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**A Klebsiella pneumoniae antibiotic resistance mechanism that subdues host defences and promotes virulence**

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**Abstract**

*Klebsiella pneumoniae* is an important cause of multidrug-resistant infections worldwide. Recent studies highlight the emergence of multidrug-resistant *K. pneumoniae* strains which show resistance to colistin, a last-line antibiotic, arising from mutational inactivation of the *mgrB* regulatory gene. However, the precise molecular resistance mechanisms of *mgrB*-associated colistin resistance and its impact on virulence remain unclear. Here, we constructed an *mgrB* gene *K. pneumoniae* mutant and performed characterisation of its lipid A structure, polymyxin and antimicrobial peptide resistance, virulence and inflammatory responses upon infection. Our data reveal that *mgrB* mutation induces PhoPQ-governed lipid A remodelling which confers not only resistance to polymyxins, but also enhances *K. pneumoniae* virulence by decreasing antimicrobial peptide susceptibility and attenuating early host defence response activation. Overall, our findings have important implications for patient management and antimicrobial stewardship, while also stressing antibiotic resistance development is not inexorably linked with subdued bacterial fitness and virulence.

**Keywords** antimicrobial peptides; *Klebsiella pneumoniae*; *mgrB*; polymyxins; virulence

**Subject Categories** Microbiology, Virology & Host Pathogen Interaction

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**Introduction**

The widespread emergence of multidrug-resistant (MDR) bacterial pathogens is an important public health challenge worldwide (World Health Organization, 2014). Infections with MDR organisms are associated with increased mortality, longer hospital stays and inflated healthcare costs (Lambert et al., 2011; Neidell et al., 2012; Martin-Loeches et al., 2015). Recent data also indicate a trend towards increased antibiotic resistance among cases of community-onset infections (Lim et al., 2014; World Health Organization, 2014; Stefaniuk et al., 2016). For many bacterial pathogens, particularly Gram-negative organisms, high rates of antimicrobial resistance present limited therapeutic options for treating serious infections.

*Klebsiella pneumoniae* is one of these MDR organisms identified as an urgent threat to human health by the World Health Organization, the US Centers for Disease Control and Prevention and the UK Department of Health. *K. pneumoniae* infections are particularly a problem among neonates, elderly and immunocompromised individuals within the healthcare setting, but this organism is also responsible for a significant number of community-acquired infections including pneumonia and sepsis (Paczosa & Mecsas, 2016; Quan et al., 2016). The lineage defined as sequence type (ST) 258 is a notorious example of MDR *K. pneumoniae*; ST-258 frequently carries the *K. pneumoniae* carbapenemase (KPC) gene, as well as numerous other acquired AMR determinants, and has been responsible for outbreaks on several continents (Paczosa & Mecsas, 2016). Colistin is now often considered as the last treatment option for KPC-producing *K. pneumoniae*, but reports of colistin-resistant *Klebsiella* isolates are on the rise (Tzouvelekis et al., 2012; Olaitan et al., 2014b; Nation et al., 2015).

Several recent studies highlight the emergence of colistin resistance in MDR *K. pneumoniae* arising from loss-of-function mutations of the *mgrB* gene, a negative regulator of the PhoPQ signalling system (Lippa & Goulian, 2009; Cannatelli et al., 2013; Olaitan et al., 2014a; Poirel et al., 2015; Wright et al., 2015; Zowawi et al., 2015). The PhoPQ two-component system is a well-known regulator of envelope remodelling, chiefly the lipopolysaccharide (LPS) lipid A section, and contributes to bacterial resistance to innate immune killing (Groisman, 2001; Llobet et al., 2011). *K. pneumoniae* PhoPQ also governs lipid A plasticity *in vivo* and *in vitro* (Llobet et al., 2015) and plays a role in virulence as assayed using the wax moth *Galleria mellonella* infection model (Insua et al., 2013).

Alarming, the evidence indicates that *mgrB*-dependent colistin resistance is not associated with a significant fitness cost *in vitro* and is stably maintained in the absence of selective pressure...
(Cannatelli et al., 2015), which may explain the rapid dissemination of strains carrying this resistance mechanism in the clinical setting (Cannatelli et al., 2013, 2014; Olaïtan et al., 2014a; Cheng et al., 2015; Poirel et al., 2015; Wright et al., 2015; Zowawi et al., 2015). However, the precise molecular resistance mechanisms of mgrB-associated colistin-resistant K. pneumoniae remain unclear. Moreover, it is currently unknown whether mgrB mutation confers any loss of virulence. This is particularly critical given the increasing number of K. pneumoniae infections caused by virulent clones and the ease with which mgrB mutations arise in the hospital setting.

To answer these clinically relevant questions, we constructed a mgrB mutant in a K. pneumoniae strain representative of the virulent Klebsiella clonal groups, therefore encoding the genetic determinants associated with severe human infections (Lery et al., 2014; Holt et al., 2015). The evidence presented in this work demonstrates that inactivation of mgrB not only results in colistin resistance, but also enhances K. pneumoniae virulence by decreasing the susceptibility to a plethora of antimicrobial peptides and attenuating the activation of host defence responses.

**Results**

**Deletion of mgrB confers increased colistin resistance and multiple lipid A modifications in a PhoPQ-dependent manner**

To solidify earlier reports indicating an association between mgrB gene mutation and colistin resistance, we constructed an mgrB mutant (referred to as 52145-mgrB herein) in the wild-type virulent K. pneumoniae strain 52145 (Kp52145 herein). Control experiments showed that the growth kinetics in rich and minimal media were similar between the wild-type and 52145-mgrB strains (Fig EV1A). The 52145-mgrB mutant showed a slightly increased capacity to form a short-term biofilm compared to the wild type (Fig EV1B).

Upon Etest® minimal inhibitory concentration (MIC) testing, we demonstrated a 128-fold increase in colistin resistance in 52145-mgrB (16.0 μg/ml) compared to the wild type (0.125 μg/ml; Fig 1A). Similar results were obtained when testing the susceptibility to polymyxin B (Appendix Fig S1), another cyclic polypeptide also used in clinical practice to treat MDR Gram-negative bacterial infections (Nation et al., 2015). When the mgrB mutant was complemented (strain 52145-mgrBCom), MICs to colistin and polymyxin B were restored to wild-type levels (0.125 μg/ml; Fig 1A and Appendix Fig S1).

Resistance to colistin and polymyxin B is associated with remodelling in the lipid A (Olaïtan et al., 2014b). To determine whether mgrB mutation results in lipid A changes, lipid A was extracted from the wild-type strain and the mgrB mutant using an ammonium hydroxide/isobutyric acid method and subjected to negative ion matrix-assisted laser desorption–ionisation time-of-flight (MALDI-TOF) mass spectrometry. Consistent with our earlier work, lipid A from the wild-type strain (Figs 1B and EV2, Appendix Table S1) showed hexa-acylated species (mass-to-charge ratio, [m/z] 1,824) corresponding to two glucosamines, two phosphates, four 3-OH-C14 and two myristate (C14), as well as two other peaks including m/z 1,840, corresponding to two glucosamines, two phosphates, four 3-OH-C14, one C14 and one -hydroxymyristate (C14:OH), and m/z 2,063 consistent with the addition of palmitate (m/z 239) to the hexa-acylated (m/z 1,824) species to produce a hepta-acylated lipid A (Llobet et al., 2011, 2015). Lipid A isolated from 52145-mgrB (Fig 1C) contained species m/z 1,824, m/z 1,840 and m/z 2,063 which were found also in the wild-type lipid A. In addition, we observed other lipid A species consistent with the addition of phosphoethanolamine (PEtN; m/z 124) and 4-amino-4-deoxy-L-arabinose (Ara4N; m/z 131) to the hexa-acylated m/z 1,824 to obtain species m/z 1,948 and m/z 1,955, respectively, and species m/z 2,079 consistent with the addition of palmitate to ion m/z 1,840 (Fig EV2).

Other ions detected in the 52145-mgrB lipid A comprised m/z 1,850 and m/z 1,866 consistent with four R-3-hydroxymyristoyl primary acyl chains, either one C14 or one C14:OH, respectively, and one palmitate (Llobet et al., 2015). Complementation of the mgrB mutant restored production of wild-type lipid A (Fig 1D).

We next investigated the lipid A of several previously published K. pneumoniae clinical strains linked to mgrB inactivation and colistin resistance (Poirel et al., 2015). We selected two isogenic ST-258 strains isolated from the same patient prior to (strain T1a) and after (strain T1b) colistin therapy and the development of mgrB-associated colistin resistance, along with four other colistin-resistant mgrB mutants (C21, C22, C2 and 1515) from individual patients comprising various geographic sources, genetic backgrounds, multidrug resistance mechanisms and mgrB sequence mutations (Appendix Table S2). Appendix Fig S2 shows that the lipid A species produced by the T1a strain were similar to those observed in the wild-type Kp52145 strain, whereas T1b lipid A showed the same modifications as that of the 52145-mgrB mutant. This latter finding was also consistent for each of the other clinical colistin-resistant mgrB mutant strains analysed.

Earlier work indicates that in K. pneumoniae, the dioxygenase LpxO is responsible for the generation of 2-hydroxymyristate, PagP is the acyltransferase required for the addition of palmitate to the lipid A, PmrC mediates the incorporation of PEtN (Llobet et al., 2011, 2015; Wright et al., 2015), whereas synthesis and addition of Ara4N is mediated by the pmrHIIKLm operon (Llobet et al., 2011). To verify that these loci were indeed responsible for mgrB-depen dent lipid A modifications, we constructed a range of double, triple, quadruple and quintuple lpxO, pagP, pmrC and pmrF mutants in the 52145-mgrB background (Appendix Table S3) and analysed their lipid A structure. As anticipated, lpxO mutants lacked lipid A species containing 2-hydroxymyristate, pagP mutants did not contain species with palmitate, pmrF mutants lacked Ara4N-modified lipid A species, and pmrC mutants did not produce lipid A species modified with PEtN (Appendix Fig S3). It should be noted that the absence of a lipid A modification did not have an impact on the others.

The increased production of modified lipid A species in the mgrB mutant led us to investigate whether the expression of the loci responsible for these modifications was upregulated in the mgrB mutant background. To quantitatively assess the transcription of these loci, we used four transcriptional fusions containing a promoterless luciferase firefly gene under the control of the relevant locus promoter region. Each fusion (i.e. lpxO::lucFF, pagP:: lucFF, pmrC::lucFF and pmrH::lucFF) was introduced into Kp52145, 52145-mgrB and 52145-mgrBCom, and then, luciferase activity was measured. Compared to the wild-type strain, we observed significantly upregulated expression of all the transcriptional fusions.
Figure 1. Deletion of $mgrB$ in *K. pneumoniae* invokes polymyxin resistance and multiple lipid A modifications in a PhoPQ-dependent manner.

A Minimal inhibitory concentrations to colistin of the *K. pneumoniae* 52145, 52145-$ mgrB$, 52145-$mgrB$Com, 52145-$mgrB$-AphoQGB, 52145-$mgrB$-ApmrAB and 52145-$mgrB$-DphoQGB strains. The broken line represents the European Committee on Antimicrobial Susceptibility Testing MIC breakpoint.

B-D Negative ion MALDI-TOF mass spectrometry spectra of lipid A purified from (B) *K. pneumoniae* 52145, (C) 52145-$mgrB$ and (D) 52145-$mgrB$Com strains. Data represent the mass-to-charge ($m/z$) ratios of each lipid A species detected and are representative of three extractions.

E-I Activity of the *lpxO* (E), *pagP* (F), *pmrC* (G) and *pmrH* (H) and *phoP* (I) promoters in *K. pneumoniae* 52145, 52145-$mgrB$, 52145-$mgrB$Com, 52145-$mgrB$-AphoQGB carrying lucFF transcriptional fusions. Values (expressed in relative luminescence units) are presented as the mean ± SD of three independent experiments measured in triplicate. ***P < 0.0005; **P = 0.0071; *P = 0.019; versus 52145 determined using two-way unpaired t-test.
in 52145−ΔmgrB (Fig 1E–H). In contrast, the luciferase activities of all transcriptional fusions in the mgrB-complemented strain were not significantly different than those observed in the wild-type background.

Based on the fact that the two-component PhoPQ and PmrAB systems govern the remodelling of Klebsiella lipid A (Llobet et al., 2011), we sought to determine the contribution of these two-component systems to mgrB-mediated lipid A changes. Lipid A produced by the mgrB-phoQ and mgrB-phoQ−pmrAB mutants resembled that of the wild type, whereas lipid A synthesised by the mgrB−pmrAB mutant and ΔmgrB−ΔphoQ complemented with phoQ (52145−ΔmgrB−ΔphoQGB−phoPQCom) were similar to the lipid A produced by the mgrB mutant (Fig EV3 and Appendix Table S1). Taken together, these data suggest that mgrB-mediated lipid A modifications are PhoPQ dependent. Further stressing the connection between MgrB and PhoPQ, phoP::lucFF activity was higher in the mgrB mutant than in the wild-type strain (Fig 1I), and the luciferase activities of lpxO, pagP, pmrF and pmrC transcriptional fusions were not significantly different between the mgrB-phoQ mutant and the wild type (Fig 1E–I). Importantly, we also confirmed that the colistin and polymyxin B resistance phenotype observed in the mgrB mutant was PhoPQ but not PmrAB dependent (Fig IA and Appendix Fig S1).

Collectively, these data demonstrate that deletion of the mgrB gene gives rise to colistin and polymyxin B resistance in a PhoPQ-dependent manner. Upregulation of the PhoPQ system results in increased expression of lpxO, pagP, pmrC and pmrF, which in turn facilitates lipid A modifications with 2-hydroxyxymristate, Ara4N, PEAN and palmitate. Significantly, these lipid A modifications were also observed in a broad selection of other clinically derived mgrB mutants showing colistin-resistant phenotypes.

**Inactivation of mgrB mediates increased resistance to antimicrobial peptides**

It is widely recognised that polymyxin antibiotics share a similar mode of action to cationic antimicrobial peptides (AMPs) with various studies demonstrating the relationship between polymyxin and AMP resistance (Groisman, 2001; McPhee et al., 2003; Campos et al., 2004). Therefore, we speculated that 52145−ΔmgrB may also show decreased susceptibility to AMPs. To test this hypothesis, we exposed Kp52145, 52145−ΔmgrB and 52145−ΔmgrBCom to four different human AMPs over 1 h and determined the proportion of surviving organisms. For human neutrophil peptide-1 (1.2 μM), we showed significantly (P = 0.0006) increased resistance in 52145−ΔmgrB when compared to the wild type (Fig 2A). Likewise, there was significantly (P ≤ 0.013) increased survival of 52145−ΔmgrB following exposure to each of three β-defensins (Fig 2B–D). The complemented strain showed similar AMP susceptibility to that of the wild type, indicating that mgrB mutation confers protection against human AMPs.

In *vivo* it is appreciated that several AMPs act in a synergistic manner in the infected tissue to combat invading pathogens (Afacan et al., 2013). Therefore, exposure to one AMP does not recapitulate the AMP challenge faced by a pathogen in *vivo*. To mimic this *in vivo* scenario, we thus turned to the *Galleria mellonella* infection model where only hours after infection, multiple AMPs are synthesised and released into the haemolymph to neutralise bacterial infection (Kavanagh & Reeves, 2004; Insua et al., 2013). Haemolymph was collected from *G. mellonella* challenged with heat-killed (HK) *Escherichia coli*, and the susceptibility of Kp52145, 52145−ΔmgrB and 52145−ΔmgrBCom to the AMPs present in the haemolymph was determined using a radial diffusion bioassay. The mgrB mutant was significantly more resistant than the wild type to AMPs present in *G. mellonella* haemolymph (Fig 2E). Complementation restored the mgrB mutant susceptibility to wild-type levels, demonstrating that mgrB mutation confers resistance to a repertoire of AMPs produced in response to bacterial infections.

**Multiple lipid A modifications contribute to mgrB-mediated colistin resistance**

To delineate the relative contributions of the lipid A modifications to the polymyxin resistance phenotype of the mgrB mutant, we undertook susceptibility testing using the Etest™ on each of the double-, triple- and quadruple-mutant Kp52145 strains. We observed a substantial reduction in MIC for both colistin and polymyxin B only in lpxO and/or pmrF mutant strains (Fig 3A and B).

To provide additional evidence for the involvement of these lipid A modifications in the mgrB-mediated resistance phenotype, we then calculated the per cent survival of the wild-type and mutant strains after a 1-h colistin (20 μg/ml) challenge. As anticipated, we observed a marked difference (P < 0.0001) in mean per cent survival between the wild-type (3.8 ± 3.8%) and 52145−ΔmgrB (83.6 ± 15.34%) strains (Fig 3C). Complementation fully restored the colistin resistance of the mgrB mutant to wild-type levels (Fig 3C), with similar results observed for polymyxin B (Fig 3D). Extended analyses of the double, triple and quadruple mutants when exposed to colistin provided confirmation of the results obtained using the Etest™. Indeed, compared to 52145−ΔmgrB, the 52145−ΔmgrB−ΔlpxO and 52145−ΔmgrB−ΔpmrF strains showed a significant (P ≤ 0.02) reduction in mean per cent survival (Fig 3C). The contribution of lipid A modifications with Ara4N and 2-hydroxyxymristate to survival was also confirmed in the 52145−ΔpmrC−ΔlpxO−ΔmgrB, 52145−ΔmgrB−ΔlpxO−ΔpmrF and 52145−ΔpmrC−ΔlpxO−ΔmgrB−ΔpmrF strains. In accordance with the colistin Etest™, we observed no difference in per cent survival between 52145−ΔpagP−ΔmgrB and 52145−ΔmgrB (P = 0.99), which corroborated earlier work showing that lipid A modification with palmitate is not involved in *K. pneumoniae* polymyxin resistance (Llobet et al., 2011). In contrast to the Etest™ MIC results, our data did reveal a significant difference in survival between 52145−ΔpmrC−ΔmgrB and 52145−ΔmgrB (P < 0.001); but with the 52145−ΔpmrC−ΔlpxO−ΔmgrB triple mutant, the anticipated amplified effect of removing both pmrC and lpxO was not evident.

In summary, we provide definitive evidence that the colistin and polymyxin B resistance phenotype of 52145−ΔmgrB occurs through at least two key modifications to the lipid A structure: the additions of Ara4N and 2-hydroxyxymristate.

**Hypervirulence of *K. pneumoniae* 52145−ΔmgrB in the *G. mellonella* infection model**

A strong correlation between the virulence of several MDR bacteria, including *K. pneumoniae*, in *G. mellonella* and mammalian virulence models has been established (Jander et al., 2000; Insua et al,
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[Image 85x393 to 512x700]

A human neutrophil peptide-1 (1.2 μM) and complemented strains into G. mellonella and monitored bacterial killing over time. No mortality was observed in the control phosphate-buffered saline (PBS)-injected G. mellonella larvae. After 72 h, 60–70% of the larvae challenged with Kp52145 and Kp52145-ΔmgrBΔphoOQGB survived. In stark contrast, only 18% of the larvae infected with Kp52145-ΔmgrB survived (Fig 4A). Complementation of the mgrB mutant restored the virulence to wild-type levels, indicating that mgrB mutation mediates the hypervirulence phenotype. This increase in virulence was also observed when the killing potential of a clinical colistin-resistant mgrB isolate was investigated. Figure 4B shows that infection with the colistin-resistant mgrB mutant (T1b) strain resulted in increased mortality as compared to the isogenic colistin-susceptible ancestral (T1a) strain.

It has been previously shown that boosting immunity in G. mellonella by pre-immunisation with HK-E. coli provides protection against subsequent K. pneumoniae infection (Insua et al., 2013). Therefore, we sought to determine whether mgrB mutation also confers hypervirulence in G. mellonella with stimulated host immunity. To this end, we inoculated larvae with 10⁶ HK-E. coli and then after 24 h infected with 10⁶ CFUs of Kp52145 or Kp52145-ΔmgrB. Confirming earlier work, larvae infected with Kp52145 showed a significant survival improvement if pre-immunised (Fig 4D). However, there was no survival difference between the Kp52145-ΔmgrB-infected larvae receiving pre-inoculation with HK-E. coli and the PBS vehicle (P = 0.76; Fig 4D), thus indicating that mgrB mutation increases virulence even in the pre-immunised host.

Earlier work from our group illustrated that K. pneumoniae capsule polysaccharide (CPS) is crucial for virulence in G. mellonella (Insua et al., 2013). To explore the contribution of CPS to the hypervirulence of the mgrB mutant, we compared the killing ability of the cps mutant (Kp52145-ΔmanC) and the double mgrB-cps mutant (Kp52145-ΔmgrB-ΔmanC). One hundred per cent of the larvae infected with 10⁶ CFUs of either Kp52145-ΔmanC or Kp52145-ΔmgrB-ΔmanC survived after 72 h (Fig 4E). Control experiments

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Figure 2. Deletion of mgrB in K. pneumoniae increases resistance to antimicrobial peptides.

A–D Per cent survival of K. pneumoniae 52145, 52145-ΔmgrB and 52145-ΔmgrBCom following 1-h exposure to the following: (A) human neutrophil peptide-1 (1.2 μM), (B) human β-defensin-1 (3 μM), (C) human β-defensin-2 (3 μM) and (D) human β-defensin-3 (7 μM). Values are presented as the mean ± SD of three independent experiments measured in triplicate. ***P = 0.0006; **P = 0.01–0.001; *P = 0.013; versus Kp52145 determined using two-way unpaired t-test.

E Resistance of K. pneumoniae 52145, 52145-ΔmgrB and 52145-ΔmgrBCom to antimicrobial factors produced by G. mellonella after 24-h exposure to 10⁶ heat-killed E. coli cells. The experiments were undertaken using a radial diffusion bioassay with data expressed as radial diffusion units (10 units = 1 mm). Values are presented as the mean ± SD of three independent experiments measured in triplicate. ***P < 0.0001; versus Kp52145 determined using two-way unpaired t-test.

2013; Jacobs et al., 2014). Importantly, we demonstrated that the G. mellonella model recapitulates key features of K. pneumoniae infection biology (Insua et al., 2013). To determine the pathogenic potential of the mgrB mutant in G. mellonella, we injected an equivalent dose of the wild-type, 52145-ΔmgrB, 52145-ΔmgrBΔphoOQGB and complemented strains into G. mellonella and monitored bacterial killing over time. No mortality was observed in the control phosphate-buffered saline (PBS)-injected G. mellonella larvae. After 72 h, 60–70% of the larvae challenged with Kp52145 and Kp52145-ΔmgrBΔphoOQGB survived. In stark contrast, only 18% of the larvae infected with Kp52145-ΔmgrB survived (Fig 4A). Complementation of the mgrB mutant restored the virulence to wild-type levels, indicating that mgrB mutation mediates the hypervirulence phenotype. This increase in virulence was also observed when the killing potential of a clinical colistin-resistant mgrB isolate was investigated. Figure 4B shows that infection with the colistin-resistant mgrB mutant (T1b) strain resulted in increased mortality as compared to the isogenic colistin-susceptible ancestral (T1a) strain.

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showed that both the wild type and mgrB mutant expressed the same amount of cell-bound CPS (187.1 ± 8.8 μg/10⁹ CFUs versus 177.7 ± 14.2 μg/10⁹ CFUs, respectively; P = 0.30). Nor was there any difference in the cps::lucFF activity determined in KpS1245 and S2145-ΔmgrB backgrounds (Fig EV4B). Overall, these data demonstrate that both mgrB mutation and CPS are necessary to increase K. pneumoniae virulence in G. mellonella, although MgrB does not control CPS expression.

Figure 3. Effect of lpxO, pmrF, pagP and pmrC mutation on the polymyxin resistance of K. pneumoniae mgrB mutant.

A, B Etest® minimal inhibitory concentrations to colistin and polymyxin B of K. pneumoniae S2145, S2145-ΔmgrB, S2145-ΔmgrBCom compared to the double, triple and quadruple S2145-ΔmgrB mutant strains.

C, D Per cent survival of the K. pneumoniae S2145 wild-type and mutant strains after exposure to 20 μg/ml colistin (C) and polymyxin B (D) over 1 h. Values are presented as the mean ± SD of three independent experiments measured in duplicate. ***P < 0.0001; *P = 0.0195; versus S2145-ΔmgrB determined using a two-way unpaired t-test.

To provide mechanistic insights into the mgrB hypervirulence phenotype, we investigated whether any of the lipid modifications found in the S2145-ΔmgrB lipid A contributed to the heightened virulence. Upon infection with the double-, triple- and quadruple-mutant strains, there was no difference in mortality when compared to S2145-ΔmgrB (Appendix Fig S4). However, infection with the mutant lacking all four lipid A modifications, strain S2145-ΔpmrC-ΔlpxO-ΔmgrB-ΔpmrF-ΔpagP, revealed a significantly different per
cent survival compared to 52145-ΔmgrpB (Fig 4C). Furthermore, there was no difference in mortality when compared to that triggered by the wild type. Collectively, these data provide evidence that 52145-ΔmgrpB pathogenicity in G. mellonella is combinatorial with all four lipid A modifications required to result in the overall virulence phenotype.

**K. pneumoniae 52145-ΔmgrpB infection is not attenuated in a murine infection model**

The hypervirulence of 52145-ΔmgrpB in G. mellonella prompted us to examine the ability of this mutant to cause infection in a mammalian infection model. C57BL/6 mice were intranasally inoculated with 3 × 10⁵ organisms, and bacterial loads in lung, spleen and nasal-associated lymphoid tissue (NALT) homogenates were determined at 24 h post-infection. Bacterial loads of 52145-ΔmgrpB in the NALT, lung and splenic samples were similar to those of the wild type (P ≥ 0.14; Fig 5A–C). We also sought to compare the level of inflammatory cytokine and antimicrobial peptide expression in the lungs of mice infected with Kp52145 and 52145-ΔmgrpB. Expression of Tnfa, Ifnb, Il1b, Il6, Il10 or Il12 was measured by real-time quantitative PCR (RT-qPCR) with levels of all cytokines, excluding Ifnb, higher in lungs of infected mice than in lungs of non-infected animals (Appendix Fig S5; P < 0.05 for all comparisons versus non-infected mice). Interestingly, the mgrpB mutant induced the same levels of all cytokines as the wild-type strain, and this was also true when the expression of murine defensins was analysed (Appendix Fig S6). Overall, these findings suggest that mgrpB mutation does not compromise *K. pneumoniae* immune-evasion strategies in the mouse model.

**K. pneumoniae 52145-ΔmgrpB attenuates the expression of antimicrobial peptides in the G. mellonella infection model**

We have previously demonstrated that there is a correlation between *K. pneumoniae* virulence and the expression of *G. mellonella* AMPs. The levels of AMPs are higher in larvae infected with an avirulent *K. pneumoniae* cps mutant than in larvae infected with the wild-type strain (Insua et al., 2013). The increased virulence of the mgrpB mutant led us to investigate whether this mutant affects the expression of AMPs in *G. mellonella*. After 8 h of infection, the expression of lysozyme, gallerimycin and galiomycin was significantly (P < 0.0001) lower in 52145-ΔmgrpB-infected larvae.
than in the wild-type-infected larvae (Fig 5D and Appendix Fig S7). Infection with the 52145-ΔpmrC-ΔlpxO-ΔmgrB, 52145-ΔmgrB-ΔlpxO-ΔpmrF and 52145-ΔpmrC-ΔlpxO-ΔmgrB-ΔpmrF triple and quadruple mutants restored lysozyme expression to wild-type levels (Fig 3D). Based on these data, we surmised that the lpxO mutation present in each of these three strains played a primary role in this phenotype. To explore this hypothesis, lysozyme expression was assessed during infections with an lpxO mutant (52145-ΔlpxO), the 52145-ΔmgrB-ΔlpxO double mutant and 52145-ΔmgrB-ΔlpxO complemented with lpxO (52145-ΔmgrB-ΔlpxO-ΔlpxOCom). Interestingly, expression of this AMP among the single and double Klebsiella mutant-infected larvae was similar to the wild type (P ≥ 0.38); however, the 52145-ΔmgrB-ΔlpxO-ΔlpxOCom strain induced an identical phenotype to the 52145-ΔmgrB mutant (P < 0.0001; Fig 5D). Likewise, expression of gallermycin and galiomycin was reduced among larvae infected with 52145-ΔmgrB-ΔlpxO-ΔlpxOCom (Fig EV5). These findings demonstrated that mgrB mutation is associated with an attenuated expression of G. mellonella AMPs upon infection. Furthermore, this phenotype is dependent on LpxO-controlled lipid A modification.

Early inflammatory responses in macrophages are subdued upon infection with 52145-ΔmgrB

The recognition of the lipid A pattern by the TLR4/MD-2 complex activates NF-κB and MAP kinase (MAPK)-regulated defence responses necessary to clear infections. The distinct lipid A produced by the mgrB mutant and the mgrB-dependent attenuation of G. mellonella defence responses led us to investigate the signalling pathways and inflammatory responses triggered by the mgrB mutant in macrophages. Immortalised bone marrow-derived macrophages (iBMMDMs) were infected with the wild type, mgrB mutant and the mgrB mutant-complemented strain with the activation of NF-κB and MAPKs assessed by immunoblotting. In the canonical NF-κB activation pathway, nuclear translocation of NF-κB is preceded by phosphorylation and subsequent degradation of IκBζ. All strains triggered the degradation of IκBζ (Fig 6A). Whereas the wild type and mgrB-complemented strain induced phosphorylation of the p38, JNK and ERK MAPKs, the mgrB mutant triggered reduced phosphorylation of the three MAPKs (Fig 6B). Similar results were obtained when MH-S macrophages were infected (Appendix Fig S8A), thus indicating that the failure to activate MAPKs by the mgrB mutant is not cell type dependent. Control experiments showed that K. pneumoniae-triggered TNF-α is dependent on MAPKs JNK and ERK but not on p38 because the levels of this cytokine were significantly lower only in the supernatants of infected macrophages treated with the ERK and JNK inhibitors (U0126 and SP600125, respectively) than in infected cells treated with vehicle solution or the p38 inhibitor (Appendix Fig S8B). As anticipated, based on the reduced activation of JNK and ERK by the mgrB mutant, levels of TNF-α in the supernatants of macrophages infected with the mgrB mutant were significantly lower than those found in the supernatants of cells infected with either the wild type or the mgrB-complemented strain. Mechanistically, the reduced activation of MAPKs JNK and ERK by the mgrB mutant was dependent on the four mgrB-controlled lipid A modifications because only the quintuple mutant triggered a consistently similar pattern of MAPK phosphorylation as the wild-type strain (Fig 6C). In good agreement with these results, the quintuple mutant induced levels of TNF-α similar to the wild-type strain (Fig 6D). Taken together, our data demonstrate that inactivation of mgrB in K. pneumoniae results in reduced activation of inflammatory signalling pathways in macrophages.

Discussion

The increasing isolation of MDR Gram-negative pathogens resistant to polymyxins, which are considered a last treatment option for these infections, is a health challenge worldwide. It is therefore important to define the molecular mechanisms responsible for polymyxin resistance and also to address whether the virulence of the pathogen is affected. The latter aspect is often neglected in those studies investigating MDR pathogens, and it is crucially important in the clinical setting where infections occur in immunocompromised patients.

Several studies have reported the emergence of colistin resistance in MDR K. pneumoniae arising from loss-of-function mutations of the mgrB gene (Cannatelli et al, 2013, 2014; Lopez-Camacho et al, 2014; Olaitan et al, 2014a; Cheng et al, 2015; Giani et al, 2015; Poirel et al, 2015; Wright et al, 2015; Zowawi et al, 2015). In this work, by combining biochemistry and genetics, we demonstrate that mgrB mutation in K. pneumoniae is associated with PhoPQ-governed lipid A remodelling which confers resistance to polymyxins and mammalian antimicrobial peptides. Mechanistically, our findings reveal that the lipid A modifications with Ara4N and 2-hydroxy-myristate mediate the resistance to polymyxins. Our results revealed that mgrB mutation increases K. pneumoniae virulence in the G. mellonella infection model, whereas it does not compromise K. pneumoniae survival in the mouse pneumonia model, hence further reinforcing the notion that the development of antibiotic resistance is not inexorably linked to decreased virulence and fitness costs.

Earlier work showed that MgrB is a small (47 amino acids), membrane-bound peptide which acts as negative feedback regulator of the PhoPQ two-component regulatory system (Lippa & Goulain, 2009). The fact that PhoPQ controls the expression of loci required for remodelling of the lipid A led to the assumption that mgrB mutation should be associated with changes in the lipid A structure, chiefly the addition of Ara4N to the lipid A. This notion was based on indirect experimental evidence showing that the transcription of the pmrF operon, responsible for the addition of Ara4N to the lipid A, is upregulated in an mgrB mutant background (Cannatelli et al, 2013; Wright et al, 2015). However, none of these assumptions have been formally proven. In this work, we demonstrate extensive remodelling of the lipid A produced by K. pneumoniae mgrB mutants, which is modified with Ara4N, 2-hydroxy-myristate, palmitate and PEtN. Mechanistically, we have demonstrated that the pmrF operon, lpxO, pagP and pmrC are the loci responsible for these lipid A modifications in the mgrB mutant. Furthermore, we provide conclusive evidence showing the crucial role of PhoPQ governing all of these lipid A modifications. PhoPQ is responsible for the upregulated expression of the pmrF operon, lpxO, pagP and pmrC observed in the mgrB mutant. However, our analysis ruled out the contribution of the PmrAB two-component system despite its previously described
contribution controlling some of these lipid A modifications in
*K. pneumoniae* and other bacteria (Gunn et al., 1998; Groisman, 2001; Mitrophanov et al., 2008; Arroyo et al., 2011).

By testing a panel of mutants constructed in the mgrB mutant, we uncovered that polymyxin resistance is mostly dependent on the lipid A modification with Ara4N and 2-hydroxymyristate, although

Figure 5. Virulence of *K. pneumoniae* mgrB mutant in a murine intranasal infection model and expression of lysozyme in *G. mellonella* upon infection.

A–C Bacterial loads of *K. pneumoniae* 52145 and 52145-DmgrB in nasal-associated lymphoid tissue (NALT), lung and spleen homogenates of infected mice after 24 h. Six mice per group were infected with log_{10} colony-forming unit values presented as the mean ± SD. Data were analysed using two-way unpaired t-test.

D Expression of lysozyme produced by *G. mellonella* after 8 h of infection with *K. pneumoniae* 52145, 52145-DmgrB, 52145-DmgrBCom, 52145-DmgrB-DphaOQGB, 52145-DmgrB-DphaOQGB-DlpxO, 52145-DmgrB-DphaOQGB-DlpxO-DpmrF, 52145-DmgrB-DphaOQGB-DlpxO-DpmrF-DlpxO, 52145-DmgrB-DphaOQGB-DlpxO-DpmrF-DlpxO-DpmrF and 52145-DmgrB-DphaOQGB-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO-DpmrF-DlpxO as determined by reverse transcriptase quantitative real-time PCR. Three larvae per group were infected, and values are presented as the mean ± SD of two independent cDNA preparations measured in duplicate. ***P < 0.0001, *P = 0.035; versus 52145 determined using two-way unpaired t-test. 
Figure 6. mgrB inactivation results in downregulation of early inflammatory responses in macrophages upon infection.

A Immunoblot analysis of IκBα and tubulin levels in lysates of iBMDM cells infected with K. pneumoniae 52145, 52145-ΔmgrB and 52145-ΔmgrBCom for the indicated times.

B Immunoblot analysis of phospho-ERK (P-ERK), phospho-p38 (P-p38), phospho-JNK (P-JNK) and tubulin levels in lysates of iBMDMs cells infected with K. pneumoniae 52145, 52145-ΔmgrB, 52145-ΔmgrBCom and for the indicated times.

C Immunoblot analysis of phospho-ERK (P-ERK), phospho-JNK (P-JNK) and tubulin levels in lysates of iBMDMs cells infected with K. pneumoniae 52145, 52145-ΔmgrB, 52145-ΔpmrC-ΔlpxO-ΔpmrF-ΔpagP, 52145-ΔpmrC-ΔpmrF, 52145-ΔpmrC, 52145-ΔlpxO and 52145-ΔmgrB-ΔpmrC for 40 min.

D TNF-α secretion by iBMDM macrophages stimulated for 6 h with 3 × 10^5 UV-killed K. pneumoniae 52145, 52145-ΔmgrB, 52145-ΔmgrBCom and 52145-ΔpmrC-ΔlpxO-ΔmgrB-ΔpmrF-ΔpagP. ***P < 0.0001; *P = 0.02; versus 52145 determined using two-way unpaired t-test (mean ± SD).

Data information: Data are representative of at least three independent experiments. Source data are available online for this figure.
a contribution of the PmrC-dependent PEtN modification was observed when the polymyxin susceptibility was assessed using a standard assay in the antimicrobial peptide field. Whereas it is well established that the substitution of the lipid A phosphates with Ara4N or PEtN limits the interaction of polymyxins with the bacterial surface, thereby mediating resistance (Olaian et al., 2014b), the role of 2-hydroxymyristate remains unknown. Nonetheless, it is becoming evident that the presence of hydroxyl groups in the lipid A provides a competitive advantage in the presence of antimicrobial peptides (Hankins et al., 2011; Llobet et al., 2015). The fact that other Gram-negative pathogens (Salmonella, Legionella, Acinetobacter, Pseudomonas) also synthesise lipid A species possessing a hydroxyl group on a secondary acyl chain (Kulshin et al., 1991; Zahringer et al., 1995; Gibbons et al., 2000; Becceiro et al., 2011) could indicate that this lipid A modification is a conserved mechanism to counteract antimicrobial peptides. It is beyond the scope of this article to precisely define the molecular mechanism underlying 2-hydroxymyristate-dependent colistin resistance. However, it has been argued by Nikaido and co-workers that LpxO-dependent lipid A modification increases the H bonding between neighbouring LPS molecules, thereby limiting polymyxins-triggered self-promoted pathway (Hancock et al., 1981; Nikaido, 2003).

Our work also highlights a connection between virulence and antimicrobial resistance. mgrB mutants were hypervirulent in the G. mellonella infection model even if the immunity of larvae was boosted prior to infection. Recently, we reported that following innate immune induction, the haemolymph of G. mellonella contains antimicrobials which inhibited K. pneumoniae growth (Insua et al., 2013). It was then plausible to speculate that the heightened virulence of the mgrB mutant was due to its increased resistance to antimicrobial peptides. Indeed, we demonstrate that mgrB mutation confers resistance to the G. mellonella antimicrobial peptides. Notably, to restore the virulence of the mgrB mutant to wild-type levels, it was necessary to mutate all loci responsible for the lipid A modifications (Fig 2E). However, since in a previous work, we demonstrated that these individual mutants are already attenuated in G. mellonella, it is possible that there are additional, yet unknown, MgrB-controlled factor(s) playing a role in Galleria virulence (Insua et al., 2013). At present, we can only speculate which are these factors. Recent transcriptional profiling analysis of six mgrB mutants revealed the upregulation of the several loci of the PhoPQ regulon but also the increased expression of two additional two-component regulatory systems, RstAB and CrrAB (Wright et al., 2015). Whereas the RstB sensor has been shown to control the expression of PhoP-regulated genes, there is no data on the CrrAB regulon and whether this system contributes to K. pneumoniae virulence. Future research efforts will be directed to understand the role of these systems in Klebsiella pathogenesis.

We demonstrated no impact of the mgrB mutation on the capacity of K. pneumoniae to establish pulmonary and systemic infection in the pneumonia mouse model. This finding is in contrast to the hypervirulence phenotype observed in G. mellonella, but this most likely reflects the complexity of mammalian models showing a more complex innate immune response dependent on several cell types. Nevertheless, in the setting of an immunocompromised host (e.g. ICU patient) coupled with increased AMP resistance, it is feasible that mgrB inactivation may play a role in the early establishment of infection, pathogenicity and subsequent patient outcomes. Supporting this notion, isolation of colistin-resistant strains has been associated with death in septic patients with K. pneumoniae infection (Falcone et al., 2016).

Another novel finding of this work is that the mgrB mutant elicited limited activation of inflammatory responses in vitro and in vivo (G. mellonella model). This is particularly relevant since more than two decades of research have established the critical importance of a controlled inflammatory response to clear K. pneumoniae infection. Therefore, the reduced response induced by the mgrB mutant could be considered another feature of increased virulence associated with this mutation. Strikingly, mgrB-dependent attenuation of G. mellonella defence responses was solely dependent on LpxO-mediated lipid A modification. This is in perfect agreement with our recent work demonstrating that Klebsiella LPS containing 2-hydroxymyristate is less inflammatory than that lacking this modification (Llobet et al., 2015). In contrast, our data revealed that mgrB-mediated attenuation of inflammatory responses in macrophages was dependent on all four lipid A modifications. This reflects the complex interaction between K. pneumoniae and macrophages and is in good agreement with published evidence demonstrating the non-redundant contribution of Klebsiella lipid A modifications to limit phagocytosis by professional phagocytes (macrophages and amoeba; March et al., 2013). MAPKs play a crucial role in governing immune responses, thereby highlighting the relevance of attenuating their activation by the mgrB mutant (Dong et al., 2002). Current evidence shows that JNK and ERK regulate the induction of inflammatory responses and production of defensins, two crucial host defence responses against K. pneumoniae (Moranta et al., 2010), and in this work, we have shown that inactivation of mgrB resulted in limited activation of both MAPKs. Nonetheless, there is still limited understanding of which MAPKs-governed responses are crucial against K. pneumoniae infections. Future studies will be directed to identify these responses.

To explain how mgrB-controlled lipid A limits the activation of inflammatory responses, it is reasonable to postulate that this extensively modified lipid A is not recognised by the TLR4/MD-2 LPS receptor complex. The work of Park and co-workers predicts that the hepta-acylated lipid A containing palmitate will not be accommodated within the active site of its MD-2 receptor, hence blocking the subsequent dimerisation of TLR4 (Park et al., 2009). In addition, the substitution of the lipid A phosphates with Ara4N or PEtN should affect the receptor–ligand interaction since the lipid A 1- and 4'-phosphate groups interact with a cluster of positively charged residues from TLR4 and MD-2 (Park et al., 2009). To rigorously validate these predictions, it will be necessary to assess individually all these lipid A species with and without their various modifications. The elegant synthetic biology approach described by Stephen Trent’s group (Needham et al., 2013) engineering of E. coli to produce each of the lipid A variants may provide a means for comparing the different lipid As.

In general, it is accepted that antibiotic resistance most often confers a cost in terms of subdued bacterial fitness and virulence (Becceiro et al., 2013). Previous studies also illustrate that microbial immune-evasive strategies contribute to the development and persistence of antimicrobial resistance (Needham & Trent, 2013; De Majumdar et al., 2015). Here, we have demonstrated a divergent
scenario whereby an antimicrobial resistance mechanism helps to counteract the activation of immune defences and enhance virulence. Overall, this evidence stresses the importance of considering antimicrobial resistance and virulence together, while also highlighting the urgent need to include the identification of virulent clones in clinical microbiology laboratories.

Finally, it is worthwhile discussing the clinical consequences and implications of our findings. Limited therapeutic options to treat MDR Gram-negative pathogens, in particular Pseudomonas aeruginosa, Acinetobacter baumannii and K. pneumoniae, led clinicians to reappraise the clinical application of colistin. This antibiotic is increasingly being used in endemic areas for KPC-producing K. pneumoniae, but reports of colistin-resistant isolates in that species are on the rise (Tzouvelekis et al, 2012; Olaitan et al., 2014b; Nation et al., 2015). The evidence presented in this study indicates that inactivation of mgrB results in colistin resistance, but also enhances K. pneumoniae virulence. The latter finding is particularly alarming because a large number of Klebsiella strains resistant to colistin carry alterations of the mgrB gene (Lippa & Goulain, 2009; Cannatelli et al., 2013; Olaitan et al., 2014a; Poirel et al., 2015; Wright et al., 2015; Zowawi et al., 2015).

In fact, the heightened virulence of these strains might be one of the explanations underlying the increased mortality associated with these infections (Capone et al., 2013). Physicians should be aware of such an occurrence for its implications on treatment and outcome. This evidence further stresses the importance of careful consideration of colistin therapies, at least for Klebsiella infections. In this context, we put forward the need to perform well-designed clinical trials to inform antibiotic regimens for treating infections caused by KPC-Klebsiella.

Materials and Methods

Ethics statement

The experiments involving mice were approved by the Queen’s University Belfast’s Ethics Committee and conducted in accordance with the UK Home Office regulations (Project Licence PPL2700). Female C57BL/6 mice (8-9 weeks of age) were mock-infected with PBS (n = 4) and infected with the wild-type strain (n = 6) or the mgrB mutant (n = 6). Animals were randomised for interventions, but researchers processing the samples and analysing the data were aware which intervention group corresponded to which cohort of animals.

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are presented in Appendix Table S3. Kp52145 is a clinical isolate, serotype O1:K2, belonging to the virulent CC65 clonal complex (Brisse et al., 2009; Lery et al., 2014). Six previously published clinical K. pneumoniae strains (T1a, T1b, C21, C22, C2 and 1515) were supplied by Prof. Patrice Nordmann, University of Fribourg, Switzerland (Poirel et al., 2015). Bacteria were grown in LB medium at 37°C, and where appropriate, antibiotics were supplemented at the following concentrations: ampicillin (Amp) 100 µg/ml, trimethoprim (Tmp) 100 µg/ml, tetracycline (Tet) 12.5 µg/ml, chloramphenicol (Cm) 25 µg/ml, kanamycin (Km) 50 µg/ml and carbenicillin (Cb) 50 µg/ml.

Virulent and resistant mgrB-disabled K. pneumoniae

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PCR primers used for the mgrB mutant construction were designed using the whole genome sequence of Kp52145 (GenBank Accession No. F0834906.1; Appendix Table S3). The primer pairs mgrB_UPFWD, mgrB_UPRVS, mgrB_DOWNFWD and mgrB_DOWNRVS (Appendix Table S4) were used in separate PCR reactions to amplify 550- to 800-bp fragments flanking the mgrB gene. BamHI restriction sites internal to these flanking regions were incorporated at the end of each amplicon. Purified mgrB UP and DOWN fragments were then polymerised and amplified as a single PCR amplicon using the primers mgrB_UPFWD and mgrB_DOWNRVS. This 1.4-kb PCR amplicon was then cloned into pGEM-T Easy (Promega) to obtain pGEM-mgrB and transformed into E. coli C600. After EcoRI digestion, the purified 1.4-kb fragment was cloned into EcoRI-digested Antarctic Phosphatase (New England Biolabs)-treated pGPl-SceI-2 suicide vector (Aubert et al., 2014) to generate pGPl-SceI-mgrB and transformed into E. coli GT115 (or SY327). pGPl-SceI-mgrB was thereafter transformed into the diaminopimelate (DAP) auxotrophic E. coli donor strain β2163 (Demarre et al., 2005) and mobilised into K. pneumoniae via conjugation. Selection of co-integrate clones was undertaken using LB agar supplemented with Tmp at 37°C. A second crossover reaction was then performed by conjugating the pDAI-SceI-SacB plasmid (Aubert et al., 2014) into a refreshed overnight culture containing three Tmp-resistant co-integrate clones. Exconjugants were selected on LB agar supplemented with Tet at 37°C. Candidate mutant clones were checked for susceptibility to Tmp and then confirmed by PCR using the mgrB_UPFWD and mgrB_DOWNRVS primers. Curing of the pDAI-SceI-SacB vector was performed by plating a refreshed overnight culture of one K. pneumoniae mutant colony onto 6% sucrose LB agar without NaCl at 30°C for 24 h. A single clone surviving the sucrose treatment was checked for susceptibility to Tet, confirmed by PCR and named 52145-ΔmgrB.

The 52145-Δapmc and 52145-ΔapgP mutants were constructed by inserting a linearised pKD4-derived Km resistance cassette (primers: pmrC_FWD, pmrC_RVS, pagP_FWD and pagP_RVS, accordingly; Appendix Table S4) into the relevant gene using the lambda Red recombinease method and pKOBEG-sacB plasmid (Datsenko & Wanner, 2000; Derbi et al., 2003). Km cassette removal was undertaken by Flp-mediated recombination using the pFLP2Tpm plasmid (Hoang et al., 1998). The 52145-Δapmc and 52145-ΔapgP strains were confirmed by PCR using the primers pmrc_checkFWD, pmrc_checkRVS, pagp_checkFWD and pagp_checkRVS, accordingly (Appendix Table S4).

The double-mutant 52145-ΔmgrB-ΔphoQGB was obtained by conjugating pMKSACaphoPOGB into 52145-ΔmgrB as previously described (Lloset et al., 2011). Double 52145-Δapmc-ΔmgrB and 52145-ΔapgP-ΔmgrB mutants were generated by conjugating pGPl-SceI-mgrB into 52145-Δapmc and 52145-ΔapgP (Lloet et al., 2015), respectively. The double 52145-Δapmc-ΔaphoQGB mutant was created by conjugating pMKSACaphoΔxO into 52145-Δapmc. The pMKSACaphoΔxO vector was constructed in accordance with the previously described mutagenesis strategy using DNA fragments amplified by the primers ΔxO_UPFWD, ΔxO_UPRVS, ΔxO_DOWNFWD and ΔxO_DOWNRVS (Appendix Table S4; Lloet et al., 2011). The triple 52145-Δapmc-ΔaphoQGB mutant was generated by conjugating the pMKSACaphoΔxO construct into 52145-Δapmc and then...
conjugating the pGPI-SceIΔmgrB into the 52145-ΔpmrC-ΔlpxO double mutant.

52145-ΔmgrB-ΔpmrAB, 52145-ΔmgrB-ΔpmrF, 52145-ΔmgrB-ΔlpxO-ΔpmrF and 52145-ΔpmrC-ΔlpxO-ΔmgrB-ΔpmrF, 52145-ΔpmrC-ΔlpxO-ΔmgrB-ΔpmrF-ΔpagP, 52145-ΔmanC and 52145-ΔmgrB-ΔmanC mutants were constructed using the pGPI-SceI-2 and pDAI-SceI-SacB plasmid methodology detailed for the mgrB mutant. PCR fragments flanking the pmrAB, pmrF, pagP and manC genes were initially generated using the relevant UPFWD, UPRVS, DWNFWD and DWNRS primers (Appendix Table S4). Gene-specific UPFWD and DWNRS primers were used to generate ΔpmrAB, ΔpmrF, ΔpagP and ΔmanC knockout fragments and confirm mutant clones. pGPI-SceI-2ΔpmrAB, pGPI-SceI-2ΔpmrF, pGPI-SceI-2ΔpagP and pGPI-SceI-2ΔmanC constructs were then conjugated with the relevant 52145-wild-type and mutant precursor strains. Confirmation of all mutants was undertaken by PCR.

**Complementation of the 52145-ΔmgrB mutant**

For complementation of the 52145-ΔmgrB mutant strain, a PCR fragment (primers: mgrB_UPFWD and mgrB_DWNRS) comprising the coding and promoter regions of the K. pneumoniae 52145 mgrB gene was amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). The 1,507-bp amplicon was gel-purified and then cloned into SmaI-digested (New England Biolabs)-treated pUC18R6KT-mini-Tn7Tkmin plasmid (Choi et al., 2005) to obtain pUC18R6KT-mini-Tn7Tkmin_Kp52145mgrBCom. pUC18R6KT-mini-Tn7Tkmin_Kp52145 mgrBCom was then transformed into E. coli SY327 and thereafter into E. coli β2163. In addition, the transposase-containing pTSNSK-Tp plasmid (Crepin et al., 2012) was introduced to the 52145-ΔmgrB strain by electroporation to give 52145-ΔmgrB/pTSNSK-Tp. 52145-ΔmgrB/pTSNSK-Tp was then conjugated overnight with E. coli β2163/pUC18R6KT-mini-Tn7Tkmin_Kp52145mgrBCom on LB agar supplemented with DAP at 30°C. The recovered culture was then serially diluted in sterile PBS, plated onto LB Km agar and incubated at 42°C for 6.5 h followed by 37°C overnight. The colonies grown were thereafter screened for resistance to Km and susceptibility to Tmp and Amp. Correct integration of the Tn7 transposon was confirmed by PCR using the primers KpmgImSup/Ptn7L and KpmgImSdown/Ptn7R as previously described (March et al., 2013). Additionally, the presence of the mgrB gene was PCR-confirmed using the mgrB_UPFWD and mgrB_DWNRS primers.

**Complementation of the 52145-ΔmgrB-ΔphoQGB and 52145-ΔmgrB-ΔlpxO mutants**

Complementation of the 52145-ΔmgrB-ΔphoQGB mutant with the 52145 phoPQ operon and 52145-ΔmgrB-ΔlpxO mutant with the 52145 lpxO gene was undertaken using pGP-Tn7-Cm_KpnPhoPQCom and pGP-Tn7-Cm_KpnLpxOCom as previously described (Llobet et al., 2015). Complemented strains were identified by resistance to Cm, susceptibility to Tmp and Amp, and PCR using the primers PhoPQ_checkFWD, PhoPQ_checkRVS, lpxO_checkFWD and lpxO_checkRVS, accordingly (Appendix Table S4). Correct Tn7 integration was PCR-confirmed using the KpmgImSup/Ptn7L and KpmgImSdown/ Ptn7R primer sets (March et al., 2013).

**Growth curve analysis**

For growth analyses, 5 µl of overnight cultures was diluted in 250 µl of LB or M9 minimal medium (5× M9 minimal salts [Sigma-Aldrich] supplemented with 2% glucose, 3 mM thiamine, 2 mM MgSO4) and incubated at 37°C with constant, manual shaking in a Bioscreen C™ Automated Microbial Growth Analyzer (MTX Lab Systems, Vienna, VA, USA). Optical density (OD; 600 nm) was measured and recorded every 20 min.

**Biofilm analysis**

Biofilms were assayed using a variation of the standard crystal-violet quantification assay. Overnight cultures were grown for 16 h in 5 ml LB broth, after which each strain was diluted to an OD600 of 0.02 in freshly prepared M9 minimal media (5× M9 minimal salts [Sigma-Aldrich] supplemented with 2% glucose, 3 mM thiamine, 2 mM MgSO4). 100 µl of each diluted strain was then added to two columns (12 internal wells) of a polystyrene U-bottomed 96-well plate (Greiner Bio-One), and the plate was incubated statically at 37°C for 24 h. Wells were stained with the addition of 25 µl of 0.5% crystal violet (Sigma-Aldrich) to each well for 1 h. The plates were washed by submersion in distilled H2O, and the crystal violet in each well was dissolved in 150 µl 95% ethanol for 1 h. Biofilms were then quantified by measuring the optical density at 595 nm of each well. Three independent cultures of each strain were tested per day, and the data shown are the average of three independent days.

**Polymyxin and antimicrobial peptide susceptibility assays**

MICs for colistin sulphate and polymyxin B were determined by Etest® (bioMérieux) using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for colistin (the European Committee on Antimicrobial Susceptibility Testing, 2016).

To assay K. pneumoniae strains for resistance to polymyxin antibiotics and AMPs, we used a modified version of the sensitivity assay described by Llobet et al (2011). Briefly, each strain was grown to early exponential phase in LB broth, washed once in PBS and diluted in liquid testing media (1% v/v Tryptone soy broth, 10% v/v 100 mM phosphate buffer [pH 6.5], 2% v/v 5 M NaCl) to an approximate concentration of 4 × 10³ colony-forming units (CFUs) per millilitre. Twenty-five microtitre plates of each diluted strain was then mixed with 5 µl of antibiotic (or AMP) and incubated at 37°C for 1 h. Fifteen microtitrets of the suspension was thereafter spread onto LB agar and incubated overnight at 37°C. Per cent survival of the cells exposed to the antibiotics (or AMPs) was determined through comparison with the unexposed (sterile PBS) controls.

**Lipid A isolation and mass spectrometry**

Lipid A was extracted using the ammonium hydroxide/isobutyric acid method described earlier (El Hamidi et al., 2005). Negative ion MALDI-TOF mass spectrometry analysis (Bruker Daltonics) of the samples was undertaken using an equal volume of dihydroxybenzoic acid matrix (Sigma-Aldrich) dissolved in (1:2) acetonitrile-0.1% trifluoroacetic acid.
Generation of the lucFF reporter fusion K. pneumoniae strains

An 868-bp amplicon comprising the pagP gene promoter region was amplified by Phusion High-Fidelity DNA Polymerase using the primers pagP_Pro_FWD and pagP_Pro_RVS (Appendix Table S4). The amplicon was digested with EcoRI, gel-purified, cloned into an EcoRI-Smal-digested pGPL01 suicide vector and then transformed into E. coli GT115 cells to obtain pGPLKpnProPagP. Correct insertion of the amplicon was verified by restriction digestions with EcoRI and HindIII.

pGPLKpnProPhoP, pGPLKpnProPmrH, pGPLKpnProPmrC, pGPLKpnProPagP and pGPLKpnProPhoO (Cellobi, 2011, 2015; Insua et al., 2013) were each introduced into E. coli β2163 and then mobilised into the Kp52145, 52145-ΔmgrB, 52145-ΔmgrBCom and 52145-ΔmgrB-ΔphoQGB strains via conjugation. Cultures were then serially diluted and checked for Amp resistance by plating on LB Cb agar at 37°C. Correct insertion of the vectors into the chromosome was confirmed by PCR using the relevant lucFF check and promoter sequence primers (Appendix Table S4; data not shown).

Luciferase activity

Overnight cultures of the K. pneumoniae reporter strains were refreshed for 2.5 h in LB containing Cb at 37°C and 180 rpm. The cells were then pelleted, washed once in sterile PBS and adjusted to an OD\text{\textsubscript{600}} of 1.0. One hundred microlitres of each suspension was added to an equal volume of luciferase assay reagent (1 mM β-luciferin [Synchem] in 100 mM sodium citrate buffer pH 5.0), vortexed for 5 s and then immediately measured for luminescence (expressed as relative light units [RLU]) using a GloMax 20/20 Luminometer (Promega). All strains were tested in triplicate from three independent cultures.

G. mellonella larvae and infections

G. mellonella larvae were obtained from UK Waxworms Limited. Upon receipt, larvae were stored in reduced light at 13°C with nil dietary supplementation. All experiments were performed within 14 days of receipt comprising larvae showing a healthy external appearance of 250–350 mg weight as previously described (Insua et al., 2013).

K. pneumoniae strains for G. mellonella infections were prepared by harvesting refreshed 5 ml of exponential phase LB cultures (37°C, 180 rpm, 2.5 h), washing once in sterile PBS and then adjusting to an OD\text{\textsubscript{600}} of 1.0 (i.e. \(5 \times 10^8\) CFUs/ml). Each suspension was thereafter diluted to the desired working concentration (i.e. \(1 \times 10^7\) and \(1 \times 10^8\) CFUs/ml). Larvae were surface-disinfected with 70% (v/v) ethanol and then injected with 10 μl of working bacterial suspension at the right last proleg using a Hamilton syringe equipped with a 27-gauge needle. Experiments involving dual injections were undertaken in the right and then the left proleg. For each experiment, 10 larvae injected with sterile PBS were included as combined trauma and vehicle controls. Injected larvae were placed inside Petri dishes at 37°C in the dark. Per cent survival following gentle physical stimulation was recorded at 24-h intervals over 72 h.

G. mellonella killing assay

Based on earlier 50% lethal dose (LD\textsubscript{50}) data, the virulence of each K. pneumoniae 52145 strain was explored using a working concentration of \(10^7\) CFUs per larvae (Insua et al., 2013). For G. mellonella infections using the K. pneumoniae T1a and T1b ST-258 clinical strains, an infection dose of \(2 \times 10^8\) per larvae was performed (Poiriel et al., 2015). Insects were considered dead if they did not respond to physical stimuli. Larvae were examined for pigmentation, and time of death was recorded. Assays were allowed to proceed for only 3 days as pupa formation was occasionally observed by day 4. A total of 40 larvae over three independent experiments were investigated for each strain.

G. mellonella with boosted immunity killing assay

Larvae were injected with \(1 \times 10^6\) CFUs of heat-killed (65°C, 20 min) E. coli MG1655 to elicit the production of antimicrobial factors (Insua et al., 2013). After 24 h of incubation at 37°C, larvae were then infected with \(1 \times 10^6\) CFUs of selected K. pneumoniae 52145 strains (n = 30 larvae/group over three independent experiments) and returned to 37°C for 72 h. Per cent survival was recorded at 24-h intervals.

Radial diffusion bioassay to assess G. mellonella antimicrobial peptides

We used a previously described radial diffusion assay to assess the in vitro resistance of the K. pneumoniae 52145 strains to antimicrobial factors produced by G. mellonella (Insua et al., 2013). Briefly, production of G. mellonella antimicrobial factors was elicited through injection of \(1 \times 10^8\) CFUs of heat-killed E. coli MG1655. After 24 h of incubation at 37°C, 15 μl of haemolymph from three surface-disinfected larvae was pooled with 10 μl of saturated N-phenylthiourea (Sigma-Aldrich). Ten microlitres of each haemolymph preparation (or sterile H2O) were then inoculated into the wells of radial diffusion underlay gels containing either 52145, 52145-ΔmgrB or 52145-ΔmgrBCom. Plates were incubated at 37°C for 3 h, overlaid with 1% agarose containing 6% TSB powder and returned to 37°C for 18 h. Zones of inhibition were measured and expressed as inhibition units (10 units = 1 mm). Haemolymph samples from larvae injected with sterile PBS were used as uninfected controls. Colistin sulphate and polymyxin B (20 μg/ml) plus sterile H2O were used as positive and negative bioassay controls, respectively.

G. mellonella RNA extraction and quantitative real-time PCR analysis

Larvae were infected with \(1 \times 10^5\) CFUs of the selected K. pneumoniae 52145 strains, incubated at 37°C for 8 h and then homogenised in 1 ml ice-cold TRIzol (Ambion\textsuperscript{TM}) using a VDI 12 tissue homogeniser (VWR). Total RNA was extracted according the manufacturer’s instructions with minor modifications including the use of Phase Lock Gel Heavy 2-ml tubes (VWR) for phase separation. Five micrograms of RNA was treated with recombinant DNase I (Roche Diagnostics Ltd) at 37°C for 30 min and then purified using a standard phenol-chloroform method and Phase Lock Gel Heavy 1.5-ml tubes (VWR). The RNA was precipitated overnight with 20 μl 3 M sodium chloride and then resuspended in 30 μl deionised H2O.
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Left lung samples for RNA extraction were removed from RNA infected murine lung (M-MLV) reverse transcriptase (Sigma-Aldrich) according to the manufacturer’s instructions. Quantitative real-time PCR analysis of G. mellonella antimicrobial peptide expression was undertaken using the KAPA SYBR® FAST qPCR Kit, previously described oligonucleotide primers (Insua et al, 2013) and Stratagene Mx3005P qPCR System (Agilent Technologies). Thermal cycling conditions were as follows: 95°C for 3 min for enzyme activation, 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 20 s. cDNA samples were tested in duplicate, and relative mRNA quantity was determined by the comparative threshold cycle (ΔΔCt) method using 18S rRNA normalisation.

Capsule polysaccharide purification and quantification

Bacteria were grown overnight in 3 ml of LB medium (37°C, 180 rpm) with viable counts determined by dilution plating. The cultures were then centrifuged, and the cell pellet was resuspended in 500 μl of sterile H2O. Each sample was then treated with 1% 3-(N,N-dimethyltetradecylammonio)propanesulphonate (Sigma-Aldrich; in 100 mM citric acid, pH 2.0) at 50°C for 20 min. Bacterial debris was pelleted (3,220 x g, 10 min), and 250 μl of the supernatant was transferred to a clean 15 ml glass tube. The CPS was then washed and precipitated at −20°C for 20 min and pelleted (9,447 x g, 10 min, 4°C). After removal of the supernatant, the pellet was then dried (5 min, 90°C) and resuspended in 200 μl of sterile water. CPS quantification was undertaken by determining the concentration of uronic acid in the samples, using a modified carbazole assay as previously described (Rahn & Whitfield, 2003). All samples were tested in triplicate.

Intranasal murine infection model

Female mice were infected intranasally with 3 x 10^5 Kp52145 and 52145-ΔmgrB in 30 μl PBS (n = 6 per strain). Control mice were inoculated with 30 μl sterile PBS (n = 4). After 24 h, mice were euthanised using a Schedule 1 method according to UK Home Office-approved protocols. Left lung samples from infected and uninfected control mice were immersed in 1 ml of RNA stabilisation solution (50% [w/v] ammonium sulphate [Fisher Scientific], 2.9% [v/v] 0.5 M ethylenediaminetetraacetic acid [Sigma-Aldrich], 1.8% [v/v] 1 M sodium citrate [Sigma-Aldrich]) on ice and then stored at 4°C for at least 24 h prior to RNA extraction. Right lung, spleen and NALT samples from infected mice were immersed in 1 ml sterile PBS on ice and processed for quantitative bacterial culture immediately. Samples were homogenised using a VDI 12 tissue homogeniser, serially diluted in sterile PBS and plated onto Salmonella Shigella agar (Oxoid Limited), and the colonies were enumerated after overnight incubation at 37°C. Data were expressed as CFUs per sample.

RNA extraction and quantitative real-time PCR analysis of infected murine lung

Left lung samples for RNA extraction were removed from RNA stabilisation solution, homogenised in 1 ml ice-cold TRIzol (Ambion™) using a VDI 12 tissue homogeniser and then subjected to five rounds of bead beating for 10 s followed by ice-cooling for 1 min using 100-μm acid-washed glass beads (Sigma-Aldrich) and a Mini-Beadbeater-1 (BioSpec Products). RNA was extracted, and duplicate cDNA preparations were generated from each sample using the protocol described for the infected G. mellonella samples. Quantitative real-time PCR analysis of inflammatory cytokine and murine antimicrobial peptide expression was undertaken using the KAPA SYBR® FAST qPCR Kit, previously described oligonucleotide primers (Appendix Table S4) and Stratagene Mx3005P qPCR System (Agilent Technologies). Thermal cycling conditions were as follows: 95°C for 3 min for enzyme activation, 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 20 s. Each cDNA sample was tested in duplicate, and relative mRNA quantity was determined by the comparative threshold cycle (ΔΔCt) method using hypoxanthine phosphoribosyltransferase 1 (mHprt) gene normalisation.

Cell culture and infections

Immortalised BMDM (iBMDM) cells (BEI Resources, NIAID, NIH: Macrophage Cell Line Derived from Wild Type Mice, NR-9456) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco® 41965) supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco) at 37°C in a humidified 5% CO2 incubator. Murine alveolar macrophages MH-S (ATCC, CRL-2019) were grown in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco), and 10 mM HEPES (Sigma-Aldrich). iBMDMs and MH-S were grown at 37°C in humidified 5% CO2 atmospheric conditions. Cells were routinely tested for Mycoplasma contamination.

For infections, bacteria were adjusted to an OD_{600} of 1.0 in PBS, and infections were performed using a multiplicity of infection of 100 bacteria per cell. To synchronise infection, plates were centrifuged at 200 x g for 5 min.

Immunoblot analysis

Proteins were resolved by standard 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 4% bovine serum albumin (w/v) in TBST, and protein bands were detected with specific antibodies using chemiluminescence reagents and a G:BOX Chemi XRQ chemiluminescence imager (Syngene).

The following rabbit antibodies were used: anti-ixBα (1:1,000; Cell Signaling), anti-phospho-p38 (1:1,000; Cell Signaling), anti-phospho-SAPK/JNK (1:1,000; Cell Signaling) and anti-phospho-ERK (1:1,000; Santa Cruz Biotechnology). Immunoreactive bands were visualised by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (1:5,000) or goat anti-mouse immunoglobulins (1:1,000; Bio-Rad). To ensure that equal amounts of proteins were loaded, blots were re-probed with α-tubulin (1:3,000; Sigma-Aldrich).

To detect multiple proteins, membranes were re-probed after stripping of previously used antibodies using a pH 2.2 glycine-HCl/SDS buffer.
Quantification of cytokines

Infections were performed in 96-well plates (5 × 10^4 cells per well) using 1 × 10^5 UV-killed bacteria (1 ml of bacterial suspension adjusted to an OD600 of 1.0) subjected to 10 J UV light for 30 min, and bacterial killing was confirmed by plating (in LB). TNF-α in the supernatants was determined at 6 h of infection using a Murine TNF-α Standard TMB ELISA Development Kit (PeproTech, catalogue number 900-T54), according to the manufacturer’s instructions. Experiments were performed in duplicate and repeated at least three times.

Statistical analyses

Statistical analyses were performed using the two-tailed t-test, or when the requirements were not met, by the Mann–Whitney U-test. P-values of < 0.05 were considered statistically significant. Normality and equal variance assumptions were tested with the Kolmogorov–Smirnov test and the Brown–Forsythe test, respectively. Survival analyses were undertaken using the log-rank (Mantel–Cox) test with Bonferroni correction for multiple comparisons (α = 0.008). All analyses were performed using GraphPad Prism for Windows (version 5.03) software.

Expanded View for this article is available online.

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Author contributions

TJK and JAB conceived the study and wrote the first draft of the manuscript. TJK, GM, JS-P, CGF, AD, JLI and LH performed the experiments and contributed data for this work. TJK, GM, JS-P, CGF, AD, JLI, RI, LH and JAB contributed to and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.


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Virulent and resistant mgrB-disabled K pneumoniae

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