Serum-Induced Iron-Acquisition Systems and TonB Contribute to Virulence in *Klebsiella pneumoniae* Causing Primary Pyogenic Liver Abscess

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Background. *Klebsiella pneumoniae* has become the predominant pathogen causing primary pyogenic liver abscess (PLA).

Methods. *K. pneumoniae* was stimulated by human serum, and gene expression was analyzed by microarray.

Results. Three putative iron acquisition systems, *Yersinia* high-pathogenicity island (HPI), *iucABCDiutA,* and *iroA(iroNDCB),* that increased in expression and predominated in PLA-associated *K. pneumoniae* strains were identified. By use of siderophore uptake assays, these 3 systems were confirmed to be siderophore-dependent iron acquisition systems. Only the *irp2-iuc-iroA* triple mutant showed decreased virulence in mice. Full-genome analysis of *K. pneumoniae* strain NTUH–K2044 identified 10 putative iron uptake systems. Seven of these 10 systems were TonB dependent, including *Yersinia* HPI, *iucABCDiutA,* and *iroA.* A *tonB* deletion mutant was demonstrated to have profound attenuation of virulence. Immunization with the *tonB* mutant resulted in seroconversion of extracellular polysaccharide antibodies and protective efficacy against subsequent exposure to the parental strain.

Conclusions. Iron uptake systems were the genes in *K. pneumoniae* that were highly up-regulated in response to sera. Although there are multiple iron transporter systems in NTUH-K2044, a mutation in all 3 loci *(irp2, iuc,* and *iroA)* is necessary to decrease virulence. The *tonB* mutant is a potential vaccine candidate because it can induce a significant protective immune response against challenge with a wild-type strain.

Community-acquired pyogenic liver abscess (PLA) is an emerging infectious disease that is endemic in Asia, North America, and Europe [1–5]. Over the past half century, *Klebsiella pneumoniae* has become the predominant pathogen causing PLA in Korea, according to a recent epidemiologic study. From 2004 to 2005, 78% of cases of PLA were caused by *K. pneumoniae,* and 59% of the *K. pneumoniae* isolates causing these cases belonged to the K1 serotype [6]. Similar trends were observed in a prospective study in Taiwan, with ~2000 cases of PLA reported in 1996 and ~4000 cases reported in 2004 (The National Health Insurance Database [Taiwan], unpublished data). In 2004, at National Taiwan University Hospital (NTUH; Taipei), ~80% of cases of PLA were caused by *K. pneumoniae,* and 80% of the *K. pneumoniae* isolates causing these cases belonged to the K1 serotype (our unpublished data). Therefore, the K1 serotype is predominant among *K. pneumoniae* isolates causing PLA in Korea and Taiwan, and, in the future, the disease may possibly be prevented by immunization.

Capsular serotype K1 is considered to be the most virulent strain of *K. pneumoniae* [7], and the *magA* gene has been found to be prevalent among PLA-associated strains [8, 9]. Mutations in *magA* have been assessed in NTUH-K2044, a serotype K1 strain of *K. pneumoniae* that causes PLA; these mutations result in the loss of K1 capsular polysaccharides (CPS) and the development of an avirulent phenotype in mouse models [8]. An effective, live attenuated vaccine strain should be nonpathogenic but still immunogenic. Therefore, the *magA* mutant is not considered to be an ideal vaccine strain.

The identification of genes with increased expression in vivo may lead to the discovery of novel virulence traits involved in bacterial pathogenesis. In the present study,

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Table 1. Bacterial strains, plasmids, and primers used in the present study.

Bacterial strain, plasmid, or primer	Genotype, sequence, and/or relevant description	Sequence analysis	Reference or source
Bacteria			
K. pneumoniae			
74 isolates	Clinical isolates collected from National Taiwan University Hospital during 1997–2003		[9, 10]
NTUH-K2044	Clinical isolate, the parent strain for generation of isogenic mutants		[8]
MGH 78578	ATCC700721, the strain chosen for full-genome sequencing[a]		ATCC
Y. enterocolitica			
5030	Deficient in yersiniabactin synthesis		K. Hantke [11]
5092	Deficient in both yersiniabactin uptake and synthesis		K. Hantke [11]
E. coli strains			
DH10B	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ*80* δλαχZ ΔM15 Δ*lacX74 endA1 recA1 deoR (ara leu)7697 ara*Δ*139 galU galK nupG rpsL* λ$^-$		Invitrogen
EPI300	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ*80* d*lacZ* ΔM15 Δ*lacX74 endA1 recA1 ara*Δ*139* Δ*(ara leu)7697 galU galK* λ$^-$*nupG rpsL trfA*		Epicentre
LG1522	*ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi (pColV-K30) iuc*		R. Reissbrodt [12]
H5058	*aroB malT tsx thi cir fiu fepA*		K. Hantke and [13, 14]
Plasmid			
pGEM-T Easy	T-A cloning vector		Promega
pBR322	Ap^R Tc^R rep$_{pMB1}$		[15]
CopyControl pCC1	Cm^R long-PCR-product cloning; low-copy-number vector		Epicentre
pKO3-Km	pKO3-derived plasmid, with insertion of the Km resistance cassette from pUC4K into the AccI site		Present study
Primer			
1040-F 936-R	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	*magA* prevalence analysis	[8]
10E4-2-5F 10E4-2-475R	AGTCGGCCTGGGGTTTAAGG CAGTCAACGTGGCGATTCGC	*allS* prevalence analysis	[16]
kfu-F1179 kfuC-R649	GAAGTGACGCTGTTTCTGGC TTTCGTGTGTGGCCAGTGACTC	*kfu*/PTS prevalence analysis	[9]
ybtU-F ybtU-R ybtA-F ybtA-R	TTGTGCGCAACACATTACGC TCACAGCGCCTCCTTATCAT ATGACGGAGTCACCGCAAAC TTACATCACGCGTTTAAAGG	*Yersinia* HPI prevalence analysis	Present study
iucB-F iucB-R	ATGTCTAAGGCAAACATCGT TTACAGACCGACCTCCGTGA	*iucABCDiutA* prevalence analysis and *iucB* qPCR	Present study
iroN-F iroN-P-R	GTCCGGCGGTAACTTCAGCC TCAGAATGAAACTACCGCCC	*iroA(iroNDCB)* prevalence analysis and *iroN* qPCR	Present study
iroNB-F iroNB-R	GGCTACTGATACTTGACTATTC CAGGATACAATAGCCCATAG	*iroA(iroBCDN)* prevalence analysis	Present study
hmuR-F hmuR-R	GTGGCGACTATGTACAAATC GCTGTTGTTTTCAGTTTCCT	*hmuRSTUV* prevalence analysis and *hmuR* qPCR	Present study
KP-23-F KP-23-R	GGTTAAGCGACTAAGCGTACACGGT ACGAGGCGCTACCTAAATAGCTTTC	23 rRNA qPCR	Present study
irp2-2200F irp2-2899R	GCATGACAGGGTGCTGGCCC CTCCGACTTTGACCTGCTTGTC	*irp2* qPCR	Present study
ybtA-F irp1-R	ATGACGGAGTCACCGCAAAC CGGTATAGCCGACCTTTCTG	*irp2* deletion construct	Present study

(continued)

Table 1. ***(Continued.)***

Bacterial strain, plasmid, or primer	Genotype, sequence, and/or relevant description	Sequence analysis	Reference or source
ybtA-R	TTACATCACGCGTTTAAAGG		
irp1-F	ATGGATAACTTGCGCTTCTC		
Iuc-FR	GAGCCGCCCCAAACGACAGC	*iucABCDiutA* deletion construct	
Iuc-RR	GGCTTTTCTGATACCAATCT		Present study
iuc-Fout	AAGCACAATCAATATATGG		
iuc-Rout	CCGGTATTCCTTTACAACAA		
iro-PF	TCCTGTTGGCCAGCGTCTAT	*iroA(iroNDCB)* deletion construct	
iro-PR	CTCCTTCAGCCCGAACAAAC		Present study
iro-P-Fout	TCGTAATTATTAGGACTAAG		
iro-P-Rout	GCTCCTGTATACTATGGCAG		
iroN-R	TCAGAATGATGCGGTGACAC		
iroD-R	TCAACCTTTTAGTAAACC	*iroA(iroBCDN)* deletion construct	
iroN-F	GTCCGGCGGTAACTTCAGCC		
iroD-F	ATGCTGAACATGCAACAAC		Present study
kfu-FR	GCAGCAGATGAATATTCTGG		
kfu-RR	TTCCGACGCCAATGCTGATC		
kfu-Fout	TCTGGGTGCAGAACCAAATG	*kfuABC* deletion construct	
kfu-Rout	ATGGAGTGGTAGTACGTTGG		Present study
tonB-FR	AGCAACTTAACGCTGGCAGC	*tonB* deletion construct	
tonB-RR	TGAGCTGGGTACCAACACC		Present study
tonB-Fout	GCAATCATATTCAATAAGG		
tonB-Rout	TAAAACGCTGCGGCGGACCG		
iro-pP	GAAAATCCCTCTTTTAACGC	Salmochelin receptor IroN from *iroA(iroBCDN)*	
iroN-P-R	TCAGAATGAAACTACCGCCC		Present study
iro-pC	TCGATCCGGTTGTTTGCAGG	Salmochelin receptor IroN from *iroA(iroBCDN)*	
iroN-R	TCAGAATGATGCGGTGACAC		Present study
irp2-p	CCCCTTCGACCTTTAAACGC	*irp2* complementation	
irp2-R	CTATATCCGCCGCTGACGAC		Present study
pluc-F	CCAGTACAGGGATCGCGACC	*iucABCDiutA* complementation	
iutA-R	TCAGAACAGCACAGAGTAGTTCAG		Present study
tonB-F	CGTAAAGCACGGCAAAGCTC	*tonB* complementation	
tonB-R	TCAGTTAATCTCGACGCCG		Present study

NOTE. Ap, ampicillin; ATCC, American Type Culture Collection; Cm, chloramphenicol; *E. coli, Escherichia coli;* HPI, high-pathogenicity island; *K. pneumoniae, Klebsiella pneumoniae;* Km, kanamycin; PCR, polymerase chain reaction; qPCR, quantitative PCR; Tc, tetracycline; *Y. enterocolitica, Yersinia enterocolitica.*

[a] Genome Sequencing Center Web site. Available at: http://genome.wustl.edu/genome.cgi?GENOME=klebsiella%20pneumoniae.

we used a *K. pneumoniae* microarray to investigate bacterial adaptation in the host on a genomewide scale. We compared transcriptional profiles in different growth conditions and identified 3 siderophore-dependent iron acquisition systems. Therefore, this study was undertaken to identify the role of these iron uptake systems in bacterial virulence and to select a candidate strain for vaccine development.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in the present study are listed in table 1. Clinically isolated *K. pneumoniae* strains were collected at NTUH. A total of 42 PLA-associated strains and 32 non–PLA-associated strains were collected from 1997 to 2003, as described elsewhere [9, 10]. *K. pneumoniae* and *Escherichia coli* isolates were cultured in Luria-Bertani (LB) medium and in nutrient broth (NB) supplemented with appropriate antibiotics, including 100 mg/mL ampicillin, 50 mg/mL kanamycin, or 100 mg/mL chloramphenicol. For iron depletion, NB was supplemented with 100–200 μg/mL 2,2-dipyridyl.

Microarray construction. The genomic library was constructed from a clinical isolate (NTUH-K2044) obtained from a patient with PLA plus meningitis and endophthalmitis for use in a KP genome shotgun sequencing project [8, 17]. DNA frag-

ments of *K. pneumoniae* in pUC18 were amplified by polymerase chain reaction (PCR) performed using primers to vector sequences, and they were spotted onto a nylon membrane (Roche) by use of a computer-controlled XYZ translation system (PM500; Newport) [18].

RNA isolation, probe preparation, and hybridization. Total RNA was purified from log-phase cultures of *K. pneumoniae* in LB medium or in LB medium supplemented with 50% human serum (HS), by use of the RNeasy Mini Kit (Qiagen). The serum samples were obtained from 3 healthy volunteers. Aliquots of 40 μg of total RNA were labeled as described elsewhere [16]. Microarray hybridization and colorimetric detection were performed as described elsewhere [16, 19]. Densitometry was performed, and findings were analyzed using Image J software, and the 23S rRNA signal was used as an internal standard.

Microarray validation. A randomly selected subgroup of genes that were highly up-regulated, as determined by microarray hybridization, was analyzed by reverse-transcription (RT) quantitative (q) polymerase chain reaction (PCR) and real-time RT-PCR. The same RNA samples that were used for microarray hybridization were reverse transcribed using random primers and SuperScript II polymerase (Stratagene), as outlined by the manufacturer. In the qPCR assay, a total of 4 μg of total RNA was reverse transcribed, and the resulting cDNA was used as template for PCR. All reactions were normalized to the 23S rRNA gene. In real-time RT-PCR, amplification and detection of the cDNAs were monitored using SYBR green dye (Invitrogen) in an ABI 7900 thermocycler (Applied Biosystems). For each gene, the calculated threshold cycle (Ct) was normalized to the Ct of the 23S rRNA gene from the same cDNA sample, before the fold change was calculated by use of the $\Delta\Delta$Ct method [20].

Determination of* Yersinia *high-pathogenicity island (HPI),* iucABCDiutA, *and* iroA *gene clusters. To detect the iron acquisition gene clusters *Yersinia* HPI, *iucABCDiutA, iroA(iroNDCB), iroA(iroBCDN),* and *hmuRSTUV,* PCR was performed using primer pairs specific for *ybtA*/*ybtU, iucB, iroN* in *iroA(iroNDCB)* or *iroA(iroBCDN),* and *hmuR.* The primers used in the present study are listed in table 1. PCR was performed as described elsewhere [10].

Construction of mutant strains. To generate the NTUH-K2044 *irp2* mutant, a PCR fragment amplified by primers ybtA-F and irp1-R was cloned into a pGEM-T Easy vector (Promega). ybtA-R and irp1-F primers were used for inverse PCR performed with LA Taq polymerase (Takara). PCR-amplified fragments were blunted by a T4 DNA polymerase and phosphorylated by use of a polynucleotide kinase (New England Biolabs) for self-ligation. Then, the inverse PCR-amplified plasmid was cut with *Not*I (New England Biolabs), to generate fragments to the left and right of the sequences targeted for deletion. We cloned the fragment into temperature-sensitive vector pKO3-Km [21], to generate an *irp2* deletion mutant by electroporation [10]. The same procedures were used for *iuc, iroA, irp2-iuc-iroA, irp2-iuc-iroA-kfu,* and *tonB* deletion constructs. All of the deletion mutants were confirmed by PCR and sequence determination.

Transcomplementation. For complementation, intact *irp2* gene, *iuc* gene cluster, or *tonB* gene was amplified by PCR and cloned into a CopyControl pCC1 vector (Epicentre) (table 1). The resulting plasmids were transformed into their corresponding isogenic mutant strains by electroporation. Complementation strains were then selected on LB agar plates supplemented with 100 μg/mL chloramphenicol.

Siderophore uptake assays. Production of yersiniabactin or aerobactin or assimilation of salmochelin was detected by performing cross-feeding assays, which test the ability of bacteria to promote growth of indicator strains under iron-deficient conditions. Detection of yersiniabactin was done using *Yersinia enterocolitica* 5030 and *Y. enterocolitica* 5092 [11]. Aerobactin production was detected using *E. coli* LG1522, and salmochelin growth promotion assays were determined using *E. coli* H5058 (table 1) [12–14].

Mouse experiments. Groups of 5-week-old female BALB/cByl mice were infected intraperitoneally or intragastrically with isogenic *K. pneumoniae* NTUH-K2044 mutants in 0.1 mL of 0.95% saline solution (10^2–10^7 cfu; 4 mice were given each dose). The exact inoculation dose was confirmed by quantifying colony-forming units on LB agar plates. Mice were monitored for 4 weeks; the liver and brain were removed for histopathological examination either at death or after surviving mice were euthanized at the end of the 4 weeks. The 50% lethal dose (LD_{50}) was calculated as described by Reed and Muench [22]. To determine the bacterial load in vivo, the same inoculation dose (2×10^3 cfu) of wild-type or *tonB* mutant bacteria was administered to each of 4 mice by intraperitoneal injection. Surviving animals were euthanized at 24 h after challenge, and organ (including liver, spleen, and brain) homogenates were cultured for quantitation of colony-forming units. The number of colony-forming units detected in the organs were standardized per 0.1 g of wet organ weight.

To further evaluate the protective efficacy of attenuated strains, mice were intraperitoneally inoculated with the indicated dose of triple mutant, quadruple mutant, and *tonB* mutant or *magA* mutant, respectively (4–8 mice received each dose). Age-matched, nonimmunized control mice were inoculated with saline. After 4 weeks, immunized and nonimmunized control mice were challenged with 1×10^3 cfu of NTUH-K2044; this dose is greater than the wild-type LD_{50} value. For 28 days, the mice that were challenged were observed for death and clinical signs of disease. Survival was assessed by Kaplan-Meier analysis conducted with a log-rank test; $P < .05$ was considered to be statistically significant.

ELISA. Titers of anti–extracellular polysaccharide (EPS) IgG of *K. pneumoniae* were determined using EPS antigen containing 2 μg/mL EPS in 50 μL of 0.154 mol/L saline per well and goat anti–mouse IgG horseradish peroxidase conjugate (Sigma).

Table 2. Serum-induced genes ("sigs") in *Klebsiella pneumoniae* strain NTUH-K2044.

Gene	RNA level, average, fold[a]
With increased expression in 50% HS	
Yersiniabactin biosynthetic protein *(irp1)*	19.3
Putative ABC transporter protein *(ybtQ)*	12.9
Yersiniabactin biosynthetic protein *(irp2)*	12.6[b,c]
Transcriptional regulator *(ybtA)*	10.8
Thiazolinyl-S-HMWP1 REDUCTASE *(YBTU)*	10.3
Yersiniabactin biosynthetic protein *(ybtT)*	10.3
Yersiniabactin outer membrane receptor *(fyuA)*	9.3
Siderophore biosynthesis protein *(iucB)*	9.0[b,c]
Siderophore biosynthesis protein *(iucA)*	9.0
Hypothetical protein–TonB-dependent outer membrane heme receptor *(hmuR)*	8.8[b]
Putative heme/hemoglobin transport protein *(chuS)*	8.5
Hemin-binding periplasmic protein *(hmuT)*	8.5
Siderophore receptor *(iroN)*	7.7[b]
ABC transporter homologue *(iroC)*	7.4
Reduced expression in 50% HS	
Putative POT family, di-/tripeptide transport protein *(yjdL)*	−24.5
Cadaverine/lysine decarboxylase operon genes *(cadAB)*	−11.0[c]
Threonine dehydratase catabolic operon genes *(tdcDE)*	−9.9
Cadaverine/lysine decarboxylase operon genes *(cadC)*	−9.3
Threonine dehydratase catabolic operon genes *(tdcABC)*	−8.8

NOTE. ABC, ATP-binding cassette; HS, human serum; POT, proton-dependent oligopeptide transporter.

[a] Defined by a densitometry ratio of ⩾5, compared with the 23S rRNA internal standard.

[b] Validation by reverse-transcription (RT)–quantitative polymerase chain reaction (PCR) assay.

[c] Validation by real-time RT-PCR.

EPS antigens were isolated from NTUH-K2044 by use of a modified hot water–phenol extraction method, as described elsewhere [10], and they were quantified using a modified carbazole assay [23].

Immunoblot assays. The EPS of *K. pneumoniae* NTUH-K2044 were blotted to a Hybond C nitrocellulose membrane (Amersham) by use of a vacuum-driven slot blot filtration manifold (Hoefer). Serum samples from both immunized and nonimmunized control mice were diluted 1/100 and were used for immunoblot assays.

RESULTS

Transcriptome analysis. We compared the transcriptional profiles of *K. pneumoniae* NTUH-K2044 in 50% HS with those of strain in LB medium, by use of a custom microarray. There were 14 cloned sequences that had at least 5-fold increases in RNA expression levels in medium containing HS, and these comprised 4 gene clusters. *Yersinia* HPI, *iucABCDiutA,* and *iroA* belong to the siderophore-dependent iron acquisition systems, which encode one kind of siderophore and possess specific receptors, and *hmuRSTUV* (GenBank accession number AB358975) is an ATP-binding cassette (ABC) hemoprotein transport system [24–28]. In contrast, there were 5 clones that revealed a ⩾5-fold decrease in the level of RNA expression, including expression of *yjdL, cadABC,* and *tdcABCDE* gene loci (table 2). These results were reconfirmed by RT-qPCR assay (figure 1*A*) and by real-time RT-PCR (table 2).

Frequencies of* Yersinia *HPI,* iucABCDiutA, *and* iroA *gene clusters in PLA–*K. pneumoniae *clinical isolates. PLA-associated and non–PLA-associated *K. pneumoniae* isolates were examined by PCR for the existence of these serum-induced genetic loci. The frequencies of *Yersinia* HPI, *iucABCDiutA,* and *iroA(iroNDCB)*, but not *iroA(iroBCDN)* and *hmuRSTUV,* were significantly higher in PLA-associated isolates than in non–PLA-associated isolates: for the *Yersinia* HPI region, the frequency was 38 of 42 vs. 7 of 32 isolates, respectively [$P < .0001$, by χ^2 test]; for the *iuc* region, 39 of 42 vs. 6 of 32 isolates, respectively [$P < .0001$, by χ^2 test]; and, for the *iroA(iroNDCB)* region, 39 of 42 vs. 7 of 32 isolates, respectively [$P < .0001$, by χ^2 test]. Figure 1*B* shows the locations and sequences of the primers used in this analysis. Thirty-two (76%) of 42 PLA-associated isolates were positive for all 3 gene clusters.

Correlation of the* Yersinia *HPI,* iucABCDiutA, iroA (iroNDCB), kfu/*PTS, *magA, and* allS *regions with PLA in* K. pneumoniae *clinical isolates. The frequencies of the *kfu*/PTS, *magA,* and *allS* regions were compared and were found to be

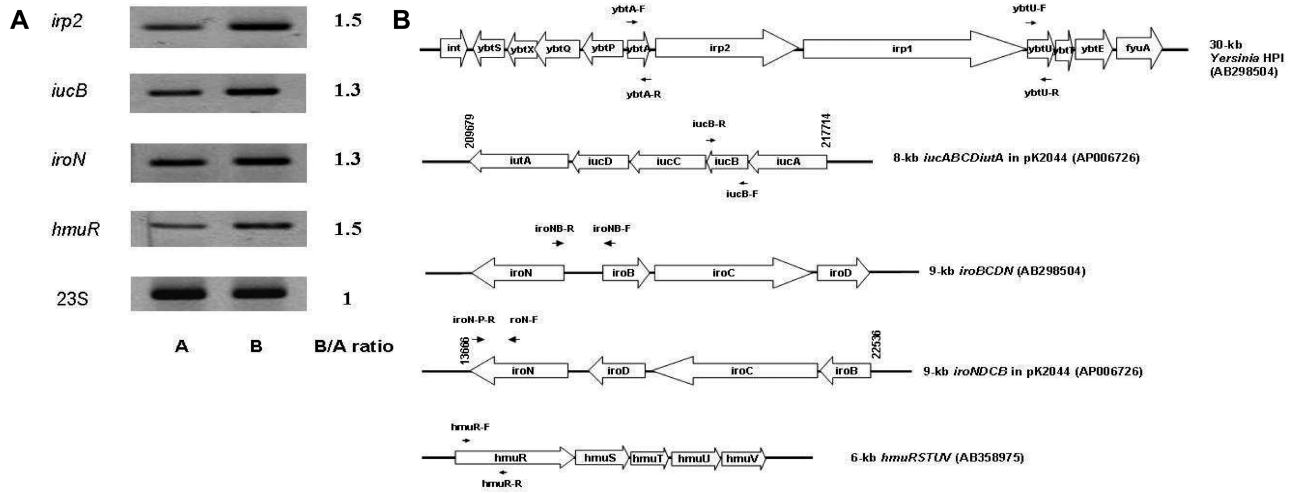

Figure 1. *A,* Selected reverse-transcription–quantitative polymerase chain reaction (PCR) assays. The primers presented in table 1 were used to amplify a DNA fragment from each gene indicated. Total RNAs were purified from *Klebsiella pneumoniae* strain NTUH-K2044 cultured in Luria-Bertani (LB) medium *(A)* or in LB medium supplemented with 50% human serum *(B).* After normalization to the 23S rRNA gene, the B/A ratio was considered to be the fold increase in the up-regulation of each gene, as indicated. *B,* Genetic organization of the 30-kb *Yersinia* high-pathogenicity island (HPI), 8-kb *iucABCDiutA,* 9-kb *iroA(iroBCDN), iroA(iroNDCB),* and 6-kb *hmuRSTUV* chromosomal regions. The PCR primer alignments used to study the frequency of these regions in pyogenic liver abscess (PLA)–associated and non–PLA-associated *K. pneumoniae* strains are shown. Arrows denote the orientations of the open-reading frames.

higher in PLA-associated isolates than in non–PLA-associated isolates, in a study published elsewhere [9]. The presence of these 6 regions was strongly associated with PLA strains but not with non-PLA strains. All 6 chromosomal regions correlated with PLA in 43 (58%) of 74 isolates; of these isolates, 28 contained all 6 regions, and 15 lacked all 6 regions.

Analysis of deletion mutants. Five deletion mutants (*irp2, iuc,* or *iroA* single mutants, the *irp2-iuc-iroA* triple mutant, and the *irp2-iuc-iroA-kfu* quadruple mutant) were generated using a pKO3-Km vector (figure 2*A*) [21]. All mutant strains grew as well as the wild-type parental strain in both iron-replete and iron-deficient conditions in vitro (figure 2*B*). Wild-type NTUH-K2044 secreted yersiniabactin and aerobactin into the culture supernatants, and, as expected, the isogenic deletion mutants *irp2* or *iuc* single mutants, the triple mutant, and the quadruple mutant produced neither yersiniabactin nor aerobactin, as determined by cross-feeding assays. A plasmid carrying either the *irp2* gene or the *iucABCDiutA* gene cluster restored the ability to produce yersiniabactin or aerobactin in low-iron conditions when introduced into *irp2* or *iuc* single mutants (table 3). There are 2 copies of the *iroA* loci, which are grouped in 2 distinct gene clusters, and their alignments are also different. *iroBCDN* is located on the chromosome of *K. pneumoniae* NTUH-K2044, and *iroNDCB* is located on its large plasmid pK2044 (figure 1*B*). Two iroN homologues, one in each *iroA* gene cluster, enable uptake of salmochelin siderophores (data not shown).

Mice intraperitoneally infected with *irp2, iuc, iroA,* or *kfu* single mutants or with the wild-type strain yielded the same LD_{50} ($<1 \times 10^2$ cfu), whereas mice inoculated with 10^3 cfu of either the triple mutant or the quadruple mutant remained healthy 4 weeks after inoculation. Triple and quadruple mutants were associated with LD_{50} values of 1.3×10^4 cfu and 5.5×10^4 cfu, respectively. When mice were challenged by intragastric infection, the triple mutant revealed only a 5-fold increase in the LD_{50}, compared with the wild-type strain, whereas the quadruple mutant was less virulent than either the wild-type or the *kfu* mutant (figure 2*C* and table 3) [9].

Iron transport systems in* K. pneumoniae *NTUH-K2044. Analysis of the genomic sequence of *K. pneumoniae* NTUH-K2044 (Whole Genome Sequencing Project of *Klebsiella pneumoniae* Web site [available at: http://genome.nhri.org.tw/kp/index.php], unpublished data) identified 10 putative iron transport systems, whereas *K. pneumoniae* strain MGH 78578 possesses only 6 of these systems. *Yersinia* HPI, *iucABCDiutA, iroA,* and *kfu* are present in NTUH-K2044 but not in MGH 78578. One putative TonB (GenBank accession number AB334778) encoded by the NTUH-K2044 genome was revealed by prediction of the protein sequence. Moreover, 7 of 10 putative iron transport systems in NTUH-K2044, including *Yersinia* HPI, *iucABCDiutA,* and *iroA,* contain putative TonB-dependent domains (table 4). Expression of the *tonB* gene in NTUH-K2044 was no different when growth occurred in medium containing HS, compared with growth occurring in serum-free conditions.

***Functional analysis of the TonB protein in* K. pneumoniae.** The *tonB* mutant was unable to grow in iron-deficient media, whereas the triple mutant and the quadruple mutant were not growth-impaired, compared with the parental strain. Comple-

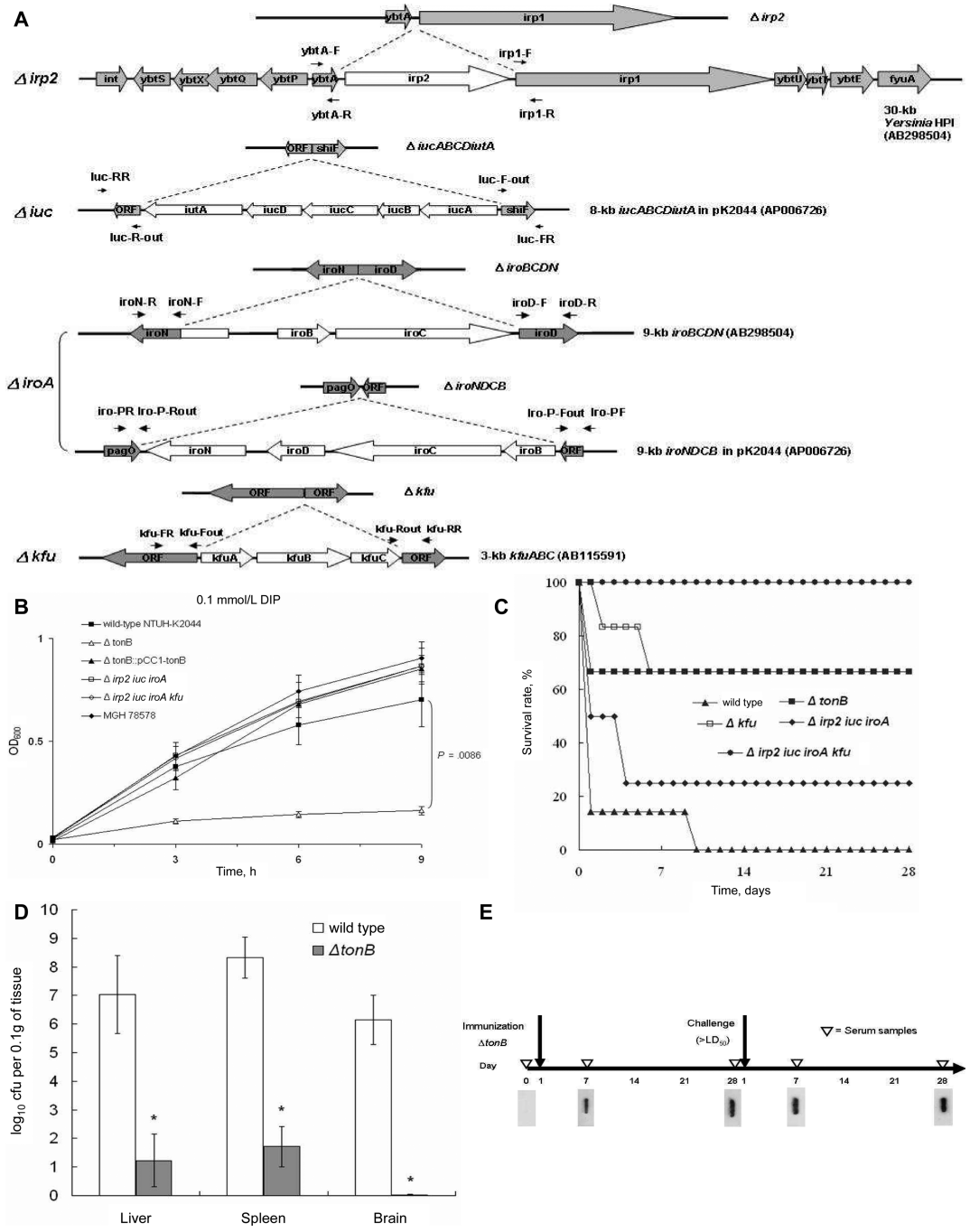

Figure 2. *A,* Schematic diagram of the constructs of deletion mutations in *Klebsiella pneumoniae* strain NTUH-K2044. Arrows denote the locations and orientation of the open-reading frames described in the present study; dashed lines, joining of the franking regions between deleted target; and primers, the relative positions in different deletion mutant strains. *B,* Growth experiments performed with the wild-type strain, the triple mutant, the quadruple mutant, the *tonB* mutant, *tonB* complemented strain, and MGH 78578 in nutrient broth (NB) containing 0.1 mmol/L 2,2'-dipyridyl. Growth was monitored spectrophotometrically at 600 nm every 3 h. Cultures were inoculated with fresh precultures to an OD of 0.01 (as measured at 600 nm). The optical density values are the mean values for data from 3 experiments, and error bars denote SDs (for comparison of the *tonB* mutant vs. the wild-type strain, $P = .0086$ [by Student's *t* test]). *C,* Survival of BALB/cByl mice intragastrically inoculated with various deletion mutants at a dose of 10^6 cfu. ▲, wild-type strain; □, *kfu* single mutant; ♦, triple mutant; ●, quadruple mutant; ■, *tonB* mutant (for comparison of the quadruple mutant with the wild-type strain, $P < .00001$ [by log-rank test]; for comparison of the *tonB* mutant or *kfu* single mutant with the wild-type strain, $P = .0082$ [by log-rank test]; and for comparison of the triple mutant with the wild-type strain, $P = .19$ [by log-rank test]). *D,* Inoculation of equivalent doses of wild-type *(white bars)* and *tonB* mutant *(gray bars) K. pneumoniae* into mice. Bacteria levels in tissue were measured at $t = 24$ h. The number of $\log_{10}$ colony-forming units was standardized per 0.1 g of wet organ weight. Bars denote the mean value for each tissue from these 2 groups, and error bars denote SDs. * $P = .0003$, for comparison of the *tonB* mutant group with the wild-type group [by Student's *t* test]). *E,* Immune response of anti–extracellular polysaccharides IgG in mice before and after immunization with *tonB* mutant.

Table 3. Phenotypic characterization of iron transport system mutants of *Klebsiella pneumoniae* strain NTUH-K2044.

Strain	Genotype or phenotype	Ybt synthesis[a]	Aerobactin synthesis[a]	K1 CPS Ag	Serum resistance	LD_{50} values,[b] by inoculation type	
						ip	ig
NTUH-K2044	*wt*/m^+	+	+	+	R	$<1 \times 10^2$	1×10^5
Δ*irp2*	*irp2*/m^+	−	ND	ND	ND	$<1 \times 10^2$	≈*wt*
Δ*iuc*	*iuc*/m^+	ND	−	ND	ND	$<1 \times 10^2$	≈*wt*
Δ*iroA*	*iroA*/m^+	ND	ND	ND	ND	$<1 \times 10^2$	≈*wt*
Δ*kfu*	*kfu*/m^+	ND	ND	ND	ND	$<1 \times 10^2$	6.3×10^6
Δ*irp2 iuc iroA*	*irp2 iuc iroA*/m^+	−	−	+	ND	1.3×10^4	5.6×10^5
Δ*irp2 iuc iroA kfu*	*irp2 iuc iroA kfu*/m^+	−	−	+	R	5.5×10^4	$>1 \times 10^7$
Δ*irp2*::pCC1-irp2	Irp2/m^+	+	ND	ND	ND	ND	ND
Δ*iuc*::pCC1-iuc	Iuc/m^+	ND	+	ND	ND	ND	ND
Δ*tonB*	*tonB*/m^+	ND	ND	+	R	2.0×10^6	7.5×10^6
Δ*tonB*::pCC1-tonB	TonB/m^+	ND	ND	+	ND	$<1 \times 10^3$	≈*wt*

NOTE. Ag, antigen; CPS, capsular polysaccharide; ig, intragastrically; ip, intraperitoneally; m^+, mucoid phenotype; ND, not determined; R, resistant; *wt,* wild type.

[a] Bioassays were performed as described in Materials and Methods; zone of stimulation, ⩾5 mm.

[b] Expressed as the no. of colony-forming units.

mentation of the *tonB* mutations restored growth in iron-restricted media (figure 2*B*). In comparison with the parental strain, the *tonB* mutant formed smaller colonies on either blood agar or LB media. A string test [8] revealed that all of the deletion mutants, including the *tonB* mutant, remained hyperviscous (table 3). CPS antigen serotyping was done using double immunodiffusion [10] and serum sensitivity [8] assays, and the results indicated no significant difference when the *tonB* mutant or the

Table 4. Iron transport systems in *Klebsiella pneumoniae* strain NTUH-K2044.

Transport system, siderophore dependency, and gene	Protein	Function (substrate)	Location	TonB dependency
Fe^{3+}				
Dependent				
fhuABCD	FhuA	Outer membrane transport (ferrichrome)	Chromosome	Dependent
	FhuBCD	Inner membrane transport (ferrichrome)	Chromosome	
iutA[a]	IutA	Outer membrane transport (aerobactin)	Chromosome and plasmid	Dependent
iucABCD[a]	IucABCD	Aerobactin biosynthesis	Plasmid	
fepABCDG	FepA, Cir	Outer membrane transport (enterobactin)	Chromosome	Dependent
	FepBCDG	Inner membrane transport (enterobactin)	Chromosome	
entABCDEF	EntABCDEF	Enterobactin biosynthesis	Chromosome	
fecABCDE	FecA	Outer membrane transport (ferric citrate)	Chromosome and plasmid	Dependent
	FecBCDE	Inner membrane transport (ferric citrate)	Chromosome	
iroN[a]	IroN	Outer membrane transport (salmochelin)	Chromosome and plasmid	Dependent
iroBCDE[a]	IroBCDE	Salmochelin biosynthesis (salmochelin)	Chromosome and plasmid	
fyuA[a]	FyuA	Outer membrane transport (yersiniabactin)	Chromosome	Dependent
Yersinia HPI[a]		Yersiniabactin biosynthesis	Chromosome	
Independent				
kfuABC[a]	KfuABC	Ferric iron uptake (Fe^{3+})	Chromosome	Independent
hmuRSTUV	HmuRSTUV	Heme	Chromosome	Dependent
Fe^{2+}				
feoAB	FeoAB	Ferrous iron uptake (Fe^{2+})	Chromosome	Independent
sitABCD	SitABCD	Ferrous iron uptake (Fe^{2+})	Chromosome	Independent

NOTE. HPI, high-pathogenicity island.

[a] Absent in the genome of *K. pneumoniae* strain MGH 78578.

Table 5. Intraperitoneal immunization with *Klebsiella pneumoniae* strain NTUH-K2044 mutants and challenge with the wild-type *(wt)* strain.

Mouse status	Immunization dose, cfu	Survival after immunization, *n/N*[a]	Challenge dose, cfu	Survival after *wt* challenge, *n/N*[b]	Time to death, days	*P*[c]
Immunized, by mutant strain						
Δ*irp2 iuc iroA*	1×10^3	4/4	1×10^3	3/4	1	.21
Δ*irp2 iuc iroA kfu*	1×10^3	4/4	1×10^3	2/4	3, 6	.25
Δ*tonB*	1×10^4	8/8	1×10^3	8/8	. . .	.0024
Δ*tonB*	1×10^5	8/8	1×10^3	8/8	. . .	.0024
Δ*magA*	1×10^6	8/8	1×10^3	2/8	1, 1, 3, 3, 3, 3	. . .
Control	Saline[d]	8/8	1×10^3	2/8	1, 2, 2, 2, 2, 5	. . .

[a] No. of mice that survived immunization/total no. of mice immunized.
[b] No. of mice that survived against challenge with the wild-type strain/total no. of mice.
[c] For comparison with nonimmunized control mice or mice immunized with the *magA* mutant strain, by use of the log-rank test.
[d] 0.1 mL of 95% saline solution.

quadruple mutant was compared with the wild-type strain. As expected, intraperitoneal inoculation of mice with the *tonB* mutant resulted in severely attenuated virulence, with an LD_{50} of $\sim 2 \times 10^6$ cfu. Moreover, transcomplementation of the *tonB* mutation almost fully restored virulence in mice (LD_{50}, $<1 \times 10^3$ cfu) (table 3). To assess bacterial growth in vivo, we examined the bacterial load in mice challenged with equivalent doses of wild-type or *tonB* mutant strains. When the organs of surviving animals were examined 24 h after bacterial challenge, the *tonB* mutant group had significantly reduced levels of colony counts in the liver, spleen, and brain, compared with the wild-type group ($P = .0003$) (figure 2*D*).

To further evaluate the protective efficacy of these attenuated strains, we challenged mice that were intraperitoneally immunized with the triple, quadruple, *tonB*, or *magA* mutant strains with a sublethal dose of NTUH-K2044. Four weeks later, all mice immunized with the *tonB* mutant survived without any symptoms of disease, whereas 75% of the nonimmunized control mice or mice immunized with the *magA* mutant died within 5 days of infection (table 5). Compared with the triple, quadruple, or *magA* single mutants in immunization studies, the *tonB* mutant was more attenuated in mice and conferred better protection against challenge with the wild-type strain. Mice immunized with the *tonB* mutant demonstrated serum EPS IgG responses on ELISA, whereas control mice or mice immunized with the *magA* mutant did not. Immunized mice with an anti-EPS IgG titer $\geqslant$1024 were fully protected against challenge with a sublethal dose of the wild-type strain (data not shown). In these mice immunized with the *tonB* mutant, seroconversion was further confirmed by slot immunoblot assays (figure 2*E*).

DISCUSSION

To investigate the transcriptional profiles of PLA-associated *K. pneumoniae* strains in response to serum, which mimic the in vivo situation, the NTUH-K2044 strain was stimulated by HS in LB medium or by LB medium alone. Iron uptake systems were the genes in *K. pneumoniae* that were highly up-regulated in response to the iron-restricted condition. Three of 4 siderophore-dependent iron acquisition systems—yersiniabactin (*Yersinia* HPI), aerobactin *(iucABCDiutA)*, and salmochelin *iroA (iroNDCB)*—were prevalent in the PLA-associated *K. pneumoniae* strains. The isogenic triple mutant of NTUH-K2044 revealed significantly attenuated virulence in mice. Because 7 of 10 putative iron transport systems in NTUH-K2044 were TonB dependent, a *tonB* mutant was created and showed severely impaired virulence. In immunization studies, mice immunized with the *tonB* mutant were protected against a challenge with the wild-type strain, whereas mice immunized with the CPS-deficient *magA* mutant were not. These results demonstrated that the *tonB* mutant is a potential vaccine candidate.

Among the genetic loci that are down-regulated by serum, *yjdL* encodes a putative di-/tripeptide transport protein, which is closely linked to the *cadABC* gene cluster on the chromosomes of pathogenic *E. coli, Shigella* species, and *K. pneumoniae* NTUH-K2044 [29]. *cadABC* contains the genes coding for lysine decarboxylase, cadaverine-lysine antiporter, and their transcriptional regulator. In addition to its role in metabolizing lysine, *cadA* has been proposed as an antivirulence gene in enteroinvasive *E. coli* and *Shigella* species [30]. The *tdcABCDE* region has been implicated in the control of genes responsible for the transport and anaerobic degradation of L-threonine. Both *cadA* and *tdcA* mutant strains had hyperadherent phenotypes, compared with the parent strain, in enterohemorrhagic *E. coli* (EHEC), suggesting a direct or indirect role of these genes in adherence [31, 32]. However, the biological functions of the *yjdL, cadABC,* or *tdcABCDE* gene clusters in PLA-associated *K. pneumoniae* strains are not clear.

To cause infections, pathogenic bacteria must be able to scavenge iron, an essential trace element, from host iron- and/or heme-binding proteins. A widespread mechanism for iron acquisition by microbes is the production of siderophores and

their cognate transport systems [33]. Siderophores contribute to the virulence of a wide variety of bacterial pathogens [34], including *K. pneumoniae* [27, 35].

In our investigation, only the isogenic triple mutant of NTUH-K2044 showed attenuation of virulence in mice. These results suggest that yersiniabactin, aerobactin, and salmochelin act synergistically on virulence of this bacterium in systemic infection due to PLA. Interestingly, in mice intraperitoneally infected with the quadruple mutant, virulence of the bacterium was similar to that demonstrated in mice infected with the triple mutant (as assessed by the LD_{50} value), indicating that the Kfu system is not involved in the pathogenesis of *K. pneumoniae* through this route of infection. However, *kfu* conferred virulence via an intragastric route that mimics natural infection. On the basis of these findings, we concluded that different iron transport systems function effectively in different organ systems of the host during the disease process of PLA due to *K. pneumoniae*. The multiple types of iron uptake systems produced by PLA-associated *K. pneumoniae* strains may function in different microenvironmental conditions within the host or at different times during the course of an infection.

The presence of multiple, perhaps redundant, iron transport systems in *K. pneumoniae* suggests that iron acquisition systems are needed during infection. Four iron transport systems, *Yersinia* HPI, *iucABCDiutA, iroA,* and *kfu,* are present in the genome of *K. pneumoniae* NTUH-K2044 but not in MGH 78578. This difference suggests that PLA-associated strains are able to use a wider range of iron sources than are non–PLA-associated strains. These findings suggest that, although a single siderophore-dependent iron acquisition system may be sufficient for bacterial colonization, the presence of additional systems could improve growth efficiency in the host by allowing the pathogen to import iron from multiple sources.

The prevalence of these iron acquisition genes among PLA-associated isolates and their correlation to virulence appear to be distinguishing traits of *K. pneumoniae* strains causing PLA. A variety of factors contributing to virulence among PLA-associated *K. pneumoniae* strains have been reported, including specific *magA*-associated K1 CPS antigens [8, 10], allantoin metabolism [16], ABC iron transport system Kfu [9], and EPS synthesis regulator *rmpA* [36]. Therefore, it is probable that these PLA-associated strains harbor a specific genotype resulting in expression of specific transcriptional profiles involved in pathogenesis.

Enteric bacteria require TonB for utilization of siderophores and heme [37, 38]. Mutations in *tonB* have been assessed in several gram-negative pathogens, and they confer an avirulent phenotype in animal models [39]. In our study, the *tonB* mutant was impaired for bacterial growth both in an iron-limiting medium and in vivo, when conditions were considered to be iron deficient. This finding was probably the result of the *tonB* mutant losing the ability to acquire iron through all TonB-dependent iron uptake systems. However, EPS released from the *tonB* mutant were sufficient to induce production of protective anti-EPS IgG antibodies. It is also possible that TonB is required in vivo for a function other than iron transport.

The ideal, live vaccine strain is one that produces asymptomatic infection and provides complete protection against subsequent exposure to the wild-type organism. In the present study, intraperitoneal immunization of mice with a single dose of the isogenic *tonB* mutant was sufficient to provide complete protection against challenge with wild-type *K. pneumoniae* NTUH-K2044, indicating that the *tonB* mutant can stimulate a protective memory immune response. Thus far, purified *K. pneumoniae* capsule [40] and other noncapsular components, including lipopolysaccharide, type 3 fimbriae, and an acapsular mutant [41–43], have been used as immunizing agents against *K. pneumoniae* infection in animals. Passive transfer to mice of human IgG to K1 CPS was only partially effective in protecting against a sublethal dose of *K. pneumoniae* [40]. In general, acapsular mutants lose immunogenicity and cannot induce effective protection immunity. Importantly, in contrast to the *magA* mutant, the *tonB* mutant retains intact K1 CPS antigen and is resistant to serum killing, resulting in anti-EPS antibody production and immediate resistance to the host innate immune response. Therefore, further experiments will be required to investigate the ability of the *K. pneumoniae tonB* mutant to stimulate protective immunity by more relevant routes of infection and at various times after immunization.

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