ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 1028–1036
0066-4804/93/051028-09$02.00/0
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Vol. 37, No. 5

Genetic and Biochemical Analysis of a Novel Ambler Class A β-Lactamase Responsible for Cefoxitin Resistance in *Bacteroides* Species

ANITA C. PARKER AND C. JEFFREY SMITH*

Department of Microbiology and Immunology, East Carolina University, Greenville, North Carolina 27858-4354

Received 10 September 1992/Accepted 2 March 1993

A clinical isolate of *Bacteroides vulgatus* was resistant to tetracycline, clindamycin, ampicillin, cephaloridine, cefoxitin, and other β-lactam antibiotics except imipenem. β-Lactam resistance was mediated by a membrane-associated, clavulanate-sensitive cephalosporinase capable of degrading cephalosporins and penicillins. Cefoxitin also was degraded but at a slow rate. The cefoxitin resistance (Fx^r) determinant was cloned from *B. vulgatus* genomic libraries that were prepared in *Escherichia coli* and then mated with *Bacteroides fragilis* for the identification of Fx^r strains. Analysis of *B. fragilis* strains with the cloned Fx^r determinant revealed the presence of a new β-lactamase protein with the physical and enzymatic properties of the β-lactamase found in the original *B. vulgatus* isolate. The β-lactamase gene (*cfxA*) was subcloned on a 2.2-kb *Dra*I-*Hin*dIII fragment, and the nucleotide sequence was determined. These results showed that *cfxA* encoded a protein of 321 amino acids and 35,375 molecular weight. Mutant strains in which the *cfxA* structural gene was disrupted by insertional inactivation lost both Fx^r and β-lactamase activity. Comparison of CfxA with other β-lactamases showed a relationship with the active-site serine β-lactamases in the Ambler molecular class A, although CfxA had apparently diverged significantly. This was exemplified by the substitution in CfxA at 13 of 25 amino acid residues previously identified as being invariant in class A β-lactamases. These results suggest that CfxA may represent a new class A homology group which diverged very early.

β-Lactamase production is the most important mechanism of resistance to β-lactam antibiotics in gram-negative bacteria, and the *Bacteroides fragilis* group possesses a wide array of these enzymes. In general, the organisms are moderately or highly resistant to many cephalosporins and penicillins, but α-methoxyl cephamycins such as cefoxitin and the carbapenems have been highly active against *Bacteroides* species. In the United States, for example, imipenem resistance rates are presently low, at about 0.2%, and cefoxitin resistance (Fx^r) rates have ranged up to 16% during the past decade (37, 38). High rates of Fx^r are cause for concern since Fx^r strains usually encountered are cross-resistant to most other β-lactams (38). In the case of strains that possess metallo-β-lactamases, the cross-resistance includes the carbapenems (8, 39).

Resistance of *Bacteroides* species to cefoxitin may involve a number of mechanisms including altered drug permeability, alterations of penicillin binding proteins, β-lactamase production, or a combination of mechanisms (8, 10, 41). Excluding the relatively rare imipenem-hydrolyzing metalloenzymes, several β-lactamases capable of cefoxitin degradation have been described. These are generally cephalosporinases with a slow rate of cefoxitin hydrolysis, and they are sensitive to inhibition by clavulanate (9, 13). Recently, Aldridge et al. (1) have found that >93% of Fx^r strains were still sensitive to β-lactam–clavulanate combinations. Thus, based on these criteria, it is possible that the slow cefoxitin-hydrolyzing cephalosporinases are one of the most widely disseminated mechanisms of Fx^r in the *Bacteroides* species. In this regard, it has been suggested that these β-lactamases are not novel enzymes but rather that the strains are Fx^r because of the production of much greater than normal levels of the conventional *Bacteroides* enzyme (36).

The occurrence of high regional Fx^r rates indicates the potential for clonal dissemination and/or horizontal transfer of the resistance phenotype. The transfer of Fx^r by a conjugation-like mechanism has been demonstrated; however, plasmids were not involved in the transfer (10). The Fx^r phenotype was mediated by the acquisition of a new β-lactamase. The present study was initiated to define the genetic basis for transmissible Fx^r in *Bacteroides* species. The results revealed a novel genetic locus designated *cfxA*, which encodes an Ambler molecular class A β-lactamase that appears to have diverged significantly from all other class A enzymes.

MATERIALS AND METHODS

Bacterial strains and growth. *Bacteroides* cells were cultured anaerobically in supplemented brain heart infusion broth as described previously (31). The following antibiotic concentrations were used unless noted in the text: clindamycin, 5 μg/ml; tetracycline, 5 μg/ml; rifampin, 20 μg/ml; gentamicin, 25 μg/ml; cefoxitin, 20 μg/ml; ampicillin, 50 μg/ml; and spectinomycin, 40 μg/ml. Antibiotic MIC values were measured by the standard agar dilution method with Wilkins-Chalgren medium (Difco Laboratories, Detroit, Mich.). Values were determined after 48 h of growth.

B. vulgatus CLA341, a cefoxitin-, tetracycline-, and clindamycin-resistant (Fx^r Tc^r Cc^r) clinical isolate, was obtained from P. C. Appelbaum at the Hershey Medical Center. *B. fragilis* 638 and *B. uniformis* V528 are Fx^s, rifampin-resistant (Rf^r) laboratory strains used in routine analysis. Strains IB246 and IB247 are Fx^r Tc^r Rf^r transconjugants obtained by mating the *B. vulgatus* CLA341 donor with 638 and V528,

* Corresponding author.

respectively. The strains 638(pJST61.cfx), 638(pFD351), and 638(pFD288) are plasmid-containing derivatives of 638 with the original cloned *cfxA* gene fragment, a subclone, or the subcloning vector, respectively. All other *Bacteroides* strains are referenced in the appropriate text. *Escherichia coli* DH5α (*recA hsdR17 lac*) was used as the host for all subcloning experiments, and *E. coli* HB101 (*recA rpsL*) was the primary host used for library construction. These strains were grown aerobically in L broth (agar) supplemented with ampicillin or spectinomycin where appropriate.

Bacterial conjugation. Standard filter mating protocols were used to transfer plasmids in triparental matings from *E. coli* donors to *Bacteroides* recipients. These used *E. coli* donor strains containing RK231 as a helper plasmid, and the mating plates were incubated aerobically as described previously (29). After 18 h of growth, the filters were washed with supplemented brain heart infusion broth, the cell suspensions were plated on selective media, and the plates were incubated anaerobically for selection of *Bacteroides* transconjugants.

DNA isolation and analysis. Purified plasmid DNA preparations from *Bacteroides* cells were obtained by CsCl-ethidium bromide ultracentrifugation of lysates prepared by alkaline denaturation (31). Genomic DNAs from *Bacteroides* strains were prepared from 50-ml overnight cultures and purified by CsCl-ethidium bromide ultracentrifugation as described previously (34). Screening and large-scale plasmid DNA preparations from *E. coli* transformants were performed as described previously (3). Plasmids were analyzed by agarose gel electrophoresis with Tris-borate or Tris-acetate buffers containing ethidium bromide, and other routine DNA manipulations have been described previously (31).

DNA hybridization analyses were performed essentially as described previously (34). Genomic or plasmid DNA samples were digested with the appropriate restriction endonuclease and electrophoresed on 0.8% agarose gels in Tris-acetate buffer. DNA was transferred from the gels to nitrocellulose filters by capillary action. DNA probes were labeled with ^{32}P by the random primer reaction with a commercial kit (Pharmacia LKB Inc., Piscataway, N.J.). Hybridizations were performed overnight at 67°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–4× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll 400 in H_2O)–1 mg of sheared salmon sperm DNA per ml. Filters were then washed three times for 30 min at 52°C in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) followed by three 30-min washes in 0.1× SSC at 52°C.

Nucleotide sequence analysis of the pFD351 *cfxA* gene fragment was performed by Sanger dideoxy sequencing reactions with modified T7 polymerase (Sequenase; U.S. Biochemical, Cleveland, Ohio), and reaction mixtures were analyzed on 6% polyacrylamide gels containing urea. The DNA fragment was sequenced in the first direction (*Dra*I to *Hin*dIII) by analyzing a set of nested deletions (16) derived directly from pFD351. Sequence of the second strand was determined by primer walking of density gradient-purified pFD351 utilizing oligonucleotide primers deduced from the sequence of the first DNA strand. The University of Wisconsin GCG DNA sequence analysis software (12) was used for computer analysis of DNA and protein sequences. The Pileup program from this package was used to generate the progressive multiple alignment of the β-lactamase protein sequences. The *cfxA* nucleotide sequence has been submitted to GenBank and assigned the accession number M72418. Other sequences used for analysis together with their abbreviations and GenBank accession numbers are as follows: *Staphylococcus aureus* (PC1), M15526 (5); *Yersinia enterocolitica* (YER), X57074 (28); *Bacillus cereus* β-lactamase III (BCIII), M15195 (20); *B. cereus* β-lactamase I (BCI), X06599 (30); *Actinomadura* strain R39 (ACT), X53650 (18); *Streptomyces albus* G (ALBUS), M28303 (11); and *E. coli* TEM-1 from pBR322, VB0001 (35).

Library and plasmid constructions. A genomic library of CLA341 was prepared by partial digestion of genomic DNA with *Sau*3A. DNA fragments in the 5- to 15-kb size range were purified on neutral sucrose gradients, pooled, and ligated to the *Bgl*II site of pJST61 (ampicillin resistance [Ap^r] in *E. coli* and Cc^r in *Bacteroides* species [39]). Ligation mixtures were transformed into *E. coli* HB101, and transformants were selected on ampicillin-containing medium. These recombinants were then mated with *B. fragilis* 638 for the identification of Fx^r clones as described in the text below. The Fx^r gene was subcloned with the pUC19-based shuttle vector pFD288 (8.8 kb; *oriT* Sp^r in *E. coli* and Cc^r in *Bacteroides* species [32]). The suicide vector pFD280.erm was similar to pFD288 except that is lacked the pBI143 replicon portion of the molecule, rendering it incapable of replication in *Bacteroides* species. The construct used for insertional inactivation of Fx^r, pSUC2, was formed by ligation of the 0.6-kb *Hae*III-*Pvu*II fragment (see Fig. 1) into the *Sma*I site of pFD280.erm.

Analysis of β-lactamase activity. Cell extracts were prepared from 200-ml cultures of mid- to late-logarithmic-phase cells. Cells were harvested by centrifugation, washed in 20 mM phosphate buffer (pH 7), and then suspended in 3 ml of the same buffer. Cells were disrupted by two passages through a French pressure cell at 12,000 lb/in^2, and cellular debris were removed by centrifugation at 12,000 × *g* for 30 min at 4°C. β-Lactamase activity was routinely measured with the chromogenic cephalosporin nitrocefin (10^{-4} M) in a 1-ml reaction mixture with a DU-65 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. [25]). Substrate profiles were determined by monitoring the hydrolysis of various β-lactam antibiotics by crude extracts in 1-ml reaction mixtures containing 0.1 mM substrate. Reactions were measured at the following wavelengths: cephaloridine, 260 nm; cefotaxime, 267 nm; cephalothin, 260 nm; ampicillin, 235 nm; benzylpenicillin, 233 nm (8). The apparent K_m values were estimated from double-reciprocal plots of velocity versus substrate concentration. For all enzymatic analysis, one unit of activity was defined as the amount of cell extract that could degrade 1 μmol of substrate per min. To test the effects of various inhibitors on β-lactamase activity, crude cell extract was incubated with inhibitor for 5 min and the reaction was then initiated by the addition of nitrocefin. Protein concentrations for all assays were estimated as described previously (24).

Molecular weight determinations. The apparent molecular weight of β-lactamase was determined by SDS-polyacrylamide gel electrophoresis (PAGE) with a discontinuous buffer system (23). β-Lactamase activity in the gels was detected after renaturing the proteins by soaking the gels in several changes of sodium phosphate buffer as described by Foweraker et al. (14). Activity was detected by overlaying the gels with nitrocefin-containing agarose and photographed with a green filter. The relative migration of the β-lactamase bands was compared with the mobilities of protein standards visualized after staining with Coomassie blue (Coomassie Brilliant Blue G; Sigma Chemical Co., St. Louis, Mo.).

Location of enzymatic activity. Bacterial cells were frac-

tionated by a modification of the osmotic shock method described by Huang and Forsberg (19). Cultures (300 ml) were centrifuged at 10,000 × g for 15 min at 4°C, and a sample of the supernatant was saved for analysis of extracellular proteins. Cells were then washed twice in 50 mM sodium phosphate buffer (pH 7) containing 0.8% NaCl, suspended in 0.5 M sucrose with 30 mM Tris (pH 7.2) (50 ml of buffer per g of cells), and then gently shaken for 10 min at room temperature. Cells were then centrifuged at 20,000 × g for 20 min at 22°C, quickly suspended in ice-cold H_2O (30 ml of H_2O per g of cells), and shaken gently for 10 min at 4°C. This cell suspension was then centrifuged for 20 min at 4°C, and the resulting supernatant contained the periplasmic fraction. The cell pellet was suspended in 5 ml of 20 mM sodium phosphate buffer (pH 7), and the cells were ruptured in a French pressure cell. Cellular debris were removed by low-speed centrifugation, and the supernatant was then centrifuged at 200,000 × g for 2 h at 4°C to separate the cytoplasmic and membrane fractions. The membranes then were suspended in 5 ml of 20 mM sodium phosphate buffer (pH 7).

Acid phosphatase and succinate dehydrogenase were used as marker enzymes for the periplasmic and membrane fractions, respectively. Acid phosphatase was assayed as described by the supplier (Sigma procedure no. 104). Succinate dehydrogenase was measured by observing the oxidation of NADH in the presence of fumarate. One-milliliter reaction mixtures containing cell extract in 50 mM sodium phosphate buffer (pH 7), 0.2 μmol of NADH, and 2.5 μmol of disodium fumarate were incubated at 30°C, and the change in A_{340} was recorded. One unit of activity is expressed as micromoles NADH oxidized per minute.

HPLC assay of cefoxitin in culture supernatants. Cefoxitin was detected in culture supernatants essentially as described previously (9). Briefly, cultures were grown overnight and diluted into fresh broth containing 100 μg of cefoxitin per ml to about 10^8 cells per ml. These cultures were incubated for 24 h and centrifuged, and the supernatant was retained for high-pressure liquid chromatographic (HPLC) (Beckman Instruments) analysis. Samples of supernatant were analyzed directly on a Supelcosil LC-18-DB (Supelco Inc., Bellefonte, Pa.) reverse-phase column (25 cm by 4.6 mm) with a 25% methanol–75% 10 mM KH_2PO_4 mobile phase. Elution was at 1 ml/min and monitored at A_{234} and A_{260} for identification of the drug and possible metabolites. Cefoxitin was identified and quantified by comparison to known cefoxitin standards prepared in supplemented brain heart infusion broth. Under these conditions, cefoxitin eluted at 9.6 ml and the putative cefoxitin breakdown product (A_{234} chromophore) eluted at 12 ml.

RESULTS

Cloning and genetic analysis of *cfxA*. *B. vulgatus* CLA341 was resistant to tetracycline, clindamycin, and to high levels of ampicillin, piperacillin, cefoxitin, and other β-lactam antibiotics except for the carbapenems such as imipenem (Table 1) (26). Resistance to β-lactam antibiotics was transmissible to other *Bacteroides* species via a conjugation-like mechanism (26). To clone this conjugative resistance gene, we prepared CLA341 genomic libraries using the positive selection vector pJST61 (39). A total of 6×10^3 *E. coli* clones were placed into 20 pools, and these were mated with the recipient *B. fragilis* 638 in triparental matings. Following selection for transconjugants on a medium containing rifampin, gentamicin, clindamycin, and cefoxitin, a single Fx^r colony was obtained, and this recombinant plasmid containing the *cfxA* gene was designated pJST61.cfx. Relative to the 638 parent strain, this transconjugant had a 200-fold increase in β-lactamase activity and an increase in both the cefoxitin and ampicillin MIC (Table 1).

TABLE 1. β-Lactamase activity and in vivo cefoxitin degradation by *Bacteroides* strains containing *cfxA*

Strain/(Plasmid)	MIC (μg/ml)		% Cefoxitin degradation[a]	β-Lactamase sp act[b]
	Cefoxitin	Ampicillin		
CLA341	256	>256	82	2.1
638(pJST61.cfx)	128	256	91	2.0
638(pFD351)	128	>256	91	5.3 ± 2.0
638(pFD288)	8	16	5	0.007
IB246	128	256	89	4.3
IB246ΩpSUC2[c]	8	16	2	0.007
638	8	16	11	0.009
IB247	128	256	ND[d]	1.8
V528	2	2	ND	0.02

[a] Cefoxitin degradation was determined by HPLC as described in the text, and results are the average of two trials. There was an average of 1% cefoxitin degradation in uninoculated controls.
[b] β-Lactamase activity was determined by nitrocefin assays of crude extracts. Results are in units per milligram.
[c] The *cfxA* gene of strain IB246ΩpSUC2 has been inactivated by insertion of the pSUC2 plasmid (see text).
[d] ND, not determined.

Restriction analysis of pJST61.cfx revealed the presence of a single 7.5-kb *Sau*3A fragment (Fig. 1). This fragment was excised with *Sau*3A and subcloned into the *Bam*HI site of the Ap^s shuttle vector pFD288. There was no β-lactamase activity detected in the resulting *E. coli* clones. The β-lactamase activity was localized by further subcloning into pFD288 and testing recombinants for Fx^r in *B. fragilis* 638. The smallest active subclone was pFD351 with a 2.2-kb insert (Fig. 1). *B. fragilis* 638(pFD351) displayed high Fx^r and Ap^r levels, and β-lactamase activity increased 3.6-fold above the activity seen with pJST61.cfx. This high level of activity was somewhat variable in the subclones and ranged from 3 to 7 U/mg of protein (Table 1). No β-lactamase activity was observed in the smaller subclones (pFD352, -353, and -354) which were deleted in the central region of the DNA fragment containing the *Hae*III, *Cla*I, and *Pvu*II sites.

Identification and inactivation of the Fx^r determinant. CLA341 contains several plasmids, two of which are usually found in the Fx^r progeny that result from matings with CLA341 as the donor. Genomic DNA from CLA341, IB246 (Fx^r transconjugant), and 638, and a CLA341 CsCl-purified plasmid preparation, were examined for the presence of *cfxA* in Southern hybridizations. The results shown in Fig. 2 were obtained with a 0.6-kb *Hae*III-*Pvu*II probe which lies within the *cfxA* gene (Fig. 1). There were no *cfxA*-homologous fragments observed in the CLA341 plasmid preparation or in the *B. fragilis* 638 control; however, a single 7.5-kb *Sau*3A fragment from genomic CLA341 and IB246 DNA hybridized strongly to the probe.

To demonstrate the dependence of Fx^r on the β-lactamase, we constructed a *cfxA* mutant by insertional inactivation. A suicide vector (pFD280.erm) unable to replicate in *Bacteroides* species was ligated to the 0.6-kb *Hae*III-*Pvu*II *cfxA* gene fragment. This construct, pSUC2, was mobilized into the Fx^r transconjugant IB246 with selection for the pSUC2 *ermF* (Cc^r) determinant. Presumably, homologous recombination with the *cfxA* gene should mediate the inser-

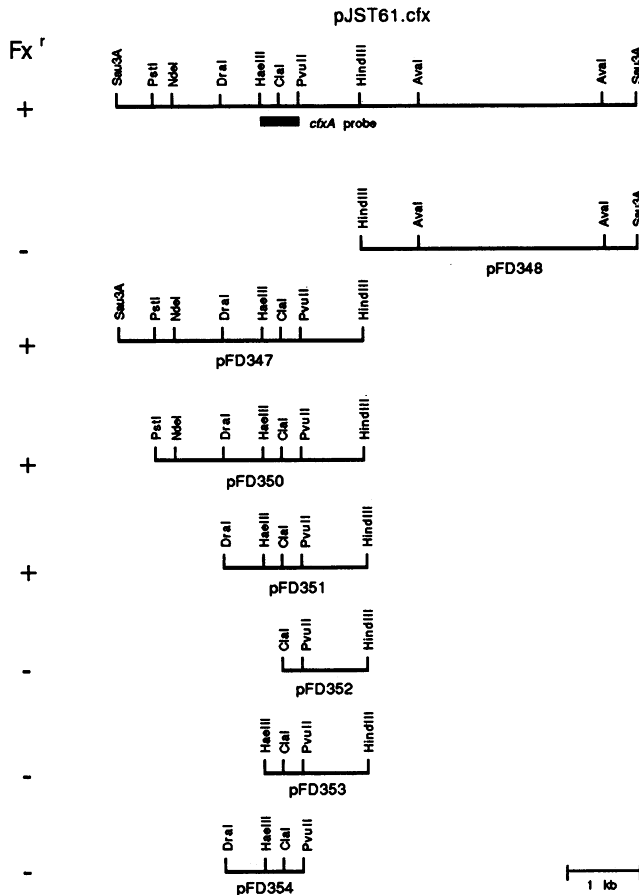

FIG. 1. Restriction endonuclease maps of clones containing *cfxA* gene fragments. In all cases, only the CLA341 DNA fragments are presented and no vector sequences are shown. pJST61.cfx was the original 7.5-kb insert cloned into pJST61, and all other plasmids were constructed in the vector pFD288 as described in the text. Fx^r denotes the phenotype of each plasmid in *B. fragilis* 638. The bar under the map of pJST61.cfx indicates the region used as probe in Fig. 2 and 6 and for the construction of IB246ΩpSUC2.

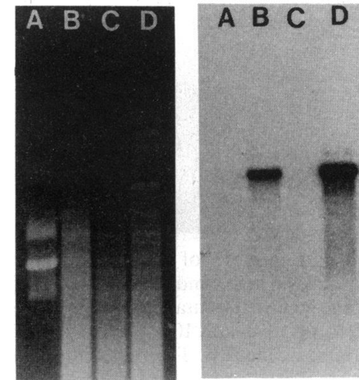

FIG. 2. Agarose gel analysis and autoradiograph of DNAs probed with the 0.6-kb *cfxA* gene fragment. Each lane contains either 1 μg of undigested plasmid DNA or 1 μg of total genomic DNA digested with *Sau*3A. Lanes: A, CLA341 purified plasmids; B, CLA341 genomic DNA; C, 638 genomic DNA; D, IB246 genomic DNA.

tion of pSUC2 into the chromosome by a single crossover event leading to the inactivation of *cfxA* (15). Thirty-two Cc^r transconjugants were obtained in two independent matings, and all those tested had pSUC2 inserted into the chromosome. Several of the transconjugants were tested for β-lactamase activity and were found to have low levels of activity similar to those of the original 638 parental strain (e.g., IB246ΩpSUC2, Table 1). These strains also were now sensitive to low concentrations of cefoxitin.

Characterization of CfxA β-lactamase. Preliminary experiments with a bioassay system indicated that crude extracts of CLA341 or IB246 but not 638 were capable of inactivating cefoxitin. These results were confirmed and extended by HPLC analysis. Results shown in Table 1 indicate that incubation of cefoxitin (100 μg/ml) with CLA341, IB246, 638(pJST61.cfx), or 638(pFD351) for 24 h resulted in the disappearance of >80% of the drug. In all cases, the disappearance of the drug coincided with the appearance of a new metabolite which had a longer retention time and absorbed strongly at 234 nm. When cefoxitin was incubated with 638, 638(pFD288), or IB246ΩpSUC2 cells, there was only a slight decrease in the concentration of drug and no new metabolites were observed.

Typical substrate and inhibitor profiles for CfxA in crude extracts of CLA341 are presented in Table 2. These results indicated that the enzyme was a cephalosporinase that had high hydrolysis activity for cephaloridine and cephalothin. Activity with penicillin substrates was less than 11% of the activity with cephalosporins. The apparent K_m values of the enzyme for cephaloridine and penicillin were 113 and 31 μM, respectively. Although the bioassay and HPLC analysis clearly indicated degradation of cefoxitin, the rate was too slow to observe in spectrophotometric assays and no enzymatic hydrolysis was detected. The inhibitors clavulanic acid and sulbactam inhibited the enzyme, causing about 50% inhibition at 1 μM. The enzyme was not significantly inhibited by EDTA, but *p*-chloromercuribenzoate (PCMB) and iodine were potent inhibitors.

The molecular mass of CfxA was estimated by SDS-

TABLE 2. Substrate and inhibitor profiles for CfxA

Compound	Concn (μM)[a]	% Activity[b]
Substrates		
Cephaloridine	100	100
Cephalothin	100	67.5
Penicillin G	100	10.7
Ampicillin	100	7.2
Cefotaxime	100	0.96
Cefoxitin	100	<0.01
Nitrocefin	100	290
Inhibitors		
Clavulanate	1	51
Sulbactam	1	35
PCMB	100	0
EDTA	100	84
Iodine	100	0

[a] Concentration is the concentration of substrate used for substrate assays or the concentration of inhibitor used in inhibitor assays with nitrocefin as substrate.

[b] The percent activity for substrate assays is the percent activity relative to that of cephaloridine (100%). In inhibitor assays, the percent activity is relative to nitrocefin assays with no inhibitor added.

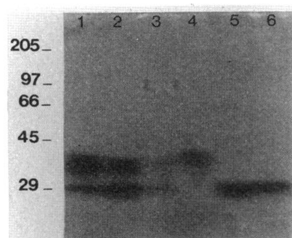

FIG. 3. SDS-PAGE analysis of crude extracts from *B. vulgatus* CLA341, Fx[r] transconjugants, and the 638(pFD351) subclone containing *cfxA*. Crude extracts containing between 80 and 130 μg of protein were electrophoresed on 10% polyacrylamide gels and then stained for β-lactamase activity. The molecular masses (in kilodaltons) of protein standards are indicated to the left of the panel. Lanes: 1, 638(pFD351); 2, IB246; 3, IB247; 4, CLA341; 5, 638; 6, V528.

PAGE of crude extracts, and the results are shown in Fig. 3. As seen in lane 4, extracts from CLA341 had a single, but broad, nitrocefin-reactive band that measured between 35,000 and 38,000 Da. The CfxA β-lactamase was clearly distinct from the common β-lactamases seen in *B. fragilis* 638 or *B. uniformis* V528 (lanes 5 and 6), which were about 30,000 Da. The transconjugants IB246 and IB247 and the clone 638(pFD351) each possessed the common β-lactamase, but in addition they had acquired a new β-lactamase. This new protein comigrated with CfxA from CLA341. Isoelectric focusing is another important analytical tool used for the analysis of β-lactamases, but several attempts by a variety of methods failed to result in clear, discrete nitrocefin-reactive bands for CfxA, although other *Bacteroides* β-lactamases were easily visualized.

The cellular location of CfxA activity in *B. vulgatus* CLA341 was determined by fractionation of cellular components with succinate dehydrogenase and acid phosphatase as markers for the membrane and periplasmic fractions, respectively. The results in Table 3 show that CfxA activity closely followed the membrane fraction, with 58% of the total activity being membrane associated and only 17% of the activity located in the periplasm. A fraction of the total succinate dehydrogenase activity also appeared in the cytoplasmic and periplasmic fractions, indicating that there may have been some partitioning of membrane into these fractions. However, only 2% of the acid phosphatase activity was found in the membrane fraction, indicating that there was little or no contamination of the membranes with periplasmic contents. Similar results were observed for the *B. fragilis* transconjugant IB246, for which 66 to 78% of the β-lactamase activity was membrane associated, and in these experiments, 93% of succinate dehydrogenase was in the membrane fraction. In contrast to these results, 61 to 87% of the total activity of common β-lactamase was found in the periplasmic fraction of *B. fragilis* ATCC 25285 (33).

TABLE 3. Cellular location of CLA341 β-lactamase activity

Fraction	% Total enzyme activity[a]		
	β-Lactamase	Acid phosphatase	SDH[b]
Extracellular	6.0	0	0
Periplasmic	17.0	83.6	18.6
Cytoplasmic	19.0	13.6	15.7
Membrane	58.0	2.8	65.6

[a] The results are the percentage of total activity in crude cell extract, and values are the average of three replicates from two experiments.
[b] SDH, succinate dehydrogenase.

Nucleotide sequence analysis of *cfxA*. The complete nucleotide sequence of the 2,141-bp pFD351 *cfxA* gene fragment was determined, and the results are summarized in Fig. 4A. The sequence information revealed three open reading frames greater than 500 bp. Overall, the region had a 39% G+C content similar to the *Bacteroides* genome, and *cfxA* was slightly lower, being 35% G+C. The assignment of *cfxA* to the largest open reading frame was consistent with the subcloning results described above.

The DNA sequence of *cfxA* revealed a potential coding region of 963 bp starting at the first ATG codon (bp 150) and ending at the nonsense codon TAA (bp 1112; Fig. 4B). This could encode a protein of 321 amino acid residues with a predicted molecular weight of 35,375. A minimal ribosome binding site (AAA) was adjacent to the ATG start site, but it is possible that translation may start further upstream within the leader sequence preceding the ATG. There is a ribosome binding site (AAAGA) located in this region with four of five bases complementary to sequences near the *B. fragilis* 16S rRNA terminus (3′ end, OH-UCUUUCC [40]). Downstream from the *cfxA* translation stop codon, at bp 1156, there was a region of dyad symmetry with the major features of a transcription terminator.

Relationship between CfxA and Ambler class A β-lactamases. There was no significant homology between the *cfxA* nucleotide sequence and DNA sequences in the GenBank data base. However, when the CfxA amino acid sequence was used in data base searches, homology to several Ambler class A (2) β-lactamases from a variety of species was observed. The homologies ranged from 22% identity (38% similarity) for the *S. albus* enzyme to 27% identity (49% similarity) for *blaZ* from *S. aureus* p1258. Several key features plus the global sequence alignment (described below) clearly placed CfxA in the class A group. First was the presence and location of the active-site residues TyrProMetMetSerValPheLys (Fig. 4B), which are similar to TEM-1 and also correspond closely to the class A consensus Phe-$(X)_3$-Ser-$(X)_2$-Lys for active-site serine β-lactamases. Second, the invariant SDN loop (21) which may contribute to the integrity of the catalytic site was present in CfxA.

Based on these preliminary observations, the amino acid sequences of seven class A enzymes representing different homology groups (7, 27) were aligned with CfxA by a progressive alignment program, and the results are shown in Fig. 5. This alignment agrees closely with the results of Couture et al. (7) in which 26 class A β-lactamases were aligned, and most of their previously found regions of homology could be identified in our alignment. The pairwise identity scores for each of these aligned sequences also were determined, and it was found that CfxA was not closely related to any specific member of the class A group (Table 4). This analysis suggested that CfxA was most closely related to the *S. aureus*, *Y. enterocolitica*, and *S. albus* enzymes, and the least homology was seen with the *B. cereus* type III and *Actinomadura* group.

Homology of *cfxA* to other Fx[r] *Bacteroides* species. An initial assessment of *cfxA* dissemination was determined by Southern analysis of a panel of Fx[r] strains. For these analyses, the internal *Hae*III-*Pvu*II gene fragment was used to probe *Eco*RI-restricted genomic DNA from four different *Bacteroides* species. The results in Fig. 6 show that seven of the strains tested contained *cfxA* homologous sequences and that the only negative isolate was another *B. vulgatus* strain.

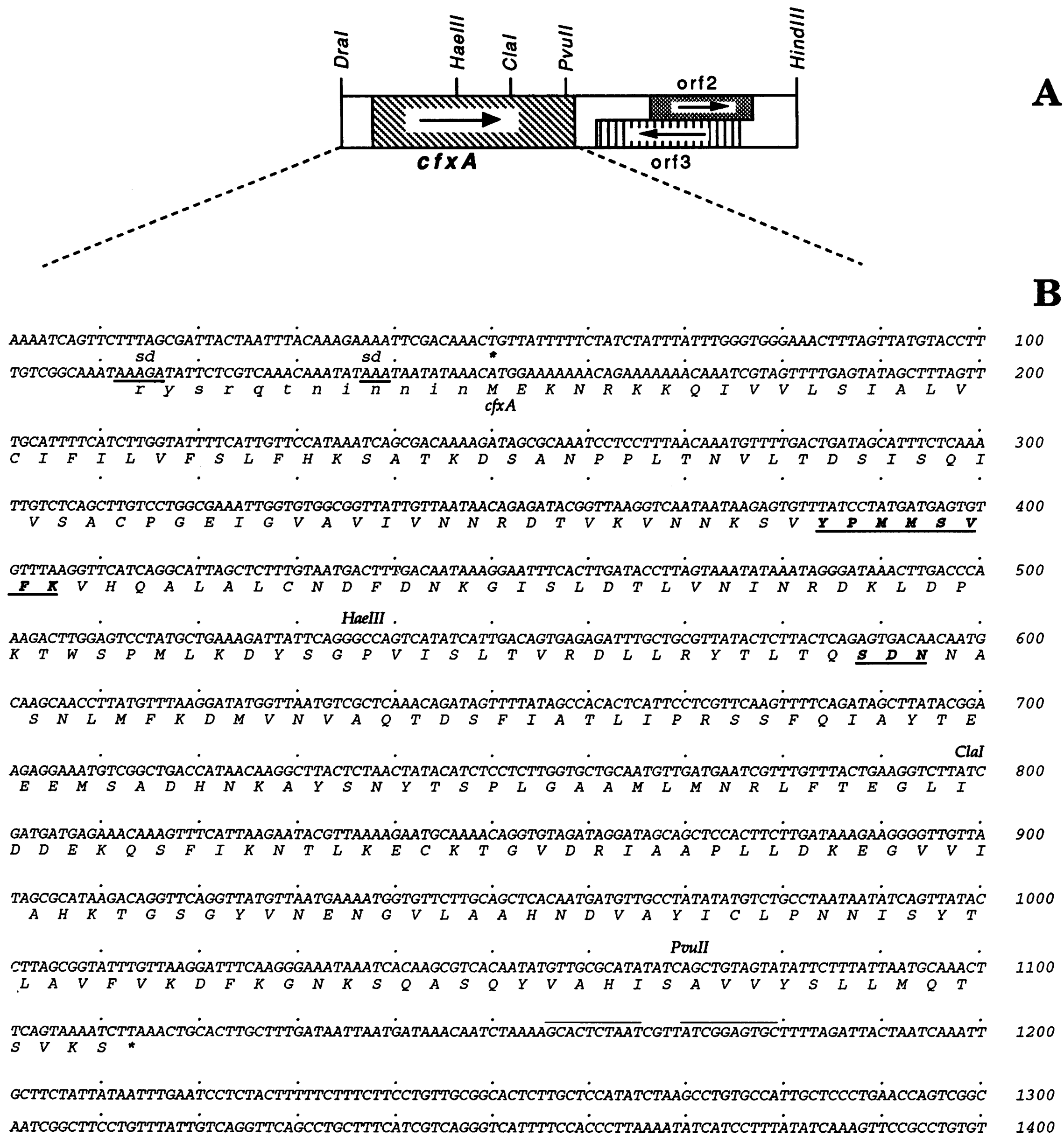

FIG. 4. Open reading frames (orf) located on the *B. vulgatus* pFD351 DNA insert and nucleotide and protein sequence of the region surrounding the *cfxA* gene. (A) Major open reading frames of the pFD351 2.2-kb cloned fragment are shown by the hatched marked boxes. (B) Nucleotide sequence from the region shown in panel A is presented, and below this is the deduced CfxA protein sequence. The first ATG codon is indicated by the asterisk over the sequence, and two possible ribosome binding sites are underlined and labeled sd. The *Cla*I, *Hae*III, and *Pvu*II sites are labeled, and the putative transcription terminator is shown by the lines over the sequence. The CfxA active-site residues and the SDN loop are underlined and shown in boldface.

Most of the strains tested were relatively recent isolates obtained from the Hershey Medical Center or Wadsworth Anaerobe Laboratory; however, *B. fragilis* V503 (VPI12256) was isolated prior to 1978 and this also hybridized strongly to the probe. It is interesting that four of the strains displayed more than one homologous fragment, but it was not determined whether this was due to multiple copies of the gene. Control hybridization experiments with Fx^s strains were invariably negative when probed with the *Hae*III-*Pvu*II *cfxA* gene fragment (Fig. 2) (33).

DISCUSSION

The results presented in this report demonstrate that the transmissible CLA341 Fx^r phenotype was mediated by a novel β-lactamase, CfxA. The results in Fig. 2 document that the *cfxA* gene was transferred to recipients strains during conjugation but that it was not located on plasmid DNA. Thus, *cfxA* may be similar to the transmissible Fx^r determinants described previously which were thought to be chromosomally located (9, 10). Except for the cellular location, CfxA was typical of conventional *Bacteroides* ceph-

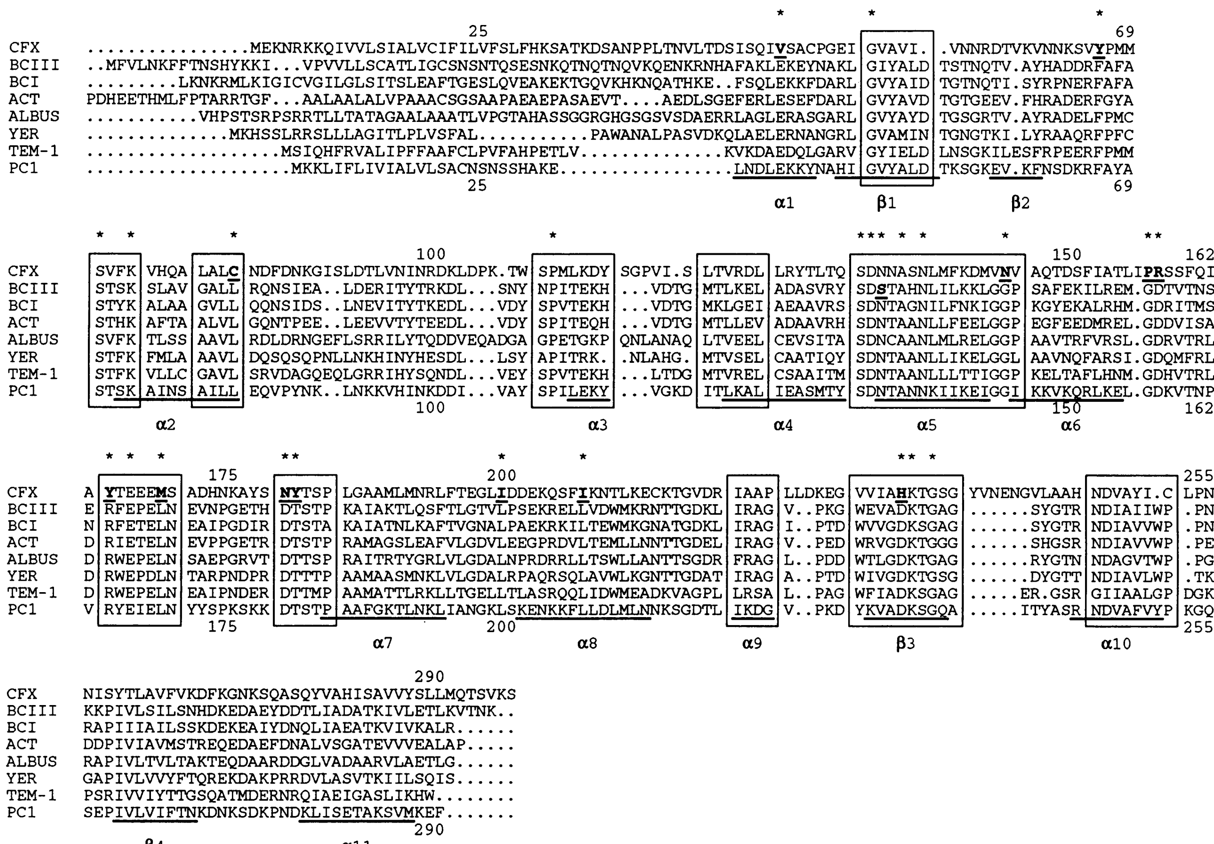

FIG. 5. Multiple sequence alignment of CfxA with class A β-lactamases representing the major homology groups. Sequences are numbered so that the active-site Ser equals position 70 according to the scheme of Ambler et al. (2) and using the PC1 sequence as a reference. PC1 secondary structures involving four or more residues are underlined and labeled α1 to α11 for α helix and β1 to β4 for β strand. Highly conserved regions are bracketed, and the invariant residues (7) are denoted by the asterisk over the sequence. Substitutions in the invariant residues are underlined and boldfaced. Abbreviations are as follow: *S. aureus*, PC1; *Y. enterocolitica*, YER; *B. cereus* β-lactamase III, BCIII; *B. cereus* β-lactamase I, BCI; *Actinomadura* strain R39, ACT; *S. albus* G, ALBUS; and *E. coli* from pBR322 (Tn*3*), TEM-1.

alosporinases (Table 2), and these enzymes have been categorized as members of group 2e in the Bush (4) classification scheme (clavulanic acid-inhibited cephalosporinases).

Although CfxA was similar to conventional β-lactamase, several lines of evidence suggest this is a novel enzyme that is widely disseminated among Fx^r (imipenem sensitive) *Bacteroides* species. First, CfxA was distinguished by the ability to slowly degrade cefoxitin (Table 1), which is not a property reported for the most common, conventional *Bacteroides* enzymes. In this regard, Yotsuji et al. (42) have shown that β-lactamases from strains resistant to high levels of cephaloridine and benzylpenicillin did not degrade cefoxitin even though many of these strains had high specific activity (0.15 to 2.43 U/mg) of the enzymes. Second, DNA sequences homologous to *cfxA* were found only in other Fx^r strains, and these represented four different species. If CfxA was the common chromosomal β-lactamase, one would predict that it would be species specific, with each species overproducing its own β-lactamase. Finally, we have shown previously (34) that the Fx^r *B. vulgatus* strains, WAL7062 and CLA341, are bona fide *B. vulgatus* species, and the results presented here indicate that no *cfxA*-homologous sequences were present in WAL7062. If Fx^r in CLA341 was due to overproduction of the conventional enzyme, then WAL7062 should have *cfxA*-homologous sequences.

Based on enzymatic properties, it was predicted that the group 2e enzymes would fall in the Ambler molecular class A (4). The protein sequence deduced from *cfxA* revealed homology to several class A enzymes. No class D and class C enzymes were identified in the data base searches, and when representatives of these classes were compared individually to CfxA, identity or similarity was extremely weak.

TABLE 4. Percent pairwise identities between aligned class A β-lactamase protein sequences

Enzyme[a]	% Identity[b]							
	TEM-1	BCIII	BCI	ACT	PC1	ALBUS	YER	CFXA
TEM-1	100	32	34	30	30	36	35	17
BCIII		100	54	46	40	35	36	16
BCI			100	50	37	34	37	16
ACT				100	34	36	37	15
PC1					100	30	30	21
ALBUS						100	43	20
YER							100	20
CFXA								100

[a] Abbreviations for enzymes are the same as defined in the legend to Fig. 5.
[b] The percent identity was determined from the aligned sequences shown in Fig. 5.

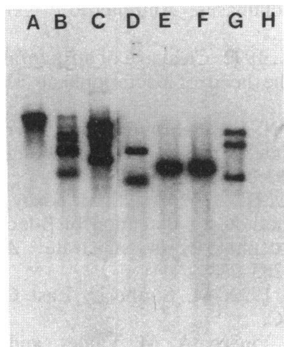

FIG. 6. Homology of the *cfxA* gene to other Fx^r *Bacteroides* strains. Genomic DNAs (1 μg) digested with *Eco*RI were electrophoresed on a 0.6% agarose gel and then transferred to nitrocellulose filters. The filters were probed with the 0.6-kb *Hae*III-*Pvu*II fragment from *cfxA*. Lanes: A, *B. vulgatus* CLA341; B, *B. fragilis* CLA262; C, *B. fragilis* CLA276; D, *B. uniformis* WAL7088; E, *B. fragilis* WAL7505; F, *B. ovatus* WAL7606; G, *B. fragilis* V503 (VPI12256); H, *B. vulgatus* WAL7062.

However, homology to class A also was relatively low, and the highest degree of protein sequence identity in individual comparisons was 27% to the *S. aureus* enzyme. The overall weak identity suggests that *cfxA* diverged early from the group, and in fact, CfxA did not fall within any of the established class A homology groups (7, 27). This idea was supported by the pair-wise identities determined from the multiple sequence alignment (Table 4 and Fig. 5). This analysis showed that representatives of each homology group shared between 30 and 54% identity, but clearly CfxA, with a maximum of 21% identity, was only distantly related. CfxA then appears to belong to a new homology group of class A enzymes, and further studies with other *Bacteroides* β-lactamases will prove useful in establishing the identity of this group.

The multiple sequence alignment also was interesting with respect to the analysis of β-lactamase structure and function. Eleven regions were identified that had a high degree of similarity between all the sequences examined (Fig. 5). These for the most part encompassed conserved secondary structures or amino acid residues involved in the active site or maintenance of the active-site depression (17). Previous studies have identified 25 invariant amino acid residues in class A sequences (7), and the multiple alignment generated in the present report accurately aligned these residues for the previously described enzymes. Inspection of the alignment revealed that 13 of 25 invariant amino acid residues were substituted in CfxA. A recent crystal structure analysis of the *S. aureus* PC1 enzyme reported on 22 of these invariant residues, and it appears that 6 of the CfxA substitutions are in replaceable positions (17). For example, the Cys for Leu-81 change is a conservative change consistent with its location in α-helix 2, and the Met for Leu-169 change is also very conservative. The other substitutions in replaceable sites include Pro for Gly-156, Arg for Asp-157, Tyr for Thr-180, and Ile for Leu-207, none of which are directly involved in active-site structures.

The highly conserved active-site region near Ser-70 was maintained in CfxA except for the notable substitution of Tyr for Phe-66. Although CfxA is clearly not a class D enzyme, it is interesting that four of five class D β-lactamase sequences also have a Tyr-66 residue (7). The SDN loop also is involved in catalysis, and these sequences were highly conserved in all class A β-lactamases including CfxA. In contrast, many other invariant positions involved in the maintenance of the active site (of PC1) were altered in CfxA, including Arg-164, which was replaced by a Tyr. It may be significant that TEM-5, -7, -8, -9, -10, -12, and -26 have Ser for Arg-164 substitutions leading to broader substrate specificity (6, 22).

Analysis of CfxA by SDS-PAGE indicated that the enzyme was between 35,000 and 38,000 Da (Fig. 3), and this estimate was close to the size (35,375 Da) deduced from the amino acid sequence. However, the molecular weight obtained from the deduced amino acid sequence did not take into account potential processing of a signal peptide. It is possible that either the enzyme is not processed or it is covalently modified, causing it to run aberrantly during SDS-PAGE. An example of the latter is the *B. cereus* BCIII β-lactamase, which is a covalently modified lipoprotein with about half of the total activity membrane bound (20). The close association of CfxA with the membrane fraction may favor the possibility that CfxA is a covalently modified lipoprotein.

β-Lactamases of gram-negative organisms are not generally membrane associated, but one that has been described is from *Capnocytophaga* species (14). This was a clavulanic acid-inhibitable cephalosporinase with many features similar to CfxA including its apparent molecular weight of 38,000. The cytophagas are gliding bacteria that appear to be related to the *Bacteroides/Flavobacteria* group based on 16S rRNA similarities (40). This phylogenetic group is apparently ancient, having branched very early from the main eubacterial line of descent, and it will be interesting to see whether the relationship between their β-lactamases supports this phylogenetic scheme.

In many Fx^r *Bacteroides* species, there may be more than one mechanism of resistance operating simultaneously. For example, the situation with *B. uniformis* WAL7088 clearly indicates two mechanisms, an Fx^r hydrolyzing β-lactamase (which could be CfxA, Fig. 6), and altered penicillin binding proteins (41). Our cloning results together with the insertion mutant IB246ΩpSUC2 clearly showed that acquisition of *cfxA* is sufficient for expression of the Fx^r phenotype. In fact, β-lactamase of the CfxA type can lead to high-level resistance (128 μg/ml) in the absence of other mechanisms, and this could be the most important resistance mechanism. This is supported by the analysis of 101 Fx^r *Bacteroides* strains with drug-inhibitor combinations (1). More than 90% of these strains remained sensitive to clavulanate combinations, indicating that β-lactamase was primarily responsible for resistance. While CfxA seems to be widely disseminated based on our limited sample size, other slow cefoxitin-hydrolyzing β-lactamases are present in the *Bacteroides* species as suggested by the variety of pI values observed for these enzymes from different sources (10, 13). As these are studied in more detail, it should be possible to determine their relationship to CfxA so that we can develop a better understanding of antibiotic resistance dissemination in the *Bacteroides* species.

ACKNOWLEDGMENTS

We thank P. C. Appelbaum and H. M. Wexler for kindly providing many of the strains used in this study. We also thank T. Ganey for construction on IB246ΩpSUC2.

This work was supported by Public Health Service grant AI-28884.

REFERENCES

1. **Aldridge, K. E., A. Henderberg, and C. V. Sanders.** 1990. In-vitro study of the susceptibility of cefoxitin/cefotetan resistant *Bacteroides fragilis* group strains to various other antimicrobial agents. J. Antimicrob. Chemother. **26:**353–359.
2. **Ambler, R. P., A. F. W. Coulson, J. Frere, J. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–270.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Bush, K.** 1989. Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob. Agents Chemother. **33:**271–276.
5. **Chan, P. I.** 1986. Nucleotide sequence of the *Staphylococcus aureus* PC1 β-lactamase gene. Nucleic Acids Res. **14:**5940.
6. **Collatz, E., R. Labia, and L. Gutmann.** 1990. Molecular evolution of ubiquitous beta-lactamases towards extended-spectrum enzymes active against newer beta-lactam antibiotics. Mol. Microbiol. **4:**1615–1620.
7. **Couture, F., J. Lachapelle, and R. C. Levesque.** 1992. Phylogeny of LCR-1 and OXA-5 with class A and class D β-lactamases. Mol. Microbiol. **6:**1693–1705.
8. **Cuchural, G. J., F. P. Tally, J. Storey, and M. H. Malamy.** 1986. β-Lactamase-mediated imipenem resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **30:**645–648.
9. **Cuchural, G. J., Jr., F. P. Tally, N. V. Jacobus, P. K. Marsh, and J. W. Mayhew.** 1983. Cefoxitin inactivation by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **24:**936–940.
10. **Cuchural, G. J., Jr., F. P. Tally, J. R. Storey, and M. H. Malamy.** 1986. Transfer of β-lactamase-associated cefoxitin resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **29:**918–920.
11. **Dehottay, P., J. Dusart, F. De Meester, B. Joris, J. Van Beeumen, T. Erpicum, J. Frere, and J. Ghuysen.** 1987. Nucleotide sequence of the gene encoding *Streptomyces albus* G β-lactamase precursor. Eur. J. Biochem. **166:**345–350.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Edwards, R., and D. Greenwood.** 1992. An investigation of β-lactamases from clinical isolates of *Bacteroides* species. J. Med. Microbiol. **36:**89–95.
14. **Foweraker, J. E., P. M. Hawkey, J. Heritage, and H. W. Van Landuyt.** 1990. Novel β-lactamase from *Capnocytophaga* sp. Antimicrob. Agents Chemother. **34:**1501–1504.
15. **Guthrie, E. P., and A. A. Salyers.** 1986. Use of targeted insertional mutagenesis to determine whether chondroitin lyase II is essential for chondroitin sulfate utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. **166:**966–971.
16. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
17. **Herzberg, O.** 1991. Refined crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.0 Å resolution. J. Mol. Biol. **217:**701–719.
18. **Houba, S., S. Willem, C. Duez, C. Molitor, J. Dusart, J. Frere, and J. Ghuysen.** 1989. Nucleotide sequence of the gene encoding the active-site serine β-lactamase from *Actinomadura* R39. FEMS Microbiol. Lett. **65:**241–246.
19. **Huang, L., and C. W. Forsberg.** 1987. Isolation of a cellodextrinase from *Bacteroides succinogenes*. Appl. Environ. Microbiol. **53:**1034–1041.
20. **Hussain, M., F. I. J. Pastor, and J. O. Lampen.** 1987. Cloning and sequencing of the *blaZ* gene encoding β-lactamase III, a lipoprotein of *Bacillus cereus* 569/H. J. Bacteriol. **169:**579–586.
21. **Jacob, F., B. Joris, S. Lepage, J. Dusart, and J. Frere.** 1990. Role of the conserved amino acids of the 'SDN' loop (Ser130, Asp131 and Asn132) in a class A β-lactamase studied by site-directed mutagenesis. Biochem. J. **271:**399–406.
22. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
23. **Laemmli, U. K.** 1992. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
25. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
26. **Parker, A. C.** 1992. M. S. thesis. East Carolina University, Greenville, N.C.
27. **Pastor, N., D. Pinero, A. M. Valdes, and X. Soberon.** 1990. Molecular evolution of class A beta-lactamases: phylogeny and patterns of sequence conservation. Mol. Microbiol. **4:**1957–1965.
28. **Seoane, A., and J. M. G. Lobo.** 1991. Nucleotide sequence of a new class A β-lactamase gene from the chromosome of *Yersinia enterocolitica*: implications for the evolution of class A β-lactamases. Mol. Gen. Genet. **228:**215–220.
29. **Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers.** 1986. Tn*4351* transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. J. Bacteriol. **165:**929–936.
30. **Sloma, A., and M. Gross.** 1983. Molecular cloning and nucleotide sequence of the type I β-lactamase gene from *Bacillus cereus*. Nucleic Acids Res. **11:**4947–5004.
31. **Smith, C. J.** 1985. Characterization of *Bacteroides ovatus* plasmid pBI136 and structure of its clindamycin resistance region. J. Bacteriol. **161:**1069–1073.
32. **Smith, C. J.** 1989. Clindamycin resistance and the development of genetic systems in the *Bacteroides*. Dev. Ind. Microbiol. **30:**23–33.
33. **Smith, C. J.** 1992. Unpublished data.
34. **Smith, C. J., and D. R. Callihan.** 1992. Analysis of rRNA restriction fragment length polymorphisms from *Bacteroides* spp. and *Bacteroides fragilis* isolates associated with diarrhea in humans and animals. J. Clin. Microbiol. **30:**806–812.
35. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43:**77–90.
36. **Tally, F. P., and G. J. Cuchural.** 1988. Antibiotic resistance in anaerobic bacteria. J. Antimicrob. Chemother. **22**(Suppl. A)**:**63–71.
37. **Tally, F. P., G. J. Cuchural, N. V. Jacobus, S. L. Gorbach, K. Aldridge, T. Cleary, S. M. Finegold, G. Hill, P. Iannini, P. O'Keefe, and C. Pierson.** 1985. Nationwide study of the susceptibility of the *Bacteroides fragilis* group in the United States. Antimicrob. Agents Chemother. **28:**675–677.
38. **Tally, F. P., G. J. Cuchural, N. V. Jocobus, S. L. Gorbach, K. E. Aldridge, T. J. Cleary, S. M. Finegold, G. B. Hill, P. B. Iannini, R. V. McCloskey, J. P. O'Keefe, and C. L. Pierson.** 1983. Susceptibility of the *Bacteroides fragilis* group in the United States in 1981. Antimicrob. Agents Chemother. **23:**536–540.
39. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* β-lactamase II. J. Bacteriol. **172:**2584–2593.
40. **Weisburg, W. G., Y. Oyaizu, H. Oyaizu, and C. R. Woese.** 1985. Natural relationship between *Bacteroides* and flavobacteria. J. Bacteriol. **164:**230–236.
41. **Wexler, H. M., and S. Halebian.** 1990. Alterations to the penicillin-binding proteins in the *Bacteroides fragilis* group: a mechanism for non-β-lactamase mediated cefoxitin resistance. J. Antimicrob. Chemother. **26:**7–20.
42. **Yotsuji, A., S. Minami, H. Kakizawa, T. Yasuda, A. Takai, I. Saikawa, M. Inoue, and S. Mitsuhashi.** 1985. Cephamycin inactivation due to enzymatic hydrolysis by β-lactamase from *Bacteroides fragilis*. Antimicrob. Agents Chemother. **28:**773–777.