

Sequence of the Gene Encoding a Plasmid-Mediated Cefotaxime-Hydrolyzing Class A β -Lactamase (CTX-M-4): Involvement of Serine 237 in Cephalosporin Hydrolysis

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The sequence of the gene encoding a novel cefotaxime-hydrolyzing β -lactamase (CTX-M-4) was determined. It was located in a plasmid harbored by a *Salmonella typhimurium* strain. CTX-M-4 was similar to the plasmidic cefotaxime-hydrolyzing β -lactamases CTX-M-2 and Toho-1 and related to the chromosomal β -lactamase of *Klebsiella oxytoca*. A Ser-237→Ala substitution, introduced by site-directed mutagenesis, caused minor alterations in the interaction of CTX-M-4 with β -lactams, reducing slightly the relative hydrolytic activity against cefotaxime and the susceptibility to inhibition by clavulanate.

Resistance to expanded-spectrum cephalosporins in enterobacteria is usually due to the expression of extended-spectrum TEM and SHV β -lactamases (14). The emergence of plasmid-mediated β -lactamases that are related to the chromosomal class A enzyme of *Klebsiella oxytoca* and that confer resistance to cephalosporins has recently been noted. The enzymes MEN-1 (3) and Toho-1 (11) were found in *Escherichia coli* clinical strains isolated in France and Japan, respectively; CTX-M-1, with an amino acid sequence identical to that of MEN-1, was found in an *E. coli* clinical isolate in Germany (5). β -Lactamase CTX-M-2 was from a *Salmonella typhimurium* strain isolated in Argentina (4). The β -lactamases described above have a distinct substrate profile in that they efficiently hydrolyze cefotaxime but spare ceftazidime (3–5, 11). It has been hypothesized that the extended-spectrum hydrolytic properties of the β -lactamases mentioned above are due, at least in part, to a serine residue located at position 237 (Ambler's numbering scheme [1]) (3). In other studies, however, this hypothesis has been considered uncertain (11).

In a previous work, we described a plasmid-mediated β -lactamase, not belonging to either the TEM or SHV family or to AmpC cephalosporinases, that preferentially hydrolyzed cefotaxime. The plasmid encoding this enzyme was harbored by an outbreak strain of *S. typhimurium* isolated in St. Petersburg, Russia (8). In the present work, it is shown that the enzyme, designated CTX-M-4, is closely related to the above-mentioned plasmid-mediated cefotaxime-hydrolyzing β -lactamases. We also examined the role of the Ser-237 residue in determining the substrate specificity of CTX-M-4 β -lactamase.

The cefotaxime-resistant *E. coli*(pMSL) transconjugant was obtained after mating *E. coli* 14R525 (Nal^r lac⁺) with an *S. typhimurium* clinical isolate (8). The *E. coli* strain C600 was used for the preparation of β -lactamase extracts. The *E. coli* strains JM109 and ES1301 *mutS* (Promega) were used for cloning and mutagenesis. The latter strain suppresses mismatch repair in vivo. The plasmid pBCSK(+) (Stratagene) was used for cloning

and sequencing. The plasmid pAlter-Ex2 (Promega) was used for site-directed mutagenesis.

MICs of β -lactam antibiotics were determined by a microdilution method using Mueller-Hinton broth (15). Screening for cefotaxime-resistant clones was performed by using standard antibiograms.

A 2.7-kbp *EcoRI*-*Bam*HI fragment from plasmid pMSL was ligated to pBCSK(+). The resulting plasmid, pMSL1, conferred cefotaxime resistance when used to transform *E. coli* C600 and JM109 cells. Several overlapping fragments of the *EcoRI*-*Bam*HI insert were subcloned to pBCSK(+), and the DNA sequence was determined by the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemicals—Amersham).

An intragenic *Pst*I-*Bam*HI fragment of 0.9 kbp from pMSL1 was subcloned into pAlter-Ex2, and the resulting plasmid (pMSL2) was used for site-specific mutagenesis with the pAlter-Ex2 mutagenesis kit (Promega) as recommended by the manufacturer. The mutagenic primer was 30 bp and contained two mismatches in the triplet corresponding to codon 237 (GCC [Ala] instead of AGC [Ser]; mismatches underlined). The mutagenized insert was reintroduced into *Pst*I-*Bam*HI-digested pMSL1, resulting in plasmid pMSL1-M. The mutant gene and the promoter region were entirely sequenced to confirm the desired exchanges in the nucleotide sequence.

For β -lactamase preparations, *E. coli* C600 strains transformed with pMSL1 and pMSL1-M were grown overnight in tryptone soy broth. β -Lactamases were released after ultrasonic treatment of cell suspensions. The extracts were clarified by ultracentrifugation, desalted, and concentrated. One unit of β -lactamase activity was defined as the amount of enzyme hydrolyzing 1 μ mol of nitrocefin per min per mg of protein at 37°C and pH 7. Isoelectric focusing was performed on polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5) (Pharmacia-LKB).

Hydrolytic activity against β -lactams was evaluated by UV spectrophotometry as described previously (9). The maximum rate of hydrolysis (relative V_{max}) and K_m values were determined by Lineweaver-Burk plots. The V_{max} values were expressed as hydrolysis rates relative to that of cephalothin, which was set at 100. Inhibition of β -lactamase activity by

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TABLE 3. Inhibition profiles of CTX-M-4 wild-type and S237A mutant β -lactamases

| β -Lactamase ^a | IC ₅₀ (μ M) of inhibitor ^b | | |
|---------------------------------|---|-----------|------------|
| | Clavulanate | Sulbactam | Tazobactam |
| CTX-M-4 | 1.0 | 14 | 0.10 |
| S237A mutant | 2.5 | 17 | 0.12 |

^a Two hundred fifty units of β -lactamase was used.

^b IC₅₀s were the mean values of three measurements not differing by more than 10%.

influence the susceptibility of the enzyme to inhibition by the penam sulfones sulbactam and tazobactam (Table 3).

The DNA sequence data showed that CTX-M-4 belongs to the group of the recently emerged plasmid-mediated class A β -lactamases that preferentially hydrolyze cefotaxime. The amino acid substitutions in the CTX-M-4 β -lactamase sequence as compared with the sequences for Toho-1 and CTX-M-2 enzymes were located at positions that are not considered important for the hydrolytic activity of class A β -lactamases (Table 4). As can also be seen from the reported β -lactamase resistance phenotypes and substrate profiles, substantial differences in their hydrolytic activity would not be expected. These enzymes are related to the species-specific β -lactamases of *K. oxytoca* (2, 18), *Citrobacter diversus* (17), and *Proteus vulgaris* (16), but the similarity does not exceed 80% and their chromosomal ancestor cannot be determined with certainty.

The process of hydrolysis of expanded-spectrum β -lactams by CTX-M-4 and the other plasmid-mediated class A cefotaxime-hydrolyzing β -lactamases may be different from that of the extended-spectrum derivatives of TEM-1, TEM-2, and SHV-1 penicillinases. Amino acid substitutions at positions 104, 164, 238, and 240, which have been associated with the expansion of the activity of TEM and SHV β -lactamases towards oxymino-cephalosporins (13), are not present in CTX-M-4 and the similar enzymes. It has been observed that the hydrolytic activity of MEN-1 (CTX-M-1) against cephalosporins is greater than that of the chromosomally encoded β -lactamase of *K. oxytoca* (3). The reported data also suggest that the plasmid-mediated cefotaxime-hydrolyzing enzymes CTX-M-2, Toho-1, and CTX-M-4 hydrolyze cephalosporins more efficiently than the related chromosomal β -lactamases (2, 4, 5, 11, 16, 18). A serine residue at position 237 is present in all the former β -lactamases, whereas the same position is occupied by alanine in most of the class A enzymes, including the related chromosomal β -lactamases of *K. oxytoca* and *C. diversus*. The minimal changes in the kinetic parameters of cefotaxime hydrolysis caused by the substitution of Ser-237 by Ala, along with the moderate decrease in the MIC of the antibiotic, indicate that Ser-237 may be involved in the hydro-

TABLE 4. Amino acid differences between CTX-M-4 β -lactamase and the related cefotaxime-hydrolyzing enzymes Toho-1 and CTX-M-2^a

| β -Lactamase | Amino acid residue at position: | | | | | | | | | |
|--------------------|---------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 39 | 48 | 61 | 98 | 99 | 125 | 171 | 225 | 230 | 274 |
| CTX-M-4 | Ser | Gln | Val | Arg | Ala | Gly | Ser | Met | Gly | Ser |
| CTX-M-2 | Ser | Leu | Arg | Lys | Lys | Ala | Thr | Leu | Val | Ser |
| Toho-1 | Arg | Leu | Arg | Lys | Lys | Ala | Thr | Leu | Val | Arg |

^a Only the differences in the mature peptides are indicated. The amino acid residues were numbered according to Ambler's system (1).

lytic process but that its role is not critical. In class A β -lactamases, the backbone NH and CO groups of residue 237 participate in the hydrogen bond network that stabilizes binding of the substrate within the active site (13). Cefotaxime and cefuroxime may not be able to form the expected hydrogen bonds but interact, through the NH of the C-7 acylamido linkage, with the side chain OH group of threonine (as in TEM-5 and TEM-24) (13). Ser-237 in CTX-M-4, as well as in *P. vulgaris* β -lactamase (19), may act in an analogous mode. A similar interaction may account for the slight reduction of the inhibitory activity of clavulanate after replacement of Ser-237 by Ala. It has also been suggested that substitutions at position 237 in class A β -lactamases may alter the conformational flexibility of the B3 strand of the enzymes (7, 10). Such modifications may be responsible for the minor changes in the interaction of the S237A mutant enzyme with several β -lactam compounds.

Nucleotide sequence accession number. The accession number of the nucleotide sequence of the *bla*_{CTX-M-4} gene in the EMBL database is Y14156.

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