

# Investigation of the Mechanism of Resistance to Third-Generation Cephalosporins by Class C $\beta$ -Lactamases by Using Chemical Complementation

Brian T. Carter,<sup>[a, b]</sup> Hening Lin,<sup>[a, c]</sup> Shalom D. Goldberg,<sup>[a, d]</sup> Eric A. Althoff,<sup>[a, e]</sup> Jessica Raushel,<sup>[a, f]</sup> and Virginia W. Cornish<sup>\*[a]</sup>

The widespread use of antibiotics to treat bacterial infections has led to the continuing challenge of antibiotic resistance. For  $\beta$ -lactam antibiotics, the most common form of resistance is the expression of  $\beta$ -lactamase enzymes, which inactivate the antibiotics by cleavage of the  $\beta$ -lactam core. In this study, chemical complementation, which is a general method to link the formation or cleavage of a chemical bond to the transcription of a reporter gene *in vivo*, was employed in combination with combinatorial mutagenesis to study the mechanism by which the class C  $\beta$ -lactamase P99 might evolve resistance to the commonly administered third-generation cephalosporin cefotaxime. The chemical complementation system was first shown to be able to distinguish between the wild-type (wt) class C  $\beta$ -lactamase P99 and the clinically isolated extended-spectrum class C  $\beta$ -lactamase GC1 in the presence of cefotaxime. The system was then employed to evaluate the activity of mutants of wt P99 towards cefotaxime. A number of single-point mutations at position 221 (Tyr

in wt P99) were identified that conferred resistance towards inhibition by cefotaxime, with as much as a 2000-fold increase in  $k_{cat}$  and a 100-fold increase in  $k_{cat}/K_M$  ( $k_{cat}$  = the rate of catalysis;  $K_M$  = the Michaelis constant), as compared to those of the wt enzyme. Finally, the chemical complementation system was employed in a high-throughput screen to identify a number of mutants of P99 that have multiple mutations around the substrate-binding pocket that increase resistance towards cefotaxime inhibition. The catalytic turnover of cefotaxime by the most active mutant identified was 5500 times higher than that of the wt P99. The resistant mutants suggest a mechanism by which a number of mutations can confer resistance by increasing the flexibility of the  $\Omega$  loop and altering the positioning of residue 221. Thus, as illustrated in this study, chemical complementation has the potential to be used as a high-throughput screen to study a wide range of enzyme–drug interactions.

## Introduction

$\beta$ -Lactam antibiotics are the largest class of antibacterial agents administered worldwide. However, shortly after the introduction of the first  $\beta$ -lactam antibiotics, antibiotic resistance emerged as a real threat to the use of these drugs. The most common bacterial resistance mechanism to these drugs is the expression of  $\beta$ -lactamase enzymes, which inactivate the antibiotics by hydrolyzing the drug's  $\beta$ -lactam core.<sup>[1]</sup> Significant resources have been devoted to the development of new generations of  $\beta$ -lactams that cannot be inactivated by the  $\beta$ -lactamases. Currently, so-called third-generation cephalosporins are normally reserved for the most resistant strains of bacteria, since these drugs are not inactivated by either class A or class C  $\beta$ -lactamases. Recently however, clinical isolates of both the class A<sup>[2–6]</sup> and class C<sup>[7–10]</sup> enzymes have been identified with increased activity towards the once-dependable oxyimino-containing third-generation cephalosporins, such as cefotaxime and ceftazidime.

Crystallographic studies have helped to shed light on the mechanism of resistance in the class C enzymes. As illustrated in Figure 1, Shoichet and co-workers proposed that ceftazidime was a potent inhibitor of the wild-type (wt) class C enzymes because of the fact that the tetrahedral intermediate formed in

[a] B. T. Carter, Dr. H. Lin, Dr. S. D. Goldberg, E. A. Althoff, J. Raushel, Prof. Dr. V. W. Cornish  
Department of Chemistry, Columbia University  
New York, NY 10027 (USA)  
Fax: (+1) 212-932-1289  
E-mail: vc114@columbia.edu

[b] B. T. Carter  
Current address:  
Department of Science, Bard High School Early College  
New York, NY 10002 (USA)

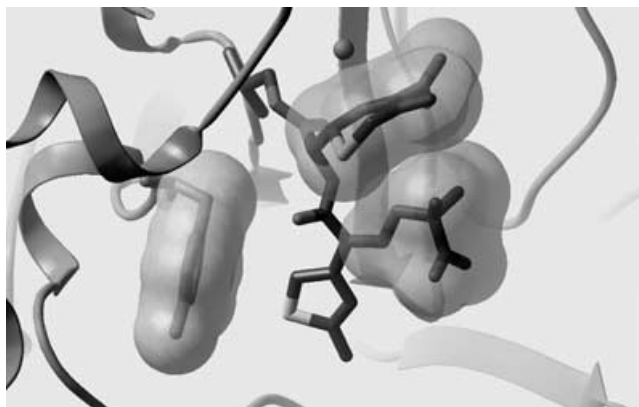
[c] Dr. H. Lin  
Current address:  
Department of Biological Chemistry and Molecular Pharmacology  
Harvard Medical School, Boston, MA 02115 (USA)

[d] Dr. S. D. Goldberg  
Current address:  
Department of Biochemistry and Biophysics, University of Pennsylvania  
Philadelphia, PA 19104 (USA)

[e] E. A. Althoff  
Current address:  
Department of Biochemistry, University of Washington  
Seattle, WA 98195 (USA)

[f] J. Raushel  
Current address:  
Department of Chemistry, The Scripps Research Institute  
La Jolla, CA 92037 (USA)

Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.



**Figure 1.** Ceftazidime bound in the active site of the wt AmpC class C  $\beta$ -lactamase. The structure was obtained by Powers et al. (PDB code: 1iel).<sup>[11]</sup> Tyr221 and the enzyme acylated form of ceftazidime are shown in stick representations. A space-filling representation is used to highlight the steric clash between the oxyimino side chain of ceftazidime and the dihydrothiazine ring of the cleaved cephem core, which prevents a conformation that is accessible to the nucleophilic water, shown as a ball, to deacylate the antibiotic. The steric bulk of Tyr221, also shown in the space-filling representation, is positioned based on its quadrupolar interaction with the aminothiazole ring of ceftazidime and prevents the oxyimino group from rotating into the binding pocket and accessing a deacylation-proficient enzyme intermediate. In the extended-spectrum GC1 mutant, it has been shown that an opening of the active site allows the oxyimino side chain to assume the position occupied by Tyr221 in the wt enzyme, thereby relieving the steric interference with the dihydrothiazine ring. The protein structure was generated by using the Mistro software.

the enzyme's acylated state cannot achieve a conformation that is competent for deacylation due to steric clashes between the oxyimino side chain and the dihydrothiazine ring of ceftazidime.<sup>[11]</sup> Knox and co-workers solved the crystal structures of a wt class C  $\beta$ -lactamase and the extended-spectrum class C  $\beta$ -lactamase GC1, both with a phosphonate transition-state analogue containing the C7 side chain of cefotaxime.<sup>[12]</sup> Based on their crystallographic data, the authors propose that the active site of the wt  $\beta$ -lactamase assumes a "closed" conformation, where the aminothiazole ring of the cefotaxime analogue has a perpendicular quadrupolar interaction with the phenol ring of the tyrosine at position 221 (Tyr221), which leads to the deacylation-deficient enzyme-bound intermediate. On the other hand, the GC1 enzyme, which has a three amino acid repeat inserted in its  $\Omega$  loop, can assume two conformations upon substrate binding. The less occupied conformation is the "closed" conformation, while the majority of the bound enzyme is found in an "open" conformation, where Tyr221 is fully removed by 10 Å from its position in the wt enzyme, allowing a catalytically competent transition state to be accessed.

Mutagenic analysis by Zhang et al. has reinforced the view of the importance played by the Tyr221 residue in the inhibition of the class C enzymes by oxyimino-containing third-generation cephalosporins.<sup>[13]</sup> Cassette mutagenesis of the 21 amino acid residues that make up the active site of the class C  $\beta$ -lactamase P99 from *Enterobacter cloacae* identified several single amino acid mutations that conferred resistance towards the third-generation cephalosporin ceftazidime. Mutants that

conferred the highest degree of resistance towards ceftazidime were those that replaced Tyr221 with smaller amino acids, such as Ala or Gly. The authors hypothesized that the Y221A and Y221G mutations not only remove the quadrupolar interaction between Tyr221 and the aminothiazole ring but also create more space to allow the oxyimino group to rotate into the position originally occupied by the phenol group of Tyr221, which is similar to the position of the oxyimino side chain found in Knox's GC1 structure.

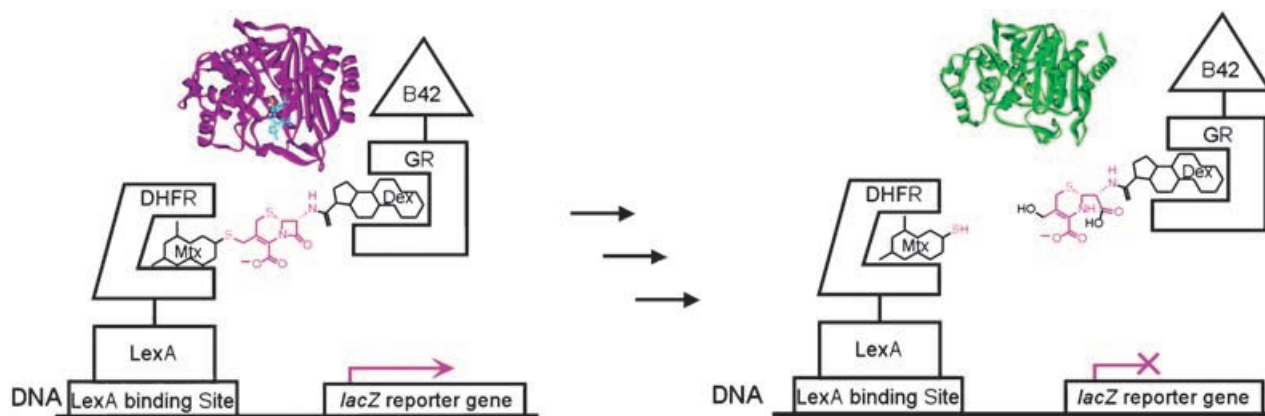
In this study, chemical complementation was employed to investigate the evolution of resistance to a third-generation cephalosporin by a class C  $\beta$ -lactamase. Our laboratory has pioneered the use of chemical complementation as a general assay for enzyme catalysis. Chemical complementation links enzyme catalysis of bond cleavage or bond formation to transcription of a reporter gene in vivo. As our laboratory has shown, one can study a number of different enzyme/substrate pairs by simply expressing the desired enzyme within the yeast cells and making the enzymatic substrate the bond between the chemical inducers of protein dimerization.<sup>[14–16]</sup>

In this study, chemical complementation was first shown to be able to distinguish between the wt P99 class C  $\beta$ -lactamase and the extended-spectrum GC1 variant in the presence of different cephalosporin antibiotics. Next, by using saturation mutagenesis at position 221 in P99, the system was used to evaluate the role this residue plays in conferring resistance towards inhibition by the third-generation oxyimino-containing cephalosporin cefotaxime. Finally, larger libraries exploring multiple residues surrounding the C7 position of the cephalosporin were screened in a high-throughput fashion to shed light on the mechanism and likelihood of resistant mutations.

## Results

### Chemical complementation screen

It was envisioned that chemical complementation could be adapted to evaluate enzyme inhibition (Figure 2). The chemical complementation system originally reported by our laboratory linked  $\beta$ -lactamase activity to transcription of a *lacZ* reporter gene in vivo through a methotrexate–cephem–dexamethasone (Mtx–Cephem–Dex) heterodimer substrate.<sup>[14]</sup> In this system, a heterodimeric Mtx–Cephem–Dex small molecule dimerizes dihydrofolate reductase (DHFR), which binds to Mtx,<sup>[17]</sup> and the hormone-binding domain of the glucocorticoid receptor (GR), which binds to Dex.<sup>[18]</sup> DHFR is fused to a DNA-binding domain (DBD) and GR is fused to a transcription activation domain (AD), such that Mtx–Cephem–Dex effectively reconstitutes the transcriptional activator (DBD–AD) and activates transcription of a downstream reporter gene. To detect  $\beta$ -lactamase activity, the Mtx and Dex molecules are linked by a cephem bond, such that cleavage of the cephem bond disrupts dimerization of the transcriptional activator, thereby ablating activation of the *lacZ* reporter gene. As the third-generation cephalosporins form a long-lived acyl-enzyme intermediate with the  $\beta$ -lactamase, these inhibitors should block cleavage of the Mtx–Cephem–Dex heterodimer by the  $\beta$ -lactamase.



**Figure 2.** Chemical complementation as a screen for active enzymes resistant to inhibition. The wt  $\beta$ -lactamase (purple) is unable to cleave the cephem bond in Mtx-Cephem-Dex due to the presence of an inhibitor (cyan) in its active site; thus, Mtx-Cephem-Dex is able to bring together a DNA-binding-domain-receptor fusion protein and an activation-domain-receptor fusion protein, thereby activating transcription of a downstream reporter gene in vivo. However, by using mutagenic techniques, such as error-prone PCR, saturated mutagenesis, or DNA shuffling, indicated by the three arrows, active mutants (green) that are resistant to inhibition can be identified by using the chemical complementation assay since their active site is available to cleave the cephem bond of Mtx-Cephem-Dex and thus turn off transcription of the downstream reporter gene. The wt class C  $\beta$ -lactamase AmpC is shown as a ribbon diagram in purple bound to ceftazidime (PDB code: 1iel),<sup>[11]</sup> while the extended spectrum class C enzyme GC1 is shown in green (PDB code: 1gc1).<sup>[38]</sup>

mase, thereby resulting in an increase in transcription of the *lacZ* reporter gene in the chemical complementation assay.  $\beta$ -Lactamase variants that are not inhibited by the third-generation cephalosporins would cleave the Mtx-Cephem-Dex heterodimer and could thus be detected based on a decrease in transcription of the *lacZ* reporter gene.

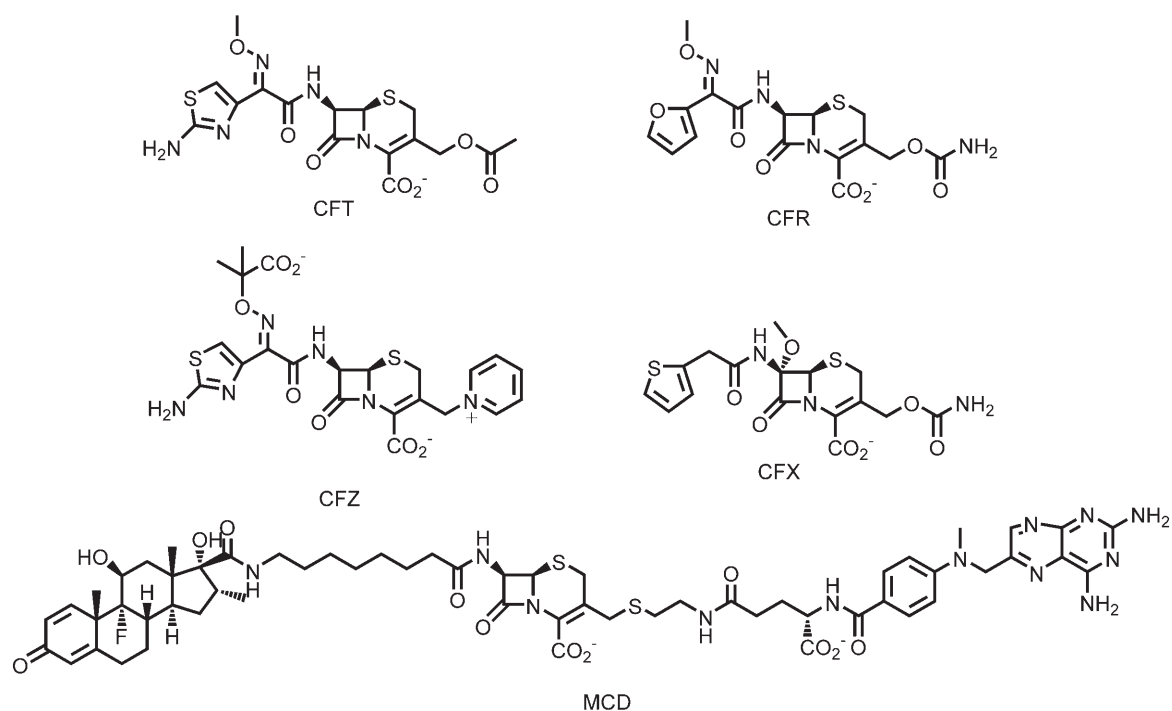
Several changes were made to the original chemical complementation screening strain used by Baker et al.<sup>[14]</sup> to optimize the transcription read-out for the enzyme inhibition screen. The original chemical complementation strain expressed both DBD-DHFR and AD-GR from the galactose-inducible *GAL1* promoter integrated into the yeast chromosome. To increase the absolute levels of *lacZ* reporter gene transcription, strain BC1187Y was used here instead; this increases the expression levels of both the AD-GR fusion protein and the *lacZ* reporter gene by placing the genes encoding these proteins on high-copy  $2\mu$  plasmids. Strain BC1187Y also had its *PDR1* gene knocked-out to increase the permeability of the yeast to the small-molecule antibiotics. The *PDR1* product Pdr1p positively controls the expression of *PDR5*, *SNQ2*, and *YOR1*, which encode ATP binding cassette (ABC) transporters that pump out a wide variety of different small molecules.<sup>[19–21]</sup> *PDR1* knockout strains have been used in a number of studies to increase the permeability of yeast to various small molecules.<sup>[22]</sup> To further increase the sensitivity of the yeast to the small-molecule antibiotics the medium was supplemented with 1% dimethyl sulfoxide (DMSO). Finally, it was found that, for strain BC1187Y, use of 0.5% galactose, 1.5% glucose, and 2% raffinose gave the optimal signal-to-noise ratio for the *lacZ* reporter gene.

Several control experiments also had to be performed to eliminate false positives and negatives. To insure that the reduction in *lacZ* expression level was the result of enzymatic cleavage of the cephem bond and was not due to the loss of one or both of the AD-GR- or *lacZ*-containing plasmids, each strain was further tested by using a Mtx-Dex small molecule with a noncleavable linker. Furthermore, to eliminate strain-to-

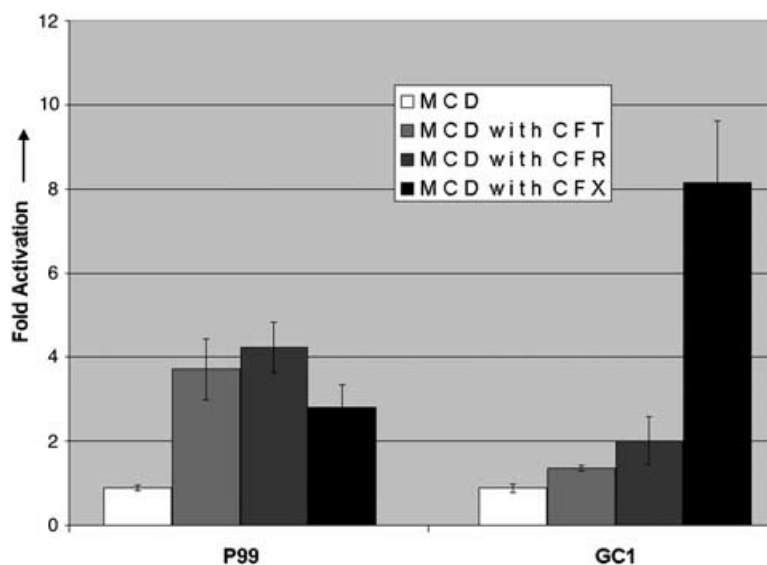
strain variations in background *lacZ* expression levels, each strain was tested in the absence of the small molecule. Thus, the *lacZ* transcription was evaluated as a fold activation by dividing the strain's *lacZ* signal in the presence of small molecule by the strain's background signal. Additionally, plasmids containing mutant enzymes that were found to be resistant to inhibition by cefotaxime were extracted from their yeast strains and retransformed into a new strain to validate that it was the mutant-enzyme-containing plasmid, and not a mutation in the yeast strain, that was causing the decrease in the *lacZ* signal.

#### Detection of a known $\beta$ -lactamase-resistant variant

The first test of the chemical complementation system was its ability to distinguish the inhibition of wt P99 from that of the extended-spectrum GC1 variant. Thus, plasmid expressing either P99 or GC1 was transformed into screening strain BC1187Y and then assayed for the levels of *lacZ* transcription by using standard conditions in liquid culture with *O*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) as the substrate after four days of growth with  $5\ \mu\text{M}$  Mtx-Cephem-Dex alone or in the presence of  $100\ \mu\text{M}$  cefotaxime, cefuroxime, or ceftaxitin (Scheme 1). P99 is known to be inhibited by all three cephalosporins, while GC1 is inhibited by ceftaxitin but is resistant to cefotaxime and cefuroxime. As shown in Figure 3, in the absence of inhibitor, expression of both P99 and GC1 disrupted *lacZ* transcription, presumably because they efficiently cleaved Mtx-Cephem-Dex inside the cell. Upon addition of any one of the three inhibitors to cells expressing P99, the levels of *lacZ* transcription increased, presumably because of inhibition of P99 and hence Mtx-Cephem-Dex cleavage. In the case of cells expressing GC1, only addition of ceftaxitin resulted in a significant increase in *lacZ* transcription.



**Scheme 1.** Structures of the small molecules used in this study, along with the structure of ceftazidime (CFZ), which was not used in this study but is shown for comparison to cefotaxime.



**Figure 3.** Chemical complementation can distinguish the wt class C  $\beta$ -lactamase P99 from the extended spectrum GC1 variant by using a series of second- and third-generation cephalosporins. Three separate transformants of the BC1187Y screening strain containing either P99 or GC1 were grown in SC media containing galactose (1%), glucose (1%), raffinose (2%), and DMSO (1%) buffered at pH 7.0 and containing the appropriate selective nutrients with 5  $\mu$ M Mtx-Cephem-Dex (MCD) in the presence or absence of 100  $\mu$ M cefotaxime (CFT), cefuroxime (CFR), or ceftaxime (CFX). After 4 days of growth at 30 °C with shaking (80 rpm), the strains are assayed for *lacZ* transcription by using a liquid ONPG assay. Controls are performed to assess the basal level transcription of the *lacZ* reporter gene with no Mtx-Cephem-Dex. Signals are reported as fold activations relative to this basal level transcription. While both P99 and GC1 effectively cleave Mtx-Cephem-Dex in the absence of any added inhibitors, thereby reducing the *lacZ* signal down to its background level, P99 is clearly inhibited by all three inhibitors tested, as seen by the fourfold increase in its *lacZ* transcription signal, while GC1 is only inhibited by the presence of ceftaxime. Error bars represent plus or minus the standard error obtained for three separate transformants.

#### Tyr221 saturation library

Crystallographic, as well as mutagenic, data had already suggested the importance of Tyr221 in preventing the class C  $\beta$ -lactamases from being able to effectively deacylate oxyimino containing third-generation cephalosporins.<sup>[12,13]</sup> From the work of Zhang et al., Y221G and Y221A mutants of P99 had been identified that conferred 8- and 21-fold increases, respectively, in the minimum inhibitory concentration (MIC) towards ceftazidime, as compared to that of the wt  $\beta$ -lactamase.<sup>[13]</sup> This mutagenic data combined with the crystallographic data from Knox and co-workers suggested that the Tyr221 might need to be mutated to a much smaller amino acid in order to prevent the steric crowding that forces the oxyimino side chain of the third-generation cephalosporins to protrude out from the enzyme pocket and assume a deacylation-deficient conformation in the enzyme's active site. Thus, in order to gain a better understanding of how mutations at position 221 impart resistance to third-generation cephalosporins and to evaluate the precision and accuracy of the chemical complementation screen, saturation mutagenesis with a degenerate NNS codon (N = A, C, G, or T; S = C or G) was used to mutate residue Tyr221 in P99 to all 20 amino acids. A vector encoding the P99 Tyr221 saturation mutagenesis library was transformed into selection strain BC1187Y; 95 individual transformants were randomly selected and assayed by using the chemical complementation *lacZ* transcription assay. With codon redun-



dancy and library integrity taken into account, it was estimated that 95 transformants would be sufficient to cover all 20 amino acids with 95% confidence. In addition to the 95 Y221X mutants, three wt P99 transformants were included as controls.

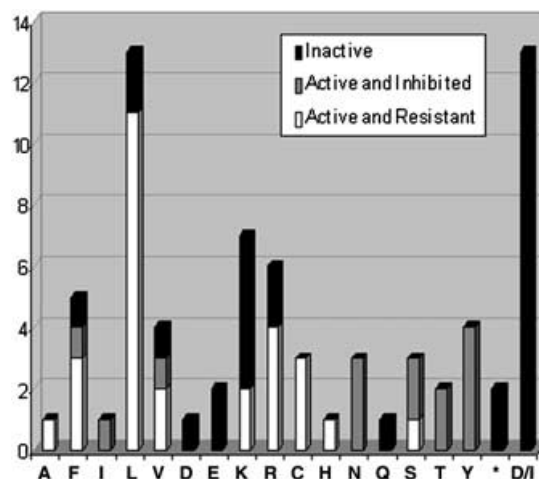
The 95 Y221X library members and 3 P99 controls were assayed in duplicate for *lacZ* transcription levels after 4 days of growth in the presence of 1  $\mu\text{M}$  Mtx–Cephem–Dex with and without 100  $\mu\text{M}$  cefotaxime by using standard *lacZ* liquid assay conditions. By using the noncleavable Mtx–Dex control, it was found that 10 of the 95 library members were false positives and showed no activation in the presence of Mtx–Dex, presumably due to loss of one or both of the AD–GR- or *lacZ*-containing plasmids.

The remaining 85 transformants could be grouped into one of three categories based on comparison of their fold activations in the *lacZ* assay in the presence of Mtx–Cephem–Dex with and without cefotaxime with the fold activations found for the P99 control strains. For reference, the P99 controls gave fold activations of  $1.0 \pm 0.3$  in the absence of cefotaxime and  $2.4 \pm 0.1$  in the presence of cefotaxime. As judged by the chemical complementation system, 34 (40%) of the 85 transformants contained inactive  $\beta$ -lactamases, with fold activations greater than 1.3, which is above one standard deviation of those obtained for wt P99 in the presence of 1  $\mu\text{M}$  Mtx–Cephem–Dex and in the absence of cefotaxime. The assay identified 20 ( $\approx 25\%$ ) of the 85 library members that behaved just as the wt enzyme, that is, giving fold activations that were below 1.3 in the absence of cefotaxime but then showing an increase in their fold activation above 1.3 upon addition of cefotaxime. The final category of Tyr221 mutations were those deemed to be both active  $\beta$ -lactamases and not inhibited by cefotaxime, that is, giving fold activation below 1.3 regardless of the presence or absence of cefotaxime. Over 35%, 31 of the 85 library members, were grouped into this category.

To test the precision of the chemical complementation system, P99 variants from each of the three categories were retested. The P99 encoding plasmids from these colonies were isolated and retransformed into BC1187Y; then 3 separate transformants were assayed under identical conditions to those used in the initial screen. It was found that 7 of the 8 selected colonies would have been placed into their original categories (data not shown). The one colony that gave inconsistent results was found to contain multiple plasmids.

To determine the sequence of the P99 variants, the region surrounding the Tyr221 mutation for the 85 colonies was sequenced by using a 96-capillary MegaBACE 1000 DNA sequencing system by the energy-transfer dideoxy-terminator method, which yielded reliable sequence data for 80 of the 85 mutants.<sup>[23]</sup> Of the 80 sequences obtained, 8 were shown to contain multiple plasmids and thus were removed from further analysis. Sequencing showed that 13 of the 80 sequences ( $\approx 15\%$ ) contained deletions or insertions in the region surrounding the Tyr221 mutation. Two colonies introduced stop codons into the  $\beta$ -lactamase sequence. All 15 of these colonies failed to express  $\beta$ -lactamases and were all easily identified as inactive  $\beta$ -lactamases in the chemical complementation system by their high fold activations in the presence of Mtx–Cephem–

Dex. The library was also found to contain 4 wt P99 enzymes. All four were correctly characterized as active  $\beta$ -lactamases inhibited by cefotaxime. The library showed a substantial bias towards Tyr221 being mutated to Leu, with approximately 15% of the library containing this mutation. The consistency of the assay was confirmed by the fact that 11 of the 13 Y221L mutants were placed in the same category and deemed to be both active and not inhibited by cefotaxime. As illustrated by the 13 Y221L mutants identified in the screen and shown in Figure 4 for Y221F, Y221V, Y221K, Y221R, and Y221S, not all



**Figure 4.** Y221X mutants of P99 characterized by using the chemical complementation screen. Mutations are grouped as hydrophobic amino acids (A, F, I, L, V), negatively and positively charged amino acids (D, E, K, R), polar amino acids (C, H, N, Q, S, T, Y), stop codons (\*), or deletions and insertions (D/I). The height of the bar represents the number of each mutant found in the 85 member library screen and the color of the bar represents which of the three categories the mutant was placed in according to its fold activation in the *lacZ* assay. Each mutant was categorized as either an inactive  $\beta$ -lactamase (fold activation  $> 1.3$ ), or an active  $\beta$ -lactamase (fold activation  $\leq 1.3$ ), based on the signals obtained from the liquid ONPG assay after 4 days of growth in the presence of 1  $\mu\text{M}$  Mtx–Cephem–Dex. Active  $\beta$ -lactamases were further categorized as inhibited (fold activations  $> 1.3$ ) or resistant (fold activation  $\leq 1.3$ ) to cefotaxime, based on the signals obtained from the liquid ONPG assay after four days of growth in the presence of Mtx–Cephem–Dex (1  $\mu\text{M}$ ) and cefotaxime (100  $\mu\text{M}$ ).

mutants with the same mutation at this position grouped together in the same category. With the high DNA copy error rate observed just in the region around position 221 that was sequenced, our assumption is that these discrepancies are due to bonus mutations occurring outside position 221 and causing mutants that have the same Tyr221 mutation to behave differently. This phenomenon was observed clearly with the three Y221S mutants identified. Two of these mutants were classified as active and inhibited, while one was classified as active and resistant. Upon examining the sequence of these three enzymes outside position 221, the two active and inhibited enzymes were both found to have bonus mutations at different positions within the  $\Omega$  loop, while no such mutations were found in the active and resistant Y221S mutant.

While the library size was selected to obtain a 95% confidence of selecting every amino acid mutation at position 221, the large number of deletions and insertions, combined with the bias towards the Leu mutation and the colonies that showed no Mtx–Dex-induced *lacZ* transcription, reduced this confidence interval and the sequencing data showed that the library possessed no Gly, Trp, Met, or Pro mutations. Mutants were found possessing the remaining 16 amino acids at position 221 and the activities of these mutants, as determined by the chemical complementation system, are shown in Figure 4.

While it was mostly the introduction of deletions, insertions, or stop codons that led to the production of inactive  $\beta$ -lactamases in the library screen, a few amino acid mutations at position 221 also produced inactive P99 mutants. Placement of a Gln residue or one of the negatively charged amino acids, Asp or Glu, seems to lead to an inactive, or at least a less active,  $\beta$ -lactamase, as one of each of these mutants was found. In addition to the four strains containing wt P99 that were properly identified in the library screen, mutants changing the Tyr221 residue to Asn, Thr, or Ile were also identified as active  $\beta$ -lactamases but were inhibited by the presence of cefotaxime. Most surprising were the large number of single amino acid mutants and the diversity of these mutations that allowed P99 to effectively avoid the cefotaxime inhibitor. From the early mutagenic studies of P99, it was expected that only small amino acid substitutions would be identified at position 221; instead, both large and small hydrophobic amino acids, namely, Ala, Val, Leu, and Phe, as well as polar amino acids, Cys and Ser, and positively charged amino acids, Arg, His, and Lys, were found to confer resistance when substituted for Tyr at position 221.

Mutants from each of the three categories, as well as wt P99, were purified by Ni-affinity chromatography for further in vitro characterization. Each protein was judged to be >95% pure based on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue. Furthermore, each single amino acid mutation was shown not to cause any major perturbations in the protein structure, as CD wavelength scans of each mutant protein were obtained and shown to be similar to that obtained for wt P99, with a minimum at 217 nm (data not shown). Sequencing of these genes in full confirmed that they contained only the single mutation at position 221.

Kinetic characterization of these mutants with cefotaxime is shown in Table 1. Steady-state kinetic constants were determined for each purified enzyme with cefotaxime by monitoring the decrease in UV absorbance upon cleavage of the  $\beta$ -lactam bond at 260 nm ( $\Delta\varepsilon_{260} = 6510 \text{ cm}^{-1} \text{ M}^{-1}$ )<sup>[24]</sup> at several different substrate concentrations around the Michaelis constant ( $K_M$ ). Inhibition constants ( $K_I$ ) were obtained for at least one mutant from each category with cefotaxime by monitoring the increase in absorbance upon cleavage of the  $\beta$ -lactam bond of a fixed amount of nitrocefin at 486 nm ( $\Delta\varepsilon_{486} = 16000 \text{ cm}^{-1} \text{ M}^{-1}$ )<sup>[25]</sup> with various amounts of cefotaxime. Furthermore, steady-state kinetic constants were obtained for at least one mutant from each category with Mtx–Cephem–Dex (Table 2) by monitoring the decrease in UV absorbance upon cleavage of the  $\beta$ -lactam bond at 265 nm ( $\Delta\varepsilon_{265} =$

**Table 1.** Kinetic characterization of selected mutants from the Y221X screen with cefotaxime.<sup>[a]</sup>

Enzyme	Cefotaxime			
	$k_{\text{cat}}$ [ $\text{s}^{-1}$ ]	$K_M$ [ $\mu\text{M}$ ]	$k_{\text{cat}}/K_M$ [ $\text{s}^{-1} \text{ M}^{-1}$ ]	$K_I$ [ $\mu\text{M}$ ]
wild type:				
P99	$0.0253 \pm 0.0008$	$8 \pm 1$	$3 \times 10^3$	$0.0115 \pm 0.0005$
active and resistant:				
Y221A	$69 \pm 4$	$130 \pm 20$	$5.3 \times 10^5$	$184 \pm 8$
Y221L	$10.9 \pm 0.5$	$100 \pm 10$	$1.1 \times 10^5$	$170 \pm 30$
Y221R	$64 \pm 5$	$610 \pm 70$	$1.0 \times 10^5$	N.D.
Y221H	$48 \pm 1$	$95 \pm 5$	$5.1 \times 10^5$	N.D.
active and inhibited:				
Y221T	$0.66 \pm 0.01$	$1.9 \pm 0.2$	$3.5 \times 10^5$	$0.93 \pm 0.04$
Y221N	$0.43 \pm 0.01$	$2.5 \pm 0.3$	$1.7 \times 10^5$	$1.8 \pm 0.2$
inactive:				
Y221E	$11 \pm 1$	$460 \pm 80$	$2.4 \times 10^4$	$590 \pm 20$

[a] N.D. = not determined.

**Table 2.** Kinetic characterization of select mutants from the Y221X screen with Mtx–Cephem–Dex.

Enzyme	Mtx–Cephem–Dex		
	$k_{\text{cat}}$ [ $\text{s}^{-1}$ ]	$K_M$ [ $\mu\text{M}$ ]	$k_{\text{cat}}/K_M$ [ $\text{s}^{-1} \text{ M}^{-1}$ ]
wild type:			
P99	$150 \pm 30$	$100 \pm 30$	$1.5 \times 10^6$
active and resistant:			
Y221A	$60 \pm 10$	$110 \pm 40$	$5 \times 10^5$
active and inhibited:			
Y221T	$26 \pm 2$	$70 \pm 10$	$4 \times 10^5$
inactive:			
Y221E	$1.2 \pm 0.1$	$100 \pm 20$	$1.2 \times 10^4$

$7600 \text{ cm}^{-1} \text{ M}^{-1}$ )<sup>[16]</sup> with several different concentrations of Mtx–Cephem–Dex around the  $K_M$  value.

Of the original 85-member library screened, 35% of the colonies yielded mutants that were both active and resistant to inhibition by cefotaxime as judged by the chemical complementation screen. All 4 of the mutants selected from this category for in vitro characterization showed a substantial increase in their rate of catalysis ( $k_{\text{cat}}$ ) values with cefotaxime, as compared to that of wt P99, with changes ranging from a 430-fold increase in the  $k_{\text{cat}}$  value with Y221L to a 2700-fold increase with Y221A. In addition, the  $K_M$  values for all four of these mutants were also increased by at least tenfold over that for the wt enzyme, a result leading to an overall improvement of 100-fold or more in their catalytic efficiency ( $k_{\text{cat}}/K_M$ ).

Of the original 85-member library, 25% of the mutants were deemed to be active but inhibited by cefotaxime according to the results of the chemical complementation screening. While the two mutants selected from this category, Y221N and Y221T, showed an increase in the  $k_{\text{cat}}$  value with cefotaxime over wt P99, this increase was only 15- to 30-fold, while their  $K_M$  values were decreased by 4-fold. While the  $k_{\text{cat}}$  values towards cefotaxime for both Y221N and Y221T showed substan-



than or equal to 1.4 in the presence of Mtx–Cephem–Dex and cefotaxime during this second screening. Sequences of the mutated regions of these 67 members of the library were obtained by using a 96-capillary MegaBACE 1000 DNA sequencing system. The sequences of mutants selected from the library that were deemed to be both active and resistant to inhibition can be found in the Supporting Information.

Every single amino acid mutant that was selected from the Y221X library as being resistant to inhibition by cefotaxime was also found in the Asp217/Ala220/Tyr221 library in combination with other mutations at positions 217 and 220. Furthermore, several Y221X mutants that were not present in the original library, due to the fact that complete amino acid coverage was not obtained, were found to be resistant in the three-member library, including Y221M and Y221W. Y221T, which was clearly shown to allow inhibition as a single-point mutation, was found to be in a resistant mutant in the three-member library when in combination with D217I. Perhaps most interesting, due to the importance of the Tyr221 residue in conferring inhibition towards cefotaxime, was the presence of 4 selected mutants that maintained Tyr at position 221.

The two mutants A220N/Y221V and D217W/A220S/Y221L, which gave the lowest signals in the *lacZ* assay in the presence of both Mtx–Cephem–Dex and cefotaxime, were purified by Ni-affinity chromatography by using His<sub>6</sub> tags. In addition to these mutants, one mutant possessing the P99 triple mutant D217L/A220F/Y221A and one possessing the P99 double mutant A220N/Y221H were also selected and purified under the same conditions, since these single amino acid mutations had been selected from the Y221X screening and shown to have the highest catalytic turnover with cefotaxime. Furthermore, two of the four mutants selected as both active and resistant to inhibition that still retained Tyr at position 221 were also selected for in vitro characterization. Each protein was judged to be >95% pure based on SDS-PAGE and staining with Coomassie blue. CD wavelength scans of each mutant protein were obtained and shown to be similar to that obtained for wt P99 (data not shown). Sequencing of the entire P99 genes revealed that four of the six selected enzymes contained an additional mutation outside the targeted region, as shown in Table 3.

Kinetic characterization of the selected mutants with cefotaxime is shown in Table 3. Kinetic characterization was carried out essentially as described for the Y221X mutants. Overall, the 6 selected P99 mutants showed kinetic constants for cefotaxime that were comparable to those obtained for the Y221X library, with both increases in their  $k_{\text{cat}}$  values of 100- to over 5000-fold, as well as increases in their  $K_{\text{M}}$  values of 2- to 100-fold as compared to those of wt P99. In comparing the single mutants to those obtained from the three-member library, the D217W/A220S/Y221L/I263V mutant showed a fourfold decrease in the  $k_{\text{cat}}$  value as well as a fivefold decrease in the  $K_{\text{M}}$  value as compared to those of the Y221L single mutant, resulting in a comparable catalytic efficiency. The kinetic constants obtained for the D88N/D217L/A220F/Y221A mutant were comparable to those obtained for the Y221A mutant. Overall, the A220N/Y221H/N226D mutant gave both the highest  $k_{\text{cat}}$  and

**Table 3.** Kinetic characterization of selected mutants from the D217/A220/Y221 library.

Enzyme	Cefotaxime			
	$k_{\text{cat}}$ [s <sup>-1</sup> ]	$K_{\text{M}}$ [μM]	$k_{\text{cat}}/K_{\text{M}}$ [s <sup>-1</sup> M <sup>-1</sup> ]	$K_{\text{I}}$ [μM]
wild type:				
P99	0.0253 ± 0.0008	8 ± 1	3 × 10 <sup>3</sup>	0.0115 ± 0.0005
active and resistant:				
A220N/Y221H/ N226D	140 ± 20	800 ± 100	2 × 10 <sup>5</sup>	800 ± 100
D88N/D217V/ A220F/Y221A	78 ± 6	210 ± 40	3.7 × 10 <sup>5</sup>	160 ± 15
D217K/A220P/ K290R	106 ± 8	470 ± 60	2.3 × 10 <sup>5</sup>	500 ± 100
D217T/A220G	12.7 ± 0.9	62 ± 13	2.0 × 10 <sup>5</sup>	83 ± 4
A220N/Y221V	11.5 ± 0.3	43 ± 3	2.7 × 10 <sup>5</sup>	N.D.
D217W/A220S/ Y221L/I263V	2.4 ± 0.06	20 ± 2	1.2 × 10 <sup>5</sup>	N.D.

highest  $K_{\text{M}}$  values obtained for any mutant tested, with a  $k_{\text{cat}}$  value that was 5600-fold higher than that obtained for wt P99 and a  $K_{\text{M}}$  value that was 100-fold higher than that obtained for wt P99. Characterization of the mutants that retained Tyr221 showed that, just as in the case with GC1, it is not necessary to mutate Tyr221 at all to obtain highly active mutant β-lactamases towards cefotaxime, as both of these mutants showed large increases in their  $k_{\text{cat}}$  values (up to 4000-fold for one of the mutants), as well as large increases in their  $K_{\text{M}}$  values, as compared to those of wt P99.

Thus far, all of the mutants that lacked Tyr at position 221 had failed to show the large discrepancy between their  $K_{\text{M}}$  and  $K_{\text{I}}$  values towards cefotaxime that is observed for the wt enzyme. One wonders if mutant enzymes that retain the tyrosine, but possess other mutations might also cause P99 not to exhibit this discrepancy. As shown in Table 3, the  $K_{\text{I}}$  values for all of the mutants tested were nearly identical to their  $K_{\text{M}}$  values, even for the two mutants that retained Tyr at position 221.

## Discussion

Perhaps the most surprising result to emerge from this study is the large number of mutations at position 221 or positions 217/220/221 that confer resistance toward cefotaxime without impairing β-lactamase activity. First and foremost, this result is of concern because it suggests that it is in fact quite easy for β-lactamase enzymes to evolve resistance to third-generation cephalosporin antibiotics, which differ in their C7 substituents. Combinatorial mutagenesis has identified the 217/220/221 loop as a region that the enzyme can afford to mutate without losing β-lactamase activity. Thus, this region perhaps should not be targeted in future drug discovery efforts. Combinatorial mutagenesis could be further used to identify regions of the enzyme that are intolerant to substitution on which to focus future drug discovery efforts.

One explanation for the impaired deacylation of third-generation cephalosporins by class C β-lactamases is that the C7



side chain of these cephalosporins blocks formation of the conformation needed for stabilization of the high-energy deacylation tetrahedral intermediate by the enzyme.<sup>[11]</sup> The large percentage of mutations at position 221 that appear to relieve this inhibition is quite surprising. These results were particularly unexpected because a previous resistance study identified only the Y221A and Y211G variants from a selection with ceftazidime.<sup>[13]</sup> The fact that not only small amino acid mutations, but also large hydrophobic, as well as polar and basic amino acids, relieve inhibition is consistent with a hypothesis that it is loop dynamics, rather than the specific nature of any one of these amino acid mutations, that determines this relief of inhibition. The isolation of several multiple mutants with Tyr221 further supports such a hypothesis. The difference in position 221 variants identified in the two studies could simply reflect the different methodologies, a selection intended only to identify the most active variants versus a screen covering the entire library of position 221 mutants here. Alternatively, the position 221 variants may differ in their activity with cefotaxime versus the bulkier C7 of ceftazidime used in the previous study.

This study was also undertaken to evaluate the suitability of chemical complementation for high-throughput applications. While a number of other assays are certainly available to study  $\beta$ -lactamase activity, we focused on the chemical complementation assay here because of its potential generality. The chemical complementation assay has been designed to detect enzyme catalysis of both bond-cleavage and -formation reactions. As has been shown in our laboratory, one can readily adapt the chemical complementation assay to study a wide variety of different enzyme/substrate pairs, which requires simply subcloning the gene for the desired enzyme into a yeast expression vector and synthesizing a new Mtx-linker-Dex enzyme substrate, with the desired substrate functionality incorporated as the linker.<sup>[14–16]</sup> Our laboratory has strategically planned the synthetic route of the Mtx-linker-Dex small molecules to readily incorporate different substrates as the linker between Mtx and Dex.<sup>[26,27]</sup> Our laboratory has recently been able to adapt the chemical complementation system to study glycosynthase enzymes, which are difficult enzymes to work with but which have proven readily amenable to the chemical complementation system.<sup>[15]</sup>

This study expands and verifies the use of the chemical complementation system in two very important ways. For one, it shows that chemical complementation can be used to evaluate enzyme inhibition and that a variety of inhibitors can be evaluated by using the same chemical dimerizer. By using the Mtx-Cephem-Dex small-molecule dimerizer, this study was able to evaluate a given  $\beta$ -lactamase's ability to hydrolyze cefotaxime; however, as shown in Figure 3, one could have just as easily studied cefuroxime, cefoxitin, or any other number of  $\beta$ -lactam drugs that work in a similar manner. Thus, the chemical complementation assay holds the power to evaluate and distinguish between a number of different protein–drug interactions by using a single chemical dimerizer.

The second advance made in this study was to show that the chemical complementation system was adaptable to a

high-throughput screening application. Not only was the method able to correctly evaluate 16 different amino acid mutations at the Tyr221 position, but it was also shown to be able to screen libraries that approach thousands of compounds and it could easily be expanded to rapidly evaluate  $10^4$ – $10^5$  different protein mutants or different drug targets, by simply scaling up the procedure followed in this study. The procedures used here lend themselves readily to automation, where greater than  $10^6$  different proteins or drug targets could be evaluated on a daily basis.

One of the limitations of the chemical complementation assay that is illustrated in this study is the inability to distinguish between P99 mutants that were placed into the same category to see which enzyme was most inhibited or resistant to cefotaxime. As was found by our laboratory in testing the dynamic range of the chemical complementation assay, although the assay could distinguish between enzymes that differed in their catalytic turnovers by over four orders of magnitude, the assay was unable to distinguish between enzymes that possessed catalytic turnovers within the same order of magnitude of each other.<sup>[16]</sup> One notices that all of the mutants that were grouped together had kinetic properties with cefotaxime that were all within an order of magnitude of each other. However, we believe that this limitation is due more to the *lacZ* reporter gene and, as more sensitive reporter genes become available, this limitation should be eliminated.

## Conclusion

In this study, chemical complementation has shown to be a powerful tool for studying enzyme–drug interactions in vivo. The screen was not only able to distinguish two enzymes, P99 and GC1, based on their activity profiles with a number of different antibiotics but was also applied in a high-throughput fashion to identify a number of mutant enzymes that were shown to have increased catalytic activity towards the third-generation cephalosporin cefotaxime. Interestingly, it appears that multiple mutations in the  $\Omega$  loop region of P99 lead to mutants that are not inhibited by cefotaxime, which could lead to potential problems for the continued use of the oxyimino-containing third-generation cephalosporins.

## Experimental Section

**Chemical synthesis:** The synthesis of Mtx-Cephem-Dex has been previously described.<sup>[14,16]</sup> Mtx-Dex is described in full elsewhere.<sup>[28]</sup> NMR spectra of both compounds can be found in the Supporting Information.

**Biological methods:** A more complete description of the general methods for molecular biology, as well as for the construction of plasmids used in this study, is given in the Supporting Information.

**Construction of P99 mutant libraries:** Cassette mutagenesis by using a two-step PCR fusion method was used to completely randomize P99 at position 221. Two halves of the P99 gene were generated by using primers VWC1264 5'-GCATACGTCCTGCAGATGACGCCAGTTAGTGAAAAGCAGCTGGCGGAG (PstI) and VWC1277 3'-GACCGTTGGTTTTCACGCCSNNGGCTTGTGCATCCAGCATAC (N-



agitation (80 rpm) for 3 days. The saturated yeast cultures (5  $\mu\text{L}$ ) were used to inoculate SC media (95  $\mu\text{L}$ ) containing the appropriate galactose, glucose, and raffinose concentration and lacking the appropriate selective nutrients. The media was supplemented with the appropriate concentrations of Mtx–Cephem–Dex with or without cefotaxime; alternatively other  $\beta$ -lactamase inhibitors or non-enzymatically cleavable Mtx–Dex small molecules were used as supplements. Optimized screening conditions were found to be SC media possessing 0.5% galactose, 1.5% glucose, 2% raffinose, and 1% DMSO, buffered at pH 7.0 by using phosphate buffer with the appropriate selective nutrients and containing either no small molecule, or 1  $\mu\text{M}$  Mtx–Cephem–Dex with or without 100  $\mu\text{M}$  cefotaxime, or 1  $\mu\text{M}$  Mtx–Dex. The cultures were allowed to grow for 4 days at 30 °C with shaking at 80 rpm. After this time, the cells were harvested by centrifugation at 3000 rpm for 5 min by using a Sorvall RT7 Plus centrifuge; the pellets were subsequently resuspended in distilled water (100  $\mu\text{L}$ ). Next, the cultures were transferred to a flat-bottomed 96-well plate for reading the absorbance at 600 nm ( $A_{600}$ ). The cultures were centrifuged and the pellets were resuspended in the Y-Per Protein Extraction Reagent (100  $\mu\text{L}$ ). Lysis was allowed to proceed for 30 min. Then, a 10 mg mL<sup>-1</sup> ONPG solution (8.5  $\mu\text{L}$ ) was added to the extracts and the mixture was allowed to incubate for 60 min at 37 °C and with shaking at 240 rpm. The  $\beta$ -galactosidase activity reaction was stopped with the addition of 1 M sodium carbonate (130  $\mu\text{L}$ ). The extracts were centrifuged and the supernatant was transferred to a flat-bottomed 96-well plate, where the  $A_{420}$  value was measured. The following equation was used to calculate  $\beta$ -galactosidase units:  $\beta$ -galactosidase =  $1000 \times (A_{420}/[A_{600} \times \text{time in minutes} \times \text{volume assayed in mL}])$ . Data are reported as fold activation values, which are the  $\beta$ -galactosidase signal obtained from a given yeast strain under a set of given conditions, divided by the basal level transcription of the *lacZ* gene, as determined by assaying the strain under identical conditions without small molecule.

**High-throughput DNA sequencing:** All 85 members screened in the P99 Y221X library, as well as the 67 members of the P99217.4 library selected from the first round of screening, were sequenced in mass by using the MegaBACE 1000 capillary sequencer by the energy-transfer dideoxy terminator method. Yeast containing the different P99 mutants were phrogged from 96-well 20% glycerol stock plates into 96-well plates containing SC media (150  $\mu\text{L}$ ) containing glucose and the appropriate selective nutrients and were grown to saturation at 30 °C and shaking at 80 rpm. The yeast strains were then phrogged onto SC agarose plates containing glucose and the appropriate selective nutrients and were incubated at 30 °C for 3 days, effectively creating patches of all of the yeast, with each patch containing a different P99 mutant. The entire P99 gene for each mutant was amplified by using Taq Polymerase by placing a small amount of each patch into a PCR reaction mix (20  $\mu\text{L}$ ) with primers VWC1264 and VWC1265 for the Y221X library and primers VWC1051 5'-CGT GTA ATACAGGGTCGTC and VWC1052 5'-GGGACCTAGACTTCA GGTG for the three-member libraries. The cells were lysed by heating at 95 °C for 5 min and the DNA was amplified by using 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The PCR reaction was quantitated by running 2  $\mu\text{L}$  of the reaction on a 1% agarose gel with known standards. Normal yields were around 50 ng per  $\mu\text{L}$  of reaction. Cycle sequencing was performed by using PCR product (100 ng), DYEnamic ET terminator premix (8  $\mu\text{L}$ ; Amersham Biosciences), and primer VWC649 5'-GGCAGCCG-CAGTGGAAGCC (5 pmol) and diluting the final volume to 20  $\mu\text{L}$  with deionized water. Amplification was performed by using 30 cycles of 95 °C for 30 s and 60 °C for 90 s. After isopropanol pre-

cipitation and 70% ethanol washes, DNA sequencing samples were suspended in MegaBACE formamide loading solution (20  $\mu\text{L}$ ) and loaded onto a MegaBACE 1000 capillary sequencer through electrokinetic injection by applying 3000 V for 60 s. The samples were separated electrophoretically by applying 9000 V for 120 min. The resulting sequencing data were transformed into electropherograms; base calling was performed by using the Cimmaron Slim Phredify 1.53 Basecaller (Amersham Biosciences). Quality scores for the resulting electropherograms were calculated by using the Phred method.<sup>[33;34]</sup> All sequences with a read length < 100 bp or not maintaining an average Phred score of at least 14 were removed from subsequent analysis. By using these criteria, 75% of the selected members of the P99217.4 library and greater than 90% of the 85 members of the Y221X library were sequenced with an accuracy of greater than 96%. For the Y221X and P99217.4 libraries, the average Phred score was 21.7 and 16.1 and the average read length was 474 and 250 nucleotides per sequence sample, respectively.

**Protein purification:** Protein purification of P99 and selected mutants was carried out by overexpression in *Escherichia coli* Tuner (DE3) cells, followed by purification through the carboxy-terminal His<sub>6</sub> tag contained by each protein by using Ni-NTA affinity chromatography essentially as described.<sup>[35]</sup> A cell pellet from culture (750  $\mu\text{L}$ ) known to express protein based on analysis by SDS PAGE was used to inoculate LB medium (100 mL) containing 30  $\mu\text{g mL}^{-1}$  kanamycin. This culture was grown at 37 °C and with shaking (240 rpm) until it reached mid-log phase ( $A_{600} \approx 0.6$ –0.8), at which time expression of the  $\beta$ -lactamase was induced by adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growth for an additional 3 h, the cells were divided into 2 cultures (50 mL each) and harvested by centrifugation at 3000 rpm for 15 min by using a Sorvall centrifuge; the cell pellets were stored at -80 °C. The pellet was thawed and cells lysis was performed by adding BugBuster Protein Extraction Reagent (1 mL) and allowing the cell suspension to sit at room temperature for 30 min. The proteins were purified from the soluble portion of the cell lysis under native conditions by using a Ni-nitrilotriacetate (Ni-NTA) Spin Kit from QIAGEN. Elution fractions containing protein were then immediately dialyzed against phosphate-buffered saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.4 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.0). The enzyme concentration was determined on the basis of the  $A_{280}$  value with  $\epsilon = 71\,000 \text{ cm}^{-1} \text{ M}^{-1}$  for wt P99.<sup>[36]</sup> Since most of the selected mutants of P99 replaced the Tyr residue at position 221, assumptions were made that a tryptophan contributed  $5690 \text{ cm}^{-1} \text{ M}^{-1}$  to the extinction coefficient, while a tyrosine residue contributed  $1280 \text{ cm}^{-1} \text{ M}^{-1}$  and a cysteine residue contributed  $120 \text{ cm}^{-1} \text{ M}^{-1}$ .<sup>[37]</sup> The proteins were judged to be > 95% pure based on Coomassie staining of an SDS polyacrylamide gel. Proteins were stored at 4 °C at concentrations of 50–120  $\mu\text{M}$ .

**Circular dichroism:** Wavelength scans were measured for each purified protein to ensure the mutants' overall structures were similar to wt P99. Proteins were diluted to a final concentration of 1  $\mu\text{M}$  by using a solution of PBS buffer (pH 7.0) and deionized water (1:10). CD wavelength scans were measured on an Aviv Circular Dichroism Spectrometer Model 215. Spectra were recorded from 190–300 nm, every 1 nm, with 3 accumulations for each sample.

**Kinetics:**  $\beta$ -Lactamase activity with cefotaxime, Mtx–Cephem–Dex, and nitrocefin in the presence of cefotaxime was detected by using a Spectramax 384 UV/Vis 96-well plate reader in 96-well Costar UV plates by monitoring a decrease in the  $A_{260}$  or  $A_{265}$  or an increase in  $A_{486}$ , respectively. Extinction coefficient changes for the substrates that were employed were  $6510 \text{ cm}^{-1} \text{ M}^{-1}$  for cefotax-



ime,<sup>[24]</sup> 7600 cm<sup>-1</sup> M<sup>-1</sup> for Mtx–Cephem–Dex,<sup>[16]</sup> and 16000 cm<sup>-1</sup> M<sup>-1</sup> for nitrocefin.<sup>[25]</sup> Activity was assayed in a solution composed of a 1:1 mixture of enzyme diluted in PBS buffer (pH 7.0) and substrate in 10 mM sodium phosphate buffer (pH 7.0) with a final reaction volume of 200 µL for the determination of the  $k_{cat}$ ,  $K_M$ , and  $K_I$  values for cefotaxime and a final reaction volume of 150 µL for the determination of the  $k_{cat}$  and  $K_M$  values for Mtx–Cephem–Dex. All enzyme dilutions were supplemented with 0.1 mg mL<sup>-1</sup> bovine serum albumin. Reactions were initiated by the addition of 100 µL of enzyme solution to 100 µL of substrate solution for the determination of kinetic constants for cefotaxime and 75 µL of enzyme solution to 75 µL of substrate solution for the determination of kinetic constants for Mtx–Cephem–Dex; the progress was monitored over several minutes. Enzyme concentrations were adjusted to give rates that would be linear over the first several minutes, except for P99 with cefotaxime, which was monitored over an hour due to its extremely low  $k_{cat}$  rate. For the kinetic characterization of P99 and its mutants with cefotaxime, the final enzyme concentrations used were 5 nM for Y221A and Y221H, 10 nM for all of the selected 217.4 mutants, except the D217T/A220G and D217W/A220S/Y221L/I263V mutants, which were used in 20 nM and 50 nM concentrations, respectively, 20 nM for Y221L and Y221R, 50 nM for Y221T, 100 nM for Y221N and Y221E, and 1 µM for P99. For the kinetic characterization of P99 and selected mutants with Mtx–Cephem–Dex, the final enzyme concentrations used were 2.5 nM for P99, 10 nM for Y221A and Y221T, and 100 nM for Y221E. The final concentrations of cefotaxime ranged from 1 to 500 µM and were adjusted in attempts to have several readings above and below the  $K_M$  value of the enzyme; this condition was hard to meet for mutants with extremely high  $K_M$  values, such as the Y221R, Y221E, A220N/Y221H/N226D, and D217K/A220P/K290R mutants, as well as mutants with extremely low  $K_M$  values, such as Y221T and Y221N. The final concentrations of Mtx–Cephem–Dex ranged from 1 to 80 µM. Initial velocities were measured for seven different substrate concentrations for cefotaxime and six different substrate concentrations of Mtx–Cephem–Dex, in triplicate, and were fitted to the Michaelis–Menten equation,  $v_o = V_{max} \times \{[S]/(K_M + [S])\}$ , where  $v_o$  is the initial velocity,  $V_{max}$  is the maximal velocity for a given enzyme concentration,  $K_M$  is the Michaelis constant, and  $[S]$  is the substrate concentration, by nonlinear regression analysis with the Kaleidagraph software (Synergy Software). Representative kinetic graphs for both cefotaxime and Mtx–Cephem–Dex can be found in the Supporting Information.

Inhibition constants for cefotaxime were also obtained for P99 and a subset of the selected mutants by using nitrocefin. Kinetic constants were first obtained for each enzyme with nitrocefin as a substrate. By using this same enzyme concentration and a fixed nitrocefin concentration, normally threefold higher than the  $K_M$  value of the enzyme for nitrocefin, the amount of cefotaxime in the reaction was varied from no inhibition to almost complete inhibition of the initial velocity of the enzyme for nitrocefin. Enzyme concentrations used were 100 pM for P99, 1 nM for Y221A, Y221T, and D217T/A220G, 10 nM for Y221L, Y221N, A220N/Y221H/N226D, and D217K/A220P/K290R, 20 nM for D88N/D217L/A220F/Y221A, and 50 nM for Y221E. The cefotaxime concentration was varied from 0–2.5 mM depending on the enzyme used. Initial velocities were measured for seven different substrate concentrations, in triplicate, and fitted to the following equation:  $v_o = V_{max} \times \{[S]/(K_M(1 + [I]/K_I) + [S])\}$ , where  $v_o$  is the initial velocity,  $V_{max}$  is the maximal velocity for a given enzyme concentration with nitrocefin,  $K_M$  is the Michaelis constant for nitrocefin,  $[S]$  is the nitrocefin concentration used,  $[I]$  is the cefotaxime concentration used, and  $K_I$  is the inhibition constant, by nonlinear regression analysis with the Kaleidagraph soft-

ware (Synergy Software). Representative kinetic graphs for the determination of the  $K_I$  values can be found in the Supporting Information.

## Acknowledgements

The authors would like to thank the Columbia Genome Sequencing Center, especially B. Iannuccilli, M. Ulz, and Professor J. Ju for use of the MegaBACE 1000 capillary sequencer, as well as for their expertise in sequencing DNA in mass. We are also grateful to Dr. R. Reid for helpful discussions on yeast genetics. We are grateful for financial support for this work from the National Institutes of Health (Grant no.: RO1-GM62867). V.W.C. is a Sloan Foundation Fellow. B.T.C. was a NDSEG Pre-Doctoral Fellow, and E.A.A. was a Howard Hughes Medical Institute Pre-Doctoral Fellow.

**Keywords:** antibiotic resistance • chemical complementation • drug research • high-throughput screening • lactamases

- [1] J. Ghuysen, *Annu. Rev. Microbiol.* **1991**, *45*, 37.
- [2] G. A. Jacoby, A. A. Medeiros, T. F. O'Brien, M. E. Pinto, H. Jiang, *N. Engl. J. Med.* **1988**, *319*, 723.
- [3] G. A. Jacoby, A. A. Medeiros, *Antimicrob. Agents Chemother.* **1991**, *35*, 1697.
- [4] E. Collatz, L. Gutmann, R. Williamson, J. F. Acar, *J. Antimicrob. Chemother.* **1984**, *14(Suppl. B)*, 13.
- [5] L. B. Rice, S. H. Willey, G. A. Papanicolaou, A. A. Medeiros, G. M. Eliopoulos, R. C. Moellering, Jr., G. A. Jacoby, *Antimicrob. Agents Chemother.* **1990**, *34*, 2193.
- [6] A. Philippon, R. Labia, G. Jacoby, *Antimicrob. Agents Chemother.* **1989**, *33*, 1131.
- [7] K. Tsukamoto, R. Kikura, R. Ohno, T. Sawai, *FEBS Lett.* **1990**, *264*, 211.
- [8] M. Nukaga, K. Tsukamoto, H. Yamaguchi, T. Sawai, *Antimicrob. Agents Chemother.* **1994**, *38*, 1374.
- [9] M. Nukaga, S. Haruta, K. Tanimoto, K. Kogure, K. Taniguchi, M. Tamaki, T. Sawai, *J. Biol. Chem.* **1995**, *270*, 5729.
- [10] M. Nukaga, K. Taniguchi, Y. Washio, T. Sawai, *Biochemistry* **1998**, *37*, 10461.
- [11] R. A. Powers, E. Caselli, P. J. Focia, F. Prati, B. K. Shoichet, *Biochemistry* **2001**, *40*, 9207.
- [12] M. Nukaga, S. Kumar, K. Nukaga, R. F. Pratt, J. R. Knox, *J. Biol. Chem.* **2004**, *279*, 9344.
- [13] Z. Zhang, Y. Yu, J. M. Musser, T. Palzkill, *J. Biol. Chem.* **2001**, *276*, 46568.
- [14] K. Baker, C. Bleczynski, H. Lin, G. Salazar-Jimenez, D. Sengupta, S. Krane, V. W. Cornish, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16537.
- [15] H. Lin, H. Tao, V. W. Cornish, *J. Am. Chem. Soc.* **2004**, *126*, 15051.
- [16] D. Sengupta, H. Lin, S. D. Goldberg, J. J. Mahal, V. W. Cornish, *Biochemistry* **2004**, *43*, 3570.
- [17] S. Benkovic, C. Fierke, A. Naylor, *Science* **1988**, *239*, 1105.
- [18] K. Yamamoto, *Annu. Rev. Genet.* **1985**, *19*, 209.
- [19] H. B. van den Hazel, H. Pichler, M. A. do Valle Matta, E. Leitner, A. Goffeau, G. Daum, *J. Biol. Chem.* **1999**, *274*, 1934.
- [20] A. Nourani, M. Wesolowski-Louvel, T. Delaveau, C. Jacq, A. Delahodde, *Mol. Cell. Biol.* **1997**, *17*, 5453.
- [21] T. Delaveau, A. Delahodde, E. Carvajal, J. Subik, C. Jacq, *Mol. Gen. Genet.* **1994**, *244*, 501.
- [22] J. Kato-Stankiewicz, I. Hakimi, G. Zhi, J. Zhang, I. Serebriiskii, L. Guo, H. Edamatsu, H. Koide, S. Menon, R. Eckl, S. Sakamuri, Y. Lu, Q. Z. Chen, S. Agarwal, W. R. Baumbach, E. A. Golemis, F. Tamanoi, V. Khazak, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14398.
- [23] I. Kheterpal, R. A. Mathies, *Anal. Chem.* **1999**, *71*, 31 A.
- [24] S. Kumar, S. A. Adediran, M. Nukaga, R. F. Pratt, *Biochemistry* **2004**, *43*, 2664.



