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Identification of Amino Acid Substitutions That Alter the Substrate Specificity of TEM-1 β-Lactamase

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TEM-1 β-lactamase is the most prevalent plasmid-mediated β-lactamase in gram-negative bacteria. Recently, TEM β-lactamase variants with amino acid substitutions in the active-site pocket of the enzyme have been identified in natural isolates with increased resistance to extended-spectrum cephalosporins. To identify other amino acid substitutions that alter the activity of TEM-1 towards extended-spectrum cephalosporins, we probed regions around the active-site pocket by random-replacement mutagenesis. This mutagenesis technique involves randomizing the DNA sequence of three to six codons in the bla_{TEM-1} gene to form a library containing all or nearly all of the possible substitutions for the region randomized. In total, 20 different residue positions that had been randomized were screened for amino acid substitutions that increased enzyme activity towards the extended-spectrum cephalosporin cefotaxime. Substitutions at positions 104, 168, and 238 in the TEM-1 β-lactamase that resulted in increased enzyme activity towards extended-spectrum cephalosporins were found. In addition, small deletions in the loop containing residues 166 to 170 drastically altered the substrate specificity of the enzyme by increasing activity towards extended-spectrum cephalosporins while virtually eliminating activity towards ampicillin.

β-Lactam antibiotics such as penicillins and cephalosporins are among the most often used antimicrobial agents. Because of the selective pressure resulting from the prevalence of β-lactam antibiotic use, bacterial resistance has increased and now represents a serious threat to antibiotic therapy (14, 19). The production of β-lactamase enzymes that cleave the amide bond in the β-lactam ring to generate inactive products is the most common mechanism of bacterial resistance to β-lactam antibiotics (27).

Genes that encode β-lactamases can be found on the bacterial chromosome or on plasmids (26, 39). Expression of chromosomal enzymes is often inducible, with β-lactam antibiotics serving as the inducers (39). Plasmid-mediated enzymes are commonly expressed constitutively (27). Plasmid-mediated enzymes are a particular concern because they can be transferred to distantly related bacteria by conjugation or transduction. On the basis of primary sequence homology, β-lactamases have been grouped into four classes (A, B, C, and D) (2). Classes A, C, and D involve a serine residue at the active site (7, 16). Class B enzymes are less abundant and require a catalytic zinc for activity (45). The active-site serine β-lactamases belong to a larger family of penicillin-recognizing enzymes that includes the penicillin-binding proteins that are involved in cell wall biosynthesis and are the lethal targets of β-lactam antibiotics (20). All of these enzymes contain the active-site serine and a conserved lysine 3 residues downstream from the serine, as well as a conserved triad of K(S/T)G between the active-site serine and the C terminus (20). The homology suggests a similar enzyme mechanism and is consistent with the suggestion that β-lactamases have evolved from cell wall peptidases (47). The crystal structures of several class A enzymes (15, 18, 29, 38), a class C enzyme (32), and a cell wall transpeptidase-carboxypeptidase (21) are available, and they have similar three-dimensional structures, which also supports a common evolutionary origin for the enzymes.

TEM-1 β-lactamase is a class A enzyme encoded by the bla_{TEM-1} gene that is present on transposons Tn*2* and Tn*3* (8, 12, 44). Epidemiological studies have shown that TEM-1 is the most common plasmid-mediated β-lactamase (11, 27, 36). It comprises as much as 72% of the population of β-lactamases that have been identified in penicillin-resistant gram-negative bacteria (27). TEM-1 β-lactamase is considered to be a broad-spectrum β-lactamase because it hydrolyzes both penicillins and cephalosporins (6). However, it cannot efficiently hydrolyze recently developed extended-spectrum cephalosporins, such as cefotaxime and ceftazidime. Extended-spectrum antibiotics were developed, in part, because of their resistance to plasmid-mediated β-lactamases such as TEM-1. However, transferable resistance to the antibiotics was identified soon after their introduction into clinical use (23). The resistance proved to be mediated by derivatives of the TEM and SHV β-lactamases (3, 10, 22, 31, 34, 42). SHV-1 β-lactamase is a class A enzyme that is 68% identical in amino acid sequence to TEM-1 (4, 44). The novel TEM- and SHV-derived enzymes have been termed extended-spectrum β-lactamases (35). The amino acid sequences of the altered enzymes contain one to three amino acid substitutions in the active-site pocket of the enzymes, suggesting that the newly introduced changes influence the binding and catalysis of the antibiotics (Table 1) (19). Specifically, substitution of lysine at position 104, serine at position 164, serine at position 238, or lysine at position 240, either alone or in combination, has been shown to increase the activity of TEM β-lactamase towards extended-spectrum cephalosporins (33, 41) (amino acids are numbered as in reference 2). The naturally occurring substitutions identify residue positions that influence the substrate specificity of the enzyme. It is not known whether the naturally occurring substitutions identified to date are the only substitutions possible that result in increased enzyme activity towards extended-spectrum cephalosporins.

Random-replacement mutagenesis was used for a more

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TABLE 1. Amino acid substitutions of TEM β-lactamases found in natural isolates of enterobacteria with increased resistance to extended-spectrum cephalosporins

Enzyme	Amino acid[a] at the following position:								Reference
	39	104	164	205	237	238	240	265	
TEM-1	Gln	Glu	Arg	Gln	Ala	Gly	Glu	Thr	44
TEM-2	Lys	Glu	Arg	Gln	Ala	Gly	Glu	Thr	5
TEM-3	Lys	Lys	Arg	Gln	Ala	Ser	Glu	Thr	41
TEM-4	Gln	Lys	Arg	Gln	Ala	Ser	Glu	Met	42
TEM-5	Gln	Glu	Ser	Gln	Thr	Gly	Lys	Thr	42
TEM-7	Lys	Glu	Ser	Gln	Ala	Gly	Glu	Thr	10
TEM-9	Gln	Lys	Ser	Gln	Ala	Gly	Glu	Met	25

[a] Amino acid residues are numbered as in reference 2.

comprehensive search for residue positions that, when substituted, alter the substrate specificity of the enzyme (33). The technique involves randomization of three to six contiguous codons to form a library of random substitutions. The random library is then screened for substitutions that result in increased enzyme activity towards extend-spectrum cephalosporins.

In this report, we describe the isolation of mutants with increased activity towards extended-spectrum cephalosporins from three random libraries in which the codons for residues 103 to 105, 168 to 170, and 238 to 241, respectively, of TEM-1 β-lactamase were randomized. The findings indicate that additional TEM-1 β-lactamase amino acid substitutions not identified among natural isolates can provide increased resistance to extended-spectrum β-lactam antibiotics. The positions of the amino acid substitutions in the TEM-1 sequence identify regions of the enzyme that are important for substrate recognition.

MATERIALS AND METHODS

Oligonucleotides and random-replacement mutagenesis. Oligonucleotide primers used for DNA sequencing were custom synthesized by the oligonucleotide synthesis facility at Genentech, Inc., and by the PAN facility at Stanford University Medical School. The construction of the random libraries screened here was described previously (33).

***Escherichia coli* strains and plasmids.** *E. coli* TG1 [K-12 Δ(*lac-pro*) *supE thi hsdDS*/F'*traD36 proA*$^+$*B*$^+$ *lacI lacZM15*] was used to assay antibiotic susceptibility and for single-stranded DNA preparations. Plasmid pBG66 was used in all of the experiments described here (33). Plasmid pBG66 is a 4.8-kb derivative of pBR322 and pBR325 that contains the bla_{TEM-1} gene that encodes TEM-1 β-lactamase, as well as the *cat* gene that encodes choloramphenicol acetyltransferase. The plasmid also contains the ColE1 and f1 origins of DNA replication.

Media and reagents. *E. coli* TG1 was maintained on glucose minimal medium plates, and growth in liquid culture for single-stranded DNA isolation was in M9 minimal medium and also 2× YT medium (28). All enzymes were purchased from New England BioLabs, except T7 DNA polymerase, which was purchased from United States Biochemical. The antibiotics ampicillin and cefotaxime were obtained from Sigma Chemical Company. The antibiotic ceftazidime was a gift from John Quinn. All antibiotics were put in solution with water and stored at −20°C until use. LB agar plates containing antibiotics were used for selections and to determine MICs of antibiotics (28).

Plasmid isolation and DNA sequencing reactions. Single-stranded plasmid DNA was prepared for DNA sequencing as described by Sambrook et al. (37). The dideoxy-chain termination method of DNA sequencing was applied to single-stranded plasmid DNA templates (40). Custom-synthesized oligonucleotides were used to prime synthesis from defined sites within the bla_{TEM-1} gene.

Random library screens. The random libraries were screened by transforming *E. coli* TG1 with a plasmid library containing the random substitutions for any given region by $CaCl_2$ transformation (37). The transformation mixtures were plated on LB agar containing 0.05 μg of cefotaxime per ml. This concentration of antibiotic inhibited the growth of *E. coli* TG1 containing the parent plasmid with a wild-type bla_{TEM-1} gene. The parent plasmid with the wild-type bla_{TEM-1} gene was also transformed into *E. coli* TG1 and plated separately on LB plates containing 0.05 μg of cefotaxime per ml as a control.

Antibiotic susceptibility testing. MIC determinations were performed by agar dilution on LB agar with an inoculum of 10^4 CFU per spot. Spotting of cells was done with a multipronged replica-plating device.

RESULTS AND DISCUSSION

Random-substitution libraries. Recently we described a mutagenesis technique, random-replacement mutagenesis, that involves randomization of three to six contiguous codons in a gene of interest to form a library containing all or nearly all of the possible amino acid substitutions for the region randomized (33). Briefly, the technique makes use of the type IIs restriction endonuclease *Bsp*MI, which recognizes a six-nucleotide sequence and cleaves the DNA four nucleotides 3' of the sequence (46). A short linker containing *Bsp*MI sites at each end was inserted into the gene at the position of the codons to be randomized. Release of the linker by *Bsp*MI digestion created a defined eight-nucleotide deletion in the gene (30). A library was formed by using a second linker to replace the eight deleted bla_{TEM-1} nucleotides with random nucleotides.

The mutagenesis was applied to 66 codons of the gene that encodes TEM-1 β-lactamase in 19 separate experiments (i.e., 19 unique libraries). It was found that TEM-1 β-lactamase is extremely tolerant of amino acid substitutions: on average, 44% of all mutants with random substitutions retained some β-lactamase activity (33). The tolerance of β-lactamase to substitutions has important implications for antibiotic resistance. Use of β-lactam antibiotics places selective pressure on β-lactamases to increase or change their hydrolytic capacity towards those antibiotics. The tolerance to substitutions allows many modified forms of the enzyme to be sampled for altered properties.

We screened the random libraries for mutants with increased resistance to β-lactam antibiotics to identify residue positions that contribute critically to the recognition and cleavage of the antibiotics by TEM-1 β-lactamase. Because each of the libraries contains all or nearly all of the possible amino acid substitutions for a given region of TEM-1 β-lactamase, the random libraries are useful for screening exhaustively for variants that contain substitutions that change the structural or functional properties of the enzyme. In this study, a set of seven random libraries that each have the nucleotide sequence of three codons of the bla_{TEM-1} gene randomized was used to identify amino acid substitutions that result in increased enzyme activity towards the extended-spectrum cephalosporin cefotaxime. Although a high-resolution structure of TEM-1 β-lactamase has not been

L69-71

	69	70	71	
M	M	S	T	F
ATG	ATG	AGC	ACT	TTT
ATG	ANN	NNN	NNN	TTT

L72-74

	72	73	74	
T	F	K	V	L
ACT	TTT	AAA	GTT	CTG
ACN	NNN	NNN	NTT	CTG

L103-105

	103	104	105	
L	V	E	Y	S
TTG	GTT	GAG	TAC	TCA
TTG	NNN	NNN	NNC	TCA

L168-170

	168	169	170	
P	E	L	N	E
CCG	GAG	CTG	AAT	GAA
CCG	GNN	NNN	NNN	GAA

L233-235

	233	234	235	
A	D	K	S	G
GCT	GAT	AAA	TCT	GGA
GCN	NNN	NNN	NCT	GGA

L235-237

	235	236	237	
K	S	G	A	G
AAA	TCT	GGA	GCC	GGT
AAA	TNN	NNN	NNN	GGT

L238-241

	238	240	241	
A	G	E	R	G
GCC	GGT	GAG	CGT	GGG
GCC	NNN	NNN	NNT	GGG

FIG. 1. Positions of the nucleotides randomized in the random-substitution libraries. For each library, the upper line shows the nucleotide and corresponding amino acid sequences of the wild-type bla_{TEM-1} gene. Randomized nucleotides are indicated in the lower line by the letter N. The amino acid residue positions are numbered in accordance with reference 2.

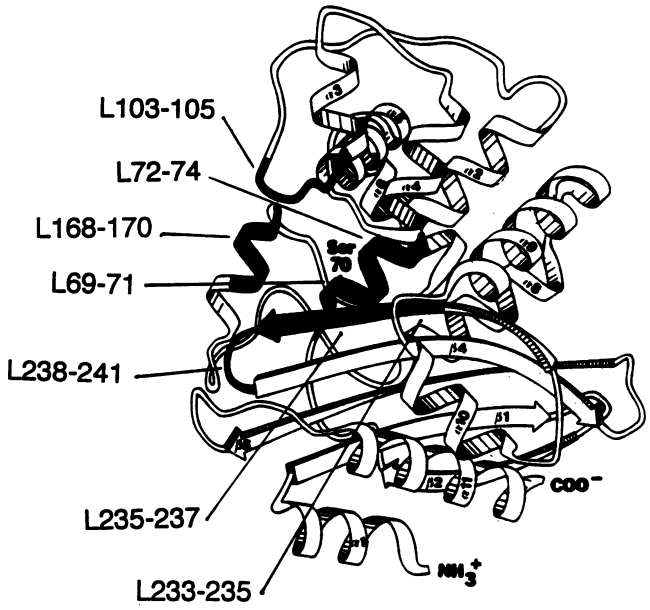

FIG. 2. Positions of random libraries on a ribbon diagram of the homologous *S. aureus* β-lactamase (18). Darkened regions correspond to the positions of the libraries. Lines point to the positions of individual libraries.

reported, high-resolution structural analyses of the homologous class A β-lactamases from *Staphylococcus aureus* and *Bacillus licheniformis* have located the active-site pocket (18, 29). On the basis of these analyses, the amino acid positions substituted in the seven libraries are located in the active-site pocket region. The nucleotides randomized for the seven libraries are shown in Fig. 1. The positions of the libraries on the three-dimensional structure of the homologous *S. aureus* β-lactamase are shown in Fig. 2 (18). The random libraries are designated by a label consisting of the letter L (library) followed by the positions of the amino acid sequences randomized.

Screening of random libraries. The screening experiments were done by first determining the MIC of cefotaxime for *E. coli* TG1 containing a plasmid with the wild-type bla_{TEM-1} gene. This plasmid was the parent plasmid from which the random libraries were constructed (see Materials and Methods). The MIC of cefotaxime was found to be 0.04 μg/ml. Agar plates containing a concentration of cefotaxime slightly higher than the MIC (0.05 μg/ml) were then made to ensure that *E. coli* containing the wild-type bla_{TEM-1} gene did not survive when spread on the plates. Each random library was then transformed into *E. coli* and spread onto agar plates containing cefotaxime. Because of the selection, only mutants with enhanced activity towards cefotaxime survived. Survivors of the selection were then picked and retested for growth on agar plates containing 0.05 μg of cefotaxime per ml to confirm the resistance phenotype. The transformants were also tested for resistance to chloramphenicol. The plasmid containing the mutagenized bla_{TEM-1} gene also contains the gene for chloramphenicol acetyltransferase (*cat*), and thus cells containing a plasmid should be chloramphenicol resistant. Plasmid DNA was isolated from cefotaxime-resistant, chloramphenicol-resistant transformants and electrophoresed on agarose gels along with the parent plasmid to ensure that the plasmid had not incurred rearrangements or gross changes in copy number.

Of the seven libraries that were tested, only three (L103-105, L168-170, and L238-241; Fig. 1) contained bla_{TEM-1} mutations that provide *E. coli* with increased resistance to cefotaxime. This result suggests that there are a limited number of amino acid substitutions that can yield an enzyme with increased activity towards cefotaxime. The L103-105 and L238-241 libraries include residue positions 104 and 238, at which amino acid substitutions are known, on the basis of natural isolates, to increase the activity of the TEM β-lactamase towards cefotaxime (Table 1). In contrast, the residues mutagenized in the L168-170 library have not previously been associated with changes in the specificity of the enzyme.

DNA sequences of cefotaxime-resistant bla_{TEM-1} mutants. To determine the nature of the substitutions that increase the activity of TEM-1 β-lactamase towards cefotaxime, the DNA sequences of a number of the cefotaxime-resistant mutants from each of the libraries were determined. The sequencing results for the L103-105 library are shown in Table 2. To gain more detailed information about the phenotypes of the bla_{TEM-1} mutants, the MIC of cefotaxime, that of another extended-spectrum cephalosporin, ceftazidime, and that of the penicillin ampicillin were also determined for each mutant and are also presented in Table 2. For comparison, the sequences of a number of mutants that were selected from the libraries on the basis of their resistance to ampicillin are also included (33). As indicated by the MICs, these mutants do not have altered resistance to either cefotaxime or ceftazidime. Among the mutants that do have increased resistance to cefotaxime, a common characteristic is a lysine or arginine at position 104. The L103-105 2-33 mutant has only one amino acid difference from the wild

TABLE 2. Amino acid sequences of substitutions from the L103-105 library with increased resistance to cefotaxime[a]

Mutant	Amino acid at the following position:					MIC (μg/ml) of:		
	102	103	104	105	106	CTX	CAZ	AMP
None (wild type)	L	V	E	Y	S	0.04	0.16	1,280
2-33	L	V	K	Y	S	0.08	0.32	1,280
7-16	L	F	R	Y	S	0.32	1.28	1,280
1-12	L	C	R	G	S	0.08	1.28	320
4-3	L	L	G	Y	S	0.04	0.16	1,280
6-36	L	I	L	Y	S	0.04	0.16	1,280
6-6	L	V	S	Y	S	0.04	0.16	1,280
2-35	L	L	A	Y	S	0.04	0.16	1,280
1-9	L	L	F	Y	S	0.04	0.16	1,280
1-4	L	A	L	H	S	0.04	0.16	640
1-7	L	V	Y	N	S	0.04	0.16	640
5-18	L	L	Y	H	S	0.04	0.16	640
6-31	L	V	H	H	S	0.04	0.16	640
1-2	L	V	Y	N	S	0.04	0.16	320
2-28	L	F	R	N	S	0.04	0.16	320
3-11	L	T	R	N	S	0.04	0.16	320
1-23	L	S	W	P	S	0.04	0.16	320
1-5	L	H	R	H	S	0.04	0.16	320
6-3	L	Y	Q	S	S	0.04	0.16	160
3-6	L	L	R	C	S	0.04	0.16	160
1-15	L	V	L	G	S	0.04	0.16	80

[a] The amino acid residue positions randomized in the library are in bold type. The sequence of TEM-1 β-lactamase is at the top. Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; AMP, ampicillin. The sequences of the substitutions with unaltered cefotaxime resistance are from reference 33.

TABLE 3. Amino acid sequences and MICs for substitutions from the L168-170 library[a]

Mutant	Amino acid at the following position:						MIC (μg/ml) of:		
	166	167	168	169	170	171	CTX	CAZ	AMP
None (wild type)	E	P	E	L	N	E	0.04	0.16	1,280
3-19	E	P	G	L	N	E	0.08	0.32	1,280
1-20	E	P	G	A	G	E	0.08	1.28	160
4-2	E	P	G	G	P	E	0.08	2.56	20
1-19	E	P	G	K	G	E	0.08	5.12	<10
1-23	E	P	G	K	A	E	0.08	2.56	<10
1-32	E	P	G	S	K	E	0.08	2.56	<10
4-5	E	P	G	K	K	E	0.08	2.56	<10
1-24	E	P	G	K		E	0.08	2.56	<10
1-33	E	P	G	Q		E	0.08	2.56	<10
4-8	E	P	G	S		E	0.08	2.56	<10
1-18			A	G	A	E	0.08	2.56	<10
1-21	E	P					0.08	1.28	<10
2-25	E	P	V	L	N	E	0.04	0.16	1,280
2-29	E	P	V	L	N	E	0.04	0.16	1,280
3-15	E	P	A	C	T	E	0.04	0.64	320
3-23	E	P	A	M	T	E	0.04	0.64	320
3-4	E	P	A	V	D	E	0.04	0.32	160
3-5	E	P	A	F	Q	E	0.04	0.32	40

[a] For details, see the footnote to Table 2.

type, and this Lys-for-Glu substitution is the same as that found in the TEM-3, TEM-4, and TEM-9 enzymes identified in ceftazidime-resistant natural isolates (Table 1) (25, 41, 42). The MIC results indicate that *E. coli* containing the 2-33 mutant is slightly more resistant to both cefotaxime and ceftazidime and also retains high levels of resistance to ampicillin. The L103-105 7-16 mutant is a two-amino-acid substitution that has significantly higher activity towards both cefotaxime and ceftazidime while retaining high activity towards ampicillin. It has a conservative substitution of Phe for Val at position 103 and retains the Tyr at position 105 but, like mutant 2-33, has a positively charged group at position 104. All three residues of the 1-12 mutant differ from the wild type; however, there is also an arginine at position 104. This mutant has higher activity towards both cefotaxime and ceftazidime but substantially reduced activity towards ampicillin. Although lysine and arginine are found in common in the cefotaxime-resistant mutants, it is apparent from L103-105 mutants 2-28, 3-11, and 1-5 that arginine at position 104 is not always sufficient for high resistance to cefotaxime. This may be due to perturbations in the overall function of the enzyme caused by the substitutions at positions 103 and 105. For example, on the basis of a comparison of mutants 7-16 and 2-28, it is apparent that asparagine at position 105 is deleterious to the function of the enzyme.

If an arginine substitution at position 104 can increase enzyme activity towards extended-spectrum cephalosporins, why has this substitution not been identified among natural isolates? A possible explanation is that the GAG codon for glutamate in the bla_{TEM-1} gene cannot be converted to an arginine codon by a single-nucleotide substitution. Thus, two separate point mutations are required to convert Glu to Arg at position 104, which would be a rare event. In contrast, Glu can be converted to Lys by a single nucleotide change.

It has been suggested that the Lys-104 substitution results in increased enzyme activity towards ceftazidime because of an electrostatic interaction between the ε-amino group of lysine and the carboxylic group of the oxime side chain of ceftazidime (9, 42). The presence of Arg-104 among mutants with increased activity towards ceftazidime is consistent with the formation of a salt bridge. However, the enzymes with lysine or arginine at position 104 also have increased activity towards cefotaxime, which does not have a carboxylic group on the oxime side chain. Therefore, a more subtle change in the geometry of the active-site pocket owing to the substitutions may also contribute to the increased activity towards ceftazidime and cefotaxime.

The results of sequencing and MIC determinations for cefotaxime-resistant mutants from the L168-170 library are shown in Table 3. As in Table 2, a number of mutants without increased activity for cefotaxime are included for comparison (33). Glycine is conserved at position 168 among the cefotaxime-resistant mutants. That a glycine-for-glutamate substitution at position 168 can provide increased resistance to both cefotaxime and ceftazidime is proven by mutant 3-19, which is a single amino acid substitution compared with the wild type. This mutant also retains a high level of resistance to ampicillin. It should be noted that only two of the three nucleotides of codon 168 were randomized for the L168-170 library (Fig. 1); therefore, only five different amino acids (Val, Ala, Asp, Glu, and Gly) are sampled in the library for position 168. The fact that only glycine at position 168 was found among the cefotaxime-resistant mutants suggests that this is the only substitution among the five possible at position 168 that can provide for increased activity towards cefotaxime. However, it is apparent from mutants 3-15, 3-23, 3-4, and 3-5 that an alanine substitution at position 168 results in a slight increase in activity towards ceftazidime. Mutants 2-25 and 2-29 demonstrate that a valine

substitution at position 168 does not alter the activity of the enzyme substantially. The nucleotide sequence of the Glu-168 codon is GAG, which can be changed to a glycine or alanine codon by a single nucleotide substitution. This result predicts that TEM-derived β-lactamases with glycine or alanine at position 168 may be found in natural isolates resistant to extended-spectrum cephalosporins.

A number of small deletions were found among the cefotaxime-resistant mutants. The deletion mutants all have higher-than-wild-type levels of resistance to both cefotaxime and ceftazidime and are invariably associated with little or no resistance to ampicillin. Thus, these enzymes appear to have drastically altered substrate profiles. Of particular interest is mutant 1-18, which contains a deletion of codons 166 and 167. This is a surprising result in that Glu-166 is presumed to be essential for catalysis of β-lactam antibiotics (17). On the basis of the crystal structures of class A β-lactamases and site-directed mutagenesis, Glu-166 has been proposed to be essential for the deacylation of β-lactam antibiotics during catalysis (1, 13, 18). Perhaps the structure of the enzyme has been altered in this mutant such that some other amino acid residue has moved into a position to catalyze deacylation. One candidate for this role is Glu-171, whose position could be shifted into the active-site pocket by the deletion.

The cefotaxime-resistant mutants from the L168-170 library are interesting in reference to the previously identified Ser-for-Arg substitution at position 164 and the three-dimensional structure of class A β-lactamases. The Arg-164–Ser substitution has been identified in the TEM-5, TEM-7, and TEM-9 enzymes (Table 1) present in natural isolates with increased resistance to ceftazidime (10, 25, 42). In the structure of the homologous class A β-lactamases from *S. aureus* and *B. licheniformis*, the side chain of residue 164 is not in a position to interact directly with the bound substrate (17, 18, 24, 29). Rather, it participates in a salt bridge with the aspartate 179 side chain to form the base of an omega loop (17, 24). The loop includes residues 166 to 170, which form a significant portion of the substrate-binding pocket. The Arg-164–Ser substitution could therefore change the conformation or orientation of the omega loop and thus indirectly alter the substrate profile of the enzyme. Although the Glu-168 residue is on the loop in the vicinity of the binding pocket, its side chain, like that of Arg-164, is not in a position to interact directly with the bound substrate in the *S. aureus* and *B. licheniformis* structures (17, 18, 24, 29). Perhaps the glycine at position 168, with its expanded range of permissible dihedral angles, results in an alteration in the conformation of the loop and thereby indirectly alters the substrate profile of the enzyme. The deletion mutants may also alter the conformation of the region and thereby change the substrate profile of the enzyme. The deletion mutants are also interesting with regard to the evolution of enzyme activity in that they represent a means of drastically altering the function of an enzyme by a single mutational step.

The pattern of amino acid substitutions among the cefotaxime-resistant mutants from the L238-241 library is complex (Table 4). It is evident from the sequences that a wide variety of substitutions result in increased resistance to cefotaxime and ceftazidime. A number of the mutants with increased resistance to cefotaxime also have increased resistance to ceftazidime, although this is not always true. For example, mutants 1-24, 3-20, and 4-13 have increased resistance to cefotaxime but not ceftazidime, while mutants 3-25, 3-27, and 5-21 have increased resistance to ceftazidime but not cefotaxime. The 1-9, 1-10, 4-13, 3-25, 3-27, and 5-21 mutants were initially selected only for resistance to ampicillin (33) and were subsequently found to have increased resistance to cefotaxime and/or ceftazidime. This result suggests that a large fraction of the substitutions in the L238-241 library provide increased resistance to extended-spectrum cephalosporins. This is consistent with the lack of strongly conserved side-chain characteristics among the resistant mutants. One pattern that is evident among the cefotaxime-resistant mutants is that serine and cysteine are overrepresented among substitutions at position 238. It is noteworthy that a serine-for-glycine substitution at position 238 has been found in the TEM-3 and TEM-4 enzymes from cefotaxime-resistant natural isolates (Table 1) (41, 42). The mutant with the highest level of resistance to cefotaxime, however, has an asparagine-for-glycine substitution at position 238. This mutant also has substitutions at positions 240 and 241, so it is not known whether an Asn substitution alone can mediate altered activity.

TABLE 4. Amino acid sequences and MICs of substitutions from the L238-241 library[a]

Mutant	Amino acid at the following position:					MIC (μg/ml) of:		
	237	238	241	242	243	CTX	CAZ	AMP
None (wild type)	A	G	E	R	G	0.04	0.16	1,280
1-9	A	N	H	C	G	0.64	1.28	320
1-34	A	S	K	D	G	0.32	20.48	640
2-21	A	S	E	N	G	0.16	0.32	640
4-5	A	S	S	A	G	0.16	2.56	640
3-30	A	S	K	R	G	0.16	0.32	320
1-24	A	C	D	S	G	0.16	0.16	1,280
1-10	A	G	Q	P	G	0.08	0.32	1,280
3-4	A	S	S	P	G	0.08	0.64	160
7-23	A	C	R	D	G	0.08	5.12	160
4-15	A	C	Y	N	G	0.08	0.16	80
3-20	A	C	N	S	G	0.08	0.16	80
4-13	A	A	C	A	G	0.08	0.16	640
3-25	A	G	R	H	G	0.04	0.64	1,280
3-27	A	G	Y	D	G	0.04	0.32	640
5-21	A		C	P	G	0.04	0.64	640
1-1	A	L	E	Y	G	0.04	0.16	160
3-1	A	C	C	H	G	0.04	0.16	40
1-6	A	V	Q	P	G	0.04	0.16	20
1-2	A	L	R	H	G	0.04	0.16	10

[a] For details, see the footnote to Table 2.

It is known from the TEM-5 enzyme (Table 1), as well as site-directed mutagenesis experiments, that a Lys-240 substitution results in increased enzyme activity towards ceftazidime (42, 43). It has been suggested that the ε-amino group of Lys-240 increases activity by an electrostatic interaction with the carboxylic group on the oxime side chain of ceftazidime (9). Consistent with this suggestion, the mutant with the highest level of resistance towards ceftazidime is 1-34, with lysine at position 240. This mutant has combined the Ser-238 and Lys-240 substitutions, which possibly results in the observed resistance to both cefotaxime and ceftazidime. However, a comparison of the 1-34 mutant with the 3-30 mutant indicates that the Asp-241 substitution enhances enzyme activity towards both cefotaxime and ceftazidime, which suggests that substitutions at position 241 can also alter enzyme activity. Taken together, the results from the L238-241 library suggest that there are a number of different molecular mechanisms for increasing enzyme ac-

tivity by substitutions in the 238-to-241 region. For example, a number of the L238-241 mutants may have increased activity because of simultaneous substitutions at two positions that, when present individually, would not have an effect. Production of such variants is possible because three codons are randomized simultaneously to form the library.

Prediction of the β-lactamase mutational response to selective pressure. The discovery of mutant derivatives of TEM and SHV β-lactamases that have altered substrate spectrums emphasizes the role of β-lactamase mutation as a mechanism for altering antibiotic resistance. Therefore, to design better β-lactam antibiotics it is important to consider not only the hydrolytic capacity of existing β-lactamases but also their ability to expand their spectrum by mutation. The use of random libraries containing all or nearly all of the possible substitutions for a region of β-lactamase provides an empirical approach to determining how β-lactamase will respond to the selective pressure of antibiotic therapy. For example, in this study, screens of the random libraries identified substitutions that increased the activity of the enzyme towards extended-spectrum cephalosporins. Among the substitutions identified were substitutions at two residue positions (residues 104 and 238) that had previously been identified in natural isolates with increased resistance to extended-spectrum cephalosporins. The random library screens thus could have been used to predict the development of resistance by these mutational pathways. Additionally, the random library screens predict that glycine or alanine substitutions at position 168 may be found among natural isolates with increased resistance to cefotaxime or ceftazidime.

Of the 20 different residue positions that were randomized in the seven libraries, substitutions at only a few positions increase enzyme activity towards cefotaxime or ceftazidime. Presumably, most of the substitutions are either neutral or deleterious. The random libraries are currently being screened for substitutions with altered activity towards other β-lactam antibiotics and β-lactamase inhibitors to determine whether different sets of substitutions affect enzyme activity for different classes of β-lactam antibiotics. The rational design of β-lactam antibiotics that are insensitive to mutational changes in β-lactamase will require a detailed knowledge of not only the identity of substitutions that change enzyme specificity but also how the specificity is altered biochemically. In this regard, it will be important to purify and characterize in detail those variant enzymes that have been identified in the random library screens. This combination of approaches should yield an understanding of the basis of β-lactamase substrate specificity.

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