Substrate Selectivity and a Novel Role in Inhibitor Discrimination by Residue 237 in the KPC-2 β-Lactamase

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β-Lactamase-mediated antibiotic resistance continues to challenge the contemporary treatment of serious bacterial infections. The KPC-2 β-lactamase, a rapidly emerging Gram-negative resistance determinant, hydrolyzes all commercially available β-lactams, including carbapenems and β-lactamase inhibitors; the amino acid sequence requirements responsible for this versatility are not yet known. To explore the bases of β-lactamase activity, we conducted site saturation mutagenesis at Ambler position 237. Only the T237S variant of the KPC-2 β-lactamase expressed in Escherichia coli DH10B maintained MICs equivalent to those of the wild type (WT) against all of the β-lactams tested, including carbapenems. In contrast, the T237A variant produced in E. coli DH10B exhibited elevated MICs for only ampicillin, piperacillin, and the β-lactam–β-lactamase inhibitor combinations. Residue 237 also plays a novel role in inhibitor discrimination, as 11 of 19 variants exhibit a clavulanate-resistant, sulfone-susceptible phenotype. We further showed that the T237S variant displayed substrate kinetics similar to those of the WT KPC-2 enzyme. Consistent with susceptibility testing, the T237A variant demonstrated a lower $k_{cat}/K_m$ for imipenem, cephalexin, and ceftaxime; interestingly, the most dramatic reduction was with ceftaxime. The decreases in catalytic efficiency were driven by both elevated $K_m$ values and decreased $k_{cat}$ values compared to those of the WT enzyme. Moreover, the T237A variant manifested increased $K_s$ for clavulanic acid, sulbactam, and tazobactam, while the T237S variant displayed $K_s$ similar to those of the WT. To explain these findings, a molecular model of T237A was constructed and this model suggested that (i) the hydroxyl side chain of T237 plays an important role in defining the substrate profile of the KPC-2 β-lactamase and (ii) hydrogen bonding between the hydroxyl side chain of T237 and the $sp^2$-hybridized carboxylate of imipenem may not readily occur in the T237A variant. This stringent requirement for selected cephalosporinase and carbapenemase activity and the important role of T237 in inhibitor discrimination in KPC-2 are central considerations in the future design of β-lactam antibiotics and inhibitors.

Antibiotic resistance is a critical challenge to clinicians treating complex bacterial infections. Moreover, the continued evolution of bacterial proteins responsible for mediating antibiotic resistance is alarming. The most notable resistance determinants in nature are the β-lactamases present in Gram-negative bacteria. Presently, the β-lactamases (EC 3.5.2.6) are classified into four distinct classes based on structural similarities (A, B, C, and D) or four groups based on hydrolytic profiles (1, 2, 3, and 4) (1, 6, 46, 53). Class A, C, and D β-lactamases use a serine as the nucleophile in the active site to hydrolyze the β-lactam, while class B β-lactamases employ either one or two reactive Zn$^{2+}$ ions. In general, class A, C, and D β-lactamases hydrolyze β-lactams through a three-step reaction mechanism represented as follows:

$$E + S \stackrel{k_1}{\rightarrow} E:S \stackrel{k_2}{\rightarrow} E-S \stackrel{k_3}{\rightarrow} E + P$$

$$H_2O$$

In this reaction scheme, $E$ corresponds to the β-lactamase, $S$ is the β-lactam substrate, $E:S$ is the Michaelis complex, $E-S$ is the acylated β-lactamase, $P$ is the inactive product; $k_1$ and $k_{-1}$ represent the on and off rates, $k_3$ is the acylation rate constant, and $k_2$ is the deacylation rate constant.

Interestingly, single amino acid substitutions allow β-lactamases to expand their substrate profile and dramatically alter the ability of β-lactamases to hydrolyze β-lactams before they reach their targets, the penicillin binding proteins. Such β-lactamases include the extended-spectrum β-lactamases (ESBLs), inhibitor-resistant TEMs and SHVs (IRTs and IRSs), and extended-spectrum AmpCs (ESACs) (4, 11, 29, 30, 37, 39). As a result of this progression, each new, improved β-lactam is eventually greeted with a new β-lactamase that threatens the efficacy of the drug (31, 33).

Resistance to carbapenems, which have been considered the last line of therapy for many types of infections, is one of the greatest threats in clinical medicine (23, 24). The serine class A β-lactamases responsible for carbapenem resistance include KPC-2-10, SME-1-3, IMI-1-2, SFC-1, BIC-1, NmcA, and GES-
Among these class A β-lactamases, KPC-2 is a clinically important and unique enzyme, as it is the most prevalent carbapenemase in enteric bacteria in the United States (12, 24, 45). In addition, the bla\textsubscript{KPC-2} gene is located on a mobile transposon designated Tn\textsubscript{4401} (36, 58, 59). As the structural basis for β-lactam resistance mediated by KPC-2 is elusive, we endeavored to explore the sequence determinants of carbapenem resistance mediated by KPC-2.

Comparing the amino acid sequence and crystal structure of KPC-2 to those of other class A β-lactamases (i.e., CTX-M-1, SHV-1, and TEM-1) shows that nine residues near or in the active site are unique and/or in distinctive positions in KPC-2 (e.g., W105, S130, N132, N170, R220, K234, T235, T237, and H274) (24). Four of these amino acids (S130, N132, N170, K234, and T235) are located in the following four conserved elements: the SSFK motif (Ambler positions 70 to 73), the SDN loop (residues 130 to 132), the omega loop (positions 164 to 166), and the KTG motif (residues 234 to 236) (Fig. 1). The SDN loop plays a role in forming a hydrogen bond to the carbohydrate moiety of imipenem.

Materials and Methods

Bacterial strains and cloning. Klebsiella pneumoniae 1534 possessing bla\textsubscript{KPC-2} and Escherichia coli containing bla\textsubscript{KPC-2} in the pBR322-\textit{cat} vector were given to us by Fred Tenover, Centers for Disease Control and Prevention, Atlanta, GA (36, 59). bla\textsubscript{KPC-2} was cloned into the pBSCK (+) phagemid (Stratagene, La Jolla, CA) using restriction sites XbaI and BamHI. E. coli DH10B (Invitrogen, Carlsbad, CA) was used as a host strain for pBR322-\textit{cat}-bla\textsubscript{KPC-2}-PBCSK (+) and pBCSK (+) -bla\textsubscript{KPC-2} T237 variants. bl\textsubscript{KPC-2} lacking the leader sequence starting at either nucleotide position 65 (corresponding to Ambler position 22) or 73 (corresponding to Ambler position 25) was subcloned into pET24a(+) (Novagen, Darmstadt, Germany) using Ndel and BamHI restriction enzymes with a stop codon to prevent fusion of the C-terminal His tag.

Antibiotic susceptibility. K. pneumoniae 1534 expressing bla\textsubscript{KPC-2}, E. coli DH10B and E. coli DH10B cells were electroporated with 1.0 μl of each reaction mixture and plated on lysogeny broth (LB) agar plates with 20 mg/liter chloramphenicol. Single colonies were selected for plasmid purification, and DNA sequencing was conducted by the Genomics Core Facility at Case Western Reserve University (Cleveland, OH).

To investigate the role residue 237 plays in KPC-2, we conducted site saturation mutagenesis at position 237 in KPC-2. Our results demonstrate that this residue is necessary to maintain carbapenemase and selected cephalosporinase activities of the enzyme and is involved in the discrimination of clavulanic acid from sulfonyl inhibitors. In addition, our study suggests that a hydroxyl side chain is necessary at position 237 for hydrogen bonding to the carbohydrate moiety of imipenem.

FIG. 1. Model of KPC-2 (PDB entry 2OV5) highlighting the four conserved regions (the SSFK motif [purple], the SDN loop [blue], the omega loop [green/pink], and the KTG motif [gray]) found in class A β-lactamases, the three main epitopes (A [light blue], B [pink], and C [orange]) of our polyclonal anti-KPC-2 antibody, and T237 (yellow).
Figure 2 shows the chemical structures of the compounds focused on in this study.

**β-Lactamase purification.** The WT KPC-2 β-lactamase was purified from *E. coli* DH10B cells carrying the pBR322-cat- bla*KPC-2* vector and *E. coli* Origami2 DE3 pLys cells carrying either pET24a(+) bla*KPC-2A22* or pET24a(+) bla*KPC-2L25*. These strains were chosen as they expressed the most β-lactamase. The T237S, T237A, and T237E variants were purified from *E. coli* DH10B cells carrying the plasmid pBC SK(+) bla*KPC-2* with the T237S, -A, and -E mutations and *E. coli* Origami2 DE3 pLys cells carrying pET24a(+) bla*KPC-2A22* with the T237S, -A, and -E mutations. *E. coli* DH10B cells carrying the various plasmids were grown in super optimal broth (SOB) containing 20 mg/liter chloramphenicol for 18 h. *E. coli* Origami2 DE3 pLys cells expressing the various plasmids were grown for 2 h in SOB, and then 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for induction and the cells were grown for an additional 2 h. All cells were pelleted and frozen for 18 h at −20°C. Pellets were resuspended in 50 mM Tris-Cl at pH 7.4 with 1 mM magnesium sulfate. Pellets were lysed with 40 mg/liter lysozyme, and 1.0 U/ml benzonase nuclease (Novagen) was added to digest nucleic acids. A 2.0 mM concentration of EDTA was added to complete the periplasmic fractionation. The lysed cells were centrifuged at 12,000 rpm for 10 min to remove the cellular debris. The crude extract was used for preparative isoelectric focusing as previously described (27, 55). Preparative isoelectric focusing fractions were processed as previously described (36).

**Steady-state expression.** *E. coli* DH10B cells carrying pBC SK(+) bla*KPC-2* with all 19 variants and the WT were grown in LB broth to mid-log phase (optical density at 600 nm [OD600] of 0.7) and 0.5 OD600 U of cells was pelleted at 10,000 rpm for 10 min. The supernatant was removed, and the pellet was directly
resuspended in 50 μl of 5% sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) loading dye to a concentration of 0.01 OD_{600} μg/ml. Cells (0.1 OD_{600} U or a 10-μl sample) were loaded into each lane of an SDS-PAGE gel. The gel was run and transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight in 5.0% bovine serum albumin (BSA; Amresco, Solon, OH) in 20 mM Tris-Cl (pH 7.4) with 150 mM NaCl (TBS). The membrane was washed five times for 10 min each with TBS and incubated for 2 h at room temperature while shaking with polycolonal anti-KPC-2 rabbit antibody at 1.0 μg/ml in 5.0% BSA in TBS. The polycolonal anti-KPC-2 rabbit antibodies were raised by Sigma-Genosys (The Woodlands, TX) and isolated from serum using protein G column purification (GE Healthcare Life Sciences) (19). The membrane was washed with TBS containing 0.05% Tween 20 (TBST) five times for 10 min each and then incubated with protein G-horseradish peroxidase conjugate (Bio-Rad) for 1 h at room temperature with shaking. The blot was washed again with TBST five times for 10 min each and then developed using the ECL developing kit (GE Healthcare Life Sciences) according to the manufacturer's instructions.

**SPOTS membrane synthesis and probing with anti-KPC antibodies.** A total of 85 peptides were synthesized and immobilized on a synthetic SPOTS membrane (Sigma-Genosys). The amino acid sequence of the mature KPC-2 β-lactamase (i.e., starting with amino acid Ala30; GenBank accession number AY648950) was employed to obtain the steady-state kinetic parameters at an excess molar concentration to establish pseudo-first-order kinetics. A nonlinear least-squares fit of the data (Henri Michaelis-Menten equation) using Enzfitter (Biosoft Corporation, Ferguson, MO) was employed to obtain the steady-state kinetic parameters $V_{max}$ and $K_m$ as follows: $v = \frac{V_{max} \cdot [S]}{[S] + K_m}$. To determine the $K_m$ of the inhibitors, a direct competition assay under steady-state conditions was performed as previously described (36). Each enzyme was maintained at 10 μM, while the inhibitor concentrations were varied from 5 μM to 5 mM. Nitrocefin (NCF) was used as the reporter substrate at a final concentration of 100 μM. The steady-state kinetic parameters were determined using an Agilent (Santa Clara, CA) 8453 Diode Array spectrophotometer as previously described (36). Briefly, each assay was performed in 10 mM phosphate-buffered saline at pH 7.4 at room temperature with the enzyme maintained at 10 nM and each substrate at an excess molar concentration to establish pseudo-first-order kinetics. A nonlinear least-squares fit of the data (Henri Michaelis-Menten equation) using Enzfitter (Biosoft Corporation, Ferguson, MO) was employed to obtain the steady-state kinetic parameters $V_{max}$ and $K_m$ as follows: $v = \frac{V_{max} \cdot [S]}{[S] + K_m}$. To determine the $K_m$ of the inhibitors, a direct competition assay under steady-state conditions was performed as previously described (36). Each enzyme was maintained at 10 μM, while the inhibitor concentrations were varied from 5 μM to 5 mM. Nitrocefin (NCF) was used as the reporter substrate at a final concentration of 100 μM. The binding energies from free enzyme to the acylation transition state were calculated using the following equation, where $R$ is the gas constant and $T$ is the absolute temperature: $\Delta G_{Ti} = -RT \ln \left( \frac{k_{cat}}{k_{cat}(T237A)} \right) = -RT \ln \left( \frac{[K_m]_{cat}(T237A) \cdot V_{max}}{[K_m]_{cat}(T237A) \cdot V_{max}} \right)$. Molecular modeling. The crystal structure coordinates of KPC-2 (PDB entry 1R0V) were used to construct acyl enzyme models of imipenem with the WT KPC-2 β-lactamase and the T237A variant. Discovery Studio 2.1 (DS 2.1; Accelrys, Inc., San Diego, CA) was used for modeling as previously described (36). The T237A variant of KPC-2 was built by substituting the residue at position 237. The imipenem structure was constructed using Fragment Builder Tools and was minimized using a Standard Dynamics Cascade protocol of DS 2.1. The hydrolyzed imipenem was automatically docked into the active site of KPC-2 β-lactamase using the Flexible Docking module of DS 2.1. The protocol allowed docking of flexible imipenem in the active site of KPC-2 with residues S70, R73, W105, N132, R220, T235, and T-A237 set as flexible. Knowledge of the kinetics of this β-lactamase helped us to choose the most favorable conformation of imipenem. The complex consisting of the enzyme and imipenem was covalently binding the carbonyl carbon of imipenem and the oxygen of S70. The acyl enzyme complex of the T237A variant with imipenem was created in the same way as for the WT enzyme. The docking protocol provides a reasonable prediction of the binding mode for the β-lactam given that the major interactions that we observed are also present in another β-lactamase, specifically, the AmpC-imipenem crystal structure (2). Wu and colleagues have previously confirmed the accuracy and efficiency of this docking method (57).

**RESULTS**

**Mutagenesis, DNA sequencing, and susceptibility testing.** Seven mutagenic primer sets were designed to capture the 19 most frequently used codons that encode Ambler position 237 in bla<sub>KPC-2</sub>. After mutagenesis and transformation into <i>E. coli</i> DH10B, a total of 100 colonies were randomly selected and screened via DNA sequencing; all of the 19 variants were obtained.

To assess the impact of single amino acid substitutions at position 237 on the activity of KPC-2 β-lactamase, we performed susceptibility testing of <i>E. coli</i> DH10B carrying the 19 <i>bla<sub>KPC-2</sub></i> variants at position 237 in the pBC SK(+) vector (Table 1). We compared these MICs to those for the parental strain <i>K. pneumoniae</i> 1534 and to those for <i>E. coli</i> DH10B strains containing <i>bla<sub>KPC-2</sub></i> on two different plasmids, pBR322-<i>cat</i> and pBC SK(+). <i>E. coli</i> DH10B with and without the pBC SK (+) plasmid served as controls.

The strains containing WT <i>bla<sub>KPC-2</sub></i> demonstrated elevated MICs for all of the β-lactams tested. For ampicillin, <i>E. coli</i> strains producing the T237A, T237Q, and T237S variants displayed MICs similar (256 to 512 mg/liter) to pBC SK(+)–producing <i>E. coli</i> DH10B (WT) (256 mg/liter). The T237G, T237M, and T237V variants expressed in <i>E. coli</i> exhibited ampicillin MICs within 1 dilution (128 mg/liter) of that for the WT. For piperacillin, only <i>E. coli</i> carrying the T237S variant demonstrated the same MIC as the WT of 128 mg/liter, while the T237A and T237Q variants expressed in <i>E. coli</i> were within 1 dilution (64 mg/liter).

Regarding cephalosporins (cephalothin and cefotaxime) and carbapenems (imipenem, ertapenem, doripenem, and meropenem), only the serine 237 variant produced in <i>E. coli</i> displayed MICs equivalent to or within 1 dilution of the WT MICs. Specifically, the T237S variant in <i>E. coli</i> demonstrated MICs of 256 mg/liter for cefalothin, 1 mg/liter for cefotaxime and imipenem, 0.5 mg/liter for ertapenem and meropenem, and 0.25 mg/liter for doripenem (Table 1). Other variants expressed in <i>E. coli</i> did not display MICs comparable to those displayed by the T237S variant expressed in <i>E. coli</i>. MICs were also determined for β-lactam–β-lactam inhibitor (ampicillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam) combinations (Table 1). <i>E. coli</i> producing the T237A, -D, -E, -H, -L, -M, -N, -Q, -S, and -V variants exhibited MICs of 4 to 8 mg/liter for ampicillin-clavulanic acid, which are comparable to the WT level of 4 mg/liter. The T237S variant in <i>E. coli</i> displayed MICs similar to those of the WT for ampicillin-sulbactam (128 mg/liter sulbactam, 50 mg/liter ampicillin) and piperacillin-tazobactam (8 mg/liter tazobactam, 64 mg/liter piperacillin). <i>E. coli</i> expressing the T237A and T237Q variants exhibited MICs within 1 dilution of the WT for ampicillin-sulbactam and piperacillin-tazobactam.

**KPC-2 β-lactamase expression.** We used a polyclonal antibody to assess steady-state production of the KPC-2 variants produced in DH10B using the ECL developing kit (GE Healthcare Life Sciences) according to the manufacturer's instructions.
expressed from pBC SK(+) in *E. coli* DH10B cells. Before measuring levels of expression, we ensured that specific amino acid changes would not alter the recognition of our polyclonal antibodies of the KPC-2 position 237 variants studied. Therefore, we first mapped the linear immunogenic epitopes of the KPC-2 protein (Fig. 1). This mapping shows that the polyclonal antibody we used recognized multiple linear epitopes of the KPC-2 protein (Fig. 1). By understanding the impact of single amino acid substitutions on kinetic behavior, the WT and the T237S, T237A, and T237E variants behaved identically in kinetic assays (data not shown).

**Kinetics of KPC-2 with substrates and inhibitors.** To better understand the impact of single amino acid substitutions on kinetic behavior, the WT and the T237S, T237A, and T237E variants were purified and steady-state kinetic parameters were determined with various substrates and inhibitors. The T237S-substituted enzyme was purified because this variant in *E. coli* had the same or increased MICs of all of the substrates tested compared to the WT. The T237A variant was also selected, as it maintained WT MICs against ampicillin, piperacillin, and β-lactam–β-lactamase inhibitor combinations, but not against imipenem, when produced by *E. coli*. Our rationale for choosing the T237E variant is that this variant in *E. coli* had low MICs for all of the β-lactams and combinations tested.

Overall, the T237S variant exhibited a kinetic profile very similar to that of the WT enzyme for the substrates piperacillin, cephalothin, cefotaxime, NCF, and imipenem (Table 2). The catalytic efficiencies ($k_{cat}/K_m$) of the T237S variant varied by a maximum of 2.7-fold from those of the WT. Also, supporting the MIC observations, the T237A variant possessed a hydrolytic profile similar to that of the WT for all of the β-lactams and combinations tested. The catalytic efficiencies ($k_{cat}/K_m$) of the T237S variant varied by a maximum of 2.7-fold from those of the WT. Also, supporting the MIC observations, the T237A variant possessed a hydrolytic profile similar to that of the WT for all of the β-lactams and combinations tested.
Notably, the T237A variant did not hydrolyze cefotaxime measurably, even when excess enzyme was used. The decrease in $k_{\text{cat}}$ and an increase in $K_m$ for imipenem and cephalothin suggest that the rate of acylation is decreased in the T237A variant. In turn, the binding energies from free enzyme to the acylation transition state are increased by $900 \text{ cal/mol}$ and $770 \text{ cal/mol}$ for the T237A variant with imipenem and cephalothin, respectively.

![FIG. 3. SPOT membrane (top) labeled with yellow numbers which correspond to the primary amino acid sequence in the bottom panel. SPOT membrane probing reveals that the polyclonal anti-KPC-2 antibodies recognize three main primary amino acid epitopes (outlined red boxes): epitope A (peptide 2 = F33 to G45, peptide 3 = L36 to A48), epitope B (peptide 47 = L169 to S181, peptide 48 = A172 to R184, peptide 49 = G175 to T187), and epitope C (peptide 83 = V278 to L290, peptide 84 = A281 to N293) (A). Immunoblot assays measuring protein expression in the position 237 variants expressed from pBC SK($\text{bla}_{KPC-2}$) in $E.\ coli$ DH10B indicate that the levels of KPC-2 protein differ in the 19 variants. $E.\ coli$ DH10B carrying WT pBC SK($\text{bla}_{KPC-2}$) indicates the basal level of expression (B).]
not that the $k_{cat}/K_m$ for NCF, a chromogenic cephalosporin substrate, is not decreased by loss of a hydroxyl side chain at position 237. Conversely, the T237E variant demonstrated increased $K_m$ for substrates, making it difficult to accurately determine other kinetic parameters of this variant. We were only able to accurately measure kinetic parameters of NCF with the T237E-substituted enzyme ($K_m = 167 \pm 16 \mu M$, $k_{cat} = 92 \pm 1 s^{-1}$, $k_{cat}/K_m = 0.6 \pm 0.1 \mu M^{-1} s^{-1}$).

Steady-state inhibitor kinetic reveals that the T237S variant of KPC-2 has $K_S$ for the inhibitors clavulanic acid, sulbactam, and tazobactam comparable to those of the WT, again varying, at most, by 1.7-fold (Table 3). In contrast to the T237S variant, the T237A-substituted $\beta$-lactamase displayed increased $K_S$ for clavulanic acid, sulbactam, and tazobactam by 5.0-, 4.2-, and 2.2-fold, respectively, supporting MIC observations. Since the $K_m$s of the substrates were elevated for the T237E variant, inhibitor kinetics were not determined.

**Molecular representations of KPC-2 and the T237A variant.**

As our phenotype and kinetic investigations revealed the importance of the hydroxyl side chain at residue 237 in KPC-2, we developed molecular representations of KPC-2 and the T237A variant as acyl enzymes to provide insights into the differences in the hydrolysis of imipenem (Fig. 4A and B). Our work centers on the understanding of carbapenemase and cephalosporinase activities abrogated extending-spectrum cephalosporins but not carbapenemases (e.g., PER-1, CTX-M-4), cephalosporinase activity is lessened when serine is changed to alanine, while penicillinase activity is maintained (5, 15, 54). In contrast, SME-1, a class A carbapenemase which has distinct active-site attributes compared to ESBLSs, a serine is present at Ambler position 237. When S237 is changed to alanine in SME-1, carbapenemase and cephalosporinase activities are abrogated (28, 52). However, if Ser at position 237 in SME-1 is changed to threonine, activity against carbapenems and cephalosporins is maintained (28, 52).

Our results are in accord with observations on SME-1, as serine and alanine were both able to substitute for threonine at

**DISCUSSION**

As seen in another class A carbapenemase, our data show that T237 in the KPC-2 $\beta$-lactamase also plays a role in the hydrolysis of carbapenems and selected cephalosporins. We show here for the first time that this residue also impacts $\beta$-lactamase inhibitor resistance in this class A carbapenemase. Only one conservative amino acid change (T$\rightarrow$S) maintains carbapenem resistance in KPC-2 when $\beta$-lactamase variants are expressed in E. coli; analyses of other amino acid biophysical properties (e.g., size of the residue at position 237, etc.; Table 1) do not bear as strong a relationship as the specific substitution. Below, we discuss the role of T237 among class A serine $\beta$-lactamases and the intricacy of $\beta$-lactamase inhibitor (clavulanic acid versus the sulfones) resistance by KPC-2 and its position 237 variants. In addition, we examine what is learned from study of the T237A variant of KPC-2. Based upon these findings, insights into future $\beta$-lactam and $\beta$-lactamase inhibitor design are presented.

**The role of T237 in class A $\beta$-lactamases.**

In the widespread class A enzyme TEM-1, a penicillinase that does not hydrolyze carbapenems readily, residue 237 is described as having an ancillary or modulating role in resistance to $\beta$-lactams (3, 16). By this we mean that other amino acid changes are required in TEM for the A237T substitution to improve catalytic activity toward cephalosporins. In other class A $\beta$-lactamases that readily hydrolyze extended-spectrum cephalosporins but not carbapenems (e.g., PER-1, CTX-M-4), cephalosporinase activity is lessened when serine is changed to alanine, while penicillinase activity is maintained (5, 15, 54). In contrast, SME-1, a class A carbapenemase which has distinct active-site attributes compared to ESBLSs, a serine is present at Ambler position 237. When S237 is changed to alanine in SME-1, carbapenemase and cephalosporinase activities are abrogated (28, 52). However, if Ser at position 237 in SME-1 is changed to threonine, activity against carbapenems and cephalosporins is maintained (28, 52).

Our results are in accord with observations on SME-1, as serine and alanine were both able to substitute for threonine at
position 237 in KPC-2 to maintain penicillin MICs, but only serine permitted carbapenemase and cephalosporinase activities. We note that the most dramatic reduction in steady-state kinetics was with cefotaxime, perhaps suggesting an important role in cefotaximase activity, as described above for other class A β-lactamases. More importantly, we propose that this finding also points to a general role for T237 in class A carbapenemases. We note that in the GES β-lactamase family, an enzyme that broadens its substrate spectrum from an ESBL to a carbapenemase with a substitution in the Ω loop and at Amblcr position 243, T237, is present as in WT KPC-2 (34, 42, 43). A comparative analysis of the pre-steady- and steady-state contributions of these residues to carbapenem hydrolysis in different class A enzymes is warranted (14).

**The complexity of β-lactamase inhibitor resistance.** When studying the β-lactamase inhibitors, we made two intriguing observations. First, we found that clavulanic acid behaves differently than the sulfones, as evidenced by the elevated MICs for clavulanic acid when combined with a partner β-lactam, as well as the lower $K_i$s for clavulanic acid. Sec-
ond, we observed that the sulfone inhibitors also act differently from one another, as supported by lower $K_I$s for tazobactam than sulbactam.

We found that flexibility exists for retaining ampicillin-clavulanic acid resistance. This phenotype was not seen with the sulfone inhibitors when combined with ampicillin and piperacillin. Based upon our findings with the WT enzyme and its inherent inhibitor-resistant phenotype (36), we were surprised to observe that substitutions at position 237 further enhanced inhibitor resistance by increasing the β-lactam-clavulanic acid MICs. Although the T237S variant of E. coli has MICs similar to those of the WT against ampicillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam, other position 237 variants (8 out of 19) expressed in E. coli demonstrate elevated ampicillin-clavulanic acid MICs but not ampicillin-sulbactam or piperacillin-tazobactam inhibitor MICs. Supporting the role of position 237 in inhibitor resistance, the S237A substitution in CTX-M-4 also increases resistance to clavulanic acid (15). The T237A variant of KPC-2, when expressed in E. coli, also has elevated MICs of ampicillin-clavulanic acid; in addition, the T237A variant has an increased $K_I$ for clavulanic acid. This clavulanate-resistant, sulfone-susceptible phenotype recalls what is observed in some complex mutants of TEM (CMTs); these TEM variants are ESBLs that have increased resistance to inactivation by clavulanic acid but not to inactivation by sulbactam and tazobactam (7, 13, 47, 48, 51). In a similar manner, selectivity toward β-lactamase inhibitors is also seen in non-ESBLs, specifically, IRS β-lactamase variants (10, 56).

**Insights from modeling.** Our molecular representations of KPC-2 and T237A variants with imipenem support the importance of the hydroxyl side chain as imipenem changes conformation in the T237A variant’s active site. We were surprised to find that our T237A model with imipenem mimics the E. coli AmpC-imipenem crystal structure done by Beadle and Shoichet (2). In another class A ESBLs, Toho-1, residue Ser237 has also been shown to form a unique hydrogen bond to the carboxylate of substrates and CTX-M-9 forms a charged interaction more than catalysis is an important and challenging endeavor.

In closing, this work tells us that the evolution of substrate specificity and inhibitor resistance among KPC β-lactamases remarkably parallels what is known in other β-lactamases. Among β-lactamases, a novel enzyme evolves as a result of the interplay of multiple factors: the primary sequence, selective pressure by β-lactam antibiotics, mutation rates, three-dimensional structure, and protein stability (41). By developing an understanding of carbapenem, cephalosporin, and inhibitor resistance in this versatile class A β-lactamase (45), we develop an appreciation of the details and interactions of future substrates or inactivators with the active sites of these potent drug-inactivating enzymes. This work is a first step in determining how KPC β-lactamases can hydrolyze carbapenems while most class A enzymes cannot.

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