The role of *Klebsiella pneumoniae rmpA* in capsular polysaccharide synthesis and virulence revisited

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*Klebsiella pneumoniae* community-acquired pyogenic liver abscess (PLA) is an emerging infectious disease. The *rmpA* gene (for regulator of mucoid phenotype A) has been reported to be associated with PLA in prevalence studies. NTUH-K2044, a K1 PLA isolate, carries three *rmpA/A2* genes: two large-plasmid-carried genes (*p-rmpA* and *p-rmpA2*) and one chromosomal gene (*c-rmpA*). In this study, we re-examined the role of *rmpA/A2* in PLA pathogenesis to clarify the relationship of *rmpA/A2* and capsular serotype to virulence. Using isogenic gene deletion strains and complemented strains of NTUH-K2044, we demonstrated that only *p-rmpA* enhanced expression of capsular polysaccharide synthesis (*cps*) genes and capsule production.

Nevertheless, the lethal dose and in vivo competitive index indicated that *p-rmpA* does not promote virulence in mice. The prevalence of these three *rmpA/A2* and capsular types in 206 strains was investigated. This revealed a correlation of *rmpA/A2* with six PLA-related capsular types (K1, K2, K5, K54, K57 and KN1). However, the correlation of *rmpA/A2* with K1 strains from the West was less obvious than with the strains from Asia (17/22 vs 39/39, *P* = 0.0019). Among the three *rmpA/A2* genes, *p-rmpA* was the most prevalent. Due to the strong correlation with PLA-related capsular types, *p-rmpA* could serve as a surrogate marker for PLA. We found an association of *p-rmpA* with three widely spaced loci in a large plasmid (30/32). Therefore, *rmpA* could be co-inherited together with virulence genes carried by this plasmid.

INTRODUCTION

*Klebsiella pneumoniae* can cause hospital-acquired urinary tract infections, sepsis and pneumonia (Abbott, 2003). A new type of community-acquired *K. pneumoniae* associated with pyogenic liver abscess (PLA) and metastatic complications has emerged globally, especially in Asia, in the past 20 years (Chang et al., 2000; Cheng et al., 1991; Chiu et al., 1988; Chuang et al., 2006; Chung et al., 2007; Fang et al., 2004, 2007, 2005; Fung et al., 2002; Keynan et al., 2007; Ko et al., 2002; Liu et al., 1986; Nadasy et al., 2007; Wang et al., 1998; Yang et al., 2004). A major virulence factor is the capsule, which protects *K. pneumoniae* from lethal serum factors and phagocytosis. With at least 78 capsular serotypes defined, serotype-related variation in infection severity has been observed. Strains with capsular serotypes K1 and K2 have been identified as the predominant virulent strains, and their virulence has been confirmed in mouse models (Fung et al., 2002; Mizuta et al., 1983; Struve et al., 2005). In our studies, the capsules of *K. pneumoniae* strains causing PLA were K1 (~80 %), K2, K5, K20, K54, K57 and a new type (Fang et al., 2007; Pan et al., 2008). Capsular types K1, K2, K5, K20, K54, K57 and an unidentified type were also prevalent in southern Taiwan (Yu et al., 2008). In addition, 59.4 % of *K. pneumoniae* strains causing PLA in Korea were serotype K1 (Chung et al., 2007). Therefore, K1 is considered to be the most common *K. pneumoniae* capsular type causing PLA.

Prevalence studies showed an association of *rmpA* with *K. pneumoniae* virulence (Cheng et al., 2010; Fang et al., 2004; Ku et al., 2008; Nassif et al., 1989a; Yu et al., 2006, 2008). The prevalence of *rmpA* has been documented to be greater in liver abscess strains (87.5 %) than in bacteraemic strains (Yu et al., 2006). In K2 strains, *rmpA* and an isofrom *rmpA2* have been shown to regulate capsular polysaccharide (CPS) biosynthesis (Cheng et al., 2010; Lai et al., 2003; Nassif et al., 1989a; Wacharotayankun et al., 1993). The *rmpA* gene was first reported to be located on the 180 kb plasmid in the K2 strain 52145, and to positively control the mucoid phenotype (Nassif et al., 1989a). The *rmpA2* gene, sharing 80 % identity with the 3′ DNA sequences of *rmpA*, was later identified on the large plasmid in a K2...
strain Chedid and shown to enhance CPS (Wacharotayankun et al., 1993). Sequence analysis of RmpA2 showed that the C-terminal region shared considerable similarity with the putative helix–turn–helix motif of transcription regulators NtrC and FixJ. The N- and C-terminal regions also exhibited some homology to RcsA, a transcription activator for colonic acid synthesis in Escherichia coli (Wacharotayankun et al., 1993). K. pneumoniae CPS requires the chromosomal cps gene cluster encoding proteins mainly for translocation and surface assembly of polysaccharides (Chuang et al., 2006). The rmpA and rmpA2 genes in the K2 strain CG43 have been reported to activate CPS transcription and increase the virulence of this K2 strain in mice (Cheng et al., 2010; Lai et al., 2003).

Although associated with PLA (Yu et al., 2006), rmpA has been suggested to be a less important determinant of liver abscess formation than capsular serotype K1 or K2 (Yeh et al., 2007), raising the issue of whether rmpA is the actual virulence determinant. Studies by Yeh et al. (2007) demonstrated that K1 or K2 isolates were more phagocytosis-resistant and virulent in mice than rmpA-positive and -negative non-K1/K2 isolates. They showed that the virulence of rmpA-positive non-K1/K2 strains was different, suggesting that factors other than rmpA contributed to virulence. The relationship between rmpA/A2, capsular serotype and virulence has yet to be elucidated.

NTUH-K2044, a K1 strain with the hypermucoviscosity phenotype, was originally isolated from a patient with community-acquired PLA and metastatic meningitis (Fang et al., 2004). Our prior work using transposon mutagenesis in NTUH-K2044 identified magA in the cps cluster as being necessary for mucoviscosity, resistance to serum killing and phagocytosis, and virulence in mice (Fang et al., 2004). We also obtained a mutant by transposon insertion into chromosomal rmpA causing reduction of mucoviscosity. Whole-genome sequencing revealed that NTUH-K2044 carried three rmpA/A2 genes: rmpA (p-rmpA) and rmpA2 (p-rmpA2) on the large plasmid, and a second rmpA on the chromosome (c-rmpA). This is different from all previous reports, which found rmpA/A2 on the large plasmid of K2 strains (Wu et al., 2009). In this study, we re-examined the functions of the three rmpA/A2 genes in NTUH-K2044. By gene deletion and complementation, we differentiated between the effects of rmpA/A2 on CPS and on virulence in mice. We also investigated rmpA/A2 prevalence in K. pneumoniae PLA isolates and clarified their relationship to capsular types and virulence.

METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids and primers used in this study are listed in Supplementary Table S1, available with the online version of this paper. One hundred and three K. pneumoniae clinical isolates were collected from Asia: 74 strains from the National Taiwan University Hospital (NTUH) (Chuang et al., 2006), 13 strains from En Chu Kong Hospital (ECKH, Taiwan), 15 strains from Hong Kong (Fang et al., 2004) and one PLA strain from Japan (Kohayagawa et al., 2009). A further 103 K. pneumoniae strains were from North America and Europe (the West): 80 non-PLA strains from Canada (Pan et al., 2008), one PLA strain each from Finland (Pan et al., 2008), Belgium (Karama et al., 2008), the USA (Rahimian et al., 2004) and Canada (Keynan et al., 2007), 15 K1 strains from France (Brisse et al., 2009), including one causing liver abscess, and four other K1 strains, A5054, ATCC 8045, ATCC 8047 and ATCC 35593, purchased from the Statens Serum Institute (SSI, Copenhagen, Denmark) and American Type Culture Collection (ATCC). Prevalence of rmpA/A2 was investigated using PCR with primers designed to distinguish the three rmpA/A2 genes. By comparing rmpA/A2 DNA sequences in strain NTUH-K2044, we used the regions with diverged sequences to design specific primer sets for each of the three rmpA/A2 genes. These primer sets were tested in NTUH-K2044, and amplified PCR products were confirmed by DNA sequencing. Capsular types were determined using cps-PCR genotyping (Pan et al., 2008). K. pneumoniae and E. coli were cultured at 37°C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics: ampicillin (100 μg ml⁻¹) or kanamycin (50 μg ml⁻¹).

Construction of gene deletion, complementation and replacement strains. Isogenic deletion mutants of NTUH-K2044 were constructed using an unmarked deletion method with the temperature-sensitive plasmid pKO3-Km (Hsieh et al., 2008; Link et al., 1997). For chromosomal complementation, a single copy of the p-rmpA gene including the promoter was cloned into the intergenic region of pgpA and yajO using vector pKO3-Km-pgpAyajO (Hsieh et al., 2010), followed by transformation into corresponding mutants. A replacement mutant in which the coding region of p-rmpA (ORF KP020) was replaced by that of c-rmpA (ORF KP3619) was constructed using a method similar to the unmarked deletion method. After integration and excision of pKO3-Km carrying the c-rmpA coding region with upstream and downstream flanking sequences of p-rmpA, the replacement construct was selected out by PCR. All mutant and complemented strains were confirmed by PCR and DNA sequencing.

Quantitative real-time RT-PCR. Using the method described by Hsieh et al. (2008), 400 ng purified K. pneumoniae total RNA was reverse-transcribed with random primers, followed by PCR amplification with appropriate primers monitored using SYBR Green dye (Applied Biosystems). For each gene, the calculated threshold cycle (Ct) value was normalized to the Ct of the 23S rRNA gene from the same complementary DNA sample before the fold change was calculated using the ΔΔCt method (Applied Biosystems, 2001).

Extraction and quantification of extracellular polysaccharides. K1 CPS is composed of D-glucose, D-glucuronic acid and L-fucose with structural unit →4)-β-(1-3)-D-gluco-pyruvate]-β-D-GlcA-(1→4)-3-L-fucose-(1→3)-β-D-Glc-(1→ (Erbing et al., 1976). To quantify CPS, extracted polysaccharides from equal numbers of K. pneumoniae were prepared using a hot water/phenol extraction method (Chuang et al., 2006), followed by a phenol/sulphuric acid assay to measure the hexose content (Chaplin, 1986). Fucose solutions with concentrations of 0, 12.5, 25, 50, 100, to 200 μg ml⁻¹ were used to establish a standard curve. Mixing 200 μl 1:10 diluted extracted polysaccharides or fucose with 200 μl 5% (w/v) phenol was followed by addition of 1 ml of H₂SO₄. After 10 min post-incubation. To determine K1 CPS antigenicity, a double immunodiffusion assay was used with anti-K1 antisera purchased from Statens Serum Institut (Pan et al., 2008).

Determination of mucoviscosity and serum resistance. The string test was performed and hypermucoviscosity was defined by formation of viscous strings >5 mm long (Fang et al., 2004). To
further quantitatively distinguish the subtle difference between mucoviscosity and capsule production, low-speed centrifugation was used since bacteria with thick and mucoid capsules pellet less readily (Lai et al., 2003; Pan et al., 2011). Briefly, equal numbers of bacteria in 1.2 ml LB medium were centrifuged at 2000 g for 15 min. The OD_{600} of the supernatants (1.1 ml) was then measured. Serum resistance of K. pneumoniae was analysed as previously described (Chuang et al., 2006; Fang et al., 2004) by incubating 2.5 × 10^8 bacterial c.f.u. with human serum from healthy volunteers for 3 h and then determining the number of recovered c.f.u.

**Animal inoculation.** Groups of 5-week-old female BALB/c mice were infected intraperitoneally or intragastrically with K. pneumoniae in 100 μl 0.95% saline solution (10^7–10^8 c.f.u. as indicated; four mice for each dose) and monitored for 4 weeks. The exact inoculation dose was confirmed on LB agar. The 50 % lethal dose (LD_{50}) was calculated using Kaplan–Meier analysis and differences in survival by a log-rank test; P<0.05 was considered to be statistically significant. In vivo competition of K. pneumoniae was analysed as outlined in previous work (Hsieh et al., 2010). Briefly, the test strain and the isogenic lacZ mutant with its promoter deletion (NTUH-K2044Ap lacZ) were mixed at a 1:1 ratio and a dose of 10^8 c.f.u. in 100 μl saline solution was intraperitoneally inoculated into 5-week-old BALB/c mice. No difference in competition between NTUH-K2044Ap lacZ strain and wild-type strain has been shown (Hsieh et al., 2010). The mice were killed 24 h post-inoculation and their livers were removed and homogenized in 1 × PBS. The number of LacZ-positive and LacZ-negative colonies on LB plates containing 1 mmol IPTG 1^{-1} and 50 mg X-Gal ml^{-1} were counted. The output to input ratio of test strain to virulent strain was defined as the competitive index (CI).

**RESULTS**

Only p-rmpA upregulates cps transcription and CPS synthesis in NTUH-K2044

NTUH-K2044 carries three rmpA/A2 genes: p-rmpA (KPP020) and p-rmpA2 (KPP302) located on the ~224 kb large plasmid pK2044, and c-rmpA (KP3619) located on the chromosome. The p-rmpA and c-rmpA genes are both 633 bp in length and show 92% DNA sequence identity and 89% amino-acid sequence identity. The p-rmpA2 gene shares only 83% of its DNA sequence with that of p-rmpA and 81% with that of c-rmpA. All three rmpA/A2 genes contain a poly-G tract commencing at the 276th nucleotide downstream of the start codon. The p-rmpA and c-rmpA genes were predicted to encode a 210 amino-acid product. However, an early stop codon appears in p-rmpA2, as a result of a shift in one nucleotide in the poly-G tract, resulting in a truncated product with 99 amino-acid residues, which lacks the C-terminal DNA-binding region. NTUH-K2044 p-RmpA and c-RmpA share 99% and 90% amino-acid sequence identity with CG43 RmpA. The 96 N-terminal amino-acid residues of NTUH-K2044 p-RmpA2 share 100% identity with those of CG43 RmpA2 (212 amino acids). To characterize functions of these rmpA/A2 genes in NTUH-K2044, we constructed single-gene deletion mutants using an unmarked deletion method, leaving a deletion in the target gene without insertion of an antibiotic resistance cassette (Hsieh et al., 2008; Link et al., 1997). Resulting constructs were confirmed by PCR with primers specific for each rmpA/A2. In addition, Southern blot analysis was performed to verify the presence of Δp-rmpA and Δc-rmpA (results not shown).

The expression of cps genes in NTUH-K2044ΔrmpA/A2 mutants was initially analysed. The p-rmpA locus of NTUH-K2044 is ~25 kb in length and contains 20 ORFs (Chuang et al., 2006). To examine cps expression quantitatively, real-time RT-PCR was used to determine transcription levels of galF and wzc, two conserved genes in the cps locus. Upstream of galF and wzi (upstream of wzc) are two promoter regions, which can be activated by RmpA in strain CG43 (Cheng et al., 2010) and also RmpA2 via direct binding (Lai et al., 2003). In comparison with expression in the wild type (defined as 100%), galF expression was significantly decreased to 17% in Δp-rmpA, increased to 135% in Δp-rmpA2 and decreased to 84% in Δc-rmpA (Fig. 1a, left graph). Expression of wzc was similarly decreased to 27% in Δp-rmpA and increased to 134% in Δp-rmpA2, but remained at 99% in Δc-rmpA (Fig. 1a, right graph). Therefore, the three rmpA/A2 genes had different effects on cps in NTUH-K2044 in this condition. Only p-rmpA promoted cps expression, while p-rmpA2 inhibited cps expression and c-rmpA had no obvious effect.

CPS biosynthesis of NTUH-K2044ΔrmpA/A2 mutants was next determined. The relative amount of extracted polysaccharides was reduced to 58% in Δp-rmpA (compared with wild-type) (Fig. 1b), but remained at 85% and 103% in Δc-rmpA and Δp-rmpA2, respectively. For comparison, polysaccharide biosynthesis was significantly reduced to 34% in a capsule-negative ΔmagA strain Although Δp-rmpA, Δp-rmpA2 and Δc-rmpA all retained K1 antigenicity (Fig. 1c) and appeared to form viscous strings indistinguishable from those of the wild-type in the string test (results not shown), low-speed centrifugation revealed further subtle differences in mucoviscosity and capsule production. Highly mucoid NTUH-K2044 was difficult to pellet by centrifugation. However, Δp-rmpA was easily pelleted, reflecting a significantly reduced mucoviscosity (Fig. 1d). Δp-rmpA2 and Δc-rmpA did not cause significant differences from the wild type. These results indicated that only p-rmpA promoted CPS biosynthesis and mucoviscosity.

To confirm the effect caused by deletion of p-rmpA, a complemented strain, in which a single copy of p-rmpA was introduced onto the chromosome of Δp-rmpA, was analysed. With the levels of p-rmpA expression recovered to 75% in the complemented strain (Fig. 2a), cps transcription, CPS biosynthesis and mucoviscosity were restored (Fig. 2b, c). Therefore, deficiency in Δp-rmpA was due directly to an effect of p-rmpA rather than the other genes.

**Replacement of the p-rmpA coding region with the c-rmpA coding region causes deficiency in CPS regulation**

Unlike other known K2 strains, NTUH-K2044 carries an additional rmpA on its chromosome. Although p-rmpA
Fig. 1. Expression of *cps* genes and CPS biosynthesis in NTUH-K2044ΔrmpA/A2 strains. (a) Relative transcriptional levels of *galF* (left graph) and *wzc* (right graph) in Δp-*rmpA*, Δc-*rmpA* and Δp-*rmpA2* strains were determined using real-time RT-PCR, and are shown in comparison with the wild-type. For *galF*: Δp-*rmpA* and wild-type, *P*<0.0001; Δp-*rmpA2* and wild-type, *P*=0.001; Δc-*rmpA* and wild-type, *P*=0.009. For *wzc*: Δp-*rmpA* and wild-type, *P*<0.0001; Δp-*rmpA2* and wild-type, *P*=0.0004; Δc-*rmpA* and wild-type, *P*=0.480. (b) Polysaccharide biosynthesis of *K. pneumoniae* was determined by phenol-sulphuric acid assays, and is shown in comparison with that of the wild-type. ΔmagA, a capsule-negative mutant, was also analysed for comparison. Δp-*rmpA* and wild-type, *P*<0.0001; Δp-*rmpA2* and wild-type, *P*=0.390; Δc-*rmpA* and wild-type: *P*=0.030. (c) Double immunodiffusion analysis of NTUH-K2044ΔrmpA/A2 strains. Anti-K1 antiserum was added in the centre well, while capsular extracts from overnight-cultured *K. pneumoniae* strains (as indicated) were loaded in the peripheral wells. (d) Centrifugation analysis of NTUH-K2044ΔrmpA/A2 strains. Equivalent amounts of *K. pneumoniae* strains Δp-*rmpA*, Δp-*rmpA2* and Δc-*rmpA* were subjected to centrifugation at 2000 g for 15 min. The OD$_{600}$ of supernatants was measured. Δp-*rmpA* and wild-type, *P*<0.0001; Δp-*rmpA2* and wild-type, *P*=0.022; Δc-*rmpA* and wild-type, *P*=0.603. Data are means ± SEM. All *P*-values were determined by Student’s t test.
and c-rmpA have 92% DNA sequence identity, c-rmpA has no obvious effects on CPS. In wild-type NTUH-K2044, gene expression of c-rmpA (taken as 100%) was higher than that of p-rmpA (22%), suggesting that dysfunction of c-rmpA is not due to lack of gene expression (Fig. 3a). We tried to clarify why c-rmpA was less functional than p-rmpA. We constructed an NTUH-K2044 isogenic mutant in which the coding region of p-rmpA (KPP020) was replaced by that of c-rmpA (ORF KP3619). Compared with the wild type, this replacement mutant had significantly lower levels of galF and wzc RNA, CPS biosynthesis and mucoviscosity (Fig. 3b–d). There were no significant differences between this replacement mutant and Δp-rmpA (for galF, P = 0.057; wzc, P = 0.192; polysaccharide biosynthesis, P = 0.107; centrifugation analysis, P = 0.369; Student’s t test). Therefore, the replacement of p-rmpA by c-rmpA causes inability to upregulate CPS biosynthesis. The difference in activity between p-rmpA and c-rmpA is likely to be due to sequence differences in the coding regions.

p-rmpA does not promote virulence of NTUH-K2044 in mice

Consistent with the findings showing no obvious effects of Δc-rmpA on CPS, its virulence in mice was not affected. Similar to wild-type NTUH-K2044, intraperitoneally inoculated Δc-rmpA had an LD₅₀ in mice of <1×10⁵ c.f.u. and intragastrically inoculated Δc-rmpA had a similar effect on mouse survival (Kaplan–Meier analysis, log-rank test, P = 0.8881). Unexpectedly, although p-rmpA promoted CPS biosynthesis and mucoviscosity, it did not affect serum resistance and virulence in mice or proliferation in non-immune human serum (Fig. 4a). The LD₅₀ of Δp-rmpA by intraperitoneal inoculation in mice was determined as <1×10⁵ c.f.u. The effects of Δp-rmpA and wild-type strains by intragastric inoculation on mouse survival were similar (Fig. 4b). In vivo competition analysis of intraperitoneally inoculated strains showed that Δp-rmpA did not decrease ability to compete (Fig. 4c). The competitive index of the Δp-rmpA mutant group
(7.06 ± 0.79) was higher than that of the wild-type group (1.23 ± 0.25), indicating that this mutant could be more competitive in mice (P < 0.001, Wilcoxon signed rank test). However, this advantage had no effect on mouse survival. Taken together, the results indicate that Δp-rmpA does not significantly reduce the virulence of NTUH-K2044.

Fig. 3. Replacing the p-rmpA coding region with that of c-rmpA causes CPS deficiency. (a) Transcriptional levels of p-rmpA and c-rmpA in wild-type NTUH-K2044 determined by real-time RT-PCR. DNA of plasmid pGEM-T Easy carrying p-rmpA and c-rmpA was used to establish the standard curves. Expression of p-rmpA is shown in comparison with c-rmpA, P < 0.0001. (b) Relative transcriptional levels of galF (left graph) and wzc (right graph) in the replacement strain [Δp→c rmpA] determined using real-time RT-PCR. For galF, wild-type and the replacement strain, P = 0.003; Δp-rmpA and the replacement strain, P = 0.057, Student’s t test. For wzc, wild-type and the replacement strain, P = 0.020; Δp-rmpA and the replacement strain, P = 0.192. (c) Relative levels of polysaccharide biosynthesis in the replacement strain. Wild-type and the replacement strain, P = 0.003; Δp-rmpA and the replacement strain, P = 0.107. (d) Centrifugation analysis of the replacement and other strains as indicated. After centrifugation at 2000 g for 15 min, the OD_{600} of supernatants was measured. Wild-type and the replacement strain, P < 0.0001; Δp-rmpA and the replacement strain, P = 0.369. Data are means ± SEM. All P-values were determined by Student’s t test.

Prevalence of three rmpA/A2 genes in PLA isolates

We further analysed prevalence of p-rmpA, p-rmpA2 and c-rmpA in 206 K. pneumoniae clinical isolates from Asia (including Taiwan, Hong Kong and Japan) and from the
Fig. 4. Virulence properties of NTUH-K2044Δp-rmpA strain. (a) Serum resistance of Δp-rmpA. After incubation with non-immune human serum, K. pneumoniae strains were recovered on LB agar plates and c.f.u. counted. Results are the mean ± SD of three independent experiments. Percentage survival rate in human serum is expressed as 100 × (number of recovered bacteria after treatment with human serum/number of bacteria originally inoculated). *P* = 0.421, Student’s *t* test. (b) Virulence of Δp-rmpA in mice. Following intragastric inoculation of 10⁶ c.f.u. Δp-rmpA (□) or wild-type (△) strains in 5-week-old female BALB/c mice, relative survival rates (number of live mice/total number of mice) were observed over 30 days. Results are from a total of 20 mice for each K. pneumoniae strain. *P* = 0.213, log-rank test. (c) In vivo competition of Δp-rmpA by intraperitoneal inoculation into BALB/c mice. The wild-type and the Δp-rmpA strain were compared with the fully virulent *placZ* mutant strain, and the ratio of LacZ-positive to LacZ-negative colonies in the contents of the liver of each mouse was determined. Each symbol represents the competitive index (CI) for each inoculum, with the medians shown by bars. Wild-type and Δp-rmpA, *P* = 0.001, Wilcoxon signed rank test.

To investigate possible variability of *rmpA/A2* sequences, we compared sequences of *rmpA/A2* in 33 randomly selected strains: 10 K1 (including NTUH-K2044) and 10 non-K1 strains collected from Asia, and 10 K1 and 3 non-K1 strains collected from the West. The DNA sequences of *p-rmpA* and *c-rmpA* were highly similar in these strains, but the *p-rmpA/A2* genes were more variable in length (Supplementary Table S2). In 33 *p-rmpA/A2* strains, 22 strains had *p-rmpA DNA* sequences that were identical to those in NTUH-K2044. In the other 10 strains (alignments shown in Supplementary Fig. S2), frame-shift mutations appeared in three strains (A5918, N7205 and N6128), resulting in predicted products with 113, 26 and 99 amino acids respectively. As there were a total of 10 strains positive for c-*rmpA* in all 206 strains, we sequenced and compared all of these c-*rmpA* genes. They were all full-length (633 bp). Nine K1 strains (including NTUH-K2044) shared identical c-*rmpA* DNA sequences; the other strain, which belonged to the new capsular type KN1 (A1517) and was the only strain carrying c-*rmpA* only, had a single nucleotide difference in the *p-rmpA* forward priming site for prevalence studies (Supplementary Fig. S2). However, in the other strains, the PCR priming sites for *p-rmpA*, *p-rmpA/A2* and c-*rmpA* in the prevalence studies were all conserved.

West, mainly Canada and France (Table 1). Consistent with previous findings (Yu et al., 2006), *rmpA/A2* was significantly associated with PLA. While 100 % (48/48) of PLA strains carried at least one *rmpA/A2* gene, 19.6 % (31/158) non-PLA strains carried *rmpA/A2* (*P* < 0.001, χ² test). Analysis of the *rmpA/A2* genes revealed that *p-rmpA* and *p-rmpA/A2* were the predominant type in both PLA (35/48, 72.9 %) and non-PLA strains (18/31, 58.1 %). Only PLA strains carried all three *rmpA/A2* genes and appeared to carry a wider variety of *rmpA/A2* than non-PLA strains (*P* < 0.001, Student’s *t* test). The *p-rmpA* gene (78/79) occurred more commonly than *p-rmpA/A2* (62/79) and c-*rmpA* (10/79) in *rmpA/A2*-positive clinical isolates, with no difference between PLA and non-PLA strains. Sixty randomly selected PCR-negative strains were negative in dot-blot hybridization using the probes recognizing three *rmpA/A2* genes (Supplementary Fig. S1.). In addition, DNA sequencing of PCR products from 33 strains (see below) confirmed the identities of these *rmpA/A2* genes using primers annealing to the upstream and downstream regions of the genes (Supplementary Table S1). The sequencing results showed that six strains had one nucleotide difference in the *p-rmpA* forward priming site for prevalence studies (Supplementary Fig. S2). However, in the other strains, the PCR priming sites for *p-rmpA*, *p-rmpA/A2* and c-*rmpA* in the prevalence studies were all conserved.
Correlation of rmpA/A2 with six PLA-related capsular types

Although rmpA associated with PLA in prevalence studies, whether rmpA is a virulence determinant for K. pneumoniae liver abscess remained to be elucidated. Capsular-serotype-related variation in K. pneumoniae infection has been observed. Our previous work demonstrated that there were six capsular serotypes in 42 PLA strains: K1, K2, K5, K54, K57 and a new capsular type KN1 (Chuang et al., 2010; Pan et al., 2008). We investigated these six PLA-related capsular types in 206 clinical strains from Asia and the West (Table 1). All 48 PLA strains belonged to these six PLA-related capsular types (35/36). Among the isolates from Asia, 56 out of 56 strains with these six PLA-related capsular types carried rmpA/A2 only; KN1, new capsular serotype identified by Pan et al. (2008).

### Table 1. Capsular types and prevalence of rmpA/A2 in K. pneumonia clinical isolates

See Methods and Supplementary Table S1 for details of the 103 Asian and 103 Western strains. Four types of rmpA/A2 combination were found in all analysed strains: (1) c-rmpA, p-rmpA and rmpA2; (2) p-rmpA and rmpA2; (3) p-rmpA only; (4) c-rmpA only. KN1, new capsular serotype identified by Pan et al. (2008).

<table>
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<th>Capsular type (no. of strains)</th>
<th>Origin (no. of strains)</th>
<th>c-rmpA, p-rmpA, rmpA2</th>
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<th>p-rmpA only</th>
<th>c-rmpA only</th>
<th>rmpA/A2-positive strains/total strains*</th>
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<td>2</td>
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<td>3</td>
<td>0</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>K2 (6)</td>
<td>PLA (2)</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>Non-PLA (4)</td>
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<td>4</td>
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<td>0</td>
<td>4/4</td>
</tr>
<tr>
<td>K5 (2)</td>
<td>PLA (1)</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>Non-PLA (1)</td>
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<td>0</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td>K54 (1)</td>
<td>PLA (1)</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td>K57 (7)</td>
<td>PLA (2)</td>
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<td>2</td>
<td>0</td>
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<td>2/2</td>
</tr>
<tr>
<td></td>
<td>Non-PLA (5)</td>
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<td>4</td>
<td>1</td>
<td>0</td>
<td>5/5</td>
</tr>
<tr>
<td>KN1 (1)</td>
<td>PLA (1)</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>1/1</td>
</tr>
<tr>
<td>Other types (47)</td>
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<td>0</td>
<td>1†</td>
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<td>1/47</td>
</tr>
<tr>
<td>West</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1 (22)</td>
<td>PLA (4)</td>
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<td>13/18</td>
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<tr>
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</tr>
<tr>
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<td>Non-PLA (2)</td>
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<td>K54 (3)</td>
<td>Non-PLA (3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/3</td>
</tr>
<tr>
<td>K57 (1)</td>
<td>PLA (1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td>KN1 (1)</td>
<td>Non-PLA (1)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0/1</td>
</tr>
<tr>
<td>Other types (70)</td>
<td>Non-PLA (70)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0/70</td>
</tr>
<tr>
<td>Subtotals</td>
<td></td>
<td>9</td>
<td>53</td>
<td>16</td>
<td>1</td>
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</tbody>
</table>

*The totals for each capsular type are shown in bold in the last column.
†The capsular type was determined to be K16 using PCR specific for K16 cps (unpublished data).

9 A, while those of truncated p-rmpA2 contain 8–13 G and 8 A. Therefore, frame-shift mutation and truncated RmpA2 occur commonly in clinical strains.

To explore the possible correlation between rmpA/A2 and virulence, we used a multiplex PCR assay to detect the large-plasmid-derived loci terW, iutA and silS, which have been associated with abscess formation (Tang et al., 2010) in these 33 strains (Supplementary Table S2). Negative results were confirmed by PCR with a single primer set. Large-plasmid-derived loci were observed in three types of strains. The first was p-rmpA⁺-p-rmpA2⁺-terW⁺-iutA⁺-silS⁺ (23 strains including 13 of 17 PLA strains), which possibly represent a group harbouring a virulence genotype on large plasmids such as pK2044 and pLVPK (Wu et al., 2009; Tang et al., 2010). The second type was positive for at least one large-plasmid-derived gene (nine strains), which may represent a group harbouring large plasmids with less similar sequences. In this group, SB3188 and A5054 were p-rmpA⁺ but p-rmpA2⁻-terW⁻-iutA⁻-silS⁻, suggesting that the genotype in their large plasmids could be less similar. The third type, p-rmpA⁻-p-rmpA2⁻-terW⁻-iutA⁻-silS⁻, was represented by only one strain, A1517 (a PLA strain), carrying c-rmpA only. This strain may lack the large plasmid or harbour a plasmid with completely different sequences. As p-rmpA correlated well with at least one gene of terW, iutA and silS (30/32), p-rmpA was related to the large plasmid.
strains with other capsular types carried \( \text{rmpA/A2} \), indicating a correlation between PLA-related capsular types and carriage of \( \text{rmpA/A2} \) \((P<0.0001, \chi^2\) test; odds ratio after adding 0.5 to all cells to correct for zero cell: 3.503). Among the isolates from the West, 22 out of 33 strains with PLA-related capsular types carried \( \text{rmpA/A2} \), while 0 out of 70 strains with other capsular types carried \( \text{rmpA/A2} \). This also suggests a correlation between PLA-related capsular types and carriage of \( \text{rmpA/A2} \) \((P<0.00001, \chi^2\) test; odds ratio after adding 0.5 to all cells to correct for zero cell: 276). When comparing the presence of \( \text{rmpA/A2} \) between K1 strains from Asia and the West, carriage of \( \text{rmpA/A2} \) in K1 strains from the West was less than that from Asia. There were 17 out of 22 K1 strains from the West (77.3%) positive for \( \text{rmpA/A2} \) while all 39 K1 strains from Asia were positive for \( \text{rmpA/A2} \) (Table 1; 17/22 vs 39/39, \( P=0.0019, \chi^2\) test).

To investigate whether \( \text{rmpA/A2} \) determined virulence of K1 strains from the West, we compared \( \text{rmpA/A2} \) carriage and virulence in mice (Table 2). The LD\(_{50}\) of three PLA strains, all carrying \( \text{p-rmpA} \) and \( \text{p-rmpA2} \), was \( <10^5 \) c.f.u. Nevertheless, although all were carrying \( \text{p-rmpA} \) only, four non-PLA strains (A5054, ATCC 35593, ATCC 8045 and ATCC 8047) had LD\(_{50}\) values of \( 10^6 \), \( 10^5 \) and \( 10^2 \) c.f.u. Similarly, four \( \text{rmpA/A2} \)-positive strains from France (SB3310, SB3431, SB3181 and SB3188) had different virulence in mice. Significantly more mice survived infection by SB3180 \( ( \text{p-rmpA} \) and \( \text{p-rmpA2} \)) and SB3188 \( ( \text{p-rmpA} \) only) than by SB3181 \( ( \text{p-rmpA} \) only) \((Kaplan–Meier analysis, SB3310 and SB3181, SB3188 and SB3181, both \( P=0.0082, \log\text{-rank test})\) although they all carried \( \text{p-rmpA} \). These results suggested that carriage of \( \text{rmpA/A2} \) does not completely indicate virulence of these K1 strains from the West.

### DISCUSSION

Invasive PLA with septic complications caused by \( K. \) \( \text{pneumoniae} \) is an emerging infectious disease, first observed in Asia and then found worldwide (see Introduction). K1 PLA strain NTUH-K2044 has not only plasmid-borne \( \text{rmpA} \) and \( \text{rmpA2} \) but also a chromosomal \( \text{rmpA} \). In this study, three \( \text{rmpA/A2} \) genes were distinguished, to our knowledge for the first time, and demonstrated to have different effects on \( \text{cps} \).

The \( \text{p-rmpA} \) gene in NTUH-K2044 enhanced \( \text{cps} \) transcription, CPS biosynthesis and mucoviscosity. A similar function has been observed for \( \text{rmpA} \) on the large plasmid in two K2 strains (Nassif \textit{et al.}, 1989a; Cheng \textit{et al.}, 2010). By contrast, \( \text{p-rmpA2} \) in NTUH-K2044 inhibited \( \text{cps} \), and \( \text{c-rmpA} \) had no obvious effects, indicating that not all \( \text{rmpA/A2} \) genes were upregulators of \( \text{cps} \) expression. Notably, \( \text{p-rmpA} \) did not enhance virulence, as indicated by intraperitoneal and intragastric inoculation as well as in \textit{vivo} competition assays, which can be used to examine more subtle differences. The ability of \( \Delta \text{rmpA} \) to compete in mice was better than that of the wild-type, possibly because less capsule production promotes bacterial colonization or adherence. However, this competitive advantage had no effect on mouse survival. Although CPS in \( \Delta \text{rmpA} \) was reduced, double immunodiffusion analysis and comparison with the \( \Delta \text{magA} \) polysaccharide biosynthesis level suggested that the \( \Delta \text{rmpA} \) mutant still

### Table 2. \textit{rmpA/A2} content and virulence of K1 strains from the West

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>( \text{p-rmpA} )</th>
<th>( \text{p-rmpA2} )</th>
<th>( \text{c-rmpA} )</th>
<th>LD(_{50}) (c.f.u.)*</th>
<th>Surviving mice/total mice+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada PLA</td>
<td>PLA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(&lt;10^2)</td>
<td>ND</td>
</tr>
<tr>
<td>USA PLA</td>
<td>PLA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(&lt;10^2)</td>
<td>ND</td>
</tr>
<tr>
<td>Belgium PLA</td>
<td>PLA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(10^6)</td>
<td>ND</td>
</tr>
<tr>
<td>ATCC 8045</td>
<td>Pneumonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(10^2)</td>
<td>ND</td>
</tr>
<tr>
<td>ATCC 8047</td>
<td>Lung</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(10^6)</td>
<td>ND</td>
</tr>
<tr>
<td>ATCC 35593</td>
<td>Clinical isolate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(10^6)</td>
<td>ND</td>
</tr>
<tr>
<td>SB14</td>
<td>Blood</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>4/4</td>
</tr>
<tr>
<td>SB3309</td>
<td>Horse</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>3/4</td>
</tr>
<tr>
<td>SB3310</td>
<td>Mare, metritis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>3/4</td>
</tr>
<tr>
<td>SB3431</td>
<td>Liver abscess</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
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</tr>
<tr>
<td>SB3181</td>
<td>Sputum</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>SB3183</td>
<td>Blood</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>SB3188</td>
<td>CSF</td>
<td>+</td>
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<td>SB3193</td>
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</table>

*LD\(_{50}\) was determined by intraperitoneal inoculation of four mice for each dose.
†Survival rate was determined by intraperitoneal inoculation of \( 10^5 \) c.f.u. into four mice for each strain, monitored for 30 days.
expresses K1 capsule. The remaining CPS in Δp-rmpA still protected bacteria from killing by serum, and therefore did not obviously reduce virulence in mice. Taking these results together, in NTUH-K2044 the only rmpA/A2 gene to promote CPS was p-rmpA; however, it did not promote virulence. In the K2 strain CG43 both rmpA and rmpA2 (complete form) are activators of CPS biosynthesis and important for virulence in mice (Cheng et al., 2010; Lai et al., 2003). Thus, the effects of rmpA/A2 genes on cps regulation and virulence could be different in different strains. This is worth considering in clinical studies, and more rmpA/A2 genes in clinical strains with different capsular types should be characterized in the future.

Our results showed that the unmarked deletion of c-rmpA, which has DNA sequence similarity to p-rmpA, did not significantly affect CPS production. A transposon-inserted c-rmpA mutant with a reduced hypermucoviscosity phenotype was identified in our previous work (Fang et al., 2004), but the use of in-frame unmarked deletions as in this study should produce more reliable results because insertional mutagenesis runs the risk of introducing polar effects on downstream genes or creating secondary mutations elsewhere in the genome (Link et al., 1997). We found that dysfunction of c-rmpA was not due to the lack of gene expression, and c-rmpA, unlike p-rmpA, was unable to promote CPS. We propose that the sequence differences between the p-rmpA and c-rmpA coding regions account for this difference in activity, possibly involving DNA binding, protein stability, interaction with other proteins, downstream signalling, etc. For example, RmpA of strain CG43 directly interacts with RcsB for cps regulation via the N-terminal region (Cheng et al., 2010). Whether p-RmpA and c-Rmpa could interact with RcsB differently can be further characterized, and the differences between them in sequences of the N-terminal RcsB-interacting region or the C-terminal DNA-binding region will be studied.

Analysis of sequence variability revealed that, in different clinical strains, p-rmpA and c-rmpA exhibited a high degree of homology, but the length of p-rmpA2 was variable. In 33 p-rmpA+ strains, five K1 strains from the West (SB3181, SB3182, SB3188, ATCC 8045 and A5054) showed less homology in p-rmpA DNA sequences, and none of them were terW+/iutA+/silS+, indicating a dissimilar genotype from NTUH-K2044. Analysis of 10 c-rmpA+ strains (all from patients with PLA and from Asia) revealed that nine K1 strains share identical c-rmpA DNA sequences. Why most of the identified c-rmpA+ strains belonged to K1 remains unclear. It could be that these strains belonged to a similar genotype, which needs to be further characterized, for example by RFLP. We also demonstrated that frameshifts resulting in truncation of p-rmpA2, ranging from 99 to 126 amino-acids in length, are common in the clinical strains. The full-length p-RmpA2 (212 amino acids) acts as a cps activator in CG43 (Lai et al., 2003). Even though it was a truncated, the NTUH-K2044 p-RmpA2 (99 amino acids predicted) inhibited cps expression. The final C-terminal 101 residues of CG43 RmpA2 have been shown to be important for DNA binding (Lai et al., 2003). The details of how truncated NTUH-K2044 p-RmpA2, lacking the C-terminal DNA-binding region, inhibits cps expression are still unclear. One speculation is that it might compete at the binding site of other regulators, or the truncated rmpA2 might interfere with expression of other rmpA copies. How other truncated forms of RmpA2 function in clinical isolates also needs further investigation.

The issue of whether rmpA is a virulence determinant for K. pneumoniae liver abscess has been raised by the inconsistency observed between rmpA carriage and virulence in mice (Yeh et al., 2007), although rmpA is significantly associated with K. pneumoniae PLA. Our data provide further insights into the roles of rmpA in the disease. We demonstrated a relationship of rmpA to the PLA-related capsular types and to the large plasmid. As rmpA did not directly contribute to virulence of some strains, association of rmpA with PLA in prevalence studies is likely through its strong correlation with PLA-related capsular types of some virulent strains. Due to this correlation, rmpA can be a marker of Klebsiella PLA, without being directly responsible for virulence. Our study revealed that carriage of rmpA/A2 in K1 strains was different between Asia and the West. All K1 strains from Asia carried at least one rmpA/A2 gene while ~22.7% of K1 strains from the West were negative for rmpA/A2. Animal inoculation showed that carriage of rmpA/A2 did not completely indicate virulence of K1 strains from the West. Although the exact functions of rmpA/A2 in these strains may need further characterization, e.g. by gene deletion and in vivo competition analysis, other virulence genes in the large plasmid probably play roles in PLA pathogenesis. An association of p-rmpA with widely spaced loci terW+/iutA+/silS+ in a large plasmid was revealed. Since 47 of 48 PLA strains harboured p-rmpA (the other PLA strain had only c-rmpA), association of rmpA with PLA could be due to the large plasmid carrying rmpA homologues. Several virulence-associated regions were found to be more prevalent in PLA strains (Chou et al., 2004; Hsieh et al., 2008; Lin et al., 2008; Ma et al., 2005), implying that a specific bacterial genome was important for K. pneumoniae PLA infection. The transmission of an integrative and conjugative element (ICEKp1) among K. pneumoniae strains has been demonstrated, and it might contribute to the transmission of the pathogenicity island and result in K. pneumoniae PLA-associated genomic heterogeneity (Lin et al., 2008). Interestingly, NTUH-K2044 c-rmpA was located in the middle region of ICEKp1, which showed similarity with part (~15 kb) of the large plasmid pLVPK in K. pneumoniae CG43 (Lin et al., 2008). The 284 bp DNA sequences flanking the middle region were also found on the large plasmid. These sequences might be the repeat structures for integration of the middle region from the large plasmid. As rmpA is related to the large plasmid, rmpA could be co-inherited together with the adjacent virulence genes carried by a large plasmid in K. pneumoniae. Virulence genes might be disseminated due
to the mobilization of the large plasmid. We hope that better understanding of the roles of rmpA can help us improve detection and treatment of *K. pneumoniae*-mediated invasive diseases in the future.

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**REFERENCES**


RmpA role in *K. pneumoniae* virulence and capsular types


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