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bla_{KPC} RNA Expression Correlates with Two Transcriptional Start Sites but Not Always with Gene Copy Number in Four Genera of Gram-Negative Pathogens▽

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***Klebsiella pneumoniae* carbapenemase (KPC)-producing organisms are therapeutically and diagnostically challenging. It is possible that bla_{KPC} gene expression plays a role in the variability observed in clinical susceptibility testing. bla_{KPC} transformants together with 10 clinical isolates representing four genera were evaluated for bla_{KPC} copy number and gene expression and correlated with β-lactam MIC data. The data suggest that mechanisms other than gene copy number and expression of bla_{KPC} contribute to variability in susceptibility when testing KPC-producing isolates.**

Infections with organisms that produce the *Klebsiella pneumoniae* carbapenemase (KPC) are associated with therapeutic failure and high mortality rates. Complicating patient treatment is the inability to accurately detect KPC producers in the clinical laboratory using the guidelines suggested for susceptibility testing. Little is known about the role gene expression plays in KPC-mediated resistance or how the level of expression may affect susceptibility testing. Hydrolysis of carbapenems by KPCs has been demonstrated *in vitro* (1, 21, 22); however, multiple studies have reported variability in susceptibility testing of KPC-producing clinical isolates (2, 3, 13, 22). Although variation in KPC enzyme production could contribute to the variability seen during susceptibility testing, it is difficult to assess the influence of the KPC β-lactamase on susceptibility testing using clinical isolates which may harbor multiple β-lactamases and may have alterations in outer membrane proteins (4, 7, 9, 10, 21, 24). Therefore, we used cloning and transformation to create a panel of four different genera of Gram-negative bacteria that expressed the bla_{KPC} gene without the influence of other resistance mechanisms. We evaluated the relationship between bla_{KPC} gene expression and copy number with β-lactam MICs among the transformants and compared bla_{KPC} gene expression and copy numbers among clinical isolates known to carry bla_{KPC}.

To construct transformants expressing bla_{KPC}, a 1,314-bp fragment including the bla_{KPC-2} structural gene and 363 bp of the adjacent upstream region was amplified by PCR and sequenced. The bla_{KPC-2} product was cloned into pCR2.1 (Invitrogen) and subcloned into the broad host vector pMP220 (20). *Escherichia coli* K-12 259, *K. pneumoniae* 23, *Enterobacter cloacae* ATCC 13047, and *Pseudomonas aeruginosa* PAO1 were transformed with pMP220-KPC-2 by electroporation (22). In addition, 10 KPC-producing clinical isolates were evaluated, including two isolates of *E. coli* (233 and 236), four isolates of *K. pneumoniae* (UMM3, V110984, HUH6, and HUH40), one *Enterobacter* sp. isolate (01MGH049), and three isolates of *P. aeruginosa* (PS5, PS17B, and PS28) (Table 1).

Isoelectric focusing revealed that these clinical isolates produced multiple β-lactamases, which included enzymes with pIs equivalent to those of OXA, SHV-5, SHV-2, and TEM-1 in addition to KPC (22). Antimicrobial susceptibility testing was performed using agar dilution and interpreted using 2010 CLSI breakpoints (5, 6). The impact of KPC production on susceptibility varied with respect to the drug and transformant analyzed (Table 1). When *K. pneumoniae* pMP220-KPC-2 is evaluated, the ceftazidime and cefepime MICs are interpreted to be in the susceptible range (5) using 2010 CLSI breakpoints. These data underscore the potential difficulty in detecting KPC-producing *K. pneumoniae* using phenotypic methodologies. As expected, β-lactam MICs varied among the clinical isolates.

Prior to evaluating bla_{KPC} gene expression, relative bla_{KPC} copy number was determined (19). For the KPC-2 transformants, the copy number of the gene varied among the different genetic backgrounds despite the use of the same shuttle vector, pMP220. The copy numbers for bla_{KPC} in the transformants ranged from 2 to 26 (Table 1). Nine of 10 clinical isolates had one or two copies of bla_{KPC}, with the exception of *K. pneumoniae* HUH40, which had five copies of bla_{KPC}.

Increases in gene copy number have been correlated with increases in β-lactamase gene expression and increases in β-lactam MICs (15). To determine whether bla_{KPC} copy number correlated with bla_{KPC} gene expression, the relative expression of each bla_{KPC} transcript was evaluated for the clinical isolates and compared to the expression observed for the KPC-2 transformants using real-time reverse transcription (RT)-PCR (12, 15, 18). The clinical isolates of *E. coli* and *P. aeruginosa* and three *K. pneumoniae* isolates (UMM3, V110984, and HUH6) all had bla_{KPC-2} expression levels lower than their respective KPC-2 transformants. Surprisingly, a 1:1 ratio between copy number and bla_{KPC} expression was not

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TABLE 1. Comparisons of bla_{KPC} copy numbers, RNA expression, and β-lactam susceptibilities among transformants and clinical isolates[a]

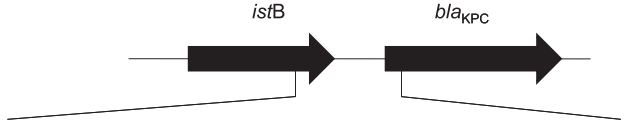
Strain[c]	KPC isoenzyme	Isoelectric focusing pI(s)	β-Lactam MIC (μg/ml)[b]					bla_{KPC} copy no.	bla_{KPC} RNA fold change[d]
			IPM	ERT	FEP	CAZ	CRO		
E. cloacae	None	ND	0.12	0.06	0.06	1	4	NA	NA
E. cloacae pMP220-KPC-2	KPC-2	ND	2	2	4	16	32	2	1
01MGH049	KPC-2	5.4, 6.9, 9.2	4	8	8	16	512	1	1
K. pneumoniae	None	ND	0.12	0.007	0.06	0.06	0.007	NA	NA
K. pneumoniae pMP220-KPC-2	KPC-2	ND	8	4	4	4	32	26	1
UMM3	KPC-2	5.4, 6.7	2	4	8	16	32	1	8↓
V110984	KPC-2	5.4, 6.9, 7.6, 8.2	1	8	8	512	64	2	3↓
HUH6	KPC-3	5.4, 6.9, 7.6, 8.2	0.5	4	8	256	64	1	10↓
HUH40	KPC-3	5.4, 6.9, 7.6, 8.2	0.5	4	16	512	128	5	5↓
E. coli	None	ND	0.12	0.007	0.03	0.12	0.015	NA	NA
E. coli pMP220-KPC-2	KPC-2	ND	8	4	8	16	64	13	1
233	KPC-3	5.4, 6.7, 7.6	8	8	32	256	512	1	2↓
236	KPC-3	5.4, 6.7, 6.9, 7.4	4	2	8	32	512	2	2↓
P. aeruginosa	None	ND	2	16	1	1	32	NA	NA
P. aeruginosa pMP220-KPC-2	KPC-2	ND	32	>512	>512	64	>512	9	1
PS5	KPC-2	6.7, ≥8.5	512	>512	256	64	>512	2	1
PS17B	KPC-2	6.7, ≥8.5	256	>512	256	64	>512	1	1
PS28	KPC-5	7.65, ≥8.5	128	256	128	256	>512	1	3↓

[a] ND, not determined; NA, not applicable.
[b] IPM, imipenem; ERT, ertapenem; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone.
[c] Strains: *E. coli* (K-12 259), *K. pneumoniae* (KP23), *E. cloacae* (ATCC 13047), *P. aeruginosa* (PAO1).
[d] Numbers next to arrows indicate a fold decrease in RNA expression of the clinical isolate compared to its genus-specific pMP220-KPC-2 transformant.

observed except for the *E. cloacae* KPC-2 transformant and 01MGH049 and the *K. pneumoniae* KPC-2 transformant and HUH40 (Table 1).

Due to the discrepancies between gene copy numbers and expression, it was possible that the entire promoter responsible for bla_{KPC} expression was not present in the transformants. Therefore, to map the bla_{KPC} promoter region, the start sites of transcription were determined for nine of the clinical isolates and compared to the start sites for the KPC-2 transformants using 5′ RACE (rapid amplification of cDNA ends) (22). These data revealed that the transcriptional start sites for the four transformants and eight of the clinical isolates were identical, indicating that the entire bla_{KPC} promoter was present in each transformant. The primary start site identified for all of the transformants and eight of the clinical isolates was an adenine residue 286 bp upstream of the translational start codon (Fig. 1). This start site did not correspond to the previously mapped start site observed by Yigit et al. or the start sites predicted by Gootz et al. (8, 23). The data presented here mapped the putative −10 and −35 promoter elements as TATCTT and TTGACA, respectively, separated by a 19-bp spacer, which correlated with σ^{70} consensus promoter elements found for *E. coli* (11, 14). A second start site not previously identified was mapped to a thymine residue 140 bp upstream of the translational start codon with putative −10 and −35 promoter elements of TACCAA and TGGAC. A 68-bp deletion in the upstream region of *K. pneumoniae* V110984 resulted in a start site of transcription with putative −10 and −35 elements that are less than consensus to those for σ^{70} of *E. coli*. Only one start site was observed for bla_{KPC-5}, which suggests a different type of transcriptional regulation (22). Taken together, these data demonstrate the level of complexity by which bla_{KPC} is regulated and could represent a means of differential expression under different selective pressures.

This study demonstrated the contribution of KPC-2 to β-lactam susceptibility in four different genera. The gene copy numbers or bla_{KPC} expression levels evaluated in the absence of selective pressure could not explain the variation in susceptibility patterns in the KPC-2 transformants or clinical isolates. These data suggest that additional mechanisms aside from gene expression and copy number contribute to the variability in susceptibility when testing KPC-producing isolates. Typical experimental approaches to understand the underlying mechanisms associated with these organisms have been unsuccess-



agtgggtcagttttcagttggtg**TTGACA**ccggcgtaccctcggtgc**TATCTT**cgcgcccc**A**atagtcgggcttggccagga
cttcctgaggccgtccgtaacgtggatgccgaggtcaggcgaggtggccgacccatgaacgccgacctgattccgttttttcaataggcc
TGGACGttgtggtgccagggact**TACCAA**cccgatgTgtgcccatccgggcagttacagccgttacagcctctggagagg
gagcggcttgccgctcggtgataatcccagctgtagcggcctgattacatccggccgctacacctagctccaccttcaaaca*aggaat*
atcgttg*ATGTCACTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTCTCATGGCCGCTGGCTGGC*

FIG. 1. Genetic organization of the bla_{KPC-2} and bla_{KPC-3} upstream region and mapping of the transcriptional start sites. The nucleotides immediately upstream of the bla_{KPC-2} and bla_{KPC-3} translational start codon are shown. The putative −10 and −35 promoter elements are shown in boldface capital letters and labeled. The primary start site of transcription is an adenine residue (capitalized, in boldface). The 68 nucleotides that are deleted in *K. pneumoniae* strain V110984 are underlined. The putative ribosomal binding site is underlined and italicized. The structural gene sequence of bla_{KPC} is capitalized and italicized. The figure is not drawn to scale.

ful. Therefore, determining the mechanisms involved in the regulation of KPC production will be critical if we are to impact the clinical needs of patients infected by KPC-producing pathogens. It is imperative that we continue to evaluate the complexity of this resistance mechanism in order to circumvent its spread to susceptible patient populations.

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