

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

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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.

## 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-1-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  $\beta$ -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  $\beta$ -lactamases and one  
73 cephalosporinase gene (*bla*<sub>CTX-M-3</sub>, *bla*<sub>SHV-12</sub> and *bla*<sub>DHA-1</sub>) (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*<sub>NDM-1</sub> and  
78 *bla*<sub>KPC-2</sub> 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  $1 \times 10^{-8}$ . 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 Kp5241 with 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* DH5 $\alpha$  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 *ScaI* and *PvuI* restriction sites of the pBR322 vector, inactivating the  $\beta$ -

188 lactamase gene, *bla*<sub>TEM-1</sub>. pBR322 is described in the literature as a medium copy vector (~  
189 15-20 copies by cell). The pGEM-T Easy<sup>TM</sup> 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<sup>10</sup> 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.

212 .

### 213 ***mgrB* gene cloning and complementation experiments**

214 After ligation into the pGEM-T Easy™ vector, PCR products of *mgrB* obtained after  
215 amplification with primer pairs: EhmgrB\_F113 and EhmgrB\_R96 (for Eh22) and  
216 KpmgrB\_F112 and KpmgrB\_R88 (for Kp5196) (**Table S3**), were used to transform *E. coli*  
217 DH5 $\alpha$  chemically competent cells. The selection of recombinant clones was carried out on LB  
218 agar medium supplemented with 100 mg/liter of ampicillin (Sigma Chemical Co.), 0.5 mM of  
219 isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG, Eurobio) and 80  $\mu$ g/mL of 5-bromo-4-chloro-3-  
220 indolyl- $\beta$ -D-galactopyranoside (X-Gal, MP Biomedical). After plasmid extraction and *EcoRI*  
221 digestion (Promega), fragments containing *mgrB* gene were purified on agarose gel and  
222 extracted using the “Gel and PCR clean-up” kit (Macherey-Nagel). The insert was cloned into  
223 the *EcoRI* site of the linearized and dephosphorylated plasmid pB-mfabI using T4 DNA  
224 ligase (Promega). After *E. coli* DH5 $\alpha$  transformation, bacteria were selected on LB agar  
225 medium with tetracycline (15 mg/liter). Recombinant plasmids, with *mgrB* inserted in the  
226 same orientation, were verified by sequencing analysis using the primers Tetra-161\_F and  
227 Tetra+95\_R (**Table S3**). Eh22-Mut (colistin resistant mutant of Eh22) and Kp5241 were  
228 transformed with *mgrB* constructs using chemically competent cells prepared by CaCl<sub>2</sub>  
229 treatment, followed by heat shock. Transformants were selected on MH agar plates containing  
230 4 mg/liter of triclosan.

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

<sup>(a)</sup> Mean of at least three independent experiments.

<sup>(b)</sup> SD, standard deviation indicated in parenthesis.

<sup>(c)</sup> 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.

<sup>(d)</sup> Values in accordance with Arpin *et al.* (29).

<sup>(e)</sup> Plasmid without or with mutated *mgrB*.

<sup>(f)</sup> 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<sup>T</sup>, NZ\_CP017183; B, *E. hormaechei steigerwaltii*, DSM 16691<sup>T</sup>, NZ\_CP017179; C, *E. hormaechei oharae*, DSM 16687<sup>T</sup>, NZ\_CP017180; D, *E. hormaechei hoffmannii*, DSM 14563<sup>T</sup>, 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<sup>T</sup>, CP017184.1; N, *E. cloacae complex*, SY-70, NZ\_JALR01000000; P, *E. cloacae complex*, JD8715, JDWG01000000; Q, *E. kobei*, DSM 13645<sup>T</sup>, 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.