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

Chun-Ru Hsu,1,2 I-Wei Chang,3,4,5 Pei-Fang Hsieh,6 Tzu-Lung Lin,6 Pei-Yin Liu,6 Chen-Hsiu Huang,2 Kun-Tzu Li,1 and Jin-Town Wang6,7

* Departments of 1Medical Research and 2Pathology, E-Da Hospital, and 3School of Medicine for International Students, I-Shou University, Kaoshiung, Taiwan, and 4Department of Pathology, College of Medicine, Taipei Medical University, 5Department of Pathology, Taipei Medical University Hospital; 6Department of Microbiology, National Taiwan University College of Medicine; and 7Internal Medicine, National Taiwan University Hospital, Taipei

*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 ΔsapA mutant was deficient for translocation across intestinal epithelial monolayers, macrophage interactions, and induction of proinflammatory cytokines. In a mouse gastrointestinal infection model, ΔsapA 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.

Received 20 June 2018; editorial decision 13 October 2018; accepted 16 November 2018; published online November 23, 2018.

Correspondence: J.-T. Wang, Department of Microbiology, National Taiwan University College of Medicine, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan (wangjt@ntu.edu.tw).

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DOI: 10.1093/infdis/jiy615
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 and 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-cm² filtration area) for 15 days to form tight polarized monolayers, assessed by transepithelial electrical resistance >300 Ω/cm² [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 × 10⁸ 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 ΔsapA) was mixed with the fully virulent isogenic lacZ promoter deletion mutant (ΔplacZ) at a 1:1 ratio. Each BALB/cByl mouse was given an intragastric inoculation of 1 × 10⁸ 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/ΔlacZ output)/(test strain input/ΔlacZ 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 mm² (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 2084069 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*+Δ*p*ABC), although complementation of Δ*sapA* with *sapA* alone (Δ*sapA*+Δ*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 | Predicted Gene Function | Accession No. | Amino Acid Sequence Similarity, % | Relative Adherence, Mean (SEM), % |
|------------|------------------|-------------------------|---------------|----------------------------------|----------------------------------|
| 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| 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_02265*      | Hypothetical protein (plasmid pKCTC2242) | AEK01010.1    | 93                               | 53.7 (11.7)                      |
| 25-12G     | *kpn_0730*       | Murein L, D-metalloprotease | WP_048333314.1 | 100                              | 67.3 (19.4)                      |
| 26-6B      | *degP*           | Serine endoprotease      | WP_004177363.1 | 100                              | 29.5 (10.2)                      |

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

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 ΔsapA 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, ΔsapA exhibited a defect in transepithelial translocation. The ΔsapA+sapABC complementation strain counteracted the translocation defect.

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

Macrophage adherence assays showed that ΔsapA 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 ΔsapA, with relative phagocytosis 0.5% that of the WT strain (Figure 3B). For both macrophage adherence and phagocytosis assays, the ΔsapA+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, ΔsapA yielded decreased...
induction (compared with WT) of both TNF-α and IL-6. Serum resistance of the ΔsapA 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 ΔsapA (compared with the WT strain). The expression of *mrkA* and *mrkD*, and that of the *cps* gene *galF* was not significantly changed in ΔsapA.

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

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**Figure 2.** The *Klebsiella pneumoniae* Sap (sensitivity to antimicrobial peptides) transporter promotes intestinal cell adherence and epithelial translocation. A, Adherence assays showing complementation of ΔsapA in *K. pneumoniae*. Human Caco-2 cells were infected with *K. pneumoniae* Ca0437 wild type (WT), ΔsapA deletion mutant, ΔsapA+sapA complementation strain, or ΔsapA+sapABC complementation strain. Genetic complementation of sapABC in *K. pneumoniae* ΔsapA strain (ΔsapA+sapABC) overcame ΔsapA-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 pCRIL-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, ΔsapA, or ΔsapA+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, ΔsapA, or ΔsapA+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.
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), ΔsapA, or ΔsapA-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, ΔsapA, and ΔsapA-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 ΔsapA (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 ΔsapA 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 [OD600]) 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, ΔsapA became sensitive (Figure 4B, left). No differences in the susceptibility to human β-defensin 2 were observed between Ca0437 WT, ΔsapA, ΔsapA+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 ΔsapA was able to use heme to grow again in the presence of heme. Expression of the heme system hmuR gene in ΔsapA did not differ significantly from that in the WT strain (Figure 4D). Outer membrane permeability was similar in WT, ΔsapA, and ΔsapA+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 ΔsapA-infected mice (survival rate, 87.5%); the virulence of ΔsapA was significantly attenuated (Figure 5A, WT vs ΔsapA). 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 ΔsapA+sapABC).

We examined bacterial loads in the infected mice (Figure 5B). Compared with those in WT-infected mice, recovered colony counts in the ΔsapA-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 ΔsapA mutant had a lower competitive index (ΔsapA/ΔplacZ) than the WT strain (WT/ΔplacZ) (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 ΔsapA mutant. These data indicate that the *K. pneumoniae* Sap transporter mediates in vivo colonization and survival. Gene complementation restored the deficiency of ΔsapA in vivo (Figure 5B–5D, ΔsapA+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 ΔsapA-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 ΔsapA-infected mice (WT vs ΔsapA, *P* = .04; Student *t* test). Therefore, the liver abscess numbers induced by ΔsapA 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 ΔsapA-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 ΔsapA+sapABC at a dose of 4 × 10⁸ colony-forming units (CFUs) per mouse. Survival of mice was monitored for 4 weeks. B, In vivo bacterial loads in liver and spleen. WT, ΔsapA or ΔsapA+sapABC was administered intragastrically to female BALB/cByl mice (4 per group) at an inoculation dose of either 4 × 10⁸ CFUs for 24 hours or 1 × 10⁸ 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 log₁₀ CFUs) were standardized per 0.1 g of wet organ weight. C, In vivo competition assays. Either test strain (WT, ΔsapA, or ΔsapA+sapABC) was compared with the Δp*lacZ* mutant strain in female BALB/cByl mice (8 per group) by intragastric inoculation with 1 × 10⁸ 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 × 10⁸ CFUs. Bacterial numbers (expressed as log₁₀ 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.
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 ΔsapA 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* ΔsapA was impaired in the ability to induce mouse death and maintain bacterial loads in liver, spleen, and intestine. Although ΔsapA 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 ΔsapA exhibited attenuated 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

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**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 ΔsapA mutant (ΔsapA). 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 ΔsapA-infected mice (lower left), only a few microabscesses were identified. The colon tissues of WT-infected mice (lower middle) and ΔsapA-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 ΔsapA-infected mice (left), only a few microabscesses were identified, and the liver capsule was intact without peritonitis. (All magnifications are original magnifications.)
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
Acknowledgments. We thank Hung Hung, PhD and Hsien-Ho Lin, MD, ScD (both from the Institute of Epidemiology and Preventive Medicine, National Taiwan University) for advice and assistance with statistical analysis. We also thank Dai-Yun Du (from the I-Shou University) for assistance with AMP experiments and the Biomedical Engineering and Biotechnology Center of the I-Shou University for assistance with confocal microscopy and 3-dimensional image analysis.

Financial support. This study was supported by the Ministry of Science and Technology, National Taiwan University, National Taiwan University Hospital, E-Da Hospital, and the E-Da Hospital-National Taiwan University Hospital Joint Research Program.

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.

References
1. Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 1998; 11:589–603.
2. Ko WC, Paterson DL, Sagnimeni AJ, et al. Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. Emerg Infect Dis 2002; 8:160–6.
3. Tsai FC, Huang YT, Chang LY, Wang JT. Pyogenic liver abscess as endemic disease, Taiwan. Emerg Infect Dis 2008; 14:1592–600.
4. Fang CT, Lai SY, Yi WC, Hsueh PR, Liu KL, Chang SC. Klebsiella pneumoniae genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clin Infect Dis 2007; 45:284–93.
5. Chung DR, Lee SS, Lee HR, et al; Korean Study Group for Liver Abscess. Emerging invasive liver abscess caused by K1 serotype Klebsiella pneumoniae in Korea. J Infect 2007; 54:578–83.
6. Wang JH, Liu YC, Lee SS, et al. Primary liver abscess due to Klebsiella pneumoniae in Taiwan. Clin Infect Dis 1998; 26:1434–8.
7. Fang CT, Chen YC, Chang SC, Sau WY, Luh KT. Klebsiella pneumoniae meningitis: timing of antimicrobial therapy and prognosis. QJM 2000; 93:45–53.
8. Tang LM, Chen ST. Klebsiella pneumoniae meningitis: prognostic factors. Scand J Infect Dis 1994; 26:95–102.
9. Fung CP, Chang FY, Lee SC, et al. A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut 2002; 50:420–4.
10. Struve C, Bojer M, Nielsen EM, Hansen DS, Krogfelt KA. Investigation of the putative virulence gene magA in a worldwide collection of 495 Klebsiella isolates: magA is restricted to the gene cluster of Klebsiella pneumoniae capsule serotype K1. J Med Microbiol 2005; 54:1111–3.
11. De Champs C, Sauvant MP, Chanal C, et al. Prospective survey of colonization and infection caused by expanded-spectrum-beta-lactamase-producing members of the
family Enterobacteriaceae in an intensive care unit. J Clin Microbiol 1989; 27:2887–90.
12. Fung CP, Lin YT, Lin JC, et al. Klebsiella pneumoniae in gastrointestinal tract and pyogenic liver abscess. Emerg Infect Dis 2012; 18:1322–5.
13. Peña C, Pujol M, Ardanuy C, et al. Epidemiology and successful control of a large outbreak due to Klebsiella pneumoniae producing extended-spectrum beta-lactamases. Antimicrob Agents Chemother 1998; 42:53–8.
14. de Almeida VC, Pessoa-Silva CL, Sampaio JL, Gontijo Filho PP, Teixeira LM, Moreira BM. Genetic relatedness among extended-spectrum beta-lactamase-producing Klebsiella pneumoniae outbreak isolates associated with colonization and invasive disease in a neonatal intensive care unit. Microb Drug Resist 2005; 11:21–5.
15. Chung DR, Lee H, Park MH, et al. Fecal carriage of serotype K1 Klebsiella pneumoniae ST23 strains closely related to liver abscess isolates in Koreans living in Korea. Eur J Clin Microbiol Infect Dis 2012; 31:481–6.
16. Hsu CR, Pan YJ, Liu YJ, Chen CT, Lin TL, Wang JT. Klebsiella pneumoniae translocates across the intestinal epithelium via Rho GTPase- and phosphatidylinositol 3-kinase/Akt-dependent cell invasion. Infect Immun 2015; 83:769–79.
17. Parra-Lopez C, Lin R, Aspedon A, Groisman EA. A Salmonella protein that is required for resistance to antimicrobial peptides and transport of potassium. EMBO J 1994; 13:3964–72.
18. Mason KM, Bruggeman ME, Munson RS, Bakaletz LO. The non-typeable Haemophilus influenzae Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD-dependent potassium acquisition. Mol Microbiol 2006; 62:1357–72.
19. Parra-Lopez C, Baer MT, Groisman EA. Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in Salmonella typhimurium. EMBO J 1993; 12:4053–62.
20. Mason KM, Munson RS Jr, Bakaletz LO. A mutation in the sap operon attenuates survival of nontypeable Haemophilus influenzae in a chinchilla model of otitis media. Infect Immun 2005; 73:599–608.
21. McCoy AJ, Liu H, Falla TJ, Gunn JS. Identification of Proteus mirabilis mutants with increased sensitivity to antimicrobial peptides. Antimicrob Agents Chemother 2001; 45:2030–7.
22. Harms C, Domoto Y, Celik C, et al. Identification of the ABC protein SapD as the subunit that confers ATP dependence to the K+-uptake systems TrkK and TrkG from Escherichia coli K-12. Microbiology 2001; 147:2991–3003.
23. Chen HY, Weng SF, Lin JW. Identification and analysis of the sap genes from Vibrio Fischeri belonging to the ATP-binding cassette gene family required for peptide transport and resistance to antimicrobial peptides. Biochem Biophys Res Commun 2000; 269:743–8.
24. López-Solanilla E, García-Olmedo F, Rodríguez-Palenzuela P. Inactivation of the sapA to sapP locus of Erwinia chrysanthemi reveals common features in plant and animal bacterial pathogenesis. Plant Cell 1998; 10:917–24.
25. Mason KM, Raffel FK, Ray WC, Bakaletz LO. Heme utilization by nontypeable Haemophilus influenzae is essential and dependent on Sap transporter function. J Bacteriol 2011; 193:2527–35.
26. Hsu CR, Lin TL, Chen YC, Chou HC, Wang JT. The role of Klebsiella pneumoniae rmpA in capsular polysaccharide synthesis and virulence revisited. Microbiology 2011; 157:3446–57.
27. Sahly H, Podschn R, Oelschlaeger TA, et al. Capsule impedes adhesion to and invasion of epithelial cells by Klebsiella pneumoniae. Infect Immun 2000; 68:6744–9.
28. Finlay BB, Falkow S. Salmonella interactions with polarized human intestinal Caco-2 epithelial cells. J Infect Dis 1990; 162:1096–106.
29. Ares MA, Fernández-Vázquez JL, Rosales-Reyes R, et al. HNS nucleoid protein controls virulence features of Klebsiella pneumoniae by regulating the expression of type 3 pil and the capsule polysaccharide. Front Cell Infect Microbiol 2016; 6:13.
30. Hsu CR, Liao CH, Lin TL, et al. Identification of a capsular variant and characterization of capsular acetylation in Klebsiella pneumoniae PLA-associated type K57. Sci Rep 2016; 6:31946.
31. Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A novel virulence gene in Klebsiella pneumoniae strains causing primary liver abscess and septic metastatic complications. J Exp Med 2004; 199:697–705.
32. National Research Council. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press. 2011.
33. Pan YJ, Lin TL, Hsu CR, Wang JT. Use of a dictyostelium model for isolation of genetic loci associated with phagocytosis and virulence in Klebsiella pneumoniae. J Infect Dis 2010; 201:52–64.
34. Hsieh PF, Lin HH, Lin TL, Wang JT. CadC regulates cad and tdc operons in response to gastrointestinal stresses and enhances intestinal colonization of Klebsiella pneumoniae. J Exp Med 2011; 190:997–1006.
35. Vornhagen J, Armistead B, Santana-Ufret V, et al. Group A streptococcus exploits vaginal epithelial exfoliation for ascending infection. J Clin Invest 2018; 128:1985–9.
36. Fader RC, Gondesen K, Tolley B, Ritchie DG, Moller P. Evidence that in vitro adherence of Klebsiella pneumoniae to ciliated hamster tracheal cells is mediated by type 1 fimbriae. Infect Immun 1998; 66:3011–3.
37. Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 2010; 135:1–11.
38. Raffel FK, Szelestey BR, Beatty WL, Mason KM. The *Haemophilus influenzae* Sap transporter mediates bacterium-epithelial cell homeostasis. Infect Immun 2013; 81:43–54.

39. 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.

40. Hsieh PF, Liu JY, Pan YJ, et al. *Klebsiella pneumoniae* peptidoglycan-associated lipoprotein and murein lipoprotein contribute to serum resistance, antiphagocytosis, and proinflammatory cytokine stimulation. J Infect Dis 2013; 208:1580–9.