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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ABSTRACT

Extensively drug-resistant *Klebsiella pneumoniae* (XDR-KP) infections cause high mortality and are disseminating globally. Identifying the genetic basis underpinning resistance allows for rapid diagnosis and treatment. XDR isolates sourced from Greece and Brazil, including nineteen polymyxin-resistant and five polymyxin-susceptible strains, underwent whole genome sequencing. 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 IS*Kpn26*-like element in the ST258 lineage and IS*Kpn13* in one ST11 isolate. Three strains acquired an IS*1* element upstream of *mgrB* and another strain had an IS*Kpn25* 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 IS*Kpn26*-like insertion were identified as potential partial suppressor mutations. Three ST258 samples were found to contain subpopulations with different resistance conferring mutations, including the IS*Kpn26*-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. These findings highlight the broad spectrum of chromosomal modifications which can facilitate and regulate resistance against polymyxins in *K. pneumoniae*.

DATA SUMMARY

1. Whole genome sequencing of the 24 clinical isolates has been deposited under BioProject PRJNA307517 (www.ncbi.nlm.nih.gov/bioproject/PRJNA307517).

IMPACT STATEMENT

Klebsiella pneumoniae contributes to a high abundance of nosocomial infections and the rapid emergence of antimicrobial resistance hinders treatment. Polymyxins are predominantly utilized to treat multidrug-resistant infections, however, resistance to the polymyxins is arising. This increasing prevalence in polymyxin resistance is evident especially in Greece and Brazil. Identifying the genomic variations conferring resistance in clinical isolates from these regions assists with potentially detecting novel alterations and tracing the spread of particular strains. This study commonly found mutations in the gene *mgrB*, the negative regulator of PhoPQ, known to cause resistance in KP. In the remaining isolates, missense mutations in *phoQ* were accountable for resistance. Multiple novel mutations were detected to be segregating with *mgrB* perturbations. This was either due to a mixed heterogeneous sample of two polymyxin-resistant strains, or because of multiple mutations within the same strain. Of interest was the validation of novel mutations in *phoPQ* segregating with a previously known IS*Kpn26*-like element in disrupted *mgrB* isolates. Complementation of these *phoPQ* mutations revealed a reduction in minimum inhibitory concentrations and suggests the first evidence of partial suppressor mutations in KP. This research builds upon our current understanding of heteroresistance, lineage specific mutations and regulatory variations relating to polymyxin resistance.

61

62 # INTRODUCTION

63

64 *Klebsiella pneumoniae* (KP) strains classified as extensively drug-resistant (XDR) are rapidly
65 emerging due to the dissemination of plasmid-encoded resistance towards aminoglycosides, β-
66 lactams, fluoroquinolones and carbapenems [1]. Notably, carbapenem-resistant KP have been
67 linked to high morbidity and an overall mortality of 48% in infected patients [2]. Polymyxin B and
68 colistin (polymyxin E) are now one of the last viable therapeutic options [3]. Unfortunately,
69 resistance to this last-line antibiotic class is an increasing global burden, with countries particularly
70 impacted including Asia (Korea [4, 5], India [6, 7]), Europe (Greece [8-10]), Italy [10, 11]) and
71 Latin America (Brazil [12, 13]). There is considerable debate regarding the mortality associated
72 with polymyxin-resistant infections. Combining several clinical cohorts has provided an overall
73 mortality estimate ranging from 20 to 100% which was dependent on early detection of the
74 outbreak [14].

75 Polymyxins infiltrate Gram-negative bacteria via initial binding to the basal component of
76 lipopolysaccharide, lipid A. This causes the displacement of Mg^{2+} and Ca^{2+}, disrupting bacterial
77 outer membrane integrity, allowing the polymyxins to traverse the inner membrane and act on
78 intracellular targets. An extended exposure in KP triggers the activation of the two-component
79 regulatory systems, PmrAB and PhoPQ [15-17]. These systems regulate a pathway that modulates
80 *pmrC* and the *pmrHFIJKLM* operon facilitating the addition of phosphoethanolamine (pEtN) and/
81 or 4-amino-4-deoxy-L-arabinose to lipid A phosphate groups, resulting in impaired polymyxin
82 binding interactions [18-20]. Disruption of *mgrB*, the negative regulator of PhoPQ, has been
83 commonly observed in isolates of clinical origin [8, 21]. The constitutive up-regulation of *pmrC*

84 and the *pmrHFIJKLM* operon incurs a minimal fitness cost and appears to be stable, with minimal
85 reports of reversions [22, 23]. Heteroresistant populations, where only a subset of bacteria are
86 resistant, have been reported in KP which complicates diagnosis [24]. The emergence of pandrug-
87 resistant KP is of grave concern [25] and this acquisition of resistance is further exacerbated by
88 the recently reported plasmid-encoded colistin resistance gene *mcr-1*, which encodes a pEtN
89 transferase enzyme, albeit currently rare in KP [26].

90 This study aimed to investigate XDR-KP clinical isolates arising in Greece and Brazil during 2012
91 to 2014 to identify and validate genetic variants contributing to resistance. These alterations were
92 compared to prior clinical isolates to ascertain if these mutations have been previously detected
93 globally.

METHODS

Bacterial isolates

KP clinical isolates were acquired from the Hygeia General Hospital, Athens, Greece and Instituto Dante Pazzanese de Cardiologia, Brazil from patients in 2012 to 2014. Cultures were supplied as stabs/slants or on agar, and were subsequently cultured in Nutrient Broth. Cultures were made to 20% (v/v) 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 sheep 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). Strains were further validated at the Institute for Molecular Bioscience (IMB) (The University of Queensland, Australia) using the standard Clinical & Laboratory Standards Institute (CLSI) approved broth microdilution (BMD) methods with cation-adjusted Mueller-Hinton Broth (caMHB). Resistance was determined as per CLSI guidelines [27] except for tigecycline and fosfomycin where The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Version 7.1, 2017) (see http://www.eucast.org) guidelines were implemented. Categorisation of drug resistance level was determined through guidelines previously outlined [28].

117 **DNA extraction**

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

121 **DNA library preparation and sequencing**

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

126 **Sequencing analysis**

127 Paired-end reads were trimmed with Trimmomatic [29] and assembled using SPAdes [30]. The
128 Rapid Annotation using Subsystem Technology (RAST) was utilized to annotate assembled
129 genomes [31]. Assemblies were also uploaded to the Centre for Genomic Epidemiology (CGE) to
130 identify sequence types (STs) (MultiLocus Sequence Typing Server 1.8 [32]) and acquired
131 antibiotic resistance genes (ResFinder 2.1 [33]). A neighbor-joining tree was constructed using the
132 2358 *Klebsiella pneumoniae*/*quasipneumoniae*/*variicola* genes known to form the core genome
133 MLST (cgMLST) using Ridom SeqSphere+ v4.0.1 software [34]. The cgMLST was compared
134 against complete assemblies of ST11 (HS11286), ST147 (MS6671), ST258 (NJST258_1,
135 NJST258_2) and a reference for *K. quasipneumoniae*, ATCC 700603 [25, 35-37].

136

137

138 **Variant detection**

139 Alterations both in and flanking the genes *pmrAB*, *phoPQ* and *mgrB* were examined and sequence
140 reads of all strains were aligned to the assembly of 20_GR_12, a polymyxin-susceptible ST258
141 strain with the least number of contigs, using BWA-MEM [38]. The alignment was analyzed
142 through FreeBayes [39] to identify single nucleotide and small indel variation, using a diploid
143 analysis in order to identify potential heterogeneity. Sites with more than 20% of reads mapping
144 to the minor allele were considered potentially heterogeneous. The effects of variations were
145 determined by snpEff [40]. The impact on protein sequence was further confirmed by the Protein
146 Variation Effect Analyzer (PROVEAN) [41]. For the analysis of large chromosome changes, the
147 gene sequences including 300 bp flanking were extracted from the assemblies. A multiple
148 alignment of each gene was constructed from the pair-wise alignment to the longest gene sequence.

149 **Insertion sequence element validation**

150 ISFinder [42] was used for the identification of insertion sequence (IS) elements. To confirm
151 disruptive IS elements, *mgrB* was amplified with primers displayed in Table S1 via 2X Phusion
152 HF master mix (Invitrogen) under the following cycling conditions: 98 °C 10 seconds, 50 °C 30
153 seconds and 72 °C 60 seconds (35X). Amplicon identity was validated via Sanger sequencing.

154 **Complementation assays**

155 The contribution of variants to resistance was validated through complementation assays as
156 previously described [43]. Briefly, genes (Table S1) were amplified from a polymyxin-susceptible
157 isolate, 20_GR_12, and cloned into the pCR-BluntII-TOPO vector via the Zero Blunt TOPO PCR
158 cloning kit (Invitrogen). Chemically competent *E. coli* TOP10 cells were transformed and selected
159 by the addition of 50 mg/L kanamycin in MHA. Isolation of plasmids were via the PureLink™

160 Quick Plasmid Miniprep Kit (Invitrogen) and transformed into KP strains via electroporation (25
161 μF, 200 Ω, 1.25 kV/cm) with a Gene Pulser (Bio-Rad Laboratories). Selection was accomplished
162 through supplementation of ≥500 mg/L zeocin in MHA plates. Transformed colonies (n=≥2) were
163 acquired and placed in MHB containing 1500 mg/L zeocin and 1 mM isopropyl *β-D*-1-
164 thiogalactopyranoside (Sigma Aldrich). If polymyxin susceptibility was not restored upon
165 complementation, genes harboring mutations were further amplified and introduced into
166 20_GR_12. To discern the impact of additional mutations in *phoPQ* and *pmrB* segregating with
167 disrupted *mgrB*, mutant genes were introduced into a polymyxin-resistant isolate only harboring
168 an IS element *mgrB* disruption, 7_GR_13. Controls included transformation of WT genes into
169 20_GR_12, sequencing of amplicon prior to introduction in vector and KP transformed strains
170 undergoing a plasmid extraction and further PCR of the multiple cloning site. Antimicrobial testing
171 against polymyxin B were conducted as described above.

172

173 # RESULTS

174

175 **Characterization of clinical isolates**

176 KP isolates were all characterized in the hospital microbiology facility using VITEK®2. Several
177 discrepancies were detected between VITEK®2 and broth microdilution (BMD) results (Table 1,
178 Table S2), predominantly the level of resistance towards aminoglycosides, tetracyclines,
179 fosfomycin and tigecycline. A major dissimilarity was polymyxin susceptibility in 6_GR_12
180 (sensitive in BMD, resistant in VITEK®2) and resistant in 23_GR_13 (resistant in BMD, sensitive
181 in VITEK®2). Polymyxin resistance was identified in 19 of the isolates. An abundance of acquired
182 resistance genes (Table 2) were detected and this presence corresponded to the antimicrobial
183 testing phenotype. This analysis did not identify *mcr-1* in these strains. Only 18_GR_14 and
184 19_GR_14 were not identified as extended-spectrum beta-lactamase producers amongst the
185 polymyxin-resistant strains. Consequently, all polymyxin-resistant strains that harbored non-
186 susceptibility to at least one antibiotic in 15 or more of the 17 antimicrobial categories hence were
187 defined as XDR.

188 **Sequence type determination**

189 Two thirds of the Greece clinical strains were found to belong to ST258 and the remaining were
190 ST11, ST147 or ST383 (Table 1). While 5_GR_13 and 6_GR_12 were both ST383, only 5_GR_13
191 was resistant to polymyxin. Among the two strains from Brazil, 11_BR_13 was ST437 and
192 12_BR_13 was ST11. 21_GR_13 had a profile previously undefined and has been newly
193 designated ST2401. Further cgMLST studies were conducted on the isolates using complete
194 assemblies of reference genomes for ST11 (HS11286), ST147 (MS6671) ST258 (NJST258_1,

195 NJST258_2) and KQ (ATCC 700603) (Fig. 1). For the ST258 isolates, these were more similar to
196 NJST258_2 rather than NJST258_1. Within this cluster, 7_GR_13, 9_GR_12 and 24_GR_13 were
197 closely related (≤15 allelic changes). Similarly, grouped together were 2_GR_12 and 23_GR_12;
198 3_GR_13 and 22_GR_12; 13_GR_14 and 14_GR_14; and 18_GR_14 and 19_GR_14. In ST11,
199 16_GR_13 and 17_GR_14 harbored only 3 allele differences and the Brazilian isolate, 12_BR_13,
200 had 206 variants apparent. ST383 isolates 5_GR_13 and 6_GR_12 only exhibited 1 allele change.
201 ST147 1_GR_13 was not clonal to the previous pandrug resistant KP, MS6671. Clustering analysis
202 revealed 21_GR_13 as *Klebsiella quasipneumoniae* (KQ) and diverged with reference genome
203 ATCC 700603 (ST489).

204 **MgrB disruption**

205 Seventeen of the nineteen polymyxin-resistant strains exhibited either missense mutations,
206 nonsense mutations or IS elements in *mgrB* (Table 3). Both 5_GR_13 and 19_GR_14 harbored a
207 truncation while an amino acid change, D31E, was apparent in 3_GR_13. IS element disruption
208 was prevalent in 53% of strains and commonly an IS*5*-like element was integrated at nucleotide
209 position 75 (Fig. S1). Sanger sequencing revealed this element was closely related to IS*Kpn26*,
210 herein known as IS*Kpn26*-like, except for 12_BR_13 which matched IS*Kpn13.* IS*1R* was detected
211 upstream of *mgrB* in 11_BR_13 and an IS*1R*-like (A>C, 393 bp; C>T, 396 bp) element in
212 16_GR_13 and 17_GR_14. Strain 15_GR_13 had a deletion of the *mgrB* locus from nucleotide
213 position 133 onwards. The 127 bp flanking region mapped to IS*Kpn25* with the transposase in the
214 same orientation as *mgrB*. All 3 of IS*1* element insertions, but only one of the 8 IS*Kpn26*-like
215 element insertions had the transposase in the same orientation as *mgrB*.

216

217 **Single, multiple and heterogeneous mutations**

218 Aberrations in genes commonly identified to confer polymyxin resistance in KP include *mgrB*,
219 *phoPQ* and *pmrAB* (Table 2). Several non-synonymous mutations were identified across the
220 isolates however, not all were predicted to be deleterious (Table S3). ST383 contained several
221 mutations in *pmrAB* although only Q30STOP in polymyxin-resistant 5_GR_13 was predicted to
222 have an impact. Similarly, neutral changes in all four of these genes were detected in polymyxin-
223 susceptible KQ strains ATCC 700603 and 21_GR_13. 8_GR_13 and 9_GR_12 harbored a single
224 detrimental missense mutation in *phoQ*. Alterations in *mgrB* were accompanied by one or more
225 missense mutations in *phoPQ* and/ or *pmrB*. Predicted deleterious variants segregating with
226 disrupted *mgrB* included *pmrB* (T140P, P158R), *phoP* (P74L, A95S) and *phoQ* (N253T, V446G),
227 which were commonly in the ST258 lineage. V446G (*phoQ*) and P158R (*pmrB*) were
228 heterogeneous in 13_GR_14 (65% (V446G), 66% (P158R) mutation allele frequency) and
229 14_GR_14 (52% (V446G) and 57% (P158R) mutation allele frequency). Assembly revealed
230 23_GR_12 harbored an IS*Kpn26*-like disrupted *mgrB* alongside the intact version with alterations
231 in *phoP* and *phoQ* in 57% of the sample.

232 **Role of *mgrB* disruptions and presence of heteroresistance via complementation assays**

233 Complementation of the WT gene elucidated the role of these mutations in resistance (Fig. 2).
234 Introduction of pTOPO-*mgrB* restored susceptibility in all resistant isolates with *mgrB* coding
235 mutations or upstream disruptions, with the exception of two strains heterogeneous for the *mgrB*
236 disruption and a *pmrB* coding mutation (13_GR_14 and 14_GR_14) (Fig. 2a). For these two
237 strains, pTOPO-*mgrB* restored susceptibility in zero of three 13_GR_14 colonies and one of three
238 14_GR_14 colonies. Transformation of 1 out of 3 colonies for both 13_GR_14 and 14_GR_14
239 strains with pTOPO-*pmrB* restored susceptibility (Fig. 2d) and *mgrB* amplification of these

240 colonies revealed an intact *mgrB* locus (data not shown). Colonies which were reverted on
241 complementation were further passaged 3 times with no antibiotic pressure in order to remove the
242 plasmid and discern if these mutations were contributing to resistance. After passaging, pTOPO-
243 *mgrB* isolates harbored an MIC of ≥64 mg/L whilst pTOPO-*pmrB* colonies were 16 mg/L to
244 confirm two resistant populations in these samples. 23_GR_12 was also observed to have a
245 heterogeneous *mgrB* disruption but did not carry a corresponding *pmrB* mutation however,
246 harbored similar mutations to 2_GR_12 in *phoPQ*. Amplification of *mgrB* identified two of three
247 23_GR_12 transformed colonies contained the IS element disruption and were reverted to
248 susceptible upon complementation with pTOPO-*mgrB*.

249 **Validation of resistance conferring mutations in *phoQ***

250 Strains 8_GR_13 and 9_GR_12 harbored a single mutation in *phoQ* potentially conferring
251 resistance (Table 2). When these isolates were transformed with pTOPO-*phoQ*, results remained
252 variable where a lack of growth was present in a susceptible range (MIC: ≤2 mg/L) however,
253 several wells containing high polymyxin B concentrations exhibited growth (Fig. 2c). To resolve
254 this, the mutated gene was introduced into a polymyxin-susceptible isolate, 20_GR_12, and
255 resistance was apparent (Fig. 2e).

256 **Potential suppressor mutations in *phoPQ***

257 Several mutations co-segregating with disrupted *mgrB* were detected including *phoP* (P74L,
258 A95S), *phoQ* (N253T, V446G) and *pmrB* (T140P). Complementation of WT genes in these
259 isolates commonly facilitated a ≥2-fold increase in MIC with the exception of 10_GR_13, which
260 had an additional predicted neutral mutation in *phoQ* (A225T) (Table S3, Fig. 2b-d). To evaluate
261 the potential influence of these mutations on polymyxin resistance, mutated genes were placed

262 into a strain only containing the *mgrB* IS element disruption, 7_GR_13 (Fig. 2f). Complementation
263 of the mutant *phoQ* (N253T) decreased the MIC by 2-fold, potentially indicating a partial
264 suppressor mutation. Initially, the *phoQ* (V446G) mutation was anticipated to segregate with the
265 *mgrB* disrupted population in 13_GR_14 and 14_GR_14 however, when *phoQ* was amplified from
266 a colony reverted to susceptible via pTOPO-*mgrB* complementation, the WT *phoQ* was observed
267 (Fig. S3). The *phoQ* (V446G) mutation was successfully amplified from a 14_GR_14 colony
268 containing the *pmrB* (T158R) mutation and upon complementation in 7_GR_13, resulted in a 2-
269 fold reduction in MIC. Although this mutation did not segregate with disrupted *mgrB*, it may act
270 as a partial suppressor mutation when a resistance conferring mutation is present in *pmrB*.
271 Mutations in *phoP* (P74L, A95S) reduced the MIC in 7_GR_13 by ≥4-fold which identifies these
272 as partial suppressor mutations. Complementation of mutant *pmrB* (T140P) into 7_GR_13 did not
273 lead to an observable corresponding reduction in MIC however, once transformed into 20_GR_12,
274 a 2-fold increase in MIC was apparent (Fig. 2e).

275

276 ## DISCUSSION

277

278 Polymyxin resistance in XDR-KP is of grave concern given that this is a last-line antibiotic, and
279 is increasingly prevalent in countries such as Greece and Brazil [10, 12-14, 44]. We evaluated the
280 genetic basis of polymyxin resistance in a series of Greek and Brazilian clinical isolates from
281 patients in 2012 to 2014 and found alterations in genes *mgrB*, *phoPQ* and *pmrAB*.

282 Inactivation of *mgrB* was highly prevalent in these strains with an IS*Kpn26*-like element being the
283 predominant cause of resistance, as confirmed by complementation restoring susceptibility in all
284 isolates. Several other studies have observed an IS*5*-like element integration in the same position,
285 including reports from Greece, Italy, France, Turkey and Colombia [8, 9, 45, 46]. The IS*Kpn26*-
286 like element resembled the same sequence from Greece isolates previously described [46]. We
287 identified that this mutation still persisted in 2014, after being first detected in 2012 [9].
288 Disruptions in *mgrB* including the IS*Kpn26*-like forward insertion at nucleotide 75 in ST147,
289 IS*Kpn13* integration at nucleotide 75 in ST11 and IS*Kpn25* in the ST258 lineage have yet to be
290 reported. We identified IS*1R* or IS*1R*-like elements positioned upstream of *mgrB* in 3 isolates
291 (11_BR_13, 16_GR_13, 17_GR_14) which were reverted upon complementation indicating an
292 impact on the promoter region.

293 Truncations identified at position 28 and 30 of *mgrB* have been previously detected, although these
294 were identified in differing STs indicating mutations potentially have arisen independently in
295 Greece [21, 47]. Complementation restored susceptibility to polymyxins for these mutations and
296 this study further revealed the amino acid change D31E in 3_GR_13 to be a resistance conferring
297 alteration. These findings support the notion that intact MgrB is required to confer negative

298 feedback on PhoPQ [8]. The inactivation of *mgrB* is prevalent in polymyxin-resistant KP and may
299 arise owing to its capacity to promote virulence and further attenuate the early host defence
300 response, with little or no fitness cost [48].

301 Single predicted detrimental mutations were observed in the *phoQ* histidine kinase region, critical
302 for phosphorylation and interaction with *phoP*, in 8_GR_13 (G385C) and 9_GR_12 (T281M). The
303 G385C mutation had previously been reported, [21] however in a differing ST. Complementation
304 revealed an inconsistent MIC for these strains, although when a polymyxin-susceptible isolate was
305 transformed with the mutated gene, full resistance was restored. Dominance of mutated *phoQ* has
306 recently been highlighted and these results may imply the inability of pTOPO-*phoQ* to override
307 the resistance caused by these mutations [49].

308 Several non-synonymous changes were identified to be not deleterious according to PROVEAN
309 analysis. Notably, these were abundant in KQ strains ATCC 700603 [37] and 21_GR_13. This
310 was further identified in KP ST383 lineages and PROVEAN detected these neutral changes. These
311 mutations represent lineage specific alterations, however, this does not negate the possibility of
312 previously resistance conferring alterations being acquired in these loci with subsequent reversion
313 mutations to give rise to a susceptible phenotype.

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

320 Several isolates harboring IS*Kpn26*-like element disrupted *mgrB* were accompanied by mutations
321 in *phoPQ* and/ or *pmrB*. These changes were present in ≥98% of reads to render the involvement
322 of heterogeneity unlikely. Once complemented, an increase in resistance was commonly recorded.
323 This potentially reflects partial suppressor mutations as strains which solely possessed this IS
324 element disruption commonly exhibited a heightened MIC of ≥64 mg/L. One variant segregating
325 with this disruption included *pmrB* T140P. This had formerly been identified in an ST258 lineage
326 but even when the resistant gene was complemented, the MIC increased by 2-fold but was not
327 defined as clinically resistant [21, 50].

328 When mutated *phoP* or *phoQ* were introduced into the *mgrB* disrupted isolate, a reduction in MIC
329 was apparent. The involvement of additional mutations in PhoPQ to influence the level of
330 polymyxin resistance has yet to be reported in KP. Previous research by Miller *et al* [51]
331 determined additional mutations in PhoPQ altered polymyxin resistance in *Pseudomonas*
332 *aeruginosa*. This prior study describes *phoP* mutations with the capacity to partially or fully
333 suppress resistance-causing mutations in *phoQ*. These mutations in *phoP* were near or within the
334 DNA binding site which differs to our results, where the alterations are impacting the response
335 regulatory region that interacts with PhoQ. Conversely, all mutations partially suppressing the
336 MIC were identified to be targeting the HAMP and histadine kinase component of PhoQ. These
337 were in regions similar to revertant *P. aeruginosa* strains identified by Lee and Ko [52]. We
338 postulate these mutations are perturbing the critical transfer of phosphoryl groups from the
339 histadine kinase of PhoQ to PhoP and subsequent *pmrD* expression. Whether these mutations
340 constitute a fitness advantage due to the reduction of metabolism required for the production of
341 LPS modifications is yet to be discerned. Furthermore, due to variability in some of the

342 complementation data, a knockout *phoPQ* background and introduction of genes that are potential
343 suppressor mutations is required.

344 Rapid and accurate detection of mutations attributed to polymyxin resistance remains a
345 longstanding burden. Our research has contributed to the current understanding of the
346 dissemination and evolution of this resistance in KP. Although the sample size is limited, this study
347 highlights several issues arising from solely interrogating genomes for resistance detection
348 including ST specific non-synonymous changes, and heterogeneity. The study provides the first
349 potential report of suppressor mutations for polymyxin resistance. Through complementation
350 assays, we have discerned the role of these modifications and have identified resistance-causing
351 alterations that can be monitored during future genome-based diagnostics.

352

353 # AUTHOR STATEMENTS

354

355 *Funding information*

356 LC is an ARC Future Fellow (FT110100972). MAC is an NHMRC Principal Research Fellow
357 (APP1059354) and currently holds a fractional Professorial Research Fellow appointment at the
358 University of Queensland with his remaining time as CEO of Inflazome Ltd. a company
359 headquartered in Dublin, Ireland that is developing drugs to address clinical unmet needs in
360 inflammatory disease by targeting the inflammasome. MEP is an Australian Postgraduate Award
361 scholar. AGE and MATB are supported in part by a Wellcome Trust Strategic Award
362 104797/Z/14/Z. Research was supported by NHMRC grants (APP1005350, APP1045326), NIH
363 grant (R21AI098731/R33AI098731) and AID sequencing Grant (2013) as well as funding from
364 the Institute for Molecular Bioscience Centre for Superbug Solutions (610246).

365 *Acknowledgements*

366 We thank Dr Aurélie Jayol and Professor Patrice Nordmann for providing their complementation
367 assay methodology. The authors thank Maite Amado and Angela M. Kavanagh for technical
368 support with susceptibility assays and Rhia Stone for the quality control of antibiotics. We
369 acknowledge the sequencing services provided by the Australian Genome Research Facility. We
370 thank the team of the curators of the Institut Pasteur MLST system (Paris, France) for importing
371 novel alleles, profiles and/or isolates at http://bigsdb.web.pasteur.fr.

372 *Conflicts of interest*

373 None.

374

375 ## ABBREVIATIONS

376

377 BMD, Broth microdilution; caMHB, cation-adjusted Mueller-Hinton broth; CLSI, Clinical &
378 Laboratory Standards Institute; EUCAST, The European Committee on Antimicrobial
379 Susceptibility Testing; IS, Insertion sequence; KP, *Klebsiella pneumoniae*; KQ, *Klebsiella*
380 *quasipneumoniae*; MHA, Mueller-Hinton agar; MHB, Mueller-Hinton broth; MIC, Minimum
381 Inhibitory Concentration; MLST, Multi-locus sequence type; pEtN, phosphoethanolamine;
382 PROVEAN, Protein Variation Effect Analyzer; ST, Sequence type; XDR, Extensively drug
383 resistant; XDR-KP, Extensively drug-resistant *Klebsiella pneumoniae.*

384

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550

551 ## FIGURES AND TABLES

Table 1. Broth microdilution and VITEK®2 antimicrobial testing for the 24 clinical isolates

Strain*	Source†	Resistance Profile‡																							
		1			2	3	4		5	6			7	8	9	10	11	12	13	14	15	16	17	
		AMK	GEN	TOB	CPT	TZP	IPM	MEM	CFZ	FEP	CTX	CAZ	FOX	CIP	SXT	TGC	ATM	AMP	SAM	CHL	FOF	CST	MIN	TET
1_GR_13	St	R	R	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2_GR_12	U	R	R	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I^{R}	R	R	R	R	R^{S}	R	R	R
3_GR_13	S	R	R	R	R^{N}	R	R	R	R^{N}	R^{I}	R	R	R	R	R	I	R	R	R	R	R	R^{I}	I^{R}	R
4_GR_12	B	R	R	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I^{R}	R	R	R	R	R	R	I^{R}	R
5_GR_13	St	S	S	I	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I^{R}	R	R	R	R	R	R	R	R
6_GR_12	St	S	S	I^{R}	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R	S^{R}	I^{R}	R
7_GR_13	St	R	S	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R	R	S^{R}	S^{I}
8_GR_13	St	R	R	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9_GR_12	Br	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I^{R}	R	R	R	R	R	R	I^{R}	S^{I}
10_GR_13	B	S^{R}	S	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I	R	R	R	R	R	R	S^{R}	S^{I}
11_BR_13	U	S	S	R^{N}	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R^{N}	I	R	R	R	S^{N}	R^{N}	R	S^{N}	S^{N}
12_BR_13	Br	S	R^{S}	I^{N}	R^{N}	R	R	R	R^{N}	R^{I}	R	R	R	R	R^{N}	I^{R}	R	R	R	R^{N}	R^{N}	R	I^{N}	S^{N}
13_GR_14	Br	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R	R^{N}	R	R	R	R	S^{R}	R	R	R	R	R	R	S^{I}	S^{I}
14_GR_14	U	I^{R}	S^{R}	R	R^{N}	R	R	R	R^{N}	R	R^{N}	R	R	R	R	S^{R}	R	R	R	R	R	R	S^{R}	S^{R}
15_GR_13	St	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R^{I}	R^{N}	R	R	R	R	I^{R}	R	R	R	R	R	R	S^{I}	S
16_GR_13	St	R	R	R	R^{N}	R	R	R	R^{N}	R	R^{N}	R	R	R	R	R^{I}	R	R	R	R	R	R	I^{R}	R
17_GR_14	St	R	R	R	R^{N}	R	R	R	R^{N}	R	R^{N}	R	R	R	R	I	R	R	R	R	R	R	S^{R}	R
18_GR_14	St	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	I	R	R	R	R	R	R	S^{I}	S
19_GR_14	St	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R	R	I^{R}	I^{R}
20_GR_12	St	R	S	R	R^{N}	R	R	R	R^{N}	R^{I}	R	R	R	R	R	R	R	R	R	R	R	S	R	R
21_GR_13	U	S	S^{R}	I	R^{N}	R	R	I^{R}	R^{N}	R^{I}	R	R	R	S	R	S	S	R	R	S	S	S	S	S
22_GR_12	S	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R^{I}	R	R	R	R	R	I	R	R	R	R	R^{S}	S	S^{I}	S
23_GR_12	St	R	R	R	R^{N}	R	R	R	R^{N}	R	R	R	R	R	R	R	R	R	R	R	R^{S}	R^{S}	R	R
24_GR_13	St	I^{R}	S	R	R^{N}	R	R	R	R^{N}	R^{I}	R	R	R	R	R	I^{R}	R	R	R	R	R	S	I^{R}	S

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

566 †Source represented as B, Blood; Br; Bronchial secretion; U, Urine; S, Sputum; St, Stool.

567 ‡Antibiotic resistance as determined by broth microdilution according to CLSI guidelines (EUCAST for fosfomycin (disk diffusion) and
568 tigecycline) and in superscript, any discrepancies identified in VITEK®2 results. Antibiotic classes tested include **1**, Aminoglycosides (Amikacin,
569 AMK; Gentamicin, GEN; Tobramycin, TOB); **2**, Anti-MRSA cephalosporins (Ceftaroline, CPT); **3**, Antipseudomonal penicillins + β-lactamase
570 inhibitors (Piperacillin-tazobactam, TZP); **4**, Carbapenems (Imipenem, IPM; Meropenem, MEM); **5**, Non-extended spectrum cephalosporins (1st
571 and 2nd generation) (Cefazolin, CFZ); **6**, Extended-spectrum cephalosporins (3rd and 4th generation) (Cefepime, FEP; Cefotaxime, CTX,
572 Ceftazidime, CAZ); **7**, Cephamycins (Cefoxitin, FOX); **8**, Fluoroquinolones (Ciprofloxacin, CIP); **9**, Folate pathway inhibitors (Trimethoprim-
573 sulfamethoxazole, SXT); **10**, Glycylcyclines (Tigecycline, TGC); **11**, Monobactams (Aztreonam, ATM); **12**, Penicillins (Ampicillin, AMP); **13**,
574 Penicillins + β-lactamase inhibitors (Amipicillin-sulbactam, SAM); **14**, Phenicols (Chloramphenicol, CHL); **15**, Phosphonic acids (Fosfomycin,
575 FOF); **16**, Polymyxins (Colistin, CST); **17**, Tetracyclines (Minocycline, MIN; Tetracycline, TET).

576 R, Resistant; I, Intermediate; S, Susceptible; N, Not tested.

Table 2. Potential mutations contributing to polymyxin resistance and acquired resistance genes

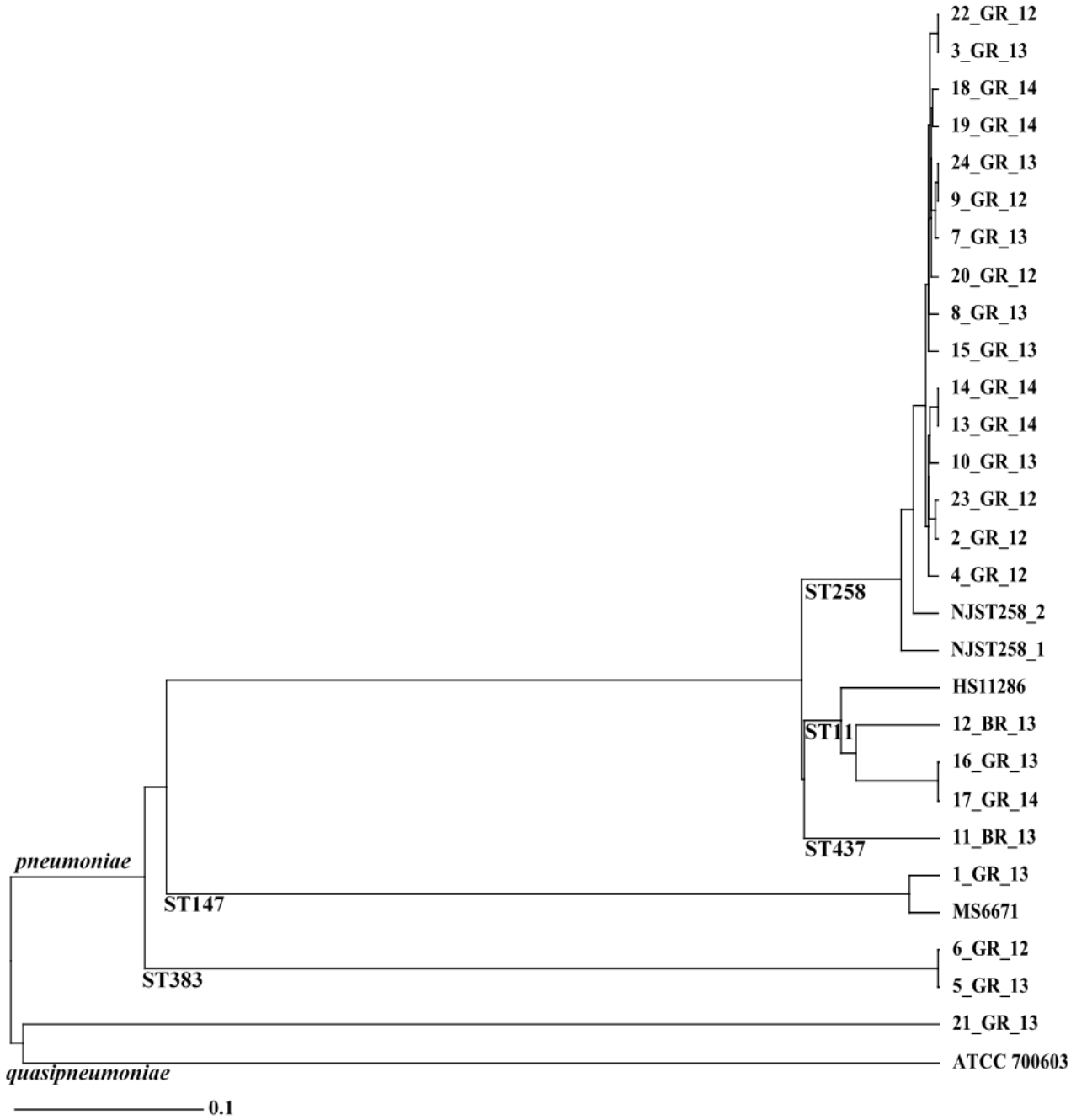
| Strain | MLST* | PMX-R† | Acquired antibiotic resistance genes‡ | | |

579 [*]Multilocus sequence type as identified through MultiLocus Sequence Typing Server 1.8.

580 [†]Variations detected in *mgrB*, *phoPQ* and *pmrAB* potentially causing polymyxin resistance (PMX-R). Significant non-synonymous changes
581 determined by PROVEAN analysis. WT (Wild-type) alleles in comparison to 20_GR_12. Displayed as gene impacted, initial amino acid, position
582 and new amino acid. If - shown in front of position, alteration is encoded upstream and if - is only displayed, no significant non-synonymous
583 changes were detected in these loci. Insertion sequences classified as Δ, identity as per ISFinder and orientation in superscript. Orientation
584 determined as forward, ^{F}, if transposase is in the same direction as *mgrB* and conversely, reverse, ^{R}, if in the opposite direction to *mgrB*.

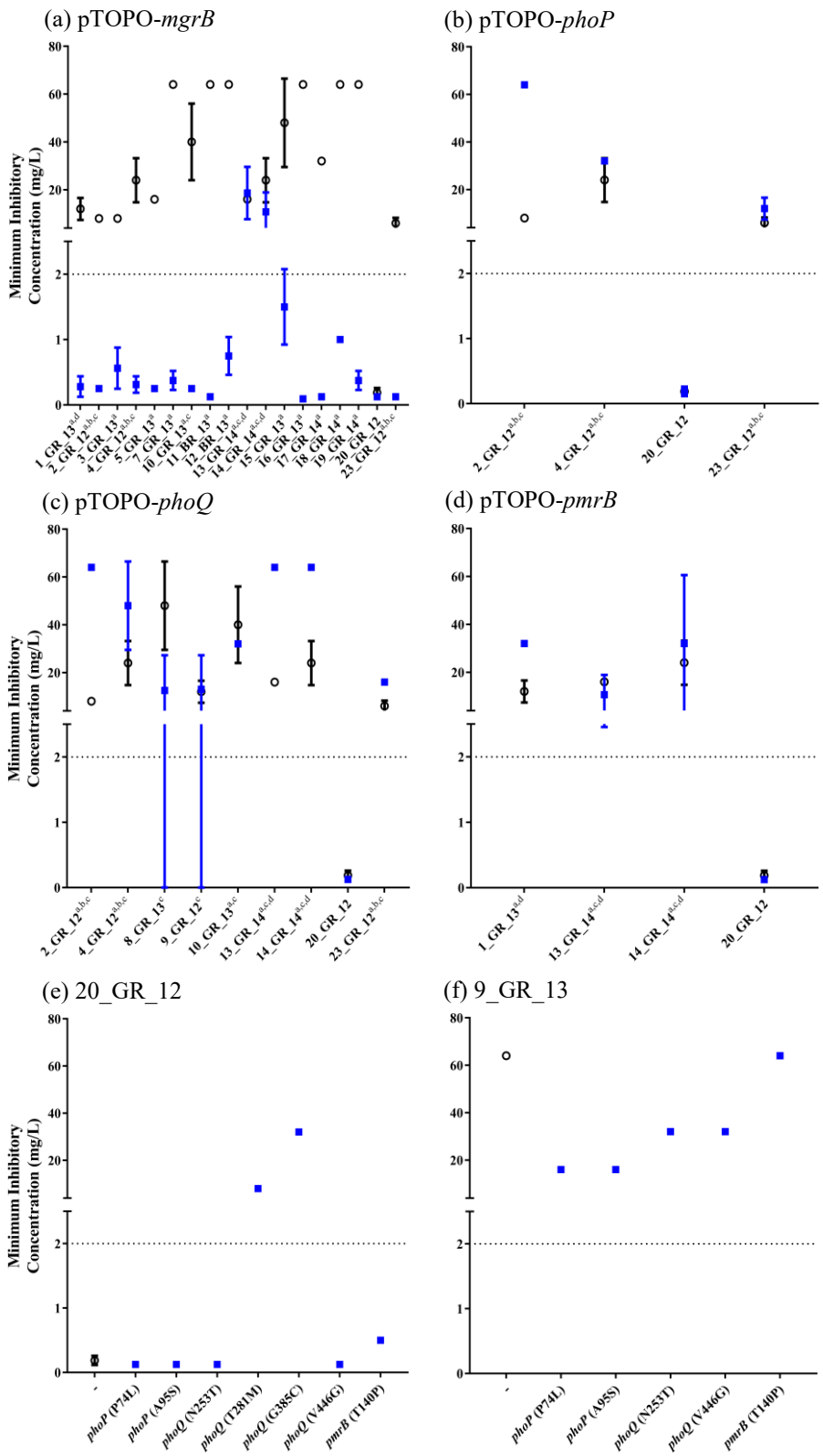
585 [‡]Acquired antibiotic resistance genes detected via ResFinder 2.1. Classes of antibiotics impacted displayed as A, Aminoglycoside; B, Beta-lactam;
586 F; Fosfomycin; M, Macrolide; P, Phenicol; Q, Quinolone; R, Rifampicin; S, Sulphonamide; T, Tetracycline; Tr, Trimethoprim. Shading indicates
587 detection of gene (≥90% homology, ≥60% sequence length).

588



589

590 **Fig. 1.** Neighbor-joining tree of core genome MLST of 24 *Klebsiella* clinical isolates. Clustering of
591 sequence type (ST) indicated at base of diverging branch. cgMLST compared to completed assemblies
592 including ATCC 700603 (*K. quasipneumoniae*), HS11286 (ST11), MS6671 (ST147) and NJST258_1
593 and NJST258_2 (ST258).


(a) pTOPO-*mgrB*
(b) pTOPO-*phoP*
(c) pTOPO-*phoQ*
(d) pTOPO-*pmrB*
(e) 20_GR_12
(f) 9_GR_13
Minimum Inhibitory Concentration (mg/L)
1_GR_13a,d
2_GR_12a,b,c
3_GR_13a
4_GR_12a,b,c
5_GR_13a
7_GR_13a
10_GR_13a,c
11_BR_13a
12_BR_13a
13_GR_14a,c,d
14_GR_14a,c,d
15_GR_13a
16_GR_13a
17_GR_14a
18_GR_14a
19_GR_14a
20_GR_12
23_GR_12a,b,c
8_GR_13c
9_GR_12c
-
phoP (P74L)
phoP (A95S)
phoQ (N253T)
phoQ (T281M)
phoQ (G385C)
phoQ (V446G)
pmrB (T140P)

618 **Fig. 2.** Complementation assays and influence of gene on polymyxin resistance. Polymyxin B
619 MIC measured before (○) and after (■) complementation of wild-type gene (a) pTOPO-*mgrB*,
620 (b) pTOPO-*phoP*, (c) pTOPO-*phoQ*, or (d) pTOPO-*pmrB* in indicated resistant isolates. (e)
621 Mutated genes complemented into 20_GR_12 (polymyxin-susceptible isolate) to determine if
622 variant induces polymyxin resistance. (f) Complementation of 9_GR_13 (IS element disrupted
623 *mgrB* control) to detect potential suppressor mutations. Strains shown on x axis for (a-d) and
624 superscript indicates variants in genes including *mgrB* (a), *phoP* (b), *phoQ* (c) and *pmrB* (d)
625 that differ from 20_GR_12. For (e, f), the x axis shows the gene complemented with amino
626 acid variation in brackets. Dotted line at 2 mg/L represents the breakpoint for polymyxin B.
627 Values indicate mean±standard deviation where no error bars display no fluctuation in MIC
628 (n≥2 colonies).