MAJOR ARTICLE

Klebsiella pneumoniae Peptidoglycan-Associated Lipoprotein and Murein Lipoprotein Contribute to Serum Resistance, Antiphagocytosis, and Proinflammatory Cytokine Stimulation

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Background. Peptidoglycan-associated lipoprotein (Pal), murein lipoprotein (LppA), and outer membrane protein A (OmpA) are dominant outer membrane proteins (OMPs) that are released by gram-negative bacteria during sepsis. OMPs are implicated in the maintenance of cell envelope integrity. Here, we characterize the roles of these OMPs in pathogenesis during bacteremia caused by *Klebsiella pneumoniae*.

Methods. *pal*-, *lppA*-, and *ompA*-deficient *K. pneumoniae* strains were constructed using an unmarked deletion method. Serum sensitivity, antiphagocytosis activity, outer membrane permeability, and sensitivity to anionic detergents and antimicrobial polypeptides were determined for these OMP gene deletion mutants. The ability of these OMP gene deletion mutants to induce immune responses was compared with that of the wild-type strain in a bacteremic mouse model.

Results. *Klebsiella pneumoniae* strains deleted for *pal* or *lppA* exhibited reduced protection from serum killing and phagocytosis; perturbation to the outer membrane permeability barrier and hypersensitivity to bile salts and sodium dodecyl sulfate. The strain mutated for *lppA* had reduced ability to activate Toll-like receptor 4. Immunization of mice with the *pal* or *lppA* mutant provided protection against infection by the wild-type strain.

Conclusions. Our findings indicate that *K. pneumoniae* Pal and LppA proteins are important in the maintenance of cell integrity, contribute to virulence, and could be used as attenuated vaccines.

Keywords. *Klebsiella pneumoniae*; outer membrane proteins; peptidoglycan-associated lipoprotein; murein lipoprotein; outer membrane protein A.

Klebsiella pneumoniae is an important pathogen in hospital-acquired and community-acquired infections [1–4]. Both the capsular polysaccharide (CPS) and the O-antigen portion of the lipopolysaccharide (LPS) components are important pathogenic determinants in *K. pneumoniae*–caused pneumonia, bacteremia, and community-acquired pyogenic liver abscess (PLA) [5–9]. The maintenance of cell envelope integrity in gram-negative bacteria requires a structural link between the murein layers and selected outer membrane proteins (OMPs). The murein lipoprotein (Lpp) is one of the most abundant OMPs. OMPs harboring a peptidoglycan-binding sequence, such as outer membrane protein A (OmpA) or peptidoglycan-associated lipoprotein (Pal), appear to interact noncovalently with the murein layer. Pal, LppA, and OmpA are structural OMPs that are highly conserved among enteric gram-negative bacteria and are released during sepsis [10, 11]. The role in sepsis of all 3 proteins in a single gram-negative bacterium has not been studied extensively. Thus, the aim of this study was to characterize the roles in pathogenesis of these 3 OMPs (Pal, LppA, and OmpA) during bacteremia caused by *K. pneumoniae*. We demonstrate here that Pal and LppA are important in *K. pneumoniae* and confer protection against serum killing and

Received 10 January 2013; accepted 5 June 2013; electronically published 2 August 2013.

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The Journal of Infectious Diseases 2013;208:1580–9

DOI: 10.1093/infdis/jit384

phagocytosis. Moreover, Pal and LppA lipoproteins, but not OmpA protein, play critical roles in maintaining cell envelope integrity and are crucial for *K. pneumoniae* virulence in vivo.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were approved under application number 20060139 of the institutional animal care and use committee of the National Taiwan University College of Medicine (NTUCM). Procedures were consistent with the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and of Taiwan's Animal Protection Act. These studies used BALB/cByl, C57BL/6JNarl wild type (WT), and C57BL/6JNarl isogenic Toll-like receptor (TLR) 2 knockout (KO) or TLR4 KO mice that were bred and housed in specific pathogen–free rooms within the animal care facilities of the NTUCM and the Laboratory Animal Center at the National Laboratory Animal Center.

Bacterial Strains and Culture Conditions

Klebsiella pneumoniae and *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics, including 100 μg/mL ampicillin or 50 μg/mL kanamycin. Bacterial strains, plasmids, and primers used in this study are listed in Table 1.

Gene Deletion and Complementation

Klebsiella pneumoniae mutated in the *pal, lppA*, or *ompA* genes (coding for the Pal, LppA, and OmpA proteins, respectively) were constructed using the previously described unmarked deletion method [12]. The primer pairs for the deletion constructs are listed in Table 1. For complementation, the intact *pal*, *lppA*, and *ompA* genes were amplified by polymerase chain reaction (PCR) and cloned into the intergenic region between 2 open reading frames (ORFs), *pgpA* and *yajO*, using a pKO3-Km-*pgpA-yajO* recombinant vector [13]. All of the deletion mutants and complementation strains were confirmed by PCR.

Bacterial Growth Assays

An 18-hour culture of each strain was used to inoculate each 5-mL tube at a ratio of 1:100. Each culture was grown at 37°C for 4 hours in LB broth. After incubation, growth was monitored spectrophotometrically at 600 nm every hour.

Characterization of LPS

The exopolysaccharide (EPS) extracts (containing both CPS and LPS) were purified by a modified hot water–phenol extraction method as described previously [9, 14]. Samples were separated by 12%-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then silver-stained [15].

Immunoblots

The extracts of EPS from each strain were separated by 12% SDS-PAGE and blotted to a Hybond C nitrocellulose membrane (Amersham). The K1 antisera were purchased from Statens Serum Institut (Denmark) and diluted 1/10 000 for CPS K1 analysis. The O1 antisera were obtained from a rabbit immunized with a *magA*-mutant *K. pneumoniae*; this serum was diluted 1/100 000 for LPS O1 analysis. The EPS extracts of *K. pneumoniae* NTUH-K2044 were blotted to a Hybond C nitrocellulose membrane (Amersham) by using a vacuum-driven slot blot filtration manifold (Hoefer). Sera from both immunized and nonimmunized control mice were diluted 1/100 for anti-EPS immunoglobulin G (IgG) analysis.

Extraction and Quantification of CPS

The amount of K1 CPS from *K. pneumoniae* NTUH-K2044 and the OMP gene mutant strains was determined by assaying uronic acid content as described by Domenico et al [16].

Serum Killing Assays

The survival of exponential-phase bacteria in nonimmune human serum was measured as previously described [9]. In brief, a log-phase inoculum of 2.5×10^4 colony-forming units (CFU) was mixed at a 1:3 vol/vol ratio with mixed nonimmune human serum donated by 5 healthy volunteers. The final mixture, comprising 75% nonimmune serum by volume, was incubated at 37°C for 3 hours. The colony count was determined by plating of serial dilutions on LB agar, and the mean survival ratio was plotted. A mean survival ratio ≥1 corresponds to serum resistance.

Dictyostelium Phagocytosis by Plaque Assays

Dictyostelium discoideum AX-2 cells were grown as previously described [17]. An aliquot of 5000 *Dictyostelium* cells was added to the bacterial lawn, and the formation of phagocytic plaques was observed after 5 days.

Phagocytosis and Killing Assays by Human Neutrophils

Human neutrophils were freshly isolated from peripheral blood donated by healthy volunteers [8]. For the phagocytosis assay, plasmid pCRII-TOPO with a gene encoding green fluorescent protein (GFP) was electroporated into the WT, mutants, and their respective complementation strains. An inoculum containing 10^8 CFU of bacteria was opsonized with 25% normal human serum for 15 minutes on ice and incubated with 10^6 human neutrophils at 37°C for 45 minutes. Cells were washed, fixed, and stained with rhodamine-phalloidin as previously described [17]. After preparation, the cells were observed by confocal microscopy under ×630 image magnification, and the numbers of intracellular bacteria in 5 fields were counted (20–40 cells in each field). The sum of the intracellular bacteria was divided by the total number of cells in these fields and to calculate the number of intracellular bacteria in 100 cells. For killing

Table 1. Bacterial Strains, Plasmids, and Primers

Bacterial Strain, Plasmid, or Primer	Genotype, Sequence, and/or Relevant Description		Reference or Source
Bacteria			
Klebsiella pneumoniae strains			
NTUH-K2044 WT	O1:K1, S^r, clinical isolate PLA strain, the parent strain for generate isogenic mutants		[8]
Δ*pal*	NTUH-K2044 isogenic mutant with deletion of *pal* gene		This study
Δ*pal*::*pal*	Δ*pal* with *pal* cassette between *pgpA* and *yajO*		This study
Δ*lppA*	NTUH-K2044 isogenic mutant with deletion of *lppA* gene		This study
Δ*lppA*::*lppA*	Δ*lppA* with *lppA* cassette between *pgpA* and *yajO*		This study
Δ*ompA*	NTUH-K2044 isogenic mutant with deletion of *ompA* gene		This study
Δ*ompA*::*ompA*	Δ*ompA* with *ompA* cassette between *pgpA* and *yajO*		This study
Δ*wbbO*	NTUH-K2044 isogenic mutant with deletion of *wbbO* gene		[9]
Δ*magA*	NTUH-K2044 isogenic mutant with deletion of *magA* gene		[9]
A5054	O1:K1, *Klebsiella* reference strain		Statens Serum Institut
Escherichia coli strains			
DH10B	*F^- mcrA (mrr-hsdRMS-mcrBC) 80* Z M15 *lacX74 endA1 recA1 deoR (ara leu)7697 ara139 galU galK nupG rpsL$^-$*		Invitrogen
Plasmids			
pGEM-T Easy	T-A cloning vector, Ap^R		Promega
pKO3-Km	pKO3 derived plasmid, with an insertion of Km resistance cassette from pUC4 K into AccI site		[12]
pKO3-Km-*pgpA-yajO*	pKO3 derived plasmid, with the *pgpA-yajO* DNA fragments for cis-complementation		[13]
TA-GFP	PCRII-TOPO TA cloning vector carrying with GFP		[8]
Primers			
pal-FR	GGTCACGCAAAATCCGGATG	*pal* mutant construct	This study
pal-RR	GTCAACTGCTCTACCAACTG		
pal-I-R	TTCTTTGATTCCTTTAATAATC		
pal-I-F	GAGAATTGCATGAGCAGTAAC		
tolB-p	ATGGTAGTCACAGGGTTTTGG	*pal* complementation	This study
pal-R	TTAGTAAACCAGTACGGCG		
tolB-p-IR	CCAAATGCTACTCGTAATGC		
pal-rbs	GATTATTAAAGGAATCAAAG		
lppA-FR	GTGGTTATCACCGCCACCCAG	*lppA* mutant construct	This study
lppA-RR	CAACAGTCGGCTGATTGGG		
lppA-FR-out	CTAGATTGAGTTAATCTCCATG		
lppA-RR-out	GAGTTCTGGTAATAAAAATGGCGC		
lppA-p	CGGAATTTACTATTTAATAAGGG	*lppA* complementation	This study
lppA-R	TTACTTACGGTAAGAGTGAGCC		
KP1958-F	TTGCAGATATTCCGGCTCTT	*ompA* mutant construct	This study
KP1958-R	CAGAAAATCGCAAGCGTTG		
KP1958-I-F	GTTTTTTTATCGGTTATAAC		
KP1958-I-R	TTAACAACAAGTCACGCCCCCG		
ompA-p	GTATCATTGAGTGAAATTAGG	*ompA* complementation	This study
ompA-R	TTAAGCCGCCGGCTGAGTTAC		

Abbreviations: Ap, ampicillin; GFP, green fluorescent protein; Km, kanamycin; S^r, resistance; S^s, sensitive.

assays, an inoculum containing 10^3 CFU of bacteria was opsonized with 25% normal human serum for 15 minutes on ice and incubated with or without 10^5 human neutrophils in 1× phosphate-buffered saline at 37°C for 45 minutes. Percentage of survival of WT and mutant strains was calculated on the basis of the viable counts relative to those for no-neutrophil controls.

Confocal Microscopy

Images were captured by a Leica SP5 confocal microscope with a 488-nm argon laser and a 543-nm laser, which emitted the excitation wavelengths of GFP and rhodamine, respectively. A representative confocal section through the middle of the cell was shown for observation of intracellular bacteria.

Outer-Membrane Permeability Assays

Leakage of β-lactamase from the periplasm into the culture supernatant of each test strain was measured. A 1-mL sample of an overnight culture of each strain was centrifuged, and the culture supernatant was kept on ice. The cell pellet was resuspended in 1 mL of cold LB medium and sonicated on ice. The unbroken cells and cell debris were removed by centrifugation. One hundred sixty microliters of supernatant and cell lysate from each strain were assayed in duplicate into 96-well polystyrene microplates. Reactions were started by the addition of 40 μL of 25 μg/mL CENTA (Calbiochem), and the absorbance at 405 nm was measured at 3 hours. A sample of 160 μL of LB growth medium with 40 μL of CENTA was used to measure background absorbance. Each strain was assayed in duplicate in multiple experiments.

Bile Challenge Assays and Sensitivity to Anionic Detergents and Polypeptide Antibiotics.

A crude ox bile extract (Sigma) was used in the bile challenge assays. The subsequent culture and detection of growth were performed as described previously [13]. For SDS sensitivity, serial 2-fold dilutions of SDS (from 3.12 to 0.095 mg/mL) were made in 5 mL of LB medium. An 18-hour culture of each strain was used to inoculate each 5-mL sample at a ratio of 1:100. After incubation for 8 hours at 37°C, a tube was rated positive for growth if the optical density at 600 nm was >0.2. The minimum inhibitory concentrations (MICs) of colistin, polymyxin B, cefotaxime, ciprofloxacin, and gentamicin were determined using an agar dilution method according to the recommendations of the European Society of Clinical Microbiology and Infectious Diseases [18].

Mouse Inoculation Experiments

Virulence was evaluated by mortality in a murine model of septicemia generated by intraperitoneal injection. Groups of 5-week-old female BALB/c mice were infected intraperitoneally with the *K. pneumoniae* NTIH-K2044 (or the isogenic mutants) in 0.1 mL of 0.95% saline (10^2–10^7 CFU; 4 mice for each dose). The exact inoculation dose was confirmed by serial dilution and plating to LB agar. Mice were monitored for 4 weeks; the 50% lethal dose (LD_{50}) was calculated as described by Reed and Muench [19]. To investigate the contribution of Pal or LppA protein in bacteremia and proinflammatory response, 4- to 6-week-old C57BL/6 WT, isogenic TLR2 KO or TLR4 KO mice were intraperitoneally given the same inoculation dose (1×10^3 CFU) of each of the WT strain or the *pal* or *lppA* mutant strain (4 mice for each group). Surviving animals were killed at 20 hours after challenge; organ homogenates (including liver and spleen) were cultured for quantification of CFU. The number of CFU detected in the organs was standardized per 0.1 g wet organ weight. Sera were collected at 20 hours and interleukin 6 (IL-6) levels were measured by enzyme-linked immunosorbent assay (R&D Systems).

Statistical Analyses

Data are presented as mean ± SD. Statistical significance was assessed by a 2-tailed Student *t* test using Prism 5 (GraphPad) software. Survival was analyzed by Kaplan-Meier analysis with a log-rank test. *P* values of <.05 were considered significant.

RESULTS

Serum Sensitivity, Antiphagocytosis, and Antineutrophil Killing Activity of the OMP Deletion Mutants

Multiple in vitro assays were used to characterize strains mutated for *pal*, *lppA*, or *ompA* in comparison to the WT parent. Each of the OMP gene deletion mutants exhibited a mucoid phenotype; each produced CPS and LPS at levels similar to those of the WT strain (Figure 1*A*–*D*). As assayed in LB broth culture, the growth rate of the OMP gene deletion mutants showed no significant difference compared to that of the WT (Figure 1*E*). Some OMPs were reported to contribute significantly to the virulence of *K. pneumoniae*, by conferring protection against the host's innate immunity [20]. To test the sensitivity to the serum's bactericidal effect of these OMP gene deletion strains, serum killing assays were performed. Killing of the *pal* and *lppA* mutants by nonimmunized healthy human serum was more efficient than killing of the WT strain or the *ompA* mutant. The serum-resistant phenotype in the *pal* and *lppA* mutants could be partially restored by complementation with the corresponding gene (Figure 2*A*). To test the opsonophagocytic activity of these mutant strains, a *Dictyostelium* model was used. *Dictyostelium* provides a useful system to study the interaction between phagocytes and bacteria because of its similarity to mammalian macrophages. Our previous study validated the correlation of the phagocytosis by *Dictyostelium* to that by human neutrophils [17]. At doses of 5000 *Dictyostelium* cells, these OMP gene deletion strains were more susceptible to phagocytosis by *Dictyostelium* cells than the WT strain. The phagocytosis-resistant phenotype in the OMP gene deletion strains could be partially restored by complementation with the corresponding gene (Figure 2*B*). Opsonin-driven phagocytosis cannot be evaluated as *Dictyostelium* lacks opsonic receptors for IgG and complement. Therefore, we analyzed the susceptibilities of these mutants to phagocytosis by healthy human neutrophils. The bacterial numbers of the *pal* and *lppA* mutants were increased in the human neutrophils. However, the *ompA* mutant, the WT, and the complemented strains were rarely present inside the human neutrophils (Figure 2*C* and 2*D*). Neutrophil-mediated killing of these OMP gene deletion strains was examined. Compared to the number of WT bacteria, the numbers of *pal* and *lppA* mutants were decreased after incubation with human neutrophils, and the

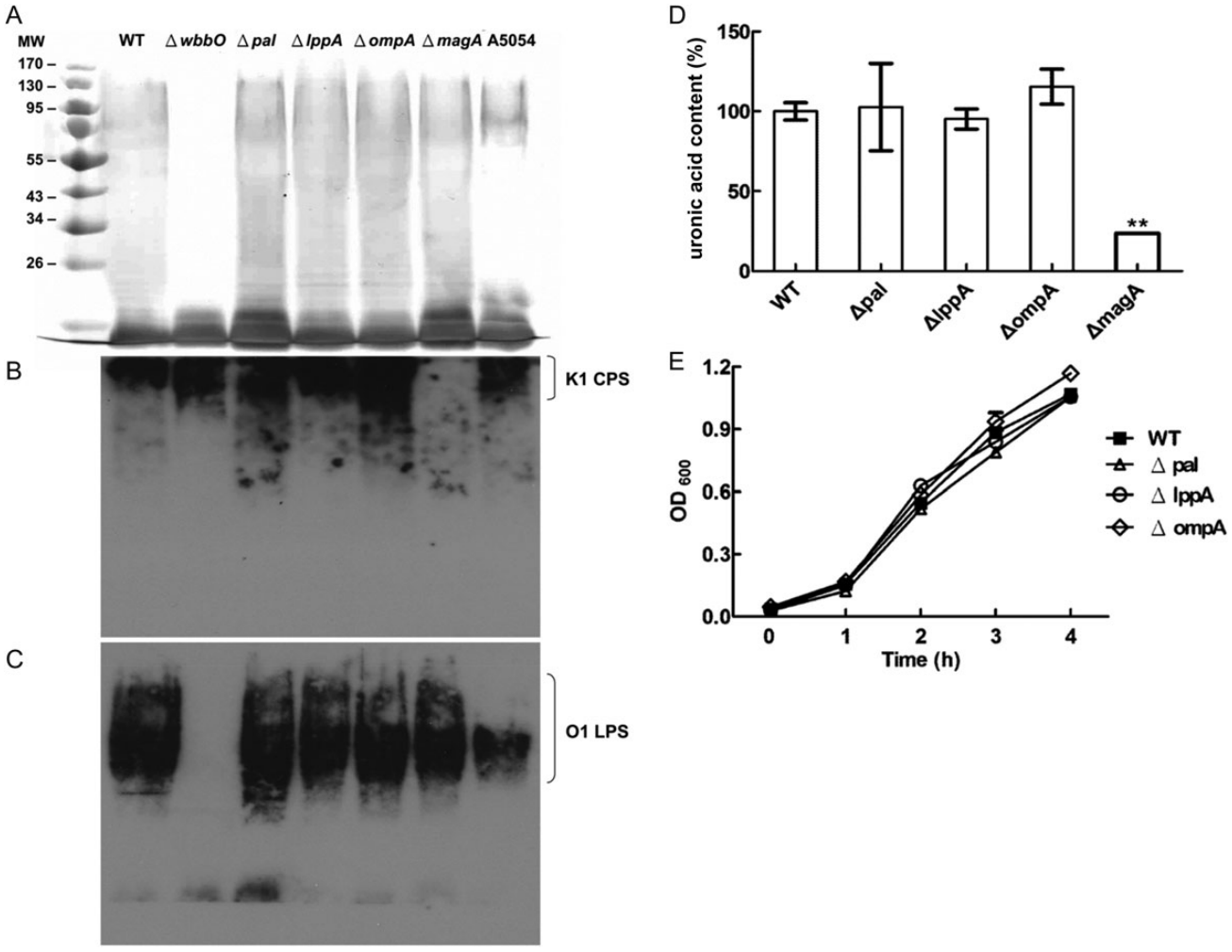

Figure 1. Exopolysaccharide (EPS) phenotype of *Klebsiella pneumoniae* NTUH-K2044 and the OMP gene mutant strains. EPS specimens were prepared from the *K. pneumoniae* NTUH-K2044, its *wbbO* mutant, and the OMP gene mutant strains. Extracts from normalized bacterial suspensions (10^8 colony-forming units) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by silver staining or immunoblotting. A5054 is an O1:K1-serotype strain and was used as a positive control. *A*, Silver staining. *B* and *C*, Immunoblots of the same material developed with rabbit anti-K1 antiserum (Statens Serum Institut; 1:10 000) (*B*); rabbit anti-O1 antiserum; 1:100 000 (*C*). *D*, The amount of K1 CPS from the NTUH-K2044 and the OMP gene mutant strains was determined by assaying uronic acid content. *E*, Growth rates in Luria-Bertani medium of the wild-type and the OMP gene mutant strains. The data represent the means of 3 independent trials; the error bars represent the standard deviations. $^{**}P < .01$ by Student *t* test (compared to the wild-type strain); other comparisons were not statistically significant ($P \geq .05$). Abbreviations: CPS, capsular polysaccharide; LPS, lipopolysaccharide; MW, molecular weight; OD_{600}, optical density at 600 nm; WT, wild-type.

complemented strains restored the resistance to neutrophil killing (Figure 2*E*). Thus, Pal and LppA proteins in *K. pneumoniae* confer protection not only against serum killing, but also against neutrophil-mediated phagocytosis and killing.

Outer Membrane Permeability and Anionic Detergent Sensitivity of the OMP Gene Deletion Mutants

Release of β-Lactamase in the Supernatant by the pal and lppA Deletion Mutants

β-Lactamase is a chromosomally encoded periplasmic enzyme of the NTUH-K2044 *K. pneumoniae* strain. Comparison of the intracellular β-lactamase activity to the extracellular (released from the periplasm to the supernatant) level was assessed to determine whether OMP gene mutants display increased leaking from the periplasmic space. Specifically, β-lactamase activity was determined using the chromogenic cephalosporin nitrocefin. Supernatant-located β-lactamase activity was significantly higher for the *pal* and *lppA* mutants than for the WT. When the *pal* or *lppA* mutants were complemented with the respective gene, the amount of β-lactamase in the supernatant was reduced. The *ompA* deletion mutant showed near-WT profiles in β-lactamase release (Figure 3*A*). These results indicate that mutation of *pal* or *lppA* perturbed the outer membrane permeability barrier, causing the release of periplasmic proteins.

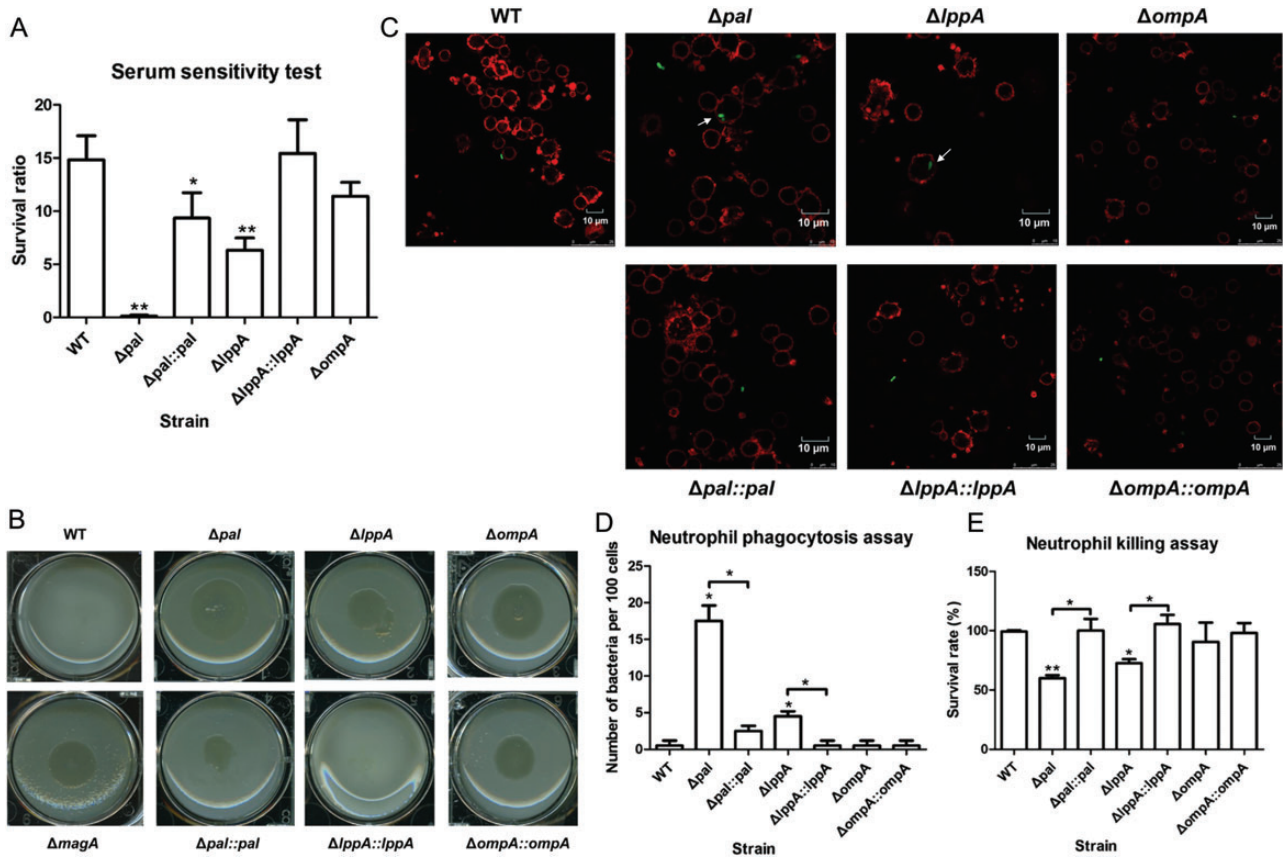

Figure 2. Sensitivities of the *Klebsiella pneumoniae* NTUH-K2044 and the OMP gene mutant strains to serum killing, phagocytosis, and human neutrophil–mediated killing. *A*, Serum sensitivity assays of resistance to killing by nonimmune healthy human serum of the NTUH-K2044 wild type (WT), the OMP gene mutant, and their complementation strains. The data represent the means of 3 independent trials; the error bars represent the standard deviations. A mean survival ratio ≥1 corresponds to serum resistance. $^{**}P < .01$ or $^{*}P < .05$ by Student *t* test (compared to the WT strain). *B*, Plaque assays of resistance to phagocytosis (5000 *Dictyostelium* cells per test) of the WT, the OMP gene mutant, and their complementation strains. The nonencapsulated *magA* mutant, which is permissive for *Dictyostelium* growth, was used as a positive control. *C*, Determination of phagocytosis resistance of the WT, the OMP gene mutant, and their complementation strains by human neutrophil. Bacteria carrying the green fluorescent protein plasmid were incubated with human neutrophils for 45 minutes and observed under a confocal microscope. A representative confocal section through the middle of the cell was shown for observation of intracellular bacteria. Image magnifications, ×630 with a zoom-in factor of 2×. Arrows denote intracellular bacteria. *D*, Bacteria phagocytosed by human neutrophils were counted under a confocal microscope. *E*, Bacterial susceptibilities to killing by human neutrophils of the WT, the OMP gene mutant, and their complementation strains after 45 minutes of incubation are presented. Survival rate indicates percentage of survival of WT or mutant strains calculated on the basis of viable counts relative to those for the no-neutrophil controls. The data represent the means of 3 independent trials; the error bars represent the standard deviations. $^{**}P < .01$ or $^{*}P < .05$ by Student *t* test (compared to the WT strain).

Sensitivity of the OMP Gene Deletion Mutants to Detergents and Polypeptide Antibiotics

The outer membrane (OM) of gram-negative bacteria is an effective permeability barrier, allowing only limited diffusion of hydrophobic compounds. To determine whether these OMP gene deletion mutants exhibited altered OM properties, the sensitivity of each mutant was tested against the anionic detergents, bile salts, SDS, and selected antimicrobial agents (including colistin, polymyxin B, and 3 other antibiotics). Our previous report indicated that NTUH-K2044 exhibits high tolerance to bovine bile [13]. To investigate the role of OMPs in bile resistance, we compared the viability of OMP gene deletion mutants with that of the WT strain after 5 hours' growth in LB medium with bile. The *pal* mutant demonstrated hypersensitivity to 1%–2% bile, which is within the range of the physiologic concentration of bile in the intestine (0.2%–2%) [21]. Resistance to bile salt in the *pal* mutant could be restored by complementation with the intact *pal* gene (Figure 3*B*). In comparison to the WT parent, the *lppA* and *pal* single mutants showed 4- to 8-fold decreases in MICs for SDS, and 2-fold decreases in MIC values for colistin and polymyxin B. Complementation of the deleted gene in these single mutants partially restored the MICs for SDS, colistin, and polymyxin B. No change in sensitivity to bile salts, SDS, colistin, or polymyxin B was seen in the

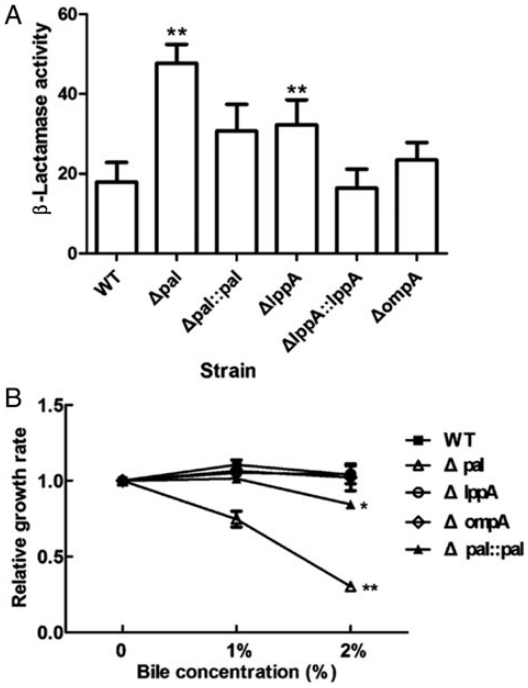

Figure 3. Outer membrane permeability and bile sensitivity of *Klebsiella pneumoniae* strains mutated for genes encoding outer membrane proteins. *A,* Outer-membrane permeability of *K. pneumoniae* strains, indicated by leakage of the periplasmic enzyme β-lactamase into the culture supernatant, as determined by nitrocephin hydrolysis. *B,* Sensitivities to bile stress of the NTUH-K2044 and the OMP gene mutant strains. Overnight cultures were inoculated into Luria-Bertani (LB) medium or LB medium containing 1% or 2% (w/v) bile salts. The cultures were incubated aerobically at 37°C for 5 hours. Cell growth was monitored by measuring the absorbance at 620 nm, with values normalized to those obtained in LB medium alone. Results are the means of 3 independent experiments, and the error bars represent the standard deviations. $^{*}P < .05$ and $^{**}P < .01$ by Student *t* test (compared to the wild-type strain); other comparisons were not statistically significant ($P \geq .05$). Abbreviation: WT, wild type.

ompA mutant. Moreover, no change in sensitivity to cefotaxime, ciprofloxacin, or gentamicin was seen in any of the OMP gene deletion mutants compared to the WT strain (Table 2). These results show that in the absence of Pal or LppA, the *K. pneumoniae* bacteria became sensitive to anionic detergents and polypeptide antibiotics.

Virulence of Strains Deleted for OMP-Encoding Genes

Although *pal* and *lppA* mutants may be impaired in cell integrity, the growth ability in vitro of these 2 mutants was the same as that of the WT. Thus, the virulence in vivo of these OMP gene deletion mutants was relatively specific compared to that of in vitro conditions. Upon intraperitoneal infection of mice, the *ompA* mutant yielded the same LD_{50} values of $<1 \times 10^2$ CFU as the WT strain. Both the *pal* and *lppA* single mutants were less virulent than the parent strain (Table 3). To explore the contribution of Pal or LppA protein in bacterial dissemination and proinflammatory response, we challenged mice with the same dose (1×10^3 CFU) of the WT and the OMP gene deletion mutant strains. The C57BL/6 mice infected with the *pal* or *lppA* mutant strains yielded significantly fewer colony counts in both the liver and spleen and lower cytokine IL-6 levels compared with those of the WT or OMP gene complemented strains ($P \leq .003$; Figure 4*A* and 4*B*). In Enterobacteriaceae, Pal and Lpp are shed into serum in vivo, where these lipoproteins act synergistically with LPS to initiate inflammation in sepsis [22, 23]. TLR2 is a receptor for bacterial Pal and other lipoproteins, and TLR4 recognizes bacterial LPS [22, 24]. To figure out the roles of the TLR-mediated immune responses in these 2 mutants, similar experiments were performed in the C57BL/6 isogenic TLR2 KO and TLR4 KO mice. Bacterial loads in both the liver and spleen were decreased and serum IL-6 concentrations were reduced in both TLR2 KO and TLR4 KO mice infected with the *pal* mutant compared to those in mice infected with the WT strain ($P \leq .004$; Figure 4*C*–4*F*). TLR2 KO mice infected with the *lppA* mutant yielded significantly lower bacterial loads and IL-6 levels compared with those of the WT strain ($P \leq .006$; Figure 4*C* and 4*D*). However, the bacterial loads in TLR4 KO mice infected with *lppA* mutant compared to those in mice infected with the WT strain showed no significant difference ($P \geq .227$; Figure 4*E*), nor did serum IL-6 levels ($P = .265$; Figure 4*F*). Meanwhile, infections with the *pal* and *lppA* complemented strains partially restored the bacterial loads and the levels of serum IL-6 in both TLR2 KO and TLR4 KO mice (Figure 4*C*–4*F*). These data indicate that the *lppA* deletion mutant had reduced ability to induce IL-6 responses than that of the WT strain, whereas no such difference could be noted when TLR4 KO mice were challenged.

To evaluate the potential protective efficacy of these attenuated strains, we challenged BALB/c mice (previously immunized intraperitoneally with *pal* or *lppA* mutant strains) with a sublethal dose of NTUH-K2044. Four weeks later, all *pal* or *lppA* mutant-immunized mice survived without any symptoms of disease, whereas 75% of the nonimmunized control mice died within 6 days of infection (Figure 4*G*). Thus, bacteria mutated for *pal* or *lppA* exhibited attenuated virulence in mouse, while conferring immune protection against challenge with the WT strain. Mice immunized with the *pal* or *lppA* mutant showed EPS serum IgG responses, whereas control mice did not. Seroconversion of these mutant-immunized mice was further confirmed by slot immunoblotting (Figure 4*H*).

DISCUSSIONS

Our previous data demonstrated that the outermost components of the *K. pneumoniae* bacterial surface, both the CPS and the O antigen of LPS, are critical virulence factors protecting *K. pneumoniae* from serum killing. Additionally, the CPS (though not the O antigen of LPS) plays an important role in *K. pneumoniae* resistance to phagocytosis [8, 9]. In the present study,

Table 2. Minimum Inhibitory Concentrations of the Anionic Detergents, the Polypeptides, and Some Antimicrobial Agents Against the *Klebsiella pneumoniae* NTUH-K2044 and Their Derivative Mutant Strains

Strain	Minimum Inhibitory Concentration[a]					
	Cefotaxime[b], μg/mL	Ciprofloxacin[b], μg/mL	Gentamicin[b], μg/mL	Colistin[b], μg/mL	Polymyxin B[b], μg/mL	SDS[c], mg/mL
WT	0.125	0.125	0.125	0.5	1	1.56
Δ*pal*	0.125	0.125	0.125	0.25	0.5	**0.19**
Δ*pal*::*pal*	. . .	. . .	. . .	0.5	1	0.78
Δ*lppA*	0.125	0.125	0.125	0.25	0.5	**0.39**
Δ*lppA*::*lppA*	. . .	. . .	. . .	0.5	1	0.78
Δ*ompA*	0.125	0.125	0.125	0.5	1	1.56

Abbreviations: SDS, sodium dodecyl sulfate; WT, wild type.

[a] Boldface numbers indicate a significant difference in the minimum inhibitory concentrations (MICs) of the OMP deletion strains and the parental NTUH-K2044 strain of at least 4-fold.

[b] MICs were determined by an agar dilution method.

[c] MICs were determined in 5 mL of Luria-Bertani broth.

we demonstrated that the *K. pneumoniae* OMPs Pal and LppA confer protection not only against serum killing, but also against neutrophil phagocytosis and killing.

In gram-negative bacteria, OM serves as the outermost barrier, extending beyond the cytoplasmic membrane and the peptidoglycan layer. Mutants with a deep-rough phenotype have been reported to release periplasmic enzymes into the extracellular space, presumably because of associated defects in the OM, which is one of the first barriers that an antimicrobial agent must overcome when interacting with its target. In this study, mutations of *K. pneumoniae pal* or *lppA* perturbed the OM permeability barrier, rendering the mutants sensitive to anionic detergents and polypeptide antibiotics. Therefore, the *K. pneumoniae* Pal and LppA proteins are implicated in the maintenance of cell envelope integrity.

Pal, Lpp, and OmpA are common contaminants of purified LPS, and these contaminants may influence the results of studies performed using purified LPS [25]. Several recent studies indicate significant roles for Pal and LppA proteins in the pathogenesis of bacterial infection. Infection of LPS-responsive mice with a *pal*-truncated *E. coli* strain led to the development of a mild form of sepsis and lower levels of IL-6 in the blood [26]. Deletion of both *lpp* genes rendered *Salmonella* defective in invasion, motility, induction of cytotoxicity, and production of inflammatory cytokines/chemokines [27]. But these findings could not conclude that these results were due to the decreased in vivo survival of the bacterial mutants, or reduced ability to induce an immune response. Our data suggested that the decreased in virulence of the *K. pneumoniae pal*–deficient mutant was mainly due to killing by serum and neutrophil. TLR4-mediated inflammation played an important role in the *lppA* deletion mutant. However, additional studies are required to further dissect infection capacity from inflammatory potential in the *pal* mutant. *Klebsiella pneumoniae* LppA directly

Table 3. Phenotypic Characterization of the *Klebsiella pneumoniae* NTUH-K2044 and Their Derivative Mutant Strains

Strain	Genotype or Phenotype[a]	CPS K Ag	LPS O1 Ag	Serum Resistance[b]	LD_{50} Values (CFU) of IP Inoculation	Reference or Source
WT	Wild-type/m^+	$K1^+$	+	R	$<1 \times 10^2$	[8]
Δ*pal*	*pal*/m^+	$K1^+$	+	S	1.7×10^5	This study
Δ*pal*::*pal*	Wild-type /m^+	ND	ND	R	1.5×10^3	This study
Δ*lppA*	*lppA*/m^+	$K1^+$	+	R	4.4×10^4	This study
Δ*lppA*::*lppA*	Wild-type/m^+	ND	ND	R	5.7×10^2	This study
Δ*ompA*	*ompA*/m^+	$K1^+$	+	R	$<1 \times 10^2$	This study
Δ*wbbO*	*wbbO*/m^+	$K1^+$	–	S	1×10^3	[9]
Δ*magA*	*magA*/m^-	$K1^-$	+	S	$>1 \times 10^7$	[9]

Abbreviations: Ag, antigen; CFU, colony-forming units; CPS, capsular polysaccharide; IP, intraperitoneal; LD_{50}, 50% lethal dose; LPS, lipopolysaccharide; ND, not done.

[a] m^+, mucoid phenotype; m^-, nonmucoid phenotype.

[b] R, resistance; S, sensitive.

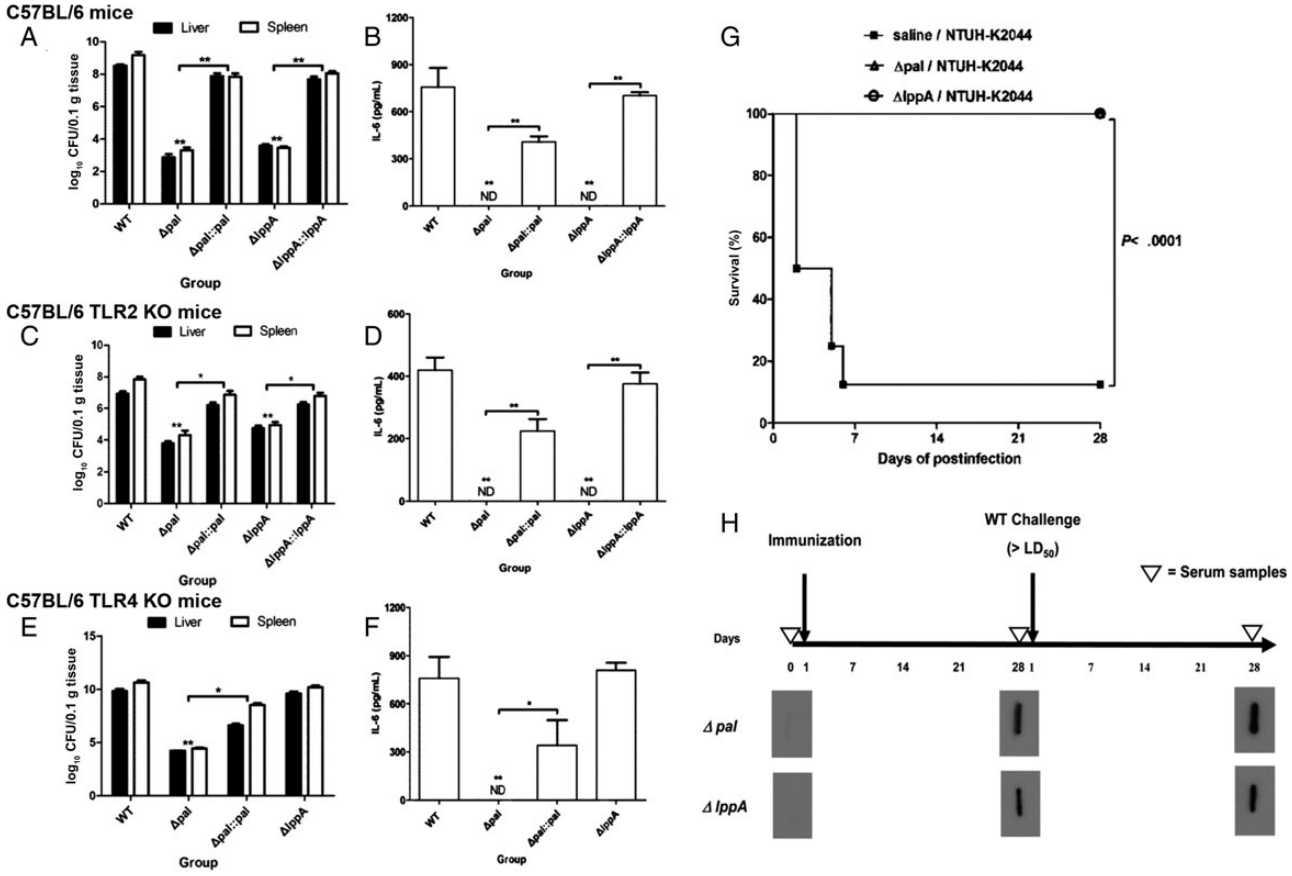

Figure 4. Immune response and survival rate following immunization with the *pal*- or *lppA*- mutant strains. C57BL/6 wild-type (WT; *A* and *B*), TLR2 knockout C57BL/6 (*C* and *D*), or TLR4 knockout C57BL/6 (*E* and *F*) mice (4 mice per group) were inoculated by intraperitoneal injection with equivalent doses (1×10^3 colony-forming units [CFU]) of the NTUH-K2044 WT, *pal*-, *lppA*- mutants, or their corresponding complemented strains. Surviving animals were killed at 20 hours after challenge. Bacterial loads were measured in the liver and spleen (*A*, *C*, and *E*); interleukin 6 (IL-6) levels were measured in the serum (*B*, *D*, and *F*). Log_{10} CFU was standardized per 0.1 g wet organ weight. IL-6 levels were measured by ELISA. Data are presented as means ± standard deviations. $^*P < .05$ and $^{**}P < .01$ by Student *t* test (compared to the WT strain); other comparisons were not statistically significant ($P \geq .05$). *G*, Survival of *Klebsiella pneumoniae*–immunized mice following challenge with NTUH-K2044. BALB/c mice (8 per group) were inoculated by intraperitoneal injection with 1×10^4 CFU of the *pal* or *lppA* mutant strain. Age-matched mice that were inoculated with an equivalent volume of saline served as unimmunized controls. At the fourth week after immunization, immunized and unimmunized animals were challenged with NTUH-K2044 (1×10^3 CFU per animal, intraperitoneal). Survival was assessed for 28 days following infection. ■, unimmunized, NTUH-K2044 challenged; △, *pal* mutant immunized, NTUH-K2044 challenged; ○, *lppA* mutant immunized, NTUH-K2044 challenged. $P < .0001$ by log-rank test (*pal*/NTUH-K2044 or *lppA*/NTUH-K2044 compared to unimmunized/NTUH-K2044). *H*, Immune response of anti-exopolysaccharide IgG in mice before and after immunized with the *pal* or *lppA* mutant strain. Abbreviations: CFU, colony-forming units; IL-6, interleukin 6; KO, knockout; LD_{50}, 50% lethal dose; ND, not detected; WT, wild type.

induces inflammation through TLR4 alone and/or synergistically with other bacterial products needs to be clarified.

Though these OMPs are highly conserved, passive immunization with murein lipoprotein or peptidoglycan-associated lipoprotein does not protect mice from sepsis [28]. Previously, we demonstrated that the acapsular *magA* mutant of *K. pneumoniae* is impaired for immunogenicity and cannot induce effective protection immunity [12]. Here, we demonstrated that the *K. pneumoniae* strains mutated in *pal* or *lppA* were attenuated for pathogenicity, retained intact K1 CPS and O1 LPS antigens and resulting in anti-EPS antibodies production. Therefore, the *K. pneumoniae pal* deletion, *lppA* deletion or double deletion mutant strains could be useful in the development of new vaccines.

In summary, peptidoglycan-associated lipoprotein and murein lipoprotein in *K. pneumoniae* confer protection against serum killing and phagocytosis. Mutation of *pal* or *lppA* perturbed the OM permeability barrier and permitted the release of periplasmic proteins. Moreover, *K. pneumoniae* strains deleted for *pal* or *lppA* became hypersensitive to bile salts and SDS. The *lppA* deletion mutant has reduced ability to activate TLR4. Immunization of mice with the *pal* or *lppA* mutant provides protection against infection with a virulent WT *K. pneumoniae* strain.

Notes

Acknowledgments. We thank Chung-Tang Chen of the Department of Microbiology for assistance in mice experiments.

Financial support. This study was supported by the National Taiwan University and the National Science Council of Taiwan.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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