Amino Acid Sequence Determinants of Extended Spectrum Cephalosporin Hydrolysis by the Class C P99 β-Lactamase*

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Class C β-lactamases are commonly encoded on the chromosome of Gram-negative bacterial species. Mutations leading to increased expression of these enzymes are a common cause of resistance to many cephalosporins including extended spectrum cephalosporins. Recent reports of plasmid- and integron-encoded class C β-lactamases are a cause for concern because these enzymes are likely to spread horizontally to susceptible strains. Because of their increasing clinical significance, it is critical to identify the determinants of catalysis and substrate specificity of these enzymes. For this purpose, the codons of a set of 21 amino acid residues that encompass the active site region of the P99 β-lactamase were individually randomized to create libraries containing all possible amino acid substitutions. The amino acid sequence requirements for the hydrolysis of ceftazidime, an extended spectrum cephalosporin commonly used to treat serious infections, were determined by selecting resistant mutants from each of the 21 libraries. DNA sequencing identified the residue positions that are critical for ceftazidime hydrolysis. In addition, it was found that certain amino acid substitutions in the ω-loop region of the P99 enzyme result in increased ceftazidime hydrolysis suggesting the loop is an important determinant of substrate specificity.

β-Lactam antibiotics such as the penicillins and cephalosporins are among the most often prescribed antimicrobial agents. These antibiotics act by inhibiting transpeptidase enzymes (also called penicillin-binding proteins or PBPs) that are essential for the synthesis of the peptidoglycan layer of the bacterial cell wall (1). Inhibition of peptidoglycan synthesis results in death of growing bacteria and accounts for the antimicrobial activity of these agents. Inhibition of peptidoglycan synthesis results in the cross-link bacterial cell walls (2). All of these enzymes contain the active site serine as well as a conserved triad of K(S/T)G that are critical for ceftazidime hydrolysis. In addition, it was found that certain amino acid substitutions in the ω-loop region of the P99 enzyme result in increased ceftazidime hydrolysis suggesting the loop is an important determinant of substrate specificity.

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† The abbreviations used are: PBP, penicillin-binding proteins; PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; MES, 4-morpholineethanesulfonic acid.
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EXPERIMENTAL PROCEDURES

Materials—Chloramphenicol and cephaloridine were purchased from Sigma. Ceftazidime was a gift from Glaxo Wellcome. All enzymes were purchased from New England Biolabs except for T7 DNA polymerase, which was purchased from U. S. Biochemical Corp. Oligonucleotide primers were custom-synthesized by Integrated DNA Technologies. E-test strips for antibiotic susceptibility testing were purchased from AB Biodisc. SP-Sepharose and G-75 gel filtration columns were purchased from Amersham Pharmacia Biotech.

Strains and Plasmids—The plasmid pGR32 was constructed previously (19). This plasmid contains a chloramphenicol resistance gene as well as a polylinker region for cloning heterologous genes. Heterologous genes are expressed under the control of a tac promoter (20). E. coli XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lacI(q)), P99 producing E. coli, and G-75 gel filtration columns were used for construction and selection of the libraries (21). E. coli RB791 (strain W3110 lacI) was used to express and purify β-lactamase mutants (20).

Construction of Random Libraries—A construct containing the wild-type P99 gene was cloned into the pGR32 plasmid as an ScaI-XhoI DNA fragment using the P99-top and P99-bottom primers used for the random library constructions described below. The template used for the original PCR was the plasmid pHUS54, which contains the wild-type P99 gene and was provided by A. Dubus (22). The resulting plasmid construct was named pYY12. The pYY12 plasmid was used as template for all library constructions. Individual codos of the E. cloacae P99 β-lactamase gene were randomized by overlap extension PCR as described previously (23). The two outside primers, P99-top 5′-CCCCCCTCT-CTAGCTGTCCATCACTGTTTATGAGTGCTG-3′ and P99-bottom 5′-GCTCCCTCTGAGACCGGAATCTTAATGATTGTATGCG-3′, were used in conjunction with overlapping primers that were designed to randomize individual codons to create a PCR product containing the P99 gene with a randomized codon. The PCR fragment was digested with the XhoI and ScaI restriction enzymes and ligated into the pGR32 vector that had been digested with XhoI and ScaI (19). The ligation reaction was electroporated into E. coli XL1-Blue cells. The cells were incubated at 37 °C for 1 h and spread on LB agar plates containing 12.5 μg/ml chloramphenicol (LB-CMP). The plates were incubated at 37 °C overnight, and the colonies were then pooled and stored at ~8 °C in 15% glycerol. Each library consisted of a minimum of 10,000 pooled colonies. Therefore, each library has a greater than 99% probability of containing all possible sequences for the codon randomized (24).

Selection of Functional Random Mutants Based on Ceftazidime Resistance—The cells from each library were diluted into LB medium and spread on LB agar plates containing 0.5 μg/ml ceftazidime (LB-CAZ). The colonies were performed in the absence of isopropyl-1-thio-β-D-galactopyranoside so that the β-lactamase gene was expressed at the low, constitutive levels of the tac promoter (20). As a control, the library was also spread on LB-CMP plates. The plates were incubated at 37 °C overnight. The colonies were counted, and the P99 gene was amplified from selected colonies by PCR. The colonies were sequenced directly from the PCR product by cycle DNA sequencing. The sequences were determined using an ABI 377 automated DNA sequencing instrument.

Minimum Inhibitory Concentration (MIC) Measurements—The MIC for ceftazidime was determined for each of the clones that were selected for DNA sequencing. The colonies were inoculated into 5 ml of LB containing 12.5 μg/ml chloramphenicol. The culture was grown at 37 °C overnight and diluted to an A600 of 0.3. A total of 100 μl of each diluted culture was spread on an LB agar plate. The plates were allowed to dry and then an E-test strip embedded with ceftazidime was applied. The MIC was read after overnight incubation at 37 °C. An E. coli strain containing the wild-type P99 gene was used as a control for all MIC measurements.

Expression and Purification of P99 Enzymes—The wild-type P99 enzyme as well as mutant derivatives were expressed and purified for the determination of kinetic parameters. The pYY12 plasmid described above was transformed into E. coli RB791 cells for large scale expression (20). A 1-liter culture was grown at 37 °C until the A600 reached 0.5 and then induced with 0.1 mg/l isopropyl-1-thio-β-D-galactopyranoside. The culture was then incubated overnight at 25 °C with shaking. The cells were collected by centrifugation and resuspended in 20 ml of sucrose buffer. The cell debris was removed by centrifugation, and the supernatant was dialyzed against 2 times 2 liters of 25 mM MES (pH 6.2). The protein lysate was fractionated on an SP-Sepharose column and eluted with a 0.5 M NaCl gradient. The enzyme was further purified using a G-75 gel filtration column in 25 mM phosphate buffer (pH 7.0). The purity of the final preparation was higher than 90% based on SDS-polyacrylamide gel electrophoresis.

Determination of Enzyme Kinetic Parameters—Enzyme kinetics measurements were carried out using a Beckman DU-40 UV spectrophotometer as described previously (26). A reaction mixture containing the antibiotic substrate, 1 mg/ml bovine serum albumin in 50 mM phosphate buffer, pH 7.0, was incubated at 30 °C for 5 min in a total volume of 0.5 ml. A total of 0.1 ml of a β-lactamase stock solution was added, and the initial reaction velocity was measured by the change in UV absorbance. For ceftazidime, the initial reaction velocity was calculated from the first 5 min of the reaction because ceftazidime is a poor substrate. Cephalosporin C hydrolysis was monitored at 280 nm, whereas ceftazidime hydrolysis was monitored at 260 nm. The change in absorbance was monitored for 5 min. The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were calculated from the initial velocity data using non-linear regression fit.

Alignment of Class C β-Lactamases—The β-lactamase sequences used for the alignment to generate the data for Fig. 5 were chosen such that none of the sequences had 90% or greater identity to any other sequence in the alignment. The sequences, the GenBank accession number, and if available, the reference number are as follows: P99 (X07274) (22), MIR-1 (27), LAT-1 (X78117) (28), Klebsiella pneumoniae (S45109), Enterobacter aerogenes (AAF18992) (29), E. coli (AA97049) (30), Yersinia enterocolitica (B48899) (31), Margarinae morganii (Y10853) (32), Serratia marcescens (X52964) (33), Providencia stuartii (CAAA7839), ACC-1 (AJ133121) (34), FOX-1 (X74755) (35), CepS (CAA56506), CMY-1 (C295580) (37), Aeromonas sobria (ACB77444) (40), Pseudomonas aeruginosa PA01 (X54719) (39), Lyssobacter lasgangenae (S54103), and Gacinobacter baumannii (CAB77444) (40). The alignment was generated using the pile-up program of the Wisconsin Package version 10.0, Genetics Computer Group (GGC), Madison, WI.

RESULTS

Systematic Randomization of Amino Acids in the Active Site of Class C β-Lactamase P99—As described above, derepression of class C β-lactamase synthesis is a widespread source of resistance of Gram-negative bacteria to extended spectrum cephalosporins such as cefotaxime and ceftazidime. Because of the significant role of P99 and highly related class C β-lactamases in antibiotic resistance, it is of interest to understand how the amino acid sequence of the enzyme determines its structure and activity. For this reason, we determined the amino acid sequence requirements for the hydrolysis of ceftazidime for a set of 21 residues that encompass the active site and substrate-binding pocket of the P99 β-lactamase (Fig. 1). Although the 21 residues under study are all in the vicinity of the active site, not all of these residues are likely to contribute equally to the structure and function of the enzyme. Some residue positions are likely to be essential, and therefore substitutions at these positions will result in a non-functional enzyme. However, other residue positions may be less important, and thus substitutions at these positions will be more freely tolerated. The location of essential residues identifies those positions in the amino acid sequence that are the most important determinants of P99 β-lactamase structure and activity.

The tolerance of each residue to amino acid substitutions was determined using saturation mutagenesis followed by a functional selection for active mutants (24, 25). The strategy consists of randomizing the DNA sequence of a single codon to create a random library containing all possible amino acid substitutions for the position randomized. The active site region of the enzyme was randomized in a set of 21 random libraries (Fig. 1). Each of the 21 random libraries was used to transform E. coli, and functional mutants were selected by spreading the transformed cells on agar plates containing 0.5 μg/ml ceftazidime. This is the minimal inhibitory concentration (MIC) of ceftazidime for an E. coli strain containing the wild-type P99 gene on the plasmid used to construct the random libraries can grow on agar plates after transformation. Thus, phenotypically wild-type mutants were selected from each of the libraries. It should be noted that this concentration is not the same as the minimal inhibitory concentration (MIC) for ceftazidime of an E. coli strain containing...
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the wild-type P99 gene, which is 3 μg/ml as determined using E-test strips (Table I; see under “Experimental Procedures”). The difference is most likely due to an inoculum effect whereby fewer plasmid-containing cells are spread on agar plates after transformation than in the E-test strip experiment. The DNA sequence of at least 10 functional mutants selected from each library were sequenced, and the results are presented in Fig. 2. In addition, to confirm that the selected mutants exhibited resistance levels similar to wild type, the minimal inhibitory concentration was determined for each non-wild-type mutant that was sequenced (Table I). The sequencing results from the naive and ceftazidime-selected libraries are also informative when compared with the sequences from the clones selected for ceftazidime resistance. For example, sequencing of the Glu272 naive library revealed a diverse collection of sequences (Fig. 2). After selection for ceftazidime resistance, however, only glutamate codons were present among the sequenced clones. This strongly suggests that glutamate is the only amino acid capable of providing wild-type levels of function at position 272. Based on this result, Glu272 is interpreted to be essential for the structure and function of the enzyme. Note, however, that this result does not indicate whether Glu272 is essential for substrate binding and catalysis. It is possible, for instance, that the glutamate residue is essential for the structural integrity of the enzyme.

In contrast to residue Glu272, a diverse set of amino acids was found at position Leu293 both before and after the selection for ceftazidime resistance. This result suggests Leu293 is not critical for the structure and function of the enzyme. Note that the Arg249 library appears from the sequencing of the unselected clones to be biased toward the wild-type arginine codon (Fig. 2). Nevertheless, useful information could still be obtained about this position due to the different spectrum of substitutions among the ceftazidime-selected clones. Despite the bias toward arginine in the starting library, the lack of lysine in the starting library combined with the multiple occurrences of lysine in the selected library indicates that a positive charge is important at position 349 (Fig. 2).

The sequencing results from the naive and ceftazidime-selected libraries were used to place the 21 mutagenized positions into four classes (Fig. 1). First, a position is considered as essential if all of the sequences of clones selected for ceftazidime resistance. For example, sequencing of the Glu272 naive library revealed a diverse collection of sequences (Fig. 2). After selection for ceftazidime resistance, however, only glutamate codons were present among the sequenced clones. This strongly suggests that glutamate is the only amino acid capable of providing wild-type levels of function at position 272. Based on

![Fig. 1. Space fill model of P99 β-lactamase showing the location of residues that were randomized. Ser64 is the active site serine directly involved in catalysis. Residues that do not tolerate substitutions are colored in red. Gly317 is not shown in the figure because it is localized immediately behind Thr316 and cannot be seen from this view. Residues that tolerate substitutions but with the wild type residue predominating among functional mutants are colored yellow. Residues that tolerate substitutions but with a non-wild-type residue predominating among functional mutants are colored blue. Residues that exhibit no strong preference for any amino acid type are colored green. The figure was made using the Molscript program (59).](http://www.jbc.org/)

### Table I

| β-Lactamase   | CAZ     | µg/ml |
|---------------|---------|-------|
| Wild-type P99 |         | 3.0   |
| L119V        |         | 2.0   |
| L119N        |         | 4.0   |
| L119I        |         | 4.0   |
| L119M        |         | 3.0   |
| Q120K        |         | 12.0  |
| Q120R        |         | 3.0   |
| Q120H        |         | 1.0   |
| R204L        |         | 4.0   |
| R204F        |         | 6.0   |
| R204Y        |         | 4.0   |
| R204V        |         | 8.0   |
| R204S        |         | 6.0   |
| D217S        |         | 6.0   |
| D217G        |         | 8.0   |
| D217E        |         | 8.0   |
| A220S        |         | 3.0   |
| A220F        |         | 4.0   |
| A220L        |         | 3.0   |
| A220R        |         | 1.5   |
| A220W        |         | 1.5   |
| Y221A        |         | 64.0  |
| Y221K        |         | 3.0   |
| Y221G        |         | 24.0  |
| Y221W        |         | 2.0   |
| Y221N        |         | 6.0   |
| S289F        |         | 6.0   |
| S289G        |         | 6.0   |
| S289Y        |         | 6.0   |
| S289V        |         | 6.0   |
| L293P        |         | 0.5   |
| L293E        |         | 4.0   |
| L293V        |         | 8.0   |
| L293C        |         | 3.0   |
| L293I        |         | 8.0   |
| L293S        |         | 8.0   |
| L293D        |         | 4.0   |
| T319L        |         | 0.5   |
| T319M        |         | 0.5   |
| S343T        |         | 6.0   |
| S343A        |         | 6.0   |
| S343R        |         | 3.0   |
| S343N        |         | 1.0   |
| N346I        |         | 3.0   |
| N346L        |         | 20.0  |
| N346V        |         | 4.0   |
| N346Y        |         | 16.0  |
| R349K        |         | 6.0   |
The Glu 272 side chain constitutes a resistance to 0.5 μg/ml ceftazidime. The finding that Thr316 is essential for hydrolysis of the ceftazidime and other cephalosporins (16). Consistent with this role, the threonine side chain is strongly conserved among functional mutants at position 319. The Thr319 side chain hydroxyl group may interact with the free carboxy group of β-lactam antibiotics either directly or through a bridging water molecule (44, 45). Previous mutagenesis results suggest that the proposed interaction is most important for binding and hydrolysis of cephalosporin antibiotics (45). The finding that Thr319 is essential for hydrolysis of the cephalosporin ceftazidime is consistent with the previous result. It is unclear, however, why a serine residue does not function at this position.

The strict conservation of glycine among functional mutants at position 317 is explained by the fact that any other residue at this position would, for steric reasons, interfere with the binding of substrate (18). A similar result was obtained after randomization and functional selection of the analogues residue in the class A TEM-1 β-lactamase (25). The main chain amide nitrogen of Ser318 forms the putative oxyanion hole of class C β-lactamases (16–18). However, it is unclear why there is a strict conservation of the serine side chains at position 318 among functional mutants. The hydroxyl group of the analogous residue (Thr319) in the Streptomyces R61 carboxypeptidase has been observed to interact with the NH group of the side chain amide of cephalothin or the carboxyl group of the dihydrothiazene ring of cefotaxime (46). By analogy, the hydroxyl group of Ser318 in the P99 enzyme may interact with a hydrogen-bonding group from the side chain of ceftazidime.

The Glu272 position was also found to be essential for ceftazidime hydrolysis (Fig. 2). The Glu272 side chain constitutes a wall of a channel at the back of the active site where it takes part in a hydrogen-bonding network with His314 and Lys315 (18). Previous mutagenesis and enzyme kinetics results suggest Glu272 is not important for acylation but may contribute to the deacylation process (41). These results are consistent with such a role. However, the exact role of the channel and Glu272 residue remains to be determined.

At four of the 21 positions randomized, the wild-type residue was the most prevalent among sequenced functional random mutants, but other residues were also observed. These positions include Leu119, Gln120, Thr319, and Arg349 (Fig. 2). The Leu119 and Gln120 residues form a wall of the active site where they can participate in substrate binding. The structure of the E. coli AmpC enzyme in complex with a boronic acid inhibitor containing the side chain of cephalothin indicates a hydrogen bond between side chain of Gln120 and the carbonyl oxygen of the amide group of cephalothin (47). In contrast, in the structure of a boronic acid inhibitor with the side chain of cloxacillin, the side chain of Gln120 is rotated away from the amide (47). Therefore, position 120 is quite versatile for substrate binding. Position 120 is also of interest because of the high percentage of lysine and arginine substitutions among the functional mutants. This result suggests lysine or arginine substitutions at position 120 may increase ceftazidime hydrolysis. Consistent with this hypothesis is that finding that an E. coli strain containing the P99 enzyme with the lysine substitution is somewhat more resistant to ceftazidime than that containing the P99 enzyme with the lysine substitution (Table I).

Residue Thr319 lies on the opposite side of the active site pocket from Leu119 and Gln120. Also, it is the first residue after the essential B3 strand (Fig. 3). Thr319 forms part of a binding pocket that could directly interact with the side chain of ceftazidime and other cephalosporins (16). Consistent with this role, the threonine side chain is strongly conserved among functional mutants at position 319.

In class A β-lactamases, the Arg244 residue is thought to assist in substrate binding and turnover by interacting with the carboxyl group on C-3 of penicillins or C-4 of cephalosporins (48). An exact counterpart does not exist in the P99 β-lactamase, but Asn346 and Arg349 are in an equivalent region of the binding site (18). It has been noted that Arg349 is not properly oriented for interaction with substrate and that Asn346 might be the better positional counterpart of Arg244 (18). The functional selection results indicate a hydrogen bonding group is not required at position 346, and thus Asn346 is not functionally
equivalent to Arg\textsuperscript{244} of the class A enzymes. In contrast, only arginine or lysine residues are consistent with function at position 349, which is consistent with this residue making an important interaction with the carboxyl group of the substrate.

To confirm that a residue that does not have hydrogen bonding potential can efficiently function at position 346, P99 enzymes containing either the Asn\textsuperscript{346} → Ala or the Asn\textsuperscript{346} → Ile substitutions were purified to homogeneity, and kinetic parameters were determined for cephalosporin C and ceftazidime hydrolysis (Tables II and III). Cephalosporin C has been shown to be an excellent substrate for the wild-type P99 enzyme (12), and it is also an excellent substrate for the Asn\textsuperscript{346} → Ala and Asn\textsuperscript{346} → Ile enzymes. The catalytic efficiency of the Asn\textsuperscript{346} → Ile enzyme for ceftazidime hydrolysis was also similar to the wild-type enzyme (Table III). The catalytic efficiency of the Asn\textsuperscript{346} → Ile enzyme is also similar to wild type, but the \( k_{cat} \) and \( K_m \) values are ~8- and 6-fold higher, respectively, than those of the wild-type enzyme. Interestingly, the Asn\textsuperscript{346} → Ile mutant also exhibits significantly higher levels of resistance to ceftazidime than wild-type (Table I) suggesting that \( k_{cat} \) strongly influences the MIC value. In addition, these results indicate that a side chain with hydrogen bonding potential is not required at position 346. Therefore, residue 346 is not the functional counterpart of Arg\textsuperscript{244} of the class A enzymes.

Ala\textsuperscript{220} and Tyr\textsuperscript{221} reside at the floor of the active site pocket within an \( \omega \)-loop region (residues 189–226) (Fig. 3) (18). At these positions, a non-wild-type residue predominated among the functional mutants selected for ceftazidime resistance (Fig. 2). Serine was the most common substitution at position 220, whereas alanine predominated at position 221. The fact that a non-wild type residue predominates among functional mutants suggests the substitution results in increased ceftazidime hydrolysis. Consistent with this hypothesis, \( E. \) coli strains containing the Tyr\textsuperscript{221} → Ala or Gly mutants exhibit significantly higher levels of resistance to ceftazidime than wild-type P99 (Table I). To confirm that substitutions at position 221 have increased catalytic efficiency, the Tyr\textsuperscript{221} → Gly enzyme was purified to homogeneity, and kinetic parameters were determined for cephalosporin C and ceftazidime hydrolysis (Tables II and III). Large increases in \( K_m \) were observed for both cephalosporin C and ceftazidime. The \( k_{cat} \) value, however, was decreased 8-fold for cephalosporin C but increased 800-fold for ceftazidime. The net result was a large decrease in \( k_{cat}/K_m \) for cephalosporin C but a large increase in catalytic efficiency for ceftazidime hydrolysis. Therefore, a non-wild-type residue was selected at position 221 because the wild-type amino acid is not the optimal residue for ceftazidime hydrolysis.

The remaining six residues, Arg\textsuperscript{244}, Asp\textsuperscript{217}, Ser\textsuperscript{289}, Leu\textsuperscript{293}, Ser\textsuperscript{343}, and Asn\textsuperscript{346}, did not exhibit a strong bias toward any specific amino acid among the mutants selected for ceftazidime resistance (Fig. 2). All of these residues are on the periphery of the active site pocket (18). The results suggest these residues are not critical for binding or hydrolysis of extended spectrum cephalosporins such as ceftazidime. It has been noted that the Asp\textsuperscript{217} residue on the \( \omega \)-loop structure is the only possible counterpart to the deacylation residue Glu\textsuperscript{166} of class A enzymes (18). Our results are consistent with previous mutagenesis studies indicating the residue does not play an important role in deacylation (49). In addition, the finding that Ser\textsuperscript{289} can be substituted and retain function is consistent with a recent study demonstrating that the kinetic parameters of substituted enzymes are not greatly different from wild-type for several cephalosporin substrates (50).

The Leu\textsuperscript{293} position has not been mutagenized previously, but the results presented in Fig. 2 indicate it is not important for ceftazidime hydrolysis. To ensure that the mutants selected from the Leu\textsuperscript{293} library do, in fact, function similar to the wild-type enzyme, the Leu\textsuperscript{293} → Cys enzyme was purified, and kinetic parameters for cephaloridine and ceftazidime hydrolysis were determined (Tables II and III). The kinetic parameters for hydrolysis of both substrates were similar to those obtained for the wild-type enzyme indicating that the mutants selected ceftazidime resistance from the Leu\textsuperscript{293} library exhibit catalytic properties similar to the wild-type enzyme.

**DISCUSSION**

**Sequence Conservation in Evolution Versus Sequence Conservation among Functional Mutants**—Sequence alignments of members of a gene family indicate the evolutionary sequence conservation of an amino acid residue position. This information is very useful in determining whether a residue is critical for the structure and function of a protein. In the experiments presented above, an indication of sequence conservation is provided by randomization of a position followed by selection and sequencing of functional random mutants. Is the information gained from these approaches the same? To answer this question, a set of 18 class C \( \beta \)-lactamases were obtained from sequence data bases and used to generate a sequence alignment. Sequences with 90% or greater identity were excluded.

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**Table II**

| \( \beta \)-Lactamase | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | Relative* |
|------------------------|-------------|-----------|-----------------|------------|
| Wild-type P99          | 1562        | 266       | 5.87            | 1.0        |
| Y221G                  | 198         | 731       | 0.27            | 0.05       |
| N346A                  | 3190        | 454       | 7.06            | 1.20       |
| N346I                  | 1606        | 336       | 4.83            | 0.82       |
| L293C                  | 1213        | 279       | 4.34            | 0.74       |

* Ratio of \( k_{cat}/K_m \) of the mutant relative to that of the wild type.

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**Table III**

| \( \beta \)-Lactamase | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | Relative* |
|------------------------|-------------|-----------|-----------------|------------|
| Wild-type P99          | 0.065       | 28        | 0.002           | 1.0        |
| Y221G                  | 59          | 1700      | 0.035           | 17.5       |
| N346A                  | 0.021       | 4         | 0.005           | 2.5        |
| N346I                  | 0.514       | 178       | 0.003           | 1.5        |
| L293C                  | 0.041       | 7         | 0.006           | 3.0        |

* Ratio of \( k_{cat}/K_m \) of the mutant relative to that of the wild type.
Fig. 4. Comparison of sequence variability among functional P99 β-lactamase mutants and 18 aligned class C β-lactamases. The residue number is indicated between the horizontal lines. Above the residue positions are the different amino acid residues that appear at these positions in the alignment of class C β-lactamas. The number of occurrences of each type is indicated. The class C sequences used are listed under “Experimental Procedures.” Below the residue positions are the different amino acid residues that were identified among the functional random mutants.

from the alignment. A comparison of sequence conservation based on the gene family alignment versus the functional selection data is shown in Fig. 4. There is a close agreement for many of the residues including Ser64, Lys87, Tyr150, Asn152, Glu272, Lys315, Thr316, Gly317, and Ser318. Many of these residues are directly involved in catalysis, and thus it is not surprising that they are strongly conserved both in the gene family and in the functional selection experiments.

At several residue positions, a wider spectrum of substitutions was observed for the ceftazidime functional selection than in the gene family. These residues include Leu119, Gin120, Arg204, Tyr217, Leu218, Thr219, and Arg349. There are two possible explanations for this observation. First, the ceftazidime selection may not be sufficiently stringent, enabling mutants with partial function to be selected. This would lead to a wider spectrum of observed substitutions but would not be indicative of the actual tolerance of the position to substitutions. The purification and characterization of the Leu293 → Cys substitution was performed to address this possibility. The finding that this enzyme exhibits catalytic properties similar to wild-type P99 suggests the ceftazidime selection is stringent. The MIC data for all of the selected mutants (Table I) is also consistent with a high stringency for the ceftazidime selection. A more likely explanation is that the class C β-lactamase family members have been under a more diverse selective pressure than simple ceftazidime resistance. Bacteria containing enzymes of the class C family have likely been under selective pressure for resistance to a number of different cephalosporins. It is known that hydrolysis of cephaloridine and cephalosporin C by the P99 enzyme is a diffusion-controlled reaction (12). The fact that the enzyme has achieved catalytic perfection for these substrates suggests that these or similar substrates have provided the selection pressure and thereby directed the evolution of the P99 enzyme (12). In contrast, ceftazidime and other extended spectrum cephalosporins are relatively poor substrates for the P99 enzyme (51). Therefore, the active site of the P99 enzyme has been highly optimized for the hydrolysis of cephalosporin C but not ceftazidime. A highly optimized active site would be more sensitive to substitution, and consequently, a wide range of substitutions at a position may not be consistent with cephalosporin C hydrolysis. However, because the P99 active site is not optimized for ceftazidime hydrolysis, many substitutions may be allowed, and in fact, several may result in increased ceftazidime hydrolysis.

ω-Loop Substitutions That Alter Class C β-Lactamase Substrate Specificity—The most striking difference between sequence conservation in the gene family versus the ceftazidime selection is at Tyr221, which is localized in the active site ω-loop structure (Fig. 3). Tyrosine is conserved at position 221 among all of the class C β-lactamases. However, tyrosine was never observed among 19 ceftazidime-resistant clones selected from the Tyr221 library. Instead, alanine was the predominant amino acid among the functional mutants. MIC measurements that the Tyr221 → Ala and Tyr221 → Gly mutants were significantly more resistant to ceftazidime than E. coli containing the wild-type P99 gene. The Tyr221 → Gly enzyme exhibits strikingly different catalytic characteristics than the wild-type P99 enzyme (Table II). The values for $k_{cat}/K_m$ with ceftazidime as a substrate are 800- and 60-fold higher, respectively, than those for the P99 enzyme. The enzyme therefore has a catalytic efficiency ($k_{cat}/K_m$) for ceftazidime hydrolysis that is 18-fold higher than the wild-type enzyme. The $k_{cat}/K_m$ value is an apparent second-order rate constant for the reaction of free enzyme and substrate to enzyme and product (52). This value has been shown to correlate strongly with MIC values, and therefore, the higher $k_{cat}/K_m$ value is consistent with the higher ceftazidime resistance of the mutant (53). The deacylation process is rate-limiting for hydrolysis of oxyimino β-lactams such as ceftazidime by class C β-lactamases (51, 54). Hence, the large increase in $k_{cat}$ may reflect an increase in the rate of deacylation. The increase in $k_{cat}$ is balanced somewhat by the large increase in $K_m$. Because of the mechanism of class C β-lactamases, however, an increase in $K_m$ does not necessarily indicate less efficient substrate binding. When deacylation is the rate-limiting step, an increased rate of deacylation will also increase the value of $K_m$ (54).

Extended spectrum cephalosporins such as ceftazidime are poor substrates for both class A and class C β-lactamases. The evolution of resistance to ceftazidime has occurred via amino acid substitutions in the class A TEM-1 and SHV-1 β-lactamases (55). A number of substitutions have been identified in enzymes from resistant clinical isolates, and these substitutions have been found to increase $k_{cat}$ and lower $K_m$ for ceftazidime (56). In contrast, only a single natural isolate with increased ceftazidime resistance due to changes in the P99 class C β-lactamase has been identified (57). This enzyme contains an insertion of three residues after position 207 in the ω-loop of the P99 β-lactamase. Replacement of the 3-residue insertion with 1–4 alanine residues indicated it is the insertion of amino acids and not the identity of the amino acids that is critical for changing the specificity of the enzyme (51). It is of interest that the insertion has a similar effect on enzyme kinetic parameters as the Tyr221 → Gly substitution; both $k_{cat}$ and $K_m$ values are greatly increased (51). This suggests that the Tyr221 → Gly substitution and the 3-residue insertion mutant act via a similar mechanism. The structure of the insertion mutant of P99 β-lactamase has been solved (58). It leads to a wider opening to the substrate binding cavity and more flexibility in the ω-loop. It has been suggested that this could facilitate hydrolysis of oxyimino β-lactams by making the acyl-
enzyme intermediate more open to attack by water and thereby increasing the rate of deacylation (58). By analogy, the Tyr\textsuperscript{221}\hspace{1em}→\hspace{1em}Gly substitution might act in a similar manner.

As stated above, only one natural mutant of P99 that leads to increased hydrolysis and thus resistance to extended spectrum cephalosporins has been identified (57). Based on the randomization and selection experiments, mutations that convert Tyr\textsuperscript{221}\hspace{1em}→\hspace{1em}Gly or Ala will lead to greatly increased ceftazidime resistance (Table I). However, these amino acid substitutions can occur only via 2-base pair changes in the coding sequence. Because this is expected to be a rare event, it may explain why mutations within the coding region of class C enzymes such as P99 leading to increased cephalosporine resistance are not commonly observed.

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