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Analysis of rRNA Restriction Fragment Length Polymorphisms from *Bacteroides* spp. and *Bacteroides fragilis* Isolates Associated with Diarrhea in Humans and Animals

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The *Escherichia coli* rRNA operon *rrnB* was used as a ^{32}P-labeled hybridization probe in Southern blots of genomic DNAs from representative strains of the saccharolytic, gram-negative, obligate anaerobes of the genus *Bacteroides*. Control experiments with the *B. fragilis* type strain ATCC 25285 established that nearly identical rRNA fragment patterns were produced when either the *E. coli rrnB* gene probe or homologous rRNA isolated from *B. fragilis* was used as the probe. In addition, it was shown that a specific 16S or 23S *rrnB* gene probe also could be used to produce fragment patterns suitable for analysis. Thirty-one strains from 8 of the 10 recognized *Bacteroides* species were then examined. The resulting autoradiographs revealed specific fragment patterns for all but one (*B. ovatus*) of the species tested. Restriction fragment length polymorphisms were observed for many of the strains tested, but these differences did not hinder species classification. The five *B. ovatus* strains examined did not form a distinct group, and their rRNA fragment patterns displayed a marked heterogeneity. The same approach was applied to a unique set of enterotoxin-producing *B. fragilis* strains isolated from animals and humans with diarrhea. The results demonstrated that these strains were in fact *B. fragilis* and that they produce rRNA fragment patterns closely related to those of the type strain ATCC 25285. This set of strains did not appear to form a separate subgroup or genotype within the *B. fragilis* species, and there were no distinguishable restriction fragment length polymorphisms that could be used to specifically separate enterotoxin-producing strains from nonenterotoxigenic strains.

Bacteroides species are obligately anaerobic, non-spore-forming, gram-negative bacilli normally found associated with the intestinal tracts of humans and animals. Historically, *Bacteroides* species was regarded as a phenotypically and phylogenetically heterogeneous group which encompassed diverse isolates from the oral cavities and intestinal tracts of animals, including ruminants (11). However, on the basis of DNA homology, RNA homology, and biochemical and molecular parameters, recently proposed revisions to taxonomic criteria restrict the genus to 10 species of saccharolytic, bile-resistant organisms that include *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. vulgatus*, *B. distasonis*, *B. eggerthii*, *B. merdae*, *B. caccae*, and *B. stercoris* (4, 13, 15, 16, 26).

In addition to their important role as part of the indigenous flora, *Bacteroides* species are significant opportunistic pathogens (primarily of humans) that are responsible for a variety of soft tissue and other infections (7, 19). Although *B. fragilis* is the most frequently isolated anaerobic pathogen, other *Bacteroides* species, such as *B. thetaiotaomicron*, are commonly encountered in clinical specimens; and these organisms display significant differences in their antimicrobial susceptibility patterns (31). Because of this variation, rapid identification to the species level is desirable for the selection of appropriate antibiotic therapy in patients with life-threatening infections. Current methods for the identification of *Bacteroides* species rely heavily on the use of biochemical tests, and little work has been done to explore the use of alternate identification strategies.

Recently, a number of reports have appeared on the characterization of a variety of genera by using restriction fragment length polymorphism (RFLP) of the rRNA operons (9, 24). Typically, these RFLPs are identified by hybridization of genomic DNA to radiolabeled rRNA or cloned rRNA genes. This approach has been used successfully to characterize members of the genera *Staphylococcus* (5), *Bacillus* (8), and *Campylobacter* (20). Furthermore, this technique has been used for epidemiological studies of closely related organisms such as uropathogenic *Escherichia coli* (1), nontypeable *Haemophilus influenzae* (30), and *Pseudomonas cepacia* (30).

In this report we describe the use of the cloned *E. coli rrnB* locus for rRNA RFLP analysis of several important *Bacteroides* species (sensu strictu). Our objective was to demonstrate the feasibility of this approach for differentiation among the species and to firmly establish the relationship among *B. fragilis* strains. We then applied this technique in an epidemiological study of a novel set of *B. fragilis* strains that elaborate an enterotoxin and that were isolated from the feces of diarrheic lambs, calves, pigs, and humans (summarized in reference 23). Considering the variety of sources and the unusual property (i.e., enterotoxin production) of these *B. fragilis* strains, it seemed possible that this group would form a distinct subset of the species.

MATERIALS AND METHODS

Bacterial strains and growth. Strain designations and origins of the *Bacteroides* isolates used in the present study are described in Tables 1 and 2. All strains were maintained at room temperature in anaerobic chopped meat medium or

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TABLE 1. *Bacteroides* strains examined in this study

Species	Strain	Source[a]
B. fragilis	ATCC 25285	A, V
B. fragilis	AM-78	W, B
B. fragilis	BF-2	D
B. fragilis	RBF-49	C
B. fragilis	638	S
B. fragilis	WAL 7505	W
B. fragilis	ATCC 29768	A, V
B. fragilis	VPI B-70	V, M
B. fragilis	ATCC 29771	A, V
B. fragilis	VPI 2393	V
B. fragilis	ATCC 23745	A
B. fragilis	CLA 262	H
B. thetaiotaomicron	ATCC 29741	A, W
B. thetaiotaomicron	ATCC 29742	A, W
B. thetaiotaomicron	VPI B1-46	V
B. thetaiotaomicron	ATCC 29148	V
B. thetaiotaomicron	VPI 11111	V
B. ovatus	ATCC 8483	A
B. ovatus	VPI C1-45	V
B. ovatus	VPI 3524	V
B. ovatus	VPI 4244	V
B. ovatus	WAL 7606	W
B. uniformis	ATCC 8492	A
B. uniformis	VPI 0061	V, M
B. uniformis	WAL 7088	W
B. vulgatus	CCLA 341	H
B. vulgatus	VPI B2-4	V
B. vulgatus	ATCC 8482	A
B. vulgatus	WAL 7062	W
B. eggerthii	VPI T3-3	V
B. caccae	ATCC 43185	A, V

[a] References and or sources for strains are as follows: A, American Type Culture Collection, Rockville, Md.; B, M. Britz, University of Melbourne; C, D. Callihan, University of Rochester; D, D. Woods, University Cape Town; H, P. Appelbaum, Hershey Medical Center; M, F. L. Macrina, Virginia Commonwealth University; S, M. Sebald, Pasteur Institute; V, Anaerobe Laboratory, Virginia Polytechnic Institute and State University; W, Anaerobe Laboratory, Wadsworth Medical Center.

TABLE 2. *B. fragilis* enterotoxin-producing strains

Strain no.	Date of isolation (mo/yr)	Designation	ATCC strain[a]	Source[b]
20428-3 Ent$^+$	2/84	C1		Calf
20147-1 Ent$^+$	2/84	C2		Calf
"X" Ent$^+$	4/83	L1		Lamb
"S" Ent$^+$	4/83	L2		Lamb
3-98B-3 Ent$^+$	2/85	P1		Piglet
3-101-1 Ent$^+$	2/85	P2		Piglet
20793-3 Ent$^+$	4/86	H1	43859	Human
207832-3 Ent$^+$	4/86	H2	43858	Human
20835-1-1 Ent$^+$	4/86	E1		Foal
20840-3-1 Ent$^+$	5/86	E2		Foal
077225-1 Ent$^-$	4/86	H3		Human
077351-1 Ent$^-$	4/86	H4		Human

[a] ATCC, American Type Culture Collection, Rockville, Md.
[b] All strains, including the two Ent$^-$ strains, were isolated from diarrheic individuals (23). Each isolate was obtained from a different animal or patient. Ent, enterotoxin.

were frozen at −70°C in the same medium containing 20% glycerol. Cultures were routinely grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin, cysteine, and $NaHCO_3$ and were incubated in a Coy anaerobic chamber as described previously (10, 27). *E. coli* HB101 (*recA leuB ara proA lacY galK rpsL xyl mtl supE* [18]) containing pKK3535 (3) was grown with shaking at 37°C in L broth containing 50 μg of ampicillin per ml.

DNA and RNA preparation. Genomic DNAs from *B. fragilis* ATCC 25285, AM78, V503, RBF49, V479-1, 638, V531, and WAL 7505 were prepared from 50-ml overnight cultures and purified by CsCl-ethidium bromide ultracentrifugation as described previously (28). A second method for the isolation of genomic DNA was used for all other strains tested. This rapid method was found to yield DNA suitable for restriction endonuclease digestion and hybridization analysis and was performed as follows. Five-milliliter overnight cultures were harvested by centrifugation (10,000 × *g*, 4°C, 15 min), washed in buffer (50 mM Tris, 5 mM EDTA, and 50 mM NaCl [pH 8]), and then stored at −20°C. The cell pellet was thawed, suspended in 0.5 ml of sucrose lysis buffer (34) containing 40 μg of RNase A (Sigma Chemical Co.; St. Louis, Mo.) per ml, and then incubated in a water bath at 37°C for 10 min. Fifty microliters of 20% sodium dodecyl sulfate (SDS) was added, incubated at room temperature for several minutes to allow lysis, and then vortexed at maximum speed for 2 min. The resulting lysate was extracted twice with equal volumes of phenol and chloroform and then precipitated with ethanol. The nucleic acid pellet was suspended in 500 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) and precipitated with ethanol for a second time. The final nucleic acid pellet was suspended in 100 μl of TE buffer containing 10 μg of RNase per ml. DNA samples were stored at −20°C until they were needed.

Plasmid pKK3535 was isolated by alkaline lysis (2) and then purified by CsCl-ethidium bromide ultracentrifugation. Restriction fragments bearing the rRNA genes were obtained by sequential digestion of pKK3535 with *Pst*I, *Bam*HI, and *Xba*I. This strategy resulted in the production of a 2.9-kb *Pst*I-*Xba*I fragment containing the 16S gene and a 4.2-kb *Xba*I-*Bam*HI fragment containing the 23S gene (3). These DNA fragments were separated by agarose gel electrophoresis, individually extracted from gel slices by absorption onto glass beads (GeneClean; Bio 101, LaJolla, Calif.), suspended in TE buffer, and stored at −20°C until they were needed.

rRNA was isolated from 100-ml mid-exponential-phase cultures of *B. fragilis* ATCC 25285 as described by Lane et al. (17), with the following modifications. Following the first ethanol precipitation, the nucleic acid pellet was suspended in 10 mM Tris–1 mM $MgCl_2$–2 M NaCl (pH 7.5) and held overnight on ice. The salt-insoluble RNA (predominately 16S and 23S rRNAs) was collected by centrifugation, dissolved in TE buffer, and precipitated with ethanol. The final preparation (1.72 mg/ml) was analyzed by formaldehyde agarose gel electrophoresis, and there was no 5S rRNA visible on the gel.

Hybridization analysis. Genomic DNA samples were digested with restriction endonuclease *Eco*RI, *Pst*I, *Hin*dIII, or *Bam*HI and electrophoresed on 0.8% agarose gels in Tris acetate buffer (18). DNA was transferred from the gels to nitrocellulose filters by capillary action (29). DNA probes were labeled with ^{32}P by the nick-translation reaction (25) with a commercial kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). RNA probes were labeled with γ-^{32}P by using the exchange reaction of T4 polynucleotide kinase (18). DNA-DNA hybridizations were performed

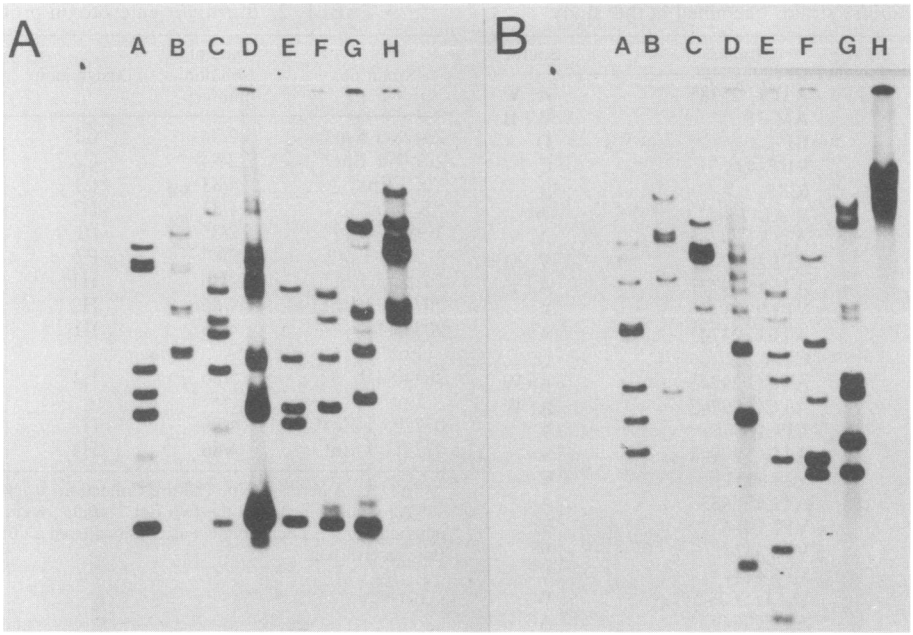

FIG. 1. Autoradiograph of *Bacteroides* species genomic digests probed with radiolabeled pKK3535. (A) Genomic DNAs digested with *Eco*RI; (B) genomic DNAs digested with *Pst*I. Lane designations are as follows: A, *B. fragilis* ATCC 25285; B, *B. thetaiotaomicron* ATCC 29148; C, *B. ovatus* ATCC 8483; D, *B. vulgatus* ATCC 8484; E, *B. uniformis* ATCC 8492; F, *B. eggerthii* VPI T3-3; G, *B. caccae* ATCC 43185; H, *B. distasonis* VPI A12-1.

overnight at 60°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–4× Denhardt solution–1 mg of sheared salmon sperm DNA per ml as described previously (32). Filters were then washed three times for 30 min each time at 60°C in 3× SSC–0.1% SDS. The conditions used for RNA-DNA hybridizations were similar to those used for DNA-DNA hybridizations, except that the hybridization was performed at 67°C and the first three washes were done in 0.1× SSC–0.1% SDS at 52°C and then three 30-min washes were done in 0.1× SSC at 52°C.

RESULTS

Specificity of rRNA gene probes. Initial hybridization experiments demonstrated discrete regions of homology between several *Bacteroides* species and the *E. coli rrnB* gene on pKK3535 (Fig. 1). The patterns appeared to be specific for each of the species and restriction enzymes tested, and the pBR322 portion of the plasmid did not hybridize with any of these *Bacteroides* strains (data not shown). This homology was examined further by probing genomic digests of the *B. fragilis* type strain ATCC 25285 with isolated DNA fragments containing either the 16S or 23S regions from the *E. coli rrnB* operon. The 2.9-kb 16S probe had strong homology with 11 *Eco*RI fragments ranging in size from 1.8 to 11.3 kb, whereas the patterns seen with the other enzymes tested (*Pst*I, *Hin*dIII, *Bam*HI) were less complex, with there being between five and eight homologous fragments (Fig. 2A). Identical digests probed with the 4.2-kb 23S fragment resulted in a simpler *Eco*RI pattern, with there being only seven homologous fragments. These seven *Eco*RI fragments were common to both the 16S and 23S probes (Fig. 2A and B, arrows). The results obtained with the other enzymes revealed that there is little difference between the two probes except for the relative intensities of some of the hybridizing bands, and in both the *Hin*dIII and *Bam*HI digests, two new small (<1-kb) fragments were seen with the 23S probe.

The results obtained with the individual 16S and 23S probes were compared with the results obtained on blots hybridized with the entire nick-translated pKK3535 (Fig. 2C). For *Eco*RI digests, the patterns that we observed resembled those seen with the 23S probe, but with prolonged exposure, all of the fragments originally seen with each of the individual probes could eventually be observed. The greater intensities of the 23S fragments may be due to the fact that these fragments are actually common to both probes and may have contained more counts than were seen on blots probed with only the 16S specific fragments. The other enzymes tested showed little difference relative to the specific probes.

In order to demonstrate that the hybridization patterns observed with the pKK3535 probes were, in fact, due to homology with the rRNA genes of *B. fragilis*, rRNA was purified from *B. fragilis* ATCC 25285 and was used as a probe. The results shown in Fig. 2D reveal nearly identical hybridization patterns obtained by using the pKK3535-derived probes. In the case of *Eco*RI, the hybridization pattern was the same as that seen with the 16S probe, although there were two weakly hybridizing high-molecular-weight bands not seen previously with the pKK3535-derived probes. Overall, use of rRNA as a hybridization probe led to greater background interference which may have been due to some contamination of the RNA samples with mRNA.

Comparison of *Bacteroides* strains. In order to establish the specificity of rRNA banding patterns for a given species, 12 *B. fragilis*, 5 *B. thetaiotaomicron*, 5 *B. ovatus*, 3 *B. uniformis*, and 4 *B. vulgatus* strains were examined in more detail by using the entire pKK3535 plasmid as a probe. The results are represented schematically in Fig. 3. *Eco*RI digests of *B. fragilis* revealed a high degree of similarity among

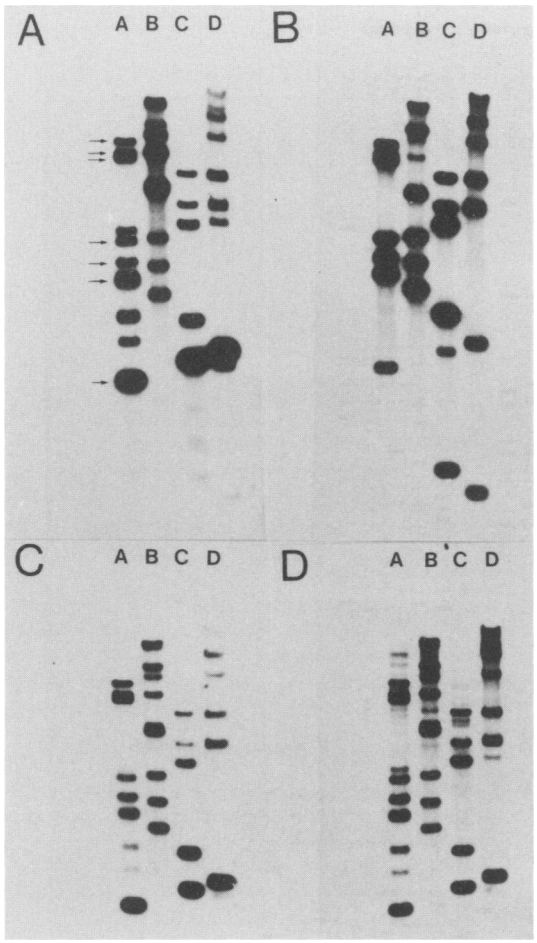

FIG. 2. Hybridization analysis of *B. fragilis* ATCC 25285 by using specific probes derived from the cloned *E. coli rrnB* gene or homologous rRNA. For each panel, genomic digests were performed by using *Eco*RI, *Pst*I, *Hin*dIII, and *Bam*HI for lanes A, B, C, and D, respectively. The following probes were used: 2.9-kb 16S probe (A); 4.2-kb 23S probe (B); entire 7.5-kb pKK3535 (C); rRNA isolated from *B. fragilis* (D). The arrows to the left of panel A indicate hybridizing fragments common to both the 16S- and 23S-specific probes. The sizes of these fragments were estimated by comparison with the migration of a radiolabeled 1-kb ladder (Bethesda Research Laboratories) and are 11.3, 10, 9.4, 4.5, 3.9, 3.6, and 1.8 kb, respectively (data not shown).

these strains of diverse geographic origin. Six of the strains tested were nearly identical to ATCC 25285, while most of the remaining strains showed only between one and three RFLPs. Similar results were obtained with the *Pst*I digests, except that each strain had at least one obvious RFLP. The two exceptions were VPI 2393, a *B. fragilis* homology group II (13) isolate, and WAL 7507, a recent cefoxitin-resistant clinical isolate. These strains were significantly different from other *B. fragilis* strains tested, sharing no more than one or two common bands in either the *Eco*RI or *Pst*I digests.

In addition to *B. fragilis*, four other *Bacteroides* species were examined. The banding patterns observed for *Eco*RI digests of *B. thetaiotaomicron* strains were all very similar and quite distinct from the *B. fragilis* pattern. This result generally was confirmed by examination of *Pst*I digests, which showed some degree of similarity among strains but was not as compelling as the *Eco*RI digests. The *B. vulgatus* and *B. uniformis* strains examined revealed species-specific patterns for *Pst*I (Fig. 3B) and also for *Eco*RI digests, when they were tested (data not shown). Although only three *B. uniformis* strains were tested, they were nearly identical, regardless of the enzyme or probe used.

In contrast to these results are the results found with the *B. ovatus* strains. Of the five strains tested, there was very little similarity among strains, nor was there similarity to any other *Bacteroides* species tested (Fig. 3). In the *Eco*RI digests, for example, only strains ATCC 8483 and VPI C1-45 shared more than two common bands, and these differences were more pronounced when the *Pst*I digests were examined.

Analysis of enterotoxin-producing strains. Enterotoxin-producing (Ent^+) *B. fragilis* strains of animal and human origin were tested by using the 16S-specific rRNA probe. This probe was chosen on the basis of a perceived need to generate a more complex pattern so that the relationships among these strains could be accessed accurately. The results in Fig. 4 show that all of the strains tested were nearly identical to each other, with no more that two RFLPs between any two isolates. Furthermore, these isolates were remarkably similar to the type strain ATCC 25285. The most consistent difference with respect to the type strain was the lack of the 5.0-kb *Eco*RI band in all strains from the enterotoxin set and the appearance of a new *Eco*RI fragment of 7.0 kb (Fig. 4A, arrows on right) in 7 of the 12 strains tested. A similar result was seen in the *Pst*I digests, with a missing doublet band at about 23 kb and the appearance of a new 8.5-kb fragment in all of the strains (Fig. 4B, arrows). Two exceptions to these observations were noted. First was the *Eco*RI digest of strain L_2; strain L_2 appeared to have many bands that shifted to a higher molecular weight. However, the *Pst*I digest of this strain was similar to those of the other strains in the enterotoxin group. Also, strain H_1 was not digested with *Pst*I, but its *Eco*RI digest was typical of those of the other strains.

Included with the enterotoxin set of strains were two non-enterotoxin-producing (Ent^+) isolates from humans (Fig. 4, strains H_3 and H_4) obtained from diarrheic patients during the same study which yielded H_1 and H_2 (Ent^+). On the basis of analysis of both the *Eco*RI and the *Pst*I digests, it was not possible to differentiate between these nonenterotoxigenic strains and those that had demonstrable toxigenic activities.

DISCUSSION

The data presented here indicate that rRNA RFLPs can be a useful taxonomic tool for *Bacteroides* species and that there is potential for the use of this technology in epidemiological studies with *Bacteroides* species. The results in Fig. 1 and 2 have established that the *E. coli rrnB* operon can be used as an effective hybridization probe for *Bacteroides* rRNA genes, even though these organisms are so far diverged (in an evolutionary sense) that the gram-positive bacteria actually are more closely related to *E. coli* than are *Bacteroides* species (33). This probe generated rRNA fragment patterns that were nearly identical to those achieved by using homologous rRNAs, with the exception of the relative intensities of some of the fragments that hybridized. The use of either *Eco*RI or *Pst*I for these analyses was found to be suitable for generating rRNA patterns that were complex enough to differentiate easily between species (e.g., Fig. 1). Other enzymes also could be used satisfactorily, as evi-

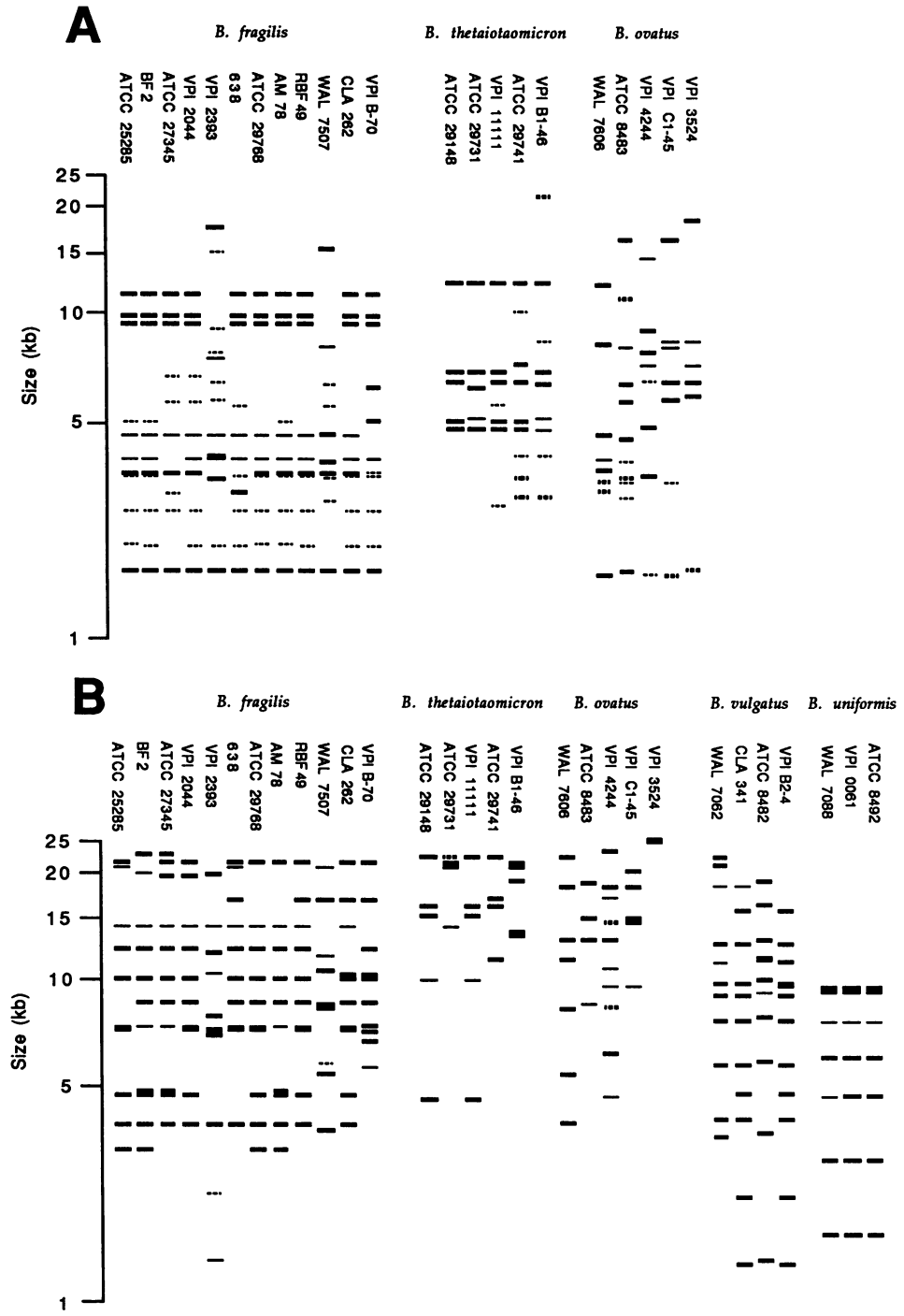

FIG. 3. Schematic of *Eco*RI- or *Pst*I-digested genomic DNA from *Bacteroides* strains probed with the entire pKK3535 plasmid probe. (A) Genomic DNA was digested with *Eco*RI. (B) Genomic DNA was digested with *Pst*I. All results are averages of size estimates obtained from at least two independent Southern blots. Strongly hybridizing fragments are indicated by the black lines, and dashed lines indicate fragments which produced weaker signals. The thickest lines represent doublet bands.

denced by the results obtained with *Hin*dIII and *Bam*HI (Fig. 2).

Five of the *Bacteroides* species were studied in some detail, with a major focus on strains of *B. fragilis*. The 12 *B. fragilis* strains examined (Fig. 3) were of broad geographic origin, but 10 of these strains displayed rRNA patterns that were comparable to that of the type strain, with generally only one to two RFLPs. The pattern of one of these strains, VPI B-70, was more disparate, especially when it was examined by using *Pst*I, but it clearly belonged within the *B. fragilis* group. Two strains, WAL 7507 and VPI 2393, diverged significantly from the other *B. fragilis* strains. VPI 2393 is known to belong to *B. fragilis* DNA homology group II (13, 14), and this strain produced an rRNA pattern quite

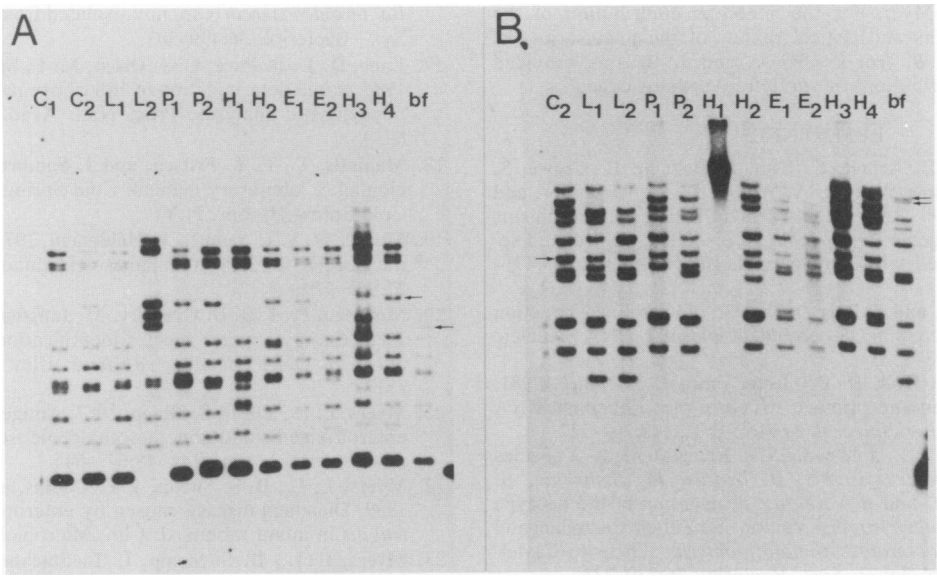

FIG. 4. rRNA banding patterns of the *B. fragilis* enterotoxin strain set. Southern blots were prepared from genomic DNAs digested with either *Eco*RI (A) or *Pst*I (B). These were probed with the 2.9-kb 16S probe as described in the text. For both panels, the strain designations are described in Table 2, and lane bf contains DNA from the *B. fragilis* type strain ATCC 25285. The arrows indicate the locations of the RFLPs described in the text.

distinct from those of the other strains. Strain WAL 7507 also produced a distinct pattern that had some features similar to those of the group II strain, but it was not closely related. Additional known group II strains need to be examined in order to assign strains to this subgroup. Generally, group II strains are difficult to differentiate on the basis of routine biochemical tests (14), but they do display some important phenotypic properties which set them apart from group I strains. These include altered susceptibilities to β-lactam antibiotics and the synthesis of a β-lactamase with greatly reduced susceptibility to clavulanate (12, 35). The clinical importance of the group II strains and their altered antimicrobial susceptibilities are not yet fully understood, but the use of rRNA RFLPs may provide a useful epidemiological tool with which to examine these strains.

Several strains each of *B. thetaiotaomicron*, *B. uniformis*, and *B. vulgatus* also were examined, and as expected, each of these species produced its own distinctive rRNA pattern, with there being only a few RFLPs between strains of the same species. However, a surprising finding came from results with the five *B. ovatus* strains. The strains chosen for testing did not form a distinct group on the basis of their rRNA patterns, and in fact, there was not even a convincing similarity between any two strains. This was especially interesting in light of previous DNA homology studies (13) that showed that at least two of the strains, VPI 3524 and ATCC 8483 (formerly VPI 0038), are more than 80% homologous. The variation we found in the *B. ovatus* rRNA patterns are, however, consistent with Johnson's (13) conclusion that this species displays more nucleotide sequence heterogeneity than any other species examined.

The ability to produce enterotoxin and to cause diarrheal disease in experimental animal models is a property only recently associated with some *B. fragilis* strains (6, 22). On the basis of limited biochemical and serological studies, enterotoxigenic strains of *B. fragilis* cannot be differentiated from nonenterotoxigenic strains, with the exception of their virulence in animal models (21–23). Thus, we examined a group of these Ent^+ strains, which were independently isolated from a variety of animals and humans over the course of 4 years, with the expectation that they might form a unique subgroup within the species analogous to homology group II. The results (Fig. 4) established that the strains were in fact *B. fragilis* (sensu strictu) in that they appeared to be closely related to the type strain ATCC 25285. However, no distinguishing feature of the rRNA patterns that would place the Ent^+ isolates within a novel group or genotype of *B. fragilis* could be identified. Several of the polymorphisms that were observed (e.g., 8.5-kb *Pst*I or 7.0-kb *Eco*RI fragments) either were not consistent within the Ent^+ strains or were also observed in the Ent^- strains that were analyzed. In general, there was no more or less heterogeneity in the rRNA patterns displayed by this group than was observed for all of the other *B. fragilis* strains examined.

These results are consistent with and extend previous work in which 69 Ent^+ and Ent^- isolates were studied by whole-cell agglutination and gel double-diffusion analysis (21). In this study it was concluded that it is not possible to distinguish between Ent^+ and Ent^- strains on the basis of their serotypes and that the strains display a marked antigenic heterogeneity, with 37 of 44 Ent^+ strains forming 13 different serogroups. The lack of distinguishing features at either the genetic or the antigenic level in these strains makes it difficult to determine the origins of the enterotoxin-producing genes. On the one hand, it is possible that these genes may be a recent acquisition and that they are located on plasmids or transposons that have become widely disseminated. On the other hand, the results could be used to support the idea that enterotoxin production is an ancient property that either was lost or lies dormant in many strains.

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