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Antibiotic Resistance and Substrate Profiles of the Class A Carbapenemase KPC-6

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The class A carbapenemase KPC-6 produces resistance to a broad range of β-lactam antibiotics. This enzyme hydrolyzes penicillins, the monobactam aztreonam, and carbapenems with similar catalytic efficiencies, ranging from 10^5 to 10^6 M^{-1} s^{-1}. The catalytic efficiencies of KPC-6 against cephems vary to a greater extent, ranging from 10^3 M^{-1} s^{-1} for the cephamycin cefoxitin and the extended-spectrum cephalosporin ceftazidime to 10^5 to 10^6 M^{-1} s^{-1} for the narrow-spectrum and some of the extended-spectrum cephalosporins.

Carbapenems are considered "last resort" antibiotics due to their broad spectrum of antimicrobial activity and resistance to hydrolysis by extended-spectrum β-lactamases (8). The emergence of carbapenem-hydrolyzing β-lactamases (carbapenemases) challenges the efficacy of carbapenem antibiotics, limits the available therapeutic options, and therefore poses a serious health threat to the community (9). Carbapenemases have been identified in all four (A, B, C, and D) classes of β-lactamases. Presently, at least eight subclasses of class A carbapenemases have been reported, including types KPC, NmcA/IMI, SME, GES, FPH, FTU, BIC, and SFC, with the KPC type being the most clinically relevant (2, 6, 9, 13, 14). In 1996, the first reported clinical isolate producing KPC-2 from *Klebsiella pneumoniae* was identified in North Carolina (16). Currently, 6 KPC variants have been characterized, with 6 more variants annotated in GenBank, and clinical isolates producing KPC are now disseminated worldwide (9; http://www.lahey.org/Studies/other.asp). Despite their utmost clinical importance, only a few KPC variants (KPC-2, KPC-3, and to some extent KPC-4 and KPC-5) have been studied kinetically (1, 10–12, 15–17). Herein we report the susceptibility profile and first steady-state kinetic characterization of the KPC-6 carbapenemase, a Val240Gly variant of KPC-2, for a panel of β-lactam antibiotics that included penicillins, cephems, carbapenems, and the monobactam aztreonam.

The gene for the mature KPC-6 β-lactamase was custom synthesized (GenScript) and fused to the leader sequence for outer membrane protein A (OmpA). Unique NdeI and HindIII sites were introduced at the 5′ and 3′ ends of the construct. This gene was then cloned into the NdeI and HindIII sites of the pET24a(+) and pHF016 vectors (5). The susceptibility profiles for the β-lactam antibiotics were determined by the microdilution method as recommended by the Clinical and Laboratory Standards Institute (3). An *Escherichia coli* JM83 strain harboring the pHF016:KPC-6 plasmid was used for the evaluation of the resistance profile of the KPC-6 β-lactamase, while the same strain harboring the pHF016 vector was used as a control. All antibiotics were purchased from Sigma (St. Louis, MO) or US Pharmacopeia (Rockville, MD), with the exception of the carbapenems, which were a generous gift from Robert Bonomo (VA Medical Center, Cleveland, OH).

The KPC-6-producing strain exhibited high-level resistance to all penicillins tested (Table 1), with MICs ranging from 512 to 16,384 μg/ml (64- to 2,048-fold above background levels). Similar to the majority of other class A enzymes, KPC-6 also produced high levels of resistance to the narrow-spectrum cephalosporins cephalothin and cefuroxime. MICs of the extended-spectrum cephalosporins cefotaxime, ceftriaxone, ceftazidime, and cefepime were also significantly elevated. The MICs of the cephamycins cefoxitin and cefmetazole were enhanced the least (a 4-fold increase in MICs above background levels), while the MIC of the monobactam aztreonam was elevated 8,192-fold above the background level, to 256 μg/ml. Although the absolute MIC values for the carbapenem antibiotics imipenem, meropenem, doripenem, and ertapenem were within the range of 1 to 4 μg/ml, they represented a significant (32- to 512-fold) increase above the background levels. The MICs for several tested penicillins against *E. coli* JM83 expressing KPC-6 were unaffected by the presence of the β-lactamase inhibitors clavulanic acid, tazobactam, or sulbactam, an indication that the enzyme is resistant to inhibition, a trait also observed for the related variant KPC-2 (10). We observed that expression of KPC-6 resulted in an 8-fold increase in the MIC for sulbactam, an indication that the enzyme is capable of hydrolyzing this inhibitor. Hydrolysis of β-lactamase inhibitors has previously been reported for the KPC-2 (10) and KPC-3 (1) carbapenemases.

For enzyme purification, *E. coli* BL21(DE3) harboring the pET24a(+):KPC-6 plasmid was grown at 37°C in LB supplemented with 60 μg/ml kanamycin to an optical density at 600 nm of 1.0. Protein expression was then induced using 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the culture was incubated at 22°C for an additional 18 h. The periplasmic fraction was isolated as previously described (7) and dialyzed against 50 mM Tris (pH 8.0). The protein was purified using a DEAE anion-exchange column equilibrated with 50 mM Tris (pH 8.0). KPC-6 eluted in the flowthrough fraction and was determined to be ≥95% pure by SDS-PAGE. The enzyme concentration was evaluated spectrophotometrically by using the predicted extinction coefficient for the mature protein ($\Delta\varepsilon_{280}$, 38,690 M^{-1} cm^{-1} [http://www.justbio

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TABLE 1 MIC profile of *E. coli* JM83 expressing the KPC-6 β-lactamase

Antimicrobial agent	MIC (μg/ml)	
	Control[a]	KPC-6
Benzylpenicillin	16	2,048
Ampicillin	2	4,096
Ampicillin-clavulanic acid[b]	2	2,048
Ampicillin-tazobactam[b]	2	2,048
Ampicillin-sulbactam[b]	1	2,048
Ampicillin-sulbactam[c]	2	256
Sulbactam	32	256
Amoxicillin[d]	4	>2,048
Amoxicillin-clavulanic acid[c]	4	32
Clavulanic acid	32	32
Oxacillin	256	16,384
Ticarcillin	4	8,192
Ticarcillin-clavulanic acid[b]	4	8,192
Piperacillin	2	512
Piperacillin-tazobactam[b]	2	512
Cephalothin	4	512
Cefuroxime	4	4,096
Ceftazidime	0.125	32
Cefotaxime	0.031	32
Ceftriaxone	0.031	16
Cefepime	0.016	8
Cefoxitin	2	8
Cefmetazole	1	4
Moxalactam	0.125	8
Aztreonam	0.031	256
Imipenem	0.125	4
Meropenem	0.031	2
Ertapenem	0.004	2
Doripenem	0.031	1

[a] The control strain was *E. coli* JM83 with the pHF016 vector.
[b] Clavulanic acid was used at a constant concentration of 2 μg/ml. Sulbactam and tazobactam were used at a constant concentration of 4 μg/ml.
[c] A 2:1 ratio was maintained for the β-lactam and β-lactamase inhibitor (clavulanic acid or sulbactam).
[d] In Mueller-Hinton II broth, the maximum solubility of amoxicillin is 2,048 μg/ml.

TABLE 2 Kinetic parameters for hydrolysis of β-lactam substrates by KPC-6

β-Lactam	k_{cat} (s^{-1})[a]	K_m or K_i (μM)[a]	k_{cat}/K_m (M^{-1} s^{-1})
Ampicillin	280 ± 10	130 ± 10	(2.2 ± 0.2) × 10^6
Oxacillin	44 ± 1	33 ± 3	(1.3 ± 0.1) × 10^6
Ticarcillin	13 ± 1	49 ± 5	(2.7 ± 0.3) × 10^5
Piperacillin	34 ± 2	50 ± 10	(6 ± 1) × 10^5
Cephalothin[b]	>100	>100	(2.4 ± 0.1) × 10^6
Cefuroxime[b]	>50	>75	(6.9 ± 0.1) × 10^5
Ceftazidime[b]	>0.6	>80	(9.1 ± 0.1) × 10^3
Cefotaxime[b]	>39	>150	(3.3 ± 0.1) × 10^5
Ceftriaxone[b]	>54	>100	(7.5 ± 0.1) × 10^5
Cefepime[b]	>4.2	>100	(4.8 ± 0.1) × 10^4
Cefoxitin[b]	>0.2	>100	(1.9 ± 0.1) × 10^3
Aztreonam[b]	>150	>500	(3.4 ± 0.1) × 10^5
Imipenem	18 ± 1	42 ± 4	(4.4 ± 0.4) × 10^5
Meropenem	3.3 ± 0.1	7 ± 1	(4.5 ± 0.7) × 10^5
Ertapenem	2.8 ± 0.1	10 ± 1	(2.9 ± 0.3) × 10^5
Doripenem	0.38 ± 0.01	2.9 ± 0.2	(1.3 ± 0.1) × 10^5
Nitrocefin	210 ± 10	37 ± 1	(5.6 ± 0.2) × 10^6
Clavulanic acid		75 ± 8	
Sulbactam		600 ± 100	
Tazobactam		290 ± 30	

[a] Values are means ± standard deviations.
[b] Saturation could not be reached.

.com/index.php?page=protcalc]). The enzyme was stored at 4°C in 50 mM Tris, pH 8.0.

The hydrolysis of β-lactam substrates was evaluated spectrophotometrically at room temperature (5). The final reaction buffer contained 50 mM NaP_i (pH 7.5), 50 mM NaCl, and 0.5 mg/ml bovine serum albumin (for protein stabilization). The parameters k_{cat} and K_m were evaluated by nonlinear fitting of the initial velocities, at various concentrations of the substrates, with the Michaelis-Menten equation. In situations in which saturation could not be reached, the value for k_{cat}/K_m was determined as previously described (13). The extinction coefficients and wavelengths for substrates used in this study have been previously reported (2, 13).

The steady-state kinetic parameters for KPC-6 are presented in Table 2. For 8 of the 17 substrates used in the kinetic studies, saturation could not be reached. As a result, the values for the catalytic efficiency (k_{cat}/K_m) of the enzyme against these substrates were evaluated, while only the lower limits for the k_{cat} and K_m values could be determined.

The KPC-6 β-lactamase hydrolyzed penicillins, narrow-spectrum cephalosporins (cephalothin and cefuroxime), and the monobactam aztreonam with catalytic efficiencies of 10^5 to 10^6 M^{-1} s^{-1}. The enzyme also exhibited similar catalytic efficiencies against two extended-spectrum cephalosporins (cefotaxime and ceftriaxone), while the catalytic efficiency against a third, ceftazidime, was 36- and 82-fold lower, respectively. Cefoxitin was the poorest substrate tested; the catalytic efficiency of KPC-6 against this cephamycin antibiotic was 2.3 × 10^3 M^{-1} s^{-1}. While KPC-6 had a very similar catalytic efficiency (k_{cat}/K_m values from 1.3 × 10^5 to 4.5 × 10^5 M^{-1} s^{-1}) against all four tested carbapenem antibiotics, the k_{cat} and K_m values for these substrates showed a broader range of distribution. The k_{cat} value was highest for imipenem (18 ± 1 s^{-1} [mean ± standard deviation]), while this number was approximately 6-fold lower for meropenem and ertapenem and 47-fold lower for doripenem. Conversely, doripenem had a 3- to 4-fold-higher apparent affinity for the enzyme than meropenem and ertapenem and 14-fold higher than imipenem. Kinetic parameters have been reported for KPC-2 and KPC-3 with two carbapenems, imipenem and meropenem (1, 11, 16). For both antibiotics, the k_{cat} and K_m values for KPC-6 were in close agreement (within 2-fold) to those for KPC-2 and KPC-3.

The dissociation constants for the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (Table 2) were determined using the Dixon method (4). Nitrocefin, as a reporter substrate, was used at concentrations of 50 and 100 μM. Consistent with the MIC data, the β-lactamase inhibitors had low affinity for KPC-6, with dissociation constants for clavulanic acid, sulbactam, and tazobactam of 75 ± 8 μM, 600 ± 100 μM, and 290 ± 30 μM, respectively. In comparison, higher affinities for the β-lactamase inhibitors have been observed for KPC-2, with dissociation constants for clavulanic acid, sulbactam, and tazobactam of 11 ± 1 μM, 167 ± 16 μM, and 74 ± 7 μM, respectively (11). The KPC-6 β-lactamase has a Val240Gly substitution in comparison to the KPC-2 enzyme. At least 3 of the KPC variants (KPC-4, -6, and -8) have this substitution, while KPC-9 has a conservative alanine substitution. Compared to

KPC-2, our kinetic studies of the KPC-6 carbapenemase indicate that the conservative Val240Gly substitution does not result in an appreciable change in the antibiotic substrate profile or hydrolytic activity of the enzyme.

REFERENCES

1. **Alba J, Ishii Y, Thomson K, Moland ES, Yamaguchi K.** 2005. Kinetics study of KPC-3, a plasmid-encoded class A carbapenem-hydrolyzing beta-lactamase. Antimicrob. Agents Chemother. **49**:4760–4762.
2. **Antunes NT, Frase H, Toth M, Vakulenko SB.** 2012. The class A beta-lactamase FTU-1 is native to Francisella tularensis. Antimicrob. Agents Chemother. **56**:666–671.
3. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 8th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Dixon M.** 1953. The determination of enzyme inhibitor constants. Biochem. J. **55**:170–171.
5. **Frase H, Shi Q, Testero SA, Mobashery S, Vakulenko SB.** 2009. Mechanistic basis for the emergence of catalytic competence against carbapenem antibiotics by the GES family of beta-lactamases. J. Biol. Chem. **284**:29509–29513.
6. **Girlich D, Poirel L, Nordmann P.** 2010. Novel ambler class A carbapenem-hydrolyzing beta-lactamase from a Pseudomonas fluorescens isolate from the Seine River, Paris, France. Antimicrob. Agents Chemother. **54**:328–332.
7. **Neu HC, Heppel LA.** 1965. The release of enzymes from Escherichia coli by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3692.
8. **Nordmann P, Cuzon G, Naas T.** 2009. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect. Dis. **9**:228–236.
9. **Nordmann P, Dortet L, Poirel L.** 2012. Carbapenem resistance in Enterobacteriaceae: here is the storm! Trends Mol. Med. **18**:263–272.
10. **Papp-Wallace KM, et al.** 2010. Inhibitor resistance in the KPC-2 beta-lactamase, a preeminent property of this class A beta-lactamase. Antimicrob. Agents Chemother. **54**:890–897.
11. **Papp-Wallace KM, et al.** 2010. Substrate selectivity and a novel role in inhibitor discrimination by residue 237 in the KPC-2 beta-lactamase. Antimicrob. Agents Chemother. **54**:2867–2877.
12. **Papp-Wallace KM, et al.** 2010. Elucidating the role of Trp105 in the KPC-2 beta-lactamase. Protein Sci. **19**:1714–1727.
13. **Toth M, Vakulenko V, Antunes NT, Frase H, Vakulenko SB.** 2012. Class A carbapenemase FPH-1 from Francisella philomiragia. Antimicrob. Agents Chemother. **56**:2852–2857.
14. **Walther-Rasmussen J, Hoiby N.** 2007. Class A carbapenemases. J. Antimicrob. Chemother. **60**:470–482.
15. **Wolter DJ, et al.** 2009. Phenotypic and enzymatic comparative analysis of the novel KPC variant KPC-5 and its evolutionary variants, KPC-2 and KPC-4. Antimicrob. Agents Chemother. **53**:557–562.
16. **Yigit H, et al.** 2001. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. Antimicrob. Agents Chemother. **45**:1151–1161.
17. **Yigit H, et al.** 2003. Carbapenem-resistant strain of Klebsiella oxytoca harboring carbapenem-hydrolyzing beta-lactamase KPC-2. Antimicrob. Agents Chemother. **47**:3881–3889.