AAC Journals.ASM.org

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

Antonio Cannatelli,[a] Marco Maria D'Andrea,[a] Tommaso Giani,[a] Vincenzo Di Pilato,[a] Fabio Arena,[a] Simone Ambretti,[b] Paolo Gaibani,[c] Gian Maria Rossolini,[a,d,e]

Department of Medical Biotechnologies, University of Siena, Siena, Italy[a]; Bacteriology Laboratory[b] and Regional Laboratory for Microbiological Emergencies,[c] Unit of Clinical Microbiology, St. Orsola University Hospital, Bologna, Italy; Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy[d]; Clinical Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy[e]

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

Received 10 July 2013 Returned for modification 5 August 2013
Accepted 15 August 2013
Published ahead of print 26 August 2013
Address correspondence to Gian Maria Rossolini, gianmaria.rossolini@unisi.it.

doi:10.1128/AAC.01480-13

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	GAATTCCACCACCTCAAAGAGAAGGCGTTC	95°C/30 s–62°C/30 s–72°C/30 s
AvaI_mgrB_R	CTCGGGAACACGTTTTGAAACAAGTCGATGATTC	
KOmgrB1_XbaI_F[c]	TCTAGATCTTATGATGCACACCTGTCGGGCACC	95°C/30 s–67°C/30 s–72°C/30 s
KOmgrB1_XhoI_R	CTCGAGGACATTTTTCTGCCGACTGATTTCCTTCTGCGC	
KOmgrB2_XhoI_F	CTCGAGACTCTCCGCATCAGTACGCAATGTCGCCAG	95°C/30 s–69°C/30 s–72°C/30 s
KOmgrB2_BglII_R[c]	AGATCTCCGTTCTGAGGCGGCGGATACCGGGTTG	
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	GCTTGATCGGCTGGTCATTCACC	
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	CGTTCTGGTACTACATCCCGTTCCTGA	
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

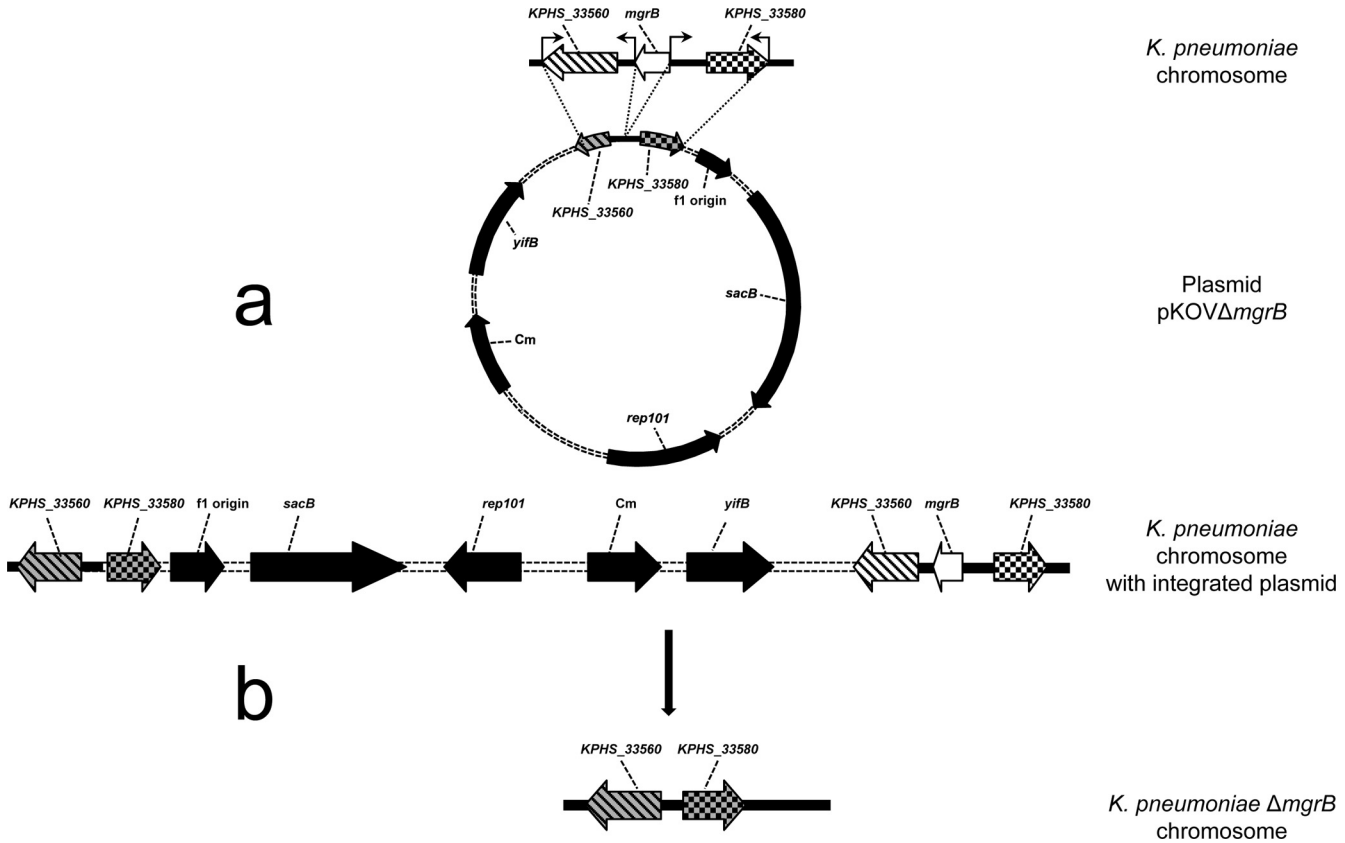

FIG 1 Construction of the *mgrB* deletion mutant of *K. pneumoniae* KKBO-1 by using plasmid pKOV via homologous recombination. (a) Outline of construction of plasmid pKOVΔ*mgrB*. The positions of primers used for amplification of the flanking regions (KOmgrB1_XbaI_F and KOmgrB1_XhoI_R; KOmgrB2_XhoI_F 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 rpsL13_F and rpsL14_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 IS*5*-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

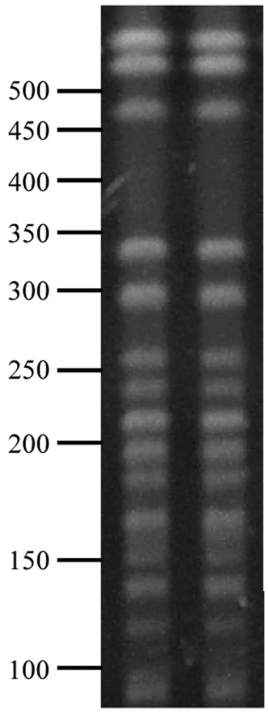

FIG 2 PFGE profiles of XbaI-digested genomic DNAs showing genomic relatedness among KKBO-1 (left) and KKBO-4 (right) *K. pneumoniae* isolates. DNA size standards are indicated (in kb) on the left.

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 IS*5*-like element) (Fig. 3) that shared 92% similarity at the nucleotide level with IS*5* (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 IS*5*-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

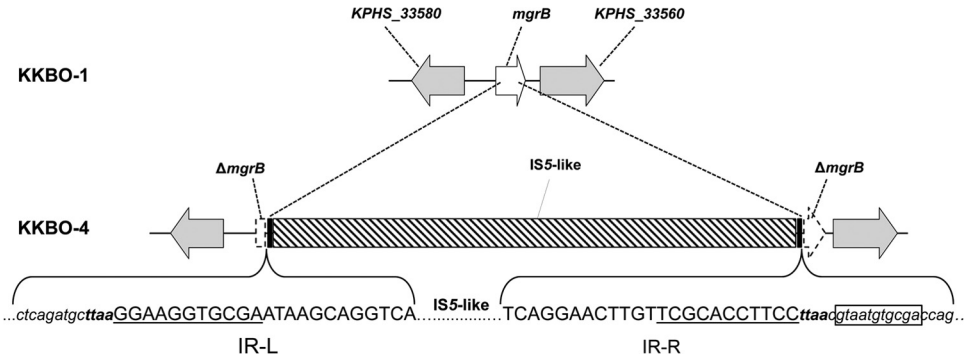

FIG 3 Schematic representation of the *mgrB* loci of *K. pneumoniae* KKBO-1 (colistin susceptible) and KKBO-4 (colistin resistant). Open reading frames are indicated by arrows. The IS*5*-like element is shown as a striped rectangle, and its inverted repeats (IR-L and IR-R) are shown as black vertical lines. The interrupted *mgrB* fragments are shown with dotted borders. Nucleotide sequence details at the insertion junctions of the IS*5*-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 IS*5*-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 IS*5*-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 *mgrB* status	Colistin MIC (μg/ml)	Expression level (mean ±SD) of:		
			phoQ	*phoP*	*pmrK*
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-*mgrB*)	WT	0.064	0.93 ± 0.18	1.10 ± 0.05	1.24 ± 0.51
KKBO-4	Interrupted by IS*5*-like element	16	2.55 ± 0.17	3.35 ± 0.69	21.1 ± 6.95
KKBO-4(pACYC184)	Interrupted by IS*5*-like element	16	2.75 ± 0.17	4.75 ± 0.72	17.3 ± 2.16
KKBO-4(pACYC-*mgrB*)	Interrupted by IS*5*-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-*mgrB*)	Deleted	0.125	0.95 ± 0.01	1.61 ± 0.13	1.39 ± 0.30
KKBO-1mut_1	Interrupted by IS*5*-like element	16	5.54 ± 0.04	3.10 ± 0.01	8.48 ± 2.24
KKBO-1mut_1(pACYC184)	Interrupted by IS*5*-like element	16	4.94 ± 0.01	4.54 ± 0.15	8.61 ± 0.12
KKBO-1mut_1(pACYC-*mgrB*)	Interrupted by IS*5*-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 IS*5*-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 IS*5*-like element into the *mgrB* gene. Interestingly, an 11-bp sequence highly similar (82%) to the outer part of the IS*5*-like inverted repeats (IR-L and IR-R) is found in the *mgrB* gene, just 1 bp downstream of the insertion site of the IS*5*-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 IS*5*-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 IS*5*-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.

ACKNOWLEDGMENTS

The precious scientific support of Cesira Galeotti is gratefully acknowledged.

This work was supported by a grant from FP7 projects TEMPOTEST-QC (no. HEALTH-2009-241742) and from EvoTAR (no. HEALTH-F3-2011-2011-282004) to G.M.R.

REFERENCES

1. **Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K, Tenover FC.** 2001. Novel carbapenem-

hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45:**1151–1161.
2. **Nordmann P, Naas T, Poirel L.** 2011. Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerg. Infect. Dis. **17:**1791–1798.
3. **Canton R, Akova M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM, Samuelsen O, Seifert H, Woodford N, Nordmann P.** 2012. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. **18:**413–431.
4. **Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL.** 2012. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. Clin. Microbiol. Rev. **25:**682–707.
5. **Giani T, Pini B, Arena F, Conte V, Bracco S, Migliavacca R, Pantosti A, Pagani L, Luzzaro F, Rossolini G.** 2013. Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. Euro Surveill. **18:**pii=20489. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20489.
6. **Borer A, Saidel-Odes L, Riesenberg K, Eskira S, Peled N, Nativ R, Schlaeffer F, Sherf M.** 2009. Attributable mortality rate for carbapenem-resistant *Klebsiella pneumoniae* bacteremia. Infect. Control Hosp. Epidemiol. **30:**972–976.
7. **Falagas ME, Karageorgopoulos DE, Nordmann P.** 2011. Therapeutic options for infections with *Enterobacteriaceae* producing carbapenem-hydrolyzing enzymes. Future Microbiol. **6:**653–666.
8. **Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, Polsky B, Adams-Haduch JM, Doi Y.** 2012. Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: superiority of combination antimicrobial regimens. Antimicrob. Agents Chemother. **56:**2108–2113.
9. **Tumbarello M, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, Spanu T, Ambretti S, Ginocchio F, Cristini F, Losito AR, Tedeschi S, Cauda R, Bassetti M.** 2012. Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy. Clin. Infect. Dis. **55:**943–950.
10. **Bogdanovich T, Adams-Haduch JM, Tian GB, Nguyen MH, Kwak EJ, Muto CA, Doi Y.** 2011. Colistin-resistant, Klebsiella pneumoniae carbapenemase (KPC)-producing *Klebsiella pneumoniae* belonging to the international epidemic clone ST258. Clin. Infect. Dis. **53:**373–376.
11. **Hrabak J, Niemczykova J, Chudackova E, Fridrichova M, Studentova V, Cervena D, Urbaskova P, Zemlickova H.** 2011. KPC-2-producing *Klebsiella pneumoniae* isolated from a Czech patient previously hospitalized in Greece and *in vivo* selection of colistin resistance. Folia Microbiol. (Praha) **56:**361–365.
12. **Mammina C, Bonura C, Di BF, Aleo A, Fasciana T, Sodano C, Saporito MA, Verde MS, Tetamo R, Palma DM.** 2012. Ongoing spread of colistin-resistant *Klebsiella pneumoniae* in different wards of an acute general hospital, Italy, June to December 2011. Euro Surveill. **17:**pii=20248. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20248.
13. **Zagorianou A, Sianou E, Iosifidis E, Dimou V, Protonotariou E, Miyakis S, Roilides E, Sofianou D.** 2012. Microbiological and molecular characteristics of carbapenemase-producing *Klebsiella pneumoniae* endemic in a tertiary Greek hospital during 2004–2010. Euro Surveill. **17:**pii=20088. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20088.
14. **Helander IM, Kato Y, Kilpelainen I, Kostiainen R, Lindner B, Nummila K, Sugiyama T, Yokochi T.** 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. Eur. J. Biochem. **237:**272–278.
15. **Cheng HY, Chen YF, Peng HL.** 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. J. Biomed. Sci. **17:**60.
16. **Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA.** 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. PLoS Genet. **4:**e1000233. doi:10.1371/journal.pgen.1000233.
17. **Groisman EA.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835–1842.
18. **Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, Klevit RE, Le MH, Miller SI.** 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell **122:**461–472.
19. **Prost LR, Daley ME, Le SV, Bader MW, Le MH, Klevit RE, Miller SI.** 2007. Activation of the bacterial sensor kinase PhoQ by acidic pH. Mol. Cell **26:**165–174.
20. **Gunn JS.** 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol. **16:**284–290.
21. **Chen HD, Groisman EA.** 17 June 2013. The biology of the PmrA/PmrB two-component system: the major regulator of lipolysaccharide modifications. Annu. Rev. Microbiol. [Epub ahead of print.] doi:10.1146/annurev-micro-092412-155751.
22. **Lippa AM, Goulian M.** 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet. **5:**e1000788. doi:10.1371/journal.pgen.1000788.
23. **Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S.** 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J. Clin. Microbiol. **43:**4178–4182.
24. **Yuan M, Aucken H, Hall LM, Pitt TL, Livermore DM.** 1998. Epidemiological typing of *klebsiellae* with extended-spectrum β-lactamases from European intensive care units. J. Antimicrob. Chemother. **41:**527–539.
25. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Chang AC, Cohen SN.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
27. **D'Andrea MM, Giani T, D'Arezzo S, Capone A, Petrosillo N, Visca P, Luzzaro F, Rossolini GM.** 2009. Characterization of pABVA01, a plasmid encoding the OXA-24 carbapenemase from Italian isolates of *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **53:**3528–3533.
28. **Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I.** 2009. ABySS: a parallel assembler for short read sequence data. Genome Res. **19:**1117–1123.
29. **Auch AF, Klenk HP, Goker M.** 2010. Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. Stand. Genomic Sci. **2:**142–148.
30. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9:**75. doi:10.1186/1471-2164-9-75.
31. **Liu P, Li P, Jiang X, Bi D, Xie Y, Tai C, Deng Z, Rajakumar K, Ou HY.** 2012. Complete genome sequence of *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286, a multidrug-resistant strain isolated from human sputum. J. Bacteriol. **194:**1841–1842.
32. **Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL.** 2009. BLAST+: architecture and applications. BMC Bioinformatics **10:**421. doi:10.1186/1471-2105-10-421.
33. **Snitkin ES, Zelazny AM, Thomas PJ, Stock F, Henderson DK, Palmore TN, Segre JA.** 2012. Tracking a hospital outbreak of carbapenem-resistant *Klebsiella pneumoniae* with whole-genome sequencing. Sci. Transl. Med. **4:**148ra116.
34. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods **25:**402–408.
35. **Kroger M, Hobom G.** 1982. Structural analysis of insertion sequence IS5. Nature **297:**159–162.