The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*

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Objectives: Alterations in the PhoPQ two-component regulatory system may be associated with colistin resistance in *Klebsiella pneumoniae.* MgrB is a small transmembrane protein produced upon activation of the PhoPQ signalling system, and acts as a negative regulator on this system. We investigated the role of the MgrB protein as a source of colistin resistance in a series of *K. pneumoniae.*

Methods: Colistin-resistant *K. pneumoniae* isolates were recovered from hospitalized patients worldwide (France, Turkey, Colombia and South Africa). The *mgrB* gene was amplified and sequenced. A wild-type *mgrB* gene was cloned and the corresponding recombinant plasmid was used for complementation assays. Clonal diversity was evaluated by MLST and Diversilab analysis.

Results: Of 47 colistin-resistant isolates, 12 were identified as having a mutated *mgrB* gene. Five clonally unrelated isolates had an *mgrB* gene truncated by an IS5-like IS, while one clone also harboured an insertional inactivation at the exact same position of the *mgrB* gene, but with IS*Kpn13*. Another clone harboured an insertional inactivation due to IS*Kpn14* at another location of the *mgrB* gene. Two clonally related isolates harboured an IS (IS*10R*) in the promoter region of *mgrB*. Finally, three clonally unrelated isolates harboured substitutions leading to anticipated stop codon in the MgrB protein. Complementation assays with a wild-type MgrB protein restored full susceptibility to colistin for all colistin-resistant isolates identified with qualitative or quantitative MgrB modifications.

Conclusion: The inactivation or down-regulation of the *mgrB* gene was shown to be a source of colistin resistance in *K. pneumoniae*. Interestingly, identical genetic events were identified among clonally unrelated isolates.

Keywords: gene inactivation, PhoPQ regulatory system, polymyxin, *Klebsiella pneumoniae*

Introduction

Colistin and other polymyxins are cationic peptides synthetized by a Gram-positive species, namely *Paenibacillus polymyxa.* Those antibiotics act by disrupting the negatively charged outer membranes of Gram-negative bacteria.[1] Colistin is now often considered as the last option to treat multidrug-resistant Gram-negatives, and in particular carbapenemase-producing Enterobacteriaceae and *Acinetobacter baumannii.*[2] *Klebsiella pneumoniae* is the enterobacterial species in which acquisition of a carbapenemase is most frequently identified, and therefore multidrug-resistant *K. pneumoniae* are increasingly reported.[3]

Colistin is consequently increasingly used in endemic areas for carbapenemase-producing *K. pneumoniae*, and reports of colistin-resistant isolates in that species are on the rise.[4]

Alterations in the two-component regulatory systems PhoPQ and PmrAB,[5,6] as well as inactivation of the *mgrB* gene,[7,8] have been reported to be involved in polymyxin resistance in *K. pneumoniae*. The sensor kinase PhoQ is an integral membrane protein whose periplasmic domain is involved in signal detection. It is activated by low extracellular magnesium (Mg^{2+}), acidic pH (pH 5.5) or the presence of cationic antimicrobial peptides.[9,10] Upon activation, it activates PhoP by phosphorylation. Then PhoP activates PmrHFIJKLM expression, directly by binding to

the *pmrHFIJKLM* promoter, and indirectly via PmrD-dependent activation of the PmrA protein also binding to the *pmrHFIJKLM* promoter.[11] The *pmrHFIJKLM* operon (also named *pmrF*, *pbg* or *arn* operon) encodes enzymes responsible for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose (LAra4N). The addition of this cationic product (LAra4N) to lipid A leads to resistance to polymyxins by modification of the lipopolysaccharide target.[10]

MgrB, a small regulatory transmembrane protein of 47 amino acids, is produced upon activation of the PhoPQ signalling system.[12] Through interactions between the MgrB protein and the periplasmic domain of PhoQ, MgrB negatively regulates the PhoP/PhoQ system. MgrB spans the inner membrane and represses PhoP phosphorylation, probably by inhibiting the PhoQ kinase activity or stimulating phosphatase activity. Phosphorylation of PhoP increases transcription of the *mgrB* gene. MgrB is consequently part of a negative feedback loop in the PhoQ/PhoP signalling circuit.

Two recent pioneering studies showed that insertional inactivations in the *mgrB* gene were the source of acquired resistance to colistin in *K. pneumoniae*.[7,8] Such inactivation leads to an up-regulation of the PhoPQ system, leading to the up-regulation of the *pmrHFIJKLM* operon, and ultimately to colistin resistance.

The aim of our study was to analyse a collection of 47 colistin-resistant *K. pneumoniae* strains recovered from different geographical areas in order to identify whether there were alterations in the *mgrB* gene that may explain the resistance to colistin.

Material and methods

Bacterial strains

Forty-seven colistin-resistant *K. pneumoniae* clinical isolates recovered from hospitalized patients in France, Turkey, Colombia and South Africa were investigated. The isolates were identified using the API 20E system and the MALDI-TOF technique (bioMérieux, La Balme-les-Grottes, France).

Antimicrobial susceptibility assays

MICs were determined using Etest strip (bioMérieux) according to the manufacturer's instructions on Mueller–Hinton agar plates (BioRad, Marnes-la-Coquette, France) with a 0.5 McFarland inoculum in sterile water, 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). Following the EUCAST breakpoints, isolates with MICs of colistin ≤2 mg/L were categorized as susceptible, although those with MICs >2 mg/L were resistant.

PCR amplification and sequencing

Whole-cell DNA was extracted using the QIAquick kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The entire *mgrB* gene was amplified with specific oligonucleotide primers *mgrB*-extF (5′-TTAAGAAGGCCGTGCTATCC-3′, position −52 with respect to the *mgrB* start codon in order to include the promoter sequences) and *mgrB*-extR (5′-AAGGCGTTCATTCTACCACC-3′, located after the stop codon) as published.[7] PCR products were analysed by gel electrophoresis and sequenced with an automated ABI 3100 sequencer (Applied Biosystems,

Foster City, CA, USA). The nucleotide and deduced protein sequences were analysed at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool program. ISs were analysed using the IS finder web site (www-is.biotoul.fr). Detection of β-lactamase genes was performed with primers as described previously.[13]

Complementation assays

The entire *mgrB* gene from the colistin-susceptible *K. pneumoniae* reference strain ATCC 53153 (MIC at 0.125 mg/L) was amplified by PCR using the 2X Phusion HF Master Mix (Finnzymes, Life Technologies, Illkirch, France) and primers *mgrB*-extF/*mgrB*-extR.

The partial and therefore non-coding *mdh* sequence (supposed to encode a malate dehydrogenase) was PCR amplified with primers *mdh*-extF (5′-CCCAACTCGCTTCAGGTTCAG-3′)/*mdh*-extR (5′-CCGTTTTTCCCCAGCAGCAG-3′).

The amplified fragments were respectively cloned into the high-copy plasmid pCR-BluntII-TOPO (Invitrogen, Illkirch, France) and the resulting plasmids pTOPO-*mgrB* and pTOPO-*mdh* (encoding resistance to zeocin) were separately transformed into the colistin-resistant isolates by electroporation. Electrotransformants were selected by overnight incubation at 37°C on Mueller–Hinton agar supplemented with 100 mg/L of zeocin. The colistin MICs of the *K. pneumoniae* transformants were measured using Etest.

Since pCR-BluntII-TOPO is a high-copy plasmid that may lead to a too high overexpression of the corresponding cloned genes and therefore constitute a bias in our experiments, we modified this plasmid backbone by replacing its pUCOri replication origin locus with the p15a replication of the low-copy plasmid pACYC184, leading to a new plasmid backbone we named pLow-TOPO. The exact same inserts were inserted into that low-copy plasmid and further experiments performed as above.

Genotyping by MLST and Rep-PCR (Diversilab®)

MLST was carried out with seven standard housekeeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) according to Diancourt *et al.*[14] Sequence types were analysed using the database provided by the Institut Pasteur, Paris, France (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

DNA was extracted using UltraClean Microbial DNA Isolation kit (Mobio, Saint-Quentin-en-Yvelines, France). Genotyping was evaluated by rep-PCR, using the Diversilab *Klebsiella* kit (bioMérieux) following the manufacturer's recommendations. Amplicons were separated by microfluidic chip with the Agilent 2100 Bioanalyzer and the rep-PCR fingerprints were analysed with the coupled software.

Results and discussion

Analysis of the mgrB gene sequences

The entire *mgrB* gene of the 47 colistin-resistant isolates was amplified by PCR, and further sequence analysis of the amplicons showed that nine isolates generated a larger-in-size amplicon compared with a wild-type isolate. Identification of different fragment lengths compared with those obtained with a

colistin-susceptible strain (T1a) suggested the insertion of DNA sequences into the coding sequence or in the upstream vicinity of the *mgrB* gene in those nine isolates. Sequencing of the amplicons revealed that insertional inactivations had occurred at different positions into the *mgrB* gene in seven isolates and into the putative *mgrB* promoter in two isolates (Table 1). In addition, sequence analysis revealed a premature stop codon occurring in the sequence of *mgrB* for three additional isolates (Table 1).

Characteristics of isolates

The nine colistin-resistant isolates for which different-in-size amplicons were obtained were of different geographical origins, with six isolates from Colombia, two isolates from France and a single isolate from Turkey (Table 1). MICs of colistin for those isolates ranged from 4 to 24 mg/L (Table 1).

Truncation of mgrB by an IS5-like IS is a key mechanism leading to colistin resistance

In five isolates, the *mgrB* was truncated by the same 1195 bp IS. This IS was closely related (93% nucleotide identity) to IS5, with both transposases sharing 99% amino acid identity (3 substitutions out of 326 amino acids).[15] Surprisingly, the IS5-like insertion identified in the *mgrB* gene of those five distinct isolates always targeted the same location, between nucleotides 74 and 75 of the *mgrB* sequence, and was always in the same orientation (Table 1). Rep-PCR (Diversilab®) analysis revealed that four out of those five isolates were clonally unrelated, with only isolates C9 and C10 from Colombia being related, but different from all others (Figure 1a, Table 1). More surprisingly, this IS5-like element was identical to that recently identified in a colistin-resistant and KPC-3-producing *K. pneumoniae* isolate.[7] In that study, Cannatelli et al.[7] showed that the insertion of this IS5-like element into the *mgrB* gene was involved in resistance to colistin. Surprisingly, this IS5-like sequence was inserted at the exact same location compared with the isolates of our study, but in the opposite orientation (Figure 2b). A 4 bp target site duplication (TTAA), being the signature of the transposition event, was systematically identified at each extremity of the IS5-like element in all isolates (Figure 2b and c).

The colistin-susceptible isolate T1a and the resistant-colistin isolate T1b recovered from a same patient, respectively, before and after colistin therapy were found to be clonally related, as expected (Figure 1a, Table 1). Isolate T1a harboured a wild-type *mgrB* gene, indicating that insertion of the IS5-like element probably occurred *in vivo* and that the resistant isolate T1b had probably been selected under antibiotic selective pressure. Altogether, these data concur with the fact that the *mgrB* inactivation mediates acquired resistance to colistin.

Truncation of the mgrB gene by diverse ISs identified in colistin-resistant isolates

In isolate C21, an IS of 1148 bp named IS*Kpn13* and belonging to the IS5 family was identified at nucleotide position 74 (Figure 2d, Table 1), which corresponds to the exact same position where the IS5-like targeted the *mgrB* gene in five other isolates (see above). IS*Kpn13* shared <30% nucleotide identity with the IS5-like element, their respective transposase proteins sharing 56% amino acid identity.[16] Detailed analysis of their inverted repeats revealed that

Table 1. Features of the colistin-resistant isolates in which disruption of the *mgrB* gene was identified

| Isolate | Country of isolation | MICs (mg/L) by Etest | | | | | | Colistin susceptibility | *mgrB* sequence | Insertion site between | Carbapenemase | Additional ESBL | Sequence type | Clone |
		ERT	IPM	MEM	DOR	CST								
T1a	Turkey	3	12	>32	3	0.094	S	wild-type	—	KPC-2	none	ST258	I
T1b	Turkey	>32	>32	>32	>32	4	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	I
11I8	France	>32	>32	>32	>32	24	R	truncated by IS5-like	+74 and +75	KPC-3	none	ST258	II
20C9	France	>32	>32	>32	>32	16	R	truncated by IS5-like	+74 and +75	KPC-3	none	ST512	III
C9	Colombia	24	>32	>32	>32	12	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	IV
C10	Colombia	24	>32	>32	>32	8	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	IV
C21	Colombia	0.25	0.19	0.094	0.064	24	R	truncated by IS*Kpn13*	+74 and +75	none	CTX-M-2	ST1271	V
C22	Colombia	0.25	0.19	0.125	0.064	6	R	truncated by IS*Kpn14*	+127 and +128	none	CTX-M-15	ST101	VI
C1	Colombia	>32	>32	>32	>32	6	R	truncated by IS*10R*	−27 and −26	KPC-2	none	ST258	VII
C2	Colombia	>32	>32	>32	>32	8	R	truncated by IS*10R*	−27 and −26	KPC-2	none	ST258	VII
C11	Colombia	24	>32	>32	>32	12	R	truncated protein (29 aa)	—	KPC-2	none	ST258	VIII
15I5	France	3	>32	6	1.5	12	R	truncated protein (29 aa)	—	none	none	ST70	IX
Sa	France	>32	>32	>32	>32	24	R	truncated protein (27 aa)	—	KPC-2	none	ST258	X

ERT, ertapenem; IPM, imipenem; MEM, meropenem; DOR, doripenem; CST, colistin; R, resistant; S, susceptible.
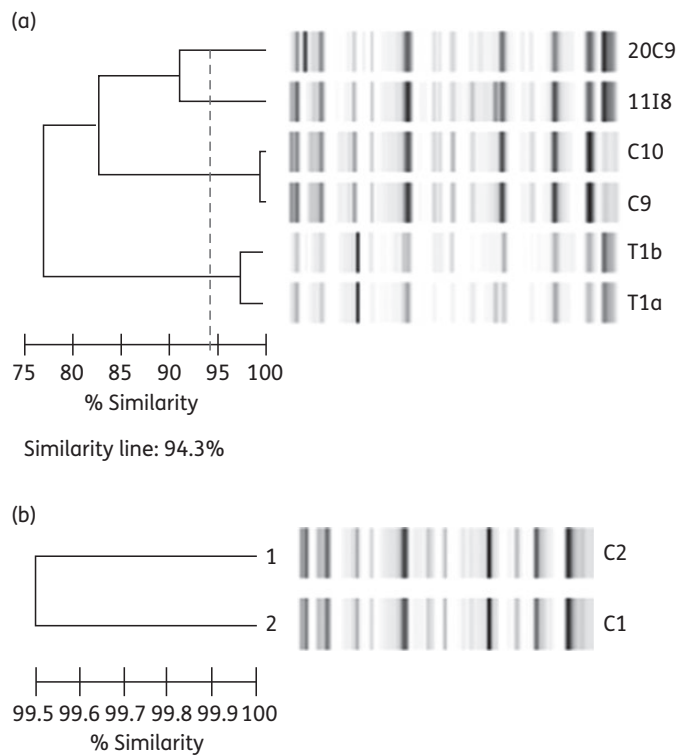
(a)

Similarity line: 94.3%

(b)

Figure 1. Dendrogram analysis and virtual gel images of rep-PCR fingerprint patterns of (a) the five *K. pneumoniae* isolates in which insertion of IS5-like into their *mgrB* gene has been identified; (b) the two *K. pneumoniae* isolates in which insertion of IS*10R* upstream of *mgrB* has been identified.

the first 11 bp were identical. In addition, the exact same target site duplication (TTAA) was identified on each extremity of IS*Kpn13*. Thus, nucleotide positions 74–75 in *mgrB* might constitute a hot spot of integration for ISs belonging to the IS*5* family.

In isolate C22, a truncation of the *mgrB* gene was also found. This truncation resulted from the insertion of IS*Kpn14*, a 768 bp IS belonging to the IS*1* family that does not share any significant identity with IS5-like elements.[17] That insertion occurred between nucleotide positions 127 and 128 in the *mgrB* gene, and generated a 9 bp target site duplication (CTATTAATA) (Figure 2e, Table 1).

Truncation of mgrB by a premature stop codon identified in three isolates

In three isolates, a premature stop codon was identified in the sequence of the *mgrB* gene, leading to truncated proteins. The full-length MgrB protein that is usually 47 amino acids in wild-type isolates was therefore only 29 amino acids long in two unrelated isolates (15I5 and C11), and it was 27 amino acids long in another isolate (Sa) (Table 1). Those amino acid substitutions were probably leading to a non-functional MgrB protein, and therefore were probably the source of colistin resistance.

Modification of the mgrB gene expression through the insertion of IS elements

In isolates C1 and C2, the IS*10R* element (also called IS*Vsa5*) was identified between nucleotide positions −27 and −26 when
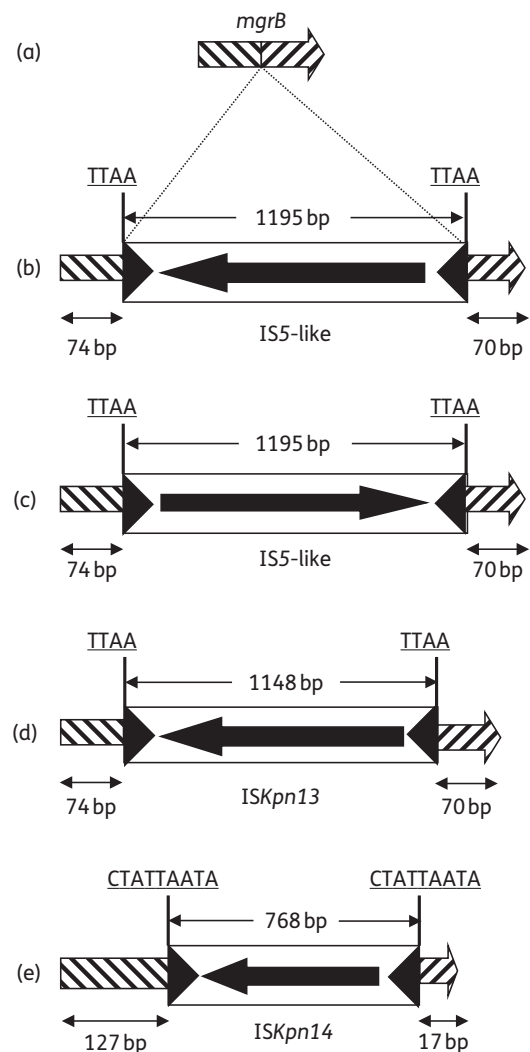
(a)

Figure 2. Schematic representation of the different insertion events identified in the *mgrB* gene. (a) The intact *mgrB* gene as found in wild-type isolates and isolate T1a; (b) *mgrB* truncated by IS5-like as identified by Cannatelli *et al.*[7]; (c) *mgrB* truncated by IS5-like in isolates 11I8, 20C9, C9, C10 and T1b; (d) *mgrB* truncated by IS*Kpn13* in isolate C21; (e) *mgrB* truncated by IS*Kpn14* in isolate C22. The target site duplications are underlined. The left and right inverted repeats of each IS are represented as black triangles.

referring to the start codon of *mgrB* as +1. This 1152 bp IS belongs to the IS*10* group, which itself belongs to the IS*4* family.[18] This suggested that insertion of IS*10R* might have influenced the expression of *mgrB* in those two isolates. Analysis of the genetic relationship of isolates C1 and C2 performed by rep-PCR (Diversilab®) showed that they were clonally related (Figure 1b, Table 1).

In silico analysis of the sequence located upstream of a wild-type *mgrB* gene revealed putative promoter sequences made from −35 [TTTTGA] and −10 [TAAACT] boxes separated by 17 bp (Figure 3). The insertion of IS*10R* actually targeted the DNA sequence located between the *mgrB* start codon and this promoter region. Therefore, it may be hypothesized that this insertion led to an inactivation of this promoter with respect to the

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TTATTTGTATGATCCCTGGCGTGATTTTGACACGAACACGTTTTGAAACAAGTCGATGATTCCTAAACTTACCTTTCGTAATACAG
                                        −35           PROM        −10
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↓

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TTAGCCGCGGTTTAAGAAGGCCGTGCTATCCTGGCGACATTGCGTACTGATGCGGAGAGTGGAGTG
                                                          -> start codon mgrB
```

Figure 3. Sequences located at the 5′-extremity of the *mgrB* gene. The putative promoter (*PROM*) is indicated, with its respective −35 and −10 boxes underlined. The arrow indicates the target site for insertion of IS*10R*. The *mgrB* start codon is in bold.

Table 2. MICs results obtained by Etest before and after complementation

Isolates	*mgrB* genotype	Colistin MIC (mg/L) before complementation	Colistin MIC (mg/L) after complementation with			
			pTOPO-*mdh*	pTOPO-*mgrB*	pLow-TOPO-*mdh*	pLow-TOPO-*mgrB*
T1b	IS in *mgrB*	4	4	0.047	4	0.064
C1	IS in *mgrB* promoter region	6	8	0.032	8	0.016
Sa	truncated protein	24	24	0.047	24	0.032

mgrB expression, the corresponding −35 and −10 sequences being too far distant from the gene (Figure 3). That might explain a decrease in or even lack of expression of the *mgrB* gene, leading to a low production of the corresponding protein and consequently the acquired resistance to colistin.

Complementation experiments

Complementation of three colistin-resistant isolates (one identified with an IS element inserted into the *mgrB* gene, one identified with an IS element inserted into the *mgrB* promoter region and one identified with a truncated *mgrB* gene) was performed. These three isolates were representative of the different isolates identified in this study. For that purpose, these three isolates were transformed with the high-copy recombinant plasmid pTOPO harbouring a wild-type *mgrB* gene (plasmid pTOPO-*mgrB*) in order to provide the wild-type MgrB protein *in trans*. As a result, a full susceptibility to colistin was fully recovered in those three isolates (Table 2). As expected, transformation with plasmid pTOPO-*mdh* used as negative control did not restore susceptibility to colistin. Similar if not identical results were actually obtained when using the low-copy plasmid pLow-TOPO harbouring the same inserts (Table 2). Those results confirmed that resistance to colistin was related to the lack of MgrB. Although we cannot exclude associated mechanisms in these *K. pneumoniae* isolates, results of this complementation assays assessed the role of MgrB in acquired colistin resistance.

Conclusions

The truncation of the *mgrB* gene was recently identified as a source of acquired colistin resistance in *K. pneumoniae*. Here we further assessed the critical role of modifications in MgrB production in colistin resistance. By identifying a series of truncated *mgrB* genes among colistin-resistant *K. pneumoniae* isolates, we further confirmed that this gene might be considered as a key target for development of colistin resistance in *K. pneumoniae*. It is noteworthy that eight of those isolates belonged to ST258, which is the most widespread clone producing KPC-type carbapenemase worldwide. Mutations were observed inside the coding sequence of the *mgrB* gene, but also in the promoter region, probably leading to a reduced expression of the *mgrB* gene in the latter case. Overall, those findings further confirm that MgrB makes a crucial contribution to the susceptibility to colistin usually observed in *K. pneumoniae*.

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Transparency declarations

None to declare.

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