Genetic Determinants of Capsular Serotype K1 of *Klebsiella pneumoniae* Causing Primary Pyogenic Liver Abscess

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Background. Primary pyogenic liver abscess (PLA) caused by *Klebsiella pneumoniae* is an emerging infectious disease. Capsular serotype K1 and the *magA* gene have been reported to be associated with this disease.

Methods. The prevalence of *magA* was determined by polymerase chain reaction (PCR). The sequences of the *magA* flanking region were completed by inverse PCR and direct sequencing. Serotyping was performed by double immunodiffusion. Insertion mutations and *trans*-complementation were used to define the K1 genetic determination region.

Results. Thirty-five of 42 strains from patients with PLA were *magA* positive, whereas only 1 of 32 non-PLA strains was *magA* positive. All 36 *magA*-positive strains were serotype K1, and the 38 *magA*-negative strains were not (36/36 vs. 0/38; $P < .0001$). Sequencing of the *magA* flanking region revealed a putative capsular polysaccharide synthesis *(cps)* region; this region was 25 kb in length and contained 20 open reading frames (ORFs); of these ORFs, 9 were cotranscribed as part of an operon and differed from both MGH78578 and the Chedid strain. Mutation of 4 genes in this region turned the mutant strains anti-K1 negative. *Trans*-complementation restored the K1 phenotype.

Conclusions. The operon containing *magA* is responsible for capsular serotype K1 of *K. pneumoniae.* Several loci in the operon are unique determinants of K1 strains.

Klebsiella pneumoniae is a common hospital-acquired, gram-negative pathogen that causes urinary tract infections, nosocomial pneumonia, and intra-abdominal infections [1–3]. However, community-acquired primary pyogenic liver abscess (PLA) caused by *K. pneumoniae* has become an emerging disease recently [4–11]. There have been >900 cases of *K. pneumoniae* liver abscess, with or without septic metastatic complications, reported from Taiwan during the past 15 years [8]. This disease has also been described in Asia, North America, and Europe [8].

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Capsular serotypes K1 and K2 are considered to be the predominant virulent strains of *K. pneumoniae;* this has been confirmed by experiments in mouse models [12]. K1 has been further investigated as the most common serotype isolated from patients with *K. pneumoniae* liver abscess and endophthalmitis [13]. Mucoviscosity has also been documented as a virulence factor of *K. pneumoniae* [12, 14]. Using transposon mutagenesis, we identified a virulence gene, *magA*, that was involved in the hypermucoviscosity phenotype and also played an important role in resistance to serum and phagocytosis [7]. A high prevalence of the *magA* gene has also been found among PLA-associated *K. pneumoniae* strains [7, 15]. The function of *magA* is still being explored. However, comparison of the *magA* flanking region between NTUH-K2044 and MGH78578 (a strain that resulted in pneumoniae infection in a 66-year-old patient; its complete genome sequence is available at http://genomeold.wustl.edu/projects/bacterial/kpneumoniae/) revealed a 31-kb *magA* flanking region that was absent from MGH78578 [7]. This region was replaced by a 26-kb fragment in MGH78578. The 5′

Table. 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Genotype or relevant description	Reference or source
Bacteria		
Klebsiella pneumoniae strains		
K. pneumoniae (74)	Clinical isolates collected from National Taiwan University Hospital during 1997–2003	Present study
NTUH-K2044	Clinical isolate; the parent strain for generate isogenic mutants	[7]
MGH78578	ATCC700721; the strain chosen for full genome sequence	ATCC
A5054	K1 reference strain from Statens Serum Institute, Denmark	[16]
Chedid	Laboratory strain; O1:K2	[16]
ATCC8052	K2 strain used for the control of serotyping	ATCC
Escherichia coli strains		
DH10B	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* ϕ*80lacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK* λ$^-$ *rpsL nupG*	Invitrogen
CC118λ*pir*	Δ*(ara-leu) araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1* λ*pir* phage lysogen	[19]
S17-1λ*pir*	*hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn*7*)(λ*pir*)	[20]
Plasmids		
pUT-Km	pUTKm1 [21] derived plasmid, with miniTn5 excised by *Eco*RI, *tnp* excised by *Sal*I, and *bla* removed by *Apa*LI, then with an insertion of Km resistance cassette from pUC4K [22] into *Pst*I site	Present study
pCRII-TOPO-CAT	pCRII-TOPO was inserted with CAT [23] cassette into *Xba*I site.	Present study

NOTE. ATCC, American Type Culture Collection; Km, kanamycin.

sequence upstream of these 2 fragments could be aligned with capsular polysaccharide synthesis *(cps)* loci of the Chedid strain (GenBank accession number D21242; an O1:K2 strain) [16]. All 3 strains contained *galF, ORF2,* and 4 conserved genes—*orfX (wzi), wza, wzb,* and *wzc*—that were considered to be responsible for the translocation and surface assembly of capsular polysaccharide [17, 18]. Therefore, *magA* and its flanking regions might be associated with capsular polysaccharide biosynthesis. In the present study, we determined the correlation between the *magA* gene and capsular serotype K1 and tried to identify the genetic determinants of capsular serotype K1.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. Bacterial strains and plasmids used in this study are listed in table 1. Clinically isolated *K. pneumoniae* strains were collected at National Taiwan University Hospital (NTUH) [15]. From 1997 to 2003, there were 42 consecutive tissue-invasive strains (i.e., strains isolated from the blood of patients with PLA with or without septic complications such as meningitis or endophthalmitis) and 32 non–tissue-invasive strains (i.e., strains isolated from the blood of patients with sepsis but without liver abscess or any tissue-invasive diseases such as endophthalmitis or meningitis) isolated [15]. *K. pneumoniae* and *Escherichia coli* were cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics, including 100 μg/mL ampicillin, 50 μg/mL kanamycin, or 100 μg/mL chloramphenicol, as described elsewhere [7].

Serotyping. Initially, antisera from the Laboratory of Health-Care Associated Infection, Health Protection Agency, were used [13]; however, cross-reactions between serotypes K1 and K2 were noted. Therefore, we used 2 other typing methods to document the capsular serotype of each *K. pneumoniae* strain. Capsular serotypes were screened using *Klebsiella* antisera SEIKEN (Denka Seiken), in accordance with the manufacturer's instructions. In brief, bacteria were cultured overnight in Worfel-Ferguson's medium (0.2% yeast extract, 0.025% magnesium sulfate, 0.1% potassium sulfate, 0.2% sodium chloride, and 2% sucrose). The bacterial cells were harvested and resuspended in saline. One loopful of dense capsular suspension of the bacteria was then mixed with serotype-specific serum (K1–K6). Within 1 min, the agglutination results were read.

To reconfirm results for strains that initially tested as serotype K1, extracellular polysaccharides were isolated by a modified hot water–phenol extraction method [24, 25]. In brief, bacteria cultured overnight in a minimal medium (supplemented with 0.2% glucose instead of 0.4% glucose) [26] were harvested and resuspended in 150 μL of water. An equal volume of hot phenol (pH 6.6; Amresco) was added, and the mixture was vortexed vigorously. The mixture was then incubated at 65°C for 20 min, followed by chloroform extraction and centrifugation. The extracts should have included both capsule and lipopolysaccharide (LPS) [24]. However, since the antiserum specifically interacted with capsule antigen only, the LPS contamination did not affect the subsequent experiments. Extracted polysaccharides were reacted with a serotype K1–specific antiserum (Sta-

Table 2. Primers used in this study.

Primer	Sequence	Nucleotide position	Purpose
1166F	GGTGCTCTTTACATCATTGC	10 to 29 of *magA*	*magA* prevalence
936R	GCAATGGCCATTTGCGTTAG	1292 to 1273 of *magA*	
18E-F613	GCTAACAGAAGACATCCCTA	105 to 124 of *ORF8*	*ORF8′* mutant construct and *ORF8′* prevalence
18E-R596	GAGTTACCCTCCACAAACTTC	470 to 450 of *ORF8*	
$wcaG_{+118}$-F	GTTGGTTCAGCAATCGTAAG	118 to 137 of *wcaG*	*wcaG* mutant construct
$wcaG_{+448}$-R	CGCAGAGTTTTATACCGGC	448 to 430 of *wcaG*	
$wcaI_{+29}$-F	ATGGTCTTGACGTTAGGGTC	29 to 48 of *wcaI*	*wcaI* mutant construct
$wcaI_{+525}$-R	GGATACTTTGTCTGCAGACG	525 to 506 of *wcaI*	
114F	GGATGTAGCAACGAAGCAAG	−302 to −283 of *ORF8*	*ORF8′* complementation
1166R	GCAATGATGTAAAGAGCACC	+60 to +41 after *ORF8*	
10111F	AGTGTTTCTGGTGAAGACGC	−187 to −168 of *wcaG*	*wcaG* complementation
wcaG-R	TTAACTCCGGAAGCTTTGCTG	513 to 493 of *wcaG*	
$wcaH_{+383}$F	GATGAGTGCGGATGAAA	−76 to −60 of *wcaI*	*wcaI* complementation
wcaI-R	CCATTCCACCTAACGTAATC	+49 to +30 after *wcaI*	
1040F	CGTTACGAACTTGAACGAGC	−118 to −99 of *magA*	*magA* complementation
936R	GCAATGGCCATTTGCGTTAG	+65 to +46 after *magA*	
orf3F	GAAACGCAATCAGTGTAGCG	170 to 189 of *wzx*	*wzx* prevalence
orf3R	CTTGCTTCGTTGCTACATCC	1009 to 990 of *wzx*	
gmd_{+72}F	AGATGGTTCATATCTCGCAG	72 to 91 of *gmd*	*gmd* prevalence
$wcaG_{+77}$R	GTCATTTCTTTACTCCAGAG	77 to 58 of *gmd*	
orf6F	CCTTGGAGGACTGTCCTTAA	226 to 245 of *ORF10*	*ORF10′-ORF11′* prevalence
23KS-431F	ATGTTGGCCCAGGTGCAAAT	137 to 156 of *ORF11*	
$wcaG_{+118}$-F	GTTGGTTCAGCAATCGTAAG	118 to 137 of *wcaG*	*wcaG-wcaH* prevalence
wcaI-R2	CACCATTTCACCACTATACT	+86 to +67 after *wcaH*	
$wcaI_{+29}$-F	ATGGTCTTGACGTTAGGGTC	29 to 48 of *wcaI*	*wcaI* prevalence
wcaI-R	CCATTCCACCTAACGTAATC	+49 to +30 after *wcaI*	
$wcaJ_{+29}$F	CGAATGCGAATGCTTCCTTG	29 to 48 of *rfbP*	*rfbP* prevalence
rfbP-R	CAATGCTTATCTTAAGCAGC	+26 to +7 after *rfbP*	

tens Serum Institute), using a double immunodiffusion assay. Each assay was performed with 1.5% Nobel agar (Difco) in normal saline. Ten microliters of anti-K1 serum (75% dilution) was loaded into the central well, and 20 μL of each polysaccharide extract was loaded into peripheral wells. After an overnight incubation at 37°C, the precipitation lines were read. The agars were then soaked in 50% normal saline for 6 h, covered with a piece of filter, and allowed to air-dry overnight. Thoroughly dried agars were treated with 1% azocarmine (dissolved in 2% glacial acid) (Chroma) for 2 h, followed by a 5% glacial acid destain. The precipitation lines were generally visible before azocarmine staining; however, the pictures were clearer after staining.

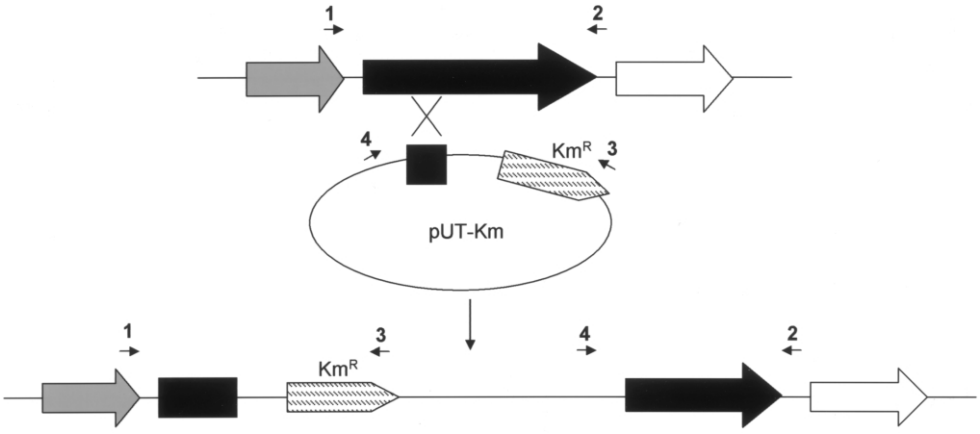

Figure 1. Constructs of the insertion mutation in NTUH-K2044. The orientations of open reading frames are represented by arrows. The "X" denotes homologous recombination. The primers shown here indicate the relative positions in different insertion mutant strains. See figure 3 for target genes. Km^R, kanamycin resistance cassette.

Table 3. Prevalence of the *magA* gene and results of capsular serotyping of *Klebsiella pneumoniae* strains.

Serotype	No. of *magA*-positive strains/ total no. of strains	
	Tissue invasive	Non–tissue invasive
K1	35/35	1/1
K2	0/1	0/1
K3	0/0	0/1
K4	0/0	0/0
K5	0/0	0/0
K6	0/1	0/0
NT	0/5	0/29
Total	35/42	1/32

NOTE. There are 3 additional control strains: A5054 (K1) is *magA* positive; ATCC8052 (K2) and MGH78578 (K52) are *magA* negative. NT, nontypeable (i.e., serotype other than K1–K6). The difference between serotype K1 and the other strains was significant (36/36 K1 strains *magA* positive, vs. 0/38 other strains; $P < .0001$, χ^2 test).

magA *prevalence.* To determine the prevalence of *magA*, polymerase chain reaction (PCR) using a primer pair specific for *magA* (1166F and 936R) was performed [7]. Primers used in this study are listed in table 2. PCR was performed as described elsewhere [7]. Briefly, 3 μL of overnight-cultured bacterial broth was added to 10 μL of water and boiled for 15 min to release DNA template. The reaction mixtures, which contained primers (0.4 μmol/L each), dNTP (0.1 mmol/L each), *Taq* polymerase (2.5 U; Bioman), and 13 μL of the above DNA template was then kept at 96°C for 3 min, followed by 30 temperature cycles of 96°C for 30 s, 56°C for 15 s, and 72°C for 1 min. The expected PCR product was 1282 bp in length.

Sequence analysis of the* magA *flanking region. The sequence of the *magA* flanking region was initially obtained by inverse PCR, and the *cps* loci were finally assembled by chromosomal walking, using the NTUH-K2044 phagemid library [27], until sequences of both ends matched those of MGH78578.

Construction of* K. pneumoniae *mutant strains. Primer pairs were designed to amplify partial regions of the following genes: *ORF8* (primers 18E-F613 and 18E-R596), *wcaG* (primers $wcaG_{+118}$-F and $wcaG_{+448}$-R), and *wcaI* (primers $wcaI_{+92}$-F and $wcaI_{+525}$-R) (table 2). PCR-amplified fragments were blunted by a T4 DNA polymerase (New England Biolabs) and ligated into a blunted *Eco*RI site of a pUT-Km vector (table 1) that contained a kanamycin-resistance gene. The resultant plasmid constructs were transformed into an *E. coli* S17-1λpir, followed by conjugation with the parent *K. pneumoniae* strain NTUH-K2044, as described elsewhere [7]. Transconjugants were selected using a minimal medium [26, 27] supplemented with 50 μg/mL kanamycin. The genotype was confirmed by PCR, using alignment-specific primer pairs (i.e., strains found to be positive by use of primer pair 1 and 3 and primer pair 2 and 4 but negative by use of primer pair 1 and 2, as shown in figure 1).

Other mutants—*magA*$^-$, *wza*$^-$, *wzc*$^-$, *rmpA2*$^-$, and *wzm*$^-$—from a mutant library of NTUH-K2044 were screened for decreased mucoviscosity by a string test and were identified by inverse PCR and sequencing [7]. *wza*$^-$ and *wzc*$^-$ mutant strains had impaired capsular antigen translocation ability [17]. *rmpA2*$^-$ and *wzm*$^-$ mutants were defective in their regulation of capsule production and O-antigen biosynthesis [12, 28], respectively.

Trans-*complementation.* Intact *ORF8*′, *wcaG*, *wcaI*, and *magA* genes were amplified by PCR and cloned into a pCRII-TOPO-CAT plasmid (table 1). These plasmids were transformed into their corresponding isogenic mutant strains by electroporation [29]; for selection of complementation strains, LB agar plates were supplemented with 50 μg/mL kanamycin and 100 μg/mL chloramphenicol. To confirm the role of this predicted *cps* region in K1 capsular synthesis by phenotype conversion, the K1-specific regions from *wzx* to *rfbP* (*wzx* to *wcaI* was unique for NTUH-K2044, whereas *rfbP* was present in both NTUH-K2044 and Chedid strains but was absent in MGH78578) were cloned into pBK-CMV vectors (Stratagene) and transformed into non-K1 *K. pneumoniae* strains.

Identifying operons by reverse-transcription PCR (RT-PCR) analysis. Total RNA was extracted from NTUH-K2044 as described elsewhere [27]. Forty micrograms of total RNA was

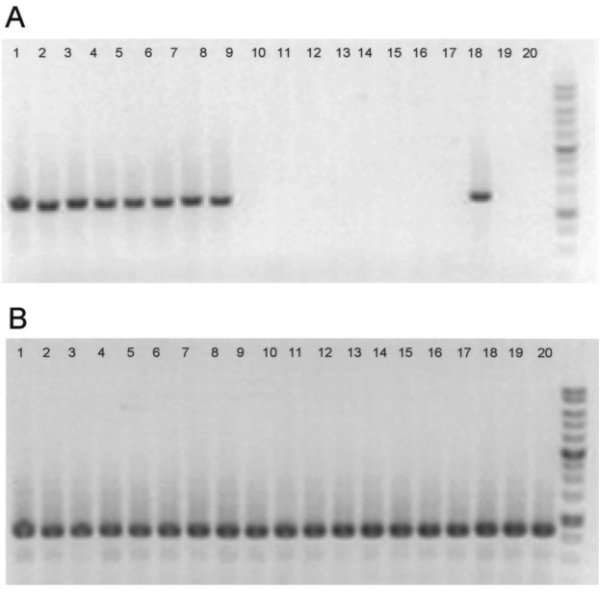

Figure 2. The prevalence of the *magA* gene in K1 and non-K1 *Klebsiella pneumoniae* strains. *A,* Polymerase chain reaction (PCR) for *magA*. *B,* PCR for *23srDNA*. *Lanes 1–8,* K1 strains (lanes 1–7 are tissue-invasive strains, and lane 8 is a non–tissue-invasive strain); *lanes 9–16,* non-K1 strains (lanes 9–11 are tissue-invasive strains, and lanes 12–16 are non–tissue-invasive strains); *lane 17,* A5054 (K1); *lane 18,* ATCC8052 (K2); *lane 19,* MGH78578 (K52); and *lane 20,* size marker.

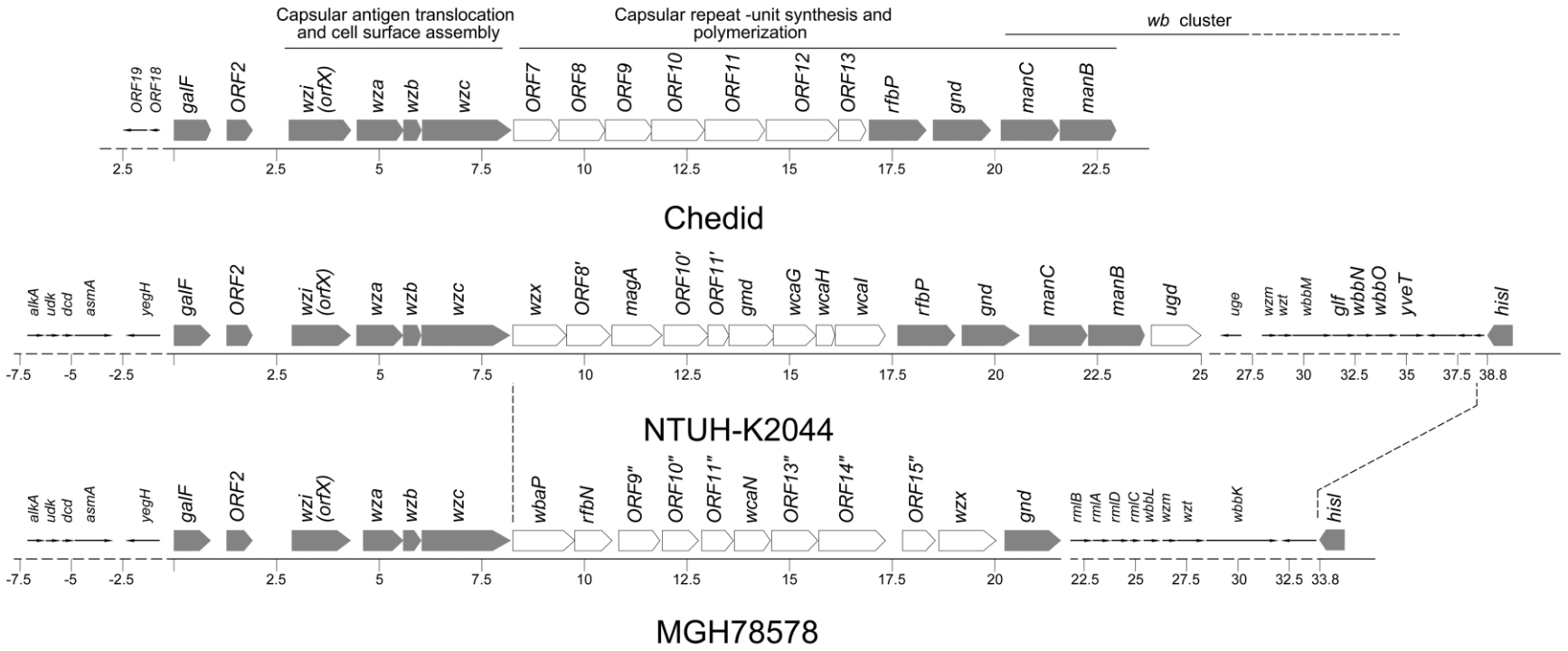

Figure 3. Comparison of capsular polysaccharide synthesis *(cps)* gene clusters between Chedid (K2), NTUH-K2044 (K1), and MGH78578 (K52). The locations and orientations of the open reading frames (ORFs) are indicated by arrows. ORFs are cited in order, starting from *galF;* ORFs with homologs are cited by putative gene names, and those without homologs are cited as ORFs and are numbered, with *galF* considered to be no. 1 (ORF in Chedid strain, ORF′ in NTUH-K2044, and ORF″ in MGH78578). Solid arrows refer to ORFs with significant similarity (the aligned genes with scores >200 and expect values $<e^{-50}$ in the BLASTP search), and unshaded arrows indicate the ORFs with low similarity between any 2 strains (the homolog could not be found even when the expect threshold was set to be 10 in the BLASTP search). The first nucleotide of *galF* defines position 1 in the sequence; nos. beneath each axis denote positions in kilobases. Dashed lines indicate the replaced region between NTUH-K2044 and MGH78578.

Table 4. Annotation of open reading frames (ORFs) of capsular polysaccharide synthesis *(cps)* loci in NTUH-K2044.

ORF no.	ORF name	Product size, aa	ORF location,[a] nt (position within GenBank sequence)	ORF homolog characteristics	Source	Sequence identity,[b] % (no. of matching amino acids/total no. of amino acids)	GenBank accession no.
1	*galF*	296	1–891 (7144–8034)	UTP–glucose-1-phosphate uridylyltransferase	*K. pneumoniae* (Chedid)	100 (296/296)	Q48447
2	*ORF2*	209	1284–1913 (8427–9056)	acidPPc, acid phosphatase homologs[c] (ORF2)	*K. pneumoniae* (Chedid)	88 (184/209)	Q48448
3	*wzi*	477	2873–4306 (10016–11449)	Capsule assembly, *orfX*	*E. coli*	92 (443/477)	AAD21561
4	*wza*	377	4449–5582 (11592–12725)	Putative capsule polysaccharide export protein precursor (ORF4)	*K. pneumoniae* (Chedid)	92 (348/377)	Q48450
5	*wzb*	144	5647–6021 (12790–13164)	Probable protein-tyrosine-phosphatase	*E. carotovora*	62 (89/142)	YP_049524
6	*wzc*	717	6056–8188 (13199–15331)	Putative transmembrane protein, tyrosine-protein kinase	*E. coli*	53 (371/692)	AAD21564.1
7	*wzx*	429	8277–9566 (15420–16709)	O–antigen flippase	*F. tularensis tularensis*	20 (89/443)	YP_170390
8	*ORF8*	356	9568–10638 (16711–17781)	Polysaccharide pyruvyl transferase[c]	…	…	…
9	*magA*	408	10670–11896 (17813–19039)	Putative O-antigen ligase, *waaL*[c]	*V. cholerae*	20 (65/319)[d]	AF443846.1
10	*ORF10*	366	11930–13030 (19073–20173)	Putative glycosyltransferase	*B. fragilis*	28 (71/250)	AAD40719
11	*ORF11*	170	13011–13523 (20154–20666)	Galactoside O-acetyltransferase	*M. acetivorans*	33 (42/127)	NP_617091
12	*gmd*	383	13520–14671 (20663–21814)	GDP-D-mannose dehydratase	*S. flexneri*	88 (329/370)	NP_837675
13	*wcaG*	346	14604–15644 (21747–22787)	GDP-4-keto-6-L-galactose reductase; GDP-fucose synthetase	*E. coli*	77 (248/318)	NP_754466
14	*wcaH*	152	15646–16104 (22789–23247)	GDP-mannose mannosylhydrolase	*E. coli*	48 (72/149)	NP_754465
15	*wcaI*	404	16116–17330 (23259–24473)	Putative colonic biosynthesis glycosyl transferase	*E. coli*	57 (233/406)	AAG57110
16	*rfbP*	467	17632–19035 (24775–26178)	Probable CPS biosynthesis glycosyltransferase (ORF14); *rfbP* homolog	*K. pneumoniae* (Chedid)	73 (345/467)	Q48460
17	*gnd*	468	19199–20605 (26342–27748)	Gluconate-6-phosphate dehydrogenase	*E. coli*	99 (464/468)	BAA28321
18	*manC*	471	20841–22256 (27984–29399)	Mannose-1-phosphate guanylyltransferase (ORF16); *cpsB* homolog	*E. coli*	97 (459/471)	BAA07745
19	*manB*	456	22279–23649 (29422–30792)	Phosphomannomutase	*E. coli*	96 (441/456)	P37755
20	*ugd*	388	23813–24979 (30956–32122)	Putative UDP-glucose dehydrogenase	*E. coli*	99 (385/388)	AAC45348.1

NOTE. *B. fragilis, Bacteroides fragilis;* CPS, capsular polysaccharide synthesis; *E. carotovora, Erwinia carotovora; E. coli, Escherichia coli; F. tularensis, Francisella tularensis; K. pneumoniae, Klebsiella pneumoniae; M. acetivorans, Methanosarcina acetivorans; S. flexneri, Shigella flexneri; V. cholerae, Vibrio cholerae.*

[a] The first nucleotide of *galF* (GenBank accession no. Q48447) defines position 1.

[b] Determined by BLAST-P.

[c] Position-specific score matrix producing significant alignments.

[d] Identity scored by PSI-PHI BLAST.

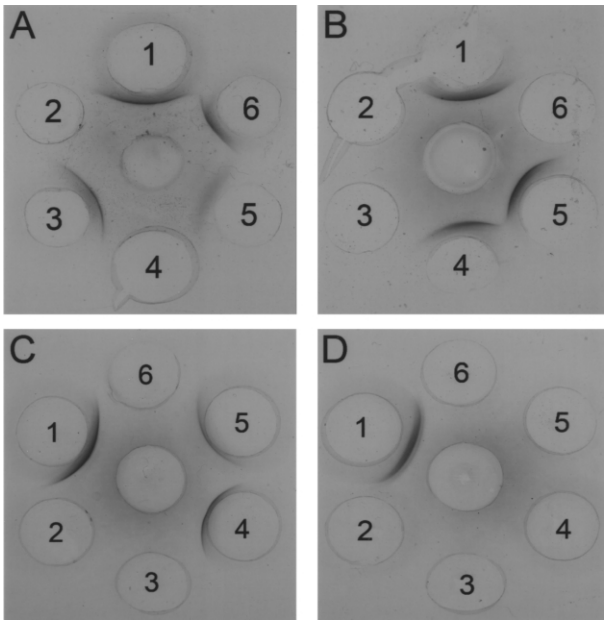

Figure 4. Double immunodiffusion with rabbit anti-K1 serum from Statens Serum Institute *(center wells)* and capsular extracts (20 μL) from 1 mL (*A* and *B*) or 5 mL (*C* and *D*) of overnight-cultured *Klebsiella pneumoniae* strains in the peripheral wells. *A, Well 1,* wild-type NTUH-K2044; *well 2, magA*$^-$ mutant; *well 3, magA*$^-$ mutant strain with *magA trans*-complementation; *well 4, wcaI*$^-$ mutant; *well 5, wcaI*$^-$ mutant strain with *wcaI trans*-complementation; *well 6,* A5054. Wells 1, 3, 5, and 6 showed a positive immunoprecipitation line, whereas wells 2 and 4 did not. *B, Well 1,* NTUH-K2044; *well 2, wza*$^-$ mutant; *well 3, wzc*$^-$ mutant; *well 4, wzm*$^-$ mutant; *well 5, rmpA2*$^-$ mutant; and *well 6,* ATCC8052. Wells 1, 4, and 5 showed a positive immunoprecipitation line, whereas wells 2, 3, and 6 did not. *C* and *D, Trans*-complementation of the *wzx–rfbP* gene fragment *(C)* and *magA* gene *(D). Well 1,* NTUH-K2044 as a positive control; *wells 2–6,* non-K1 *K. pneumoniae* strains (*well 2,* NTUH-N3529; *well 3,* NTUH-N3932; *well 4,* NTUH-N5906; *well 5,* NTUH-N2059; *well 6,* ATCC8052). Two (wells 4 and 5 in panel C) of the 5 non-K1 strains complemented with the *wzx–rfbP* fragment turned K1 positive, whereas *magA* complementation converted none of the 5 strains (wells 2–6 in panel D).

reverse transcribed using 0.1 μmol of random primer (Roche) and 600 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a reaction mixture of 60 μL. Reaction mixtures without reverse transcriptase were included as negative controls. PCR was performed with 1 μL of each reverse-transcription reaction mixture as template and subjected to the above-mentioned temperature cycles.

RESULTS

***Capsular serotype and prevalence of* magA.** Thirty-five of the 42 tissue-invasive strains were *magA* positive, whereas only 1 of the 32 non–tissue-invasive strains was found to be positive for *magA* by PCR (35/42 vs. 1/32; $P < .001$, χ^2 test) (table 3). The capsular serotypes of 74 clinical isolates were initially screened with *Klebsiella* antisera SEIKEN. Of 42 tissue-invasive strains, 35 were K1, 1 was K2, 1 was K6, and 5 were nontypeable (i.e., a serotype that was unable to react with the K1–K6 antisera in the kit). However, of 32 non–tissue-invasive strains, only 1 was K1, 1 was K2, and 1 was K3; the other 29 strains were nontypeable (table 3). The results were further confirmed with anti-K1 serum from Statens Serum Institute, using double immunodiffusion analysis. The results obtained with these 2 assays were all consistent. This showed that all 37 K1 *K. pneumoniae* strains (35 tissue-invasive strains, 1 non–tissue-invasive strain, and the reference strain A5054) were *magA* positive, whereas all 40 non-K1 *K. pneumoniae* strains (7 tissue-invasive strains, 31 non–tissue-invasive isolates, MGH78578, and ATCC8052) were *magA* negative (37/37 vs. 0/40; $P < .0001$) (table 3 and figure 2).

Comparison of* cps *loci in NTUH-K2044, MGH78578, and the Chedid strain. Because there was no sequence match between the *magA* and MGH78578 genomes, we used inverse PCR and the NTUH-K2044 phagemid library [23] to extend the upstream and downstream regions of the *magA* gene in NTUH-K2044 until matches were found. DNA sequences were then compared with MGH78578. A 31-kb fragment in NTUH-K2044 replaced a 26-kb region in MGH78578; they were both downstream of *galF, ORF2, wzi (orfX),* and the *wza-wzb-wzc* cluster, as shown in figure 3. The ORFs between *galF* and *ugd* in NTUH-K2044 (GenBank accession number AB198423) were thus considered to be *cps* loci by comparison with the K2 Chedid strain [16, 18].

The percentage of GC content in the *cps* region was 42.5%, in contrast to 57.7% of the full genome in MGH78578. Genetic loci in the *cps* region of NTUH-K2044 (K1), MGH78578 (K52), and the Chedid (K2) strain, which have different capsular serotypes, had similar functions, such as glycosyltransferase (table 4). However, sequences from *wzx* to *wcaI* of NTUH-K2044 (K1) revealed a low similarity, compared with *ORF7–ORF13* of Chedid (K2) and *wbaP–wzx* in MGH78578 (K52) (figure 3), as determined by National Center for Biotechnology Information BLAST (version 2.2.11; available at: http://www.ncbi.nlm.nih.gov/blast/; (expect, 10; word size, 3; matrix, BLOSUM62; gap costs: existence, 11 extension, 1). These comparisons suggested that the ORFs from *wzx* to *wcaI* of NTUH-K2044 might be unique for the K1 capsule, while *rfbP* was relatively specific in some serotypes.

Characterization of the K1 determinant region of* cps *loci in NTUH-K2044. Insertion mutants, including *ORF8′*$^-$, *wcaG*$^-$, and *wcaI*$^-$, as well as *magA*$^-$, were constructed in the NTUH-K2044 parent strain. These mutants turned negative for anti-K1 serum. Complementation experiments showed that K1-negative *magA*$^-$, *ORF8′*$^-$, *wcaG*$^-$, and *wcaI*$^-$ mutants turned K1 positive by transformation of a pCRII-TOPO-CAT carrying their corresponding intact genes (figure 4*A*). *wza*$^-$ and *wzc*$^-$

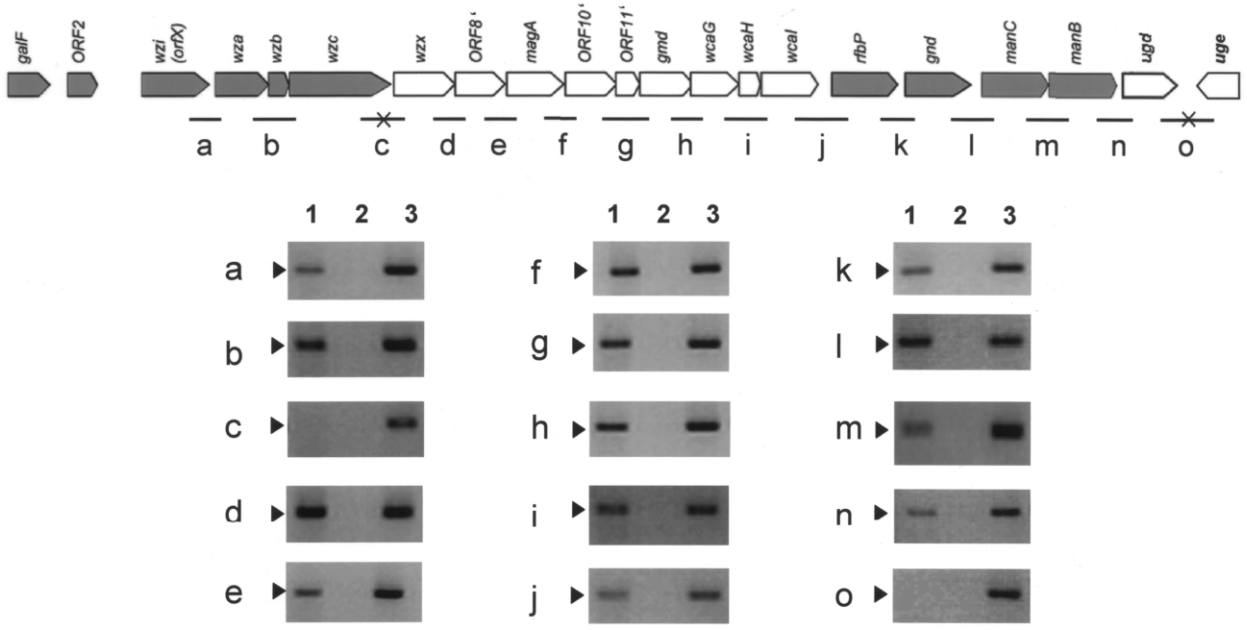

Figure 5. The transcription units of capsular polysaccharide synthesis *(cps)* loci in NTUH-K2044 defined by reverse-transcription (RT) polymerase chain reaction (PCR). The genetic organization of *cps* loci is shown in the upper part of the figure. Panels a–o show the corresponding PCR products for primers located at each open reading frame (ORF) junction. The bars denote RT-PCR–positive junctions, and bars with an X denote RT-PCR–negative junctions. The lower part of the figure shows the RT-PCR results by ethidium bromide–stained agarose gel. *Lane 1,* RT-PCR products of each junction; *lane 2,* RT-PCR without reverse transcriptase, as a negative control; *lane 3,* PCR with genomic DNA as a template, as a positive control. Arrowheads indicate the expected sizes of RT-PCR products.

mutants that were defective in capsular antigen translocation were used as negative controls (figure 4*B*).

The prevalence of genes other than *magA* in the region from *wzx* to *rfbP* was also tested between 20 K1 strains (table 2) (of the first 20 of 36 K1 strains, 19 were tissue invasive and 1 was non–tissue invasive) and 20 non-K1 strains (of the first 20 of 38 non-K1 strains, 3 were tissue invasive and 17 were non–tissue invasive). The 9 loci (*wzx, ORF8′, ORF10′-ORF11′, gmd, wcaG-wcaH, wcaI,* and *rfbP*) were all present in K1 strains (20/20). Like *magA, wzx, ORF8′, ORF10′-ORF11′, gmd,* and *wcaI* were totally absent in non-K1 strains (0/20). However, *wcaG-wcaH* and *rfbP* could still be found in 25% (5/20) and 20% (4/20) of non-K1 strains, respectively. Transformation of the plasmid containing the *wzx* to *rfbP* region were able to turn 2 of the 5 non-K1 *K. pneumoniae* strains K1 positive, whereas plasmids containing only *magA* were unable to turn any of the 5 strains K1 positive (figure 4*C* and 4*D*).

Analysis of transcriptional units by RT-PCR. The predicted promoter regions in *cps* loci of NTUH-K2044 were first analyzed using software from the Berkeley Drosophila Genome Project (available at: http://www.fruitfly.org/seq_tools/promoter.html). It revealed hundreds of putative promoters in *cps* loci. We thus determined the transcriptional unit by RT-PCR analysis. Fifteen primer pairs were designed to amplify the junction of ORFs, using DNaseI-treated total RNA as an RT-PCR template (figure 5). RT-PCR results for units a, b, d, e, f, g, h, i, j, k, l, m, and n were positive, whereas those for c and o were negative (figure 5, *lane 1*). The results suggested that the transcriptional units were *wzi* to *wzc* and *wzx* to *ugd.*

DISCUSSION

Capsule—but not LPS—of *K. pneumoniae* has been determined to be the important virulence factor against complement and phagocyte attack in vitro [30]. Serological tests have revealed that *K. pneumoniae* of capsular serotypes K1 and K2 were the predominant virulent strains among those isolated from patients with either bacteremia or liver abscess (85/134 were K1; 19/134 were K2) [13, 14, 31]. The serotype of PLA-causing *K. pneumoniae* has not yet been identified. *K. pneumoniae* PLA differs from traditional liver abscess in 3 aspects: first, it is community acquired; second, patients with *K. pneumoniae* liver abscess have no history of hepatobiliary diseases; and third, 11%–12% of patients have other septic metastatic lesions [8, 32]. *K. pneumoniae* PLA has become an important emerging infectious disease in Asia, especially in Taiwan [8]. The reason for the increased incidence of *K. pneumoniae* PLA is still not well understood. In our study, 83.3% (35/42) of bacteria causing primary liver abscess were serotype K1; however, only 2.4% (1/42) were serotype K2. The difference might be due to cross-

reactions between K1 and K2 resulting from the use of antisera from the Laboratory of HealthCare Associated Infection, Health Protection Agency, since such cross-reactions were found in our initial tests. Although the structures of capsular K1 and K2 lack mannose-α2/3-mannose and L-rhamnose-α2/3-rhamnose, which easily prompt lectinophagocytosis [33, 34, 35], it seems that the biological function of capsular K1 antigen may play a more important role in the pathogenesis of PLA or its derived metastatic complications. Actually, we found that all 9 *K. pneumoniae* strains that caused complications with metastatic lesions in our series were serotype K1 (8 caused endophthalmitis, and 1 caused meningitis).

Serotyping and studies of the bacterial genome provide clues for tracing the origin of this disease. The association of serotype K1 strains with PLA and the prevalence of *magA* prompted us to study the correlation between capsular serotype K1 and *magA*. In our clinical isolates, as well as in the reference strains, we found that all *magA*-positive strains were capsular serotype K1, whereas none of the *magA*-negative strains was serotype K1. The strong correlation suggested that *magA* was associated with K1 strains.

By comparison with sequences of the K2 Chedid strain and the K52 MGH78578 strain, we identified a *cps* region specific for capsular serotype K1. Mutant strains defective in several genes located in this region turned negative for anti-K1 serum. In addition, complementation of such mutant strains restored a positive K1 phenotype. The *wzm*$^-$ and *rmpA2*$^-$ mutants—which lacked O-antigen biosynthesis and regulation of capsule production, respectively—were isolated by decreased mucoviscosity, using a string test [7]. RmpA2 has been shown to be a transcriptional activator of *cps* loci, and mutation in *rmpA2* would result in 13%–29% lower capsule production [36]. However, these 2 mutants retained the K1-positive phenotype, suggesting that these 2 ORFs, located outside of the regions between *wzx* and *wcaI,* did not affect antigenic structure and were not K1 determinants. RT-PCR results showed that there was an operon (from *wzx* to *ugd*) specific for K1. We determined the prevalence of genes from *wzx* to *rfbP,* because they seemed to be unique on the basis of sequence comparison. The prevalences of several genes from *wzx* to *rfbP* indicated that they, like *magA,* were also very unique for K1 (*wzx, ORF8′, ORF10′-ORF11′, gmd,* and *wcaI* were present in 20/20 vs. 0/20 in K1 and non-K1 strains, respectively). The other 2 gene fragments, *wcaG-wcaH* and *rfbP,* were relatively specific but not unique for K1 (*wcaG-wcaH* was present in 20/20 vs. 5/20, and *rfbP* was present in 20/20 vs. 4/20 of K1 and non-K1 strains, respectively). We did not test the remaining ORFs in the operon, since sequence comparison showed that they were relatively conserved (figure 3). Although we could not rule out the possibility that some ORFs in this region could also play a role in LPS biosynthesis, the fact that *trans*-complementation of the *wzx-rfbP* fragment turned some (2/5) strains K1 positive confirmed that these genes were involved in capsular synthesis. These results confirmed that the operon from *wzx* to *wcaI* was responsible for capsular serotype K1. Failure of K1 conversion in the other 3 strains was very likely due to the difference in other genes of the *cps* region, although comparison of available *cps* sequences in the 3 strains suggested that they were less variable.

Capsular serotyping is a convenient method; however, some antisera, such as those from the Laboratory of HealthCare Associated Infection, Health Protection Agency, may yield cross-reactions between K1 and K2. Therefore, sequences of this *cps* region would help to confirm serotyping results that are in doubt. As shown in our study, PCR using primer pairs derived from *magA* or other genes unique for K1 is a rapid and accurate method to detect K1 strains. Serotype K1 has been shown to have a more close relationship to PLA with metastatic lesions, such as endophthalmitis [13]. Metastatic endophthalmitis can result in blindness or evisceration unless treated within 24 h with appropriate systemic and intravitreous antibiotics [37]. Rapid molecular diagnosis using the genetic determinants will help these patients.

The structural unit of capsular K1 has been resolved as →4)-[2,3-(S)-pyruvate]-β-D-GlcA-(1→4)-α-L-Fuc-(1→3)-β-D-Glc-(1→ [38]. This is in agreement with the function of some genes in *cps* loci: *ORF8′* is a putative polysaccharide pyruvyl transferase, and *wcaG* is a putative GDP-fucose synthetase. There are other sugar metabolism–associated loci, such as *gmd* and *manC,* that are involved in mannose metabolism; however, they are not included in the structural unit. Polysaccharide biosynthesis is a complex process, and many intermediates and enzyme substrates could be involved; however, only structural units remain at the end of this process. For example, Gmd dehydrates GDP-D-mannose into GDP-4-keto-6-deoxy-D-mannose, which is subsequently converted into GDP-L-fucose by WcaG, a GDP-fucose synthetase [39]. Therefore, the structural unit could not be predicted directly from the annotation of genes in *cps* loci.

In conclusion, we have identified a *magA*-containing chromosomal region that encodes capsular structure and is specific for the K1 serotype. This *cps* region contains an operon from *wzx* to *ugd* in NTUH-K2044. Among these ORFs, *magA, wzx, gmd, wcaI,* and *ORF10′-ORF11′* seem unique for K1 and can be used for rapid molecular typing. These results may help in the early detection and tracing the origin of this emerging infectious disease.

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