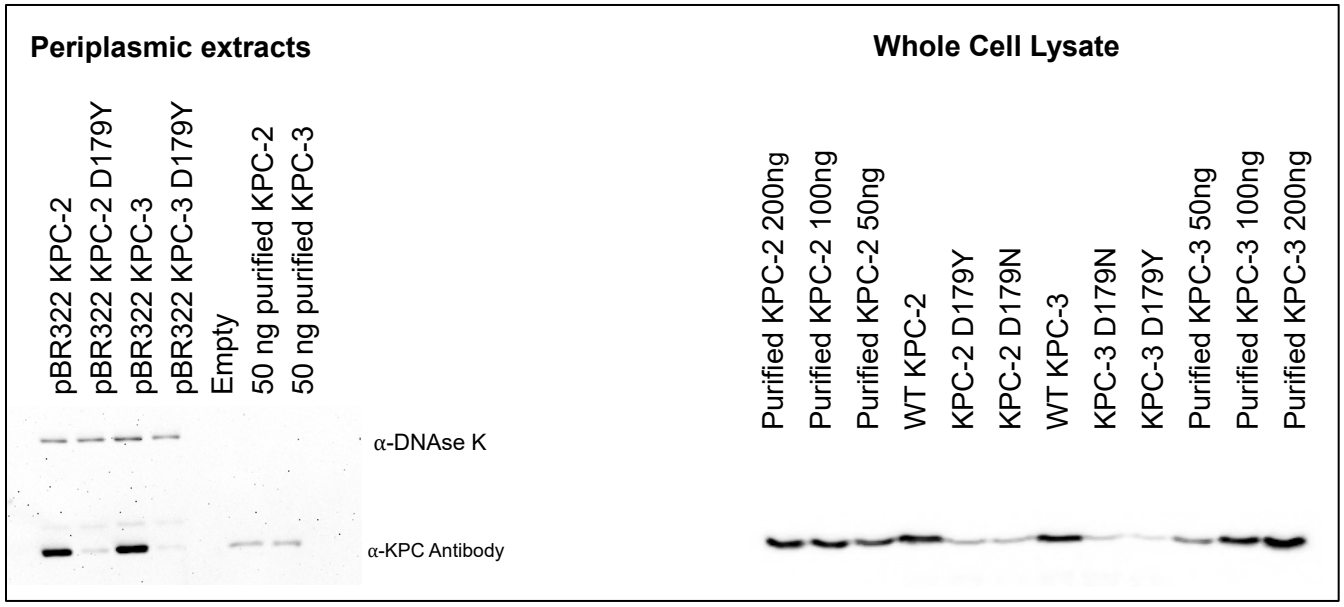
Supplementary File

Isolate*	CAZ	CZA	FEP	PIP	PIP/TAZO	IMI	IMI/REL	MEM	MEM/VAB	ATM
E. coli DH10B bla_{KPC-2}	64	≤ 0.5/4	8	512	512/4	8	0.5/4	4	0.03/8	256
E. coli DH10B $bla_{KPC-2(D179Y)}$	512	64/4	4	128	8/4	0.25	0.5/4	0.06	0.03/8	8
E. coli DH10B bla_{KPC-3}	512	2/4	16	512	512/4	4	0.5/4	4	0.03/8	512
E. coli DH10B $bla_{KPC-3(D179Y)}$	> 512	64/4	4	128	8/4	0.25	0.5/4	0.06	0.03/8	8
E. coli DH10B	≤ 0.5	≤ 0.5/4	≤ 0.5	2	2/4	0.125	0.25/4	0.03	0.03/8	≤ 0.5

Table S1. AST results of the study isolates. Mueller-Hinton (MH) agar-dilution MICs (mg/L) were interpreted according to 2020 CLSI criteria for *Enterobacterales* as follows: for aztreonam (ATM) and ceftazidime (CAZ), MIC ≤ 4 is susceptible (S), MIC = 8 is intermediate (I), and MIC ≥ 16 is resistant (R); for cefepime (FEP), MIC ≤ 2 is S, MIC = 4-8 is susceptible dose dependent (SDD), and MIC ≥ 16 is R; for piperacillin (PIP), MIC ≤ 16 is S, MIC = 32-64 is I, and MIC ≥ 128 is R; for meropenem (MEM) and imipenem (IMI), MIC ≤ 1 is S, MIC = 2 is I, and MIC ≥ 4 is R; for ceftazidime/avibactam (CZA), MIC ≤ 8/4 is S, and MIC ≥ 16/4 is R; for piperacillin/tazobactam (PIP/TAZO), MIC ≤ 16/4 is S, MIC = 32/4-64/4 is I, and MIC ≥ 128/4 is R; for meropenem/vaborbactam (MEM/VAB), MIC ≤ 4/8 is S, MIC = 8/8 is I, and MIC ≥ 16/8 is R. Imipenem/relebactam (IMI/REL) MICs were interpreted according to FDA breakpoints: MIC ≤ 2/4 is S, MIC = 4/4 is I, and MIC ≥ 8/4 is R. Relebactam was tested at fixed concentration of 4 mg/L while imipenem concentration was in doubling dilutions as a 1:1 mixture of imipenem:cilastatin. *All KPC variants cloned into pBR322 and transformed into *E. coli* DH10B cells. **AVI/TAZO/MEM/REL are kept at 4µg/ml, VAB at 8µg/ml per CLSI criteria.



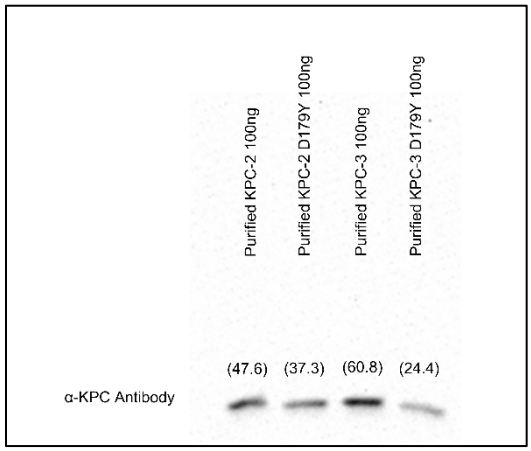
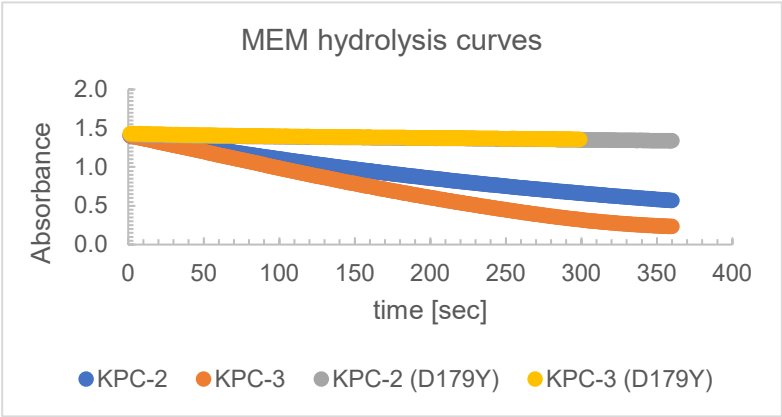
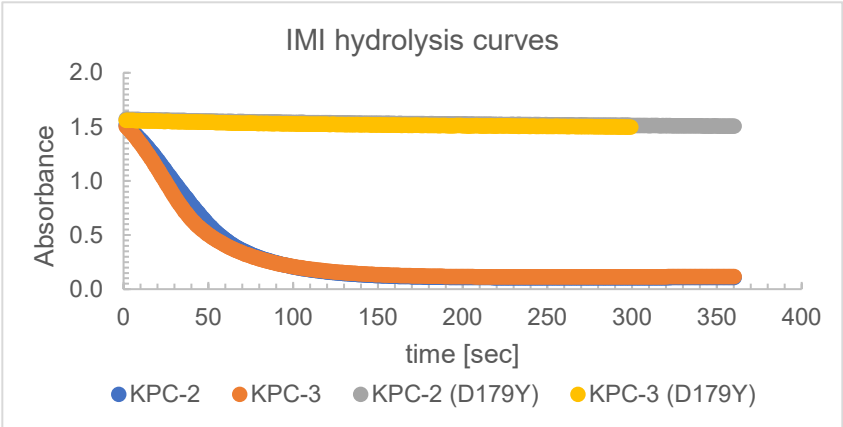


Figure S1. Immunoblots of periplasmic extracts, whole cell lysates, and 100 ngs each of purified KPC-2, KPC-2 D179Y, KPC-3, and KPC-3 D179Y. ImageJ analysis of equal amounts of enzyme (100 ngs) show varying recognition by the polyclonal anti-KPC-2 antibody (values shown in parentheses).

a.



b.



c.

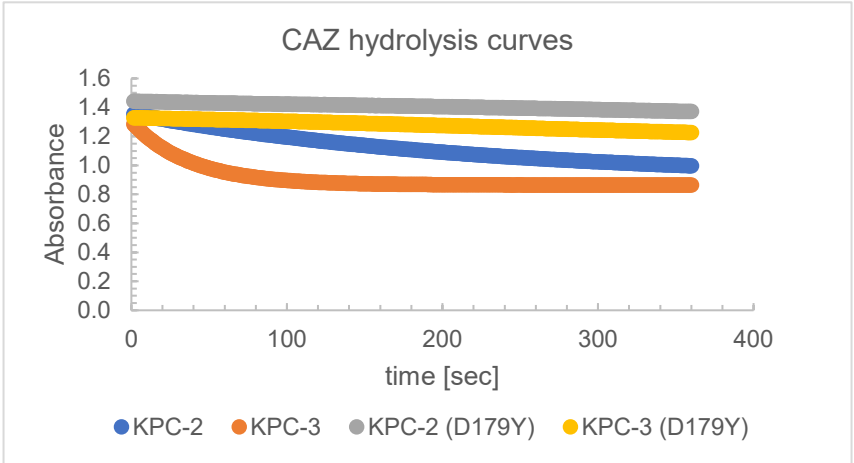
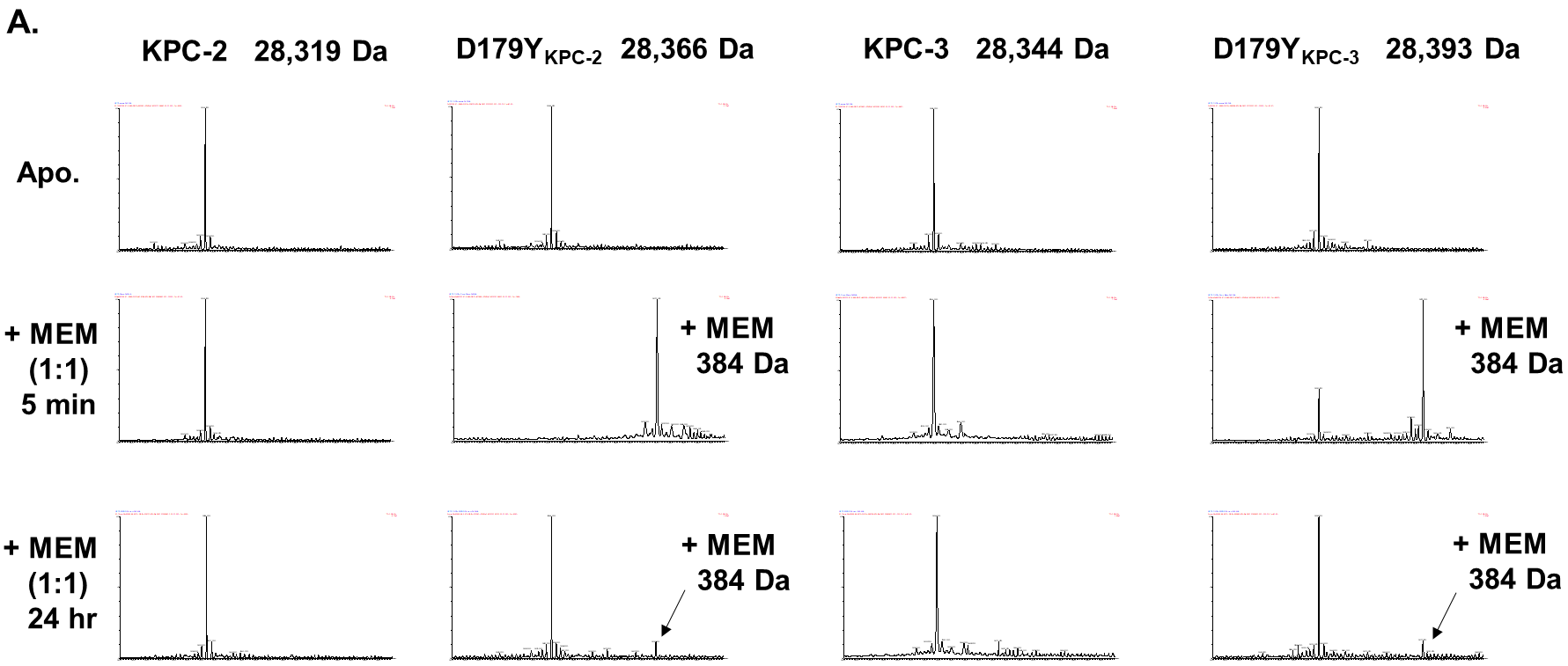
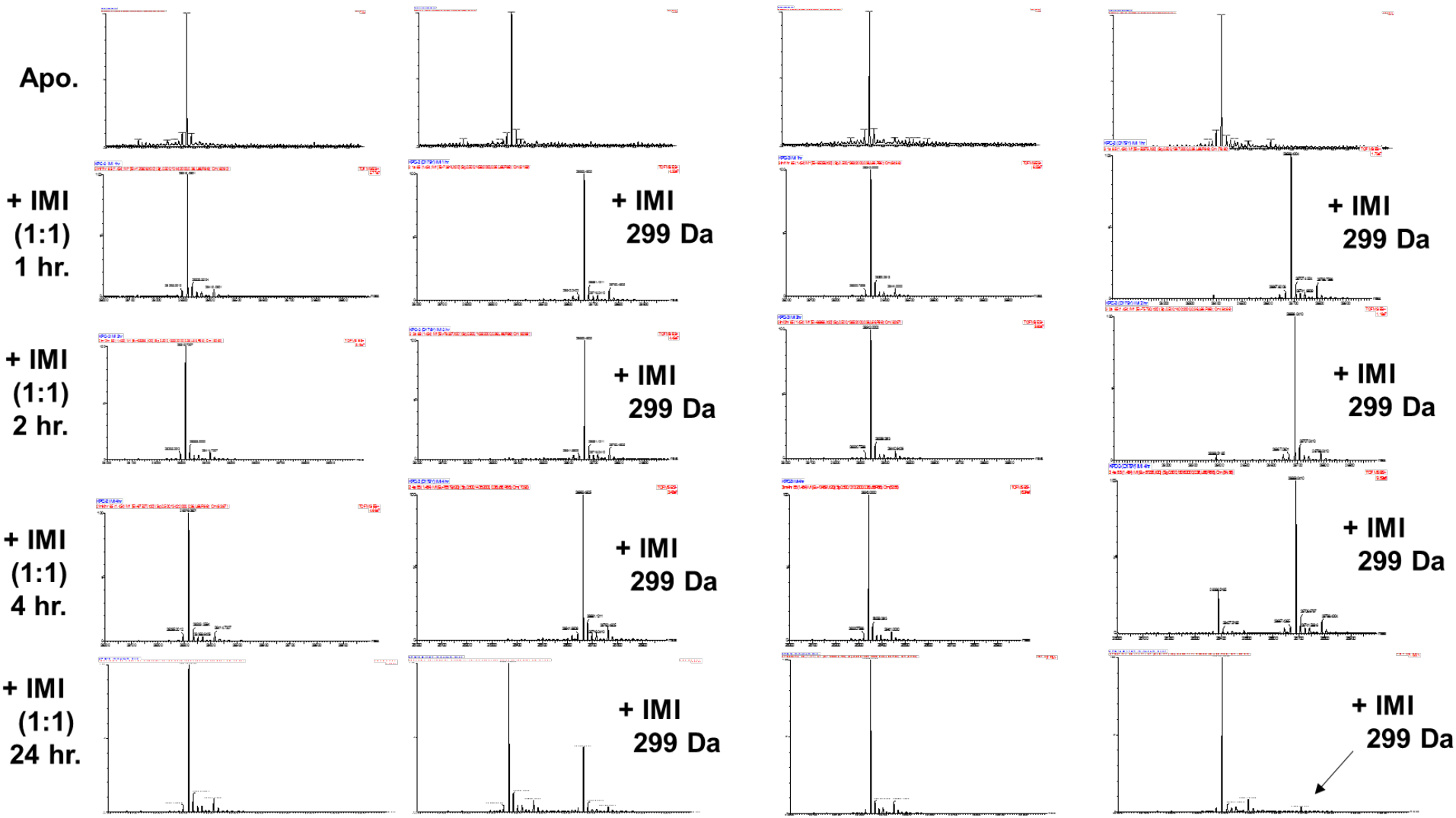


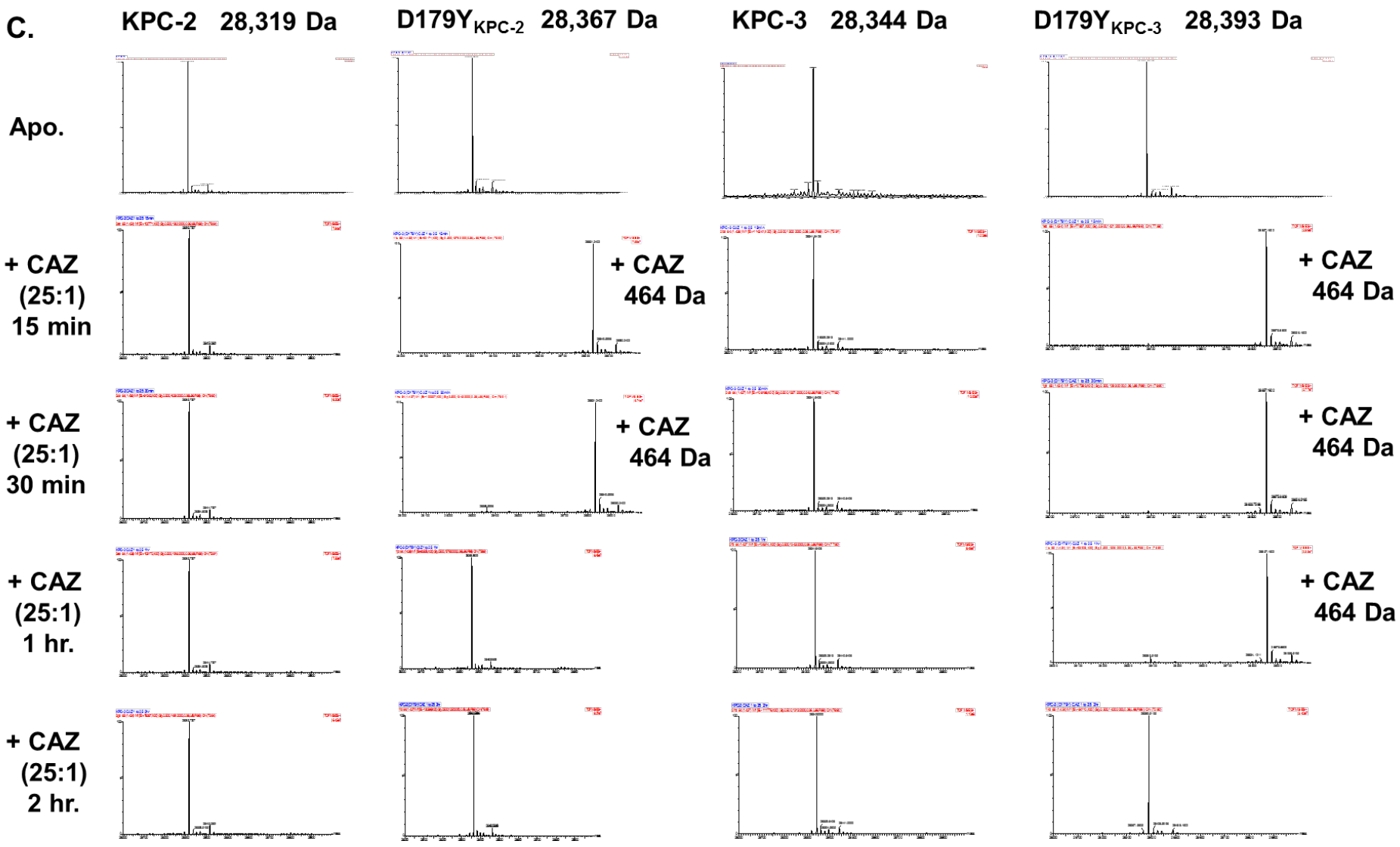
Figure S2. Steady state kinetics. (**a.**) MEM, (**b.**) IMI, and (**c.**) CAZ hydrolysis curves. WT KPC-2 and KPC-3 can hydrolyze MEM, IMI, and CAZ. D179Y variants showed no catalytic activity against IMI and MEM, and minimal hydrolysis against CAZ.


A.
KPC-2 28,319 Da
D179Y KPC-2 28,366 Da
KPC-3 28,344 Da
D179Y KPC-3 28,393 Da
Apo.
+ MEM (1:1) 5 min
+ MEM 384 Da
+ MEM (1:1) 24 hr
+ MEM 384 Da

48

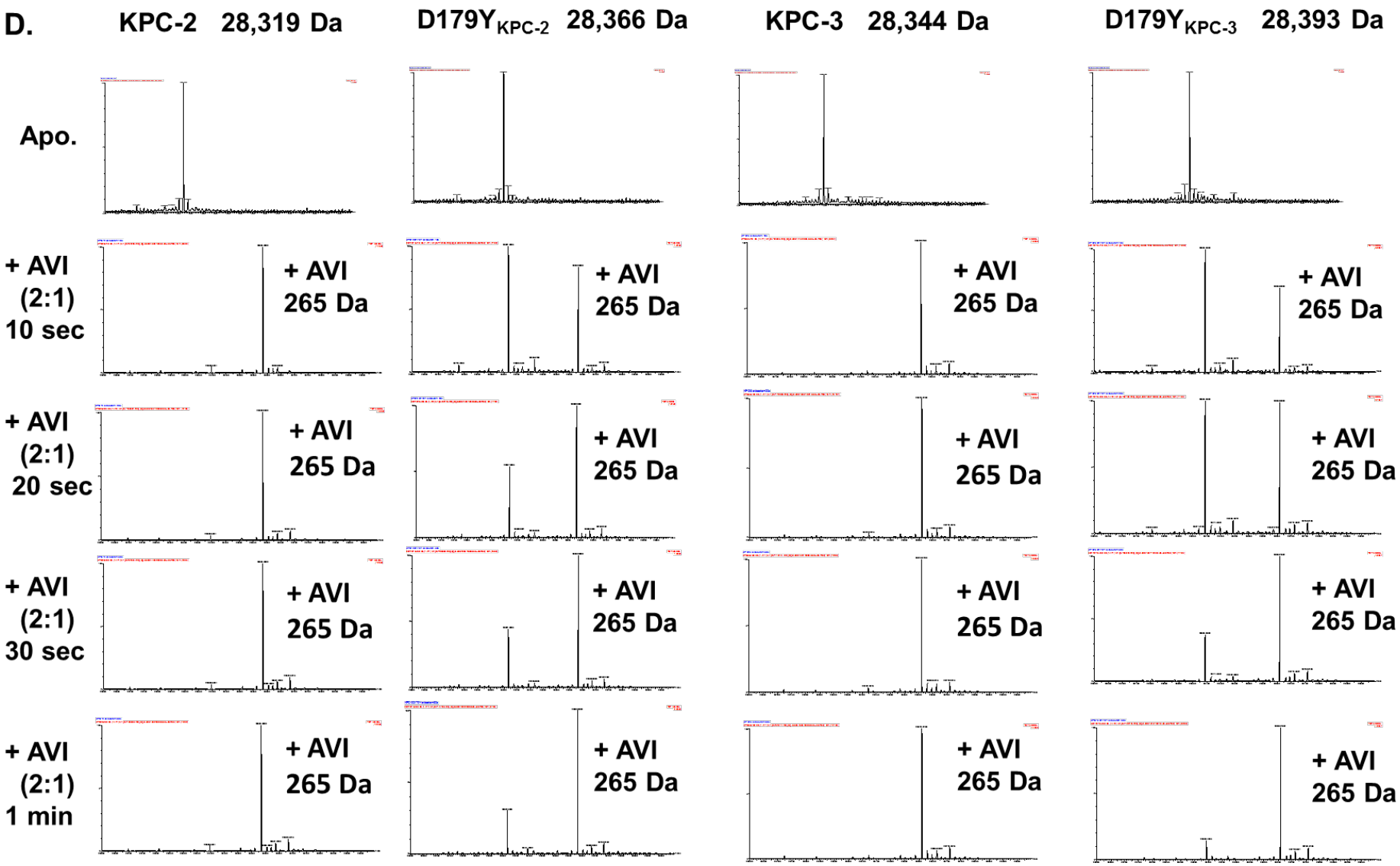
49


B.
KPC-2 28,319 Da
D179Y$_{KPC-2}$ 28,366 Da
KPC-3 28,344 Da
D179Y$_{KPC-3}$ 28,393 Da
Apo.
+ IMI (1:1) 1 hr.
+ IMI (1:1) 2 hr.
+ IMI (1:1) 4 hr.
+ IMI (1:1) 24 hr.
+ IMI 299 Da


C.
KPC-2 28,319 Da
D179Y KPC-2 28,367 Da
KPC-3 28,344 Da
D179Y KPC-3 28,393 Da
Apo.
+ CAZ (25:1) 15 min
+ CAZ 464 Da
+ CAZ (25:1) 30 min
+ CAZ 464 Da
+ CAZ (25:1) 1 hr.
+ CAZ 464 Da
+ CAZ (25:1) 2 hr.

52

53


D.
KPC-2 28,319 Da
D179Y KPC-2 28,366 Da
KPC-3 28,344 Da
D179Y KPC-3 28,393 Da
Apo.
+ AVI (2:1) 10 sec
+ AVI (2:1) 20 sec
+ AVI (2:1) 30 sec
+ AVI (2:1) 1 min
+ AVI 265 Da

55

56

Figure S3. Timed Mass Spectrometry. Both KPC-2 and KPC-3 D179Y variants form prolonged (24h) acyl-complexes with MEM (**A**) and IMI (**B**). The prolonged trapping of MEM and IMI by wild type KPCs is not evident. However, the D179Y variants of KPC-2 and KPC-3 trap MEM and IMI similarly. D179Y variants were able to form acyl-enzyme complex with CAZ (**C**) using a 25x molar excess of CAZ to enzyme, but the acyl-enzyme complex was reduced to < 2 hours ($bla_{\text{KPC-2 D179Y}}$, $k_{cat} \leq 0.014$ s^{-1}; $bla_{\text{KPC-3 D179Y}}$, $k_{cat} \leq 0.007$ s^{-1}). KPC-2 and KPC-3 formed the acyl-enzyme with AVI immediately, in the first 10 sec of incubation, and at a ratio of 2:1. For the KPC-2 D179Y variant, the complex was formed after 1 min incubation (**D**).

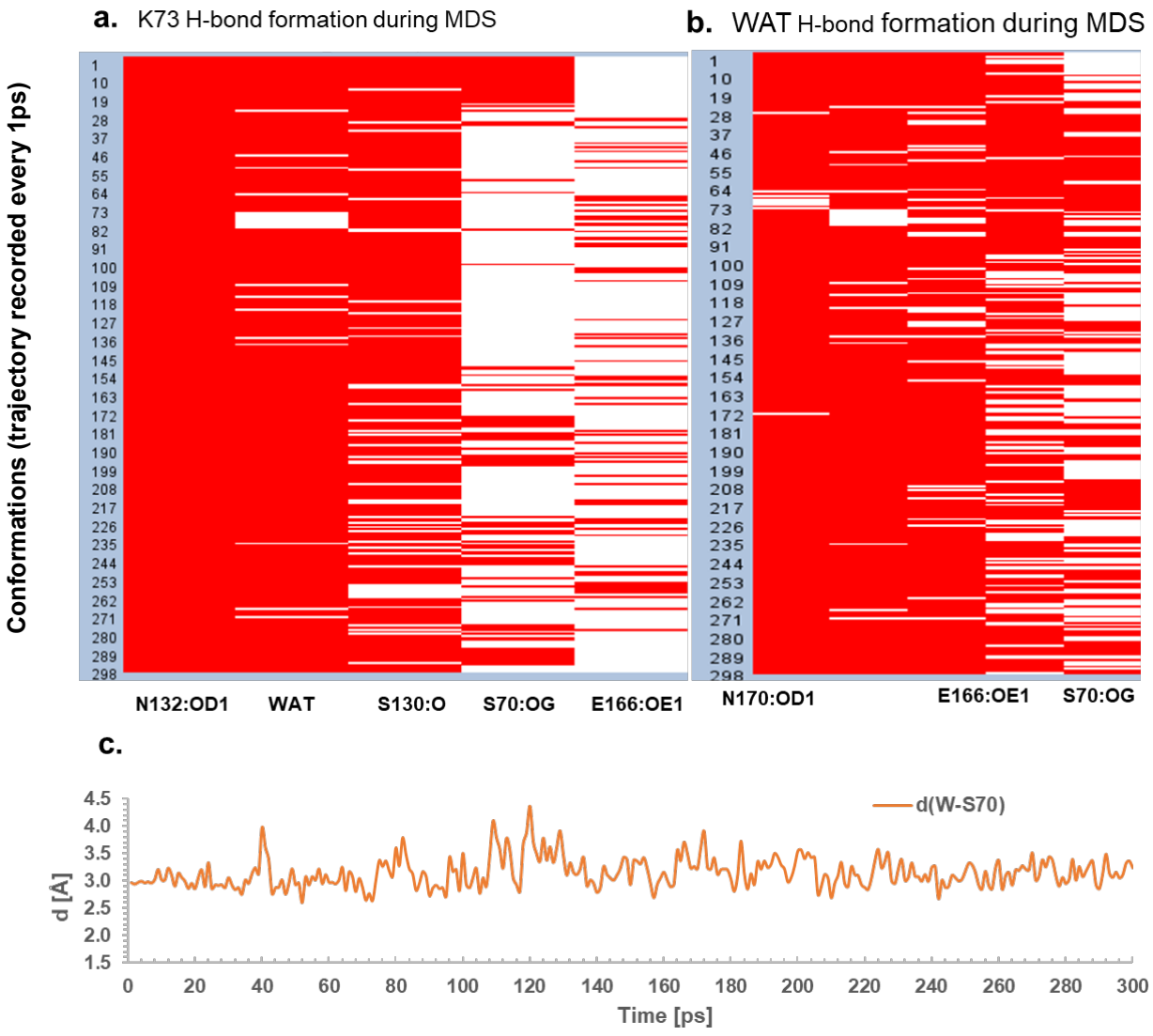


Figure S4. During the 300ps MDS, we follow the possible interactions and H-bond formation of K73 (**a**), and deacylation water (**b, c**). K73 residue is forming and maintaining a H-bonds network with N132:OD1, S70:O and a water molecule (**a**). The de-acylation water molecule (b.) Preserve the H-bonds with K73, and is positioned at H-bond distance during the simulation.

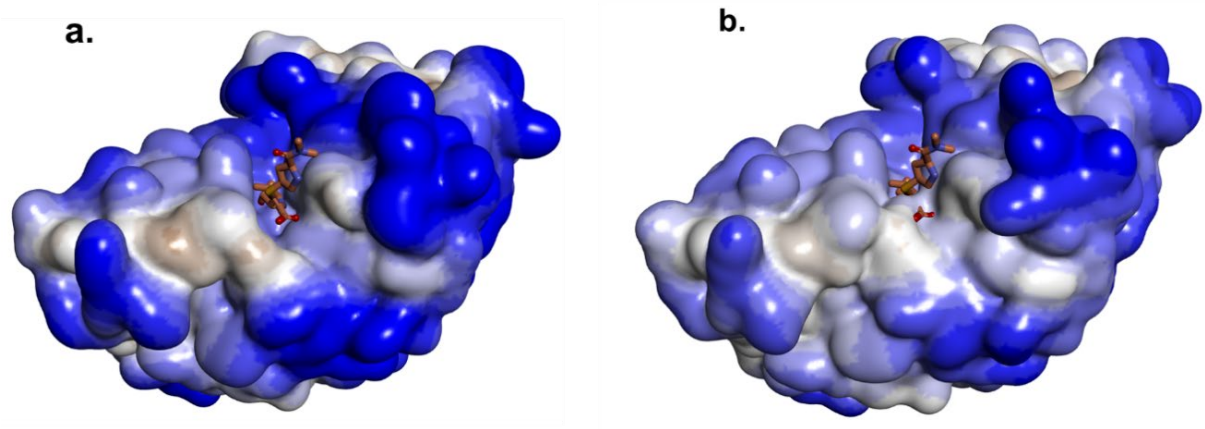


Figure S5. The Connolly electrostatic surface representation of KPC-2 (3) and D179Y variants with CAZ docked into the active site, shows the active site volumes and surface potential variability.

Supplementary Materials and Methods

MICs. Mueller-Hinton (MH) agar-dilution MIC measurements were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines as previously described (1). The MICs are reported as the concentrations at which bacterial growth was no longer observed. Avibactam, relebactam, and vaborbactam were tested at a constant 4 μg/ml in combination with their respective antibiotic partners. All MICs were performed in triplicate.

Immunoblotting. Immunoblotting was used to evaluate the expression levels of wild-type KPC-2, KPC-3, and D179Y variants as previously described (2). Five-milliliter cultures of *E. coli* DH10B cells containing pBR322 phagemids harboring the bla_{KPC} genes in Mueller-Hinton II broth containing 20 μg/mL chloramphenicol were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.8. Fifty-microliter aliquots of whole cells from these cultures were pelleted and frozen overnight. Pellets were resuspended in 20 μL of loading buffer. Whole cells were then separated via SDS-PAGE and subsequently transferred to a polyvinylidene difluoride (PVDF) membranes (Novex, Life Technologies, Carlsbad, CA) by electroblotting. After blocking for 1 h with 5% nonfat dry milk, the presence of KPC was detected by incubation in 5% nonfat dry milk with anti-KPC-2 polyclonal antibodies (1μg/ml) (3, 4) and anti-DnaK (Enzo Life Science, Farmingdale, New York) (1/10,000 dilution) overnight at 4°C. The membranes were washed four times, 15 min each, in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and subsequently incubated in 5% nonfat dry milk with a 1/10,000 dilution of horseradish peroxidase (HRP)-protein G conjugate (Bio-Rad, Hercules, California) and 1/10,000 dilution of goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, California). After four additional washes, the membranes were processed for exposure using the ECL kit (GE Healthcare, Chicago, Illinois) and FOTO/AnalystVR FX (Fotodyne, Hartland, Wisconsin). Additional immunoblotting was done with 100 ng each of KPC-2, KPC-3, KPC-2 (D179Y), and KPC-3 (D179Y). ImageJ analysis software (version 1.52, National Institutes of Health) was used to determine protein band intensities.

References

1. Clinical and Laboratory Standards Institute. 2020. M100-S30. Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition Clinical and Laboratory Standards Institute, Wayne, PA.
2. 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 beta-lactamase. Antimicrob Agents Chemother 54:2867-77.
3. Barnes MD, Winkler ML, Taracila MA, Page MG, Desarbre E, Kreiswirth BN, Shields RK, Nguyen MH, Clancy C, Spellberg B, Papp-Wallace KM, Bonomo RA. 2017. *Klebsiella pneumoniae* Carbapenemase-2 (KPC-2), Substitutions at Ambler Position Asp179, and Resistance to Ceftazidime-Avibactam: Unique Antibiotic-Resistant Phenotypes Emerge from beta-Lactamase Protein Engineering. mBio 8:e00528-17.
4. Papp-Wallace KM, Taracila MA, Smith KM, Xu Y, Bonomo RA. 2012. Understanding the molecular determinants of substrate and inhibitor specificities in the carbapenemase KPC-2: exploring the roles of Arg220 and Glu276. Antimicrob Agents Chemother 56:4428-38.