ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1990, p. 1590–1592
0066-4804/90/081590-03$02.00/0

Vol. 34, No. 8

NOTES

Cloning and Sequencing of the Class B β-Lactamase Gene (*ccrA*) from *Bacteroides fragilis* TAL3636

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Received 8 December 1989/Accepted 15 May 1990

***Bacteroides fragilis* TAL3636 produces a class B, Zn^{2+}-requiring β-lactamase. The gene, *ccrA*, was cloned and expressed in *Escherichia coli*. The gene was sequenced and shown to share greater than 33% identity with the metalloenzyme from *Bacillus cereus* 569/H.**

The unique class B β-lactamases require a metal cofactor, Zn^{2+}, are able to inactivate nearly every class of beta-lactam antibiotic, and are unaffected by β-lactamase-blocking agents such as clavulanic acid (5). A limited number of organisms, including *Bacillus cereus*, *Xanthomonas maltophilia*, *Flavobacterium odoratum*, and *Bacteroides fragilis* (3, 8, 10, 11), are known to produce this type of enzyme. Two *Bacteroides fragilis* isolates have been reported to produce such an enzyme (3). This enzyme is able to inactivate a wide variety of beta-lactams including imipenem, a carbapenem, and cefoxitin, a cephamycin, and is sensitive to inactivation by EDTA (3).

In this report we describe the cloning and sequencing of the class B β-lactamase gene, *ccrA* (carbapenem and cephamycin resistance), from *Bacteroides fragilis* TAL3636 (3). The gene product was compared with the known sequence of the *Bacillus cereus* 569/H class B enzyme (1, 5).

Cloning of *ccrA*. Chromosomal DNA (7) from TAL3636 (Tufts Anaerobic Laboratories, New England Medical Center Hospital, Boston, Mass.) was restricted with *Eco*RI and ligated into the kanamycin resistance-conferring vector pCLL2300. pCLL2300 is a pMK16 (6, 7) derivative that is deleted for the carboxy-terminal half of the tetracycline resistance marker and that contains the *Eco*RI-*Bam*HI multiple cloning site from plink 322 (7). The ligation mix was used to transform *Escherichia coli* DH5α (Bethesda Research Laboratories, Inc.) by selecting for kanamycin resistance. A clone bank composed of over 20,000 independent kanamycin-resistant colonies was created.

Escherichia coli strains harboring a β-lactamase-encoding plasmid were identified by plating cells from the clone bank on Luria broth plates containing ampicillin (25 μg/ml). Thirteen ampicillin-resistant colonies were identified. A plasmid from each of these eight isolates was purified and characterized. Restriction endonuclease analysis showed that all eight plasmids contained a 6.5-kilobase (kb) *Eco*RI insert. Further studies were confined to one plasmid, pCLL2201, which contained only the 6.5-kb fragment. Transformation of pCLL2201 into DH5α yielded ampicillin-resistant transformants whose resistance was susceptible to inhibition by EDTA (data not shown). Southern hybridization analysis (7) confirmed that the 6.5-kb *Eco*RI fragment was of *Bacteroides fragilis* origin (data not shown). Thus, the 6.5-kb fragment encoded *ccrA*.

Spectrum of beta-lactam resistance. TAL3636 is resistant to a very broad spectrum of beta-lactams because of production of the class B enzyme. The cloned enzyme was tested for its ability to confer broad-spectrum resistance to *Bacteroides thetaiotaomicron*. An *Escherichia coli* to *Bacteroides* spp. shuttle vector, pCLL2203, was constructed by ligating the large *Eco*RI-*Bam*HI fragment from pVal1 (14) to the large *Eco*RI-*Bam*HI fragment from pCLL2300. The 6.5-kb *Eco*RI fragment was inserted into the unique *Eco*RI site in pCLL2203. This construct, pCLL2204, was mated from *Escherichia coli* to *Bacteroides thetaiotaomicron* (14). The *Bacteroides thetaiotaomicron* transconjugants were screened for their susceptibility to beta-lactams and β-lactamase-blocking agents by the agar dilution method (Table 1) (12). The β-lactamase CcrA conferred increased resistance to all of the beta-lactams tested and was not susceptible to inactivation by the β-lactamase-blocking agents clavulanic acid or tazobactam.

Subcloning and sequence analysis. Restriction analysis of the *Eco*RI fragment from pCLL2201 yielded the map shown in Fig. 1. The location of the β-lactamase gene was determined by subcloning and deletion analysis (Fig. 1). These results localized the β-lactamase gene to the 2.4-kb *Pst*I-*Bgl*II restriction fragment.

DNA sequence of *ccrA*. The *Pst*I-*Bgl*II fragment was subcloned as *Pst*I-*Kpn*I and *Kpn*I-*Bgl*II fragments into pUC118 and pUC119 for the production of single-stranded DNA (15). Both strands of the DNA were sequenced by using the Sequenase system (U.S. Biochemicals) with additional oligonucleotides complementary to the single-stranded DNA. Within the sequence, a large open reading frame encoding a protein of 249 amino acids was identified (Fig. 2). Translation of this open reading frame led to its identification as *ccrA*. DNA sequences corresponding to possible −10 and −35 based on *Escherichia coli* consensus sequences (4), were identified. A potential Shine-Dalgarno sequence, AAAAGA, was identified five bases upstream of the initiation site. It shares homology, in five of six bases, with the 3′ end of *Bacteroides fragilis* 16S RNA, GAACACCUCCU UUCU (17). Because β-lactamases are secreted proteins, one would predict that the enzyme should have a signal sequence. A potential amino-terminal signal sequence was identified. The sequence has a positively charged amino

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TABLE 1. *ccrA*-conferred beta-lactam resistance[a]

Organism	MIC (μg/ml) by agar dilution						
	Cefoxitin	Imipenem	Ticarcillin	Ticarcillin-clavulanic acid	Piperacillin	Tazobactam	Piperacillin-tazobactam
Bacteroides thetaiotaomicron	32	<0.12	32	1	32	8	4
Bacteroides thetaiotaomicron(pCLL2203) (shuttle vector only)	16	<0.12	32	2	64	16	4
Bacteroides thetaiotaomicron(pCLL2204) (vector with β-lactamase gene)	>256	>16	>256	>128	>256	>256	>256
Bacteroides fragilis TAL3636	>256	>16	>256	>128	>256	>256	>256

[a] *Bacteroides thetaiotaomicron* harboring no plasmid, pCLL2203, or pCLL2204 and *Bacteroides fragilis* TAL3636 were tested for their susceptibilities to various beta-lactams and β-lactamase-blocking agents. The beta-lactam–β-lactamase-blocking agents were present in the following concentrations: ticarcillin-clavulanic acid, clavulanic acid constant at 2 μg/ml; and piperacillin-tazobactam, tazobactam constant at 4 μg/ml (cefoxitin and ticarcillin, Sigma Chemical Co., St. Louis, Mo.; imipenem, Merck Sharp & Dohme, West Point, Pa.; clavulanic acid, Beecham Laboratories, Bristol, Tenn.; piperacillin and tazobactam, Lederle Laboratories, Pearl River, N.Y.).

terminus, a hydrophobic core, and the cleavage recognition sequence valine-methionine-alanine (16). As with the *Bacillus cereus* enzyme (1, 5), no serine-X-X-lysine active site was identified.

Comparison of CcrA with the *Bacillus cereus* enzyme. Amino acid sequence comparison was performed by using the DNA Star (DNA Star) program for protein sequence alignment. The two proteins shared 33.5% identity over the 221-amino-acid overlap (Fig. 3). The strongest identity was found surrounding the three histidine residues and one cysteine residue involved in Zn^{2+} binding in the *Bacillus cereus* enzyme (2, 9) and another region between Ile-191 and Trp-202. These regions were amino acids 99 to 107 (>66% identity), 157 to 184 (>67% identity), 191 to 202 (>58% identity), and 219 to 227 (>77% identity). A fifth region of strong identity was the five-amino-acid sequence VSPNG, from amino acids 52 to 56, which was 100% identical. The amino-terminal sequences and signal sequences of the two proteins showed the weakest identity. Beginning at amino acid 50, no adjustment in the amino acid spacing was required to align the Zn^{2+}-binding histidine residues and cysteine residue of the *Bacillus cereus* enzyme with histidine residues and a cysteine residue in CcrA.

The class B β-lactamase gene from *Bacteroides fragilis* was cloned directly into *Escherichia coli*. The gene contains the signal sequences −10 and −35 of an *Escherichia coli*

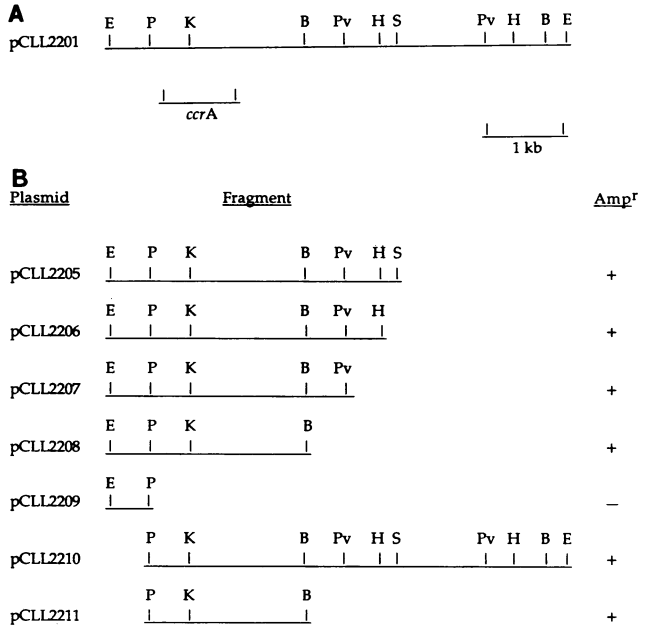

FIG. 1. (A) Restriction endonuclease map of the cloned 6.5-kb fragment from pCLL2201. The location of the β-lactamase gene within the fragment is indicated. The leftmost *Bgl*II restriction site is shown. There are several other *Bgl*II restriction sites in the remaining region. (B) Plasmids carrying subfragments of the 6.5-kb *Eco*RI fragment and their ability to confer ampicillin resistance (Amp^r) to DH5α. Restriction enzyme abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; P, *Pst*I; Pv, *Pvu*I. The enzymes *Bam*HI, *Cla*I, *Xba*I, and *Xho*I do not restrict the DNA fragment.

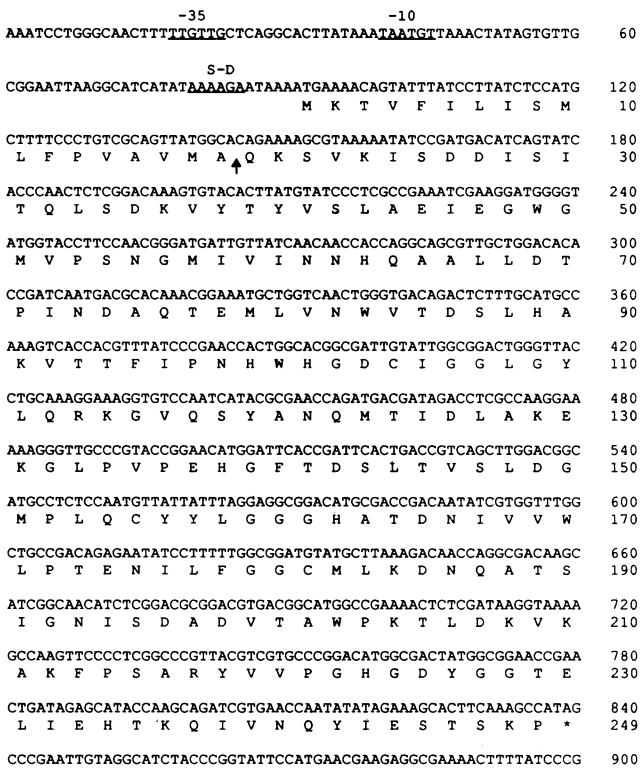

FIG. 2. DNA sequence of the sense strand and the deduced amino acid sequence of the *Bacteroides fragilis* β-lactamase gene. The −35 and −10 regions of a promoterlike sequence are underlined. The Shine-Dalgarno (S-D) sequence is also underlined. The arrow indicates the potential signal sequence cleavage site.

FIG. 3. Comparison of the amino acid sequences of the *Bacteroides fragilis* class B β-lactamase and the *Bacillus cereus* 569/H class B β-lactamase. Matching amino acids are printed between the two sequences. Amino acids that are positively related are shown with a colon on the line between the amino acids, those with a zero value relationship are shown with a period, and those that are negatively related show a blank. The three histidine residues and one cysteine residue which function in binding of the Zn^{2+} ligand in the *Bacillus cereus* enzyme are underlined. The signal sequence cleavage sites are indicated with an arrow.

transcriptional promoter and a potential *Bacteroides fragilis* Shine-Dalgarno sequence. It is not known whether these are the functional signals for *ccrA*. Comparison of CcrA with the *Bacillus cereus* enzyme revealed histidine and cysteine residues which are likely to participate in Zn^{2+} binding and other conserved amino acids that may participate either actively or structurally in enzymatic activity.

ADDENDUM

Since submission of the manuscript, a similar report has been published (13). The authors of that report were, however, unable to clone the gene directly into *Escherichia coli*.

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