

## Mutational Replacement of Leu-293 in the Class C *Enterobacter cloacae* P99 $\beta$ -Lactamase Confers Increased MIC of Cefepime

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**The class C  $\beta$ -lactamase from *Enterobacter cloacae* P99 confers resistance to a wide range of broad-spectrum  $\beta$ -lactams but not to the newer cephalosporin cefepime. Using PCR mutagenesis of the *E. cloacae* P99 *ampC* gene, we obtained a Leu-293-Pro mutant of the P99  $\beta$ -lactamase conferring a higher MIC of cefepime (MIC, 8  $\mu$ g/ml, compared with 0.5  $\mu$ g/ml conferred by the wild-type enzyme). In addition, the mutant enzyme produced higher resistance to ceftazidime but not to the other  $\beta$ -lactams tested. Mutants with 15 other replacements of Leu-293 were prepared by site-directed random mutagenesis. None of these mutant enzymes conferred MICs of cefepime higher than that conferred by Leu-293-Pro. We determined the kinetic parameters of the purified *E. cloacae* P99  $\beta$ -lactamase and the Leu-293-Pro mutant enzyme. The catalytic efficiencies ( $k_{cat}/K_m$ ) of the Leu-293-Pro mutant  $\beta$ -lactamase for cefepime and ceftazidime were increased relative to the respective catalytic efficiencies of the wild-type P99  $\beta$ -lactamase. These differences likely contribute to the higher MICs of cefepime and ceftazidime conferred by this mutant  $\beta$ -lactamase.**

Class C  $\beta$ -lactamases are the second most common class of  $\beta$ -lactam-hydrolyzing enzymes (8). Most of the class C  $\beta$ -lactamases are chromosomally encoded; they are thus less prone to mutational alteration of structure than are plasmid-encoded enzymes, whose structural genes exist in greater numbers of copies. Moreover, the development of clinically important new resistance phenotypes from chromosomally encoded  $\beta$ -lactamases would require not only mutation of the structural gene, as in the case of constitutive plasmid-mediated enzymes such as TEM-1, but also the genetic derepression of the structural gene to produce large amounts of enzyme. Recently, several bacterial strains expressing plasmid-encoded class C  $\beta$ -lactamases have been identified among clinical isolates (1, 5, 11, 14, 30, 31). The discovery of plasmid-encoded class C  $\beta$ -lactamases bodes ill for the prospects of  $\beta$ -lactam therapy in the future and may be a prelude to a potential explosion among clinical isolates of new variants of class C enzymes with extended substrate profiles, such as we have witnessed for class A  $\beta$ -lactamases.

The principal mechanism of resistance to extended-spectrum  $\beta$ -lactams in nosocomial pathogens such as *Enterobacter* is the production of class C  $\beta$ -lactamases by derepression of chromosomal genes (16, 27). This results in high-level resistance to virtually all penicillins and cephalosporins and to the monobactam aztreonam. To overcome such resistance, novel  $\beta$ -lactams that are poor substrates for these enzymes have been developed. Two classes of such  $\beta$ -lactams are currently in clinical use: newer cephalosporins (10, 17, 28), such as cefepime and ceftazidime, and carbapenems (13), such as imipenem and

meropenem. Class A  $\beta$ -lactamases, such as TEM and SHV, can mutate easily in clinical isolates to confer resistance to newer cephalosporins but not to carbapenems (<http://www.lahey.org/studies/webt.htm>). In contrast, such mutations of class C enzymes have rarely been reported to occur in clinical isolates (2, 3, 25). From the class C  $\beta$ -lactamase of *Enterobacter cloacae* P99, chosen because its X-ray crystallographic structure is known (20), we have selected a mutant that confers resistance to cefepime as a result of a single amino acid substitution, Leu-293-Pro. Site-directed mutagenesis of the corresponding codon generated mutants with 14 additional replacements of Leu-293 that resulted in alterations of conferred resistance to  $\beta$ -lactams. The kinetic properties of the  $\beta$ -lactamase from the cefepime-resistant mutant with replacement of Leu-293 by proline have been compared to those of the parental enzyme in this report.

(This work was previously presented in part [S. B. Vakulenko, B. Geryk, and S. A. Lerner, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-202, 1997].)

### MATERIALS AND METHODS

**Bacterial strains.** *Escherichia coli* JM83 [ $F^-$  *ara*  $\Delta$ (*lac-proAB*) *rpsL* (Str<sup>r</sup>) [ $\phi$ 80 *dlac*  $\Delta$ (*lacZ*)M15] *thi*] (New England Biolabs) was utilized as a recipient strain for plasmids and as a host for the determination of susceptibilities to antibiotics. *E. coli* BMH 71-18 *mutS* [*thi supE*  $\Delta$ (*lac-proAB*) [*mutS*::Tn10] [*F'* *proAB lacI*<sup>q</sup>Z $\Delta$ M15]] (Clontech) was used as the intermediate host in site-directed mutagenesis experiments. *E. coli* BL21(DE3) [ $F^-$  *ompT hsdS<sub>B</sub>*( $\tau_B^-$   $m_B^-$ ) *gal dem* (DE3)] (Novagen) was the host for target gene expression.

**Antibiotics.** Ampicillin, ceftazidime, ceftazidime, ceftriaxone, cefoperazone, cephaloridine, and kanamycin were from Sigma, aztreonam and cefepime were from Bristol-Myers Squibb, and piperacillin was from Lederle (Wyeth-Ayerst).

**Plasmids for mutagenesis and DNA sequencing.** We constructed plasmid pUC19(*ampC1*) in the following manner. We replaced the *DraI* fragments of pUC19 containing the *bla* gene for the TEM  $\beta$ -lactamase with a *HincII* fragment of pUC4K (Pharmacia) containing the kanamycin resistance marker from Tn903 (construct I). The entire structural gene (plus the Shine-Dalgarno sequence) of the *E. cloacae* P99  $\beta$ -lactamase was amplified by PCR from the genomic DNA of

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*E. cloacae* P99 (kindly provided by J.-M. Frère) with primers containing *Eco*RI sites near their 5' ends. We used the resulting blunt-end product to replace the *Ssp*I-*Hind*III fragment in construct I after filling in the *Hind*III site. Expression of the *E. cloacae* P99 *ampC*  $\beta$ -lactamase gene was then driven by the TEM  $\beta$ -lactamase promoter, and therefore, a transformant containing this gene [pUC19(*ampC*1)] was selected by growth on ceftazidime-supplemented agar.

**Mutagenesis and DNA sequencing.** We utilized *Taq* DNA polymerase and two primers containing *Eco*RI sites to reamplify the cloned *ampC* gene in order to introduce random mutations. After 30 cycles of PCR, the PCR product was digested with *Eco*RI, purified by using a QIAquick PCR purification kit (Qiagen), and substituted for the parental wild-type gene in pUC19(*ampC*1). The resulting construct was subsequently introduced into competent *E. coli* JM83 by high-efficiency electroporation, and selection was performed on the cefepime-supplemented agar. The entire sequences of the *ampC* genes from several cefepime-resistant strains were determined by the dideoxy chain termination method (29). Random site-directed mutagenesis of the *E. cloacae* P99  $\beta$ -lactamase gene at the codon corresponding to Leu-293 of the enzyme was performed by using a transformer site-directed mutagenesis kit (Clontech) with the following mutagenic primer: GCGACAGTAAGGTAGCANNNGCGCGTTGCCCGTGGCAG. After mutagenesis, transformants were selected by growth on cefepime or kanamycin, and the nucleotide sequence around and including the mutagenized codon was determined.

**Expression and purification of parental and mutant *E. cloacae* P99  $\beta$ -lactamases.** In order to facilitate  $\beta$ -lactamase secretion of both the wild-type and mutant  $\beta$ -lactamases into the growth medium, we replaced the DNA fragment corresponding to the original leader sequence of the *AmpC*  $\beta$ -lactamase with that of the leader sequence of the *OmpA* protein. To enhance the production of the parental and mutant enzymes, we cloned their genes fused to the *OmpA* leader sequence into the *Nde*I/*Hind*III sites of the pET-24a(+) vector (Novagen) under the control of the strong IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-inducible bacteriophage T7 promoter. The resulting constructs were used to transform the recipient strain of *E. coli* BL21(DE3).

For enzyme purification, *E. coli* BL21(DE3) strains producing wild-type or mutant  $\beta$ -lactamase were inoculated in Luria-Bertani medium containing kanamycin at 20  $\mu$ g/ml and grown overnight. Overnight cultures were diluted 100-fold in Terrific Broth (Difco) supplemented with 2 M sorbitol and 0.05 M betaine and incubated with shaking at 37°C until the cultures reached an optical density at 600 nm of 0.6. IPTG (0.4 mM) was added to each culture, and they were incubated overnight at 25°C. The cells were removed by centrifugation. The supernatant containing the enzyme was concentrated on an Amicon ultrafiltration device (membrane molecular size cutoff, 10,000 kDa). The concentrated protein was loaded onto a strong cation-exchange column (Macro-Prep high S support, 200 by 20 mm; Bio-Rad), equilibrated, and washed with 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 6.8). The enzyme was eluted with a linear gradient of 0 to 1 M NaCl in 10 mM TES buffer (pH 6). The wild-type and mutant enzymes were purified by this procedure, and all appeared homogeneous according to the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Determination of the amount of active enzymes in the periplasm.** Cells producing the wild-type and mutant P99  $\beta$ -lactamases were grown at 37°C overnight in Luria-Bertani broth supplemented with 30  $\mu$ g of kanamycin/ml. After 20-fold dilution into the same medium, the cells were regrown for another 2 h, and the cell densities of the two cultures were brought to the same value as determined spectrophotometrically at 600 nm. The cells were pelleted by centrifugation from 50 ml of culture, suspended in 1 ml of solution containing 27% (wt/vol) sucrose, 30 mM Tris, 5 mM EDTA, and 0.2 mg of lysozyme (pH 8.0), and incubated for 30 min at 4°C. Spheroplasts were removed by centrifugation for 5 min at 20,000  $\times$  g. Enzyme concentrations in the periplasm were determined from the hydrolysis rates of nitrocefin at the saturating concentration and turnover rate constants of both enzymes with nitrocefin.

**Kinetic analysis of  $\beta$ -lactamases.** All kinetic measurements were performed on a Hewlett-Packard 8453 diode array spectrophotometer at 25°C in 100 mM sodium phosphate buffer (pH 7.0). Hydrolysis of each substrate was monitored at the corresponding wavelength: cephaloridine at 267 nm,  $\Delta\epsilon = 1,000 \text{ cm}^{-1} \text{ M}^{-1}$ ; ceftazidime at 280 nm,  $\Delta\epsilon = 3,725 \text{ cm}^{-1} \text{ M}^{-1}$ ; cefepime at 260 nm,  $\Delta\epsilon = 750 \text{ cm}^{-1} \text{ M}^{-1}$ . The kinetic parameters for cephaloridine and ceftazidime turnover were evaluated from Hanes-Wolf plots, in which  $K_m$  values were flanked by six points. The  $K_m$  values of the wild-type enzyme for cefepime were determined as  $K_i$  by using this antibiotic as a competitive inhibitor versus nitrocefin, and the  $k_{cat}/K_m$  value was determined at substrate concentrations lower than  $K_m$  with enzyme concentrations of 2  $\mu$ M. The kinetic parameters for cefepime turnover by the mutant enzyme were evaluated from Hanes-Wolf plots, in which  $K_m$  values were flanked by six points.

## RESULTS

**Cloning of the *E. cloacae* P99 *ampC*  $\beta$ -lactamase gene.** We constructed the pUC19(*ampC*1) vector by cloning the 1,172-bp fragment containing the entire structural gene for the *E. cloacae* P99 *AmpC*  $\beta$ -lactamase into construct I, a derivative of pUC19 bearing a kanamycin resistance marker, *aph*(3')-Ia. The complete nucleotide sequence of the insert was confirmed, and restriction mapping revealed that the *ampC* and *aph*(3')-Ia genes were transcribed from the same strand.

**Generation and selection of cefepime-resistant mutants.** After PCR mutagenesis of the *E. cloacae* P99 *ampC* gene, the products were recloned into the pUC19(*ampC*1) vector. The resulting ligation mixture was used to electroporate *E. coli* JM83, and mutants were selected by growth on agar plates supplemented with 1, 2, 4, or 8  $\mu$ g of cefepime/ml. Since there was no growth at the highest concentration, we chose four colonies from the plate with 4  $\mu$ g of cefepime/ml for analysis of the  $\beta$ -lactamase gene. One mutant, with a single base change resulting in the replacement of Leu-293 with proline, was investigated further. The cefepime MIC for this mutant was 8  $\mu$ g/ml. The  $\beta$ -lactamase structural gene from the mutant was recloned into the same vector and transformed into *E. coli* JM83; MICs of cefepime for transformants selected by growth in the presence of kanamycin were the same as those for the original mutant. Thus, the observed level of resistance to cefepime was due entirely to the replacement of Leu-293 by proline.

**Mutagenic replacement of Leu-293.** Site-directed mutagenesis was carried out with a mixture of primers bearing all four nucleotides at the three positions of the codon corresponding to Leu-293. Initially, we sought mutants that conferred higher levels of resistance to cefepime by selection on cefepime plates, but all of the 20 mutants that grew in the presence of 4  $\mu$ g of cefepime/ml had the same Leu-293-Pro mutation. Sequencing of *ampC* DNA from 70 of the transformants that grew on kanamycin revealed 15 different replacements of Leu-293, including those with proline.

**Susceptibility of mutants to  $\beta$ -lactams.** Fifteen mutants, each with a different replacement of Leu-293 in the *E. cloacae* P99  $\beta$ -lactamase, were tested for susceptibility to various  $\beta$ -lactams (Table 1). The cefepime MIC for the Leu-293-Pro mutant originally selected for growth on cefepime after random mutagenesis was the highest (8  $\mu$ g/ml), 16-fold higher than that conferred by the wild-type enzyme. The same mutation increased the MIC of ceftazidime fourfold but reduced the MICs of ampicillin, aztreonam, ceftriaxone, and cephaloridine four- to eightfold. The cefepime resistance seen with the proline mutant was compromised by all of the other replacements. The highest residual MIC of cefepime (2  $\mu$ g/ml) was conferred by residues with smaller, uncharged side chains (glycine, alanine, valine, and serine) but also by aspartate. The cefepime MICs for mutants with arginine, phenylalanine, histidine, tyrosine, and lysine were at or below that for the strain with the parental *E. cloacae* P99  $\beta$ -lactamase. The profile of ceftazidime MICs for the various mutants followed closely that of cefepime MICs. In contrast to the profiles with cefepime and ceftazidime, there was no rise in resistance to ampicillin, piperacillin, ceftriaxone, cefoperazone, aztreonam, or cephaloridine. However, functional activity was retained against these  $\beta$ -lactams,

TABLE 1. MICs of  $\beta$ -lactams conferred in *E. coli* JM83 by the *E. cloacae* P99  $\beta$ -lactamase and its mutants with replacement of Leu-293

Amino acid at residue 293	MIC ( $\mu$ g/ml) of indicated $\beta$ -lactam <sup>a</sup>							
	AMP	CAZ	FEP	CRO	CFP	PIP	ATM	LOR
None <sup>b</sup>	4	0.125	<0.015	<0.03	<0.06	0.5	<0.03	4
Leu <sup>c</sup>	1,024	64	0.5	128	128	128	64	512
Pro	256	256	8	32	128	64	8	64
Gly	512	256	2	64	128	128	16	512
Ala	256	128	2	64	128	128	16	512
Val	512	128	2	64	128	256	32	256
Ser	512	128	2	64	128	128	16	256
Cys	512	128	1	64	64	128	32	256
Asn	512	128	1	32	64	64	32	512
Glu	512	64	1	32	128	128	16	256
Met	512	128	1	128	128	128	32	512
Asp	256	128	2	4	32	8	2	512
Arg	256	32	0.5	64	32	128	64	128
Phe	256	32	0.5	64	128	64	64	256
His	256	16	0.25	32	32	32	64	128
Tyr	256	32	0.25	32	128	32	64	256
Lys	256	32	0.25	64	32	64	64	128

<sup>a</sup> Abbreviations: AMP, ampicillin; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; CFP, cefoperazone; PIP, piperacillin; ATM, aztreonam; LOR, cephaloridine.

<sup>b</sup> *E. coli* JM83 recipient strain.

<sup>c</sup> Wild-type *E. cloacae* P99  $\beta$ -lactamase.

since the MICs were in the range of 12.5 to 100% of those conferred by the wild-type *E. cloacae* P99 enzyme. The results with the Leu-293-Asp mutant were somewhat anomalous. Whereas the MICs of ampicillin, ceftazidime, cefoperazone, and cephaloridine conferred by this mutant were in line with those conferred by the other mutants, the MICs of piperacillin, ceftriaxone, and aztreonam were markedly (16- to 32-fold) reduced compared to those observed with the wild-type strain.

**Relative amount of active enzymes in the periplasm and their stability.** The experiments with enzymes extracted from the periplasm revealed that there was 2.4-fold less active Leu-293-Pro mutant enzyme than there was wild-type  $\beta$ -lactamase. Both enzymes were stable for at least 8 days at 4°C.

**Kinetic studies.** We carried out kinetic studies with homogeneous preparations of the *E. cloacae* P99  $\beta$ -lactamase and its mutant Leu-293-Pro variant (Table 2). The kinetic data that we obtained for the wild-type enzyme with cephaloridine, ceftazidime, and cefepime were within ranges reported previously (6, 9, 12, 24, 26). The catalytic efficiencies ( $k_{\text{cat}}/K_m$ ) of the two enzymes with cephaloridine showed a 5.7-fold decline for the mutant  $\beta$ -lactamase, mostly resulting from the decline in  $k_{\text{cat}}$ . On the other hand, the mutant enzyme was more efficient than the wild-type  $\beta$ -lactamase against ceftazidime and cefepime: the  $k_{\text{cat}}/K_m$  values were 11- and 27-fold higher, respectively. With ceftazidime, the change was mainly due to an eightfold increase in  $k_{\text{cat}}$ , whereas with cefepime, the greater efficiency was due to the fourfold decrease in  $K_m$  and the sixfold increase in  $k_{\text{cat}}$ .

## DISCUSSION

The proliferation of extended-spectrum class A  $\beta$ -lactamases and the hyperproduction of AmpC  $\beta$ -lactamases has led to the potential compromise of the clinical utility of most of the  $\beta$ -lactam antibiotics, especially in nosocomial infections. Fur-

thermore, in contrast to the class A  $\beta$ -lactamases, the intrinsic resistance of class C enzymes to clinically available  $\beta$ -lactamase inactivators limits the option of using such inactivators in combination with penicillins or cephalosporins against strains producing these enzymes. The carbapenems, such as imipenem and meropenem, fortunately retain activity against many strains that hyperproduce AmpC-type  $\beta$ -lactamases. Moreover, the zwitterionic cephalosporin, cefepime, may retain its activity against most AmpC-producing strains of *Enterobacter*, including those with reduced permeability of their outer membrane resulting from mutational alteration of porin proteins (21). In fact, cefepime was shown to retain much greater activity than carbapenems against an AmpC-producing *Klebsiella pneumoniae* strain that lacks one of the porin proteins (22).

Unlike the prototypical TEM-1 and SHV-1 class A  $\beta$ -lactamases, the class C enzymes confer resistance broadly to cephalosporins as well as to penicillins. The resulting lack of selective pressure on class C  $\beta$ -lactamases by most  $\beta$ -lactams and the requirement for derepression of these naturally inducible enzymes to produce resistance might explain in part why mutants of class C  $\beta$ -lactamases have apparently not emerged commonly in the clinical setting. Only three cases of mutant class C  $\beta$ -lactamases in clinical isolates have been reported. Two showed unusual types of mutations: a duplication of three amino acids (25) and a deletion of six consecutive amino acid residues in the P99  $\beta$ -lactamase (2). Although the strain of *E. cloacae* from the first report was shown to be highly resistant to cefuroxime, ceftazidime, and aztreonam, the wild-type P99  $\beta$ -lactamase itself is known to confer clinically significant, though lower, resistance to these  $\beta$ -lactams. Regrettably, the strain producing this mutant enzyme was not tested for susceptibility to cefepime and other  $\beta$ -lactams that retain activity against *Enterobacter* strains which hyperproduce an AmpC  $\beta$ -lactamase. The third report revealed that a point mutation in the plasmid-encoded CMY-1  $\beta$ -lactamase had resulted in new resistance to ceftazidime (3).

The increased utilization of newer cephalosporins such as cefepime for the treatment of infections caused by AmpC-producing bacterial pathogens may now provide the setting for the selection of mutant class C enzymes that confer resistance to an even broader spectrum of  $\beta$ -lactams. With this in mind, novel cefepime resistance was previously selected in *E. cloacae*

TABLE 2. Kinetic constants for turnover of  $\beta$ -lactam substrates with the *E. cloacae* P99  $\beta$ -lactamase and its Leu-293-Pro mutant derivative<sup>a</sup>

$\beta$ -Lactamase	$K_m$ ( $\mu$ M)	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{s}^{-1}$ )
LOR			
Wild type	68 $\pm$ 8	826 $\pm$ 62	(12 $\pm$ 1) $\times$ 10 <sup>6</sup>
Leu-293-Pro	60 $\pm$ 6	126 $\pm$ 9	(2.1 $\pm$ 0.2) $\times$ 10 <sup>6</sup>
CAZ			
Wild type	15 $\pm$ 2	0.013 $\pm$ 0.001	(9 $\pm$ 1) $\times$ 10 <sup>2</sup>
Leu-293-Pro	10 $\pm$ 3	0.10 $\pm$ 0.01	(10 $\pm$ 3) $\times$ 10 <sup>3</sup>
FEP			
Wild type	100 $\pm$ 16	0.5 $\pm$ 0.1	(0.47 $\pm$ 0.03) $\times$ 10 <sup>4</sup>
Leu-293-Pro	24 $\pm$ 2	3.1 $\pm$ 0.2	(13 $\pm$ 1) $\times$ 10 <sup>4</sup>

<sup>a</sup> Abbreviations: LOR, cephaloridine; CAZ, ceftazidime; FEP, cefepime.



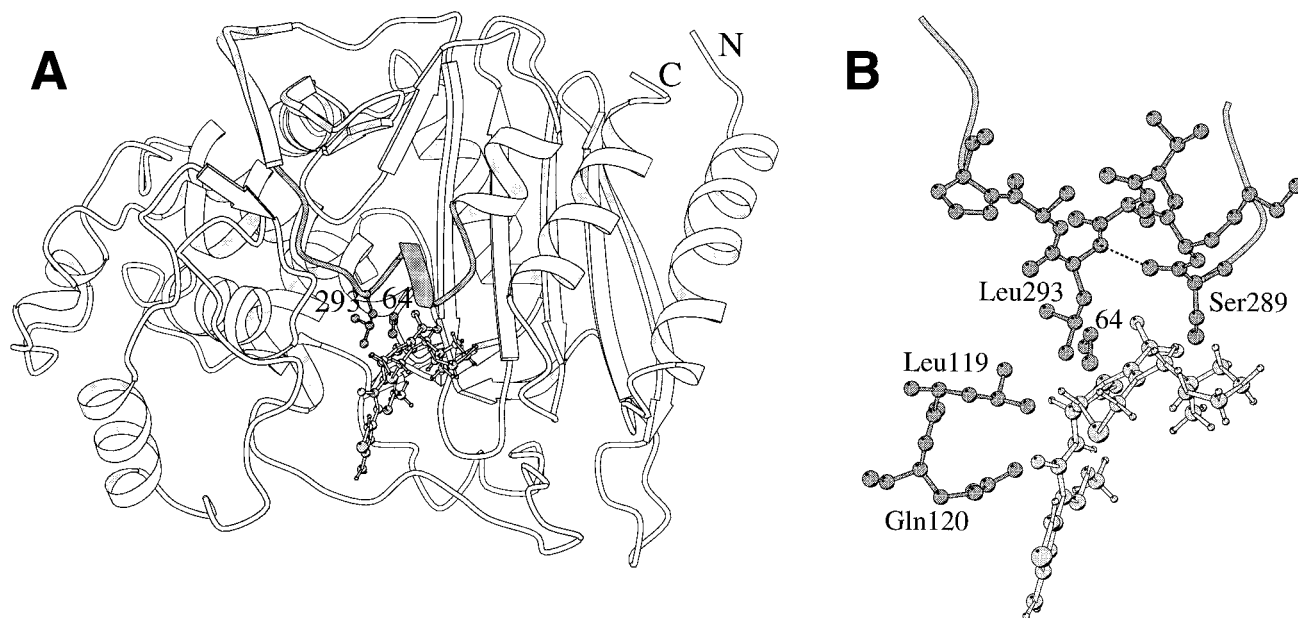


FIG. 1. (A) Ribbon representation of the crystallographic structure of the *E. cloacae* P99  $\beta$ -lactamase (20). Cefepime is modeled near the reactive Ser-64. (B) A closer view of the environment of Leu-293. The hydrogen bond from the 293-NH to the carbonyl oxygen atom of Ser-289 is drawn as a dashed line. Hydrogen atoms are omitted from the protein structure. The figure was drawn by using MOLSCRIPT (18).

P99 in vitro; the mutant enzyme was shown to bear the replacements T147R and V298E (R. Gomez, S. B. Vakulenko, and S. A. Lerner, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. A-1, 1997). The importance of the V298E mutation in producing cefepime resistance was subsequently confirmed (23).

We report here the selection of a mutant of the *E. cloacae* P99  $\beta$ -lactamase conferring a 16-fold rise in the MIC of cefepime. At the same time, the MIC of ceftazidime was enhanced fourfold, and there was little compromise of activity for a variety of other  $\beta$ -lactams. Although the  $\beta$ -lactamase genes we describe were expressed in *E. coli* from the TEM-1 promoter in a multicopy plasmid, we should point out that the levels of resistance to the various  $\beta$ -lactams conferred by the cloned wild-type gene were similar to those for *E. cloacae* P99 (data not shown).

The significant increase in the MIC of cefepime resulted from a single base pair change that replaced Leu-293 with proline. Although the insertion of proline into the primary structure of an efficient enzyme may compromise its function, in this case it is noteworthy that the efficiency of the mutant enzyme for cefepime increased along with that for ceftazidime.

The crystal structure of the *E. cloacae* P99  $\beta$ -lactamase (20) shows that the side chain of Leu-293 is located rather far (9.5 Å) from the reactive Ser-64, yet near the expected position of C<sub>3</sub> substituents of cephalosporins (Fig. 1A). The side chain is rather exposed and not intimately involved in stabilizing the folded protein. Therefore, one might expect that the enzyme could tolerate many changes at this position, even though it is fairly conserved among class C enzymes. In fact, the modest effect on resistance to various  $\beta$ -lactams resulting from other substitutions at position 293 in our set of mutants supports this expectation.

Since the relatively large side chain of Leu-293 in the wild-

type P99  $\beta$ -lactamase may possibly contact the C<sub>3</sub> substituent of cefepime, substitution with a smaller side chain, as in proline, glycine, and alanine, apparently provides more space or a better fit for cefepime. Conversely, bulky residues such as phenylalanine and tyrosine are associated with the lowest cefepime MICs conferred. In addition, one may consider the electrostatic interaction between the positively charged C<sub>3</sub> substituent of cefepime and residues in proximity such as that at position 293. Thus, positively charged residues at that site (arginine, histidine, and lysine) are also associated with the lowest levels of resistance to cefepime, perhaps because of mutual repulsion. On the other hand, negatively charged residues are associated with somewhat higher levels of conferred cefepime resistance.

The crystal structure shows that the replacement of Leu-293 by proline will have at least two structural consequences. An existing hydrogen bond (2.9 Å) from the backbone NH of Leu-293 to the CO of Ser-289 will be lost, as will a stabilizing hydrophobic interaction with the side chain of a neighboring Leu-119 (Fig. 1B), which lies in a conserved sequence: GLPL (residues 116 to 119). The first change is possible only with the substitution by proline. Combined with the narrowness of backbone conformations available to proline, both changes are highly likely to produce a conformational change somewhere in the 288 to 296 loop above the binding site. While details of the change are, of course, unknown, it is quite possible that a more flexible loop, or a more open one, results from the mutation, such that more space is available to an approaching substrate, with a reduction of  $K_m$ . It is noteworthy that a recently described mutant *E. cloacae*  $\beta$ -lactamase that was responsible in part for resistance to cefepime in a clinical isolate lacked residues 289 to 294 (2).

The MIC of a  $\beta$ -lactam for  $\beta$ -lactamase-producing bacteria is a complex observation that includes not only the catalytic

efficiency of the enzyme but also its concentration in the periplasm and the penetration of the drug through the outer membrane (6, 15, 19, 24). It should be noted (Tables 1 and 2) that the differences in catalytic efficiency between the wild-type and mutant enzymes for each substrate parallel the differences in the MICs of the respective  $\beta$ -lactam conferred by the two enzymes in the intact cells. Thus, the mutant enzyme conferred an MIC of cephaloridine eight times lower than that conferred by the P99  $\beta$ -lactamase, and its catalytic efficiency ( $k_{cat}/K_m$ ) was six times lower. The  $k_{cat}/K_m$  values of the mutant enzyme for ceftazidime and cefepime were 11 and 27 times higher, respectively, than those of the wild-type enzyme, and the MICs of these  $\beta$ -lactams conferred by the mutant enzyme were also higher, 4- and 16-fold, respectively. If one takes into account the fact that the concentration of the mutant enzyme in the periplasm is 2.4-fold lower than that of the wild type, the correlations between the MICs and kinetic parameters, especially for ceftazidime and cefepime, become even closer.

We have shown that the development of resistance to cefepime by mutational alteration of the *E. cloacae* P99  $\beta$ -lactamase can occur readily by a single mutation. Recent reports have described AmpC-type  $\beta$ -lactamases encoded by plasmid genes that confer broad  $\beta$ -lactam resistance but not to cefepime (4, 7). Concern about the evolution of class C  $\beta$ -lactamases to confer resistance to cefepime is further heightened by the possibility of the plasmid-mediated spread of such mutant enzymes.

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