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Exposing a β-Lactamase "Twist": the Mechanistic Basis for the High Level of Ceftazidime Resistance in the C69F Variant of the *Burkholderia pseudomallei* PenI β-Lactamase

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Around the world, *Burkholderia* spp. are emerging as pathogens highly resistant to β-lactam antibiotics, especially ceftazidime. Clinical variants of *Burkholderia pseudomallei* possessing the class A β-lactamase PenI with substitutions at positions C69 and P167 are known to demonstrate ceftazidime resistance. However, the biochemical basis for ceftazidime resistance in class A β-lactamases in *B. pseudomallei* is largely undefined. Here, we performed site saturation mutagenesis of the C69 position and investigated the kinetic properties of the C69F variant of PenI from *B. pseudomallei* that results in a high level of ceftazidime resistance (2 to 64 mg/liter) when expressed in *Escherichia coli*. Surprisingly, quantitative immunoblotting showed that the steady-state protein levels of the C69F variant β-lactamase were ~4-fold lower than those of wild-type PenI (0.76 fg of protein/cell versus 4.1 fg of protein/cell, respectively). However, growth in the presence of ceftazidime increases the relative amount of the C69F variant to greater than wild-type PenI levels. The C69F variant exhibits a branched kinetic mechanism for ceftazidime hydrolysis, suggesting there are two different conformations of the enzyme. When incubated with an anti-PenI antibody, one conformation of the C69F variant rapidly hydrolyzes ceftazidime and most likely contributes to the higher levels of ceftazidime resistance observed in cell-based assays. Molecular dynamics simulations suggest that the electrostatic characteristics of the oxyanion hole are altered in the C69F variant. When ceftazidime was positioned in the active site, the C69F variant is predicted to form a greater number of hydrogen-bonding interactions than PenI with ceftazidime. In conclusion, we propose "a new twist" for enhanced ceftazidime resistance mediated by the C69F variant of the PenI β-lactamase based on conformational changes in the C69F variant. Our findings explain the biochemical basis of ceftazidime resistance in *B. pseudomallei*, a pathogen of considerable importance, and suggest that the full repertoire of conformational states of a β-lactamase profoundly affects β-lactam resistance.

The expanded-spectrum (ES) cephalosporin ceftazidime is a first-line agent used to treat patients infected with *Burkholderia pseudomallei* (1). *B. pseudomallei* is the causative agent of the multisystem disease melioidosis, is a potential biothreat agent, and possesses many innate antimicrobial resistance genes (2). In some parts of the world, melioidosis is characterized by a 20 to 50% mortality rate with treatment, and the death rate increases to >80% for patients with sepsis (2, 3). Resistance to ceftazidime is common in clinical isolates of *B. pseudomallei* and is linked to increased expression of serine β-lactamases (classes A and D) and expression of different isoforms of the class A β-lactamase, as well as to deletion of penicillin binding protein 3 (ΔPBP3) (4–12).

The class A β-lactamase PenI, also referred to as PenA and BPS, produced by *B. pseudomallei* is part of the PenA family of class A β-lactamases that are widely distributed in the genus *Burkholderia* (5, 7, 13–15). Single-amino-acid substitutions at positions C69 and P167 are associated with ceftazidime resistance in PenI-expressing *B. pseudomallei* (5–7, 11). In class A β-lactamases, the evolution of ceftazidime resistance is mediated by amino acid substitutions in the Ω loop (residues 164 to 179), the B3 β-strand (residues 234 to 240), and, in rare instances, the oxyanion hole (i.e., residue 69) (Fig. 1A) (16–18). The mechanistic contributions of amino acid substitutions at these sites that enhance catalytic efficiency toward ceftazidime are poorly understood and merit further exploration. Ascertaining the details and mechanism of ceftazidime resistance is critical to identifying future successful therapies.

The focus of this work is Ambler residue 69 in PenI, which forms the "back wall" of the oxyanion hole in class A β-lactamases and is located on helix 2 (H2) proximal to the nucleophilic S70 between the B3 β-strand and the Ω loop (Fig. 1A). The oxyanion hole is a "pocket" formed by the backbone amides of S70 and T237 that creates an electrophilic center attracting the β-lactam's carbonyl for binding and subsequent hydrolysis. Conservation of amino acids at position 69 is minimal, as class A enzymes possess

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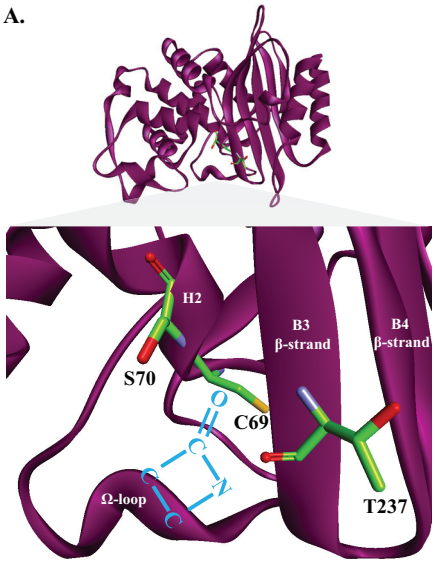
A.



B.

Position	66	67	68	69	70	71	72	73
PenI	F	P	F	C	S	T	S	K
SHV-1	F	P	M	M	S	S	F	K
KPC-2	F	P	L	C	S	S	F	K
PSE-1	F	P	L	T	S	T	F	K
PER-1	F	P	M	Q	S	V	F	K
PC1	F	A	Y	A	S	T	S	K
VEB-1	F	P	M	Q	S	V	M	K
TEM-1	F	P	M	M	S	T	F	K
CTX-M-1	F	A	M	C	S	T	S	K

FIG 1 (A) Structure of the PenI β-lactamase and PenI's active site displaying residues C69, S70, and T237 in green. The backbone amides of S70 and T237 form the electrophilic center of the β-lactamase that draws in the carbonyl of the β-lactam (blue) for nucleophilic attack by the S70 hydroxyl side chain. C69 resides on the back wall of the active site and does not participate in the catalytic mechanism. (B) Alignment of amino acid positions 66 to 73 from a variety of class A β-lactamases showing the diversity at position 69.

different amino acids (e.g., M, C, A, T, and Q) at this position (Fig. 1B). Most often, substitutions at position 69 in other class A β-lactamases (e.g., SHV, TEM, OHIO-1, and BlaC) are associated with β-lactam–β-lactamase inhibitor resistance (e.g., ampicillin-clavulanic acid) (16, 19–27). For example, M69I, -V, and –L variants of SHV-1 are resistant to inhibition by clavulanic acid. β-Lactamase inhibitor resistance in the SHV family of β-lactamases is hypothesized to occur due to the "deformation" of the oxyanion hole (25, 26).

On the other hand, studies using an entire panel of M69 variants of SHV-1 revealed that residue 69 played a larger role in determining substrate specificity, since M69K, -Y, and -F variants demonstrated increased resistance to ceftazidime (16). This phenotype was attributed to movement of the B3 β-strand and repositioning of amino acids in the active site that mimicked the case with extended-spectrum β-lactamases (ESBLs). Clearly, the mechanism that allows ceftazidime resistance due to amino acid substitutions at position 69 of class A β-lactamases is complex.

In this study, we explore the role of Ambler position C69 and its contribution to ceftazidime resistance in PenI using microbiological, biochemical, analytical, and proteomic methods. Interestingly, our data reveal that 9 of 19 substitutions at this position increase ceftazidime resistance by >2 doubling dilutions. We chose to study the C69F variant in detail as (i) it conferred an MIC of 64 mg/liter despite lower protein expression levels; (ii) the change to Phe is found in other enzymes, such as SHV; and (iii) the C69Y variant of PenI was previously studied (6, 7). Our data suggest that ceftazidime resistance in the C69F variant is mediated by unique conformational species of the C69F variant, one of which hydrolyzes ceftazidime rapidly when stabilized. Our findings provide new insight in the quest to understand β-lactamase-mediated resistance in this clinically important pathogen.

MATERIALS AND METHODS

Strains and mutagenesis. Construction of the wild-type β-lactamase vectors has been previously described (14). Site saturation and site-directed mutagenesis were performed using the Quikchange XL site-directed-mutagenesis kit (Agilent) according to the manufacturer's protocol. Briefly, primers that included all 19 amino acid substitutions at Ambler position 69 for bla_{PenI} were designed. PCR was conducted using the phagemid pBC SK(+) bla_{PenI} as the template for the C69 site saturation primer sets. To digest the template, 1.0 μl of DpnI was added to all of the reaction mixtures, and the reaction mixtures were incubated at 37°C for 1 h. *Escherichia coli* DH10B cells were electroporated with 1.0 μl of each reaction mixture and plated on lysogeny broth (LB) agar plates containing 20 mg/liter chloramphenicol as the selecting agent. Single colonies were chosen for plasmid purification, and DNA sequencing by MCLAB (Molecular Cloning Laboratories) was conducted to verify the mutations. Site-directed mutagenesis was also performed as described above, but using the pET24a(+) bla_{PenI} vector with site-directed primers containing the nucleotide sequence for the corresponding C69F substitution. After the mutations were verified, pET24a(+) plasmids were transformed into *E. coli* Origami 2(DE3) cells. All protein expression constructs carrying pET24a(+) bla_{PenI} are missing the signal peptide corresponding to the first 90 nucleotides.

Cell-based assays. *E. coli* DH10B expressing bla_{PenI} and position 69 from pBC SK(+) were phenotypically characterized using agar dilution MICs. Mueller-Hinton (MH) agar was used for the cell-based assays, according to the Clinical and Laboratory Standards Institute guidelines, using a Steers replicator (28). Ampicillin, piperacillin, cephalothin, cefotaxime, and clavulanic acid were purchased from Sigma-Aldrich. Ceftazidime was acquired from Research Products International Corp. Imipenem-cilastatin was obtained from its commercial source. Sulbactam and aztreonam were bought from Astratech Inc., while tazobactam was purchased from Chem-Impex International Inc.

Immunoblotting. Cells expressing PenI and all of the variants were grown in LB to log phase (with an optical density at 600 nm [OD_{600}] between 0.6 and 0.7). The cells were pelleted and lysed using stringent periplasmic fractionation to prepare crude extracts, as previously described (29). The extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% nonfat dry milk in 20 mM Tris-Cl with 150 mM NaCl, pH 7.4 (Tris-buffered saline [TBS]), for 1 h and probed in 5% nonfat dry milk in TBS with 1 μg/ml of polyclonal anti-PenI antibody (7) and a loading control,

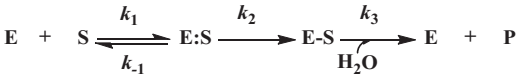


FIG 2 Interactions between the β-lactamase (E) and the β-lactam (S) were initially interpreted according to the scheme shown. Here, the formation of the noncovalent complex, E:S, is represented by the dissociation constant, K_s, which is equivalent to k_{-1}/k_1. k_2 is the first-order rate constant for the acylation step, or the formation of the E-S complex. k_3 is the rate constant for the hydrolysis of the E-S acyl-enzyme and product (P) release. The Michaelis constant, K_m, is equivalent to $K_s \times (k_3/k_2 + k_3)$.

a 1:10,000 dilution of monoclonal anti-DNAK antibody (Stressgen), for 1 h. The membranes were washed five times for 10 min each time with TBS with 0.05% Tween 20 (TBST), and for protein detection, blots were incubated for 1 h in 1:10,000 dilutions of horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies in 5% nonfat dry milk in TBS. The blots were washed five times for 10 min each time with TBST and developed using the ECL-Plus developing kit (GE Healthcare Life Sciences) or the SuperSignal West Femto Chemiluminescent Substrate (Thermoscientific) according to the manufacturers' instructions. A Fotodyne Luminary/FX was used to capture images.

Quantitative immunoblotting of β-lactamases. Previously described methods were used to determine the amount of β-lactamase protein per cell (30). Briefly, cells were grown and periplasmic extracts were prepared as described above for immunoblotting. Cells pretreated with ceftazidime at a concentration of 1 μg/ml were grown overnight in LB and then subcultured and grown to an OD_{600} of 0.6 to 0.7 in LB with ceftazidime at 1 μg/ml. An aliquot of cells was used to determine the total cell number by serial dilution, plating, and colony counts. A standard curve with known amounts of purified PenI and crude extracts was used for immunoblotting as described above. Band densities on the blot images were assigned using EZQuant gel analysis software. The standard curve and total cell number were used to calculate the amount of protein per cell.

β-Lactamase purification. The purification of PenI was described previously (14). Briefly, *E. coli* Origami 2(DE3) cells expressing PenI and the C69F variant β-lactamases expressed from the pET24a(+) plasmid were grown in superoptimal broth to an OD_{600} of 0.6, 200 μM isopropyl-β-D-1-thiogalactopyranoside was added, and the cells were grown for an additional 3 h at 37°C. The cells were pelleted and lysed. The crude extracts prepared from PenI and the variants were used for preparative isoelectric focusing (pIEF) with a pH gradient from 3.5 to 10, followed by a nitrocefin overlay for detection (31). The samples from pIEF were dialyzed against 10 mM phosphate-buffered saline (PBS), pH 7.4, for up to 48 h to remove residual nitrocefin. The purity of the fractions from all of the preparations was determined by SDS-PAGE. The gels were stained with Coomassie brilliant blue R250. The protein concentrations were determined by measuring the absorbance at λ_{280} and Beer's law using the proteins' extinction coefficients (Δε) (21,555 M^{-1} cm^{-1} for PenI and the variants at 280 nm) (14). The theoretical masses of the β-lactamases, as well as the extinction coefficients, were obtained using the ProtParam tool on the ExPASy Bioinformatics Resource Portal. To maintain full enzymatic activity, PenI and the variant β-lactamases were stored in 10 mM PBS, pH 7.4, with 25% glycerol at −20°C.

Steady-state kinetics. The steady-state kinetic parameters were determined using an Agilent 8453 Diode Array spectrophotometer. All of the assays were completed in 10 mM PBS at pH 7.4 at 25°C. The interactions between the PenI β-lactamase (E) and the β-lactam (S) substrate studies were initially interpreted according to the scheme presented in Fig. 2.

The spectroscopic properties of nitrocefin and ceftazidime, as well as the poor catalytic efficiency of the C69F variant, limited the ability to determine K_m and V_{max} values. To study ceftazidime hydrolysis, 10 μM PenI or the C69F variant was mixed with 25 μM ceftazidime. Ceftazidime hydrolysis was measured during a 2,000 s time course at an absorbance of λ_{260}.

Additionally, progress curves were obtained by inhibiting nitrocefin hydrolysis with increasing concentrations of ceftazidime for a selected time course. For these assays, ceftazidime and nitrocefin were initially mixed, and then the enzyme was diluted in the reaction mixture. PenI was maintained at 350 nM with ceftazidime concentrations ranging from 20 to 320 mM to observe inhibition of 50 μM nitrocefin hydrolysis for 300 s. The C69F variant was held at 10 μM (to obtain measurable nitrocefin hydrolysis) with a ceftazidime concentration range of 10 μM up to 1 mM with 50 μM nitrocefin for 2,200 s. Progress curves were fitted to obtain k_{obs} values using the following equation: $y = V_f \times x + (V_0 - V_f) \times [1 - \exp(-k_{obs} \times x)]/k_{obs} + A_0$, where, V_f is the final velocity, V_0 is the initial velocity, and A_0 is the initial absorbance at 482 nm. The k_{obs} values versus the ceftazidime concentration were plotted. If a saturation curve was obtained from the k_{obs} versus the ceftazidime concentration graph, then a modified Michaelis-Menten equation was used to determine K_I and k_{inact} (32). Conversely, if a line was obtained, then the slope of the line was determined to be k_2/K (33). The K_I and k_2/K values were not corrected for the use of nitrocefin, as the K_m and V_{max} values could not be determined for the C69F variant.

Pre-steady-state kinetics. The pre-steady-state kinetic parameters were determined using an Applied Photophysics SX20 stopped-flow apparatus. All of the assays were completed in 10 mM PBS at pH 7.4 at 25°C. To assess for a "burst" in ceftazidime hydrolysis, 10 μM the C69F variant was mixed with 25 μM ceftazidime, and ceftazidime hydrolysis at an absorbance of λ_{260} was measured. In order to evaluate the changes in protein conformation, 10 μM the C69F variant was preincubated for 10 min with a polyclonal anti-PenI antibody (which was also used for immunoblotting [see above]) according to the method developed by Citri et al. (34). The preincubated C69 variant-antibody complex was again mixed with 25 μM ceftazidime, and ceftazidime hydrolysis at an absorbance of λ_{260} was measured.

ESI-MS of ceftazidime. Electrospray ionization-mass spectrometry (ESI-MS) of ceftazidime and the hydrolyzed products of ceftazidime was performed on an Agilent Technologies 6460 Triple Quad mass spectrometer equipped with an electrospray ion source. A check tune was conducted in positive mode and passed on the Agilent 6460 Triple Quad using ESI-L low-concentration tuning mix (Agilent; G1969-85000). Samples consisted of 2 mM ceftazidime, 2 mM ceftazidime with 100 mM sodium hydroxide (NaOH), 2 mM ceftazidime with 100 μM PenI, and 2 mM ceftazidime with 100 μM the C69F variant. These samples were incubated at 37°C for 4 h, and then the protein was removed using centrifugation on Nanosep centrifugation columns with a 10,000 Da molecular mass cutoff (Pall Corp.) for 10 min at 10,000 rpm. One microliter of flowthrough from each centrifugation was run on an Agilent Infinity 1260 high-performance liquid chromatograph (HPLC) with an Agilent Poroshell 120 EC-C_{18} 2.7-μm, 3.0- by 50-mm column with the column temperature maintained at 25°C. The mobile phase consisted of 0.1% formic acid in water. The small molecules were eluted using a gradient with final conditions of 10% mobile phase and 90% organic phase (100% acetonitrile with 0.1% formic acid). The samples were run in MS2 positive scan mode with a starting mass of 400 atomic mass units (amu) and an ending mass of 600 amu, a scan time of 500, the fragmenter set at 70, and the cell accelerator voltage at 4. The settings for each of the data runs were as follows: capillary voltage at 4.0 kV, gas temperature at 350°C, gas flow at 10 liters/min, nebulizer at 40 lb/in^2, sheath gas temperature at 350°C, and sheath gas flow at 11 liters/min. The spectra were analyzed using the Agilent Mass Hunter Qualitative Analysis Program B.06.00.

ESI-MS of β-lactamases. ESI-MS of the intact PenI and the C69F variant with and without ceftazidime was performed on a Waters Synapt G2-Si quadrupole-time of flight mass spectrometer equipped with a LockSpray dual-electrospray ion source, using glu-1-fibrinopeptide B as the lock mass. The Synapt G2-Si was calibrated with a sodium iodide solution using a 50 to 2,000 *m/z* mass range. Each β-lactamase (10 μM) was incubated with the β-lactam (10 μM in most cases for a 1:1 β-lactam-to-β-

TABLE 1 Cell-based assays to determine MIC values of *E. coli* DH10B expressing bla_{PenI} and the C69X variants of PenI for selected β-lactams and β-lactam–β-lactamase inhibitor combinations

Strain	MIC[a] (mg/liter)								
	AMP	THIN	TAX	CAZ	AZT	IMI	AMP-CLAV[b]	AMP-SUL[b]	PIP-TAZO[c]
E. coli DH10B pBC SK(+)	4	2	0.06	0.25	1	0.5	50/0.06	50/1	2/0.25
E. coli DH10B pBC SK(+) bla_{PenI}[d]	512	256	4	2	8	0.25	50/0.06	50/4	4/0.5
C69A	512	512	2	**4**	1	0.25	50/0.125	50/4	4/0.5
C69D	8	4	0.125	0.5	1	0.25	50/0.06	50/1	4/0.5
C69E	8	16	1	**4**	1	0.25	50/0.06	50/1	4/0.5
C69F	8	16	4	**64**	1	0.25	50/0.06	50/1	4/0.5
C69G	256	64	0.25	2	1	0.25	50/0.125	50/2	8/1
C69H	64	64	2	**16**	1	0.25	50/0.06	50/1	4/0.5
C69I	256	32	0.5	**16**	1	0.25	50/0.06	50/1	8/1
C69K	8	16	1	**16**	1	0.25	50/0.06	50/1	4/0.5
C69L	512	128	0.5	**4**	1	0.5	50/0.06	50/4	8/1
C69 M	256	256	2	**16**	1	0.5	50/0.06	50/1	4/0.5
C69N	256	128	0.5	**8**	1	0.5	50/0.06	50/2	8/1
C69P	4	2	0.06	0.25	1	0.25	50/0.06	50/1	2/0.25
C69Q	64	16	1	**16**	1	0.25	50/0.06	50/1	4/0.5
C69R	8	8	1	**16**	1	0.25	50/0.06	50/1	4/0.5
C69S	256	32	0.125	0.5	1	0.5	50/0.06	50/4	8/1
C69T	512	256	0.5	1	1	0.5	50/0.06	50/4	16/2
C69V	512	64	0.5	**8**	1	0.5	50/0.25	50/4	8/1
C69W	4	8	0.125	0.5	1	0.25	50/0.06	50/1	2/0.25
C69Y	4	16	8	**64**	1	0.25	50/0.06	50/1	4/0.5

[a] β-Lactam and β-lactamase inhibitor abbreviations: AMP, ampicillin; THIN, cephalothin; TAX, cefotaxime; CAZ, ceftazidime; AZT, aztreonam; IMI, imipenem; CLAV, clavulanic acid; SUL, sulbactam; and TAZO, tazobactam. Experiments were completed in triplicate. Elevated CAZ MICs are in boldface.

[b] AMP was maintained at a constant concentration of 50 mg/liter, and the clavulanic acid and sulbactam concentrations were varied.

[c] Piperacillin and tazobactam were varied at a ratio of 8:1.

[d] All of the C69 variants were expressed from pBC SK(+) bla_{PenI} in *E. coli* DH10B.

lactamase ratio, but up to 1 mM with PenI for a 100:1 ceftazidime-to-PenI ratio) for various incubation times. The reactions were terminated by the addition of 0.1% formic acid and 1% acetonitrile. The samples were run using a Waters Acquity H class ultraperformance liquid chromatograph (UPLC) on an Acquity UPLC BEH C_{18} 1.7-μm, 2.1- by 100-mm column. The mobile phase consisted of 0.1% formic acid in water. The β-lactamase and β-lactamase–β-lactam complexes were eluted using a gradient with final conditions of 15% mobile phase and 85% organic phase (100% acetonitrile with 0.1% formic acid). Lock mass spectra were collected during the sample injections. The tune settings for each of the data runs were as follows: capillary voltage at 3.5 kV, sampling cone at 35, source offset at 35, source temperature at 100°C, desolvation temperature at 500°C, cone gas at 100 liters/h, desolvation gas at 800 liters/h, and nebulizer bar at 6.0. The spectra were analyzed using MassLynx v4.1, modified for the lock mass deviations by applying a gain factor, and deconvoluted using the MaxEnt1 program.

Molecular modeling. The crystal coordinates of PenI (Protein Data Bank [PDB] 3W4P) were used to construct the acyl-enzyme representations of PenI and the C69F variant with ceftazidime, as previously described, using the Discovery Studio 4.1 (DS 4.1) (Accelrys, Inc., San Diego, CA) molecular-modeling software (17, 35). The variant was built by substituting phenylalanine for the cysteine at position 69.

Ceftazidime was constructed for molecular modeling using the Fragment Builder tools and was minimized using a Standard Dynamics Cascade protocol of DS 4.1. Ceftazidime (missing its R2 side chain [see below]) was automatically positioned in the active site of PenI β-lactamase using the Flexible Docking module of DS 4.1. The protocol allowed for the flexible placement of a β-lactamase inhibitor in the active site of PenI. After docking, the most favorable pose of ceftazidime was chosen (i.e., demonstrating a short distance [2 to 3 Å] between Ser70: O (hydroxyl side chain of Ser70) and C_7 of ceftazidime, as well as anticipated active-site contacts), and the acyl complex was created. The complex of the C69F variant with ceftazidime was created in a similar fashion. To check the stability and to look for possible conformational changes of the complex, Molecular dynamics simulation (MDS) was conducted for 6 ps on PenI-ceftazidime and the C69F-ceftazidime complexes, as previously described and validated (35).

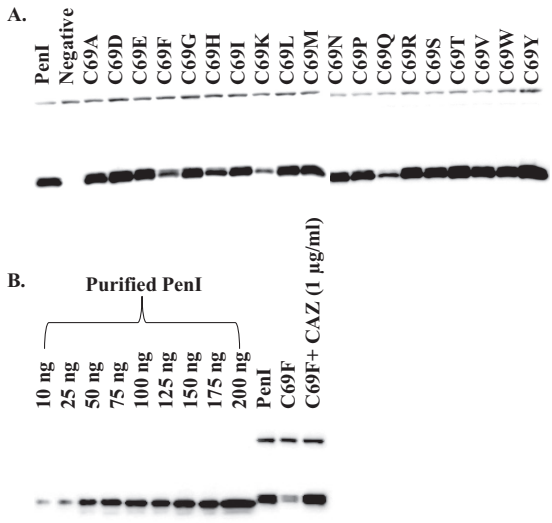


FIG 3 Immunoblots of PenI and variants. (A) PenI β-lactamase (bottom band) expression levels of *E. coli* DH10B cells carrying pBC SK(+) bla_{PenI} (PenI) and the C69 variants grown in LB to log phase; DNAK was used as a loading control (top band). Lane "Negative" is *E. coli* DH10B with an empty pBC SK(+) phagemid. (B) PenI β-lactamase expression levels of *E. coli* DH10B cells carrying pBC SK(+) bla_{PenI} (PenI) and the C69F variant grown in LB to log phase with or without ceftazidime (CAZ) at 1 μg/ml. A standard curve was derived from the densities of the bands for known concentrations of purified PenI (10 to 200 ng).

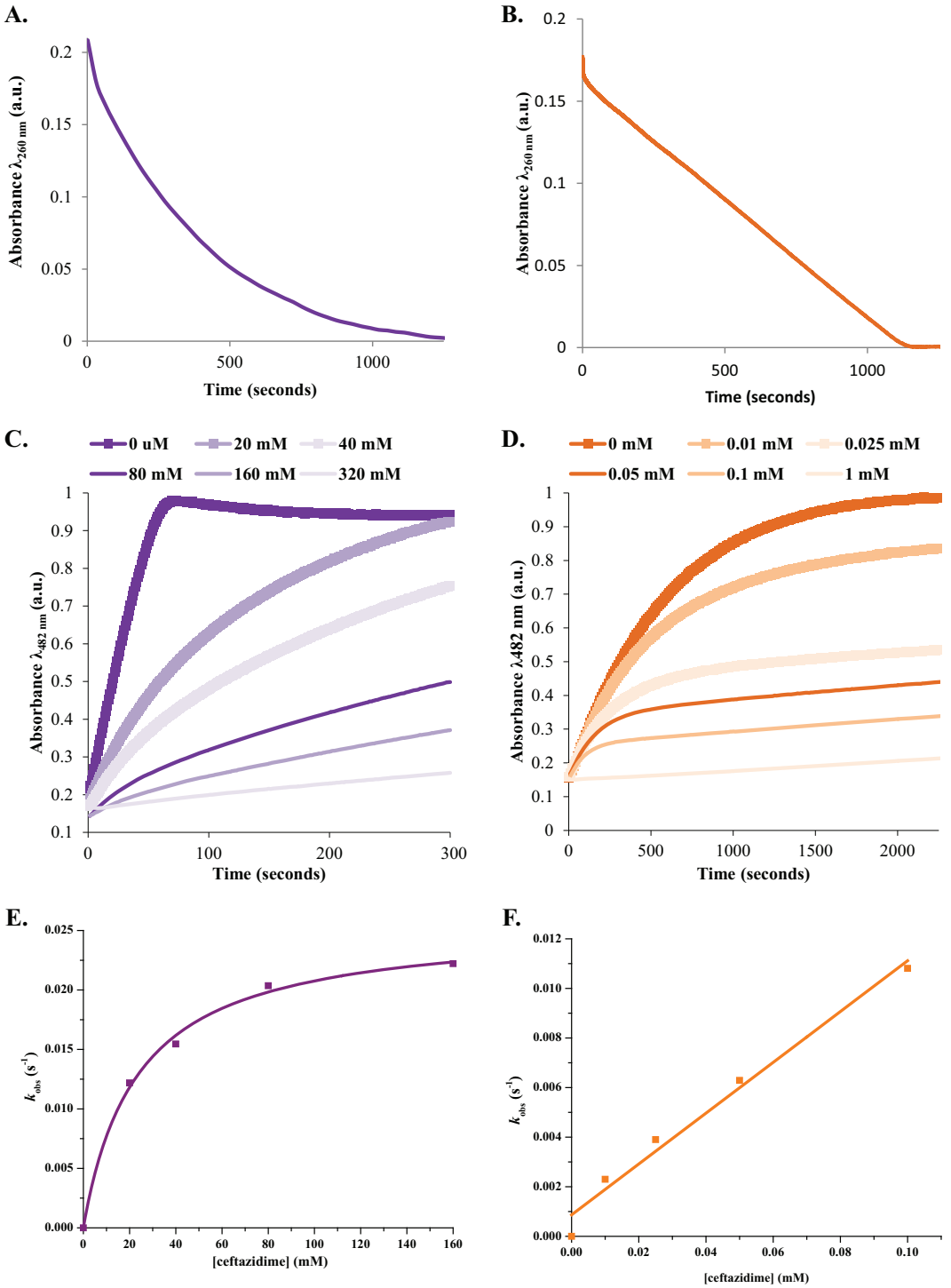


FIG 4 Kinetics of PenI and the C69F variant. (A) Hydrolysis of 25 μM ceftazidime by 10 μM PenI. (B) Biphasic hydrolysis of 25 μM ceftazidime by 10 μM the C69F variant measured on a stopped-flow apparatus. (C) Hydrolysis of 50 μM nitrocefin by 350 nM PenI inhibited by increasing concentrations of ceftazidime. (D) Hydrolysis of 50 μM nitrocefin by 10 μM the C69F variant inhibited by increasing concentrations of ceftazidime. (E) The k_{obs} values obtained by fitting the progress curves in panel C to the equation in Materials and Methods plotted versus the ceftazidime concentration. (F) The k_{obs} values obtained by fitting the progress curves in panel D to the equation in Materials and Methods plotted versus the ceftazidime concentration. a.u., arbitrary units.

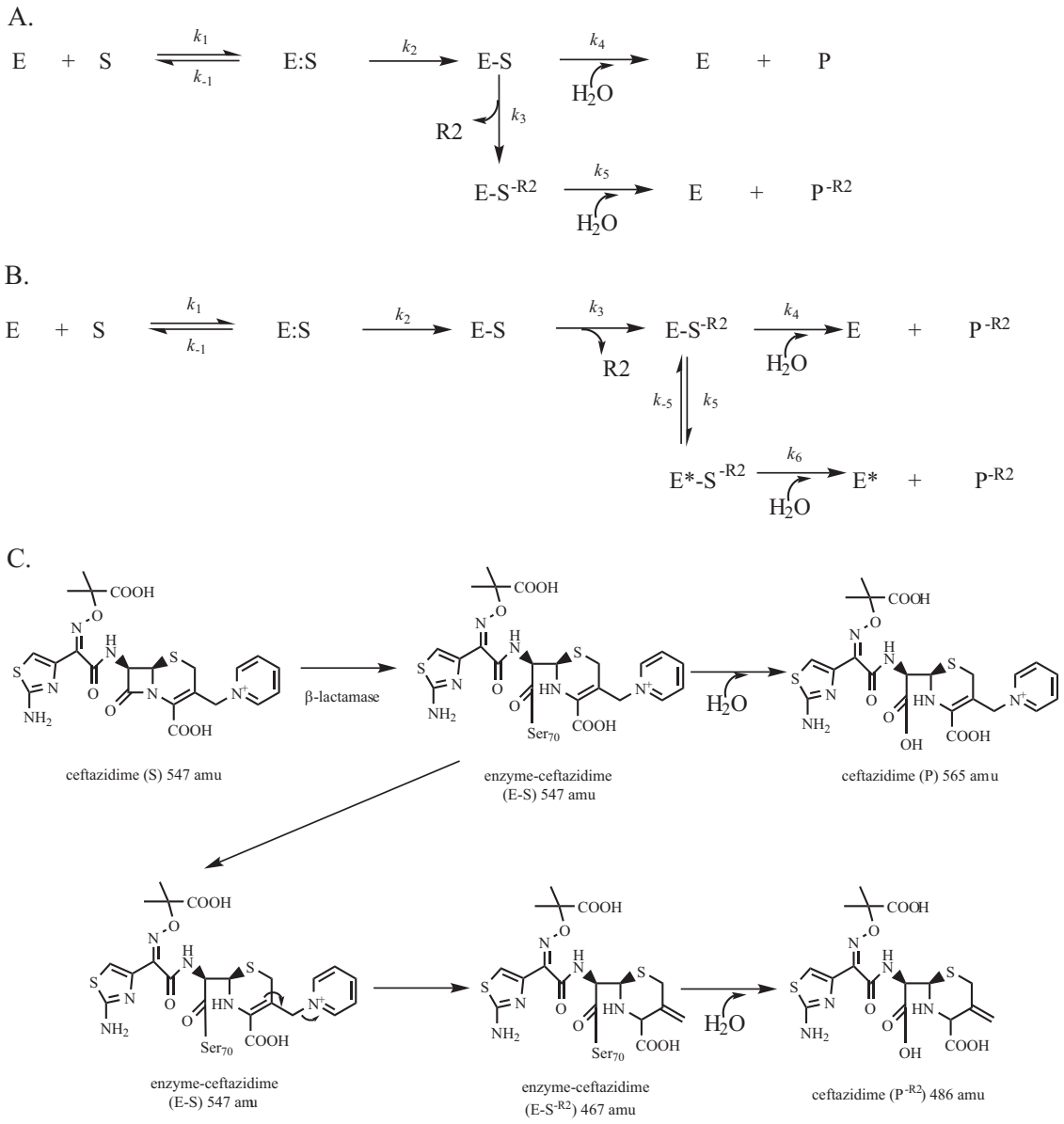


FIG 5 The biphasic kinetics observed for the C69F variant can be explained by two different branched kinetic schemes. (A) As previously described by Faraci and Pratt (36), loss of the R2 of cephalosporins is a common mechanism during cephalosporin hydrolysis that can lead to a branched mechanism, depicted by the rate constant k_3. (B) Alternatively, earlier, Citri et al. (34) found that β-lactamases can undergo a potentially reversible conformation change to a less active form, E*, here depicted by the rate constant k_5, resulting in an alternative branched mechanism. (C) Chemical structures of hydrolysis of ceftazidime (S), acyl-enzyme complexes, loss of the R2 side chain (S^{-R2}), and potential final products (P and P^{-R2}).

RESULTS AND DISCUSSION

Cell-based assays of *E. coli* expressing bla_{PenI} and the C69 variants. An isogenic *E. coli* library was created to assess the impact of single-amino-acid substitutions. We observed that, in general, changes at residue 69 in PenI increased resistance to ceftazidime, but not to other β-lactams or β-lactam–β-lactamase inhibitor combinations. As Table 1 shows, 13 of 19 variants demonstrated an increase in the ceftazidime MIC compared to the wild-type bla_{PenI} counterpart when expressed in an isogenic background, *E. coli* DH10B carrying the pBC SK(+) phagemid. The C69F and -Y variants displayed the highest levels of ceftazidime MICs at 64 mg/liter, and the former (C69F) was chosen for further study. This increase in the MIC was much more dramatic than that observed with another class A β-lactamase, SHV-1. For SHV-1, only a two-doubling-dilution increase in the ceftazidime MIC was observed for the M69F variant (8 mg/liter) compared to wild-type SHV-1 (2 mg/liter) expressed in *E. coli* DH10B (16).

Interestingly, three variants, C69D, -P, and -W, were more susceptible to both ampicillin and ceftazidime, unlike the other 16 variants. We hypothesize that the amino acid substitutions of aspartic acid, proline, and tryptophan must alter the oxyanion hole pocket due to the introduction of a negative charge, a kink in the strand, and a steric clash caused by a large hydrophobic side chain, respectively. These changes would directly impact the binding and hydrolysis of ampicillin and ceftazidime. The understanding of these mechanisms will be the focus of future study.

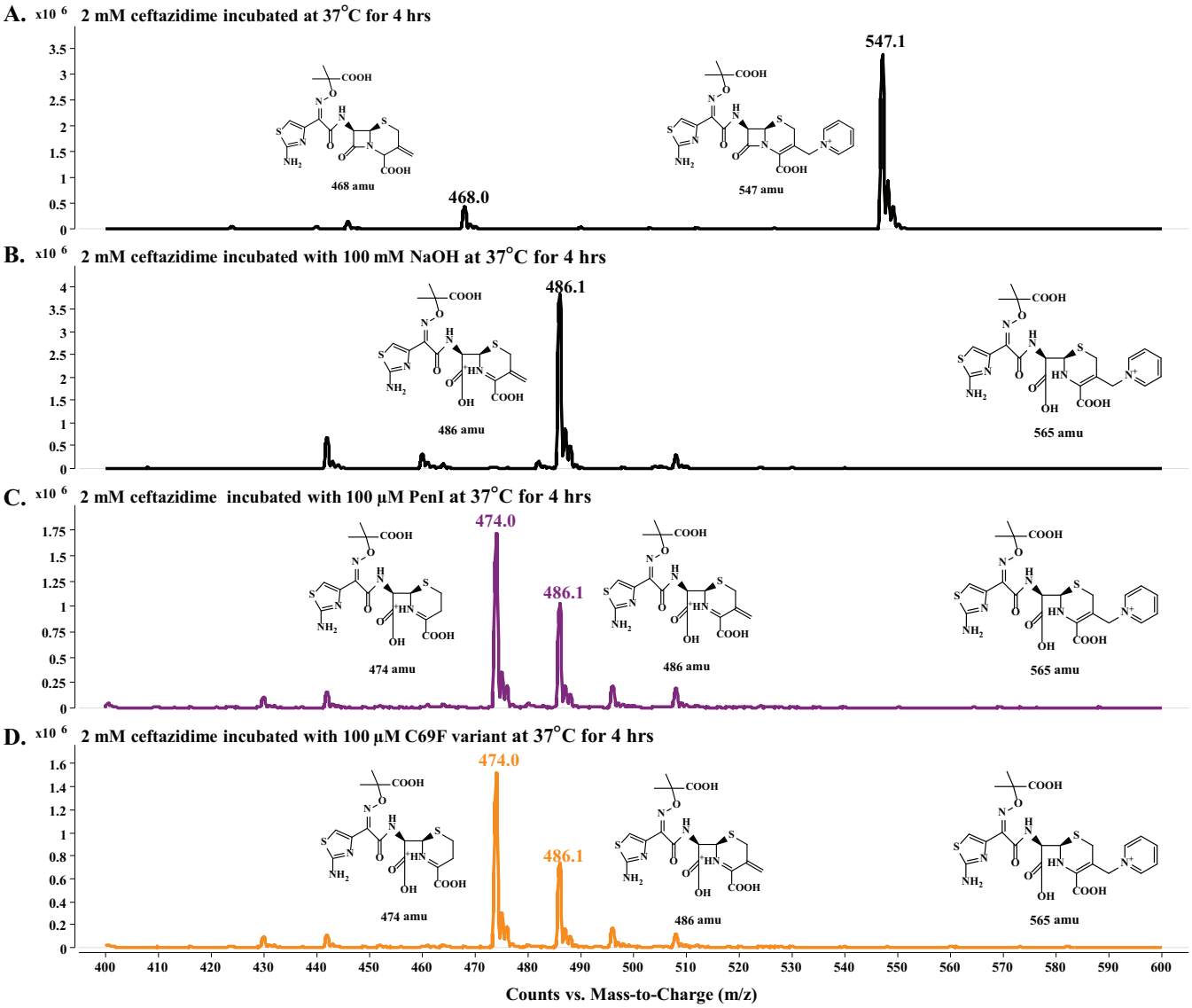


FIG 6 Small-molecule mass spectrometry revealed the observed products after incubation at 37°C for 4 h, restricting the mass range window to 400 to 600 amu. (A) Ceftazidime (2 mM) alone showed a major peak of the full-size ceftazidime at 547 amu and a very minor peak at 468 amu representative of ceftazidime with the breakdown of the R2 side chain to the exomethylene; increasing the fragmenter and cell accelerator voltage while conducting mass spectrometry can result in artifactual breakdown to the 468-amu ceftazidime. (B) Ceftazidime (2 mM) hydrolyzed by 100 mM NaOH revealed one major peak of 486 amu, corresponding to the hydrolyzed ceftazidime with breakdown of the R2 side chain to the exomethylene. (C) Ceftazidime (2 mM) hydrolyzed by 100 µM PenI identified two peaks of 474 and 486 amu, which correspond to hydrolyzed ceftazidime with complete loss of the R2 side chain and hydrolyzed ceftazidime with breakdown of the R2 side chain to the exomethylene, respectively. (D) Ceftazidime (2 mM) hydrolyzed by 100 µM the C69F variant identified two peaks of 474 and 486 amu, which correspond to hydrolyzed ceftazidime with complete loss of the R2 side chain and hydrolyzed ceftazidime with breakdown of the R2 side chain to the exomethylene, respectively. The full-size hydrolyzed ceftazidime of 565 amu was not observed under any tested condition.

Steady-state β-lactamase expression of PenI and C69 variants in *E. coli*. The pattern of susceptibility of the C69 variants prompted us to measure the β-lactamase expression levels of PenI and the C69 variants of these cells grown to log phase in LB. Here, we asked whether the differences in the ceftazidime resistance profiles of the C69 variants when expressed in *E. coli* could be the result of changes in β-lactamase expression (Fig. 3A). Interestingly, expression of the C69F variant of PenI was less than steady-state wild-type PenI expression. Growing the C69F variant with subinhibitory concentrations of ceftazidime returned the expression of C69F to steady-state levels (Fig. 3B). A number of factors (e.g., altered transcription, altered translation, protein half-life, proteolytic degradation, protein stability, and/or transport into the periplasmic space) have the potential to influence the increased protein content. Deeper analysis of these factors will be the focus of future work.

Ceftazidime turnover by PenI and the C69F variant. To assess the β-lactam resistance profile of the C69F variant compared to PenI, the β-lactamases were purified for steady-state and pre-steady-state kinetics. We also investigated the β-lactam products formed following hydrolysis by the β-lactamases.

Measuring hydrolysis of 25 µM ceftazidime by 10 µM PenI and the C69F variant revealed that the C69F variant hydrolyzed an

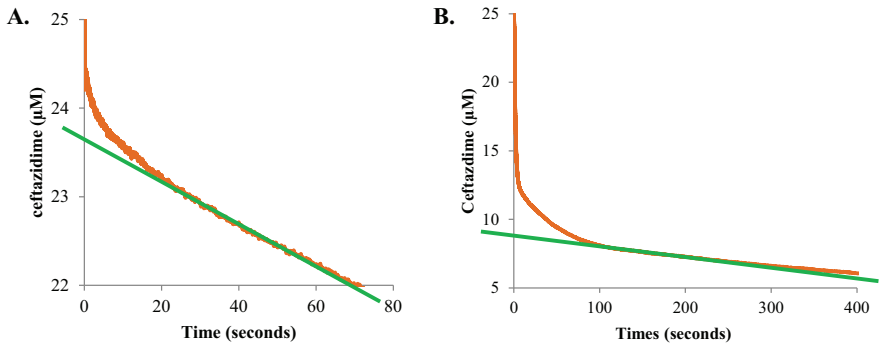


FIG 7 (A) Burst in ceftazidime (25 μM) hydrolysis observed with 10 μM the C69F variant. Extrapolation (green line) of the steady-state velocity to the *y* axis revealed a burst amplitude of ~1.5 μM. (B) Burst in ceftazidime (25 μM) hydrolysis observed with 10 μM the C69F variant that was preincubated with anti-PenI polyclonal antibody for 10 min. Extrapolation (green line) of the steady-state velocity to the *y* axis revealed a burst amplitude of ~17 μM.

amount of ceftazidime similar to that hydrolyzed by PenI within 1,500 s, but at different overall rates (Fig. 4A and B). PenI's hydrolysis of ceftazidime follows an exponential decrease that is faster than that of the C69F variant. This hydrolysis could be due to poor affinity of ceftazidime for PenI, resulting in an instantaneous velocity for the reaction that decreases as the ceftazidime is consumed. Notably, the progress curve of the C69F variant with ceftazidime was biphasic with an initial burst in hydrolysis, suggesting a branched pathway followed by a linear velocity similar to a zero-order process, which may be attributable to better affinity of ceftazidime for the C69F variant than PenI.

Two primary branched pathways for select substrates have been described for certain β-lactamases. As initially reported by Faraci and Pratt in 1985 (36), one branched pathway can occur as a result of the loss of the R2 side chain of cephalosporins (Fig. 5A). Another branched pathway can be observed due to a reversible conformational change in the enzyme during hydrolysis of a cephalosporin (Fig. 5B) (34). To aid in distinguishing between these two possible branched mechanisms in C69F turnover of ceftazidime, we performed small-molecule mass spectrometry and pre-steady-state kinetics.

If the branched mechanism is due to loss of the R2 side chain, then we would observe two ceftazidime products (P and P^{-R2}) (Fig. 5A and C). To test this possible mechanism, we incubated ceftazidime alone, ceftazidime with NaOH (as a control), ceftazidime with PenI, and ceftazidime with the C69F variant at 37°C for 4 h. In each case, the β-lactamase was removed using size exclusion column chromatography, and the products were evaluated using HPLC and triple quad mass spectrometry.

Unreacted ceftazidime produced the expected mass of unhydrolyzed substrate at 547.1 amu (Fig. 6A). The reaction that served as a control (NaOH) revealed a peak of 486.1 amu, which corresponds to hydrolyzed exomethylene ceftazidime product (P^{-R2}); the hydrolyzed product (P) of ceftazidime of 565 amu was not observed (Fig. 6B). The exomethylene product of ceftazidime is predicted to occur as a result of catalysis by the β-lactamase and was previously observed by other groups (17, 37). Both PenI and the C69F variant incubated with ceftazidime produced the same two products of 474.0 amu and 486.1 amu, corresponding to the complete removal of the R2 side chain and hydrolyzed exomethylene ceftazidime product (P^{-R2}), respectively (Fig. 6C and D). The products observed from hydrolysis of ceftazidime were missing the R2 side chain, which leads us to hypothesize that a conformation change in the β-lactamase may be major driving force behind the kinetics observed. However, we note that the hydrolyzed ceftazidime with an intact R2 side chain may be too unstable under these conditions to be detected by this method. Thus, the contribution of the loss of the R2 side chain of ceftazidime to a branched mechanism cannot be entirely ruled out.

Pre-steady-state kinetics was next used to further explore this pathway, following the method developed by Citri and colleagues (34). The burst amplitude for 10 μM the C69F variant with 25 μM ceftazidime was approximately 1.5 μM, nearly 7-fold lower than the enzyme concentration used in the assay, and represents a nonstoichiometric burst (Fig. 7A) (38). Partitioning between the parallel reactions of a branched mechanism may be the cause of this nonstoichiometric burst. Preincubating 10 μM the C69F variant with the anti-PenI polyclonal antibody for 10 min increased the burst amplitude to 17 μM when reacted with 25 μM ceftazidime (Fig. 7B). These results are consistent with the work conducted by Citri and colleagues with the *Bacillus cereus* class A β-lactamase, showing that adding an antibody stabilizes a particular conformation of a specific β-lactamase, resulting in increased hydrolysis. These data also support our contention that there is a conformational change in the β-lactamase (34).

Ceftazidime steady-state kinetic parameters were unable to be accurately measured due to the spectroscopic properties of ceftazidime and poor hydrolytic activity of the enzymes; thus, the ability of ceftazidime to inhibit PenI and the C69F variant was assessed using a competition experiment. Progress curves of nitrocefin inhibition by ceftazidime revealed that 320 mM ceftazidime was required to almost fully inhibit PenI versus only 1 mM ceftazidime for the C69F variant (Fig. 4C and D). Subsequently, the k_{obs} values were determined for each progress curve, and when the k_{obs} values were plotted versus the concentration of ceftazidime, two different graphs were obtained. A saturation curve for PenI was observed and fitted to obtain a K_I observed value of 23 ± 3 mM and a k_{inact} value of 0.026 s^{-1} (Fig. 4E). A linear plot was detected with the C69F variant corresponding to a slope or k_2/K observed value of 102 ± 10 M^{-1} s^{-1} (Fig. 4F). The linearity of the C69F variant suggests rapid acylation, as well as better affinity for ceftazidime than PenI. With SHV-1, the M69F variant possessed only a 3-fold-lower K_m value than wild-type SHV-1 (16). The different kinetic properties of the C69F variant of PenI and the M69F variant of SHV-1 were correlated with the higher ceftazidime MIC for the C69F variant.

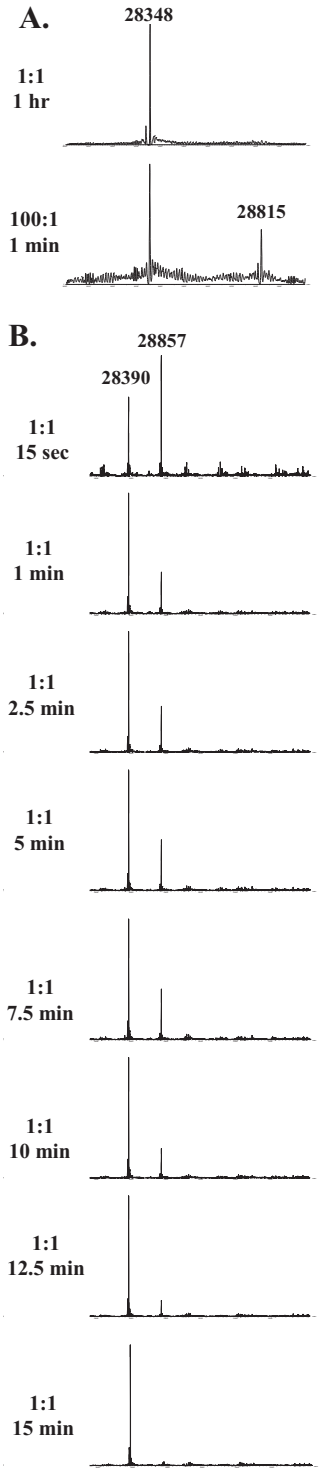


FIG 8 Mass spectrometry of PenI and the C69F variant with ceftazidime (error, ±3 amu). (A) Ceftazidime (10 μM) and PenI (10 μM) at a 1:1 ratio revealed only apo-enzyme (28,348 amu) after 1 h. However, at a 100:1 ratio of ceftazidime to PenI, the acyl-enzyme (28,348 amu + 467 amu = 28,815 amu) was observed within 1 min. (B) Ceftazidime (10 μM) and the C69F variant (10 μM) at a 1:1 ratio over a time course of 15 s to 15 min revealed acyl-enzyme formation (28,857 amu) within 15 s and returned to complete apo-enzyme (28,390 amu) by 15 min.

Protein mass spectrometry. To further investigate our kinetic observations, mass spectrometry of purified PenI missing its signal peptide sequence (theoretical mass = 28,350 amu) and the C69F variant missing its signal peptide sequence (theoretical mass = 28,394 amu) with ceftazidime (theoretical mass = 546 amu), using a 1:1 ratio of ceftazidime to β-lactamase, was conducted. Observable adducts were not detected between PenI and ceftazidime over a 15-s to 15-min time course. After longer times, up to 1 h, an acyl-enzyme adduct of PenI and ceftazidime was also not detected under these conditions (Fig. 8A). However, at higher ratios of ceftazidime to PenI—a ratio of 100:1—a small population of acyl-enzyme (28,815 ± 3 amu) could be observed after 1 min incubation (Fig. 8A). The mass of the adduct corresponded to the exomethylene form of ceftazidime (measured mass = 467 ± 3 amu) (Fig. 5C). Conversely, the C69F variant was found to rapidly acylate ceftazidime (28,857 ± 3 amu). Deacylation (28,390 ± 3 amu) occurred within 15 min; only the 467 ± 3 amu exomethylene form of ceftazidime adduct was observed with the variant (Fig. 8B). These data are in agreement with the small-molecule mass spectrometry.

Molecular modeling. X-ray crystallography of class A β-lactamases with substitutions at position 69 was previously unrevealing in fully elucidating how amino acid changes affected clavulanic acid binding (39). Earlier studies showed that structural changes were not readily observed between the TEM-1 β-lactamase and its M69L variant despite the presence of a resistance phenotype when expressed in *E. coli*. Only upon molecular modeling and MDS were any changes between the two β-lactamases detected. Thus, to assess the contributions of the substitutions at position C69 to the structure of the β-lactamase, molecular representations of PenI and the C69F variant were generated and MDS was conducted.

The apo forms of the β-lactamases revealed that the geometry of the oxyanion hole was different in the C69F variant, as the oxyanion hole water molecule was deeper in the pocket by ~1 Å than in PenI (Fig. 9A). The deeper oxyanion hole in the C69F variant may serve to draw the carbonyl of ceftazidime into the active site more readily than PenI, explaining the lower values seen with competition experiments. Next, the exomethylene form of ceftazidime was docked into the active sites of PenI and the C69F variant. An acyl bond was formed with the deprotonated oxygen of the hydroxyl side chain of S70 and the ceftazidime carbonyl. Two important differences were evident between the two acyl-enzyme models. First, the R1 side chain of ceftazidime possessed two different orientations, similar to the structure of cefotaxime in TOHO-1 (PDB 2ZQA); thus, different orientations of the R1 side chain are conceivable. Second, in the C69F variant model, ceftazidime formed 13 possible hydrogen-bonding interactions with active-site residues, mainly (8 interactions) with the B3 β-strand; a unique interaction with D240 not seen with PenI was also observed (Fig. 9B). The C-4 carboxylate formed typically observed interactions with the B3 β-strand (e.g., hydrogen bonding with amino acids at positions 234, 235, and 237). Conversely, ceftazidime formed fewer potential hydrogen bonds with active-site residues of PenI, and the β-lactam C-4 carboxylate made uncommon interactions with the left side of the active site (with residues S130 and Y105) (Fig. 9C). Given the difference between PenI and the C69F variant, the modeled form of the C69F variant may be the conformation that hydrolyzes ceftazidime rapidly.

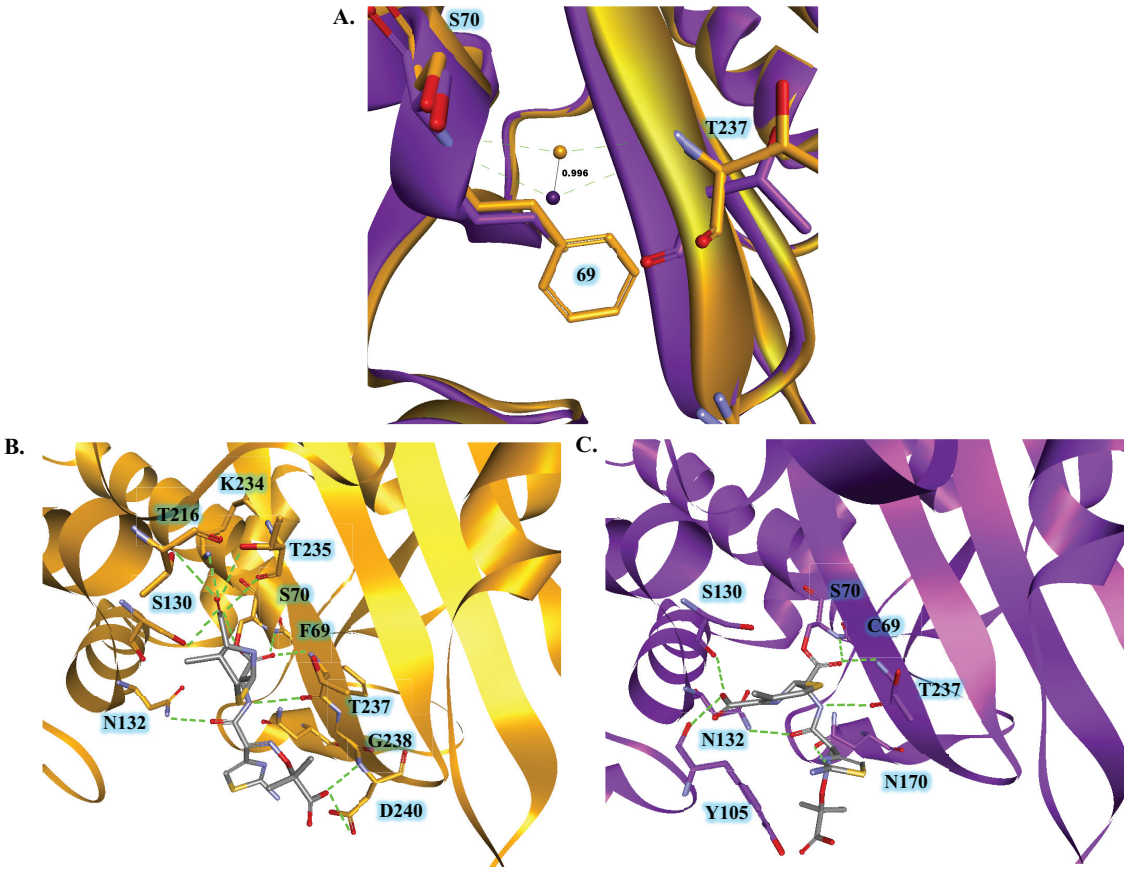


FIG 9 (A) Overlay of the PenI (purple) and C69F (orange) apo-enzyme models showing that the oxyanion hole water position was ~1 Å deeper in the electrophilic center in the C69F variant than with PenI. (B) Molecular modeling of the C69F variant acyl-enzyme with ceftazidime showing the possibility of up to 13 hydrogen-bonding partners, including residue D240. (C) Molecular modeling of the PenI acyl-enzyme with ceftazidime in the active site revealed a limited number of hydrogen-bonding interactions.

Mechanism for ceftazidime resistance. Our data led us to propose a new pathway explaining enhanced ceftazidime kinetics in the C69F variant compared to PenI (Fig. 10). Using previously published approaches as a guide (34, 36), we showed that the C69F variant hydrolyzes ceftazidime along a branched kinetic pathway compared to wild-type PenI. We found that the products formed upon ceftazidime hydrolysis by PenI and the C69F variant lacked the R2 side chain. In addition, pre-steady-state kinetics suggested that the C69F variant may be present in at least two conformations; β-lactamases possessing different conformations have previously been observed (38, 40). We propose that the isoform of the C69F variant that hydrolyzes ceftazidime rapidly was stabilized by preincubation with an anti-PenI antibody. Almost-complete hydrolysis of 25 μM ceftazidime was observed within 100 s compared to 1,500 s without the antibody preincubation. In the cell, the C69F variant may be stabilized in the "rapid form" in the periplasmic space, resulting in the high MIC of 64 mg/liter. Conversely, based upon (i) the observation that PenI possesses a K_I observed value of 23 mM and (ii) our inability to capture the PenI-ceftazidime acyl-enzyme complex on mass spectrometry unless the concentration of ceftazidime was raised, we propose that PenI possesses poor affinity for ceftazidime compared to the C69F variant.

What are the clinical and mechanistic implications of these findings? Our investigations reveal that the C69F variant of PenI is a unique β-lactamase. The wild-type enzyme, PenI, interacts with ceftazidime so poorly that ceftazidime is able to reach the PBP target, thus killing the bacterial cell. In contrast, when expressed in *E. coli*, the C69F variant confers a high ceftazidime MIC (64 mg/liter). To our surprise, we next discovered that a branched pathway exists and that this variant β-lactamase can be present in two conformations. Interestingly, one conformation of the C69F variant rapidly hydrolyzes ceftazidime and may be the dominant form expressed in the cell, resulting in high MICs; the other conformation is catalytically impaired. The presence of ceftazidime in the medium during growth also increases (e.g., stabilizes) the C69F β-lactamase present in the periplasm (41). Underexpressed β-lactamases and those with low hydrolytic activity may be overlooked in clinical isolates using nonphenotypic diagnostic procedures (e.g., mass spectrometry) for detection of antibiotic resistance mechanisms (42); this could result in poor therapeutic choices and treatment failure.

From a mechanistic standpoint, we posit that amino acid changes that affect the tertiary structure by permitting conformational changes that enhance hydrolytic activity can have a significant impact on substrate turnover. In the case of the C69F variant of PenI, changes in the oxyanion hole result in alterations in hydrogen bond formation that permit catalysis of a substrate not normally regarded as "favorable." Considerations such as this have significant impact on new β-lactam and β-lactamase inhib-

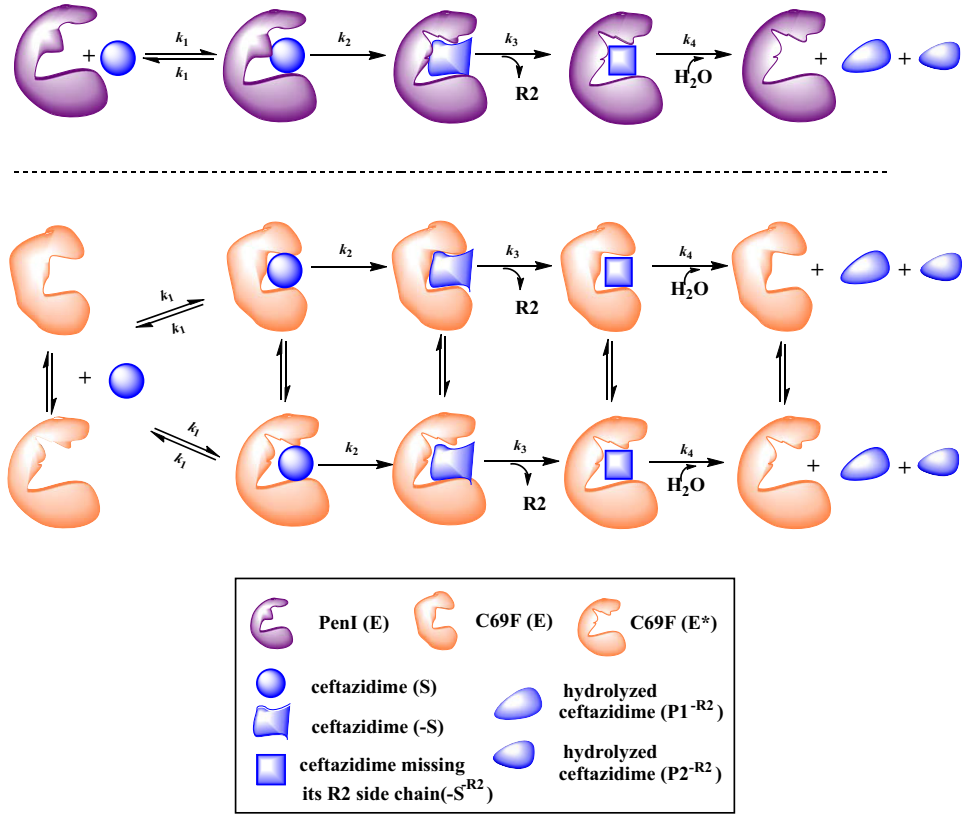


FIG 10 Proposed kinetic mechanism for different ceftazidime kinetics of the PenI (purple) and C69F variant (orange) β-lactamases. The major difference is that the C69F variant possesses two enzyme conformations, resulting in a branched pathway toward ceftazidime hydrolysis compared to PenI.

itor design. Showing that conformational changes result in higher MICs also adds a new level of complexity to understanding β-lactamase evolution. Conversely, finding ways to inhibit conformational changes in enzymes with enhanced kinetic properties can lead to new insights into allosteric inhibitor design (43). Such unanticipated findings can open "new doors" in the design of β-lactamase inhibitors.

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