Molecular and Genetic Analysis of the \textit{Bacteroides uniformis} Cephalosporinase Gene, \textit{cblA}, Encoding the Species-Specific \(\beta\)-Lactamase

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

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\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and growth.} \textit{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 \(\mu\)g/ml; tetracycline, 5 \(\mu\)g/ml; rifampin, 20 \(\mu\)g/ml; gentamicin, 25 \(\mu\)g/ml; and ampicillin (Ap), 25 \(\mu\)g/ml. \textit{B. uniformis} BU1001 and V528 are both rifampin-resistant strains derived from VPI1006-1 (24, 34), and WAL-7088 is a cefoxitin-resistant clinical isolate (35). \textit{E. coli} DH5\(\alpha\) \((\text{recA hsdR17 lac})\) was used for all cloning experiments and library construction. DH5\(\alpha\) was grown aerobically in L broth (agar) containing Ap (50 \(\mu\)g/ml) or spectinomycin (Sp; 40 \(\mu\)g/ml) when appropriate. Susceptibility testing was done by the standard agar dilution method (14).

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

\textbf{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 \textit{cblA} sequence is L08472.

The following plasmids were used or constructed for the current study. The standard \textit{Bacteroides} shuttle and cloning vector pFD288 (Sp\(^{\prime}\) Cc\(^{\prime}\)) and the suicide vector pFD280.erm (Sp\(^{\prime}\) Cc\(^{\prime}\)) have been described previously (19, 26, 29). pFD314 contained the 2.8-kb \textit{cblA} EcoRI-SmaI fragment cloned into...
TABLE 1. Ampicillin and cephaloridine MICs and \( \beta \)-lactamase activities of \( B. \) uniformis strains

| Strain      | MIC (\( \mu \)g/ml) | \( \beta \)-Lactamase activity | % Activity |
|-------------|---------------------|-------------------------------|------------|
|             | Amp | Cep | Cep | Pen | Neef |              |
| BU1001      | 32  | 64  | 0.018 | <0.002 | 0.57 | 9.8          |
| BU1001\( \Delta D483 \) | 2   | 2   | 2   | <0.001 | —   | —            |
| BU1001\( \Delta D483 \) | 4   | 8   | —   | —   | <0.001 | —            |
| (pFD544)    |     |     |     |     |     |              |
| BU1001\( \Delta D483 \) | 64  | 64  | 0.011 | <0.002 | 0.045 | 10.7        |
| (pFD548)    |     |     |     |     |     |              |
| 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 (Neef).

\( ^c \) Percent activity of nitrocefin assays containing 1 \( \mu \)M potassium clavulanate relative to that in assays to which no inhibitor was added.

\( ^d \) —, assays not performed.

library of about 7,300 clones prepared in \( E. \) coli DH5\( \alpha \) by using the \( Sp \) vector, pFD288. Four \( Ap \) colonies were initially observed after plating the library on medium containing 20 \( \mu \)g of Ap per ml and 40 \( \mu \)g 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-Smal fragment was sufficient to encode \( Ap \) in \( E. \) coli. Subsequent experiments showed that the \( Ap \) 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 \( E n v \) 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 \( a m n \) gene (12). The open reading frame encoding the \( \beta \)-lactamase activity is 891 bp and was designated \( c b l A \) for chromosomal \( \beta \)-lactamase (Fig. 2). The \( c b l A \) structural gene has a G+C content of 48%, which is 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 \( c b l A \) gene was consistent with analysis of the pFD314 deletion derivatives (Fig. 1). The strain with \( D7 \), which has its \( S \) end upstream of the \( c b lA \) ATG start site, was \( Ap \) (MIC, \( >128 \) \( \mu \)g/ml), but the strain with \( D2 \), which has its \( S \) end within the predicted \( c b lA \) structural gene, was \( Ap \).

The putative \( C b lA \) \( \beta \)-lactamase precursor would be 296 amino acid residues, with a predicted molecular weight of 33,450. The relationship of \( CblA \) to other \( \beta \)-lactamases was examined, and the results showed that it is most closely related to two other \( Bacteroides \) \( \beta \)-lactamases, forming a unique group of Ambler class A \( \beta \)-lactamases (21). The protein has the greatest homology with the indigenous \( B. \) fragilis \( \beta \)-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.

**RESULTS AND DISCUSSION**

**Cloning and nucleotide sequence of \( c b lA \).** \( B. \) uniformis WAL-7088 (Table 1) is a cefoxitin-resistant clinical isolate that uses a combination of mechanisms for \( \beta \)-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 \( c f A \) gene, which encodes a \( \beta \)-lactamase capable of cefoxitin degradation (19). In addition, a second \( \beta \)-lactamase is present in this strain, and in order to determine its role in \( \beta \)-lactam resistance, the gene for this enzyme was cloned and examined. The cloned gene was obtained from a \( B. \) uniformis WAL-7088 EcoRI

**FIG. 1.** Map of the deletions and open reading frames present on the 2.8-kb EcoRI-Smal \( Ap \) 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 \( Ap \) in \( E. \) coli, respectively. The thick black bar shows the \( c b lA \) HaeIII fragment used as a probe in hybridization experiments and for insertion inactivation in pFD483. Restriction sites are abbreviated as follows: Sm, Smal1; Sp, SphI; P, PstI; RV, EcoRV; K, KpnI; RI, EcoRI.
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 EcoRI digests of B. uniformis WAL-7088, V528, and BU1001, but only a very weak hybridization signal was observed with the chromosomally encoded 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 inserional 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 pFD280erm, 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 BU1001FD483 (Table 1). In addition, there was an 8- to 32-fold decrease in the MICs of β-lactam antibiotics for the mutant BU1001FD483.

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 EcoRI-Smal cblA fragment (from pFD314) and a tetacycline resistance gene (tetQ) for counterselection. In the mutant BU1001FD483 containing pFD548, β-lactamase activity was restored to nearly the same level that as seen in the parent strain, BU1001. This activity was cephaporphinase in nature and was inhibited by clavulanate, like BU1001 was (Table 1). In contrast, BU1001 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 ceftoxitin-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' mutant BU1001FD483 had...
The essentially by 28, session demonstrated observed. proteolytic with geneity pFD548 obtained 4.5. B-lactamase containing "normal" such band mutants (106,000). (106,000). B-lactamase BU1001 pl clB A and of 4.6 missing enzyme activity by with 1-lactamase that all three bands of 2, 3, and 4. No such band was observed in gels of BU1001FD483 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 BU1001FD483 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-lactama- mase 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 clbA 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 clbA 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 or Ap. 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 clbA gene, the Ap′ transformation frequency would be much lower than that for Sp′. Thus, expression of clbA in E. coli must be mediated by a mechanism different from that seen for ccrA.

In summary, the following evidence supports the idea that clbA 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 isolectric focusing gels with the enzyme found in several B. uniformis strains. The enzyme is a cephalospirinase, it is susceptible to clavulonate, 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 clbA 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 clbA gene fragment to mediate inser-100nal inactivation of the gene.

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