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# A Recent Fixation of *cfiA* Genes in a Monophyletic Cluster of *Bacteroides fragilis* Is Correlated with the Presence of Multiple Insertion Elements

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**Small-subunit ribosomal DNA sequences of 16 strains of *Bacteroides fragilis* were determined and compared with previously published sequences. Three phylogenetic methods (the neighbor-joining, maximum-likelihood, and maximum-parsimony methods) as well as a bootstrap analysis were used to assess the robustness of each topology. All phylogenetic analyses demonstrated that the *B. fragilis* strains were clearly divided into two robust monophyletic units which corresponded to the *cfiA*-negative and *cfiA*-positive groups. Strains of two previously identified DNA homology groups separated similarly into these two monophyletic units. According to the intensity of the hybridization signal with a *cfiA* probe, the *cfiA*-positive cluster could be further divided into two groups. This difference might reflect the existence of two, probably closely related *cfiA*-type genes. In the strongly hybridizing *cfiA*-positive strains, the gene is capable of conferring high-level resistance to the carbapenems and to most β-lactamase inhibitors as well, while in the weakly hybridizing *cfiA*-positive strains, only the latter type of resistance is known to occur. The presence of the *cfiA*-type genes within a monophyletic cluster of *B. fragilis* that apparently represents only a minority of the species *B. fragilis* is suggestive of a recent acquisition. The fact that this cluster is also the predominant pool of all known *B. fragilis* insertion elements, which have been found to play an important role in the expression of carbapenem resistance, raises the possibility that both genetic determinants, i.e., the resistance gene(s) and insertion elements, may have coevolved.**

*Bacteroides fragilis*, the anaerobic bacterial species most frequently isolated from human infections (3, 4), shows little phenotypic variability but can nevertheless be assigned to different genotypic groups (11, 22). DNA homology studies led to the distinction of two homology groups, called I and II (11). DNA relatedness between *B. fragilis* ATCC 25285$^T$, representative of homology group I, and strains of homology group II ranged from 64 to 72%, while intragroup relatedness ranged from 80 to 90% (11). About 80% of *B. fragilis* strains isolated in clinical studies can be assigned to homology group I (11). Although strains from homology groups I and II are phenotypically indistinguishable (12), strains of homology group II lack susceptibility to the synergistic effect of such β-lactamase inhibitors as clavulanic acid, sulbactam, and tazobactam (2, 40), which are potent inhibitors of the vast majority of the β-lactamases produced by clinical isolates of *B. fragilis* (3, 4, 10, 20, 40). These are active-site serine enzymes, loosely related to those of Ambler's class A (1, 17, 28). Probing with *cfiA* gene sequences led to the distinction within *B. fragilis* of the *cfiA*-negative and *cfiA*-positive groups, representing ca. 3 and 97%, respectively, of the clinical *B. fragilis* isolates (22, 23). The *cfiA* gene, which has been searched for only in *B. fragilis*, encodes a metallo-β-lactamase (5, 21, 25, 37), i.e., an Ambler class B enzyme (1). Only one-third of the *cfiA*-positive strains express *cfiA*, and these strains can be recognized easily by their high level of resistance to almost all β-lactam antibiotics, typically the carbapenems imipenem and meropenem. The gene is silent in the remaining two-thirds of the strains (21, 22). Despite an analysis of 82 metabolic traits, the *cfiA*-positive group could not be distinguished from the *cfiA*-negative group, which includes strain ATCC 25285$^T$ (22). However, *cfiA*-positive strains are genotypically particular for several reasons (22): (i) they lack the *cepA* gene encoding the cephalosporinase which is considered to be the endogenous β-lactamase of *B. fragilis* (17), (ii) they show a distinctive and highly homogeneous ribotyping pattern, (iii) they can be distinguished from the *cfiA*-negative strains by arbitrarily primed PCR, and (iv) they harbor rather preferentially most of the insertion elements described to date for *B. fragilis*, i.e., IS*4351* (27), IS*942* (26), and IS*1186* (23). Indeed, 82% of the close to 50 *cfiA*-positive strains contained at least one of these insertion elements, with less than 1% of the 250 *cfiA*-negative strains containing any of these elements (22).

In view of these data, the present study was undertaken in order to investigate the phylogenetic relationship between the *cfiA*-positive group and the *cfiA*-negative group by comparing small-subunit ribosomal DNA (rDNA) sequences (16, 39). Because initial analysis showed that available sequences of *B. fragilis* could be divided into two robust monophyletic units, the rDNA sequences of three additional *Bacteroides* species (*Bacteroides caccae*, *Bacteroides stercoris*, and *Bacteroides merdae*) were determined in order to test the possibility that the *cfiA*-positive or *cfiA*-negative group was related to *Bacteroides* species whose small-subunit rDNA sequences were still unknown.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and DNA preparation.** Strains used in this study are listed in Table 1. Strains were grown on Wilkins-Chalgren (Oxoid, Basingstoke, United Kingdom) agar plates under an aerobic atmosphere with a gas-generating kit ($CO_2$ system; Oxoid). Total DNA was prepared from bacteria grown for 48 h. One loopful of bacteria was suspended in 160 μl of a solution containing 0.5 M NaCl, 0.1 M EDTA, and 1 mg of lysozyme per ml (pH 8). After

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TABLE 1. Strains whose small-subunit rDNAs have been sequenced in this study

| Strain | *cfiA*[a] | Source or reference[b] | EMBL accession no. |
|---|---|---|---|
| *Bacteroides fragilis* | | | |
| ATCC 25285$^T$ | − | ATCC | X83935 |
| AIP 638 | − | 24 | X83936 |
| BFr 790 | − | 21 | X83937 |
| BFr 2991 | − | A. Buu-Hoï | X83938 |
| BFr 6791 | − | 21 | X83939 |
| BFr 12491 | − | 21 | X83940 |
| VPI 2393 | * | 11 | X83948 |
| VPI 4117 | * | 11 | X83949 |
| VPI 4225 | * | 11 | X83950 |
| TAL 3636 | + | 5 | X83941 |
| B 119 | + | 6 | X83942 |
| BFr 81 | + | 21 | X83943 |
| BFr 189 | + | 21 | X83944 |
| BFr 920 | + | C. Betriu | X83946 |
| BFr 901 | + | 21 | X83945 |
| KSB 1486 | + | 8 | X83947 |
| *Bacteroides caccae* ATCC 43185$^T$ | − | ATCC | X83951 |
| *Bacteroides ovatus* ATCC 8483$^T$ | − | ATCC | X83952 |
| *Bacteroides merdae* ATCC 43184$^T$ | − | ATCC | X83954 |
| *Bacteroides stercoris* ATCC 43183$^T$ | − | ATCC | X83953 |

[a] The presence (+) of *cfiA* was established by DNA-DNA hybridization and/or nucleotide sequence analysis; the absence (−) of *cfiA* was determined by hybridization under stringent conditions. A weak hybridization under stringent conditions (cf. Fig. 3A) with DNA of strains from homology group II (11) is indicated with asterisks. These strains were kindly provided by J. L. Johnson.

[b] ATCC, American Type Culture Collection, Rockville, Md. *B. stercoris* was kindly provided by R. Hamman; *B. merdae* and *B. ovatus* were kindly provided by F. Tessier.

30 min of incubation at room temperature, bacteria were lysed by the addition of 40 μl of a sodium dodecyl sulfate solution (20%, wt/vol) for 5 min; 20 μl of a proteinase K solution (20 mg/ml) was then added, and the mixture was incubated for 2.5 h at 65°C. After two extractions with phenol-chloroform, DNA was precipitated with ethanol, recovered in 200 μl, and dialyzed overnight against 1 liter of distilled water.

**DNA-DNA hybridization.** For filter hybridization, 20 μl of bacterial suspension containing ca. $10^{10}$ CFU/ml was deposited onto Hybond $N^+$ membranes (Amersham) with a slot blot apparatus (Hoefer Scientific), and the membranes were air dried. The bacteria were lysed by placing the filters for 5 min on Whatman 3MM paper soaked in 0.5 M NaOH and were then neutralized in 1 M Tris-HCl (pH 7). Hybridization for the detection of *cfiA* was carried out with a randomly $^{32}$P-labeled *cfiA* internal 729-bp fragment (21). Prehybridization, hybridization, and high-stringency washes were carried out according to the protocols provided by Amersham for Hybond $N^+$ membranes. Hybridization for the estimation of the DNA load was carried out with small-subunit rDNA generated by PCR (see below). rDNA was labeled, and hybridization was revealed by chemiluminescence with the enhanced chemiluminescence random prime labeling and detection systems of Amersham according to the instructions of the manufacturer.

**PCR amplification and small-subunit rDNA sequencing.** One hundred nanograms of DNA was used in a PCR to amplify the small-subunit rDNA genes. Two primers corresponding, respectively, to positions 8 to 28 and 1384 to 1400 of the *Escherichia coli* small-subunit rDNA sequence were used. Amplifications were carried out in a 50-μl reaction mixture containing 2 ng of each primer per μl, the four deoxynucleotide triphosphates at 250 μM each, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 2.5 U of *Taq* DNA polymerase (Stratagene Cloning Systems). The procedures for PCR amplification and sequencing of PCR products were as described previously (29).

**Phylogenetic analysis and alignment (general procedure).** The phylogenetic data described below were obtained by (i) using successive alignment and phylogeny procedures and (ii) reinvestigating deep branching patterns after the determination of close relationships. In each phylogenetic analysis, we restricted the comparisons to nucleotide positions that were aligned without doubt. All sequence alignment and species selection operations were carried out with computer programs developed by us and available on request from R. Christen.

**Phylogenetic methods.** A neighbor-joining method (30) was used as a preliminary analysis. Resulting topologies were then further investigated by maximum-likelihood and maximum-parsimony analyses. Maximum-likelihood analyses used the fdnaml program written by G. J. Olsen (University of Illinois, Urbana) and were compiled on a Hewlett-Packard model 700 workstation, while maximum-parsimony analyses were performed with the PAUP program for Macintosh (35). The robustness of each topology was evaluated under maximum parsimony through 100 bootstrap replications (heuristic search). Trees were plotted with the njplot program for Macintosh developed by M. Gouy (URA 243 CNRS, Université Claude Bernard, Lyon, France) that allows the transformation of a formal tree representation (Newick's format) into MacDraw drawings.

Recent theoretical work (9) has demonstrated that phylogenetic methods may fail when some assumptions are violated. When the same tree is found by different methods, it is therefore much more likely the true tree (unique) has been found rather than the same wrong tree (infinite). A convergence of the different methods as an indication that the correct phylogeny has been determined has also been proposed by Kim (14). We have therefore used three phylogenetic methods to assess the reliability of all of our analyses.

**Domains used.** The following sections of the small-subunit rRNA sequences (numbers relate to the *E. coli* sequence) were used for phylogenetic analysis: nucleotides 34 to 68, 100 to 132, 250 to 334, 338 to 419, 423 to 449, 492 to 539, 546 to 587, 594 to 622, 630 to 645, 664 to 734, 747 to 826, 854 to 926, 932 to 996, 1043 to 1113, 1122 to 1254, and 1275 to 1372 (see Fig. 1) and nucleotides 34 to 74, 97 to 132, 250 to 449, 479 to 587, 592 to 645, 664 to 999, and 1040 to 1372 (see Fig. 2). These domains are shorter than the total length of rDNA sequences obtained (i.e., 972 nucleotides [about 70%] [see Fig. 1] and 1,102 nucleotides [about 80%] [see Fig. 2]). One should exclude from the phylogenetic analyses nucleotides that are in domains for which a proper alignment cannot be obtained between all sequences analyzed. It is, however, also necessary to exclude positions that can be well aligned but that are hot spots for mutations; for such nucleotides, homoplasies become preponderant over the true phylogenetic signal. A robust phylogenetic analysis is therefore obtained only when these two types of positions are excluded from the analysis. The similarity values for closely related strains were calculated on the basis of the entire length of the rDNA sequenced.

**Nucleotide sequence accession numbers.** Sequences have been deposited in the EMBL sequence database under accession numbers X83935 (*B. fragilis* ATCC 25285$^T$), X83936 (*B. fragilis* AIP 638), X83937 (*B. fragilis* BFr 790), X83938 (*B. fragilis* BFr 2991), X83939 (*B. fragilis* BFr 6791), X83940 (*B. fragilis* BFr 12491), X83941 (*B. fragilis* TAL 3636), X83942 (*B. fragilis* B 119), X83943 (*B. fragilis* BFr 81), X83944 (*B. fragilis* BFr 189), X83945 (*B. fragilis* BFr 901), X83946 (*B. fragilis* BFr 920), X83947 (*B. fragilis* KSB 1486), X83948 (*B. fragilis* VPI 2393), X83949 (*B. fragilis* VPI 4117), X83950 (*B. fragilis* VPI 4225), X83951 (*B. caccae* ATCC 43185$^T$), X83952 (*Bacteroides ovatus* ATCC 8483$^T$), X83953 (*B. stercoris* ATCC 43183$^T$), and X83954 (*B. merdae* ATCC 43184$^T$).

## RESULTS AND DISCUSSION

**Phylogenetic position of *B. fragilis* within the *Cytophaga-flavobacter-Bacteroides* phylum.** General phylogenetic analyses confirmed that all *Bacteroides* strains sequenced in this study (Table 1) belonged to the *Cytophaga-flavobacter-Bacteroides* phylum (19) (data not shown). More detailed analyses were then restricted to species representative of the five clusters previously described in this phylum (18). The genera *Prevotella*, *Bacteroides*, and *Porphyromonas* formed three distinct robust monophyletic units, a result in agreement with those of all previous studies (7, 15, 18, 19, 31–34, 38). The genus *Porphyromonas* appeared as a sister group to the other two genera. *B. merdae* clustered with the genus *Porphyromonas* and was closely related to *Bacteroides distasonis*. This result was supported by all phylogenetic methods and by 85% of the bootstrap replications. *B. stercoris*, *B. caccae*, and all *B. fragilis* strains were clearly included in the genus *Bacteroides* (Fig. 1). All *B. fragilis* strains, *B. caccae*, *B. ovatus*, and *Bacteroides thetaiotaomicron* formed a robust monophyletic unit that was found by all three phylogenetic methods and supported by 96% of the bootstrap replications.

**Phylogenetic relationships among strains of *B. fragilis* and their related species.** The exact intrageneric relationships among members of the genus *Bacteroides* were difficult to determine in a general analysis such as that shown in Fig. 1. In particular, some branches were not well supported by bootstrap data in the presence of distant taxa or changed when different outgroups were used. Low bootstrap values can indi-

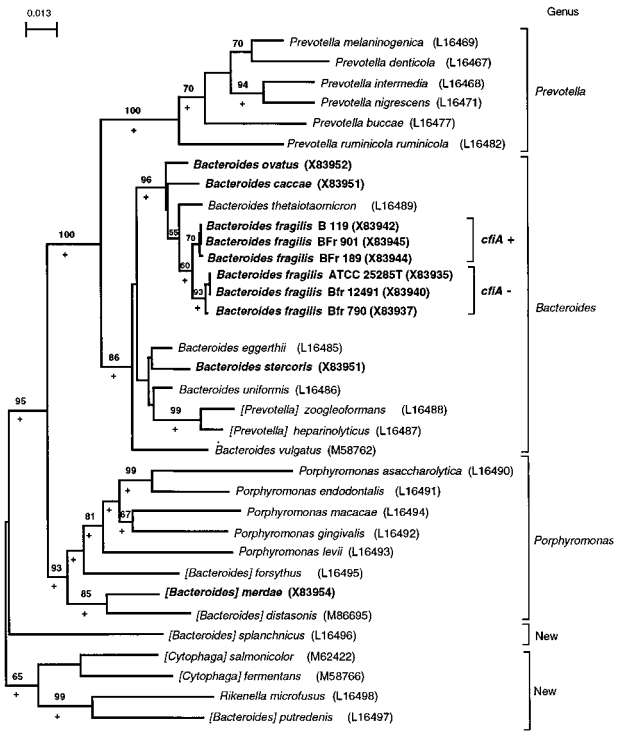


FIG. 1. Phylogenetic analysis restricted to the genera *Prevotella*, *Porphyromonas*, and *Bacteroides* and some related bacteria within the cytophaga-flavobacter-bacteroides phylum (unrooted tree obtained by the neighbor-joining method). The branches that were also found to be significantly positive at $P < 0.01$ by maximum-likelihood analysis are indicated by plus signs. Values above the lines (only values greater than 50% are shown) indicate branches also found in the most parsimonious tree and indicate how these branches were supported by a bootstrap analysis (heuristic search, 100 replications). The scale bar indicates accumulated changes per nucleotide. Strains whose sequences were determined in this study are shown in boldface. Brackets around a genus name indicate species that do not fulfill sensu stricto the definition of a genus.

cate a weakly supported relationship, but they can also result from peculiar rates of mutation in some species or from the inclusion of outgroups that are too distantly related for the phylogenetic relationships being investigated. We therefore restricted phylogenetic analyses to the genus *Bacteroides*. Also, sequences of additional *B. fragilis* strains were used, as well as longer stretches of the sequences, including more variable regions that could be well aligned only between closely related species. Figure 2 shows an unrooted tree based on the results of a maximum-likelihood analysis (topology shown on this figure) and those of the two other methods. Among the true *Bacteroides* species, there was a clear separation into several distinct groups. A well-defined group, established by all three methods and supported by more than 95% of the bootstrap replications, comprised the 16 *B. fragilis* strains analyzed as well as the type strains of *B. caccae*, *B. ovatus*, and *B. thetaiotaomicron* (Fig. 1 and 2). This result is in agreement with rDNA-DNA hybridization data (12) and with the relatedness of these species as reflected by their shared antigenicity (36). All *B. fragilis* strains formed a homogeneous taxon that comprised two robust monophyletic units. One unit contained all *cfiA*-negative strains, including bona fide homology group I strain ATCC 25285$^T$ (11). The other unit contained all *cfiA*-positive strains and the three homology group II strains for which we determined 16S rDNA sequences. The two clades were supported by all three phylogenetic methods and by bootstrap values, respectively, of 75 and 100% (Fig. 2). In both clades, all strains showed high percentages of rDNA similarities. Within each group, the number of nucleotide differences between any two sequences ranged from three to zero, i.e., from 99.8 to 100% identity. When the rDNA sequences of one group were compared with those of the other group, the number of differences ranged from 16 to 21, i.e., from 98.9 to 98.4% identity. These differences were located in regions known to be highly variable among species (7). It remains to be determined whether, as suggested by the data shown in Fig. 2, these clusters can be equated, respectively, with DNA homology groups I and II described by Johnson and by Johnson and Ault (11, 12).

In light of the apparent relatedness between the *cfiA*-positive strains and strains belonging to homology group II, the latter were tested for the presence of *cfiA*-related sequences by DNA-DNA hybridization under conditions of high stringency (22). All five strains analyzed (Fig. 2) did indeed contain such sequences, which displayed, however, a lesser degree of homology than the true *cfiA*-positive strains, as estimated on the basis of weaker hybridization signals (Fig. 3A). Differences in the intensities of these signals were not due to differences in the loads of DNA, as is demonstrated by the pattern in Fig. 3B, which shows that very similar amounts of DNA had been loaded.

If the intensity of the hybridization signal with the *cfiA* probe

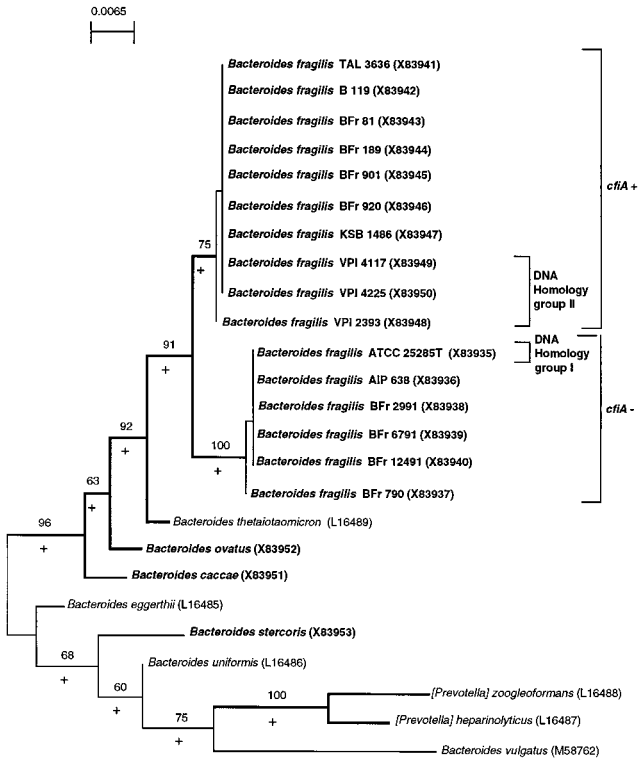


FIG. 2. Phylogenetic relationships within the genus *Bacteroides* (unrooted tree obtained by a maximum-likelihood method). Branches significantly positive at $P < 0.01$ are indicated by plus signs. Boldface lines indicate monophyletic taxa also found by a neighbor-joining analysis. Values above the lines (only values greater than 50% are shown) indicate branches also found in the most parsimonious tree and indicate how these branches are supported by a bootstrap analysis (heuristic search, 100 replications). The scale bar indicates accumulated changes per nucleotide. Strains whose sequences were determined in this study are shown in boldface. Brackets around a genus name indicate species that do not fulfill sensu stricto the definition of a genus.

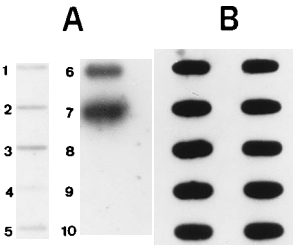


FIG. 3. Filter hybridization to *B. fragilis* strains. Bacteria were filtered onto Hybond$^+$ membrane (Amersham) with a slot blot apparatus and treated as described in Materials and Methods. (A) Hybridization with a $^{32}$P-labeled *cfiA* internal fragment (21). Autoradiographic exposure time, ca. 10 h. (B) Hybridization with a PCR-generated 16S rDNA fragment. The DNA was labeled, and hybridization was revealed by chemiluminescence. Slots 1 through 5, homology group II strains VPI 2393, VPI 3392, VPI 4117, VPI 2552, and VPI 4225 (10); slots 6 and 7, *cfiA*-positive strains BFr 81 and BFr 189; slots 8 to 10, strains AIP 638, BFr 790, and BFr 2991.

is used as a criterion, the *cfiA*-positive cluster can be further subdivided into members that hybridize strongly and those that hybridize weakly (Fig. 3). This difference might reflect the existence of two, most likely closely related *cfiA*-type genes. In the *cfiA*-positive strains that were isolated as such and that hybridize strongly (22, 23), the gene is capable of conferring a high-level resistance to the carbapenems as well as resistance to most β-lactamase inhibitors, while in the weakly *cfiA*-positive strains of DNA homology group II (11), only the latter type of resistance is known to occur (2, 40).

The presence of the *cfiA*-type genes within a monophyletic cluster of *B. fragilis* that apparently represents only a minority of the species (11, 22) is suggestive of a recent acquisition. This cluster is also the predominant pool of all known *B. fragilis* insertion elements, which have been found to play an important role in the expression of carbapenem resistance (22, 23). These data raise the possibility that both categories of genetic determinants, i.e., the resistance gene(s) and insertion elements, may have coevolved under a selective pressure for the regulation of the expression of the *cfiA* genes.

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