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MAJOR ARTICLE

A Novel Role for the *Klebsiella pneumoniae* Sap (Sensitivity to Antimicrobial Peptides) Transporter in Intestinal Cell Interactions, Innate Immune Responses, Liver Abscess, and Virulence

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Klebsiella pneumoniae is an important human pathogen causing hospital-acquired and community-acquired infections. Systemic *K. pneumoniae* infections may be preceded by gastrointestinal colonization, but the basis of this bacterium's interaction with the intestinal epithelium remains unclear. Here, we report that the *K. pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter contributes to bacterial–host cell interactions and in vivo virulence. Gene deletion showed that *sapA* is required for the adherence of a *K. pneumoniae* blood isolate to intestinal epithelial, lung epithelial, urinary bladder epithelial, and liver cells. The Δ*sapA* mutant was deficient for translocation across intestinal epithelial monolayers, macrophage interactions, and induction of proinflammatory cytokines. In a mouse gastrointestinal infection model, Δ*sapA* yielded significantly decreased bacterial loads in liver, spleen and intestine, reduced liver abscess generation, and decreased mortality. These findings offer new insights into the pathogenic interaction of *K. pneumoniae* with the host gastrointestinal tract to cause systemic infection.

Keywords. *Klebsiella pneumoniae*; Sap transporter; liver abscess; intestinal cell; virulence.

Klebsiella pneumoniae is an important human pathogen that can cause hospital-acquired and community-acquired infections [1–3]. This organism causes nosocomial infections, including septicemia, pneumonia, urinary tract infections, surgical site infections, and catheter-related infections. A type of community-acquired *K. pneumoniae* that is associated with pyogenic liver abscess (PLA) has emerged. This invasive infection is often complicated by metastatic infections, such as meningitis and endophthalmitis. Diabetes mellitus, a predisposing factor, has been detected in about 50% of patients with PLA [3–5]. Mortality rates are 10% for patients with *K. pneumoniae* PLA alone [6] and 30%–40% for those with metastatic meningitis [7, 8]. *K. pneumoniae* strains with the K1 and K2 capsular types have been identified as the most prevalent in PLA [9, 10].

K. pneumoniae is a member in the human gut microbiota. Many *K. pneumoniae* infections may be preceded by gastrointestinal colonization [11–15]. Notably, strains isolated from patients with liver abscess and bacteremia possess genotypes similar to strains obtained from gastrointestinal reservoirs [12, 13]. An earlier study by Hsu et al [16] demonstrated that *K. pneumoniae* bacteremia and PLA isolates are able to invade and translocate across the intestinal epithelium via a transcellular pathway, a process that involves manipulation of host signaling machinery. These results imply that *K. pneumoniae* that have colonized the gut use this transcellular pathway to penetrate the intestinal barrier and access extraintestinal locations, thereby causing systemic infections. However, the mechanistic and molecular details of *K. pneumoniae* interactions with host intestinal epithelial cells remain unclear. The role of cell interactions in *K. pneumoniae* virulence needs to be elucidated.

The Sap (sensitivity to antimicrobial peptides) transporter of bacteria is a multifunctional inner membrane protein complex that belongs to the adenosine triphosphate–binding-cassette (ABC) transport family [17, 18]. Sap transporters were first identified in *Salmonella* based on the ability to transport antimicrobial peptides (AMPs); these peptides, which often demonstrate bactericidal activity, serve as a component of host innate immunity [17]. The Sap transporter consists of 5 proteins. SapA is a periplasmic substrate binding protein, SapB and SapC are permease proteins that form a pore in the bacterial inner membrane, and SapD and SapF provide the adenosine triphosphatase activity of the transporter. The Sap transporter confers resistance to human AMPs in several gram-negative pathogens, such as *Salmonella*

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enterica serovar Typhimurium, nontypeable *Haemophilus influenzae, Proteus mirabilis, Escherichia coli, Vibrio fischeri,* and *Erwinia chrysanthemi* [19–24]. Moreover, in *Haemophilus,* the Sap transporter was reported to transport heme iron for heme homeostasis [25]. However, the function of the Sap transporter in *K. pneumoniae* has not, to our knowledge, been reported. Notably, it remains unclear whether the Sap transporter is involved in *K. pneumoniae*–host cell interactions or in *K. pneumoniae* virulence.

In the current study, we demonstrated that the Sap transporter contributes to *K. pneumoniae* interactions with host intestinal cells. Screening of a mutant library disclosed that *sapA* is involved in the adherence of a blood isolate to human intestinal epithelial cells. Gene deletion and complementation showed that the Sap transporter promoted *K. pneumoniae* translocation across the intestinal epithelial monolayer, macrophage interactions, and cytokine induction. A mouse model of gastrointestinal infection demonstrated that Sap mediated bacterial colonization, liver abscess formation, and mouse mortality. Collectively, the *K. pneumoniae* Sap transporter is important for epithelial interactions, host innate immune responses, and in vivo virulence.

METHODS

Bacterial Strains and Plasmids

K. pneumoniae Ca0437 is a clinical isolate originally obtained from the blood of a patient with septicemia [26]. *K. pneumoniae* and *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C. The bacterial strains, plasmids, and primers used in this study are listed in Supplementary Table 1.

Adherence, Invasion, and Translocation Assays

Caco-2 human intestinal epithelial cells and A549 human lung epithelial cells were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% nonessential amino acids (Gibco). HepG2 human hepatocytes were grown in Dulbecco's modified Eagle medium/F12 medium (1:1) supplemented with 10% FBS. T24 human urinary bladder epithelial cells were grown in McCoy's 5a medium supplemented with 10% FBS. All cells were determined to be mycoplasma free using a polymerase chain reaction (PCR) Mycoplasma Detection Kit (Biosmart). Cells at passages 15–35 were used for all assays. The adherence assays and invasion assays of *K. pneumoniae* were performed as described elsewhere [16, 27]. Details of these 2 assays and library screening via adherence assays are described in the Supplementary Methods.

Translocation assays were used to assess the potential ability of *K. pneumoniae* to penetrate the intestinal epithelium, based on the method described elsewhere [16, 28]. Caco-2 cells can form tight polarized monolayers and differentiate to generate tight junctions (TJs) and a brush border, mimicking the human intestinal epithelium [28]. Caco-2 cells were grown on Transwell inserts (3-μm pore size and 0.33-cm^2 filtration area) for 15 days to form tight polarized monolayers, assessed by transepithelial electrical resistance >300 Ω/cm^2 [28]. Differentiation of Caco-2 monolayers was also confirmed by immunofluorescence staining of TJs (Supplementary Methods). In translocation assays, *K. pneumoniae* were added to the apical side of Caco-2 monolayers in the upper chambers of the Transwell inserts and incubated at 37°C for 1 hour. The numbers of viable bacteria in the upper and lower chambers were determined by plating appropriate dilutions on LB medium.

Macrophage Adherence, Phagocytosis, Cytokine Induction, and Serum Resistance Assays

RAW 264.7 murine macrophages were grown in RPMI 1640 medium supplemented with 10% FBS. *K. pneumoniae* adherence and phagocytosis by macrophages were determined according to methods described elsewhere [29]. For cytokine induction [30], RAW 264.7 cells were infected with *K. pneumoniae* for 1 hour at 37°C, washed with phosphate-buffered saline, and then incubated in gentamicin-containing medium. Culture supernatants were collected at various time points as indicated in Figure 3C. The levels of proinflammatory cytokines tumor necrosis factor (TNF) α and interleukin 6 (IL-6) were measured using enzyme-linked immunosorbent assay kits (R&D Systems), according to the manufacturer's instructions. Serum resistance assays to determine the survival ratio of *K. pneumoniae* in human serum were performed as described elsewhere [31]. Values ≥1 were defined as serum resistance.

Mouse Inoculation

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the E-Da Hospital (IACUC-105013). All animal procedures were conducted in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* [32] from the National Institutes of Health and Taiwan's Animal Protection Act.

K. pneumoniae in vivo virulence was evaluated by means of intragastric inoculation in a murine model. To analyze the survival of infected mice [33], we inoculated 5-week-old female BALB/cByl mice intragastrically with 4×10^8 colony-forming units (CFUs) of mid–log phase *K. pneumoniae* and monitored them for 30 days. Their in vivo bacterial load was determined as described elsewhere [33], by testing at either 24 or 72 hours after inoculation. Briefly, tissues were homogenized, and appropriate dilutions were plated for counting of recovered CFUs. For in vivo competition assays [34], the test strain (wild type (WT) or Δ*sapA*) was mixed with the fully virulent isogenic *lacZ* promoter deletion mutant (Δp*lacZ*) at a 1:1 ratio. Each BALB/cByl mouse was given an intragastric inoculation of 1×10^8 CFUs in 100 μL of saline solution. At 24 hours after inoculation, the liver and spleen were removed and homogenized in 1× phosphate-buffered saline; bacteria were recovered by plating appropriate dilutions on LB plates containing 1 mmol/mL isopropyl β-D-1-thiogalactopyranoside (IPTG) and 50 μg/mL X-Gal. The number of LacZ-positive (blue) and LacZ-negative (white) colonies were counted.

The competitive index was defined as (test strain output/Δp*lacZ* output)/(test strain input/Δp*lacZ* input); the resulting ratio was interpreted as the in vivo colonization ability [34].

Histological Analysis

Five-week-old female BALB/cByl mice (4 per group) were infected intragastrically with *K. pneumoniae* at a dose of 1×10^8 CFUs per mouse for 72 hours or 6×10^8 CFUs for 120 hours. The liver and colon were retrieved, fixed in 10% formalin, and embedded in paraffin blocks. Sections were prepared at 5–10-μm thicknesses and stained with hematoxylin-eosin. Stained tissue sections were imaged under a light microscope for blinded evaluation and quantification by the pathologist. The number of abscesses was quantified in five low-power fields (×40 magnification). The total fields for observation equaled 118.79 mm^2 (diameter, 5.5 mm).

Statistical Analyses

Data are presented as means with standard errors of the mean (SEM). Statistical significance was assessed using analysis of variance followed by a post hoc multiple comparisons test performed with Prism 5 software (GraphPad). Mouse survival was analyzed by means of Kaplan-Meier analysis using a log-rank test with Bonferroni correction. Differences were considered significant at $P < .05$.

RESULTS

Identification of Adherence-Deficient Mutants From a *K. pneumoniae* Mutant Library

To identify genes involved in *K. pneumoniae* adherence to enterocytes, we constructed a transposon mutant library of bacteremia-inducing strain Ca0437 (Supplementary Methods). A total of 2450 *K. pneumoniae* mutants were collected and screened by means of adherence assays using Caco-2 cells, with a Ca0437 WT parent and an inadherent strain 2 084 069 as controls (Supplementary Methods and Supplementary Table 1). Eight adherence-deficient mutants showing reduced adherence rates (compared with WT) were identified (Supplementary Figure 1); transposon insertion sites were determined (Table 1). Mutants harboring insertions in *hns, sapA,* and *kpn_02265* were initially selected for further study, including verification by construction of in-frame unmarked deletion mutations in an isogenic background (Supplementary Methods).

Contribution of *hns* and *sapA* to *K. pneumoniae* Adherence to Different Epithelial Cells

First, we assessed host cell adherence by the Δ*hns,* Δ*sapA,* and Δ*kpn_02265* gene deletion mutants. Specifically, the bacterial mutants were tested for adherence to 4 types of cells (Figure 1). Compared with the WT strain, the Δ*hns* and Δ*sapA* strains showed significantly decreased adherence to all tested cell types. The Δ*kpn_02265* strain exhibited decreased adherence to Caco-2 cells but no significant reduction in adherence to HepG2, A549, or T24 cells. A recent parallel study of a *K. pneumoniae* pneumonia–causing isolate found that *hns,* which encodes the putative global gene regulator H-NS, contributed to adherence [29]. The remainder of the present study focused on *sapA*.

We tested whether the defects in adherence to the colonic epithelial cells could be overcome by genetic complementation (Supplementary Methods). Complementation of Δ*sapA* with *sapABC* (3 genes) provided significant restoration of adherence to Caco-2 cells (Figure 2A; Δ*sapA*+*sapABC*), although complementation of Δ*sapA* with *sapA* alone (Δ*sapA*+*sapA*) was not sufficient to restore the defective function. Reverse-transcription quantitative PCR (Supplementary Methods) showed that, in the Δ*sapA* mutant, the expression levels of *sapA, sapB,* and *sapC* were all decreased compared with WT

Table 1. Identification of Cell Adherence-Deficient Mutants from the *Klebsiella pneumoniae* Ca0437 Mutant Library

Mutant No.	Tn-Inserted Gene[a]	Predicted Gene Function[b]	Accession No.	Amino Acid Ssequence Similarity, %	Relative Adherence, Mean (SEM), %[c]
8-1G	*hflX*	GTPase HflX	CDL63628.1	100	57.3 (4.4)
9-6E	*hns*	DNA-binding protein H-NS	CP002910.1	97	0.8 (0.8)
9-10E	*sapA*	Antimicrobial peptide ABC transporter periplasmic binding protein SapA	CP002910.1	96	18.4 (5.9)
11-2A	*kpn_02265*	Putative transmembrane protein containing GGDEF domain	WP_002887653	100	18.1 (3.1)
12-1B	*trkA*	Potassium transporter peripheral membrane protein	WP_002919153.1	97	15.7 (11.5)
21-11A	*kpn_25946*	Hypothetical protein (plasmid pKCTC2242)	AEK01010.1	93	53.7 (11.7)
25-12G	*kpn_07830*	Murein L,D-transpeptidase	WP_048333314.1	100	67.3 (19.4)
26-6B	*degP/htrA*	Serine endoprotease	WP_004177363.1	100	29.5 (10.2)

Abbreviations: ABC, adenosine triphosphate–binding-cassette; GTPase, guanosinetriphosphatase; SEM, standard error of the mean; Tn, transposon.

[a]Compared with the *Klebsiella pneumoniae* KCTC2242 complete genome from the National Center for Biotechnology Information (NCBI) as the template genome.

[b]Analysis performed with the NCBI Basic Local Alignment Search Tool.

[c]Compared with the ca0437 wild-type strain (100%).

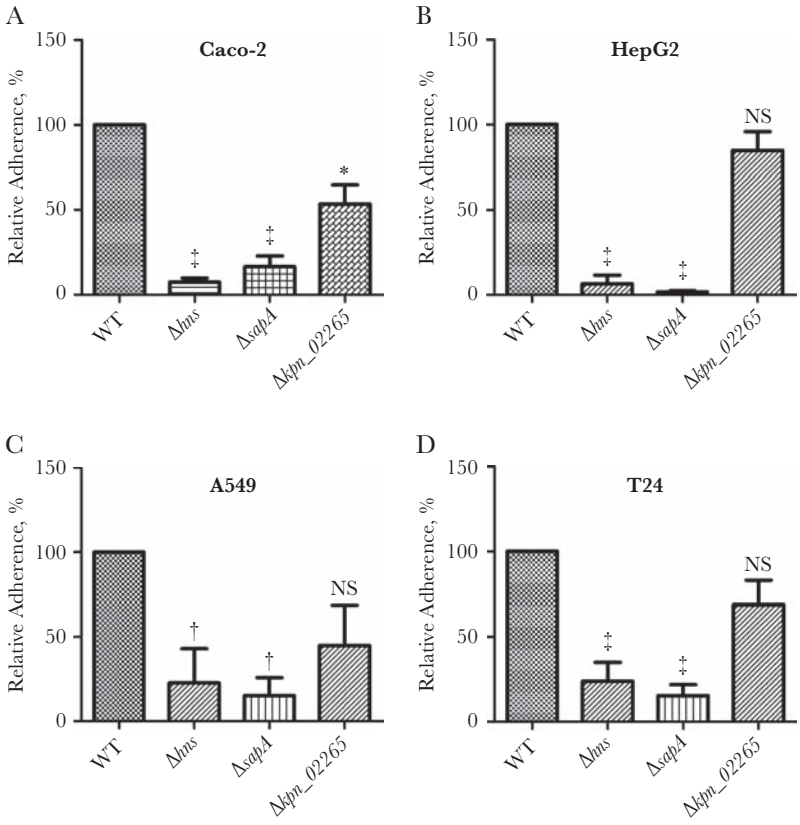

Figure 1. Adherence of *Klebsiella pneumoniae* Ca0437 wild type (WT) and Δ*hns*, Δ*sapA*, and Δ*kpn_02265* deletion mutants to various cell lines, including colonic epithelial cells Caco-2 (*A*), hepatocytes HepG2 (*B*), lung epithelial cells A549 (*C*), and urinary bladder epithelial cells T24 (*D*). Mid–log phase *K. pneumoniae* were added to cells of the indicated cell line, and the mixture was incubated for 15 minutes, washed with phosphate-buffered saline wash, lysed with Triton X-100, and plated for colony-forming unit counts. The adherence rates of the isogenic mutants were normalized to that of the WT parent strain (defined as 100%). Bars represent the mean and standard error of the mean from ≥3 independent experiments. **P* < .05; †*P* < .01; ‡*P* < .001 (comparison with WT; analysis of variance followed by Dunnett multiple comparisons test). Abbreviation: NS, not significant.

(Supplementary Figure 2*A*). Expression of these 3 genes was rescued by complementation with *sapABC* (Δ*sapA*+*sapABC*); complementation with *sapA* alone (Δ*sapA*+*sapA*) did not restore the decreased expression of *sapB* and *sapC* observed with Δ*sapA*. No significant differences in bacterial growth were observed among WT, Δ*sapA*, and Δ*sapA*+*sapABC* strains (Supplementary Figure 2*B*). Adherence assays using the differentiated Caco-2 monolayers also showed that *sapA* deletion caused the decrease of adherence, with complementation by Δ*sapA*+*sapABC* (Supplementary Figure 3*A* and 3*B*).

Effect of the Sap Transporter in Promoting *K. pneumoniae* Translocation Across Intestinal Epithelium

Confocal microscopy showed that *K. pneumoniae* Ca0437 WT invaded and was internalized into Caco-2 cells (Figure 2B). Invasion assays revealed that the invasive index (after normalization for adherence) [35] of Δ*sapA* did not differ significantly from that of the WT strain (Figure 2C), although the relative invasion rate (without normalization) was lower (Supplementary Figure 4).

Translocation assays were used to determine whether *sapA* deletion influenced *K. pneumoniae* translocation across Caco-2–polarized epithelial monolayers on the Transwell inserts. Ca0437 WT translocated across Caco-2 monolayers, such that bacteria were recovered from the medium in the lower chamber (Figure 2D). In contrast, Δ*sapA* exhibited a defect in transepithelial translocation. The Δ*sapA*+*sapABC* complementation strain counteracted the translocation defect.

Sap Transporter Enhancement of *K. pneumoniae*–Macrophage Interactions and Cytokine Induction

Macrophage adherence assays showed that Δ*sapA* was significantly less adherent to Raw 264.7 macrophages, with relative adherence 2.8% that of the WT strain (Figure 3A). *K. pneumoniae* phagocytosis by macrophages was diminished in Δ*sapA*, with relative phagocytosis 0.5% that of the WT strain (Figure 3B). For both macrophage adherence and phagocytosis assays, the Δ*sapA*+*sapABC* complementation strain exhibited a recovery from the defects (Figure 3A and 3B).

The influence of the *sapA* deletion on the proinflammatory responses was assessed. Enzyme-linked immunosorbent assay showed that Ca0437 WT induced secretion of the cytokines TNF-α and IL-6 (Figure 3C). However, Δ*sapA* yielded decreased

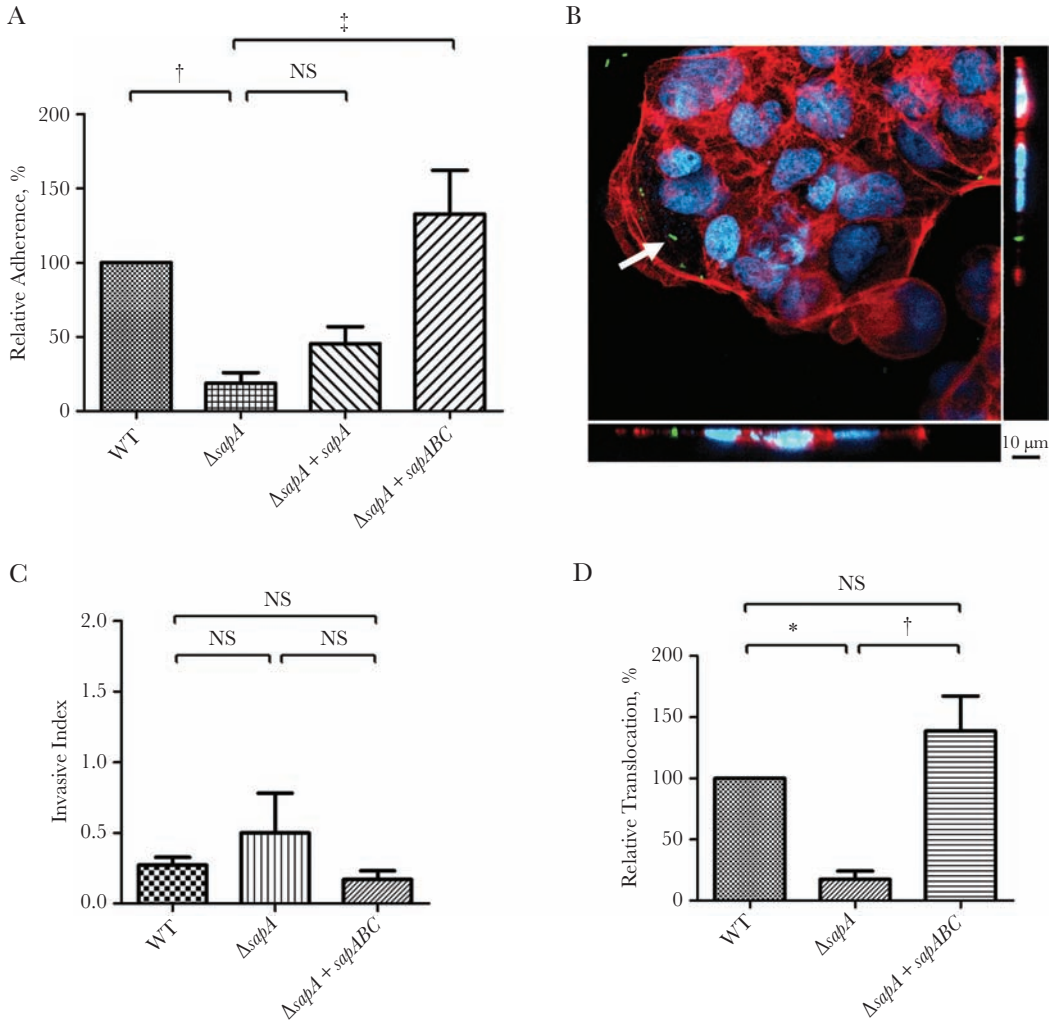

Figure 2. The *Klebsiella pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter promotes intestinal cell adherence and epithelial translocation. *A,* Adherence assays showing complementation of Δ*sapA* in *K. pneumoniae*. Human Caco-2 cells were infected with *K. pneumoniae* Ca0437 wild type (WT), Δ*sapA* deletion mutant, Δ*sapA*+*sapA* complementation strain, or Δ*sapA*+*sapABC* complementation strain. Genetic complementation of *sapABC* in *K. pneumoniae* Δ*sapA* strain (Δ*sapA*+*sapABC*) overcame Δ*sapA*-associated defects in cell adherence. Bars represent the mean and standard error of the mean (SEM) from ≥3 independent experiments. *B,* Three-dimensional confocal micrograph showing invasion into Caco-2 intestinal epithelial cells by *K. pneumoniae* Ca0437. Cells were infected with *K. pneumoniae* Ca0437 carrying pCRII-TOPO::green fluorescent protein (*green*) for 2 hours. The actin cytoskeleton and DNA were stained with rhodamine-phalloidin (*red*) and 4',6-diamidino-2-phenylindole (*blue*). Images from confocal microscopy with Z-stacking were analyzed by XY, XZ, and YZ sections. Intracellular *K. pneumoniae* is indicated by white arrows. *C,* Invasion assays into intestinal cells. Invasion of Caco-2 cells by *K. pneumoniae* Ca0437 WT, Δ*sapA*, or Δ*sapA*+*sapABC* complementation strain was quantified using a gentamicin-based assay (Supplementary Methods). The invasive index (number of invaded bacterial colony-forming units [CFUs] divided by the number of adherent bacterial CFUs) of strains was expressed [35]. Bars represent the mean and SEM from 4 independent experiments *D,* Translocation assays across intestinal epithelial monolayers. Caco-2 cells were grown on Transwell inserts for 15 days to form a tight polarized monolayer; monolayers then were infected with *K. pneumoniae* Ca0437 WT, Δ*sapA*, or Δ*sapA*+*sapABC* strains for 1 hour, followed by recovery and quantification of CFUs of in the lower chambers (translocated bacteria). Bars represent the mean and SEM from 4 independent experiments. **P* < .05; †*P* < .01; ‡*P* < .001 (analysis of variance followed by Bonferroni multiple comparisons test). Abbreviation: NS, not significant.

induction (compared with WT) of both TNF-α and IL-6. Serum resistance of the Δ*sapA* mutant did not differ significantly from that of the WT strain (Figure 3D).

Influence of the Sap Transporter on Type I Fimbriae Expression and AMP Resistance

Fimbriae (pili) and capsular polysaccharides have been implicated in *K. pneumoniae* adherence [27, 36]. We compared the expression of type I fimbrial genes (*fimA* and *fimC*), type III fimbrial genes (*mrkA* and *mrkD*), and *galF* in the capsular polysaccharide synthesis (*cps*) gene cluster (Figure 4A). Reverse-transcription quantitative PCR showed that *fimA* and *fimC* expression was significantly decreased in Δ*sapA* (compared with the WT strain). The expression of *mrkA* and *mrkD*, and that of the *cps* gene *galF,* was not significantly changed in Δ*sapA*.

We characterized the functions of the *K. pneumoniae* Sap transporter in AMPs interactions and heme use. We tested cathelicidin LL-37 and human β-defensin 2, 2 human AMPs

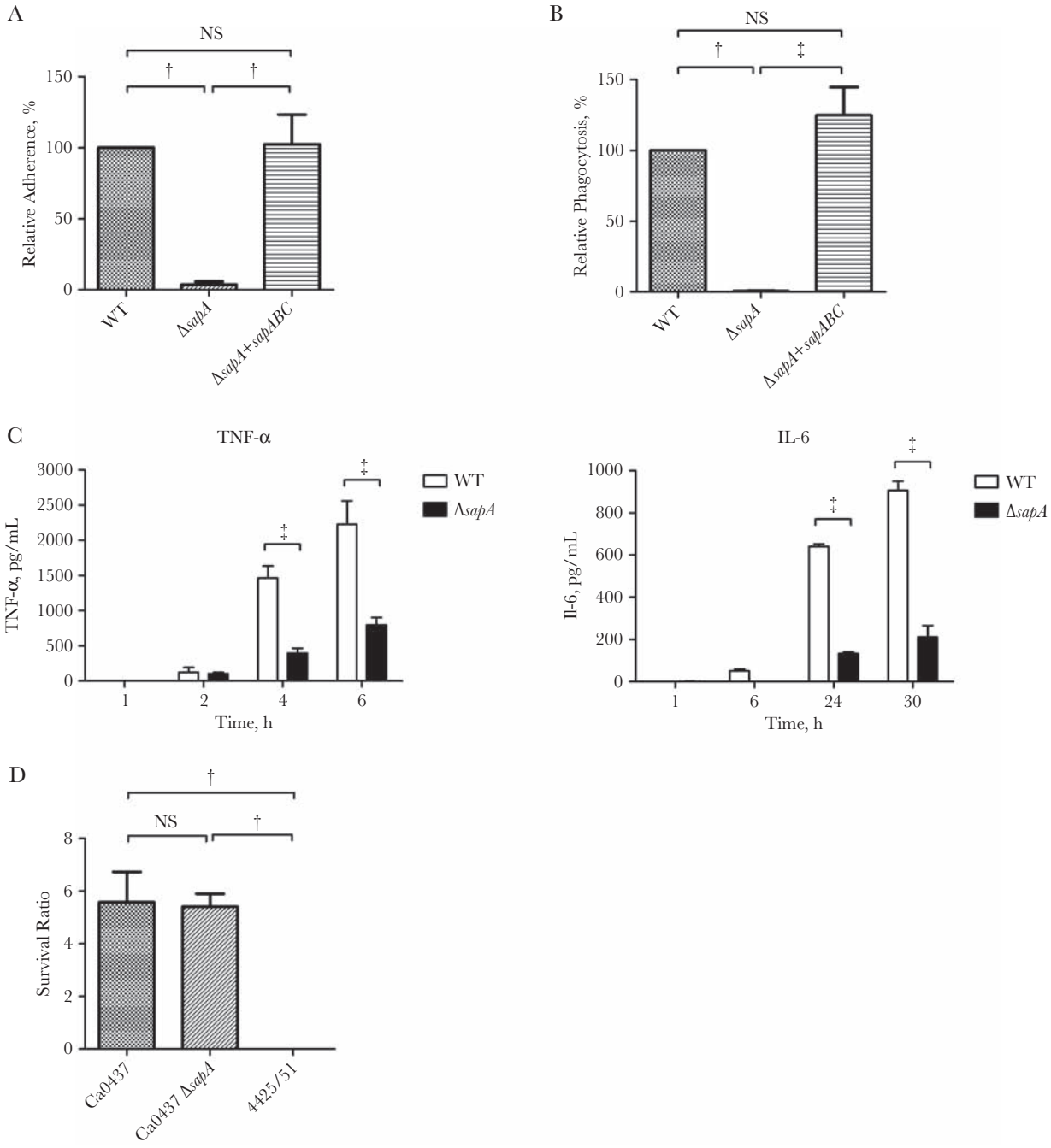

Figure 3. The *Klebsiella pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter contributes to macrophage adherence, phagocytosis, and secretion of proinflammatory cytokines. *A,* Macrophage adherence assays. Mid–log phase *K. pneumoniae* Ca0437 wild type (WT), Δ*sapA*, or Δ*sapA*+*sapABC* was added to RAW 264.7 macrophages in 24-well plates for 1-hour incubation at 4°C, followed by phosphate-buffered saline wash, Triton X-100 lysis of eukaryotic cells, and colony-forming unit (CFU) recovery for plate counts. *B,* Phagocytosis assays. RAW 264.7 macrophages were infected by mid–log phase *K. pneumoniae* Ca0437 WT, Δ*sapA*, and Δ*sapA*+*sapABC* for 2 hours at 37°C. Phagocytosed bacteria cells then were quantified using a gentamicin-based assay (see Materials and Methods). *C,* Induction of cytokines tumor necrosis factor (TNF) α and interleukin 6 (IL-6). RAW 264.7 macrophages were incubated with *K. pneumoniae* Ca0437 WT (*white bars*) or Δ*sapA* (*black bars*), and the levels of secreted cytokines in the medium were measured at the indicated time points. *D,* Serum resistance assays. The indicated strains of *K. pneumoniae* were incubated with human serum (75%) at 37°C for 1 hour. The numbers of recovered CFUs were determined, and the survival ratio was expressed as recovered CFUs/inoculum CFUs. *K. pneumoniae* Ca0437 WT strain and the Ca0437 Δ*sapA* mutant were both serum resistant. *K. pneumoniae* 4425/51 is a serum-sensitive strain for comparison [30]. Throughout figure, data represent the mean and standard error of the mean from 3 independent experiments. †$P < .01$; ‡$P < .001$ (analysis of variance followed by Bonferroni multiple comparisons test). Abbreviation: NS, not significant.

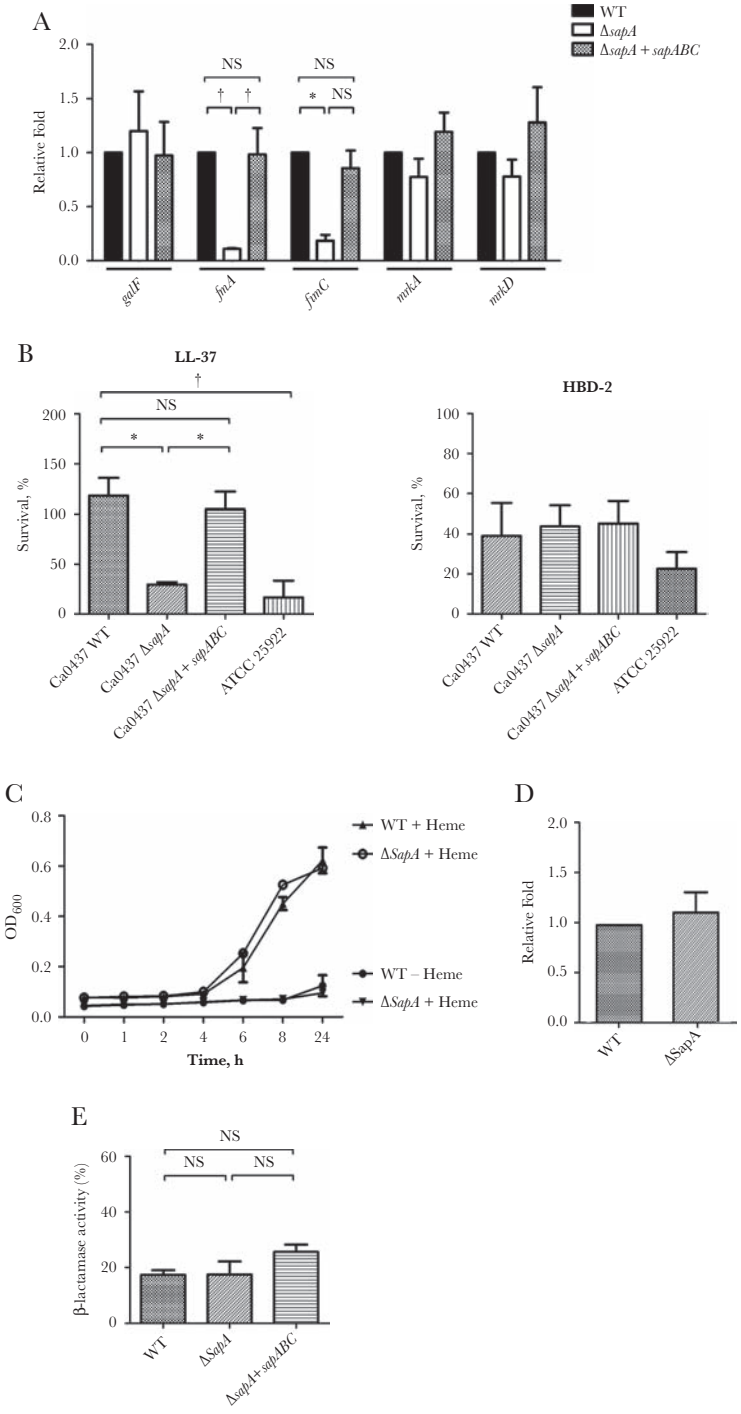

Figure 4. The *Klebsiella pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter influences transcriptional expression of type I fimbriae and antimicrobial peptide (AMP) resistance of *K. pneumoniae. A,* Quantitative real-time expression of capsular polysaccharide synthesis (*cps*) and fimbrial genes. The *cps* gene *galF,* type I fimbrial genes *fimA* and *fimC,* and type III fimbrial genes *mrkA* and *mrkD* in Ca0437 wild type (WT), Δ*sapA,* and the complementation strain Δ*sapA*+*sapABC* were determined with reverse-transcription quantitative polymerase chain reaction (RT-qPCR). *B,* Sensitivity tests of *K. pneumoniae* to human AMP LL-37 (*left*) and human β-defensin 2 (HBD-2) (*right*). *K. pneumoniae* Ca0437 WT, Δ*sapA*, Δ*sapA*+*sapABC,* and *Escherichia coli* American Type Culture Collection (ATCC) 25922 were incubated with LL-37 (2 μg/mL) or HBD-2 (0.5 μg/mL), respectively. The survival of bacteria (percentage of colony-forming units of control wells not exposed to AMP) is shown. For HBD-2, there were no significant differences between all tested strains. *C,* Heme use of *K. pneumoniae* Ca0437 WT and Δ*sapA*. Bacteria were starved for heme iron for 24 hours and then shifted to DIS cultures supplemented with (+ heme, 10 μg/mL) or without heme (– heme). Growth was assessed by monitoring the increase in culture absorbance (optical density at 600 nm [OD_{600}]) at the time points indicated. *D,* Quantitative expression of heme system gene in *K. pneumoniae* Ca0437 WT and Δ*sapA*. RNA transcripts of *hmuR* were determined with real-time RT-qPCR. There were no significant differences between WT and Δ*sapA* (Student *t* test). *E,* Outer-membrane permeability of *K. pneumoniae*. The leakage of the periplasmic enzyme β-lactamase into the culture supernatants of Ca0437 WT, Δ*sapA,* and Δ*sapA*+*sapABC* was determined. Throughout figure, data represent the mean and standard error of the mean from 3 independent experiments. **P* < .05; †*P* < .01 (analysis of variance followed by Bonferroni multiple comparisons test. Abbreviation: NS, not significant.

present in the gut epithelium [37]. Although *K. pneumoniae* Ca0437 WT was resistant against LL-37, Δ*sapA* became sensitive (Figure 4B, *left*). No differences in the susceptibility to human β-defensin 2 were observed between Ca0437 WT, Δ*sapA,* Δ*sapA*+*sapABC,* and *E. coli* American Type Culture Collection 25922 (Figure 4B, *right*). Involvement of the *K. pneumoniae* Sap transporter in heme iron use was not observed (Figure 4C). Although heme iron–starved WT bacteria failed to grow in heme iron–depleted medium, growth was restored when cultured in the presence of heme iron. Similar to WT, heme iron–starved Δ*sapA* was able to use heme to grow again in the presence of heme. Expression of the heme system *hmuR* gene in Δ*sapA* did not differ significantly from that in the WT strain (Figure 4D). Outer membrane permeability was similar in WT, Δ*sapA,* and Δ*sapA*+*sapABC* (Figure 4E), indicating that *sapA* deletion might not cause a broad disruption of the outer membrane. In conclusion, the Sap transporter in *K. pneumoniae*–mediated bacterial resistance against AMP LL-37, but its effects on heme acquisition were not observed.

Contribution of the Sap Transporter to *K. pneumoniae* Virulence and Liver Abscess Formation In Vivo

We used a murine gastrointestinal infection model to investigate the role of the Sap transporter in *K. pneumoniae* virulence. Mouse survival was monitored after intragastric inoculation. By 30 days after infection, 7 of 8 WT-infected mice had died (survival rate, 12.5%), compared with 1 of 8 Δ*sapA*-infected mice (survival rate, 87.5%); the virulence of Δ*sapA* was significantly attenuated (Figure 5A, WT vs Δ*sapA*). The complementation strain caused death in 5 of 8 mice (survival rate, 37.5%), not significantly different from the WT strain (Figure 5A, WT vs Δ*sapA*+*sapABC*).

We examined bacterial loads in the infected mice (Figure 5B). Compared with those in WT-infected mice, recovered colony counts in the Δ*sapA*-infected mice were significantly reduced in both liver and spleen, suggesting that the bacterial ability to survive and spread in the host was attenuated. Similar results were observed in male mice (Supplementary Figure 5). In addition, in vivo competition assays showed that the Δ*sapA* mutant had a lower competitive index (Δ*sapA*/Δp*lacZ*) than the WT strain (WT/Δp*lacZ*) (Figure 5C). To clarify the role of the Sap transporter in intestinal colonization in vivo, we quantified *K. pneumoniae* in the colorectum of infected mice (Figure 5D). Compared with the WT strain, intestinal colonization was significantly decreased in the Δ*sapA* mutant. These data indicate that the *K. pneumoniae* Sap transporter mediates in vivo colonization and survival. Gene complementation restored the deficiency of Δ*sapA* in vivo (Figure 5B–5D, Δ*sapA*+*sapABC*).

Histopathological evaluation of the livers from Ca0437 WT–infected mice at 72 hours after infection revealed the frequent presence of either macroabscesses (Figure 6A, *upper left*) or microabscesses (Figure 6A, *upper middle and right*). In contrast, only scattered microabscesses were observed in Δ*sapA*-infected mice (Figure 6A, *lower left*). The numbers of liver abscesses were 13, 23, 49, 27 (mean [SEM], 28 [15.2]) in 4 WT-infected mice, and 0, 7, 0, 14 (5.3 [6.7]) in 4 Δ*sapA*-infected mice (WT vs Δ*sapA*, $P = .04$; Student *t* test). Therefore, the liver abscess numbers induced by Δ*sapA* were significantly decreased. The histopathological appearance of the colon was unremarkable in these 2 groups of mice 72 hours after infection (Figure 6A, *lower middle and right*). Similar results were seen with liver histopathology 120 hours after infection (Figure 6B). In WT-infected mice, gross liver abscesses were observed (Figure 6B, *left*); microscopically, macroabscesses and microabscesses with rupture causing peritonitis were noted (Figure 6B, *middle*). In contrast, fewer microabscesses were identified in Δ*sapA*-infected mice, and the liver capsule was intact without peritonitis (Figure 6B, *right*). In conclusion, the Sap transporter promotes *K. pneumoniae* induction of liver abscess.

DISCUSSION

Results of epidemiological studies suggest that many *K. pneumoniae* infections, including bacteremia and PLA, may originate from gastrointestinal reservoirs [11–15]. However, it remains largely unknown how *K. pneumoniae* interacts with host intestinal epithelium. The present study demonstrated that a bacteremia-causing isolate induced liver abscess in a mouse gastrointestinal infection model. We revealed a novel role for the *K. pneumoniae* Sap transporter in bacterial–intestinal cell interactions and virulence, suggesting that *K. pneumoniae*–intestinal interplay contributes to pathogenesis.

The *K. pneumoniae* Sap transporter functions in interactions with the host cells. In agreement with our findings, a study of nontypeable *H. influenzae* showed that a *sapA* mutant was less adherent and invasive to cultured bronchial and middle ear epithelial cells [38]. We observed that the *K. pneumoniae* Sap transporter was required for adherence to human liver cells and a variety of epithelial cells, including intestinal, lung, and urinary bladder epithelial cells. The Sap transporter also contributed to *K. pneumoniae* translocation across the intestinal epithelium. It also promoted *K. pneumoniae*–macrophage interactions and host secretion of proinflammatory cytokines. Classically, the Sap transporter was identified based on AMP resistance in several gram-negative bacteria [17–24] and heme acquisition in *H. influenzae* [25]. In *K. pneumoniae*, we observed involvement of the Sap transporter in bacterial resistance against AMP LL-37 but not in heme iron use. Whether the *K. pneumoniae* Sap transporter mediates acquisition of other iron sources needs to be further assessed. It is involved in multiple functions relating to pathogenesis, including (at minimum) AMP resistance, epithelial cell attachment and translocation, macrophage interactions, and induction of host innate responses. This may serve as a target for drug design or vaccine development.

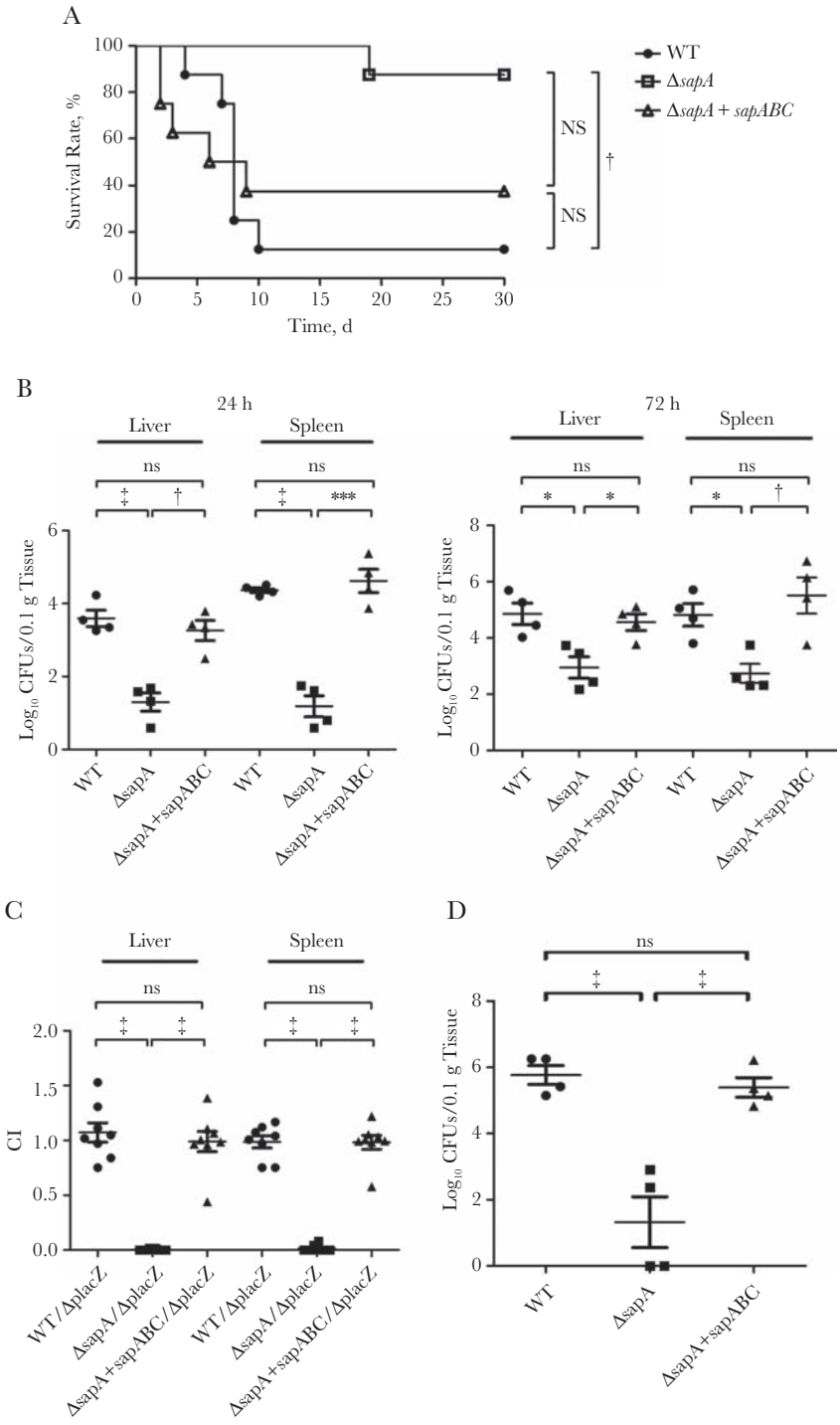

Figure 5. The *Klebsiella pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter contributes to the in vivo virulence of *K. pneumoniae* during gastrointestinal infection. *A,* Mouse survival analysis. Female BALB/cByl mice (8 per group) were infected intragastrically with *K. pneumoniae* Ca0437 wild type (WT), *sapA,* or Δ*sapA*+*sapABC* at a dose of 4×10^8 colony-forming units (CFUs) per mouse. Survival of mice was monitored for 4 weeks. *B,* In vivo bacterial loads in liver and spleen. WT, Δ*sapA* or Δ*sapA*+*sapABC* was administered intragastrically to female BALB/cByl mice (4 per group) at an inoculation dose of either 4×10^8 CFUs for 24 hours or 1×10^8 CFUs for 72 hours. Bacterial levels in liver and spleen were determined at 24 (*right*) or 72 (*left*) hours after infection. Bacterial numbers (expressed as $\log_{10}$ CFUs) were standardized per 0.1 g of wet organ weight. *C,* In vivo competition assays. Either test strain (WT, Δ*sapA,* or Δ*sapA*+*sapABC*) was compared with the Δp*lacZ* mutant strain in female BALB/cByl mice (8 per group) by intragastric inoculation with 1×10^8 CFUs per strain per mouse. After bacterial recovery on isopropyl β-D-1-thiogalactopyranoside (IPTG)/X-Gal plates, the ratio of LacZ-positive (blue) to LacZ-negative (white) colonies in the liver or spleen of each mouse was determined. The competitive index was defined as (Δp*lacZ* output/test strain output)/(Δp*lacZ* input/test strain input). Each symbol represents the index for each inoculum, and bars represent medians. *D,* Intestinal colonization in vivo. *K. pneumoniae* were administered intragastrically to each of female BALB/cByl mice (4 per group) at an inoculation dose of 4×10^8 CFUs. Bacterial numbers (expressed as $\log_{10}$ CFUs) in the colorectum were determined 72 hours after infection. **P* < .05; †*P* < .01; ‡ *P* < .001 (log-rank test [*A*] or analysis of variance [*B–D*] with Bonferroni multiple comparisons test). Abbreviation: NS, not significant.

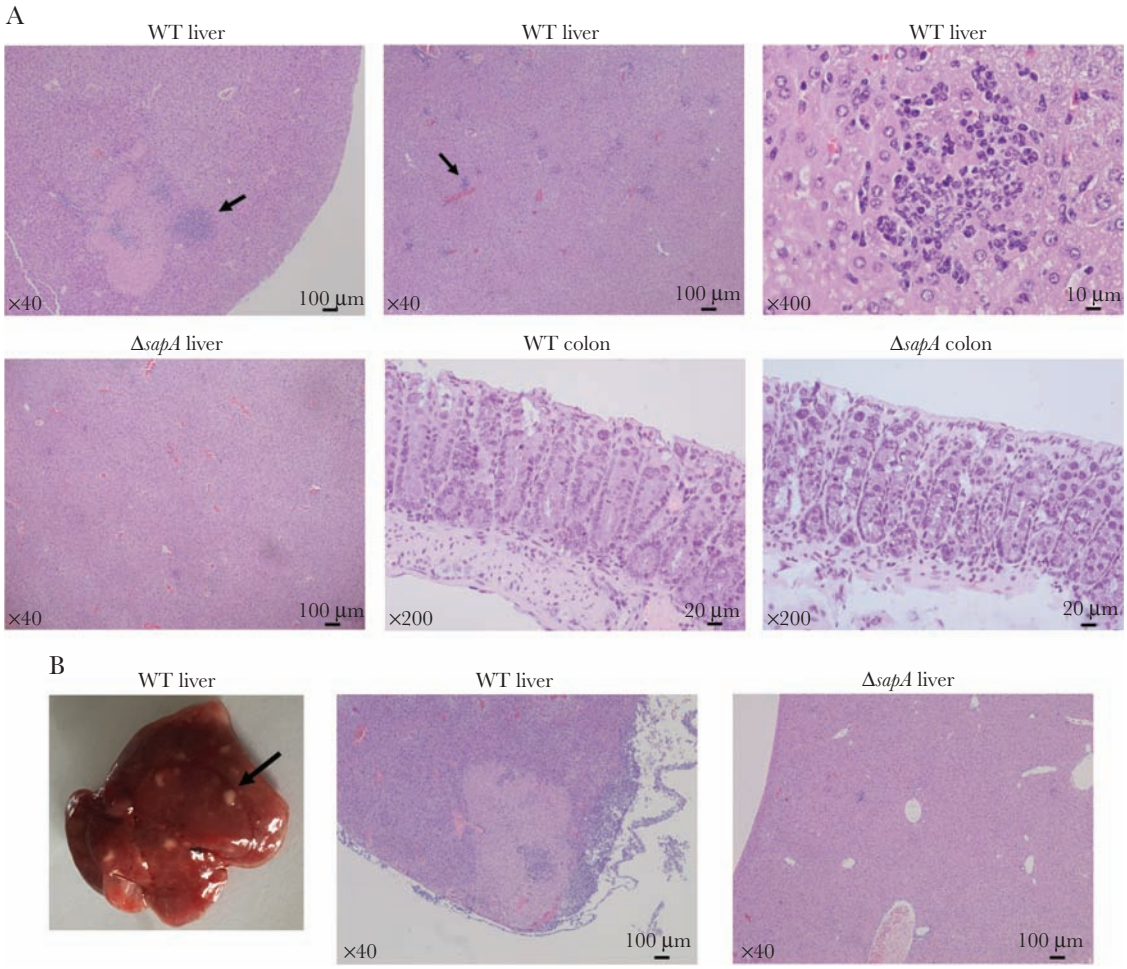

Figure 6. Histopathological examination of tissues from mice infected with *Klebsiella pneumoniae*–infected mice. BALB/cByl mice were infected intragastrically with *K. pneumoniae* Ca0437 wild type (WT) or the isogenic Δ*sapA* mutant (Δ*sapA*). Representative micrographs of hematoxylin-eosin–stained sections from the liver or colon retrieved at 72 or 120 hours after infection are shown. *A,* Findings 72 hours after infection (1×10^8 colony-forming units [CFUs] per mouse). In WT-infected mice, macroabscesses (*upper left panel,* arrow) and/or microabscesses (*upper middle,* arrow) were noted in the liver (original magnification, ×40); the high-power field (magnification, ×400) shows the microabscesses composed of neutrophils and lymphocytes (*upper right*). In the liver of Δ*sapA*-infected mice (*lower left*), only a few microabscesses were identified. The colon tissues of WT-infected mice (*lower middle*) and Δ*sapA*-infected mice (*lower right*) were both unremarkable under light microscopy. *B,* Findings 120 hours after infection (6×10^8 CFUs). In the WT-infected mice, the resected liver revealed grossly visible abscesses (*left panel, arrow*); microscopically, macroabscesses and microabscesses with rupture causing peritonitis were noted (*middle*). In the liver of Δ*sapA*-infected mice (*left*), only a few microabscesses were identified, and the liver capsule was intact without peritonitis. (All magnifications are original magnifications.)

Using a murine intragastric infection model, we demonstrated that the Sap transporter–mediated *K. pneumoniae* virulence. Consistent with our findings, the Sap transporter has been implicated in *H. influenzae* virulence [20]. In a chinchilla otitis media model, a Δ*sapA* mutant of nontypeable *H. influenzae* was significantly attenuated in its ability to survive in the nasopharynx and the middle ear. Our data demonstrated that *K. pneumoniae* Δ*sapA* was impaired in the ability to induce mouse death and maintain bacterial loads in liver, spleen, and intestine. Although Δ*sapA* is more resistant to macrophage recognition and killing, its abilities to adhere to and translocate across the intestinal epithelium and to resist AMP are attenuated. Overall in vivo virulence is reduced. Interactions with intestinal epithelium and AMP, which could happen in the earlier stage of infection, seem to be important for *K. pneumoniae* pathogenesis. Histopathological examination revealed that Δ*sapA* exhibited attenuated the ability to induce liver abscess. These data indicate that the Sap transporter is essential for *K. pneumoniae* to colonize, survive, and spread in the host, which contributes to systemic infection, liver abscess generation, and host death.

The molecular mechanisms whereby the *K. pneumoniae* Sap transporter contributes to cell adherence and cell interactions remain unclear. Our results revealed that Sap transporter promotes expression of genes encoding type I fimbriae. Thus, its effects on adherence may reflect changes in the production of other *K. pneumoniae* adhesive molecules. To our knowledge, the present work is the first study to demonstrate that a

Sap transporter affects the transcription levels of other genes, suggesting a novel role for it in the regulation of gene expression. Type I fimbria is an adhesin mediating the adherence of *K. pneumoniae* to ciliated tracheal cells in vitro [36] and a virulence factor in a mouse model of *K. pneumoniae* urinary tract infection [39, 40]. However, type I fimbriae do not affect the ability of *K. pneumoniae* to colonize the intestine or the lung in vivo [39]. Different from type I fimbriae, the Sap transporter contributed to gastrointestinal colonization and infection in vivo. We hypothesize that it may affect the production of other factors (other than type I fimbriae) involved in bacterial interactions with intestinal cells. Further investigations will be needed to identify *K. pneumoniae* genes influenced by the Sap transporter and to elucidate potential cross-talk or regulatory networks among the affected factors.

This study has some limitations. First, we used the human and mouse cell lines; primary cells were not used to confirm the results. Second, in vivo translocation of *K. pneumoniae* in intestine was not directly demonstrated. Third, the mutation of *sapA* seemed to be polar. The phenotype could be attributed to *sapA, sapB,* or *sapC*. Fourth, we did not use the differentiated Caco-2 monolayers for library screening or the pulmonary and urinary epithelial cells that generate TJs to mimic barrier functions for the adherence assays. The factors involved in the interactions with differentiated epithelium could be neglected.

In summary, we demonstrated in the current study, for the first time, that the Sap transporter contributes to *K. pneumoniae* pathogenesis. Our data showed that the *K. pneumoniae* Sap transporter mediates bacterial–host cell interactions, epithelial colonization, and virulence. This study offers new insights into the interactions of *K. pneumoniae* with host gastrointestinal cells and suggests possible mechanisms whereby gastrointestinal *K. pneumoniae* could escape the gut to cause systemic infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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