Contribution of β -Lactamases and Porin Proteins OmpK35 and OmpK36 to Carbapenem Resistance in Clinical Isolates of KPC-2-Producing *Klebsiella pneumoniae*

Ying Zhang,^a Xiaofei Jiang,^{a,b} Yanyan Wang,^a Gang Li,^a Yueru Tian,^a Hong Liu,^a Fuqi Ai,^a Yiming Ma,^a Bei Wang,^a Feiyi Ruan,^a Kumar Rajakumar^{b,c}

Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, China^a; Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom^b; Department of Clinical Microbiology, University Hospitals of Leicester NHS Trust, Leicester, United Kingdom^c

Fifty-seven carbapenem-resistant *Klebsiella pneumoniae* isolates belonging to ST11 (50 isolates), ST423 (5 isolates), and two other sequence types were studied. All were positive for *bla*_{KPC-2}, *bla*_{TEM-1}, and *bla*_{CTX-M-14}. SDS-PAGE analysis of six representative isolates demonstrated varied porin expression. Nevertheless, when *bla*_{KPC-2} was deleted, carbapenem resistance was markedly reduced. Additionally, SHV-12, DHA-1, and/or VIM-1 appeared to contribute to accessory carbapenemase activity. In contrast, OmpK35 and/or OmpK36 deficiency seemed to serve only as a minor cooperative factor.

Carbapenem MICs of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* vary widely from isolate to isolate, supporting the involvement of other factors (1–4). Indeed, Landman et al. (5) have demonstrated that carbapenem MICs are negatively correlated with OmpK36 expression, and Doménech-Sánchez et al. (6) have shown that loss of OmpK35 is associated with increased resistance to cephalosporins and carbapenems. However, other studies have shown that reduced expression of OmpK35 or OmpK36 alone does not significantly alter carbapenem MICs (7–9).

We analyzed 57 KPC-producing *K. pneumoniae* isolates collected from 2006 to 2009 at Huashan Hospital, Shanghai, China, using disc-based and/or Etest susceptibility testing. Colony PCR assays for *bla*_{IMP}, *bla*_{VIM}, *bla*_{KPC}, *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} and six plasmid-borne genes (*bla*_{ACC}, *bla*_{CIT}, *bla*_{DHA}, *bla*_{ENT/EB}, *bla*_{FOX}, and *bla*_{MOX}) that code for AmpC enzymes were performed (see Table S1 in the supplemental material for primer details). Standard multilocus sequence typing (MLST) protocols were utilized, with alternative primers for *gapA*, *mdh*, *rpoB*, and *tonB* used as required. Fifty isolates belonged to ST11, five belonged to ST423, and one each belonged to ST65 and a novel MLST type, ST977. The first carbapenem-resistant isolate, derived from a urine specimen obtained in August 2006, belonged to ST423. However, only four other ST423 isolates were detected over the study period. In contrast, ST11 remained endemic throughout this period. Intriguingly, isolates belonging to ST65 and ST977 appeared only once. Except for XJ-2 and XJ-4, which exhibited lower carbapenem MICs, all 57 isolates showed high-level resistance to ampicillin, cefotaxime, ceftazidime, imipenem, meropenem, and ertapenem. PCR analysis suggested that all but two isolates produced TEM-1, KPC-2, CTX-M-14, and SHV-12; *bla*_{SHV-12} was not detected in XJ-1 and XJ-4. In addition, all five ST423 isolates encoded DHA-1, and the XJ-5 (ST11) isolate uniquely produced VIM-1.

Isolates XJ-1, XJ-2, and XJ-3, representative of ST977, ST65, and ST423, respectively, were chosen for further analysis together with three ST11 isolates: XJ-4 because it had relatively low carbapenem MICs, XJ-5 because it had a supplementary *bla*_{VIM-1} gene, and XJ-6 because it was typical of most ST11 isolates. SDS-PAGE

analysis of outer membrane proteins extracted as described previously (10) from cells grown overnight with shaking at 37°C in nutrient broth with or without 10 g/liter NaCl showed that XJ-1 expressed smaller quantities of OmpK36, while OmpK35 production was not detected for XJ-1, XJ-4, and XJ-5 (Fig. 1). Furthermore, as the likely OmpK36 protein bands of XJ-3 and XJ-6 were shifted upwards (see below for details), the status of OmpK35 bands in these strains could not be determined (Fig. 1).

Although DNA mutations relative to that of NTUH-K2044 were detected in the *ompK35* sequences of XJ-4, XJ-5, and XJ-6, no amino acid changes were predicted (Fig. 2). In contrast, the predicted OmpK35 of XJ-1 exhibited 25 amino acid substitutions and a single insertion, while the corresponding sequence of XJ-2 varied by a single amino acid substitution only. The OmpK35 protein of XJ-3 lacked a five-amino-acid string (EIYNK), which mapped to the B1 β -sheet. This string of amino acids was strictly conserved in the OmpK35 sequences of the remaining five clinical isolates, NTUH-K2044, and MGH 78578, the last being the most divergent example analyzed (Fig. 2). The *ompK36* sequences in XJ-3 and XJ-6 showed five sets of identical in-frame insertions and deletions that were predicted to lead to 9 additional amino acid residues and an increase in mass of approximately 1 kDa. Interestingly, this variant possessed an inserted aspartic acid-glycine pair that lay directly adjacent to the highly conserved L3 loop-borne PEFXG motif. Indeed, an identically placed aspartic acid-threonine insertion has previously been proposed to lead to OmpK36

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Address correspondence to Kumar Rajakumar, Kr46@le.ac.uk, or Xiaofei Jiang, jiangxi2154@yahoo.com.cn.

Y.Z. and X.J. contributed equally to this work.

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FIG 1 SDS-PAGE-based outer membrane protein profiles of *K. pneumoniae* strains grown under high (top panel)- or low (bottom panel)-osmolarity conditions. The two most prominent bands for all strains were OmpK36 and OmpA (lower prominent band). Faint bands corresponding to OmpK35 were seen just above the OmpK36 band for strains ATCC 13883 and XJ-2. The positions of the OmpK35 (white arrowhead) and OmpK36 (black arrowhead) bands of ATCC 13883 are indicated. Bands corresponding to the variant, size-shifted OmpK36 proteins of XJ-3 and XJ-6 are indicated with asterisks.

functional deficiency (Fig. 2) (11, 12). Extending this theme, we hypothesize that other amino acid sequence changes identified in OmpK35 and OmpK36, particularly those mapping to predicted secondary structural features, may also impact carbapenem resis-

tance. Finally, we failed to amplify the entire *ompK36* XJ-1 coding sequence despite using multiple primer pairs, suggesting that it was affected by a deletion and/or rearrangement.

We next deleted *bla*_{KPC-2} to examine its contribution to car-

FIG 2 Comparison of predicted amino acid sequences of OmpK35 and OmpK36 with reference protein sequences. (a) Alignment of all regions of deduced sequences of OmpK35 which exhibited variation relative to the OmpK35 sequence of *K. pneumoniae* NTUH-K2044. Matching regions of the predicted, highly variant OmpK35 sequence of MGH 78578 are also shown. (b) Alignment of regions of deduced OmpK36 sequences which exhibited variation relative to that of Z33506.1. Reference sequence coordinates are indicated above. Schematics represent predicted secondary structures of the reference proteins, with that of OmpK36 being derived from the work of Alberti et al. (11). White rectangles represent β -strands, thick black lines indicate predicted protein loops, and gray rectangles symbolize α -helices. Deleted amino acid residues are shown as dashes, while substitutions are shaded gray. Nonconserved amino acid substitutions and insertions are underlined. The highly conserved PEFXG motif implicated in OmpK36 function is shown in bold (12).

TABLE 1 Characteristics of six clinical *Klebsiella pneumoniae* isolates and matching *bla*_{KPC-2}-minus mutants

Strain ^a	MLST	β-Lactamase profile ^b						MIC (mg/liter) ^c					
		KPC	SHV	CTX	TEM	VIM	DHA	AMP	CTX	CAZ	IMP	MEM	ETM
ATCC 13883	NA ^d	ND ^e	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 25922	NA	ND	ND	ND	ND	ND	ND	1	0.06	0.06	0.125	0.01	<0.01
XJ-1	ST977	+	—	+	+	—	—	≥32	≥32	≥256	≥32	≥32	≥32
ΔXJ-1		—	—	+	+	—	—	≥32	≥32	4	0.3	0.1	0.5
XJ-2	ST65	+	+	+	+	—	—	≥32	≥32	≥256	4	2	≥32
XJ-3	ST423	+	+	+	+	—	+	≥32	≥32	≥256	≥32	≥32	≥32
ΔXJ-3		—	+	+	+	—	+	≥32	≥32	≥256	4	1	≥32
XJ-4	ST11	+	—	+	+	—	—	≥32	≥32	64	4	4	8
ΔXJ-4		—	—	+	+	—	—	≥32	≥32	6	0.1	0.03	0.3
XJ-5	ST11	+	+	+	+	+	—	≥32	≥32	≥256	≥32	≥32	≥32
ΔXJ-5		—	+	+	+	+	—	≥32	≥32	≥256	4	4	2
XJ-6	ST11	+	+	+	+	—	—	≥32	≥32	≥256	≥32	≥32	≥32
ΔXJ-6		—	+	+	+	—	—	≥32	≥32	≥256	0.5	0.25	6

^a Strain names prefixed with Δ correspond to *bla*_{KPC-2}-minus mutants.

^b Predicted β-lactamase profile based on PCR detection of the following genes: *bla*_{KPC-2} (KPC), *bla*_{SHV-12} (SHV), *bla*_{CTX-M-14} (CTX), *bla*_{TEM-1} (TEM), *bla*_{VIM-1} (VIM), and *bla*_{DHA-1} (DHA). +, amplicon produced; —, no amplicon produced.

^c MICs were determined by Etest. AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; MEM, meropenem; ETM, ertapenem.

^d NA, not applicable.

^e ND, not determined.

bapenem resistance. Allelic replacement with a fragment tagged with the hygromycin resistance gene from pMQ300 (13) (see Fig. S1 in the supplemental material) was facilitated by λ-Red recombination as described previously (14). The XJ-2-derived mutant remained positive for *bla*_{KPC-2} despite PCR confirmation of the intended allelic exchange, suggesting the presence of a second *bla*_{KPC-2} copy. For XJ-1 and XJ-4, which lacked SHV-12, DHA-1, and VIM-1, deletion of *bla*_{KPC-2} resulted in a >8-fold decrease in carbapenem MICs, regardless of OmpK35 and OmpK36 expression levels (Fig. 1). Most remarkably, XJ-1, which exhibited MICs in excess of 32 mg/liter for all three carbapenems, no detectable OmpK35, and deficient expression of OmpK36, became sensitive to all three agents following deletion of *bla*_{KPC-2} (Table 1), suggesting that dual porin deficiency is only a cooperative factor and insufficient on its own to confer carbapenem resistance. In contrast, when *bla*_{KPC-2} was deleted from the SHV-12-encoding XJ-6, the resultant mutant exhibited reduced but relatively high imipenem (0.5 mg/liter), meropenem (0.25 mg/liter), and ertapenem (6 mg/liter) MICs. Similarly, the SHV-12- and DHA-1-encoding ΔXJ-3 exhibited reduced but still clinically significant meropenem (1 mg/liter), imipenem (4 mg/liter), and ertapenem (≥32 mg/liter) MICs (Table 1). Likewise, carbapenem MICs of the *bla*_{SHV-12}- and *bla*_{VIM-1}-encoding ΔXJ-5 were much greater than those of ΔXJ-4 (Table 1), despite both exhibiting no OmpK35 band and broadly equivalent OmpK36 expression levels, suggesting that VIM-1 (15) and/or SHV-12 was contributing to carbapenem resistance in XJ-5.

In conclusion, we found that *bla*_{KPC-2} was the key factor contributing to carbapenem resistance in the isolates studied and that deficient expression of OmpK35 and/or OmpK36 serves only as a cooperative factor. Additionally, our data support the idea that expression of two or more carbapenem-inactivating enzymes, even in the absence of porin deficiencies, leads to markedly elevated carbapenem MIC levels. Collectively, these findings add to the growing appreciation of the multiple pathways exploited by *K. pneumoniae* to evade carbapenems (4–6, 12, 15, 16).

Nucleotide sequence accession numbers. Coding sequences of *ompK35* and *ompK36* were amplified, sequenced, and submit-

ted to GenBank under the following accession numbers: JX310550, JX310551, JX310552, JX310553, JX310554, JX310555, JX310556, JX310557, JX276743, JX291113, and JX291114.

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