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Genotypic Identification of Two Groups within the Species *Bacteroides fragilis* by Ribotyping and by Analysis of PCR-Generated Fragment Patterns and Insertion Sequence Content

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Molecular typing allowed the separation of the species *Bacteroides fragilis* into two genotypically distinct groups. A unique set of 50 strains of *B. fragilis* carrying the chromosomal metallo-β-lactamase gene *cfiA* was subjected to a comparative analysis with respect to sets of up to 250 randomly collected strains devoid of this gene. The two groups were found to be distinct on the basis of the following results: (i) ribotyping, after DNA digestion with *Ava*I, revealed a practically homogeneous DNA fragment pattern for the *cfiA*-positive strains and distinct multiple patterns for the *cfiA*-negative strains; (ii) PCR, arbitrarily primed with an experimentally selected decamer, generated fragment patterns typical for the strains of each group; (iii) the three insertion sequences described to date in the species *B. fragilis*, i.e., IS*4351*, IS*942*, and IS*1186*, were all but confined to the *cfiA*-positive group, in which they were capable of providing promoter sequences for the transcription of *cfiA*; and (iv) the *cepA* gene, encoding the so-called endogenous cephalosporinase of *B. fragilis*, was found exclusively in the *cfiA*-negative group, in which it was present in ca. 70% of the strains. The *cfiA*-, *cepA*-negative fraction was not characterized further. In a natural population of 500 randomly selected strains of *B. fragilis*, the *cfiA*-positive and *cfiA*-negative groups represented ca. 3 and 97% of the strains, respectively. Analysis of 82 metabolic traits revealed no difference between the two groups.

The genus *Bacteroides* sensu stricto, which is part of the *Cytophaga-Flavobacter-Bacteroides* phylum (10), is distant, in evolutionary terms, from most other bacteria of medical interest and appears to be one that evolves rather rapidly (41). According to a proposal by Shah and Collins (34), it has been restricted to 10 closely related species, with *Bacteroides fragilis* as the type species.

B. fragilis is the anaerobe most frequently isolated from human infections (8, 33) and may constitute up to 60% of pathogenic anaerobic isolates from hospitalized patients (20). Apart from its resistance traits to various antibiotics (25), the species *B. fragilis* appears phenotypically homogeneous; however, DNA-DNA hybridization experiments have shown that it separates into two DNA homology groups, I and II, which have ca. 65 to 70% intergroup and 80 to 90% intragroup homology (15, 16). Strains belonging to group II have been reported to be less susceptible than those of group I to the synergistic inhibitory effect of clavulanic acid and β-lactam antibiotics (3, 43).

Two chromosomal cephalosporinase genes, considered to be species specific, have been described in *B. fragilis*; these are *cepA* (32), coding for an enzyme related to the β-lactamases of class A (2), and *cfiA* (38), also called *ccrA* (26), coding for a metallo-β-lactamase of class B (1). Neither gene is consistently expressed in naturally occurring strains of *B. fragilis*. High-level expression of the so-called endogenous cephalosporinase gene *cepA* has been studied recently by Rogers et al. (32) and was reported to result from transcriptional activation after insertion of an IS*21*-related nucleotide sequence 50 bp upstream of the translation start codon. Two possible consequences of this insertion were discussed, i.e., disruption of a hypothetical repressor-binding site or formation of an efficient hybrid promoter (31). Production of CepA leads to resistance to most β-lactam antibiotics, which are otherwise efficient in the treatment of *B. fragilis* infections, with the exception of the cephamycins, the carbapenems, and the β-lactamase inhibitors. These last three groups of compounds, and most typically the carbapenems imipenem and meropenem, remain without effect against strains producing the metallo-β-lactamase CfiA (7, 9, 21, 44). The corresponding gene can be silent or expressed, depending upon the promoter which drives its transcription (21, 22). Mutation to high-level resistance has been shown to result from the insertion of an insertion element (IS element), carrying an efficient promoter, immediately upstream of *cfiA* (22), a possibility which had been considered for some time (28). Intermediate-level resistance to carbapenems has also been observed (9, 27, 44), and it appears likely that this phenotype too depends upon variations in the promoter structure (27) rather than on reported variations in the *cfiA* gene itself (24).

In our previous attempts to select spontaneous high-level carbapenem-resistant mutants from susceptible clinical isolates, we were able to obtain such mutants in only a few cases (21). To evaluate the risk of their emergence, via gene activation, under natural conditions, we decided to screen a random collection of clinical isolates of *B. fragilis* for the presence of *cfiA* and of IS elements capable of its activation.

Here we present evidence for a low prevalence of the *cfiA* gene within these isolates and for the existence, within the species *B. fragilis*, of a genotypically distinct group which can be recognized by the presence of *cfiA*. This *cfiA*-positive group was differentiated from the *cfiA*-negative bulk of strains by molecular typing, such as ribotyping and analysis of fragment patterns generated by arbitrarily primed (AP) PCR, as well as by testing for *cepA* (32), which was absent, and for the *B. fragilis* IS elements IS*4351* (29), IS*942* (28), and IS*1186* (22),

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TABLE 1. Expression of the carbapenemase gene as reflected in the MIC of imipenem and prevalence of IS elements in *cfiA*-positive strains of *B. fragilis*

Strain[a]	MIC		Hybridization with		
	Imipenem (μg/ml)		IS*1186*	IS*942*	IS*4351*
BFr38	0.25				
BFr1290	0.25				
BFr113	0.5				
BFr81	2				
BFr271	2				
BFr901	64	△[b]			
BFr920*	128	△			
BFr911	128	△			
BFr189	>128	▲			
BFr912	>128	▲	+	−	+
BFr913	>128	▲			
KSB1486	>128	▲			
BFr907	128	△			
BFr209	128	△			
BFr910	>128	△			
BFr289	128	△			
BFr690	>128	▲			
BFr389	>128	▲			
BFr909	128	△	+	+	−
TAL2480	>128	◆[c]	−	+	+
BFr916	0.06		+	−	−
BFr921*	0.5				
BFr890	2				
BFr918	32				
BFr905	128	◇			
BFr904	128	◇	−	+	−
BFr908	128	◇			
BFr923*	>128	◇			
BFr929*	128	◆			
BFr12	0.5				
BFr926	0.06				
BFr925	1				
BFr927	1				
BFr914	1				
BFr915	1		−	−	+
BFr60	1				
BFr84	4				
BFr917	16	■[d]			
BFr900	16	□			
BFr930	16	□			
BFr212	0.25				
BFr253	0.25				
BFr919	0.25				
BFr922*	0.06				
BFr928	0.25		−	−	−
BFr903	2				
B119	8				
BFr902	64				
BFr906	>128				

[a] The strains labelled BFr include 5 strains collected in Spain and kindly provided by C. Betriu (marked with an asterisk) and the 12 *cfiA*-positive strains which were randomly collected in France (see Results).

which were found to be confined almost exclusively to the *cfiA*-positive group.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 500 strains of *B. fragilis* were randomly collected in 35 hospitals from all over France during a 2-year period (1990 and 1991). Most of the strains (75%) came from blood cultures, and the bulk of the remaining strains (23%) came from stool cultures.

Thirty-eight nonrandomly collected *cfiA*-positive carbapenem-resistant (Cpm^r) or carbapenem-susceptible (Cpm^s) clinical isolates were included in this study (Table 1): 34 strains, designated BFr, some of which have been described previously (21, 22), were isolated in France since 1983 or in Spain since 1992 (the latter, comprising 5 strains, were kindly provided by C. Betriu); 2 strains, TAL3636 (not shown in Table 1) and TAL2480, were isolated in the United States (7, 37); one strain, B119, was isolated in the United Kingdom (9); and one strain, KSB1468, was isolated in Sweden (13). The strains were grown in Wilkins-Chalgren (WC; Oxoid) liquid medium or on WC agar plates in an anaerobic atmosphere produced by a gas-generating kit (Anaerobic System BR38; Oxoid). The *B. fragilis* strains AIP638 (23) and ATCC25285^T were used as reference strains.

Strain identification, biochemical characterization, and antibiotic susceptibility testing. *B. fragilis* strains were identified by using the API 32A system and selected strains were additionally analyzed with API 50CH strips (API-bio-Mérieux). These strips can be used for the differentiation of bacteria from the *B. fragilis* group, provided that a pH indicator is added (bromocresol purple at a final concentration of 1%) and the culture is overlaid with a drop of oil for anaerobiosis.

MICs of imipenem on WC agar were determined by the standard procedure proposed by the National Committee for Clinical Laboratory Standards (19), and strains for which an MIC of ≥16 mg/ml was determined were considered to be resistant. Imipenem was a gift from Merck Sharp & Dohme-Chibret.

Preparation and restriction endonuclease digestion of DNA. Total DNA was prepared from bacteria grown for 48 h on WC agar plates. One loopful of bacteria was suspended in 160 μl of a solution containing NaCl (0.5 M), EDTA (0.1 M), and lysozyme (1 mg/ml) (pH 8). After 30 min of incubation at room temperature, the bacteria were lysed by the addition of 60 μl of a sodium dodecyl sulfate solution (20%, wt/vol) for 5 min; 20 μl of a proteinase K solution (20 mg/ml) was added, and the mixture was incubated for 2.5 h at 65°C and extracted twice with phenol-chloroform. The DNA was precipitated with ethanol, recovered in 200 μl, and dialyzed overnight against 1 liter of distilled water. Restriction endonucleases were purchased from Boehringer-Mannheim or Gibco-BRL and used as recommended by the suppliers.

DNA probes, filter hybridization and Southern blot analysis. A 0.73-kb fragment covering almost the entire *cfiA* structural gene (26, 38) was prepared by PCR as described previously (21). A *B. fragilis* rRNA gene probe was prepared for ribotyping (11), in diethylpyrocarbonate-treated solutions, as follows. 70S ribosomes were isolated from strain BFr81 (22) by sedimentation through a sucrose cushion as described previously (39), except that the bacteria were disintegrated by sonolysis. rRNA was extracted three times with phenol (pH 6.5) and once with phenol-chloroform and dialyzed against distilled water. DNA was obtained with the cDNA synthesis kit (Boehringer-Mannheim) following the protocol provided by the supplier.

Probes for the IS elements were prepared by PCR with three sets of oligonucleotides, oligonucleotides C and D, oligonucleotides A and B (22), and 5′-GAGAATCAAGCTTCTCGCC-3′ and 5′-CCCCGAATTCGCCTTTGCCCGTA-3′, for the amplification of IS-central fragments of 0.9 kb (IS*4351* [29]), 1.3 kb (IS*942* [28]), and 0.94 kb (IS*1186* [22]), respectively. A probe for *cepA* was prepared with oligonucleotides 5′-GGGGGGGATCCAAGACTTATACATTTATCC-3′ and 5′-GGGGGAAGCTTATTAATCTATCTGTTGCG-3′. The PCR conditions in all cases were those described previously (22). All probes were radioactively labelled with the Megaprime kit and [α-^{32}P]dCTP from Amersham.

For filter hybridization, 20 μl of culture containing ca. 3×10^6 CFU/ml was deposited onto Hybond N^+ membranes (Amersham) and the membranes were air dried. The bacteria were lysed by placing the filters for 5 min onto Whatman 3MM paper soaked in 0.5 M NaOH and then neutralized them in 1 M Tris-HCl (pH 7).

For Southern hybridization, restriction enzyme-digested DNA was transferred, after agarose gel electrophoresis, to Hybond N^+ membranes by using a vacuum blotter (Hoefer Scientific Instruments) as specified by the supplier. Prehybridization (for 2 h at 65°C) and hybridization (for 16 h at 65°C) in 5×

[b] IS*1186* inserted upstream of *cfiA* as determined by PCR mapping (△) or nucleotide sequencing (▲).

[c] IS*942* inserted upstream of *cfiA* as determined by PCR mapping (◇) or nucleotide sequencing (◆). For strain TAL2480, see references 29 and 38.

[d] IS*4351* inserted upstream of *cfiA* as determined by PCR mapping (□) or nucleotide sequencing (■).

Denhardt's solution and high-stringency washes were carried out by following the protocol provided by Amersham for Hybond N^+ membranes.

Molecular typing by AP PCR. Oligonucleotide primers for the AP PCR described by Williams et al. (42) and Mazurier and Wernars (18) were used. PCR conditions were as previously described (22), with arbitrary primers at 2 μM, except that the temperature at the annealing steps was 36°C. In preliminary experiments, 10 primers were tested, and 2 of these, AP11a and AP12b (42), were retained for subsequent typing on the basis of the number and size of the DNA fragments amplified.

PCR mapping experiments and DNA sequencing. PCR mapping experiments were carried out to estimate the distance and the orientation of the three *Bacteroides* IS elements with respect to the *cfiA* gene and to screen for the possible existence of so far unknown elements immediately upstream of the gene. The primers used were those described for amplification of the IS elements (see the discussion of DNA probes above) and oligonucleotides E and G (22) for amplification of the region into which insertion of IS*1186* has been observed. The lengths of the amplified fragments were estimated after agarose gel electrophoresis with a molecular mass standard (Raoul; Appligène, Illkirch, France). Nucleotide sequencing was performed as described previously (22).

RESULTS

Prevalence and expression of *cfiA* in randomly collected isolates of *B. fragilis*. A *cfiA* internal probe (21) was used to determine, by DNA-DNA hybridization, the fraction of strains which carried the carbapenem resistance gene *cfiA* (*cfiA* positive). Five-hundred randomly collected isolates were analyzed, and the expression of *cfiA* was tested by determining the MICs of imipenem. Twelve strains (2.4%) were *cfiA* positive, but only four of them (0.8%) expressed resistance (Cpm^r). The MICs for these four strains were 128 μg/ml, while the MICs for the eight (1.6%) remaining susceptible strains (Cpm^s) were ≤1 μg/ml, similar to those for the *cfiA*-negative strains. We concluded that the *cfiA*-positive Cpm^s strains probably carry a complete but silent *cfiA* gene, as observed in strains BFr81 and BFr271 (21, 22).

All 12 randomly collected *cfiA*-positive strains, as well as an additional 37 Cpm^s or Cpm^r *cfiA*-positive strains (Table 1), were analyzed for their biochemical and metabolic properties and for the prevalence of IS elements as well as *cepA* (32).

Biochemical and metabolic properties of *cfiA*-positive strains. Comparative analysis with the API 32A system, probing a total of 32 characteristics, and the API 50CH strip, which tests for the utilization of 50 additional sugars in minimal medium, did not reveal any difference between the *cfiA*-positive strains listed in Table 1, strains ATCC25285^T and AIP638, and 50 randomly chosen *cfiA*-negative strains (data not shown).

Prevalence of IS elements in the *cfiA*-positive strains. The prevalence of all three IS elements described so far in *B. fragilis*, i.e., IS*4351*, IS*942*, and IS*1186* (or of closely related sequences) was evaluated by DNA-DNA hybridization with IS internal probes (Table 1). Of the 49 *cfiA*-positive strains analyzed, 82% contained at least one of the elements, with IS*4351* being observed most frequently (61%), followed by IS*1186* (43%) and IS*942* (18%). The association of IS*4351* and IS*1186* was frequently observed (37%), while only one strain each was found to harbor the combination of either IS*4351* or IS*1186* and IS*942*. By contrast, similar sequences were detected in less than 1% of 250 randomly selected *cfiA*-negative strains (data not shown).

Expression of carbapenem resistance and proximity of *cfiA* and IS elements. The susceptibilities of 49 *cfiA*-positive strains to imipenem are shown in Table 1. For 80% of the imipenem-resistant strains, the MICs were ≥128 μg/ml, suggesting that *cfiA* was efficiently expressed. Whether alterations of membrane permeability or penicillin-binding proteins contributed to this resistance has not been investigated. All high-level resistant strains except one (BFr906) were probe positive for one or two of the three IS elements, IS*1186*, IS*942*, and IS*4351*, and PCR-based mapping experiments with IS and *cfiA* internal oligomers showed that in these strains *cfiA* was preceded by either IS*1186* or IS*942* but not by IS*4351*. No high-level resistance was observed in strains which hybridized with IS*4351* only (Table 1).

The precise sites of insertion were determined by nucleotide sequencing in eight strains (Table 1) and found to be 8, 20, or 22 bp upstream of the initiation codon ATG (data not shown), in agreement with previous results (22, 28). Of the 11 strains which apparently carried only IS*4351*, 3 showed a low level of resistance to the carbapenems (the MICs of imipenem were 16 μg/ml). In these three strains, IS*4351* had inserted upstream of *cfiA*. Sequence analysis, carried out for one strain, revealed that the insertion had taken place 31 bp away from the initiation codon ATG (data not shown). In all instances, the IS elements had inserted in the same orientation. The high-level resistant strain BFr906 (Table 1) apparently carried a novel element.

Prevalence of *cepA* gene sequences in *cfiA*-positive and *cfiA*-negative strains. The presence of *cepA* (32) or closely related sequences was tested for by filter hybridization with a PCR-generated fragment covering the entire structural gene. Duplicates of the filters used for hybridization with the IS probes were used. The *cepA* probe hybridized, under stringent conditions, with DNA from close to 70% of 250 *cfiA*-negative strains but not with DNA from any of the 50 *cfiA*-positive strains (data not shown).

Comparative molecular typing of *cfiA*-positive and *cfiA*-negative strains. The fragment patterns of *Ava*I-digested chromosomal DNA, as revealed by hybridization with a cDNA probe prepared by reverse transcription of *B. fragilis* rRNA, are shown in Fig. 1. Hybridization was carried out with 11 *cfiA*-positive strains, including strains isolated in the United Kingdom (B119), the United States, (TAL2480), Sweden (KSB1486), and Spain (BFr920), and 11 *cfiA*-negative strains (randomly chosen except for ATCC25285^T and AIP638). The *cfiA*-negative strains all produced five fragments in the size range between ca. 0.5 and 10 kb and were heterogeneous inasmuch as at least seven variants could be distinguished (Fig. 1A). The strains seemed to be related, since (i) all shared one common fragment (ca. 3.8 kb), (ii) every strain shared at least two fragments with one other strain, and (iii) no strain had a unique fragment. By contrast, the *cfiA*-positive strains were highly homogeneous (Fig. 1B). These strains produced four common fragments of ca. 3.5, 2.5, 0.7, and 0.5 kb. The fragments of 3.5, 2.5, and 0.7 kb did not comigrate with any of the fragments observed in the *cfiA*-negative strains. The lowest-molecular-mass fragment of ca. 0.5 kb was present in all *cfiA*-positive and most *cfiA*-negative strains analyzed (Fig. 1).

The genomic difference between the two groups of strains was confirmed when they were analyzed by AP PCR (Fig. 2). With primer AP11a, a homogeneity of fragment sizes was evident among the strains of each group, while none of the prominent fragments of the *cfiA*-positive strains (and possibly none at all) had electrophoretic mobilities identical to those of the *cfiA*-negative strains (Fig. 2A). With primer AP12h, a seemingly greater heterogeneity was revealed within both the *cfiA*-positive and the *cfiA*-negative strains. Although, again, no fragment observed in one group had a size identical to that of any fragment in the other group, the two groups could not be readily distinguished with primer AP12h.

DISCUSSION

The data presented in this study allow us to draw conclusions in two areas, one concerning the β-lactam resistance of *B. fragilis* and the other concerning the genotypic heterogeneity of

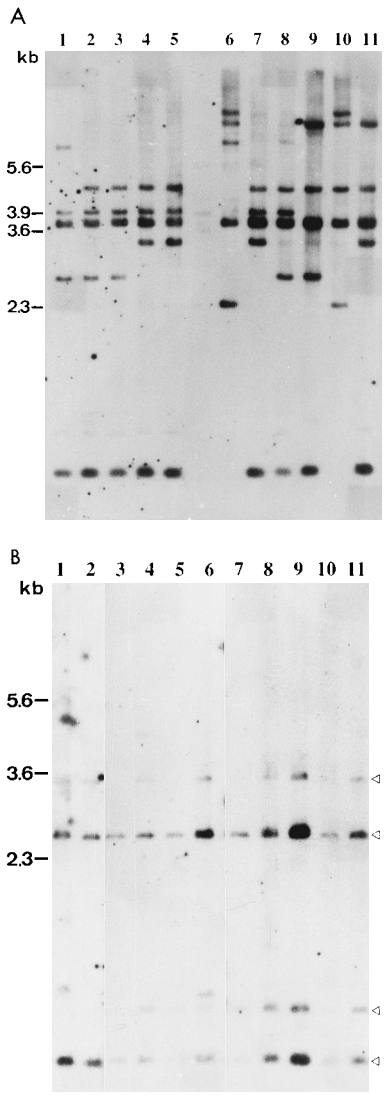

FIG. 1. Patterns of *Ava*I-generated fragments containing rDNA from *B. fragilis* strains. (A) *cfiA*-negative strains. Lanes: 1, BFr790; 2, BFr1591; 3, BFr2191; 4, BFr2391; 5, BFr2591; 6, BFr2991; 7, BFr3091; 8, BFr3991; 9, BFr6791; 10, BFr8891; 11, BFr12491. (B) *cfiA*-positive strains. Lanes: 1, BFr1290; 2, BFr920; 3, BFr209; 4, BFr189; 5, B119; 6, TAL2480; 7, BFr271; 8, BFr81; 9, BFr690; 10, KSB1486; 11, BFr890. The data shown are from one experiment but with different times of exposure during autoradiography. The four common fragments of ca. 3.5, 2.5, 0.7, and 0.5 kb (indicated by arrowheads) could be seen for all strains on the original autoradiographs.

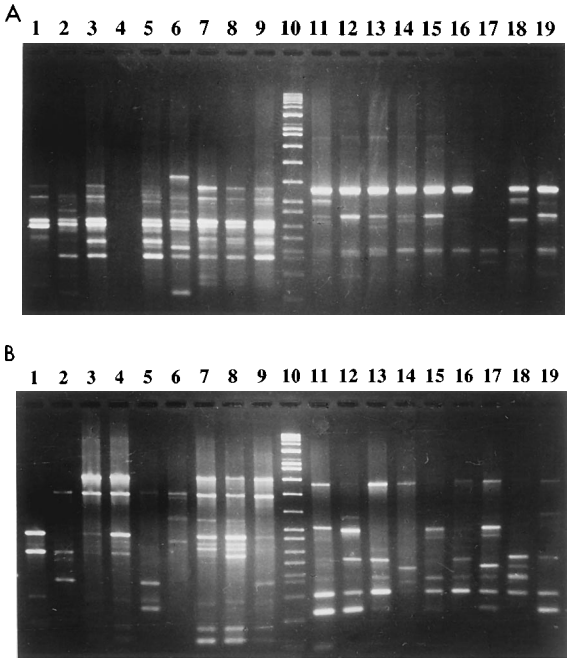

FIG. 2. AP PCR-generated fragment patterns of *B. fragilis* strains. (A) Fragments generated with primer AP11a; (B) Fragments generated with primer AP12h. Lanes 1 through 9: *cfiA*-positive strains TAL3636, B119, KSB1468, none (A)/BFr189 (B), BFr690, BFr289, BFr271, BFr81, and BFr1290; lanes 11 through 19: *cfiA*-negative strains BFr790, BFr1591, BFr2191, BFr2391, BFr2991, BFr3091, BFr3991 (A)/BFr8891 (B), BFr6791, and BFr20992. Lane 10 contains molecular size markers (48.5, 18.5, 14.9, 10.6, 9, 7.3, 5.6, 4.3, 3.9, 3.6, 2.9, 2.3, 1.8, 1.4, 1.2, 1, 0.9, 0.75, 0.68, 0.55, 0.37, and 0.23 kb; Raoul, Appligène).

this species. *B. fragilis* is one of the few bacterial species in which production of a carbapenem-hydrolyzing metallo-β-lactamase has been found (17), but this is not a common trait. In most surveys of the β-lactam susceptibility of this species, carbapenem resistance either was not observed at all (6, 14) or did not exceed 1.5 to 2.5% (4, 5). We found the carbapenem resistance rate to be 0.8% in 500 randomly collected clinical isolates, as determined for the 2-year period of 1990 to 1991. DNA of all high-level resistant strains hybridized with a *cfiA* gene probe, from which we conclude from our previous results (21, 22) that this gene was efficiently expressed and responsible for the resistance phenotype. The hybridization results, combined with the MIC determinations, also revealed that an additional 1.6% of the strains carried a silent *cfiA* gene. This observation allows the prediction that the risk of emerging resistance via gene activation (22) in the clinical setting is low in the species *B. fragilis* as it is currently defined (34) but that it is high in the *cfiA*-positive group.

Two β-lactamases, the endogenous cephalosporinase CepA (32) and the carbapenemase CfiA (26, 38), appear to be confined to the species *B. fragilis*. We found, by DNA-DNA hybridization with the respective gene probes, that the two genes did not coexist in the same strains. While ca. 70% of 250 *cfiA* probe-negative strains hybridized with *cepA*, none of the 50 *cfiA*-positive strains did. Using Southern blot hybridization, Rogers et al. (32) had found *cepA* to be present in all 13 strains which they analyzed. Whether our observation of a sizeable *cepA* probe-negative fraction among the clinical *cfiA*-negative *B. fragilis* isolates is due to the hybridization conditions used, to the possible existence of a *cepA* allele that is not close enough structurally to be recognized by hybridization under stringent conditions, or to the absence of *cepA* is not clear. We have not carried out any further genotypic analysis of the apparent *cfiA*-, *cepA*-negative fraction.

The *cfiA*-positive, *cepA*-negative strains constitute a genotypically distinct group. While ribotyping of *cfiA*-negative strains revealed a degree of heterogeneity of fragment patterns (Fig. 1A) that has been observed within many other species (36), an almost unique pattern, with only very minor variations, was observed with all *cfiA*-positive strains analyzed (Fig. 1B). The fact that the strains were isolated between 1983 and 1993 in different European countries and in North America makes it highly unlikely that they are linked epidemiologically. Analysis of rRNA gene restriction fragment length polymorphism has been used before in an attempt to distinguish between enterotoxin-producing and enterotoxin-nonproducing strains

of *B. fragilis*, but no corresponding separate genotypic groups were identified (35).

The distinctness of the *cfiA*-positive and *cfiA*-negative groups was also evident when the AP PCR-generated fragment patterns with primer AP11a were compared. Similar degrees of homology (Fig. 2A) were observed within each group, but in no case did any prominent fragment from one group comigrate with any such fragment from the other. The fragment patterns obtained with primer AP12h were more heterogeneous, but again no major fragments common to both groups were observed (Fig. 2B). Because of the greater diversity of the patterns generated with AP12h, this primer appears to be useful for determining the potential homogeneity of a given *B. fragilis* population.

A further, and rather unexpected, argument in favor of a unique *cfiA*-positive group came from the analysis of its content of IS elements. In a previous report on the activation of *cfiA* in high-level carbapenem-resistant strains, we described a novel element, IS*1186*, and its role in that process (22). To evaluate the full potential of this and other IS elements for *cfiA* activation, we carried out DNA-DNA hybridization experiments for the detection of all IS elements described to date in this species, i.e., IS*4351* (29), IS*942* (28), and IS*1186* (12, 22). Over 80% of the *cfiA*-positive strains carried one or two of the elements, as shown in Table 1. By contrast, these elements were all but absent from the *cfiA*-negative strains. Obviously, with the method of detection used, it cannot be ascertained whether there were complete and functional elements in every probe-positive strain or whether they were chromosome or plasmid borne. IS*942* has been found previously on the chromosome of two strains, TAL3636 and TAL2480 (28, 38). In 11 strains in which we have verified the site of insertion of IS*1186* upstream of *cfiA* by nucleotide sequence analysis, this element was also chromosome borne (Table 1) (reference 22 and data not shown). It has, however, been found on one plasmid, pIP417, originally isolated from *Bacteroides vulgatus* (30), where it was located immediately upstream of the 5-nitroimidazole resistance gene *nimA* (12). In three strains carrying only IS*4351* (Table 1), this element was found immediately upstream of the carbapenemase gene, suggesting that *cfiA* activation occurs by one of the three putative outward-directed promoters (29). Considering the at least eightfold-lower imipenem resistance levels observed in comparison with the high levels in strains carrying IS*1186* or IS*942* (Table 1), it appears that the promoter provided by IS*4351* is less efficient in driving *cfiA* transcription than are those of the former two elements.

Despite extensive comparative analysis of metabolic characteristics, no distinctive phenotypic trait was found for the *cfiA*-positive group other than carbapenem resistance in strains in which the gene is efficiently expressed or a discretely reduced carbapenem susceptibility in several of those in which it is not. A different group, called DNA homology group II, has been described previously within the species *B. fragilis* (15), and this group also could not be distinguished phenotypically (16). Strains of this group have been reported to be less susceptible to β-lactams combined with penicillinase inhibitors and to cefoxitin and more susceptible to broad-spectrum penicillins and cefoperazone than the strains of homology group I (3, 43), to which most *B. fragilis* strains belong (15).

All data of the present study combined led us to suspect that the *cfiA*-positive group may represent a novel species. However, while the methods of molecular typing used here, in conjunction with the screening for specific genes and other functional DNA sequences, are clearly adequate for the genotypic differentiation of the *cfiA*-positive and the *cfiA*-negative groups, they do not provide information required for the determination of their exact phylogenetic relationship. A powerful method that does provide this information is the comparative nucleotide sequence analysis of the 16S rRNA genes (40). Preliminary results of such an analysis confirmed our conclusion that the *cfiA*-positive group is genotypically distinct (32a), but full sequence data will be necessary to evaluate the phylogenetic distance.

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