Saturation mutagenesis of Asn152 reveals a substrate selectivity switch in P99 cephalosporinase

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Abstract

In class C β-lactamases, the strictly conserved Asn152 forms part of an extended active-site hydrogen-bonding network. To probe its role in catalysis, all 19 mutants of *Enterobacter cloacae* P99 cephalosporinase Asn152 were simultaneously constructed and screened in *Escherichia coli* for their in vivo activity. The screen identified the previously uncharacterized mutants Asn152Ser, Asn152Thr, and Asn152Gly, which possess significant activity and altered substrate selectivity. In vitro measurement of Michaelis-Menten kinetic constants revealed that the Asn152Ser mutation causes a selectivity switch for penicillin G versus cefoxitin. Asn152Thr showed a 63-fold increase in k_{cat} for oxacillin, a slow substrate for wild-type cephalosporinase. The results contribute to a growing body of data showing that mutation of highly conserved residues in the active site can result in substrate selectivity changes. The library screening method presented here would be applicable to substrate selectivity determination in other readily screenable enzymes.

Keywords: β-lactamase; substrate selectivity; microbial sensitivity tests; saturation mutagenesis; kinetics; enzymology

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Enzymes are extraordinary both for their ability to accelerate the rate of chemical transformations and for the exquisite specificity shown toward a particular substrate or a series of related substrates. Rational mutation of several residues contacting the substrate has been used to alter the specificity of trypsin (Hedstrom et al. 1991), subtilisin (Wells et al. 1987), carnitine acyltransferase (Hsiao et al. 2004), and *N*-acetylneuraminate lyase (Joerger et al. 2003). Directed evolution methods have increasingly been employed, as in the evolution of a branched-chain aminotransferase from an aspartate aminotransferase (Yano et al. 1998) and the alteration of specificity of aminoacyl-tRNA synthetases (Wang et al. 2001). In contrast to these studies are cases where a single-point mutation is sufficient to broaden or switch specificity (Toscano et al. 2007). A classic example is α-lytic protease Met192Ala, which is able to cleave Phe- and Leu-containing substrates in addition to those with Ala (Bone et al. 1989). More recently, the H89L mutant was shown to invert the selectivity of tyrosine amino lyase (Watts et al. 2006). These and other examples are beginning to form a picture of how substrate specificity is determined (Wilks and Holbrook 1991; Antikainen and Martin 2005).

β-lactamases present themselves as an ideal model system for studying substrate selectivity. Extensive research has resulted in the collection and cataloging of hundreds of naturally occurring β-lactamases and mutants for which we have sequence, structural, and

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biochemical data. Conveniently, dozens of closely related substrates are commercially available, and a straightforward high-throughput method of screening and selection can be employed to measure activity (Galleni et al. 1995; Fisher et al. 2005). Through evolution, β-lactamases have gained the ability to hydrolyze much of the current spectrum of antibiotics, leading to drug-resistant strains of bacteria that pose an important public health threat. The development of resistance is, at its core, a substrate selectivity problem (Petrosino et al. 1998). Comparatively few studies have attempted to describe how substrate selectivity changes upon mutation (Cantu and Palzkill 1998; Doucet et al. 2004).

Our laboratory previously identified several key residues near the active site of *Enterobacter cloacae* P99 cephalosporinase, including a conserved hydrogen-bonded network comprised of Lys315, Tyr150, Ser64, Lys67, Asn152, Gln120, and the substrate carboxamide (Goldberg et al. 2003). This network can be seen in the crystal structure of AmpC, a class C enzyme that is structurally highly similar to P99 cephalosporinase, bound to cephalothin (Fig. 1; Lobkovsky et al. 1993). Mutants Lys67Arg and Tyr150Phe, which alter the putative general base residues, could efficiently hydrolyze certain substrates (>10% wild-type activity) but not others (three to five orders of magnitude reduction in k_{cat}/K_m) (Monnaie et al. 1994; Dubus et al. 1996). The strictly conserved Asn152 has been mutated to Asp, Glu, His, Leu, or Ala, resulting in the reduction of k_{cat}/K_m by three to five orders of magnitude for a wide range of substrates (Dubus et al. 1995; Trehan et al. 2001). The loss of activity upon mutation of Asn152 is intriguing, given that this residue is relatively distant from the bond being broken.

The analogous residue Asn132 in class A β-lactamases occupies the same position as Asn152 relative to the substrate (Lobkovsky et al. 1993) and is likewise conserved among hundreds of sequences, with two known exceptions, Ser and Gly (Connolly and Waley 1983; Wang et al. 2006). Mutagenesis studies in class A enzymes have shown that Ser or Asp can substitute for Asn132 (Jacob et al. 1990a,b; Osuna et al. 1995). In the case of Asn132Ser, substitution resulted in a shift of substrate preference away from cephalosporins. In class D enzymes, a conserved Val occupies the analogous position. In class C enzymes, Asn152 is thought to play a hydrogen-bonding role, so it is somewhat surprising that variants possessing Ser, Thr, Gln, Lys, and Arg at this position have not been tested. Previous studies seem to have focused on either too few mutants or on substrates for which Asn152 variants had low activity (Dubus et al. 1995; Majiduddin and Palzkill 2005).

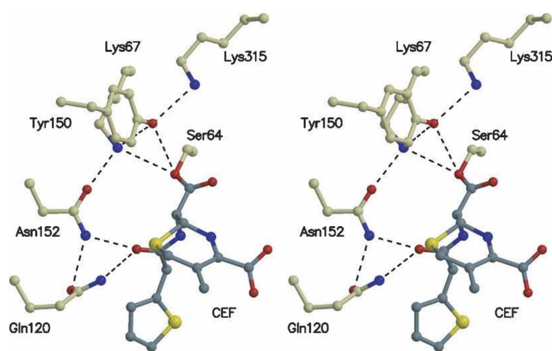


Figure 1. Stereoview of the AmpC cephalosporinase active site with the cephalothin acyl intermediate bound (PDB entry: 1KVM) (Beadle et al. 2002); (CEF) cephalothin, (dashed lines) proposed hydrogen bonds. The figure was prepared using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt and Bacon 1997).

Given the suggested role of this residue in substrate selectivity and activity, we used a modern high-throughput approach to randomize position 152 in P99 cephalosporinase to all 20 amino acids and screen for residual activity in vivo. The activity was assessed as the maximal permissive concentration for each mutant with six representative β-lactams including penicillins (penicillin G, ampicillin, and oxacillin), cephalosporins (cephalothin and cefotaxime), and a cephamycin (cefoxitin) (Fig. 2). In vitro characterization of Michaelis-Menten constants confirmed that substitution of Asn152 results in highly active mutant enzymes that show significant changes in substrate specificity.

Results

Library design

The role of Asn152 in the hydrogen-bonding network of P99 cephalosporinase is not well understood. Previously, Asn152 in class C β-lactamases has been mutated to Asp, Glu, His, Leu, and Ala; this set notably lacks hydrogen-bonding residues Ser, Thr, Gln, Lys, and Arg. In order to fully investigate the role of Asn152, this position was randomized to all 20 amino acids. Rather than making each mutation individually, we employed a library approach (used previously) to generate all mutants simultaneously and then isolate a representative set of constructs by transformation into *Escherichia coli* (Goldberg et al. 2003). A problem with our previous library efforts was low yield of the desired mutations and a high incidence of mutations outside of the randomized codons, most likely owing to the use of a PCR-based method of plasmid construction. To avoid these problems, we inserted restriction sites flanking the locus of mutation and ligated a 17-bp oligonucleotide cassette coding for NNS at position 152 into the vector (Fig. 3). Sequencing showed no additional mutations (see Materials and

Figure 2. Antibiotics used in this study. Side chains are colored to highlight differences in structure.

Methods). A test set of mutants was chosen by plating the library under nonselective conditions. Sequencing of 192 colonies showed that each of the 20 amino acid substitutions and the null mutant were represented by at least four independent colonies, except for valine (only three colonies), glutamine (three colonies), and aspartate (two colonies). Assaying several clones for each mutant reduced the probability that the results would be skewed by clonal variation or the effect of neighboring clones in the activity assay.

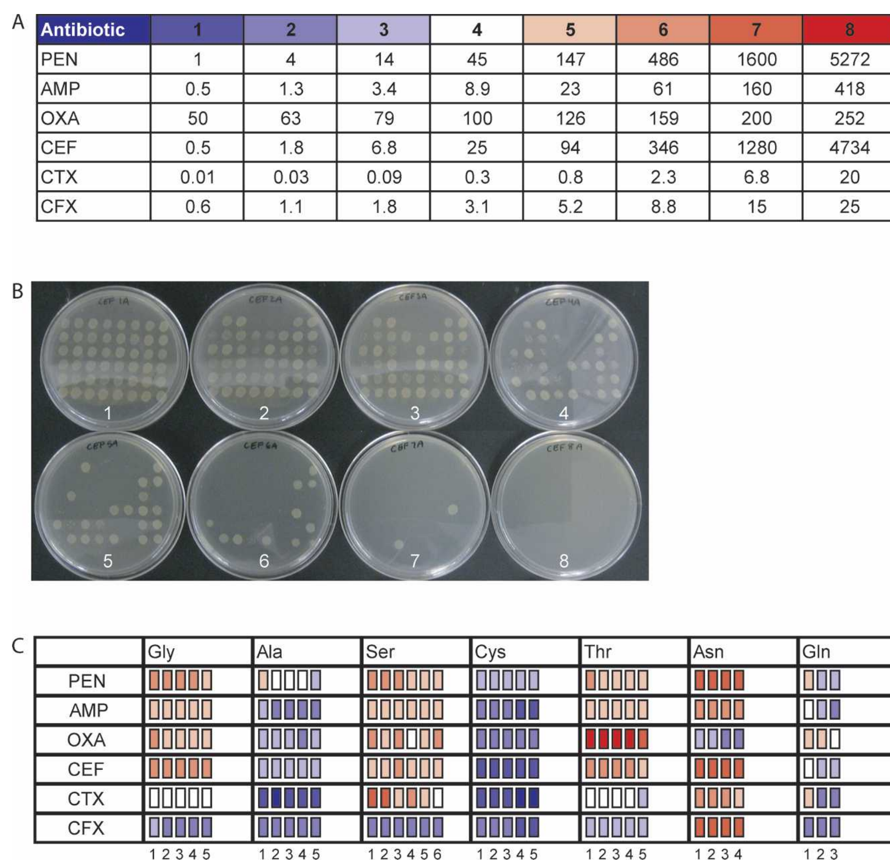
In vivo activity screen

To rapidly define the gross activity of each mutant with different antibiotic substrates, we employed a screen to detect the capacity of β-lactamase to protect bacterial cells from an antibiotic. Use of a high-throughput screen allowed us to readily test several different substrate molecules to assess substrate specificity of the different Asn152 variants. The enzyme activity was scored as the maximal permissive concentration (MPC) of antibiotic at which an *E. coli* culture will grow when expressing the mutant enzyme from a weakly constitutive promoter on a plasmid. The MPC assay allows active mutants to be identified solely on the basis of their in vivo activity, without having to purify and characterize each protein. To increase the limit of detection, we used a sensitive *E. coli* strain that lacks two native transpeptidases and the AmpC β-lactamase (Henderson et al. 1997; Denome et al. 1999). In this assay, the wild-type protein grows well at 1280 μg/mL cephalothin, 6.75 μg/mL cefotaxime, 1600 μg/mL penicillin G, 160 μg/mL ampicillin, 15 μg/mL cefoxitin, and 200 μg/mL oxacillin. Cells containing a construct with a stop codon have some intrinsic resistance and grow well on 0.5 μg/mL, 0.01 μg/mL, 1 μg/mL, 0.5 μg/mL, 0.63 μg/mL, and 50 μg/mL of the above antibiotics, respectively. A series of plates spanning these concentrations (and one additional high-concentration plate where wild type did not grow) was devised for each antibiotic following a scaled exponential increase, reminiscent of the twofold dilutions typically seen for minimum inhibitory concentration (MIC) assays (Fig. 4A). The range for oxacillin was based on the MPC of the Asn152Ser mutant as a positive control. Concentrations of antibiotics based on these controls were used rather than a standard twofold dilution so that small variations in activity could be distinguished when the concentration range between the positive and negative controls was small.

The growth patterns on increasing concentrations of antibiotic showed that only a few mutants retained activity (Fig. 4B,C). The wild type grew on high concentrations of antibiotic, as expected for the positive control. The null mutant grew only on the lowest two plates. The previously characterized mutants also served as negative controls; His, Leu, Glu, and Asp are predicted to have low activity, and the in vivo screen is consistent with this expectation (see Supplemental material). Large hydrophobic residues showed little to no activity, as did basic residues. Results for Gln showed two inactive clones with one outlier.

The small hydrogen-bonding residues showed activity approaching and sometimes exceeding wild type with several antibiotics. Asn152Ser and Asn152Thr appeared to be active against penicillins (penicillin G and ampicillin) as well as cephalosporins (cephalothin and cefotaxime) at a level far exceeding the other mutants, but less than wild type. In addition, both mutants were more active with oxacillin and less active with cefoxitin than wild type.

Figure 3. Cassette mutagenesis. A 25-bp cassette with overlapping ends was inserted into the vector at engineered SexAI and MfeI sites to introduce the NNS codon at position 152.



Antibiotic	1	2	3	4	5	6	7	8
PEN	1	4	14	45	147	486	1600	5272
AMP	0.5	1.3	3.4	8.9	23	61	160	418
OXA	50	63	79	100	126	159	200	252
CEF	0.5	1.8	6.8	25	94	346	1280	4734
CTX	0.01	0.03	0.09	0.3	0.8	2.3	6.8	20
CFX	0.6	1.1	1.8	3.1	5.2	8.8	15	25

Figure 4. In vivo assay of mutant P99 cephalosporinase activity. (*A*) Concentrations of antibiotics in plates 1–8 (μg/mL). (*B*) Mutants arrayed in 96-well plates were grown to saturation and spotted on agar (1–8) containing increasing concentrations of antibiotic. Maximal permissive concentration (MPC) was scored as the highest concentration on which the clone grew unimpaired. Sample data are shown for 48 clones on cephalothin (see Supplemental material for full data set). (*C*) MPC scores are represented as colored rectangles according to a color temperature scale, where blue indicates growth only at a low concentration of antibiotic and red indicates growth up to a high concentration. (Colors 1–8) Concentrations from *A*, (dark blue, 0) clone was impaired on the lowest antibiotic concentration tested. The MPC of an individual clone against each antibiotic is presented in a single column that is labeled at the *bottom* with the clone index number.

Asn152Gly showed significant activity against penicillin G, ampicillin, oxacillin, and cephalothin. Alanine and cysteine, which do not form hydrogen bonds through their side chains, showed activity similar to the null mutant.

In vitro characterization

In order to confirm the result of the in vivo screen, the wild-type enzyme and the Asn152Ser and Asn152Thr mutants were chosen for further characterization in vitro, due to the unexpectedly high activity of these mutants and their altered substrate profile. The results of initial rate kinetics of the purified enzymes are shown in Table 1. For cephalothin and cefotaxime, the rate of hydrolysis was measured spectroscopically at several substrate concentrations and fit to the Michaelis-Menten equation. For penicillin G, ampicillin, oxacillin, and cefoxitin, the absorbance changes were too low to measure K_m values directly, so these values were approximated as K_i (Kumar et al. 2004). The k_{cat} for cefoxitin with Asn152Ser and Asn152Thr, as well as the k_{cat} for oxacillin with the wild-type enzyme, was too slow to measure directly even at micromolar concentrations of enzyme. Since β-lactamases proceed through an acyl-enzyme intermediate for which deacylation is rate limiting (Galleni and Frère 1988; Galleni et al. 1988), the deacylation rate constant k_3 was used to estimate k_{cat} ($k_{cat} = k_2k_3/(k_2 + k_3)$, which reduces to $k_{cat} = k_3$ when $k_3 << k_2$). This rate was measured by first saturating the enzyme with the slow substrate to acylate all of the enzyme, then diluting into saturating reporter substrate (in this case nitrocefin) and measuring the first-order reappearance of activity (Fisher et al. 1980). The deacylation rate constant was extracted graphically from the absorbance trace (Glick et al. 1978).

The effect of mutation of Asn152 to Ser or Thr appears to be highly substrate dependent. Radar plots of the

Table 1. *Kinetic properties of Asn152 mutants*

Enzyme with antibiotic	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Cephalothin			
Wild type	150 ± 2	15 ± 1	$1.0 \pm 0.1 \times 10^7$
Asn152Ser	4.65 ± 0.07	3.3 ± 0.2	$1.4 \pm 0.1 \times 10^6$
Asn152Thr	14.3 ± 0.4	48 ± 4	$3.0 \pm 0.3 \times 10^5$
Cefotaxime			
Wild type	0.0201 ± 0.0004[a]	0.018 ± 0.003[b]	$1.1 \pm 0.2 \times 10^6$ [c]
Asn152Ser	0.830 ± 0.013	5.3 ± 0.3	$1.6 \pm 0.1 \times 10^5$
Asn152Thr	1.90 ± 0.06	87 ± 7	$2.2 \pm 0.2 \times 10^4$
Penicillin G			
Wild type	5.37 ± 0.25[a]	0.73 ± 0.05[b]	$7.4 \pm 0.6 \times 10^6$ [c]
Asn152Ser	1.68 ± 0.10[a]	0.096 ± 0.014[b]	$1.8 \pm 0.3 \times 10^7$ [c]
Asn152Thr	2.55 ± 0.01[a]	3.3 ± 0.3[b]	$7.7 \pm 0.8 \times 10^5$ [c]
Ampicillin			
Wild type	0.499 ± 0.014[a]	0.29 ± 0.02[b]	$1.7 \pm 0.1 \times 10^6$ [c]
Asn152Ser	0.0515 ± 0.0007[a]	0.086 ± 0.007[b]	$6.0 \pm 0.5 \times 10^5$ [c]
Asn152Thr	0.101 ± 0.003[a]	0.70 ± 0.14[b]	$1.5 \pm 0.3 \times 10^5$ [c]
Cefoxitin			
Wild type	0.0306 ± 0.0001[a]	0.038 ± 0.004[b]	$8.0 \pm 0.9 \times 10^5$ [c]
Asn152Ser	0.00161 ± 0.00017[d]	0.093 ± 0.022[b]	$1.7 \pm 0.4 \times 10^4$ [c]
Asn152Thr	0.00217 ± 0.00063[d]	0.049 ± 0.006[b]	$4.4 \pm 1.4 \times 10^4$ [c]
Oxacillin			
Wild type	0.00496 ± 0.00014[d]	0.010 ± 0.001[b]	$5.2 \pm 0.5 \times 10^5$ [c]
Asn152Ser	0.0320 ± 0.0010[a]	0.072 ± 0.011[b]	$4.4 \pm 0.7 \times 10^5$ [c]
Asn152Thr	0.313 ± 0.009[a]	1.64 ± 0.19[b]	$1.9 \pm 0.2 \times 10^5$ [c]

[a] Determined at >10*K_m substrate.
[b] Estimated as K_i.
[c] Estimated as k_3/K_i.
[d] Estimated as k_3.

kinetic constants and MPC values outline six-sided shapes that correspond to the substrate profile of a given mutant enzyme (Fig. 5). In general, mutation to a hydroxyl side chain causes k_{cat} to decrease with penicillin G, ampicillin, cephalothin, and cefoxitin, and increase for cefotaxime and oxacillin. Notably, the increase in k_{cat} for oxacillin is 6.5-fold for Asn152Ser and 63-fold for Asn152Thr. The apparent affinity for all substrates, measured as K_m or K_i, tends to decrease for the Ser and Thr mutants, with the exception of Asn152Ser with cephalothin. This decrease in affinity is more pronounced for Asn152Thr than Asn152Ser. The effect of mutation on catalytic efficiency, k_{cat}/K_m, is nearly equally dependent on changes in both constants. The catalytic efficiency of the Ser and Thr mutants is generally decreased by one to two orders of magnitude against the antibiotics tested. The two exceptions are oxacillin, for which k_{cat}/K_m is the same as wild type for Asn152Ser and 2.7 times lower for Asn152Thr, and penicillin, which is increased 2.4-fold for Asn152Ser relative to wild type.

Discussion

Site-directed mutagenesis of highly conserved active-site residues frequently results in an ablation of enzymatic activity. In class C β-lactamases, the putative general base residues Lys67 and Tyr150 follow this pattern. Likewise, previous reports have shown that mutation of Asn152 of P99 cephalosporinase to Ala, Asp, Glu, His, and Leu results in a three to five orders of magnitude decrease in k_{cat} and k_{cat}/K_m across a broad range of substrates, suggesting that this position is critical for catalysis (Dubus et al. 1995; Trehan et al. 2001). A BLAST search against P99 cephalosporinase indicated that Asn152 is invariant among known class C β-lactamases (data not shown), yet the role of this residue is not well understood. Our randomization experiment has shed light on Asn152, suggesting that mutations at this position alter the substrate selectivity.

Variants of Asn152 that have small, hydrogen-bonding side chains are highly active. Our results show that Ser, Thr, or Gly can be substituted for Asn, generating an enzyme that has significant activity toward penicillins and cephalosporins in vivo. The hydroxyl side chain of the Ser and Thr mutants could form one (or at most two) of the three hydrogen bonds in which the wild-type amide side chain participates (Lobkovsky et al. 1993). This implies that Ser or Thr can probably carry out only one function: Either it can hydrogen bond to Lys67 and support the electrostatic network, or it can hydrogen bond to the substrate to stabilize it for deacylation. Another interesting possibility is that the role that is played by the hydroxyl could change depending on the substrate or the step of the catalytic cycle. The Gly variant has a substrate profile in the in vivo screen that resembles that of Asn152Ser, with the exception of cefotaxime. This variant is structurally quite different from the wild-type enzyme, making it difficult to speculate on what the role of the Gly at position 152 might be. In the crystal structure of the *Mycobacterium tuberculosis* class A β-lactamase, which has a naturally occurring Gly at the corresponding position 132, no crystallographic water molecule is observed (Wang et al. 2006). We surmise, however, that a water molecule could occupy the place of a side chain in Asn152Gly and fulfill a hydrogen-bonding role, as is the case in the class A TEM-76 (Ser130Gly) variant (Thomas et al. 2005). The interpretation of the activity of this variant must therefore await a crystal structure. The Gln, Ala, and Cys variants, on the other hand, show reduced activity relative to Ser, Thr, and Gly, giving further evidence that hydrogen bonding is the critical function of Asn152 and that small size is a requirement.

The residue at position 152 is a determinant of substrate specificity. The substrate preference of a particular mutant can also be expressed in terms of the ratio of the specificity constants ($k_{cat}/K_{m\ substrate1}/k_{cat}/K_{m\ substrate2}$). These ratios tend to be 10 or lower for the wild-type enzyme, indicating similar preference for all substrates

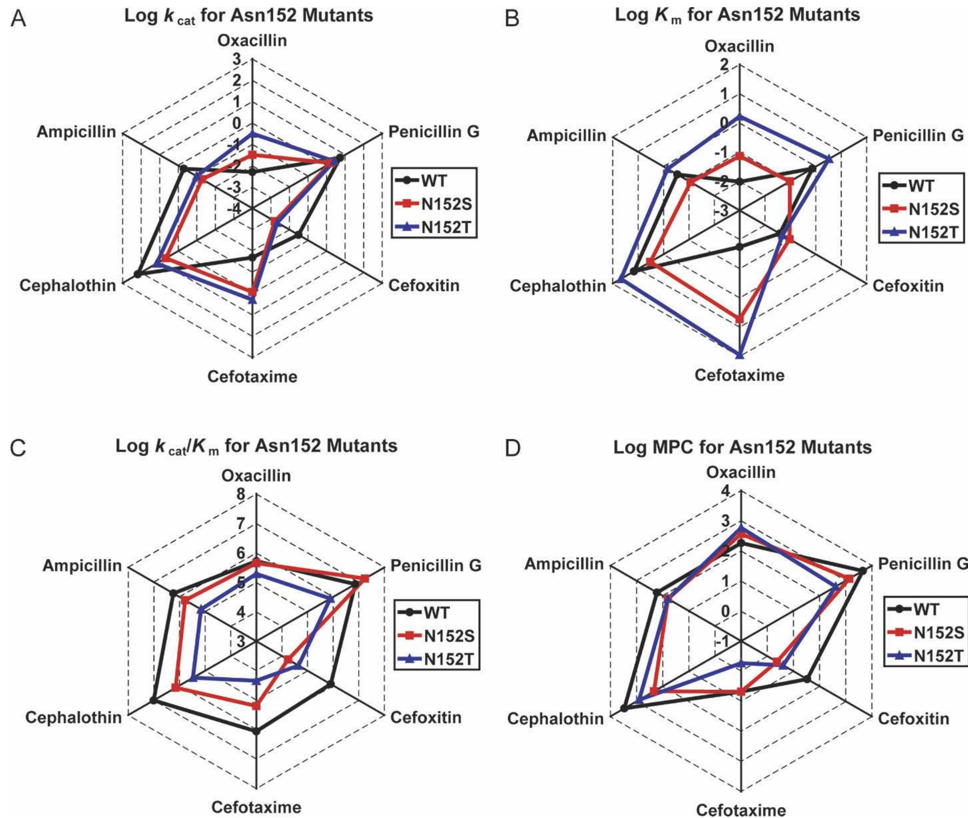


Figure 5. Radar plots of kinetic parameters k_{cat} (*A*), K_m (*B*), k_{cat}/K_m (*C*), and maximal permissive concentration (MPC) (*D*). Values from Table 1 are plotted as logarithms. MPC values were converted to micromolar units prior to taking logarithms. (Black circles) Wild type, (red squares) N152S, (blue triangles) N152T.

(Fig. 6). Asn152Ser shows a strong preference for penicillin G (a penicillin) and against cefoxitin (a cephamycin), with a specificity constant ratio ($k_{cat}/K_{m\ penicillin\ G}/k_{cat}/K_{m\ cefoxitin}$) changed by 150-fold from about nine for the wild type to ~1400 for the mutant (Fig. 6A,B). Asn152Thr, in contrast, shows substrate preferences that are more consistent with those of the wild-type enzyme, indicating that this mutation results in roughly equal decreases in catalytic efficiency with all substrates. However, the preference for oxacillin (a penicillin) relative to cefotaxime (a cephalosporin) is inverted between the wild-type and hydroxyl variants, with the Ser mutant showing a stronger selectivity (Fig. 6C). Thus, Asn152Ser acts as a switch between classes of antibiotics, while Asn152Thr does not. We note that Asn152Thr has relatively high activity against oxacillin, though this mutant is still two orders of magnitude lower in k_{cat}/K_m than class D oxacillinases (Golemi et al. 2001).

An analogous, but distinct, selectivity effect occurred upon the corresponding Asn132Ser mutation in the class A β-lactamase from *Streptomyces albus* G. Class A Asn132Ser showed a 1000-fold decrease in catalytic efficiency toward cephalosporins compared with wild type while retaining efficiency against penicillins, suggesting that this mutation acts as a switch for penicillin versus cephalosporin preference (Jacob et al. 1990a). This selectivity effect is not observed in our class C Asn152Ser enzyme, where the k_{cat}/K_m decrease is similar for ampicillin and cephalothin. Interestingly, the striking activity with oxacillin that is observed in class C Asn152Ser is not observed with class A Asn132Ser. The k_{cat}/K_m for oxacillin is reduced 79-fold for class A Asn132Ser, while in class C Asn152Ser the efficiency is maintained, but with a 6.5-fold higher k_{cat}. While these two point mutations act on analogous residues, it is reasonable to think that the effect of mutation would be different for each class of enzyme, given that the contexts of the mutations (i.e., the neighboring residues) and the catalytic mechanisms are different (Dubus et al. 1995).

The differences in substrate selectivity among the P99 cephalosporinase variants suggest that the precise positioning of the substrate could be a key role of Asn152. Oxacillin (a penicillin) is hydrolyzed 63 times more rapidly by the Asn152Thr mutant than by the wild-type enzyme. In the crystal structure of wild-type *E. coli* AmpC (a related class C enzyme) in complex with a

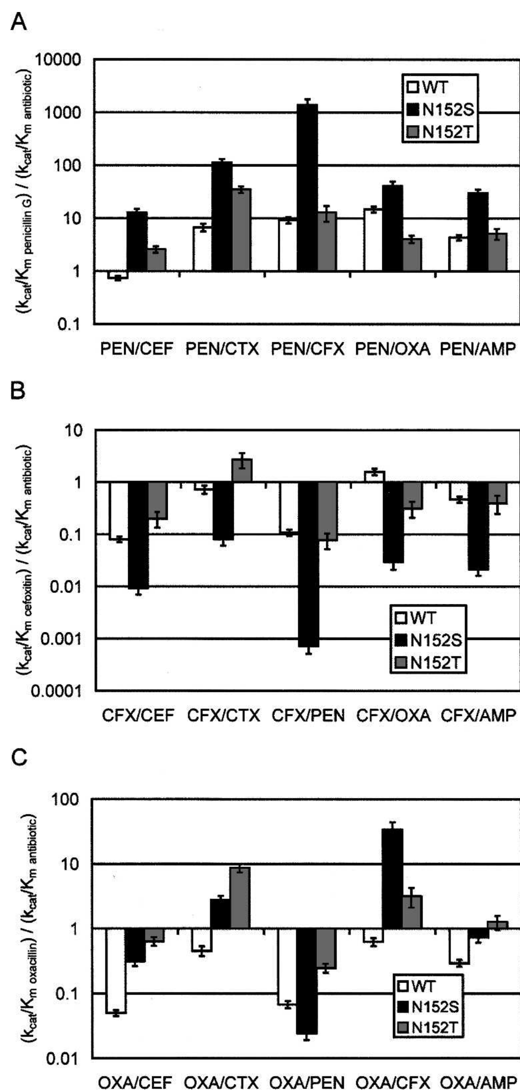


Figure 6. Selectivity of P99 cephalosporinase mutants for penicillin G (*A*), cefoxitin (*B*), and oxacillin (*C*). k_{cat}/K_m ratios for each pair of antibiotics are plotted on a logarithmic scale. (Black circles) Wild type, (red squares) N152S, (blue triangles) N152T. Positive values indicate stronger preference for the antibiotic indicated in the numerator.

boronic acid analog of the cloxacillin acyl intermediate (Caselli et al. 2001), Gln120 has moved away from its usual hydrogen-bonding partner Asn152 (Fig. 7A). Mutation of Asn152 to Thr further alters the geometry of the binding pocket, perhaps allowing the bulky phenylisoxazolyl substituent of oxacillin to adopt a more deacylation-competent conformation, thus speeding the reaction. Alternatively, replacement with Thr could create space for Gln120 to reposition its side chain and participate in substrate binding again, possibly leading to the observed increase in catalytic efficiency.

Cefoxitin (a cephamycin) appears to require the geometry and hydrogen bonding afforded by Asn, given that Asn152Ser grows at a concentration of cefoxitin that is 14 times lower than wild type, with a catalytic efficiency 47 times lower. A model of the cefoxitin acyl intermediate bound to AmpC shows that the 7α-methoxy moiety is pointing toward Asn152, suggesting that this group may contribute to positioning the acyl intermediate for optimal hydrolysis (Fig. 7B). Mutation to Ser or Thr could reduce the strain caused by the methoxy group, resulting in a more stable intermediate that deacylates more slowly. Previously, a crystal structure of AmpC bearing the Asn152Ala mutation in complex with moxalactam suggested that the 7α-methoxy substituent caused strain in the wild-type active site that was relieved upon mutation (Trehan et al. 2001). It is possible that Asn152Ser with cefoxitin behaves similarly. Crystal structures of Asn152Ser and Asn152Thr in complex with these and other substrates could assist in testing these hypotheses.

The in vivo activity screen used in this study was an excellent predictor of in vitro activity. MPC is a complex quantity that depends on membrane permeability, the susceptibility of the penicillin-binding proteins to the antibiotic, and the efficacy of drug efflux pumps, as well as β-lactamase activity. In our experiments, the change

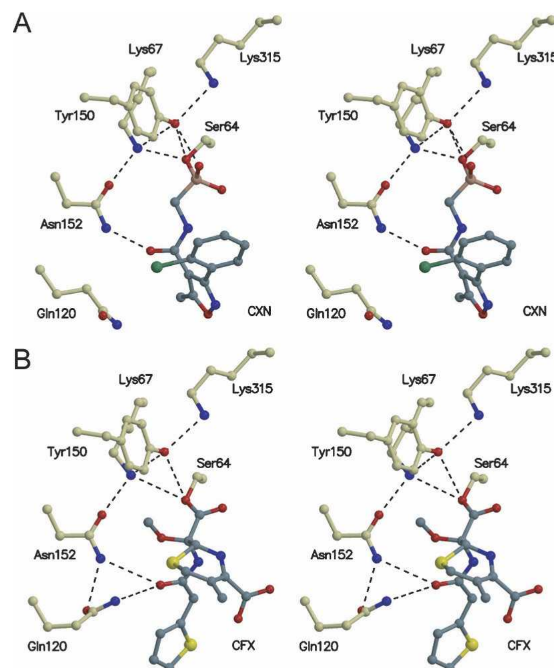


Figure 7. Stereoviews of models of class C β-lactamase with substrates. Models of AmpC, a β-lactamase that is 70% identical to P99 cephalosporinase, illustrate the steric constraints of the active site of class C enzymes with kinetically slow substrates. (*A*) AmpC β-lactamase with acyl intermediate analog cloxacillin boronic acid (CXN) (PDB entry: 1FSY) (Caselli et al. 2001). (*B*) AmpC β-lactamase with cefoxitin (CFX) manually docked in place of cephalothin (PDB entry: 1KVM) (Beadle et al. 2002). The figure was prepared using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt and Bacon 1997).

in MPC upon mutation for an enzyme–substrate pair correlated with the change in k_{cat} in every case except cefotaxime, for which the trend was reversed (Fig. 5A,D). This result was surprising, given that substrate specificity is classically defined by k_{cat}/K_m. A mathematical model by Frère and coworkers posits that the periplasmic level of antibiotic that inhibits bacterial growth should correlate with the velocity of β-lactamase at that concentration of antibiotic (Frère 1989; Frère et al. 1989). Under the conditions of our MPC assay, the external concentrations (and presumably the periplasmic concentrations) of these five antibiotics are well above their estimated K_m values with the wild-type, Asn152Ser, and Asn152Thr enzymes. Therefore, the velocity of the β-lactamase with these substrates is dependent only on k_{cat}, as is reflected by our results. By contrast, the concentration of cefotaxime that impairs growth is well below the K_m of the enzymes tested. Accordingly, the change in MPC value with cefotaxime correlates best with changes in k_{cat}/K_m for Asn152Ser and Asn152Thr. The relationship we have observed between k_{cat} and the protective effect of β-lactamases in vivo may have implications for the design of drugs that target periplasmic enzymes.

The present approach of randomization and screening for residual activity rapidly identified interesting mutants that were overlooked by previous studies of Asn152/132. In these studies, either too few mutants (Dubus et al. 1995) or a limited range of antibiotics and concentrations (Majiduddin and Palzkill 2005) were tested. Interestingly, the latter study actually identified N132S in a saturation library selection in SME-1 β-lactamase, but surmised the mutant was an artifact because it gave a low MIC with the antibiotics tested. Our study circumvented the limitations of previous studies by testing all 19 mutants for residual activity in vivo against a wide concentration range of six substrates, rapidly identifying the interesting mutants for in vitro characterization in an unbiased fashion. The availability of inexpensive high-throughput DNA sequencing greatly improved the efficiency of mutant identification and screening. This approach could be used to probe the effects of mutation of other β-lactamase residues on activity and substrate selectivity and could also be extended to other enzymes for which screens or selections exist.

Materials and Methods

General

Nitrocefin and cefotaxime were purchased from Calbiochem. Ampicillin, penicillin G, oxacillin, cephalothin, and cefoxitin were purchased from Sigma. Restriction enzymes, T4 DNA ligase, and Vent polymerase were purchased from New England Biolabs. *E. coli* TG1 was purchased from Stratagene. *E. coli* BL21(DE3)pLysS was purchased from Invitrogen. The Bug-Buster protein extraction reagent was purchased from Novagen. Oligonucleotides were purchased from Invitrogen. Falcon 96-well U-bottom plates were used for growing bacteria. The 48-pin tool used to transfer growing bacterial cultures to solid agar medium was made by Dan-Kar. Ni-NTA resin and DNA purification kits were purchased from Qiagen. Slide-a-Lyzer dialysis cassettes were purchased from Pierce. Costar UV-transparent 96-well plates were purchased from Corning. CS337–1 *E. coli* (dacA, dacC, ampC) was a gift from Kevin Young (University of North Dakota). A BLAST search (Altschul et al. 1990) of P99 cephalosporinase was carried out at the NCBI server www.ncbi.nlm.nih.gov/BLAST and aligned using ClustalW (Thompson et al. 1994) on the EMBL-EBI server www.ebi.ac.uk.

Library construction

The random library at position 152 was constructed by cassette mutagenesis. The plasmid pSG2034 containing P99:Y150F under control of the TEM-1 promoter was further modified by site-directed mutagenesis using primers SL1774 (5′-GCCT GGCACAACCAGGTCTTTTGCCAACGCCTCAATTGGTCTT TTTGG-3′) and SL1775 (5′-CCAAAAAGACCAATTGAGGC GTTGGCAAAAGACCTGGTTGTGCCAGGC-3′) to introduce unique SexAI and MfeI restriction sites flanking position 152. The modified plasmid, pSL2241, was purified from BL21(DE3) cells, which lack a *dcm* methylase, to facilitate digestion of the unmethylated plasmid by SexAI. Two degenerate oligos, SL1776 (5′-CCAGGCTGTACGCCNNSGCCTC-3′) and SL1777 (5′-AATTGAGGCSNNGGCGTACAG-3′) were annealed and ligated into pSL2241 that had been digested with SexAI and MfeI, restoring the wild-type protein sequence and randomizing position N152. Ligation removes the SexAI site from the final vector, facilitating restriction mapping of library members. One microliter of a 20-μL ligation mixture was electroporated into CS337–1 electrocompetent *E. coli* cells and grown in LB for 1 h prior to plating on LB-kanamycin (30 μg/mL). Dilutions were made to ensure growth of single colonies and to count the library size (10^5). One hundred ninety-two colonies were picked into microtiter plates and sequenced in the region of the mutation. Eight colonies were sequenced in full, confirming that no additional mutations were present in the library.

DNA sequencing

DNA sequencing was carried out on an ABI3730 capillary electrophoresis system. The entire P99 cephalosporinase coding region was amplified by PCR with AccuTaq polymerase (Sigma) using picked colonies suspended in Milli-Q water as a template and primers 1518 (5′-CACTGCGCCTAGCAGGG GCCCCCGGGGCCGGCGTAGAGGATCG-3′) and 1519 (5′-GTCAGCGCATCCCTGCGGCCGGCAGGGCCGTTAGCAGCC GGATCTC-3′). Ten microliters of a suspended colony were added to 10 μL of PCR cocktail (0.2 μL of 20 mM deoxynucleotide triphosphate mix, 0.04 μL each of forward and reverse primers, 2 μL of 10× JumpStart PCR buffer, 0.25 μL of JumpStart Red AccuTaq, 7.47 μL of Milli-Q water) in a 96-well PCR plate (Corning Thermowell Gold). Under these conditions, the dNTPs and primers are almost entirely consumed by the end of the reaction (Pfeffer et al. 2004). The PCR profile was 30 sec at 96°C; 30 cycles of 15 sec at 94°C, 30 sec at 55°C; 5 min at 72°C;

and finally 30 min at 68°C. The PCR product was diluted 2.5-fold with Milli-Q water and primed for cycle sequencing using primer VWC1488 (5′-TATCAGGGAAAACCGCAC-3′) to cover the region containing codon 152. Each reaction consisted of 0.4 μL of BigDye v.3.1 (Applied Biosystems), 7.6 μL of 2.5× buffer (175 mM Tris-HCl pH 9, 1.25 mM $MgCl_2$), 5 μL of diluted PCR product, and 7 μL of ddH2O. The mixture was cycled 25 times (10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C) in a 96-well PCR plate (Applied Biosystems). The cycle sequencing reaction was ethanol precipitated with 25 μL of 120 mM sodium acetate pH 4.5 and 100% ethanol, washed in 70% ethanol, and air dried. Hi-Di formamide (Applied Biosystems) was added (10 μL) to resuspend the DNA, and the plate was vortexed for 3 min immediately prior to denaturing (10 min, 94°C). The plate was then loaded on to the ABI3730 sequencer. Results from a single plate were obtained within 2 h.

In vivo growth screen

A test set of 96 clones comprising all 20 amino acids and a null mutant containing a stop codon was chosen such that each mutation was represented by four to six colonies, with the exceptions of Valine (three), Glutamine (three), and Aspartate (two). These clones were arrayed randomly in a master microtiter plate, with positive and negative controls included in each half of the plate (see Supplemental material). The test set was grown to saturation, and a few microliters were transferred with a 48-pin tool onto LB agar containing 30 μg/mL kanamycin and a β-lactam antibiotic. The cells were grown for 12 h at 37°C and then visually inspected for growth. Clones were scored based on the highest plate in the series of eight concentrations on which their growth was unimpaired (the full data set is presented in the Supplemental material.) The concentrations for maximal permissive concentration (MPC) determination were chosen based on control experiments with the wild-type and nonsense-mutated constructs. The highest concentration on which the nonsense mutated (negative control) grew was designated ‘‘1,’’ and the highest concentration on which wild type grew was designated ‘‘7.’’ The intervening five concentrations were distributed according to an exponential scale. Plate ‘‘8’’ contained a concentration of antibiotic on which wild type did not grow. A score of zero was assigned to clones that did not grow on the lowest plate. By using a large number of concentrations, small differences in activity could be detected among the mutants, and the relative activity of a mutant could be roughly compared from one drug to another, facilitating the detection of substrate selectivity changes.

Expression and purification of wild type, Asn152Ser, and Asn152Thr P99 cephalosporinase

The P99 cephalosporinase genes from the library plasmid of clones for wild type, Asn152Ser and Asn152Thr were amplified using primers VWC68 (5′-GCATACGTCCATATGATGAGA AAATCCCTTTG-3′) and VWC69 (5′-GCATTGCTGAAGCT TAGTGGTGGTGGTGGTGGTGCTGTAGCGCCTCGAGG-3′), which added NdeI and HindIII restriction sites to the ends of the coding region, as well as a C-terminal His_6-tag. The PCR fragments were digested and ligated into the pET26 T7 expression vector (Novagen). The constructs were sequenced in full to verify the mutations (Genewiz). Wild-type, Asn152Ser, and Asn152Thr expression vectors were electroporated into BL21-DE3 pLysS *E. coli* cells and selected on LB-Kan. Colonies were picked and grown for 5 h at 37°C with shaking. Seed pellets were made from 100-μL aliquots that were centrifuged for 3 min at 14,000 rpm and aspirated; the seeds were frozen at −80°C. Single seeds were used to inoculate 500-mL cultures of LB medium supplemented with 30 μg/mL kanamycin. These cultures were grown in a shaker incubator at 37°C and 200 rpm to an OD_{600} of 0.6 before being induced with 100 μM IPTG. The induced cultures grew for 3–4 h under the same conditions and were then harvested by centrifugation at 5000*g* for 5 min at 4°C. Pellets were frozen at −80°C. Thawed pellets were resuspended in BugBuster lysis reagent according to the manufacturer’s protocol and lysed for 20 min shaking at room temperature. Lysates were centrifuged at 15,000*g* for 20 min at 4°C. The soluble fraction was passed over 2 mL of Ni-NTA resin that had been equilibrated with lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole). Two washes (8-mL) with wash buffer (lysis buffer with 20 mM imidazole) followed. The protein was eluted by a step gradient of elution buffer, with the majority of the P99 cephalosporinase eluting at 100–150 mM imidazole. The elution fractions were pooled and dialyzed versus phosphate-buffered saline with three changes at 1 h intervals. The dialyzed protein was stored at 4°C and was stable for several weeks. The protein was >95% pure as judged from Coomassie Brilliant Blue staining. The yield from a typical purification was 2 mg/L. The concentration of purified proteins was determined spectrophotometrically at 280 nm, using an extinction coefficient of 71,000 $M^{-1}cm^{-1}$ (Joris et al. 1985).

Kinetic characterization

Measurements of hydrolysis rates for six antibiotics were carried out on a Spectramax Plus 384 microplate UV spectrophotometer (Molecular Devices). β-lactam substrates were prepared at 100 mM in DMSO (50 mM for cephalothin), aliquotted, and frozen at −80°C. For each kinetic trial, a single 10-μL aliquot was thawed. Final concentrations of DMSO in kinetic measurements were always well below 1% v/v and had no discernible effect on activity compared with phosphate buffer alone. The extinction coefficients used were: ampicillin, 236 nm, $\Delta\varepsilon = -900\ M^{-1}cm^{-1}$; penicillin G, 233 nm, $\Delta\varepsilon = -1140\ M^{-1}cm^{-1}$; cephalothin, 262 nm, $\Delta\varepsilon = -7660\ M^{-1}cm^{-1}$; cefotaxime, 264 nm, $\Delta\varepsilon = -7250\ M^{-1}cm^{-1}$; oxacillin, 260 nm, $\Delta\varepsilon = +450\ M^{-1}cm^{-1}$ (Franceschini et al. 2001); cefoxitin, 260 nm, $\Delta\varepsilon = -7700\ M^{-1}cm^{-1}$ (Tribuddharat et al. 2003); Nitrocefin, 486 nm, $\Delta\varepsilon = -17{,}400\ M^{-1}cm^{-1}$ (Crowder et al. 1998). For cephalothin and cefotaxime, hydrolysis rates were determined with 1–100 nM enzyme at seven substrate concentrations spanning $0.2 \times K_m$ to $5 \times K_m$, and these rates were fitted to the Michaelis-Menten equation $v_o = V_{max} \times [S] / (K_m + [S])$ by nonlinear regression analysis to determine k_{cat} and K_m (Kaleidagraph, Synergy Software). For penicillin G, ampicillin, oxacillin, and cefoxitin, as well as cefotaxime with the wild-type enzyme, the K_m was too low to be determined by initial rate kinetics, so the inhibitor dissociation constant (K_i) was determined by varying the concentration of these antibiotics in the presence of 200 or 400 μM of the chromogenic reporter substrate nitrocefin and 0.25–3 nM protein. The initial rates of nitrocefin hydrolysis were fit to the Michaelis-Menten equation with competitive inhibition using nonlinear regression analysis to determine K_i (Kumar et al. 2004). The k_{cat} for penicillin G, ampicillin, oxacillin, and cefoxitin was determined by measuring hydrolysis directly at a saturating concentration of substrate, typically 200–500 μM, well above the measured K_i values. In a

typical experiment, 180 μL of enzyme at 2–5 μM was added to 20 μL of substrate at 10× concentration in the microtiter plate to avoid dilution of the enzyme. When an enzyme–substrate pair did not show measurable rates by direct detection, a competition assay with the reporter substrate nitrocefin allowed determination of k_3 as described (Fisher et al. 1980). Briefly, 200 nM enzyme was pre-incubated with 2000 nM substrate for 10 min and then diluted 400-fold in 400 μM nitrocefin. Hydrolysis of nitrocefin was followed for 1 h, and k_3 was extracted from the reaction progress curve as described (Glick et al. 1978). This procedure was used for oxacillin with wild type and cefoxitin with Asn152Ser and Asn152Thr.

Molecular modeling

A model of the 70% identical AmpC β-lactamase with the antibiotic oxacillin covalently bound in the active site was made by manual docking using the XtalView software package (McRee 1999). P99 and AmpC have identical active sites, and thus crystal structures of AmpC can be considered good structural models for P99. The model of AmpC with a cefoxitin acyl intermediate bound was produced by manual docking of cefoxitin onto the structurally similar cephalothin acyl intermediate covalently bound to AmpC in the structure 1KVM (PDB entry: 1KVM) (Beadle et al. 2002). Inter- and intramolecular steric clashes of the 7α-methoxy group of cefoxitin were removed by manual rotation around its C–O torsion angle.

Electronic supplemental material

Photographs of MPC growth assay plates, MPC scores for all mutants, and Michaelis-Menten plots are included in the supplemental research data.

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