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Cloning and Characterization of the Endogenous Cephalosporinase Gene, *cepA*, from *Bacteroides fragilis* Reveals a New Subgroup of Ambler Class A β-Lactamases

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Bacteroides fragilis **CS30 is a clinical isolate resistant to high concentrations of benzylpenicillin and cephaloridine but not to cephamycin or penem antibiotics. β-Lactam resistance is mediated by a chromosomally encoded cephalosporinase produced at a high level. The gene encoding this β-lactamase was cloned from genomic libraries constructed in *Escherichia coli* and then mated with *B. fragilis* 638 for identification of ampicillin-resistant (Ap^r) strains. Ap^r transconjugants contained a nitrocefin-reactive protein with the physical and enzymatic properties of the original CS30 isolate. The β-lactamase gene (*cepA*) was localized by deletion analysis and subcloned, and its nucleotide sequence was determined. The 903-bp *cepA* open reading frame encoded a 300-amino-acid precursor protein (predicted molecular mass, 34,070 Da). A β-lactamase-deficient mutant strain of *B. fragilis* 638 was constructed by insertional inactivation with the *cepA* gene of CS30, demonstrating strict functional homology between these chromosomal β-lactamase genes. An extensive comparison of the CepA protein sequence by alignment with other β-lactamases revealed the strict conservation of at least four elements common to Ambler class A. A further comparison of the CepA protein sequence with protein sequences of β-lactamases from two other *Bacteroides* species indicated that they constitute their own distinct subgroup of class A β-lactamases.**

Bacteroides fragilis is responsible for approximately half of all human anaerobic infections and is the most common anaerobe recovered from clinical specimens (12). Numerous reports of *B. fragilis* isolates resistant to a variety of β-lactam antibiotics indicate that these organisms are becoming increasingly refractory to treatment with these drugs. The primary mechanism of β-lactam resistance in *Bacteroides* species is the production of β-lactamase (40). At least four types of β-lactamase have been described for members of the *B. fragilis* group, but the most common type is a constitutively produced, chromosomally encoded cephalosporinase having no activity against cefoxitin or imipenem. This "endogenous" β-lactamase is present in over 90% of clinical isolates tested (10). Unlike the class C chromosomally encoded β-lactamases of members of the family *Enterobacteriaceae*, the *B. fragilis* β-lactamase has an isoelectric point in the acid range and is susceptible to inhibition by clavulanic acid and sulbactam, placing it in group 2e in the Bush classification scheme (8).

Regulation of the endogenous *B. fragilis* β-lactamase has not been extensively studied, but this enzyme may be growth rate regulated, with maximal activity occurring 3 h into the stationary phase (7). With regard to the production of the endogenous cephalosporinase, others have grouped *B. fragilis* clinical isolates into three expression classes (18). Low-level β-lactamase producers are susceptible to all β-lactams, the MICs of benzylpenicillin and cephaloridine being <2 and <16 μg/ml, respectively. For intermediate-level β-lactamase producers, the most frequently encountered group, the MICs of benzylpenicillin and cephaloridine are 16 and 32 μg/ml, respectively. High-level β-lactamase producers are resistant to both drugs at >256 μg/ml. In all cases, the levels of β-lactamase produced correlate closely with MICs of both cephalosporins and penicillins (18).

In 1977, it was shown that 87% of all *B. fragilis* strains tested produced small amounts of the endogenous cephalosporinase constitutively and that 6% produced large amounts (42). More recent surveys have shown that at least 90% of all *B. fragilis* group strains produce β-lactamase and that 25% produce high levels (10). The existence of these classes and the increasing frequency of isolation of high-level β-lactamase-producing strains suggest a trend towards high-level β-lactamase production in *Bacteroides* species.

As a first step in identifying possible differences in the regulation of β-lactamase production between expression classes, we have cloned and sequenced the chromosomal cephalosporinase gene (*cepA*) from a high-level β-lactamase-producing clinical isolate, *B. fragilis* CS30. In contrast to the class C chromosomal β-lactamases of members of the family *Enterobacteriaceae*, we present evidence that CepA is a β-lactamase containing at least four amino acid motifs characteristic of the class A active-site serine β-lactamases described by Ambler (1). A comparison of the CepA amino acid sequence with those of other class A β-lactamases, including two from other *Bacteroides* species, indicates that the enzymes from *Bacteroides* species are most closely related to each other, forming a distinct homology group that is significantly different from other class A enzymes.

MATERIALS AND METHODS

Strains and media. The *B. fragilis* clinical isolates used in this study are listed in Table 1. *B. fragilis* 638 (made resistant to rifampin [47]) and *B. uniformis* 1001 (MICs of ampicillin and cephaloridine, 16 and 32 μg/ml, respectively) (51) were the standard recipient strains. *Bacteroides* strains were grown at 37°C anaerobically in supplemented brain heart

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TABLE 1. β-Lactamase activities of and MICs for cefoxitin-susceptible *B. fragilis* strains[a]

B. fragilis strain	MIC (μg/ml) of:		β-Lactamase activity (U/mg of protein)[b] against:			Origin and yr of original isolation[c]
	Cephaloridine	Ampicillin	Nitrocefin	Cephaloridine	Penicillin G	
CS29	32	8	0.004	0.007	<0.001	PCMH, 1989
CS14	128	16	0.006	0.013	<0.001	PCMH, 1989
638	128	16	0.007	0.015	<0.001	MS, 1974
ATCC 25285	128	32	0.013	0.025	<0.001	ATCC, 1955
CS44	256	32	0.010	0.025	<0.001	PCMH, 1990
RBF49	750	500	0.110	0.86	0.014	URAML, 1979
RBF43	750	500	0.150	0.836	0.009	URAML, 1979
CS30	1,000	750	0.230	1.229	0.012	PCMH, 1990
RBF103	1,250	750	0.270	1.336	0.028	URAML, 1981

[a] Cefoxitin MICs were ≤16 μg/ml. All strains contained a single β-lactamase with an isoelectric point of 4.9.

[b] β-Lactamase was measured in crude cell extracts with the indicated substrate. All β-lactamase activities were inhibited by >50% by 1 μM clavulanate and 1 μM cefoxitin.

[c] PCMH, D. Callihan, Pitt County Memorial Hospital, Greenville, N.C.; ATCC, American Type Culture Collection; URAML, University of Rochester Anaerobic Microbiology Laboratory; MS, M. Sebald, Pasteur Institute, Paris, France.

infusion broth as described previously (53). Antibiotic MICs were measured by the standard agar dilution method with Wilkins-Chalgren agar (Difco Laboratories, Detroit, Mich.). Values were determined after 48 h of growth. The following antibiotic concentrations were used unless noted otherwise: clindamycin, 5 μg/ml; tetracycline, 5 μg/ml; rifampin, 20 μg/ml; gentamicin, 25 μg/ml; and ampicillin, 50 μg/ml. *Escherichia coli* DH5αMCR (F^- *mcrA deoR recA1 endA1 relA1 lac*) (GIBCO/BRL, Inc., Gaithersburg, Md.) was grown aerobically in Luria-Bertani broth (agar) supplemented with 50 μg of ampicillin per ml, 50 μg of spectinomycin per ml, or 50 μg of kanamycin per ml as appropriate.

Bacterial conjugation. Standard filter mating protocols were used to transfer plasmids in triparental matings from *E. coli* donors to *Bacteroides* recipients. The *E. coli* donors contained helper plasmid RK231, and the filters were incubated aerobically (50).

DNA isolation and analysis. A salt (high concentration)-sodium dodecyl sulfate (SDS) lysis method was used to screen *Bacteroides* strains for plasmid content (61). Purified plasmid DNA preparations from *Bacteroides* strains were obtained by CsCl-ethidium bromide ultracentrifugation of lysates prepared by alkaline denaturation (53). Genomic DNAs from *Bacteroides* strains were prepared as described previously (55). Screening and large-scale preparations of plasmid DNAs from *E. coli* transformants were performed by the alkaline lysis method (5). Plasmids were analyzed by agarose gel electrophoresis with Tris-borate or Tris-acetate buffer containing ethidium bromide. Restriction endonuclease digestion was performed according to supplier instructions. Other routine DNA manipulations, such as ligation and Klenow reactions, have been described elsewhere (36).

Southern hybridization analysis was performed on genomic DNA samples digested with the appropriate restriction endonuclease and electrophoresed on 0.8% agarose gels. DNA was transferred to nylon membranes (Hybond N; Amersham Corp., Arlington Heights, Ill.) by either capillary action or vacuum (Hoefer Scientific Instruments, San Francisco, Calif.). 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 with 3× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0])–4× Denhardt's solution (1× Denhardt's solution contains, per liter, 0.2 g each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin [Pentex fraction V; Miles Laboratories])–50 μg of yeast RNA per ml. Blots prepared on nylon membranes were washed in 0.1× SSC–0.1% SDS for 30 min each at room temperature, 50°C, and 65°C (high stringency). A lower stringency was achieved by omitting the 65°C wash.

Nucleotide sequence analysis of the *cepA* gene was performed by first cloning the 2,900-bp *Bam*HI-*Bgl*II fragment of pFD396 into pUC19 and then creating nested sets of deletions in both orientations as previously described (25). The forward primer of pUC19 was used in sequencing reactions with double-stranded DNA templates and modified T7 polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio). Reaction mixtures were analyzed on 6% polyacrylamide gels containing urea.

Library and plasmid constructions. A library of CS30 was prepared by partial digestion of genomic DNA with *Sau*3AI. Fragments were separated on sucrose density gradients, and fractions of between 5 and 15 kb were isolated, pooled, and ligated to the *Bgl*II site of pJST61.kan (Kn^r in *E. coli* strains and Cc^r in *Bacteroides* strains). This vector was a derivative of pJST61 (58) in which the *bla* gene was replaced with a kanamycin resistance cassette (on a 1,486-bp *Cla*I fragment) (59). Ligation mixtures were transformed into *E. coli* DH5αMCR. Transformants resistant to 30 μg of kanamycin per ml were scraped from plates and either flash frozen or used immediately in aerobic filter matings with *B. fragilis* 638. Subcloning of the Ap^r gene was performed with pFD288 (8.8 kb; *oriT* Sp^r in *E. coli* strains and Cc^r in *Bacteroides* strains [56]). Suicide vector pFD434 was derived from pFD280 (56) by insertion of the *Bacteroides tetQ* gene (38) into the unique *Sst*I site. The construct used for insertional inactivation of *cepA*, pFD439, contained the 400-bp *Hha*I-*Eco*RI fragment of *cepA* (bp 502 to 902) ligated into the *Sma*I site of pFD434.

Computer analysis. Computer analysis of nucleotide and amino acid sequence data was performed with University of Wisconsin Genetics Computer Group DNA sequence analysis software (16). The FastA program was used to search the GenBank-EMBL data base for protein sequences having similarity to CepA. The Pileup program was used to generate the progressive multiple alignment of the β-lactamase protein sequences. Individual comparisons to the CepA amino acid sequence were made by use of the GAP program with a gap weight of 3.0 and a gap length weight of 0.1. The Testcode program was used to identify protein coding sequences within open reading frame (ORF) 1 (ORF1). Phylogenetic relationships were inferred from the multiple sequence alignment by the parsimony method with the PHYLIP phylogeny inference package (version 3.5), and a consensus tree was constructed from 100 bootstrap estimates of the tree (19).

The *cepA* nucleotide sequence has been submitted to GenBank and assigned accession number L13472. Other sequences used for the analysis, together with their designations and GenBank accession numbers, are as follows: *Staphylococcus aureus* (PC1), M15526 (9); *Yersinia enterocolitica* (YER), X57074 (49); *Bacillus cereus* β-lactamase III (BCIII), M15195 (29); *B. cereus* β-lactamase I (BCI), X06599 (52); *Actinomadura* sp. strain R39 (ACT), X53650 (26); *Streptomyces albus* G(ALBUS), M28303 (15); *S. aureofaciens* β-lactamase, X13597; *E. coli* TEM-1 from pBR322 (TEM-1), VB0001 (57); *B. vulgatus* CLA341 (CFXA), M72418 (45); and *B. uniformis* 7088 (CBLA), L08472 (54). Other designations and accession numbers used (see Fig. 5) are as follows: *Klebsiella oxytoca* E23004 (KOXY), M27459 (4); *S. fradiae* DSM40063 (FRAD), M34179 (20); *K. pneumoniae* LEN-1 (LEN-1), X04515 (3); *K. ozaenae* pBP60-1-2 SHV-2 β-lactamase (SHV-2), X53433 (46); *B. licheniformis* PenP penicillinase (BLIP), J01545 (37); *E. coli* RGN238 (Tn*2603*) OXA-1 β-lactamase (OXA1), J02967 (44); *Salmonella typhimurium* type 1a R46 OXA-2 β-lactamase (OXA2), M25261 (14); and *Pseudomonas aeruginosa* PU21 R151 (Tn*1404*) PSE-2 β-lactamase (PSE2), J03427 (27).

Analysis and purification of β-lactamase. Cell extracts were prepared by use of a French pressure cell from mid- to late-logarithmic-phase cells and tested for β-lactamase activity with nitrocefin as described previously (41, 45). The degradation of cephaloridine and benzylpenicillin was determined by monitoring the decrease at 260 and 233 nm, respectively (45). Specific activity is expressed as the number of micromoles of substrate consumed per minute per milligram of protein. Protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as the standard. The cellular location of β-lactamase activity was determined by an osmotic shock method as described previously (45).

Isoelectric focusing was performed by loading cell extracts onto polyacrylamide gels containing 2% ampholytes at a pH range of 3.0 to 10.0. Predetermined pI markers were used as standards. Gels were run at 5°C and 25 W for 1.5 h. The gradient was measured after electrophoresis by use of a flat-end surface electrode, and β-lactamases were visualized by overlaying the polyacrylamide gels with 0.8% agarose in 20 mM sodium phosphate buffer (pH 7.0) containing 50 μg of nitrocefin per ml and photographed with a green filter.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed as described previously (35). β-Lactamase proteins were identified in gels by activity staining with nitrocefin (21, 45). The estimated molecular weight of the CS30 β-lactamase was determined by SDS-PAGE, by comparison with appropriate standards.

The CS30 β-lactamase was purified as follows. Crude cell extracts were clarified by centrifugation at 115,000 × *g* for 2 h (4°C), and the supernatant was applied to a DEAE–Bio-Gel column (5 by 10 cm; Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 5 column volumes of 50 mM sodium phosphate buffer (pH 7), and protein was eluted with a linear 0 to 0.3 M NaCl gradient in the same buffer. Active fractions were pooled, concentrated by ultrafiltration (10,000-molecular-weight cutoff), dialyzed against 25 mM histidine-HCl buffer (pH 6.2), and applied to a PBE 94 chromatofocusing column (1 by 42 cm; Pharmacia LKB, Uppsala, Sweden). The column was developed with pH 4.0 Polybuffer 74-HCl, and active fractions were eluted in the pH range of 4.6 to 5.2. Active fractions were concentrated as described above and then dialyzed against 1.4 M NH_2SO_4 in 0.1 M sodium phosphate buffer (pH 7.2). The resulting material was applied to a SynChropak Propyl hydrophobic-interaction high-pressure liquid chromatography column (250 by 4.6 mm; SynChrom, Inc., Lafayette, Ind.), and active fractions were eluted with a reverse-phase 2 to 0 M NH_2SO_4 gradient. The resulting β-lactamase fractions were then pooled and purified to apparent homogeneity by preparative SDS-PAGE on a 10-cm gel (model 491 Prep Cell; Bio-Rad). The purified protein was submitted to the UCLA Protein Microsequencing Facility for analysis of the N-terminal amino acid sequence and total amino acid composition. The total amino acid composition analysis did not identify tryptophan or cysteine residues and did not differentiate between glutamate and glutamine or aspartate and asparagine.

RESULTS

Characterization of clinical strains. β-Lactam MICs and β-lactamase specific activities were determined for 39 clinical *B. fragilis* (sensu strictu) strains and compared with those for the type strain, *B. fragilis* ATCC 25285. Representative strains are listed in Table 1. Cell extracts from all of these strains were able to hydrolyze the cephalosporins nitrocefin and cephaloridine and displayed only weak activity against penicillin, indicating that the β-lactamases were cephalosporinases. β-Lactamase specific activities ranged from 0.004 to 0.270 (67.5-fold) with nitrocefin as a substrate, and all β-lactamases were susceptible to inhibition by clavulanate and cefoxitin, as expected for the *Bacteroides* endogenous cephalosporinases. Furthermore the enzymatic activities for all strains comigrated on SDS gels (mass, 31,500 Da) and isoelectric focusing gels (pI, 4.9). For both ampicillin and cephaloridine, there was a high correlation (R = 0.96) between β-lactamase activities and MICs. On the basis of these strain characteristics, strains were placed into two expression classes: low (specific activity, 0.004 to 0.013 U/mg of protein) and high (specific activity, 0.110 to 0.270 U/mg of protein). Attempts to induce low-level β-lactamase-producing strains to express higher β-lactamase activities were unsuccessful, and no spontaneous mutants to a high-level phenotype were detected.

Cloning of the cephalosporinase gene, *cepA*. Because antibiotic resistance genes from *Bacteroides* strains are poorly expressed or are not expressed in *E. coli* (22), it was important to use a cloning strategy that would select for the gene in *Bacteroides* strains. This was accomplished by use of a derivative of the positive selection vector pJST61 (58). pJST61 was modified by replacing the TEM-1 β-lactamase gene with a kanamycin resistance determinant (59), because the level of β-lactamase activity was high enough to interfere with the selection of *B. fragilis* transconjugants. CS30 was chosen as the source of the DNA, because it produced the cephalosporinase at a high level, facilitating selection. A genomic library of approximately 7,650 Kn^r *E. coli* colonies was conjugated with *B. fragilis* 638, and transconjugants selected on Wilkins-Chalgren agar containing rifampin, gentamicin, and clindamycin were tested for growth on plates containing ampicillin (150 μg/ml). A total of 24 *B. fragilis* 638 clones were obtained. The stability of the Ap^r phenotype was demonstrated by the ability to be restreaked onto medium containing rifampin, gentamicin, clindamycin, and ampicillin (150 μg/ml). Several clones were examined in

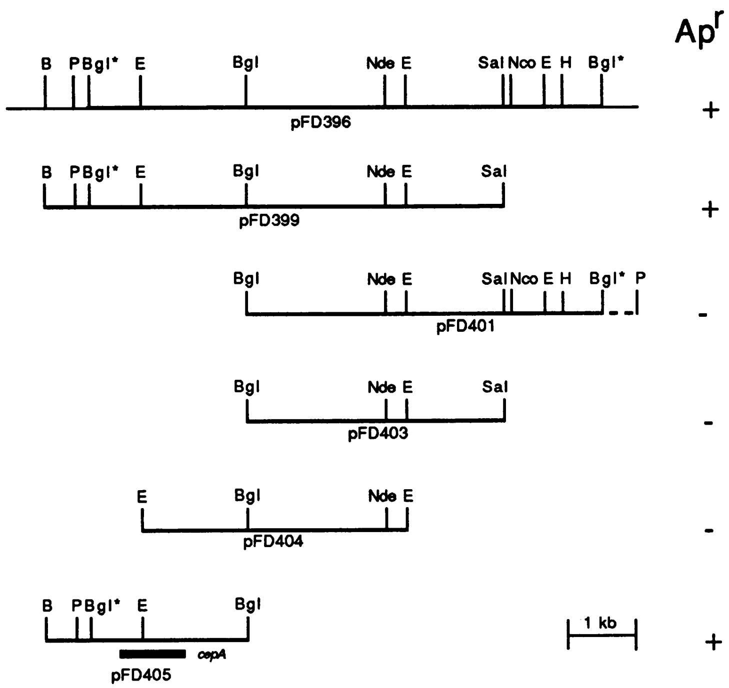


FIG. 1. Restriction endonuclease maps of clones containing *cepA* gene fragments. pFD396 was the original 6.65-kb insert cloned into pJST61.kan, and all other plasmids were constructed in shuttle vector pFD288 as described in the text. Ap^r denotes the phenotype of each plasmid in *B. fragilis* 638 or *B. uniformis* 1001. The junction between the CS30 insert fragment and pJST61.kan is indicated by an asterisk. The bar under pFD405 represents the location of the *cepA* ORF. Restriction endonuclease abbreviations: B, *Bam*HI; P, *Pst*I; E, *Eco*RI; H, *Hin*dIII.

more detail; β-lactamase specific activities ranged from 0.149 to 0.506 U/mg of protein, and ampicillin MICs for all the clones were >800 μg/ml. Plasmid DNAs from these clones were isolated, transformed into *E. coli*, and analyzed by digestion with *Sau*3AI. These plasmids shared many common fragments, and one clone, pFD396, with a 6.65-kb chromosomal DNA insert, was chosen for further study. *E. coli* donors containing pFD396 could transfer the Ap^r phenotype to *B. fragilis* 638, whereas donors containing pJST61.kan alone could not confer Ap^r. The genetic locus for this β-lactamase gene was designated *cepA* (cephalosporinase), and the location of this gene within the CS30 DNA insert is shown in Fig. 1.

Localization of the *cepA* gene. The *B. fragilis cepA* gene was not phenotypically expressed in *E. coli*; therefore, all plasmid constructs were first made in *E. coli* and then transferred into *Bacteroides* strains to test for expression. Partial restriction maps of subfragments cloned from pFD396 are shown in Fig. 1. Each construct was tested for the ability to confer Ap^r to either *B. fragilis* 638 or *B. uniformis* 1001, as measured by selection on Wilkins-Chalgren agar containing 300 μg of ampicillin per ml. These experiments revealed that the subcloned gene was unstable in *B. fragilis* 638, and deletions often occurred. However, these subclones were stable in *B. uniformis* 1001, which does not contain a copy of *cepA* in its chromosome. Constructs pFD401, pFD403, and pFD404 did not confer Ap^r, suggesting that an essential portion of the gene was located beyond the first *Eco*RI site. The 2,485-bp *Pst*I-*Bgl*II fragment, which encompasses this *Eco*RI site, was sufficient to confer Ap^r to both *B. fragilis* 638 and *B. uniformis* 1001.

Evidence that the cloned *cepA* gene encoded the CS30 β-lactamase was obtained by isoelectric focusing analysis. Figure 2a shows an isoelectric focusing gel of β-lactamases from several *cepA* clones and *B. fragilis* CS30, ATCC 25285, and 638. Each of the wild-type strains showed only a single nitrocefin-reactive band, and all bands comigrated with a pI of about 4.9, in agreement with previous studies (43). Strains containing the cloned gene (lanes B to D) also produced a β-lactamase focusing at the same pI as the CS30 enzyme. Although *B. fragilis* 638 and ATCC 25285 also produced the endogenous CepA β-lactamase, the amount produced was much smaller, and 6- to 20-fold more total protein was required to visualize an activity band (lanes E and F).

Distribution of *cepA* among *Bacteroides* species. The distribution of *cepA* sequences among other *Bacteroides* strains was examined by Southern hybridization analysis. Figure 2b shows an autoradiograph of a Southern blot containing chromosomal DNAs from several *B. fragilis* strains, restricted to completion with *Eco*RI-*Hin*dIII, blotted onto nylon membranes, and hybridized with a DNA probe encompassing the entire *cepA* structural gene (bp 407 to 1518). The probe hybridized to chromosomal DNAs from all *B. fragilis* strains tested. The *B. fragilis* strains shown represent isolates from both β-lactamase expression classes, as well as a penicillinase-producing isolate, RBF78 (7), and a cefoxitin-resistant isolate, V503. In general, members of the low-level expression class shared common hybridizing fragments of 0.62 and 4.0 kb. For example, *B. fragilis* CS44, ATCC 25285, CS14, CS29, BF-2 (55), TM4000 (identical to strain 638), and V503 (55) shared these two common fragments (with a slight restriction fragment length polymorphism in the larger BF-2 fragment); members of the high-level expression class also shared common fragments in addition to the 4.0-kb fragment, but some variations were observed, most notably the presence of an additional 2.0-kb band and/or a 5.4-kb band.

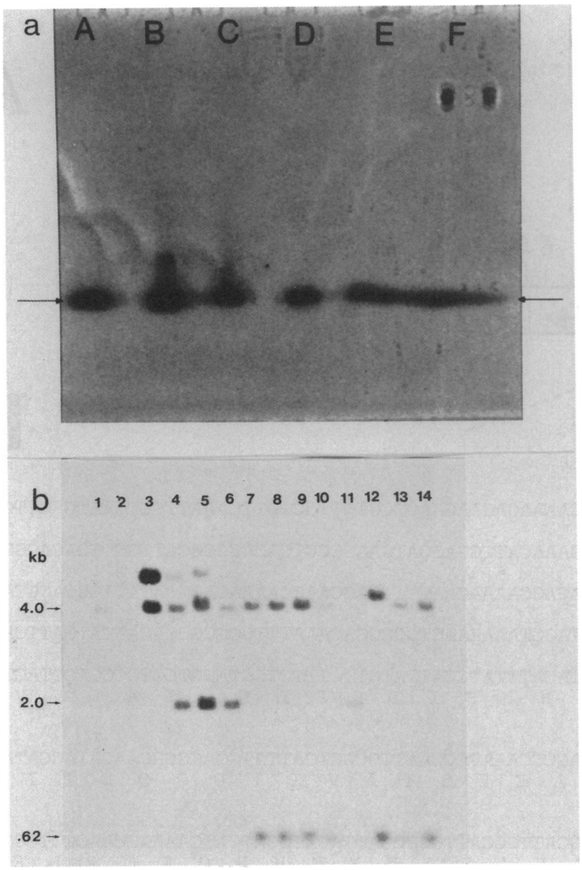


FIG. 2. Presence of CepA β-lactamase and *cepA* in wild-type strains of *B. fragilis*, as detected by isoelectric focusing and Southern blot analysis. (a) Isoelectric focusing gel (pH 3 to 10) of *Bacteroides* cell extracts showing nitrocefin-reactive proteins. The strain and total amount of protein applied to the gel were as follows: A, *B. fragilis* CS30, 8 μg; B, *B. fragilis* 638(pFD396), 16 μg; C, 638(pFD399), 8 μg; D, *B. uniformis* 1001(pFD405), 15 μg; E, *B. fragilis* ATCC 25285, 152 μg; F, *B. fragilis* 638, 92 μg. The arrows indicate the location of the gradient at which the pH was 4.9. (b) Autoradiograph of a Southern blot containing *Bacteroides* chromosomal DNAs, illustrating the distribution of *cepA* among different *B. fragilis* strains. Lanes: 1, 638; 2, *B. uniformis* 7088; 3, CS30; 4, RBF43; 5, RBF103; 6, RBF49; 7, CS44; 8, ATCC 25285; 9, CS14; 10, CS29; 11, RBF78; 12, BF-2; 13, TM4000; 14, V503.

There appears to be only one *cepA*-specific sequence in all of the *B. fragilis* strains tested, resulting in two hybridizing bands due to the internal *Eco*RI site present in *cepA* (see pFD405 in Fig. 1) but only one hybridizing band when an enzyme that does not digest within the *cepA* gene is used.

Chromosomal DNAs from other *Bacteroides* species also were tested: *B. vulgatus* WAL 7062, CLA 341, VPI B2-4, and ATCC 8482; *B. uniformis* ATCC 8492, 006-1, and 7088; *B. ovatus* ATCC 8483, VPI 4244, VPI 3524, and WAL 7606; *B. thetaiotaomicron* VPI 11111, ATCC 29148, and ATCC 29741; *B. distasonis* ATCC 8503; *B. eggerthii* VPI T3-3; and *B. caccae* ATCC 43185. No sequences in these strains hybridized to the *cepA*-specific probe at 67°C.

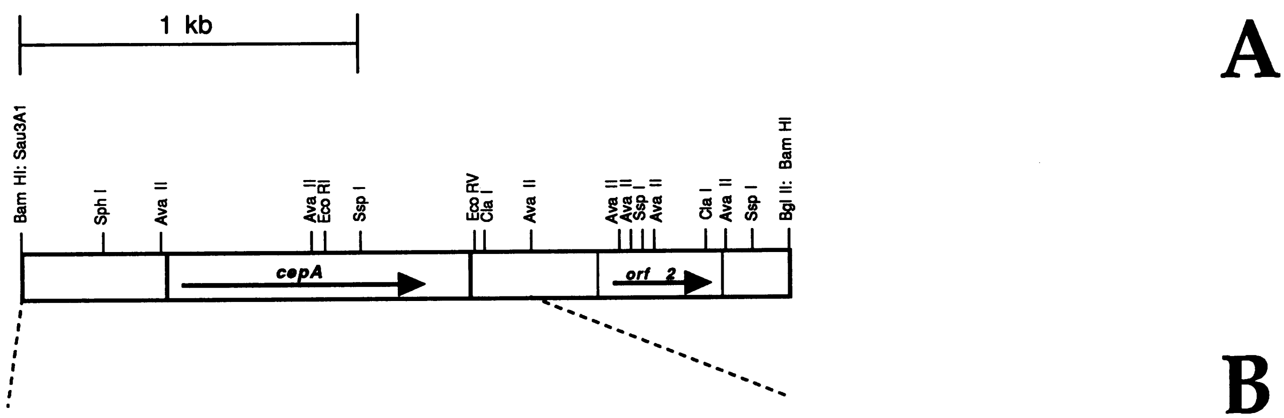
Construction and analysis of a *cepA* mutant. To establish the relationship between the cloned *cepA* gene and the endogenous *B. fragilis* β-lactamase, a mutant strain of *B. fragilis* 638 was constructed by targeted insertional inactivation (23) with suicide vector pFD439. A central portion of the *cepA* structural gene was used to mediate insertion of the plasmid into the 638 chromosome via homologous recombination. All of the Tc^r 638 transconjugants were phenotypically Ap^s. Integration of the plasmid into the chromosome of *B. fragilis* 638 was detected by restricting total DNA with *Bgl*II (which does not cleave pFD439 or *cepA*) and probing blots with a ^{32}P-labeled *cepA*-specific *Sph*I-*Eco*RI fragment (bp 247 to 902). In Tc^r transconjugants, the hybridizing band increased in size 7.4 kb relative to that in 638, resulting in a 10.4-kb band in the transconjugants. Other Southern blots with different restriction endonucleases confirmed these results. Cell extracts from Tc^r transconjugants were examined on isoelectric focusing gels together with an extract from *B. fragilis* 638. The transconjugants failed to display any nitrocefin-reactive proteins, even when excess protein was loaded, indicating that they were indeed deficient for β-lactamase production.

DNA sequence analysis of *cepA*. The 2,300-bp region of the CS30 chromosomal insert was sequenced in both directions, and two ORFs were found (Fig. 3A). No other ORFs larger than 300 bp were observed in any reading frame. The assignment of *cepA* to ORF1 is consistent with the subcloning analysis and the results of data base searches described below.

The nucleotide sequence of the *cepA* coding region is shown in Fig. 3B. The moles percent G+C content of the 2,300-bp fragment was 41.6%, and that of the 903-bp *cepA* coding region was 40.5%, consistent with the 43% overall moles percent G+C content of *B. fragilis* chromosomal DNA (32). *cepA* had two possible translational start sites, ATGs at bp 328 and 436. Statistical analysis of the 2,300-bp nucleotide sequence was used to determine likely coding regions by plotting a measure of the nonrandomness of the sequence at every third base (Testcode [16]). With a 95% level of confidence, the coding region began at bp 436, and the region between bp 328 and 436 was a noncoding region. A possible Shine-Dalgarno ribosome-binding site present at bp 417 to 424 was complementary to the 3′ terminus of *B. fragilis* 16S rRNA (60) in 6 of 8 bp. On the basis of this analysis, *cepA* could encode a protein of 300 amino acids (34,070 Da). With established criteria (13), a signal peptide cleavage site after Ala-22 was predicted; this cleavage site would result in a mature protein of 278 residues with a predicted molecular mass of 31,562 Da.

Results from an analysis of the CS30 β-lactamase agreed closely with the nucleotide sequence predictions. When CS30 cells were fractionated, 87% of the total β-lactamase activity was associated with the periplasmic fraction, the remaining activity being equally divided between the cytoplasmic and extracellular fractions. SDS-PAGE revealed that this periplasmic β-lactamase had a molecular mass of 31,500, and isoelectric focusing gels showed an acid isoelectric point of about 4.9. The purified CS30 β-lactamase had identical properties, and the total amino acid composition of the purified protein was very similar to that predicted for CepA, with a cleavage site at Ala-22. Repeated attempts to obtain N-terminal sequence data from the protein were not successful because of extensive acid splitting of the sample. Although the actual Asp and Asn values were slightly higher than predicted, the total amino acid composition data for purified CepA are strong evidence supporting the hypothesis that *cepA* is the gene responsible for the production of this enzyme.

Relationship of CepA to other β-lactamases. GenBank-EMBL data base searches with the CepA protein sequence as a query revealed amino acid similarities to 33 different



B

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   1 GATCATGAAGGCCCGGCTGCTGGCCATAGACGATGCCACACTGTTCCCTTTGAAAAGAGAAGAAGCCGTGTTGCTGTTCAAACTGGTCAATGACTTCCAG

 101 GAAAGGACATCACTCATCATCACGGCAAACAAGGCACTCACCCGTTGGCTGGAAACATTGGAGGATGAAGCGGTCACAGCCGCCTTGCTTGACAGGCTGC
                                                  SphI
 201 TCTACTGCTGCGAGATTATCAGGCTCGGAGGAACAAGCTATCGCATGCAAAACAGGAAAACAATTTTTAGCAACCAAAACACGGATATAGGCACGTAAAA

 301 AGAGTTAAGGAAAGTGAAGCATCTTCGATGCTGGAGTGGGATATACTAAATTACCTAAAAAAGTGGCGCACAAATTTGCGCGCCACATTATTATTCATAC
           AvaII
 401 CTTTGTGGACCGTATTACAAAGAACCCAATCATATATGCAAAAAAGACTTATACATTTATCCATTATCTTCTTTCTGCTATGTCCTGCCCTGGTAGTTGC
                          S-D                M  Q  K  R  L  I  H  L  S  I  I  F  F  L  L  C  P  A  L  V  V  A↑
   HhaI
 501 GCAGAACAGTCCTCTTGAAACTCAACTCAAGAAAGCCATAGAAGGGAAAAAAGCCGAAATAGGAATTGCAGTCATTATCGACGGGCAAGATACGATAACA
       Q  N  S  P  L  E  T  Q  L  K  K  A  I  E  G  K  K  A  E  I  G  I  A  V  I  I  D  G  Q  D  T  I  T

 601 GTCAATAATGATATTCATTATCCCATGATGAGTGTTTTCAAATTTCATCAGGCATTGGCATTGGCCGATTACATGCATCATCAAAAGCAACCTTTGGAAA
      V  N  N  D  I  H  Y  P  M  M  S  V  F  K  F  H  Q  A  L  A  L  A  D  Y  M  H  H  Q  K  Q  P  L  E  T

 701 CCCGGTTATTGATTAAAAAGTCGGATTTAAAGCCGGACACCTATAGTCCGCTTCGAGAAACATACCCACAGGGAGGAATCGAAATGAGCATTGCCGATCT
        R  L  L  I  K  K  S  D  L  K  P  D  T  Y  S  P  L  R  E  T  Y  P  Q  G  G  I  E  M  S  I  A  D  L
                                                                             AvaII
 801 ACTGAAATATACGCTTCAGCAAAGTGACAATAATGCCTGCGATATTCTTTTTAATTATCAAGGTGGTCCGGATGCCGTGAATAAGTATCTTCATTCATTG
       L  K  Y  T  L  Q  Q  S  D  N  N  A  C  D  I  L  F  N  Y  Q  G  G  P  D  A  V  N  K  Y  L  H  S  L
    EcoRI
 901 GGAATTCGTGAATGTGCTGTCATCCATACAGAAAACGATATGCATAAGAATCTGGAGTTCTGTTACCAAAACTGGACTACTCCATTAGCAGCCGCCAAAT
      G  I  R  E  C  A  V  I  H  T  E  N  D  M  H  K  N  L  E  F  C  Y  Q  N  W  T  T  P  L  A  A  A  K  L

1001 TACTGGAAATATTTCGCAATGAAAACCTTTTTGACAAAGAATACAAGAATTTCATTTATCAAACAATGGTGGAATGTCAGACAGGACAAGACCGCCTGAT
        L  E  I  F  R  N  E  N  L  F  D  K  E  Y  K  N  F  I  Y  Q  T  M  V  E  C  Q  T  G  Q  D  R  L  I

1101 TGCTCCACTGCTCGATAAAAAAGTAACAATGGGGCATAAAACCGGAACAGGCGACCGTAATGCGAAAGGACAACAGATCGGTTGCAATGACATCGGGTTT
       A  P  L  L  D  K  K  V  T  M  G  H  K  T  G  T  G  D  R  N  A  K  G  Q  Q  I  G  C  N  D  I  G  F

1201 ATTCTTCTTCCCGACGGACATGCCTATAGTATAGCCGTCTTCGTGAAAGATTCCGAAGCAGATAACAGAGAAAACAGTGAGATTATCGCAGAAATTTCGC
      I  L  L  P  D  G  H  A  Y  S  I  A  V  F  V  K  D  S  E  A  D  N  R  E  N  S  E  I  I  A  E  I  S  R
                                                  EcoRV                     ClaI
1301 GCATCGTTTACGAATACGTAACGCAACAGATAGATTAACGCAAAGATATCAAAGAATACACCTCCGTTTACAATATCGATGCCCGATAACAAAACCGGCT
       I  V  Y  E  Y  V  T  Q  Q  I  D  *

1401 ACAGATACAACCGTTCACTATCACTATCGGTGACTTGAAAAATCCAGTATCTGTAGCCAGTTTGTAAAATCAGCTTTGTTTGTGCAACCTTTTGTTTCTA
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FIG. 3. ORFs located in the pFD405 insert fragment and nucleotide and protein sequences of the *cepA* region. (A) Restriction endonuclease map of the 2,300-bp *Bgl*II fragment containing *cepA*. Major ORFs are depicted as labeled boxes with arrows indicating the direction of transcription. (B) Nucleotide sequence of the *cepA* region, with the deduced CepA protein sequence below it. The *cepA* coding region begins at bp 436 and terminates at bp 1338, as indicated by an asterisk. The putative ribosome-binding site is underscored and labeled S-D. The vertical arrow after Ala-22 represents the predicted signal peptide cleavage site. Key restriction endonuclease sites useful for the isolation of probe fragments are also labeled. The four Ambler class A signature elements (active-site serine residues, the SDN loop, E-166, and KTG) are shown in boldface type.

```
                                                                                   *        *                      *
                           25                                                                                     69
CFXA   ................MEKNRKKQIVVLSIALVCIFILVFSLFHKSATKDSANPPLTNVLTDSISQIVSACPGEI GVAVI. .VNNRDTVKVNNKSVYPMM
CEPA   ................................MQKRLIHLSIIFFLLCPALVVAQNSPLETQLKKAIEGKKAEI GIAVI. .IDGQDTITVNNDIHYPMM
CBLA   ................................MKAYFIAILTLFTCIATVVRAQQMSELENRIDSLLNGKKATV GIAVW. TDKGDML.RYNDHVHFPLL
BCIII  ..MFVLNKFFTNSHYKKI...VPVVLLSCATLIGCSNSNTQSESNKQTNQTNQVKQENKRNHAFAKLEKEYNAKL GIYALD TSTNQTV.AYHADDRFAFA
BCI    .........LKNKRMLKIGICVGILGLSITSLEAFTGESLQVEAKEKTGQVKHKNQATHKE..FSQLEKKFDARL GVYAID TGTNQTI.SYRPNERFAFA
ACT    PDHEETHMLFPTARRTGF...AALAALALVPAAACSGSAAPAEAEPASAEVT....AEDLSGEFERLESEFDARL GVYAVD TGTGEEV.FHRADERFGYA
ALBUS  ...........VHPSTSRPSRRTLLTATAGAALAAATLVPGTAHASSGGRGHGSGSVSDAERRLAGLERASGARL GVYAYD TGSGRTV.AYRADELFPMC
YER    ..............MKHSSLRRSLLLAGITLPLVSFAL...........PAWANALPASVDKQLAELERNANGRL GVAMIN TGNGTKI.LYRAAQRFPFC
TEM-1  ...................MSIQHFRVALIPFFAAFCLPVFAHPETLV..............KVKDAEDQLGARV GYIELD LNSGKILESFRPEERFPMM
PC1    ...................MKKLIFLIVIALVLSACNSNSSHAKE................LNDLEKKYNAHI GVYALD TKSGKEV.KFNSDKRFAYA
                           25                                                                                     69
                                                                                α1       β1           β2

        *  *         *                                   *                               *** * *      *          **
                                   100                                                                        150       162
CFXA   SVFK VHQA LALC NDFDNKGISLDTLVNINRDKLDPK.TW SPMLKDY SGPVI.S LTVRDL LRYTLTQ SDNNASNLMFKDMVNV AQTDSFIATLIPRSSFQI
CEPA   SVFK FHQA LALA DYMHHQKQPLETRLLIKKSDLKPD.TY SPLRETY PQGGI.E MSIADL LKYTLQQ SDNNACDILFNYQGGP DAVNKYLHSL.GIRECAV
CBLA   SVFK FHVA LAVL DKMDKQSISLDSIVSIKASQMPPN.TY SPLRKKF PDQDF.T ITLREL MQYSISQ SDNNACDILIEYAGGI KHINDYIHRL.SIDSFNL
BCIII  STSK SLAV GALL RQNSIEA..LDERITYTRKDL...SNY NPITEKH ...VDTG MTLKEL ADASVRY SDSTAHNLILKKLGGP SAFEKILREM.GDTVTNS
BCI    STYK ALAA GVLL QQNSIDS..LNEVITYTKEDL...VDY SPVTEKH ...VDTG MKLGEI AEAAVRS SDNTAGNILFNKIGGP KGYEKALRHM.GDRITMS
ACT    STHK AFTA ALVL GQNTPEE..LEEVVTYTEEDL...VDY SPITEQH ...VDTG MTLLEV ADAAVRH SDNTAANLLFEELGGP EGFEEDMREL.GDDVISA
ALBUS  SVFK TLSS AAVL RDLDRNGEFLSRRILYTQDDVEQADGA GPETGKP QNLANAQ LTVEEL CEVSITA SDNCAANLMLRELGGP AAVTRFVRSL.GDRVTRL
YER    STFK FMLA AAVL DQSQSQPNLLNKHINYHESDL...LSY APITRK. .NLAHG. MTVSEL CAATIQY SDNTAANLLIKELGGL AAVNQFARSI.GDQMFRL
TEM-1  STFK VLLC GAVL SRVDAGQEQLGRRIHYSQNDL...VEY SPVTEKH ...LTDG MTVREL CSAAITM SDNTAANLLLTTIGGP KELTAFLHNM.GDHVTRL
PC1    STSK AINS AILL EQVPYNK..LNKKVHINKDDI...VAY SPILEKY ...VGKD ITLKAL IEASMTY SDNTANNKIIKEIGGI KKVKQRLKEL.GDKVTNP
                                   100                                                                        150       162
            α2                                     α3                 α4                α5              α6

         * *  *          **                    *          *                                        ** *
                  175                        200                                                                                   255
CFXA   A YTEEEMS ADHNKAYS NYTSP LGAAMLMNRLFTEGLIDDEKQSFIKNTLKECKTGVDR IAAP LLDKEG VVIAHKTGSG YVNENGVLAAH NDVAYIC. LPN
CEPA   I HTENDMH KNLEFCYQ NWTTP LAAAKLLEIFRNENLFDKEYKNFIYQTMVECQTGQDR LIAP LLDKK. VTMGHKTGTG DRNAKGQQIGC NDIGFIL. LPD
CBLA   S ETEDGMH SSFEAVYR NWSTP SAMVRLLRTADEKELFSKELKDFLWQTMIDNTETGAN KLKG MLPAK. TVVGHKTGSS DRNADGMKTAD NDAGLVI. LPD
BCIII  E RFEPELN EVNPGETH DTSTP KAIAKTLQSFTLGTVLPSEKRELLVDWMKRNTTGDKL IRAG V..PKG WEVADKTGAG ......SYGTR NDIAIIWP .PN
BCI    N RFETELN EAIPGDIR DTSTA KAIATNLKAFTVGNALPAEKRKILTEWMKGNATGDKL IRAG I..PTD WVVGDKSGAG ......SYGTR NDIAVVWP .PN
ACT    D RIETELN EVPPGETR DTSTP RAMAGSLEAFVLGDVLEEGPRDVLTEMLLNNTTGDEL IRAG V..PED WRVGDKTGGG ......SHGSR NDIAVVWP .PE
ALBUS  D RWEPELN SAEPGRVT DTTSP RAITRTYGRLVLGDALNPRDRRLLTSWLLANTTSGDR FRAG L..PDD WTLGDKTGAG ......RYGTN NDAGVTWP .PG
YER    D RWEPDLN TARPNDPR DTTTP AAMAASMNKLVLGDALRPAQRSQLAVWLKGNTTGDAT IRAG A..PTD WIVGDKTGSG ......DYGTT NDIAVLWP .TK
TEM-1  D RWEPELN EAIPNDER DTTMP AAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPL LRSA L..PAG WFIADKSGAG .....ER.GSR GIIAALGP DGK
PC1    V RYEIELN YYSPKSKK DTSTP AAFGKTLNKLIANGKLSKENKKFLLDLMLNNKSGDTL IKDG V..PKD YKVADKSGQA .....ITYASR NDVAFVYP KGQ
                  175                        200                                                                                   255
                              α7                      α8                   α9          β3                      α10

                                      290
CFXA   NISYTLAVFVKDFKGNKSQASQYVAHISAVVYSLLMQTSVKS
CEPA   GHAYSIAVFVKDSEADNRENSEIIAEISRIVYEYVTQQID..
CBLA   GRKYYIAAFVMDSYETDEDNANIIARISRMVYDAMR......
BCIII  KKPIVLSILSNHDKEDAEYDDTLIADATKIVLETLKVTNK..
BCI    RAPIIIAILSSKDEKEAIYDNQLIAEATKVIVKALR......
ACT    DDPIVIAVMSTREQEDAEFDNALVSGATEVVVEALAP.....
ALBUS  RAPIVLTVLTAKTEQDAARDDGLVADAARVLAETLG......
YER    GAPIVLVVYFTQREKDAKPRRDVLASVTKIILSQIS......
TEM-1  PSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW........
PC1    SEPIVLVIFTNKDNKSDKPNDKLISETAKSVMKEF.......
                                      290
              β4                α11
```

FIG. 4. Deduced amino acid sequence of CepA (CEPA) multiply aligned with sequences of class A β-lactamases representing various homology groups. Numbering is according to Ambler et al. (2) and is based on the PC1 sequence as a reference (9). PC1 secondary structures involving four or more residues are underlined and labeled α1 to α11 for α helices and β1 to β4 for β strands (34). Highly conserved regions are boxed, and previously identified invariant residues (11) are denoted by an asterisk over the sequence. Substitutions in the invariant residues are underlined and boldfaced. Abbreviations for each sequence are explained in Materials and Methods. The four conserved elements (34) typical of most class A β-lactamases are as follows: element 1 (ABL 70 to 73); element 2 (ABL 130 to 132); element 3 (ABL 166); and element 4 (ABL 234 to 236).

proteins, all of which were β-lactamases, most belonging to Ambler class A (1). When class A β-lactamases from other genera were compared individually with CepA, with a similarity threshold of <0.5 and minimal gapping (five or fewer gaps), the amino acid homology ranged from 19.9% identity (44.7% similarity) for the *S. aureofaciens* β-lactamase to 26.4% identity (49.6% similarity) for the *K. oxytoca* β-lactamase. Of the six non-*Bacteroides* sequences aligned to CepA with five gaps or fewer, four were β-lactamases from *Streptomyces* species. The most striking homology, however, was to two other chromosomal β-lactamases from *Bacteroides* species. CepA shared 39.9% identity (58.8% similarity) with the *B. vulgatus* CLA341 β-lactamase, CfxA (45), and 42.5% identity (65.8% similarity) with the *B. uniformis* 7088 β-lactamase, CblA (54). The degree of similarity between CepA and β-lactamases of Ambler classes B, C, and D was much smaller than that between CepA and the class A enzymes, as more gaps had to be introduced in the comparisons to detect an overall similarity. For example, the best similarity between CepA and a class B β-lactamase was to the *B. cereus* 569/H (28) enzyme (26.0% identity and 51.2% similarity with 10 gaps); for class C enzymes, the best similarity was to *E. coli* AmpC (31) (19.1% identity and 42.4% similarity with 11 gaps); and for class D enzymes, the OXA-1 β-lactamase of Tn*2603* showed the best similarity (44) (17.4% identity and 38.9% similarity with 6 gaps). Several key features of the overall protein sequence alignment shown in Fig. 4 clearly placed CepA in Ambler class A. The alignment was originally generated with at least two representatives of each major class A homology group, but five of the sequences were removed from the figure for clarity. Also included were the chromosomal β-lactamases from *B. uniformis* and *B.*

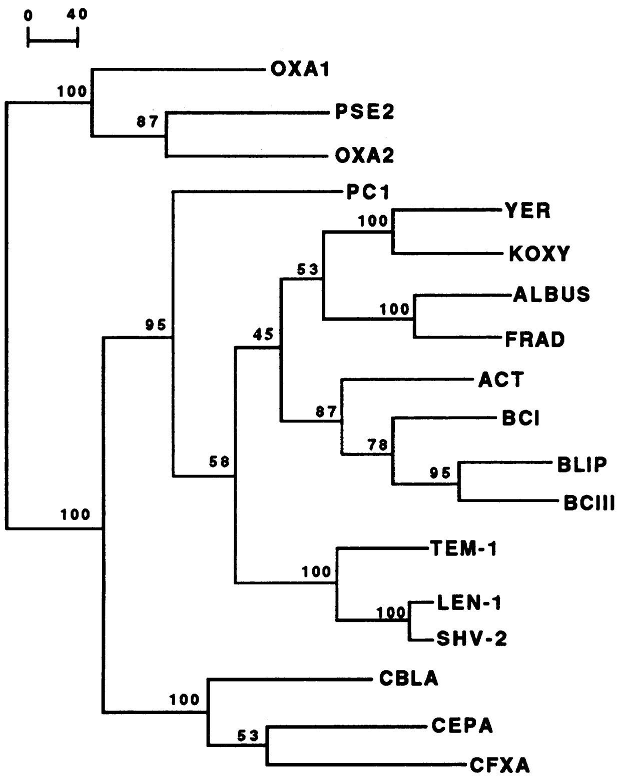

FIG. 5. Computer-generated homology distance tree illustrating the relationship among class A β-lactamases, including the *Bacteroides* enzymes and enzymes from class D for comparison. This unrooted tree was constructed from a multiple alignment similar to that shown in Fig. 4, except that OXA-1, OXA-2, and PSE-2 (class D) were included. See Materials and Methods for abbreviations and accession numbers. The tree topology was obtained by use of the parsimony program (Protpars) of Felsenstein (19), and the numbers represent how often the groups of species to the right of a fork occurred in 100 trees tested. Branch lengths are proportional to the number of amino acid changes. CEPA, CepA.

vulgatus. Our data agree closely with the extensive alignment of Couture et al. (11), which included 26 enzyme sequences. The four major class A signature elements were conserved among all of the enzymes (including the *Bacteroides* enzymes), and these were readily identified in our alignment. These elements included the consensus active-site serine residues Ser-$(X)_2$-Lys starting at residue Ser-70 (numbering according to Ambler et al. [2]), the SDN loop starting at Ser-130 (30), the conserved Glu-166 (34), and the KTG sequence starting at Lys-234 (34).

The phylogenetic relationship between the *Bacteroides* enzymes and the other class A β-lactamases was visualized as a dendogram constructed by parsimony analysis of the multiple alignment and included the class D enzymes for comparison (Fig. 5). Four subgroups were observed within the class A enzymes. The TEM-1–LEN-1–SHV-2 subgroup constitutes the plasmid- or transposon-encoded broad-spectrum β-lactamases of gram-negative bacteria; the *Actinomadura*, *Staphylococcus*, and *Bacillus* enzymes form a gram-positive subgroup; and, surprisingly, the *Streptomyces* enzymes are grouped with *K. oxytoca* and *Y. enterocolitica*. The fourth major subgroup is composed of the three *Bacteroides* enzymes, which were found in all of the bootstrap replications, clearly establishing a unique homology group within the realm of Ambler class A β-lactamases.

DISCUSSION

Recent surveys have shown that over 90% of *B. fragilis* group clinical isolates produce a species-specific chromosomally encoded β-lactamase and that 25% of these isolates produce high levels of this enzyme (10). Others have noted that isolates can be classified on the basis of the amount of β-lactamase produced (specific activity) (18), yet the basis for the differential expression of this enzyme has not been studied at the molecular level. On the basis of the criteria of ampicillin, cephaloridine, and cefoxitin MICs, the ability to hydrolyze cephaloridine and nitrocefin more rapidly than benzylpenicillin, the production of a single β-lactamase, and the β-lactamase isoelectric point, apparent molecular mass, and inhibition profile, we have described a *B. fragilis* strain set producing either high or low levels of this endogenous cephalosporinase. As a first step towards understanding β-lactamase expression in *Bacteroides* strains, we have cloned the *cepA* gene responsible for conventional cephalosporinase production from a high-level β-lactamase producer, CS30.

On the basis of the results of DNA hybridizations with *cepA*, the strain set fell into at least two definitive classes, with all of the low-level producers sharing two common fragments. The high-level producers also shared some common homologous fragments, but there seemed to be greater variability, depending on the restriction enzyme used for analysis. Additional evidence that *cepA* is the structural gene for the endogenous β-lactamase in low- and high-level producers was obtained by constructing a β-lactamase-deficient mutant of 638. The ability of the 400-bp internal portion of the CS30 *cepA* gene to mediate insertional inactivation of the *cepA* gene of *B. fragilis* 638 illustrates the functional homology of the *cepA* gene between members of different expression classes, as well as the dependence of the Ap^r phenotype on the presence of a functional copy of *cepA*.

DNA sequence analysis of the 2,300-bp *Sau*3AI-*Bgl*II fragment revealed the presence of two ORFs. ORF1 (903 bp) was designated *cepA* on the basis of its size (ORF2 is only 384 bp) and its striking similarity at the amino acid level to other β-lactamases found in the GenBank-EMBL data base. The 300-amino-acid CepA protein sequence predicted from the nucleotide sequence closely matched the purified protein sequence with regard to size and amino acid composition. A signal peptide cleavage site was predicted after Ala-22; this cleavage site would result in a mature protein of 278 amino acids (31,562 Da), a result that agrees well with the 31.5-kDa nitrocefin-reactive band seen in an SDS-PAGE analysis of the purified CS30 CepA protein. The predicted pIs of the pre-β-lactamase and the mature protein were 6.0 and 5.4, respectively. The pI of purified CepA β-lactamase or crude extracts of our clinical isolates was 4.9. It is clear that this value is closer to the predicted value for the mature form, and there may be charged amino acids buried within the enzyme that can account for the difference. Others have reported pIs of 4.9 to 5.4 for the endogenous *B. fragilis* enzyme (17, 39).

Other workers have described four conserved elements (34) and seven "boxes" (33) in alignments of class A β-lactamases. All three *Bacteroides* enzymes are identical to each other with regard to the four elements and to four of the seven boxes. With regard to individual substitutions of the 25 conserved residues identified by Couture et al. (11), only

11 of these 25 "invariant" residues are conserved in all three *Bacteroides* β-lactamases, indicating that these enzymes, while still maintaining the majority of characteristic class A elements, have diverged considerably from other class A enzymes. CepA from *B. fragilis* CS30 has 12 substitutions of the 25 invariant residues, and except for the possible conservative tyrosine-for-phenylalanine change at residue 67, none of the substitutions seem strictly conservative. Interestingly, all three *Bacteroides* enzymes have a threonine residue substituted for the normally conserved tryptophan residue (ABL Trp-210 [ABL denotes the standard numbering scheme of Ambler et al. (2)]) in box 6. Also of note is the substitution of a threonine residue in CS30 (ABL Thr-237) for the normally conserved alanine residue. It is known that the increased cephalosporinase activity of mutant H1 of the pBR322 TEM-1 β-lactamase (24) results from a change just after box 7, whereby Ala-237 (ABL) is replaced by a threonine residue (33). The β-lactamases of *B. vulgatus* CLA341 and *B. uniformis* 7088 both contain a serine residue in this position (Ser-237), and both have high cephalosporinase activity.

In summary, this work provides the first report of a class A cephalosporinase from a high-level β-lactamase-producing clinical isolate of *B. fragilis*, CS30. The basis for the increased specific activity in this strain is unknown, but it is not due to an increase in gene copy number, since only a single hybridization band appears on Southern blots of total chromosomal DNA cleaved with an endonuclease that does not digest within the *cepA* structural gene (data not shown). The increase in specific activity is also not due to the presence of additional β-lactamases in this strain, as only one nitrocefin-reactive band can be detected in cell extracts on isoelectric focusing or SDS-polyacrylamide gels. The gene appears to be of *Bacteroides* origin, on the basis of its moles percent G+C content. We have also shown at the DNA level that *cepA* is specific to *B. fragilis* species, extending conclusions drawn by others on the basis of the presence of β-lactamases with common isoelectric points (40).

This study also shows the extensive similarity at the amino acid sequence level of chromosomally encoded β-lactamases from three different *Bacteroides* species. While others have shown that β-lactamases from different *Bacteroides* species can be differentiated with regard to size, pI, and enzyme kinetics (17, 43), we show here that at least three of these β-lactamases share overall similarity in the sense that they are all class A enzymes and are all more related to each other than to all other class A β-lactamases so far discovered. These results support and extend previous conclusions drawn about the unique cephalosporinases of *Bacteroides* species when antisera made to individual purified endogenous cephalosporinases from *B. fragilis*, *B. vulgatus*, and *B. thetaiotaomicron* showed some cross-reactivity with one another but not with antisera made to enzymes from other genera (48). Furthermore, the phylogenetic analysis clearly indicates that the *Bacteroides* enzymes evolved much earlier than the others, consistent with the taxonomic standing of the genus (60). In addition, these results suggest that CfxA, responsible for widespread cefoxitin resistance in *Bacteroides* species (45), clearly evolved from a *Bacteroides* β-lactamase gene and was not the result of a recent acquisition from another species. Future studies will examine at the DNA level the *cepA* genes from other high- and low-level β-lactamase producers, allowing the determination of the molecular mechanisms that could lead to high-level β-lactamase production in these clinically important anaerobes.

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