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Unravelling ceftazidime/avibactam resistance of KPC-28, a KPC-2 variant lacking carbapenemase activity

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Background: KPC-like carbapenemases have spread worldwide with more than 30 variants identified that differ by single or double amino-acid substitutions.

Objectives: To describe the steady-state kinetic parameters of KPC-28, which differs from KPC-2 by a H274Y substitution and the deletion of two amino acids (Δ242-GT-243).

Methods: The bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-14} and bla_{KPC-28} genes were cloned into a pTOPO vector for susceptibility testing or into pET41b for overexpression, purification and subsequent kinetic parameter (K_m, k_{cat}) determination. Molecular docking experiments were performed to explore the role of the amino-acid changes in the carbapenemase activity.

Results: Susceptibility testing revealed that *Escherichia coli* producing KPC-28 displayed MICs that were lower for carbapenems and higher for ceftazidime and ceftazidime/avibactam as compared with KPC-2. The catalytic efficiencies of KPC-28 and KPC-14 for imipenem were 700-fold and 200-fold lower, respectively, than those of KPC-2, suggesting that Δ242-GT-243 in KPC-28 and KPC-14 is responsible for reduced carbapenem hydrolysis. Similarly, the H274Y substitution resulted in KPC-28 in a 50-fold increase in ceftazidime hydrolysis that was strongly reversed by clavulanate.

Conclusions: We have shown that KPC-28 lacks carbapenemase activity, has increased ceftazidime hydrolytic activity and is strongly inhibited by clavulanate. KPC-28-producing *E. coli* isolates display an avibactam-resistant ESBL profile, which may be wrongly identified by molecular and immunochromatographic assays as the presence of a carbapenemase. Accordingly, confirmation of carbapenem hydrolysis will be mandatory with assays based solely on bla_{KPC} gene or gene product detection.

Introduction

In Gram-negative bacteria, acquired resistance to β-lactams may be mediated by various non-enzymatic mechanisms, such as decreased permeability of the outer membrane, or active efflux, but enzymatic inactivation by β-lactamases is the main mechanism. These enzymes, which are able to hydrolyse β-lactams, are divided into four molecular classes based on structural homologies (Ambler classification). Ambler's classes A, C and D are β-lactamases possessing a serine in their active site, whereas the class B enzymes are MBLs that use divalent Zn^{2+} ion(s) for their hydrolytic activity. It has been suggested that the rising use of carbapenems (imipenem and meropenem) for the treatment of infections caused by MDR Gram-negative pathogens during the last decade has contributed to the emergence of carbapenem-resistant bacteria that may be the result of outer membrane permeability alteration associated with overproduction of a cephalosporinase and/or production of an ESBL or of carbapenem-hydrolysing β-lactamases called carbapenemases.[1] These enzymes belong to Ambler's classes A, B and D.[2] Within class A, several enzymes have been described in Enterobacteriaceae (NMC-A, IMI-1, SME-1, GES-2 and FRI-1), but *Klebsiella pneumoniae* carbapenemase (KPC) is the most prevalent across the globe. KPC is regarded as the most

worrying class A carbapenemase because of its location on self-conjugative plasmids and its frequent association with a highly successful *K. pneumoniae* clone, the clonal complex (CC) 258.[3] Until 2005, the geographical distribution of KPC-producing Enterobacteriaceae remained limited to the eastern USA.[4] Today, KPC producers have disseminated worldwide and are endemic in the USA, South America, Greece, Italy, Poland, China and Israel. Since its initial description in a *K. pneumoniae* clinical isolate from North Carolina,[5] 39 KPC variants have been reported (www.bldb.eu).[6] Among these variants, a mono-substitution (KPC 3–6 and 9–11) resulted in increased ceftazidime hydrolysis, with carbapenem hydrolysis being unaffected.[7–12] Recently, *in vitro* selection of ceftazidime/avibactam resistance in Enterobacteriaceae with KPC-3 carbapenemase revealed modifications in the Ω loop that are also responsible for carbapenem susceptibility. The most prevalent modification occurs at position 179 of the KPC enzyme,[13,14] but a few other substitutions at different positions were also involved, such as V240G and T243A.[13] Ceftazidime/avibactam resistance also occurred *in vivo,* usually following ceftazidime/avibactam treatment for prolonged periods (>2 weeks).[15–18] The D179 modification is always reported in cases of *in vivo* selection of ceftazidime/avibactam-resistant KPC-producing isolates.[15,17] Recently, KPC-28, a new variant of KPC-2 with a two-amino-acid deletion (Δ242-GT-243, according to the ABL numbering scheme)[19] and a substitution, H274Y, was reported.[20] In that study, a KPC-28-producing clone was found to be more resistant to ceftazidime as compared with KPC-2 or KPC-3 producers, but was fully susceptible to carbapenems, suggesting a complete loss of carbapenemase activity. Here, we report the susceptibility profile and the steady-state kinetic characterization of the KPC-28 β-lactamase (a variant of KPC-3 with a two-amino-acid deletion, Δ242-GT-243) compared with KPC-2, KPC-3 and KPC-14.

Materials and methods

Bacterial strains

The clinical strain *Escherichia coli* WI2 expressing the KPC-28 β-lactamase was used for cloning of the bla_{KPC-28} gene.[20] *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning and mutagenesis experiments and *E. coli* BL21 Rosetta-gami™ DE3 (Novagen, Fontenay-sous-Bois, France) was used for overexpression experiments.

Susceptibility testing

Antimicrobial susceptibilities were determined by the disc diffusion technique on Mueller–Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in May 2018 (http://www.eucast.org). MICs were determined using the Etest technique (bioMérieux, La Balme les Grottes, France).

PCR, cloning experiments, site-directed mutagenesis and DNA sequencing

Whole-cell DNA of *E. coli* isolates producing KPC-2, KPC-3 and KPC-28 were extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were then used as a template to amplify the bla_{KPC-2}-like genes. The gene encoding KPC-14 was obtained by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies), using the primer KPC-Y274H (5′-CTAACAAGGATGACAAGCACAGCGAGGCCGTCATC-3′) and the plasmid pTOPO-bla_{KPC-28} as a template. The PCR, using the primers Kpc-rbs (5′-CTCCACCTTCAAACAAGGAAT-3′) and Kpc-rev (5′-ATCTGCAGAATTCGCCCTTCGCCATCGTCAGTGCTCTAC-3′), was able to amplify bla_{KPC-3} and bla_{KPC-28} genes. The amplicons obtained were then cloned into the pCR-Blunt II-Topo plasmid (Invitrogen) downstream from the pLac promoter, in the same orientation as for the phenotypic studies. The recombinant pTOPO-KPC plasmids were electroporated into the *E. coli* TOP10 strain. For protein production, the sequences without the peptide signal of the bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-14} and bla_{KPC-28} genes were obtained by PCR amplification using primers NdeI-KPC-$2_{30–293}$ (5′-CATATGGCGGAACCATTCGCTAAC-3′) and KPC-$2_{\Delta STOP}$ (5′-CTCGAGCTGCCCGTTGACGCCAAT-3′) and were then inserted into plasmid pET41b (Novagen). The recombinant plasmids were transformed into *E. coli* BL21 Rosetta-gami™ DE3 (Novagen). All the recombinant plasmids were sequenced using a T7 promoter and M13 reverse primers or T7 terminator (depending on the plasmid) with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide sequences were analysed using software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Detection of KPC producers and carbapenemase activity

The detection of the KPC variants was performed with *E. coli* TOP10 harbouring the recombinant vector pTOPO-KPC. The carbapenemase activity was detected using six techniques: the Carba NP test as previously described,[21] the CIM test,[22] the modified Hodge test[23] and the β CARBA™ test (Bio-Rad),[24] the RAPIDEC® CARBA NP (bioMérieux)[25] and the MBT STAR®-Carba IVD Kit (Bruker Daltonics, Bremen, Germany)[26] in accordance with the manufacturers' recommendations. Molecular tests were performed with the Xpert® Carba-R kit (Cepheid, Sunnyvale, USA)[27] and by standard in-house PCR using the primers KPC-A (5′-CTGTCTTGTCTCTCAT GGCC-3′) and KPC-B (5′-CCTCGCTGTGCTTGT-CATCC-3′). Immunoenzymatic tests were carried out using two techniques: NG-Test CARBA 5 (NG Biotech, Guipry, France)[28] and the Resist-4 O.K.N.V. K-Set (CORIS BioConcept, Gembloux, Belgium).[29]

Protein purification

An overnight culture of *E. coli* BL21 Rosetta-gami™ DE3 harbouring recombinant pET41b-KPC plasmids was used to inoculate 2 L of LB medium broth containing 50 mg/L kanamycin and 30 mg/L chloramphenicol. Bacteria were cultured at 37°C until an OD of 0.6 at 600 nm was reached. The expression of the β-lactamase genes was carried out overnight at 22°C with 1 mM IPTG as inducer. Cultures were centrifuged at 6000 **g** for 15 min and then the pellets were resuspended with the binding buffer (10 mM imidazole, 25 mM sodium phosphate pH 7.4 and 300 mM NaCl). Bacterial cells were disrupted by sonication and the bacterial pellet was removed by two consecutive centrifugation steps at 10000 **g** for 1 h at 4°C; the supernatant was then centrifuged at 96000 **g** for 1 h at 4°C. The soluble fractions were filtered and then passed through a HisTrap™ HP column (GE Healthcare) and proteins were eluted with the elution buffer (500 mM imidazole, 25 mM sodium phosphate pH 7.4 and 300 mM NaCl). Finally, a gel filtration step was performed with 100 mM sodium phosphate buffer pH 7 and 150 mM NaCl with a Superdex 75 column (GE Healthcare). The protein purity was estimated by SDS–PAGE and the pooled fractions were dialysed against 10 mM Tris-HCl pH 7.6 and concentrated using Vivaspin columns. The concentrations were determined by measuring the OD at 280 nm and with the extinction coefficients obtained from the ProtParam tool (Swiss Institute of Bioinformatics online resource portal).[30]

Steady-state kinetic parameters

Kinetic parameters were determined using purified KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases in 100 mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined by analysing β-lactam hydrolysis under initial-rate conditions with an ULTROSPEC 2000 UV spectrophotometer and the SWIFT II software (GE Healthcare, Velizy-Villacoublay, France)

using the Eadie–Hoffstee linearization of the Michaelis–Menten equation. The different β-lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). For some cephalosporins, saturation could not be reached. Thus, the values for the catalytic efficiency (k_{cat}/K_m) of the enzymes KPC-2 and KPC-3 against these substrates were evaluated with the lower limits for the k_{cat} and K_m determined. The IC_{50} of the β-lactamase inhibitors clavulanate and avibactam was determined in 100 mM sodium phosphate buffer (pH 7) and with 100 μM piperacillin and 100 μM ceftazidime (respectively) as a reporter substrate.

Molecular modelling

Molecular models of KPC-3, KPC-14 and KPC-28 were generated by comparative modelling using MODELLER version 9.16[31] with the KPC-2 structure[32] (PDB code 5UJ3) as template. Three-dimensional structures of the ligands were generated using CORINA version 3.60 (Molecular Networks GmbH, Erlangen, Germany; http://www.molecular-networks.com). Covalent docking calculations were carried out using GOLD version 5.2[33] and the GoldScore scoring function. The binding site was defined as a sphere with a 20 Å radius around the OG atom of the Ser70 residue. The covalent connection was made between the OG atom of the Ser70 residue and the open form of the β-lactam ring in order to generate the acyl-enzyme complex. Molecular modelling images were generated using UCSF CHIMERA.[34]

Results

Clinical isolate

E. coli WI2 was recovered from a faecal sample from a Portuguese patient upon admission to a French hospital subsequent to a medical repatriation. This isolate was resistant to broad-spectrum cephalosporins, aminoglycosides and colistin and of reduced susceptibility to carbapenems.[20] As previously described, this isolate possessed the bla_{OXA-48} and bla_{KPC-28} genes and the acquired colistin resistance determinant *mcr-1*.[20] KPC-28 is a variant of KPC-3 with a deletion of two amino acids (Δ242-GT-243) and KPC-14 is a variant of KPC-2 with the same deletion of these two amino acids (Figure 1).

Antimicrobial susceptibilities of transformants with KPC-2, KPC-3, KPC-14 and KPC-28

To evaluate and compare the antimicrobial susceptibility profiles conferred by KPC-28, the bla_{KPC-2}, bla_{KPC-3}, bla_{KPC-14} and bla_{KPC-28} genes were cloned into pTOPO vector and expressed in *E. coli* TOP10 (Table 1). Although the different KPC variants exhibited similar MICs of amoxicillin, temocillin, cefixime and cefepime, KPC-28- and KPC-14-producing *E. coli* TOP10 possessed lower MICs of cefotaxime and carbapenems (being fully susceptible), but exhibited higher MICs of ceftazidime as compared with KPC-2-producing *E. coli* TOP10 (Table 1). The highest MICs of cefotaxime and aztreonam were found for the KPC-3-producing *E. coli* TOP10 clones. The addition of clavulanate restored susceptibility to amoxicillin for KPC-28- and KPC-14-producing *E. coli* clones, while inhibition by avibactam seemed to be less efficient, as both KPC-28- and KPC-14-producing *E. coli* isolates were resistant to the combination ceftazidime/avibactam. Thus, MIC values suggested that Δ242-GT-243 (in KPC-14 and KPC-28) resulted in the loss of carbapenemase activity, but in increased hydrolytic activity

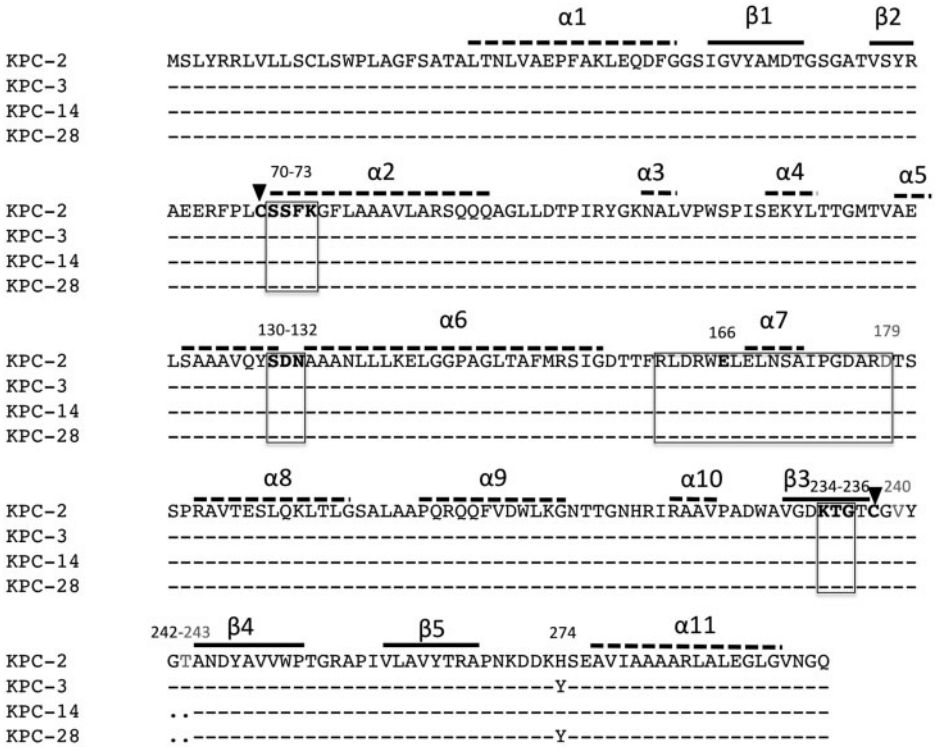

Figure 1. Sequence alignment of KPC variants. Alpha helices are indicated by dashed lines and β strands by continuous black lines. Cysteines involved in disulphide bonds are indicated by inverted black triangles. Key residues known to be implicated in ceftazidime/avibactam resistance are indicated in grey. Conserved residues among class A β-lactamases are indicated by boxes.

Table 1. MIC profile of *E. coli* TOP10 expressing KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases determined by Etests

	MIC (mg/L)				
Antimicrobial agent	*E. coli* TOP10 pTOPO KPC-2	*E. coli* TOP10 pTOPO KPC-3	*E. coli* TOP10 pTOPO KPC-14	*E. coli* TOP10 pTOPO KPC-28	*E. coli* TOP10
Amoxicillin	>256	>256	>256	>256	6
Amoxicillin/clavulanate[a]	24	48	6	6	6
Temocillin	12	16	16	16	6
Ceftriaxone	12	48	6	4	0.032
Cefotaxime	8	>32	6	4	0.064
Ceftazidime	4	>256	>256	>256	0.125
Ceftazidime/avibactam[b]	0.38	0.75	24	12	0.125
Cefixime	3	12	12	8	0.38
Cefepime	2	6	4	4	0.0064
Aztreonam	24	>256	32	24	0.047
Imipenem	8	8	0.25	0.25	0.25
Meropenem	3	3	0.032	0.032	0.032
Ertapenem	1	1	0.006	0.008	0.004

[a]Clavulanate at a fixed concentration of 4 mg/L.
[b]Avibactam at a fixed concentration of 4 mg/L.

Table 2. Steady-state kinetic parameters[a] for hydrolysis of β-lactam substrates by KPC-2 and KPC-2-like β-lactamases

	K_m (μM)				k_{cat} (s^{-1})				k_{cat}/K_m ($mM^{-1}\ s^{-1}$)			
Substrate	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28
Piperacillin	137	255	6	11	59	38	0.8	0.95	434	150	127	86
Cefoxitin	>1000	>1000	NH	NH	>12	>4	ND	ND	2	1.6	ND	ND
Cefotaxime	>1000	335	77	75	>163	403	4	5	95	1202	52	67
Ceftazidime	>1000	656	41	125	>16	9	1	4	0.6	14	24	32
Cefepime	>1000	491	34	30	>48	14	1.7	2.5	13	29	50	83
Imipenem	198	235	5	18	47	31	0.006	0.006	237	131	1.2	0.34
Meropenem	45	18	7	NH	3	2	0.003	ND	67	103	0.4	ND
Ertapenem	30	25	5	9	4	3	0.002	0.004	133	114	0.47	0.42

ND, not determined; NH, no detectable hydrolysis.
[a]Data are the mean of three independent experiments. Standard deviations were within 10% of the mean value.

towards ceftazidime. This increased ceftazidime hydrolytic activity was potentiated by the H274Y substitution in KPC-28, as already shown for KPC-3, but, in the latter case, carbapenem resistance was not affected.[12]

Biochemical properties of KPC-28

To characterize and compare kinetic parameters of KPC-28, the four variants were purified and kinetic studies were performed (Table 2). KPC-2 was used as a control for comparison. Overall, the steady-state kinetics revealed three patterns. KPC-3 exhibited a higher hydrolytic activity towards extended-spectrum cephalosporins (cefotaxime, ceftazidime and cefepime), but a similar hydrolysis rate for carbapenems to KPC-2. KPC-14 and KPC-28 possessed a higher affinity for ceftazidime, which increased their catalytic efficiencies 40-fold and 50-fold, respectively. However, their carbapenemase activities were reduced. Imipenem catalytic efficiencies were reduced 200-fold and 700-fold, respectively, compared with KPC-2. Thus, the reduced carbapenem hydrolysis can be linked to Δ242-GT-243 that results in a 10-fold increase in affinity, but also a 1000-fold decrease in k_{cat} values. Similarly, KPC-14 and KPC-28 completely lost hydrolytic activity for cefoxitin. IC_{50} values of clavulanate for KPC-2, KPC-3, KPC-14 and KPC-28 were 47, 16, 0.1 and 0.09 μM, respectively. Taken together, our results suggest that KPC-28 has a clavulanate-inhibited ESBL profile and no longer displays that of a carbapenemase. Finally, our data suggest that the increased ceftazidime hydrolysis of KPC-28 is due to the H274Y substitution (Table 2) confirming previous results,[12] but is potentiated by the two-amino-acid deletion Δ242-GT-243. The H274Y substitution does not affect carbapenem hydrolysis, unlike the Δ242-GT-243 deletion (Table 2). The activity of avibactam against the different KPCs was determined by IC_{50} measurements.

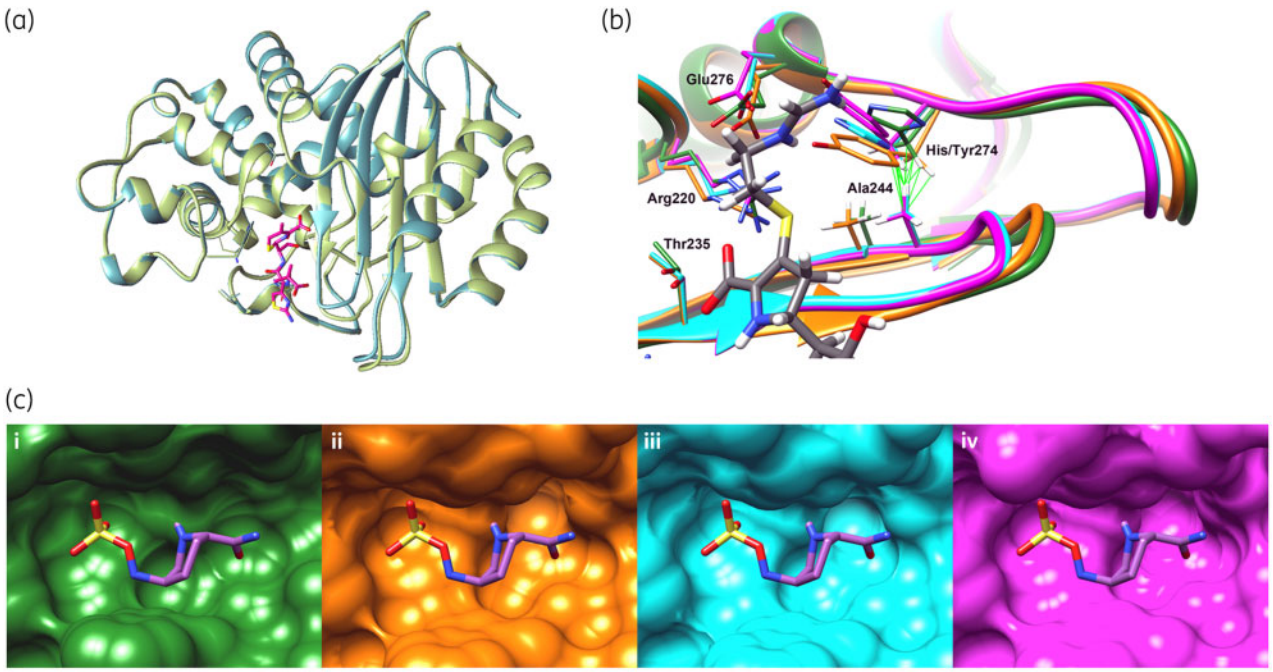

Figure 2. Models of interaction of KPC-3 variant (KPC-14 and KPC-28) with ceftazidime and imipenem. (a) Superposition of the molecular model of KPC-3 and KPC-28 with ceftazidime. KPC-3 is in light green, KPC-28 in dark green and ceftazidime in magenta. (b) Crystal structure of KPC-2 (PDB code 5UJ3, green) superposed with homology models of KPC-3 (orange), KPC-14 (cyan) and KPC-28 (magenta). The imipenem conformation obtained by covalent docking on KPC-2 is shown as grey sticks. The steric clashes between the side chains of A244 and of the residue at position 274 are highlighted in light green. (c) Crystal structure of KPC-2 in complex with avibactam (i, PDB code 4ZBE, green) superposed with the KPC-3 (ii, orange), KPC-14 (iii, cyan) and KPC-28 (iv, magenta) homology models, showing no significant clashes between the protein and the ligand.

The IC_{50} values for KPC-2, KPC-3, KPC-14 and KPC-28 were 230, 350, 107 and 586 nM, respectively. These values are very similar, which suggests that the two-amino-acid deletion Δ242-GT-243 has no impact on the inhibition properties of avibactam.

Molecular modelling

We performed a molecular modelling study to identify the structural determinants that could explain the experimentally determined differences between the hydrolytic profiles of KPC-2, KPC-3, KPC-14 and KPC-28. In the absence of structural data for the KPC-2 variants, we generated molecular models of KPC-3, KPC-14 and KPC-28 by comparative modelling using MODELLER version 9.16[31] with the KPC-2 structure (PDB code 5UJ3) as template (Figure 2a and b).[32] The resulting models showed that the Δ242-GT-243 deletion did not modify the overall structure of the protein, but resulted in a shorter 238–243 loop, giving rise to a 2.4 Å shift of A244 in KPC-14 and KPC-28 (Figure 2a). This new position of A244 led to a clash between the side chains of A244 and of the residue at position 274 (H and T for KPC-14 and KPC-28, respectively) that may expand the active site and allow better access for the substrates. In addition, the covalent complex of KPC-2 with imipenem obtained by docking showed a stabilizing interaction between H274 and the positively charged R2 substituent of imipenem (Figure 2b). In the case of KPC-14 and KPC-28, the above-mentioned clash may prevent this stabilizing interaction with imipenem. Under these conditions, the substrate may interact with the binding site in a slightly different way and therefore explain the 10-fold increase in K_m and the 1000-fold decrease in k_{cat}, which ultimately lead to the loss of the carbapenemase activity in these mutants. Additional theoretical calculations, and especially molecular dynamics simulations, will be needed to understand these details.

Detection methods for KPC variants

Four carbapenemase detection tests based on imipenem hydrolysis were evaluated with respect to their ability to detect these four variants when expressed in *E. coli* TOP10 (Table 3). Thus, the Carba NP test,[21] the RAPIDEC® CARBA NP, the MBT STAR®-Carba IVD Kit, the β CARBA™ test, the CIM test and the modified Hodge test were able to detect KPC-2 and KPC-3, but failed to detect KPC-14 and KPC-28.[21–26] These results are in line with the kinetic studies, which showed a loss of carbapenemase activity for KPC-14 and KPC-28. On the other hand, the immunochromatographic assays Resist-4 O.K.N.V. K-Set (CORIS BioConcept) and the NG-Test CARBA 5 (NG Biotech) and molecular Xpert® Carba-R test (Cepheid) and in-house PCR were positive for all four enzymes.[27–29,35] The positive results of the immunochromatographic assays for KPC-14 and KPC-28 confirmed our molecular modelling results, suggesting that Δ242-GT-243 did not affect the overall conformation of these proteins.

Discussion

KPC-producing Enterobacteriaceae are now endemic in the USA and have spread worldwide. So far, 39 variants of KPC have been

Table 3. Diagnostic tests performed on *E. coli* TOP10 harbouring the vector pTOPO expressing KPC-2, KPC-3, KPC-14 and KPC-28

	Biochemical tests						Molecular tests		Immunoenzymatic tests	
	Carba NP test	RAPIDEC® CARBA NP	CIM test	modified Hodge test	β CARBA™ test	MBT STAR®-Carba IVD Kit	standard bla_{KPC} PCR	Xpert® Carba-R	NG-Test CARBA 5	CORIS BioConcept Resist-4 O.K.N.V. K-Set
KPC-2	+	+	+	+	+	+	+	+	+	+
KPC-3	+	+	+	+	+	+	+	+	+	+
KPC-14	−	−	−	−	−	−	+	+	+	+
KPC-28	−	−	−	−	−	−	+	+	+	+

Key: +, positive test result; −, negative test result.

reported,[6] with KPC-2 and KPC-3 appearing to be the most prevalent.[36] In this study, we have characterized the biochemical properties of KPC-28, which has the same H274Y substitution as KPC-3 and possesses in addition a Δ242-GT-243 deletion previously identified in KPC-14. Steady-state kinetics of KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases revealed that the unique substitution of KPC-3 (H274Y) conferred an ∼20-fold increase in the catalytic efficiency towards ceftazidime as compared with KPC-2. This substitution has also been identified in other variants, such as KPC-7 (M49I; H274Y), KPC-8 (V240G; H274Y), KPC-9 (V240A; H274Y) and KPC-10 (P104R; H274Y), which are associated with increased ceftazidime resistance.[9] The study of KPC-5 showed that substitution of residue 104 can also confer increased ceftazidime hydrolysis.[11] These studies highlighted that a single or a double substitution can affect the hydrolysis rate of cephalosporins, but do not significantly change the carbapenemase activity. More recently, Shields *et al.*[13] reported that the T243A substitution increased ceftazidime hydrolysis of the KPC-3 enzyme with little impact on carbapenemase activity. Here, we demonstrated that the Δ242-GT-243 deletion also leads to an increase in ceftazidime hydrolysis, but, unlike the T243A substitution, has a drastic impact on carbapenemase activity. For example, compared with KPC-2, the catalytic efficiencies (k_{cat}/K_m) towards imipenem were 700-fold and 200-fold lower for KPC-28 and KPC-14, respectively. This deletion is linked with a 10-fold higher affinity (lower K_m) but a 1000-fold decrease in k_{cat} for carbapenems. The loss of the carbapenemase activity can be explained by the loss of the interaction between the residue H274 in KPC-14 and Y274 in KPC-28 and carbapenems due to a 2 Å shift of the A244. Additionally, the IC_{50} of clavulanate was 500-fold lower for KPC-28 as compared with KPC-2. The most worrying result is that this two-amino-acid deletion also has an impact on ceftazidime/avibactam susceptibility. Indeed, while KPC-2- and KPC-3-producing *E. coli* isolates remain susceptible to ceftazidime/avibactam, *E. coli* isolates producing KPC-14 (and those producing KPC-28) are resistant. Several mutations in KPC enzymes have been shown to yield ceftazidime/avibactam resistance. The most common *in vivo* mutation described is D179Y, especially when associated with the H279Y mutation that yields increased ceftazidime hydrolysis.[14,37] Other mutations with minor phenotypic expressions have also been identified, such as S130G, T243A or T243M.[38] In KPC-28, T243 has been deleted and may thus play an additional role in avibactam resistance. It is very likely that avibactam resistance in KPC-28 is the result of the increase in the catalytic efficiency for ceftazidime, since no clashes were evidenced between avibactam and the homology models of the KPC-3, KPC-14 and KPC-28 variants (Figure 2c). This hypothesis is reinforced by the fact that the IC_{50} values of avibactam for the different KPC variants are very similar.

Conclusions

This study underlines that KPC-type β-lactamases are more complex and diverse than expected. As exemplified by KPC-28 and KPC-14, they are not all true carbapenemases, a scenario well known for OXA-48-like enzymes.[39] Unfortunately, molecular detection assays and immunochromatographic tests are not able to distinguish KPC variants with carbapenem hydrolytic capacities from those lacking any carbapenemase activity. Therefore, the first-line screening of carbapenemase producers in Enterobacteriaceae must include a test able to detect any carbapenemase activity, such as biochemical tests (e.g. Carba NP test and derivatives, β CARBA™ test), or MALDI-TOF-based assays (e.g. MBT STAR®-Carba IVD Kit, Bruker Daltonics). Finally, as KPC-producing organisms cause infections with a high morbidity and mortality, avibactam was designed as a powerful inhibitor of KPC enzymes.[40–43] However, several studies now report the resistance of KPC-producing isolates to ceftazidime/avibactam as a consequence of the selection of point-mutation derivatives.[43] KPC-28-producing bacterial isolates are resistant to ceftazidime/avibactam as a consequence of increased ceftazidime hydrolysis, rather than intrinsic avibactam resistance. Finally, as KPC-28 lacks carbapenemase activity, has increased ceftazidime hydrolytic activity and is strongly inhibited by clavulanate, KPC-28-producing bacterial isolates may be identified as ESBL producers.

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Transparency declarations

L. D. is co-inventor of the Carba NP test, the patent for which has been licensed to bioMérieux (La Balmes les Grottes, France). All other authors: none to declare.

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