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Sequencing the Gene for an Imipenem-Cefoxitin-Hydrolyzing Enzyme (CfiA) from *Bacteroides fragilis* TAL2480 Reveals Strong Similarity between CfiA and *Bacillus cereus* β-Lactamase II

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Using a newly constructed *Bacteroides fragilis-Escherichia coli* cloning shuttle vector, pJST61, we have cloned the cefoxitin (FOX)-imipenem (IMP) resistance determinant from *B. fragilis* TAL2480. FOX-IMP resistance in this strain results from the production of a periplasmic, Zn^{2+}-containing β-lactamase which hydrolyzes carbapenems and cephamycins and whose activity is resistant to clavulanic acid but sensitive to Zn^{2+}-binding reagents, including EDTA. The pJST61 vector permits efficient library construction in *E. coli* and allows for the transfer of the library to *B. fragilis* recipients for the screening or selection of specific phenotypes. The library clone containing the FOX-IMP resistance gene was detected after transfer to *B. fragilis* TM4000 (Fox-Imp^s) selecting for Fox^r. One of the isolates carrying plasmid pJST241 is resistant to FOX and IMP and synthesizes a periplasmic protein with substrate and inhibitor properties identical to those of strain TAL2480. On the basis of deletion analysis, Tn*1000* insertion mutations, and DNA sequencing, we have defined the 747-base *cfiA* (FOX-IMP resistance) gene within the 3.6-kilobase cloned insert in pJST241. The *cfiA* gene contains an open reading frame that could code for a precursor protein of 249 amino acids and with a molecular mass of 27,260 daltons. A potential signal sequence has been identified at the N terminus of this protein; cleavage within this sequence would result in a protein of 231 amino acids with a molecular mass of 25,249 daltons. The CfiA protein shows remarkable similarities to the exported, Zn^{2+}-requiring, type II β-lactamase Blm proteins from *Bacillus cereus* 569/H and 5/B/6. Although overall amino acid identity is only 32%, the Zn ligand-binding His and Cys residues are precisely conserved and the amino acids in the vicinity of these sites show strong similarities (>80%) when the CfiA and Blm proteins are compared.

Bacteroides fragilis is one of the most important anaerobic pathogens of humans. Since members of the *B. fragilis* group possess endogenous β-lactamase enzymes with activities against many narrow- and broad-spectrum penicillins and cephalosporins (18, 26), the preferred drugs for treatment of *B. fragilis*-associated infections include imipenem (IMP), a carbapenem, and cefoxitin (FOX), a cephamycin. Indeed, recent drug survey studies have shown IMP and FOX to be the most active β-lactam antibiotics against *B. fragilis* (6, 37).

We have previously described two *B. fragilis* clinical isolates, TAL2480 and TAL3636, that are highly resistant to both IMP and FOX as well as most other β-lactam antibiotics (5, 7). Both strains possess a periplasmic β-lactamase that hydrolyzes β-lactam substrates, including cephamycins and carbapenems. In contrast to the endogenous β-lactamases of *B. fragilis*, the purified enzyme from strain TAL2480 was not inhibited by clavulanic acid. β-Lactamase activity from TAL2480 was completely inhibited in the presence of EDTA, but the inhibition could be reversed by the addition of Zn^{2+}, suggesting that this β-lactamase is a Zn-metalloenzyme. The substrate profile and zinc requirement identify this *B. fragilis* enzyme as a class B β-lactamase (1). The *Bacillus cereus* type II β-lactamases of strains 569/H/9 (16, 17) and 5/B/6 (19) are the most extensively studied enzymes of this class, which may also include the L1 β-lactamase of *Pseudomonas maltophilia* GN12873 (29) and the β-lactamase of *Flavobacterium odoratum* GN14053 (31).

To provide an additional drug resistance marker for use in genetic experiments as well as to aid our studies on the distribution of the genes for the FOX-IMP resistance determinant in *B. fragilis* and other organisms, we have cloned the gene for this enzyme from *B. fragilis* TAL2480. The genetic locus in *B. fragilis* TAL2480 has been designated *cfiA* (FOX-IMP resistance). For these experiments, we have relied on a new series of shuttle cloning vectors which contain replicons for both *E. coli* and *B. fragilis*, antibiotic resistance determinants for each host, *oriT* regions that allow plasmid transfer by conjugation between *E. coli* and *B. fragilis*, and a convenient cloning target that allows efficient isolation only of plasmids containing cloned inserts.

We have sequenced the *cfiA* gene and deduced the primary sequence of its β-lactamase protein. The deduced CfiA protein of *B. fragilis* shows remarkable similarities to the Blm type II β-lactamases from *Bacillus cereus*.

MATERIALS AND METHODS

Media and growth conditions. ML broth or Luria agar was used as the nutrient source for all *E. coli* strains; brain heart infusion (BBL Microbiology Systems, Cockeysville, Md.) broth (BHIS) supplemented with 5 μg of hemin per ml and 5 mg of yeast extract per ml was used for all *B. fragilis* cultures. Bacto-Agar (Difco Laboratories, Detroit, Mich.) was added at 1.5% for plates. Antibiotics were added when required in the following concentrations (μg/ml), unless otherwise indicated: ampicillin (AMP), 200; kanamycin, 50; tetracycline, 5; streptomycin, 200; gentamicin, 50; ciprofloxacin, 1; spectinomycin, 50; clindamycin (CLN), 6; FOX, 25 or 50; and IMP, 8. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.), except for ciprofloxacin

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
E. coli		
HB101	*recA rpsL*	21
KS303	KS272 *lpp-5508*	35
DW1030	Δ*lacX74 rpsE recA13*	14, 28
JF270	Δ*lacX74 recA rpsL*	14, 28
B. fragilis		
TM4000	Standard recipient, endogenous β-lactamase	14, 38
TAL2480	Cln^r Tet^r Fox-Imp^r	TAL[a] clinical isolate (5, 7)
TAL3636	Cln^r Tet^r Fox-Imp^r	TAL clinical isolate (5, 7)
TAL4170	Tet^r Fox^r Imp^s	TAL clinical isolate (8)
TMP14	Amp^r	TAL clinical isolate (22)
TMP230	Cln^r Tet^r	TAL clinical isolate (22)
B. distasonis		
TAL7860	Fox-Imp^r	TAL clinical isolate (15a)
E. coli		
F′ *lac pifC21*		23
pDG5	pBR322 replicon, RK2*oriT* Amp^r	13
pBR322	Amp^r Tet^r	36
pRK231	RK2 derivative, Kan^r Tet^r	13
pEcoR251	pBR322 replicon, λ p_R-*endRI*; Amp^r	34
pJST51	pEcoR251 containing RK2*oriT*; Amp^r	This study
pJST54	pJST51 with 4.4-kb *Eco*RI fragment of pBFTM10; Cln^r Tet^r (*Tc^r)	This study
PJST55	pJST54 *Cla*I deletion; Cln^r Tet^s (*Tc^s)	This study
pRWH012	Contains *blm* gene	16
B. fragilis		
pBFTM10	Tra$^+$ Tn*4400* Cln^r	
pBFTM2006	Cryptic plasmid	38
Shuttle vectors		
pGAT400	pBFTM10-pDG5 chimera, Amp^r Tet^r (*Tc^r) Cln^r	14
pJST61	pJST55-pBFTM2006, Amp^r Cln^r	This study
pJST62	pJST61 with pBFTM2006 in opposite orientation	This study
pJST241	pJST61 with 3.5-kb TAL2480 insert; Cln^r Fox-Imp^r	This study
pJST2411	pJST241 with 1.0-kb *Bgl*II deletion; Cln^r Fox-Imp^r	This study
pJST2414	pJST61 with 1.5-kb *Bst*YI fragment from pJST214 Cln^r Fox-Imp^r (reduced)	This study
pJST241GD08	pJST241::Tn*1000*; Cln^r Fox-Imp^r	This study
pJST241GD12	pJST241::Tn*1000*; Cln^r Fox-Imp^s	This study
pJST241GD023	pJST241::Tn*1000*; Cln^r Fox-Imp^r (reduced)	This study
pJST241GD017	pJST241::Tn*1000*; Cln^r Fox-Imp^r	This study
pJST241GD039	pJST241::Tn*1000*; Cln^r Fox-Imp^s	This study

[a] TAL, Tufts Anaerobe Laboratory.

(Miles Pharmaceutical Co., West Haven, Conn.), CLN and spectinomycin (The Upjohn Co., Kalamazoo, Mich.), and FOX and IMP (Merck Sharp & Dohme, Rahway, N.J.).

E. coli cultures were grown aerobically at 37°C with vigorous aeration. *B. fragilis* cultures were grown at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) with an atmosphere composed of 85% N_2, 10% CO_2, and 5% H_2.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pEcoR251 (provided by D. Woods [34]) is a pBR322 derivative containing the *E. coli* *Eco*RI endonuclease gene, *endRI*, under the control of the rightward promoter of bacteriophage lambda. The *endRI* gene product is lethal to *E. coli* unless its synthesis is repressed by the lambda cI repressor or insertionally inactivated as by the cloning of DNA into the unique *Bgl*II site within the gene (24, 34).

Construction of plasmids pJST51, pJST54, pJST55, and pJST61. pJST51 was constructed by replacing the 0.8-kilobase (kb) *Pst*I-*Eco*RI restriction fragment of pDG5 with the 1.9-kb *Pst*I-*Bam*HI fragment from pEcoR251 containing the λ p_R-*endRI* gene fusion; the 0.4-kb *Eco*RI-*Bam*HI fragment from the tetracycline resistance gene of pBR322 was used as a linker. The resultant 5.9-kb plasmid, pJST51 (Fig. 1), was isolated as an AMP-resistant colony after transformation into HB101 (λ). pJST51 has a fully functional RK2 *oriT* from pDG5, allowing mobilization in *trans* by pRK2 derivatives, and the λ p_R-*endRI* fusion produces a lethal product in the absence, but not in the presence, of cI repressor. The *endRI* gene contains a *Bgl*II site unique to pJST51; cloning into this site inactivates the *endRI* gene.

To construct pJST54 with a marker selectable in *B. fragilis*, the 4.4-kb *Eco*RI B fragment of pGAT400 which contains most of Tn*4400*, except for the outside ends of both IS*4400*$_L$ and IS*4400*$_R$ (28), and expresses resistance to CLN in *B. fragilis* and tetracycline (*Tc^r) in *E. coli* (12) was ligated to an *Eco*RI partial digest of pJST51. Upon transformation to HB101(λ) and selection for Amp^r and *Tc^r, the 10.3-kb

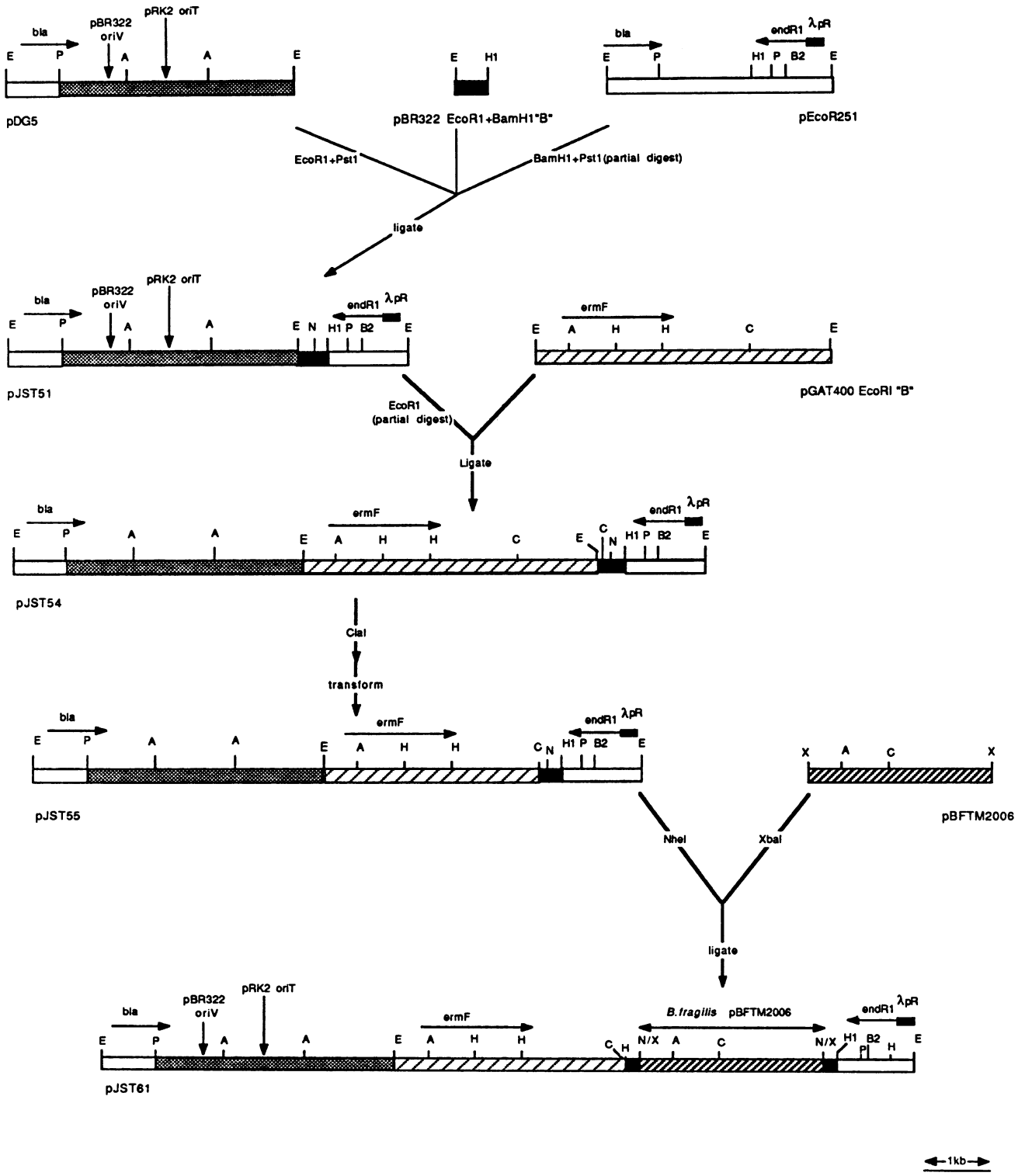

FIG. 1. Construction of the *B. fragilis* cloning shuttle vector, pJST61. The starting plasmids are shown by using different patterns and fillings to emphasize the origins of the DNA components making up the final vector. Construction details are discussed in the text. The length and direction of transcription of the genes coding for *bla* (AMP resistance), *ermF* (CLN resistance), and *endRI* (endonuclease *Eco*RI) are designated by horizontal arrows. Vertical arrows indicate the locations of the indicated components. The λ p_R region of the λ p_R-*endRI* gene fusion is indicated by a black box at the end of the arrow. Restriction endonuclease abbreviations are as follows: A, *Ava*I; B2, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; H1, *Bam*HI; N, *Nhe*I; P, *Pst*I; and X, *Xba*I. N/X denotes an *Nhe*I-*Xba*I fusion site. All drawings are to scale as shown.

plasmid, pJST54, was isolated. Since the two intact inside ends of IS*4400*$_L$ and IS*4400*$_R$ possess a substantial inverse transposition activity, the remainder of IS*4400*$_R$ was removed from pJST54 by complete digestion of this plasmid with *Cla*I. The linear DNA was used to transform HB101(λ) to Amp^r, and candidates were verified by testing for tetracycline sensitivity under aerobic growth conditions. The resultant 9.3-kb plasmid, pJST55, possesses the active CLN resistance gene *ermF* originally found in Tn*4400*.

pJST61 was constructed by adding a *B. fragilis* replicon to pJST55. The *B. fragilis* cryptic plasmid pBFTM2006 originally isolated from *B. fragilis* TMP10 (38) was digested with *Xba*I and ligated into the *Nhe*I site of pJST55. The ligation mix was used to transform HB101(λ)(pRK231) selecting for resistance to AMP and kanamycin. Colonies were pooled, cultured in ML broth, and mated with *B. fragilis* TM4000. Transconjugants were selected by plating the mating mix on BHIS containing CLN and ciprofloxacin. All CLN- and ciprofloxacin-resistant *B. fragilis* colonies must contain the pBFTM2006 replicon inserted into pJST55. The pBFTM2006 replicon was inserted in both orientations to yield plasmids pJST61 and pJST62.

Genetic techniques. The protocols for transformation of *E. coli* and the mobilization of shuttle plasmids from *E. coli* to *B. fragilis* have been previously described (14, 21).

DNA manipulations and analysis. Rapid DNA preparations were made by an alkaline lysis method described previously (21). Large-scale DNA purifications were performed by the method of Clewell and Helinski for plasmids (3) or of Deonier and Mirels for chromosomal DNA (9). All restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs, Inc. (Beverly, Mass.) and used under conditions specified by the supplier. Following digestion, DNA fragments were separated by electrophoresis through agarose (SeaKem; FMC Corp., Marine Colloids Div., Rockland, Maine) horizontal gels. Individual DNA fragments to be used in plasmid constructions were isolated by excision and purification from 0.5% low-melting-point agarose (SeaPlaque; FMC Corp.). Unless otherwise noted, ligation reactions were performed by mixing linear DNA fragments at a molar ratio of free ends of 2:1 (insert:vector).

DNA-DNA hybridization. Restriction endonuclease digests resolved on 0.6% agarose horizontal gels were transferred to GeneScreen (New England Nuclear Corp., Boston, Mass.) membranes by the method of Southern (33) as modified by the supplier. The filters were hybridized with [α-^{32}P]dCTP probes labeled by the random primer method under conditions recommended by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The washed filters were exposed to XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Construction of TAL2480 chromosomal DNA libraries in pJST61. Purified chromosomal DNA from *B. fragilis* TAL2480 was partially digested with *Sau*3A. DNA fragments were then separated by size by sucrose density centrifugation (10 to 40%, wt/vol). Two DNA pools containing fragments with sizes of >15 kb or 1 to 15 kb were prepared. The *B. fragilis* chromosomal DNA fragments were ligated into the unique *Bgl*II site of the shuttle cloning vector pJST61 by using a molar ratio of free ends of 1:4 (insert: vector). Ligation mixes were used to transform the nonlysogenic *E. coli* HB101(pRK231) and KS303(pRK231) with selection for AMP and kanamycin resistance. Colonies were pooled by flooding the plates with ML broth. These pools constituted the pJST61-TAL2480 libraries in *E. coli* and were stored at −20°C in 50% glycerol.

Tn*1000* mutagenesis. For Tn*1000* mutagenesis, plasmids were first introduced by transformation into *E. coli* JF270 cells containing F′ *lac pifC21*. (The F′ *lac* derivative F′ *lac pifC21* was used as the source of Tn*1000*, because the active PifC product inhibits transfer of pRK2 and its derivatives as well as the mobilization in *trans* of plasmids containing the *oriT* region of RK2 [23]. In the presence of F′ *lac pifC21*, RK231-mediated mobilization of mutagenized shuttle cloning vectors to *B. fragilis* is efficient.) In the second step, these plasmids were mobilized into the recipient DW1030 (pRK231) selecting for resistance to spectinomycin, kanamycin, and AMP. DNA analysis revealed that >90% of the transconjugants had been mobilized via Tn*1000*-mediated cointegration with F′ *lac pifC21*. Colonies were pooled by flooding the plates with ML broth, subcultured, and mated with *B. fragilis* TM4000. Transconjugants were selected on BHIS CLN-gentamicin-streptomycin plates and then tested for the loss or retention of the FOX-IMP phenotype by replica plating to BHIS plates containing FOX or IMP.

β-Lactamase assays. Plasmid-containing *B. fragilis* strains were grown overnight from a single colony in BHIS CLN broth; TAL2480 was grown without antibiotic. Crude cell extracts were obtained as previously described (5). β-Lactamase activity of the crude enzyme extracts towards nitrocefin (NTC; Becton Dickinson Microbiology Systems, Hunt Valley, Md.) was determined spectrophotometrically at A_{482}, as previously described (5, 25). β-Lactamase activity is reported as the change in A_{482} due to the destruction of NTC per minute per milligram of protein (for sonic extracts) or per A_{600} unit (for cells), in the initial linear phase of the reaction at pH 7.0 and 22°C. β-Lactamase inhibition studies were performed by preincubating cell extracts with 1 mM EDTA or 2 mM clavulanic acid for 1 h at 4°C before assaying with NTC.

Cell fractionation and enzyme localization. Overnight *B. fragilis* cultures were harvested by centrifugation, and the supernatants were recovered. The cells were washed twice in 0.1 M Tris buffer (pH 7.2) (10 ml/g of cells) and suspended in 0.5 M sucrose–0.03 M Tris (pH 7.2) (50 ml/g of cells). After standing at room temperature for 5 min, the cells were collected by centrifugation, shocked by suspension in ice-cold deionized water (30 ml/g of cells), and shaken for 10 min in an ice bath. Cells were removed by centrifugation, and the supernatant was recovered; this fraction is called the shockate. The cell pellet was suspended in 0.1 M KPO_4 buffer, pH 7.0 (30 ml/g of cells), and sonicated for 1 min in an ice slush bath by using a sonicator (Branson Sonic Power Co., Danbury, Conn.) at a high power setting; this fraction is called the postshock sonic extract and contains membrane and cytoplasmic enzymes.

DNA sequencing. Sequencing was performed by the dideoxynucleotide-chain termination method of Sanger et al. (30) by using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Oligonucleotides for use as sequencing primers were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.). The following primers were used: Tn*1000*$_R$, 5′CAACGAATTATCTCCTTAAC; Tn*1000*$_L$, 5′TCAATAAGTTATACCAT; Pjt2 (from the *endRI* gene [24]), 5′GGAAAGTGGCTCTCAGAGAGC; Pjt3 (*cfiA* internal), 5′TCCATGAACGAAGAGGCGAAA; Pjt4 (*cfiA* internal), 5′GTTAAGCGTTTCTTCCCG; and Pjt5 (*cfiA* internal), 5′CGGGAAGAAACGCTTAAC.

MIC determinations. MICs were determined by an agar dilution technique by using a Steers replicator (6).

RESULTS

Construction of shuttle cloning vector pJST61. To clone the FOX-IMP resistance gene, it was first necessary to construct an *E. coli-B. fragilis* shuttle vector into which inserts of additional DNA would provide a selectable phenotype in both organisms. Because there are no efficient systems for transduction or transformation of *B. fragilis*, *B. fragilis* chromosomal libraries cannot be directly isolated in *B. fragilis* but may be isolated in *E. coli*. However, in general, *B. fragilis* genes are poorly expressed in *E. coli*, making library selection and screening in this background inefficient. Guiney et al. (13) introduced the use of pDG5, a plasmid containing the *oriT* region of RK2, to construct chimeric plasmids with *B. fragilis* replicons. These plasmids can be efficiently mobilized by conjugation from *E. coli* to *B. fragilis*. If the *B. fragilis* replicon is also Tra^+ (pBF4 or pBFTM10), then retransfer of the plasmids from *B. fragilis* to *B. fragilis* or to *E. coli* recipients can occur. Libraries constructed in a shuttle vector could be introduced into *E. coli* by transformation and then mobilized to *B. fragilis* for expression of the cloned genes. However, as in all library constructions, a large percentage of the transformants may not contain any cloned DNA. Therefore, the process of screening for a gene for which there is no direct selection is made even more difficult. In the construction of the cloning shuttle vector pJST61, we overcame these difficulties. An important feature of pJST61 is the presence of a target gene that must be inactivated by insertion of cloned DNA for survival of transformants in certain *E. coli* hosts. In addition, this lethal target gene is located distal to a regulated strong *E. coli* promoter, so that expression of the cloned genes in *E. coli* may be tested.

Isolation of *B. fragilis* FOX-IMP resistance determinant. Libraries of 2.2×10^4 independent clones from the TAL2480 chromosome were prepared in pJST61 as described in Materials and Methods. Direct plating of these libraries in *E. coli* on selective media with IMP or FOX failed to reveal resistant candidates. The libraries were subcultured, grown to mid-exponential phase, and mated with *B. fragilis* TM4000, and transconjugants were selected anaerobically on BHIS with FOX and ciprofloxacin. Three *B. fragilis* candidates originating from the library in KS303(pRK231) (pJST61-TAL2480) were isolated in two independent matings. Plasmid DNAs from the three FOX-resistant isolates each contained an insert of 3.6 kb located at the *Bgl*II site of pJST61. All three plasmids seemed to be identical on the basis of restriction enzyme analysis. One of the plasmids, designated pJST241, was chosen for further study. When pJST241 was retransferred to *E. coli* HB101 and these cells were tested for FOX-IMP resistance, no significant differences in plating with respect to the parental HB101 strain were observed. Because of the presence of the *bla* gene on the pDG5 portion of pJST61, we could not use AMP resistance as an indicator of the expression of the *cfiA* gene in *E. coli* HB101. A detailed restriction map of pJST241 is shown in Fig. 2A.

Relationship of the β-lactamase enzymes synthesized by TAL2480 and TM4000 containing pJST241. We have previously found that strain TAL2480 synthesizes a periplasmic, Zn^{2+}-requiring β-lactamase which is responsible for the inactivation of FOX, IMP, and many other β-lactam antibiotics (11). The activity of this enzyme is resistant to clavulanic acid and sensitive to EDTA. Cell extracts from TM4000 containing pJST241 had approximately the same specific activity against NTC as did extracts prepared from TAL2480 (Table 2). Small amounts of NTC-degrading activity could be detected in cell extracts prepared from cells of TM4000 containing pJST61; this is probably due to the endogenous β-lactamase of TM4000. The ability of TM4000(pJST241) extracts to hydrolyze NTC was not inhibited by 2 mM clavulanic acid but was almost completely inhibited by 1 mM EDTA. This inhibition could be reversed by the addition of 1 mM Zn^{2+} (Table 2). These characteristics indicate that pJST241 contains a gene for a FOX-IMP-degrading enzyme with properties identical to those of the enzyme from TAL2480.

Although the specific activity of NTC hydrolysis of the enzyme preparation from TM4000 containing pJST241 was comparable to that of the TAL2480 parental strain (Table 2), the MICs of FOX and IMP for the two strains were different: 64 μg/ml for TM4000(pJST241) versus 256 μg/ml for TAL2480 for FOX and 16 μg/ml for TM4000(pJST241) versus 128 μg/ml for TAL2480 for IMP. This discrepancy could be explained by differential permeability of the antibiotics through the outer membranes of the two strains, which is often defined as crypticity (4).

When TM4000 cells containing pJST241 were subjected to the osmotic shock fractionation procedure, >90% of the β-lactamase activity was found associated with the periplasmic fraction, in contrast to the cytoplasmic enzymes β-galactosidase and glucose-6-P dehydrogenase, which were retained by the shocked cells (95 and 100%, respectively). Similar results were obtained with the parental strain, TAL2480. Thus, the cloned *cfiA* protein from TAL2480 can be properly localized in TM4000 cells.

Identifying the *cfiA* gene in the insert of pJST241. In order to localize the gene(s) responsible for FOX-IMP resistance within the TAL2480 chromosomal fragment of pJST241, a deletion and a subclone were generated from the plasmid in the *E. coli* background (Fig. 2A). The resulting plasmids were then transferred to TM4000 and tested for FOX-IMP resistance. Removal of the 1.1-kb *Bgl*II fragment of pJST241 to create pJST2411 had no effect on the MIC of either FOX or IMP in whole cells and showed only a small decrease (40%) in the specific activity of the enzyme in extracts prepared from TM4000(pJST2411) cells (Table 2). Thus, the remaining 2.5 kb of the insert should contain the structural gene for FOX-IMP resistance. This 2.5-kb region was separated into two *Bst*YI fragments of 1.5 and 1.0 kb each. pJST2414 contains the larger *Bst*YI fragment (Fig. 2A). The resistance to FOX-IMP and the β-lactamase activity of TM4000(pJST2414) were substantially lower than those of TM4000(pJST241); the specific activity was only 25% of that of TM4000(pJST241) (Table 2). This suggests that while the *cfiA* structural gene is contained in the 1.5-kb *Bst*YI fragment, the presence of the 1.0-kb adjacent DNA fragment is required for high levels of FOX-IMP resistance and β-lactamase activity.

The β-lactamase structural gene was localized more precisely within the *B. fragilis* DNA of pJST241 by transposon mutagenesis. That is, loss or retention of the FOX-IMP phenotype was determined after random Tn*1000* insertion, and then the sites of the various insertions were mapped by restriction analysis. In several experiments, independent Tn*1000* insertions were mapped to 15 different sites within the 2.5-kb *Bam*HI-*Bgl*II fragment of pJST241 (Fig. 2B). Insertions resulting in a Fox-Imp^s phenotype were mapped to an 0.8-kb portion of the insert. To determine the extent of the region coding for FOX-IMP resistance, we mapped the site of the Tn*1000* insertion giving a FOX-IMP-resistant phenotype closest to the site of insertion resulting in a

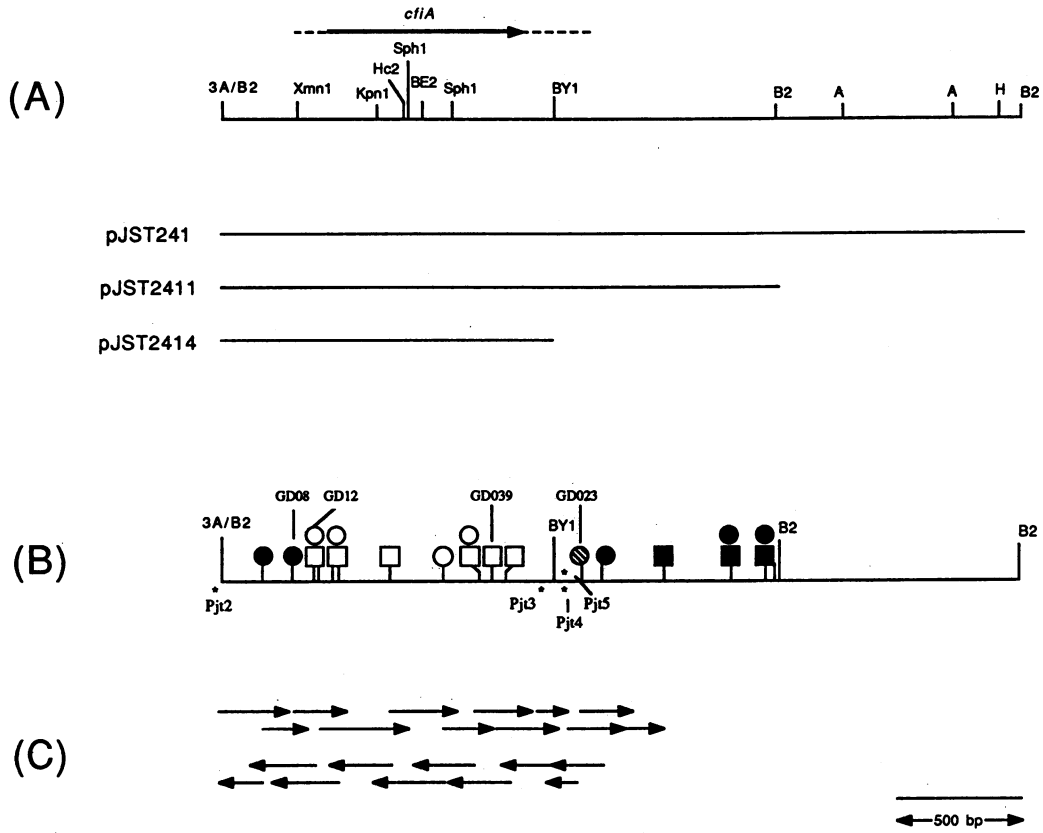

FIG. 2. Maps and sequencing strategy. (A) Partial restriction endonuclease map of the TAL2480 chromosomal DNA insert cloned into pJST241. The solid arrow indicates the position and direction of transcription of the ORF encoding the *cfiA* structural gene. The dashed lines on either end of the arrow indicate upstream and downstream regions required for the high level activity of the enzyme. Lines underneath the map show the extent of the TAL2480 insert in pJST241 and subclones pJST2411 and pJST2414. Abbreviations: A, *Ava*I; B2, *Bgl*II; BE2, *Bst*EII; BY1, *Bst*YI; H, *Hin*dIII; Hc2, *Hin*cII; and 3A, *Sau*3AI. (B) Tn*1000* insertion map of pJST241. Squares indicate Tn*1000* insertions in the left-to-right orientation, while circles indicate insertions in the opposite orientation with respect to the 3A/B2 fusion site that marks the left end of the TAL2480 insert. Open symbols indicate no detectable enzyme activity, closed symbols indicate high levels of enzyme activity, and hatched symbol indicates intermediate levels of enzyme activity. Tn*1000* insertions of special interest are indicated by their insertion number. Asterisks indicate areas to which complementary oligonucleotides were synthesized for use as primers in DNA sequencing; those asterisks below the line (Pjt2, Pjt3, and Pjt4) are complementary to the *cfiA* sense strand, while that above the line (Pjt5) is complementary to the antisense strand. (C) Sequencing strategy for *cfiA*. The arrows indicate the site of initiation of sequencing, the length of sequence, and the strand sequenced. Figures are drawn to scale as indicated. bp, Base pairs.

sensitive phenotype. On one end, a Fox-Imp^r mutant (pJST241GD08) was placed within approximately 100 bases of a sensitive mutant (pJST241GD12). On the other end, the Tn*1000* insertions that either retain full resistance or lose all resistance can be mapped either 200 bases (pJST241GD017) or 300 bases (pJST241GD039) away from the internal *Bst*YI site. One insertion, pJST241GD023, that resulted in a reduced but not completely sensitive phenotype, much like pJST2414 (Table 2), was localized close to the internal *Bst*YI site. This information localizes the FOX-IMP determinant to an 800- to 1,200-base-pair region within the 2.5-kb *Bam*HI-*Bgl*II segment. The internal *Bst*YI site is very close to one end of the gene, since the exclusion of genetic information beyond it, as in pJST2414 and pJST241GD023, still allows expression of a Fox-Imp^r phenotype, albeit at a reduced level.

TABLE 2. β-Lactamase specific activities of cell extracts

Strain or plasmid[a]	Sp act[b]	% of NTC specific activity retained after pretreatment with:		
		Clavulanic acid	EDTA	EDTA[c]
pJST61	6 (4)	ND	ND	ND
pJST241	159 (8)	108	8	106
pJST2411	94 (5)	132	5	115
pJST2414	38 (6)	46	18	128
pJST241GD023	71 (5)	102	10	70
TAL2480	189 (8)	73	8	60

[a] All plasmids were present in TM4000.
[b] Reported as A_{482} units per milligram of protein. Numbers in parentheses indicate number of repeated experiments. All assays were performed in 0.1 M KPO_4 (pH 7.0) unless otherwise noted.
[c] Assay buffer was 0.1 M KPO_4 plus 0.5 mM $ZnSO_4$.

Southern blot hybridizations establish that the insert in pJST241 was derived from the chromosome of TAL2480. A 5.2-kb fragment containing 5 kb of sequence internal to Tn*1000* and 200 base pairs of sequences internal to *CfiA* was isolated as a *Sph*I fragment from pJST241GD039 and used as a hybridization probe. Since Tn*1000* shows no homology with *B. fragilis* chromosomal DNA, any hybridization would result from homology to the 200-base-pair *cfiA* internal fragment. The probe hybridized strongly under high-stringency conditions to a 6.8-kb fragment of the chromosome

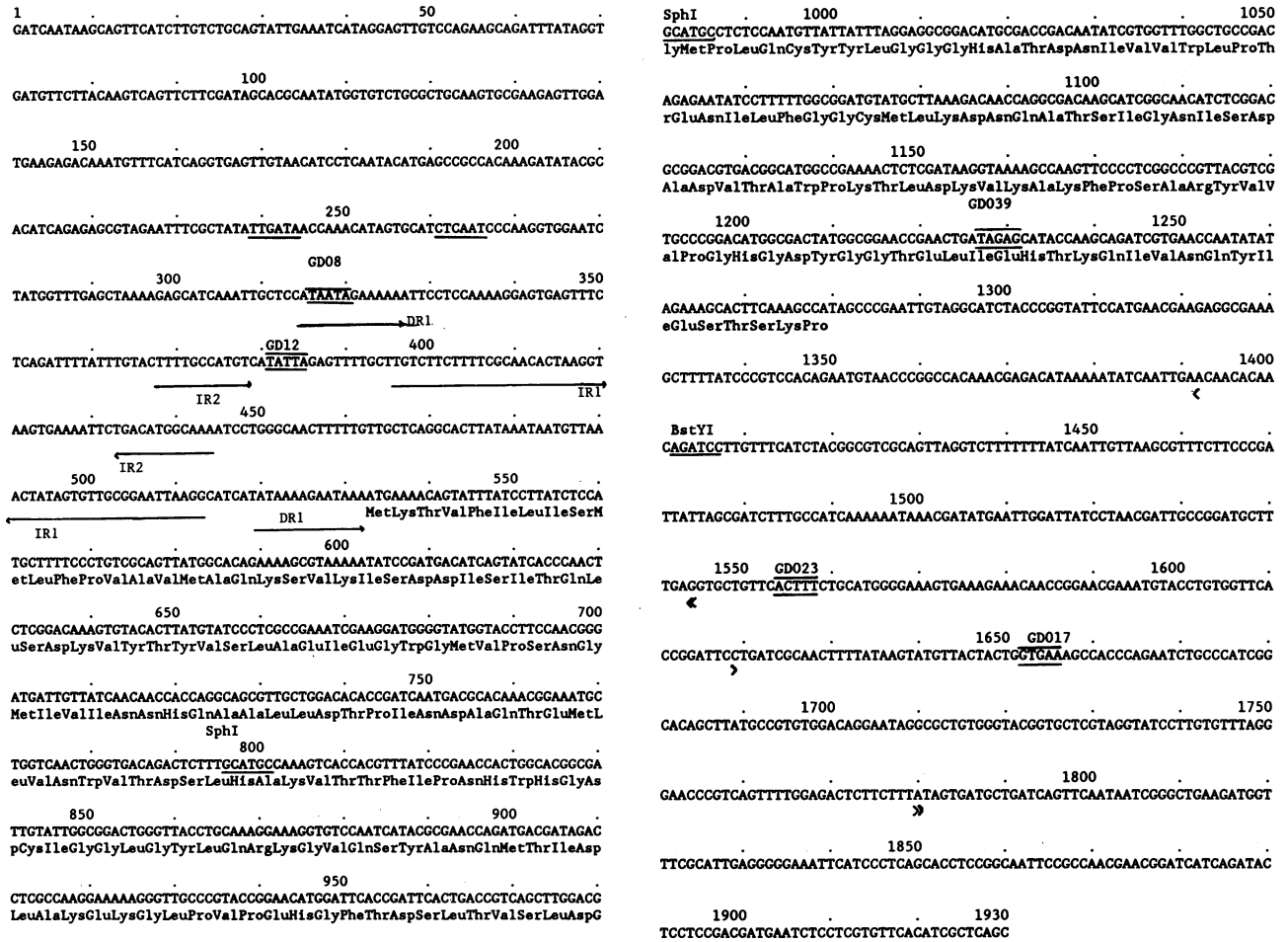

FIG. 3. DNA sequence of the sense strand and deduced amino acid sequence of the *B. fragilis* TAL2480 *cfiA* gene and protein. Numbers corresponding to the nucleotide base of the DNA insert sequenced in pJST241 are shown above the sequence. Number 1 represents the first base of the cloned insert at the *Bgl*II-*Sau*3A fusion site. The CfiA precursor protein begins at nucleotide 533 and terminates at nucleotide 1280. Two potential ORFs distal to the *cfiA* gene are indicated by < to > and << to >>. →, Positions of direct and inverted repeats in the putative control region. The sites of important Tn*1000* insertions are boxed. Important restriction endonuclease sites are also indicated.

restricted with *Pst*I (data not shown). The probe also hybridized strongly with *Pst*I-treated chromosomal DNA from TAL3636, producing a band identical in size to that seen with TAL2480. Chromosomal DNA of these two strains was restricted with several other enzymes. In every case, no differences in their hybridization profiles were detectable when probed with the *cfiA* internal fragment. TAL3636 and TAL2480 both possess a Zn^{2+}-requiring broad-spectrum β-lactamase (5, 7).

Sequencing the *cfiA* gene and determination of the primary sequence of the CfiA protein. pJST241::Tn*1000* isolates that were Fox-Imp^s or resistant isolates with insertions mapping close to the site of sensitive insertions were sequenced by utilizing the ends of Tn*1000* as sites of primer annealing (Fig. 2B and C) (20). This strategy yields overlapping sequences on both the sense and antisense strands (Fig. 2C). Oligonucleotides complementary to sequences surrounding the *Bgl*II site in the *endRI* gene and to sequences within the *cfiA* gene were synthesized as needed to fill any gaps in the sequence.

The DNA sequence of 1,930 bases of the sense strand is shown in Fig. 3; these data have been submitted to GenBank and have been assigned accession number M31518. There is a 747-base open reading frame (ORF) initiating at ATG (nucleotides 533 to 535) and terminating at TAG (nucleotides 1280 to 1282); all Tn*1000* insertions within this ORF eliminate FOX-IMP resistance. This ORF has the potential to code for a protein of 249 amino acids with a predicted molecular mass of 27,260 daltons (Da). No other significant ORF can be detected in the region proximal to position 533. The signals for *cfiA* expression should be contained in the region distal to position 316, the site of a Tn*1000* insertion that does not alter FOX-IMP resistance. Potential promoter regions showing strong homology to the *E. coli* promoter consensus sequences were not apparent in this region. However, a potential −35 region occurs at positions 238 to 244, 16 bases from a potential −10 sequence at positions 261 to 266. These sites are well upstream of the proposed regulatory region defined by Tn*1000* insertion pJST241GD08.

No clear Shine-Dalgarno sequence resembling that of *E. coli* or *B. fragilis* (27) is apparent in the region just upstream of the ATG codon at position 533. This raises questions as to the actual start site of translation. It cannot be upstream of position 533, since several stop codons upstream interrupt the relevant reading frames. Translation could begin further downstream, e.g., at position 560 (ATG), 581 (ATG), 641 (GTG), or 683 (ATG). Of these, only position 683 is preceded by an apparent Shine-Dalgarno sequence.

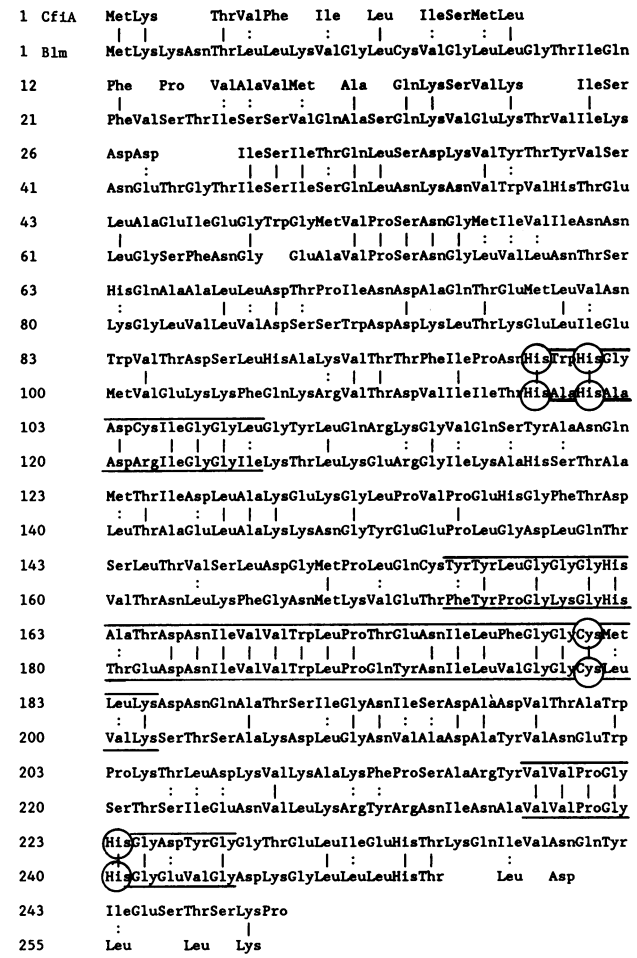

FIG. 4. Comparison of the deduced primary sequences of the CfiA enzyme from *B. fragilis* TAL2480 and the Blm enzyme from *Bacillus cereus* 569/H. The CfiA sequence is listed on top of each pair of lines. Numbers located at the left correspond to the residue numbers of the respective proteins. Aligned identical residues are indicated by a vertical line; residues with similar R groups are indicated by a colon. Amino acids in the Blm enzyme known to be involved in Zn^{2+} binding are circled; note that these residues occur at the same position in the CfiA protein. Regions of extensive similarity are underlined and overlined.

Comparison of the *B. fragilis* TAL2480 and *Bacillus cereus* 569/H β-lactamase type B proteins. Computer analysis (Microgenie; Beckman Instrument Co., Palo Alto, Calif.) failed to find any regions of significant homology in the DNA sequences of the genes coding for the *B. fragilis* (*cfiA*) and *Bacillus cereus* class B (*blm*) (16, 19) β-lactamase enzymes. However, the proteins show similar enzymatic and physical properties. The molecular masses of the deduced proteins are 27,260 Da for the *B. fragilis* enzyme (assuming translation initiation at position 533) and 28,060 Da for the *Bacillus cereus* protein. This similarity is reinforced by a remarkable degree of similarity in the amino acid sequences: overall amino acid identity of the two proteins is 32%, with the degree of similarity increasing to 48% after allowing for conservative R group substitutions. Four residues in the *Bacillus cereus* enzyme known to be involved in the binding of the Zn^{2+} cofactor (2, 15, 16) are circled in Fig. 4. Each of these ligand-binding residues can be aligned with identical residues in the *B. fragilis cfiA* enzyme. The areas surrounding these residues in the aligned sequences show very strong similarity. There is a 67% perfect region of similarity (6 of 9 matches, or 7 of 10 matches with conservative substitution) around the *Bacillus cereus* enzyme residues His-116 and His-118, involving *B. fragilis* residues 99 to 108 and *Bacillus cereus* residues 116 to 125. A more striking region of similarity showing 19 of 29 (66%) matches (or 23 of 29 [79%] matches allowing for conservative substitutions) surrounds the ligand-binding Cys (*Bacillus cereus* Cys-198), involving residues 156 to 184 of the *B. fragilis* protein and residues 173 to 201 of the *Bacillus cereus* protein. There is also a perfect region of similarity (7 of 9 matches, or 8 of 9 [89%] matches with conservative substitutions) around the *Bacillus cereus* C-terminal His residue (His-240), involving residues 219 to 227 of the *B. fragilis* protein and amino acids 236 to 244 of the *Bacillus cereus* enzyme. There are several other areas of significant similarity between the two proteins in regions of unknown function.

The primary *Bacillus cereus* 569/H β-lactamase II translation product is known to contain a 30-amino-acid signal peptide sequence (16); processing occurs at Ser-31 to yield a processed protein of 24,964 Da. Indeed, the signal peptide of this enzyme obeys all the established criteria (39) for this region: a basic NH_2 terminus, a long uninterrupted hydrophobic stretch (17 residues in this case), a small number of neutral amino acids close to the cleavage site with Gly or Ala highly preferred (Ala-30), and the presence of a Thr or Ser residue −5 from the cleavage site (Ser-26 and Ser-27). The *B. fragilis* sequence contains a potential signal sequence involving amino acids 1 to 18: there is a basic NH_2-terminal region, a long uninterrupted hydrophobic stretch (15 residues), and several small neutral residues and Ala (Ala-18) at the proposed −1 position relative to the cleavage site. The processed *cfiA* protein determined by these criteria would have a molecular mass of 25,249 Da. The sequence lacks a Thr or Ser residue five amino acids upstream of Ala-18, but the absence of these amino acids at this site has been observed in the signal peptides of other exported proteins (39), including the periplasmic TEM β-lactamase (36).

Homology of *cfiA* with other β-lactam-resistant *B. fragilis* strains. When the 1.5-kb *Bst*YI fragment from pJST241 containing the entire *cfiA* gene was used as a probe with chromosomal DNA from several *B. fragilis* strains (data not shown), it did not hybridize even under low stringency conditions with the chromosomes of other β-lactam-resistant *B. fragilis* strains, such as TAL4170, TMP14, or TM4000 (endogenous penicillinase-cephalosporinase) (Table 1). Under similar conditions, no hybridization was seen with TAL7860, a Fox-Imp^r *Bacteroides distasonis* strain (15a).

A purified *Hin*dIII fragment containing the cloned *Bacillus cereus* 569/H β-lactamase II gene (*blm*) was obtained from plasmid pRWH012, a kind gift from J. Lampen (16). No homology between the *cfiA* probe and this fragment was detected even at low stringency.

DISCUSSION

Using a new *B. fragilis-E. coli* shuttle vector, pJST61, we have cloned and sequenced the *cfiA* gene which encodes a Zn^{2+}-requiring broad-spectrum β-lactamase from TAL2480. Plasmid pJST241, a derivative of pJST61 with a 3.6-kb insert of chromosomal DNA from TAL2480, when present in *B. fragilis* TM4000, confers high-level resistance to FOX and IMP. Extracts prepared from such cells contain β-lactamase activity with the same substrate and inhibitor profiles as those of the parental TAL2480 enzyme. The location of the

cfiA gene within the cloned insert was determined by the generation of deletions, isolation of transposon insertions, and subcloning the original insert. The DNA sequence of the *cfiA* gene reveals an ORF of 747 bases beginning at the ATG codon at position 533 and terminating at the TAG codon at position 1282. The gene would code for a protein of 249 amino acids with a molecular mass of 27,260 Da. However, if the first 18 amino acids comprise a signal peptide which is removed by processing, the final protein would be 231 amino acids with a molecular mass of 25,249 Da. While we have not yet proved that translation begins at position 533, the similarity of this putative product to the β-lactamases of *Bacillus cereus* argues that it does.

Tn*1000* insertions upstream of position 316 in pJST241 (such as pJST241GD08) do not interfere with FOX-IMP resistance in *B. fragilis*; insertion of Tn*1000* at position 381 (such as in pJST241GG12) causes a drug-sensitive phenotype. Thus, this region may be involved in transcription or translation in *B. fragilis*, since it lies upstream of the putative start codon at position 533. Tn*1000* insertions distal to the TAG terminator at position 1282 result in a reduced level of FOX-IMP resistance in *B. fragilis* and decreased levels of enzyme activity; pJST2414, in which all DNA distal to 1,406 base pairs is deleted, and pJST241GD023 with Tn*1000* at position 1554 are such examples. Tn*1000* insertion in pJST241GD017 retains full FOX-IMP resistance. This suggests that the region between positions 1282 and 1652 is required for full expression of FOX-IMP resistance. This region may code for a protein product(s) (there are two ORFs between bases 1392 and 1619 and bases 1544 and 1780 that could code for proteins of 76 and 79 amino acids, respectively) or may affect the amount or stability of *cfiA* mRNA. Antipolar effects of Tn*1000* within distal regions of a procaryotic operon have been observed (20), suggesting that this property of Tn*1000* may also be responsible for decreased levels of *cfiA* expression. However, pJST2414, lacking the distal 2.1 kb of pJST241 but containing the entire *cfiA* gene, also leads to similar, lower levels of *cfiA* expression as seen with the distal Tn*1000* insertions (Table 2).

The complete nucleotide sequence of the class B β-lactamase genes from *Bacillus cereus* 569/H (*blm*) and 5/B/6 have been determined, and the amino acid sequences of the corresponding proteins have been derived. The N-terminal 32 amino acids of the Zn^{2+}-requiring β-lactamase from *P. maltophilia* are also known (10), and the possible corresponding DNA sequence has been deduced. The nucleotide sequence of the *B. fragilis cfiA* gene shows no homology with these genes. However, the primary sequences of the deduced *Bacillus cereus* and *B. fragilis* proteins show a high degree of similarity. No similarity between the N-terminal sequence of the *P. maltophilia* enzyme and either the *Bacillus cereus* or *B. fragilis* proteins could be detected. Overall, the *Bacillus cereus* and *B. fragilis* proteins show 32% identity, or 48% similarity. However, the similarity within regions in the vicinity of residues known to be involved in Zn^{2+} binding is much higher. If the enzymes share a common origin, it would be expected that the amino acid sequences in catalytically important regions would be conserved. The *B. fragilis* and *Bacillus cereus* enzymes also show strong similarities in several other regions, suggesting that these regions may define additional important functional domains. Now that the primary amino acid sequences of two Zn-requiring β-lactamases from unrelated sources have been shown to possess significant regions of similarity, it can be expected that detailed comparisons of their structures may reveal the details of these functionally important regions. Further characterization of the CfiA and Blm proteins should lead to an increased understanding of the mechanism of action of the metallo-β-lactamases. An important question requiring further study is why the CfiA protein is capable of hydrolyzing FOX, while the closely related Blm protein cannot (G. Cuchural, personal communication).

The finding of β-lactamases with similar primary sequences in such diverse organisms as *B. fragilis* and *Bacillus cereus* raises the question as to the origin and dissemination of the ancestral structural gene. In this regard, it should be pointed out that the CLN resistance determinant found in several *B. fragilis* strains and transposons has been shown to be closely related to the macrolide-lincosamide-streptogramin B determinants present in certain staphylococci, streptococci, and bacilli (27). Thus, the *ermF* gene found in *B. fragilis* transposons Tn*4351* and Tn*4000* may have originated from the *ermC* or *ermA* gene of *Staphylococcus aureus* or the *ermD* gene of *Bacillus licheniformis*, or vice versa. It seems reasonable to suggest that DNA exchanges between the gram-negative *Bacteroides* spp. and gram-positive bacteria have occurred and may account for the acquisition of these antibiotic resistance determinants, although the mechanism(s) of exchange remains unknown.

The cloned *cfiA* gene will be used as a probe to monitor the occurrence of this determinant in *B. fragilis* clinical isolates resistant to FOX-IMP. To date, only three Imp^r *Bacteroides* strains have been isolated by the Tufts Anaerobe Laboratory (TAL) (6, 37); two of these are TAL2480 and TAL3636, described in this report. The third strain is *B. distasonis* TAL7860, which shows broad-spectrum resistance to β-lactam antibiotics, including IMP and FOX. No homology between the *cfiA* probes and the chromosomal DNA obtained from TAL7860 could be detected even at low stringency. In addition, the enzyme synthesized by TAL7860 is insensitive to EDTA but sensitive to clavulanic acid (15a); it thus does not represent a class B β-lactamase.

An immediate application of the cloned *cfiA* gene will be its use as a marker for genetic studies with *Bacteroides* spp. At present, CLN resistance is the most commonly used selective marker, although Shoemaker et al. (32) and Guiney et al. (11) have recently described the isolation of a tetracycline resistance gene from *Bacteroides* spp. FOX-IMP resistance can be added to existing *Bacteroides* shuttle vectors to create plasmids that can be used in complementation experiments with vectors marked with CLN or tetracycline.

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