



# Colistin Resistance in Carbapenem-Resistant *Klebsiella pneumoniae* Mediated by Chromosomal Integration of Plasmid DNA

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**ABSTRACT** Here we describe the spread of colistin resistance in clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* in Medellín, Colombia. Among 32 isolates collected between 2012 and 2014, 24 showed genetic alterations in *mgrB*. Nineteen isolates belonged to sequence type 512 (ST512) (or its single locus variant [SLV]) and harbored an 8.1-kb *hsdMSR* insertion corresponding to *ISKpn25*, indicating a clonal expansion of the resistant strain. The insertion region showed 100% identity to several plasmids, suggesting that the colistin resistance is mediated by chromosomal integration of plasmid DNA.

**KEYWORDS** *Klebsiella pneumoniae*, carbapenem resistance, colistin resistance, *mgrB*

Resistance to colistin in *Klebsiella pneumoniae* is frequently associated with the inactivation of MgrB, a small protein that acts as a negative regulator of the PhoP/PhoQ two-component system. The upregulation of this pathway leads to lipopolysaccharide (LPS) modifications by the addition of phosphoethanolamine, 4-amino-4-arabinose or 2-hydroxymyristate to lipid A (1), resulting in the overall decrease in negative charge and the decrease in affinity of colistin to its target (2). Genotypic analysis of the *mgrB* gene regulator among colistin-resistant isolates revealed a high degree of plasticity and genetic disruptions (3, 4). Complementation experiments with a cloned wild-type *mgrB* gene restored susceptibility to the drug and reduced *PhoQ* and *PmrK* expression in resistant isolates to colistin-susceptible levels, in support of the role of *mgrB* in colistin resistance (2–4).

Here we describe the emergence and clonal expansion of a colistin- and carbapenem-resistant *K. pneumoniae* strain in Medellín, Colombia, where a unique disruption of the *mgrB* gene was caused by a chromosomal insertion of *ISKpn25*.

In a study conducted in four referral centers between 2012 and 2014 in Medellín, Colombia, we found 32 patients infected with carbapenem- and colistin-resistant *K. pneumoniae*, of which 27 belonged to sequence type 512 (ST512) or its single locus variant (SLV) (Table 1). Genotyping the *mgrB* gene among the 32 *K. pneumoniae* isolates showed that 8 isolates carried the wild-type gene, 1 isolate had a stop codon, another isolate had a frameshift, and 3 isolates had insertion sequence (IS) insertions; whereas 19 isolates were negative for the PCR amplification of *mgrB* (all belonged to ST512 or its SLV) (Table 1). Three *mgrB*-PCR negative ST512 (*K. pneumoniae* carbapenemase [KPC]-3 positive) isolates and one colistin-susceptible ST512 *K. pneumoniae* (KPC-3 positive) isolate were then selected for whole-genome sequencing analysis. Genome sequencing was done using the platform NextSeq (Illumina). The quality of the readings (150-bp insert size) was assessed using FastQC software ([www](http://www)

Received 23 February 2017 Returned for modification 19 March 2017 Accepted 29 April 2017

Accepted manuscript posted online 15 May 2017

**Citation** Cienfuegos-Gallet AV, Chen L, Kreiswirth BN, Jiménez JN. 2017. Colistin resistance in carbapenem-resistant *Klebsiella pneumoniae* mediated by chromosomal integration of plasmid DNA. *Antimicrob Agents Chemother* 61:e00404-17. <https://doi.org/10.1128/AAC.00404-17>.

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**TABLE 1** Characteristics of colistin- and carbapenem-resistant *K. pneumoniae* isolates from Medellín, Colombia, 2012–2014<sup>a</sup>

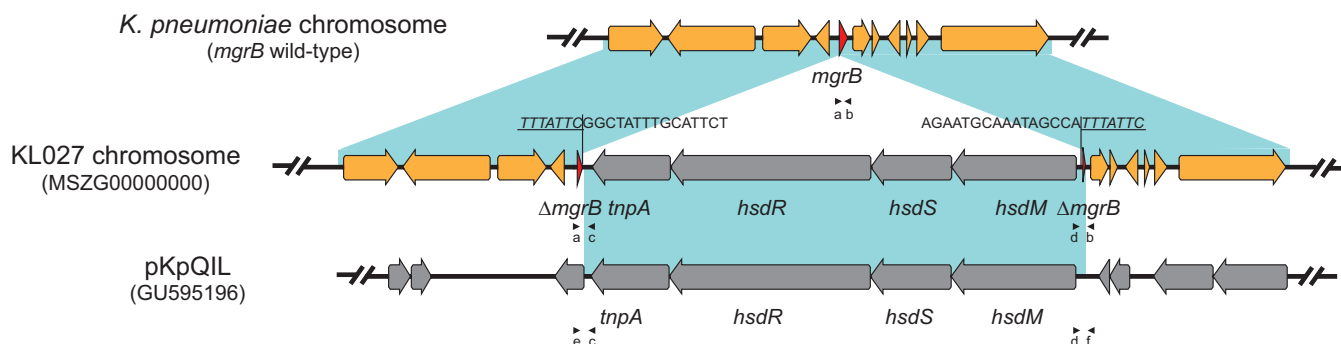
Isolate	Hospital	Date of sampling (month/year)	ST	KPC variant	<i>mgrB</i>	Co-resistance	Infection type
KL004	C	6/2012	512	KPC-3	<i>ISKpn25</i>	Amk-Cip	Urinary tract
KL008	C	7/2012	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Intraabdominal
KL011	C	8/2012	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Urinary tract
KL015	C	11/2012	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Urinary tract
KL026	C	3/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Urinary tract
KL027	C	3/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Surgical site
KL029	C	4/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Pneumonia
KL030	C	4/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip	Surgical site
KL038	C	7/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Bacteremia
KL041	C	8/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Bacteremia
KL043	C	10/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Intraabdominal
KL046	C	10/2013	512	KPC-3	<i>ISKpn25</i>	Gen-Cip-Tig	Bacteremia
KL050	C	11/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Urinary tract
KL051	C	12/2013	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Bacteremia
KL052	C	1/2014	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip	Bacteremia
KL053	C	1/2014	512	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Urinary tract
KL056	C	2/2014	512	KPC-2	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Urinary tract
KL061	C	5/2014	512	KPC-3	<i>ISKpn25</i>	Amk-Cip-Tig	Urinary tract
KL064	C	5/2014	868 <sup>b</sup>	KPC-3	<i>ISKpn25</i>	Amk-Gen-Cip-Tig	Intraabdominal
KP018	B	11/2012	512	KPC-3	Frameshift	Amk-Gen-Cip-Tig	Osteomyelitis
KP032	B	4/2013	14	KPC-2	Stop codon	Gen-Cip-Tig	Intraabdominal
KL014	C	11/2012	512	KPC-3	<i>IS5-like</i> (+74 nt)	Amk-Cip-Tig	Urinary tract
KP001	B	6/2012	512	KPC-3	<i>IS5-like</i> (+74 nt)	Amk-Cip-Tig	Urinary tract
KP008	B	9/2012	ND	Negative	<i>IS903</i> (+83 nt)	Cip-Tig	Intraabdominal
KH036	A	1/2014	512	KPC-3	WT	Amk-Gen-Cip-Tig	Urinary tract
KL016	C	11/2012	512	KPC-3	WT	Amk-Cip-Tig	Urinary tract
KL039	C	7/2013	512	KPC-3	WT	Amk-Cip	Bacteremia
KL045	C	10/2013	512	KPC-3	WT	Amk-Gen-Cip-Tig	Intraabdominal
KP007	B	8/2012	1708	KPC-2	WT	Ciip	Intraabdominal
KP011	B	9/2012	636	Negative	WT	Gen-Cip-Tig	Bacteremia
KP025	B	1/2013	1706	KPC-2	WT	Cip-Tig	Intraabdominal
KP028	B	12/2012	512	KPC-3	WT	Amk-Cip	Urinary tract

<sup>a</sup>ST, sequence type; WT, wild type; Amk, amikacin; Gen, gentamicin; Cip, ciprofloxacin; Tig, tigecycline.

<sup>b</sup>Single-locus variant of ST512.

.bioinformatics.babraham.ac.uk/projects/), and *de novo* assembly was done using SPAdes assembler (5). The plasmid replicons and resistance genes were mined using PlasmidFinder (6) and ResFinder (7), respectively. The sequence of *mgrB* and flanking regions reported in GenBank by Cannatelli (3) (GenBank accession number [AVFC01000053](#), 155,512-155,655; 253 bp) was used to locate the gene in the *de novo* assembly. The whole-genome sequences (WGS) of four *K. pneumoniae* isolates have been deposited into GenBank under Bioproject PRJNA353361. In addition, two sets of primers (junction 1: a-F 5'-GCTCAGCCACATCCTCTTTC-3' and c-R 5'-CTTGAAGTACCC CACAGGT-3', junction 2: d-F 5'-CCAGAAGCAGCTTTTGTCC-3' and b-R 5'-CCGCTCATT AATACGCCAAT-3') targeting the junctions between chromosomal and plasmid regions identified in the whole-genome sequencing analysis were designed to investigate the presence of this element. The PCR products for both junctions were sequenced in both the sense and anti-sense directions. pKpQIL-like plasmids were screened by PCR (8), and *bla*<sub>KPC</sub> plasmid identification was confirmed by S1-pulsed-field gel electrophoresis (PFGE) and *bla*<sub>KPC</sub>-probe hybridization in one strain (KL027).

Whole-genome sequence analysis of 3 (KL027, GenBank accession number [MSZG000000000](#); KL015, GenBank accession number [MSZL000000000](#); and KL064, GenBank accession number [MSZM000000000](#)) of the 19 PCR-negative *mgrB* isolates identified an 8.1-kb fragment insertion into the *mgrB* gene. No additional mutations were detected in other genes associated with colistin resistance (*phoPQ*, *pmrCAB*, and *crrAB*) when they were compared to a colistin-susceptible strain (KL033, GenBank accession number [MSZH000000000](#)). Analyses of the inserted region identified four open reading frames (ORFs), corresponding to predicted proteins from a type I restriction-methylation system (*hsdM*, *hsdS*, *hsdR*) and one corresponding to a transposase (*tnpA*).



**FIG 1** Schematic representation of the plasmid sequence (*ISKpn25*) inserted into *mgrB* in the KL027 *K. pneumoniae* chromosome and comparison of the inserted region to plasmid pKpQIL. Direct repeats are underlined and inverted repeats are in italic type. Primers amplifying *mgrB* (a b), the junctions between the chromosome and the 8.1-kb element (a c and d b), and the junctions between the plasmid backbone and the 8.1-kb element (e c and d f) are indicated by black arrowheads.

A BLAST search of the inserted region in ISfinder (9) revealed that this element corresponded to *ISKpn25*. Further PCR and Sanger sequencing, targeting the chromosome insertion junction, showed that all 19 *mgrB* “negative” isolates carried the same *ISKpn25* insertion. This insertion showed >99% identity and query coverage to a number of plasmids in GenBank, ranging in size from 48 to 307 kb, including the *bla*<sub>KPC</sub>-harboring plasmid pKpQIL which has been frequently identified in ST258 and ST512 isolates. In our study, pKpQIL-like plasmids were detected in 79% (15/19) of isolates harboring the *ISKpn25* insertion. The presence of plasmid-borne *ISKpn25* was further examined by two primers (junction 3: e-F: CCTACTGGATCCGTGTCGTT, c-R 5'-CTTGAAGTACCCACAGGT; junction 4: d-F 5'-CCAGAAGCAGCTTTTGTCC-3'; f-R: TTTCCCCTGGCATAAACAAC) targeting the junctions between the 8.1-kb *hsdMSR* locus and the plasmid backbone (Fig. 1). The results showed that all 15 isolates carrying pKpQIL-like plasmids harbored a second copy of *ISKpn25* on the plasmids. Moreover, S1-PFGE and *bla*<sub>KPC</sub>-hybridization to KL027 showed that *bla*<sub>KPC</sub> was present in a 113-kb plasmid, consistent with the size reported for pKpQIL. Plasmid analysis revealed sequences from incompatibility groups IncFII(K), IncFIB(K), IncFIB (pQIL), and IncX3 of plasmids pKpQIL, pKpn3, and plncX. In addition to KPC genes, other resistant genes were detected, including those for TEM, SHV, and OXA beta-lactamases; aminoglycoside-, fluoroquinolone-, and phenicol-modifying enzymes; and genes conferring resistance to fosfomycin and quinolones. It is clinically significant to note that the 19 isolates were all the same ST (or its SLV) and were recovered from the same hospital between June 2012 and May 2014; also, only one patient had a history of using colistin previously. Additionally, PFGE examination showed that these isolates had indistinguishable profiles, in support of the clonal expansion of this strain.

Insertional inactivation of *mgrB* gene has been shown to be a predominant cause of colistin resistance in *K. pneumoniae*. The role of IS elements in the inactivation of *mgrB* gene was described first by Cannatelli et al. (2) in one colistin resistant KPC-3 producing *K. pneumoniae* strain belonging to ST258 and selected *in vivo* after multiple rounds of antibiotic therapy, including colistin. The inactivation proved to be an insertion of an IS5-like element at nucleotide 75 in the *mgrB* gene, similar to two strains in our collection. Additional studies have confirmed the role of several IS sequences disrupting *mgrB* (IS5-like, *IS1F* and *ISKpn14*, *ISKpn13* and *IS10R*) in colistin resistance (3, 4).

In this study, we described the largest insertional inactivation of *mgrB* reported, which includes an 8.1-kb element corresponding to *ISKpn25*. Complementation experiments with the wild-type *mgrB* gene are still needed to confirm that this insertion and the disruption of *mgrB* is the cause of colistin resistance. However, both the current literature regarding IS-element disruptions of the *mgrB* gene among colistin-resistant strains and whole-genome sequencing failing to find any mutations in the numerous two-component regulatory genes associated with colistin resistance give support to

our hypothesis that colistin resistance is likely due to the *ISKpn25* insertion in the *mgrB* gene.

The *ISKpn25* region had an *hsdMSR* locus that encodes a type I restriction-modification (R-M) system. Most R-M systems are present in the bacterial accessory genome, and they are also frequently found associated with mobile elements, such as plasmids, phages, conjugative elements, transposons, and integrons (10). Our results showed that the *ISKpn25* is present in several plasmids, including *bla*<sub>KPC</sub>-harboring plasmids, such as pKpQIL. The presence of two copies of *ISKpn25* on the chromosome and the plasmid suggested that the chromosomal insertion of the *hsdMSR* locus in *mgrB* was likely acquired from the plasmid through transposition or homologous recombination. Similarly, it has been suggested *ISKpn25* is involved in DNA rearrangements among pKpQIL-like and possibly other plasmids (11). An additional study showed that a mobile element containing the carbapenemase OXA-181 disrupted the *mgrB* gene in a carbapenem- and colistin-resistant *K. pneumoniae* isolate from a patient from the United Arab Emirates (12). In the same isolate, a copy of a second element harboring CTX-M-15 (ISEcp1-*bla*<sub>CTX-M-15</sub>) was inserted into *ompK35*, inactivating the gene. These findings underscore the potential role of mobile elements associated with carbapenemase genes in the evolution of colistin-resistant clones.

In addition, the presence of the same insertion element in a cluster of 19 isolates from the same clonal group isolated from the same hospital support the hypothesis that this spread was the result of clonal expansion and that the inactivation of the *mgrB* gene was a unique event. Consistent with the clonal spread reported in this study, large outbreaks of colistin-resistant, KPC-3-harboring *K. pneumoniae* from ST258 and ST512 (SLV of ST258) were reported in Italy, Greece, and the United States (13, 14), and at least one of the outbreaks was caused by the clonal expansion of a mutant with a deletion of 11 bp in the *mgrB* coding sequence (15).

The repeated observation that the *mgrB* gene is a hot spot for insertions and that its loss is associated with colistin resistance, plus the recent finding that *mcr-1* genes that confer colistin resistance are plasmid encoded (16), raises significant clinical and public health issues in a background where the pipeline for new and novel antibiotics is limited. As a consequence, close monitoring of colistin- and carbapenem-resistant strains should be maintained to control their spread.

**Accession number(s).** Whole-genome sequence analysis of 3 PCR-negative *mgrB* isolates, KL027, KL015, and KL064, have been deposited at GenBank under the respective accession numbers [MSZG000000000](https://www.ncbi.nlm.nih.gov/nuccore/MSZG000000000), [MSZL000000000](https://www.ncbi.nlm.nih.gov/nuccore/MSZL000000000), and [MSZM000000000](https://www.ncbi.nlm.nih.gov/nuccore/MSZM000000000). A colistin-susceptible strain, KL033, has been deposited in GenBank under the accession number [MSZH000000000](https://www.ncbi.nlm.nih.gov/nuccore/MSZH000000000).

## ACKNOWLEDGMENTS

This work was supported by Colciencias project 111565741641 (to J.N.J.). This work was also supported by the National Institute of Allergy and Infectious Diseases (grants R01AI090155 [to B.N.K.] and R21AI117338 [to L.C.]).

We declare no competing interests.

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