

Klebsiella pneumoniae FimK Promotes Virulence in Murine Pneumonia

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Klebsiella pneumoniae, a chief cause of nosocomial pneumonia, is a versatile and commonly multidrug-resistant human pathogen for which further insight into pathogenesis is needed. We show that the pilus regulatory gene *fimK* promotes the virulence of *K. pneumoniae* strain TOP52 in murine pneumonia. This contrasts with the attenuating effect of *fimK* on urinary tract virulence, illustrating that a single factor may exert opposing effects on pathogenesis in distinct host niches. Loss of *fimK* in TOP52 pneumonia was associated with diminished lung bacterial burden, limited innate responses within the lung, and improved host survival. FimK expression was shown to promote serum resistance, capsule production, and protection from phagocytosis by host immune cells. Finally, while the widely used *K. pneumoniae* model strain 43816 produces rapid dissemination and death in mice, TOP52 caused largely localized pneumonia with limited lethality, thereby providing an alternative tool for studying *K. pneumoniae* pathogenesis and control within the lung.

Keywords. *Klebsiella pneumoniae*; pneumonia; capsule; *fimK*; EAL domain; murine model.

Klebsiella pneumoniae is a leading cause of nosocomial infections, including pneumonia, urinary tract infection (UTI), and sepsis [1, 2]. *K. pneumoniae* is ubiquitous in the environment and is often found in the gastrointestinal tract and on medical devices [2, 3]. The emergence of *K. pneumoniae* carbapenemases and extended-spectrum β -lactamases renders this pathogen increasingly difficult to treat [4–6]. Identification of critical virulence factors in *K. pneumoniae* will support development of new approaches to combat these daunting infections.

The virulence repertoire of *K. pneumoniae* is incompletely defined, although animal models and genomic studies have identified several important determinants. The capsule protects against phagocytosis, antimicrobial peptides, and serum bactericidal activity [7–10]. Adhesins, iron-scavenging systems, lipopolysaccharide, OmpA, phospholipase D1, and urease also promote successful *K. pneumoniae* infections [2, 11–18]. Type 1 pili, as in *Escherichia coli*, are critical for UTI pathogenesis; however, unlike uropathogenic *E. coli*, *K. pneumoniae* encodes a type 1 pilus regulatory gene, *fimK* [16, 18–21]. Deletion of *fimK* results in hyperfimbriate bacteria with augmented virulence in murine UTI [21]. We therefore investigated whether this conserved gene may be important for enhancing virulence in other niches.

Here, using a murine model of intratracheal inoculation, we demonstrate that FimK promotes *K. pneumoniae* pathogenesis in the lung. While no single approach to modeling *K. pneumoniae* pulmonary infection has been adopted, the most commonly used *K. pneumoniae* model strain, ATCC 43816, causes bacteremia and death in mice within 5 days of lung inoculation, with median lethal doses (LD₅₀) of 10¹–10³ colony-forming units (CFU) [11, 22–24]; capsule and O antigen are critical for 43816 dissemination and lethality [10, 17]. The present study identifies TOP52, a *K. pneumoniae* strain originally isolated from the human urinary tract, as also pathogenic in the murine respiratory tract. We demonstrate that loss of *fimK* attenuates lung infection, diminishes capsule production, and renders *K. pneumoniae* more susceptible to phagocytosis by host immune cells. Our work also introduces an alternative model strain of *K. pneumoniae* that produces infection localized to the respiratory tract.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

K. pneumoniae strains included TOP52 (strain 1721), a K6 isolate from a woman with acute cystitis [19, 21]; TOP52 Δ *fimK*, a deletion mutant that exhibits growth equivalent to the parent strain [21]; and ATCC 43816, a K2 isolate lethal in murine pneumonia models [1, 10, 17]. Plasmids included the empty arabinose-inducible expression vector pBAD33 and the *pfimK* vector for expression of TOP52 *fimK* [21]. Bacteria were grown statically in 20-mL cultures at 37°C for 16 hours in Luria-Bertani (LB) broth containing, as appropriate, 20 μ g/mL chloramphenicol and 0.2% arabinose. Overnight cultures were centrifuged at 7000g for 10 minutes. Bacteria were resuspended in sterile phosphate-buffered saline (PBS) and diluted to the

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desired inoculum concentration according to OD₆₀₀. Inocula were verified by serial dilution and plating.

Mouse Infections

Female C57BL/6J mice and complement component 3 (C3)-deficient female B6.129S4-C3^{tml¹Crr}/J mice (Jackson Laboratories, Bar Harbor, Maine) aged 7–8 weeks were used. For respiratory tract infections, an intratracheal inoculation procedure was adapted from those previously described [25]. Briefly, each mouse was anesthetized with inhaled isoflurane, and the trachea was exposed via surgical dissection. Inoculum (20 µL containing 1–2 × 10⁷ colony-forming units [CFU] unless otherwise indicated) was injected intratracheally using a 30-gauge, caudally directed needle. Overlying tissues were replaced, and skin was closed using Vetbond (3M Animal Care Products, St. Paul, Minnesota). Control mice were inoculated with sterile PBS in identical fashion. For UTI, 50 µL of suspension (1–2 × 10⁷ CFU) was inoculated into mouse bladders by transurethral catheterization [26]. All animal procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Mouse Survival, Weights, Organ Titters, Cytokines, and Histologic Analysis

K. pneumoniae-infected or PBS-inoculated mice were weighed daily for up to 2 weeks. Weights and organ titters were obtained only for surviving mice. Mouse organs (lungs, spleen, bladder, or kidneys) were harvested and homogenized in sterile PBS via Bullet Blender (Next Advance, Averill Park, New York) for 5 minutes. Aliquots of homogenates were serially diluted and plated on LB agar. Remaining homogenates were stored at –80°C before cytokine analysis, performed with a 23-plex magnetic bead cytokine array (Bio-Plex Pro, Mouse Group I, Bio-Rad, Hercules, California); duplicate samples were assayed at 1:20 dilution per the manufacturer's instructions. Organs removed for histologic analysis were washed in PBS, fixed in 10% neutral buffered formalin, dehydrated in ethanol, and embedded in paraffin; 5-µm sections were stained with hematoxylin and eosin. Images were obtained using an Olympus DP25 camera and BX40 light microscope.

Blood Cultures

Blood cultures were performed on a subset of mice 24 hours after infection. Mice were anesthetized with isoflurane, and cardiac puncture was performed with a 26-gauge needle. A total of 10 µL of blood was plated onto LB agar; samples yielding >100 CFU/mL (limit of detection) were considered positive.

Flow Cytometry

Lungs were harvested and placed into Incomplete Medium composed of Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, New York), 10 mM HEPES buffer (Corning, Manassas, Virginia), 1 mM sodium pyruvate (Gibco), 5% MEM Nonessential Amino Acids (Corning), 1% penicillin/streptomycin (Gibco), and 2 mM L-glutamine (Corning). Single

cell suspensions were prepared by dicing lungs into Hank's balanced salt solution (Gibco) containing 1 mg/mL collagenase D (Roche, Indianapolis, Indiana), 2% fetal bovine serum (Gibco), and 25 mM HEPES buffer; incubating the material for 1 hour at room temperature with shaking; and passing the material through a 70-µm nylon cell strainer (Falcon Corning Life Sciences, Tewksbury, Massachusetts). Erythrocytes were lysed using sterile Gey's solution (500 mL sterile water, 4.15 g ammonium chloride, and 0.5 g potassium bicarbonate). Cells were counted by hemacytometer. For flow cytometry, 10⁶ cells were incubated with 0.5 µg of Fc Block (BD Biosciences, San Jose, California) for 15 minutes at 4°C prior to staining. Surface antibody markers included GR1-PerCP-Cy5.5 (RB6-8C5; BD Biosciences) and CD11b-eFluor450 (M1/70; eBioscience, San Diego, California). Live cells were determined by forward scatter/side scatter gating. Cells were analyzed on a BD LSR II cytometer; data were collected using BD FACS Diva software and analyzed using FlowJo software (FlowJo, Ashland, Oregon).

Serum Resistance

Serum bactericidal assays were adapted from those previously described [27]. Blood specimens were collected by venipuncture from healthy adult donors (the protocol was approved by the Human Research Protection Office at Washington University). Briefly, 25 µL of a suspension of statically grown bacteria (10⁶ CFU/mL in PBS) was mixed in 96-well microplates with 75 µL of pooled human serum and incubated for 3 hours at 37°C. Serum was either active or inactivated by preincubation at 65°C for 30 minutes. Samples were serially diluted and plated to calculate the percentage survival as a ratio of the 3-hour CFU to the input CFU.

Capsule Quantification

Capsule extraction and uronic acid quantification were performed using a modified protocol [28–30]. A total of 500 µL of overnight static LB cultures was mixed with 100 µL of 1% Zwittergent 3–14 (Sigma, St. Louis, Missouri) in 100 mM citric acid and incubated at 50°C for 20 minutes. Following centrifugation, supernatants were precipitated with cold ethanol. After recentrifugation, the pellet was dissolved in 200 µL of water, and 1200 µL of 12.5 mM tetraborate in concentrated H₂SO₄ was added. After vortexing, samples were boiled at 95°C for 5 minutes and then mixed with 20 µL of 0.15% 3-hydroxydiphenol (Sigma) in 0.5% NaOH. Absorbance was measured at 520 nm. The uronic acid concentration in each sample was determined from a standard curve of glucuronic acid (Sigma).

In Vivo Phagocytosis Assay

In vivo phagocytosis was assessed as previously described [31]. Briefly, bacteria were incubated with 0.2 mg/mL fluorescein 5(6)-isothiocyanate (FITC, Sigma) for 30 minutes at 37°C. Uniform FITC labeling was confirmed by fluorescence-activated cell-sorting analysis. Bronchoalveolar lavage (BAL) fluid was obtained 3 hours after infection with 1–2 × 10⁷ CFU *K. pneumoniae*.

Fluorescence of extracellular FITC was quenched by washing with trypan blue (Sigma). Alveolar macrophages (CD45⁺ CD11c^{hi}) and neutrophils (CD45⁺ GR-1⁺) were quantified by flow cytometry; phagocytosis of bacteria was measured by the shift in FITC fluorescence. Samples were blocked with 1 µg Fc block (2.4G2 hybridoma; ATCC). Surface antibody markers included CD11c-PECy7 (N418), GR1-Pacific blue (RB6-8C5), and CD45-Pacific blue (30-F11; BioLegend, San Diego, California) or CD45-BV510 (30-F11; BD Biosciences). Cells were acquired with a BD FACScan flow cytometer with DXP multicolor upgrades (Cytex Development, Woodland Park, New Jersey) and analyzed using FlowJo software.

Statistical Analysis

Comparisons between 2 groups of continuous variables were analyzed using the Mann–Whitney *U* test, as these values were not all normally distributed. For Kaplan–Meier survival analysis, the Mantel–Cox log-rank test was used. The Fisher exact test was used for blood culture comparisons. All tests were 2 tailed; *P* values of <.05 were considered statistically significant. Analyses were performed using GraphPad Prism, version 6.04.

RESULTS

FimK Promotes Mortality and Morbidity in *K. pneumoniae* TOP52 Infection of the Murine Respiratory Tract

While past studies have shown that genomic carriage of *fimK* attenuates pathogenesis of *K. pneumoniae* TOP52 in the urinary tract [21], the role of *fimK* in other niches remains undefined. To evaluate whether *fimK* promotes virulence in the respiratory tract, we intratracheally inoculated C57BL/6 mice with 10⁷ CFU of TOP52 or TOP52Δ*fimK* and monitored weight and survival (Figure 1). While no mice infected with TOP52Δ*fimK* died over 14 days, 36% of mice infected with wild-type TOP52 died between 3 and 9 days after infection (Figure 1A, *P* = .0013). TOP52-infected surviving mice had significantly lower weights than TOP52Δ*fimK*-infected mice from days 2 through 14 after infection (days 2–7, *P* < .0001; days 8–14, *P* < .01; Figure 1B). These data may understate morbidity, as only surviving mice are represented on each day. PBS–mock-infected mice lost an average of 5% body weight on day 1 after inoculation but recovered quickly and gained weight each subsequent day. TOP52Δ*fimK*-infected mice recovered by day 5 after infection; their weights were not statistically different from those of mock-infected mice after this interval. These data indicate that loss of *fimK* decreases morbidity and mortality in *K. pneumoniae* pneumonia.

Infection With *K. pneumoniae* TOP52 Yields a Higher Lung Bacterial Burden Than Infection With TOP52Δ*fimK*

To evaluate whether *fimK* was important for *K. pneumoniae* pathogenesis within the lung itself, organs were harvested and bacterial titers quantified after infection with TOP52 or

TOP52Δ*fimK* (Figure 2A). At 6, 24, and 48 hours after infection, TOP52Δ*fimK* was attenuated in the lungs (*P* < .0001 vs TOP52). Lung bacterial titers in surviving TOP52-infected mice continued to trend higher than those in TOP52Δ*fimK*-infected mice 1 week after infection (*P* = .0567). By 2 weeks after infection, surviving mice had substantially cleared either TOP52 or TOP52Δ*fimK*. Confirming that the observed differences were attributable to *fimK* loss, TOP52Δ*fimK*/p*fimK* yielded higher lung titers than TOP52Δ*fimK*/pBAD33 both 6 and 24 hours after infection (*P* = .0051 and .0003, respectively; Figure 2B and 2C).

To verify that the increased urovirulence of TOP52Δ*fimK* previously demonstrated in C3H/HeN mice [21] was not specific to that murine background, we transurethrally infected C57BL/6 female mice with TOP52 or TOP52Δ*fimK*. Indeed, C57BL/6 bladders infected with TOP52Δ*fimK* bore significantly

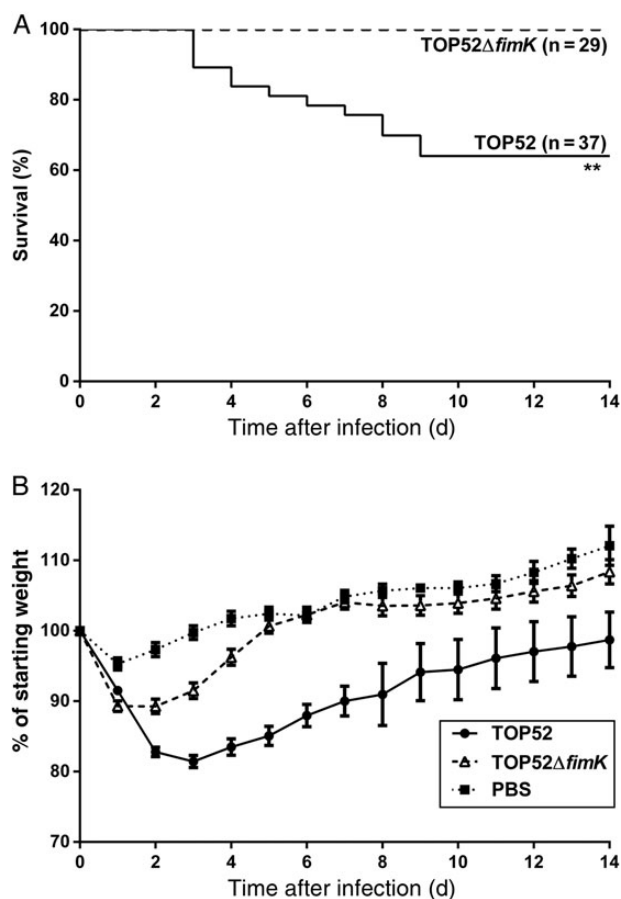


Figure 1. Kaplan–Meier survival and weights of mice intratracheally infected with *Klebsiella pneumoniae* TOP52 or TOP52Δ*fimK*. *A*, TOP52Δ*fimK*-infected mice exhibited a significant survival advantage relative to TOP52-infected mice (***P* < .01, by the Mantel–Cox log-rank test). *B*, By day 2 after infection, TOP52-infected surviving mice (circles) had lost significantly more weight than TOP52Δ*fimK*-infected mice (triangles) and remained significantly lower in weight through the 14-day analysis. Data are shown as mean ± standard error of the mean and are combined from at least 3 independent experiments. Abbreviation: PBS, phosphate-buffered saline.

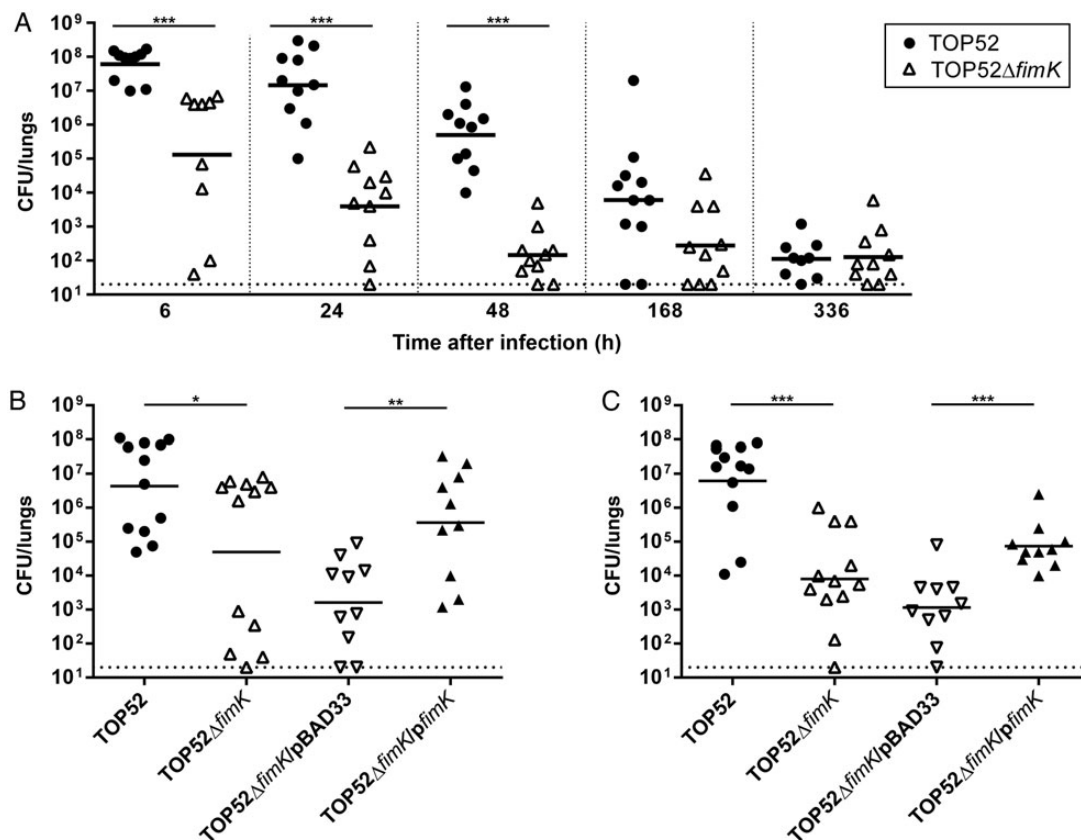


Figure 2. Time course of *Klebsiella pneumoniae* TOP52 and TOP52 Δ *fimK* murine pneumonia and complementation with *pfimK*. *A*, Female C57BL/6 mice were infected with 10^7 TOP52 (circles) or TOP52 Δ *fimK* (triangles) by intratracheal inoculation. Lung bacterial loads were significantly higher in TOP52-infected mice than in TOP52 Δ *fimK*-infected mice at 6, 24, and 48 hours after infection. *B* and *C*, This phenotype was complemented with *fimK* in trans at both 6 hours after infection (*B*) and 24 hours after infection (*C*). Data are combined from at least 3 independent experiments. Each symbol represents 1 animal, short bars represent geometric means of each group, and full dotted horizontal lines represent limits of detection (* $P < .05$, ** $P < .01$, and *** $P < .001$, by the Mann–Whitney *U* test). Abbreviation: CFU, colony-forming units.

higher bacterial burdens 6 and 24 hours after infection than TOP52-infected bladders (Supplementary Figure 1). Taken together, these data indicate that, while *fimK* diminishes TOP52 virulence in the urinary tract, it is required for optimal virulence in *K. pneumoniae* murine pneumonia.

Intratracheal Inoculation With TOP52 Results in Limited Bacteremia and Dissemination

To test whether TOP52 murine pneumonia results in dissemination of infection beyond the respiratory tract, we first evaluated spleen bacterial titers. Spleen titers in TOP52-infected mice were very low but significantly higher than in TOP52 Δ *fimK*-infected mice 24 and 48 hours after infection ($P = .0027$ and $P = .0007$, respectively; Figure 3A). At 1 and 2 weeks after intratracheal infection with TOP52 or TOP52 Δ *fimK*, spleen titers were extremely low or absent and did not differ significantly (data not shown). In contrast, intratracheal inoculation of an equivalent inoculum (10^7 CFU) of the commonly used *K. pneumoniae* model strain 43816 yielded markedly higher spleen titers 24 hours after infection ($P = .0002$; Figure 3A).

Intratracheal inoculation with 10^5 CFU of 43816, a typical experimental dose of this strain, similarly resulted in uniformly high ($>10^4$ CFU) spleen titers 24 hours after infection (data not shown). Cultures of blood specimens obtained 24 hours after infection were uniformly negative in mock-infected and TOP52- Δ *fimK*-infected mice (Figure 3B). A total of 11.1% of TOP52-infected mice exhibited positive blood cultures, compared with 100% of 43816-infected mice ($P < .0001$). Intratracheal infection with the lower inoculum (10^5 CFU) of 43816 also yielded 100% bacteremia 24 hours after infection (data not shown). These data demonstrate that, while respiratory tract infection with TOP52 may be associated with bacteremia more often than its isogenic *fimK* mutant, TOP52 has substantially less propensity to disseminate than 43816.

Murine TOP52 Pneumonia Features Increased Inflammation Compared With TOP52 Δ *fimK*

To gain insight into host response during TOP52 versus TOP52 Δ *fimK* pneumonia, histologic analysis was performed on lung sections prepared 24 hours after infection. Mouse

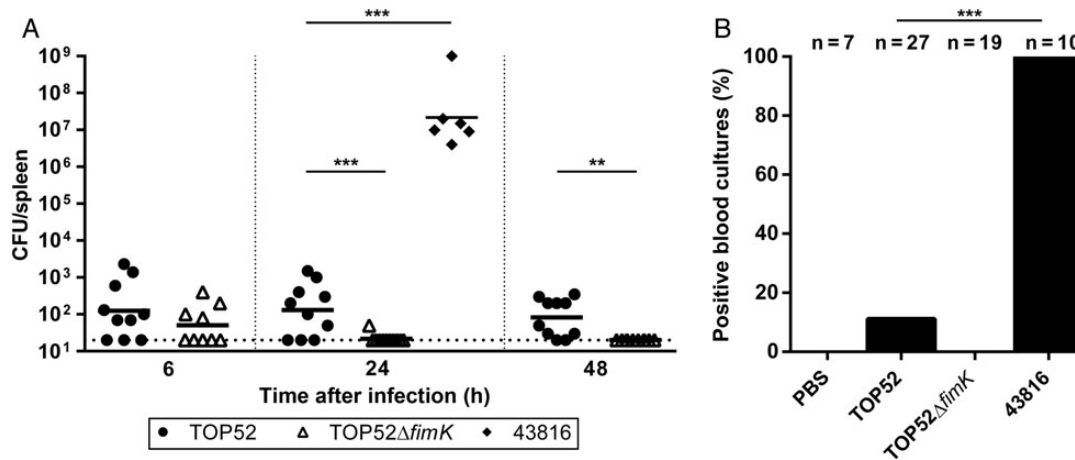


Figure 3. *Klebsiella pneumoniae* TOP52 and TOP52ΔfimK display limited dissemination and bacteremia. **A**, Female C57BL/6 mice were inoculated intratracheally with 10⁷ TOP52 (circles), TOP52ΔfimK (triangles), or ATCC 43816 (diamonds). Spleen bacterial loads were measured at indicated time points. Data are combined from at least 3 independent experiments. Each symbol represents 1 animal, short bars represent geometric means of each group, and full dotted horizontal lines represent limits of detection (***P* < .01 and ****P* < .001, by the Mann–Whitney *U* test). **B**, Blood cultures at 24 hours after infection were positive in only 11.1% of TOP52-infected mice and 0% of TOP52-ΔfimK-infected mice, compared with 100% of ATCC 43816–infected mice (****P* < .001, by the Fisher exact test). Abbreviations: CFU, colony-forming units; PBS, phosphate-buffered saline.

lungs infected with TOP52 demonstrated significant consolidation throughout, with few remaining morphologically normal alveoli (Figure 4A). A marked acute inflammatory infiltrate (neutrophils and macrophages) and areas of hemorrhage were evident. In contrast, TOP52ΔfimK-infected lungs exhibited only sporadic areas of acute inflammation and mild hemorrhage, with sparing of most distal airways. These differences in lung inflammation between TOP52-infected mice and TOP52-ΔfimK-infected mice were amplified 48 hours after infection; TOP52-infected lungs were completely consolidated at this time point, while TOP52ΔfimK histologic findings were nearly normal (data not shown).

We quantified neutrophilic inflammation by flow cytometry in the lungs of mice inoculated with TOP52, TOP52ΔfimK, or PBS. In mock-infected lungs, neutrophils (Gr-1⁺, CD11b⁺) comprised on average 11.4% of total live cells 24 hours after infection (Figure 4B). TOP52-infected lungs harbored a greater mean percentage of neutrophils 24 hours after infection, compared with TOP52ΔfimK-infected lungs (65% vs 38%; *P* = .0005). In addition, absolute neutrophil numbers in TOP52-infected mouse lungs were >2-fold higher than those in TOP52ΔfimK-infected lungs 24 hours after infection (*P* = .0016) and >3-fold higher at 1 and 2 weeks after infection (*P* = .0207 and *P* = .0022, respectively; Figure 4C).

Quantification of 23 cytokines in lung homogenates was performed 6 and 24 hours after infection (Figure 5). There were few significant differences in lung cytokine levels 6 hours after infection with TOP52 or TOP52ΔfimK, notably including higher IFN-γ and TNF-α levels in TOP52-infected samples (*P* = .0022 and *P* = .0152, respectively). However, TOP52-infected lungs 24 hours after infection contained significantly higher levels of

many proinflammatory cytokines (IL-1β, IL-6, IL-17, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, and TNF-α) and chemokines (KC, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α [MIP-1α], and RANTES) than TOP52ΔfimK-infected lungs. Together, these data indicate that the higher-titer infection achieved by TOP52 is accompanied by a more robust immune response (involving inflammatory mediators and immune cells), compared with TOP52ΔfimK.

FimK Promotes *K. pneumoniae* Serum Resistance, Capsule Production, and Resistance to Phagocytosis

To test the hypothesis that differences in pathogenesis of TOP52 and TOP52ΔfimK reflect an influence of FimK on the expression of virulence factors important in resisting innate defenses, we first performed serum resistance assays (Figure 6A). The serum-resistant, heavily encapsulated *K. pneumoniae* strain 43816 served as a comparator. TOP52 titers were approximately 100% of input CFU after 3-hour incubation in active serum, while TOP52ΔfimK showed <1% survival (*P* = .0006). Meanwhile, 43816 exhibited 10-fold higher titers than TOP52 after 3 hours in active serum (*P* = .0041). Interestingly, while inactivation of serum did not alter TOP52 and 43816 survival, TOP52ΔfimK grew to 300% of input in inactivated serum (vs <1% survival in active serum; *P* = .0012), indicating that lack of FimK confers marked susceptibility to complement-mediated killing in vitro.

To test whether increased susceptibility to complement was responsible for the attenuated in vivo virulence of TOP52ΔfimK, we intratracheally inoculated mice lacking the complement component 3 (C3) gene. Lungs and spleens of C3-deficient mice infected with TOP52 harbored increased bacterial loads relative to

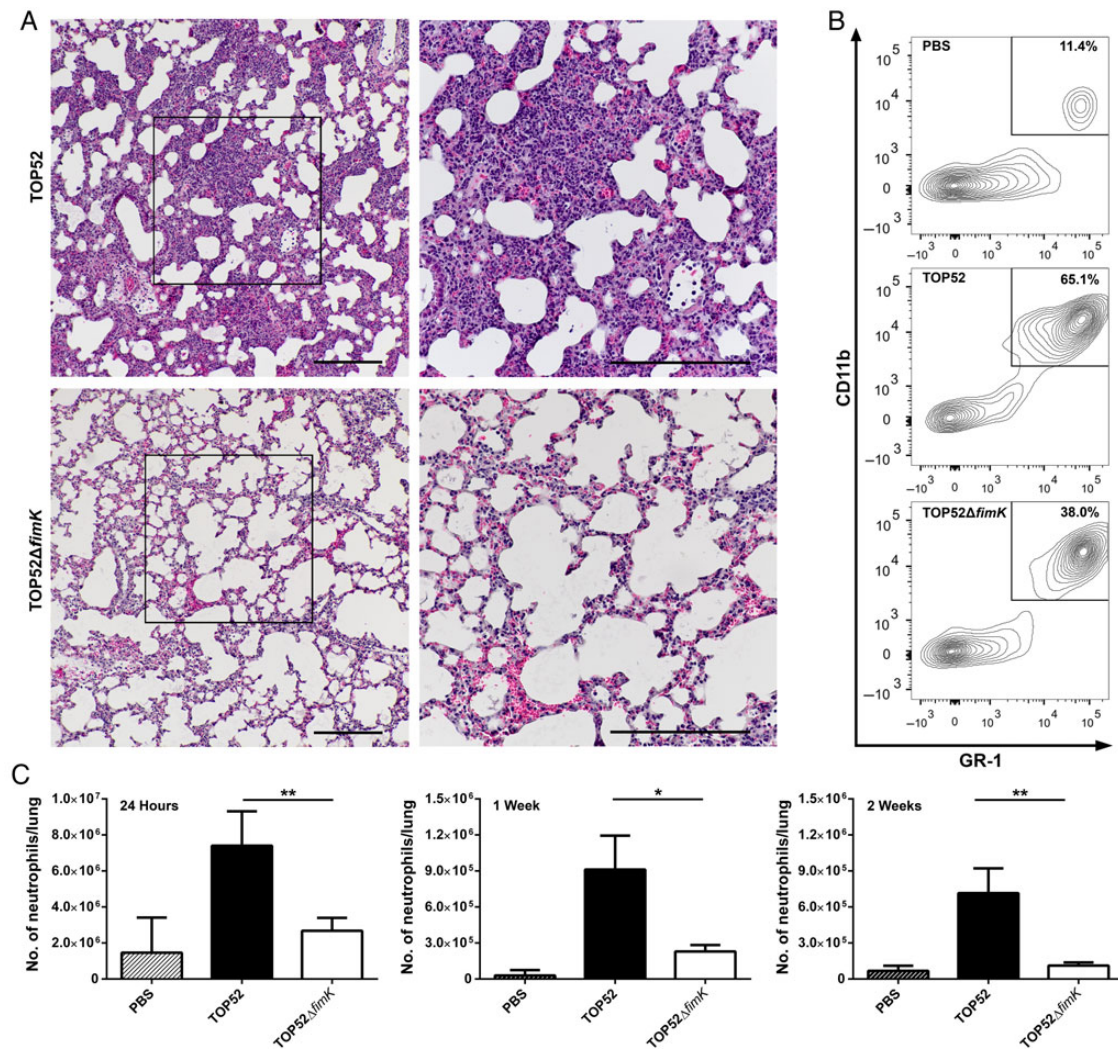


Figure 4. *Klebsiella pneumoniae* TOP52 elicits a more robust innate immune response in the lungs at 24 hours after infection than TOP52 Δ fimK. **A**, Representative histologic staining of murine lungs at 24 hours after infection with TOP52 (top panels) or TOP52 Δ fimK (bottom panels) reveals marked differences in consolidation and acute inflammatory infiltrate. Boxes indicate areas of increased magnification in right panels (scale bars, 200 μ m). **B**, Representative flow cytometry shows a higher proportion of neutrophils in lungs after TOP52 infection, compared with TOP52 Δ fimK infection. One representative plot shown for each group, with mean neutrophil percentages indicated from 33 mice across 3 independent experiments. **C**, Flow cytometric quantification demonstrates significantly higher absolute numbers of neutrophils in TOP52-infected mouse lungs (solid bars) than in TOP52 Δ fimK-infected lungs (open bars) at 24 hours, 1 week, and 2 weeks after infection. Data are shown as mean \pm standard error of the mean (* P < .05 and ** P < .01, by the Mann-Whitney U test). Abbreviation: PBS, phosphate-buffered saline.

those infected with TOP52 Δ fimK (Supplementary Figure 2A). Additionally, a significantly higher rate of bacteremia (80%) in C3^{-/-} mice 24 hours after infection was noted after infection with TOP52, while TOP52 Δ fimK blood cultures were all sterile (Supplementary Figure 2B). Thus, increased susceptibility of TOP52 Δ fimK to complement activity in vitro does not account for its diminished virulence within the murine lung.

Beyond its role in serum resistance, capsule mediates interactions between bacteria and host epithelial and immune cells [8, 9, 32]. To test for alterations in capsule production, TOP52, TOP52 Δ fimK, and 43816 were grown with and without the capsule inhibitor sodium salicylate [29], and uronic acid was extracted and quantified (Figure 6B). TOP52 produced

significantly more capsule than TOP52 Δ fimK and significantly less than 43816 ($P = .0022$ for each comparison). Both TOP52 and 43816 exhibited lower capsule production in the presence of sodium salicylate, as expected ($P = .0022$). However, sodium salicylate induced no further decrement in capsule production by TOP52 Δ fimK.

To determine whether decreased capsule in TOP52 Δ fimK might allow early immune clearance in vivo, we quantified phagocytosis by host leukocytes in BAL fluid of mice 3 hours after infection with FITC-labeled TOP52 or TOP52 Δ fimK (Figure 6C). Only 2.3% of alveolar macrophages isolated from TOP52 infections were FITC positive (indicating uptake of bacteria), compared with 15.3% of macrophages from

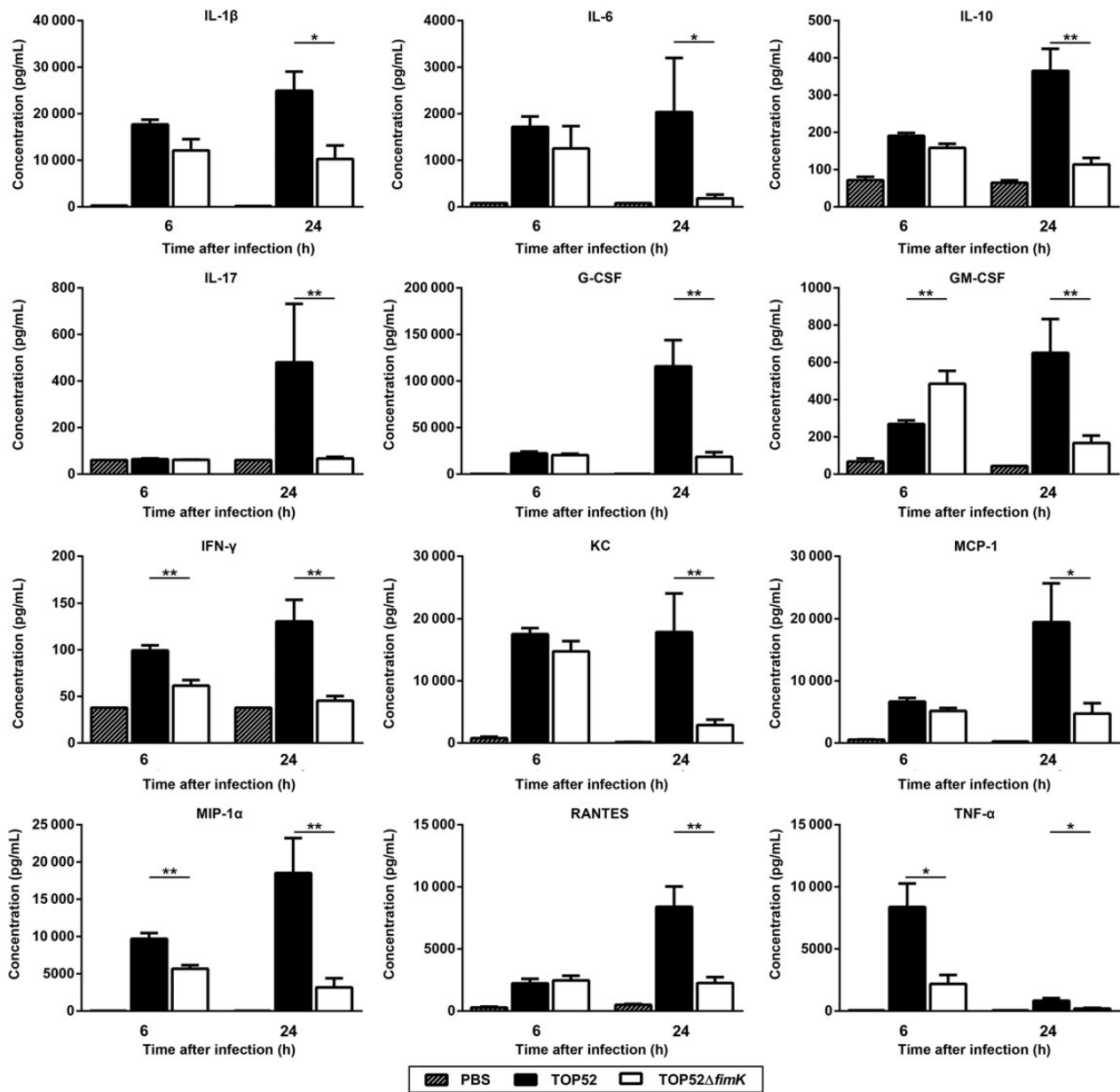


Figure 5. *Klebsiella pneumoniae* TOP52-infected lungs have an increased proinflammatory cytokine response at 24 hours after infection, relative to TOP52ΔfimK-infected lungs. TOP52-infected (solid bars), TOP52ΔfimK-infected (open bars), or phosphate-buffered saline (PBS) mock-infected (hatched bars) mouse lungs were harvested at 6 or 24 hours after infection, and homogenates were analyzed by multiplex bead array for cytokine content. Data were collected using organ homogenates from at least 3 independent mouse experiments and are shown as mean ± standard error of the mean (* $P < .05$ and ** $P < .01$, by the Mann–Whitney U test). Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN- γ , interferon γ ; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-10, interleukin 10; IL-17, interleukin 17; MCP-1, monocyte chemoattractant protein 1; MIP-1 α , macrophage inflammatory protein 1 α ; TNF- α , tumor necrosis factor α .

TOP52ΔfimK infections ($P < .0001$). A similar disparity was observed in neutrophils (1.8% vs 9.6%; $P = .0159$; data not shown). These data indicate that FimK positively influences production of capsule and impedes phagocytosis of *K. pneumoniae* in the lung.

DISCUSSION

K. pneumoniae is a versatile pathogen able to infect multiple host sites. *K. pneumoniae* as a species harbors substantial genomic diversity, but in contrast to *E. coli*, genomic content in most

K. pneumoniae strains does not reliably predict host tissue tropism [33]. Although pyogenic liver abscesses with systemic sequelae have been associated with *K. pneumoniae* expressing *magA*, *rmpA*, and other capsular determinants [34, 35], the genomic attributes required for virulence in other sites or for gastrointestinal tract colonization remain largely unknown. Hyperexpression of type 1 pili by *E. coli* may reduce bowel colonization [36], but this has not been shown for *K. pneumoniae*. In addition, *K. pneumoniae* environmental isolates may colonize the

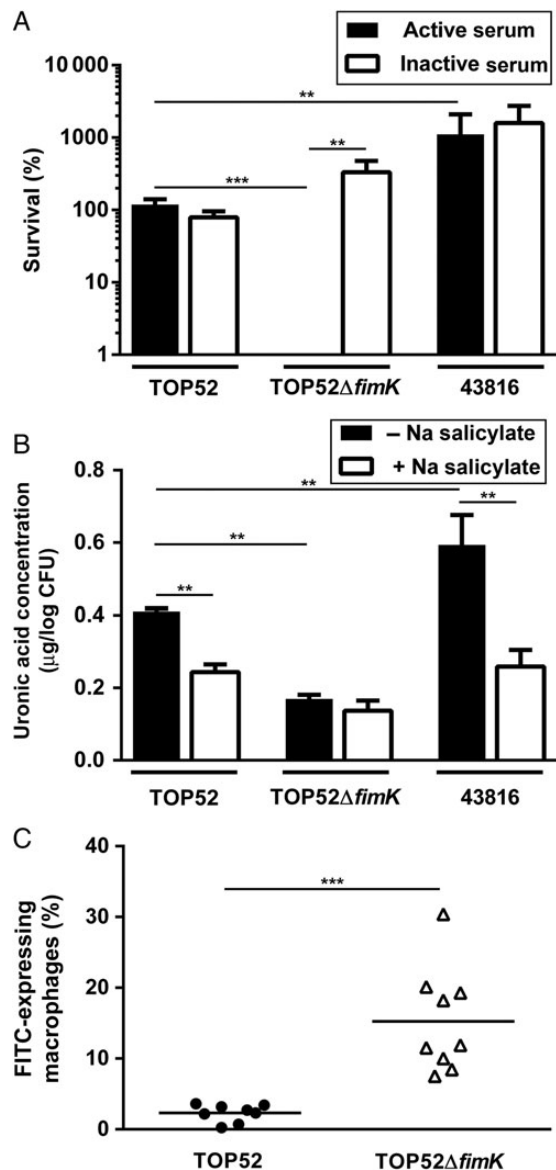


Figure 6. *Klebsiella pneumoniae* *fimK* promotes serum resistance, capsule production, and resistance to phagocytosis. *A*, In serum resistance assays, bacteria were incubated with pooled human active (solid bars) or inactivated (open bars) serum for 3 hours. Bacterial survival is expressed as a percentage of input colony-forming units (CFU). *B*, Capsule extraction and quantification was performed on bacteria grown with (open bars) and without (solid bars) the capsule inhibitor sodium (Na) salicylate. *C*, Mice were intratracheally inoculated with fluorescein isothiocyanate (FITC)-labeled bacteria, and bronchoalveolar lavage was performed at 3 hours after infection. The percentage of macrophages expressing FITC was determined by flow cytometry after infection with TOP52 (circles) or TOP52Δ*fimK* (triangles). All data are combined from 2 independent experiments and shown as mean or mean ± standard error of the mean (***P* < .01 and ****P* < .001, by the Mann-Whitney *U* test).

murine gastrointestinal tract and infect the urinary tract just as effectively as clinical isolates [37]. The present study demonstrates that a strain derived from the human urinary tract can cause murine pneumonia. Many *K. pneumoniae* isolates likely retain the genetic machinery required to thrive in multiple environments

into which they may be introduced. Past work demonstrated that the terminal gene in the type 1 pilus operon, *fimK*, reduces the virulence of *K. pneumoniae* in UTI [21]. Here we show that, in contrast, *fimK* carriage is imperative for virulence in the respiratory tract. Thus, *fimK* is not broadly deleterious and, in fact, confers a niche-specific survival advantage in the lung.

Deletion of *fimK* from *K. pneumoniae* TOP52 resulted in decreased bacterial loads, lower morbidity and mortality, and attenuated lung pathology and cytokine response during murine pneumonia. TOP52Δ*fimK* produced less capsule, which rendered it more susceptible to phagocytosis in vivo. This likely favors earlier clearance and diminished bacterial loads and, in turn, a less dramatic cellular inflammatory response. In contrast, lung titers of TOP52 at early time points were modestly higher than in the inoculum, reflecting replication (as opposed to simple persistence) of TOP52 in the lung. IFN-γ and TNF-α, levels of which were elevated in TOP52-infected lungs, represent important stimulators of activated proinflammatory macrophages [38, 39], which subsequently may fuel the globally increased proinflammatory milieu observed 24 hours after infection with TOP52. In turn, several locally produced chemokines promote neutrophil recruitment to the lungs (eg, KC and MIP-2) [40–42]. The amplified cellular response elicited by TOP52 persisted through 2 weeks, at which time it likely contributes to tissue repair.

The mechanisms by which FimK promotes pathogenesis in the lungs are not yet fully elucidated. FimK enhances production of capsule, an important virulence determinant in the lung [8–10]. As host C3 deficiency did not rescue TOP52Δ*fimK*, the contribution of capsule within the lung is likely in resistance to phagocytosis rather than to complement activity. Of note, previous work has proposed that fimbria and capsule may be counterregulated or exhibit steric interference in *K. pneumoniae* [43, 44]. FimK decreases expression of type 1 pili in TOP52 [21], which are critical for uropathogenesis but dispensable for virulence in the lung [18]. Wang et al showed that FimK in *K. pneumoniae* CG43S3 may instead increase expression of type 1 pili; however, *fimB* was overexpressed in this strain [45], which would bypass the known ability of FimK to influence orientation of the *fimS* promoter [21]. In our new studies, we confirmed that *fimK* deletion yields increased FimA expression (by Western blot; data not shown) in TOP52, as was shown previously [21]. FimK might also regulate other virulence factors important for pneumonia pathogenesis. FimK contains a predicted N-terminal helix-turn-helix domain, which may bind regions of DNA, and a C-terminal EAL domain, characteristic of phosphodiesterases that cleave the second messenger cyclic di-GMP [45, 46]. Ongoing work continues to investigate the broader range of *K. pneumoniae* virulence properties influenced by FimK.

Murine pulmonary infection with TOP52 provides an alternative preclinical model organism that contrasts with 43816.

First, the histologic features of TOP52-infected lungs 24 hours after infection parallel those observed in human disease [47, 48]. Additionally, infection after intratracheal inoculation with TOP52 is largely survivable and contained to the respiratory tract; conversely, inoculation with 43816 (which causes mortality likely due to sepsis rather than respiratory failure) may be more valuable in studying disseminated *K. pneumoniae* infection. Compared with 100% bacteremia 24 hours after infection with 43816, only 11% of TOP52-infected mice were bacteremic, a proportion similar to that observed in human pneumonia (7%–15%) [49, 50]. Of note, the TOP52 genome was recently sequenced, facilitating additional genetic manipulation and genomic comparisons [19]. TOP52 may thus prove a more useful model strain for studying specific aspects of pneumonia pathogenesis (eg, adaptive immune responses). Continued work will provide an improved understanding of *Klebsiella* virulence mechanisms and avenues for inhibition, informing efforts to combat this versatile and increasingly antibiotic-resistant pathogen.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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References

- Bakker-Woudenberg IA, van den Berg JC, Vree TB, et al. Relevance of serum protein binding of cefoxitin and cefazolin to their activities against *Klebsiella pneumoniae* pneumonia in rats. *Antimicrob Agents Chemother* **1985**; 28:654–9.
- Podschn R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Micro Rev* **1998**; 11:589–603.
- Rock C, Thom KA, Masnick M, et al. Frequency of *Klebsiella pneumoniae* carbapenemase (KPC)-producing and non-KPC-producing *Klebsiella* species contamination of healthcare workers and the environment. *Infect Cont Hosp Epidemiol* **2014**; 35:426–9.
- Munoz-Price LS, Poirer L, Bonomo RA, et al. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis* **2013**; 13:785–96.
- Paterson DL, Ko WC, Von Gottberg A, et al. Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. *Clin Infect Dis* **2004**; 39:31–7.
- Santino I, Bono S, Nuccitelli A, et al. Microbiological and molecular characterization of extreme drug-resistant carbapenemase-producing *Klebsiella pneumoniae* isolates. *Int J Immunopathol Pharmacol* **2013**; 26:785–90.
- Campos MA, Vargas MA, Regueiro V, et al. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* **2004**; 72:7107–14.
- Tomas JM, Benedi VJ, Ciurana B, et al. Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect Immun* **1986**; 54:85–9.
- Kabha K, Nissimov L, Athamna A, et al. Relationships among capsular structure, phagocytosis, and mouse virulence in *Klebsiella pneumoniae*. *Infect Immun* **1995**; 63:847–52.
- Lawlor MS, Handley SA, Miller VL. Comparison of the host responses to wild-type and *cpsB* mutant *Klebsiella pneumoniae* infections. *Infect Immun* **2006**; 74:5402–7.
- Fodah RA, Scott JB, Tam HH, et al. Correlation of *Klebsiella pneumoniae* comparative genetic analyses with virulence profiles in a murine respiratory disease model. *PLoS One* **2014**; 9:e107394.
- Lery LM, Frangeul L, Tomas A, et al. Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biol* **2014**; 12:41.
- Llobet E, March C, Gimenez P, et al. *Klebsiella pneumoniae* OmpA confers resistance to antimicrobial peptides. *Antimicrob Agents Chemother* **2009**; 53:298–302.
- Maroncle N, Rich C, Forestier C. The role of *Klebsiella pneumoniae* urease in intestinal colonization and resistance to gastrointestinal stress. *Res Microbiol* **2006**; 157:184–93.
- Murphy CN, Mortensen MS, Krogfelt KA, et al. Role of *Klebsiella pneumoniae* type 1 and type 3 fimbriae in colonizing silicone tubes implanted into the bladders of mice as a model of catheter-associated urinary tract infections. *Infect Immun* **2013**; 81:3009–17.
- Rosen DA, Pinkner JS, Walker JN, et al. Molecular variations in *Klebsiella pneumoniae* and *Escherichia coli* FimH affect function and pathogenesis in the urinary tract. *Infect Immun* **2008**; 76:3346–56.
- Shankar-Sinha S, Valencia GA, Janes BK, et al. The *Klebsiella pneumoniae* O antigen contributes to bacteremia and lethality during murine pneumonia. *Infect Immun* **2004**; 72:1423–30.
- Struve C, Bojer M, Krogfelt KA. Characterization of *Klebsiella pneumoniae* type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence. *Infect Immun* **2008**; 76:4055–65.
- Johnson JG, Spurbeck RR, Sandhu SK, et al. Genome sequence of *Klebsiella pneumoniae* urinary tract isolate Top52. *Genome Announc* **2014**; 2:e00668–14.
- Rosen DA, Hung CS, Kline KA, et al. Streptozocin-induced diabetic mouse model of urinary tract infection. *Infect Immun* **2008**; 76:4290–8.
- Rosen DA, Pinkner JS, Jones JM, et al. Utilization of an intracellular bacterial community pathway in *Klebsiella pneumoniae* urinary tract infection and the effects of FimK on type 1 pilus expression. *Infect Immun* **2008**; 76:3337–45.
- Lau HY, Clegg S, Moore TA. Identification of *Klebsiella pneumoniae* genes uniquely expressed in a strain virulent using a murine model of bacterial pneumonia. *Microb Pathog* **2007**; 42:148–55.
- Lavender HF, Jagnow JR, Clegg S. Biofilm formation in vitro and virulence in vivo of mutants of *Klebsiella pneumoniae*. *Infect Immun* **2004**; 72:4888–90.
- Lawlor MS, Hsu J, Rick PD, et al. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. *Mol Micro* **2005**; 58:1054–73.
- Deng JC, Zeng X, Newstead M, et al. STAT4 is a critical mediator of early innate immune responses against pulmonary *Klebsiella* infection. *J Immunol* **2004**; 173:4075–83.
- Hung CS, Dodson KW, Hultgren SJ. A murine model of urinary tract infection. *Nat Protoc* **2009**; 4:1230–43.
- Podschn R, Sievers D, Fischer A, et al. Serotypes, hemagglutinins, siderophore synthesis, and serum resistance of *Klebsiella* isolates causing human urinary tract infections. *J Infect Dis* **1993**; 168:1415–21.
- Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. *Anal Biochem* **1973**; 54:484–9.
- Domenico P, Schwartz S, Cunha BA. Reduction of capsular polysaccharide production in *Klebsiella pneumoniae* by sodium salicylate. *Infect Immun* **1989**; 57:3778–82.
- Lin TL, Yang FL, Yang AS, et al. Amino acid substitutions of MagA in *Klebsiella pneumoniae* affect the biosynthesis of the capsular polysaccharide. *PLoS One* **2012**; 7:e46783.
- Deady LE, Todd EM, Davis CG, et al. L-plastin is essential for alveolar macrophage production and control of pulmonary pneumococcal infection. *Infect Immun* **2014**; 82:1982–93.
- Favre-Bonte S, Joly B, Forestier C. Consequences of reduction of *Klebsiella pneumoniae* capsule expression on interactions of this bacterium with epithelial cells. *Infect Immun* **1999**; 67:554–61.
- Kumar V, Sun P, Vamathevan J, et al. Comparative genomics of *Klebsiella pneumoniae* strains with different antibiotic resistance profiles. *Antimicrob Agents Chemother* **2011**; 55:4267–76.

34. Fang CT, Chuang YP, Shun CT, et al. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med* **2004**; 199:697–705.
35. Yu WL, Ko WC, Cheng KC, et al. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. *Clin Infect Dis* **2006**; 42:1351–8.
36. McCormick BA, Klemm P, Krogfelt KA, et al. *Escherichia coli* F-18 phase locked 'on' for expression of type 1 fimbriae is a poor colonizer of the streptomycin-treated mouse large intestine. *Microb Pathog* **1993**; 14:33–43.
37. Struve C, Krogfelt KA. Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. *Environ Microbiol* **2004**; 6:584–90.
38. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* **2004**; 21:467–76.
39. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **2008**; 8:958–69.
40. Craig A, Mai J, Cai S, Jeyaseelan S. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect Immun* **2009**; 77:568–75.
41. Driscoll KE, Hassenbein DG, Howard BW, et al. Cloning, expression, and functional characterization of rat MIP-2: a neutrophil chemoattractant and epithelial cell mitogen. *J Leukoc Biol* **1995**; 58:359–64.
42. Wolpe SD, Sherry B, Juers D, et al. Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci USA* **1989**; 86:612–6.
43. Schembri MA, Blom J, Krogfelt KA, et al. Capsule and fimbria interaction in *Klebsiella pneumoniae*. *Infect Immun* **2005**; 73:4626–33.
44. Matatov R, Goldhar J, Skutelsky E, et al. Inability of encapsulated *Klebsiella pneumoniae* to assemble functional type 1 fimbriae on their surface. *FEMS Microbiol Lett* **1999**; 179:123–30.
45. Wang ZC, Huang CJ, Huang YJ, et al. FimK regulation on the expression of type 1 fimbriae in *Klebsiella pneumoniae* CG43S3. *Microbiol* **2013**; 159:1402–15.
46. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **2009**; 7:263–73.
47. Okada F, Ando Y, Honda K, et al. Clinical and pulmonary thin-section CT findings in acute *Klebsiella pneumoniae* pneumonia. *Eur Radiol* **2009**; 19:809–15.
48. Bullowa JGM, Chess J, Friedman NB. Pneumonia due to *Bacillus friedlanderi*. *Arch Intern Med* **1937**; 60:735–52.
49. Metersky ML, Ma A, Bratzler DW, et al. Predicting bacteremia in patients with community-acquired pneumonia. *Am J Resp Crit Care* **2004**; 169:342–7.
50. Waterer GW, Wunderink RG. The influence of the severity of community-acquired pneumonia on the usefulness of blood cultures. *Resp Med* **2001**; 95:78–82.