

Regulation of the *Klebsiella pneumoniae* Kpc fimbriae by the site-specific recombinase Kpcl

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In the genome of *Klebsiella pneumoniae* NTUH-K2044, nine fimbrial gene clusters were identified. Besides type 1 and type 3 fimbriae, the others are novel and were named Kpa, Kpb, Kpc, Kpd, Kpe, Kpf and Kpg fimbriae. Prevalence analysis among 105 *K. pneumoniae* clinical isolates revealed that the *kpc* genes were highly associated with the K1 serotype isolates. Induced expression of the recombinant *kpcABCD* genes in *Escherichia coli* resulted in Kpc fimbriation and increased biofilm formation. A putative site-specific recombinase encoding gene *kpcl* and a 302 bp intergenic DNA flanked by 11 bp inverted repeats, namely *kpcS*, were identified in the upstream region of the *kpcABCD* genes. Using LacZ as the reporter, a dramatic difference in promoter activity of *kpcS* in two different orientations was observed and accordingly assigned as ON and OFF phase. *kpcl* expression was found to be able to invert *kpcS* *in trans* from phase ON to OFF and vice versa. Using the two-plasmid system, expression of *kpcA*, encoding the major component of the Kpc fimbriae, could be observed upon the induced expression of *kpcl*. These results indicate that Kpcl is involved in the regulation of Kpc fimbriation in a phase-variable manner.

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INTRODUCTION

Klebsiella pneumoniae is a Gram-negative pathogen which causes suppurative lesions, bacteraemia, and urinary as well as respiratory tract infections mostly in patients with underlying diseases (Podschn & Ullmann, 1998). In Taiwan, *K. pneumoniae* has surpassed *Escherichia coli* as the predominant isolate from patients with hepatic abscesses. Although more than 77 capsular serotypes have been reported for this micro-organism (Podschn & Ullmann, 1998), isolates of the K1 serotype have been shown to be significantly associated with liver abscesses in diabetic patients (Fung *et al.*, 2002; Lee *et al.*, 2005). Reports of *Klebsiella* liver abscess (KLA) in Western countries have also been accumulating in recent years (Lederman & Crum, 2005). Besides the K1 serotype, other serotypes (mainly K2) and other virulence factors, including the mucoid factor RmpA and the siderophore aerobactin, have been reported to be important determinants of KLA (Yu *et al.*, 2008). However, the correlation

between KLA and the K1 serotype remains to be investigated.

Successful establishment of infection by bacterial pathogens requires adhesion to host cells, colonization of tissues, and in certain cases cellular invasion, followed by intracellular multiplication, dissemination to other tissues, or persistence (Pizarro-Cerda & Cossart, 2006). Fimbriae, also called pili, are hair-like appendages that extend out of the bacterial cell surface as an adherence factor. Generally, the fimbrial rod is composed of repeating subunits of pilin, the major component, and of adhesin, the minor subunit, located at the tip to mediate its specific binding activity. There are at least four mechanisms that account for the assembly of fimbriae: the chaperone–usher-dependent pathway, the general secretion pathway, the extracellular nucleation-precipitation pathway, and the alternate chaperone pathway (Soto & Hultgren, 1999).

Several different types of fimbriae have been identified in *K. pneumoniae*. Type 1 fimbriae, found in virtually all members of the family *Enterobacteriaceae* (Klemm & Schembri, 2000), are well known for their ability to bind to mannose-containing trisaccharides of host glycoproteins. Expression of type 1 fimbriae has been closely associated

Abbreviation: KLA, *Klebsiella* liver abscess.

A supplementary table and five supplementary figures are available with the online version of this paper.

with urinary tract infections (Kil *et al.*, 1997; Struve *et al.*, 2008). Type 3 fimbriae, encoded by the *mrk* gene cluster, are produced by the majority of *K. pneumoniae* strains (Allen *et al.*, 1991) and are involved in biofilm-associated infections (Struve *et al.*, 2009). The afimbrial adhesin CF29K, KPF-28 fimbriae, and a capsule-like extracellular afimbrial adhesin have also been reported for some *K. pneumoniae* strains (Di Martino *et al.*, 1995; Favre-Bonte *et al.*, 1995).

Many bacteria differentially express multiple types of fimbriae in order to adhere to different receptors during infection (Humphries *et al.*, 2001; van der Velden *et al.*, 1998; Weening *et al.*, 2005). In *Salmonella enterica* serovar Typhi (*S. Typhi*) CT18, 12 fimbrial gene clusters of the chaperone-usher family have been found. This repertoire of fimbrial sequences has been found to be unique to the serotype Typhi, suggesting a role in the determination of specific host adaptation (Townsend *et al.*, 2001). Several of the fimbrial gene clusters identified in the genome of enterohaemorrhagic *E. coli* have been shown to be more prevalent in the O157 serotype (Low *et al.*, 2006).

In order to investigate whether fimbrial type is associated with KLA pathogenesis, we initiated the study using bioinformatic tools to identify the fimbrial genes in *K. pneumoniae* NTUH-K2044, a liver abscess isolate of serotype K1 (Wu *et al.*, 2009). The prevalence of the fimbrial genes was then employed in the analysis of 105 *K. pneumoniae* clinical isolates from different infection sites. Subsequently, the genes encoding Kpc fimbriae which appeared to be most prevalent in the K1 serotype isolates were selected for further study.

METHODS

Bacterial strains, growth conditions and serotyping. *K. pneumoniae* NTUH-K2044, a highly invasive and hypermucous strain of K1 serotype (Fang *et al.*, 2004), was provided by Dr Jin-Town Wang, National Taiwan University Hospital. The 105 *K. pneumoniae* clinical isolates were recovered from different tissue specimens of patients with a variety of infections at the Veterans General Hospital, Taipei, Taiwan, from 1991 to 1998. These strains have been identified and their serotypes determined, as previously described (Fung *et al.*, 2002). Fourteen of the 18 isolates from liver abscess patients were capsular serotype K1. Bacteria were generally propagated overnight in Luria-Bertani (LB) broth with agitation at 37 °C. M9 minimal medium and tryptic soy broth (TSB) were also used. The antibiotics used included ampicillin (100 µg ml⁻¹), chloramphenicol (35 µg ml⁻¹) and kanamycin (25 µg ml⁻¹). All the tissue culture media used were purchased from Gibco.

Bioinformatics. The 5.5 Mb *K. pneumoniae* NTUH-K2044 genome sequence (GenBank accession no. AP006725.1) has been determined and annotated (Wu *et al.*, 2009). The fimbriae proteins were identified using the Pfam database (accession no. PF00419) and HMMER on the basis of a hidden Markov model (Bateman *et al.*, 2004; Eddy, 1998). The fimbrial gene clusters and the neighbouring genes were analysed by homology search using the BLAST program provided online by the National Center for Biotechnology Information.

PCR detection of fimbrial genes. All the primer pairs used in this study are listed in Supplementary Table S1. The PCR mixture

contained 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP) and 1 U recombinant *Taq* DNA polymerase (Violet Bioscience Inc.), along with *K. pneumoniae* genomic DNA and specific primers. The amplification cycle consisted of an initial 5 min hold at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min, and finally an elongation step for 10 min at 72 °C. The amplified PCR product was then analysed by electrophoresis on a 1% agarose gel.

Construction of the expression plasmid pETQ. The DNA fragment containing the T5lac promoter, multiple cloning sites and the *rrnB* T1 transcription terminator from pQE30 (Qiagen) was inserted into pET30a (Novagen) using restriction enzymes *Xba*I and *Xho*I. The 6His-tag-coding sequence was subsequently removed by inverse PCR to yield plasmid pETQ.

Construction of fimbriae expression plasmids. The *kpcABCD* genes were PCR-amplified using primers pcc053 and pcc056, and the PCR product was cloned into the expression plasmids pET30a and pETQ to yield pKPC-7 and pKPC-36, respectively. The *kpcABC* genes were PCR-amplified using primers pcc053 and pcc203 and cloned into pETQ to yield pAW67. Genes encoding type 1 fimbriae (*fimAICDEFGH*) were PCR-amplified using primers pcc167 and pcc169 and cloned into pETQ to yield pAW69. The DNA containing *kpcS_{OFF}-kpcABCD* was PCR-amplified by primers pcc081 and pcc056 and then cloned into γ T&A (Yeastern Biotech Co.) to yield pAW73.

Transmission electron microscopy (TEM). After IPTG (0.05 mM) induction of exponential-phase *E. coli* Novablue (DE3) harbouring the expression plasmids pKPC-7 or pET30a for 3 h, the bacteria were collected by centrifugation, washed once with PBS, and subjected to TEM observation performed as described by Huang *et al.* (2006).

Kpca antiserum preparation. The coding region of *kpcA* was PCR-amplified using primers pcc023 and pcc024 and cloned into pET30 to yield pKPCA. Plasmid pKPCA was then introduced into *E. coli* Novablue (DE3) for overexpression of the His₆::Kpca recombinant protein. The recombinant protein was expressed and purified according to the protocol in the pET manual (Novagen). Kpca antiserum was prepared by immunizing a New Zealand white rabbit with 0.5 mg of the purified His₆::Kpca recombinant protein and the immunized rabbit was exsanguinated on day 45. The specificity of the obtained antibody was confirmed using Western blotting (Supplementary Fig. S1).

Anti-Kpca Western blot hybridization and immunofluorescence microscopy. Induction of the *kpc* genes was carried out by adding 0.5 mM IPTG to an exponential-phase *E. coli* HB101 [pKPC-36] culture and incubating for an additional 3 h. Bacterial lysates were resolved by SDS-PAGE; the Western blot analysis and immunofluorescence microscopy using anti-Kpca antiserum were performed as previously described (Huang *et al.*, 2009).

Biofilm formation assay. Bacteria diluted 1:100 in LB broth supplemented with 25 µg kanamycin ml⁻¹ and 0.5 mM IPTG were inoculated into each well of a 96-well microtitre dish (Orange Scientific) and statically incubated at 37 °C for 48 h. The biofilm-forming ability of bacteria was analysed as described by Huang *et al.* (2009).

Construction of the Kpcl expression plasmid. The DNA fragment encoding Kpcl₁₉₆ was PCR-amplified from *K. pneumoniae* NTUH-K2044 by primers pcc183 and pcc150, and cloned into pBAD33 (Guzman *et al.*, 1995) to generate pKPCl₁₉₆. pKPCl₁₉₆ was transformed

into *K. pneumoniae* NTUH-K2044, and 0.4% L-arabinose was added to the culture of the transformant for the induction of KpcI₉₆ expression.

Switch orientation assay. The *kpcS* (switch region) was PCR-amplified from the tested strain using the primers pcc081 and pcc082. The amplified product was then purified and digested with *Afl*III. The restricted fragments were separated on 2% agarose gels and the pattern was visualized for the determination of orientation by staining with ethidium bromide. The DNA fragments containing *kpcS*_{ON} and *kpcS*_{OFF} were PCR-amplified from L-arabinose-induced *K. pneumoniae* NTUH-K2044 [pKPC_{I96}] using primers pcc081 and pcc082, and subsequently cloned into γ T&A to yield the pKPC-ON and pKPC-OFF plasmids, respectively.

β -Galactosidase assay. *kpcS* DNA was PCR-amplified from *K. pneumoniae* NTUH-K2044 by primers YCY001 and YCY002, and cloned into the promoter reporter plasmid placZ15 (Lin *et al.*, 2006) to yield plasmids pSY003 and pSY004, containing *kpcS* without the two inverted repeats in opposite orientations. pSY003 and pSY004 were introduced into the *K. pneumoniae* NTUH-K2044 Δ lacZ strain CCW01, which was constructed by using the gene replacement vector pKOV (Link *et al.*, 1997). One-tenth of an overnight culture of the bacteria carrying either plasmid was subcultured in LB broth to OD₆₀₀ ~0.6–0.7. The β -galactosidase activity assay was carried out as described by Lin *et al.* (2006).

Statistical methods. An unpaired *t* test was used to determine statistical significance, and values of $P < 0.001$ were considered significant. The results of the biofilm-forming activity and β -galactosidase activity assays were derived from a single experiment that was representative of three independent experiments. Each sample was assayed in triplicate and the mean activity and standard deviation are presented.

RESULTS

Identification of the fimbrial gene clusters in *K. pneumoniae* NTUH-K2044

Nine fimbrial gene clusters were identified using the HMMER search of the genome of *K. pneumoniae* NTUH-K2044. Each contained at least four genes, encoding a putative major pilin, a chaperone, an usher and an adhesin for the biosynthesis of fimbriae belonging to the chaperone–usher-dependent assembly. As shown in Fig. 1, these included the type 1 and type 3 fimbrial gene clusters *fim* and *mrk*, and seven novel ones, namely *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf* and *kpg*. Multiple sequence alignment by CLUSTAL W showed that the amino acid sequences of these pilins and adhesins shared 26.5–36.4% similarity; chaperones and ushers shared a higher similarity ranging from 49.3 to 55.4%. The *mrk*–*fim* fimbrial genes are clustered and transcribed divergently (Fig. 1). This gene organization including the *pecM*, *pecS* and *nicO* homologues has been found to be conserved in the genomes of *K. pneumoniae* C3091, MGH78578 and 342 (Fouts *et al.*, 2008; Ogawa *et al.*, 2005; Struve *et al.*, 2009). Interestingly, the gene clusters *kpf* and *kpg* are also linked physically but transcribed convergently.

BLAST analysis, using the sequences of the nine fimbrial gene clusters identified in *K. pneumoniae* NTUH-K2044 as templates, of the genome of *K. pneumoniae* strains

MGH78578 and 342 (Fouts *et al.*, 2008; Ogawa *et al.*, 2005) showed that, except for *kpc* and *kpf*, the genes were conserved in the three genomes. No homologue of the *kpc* genes was found in strains MGH78578 and 342, while homologues of the *kpf* genes were found in the genome of MGH78578 but not that of 342.

PCR screening for the presence of the fimbrial genes

To investigate the prevalence of the nine fimbrial gene clusters among *K. pneumoniae* strains, a total of 105 *K. pneumoniae* clinical isolates from different infection sites were collected. Two specific primer pairs corresponding to the pilin- and adhesin-encoding genes were designed for PCR detection. Prevalence was determined on the basis of the presence of the PCR amplicons (Supplementary Fig. S2). The analysis revealed the presence of the *kpa*, *kpd*, *kpe*, *kpg*, *fim* and *mrk* genes in most of the isolates, and the prevalence percentages were 99, 82, 93, 97, 84 and 100%, respectively. The prevalence percentages for the *kpb*, *kpc* and *kpf* genes were lower at 52, 33 and 70% of the isolates, respectively. No obvious correlation between fimbrial type and disease could be identified. However, the *kpb* and *kpc* genes were shown to be more prevalent in K1 isolates ($P < 0.0001$). As shown in Table 1, most of the clinical isolates of the K1 serotype harboured *kpb* and *kpc* genes, while non-K1 isolates carrying the *kpb* and *kpc* genes were much less frequent (32 and 1%, respectively). The close association of *kpc* fimbriae with the K1 serotype prompted the selection of the *kpc* fimbriae for further study of the KLA pathogenic mechanism.

Display of the Kpc fimbriae on the *E. coli* surface

In order to determine whether the *kpcABCD* genes encode a fimbrial apparatus, an *E. coli* fimbriae display system was used. The *kpcABCD* genes were PCR-amplified and cloned into pET30a, designated pKPC-7, to allow controlled expression by IPTG induction. Numerous thin and rigid fimbriae on the surface of *E. coli* Novablue (DE3) harbouring pKPC-7 could be observed, while *E. coli* harbouring pET30a was afimbriate (Fig. 2). Expression of KpcA, the putative major pilin, could also be detected by Western blot analysis (Supplementary Fig. S1). These results indicated that the *kpcABCD* genes are sufficient to produce a fimbrial apparatus. However, the growth rate of *E. coli* harbouring pKPC-7, in comparison with that of *E. coli* Novablue (DE3) [pET30a], was obviously decreased even without IPTG induction. This could be a result of the overexpression of *kpc* genes, especially *kpcA* (Supplementary Fig. S1, lane 3). In case the biased growth rate impeded the characterization of the Kpc fimbriae, another expression plasmid, pETQ, which can be used for protein expression under T5lac promoter control in *E. coli* and *K. pneumoniae*, was used. The pETQ plasmid carrying the *kpcABCD* genes, pKPC-36, was then transformed into *E. coli* HB101, which is an afimbriate bacterium. Expression of the *kpcABCD* genes

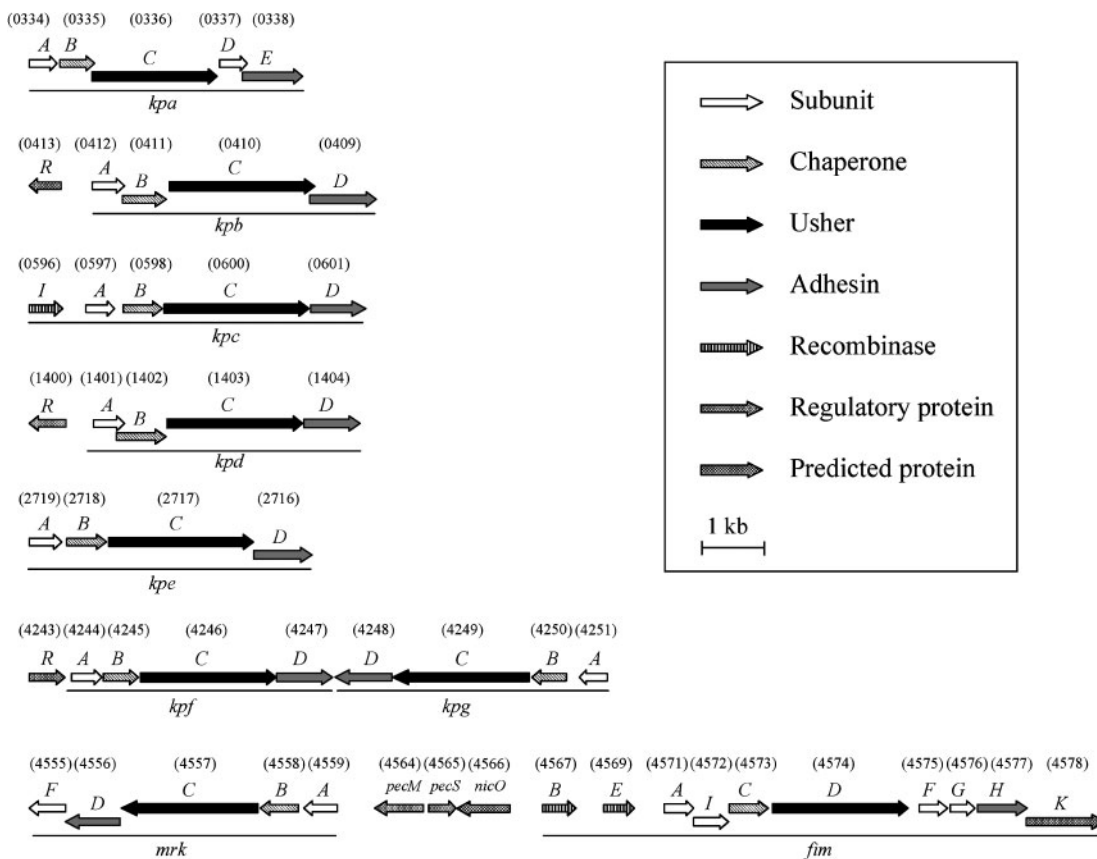


Fig. 1. Fimbrial gene clusters of the chaperone–usher-dependent assembly class in *K. pneumoniae* NTUH-K2044. The designation of putative fimbrial genes and the locus tag (KP1_number) of ORFs annotated in the *K. pneumoniae* NTUH-K2044 genome are indicated. A total of nine fimbrial gene clusters and genes encoding putative regulators are shown. Each of the putative fimbrial operons is underlined. The putative functions of the ORFs are also shown.

was tightly controlled under the T5*lac* promoter, and no obvious difference in growth rate between *E. coli* HB101 harbouring pKPC-36 and *E. coli* HB101 harbouring pETQ

was noted. Using polyclonal anti-KpcA antibodies, Western blot analysis revealed that KpcA was expressed (Fig. 2b), and fimbriation on the surface of the recombinant *E. coli* HB101

Table 1. Prevalence of *kpb* and *kpc* genes in *K. pneumoniae* isolates

<i>K. pneumoniae</i> isolate	No. of strains containing <i>kpb</i> /total no. of strains isolated (%)		No. of strains containing <i>kpc</i> /total no. of strains isolated (%)	
	K1 serotype	Other serotype	K1 serotype	Other serotype
Liver abscess	14/14	3/4	14/14	1/4
Bile		2/7		1/7
Urine	1/1	3/13	1/1	0/13
Sputum	3/3	3/11	2/3	1/11
Wound	6/6	5/15	6/6	1/15
Blood	5/5	4/17	5/5	2/17
Ascites		4/9		1/9
Total	29/29 (100)*	24/76 (32)*	28/29 (97)*	7/76 (1)*

**P*<0.0001.

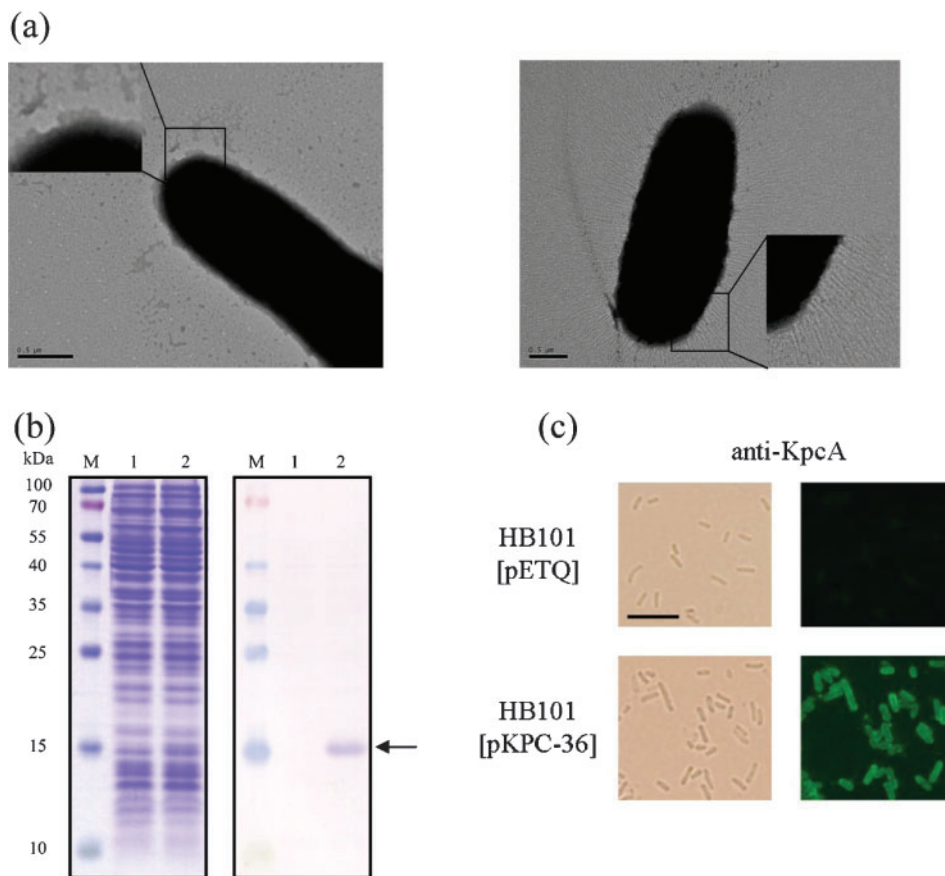


Fig. 2. Expression of Kpc fimbriae on recombinant *E. coli*. (a) Transmission electron micrographs of recombinant Kpc fimbriae. Left panel, *E. coli* Novablue (DE3) [pET30a]; right panel, *E. coli* Novablue (DE3) [pKPC-7]. Bars, 0.5 μm. (b) Anti-KpcA Western blot analysis of *E. coli* HB101 harbouring pETQ (lane 1) or pKPC-36 (lane 2). The expressed KpcA is indicated by an arrow. (c) Bright-field (left panel) and anti-KpcA immunofluorescence (right panel) microscopic analysis of *E. coli* HB101 harbouring pETQ or pKPC-36 (magnification $\times 630$). Bar, 10 μm.

was also observed by fluorescence microscopy (Fig. 2c). *E. coli* HB101 [pKPC-36] was thus used for the characterization of the recombinant Kpc fimbriae.

Expression of Kpc fimbriae increased biofilm-forming activity

Activity assessment of the recombinant Kpc fimbriae, including haemagglutination, cell adherence and biofilm formation, was subsequently carried out. However, no haemagglutination activity against red blood cells from guinea pig, rabbit or human (type A and type B) could be observed for the recombinant bacteria. Besides human epithelial cell lines Int407 (intestine), HCT-8 (intestine), Hep-2 (larynx) and T24 (bladder), two human hepatocellular liver carcinoma cell lines, HepG2 and SK-HEP-1, were used to assess the cell adherence activity of the Kpc fimbriae. The cell adherence assay was performed as described by Huang *et al.* (2006). Although the recombinant *E. coli* HB101 exhibited differential adherence to these cell lines, Kpc fimbriation did not increase the adherence

activity of the bacteria to any of the cells. On the other hand, as shown in Fig. 3, *E. coli* HB101 harbouring pKPC-36 exhibited a higher level of biofilm-forming activity on the abiotic surface than that observed for *E. coli* HB101 [pETQ], as assessed by direct observation after crystal violet staining or by quantitative measurement ($P < 0.001$). However, *E. coli* HB101 harbouring the type 1 fimbriae expression plasmids pAW69 or pAW67 (pETQ carrying the *kpcABC* genes) exhibited levels of biofilm-forming activity similar to those of the bacteria carrying pETQ. This suggested a specific binding of the Kpc fimbriae to polystyrene wells and a positive role of Kpc fimbriae in biofilm formation. The expression of type 1 fimbriae encoded on pAW69 was also confirmed by yeast agglutination analysis (Supplementary Fig. S3b).

The KpcI recombinase is probably the regulator for the Kpc fimbriae

A gene encoding a putative DNA recombinase of the lambda integrase family (Esposito & Scocca, 1997; Nunes-

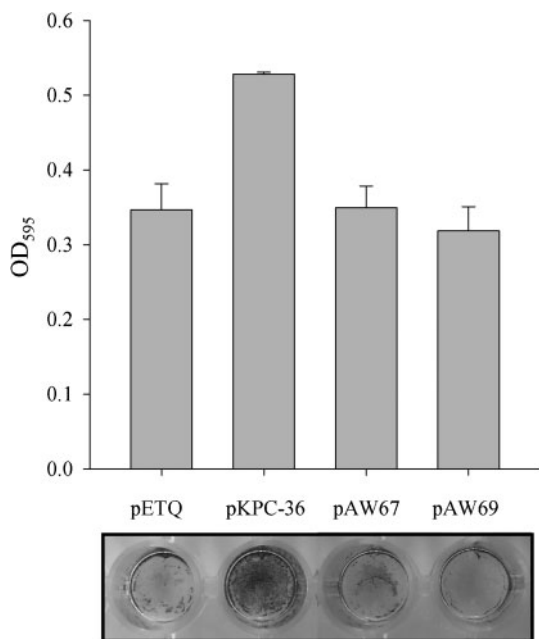


Fig. 3. Biofilm-forming ability of *E. coli* expressing the Kpc fimbriae. The development of biofilms of *E. coli* HB101 harbouring pETQ, pKPC-36, pAW67 and pAW69 was observed (lower panel) and quantified (upper panel) as described in Methods. A higher biofilm-forming activity could be observed for *E. coli* HB101 [pKPC-36].

Duby *et al.*, 1998) was identified in the upstream region of the *kpcABCD* genes (Fig. 1) and was named *kpcI*. Multiple sequence alignment of the family of site-specific recombinases, including FimB/E of the type 1 fimbriae, MrpI of the MR/P fimbriae, FotS/T of the CS18 fimbriae and KpcI, revealed four conserved residues, R42, H136, R139 and Y171, of the tyrosine recombinase family (Supplementary Fig. S4) (Abremski & Hoess, 1992; Han *et al.*, 1994). Between *kpcI* and *kpcA*, a 302 bp region flanked by a pair of 11 bp inverted repeats (IRs) was identified (Fig. 4). These findings suggested a recombinase-mediated phase variation

control of Kpc fimbriae, as reported for type 1 fimbriae, MR/P fimbriae and CS18 fimbriae (Honarvar *et al.*, 2003; Klemm, 1986; Li *et al.*, 2002). KpcI is thus predicted to be able to invert the DNA segment between the two inverted repeats to regulate the expression of Kpc fimbriae. The putative invertible DNA segment was designated *kpcS* (switch region).

Expression of *kpcI* leads to inversion of *kpcS*

A PCR-based switch orientation assay (Fig. 5a) was subsequently employed to detect the *kpcS* inversions in *K. pneumoniae* NTUH-K2044 grown in various growth conditions, including LB broth, TSB and M9 minimal medium with or without agitation at 25, 30 and 37 °C. Since enteropathogenic *E. coli* bundle-forming pili are expressed when the bacteria are grown in Dulbecco's Modified Eagle's Medium (DMEM) (Vuopio-Varkila & Schoolnik, 1991), several tissue-culture media, including DMEM, Roswell Park Memorial Institute (RPMI) medium, minimum essential medium (MEM), Basal Medium Eagle (BME) and McCoy's 5a medium, were also used to grow *K. pneumoniae* NTUH-K2044 (statically incubated at 37 °C in 5% CO₂). However, the *kpcS* inversion was not observed under all the above growth conditions. The expression of KpcA was also not detected by Western blot analysis, suggesting an 'OFF' phase of *kpcS* (*kpcS*_{OFF}) in *K. pneumoniae* NTUH-K2044. In the *kpcS*_{OFF} phase, a polypeptide of 196 amino acid residues, KpcI₁₉₆, could be deduced (Supplementary Fig. S4). To determine whether expression of the KpcI recombinase could invert the DNA, pKPCI₁₉₆, an expression plasmid encoding KpcI₁₉₆ driven by an *araBAD* promoter, was transformed into *K. pneumoniae* NTUH-K2044. As shown in Fig. 5(b), the addition of L-arabinose to induce expression of KpcI₁₉₆ was able to invert the DNA. After the inversion, the DNA was isolated, sequenced and named *kpcS*_{ON} (Fig. 4). To assess the promoter activity of *kpcS*_{ON} or *kpcS*_{OFF}, each DNA was cloned in front of the promoterless *lacZ* gene in pLacZ15 by transcriptional fusion, and the resulting plasmids were named pSY003 and pSY004, respectively. After the transformation of pSY003 or pSY004 into *K. pneumoniae*

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1          50
CGTCTGATACAGGATTTATCTGGGGCACCGGAACATCCGGCATAAGGTTATACGGGACAAATACGCAGAGGTTTATGAATGTCTGGGGGAACATGA
101        150
GAGAAAAACACACAAATTAGTGCCAGATGTGGTGTGAGTTCAGGAGAAAAATTTGGTGGAAAAAGAAAGTCCAGAAATAAAGCGTTGGTGAAGCGGAATACCGCT
201        250
TAAGGAACCGGCATAAAAACCATAGAAGGTGAGCAAAATGAAAACTGGCAACGTGAACAACCTCAGCACGTTGCCAGCAGGAGCCAGAGGTTGTGTAGACAA
301        350        -35        -10
TATTTCGCGCAGATAAAATTTAAAAACAGCGAGATAATCCGTAACCTAAAGGGGTCTCAGAAAAATTTTGACACTTTGCTAAATTTTCTGTGATACTACT
401        450
TTACAACTCTGGCACTAAACGGGAAAGTCTAATGCGGATTTTTTTCACGCGCGAGTGAAGCAGGGCAGACCCTGGAGTGATTTTTTCATCAAGGAGAAAGGT
501        550
ATGAAAAAAACGATAAACAATCGTGTGTTTTATGCTGGCGCGCGCAGTGC

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Fig. 4. Sequence analysis of the putative promoter region of the *kpc* gene cluster. The 500 bp upstream and 50 bp downstream regions of the *kpcA* translation start codon ATG (underlined) in the *kpcS*_{ON} phase are shown. The 11 bp inverted repeats are outlined by square boxes. The predicted -10 and -35 promoter regions are shaded. The *kpcI*₂₁₀ translation stop codon TAA is also underlined.

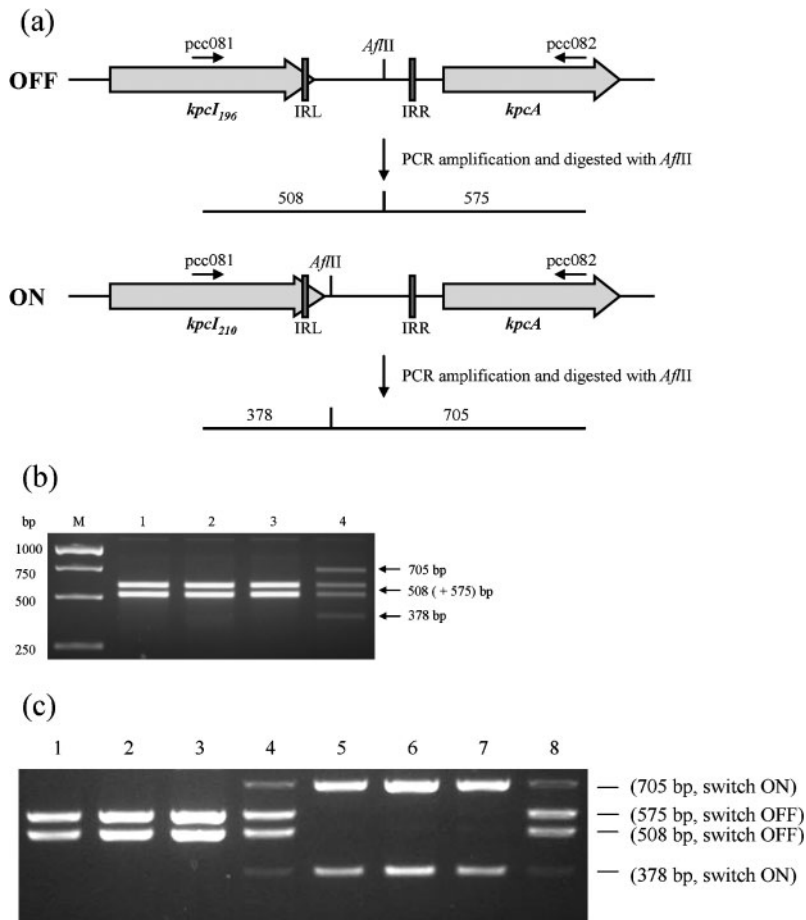


Fig. 5. KpcI₁₉₆-mediated inversion of *kpcS*. (a) Map of the invertible region, *kpcS*, in both OFF and ON orientations. The positions of the primers used in the PCR amplification, pcc081 and pcc082, and the sizes of the DNA fragments resulting from *Afl*III digestion are as indicated. IRL, inverted repeat left; IRR, inverted repeat right. (b) Expression of recombinant KpcI₁₉₆ resulted in a switch from the OFF to the ON phase. *K. pneumoniae* NTUH-K2044 transformed with pBAD33 or pKPC₁₉₆ was grown in M9 broth supplemented with 0.4% glucose (lanes 1 and 3) or L-arabinose (lanes 2 and 4) for 16 h with agitation at 37 °C. Lanes: M, DNA molecular size markers; 1 and 2, pBAD33 vector as a control; 3 and 4, pKPC₁₉₆. (c) *E. coli* JM109 transformed with two plasmids was grown in LB broth, supplemented with 0.4% glucose (lanes 1, 3, 5 and 7) or 0.4% L-arabinose (lanes 2, 4, 6 and 8), for 16 h with agitation at 37 °C. Lanes: 1 and 2, pBAD33 + pKPC-OFF; 3 and 4, pKPC₁₉₆ + pKPC-OFF; 5 and 6, pBAD33 + pKPC-ON; 7 and 8, pKPC₁₉₆ + pKPC-ON. The fragment sizes corresponding to the position of the switches are shown to the right of the panel.

NTUH-K2044 Δ *lacZ* strain CCW01, the LacZ activity of the transformants was measured. The *kpcS*_{ON} phase activity ($21\,359.5 \pm 233.4$ Miller units) was much higher than that of *kpcS*_{OFF} (109.2 ± 3.2 Miller units) and pLacZ15 (111.6 ± 1 Miller units), implying that the orientation of *kpcS* determines the transcription efficiency of the *kpc* genes.

Effect of the recombinant KpcI₁₉₆ on the switching of *kpcS*

The fimbrial recombinases have been shown to have different activities towards the orientation control of the invertible element. The FimB recombinase inverts the *fim* switch in the ON-to-OFF and OFF-to-ON directions, whereas FimE inverts it predominantly in the ON-to-OFF direction (Blomfield *et al.*, 1991; Gally *et al.*, 1993; McClain *et al.*, 1991); MrpI is able to invert the *mrp* switch in both directions (Zhao *et al.*, 1997). As shown in Fig. 5(b), the induced expression of KpcI₁₉₆ inverted *kpcS* in the OFF-to-ON direction. Since the growth condition for *K. pneumoniae* to switch ON *kpcS* is yet to be identified, a two-plasmid system in *E. coli* was used to analyse the activity of KpcI₁₉₆ towards *kpcS* inversion in both directions. pKpcI₁₉₆ was introduced into *E. coli* JM109 harbouring either pKPC-ON or pKPC-OFF. As shown in Fig. 5(c), the induced

expression of KpcI₁₉₆ in the recombinant *E. coli* was able to invert *kpcS* in both the ON-to-OFF and OFF-to-ON directions.

KpcI-mediated expression of KpcA

Although the recombinant KpcI₁₉₆ was able to switch ON *kpcS*, the induced expression of KpcI₁₉₆ in *K. pneumoniae* did not lead to the expression of KpcA (data not shown). This result implies the involvement of further regulators in regulation of the expression of Kpc fimbriae. To avoid possible factors in *K. pneumoniae* that impede the expression of KpcA, another two-plasmid system was constructed to analyse the KpcI-mediated phase variation in *E. coli*. The DNA fragment containing the *kpcS*_{OFF}-*kpcABCD* region was PCR-amplified and cloned into plasmid γ T&A to yield pAW73. Subsequently, pKPC₁₉₆ was introduced into *E. coli* harbouring pAW73. Upon induction with L-arabinose, the induced expression of KpcI₁₉₆ was able to switch ON the expression of KpcA as detected by anti-KpcA antiserum (Fig. 6). This indicated that the KpcI₁₉₆-mediated phase variation was able to control the expression of KpcA.

To investigate whether the production of KpcA could only be detected in the *E. coli* system (Fig. 6) but not in the

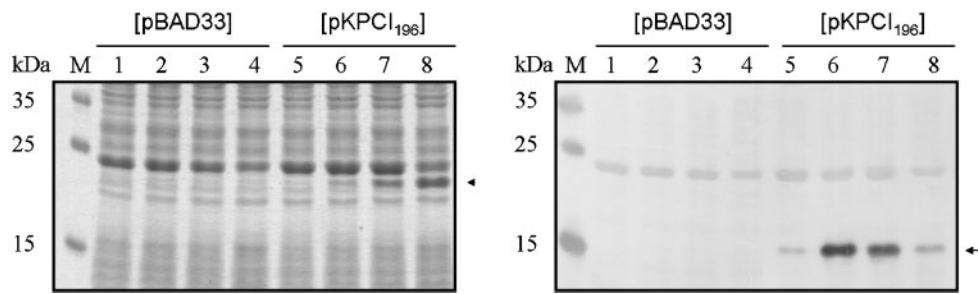


Fig. 6. KpcI₁₉₆-mediated expression of KpcA in *E. coli*. Plasmid pBAD33 or pKPC₁₉₆, as marked above the panels, was introduced into *E. coli* JM109 [pAW73]. *E. coli* carrying the two plasmids was grown in LB broth, and when growth reached mid-exponential phase, the expression of KpcI₁₉₆ was induced by varying concentrations of L-arabinose. Lanes: 1 and 5, no induction; 2 and 6, 0.002%; 3 and 7, 0.02%; 4 and 8, 0.2% L-arabinose induction. After 3 h induction, the bacteria were analysed by SDS-PAGE (left panel) and anti-KpcA Western blot hybridization (right panel). The expression of KpcI₁₉₆ (arrowhead) and KpcA (arrow) is marked.

phase ON *K. pneumoniae* cells, which may be due to no transcription of mRNA, or the instability of *kpcA* mRNA or the KpcA protein, plasmid pKPC-36 was introduced into *K. pneumoniae* NTUH-K2044. KpcA production was readily observed upon IPTG induction (Supplementary Fig. S5), which implied that transcription of *kpcA* was low in the ON phase of *K. pneumoniae* cells. Further studies will be needed to identify regulators of Kpc fimbriae expression.

DISCUSSION

A large number of fimbrial gene clusters are known to be present in a bacterial genome (Low *et al.*, 2006; Townsend *et al.*, 2001; Vallet *et al.*, 2001). It has been demonstrated that multiple types of fimbriae are required for the full virulence and long-term persistence of *Salmonella enterica* serovar Typhimurium in mice (Humphries *et al.*, 2005; van der Velden *et al.*, 1998; Weening *et al.*, 2005). Although these fimbrial gene clusters identified using bioinformatic tools should be considered as putative ones, we speculate that, in addition to the type 1 and type 3 fimbriae, the others may also be required for *K. pneumoniae* infection in different environments.

A prevalence study has indicated that *S. Typhi* possesses a unique repertoire of fimbrial gene sequences (Townsend *et al.*, 2001). A specific repertoire of fimbrial operons was therefore proposed as a complex virulence factor involved in infections (Humphries *et al.*, 2001). Although no obvious correlation between disease types and fimbrial types was noted in the *K. pneumoniae* clinical isolates, we found that out of 29 *K. pneumoniae* K1 isolates, 28 possessed an identical repertoire of fimbrial gene clusters. Since a correlation of the K1 serotype with KLA has been reported, the conserved fimbrial repertoire possessed by the *K. pneumoniae* strains of serotype K1 may also play a role in pathogenesis.

Like many of the fimbrial operons identified that are not expressed *in vitro* (Nuccio *et al.*, 2007), the expression of Kpc fimbriae also could not be observed with various environmental stimuli, including temperature, starvation and aeration. Western blot analysis using the anti-KpcA antiserum was employed to investigate whether the 35 *K. pneumoniae* clinical isolates which possessed *kpc* genes (Table 1) expressed Kpc fimbriae. However, no expression of Kpc fimbriae could be identified for the bacteria grown statically overnight in LB broth or M9 medium at 25 or 37 °C (data not shown). To further characterize the Kpc fimbriae, the *kpcABCD* genes were heterologously expressed in the afimbriate *E. coli* HB101. The displayed Kpc fimbriae were shown to confer upon the recombinant *E. coli* a higher biofilm-forming activity. Bacterial biofilm formation on indwelling devices, such as catheters and endotracheal tubes, is a significant medical problem. The Kpc fimbriae may play a role in the development of infections in catheterized patients. However, this possibility awaits further investigation.

As shown in Fig. 5(c), the recombinant KpcI₁₉₆ possessed activity to switch *kpcS* in both directions; therefore, the induced expression of KpcI₁₉₆ in *K. pneumoniae* may lead to both ON-to-OFF and OFF-to-ON inversions that occur simultaneously in different cells in the bacterial population (Fig. 5b, lane 4). However, whether KpcI₁₉₆ could invert *kpcS* in the ON-to-OFF direction in *K. pneumoniae* remains to be investigated. Unlike all the characterized 'fimbrial recombinases', in which the inverted repeat left (IRL) is located in the non-coding region between the recombinase-encoding gene and the fimbrial operon, the IRL of *kpcS* is located in the coding region of *kpcI* (Fig. 4). The inversion of *kpcS*_{OFF} could result in a polypeptide of 210 residues, KpcI₂₁₀ (Supplementary Fig. S4). The DNA fragment containing *kpcI*₂₁₀ could be amplified by PCR from the genomic DNA of the L-arabinose-induced *K. pneumoniae* NTUH-K2044 [pKPC₁₉₆]. However, when the plasmid containing the PCR amplicon of *kpcI*₂₁₀ was

introduced into *E. coli* JM109, unexpected point mutations and recombination adjacent to the IRL were found in the *kpcI*₂₁₀-coding region. In contrast, we found no evidence of mutations or recombination in pKPCI₁₉₆. *recA*-independent recombination has also been described in the study of FimE recombinase (McClain *et al.*, 1991). Since the IRL of *kpcS* is located in the coding region of *kpcI*₂₁₀, and the expression of *kpcI*₂₁₀ changes its own coding sequence, the activity of Kpcl₂₁₀ is difficult to assess and remains to be studied.

In this study, we report the identification of nine fimbrial gene clusters in the genome of *K. pneumoniae* NTUH-K2044. The Kpc fimbria was found to be most prevalent in the clinical isolates of serotype K1. The heterologous expression of the *kpcABCD* genes in *E. coli* resulted in Kpc fimbriation and an increased biofilm-forming activity. Moreover, the involvement of Kpcl in the regulation of Kpc fimbriation in a phase-variable manner was elucidated.

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