Polymyxin Resistance Caused by mgrB Inactivation Is Not Associated with Significant Biological Cost in Klebsiella pneumoniae

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The inactivation of the mgrB gene, which encodes a negative-feedback regulator of the PhoPQ signaling system, was recently shown to be a common mutational mechanism responsible for acquired polymyxin resistance among carbapenemase-producing Klebsiella pneumoniae strains from clinical sources. In this work, we show that mgrB mutants can easily be selected in vitro from different K. pneumoniae lineages, and mgrB inactivation is not associated with a significant biological cost.

Global-scale dissemination of carbapenem-resistant Enterobacteriaceae (CRE) and carbapenem-resistant Acinetobacter baumannii strains has occurred in recent years, resulting in the renewed popularity of polymyxins, which are among the few drugs that retain activity against these resistant Gram-negative organisms (1–3). As a consequence, polymyxin resistance is increasingly being reported among these resistant pathogens (4–8). In A. baumannii, however, polymyxin resistance has been shown to be associated with a relevant biological cost, which would reduce the risk of dissemination of resistant strains and explain why polymyxin resistance has remained relatively uncommon among clinical isolates of this species (9, 10).

We recently reported that the inactivation of the mgrB gene can be a mutational mechanism responsible for acquired polymyxin resistance in carbapenem-resistant (CR) Klebsiella pneumoniae organisms producing the K. pneumoniae carbapenemase (KPC) (11). MgrB is a small membrane protein that acts as a negative-feedback regulator of the PhoPQ two-component signaling system (12). The inactivation of the mgrB gene is associated with an upregulation of that system, which in turn activates the polymyxin resistance (Pmr) system responsible for the biochemical modification of lipid A, which reduces its affinity for polymyxins (11, 13). Further epidemiological investigations revealed that mgrB inactivation is a common mechanism of resistance among polymyxin-resistant clinical isolates of CR K. pneumoniae (14–16). Inactivation can be mediated by mutations in mgrB or through the integration of an insertion sequence (IS), with the IS integration being more common (14–16). The clonal expansion of mgrB-inactivated mutant strains has also been observed in some health care settings (14). A similar epidemiological scenario suggests that mgrB inactivation does not impose a relevant fitness cost on the bacterial host, unlike what is observed with pmr or lpx mutations responsible for polymyxin resistance in A. baumannii (9).

In this work, we investigated the stability and the fitness cost of

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Clonal lineage</th>
<th>Carbapenemase gene</th>
<th>Phenotype</th>
<th>Mutation frequency</th>
<th>No. (%) of IS in mgrB</th>
<th>IS type, position (orientation) (n)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKBO-1</td>
<td>11</td>
<td>ST258 (clade 2)</td>
<td>0.12</td>
<td>blaKPC-3</td>
<td>7.0 × 10^{-7}</td>
<td>12 (60)</td>
<td>IS5-like, nt 75 (R) (12)</td>
</tr>
<tr>
<td>KPB-1</td>
<td>19</td>
<td>ST512</td>
<td>0.06</td>
<td>blaKPC-3</td>
<td>3.0 × 10^{-7}</td>
<td>12 (60)</td>
<td>IS3-like, nt 75 (R) (11); IS5p26-like, nt 75 (R) (1)</td>
</tr>
<tr>
<td>KPGP1</td>
<td>20</td>
<td>ST16</td>
<td>0.12</td>
<td>blaKPC-3</td>
<td>4.7 × 10^{-8}</td>
<td>9 (45)</td>
<td>ISkp1-like, nt 124 (F) (6); ISkp1-like, nt 132 (F) (3)</td>
</tr>
</tbody>
</table>

a MDR, multidrug resistant, i.e., nonsusceptible to carbapenems (imipenem, meropenem, and ertapenem), b-lactamase-inhibitor combinations (amoxicillin-clavulanate and piperacillin-tazobactam), expanded-spectrum cephalosporins (cefotaxime, cefazidime, and cefepime), trimethoprim-sulfamethoxazole, and fluoroquinolones.

b Frequency of COL1 colonies obtained plating 1 × 10^6 CFU from an early stationary phase.

c Nature and proportion (%) of 20 analyzed COL1 mutants with an IS in the mgrB gene. The remaining mutants in all cases carried a wild-type mgrB gene.

d Nature of the IS found in the mgrB gene in COL1 mutants. The nucleotide (nt) numbers indicate the positions of the ISs; the numbering is based on the coding sequence of the mgrB open reading frame (ORF) (GenBank accession no. AVF010000053, region 155512 to 155655), with number 1 as the first base of the GTG start codon. F indicates that the transposase gene is in the same orientation as the mgrB gene, while R indicates that the transposase gene is in the opposite orientation.
**TABLE 2** Biological cost of *mgrB* insertional inactivation in *K. pneumoniae* strains belonging to different STs

<table>
<thead>
<tr>
<th>COL′ strain</th>
<th>Clonal lineage</th>
<th>Chromosomal <em>mgrB</em> interruption, position (orientation)</th>
<th>Colistin MIC (mg/liter)</th>
<th>Selection coefficient ± SE&lt;sup&gt;α&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKBO-1</td>
<td>ST258 (clade 2)</td>
<td>IS5-like, nt 75 (R)</td>
<td>8</td>
<td>1.001 ± 0.004</td>
</tr>
<tr>
<td>KPB-1</td>
<td>ST512</td>
<td>IS3-like, nt 75 (R)</td>
<td>16</td>
<td>1.006 ± 0.0018</td>
</tr>
<tr>
<td>KPGP1</td>
<td>ST16</td>
<td>IS5pnr14-like, nt 124 (F)</td>
<td>4</td>
<td>0.999 ± 0.0017</td>
</tr>
<tr>
<td>KFPan</td>
<td>ST674</td>
<td>IS102-like, nt 69 (F)</td>
<td>4</td>
<td>1.005 ± 0.003</td>
</tr>
</tbody>
</table>

<sup>α</sup> The value of the selection coefficient of the mutant is relative to the progenitor COL′ strain.

The *mgrB* inactivation in polymyxin-resistant mutants of different lineages of *K. pneumoniae*.

Four different *K. pneumoniae* strains of a clinical origin were investigated in this study. The strains belonged to different sequence types (STs) and included representatives of the ST258 clonal lineage, which is responsible for the recent global dissemination of KPC-producing *K. pneumoniae* strains, and also included an OXA-48-producing ST16 strain (Table 1). The MICs of colistin and other antimicrobial agents (including carbapenems, cephalosporins, trimethoprim-sulfamethoxazole, and fluoroquinolones) were determined by reference broth microdilution (BMD) (17) and interpreted according to the EUCAST guidelines (http://www.eucast.org). Escherichia coli strain ATCC 25922 and *K. pneumoniae* strain KKBO-4 (11) were used as the control strains for antimicrobial susceptibility testing. Colistin-resistant (COL′) colonies were selected from each colistin-susceptible (COL′) strain by plating approximately 1 × 10⁶ CFU from an early stationary-phase culture onto Mueller-Hinton agar (MHA) (Oxoid Ltd., United Kingdom) containing 16 mg/liter colistin sulfate (AppliChem GmbH, Germany). The stability of the COL′ phenotype was tested by growing selected mutants in antibiotic-free MHA broth aerobically at 37°C for approximately 50 generations (by diluting the culture 1:200 in fresh medium every day for 6 days); at the end of growth, each culture was plated onto MHA to obtain isolated colonies, and 50 randomly selected colonies were checked by replica plating onto MHA supplemented with colistin (12 μg/ml, a concentration that allowed the growth of the COL′ mutants used in these experiments but not of the COL′ progenitors). With each mutant, the stability experiments were carried out in triplicate. The biological cost assay was performed by coculturing each COL′ strain together with the corresponding COL′ mutant under the same conditions described for the stability assays. At the end of growth, the ratio of COL′ to COL′ cells was measured as described for the stability assays. For each pair of strains, four independent competition experiments were carried out. The selection coefficient (s) was calculated as the slope of the linear regression model: \( s = \log(CI)/\log(d) \), where CI is the competition index, calculated as the ratio between the CFU counts of the resistant and susceptible populations every day divided by the same ratio at time zero (\( t_0 \)), and (d) is the dilution factor (18).

The COL′ colonies were obtained from each COL′ strain at various frequencies between 7 × 10⁻⁷ and 4.7 × 10⁻⁸ (Table 1). The characterization of the *mgrB* mutations, which was carried out by PCR and sequencing, as described previously (14), with a random sample of 20 COL′ colonies obtained from each strain revealed an insertional inactivation by an IS in 45 to 65% of the mutants, with some diversity in the IS types and sites of insertion. All the remaining analyzed COL′ mutants exhibited a wild-type *mgrB* gene (Table 1).

Of the insertional *mgrB* mutants obtained from each strain, one representative of the most frequent type of insertion event was selected to test the stability and biological cost of the resistance mechanism. The ISs carried by these four mutants were different (IS5-like, IS3-like, IS5pnr14-like, and IS102-like) and inserted at various positions within the *mgrB* gene (Table 2). The colistin MICs varied from 4 to 16 mg/liter. Despite the MIC values determined by the BMD, all mutants were able to grow onto MHA containing colistin at a concentration of 12 mg/liter. The stability assays revealed that after ca. 50 generations in the absence of antibiotics, the resistance phenotype was stable with each selected resistant mutant. PCR and a sequence analysis of the *mgrB* locus carried out with end-stage cultures confirmed the presence of the original *mgrB* alteration in all cases (data not shown).

The competition assays, which were carried out with each pair of COL′ and COL′ isogenic strains, did not detect any significant biological cost for the COL′ mutants (Table 2). Together, the present findings indicated that (i) COL′ mutants created by insertional inactivation of the *mgrB* gene can be selected from different strains upon exposure to colistin, (ii) the resistance mechanism based on *mgrB* insertional inactivation was stable even in the absence of antibiotic selection, and (iii) the *in vitro* competition experiments between the COL′ and COL′ strains did not show any significant biological cost associated with the insertional inactivation of the *mgrB* gene. Although this behavior was observed *in vitro*, these findings are consistent with the epidemiological observations that indicate a major role for *mgrB* inactivation in the evolution of polymyxin resistance in the clinical setting. Further studies will be necessary to investigate the potential effect of *mgrB* inactivation on virulence and the ability to establish persistent colonization in humans.

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**REFERENCES**


