AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

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

Astrid V. Cienfuegos-Gallet,[a] Liang Chen,[b] Barry N. Kreiswirth,[b] J. Natalia Jiménez[a]

Línea de Epidemiología Molecular Bacteriana, Grupo de Microbiología Básica y Aplicada, Universidad de Antioquia, Medellín, Colombia[a]; Public Health Research Institute TB Center, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, New Jersey, USA[b]

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 IS*Kpn25*, 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*

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.

Address correspondence to J. Natalia Jiménez, jnatalia.jimenez@udea.edu.co.

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 IS*Kpn25*.

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

TABLE 1 Characteristics of colistin- and carbapenem-resistant *K. pneumoniae* isolates from Medellín, Colombia, 2012–2014[a]

Isolate	Hospital	Date of sampling (month/year)	ST	KPC variant	*mgrB*	Coresistance	Infection type
KL004	C	6/2012	512	KPC-3	IS*Kpn25*	Amk-Cip	Urinary tract
KL008	C	7/2012	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Intraabdominal
KL011	C	8/2012	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Urinary tract
KL015	C	11/2012	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Urinary tract
KL026	C	3/2013	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Urinary tract
KL027	C	3/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Surgical site
KL029	C	4/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Pneumonia
KL030	C	4/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip	Surgical site
KL038	C	7/2013	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Bacteremia
KL041	C	8/2013	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Bacteremia
KL043	C	10/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Intraabdominal
KL046	C	10/2013	512	KPC-3	IS*Kpn25*	Gen-Cip-Tig	Bacteremia
KL050	C	11/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Urinary tract
KL051	C	12/2013	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Bacteremia
KL052	C	1/2014	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip	Bacteremia
KL053	C	1/2014	512	KPC-3	IS*Kpn25*	Amk-Gen-Cip-Tig	Urinary tract
KL056	C	2/2014	512	KPC-2	IS*Kpn25*	Amk-Gen-Cip-Tig	Urinary tract
KL061	C	5/2014	512	KPC-3	IS*Kpn25*	Amk-Cip-Tig	Urinary tract
KL064	C	5/2014	868[b]	KPC-3	IS*Kpn25*	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	IS*5-like* (+74 nt)	Amk-Cip-Tig	Urinary tract
KP001	B	6/2012	512	KPC-3	IS*5-like* (+74 nt)	Amk-Cip-Tig	Urinary tract
KP008	B	9/2012	ND	Negative	IS*903* (+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

[a]ST, sequence type; WT, wild type; Amk, amikacin; Gen, gentamicin; Cip, ciprofloxacin; Tig, tigecycline.
[b]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′-CTTGAAGTCACCC CACAGGT-3′, junction 2: d-F 5′-CCAGAAGCAGCTTTTTGTCC-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_{KPC} plasmid identification was confirmed by S1-pulsed-field gel electrophoresis (PFGE) and bla_{KPC}-probe hybridization in one strain (KL027).

Whole-genome sequence analysis of 3 (KL027, GenBank accession number MSZG00000000; KL015, GenBank accession number MSZL00000000; and KL064, GenBank accession number MSZM00000000) 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 MSZH00000000). 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*).

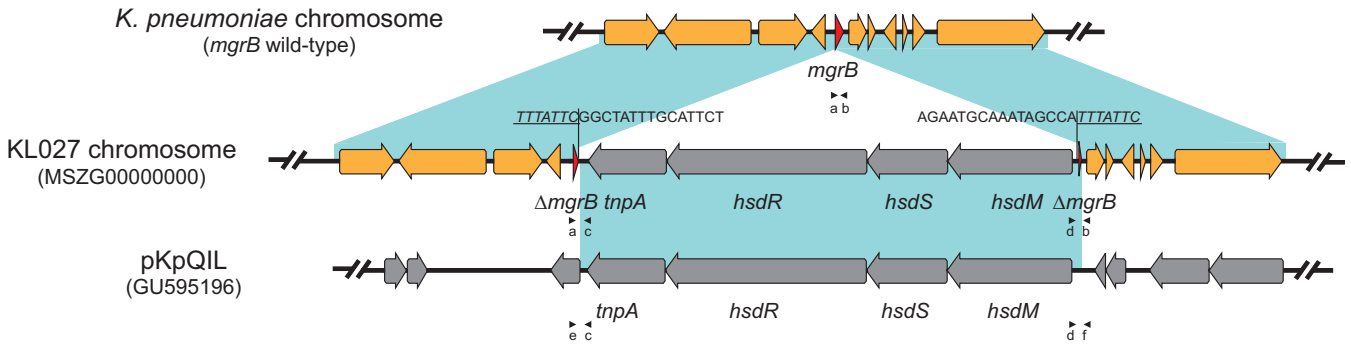

FIG 1 Schematic representation of the plasmid sequence (IS*Kpn25*) inserted into *mgrB* in the KL027 *K. pneumoniae* chromosome and comparison of the inserted region to plasmid pKpQIL. Direct repeats are underlined and inverted repeats are in italic type. Primers amplifying *mgrB* (a b), the junctions 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 IS*Kpn25*. Further PCR and Sanger sequencing, targeting the chromosome insertion junction, showed that all 19 *mgrB* "negative" isolates carried the same IS*Kpn25* 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_{KPC}-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 IS*Kpn25* insertion. The presence of plasmid-borne IS*Kpn25* was further examined by two primers (junction 3: e-F: CCTACTGGATCCGTGTCGTT, c-R 5′-CTTGAAGTCACCCCACAGGT; junction 4: d-F 5′-CCAGAAGCAGCTTTTTGTCC-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 IS*Kpn25* on the plasmids. Moreover, S1-PFGE and bla_{KPC}-hybridization to KL027 showed that bla_{KPC} 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 pIncX. 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 IS*5*-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* (IS*5*-like, IS*1F* and IS*Kpn14*, IS*Kpn13* and IS*10R*) 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 IS*Kpn25*. 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 IS*Kpn25* insertion in the *mgrB* gene.

The IS*Kpn25* 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 IS*Kpn25* is present in several plasmids, including bla_{KPC}-harboring plasmids, such as pKpQIL. The presence of two copies of IS*Kpn25* 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 IS*Kpn25* 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-$bla_{CTX\text{-}M\text{-}15}$) 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 MSZG00000000, MSZL00000000, and MSZM00000000. A colistin-susceptible strain, KL033, has been deposited in GenBank under the accession number MSZH00000000.

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.

REFERENCES

1. Kidd TJ, Mills G, Sá-Pessoa J, Dumigan A, Frank CG, Insua JL, Ingram R, Hobley L, Bengoechea JA. 2017. A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. EMBO Mol Med 9:430–447. https://doi.org/10.15252/emmm.201607336.
2. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother 57:5521–5526. https://doi.org/10.1128/AAC.01480-13.
3. Cannatelli A, Giani T, D'Andrea MM, Pilato Di V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini GM. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother 58:5696–5703. https://doi.org/10.1128/AAC.03110-14.
4. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The mgrB gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. J Antimicrob Chemother 70:75–80. https://doi.org/10.1093/jac/dku323.
5. Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A, Lapidus A, Prjibelski AD, Pyshkin A, Sirotkin A, Sirotkin Y, Stepanauskas R, Clingenpeel SR, Woyke T, McLean JS, Lasken R, Tesler G, Alekseyev MA, Pevzner PA. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. J Comput Biol 20:714–737. https://doi.org/10.1089/cmb.2013.0084.
6. Carattoli A, Zankari E, Garciá-Fernández A, Larsen MV, Lund O, Villa L,

Aarestrup FM, Hasman H. 2014. In silico detection and typing of plasmids using Plasmid Finder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895–3903. https://doi.org/10.1128/AAC.02412-14.24777092.
7. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644. https://doi.org/10.1093/jac/dks261.
8. Chen L, Chavda KD, Melano RG, Jacobs MR, Koll B, Hong T, Rojtman AD, Levi MH, Bonomo RA, Kreiswirtha BN. 2014. Comparative genomic analysis of KPC-encoding pKpQIL-like plasmids and their distribution in New Jersey and New York hospitals. Antimicrob Agents Chemother 58:2871–2877. https://doi.org/10.1128/AAC.00120-14.
9. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 2006:D32–D36. https://doi.org/10.1093/nar/gkj014.
10. Furuta Y, Kobayashi I. 2013. Restriction-modification systems as mobile epigenetic elements, p 1–19. *In* Roberts AP, Mullany P (ed), Bacterial integrative mobile genetic elements. Landes Bioscience, Austin, TX.
11. He S, Chandler M, Varani AM, Hickman AB, Dekker JP, Dyda F. 2016. Mechanisms of evolution in high-consequence drug resistance. MBio 2016:e01987-16.
12. Zowawi HM, Forde BM, Alfaresi M, Alzarouni A, Farahat Y, Chong T-M, Yin W-F, Chan K-G, Li J, Schembri MA, Beatson SA, Paterson DL. 2015. Stepwise evolution of pandrug-resistance in *Klebsiella pneumoniae*. Sci Rep 5:15082. https://doi.org/10.1038/srep15082.
13. Marchaim D, Chopra T, Pogue JM, Perez F, Hujer AM, Rudin S, Endimiani A, Navon-Venezia S, Hothi J, Slim J, Blunden C, Shango M, Lephart PR, Salimnia H, Reid D, Moshos J, Hafeez W, Bheemreddy S, Chen TY, Dhar S, Bonomo RA, Kaye KS. 2011. Outbreak of colistin-resistant, carbapenem-resistant *Klebsiella pneumoniae* in metropolitan Detroit, Michigan. Antimicrob Agents Chemother 55:593–599. https://doi.org/10.1128/AAC.01020-10.
14. Mezzatesta ML, Gona F, Caio C, Petrolito V, Sciortino D, Sciacca A, Santangelo C, Stefani S. 2011. Outbreak of KPC-3-producing, and colistin-resistant, *Klebsiella pneumoniae* infections in two Sicilian hospitals. Clin Microbiol Infect 17:1444–1447. https://doi.org/10.1111/j.1469-0691.2011.03572.x.
15. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, De Angelis LH, Fornaini R, Grazzini M, Niccolini F, Pecile P, Rossolini GM. 2015. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to clonal expansion of an *mgrb* deletion mutant. J Clin Microbiol 53:3341–3344. https://doi.org/10.1128/JCM.01017-15.
16. Liu Y, Wang Y, Walsh TR, Yi L, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Park H. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 16:161–168. https://doi.org/10.1016/S1473-3099(15)00424-7.