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MgrB Inactivation Is a Common Mechanism of Colistin Resistance in KPC-Producing *Klebsiella pneumoniae* of Clinical Origin

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***Klebsiella pneumoniae* strains producing KPC-type carbapenemases (KPC-KP) are challenging multidrug-resistant pathogens due to their extensively drug-resistant phenotypes and potential for epidemic dissemination in health care settings. Colistin is a key component of the combination antimicrobial regimens used for treatment of severe KPC-KP infections. We previously reported that insertional inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the PhoQ-PhoP signaling system, can be responsible for colistin resistance in KPC-KP, due to the resulting upregulation of the Pmr lipopolysaccharide modification system. In this work we investigated the status of the *mgrB* gene in a collection of 66 colistin-resistant nonreplicate clinical strains of KPC-KP isolated from different hospitals in Italy and Greece. Overall, 35 strains (53%) exhibited alterations of the *mgrB* gene, including insertions of different types of mobile elements (IS*5*-like, IS*1F*-like, or IS*Kpn14*), nonsilent point mutations, and small intragenic deletions. Four additional strains had a larger deletion of the *mgrB* locus, while the remaining 27 strains (41%) did not show *mgrB* alterations. Transcriptional upregulation of the *phoQ* and *pmrK* genes (part of the *phoPQ* and *pmrHFIJKLM* operon, respectively) was observed in all strains with *mgrB* alterations. Complementation experiments with a wild-type *mgrB* gene restored colistin susceptibility and basal expression levels of *phoQ* and *pmrK* genes in strains carrying different types of *mgrB* alterations. The present results suggest that *mgrB* alteration can be a common mechanism of colistin resistance among KPC-KP in the clinical setting.**

The dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) is an emerging global problem (1, 2). Among CPE, *Klebsiella pneumoniae* producing KPC-type carbapenemases (KPC-KP) are likely the most challenging pathogens due to their extensively drug-resistant phenotypes and potential for rapid dissemination in health care settings, with a remarkable impact on morbidity and mortality (2–4).

Polymyxins (polymyxin B and colistin) are among the few antimicrobial agents that retain activity against KPC-KP, being a key component of the combination antimicrobial regimens used for the treatment of severe KPC-KP infections (2, 5). The emergence of polymyxin-resistant KPC-KP, however, has repeatedly been reported (6–9) and is a matter of major concern. In some settings, polymyxin resistance has achieved alarming proportions among KPC-KP, 18.6% among isolates from a Greek hospital in 2007 to 2010 (7) and 22.4% among isolates from the first Italian countrywide survey on carbapenem-resistant *Enterobacteriaceae* (CRE), carried out in 2011 (9).

We recently showed that inactivation of the *mgrB* gene, encoding a negative feedback regulator of the PhoQ-PhoP signaling system, is responsible for acquired colistin resistance in KPC-KP by upregulating PhoQ-PhoP, which, in turn, activates the Pmr system responsible for modification of the lipopolysaccharide polymyxin target (10). This resistance mechanism was identified in a colistin-resistant isolate from a patient with bacteremia who had been exposed to colistin treatment, and it was easily reproduced *in vitro* after exposure to colistin of the susceptible progenitor strain (10). The same resistance mechanism has also been detected in a non-KPC-producing carbapenemase- and colistin-resistant *K. pneumoniae* isolate from Spain (11).

In this work we investigated the occurrence of *mgrB* gene alterations as a mechanism of resistance in a large collection of colistin-resistant clinical isolates of KPC-KP collected during the ongoing Greek and Italian KPC-KP epidemics.

MATERIALS AND METHODS

Bacterial strains. The strains studied in this work included 66 nonreplicate colistin-resistant KPC-KP clinical isolates obtained during the period from 2010 to 2012 from 23 centers (19 in Italy and 4 in Greece) (Fig. 1 and Table 1). Some of these isolates ($n = 41$) were from the first Italian nationwide survey on carbapenem-resistant *Enterobacteriaceae*, carried out in mid-2011 (9). The remaining isolates ($n = 25$) were collected outside that survey by participants in the COLGRIT Study Group. Identification of the isolates was always confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Vitek MS; bioMérieux, Marcy l'Etoile, France). The presence of the bla_{KPC} gene was confirmed by PCR, and the bla_{KPC} gene variant was determined by direct amplicon sequencing, as previously described (9).

Antimicrobial susceptibility testing. MICs of colistin and other antimicrobial agents (including carbapenems, cephalosporins, trimethoprim-sulfamethoxazole, ciprofloxacin, levofloxacin, amikacin, gentamicin, and tigecycline) were determined by reference broth microdilution (12) and interpreted according to the EUCAST guidelines (EUCAST breakpoint tables for interpretation of MICs and zone diameters, version 4.0, 2014) (see http:

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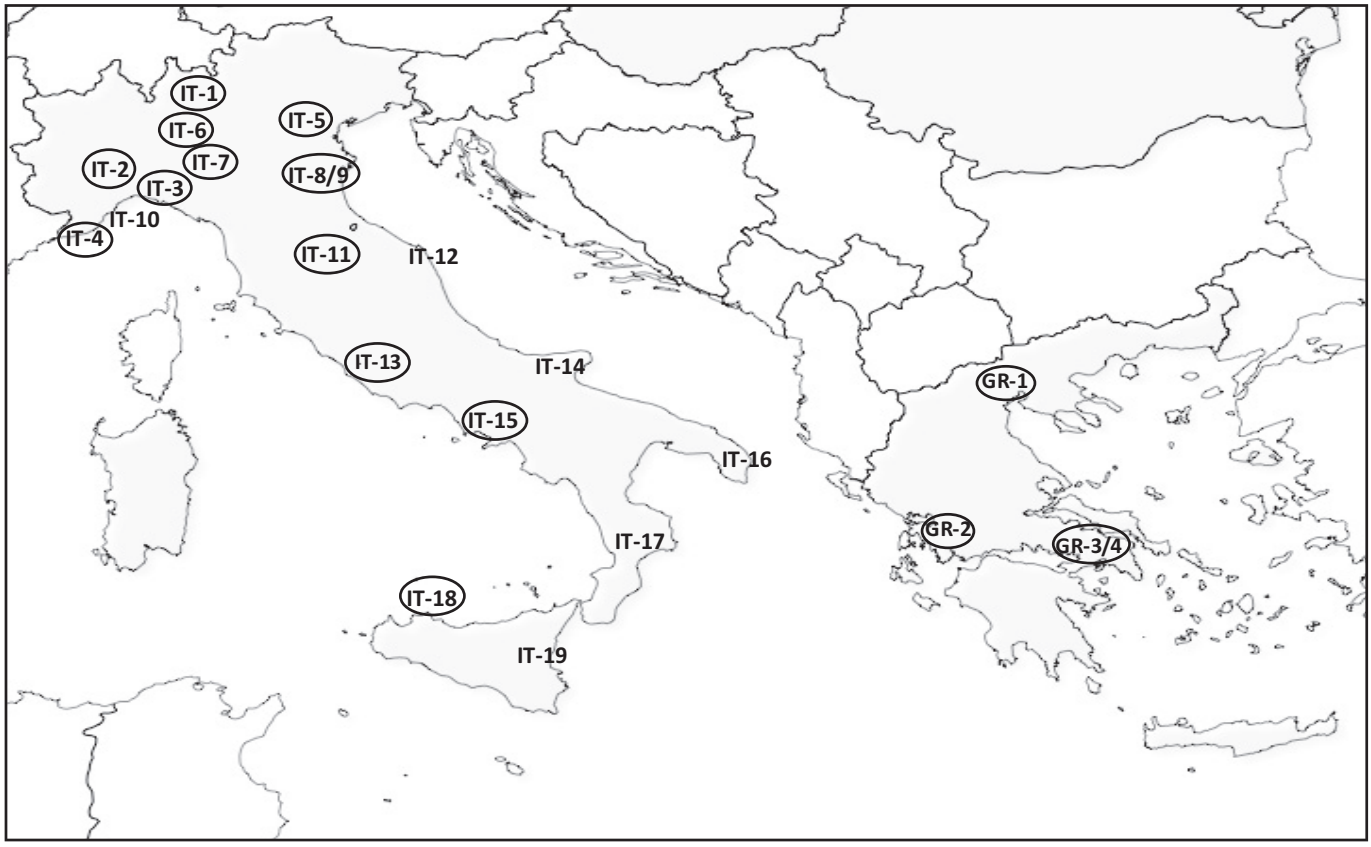

FIG 1 Geographical distribution of the Greek and Italian centers from which COL-R strains were originated. Centers where strains carrying *mgrB* lesions were detected are circled. The number of each center corresponds to the location as follows: IT-1, Lecco; IT-2, Turin; IT-3, Genoa; IT-4, Pietra Ligure; IT-5, Verona; IT-6, Milan; IT-7, Pavia; IT-8-9, Modena; IT-10, San Remo; IT-11, Florence; IT-12, Ancona; IT-13, Rome; IT-14, Foggia; IT-15, Naples; IT-16, Lecce; IT-17, Cosenza; IT-18, Palermo; IT-19, Catania; GR-1, Salonika; GR-2, Agrinio; GR-3-4, Athens.

//www.eucast.org). *Escherichia coli* ATCC 25922 and *K. pneumoniae* KKBO-4 (colistin resistant) (10) were used as control strains for antimicrobial susceptibility testing.

Bacterial genotyping. Genotyping of the *K. pneumoniae* isolates by pulsed-field gel electrophoresis (PFGE) was carried out as described previously (9), and results were interpreted as recommended by van Belkum et al. (13). Multilocus sequence typing (MLST) was performed as previously described (14), and sequence type (ST) was assigned according to the *K. pneumoniae* MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Analysis of the *mgrB* locus. PCR amplification of the *mgrB* locus was carried out using primers mgrB_Ext_F and mgrB_Ext_R, which target amplification of the *mgrB* coding sequence and some flanking regions (Fig. 2 and Table 2). A set of primers external to *mgrB*, covering a larger portion of the *mgrB* locus, and a set of primers targeting amplification of an internal region of the *mgrB* coding sequence were also used with some strains (Fig. 2 and Table 2). *K. pneumoniae* KKBO-1 (10) was used as a control strain carrying wild-type *mgrB* in PCR mapping experiments. DNA sequences were determined on both strands of the amplification products at an external sequencing facility (Macrogen, Seoul, South Korea).

Complementation experiments. For complementation experiments, a pACYC184 derivative carrying the wild-type *mgrB* gene with its putative promoter and terminator (named pACYC-*mgrB*) was used (10). The pACYC-*mgrB* plasmid was introduced into the *K. pneumoniae* strains by electroporation, and transformants were selected on Mueller-Hinton agar plates supplemented with 40 μg/ml of tetracycline.

Transcriptional analysis by real-time quantitative PCR. RNA extraction and retrotranscription were carried out as previously described (10). Real-time quantitative PCR (RT-qPCR) was used to measure the expression of the *phoQ* and *pmrK* genes using the primers and conditions reported in Table 2. Expression of the *rpsL* gene was used as an internal standard (primers and conditions reported in Table 2). Normalization was performed against *rpsL* gene using the $\Delta\Delta CT$ method (relative) (15), and the obtained values were then normalized against those obtained with the colistin-susceptible strain KKBO-1 (10).

Nucleotide sequence accession numbers. The nucleotide sequences of the altered *mgrB* genes described in this work have been deposited at DDBJ/EMBL/GenBank under the accession numbers KJ129594 (isolate KP04C62), KJ129595 (isolate KP04C83), KJ129596 (isolate KP06C01), KJ129597 (isolate KP08C16), KJ129598 (isolate KP08C24), KJ129599 (isolate KPL171), KJ129600 (isolate KP23C12), KJ129602 (isolate KP4707), KJ129603 (isolate KPIAC), KJ129604 (isolate KP4554), and KJ586580 (isolate KP12C48).

RESULTS

Characteristics of the investigated clinical isolates. All isolates exhibited complex multidrug-resistant phenotypes, including carbapenems and other β-lactams, fluoroquinolones, and amikacin. Some of them retained susceptibility to gentamicin (71%), tigecycline (64%), and/or trimethoprim-sulfamethoxazole (12%). Colistin MICs varied from 4 to 64 μg/ml (MIC_{90}, 32 μg/ml; median MIC, 32 μg/ml) (Table 1).

Most of the isolates belonged in ST258 and ST512, but isolates of other STs (ST101 and ST147) were also represented (Table 1).

Occurrence of *mgrB* alterations in colistin-resistant clinical isolates of KPC-KP. A PCR mapping and sequencing strategy for

TABLE 1 Characteristics of the colistin-resistant *K. pneumoniae* strains investigated in this work

Strain	Yr	Center[b]	Source[c]	ST	KPC type	Colistin MIC (μg/ml)	Additional resistance trait(s)[d]	*mgrB* status[e]
KP6303	2010	IT-1	Blood	258	KPC-2	16	SXT, TGC	Insertional inactivation, IS*5*-like element at nt 75 (FW)
KPENA	2010	IT-2	CVC	258	KPC-2	4	SXT, TGC	WT
KP04C83[a]	2011	IT-2	AF	512	KPC-3	32	SXT, TGC	Δ18/27 (frameshift and premature termination)
KP04C93[a]	2011	IT-2	Blood	512	KPC-3	32	GEN, SXT, TGC	Δ18/27 (frameshift and premature termination)
KP04C62	2011	IT-2	Blood	512	KPC-3	32	SXT	Insertional inactivation, IS*5*-like element at nt 126 (FW)
KP06C01[a]	2011	IT-3	Urine	512	KPC-3	16	SXT	g109a (G37S)
KP06C19[a]	2011	IT-3	Blood	512	KPC-3	32		g109a (G37S)
KP06C02	2011	IT-3	Urine	258	KPC-2	16	SXT	WT
KP06C05	2011	IT-3	Urine	258	KPC-3	32	TGC	g109a (G37S)
KP06C16[a]	2011	IT-3	Urine	258	KPC-3	16	SXT, TGC	g109a (G37S)
KP06C18[a]	2011	IT-3	Blood	258	KPC-3	16		g109a (G37S)
KP06C07	2011	IT-3	LRS	258	KPC-2	32	GEN, TGC	WT
KP207-2	2010	IT-4	CVC	258	KPC-3	16	GEN, SXT, TGC	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP08C01	2011	IT-5	Urine	512	KPC-3	16	SXT	WT
KP08C02	2011	IT-5	Urine	512	KPC-3	32	SXT, TGC	WT
KP08C08	2011	IT-5	Urine	512	KPC-3	32	SXT	WT
KP08C11	2011	IT-5	Urine	512	KPC-3	8	SXT	WT
KP08C16	2011	IT-5	Urine	512	KPC-3	32	SXT	a7t (nonsense, premature termination)
KP08C24	2011	IT-5	Urine	512	KPC-3	32	SXT	Insertional inactivation, IS*5*-like element at nt 75 (FW)
KP08C32	2011	IT-5	LRS	512	KPC-3	8	SXT	WT
KPKL1HU	2010	IT-6	Bile	258	KPC-3	16	SXT, TGC	Insertional inactivation, IS*5*-like element at nt 75 (FW)
KPKL52MG11	2011	IT-7	Blood	512	KPC-3	8	SXT, TGC	Δ*mgrB locus*[f]
KPL171	2011	IT-8	Urine	512	KPC-2	8	GEN, SXT, TGC	Δg47 (frameshift and premature termination)
KP10C06	2011	IT-8	Urine	258	KPC-2	32	TGC	Δg47 (frameshift and premature termination)
KP11C07	2011	IT-9	Urine	512	KPC-3	16	SXT, TGC	Insertional inactivation, IS*5*-like element at nt 75 (FW)
KP07C07	2011	IT-10	LRS	258	KPC-2	16	GEN, SXT	WT
KP12C48	2011	IT-11	Urine	512	KPC-3	32	SXT	Δ109/119 (frameshift and premature termination)
KP12C69	2011	IT-11	WEX	101	KPC-2	8	GEN	WT
KPAN-3	2011	IT-12	Blood	512	KPC-3	32	SXT, TGC	WT
KP15C05	2011	IT-12	LRS	512	KPC-3	32	SXT	WT
KP15C10	2011	IT-12	Urine	258	KPC-3	32	SXT	WT
KP15C15	2011	IT-12	LRS	512	KPC-3	32	SXT, TGC	WT
KP15C16	2011	IT-12	Urine	512	KPC-3	32	SXT, TGC	WT
KP15C17	2011	IT-12	LRS	512	KPC-3	32	SXT	WT
KP15C18	2011	IT-12	Urine	258	KPC-3	32	SXT	WT
KP15C23	2011	IT-12	CVC	258	KPC-3	16	SXT, TGC	WT
KP15C24	2011	IT-12	Urine	512	KPC-3	32	SXT	WT
KP15C30	2011	IT-12	WEX	258	KPC-3	32	SXT	WT
KP9	2011	IT-13	Urine	258	KPC-3	16	GEN, SXT, TGC	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP16C05	2011	IT-13	Blood	512	KPC-3	32	SXT	WT
KP16C08	2011	IT-13	Blood	258	KPC-3	16	SXT, TGC	WT
KP16C12	2011	IT-13	WEX	258	KPC-3	8	SXT	WT
KP18C37	2011	IT-14	Urine	258	KPC-3	4	SXT	WT
KP20C14	2011	IT-15	Blood	258	KPC-3	32	SXT	Insertional inactivation, IS*5*-like element at nt 75 (FW)

(Continued on following page)

TABLE 1 (Continued)

Strain	Yr	Center[b]	Source[c]	ST	KPC type	Colistin MIC (μg/ml)	Additional resistance trait(s)[d]	*mgrB* status[e]
KP20C26	2011	IT-15	Blood	258	KPC-3	32	SXT	Insertional inactivation, IS*5*-like element at nt 75 (FW)
KP19C07	2011	IT-16	CVC	512	KPC-3	32	SXT	WT
KP22C22	2011	IT-17	Urine	258	KPC-3	32	SXT	WT
KP23C07[a]	2011	IT-18	Urine	512	KPC-3	8		Insertional inactivation, IS*1F*-like element at nt 105 (FW)
KP23C12[a]	2011	IT-18	LRS	512	KPC-3	32	SXT, TGC	Insertional inactivation, IS*1F*-like element at nt 105 (FW)
KPTRAV	2010	IT-18	WEX	258	KPC-3	16	SXT, TGC	Δ*mgrB* (from −400 to +599)
KPIAC	2010	IT-18	Blood	258	KPC-3	32	GEN, SXT, TGC	t71a (L24H)
KPCAT	2011	IT-19	Blood	512	KPC-3	4	GEN, SXT, TGC	WT
KP4603[a]	2011	GR-1	Urine	258	KPC-2	64	GEN, SXT	Δ*mgrB* locus[f]
KP4623[a]	2011	GR-1	Urine	258	KPC-2	64		Δ*mgrB* locus[f]
KP4566	2012	GR-2	LRS	258	KPC-2	32	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4495[a]	2012	GR-2	Blood	258	KPC-2	64	SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4569[a]	2012	GR-2	Blood	258	KPC-2	8	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4579[a]	2012	GR-2	LRS	258	KPC-2	4	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4594[a]	2012	GR-2	Blood	258	KPC-2	4	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4619[a]	2012	GR-2	LRS	258	KPC-2	4	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4620[a]	2012	GR-2	LRS	258	KPC-2	4	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4657[a]	2012	GR-2	LRS	258	KPC-2	4	GEN, SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4697	2012	GR-3	CVC	258	KPC-2	32	SXT	Insertional inactivation, IS*5*-like element at nt 75 (RW)
KP4707	2012	GR-3	Blood	258	KPC-2	32	SXT	g83a (C28Y)
KP4553[a]	2012	GR-4	CVC	147	KPC-2	16	GEN, SXT	Insertional inactivation, IS*Kpn14* at nt 124 (FW)
KP4554[a]	2012	GR-4	Blood	147	KPC-2	8	GEN, SXT	Insertional inactivation, IS*Kpn14* at nt 124 (FW)

[a] For each center, these strains exhibited identical PFGE profiles and likely reflected phenomena of clonal expansion.
[b] IT, Italy; GR, Greece; the center number corresponds to that reported in Fig. 1.
[c] Clinical specimens from which the strains were isolated. CVC, central venous catheter; AF, ascitic fluid; LRS, lower respiratory secretions; WEX, wound exudate.
[d] GEN, gentamicin; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole; all strains were resistant to carbapenems (imipenem, meropenem, and ertapenem), expanded-spectrum cephalosporins (cefotaxime, ceftazidime, and cefepime), β-lactamase inhibitor combinations (amoxicillin-clavulanate and piperacillin-tazobactam), fluoroquinolones, and amikacin.
[e] Nucleotide (nt) numbers indicate the positions of mutations or of the insertion sites of ISs; numbering refers to the coding sequence of the *mgrB* open reading frame (ORF) (GenBank accession no. AVFC01000053, region 155512 to 155655), considering number 1 as the first base of the GTG start codon. FW indicates that the transposase gene is in the same orientation as the *mgrB* gene, while RW indicates that the transposase gene has the opposite orientation. Wild type (WT) indicates that the sequence of the *mgrB* locus was identical to that of the colistin-susceptible KKBO-1 KPC-KP strain (accession no. AVFC01000053, region 155512 to 155655) (10). Δ, deletion; the numbers refer to the *mgrB* start codon.
[f] Δ*mgrB* locus, not amplifiable with all of the primers used in this work (Fig. 2).

the *mgrB* locus was carried out, using primers designed on regions flanking the *mgrB* gene for each strain (Fig. 2).

Using primers mgrB_Ext_F and mgrB_Ext_R, an amplification product was obtained with 62 of the 66 KPC-KP strains. Of these, 35 strains exhibited nonsilent alterations in the *mgrB* coding sequence, while 27 strains carried a wild-type *mgrB* gene (Table 1). Of the four strains that yielded no amplification product with the former primers (KPKL52MG11, KPTRAV, KP4603, and KP4623), one exhibited a large deletion of the *mgrB* locus that was mapped with the EE_mgrB_F and EE-_mgrB_R primers, while the remaining three strains did not yield any amplification product even with the latter primers, suggesting a larger deletion of the locus (Table 1). In these four strains, the absence of *mgrB* sequences from genomic DNA was confirmed by PCR with the internal Int_mgrB_F and Int_mgrB_R primers.

Several different nonsilent alterations of the *mgrB* coding sequence were detected, including inactivation by an insertion sequence (IS), missense or nonsense point mutations, and small deletions (Table 1). The presence of an IS-inactivated *mgrB* was detected in 22 of the 35 strains with alterations in the *mgrB* coding sequence. The ISs were of three different types, namely, an IS*5*-like

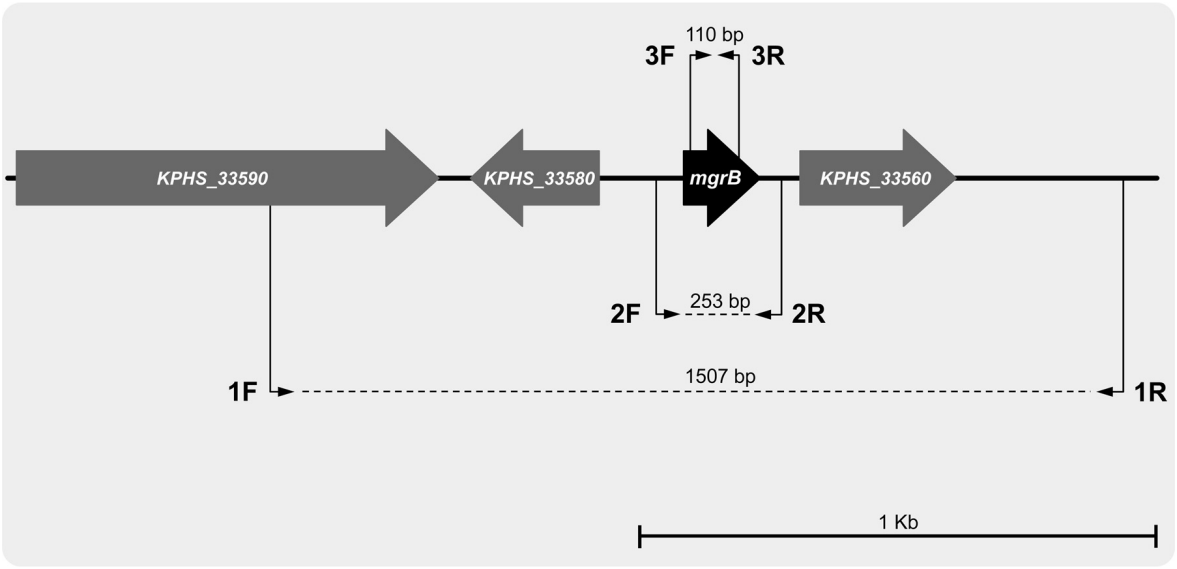

FIG 2 Map of the *K. pneumoniae* chromosomal region carrying the *mgrB* gene. The map is drawn based on sequence information from the *K. pneumoniae* HS11286 chromosome (GenBank accession no. CP000649) (19). Open reading frames are indicated by arrows (arrow direction indicates orientation). The locations of primers used for PCR mapping of the *mgrB* locus are indicated by thin black arrows (1F, EE_mgrB_F; 1R, EE_mgrB_R; 2F, mgrB_Ext_F; 2R, mgrB_Ext_R; 3F, Int_mgrB_F; 3R, Int_mgrB_R). The expected sizes of the PCR products are also shown.

element identical to that previously found to inactivate *mgrB* in strain KKBO-4 (10), an IS*1F*-like element 98% identical to IS*1F* (GenBank accession no. X52538) (16), and an IS*Kpn14* element (accession no. CP000649, from 28285 to 29052) (17). The different ISs were inserted at different sites, and the *IS5*-like element was found to be inserted at two different positions and in different orientations (Table 1). The presence of nonsilent point mutations or small deletions was detected in 13 of the 35 strains with alterations in the *mgrB* coding sequence. In some cases they resulted in truncated proteins, while in other isolates the alterations yielded a mutated MgrB protein with amino acid substitutions at different positions (Table 1).

Analysis of the distribution of colistin MICs among strains with different resistance mechanisms did not reveal significant differences. Geometric mean MICs were 18.1 and 19.6 μg/ml, MIC_{50}s were 16 and 32 μg/ml, MIC_{90}s were 32 and 32 μg/ml, and MIC ranges were 4 to 64 and 4 to 32 μg/ml in strains with or without *mgrB* alterations, respectively (Table 1).

Significance of the *mgrB* alterations detected in colistin-resistant strains. The significance of the nonsilent alterations of the

TABLE 2 Primers and PCR conditions used in this work

PCR and primer	Sequence (5′ to 3′)	Cycling conditions (°C/s)[a]	Reference or source
Conventional PCR			
mgrB_ext_F	AAGGCGTTCATTCTACCACC	D (95/30), A (54/30), E (72/105)	10
mgrB_ext_R	TTAAGAAGGCCGTGCTATCC		
EE_mgrB_F	GGCTATGGCGAGGATAATGAG	D (95/30), A (54/30), E (72/120)	This work
EE_mgrB_R	GCTGTGATGTAAGCGTCTGGTG		
Int_mgrB_F	CGGTGGGTTTTACTGATAGTCA	D (95/30), A (54/30), E (72/30)	This work
Int_mgrB_R	ATAGTGCAAATGCCGCTGA		
RT-qPCR			
pmrK_F2	CGCTGAATATGCTCGACCCAGAAG	D (95/10), A (52/5), E (72/5)	This work
pmrK_R2	GCTGGCGGTAATCGTCTGTACG		
phoQ_F	ATATGCTGGCGAGATGGGAAAACGG	D (95/10), A (52/5), E (72/5)	10
phoQ_R	CCAGCCAGGGAACATCACGCT		
rpsL13_F	GCCGTACTTGGAGCGAGCCTG	D (95/10), A (52/5), E (72/5)	10
rpsL14_F	CCGTGGCGGTCGTGTTAAAGA		

[a] 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 and times, and a final extension step of 300 s at 72°C; all RT-qPCRs included an initial denaturation step of 300 s at 95°C and 40 cycles of denaturation, annealing, and extension at the reported temperatures and times. D, denaturation; A, annealing; E, extension.

TABLE 3 Different *mgrB* lesions detected in the colistin-resistant strains and results of complementation experiments with strains representative of each type of lesion[a]

Strain	*mgrB* lesion[b]	Colistin MIC (μg/ml)	Colistin MIC after complementation (μg/ml)[c]	*pmrK* expression (mean ± SD)	*pmrK* expression after complementation (mean ± SD)	*phoQ* expression (mean ± SD)	*phoQ* expression after complementation (mean ± SD)
KKBO-1	WT	0.125	0.064	1	1.24 ± 0.51	1	0.93 ± 0.18
KP207-2	Insertional inactivation, IS*5*-like element at nt 75 (RW)	16	0.5	6.19 ± 0.19	1.34 ± 0.13	3.56 ± 0.15	0.92 ± 0.17
KP04C62	Insertional inactivation, IS*5*-like element at nt 126 (FW)	32	0.03	7.97 ± 0.12	1.81 ± 0.11	4.42 ± 0.25	1.46 ± 0.35
KP23C12	Insertional inactivation, IS*1F*-like element at nt 105 (FW)	32	0.25	8.54 ± 0.09	2.04 ± 0.23	8.81 ± 0.44	1.11 ± 0.14
KPIAC	t71a (L24H)	32	0.03	3.25 ± 0.23	1.87 ± 0.37	2.98 ± 0.77	1.24 ± 0.26
KP04C83	Δ18/27 (frameshift and premature termination)	32	0.03	5.15 ± 0.04	1.59 ± 0.34	7.23 ± 0.30	2.18 ± 0.45
KP06C01	g109a (G37S)	16	0.03	6.61 ± 0.07	1.97 ± 0.14	6.65 ± 0.21	0.48 ± 0.34
KP08C16	a7t (nonsense, premature termination)	32	0.03	5.44 ± 0.11	1.82 ± 0.22	4.22 ± 0.29	2.18 ± 0.33
KP10C06	Δg47 (frameshift and premature termination)	32	0.03	10.39 ± 1.12	1.10 ± 0.04	11.45 ± 1.23	1.08 ± 0.45
KP4707	g83a (C28Y)	32	0.03	9.51 ± 0.32	1.48 ± 0.20	14.02 ± 1.17	3.71 ± 0.75
KP12C48	Δ109/119 (frameshift and premature termination)	32	0.03	5.37 ± 0.15	1.69 ± 0.17	9.61 ± 0.55	1.59 ± 0.15
KP4553	Insertional inactivation, IS*Kpn14* at nt 124 (FW)	16	0.12	5.97 ± 0.18	1.99 ± 0.18	4.72 ± 0.35	1.20 ± 0.39
KPTRAV	Δ*mgrB* (from −400 to +599)	16	0.06	7.86 ± 0.87	2.08 ± 0.34	4.08 ± 0.23	0.97 ± 0.11
KPKL52MG11	Δ*mgrB locus*	8	0.12	3.26 ± 0.07	0.78 ± 0.24	7.54 ± 0.54	1.75 ± 0.65
KP4603	Δ*mgrB locus*	64	0.12	4.21 ± 0.13	1.77 ± 0.13	5.05 ± 0.05	1.02 ± 0.34
KP4623	Δ*mgrB locus*	64	0.12	5.75 ± 0.52	1.08 ± 0.36	4.51 ± 0.39	0.77 ± 0.43

[a] The expression levels of *pmrK* and *phoQ* genes (presented as fold increase) were normalized against the value obtained with the colistin-susceptible strain KKBO-1 (10).
[b] WT, wild type; for explanation of the nature of *mgrB* lesions, see Table 1.
[c] MIC testing of the complemented strains was performed in medium supplemented with 40 μg/ml of tetracycline, to avoid plasmid loss.

mgrB gene detected in colistin-resistant strains was investigated by complementation experiments with plasmid pACYC-*mgrB*, which carries a cloned copy of wild-type *mgrB* and its putative promoter. These experiments were carried out with 15 strains, representative for the various types of *mgrB* alteration, including the four isolates with large deletions (Table 3)

In all cases complementation with wild-type *mgrB* restored full colistin susceptibility and reduced the level of *pmrK* expression to a value similar to those of colistin-susceptible strains (Table 3), supporting a key role of the corresponding *mgrB* alterations in determining colistin resistance.

Distribution of the *mgrB* alterations associated with colistin resistance. The *mgrB* alterations associated with colistin resistance were detected in strains from 13 of the 19 Italian centers and from 4 of 4 Greek centers (Table 1, Fig. 1). Insertional inactivation by IS*5*-like elements was detected in strains from several different centers in Italy and Greece, while the other alterations exhibited a more restricted geographical distribution (Table 1).

Identical alterations were sometimes detected in strains of the same ST and PFGE profile from the same center (Table 1), suggesting the occurrence of clonal expansion and cross-transmission of the resistant strain within a center. However, identical alterations were also detected in strains of different STs isolated from different centers (Table 1), suggesting an independent origin of these alterations.

DISCUSSION

We have previously shown that inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the PhoQ-PhoP signaling system, is responsible for acquired colistin resistance in KPC-KP by activation of the Pmr system responsible for modification of the lipopolysaccharide polymyxin target (10). In this work, we investigated a large collection of nonreplicate KPC-KP strains of clinical origin obtained from several different Greek and Italian centers, and we found that the majority of them (39 of 66, 59%) carried alterations of the *mgrB* gene that were apparently responsible for the colistin-resistant phenotype. These findings underscore the role of this genetic mechanism in acquired colistin resistance by KPC-KP in the studied clinical setting. It will be of interest to investigate whether a similar situation is also found in other epidemiological settings.

A number of different genetic alterations were actually observed, including insertional inactivation by various insertion sequences, point mutations, and small or even large deletions of the *mgrB* locus. These findings reflect the occurrence of several independent mutational events and suggest that genetic alteration of *mgrB* may occur at relatively high frequency and without major consequences for fitness and virulence of the KPC-KP strain. A similar hypothesis is further underscored by the phenomena of clonal expansions of colistin-resistant strains carrying *mgrB* alterations observed in some centers and will be the subject of future investigation.

Altogether, inactivation of *mgrB* by insertion sequences, and especially by IS*5*-like elements, appeared to be the most common mechanism of *mgrB* alteration in KPC-KP. This observation reinforces the hypothesis regarding the existence of a specific hot spot for IS*5* insertion in the *mgrB* gene (10), although IS*5*-like elements were also detected at different positions and in different orientations. It will also be interesting to assess whether this mechanism of *mgrB* inactivation is facilitated by the presence of similar ISs on KPC-encoding plasmids.

Regarding the missense mutations in the *mgrB* gene, observed in 7 isolates, the complementation of the mutant restored the susceptibility to colistin in all cases, suggesting that the mutated protein was not or was only partially functional. However, given the experimental approach performed in this work (complementation was carried out with a plasmid-borne wild-type *mgrB* gene expressed under its own promoter), we cannot completely rule out that, in these cases, the mutated MgrB protein was functional and the resistance was due to an alteration of the *mgrB* promoter leading to downregulated expression.

Although many colistin-resistant strains exhibited an alteration of *mgrB*, more than one-third of them carried a wild-type *mgrB* gene, revealing that *mgrB* alterations are not the sole mechanism of colistin resistance in the clinical setting. Additional mechanisms of colistin resistance might be related to promoter mutations leading to a reduction of *mgrB* expression or to mutations in other loci leading to upregulation of the Pmr system responsible for modification of the lipopolysaccharide polymyxin target. A recent report on *in vitro* selection of colistin-resistant *K. pneumoniae* isolates has described the occurrence of *pmrB* and *phoQ* mutations associated with colistin-resistant phenotypes (18), although the role of the observed mutations was not experimentally confirmed. Other recent reports have demonstrated the role of *pmrB* mutations in colistin resistance among clinical isolates of KPC-KP (20, 21). Further studies are ongoing to characterize the colistin resistance mechanisms in strains carrying a wild-type *mgrB* gene.

Alterations of *mgrB* were associated with a relatively broad range of colistin MICs (4 to 64 μg/ml), and no significant differences in the distribution of colistin MICs were observed between strains carrying *mgrB* alterations or other resistance mechanisms. Further investigations will be necessary to understand how the expression of colistin resistance is modulated in mutants carrying similar or different resistance mechanisms.

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