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Systematic Mutagenesis of the Active Site Omega Loop of TEM-1 β-Lactamase

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β-Lactamase is a bacterial protein that provides resistance against β-lactam antibiotics. TEM-1 β-lactamase is the most prevalent plasmid-mediated β-lactamase in gram-negative bacteria. Normally, this enzyme has high levels of hydrolytic activity for penicillins, but mutant β-lactamases have evolved with activity toward a variety of β-lactam antibiotics. It has been shown that active site substitutions are responsible for changes in the substrate specificity. Since mutant β-lactamases pose a serious threat to antimicrobial therapy, the mechanisms by which mutations can alter the substrate specificity of TEM-1 β-lactamase are of interest. Previously, screens of random libraries encompassing 31 of 55 active site amino acid positions enabled the identification of the residues responsible for maintaining the substrate specificity of TEM-1 β-lactamase. In addition to substitutions found in clinical isolates, many other specificity-altering mutations were also identified. Interestingly, many nonspecific substitutions in the N-terminal half of the active site omega loop were found to increase ceftazidime hydrolytic activity and decrease ampicillin hydrolytic activity. To complete the active site study, eight additional random libraries were constructed and screened for specificity-altering mutations. All additional substitutions found to alter the substrate specificity were located in the C-terminal half of the active site loop. These mutants, much like the N-terminal omega loop mutants, appear to be less stable than the wild-type enzyme. Further analysis of a 165-YYG-167 triple mutant, selected for high levels of ceftazidime hydrolytic activity, provides an example of the correlation which exists between enzyme instability and increased ceftazidime hydrolytic activity in the ceftazidime-selected omega loop mutants.

β-Lactam antibiotics, such as the penicillins and cephalosporins, are extensively used for antimicrobial therapy. Because of their widespread use, the problem of bacterial resistance to these antimicrobial agents is growing. The most common mechanism for resistance to β-lactam antibiotics is the bacterial production of β-lactamase enzymes (18). These enzymes act by hydrolyzing the β-lactam antibiotic to create an inactive product (1). There are four classes of β-lactamases based on the primary sequence (3). Classes A, C, and D involve a serine residue in the active site (9). Class B enzymes are less abundant and require a catalytic zinc for activity (33).

TEM-1 β-lactamase, the most common plasmid-mediated β-lactamase, is a class A serine hydrolase encoded by the bla_{TEM-1} gene in bacteria (6, 18, 26, 32). Wild-type TEM-1 β-lactamase has strong hydrolytic activity for penicillins and weak hydrolytic activity for many cephalosporins (30). However, mutations in the bla_{TEM-1} gene enable the enzyme to hydrolyze both penicillins and cephalosporins (5, 7). Extended-spectrum cephalosporins, such as ceftazidime and cefotaxime, were developed, in part, because plasmid-mediated β-lactamases such as TEM-1 are unable to hydrolyze these antibiotics. However, shortly after the introduction of these extended-spectrum drugs, mutations which allowed TEM β-lactamase to hydrolyze the extended-spectrum cephalosporins arose (14, 24). Amino acid substitutions in TEM β-lactamase at positions 104, 164, 238, and 240 were shown to increase hydrolytic activity toward extended-spectrum cephalosporins (4, 12, 30). The fact that specificity-altering mutations have occurred near the active site pocket in response to the selective pressure of antibiotic therapy indicates that when designing new β-lactam antibiotics, not only the activity of the existing β-lactamase but also its ability to alter substrate specificity by mutation must be considered.

The purpose of this study is to understand how amino acid substitutions in the active site of TEM-1 β-lactamase can alter its substrate specificity. The strategy in understanding how amino acid substitutions affect substrate specificity has been to first use random replacement mutagenesis to randomize three or four contiguous codons to form random libraries containing all or nearly all possible amino acid combinations for the region being randomized (19–22). Then, functional mutants able to catalyze the hydrolysis of a poor substrate, ceftazidime (an extended-spectrum cephalosporin), and an excellent substrate, ampicillin (a penicillin), are isolated and sequenced. Comparing the sequences of mutants selected for various hydrolytic capacities allows the identification of residues responsible for altering the substrate specificity of the enzyme (22, 36).

Previously, random replacement mutagenesis was used to create 10 random libraries containing residues in or adjacent to the active site of TEM-1 β-lactamase, as determined in crystallography studies (10, 13, 15, 31). Several substitutions responsible for altering the substrate specificity of the enzyme were identified. As expected, substitutions at positions 104, 164, 238, and 240 were found to alter substrate specificity (20, 21). However, it was also discovered that substitutions at many positions within the active site omega loop are able to change the substrate specificity of TEM-1 β-lactamase as well (22).

The omega loop region, which includes residues 164 to 179, forms the bottom wall of the active site pocket (13). This region also contains Glu-166, which has been shown to be important in the deacylation step of β-lactam hydrolysis (2, 8, 31). Sequencing of functional random mutants indicated that several positions from the 161 to 170 region have stringent

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sequence requirements for wild-type ampicillin hydrolysis. In contrast, many substitutions in the 161 to 170 domain were found to confer a threefold or greater increase in ceftazidime hydrolysis relative to that of wild-type TEM-1 β-lactamase. Furthermore, mutants possessing greater than 100 times the wild-type activity for ceftazidime hydrolysis were isolated. Many mutants having increased ceftazidime hydrolytic activity were expressed at levels much lower than that of the wild-type enzyme. It was concluded from these observations that a direct correlation exists between high levels of ceftazidime hydrolytic activity and enzyme instability (22).

In this study, random replacement mutagenesis was used to study the sequence requirements of the remainder of the active site residues including omega loop amino acid residues 171 to 179. Eight random libraries were constructed in order to complete this active site study. No further substitutions outside the omega loop were found to alter the enzyme specificity toward ceftazidime. The results show that the correlation between hydrolytic activity for ceftazidime and enzyme instability extends throughout the omega loop. A 165-YYG-167 triple mutant isolated from an earlier study is characterized to further investigate the correlation between ceftazidime activity and enzyme instability.

MATERIALS AND METHODS

***Escherichia coli* strains and plasmids.** *E. coli* BW313 [Hfr *lysA*(61-62) *dut-1 ung-1 thi-1 recA1 spoT1*] was used to propagate plasmid DNA prior to mutagenesis (17). Mutagenized DNA was initially introduced into *E. coli* ES1301 [*lacZ53 mutS201*::Tn*5 thyA36 rha-5 metB1 deoC* IN(*rrnD-rrnE*)] (29). *E. coli* XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′::Tn*10* (Tet^r)*proAB*, Δ*lacI*^{q} (*lacZ*)*M15*]} was used to assay antibiotic susceptibility and to prepare single-stranded DNA (Stratagene, Inc.). Mutagenesis was performed on plasmid pBG66, which was used in previous studies (21). The pBG66 plasmid contains the wild-type bla_{TEM-1} gene and a *cat* gene encoding chloramphenicol acetyltransferase. This 4.8-kb plasmid also contains the ColE1 and f1 origins of DNA replication.

Oligonucleotides and random replacement mutagenesis. Oligonucleotide primers used for mutagenesis and DNA sequencing were synthesized by the oligonucleotide synthesis facility at Genentech, Inc., at Stanford University Medical School, and at Genosys Biotechnologies, Inc.. The construction of random libraries L69-71 (library [L] including amino acid positions 69 to 71), L72-74, L103-105, L130-132, L232-234, L235-237, L238-241, L161-164, L165-167, and L168-170 has been described previously (21). Libraries L211-213, L214-216, L217-219, L242-244, L245-247, L171-173, L174-176, and L177-179 were randomized for this study by a modified mutagenesis protocol.

The strategy behind the modified mutagenesis was to first insert a unique *Sal*I restriction site into a location within the *bla* gene, which has been targeted for mutagenesis. A frameshift mutation, resulting from this insertion, renders the *bla* gene nonfunctional. Subsequent randomization is achieved by replacing the unique restriction site with a 9-base randomized DNA sequence. The *Sal*I restriction site was introduced by site-directed mutagenesis by the method of Kunkel et al. and an oligonucleotide containing the *Sal*I recognition sequence, 5′-GTCGAC-3′ (17). The *Sal*I recognition sequence is flanked by two 12-base arms which are complementary to the sequence adjacent to the site targeted for mutagenesis. The restriction site was positioned at or near the middle of the three codons to be randomized, and the second base of the middle codon was deleted to create a frameshift mutation. Phosphorylation of the oligonucleotides was done with 200 pmol of the primers in the presence of 0.1 M Tris-Cl (pH 7.5), 0.01 M $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, and 5 U of T4 polynucleotide kinase (New England Biolabs) at 37°C for 45 min. The T4 kinase was inactivated at 65°C for 10 min. The oligonucleotide was then annealed to 200 ng of the single-stranded DNA pBG66 template in the presence of 20 mM Tris-Cl (pH 7.5), 2 mM $MgCl_2$, and 50 mM NaCl. This mixture was heated to 70°C for 10 min and was slowly cooled to 30°C. The annealed DNA was then placed on ice. Second-strand synthesis was done with 5 U of T7 DNA polymerase (United States Biochemical) in the presence of 0.5 mM deoxynucleoside triphosphates (Pharmacia), 1 mM ATP, 10 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, and 400 U of T4 DNA ligase (New England Biolabs). The synthesis reaction was carried out at 4°C for 5 min, at 25°C for 5 min, and finally at 37°C for 30 min. After the 37°C incubation, 90 μl of a TE stop buffer containing 10 mM Tris-Cl (pH 8) and 10 mM EDTA was added to the reaction mixture to bring the volume up to 100 μl. Following phenol extraction and ethanol precipitation, the mutagenized DNA was transformed into *E. coli* ES1301 cells by electroporation (27). Mutants were screened on separate Luria-Bertani (LB) plates containing 12.5 μg of chloramphenicol ml^{-1} or 1 mg of ampicillin ml^{-1}. Plasmid DNA containing the *Sal*I insert was identified by selecting clones which were chloramphenicol resistant and ampicillin susceptible. The plasmid DNA was isolated by the alkaline lysis procedure, and proper insertion of the *Sal*I recognition sequence was confirmed by DNA sequencing (27).

In order to randomize the targeted regions, plasmid DNA containing the *Sal*I site was electroporated into BW313. Single-stranded DNA for random replacement mutagenesis was prepared from BW313 transformants as described previously (27). An oligonucleotide designed to replace the 9-base window (including the *Sal*I site) with the random sequence 5′-NNS NNS NNS-3′ (where N indicates an equal probability of any base, and S indicates an equal probability of either C or G) was used in a second round of mutagenesis. This ensured that all amino acids would be sampled in the window. Two 14-base complementary arms flank the random sequence. The second round of mutagenesis reactions was carried out as described for the first. However, after transformation into *E. coli* ES1301, the number of transformants needed to be greater than 75,000 to ensure that all possible sequences in the three-amino-acid window have a 99.9% chance of being generated. The calculations involved in the determination of the percentage of randomization have been described previously (21). Library DNA was isolated from the *E. coli* ES1301 cells by alkaline lysis and electroporated into *E. coli* XL1-Blue for further screening.

Site-directed mutagenesis. Construction of the 165-WYP-167 and 165-WEG-167 single-substitution mutants and the 165-WYP-167 and 165-WYG-167 double mutants was accomplished by using the L165-167 excision linker insert (21, 22). Digestion of this plasmid with the restriction endonuclease *Bsp*MI created a deletion in the bla_{TEM-1} gene with the following sticky ends:

```
  162 613 164     168 168 170
5′-CTT GAT         GAG CTG AAT-3′
3′-GAA CTA GCA A         AC TTA-5′
```

Double-stranded DNA fragments having the appropriate sticky ends were constructed from complementary oligonucleotides and were ligated into the L165-167 excision linker insert plasmid following restriction with *Bsp*MI. The double-stranded insert fragments were constructed with the necessary nucleotide substitutions to generate the desired single or double mutant. The sequence of each insert fragment used to construct the site-directed mutants (in lowercase, with the nucleotide substitutions made underlined) is as follows:

165-WYP-167:

```
  162 163 164 165 166 167 168 169 170
5′-CTT GAT cgt tgg tac ccg GAG CTG AAT-3′
3′-GAA CTA GCA Acc atg ggc ctc gAC TTA-5′
```

164-WEG-167:

```
  162 163 164 165 166 167 168 169 170
5′-CTT GAT cgt tgg gaa ggg GAG CTG AAT-3′
3′-GAA CTA GCA Acc ctt ccc ctc gAC TTA-5′
```

165-YYP-167:

```
  162 163 164 165 166 167 168 169 170
5′-CTT GAT cgt tac tac ccg GAG CTG AAT-3′
3′-GAA CTA GCA Atg atg ggc ctc gAC TTA-5′
```

165-WYG-167:

```
  162 163 164 165 166 167 168 169 170
5′-CTT GAT cgt tgg tac ggg GAG CTG AAT-3′
3′-GAA CTA GCA Acc atg ccc ctc gAC TTA-5′
```

Construction of the 165-YEP-167 and 165-YEG-167 site-directed mutants was accomplished by a strategy similar to that of the modified random replacement mutagenesis protocol. However, the second round of mutagenesis was performed with oligonucleotides made with specific substitutions to create the appropriate site-directed mutations. The following modifications (underlined) to the wild-type sequence were made in constructing these two mutants:

165-YEP-167

```
    164 165 166 167 168
5′-CGT TAC GAA CCG GAG-3′
3′-GCA ATG CTT GGC CTC-5′
```

165-YEG-167

```
    164 165 166 167 168
5′-CGT TAC GAA GGG GAG-3′
3′-GCA ATG CTT CCC CTC-5′
```

The final products of all the above-described site-directed mutagenesis reactions were confirmed by DNA sequencing.

Single-stranded DNA preparation and DNA sequencing. Single-stranded plasmid DNA was isolated for sequencing as previously described (27). DNA se-

L171-173

	171	172	173
	N	E	A	I	P
5′-	AAT	GAA	GCC	ATA	CCA-3′
5′-	AAT	NNS	NNS	NNS	CCA-3′

L174-176

	174	175	176
	I	P	N	D	E
5′-	ATA	CCA	AAC	GAC	GAG-3′
5′-	ATA	NNS	NNS	NNS	GAG-3′

L177-179

	177	178	179
	D	E	R	D	T
5′-	GAC	GAG	CGT	GAC	ACC-3′
5′-	GAC	NNS	NNS	NNS	ACC-3′

L211-213

	211	212	213
	W	M	E	A	D
5′-	TGG	ATG	GAG	GCG	GAT-3′
5′-	TGG	NNS	NNS	NNS	GAT-3′

L214-216

	214	215	216
	A	D	K	V	A
5′-	GCG	GAT	AAA	GTT	GCA-3′
5′-	GCG	NNS	NNS	NNS	GCA-3′

L217-219

	217	218	219
	V	A	G	P	L
5′-	GTT	GCA	GGA	CCA	CTT-3′
5′-	GTT	NNS	NNS	NNS	CTT-3′

L242-244

	242	243	244
	R	G	S	R	G
5′-	CGT	GGG	TCT	CGC	GGT-3′
5′-	CGT	NNS	NNS	NNS	GGT-3′

L245-247

	245	246	247
	R	G	I	I	A
5′-	CGC	GGT	ATC	ATT	GCA-3′
5′-	CGC	NNS	NNS	NNS	GCA-3′

FIG. 1. Positions of nucleotides randomized during construction of the random libraries. For each library, the top line shows the amino acid and corresponding nucleotide sequences of the wild-type bla_{TEM-1} gene. The bottom line shows the positions of the random nucleotides of the mutagenic primers (the letter N for A, C, G, or T and the letter S for either G or C). The 5′-NNS-3′ random DNA construct allows all 20 amino acids to be sampled at the corresponding amino acid position.

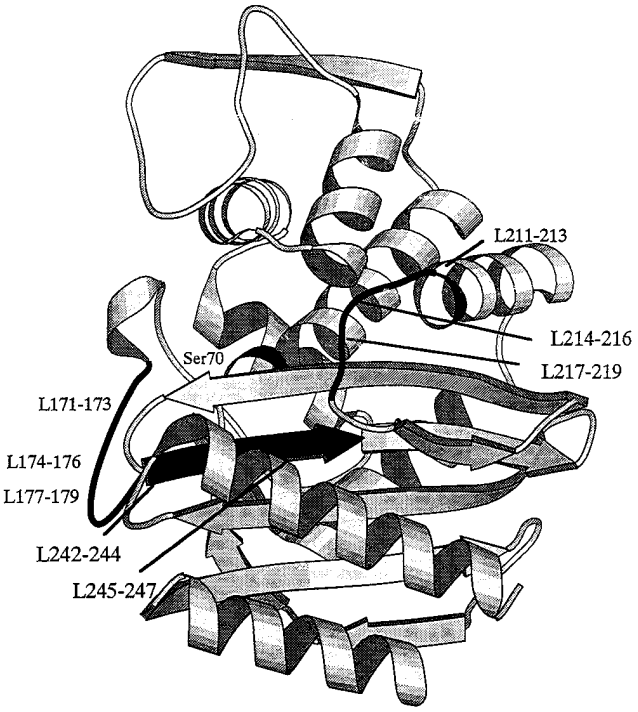

FIG. 2. Ribbon diagram of TEM-1 β-lactamase. The locations of the randomized library positions are indicated. The figure was prepared by using MOLSCRIPT (16).

quencing was performed by the dideoxy chain termination method (28). Oligonucleotides were designed to prime specific sites within the bla_{TEM-1} gene.

Antibiotic susceptibility. MICs were determined by broth microdilution. *E. coli* XL1-Blue cells (10^4) containing a selected mutant β-lactamase were inoculated into microtiter wells containing 100 μl of LB medium with twofold increases of each antibiotic tested. After an 18-h incubation, the plates were examined, and the lowest concentration of antibiotic which inhibited visual growth was recorded as the MIC.

Immunoblots of β-lactamases. Steady-state expression levels of β-lactamase were determined by immunoblotting. *E. coli* XL1-Blue cells containing mutant β-lactamase enzymes were grown to mid-log phase (A_{600} of 0.35) in LB broth supplemented with 12.5 μg of chloramphenicol ml^{-1}. Each culture (1.5 ml) was pelleted in a microcentrifuge for 30 s. The pellets were resuspended in 0.1 ml of 50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol. The cells were then incubated at 100°C for 10 min. Twenty microliters of lysate was resolved by SDS-polyacrylamide gel electrophoresis containing 12% acrylamide. Transfer of the proteins to nitrocellulose was achieved by electroblotting (27). The blot was probed with anti-β-lactamase polyclonal antibodies. Bound antibody was visualized by using anti-rabbit antibodies labeled with horseradish peroxidase and the Amersham ECL Western blot kit.

RESULTS

Random replacement mutagenesis. The three-dimensional structure of TEM-1 β-lactamase allows the identification of those amino acids which may influence substrate specificity, namely, those residues located in the active site pocket. The target of the initial random replacement mutagenesis experiments has been on the 55 amino acids located in and around the active site pocket. In previous studies, a set of 10 random libraries, encompassing 31 of the 55 active site amino acids, were constructed and screened for substrate specificity mutants (20–22). The remaining 24 active site amino acids were randomized by using the modified mutagenesis protocol described in Materials and Methods. As a result, the following libraries were constructed: L171-173, L174-176, L177-179, L211-213, L214-216, L217-219, L242-244, and L245-247. The random libraries are labeled L, for library, followed by the numbers of the amino acid residues which were randomized (numbering according to Ambler et al. [4]) (Fig. 1). The positions of these libraries on the TEM-1 β-lactamase three-dimensional structure is shown in Fig. 2. Since over 75,000 transformants were obtained for each construction, there is a 99.9% probability that all possible amino acid substitutions are contained in each library.

Selection of mutants. The purpose of the first set of experiments was to identify, from each of the newly constructed random libraries, those positions responsible for altering the substrate specificity of TEM-1 β-lactamase. Since ceftazidime is a poor substrate for wild-type TEM-1 β-lactamase, mutants selected for increased ceftazidime hydrolytic activity are considered to have an altered substrate specificity. Mutants with at least three times the wild-type activity for ceftazidime hydrolysis were selected by transforming *E. coli* XL1-Blue cells with each random library and spreading the transformed cells on agar plates containing 0.5 μg of ceftazidime ml^{-1}. *E. coli* XL1-Blue cells, containing pBG66 with a copy of the wild-type bla_{TEM-1} gene are unable to grow in the presence of this concentration of ceftazidime. Therefore, only mutants with greater than wild-type hydrolytic activity for ceftazidime were identified. A concentration of ceftazidime higher than at which *E. coli* containing wild-type β-lactamase will grow was chosen to clearly identify residue positions that, when mutated, display increased ceftazidime hydrolysis. Of the eight random libraries constructed, only L171-173, L174-176, and L177-179 contained mutants with increased hydrolytic activity for ceftazidime. These three libraries all contain residues located in the C-terminal half of the active site omega loop (Fig. 2).

To identify the individual positions responsible for altering enzyme specificity in the three omega loop libraries, the sequence requirements for wild-type ampicillin hydrolysis were determined. Ampicillin is an excellent substrate for TEM-1 β-lactamase with a k_{cat}/K_m ratio on the order of 10^7 M^{-1} s^{-1} (22). This high level of activity can be attributed to precise

	171	172	173	174	175	176	177	178	179	MIC (μg/ml) AMP	CAZ
w.t.	E	A	I	P	N	D	E	R	D	2048	.25
1	**E**	**R**	**R**	P	N	D	E	R	D	2048	2.0
1	**H**	**K**	**A**	P	N	D	E	R	D	512	1.0
1	**D**	**Q**	**R**	P	N	D	E	R	D	1024	1.0
1	**P**	**R**	**A**	P	N	D	E	R	D	512	2.0
1	**S**	**L**	**D**	P	N	D	E	R	D	2048	1.0
1	**S**	**K**	**N**	P	N	D	E	R	D	512	1.0
1	**A**	**R**	**H**	P	N	D	E	R	D	1024	1.0
1	E	A	I	**K**	**N**	**L**	E	R	D	32	.50
1	E	A	I	**D**	**V**	**D**	E	R	D	1024	8.0
1	E	A	I	**D**	**D**	**N**	E	R	D	1024	2.0
1	E	A	I	**L**	**L**	**L**	E	R	D	16	1.0
1	E	A	I	**E**	**N**	**F**	E	R	D	256	8.0
1	E	A	I	**S**	**A**	**D**	E	R	D	32	4.0
1	E	A	I	**M**	**H**	**S**	E	R	D	512	1.0
1	E	A	I	P	N	D	**Q**	**W**	**Y**	8	2.0
1	E	A	I	P	N	D	**C**	**W**	**N**	1024	2.0
1	E	A	I	P	N	D	**A**	**F**	**N**	64	2.0
1	E	A	I	P	N	D	**D**	**A**	**N**	256	4.0
1	E	A	I	P	N	D	**R**	**S**	**N**	128	4.0
1	E	A	I	P	N	D	**G**	**W**	**A**	8	1.0
1	E	A	I	P	N	D	**T**	**I**	**F**	8	2.0

FIG. 3. Amino acid sequences found to confer resistance to 0.5 μg of ceftazidime ml^{-1} are listed under the corresponding position numbers and wild-type (w.t.) sequence. Mutants were selected from the L171-173, L174-176, and L177-179 random libraries. Positions randomized prior to selection are boxed and in bold type. The number of times each sequence occurred is indicated on the left. The MICs of ampicillin (AMP) and ceftazidime (CAZ) for each sequence are listed on the right.

	171	172	173	174	175	176	177	178	179	MIC (μg/ml) AMP	CAZ
w.t.	E	A	I	P	N	D	E	R	D	2048	.25
1	**H**	**A**	**T**	P	N	D	E	R	D	2048	.25
1	**F**	**T**	**S**	P	N	D	E	R	D	2048	.50
1	**G**	**A**	**A**	P	N	D	E	R	D	2048	.25
1	**K**	**S**	**I**	P	N	D	E	R	D	2048	.50
1	**D**	**A**	**I**	P	N	D	E	R	D	2048	.25
1	**A**	**A**	**N**	P	N	D	E	R	D	2048	.25
1	**D**	**V**	**N**	P	N	D	E	R	D	1024	.25
1	**E**	**C**	**V**	P	N	D	E	R	D	2048	.50
1	**Y**	**R**	**M**	P	N	D	E	R	D	1024	.25
1	E	A	I	**L**	**L**	**D**	E	R	D	2048	.25
1	E	A	I	**A**	**E**	**D**	E	R	D	2048	.25
1	E	A	I	**P**	**C**	**D**	E	R	D	4096	.25
1	E	A	I	**I**	**S**	**D**	E	R	D	4096	.25
1	E	A	I	**P**	**N**	**D**	E	R	D	4096	.25
1	E	A	I	**D**	**T**	**D**	E	R	D	2048	2.0
1	E	A	I	**P**	**L**	**D**	E	R	D	2048	.25
1	E	A	I	**D**	**G**	**D**	E	R	D	2048	.25
1	E	A	I	**P**	**A**	**D**	E	R	D	2048	.25
1	E	A	I	**P**	**T**	**D**	E	R	D	2048	.25
1	E	A	I	P	N	D	**E**	**Q**	**D**	2048	.25
1	E	A	I	P	N	D	**V**	**N**	**D**	2048	.25
1	E	A	I	P	N	D	**T**	**Q**	**D**	2048	.25
1	E	A	I	P	N	D	**A**	**Q**	**D**	2048	2.0
1	E	A	I	P	N	D	**F**	**R**	**D**	2048	2.0
2	E	A	I	P	N	D	**A**	**R**	**D**	4096	.25
1	E	A	I	P	N	D	**P**	**R**	**D**	2048	.25

FIG. 4. Amino acid sequences found to confer wild-type ampicillin hydrolytic activity are listed under the corresponding position numbers and wild-type (w.t.) sequence. Details are as in the legend to Fig. 3.

interactions between the amino acids in the active site pocket and the substrate (22). As a result, it is expected that stringent sequence requirements exist for wild-type levels of ampicillin hydrolytic activity. Ampicillin selections were performed by transforming each of the random libraries into *E. coli* XL1-Blue cells and then plating the transformed cells on agar plates containing 1 mg of ampicillin ml^{-1}. This is the maximum concentration on which *E. coli* XL1-Blue cells, containing pBG66 with a copy of the wild-type bla_{TEM-1} gene, will grow. Thus, only mutants with wild-type levels of hydrolytic activity for ampicillin are identified in this selection. A comparison of the sequence requirements for increased ceftazidime hydrolytic activity and for wild-type ampicillin hydrolytic activity can identify the positions responsible for altering enzyme specificity.

Sequencing of omega loop mutants. Seven to ten ceftazidime-selected mutants from each of the three omega loop libraries were sequenced in order to determine the amino acid sequence requirements for increased ceftazidime hydrolysis (Fig. 3). For comparison, 7 to 10 ampicillin-selected mutants from these three libraries were sequenced to determine the amino acid sequence requirements for wild-type ampicillin hydrolysis (Fig. 4).

Sequencing of ampicillin-selected mutants showed that D-176 and D-179 are conserved among mutants with wild-type ampicillin resistance. Therefore, these amino acids must be important for wild-type activity towards ampicillin. Furthermore, only amino acids known to be hydrogen bond donors were among the residues substituted at position 178 in mutants selected for wild-type ampicillin resistance. This finding would suggest that, although a specific amino acid is not required, hydrogen bond interactions at this position may help maintain wild-type activity for ampicillin.

In contrast, many substitutions observed at omega loop positions 171 to 179 resulted in greater than wild-type activity for ceftazidime. No amino acid was conserved at any position. These results correlate with the information gathered in studies of the N-terminal half of the omega loop (positions 164 to 170), where it was found that many nonspecific substitutions appear to increase hydrolytic activity for ceftazidime (22).

A comparison of the sequence requirements for wild-type ampicillin activity and greater than wild-type ceftazidime activity shows that there are two positions with drastically different sequence requirements for hydrolysis of the two substrates (Fig. 5). D-176 and D-179 show no tolerance for substitution when selected for wild-type ampicillin hydrolysis, while many different amino acid substitutions are tolerated at these positions for greater than wild-type hydrolytic activity for ceftazidime.

Antibiotic resistance. To more precisely determine the extent to which the mutant enzymes confer resistance to ampicillin and ceftazidime, the MICs of both antibiotics were determined for *E. coli* producing each mutant enzyme (Fig. 3 and 4). As expected, those mutants selected at 1 mg of ampicillin ml^{-1} were resistant to ampicillin at wild-type or greater levels. In addition, most ampicillin-selected mutants were at least as

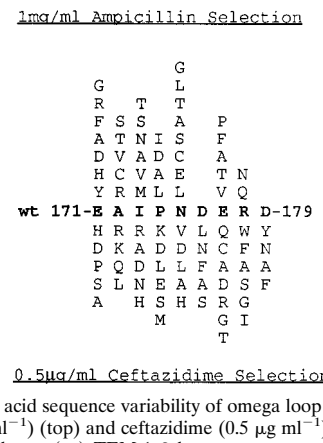

FIG. 5. Amino acid sequence variability of omega loop mutants selected for ampicillin (1 mg ml^{-1}) (top) and ceftazidime (0.5 μg ml^{-1}) (bottom) hydrolytic activities. The wild-type (wt) TEM-1 β-lactamase sequence between positions 171 and 179 is listed in bold type. Above the wild-type sequence, at their respective positions, are those amino acids found in mutants selected for function at 1 mg of ampicillin ml^{-1}. Below the wild-type sequence are those amino acids found in mutants selected for function at 0.5 μg of ceftazidime ml^{-1}.

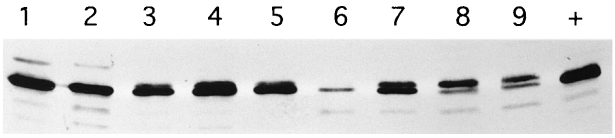

FIG. 6. Western blot of TEM-1 β-lactamase and mutants selected for ampicillin (1 mg ml^{-1}) hydrolytic activity. Three mutants from each random library selection were selected for expression studies. Lanes: 1, 177-PRD-179; 2, 177-VND-179; 3, 177-EQD-179; 4, 174-PCD-176; 5, 174-AED-176; 6, 174-LLD-176; 7, 171-DAI-173; 8, 171-GAA-173; 9, 171-HAT-173; +, wild type.

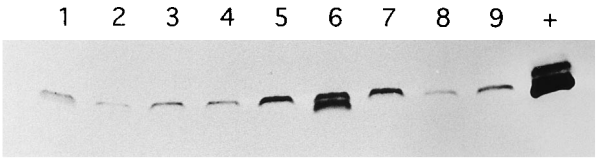

FIG. 7. Western blot of TEM-1 β-lactamase and mutants selected for ceftazidime (0.5 μg ml^{-1}) hydrolytic activity. Three mutants from each random library selection were selected for expression studies. Lanes: 1, 177-DNA-179; 2, 177-CWN-179; 3, 177-QWY-179; 4, 174-ENF-176; 5, 174-LLL-176; 6, 174-DDN-176; 7, 171-DQR-173; 8, 171-HKA-173; 9, 171-ERR-173; +, wild type.

resistant to ceftazidime as wild-type TEM-1 β-lactamase. This observation reinforces the sequencing result that many substitutions in the omega loop region confer greater than wild-type hydrolytic activity for ceftazidime. Since many substitutions were tolerated in the selection for three times greater than wild-type activity for ceftazidime, even more substitutions would be expected to be tolerated for only wild-type levels of hydrolysis. Therefore, it is not surprising that mutants having wild-type levels of ceftazidime hydrolytic activity were identified among mutants selected for ampicillin hydrolytic activity.

All mutants selected for increased ceftazidime hydrolytic activity exhibited MICs higher than the wild-type MIC of ceftazidime (Fig. 3). In mutants selected from libraries L174-176 and L177-179, this increase in ceftazidime hydrolytic activity was often accompanied by a decrease in ampicillin hydrolytic activity. This observation is presumably due to the strong functional constraints on the sequences at positions 176, 179, and, to a lesser extent, 178. Many of the mutants selected for increased ceftazidime hydrolytic activity from the L171-173 library also had high levels of ampicillin hydrolytic activity. The lack of strong sequence requirements for wild-type ampicillin hydrolysis in the 171 to 173 region, as determined in the sequencing results, accounts for this observation.

Expression of omega loop mutant enzymes. In vivo β-lactamase expression levels of omega loop mutants from both selections with 1 mg of ampicillin ml^{-1} and 0.5 μg of ceftazidime ml^{-1} were examined by immunoblotting. Three mutants from each selection were examined for each library. Total protein from *E. coli* XL1-B cells possessing the mutant β-lactamase was screened with anti-TEM-1 β-lactamase polyclonal antibodies.

Screens of mutants selected for ampicillin (1 mg ml^{-1}) activity showed that mutant β-lactamases in these mutants are produced at wild-type levels (Fig. 6). β-Lactamase precursors and partial degradation products are also observed above and below the native protein bands in several samples. Since a correlation between in vivo turnover of proteins in *E. coli* and thermal stability exists, the expression levels of the ampicillin mutants suggest that they are, in general, stable enzymes (23). However, there are mutants that are expressed at levels slightly lower than the wild-type TEM-1 β-lactamase level, suggesting that these mutants are less stable than the wild-type enzyme. Therefore, 100% wild-type activity (and stability) may not be required for growth on LB plates containing 1 mg of ampicillin ml^{-1}.

The level of expression of β-lactamase in mutants selected for ceftazidime (0.5 μg ml^{-1}) activity was much lower than that of the wild-type enzyme (Fig. 7). Therefore, as found in earlier studies of the N-terminal half of the omega loop, substitutions in the omega loop region that increase activity towards ceftazidime, in general, lead to an enzyme with poor stability (22).

Examination of an omega loop mutant with high-level ceftazidime resistance. To investigate the correlation between instability and ceftazidime hydrolysis among omega loop mutants, a 165-YYG-167 triple mutant (mutant 16-1 in reference 21) that was selected for function at 15 μg of ceftazidime ml^{-1} was further examined (22). This mutant was selected for a 100-fold increase in ceftazidime hydrolytic activity from a random library spanning amino acids 165 to 167 in the N-terminal half of the omega loop. Immunoblot analysis showed that the triple mutant was expressed at low levels compared with the wild-type enzyme (22). Therefore, as with the mutants selected from the random libraries for increased ceftazidime hydrolytic activity, these mutants do not appear to confer increased ceftazidime resistance by increasing expression. Rather, these mutants contain substitutions that improve the specific activity for ceftazidime while simultaneously destabilizing the enzyme.

To further show how increased ceftazidime hydrolytic activity and enzyme instability are correlated, the W165Y (W at position 165 replaced with Y) E166Y P167G triple substitution was dissected. All single substitutions, W165Y, E166Y, P167G, and doubled substitutions, W165Y E166Y, W165Y P167G, and E166Y P167G, were constructed by site-directed mutagenesis. To show a correlation between activity and stability, relative levels of expression were examined by immunoblotting, and relative ampicillin and ceftazidime susceptibilities were quantitated by MIC determination (Fig. 8).

Results from immunoblotting show that the single substitu-

A

	164	165	166	167	168	MIC (μg/ml) AMP	CAZ
w.t.	R	W	E	P	E	2048	.25
single	R	W	E	G	E	512	8
	R	W	Y	P	E	64	4
	R	Y	E	P	E	2048	.25
double	R	W	Y	G	E	16	16
	R	Y	E	G	E	512	8
	R	Y	Y	P	E	32	8
triple	R	Y	Y	G	E	16	64

B

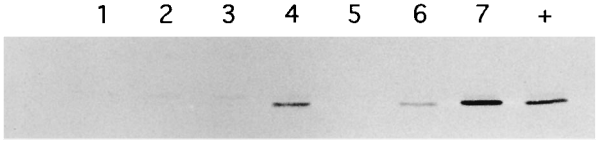

FIG. 8. Expression and resistance characteristics of the mutations which make up the 165-YYG-167 triple mutant. This mutant was isolated previously by selecting for mutants resistant to 15 μg of ceftazidime ml^{-1}. (A) MICs for 165-YYG-167 triple mutant and its elemental single and double mutants. The MICs of ampicillin (AMP) and ceftazidime (CAZ) are shown on the right. The type of mutant (single, double, or triple) is indicated on the left. (B) Expression of 165-YYG-167 triple mutant and its elemental single and double mutants demonstrated by Western blot analysis. Lanes: 1, 165-YYG-167 triple mutant; 2, 165-WYG-167 double mutant; 3, 165-YEG-167 double mutant; 4, 165-YYP-167 double mutant; 5, 165-WEG-167 single mutant; 6, 165-WYP-167 single mutant; 7, 165-YEP-167 mutant; +, 165-WEP-167 wild-type TEM-1 β-lactamase.

tion P167G leads to reduced β-lactamase expression relative to that of wild-type TEM-1 β-lactamase. The E166Y substitution, to a lesser extent, also seems to reduce the expression of the enzyme. The lowered expression levels in these two mutants coincides with an increase in ceftazidime resistance and a loss of ampicillin resistance. In contrast, the W165Y mutation seems to have little effect on expression or resistance levels.

The W165Y substitution was added to the E166Y and P167G substitutions to form the W165Y E166Y and the W165Y P167G double mutants. Little change in expression or resistance was observed for either double mutant compared with the single mutant. However, combining the E166Y and P167G substitutions to form the E166Y P167G double mutant resulted in a drop in ampicillin hydrolytic activity compared with that of the wild-type enzyme and both single mutants. A slight increase in ceftazidime resistance compared with that of either the E166Y or P167G single mutants was also observed. Since both single substitutions lowered enzyme expression levels significantly, the observed instability of the E166Y P167G double mutant was expected.

As anticipated, the W165Y E166Y P167G triple mutation confers high levels of resistance to ceftazidime and low levels of resistance to ampicillin. As previously shown, the expression level of the triple mutant is low, which suggests that the triple mutant is not very stable. The level of ceftazidime hydrolytic activity is slightly higher for the triple mutant than for any of the single or double mutants. Therefore, each individual substitution appears to contribute to the activity of the triple mutant. This dissection of the W165Y E166Y P167G triple mutation again shows that a correlation exists between enzyme destabilization and increased ceftazidime hydrolytic activity.

DISCUSSION

In order to understand how enzyme specificity can be altered by mutation, we conducted a systematic search for specificity-altering mutations over 24 of the 55 amino acid positions located within the active site of the TEM-1 β-lactamase. A functional selection strategy was used to carry out this search. Random replacement mutagenesis was used to construct eight random libraries each containing all possible amino acid substitutions for a three-amino-acid window (19, 20). Together the eight random libraries contain all 24 active site amino acid positions targeted for this study. Each library was selected for mutants with increased hydrolytic activity for the cephalosporin ceftazidime. Since ceftazidime is a poor substrate for TEM-1 β-lactamase, mutants having an increased hydrolytic activity for this antibiotic have an altered substrate specificity. Libraries possessing mutants with increased ceftazidime activity were then selected for mutants having wild-type hydrolytic activity for ampicillin, an excellent substrate for TEM-1 β-lactamase. Contrasting the sequence requirements for these two different activities identified individual positions responsible for altering the substrate specificity of the enzyme.

No mutants grew when the L211-213, L214-216, L217-219, L242-244, and L245-247 libraries were selected in the presence of 0.5 μg of ceftazidime ml^{-1}. Therefore, it was concluded that substitutions at these positions do not increase enzyme-mediated ceftazidime hydrolysis. However, many mutants with increased hydrolytic activity for ceftazidime were isolated from the L171-173, L174-176, and L177-179 libraries. Contrasting the sequence requirements for increased ceftazidime hydrolysis and wild-type ampicillin hydrolysis showed that substitutions at positions 176, 179, and possibly 178 alter enzyme specificity. In wild-type TEM-1 β-lactamase, these three residues are involved in salt bridge interactions which help to stabilize the omega loop (13). The conservation of these residues in mutants selected for wild-type ampicillin activity suggests that these interactions are important for maintaining this level of ampicillin hydrolysis. In the ceftazidime-selected mutants, these residues are freely substituted. Therefore, these ionic interactions do not appear necessary for increased ceftazidime hydrolytic activity. In fact, the disruption of these interactions may be important for increasing ceftazidime hydrolytic activity.

The ionic bond between D-179 and R-164, which effectively links the two ends of the omega loop together, is the only omega loop ionic bond present in every class A β-lactamase (13). However, in TEM-1, three other omega loop ionic bonds are present and are believed to increase the structural constraint in this region (13). D-179 is believed to be conserved for wild-type levels of ampicillin hydrolysis because the bond that this residue forms with R-164 appears important in stabilizing the omega loop so that critical interactions between the substrate and catalytic residues, such as E-166, are maintained. Other mutational studies of D-179 have shown that disruption of the ionic bond between D-179 and R-164 leads to decreased ampicillin hydrolysis (34).

The ionic bond between positions 176 and 178 also appears to play a role in maintaining critical enzyme-substrate interactions needed for ampicillin hydrolysis. However, since amino acids known to be hydrogen bond donors were found to be substituted at position 178, it appears that an ionic bond is not absolutely necessary. A hydrogen bond may be substituted in the same capacity to maintain wild-type levels of ampicillin hydrolysis. Since D-176, R-178, and D-179 are substituted freely in mutants with increased hydrolytic activity toward ceftazidime, it is possible that the removal of the intramolecular interactions releases structural constraints imposed on the omega loop. Therefore, the omega loop may become mobile, and important side chains may be repositioned so they can better interact with a different substrate. In this instance, removal of constraints in the omega loop could allow the bulkier ceftazidime side chain to bind more favorably in the active site pocket. This particular hypothesis was introduced in an earlier study in which similar results were found among residues in the N-terminal half of the TEM-1 β-lactamase omega loop (22).

To further investigate this hypothesis, the stability of ceftazidime and ampicillin mutants from the L171-173, L174-176, and L177-179 libraries was examined. Since wild-type sequence constraints appear to be conserved in the ampicillin mutants, it was anticipated that these mutants would generally be expressed as stably as the wild-type enzyme. It was also predicted that since mutations in the ceftazidime mutants appear to lift wild-type constraints, these mutants would not be expressed as stably as the wild-type enzyme. According to the immunoblot data, these predictions appear to be correct. Therefore, a direct correlation between instability and ceftazidime hydrolytic activity exists because of the removal of wild-type constraints in the active site omega loop.

A specific example of this correlation between ceftazidime hydrolytic activity and enzyme instability is illustrated by dissecting the 165-YYG-167 triple mutant selected for high levels of ceftazidime activity (22). The triple mutant was broken down into each of its single and double mutants in order to determine which position(s) contributes most to the altered specificity of the triple mutant. The P167G and the E166Y substitutions appear to play the largest role in destabilizing the enzyme, and according to the MIC results, they appear to make important contributions to the substrate specificity of the enzyme as well. Studies have shown that a residue other than proline at position 167 destabilizes class A β-lactamases be-

cause of the presence of a *cis* peptide bond between positions 166 and 167 (35). Also, E-166 has been shown to form an ionic bond with K-73 (13). It is possible that removal of this ionic bond further perturbs the omega loop.

The W165Y single mutation appears to have little effect on enzyme expression and activity (Fig. 7). However, when the W165Y mutation is added to the E166Y P167G double mutant, it improves the enzyme's hydrolytic activity for ceftazidime. This result may represent a new additive interaction between the enzyme and substrate; however, further experiments are needed to confirm this possibility.

Unfortunately, the unstable nature of most of the ceftazidime mutants makes further kinetic and structural studies of these mutants difficult. Attempts to purify these enzymes have not been successful. A stably expressed mutant with a perturbed omega loop would be ideal for further study. Such mutants are rare, yet they appear to exist. The P-54 mutant from the class A PC1 β-lactamase of *S. aureus* has a D179N substitution, which has been shown, by crystallographic analysis, to cause a perturbation in the omega loop of this enzyme (11). Alternate intramolecular interactions appear to be responsible for maintaining stability in this mutant. β-Lactamase molecular modeling studies of the R164S mutation, found in many clinical isolates resistant to extended-spectrum cephalosporins, have shown that this mutation also perturbs the structure of the omega loop. Although the salt bridge between D-179 and R-164 is eliminated, it was shown that new hydrogen bonds between D-179 and S-164 are possible (25). A hydrogen bond between these two positions would help to stabilize the perturbed form of the omega loop, resulting in a sharp increase in ceftazidime activity.

A recent mutagenesis study of position 179 in TEM β-lactamase supports the hypothesis that removal of functional constraints in the omega loop increases ceftazidime activity (22, 34). In the study, it was postulated that the substitutions at position 179 would disrupt the ionic bond between R-164 and D-179 and that this disruption would affect enzymatic activity. Antibiotic susceptibility data showed that the replacement of D-179 with virtually any amino acid increases ceftazidime hydrolytic activity and reduces ampicillin hydrolytic activity (34).

So far, R164S is the only omega loop substitution found in isolated clinical mutants with resistance toward extended-spectrum antibiotics. The fact that this substitution appears to stabilize a perturbed form of the omega loop partly explains why mutants with this substitution have such a high level of hydrolytic activity for ceftazidime. Clearly, it would be more favorable if a mutation could not only improve enzyme-substrate interactions but also maintain enzyme stability. For substitutions in a mutant enzyme to be clinically relevant, the number of DNA base substitutions needs to be minimal so that there is a reasonable probability the substitution(s) can occur in vivo. Since the 165-YYG-167 triple mutant is not expressed stably, and because it would take three consecutive amino acid substitutions (at least six DNA base substitutions) to construct this mutant, this enzyme is not likely to be found in a clinical setting. Many other ceftazidime mutants isolated in the random library screens also have a low probability of being found in a clinical setting because the activity levels of these mutants appear to be too low to be clinically relevant. Usually, additional substitutions at positions outside the omega loop, such as positions 104 and 238, are necessary for a mutant enzyme to provide clinically relevant levels of ceftazidime resistance (30).

Nevertheless, substitutions confined to the active site omega loop region of TEM-1 β-lactamase are capable of altering enzyme specificity. The random library study performed on the N-terminal half of the TEM-1 β-lactamase active site loop showed that substitutions at positions 164, 166, and 170 increase ceftazidime hydrolytic activity and decrease ampicillin hydrolytic activity. Mutants having substitutions at these positions were also expressed at levels lower than that of wild-type β-lactamase (22). These results led to the hypothesis that removal of functional constraint in the omega loop through substitution increases ceftazidime hydrolytic activity (22). The current random library study has shown that mutants with substitutions at positions 176, 178, and 179 behave like the mutants with substitutions at positions 164, 166, and 170. Therefore, the results from this study of the C-terminal half of the omega loop are consistent with the earlier hypothesis. However, alternate explanations are possible. For example, the fact that mutants isolated from the ceftazidime selections are poorly expressed suggests that these mutant enzymes may be toxic to *E. coli*. Experiments are in progress to determine the exact mechanism of action of the amino acid substitutions in the omega loop of TEM-1 β-lactamase.

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REFERENCES

1. **Abraham, E. P., and E. Chain.** 1940. An enzyme from bacteria able to destroy penicillin. Nature (London) **146:**837.
2. **Adachi, H., T. Ohta, and H. Matsuzawa.** 1991. Site-directed mutants, at position 166, of RTEM-1 β-lactamase that form a stable acyl-enzyme intermediate with penicillin. Biol. Chem. **266:**3186–3191.
3. **Ambler, R. P.** 1980. The structure of beta-lactamases. Philos. Trans. R. Soc. Lond. Ser. B **289:**321–331.
4. **Ambler, R. P., F. W. Coulson, J.-M. Frere, J.-M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–272.
5. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
6. **Cooksey, R., J. Swenson, N. Clark, E. Gay, and C. Thornsberry.** 1990. Patterns and mechanisms of β-lactam resistance among isolates of *Escherichia coli* from hospitals in the United States. Antimicrob. Agents Chemother. **34:**739–745.
7. **Du Bois, S. K., M. S. Marriott, and S. G. B. Amyes.** 1995. TEM- and SHV-derived extended-spectrum beta-lactamases: relationship between selection, structure and function. J. Antimicrob. Chemother. **35:**7–22.
8. **Escobar, W. A., A. K. Tan, and A. L. Fink.** 1991. Site-directed mutagenesis of β-lactamase leading to accumulation of an acyl-enzyme intermediate. Biochemistry **30:**10783–10787.
9. **Fisher, J., J. Belasco, S. Khosla, and J. Knowles.** 1980. β-Lactamase proceeds via an acyl-enzyme intermediate: interaction of Escherichia coli rTEM enzyme with cefoxitin. Biochemistry **19:**2895–2901.
10. **Herzberg, O.** 1991. Refined crystal structure of β-lactamase from *S. aureus* at 2 A resolution. J. Mol. Biol. **217:**701–719.
11. **Herzberg, O., G. Kapadia, B. Blanco, T. S. Smith, and A. Coulson.** 1991. Structural basis for the inactivation of the P54 mutant of β-lactamase from *Staphylococcus aureus* PC1. Biochemistry **30:**9503–9509.
12. **Huletsky, A., J. R. Knox, and R. C. Levesque.** 1993. Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and three-dimensional modeling. J. Biol. Chem. **268:**3690–3697.
13. **Jelsch, C., L. Mourey, J.-M. Masson, and J.-P. Samama.** 1993. Crystal structure of *Escherichia coli* TEM1 β-lactamase at 1.8 A resolution. Proteins **16:**364–383.
14. **Knothe, H., P. Shah, V. Krcmery, M. Antal, and S. Mitsuhashi.** 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole, and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection **11:**315–317.
15. **Knox, J. R., and P. C. Moews.** 1991. β-Lactamase of Bacillus licheniformis 749/C: refinement at 2 A resolution and analysis of hydration. J. Mol. Biol. **220:**435–455.
16. **Kraulis, P. J.** 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structure. J. Appl. Crystallogr. **24:**946–950.
17. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.

18. **Medeiros, A. A.** 1984. β-Lactamases. Br. Med. Bull. **40:**18–27.
19. **Palzkill, T., and D. Botstein.** 1991. Extracting information from protein sequences using random replacement mutagenesis. Methods **3:**155–164.
20. **Palzkill, T., and D. Botstein.** 1992. Identification of amino acid substitutions that alter the substrate specificity of TEM-1 β-lactamase. J. Bacteriol. **174:** 5237–5243.
21. **Palzkill, T., and D. Botstein.** 1992. Probing β-lactamase structure and function using random replacement mutagenesis. Proteins **14:**29–44.
22. **Palzkill, T., Q.-Q. Le, K. V. Venkatachalam, M. LaRocco, and H. Ocera.** 1994. Evolution of antibiotic resistance: several different amino acid substitutions in an active site loop alter the substrate profile of β-lactamase. Mol. Microbiol. **12:**217–229.
23. **Parsell, D. A., and R. T. Sauer.** 1989. The structural stability of a protein is an important determinant of its proteolytic susceptibility in *Escherichia coli*. J. Biol. Chem. **264:**7590–7595.
24. **Philippon, A., R. Labia, and G. Jacoby.** 1989. Extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **33:**1131–1136.
25. **Raquet, X., J. Lamotte-Brasseur, E. Fonze, S. Goussard, P. Courvalin, and J. M. Frere.** 1994. TEM beta-lactamase mutants hydrolysing third-generation cephalosporins—a kinetic and molecular modeling analysis. J. Mol. Microbiol. **244:**625–639.
26. **Roy, C., C. Segura, M. Tirado, R. Reig, M. Hermida, D. Teruel, and A. Foz.** 1985. Frequency of plasmid-determined β-lactamases in 680 consecutively isolated strains of *Enterobacteriaceae*. Eur. J. Clin. Microbiol. **4:**146–147.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2 ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Sanger, T., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Siegel, E. C., S. L. Wain, S. F. Meltzer, M. L. Binon, and J. L. Steinberg.** 1982. Mutator mutations in *Escherichia coli* induced by the insertion of phage Mu and the transposable resistance elements Tn*5* and Tn*10*. Mutat. Res. **93:**25–33.
30. **Sowek, J. A., S. B. Singer, S. Ohringer, M. F. Malley, T. J. Dougherty, J. Z. Gougoutas, and K. Bush.** 1991. Substitution of lysine at position 104 or 240 of TEM-1_{pTZ18R} β-lactamase enhances the effect of serine-164 substitution on hydrolysis or affinity for cephalosporins and the monobactam aztreonam. Biochemistry **30:**3179–3188.
31. **Strynadka, N. C. J., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh, and M. N. G. James.** 1992. Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 A resolution. Nature (London) **359:**700–705.
32. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pBr322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
33. **Sutton, B. J., P. J. Artymiak, A. E. Cordero-Borboa, C. Little, D. C. Phillips, and S. G. Waley.** 1987. An X-ray crystallographic study of beta-lactamase II from *Bacillus cereus* at .35nm resolution. Biochem. J. **248:**181–188.
34. **Vakulenko, S. B., M. Toth, P. Taibi, S. Mobashery, and S. Lerner.** 1995. Effects of Asp-179 mutations in TEM_{pUC19} β-lactamase on susceptibility to β-lactams. Antimicrob. Agents Chemother. **39:**1878–1880.
35. **Vanhove, M., S. Houba, J. Lamotte-Brasseur, and J.-M. Frere.** 1995. Probing the determinants of protein stability: comparison of class A β-lactamases. Biochem. J. **308:**859–864.
36. **Venkatachalam, K. V., W. Huang, M. LaRocco, and T. Palzkill.** 1994. Characterization of TEM-1 β-lactamase mutants from positions 238 to 241 with increased catalytic efficiency for ceftazidime. J. Biol. Chem. **269:**23444–23450.