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Secondary Acylation of *Klebsiella pneumoniae* Lipopolysaccharide Contributes to Sensitivity to Antibacterial Peptides*

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***Klebsiella pneumoniae* is an important cause of nosocomial Gram-negative sepsis. Lipopolysaccharide (LPS) is considered to be a major virulence determinant of this encapsulated bacterium and most mutations to the lipid A anchor of LPS are conditionally lethal to the bacterium. We studied the role of LPS acylation in *K. pneumoniae* disease pathogenesis by using a mutation of *lpxM* (*msbB/waaN*), which encodes the enzyme responsible for late secondary acylation of immature lipid A molecules. A *K. pneumoniae* B5055 (K2:O1) *lpxM* mutant was found to be attenuated for growth in the lungs in a mouse pneumonia model leading to reduced lethality of the bacterium. B5055Δ*lpxM* exhibited similar sensitivity to phagocytosis or complement-mediated lysis than B5055, unlike the non-encapsulated mutant B5055nm. *In vitro*, B5055Δ*lpxM* showed increased permeability of the outer membrane and an increased susceptibility to certain antibacterial peptides suggesting that *in vivo* attenuation may be due in part to sensitivity to antibacterial peptides present in the lungs of BALB/c mice. These data support the view that lipopolysaccharide acylation plays a important role in providing Gram-negative bacteria some resistance to structural and innate defenses and especially the antibacterial properties of detergents (*e.g.* bile) and cationic defensins.**

Klebsiella pneumoniae is a common cause of nosocomial pneumonia, septicemia, and urinary tract infections; a recent US study found the bacterium to be the third most commonly isolated organism from intensive care wards and the most common species identified in blood cultures (1), with a similar trend reported among European hospitals (2). This rising prevalence combined with the extensive spread of antibiotic-resistant strains, especially extended spectrum β-lactamase (ESBL)-producing strains, will drive the search for alternative treatments and/or an effective vaccine against the bacterium.

K. pneumoniae is known to express a number of virulence determinants, including a thick polysaccharide capsule. The capsule protects the bacterium from phagocytosis by polymorphonuclear leukocytes (3) and is thought to prevent killing of the bacterium by bactericidal serum factors (4). Other, well studied virulence factors of *K. pneumoniae* include the pili/fimbriae and lipopolysaccharide (LPS).[6] LPS consists of an outer membrane-embedded lipid A molecule, partially conserved inner core carbohydrate structure, attached to an outer core (of which there are at least two variants, conferred by the waa operon (5)), and a repeated polysaccharide or O-antigen (O-Ag). The O-Ag region specifically is thought to play a role in resistance to complement killing (6) and to contribute to bacteremia and lethality during murine pneumonia infections (7) although this contribution is still disputed (8). The comparatively small number of Klebsiella LPS or O-types (9 serotypes) compared with capsule or K-types (>77 serotypes) suggests that LPS could be utilized for vaccination. However any LPS-based vaccines would need to be detoxified before use in humans.

The lipid A (endotoxic) region of LPS has been studied as a possible virulence determinant in a number of Gram-negative bacteria (9–11) but has not yet been studied in detail in *K. pneumoniae in vivo*. Lipid A is thought to be the primary inflammatory component of LPS because of specific and sensitive recognition by the innate immune system (12). The structure of *K. pneumoniae* lipid A differs from the classical lipid A form (*i.e. Escherichia coli*) only by the presence of a secondary

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[6] The abbreviations used are: LPS, lipopolysaccharide; MIC, minimal inhibitory concentrations; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

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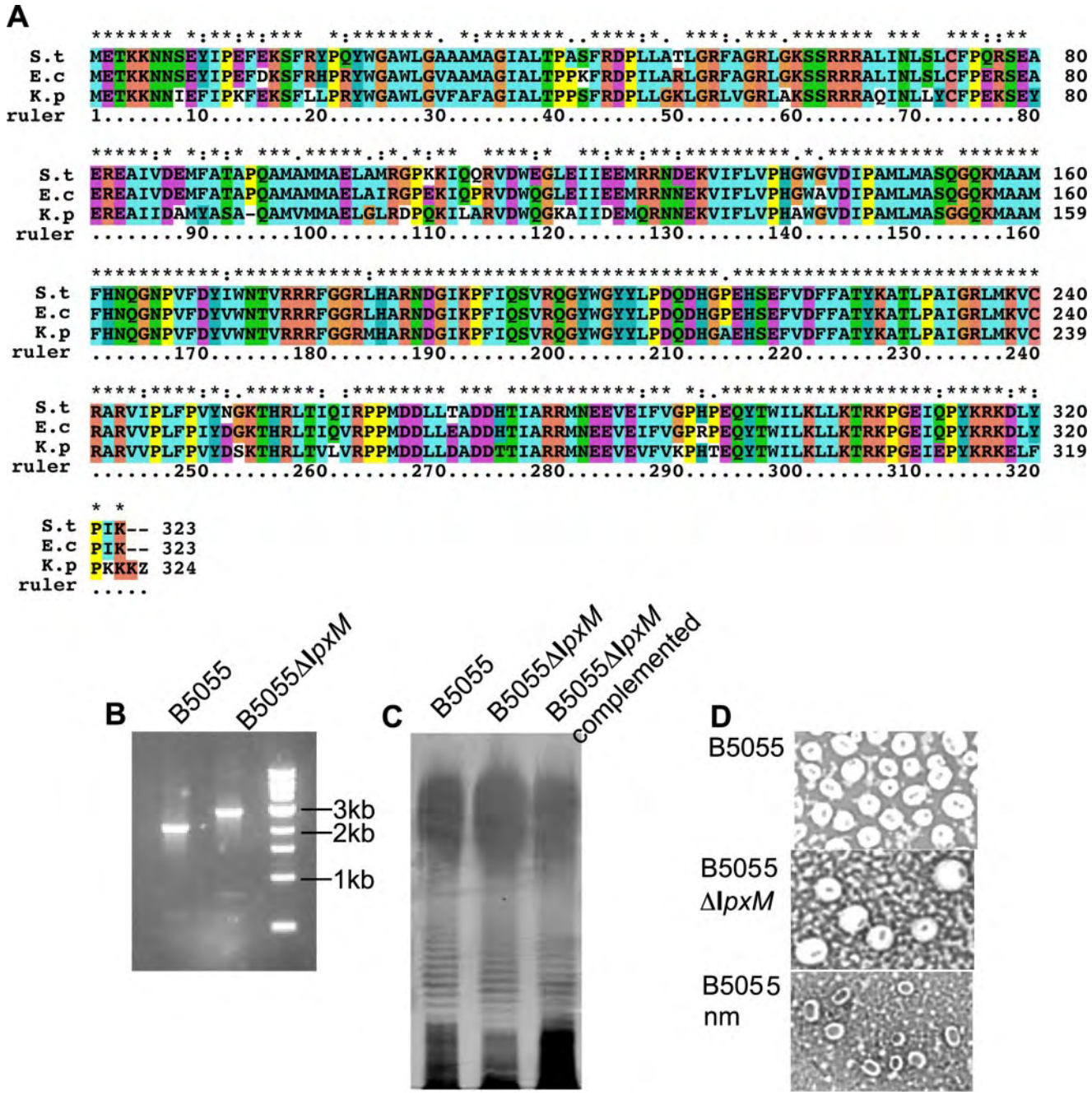

FIGURE 1. **Characterization of *lpxM* from *K. pneumoniae*.** *A*, ClustalX protein alignment of *lpxM* from *S. typhimurium, E. coli* K12, and *K. pneumoniae* MGH78578 showing significant sequence homology. *B*, PCR amplification of *lpxM* from wild type B5055 and the *lpxM* mutant B5055Δ*lpxM* (142 bp removed; *cat* cassette inserted). *C*, visualization of silver-stained LPS prepared from B5055, B5055Δ*lpxM*, and B5055Δ*lpxM* complemented (pGEM-*lpxM*) and separated by SDS-PAGE. *D*, visualization of capsule by Maneval's stain of B5055, B5055Δ*lpxM*, and B5055nm (non-mucoid mutant).

myristoyl residue mainly taking the place of the lauroyl group, resulting in two secondary myristoyl chains for *K. pneumoniae* (13), see Fig. 1. Lipid A is generally considered essential for normal growth of Gram-negative bacteria albeit an LPS-deficient mutant of *Neisseria meningitidis* has been constructed which shows a reduced growth rate (14). There are only a small number of mutations that can be made in the lipid A molecule that do not affect the growth of the bacterium. One mutation that has been studied in a number of Enterobacteriacceae is in *lpxM* (formally *msbB* or *waaN*) which encodes the enzyme responsible for the addition of one of the secondary acyl chains (10, 15). Mutation of *lpxM* has had varying and inconclusive effects in animal models for different pathogens: attenuation of an *lpxM* mutant in a mouse model of *Salmonella enterica var* Typhimurium (10) was consequently attributed to secondary mutations induced by the *lpxM* defect (16); *Shigella flexneri lpxM* mutants showed attenuated inflammation in a rabbit ligation loop model (11), while *N. meningitidis lpxM* mutants showed defects in assembly and incorporation of the lipid A into the outer membrane thereby precluding *in vivo* analysis (17). The only attenuation in a murine model of infection directly linked to the *lpxM* mutation has been in a clinical iso-

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TABLE 1
Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic	Reference or source
***K. pneumoniae* strains**		
B5055	K2:O1, mouse lethal clinical isolate	D.HansenSSI
B5055nm	B5055, mutation in *wza/wzb*, non-mucoid	A. Jenney, T. Uren
B5055Δ*lpxM*	B5055, mutation in *lpxM*, lipid A	This study
B5055nmΔ*lpxM*	Double mutant, non-mucoid, lipidA mutant	This study
***E. coli* strains**		
JM109	Cloning strain (F′ *traD36 proA+B+ lacIq* Δ *(lacZ)M15/* Δ*(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17*	(61)
Plasmids		
pBR322	Ap^r , Tc^r medium copy plasmid	Promega
pGEM T-EASY	Ap^r high copy plasmid	Promega
pACYC184	Cm^r, source of Cm^r cassette	Promega
pKD46	λ red recombinase	(19)
pBR-*lpxM*	pBR322 plus *lpxM* from B5055	This study
pGEM-*lpxM*	pGEM T-EASY plus *lpxM* from B5055	This study

late of *E. coli*, however secondary effects such as capsule reduction and filament formation were also seen in this bacterium (9) making data interpretation difficult. The effect of underacylated lipid A on human cells is better understood as purified pentaacyl and tetraacyl LPS has been shown to act as an endotoxin antagonist in the induction of cytokines because of differential recognition by human MD2·TLR4 complexes (18). However, this is not the case for murine cells as mouse MD2:TLR4 do not appear to differentiate between acylation states of lipid A (18).

The aim of this study was therefore to analyze an *lpxM* mutant of *K. pneumoniae* for *in vivo* attenuation and to understand the mechanisms leading to any observed attenuation, with a view to increasing our global understanding of the role of LPS.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The *K. pneumoniae* strain used was B5055, a mouse virulent clinical isolate (serotype K2:O1) or non-encapsulated mutant designated B5055 nm, produced by Tania Uren and Adam Jenney through the inactivation of *wza* and *wzb*, data not shown. *E. coli* JM109 and the plasmid pGEM T-Easy or pBR322 (Promega) were used for cloning work (Table 1). The temperature-sensitive plasmid pKD46 (19) was used to induce λ red recombinase production in the host cell to enhance homologous recombination. Bacteria were grown in Luria broth (LB) or on Luria agar (LA) at 37 °C (except for induction of λ red recombinase when bacteria were cultured at 30 °C). Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 60 μg/ml, and chloramphenicol at 30 μg/ml.

Generation of the K. pneumoniae lpxM Mutant—The *lpxM* gene of *K. pneumoniae* was identified by homology to *E. coli* K12 *lpxM/msbB* (AAC74925) and *S. enterica var Typhimurium* (AAL20805). The entire gene plus 500 bp at either end of the open reading frame was cloned from B5055 using oligonucleotide primers (5′- GTCACACTAGTGTCCAGTTCGACAACGTGGAAG and 5′-CTAGTGGGCCCGGTAAGGATTACTATGCCATCC, which include the underlined restriction enzyme sites SpeI and ApaI, respectively. The gene was ligated using these sites into pGEM T-EASY (to form pGEM-*lpxM*) and digested with BssHII and SphI to remove a central 142-bp fragment. A chloramphenicol resistance cassette (*cat*) from pACYC184 was amplified with primers 5′-ACTGTGCATGCCGTAAGAGGTTCCAACTTTCAC and 5′-GATCAGCGCGCGTAGCAGCACCAGGCGTTTAAGGG and cloned into pGEM-*lpxM* using the underlined SphI and BssHII sites (to form pGEM-*lpxM*/Chl). pKD46 was electroporated into B5055 (2.5 kV, 25 μF, 200 Ω) and grown at 30 °C with L-arabinose to induce λ red recombinase production. The *lpxM*-Chl fragment was then amplified as a 2755-bp linear fragment, gel-purified, and digested with NotI to remove any residual intact plasmid. The linear DNA fragment was then electroporated into B5055 + pKD46 or B5055nm + pKD46 and grown at 37 °C on chloramphenicol to remove pKD46 and select for recombinants, respectively. The anticipated disrupted *lpxM* gene size was confirmed by PCR and confirmed recombinants designated B5055Δ*lpxM* and B5055nmΔ*lpxM* (for capsulated and unencapsulated strains, respectively). pGEM-*lpxM* and pBR-*lpxM* (*lpxM* gene amplified with SalI and EcoRI sites and ligated into pBR322 using these sites) were used as high and low copy number constructs for complementation studies.

SDS-PAGE Analysis of the Mutant—Crude LPS preparations were made by pelleting cells from 3 ml of overnight (o/n) cultures. The pellets were resuspended in Laemmli buffer (2.3% SDS, 0.8% Tris, 10% glycerol, 5% dithiothreitol) and samples boiled for 10 min. Samples were digested with proteinase K (0.417 μg/μl) for 2 h at 56 °C. SDS-PAGE was performed on 15% polyacrylamide gels (20) and stained with alkaline silver stain to visualize LPS (21).

Extraction and Quantification of Capsule—Cell-associated capsule was extracted from mid-log and static cultures by the phenol-extraction method (22). Briefly, 5-ml samples were centrifuged (5000 × *g*, 15 min, 4 °C), washed, resuspended in 500 μl of dH_2O, and viable counts determined. Samples were then incubated at 68 °C for 2 min before addition of 500 μl of phenol, incubation was then continued for 30 min. Mixture was cooled, and 500 μl of chloroform added, centrifuged, and aqueous phase taken. Capsular material was precipitated at −20 °C for 20 min and resuspended in 500 μl of dH_2O.

Uronic acid content was determined as previously described (23). Briefly, 1.2 ml of 12.5 mM tetraborate in concentrated H_2SO_4 was added to 200 μl of sample and vortexed vigorously. Samples were then boiled for 5 min and cooled before addition of 20 μl of 0.15% 3-hydroxydiphenol in 0.5% NaOH. Absorbance was measured at 520 nm. Standard curves were con-

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structed with glucuronic acid. Bacterial capsules were also visualized by Maneval's stain (24).

Thin Layer Chromatography of ^{32}P-labeled Lipid A—Strains were grown to mid-log with 5 μCi/ml $^{32}P_i$ in the presence and absence of EDTA to activate *pagP* (25). Lipid A was extracted from cells by the mild acid hydrolysis method as described in Ref. 26 with slight modifications: initial single phase Bligh/Dyer incubation was at room temperature for 10 min rather than 60 min and the lipid A recovered after two-phase Bligh/Dyer was washed only once with fresh two-phase Bligh/Dyer mixture. 1000 cpm of each lipid preparation was applied to the origin of a Silica Gel 60 TLC. The plate was developed using the solvent chloroform:pyridine:88% formic acid:water (50:50:16:5, v/v) and exposed to a PhosPhorImage Screen overnight for visualization.

MALDI-MS Analysis of Lipid A—Lipid A samples were resuspended in chloroform/methanol (4:1 v/v) and analyzed by MALDI-TOF-MS. Mass spectra were acquired on a Voyager-DE STR mass spectrometer (PerSpective Biosystems) in the negative reflector mode, scanning over a mass/charge range of 1000–3000, and using an extraction delay time of 92 ns and an accelerating voltage of 21 kV. Saturated α-cyano-4-hydroxycinnamic acid (Sigma) in 60% 1-propyl alcohol was used as the matrix, and free glycosylphosphatidylinositols of known molecular weights (27) were used as standards.

GC-MS Analysis of Lipid A—Lipid A samples containing scyllo inositol standard (1nM) were resuspended in methanol, dried under vacuum, and then subjected to methanolysis in 0.5 M methanolic HCl (50 μl, Sigma) at 80 °C for 16 h. The samples were *N*-acetylated with pyridine (10 μl) and acetic anhydride (10 μl) for 15 min at 25 °C, dried under vacuum, and derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide + 1% trichloromethylsilane (50 μl; Pierce) for 1 h at 25 °C. Samples were injected directly onto the GC-MS and analyzed as previously described (27).

To investigate modification of lipid A with amino-arabinose, dried lipid A samples were resuspended in either water (200 μl) or 10 mM HCl (200 μl) and incubated at 100 °C for 10 min. The mixture was subjected to biphasic 1-butyl alcohol:water (1:1 v/v) partitioning. The lipid A species recovered in the butanol fraction were dried under vacuum, subjected to methanolysis, and GC-MS analysis as described above.

In Vivo Growth—B5055 and B5055Δ*lpxM* were grown to mid-log and washed twice with phosphate-buffered saline and diluted to ~10^4 cfu/dose. BALB/c mice (6–8 week old) were inoculated intravenously with 200 μl, or intranasally with 50 μl of bacterial suspension. Five mice for each bacterial strain tested were euthanased at 4, 24, and 72 h time points, and lungs, livers, and spleens were aseptically removed. Organs were homogenized in sterile phosphate-buffered saline and plated on LA with appropriate antibiotics at several dilutions to obtain a viable count.

Complement-mediated Lysis Assay—Complement-mediated lysis assay was performed as described previously (6). Briefly, 400 μl of human sera (Sigma-Aldrich) was added to ~10^6 cfu/100 μl of bacteria (mid-log phase) and incubated at 37 °C, with rotation. Samples were taken every 30 min and plated for counts. Heat-inactivated sera (56 °C, 30 min) was used as a negative control for all strains.

Whole Blood Phagocytosis Assay—300 μl of fresh (used within 30 min of removal) whole mouse or human blood was mixed with ~10^7 cfu/100 μl of B5055, B5055nm, B5055Δ*lpxM*, and B5055nmΔ*lpxM* (grown to mid-log phase) and incubated for 3 h with rotation at 37 °C. Bacterial dilutions were plated to obtain viable counts. The bacterial counts recovered were divided by the initial counts to give a survival index, with <1 indicating a susceptible organism (*i.e.* bacteria killed) and >1 indicating a resistant organism (*i.e.* bacteria proliferated).

Outer Membrane Permeability Assay—All strains were transformed with pGEM T-EASY and pBR322 (except complemented strains) to enable β-lactamase production. β-lactamase leakage from the periplasm into the culture supernatant was measured using a nitrocephin (Oxoid) spectrophotometric assay exactly as described previously (28, 29).

Sensitivity to Antimicrobial Peptides and Antibiotics—MICs for B5055, B5055Δ*lpxM*, and the complemented mutant (B5055Δ*lpxM* + pGEM-*lpxM*) were determined for a range of antimicrobial peptides and antibiotics as described previously (30). Briefly, 96-well microtiter plates containing 0.125–64 μg/ml peptides or antibiotics were prepared by 2-fold dilution in LB and wells inoculated with ~5×10^5 cfu/ml of mid-log bacterial cultures. Growth was scored after 18–24 h. Peptides were synthesized by standard f-moc chemistry at the Nucleic Acids and Protein Synthesis Unit, University of British Columbia and reconstituted in deionized water at 2 mg/ml.

C18G Sensitivity Assay—Mid-log bacterial cultures were diluted to ~5×10^4 cfu/ml, and 50 μl of bacteria added to 250 μl of normal human sera plus C18G (synthesized by Jackson Laboratory, University of Melbourne from published sequence (31)) at 0, 50, 100, or 200 μg. Samples were rotated at 37 °C for 3 h, and aliquots diluted to obtain viable counts.

RESULTS

Construction and Analysis of a K. pneumoniae lpxM Mutant

The *lpxM* gene was identified in the sequenced *K. pneumoniae* MGH75878 genome by homology to the *lpxM* genes from *E. coli* (AAC74925) and *S. enterica var Typhimurium* (AAL20805). 76% nucleotide homology exists between *E. coli* and *K. pneumonaie lpxM* genes with a probability score of $4.4e^{-119}$. ClustalW protein analysis is shown in Fig. 1*A*. A second region of homology (55% nucleotide homology, $p = 2.6e^{-8}$) was found to have higher homology to *lpxL* (71%, $p = 1.2e^{-93}$) and is therefore believed to be one of two *lpxL* homologs present in MGH75878. Primers were designed from the MGH75878 sequence to amplify the *lpxM* gene from *K. pneumoniae* B5055 (K2:O1). The gene was disrupted by removal of an internal 142 bp and addition of a *cat* (chloramphenicol acetyl transferase) gene and then re-introduced into B5055 and B5055nm (a non-encapsulated mutant of B5055 containing a deletion in *wza-wzc*). Mutants with the disrupted gene (B5055Δ*lpxM* or B5055nmΔ*lpxM*) incorporated were selected on chloramphenicol plates and resultant colonies confirmed by PCR (Fig. 1*B*) and Southern blotting.

Wild type and B5055Δ*lpxM* cells synthesized equivalent levels of LPS, as shown by SDS-PAGE (Fig. 1*C*) and monosaccharide analysis of total cell extracts (data not shown). Analysis of

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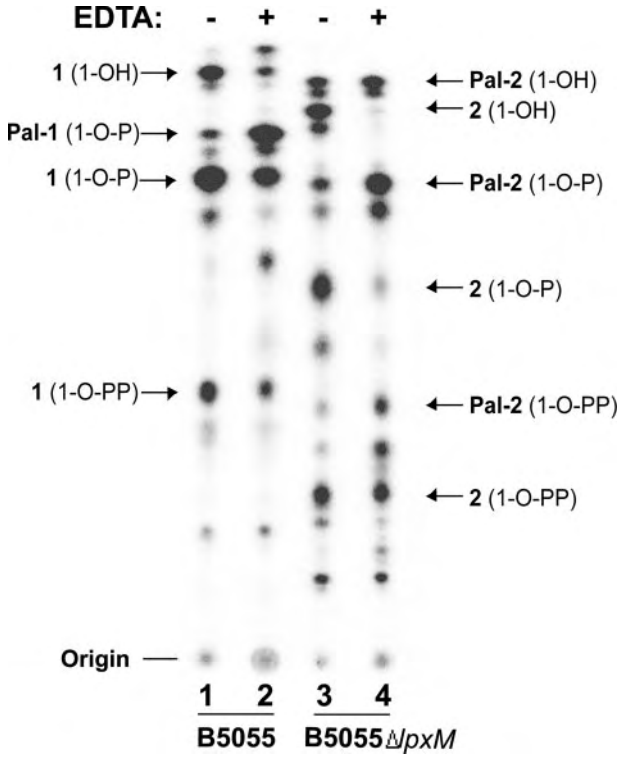

FIGURE 2. **Thin layer chromatography of ^{32}P-labeled lipid A species extracted from *K. pneumoniae* wild type and *lpxM* mutant grown in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of EDTA.** Species **1** is hexaacyl lipid A and **2** is pentaacyl lipid A; palmitoylated variations are prefixed with *pal* (*i.e.* **Pal-1** is palmitoylated hexaacyl lipid A and **Pal-2** is palmitoylated pentaacyl lipid A. 1-OH, 1-O-P, and 1-O-PP contain 0, 1, or 2 phosphate groups at the reducing termini of the lipid A chitobiose core (see Fig. 3 for structure).

uronic acids levels in the wild type and the B5055Δ*lpxM* mutant indicated that both strains synthesized a cell-associated capsule. Total capsule uronic acid in the mutant was approximately half that of the wild type (12.61 ± 0.43 ng/10^6 cfu *versus* 21.13 ± 4.6 ng/10^6 cfu), but much higher than in the non-encapsulated B5055nm mutant ($p < 0.0001$). The cell-associated capsule of B5055Δ*lpxM* appeared ultrastructurally similar to the capsule of wild type cells when stained with Maneval's stain (Fig. 1*D*) and appeared to be functionally intact as shown by the resistance of the *lpxM* mutant to innate immune mechanisms for which the capsule is required (Figs. 5 and 6).

Deletion of the *lpxM* gene is expected to result in altered acylation of the lipid A moiety of LPS. This was confirmed by TLC analysis of the ^{32}P-labeled lipid A moieties, GC-MS and MALDI-MS. (Fig. 3) and thin layer chromatography (TLC) of ^{32}P-labeled lipid A (Fig. 2). The TLC analysis showed that the lipid A of wild type LPS was primarily hexaacylated (see species labeled 1(1-OH), 1(1-O-P), and 1(1-O-PP)), while the majority of the *lpxM* mutant was predominantly pentaacylated (species labeled 2(1-OH), 2(1-O-P), and 2(1-O-PP) (Fig. 2). The lipid A moieties from both wild type and mutant cells were similarly phosphorylated at the reducing termini of the chitobiose backbone. A significant pool of lipid A in the mutant contained molecular species that corresponded to palmitoylated pentaacyl-lipid A (Pal-2 (1-OH). The abundance of this species and several other species with the expected migration position of palmitoylated pentaacyl-lipid A increased dramatically when cultures were treated with EDTA prior to harvesting, conditions known to induce palmitoylation of the lipid A by *pagP* (25). A *pagP* homolog has not been published for *K. pneumoniae* however BLAST analysis revealed a region of homology to *pagP* from *E. coli* K12 (GI: 946360) in MGH78578. ClustalW analysis shows the translated genes to have 72% amino acid homology, with the C-terminal half showing higher amino acid homology (86%) than the N-terminal half (58%). The absence of heptaacylated lipid A in the EDTA-treated *lpxM* mutant, indicated that very little, if any of the lipid A in this mutant is hexacylated.

To confirm the TLC-based assignments of lipid A structure, the lipid A moieties of wild type and *lpxM* mutant LPS was analyzed by MALDI-TOF (Fig. 3). The negative ion MALDI-TOF mass spectrum of wild type lipid A contained a series of prominent molecular ions that indicated hexaacylated, bisphosphoylated structure with a variable 2′ secondary acyl chain. The secondary acyl chain comprised (in order of abundance) myristate (*m*/*z* 1822), laurate (*m*/*z* 1794), or hydroxymyristate (*m*/*z* 1838), consistent with previous reports for *K. pneumoniae* lipid A (13, 32). Minor lipid A species containing an additional palmitate (*m*/*z* 2062) or aminoarabinose (*m*/*z* 1952) were also detected. In contrast, the lipid A from the *lpxM* mutant was separated by MALDI-MS into two major clusters; pentaacylated, bisphosphorylated lipid A (with a variable 2′ secondary acyl substitution) or hexacylated bisphosphorylated lipid A (with a variable 2′ secondary acyl substitution and a secondary palmitate addition). These analyses are consistent with the TLC analyses, in indicating that the lipid A moiety of the *lpxM* mutant is under-acylated compared with the wild type lipid A, due to loss of the conserved myristate group. They also reveal that loss of myristate is partially compensated for by increased palmitoylation of the lipid A. The deletion of the *lpxM* gene had no apparent effects on other modification, such as substitution with aminoarabinose, as shown by GC-MS analysis of the lipid A fractions (33–35) (data not shown).

In Vivo Growth of lpxM Mutant

The *in vivo* growth of the *lpxM* mutant was initially assessed by bacterial counts in organs (Fig. 4*A*) of BALB/c mice after intravenous inoculation. The spleen counts in animals infected intravenously with the parent bacterium, B5055, were indicative of a fulminant bacteremia with mice reaching counts of 10^9 cfu/spleen before being euthanased on day 3, however no significant difference was observed between wild type and mutant counts in the spleen at any time point (data not shown). The only significant difference in counts was observed in the lungs 24 h after inoculation when the mutant showed decreased proliferation compared with wild type *K. pneumoniae* (Fig. 4*A*). When inoculated intranasally, a 30% reduction in lethality was observed for the *lpxM* mutant after 7 days of infection (Fig. 4*B*). To determine whether this was due to a reduction in bacterial load, mice were inoculated intranasally with B5055, B5055Δ*lpxM* and the complemented mutant and organs harvested at three time points. Statistically significant differences in counts were observed at all timepoints in the lungs (Fig. 4*C*), (all $p \leq 0.01$) as well as at the final time point in the spleen (Fig. 4*D*). Both infection routes suggest that an early innate immune mechanism in the lungs is able to limit growth of an *lpxM*

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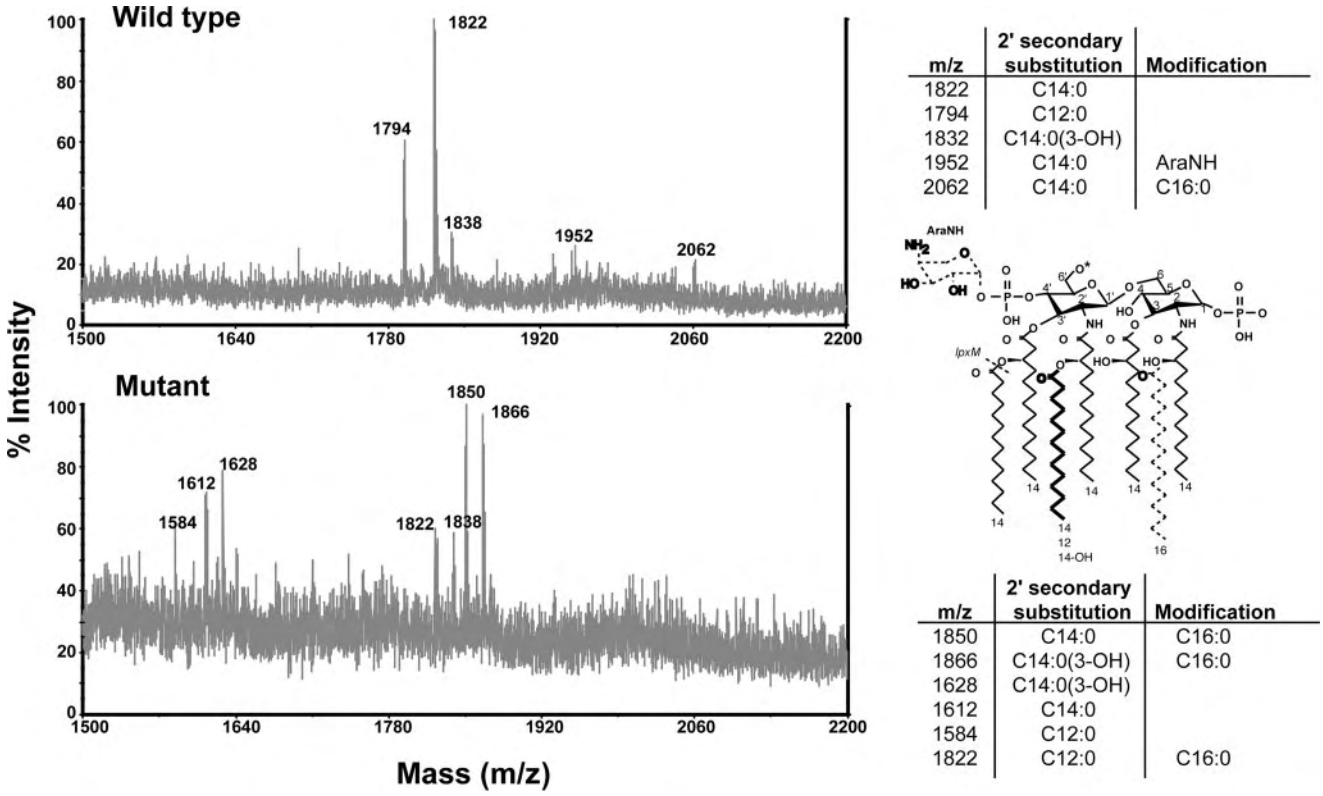

m/z	2' secondary substitution	Modification
1822	C14:0	
1794	C12:0	
1832	C14:0(3-OH)	
1952	C14:0	AraNH
2062	C14:0	C16:0

m/z	2' secondary substitution	Modification
1850	C14:0	C16:0
1866	C14:0(3-OH)	C16:0
1628	C14:0(3-OH)	
1612	C14:0	
1584	C12:0	
1822	C12:0	C16:0

FIGURE 3. **Negative ion MALDI-TOF mass spectrum of lipid A from *K. pneumoniae* wild type and *lpxM* acyl chain mutant.** The major molecular ions detected are consistent with variable 2′ secondary chain modifications (bold aliphatic chain), substitution of the 3′ myristate with palmitoyl group or modification of chitobiose core with aminoarabinose-phosphate (*AraNH*) (modifications by *dashed lines*).

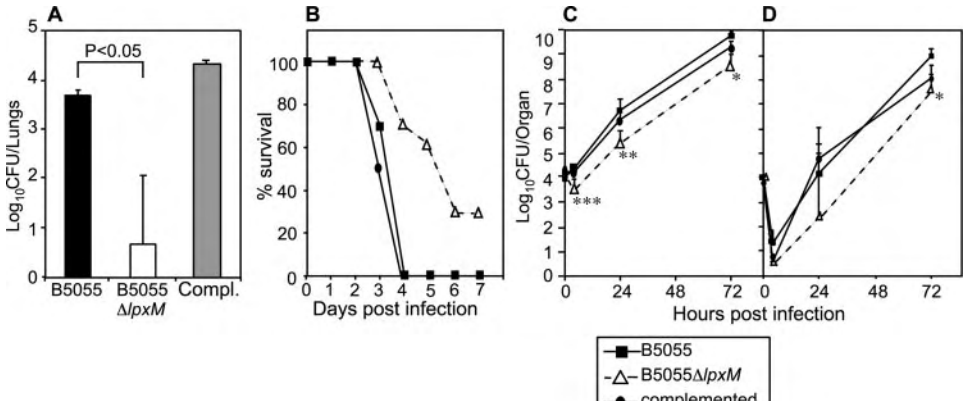

FIGURE 4. **Comparison of *in vivo* growth and lethality of *K. pneumoniae* strains.** *A*, bacterial counts in lungs from mice infected intravenously with ~ 1–2 × 10^4 cfu wild type *K. pneumoniae* ($n = 15$), the *lpxM* mutant ($n = 15$), or the complemented mutant ($n = 5$), 24 h after inoculation. *B*, survival curve of mice infected intranasally with ~1–2 × 10^4 cfu of wild type *K. pneumoniae*, the *lpxM* mutant or the complemented mutant ($n = 10$). *C* and *D*, bacterial counts in lungs (*C*) and spleen (*D*) from mice infected intranasally with ~1–2 × 10^4 cfu and organs harvested at 4, 24, and 72 h ($n = 10$ for all strains). *, $p < 0.01$; **, $p < 0.001$; and ***, $p < 0.0001$ calculated by the Student's *t* test.

mutant but not wild type bacteria. In the intranasal infection model, reduction of the bacterial load in the spleen could be due either to killing in the spleen or a secondary effect of the reduced bacterial load in the lungs.

Phagocytosis of K. pneumoniae B5055 and B5055ΔlpxM in Vitro

Two assays were performed to determine whether the reduced *in vivo* growth rate of B5055Δ*lpxM* was caused by increased killing of the bacterium by the innate immune system. The complement-mediated lysis assay showed the B5055Δ*lpxM* had equivalent resistance to human complement compared with B5055 (Fig. 5). Both B5055 and B5055Δ*lpxM* counts increased over the 3-h time course indicating that these strains were resistant to the effect of human complement. By contrast, B5055nm decreased by ~100-fold over the same time period showing that the presence of capsule is necessary to resist complement-mediated lysis. B5055 nmΔ*lpxM* also decreased over time with an equivalent kinetics to B5055nm supporting the previous results that capsule, but not a fully acylated lipid A, is necessary for resistance to human complement. Bacterial growth in heat-inactivated sera was also analyzed for each strain and all strains increased in number over the three hours, ruling out differences in growth in sera as a possible reason for differences in sensitivity.

Phagocyte-mediated killing of B5055Δ*lpxM* was analyzed using the whole blood phagocytosis assay originally used to detect resistance conferred by M protein to phagocytosis of Group A Streptococci (36). This assay analyses the survival of bacteria in whole blood, measured by viable count, and was

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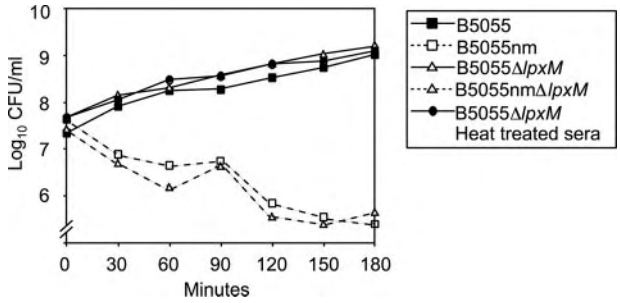

FIGURE 5. **Complement-mediated lysis of *K. pneumoniae* mutant strains.** 400 μl of human sera (Sigma-Aldrich) was added to ~10^6 cfu/100 μl of bacteria and incubated at 37 °C, shaking. Samples were taken every 30 min and plated for counts. Heat-inactivated sera was used as a negative control for all strains to confirm growth in sera. Presented are results of a single experiment, representative of duplicate experiments.

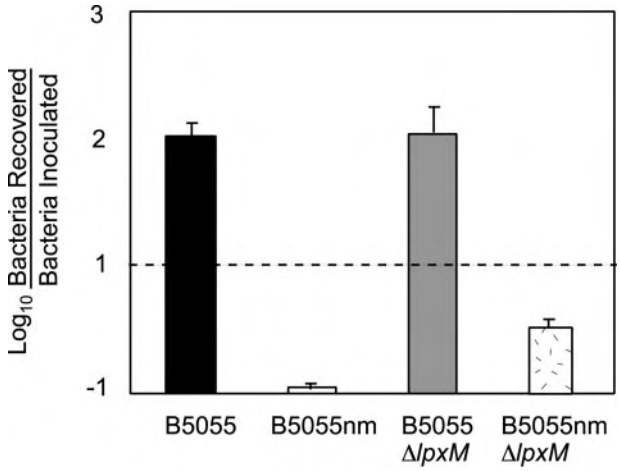

FIGURE 6. **Whole blood phagocytosis of *K. pneumoniae* mutant strains.** 300 μl of fresh whole (murine) blood was mixed with 10^7 cfu/100 μl of the labeled bacterial strains and incubated for 3 h with rotation, 37 °C. The bacterial counts recovered were divided by the initial counts to give a survival index, with <1 indicating a susceptible organism (*i.e.* bacteria killed) and >1 indicating a resistant organism (*i.e.* bacteria proliferated). Presented are means and standard errors of duplicate samples, representative of duplicate experiments.

conducted using murine whole blood (Fig. 6). The assay showed that in murine blood, growth of both B5055 and B5055Δ*lpxM* increased 10-fold over the course of the experiment, while the number of B5055nm and B5055nmΔ*lpxM* decreased 10-fold, again showing that the capsule, rather than fully acylated lipid A, is necessary for *K. pneumoniae* to resist phagocytic killing by the murine innate immune system.

Outer Membrane Integrity of lpxM Mutant

Leakage of the Periplasmic Protein β-Lactamase—Many *K. pneumomiae* strains have naturally acquired β-lactamase genes, encoded either chromosomally or on plasmids, however B5055 remains sensitive to β-lactam antibiotics. In Gram-negative bacteria TEM β-lactamase is a periplasmic enzyme (37) and leakage of this enzyme into the culture supernatant can therefore be used as a measure of the integrity of the outer membrane. All strains were transformed with plasmids containing β-lactamase to allow release of the enzyme into the culture medium to be measured. Analysis of wild type *K. pneumoniae* showed ~3% leakage of β-lactamase into the culture medium (Table 2) while the acyl chain mutant showed an ~10-fold increased leakage of β-lactamase into the culture supernatant, indicating increased permeability of the outer membrane in this mutant. The permeability was reduced to near wild type levels (~3% leakage) in the complemented mutant.

TABLE 2

Outer membrane permeability of *K. pneumoniae* strains, indicated by leakage of the periplasmic protein TEM β-lactamase into the culture supernatant

Strain	% β-Lactamase activity in	
	Culture supernatant[a]	Cell lysate[a]
B5055	2.98	97.02
B5055Δ*lpxM*	29.1	70.9
B5055Δ*lpxM* pBR-*lpxM*	3.12	96.88

[a] Results are averages of triplicate samples.

TABLE 3

MICs of a number of antibiotics and antimicrobial peptides against wild type *K. pneumoniae* (B5055), a lipid A mutant (B5055Δ*lpxM*), and the complemented mutant (B5055Δ*lpxM* complemented)

The modal MIC of at least four experiments is given.

	Strain			Fold reduction
	B5055	B5055Δ*lpxM*	B5055Δ*lpxM* complemented	
Poly-L-lysine	>320	>320	>320	0
Indolicidin	>32	>32	>32	0
CP10A	>32	>32	>32	0
CP11CN	>32	>32	>32	0
Gramicidin S	8–16	16	8–16	0
Polymyxin B[a]	4–8	0.5	4	8–16
Colistin[a]	16–32	2	16–32	8–16
LL-37	>32	>32	>32	0
CP28 (CEMA)[a,b]	>32	4	32	8
CP26[c]	16	32	32	0
BacCN	>32	>32	>32	0
Gentamicin	2–4	2	2	0

[a] Substances demonstrated increased inhibitory ability against the lipid A mutant, which was restored to wild type levels by complementation.
[b] CP28 – KWKLFKKIGIGAVLKVLTTGLPALKLTK.
[c] CP26 – KWKSFIKKLTSAAKKVVTTAKPLISS.

MICs of Antimicrobial Peptides and Antibiotics—Cationic antimicrobial peptides are a major component of innate immunity as they are released at mucosal and skin surfaces as part of the phagocytic vacuole microbicidal mechanism (38), and kill bacteria by permeabilization of lipid bilayers. The *lpxM* mutant showed increased sensitivity to three of the panel of peptides tested: polymyxin B, colistin, or polymyxin E, and CP28, (Table 3); as well as C18G (Fig. 7), which requires the presence of serum for optimal bactericidal effect (31). This demonstrates that the permeability of the outer membrane shown by β-lactamase release is of physiological importance as it imparts increased susceptibility to a select number of antimicrobial peptides.

DISCUSSION

The viability *of K. pneumoniae lpxM* mutants and their ability to express known virulence determinants (capsule and LPS) at wild type levels allows a unique opportunity to study the role of lipid A in murine models of bacteremia and pneumonia. Although *K. pneumoniae* virulence is heavily dependent on the polysaccharide capsule (8, 39), the attenuation of *in vivo* growth of the acyl chain mutant indicated a role for lipid A in *K. pneumoniae* infection. The *lpxM* mutant showed an increased permeability of the outer membrane, which did not increase sensitivity of the bacterium to complement-mediated lysis or phagocytosis. Given that capsule and LPS O-polysaccharide

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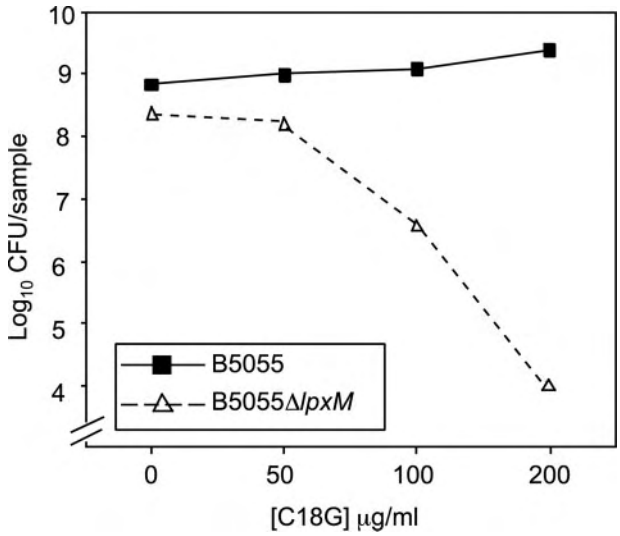

FIGURE 7. **Increased sensitivity of *K. pneumoniae lpxM* mutant to the cationic peptide C18G.** Increasing concentrations of the α-helical cationic peptide C18G were added to ~5 × 10^4 cfu/ml of B5055 or B5055Δ*lpxM* and rotated at 37 °C for 3 h after which viable counts were determined.

have previously been shown to protect against complement-mediated killing (7, 40), and the *lpxM* mutant retains wild type levels of these virulence determinants, it is not surprising that innate resistance to complement and phagocytosis is retained. The sensitivity of the acyl chain mutant to a small selection of α-helical cationic antimicrobial peptides indicates that the capsule and LPS are unable to prevent these peptides from interacting with the outer membrane, as they appear to be able to do for complement factors. From this evidence it is hypothesized that increased sensitivity to cationic antimicrobials may be one mechanism through which growth of the *K. pneumoniae lpxM* mutant is attenuated in murine bacteremia and pneumonia models.

Lipid A has been shown to be an important virulence determinant of many Gram-negative pathogens and modifications in its structure have been specifically demonstrated in response to host defense mechanisms for a number of bacteria (*Salmonella enterica var* Typhimurium (41) *Brucella abortus* (42), *Pseudomonas aeruginosa* (43)). In *Salmonella* two two-component regulatory systems, PhoP/PhoQ and PmrA/PmrB, induce modifications to the lipid A backbone (aminoarabinose or phosphoethanolamine) as well as acyl chain substitutions (secondary chain myristate to 2-OH myristate) and additions (*pagP*-mediated palmitate addition at the 3′ position) (41). Similarly, the *B. abortus* BvrS/BvrR system appears to alter acylation patterns as well as controlling outer membrane protein expression (42, 44). Mutants in these systems were found to increase the bacterium susceptibility to antimicrobial peptides, indicating a pivotal role for lipid A in antimicrobial peptide resistance. However, as these mutants had multiple outer membrane/lipid alterations, the antimicrobial peptide resistance could not be directly attributed to acyl chain modifications. Most of these changes could be attributed to charge alterations by changes to aminoarabinose and phosphoethanolamine additions. This was addressed by studying the salmonella *pagP* mutant (45) which prevented the addition of a seventh acyl chain, palmitoyl, to the lipid A moiety. This hexaacyl-restricted mutant had increased sensitivity to antimicrobial peptides, thus directly linking antimicrobial peptide resistance to acyl chain numbers.

Interestingly, the Salmonella *pagP* mutant showed increased sensitivity to antimicrobial peptides from a number of different classes (α-helical and β-sheet structures). In the current study, the *K. pneumoniae lpxM* mutant was more sensitive to a restricted repertoire of peptides. For example, CP28 showed increased anti-bacterial activity while its derivative CP26 (46) showed no alteration in activity. The effective peptides were α-helical amphipathic peptides that are proposed to act subsequent to self promoted uptake, interacting with the LPS divalent cation binding sites on the outer membrane surface and competitively displacing these cations (47, 48). The exact mechanisms of lethality of these peptides are still disputed, whether through destabilization and permeabilization of the cytoplasmic membrane (49), or passage across the membrane to internal cytoplasmic targets (as proposed for the polymyxins although the exact targets have not been identified (50)). The two active antimicrobial peptides; CP28 and C18G, both contain 50% hydrophobic residues (51), which is a distinct property of these peptides among the panel tested. It is therefore suggested that very specific structural configurations and/or amphipathicity are necessary for activity against *lpxM* mutants. Remembering that *pagP* is present and inducible in *K. pneumoniae* in response to outer membrane stress (Fig. 2), it is possible that the restricted repertoire of peptides active against the *lpxM* mutant may be those specifically obstructed by heptaacylated lipid, while those active against the *pagP* mutant, but not the *lpxM* mutant, may be hindered by the presence of palmitate. Other differences between *Salmonella* and *Klebsiella* lipid structures cannot be ruled out in contributing to differential peptide sensitivities.

The *K. pneumoniae lpxM* mutant also showed increased susceptibility to polymyxins B and E (colistin). In *Salmonella* spp polymyxin resistance has been shown to be dependent on charge-altering modifications; aminoarabinose and ethanolamine additions to the lipid, and phosphate and ethanolamine additions to the core (52, 53). The *K. pneumoniae* core is not phosphorylated but constitutively contains galacturonic acid residues to maintain a negatively charged core (54). The absence of inducible modifications to the core may place more importance on the integrity of the lipid A for polymyxin resistance, as there are no modifications to divert the peptide from this lipid. The action of polymyxin relies on a number of processes. Those important for our work are the initial binding to the negative charges of the bacterial membrane (the cyclic peptide head group of polymyxin B has a net positive charge of 4) and secondly insertion of either the fatty acid tail or the head group into the membrane (the exact mechanism is undetermined (55, 56)). As the *lpxM K. pneumoniae* mutant does not appear to have any charge alterations it is proposed that the method of increased susceptibility of the *lpxM* mutant is not due to increased binding, but rather increased insertion of either the head group or fatty acid tail into the membrane due to the reduced acyl chain numbers.

K. pneumoniae resistance to polymyxin B (and a number of antimicrobial peptides) was previously shown to be mediated by capsule (57). In the current study capsule did not appear to play a role in resistance to antimicrobial peptides as B5055nm, a mutant with ~1/10^{th} wild type capsule levels, showed no

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increase in sensitivity to polymyxin B (data not shown). Campos *et al.* did show that in the absence of capsule, O-polysaccharide was able to confer some resistance to polymyxin B which could explain the resistance of B5055nm (which has wild type O-polysaccharide levels) to polymyxins. However, a number of other studies have also shown that capsule does not contribute to resistance to antimicrobial peptides (58, 59) which is in agreement with the current observations, and these fundamental discrepancies need further investigation.

The role of the ubiquitous LPS molecule in the pathogenesis of Gram-negative bacteria remains somewhat enigmatic. LPS O-Ag is a major and effective target for the acquired immune system yet the lipid A region seems to provide protection against a widely conserved innate response as well as resistance to detergents and bile (60). The comparative role of each LPS region may vary greatly depending on the organism and infection route studied, making a global identification of its importance problematic.

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