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MOLECULAR PATHOGENESIS

Identification and Characterization of Two *Klebsiella pneumoniae lpxL* Lipid A Late Acyltransferases and Their Role in Virulence

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ABSTRACT *Klebsiella pneumoniae* causes a wide range of infections, from urinary tract infections to pneumonia. The lipopolysaccharide is a virulence factor of this pathogen, although there are gaps in our understanding of its biosynthesis. Here we report on the characterization of *K. pneumoniae lpxL*, which encodes one of the enzymes responsible for the late secondary acylation of immature lipid A molecules. Analysis of the available *K. pneumoniae* genomes revealed that this pathogen's genome encodes two orthologues of *Escherichia coli* LpxL. Using genetic methods and mass spectrometry, we demonstrate that LpxL1 catalyzes the addition of laureate and LpxL2 catalyzes the addition of myristate. Both enzymes acylated *E. coli* lipid A, whereas only LpxL2 mediated *K. pneumoniae* lipid A acylation. We show that LpxL1 is negatively regulated by the two-component system PhoPQ. The lipid A produced by the *lpxL2* mutant lacked the 2-hydroxymyristate, palmitate, and 4-aminoarabinose decorations found in the lipid A synthesized by the wild type. The lack of 2-hydroxymyristate was expected since LpxO modifies the myristate transferred by LpxL2 to the lipid A. The absence of the other two decorations is most likely caused by the downregulation of *phoPQ* and *pmrAB* expression. LpxL2-dependent lipid A acylation protects *Klebsiella* from polymyxins, mediates resistance to phagocytosis, limits the activation of inflammatory responses by macrophages, and is required for pathogen survival in the wax moth (*Galleria mellonella*). Our findings indicate that the LpxL2 contribution to virulence is dependent on LpxO-mediated hydroxylation of the LpxL2-transferred myristate. Our studies suggest that LpxL2 might be a candidate target in the development of anti-*K. pneumoniae* drugs.

KEYWORDS *Klebsiella pneumoniae*, lipid A, LpxL, pathogenesis, virulence factors

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Lipopolysaccharide (LPS) is the major constituent in the outer membrane of Gram-negative bacteria, and it is composed of three regions: the lipid A domain, the core oligosaccharide, and the O-antigen polysaccharide. The canonical hexa-acylated lipid A structure is expressed by *Escherichia coli* K-12 and consists of a $\beta(1'\text{-}6)$-linked disaccharide of glucosamine phosphorylated at the 1 and 4′ positions, with positions 2, 3, 2′, and 3′ being acylated with R-3-hydroxymyristoyl groups, the so-called lipid IV_A. The 2′ and 3′ R-3-hydroxymyristoyl groups are further acylated with laureate (C_{12}) and myristate (C_{14}) (Fig. 1A) (1). Five enzymes are required to assemble the $\beta(1'\text{-}6)$-linked disaccharide that is characteristic of all lipid A molecules, whereas LpxK, KdtA, LpxL (HtrB), and LpxM (MsbB) catalyze the last four enzymatic steps required to assemble the Kdo2–hexa-acylated lipid A (1). LpxK phosphorylates the 4′ position of the disaccharide 1-phosphate to form lipid IVa; the next two Kdo residues are incorporated by the

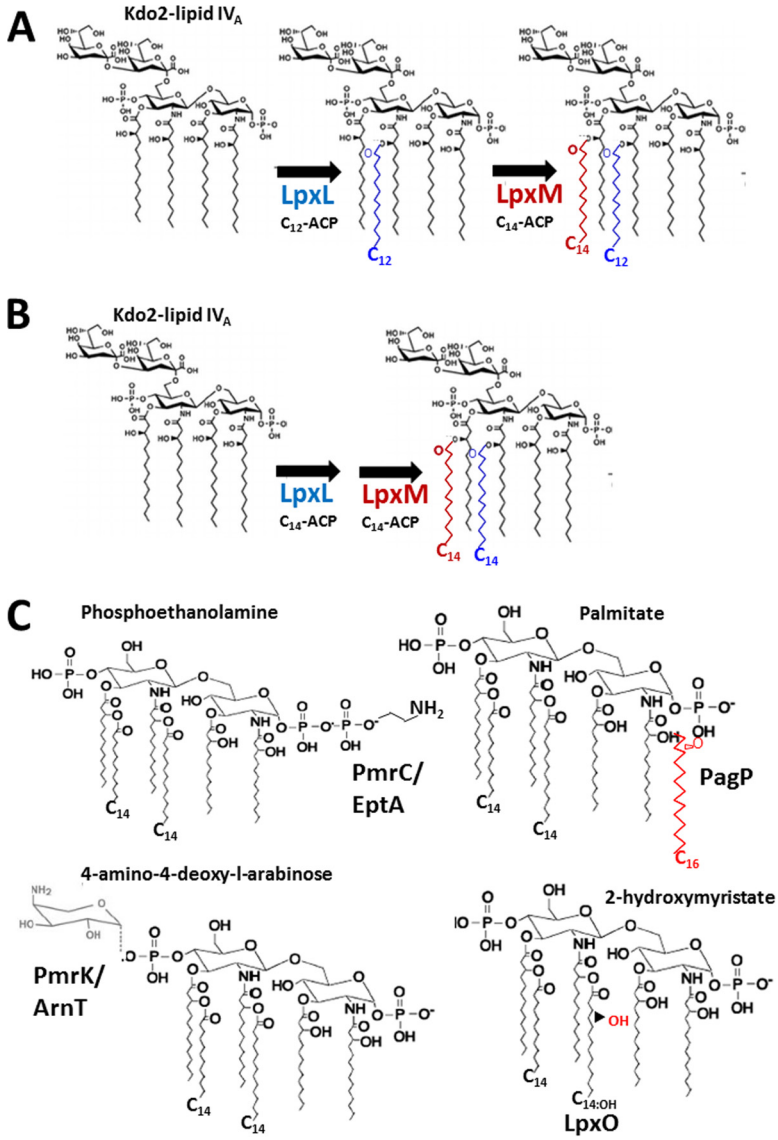

FIG 1 Synthesis of hexa-acylated lipid A in *E. coli* and *K. pneumoniae*. (A) In *E. coli*, LpxL transfers a laurate (C_{12}) group from an acyl carrier protein (ACP) onto the R-2′-hydroxymyristate acyl chain of Kdo2-lipid IV_A. Subsequently, LpxM-dependent addition of myristate (C_{14}) onto the R-3′-hydroxymyristate residue results in the synthesis of hexa-acylated Kdo2-lipid A. (B) In *K. pneumoniae*, LpxL (which we named LpxL2 in this work) and LpxM transfer myristate onto the R-3′-hydroxymyristate residue to complete the synthesis of hexa-acylated Kdo2-lipid A. (C) Proposed lipid A structures follow previously reported structures for *K. pneumoniae* (23–27). The modifications and genes responsible for each of them are indicated.

enzyme KdtA to generate the molecule Kdo2-lipid IV_A (Fig. 1A). The last steps involve the addition of the secondary lauroyl and myristoyl residues to the distal glucosamine unit by LpxL and LpxM, respectively (Fig. 1A), which require the Kdo disaccharide moiety in their substrates for activity (1). When *E. coli* is grown at 12°C, LpxP, the cold temperature-specific late acyltransferase, acts in place of LpxL by adding palmitoleate ($C_{16:1}$) (1).

There is lipid A diversity among Gram-negative bacteria. This is dependent on the type and length of fatty acids, on the presence of chemical moieties, or even on the lack of groups, such as phosphates or fatty acids, from lipid A (1, 2). Well-characterized modifications comprise the addition of phosphoethanolamine (3), 4-amino-4-deoxy-L-arabinose (4), or palmitate (5) and hydroxylation by the Fe^{2+}/α-ketoglutarate-dependent dioxygenase enzyme (LpxO) (6). These modifications provide resistance to

stresses, such as harsh pH and antimicrobial peptides (APs) (2). APs are directed to LPS on the bacterial surface by electrostatic interactions. Colistin (polymyxin E) and polymyxin B are two antimicrobials which share the interaction with the anionic LPS with mammalian APs (7). Colistin is one of the last-line antimicrobials used to treat infections caused by multidrug-resistant strains of Gram-negative bacteria (8). Additionally, these lipid A changes may help pathogens to modulate the activation of host defense mechanisms by altering the activation of the Toll-like receptor 4 (TLR4)/MD-2 receptor complex (9).

Klebsiella pneumoniae is a capsulated Gram-negative bacterial pathogen which causes a wide range of infections, from urinary tract infections to pneumonia, with the latter being particularly devastating among immunocompromised patients (10, 11). Of particular concern is the increasing isolation of multidrug-resistant strains worldwide, hence narrowing the therapeutic options for the treatment of *Klebsiella* infections. However, despite the clinical relevance, there is still scant evidence on *K. pneumoniae* pathogenesis at the molecular and cellular levels.

The polysaccharide sections of *K. pneumoniae* LPS have been extensively studied. The core and O-antigen polysaccharides from several *Klebsiella* strains were characterized chemically and genetically, and their contribution to virulence was assessed in an animal model of infection (12–22). In contrast, little attention has been given to *K. pneumoniae* lipid A biosynthesis. We and others have demonstrated that *K. pneumoniae* lipid A is hexa-acylated, with the 2′ and 3′ R-3-hydroxymyristoyl groups being acylated with C_{14} (Fig. 1B) (23–27). The enzymatic activity of *K. pneumoniae* LpxM, which catalyzes the transfer of C_{14} to the 3′ R-3-hydroxymyristoyl group, has been confirmed experimentally (23, 26). We have uncovered that *K. pneumoniae* lipid A could be decorated with palmitate, 4-amino-4-deoxy-L-arabinose, phosphoethanolamine, and 2-hydroxymyristate ($C_{14:OH}$) (Fig. 1C) (23–25). The gene encoding the acyltransferase *pagP* is required for the addition of palmitate to lipid A, the *pmrHFIJKLM* (*arnBCADTEF*) loci (here referred to as the *pmrF* operon) are required for the synthesis and addition of aminoarabinose to lipid A, *eptA* (*pmrC*) is necessary for the modification of the lipid A with phosphoethanolamine, and *lpxO* is responsible for modification of the 2′-linked secondary acyl chain with a hydroxyl group at the 2 position (Fig. 1C) (23–25). We have demonstrated that these lipid A decorations provide resistance to APs (23–25) and *K. pneumoniae* mutants lacking them are attenuated for virulence in the mouse pneumonia model (23–25).

The function of the *K. pneumoniae* LpxL late acyltransferase has not yet been investigated. On the basis of the structure of the lipid A produced by the *lpxM* mutant (23, 26), the current widely held belief is that *K. pneumoniae* LpxL catalyzes the transfer of C_{14} to the 2′ R-3-hydroxymyristoyl group (Fig. 1B). However, the presence of hexa-acylated *K. pneumoniae* lipid A species has been reported, consistent with the presence of C_{12} and C_{14} acylating the 2′ and 3′ R-3-hydroxymyristoyl groups, respectively (23, 26). Moreover, a recent report suggests that the *K. pneumoniae* genome may encode two LpxL acyltransferases responsible for late secondary acylation of immature lipid A molecules (28). Altogether, these observations question the previously assigned function to the lipid A late acyltransferases, chiefly, LpxL. The aim of this work was to provide mechanistic insights into the function of *K. pneumoniae* LpxL and to investigate its role in *K. pneumoniae* virulence.

RESULTS

The *K. pneumoniae* genome encodes two *lpxL* late acyltransferases. *In silico* analysis of the available *K. pneumoniae* genomes revealed that this pathogen encodes two orthologues of *E. coli* LpxL. Analysis of the genome of wild-type virulent *K. pneumoniae* strain 52.145 (referred to here as strain Kp52145) (Table 1) revealed that LpxL1 (locus tag BN49_2155) and LpxL2 (locus tag BN49_1538) are 77 and 71% identical to *E. coli* LpxL, respectively. Previously, we identified the Kp52145 orthologue of *E. coli* LpxM (locus tag BN49_3476) and demonstrated that it catalyzes the transfer of C_{14} to the 3′ R-3-hydroxymyristoyl group (23). Each Kp52145 LpxL acyltransferase was mu-

TABLE 1 Strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or comments	Source or reference
Strains		
Escherichia coli		
C600	*thi thr leuB tonA lacY supE*	Laboratory collection[a]
SY327	λ(*lac pro*) *argE*(Am) *rif nalA recA56* (λ *pir*)	Laboratory collection
*β*2163	F$^-$ RP4-2-Tc::Mu d *dapA*::(*erm-pir*) [Km^r Em^r]	62
BN1	W3110 Δ*eptA* Δ*lpxT* Δ*pagP*	57
BN1 Δ*lpxL*	BN1 Δ*lpxL*::FRT; the *lpxL* gene was inactivated	This work
BN1 Δ*lpxL*::Tn*7*-T-Km-*lpxL1*	BN1 Δ*lpxL* Km^r; Tn*7*-T-Km-*lpxL1* was integrated into the *att* Tn*7* site	This work
MG1655	F$^-$ λ$^-$ *ilvG rfb-50 rph-1*	Laboratory collection
Klebsiella pneumoniae		
52.145	Clinical isolate; serotype O1:K2, sequence type 66	63
52145-Δ*lpxL1*	Kp52145 Δ*lpxL1*::FRT; the *lpxL1*gene was inactivated	This study
52145-Δ*lpxL2*	Kp52145 Δ*lpxL2*::FRT; the *lpxL2* gene was inactivated	This study
52145-Δ*lpxL1*Δ*lpxL2*	Kp52145 Δ*lpxL1*::FRT Δ*lpxL2*::FRT; the *lpxL1* and *lpxL2* genes were inactivated	This study
52145-Δ*lpxL2*Com2	Kp52145 Δ*lpxL2*::FRT Km^r; Tn*7*-T-Km-*lpxL2* was integrated into the *att* Tn*7* site	This study
52145-Δ*lpxL1*Δ*lpxL2*Com2	Kp52145 Δ*lpxL1*::FRT Δ*lpxL2*::FRT Km^r; Tn*7*-T-Km-*lpxL2* was integrated into the *att* Tn*7* site	This study
52145-Δ*lpxL1*Δ*lpxL2*Com1	Kp52145 Δ*lpxL1*::FRT Δ*lpxL2*::FRT Km^r; Tn*7*-T-Km-*lpxL1* was integrated into the *att* Tn*7* site	This study
52145-Δ*lpxO*	Kp52145 Δ*lpxO*::FRT; the *lpxO* gene was inactivated	This study
52145-Δ*lpxO*Com	Kp52145 Δ*lpxO*::FRT; Tn*7*-Cm_KpnLpxOCom was integrated into the *att* Tn*7* site	This study
52145-Δ*phoQ*GB	Kp52145 Δ*phoQ*::Km-GenBlock Km^r; the *phoQ* gene was inactivated	24
52145-Δ*phoQ*GBCom	Kp52145 Δ*phoQ*::Km-GenBlock Cm^r, Km^r; Tn*7*-Cm_KpnPhoPQCom was integrated into the *att* Tn*7* site	23
52145-Δ*lpxL1*Δ*phoQ*GB	Kp52145 Δ*lpxL1*::FRT Δ*phoQ*::Km-GenBlock Km^r; the *lpxL1* and *phoQ* genes were inactivated	This study
52145-Δ*pmrF*	Kp52145 Δ*pmrF*::FRT; the *pmrF* gene was inactivated	24
52145-Δ*pagP*	Kp52145 Δ*pagP*; the *pagP* gene was inactivated	25
Plasmids		
pGEM-T Easy	Cloning plasmid; Amp^r	Promega
pGEMTFRTKm	Km resistance cassette source for mutagenesis flanked by BamHI-FRT sites	24
pJTOOL-1	λ *pir*-based suicide vector with NotI cloning site; derived from pDS132; Cm^r	51
pSTNSK-Tp	pSTNSK-Tp containing a transposase for Tn*7* insertion; Km^r Tp^r	54
pKD46	λ phage *red*γβα, arabinose inducible; Amp^r	56
pUC18R6kT-mini-Tn*7*-T-Km	pUC18R6kT-mini-Tn*7*-T complementation vector; Amp^r Km^r	53
pGP-Tn*7*-Cm	pGP-Tn*7* complementation vector; Amp^r Cm^r	54
pFLP2	Plasmid encoding FLP to remove cassettes between FRT sites, *sacB* gene; Tp^r	52
pGPL01	Firefly luciferase (*lucFF*) transcriptional fusion suicide vector that carries the R6K origin of replication; Amp^r	58
pGEMTlpxL1	pGEM-T Easy containing Δ*lpxL1*; Amp^r	This study
pGEMTlpxL2	pGEM-T Easy containing Δ*lpxL2*; Amp^r	This study
pGEMTlpxL1Km	pGEM-T Easy containing Δ*lpxL1*::Km; Km^r Amp^r	This study
pGEMTlpxL2Km	pGEM-T Easy containing Δ*lpxL2*::Km; Km^r Amp^r	This study
pJTOOL-1-lpxL1Km	pJTOOL-1 containing Δ*lpxL1*::Km; Km^r Cm^r	This study
pJTOOL-1-lpxL2Km	pJTOOL-1 containing Δ*lpxL2*::Km; Km^r Cm^r	This study
pMAKSACBΔlpxO	pMAKSACB containing Δ*lpxO*::Km-GenBlock; Cm^r Km^r	25
pMAKSACBΔphoQGB	pMAKSACB containing Δ*phoQ*::Km-GenBlock; Cm^r Km^r	24
pGEMTlpxL1Com	pGEM-T Easy containing the *lpxL1* gene for complementation; Amp^r	This study
pGEMTlpxL2Com	pGEM-T Easy containing the *lpxL2* gene for complementation; Amp^r	This study
pUC18R6kT-mini-Tn*7*-T-Km-lpxL1Com	pUC18R6kT-mini-Tn*7*-T-Km containing the *lpxL1* gene for complementation; Amp^r Km^r	This study
pUC18R6kT-mini-Tn*7*-T-Km-lpxL2Com	pUC18R6kT-mini-Tn*7*-T-Km containing the *lpxL2* gene for complementation; Amp^r Km^r	This study
pGP-Tn*7*-Cm_KpnLpxOCom	pGP-Tn*7*-Cm containing the *lpxO* gene for complementation; Amp^r Cm^r	23
pGP-Tn*7*-Cm_KpnPhoPQCom	pGP-Tn*7*-Cm containing the *phoPQ* operon for complementation; Amp^r Cm^r	23
pGPLKpnProlpxL1	pGPL01 containing the *lpxL1* promoter region; Amp^r	This study
pGPLKpnProlpxL2	pGPL01 containing the *lpxL2* promoter region; Amp^r	This study
pGPLKpnPmrH	pGPL01 containing the *pmrH* promoter region; Amp^r	24
pGPLKpnProPagP	pGPL01 containing the *pagP* promoter region; Amp^r	25

[a]Laboratory collection, frozen bacterial stocks kept at the Bengoechea laboratory.

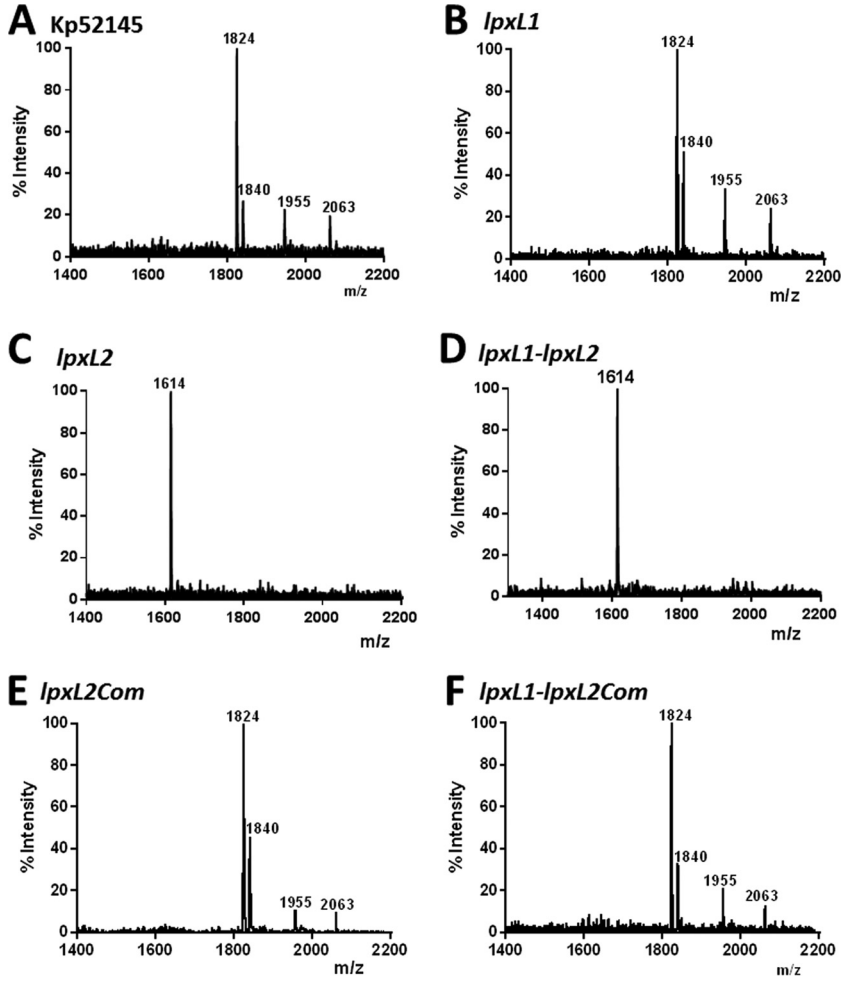

FIG 2 *K. pneumoniae* LpxL2 acylates the 2′ R-3-hydroxymyristoyl group with C_{14}. Negative-ion MALDI-TOF mass spectrometry spectra of lipid A purified from *K. pneumoniae* strains 52.145 (Kp52145) (A), 52145-Δ*lpxL1* (*lpxL1*) (B), 52145-Δ*lpxL2* (*lpxL2*) (C), 52145-Δ*lpxL1*Δ*lpxL2* (*lpxL1-lpxL2*) (D), 52145-Δ*lpxL2*Com2 (*lpxL2Com*) (E), and 52145-Δ*lpxL1*Δ*lpxL2*Com2 (*lpxL1-lpxL2Com*) (F) are shown. The data represent the mass-to-charge ratio (*m/z*) of each lipid A species detected and are representative of those from three extractions.

tated to determine whether it contributes to the acylation of *K. pneumoniae* lipid A. Control experiments showed that the growth kinetics in both rich and minimal media were similar between the wild type and the *lpxL* mutant strains (see Fig. S1 in the supplemental material). We sought to determine whether capsule levels in the *lpxL* mutants were affected. This is an important control, given the crucial role played by the *K. pneumoniae* capsule to thwart host defenses (20, 29–35). The levels of surface-attached capsule were not significantly different between the *lpxL1* and *lpxL2* mutant strains and the wild type (168 $\pm$ 5.7 μg/10^9 CFU, 170.5 $\pm$ 4.4 μg/10^9 CFU, and 176.4 $\pm$ 7.8 μg/10^9 CFU, respectively; $P > 0.05$ for any comparison between the mutants and the wild type).

Lipid A was extracted from the wild type and the *lpxL* mutant strains using an ammonium hydroxide-isobutyric acid method and subjected to negative-ion matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry. The *lpxL1* mutant (strain 52145-Δ*lpxL1*) produced a lipid A similar to that previously reported for the wild-type strain (23, 24) (Fig. 2A and B). Lipid A molecules contained predominantly hexa-acylated species with a mass-to-charge ratio (*m/z*) of *m/z* 1824, corresponding to two glucosamines, two phosphates, four 3-OH-C_{14} groups, and two C_{14} groups. Hexa-acylated species of *m/z* 1840 corresponded to two glucosamines, two

phosphates, four 3-OH-C_{14} groups, one C_{14} group, and one $C_{14:OH}$ group. Other species detected were consistent with the addition of 4-amino-4-deoxy-L-arabinose (*m/z* 131) or palmitate (*m/z* 239) to the hexa-acylated species (*m/z* 1824) to produce lipid A species of *m/z* 1955 and *m/z* 2063, respectively.

In contrast, the lipid A produced by the *lpxL2* mutant (strain 52145-Δ*lpxL2*) (*m/z* 1614) was consistent with a penta-acylated lipid A corresponding to two glucosamines, two phosphates, four 3-OH-C_{14} groups, and one C_{14} group (Fig. 2C). The lipid A produced by the *lpxL1 lpxL2* double mutant was similar to that of the *lpxL2* mutant (Fig. 2D). Complementation of the *lpxL2* single mutant and the *lpxL1 lpxL2* double mutant with *lpxL2* restored the production of wild-type lipid A (Fig. 2E and F), hence suggesting that LpxL2 is the acyltransferase responsible for the transfer of C_{14} to the acyl chain linked at the 2′ position of *K. pneumoniae* lipid A.

To provide additional evidence that LpxL2 acylates the 2′ R-3-hydroxymyristoyl group with C_{14}, we determined the lipid A produced by *E. coli lpxL*-deficient strain BN1 Δ*lpxL* (Table 1) harboring *lpxL2* cloned into the pGEM-T Easy plasmid (pGEMTlpxL2Com) (Table 1). The BN1 strain produced bisphosphorylated, hexa-acylated lipid A containing C_{12} and C_{14} (Fig. S2A). In contrast, MALDI-TOF analysis of the lipid A isolated from BN1 Δ*lpxL* yielded an expected peak at *m/z* 1614 (Fig. S2B), indicating bisphosphorylated, penta-acylated lipid A lacking C_{12}. However, mass spectrometry analysis of the lipid A isolated from BN1 Δ*lpxL*/pGEMTlpxL2Com produced a peak at *m/z* 1824 (Fig. S2C), thereby demonstrating that LpxL2 catalyzes the transfer of C_{14} to *E. coli* lipid A.

Notably, no lipid A modifications were detected in the lipid A produced by the *lpxL2* mutant (Fig. 2C). The lack of 2-hydroxymyristate was expected since LpxO modifies the C_{14} transferred by LpxL2 to the 2′ R-3-hydroxymyristoyl group (23, 25). However, we did not anticipate the lack of 4-amino-4-deoxy-L-arabinose and palmitate. To provide mechanistic insights, we sought to determine if the expression of the *pmrF* operon and *pagP* was downregulated in the *lpxL2* mutant background. To quantitatively assess the transcription of these loci, we used two transcriptional fusions containing a promoterless firefly luciferase gene (*lucFF*) under the control of the relevant locus promoter region. Each fusion (*pmrH*::*lucFF* and *pagP*::*lucFF*; Table 1) was introduced into Kp52145, 52145-Δ*lpxL2*, and 52145-Δ*lpxL2*Com2, and then the luciferase activity was measured. Figure 3 shows that the activities of the *pmrH*::*lucFF* and *pagP*::*lucFF* fusions were significantly lower in the *lpxL2* mutant background than in the wild-type background. Complementation of the *lpxL2* mutant restored the activities of the transcriptional fusions to wild-type levels (Fig. 3). The fact that the two-component systems PhoPQ and PmrAB regulate the expression of the *pmrF* operon and *pagP*, respectively, in *K. pneumoniae* (24) prompted us to evaluate whether the expression of *phoPQ* and *pmrAB* is affected in the *lpxL2* mutant. Reverse transcription (RT)-quantitative PCR (qPCR) experiments showed that *phoPQ* and *pmrAB* mRNA levels were significantly lower in the *lpxL2* mutant than in the wild type and the levels of these two mRNAs were not different from those found in the complemented strain (Fig. 3). Altogether, these findings suggest that the absence of 4-amino-4-deoxy-L-arabinose and palmitate modifications in the lipid A produced by the *lpxL2* mutant is most likely caused by downregulation of the expression of *phoPQ* and *pmrAB*, the positive transcriptional regulators of the *pmrF* operon and *pagP*.

Heterologous expression of LpxL1 in *E. coli* revealed its C_{12} transferase activity. Sequence analysis of the *K. pneumoniae lpxL1* gene did not reveal any frameshift or point mutations consistent with a deleterious effect. Therefore, we investigated the activity of *K. pneumoniae* LpxL1 by determining the lipid A produced by *E. coli* BN1 Δ*lpxL* harboring *lpxL1* cloned into the pGEM-T Easy plasmid (pGEMTlpxL1Com). Interestingly, *K. pneumoniae* LpxL1 restored the production of wild-type *E. coli* lipid A in BN1 Δ*lpxL*, hence indicating that LpxL1 transfers C_{12} to the 2′ R-3-hydroxymyristoyl group (Fig. 4A). This was also true in the *Klebsiella* background because MALDI-TOF analysis of the lipid A extracted from 52145-Δ*lpxL1*Δ*lpxL2*/pGEMTlpxL1Com yielded a peak at *m/z* 1797 indicating the presence of bisphosphorylated, hexa-acylated lipid A containing C_{12} and C_{14} (Fig. 4B). Further, this lipid A species was modified with palmitate (*m/z*

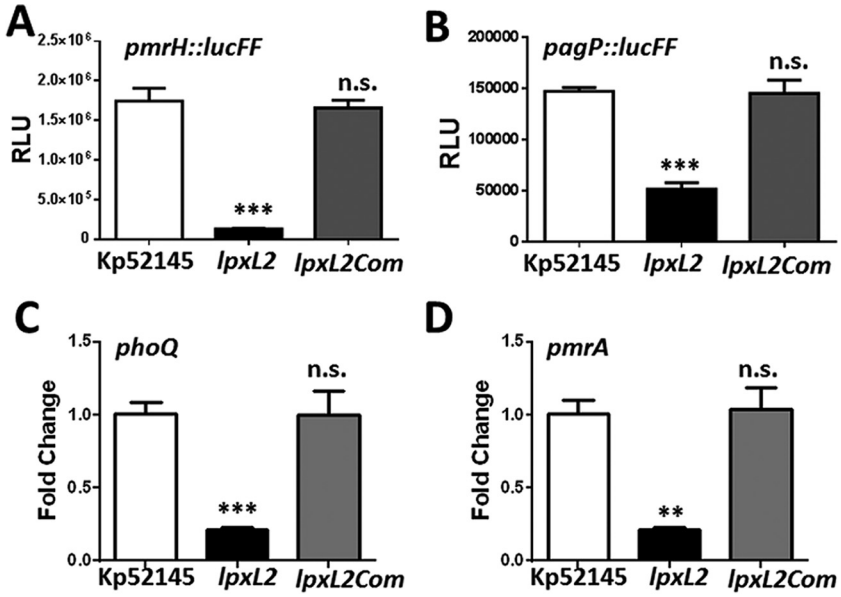

FIG 3 Deletion of *lpxL2* reduces the transcription of *phoPQ* and *pmrAB*. (A and B) Activity of the *pmrH* (A) and *pagP* (B) promoters in *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL2* (*lpxL2*), and 52145-Δ*lpxL2*Com2 (*lpxL2Com*) carrying *lucFF* transcriptional fusions. Values (expressed in RLU) are presented as the mean ± SD from three independent experiments measured in quintuplicate. (C and D) The transcription levels of *phoP* (C) and *pmrA* (D) in *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL2* (*lpxL2*), and 52145-Δ*lpxL2*Com2 (*lpxL2Com*) were determined by RT-qPCR and are shown relative to the expression levels in wild-type bacteria (white bars). Results represent means ± SDs. *P* values indicate the significance of the differences versus Kp52145 determined using one-way ANOVA with Bonferroni contrasts. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

2036) (Fig. 3B). However, single-copy chromosomal complementation of the double mutant with Tn*7*-T-Km-*lpxL1* did not yield the peak at *m/z* 1797. Control experiments confirmed that *E. coli* BN1 Δ*lpxL* was complemented when this construct was used (Fig. 4D). Collectively, these results indicate that the lack of LpxL1-dependent acylation in *K. pneumoniae* lipid A cannot be attributed to a general deficient function of the enzyme.

PhoPQ negatively regulated *lpxL1*. To explain why LpxL1-dependent lipid A was not observed in *K. pneumoniae*, we speculated, among other possibilities, that *lpxL1* expression could be repressed in *Klebsiella*. To monitor the transcription of *lpxL1* and *lpxL2*, two transcriptional fusions in which a promoterless *lucFF* gene was under the control of the acyltransferase promoters were constructed. These fusions were introduced into wild-type *Klebsiella*, and the amount of light was determined. The expression of *lpxL1*::*lucFF* was lower than that of *lpxL2*::*lucFF* (Fig. 5A). Likewise, when *lpxL* mRNA levels were analyzed by RT-qPCR, the transcript levels of *lpxL1* were lower than those of *lpxL2* (Fig. 5B). These results gave initial support to our hypothesis that the expression of *lpxL1* might be repressed in *K. pneumoniae*.

In silico analysis of the *lpxL1* promoter region revealed the presence of a PhoP box-like sequence (36) 120 bp upstream of the initiation codon. In contrast, no PhoP consensus motif was found in the *lpxL2* promoter region. Since PhoPQ governs *K. pneumoniae* loci implicated in lipid A remodeling (24), we hypothesized that PhoPQ represses the expression of *lpxL1*. Indeed, the activity of the transcriptional fusion *lpxL1*::*lucFF* was higher in the *phoQ* mutant background than in the wild-type one (Fig. 5C). Complementation of the *phoQ* mutant restored the activity of the transcriptional fusion to wild-type levels. Similar results were obtained when the *lpxL1* mRNA levels were analyzed (Fig. 5). Of note, neither the activity of *lpxL2*::*lucFF* nor *lpxL2* mRNA levels were affected in the *phoQ* mutant background (Fig. S3).

These findings led us to hypothesize that LpxL1-dependent lipid A acylation should be apparent in the *phoQ* mutant. Supporting this hypothesis, MALDI-TOF analysis of lipid A purified from the *phoQ* mutant yielded a peak at *m/z* 1797 (Fig. 5E). Comple-

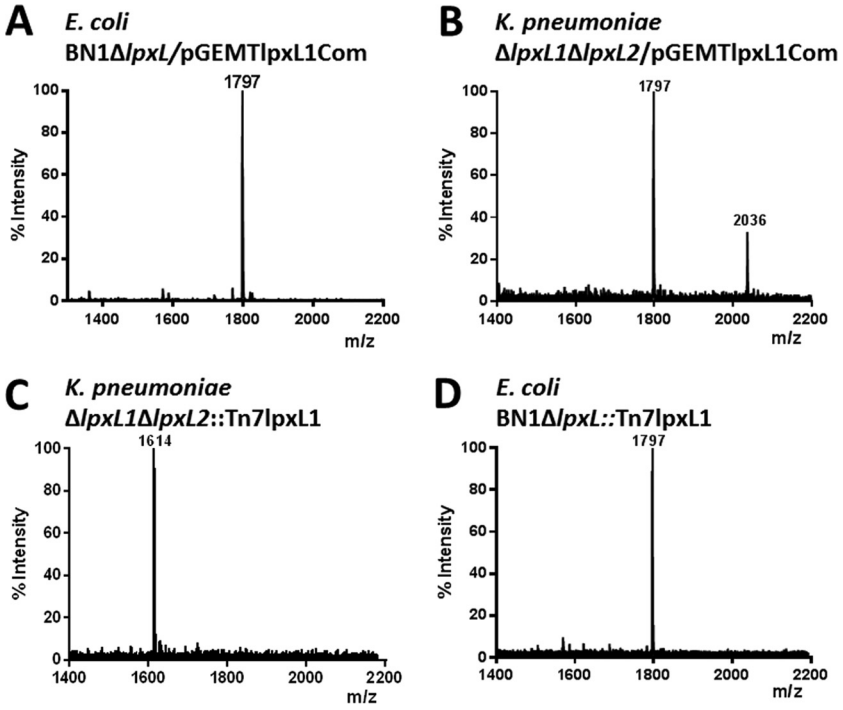

FIG 4 *K. pneumoniae* LpxL2 acylates the 2′ R-3-hydroxymyristoyl group with C_{12}. The negative-ion MALDI-TOF mass spectrometry spectra of lipid A purified from *E. coli* BN1 Δ*lpxL* complemented with *K. pneumoniae lpxL2* (BN1 Δ*lpxL*/pGEMTlpxL2Com) (A), 52145-Δ*lpxL1*Δ*lpxL2* complemented with *K. pneumoniae lpxL1* cloned into pGEM-T Easy plasmid (Δ*lpxL1*Δ*lpxL2*/pGEMTlpxL1Com) (B), 52145-Δ*lpxL1*Δ*lpxL2* complemented with *K. pneumoniae lpxL2* cloned into the Tn*7* transposon (Δ*lpxL1*Δ*lpxL2*::Tn*7*lpxL1) (C), *E. coli* BN1 Δ*lpxL* complemented with *K. pneumoniae lpxL1* cloned into the Tn*7* transposon (BN1 Δ*lpxL*::Tn*7*lpxL1) (D) are shown. Data represent the *m/z* of each lipid A species detected and are representative of those from three extractions.

mentation of the *phoQ* mutant restored the production of wild-type lipid A (Fig. 5F). The *m/z* 1797 species in the *phoQ* mutant was dependent on LpxL1 activity since the peak was absent in the lipid A from the *lpxL1 phoQ* double mutant (Fig. 5G).

On the whole, these results are consistent with the notion that PhoPQ negatively regulates LpxL1-dependent lipid A acylation.

Resistance to antimicrobial peptides and *Klebsiella* LpxL2 late acyltransferase. We sought to determine whether Lpx2-dependent acylation protects *K. pneumoniae* from polymyxins. The *lpxL2* mutant showed increased sensitivity to both polymyxins (Table 2). Similar findings were obtained when the susceptibility to polymyxins was evaluated after a 1-h challenge with the peptides (3 μg/ml) (Fig. 6). Complementation fully restored the resistance of the *lpxL2* mutant to wild-type levels (Fig. 6), indicating that LpxL2-dependent acylation is associated with resistance to polymyxins.

In *K. pneumoniae*, the lipid A modifications with 4-amino-4-deoxy-L-arabinose and 2-hydroxymyristate mediate resistance to polymyxins (23–25). The fact that the lipid A of the *lpxL2* mutant lacked both modifications (Fig. 3) may suggest that the LpxL2 contribution to AP resistance could be indirect. The MICs of the polymyxins for the *lpxO* mutant were the same as those for the *lpxL2* mutant, whereas the MICs of the polymyxins for the *pmrF* mutant were not significantly different from those for the wild-type strain (Table 2). The *lpxO* mutant also showed an increased susceptibility to the polymyxins after the 1-h challenge (Fig. 6). Complementation of the *lpxO* mutant restored the MIC value to the wild-type level, suggesting that the increased susceptibility of the *lpxL2* mutant to polymyxins is associated with the lack of 2-hydroxymyristate. Control experiments confirmed that the lipid A produced by the *lpxO* mutant still contained the *m/z* 1955 and *m/z* 2063 species, consistent with

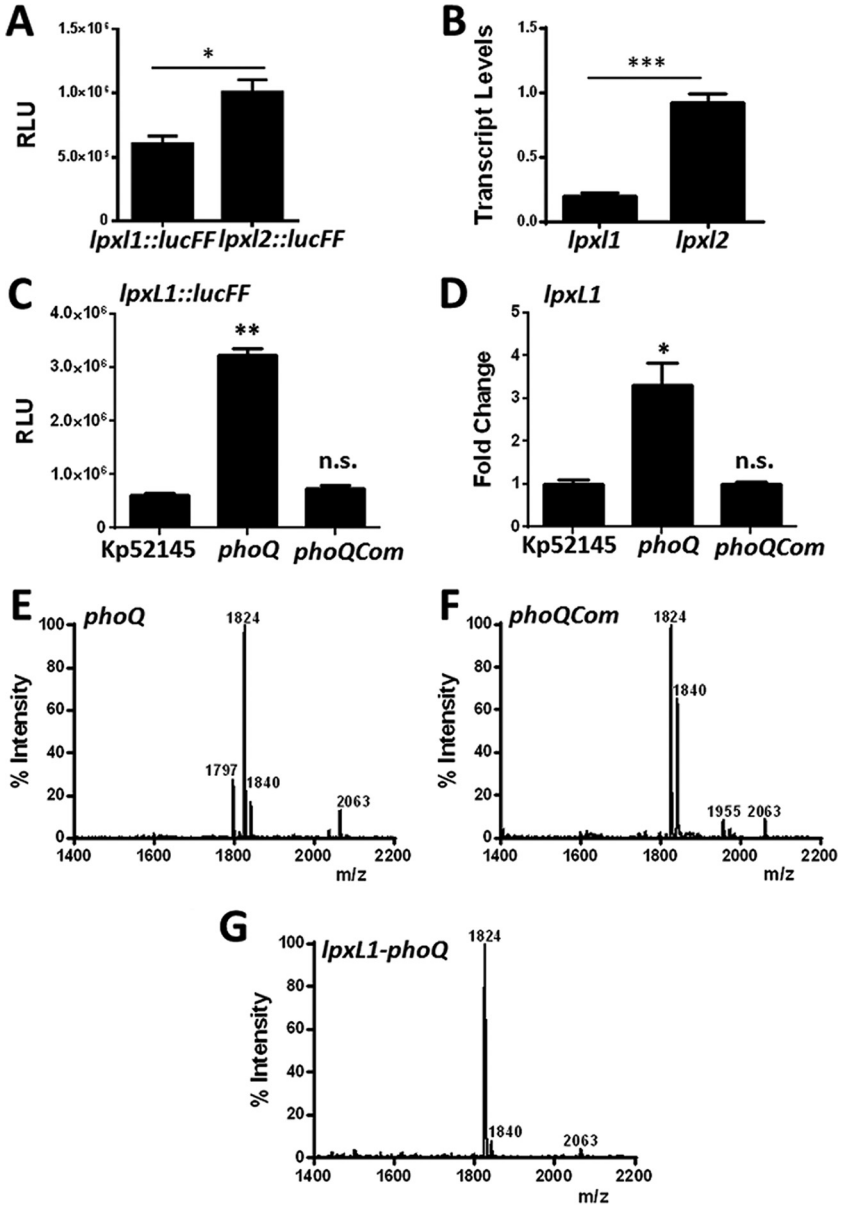

FIG 5 PhoPQ negatively regulates *K. pneumoniae lpxL1*. (A) Activity of the *lpxL1* and *lpxL2* promoters in *K. pneumoniae* 52.145 (Kp52145) carrying *lucFF* transcriptional fusions. Values (expressed in RLU) are presented as the means ± SDs from three independent experiments measured in quintuplicate. (B) The transcription levels of *lpxL1* and *lpxL2* in *K. pneumoniae* Kp52145 were determined by RT-qPCR and are shown relative to the expression levels in wild-type bacteria. The results represent means ± SDs. (C) Activity of the *lpxL1* promoter in *K. pneumoniae* Kp52145, 52145-Δ*phoQ*GB (*phoQ*), and 52145-Δ*phoQ*GBCom (*phoQCom*) carrying *lucFF* transcriptional fusions. Values (expressed in RLU) are presented as the means ± SDs from three independent experiments measured in quintuplicate. (D) The transcription levels of *lpxL1* in *K. pneumoniae* Kp52145, 52145-Δ*phoQ*GB (*phoQ*), and 52145-Δ*phoQ*GBCom (*phoQCom*) were determined by RT-qPCR and are shown relative to the expression levels in wild-type bacteria. Results represent means ± SDs. (E to G) Negative-ion MALDI-TOF mass spectrometry spectra of lipid A purified from 52145-Δ*phoQ*GB (*phoQ*) (E), 52145-Δ*phoQ*GBCom (*phoQCom*) (F), and 52145-Δ*lpxL1*Δ*phoQ*GB (*lpxL1-phoQ*) (G). Data represent the *m/z* of each lipid A species detected and are representative of those from three extractions. *P* values indicate the significance of the differences versus Kp52145 or between the indicated comparisons determined using one-way ANOVA with Bonferroni contrasts. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

the addition of 4-amino-4-deoxy-L-arabinose and palmitate to the hexa-acylated species (*m/z* 1824), respectively (Fig. S4). The growth kinetics in both rich and minimal media were similar among the wild-type, the *lpxO* mutant, and the *lpxL* mutant strains (Fig. S1).

TABLE 2 MICs of polymyxin B and colistin for *K. pneumoniae* strains

Strain	MIC[a] (μg/ml)	
	Polymyxin B	Colistin
Kp52145	0.5	0.25
52145-Δ*lpxL1*	0.5	0.25
52145-Δ*lpxL2*	0.125[b]	0.094[b]
52145-Δ*lpxL1*Δ*lpxL2*	0.125[b]	0.094[b]
52145-Δ*lpxL2*Com2	0.5	0.25
52145-Δ*lpxL1*Δ*lpxL2*Com2	0.5	0.25
52145-Δ*lpxO*	0.125[b]	0.094[b]
52145-Δ*lpxO*Com	0.5	0.25
52145-Δ*pmrF*	NT	0.25
52145-Δ*pagP*	NT	0.25

[a]Experiments were repeated two independent times. NT, not tested.
[b]The MIC was significantly different ($P < 0.05$, one-tailed *t* test) from the MIC for the wild-type strain.

Phagocytosis of *K. pneumoniae lpxL2* mutant. Phagocyte-mediated killing of the *lpxL2* mutant was analyzed using a whole-blood phagocytosis assay which measures bacterial viability following exposure to whole human blood (Fig. 7). The assay showed that the *lpxL2* mutant was recovered in significantly lower numbers than the wild type. Interestingly, the survival of the *lpxO* mutant was not significantly different from that of the *lpxL2* mutant ($P > 0.05$) (Fig. 6), whereas the viability of the *pmrF* and *pagP* mutants did not differ from that of the wild-type strain (Fig. S5). Complementation of the *lpxO* mutant restored the level of survival to wild-type levels, indicating that the decreased survival of the *lpxL2* mutant is associated with the lack of 2-hydroxymyristate in lipid A (Fig. 7).

Inflammation induced by the *K. pneumoniae lpxL2* mutant. Lipid A is bound by the TLR4/MD-2 complex, hence leading to the activation of MyD88 and TIR-domain-containing adapter-inducing beta interferon (TRIF)-dependent innate signaling pathways, resulting in inflammation and clearance of the infection. Not surprisingly, TLR4-governed responses are essential to clear *K. pneumoniae* infections (37, 38). We sought to determine the responses induced by the *lpxL2* mutant in macrophages. Figure 8 shows that the *lpxL2* mutant induced higher levels of the MyD88-dependent cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) and the TRIF-dependent cytokines RANTES and monocyte chemotactic protein 1 (MCP-1) in the supernatants of bone marrow-derived macrophages than those triggered by the wild type. The strain complemented with *lpxL2* induced levels of cytokines similar to those induced by the wild-type strain (Fig. 8). The *lpxO* mutant also induced higher levels of cytokines than

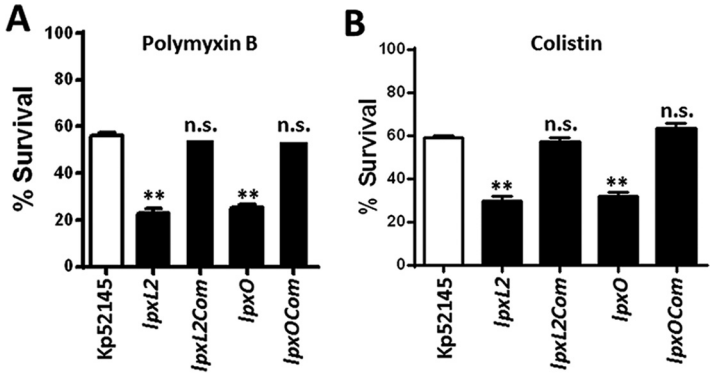

FIG 6 Deletion of *lpxL2* decreases *K. pneumoniae* resistance to polymyxins. The percent survival of *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL2* (*lpxL2*), 52145-Δ*lpxL2*Com2 (*lpxL2Com*), 52145-Δ*lpxO* (*lpxO*), and 52145-Δ*lpxO*Com (*lpxOCom*) following 1 h of exposure to polymyxin B (A) and colistin (B) is shown. Values are presented as the means ± SD from three independent experiments measured in duplicate. *P* values indicate the significance of the differences versus Kp52145 determined using one-way ANOVA with Bonferroni contrasts. **, $P < 0.01$; n.s., not significant.

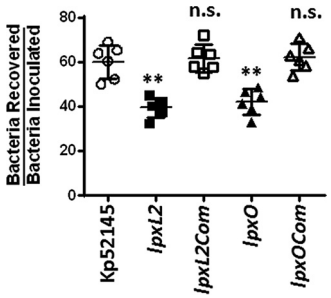

FIG 7 Deletion of *lpxL2* increases human phagocyte-mediated killing of *K. pneumoniae*. Three hundred microliters of fresh human blood (from three different donors) was mixed with 1×10^7 CFU of *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL2* (*lpxL2*), 52145-Δ*lpxL2*Com2 (*lpxL2Com*), 52145-Δ*lpxO* (*lpxO*), and 52145-Δ*lpxO*Com (*lpxOCom*) and incubated at 37°C for 3 h. The bacterial counts recovered were then divided by the initial counts. Experiments were performed with duplicate samples on three independent occasions. *P* values indicate the significance of the differences versus Kp52145 determined using one-way ANOVA with Bonferroni contrasts. **, $P < 0.01$; n.s., not significant.

the wild type, and the levels were not significantly different from those induced by the *lpxL2* mutant ($P > 0.05$) (Fig. 8). Complementation of the *lpxO* mutant restored the level of cytokine production to wild-type levels in infected macrophages (Fig. 8), suggesting that the increased inflammatory response triggered by the *lpxL2* mutant is due to the lack of 2-hydroxymyristate in the lipid A.

Virulence of *K. pneumoniae lpxL* mutants in the *Galleria mellonella* model. The wax moth (*G. mellonella*) infection model has recently been established to model the

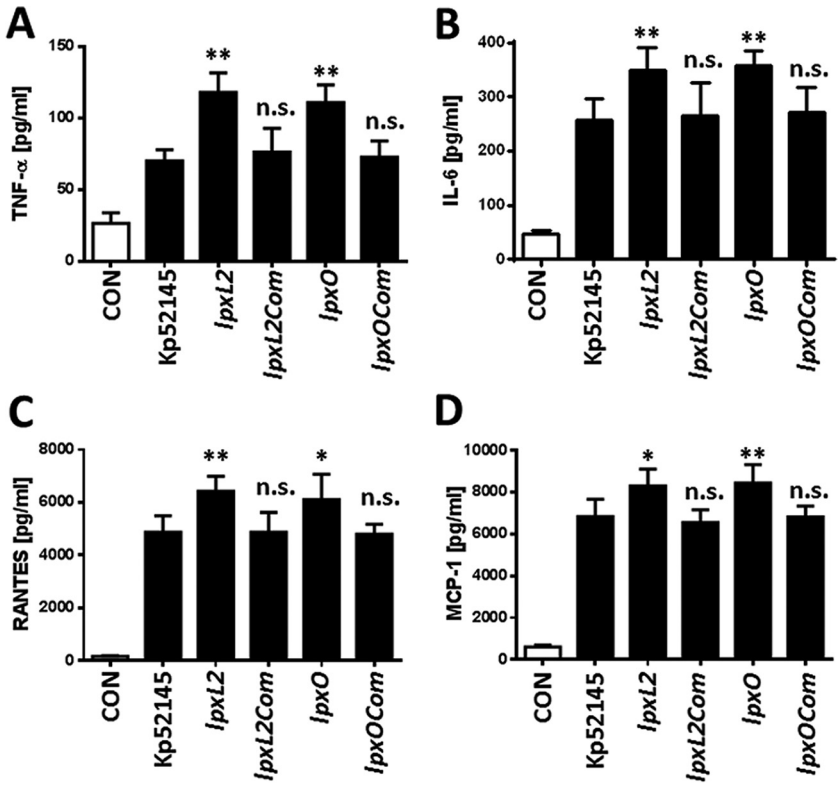

FIG 8 *lpxL2* deletion results in the upregulation of inflammatory responses in macrophages upon infection. The levels of TNF-α (A), IL-6 (B), RANTES (C), and MCP-1 (D) secretion by iBMDM macrophages stimulated for 6 h with UV-killed *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL2* (*lpxL2*), 52145-Δ*lpxL2*Com2 (*lpxL2Com*), 52145-Δ*lpxO* (*lpxO*), and 52145-Δ*lpxO*Com (*lpxOCom*) are shown. *P* values indicate the significance of the differences versus Kp52145 determined using one-way ANOVA with Bonferroni contrasts. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant. CON, uninfected cells.

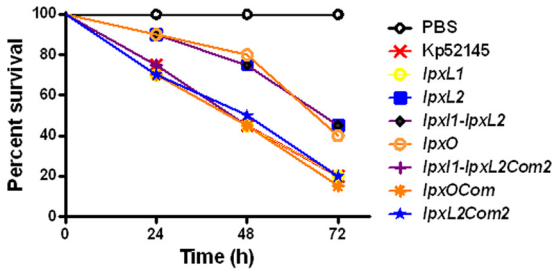

FIG 9 The *K. pneumoniae lpxL2* mutant displays decreased virulence in the *G. mellonella* wax worm infection model. The percent survival of *G. mellonella* over 72 h postinfection with 10^5 organisms of *K. pneumoniae* 52.145 (Kp52145), 52145-Δ*lpxL1* (*lpxL1*), 52145-Δ*lpxL2* (*lpxL2*), 52145-Δ*lpxL1*Δ*lpxL2* (*lpxL1-lpxL2*), 52145-Δ*lpxO* (*lpxO*), 52145-Δ*lpxL1*Δ*lpxL2*Com2 (*lpxL1-lpxL2Com2*), 52145-Δ*lpxO*Com (*lpxOCom*), and 52145-Δ*lpxL2*Com2 (*lpxL2Com2*) is shown. Thirty larvae were infected in each group. The level of significance was determined using the log-rank (Mantel-Cox) test with the Bonferroni correction for multiple comparisons.

virulence of *K. pneumoniae* (30). Importantly, a strong correlation between virulence in *Galleria* wax moths and that in mice (pneumonia model) has been demonstrated (30). To determine the virulence of the *lpxL* mutants, we injected equivalent numbers of CFU from the wild-type, mutant, and complemented strains into *G. mellonella* wax moths and monitored bacterial killing over time. No mortality was observed in *Galleria* moths into which phosphate-buffered saline (PBS) was injected (Fig. 9). After 72 h, only 20% of the larvae injected with the wild type and the *lpxL1* mutant survived. In contrast, 40% of the larvae injected with the *lpxL2* and the *lpxL1 lpxL2* mutants survived ($P < 0.05$ for any comparison versus wild-type survival). Complementation of these strains with *lpxL2* restored the virulence to wild-type levels, indicating that LpxL2-dependent acylation is important for *K. pneumoniae* survival and virulence in invertebrates. Interestingly, the killing induced by the *lpxO* mutant was similar to that triggered by the *lpxL2* mutant ($P > 0.05$), suggesting that the reduced virulence of the *lpxL2* mutants is due to the absence of 2-hydroxymyristate in the lipid A. Control experiments showed that the *pmrF* and *pagP* mutants were as virulent as the wild-type strain (Fig. S6).

DISCUSSION

The work described in this study demonstrates that the *K. pneumoniae* genome encodes two LpxL late acyltransferases; LpxL1 catalyzes the addition of laureate (C_{12}), and LpxL2 catalyzes the addition of myristate (C_{14}). Our data establish that both enzymes catalyze the transfer of these fatty acids to the lipid A in the *E. coli* background, whereas only LpxL2 mediates *K. pneumoniae* lipid A acylation when *Klebsiella* is grown in standard laboratory medium. This study demonstrates that LpxL2 plays an important role in *K. pneumoniae* infection biology since LpxL2-dependent lipid A acylation protects the pathogen from APs, mediates resistance to phagocytosis, and limits the activation of inflammatory responses by macrophages. Our results also show that deletion of *lpxL2* attenuates *K. pneumoniae* virulence in the *G. mellonella* infection model. The fact that there is a strong correlation between the virulence of *K. pneumoniae* in the *G. mellonella* wax moth and mammalian models of infection (30) suggests that LpxL2-mediated lipid A acylation is important for *K. pneumoniae* virulence beyond invertebrates.

The synthesis of hexa-acylated lipid A occurs via a nine-step enzymatic pathway, which is generally well conserved throughout all Gram-negative bacteria (1). In *Enterobacteriaceae*, the last steps of lipid A biosynthesis are dependent on LpxL and LpxM late acyltransferases, which utilize acyl carrier proteins (ACPs) exclusively as donors (1). *lpxL* and *lpxM* display significant sequence similarity among *Enterobacteriaceae*, although they may catalyze the transfer of a different fatty acid. In this context, it was unexpected to note that the *K. pneumoniae* chromosome encodes two orthologues of *E. coli* LpxL. Our genetic and biochemical analysis conclusively assigned the functions of LpxL1 and LpxL2 to be laureate and myristate acyltransferases, respectively. Initially,

we were puzzled by the lack of LpxL1-mediated lipid A acylation in the *K. pneumoniae* background. However, the fact that we observed LpxL1-dependent lipid A species *m/z* 1797 in the *lpxL1 lpxL2* double mutant only when *lpxL1* was carried by a high-copy-number plasmid led us to hypothesize that *lpxL1* expression is repressed in *K. pneumoniae*. Indeed, we uncovered that LpxL1 is negatively regulated by the two-component system PhoPQ. Interestingly, we have recently demonstrated that this is also true in *K. pneumoniae* isolates with colistin resistance arising from mutational inactivation of the *mgrB* regulatory gene (25). MgrB is a negative feedback regulator of PhoPQ (25, 39); therefore, the *mgrB* mutation is associated with the upregulation of *phoPQ*, resulting in increased expression of *lpxO*, *pagP*, *pmrC*, and the *pmrF* operon, which in turn facilitates lipid A modifications with 2-hydroxymyristate, 4-amino-4-deoxy-L-arabinose, phosphoethanolamine, and palmitate (25). As anticipated, these modifications were absent in the lipid A produced by the *mgrB phoPQ* double mutant, but, in perfect agreement with the findings reported in this work, we detected the LpxL1-dependent species *m/z* 1797 in the lipid A produced by this mutant (25).

Our findings are reminiscent of those reported for *Shigella flexneri* and *E. coli* O157:H7 strains whose genomes encode two copies of *lpxM* (40–42). However, in stark contrast to the findings for *Klebsiella*, in both of these pathogens one of the two LpxM paralogues is carried by a plasmid, both enzymes catalyze the transfer of the same fatty acid to the lipid A, and PhoPQ is a positive regulator (40–42).

The fact that PhoPQ is activated by different signals *in vivo* and *in vitro* raises the intriguing notion that *K. pneumoniae* may regulate the acylation of its 2′ R-3-hydroxymyristoyl group in response to environmental signals. Furthermore, our data suggest that the presence of lipid A species *m/z* 1797 may indicate an environment where PhoPQ activity is repressed. We have recently reported the detection of lipid A species *m/z* 1797 in *K. pneumoniae* isolates that reached the spleens of intranasally infected mice (23), hence suggesting that PhoPQ activity may be diminished in the spleens of infected mice. On the other hand, it is interesting to consider why *K. pneumoniae* may prefer LpxL2-mediated acylation versus LpxL1-controlled acylation. Although there are potentially several explanations, it is worth noting that in *K. pneumoniae* LpxO-dependent hydroxylation occurs only in the C_{14} transferred to the 2′ R-3-hydroxymyristoyl group by LpxL2 (23; this work), and we have demonstrated that deletion of *lpxO* attenuates *K. pneumoniae* virulence *in vivo* (23, 30). The fact that PhoPQ is a positive regulator of *lpxO* (23) further underscores the importance of PhoPQ-mediated negative regulation of *lpxL1*.

It was unexpected to observe the lack of 4-amino-4-deoxy-L-arabinose and palmitate decorations in the lipid A produced by the *lpxL2* mutant. Notably, this phenotype was specific for *lpxL2* inactivation since we did not observe any effect on lipid A decorations of the lipid A molecules produced by *lpxL1* and *lpxM* mutants (23, 26; this work). This is in stark contrast to the findings for *E. coli* and *Salmonella enterica serovar* Typhimurium, where *lpxM* inactivation results in the loss of 4-amino-4-deoxy-L-arabinose (43). We cannot rule out the possibility that the lack of myristoylation in *K. pneumoniae* lipid A impairs the enzymatic activity of PmrK/ArnT and PagP, responsible for transferring 4-amino-4-deoxy-L-arabinose and palmitate to the lipid A, respectively. However, our data demonstrate that transcription of *phoPQ* and *pmrAB*, which encode two-component systems which govern the expression of *pagP* and the *pmrF* (*arn*) operon, respectively (24), were downregulated in the *lpxL2* mutant with a concomitant downregulation of the expressions of *pagP* and the *pmrF* (*arn*) operon. Interestingly, we did not observe an increase in the level of *lpxL1* transcription in the *lpxL2* mutant background (see Fig. S7 in the supplemental material), despite the decrease in the level of *phoPQ* expression in this mutant. This result may indicate that the low levels of *phoP* in the *lpxL2* mutant background are enough to repress *lpxL1* expression but are not sufficient to increase the levels of expression of *pagP* and the *pmrF* operon. However, by no means do we rule out the possibility that there might be other regulatory systems controlling *lpxL1* expression. Future research efforts will be devoted to the

detailed characterization of *lpxL1* expression and to the identification of any additional putative *lpxL1* regulator.

Another novel finding of this work is that the *lpxL2* mutant elicited higher inflammatory responses than the wild type. This observation challenges the conventional wisdom that *Enterobacteriaceae* late acyltransferase lipid A mutants elicit limited activation of inflammatory responses (40, 44, 45). Mechanistically, our data are consistent with the notion that the *lpxL2* mutant-induced heightened inflammation is due to the lack of the LpxO-mediated lipid A modification. This is in perfect agreement with our recent work demonstrating that *K. pneumoniae* LPS containing 2-hydroxymyristate is less inflammatory than that lacking this modification (23). Our data also suggest that the contribution of *lpxL2* to polymyxin resistance, phagocytosis evasion, and virulence in the *G. mellonella* infection model is dependent on LpxO-mediated hydroxylation of the LpxL2-transferred myristate. These findings are consistent with those of previous studies demonstrating the role of the *K. pneumoniae* LpxO-mediated lipid A modification on AP resistance and phagocytosis evasion by professional phagocytes (macrophages and amoebae) (23, 25, 46). Altogether, this evidence sustains the notion that LpxO-controlled lipid A hydroxylation plays a major role in *K. pneumoniae* strategies to counter host defense mechanisms. The generalization that the presence of a hydroxyl group on a lipid A secondary acyl chain is a bacterial mechanism to evade innate immune defenses warrants further studies. However, providing additional support to this notion, hydroxylation on the 3′-linked secondary acyl chain of *Vibrio cholerae* also promotes resistance to APs (47). The fact that several other Gram-negative bacterial pathogens synthesize lipid A species that possess a hydroxyl group on a secondary acyl chain (*Salmonella*, *Pseudomonas*, *Legionella*, *Acinetobacter*, *Vibrio*) (6, 48–50) might suggest that this lipid A modification is a conserved microbial anti-immune mechanism.

Finally, the clinical implications of our findings are worth discussing. *K. pneumoniae* has recently been singled out to be an urgent threat to human health by the World Health Organization, the U.S. Centers for Disease Control and Prevention, and the UK Department of Health due to the increasing number of multidrug-resistant isolates. *K. pneumoniae* infections are particularly a problem among neonates, elderly individuals, and immunocompromised individuals within the health care setting, but this organism is also responsible for a significant number of community-acquired infections, including pneumonia and sepsis (10, 11). The evidence presented in this work warrants further investigation of the *lpxL2* gene product as a novel target for antimicrobial therapy. Even though compounds targeting LpxL2 might not inhibit the growth of *Klebsiella*, since the growth kinetics of *lpxL2* and *lpxO* mutants were not affected, our data suggest that they will render the bacterium susceptible to APs and will also increase inflammatory responses. Both actions should facilitate the clearance of the pathogen from the airways, which might be further enhanced with the aid of antibiotics. However, the clinical utility of any molecule active against *lpxL2*, when and if such a molecule is developed, remains to be determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in lysogeny broth (LB) medium at 37°C on an orbital shaker (180 rpm). When appropriate, the following antibiotics were added to the growth medium at the indicated concentrations: ampicillin (Amp) at 100 μg/ml, kanamycin (Km) at 50 μg/ml, chloramphenicol (Cm) at 25 μg/ml, and trimethoprim (Tp) at 100 μg/ml.

To assess the growth of *K. pneumoniae* strains, bacteria were grown at 37°C in 5 ml of LB medium on an orbital shaker (180 rpm) overnight, and 5 μl of this culture was added to 250 μl of either LB or M9 minimal medium. Absorbance readings at an optical density of 600 nm (OD_{600}) were measured at 20-min intervals over a 24-h period using a Bioscreen C automated microbial growth analyzer (MTX Lab Systems, Vienna, VA, USA). A total of 5 independent growth curves were obtained for each strain.

***K. pneumoniae* mutant construction.** Primers for mutant construction (see Table S1 in the supplemental material) were designed on the basis of the whole-genome sequence of *K. pneumoniae* 52.145 (GenBank accession no. FO834906.1). Two sets of primers, LpxI1UP and LpxL1DOWN, were used to amplify two *lpxL1* fragments by PCR using *Ex Taq* polymerase (TaKaRa). These fragments were annealed at their overlapping regions, amplified as a single fragment, and cloned into the pGEM-T Easy vector (Promega) to obtain pGEMTlpxL1. A similar approach was followed to obtain pGEMTlpxL2. A kanamycin

resistance cassette was obtained as a BamHI fragment from pGEMTFRTKm (24) and cloned into BamHI-digested pGEMTΔlpxL-1 and pGEMTΔlpxL-2 to generate pGEMTΔlpxL1Km and pGEMTΔlpxL2Km, respectively. The lpxL-1Km and lpxL-2Km fragments (~3.5 kb each) were subsequently obtained by NotI digestion, gel purified, and cloned into NotI-digested pJTOOL-1. pJTOOL-1 is a suicide vector that carries an R6K origin of replication, an oriT sequence for conjugational transfer, and a Cm resistance marker (51). It also carries the *sacB* gene, which mediates sucrose sensitivity as a positive selection for the excision of the vector after double crossing-over (51). pJTOOL-1-lpxL1Km and pJTOOL-1-lpxL2Km were transformed into *E. coli* β2163, from which the plasmids were mobilized into *K. pneumoniae* 52.145 by conjugation. Transconjugants were selected after growth on LB plates supplemented with Cm at 37°C. A total of 10 merodiploids were serially diluted in PBS, spread on LB plates without NaCl containing 6% sucrose, and incubated at 25°C. The recombinants that survived 6% sucrose were checked for their antibiotic resistance, and the appropriate replacement of the wild-type alleles by the mutant ones was confirmed by PCR. The recombinants selected were named 52145Δ*lpxL1*Km and 52145Δ*lpxL2*Km. The Km resistance cassettes were excised by FLP-mediated recombination using plasmid pFLP2 (52), as we have previously described (24). The mutants selected were named 52145-Δ*lpxL1* and 52145-Δ*lpxL2*, respectively.

The *lpxL1 lpxL2* double mutant strain 52145-Δ*lpxL1*Δ*lpxL2* was obtained by mobilizing pJTOOL-1-lpxL2Km into 52145-Δ*lpxL1*. The replacement of the wild-type allele by the mutant one was done as described above and confirmed by PCR.

The *lpxL1 phoQ* double mutant strain 52145-Δ*lpxL1*Δ*phoQ* was obtained by mobilizing pMAKSACBΔphoQGB (24) into 52145-Δ*lpxL1*. The *lpxO* mutant strain 52145-Δ*lpxO* was constructed by mobilizing pMAKSACBΔlpxO (25) into *K. pneumoniae* 52.145. The replacement of the wild-type alleles by the mutant one was done as described above and confirmed by PCR.

Complementation of *K. pneumoniae* mutants. To complement the *lpxL* mutants, DNA fragments containing the putative promoter region and coding region of *lpxL1* and *lpxL2* were PCR amplified using *Ex Taq* polymerase, gel purified, and cloned into pGEM-T Easy to obtain pGEMTlpxL1Com and pGEMTlpxL2Com, respectively. These plasmids were then electroporated into *K. pneumoniae* and *E. coli* mutant strains, and clones were selected after growth on LB plates supplemented with Amp at 37°C. For single-copy chromosomal complementation, PvuII fragments from pGEMTlpxL1Com and pGEMTlpxL2Com were gel purified and cloned into SmaI-digested pUC18R6kT-mini-Tn*7*-T-Km (53) to obtain pUC18R6kT-mini-Tn*7*-T-Km-lpxL1Com and pUC18R6kT-mini-Tn*7*-T-Km-lpxL2Com, respectively. The pSTNSK-Tp plasmid, which contains the transposase *tnsABCD* necessary for Tn*7* transposition (54), was electroporated into the *K. pneumoniae lpxL* mutants. The pUC18R6kT-mini-Tn*7*-T-Km-lpxL1 and pUC18R6kT-mini-Tn*7*-T-Km-lpxL2 plasmids were then mobilized by conjugation into the mutants harboring pSTNSK-Tp. Colonies were checked for resistance to Km and sensitivity to Amp. As the Amp resistance cassette is located outside the Tn*7* region of the vector, Amp sensitivity denotes the integration of the Tn*7* derivative at the *att* Tn*7* site instead of incorporation of the vector into the chromosome. Confirmation of integration of the Tn*7* transposon at the established *att* Tn*7* site located downstream of the *glmS* gene was verified by PCR, as we have previously described (25, 55). Because the origin of replication of plasmid pSC101 is thermosensitive, the recipient strains were cured of pSTNSK-Tp by growing the bacteria at 37°C. Plasmid removal was confirmed by susceptibility to Tp.

***E. coli lpxL* mutant construction.** The bacterial mutant BN1 Δ*lpxL* was created using the Red recombinase system (56). Strain BN1 is an *lpxT*, *eptA*, and *pagP* mutant (57). A Km resistance cassette flanked by FLP recombination target (FRT) sites was PCR amplified using the pKD46 plasmid as a template (56). The primers used for amplification incorporated homologous extensions to the *lpxL* gene (Table S1). The PCR product was gel purified and treated with DpnI, and 1 μg of the DNA was electroporated into *E. coli* BN1 harboring the pKD46 plasmid (56). Mutants were selected on LB agar containing Km, and a recombinant in which the wild-type allele was replaced by the mutant one was selected. The appropriate replacement of the wild-type allele by the mutant one was confirmed by PCR, and the Km cassette was subsequently excised by FLP-mediated recombination using plasmid pFLP2. The strain generated was named BN1 Δ*lpxL*.

Construction of reporter fusions. DNA fragments containing the promoter regions of the *lpxL1* and *lpxL2* genes were amplified by PCR using Phusion polymerase (NEB), EcoRI digested, gel purified, and cloned into the EcoRI-SmaI-digested pGPL01 suicide vector (58). This vector contains a promoterless firefly luciferase gene (*lucFF*) and an R6K origin of replication. Plasmids in which *lucFF* was under the control of the *Klebsiella* promoters were identified by restriction digestion analysis and named pGPLK-pnProlpxL1 and pGPLKpnProlpxL2, respectively. Reporter plasmids pGPLKpnPmrH and pGPLKpnPagP have been described previously (24, 25).

Plasmids were conjugated into the different *K. pneumoniae* strains, and strains in which the suicide vector was integrated into the genome by homologous recombination were selected. Correct insertion of the vectors into the chromosome was confirmed by PCR using the relevant *lucFF*_check and promoter sequence primers (Table S1).

Luciferase activity. The reporter strains were grown on an orbital shaker (180 rpm) at 37°C until the exponential phase and harvested (3,000 × *g*, 20 min, 22°C). The bacteria were then washed once with sterile PBS and the OD_{600} was adjusted to 1.0. A 100-μl aliquot of the bacterial suspension was mixed with 100 μl of luciferase assay reagent (1 mM D-luciferin [Synchem] in 100 mM citrate buffer [pH 5]). Luminescence was immediately measured with a Glomax 20/20 luminometer (Promega) and expressed as the number of relative light units (RLU). All measurements were carried out in quintuplicate on at least three separate occasions.

Capsule purification and quantification. Bacterial strains were grown overnight at 37°C overnight in 3 ml LB medium, and viable counts were determined by dilution plating. The bacteria were then

harvested (3,000 × *g*, 20 min, 22°C), and the cell pellet was resuspended in 500 μl of sterile water. Samples were then treated with 1% 3-(*N*,*N*-dimethyltetradecylammonio)propanesulfonate (Sigma) in 100 mM citric acid, pH 2.0, at 50°C for 20 min. The bacterial debris was pelleted (3,000 × *g*, 10 min, 22°C), and 250 μl of the supernatant was transferred into a clean 15-ml glass tube. The capsule polysaccharide was ethanol precipitated at −20°C for 20 min and recovered by centrifugation (9,000 × *g*, 10 min, 4°C). The pellets were dried (5 min, 90°C) and resuspended in 200 μl of sterile water. The capsule was quantified by determining the concentration of uronic acid in the samples, using a modified carbazole assay, as described by Rahn and Whitfield (59). All extractions and quantifications were carried out on three independent occasions.

Isolation and analysis of lipid A. Lipid A molecules were extracted using an ammonium hydroxide-isobutyric acid method (60) and subjected to negative-ion matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry analysis. Briefly, the bacteria were grown in 10 ml of LB medium until the exponential phase and washed once with PBS, and the pellet was resuspended in 400 μl of isobutyric acid–1 M ammonium hydroxide (5:3 [vol/vol]) in a screw-cap test tube. A sample was then incubated at 100°C for 2 h with occasional vortexing before being cooled in ice water and centrifuged (2,000 × *g* for 15 min). The supernatant was transferred to a new tube, diluted with an equal volume of water, and lyophilized. The lyophilized material was washed twice with 400 μl of methanol and centrifuged (2,000 × *g* for 15 min). The insoluble lipid A was solubilized in 50 to 100 μl of chloroform-methanol-water (3:1.5:0.25 [vol/vol/vol]). To analyze the samples, a few microliters of the lipid A suspension (1 mg/ml) was desalted with a few grains of ion-exchange resin (H^+; Dowex 50W-X8) in a 1.5-ml microcentrifuge tube. A 1-μl aliquot of the suspension (50 to 100 μl) was deposited on the target and covered with the same amount of dihydroxybenzoic acid matrix (Bruker Daltonics Inc.) dissolved in acetonitrile–0.1% trifluoroacetic acid (1:2 [vol/vol]). Different ratios between the samples and dihydroxybenzoic acid were used when necessary. Analyses were performed on a Bruker Autoflex speed TOF/TOF mass spectrometer (Bruker Daltonics Inc.) in negative reflective mode with delayed extraction. The ion-accelerating voltage was set at 20 kV. Each spectrum was an average of 300 shots. A peptide calibration standard (Bruker Daltonics Inc.) was used to calibrate the MALDI-TOF/TOF mass spectrometer. Further calibration for lipid A analysis was performed externally using lipid A extracted from *E. coli* strain MG1655 grown in LB medium at 37°C. Interpretation of the negative-ion spectra was based on earlier studies showing that ions with masses higher than 1,000 give signals proportional to the signals of the corresponding lipid A species present in the preparation (23, 24, 61). Important theoretical masses for the interpretation of peaks found in the present study are as follows: $C_{14:OH}$, 226; C_{12}, 182; C_{14}, 210; 4-amino-4-deoxy-L-arabinose, 131; and C_{16}, 239. The spectra are representative of those from at least three independent lipid A extractions.

Infection of *Galleria mellonella* larvae. *G. mellonella* larvae were acquired from UK Waxworms Ltd. and kept at 21°C in the dark. The larvae were used within 3 days of receipt. Larvae of approximately 250 to 350 mg were selected for the experiments.

Infections were performed as we have described previously with minor modifications (30). Briefly, bacteria were grown in 5 ml LB medium until exponential phase and harvested (3,000 × *g*, 20 min, 22°C). The bacteria were subsequently washed once with 10 mM PBS (pH 6.5) and diluted in PBS to an OD_{600} of 1.0, which corresponds to approximately 5×10^8 CFU/ml. After surface disinfection using ethanol (70% [vol/vol]), the larvae were injected in the last right proleg with 10 μl of the bacterial suspension containing approximately 1×10^5 CFU using a Hamilton syringe with a 30-gauge needle. A group of 10 larvae was injected with 10 μl of PBS in parallel to ensure that death was not due to trauma from the injection. The larvae were placed in 9.2-cm petri dishes and kept at 37°C in the dark. Insects were considered dead when they did not respond to physical stimuli. The larvae were examined for pigmentation, and the time of death was recorded. Assays were allowed to proceed for only 3 days, as pupa formation could occasionally be seen by day 4. At least three independent experiments (30 larvae per strain) were performed.

RNA isolation and RT-qPCR. Bacteria were grown in 5 ml LB medium until they reached an OD_{600} of 1.0. An equal volume of RNAlater stabilization solution (Ambion) was added to the culture, and the culture was incubated at room temperature (22°C) for 30 min. The bacteria were then centrifuged (3,000 × *g*, 20 min, 22°C), and the supernatant was removed. Total RNA was extracted from the bacterial pellets using the TRIzol reagent (Ambion). Extracted RNA was treated with DNase I (Roche) and precipitated with sodium acetate (Ambion) and ethanol. RNA was quantified using a Nanovue Plus spectrophotometer (GE Healthcare Life Sciences).

cDNA was obtained by retrotranscription of 1 μg of total RNA using commercial Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers (Invitrogen). Twenty nanograms of cDNA was used as a template in 20 μl of the reaction mixture from a KapaSYBR Fast qPCR kit (Kapa Biosystems) and primer mix. RT-qPCR analyses were performed using an Mx3005P instrument (Agilent) and the following thermocycling protocol: 95°C for 3 min for hot-start polymerase activation, followed by 40 cycles of 95°C for 10 s and 56°C for 30 s. The fluorescence of the SYBR green dye was measured at 521 nm. Relative quantities of mRNAs were obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method by normalization to the amount of *rpoD*. cDNAs were obtained from three independent extractions of RNA, and each cDNA was amplified by RT-qPCR.

Antimicrobial peptide susceptibility assays. Bacteria were grown at 37°C in 5 ml of LB medium and harvested (3,000 × *g*, 20 min, 22°C) when they were in the exponential growth phase. The bacteria were then washed once with PBS, and a suspension containing 4×10^4 CFU/ml was prepared in 10 mM PBS (pH 6.5), 1% tryptone soy broth (Oxoid), and 100 mM NaCl. Aliquots of the suspension (25 μl) were mixed in 0.2-ml tubes with the appropriate antibiotic at a final concentration of 3 μg/ml to give a final volume

of 30 μl. After 1 h of incubation at 37°C, 15 μl was plated on LB agar and colony counts were determined after incubation at 37°C for 24 h. The results were expressed as a percentage of the colony count of bacteria not exposed to antibiotics. All experiments were performed with duplicate samples on three independent occasions.

MICs were determined by Etest (bioMérieux) according to the manufacturer's instructions. MIC testing was performed on two independent occasions.

Whole-blood phagocytosis assay. Bacteria were grown at 37°C in 5 ml of LB medium on an orbital shaker (180 rpm) until the exponential phase and harvested (3,000 $\times$ *g*, 20 min, 22°C). The bacteria were subsequently washed once and diluted in PBS to an OD_{600} of 1.0. Further dilutions in PBS were made to obtain 1×10^7 CFU/100 μl bacterial suspensions. Three hundred microliters of fresh human blood (used within 30 min of removal) was mixed with 1×10^7 CFU/100 μl bacterial suspension and incubated at 37°C on an orbital shaker (180 rpm) for 3 h. After incubation, dilutions were plated to obtain viable counts. The bacterial counts recovered were then divided by the initial counts. Experiments were performed using blood from three individual blood donors, and for each blood sample, each strain was tested in duplicate. Ethical approval for the use of blood from healthy volunteers to study bacterial killing was from the Research Ethics Committee of the School of Medicine, Dentistry, and Biomedical Sciences (Queen's University Belfast).

Macrophage infections. Immortalized murine bone marrow-derived macrophages (iBMDMs; the macrophage cell line was derived from wild-type NR-9456 mice; BEI Resources, NIAID, NIH) were grown in Dulbecco's modified Eagle medium (DMEM; catalog number 41965; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco) at 37°C in a humidified 5% CO_2 incubator. Cells were routinely tested for *Mycoplasma* contamination. For infections, iBMDMs were seeded at 2.5×10^5 cells per well in 96-well plates.

To prepare the inocula for infections, bacteria were grown until exponential phase in 5 ml LB medium, supplemented with the appropriate antibiotics, when required, at 37°C on an orbital shaker (180 rpm). Bacteria were recovered by centrifugation (3,000 $\times$ *g*, 20 min, 22°C), washed once with PBS, and diluted in PBS to an OD_{600} of 1.0, which corresponds to approximately 5×10^8 CFU/ml. This bacterial suspension was UV irradiated at 1 J for 20 min, and bacterial killing was confirmed by plating in LB medium. Bacterial suspensions were stored at −80°C. iBMDMs were challenged with 1×10^5 bacteria for 6 h, and supernatants were collected for cytokine determination. Experiments were performed in duplicate and repeated three independent times.

Cytokine quantification. Cytokines in the supernatants were determined using Luminex multiplex assays (R&D Systems, Abingdon, UK) as recommended by the manufacturer. Analyses were performed using a Bio-Plex 100 system with the xMAP technology (Bio-Rad, Hertfordshire, UK).

Statistical analysis. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni contrasts, the one-tailed *t* test, or, when the requirements were not met, the Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant. Survival analyses were undertaken using the log-rank (Mantel-Cox) test with the Bonferroni correction for multiple comparisons ($\alpha = 0.008$). The analyses were performed using Prism (version 4) software for the personal computer (GraphPad Software).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00068-17.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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REFERENCES

1. Raetz CR, Whitfield C. 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700. https://doi.org/10.1146/annurev.biochem.71.110601.135414.
2. Needham BD, Trent MS. 2013. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 11:467–481. https://doi.org/10.1038/nrmicro3047.
3. Lee H, Hsu FF, Turk J, Groisman EA. 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J Bacteriol 186:4124–4133. https://doi.org/10.1128/JB.186.13.4124-4133.2004.
4. Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI. 1997. Regulation of lipid A modifications by Salmonella typhimurium

virulence genes *phoP-phoQ*. Science 276:250–253. https://doi.org/10.1126/science.276.5310.250.
5. Guo L, Lim KB, Poduje CM, Daniel M, Gunn JS, Hackett M, Miller SI. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95:189–198. https://doi.org/10.1016/S0092-8674(00)81750-X.
6. Gibbons HS, Lin S, Cotter RJ, Raetz CR. 2000. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, A new Fe^{2+}/alpha-ketoglutarate-dependent dioxygenase homologue. J Biol Chem 275:32940–32949.
7. Nizet V. 2006. Antimicrobial peptide resistance mechanisms of human bacterial pathogens. Curr Issues Mol Biol 8:11–26.
8. Nation RL, Li J, Cars O, Couet W, Dudley MN, Kaye KS, Mouton JW, Paterson DL, Tam VH, Theuretzbacher U, Tsuji BT, Turnidge JD. 2015. Framework for optimisation of the clinical use of colistin and polymyxin B: the Prato polymyxin consensus. Lancet Infect Dis 15:225–234. https://doi.org/10.1016/S1473-3099(14)70850-3.
9. Maeshima N, Fernandez RC. 2013. Recognition of lipid A variants by the TLR4-MD-2 receptor complex. Front Cell Infect Microbiol 3:3. https://doi.org/10.3389/fcimb.2013.00003.
10. Quan TP, Fawcett NJ, Wrightson JM, Finney J, Wyllie D, Jeffery K, Jones N, Shine B, Clarke L, Crook D, Walker AS, Peto TE, Infections in Oxfordshire Research Database (IORD). 2016. Increasing burden of community-acquired pneumonia leading to hospitalisation, 1998-2014. Thorax 71:535–542. https://doi.org/10.1136/thoraxjnl-2015-207688.
11. Paczosa MK, Mecsas J. 2016. *Klebsiella pneumoniae*: going on the offense with a strong defense. Microbiol Mol Biol Rev 80:629–661. https://doi.org/10.1128/MMBR.00078-15.
12. Regue M, Izquierdo L, Fresno S, Pique N, Corsaro MM, Naldi T, De Castro C, Waidelich D, Merino S, Tomas JM. 2005. A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. J Bacteriol 187:4198–4206. https://doi.org/10.1128/JB.187.12.4198-4206.2005.
13. Fresno S, Jimenez N, Canals R, Merino S, Corsaro MM, Lanzetta R, Parrilli M, Pieretti G, Regue M, Tomas JM. 2007. A second galacturonic acid transferase is required for core lipopolysaccharide biosynthesis and complete capsule association with the cell surface in *Klebsiella pneumoniae*. J Bacteriol 189:1128–1137. https://doi.org/10.1128/JB.01489-06.
14. Izquierdo L, Coderch N, Pique N, Bedini E, Corsaro MM, Merino S, Fresno S, Tomas JM, Regue M. 2003. The *Klebsiella pneumoniae wabG* gene: role in biosynthesis of the core lipopolysaccharide and virulence. J Bacteriol 185:7213–7221. https://doi.org/10.1128/JB.185.24.7213-7221.2003.
15. Frirdich E, Bouwman C, Vinogradov E, Whitfield C. 2005. The role of galacturonic acid in outer membrane stability in *Klebsiella pneumoniae*. J Biol Chem 280:27604–27612. https://doi.org/10.1074/jbc.M504987200.
16. Frirdich E, Vinogradov E, Whitfield C. 2004. Biosynthesis of a novel 3-deoxy-D-manno-oct-2-ulosonic acid-containing outer core oligosaccharide in the lipopolysaccharide of *Klebsiella pneumoniae*. J Biol Chem 279:27928–27940. https://doi.org/10.1074/jbc.M402549200.
17. Vinogradov E, Cedzynski M, Ziolkowski A, Swierzko A. 2001. The structure of the core region of the lipopolysaccharide from *Klebsiella pneumoniae* O3. 3-Deoxy-alpha-D-manno-octulosonic acid (alpha-Kdo) residue in the outer part of the core, a common structural element of *Klebsiella pneumoniae* O1, O2, O3, O4, O5, O8, and O12 lipopolysaccharides. Eur J Biochem 268:1722–1729.
18. Vinogradov E, Perry MB. 2001. Structural analysis of the core region of the lipopolysaccharides from eight serotypes of *Klebsiella pneumoniae*. Carbohydr Res 335:291–296. https://doi.org/10.1016/S0008-6215(01)00216-6.
19. Vinogradov E, Frirdich E, MacLean LL, Perry MB, Petersen BO, Duus JO, Whitfield C. 2002. Structures of lipopolysaccharides from *Klebsiella pneumoniae*. Elucidation of the structure of the linkage region between core and polysaccharide O chain and identification of the residues at the non-reducing termini of the O chains. J Biol Chem 277:25070–25081.
20. Cortes G, Borrell N, de Astorza B, Gomez C, Sauleda J, Alberti S. 2002. Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a murine model of pneumonia. Infect Immun 70:2583–2590. https://doi.org/10.1128/IAI.70.5.2583-2590.2002.
21. Hsieh PF, Lin TL, Yang FL, Wu MC, Pan YJ, Wu SH, Wang JT. 2012. Lipopolysaccharide O1 antigen contributes to the virulence in *Klebsiella pneumoniae* causing pyogenic liver abscess. PLoS One 7:e33155. https://doi.org/10.1371/journal.pone.0033155.
22. Shankar-Sinha S, Valencia GA, Janes BK, Rosenberg JK, Whitfield C, Bender RA, Standiford TJ, Younger JG. 2004. The *Klebsiella pneumoniae* O antigen contributes to bacteremia and lethality during murine pneumonia. Infect Immun 72:1423–1430. https://doi.org/10.1128/IAI.72.3.1423-1430.2004.
23. Llobet E, Martinez-Moliner V, Moranta D, Dahlstrom KM, Regueiro V, Tomas A, Cano V, Perez-Gutierrez C, Frank CG, Fernandez-Carrasco H, Insua JL, Salminen TA, Garmendia J, Bengoechea JA. 2015. Deciphering tissue-induced *Klebsiella pneumoniae* lipid A structure. Proc Natl Acad Sci U S A 112:E6369–E6378. https://doi.org/10.1073/pnas.1508820112.
24. Llobet E, Campos MA, Gimenez P, Moranta D, Bengoechea JA. 2011. Analysis of the networks controlling the antimicrobial-peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect Immun 79:3718–3732. https://doi.org/10.1128/IAI.05226-11.
25. Kidd TJ, Mills G, Sa-Pessoa J, Dumigan A, Frank CG, Insua JL, Ingram R, Hobley L, Bengoechea JA. 2017. A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. EMBO Mol Med 9:430–447. https://doi.org/10.15252/emmm.201607336.
26. Clements A, Tull D, Jenney AW, Farn JL, Kim SH, Bishop RE, McPhee JB, Hancock RE, Hartland EL, Pearse MJ, Wijburg OL, Jackson DC, McConville MJ, Strugnell RA. 2007. Secondary acylation of *Klebsiella pneumoniae* lipopolysaccharide contributes to sensitivity to antibacterial peptides. J Biol Chem 282:15569–15577. https://doi.org/10.1074/jbc.M701454200.
27. Helander IM, Kato Y, Kilpelainen I, Kostiainen R, Lindner B, Nummila K, Sugiyama T, Yokochi T. 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. Eur J Biochem 237:272–278. https://doi.org/10.1111/j.1432-1033.1996.0272n.x.
28. Li Y, Yun J, Liu L, Li Y, Wang X. 2016. Identification of two genes encoding for the late acyltransferases of lipid A in *Klebsiella pneumoniae*. Curr Microbiol 73:732–738. https://doi.org/10.1007/s00284-016-1117-6.
29. Tomas A, Lery L, Regueiro V, Perez-Gutierrez C, Martinez V, Moranta D, Llobet E, Gonzalez-Nicolau M, Insua JL, Tomas JM, Sansonetti PJ, Tournebize R, Bengoechea JA. 2015. Functional genomic screen identifies *Klebsiella pneumoniae* factors implicated in blocking nuclear factor kappaB (NF-kappaB) signaling. J Biol Chem 290:16678–16697. https://doi.org/10.1074/jbc.M114.621292.
30. Insua JL, Llobet E, Moranta D, Perez-Gutierrez C, Tomas A, Garmendia J, Bengoechea JA. 2013. Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. Infect Immun 81:3552–3565. https://doi.org/10.1128/IAI.00391-13.
31. Moranta D, Regueiro V, March C, Llobet E, Margareto J, Larrarte E, Garmendia J, Bengoechea JA. 2010. *Klebsiella pneumoniae* capsule polysaccharide impedes the expression of beta-defensins by airway epithelial cells. Infect Immun 78:1135–1146. https://doi.org/10.1128/IAI.00940-09.
32. Llobet E, Tomas JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154:3877–3886. https://doi.org/10.1099/mic.0.2008/022301-0.
33. Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72:7107–7114. https://doi.org/10.1128/IAI.72.12.7107-7114.2004.
34. Regueiro V, Campos MA, Pons J, Alberti S, Bengoechea JA. 2006. The uptake of a *Klebsiella pneumoniae* capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells. Microbiology 152:555–566. https://doi.org/10.1099/mic.0.28285-0.
35. Alvarez D, Merino S, Tomas JM, Benedi VJ, Alberti S. 2000. Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient *Klebsiella pneumoniae* clinical isolates. Infect Immun 68:953–955. https://doi.org/10.1128/IAI.68.2.953-955.2000.
36. Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. PLoS Genet 4:e1000233. https://doi.org/10.1371/journal.pgen.1000233.
37. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, Florquin S, van der Poll T. 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun 72:788–794. https://doi.org/10.1128/IAI.72.2.788-794.2004.
38. Wieland CW, van Lieshout MH, Hoogendijk AJ, van der Poll T. 2011. Host defence during *Klebsiella* pneumonia relies on haematopoietic-expressed Toll-like receptors 4 and 2. Eur Respir J 37:848–857. https://doi.org/10.1183/09031936.00076510.
39. Lippa AM, Goulian M. 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet 5:e1000788. https://doi.org/10.1371/journal.pgen.1000788.

40. D'Hauteville H, Khan S, Maskell DJ, Kussak A, Weintraub A, Mathison J, Ulevitch RJ, Wuscher N, Parsot C, Sansonetti PJ. 2002. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. J Immunol 168:5240–5251. https://doi.org/10.4049/jimmunol.168.10.5240.
41. Goldman SR, Tu Y, Goldberg MB. 2008. Differential regulation by magnesium of the two MsbB paralogs of Shigella flexneri. J Bacteriol 190:3526–3537. https://doi.org/10.1128/JB.00151-08.
42. Kim SH, Jia W, Bishop RE, Gyles C. 2004. An *msbB* homologue carried in plasmid pO157 encodes an acyltransferase involved in lipid A biosynthesis in Escherichia coli O157:H7. Infect Immun 72:1174–1180. https://doi.org/10.1128/IAI.72.2.1174-1180.2004.
43. Tran AX, Lester ME, Stead CM, Raetz CR, Maskell DJ, McGrath SC, Cotter RJ, Trent MS. 2005. Resistance to the antimicrobial peptide polymyxin requires myristoylation of *Escherichia coli* and *Salmonella typhimurium* lipid A. J Biol Chem 280:28186–28194. https://doi.org/10.1074/jbc.M505020200.
44. Khan SA, Everest P, Servos S, Foxwell N, Zahringer U, Brade H, Rietschel ET, Dougan G, Charles IG, Maskell DJ. 1998. A lethal role for lipid A in *Salmonella* infections. Mol Microbiol 29:571–579. https://doi.org/10.1046/j.1365-2958.1998.00952.x.
45. Somerville JE, Jr, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP. 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. J Clin Invest 97:359–365. https://doi.org/10.1172/JCI118423.
46. March C, Cano V, Moranta D, Llobet E, Perez-Gutierrez C, Tomas JM, Suarez T, Garmendia J, Bengoechea JA. 2013. Role of bacterial surface structures on the interaction of *Klebsiella pneumoniae* with phagocytes. PLoS One 8:e56847. https://doi.org/10.1371/journal.pone.0056847.
47. Hankins JV, Madsen JA, Giles DK, Childers BM, Klose KE, Brodbelt JS, Trent MS. 2011. Elucidation of a novel *Vibrio cholerae* lipid A secondary hydroxy-acyltransferase and its role in innate immune recognition. Mol Microbiol 81:1313–1329. https://doi.org/10.1111/j.1365-2958.2011.07765.x.
48. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. Antimicrob Agents Chemother 55:3370–3379. https://doi.org/10.1128/AAC.00079-11.
49. Kulshin VA, Zahringer U, Lindner B, Jager KE, Dmitriev BA, Rietschel ET. 1991. Structural characterization of the lipid A component of *Pseudomonas aeruginosa* wild-type and rough mutant lipopolysaccharides. Eur J Biochem 198:697–704. https://doi.org/10.1111/j.1432-1033.1991.tb16069.x.
50. Zahringer U, Knirel YA, Lindner B, Helbig JH, Sonesson A, Marre R, Rietschel ET. 1995. The lipopolysaccharide of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. Prog Clin Biol Res 392:113–139.
51. van Aartsen JJ, Rajakumar K. 2011. An optimized method for suicide vector-based allelic exchange in *Klebsiella pneumoniae*. J Microbiol Methods 86:313–319. https://doi.org/10.1016/j.mimet.2011.06.006.
52. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212:77–86. https://doi.org/10.1016/S0378-1119(98)00130-9.
53. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP. 2005. A Tn*7*-based broad-range bacterial cloning and expression system. Nat Methods 2:443–448. https://doi.org/10.1038/nmeth765.
54. Crepin S, Harel J, Dozois CM. 2012. Chromosomal complementation using Tn*7* transposon vectors in Enterobacteriaceae. Appl Environ Microbiol 78:6001–6008. https://doi.org/10.1128/AEM.00986-12.
55. March C, Moranta D, Regueiro V, Llobet E, Tomas A, Garmendia J, Bengoechea JA. 2011. *Klebsiella pneumoniae* outer membrane protein A is required to prevent the activation of airway epithelial cells. J Biol Chem 286:9956–9967. https://doi.org/10.1074/jbc.M110.181008.
56. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.120163297.
57. Needham BD, Carroll SM, Giles DK, Georgiou G, Whiteley M, Trent MS. 2013. Modulating the innate immune response by combinatorial engineering of endotoxin. Proc Natl Acad Sci U S A 110:1464–1469. https://doi.org/10.1073/pnas.1218080110.
58. Gunn JS, Hohmann EL, Miller SI. 1996. Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. J Bacteriol 178:6369–6373. https://doi.org/10.1128/jb.178.21.6369-6373.1996.
59. Rahn A, Whitfield C. 2003. Transcriptional organization and regulation of the *Escherichia coli* K30 group 1 capsule biosynthesis (*cps*) gene cluster. Mol Microbiol 47:1045–1060. https://doi.org/10.1046/j.1365-2958.2003.03354.x.
60. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46:1773–1778. https://doi.org/10.1194/jlr.D500014-JLR200.
61. Lindner B. 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipopolysaccharides. Methods Mol Biol 145:311–325.
62. Demarre G, Guerout AM, Matsumoto-Mashimo C, Rowe-Magnus DA, Marliere P, Mazel D. 2005. A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate Escherichia coli host strains. Res Microbiol 156:245–255. https://doi.org/10.1016/j.resmic.2004.09.007.
63. Nassif X, Sansonetti PJ. 1986. Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. Infect Immun 54:603–608.