MgrB Inactivation Is Responsible for Acquired Resistance to Colistin in Enterobacter hormaechei subsp. steigerwaltii

Amel Mhaya, Dominique Bégu, Slim Tounsi, Corinne Arpin

▶ **To cite this version:**

Amel Mhaya, Dominique Bégu, Slim Tounsi, Corinne Arpin. MgrB Inactivation Is Responsible for Acquired Resistance to Colistin in Enterobacter hormaechei subsp. steigerwaltii. Antimicrobial Agents and Chemotherapy, 2020, 64 (6), pp.e00128-20. 10.1128/AAC.00128-20 . hal-02980500

HAL Id: hal-02980500
https://cnrs.hal.science/hal-02980500

Submitted on 6 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Submission to *Antimicrobial Agents and Chemotherapy*

Section: ''Mechanisms of resistance''

Date of submission: 17 January 2020

Date of re-submission: 27 March 2020

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

Authors: Amel MHAYA[a,b], Dominique BÉGU[a], Slim TOUNSI[b], Corinne ARPIN[a,#]

Adresses and authors' affiliations:

[a], Laboratoire de Microbiologie Fondamentale et Pathogénicité (MFP), Université de Bordeaux, CNRS UMR-5234, Bordeaux, France

[b], Laboratoire des Biopesticides, Centre de Biotechnologie de Sfax, BP 1177, Sfax, Tunisia

[#], **Corresponding author:** Corinne ARPIN

Electronic mail address: corinne.arpin@u-bordeaux.fr

Running title: MgrB inactivation in *Enterobacter* spp.

Keywords: *Enterobacter cloacae* complex, *Enterobacter hormaechei* subsp. *steigerwaltii*, colistin resistance, MgrB, PhoPQ regulatory system.

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 g*ene 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.

16 **INTRODUCTION**

17 The genus *Enterobacter* is a member of the ESKAPE group (*Enterococcus faecium*,
18 *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*
19 *aeruginosa*, and *Enterobacter* spp.), and is grouped within the *Enterobacter cloacae* complex
20 (ECC) group (1). In a seminal work, Hoffmann and Roggenkamp defined 13 genetic clusters
21 (C-I to C-XIII) of ECC according to their *hsp60* sequences (*hsp*, for heat shock protein) (2).
22 More recently, based on the analysis of 360 whole-genome sequences, ECC was placed into
23 18 phylogenomic groups (A to R) and two meta-clusters, *i.e*. *Enterobacter hormaechei* and
24 *Enterobacter cloacae* (3, 4). At present, more than 3,000 *Enterobacter* genomes are available
25 in GenBank databases and the taxonomy of this genus is still evolving (5). ECC species are
26 opportunistic pathogens and, due to their natural and acquired resistance to many antibiotics,
27 they are described as the leading cause of resistant nosocomial infections (1).

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

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

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

51

52 RESULTS AND DISCUSSION

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

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

65 genome sequencing had been performed (21, 22). *Enterobacter* spp. are one of the most
66 common *Enterobacteriaceae* resistant to third-generation cephalosporins, along with
67 *Escherichia coli* and *K. pneumoniae* (1). They are able to produce a low level of a
68 chromosomal AmpC β-lactamase-type cephalosporinase that generates resistance to first-
69 generation cephalosporins. *Enterobacter* spp. also often contain multiple resistance genes
70 mediated by conjugative plasmids (1). In our study, Eh22 was multidrug resistant due, in part,
71 to the presence of a conjugative plasmid of incompatibility group IncHI2, which carried
72 various antibiotic resistance genes, including two extended-spectrum β-lactamases and one
73 cephalosporinase gene (*bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{SHV\text{-}12}$ and *bla*$_{DHA\text{-}1}$) (data not published, whole-plasmid
74 sequencing in progress). Eh22 remained sensitive to carbapenems (data not shown) (data not
75 shown).. However, in recent years clinical ECC isolates resistant to carbapenems have been
76 increasingly reported through the production of plasmid-mediated carbapenemases (1). In
77 particular, *E. hormaechei* subsp. *steigerwaltii* strains of ST177 carrying the *bla*$_{NDM\text{-}1}$ and
78 *bla*$_{KPC\text{-}2}$ carbapenemases were described (21, 22). Eh22 also remained sensitive to colistin
79 (see below) and to fosfomycin (MIC, 32 mg/liter), despite the presence of the *fosA2* gene
80 (with identical nucleotide sequence to that of GenBank accession number CP041733, except
81 for a T393C silent mutation). FosA is widely distributed among various Gram-negative
82 bacilli, including *Enterobacter* spp. (24).
83 Thus, colistin is considered as one of the last resort treatment against these multidrug-resistant
84 bacteria. Unfortunately, colistin resistance in ECC has been increasingly reported and the
85 resistance molecular mechanisms are less known than in *K. pneumoniae* (25). Very recently,
86 *mgrB* mutations have been found in clinical strains of *E. cloacae* (15, 16). A missense
87 mutation in MgrB (I10V) (13), as well as C39G, N42S, I45Y, W47V, W47S, *48K and *48Y
88 changes were identified (12). Thus, it is important to confirm the role of MgrB in colistin

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

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

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

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

102 The inactivation of the PhoQ/PhoP negative regulator encoded by the *mgrB* gene has been
103 identified to play a prominent role in polymyxin resistance in clinical isolates of *K.*
104 *pneumoniae* and *Klebsiella oxytoca* (13). In the literature, complementation assays with *mgrB*
105 genes from wild-type *K. pneumoniae* or *K. oxytoca* restored full susceptibility to colistin, and
106 confirm that MgrB expression was the key factor for this acquired resistance to colistin (12,
107 27, 28). These complementation assays with wild-type *mgrB* in *K. pneumoniae* were
108 performed with recombinant plasmids as well as low or high copies, without any significant
109 differences (27). However, to check our procedure of MgrB complementation in *E.* subsp.
110 *steigerwaltii* *hormaechei* with recombinant constructions using a medium copy vector (pB-
111 mfabI), we performed in parallel and under the same conditions, the *mgrB* complementation
112 of *K. pneumoniae*. In a previous study, we have described two carbapenem-resistant strains of
113 *K. pneumoniae*, Kp5196 and Kp5241, which were respectively susceptible and resistant to

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

127 **Complementation of Eh22-Mut and Kp5241with the wild-type *mgrB* gene**

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

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

143 **Comparison of the *mgrB* gene sequences**

144 Alignment of MgrB sequences of Eh22 with 15 out of 18 ECC phylogroups (A to R) publicly
145 available in Genbank databases (3), showed a high conservation of amino acids, in particular
146 among the *E. hormaechei* meta-cluster to which Eh22 belongs (**Fig. 2A**)(4).
147 The alignment from colistin-susceptible *K. pneumoniae*, Kp5196 and *E. hormaechei*, Eh22,
148 showed a difference of 12 amino acids with 74% identity and 93% similarity; the N-terminal
149 sequence being the least conserved (**Fig. 2B**). This region with a hydrophobic stretch may
150 function as a transmembrane domain (33). Chavda *et al.* (16) identified a missense mutation
151 in MgrB (I10V), together with PmrA/PmrB modifications in *E. cloacae*, suggesting that these
152 modifications may be responsible for colistin resistance (15, 16). Although a valine instead of
153 an isoleucine is present in position 10 in the wild-type MgrB of *E. coli* K12 **(Fig. 2B)**, we
154 wanted to verify the possible consequence of such minor modifications in resistance
155 acquisition ~~(**Fig. 2**)~~. As indicated in **Table 1**, the cross-complementation of *mgrB* wild-type
156 genes between Eh22-Mut (with its truncated chromosomally-encoded MgrB of 6 amino acids)
157 and Kp5241 (with its chromosomally-encoded deleted MgrB) restored similar colistin
158 susceptibility in both species, confirming that the amino acid differences found between their
159 respective MgrB have no impact on the structure and activity of the PhoP/PhoQ regulator
160 system, and suggesting that the I10V change previously found in MgrB of *E. cloacae* is
161 probably not the source of acquired resistance to colistin in *E. cloacae.*
162
163

164 **Concluding remarks**

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

170

171 **MATERIALS AND METHODS**

172 **Bacterial strains and plasmids**

173 The ECC strain, Eh22 was identified by using the commercialized system Api20E gallery
174 (bioMerieux), analysis by rDNA16S sequencing with the universal primers 27F/1492R
175 (sequence overlapping the region from bases 71 to 1,482 of the complete sequence of 1,552
176 bp) (20), and the matrix-assisted laser desorption ionization–time of flight mass spectrometry
177 identification (MALDI-TOF MS, Bruker Daltonics). Cluster *Enterobacter* membership was
178 determined by partial sequencing of the *hsp60* gene (2, 17). The ST was determined using the
179 genomic sequence to query the MLST database of *E. cloacae* (http://pubmlst.org/ecloacae/)
180 (18), and MLSA was performed using the primers and conditions as described previously
181 (19). Two previously described *K. pneumoniae*, Kp5196 and Kp5241, respectively susceptible
182 and resistant to colistin, were included in our study (29). *E. coli* DH5α was used for cloning
183 and sub-cloning experiments of *mgrB* genes.

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

188 lactamase gene, bla_{TEM-1}. pBR322 is described in the literature as a medium copy vector (~
189 15-20 copies by cell). The pGEM-T Easy™ vector (Promega) was used for PCR product
190 cloning.

191 **Antimicrobial susceptibility testing**

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

200 **Selection of *in vitro* colistin mutants**

201 Selection of mutants from Eh22 were carried out by plating a high inoculum (~ 10^{10} colony
202 forming units, CFU) on Luria-Bertani (LB) agar plates containing 2 to 32 mg/liter of colistin,
203 as previously described for the analysis of PAP, analysis, as described elsewhere (25).

204 **PCR amplification, sequencing and plasmid purification**

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

.

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 $CaCl_2$ treatment, followed by heat shock. Transformants were selected on MH agar plates containing 4 mg/liter of triclosan.

ACKNOWLEDGMENTS

We warmly thank Sabine Aillerie and Nathalie Peyron for their technical assistance. We thank Véronique Dubois for allowing us access to the device (MALDI-TOF) of the Bacteriology Laboratory of University Hospital of Bordeaux. We thank Christine Reix for the English correction.

236 This work was supported by the University of Bordeaux and the National Center of Scientific
237 Research National (France), and by the Ministry of Higher Education and Research Scientific
238 (Tunisia).

239 We have no relevant financial disclosures or funding to declare.

240 **REFERENCES**

241 1. Davin-Regli A, Lavigne J-P, Pagès J-M. 2019. Enterobacter spp.: Update on Taxonomy,
242 Clinical Aspects, and Emerging Antimicrobial Resistance. Clin Microbiol Rev 32.

243 2. Hoffmann H, Roggenkamp A. 2003. Population genetics of the nomenspecies
244 Enterobacter cloacae. Appl Environ Microbiol 69:5306–5318.

245 3. Chavda KD, Chen L, Fouts DE, Sutton G, Brinkac L, Jenkins SG, Bonomo RA, Adams
246 MD, Kreiswirth BN. 2016. Comprehensive Genome Analysis of Carbapenemase-
247 Producing Enterobacter spp.: New Insights into Phylogeny, Population Structure, and
248 Resistance Mechanisms. mBio 7.

249 4. Beyrouthy R, Barets M, Marion E, Dananché C, Dauwalder O, Robin F, Gauthier L,
250 Jousset A, Dortet L, Guérin F, Bénet T, Cassier P, Vanhems P, Bonnet R. 2018. Novel
251 Enterobacter Lineage as Leading Cause of Nosocomial Outbreak Involving
252 Carbapenemase-Producing Strains. 8. Emerging Infect Dis 24:1505–1515.

253 5. Wang C, Wu W, Wei L, Feng Y, Kang M, Xie Y, Zong Z. 2020. Enterobacter
254 wuhouensis sp. nov. and Enterobacter quasihormaechei sp. nov. recovered from human
255 sputum. Int J Syst Evol Microbiol 70:874–881.

256 6. Baron S, Hadjadj L, Rolain J-M, Olaitan AO. 2016. Molecular mechanisms of polymyxin
257 resistance: knowns and unknowns. Int J Antimicrob Agents 48:583–591.

258 7. Kieffer N, Royer G, Decousser J-W, Bourrel A-S, Palmieri M, Ortiz De La Rosa J-M,
259 Jacquier H, Denamur E, Nordmann P, Poirel L. 2019. mcr-9, an Inducible Gene Encoding
260 an Acquired Phosphoethanolamine Transferase in Escherichia coli, and Its Origin.
261 Antimicrob Agents Chemother 63.

262 8. Yuan Y, Li Y, Wang G, Li C, Xiang L, She J, Yang Y, Zhong F, Zhang L. 2019.
263 Coproduction Of MCR-9 And NDM-1 By Colistin-Resistant Enterobacter hormaechei
264 Isolated From Bloodstream Infection. Infect Drug Resist 12:2979–2985.

265 9. Baron S, Hadjadj L, Rolain J-M, Olaitan AO. 2016. Molecular mechanisms of polymyxin
266 resistance: knowns and unknowns. 6. Int J Antimicrob Agents 48:583–591.

267 10. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P,
268 Rossolini GM. 2013. In vivo emergence of colistin resistance in Klebsiella pneumoniae
269 producing KPC-type carbapenemases mediated by insertional inactivation of the
270 PhoQ/PhoP mgrB regulator. 11. Antimicrob Agents Chemother 57:5521–5526.

271 11. Kang KN, Klein DR, Kazi MI, Guérin F, Cattoir V, Brodbelt JS, Boll JM. 2019. Colistin
272 heteroresistance in Enterobacter cloacae is regulated by PhoPQ-dependent 4-amino-4-
273 deoxy-l-arabinose addition to lipid A. Mol Microbiol 111:1604–1616.

274 12. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K,
275 Vatopoulos A, Rossolini GM, COLGRIT Study Group. 2014. MgrB inactivation is a
276 common mechanism of colistin resistance in KPC-producing Klebsiella pneumoniae of
277 clinical origin. Antimicrob Agents Chemother 58:5696–5703.

278 13. Olaitan AO, Morand S, Rolain J-M. 2014. Mechanisms of polymyxin resistance: acquired
279 and intrinsic resistance in bacteria. Front Microbiol 5.

280 14. Jayol A, Poirel L, Dortet L, Nordmann P. 2016. National survey of colistin resistance
281 among carbapenemase-producing Enterobacteriaceae and outbreak caused by colistin-
282 resistant OXA-48-producing Klebsiella pneumoniae, France, 2014. Euro Surveill 21.

283 15. Nawfal Dagher T, Al-Bayssari C, Chabou S, Baron S, Hadjadj L, Diene SM, Azar E,
284 Rolain J-M. 2019. Intestinal Carriage of Colistin Resistant Enterobacteriaceae at Saint
285 Georges Hospital in Lebanon. J Glob Antimicrob Resist.

286 16. Chavda B, Lv J, Hou M, Chavda KD, Kreiswirth BN, Feng Y, Chen L, Yu F. 2018.
287 Coidentification of mcr-4.3 and blaNDM-1 in a Clinical Enterobacter cloacae Isolate from
288 China. Antimicrob Agents Chemother 62.

289 17. Hoffmann H, Stindl S, Ludwig W, Stumpf A, Mehlen A, Monget D, Pierard D, Ziesing S,
290 Heesemann J, Roggenkamp A, Schleifer KH. 2005. Enterobacter hormaechei subsp.
291 oharae subsp. nov., E. hormaechei subsp. hormaechei comb. nov., and E. hormaechei
292 subsp. steigerwaltii subsp. nov., three new subspecies of clinical importance. J Clin
293 Microbiol 43:3297–3303.

294 18. Miyoshi-Akiyama T, Hayakawa K, Ohmagari N, Shimojima M, Kirikae T. 2013.
295 Multilocus sequence typing (MLST) for characterization of Enterobacter cloacae. PLoS
296 ONE 8:e66358.

297 19. Brady C, Cleenwerck I, Venter S, Coutinho T, De Vos P. 2013. Taxonomic evaluation of
298 the genus Enterobacter based on multilocus sequence analysis (MLSA): proposal to
299 reclassify E. nimipressuralis and E. amnigenus into Lelliottia gen. nov. as Lelliottia
300 nimipressuralis comb. nov. and Lelliottia amnigena comb. nov., respectively, E. gergoviae
301 and E. pyrinus into Pluralibacter gen. nov. as Pluralibacter gergoviae comb. nov. and
302 Pluralibacter pyrinus comb. nov., respectively, E. cowanii, E. radicincitans, E. oryzae and
303 E. arachidis into Kosakonia gen. nov. as Kosakonia cowanii comb. nov., Kosakonia
304 radicincitans comb. nov., Kosakonia oryzae comb. nov. and Kosakonia arachidis comb.
305 nov., respectively, and E. turicensis, E. helveticus and E. pulveris into Cronobacter as
306 Cronobacter zurichensis nom. nov., Cronobacter helveticus comb. nov. and Cronobacter

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

309 20. Usadel, B. 16S/23S rRNA sequencing, p. 115–175. *In* Nucleic acid techniques in bacterial
310 systematics.Stackebrandt E and Goodfellow M (editors). John Wiley and Sons,
311 Chichester, UK.

312 21. Yang B, Feng Y, McNally A, Zong Z. 2018. Occurrence of Enterobacter hormaechei
313 carrying blaNDM-1 and blaKPC-2 in China. Diagn Microbiol Infect Dis 90:139–142.

314 22. Wu W, Feng Y, Carattoli A, Zong Z. 2015. Characterization of an Enterobacter cloacae
315 Strain Producing both KPC and NDM Carbapenemases by Whole-Genome Sequencing.
316 Antimicrob Agents Chemother 59:6625–6628.

317 23. Huang L, Hu YY, Zhang R. 2017. Prevalence of fosfomycin resistance and plasmid-
318 mediated fosfomycin-modifying enzymes among carbapenem-resistant
319 Enterobacteriaceae in Zhejiang, China. J Med Microbiol 66:1332–1334.

320 24. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, Shanks
321 RMQ, Sluis-Cremer N, Doi Y. 2017. Widespread Fosfomycin Resistance in Gram-
322 Negative Bacteria Attributable to the Chromosomal fosA Gene. mBio 8.

323 25. Guérin F, Isnard C, Sinel C, Morand P, Dhalluin A, Cattoir V, Giard J-C. 2016. Cluster-
324 dependent colistin hetero-resistance in *Enterobacter cloacae* complex. J Antimicrob
325 Chemother 71:3058–3061.

326 26. Morand PC, Billoet A, Rottman M, Sivadon-Tardy V, Eyrolle L, Jeanne L, Tazi A,
327 Anract P, Courpied J-P, Poyart C, Dumaine V. 2009. Specific distribution within the

328 Enterobacter cloacae complex of strains isolated from infected orthopedic implants. 8. J
329 Clin Microbiol 47:2489–2495.

330 27. Poirel L, Jayol A, Bontron S, Villegas M-V, Ozdamar M, Türkoglu S, Nordmann P. 2015.
331 The mgrB gene as a key target for acquired resistance to colistin in Klebsiella
332 pneumoniae. J Antimicrob Chemother 70:75–80.

333 28. Jayol A, Poirel L, Villegas M-V, Nordmann P. 2015. Modulation of mgrB gene
334 expression as a source of colistin resistance in Klebsiella oxytoca. 1. Int J Antimicrob
335 Agents 46:108–110.

336 29. Arpin C, Noury P, Boraud D, Coulange L, Manetti A, André C, M'Zali F, Quentin C.
337 2012. NDM-1-producing Klebsiella pneumoniae resistant to colistin in a French
338 community patient without history of foreign travel. Antimicrob Agents Chemother
339 56:3432–3434.

340 30. Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P,
341 Rossolini GM. 2013. In vivo emergence of colistin resistance in Klebsiella pneumoniae
342 producing KPC-type carbapenemases mediated by insertional inactivation of the
343 PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother 57:5521–5526.

344 31. Jayol A, Poirel L, Brink A, Villegas M-V, Yilmaz M, Nordmann P. 2014. Resistance to
345 colistin associated with a single amino acid change in protein PmrB among Klebsiella
346 pneumoniae isolates of worldwide origin. Antimicrob Agents Chemother 58:4762–4766.

347 32. Lippa AM, Goulian M. 2009. Feedback Inhibition in the PhoQ/PhoP Signaling System by
348 a Membrane Peptide. PLoS Genet 5.

349 33. Lippa AM, Goulian M. 2009. Feedback Inhibition in the PhoQ/PhoP Signaling System by
350 a Membrane Peptide. PLoS Genet 5.

351 34. Jang C-W, Magnuson T. 2013. A novel selection marker for efficient DNA cloning and
352 recombineering in E. coli. PLoS ONE 8:e57075.

353

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)]			
	E. hormaechei subp. *steigerwaltii*		*K. pneumoniae*	
	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-*mgrB*/Eh	Nd	0.062 (0.045) [(f)]	Nd	0.11 (0.026)
pB-*mgrB*/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 Lippa 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.