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MgrB inactivation is responsible for acquired resistance to colistin in *Enterobacter hormaechei* subsp. *steigerwaltii*

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1 ABSTRACT

2 Multidrug resistant strains belonging to the *Enterobacter cloacae* complex (ECC) group, and
3 especially those belonging to clusters C-III, C-IV and C-VIII, have increasingly emerged as a
4 leading cause of healthcare-associated infections, with colistin used as one of the last line of
5 treatment. However, colistin-resistant ECC strains have emerged. The aim of this study was to
6 prove that MgrB, the negative regulator of PhoP/PhoQ two-component regulatory system, is
7 involved in colistin resistance in ECC of cluster C-VIII, formerly referred to as *Enterobacter*
8 *hormaechei* subsp. *steigerwaltii*. An *in vitro* mutant (Eh22-Mut) was selected from a clinical
9 isolate of Eh22. The sequencing analysis of its *mgrB* gene showed the presence of one
10 nucleotide deletion leading to the formation of a truncated protein of six instead of 47 amino
11 acids. Wild-type *mgrB* gene from Eh22, as well as that of a clinical strain of *Klebsiella*
12 *pneumoniae* used as controls, were cloned and the corresponding recombinant plasmids were
13 used for complementation assays. Results showed a fully restored susceptibility to colistin,
14 and confirmed for the first time that *mgrB* gene expression plays a key role in acquired
15 resistance to colistin in ECC strains.

INTRODUCTION

The genus *Enterobacter* is a member of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), and is grouped within the *Enterobacter cloacae* complex (ECC) group (1). In a seminal work, Hoffmann and Roggenkamp defined 13 genetic clusters (C-I to C-XIII) of ECC according to their *hsp60* sequences (*hsp*, for heat shock protein) (2). More recently, based on the analysis of 360 whole-genome sequences, ECC was placed into 18 phylogenomic groups (A to R) and two meta-clusters, *i.e.* *Enterobacter hormaechei* and *Enterobacter cloacae* (3, 4). At present, more than 3,000 *Enterobacter* genomes are available in GenBank databases and the taxonomy of this genus is still evolving (5). ECC species are opportunistic pathogens and, due to their natural and acquired resistance to many antibiotics, they are described as the leading cause of resistant nosocomial infections (1).

Colistin (polymyxin E) is considered as one of the last-resort therapeutic agents for treatment of multidrug-resistant Gram-negative bacteria (6). It is a cationic antimicrobial peptide that targets the anionic lipid A phosphate moiety of bacterial lipopolysaccharide (LPS), leading to an altered cell permeability and thereafter to cell death (6). The most common resistance mechanism in *Enterobacteriaceae* is attributed to the covalent modifications of LPS through the incorporation of positively charged groups such as phosphoethanolamine (pEtN) and 4-amino-4-deoxy-1-arabinose. These modifications neutralize the negative charges of LPS and subsequently reduce binding affinity of colistin to its target (6). Plasmid-mediated colistin resistance, *i.e.* MCR genes encoding pEtN transferases, were described (7, 8). However, colistin resistance mechanisms are mostly attributed to chromosomal modifications in two-component regulatory systems (TCRS), namely PmrA/PmrB and PhoP/PhoQ, which can cause constitutive expression of LPS modifications and consequently colistin resistance. Such chromosomal modifications were well described in *Klebsiella pneumoniae* (9, 10), but also in

E. cloacae complex (11). Moreover, substitution, disruption and inactivation changes in *mgrB* gene encoding for the negative regulator of PhoP/Q have been identified to play a prominent role in polymyxin resistance in clinical *K. pneumoniae* isolates (12–14). Very few reports have mentioned *mgrB* mutations and their possible role in colistin resistance of clinical *Enterobacter* spp. (15, 16), and no studies were performed to confirm its involvement in this genus.

Thus, the aim of this study was to analyze an *in vitro* mutant of *Enterobacter hormaechei* subsp. *steigerwaltii* containing a mutated *mgrB* gene, and to confirm its role in colistin resistance. Our procedure was validated by comparison of results with those obtained with a clinical colistin resistant *K. pneumoniae* deleted for *mgrB*.

RESULTS AND DISCUSSION

Eh22, a multidrug resistant strain of *E. hormaechei* subsp. *steigerwaltii*

A multidrug resistant strain of ECC (named Eh22) was isolated from a patient urine sample and provided by an urban health care system (community laboratory of Djerba, Tunisia). Analysis of the *hsp60* sequence revealed that Eh22 is included in the cluster C-VIII, referred to as *E. hormaechei* subsp. *steigerwaltii* (2, 17). Furthermore, *in silico* multilocus sequence typing (MLST) of seven house-keeping genes (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB* (18), *in silico* multilocus sequence analysis (MLSA) of four other target genes and/or other target gene regions (*gyrB*, *rpoB*, *infB*, and *atpD*) (19), as well as the nearly complete (1,412 bp) 16S rRNA gene sequence (20), confirmed this species assignation (Tables S1 and S2, Supplementary information). MLST analysis also showed that Eh22 belongs to the sequence type, ST177 which has a single-allele different from ST93, ST294 and ST828, similarly to strains of *E. hormaechei* subsp. *steigerwaltii* for those of which the whole-

genome sequencing had been performed (21, 22). *Enterobacter* spp. are one of the most common *Enterobacteriaceae* resistant to third-generation cephalosporins, along with *Escherichia coli* and *K. pneumoniae* (1). They are able to produce a low level of a chromosomal AmpC β -lactamase-type cephalosporinase that generates resistance to first-generation cephalosporins. *Enterobacter* spp. also often contain multiple resistance genes mediated by conjugative plasmids (1). In our study, Eh22 was multidrug resistant due, in part, to the presence of a conjugative plasmid of incompatibility group IncHI2, which carried various antibiotic resistance genes, including two extended-spectrum β -lactamases and one cephalosporinase gene (*bla*_{CTX-M-3}, *bla*_{SHV-12} and *bla*_{DHA-1}) (data not published, whole-plasmid sequencing in progress). Eh22 remained sensitive to carbapenems (data not shown) (data not shown). However, in recent years clinical ECC isolates resistant to carbapenems have been increasingly reported through the production of plasmid-mediated carbapenemases (1). In particular, *E. hormaechei* subsp. *steigerwaltii* strains of ST177 carrying the *bla*_{NDM-1} and *bla*_{KPC-2} carbapenemases were described (21, 22). Eh22 also remained sensitive to colistin (see below) and to fosfomycin (MIC, 32 mg/liter), despite the presence of the *fosA2* gene (with identical nucleotide sequence to that of GenBank accession number CP041733, except for a T393C silent mutation). FosA is widely distributed among various Gram-negative bacilli, including *Enterobacter* spp. (24).

Thus, colistin is considered as one of the last resort treatment against these multidrug-resistant bacteria. Unfortunately, colistin resistance in ECC has been increasingly reported and the resistance molecular mechanisms are less known than in *K. pneumoniae* (25). Very recently, *mgrB* mutations have been found in clinical strains of *E. cloacae* (15, 16). A missense mutation in MgrB (I10V) (13), as well as C39G, N42S, I45Y, W47V, W47S, *48K and *48Y changes were identified (12). Thus, it is important to confirm the role of MgrB in colistin

resistance, and particularly in isolates of the C-VIII cluster, which are most frequently recovered from human clinical samples, with those of clusters C-III and C-VI (25, 26).

Selection of an *mgrB* mutant from *E. hormaechei* subsp. *steigerwaltii*

The colistin MIC of Eh22 was 0.125 mg/liter without any heteroresistance phenomenon as described elsewhere (25). Using the Population Analysis Profile (PAP) analysis (ref Guérin), a colistin resistant mutant named Eh22-Mut was selected on agar medium containing 8 mg/liter of colistin with a frequency of 1×10^{-8} . The colistin MIC of Eh22-Mut was 32 mg/liter. Sequence analysis of *pmrA*, *pmrB*, *phoP* and *phoQ* genes did not reveal any differences between Eh22 and Eh22-Mut. In contrast, the amplified region of *mgrB* (353 pb) spanning from 113 pb upstream of the initiation codon to 96 pb downstream of the stop codon (**Fig. 1**), showed one adenine deletion in the *mgrB* open reading frame of Eh22-Mut, leading to a truncated protein of 6 amino acids (VKKYAG) instead of 47 for the wild type MgrB.

Detection of *mgrB* inactivation in a clinical strain of *K. pneumoniae*

The inactivation of the PhoQ/PhoP negative regulator encoded by the *mgrB* gene has been identified to play a prominent role in polymyxin resistance in clinical isolates of *K. pneumoniae* and *Klebsiella oxytoca* (13). In the literature, complementation assays with *mgrB* genes from wild-type *K. pneumoniae* or *K. oxytoca* restored full susceptibility to colistin, and confirm that MgrB expression was the key factor for this acquired resistance to colistin (12, 27, 28). These complementation assays with wild-type *mgrB* in *K. pneumoniae* were performed with recombinant plasmids as well as low or high copies, without any significant differences (27). However, to check our procedure of MgrB complementation in *E. subsp. steigerwaltii* *hormaechei* with recombinant constructions using a medium copy vector (pB-mfabI), we performed in parallel and under the same conditions, the *mgrB* complementation of *K. pneumoniae*. In a previous study, we have described two carbapenem-resistant strains of *K. pneumoniae*, Kp5196 and Kp5241, which were respectively susceptible and resistant to

colistin (29). Both strains, isolated from the same patient at a two month interval, belonged to the sequence type ST15 and were clonal as demonstrated by analysis of their pulsotype (29). At that time, molecular analysis of the colistin resistance mechanism of Kp5241 had not been studied. Thus, given the data from the literature, *pmrA/pmrB* and *phoP/phoQ* genes were amplified and sequenced as described elsewhere (12, 30, 31). No changes were detected in both TCRS. The *mgrB* locus was targeted using three pairs of primers (**Table S3, supplementary material**) designed to amplify the coding region and the regions flanking the *mgrB* gene (12). Kp5196 showed amplicons with expected sizes of 110, 250 and 1,507 bp (PCR amplifications with the primer pair mgrB_ext_F/mgrB_ext_R is depicted in **Fig. S1, supplementary material**). In contrast, Kp5241 yielded no amplification products with the former primers, suggesting a larger deletion of the locus carrying the *mgrB* gene (**Fig. S1**). Such large deletions have already been showed to be responsible for colistin resistance in *K. pneumoniae* (32).

Complementation of Eh22-Mut and Kp5241 with the wild-type *mgrB* gene

Lippa and Goulian identified MgrB homologues in the genome sequences of various enterobacteria (32). However, regulation of TCRS, PhoP/PhoQ and PmrA/pmrB, may be different according to *Enterobacteriaceae* species. Indeed, examination of the available genomes of ECC revealed the absence of an homologous gene encoding for the PmrD connector, suggesting no cross-talk between both TCRS (25). Thus, it is important to confirm the role of MgrB in ECC. In order to confirm whether the *mgrB* mutation found in Eh22-Mut is responsible for the colistin resistance in *E. hormaechei* subsp. *steigerwaltii*, the wild-type *mgrB* gene of Eh22 was inserted into pB-mfabI, giving the recombinant plasmid pB-mgrB/Eh which was used to transform Eh22-Mut. The colistin MICs of transformants showed a decreased resistance of 258-fold (0.062 mg/liter *versus* 16 mg/ liter without the *mgrB* gene or with the mutated *mgrB* gene) (**Table 1**). The decrease in MIC was comparable (290-fold, 0.11

mg/ liter vs 32 mg/ liter) for transformants from Kp5241 with the plasmid pB-mgrB/Kp (wild-type *mgrB* gene of Kp5196), allowing validation of our procedure (**Table 1**). Thus, our results show, for the first time, that MgrB is a key target for colistin resistance in *Enterobacter* species.

Comparison of the *mgrB* gene sequences

Alignment of MgrB sequences of Eh22 with 15 out of 18 ECC phylogroups (A to R) publicly available in Genbank databases (3), showed a high conservation of amino acids, in particular among the *E. hormaechei* meta-cluster to which Eh22 belongs (**Fig. 2A**)(4).

The alignment from colistin-susceptible *K. pneumoniae*, Kp5196 and *E. hormaechei*, Eh22, showed a difference of 12 amino acids with 74% identity and 93% similarity; the N-terminal sequence being the least conserved (**Fig. 2B**). This region with a hydrophobic stretch may function as a transmembrane domain (33). Chavda *et al.* (16) identified a missense mutation in MgrB (I10V), together with PmrA/PmrB modifications in *E. cloacae*, suggesting that these modifications may be responsible for colistin resistance (15, 16). Although a valine instead of an isoleucine is present in position 10 in the wild-type MgrB of *E. coli* K12 (**Fig. 2B**), we wanted to verify the possible consequence of such minor modifications in resistance acquisition (**Fig. 2**). As indicated in **Table 1**, the cross-complementation of *mgrB* wild-type genes between Eh22-Mut (with its truncated chromosomally-encoded MgrB of 6 amino acids) and Kp5241 (with its chromosomally-encoded deleted MgrB) restored similar colistin susceptibility in both species, confirming that the amino acid differences found between their respective MgrB have no impact on the structure and activity of the PhoP/PhoQ regulator system, and suggesting that the I10V change previously found in MgrB of *E. cloacae* is probably not the source of acquired resistance to colistin in *E. cloacae*.

Concluding remarks

Our data confirm for the first time the role of MgrB inactivation in colistin resistance in *E. hormaechei* subsp. *steigerwaltii* (cluster C-VIII); ECC being increasingly reported as the leading cause of resistant nosocomial infections. The knowledge of colistin resistance mechanisms is important because very few alternative therapeutic options are available for treatment of infection.

MATERIALS AND METHODS

Bacterial strains and plasmids

The ECC strain, Eh22 was identified by using the commercialized system Api20E gallery (bioMérieux), analysis by rDNA16S sequencing with the universal primers 27F/1492R (sequence overlapping the region from bases 71 to 1,482 of the complete sequence of 1,552 bp) (20), and the matrix-assisted laser desorption ionization–time of flight mass spectrometry identification (MALDI-TOF MS, Bruker Daltonics). Cluster *Enterobacter* membership was determined by partial sequencing of the *hsp60* gene (2, 17). The ST was determined using the genomic sequence to query the MLST database of *E. cloacae* (<http://pubmlst.org/ecloacae/>) (18), and MLSA was performed using the primers and conditions as described previously (19). Two previously described *K. pneumoniae*, Kp5196 and Kp5241, respectively susceptible and resistant to colistin, were included in our study (29). *E. coli* DH5 α was used for cloning and sub-cloning experiments of *mgrB* genes.

pB-mfabI is a pBR322 vector (4.4 kb, New England Biolabs), which carries the *mfabI* gene (mutant *fabI*) affecting the binding of triclosan to FabI allow the bacteria (*E. coli* or *E. cloacae* complex) to grow in the presence of 4 mg/liter of triclosan (34). The *mfabI* gene was inserted between the *ScaI* and *PvuI* restriction sites of the pBR322 vector, inactivating the β -

lactamase gene, *bla*_{TEM-1}. pBR322 is described in the literature as a medium copy vector (~15-20 copies by cell). The pGEM-T EasyTM vector (Promega) was used for PCR product cloning.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of colistin (Sigma Chemical co.) were determined by the microdilution reference method using Mueller–Hinton broth adjusted for divalent cations (CA-MHB, BioRad) in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<http://www.eucast.org/>). Fosfomycin MIC was determined by the E-test method (BioMérieux). Fosfomycin and colistin MICs were interpreted as indicated by the EUCAST breakpoint tables; For colistin MICs, isolates with MICs of ≤2 mg/liter were categorized as susceptible, whereas those with MICs of >2 mg/liter were categorized as resistant.

Selection of *in vitro* colistin mutants

Selection of mutants from Eh22 were carried out by plating a high inoculum (~ 10¹⁰ colony forming units, CFU) on Luria-Bertani (LB) agar plates containing 2 to 32 mg/liter of colistin, as previously described for the analysis of PAP, analysis, as described elsewhere (25).

PCR amplification, sequencing and plasmid purification

Plasmids were purified by using the Macherey-Nagel Nucleospin plasmid kit according to the supplier recommendations. PCR experiments of *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* genes using specific primers (Table S3, Supplementary information), and amplicons were verified by Sanger's sequencing (MWG operon society, <https://www.eurofinsgenomics.eu>). The nucleotide and deduced protein sequences were analyzed at the NCBI website (<http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Search Tool (BLAST) program.

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***mgrB* gene cloning and complementation experiments**

After ligation into the pGEM-T Easy™ vector, PCR products of *mgrB* obtained after amplification with primer pairs: EhmgrB_F113 and EhmgrB_R96 (for Eh22) and KpmgrB_F112 and KpmgrB_R88 (for Kp5196) (**Table S3**), were used to transform *E. coli* DH5α chemically competent cells. The selection of recombinant clones was carried out on LB agar medium supplemented with 100 mg/liter of ampicillin (Sigma Chemical Co.), 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Eurobio) and 80 μg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, MP Biomedical). After plasmid extraction and *Eco*RI digestion (Promega), fragments containing *mgrB* gene were purified on agarose gel and extracted using the “Gel and PCR clean-up” kit (Macherey-Nagel). The insert was cloned into the *Eco*RI site of the linearized and dephosphorylated plasmid pB-mfabI using T4 DNA ligase (Promega). After *E. coli* DH5α transformation, bacteria were selected on LB agar medium with tetracycline (15 mg/liter). Recombinant plasmids, with *mgrB* inserted in the same orientation, were verified by sequencing analysis using the primers Tetra-161_F and Tetra+95_R (**Table S3**). Eh22-Mut (colistin resistant mutant of Eh22) and Kp5241 were transformed with *mgrB* constructs using chemically competent cells prepared by CaCl₂ treatment, followed by heat shock. Transformants were selected on MH agar plates containing 4 mg/liter of triclosan.

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pulveris comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Syst Appl Microbiol* 36:309–319.

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TABLE and FOOTNOTES

Table 1. Colistin MIC of strains and transformants of *E. hormaechei* and *K. pneumoniae*.

Plasmid	Mean of colistin MIC (mg/liter)(SD) ^(a, b)			
	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>		<i>K. pneumoniae</i>	
	Eh22	Eh22-Mut	Kp5196	Kp5241
-	0.125 ^(c)	32 ^(c)	2 ^(c, d)	64 ^(c, d)
pB-mfabI	Nd	16 (0) ^(e)	Nd	32 (0)
pB- <i>mgrB</i> /Eh	Nd	0.062 (0.045) ^(f)	Nd	0.11 (0.026)
pB- <i>mgrB</i> /Kp	Nd	0.043 (0.023) ^(f)	Nd	0.11 (0.031)

^(a) Mean of at least three independent experiments.

^(b) SD, standard deviation indicated in parenthesis.

^(c) Values obtained without triclosan. For the other experiments, the values are obtained with a final concentration of 0.5 mg/liter of triclosan used for plasmid maintenance.

^(d) Values in accordance with Arpin *et al.* (29).

^(e) Plasmid without or with mutated *mgrB*.

^(f) Non-significant differences (*P*-value of 0.2) by using a Student unpaired bilateral t-test.

- = without plasmid.

Nd = not determined.

FIGURE LEGENDS

FIG 1. Sequences of the Eh22 *mgrB* open reading frame and upstream/downstream region -113/+96

The stretch of seven adenines is boxed (a stretch of six adenines was obtained for Eh22-Mut, data not shown). The *mgrB* start codon is indicated in bold, the sequences of the -10 hexamer of the putative promoter and ribosome-binding site (RBS) are underlined. The open reading frame of MgrB is showed in italic.

FIG 2. MgrB alignment comparison of Eh22.

(A) MgrB alignments of Eh22 with peptidic sequences from 15 out of 18 *Enterobacter cloacae* complex groups as defined by Chavda *et al.* (3).

MgrB sequences used for alignments are selected as follows: group, name of species, name of strain (strain type, when available, is designated by the superscript T), and GenBank accession number.

In *E. hormaechei* meta-cluster (A to E groups): A, *E. xiangfangensis*, LMG 27195^T, NZ_CP017183; B, *E. hormaechei steigerwaltii*, DSM 16691^T, NZ_CP017179; C, *E. hormaechei oharae*, DSM 16687^T, NZ_CP017180; D, *E. hormaechei hoffmannii*, DSM 14563^T, NZ_CP017186; E, *E. hormaechei*, ATCC 49162, MKEQ01000000.

In *E. cloacae* meta-cluster (G to N, P and Q groups): G, *E. cloacae cloacae*, ATCC 13047, NC_014121; H, *E. cloacae dissolvens*, SDM, NC_018079; I, *E. cloacae ludwigii*, EN-119, CP017279; J, *E. cloacae asburiae*, ATCC 35953, CP011863; K, *E. cloacae complex*, DC4, AZUB01000000; L, *E. cloacae complex*, BWH 43, JMUR01000000; M, *E. cloacae complex*, DSM 16690^T, CP017184.1; N, *E. cloacae complex*, SY-70, NZ_JALR01000000; P, *E. cloacae complex*, JD8715, JDWG01000000; Q, *E. kobei*, DSM 13645^T, CP017181.

In *E. hormaechei* meta-cluster, three groups have unavailable MgrB sequences: F (*E. mori*), O and R (*E. cloacae complex*).

(B) MgrB alignments of *E. coli* K12, Eh22 and *K. pneumoniae* Kp5196.

. The putative transmembrane domain of *E. coli* MgrB is underlined according to Lipka et Goulian (33), and the amino acid at position 10 is indicated in bold.

The MgrB sequences of Eh22 (**A**) and *E. coli* K12 (**B**) are given and only different residues are shown for the other alignments.