Modifying the Specificity and Activity of the *Enterobacter cloacae* P99 β-Lactamase by Mutagenesis within an M13 Phage Vector

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ABSTRACT: A library of *Enterobacter cloacae* P99 β-lactamase mutants was produced to investigate the importance of residues 286−290 for substrate binding and catalysis and to characterize mutants with altered specificities and activities for various 3′-substituted cephalosporins. This region of the enzyme is a component of the active site that has not been implicated as participating in the catalytic mechanism but, based on molecular modeling, should contact the 3′ substituents of cephalosporins. Random mutagenesis was carried out within an M13 phage vector by hybridization mutagenesis, and the phage library could be highly enriched for active β-lactamase genes by incubation of infected bacteria with β-lactam antibiotics. The mutants were characterized by Michaelis−Menten kinetic analyses with several cephalosporin substrates and spanned a 25-fold range of k_{cat}, 24-fold range of K_m, and 6-fold range of k_{cat}/K_m values. All five amino acid positions were found to be permissive to substitution, but the active mutant proteins carried substitutions that likely maintained the structure of the region. Serine 287 was the least permissive to change, requiring small, uncharged residues for retention of catalytic activity. The variation of Michaelis−Menten kinetic parameters observed in these enzymes was shown to be significant in the context of *in vitro* cytotoxicity assays with the cephalosporin−doxorubicin prodrug C-Dox and is suitable for experiments to probe the relationship between enzyme kinetics and efficacy in enzyme−prodrug approaches to targeted therapy.

β-Lactamases (EC 3.5.2.6) are bacterial enzymes that carry out the degradation of β-lactam antibiotics. They function by breaking the amide bond of the β-lactam ring, irreversibly destroying antibiotic activity (Figure 1). The dissemination of the genes for these enzymes throughout the bacterial kingdom (often plasmid borne) over the past few decades has seriously compromised the efficacy of β-lactam antibiotic therapy for bacterial infections (Norrby, 1992; Urban *et al*., 1994). β-Lactamases have been classified on the basis of sequence and structure and also by their ability to hydrolyze particular cephalosporin, penicillin, or carbapenem substrates (Ambler, 1980; Bauernfeind, 1986; Bush, 1989a,b). Structurally, the enzymes have been grouped into four classes, A−D. Classes A, C, and D (Ledent *et al*., 1993) employ a catalytic serine hydroxyl group as the nucleophilic agent that breaks the β-lactam ring, while the class B β-lactamases contain zinc at the active site (Felici *et al*., 1993).

Class A enzymes have been the most thoroughly studied β-lactamases and are termed penicillinases due to their ability to efficiently hydrolyze penem and penam antibiotics. Both evolution and directed mutagenesis studies have demonstrated the ability of these ≅30 kDa enzymes to broaden their substrate spectra and have provided insight into the mechanism of hydrolysis [for review, see Matagne and Frère (1995)]. Mechanistic studies have been complemented by several X-ray structures of these enzymes and their inhibitor complexes (Herzberg & Moult, 1987; Jelsch *et al*., 1993; Strynadka *et al*., 1992), as well as molecular modeling studies (Lamotte-Brasseur *et al*., 1991).

Class C enzymes are not characterized as well as their class A counterparts (Galleni *et al*., 1988a; Joris *et al*., 1985, 1986; Nukaga *et al*., 1994; Tsukamoto *et al*., 1992). These cephalosporinases are larger (40 kDa) and display only 30% similarity to the A class enzymes. They are usually encoded chromosomally in Gram-negative bacteria (*Escherichia coli*, *Citrobacter freundii*, *Enterobacter cloacae*, etc.), although plasmid-borne members of this class have been reported (Fosberry *et al*., 1994). The reported X-ray structures of class C β-lactamases have shown that, along with the larger size, there are significant differences in the arrangement of secondary structure elements when compared to the class A enzymes, although the geometries of the active sites are very similar (Lobkovsky *et al*., 1993, 1994; Oefner *et al*., 1990). Frère and co-workers have undertaken the majority of mutational studies, producing point mutations to probe the substrate binding and reaction mechanism of the *E. cloacae* enzymes (Dubus *et al*., 1994; Jacobs *et al*., 1992). Mutation at K67, proximal to the active site S64, greatly decreased catalytic activity, although the K67R mutant showed increased activity toward some poor substrates of the wild-type enzyme (Monnaie *et al*., 1994a). K315 was shown to be necessary for efficient catalysis (Monnaie *et al*., 1994b). S64 was mutated to cysteine, resulting in decreased enzymatic activity and an apparent shift of the rate-limiting step of catalysis from deacylation to initial acylation (Dubus *et al*., 1993).

We have previously studied the *E. cloacae* P99 β-lactamase because this enzyme can efficiently cleave cephalosporin prodrugs such as the cephalosporin−doxorubicin conjugate C-Dox (Figure 1) (Vrudhula *et al*., 1993). Hydrolysis of cephalosporin β-lactam rings leads to a secondary elimination reaction, resulting in expulsion of the 3′ sub-

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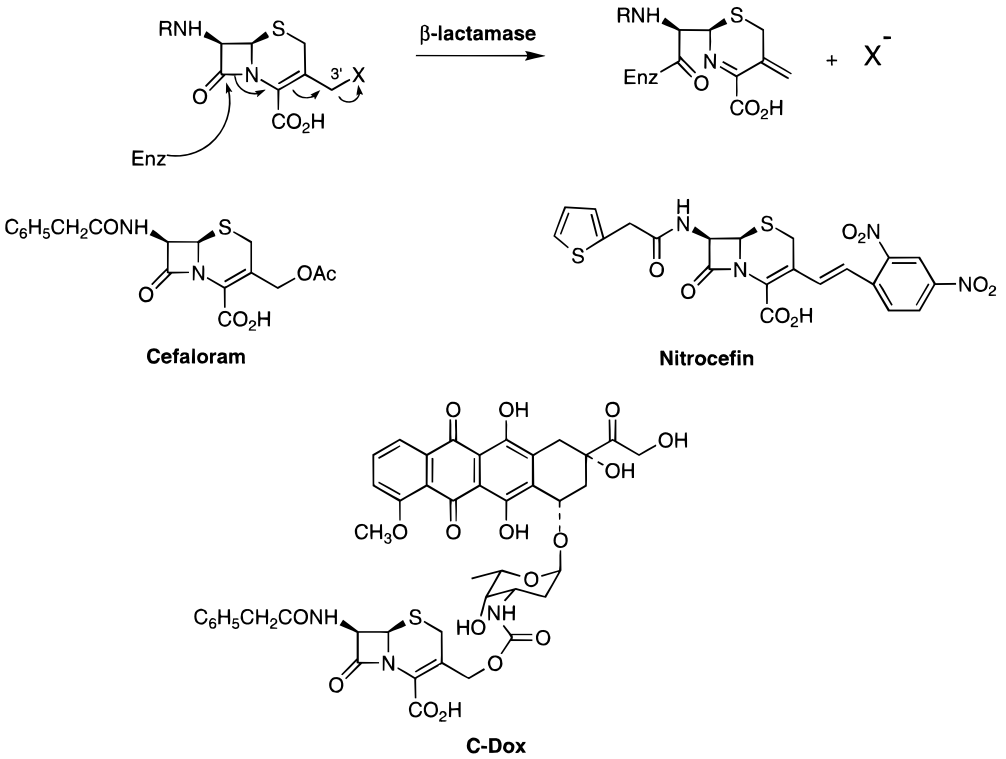

FIGURE 1: Simplified mechanism of β-lactamase-mediated hydrolysis of β-lactams and structures of cephalosporin substrates. β-Lactam cleavage results in expulsion of any 3′ leaving group, producing acetate (cefaloram) or doxorubicin (C-Dox).

stituent of the molecule. In the case of C-Dox, this elimination releases doxorubicin, a cytotoxic compound used in cancer chemotherapy. This strategy is also used in the design of bifunctional antibiotics for treatment of bacterial infections (Albrecht *et al*., 1991), where destruction of the β-lactam ring by resistant bacteria will release a second (usually fluoroquinoline) antibiotic.

β-Lactamase-mediated release of cytotoxic agents from cephalosporin prodrugs (along with other enzyme−prodrug combinations) has been used in two-step approaches to cancer therapy (Bagshawe *et al*., 1991; Senter *et al*., 1993). Antitumor antibody β-lactamase conjugates, administered systemically, have been shown to localize preferentially to tumors in animal models of human cancer. Following tumor localization of the antibody−enzyme conjugate, a cephalosporin prodrug is administered. Under the action of the β-lactamase the prodrug is cleaved, resulting in tumor-selective delivery of chemotherapeutic agents.

The *E. cloacae* P99 β-lactamase displays versatility and very high activity toward hydrolysis of cephalosporins and cephalosporin prodrug substrates. However, ideal kinetic parameters for an enzyme used in prodrug release are currently not known (Baxter *et al*., 1992). A conclusive way to address the issue of enzyme kinetics vs efficacy would be to produce and compare a panel of enzymes that are nearly identical except for kinetic parameters.

In this study, the P99 β-lactamase residues 286−290 (GSDSK) were subjected to random mutagenesis using an M13 phage display vector. This region of the enzyme, a helix strand connection proximal to the active site, is the only component of the active site that has not been identified as participating in the catalytic mechanism. We felt this region could participate in the formation of a secondary substrate binding site, since it is expected to intimately contact cephalosporins with large 3′ substituents. The goal of the present study was twofold: (A) to determine if mutations at positions 286−290 affect enzyme specificity for 3′-substituted cephalosporins and (B) to obtain a panel of β-lactamase mutants with various kinetic parameters, in order to study their effects on the efficacy of prodrug release in cancer therapy.

MATERIALS AND METHODS

General. The *E. cloacae* P99 β-lactamase gene was obtained from plasmid pNU363 (Galleni *et al*., 1988b). Polymerase chain reactions were performed with the recombinant Pfu polymerase (Stratagene) in a Perkin-Elmer thermal cycler, using the supplied buffer with 10% added dimethyl sulfoxide under hot start conditions (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min; 25 cycles). Creation of the mutagenesis library was performed with materials from the Bio-Rad Laboratories M13 mutagenesis kit, using bacterial strain CJ236 for isolation of uracil-containing DNA. Isolation of DNA from agarose gels utilized Gel-Ase (Epicentre Technologies) digestion of the agarose followed by ethanol precipitation. Transfections were accomplished by electroporation in a Bio-Rad Laboratories Gene Pulser. Selection rounds, propagation of libraries, plating of phage, and protein expressions were all performed with XL1-Blue bacteria in LB[1] or 2×YT media. Oligodeoxynucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer using cyanoethyl phosphoramidites on a 0.2 μmol scale. The antibody 7F11 to the decapeptide tag YPYDVPDYAS was kindly provided by G. Valkirs (Biosite Diagnostics), and an alkaline phosphatase conjugate of the antibody was used for nitrocellulose filter lift assays (Huse *et al*., 1992).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria−Bertani media; PBS, 40 mM sodium phosphate and 0.15 M NaCl, pH 7.4.

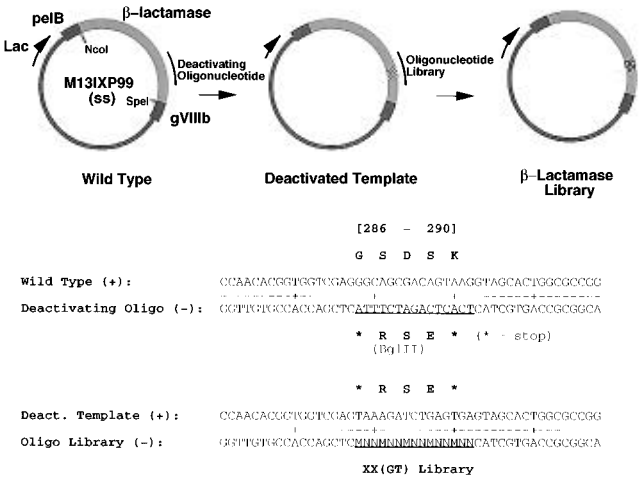

FIGURE 2: The M13IXP99 vector and outline of the procedure for library creation. The *E. cloacae* β-lactamase gene is fused to the pelB leader sequence, to drive export of enzyme into the periplasmic space of *E. coli*. The 3′ end of the β-lactamase gene is fused to a short sequence encoding the decapeptide YPYDVPDYAS (not shown), followed by fusion to a second M13 gVIII coat protein gene. Mutagenesis was performed by M13 hybridization, first with an oligonucleotide producing a crippled β-lactamase template (containing two stop codons in the mutagenesis region). The oligonucleotide library was then hybridized onto the uracil-containing deactivated template.

ELISA analyses were performed by coating 96-well plates with a 1 μg/mL solution of a rabbit polyclonal antisera raised to the P99 β-lactamase. After being blocked with specimen diluent (Genetic Systems Corp.), samples were applied in specimen diluent, followed by 1 μg/mL biotinylated antisera in conjugate diluent (Genetic Systems Corp.), and Vectastain avidin-HRP reagents (Vector Laboratories Inc.).

Construction of the M13IXP99 Vector. Construction of the M13IXP99 vector was accomplished by restriction digest of M13IXBR96 and ligation of the β-lactamase gene into *Spe*I and *Nco*I sites. M13IXBR96 contains the Fab fragment of the antitumor antibody BR96 and is based on the M13 phage display vectors M13IX31 and M13IX12 (Huse *et al*., 1992). Briefly, this M13 vector contains a second, modified gVIII encoding the mature gVIII coat protein linked via a 5′ amber stop codon to nucleotides encoding the decapeptide YPYDVPDYAS, followed by the inserted gene of interest (Figure 2). The vector contains the Lac operon, which allows control of expression with IPTG, and the 5′ end of the inserted gene is fused to the PelB leader, directing export of the protein to the periplasmic space of *E. coli*. PCR amplification of the *E. cloacae* P99 β-lactamase gene was performed with forward primer 5′-CATGGCCACGCCAGTGTCAGAAAAACAGCTGGCG-3′ and reverse primer 5′-ACATCAACTAGTCTGTAGCGCCTCGAGGATATG-3′.

Library Creation. An inactivated M13IXP99 vector with an added *Bgl*II restriction site and two stop codons in the mutagenesis region was prepared as starting template for mutagenesis. Single-stranded uridinylated M13IXP99 DNA was annealed with the phosphorylated oligonucleotide 5′-ACGGCGCCAGTGCTACTCACTCAGATCTTTACTCGACCACCGTGTTGG-3′ (noncomplementary region is underlined). The mutant library was prepared by annealing the deactivated single-stranded template to a phosphorylated oligonucleotide library, 5′-ACGGCGCCAGTGCTACMNNMNNMNNMNNMNNCTCGACCACCGTGTTGG-3′. Extension, ligation, and electroporations into electrocompetent DH10B cells (Gibco BRL) were followed by the immediate addition of an overnight XL1-Blue culture (200 μL per electroporation). The electroporation cultures were combined, and a small sample was taken for plaque counting. The rest of the mixture was split into 20 portions, and each portion was incubated in LB media (4 mL, 4 h, 37 °C) with shaking. The cultures were combined and centrifuged, and the supernatant was collected.

Cefaloram Selection of Clones. A log phase XL1-Blue culture (OD_{660} 0.7−1) was diluted (1:10) into LB media (5 mL) in six-well tissue culture plates. The culture was infected with portions of the phage library (multiplicity of infection $\cong$ 1). The infected culture was incubated without shaking (45 min, 37 °C), and cefaloram was added (200 μg/mL). After further incubation (40 h, 37 °C, 100 rpm), the cells were removed by centrifugation, and the selected phage library was obtained from the supernatant (stored at 4 °C).

Protein Expression. A log phase XL1-Blue culture (OD_{660} 0.7−1) was diluted (1:30) into 2 × YT media in 4 L baffled culture flasks (1 L maximum volume per flask). Cells were infected with phage (multiplicity of infection $\cong$ 1) and incubated with shaking (5 h, 37 °C). IPTG (1 mM) was added, and the culture was incubated with shaking (overnight, 37 °C). Cells were pelleted by centrifugation and resuspended in 30 mM Tris-HCl, pH 8.0, 2 mM EDTA, 20% sucrose, and 1 mg/mL lysozyme (1/50 initial culture volume) while being cooled in an ice bath (15−20 min). The supernatant was collected by centrifugation, filtered through a 5 μm filter, and dialyzed against PBS (1−2 L, two buffer changes).

The crude periplasmic preparation was applied to an agarose−phenylboronate affinity column (Cartwright & Waley, 1984) at a flow rate of 0.5 mL/min. The column was washed with 10 column volumes of 20 mM triethanolamine hydrochloride and 0.5 M NaCl, pH 7, and the enzyme was eluted with 0.5 M sodium borate and 0.5 M NaCl, pH 7. Fractions containing β-lactamase activity were pooled and dialyzed against PBS (1 L, two buffer changes). Yields of β-lactamase (determined by UV absorbance, λ = 280−320 nm, ϵ = 25 800 M^{-1}) ranged from 0.05 to 3 mg/L of culture. Purity was assessed by SDS−PAGE and TSK-G200SWXL size exclusion HPLC (1 mL/min flow rate, PBS−0.02% azide solvent).

Kinetics. Nitrocefin hydrolysis (O'Callaghan *et al*., 1972) was measured in a microtiter plate assay at 22 °C. β-Lactamase samples were serially diluted (1:4) into 100 μL of PBS containing bovine serum albumin (0.1 mg/mL), followed by addition of 100 μM nitrocefin (100 μL) in PBS/1% dimethylformamide. Absorbance measurements (λ = 490−630 nm) were taken every 60 s with an EL 312 BioKinetics Reader (Bio-Tek Instruments, Inc.), and the results were plotted to calculate the slope of each line. Enzymatic hydrolysis rates of other substrates were measured by observing the change in absorbance (C-Dox, λ = 230 nm, $\Delta\epsilon$ = 9310/M; cefaloram, λ = 266 nm, $\Delta\epsilon$ = −6360/M) over time in a UV spectrophotometer, using 50−100 ng of β-lactamase in 0.8 mL of PBS at 22 °C.

Michaelis−Menten kinetic parameters were obtained by analysis of progress curves from a concentration of 20−200 μM (cefaloram), 10−50 μM (C-Dox), or 5−100 μM (nitrocefin, λ = 490 nm, $\Delta\epsilon$ = 19 500/M) at 37 ± 0.3 or 22 ± 0.3 °C using a water-jacketed UV cell holder and Haake water bath to maintain sample temperature. Absorbance

readings were taken every 6 s for the duration of the assay. The concentration of substrate at each point was determined by calculating up from the absorption reading at the end of hydrolysis. Hydrolysis activity was calculated at each point by taking the slope of the three surrounding data points. The resulting set of substrate concentrations and hydrolysis rates was plotted in Eadie−Hofstee fashion, and a linear regression afforded V_{max} and K_m parameters.

Cell Cytotoxicity Assays. H2981 cells (8000 cells/0.1 mL) (Hellström *et al*., 1986) in IMDM/FBS (Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, v/v, 0.1 mg/mL streptomycin, and 60 μg/mL penicillin G) were plated into 96-well tissue culture plates and incubated for 24 h at 37 °C. The cells were washed three times with RPMI/FBS (Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, v/v), followed by treatment with 1 μM C-Dox and various amounts of β-lactamase enzymes in 200 μL RPMI/FBS (37 °C, 60 min). The cells were washed three times with IMDM/FBS and allowed to incubate overnight at 37 °C. The cells were then pulsed with [^{3}H]thymidine (1 μCi per well, 6 h), washed with PBS, detached with a trypsin/EDTA solution, harvested onto filtermats (TOMTEC Harvester 96), and counted on a LKB WALLAC 1205 liquid scintillation counter.

RESULTS

Selection of β-Lactamases in a Phage Expression Vector. At the outset of this study, it was critical to determine if antibiotic selection would function in a system where the β-lactamase resistance gene was carried by an M13 phage rather than a plasmid. To determine this, the wild-type P99 β-lactamase gene was inserted into an M13 phage vector and control studies were performed to assess the selective propagation of phage containing the β-lactamase gene (M13IXP99, Figure 2). The prodrug substrate C-Dox is inappropriate for selection, as the products of hydrolysis (doxorubicin) is also highly toxic to bacteria. The antibiotic cefaloram (containing an acetate at the 3′ position) was therefore used for selection. Dilutions (1:10^3, 1:10^6) were made of M13IXP99 into M13 phage containing no insert. XL1-Blue bacteria were infected with these mixtures of phage, followed by incubation with various amounts of cefaloram. After a single incubation with cefaloram at 50−200 μg/mL, 1:10^3 dilutions of M13IXP99 were converted to 100% M13IXP99. Plated phage from these mixtures could be conveniently analyzed for β-lactamase activity by overlaying a solution of 0.5 mM nitrocefin (plaques containing active β-lactamase turn red); 1:10^6 mixtures submitted to two rounds of this procedure afforded a final phage mixture that was again 100% M13IXP99. Thus, antibiotic selection is powerful in this system, and successive rounds offer additional selective power.

Library Creation. When creating the mutant library, we wanted to eliminate competition from disproportionate amounts of wild-type lactamase in the library of phage. To do this, we prepared a deactivated M13IXP99 template that contained stop codons in the target region for mutagenesis. Hybridization of the oligonucleotide library (3.2 × 10^6 possible codon degeneracies) onto this deactivated template, followed by polymerase extension and ligation, gave double-stranded M13 DNA, which was introduced into DH10B bacteria by electroporation. A total of 3.5 × 10^6 phage were obtained, 30% of which contained template sequence. This yields 2.5 × 10^6 transfectants containing mutant sequences, with a 53% probability of any particular mutant being present in the library (the total number of transfectants needed to include the entire library with 95% confidence would be 9.6 × 10^6). A total of 22% of the phage in this initial library produced β-lactamases with detectable activity toward nitrocefin, suggesting that many substitutions in the mutated region of the enzyme were permitted.

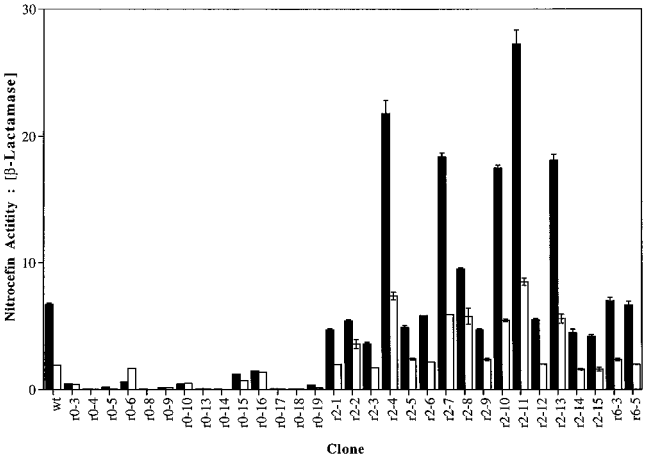

FIGURE 3: Nitrocefin activity (black bars, 50 μM substrate, 22 °C) and β-lactamase concentrations (white bars) in periplasmic preparations of infected bacteria. The scale for nitrocefin activity is in micromoles per minute; the β-lactamase concentrations (determined by ELISA) are given in micrograms per milliliter. The error bars indicate standard deviations of the measurements.

Selection and Analysis of Active Enzymes from the Library. The original phage library was submitted to a total of six successive rounds of selection with cefaloram. Individual library members before and after selection were analyzed by measuring the β-lactamase activity of 1 mL periplasmic preparations of cultures infected with the phage. Screening of phage from the original library with the nitrocefin substrate (50 μM, 22 °C) suggested that while many of the clones expressed active β-lactamases, the average level of activity was only 5% that of wild type incubated under the same conditions (Figure 3). Measurement of β-lactamase concentrations in the periplasmic preparations by an anti-P99 EIA revealed that these differences in activity were partly due to decreased activities of the enzymes and partly due to lower levels of expression or transport into the periplasmic space of *E. coli*.

Submitting the original library to antibiotic selection with cefaloram greatly increased the activity of the library (Figure 3 and Table 1). By the second round of selection, β-lactamase activity from mutant clones had increased to become roughly comparable to wild-type activity toward nitrocefin and C-Dox, in terms of both absolute activities of the enzyme preparations and specific activities (only the enzymes from the unselected library that had both expression and activity levels above background were included in Table 1). In the case of C-Dox, several enzymes were modestly higher in activity than wild type. Comparing the relative activity of these enzymes to hydrolyze C-Dox vs nitrocefin indicated that there was a 10-fold range of selectivity for C-Dox vs nitrocefin among these enzymes (Table 1). Clone r0-6 (the sixth clone from the unselected library) displayed a 5.9-fold

Table 1: Nitrocefin and C-Dox Hydrolysis Activity of Periplasmic Preparations[a]

clone	nitrocefin specific activity	C-Dox specific activity	ratio of C-Dox/nitrocefin activity (normalized)
wt	355 (14)	238 (11)	1.00
r0-3	118 (2.4)	132 (36)	1.67
r0-6	35.2 (1.2)	140 (3.1)	5.89
r0-10	88.2 (2.3)	242 (27)	4.09
r0-15	175 (1.9)	122 (25)	1.03
r0-16	109 (4.5)	58.3 (9.8)	0.79
r2-1	236 (8.4)	306 (11)	1.93
r2-2	150 (15)	131 (13)	1.30
r2-3	207 (9.3)	179 (14)	1.28
r2-4	295 (17)	201 (13)	1.01
r2-5	203 (9.4)	209 (26)	1.53
r2-6	271 (3.5)	353 (46)	1.94
r2-7	310 (5.3)	239 (2.4)	1.15
r2-8	164 (18)	153 (20)	1.38
r2-9	199 (8.5)	246 (18)	1.84
r2-10	321 (7.7)	190 (11)	0.88
r2-11	321 (16)	213 (8.1)	0.99
r2-12	274 (5.9)	153 (16)	0.83
r2-13	325 (22)	218 (20)	1.00
r2-14	279 (24)	268 (16)	1.43
r2-15	261 (27)	185 (28)	1.06
r6-3	298 (19)	248 (16)	1.24
r6-5	337 (16)	135 (8.4)	0.59

[a] All assays were performed at 22 °C with 50 μM substrate. Specific activity is reported in μmol/(min·mg). Standard deviations are given in parentheses and were calculated by the equation $S = [(S_{activity}^2/[substrate]^2) + ((activity^2 \times S_{[substrate]}^2)/[substrate]^4)]^{1/2}$. The members of the phage library have been named r*x*-*y*, indicating that the clone is the *y*th pick of the library that was submitted to *x* rounds of selection.

Table 2: Sequences of Inactive β-Lactamase Mutants[a]

clone	286	287	288	289	290
I-1	AAG	TAT	GAT	AAT	CCT
	K	Y	D	K	P
I-2	GTT	CGG	AGT	GTG	AGT
	V	R	S	V	S
I-3	TGT	AAG	TTT	GTT	TGT
	C	K	F	V	C
I-4	ATT	AAT	CCT	CGG	CTT
	I	N	P	R	L

[a] The periplasmic preparations of these enzymes were found to possess no nitrocefin hydrolysis activity above the background level of the measurement (0.4% of wild-type activity).

greater preference for C-Dox than nitrocefin (relative to wild type), while clone r6-5 (the fifth clone from the sixth round of selection) displayed a 1.7-fold preference for nitrocefin. Thus, the mutations in this region alter the selectivity profiles of the β-lactamase enzymes.

In addition to selecting for the high-activity enzymes in the library, we also wanted to identify which amino acid substitutions lead to inactive β-lactamases. Plaque lifts on plated phage from the original library were probed with a monoclonal antibody to determine which were expressing full-length protein, including the C-terminal decapeptide recognized by the antibody. A total of 17% of the phage expressing P99 exhibited undetectable (<0.4% of wild type) activity toward nitrocefin and were isolated for sequence analysis (Table 2).

Kinetic Parameters. Michaelis−Menten kinetic analysis of the mutant β-lactamases showed a range of k_{cat}, K_m, and k_{cat}/K_m values toward the cefaloram, C-Dox, and nitrocefin substrates (Table 3). The least active enzymes isolated were from the unselected library. Two enzymes, r2-1 and r6-3, were the most active toward C-Dox. However, because of the higher K_m of r6-3, only the r2-1 enzyme showed a significantly higher k_{cat}/K_m ratio than wild type. The average k_{cat} for cefaloram hydrolysis among enzymes submitted to the selection procedure was 1.65 times that of wild type, while the K_m values also increased to 1.91 times the wild-type value. For C-Dox and nitrocefin, the average k_{cat} and K_m values were roughly equivalent to wild-type parameters.

Sequences of Mutant β-Lactamases. Sequencing revealed that no residue was strictly conserved in the mutant β-lactamases but that several trends were present in those that were submitted to the selection procedure (Table 3). Residue 286 (G in wild type) almost always remained uncharged but was often replaced with somewhat larger residues, such as leucine, threonine, and valine. Residue 287 (S in wild type), when mutated, remained uncharged and replaced with a residue of similar size. The charged residues D288 and K290 retained charge in 67% of the observed mutations in the selected libraries. S289N mutations occur in 4/9 of the selected mutants. The above trends were absent in the inactive mutant enzymes isolated from the original library (Table 2). Among the full-length proteins having no detectable activity, the major difference in sequence from the active mutants was the presence of larger residues at S287, such as S287R and S287N mutations.

Activity of β-Lactamase Mutants in Enzyme/Prodrug-Mediated Cytotoxicity. β-Lactamases possessing a range of activities were tested in an *in vitro* assay of DNA synthesis inhibition due to release of doxorubicin by the enzymes' hydrolysis of C-Dox (Figure 4). H2981 lung adenocarcinoma cells were incubated for 1 h with a subcytotoxic concentration of C-Dox and varied amounts of the β-lactamase enzymes. Enzyme from r2-1, having both a higher k_{cat} and lower K_m than wild type, was significantly more potent than wild type in inhibiting thymidine uptake by cells treated with β-lactamase and C-Dox. The low-activity enzyme from r0-6 displayed a corresponding lower potency. Finally, the enzyme from r6-3, having a higher k_{cat} but also a higher K_m, showed comparable potency to wild type under these sub-K_m conditions. The IC_{50} values of these enzymes span a 9-fold range of β-lactamase concentrations. The results are consistent with the kinetic characteristics determined by Michaelis−Menten analysis and demonstrate that the activity differences translate into modulation of cytotoxicity.

DISCUSSION

Phage display systems have been highly successful for screening libraries of antibody fragments, small peptides, and other proteins that bind to target ligands (Crameri *et al*., 1994; Glaser *et al*., 1992; Huse *et al*., 1992; Kishchenko *et al*., 1994; Roberts *et al*., 1992). Successive rounds of panning with ligand or other affinity methods can identify active agents displayed on filamentous phage in libraries larger than 10^{10} members. Recently, the gene for the TEM-1 β-lactamase has been placed in a phage display vector, along with a crippled TEM-1 mutant lacking the catalytic serine (Soumillion *et al*., 1994). These workers demonstrated the ability to pan a mixture of the two phages, enriching by approximately 50-fold at each step, by trapping active TEM-1 phage with an affinity matrix containing a suicide inhibitor of β-lactamase.

Table 3: Sequences (Residues 286−290) and Michaelis−Menten Kinetic Parameters of Selected β-Lactamases[a]

clone	sequence 286−290	cefaloram			C-Dox			nitrocefin		
		k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m
wild type	GSDSK	285 (35)	87 (8)	3.3	383 (23)	58 (12)	6.9	439 (14)	31 (0.2)	14.2
r0-6	ASARR	nd[c]	nd[c]	nd[c]	74 (11)	28 (8)	2.6	21 (0.1)	10 (0.4)	2.1
r0-16	NNAGY	29 (0.5)	32 (2)	1.0	33 (0.6)	5 (0.6)	6.2	53 (10)	31 (11)	1.7
r2-1	TSFGN	519 (102)	197 (44)	2.9	446 (3)	46 (2)	9.8	261 (7)	19 (0.5)	13.7
r2-3	EVEIK	218 (18)	121 (4)	1.8	148 (46)	32 (18)	5.1	133 (0.7)	23 (0.8)	5.8
r2-9	LTSNR	935 (181)	224 (39)	4.3	420[b]	66[b]	6.4[b]	330 (11)	43 (2)	7.7
r2-14	LVYNQ	978 (210)	191 (41)	5.1	289 (96)	48 (26)	6.5	307 (9)	47 (0.2)	6.5
r6-1	GSKSH	168 (15)	106 (2)	1.6	259 (1)	42 (3)	6.2	411 (4)	30 (2)	13.7
r6-3	VTRNQ	475 (82)	80 (15)	6.2	893 (225)	122 (40)	7.7	703 (10)	46 (0.9)	15.3
r6-4	IVNNK	486 (66)	152 (23)	3.2	312 (68)	72 (21)	4.4	200 (12)	33 (2)	6.1
r6-5	TAIPD	198 (43)	221 (69)	0.9	139 (3)	46 (1)	3.0	288 (11)	55 (5)	5.2
r6-6	ITKPD	244 (46)	202 (38)	1.2	254[b]	42[b]	6.0[b]	362 (10)	27 (2)	13.4

[a] Kinetic analyses for cefaloram and C-Dox were performed at 37 ± 0.3 °C; nitrocefin was at 22 ± 0.3 °C. Standard deviations ($n = 2-5$) are given in parentheses. Units of k_{cat}/K_m are s^{-1} μM^{-1}. [b] Experiment was performed once. [c] Not determined.

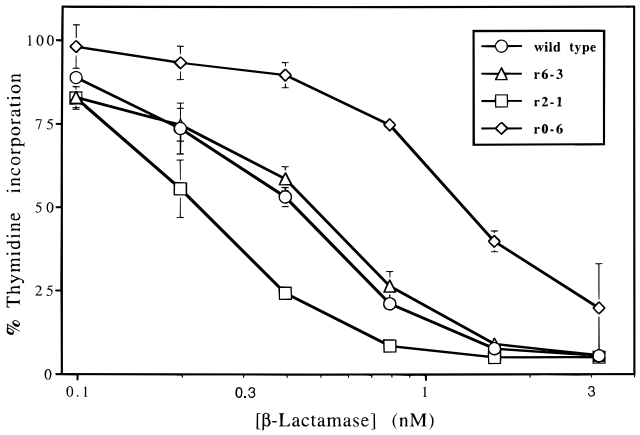

FIGURE 4: Cell cytotoxicity due to β-lactamase-mediated hydrolysis of C-Dox. H2981 cells were exposed to a mixture of β-lactamase and 1 μM C-Dox (1 h, 37 °C). Twenty-four hours later, the cells were pulsed with [^{3}H]thymidine (6 h, 37 °C), harvested, and counted. The incorporation of radioactivity was calculated as a percentage of treated cells to controls. Symbols: diamonds, r0-6 enzyme; triangles, wild type; circles, r6-3; squares, r2-1.

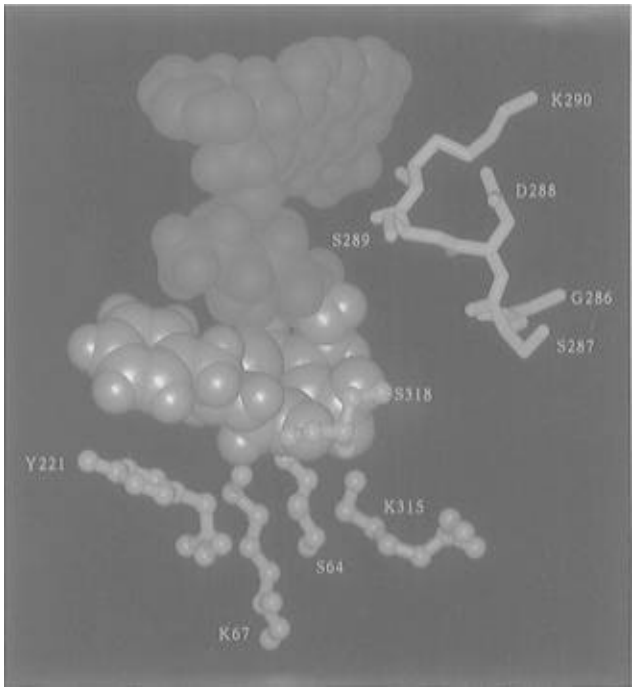

FIGURE 5: Hypothetical binding of C-Dox (gray and red space-filling model) into the active site of the *E. cloacae* P99 β-lactamase. Low-energy conformations of C-Dox were found by conformational search and molecular mechanics minimization. C-Dox was then docked into the *E. cloacae* structure, in accordance with the structures of known inhibitor complexes and molecular modeling studies of the class A β-lactamases. The cephalosporin nucleus of C-Dox is shown in gray, and the doxorubicin portion is shown in red. Side chains of active site residues are shown in dark blue. Residues 286−290 (light blue) were altered by random mutagenesis.

We have investigated an alternate method for identifying active β-lactamases in a phage library, based on simple antibiotic selection for enrichment of the phage population. The use of selection for identifying active species in a phage library is uncommon but has precedence in the reports of phage cloning vectors that contain resistance genes (Barnes, 1979; Herrmann *et al*., 1980; Zacher *et al*., 1980). In the work reported here, selection afforded greater than 1000-fold enrichment of active phage during each round, an amplification factor not generally achievable with phage panning techniques. This selection scheme was undertaken at relatively high (~400 μM) cefaloram concentrations, which may explain the selected mutants' tendencies for increased k_{cat} values at the expense of K_m with cefaloram, yielding more active enzymes at the relevant antibiotic concentrations. Other schemes that could be devised to exploit selection of phage libraries by catalytic activity might include complementation of an auxotrophic defect or selecting for an alternate detoxifying enzyme.

One goal of this study was to see if mutagenesis of amino acids 286−290 of the P99 β-lactamase could affect the enzyme's selectivity for 3′-substituted cephalosporins. Hypothetical binding of C-Dox into the active site of the P99 β-lactamase crystal structure (Lobkovsky *et al*., 1993) (Figure 5) suggests that interaction of residues 286−290 with larger 3′ substituents is likely, although in the case of C-Dox, the doxorubicin portion of the molecule should be able to adopt several relatively unrestricted conformations. The 10-fold range of preference for C-Dox vs nitrocefin exhibited by the 23 enzymes screened (Table 1) suggests that the mutations are modestly affecting selectivity for these 3′-substituted cephems.

Sequencing of the β-lactamase variants revealed a variety of permitted mutations. This indicates that despite the proximity of the loop to the active site, no residues in the region 286−290 of the *E. cloacae* enzyme are intimately involved in the catalytic mechanism. Despite gross sequence variability (all enzymes isolated in this study contained multiple mutations), there are clear residue preferences

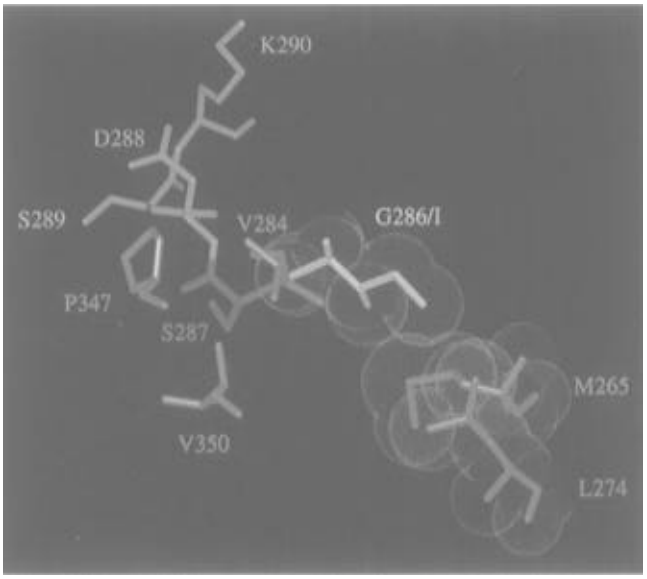

FIGURE 6: Ball and stick structures of residues neighboring S287 (red) and a G286I mutant (yellow). Mutation of G286 to a larger residue is thought to fill a hydrophobic pocket within the enzyme, mainly formed by M265 and L274 (gold). Space available to S287 mutants is much more limited due to the proximity of P347, and V350, and more conservative mutations, such as to threonine or alanine, are preferred.

observed. Inspection of the X-ray structure suggests that these residue preferences are consistent with structural characteristics of the region and suggest a few possible structure−activity relationships for the mutations at individual positions.

One trend seen in the selected libraries is the favoring of charged residues at positions 288 and 290. The side chains of these residues are fully exposed to solvent and should therefore be permissive to change, as well as favor charged amino acid substitutions, which occur in 67% of the mutations. An exception to this trend is the D288F mutation of the r2-1 enzyme, the only phenylalanine mutation present in the enzymes from the selected libraries. This suggests that the increased k_{cat}/K_m ratio of r2-1 for C-Dox could be partially due to hydrophobic interactions of the phenylalanine with the anthracycline ring of C-Dox.

Another trend observed is the favoring of small, uncharged residues in positions 286 and 287. Glycine 286 shows regular main-chain torsion angles, and mutation to other residues should, in principle, be permitted. Mutations at this position fill a hydrophobic pocket on the surface of the enzyme, mainly formed by L274 and M264 (Figure 6). The mutations observed at position 286 could therefore be expected to stabilize the region of the P99 β-lactamase. Mutations at S287 are less frequent and restricted to threonine, valine, and alanine, uncharged residues of comparable size. Examination of the crystal structure shows that position 287 is sterically restricted due to the proximity of several other residues (Figure 6). Accordingly, substitution of larger residues is disfavored, and every inactive mutant isolated and characterized contains a larger residue at this position.

Serine 289 is the only residue in the region whose side chain points into the active site. Residue changes at this position have the potential to favorably interact with 3′-substituted cephalosporins, although larger residues could interfere with binding. It is noteworthy that the four mutant enzymes possessing the highest k_{cat}/K_m values for cefaloram hydrolysis share a common S289N mutation. The side-chain amide of the S289N mutants partially fills the active site cavity and should be well situated for hydrogen-bonding interactions with the small 3′-acetate substituent of cefaloram.

The major goal of this study was to identify a panel of β-lactamase mutants possessing a range of activity toward 3′-substituted cephalosporins. The initial selection procedure guaranteed that the library members were proficient at recognizing the cephalosporin nucleus and were catalytically active. The particularly efficient or inefficient enzymes were subsequently discovered by screening the selected libraries or unselected libraries, respectively. Changing these five amino acids produced approximately a 25-fold variation in k_{cat} and K_m parameters for the substrates tested, while the range of k_{cat}/K_m values varied by about a factor of 6 (these ranges do not include the enzymes that were totally inactive within the level of detection). Included among these enzymes were also a few with modestly improved kinetic parameters compared to wild type.

For the comparison of enzyme kinetics vs efficacy in β-lactamase-mediated prodrug conversion there is no substitute for *in vivo* experiments. However, we wanted to at least assess whether or not the variations in kinetic parameters of these mutant β-lactamases would be significant in the context of an *in vitro* cytotoxicity assay. A panel of four β-lactamases from this study, incubated with the cephalosporin prodrug C-Dox and the carcinoma cell line H2981, gave a 9-fold range of IC_{50} values in a thymidine incorporation assay (Figure 4). The different effects among the enzymes tested were statistically significant, and the range of activities should allow for practical design of *in vivo* trials to compare efficacy. Further studies in this laboratory will investigate the therapeutic potential of antibody conjugates of a panel of these enzymes in order to ascertain the influence of k_{cat}, K_m, and k_{cat}/K_m on the selective activation of prodrugs such as C-Dox in animal models of human cancer.

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