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N152G, -S, and -T Substitutions in CMY-2 β-Lactamase Increase Catalytic Efficiency for Cefoxitin and Inactivation Rates for Tazobactam

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Class C cephalosporinases are a growing threat, and clinical inhibitors of these enzymes are currently unavailable. Previous studies have explored the role of Asn152 in the *Escherichia coli* AmpC and P99 enzymes and have suggested that interactions between C-6′ or C-7′ substituents on penicillins or cephalosporins and Asn152 are important in determining substrate specificity and enzymatic stability. We sought to characterize the role of Asn152 in the clinically important CMY-2 cephalosporinase with substrates and inhibitors. Mutagenesis of CMY-2 at position 152 yields functional mutants (N152G, -S, and -T) that exhibit improved penicillinase activity and retain cephamycinase activity. We also tested whether the position 152 substitutions would affect the inactivation kinetics of tazobactam, a class A β-lactamase inhibitor with *in vitro* activity against CMY-2. Using standard assays, we showed that the N152G, -S, and -T variants possessed increased catalytic activity against cefoxitin compared to the wild type. The 50% inhibitory concentration (IC_{50}) for tazobactam improved dramatically, with an 18-fold reduction for the N152S mutant due to higher rates of enzyme inactivation. Modeling studies have shown active-site expansion due to interactions between Y150 and S152 in the apoenzyme and the Michaelis-Menten complex with tazobactam. Substitutions at N152 might become clinically important as new class C β-lactamase inhibitors are developed.

Class C β-lactamases such as CMY-2, found in Gram-negative pathogens, confer resistance to a wide variety of β-lactam antibiotics, including narrow- and extended-spectrum cephalosporins and penicillins (1). When combined with other resistance mechanisms, such as porin loss or efflux (2–5), or when increased expression occurs in derepressed strains (1, 6), organisms expressing class C β-lactamases become resistant to cefepime and carbapenems. Point mutations and deletions in the omega loop or helix H2 or H10 and near the C terminus of the AmpC β-lactamases that cause an extended-spectrum AmpC (ESAC) phenotype have been described (1, 7). Of the CMY enzymes, 97 unique types have been described to date (see http://www.lahey.org/Studies), including enzymes such as CMY-30, CMY-32, CMY-33, CMY-37, and CMY-44 (8–12), with alterations of the omega loop or the H10 helix. Recently, Dahyot and Mammeri described a ceftazidime- and cefepime-hydrolyzing CMY-2 laboratory variant based on a clinical mutant in which a Y-X-N loop mutation (R148H) accounted for the ESAC phenotype (13). Little has been written about the behavior of the ESAC variants in regard to β-lactamase inhibitors, although a tazobactam-susceptible H-10 helix variant of *Escherichia coli* AmpC has been described (14).

Previous studies on the Y-X-N loop of class C β-lactamases explored the role of N152 in the *E. coli* AmpC (15) and P99 (16) enzymes and suggested that interactions between C-6′ or C-7′ substituents of penicillins or cephalosporins and N152 are important in determining substrate specificity and enzymatic stability. We sought to characterize the role of N152 in the clinically important CMY-2 cephalosporinase to help us anticipate new resistance phenotypes. Based upon an examination of the current structural evidence, we hypothesized that, since N152 makes important interactions with substrates and inhibitors and is essential for enzymatic function, inhibitors with unique C-6′ and C-7′ substituents might enhance affinity and be more durable against resistance mutations. Since N152 is a highly conserved residue in class C β-lactamases, it was necessary to determine first whether there were functional variants of CMY-2 at position 152 and, second, whether any of the variants showed altered phenotypes with substrates and inhibitors such as carbapenems and tazobactam. The studies presented in this paper reveal that N152 substitutions indeed are tolerated in CMY-2 β-lactamase. In addition, the N152G, -S, and -T variants of CMY-2 possess increased cephamycinase activity as well as an unusual sensitivity to tazobactam. Molecular modeling suggests that movement of other proximate active-site residues is responsible for the "extended-spectrum β-lactamase"-like phenotype demonstrated by these enzymes.

MATERIALS AND METHODS

Mutagenesis. Variants at position 152 of CMY-2 β-lactamase were prepared by site saturation mutagenesis using a Stratagene QuikChange kit with bla_{CMY-2} directionally subcloned in the pBCSK(−) vector, as described previously (17, 18), and confirmed by DNA sequencing of 96 randomly selected clones. Mutagenesis and sequencing primers are shown in Table S1 in the supplemental material. The entire bla_{CMY-2} gene was sequenced for each variant. This initial screening yielded 12 of the 19 variants. Mutagenesis primers were designed to prepare the N152A, -C,

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-F, -L, -P, -Q, and -Y variants by site-directed mutagenesis and were confirmed by DNA sequencing.

MIC determinations. MICs were determined for all 19 variants at position 152 by agar dilution using ampicillin, piperacillin, cefoxitin, ceftriaxone, cephalothin, and cefotaxime (all from Sigma), ceftazidime (Hoffman-LaRoche), cefepime and aztreonam (Bristol Meyers Squibb), and piperacillin with tazobactam (Pfizer) in Mueller-Hinton agar according to Clinical and Laboratory Standards Institute guidelines (19). Tazobactam was used with piperacillin in a fixed 8:1 ratio. Modal MIC values are reported for 3 to 5 determinations.

Immunoblots. To prepare samples, *E. coli* DH10B cells transformed with pBCSK(−) bla_{CMY-2} with all 19 N152 variants and the wild type (WT) were grown in lysogeny broth to an optical density at 600 nm (OD_{600}) of 0.8. Five milliliters at this cell density was pelleted at 12,000 rpm for 2 min. After discarding the supernatant, the pellet was resuspended in 80 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye and boiled at 100°C for 10 min. Immunoblotting was performed with the samples as described previously (20). The membrane was probed with rabbit polyclonal anti-CMY-2 antibody at 1.0 μg/ml in blocking buffer, and bands were detected with protein G-horseradish peroxidase conjugate (Bio-Rad). The blot was developed using an ECL developing kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. The gels were analyzed using ImageJ software (http://rsb.info.nih.gov/ij), and expression levels were normalized to the WT expression level using the major band with a molecular mass of 39.7 kDa.

Protein purification. CMY-2 and N152G, -S, and -T CMY-2 β-lactamases were expressed in *E. coli* AS226-51 cells (Invitrogen), derived from a strain that does not produce a chromosomal AmpC β-lactamase. As described previously, crude extracts were purified to homogeneity by preparative isoelectric focusing and gel filtration or cation exchange chromatography using a GE Superdex 75 10/300 column with 1× phosphate-buffered saline (pH 7.4) or GE HiTrap Q HP columns with 50 mM HEPES (pH 7.0) and elution buffer consisting of 50 mM HEPES and 1 M NaCl (pH 7.0) (20). Protein purity was assessed with SDS-PAGE and was >95%. Samples were concentrated and then used directly in kinetic assays. Protein concentrations were determined with the Bradford assay (21) using bovine serum albumin as the standard.

Kinetic assays. Standard multiple-turnover kinetic analyses of reaction velocity versus substrate concentration were performed using 5 nM CMY-2 or N152G, -S, and -T CMY-2 in phosphate-buffered saline (pH 7.4). β-Lactam hydrolysis was monitored at ambient temperature (23 to 26°C) using an Agilent 8453 diode array spectrophotometer in a total reaction volume of 500 μl. Assays were performed with nitrocefin (NCF) (Becton-Dickson, Cockeysville, MD) ($\Delta\varepsilon_{482}$ = 17,400 M^{-1} cm^{-1}), cefoxitin (Sigma) ($\Delta\varepsilon_{260\ nm}$ = −7,700 M^{-1} cm^{-1}), and cefoperazone (Sigma) ($\Delta\varepsilon_{275\ nm}$ = −8,640 M^{-1} cm^{-1}). The steady-state kinetic parameters V_{max} and K_m were obtained by fitting reaction curves to the Michaelis-Menten equation using Origin 8.0 (OriginLab, Northampton, MA). When hydrolysis could not be measured directly, a competition assay using NCF as a reporter substrate was performed; an apparent K_m is reported for the compound, correcting for the NCF K_m (22). We initiated the reaction with the addition of enzyme to a mixture of NCF and the poor substrate and took initial velocity measurements during the first 5 to 10 s of the progress curves to better approximate formation of the Michaelis-Menten complex (23).

Inhibitor assays. We determined the 50% inhibitory concentration (IC_{50}) ratios (with 5-min preincubation) for tazobactam using 4 nM CMY-2 protein and 200 μM nitrocefin as a reporter substrate. Data were fitted to the following equation to plot the fractional velocity versus [I] (the inhibitor concentration) as follows: $V_i/V_o = 1/[1 + ([I]/IC_{50})]$ (24). The IC_{50} is time dependent for suicide inhibitors, and the most commonly used time point in the literature (5 min) was selected to enable comparisons with values in the literature. K_I (the inhibitor concentration required for half-maximal inactivation) and k_{inact} were determined by using timed inactivation, i.e., mixing fixed amounts of enzyme (4 nM) and variable concentrations of inhibitor and, at various time points (0, 15, 30, 60, 120, 300, and 600 s), assaying an aliquot of the mixture with NCF as the substrate (20, 25). The timed-inactivation plots were fitted with a single-exponential decay to obtain the apparent inhibition constant (k_{app}) for each inhibitor concentration. The k_{app} values were plotted versus the inhibitor concentration to obtain the k_{inact} and K_I values using the equation $k_{app}[I] = k_{inact}/(1 + K_I/[I])$. Tazobactam follows a complex inhibition pathway given by the following reaction (according to the method of Stachyra et al. [26]):

$$E + I \underset{K_i = k_{-1}/k_1}{\rightleftharpoons} EI \xrightarrow{k_2} EI^{*} \xrightarrow{k_3} E + P \qquad (1)$$
$$\Big\Downarrow k_4$$
$$EI^{**}$$

EI* represents the imine that forms following the initial acylation step of serine β-lactamases and undergoes formation of a stable inhibited complex, EI**, at a rate k_4, which represents, for most serine enzymes, formation of the stable (albeit not the final) inhibition intermediate, the *trans*-enamine (26–28). Here, k_{inact}/K_I represents the inactivation efficiency, where $k_{inact} = k_2k_4/(k_2 + k_3 + k_4)$ (26).

Molecular modeling. The CMY-2 β-lactamase structure (Protein Data Bank entry 1ZC2) was used to generate the model of N152S with Discovery Studio molecular modeling software (DS 2.1) (Accelrys, Inc., San Diego, CA) as described previously (29). The protein report and utility tools were used to correct crystallographic disorder, remove all alternative conformations, add missing atoms, and correct connectivity and bond order. The crystallographic waters were removed, and the enzyme was immersed in a box of water, 7 Å from any face of the box (solvation module of DS 2.1), using explicit periodic boundary conditions. The complex was minimized in several steps using the steepest-descendant and conjugate gradient algorithms to reach the minimal convergence (0.01 kcal/[mol · Å]). All energy minimizations and molecular dynamics simulations of the enzyme and enzyme complexes were carried out using the force field parameters of CHARMM. The particle mesh Ewald (PME) method was used to treat long-range electrostatics, and bonds that involved hydrogen atoms were constrained with the SHAKE algorithm.

For the tazobactam Michaelis-Menten complex, the minimized and equilibrated N152S CMY-2 variant model was used. The ligand structure was built using the fragment builder tools of DS 3.1. The minimized ligand was docked in the active site of the enzyme model using the flexible docking algorithm. The protocol allowed placement of a flexible ligand in the flexible active site of the CMY-2 variant. The conformations generated were visually inspected, and the most favorable ones were chosen (tazobactam carbonyl in the oxyanion hole formed by the Ser64 and Ser318 backbone amide NH groups). The complex was created and minimized using the conjugate gradient algorithm, with the generalized Born model for the solvent.

RESULTS AND DISCUSSION

Mutagenesis and expression of N152 CMY-2 variants: substitution reduces expression levels of N152 CMY-2 variants. Screening of 96 clones by DNA sequencing yielded 12 variants. The remaining variants (N152C, -A, -P, -Q, -L, -Y, and -F) were obtained by site-directed mutagenesis. We used a polyclonal antibody to assess steady-state production of β-lactamase by the N152 variants to help interpret MIC findings (18, 29–32). Notably, the anti-CMY-2 antibody detects *E. coli* CMY-2, *Enterobacter cloacae* P99, *Klebsiella pneumoniae* ACT-1, and the AmpC β-lactamases of *Enterobacter aerogenes*, *Morganella morganii*, and *Citrobacter freundii*. We saw no cross-reactivity of the anti-CMY-2 antibody against laboratory strains of *E. coli* possessing TEM-1, SHV-1, K-1, or OXA-1 β-lactamases (32), and there was no nonspecific binding to other cellular proteins. In these systems, which

TABLE 1 MICs for N152 CMY-2 variants and relative expression levels of N152 variants, as determined in Western blots probed with a polyclonal anti-CMY-2 antibody, relative to WT CMY-2 β-lactamase expression levels

Variant	MIC (μg/ml)[a]										Relative expression level (±10%)
	ATM	AMP	PIP	TZP 8:1	CAZ	FOX	CRO	CEF	CTX	FEP	
DH10B	≤0.06	≤4	≤2	≤2/0.25	≤0.25	≤4	≤0.015	≤4	≤2	≤0.015	0
WT	2	128	8	4/0.5	16	32	≤0.015	1,024	4	≤0.015	100
N152A	≤0.06	≤4	≤2	≤2/0.25	0.5	8	≤0.015		≤2	≤0.015	66
N152C	≤0.06	8	4	≤2/0.25	1	8	≤0.015			≤0.015	39
N152D	≤0.06	≤4	≤2	≤2/0.25	0.5	8	≤0.015			≤0.015	25
N152E	≤0.06	8	≤2	≤2/0.25	≤0.25	8	≤0.015			≤0.015	33
N152F	≤0.06	≤4	≤2	≤2/0.25	≤0.25	8	≤0.015			≤0.015	35
N152G	0.25	64	16	≤2/0.25	4	8	≤0.015	64	≤2	≤0.015	49
N152H	≤0.06	≤4	≤2	≤2/0.25	1	8	≤0.015			≤0.015	27
N152I	≤0.06	8	8	≤2/0.25	0.5	8	≤0.015	≤4	≤2	≤0.015	14
N152K	≤0.06	≤4	≤2	≤2/0.25	1	8	≤0.015	≤4	≤2	≤0.015	10
N152L	≤0.06	≤4	4	≤2/0.25	0.5	≤4	≤0.015	≤4	≤2	≤0.015	65
N152M	≤0.06	8	4	≤2/0.25	0.5	8	≤0.015			≤0.015	25
N152P	≤0.06	≤4	≤2	≤2/0.25	≤0.25	≤4	≤0.015	≤4	≤2	≤0.015	60
N152Q	≤0.06	8	≤2	≤2/0.25	≤0.25	≤4	≤0.015	≤4	≤2	≤0.015	45
N152R	≤0.06	8	≤2	≤2/0.25	≤0.25	8	≤0.015			≤0.015	33
N152S	0.25	32	16	≤2/0.25	8	≤4	≤0.015	128	≤2	≤0.015	51
N152T	0.25	64	32	≤2/0.25	4	8	≤0.015	64	≤2	≤0.015	59
N152V	≤0.06	≤4	2	≤2/0.25	≤0.25	8	≤0.015			≤0.015	32
N152W	0.125	≤4	2	≤2/0.25	≤0.25	8	≤0.015	≤4	≤2	≤0.015	27
N152Y	≤0.06	≤4	2	≤2/0.25	0.5	8	≤0.015			≤0.015	45

[a] MICs are the mode of 3 to 5 determinations. ATM, aztreonam; AMP, ampicillin; PIP, piperacillin; TZP 8:1, piperacillin and tazobactam in a fixed ratio of 8:1; CAZ, ceftazidime; FOX, cefoxitin; CRO, ceftriaxone; CEF, cephalothin; CTX, cefotaxime; FEP, cefepime.

possess intact secretion systems, the protein detected is the fully processed β-lactamase enzyme, which correlates with MIC values (33).

The reactivity of the anti-CMY-2 antibody has been well characterized and described (30, 32). The facts that the antibody is polyclonal in nature and recognizes multiple epitopes ensure that a single amino acid substitution will not influence recognition and allows one to use the antibody to assess the overall expression of these CMY-2 variant proteins. Additionally, for the N152 variants, this amino acid is not included in the major epitopes (30). The N152 CMY-2 variants had variable expression levels, ranging from 10% (N152I) to 65% (N152A) of WT levels (Table 1), as determined by Western blotting using the anti-CMY-2 polyclonal antibody. These results suggest that N152 substitution has an effect on enzyme stability, as previously suggested (34).

MIC testing: N152G, -S, and -T CMY-2 variants retain activity against penicillins and cephalosporins. Previous investigations of the N152 site in class C β-lactamases identified the importance of this residue in conferring a substrate "selectivity switch" in bacteria expressing AmpC enzymes. Lefurgy et al. examined the MICs of all 19 variants of P99 β-lactamase at the N152 position and found that the N152G, -S, and -T variants demonstrated higher MICs against penicillins (penicillin G and ampicillin) and cephalothin, but not against cefoxitin or cefotaxime, than the other variants (16), indicating a selectivity switch from cephalosporinase to penicillinase activity in bacteria expressing N152 P99 variant β-lactamases.

In the case of CMY-2, MIC testing showed that the N152G, -S, and -T CMY-2 variants retained activity versus ampicillin (32 to 64 μg/ml), penicillin G (>64 μg/ml) (data not shown), and piperacillin (8 to 64 μg/ml) despite reduced levels of β-lactamase expression (~50% of the WT level) (Table 1). The N152T strain exhibited 4-fold-higher MICs against piperacillin than the WT (64 versus 16 μg/ml). N152G, -S, and -T retained WT activity versus cefoperazone (0.5 to 1 μg/ml) (data not shown). All of the variants were susceptible to ceftazidime, although N152G, -S, and -T had higher MICs than the other variants, especially N152S, with an MIC of 8 μg/ml. Similar findings were observed with aztreonam, with higher MICs for the N152G, -S, and -T variants (0.25 μg/ml) than the variants with other substitutions. With the exception of the N152L, -P, -Q, and -S variants, all of the constructs had an MIC of 8 μg/ml for cefoxitin. All the variants were susceptible to ceftriaxone, cefepime, meropenem, imipenem, ertapenem (data not shown for the latter three antibiotics), and piperacillin in combination with tazobactam (≤2/0.5 μg/ml). Thus, the selectivity switch seen for P99 with position 152 variants (16) does not appear to occur in CMY-2.

Kinetics of substrate turnover: retention of cephalosporinase activity in N152 CMY-2 variants. Standard multiple-turnover kinetics (steady state) (Table 2) showed that the N152G, -S, and -T variants were able to hydrolyze nitrocefin with catalytic efficiencies 28 to 57% of the WT enzyme values. However, the catalytic efficiency against cefoxitin was increased in the variants, especially N152S CMY-2, which showed a 3.6-fold increase in k_{cat}/K_m. There was also a 1.8-fold increase in the catalytic efficiency against cefoperazone, an extended-spectrum cephalosporin with a bulky piperacillin-like R1 side chain. Comparing the N152S variants of CMY-2 and N152S P99, the difference in catalytic efficiency against cefoxitin increased, with N152S CMY-2 having an ~500-fold-higher k_{cat}/K_m than the P99 variant (16). Similar results were noted for the hydrolysis of extended-spectrum cephalosporins such as cefoperazone. Despite the increased MICs against penicillins, these proved to be difficult substrates with which to monitor hydrolysis directly, similar to findings seen with

TABLE 2 Kinetic constants for selected N152 variants and substrates[a]

β-Lactamase	Nitrocefin			Cefoxitin			Cefoperazone		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
WT CMY-2	$(4 \pm 1) \times 10^2$	32 ± 8	12 ± 2	35 ± 8	14 ± 2	2.5 ± 0.2	40 ± 10	10 ± 2	3.9 ± 0.5
N152G	$(4 \pm 1) \times 10^2$	90 ± 20	3.9 ± 0.4	40 ± 20	16 ± 4	2.3 ± 0.4	39 ± 7	11 ± 2	3.5 ± 0.5
N152S	$(3.2 \pm 0.6) \times 10^2$	46 ± 5	6.9 ± 0.5	53 ± 7	6.0 ± 0.4	8.9 ± 5	40 ± 10	5 ± 1	7 ± 1
N152T	$(4 \pm 1) \times 10^2$	120 ± 20	3.2 ± 0.6	40 ± 20	13 ± 3	3.2 ± 0.6	30 ± 10	8 ± 2	4.2 ± 0.6

[a] Determinations of kinetic parameters were performed in triplicate, and values are reported as means ± standard deviations.

the P99 enzymes (16). An earlier study (35) measured ampicillin K_m values for CMY-2 (0.16 ± 0.02 μM for a plasmid-derived enzyme and 0.2 μM for a chromosomally derived enzyme), which are in good agreement with the value reported here. In competition assays, the N152G, -S, and -T variants demonstrated significantly lower apparent K_m values for ampicillin, especially the N152S variant (Table 3), similar to findings seen with the P99 enzyme [N152S P99, $K_m(K_i)$ = 86 nM; N152S CMY-2, $K_m(K_i)$ = 19 nM] (16).

Dubus et al. (15) showed that the N152E, -D, -H, and -L variants of *Escherichia coli* AmpC β-lactamase lost activity versus substrates and inhibitors, with effects on both acylation and deacylation rates. Similar results were found by Trehan et al., who examined the N152A-substituted AmpC of *E. coli* (36). They concluded that some mutations at position 152 led to less destabilization of AmpC when it was inhibited by poor substrates, such as imipenem and moxalactam, that possess bulky C-6′ or C-7′(α) substituents that interfere sterically with N152 in class C β-lactamases or N132 in class A β-lactamases. However, for the N152A AmpC variant, there was also an overall catalytic cost to the N152-substituted enzyme. Lefurgy et al. examined the kinetic properties of N152S and -T P99 variants with penicillin and cephalosporin substrates and concluded that hydrogen bonding of S152 or T152 to K67 or to the substrate accounted for the preservation of catalytic activity (16). They hypothesized that a water molecule can play a catalytic role in the N152G variant of P99, as has been shown for S130G SHV (25, 37, 38). The recently reported crystal structure of the N152G P99 variant shows considerable space left by the N152G mutation, occupied by the His_6 tag at the C terminus of the protein (39).

Overall, the WT P99 enzyme is less active than CMY-2 for its signature substrate, cefoxitin, with an ~20-fold-higher k_{cat}/K_m for the CMY-2 enzyme, as shown in our study and in previous reports (35). The increased catalytic efficiency of the N152S and -T variants for cefoxitin is likely a consequence of improved H-bonding to the R1 side chain of cefoxitin or a reduction in steric hindrance at this site in the protein, as suggested by the model of N152S CMY-2. Expansion of the active site might also occur with the N152G CMY-2 variant.

TABLE 3 Apparent $K_m(K_i)$ and inhibition values for select N152 variants[a]

β-Lactamase	Ampicillin, $K_m(K_i)$ (nM)	Tazobactam		
		IC_{50} (nM)	K_I (μM)	k_{inact} (s^{-1})
WT CMY-2	240 ± 20	3,200 ± 400	50 ± 20	0.050 ± 0.006
N152G	120 ± 10	900 ± 200	60 ± 20	0.25 ± 0.04
N152S	19 ± 2	180 ± 30	50 ± 20	0.30 ± 0.06
N152T	47 ± 5	330 ± 30	30 ± 20	0.12 ± 0.03

[a] Values are reported as means ± standard deviations.

The individual k_{cat} and K_m values for CMY-2 with cefoxitin reported here vary from previously reported values (35); nonetheless, there is excellent agreement in the reported catalytic efficiency, k_{cat}/K_m, which is a more robust parameter in steady-state kinetic determinations. The differences in k_{cat} and K_m are likely due to the limitations of standard kinetic measurements with poor substrates such as cefoxitin, as well as differences in expression, purification, and quantification of protein concentrations. Without the use of stopped-flow methods, initial lag-phase kinetics might be missed, resulting in larger k_{cat} values. Small absorbance changes at low cefoxitin concentrations might also affect determinations of K_m.

N152G, -S, and -T CMY-2 variants are more susceptible to inhibition by tazobactam because of more rapid inactivation. When examining the inhibition properties of the N152G, -S, and -T variants, it was noted that the IC_{50}s for tazobactam were decreased significantly for the variants (Table 3). Timed-inactivation experiments using nitrocefin as a reporter substrate revealed that, for tazobactam, the K_I values for the variants were similar to those for the WT enzyme, but the rates of inactivation were greater.

Previously, the inhibition complex of CMY-2 with tazobactam was examined and found to have a K_i of 40 μM for this inhibitor (40). The UV absorbance characteristics of the tazobactam intermediate suggested the presence of an enamine intermediate. An intact adduct of 266 Da was demonstrated by mass spectrometry. Subsequent studies with other serine β-lactamases have demonstrated the importance of the *trans*-enamine as the stable inhibition intermediate that predominates (27, 28, 41, 42). Preliminary X-ray crystallographic data for the N152S CMY-2 variant with tazobactam indicate formation of a stable *trans*-enamine intermediate (34).

Our study is the first to examine the role of the N152 residue in the kinetics of inactivation with tazobactam. Inactivation by mechanism-based inhibitors specifically designed to inhibit class A serine β-lactamases is a complex chemical process that has been well reviewed in the literature (43). Depending on the time point at which one examines the complex, different inhibitor intermediates predominate in the inactivation. Each intermediate might be deacylated from the enzyme, with the possible exception of the final inhibition product, which is covalently attached to Ser130 in the class A β-lactamases. Furthermore, the reaction is rapid and irreversible so that kinetic parameters for early reversible events of inhibition are influenced by irreversible events. Thus, parameters that describe reversible processes might be difficult to capture cleanly, and time-dependent parameters such as the IC_{50} might be

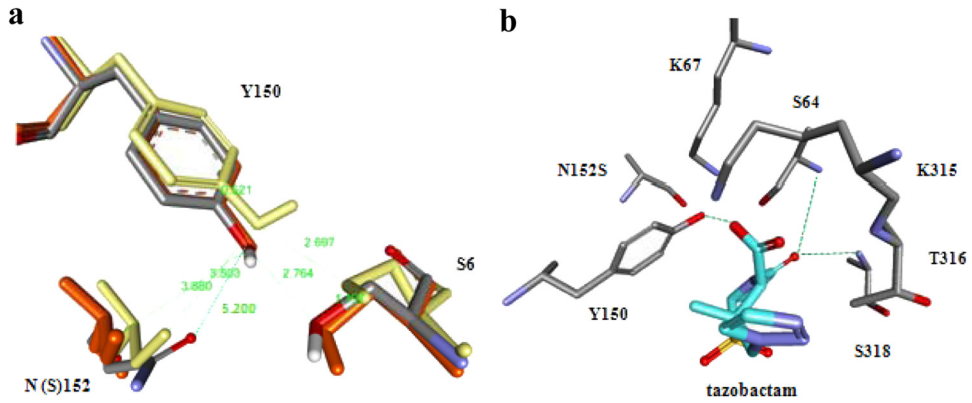

FIG 1 (a) Molecular model of CMY-2 (atoms colored by type) superimposed on the N152S variant (orange) and the N152S CMY-2:tazobactam Michaelis-Menten complex (yellow, shown without tazobactam), demonstrating a decrease from the N152-O-δ–Y150-OH distance (5.2 Å) to the S152-O-γ–Y150-OH distance (3.9 Å), enlarging the active site to accommodate R1 side chains. In the Michaelis-Menten complex, this distance decreases further to 3.5 Å. The distance between the catalytic serine at position 64 and Tyr150, thought to be the general base that activates the serine OH for attack on the β-lactam carbonyl, does not vary in these constructs. (b) Michaelis-Menten complex of tazobactam with the N152S CMY-2 variant, with the inhibitor carbonyl moiety in the oxyanion hole formed by the backbone nitrogens of S64 and S318. The S152-O-γ–Y150-OH distance is decreased further to 3.5 Å, from 4 Å, when tazobactam is docked in the active site.

hard to compare among different inhibitors and different enzymes. In comparing CMY-2 with other class C β-lactamases, one finds that the IC_{50} for tazobactam is higher for CMY-2 than for P99 or the AmpC β-lactamase of *E. coli* (IC_{50}s for tazobactam, 32, 1.3, and 4.6 μM, respectively) (26). However, the N152G, -S, and -T mutants show nanomolar IC_{50}s, which is difficult to rationalize from a structural standpoint, given that there is no C-6 substituent to interact with position 152. When timed-inactivation experiments are performed to obtain K_I and k_{inact} for tazobactam over a 10-min time course, the rates of inactivation of WT CMY-2 with tazobactam are similar to values reported in the literature for P99 with these inhibitors (26). For tazobactam, the rates of inactivation for the N152 variants increase 2- to 6-fold, with no measurable change in the K_I value, compared to that for the WT CMY-2. Thus, changes in IC_{50} for this irreversible rapid mechanism-based inhibitor reflect higher rates of inactivation for the inhibitor. Previous studies of tazobactam inhibition of class A and C β-lactamases suggested that K_I values for tazobactam were similar among enzymes but that the rates of inactivation varied dramatically (44).

Molecular modeling. Superimposition of the 2.09-Å crystal structure of CMY-2 (1ZC2) on the models of the N152S mutant and the N152S CMY-2:tazobactam Michaelis-Menten complex is shown in Fig. 1. The model suggests that there are very minimal changes in the overall backbone structure of the N152S CMY-2 mutant and the Michaelis-Menten complex (root mean square deviation, 0.5 Å) and very small changes in the positioning of the key catalytic residues S64, K67, and Y150, as well as other residues of potential importance in substrate and inhibitor interactions, i.e., K316, Q120, T315, and A220. However, the S152-O-γ–Y150-OH distance is 3.9 Å, compared to the N152-O-δ–Y150-OH distance of 5.2 Å in the wild-type enzyme. This expands the active-site region around residue 152. In the Michaelis-Menten complex with tazobactam shown in Fig. 1b, the S152-O-γ–Y150-OH distance is decreased further to 3.5 Å.

The molecular models of the N152S variant compared to those of the WT CMY-2 suggest that the active site is expanded in the position 152 region, similar to what is seen in N152G P99 (39). This expansion would allow for improved accommodation of specific R1 groups of β-lactams such as cefoxitin and cephalothin ([2-thienylacetyl]amino containing), piperacillin and cefoperazone (piperazinyl containing), and aztreonam and ceftazidime (oxyimino containing).

N152S CMY-2, a class C "ESBL." Similar to the phenotype observed for the class A family of β-lactamase enzymes, where single point mutations (e.g., G238S SHV) cause a shift in substrate specificity from penicillinase to cephalosporinase and enhanced susceptibility to mechanism-based inhibitors like tazobactam, the N152S variant of CMY-2 demonstrates an extended-spectrum β-lactamase (ESBL)-like phenotype. MICs against cephalothin and ceftazidime are preserved. The enzyme can hydrolyze cefoxitin and cefoperazone more readily and is easily inhibited by tazobactam. This phenotype results from the increased rates of inactivation that are observed rather than the lower K_I values for the inhibitor seen with the class A ESBLs. Such increased susceptibility to tazobactam in the setting of extended-spectrum activity has been described for an H10 deletion mutant (ΔG286-S287-D288) of *E. coli* AmpC (14), although the mechanistic basis was not determined.

In conclusion, N152 substitutions in the CMY-2 class C β-lactamase result in a novel phenotype which is of interest, as inhibitors of the class C enzymes are awaited in the clinic. The stability and expression of these variants might be sufficient to result in altered susceptibility of Gram-negative pathogens to commonly prescribed antibiotics, e.g., resistance to cephalosporins but enhanced susceptibility to β-lactam–β-lactamase inhibitor combinations. The relationship between MICs, the expression of resistance determinants like plasmid and chromosomal β-lactamases, and the kinetic properties of these enzymes is complex (35), as evidenced by the results presented here. Further structural and kinetic investigations are under way to elucidate the role of residue 152 in CMY-2 in inhibition reactions. These important mechanistic insights will help us to better understand the basis for the highly efficient inhibition of both class A and class C β-lactamases by tazobactam and to extend the life of this important sulfone inhibitor.

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REFERENCES

1. **Jacoby GA.** 2009. AmpC β-lactamases. Clin. Microbiol. Rev. **22**:161–182.
2. **Gutiérrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Pérez JL, Oliver A.** 2007. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. Antimicrob. Agents Chemother. **51**:4329–4335.
3. **Kohler T, Michea-Hamzehpour M, Epp SF, Pechere JC.** 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. **43**:424–427.
4. **Livermore DM.** 1992. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36**:2046–2048.
5. **Quale J, Bratu S, Gupta J, Landman D.** 2006. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **50**:1633–1641.
6. **Mark BL, Vocadlo DJ, Oliver A.** 2011. Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. Future Microbiol. **6**:1415–1427.
7. **Rodríguez-Martínez JM, Nordmann P, Ronco E, Poirel L.** 2010. Extended-spectrum cephalosporinase in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **54**:3484–3488.
8. **Ahmed AM, Shimamoto T.** 2008. Emergence of a cefepime- and cefpirome-resistant *Citrobacter freundii* clinical isolate harbouring a novel chromosomally encoded AmpC β-lactamase, CMY-37. Int. J. Antimicrob. Agents **32**:256–261.
9. **Doi Y, Paterson DL, Adams-Haduch JM, Sidjabat HE, O'Keefe A, Endimiani A, Bonomo RA.** 2009. Reduced susceptibility to cefepime among clinical isolates producing novel variants of CMY-2 β-lactamase. Antimicrob. Agents Chemother. **53**:3159–3161.
10. **Endimiani A, Doi Y, Bethel CR, Taracila M, Adams-Haduch JM, O'Keefe A, Hujer AM, Paterson DL, Skalweit MJ, Page MG, Drawz SM, Bonomo RA.** 2010. Enhancing resistance to cephalosporins in class C β-lactamases: impact of Gly214Glu in CMY-2. Biochemistry **49**:1014–1023.
11. **Kotsakis SD, Papagiannitsis CC, Tzelepi E, Tzouvelekis LS, Miriagou V.** 2009. Extended-spectrum properties of CMY-30, a Val211Gly mutant of CMY-2 cephalosporinase. Antimicrob. Agents Chemother. **53**:3520–3523.
12. **Kotsakis SD, Tzouvelekis LS, Petinaki E, Tzelepi E, Miriagou V.** 2011. Effects of the Val211Gly substitution on molecular dynamics of the CMY-2 cephalosporinase: implications on hydrolysis of expanded-spectrum cephalosporins. Proteins **79**:3180–3192.
13. **Dahyot S, Mammeri H.** 2012. Hydrolysis spectrum extension of CMY-2-like β-lactamases resulting from structural alteration in the Y-X-N loop. Antimicrob. Agents Chemother. **56**:1151–1156.
14. **Doi Y, Wachino J, Ishiquro M, Kurokawa H, Yamane K, Shibata N, Shibayama K, Yokoyama K, Kato H, Yaqi T, Arakawa Y.** 2004. Inhibitor sensitive AmpC β-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob. Agents Chemother. **48**:2652–2658.
15. **Dubus A, Normark S, Kania M, Page MG.** 1995. Role of asparagine 152 in catalysis of β-lactam hydrolysis by *Escherichia coli* AmpC β-lactamase studied by site-directed mutagenesis. Biochemistry **34**:7757–7764.
16. **Lefurgy ST, de Jong RM, Cornish VW.** 2007. Saturation mutagenesis of Asn152 reveals a substrate selectivity switch in P99 cephalosporinase. Protein Sci. **16**:2636–2646.
17. **Hoyen CM, Hujer AM, Hujer KM, Marshall SH, Carias L, Toltzis P, Rice LB, Bonomo RA.** 2002. A clinical strain of *Escherichia coli* possessing CMY-2 plasmid-mediated amp C β-lactamase: an emerging concern in pediatrics? Microb. Drug Resist. **8**:329–333.
18. **Helfand MS, Hujer AM, Sönnichsen FD, Bonomo RA.** 2002. Unexpected advanced generation cephalosporinase activity of the M69F variant of SHV β-lactamase. J. Biol. Chem. **277**:47719–47723.
19. **Clinical and Laboratory Standards Institute, CLSI.** 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard—ninth edition. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
20. **Li M, Conklin BC, Taracila MA, Hutton RA, Skalweit MJ.** 2012. Substitutions at position 105 in SHV-family β-lactamases decrease catalytic efficiency and cause inhibitor resistance. Antimicrob. Agents Chemother. **56**:5678–5686.
21. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
22. **Drawz SM, Bethel CR, Hujer KM, Hurless KN, Distler AM, Caselli E, Prati F, Bonomo RA.** 2009. The role of a second-shell residue in modifying substrate and inhibitor interactions in the SHV β-lactamase: a study of Ambler position Asn276. Biochemistry **48**:4557–4566.
23. **Thomson JM, Distler AM, Prati F, Bonomo RA.** 2006. Probing active site chemistry in SHV β-lactamase variants at Ambler position 244: understanding unique properties of inhibitor resistance. J. Biol. Chem. **281**:26734–26744.
24. **Copeland RA.** 2005. Evaluation of enzyme inhibitors in drug discovery, 1st ed. John Wiley & Sons, Hoboken, NJ.
25. **Helfand MS, Bethel CR, Hujer AM, Hujer KM, Anderson VE, Bonomo RA.** 2003. Understanding resistance to β-lactams and β-lactamase inhibitors in the SHV β-lactamase: lessons from the mutagenesis of Ser-130. J. Biol. Chem. **278**:52724–52729.
26. **Stachyra T, Péchereau MC, Bruneau JM, Claudon M, Frère JM, Miossec C, Coleman K, Black MT.** 2010. Mechanistic studies of the inactivation of TEM-1 and P99 by NXL104, a novel non-β-lactam β-lactamase inhibitor. Antimicrob. Agents Chemother. **54**:5132–5138.
27. **Helfand MS, Totir MA, Carey MP, Hujer AM, Bonomo RA, Carey PR.** 2003. Following the reactions of mechanism-based inhibitors with β-lactamase by Raman crystallography. Biochemistry **42**:13386–13392. (Erratum, **42**:15398.)
28. **Kalp M, Totir MA, Buynak JD, Carey PR.** 2009. Different intermediate populations formed by tazobactam, sulbactam and clavulanate reacting with SHV-1 β-lactamases: Raman crystallographic evidence. J. Am. Chem. Soc. **131**:2338–2347.
29. **Papp-Wallace KM, Taracila M, Hornick JM, Hujer AM, Hujer KM, Distler AM, Endimiani A, Bonomo RA.** 2010. Substrate selectivity and a novel role in inhibitor discrimination by residue 237 in the KPC-2 β-lactamase. Antimicrob. Agents Chemother. **54**:2867–2877.
30. **Hujer AM, Bethel CR, Bonomo RA.** 2004. Antibody mapping of the linear epitopes of CMY-2 and SHV-1 β-lactamases. Antimicrob. Agents Chemother. **48**:3980–3988.
31. **Hujer AM, Hujer KM, Bonomo RA.** 2001. Mutagenesis of amino acid residues in the SHV-1 β-lactamase: the premier role of Gly238Ser in penicillin and cephalosporin resistance. Biochim. Biophys. Acta **1547**:37–50.
32. **Hujer AM, Page MG, Helfand MS, Yeiser B, Bonomo RA.** 2002. Development of a sensitive and specific enzyme-linked immunosorbent assay for detecting and quantifying CMY-2 and SHV β-lactamases. J. Clin. Microbiol. **40**:1947–1957.
33. **Rholl DA, Papp-Wallace KM, Tomaras AP, Vasil ML, Bonomo RA, Schweizer HP.** 2011. Molecular investigations of PenA-mediated β-lactam resistance in *Burkholderia pseudomallei*. Front. Microbiol. **2**:139. doi: 10.3389/fmicb.2011.00139.
34. **Timkovich N, Skalweit MJ, Powers RA.** 2012. Exploring the role of Asn 152 in altering the resistance spectrum of the class C β-lactamase CMY-2, poster 482. Abstr. 243rd American Chemical Society National Meeting & Exposition. American Chemical Society, Washington, DC.
35. **Bauvois C, Ibuka AS, Celso A, Alba J, Ishii Y, Frere JM, Galleni M.** 2005. Kinetic properties of four plasmid-mediated AmpC β-lactamases. Antimicrob. Agents Chemother. **49**:4240–4246.
36. **Trehan I, Beadle BM, Shoichet BK.** 2001. Inhibition of AmpC β-lactamase through a destabilizing interaction in the active site. Biochemistry **40**:7992–7999.
37. **Sun T, Bethel CR, Bonomo RA, Knox JR.** 2004. Inhibitor-resistant class A β-lactamases: consequences of the Ser130-to-Gly mutation seen in Apo and tazobactam structures of the SHV-1 variant. Biochemistry **43**:14111–14117.
38. **Thomas VL, Golemi-Kotra D, Kim C, Vakulenko SB, Mobashery S, Shoichet BK.** 2005. Structural consequences of the inhibitor-resistant Ser130Gly substitution in TEM β-lactamase. Biochemistry **44**:9330–9338.
39. **Ruble JF, Lefurgy ST, Cornish VW, Powers RA.** 2012. Structural analysis of the Asn152Gly mutant of P99. Acta Crystallogr. D Biol. Crystallogr. **68**:1189–1193.
40. **Bonomo RA, Liu J, Chen Y, Ng L, Hujer AM, Anderson VE.** 2001.

Inactivation of CMY-2 β-lactamase by tazobactam: initial mass spectroscopic characterization. Biochim. Biophys. Acta **1547**:196–205.
41. **Pattanaik P, Bethel CR, Hujer AM, Hujer KM, Distler AM, Taracila M, Anderson VE, Fritsche TR, Jones RN, Pagadala SR, van den Akker F, Buynak JD, Bonomo RA.** 2009. Strategic design of an effective β-lactamase inhibitor: LN-1-255, a 6-alkylidene-2′-substituted penicillin sulfone. J. Biol. Chem. **284**:945–953.
42. **Totir MA, Padayatti PS, Helfand MS, Carey MP, Bonomo RA, Carey PR, van den Akker F.** 2006. Effect of the inhibitor-resistant M69V substitution on the structures and populations of *trans*-enamine β-lactamase intermediates. Biochemistry **45**:11895–11904.
43. **Drawz SM, Bonomo RA.** 2010. Three decades of β-lactamase inhibitors. Clin. Microbiol. Rev. **23**:160–201.
44. **Bush K, Macalintal C, Rasmussen BA, Lee VJ, Yang Y.** 1993. Kinetic interactions of tazobactam with β-lactamases from all major structural classes. Antimicrob. Agents Chemother. **37**:851–858.