains varied between 38 and 51 mol%, while 8 strains exhibited less than 30% relatedness with the type strain of the species in total DNA-DNA hybridization experiments. These authors concluded that as many as nine

distinct species might be represented by these bacteria. In this study we investigated genetic variation in and phylogenetic relatedness among *P. ruminicola* strains of rumen origin. Our results fully support previous evidence indicating that there is a high degree of genetic divergence among isolates regarded as *P. ruminicola* strains and, together with other recent evidence, provide the basis for redefining new species within the group.

MATERIALS AND METHODS

Strains. The bacterial strains which we used and their origins are shown in Table 1. The isolate of strain 23 which we used gave total-protein sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) profiles identical to the profiles of ATCC 19189 and is the *P. ruminicola* type strain. M2 medium (17) was prepared anaerobically under O₂-free CO₂ by using the methods of Bryant (4).

DNA extraction. The method used to extract chromosomal DNA was based on a method described by Ausubel et al. (1). Cultures were grown to the stationary phase in M2 broth in 100-ml crimp-sealed bottles under anaerobic conditions at 38°C. The cells were centrifuged at 4,000 × g for 10 min and resuspended in 9.5 ml of TE buffer. A 0.5-ml portion of 10% SDS and 50 µl of a 20-mg/ml proteinase K solution were added, and the mixture was incubated for 1 h at 37°C. After addition of 1.8 ml of 5 M NaCl and 1.5 ml of a solution containing 10% hexadecyltrimethyl ammonium bromide in 0.7% NaCl, the mixture was incubated for 20 min at 65°C and then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acids were recovered from the aqueous phase by spooling after precipitation with 0.6 volume of isopropanol and were purified further by CsCl-ethidium bromide density gradient centrifugation. The ethidium bromide was removed by sequential extraction with water-saturated *n*-butanol, samples were dialyzed extensively against TE buffer at 4°C, and the DNA was concentrated by ethanol precipitation.

G+C determinations. DNA base compositions (G+C con-

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TABLE 1. Origins and characteristics of the 29 *P. ruminicola* strains examined in this study^a

Strain	Origin ^b	SDS-PAGE group	16S ribosomal DNA restriction pattern	DNA G+C content (mol%) ^c		PCR amplification with the following signature oligonucleotide primers ^d :					Overall group assignment
				This study	Previous studies	BacPre	23	GA33	B ₁ 4	M384	
23 ^T	1		1	49.2	50.6, ^e 49.4 ^f	+	+	—	—	—	A
118B	1		1	ND ^g	50.6, ^e 50.3 ^f	+	+	—	—	—	A
TC18	2		2	46.5		+	+	—	—	—	A
TC35	2		2	47.7		+	+	—	—	—	A
TC44	2		3	48.9		+	+	—	—	—	A
TC27	2		3	47.8		+	+	—	—	—	A
TS4-6	2	1	ND	49.9		+	+	—	—	—	A
TF1-10	2	1	4	ND				—	—	—	A
TF1-5	2	1	4	ND				—	—	—	A
TF1-2	2	1	4	47.0		+	+	—	—	—	A
TC2-3	2			47.7		+	+	—	—	—	A
TS1-2	2			ND		+	+	—	—	—	A
TC2-24	2			48.9		+	—	—	—	—	A
TF2-5	2			47.0		+	—	—	—	—	A
GA33 ^T (= ATCC 19188 ^T)	5	2		ND	50.9, ^e 50.3 ^f	+	—	+	—	—	A
FC2	6	2	5	48.5				+	—	—	A
FC4	6	2	5	48.9		+		+	—	—	A
FC6	6	2	ND	ND					—	—	A
223/M2/7	3		ND	49.1		+	—	—	—	—	B
TC2-28	2			50.2		+	—	—	—	—	
TS2-7	2		ND	46.3		+	—	—	—	—	
9958/78	4			45.3		+	—	—	—	—	
TC20	2			44.1		—	—	—	—	—	
2202	7			ND	43.5 ^h	+	—	—	—	—	
M384	6			42.8	43.8 ^e	+	—	—	—	+	B
B ₁ 4	8	3	6	40.7	41.6 ^e	+	—	—	+	—	B
TC1-1	2	3	6	42.1		+			+	—	B
TF1-3	2	3	6	39.2		+			+	—	B
TS1-5	2	3		39.7		+			+	—	B

^a Strains were grouped as far as possible on the basis of SDS-PAGE and 16S ribosomal DNA data (see text).

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^c The probable error for DNA G+C content determinations is $\pm 1\%$. Strains TC20, 2202, M384, B₁4, TC1-1, TF1-3, and TS1-5 have G+C contents that are less than 45 mol%.

^d BacPre, 23, GA33, B₁4, and M384 are oligonucleotides that were used as forward primers in PCR; these primers were used together with a universal reverse primer (see Fig. 7).

^e Data from reference 23.

^f Data from reference 29.

^g ND, not determined.

^h Data from reference 22a.

tents) were estimated by the thermal denaturation method (24), using 0.15 M NaCl–0.015 M trisodium citrate and a Gilford model 240 spectrophotometer (38). The DNA base ratio was calculated with the following equation: G+C content = 50.9 + 2.44 (melting temperature of unknown DNA – melting temperature of *Escherichia coli* DNA). *E. coli* B DNA was used as the standard.

16S ribosomal DNA restriction fragment length polymorphism. Chromosomal DNA was digested to completion with *Eco*RI (Boehringer Mannheim), and fragments were separated in a 0.6% (wt/vol) agarose gel. The DNA fragments were transferred to a GeneScreen Plus hybridization membrane (Amersham, Little Chalfont, United Kingdom) by Southern blotting. The DNA used for probe preparation was an equimolar mixture of *Bst*EII–*Hind*III fragments (approximately 650 and 950 bp) from plasmid pKK3535 (3), which represented the majority of the *E. coli* 16S rRNA gene. The fragments were separated on a 0.8% agarose gel, purified by using GeneClean (Bio 101, La Jolla, Calif.), and labelled with [³²P]dCTP by the oligonucleotide primer extension method (9, 10), using a kit

supplied by Boehringer Mannheim, Mannheim, Germany. Unincorporated label was removed by spun column chromatography, and hybridizations were performed at 65°C as described previously (11).

PCR amplification of 16S ribosomal DNA regions V4 and V5. Chromosomal DNAs from 10 *P. ruminicola* strains were used as target DNAs in PCR reactions by using procedures described by Sambrook et al. (34). *Taq* polymerase, reaction buffers, and deoxyribonucleoside triphosphates were obtained from Boehringer Mannheim and were used according to the manufacturer's recommendations. Reaction mixtures (100 µl) containing approximately 100 ng of target DNA and 200 pmol each of the forward and reverse primers were heated to 94°C for 5 min before *Taq* polymerase was added. They were then subjected to 30 cycles of amplification as follows: denaturation at 95°C for 1 min (5 min in the first cycle), annealing at 47 or 51°C for 2 min, and synthesis at 72°C for 3 min (10 min in the last cycle). DNA products were recovered by chloroform extraction and electrophoresed on horizontal agarose gels.

Cloning and sequencing of PCR products. The PCR prod-

ucts were subjected to double digestion with restriction endonucleases *Bam*HI and *Sal*I (Northumbria Biologicals, Ltd.) and then cloned into pUC13 (27). *E. coli* DH5 α transformants carrying inserted plasmids were identified on the basis of formation of white colonies on Luria-Bertani agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) and ampicillin. DNA sequencing was performed with at least two independent clones by the dideoxy chain termination method (35) from double-stranded templates, using Sequenase 2 in the presence of 7-deaza-dGTP and M13 forward (-40) and reverse sequencing primers (United States Biochemical). Internal oligonucleotide primers were also prepared with a Cruachem synthesizer.

Sequence analysis and phylogenetic tree construction. Sequences were analyzed and manipulated by using the GCG (University of Wisconsin) and CLUSTAL V packages available through the Daresbury SEQNET Facility, Daresbury, United Kingdom. The 16S rRNA sequences which were not determined in this study were obtained from the GenBank data base. Sequences were multiply aligned by using the CLUSTAL V package (16) and the default parameters provided for this purpose; the resulting alignment was then checked manually. The pairwise distances between pairs of aligned sequences were calculated, and regions which aligned with gaps in the other sequences were ignored. The distances were corrected for multiple substitutions by using Kimura's two-parameter method (21). This correction had no effect on the final tree topology because the substitution rate in 16S rRNA is low. A neighbor-joining tree (33) was then constructed from the matrix of paired distances. In order to statistically evaluate the tree produced by the neighbor-joining method, the technique known as bootstrapping was used. The original data were resampled 2,000 times as recommended by Hedges (15). The resulting P value given at each node of the tree has an accuracy of $\pm 1\%$. Thus, a bootstrap P value of 95% at a tree node indicates that 94 to 96% of samples confirmed the node. Bootstrap P values derived from 16S rRNA phylogenetic trees must be treated with some caution (25) because the substantial secondary structure of 16S rRNA violates the bootstrap assumption of independence.

SDS-PAGE. SDS-PAGE of *P. ruminicola* cell extracts was performed by the method of Laemmli (22). Cells were grown to the stationary phase in M2 medium, harvested by centrifugation at $2,500 \times g$ in sealed tubes, and washed twice in distilled water. After SDS sample buffer (22) was added, samples were boiled for 10 min and then subjected to mild sonication in a Nusonic bath (three times for 60 s, with cooling on ice).

RESULTS

Total-protein SDS-PAGE comparisons. A total of 29 *P. ruminicola* strains were examined; 18 of these (those whose designations begin with TS, TF, or TC) are strains that were isolated recently from the rumina of silage-fed cows in Sweden by Van Gylswyk (43). Six isolates were from the Rowett Research Institute, while the remaining five strains were from stock collections (Table 1).

A comparison of cell extracts by SDS-PAGE led to identification of three groups of related strains (Fig. 1). Three recent Rowett Research Institute isolates produced total-protein patterns almost identical to the *P. ruminicola* subsp. *brevis* type strain GA33 pattern, while three of the Swedish isolates produced patterns almost identical to the strain B₁₄ pattern. A third group consisted of four similar Swedish strains. For the

FIG. 1. SDS-polyacrylamide gel separations of total-cell proteins from 27 *P. ruminicola* strains. Three groups of strains can be distinguished in panel A (see Table 1). The strains included in panel B and two additional strains (223/M2/7 and TS2-7), which were compared on other gels (data not shown), did not group with any of the other strains.

remaining 17 strains, however, no two profiles were sufficiently similar to allow certain grouping.

Restriction fragment polymorphism in 16S rRNA genes. As an alternative approach to establish strain relationships, we examined the patterns obtained when we hybridized cloned fragments of the *E. coli* 16S rRNA gene to restriction enzyme digests of total *P. ruminicola* chromosomal DNA. This approach has recently proved to be valuable for many groups of bacteria, including *Bacteroides* spp. (40). Strains in the three groups previously established on the basis of SDS-PAGE comparisons generally, but not invariably, produced similar *Eco*RI restriction fragment patterns (Fig. 2 and Table 1). Three other groups of strains, one of which included type strain 23, exhibited internal similarities in their *Eco*RI digestion patterns. The remaining 13 strains examined exhibited a remarkable degree of variation in their restriction fragment patterns.

G+C contents. Mannarelli et al. (23) found that the G+C contents of the 14 *P. ruminicola* strains which they examined ranged from 38 to 51 mol%. A similar range was found in this

FIG. 2. Hybridization of 32 P-labelled DNA fragments carrying *E. coli* 16S ribosomal DNA sequences to *Eco*RI-digested chromosomal DNAs from different *P. ruminicola* strains. Chromosomal DNAs from 24 strains and from *E. coli* B were completely restricted with *Eco*RI, and the fragments were transferred to a nylon filter after separation on a 0.8% agarose gel. Fragments carrying approximately 1,600 bp of the *E. coli* 16S rRNA genes were used as probes (see Materials and Methods). The values on the left are the sizes (in base pairs) of markers (1-kb ladder [Bethesda Research Laboratories]).

study (Table 1). In agreement with Mannarelli et al. (23), B₁₄ had a G+C content close to 40 mol%, and the G+C content of type strain 23 was close to 50 mol%. The three strains which produced SDS-PAGE patterns similar to the B₁₄ pattern also had similarly low G+C contents. Only three other strains had G+C values of less than 45 mol%; the values for the remaining 17 strains for which data were obtained were between 45 and 51 mol%.

Comparison of 16S rRNA gene sequences. In order to obtain more precise information concerning the phylogenetic relationships of the strains, we compared the sequences of variable regions of their 16S rRNA genes. Ten strains were chosen for this study. Strain 23 was included as the current *P. ruminicola* type strain, on which any narrow definition of the species *P. ruminicola* should be based (see reference 23). Strains TF1-2, B₁₄, and GA33^T (T = type strain) were chosen as representatives of the three SDS-PAGE groups described above; we also included strain M384, which appears to typify another SDS-PAGE group, as determined in a separate study (26). B₁₄ and GA33^T were previously identified as members of *Bacteroides ruminicola* subsp. *brevis* (19). 223/M2/7 was chosen because of its importance in previous genetic studies (13, 39). The remaining four strains examined, TC18, TC27, TC2-5 and TC2-24, all had G+C contents (46.5 to 49 mol%) reasonably close to the G+C content of type strain 23 (49 to 50 mol%) but could not

forward 5' gaattcgtcgacTGCCAGCAGCCGCGTAATA 3'
*Eco*RI *Sal*I
reverse 5' aagcttggatcCCCGTCAATTCCTTTGAGTT 3'
*Hind*III*Bam*HI

FIG. 3. Oligonucleotide primers used for PCR amplification of a 389-bp region of the 16S rRNA gene from rumen *Prevotella* strains. The bases in lowercase letters are bases that were added to create restriction endonuclease sites for use in cloning amplified fragments. The bases in uppercase letters are complementary to bases in conserved regions of the 16S rRNA gene.

be grouped definitively with it or with each other on the basis of the other criteria used.

Chromosomal DNA was prepared from each of the 10 strains and used as a template in amplification reactions with *Taq* polymerase by using primers that were designed and synthesized to recognize conserved regions some 389 bp apart in the 16S rRNA gene (Fig. 3). The forward primer (residues 516 to 535 in *E. coli* [see reference 3]) was from a region believed to be strongly conserved among eubacteria (46). The reverse primer (residues 908 to 928 in *E. coli* [3]) was also from a highly conserved region but contained a residue known to be different in *E. coli* and *B. fragilis*, and the *B. fragilis* sequence was assumed to be more likely conserved in *Prevotella* strains. The amplified sequence included variable regions 4 and 5. Each primer included additional residues at the 5' end, which provided sites for restriction enzyme cleavage to allow cloning of the amplified product into pUC vectors. Not all of the strains gave amplified products when an annealing temperature of 51°C was used, and therefore a temperature of 47°C was used routinely; this was almost certainly due to variations in the sequence recognized by the reverse primer. The sequence of the amplified region was determined on the basis of at least two independent clones for each strain (Fig. 4).

Estimates of genetic distances for all 10 *P. ruminicola* strains and representatives of several other bacterial genera, based on the data in Fig. 4, are shown in Table 2. Multiple sequence comparisons were also made for the same group of strains, and a phylogenetic tree was constructed on the basis of the genetic distance measurements (Fig. 5). Our conclusions are summarized as follows. First, the 10 *P. ruminicola* strains, which were classified as *Bacteroides* strains until recently, all fall in a broad group that also includes the *Bacteroides* species *Bacteroides vulgatus*, *B. fragilis*, *Bacteroides thetaotaomicron*, and *Bacteroides distasonis*. All of these organisms possess sequence elements that are characteristic of the *Bacteroides-Flavobacterium* phylum defined by Woese (48) (Table 3). The 10 strains form a separate cluster distinct from *Bacteroides* species (Fig. 5), which supports the hypothesis that they should be reclassified in new genera (36, 37). The variation within this cluster is considerable, however. Strains 23^T, TC27, TC18, and TF1-2 have almost identical sequences and clearly belong to the narrowly defined species *P. ruminicola* as exemplified by type strain 23. On the other hand, the genetic distances between other strains of *P. ruminicola* are often greater than the genetic distances between representatives of two different genera of gram-positive bacteria (*Clostridium perfringens* and *Bacillus subtilis*). This is true, for example, for strains GA33^T and B₁₄, which were once regarded as members of the same subspecies.

The 16S rRNA sequence comparisons revealed an unambiguous division between the group of strains represented by B₁₄,

23	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
TF12	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
TC18	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
TC27	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
TC2-24	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
TF2-5	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
GA33	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
B14	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
223/M2/7	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
M384	TGCCAGCAGC	CGCGGTAATA	CGGAAGGTCC	AGGCGTTATC
23	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
TF12	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
TC18	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
TC27	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
TC2-24	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
TF2-5	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
GA33	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
B14	CGGATTTTATT	GGGTTTAAAG	GGAGCGCAGG	CTGATGATT
223/M2/7	CGGATTTTATT	GGGTTTAAAG	GGAGCGT	CTGATGATT
M384	CGGATTTTATT	GGGTTTAAAG	GGAGCGT	CTGATGATT
23	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
TF12	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
TC18	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
TC27	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
TC2-24	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
TF2-5	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
GA33	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
B14	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
223/M2/7	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
M384	AGCGTGACGT	GAAATGTAGC	CGCTCAACGG	CTGAACCTGC
23	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
TF12	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
TC18	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
TC27	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
TC2-24	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
TF2-5	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
GA33	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
B14	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
223/M2/7	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
M384	GTCGCGAACT	GGTTATCTTG	AGTGAGTTTCG	ATGTTTGGCGG
23	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
TF12	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
TC18	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
TC27	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
TC2-24	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
TF2-5	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
GA33	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
B14	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
223/M2/7	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
M384	AATTCGTGGT	GTAGCGGTGA	AATGCTTAGA	TATCACGAAG
23	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
TF12	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
TC18	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
TC27	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
TC2-24	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
TF2-5	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
GA33	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
B14	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
223/M2/7	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC
M384	AACCTCCGATT	GCGAAGGCAG	CCAACAAAGGC	CTTTACTGAC

FIG. 4. Multiple sequence alignment of 16S ribosomal DNAs from 10 *P. ruminicola* rumen strains. Sequences were determined as described in Materials and Methods for both strands of the region shown. A black background indicates that a residue was conserved in at least 6 of the 10 aligned sequences. The region shown corresponds to positions 516 to 907 of the *E. coli* 16S rRNA gene (3; also see text).

M384, and 223/M2/7A, which is referred to below as group B, and the remaining seven strains, which is referred to below as group A. Group A was unambiguously subdivided into strain 23^T-like strains (five strains, including the somewhat distantly related organism strain TC2-24) and the strain GA33^T-like strains (GA33^T and TF2-5) (Fig. 5).

Signature oligonucleotides. Potential signature sequences in the aligned 16S rRNA sequences were identified for strains 23^T, GA33^T, B14, and M384 (Fig. 6). Oligonucleotides having these sequences were used as forward primers in PCR reactions along with a "universal" reverse primer (5'-ACGGGCG GTGTGTACAAGGCC) (41). Amplification occurred with template DNAs from strains belonging to the appropriate group and not with DNAs from strains belonging to other groups. Of the 25 strains tested, 10 gave positive amplification results with the strain 23^T primer, 4 gave positive results with the B14 primer, 2 gave positive results with the GA33^T primer,

and 1 gave positive results with the M384 primer (Table 1 and Fig. 7). The remaining eight strains (2202, 223/M2/7, 9958/78, TC2-24, TC2-28, TC20, TS2-7, and TF2-5) did not give positive amplification results with any of the four group-specific primers. In addition, we identified an oligonucleotide that was conserved in all of the *Prevotella* and *Bacteroides* strains shown in Fig. 5 (Fig. 6). This oligonucleotide, designated BacPre, did not occur in the 16S ribosomal DNA of any other bacterial group examined in our data base searches. When used as a forward primer in conjunction with the universal reverse primer mentioned above, BacPre gave positive amplification results with all 25 strains tested except TC20 (Table 1).

As an additional test of the efficacy of the group-specific primers, we examined five strains isolated recently from rumina (26). Three strains (79/1, 79/2, and 52/3), which have been reported to produce total-protein SDS-PAGE profiles similar to the M384 profile, were recognized by the M384 group

TABLE 2. Genetic distances for 10 *P. ruminicola* strains from rumina and other bacterial species based on comparisons of partial 16S rRNA sequences

Organism	No. of nucleotide substitutions per position ^a											
	<i>P. ruminicola</i> strains											
	23 ^T	TC18	TC27	TF1-2	TF2-5	TC2-24	GA33 ^T	B ₁ 4	M384	223/M2/7		
<i>P. ruminicola</i> 23 ^T												
<i>P. ruminicola</i> TC18	0.0											
<i>P. ruminicola</i> TC27	0.0	0.0										
<i>P. ruminicola</i> TF1-2	0.0	0.0	0.0									
<i>P. ruminicola</i> TF2-5	0.052	0.052	0.052	0.052								
<i>P. ruminicola</i> TC2-24	0.052	0.052	0.052	0.052	0.043							
<i>P. ruminicola</i> GA33 ^T	0.067	0.067	0.067	0.067	0.034	0.058						
<i>P. ruminicola</i> B ₁ 4	0.130	0.130	0.130	0.130	0.130	0.127	0.120					
<i>P. ruminicola</i> M384	0.127	0.127	0.127	0.127	0.124	0.131	0.124	0.111				
<i>P. ruminicola</i> 223/M2/7	0.144	0.144	0.144	0.144	0.144	0.144	0.147	0.127	0.074			
<i>B. vulgatus</i>	0.180	0.180	0.180	0.180	0.200	0.206	0.174	0.187	0.201	0.197		
<i>B. thetaiotaomicron</i>	0.184	0.184	0.184	0.184	0.188	0.174	0.174	0.176	0.175	0.190		
<i>B. fragilis</i>	0.187	0.187	0.187	0.187	0.191	0.179	0.176	0.162	0.175	0.189		
<i>B. distasonis</i>	0.230	0.230	0.230	0.230	0.211	0.237	0.218	0.244	0.249	0.265		
<i>Cytophaga lytica</i>	0.289	0.289	0.289	0.289	0.277	0.280	0.297	0.294	0.296	0.284	0.219	
<i>Clostridium perfringens</i>	0.323	0.323	0.323	0.323	0.320	0.333	0.290	0.285	0.305	0.305	0.252	0.309
<i>Fusobacterium nucleatum</i>	0.360	0.360	0.360	0.360	0.338	0.334	0.347	0.343	0.351	0.388	0.309	0.351
<i>Bacillus subtilis</i>	0.379	0.379	0.379	0.379	0.352	0.370	0.334	0.317	0.356	0.360	0.322	0.356
<i>E. coli</i>	0.383	0.383	0.383	0.383	0.370	0.393	0.383	0.322	0.388	0.393	0.313	0.388
<i>Haemophilus influenzae</i>	0.422	0.422	0.422	0.422	0.412	0.442	0.422	0.393	0.407	0.408	0.353	0.407
											0.297	0.249
											0.301	0.249
											0.241	0.214
											0.233	0.207
											0.127	0.249
											0.305	0.374
											0.288	0.374
											0.296	0.305
											0.322	0.313
											0.351	0.353
											0.309	0.309
											0.288	0.288
											0.252	0.252
											0.148	0.148
											0.011	0.011
											0.064	0.064
											0.134	0.134
											0.254	0.254
											0.265	0.265
											0.305	0.305
											0.388	0.388
											0.351	0.351
											0.360	0.360
											0.393	0.393
											0.407	0.407
											0.408	0.408
											0.379	0.379
											0.340	0.340
											0.317	0.317
											0.292	0.292
											0.280	0.280
											0.241	0.241
											0.222	0.222
											0.134	0.134
											0.064	0.064
											0.190	0.190
											0.189	0.189
											0.175	0.175
											0.249	0.249
											0.265	0.265
											0.284	0.284
											0.305	0.305
											0.388	0.388
											0.351	0.351
											0.360	0.360
											0.393	0.393
											0.407	0.407
											0.408	0.408

^a Corrected for multiple changes by the Kimura two-parameter method (see Materials and Methods).

FIG. 5. Phylogenetic relationships of the *P. ruminicola* strains. The values in italics are the genetic distances (expressed as number of substitutions per 100 nucleotides) between strains based on a comparison of a 389-bp region of 16S ribosomal DNA including regions V4 and V5 (Fig. 4; see text). The length of the horizontal lines are proportional to genetic distances; the lengths of the vertical lines are arbitrary. The values in boldface type are the percentages of trials in which the branch points indicated occurred when repeated bootstrapping procedures were performed.

primer, while the other two strains (92/1 and 92/2), which have been reported to resemble B₁4, were recognized by the B₁4 group primers.

DISCUSSION

Mannarelli et al. (23) concluded previously that *P. ruminicola* is genetically a highly diverse taxon. These authors showed that the previous subdivision of *B. ruminicola* into subspecies

Strain 23	5' ATCTTGAGTGAGTTCGATGTTGG 3'
Strain GA33	5' TCCTTGAGTGATTCGACGTCAG 3'
Strain M384	5' TACTTGAGTACGCACAACGTAGG 3'
Strain B ₁ 4	5' ACTTGAGTGACAGGAAGCG 3'
<i>Bacteroides/Prevotella</i> (BacPre)	5' GAGTACGCCGGCAACGGTGA 3'

FIG. 6. Oligonucleotides that differentiate *P. ruminicola* strains. These oligonucleotides were selected from multiple alignments as oligonucleotides that distinguish four groups of *P. ruminicola* strains (23^T, GA33^T, M384, B₁4) or all *Bacteroides* and *Prevotella* strains (BacPre). No corresponding sequences were found in FASTA searches of the whole DNA data base. Used in conjunction with a universal reverse primer (see text), these oligonucleotides produced group-specific amplification of 16S ribosomal DNA sequences from chromosomal DNAs (see Table 1 and Fig. 7).

B. ruminicola subsp. *brevis* and *B. ruminicola* subsp. *ruminicola* on the basis of morphology and hemin requirement was largely meaningless and proposed that the species *P. ruminicola* should be limited to strains that are closely related to type strain 23, have G+C contents between 49 and 51 mol%, and are more than 60% related to strain 23 as determined by total-DNA hybridization experiments. They concluded that of the strains included in this study, M384, B₁4, and GA33^T differed enough in G+C content or DNA-DNA hybridization characteristics from each other and from strain 23^T that they should be placed in different species. The pairwise DNA-DNA hybridization values for strains 23^T, M384, B₁4, and GA33^T all ranged from 19 to 22%, whereas the value for 23^T and 118B was 83% (23). Since the potential species were represented by single strains, however, it was difficult to reclassify them.

On the basis of our comparison of 16S ribosomal DNA sequences and on the basis of recognition by signature oligonucleotides, together with other criteria, we assigned 18 of the 29 strains which we studied to one broad genotypic group, group A, and 6 strains to a second broad genotypic group, group B, which left the status of five strains (2202, TC20, 9958/78, TC2-28, and TS2-7) uncertain (Table 1). As noted previously (42), strain 2202, which is distributed by the National Collection of Food Bacteria (Shinfield, United Kingdom) as strain 23, is clearly distinct from *P. ruminicola* 23^T; this strain has been used in some genetic and physiological studies

TABLE 3. Comparison of sequences of *P. ruminicola* strains at signature positions which characterize the *Bacteroides-Flavobacterium* phylum^a

Position of base ^b	<i>Bacteroides</i> spp.	23 ^T	B ₁ 4	GA33 ^T	M384	TC2-24	TF2-5	223/M2/7A	Ancestral position ^c
564	U	U	U	U	U	U	U	U	— ^d
570	U	U	U	U	U	U	U	U	G
684	U	U	U	U	U	U	U	U	U
698	G	G	G	G	G	G	G	G	G
718	U	U	U	U	U	C	C	C	A
809	A,G	A	A	A	A	A	A	A	G
812	G	G	G	G	G	G	G	G	G
837	C,G	C	C	C	U	C	C	G	U,G
849	C,G	—	—	—	—	—	—	—	G,G
868	C	C	C	C	C	C	C	C	C
870	U	U	U	U	U	U	U	U	U
906	G	G	G	G	G	G	G	G	G

^a See reference 14. The sequences of strains TC27, TC18, and TF1-2 (data not shown) were identical to the sequence of strain 23^T at these positions.

^b Position number in the *E. coli* sequence (3).

^c Ancestral composition was defined as the dominant composition in the majority of bacterial phyla (14).

^d —, deletion compared with *E. coli* sequence in the region between positions 832 and 850 in the majority of aligned sequences.

FIG. 7. Specific amplification of an approximately 800-bp region of 16S ribosomal DNA by using the B₁4 oligonucleotide together with the universal reverse primer. Lanes of an agarose gel were loaded with PCR mixtures from experiments performed with chromosomal DNAs from *P. ruminicola* B₁4 (lane 2), TC1-1 (lane 3), TS1-5 (lane 4), TF1-3 (lane 5), 92/1 (lane 6), 92/2 (lane 7), TC18 (lane 8), M384 (lane 9), and GA33^T (lane 10) and *E. coli* B (lane 11). Strains 92/1 and 92/2 are described in reference 26; other strains are listed in Table 1. Lane 1 contained molecular size markers (1-kb ladder [BRL]).

(12, 47). We propose that the species *P. ruminicola* should be redefined more narrowly so that it includes only those strains likely to be recognized by the strain 23^T signature oligonucleotide. On this basis 12 of the strains which we studied would belong to this species; the G+C contents of these strains range from 46 to 51 mol%, which is a slightly wider range than the range proposed by Mannarelli et al. (23). The four strains related to GA33^T were clearly distinct from the strains in group A related to strain 23^T, and we believe that these four strains should form a separate species created by elevating the *P. ruminicola* subsp. *brevis* strains related to GA33^T to the species level. The two remaining group A strains, TC2-24 and TF2-5, are probably best regarded as strain 23^T-like and strain GA33^T-like, respectively.

The finding that three recently isolated strains from Sweden (Table 1) and two strains from Scotland (26) are genotypically closely related to strain B₁4 (presently regarded as a *P. ruminicola* subsp. *brevis* strain) suggests that it is appropriate to designate an additional species characterized by low G+C contents (39 to 42 mol%). The genotypic differences among B₁4, M384, and 223/M2/7 (group B) were too large for all three strains to be considered members of the same species, and separate species status may be appropriate for M384 and 223/M2/7 once additional related strains have been characterized. In the case of M384, a signature oligonucleotide has already confirmed its close relationship with three strains recently isolated from rumina (26).

Although we believe that there is now compelling evidence for the creation of at least three new genotypic species for *P. ruminicola* strains, based on strains 23^T, GA33^T, and B₁4, we believe that it is premature to propose naming these taxa formally as new species until convenient phenotypic criteria can be provided to distinguish them. There are already indications that the genetic differences are reflected in functional differences with respect to fermentation patterns, peptide or protein breakdown, and polysaccharidase activities (2, 2a, 26).

It will also be important to establish whether nonrumen isolates (e.g., isolates from pig guts) regarded as *P. ruminicola* strains correspond to any of the new species recognized mentioned above or whether these strains represent entirely different groups. In the future, more rapid and extensive surveys of strains should be possible with the signature oligonucleotides designed in this study.

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