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Insertional Activation of *cepA* Leads to High-Level β-Lactamase Expression in *Bacteroides fragilis* Clinical Isolates

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Bacteroides fragilis is an important opportunistic pathogen of humans and is resistant to many drugs commonly used to treat anaerobic infections, including β-lactams. A strain set comprised of _B. fragilis_ isolates producing either low or high levels of the endogenous cephalosporinase activity, CepA, has been described previously (M. B. Rogers, A. C. Parker, and C. J. Smith, Antimicrob. Agents Chemother. 37:2391–2400, 1993). Clones containing _cepA_ genes from each of seven representative strains were isolated, and the DNA sequences were determined. Nucleotide sequence comparisons revealed that there were few differences between the _cepA_ coding sequences of the low- and high-activity strains. The _cepA_ coding sequences were cloned into an expression vector, pFD340, and analyzed in a _B. fragilis_ 638 _cepA_ mutant. The results of β-lactamase assays and ampicillin MICs showed that there was no significant difference in the enzymatic activity of structural genes from the high- or low-activity strains. Comparison of sequences upstream of the _cepA_ coding region revealed that 50 bp prior to the translation start codon, the sequence for high-activity strains change dramatically. This region of the high-activity strains shared extensive homology with IS_21_, suggesting that an insertion was responsible for the increased expression of _cepA_ in these isolates. Northern (RNA) blot analysis of total RNA by using _cepA_-specific DNA probes supported the idea that differential _cepA_ expression in low- and high-activity strains was controlled at the level of transcription. However, the insertion did not alter the _cepA_ transcription start site, which occurred 27 bp upstream of the ATG translation start codon in both expression classes. Possible mechanisms of _cepA_ activation are discussed.

The anaerobe *Bacteroides fragilis* contains an endogenous, chromosomally encoded β-lactamase which preferentially hydrolyzes cephalosporins and is responsible for the intrinsic resistance to most penicillins and cephalosporins (6, 15). These organisms are generally susceptible to some of the newer β-lactams such as cephamycins (cefoxitin) and carbapenems (imipenem), although newly acquired β-lactamases capable of degrading these antibiotics have been described (18, 21, 38). The indigenous β-lactamase is present in between 90 and 99% of *B. fragilis* strains and at the biochemical level, this β-lactamase has been shown to be species specific (8, 16). Recently, the gene for this enzyme, *cepA*, was cloned and the nucleotide sequence was determined (27). Southern hybridization analyses with a *cepA* probe showed that there was homology only with other *B. fragilis* strains, and construction of a *cepA* mutant provided evidence that this gene did in fact encode for the endogenous β-lactamase. Comparison of the predicted CepA amino acid sequence with other β-lactamase sequences indicated that it was not in the Ambler molecular class C like the chromosomal β-lactamases of most other gram-negative bacteria, but rather CepA belonged to the class A β-lactamases. The CepA enzyme together with two other *Bacteroides* β-lactamases formed a unique group that diverged very early in the evolution of the class A enzymes.

B. fragilis clinical isolates producing high levels of the endogenous β-lactamase activity are being isolated more frequently, and these strains are often grouped on the basis of the level of enzymatic activity (reviewed in references 9, 15, and 20). We have described a set of strains that possess only the endogenous β-lactamase, and these strains clearly fall into two expression classes (27). Low-activity strains (0.004 to 0.013 U mg^{-1}) and high-activity strains (>0.1 U mg^{-1}) display a 10-fold difference in activity, but the enzymes have the same pI (4.9) and molecular weight (31,500). On the basis of Southern filter hybridizations, the two expression classes could be distinguished at the DNA level by their different patterns of homology with a *cepA* gene probe (27). The nature of these structural differences and the mechanisms responsible for high or low activity were not known and are the subject of this report.

Unlike the chromosomal β-lactamases of many other gram-negative eubacteria, the *B. fragilis* cephalosporinase appears to be constitutively expressed at a low level and is not inducible by subinhibitory concentrations of β-lactam drugs (15). There is some evidence that the enzyme may be moderately growth rate regulated, with activity steadily increasing during logarithmic growth and reaching a maximum in early stationary phase (5). Little additional information on β-lactamase regulation in *B. fragilis* is available, so in order to address the possibility of differential *cepA* regulation in the high- and low-activity classes, we cloned *cepA* homologs from several representatives of each class. Analysis of the DNA sequences showed few amino acid substitutions in the structural genes, and these did not account for the altered enzymatic activity. However, high-activity strains produced significantly higher levels of *cepA* mRNA, and we provide evidence that this activation of transcription is due to the presence of an insertion sequence element. The insertion was observed 50 or 51 bp upstream of the *cepA* ATG start codon in all of the high-activity strains examined but not in the low-activity strains. The mechanism of

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TABLE 1. Plasmids used for analysis of *cepA* clones and their relevant properties

Plasmid	Relevant characteristics[a]	Reference
Vectors		
pFD288	(Sp[r]) Cc[r], *oriT*, pUC19::pBI143 8.8-kb shuttle vector	35
pFD340	(Ap[r]) Cc[r], *oriT*, IS*4351* promoter	36
pFD395	(Sp[r]) Cc[r], *oriT*, *rrnB* terminators, CAT reporter gene	36
Plasmids containing *cepA* homologs		
pFD457	(Sp[r]) Cc[r], 1.2-kb RBF49 *cepA* gene fragment in pFD288	T[b]
pFD470	(Sp[r]) Cc[r], 1.4-kb CS44 *cepA* gene fragment in pFD288	T
pFD471	(Sp[r]) Cc[r], 1.4-kb ATCC 25285 *cepA* gene fragment in pFD288	T
pFD480	(Sp[r]) Cc[r], 1.2-kb RBF103 *cepA* gene fragment in pFD288	T
pFD488	(Sp[r]) Cc[r], 1.4-kb CS29 *cepA* gene fragment in pFD288	T
pFD528	(Sp[r]) Cc[r], 2.8-kb CS14 *cepA* gene fragment in pFD288	T
Plasmid clones for *cepA* structural gene analyses		
pFD512	(Ap[r]) Cc[r] pFD340:ATCC 25285 *cepA* fusion	T
pFD513	(Ap[r]) Cc[r] pFD340:CS29 *cepA* fusion	T
pFD514	(Ap[r]) Cc[r] pFD340:RBF103 *cepA* fusion	T
pFD515	(Ap[r]) Cc[r] pFD340:CS14 *cepA* fusion	T

[a] Antibiotic resistance designations in parentheses are expressed only in *E. coli*; the other determinants are expressed in *Bacteroides* species.
[b] T, this study.

insertional activation with regard to increased β-lactamase production in *B. fragilis* is discussed.

MATERIALS AND METHODS

Strains and media, MICs, and DNA transfer. The various *B. fragilis* vectors used in this study are listed in Table 1. The standard laboratory strain used in these studies was a rifampin-resistant derivative of *B. fragilis* 638 (19). *Bacteroides* strains were grown at 37°C anaerobically in supplemented brain heart infusion (Difco Laboratories, Detroit, Mich.) broth or agar as described previously (33). Antibiotic MICs were measured by the standard agar dilution method with Wilkins-Chalgren agar (Difco) after 48 h of growth. The following antibiotic concentrations were used unless noted otherwise: ampicillin, 50 μg/ml; clindamycin, 5 μg/ml; gentamicin, 25 μg/ml; rifampin, 20 μg/ml; and tetracycline, 5 μg/ml. *Escherichia coli* DH5α MCR [F$^-$ *lacZ deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 mcrA* (*mrr-hsdRMS-mcrBC*)] was grown aerobically at 37°C in Luria-Bertani broth (agar) supplemented with kanamycin, spectinomycin, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 50 μg/ml as appropriate.

Standard filter mating protocols were used to transfer plasmids in triparental matings from *E. coli* donors to *Bacteroides* recipients. The *E. coli* donors contained the helper plasmid RK231, and filters were incubated aerobically at 37°C overnight (32). *E. coli* transformations were done by the method of Hanahan (11).

Isolation of *cepA* homologs from low- and high-activity strains. Chromosomal DNA preparations of *B. fragilis* strains in Table 2 (excluding CS30) were purified by CsCl-ethidium bromide density gradient ultracentrifugation and partially digested with *Sau*3A1, and fragments of between 5 and 15 kb were pooled from linear sucrose density gradients. The DNA fragments were ligated into the *Bgl*II site of positive selection vectors pJST61.kan (27) or pEcoR251 (37), and then *E. coli* HB101 was transformed by electroporation and plated on L agar with ampicillin or kanamycin. The *cepA*-containing clones were identified by colony hybridizations (28) using a *cepA*-specific DNA probe (*cepA* bp 247 to 902 [27]). Colonies that hybridized to the probe were purified, and plasmid DNA was extracted and hybridized again to the *cepA*-specific probe.

DNA manipulations, sequence analysis, and PCR. Large-scale plasmid DNA preparations from *Bacteroides* strains were obtained by CsCl-ethidium bromide ultracentrifugation of crude lysates prepared by alkaline denaturation (33). Plasmid DNA preparations from *E. coli* transformants were performed by the alkaline lysis method (2). Routine DNA ligation, restriction endonuclease digestion, Klenow reactions, radiolabeling of DNA probes, and agarose gel electrophoresis have been described elsewhere (28). Individual restriction fragments or PCR products were excised from Tris acetate-EDTA agarose gels and purified by adsorption to glass beads, using a Gene Clean kit (Bio 101, La Jolla, Calif.) according to the supplied instructions.

DNA sequence analysis of the *cepA* homologs was performed by dideoxy nucleotide sequencing (29) of the recombinant plasmid clones, using modified T7 polymerase (Sequenase 2.0; U.S. Biochemical Corp., Cleveland, Ohio). DNA primers used for sequencing were based on sequence obtained for *cepA* of *B. fragilis* CS30 (27). Reaction mixtures were analyzed on 0.2-mm-thick 6% polyacrylamide gels (6% T, 5% C) containing 42% (wt/vol) urea (28), and sequence information was analyzed with a MicroVAX computer system and University of Wisconsin Genetics Computer Group DNA sequence analysis software (7).

DNA amplification of *cepA* coding or promoter regions by PCR was performed with the corresponding primers described in the appropriate text. Generally, plasmid DNA template (100 ng) was amplified with 2 U of Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) according to supplied instructions, using a twin-block thermal cycler (Ericomp, Inc., San Diego, Calif.) set for 17 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C, 1 cycle of 10 min at 72°C, and then 1 cycle of 1 min 4 s at 27°C. The amplified products were phenol-chloroform extracted and electrophoresed in Tris acetate-EDTA agarose gels for subsequent purification of amplified fragments. Unless otherwise noted, PCR products were first cloned in pUC19 and then subcloned into the appropriate vector. All PCR fragments were sequenced to verify their structures.

The copy number of pFD340 constructs bearing *cepA* genes was estimated by Southern hybridization. Total genomic DNA from plasmid-containing strains was digested with *Bam*HI, serial dilutions were electrophoresed, and the gels were blotted onto nitrocellulose filters. These were then probed with the *cepA* structural gene probe (Fig. 1), and the resulting autoradiographs were analyzed by densitometry as described below.

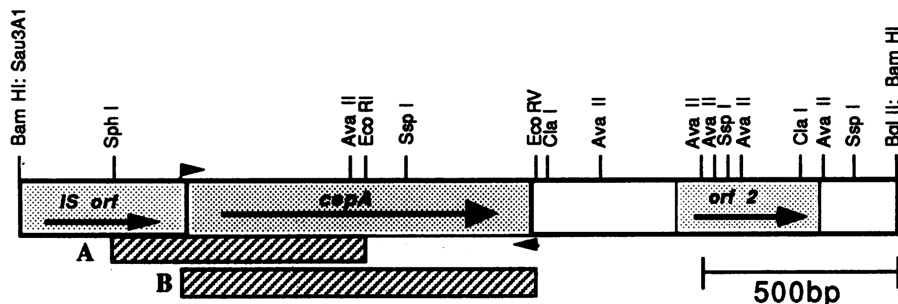


FIG. 1. Restriction map and open reading frames of $cepA_{30\text{-}H}$. The restriction sites and open reading frames (*orf*) were deduced from DNA sequence analysis of the cloned *cepA* gene from *B. fragilis* CS30 (27). The probe used for cloning additional *cepA* homologs is shown by the hashed box A, and the *cepA* structural gene probe is shown by the hashed box B. The location of primers used for PCR amplification of structural genes are shown by the arrowheads.

The chromosomal and plasmid copies of *cepA* migrate differently in *Bam*HI-digested samples; thus, it was possible to estimate a copy number from the relative hybridization intensities of the bands.

RNA isolation from *B. fragilis* and Northern (RNA) blot analysis. Total RNA was isolated by the hot phenol method of Aiba et al. (1). Briefly, chloramphenicol (to 100 μg/ml [14]) was added to *Bacteroides* cultures in late logarithmic phase, and the cultures were then immediately centrifuged at 4°C. The cell pellet was suspended in AE buffer (20 mM sodium acetate [pH 5.5], 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM EDTA) and quickly extracted with 3 ml of phenol. Phenol (U.S. Biochemical) was equilibrated with 20 mM sodium acetate (pH 5.5) until the pH of the phenol was 5.5. Before extraction of the cell suspension, phenol was heated to 65°C. Cells were extracted with phenol for 5 min at 65°C and centrifuged. Phenol extraction was repeated, and the final aqueous phase was precipitated a total of three times with 3 vol of ethanol at −70°C. The final RNA pellet was dissolved in deionized formamide and stored at −70°C. Concentration was determined by measuring A_{260}.

RNA samples (5 to 50 μg) and size standards (0.24- to 9.5-kb RNA ladder) were electrophoresed in large gels (25.3 by 15.1 cm^2 and 1 cm thick) containing 1.1% (wt/vol) agarose, 1× MOPS buffer (40 mM 3-[*N*-morpholino]propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA [pH 7]), and 2.2 M formaldehyde. RNA was transferred to nylon membranes (Hybond N; Amersham Corp., Arlington Heights, Ill.) by capillary action in 10× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7]), and cross-linked by UV irradiation. DNA probes were labeled with [α-^{32}P]dCTP by the random primer reaction. Prehybridization (4 h) and overnight hybridization were performed at 50°C. Prehybridization buffer contained 50% deionized formamide, 4× SSC, 5× 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 mM Na_2HPO_4 (pH 7.0), 0.1 mg of yeast RNA per ml, 1% SDS, and 0.5 mg of $NaPP_i$ per ml. Hybridization buffer was identical except Denhardt's solution was used at 1× concentration. Nylon blots were washed in 0.1× SSC–0.1% SDS for 20 min each at room temperature, 50°C, and 65°C.

Primer extension analysis of total RNA. Primer extension analysis of total RNA using *cepA*-specific oligonucleotide primers was performed as described previously (3, 28), with slight modifications. Primers (400 pmol of 5′ ends) were labeled with [γ-^{32}P]ATP, and 10^5 cpm (0.045 μCi) of oligonucleotide was precipitated with total RNA (up to 50 μg for high-activity strains and 100 μg for low-activity strains) in diethyl pyrocarbonate-treated tubes. The resulting pellet was dried, resuspended in hybridization buffer {80% formamide, 0.4 M NaCl, 1 mM EDTA (pH 8), 40 mM PIPES [piperazine-*N,N*′-bis-(2-ethanesulfonic acid); pH}, incubated at 85°C for 10 min, and then annealed overnight (8–12 h) at 40°C. After ethanol precipitation and centrifugation, the pellet was dried, resuspended in 20 μl of RT buffer (50 mM Tris [pH 7.6], 60 mM KCl, 10 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate, 1 mM dithiothreitol, 1 U of RNasin RNase inhibitor [Promega, Madison, Wis.] per ml, 50 μg of actinomycin D per ml, 50 U of Moloney murine leukemia virus reverse transcriptase [Gibco/BRL]), and incubated 2 h at 37°C. Reverse transcriptase was inactivated by addition of EDTA, and then RNase A (DNase free; 5 μg/ml) was added to digest the RNA templates. The volume was brought up to 150 μl with Tris-EDTA, and the reaction mixtures were phenol-chloroform extracted. The supernatants were precipitated, dried, resuspended in 4 μl of formamide loading buffer, and electrophoresed on 8% polyacrylamide gels containing urea. A sequencing ladder was prepared with a template covering the transcription start site region, using the same oligonucleotides that were used for the reverse transcription reactions.

Densitometry analysis of autoradiographs was performed with a Hewlett-Packard ScanJet Plus flatbed scanner interfaced with a Macintosh IIci computer. Collage imaging software (Fotodyne, Inc.) was used to quantitate the band intensities.

β-Lactamase analysis. Cell extracts for β-lactamase activity assays and isoelectric focusing were prepared with a French pressure cell (American Instrument Company, Inc., Silver Spring, Md.) in 20 mM sodium phosphate [pH 7] as described previously (18). Activity was measured spectrophotometrically with nitrocefin, and specific activity is expressed as micromoles of substrate consumed per minute per milligram of protein (17, 18). Protein concentrations were determined by the method of Bradford (4).

Nucleotide sequence accession numbers. The gene designations and GenBank accession numbers for the DNA sequences of the high-activity strains are as follows: CS30, $cepA_{30\text{-}H}$ L13472 (27); RBF103, $cepA_{103\text{-}H}$, U05888; and RBF49, $cepA_{49\text{-}H}$, U05886. Those for the low activity strains are as follows: ATCC

TABLE 2. β-Lactamase and ampicillin MICs for *B. fragilis* wild-type strains and pFD288 recombinant plasmids containing *cepA*

B. fragilis strain[a]	β-Lactamase activity (U/mg of protein)[b]	Ampicillin MIC (μg/ml)
Wild type		
CS29	0.004 (low)	8
CS14	0.006 (low)	16
638	0.007 (low)	16
CS44	0.010 (low)	32
ATCC 25285	0.013 (low)	32
RBF49	0.110 (high)	500
RBF43	0.150 (high)	500
CS30	0.230 (high)	750
RBF103	0.270 (high)	750
With cloned *cepA* genes		
638(pFD288)	0.009	16
$cepA_{14\text{-L}}$	0.019	32
$cepA_{29\text{-L}}$	0.014	16
$cepA_{85\text{-L}}$	0.014	16
$cepA_{103\text{-H}}$	0.415 ± 0.15	>800

[a] All strains are *B. fragilis* sensu strictu and contain a single β-lactamase with an isoelectric point of 4.9. Cefoxitin MICs were ≤16 μg/ml for all strains.

[b] Measured in crude cell extracts with nitrocefin as a substrate. All β-lactamase activities were inhibited >50% by 1 μM clavulanate and 1 μM cefoxitin.

25285, $cepA_{85\text{-L}}$, U05887; CS14, $cepA_{14\text{-L}}$, U05883; CS29, $cepA_{29\text{-L}}$, U05884; and CS44, $cepA_{44\text{-L}}$, U05885.

RESULTS

Analysis of cloned *cepA* genes. *cepA* homologs from *B. fragilis* strains representing the high- and low-activity classes (Table 2) were cloned in *E. coli* and identified by hybridization to the *cepA* DNA probe shown in Fig. 1 (probe A). Several positive clones were obtained from each of the strains, and the cloned inserts ranged in size from 3 to 16 kb. Subsequently, the *cepA* regions were sequenced as described in Materials and Methods, using oligonucleotide primers derived from the CS30 *cepA*.

Several of the *cepA* homologs were subcloned into the shuttle vector pFD288, transferred to *B. fragilis* 638, and assayed for β-lactam resistance (Table 2). The results show that the low-activity class *cepA* homologs were on average no more resistant to ampicillin than the parent strain 638, and the β-lactamase activities were only slightly higher than background. In contrast to this, strains with the cloned $cepA_{30\text{-H}}$ (27) or $cepA_{103\text{-H}}$ (Table 2) were highly resistant to ampicillin, and the β-lactamase activity was 40-fold greater than seen in 638 containing just the vector and no insert. As noted previously (27), clones containing the high-activity *cepA* homologs were very unstable and yielded widely varying β-lactamase activities.

The DNA sequences of the *cepA* homologs were analyzed for clues to the differential expression. Examination of the sequences showed that relative to $cepA_{85\text{-L}}$, there were a total of 16 unique nucleotide changes in the seven sequences (Fig. 2 and data not shown). These corresponded to eight different amino acid substitutions, but only one (in $cepA_{29\text{-L}}$) was within any of the highly conserved β-lactamase structural motifs. Also, in the case of $cepA_{85\text{-L}}$ and $cepA_{103\text{-H}}$, the predicted amino acid sequences were the same. These observations suggested that high β-lactamase activity was not the result of a structural gene point mutation leading to altered activity as exemplified by many of the TEM enzymes (12).

Expression of *cepA* structural genes in pFD340. Further experiments on the *cepA* structural genes confirmed the DNA sequence findings. *cepA* homologs from high- and low-activity strains were cloned into the *Bacteroides* expression vector pFD340 so that β-lactamase activities could be compared for these genes under control of the same promoter. The precise cloning was accomplished by PCR amplification using oligonucleotide primers shown in Fig. 1 and 2. DNA sequence analysis of the amplified fragments confirmed that no nucleotide substitutions had occurred during the amplification procedure. The pFD340 constructs were transferred by conjugation into a *B. fragilis* 638 *cepA* mutant (27) for determination of both ampicillin MICs and β-lactamase specific activities (Fig. 3). Generally, the *cepA* fusions displayed similar β-lactamase activities and ampicillin MICs when controlled from the IS*4351* promoter. One exception, the $cepA_{29\text{-L}}$ gene fusion, consistently produced β-lactamase activities and ampicillin MICs about half of the values for the other *cepA* homologs. This finding suggested that the amino acid substitutions in the CS29 enzyme directly influenced the intrinsic activity of this β-lactamase. For the other *cepA* homologs, however, both ampicillin MICs and β-lactamase specific activities were very similar, and specific activities were more than 100-fold greater than for the pFD340 control. Isoelectric focusing gels of cell extracts from these strains all displayed a nitrocefin-reactive protein which focused at pH 4.9, while the 638 *cepA* mutant strain containing only pFD340 did not (data not shown). There was some variation in the estimated copy number of the various pFD340 constructs (Fig. 3), but these differences in gene copy did not significantly influence the results. These data show that the major differences in β-lactamase activity seen between the two β-lactamase expression classes are not due to amino acid substitutions in the *cepA* structural genes and that β-lactamase activity apparently can be influenced by promoter strength.

Comparison of upstream DNA sequences of *cepA* genes from low- and high-activity strains. Subsequent examination of the *cepA* DNA sequences focused on regions flanking the structural gene. Downstream from the TAA translation stop codon, the nucleotide sequences for all strains were nearly identical for the ~400 bp of available sequence data. In Fig. 2, 390 and 238 bp of sequence are shown for regions upstream of the ATG start site of high- and low-activity strains, respectively. Comparison of these upstream sequences yielded a notable discovery. Exactly 50 bp (or 51 bp for RBF103) upstream of the ATG translation start codon, the DNA sequences diverged completely between the low- and high-activity strains. All of the low-activity class sequences were nearly identical to each other. The high-activity class sequences from CS30 and RBF103 were identical to each other, and RBF49, though not identical, shared 67% homology.

The nucleotide sequences for the upstream regions have been compared against the nucleic acid and protein databases, and for the low-activity strains, no significant matches were found. On the other hand, DNA sequence from the high-activity strains revealed an open reading frame starting at the 5′ end of the available data. When this peptide was compared against the databases, we observed a high degree of similarity to the insertion sequences related to IS*21* (25, 26). More specifically, the partial open reading frames from CS30, RBF103, and RBF49 shared about 30% amino acid identity with the *istB* gene product, and a comparison with IstB from IS*21* and IS*5376* is shown in Fig. 4. Remarkably, RBF49, while clearly diverged from CS30 and RBF103, shared the same level of homology to the IS*21* family. These results suggested that an insertion 50 bp upstream from the *cepA* start codon activated

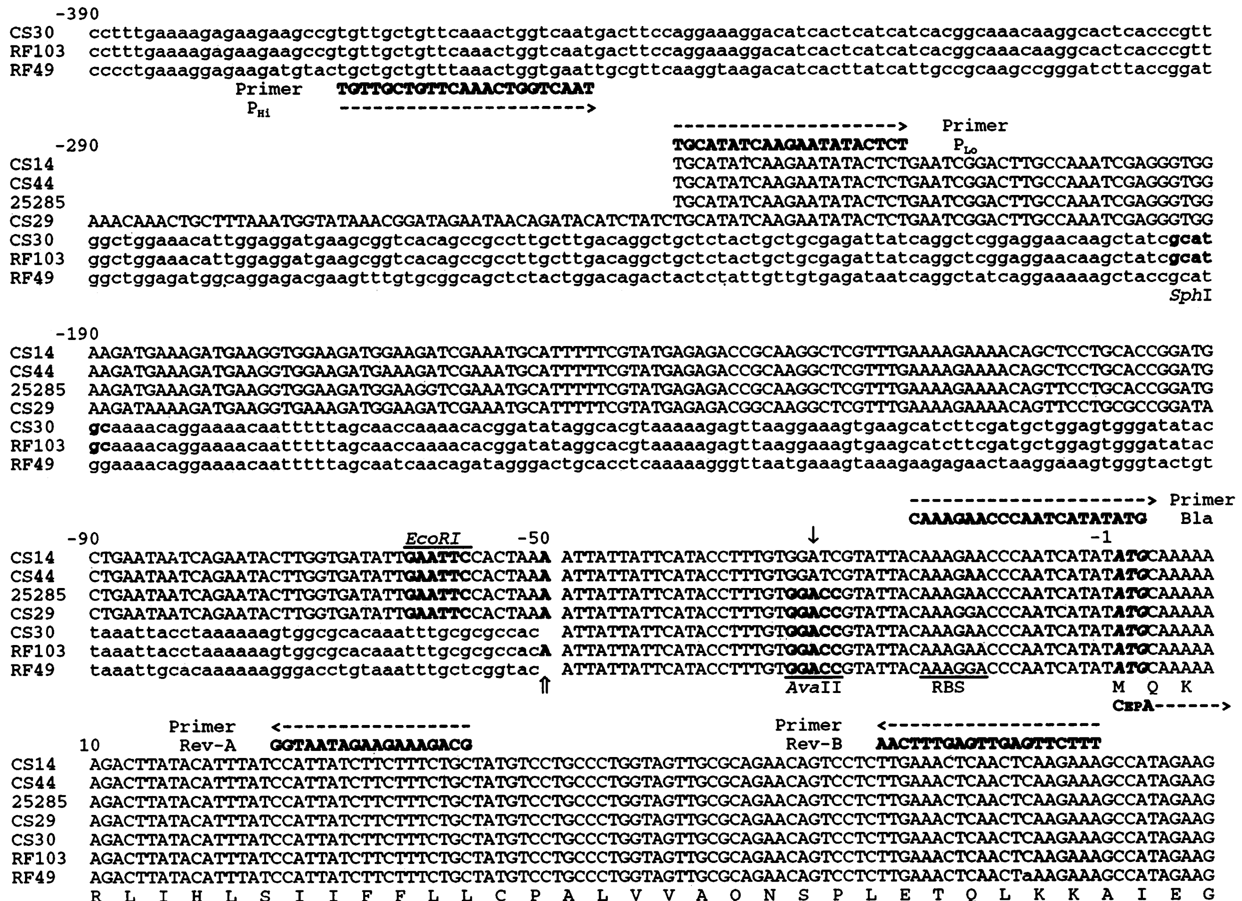
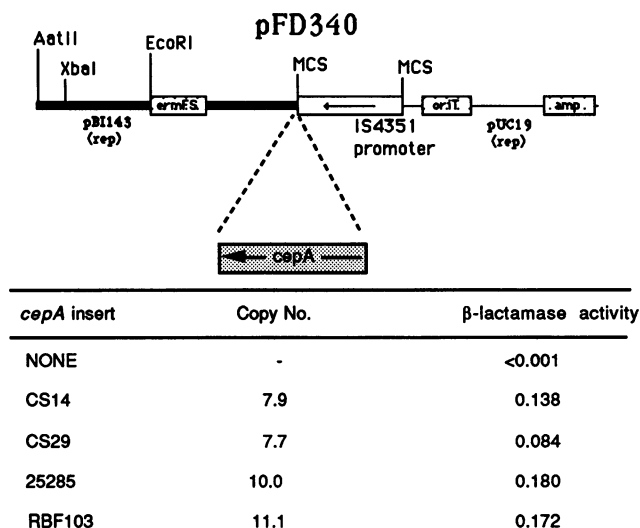


FIG. 2. Alignment of DNA sequences upstream of seven *cepA* homologs. The *cepA* ATG start codon is in boldface, and the first base prior to this is numbered −1. The ribosome binding site (RBS) is underlined, and several relevant restriction endonuclease sites are in boldface. The point of sequence divergence between the low- and high-expression classes is indicated by the upward-pointing arrow at bp −50. Upstream of this point, the sequences of low-activity strains are shown in capital letters and the high-activity strain sequences are in lowercase. The downward-pointing arrow at the −27 A residue represents the *cepA* transcriptional start site for both expression classes. Primers used for PCR amplifications described in the text are in boldface.

expression of β-lactamase in the high-activity class strains. Since it was likely that this insertion provided increased promoter activity or release from attenuation or some other negative regulatory mechanism, we examined *cepA* transcription.

Analysis of *cepA*-specific mRNA. Northern blot experiments were performed to examine *cepA* transcription in the two *B. fragilis* β-lactamase expression classes. Total cellular RNA was isolated from *B. fragilis* strains in late logarithmic phase, fractionated on formaldehyde gels, and blotted to nylon filters. Preliminary experiments showed strong *cepA*-specific hybridization to RNA isolated from high-activity class strains but not to RNA preparations from low-activity strains. This problem was overcome by altering the RNA isolation procedure so that chloramphenicol was added to cultures immediately prior to harvesting (14). Typical results in Fig. 5 show an autoradiograph of a Northern blot hybridized to the *cepA* structural gene probe. A single *cepA*-specific transcript was found in RNA preparations from all of the *B. fragilis* strains tested, including the low-activity class strains CS44, CS29, CS14, ATCC 25285, and 638. There was no signal from RNA isolated from *B. uniformis* (Fig. 5, lane 6). The transcript was estimated to be between 1,060 and 1,100 nucleotides. Additional experiments were performed with different samples loaded in a different order, and these all displayed a *cepA*-specific transcript of equal size (data not shown). It is readily apparent that there was a great difference in the intensity of the bands between the low- and high-activity strains, especially considering that 5- to 10-fold more total RNA was loaded for the low-level β-lactamase producers than for CS30, RBF49, or RBF103. This finding suggested that the mechanism of differential expression of the *cepA* gene might be at the level of transcription, so the transcription start sites were determined.

Primer extension analysis of *cepA* RNA from low- and high-activity strains. Primer extension was used to identify the exact start site of transcription. Total RNA from *B. fragilis* was annealed to γ-^{32}P-5′-end-labeled primer Rev-A, hybridized, extended with reverse transcriptase, and digested with RNase (Materials and Methods). Figure 6A shows an autoradiograph of a typical primer extension experiment performed with 50 μg of RNA isolated from the high-activity strains RBF103, RBF49, and CS30 (with two independent RNA preparations). All of the transcripts extended to the same transcription start site, the T residue labeled +1. This residue corresponds to an A residue located just 27 bp upstream of the *cepA* ATG translation start site (Fig. 2). This result was highly reproduc-



cepA insert	Copy No.	β-lactamase activity
NONE	-	<0.001
CS14	7.9	0.138
CS29	7.7	0.084
25285	10.0	0.180
RBF103	11.1	0.172

FIG. 3. β-Lactamase activities and ampicillin MICs for pFD340::*cepA* constructs in a *B. fragilis cepA* mutant. The restriction map of the expression vector pFD340 shows the cloning strategy for the *cepA* structural gene inserts. The ampicillin MICs were determined after growth for 48 h at 37°C on Wilkins-Chalgren agar containing ampicillin. β-Lactamase specific activities are averages of at least two experiments performed in triplicate.

ible, and control reactions with just the primer or RNA template yielded no reaction products. The experiments were repeated with RNA prepared from low-activity strains, using 100 μg of RNA in each reaction, and the results are shown in Fig. 6B. The low-activity class *cepA*-specific transcripts (lanes 2 to 6) were extended to the same residue as the RBF103 transcript (lane 1), corresponding to *cepA* bp −27. Results obtained using the Rev-A primer, which annealed 25 bp downstream of the ATG translation start codon, were confirmed by using a different primer, Rev-B, which annealed 79 bp downstream of the ATG codon.

In addition, the amount of radiolabeled primer used in the extension reactions was in excess; thus, it was possible to quantitate the total amount of *cepA*-specific message in the samples and compare them with each other (28). Hybridization intensities from the Rev-A primer autoradiographs over a range of exposures were quantitated by measuring pixel intensity of a scanned image. The results of these analyses are listed in Table 3. Assuming that the starting amount of total RNA was 100 μg per reaction (50 μg for the RBF103 reaction), it was concluded that the $cepA_{103\text{-H}}$ transcripts were 40-fold more abundant than the $cepA_{44\text{-L}}$ transcripts, 35-fold more abundant than the 638 *cepA* transcripts, 25-fold more abundant than the $cepA_{14\text{-L}}$ transcripts, 18-fold more abundant than the $cepA_{85\text{-L}}$ transcripts, and 13-fold more abundant than the $cepA_{29\text{-L}}$ transcripts.

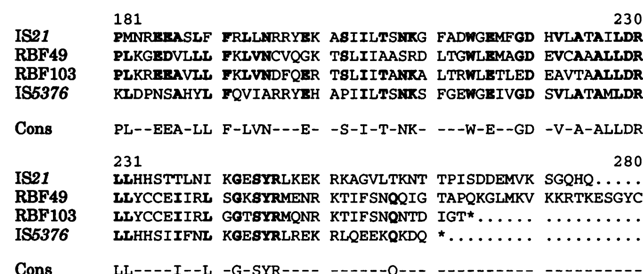


FIG. 4. Alignment of sequences of the IstB proteins from IS*21* and IS*5376* with sequences from *B. fragilis* RBF103 and RBF49. Amino acid sequence from the C terminus of two IstB proteins was obtained from GenBank and aligned with the protein coding sequences found upstream of *cepA* in RBF103 and RBF49. Sequences were aligned by using the Genetics Computer Group Pileup program, and a three-of-four consensus (Cons) is presented below the aligned sequences. Residues contributing to the consensus are in boldface.

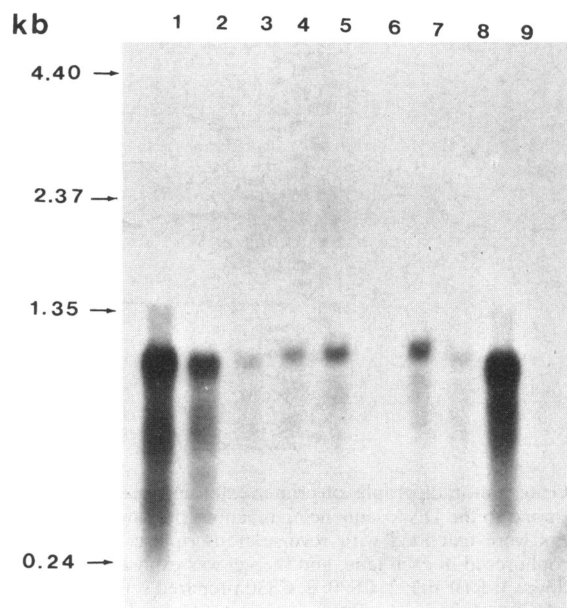


FIG. 5. Autoradiograph of a Northern blot of total RNA from *Bacteroides* strains. The blot was hybridized to an α-^{32}P-labeled DNA probe encompassing the $cepA_{85\text{-L}}$ structural gene. Exposure was for 48 h at −70°C. Sources and amounts of total RNA loaded per lane are as follows: 1, RBF103, 10 μg; 2, RBF49, 10 μg; 3, CS44, 50 μg; 4, CS29, 50 μg; 5, CS14, 50 μg; 6, *B. uniformis* 1001, 50 μg; 7, ATCC 25285, 50 μg; 8, 638, 50 μg; and 9, CS30, 5 μg. Size markers from a 0.24- to 9.5-kb RNA ladder are indicated by the arrows. The size of the *cepA*-specific transcript is approximately 1,100 nucleotides.

***cepA* promoter activity in the CAT fusion vector pFD395.** Another approach to look at differences in *cepA* transcription was to clone the low- and high-activity promoters into a vector with the chloramphenicol acetyltransferase (CAT) reporter gene. PCR amplification with primers P_{Hi} and P_{Lo} (Fig. 2) was used to isolate the promoter regions, and these were cloned into pFD395. The resulting plasmids were transferred into *B. fragilis* 638 and tested for CAT activity. Transconjugants from matings with plasmid containing the P_{Lo} constructs were found to have little or no CAT above the background levels measured for *B. fragilis* 638 containing just the vector (data not shown). These results were similar to the low levels of β-lactamase activity seen with the cloned low-level *cepA* homologs (Table 2).

In contrast to the P_{Lo} results, no results were obtained with pFD395 constructs bearing the P_{Hi} regions. These recombinant plasmids failed to transfer from *E. coli* to *B. fragilis* 638. The infrequent transconjugants that were obtained had been deleted for the cloned P_{Hi} DNA. A modified version of pFD395, pFD551, containing the *trp* terminator inserted downstream of the *cat* gene, was tested for cloning these promoters, but this vector also failed to yield transconjugants with intact

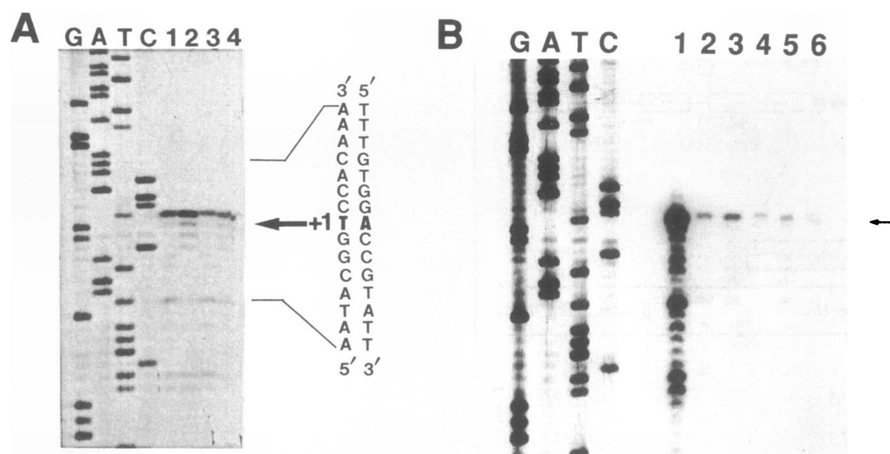


FIG. 6. Autoradiographs of primer extension reactions with total RNA prepared from high- and low-activity strains. Lanes G, A, T, and C correspond to the DNA sequencing reactions prepared using the same Rev-A primer (see Fig. 2) as used for the primer extension reactions. (A) Primers were extended with reverse transcriptase after hybridization to 50 μg of total RNA. The entire contents of each reaction were electrophoresed in each lane, and the gel was exposed for 24 h at room temperature. The sources of starting RNAs for the numbered lanes are as follows: 1, RBF103; 2, CS30; 3, CS30 prepared separately from that of lane 2; and 4, RBF49. The arrow points to the T residue corresponding to the 5′ end of the *cepA* mRNA transcript, which is in boldface, and corresponds to the A residue at bp −27 (Fig. 2). (B) The sources and amounts of starting RNA per reaction for the numbered lanes are as follows: 1, RBF103, 50 μg; 2, ATCC 25285, 100 μg; 3, CS29, 100 μg; 4, 638, 100 μg; 5, CS14, 100 μg; and 6, CS44, 100 μg. This gel was exposed for 71.6 h, but all other conditions were as described above.

plasmids. The P_{Hi} regions proved to be deleterious to *B. fragilis* (but not *E. coli*) when cloned into the standard shuttle/cloning vector pFD288. This was shown by cloning the $cepA_{30\text{-}H}$ and $cepA_{103\text{-}H}$ promoter regions into the multiple cloning site of pFD288 and mating these constructs with 638. As seen with the CAT fusions, transconjugants with intact plasmids were not obtained.

DISCUSSION

We have begun to elucidate the basis for the increased β-lactamase activity in *B. fragilis* CS30, RBF103, and RBF49. Previously, results obtained by Southern hybridizations indicated that high levels of activity were not due to an increase in gene copy number, but the two expression classes could be differentiated on the basis of their hybridization patterns (27). Also, the increased specific activities are not due to the presence of additional β-lactamases in these strains, as only one nitrocefin-reactive band can be detected in cell extracts on isoelectric focusing or renatured SDS-polyacrylamide gels (27). Comparison of the DNA sequences upstream of the *cepA* genes revealed that in high-activity strains an insertion had occurred 50 bp (or 51 bp for RBF103) before the ATG translation start site (Fig. 2). This insertion event was found to be responsible for the different hybridization patterns of the two classes. Analysis of the insertions showed a similarity to the *istB* gene of insertion sequence (IS) elements in the IS*21* family (Fig. 4).

TABLE 3. Quantitation of *cepA*-specific transcripts from primer extension analysis of RNAs from *B. fragilis* strains[a]

RNA source (μg)	Pixel intensity	Relative amt[b]
RBF103 (50)	9,355,120	39.6
CS29 (100)	1,433,520	3.0
ATCC 25285 (100)	1,040,640	2.2
CS14 (100)	754,960	1.6
638 (100)	534,032	1.1
CS44 (100)	472,976	1.0

[a] Quantitation was determined by counting pixel intensity of a scanned image of the autoradiograph from Fig. 6B.

[b] Calculated by comparison of pixel intensity values with the lowest value, that of CS44. Analysis of other exposures of the same autoradiograph gave comparable results.

The discovery of an IS-like element upstream of *cepA* suggests several possible mechanisms for the up-regulation of expression: (i) there may be strong outward-directed promoters present on the IS*21*-like element, and these promote high-level *cepA* transcription; (ii) the insertion event could have created a stronger promoter for *cepA* transcription by providing new −35 sequences; (iii) the insertion event displaces or disrupts a regulatory region, allowing increased *cepA* transcription; and (iv) any combination of these mechanisms. The importance of gene activation by IS elements is being recognized more and more, and there are examples of these activation mechanisms in the literature. The classic example of activation of prokaryotic gene transcription by promoters present on IS elements is the cryptic *bgl* operon of *E. coli*, which encodes enzymes required for β-glucoside utilization (30). In *Bacteroides* species, IS elements have been implicated in the activation of antibiotic resistance genes. These include the activation of *ermF* by IS*4351* in Tn*4351* on pBF4 plasmid (23, 24) and activation of *ermFS* by IS*4351* in the opposite orientation in Tn*4551* on pBI136 (34). In addition, a 1,598-bp IS element, IS*942*, has been shown to have integrated 19 bp upstream of the proposed initiation codon for *ccrA*, the gene encoding the class B metallo-β-lactamase of *B. fragilis* TAL3636 (22). However, in none of these *Bacteroides* examples has the mechanism of activation been established.

IS*21* has also been shown to be a source of mobile promoters; one active promoter has been localized to within 170 bp of the left end of IS*21*, and the other promoter located closer to the center of IS*21* reads into the element (31). IS*2* has been shown to form a new promoter upon insertion into target DNA and to up-regulate transcription of the *E. coli ampC* β-lactamase gene 20-fold (13). The −10 region from the original

ampC promoter was retained in these insertion mutants, but the −35 region(s) was IS*2* derived. Importantly, the transcription initiation site in the *ampC*:IS*2* mutant was identical with that of the wild-type *ampC* promoter (13). In *E. coli* HB251, a 116-bp insert with similarity to IS*1* was found to have inserted in the native promoter region of the *blaT-6* gene encoding TEM-6 β-lactamase. The original −10 region was retained, and the new −35 sequences were provided by the IS*1*-like element, increasing activity of the promoter 10-fold (10).

These latter examples are analogous to the situation observed with the IS*21*-like element present upstream of *cepA* in the high-activity strains. This insertion does not alter the location of the *cepA* transcription start site from that observed for low-activity β-lactamase strains. However, one needs to keep in mind the differences between *E. coli* and *B. fragilis*. We have shown previously that the consensus −10 and −35 regions of *E. coli* promoters are not recognized as such by *B. fragilis* transcriptional machinery (36). It is possible that *Bacteroides* genes contain their own versions of −10 and −35 recognition sequences, of which the latter has been disrupted and perhaps altered by the IS*21*-like element. Regardless of the specific structures required of a *Bacteroides* promoter, these hybrid promoters from the high-β-lactamase-activity strains are very strong, resulting in up to 40-fold-higher levels of *cepA* message. It does not seem likely that message stability plays a significant role in differential regulation of *cepA* because the transcription start sites were the same and nucleotide sequences downstream of *cepA* were nearly identical for both expression classes.

The results presented above strongly support a role for the ISs in transcriptional activation of *cepA*. However, there is evidence that the insertion also may have disrupted normal *cepA* regulation. This evidence stems from the first experiments, in which the increase in β-lactamase activity observed for the subcloned low-activity *cepA* genes was not consistent with the high copy number of the vector (Table 2). This observation was confirmed latter by the lack of detectable CAT activity in *B. fragilis* 638 extracts containing the pFD395:low-activity class *cepA* promoter constructs. We had expected to see low-level CAT activity, similar to the relatively low amount of β-lactamase activity seen in wild-type *B. fragilis* 638 or 25285 cells, especially since these plasmids are present in copy numbers greater than one. It is possible that some form of negative regulation maintains the expression of *cepA* at a very low level and that the insertion disrupts a repressor binding site and provides an improved promoter structure, leading to the 20- to 60-fold increases in β-lactamase activity.

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