Changes in the substrate specificity of TEM-1 β-lactamase due to amino acid substitutions in the enzyme have been observed among resistant clinical isolates in response to selective pressure applied by extended-spectrum antibiotics (6). The most common amino acid substitutions occur at residues 104, 164, and 237–240, both as individual and as combinatorial substitutions (numbering of amino acids according to Ambler et al. (7)). The substitutions are thought to provide more favorable interactions between the enzyme and the bulkier side chains of the extended-spectrum cephalosporins, thus increasing the catalytic efficiency of the enzyme (6).

Because of the importance of mutation-mediated changes in TEM-1 β-lactamase substrate specificity during the development of antibiotic resistance, it is of interest to determine how mutations can alter the substrate profile of the enzyme toward various β-lactam antibiotics. Previously, saturation mutagenesis was used to study the amino acid residues critical for the function of TEM-1 β-lactamase with ampicillin as a substrate (8). This was accomplished by randomizing three contiguous codons to create random libraries containing all possible amino acid substitutions for a given three residue window. The entire coding sequence of TEM-1 β-lactamase has been randomized in a set of eighty-eight libraries (8). Functional random mutants which conferred resistance to ampicillin were selected from each of the libraries and sequenced to identify the critical residues (8). It was found that 43 out of the 263 amino acid residues of TEM-1 β-lactamase are essential for function of the enzyme with ampicillin as a substrate. Conversely, 220 residues of the enzyme could be substituted to varying degrees and still maintain ampicillinase activity. This data set can now be used to identify residue positions that are important determinants of β-lactamase substrate specificity by comparing the sequence requirements for efficient ampicillin hydrolysis with those for other β-lactam antibiotics.

In this report, eleven random libraries, including the residues that constitute the active site of TEM-1 β-lactamase, have been used to select functional mutants with cephaloridine as a substrate. Cephaloridine is a second generation cephalosporin, such as ceftaxidime and cefotaxime, however, are poor substrates for the TEM-1 enzyme (6).

β-lactam antibiotics, such as the penicillins and cephalosporins, are among the most commonly used antimicrobial agents. The production of β-lactamases, which catalyze β-lactam hydrolysis, is the predominant mechanism of bacterial resistance to these antibiotics (1). β-lactamases are grouped into 4 classes (A, B, C, and D) based on primary sequence homology (2, 3). Classes A, C, and D are serine hydrolases (3) while class B consists of zinc metalloenzymes (4). TEM-1 β-lactamase is a plasmid encoded, class A enzyme that can efficiently cleave the penicillins and some cephalosporins (5). The third generation or extended-spectrum cephalosporins, such as ceftazidime and cefotaxime, however, are poor substrates for the TEM-1 enzyme (6).
minded by introducing the substitutions individually and in combination. In particular, the A237T and E240C substitutions alone or in combination exhibit increased cephalosporinase activity and decreased penicillinase activity relative to the wild-type enzyme.

EXPERIMENTAL PROCEDURES

Materials—All enzymes were purchased from New England Biolabs, except T7 DNA polymerase, which was purchased from U. S. Biochemical Corp. Ampicillin, benzylpenicillin, cephaloridine, cephalothin, and cefotaxime were purchased from Sigma. Nitrocefin and blank antibiotic discs were purchased from Beaston Dickinson (Cockeye Valley, MD). G-75 Sephadex was obtained from Pharmacia Biotech Inc.

Escherichia Coli Strains and Plasmids—Prior to mutagenesis, the E. coli BW313 (Hfr lysA61–62, dut1, ung1, thi1, relA spotT1) strain was used for the propagation of plasmid DNA (10). For the introduction of mutagenized DNA, the E. coli ES1390 (lacZ53, mutS201::Tn5, thyA36, rfa5, metB1, deoC, IN (rnmD-rnmE)) strain was used (11). For the determination of antibacterial susceptibility and for the preparation of single-stranded DNA, the E. coli XL1-Blue (recA1, endA1, gviA96, thi-1, hsdRI17, supE44, relA1, lac, [F::Tn10(Tet’’’’’proA8, DlacP (lacZIM15)]) strain was used (12).

The parent plasmid of all random library constructions is plasmid pB666. The pB666 plasmid contains the wild-type blaTEM-1 gene and a cat gene, which encodes for chloramphenicol acetyltransferase. The 4.8-kilobase plasmid also contains the CoIE1 and Fl 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 random library L234–236 was constructed as described previously (8).

Plasmid Isolation and DNA Sequencing—Single-stranded plasmid DNA was prepared for sequencing as described (14). DNA sequencing was performed using the deoxy chain termination method (15). Oligonucleotides were designed to prime synthesis from specific sites within the blaTEM-1 gene.

Selection of Active Mutants—Selections for ampicillin resistance were done on LB agar plates containing 1.0 mg/ml ampicillin. Selections for cephaloridine resistance were done using the disc diffusion method. Antibiotic paper discs, which had been soaked for 2 h in a 15 mM sodium phosphate buffer, pH 7.0, were applied to the surface of the plates and were incubated overnight at 37 °C. A 1.2 cm diameter inhibition zone around each antibiotic paper disc was used as a relative indicator for wild-type level activity and was used as a control for the disc forming a circular concentration gradient on the agar plate, and each antibiotic disc was used in this study (Fig. 1). The kinetics of TEM-1 β-lactamase and the mutant enzymes were determined by nitrocefin hydrolysis and SDS-polyacrylamide gel electrophoresis. The results were expressed as the number of times each sequence occurred is indicated in parentheses next to each sequence.

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\text{Kinetic Parameters: } k_{\text{cat}} / K_m = \frac{V_{\text{max}}}{K_m} = \frac{\text{velocity}}{[\text{Substrate}]}, \quad \text{where } [\text{Substrate}] = [S] \\
\text{for each region is shown in } \text{bold italic type.}
\]

RESULTS

Selection of Mutants from the Active Site Random Libraries—To determine the amino acid residues in the TEM-1 β-lactamase active site critical for cephalosporin hydrolysis, saturation mutagenesis was used followed by a functional selection for active mutants. A set of random libraries that each contain all possible amino acid residue combinations for three to four contiguous codons that had previously been constructed (8, 9, 13). Eleven of these libraries that encompass all residues in or near the active site were used in this study (Fig. 1). Plasmid DNA from each library was transformed into E. coli XL1-Blue cells, and functional random mutants were identified based on their ability to confer high level bacterial resistance to cephaloridine using a disc diffusion method (see “Experimental Procedures”).

To identify those residue positions that are important for cephaloridine hydrolysis, the DNA sequence of the relevant region of the blaTEM gene was determined for 6 to 11 cephaloridine-resistant mutants selected from each random library (Fig. 1). A total of 30 residue positions examined from the 11 random libraries, 15 positions do not tolerate amino acid substitutions in that only codons representing the wild-type amino acid were found among functional mutants selected from these libraries including those positions. Those residues are inferred to be critical for cephaloridine hydrolysis and include: Pro-67, Met-69, Ser-70, Thr-71, Lys-73, Tyr-105, Ser-130, Asp-131, Asn-132, Arg-164, Ghu-166, Asn-170, Lys-234, Gly-236, and Gly-238 (Fig. 2). Among these residues, several (Ser-70, Lys-73, Ser-130, Ghu-166, and Lys-234) have previously been shown to
be essential for catalysis of β-lactam antibiotics (20). The remaining residues, Pro-67, Met-69, Thr-71, Tyr-105, Asp-131, Asn-132, Arg-164, Asn-170, Gly-236, and Gly-238 are presumably important for enzyme stability or active site conformation related to cephaloridine binding and hydrolysis (6, 20–22).

Fifteen residue positions in the active site region of β-lactamase are able to tolerate amino acid substitutions and maintain cephaloridine hydrolysis as indicated by multiple amino acid types at these positions among the sequenced functional mutants (Fig. 1). The residues that tolerate substitutions include: Met-68, Phe-72, Val-74, Val-103, Glu-104, Arg-161, Leu-162, Asp-163, Trp-165, Pro-167, Glu-168, Leu-169, Ser-235, Ala-237, and Glu-240 (Fig. 2). Substitutions identified at these residues may be neutral with respect to cephaloridine hydrolysis, indicating the position is not contributing to cephaloridine hydrolysis; alternatively, substitutions at these positions may increase the efficiency of cephaloridine hydrolysis, arguing the residue position is important for controlling the substrate specificity of the enzyme. An indication that substitutions at residues Trp-165, Pro-167, Ala-237, and Glu-240 may alter the substrate specificity of the enzyme is that a single amino acid type predominates among the selected functional mutants, and this amino acid is not the wild-type residue. For example, threonine is the predominant substitution at position 237 among functional mutants even though alanine is the wild-type residue at this position (Fig. 1). It is possible that threonine predominates among the selected mutants because the enzyme with threonine at position 237 hydrolyzes cephaloridine more efficiently than the wild-type enzyme.

Another means of identifying residues that, when mutated, alter the substrate specificity of the enzyme is to compare the patterns of substitutions from mutants selected for function on different antibiotics. A database of functional mutants previously selected for ampicillin hydrolysis allows for a comparison in sequence variability between amino acid substitutions seen in the functional mutants from the cephaloridine selection and those seen in the ampicillin selection (Fig. 3). In the comparison, 10 out of 30 residues tolerate no substitutions in either selection (Pro-67, Ser-70, Thr-71, Lys-73, Ser-130, Asp-131, Asn-132, Arg-164, Glu-168, Lys-234) (Fig. 2). The residues essential for catalysis are conserved among functional mutants selected with either ampicillin or cephaloridine. Also, another six residues (Met-68, Phe-72, Val-74, Pro-167, Glu-168, Ser-235) have similar patterns of substitutions in mutants from both selections. Therefore, these sixteen residues may have the same requirements for cephaloridine hydrolysis as for ampicillin hydrolysis.

As mentioned above, residues Trp-165, Pro-167, Ala-237, and Glu-240 exhibit a strong bias among cephaloridine selected mutants for an amino acid other than the wild-type residue. Of these residues, substitutions at Pro-167 display a strong bias for threonine among mutants selected for both ampicillin and cephaloridine hydrolysis. This suggests that threonine at position 167 is not specific for cephalosporin hydrolysis but rather effects a more general property of the enzyme, such as folding or stability. In this regard, it has been shown that proline isomerization of residue 167 is a rate-limiting step in the folding of wild-type β-lactamase and that substitution of this residue with a threonine increases the rate of folding (23). Still, the cis conformation of the peptide bond between residues 166 and 167, necessary for proper orientation of the catalytic residue Glu-166, is maintained with the P167T substitution (23). Interestingly, threonine is the second most common amino acid seen at position 167 in other naturally occurring class A β-lactamases (7). In contrast to the P167T substitution, the predominant substitutions at positions 165 (W165S), 237 (A237T), and 240 (E240C) were never found in the collection of functional mutants selected on ampicillin (8). This result suggests that these substitutions are cephaloridine-specific; that is, they result in wild-type or higher levels of cephaloridine hydrolysis but decreased efficiency of ampicillin hydrolysis since they are never observed in the ampicillin selection.

Kinetic Analysis—The hypothesis that W165S, A237T, and E240C alter the substrate specificity of the enzyme such that it discriminates more effectively between ampicillin and cephaloridine was tested by purifying the enzymes and performing kinetic analysis. Kinetic parameters were determined for two penicillins (ampicillin and benzylpenicillin) and three cephalosporins (cephaloridine, cephalothin, and cefotaxime) (Fig. 4). With cefotaxime as substrate, a high $K_m$ value (>1000 μM) prevented the individual values of $k_{cat}$ and $K_m$ from being determined; the $k_{cat}/K_m$ values were estimated by examining the reaction rate at [S] ≪ $K_m$ (19).

Kinetic analysis of the W165S enzyme revealed that its substrate specificity is not substantially different from the wild-type enzyme (Tables I). The catalytic efficiency ($k_{cat}/K_m$) was reduced 2- to 4-fold for all of the substrates tested. In addition, the W165S enzyme does not differ from wild-type in the ability to discriminate between cephalosporins and penicillins (Fig. 5). For example, the catalytic efficiency of the W165S enzyme with ampicillin as substrate is 25% of wild-type activity while it is 29% of wild-type activity with cephaloridine as substrate.

If the W165S enzyme does not exhibit increased hydrolysis of cephaloridine, why was serine the predominant substitution at position 165 in the selection with cephaloridine as substrate? It is possible that an insufficient number of sequences of functional mutants were determined. If more sequences had been
obtained, the serine substitution may not have predominated. Alternatively, the random library may be biased for codons containing serine at position 165. This possibility is unlikely in that serine was never observed at position 165 among functional mutants selected from the same library with ampicillin as substrate (8).

The kinetic data clearly indicate the A237T enzyme has altered substrate specificity. The enzyme exhibits a 2-fold increase in catalytic efficiency with cephaloridine as substrate and a 5-fold decrease in efficiency with ampicillin as substrate (Table I). The increase in $k_{cat}/K_m$ for cephaloridine is completely accounted for by a 2-fold reduction in $K_m$ (Table I). In contrast, the reduction in catalytic efficiency with ampicillin as substrate is due almost entirely to a decrease in $k_{cat}$ (Table I). The observed increase in catalytic efficiency appears to generalize to other cephalosporins since the enzyme exhibited an increase in catalytic efficiency for all cephalosporins tested. In addition, the enzyme exhibited a decrease in catalytic efficiency with benzylpenicillin as substrate similar to that seen with ampicillin. Therefore, the A237T enzyme is more of a cephalosporinase than the wild-type enzyme (Fig. 5).

The E240C enzyme also has an altered substrate specificity (Fig. 5). The enzyme displays wild-type levels of catalytic efficiency with cephaloridine, but has a 10-fold reduc-

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**FIG. 3.** Comparison of sequence variability of active site mutants. The wild-type TEM-1 β-lactamase sequence for each region is shown in the center row. Mutants were selected for at least wild-type level or greater hydrolytic activity against cephaloridine (top) or ampicillin (bottom). The ampicillin selection data is from Huang et al. (8; and C. Cantu, III, W. Huang, and T. Palzkill, unpublished data.)

**FIG. 4.** Structures of β-lactam antibiotics used in this study.
specificity determinants of \( \beta \)-lactamase. The cephalosporinase selection data set (Fig. 1) was then used to compare and contrast the sequence preferences of functional mutants selected for cephalosporin hydrolysis with those previously selected for ampicillin hydrolysis (8) and unpublished results). These two antibiotics are representatives of the cephalosporin and penicillin classes of \( \beta \)-lactam antibiotics, respectively. Therefore, differences seen in the sequence comparison will reflect not only critical interactions required by the different antibiotic side groups, but also those interactions required by the different ring structures of penicillins and cephalosporins.

16 out of 30 residues examined in this study display similar sequence requirements for both cephalosporin and ampicillin hydrolysis (Fig. 3). The similarity indicates that the interactions required of these residues are identical for both classes of \( \beta \)-lactams, irrespective of the differences in structure for each antibiotic class. The remaining 14 residues exhibit little sequence variation among the functional mutants from the cephalosporin selection despite the fact that many different amino acid types were observed at the same residue positions in functional mutants from the ampicillin selection (Fig. 3). For example, no substitutions were found at residue Gly-236 among the mutants selected for cephalosporin resistance. It is thought that any substitution at residue Gly-236, which is invariant in the class A \( \beta \)-lactamase family, would introduce steric conflicts with Ser-70 (20), yet the TEM-1 enzyme is able to tolerate a glycine to alanine substitution and retain wild-type levels of ampicillin hydrolysis. Another example of the difference in sequence variation involves residues 69 and 238. In class A \( \beta \)-lactamases, excluding extended-spectrum \( \beta \)-lactamases, an inverse correlation exists between the sizes of the side chains of residue 69 and residue 238. For instance, while the \( E. coli \) TEM-1 \( \beta \)-lactamase has the large methionine at position 69 and small glycine at position 238, the \( Streptomyces cacaoi \) ULg \( \beta \)-lactamase has the small glycine at position 69 and the large valine at position 238 (24). Because both residues are involved in positioning of the B3 \( \beta \)-strands and formation of the oxyanion pocket, a substitution at one residue without a compensating substitution at the other residue could introduce steric conflicts that would alter the \( \beta \)-lactam binding configuration. Both residues are absolutely conserved in the functional mutants from the cephalosporin selection, yet several amino acid types are observed at each residue among the functional mutants from the ampicillin selection. Thus, the decreased number of substitutions seen in the functional mutants from the cephalosporin selection suggests a greater restriction on the enzyme in maintaining cephalosporinase activity versus penicillinase activity. Because these conserved residues are involved both in substrate binding and proper positioning of catalytic residues, their conservation only with respect to cephalosporin hydrolysis may reflect different interactions required by the dihydrothiazine ring of cephalosporins than by the thiazolidine ring of penicillins.

Mutational studies have demonstrated that substitutions at residues Ser-130 and Ser-235 result in a significant decrease in cephalosporinase activity, and yet do not greatly alter penicillinase activity (25, 26). Each study proposed that the loss of a hydrogen bond to the substrate carboxylate from the serine side chain was detrimental only for cephalosporin hydrolysis.
The conclusion from these studies was that the hydrogen bond network in the active site is developed to such an extent for penicillinase activity that the enzyme can afford the mutational loss of one or two hydrogen bonds and retain function. For example, it was proposed that the S130G mutation was compensated for in penicillin hydrolysis by a hydrogen bond to the substrate carboxylate from Arg-244 that is shorter (stronger) for penicillins than for cephalosporins (26). Therefore, the substrate carboxylate from Arg-244 that is shorter (stronger) can be compensated for in penicillin hydrolysis by a hydrogen bond to the side chain of the substrate and residue 240 faces outward. The TEM enzyme has more interactions with penicillin, cephaloridine, cephalothin, and cefotaxime than for cephalosporins (26). Therefore, the substrate carboxylate from Arg-244 that is shorter (stronger) can be compensated for in penicillin hydrolysis by a hydrogen bond to the side chain of the substrate and residue 240 faces outward. The conclusion from these studies was that the hydrogen bond network in the active site is developed to such an extent for penicillinase activity that the enzyme can afford the mutational loss of one or two hydrogen bonds and retain function.

A comparison of the binding of penicillins and cephalosporins in the DD-peptidase binding site (27), showed that hydrogen bonding to the carboxylate of the respective 5- or 6-member ring of each class of substrate was stronger for the cephalosporins due to a closer proximity of threonine residues at position 299 and 301 (residues Ser-235 and Ala-237 on the B3 β-strand of TEM-1 β-lactamase). Previous studies with TEM-1 have shown the ability of the A237T substitution to enhance cephalosporinase activity of TEM-1 by introducing another hydrogen-bonding group in the active site. The A237T substitution was isolated in 1976 by Hall and Knowles in a selection screen for cephalosporinase activity (30).

Although the TEM-1 enzyme does not tolerate amino acid substitutions at several residue positions with cephaloridine as substrate as compared with ampicillin, there are specific substitutions that decrease cephaloridine hydrolysis and that increase or do not affect cephaloridine hydrolysis. Based on the sequence results, it was found that individual substitutions of serine for tryptophan at position 165, threonine for alanine at 237, and cysteine for glutamic acid at 240 were cephaloridine specific. To better understand the role of each identified substitution in altering specificity, the contributions of each single substitution as well as the A237T:E240C and W165S:A237T:E240C substitutions at several residue positions with cephaloridine as substrate were determined with ampicillin, benzylpenicillin, cephaloridine, cephalothin, and cefotaxime as substrates.

Characterization of these enzymes for the cephalosporins indicate that the A237T and E240C single and combinatorial mutants either maintain or improve the $K_m$ and/or $k_{cat}$ values for the cephalosporins resulting in $k_{cat}/K_m$ values equal to or greater than wild-type (Table I). As expected, the $k_{cat}/K_m$ values of these mutants are decreased for the penicillins. The W165S mutant showed no distinction in its effect on catalytic activity and lowered the $k_{cat}/K_m$ values for all substrates.

A comparison of the binding of penicillins and cephalosporins in the DD-peptidase binding site (27), showed that hydrogen bonding to the carboxylate of the respective 5- or 6-member ring of each class of substrate was stronger for the cephalosporins due to a closer proximity of threonine residues at position 299 and 301 (residues Ser-235 and Ala-237 on the B3 β-strand of TEM-1 β-lactamase). Previous studies with TEM-1 have shown the ability of the A237T substitution to enhance cephalosporinase activity of TEM-1 by introducing another hydrogen-bonding group in the active site to anchor the carboxylate group of the dihydrothiazine ring of a cephalosporin (28, 29). In fact, the A237T substitution was first isolated in 1976 by Hall and Knowles in a selection screen for TEM mutants with increased cephalosporin C hydrolytic activity (30).
into solvent makes it difficult to understand how the E240C enzyme is establishing any specific interaction that results in greater discrimination between cephalosporins and penicillins. In summary, a sequence comparison between functional mutants selected for ampicillin hydrolysis (8) and cephaloridine hydrolysis indicates that amino acid substitutions at many positions in the active site of TEM-1 \(\beta\)-lactamase are detrimental for cephaloridine hydrolysis but not for ampicillin hydrolysis. The limited tolerance to amino acid substitutions in the active site suggests that \(\beta\)-lactamase has greater restrictions in maintaining cephalosporinase activity than in maintaining penicillinase activity. In addition, screening of the active site for functional mutants with substitutions that provide wild-type or greater levels of hydrolysis for cephaloridine revealed that the A237T and E240C substitutions, alone or in combination, exhibit increased cephalosporinase activity and decreased penicillinase activity relative to the wild-type enzyme.

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