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Genetic Factors Associated with Elevated Carbapenem Resistance in KPC-Producing *Klebsiella pneumoniae*▽

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In the United States, the most prevalent mechanism of carbapenem resistance among *Enterobacteriaceae* is the production of a *Klebsiella pneumoniae* carbapenemase (KPC). KPC-producing isolates often exhibit a range of carbapenem MICs. To better understand the factors that contribute to overall carbapenem resistance, we analyzed 27 KPC-producing *K. pneumoniae* isolates with different levels of carbapenem resistance, 11 with low-level (i.e., meropenem or imipenem MIC ≤ 4 μg/ml), 2 with intermediate-level (i.e., meropenem and imipenem MIC = 8 μg/ml), and 14 with high-level (i.e., imipenem or meropenem MIC ≥ 16 μg/ml) carbapenem resistance, that were received from throughout the United States. Among 14 isolates that exhibited high-level carbapenem resistance, Western blot analysis indicated that 10 produced an elevated amount of KPC. These isolates either contained an increased bla_{KPC} gene copy number ($n = 3$) or had deletions directly upstream of the bla_{KPC} gene ($n = 7$). Four additional isolates lacked elevated KPC production but had high-level carbapenem resistance. Porin sequencing analysis identified 22 isolates potentially lacking a functional OmpK35 and three isolates potentially lacking a functional OmpK36. The highest carbapenem MICs were found in two isolates that lacked both functioning porins and produced elevated amounts of KPC. The 11 isolates with low-level carbapenem resistance contained neither an upstream deletion nor increased bla_{KPC} copy number. These results suggest that both bla_{KPC} copy number and deletions in the upstream genetic environment affect the level of KPC production and may contribute to high-level carbapenem resistance in KPC-producing *K. pneumoniae*, particularly when coupled with OmpK36 porin loss.

The occurrence of Gram-negative bacterial infections that are resistant to extended-spectrum β-lactam antimicrobial agents forces clinicians to rely on carbapenems as a "last resort" to combat these resistant pathogens. However, as carbapenems are more frequently utilized, an increasing number of bacteria with various mechanisms of resistance to this class of antimicrobial agents are identified. The most widespread resistance mechanisms include the production of a carbapenemase and the combination of porin loss with the production of either an AmpC enzyme or an extended-spectrum β-lactamase (4, 15). *Klebsiella pneumoniae* carbapenemase (KPC), an Ambler class A β-lactamase that can hydrolyze most β-lactam agents, including carbapenems, is now the most prevalent carbapenemase found among clinical Gram-negative isolates in the United States (22).

KPC was first reported in a *K. pneumoniae* isolate from North Carolina in 1996 (28). However, recent reports indicate that KPC-producing Gram-negative isolates are being identified throughout the United States as well as parts of Europe, Asia, and South America (13, 20, 22). Although these β-lactamases occur most commonly in *K. pneumoniae*, they have also been identified in other members of the *Enterobacteriaceae* family and in *Pseudomonas* and *Acinetobacter* species (3, 21, 24, 26, 27). The bla_{KPC} gene is plasmid mediated and is carried on a Tn*3*-based transposon, Tn*4401* (17), which may account for the high mobility of this resistance mechanism.

KPC-producing isolates can exhibit a range of carbapenem MICs, thus making their detection a significant challenge for clinical laboratories. By using 2009 Clinical and Laboratory Standards Institute (CLSI) breakpoints and testing methods (1, 6), some KPC-producing isolates may be identified as susceptible to carbapenems. The clinical significance of carbapenem-susceptible isolates with elevated carbapenem MICs is unclear (6), and the cellular changes that may convert a susceptible KPC-producing isolate to one with MICs indicating resistance to carbapenem are not well described. From previous reports, we know that KPC production combined with porin loss can result in higher carbapenem MICs (10, 14, 29). This finding suggests that the KPC enzyme alone is not always sufficient to confer carbapenem resistance, as defined by the 2009 CLSI breakpoints.

Other factors likely result in higher carbapenem MICs for KPC-producing isolates. For example, isolates with an increased expression of bla_{KPC} were previously shown to have increased rates of hydrolysis of imipenem and meropenem (14). Directly upstream of the bla_{KPC} gene is a nonconserved region of the Tn*4401* transposon, located between the *istB* and the bla_{KPC} genes (17). Previous reports describe four isoforms in this variable region: Tn*4401a* contains a 100-bp deletion, Tn*4401b* contains no deletion (17), and isoforms with 215-bp (GenBank accession no. DQ989640) and 255-bp (13) deletions

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were recently reported. Additional studies of this variable region suggest that the 100-bp deletion may result in a different −35 promoter region of the bla_{KPC} gene (11). Upstream deletions that affect the promoter may impact the level of bla_{KPC} expression and thus would influence the overall level of carbapenem resistance. Also, KPC-producing isolates may contain different levels of bla_{KPC} dosage based on the presence of multiple copies of a bla_{KPC}-carrying plasmid, multiple bla_{KPC}-carrying plasmids, or multiple copies of the bla_{KPC} gene located within the same plasmid (11). Increasing the bla_{KPC} gene copy number could result in increased enzyme production and higher carbapenem MICs. Understanding the impact of these factors may help to predict the potential for KPC-producing isolates susceptible to carbapenems to convert to isolates resistant to carbapenems.

In this study, we examined genetic factors that may enhance the level of carbapenem resistance. We selected 27 KPC-producing *K. pneumoniae* isolates that were obtained from clinical patients in different areas of the country and exhibited a range of carbapenem MICs. These isolates were characterized by determining the sequences of the two main porins, OmpK35 and OmpK36 (9), examining levels of KPC production by Western blot analysis, comparing relative bla_{KPC} copy numbers using quantitative real-time PCR, and analyzing sequence variations in the genetic environment directly upstream of the bla_{KPC} gene.

MATERIALS AND METHODS

Selection of bacterial strains. *K. pneumoniae* isolates sent to the Centers for Disease Control and Prevention (CDC) for reference antimicrobial susceptibility testing were analyzed by PCR for bla_{KPC} if the MIC was ≥2 μg/ml for any of the carbapenems (i.e., imipenem, meropenem, or ertapenem) or if the isolate tested positive by the modified Hodge test (1, 6). For this study, KPC-producing *K. pneumoniae* isolates (n = 27) were selected from diverse geographic locations and represented isolates with MICs that spanned the 2009 CLSI breakpoints for imipenem and meropenem (i.e., susceptible, ≤4 μg/ml; intermediate, 8 μg/ml; resistant, ≥16 μg/ml) (6). These isolates were recovered from patients located in 17 cities in 9 states representing each major geographic region of the continental United States. We defined 11 isolates as having "low-level carbapenem resistance" (i.e., MIC ≤ 4 μg/ml for either imipenem or meropenem) and 14 isolates as having "high-level carbapenem resistance" (i.e., MIC ≥ 16 μg/ml for either imipenem or meropenem). Also, two additional isolates that had an intermediate level of resistance (MIC = 8 μg/ml for both imipenem and meropenem) were selected.

BMD. Carbapenem MICs were measured using broth microdilution (BMD) on panels made in-house according to CLSI guidelines (5, 6).

PFGE. The CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA) was used to type KPC-positive *K. pneumoniae* isolates selected for this study by pulsed-field gel electrophoresis (PFGE) of XbaI-digested DNA as described for *Escherichia coli* (http://www.cdc.gov/pulsenet/protocols.htm) and compared to the CDC's KPC-producing *K. pneumoniae* PFGE database (n > 430).

Sequencing of the bla_{KPC} gene. The KPC subtypes of the 27 isolates were determined by amplification of a 1,011-bp PCR product and bidirectional DNA sequence analysis using previously described primers (23).

Western blot analysis of KPC production. Rabbit polyclonal antibodies were raised against the KPC type 2 (KPC-2) β-lactamase in order to measure bla_{KPC} expression. The bla_{KPC-2} gene was expressed from pBR322-*catI*-bla_{KPC-2} in *E. coli* DH10B. The KPC-2 β-lactamase was then isolated and purified as previously described (18). Anti-KPC-2 polyclonal antibodies were raised by Sigma-Genosys (The Woodlands, TX) and isolated from serum using protein G column purification (Sigma-Genosys) (19). KPC-producing *K. pneumoniae* study isolates were grown in Luria-Bertani broth to an optical density at 600 nm (OD_{600}) of 0.8. Preparation of samples, immunoblotting, and recognition of KPC expression were performed as previously reported (19).

Relative bla_{KPC} copy number. The quantity of the bla_{KPC} gene was measured relative to an internal *K. pneumoniae* housekeeping gene, *rpoB*, using real-time PCR on the 7500 Fast system (Applied Biosystems Inc., Carlsbad, CA). Cell lysates were prepared as previously described (7) except that 50 μl of heated lysate was neutralized with 18 μl of 0.5 M Tris-HCl, pH 8, diluted with 400 μl of cold, sterilized reagent grade water, and stored at −20°C. DNA concentrations of cell lysates were subsequently normalized using a Nanodrop spectrophotometer (Thermo Scientific, West Palm Beach, FL). PCRs were performed in triplicate using QuantiFast reagents (Qiagen, Valencia, CA), 1 ng/μl of template, 500 nM each primer, and 250 nM each probe (listed in Table 1). Cycling conditions included a 3-min enzyme activation step at 95°C, followed by 40 cycles of melting (95°C for 3 s) and annealing/extension (60°C for 20 s). Standard curves were generated for both the target (bla_{KPC}) and the endogenous control (*rpoB*) using 10-fold dilutions of template DNA at known concentrations and by plotting the logarithm of initial quantity of template (along the x axis) versus the respective cycle threshold (C_T) values (along the y axis) (12). Absolute quantification analysis of gene copy number was performed using the following equations derived from these standard curves (rearrangement of $y = mx + b$): quantity of bla_{KPC} (ng/μl) = $10^{(CT - 22.43)/-3.4665}$ (r^2 = 0.994) and quantity of *rpoB* (ng/μl) = $10^{(CT - 22.05)/-3.2561}$ (r^2 = 0.996). The ratio of bla_{KPC} copy number to *rpoB* copy number was calculated in order to determine the relative bla_{KPC} gene dosage in each isolate. For each isolate, an average of these ratios and their standard deviation are shown in Table 2.

TABLE 1. Sequences of primers and probes utilized for bla_{KPC} gene dosage studies and analysis of Tn*4401* deletions (located between the *istB* and bla_{KPC} genes)

Primer or probe	Sequence (5′–3′) (reference)
KPC forward primer	GGC CGC CGT GCA ATA C
KPC reverse primer	GCC GCC CAA CTC CTT CA
KPC probe (FAM)[a]	TG ATA ACG CCG CCG CCA ATT TGT
RPOB forward primer	CTG ATG CCT CAG GAT ATG ATC AAC
RPOB reverse primer	CTG GCT GGA ACC AAA GAA CTC T
RPOB probe (Cy5)[a]	CA AGC CGA TTT CCG CAG CAG TGA
Tn*4401* forward primer	TGA CCC TGA GCG GCG AAA GC (17)
Tn*4401* reverse primer	CAC AGC GGC AGC AAG AAA GC

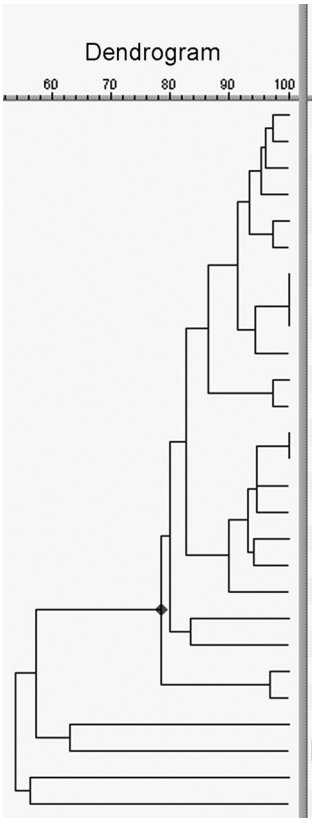
[a] KPC probes were labeled with 6-carboxyfluorescein (FAM), and RPOB probes were labeled with Cy5 on their 5′ ends. Each contained a black hole quencher (BHQ) on its 3′ end.

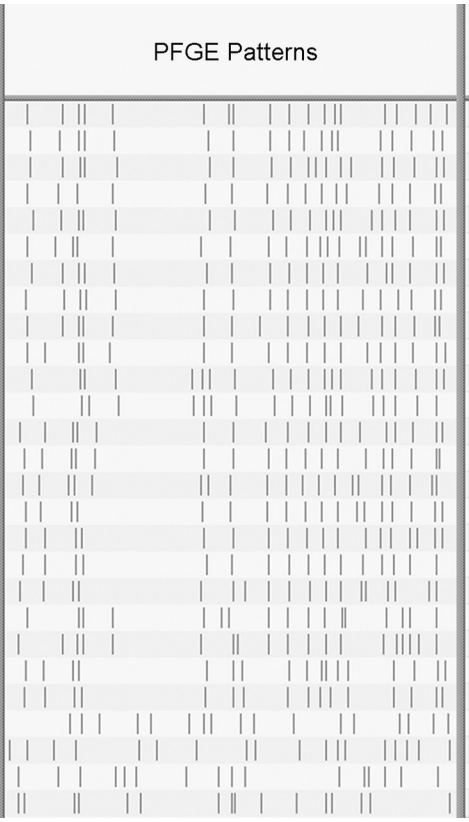
Analysis of upstream genetic environment. Directly upstream of the bla_{KPC} gene is a variable region of the encompassing Tn*4401* transposon structure. PCR and subsequent bidirectional DNA sequence analysis were performed using primers shown in Table 1 in order to analyze the region located between the *istB* and bla_{KPC} genes (17).

Sequencing of *ompK35* and *ompK36* genes. PCR for *ompK35* and *ompK36* was performed using previously described primers and conditions (16). DNA sequence analysis was performed using Lasergene 7.2 (DNASTAR, Madison, WI). The final amino acid sequences were determined using the ExPASy proteomics server (http://ca.expasy.org) and compared with those previously described in GenBank (i.e., accession numbers FJ577672, AJ011501, ADG27478, FJ577673, FJ577675, BAH64431, and ACI07318) (see Table 3).

RESULTS AND DISCUSSION

Characterization of isolates. Isolates were selected to represent a diverse population of KPC-producing *K. pneumoniae* isolates seen in the United States. Notably, the majority of the isolates (n = 23) had ≥80% PFGE pattern similarity using the Dice coefficient and clustering by the unweighted-pair group method using average linkages (UPGMA) (Bionumerics 5.10; Applied Maths Inc., Austin, TX) (Fig. 1). Since six of these 23 isolates were previously confirmed to be multilocus sequence type 258 (ST 258), the dominant strain of KPC-producing *K. pneumoniae* in the United States (13), the remaining isolates whose PFGE patterns cluster with this group are also likely ST 258 (2). Sequencing the bla_{KPC} genes for all 27 isolates showed that the majority (n = 22) produced KPC-3, while only five produced KPC-2 (Table 2). These are the most common KPC subtypes reported in the United States. The





Isolate	Year	Location		MIC (μg/ml)		
				IPM	MEM	ETP
ART2008226	2006	AZ	Phoenix	<1	<0.25	<0.5
ART2008027	2006	AZ	Phoenix	512	512	1024
ART2008142	2006	AZ	Phoenix	<1	4	8
ART2008026	2006	NJ	Livingston	64	64	256
ART2008140	2006	AZ	Phoenix	4	2	8
AIS081042	2008	FL	Ft. Lauderdale	64	128	256
ART2008137	2005	PA	Philadelphia	4	8	16
ART2008022	2005	PA	Hershey	8	16	32
AIS070654	2007	NJ	Trenton	4	1	2
AIS081072	2008	PA	Wilkes-Barre	2	2	8
ART2008141	2006	AZ	Phoenix	256	256	256
ART2008028	2006	AZ	Phoenix	4	8	8
AIS080949	2008	GA	Decatur	32	16	64
ART2008136	2005	NY	NY	8	8	16
HIP14358	2005	NY	NY	32	16	64
ART2008135	2003	NJ	Hackensack	512	256	1024
HIP14474	2005	PA	Philadelphia	8	8	16
ART2008138	2005	NJ	Livingston	32	32	128
AIS080792	2008	VA	Norfolk	32	32	64
AIS070446	2007	PA	Exton	4	4	4
AIS080571	2008	IL	Naperville	128	128	256
ART2008143	2007	NJ	Flemington	8	16	64
ART2008139	2005	NJ	Flemington	32	16	16
HIP10924	2001	PA	Pittsburgh	4	4	4
AIS081058	2008	FL	Ft. Lauderdale	1	4	2
ART2008024	2006	MO	St. Louis	32	64	128
AIS080884	2008	VA	Charlotteville	2	4	8

FIG. 1. Dendrogram showing the relatedness of isolates based on PFGE patterns. Isolates were selected to represent a broad range of carbapenem MICs and geographic locations of isolation. The similar PFGE patterns observed for the majority of these isolates (sharing ≥80% similarity) correspond to a PFGE pattern associated with multilocus sequence type 258, the dominant strain of KPC-producing *K. pneumoniae* seen throughout the United States (13). IPM, imipenem; MEM, meropenem; ETP, ertapenem.

carbapenem MICs for these isolates are shown in Tables 2 and 3.

Levels of KPC production. To evaluate the relative levels of steady-state KPC expression in the 27 isolates, Western blot analysis was performed. Immunoblotting experiments were conducted in duplicate and produced nearly identical results (Fig. 2). Only one isolate (ART2008027) had results that varied, demonstrating a relatively more intense band in the second blot (Fig. 2, blot 2b). All isolates exhibiting elevated KPC production (i.e., ≥4 pluses in Table 2) were associated with high-level carbapenem resistance. Conversely, only four of the isolates with high-level carbapenem resistance (ART2008022, ART2008026, AIS080571, and ART2008141) lacked elevated KPC production. All of the isolates with low and intermediate levels of carbapenem resistance ($n = 13$) exhibited lower KPC production (i.e., ≤2 pluses in Table 2). These results demonstrate that elevated KPC production is associated with high-level carbapenem resistance.

Analysis of relative bla_{KPC} copy number. We hypothesized that the amount of KPC expression could be directly affected by bla_{KPC} copy number. To analyze the relative bla_{KPC} copy number, we used real-time PCR to measure the quantity of bla_{KPC} DNA relative to that of an internal *K. pneumoniae* housekeeping gene, *rpoB*. Three of the isolates (AIS080949, ART2008135, and ART2008027) exhibited an elevated relative bla_{KPC} copy number compared to the other isolates; all three of these isolates demonstrated elevated KPC production and high-level carbapenem resistance (Table 2). Of the remaining 24 isolates, 7 (ART2008143, HIP14358, ART2008024, AIS080792, AIS081042, ART2008138, and ART2008139) exhibited elevated KPC production but lacked an increased bla_{KPC} copy number. This suggests that factors other than bla_{KPC} copy number may contribute to the elevated KPC production and high-level carbapenem resistance observed in these isolates.

Analysis of the genetic environment upstream of the bla_{KPC} gene. The upstream Tn*4401* element was analyzed for the presence of deletions by PCR, and its sequence was analyzed for 26 of the 27 isolates. One isolate, ART2008226, did not produce a PCR product because its bla_{KPC-3}-carrying plasmid was lost during passage of the isolate *in vitro*. Four different isoforms of Tn*4401* were detected in this study, including sequences with no deletion ($n = 18$), a novel 68-bp deletion ($n = 3$), a 100-bp deletion ($n = 4$), and a 255-bp deletion ($n = 1$). Sequence analysis of these deletions indicated that the 68-bp and 100-bp deletions both end upstream of the −35 region of the putative promoter identified by Yigit et al. (28). However, the 255-bp deletion observed in this study eliminates the entire putative promoter region (28), including the transcription start site (Fig. 3).

Seven of the eight isolates with deletions demonstrated high-level carbapenem resistance and elevated KPC production

TABLE 2. Characteristics of KPC-producing *Klebsiella pneumoniae* isolates with low-, intermediate-, and high-level carbapenem resistance

Carbapenem resistance category and isolate[a]	MIC (μg/ml) of[i]:			KPC variant	Western blot result[d]	Gene dosage[f]	Tn*4401* variable region
	IPM	MEM	ETP				
Low							
ART2008226[b]	<1	<0.25	<0.5	3	+	NA[g]	NA
ART2008142[b]	<1	4	8	3	+	0.62 ± 0.07	No deletion
AIS081058	1	4	2	2	+	0.05 ± 0.07	No deletion
AIS070654[b]	4	1	2	3	+	0.27 ± 0.02	No deletion
AIS081072[b]	2	2	8	3	+	0.35 ± 0.03	No deletion
AIS080884	2	4	8	3	++	0.49 ± 0.05	No deletion
ART2008140[b]	4	2	8	3	++	0.51 ± 0.01	No deletion
AIS070446[b]	4	4	4	3	++	0.64 ± 0.11	No deletion
HIP10924	4	4	4	3	++	0.45 ± 0.01	No deletion
ART2008028	4	8	8	3	++	0.52 ± 0.09	No deletion
ART2008137[b]	4	8	16	3	+	0.57 ± 0.04	No deletion
Intermediate							
HIP14474[b]	8	8	16	3	+	0.48 ± 0.02	No deletion
ART2008136[b]	8	8	16	2	+	0.05[h]	100-bp deletion
High							
ART2008022[b]	8	16	32	3	++	0.64 ± 0.01	No deletion
ART2008139[b]	32	16	16	3	+++++	0.37 ± 0.14	68-bp deletion
ART2008143[b]	8	16	64	3	++++	0.38 ± 0.02	68-bp deletion
AIS080949[b]	32	16	64	3	++++	2.03 ± 0.01	No deletion
HIP14358[b]	32	16	64	2	+++++	0.12 ± 0.05	100-bp deletion
AIS080792[b]	32	32	64	2	+++++++	0.55 ± 0.18	100-bp deletion
ART2008138[b]	32	32	128	3	++++	0.28 ± 0.19	68-bp deletion
ART2008024	32	64	128	2	+++++++	0.18 ± 0.06	255-bp deletion
ART2008026[b]	64	64	256	3	++	0.56 ± 0.29	No deletion
AIS081042[b]	64	128	256	3	+++++	0.25 ± 0.09	100-bp deletion
AIS080571[b]	128	128	256	3	++	0.59 ± 0.13	No deletion
ART2008141[b,c]	256	256	256	3	++	0.47 ± 0.08	No deletion
ART2008135[b,c]	512	256	1,024	3	++++	1.81 ± 0.46	No deletion
ART2008027[b,c]	512	512	1,024	3	++++[e]	3.03 ± 0.29	No deletion

[a] Isolates are arranged in order of increasing carbapenem resistance. Classification of isolates as having low-, intermediate-, and high-level carbapenem resistance was as defined in this study.
[b] Isolate that potentially lacks a functional OmpK35 porin (Table 3).
[c] Isolate that potentially lacks a functional OmpK36 porin (Table 3).
[d] KPC production is interpreted with plus signs to represent relative intensity of bands from the Western blot analysis (Fig. 2).
[e] The only result that varied between repeated Western blots as seen in Fig. 2.
[f] Gene dosage results are shown as a ratio of quantity of bla_{KPC} (ng/μl) to that of *rpoB* (ng/μl).
[g] NA, not applicable because the isolate lost its bla_{KPC}-carrying plasmid upon passage *in vitro*.
[h] bla_{KPC} gene dosage result from only the first experimental repetition due to loss of the bla_{KPC}-carrying plasmid prior to subsequent repetitions.
[i] IPM, imipenem; MEM, meropenem; ETP, ertapenem.

(i.e., ≥4 pluses in Table 2), including ART2008024 (255-bp deletion), which produced one of the highest levels of KPC expression. One isolate with a 100-bp deletion, ART2008136, demonstrated an intermediate level of carbapenem resistance and did not exhibit elevated KPC production. The bla_{KPC}-carrying plasmid in this isolate was likely unstable since the initial bla_{KPC} gene dosage assay indicated a decreased amount of relative gene dosage (i.e., 0.05) (Table 2) and since in a

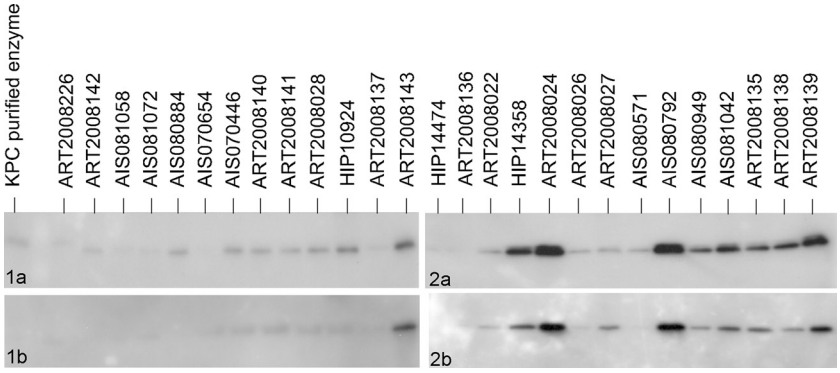


FIG. 2. Western blot analysis of KPC production within each of the 27 isolates. Results from blots 1a and 2a were repeated (seen below as blots 1b and 2b, respectively). Comparison of each blot was crucial in determining the relative level of KPC production notated in Table 2.

TABLE 3. Sequencing profiles of *ompK35* and *ompK36*

Carbapenem resistance category and isolate[f]	MIC (μg/ml) of:			OmpK35		OmpK36	
	IPM	MEM	ETP	Sequence accession no.[a]	Modification[b]	Sequence accession no.	Modification(s)
Low							
ART2008226	<1	<0.25	<0.5	FJ577672[c]	—	FJ577673	Thr192 del
ART2008142	<1	4	8	FJ577672[c]	—	FJ577673	—
AIS081058	1	4	2	AJ011501	—	BAH64431	—
AIS070654	4	1	2	FJ577672[c]	—	FJ577673	—
AIS081072	2	2	8	FJ577672[c]	—	FJ577673	—
AIS080884	2	4	8	AJ011501	Glu132Lys	BAH64431	Ser255Thr
ART2008140	4	2	8	FJ577672[c]	—	FJ577673	—
AIS070446	4	4	4	FJ577672[c]	—	FJ577673	—
HIP10924	4	4	4	ADG27478	—	ACI07318	Asp91Asn, Tyr198Phe, Leu311Ile
ART2008028	4	8	8	AJ011501	—	FJ577675	Gly136 and Asp137 del, Ala305Pro
ART2008137	4	8	16	FJ577672[c]	—	FJ577673	—
Intermediate							
HIP14474	8	8	16	FJ577672[c]	—	FJ577673	—
ART2008136	8	8	16	FJ577672[c]	—	FJ577673	—
High							
ART2008022	8	16	32	FJ577672[c]	—	FJ577673	—
ART2008139	32	16	16	FJ577672[c]	—	FJ577673	—
ART2008143	8	16	64	FJ577672[c]	—	FJ577673	—
AIS080949	32	16	64	FJ577672[c]	—	FJ577673	—
HIP14358	32	16	64	FJ577672[c]	—	FJ577673	—
AIS080792	32	32	64	FJ577672[c]	—	FJ577673	—
ART2008138	32	32	128	FJ577672[c]	—	NA[d]	ND[e]
ART2008024	32	64	128	AJ011501	—	BAH64431	135 and 136 ins Asp
ART2008026	64	64	256	FJ577672[c]	—	FJ577673	—
AIS081042	64	128	256	FJ577672[c]	—	FJ577673	135 and 136 ins GlyAsp
AIS080571	128	128	256	FJ577672[c]	—	NA	ND
ART2008141	256	256	256	FJ577672[c]	—	FJ577673	Thr261fsX
ART2008135	512	256	1,024	FJ577672[c]	—	FJ577673	Thr126fsX
ART2008027	512	512	1,024	FJ577672[c]	—	FJ577673	Glu78fsX

[a] Accession numbers indicate most similar reference sequences in GenBank.
[b] Predicted translational modifications, based on nucleotide sequencing data, that deviate from the sequence of the listed accession number. —, no modification; del, deletion; ins, insertion of; fsX, frameshift resulting in a premature stop codon.
[c] Previously described sequence with a frameshift that ultimately results in a stop codon after the amino acid 88 codon.
[d] NA, unable to generate a quality PCR product for sequencing despite multiple attempts.
[e] ND, not determined.
[f] Listing of isolates is as for Table 2.

repeat experiment bla_{KPC} could not be detected. With the exception of this isolate, the observed upstream deletions corresponded with increased KPC production, suggesting that bla_{KPC} expression is driven by a second upstream promoter.

Porin analysis. Genes encoding the two major porins, *ompK35* and *ompK36*, were sequenced. Most of the isolates ($n = 22$ out of 27) contained *ompK35* genes that had a sequence similar to that of GenBank accession number FJ577672 (Table 3). This previously described *ompK35* sequence has a G insertion after base pair 122 (in relation to the start of translation), resulting in an early frameshift and a premature stop codon following the amino acid 88 codon, producing a truncated protein that was nonfunctional (14). Specifically, genes with this sequence were found in 22 of the 23 ST 258-like isolates from this study, suggesting that this may be a common characteristic of ST 258 strains. Even though most isolates in this study appear to lack a functional OmpK35, previous reports suggest that OmpK36 likely plays a larger role in carbapenem resistance (8, 14).

DNA sequence analysis of *ompK36* could not be performed in two isolates (ART2008138 and AIS080571) because quality PCR products could not be generated after multiple attempts; this may be attributed to unknown sequence modifications that interfered with primer binding. The majority of the other 25 isolates ($n = 15$) were found to possess *ompK36* genes that were identical in sequence to a previously described *ompK36* (GenBank accession no. FJ577673), which previously produced a functional protein (14). These 15 isolates had carbapenem MICs that spanned low, intermediate, and high levels of carbapenem resistance (Table 3). Four isolates with low-level carbapenem resistance and two isolates with high-level resistance also had OmpK36 sequences that contained modifications additional to those previously reported (e.g., single amino acid substitutions, insertions, or deletions) (Table 3). The significance of these modifications is unclear, and their impact on OmpK36 porin function is unknown. The three isolates that produced the highest carbapenem MICs (i.e., ≥256 μg/ml for each of the carbapenems) (ART2008141, ART2008135, and ART2008027) each contained *ompK36* modifications that resulted in a frameshift (starting after amino acids 261, 126, and 78, respectively) and ultimately produced a premature stop codon (Table 3). The resulting truncated OmpK36 proteins

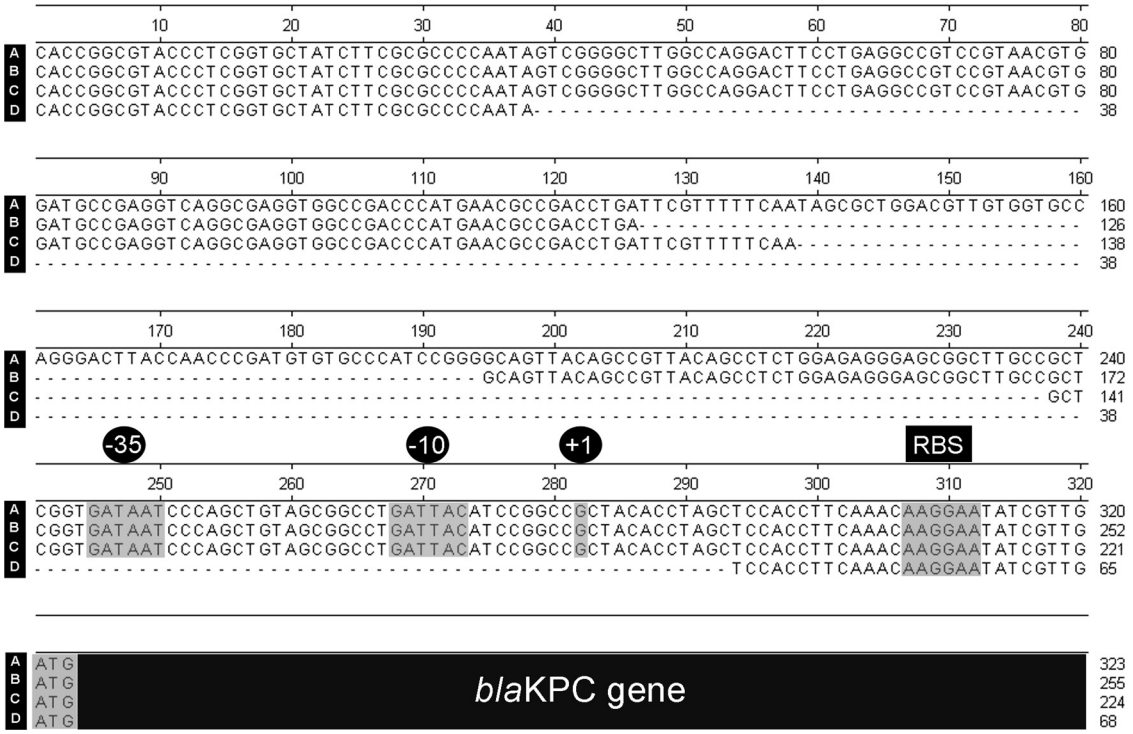


FIG. 3. Nucleotide sequence illustrating observed variations in the genetic environment directly upstream of the bla_{KPC} gene (seen in black at the bottom). Highlighted regions include the putative −35 and −10 regions of the promoter, the transcription start site (marked as +1), the potential ribosome-binding site (RBS), and the bla_{KPC} start codon (ATG), as previously reported by Yigit et al. (28). Sequences of isolates with no deletion (A), a 68-bp deletion (B), a 100-bp deletion (C), and a 255-bp deletion (D) were observed in this study.

would likely be nonfunctional (8, 25). The potential loss of functional OmpK35 and OmpK36 porins in these three isolates likely contributes in part to their high level of carbapenem resistance (Table 3).

Isolates with low-level carbapenem resistance. Western blot analysis of the 11 isolates with low-level carbapenem resistance revealed relatively low-level KPC production for all of these isolates (i.e., ≤2 pluses in Table 2). Porin sequence analysis identified seven of these isolates with critically modified *ompK35* genes (Table 3); however, all *ompK36* genes in these isolates appear to lack significant modifications. One isolate with very low level carbapenem resistance, ART2008226, completely lost its bla_{KPC-3}-carrying plasmid as described above. Evidence of unstable bla_{KPC}-carrying plasmids may also be observed in other isolates with low-, intermediate-, and high-level carbapenem resistance (e.g., <0.2 relative bla_{KPC} gene dosage) (Table 2). Gene dosage studies for each of the isolates with low-level carbapenem resistance did not detect any significant elevation in bla_{KPC} copy number relative to those of the other study isolates (e.g., >0.8) (Table 2). Also, all isolates with low-level carbapenem resistance were found to have the Tn*4401b* isoform (containing no deletions upstream of the bla_{KPC} gene). The lack of these two genetic factors in the 11 isolates with low-level carbapenem resistance supports our hypothesis that both elevated bla_{KPC} copy number and deletions upstream of the bla_{KPC} gene contribute to increased KPC production.

Isolates with high-level carbapenem resistance. Ten of the 14 isolates with high-level carbapenem resistance exhibited elevated KPC production (i.e., ≥4 pluses in Table 2) and had either an elevated relative bla_{KPC} copy number ($n = 3$) or deletions directly upstream of the bla_{KPC} gene ($n = 7$). Seven of these 10 isolates with elevated KPC production (excluding ART2008138, ART2008135, and ART2008027) contained *ompK36* genes that have either been previously shown to be functional (14) or appear to lack significant modifications (Table 3), suggesting that high-level carbapenem resistance can be achieved without OmpK36 porin loss. However, when critical *ompK36* modifications were observed in isolates with elevated KPC production (i.e., ART2008135 and ART2008027), the level of carbapenem MICs incrementally increased. Four isolates with high-level carbapenem resistance (ART2008022, ART2008026, AIS080571, and ART2008141) did not exhibit an elevated bla_{KPC} copy number, an upstream deletion, or increased KPC production (Table 2). The high-level carbapenem resistance in these four isolates may likely be attributable to identified porin modifications (Table 3) or other factors not examined in this study. For example, ART2008026 had high-level carbapenem resistance and contained an *ompK36* sequence previously shown to encode a functional porin (GenBank accession no. FJ577673) but lacked upstream deletions, increased bla_{KPC} copy number, and elevated KPC production.

In conclusion, our results suggest that both bla_{KPC} copy number and deletions in the upstream genetic environment may affect the level of KPC production and thus contribute to high-level carbapenem resistance in KPC-producing *K. pneumoniae.* In addition, the combination of OmpK36 porin loss with elevated KPC production appears to contribute to incre-

mental carbapenem MIC increases. These results indicate that KPC-positive isolates susceptible to carbapenems but with elevated carbapenem MICs may have multiple pathways to achieve high-level carbapenem resistance. Also, the identification of plasmid-based factors that elevate carbapenem MICs suggests that KPC-producing isolates with high-level carbapenem resistance may become more common as plasmids continue to disseminate within the *Enterobacteriaceae* population. Our analysis confirms the complexity of the carbapenem resistance phenotype and highlights the threat this continues to present to patients.

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