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

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Bacteroides fragilis is a clinical isolate resistant to high concentrations of benzylpenicillin and cephalexin but not to cefoxitin or penem antibiotics. ß-Lactam resistance is mediated by a chromosomally encoded cephalosporinase produced at a high level. The gene encoding this ß-lactamase was cloned from genomic libraries constructed in Escherichia coli and then mated with B. fragilis 638 for identification of ampicillin-resistant (Ap') strains. Ap' transconjugants contained a nitrocefin-reactive protein with the physical and enzymatic properties of the original CS30 isolate. The ß-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 ß-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 ß-lactamase genes. An extensive comparison of the CepA protein sequence by alignment with other ß-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 ß-lactamases from two other Bacteroides species indicated that they constitute their own distinct subgroup of class A ß-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 ß-lactam antibiotics indicate that these organisms are becoming increasingly refractory to treatment with these drugs. The primary mechanism of ß-lactam resistance in Bacteroides species is the production of ß-lactamase (40). At least four types of ß-lactamases 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” ß-lactamase is present in over 90% of clinical isolates tested (10). Unlike the class C chromosomally encoded ß-lactamases of members of the family Enterobacteriaceae, the B. fragilis ß-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 ß-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 ß-lactamase producers are susceptible to all ß-lactams, the MICs of benzylpenicillin and cephalexin being <2 and <16 μg/ml, respectively. For intermediate-level ß-lactamase producers, the most frequently encountered group, the MICs of benzylpenicillin and cephalexin are 16 and 32 μg/ml, respectively. High-level ß-lactamase producers are resistant to both drugs at >256 μg/ml. In all cases, the levels of ß-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 ß-lactamase and that 25% produce high levels (10). The existence of these classes and the increasing frequency of isolation of high-level ß-lactamase-producing strains suggest a trend towards high-level ß-lactamase production in Bacteroides species.

As a first step in identifying possible differences in the regulation of ß-lactamase production between expression classes, we have cloned and sequenced the chromosomal cephalosporinase gene (cepA) from a high-level ß-lactamase-producing clinical isolate, B. fragilis CS30. In contrast to the class C chromosomal ß-lactamases of members of the family Enterobacteriaceae, we present evidence that CepA is a ß-lactamase containing at least four amino acid motifs characteristic of the class A active-site serine ß-lactamases described by Ambler (1). A comparison of the CepA amino acid sequence with those of other class A ß-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 cephalexin, 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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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 rcsA1 endA1 relA 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 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 32P 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). 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 BamHI-BglII 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 Sau3AI. Fragments were separated on sucrose density gradients, and fractions of between 5 and 15 kb were isolated, pooled, and ligated to the BglII site of pJST61kan (Kn' in E. coli strains and Cc' 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 Clal 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' gene was performed with pFD288 (8.8 kb; oriT Sp' in E. coli strains and Cc' in Bacteroides strains [56]). Suicide vector pFD434 was derived from pFD280 (56) by insertion of the Bacteroides tetQ gene (38) into the unique SsuI site. The construct used for insertional inactivation of cepA, pFD439, contained the 400-bp HhaI-EcoRI fragment of cepA (bp 502 to 902) ligated into the SmaI 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 se-
quence 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 (PCE), M15526 (9); Verrinia enterocolitica (YER), X57074 (49); Bacillus cereus ß-lactamase III (BCIII), M15195 (29); B. cereus ß-lactamase I (BCI), X06599 (52); Actinomadura sp. strain R39 (ACT), X53650 (26); Streptomyces albus G(ALBUS), M28303 (15); Saurofaciens ß-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 pB60-1-2 SHV-2 ß-lactamase (SHV-2), X53433 (46); B. licheniformis PenP penicillinase (PENP), X54772 (37); E. coli RGN238 (Tn2603) OXA-1 ß-lactamase (OXAX), J02967 (44); Paracoccus taiwanensis type 1a R64 OXA-2 ß-lactamase (OXA2), M25261 (14); and Pseudomonas aeruginosa PU21 R515 (Tn1404) PSE-2 ß-lactamase (PSEX), J03427 (27).

Analysis and purification of ß-lactamase. Cell extracts were prepared by use of a French pressure cell from mid- to late-logarithmic-phase cells and tested for ß-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 ß-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 pH 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 ß-lactamases were visualized by overlaying the polyacrylamide gels with 0.8% agarose in 20 mM sodium phosphate buffer (pH 7.0) containing 50 ìg of nitrocefin per ml and photographed with a green filter.

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

The CS30 ß-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 7.0), 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 NH₄SO₄ 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 NH₄SO₄ gradient. The resulting ß-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. ß-Lactam MICs and ß-lactamase specific activities were determined for 39 clinical B. fragilis (sensu strictu) strains and 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 cefalosporins nitrocefin and cephodin and displayed only weak activity against penicillin, indicating that the ß-lactamases were cephalosporinases. ß-Lactamase specific activities ranged from 0.004 to 0.270 (67.5-fold) with nitrocefin as a substrate, and all ß-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 (pl, 4.9). For both ampicillin and cefaloridine, there was a high correlation (R = 0.96) between ß-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 ß-lactamase-producing strains to express higher ß-lactamase activities were unsuccessful, and no spontaneous mutants to a high-level phenotype were detected.

Cloning of the cephalosporinase gene, cepA. Because antibiopic 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 ß-lactamase gene with a kanamycin resistance determinant (59), because the level of ß-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 Kbp 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 mU/ml). A total of 24 B. fragilis 638 clones were obtained. The stability of the Ap phenotype was demonstrated by the ability to be restreaked onto medium containing rifampin, gentamicin, clindamycin, and ampicillin (150 mU/ml). Several clones were examined in
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 Sau3AI. 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<sup>+</sup> phenotype to B. fragilis 638, whereas donors containing pJST61.kan alone could not confer Ap<sup>+</sup>. 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<sup>+</sup> to either B. fragilis 638 or B. uniformis 1001, as measured by selection on Wilkins-Chal- gren 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<sup>+</sup>, suggesting that an essential portion of the gene was located beyond the first EcoRI site. The 2,485-bp PstI-BglII fragment, which encompasses this EcoRI site, was sufficient to confer Ap<sup>+</sup> 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-lactamas 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 EcoRI-HindIII, 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 ceproxin-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.
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 EcoRI 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 1111, ATCC 29148, and ATCC 29741; B. distasonis ATCC 8503; B. eggerthii VPI T3-3; and B. cacae 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 TeC 638 transconjugants were phenotypically ApR. Integration of the plasmid into the chromosome of B. fragilis 638 was detected by restricting total DNA with BglII (which does not cleave pFD439 or cepA) and probing blots with a 22P-labeled cepA-specific Sphi/EcoRI fragment (bp 247 to 902). In TeC 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 TeC 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 ORF I 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 acidic 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...
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 BglII 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.
proteins, all of which were \( \beta \)-lactamases, most belonging to Ambler class A (1). When class A \( \beta \)-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 \( \beta \)-lactamase to 26.4% identity (49.6% similarity) for the \( K. \) oxytoca \( \beta \)-lactamase. Of the six non-Bacteroides sequences aligned to CepA with five gaps or fewer, four were \( \beta \)-lactamases from \( S. \) intermedius species. The most striking homology, however, was to two other chromosomal \( \beta \)-lactamases from different species. CepA shared 39.9% identity (58.8% similarity) with the \( B. \) vulgatus CLA341 \( \beta \)-lactamase, CfxA (45), and 42.5% identity (65.8% similarity) with the \( B. \) uniformis 7088 \( \beta \)-lactamase, CblA (54). The degree of similarity between CepA and \( \beta \)-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 \( \beta \)-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 \( OXA-1 \) \( \beta \)-lactamase of \( Tn2603 \) showed the best similarity (44) (17.4% identity and 38.9% similarity with 6 gaps). Several key features of the overall amino acid sequence alignments, as 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 \( \beta \)-lactamases from \( B. \) uniformis and \( B. \)
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 cephapirin 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 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' phenotype on the presence of a functional copy of cepA.

DNA sequence analysis of the 2,300-bp Sau3AI-BglII 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 pl of the pre-β-lactamase and the mature protein were 6.0 and 5.4, respectively. The pl 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 pl 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 HI of the pBR322 TEM-1 β-lactamase (24) results from a change just after box 7, whereby A1a-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 cephalospo- rinase 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 mole percent G+C content. We have also shown that 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 endoge- nous cephalosporinases from B. fragilis, B. vulgatus, and B. thetaiotamicron 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 CxxA, responsible for widespread cefoxitin resistance in Bacte- roides species (45), clearly evolved from a Bacteroides β-lactamase gene and was not the result of a recent acquisi- tion 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-lacta- mase production in these clinically important anaerobes.

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REFERENCES


