In Vivo Emergence of Colistin Resistance in *Klebsiella pneumoniae* Producing KPC-Type Carbapenemases Mediated by Insertional Inactivation of the PhoQ/PhoP *mgrB* Regulator

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Colistin is one of the few agents that retain activity against extensively drug-resistant strains of *Klebsiella pneumoniae* producing KPC-type carbapenemases (KPC-KP). However, resistance to colistin is increasingly reported among KPC-KP. Comparative genomic analysis of a pair of sequential KPC-KP isolates from the same patient including a colistin-susceptible isolate (KKBO-1) and a colistin-resistant isolate (KKBO-4) selected after colistin exposure revealed that insertional inactivation of the *mgrB* gene, encoding a negative regulator of the PhoQ/PhoP signaling system, is a genetic mechanism for acquired colistin resistance. The role of *mgrB* inactivation in acquired colistin resistance was confirmed by complementation experiments with wild-type *mgrB*, which restored colistin susceptibility in KKBO-4, and by construction of an *mgrB* deletion mutant from KKBO-1, which exhibited a colistin-resistant phenotype. Insertional *mgrB* inactivation was also detected in 60% of colistin-resistant mutants selected from KKBO-1 *in vitro*, following plating on colistin-containing medium, confirming the role (although not unique) of this mechanism in the emergence of acquired colistin resistance. In colistin-resistant mutants carrying insertional inactivation or deletion of the *mgrB* gene, upregulated transcription of *phoP*, *phoQ*, and *pmrK* (which is part of the *pmrHFIJKLM* operon) was detected. These findings confirmed the MgrB regulatory role in *K. pneumoniae* and were in agreement with the known association between upregulation of the PhoQ/PhoP system and activation of the *pmrHFIJKLM* operon, which eventually leads to resistance to polymyxins by modification of the lipopolysaccharide target.

Klebsiella pneumoniae strains producing KPC-type carbapenemases (KPC-KP) have become a major clinical challenge. Emerged in the late 1990s (1), KPC-KP strains have undergone a remarkable epidemic dissemination in health care settings, with large outbreaks reported in the Americas, Europe, and Asia (2–4). In some areas (e.g., some U.S. states, Colombia, Israel, Greece, Italy, and some provinces of China), KPC-KP have become endemic and represent a significant proportion of *K. pneumoniae* isolates (2, 5).

Infections caused by KPC-KP are typically difficult to treat, due to the extended antibiotic resistance phenotypes of these strains, and are associated with high mortality rates (6). Polymyxins (polymyxin B and colistin) are among the few antimicrobials that retain activity against KPC-KP and represent a key component of anti-KPC-KP antimicrobial regimens (7–9). However, the emergence of colistin-resistant KPC-KP has been repeatedly reported, and in some areas, the problem has achieved remarkable proportions (5, 10–13).

In *K. pneumoniae*, resistance to polymyxins and other cationic antimicrobial peptides is known to be mediated by modification of the lipopolysaccharide (LPS) following the addition of 4-amino-4-deoxy-L-arabinose to lipid A, which decreases the affinity of polymyxins to the LPS target (14). This modification is carried out by the products of the *pmrHFIJKLM* (also named *pbgPE* or *arn*) operon, which is conserved among *Enterobacteriaceae* and is positively regulated by the PhoQ/PhoP and PmrAB signaling systems (15–17), which are responsive to various environmental parameters including low pH, cationic antimicrobial peptides, and the concentration of some cations (17–21).

MgrB is a small regulatory transmembrane protein whose production is induced upon activation of the PhoQ/PhoP signaling system and which exerts a negative feedback on the same system by direct interaction with the PhoQ sensor kinase at the periplasmic level (22). This regulatory function of MgrB has been demonstrated in *Escherichia coli*, *Salmonella enterica*, and *Yersinia pestis*, but the remarkable conservation of this protein among *Enterobacteriaceae* suggests that its function might be conserved also in other species, including *K. pneumoniae* (22).

In this work, we demonstrate that insertional inactivation of the *mgrB* gene in *K. pneumoniae* leads to upregulation of the PhoQ/PhoP system and of the *pmrHFIJKLM* operon, being a genetic mechanism responsible for the emergence of colistin resistance in clinical isolates of KPC-KP, following colistin exposure.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. *K. pneumoniae* strains investigated in this work included a pair of sequential clinical isolates of KPC-KP obtained from blood cultures of an inpatient admitted to the intensive care unit (ICU) of St. Orsola University Hospital of Bologna, Italy. The

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TABLE 1 List of primers and PCR conditions used in this work

Type of reaction and primer name	Sequence (5'–3') ^a	Cycling conditions (temp/time) ^b
Conventional PCR		
EcoRI_mgrB_F	<u>GAATTC</u> CACCACCTCAAAGAGAAGGCGTTC	95°C/30 s–62°C/30 s–72°C/30 s
AvaI_mgrB_R	CTCGGGAACACGTTTTGAAACAAGTCGATGATTC	
KOmgrB1_XbaI_F ^c	<u>TCTAGAT</u> CTTATGATGCACACCTGTCGGGCACC	
KOmgrB1_XhoI_R	<u>CTCGAGG</u> ACATTTTCTGCCGACTGATTTCTTCTGCGC	
KOmgrB2_XhoI_F	<u>CTCGAG</u> ACTCTCCGCATCAGTACGCAATGTCGCCAG	95°C/30 s–69°C/30 s–72°C/30 s
KOmgrB2_BglII_R ^c	<u>AGATCT</u> CCGTTCTGAGGCGGCGGATACCGGGTTG	
mgrB_ext_F ^d	AAGGCGTTCATTCTACCACC	95°C/30 s–54°C/30 s–72°C/105 s
mgrB_ext_R	TTAAGAAGGCCGTGCTATCC	
pKOV_F ^d	CCATGACGCTCAAGCAGTTGTGCC	95°C/30 s–54°C/30 s–72°C/105 s
qRT-PCR		
phoP_F	ATTGAAGAGGTTGCCGCCCGC	95°C/1 s–52°C/5 s–72°C/7 s
phoP_R	GCTTGATCGGCTGGTCATTACCC	
phoQ_F	ATATGCTGGCGAGATGGGAAAACGG	95°C/1 s–52°C/5 s–72°C/7 s
phoQ_R	CCAGCCAGGGAACATCACGCT	
pmrK_FT	GCGGGCCATCAGGATCGACAGCG	95°C/1 s–65°C/5 s–72°C/7 s
pmrK_RT	CGTTCTGTTACTACATCCCGTTCTCTGA	
rpsL13_F	GCCGTACTTGGAGCGAGCCTG	95°C/1 s–52°C/5 s–72°C/7 s
rpsL14_F	CCGTGGCGGTCGTGTTAAAGA	

^a Restriction sites added for cloning purposes in some primers are underlined.

^b All conventional PCRs included an initial denaturation step of 180 s at 95°C, 30 cycles of denaturation, annealing, and extension at the reported temperatures for the reported times, and a final extension step of 300 s at 72°C; all qRT-PCRs included an initial denaturation step of 30 s at 95°C, 35 cycles of denaturation, annealing, and extension at the reported temperatures for the reported times.

^c For conventional PCR with primers KOmgrB1_XbaI_F and KOmgrB2_BglII_R, the cycling conditions were 95°C for 30 s, 67°C for 30 s, and 72°C for 30 s.

^d For conventional PCR with primers pKOV_F and mgrB_ext_F, the cycling conditions were 95°C for 30 s, 54°C for 30 s, and 72°C for 105 s.

first isolate (KKBO-1) was susceptible to colistin; the second isolate (KKBO-4) was resistant to colistin and was isolated 30 days after KKBO-1. In the intervening period, the patient had received multiple antibiotics including tigecycline, gentamicin, meropenem, and colistin, the last both intravenously and aerosolized. KKBO-4 was isolated after the patient had received aerosolized colistin for 25 days and intravenous colistin for 14 days. Multilocus sequence typing (MLST) of the *K. pneumoniae* isolates was carried out as previously described (23), and sequence type (ST) was assigned according to the *K. pneumoniae* MLST database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>). Pulsed-field gel electrophoresis (PFGE) profiling of genomic DNA of *K. pneumoniae* isolates was carried out as previously described (24). *Escherichia coli* DH5α (25) was used as the host for recombinant plasmids. Plasmid pACYC184 (26) was used as a genetic vector for complementation experiments. Plasmid pKOV (Addgene, Cambridge, MA, USA), which has a temperature-sensitive pSC101 replication origin, was used for the gene knockout experiment.

Antimicrobial susceptibility testing. MICs of colistin were determined by Etest (bio-Mérieux, Marcy l'Etoile, France) according to the manufacturer's instructions and interpreted as indicated by the EUCAST guidelines (EUCAST breakpoint tables for interpretation of MICs and zone diameters, version 3.1, 2013; <http://www.eucast.org>). *E. coli* ATCC 25922 (colistin susceptible) and *Acinetobacter baumannii* N50 (colistin resistant) (27) were used as control strains for antimicrobial susceptibility testing.

HTGS and analysis. KKBO-1 and KKBO-4 were subjected to high-throughput genome sequencing (HTGS) at an external facility (IGA Technology Services, Udine, Italy), using the HiSeq 2000 Illumina platform (Illumina Inc., San Diego, CA, USA) and a paired-end protocol with an average insert size of 350 bp. Reads were assembled into contigs using the ABySS *De novo* assembler (28). GGDC software (29) was used to assess genomic diversity. The RAST web server (30) was employed for the annotation of the obtained contigs. The chromosome of *K. pneumoniae* HS11286 (31), belonging to ST11, a single-locus variant (SLV) of ST258,

was used as a comparator genome because it was the most similar complete *K. pneumoniae* genome publicly available. Differences in chromosomally carried genes of KKBO-1 and KKBO-4 were identified by comparison of the contigs obtained with ABySS to the genome of HS11286 (NC_016845.1) by using BLAST+ (32). Genes that, following this analysis, were found to be different between KKBO-1 and KKBO-4 ($n = 73$) were further filtered out to remove coding sequences for which the differences were likely due to alignment or sequencing errors, as previously described (33). The criteria used in this filtering step were as follows: (i) removal of genes annotated as phage related, insertion sequences, or encoding transposases; (ii) removal of genes that were present in more than one copy in the HS11286 genome; and (iii) removal of genes with degenerate bases in their sequence.

Recombinant DNA methodology. Plasmid DNA was purified as previously described (25). Genomic DNA was purified by the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany). DNA sequences were determined on both strands at an external sequencing facility (MacroGen Inc., Seoul, South Korea). Electroporation of plasmids into *E. coli* was carried out as previously described (25), and the same procedure was performed also for *K. pneumoniae*.

Complementation experiments. For complementation experiments, plasmid pACYC-mgrB was used. The plasmid is a pACYC184 derivative carrying a cloned copy of wild-type *mgrB* gene and some flanks, amplified by PCR using primers EcoRI_mgrB_F and AvaI_mgrB_R (Table 1) and the genomic DNA of strain KKBO-1 as the template. The amplicon was digested with EcoRI and AvaI and cloned into pACYC184 restricted with the same enzymes. The authenticity of the cloned fragment was confirmed by sequencing. The pACYC-mgrB plasmid and pACYC184 (as a control) were introduced into *K. pneumoniae* strains by electroporation. Transformants were selected on Mueller-Hinton agar plates supplemented with 40 μg/ml of tetracycline.

Gene disruption experiment. Chromosomal deletion of *mgrB* in strain KKBO-1 was obtained using the following procedure. PCR amplicons corresponding to the regions upstream and downstream of the

FIG 1 Construction of the *mgrB* deletion mutant of *K. pneumoniae* KKBO-1 by using plasmid pKOV via homologous recombination. (a) Outline of construction of plasmid pKOVΔ*mgrB*. The positions of primers used for amplification of the flanking regions (KOMgrB1_XbaI_F and KOMgrB1_XhoI_R; KOMgrB2_XhoI_F and KOMgrB2_BglII_R) are indicated by thin black arrows. (b) Chromosomal integration of pKOVΔ*mgrB* in *K. pneumoniae* KKBO-1 followed by excision of plasmid and generation of *K. pneumoniae* ΔKKBO-1, carrying a deletion of *mgrB*. Open reading frames are indicated by arrows. Black backbone lines represent chromosomal regions; dashed backbone lines represent plasmid regions. Gene identifications (IDs) refer to the *K. pneumoniae* HS11286 chromosome (31).

mgrB locus, generated using primers KOMgrB1_XbaI_F and KOMgrB1_XhoI_R (upstream region) and KOMgrB2_XhoI_F and KOMgrB2_BglII_R (downstream region) (Table 1), were digested with XhoI, ligated, and further amplified by PCR as a single fragment using primers KOMgrB1_XbaI_F and KOMgrB2_BglII_R. The corresponding amplicon was digested with XbaI and BglII and cloned into the pKOV vector digested with the same enzymes to obtain plasmid pKOVΔ*mgrB* (Fig. 1a). The authenticity of the cloned fragment was confirmed by sequencing. This plasmid was then introduced by electroporation into KKBO-1, and transformants were selected on LB agar plates containing chloramphenicol (125 µg/ml) at 30°C. To obtain plasmid integration into the chromosome (Fig. 1b), KKBO-1(pKOVΔ*mgrB*) was grown in LB broth supplemented with chloramphenicol (400 µg/ml) at 43°C for approximately 30 generations. The integration events were confirmed by PCR using primers pKOV_F and mgrB_ext_F (Table 1). Four randomly selected integrants were then inoculated in LB broth and grown at 30°C for approximately 50 generations, to promote plasmid excision by homologous recombination (Fig. 1b). Plasmid excision was confirmed by replica plating of colonies onto LB agar supplemented with chloramphenicol (400 µg/ml). The colonies that did not grow on that medium were then analyzed by PCR using primers flanking *mgrB* (mgrB_Ext_F and mgrB_Ext_R [Table 1]), in combination with amplicon sequencing, to confirm that correct excision of the plasmid and the expected deletion of *mgrB* had occurred.

Transcriptional analysis by qRT-PCR. Quantitative real-time PCR (qRT-PCR) was used to measure the expression of the *phoP*, *phoQ*, and

pmrK genes, using the *phoP*_F and *phoP*_R, *phoQ*_F and *phoQ*_R, and *pmrK*_FT and *pmrK*_RT primers, respectively (Table 1). RNA and cDNA preparations were obtained using the SV Total RNA Isolation kit (Promega, Madison, USA) and the Improm-II Reverse Transcriptase kit (Promega) according to the manufacturer's instructions. qRT-PCR was carried out using a LightCycler instrument (Roche Applied Science, Mannheim, Germany). Expression of the *rpsL* gene, evaluated by primers *rpsL*13_F and *rpsL*14_R (Table 1), was used as an internal standard, and DNA Master Sybr green (Roche) was used as a signal reporter. Normalization was performed against the *rpsL* gene using the $\Delta\Delta C_T$ (delta delta threshold cycle) method (relative), and the obtained values were then normalized against the value obtained for the susceptible isolate (34).

Nucleotide sequence accession numbers. The nucleotide sequence of the *mgrB* gene interrupted by an IS5-like element has been deposited at DDBJ/EMBL/GenBank under the accession no. [HG008893](#). Genome projects for strains KKBO-1 and KKBO-4 have been registered as NCBI Bioprojects PRJNA214751, and PRJNA214752, respectively. The draft genome projects of strains KKBO-1 and KKBO-4 have been deposited at DDBJ/EMBL/GenBank under the accession no. [AVFC00000000](#) and [AVFD00000000](#), respectively. The versions described in this paper are the first versions of these entries: AVFC01000000 and AVFD01000000.

RESULTS AND DISCUSSION

Genomic comparison of colistin-susceptible and colistin-resistant KPC-KP isolates: identification of *mgrB* inactivation as a candidate mechanism for colistin resistance. The two sequential

FIG 2 PFGE profiles of XbaI-digested genomic DNAs showing genomic relatedness among KKBO-1 (left) and KKBO-4 (right) *K. pneumoniae* isolates. DNA size standards are indicated (in kb) on the left.

isolates carried a *bla*_{KPC-3} gene, belonged in ST258, and exhibited identical XbaI PFGE profiles (Fig. 2).

Comparative analysis of the draft genomes with the chromosome of HS11286 revealed an overall highly conserved genomic structure. After comparative analysis of the two draft genomes performed as described in Materials and Methods, the only significant difference between KKBO-1 and KKBO-4 was found in the *mgrB* gene, which in KKBO-4 presented an insertional inactivation at nucleotide 75 by an insertion sequence (an IS5-like element) (Fig. 3) that shared 92% similarity at the nucleotide level with IS5 (accession no. J01735) (35). MgrB is a small membrane

protein that, in *E. coli* and other *Enterobacteriaceae*, is known to be involved in negative regulation of the PhoQ/PhoP signaling system (22), which controls, among others, the modification of the polymyxin LPS target (15–17).

Based on this, we hypothesized that insertional inactivation of *mgrB* could be associated with colistin resistance in the KKBO-4 isolate.

Complementation of the *mgrB* mutant restored colistin susceptibility. To confirm the role of *mgrB* inactivation in the expression of colistin resistance by KKBO-4, a complementation experiment was performed with plasmid pACYC-*mgrB*, which carries a cloned copy of the *K. pneumoniae mgrB* gene along with its own putative promoter.

The introduction of a functional *mgrB* gene in KKBO-4 was able to reduce the colistin MIC to the same level as that for KKBO-1 (Table 2). The colistin MIC for KKBO-1 (pACYC-*mgrB*) was 1 dilution lower than that for KKBO-1, probably due to a gene dosage effect of *mgrB*.

***mgrB* disruption conferred colistin resistance.** To further confirm the role of *mgrB* inactivation in colistin resistance, a KKBO-1 derivative was constructed, which carried a chromosomal deletion of 144 bp, corresponding to the complete *mgrB* gene (Fig. 1).

The KKBO-1Δ*mgrB* mutant exhibited a colistin-resistant phenotype similar to that of KKBO-4, and complementation of the mutant with plasmid pACYC-*mgrB* was able to restore colistin susceptibility (Table 2).

In vitro selection of colistin-resistant mutants from the colistin-susceptible strain. Colistin-resistant mutants were selected *in vitro* from colistin-susceptible strain KKBO-1 by plating cells on colistin-containing medium (concentration, 8 μg/ml). With this approach, colistin-resistant mutants were selected at a frequency of approximately 7 × 10^{−7}.

Analysis of 10 randomly selected mutants by PCR and sequencing, using primers mgrB_Ext_F and mgrB_Ext_R (Table 1), revealed the presence of an insertional inactivation of *mgrB* in six of them. In all these, the inactivation was mediated by insertion of the same IS5-like element as that present in KKBO-4, located at the same position. In the remaining mutants, the sequence of *mgrB* and flanking regions was identical to that of KKBO-1. The

FIG 3 Schematic representation of the *mgrB* loci of *K. pneumoniae* KKBO-1 (colistin susceptible) and KKBO-4 (colistin resistant). Open reading frames are indicated by arrows. The IS5-like element is shown as a striped rectangle, and its inverted repeats (IR-L and IR-R) are shown as black vertical lines. The interrupted *mgrB* fragments are shown with dotted borders. Nucleotide sequence details at the insertion junctions of the IS5-like element are also shown: *mgrB* sequences flanking the insertion point are shown in italics, and the 4-bp direct repeats generated by transposition are boldfaced; the external portions of the IR-L and IR-R of the IS5-like element are in uppercase letters. The 11-bp segment of *mgrB* representing the putative recombination hot spot is boxed, and the highly similar sequences within the IRs of the IS5-like element are underlined. Gene IDs refer to the *K. pneumoniae* HS11286 chromosome (31).

TABLE 2 Colistin MICs and expression levels of *phoQ*, *phoP*, and *pmrK* genes of KKBO-1, KKBO-4, ΔKKBO-1, KKBO-1mut_1 and of the corresponding transformants carrying either the pACYC184 or the pACYC-*mgrB* plasmids^a

Strain	Chromosomal <i>mgrB</i> status	Colistin MIC (μg/ml)	Expression level (mean ± SD) of:		
			<i>phoQ</i>	<i>phoP</i>	<i>pmrK</i>
KKBO-1	WT	0.125	1	1	1
KKBO-1(pACYC184)	WT	0.125	0.97 ± 0.21	1.20 ± 0.15	1.30 ± 0.66
KKBO-1(pACYC- <i>mgrB</i>)	WT	0.064	0.93 ± 0.18	1.10 ± 0.05	1.24 ± 0.51
KKBO-4	Interrupted by IS5-like element	16	2.55 ± 0.17	3.35 ± 0.69	21.1 ± 6.95
KKBO-4(pACYC184)	Interrupted by IS5-like element	16	2.75 ± 0.17	4.75 ± 0.72	17.3 ± 2.16
KKBO-4(pACYC- <i>mgrB</i>)	Interrupted by IS5-like element	0.125	0.87 ± 0.16	0.97 ± 0.16	1.49 ± 0.87
ΔKKBO-1	Deleted	8	2.62 ± 0.11	3.87 ± 0.04	19.6 ± 0.17
ΔKKBO-1(pACYC184)	Deleted	8	2.61 ± 0.18	5.73 ± 0.21	21.9 ± 2.28
ΔKKBO-1(pACYC- <i>mgrB</i>)	Deleted	0.125	0.95 ± 0.01	1.61 ± 0.13	1.39 ± 0.30
KKBO-1mut_1	Interrupted by IS5-like element	16	5.54 ± 0.04	3.10 ± 0.01	8.48 ± 2.24
KKBO-1mut_1(pACYC184)	Interrupted by IS5-like element	16	4.94 ± 0.01	4.54 ± 0.15	8.61 ± 0.12
KKBO-1mut_1(pACYC- <i>mgrB</i>)	Interrupted by IS5-like element	0.125	1.22 ± 0.04	0.50 ± 0.03	1.26 ± 0.02

^a Expression levels for the *phoQ*, *phoP*, and *pmrK* genes were normalized against the value obtained with KKBO-1. For each strain, the chromosomal status of *mgrB* is also reported. WT, wild type.

colistin MIC of a randomly selected mutant carrying the IS5-like insertion (KKBO-1mut_1) was identical to that of KKBO-4, and complementation with pACYC-*mgrB* of the mutant restored susceptibility to colistin (Table 2).

Taken together, these results indicated that insertional inactivation of *mgrB* could be selected at a relatively high frequency upon colistin exposure and was associated with the expression of colistin resistance, although additional mechanisms of colistin resistance also exist.

Results of these experiments also suggested a marked preference for the insertional site of the IS5-like element into the *mgrB* gene. Interestingly, an 11-bp sequence highly similar (82%) to the outer part of the IS5-like inverted repeats (IR-L and IR-R) is found in the *mgrB* gene, just 1 bp downstream of the insertion site of the IS5-like element (Fig. 3), which could act as a recombination hot spot for the integration of such mobile elements. However, further experiments will be needed to confirm this hypothesis.

***mgrB* inactivation is associated with *phoPQ* and *pmrHFIJKLM* overexpression.** Since MgrB is known to act as a negative regulator of the *phoPQ* expression in *E. coli* (22), the insertional inactivation of *mgrB* was expected to be associated with overexpression of the *phoPQ* operon and also of the *pmrHFIJKLM* operon, which is positively regulated by the PhoQ-PhoP signaling system and whose products are eventually responsible for modification of the LPS target.

Indeed, analysis of *phoP* and *phoQ* transcription by qRT-PCR revealed a 3- to 5-fold increase in all strains carrying an inactivated or deleted *mgrB* allele (KKBO-4, ΔKKBO-1, and KKBO-1mut_1) in comparison with KKBO-1 and with the *mgrB* mutants complemented with a cloned copy of wild-type *mgrB* (Table 2). Moreover, analysis of transcription of the *pmrK* gene, which belongs to the *pmrHFIJKLM* operon, confirmed that this operon was also activated in all strains carrying an inactivated or deleted *mgrB* allele (Table 2).

All together, these data showed that *mgrB* inactivation leads to an upregulation of the PhoQ/PhoP signaling system and of the *pmrHFIJKLM* operon, which was apparently responsible for the acquisition of colistin resistance. To our best knowledge, this is also the first experimental demonstration of the conserved MgrB regulatory function in *K. pneumoniae*.

Concluding remarks. The emergence of colistin-resistant KPC-KP strains is a matter of major concern due to the very few therapeutic options for treatment of infections caused by similar strains.

Results of this work identified a new genetic mechanism responsible for the emergence of colistin resistance in KPC-KP following exposure to colistin, either *in vivo* or *in vitro*. The mechanism consists of the inactivation of the *mgrB* gene, which encodes a transmembrane regulator that negatively controls the PhoQ/PhoP signaling system (22). Once upregulated, PhoQ/PhoP activates the LPS modification system that is eventually responsible for polymyxin resistance.

The IS5-like element responsible for insertional inactivation of *mgrB* in KKBO-4 was already present in the genome of KKBO-1 (as shown by the genome sequence data), and therefore its origin was most likely endogenous (i.e., mobilized from a preexisting location). That such a possibility could occur was also demonstrated by results of the experiment on *in vitro* selection of colistin-resistant mutants from KKBO-1, which returned a majority of resistant mutants in which *mgrB* was inactivated by the same mechanism. Since IS5-like elements that are identical or very similar to that involved in insertional inactivation of *mgrB* are found on several KPC-encoding plasmids (see, for instance, accession no. JX430448.1, JN233704.1, CP000648.1, and CP002474.1), it could be speculated that acquisition of these plasmids could facilitate the emergence of colistin resistance by providing a source of similar insertion sequences capable of targeting the *mgrB* gene. Further experiments are under way to verify this hypothesis.

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