Multifactorial Chromosomal Variants Regulate Polymyxin Resistance in Extensively Drug-Resistant *Klebsiella pneumoniae*

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Running Title: Polymyxin Resistance in XDR *K. pneumoniae*

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SYNOPSIS

Objectives: Infections facilitated by extensively drug-resistant Klebsiella pneumoniae (XDR-KP) cause high mortality and are disseminating globally. Identifying the genetic basis underpinning resistance allows for rapid diagnosis and treatment.

Methods: XDR isolates sourced from Greece and Brazil, including nineteen polymyxin-resistant and five polymyxin-susceptible strains, underwent whole genome sequencing.

Results: Approximately 90% of polymyxin resistance was enabled by alterations upstream or within mgrB. The most common mutation identified was an insertion at nucleotide position 75 in mgrB via an ISKpn26-like element in the ST258 lineage and ISKpn13 in one ST11 isolate. Three strains acquired an IS1 element upstream of mgrB and another strain had an ISKpn25 insertion at 133 bp. Other isolates had truncations (C28STOP, Q30STOP) or a missense mutation (D31E) affecting mgrB. Complementation assays revealed all mgrB perturbations contributed to resistance. Missense mutations in phoQ (T281M, G385C) were also found to facilitate resistance. Several variants in phoPQ co-segregating with the ISKpn26-like insertion were identified as partial suppressor mutations. Three ST258 samples were found to contain subpopulations with different resistance conferring mutations, including the ISKpn26-like insertion colonising with a novel mutation in pmrB (P158R), both confirmed via complementation assays. We also characterized a new multi-drug resistant Klebsiella quasipneumoniae strain ST2401 which was susceptible to polymyxins.

Conclusions: These findings highlight the broad spectrum of chromosomal modifications which can facilitate and regulate resistance against polymyxins in KP.
INTRODUCTION

*Klebsiella pneumoniae* (KP) strains classified as extensively drug-resistant (XDR) are rapidly emerging due to the dissemination of aminoglycoside, β-lactam, fluoroquinolone and carbapenem plasmid-encoded resistance genes.\(^1\) Notably, carbapenem-resistant KP (KPC) have been linked to high morbidity and an overall mortality of 48% in infected patients.\(^2\) Polymyxin B and colistin (polymyxin E) are now one of the last viable therapeutic options.\(^3\) Unfortunately, resistance to this last line antibiotic class is an increasing global burden, with high prevalence reported in Asia (Korea\(^4,5\), India\(^6,7\), Europe (Greece\(^8,10\), Italy\(^10,11\)) and Latin America (Brazil\(^12,13\)). Depending on early detection, mortality associated with polymyxin-resistant KP infections ranges from 20 to 100%.\(^14\)

Polymyxins infiltrate Gram-negative bacteria via initial binding to the basal component of lipopolysaccharide, lipid A. This causes the displacement of Mg\(^{2+}\) and Ca\(^{2+}\), disrupting bacterial outer membrane integrity and allowing the polymyxins to enter and act on intracellular targets. An extended exposure in KP triggers the activation of the two-component regulatory systems, PmrAB and PhoPQ.\(^15-17\) This pathway modulates *pmrC* and the *pmrHFIJKLM* operon facilitating the addition of phosphoethanolamine (pEtN) and/ or 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A phosphate groups, impairing polymyxin binding interactions.\(^18-20\) Disruption of *mgrB*, the negative regulator of PhoPQ, has been commonly observed in isolates of clinical origin.\(^8,21\) This constitutive up-regulation incurs no apparent fitness cost and appears to be stable, with minimal reports of reversions.\(^22,23\) Heteroresistant populations, where only a subset of bacteria are resistant, are prevalent in KP which complicates diagnosis.\(^24\) This situation is further exacerbated by the recently reported plasmid gene *mcr-1*, which encodes a pEtN transferase enzyme\(^25\) and identification of pandrug-resistant KP.\(^26\)
This study aimed to investigate XDR-KP clinical isolates arising in Greece and Brazil during 2012 to 2014 to identify and validate genetic variants contributing to resistance. These alterations were further compared to prior clinical isolates to decipher if these mutations have been previously detected.
MATERIALS AND METHODS

Bacterial isolates

KP clinical isolates were acquired from Hygeia General Hospital, Athens, Greece and Instituto Dante Pazzanese de Cardiologia, Brazil from patients in 2012 to 2014. Cultures were supplied as stab/slants or on agar, and were subsequently cultured in Nutrient Broth. Cultures were made to 20% glycerol and stored at -80 °C. When required for assay or extraction, glycerol stocks were struck out to obtain single colonies on either Nutrient Agar or Tryptic Soy Agar with 5% defibrinated sheeps blood. Reference strains included Escherichia coli (ATCC 25922) and Klebsiella spp. (ATCC 13883, ATCC 700603, ATCC BAA-2146), which were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Antimicrobial susceptibility assays

Species identification and susceptibility profiles of clinical isolates from Greece and Brazil were evaluated in the clinic using VITEK®2 (bioMérieux) and further validated at the Institute for Molecular Bioscience (IMB) (The University of Queensland, Australia) by determination of MICs using the standard Clinical & Laboratory Standards Institute (CLSI) approved broth microdilution (BMD) methods with Mueller Hinton Broth (MHB). Resistance was determined as per The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Version 5.0, 2015) (see http://www.eucast.org). The break point for tetracycline was in concordance to CLSI guidelines. Categorisation of drug resistance level was determined through guidelines previously outlined.
DNA extraction

DNA was extracted from overnight cultures using the DNeasy Blood and Tissue Kit (Qiagen) with the additional enzymatic lysis buffer pre-treatment as per manufacturer’s instructions. DNA was quantified with Qubit®3.0 (ThermoFisher Scientific).

DNA library preparation and sequencing

Library preparation was accomplished using the Nextera XT kit (Illumina) with 1 ng input of DNA as per manufacturer’s instructions. Quality of libraries were checked using a 2100 Bioanalyzer (Agilent Technologies). Libraries were sequenced on an Illumina MiSeq with 300 bp paired end sequencing reads and >100X coverage per sample.

Sequencing analysis

Paired end reads were trimmed with Trimmomatic and assembled using SPAdes. The Rapid Annotation using Subsystem Technology (RAST) was utilised to annotate assembled genomes. Assemblies were also uploaded to the Centre for Genomic Epidemiology (CGE) to identify STs (MultiLocus Sequence Typing Server 1.8) and acquired resistance which included mcr-1 detection (ResFinder 2.1). Phylogroups of STs were established by constructing a phylogenetic tree along with 117 KP STs as reported previously. The 7 MLST defining loci were aligned with MUSCLE and used to build a tree using Neighbour-Joining implemented in MEGA6.

Variant detection

Alterations both in and flanking the genes pmrA, pmrB, phoP, phoQ and mgrB were examined and sequence reads of all strains were aligned to the assembly of 20_GR_12, a polymyxin-susceptible ST258 strain with the least number of contigs, using BWA-MEM. The alignment was analysed through FreeBayes to identify single nucleotide and small indel
variation, using a diploid analysis in order to identify potential heterogeneity. Sites with more than 20% of reads mapping to the minor allele were considered potentially heterogeneous. The effects of variations were determined by snpEff.\textsuperscript{39} The impact on protein sequence was further confirmed by the Protein Variation Effect Analyzer (PROVEAN).\textsuperscript{40} For the analysis of large chromosome changes, the gene sequences including 300 bp flanking were extracted from the assemblies. A multiple alignment of each gene was constructed from the pair-wise alignment to the longest gene sequence.

**Insertion sequence element validation**

ISFinder\textsuperscript{41} was used for the identification of IS elements. To confirm disruptive IS elements, \textit{mgrB} was amplified with primers displayed in Table S1 via 2X Phusion HF master mix (Invitrogen) under the following cycling conditions: 98 °C 10 seconds, 50 °C 30 seconds and 72 °C 60 seconds (35X). Amplicons were examined using Sanger Sequencing, aligned through Clustal Omega\textsuperscript{42} and a tree constructed as described above.

**Complementation assays**

Contribution of variants to resistance were validated through complementation assays as previously described.\textsuperscript{43} Briefly, genes (Table S1) were amplified from a polymyxin-susceptible isolate, 20\_GR\_12, and cloned into the pCR-BluntII-TOPO vector via the Zero Blunt TOPO PCR cloning kit (Invitrogen). Chemically competent \textit{E. coli} TOP10 cells were transformed and selected by the addition of 50 mg/L kanamycin in MHB agar. Isolation of plasmids were via the PureLink\textsuperscript{TM} Quick Plasmid Miniprep Kit (Invitrogen) and transformed into KP strains via electroporation (25 \textmu F, 200 \textOmega , 1.25 kV/cm) with a Gene Pulser (Bio-Rad Laboratories). Selection was accomplished through supplementation of \textgreater=500 mg/L zeocin in MHB agar plates. Transformed colonies (n=\textgreater=2) were acquired and placed in MHB containing 1500 mg/L zeocin and 1 mM isopropyl \textbeta-D-1-
thiogalactopyranoside (Sigma Aldrich). If polymyxin susceptibility was not restored upon complementation, genes harbouring mutations were further amplified and introduced into 20_GR_12. To discern the impact of additional mutations in *phoPQ* and *pmrB* segregating with disrupted *mgrB*, mutant genes were introduced into a polymyxin-resistant isolate only harbouring an IS element *mgrB* disruption, 7_GR_13. Controls included transformation of WT genes into 20_GR_12, sequencing of amplicon prior to incision in vector and KP transformed strains undergoing a plasmid extraction and further PCR of the multiple cloning site. Antimicrobial testing against polymyxin B were conducted as above however, using cation-adjusted MHB.

**Nucleotide sequence accession numbers**

The nucleotide sequences obtained in this study have been deposited under BioProject PRJNA307517.
RESULTS

Characterisation of clinical isolates

KP isolates were all characterised in the hospital microbiology facility using VITEK®2 (Table 1). Subtle variations were detected between VITEK and broth microdilution (BMD) results (Table S2), predominantly the level of resistance towards gentamicin, tetracycline and tigecycline. A major dissimilarity was polymyxin susceptibility in 6_GR_12 (sensitive in BMD, resistant in VITEK) and resistant in 23_GR_13 (resistant in BMD, sensitive in VITEK). Polymyxin resistance was identified in 19 of the isolates. An abundance of acquired resistance genes (Table 2) were detected and this presence corresponded to the antimicrobial testing phenotype. This analysis did not identify mcr-1 in these strains. Only 18_GR_14 and 19_GR_14 were not identified as extended-spectrum beta-lactamase producers amongst the polymyxin-resistant strains. Consequently, all polymyxin-resistant strains harboured non-susceptibility to at least one antibiotic in 14 or more of the 16 antimicrobial categories hence were defined as XDR.

Sequence type determination

Two thirds of the Greece clinical strains were found to belong to ST258 and the remaining were ST11, ST147 or ST383 (Table 1). While 5_GR_13 and 6_GR_12 were both ST383, only 5_GR_13 was resistant to polymyxin. Among the two strains from Brazil, 11_BR_13 was ST437 and 12_BR_13 was ST11. 21_GR_13 had a profile previously undefined and has been newly designated ST2401. Clustering analysis further revealed this multidrug-resistant polymyxin-susceptible strain as Klebsiella quasipneumoniae (KQ) (Figure S1).

MgrB disruption

In approximately 90% of strains, mgrB was impacted either by missense mutations, nonsense mutations or insertion sequence (IS) elements (Table 3). Both 5_GR_13 and 19_GR_14
harboured a truncation while an amino acid change, D31E, was apparent in 3_GR_13. IS element
disruption was prevalent in 53% of strains and commonly an IS5-like element was integrated at
nucleotide position 75 (Figure S2). Sequencing revealed this element was closely related to
ISKpn26, herein known as ISKpn26-like, except for 12_BR_13 which matched ISKpn13 (Figure 1).
ISIR was detected upstream of mgrB in 11_BR_13 and an ISIR-like (A>C, 393 bp; C>T, 396
bp) element in 16_GR_13 and 17_GR_14. Strain 15_GR_13 had a deletion of the mgrB locus from
nucleotide position 133 onwards. The 127 bp flanking region mapped to ISKpn25 with the
transposase in the same orientation as mgrB. All 3 of IS1 element insertions, but only one of the 8
ISKpn26-like element insertions had their transposases in the same orientation as mgrB.

**Single, multiple and heterogeneous mutations**

Aberrations in genes commonly identified to confer polymyxin resistance in KP include
mgrB, phoPQ and pmrAB. Several non-synonymous mutations were identified across the isolates,
however, not all were predicted to be deleterious (Table S3). ST383 contained several mutations
in pmrA and pmrB although only Q30STOP in polymyxin-resistant 5_GR_13 was predicted to have an impact. Similarly, neutral changes in all four of these genes were detected in polymyxin-
susceptible KQ strains ATCC 700603 and 21_GR_13. 8_GR_13 and 9_GR_12 harboured a single
detrimental missense mutation in phoQ. Alterations in mgrB were accompanied by one or more
missense mutations in phoP, phoQ and/ or pmrB. Predicted deleterious variants segregating with
disrupted mgrB included pmrB (T140P, P158R), phoP (P74L, A95S) and phoQ (N253T, V446G),
which were commonly in the ST258 lineage. V446G (phoQ) and P158R (pmrB) were
heterogeneous in 13_GR_14 (65, 66% variant allele frequency) and 14_GR_14 (52, 57% variant
allele frequency). Assembly revealed 23_GR_12 harboured an ISKpn26-like disrupted mgrB
alongside the intact version with alterations in phoP and phoQ in 57% of the sample.
Role of mgrB disruptions and presence of heteroresistance via complementation assays

Complementation of the WT gene elucidated the role of these mutations in resistance (Table 3). Introduction of pTOPO-mgrB restored susceptibility in all resistant isolates with mgrB coding mutations or upstream disruptions, with the exception of two strains heterogeneous for the mgrB disruption and a pmrB coding mutation (13_GR_14 and 14_GR_14). For these two strains, pTOPO-mgrB restored susceptibility in zero of three 13_GR_14 colonies and one of three 14_GR_14 colonies. Transformation of 1 out of 3 colonies for both 13_GR_14 and 14_GR_14 strains with pTOPO-pmrB restored susceptibility and mgrB amplification of these colonies revealed an intact mgrB locus (data not shown). This indicates that the mutation in pmrB (P158R) is conferring resistance to polymyxin and coexisting with the mgrB disrupted population. 23_GR_12 was also observed to have a heterogeneous mgrB disruption but did not carry a corresponding pmrB mutation however, harboured similar mutations to 2_GR_12 in phoPQ. Amplification of mgrB identified two of three 23_GR_12 transformed colonies contained the IS element disruption and were reverted to susceptible upon complementation with pTOPO-mgrB.

Validation of resistance conferring mutations in phoQ

Strains 8_GR_13 and 9_GR_12 harboured a single mutation in phoQ potentially conferring resistance (Table 3). When these isolates were transformed with pTOPO-phoQ, results remained variable where a lack of growth was present in a susceptible range (MIC: ≤2 mg/L) however, several wells containing high PMB concentrations exhibited growth. To resolve this, the mutated gene was introduced into a polymyxin-susceptible isolate, 20_GR_12, and resistance was apparent.
Identification of suppressor mutations in *phoPQ*

Several mutations co-segregating with disrupted *mgrB* were detected including *phoP* (P74L, A95S), *phoQ* (N253T, V446G) and *pmrB* (T140P). Complementation of WT genes in these isolates commonly facilitated a ≥2-fold increase in MIC with the exception of 10_GR_13, which had an additional predicted neutral mutation in *phoQ* (A225T) (Table S2). To evaluate the potential influence of these mutations on polymyxin resistance, mutated genes were placed into a strain only containing the *mgrB* IS element disruption, 7_GR_13. Complementation of the mutant *phoQ* (N253T) decreased the MIC by 2-fold (Table 3), confirming the role of this mutation as a resistance suppressor. Initially, the *phoQ* (V446G) mutation was anticipated to segregate with the *mgrB* disrupted population in 13_GR_14 and 14_GR_14 as a suppressor mutation however, when *phoQ* was amplified from a colony reverted to susceptible via pTOPO-*mgrB* complementation, the WT *phoQ* was observed (Figure S3). The *phoQ* (V446G) mutation was successfully amplified from a 14_GR_14 colony containing the *pmrB* (T158R) mutation and upon complementation in 7_GR_13, resulted in a 2-fold reduction in MIC. Although this mutation did not segregate with disrupted *mgrB*, it may act as a partial suppressor mutation when a resistance conferring mutation is present in *pmrB*. Mutations in *phoP* (P74L, A95S) reduced the MIC in 7_GR_13 by ≥8-fold which also identifies these as partial suppressor mutations. Complementation of mutant *pmrB* (T140P) into 7_GR_13 did not lead to an observable corresponding reduction in MIC however, once transformed into 20_GR_12, a 2-fold increase in MIC was apparent implying this is not a suppressor mutation.
DISCUSSION

Polymyxin resistance in XDR-KP is of grave concern, especially the alarming increase reported in Greece and Brazil.\textsuperscript{10-12,44} We evaluated the genetic basis of polymyxin resistance in a series of Greek and Brazilian clinical isolates from patients in 2012 to 2014 and found alterations in genes \textit{mgrB}, \textit{phoPQ} and \textit{pmrAB}.

Inactivation of \textit{mgrB} was highly prevalent in these strains with an IS\textit{Kpn26}-like element being the predominant cause of resistance, as indicated by complementation restoring susceptibility in all isolates. Several other studies have observed an IS\textit{5}-like element integration in the same position, including reports from Greece, Italy, France, Turkey and Colombia.\textsuperscript{8,9,45,46} The IS\textit{Kpn26}-like element resembled the same sequence from Greece isolates previously described.\textsuperscript{46} We identified that this mutation still persisted in 2014, after being first detected in 2012.\textsuperscript{9} The IS\textit{Kpn26}-like forward insertion at nucleotide 75 in ST147, IS\textit{Kpn13} integration at nucleotide 75 in ST11 and the potential \textit{mgrB} disruption with IS\textit{Kpn25} was not previously reported. We identified IS\textit{IR} or IS\textit{IR}-like elements positioned upstream of \textit{mgrB} in 3 isolates (11\_BR\_13, 16\_GR\_13, 17\_GR\_14) which were reverted upon complementation indicating an impact on the promoter region.

Truncations identified at position 28 and 30 of \textit{mgrB} have also been previously detected, although these were identified in differing STs indicating mutations potentially have arisen independently in Greece.\textsuperscript{21} Complementation of these changes had not previously been conducted and with our study now providing definitive evidence that these mutations to cause resistance. This study further identified that the amino acid change D31E in 3\_GR\_13 is a polymyxin resistance conferring change. These findings support the notion that intact MgrB is required to confer negative feedback on PhoPQ.\textsuperscript{8}
Single predicted detrimental mutations were observed in the *phoQ* histidine kinase region, critical for phosphorylation and interaction with *phoP*, in 8_GR_13 (G385C) and 9_GR_12 (T281M). The G385C mutation had been detected however, in a differing ST. Complementation revealed an inconsistent MIC for these strains, although when a polymyxin-susceptible isolate was transformed with the mutated gene, full resistance was restored. Dominance of mutated *phoQ* has recently been highlighted and these results may imply the inability of pTOPO-*phoQ* to override the resistance caused by these mutations. 

Several non-synonymous changes were identified to be not deleterious according to PROVEAN analysis. Notably, these were abundant in KQ strains ATCC 700603 and 21_GR_13. This was further identified in KP ST383 lineages and PROVEAN accurately detected these neutral changes. However, this does not negate the possibility of previously resistance conferring alterations being acquired in these loci with subsequent reversion mutations to give rise to a susceptible phenotype.

Heterogeneity was apparent in several isolates. In near equal ratios, 13_GR_14 and 14_GR_14 possessed the IS*Kpn26*-like *mgrB* disruption and a new alteration conferring resistance in *pmrB*, P158R as determined by complementation. 23_GR_12 consisted of approximately half the reads mapping to the undisrupted genes and the other to the IS*Kpn26*-like strain with several additional predicted deleterious mutations. This heterogeneity may explain the initial clinical detection for this isolate to be polymyxin-susceptible.

Several isolates harbouring IS*Kpn26*-like element disrupted *mgrB* were accompanied by mutations in *phoP*, *phoQ* and/or *pmrB*. These changes were present in ≥98% of reads to render the involvement of heterogeneity unlikely. Once complemented, an increase in resistance was commonly recorded. This potentially reflects partial suppressor mutations as strains which solely
possessed this IS element disruption commonly exhibited a heightened MIC of ≥64 mg/L. Once these mutations were introduced into a strain only harbouring the $mgrB$ disruption, a reduction in MIC was apparent especially for $phoP$ and $phoQ$. The involvement of additional mutations in PhoPQ to influence the level of polymyxin resistance has yet to be reported in KP. Previous research by Miller et al. determined additional mutations in PhoPQ alters polymyxin resistance in *Pseudomonas aeruginosa*. This prior study describes $phoP$ mutations with the capacity to partially or fully suppress resistance causing mutations in $phoQ$. These mutations in $phoP$ were near or within the DNA binding site which differs to our results where the alterations are impacting the response regulatory region that interacts with PhoQ. Conversely, all mutations partially suppressing the MIC were identified to be targeting the HAMP and histidine kinase component of PhoQ. These were in regions similar to revertant *P. aeruginosa* strains identified by Lee and Kwan. We postulate these mutations are perturbing the critical transfer of phosphoryl groups from the histidine kinase of PhoQ to PhoP and subsequent $pmrD$ expression. Whether these mutations constitute to a fitness advantage due to the reduction of metabolism required for the production of LPS modifications is yet to be discerned.

Interestingly, the $pmrB$ T140P had formerly been identified in an ST258 lineage but even when the resistant gene was complemented, the MIC increased by 2-fold but was not defined as clinically resistant. When this mutation was introduced into a strain with only the disrupted $mgrB$, an elevation in MIC was witnessed, however, the original MIC for this isolate is 8-fold lower. This may suggest the presence of additional suppressor mutations in other yet to be identified loci.
Rapid and accurate detection of mutations attributed to polymyxin resistance remains a longstanding burden. Our research has contributed to the current understanding of the dissemination and evolution of this resistance in KP. This study highlights several issues of solely interrogating genomes for resistance detection including ST specific non-synonymous changes, heterogeneity and provides the first report of suppressor mutations. Through complementation assays, we have discerned the role of these modifications and have identified resistance-causing alterations for future genome-based diagnostics.
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Transparency declarations

None to declare.

Author Contributions

AGE, LC, MAC, MATB conceived the study. MEP, AGE, MDC, DG performed the experiments and analysed the data. MEP and MDC performed the sequencing analysis. IK, HG, CSA provided the bacterial isolates, clinical information on the strains and clinical perspective in writing the paper. MEP wrote the paper with input from all authors.
REFERENCES


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Strain identification, numerical order catalogued at IMB_Country (GR: Greece, BR: Brazil)_last two digits of isolation year.

Multilocus sequence type as identified through MultiLocus Sequence Typing Server 1.8.

Antibiotic resistance as determined by VITEK®2 according to EUCAST guidelines (CLSI for tetracycline). Resistance called if identified for one antibiotic in class. AMG, Aminoglycosides; APβI, Antipseudomonal penicillins + β-lactamase inhibitors; CARB, Carbapenems; CEPH1/2, Non-extended spectrum cephalosporins (1st and 2nd generation); CEPH3/4, Extended-spectrum cephalosporins (3rd and 4th generation); CEPH, Cephamycins; QUIN, Fluoroquinolones; FOL, Folate pathway inhibitors; GLY, Glycylcyclines; MON, Monobactams; PEN, Penicillins; PENβI, Penicillins + β-lactamase inhibitors; PHEN, Phenicols; PHOS, Phosphonic acids; POL, Polymyxins; TET, Tetracyclines; R, Resistant; I, Intermediate; S, Susceptible; N, Not tested.
<table>
<thead>
<tr>
<th>Strain ID</th>
<th>A</th>
<th>B</th>
<th>F</th>
<th>M</th>
<th>P</th>
<th>Q</th>
<th>R</th>
<th>S</th>
<th>T</th>
<th>Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>oseB(6)/a</td>
<td>oseB(6)/b</td>
<td>oseB(6)/c</td>
<td>oseA1</td>
<td>oseA2</td>
<td>mph(3)/a</td>
<td>mph(3)/c</td>
<td>rmtB</td>
<td>mph(3)/c</td>
<td>mph(3)/c</td>
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<tr>
<td>ATCC 700603</td>
<td>btaA</td>
<td>btaB</td>
<td>btaC</td>
<td>btaA</td>
<td>btaB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
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<tr>
<td>ATCC 13883</td>
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<td>btaB</td>
<td>btaC</td>
<td>btaA</td>
<td>btaB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
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<tr>
<td>ATCC BAA-2146</td>
<td>btaA</td>
<td>btaB</td>
<td>btaC</td>
<td>btaA</td>
<td>btaB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
<td>rmtB</td>
</tr>
</tbody>
</table>

A, Aminoglycoside; B, Beta-lactam; F; Fosfomycin; M, Macrolide; P, Phenicol; Q, Quinolone; R, Rifampicin; S, Sulphonamide; T, Tetracycline; Tr, Trimethoprim. Shading indicates detection of gene (≥98% homology, ≥60% sequence length).
TABLE 3 Complementation of WT genes from polymyxin-susceptible isolate 20_GR_12 into resistant strains and mutated genes identified in resistant isolates also transformed into mgrB disrupted polymyxin-resistant strain 7_GR_13

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>MLST</th>
<th>Genotype$^a$</th>
<th>Polymyxin B MIC (mg/L)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mgrB phoP phoQ pmrB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT WT P74L A95S WT N253T T281M G385C V446G WT T140P</td>
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<tr>
<td>1_GR_13</td>
<td>147</td>
<td>mgrB (N25ΔISKpn26-like$^8$), pmrB (T140P)</td>
<td>8-16 0.25 64</td>
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<tr>
<td>2_GR_12</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$), pmrB (A95S), phoQ (N253T)</td>
<td>8 0.25 64</td>
</tr>
<tr>
<td>3_GR_13</td>
<td>258</td>
<td>D31E</td>
<td>8 0.5-1</td>
</tr>
<tr>
<td>4_GR_12</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$), pmrB (P74L), phoQ (N253T)</td>
<td>16-32 0.25 32</td>
</tr>
<tr>
<td>5_GR_13</td>
<td>383</td>
<td>mgrB (Q30STOP)</td>
<td>16 0.25</td>
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<tr>
<td>7_GR_13</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$)</td>
<td>64-64 0.25-0.5 64 16 16 64 32</td>
</tr>
<tr>
<td>8_GR_13</td>
<td>258</td>
<td>phoQ (G385C)</td>
<td>32-64</td>
</tr>
<tr>
<td>9_GR_12</td>
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<td>phoQ (T281M)</td>
<td>8-16</td>
</tr>
<tr>
<td>10_GR_13</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$), phoQ (N253T)</td>
<td>32-64 0.25 32</td>
</tr>
<tr>
<td>11_BR_13</td>
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<td>mgrB (-35ΔISR)</td>
<td>64 0.125</td>
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<tr>
<td>12_BR_13</td>
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<td>mgrB (N25ΔISKpn13$^9$)</td>
<td>&gt;64 0.5-2</td>
</tr>
<tr>
<td>13_GR_14</td>
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<td>mgrB (N25ΔISKpn26-like$^8$), phoQ (V446G), pmrB (P158R)</td>
<td>16 16</td>
</tr>
<tr>
<td>14_GR_14</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$), phoQ (V446G), pmrB (P158R)</td>
<td>16-32 0.125 32</td>
</tr>
<tr>
<td>15_GR_13</td>
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<td>mgrB (H45ΔISKpn25$^5$)</td>
<td>32-64 2-4</td>
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<tr>
<td>16_GR_13</td>
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<td>mgrB (-19ΔISR-like)</td>
<td>64 0.06-0.125</td>
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<tr>
<td>17_GR_14</td>
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<td>mgrB (-19ΔISR-like)</td>
<td>32 0.125</td>
</tr>
<tr>
<td>18_GR_14</td>
<td>258</td>
<td>mgrB (N25ΔISKpn26-like$^8$)</td>
<td>64 1</td>
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<tr>
<td>19_GR_14</td>
<td>258</td>
<td>mgrB (C28STOP)</td>
<td>64 0.25-0.5</td>
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<tr>
<td>20_GR_12</td>
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<td>WT</td>
<td>0.125-0.25 0.125 0.125 0.125 0.125 0.125 8 32 0.125 0.125 0.5</td>
</tr>
<tr>
<td>23_GR_12</td>
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<td>mgrB (N25ΔISKpn26-like$^8$), phoP (A95S), phoQ (N253T)</td>
<td>4-8 0.125 8 16</td>
</tr>
</tbody>
</table>
Predicted deleterious mutations in genes when compared against polymyxin-susceptible isolate, 20_GR_12; represented as gene (change); change defined as initial amino acid, position and new alteration; -, upstream nucleotide position from gene start where first dissimilarity occurs; F/R, IS element in forward (F) or reverse (R) orientation to mgrB defined by transposase direction.

Minimal inhibitory concentration of PMB before and after complementation with specified WT and mutated genes; -, MIC range as per at least 2 transformed colonies and tested in duplicate; Susceptible ($\leq 2$ mg/L); 2 to 8-fold above EUCAST clinical breakpoint; $\geq 16$-fold above EUCAST clinical breakpoint (Het), Heterogeneity detected in isolate.
Figure 1. Clustering of IS elements disrupting \( mgrB \) detected in clinical strains together with reference IS element sequences. Neighbour-joining clustering of the IS element amplicon in relation to reference sequences for \( IS^Kpn13 \), \( IS^Kpn26 \) and \( IS^1R \) with transposase in the forward orientation. Scale represents number of base substitutions per site.