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Klebsiella pneumoniae Type VI Secretion System Contributes to Bacterial Competition, Cell Invasion, Type-1 Fimbriae Expression, and In Vivo Colonization

Pei-Fang Hsieh,[1] Yi-Rou Lu,[1] Tzu-Lung Lin,[1] Li-Yin Lai,[1] and Jin-Town Wang[1,2]

[1]Department of Microbiology, National Taiwan University College of Medicine, Taipei; [2]Department of Internal Medicine, National Taiwan University Hospital, Taipei

Background. We previously isolated a *Klebsiella pneumoniae* strain, NTUH-K2044, from a community-acquired pyogenic liver abscess (PLA) patient. Analysis of the NTUH-K2044 genome revealed that this strain harbors 2 putative type VI secretion system (T6SS)-encoding gene clusters.

Methods. The distribution of T6SS genes in the PLA and intestinal-colonizing *K pneumoniae* clinical isolates was examined. *icmF1*-, *icmF2*-, *icmF1*/*icmF2*-, and *hcp*-deficient *K pneumoniae* strains were constructed using an unmarked deletion method. The roles of T6SSs in antibacterial activity, type-1 fimbriae expression, cell adhesion, and invasion and intestinal colonization were determined.

Results. The prevalence of T6SSs is higher in the PLA strains than in the intestinal-colonizing strains (37 of 42 vs 54 of 130). Deletion of *icmF1*/*icmF2* and *hcp* genes significantly reduced interbacterial and intrabacterial killing. Strain deleted for *icmF1* and *icmF2* exhibited decreased transcriptional expression of type-1 fimbriae and reduced adherence to and invasion of human colorectal epithelial cells and was attenuated for in vivo competition to enable colonization of the host gut. Finally, Hcp expression in *K pneumoniae* was silenced by the histone-like nucleoid structuring protein via direct binding.

Conclusions. These results provide new insights into T6SS-mediated bacterial competition and attachment in *K pneumoniae* and could facilitate the prevention of *K pneumoniae* infection.

Keywords. H-NS protein; intestinal colonization; *Klebsiella pneumoniae*; type-1 fimbriae; type VI secretion system (T6SS).

Community-acquired pyogenic liver abscess (PLA) caused by *Klebsiella pneumoniae* has recently emerged [1, 2]. For patients with *K pneumoniae* PLA, the mortality rate is 10%, and for patients who also have metastatic meningitis, the mortality rate is 30%–40% [3, 4]. Therefore, early diagnosis and appropriate therapy are critical.

Many Gram-negative bacteria encode a molecular machine called the type VI secretion system (T6SS), which appears to function in bacterial pathogenesis as a nano-syringe that translocates effector proteins into eukaryotic and prokaryotic target cells [5–7]. For some enteric pathogens, such as *Salmonella* Typhimurium and *Vibrio cholerae*, T6SSs are required for overcoming microbiota-mediated colonization resistance, leading to successful host infection [8, 9].

Intracellular multiplication F (IcmF) family proteins are conserved integral inner membrane proteins of T6SS that are involved in effector proteins delivery into target cells [10]. Although the exact functions of most T6SS proteins are unknown, many are not secreted but are required for the secretion of T6SS substrates, such as hemolysin-coregulated protein (Hcp) and valine-glycine repeat protein (VgrG) [11, 12]. Some evidence suggests the existence of an organized T6SS mechanism that likely accounts for the host-pathogen interaction of *K pneumoniae*. Lawlor et al [13] demonstrated that *K pneumoniae* strains with mutations in the core proteins of the T6SS showed decreased ability to infect mouse spleen. *Klebsiella pneumoniae* Kp52.145, which harbors a deletion in a gene encoding a phospholipase D family protein (PLD1) located within a T6SS locus, was avirulent in a mouse pneumonia model [14].

Type-1 fimbriae are produced by many strains of *K pneumoniae* and are involved in adhesion to eukaryotic cells [15]. We have shown that a PLA *K pneumoniae* NTUH-K2044 strain expressed type-1 fimbriae-encoding genes when cultivated in Luria-Bertani (LB) broth with shaking. In addition, inversion of a putative invertible deoxyribonucleic acid (DNA) element containing the *fimA* promoter regulates the expression of type-1 fimbriae-encoding genes in *K pneumoniae* [16, 17].

The histone-like nucleoid-structuring protein (H-NS) is a DNA-binding protein and functions as a silencer by directly

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Correspondence: J.-T. Wang, MD, PhD, Department of Microbiology, National Taiwan University College of Medicine, 1, Sec 1, Jen-Ai Rd., Taipei, Taiwan (wangjt@ntu.edu.tw).

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binding to target AT-rich DNA [18]. Previous studies have noted that T6SS gene clusters are tightly regulated in several Gram-negative pathogens [19], and H-NS has been reported to be a negative regulator of T6SS gene transcription [20–22].

In this study, we (1) examined the distribution of T6SS genes in PLA and intestinal-colonizing *K pneumoniae* clinical isolates, (2) investigated the role of T6SSs in interbacterial and intrabacterial competition, (3) demonstrated the roles of T6SSs in type-1 fimbriae expression, cell adhesion, and invasion, (4) explored the function of T6SSs in intestinal colonization of mice, and (5) confirmed that H-NS functions as a silencer of T6SS expression in *K pneumoniae*.

METHODS

Ethics Statement

All animal procedures were approved (application number 20150487) by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine. All animal procedures were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Taiwan's Animal Protection Act.

Bacterial Strains and Culture Conditions

A total of 42 clinical PLA *K pneumoniae* isolates and 130 intestinal-colonizing strains were collected as described previously [23, 24]. The bacterial strains, plasmids, and primers used in this study are listed in Supplementary Table S1.

Type VI Secretion System Genetic Loci Determination

To identify the T6SS genetic loci in *K pneumoniae*, polymerase chain reaction (PCR) was performed using primer pairs designed specifically for *hcp*, *icmF1*, and *icmF2*. Polymerase chain reaction was performed as described previously [25].

Gene Deletion and Complementation

Klebsiella pneumoniae strains with *hcp*, *icmF1*, and *icmF2* single gene deletions, the *icmF1*/*icmF2* double gene deletion, and the *hns*/*icmF1*/*icmF2* triple gene deletion were constructed using a previously described unmarked deletion method [23]. For complementation, *hcp*, *icmF2*, and *icmF1* genes were amplified by PCR and cloned into the pGEM-T or pACYC184 plasmid, respectively. These resulting plasmids were transformed into their corresponding deletion mutants by electroporation. All of the deletion mutants and complementation strains were confirmed by PCR and sequencing.

Interbacterial and Intrabacterial Competitive Growth Assays

Cultures of predator and prey strains were mixed together at a multiplicity of infection of 10:1, and 25 mL of the mixed bacterial culture were spotted onto LB agar for 5 hours. Bacterial spots were harvested, and the colony-forming units (CFU) per milliliter of surviving prey and predator strains were measured by plating serial dilutions on selective agar. The output/input ratio of the prey to predator strains was interpreted as survival.

Adhesion and Invasion Assays

Human colorectal epithelial Caco-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% nonessential amino acids (Gibco/BRL). Adhesion and invasion assays were performed as described previously [17, 26].

Fimbrial Switch Orientation Assays

A modification of a previously described method was used to determine the orientation of the *K pneumoniae fim* switch [17, 27].

Mouse Inoculation Experiments

For in vivo competition, the mice experiments were followed according to previously described methods [28]. The output/input ratio of the test/virulent strains, the competitive index (CI) was interpreted as colonization ability. To investigate the contribution of T6SSs in the intestinal colonization and systemic dissemination, mice were infected by intragastric inoculation (3×10^6 CFU each) of the wild-type or the *icmF1*/*icmF2* mutant strain. Surviving animals were sacrificed on the second day or the sixth day after challenge; organ homogenates (including liver, spleen, and intestine) were cultured for quantification of CFU.

Reverse-Transcription Polymearase Chain Reaction Analysis

To identify the transcriptional unit of T6SS locus I, total ribonucleic acid (RNA) was extracted from NTUH-K2044 [23], complementary DNA was prepared, and PCR was performed as described previously [25].

Quantitative Real-Time Reverse-Transcription Polymearase Chain Reaction Analysis

To measure the expression of the T6SS locus, total RNA from cultures of *K pneumoniae* strains was extracted, and reverse-transcription PCR was performed as described previously [28].

Gel Retardation Assays

The DNA fragments of the T6SS locus I were generated by PCR from NTUH-K2044 chromosomal DNA. The PCR fragments were purified, and 200 ng of DNA was incubated with 1 or 1.5 μg of recombinant H-NS in binding buffer, and these assays were performed as described previously [29]. Supershift assays were conducted by adding anti-His antibodies (Sigma-Aldrich) for 30 minutes at room temperate.

Preparation and Analysis of Proteins in Whole-Cell Extracts and Culture Supernatant

Equal amounts of cell lysates were generated by sonicating the cell pellets (~1×10^9 CFU). Supernatants and secreted proteins

were collected as described previously [30] and verified by Western blotting.

Statistical Analyses

Statistical significance was assessed by 1-way analysis of variance or 2-tailed Student's *t* tests using Prism 5 (GraphPad) software.

RESULTS

Frequencies of Type VI Secretion System Loci in *Klebsiella pneumoniae* Clinical Isolates

From the previously sequenced genome of *K pneumoniae* NTUH-K2044, it was shown that this strain possesses 2 contiguous putative T6SS loci: locus I and locus III [31]. Locus I appears to be complete, because it contains both the putative *hcp* and *vgrG* secreted protein-encoding genes as well as *icmF*, a structural protein-encoding gene, which is designated as *icmF1*. In locus III, the *hcp*, *impB*, and *impC* genes are missing; however, it does contain putative *vgrG* and *icmF* genes, the latter of which is designated as *icmF2*.

To assess the prevalence of these T6SS loci, we focused on 3 genes: *icmF1*, *icmF2*, and *hcp*. Pyogenic liver abscess and intestinal-colonizing *K pneumoniae* isolates were examined for the presence of these genes by PCR. The frequency of the *icmF1*, *icmF2*, and *hcp* genes was significantly higher in the PLA isolates (37 of 42, 88.1%) than in the intestinal isolates (54 of 130, 41.5%). *icmF1* and *icmF2* genes-deficient strains were more prevalent among the intestinal isolates (29 of 130, 22.3%) than those among the PLA isolates (0 of 42, 0%) (Table 1).

IcMF1, IcmF2, and Hcp Are Required for Interbacterial and Intrabacterial Killing

To examine the contributions of the 2 distinct IcmF proteins and the Hcp effector protein toward antibacterial activity, we isolated spontaneous streptomycin-resistant prey and performed in vitro competition assays. Growth of *Salmonella* Typhimurium 14028 or *Escherichia coli* MG1655 was significantly reduced (3-fold) in the presence of wild-type NTUH-K2044 when compared with both the *icmF1*/*icmF2* mutant and *hcp* mutant strains (Figures 1A and C and 2C). The ability of the *hcp* mutant strain to kill either *Salmonella* Typhimurium 14028 or *E coli* MG1655 was restored by complementation with a plasmid containing the *hcp* gene under a *lac* promoter (Figure 1B and D).

To test whether the T6SSs are involved in intrabacterial competition, 4 spontaneous streptomycin-resistant *K pneumoniae* strains (a T6SS-null colonization strain [100-K79] and the NTUH-K2044 *icmF1*, *icmF2* and *hcp* mutants) were used as prey in a competition assay with wild-type NTUH-K2044. We obtained similar results to the previous assay; the wild-type strain was better able to eliminate the T6SS-null intestinal *K pneumoniae* isolate 100-K79 when compared with the T6SS-related mutant strains. The ability of the *hcp* mutant strain to kill *K pneumoniae* 100-K79 was restored by complementation (Figure 2A–C). The growth of the *icmF1*, *icmF2* and *hcp* mutant strains was significantly reduced in the presence of the wild-type strain by more than 1 log (Figure 2D). In these competition assays, survival of predator strains was similar for each strain (Supplementary Figure S1). Taken together, these results show that *K pneumoniae* competes against *Salmonella* Typhimurium, *E coli*, and T6SS-null *K pneumoniae* in a T6SS-dependent manner, suggesting that the Hcp-mediated antibacterial activity of T6SSs confers a competitive advantage to *K pneumoniae*.

Type VI Secretion Systems in *Klebsiella pneumoniae* Is Involved in Adhesion to and Invasion of Human Caco-2 Colonic Epithelial Cells

Several studies in *E coli* and *Campylobacter jejuni* have demonstrated that T6SSs promote host cell adhesion and invasion, 2 crucial processes required for host colonization [32–34]. To explore the roles of T6SSs in *K pneumoniae* in these processes, we evaluated the cell adherence and internalization potential of the wild-type and T6SS-related gene deletion strains in Caco-2 cells. The *icmF1*/*icmF2* mutant exhibited a highly significant, 3-fold decrease in adherence to Caco-2 cells. Moreover, the capacity of the *icmF1*/*icmF2* mutant to invade Caco-2 cells was significantly reduced, by approximately 60%, compared with that of the wild-type strain. In contrast, the adherence and invasion capacity of the *hcp* mutant strain were similar to those of the wild-type strain (Figure 3A). These results suggest that the T6SSs machinery contributes to *K pneumoniae* adherence to and invasion of Caco-2 cells.

Transcriptional Regulation of the *Klebsiella pneumoniae fim* Gene Cluster by the Type VI Secretion System

A previous study reported that 2 T6SS genes, *hcp* and *clpV*, influence the expression of type-1 fimbriae and contribute to the pathogenesis of avian-pathogenic *E coli* [35]; therefore, we

Table 1. Prevalence of T6SS Genetic Loci in PLA-Associated and Intestinal-Colonizing *Klebsiella pneumoniae* Strains

	T6SS Genetic Loci					
Group	*hcp*[a]	*icmF1*[b]	*icmF2*[b]	*hcp*, *icmF1*, *icmF2* (+)[b]	*hcp*, *icmF1*, *icmF2* (–)[c]	*icmF1*, *icmF2* (–)[a]
PLA	42 of 42	39 of 42	40 of 42	37 of 42 (88.10%)	0 of 42	0 of 42
Intestinal colonizing	104 of 130	70 of 130	86 of 130	54 of 130 (41.54%)	14 of 130	29 of 130

Abbreviations: PLA, pyogenic liver abscess; T6SS, type VI secretion system.

[a]$P < .01$, [b]$P < .001$, and [c]$P < .05$ by χ^2 test.

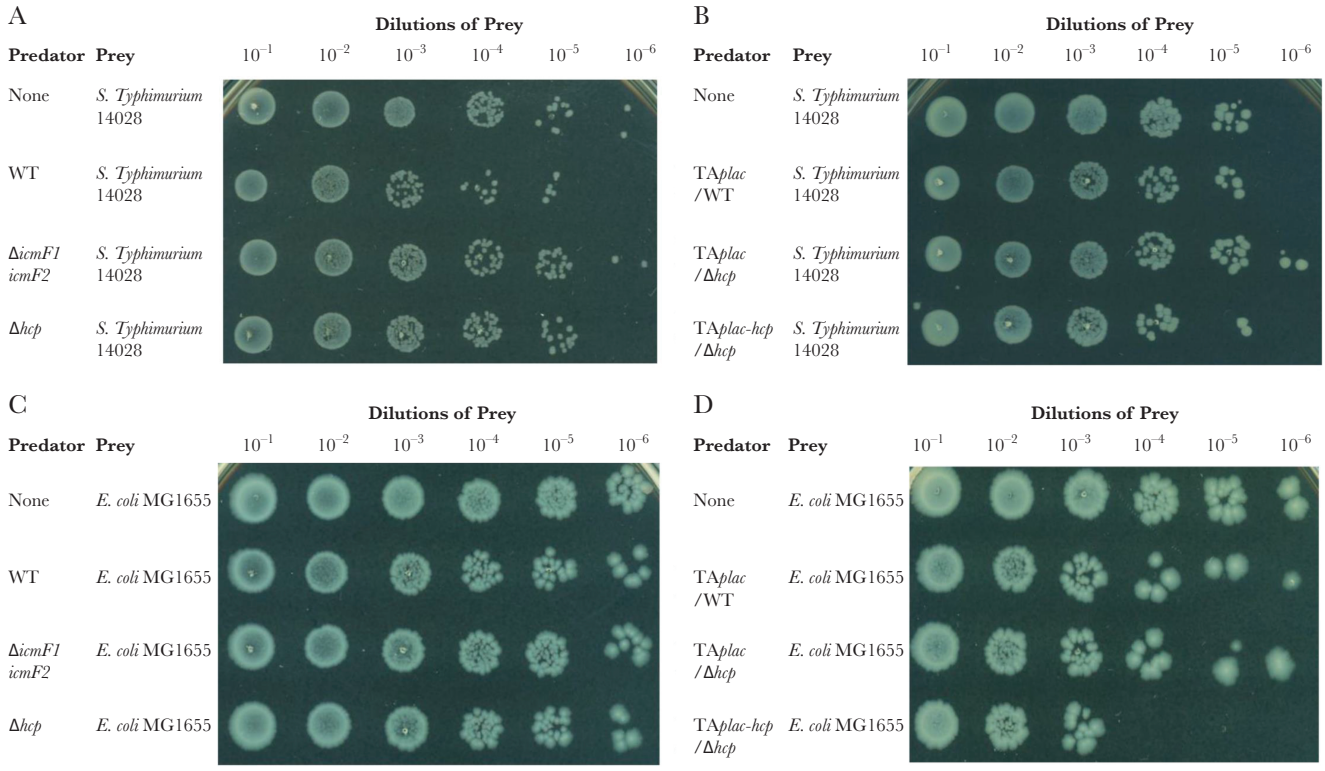

Figure 1. Interspecies killing by *Klebsiella pneumoniae* NTUH-K2044 in a T6SS-dependent manner. (A–D) Interbacterial competitive growth assays showing that a functional T6SS is essential for interbacterial killing by *K pneumoniae* NTUH-K2044. Surviving *Salmonella* Typhimurium 14028 (A) and *Escherichia coli* MG1655 (C) after 5 hours of coincubation with *K pneumoniae* NTUH-K2044 wild-type (WT), T6SS-related gene mutants, the *hcp* mutant carrying a functional copy of *hcp* on a plasmid (TAplac-*hcp*), and the wild-type or *hcp* mutant containing the empty vector (TAplac) (B and D). Recovered mixtures were plated onto Luria-Bertani medium supplemented with 100 μg/mL streptomycin.

compared the *fim* phase switching of our T6SS-related gene deletion strains with that of the wild-type strain in a fimbrial switch orientation assay. When either *icmF1* or *icmF2* was deleted, it appeared that there was as much in the "off" orientation as in the "on" orientation, indicating that type-1 fimbriae expression was downregulated in both the *icmF1* and *icmF2* mutants (Figure 3B). Compared with the wild-type strain, more DNA fragments corresponding to the off orientation were detected in the *icmF1*/*icmF2* mutant, indicating a significant reduction in type-1 fimbriae expression in this mutant. In contrast, more DNA fragments corresponding to the on orientation were detected in the wild-type and *hcp* mutant strains. We obtained similar results in the mannose-sensitive yeast agglutination (MSYA) assay. Consistent with the *fim* transcription results, the MSYA assays demonstrated that the *icmF1* and *icmF2* mutants synthesized 8-fold lower titers of type-1 fimbriae than the wild-type strain (Supplementary Table S2). Likewise, the *icmF1*/*icmF2* mutant synthesized 16-fold lower titers of type-1 fimbriae than the wild-type parent strain. In contrast, the level of type-1 fimbriae expressed in the *hcp* mutant was similar to that of the wild-type strain. Therefore, the attenuated adherence of the *icmF1*/*icmF2* mutant might partly be due to a reduction in the production of type-1 fimbriae.

Klebsiella pneumoniae Type VI Secretion System Is Important for Bacterial Colonization and Dissemination In Vivo During Infection

In a previous study, we generated an isogenic *lacZ* mutant with a promoter deletion, which was used as the wild-type strain in a competition assay [28]. Using the same assay, the *icmF1*/*icmF2* mutant strain showed a lower competitive index than the wild-type or p*lacZ* mutant. Complementation of the *icmF1*/*icmF2* mutant with 2 plasmids containing the *icmF1* and *icmF2* genes partially restored the ability for in vivo competition ($P < .05$) (Figure 3C). To determine the kinetics of the gastrointestinal colonization and systemic dissemination, we examined the bacterial load in mice challenged with equivalent doses (3×10^6 CFU) of the wild-type and the *icmF1*/*icmF2* mutant strains. The second day after bacterial inoculation, bacterial loads in mice infected with *icmF1*/*icmF2* mutant compared with those in mice infected with the wild-type strain showed no significant difference. When organs were examined in surviving animals on the sixth day after bacterial inoculation, the mice infected with the *icmF1*/*icmF2* yielded significantly fewer colony counts in the intestine, spleen, and liver compared with those of the wild-type ($P < .05$) (Figure 3D). These data demonstrate that mutants impaired for T6SSs have decreased virulence, with reduced the intestinal colonization and decreased bacterial dissemination.

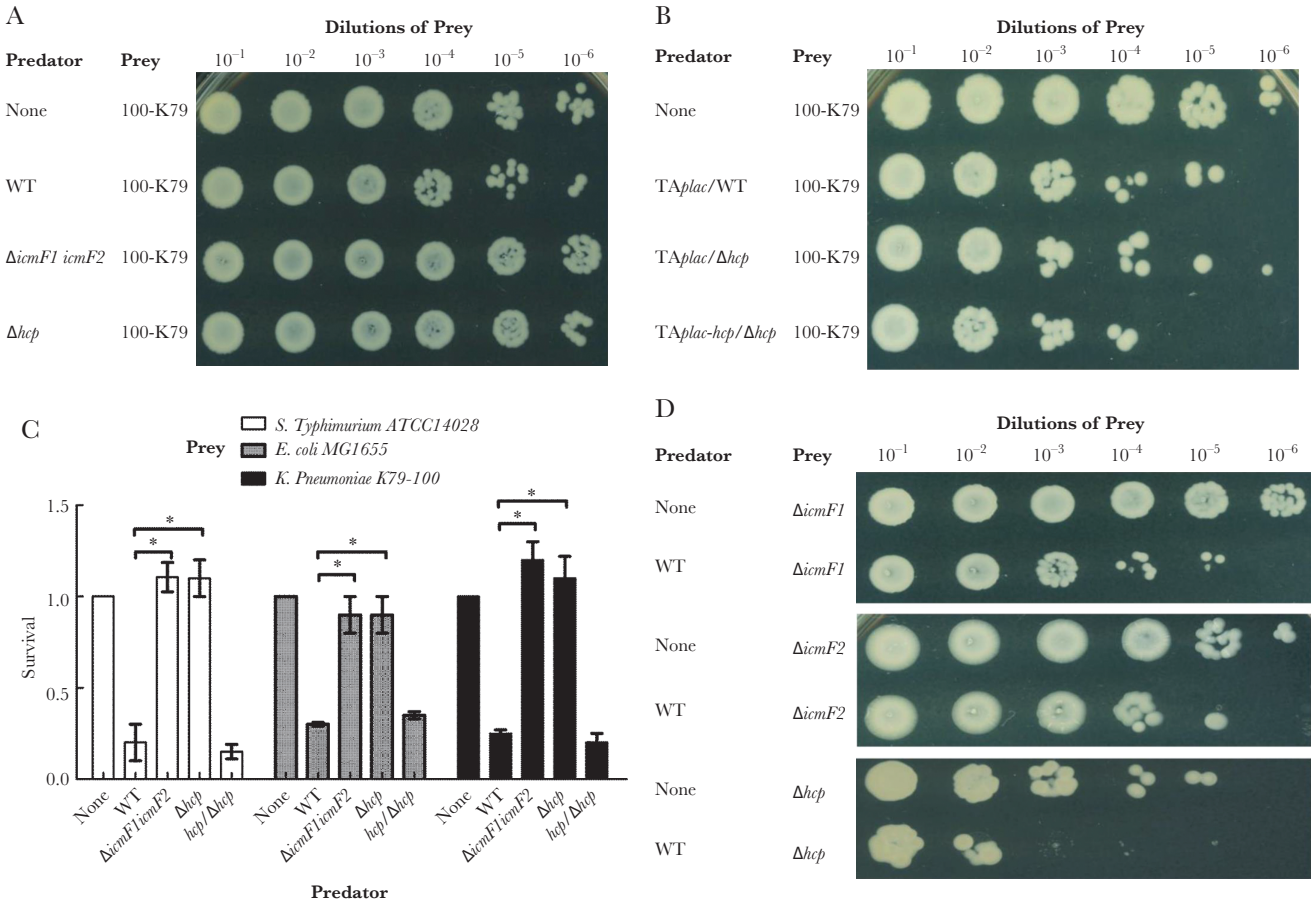

Figure 2. The role of the NTUH-K2044 T6SS in intraspecies and interspecies competitive growth. (A and B) Intrabacterial competitive growth assays showing that a functional T6SS is essential for intrabacterial killing. Surviving *Klebsiella pneumoniae* 100-K79 (A) after 5 hours of coincubation with various *K pneumoniae* NTUH-K2044 strains, including wild-type (WT), T6SS-related gene mutant, *hcp* mutant carrying a functional copy of *hcp* on a plasmid (TAplac-*hcp*), and wild-type or *hcp* mutant containing the empty vector (TAplac) strains (B). (C) Survival rates of the prey strains *Salmonella* Typhimurium 14028 (white bars), *Escherichia coli* MG1655 (gray bars), or *K pneumoniae* 100-K79 (black bars) after 5 hours of coincubation with the predator strains: *K pneumoniae* NTUH-K2044 wild-type, T6SS-related gene mutants, and *hcp* complemented strains. The data represent the means of 3 independent trials, and the error bars represent the standard deviations. *, $P < .05$ by 1-way analysis of variance (compared with the wild-type strain). (D) Survival of the T6SS-related gene mutant strains after 5 hours of coincubation with NTUH-K2044 wild-type. The recovered mixtures were plated on Luria-Bertani medium supplemented with 100 μg/mL streptomycin.

Type VI Secretion System Locus I Transcriptional Unit in *Klebsiella pneumoniae* NTUH-K2044

Reverse-transcription PCR to determine the transcriptional unit of the T6SS locus I was performed with total RNA from the NTUH-K2044 strain as template and several primer pairs (a–h) that hybridize within 2 consecutive open reading frames (ORFs). In the analysis, positive results were obtained for pairs b, c, d, e, and f (Figure 4A), suggesting that 6 ORFs (*impB*, *impC*, *impJ*, *dotU*, *ompA*, and *hcp*) formed a single transcriptional unit, whereas *clpV and vgrG1* were transcribed independently.

Histone-Like Nucleoid-Structuring Silences the Transcriptional Expression of Type VI Secretion System Locus I in NTUH-K2044

We sought to determine whether an *hns* deletion impacts the transcriptional expression of the T6SS loci in the NTUH-K2044 strain. Deletion of *hns* resulted in increased *hcp* gene transcription when compared with that of the parental strain when the strain was cultivated in LB broth (Figure 4B). To determine whether bacteria-host interaction affects the transcriptional expression of *hns* and *hcp* in *K pneumoniae*, we measured expression with or without Caco-2 cells. *hns* transcript levels in the wild-type strain were lower (<10%) during coculture with Caco-2 cells than during culture in medium only without Caco-2 cells (Figure 4C). In contrast, *hcp* transcript levels were higher (approximately 2-fold) when cultured with Caco-2 cells, than when this strain was cultured in medium only. These results demonstrate that H-NS silences the transcriptional expression of *hcp* in *K pneumoniae*.

Binding of Histone-Like Nucleoid Structuring to the Type VI Secretion System Locus I in NTUH-K2044

In silico analyses using Virtual Footprint software [36] predicted a large number of putative H-NS binding sites within the NTUH-K2044 T6SS loci distributed throughout the promoter

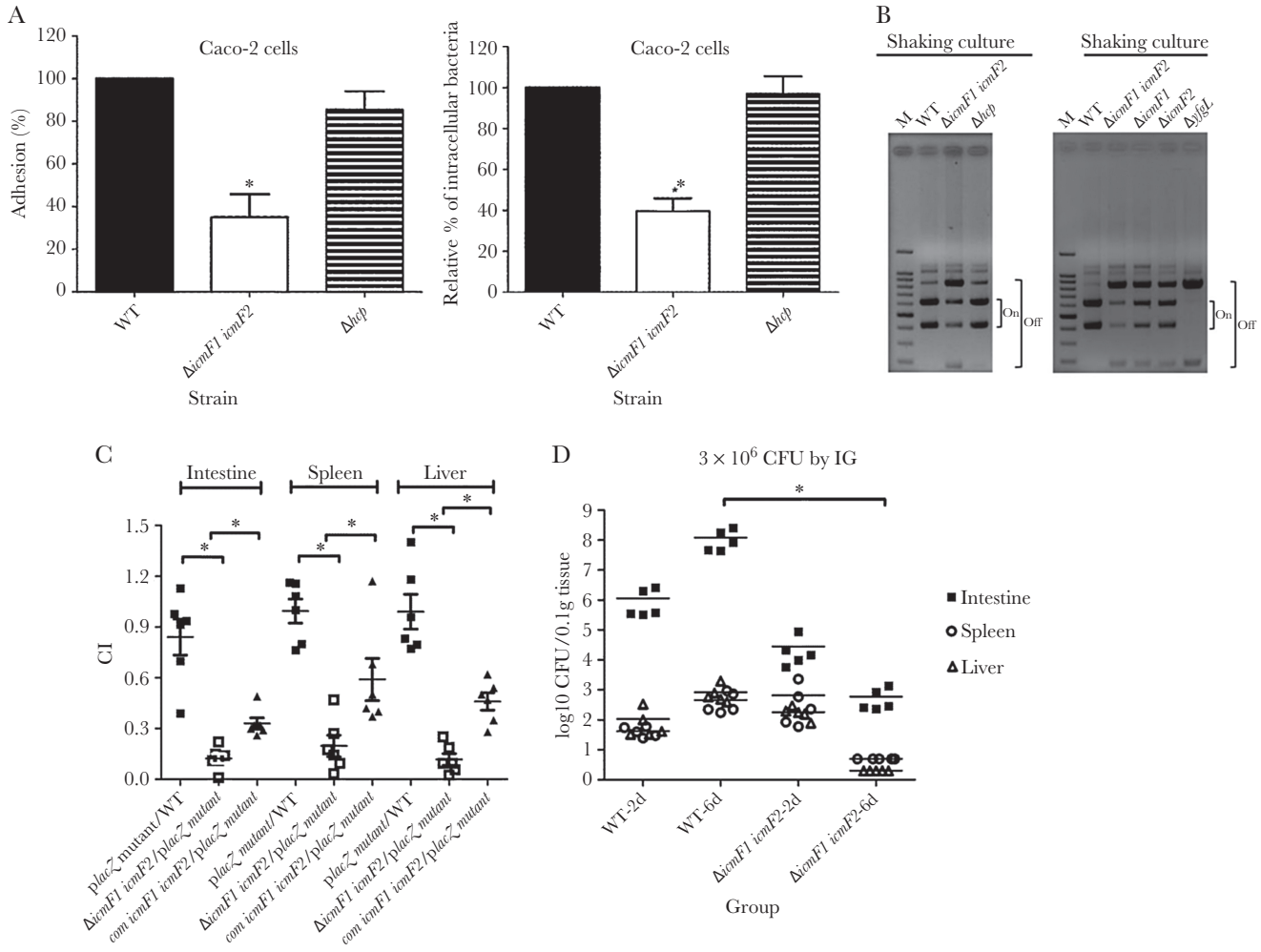

Figure 3. The function of the T6SS of *Klebsiella pneumoniae* in adhesion to and invasion of Caco-2 cells, type-1 fimbriae production, and in vivo colonization. (A) Adhesion and invasion of Caco-2 cells by the *K pneumoniae* NTUH-K2044 wild-type (WT) and derived T6SS-related gene mutant strains. The adhesion rate is expressed as the proportion of the NTUH-K2044 wild-type strain that adhered. The invasion rate is expressed as the proportion of the NTUH-K2044 wild-type strain that invaded. The data represent the means of 3 independent trials, and the error bars represent the standard deviations (SDs). *, $P < .05$ by 1-way analysis of variance (compared with the wild-type strain). (B) Orientation of the *fim* phase-switch element in the NTUH-K2044 and T6SS-related gene mutant strains after overnight culture in Luria-Bertani broth with shaking. Depending on the orientation of the *fim* invertible element, paired fragments of different sizes are amplified (613 and 404 base pairs [bp] when in the "on" orientation, and 840 and 177 bp when in the "off" orientation). Lane M contains the deoxyribonucleic acid molecular size marker. The *yfgL*-deletion mutant was included as a negative control and showed downregulated type-1 fimbriae expression. (C) Survival of the *K pneumoniae icmF1/icmF2* mutant and its complementation strains in the in vivo bacterial competition assay against a fully virulent p*lacZ* mutant. The *icmF1/icmF2* mutant or its complementation strain and the fully virulent p*lacZ*-deletion mutant were mixed at a 1:1 ratio (1×10^6 colony-forming units [CFU] for each mice of the wild-type group and *icmF1/icmF2* mutant group; 1×10^7 CFU for each mice of the *icmF1/icmF2* complementation group) and inoculated intragastrically into 5-week-old BALB/c mice (6 mice for each group). The ratio of *lacZ*+/*lacZ*– *K pneumoniae* in the spleen, liver, and intestine was determined from a single mouse when it either died or was sacrificed on the seventh day after infection. Each symbol represents the competitive index (CI) for each inoculum; and the medians and SDs of the values are shown (closed squares for the p*lacZ* mutant group vs the wild-type group, the CI of intestine, spleen, and liver were 0.841 ± 0.261, 0.995 ± 0.175, and 0.990 ± 0.252, respectively; open squares for the *icmF1/icmF2* mutant vs p*lacZ* mutant, the CI of the intestine, spleen, and liver were 0.123 ± 0.069, 0.197 ± 0.155, and 0.118 ± 0.085, respectively; $P = .031$, compared with the wild-type group, Wilcoxon's signed-rank test; closed triangles for the *icmF1/icmF2* complementation strain vs p*lacZ* mutant, the CI of the intestine, spleen, and liver were 0.330 ± 0.082, 0.590 ± 0.305, and 0.460 ± 0.125, respectively; $P = .031$, compared with the *icmF1/icmF2* mutant group, Wilcoxon's signed-rank test). (D) Five-week-old BALB/c mice (5 mice for each group) were inoculated by intragastric inoculation with equivalent doses (3×10^6 CFU) of the NTUH-K2044 wild type or its isogenic *icmF1/icmF2* mutant strain. Surviving animals were sacrificed on the second day or sixth day after challenge. Bacterial loads were measured in the liver, spleen, and intestine. Log_{10} CFU was standardized per 0.1 gram wet organ weight. The medians and SDs of the values are shown; *, $P < .05$ by Wilcoxon's signed-rank test.

region and within the coding sequences. To test whether H-NS binds to T6SS locus I in vitro, a His-tagged version of the Hcp protein of NTUH-K2044 was expressed and purified (Figure 5A). We then selected 2 DNA fragments that bear putative H-NS binding sites, including a fragment encompassing the partial promoter region of T6SS locus I (F1) and a fragment within the *hcp* gene (F2), as well as a control fragment within the junction of the *dotU* and *ompA* genes, for which no H-NS binding site is predicted (Figure 5B). These 3 fragments were PCR amplified and used in gel retardation assays with purified

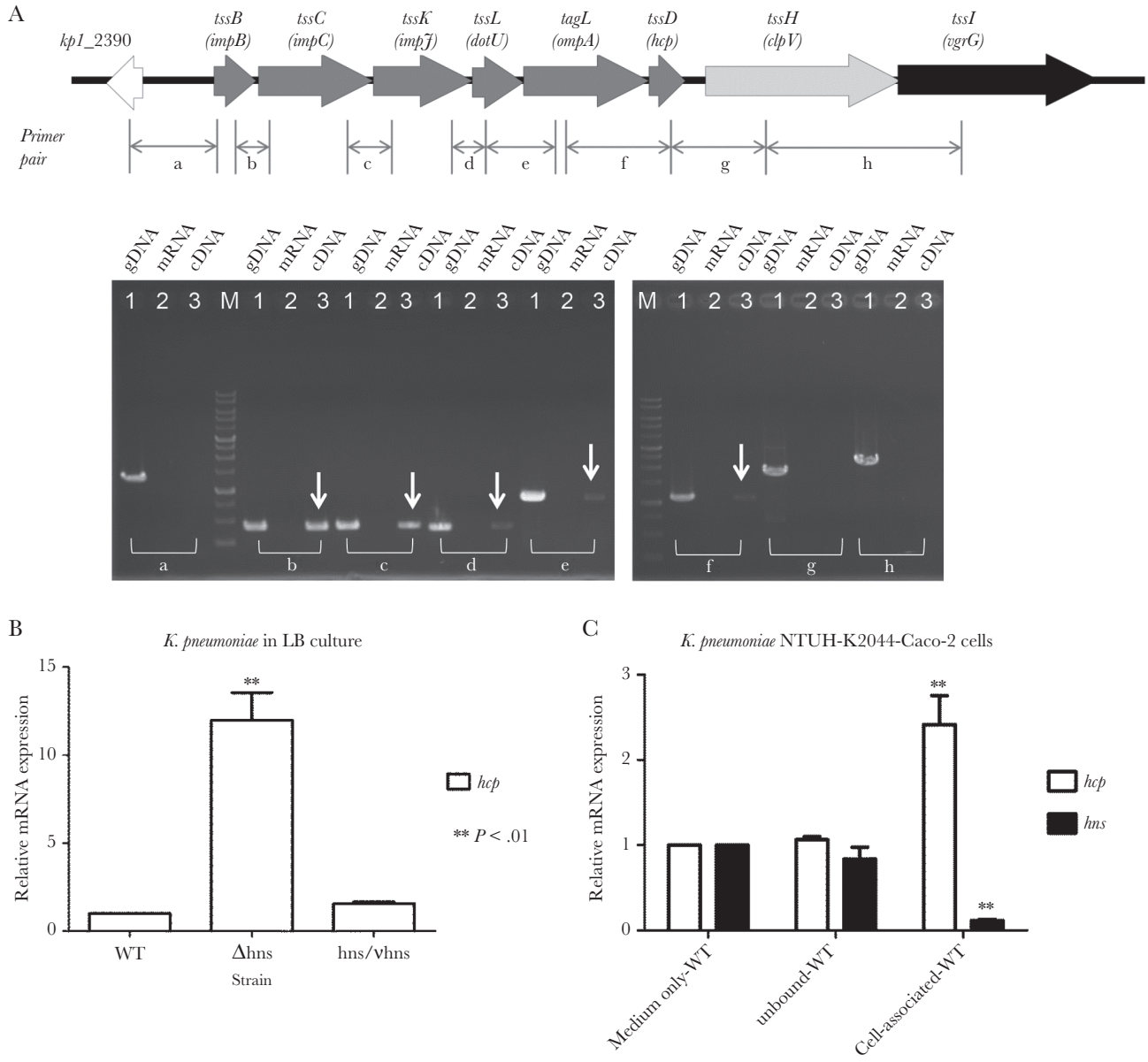

Figure 4. Histone-like nucleoid-structuring protein (H-NS) represses the transcription of the type VI secretion gene clusters. (A) Schematic representation of T6SS locus I in *Klebsiella pneumoniae* NTUH-K2044 (top). The arrow heads indicate the direction of transcription. The primer pairs (a–h) used to amplify the junctions between the open reading frames by reverse-transcription polymerase chain reaction (RT-PCR) are shown. The bottom part of the panel shows the RT-PCR products obtained in an ethidium bromide-stained agarose gel, with genomic deoxyribonucleic acid (DNA) as a positive control. The assay described above was repeated 3 times, and representative gels are shown. (B and C) Downregulation of T6SS gene transcription levels by H-NS. (B) Transcription levels of *hcp* were measured in the NTUH-K2044 wild-type (WT) and isogenic *hns* deletion mutant strains after culture in Luria-Bertani broth with shaking by quantitative RT-PCR (qRT-PCR). (C) Transcription levels of *hcp* and *hns* were measured in the NTUH-K2044 wild-type strain during coculturing with Caco-2 cells by qRT-PCR. The data represent the means of 3 independent trials, and the error bars represent the standard deviations. **, $P < .01$ by 1-way analysis of variance (compared with the wild-type strain cultured in medium only or the unbound wild-type strain).

NTUH-K2044 H-NS. Figure 5C showed that the F1 and F2 fragments were retarded in the presence of H-NS, whereas the negative control fragment was not retarded. Image analyses of the shifts estimated that H-NS binds to fragments F1 and F2 in a dose-dependent manner (Figure 5D). Therefore, we concluded that H-NS specifically binds to different regions in *K pneumoniae* NTUH-K2044 T6SS locus I.

Histone-Like Nucleoid-Structuring Represses Hcp Expression and Secretion in NTUH-K2044

Finally, we tested the effects of an *hns* deletion on the expression and secretion of Hcp. The Hcp protein was absent from lysates prepared from wild-type NTUH-K2044 cells. Deletion of *hns* resulted in increased expression of Hcp compared with that in the parental strain, whereas deletion of another *hns*

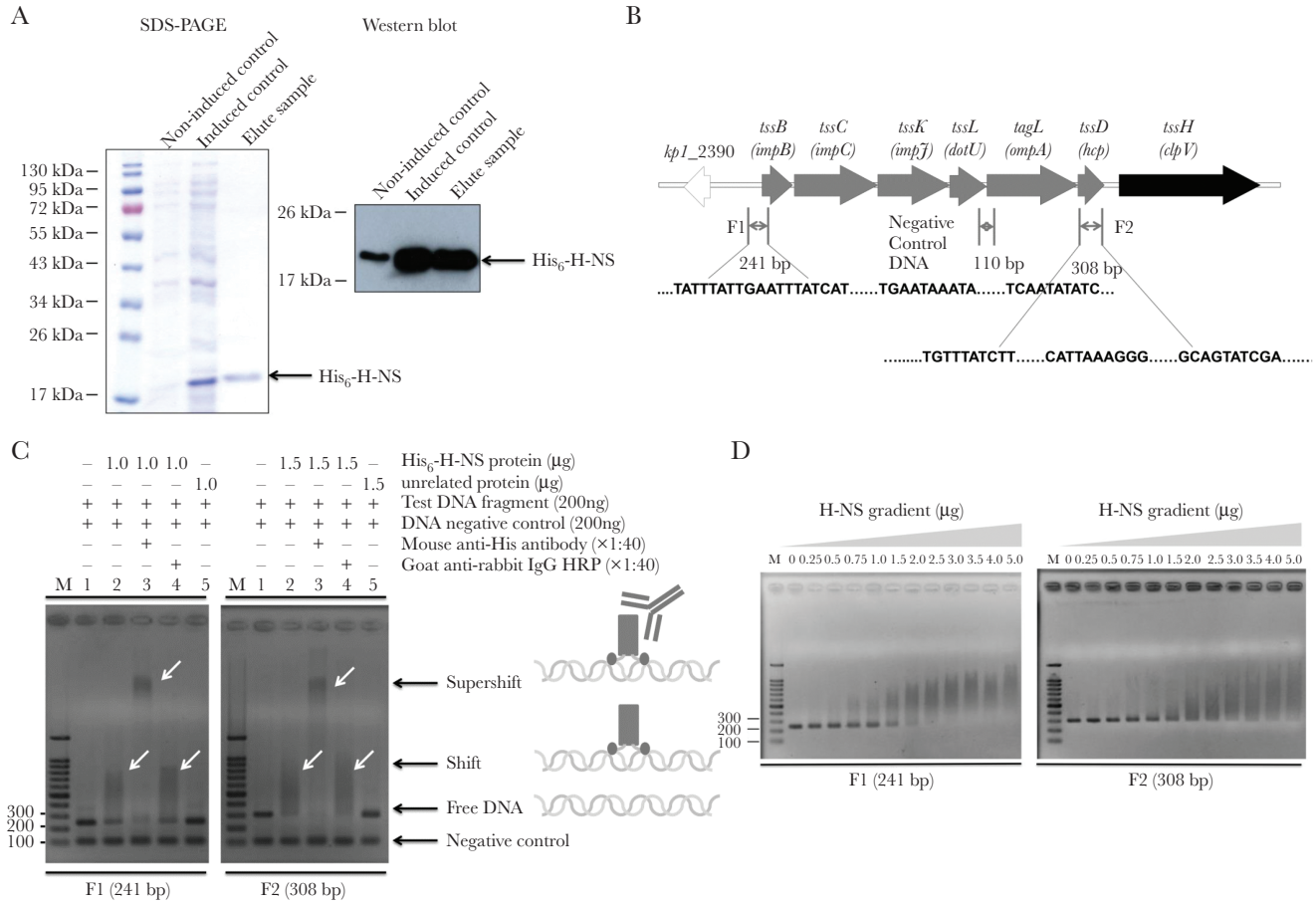

Figure 5. Histone-like nucleoid-structuring protein (H-NS) binds to several regions in the T6SS locus I of NTUH-K2044. (A) Purity of the recombinant NTUH-K2044 H-NS protein. The purified H-NS protein was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining, and the molecular weight is indicated beside the protein markers. Western blot showing the purified recombinant Hcp protein separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected using a mouse anti-His antibody (1:5000) and rabbit antimouse immunoglobulin G (IgG)-horseradish peroxidase ([HRP] 1:10 000). (B) Schematic representation of T6SS locus I of NTUH-K2044. Gel retardation assays using 2 deoxyribonucleic acid (DNA) fragments corresponding to regions of the T6SS gene cluster (F1 and F2). The 110 base-pair (bp) fragment corresponds to a negative control (no H-NS binding site predicted). (C) The different substrates used in the gel retardation assays are represented. Binding reactions (indicated at the top of each lane) were resolved in a 1.5% agarose gels at 100 V. Deoxyribonucleic acid was detected after staining with ethidium bromide. Free DNA and DNA-protein complexes are indicated. (D) The DNA fragments were mixed with increasing concentrations of purified the NTUH-K2044 H-NS (0–5 µg). Binding reactions using increasing concentrations of H-NS (indicated at the top of each lane) were resolved in a 1.5% agarose gel. The assays described above were repeated 3 times, and representative gels are shown.

homolog, *kpp056*, did not increase expression. Consistently, we obtained similar results with another PLA-associated *K pneumoniae* K2 strain, NTUH-A4528 [37] (Figure 6A). A slight increase in Hcp protein levels was observed in the culture supernatant of the *hns* mutant compared with the levels in the wild-type NTUH-K2044, indicating that it is unable to secrete the T6SS substrate Hcp, even in the absence of the repressor H-NS (Figure 6B). These results suggest that Hcp expression is repressed by H-NS in *K pneumoniae*.

DISCUSSION

Type VI secretion system has been found in more than 25% of all sequenced Gram-negative bacteria [38]. In a previous study, we demonstrated that PLA-related strains have a pathogenesis-specific genotype and transcriptional profile [23, 39]. Strains of the K1 and K2 capsular types have been identified as the predominant virulent types and are prevalent among *K pneumoniae* PLA isolates [2, 40]. Transcellular translocation is exploited by *K pneumoniae* to migrate from the gut flora into other tissues, resulting in systemic infections [41]. Recent studies have also demonstrated that several pathogenic bacteria use a T6SS to interact with and compete against bacterial competitors [8, 42–44]. It has been speculated that these pathogens have evolved mechanisms to kill bacterial competitors and establish infection. In this study, we demonstrated the higher prevalence of T6SSs in the PLA strains when compared with that in the intestinal-colonizing isolates (88.1% vs 41.5%), and we showed that deletion of *icmF1* and *icmF2* in a PLA *K pneumoniae* strain reduced interspecies and intraspecies antibacterial competitiveness in vitro. Therefore, our findings suggest that *K pneumoniae*

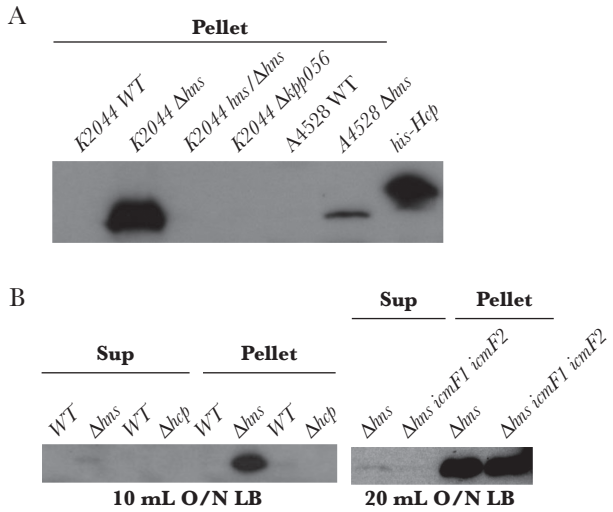

Figure 6. Effects of *Klebsiella pneumoniae* histone-like nucleoid-structuring protein (H-NS) protein on Hcp expression. (A) Western blot analysis to compare the expression of Hcp protein in whole-cell lysates of different *K pneumoniae* strains (as indicated). Hcp protein was expressed in the *hns*-deletion mutants. Anti-Hcp antisera were obtained from rabbits immunized with the recombinant Hcp protein (1:100 000; LTK BioLaboratories) and used as the primary antibodies, and rabbit horseradish peroxidase-conjugated antigoat immunoglobulin G antibody (1:10 000; Jackson ImmunoResearch) was used as the secondary antibody. Blots were visualized and developed with an enhanced chemiluminescence system (Amersham Biosciences). (B) Western blot analysis to compare the expression of Hcp protein in supernatants (Sup) and whole-cell lysates of different *K pneumoniae* strains (as indicated). Hcp expression was repressed by H-NS in *K pneumoniae*. LB, Luria-Bertani; WT, wild type.

outcompetes *Salmonella* Typhimurium and *E coli* in a T6SS-dependent manner and that T6SSs may aid bacterial competition in PLA strains.

IcmF is necessary for the efficient functioning of T6SS in Legionella *pneumophila*, and a protein homolog is required for Hcp secretion from *Agrobacterium tumefaciens* [45, 46]. Comparison of the amino acid sequences of the 2 IcmF proteins from NTUH-K2044 by using Basic Local Alignment Search Tool (BLAST) revealed 41.1% sequence identity. Analysis using the Simple Modular Architecture Research tool (SMART) showed that these 2 predicted IcmF proteins belong to the IcmF-related protein family and contain 2 conserved Pfam domains. Wild-type NTUH-K2044 showed intrabacterial killing ability and could compete against the *icmF1*, *icmF2*, and *hcp* mutant strains. In addition, compared with the *icmF1* or *icmF2* mutant, a significant reduction in type-1 fimbriae expression was observed in the *icmF1*/*icmF2* mutant. Thus, we concluded that these 2 IcmF proteins are important for a fully functioning T6SS in NTUH-K2044. Hcp has been proposed as a core component and hallmark secreted protein of T6SS [11]. The Hcp protein from NTUH-K2044 was predicted to be a T6SS effector Hcp1 family protein, and analysis of the amino acid sequence of this protein revealed high sequence identity (99%–100%) to Hcp proteins in other *Klebsiella* spp *and Enterobacteriaceae*. When the suppressor H-NS was deleted from NTUH-K2044, Hcp was expressed. Therefore, we suggest that Hcp is a hallmark secreted protein of T6SSs in *K pneumoniae*.

Bacterial adherence to epithelial cell surfaces is believed to be an important first step in the initiation of infection. Our recent study demonstrated that type-1 fimbriae are involved in the adherence of PLA *K pneumoniae* [17]. We demonstrated here that deletion of *icmF1* and *icmF2* in a PLA *K pneumoniae* also led to decreased expression of *fim* transcripts. Hence, T6SSs mediate in the transcriptional expression of type-1 fimbriae and are essential for adhesion to and invasion of host cells in PLA *K pneumoniae*.

Upon in vivo infection, the competition index of the *icmF1*/*icmF2* mutant was reduced when compared with the parental strain. Moreover, the ability for the intestinal colonization and systemic dissemination of the *icmF1*/*icmF2* mutant was attenuated during the first 6 days of infection. These findings indicate that deletion of the *K pneumoniae* genes encoding the T6SS biosynthetic machinery results in decreased bacterial colonization and restricted bacterial dissemination into distant organs. We conclude that T6SSs confer a competitive advantage to PLA *K pneumoniae* strains against the gut microbiota and facilitates the establishment of infection in the host tissues, thus enhancing pathogenicity.

The H-NS functions as an architectural component of the nucleoid and a pleiotropic regulator of gene expression [47, 48]. In a recent study, Ares et al [49] demonstrated that the H-NS nucleoid protein is an activator of type 3 fimbriae and a repressor of capsular polysaccharide expression in *K pneumoniae*. In this study, we showed that transcript levels of *hcp* were downregulated by H-NS and that this global regulator could interact directly with the promoter region and the coding sequences of T6SS locus I in *K pneumoniae*. These findings indicate that H-NS functions as a repressor of the T6SS secretory protein Hcp in *K pneumoniae*.

CONCLUSIONS

In conclusion, we report the following in this study: the prevalence of T6SSs is higher in the PLA *K pneumoniae* strains than in the intestinal-colonizing strains; T6SS antibacterial activity is essential for *K pneumoniae* survival and competition against host microbiota; T6SS-mediated type-1 fimbriae expression, cell adherence, invasion, and subsequent in vivo colonization are critical for establishing an infection; and H-NS functions as a silencer of T6SS expression in *K pneumoniae*. This report demonstrates that the antibacterial activity and cell invasiveness conferred by T6SS is important for *K pneumoniae* to establish itself within the host gut, thereby opening new and exciting research perspectives.

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

Author contributions. P.-F. H. and J.-T. W. conceived and designed the experiments. P.- F. H., Y.-R. L., T.-L. L., and L.-Y. L. performed the experiments. P.- F. H., Y.-R. L., and L.-Y. L. analyzed the data. P.-F. H. and J.-T. W. wrote the paper.

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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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