

## Selection and Characterization of $\beta$ -Lactam- $\beta$ -Lactamase Inactivator-Resistant Mutants following PCR Mutagenesis of the TEM-1 $\beta$ -Lactamase Gene

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**Mechanism-based inactivators of  $\beta$ -lactamases are used to overcome the resistance of clinical pathogens to  $\beta$ -lactam antibiotics. This strategy can itself be overcome by mutations of the  $\beta$ -lactamase that compromise the effectiveness of their inactivation. We used PCR mutagenesis of the TEM-1  $\beta$ -lactamase gene and sequenced the genes of 20 mutants that grew in the presence of ampicillin-clavulanate. Eleven different mutant genes from these strains contained from 1 to 10 mutations. Each had a replacement of one of the four residues, Met69, Ser130, Arg244, and Asn276, whose substitutions by themselves had been shown to result in inhibitor resistance. None of the mutant enzymes with multiple amino acid substitutions generated in this study conferred higher levels of resistance to ampicillin alone or ampicillin with  $\beta$ -lactamase inactivators (clavulanate, sulbactam, or tazobactam) than the levels of resistance conferred by the corresponding single-mutant enzymes. Of the four enzymes with just a single mutation (Ser130Gly, Arg244Cys, Arg244Ser, or Asn276Asp), the Asn276Asp  $\beta$ -lactamase conferred a wild-type level of ampicillin resistance and the highest levels of resistance to ampicillin in the presence of inhibitors. Site-directed random mutagenesis of the Ser130 codon yielded no other mutant with replacement of Ser130 besides Ser130Gly that produced ampicillin-clavulanate resistance. Thus, despite PCR mutagenesis we found no new mutant TEM  $\beta$ -lactamase that conferred a level of resistance to ampicillin plus inactivators greater than that produced by the single-mutation enzymes that have already been reported in clinical isolates. Although this is reassuring, one must caution that other combinations of multiple mutations might still produce unexpected resistance.**

The hydrolytic activities of  $\beta$ -lactamases that inactivate  $\beta$ -lactam antibiotics are the principal mechanism of acquired resistance to these compounds in gram-negative bacterial pathogens such as *Escherichia coli* and *Klebsiella pneumoniae*. One strategy that has been used successfully to circumvent resistance mediated by class A  $\beta$ -lactamases in these and other bacteria has been the development of mechanism-based inactivators of these enzymes, such as clavulanic acid, sulbactam, and tazobactam, to protect the  $\beta$ -lactam antibiotics with which they are coadministered from inactivation. Inevitably, the use of such drug combinations has selected mutant derivatives of the TEM and SHV families of class A  $\beta$ -lactamases that have become relatively resistant to inactivation by mechanism-based inactivators and thereby confer resistance to  $\beta$ -lactam- $\beta$ -lactamase inactivator combinations (3, 5, 10, 19, 23, 29, 31). Although in some cases such resistance appears to have resulted from hyperproduction of wild-type  $\beta$ -lactamase (20, 24, 25), it has been demonstrated that many inhibitor-resistant clinical isolates and also laboratory mutants that display such resistance have emerged as the result of single or multiple mutations in the structural genes for their  $\beta$ -lactamases (2, 7, 11, 28). Amino acid replacements at positions 69, 130, 244, and 276 of TEM or SHV  $\beta$ -lactamases (residues are numbered according to Ambler et al. [1]) have been recognized as major contributors to clinically significant levels of resistance to  $\beta$ -

lactamase inhibitors. Although critical substitutions at positions 69, 244, and 276 have been reported by themselves, generally they occur in various clinical isolates in combination with another critical substitution or with mutational replacements at other positions. Therefore, the specific effects of these mutations on the MICs of  $\beta$ -lactam antibiotics in combination with inhibitors have been difficult to compare among different strains.

We sought to explore the range of mutations that might yet arise to confer resistance of the TEM-1  $\beta$ -lactamase to inactivation by clavulanate, using mutagenesis of its gene and selection of resistance to the combination of ampicillin and clavulanate in vitro. Since our mutant genes would all be in the same genetic environment (host, strain, plasmid, and promoter), we intended to compare the effects of the mutations that we would find on the susceptibilities to various  $\beta$ -lactam antibiotics, alone and in combination with clavulanate and other  $\beta$ -lactamase inactivators.

### MATERIALS AND METHODS

**Antibiotics.** Ampicillin, kanamycin, cephalothin, cephaloridine, and ceftriaxone were obtained from Sigma. Ceftazidime was provided by Glaxo, cefepime and aztreonam were provided by Bristol-Myers Squibb, clavulanic acid was provided by SmithKline Beecham, sulbactam was provided by Pfizer, and tazobactam was provided by Wyeth-Ayerst.

**Antibiotic and inhibitor susceptibility testing.** Stock solutions of antibiotics and  $\beta$ -lactamase inhibitors were freshly prepared from the powders. The MICs for each strain were determined at least three times in Mueller-Hinton broth by a two-fold dilution method in microtiter plates. For assessment of the susceptibilities of the transformants'  $\beta$ -lactamases to inhibition, we determined the MICs of ampicillin in the presence of designated fixed concentrations of the inhibitors.

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TABLE 1. Types of mutant  $\beta$ -lactamases selected by growth of transformants in the presence of ampicillin plus clavulanate

Mutant type <sup>a</sup>	Amino acid <sup>b</sup> substitutions for the following amino acids of the wild-type TEM-1 $\beta$ -lactamase:													Silent mutation(s) <sup>c</sup>
	A42	N52	<b>M69<sup>d</sup></b>	K111	<b>S130</b>	H153	K192	T195	L201	G238	<b>R244</b>	T272	<b>N276</b>	
1		N52Y	<b>M69L</b>									T272A		A42A, T281T
2			<b>M69L</b>				K192R							R9R, Q206Q
3					<b>S130G</b>									
4	A42G				<b>S130G</b>			T195A		G238S				T265T
5											<b>R244C</b>			
6	A42G									L201P	<b>R244C</b>			L73L
7	A42G					H153D					<b>R244C</b>			R9R, K34K, L49L, R61R, Q88Q, K111K
8	A42G			K111R						L201P	<b>R244C</b>			R9R, K34K, L49L, R61R, G87G, I260I
9											<b>R244S</b>			G45G, P257P
10										L201P	<b>R244S</b>			R65R, P67P, I260I
11													<b>N276D</b>	

<sup>a</sup> The wild type is TEM-1.

<sup>b</sup> Amino acids are indicated by conventional single-letter nomenclature.

<sup>c</sup> Mutations in the base sequence of the gene that do not alter the amino acid sequence of the enzyme.

<sup>d</sup> The boldface font indicates sites at which mutations have previously been reported to confer resistance to  $\beta$ -lactam-clavulanate combinations.

**Vector construction.** Plasmid pTZ19 (Bio-Rad) was digested with restriction endonucleases *Pst*I and *Hind*III. This DNA was blunt ended by a standard procedure with the Klenow fragment of DNA polymerase I (New England Biolabs) and a mixture of four deoxynucleoside triphosphates. A *Pst*I-*Pst*I fragment containing the kanamycin resistance marker from Tn903 (Pharmacia) was also blunt ended and ligated with pTZ19. The ampicillin resistance gene of pTZ19 was substituted by the wild-type TEM-1  $\beta$ -lactamase gene of pBR322 (plasmid pTZ19-1). The *Bam*HI site in the polylinker of pTZ19-1 was destroyed by *Bam*HI digestion followed by treatment with the Klenow fragment of DNA polymerase I and religation. A new *Bam*HI site was constructed by site-directed mutagenesis 97 to 102 bp upstream of the ATG start codon of the ampicillin resistance gene. A *Hind*III site was created 11 to 16 bp downstream of the stop codon of the ampicillin resistance gene, and the resulting plasmid was designated pTZ19-2. Plasmid pTZ19-2 was further modified to construct two additional plasmids. In plasmid pTZ19-3 the recognition sequence for the *Hind*III restriction endonuclease in the kanamycin resistance gene was altered by site-directed mutagenesis (without changing the amino acid sequence). In plasmid pTZ19-4 the recognition sequence for the restriction endonuclease *Hind*III downstream of the TEM-1  $\beta$ -lactamase gene was removed by mutation back to the original sequence, and this plasmid was used in site-directed mutagenesis experiments.

**PCR mutagenesis.** The nonmutagenic PCR mixture contained (per 100  $\mu$ l) 0.2 mM dATP and dGTP and 0.4 mM dTTP and dCTP, 2.5 mM MgCl<sub>2</sub>, 1 $\times$  PCR buffer (Bethesda Research Laboratories), 50 ng of DNA, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Bethesda Research Laboratories). The mutagenic PCR mixture contained a higher concentration (7 mM) of MgCl<sub>2</sub> and also contained 0.5 mM MnCl<sub>2</sub>. The following PCR program was used in a Perkin-Elmer Cetus 480 Thermal Cycler: 35 cycles of 94°C for 30 s, 60°C for 30 s, and 73°C for 60 s.

**Cloning and selection.** The PCR product was isolated from a gel by electroelution, digested with *Bam*HI and *Hind*III restriction enzymes, and reisolated from a gel. Plasmid pTZ19-3 was digested with *Bam*HI and *Hind*III, and the resulting fragments were separated on an agarose gel. The PCR product was cloned into the pTZ19-3 vector, substituting the gene for the TEM-1  $\beta$ -lactamase gene. DNA from the ligation mixture was precipitated with ethanol and was used to electroporate competent *E. coli* JM83 cells. After 1 h of incubation in Luria-Bertani broth cells were plated onto Mueller-Hinton agar (Difco) containing 5  $\mu$ g of clavulanate per ml and 25, 50, 100, or 200  $\mu$ g of ampicillin per ml. The MICs of ampicillin and ampicillin plus 5  $\mu$ g of clavulanate per ml were determined in microtiter plates. DNA from resistant isolates was used to retransform competent cells of *E. coli* JM83, and the MICs of antibiotics for the retransformants were determined.

**Site-directed mutagenesis.** Site-directed mutagenesis of the TEM-1  $\beta$ -lactamase gene was performed with double-stranded DNA of pTZ19-4 with the Transformer Site-Directed Mutagenesis Kit (Clontech). Two primers were used for this purpose: a selection primer (GCATAAGCTATTGCCATTCTC), which mutates the recognition sequence for a unique *Hind*III restriction endonuclease site in the kanamycin resistance gene of the plasmid, and mutagenic primer TEM-130 (CGCAGTGTATCANNNCATGGTTATGGC), which promotes random mutation of all three bases of the Ser130 triplet codon. Following mutagenesis, DNA was electroporated into *E. coli* JM83, and mutants were selected on agar containing either kanamycin or clavulanate and ampicillin.

**DNA sequencing.** Double-stranded DNA was isolated with the QIAprep spin miniprep kit (Qiagen), and DNA sequencing was performed with the Sequenase,

version 2.0, DNA Sequencing Kit (United States Biochemicals) and a set of custom-made internal primers.

**Molecular modeling.** The crystal structure for *Z-p*-nitrobenzyl clavulanate (CLAVBB10) was obtained from the Cambridge Structural Database, version 5.13, and the clavulanate structure was extracted from it for use in molecular modeling. The crystal structure of the native TEM-1  $\beta$ -lactamase (15) was used in this study. Both the preacylation complex and the immediate acyl-enzyme intermediate for clavulanate with the TEM-1  $\beta$ -lactamase were constructed, and the energies of the complexes were minimized by using the AMBER force field by the protocol described previously (8).

## RESULTS

**Mutations in TEM-1  $\beta$ -lactamase.** After PCR mutagenesis of the TEM-1  $\beta$ -lactamase gene, we selected clones resistant to ampicillin in combination with clavulanate. In order to reconfirm this phenotype, we retransformed *E. coli* JM83 with DNA from individual colonies and determined the MICs of ampicillin in the presence of clavulanate for these strains. The entire nucleotide sequence of the TEM-1  $\beta$ -lactamase gene was determined for 20 individual colonies that were resistant to this combination. A total of 11 different types of mutants were recovered (Table 1). We found among the first seven mutants examples of mutations at residues 69, 130, 244, and 276, all in a single mutagenic experiment under nonmutagenic PCR conditions. Mutant enzymes with replacements of Met69, Arg244, and Asn276 had been reported at that time to confer resistance to  $\beta$ -lactam-clavulanate combinations (2, 7, 11, 26), but only recently has replacement of Ser130 been associated with such resistance (23). The sites of these mutations are shown in the preacylation complex of the clavulanate in the active site of the TEM-1  $\beta$ -lactamase (Fig. 1). In order to look for additional mutations that would confer resistance to ampicillin-clavulanate, we sequenced the TEM-1  $\beta$ -lactamase gene from another three clones selected following PCR under nonmutagenic conditions. Additionally, we sequenced the gene from 10 more resistant mutants obtained following PCR under mutagenic conditions. Each of these 13 mutants had a mutation of one of the above-mentioned sites that could account for resistance to ampicillin-clavulanate. However, most had mutations of other residues as well, and we could not rule out their contribution to the observed resistance. In addition to the amino acid changes, individual mutants had up to six silent mutations in the TEM-1 structural gene. To determine whether other

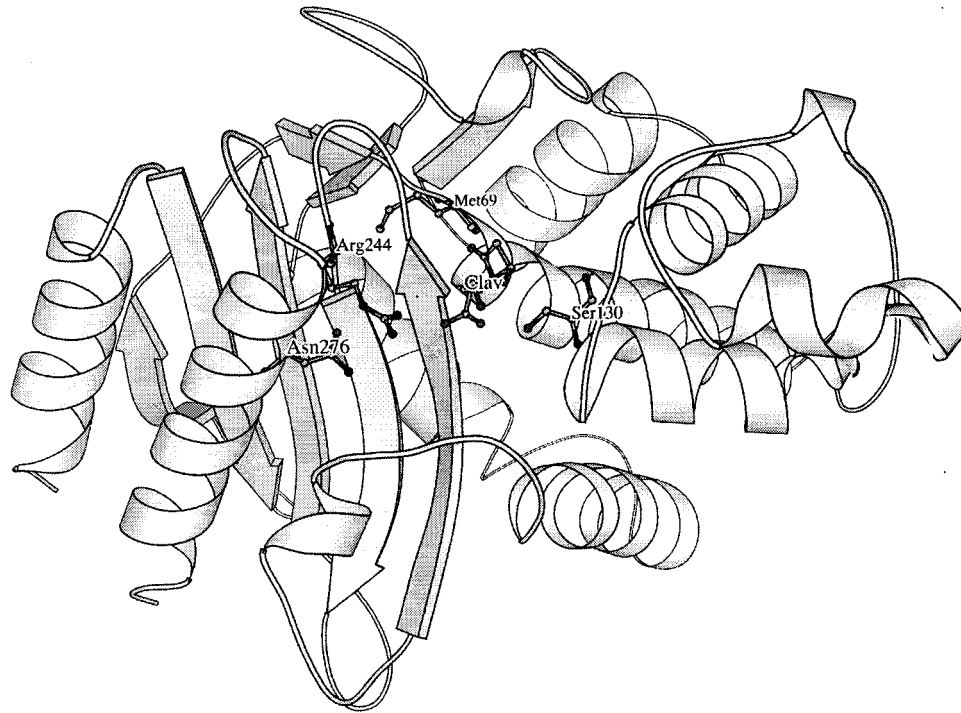


FIG. 1. TEM-1  $\beta$ -lactamase with clavulanic acid bound in the active site. The side chains of the residues Met69, Ser130, Arg244, and Asn276 are shown as a ball and stick representation; Clav, clavulanic acid. The figure was prepared by using the MOLSCRIPT program (17).

replacements of Ser130 besides glycine could produce ampicillin-clavulanate resistance, we performed site-directed random mutagenesis at this position. After selection of mutants on plates containing 5  $\mu$ g of clavulanate per ml together with 50, 100, or 200  $\mu$ g of ampicillin per ml, we determined the nucleotide sequence of the codon corresponding to residue 130 in the  $\beta$ -lactamase gene of 10 individual isolates. Each contained a mutation resulting in the replacement of Ser130 by glycine. Several additional replacements of Ser130 were detected among transformants grown without selection on ampicillin-

clavulanate. These mutations resulted in the substitution of leucine, cysteine, tyrosine, or valine for Ser130.

**Phenotypic characterization of mutants.** The MICs of ampicillin, clavulanate, sulbactam, and tazobactam alone and also the MICs of ampicillin in combination with these inhibitors at fixed concentrations are presented in Table 2. Replacement of Asn276 by aspartate (type 11) had no effect on the MIC of ampicillin. Two other replacements, Met69Leu and Arg244Ser (types 1 and 9, respectively), resulted in only a twofold reduction in the level of resistance to ampicillin, although interpre-

TABLE 2. MICs of ampicillin,  $\beta$ -lactam inhibitors, and their combinations conferred by the TEM-1  $\beta$ -lactamase and various types of mutant derivatives for *E. coli* JM83

$\beta$ -Lactamase or mutant derivative	MIC ( $\mu$ g/ml) <sup>a</sup>													
	Ap	Cl	Ap + Cl 1 <sup>b</sup>	Ap + Cl 2	Ap + Cl 5	Ap + Cl 10	Sb	Ap + Sb 5	Ap + Sb 10	Ap + Sb 20	Tb	Ap + Tb 5	Ap + Tb 10	Ap + Tb 50
— <sup>c</sup>	4	32	4	4	4	2	32	2	1	NG	256	4	4	4
TEM-1	16,000	32	250	125	64	16	64	16,000	8,000	4,000	256	32	16	4
1	8,000	32	1,000	500	250	125	32	4,000	2,000	NG	256	64	32	4
3	1,000	32	1,000	1,000	500	250	32	250	64	NG	256	125	64	16
4	500	32	500	500	250	125	32	125	32	NG	256	125	64	16
5	2,000	32	2,000	1,000	500	125	32	500	125	NG	256	64	32	8
6	2,000	32	2,000	1,000	500	125	32	500	125	NG	256	125	64	8
7	500	32	500	250	125	64	32	125	32	NG	256	32	16	4
8	2,000	32	2,000	1,000	500	125	32	500	125	NG	256	125	64	8
9	8,000	32	4,000	2,000	1,000	250	32	2,000	500	NG	256	500	64	8
10	2,000	32	2,000	1,000	500	125	32	500	125	NG	256	64	32	8
11	16,000	32	4,000	2,000	1,000	250	64	16,000	16,000	8,000	256	2,000	64	8

<sup>a</sup> Abbreviations: Ap, ampicillin; Cl, clavulanate; Sb, sulbactam; Tb, tazobactam.

<sup>b</sup> The numbers indicate the fixed concentration (in micrograms per milliliter) of the indicated inhibitor in combination with ampicillin for determination of ampicillin MICs.

<sup>c</sup> —, *E. coli* JM83 without a plasmid.

<sup>d</sup> NG, no growth in the presence of the lowest concentration of ampicillin tested (0.06  $\mu$ g/ml for *E. coli* JM83 and 8  $\mu$ g/ml for the transformants bearing a  $\beta$ -lactamase).

tation of the consequences of the Met69Leu mutation is complicated by the presence of two additional mutations. The most profound decrease in ampicillin resistance (to an MIC of 1,000  $\mu\text{g/ml}$ ) resulting from a single amino acid replacement was noticed for the Ser130Gly mutant (type 3). The presence of additional mutations in combination with Ser130Gly, Arg244Cys, or Arg244Ser (types 4, 7, and 10, respectively) resulted in a decline of ampicillin resistance to less than that observed with the Ser130Gly, Arg244Cys, or Arg244Ser mutation alone.

Neither the TEM-1  $\beta$ -lactamase nor any of the mutant derivatives produced a level of resistance to clavulanate or tazobactam alone greater than that of the plasmidless host strain. In contrast, both TEM-1 and the Asn276Asp mutant enzyme consistently conferred a twofold rise in the sulbactam MIC over that for *E. coli* JM83. The other mutant enzymes had no effect on the susceptibility of the strain to this  $\beta$ -lactamase inactivator.

For most of the mutant strains, the addition of 1 or 2  $\mu\text{g}$  of clavulanate per ml had little or no effect on the MICs of ampicillin, and even with 5  $\mu\text{g/ml}$ , the MICs declined only two- to fourfold for all but types 1 (32-fold), 9 (8-fold), and 11 (16-fold). In contrast, in the presence of only 1  $\mu\text{g}$  of clavulanate per ml, there was a marked (64-fold) decline in the MIC of ampicillin for the isolate producing wild-type (TEM-1)  $\beta$ -lactamase. For most mutants, sulbactam behaved similarly to clavulanate, gradually reducing the MICs of ampicillin as the concentration of inhibitor used in the combination was increasing. On the other hand, the strains producing the TEM-1 and Asn276Asp mutant  $\beta$ -lactamases maintained the original high level of resistance to ampicillin with a sulbactam concentration of 10  $\mu\text{g/ml}$ ; even with sulbactam at 20  $\mu\text{g/ml}$ , the MICs of ampicillin were still 4,000 and 8,000  $\mu\text{g/ml}$  for the two strains, respectively. It should be noted that only for these two strains was there a consistent twofold increase in the MIC of sulbactam alone above that for the background strain, so the sulbactam concentration of 20  $\mu\text{g/ml}$  had little inhibitory effect on those strains. Also for the strain producing the type 1 mutant enzyme, sulbactam had a relatively poor influence on the MIC of ampicillin, even though this enzyme had no detectable effect on the MIC of sulbactam alone.

From the observed MICs, tazobactam appeared to be an efficient inhibitor of the TEM-1 enzyme and reduced the MICs of ampicillin for most of the mutants even more efficiently than clavulanate. The type 11 mutant strain remained highly resistant to ampicillin (2,000  $\mu\text{g/ml}$ ) in the presence of 5  $\mu\text{g}$  of tazobactam per ml. However, in the presence of higher tazobactam concentrations the MIC of ampicillin for the type 11 mutant strain dropped drastically.

The presence of additional amino acid replacements besides those at residue 130 or 244 (mutant types 4, 6, 7, 8, and 10) in no case improved the level of ampicillin resistance that was conferred by the corresponding single-mutation enzyme, type 3, 5, or 9. Likewise, none of the additional substitutions improved the level of resistance to ampicillin plus any inhibitor over that produced by the corresponding enzymes with just the single replacements.

We also determined the MICs of several cephalosporins and aztreonam for isolates harboring the 11 types of mutant  $\beta$ -lactamases and compared them with the MICs for the plasmidless host *E. coli* JM83 and the strain producing the TEM-1  $\beta$ -lactamase (Table 3). All strains were uniformly susceptible to ceftriaxone (MIC, 0.03  $\mu\text{g/ml}$ ) and aztreonam (MIC, 0.06  $\mu\text{g/ml}$ ). Only TEM-1 and the Asn276Asp mutant enzyme conferred resistance to cephaloridine and cephalothin; for strains producing the other mutant enzymes the MICs of these cepha-

TABLE 3. MICs of cephalosporins and aztreonam conferred by TEM-1  $\beta$ -lactamase and various types of mutant derivatives for *E. coli* JM83

$\beta$ -Lactamase or mutant derivative	MIC ( $\mu\text{g/ml}$ )				
	Cephalothin	Cephaloridine	Ceftazidime	Ceftriaxone	Aztreonam
— <sup>a</sup>	4	2	0.125	0.03	0.06
TEM-1	32	128	0.25	0.03	0.06
1	4	2	0.125	0.03	0.06
3	4	2	0.125	0.03	0.06
4	4	2	0.125	0.03	0.06
5	4	2	0.125	0.03	0.06
6	4	2	0.125	0.03	0.06
7	4	2	0.125	0.03	0.06
8	4	2	0.125	0.03	0.06
9	4	2	0.125	0.03	0.06
10	4	2	0.125	0.03	0.06
11	16	128	0.5	0.03	0.06

<sup>a</sup> —, *E. coli* JM83 without a plasmid.

alosporins were at background levels. The resistance to cephaloridine conferred by the strains with the TEM-1 and Asn276Asp mutant  $\beta$ -lactamases was especially high (64-fold increase over the background MIC). For the same two strains there were reproducible two- and fourfold increases in the MICs of ceftazidime, respectively.

Since the finding of a Ser130Gly mutant had not yet been reported to confer resistance to  $\beta$ -lactam-inhibitor combinations and the resistant mutants that we selected had only the glycine replacement at that position, we also determined the MICs for strains with several other amino acid substitutions at residue 130 in the TEM  $\beta$ -lactamase. In contrast to the results with the Ser130Gly mutant, replacement of Ser130 by leucine, cysteine, tyrosine, or valine drastically reduced the activity of the enzyme against  $\beta$ -lactam substrates (e.g., MICs of ampicillin, <32  $\mu\text{g/ml}$ ); hence, the effect of the inactivators on  $\beta$ -lactam resistance could not be assessed.

## DISCUSSION

Mutations at only four sites in the TEM  $\beta$ -lactamase have been implicated in resistance to inactivation by  $\beta$ -lactamase inactivators. In an attempt to identify other sites where mutations would produce similar levels of resistance, we exploited the inherent production of random replicative errors by *Taq* polymerase in the course of PCR. By regulating the fidelity of *Taq* polymerase with various PCR conditions, we hoped to be able to introduce either single or multiple mutations throughout the TEM-1  $\beta$ -lactamase gene that would produce resistance to  $\beta$ -lactam-inhibitor combinations. In fact, after selection of mutants on ampicillin-clavulanate and sequencing of the gene from 20 of them, we found 11 different types of mutants containing from 1 to 10 mutations. Even more impressive is the fact that among the first seven mutants obtained after PCR under nonmutagenic conditions we found mutations at all of the four important residues where mutations have been reported to produce resistance to inactivators. Four mutants had just a single amino acid substitution: Ser130Gly, Arg224Cys, Arg244Ser, or Asn276Asp. In fact, these are the only mutations at these residues that have been reported to produce significant levels of resistance to  $\beta$ -lactam-inhibitor combinations. Thus, our results indicate the power and random nature of PCR mutagenesis, so one would expect under appropriate conditions to obtain any mutation in the gene of interest.

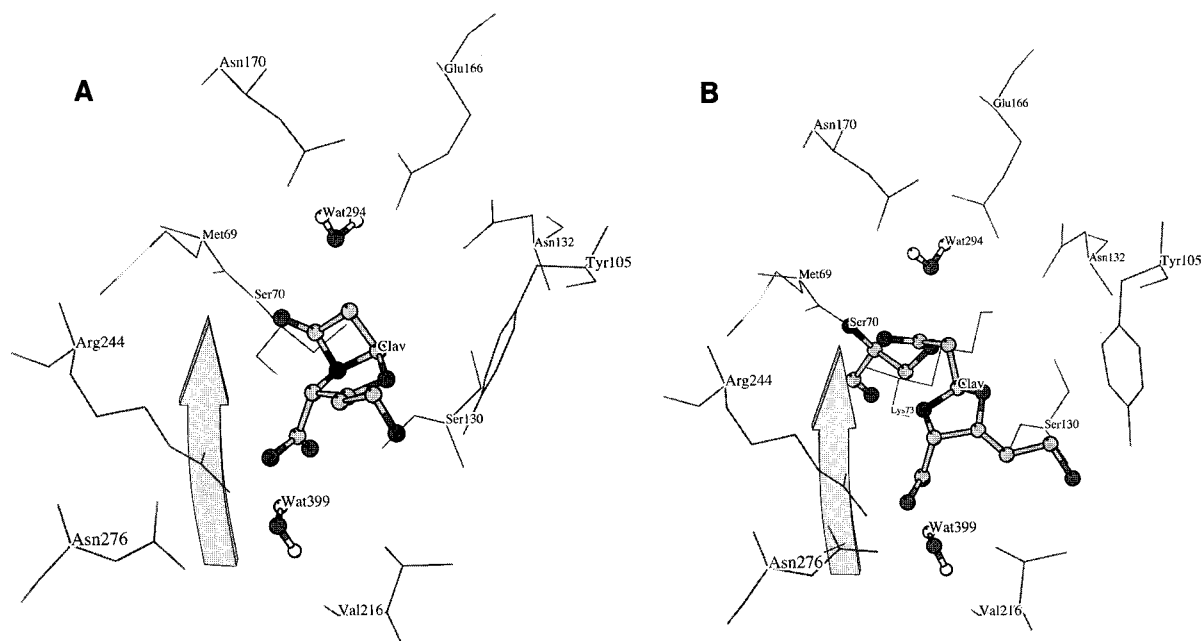


FIG. 2. (A) Preacylation complex of TEM-1  $\beta$ -lactamase and clavulanic acid. (B) The active site is shown for the regions within the vicinity of the acyl-enzyme intermediate of TEM-1  $\beta$ -lactamase and clavulanic acid. Clavulanic acid (Clav), water molecules, and Ser70 are shown as ball and stick representations. The arrow indicates the B3 strand, which forms a portion of the active-site surface. The figure was prepared by using the MOLSCRIPT program (17).

We designed our search for mutants resistant to  $\beta$ -lactam-inhibitor combinations to use a high-copy-number plasmid (pUC19 derivative) that would be expected to amplify small effects of mutations on MICs because of hyperproduction of the mutant enzyme. Hyperproduction of wild-type  $\beta$ -lactamase has been reported as one of the mechanisms responsible for resistance to  $\beta$ -lactam-inhibitor combinations (20, 24, 25). In fact, this may account for the relatively high  $\beta$ -lactam MICs that we observed for the strain producing the TEM-1  $\beta$ -lactamase from our plasmid construct.

With our construct we were able to select by growth on ampicillin-clavulanate mutants with amino acid replacements at each of the sites identified in inhibitor-resistant  $\beta$ -lactamases. This enabled us to compare the effects of these mutations on the MICs of different  $\beta$ -lactam antibiotics,  $\beta$ -lactamase inhibitors, and combinations of these drugs for the *E. coli* JM83 transformants harboring the wild-type or mutant TEM  $\beta$ -lactamase genes on the same multicopy plasmid and under the control of the same promoter.

The models for the preacylation complex and the immediate acyl-enzyme intermediate for clavulanate in the active site of the TEM-1 enzyme were generated to gain insight into the nature of the effect of mutations with reference to the interactions with the inactivator (for a discussion of mechanisms of action for clavulanate and sulbactam, consult references 12 and 13). These two models are shown in Fig. 2A and B, respectively. The sites of mutations associated with the resistance phenotype are clearly indicated. Specifically, modeling was intended to show whether these residues actually make contact with the clavulanate species or whether the effects are indirect. The contribution of each of these mutated sites to understanding of the chemistry of clavulanate is described in the following paragraphs.

We obtained two distinct mutants with the Met69Leu mutation. Unfortunately, each of our mutants also has other mutations, including one or two additional amino acid substitutions, so the relative contribution of each mutation to the

observed susceptibility patterns is unclear. The first report of inhibitor-resistant TEM-1  $\beta$ -lactamases described laboratory mutants in which Met69 was replaced by leucine, isoleucine, or valine (22). Since that time, clinical isolates in which Met69 was replaced by each of these branched-chain aliphatic amino acids have been reported (27, 31). It has been proposed that such replacements alter the structure of the oxyanion pocket of the  $\beta$ -lactamase, which impairs the binding of  $\beta$ -lactams in the active site (4, 16). The binding of small  $\beta$ -lactams, such as clavulanate, sulbactam, and tazobactam, is especially sensitive to such perturbation, since they lack other substituents at the C-6 position which would provide additional anchoring in the active site.

The Ser130Gly mutant that was resistant to ampicillin-clavulanate had not been reported at the time that we encountered it, so we pursued our investigation by site-directed random mutagenesis at that site. Our results suggest that other amino acids besides glycine at residue 130 are unlikely to produce  $\beta$ -lactam- $\beta$ -lactamase inhibitor resistance. Recently, a mutant derivative of the SHV  $\beta$ -lactamase which confers  $\beta$ -lactam-inhibitor resistance was reported to contain Ser130Gly, but two additional amino acid substitutions were also present (23), thus complicating the analysis of the effect of the Ser130Gly replacement alone. Ser130 participates in multiple interactions, including potentially a proton shuttle in catalysis, stabilization of the active site, and anchoring of  $\beta$ -lactams to the active site (12–14, 18, 20, 21). Therefore, although we were able to show that the Ser130Gly mutation by itself was sufficient to confer  $\beta$ -lactam-inhibitor resistance, it is difficult to dissect out the precise reason why the replacement of Ser130 by just glycine resulted in such resistance while preserving significant residual resistance to ampicillin. In fact, the other replacements of Ser130 dramatically compromised resistance to ampicillin. However, we had predicted that Ser130 was the site of covalent modification by clavulanate (13), a fact which has recently been verified experimentally (6). Therefore, the Ser130Gly mutation would be incapable of irreversible modi-

fication by clavulanate. Furthermore, we have shown previously (12, 13) and also in the present report (Fig. 2) that Ser130 makes hydrogen bonds to the carboxylate of the enzyme inactivator. The absence of the side chain of serine in the glycine mutant would deprive the complex of this hydrogen bonding interaction; hence, the recognition of the inactivator by the enzyme should be impaired.

Among our resistant mutants of TEM-1  $\beta$ -lactamase we found Arg244Cys and Arg244Ser as single replacements. The level of resistance to ampicillin in the Arg244Ser mutant hardly declined from that of the wild type, whereas the residual level of resistance in the Arg244Cys mutant was reduced eightfold. The profile of declining MICs of ampicillin in the presence of increasing concentrations of clavulanate and sulbactam was similar for these two mutants and for the Ser130Gly mutant. With tazobactam, there was a sharper drop-off in the ampicillin MICs for the Arg244Ser mutant. In addition to the single substitutions with serine or cysteine, four other mutant types also had other amino acid replacements and silent mutations. Both cysteine and serine substitutions at position 244 in TEM-1  $\beta$ -lactamase have been reported in laboratory mutants and clinical isolates that were resistant to combinations of  $\beta$ -lactams and  $\beta$ -lactamase inactivators (2, 13, 29, 30). It has been proposed that Arg244 anchors the carboxylate of  $\beta$ -lactams in the active site (30). A water molecule coordinated to Arg244 (Wat399; according to the numbering method of Jelsch et al. [15] for crystallographic water molecules) serves as the source of a critical proton for the inactivation process with clavulanate (13). The side chains of cysteine or serine in the mutant enzymes are unable to retain this water molecule in the active site, so they are resistant to inactivation by clavulanate. Furthermore, similarly to the role of Ser130, Arg244 also makes important hydrogen bonds to the carboxylate of the inactivators (12, 13) (Fig. 2). The mutant enzymes possessing shorter side chains at this position would not have the benefit of this important hydrogen bond to the inactivators and would, as a consequence, show reduced affinities toward these molecules.

Substitution of Asn276 by aspartate has not been reported by itself in a TEM-1-producing clinical isolate resistant to  $\beta$ -lactam-inhibitor combinations, but it has been found in a number of mutants that also include replacements of Met69 by leucine, isoleucine, or valine (11, 31). However, when 15 different substitutions of Asn276 were introduced alone into the TEM-1  $\beta$ -lactamase, only the aspartate mutant conferred significant resistance to such combinations (26). Among our resistant mutants, we found only the aspartate replacement of Asn276. Enzymologic study of such a mutant revealed marked elevation of the  $K_i$  of clavulanate (26). Mutations at position 276 would alter the intimate interaction between this residue and Arg244 (Fig. 2). Such a tampering in the structural integrity of the enzyme in this important region would reposition the side chain of Arg244 and also would affect the location of Wat399. Both of these effects would have deleterious consequences for binding of the enzyme to the inactivators.

In contrast to the other mutants with a single amino acid substitution, the Asn276Asp mutation in TEM-1  $\beta$ -lactamase did not alter the MICs of ampicillin, cephalothin, or cephaloridine. Although the wild-type TEM-1  $\beta$ -lactamase conferred no resistance to clavulanate or tazobactam, its production from our multicopy vector consistently raised the MIC of sulbactam twofold. Likewise, the Asn276Asp mutant conferred a similar level of resistance to sulbactam. This doubling of the sulbactam MIC significantly raised the levels of resistance of these strains to combinations of ampicillin and sulbactam. The Asn276Asp mutant also exhibited a somewhat unusual pattern of resistance to ampicillin in combination with tazobactam. In the

presence of tazobactam at 5  $\mu$ g/ml, the MIC of ampicillin was still 2,000  $\mu$ g/ml, whereas in the presence of tazobactam at 10  $\mu$ g/ml, the ampicillin MIC was already diminished to 64  $\mu$ g/ml, in the range of those for all of the other mutants. It has been shown that  $\beta$ -lactamases exhibit reversible inactivation with tazobactam, and the extent of inactivation depends on the type of enzyme and the amount of inhibitor present in combination with the  $\beta$ -lactam antibiotic (9). Therefore, it would appear that a minimum level of tazobactam greater than 5  $\mu$ g/ml is required to block the large amounts of the Asn276Asp mutant enzyme produced from the multicopy plasmid in this strain.

A major strategy for overcoming the problem of high-level resistance to  $\beta$ -lactams produced by  $\beta$ -lactamases has been to develop effective  $\beta$ -lactamase inactivators, which are coadministered with the  $\beta$ -lactam antibiotics which they protect. The emergence of inactivator-resistant enzymes is a serious threat to this approach. The TEM-1  $\beta$ -lactamase is a very plastic enzyme and is capable of tolerating numerous mutations that enhance its activity or broaden its spectrum. Fourteen inhibitor-resistant TEM-1  $\beta$ -lactamases produced by clinical isolates have been reported so far. Although some of these enzymes had multiple mutations, replacements of only four individual residues were unambiguously shown to produce resistance of the enzyme to inactivation by drugs such as clavulanate. We used a powerful mutagenic tool to generate as broad a range of mutants as possible from which we could select for resistance to ampicillin-clavulanate to probe the possibility that other mutants might yet emerge in clinical strains. It is thus noteworthy that we found no new single-mutation enzymes that have not yet been recognized to confer significant levels of resistance in clinical isolates. Furthermore, none of the other mutant enzymes with additional replacements conferred greater levels of resistance to ampicillin plus inactivators than those conferred by these single-mutation enzymes. Although this is reassuring, one must caution that other combinations of multiple mutations might still produce unexpected resistance to clavulanate. Moreover, selection of mutants with other  $\beta$ -lactamase inactivators besides clavulanate might also have yielded other mutations that confer inactivator resistance.

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