Molecular and Genetic Analysis of the *Bacteroides uniformis* Cephalosporinase Gene, *cblA*, Encoding the Species-Specific β-Lactamase

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The gene, *cblA*, encoding the species-specific, clavulanate-susceptible, endogenous cephalosporinase was cloned from *Bacteroides uniformis* WAL-7088. The nucleotide sequence was determined, and the *cblA* structural gene was found to be 891 nucleotides, with a 48% G+C composition, which is similar to that of the *B. uniformis* genome. The *cblA* open reading frame encoded an Ambler class A β-lactamase polypeptide precursor of 296 amino acid residues with a predicted molecular weight of 33,450. A β-lactamase-deficient *B. uniformis* mutant with increased β-lactam susceptibility was constructed by insertional inactivation of the chromosomal gene. This mutant was complemented by plasmids bearing the *cblA* gene, and the resulting strains were resistant to cephaloridine and had a β-lactamase that comigrated with the parental β-lactamase on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30,500 Da) and in isoelectric focusing gels (pI 4.6), confirming a role for this β-lactamase in resistance.

In *Bacteroides* species, β-lactamases have been known since the early 1950s (5) and are probably the most important mechanism of resistance to β-lactam antibiotics (16). The enzymes found among members of this genus show a remarkable degree of diversity and span most of the biochemical classes described by Bush (2, 3). These include both cephalosporinases and penicillinases, some of which can hydrolyze cefoxitin or carbapenems. However, the majority of *Bacteroides* isolates seem to have at least one β-lactamase with activity that is species specific, and these are generally found to be cephalosporinases, which are inhibited by clavulanate, with pIs of from 4.5 to 5.5, and with molecular weights in the range of 30,000 (2, 16, 21, 23, 31, 33). In order to understand the contributions of these indigenous β-lactamases to overall β-lactam resistance in *Bacteroides* species, we have begun to characterize the β-lactamase genes and their regulation. The endogenous β-lactamase from *Bacteroides fragilis* (CepA) was recently cloned and sequenced and was found to belong to Ambler molecular class A (21). Hybridization studies with a *cepA* gene probe showed that this β-lactamase gene is specific for *B. fragilis* and that there is little or no nucleotide homology with other *Bacteroides* species, including *Bacteroides uniformis*. Thus, it is of interest to determine the relationship among the different *Bacteroides* β-lactamases.

In this report we describe the cloning and nucleotide sequence of *cblA*, which encodes a species-specific β-lactamase in *B. uniformis*. The gene was most closely related to other *Bacteroides* β-lactamase genes, and the protein belonged to Ambler class A. The cloning was somewhat notable because high-level β-lactamase expression was obtained in *Escherichia coli*. Most previous reports of the cloning of *Bacteroides* antibiotic resistance genes have resulted in low-level or no expression of the resistance phenotype in *E. coli* (6, 15, 19–21, 25, 26, 32).

MATERIALS AND METHODS

Bacterial strains and growth. *Bacteroides* strains were grown anaerobically in supplemented brain heart infusion broth (19), and the following antibiotics at the indicated concentrations were used routinely: clindamycin (Cc), 5 μg/ml; tetracycline, 5 μg/ml; rifampin, 20 μg/ml; gentamicin, 25 μg/ml; and ampicillin (Ap), 25 μg/ml. *B. uniformis* BU1001 and V528 are both rifampin-resistant strains derived from VPI006-1 (24, 34), and WAL-7088 is a cefoxitin-resistant clinical isolate (35). *E. coli* DH5α (*recA hsdR17 lac*) was used for all cloning experiments and library construction. DH5α was grown aerobically in L broth (agar) containing Ap (50 μg/ml) or spectinomycin (Sp; 40 μg/ml) when appropriate. Susceptibility testing was done by the standard agar dilution method (14).

Plasmids were transferred in triparental matings from *E. coli* donors to *Bacteroides* recipients by standard filter mating protocols (29). These used *E. coli* donor strains containing RK231 as a conjugation helper plasmid, and the mating plates were incubated aerobically to enhance the growth of donor cells. *E. coli* transformations were done by the method of Hanahan (7).

DNA manipulations and sequence analysis. Routine DNA ligations, endonuclease restrictions, plasmid screening, plasmid purification, agarose gel electrophoresis, and Southern hybridizations were done as described previously (1, 13, 21). The DNA sequence of the 2.8-kb pFD314 cloned insert was determined in one direction by constructing nested deletions with exonuclease III (8); this was followed by dideoxy sequencing (22) by using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio). The nucleotide sequence of the opposite DNA strand was elucidated by primer walking by using sequencing primers deduced from the first DNA strand. Analysis of the sequence was performed on a VAX computer by using the GCG analysis software (4), and the GenBank accession number for the *cblA* sequence is L08472.

The following plasmids were used or constructed for the current study. The standard *Bacteroides* shuttle and cloning vector pFD288 (Sp^r Cc^r) and the suicide vector pFD280.erm (Sp^r Cc^r) have been described previously (19, 26, 29). pFD314 contained the 2.8-kb *cblA* EcoRI-*SmaI* fragment cloned into

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TABLE 1. Ampicillin and cephaloridine MICs and β-lactamase activities of *B. uniformis* strains

Strain	MIC (μg/ml)[a]		β-Lactamase activity[b]			% Activity[c]
	Amp	Cep	Cep	Pen	Ncef	
BU1001	32	64	0.018	<0.002	0.057	9.8
BU1001ΩFD483	2	2	—[d]	—	<0.001	—
BU1001ΩFD483 (pFD544)	4	8	—	—	<0.001	—
BU1001ΩFD483 (pFD548)	64	64	0.011	<0.002	0.045	10.7
WAL-7088	>256	>256	1.796	0.179	4.440	69

[a] Amp, ampicillin; Cep, cephaloridine.

[b] Units of activity are micromolar substrate degraded minute[−1] milligram of protein[−1] for cephaloridine (Cep), benzylpenicillin (Pen), and nitrocefin (Ncef).

[c] Percent activity of nitrocefin assays containing 1 μM potassium clavulanate relative to that in assays to which no inhibitor was added.

[d] —, assays not performed.

the multiple cloning site of pFD288. The suicide plasmid used for mutagenesis, pFD483, was constructed by insertion of a 560-bp *cblA* HaeIII fragment (base pairs 1071 to 1630) from *cblA* into the SmaI site of pFD280.erm. In order to perform complementation experiments with the *cblA* insertion mutant, BU1001ΩFD483 (Cc[r]), a tetracycline resistance shuttle vector, was constructed. This plasmid, pFD544, was similar to pFD288 except that the *ermF* gene conferring Cc[r] was replaced by a 2.7-kb SstI fragment bearing the *Bacteroides tetQ* gene (15). The 2.8-kb *cblA* gene fragment was then inserted to make pFD548, which conferred Sp[r] Ap[r] in *E. coli* and tetracycline resistance and Ap[r] in *B. uniformis*.

β-Lactamase analysis. Cell extracts for β-lactamase assays were prepared in 20 mM sodium phosphate buffer (pH 7.0) with a French pressure cell and were centrifuged at 12,000 × *g* for 30 min as described previously (19). Activity was measured spectrophotometrically with either nitrocefin (482 nm), benzylpenicillin (233 nm), or cephaloridine (260 nm) as the substrate (17, 19), and the results are given in units in which 1 U equals 1 μmol of substrate degraded min^{−1} mg of protein[−1]. Studies testing inhibition with clavulanate were performed by preincubating cell extracts with 1 μM clavulanate for 5 min prior to the addition of the nitrocefin substrate as described previously (19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using a discontinuous buffer system (11). The β-lactamase activity in the gels was detected after renaturing the proteins in sodium phosphate buffer by overlaying the gels with nitrocefin-containing agarose (19). The isoelectric focusing gels were run in polyacrylamide gels with ampholytes (pH 3 to 10) exactly as described previously (21).

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of *cblA*. *B. uniformis* WAL-7088 (Table 1) is a cefoxitin-resistant clinical isolate that uses a combination of mechanisms for β-lactam resistance. Wexler and Halebian (35) showed that there are changes in the WAL-7088 penicillin-binding proteins and that the strain produces a cefoxitin-inactivating activity. During our studies of WAL-7088, we demonstrated the presence of the *cfxA* gene, which encodes a β-lactamase capable of cefoxitin degradation (19). In addition, a second β-lactamase is present in this strain, and in order to determine its role in β-lactam resistance, the gene for this enzyme was cloned and examined. The cloned gene was obtained from a *B. uniformis* WAL-7088 EcoRI

library of about 7,300 clones prepared in *E. coli* DH5α by using the Sp[r] vector, pFD288. Four Ap[r] colonies were initially observed after plating the library on medium containing 20 μg of Ap per ml and 40 μg of Sp per ml. Restriction endonuclease analysis of the recombinant plasmids showed that these clones all share a common 7.8-kb EcoRI fragment.

One clone was chosen for further examination, and subcloning experiments indicated that a 2.8-kb EcoRI-SmaI fragment was sufficient to encode Ap[r] in *E. coli*. Subsequent experiments showed that the Ap[r] phenotype was independent of orientation of the cloned fragment. This DNA fragment present on pFD314 was subjected to exonuclease III treatment, and a set of nested deletions was obtained for DNA sequence determination. Analysis of the nucleotide sequence revealed three open reading frames of greater than 400 bp (Fig. 1). Two of the protein-coding sequences were truncated, but they shared significant homology with predicted proteins of genes in GenBank. The deduced amino acid sequence from the open reading frame designated Env shared 29% identity with the C terminus of the *E. coli* EnvD protein, which is involved in the cell envelope structure (10). The amino acid sequence deduced from ORF3 was about 31% identical to the AMP nucleosidase predicted from the *E. coli amn* gene (12). The open reading frame encoding the β-lactamase activity is 891 bp and was designated *cblA* for chromosomal β-lactamase (Fig. 2). The *cblA* structural gene has a G+C content of 48%, which was identical to the composition of the entire cloned 2.8-kb fragment and is consistent with the 46 to 48% G+C content of the *B. uniformis* species (9). Assignment of the *cblA* gene was consistent with analysis of the pFD314 deletion derivatives (Fig. 1). The strain with Δ7, which has its 5′ end upstream of the *cblA* ATG start site, was Ap[r] (MIC, >128 μg/ml), but the strain with Δ2, which has its 5′ end within the predicted *cblA* structural gene, was Ap[s].

The putative CblA β-lactamase precursor would be 296 amino acid residues, with a predicted molecular weight of 33,450. The relationship of CblA to other β-lactamases was examined, and the results showed that it is most closely related to two other *Bacteroides* β-lactamases, forming a unique group of Ambler class A β-lactamases (21). The protein has the greatest homology with the indigenous *B. fragilis* β-lactamase, CepA. There was 43% identity between the aligned protein sequences of CepA and CblA, and there was 51% identity between nucleotide sequences of the aligned structural genes
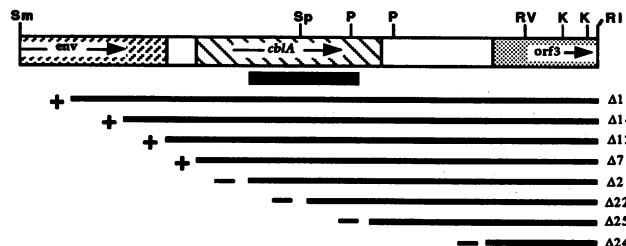
FIG. 1. Map of the deletions and open reading frames present on the 2.8-kb EcoRI-SmaI Ap[r] fragment cloned in pFD314 and pFD548. The relevant open reading frames are indicated by the shaded boxes, with arrows showing the direction of translation. Some of the deletions used for phenotypic testing and DNA sequencing are shown below the map, with a plus or a minus sign indicating the ability or lack of ability to confer Ap[r] in *E. coli*, respectively. The thick black bar shows the *cblA* HaeIII fragment used as a probe in hybridization experiments and for insertional inactivation in pFD483. Restriction sites are abbreviated as follows: Sm, SmaI; Sp, SphI; P, PstI; RV, EcoRV; K, KpnI; RI, EcoRI.

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                                                                                            rbs    .
  748  GCTATCACACTTATAGCCCATAATCCATAACTCATTTTAGCAACTTGCTGCTAAAAGTTGTATCTTTACGTGCGAATTGCCTCTTAAAGAAATTGAAGTT


  848  ATGAAAGCATATTTCATCGCCATACTTACCTTATTCACTTGTATAGCTACCGTCGTCCGGGCGCAGCAAATGTCTGAACTTGAAAACCGGATTGACAGTC
       M  K  A  Y  F  I  A  I  L  T  L  F  T  C  I  A  T  V  V  R  A  Q  Q  M  S  E  L  E  N  R  I  D  S  L
                                                                  ⇑BU


  948  TGCTCAATGGCAAGAAAGCCACCGTTGGTATAGCCGTATGGACAGACAAAGGAGACATGCTCCGGTATAACGACCATGTACACTTCCCCTTGCTCAGTGT
       L  N  G  K  K  A  T  V  G  I  A  V  W  T  D  K  G  D  M  L  R  Y  N  D  H  V  H  F  P  L  L  S  V
                                        ⇑EC

                      .HaeIII                                                         ‾
 1048  ATTCAAATTCCATGTGGCACTGGCCGTACTGGACAAGATGGATAAGCAAAGCATCAGTCTGGAC│AGCATTGTTTCCATAAAGGCATCCCAAATGCCGCCC
       F  K  F  H  V  A  L  A  V  L  D  K  M  D  K  Q  S  I  S  L  D──┘S  I  V  S  I  K  A  S  Q  M  P  P
                                                                 ←Δ2


 1148  AATACCTACAGCCCCCTGCGGAAGAAGTTTCCCGACCAGGATTTCACGATTACGCTTAGGGAACTGATGCAATACAGCATTTCCCAAAGCGACAACAATG
       N  T  Y  S  P  L  R  K  K  F  P  D  Q  D  F  T  I  T  L  R  E  L  M  Q  Y  S  I  S  Q  S  D  N  N  A


 1248  CCTGCGACATCTTGATAGAATATGCAGGAGGCATCAAACATATCAATGACTATATCCACCGGTTGAGTATCGACTCCTTCAACCTCTCGGAAACAGAAGA
       C  D  I  L  I  E  Y  A  G  G  I  K  H  I  N  D  Y  I  H  R  L  S  I  D  S  F  N  L  S  E  T  E  D

       SphI .
 1348  CGGCATGCACTCCAGCTTCGAGGCTGTATACCGCAACTGGAGTACTCCTTCCGCTATGGTCCGACTACTGAGAACGGCTGATGAAAAAGAGTTGTTCTCC
       G  M  H  S  S  F  E  A  V  Y  R  N  W  S  T  P  S  A  M  V  R  L  L  R  T  A  D  E  K  E  L  F  S


 1448  AACAAGGAGCTGAAAGACTTCTTGTGGCAGACCATGATAGATACTGAAACCGGTGCCAACAAACTGAAAGGTATGTTGCCAGCCAAAACCGTGGTAGGAC
       N  K  E  L  K  D  F  L  W  Q  T  M  I  D  T  E  T  G  A  N  K  L  K  G  M  L  P  A  K  T  V  V  G  H

                                                   PstI .                                . HaeIII .
 1548  ACAAGACCGGCTCTTCCGACCGCAATGCCGACGGTATGAAAACTGCAGATAATGATGCCGGCCTCGTTATCCTTCCCGACGGCCGGAAATACTACATTGC
       K  T  G  S  S  D  R  N  A  D  G  M  K  T  A  D  N  D  A  G  L  V  I  L  P  D  G  R  K  Y  Y  I  A


 1648  CGCCTTCGTCATGGACTCATACGAGACGGATGAGGACAATGCGAACATCATCGCCCGCATATCACGCATGGTATATGATGCGATGAGATGAGAGAGGCAA
       A  F  V  M  D  S  Y  E  T  D  E  D  N  A  N  I  I  A  R  I  S  R  M  V  Y  D  A  M  R  *

                                              PstI .
 1748  GGGGGCTATTGGTAGCTCCTCCTATAAGTTTTGATACTCGGGTATATCCTGCAGAAAAGTATAGCTGTTGTGCTCGTAATCTATGCGGCAACAATAATGC
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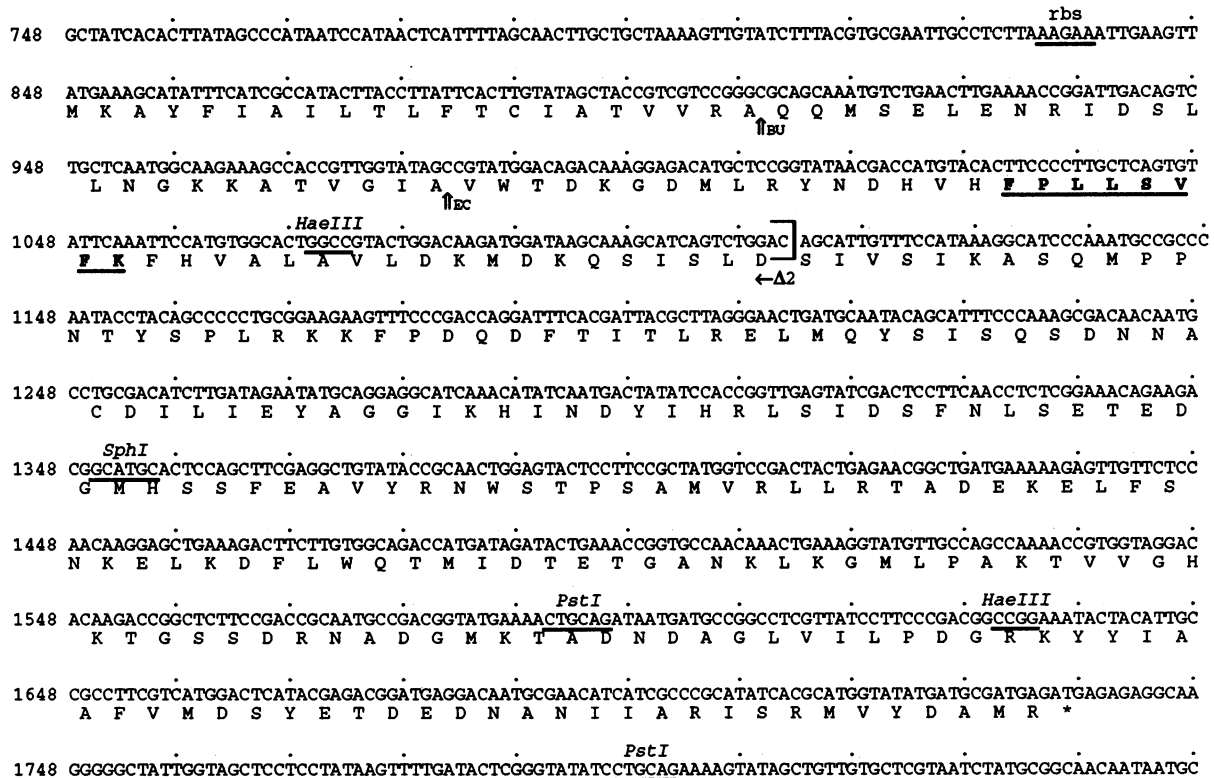
FIG. 2. Nucleotide sequence showing the region coding for *cblA*. The base pair numbering corresponds to that of the GenBank sequence L08472, which encompasses the entire 2.8-kb fragment shown in Fig. 1. The proposed signal sequence cleavage sites for *B. uniformis* (BU) or *E. coli* (EC) are indicated by vertical arrows, and the putative serine active site residues are in boldface type. The endpoint of the pFD314 deletion, Δ2, is shown by the bracket.

for these two peptides. Upstream of the *cepA* and *cblA* ATG start sites there was only 36% identity in the nucleic acid sequences for a distance of 500 bp. By comparison, CblA shared just 24% identity with the TEM1 protein and 22% identity with the *Bacillus cereus* β-lactamase III amino acid sequence.

Analysis and expression of *cblA* in *B. uniformis*. The possibility that *cblA* encoded the indigenous *B. uniformis* chromosomal β-lactamase was supported by Southern hybridization experiments by using an internal *cblA* fragment (Fig. 1) as the probe. These results showed that there was a homologous fragment present in *Eco*RI digests of *B. uniformis* WAL-7088, V528, and BU1001, but only a very weak hybridization signal was observed with the chromosomes of *B. fragilis*, *Bacteroides ovatus*, and *Bacteroides vulgatus* (27).

Additional evidence for the role of *cblA* was obtained by constructing a β-lactamase-deficient *B. uniformis* mutant by targeted insertional inactivation. The strain chosen for mutagenesis was BU1001, which contained a cephalosporinase which was inhibited by >50% by 1 μM clavulanate (Table 1). For the experiment, an internal *cblA* gene fragment (Fig. 1) was inserted into the suicide vector pFD280.erm, and the resulting construct, designated pFD483, was then conjugated into BU1001. The clindamycin-resistant transconjugants obtained from this mating were found to have pFD483 inserted into the chromosomal copy of *cblA* (27). This insertion by a single crossover event was mediated by homologous recombination, and disruption of the gene resulted in a complete loss of β-lactamase activity, as was seen with strain BU1001Ω FD483 (Table 1). In addition, there was an 8- to 32-fold

decrease in the MICs of β-lactam antibiotics for the mutant BU1001ΩFD483.

β-Lactamase activity could be restored in mutant strains when plasmids containing the cloned *cblA* gene were introduced into the mutants. This was shown with pFD548, which contained the 2.8-kb *Eco*RI-*Sma*I *cblA* fragment (from pFD314) and a tetracycline resistance gene (*tetQ*) for counterselection. In the mutant BU1001ΩFD483 containing pFD548, β-lactamase activity was restored to nearly the same level as that seen in the parent strain, BU1001. This activity was cephalosporinase in nature and was inhibited by clavulanate, like BU1001 was (Table 1). In contrast, BU1001Ω FD483 cells containing only the vector pFD544 displayed no detectable β-lactamase activity, and there was no increase in the β-lactam MICs for those cells (Table 1). Experiments similar to these were attempted with the strain *B. uniformis* WAL-7088. However, repeated attempts to transfer any plasmids into this strain via conjugation were unsuccessful. The underlying reasons for this inability to function as a recipient strain were not investigated.

Isoelectric focusing and SDS-PAGE were used to characterize the cloned *cblA* gene product. On the basis of staining of SDS-polyacrylamide gels for nitrocefin activity, strain WAL-7088 had two β-lactamases (Fig. 3). One was 38,000 to 40,000 Da and corresponded to CfxA, the β-lactamase with cefoxitin-degrading activity (19). The second nitrocefin-reactive protein was about 30,500 Da and comigrated with the single band of a protein with β-lactamase activity present in BU1001 cells. Consistent with the idea that *cblA* encoded the indigenous *B. uniformis* β-lactamase, the Ap[s] mutant BU1001ΩFD483 had
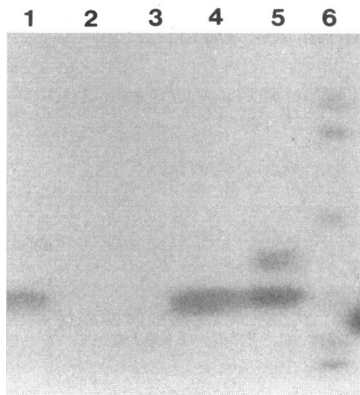
FIG. 3. SDS-polyacrylamide gel of crude cell extracts stained for β-lactamase activity by overlaying with nitrocefin as described in the text. Lanes: 1, BU1001 (235 μg); 2, BU1001ΩFD483 (409 μg); 3, BU1001ΩFD483(pFD544) (319 μg); 4, BU1001ΩFD483(pFD548) (317 μg); 5, WAL-7088 (212 μg); 6, prestained standards. Estimated molecular weights of the dye-containing proteins: lysozyme (18,500), trypsin inhibitor (27,500), carbonic anhydrase (32,500), ovalbumin (49,500), bovine serum albumin (80,000), and phosphorylase B (106,000).

no detectable band of a protein with β-lactamase activity, but a mutant containing the cloned gene (pFD548) displayed a protein with β-lactamase activity that comigrated with the band of a protein from BU1001 with β-lactamase activity. No such band was observed in gels of BU1001ΩFD483 cells containing just the vector pFD544 (Fig. 3; compare lanes 1 to 4). Isoelectric focusing experiments yielded results consistent with those of SDS-PAGE. Examination of BU1001 revealed one major band with a pI of 4.6 and minor bands at pIs 4.8 and 5.2. Other strains of B. uniformis also have β-lactamases with pIs in the same range (18), and one study of six strains with a "normal" substrate profile showed that all had a major band with a pI of 4.5 (33). Interestingly, all three bands of proteins with β-lactamase activity were missing from the mutant BU1001ΩFD483 but were restored in mutants containing pFD548 (27). This suggests that there might be some heterogeneity in the processing of the β-lactamase precursor or some proteolytic degradation of the enzyme upon storage. Results obtained with cell-free extracts of strain WAL-7088 showed that there was consistently one major band of a protein with β-lactamase activity that comigrated with the band of a protein from BU1001 with β-lactamase activity (pI 4.6). In addition, a second minor band of a β-lactamase with a pI of 4.8 was observed. We have shown previously that the CfxA β-lactamase in B. vulgatus CLA341 does not focus well (19). Similarly, results with B. uniformis WAL-7088 demonstrated a large nitrocefin-reactive region in the alkaline region of the gel, but this did not form a definitive band of activity.

Expression of cblA in E. coli. A variety of studies have demonstrated the differential expression or the lack of expression of Bacteroides antibiotic resistance genes in E. coli (6, 20, 28, 30). The ability of E. coli to express cblA was not the result of a mutation, as was shown previously for the Bacteroides metallo-β-lactamase CcrA (20). This possibility was excluded by transformation experiments with pFD314 in which the antibiotic selection for transformants was either Sp[r] or Ap[r]. The results showed that transformation frequencies were essentially identical regardless of the antibiotic used for selection. If a mutation was required for expression of the B. uniformis cblA gene, the Ap[r] transformation frequency would

be much lower than that for Sp[r]. Thus, expression of cblA in E. coli must be mediated by a mechanism different from that seen for ccrA.

In summary, the following evidence supports the idea that cblA encodes the species-specific endogenous β-lactamase of B. uniformis. The gene encodes a protein with β-lactamase activity that comigrates in SDS-polyacrylamide gels and isoelectric focusing gels with the enzyme found in several B. uniformis strains. The enzyme is a cephalosporinase, it is susceptible to clavulanate, and it has a pI of 4.6, all of which are characteristics similar to those previously reported for the low-level, endogenous B. uniformis β-lactamases (Table 1) (18, 31, 33). Hybridization studies showed that cblA is homologous to DNA fragments in B. uniformis but not other Bacteroides species. Finally, B. uniformis mutants lacking β-lactamase were constructed by using a cblA gene fragment to mediate insertional inactivation of the gene.

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