INTRODUCTION

*Klebsiella pneumoniae* is an important nosocomial pathogen that causes suppurative lesions, sepsis, and urinary and respiratory tract infections in immunocompromised patients (Han, 1995; Schelenz et al., 2007). A steady increase in the incidence of *Klebsiella* liver abscesses (KLAs) in patients with diabetes, malignancy, renal disease or pneumonia has been observed in Taiwan (Fung et al., 2002). Reports of KLAs have also increased in Western and other Asian countries (Pope et al., 2011). Although KLA pathogenic mechanisms remain obscure, several virulence traits, including the vast amount of K1 capsular polysaccharide surrounding the bacteria surface (Fung et al., 2002), *magA* (Chuang et al., 2006), iron acquisition loci on pLVPK (Tang et al., 2010) and type 1 fimbriae (Struve et al., 2008, 2009; Stahlhut et al., 2012), have been reported. Fimbriae (also called pili) allow bacteria to attach to host cells to establish infection. Type 1 fimbriae expressed by most members of the family *Enterobacteriaceae* are commonly associated with urinary tract infections and bind to the mannose-containing structure present on host cells or in the extracellular matrix (Ishikawa, 1991; Jones et al., 1995). The expression of type 1 fimbriae is phase-variable and is mediated by an invertible 314 bp *cis* element, *fimS*, located upstream of the type 1 fimbrial major subunit gene, *fimA*. The *fimS* switching, which alternates *Escherichia coli* between type 1 fimbriated and non-fimbriated states, is controlled by site-specific recombinases FimB and FimE. In addition, DNA-binding proteins IHF and Lrp can specifically bend *fimS* DNA, enabling proper positioning of the inverted repeat sequences to facilitate recombination (Schwan, 2011). Besides *fimB*, *fimE* and the *fimAIICDFGH* gene cluster, a unique gene, *fimK*, was found immediately downstream of the *fimH* gene in the *K. pneumoniae* genome. The *fimK* gene has also been shown to be transcribed as part of the

FimK regulation on the expression of type 1 fimbriae in *Klebsiella pneumoniae* CG43S3

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*Klebsiella pneumoniae* CG43, a heavy encapsulated liver abscess isolate, mainly expresses type 3 fimbriae. Type 1 fimbriae expression was only apparent in CG43S3ΔmrkA (the type 3 fimbriae-deficient strain). The expression of type 1 fimbriae in CG43S3ΔmrkA was reduced by deleting the *fimK* gene, but was unaffected by removing the 3’ end of *fimK* encoding the C-terminal EIL domain (EIL<sub>mrkK</sub>). Quantitative RT-PCR and promoter activity analysis showed that the putative DNA-binding region at the N terminus, but not the C-terminal EIL domain, of FimK positively affects transcription of the type 1 fimbrial major subunit, *fimA*. An electrophoretic mobility shift assay demonstrated that the recombinant FimK could specifically bind to *fimS*, which is located upstream of *fimA* and contains a vegetative promoter for the *fim* operon, also reflecting possible transcriptional regulation. EIL<sub>fimK</sub> was shown to encode a functional phosphodiesterase (PDE) via enhancing motility in *Escherichia coli* JM109 and *in vitro* using PDE activity assays. Moreover, EIL<sub>fimK</sub> exhibited higher PDE activity than FimK, implying that the N-terminal DNA-binding domain may negatively affect the PDE activity of FimK. *FimA* expression was detected in CG43S3 expressing EIL<sub>fimK</sub> or AIL<sub>fimK</sub>, suggesting that FimA expression is not directly influenced by the c-di-GMP level. In summary, FimK influences type 1 fimbriation by binding to *fimS* at the N-terminal domain, and thereafter, the altered protein structure may activate C-terminal PDE activity to reduce the intracellular c-di-GMP level.

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**Abbreviations**

bis-pNPP, bis(p-nitrophenol) phosphate; c-di-GMP, cyclic di-GMP; EMSA, electrophoretic mobility shift assay; KLA, *Klebsiella* liver abscess; PDE, phosphodiesterase.

Two supplementary figures are available with the online version of this paper.

†These authors contributed equally to this paper.

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single fimAICDFGH operon (Rosen et al., 2008). The FimK protein of a K. pneumoniae urinary tract infection isolate negatively affects the expression of type 1 fimbriae. This may be caused by the involvement of cyclic di-GMP (c-di-GMP) phosphodiesterase (PDE) activity (Rosen et al., 2008). This possibility is supported by the multiple sequence alignment data of Fig. S1, available in Microbiology online, which reveals a conserved EAL domain at the C terminus of FimK. In addition to the EAL domain, a DNA-binding domain has been predicted in FimK (Struve et al., 2008). Amino acid sequence analysis on the basis of Pfam classification also showed a putative helix–turn–helix DNA-binding motif from the HTH_23 family (http://pfam.sanger.ac.uk/) at the N-terminal region of FimK.

Analysis of the genome sequence of K. pneumoniae CG43 (unpublished data), a liver abscess isolate that belongs to the K2 serotype (Chang et al., 1996), showed that the type 1 fimbriae gene cluster is physically linked and divergently transcribed to the type 3 fimbriae operon. This study reports FimK-mediated regulation of type 1 fimbriae expression at the transcriptional level in K. pneumoniae CG43S3, which may be achieved by binding with the HTH domain of FimK to the fimS DNA. The changed protein

Table 1. Bacterial strains and plasmids used in this study

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<th>Strain or plasmid</th>
<th>Properties*</th>
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<td>Lai et al. (2003)</td>
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<td>CG43S3 with deletion of mrkA and HTH_fimK coding region</td>
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<td>CG43S3 with deletion of lacZ gene</td>
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<td>CG43S3 with deletion of lacZ gene</td>
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<td>Laboratory stock</td>
</tr>
<tr>
<td>NovaBlue BL21(DE3)</td>
<td>Recombinant protein overexpression host</td>
<td>Laboratory stock</td>
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<tr>
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<td>Km', expression vector</td>
<td>Wu et al. (2010)</td>
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<td>Keen et al. (1988)</td>
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<tr>
<td>pBAD33-yeDEH</td>
<td>Cm'; yeDEH coding region cloned into pBAD33</td>
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</table>

*Cm', chloramphenicol resistance; Tc', tetracycline resistance; Ap', ampicillin resistance; Sm', streptomycin resistance; Km', kanamycin resistance.
Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
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| Gene deletions | mrkA | SL0141 CGAGCTCAGGCTGATGTCTATCCAG  
SL0142 CGCGGATCCCGAATCAATGACGACACT  
SL0143 CGCGGATCCACAATAATAAAGCGGCAAT  
SL0158 TGCTCTAGAGACTGCCGACAATAAAGC |
| | fimK | K1 TCGCTTCGCCGTCAGGCC  
K2 CTTCGCCGCGGCCGAGCATC  
K3 AGATCTTGTATTCCCGGGGTG  
K4 CATGATCTGCGCGTCGAGG |
| | EII<sub>fimK</sub> | WCC32 AGGCTACTCGACGAGGCCTTGC  
WCC33 GGATCTTAAAACGCCGTCAGTG  
WCC34 GGATCCGGCGGAGTCCGAGG  
WCC35 GGTACCGCGAGTAAGTGATGTCG |
| | HTH<sub>fimK</sub> | WCC127 ACCTCTAGAGGCCGGTGATATTACGTACCTATAC  
WCC128 ATTAAGCTTCGAGCAGGGCGAGAGGATATAAT  
WCC133 ATTAAGCTTAACGCACTGACGGCGTTGAAA  
WCC130 ATTAAGCTCAAGATTATCCCTCTCTGCG |
| Gene expression | fimK/fimKE245A | WCC71 GATGACCGAGATTATATCCTCTGCGG  
WCC74 GAATCTCAGGGAGAATGAGTGACGATCGGTATATCAC  
WCC81 ACCCTCTAAGGAGAGAAAGGATGCGCACG  
WCC84 ATTAAGCTTAAACGCCGTCAGTG |
| | HTH<sub>fimK</sub> | WCC72 GAATCTCAGGGAGAATGAGTGACGATCGGTATATCAC  
WCC82 ACCCTCTAAGGAGAGAAAGGATGCGCACG  
WCC83 ATTAAGCTTAAACGCCGTCAGTG |
| | EII<sub>fimK</sub>/AIL<sub>fimK</sub> | WCC73 GATGACCGAGATTATATCCTCTGCGG  
WCC83 ATTAAGCTTAAACGCCGTCAGTG |
| | ydeH | WCC75 GATGACCGAGATTATATCCTCTGCGG  
WCC76 ATTAAGCTTAAACGCCGTCAGTG |
| | mrkJ | WCC77 GATGACCGAGATTATATCCTCTGCGG  
WCC78 ATTAAGCTTAAACGCCGTCAGTG |
| | fimB | B1 GGATCCGAAAAGCCTGGGATCTGACCGGAG  
B2 CCTTGATCGACCGACGATCG |
| Site-directed mutation | fimKE245A | M1 CTATCTGCGACAGTCGCTACAGATGCGGAGGAGGATCTG  
M2 CAGGATCAGCGCCACCCCTGCTAGCTGAGCGACTGTCGAGTAG |
| EMSA | | WCC89 GCCATATTCTCCGACAAAAAAAAT  
WCC90 GATCCGTCGCACCCCTGCTAGCTGAGCGACTGTCGAGTAG  
WCC91 CCACATGATCCGACAAAAAAAATA  
WCC92 biotin-TTGGGCGGCGCCGACAAAAAAAATA  
WCC93 biotin-TTGGGCGGCGCCGACAAAAAAAATA  
WCC94 biotin-TGCGACGACTGTCGAGTAG |
| qRT-PCR | fimA | fimA-F GAACGATGTCGAAATAACGAACCGG  
fimA-R AACAATGACCTGACGCAGCG |

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STRUCTURING THE GENE DELETION MUTANTS.

IMMUNOELECTRON MICROSCOPY. *K. pneumoniae* CG43S3 was grown overnight in LB broth with shaking at 37 °C, unless otherwise indicated. Antibiotics used included ampicillin (100 µg ml⁻¹), kanamycin (25 µg ml⁻¹), streptomycin (500 µg ml⁻¹), chloramphenicol (35 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹).

**METHODS**

**Bacterial strains, plasmid, primer and growth conditions.** Table 1 list the bacterial strains and plasmids used in this study, and Table 2 lists the primers. Bacteria were grown in Luria–Bertani (LB) broth with shaking at 37 °C, unless otherwise indicated. Antibiotics used included ampicillin (100 µg ml⁻¹), kanamycin (25 µg ml⁻¹), streptomycin (500 µg ml⁻¹), chloramphenicol (35 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹).

**Immunoelectron microscopy.** *K. pneumoniae* CG43S3 was grown overnight in LB broth with shaking, and 20 µl of the bacterial suspension (10⁶ c.f.u. ml⁻¹) was added to collodion-coated 300-mesh copper grids. The bacteria coated on the grids were then incubated with a 1:50 dilution of the previously prepared anti-MrkA polyclonal antibody (Huang et al., 2009), followed by incubation with a 1:65 dilution of 10 nm gold particles conjugated with protein A (Sigma- Aldrich P9660). After negatively staining with 2 % (w/v) phosphotungstic acid, pH 7.2, the grids were examined under a JEOL JEM 2000EXII transmission electron microscope at an operating voltage of 100 kV.

**FimA antisera preparation.** The FimA coding sequence was amplified by PCR from *K. pneumoniae* CG43S3 and ligated into expression vector pET30a. Recombinant plasmid pET30a-fimA was then transformed into *E. coli* NovaBlueBL21(DE3), and overexpression of the recombinant protein His6-FimA was induced with 0.5 mM IPTG for 5 h at 37 °C. The insoluble fraction was denatured using 6 M urea and then the protein purified using a nickel column (Novagen) saturated with 6 M urea. Finally, 3 mg of the purified His6-FimA was used to immunize rabbits for the anti-FimA antibody.

**Constructing the gene deletion mutants.** Specific gene deletion was introduced into the chromosome of *K. pneumoniae* CG43S3 using an allelic-exchange strategy (Lai et al., 2003). Briefly, DNA fragments of 1 kb flanking both ends of *mrkA, fimK, EILfimK*, and *HTHfimK* DNA were amplified by PCR with primer sets SL0141/ SL0142 and SL0143/SL0158, K1/K2 and K3/K4, WCC32/WCC33 and WCC34/WCC35, and WCC127/C128 and WCC133/WCC130, respectively. The amplified DNA fragments were individually cloned into suicide vector pKAS46 (Skorupski & Taylor, 1996). The resulting plasmid was transformed into *E. coli* S17-1pir and then mobilized by conjugating to the streptomycin-resistant strain, *K. pneumoniae* CG43S3 (Lai et al., 2003). Several kanamycin-resistant transconjugants, with the plasmid integrated into the chromosome by homologous recombination, were selected from the M9 agar plates containing 500 µg streptomycin ml⁻¹. Colonies susceptible to kanamycin were isolated, and the specific gene deletions were verified by PCR analysis.

**Quantitative reverse-transcription PCR (qRT-PCR).** Total RNA was isolated from bacteria using a High Pure RNA isolation kit (Roche), and residual DNA was eliminated with RNase-free DNase 1 according to the manufacturer’s instructions. The cDNAs used for PCR were synthesized from 1.5 µg RNA using a random hexamer primer form RevertAid H Minus First-strand cDNA synthesis kit (Fermentas). PCR was performed using an ABI Prism 7000 Detection system according to manufacturer’s instructions, and products were detected using SYBR Green PCR Master Mix (Roche). The RNA samples were normalized to the 23S rRNA level. Analysis was performed in triplicate in a reaction volume of 25 µl containing 12.5 µl SYBR Green PCR Master Mix, 300 nM primer pair, 9.5 µl distilled H₂O and 1 µl cDNA. Samples were heated for 10 min at 95 °C and amplified for 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Quantification was performed using the 2⁻ΔΔCt method (Tsai et al., 2009).

**Western blot analysis of FimA and MrkA expression.** Aliquots of total cellular lysates were resolved by SDS-PAGE, and proteins were electrophoretically transferred onto a PVDF membrane (Millipore). After incubation with 5 % skimmed milk at room temperature for 1 h, the membrane was washed three times in PBS with Tween 20 (PBST). The membrane was then incubated with anti-GAPDH (GeneTex), anti-FimA or anti-MrkA antiserum at room temperature for 2 h. After three washes with 1 × PBST, the PVDF membrane was incubated with a 1:5000 dilution of the secondary antibody, alkaline phosphatase-conjugated anti-rabbit immunoglobulin G, at room temperature for 1 h. Finally, the blot was washed, and the secondary antibodies bound on the PVDF membrane were detected using chromogenic reagents 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Yeast-cell agglutination.** Agglutination of yeast *Saccharomyces cerevisiae* AH109 was conducted as described by Blumer et al. (2005). Bacteria (10⁶ c.f.u. ml⁻¹) were suspended in PBS with or without mannose and then mixed with yeast (10 mg ml⁻¹) on a glass slide. The degree of clumping was assessed by observation.

**Biofilm formation assay.** As described previously (Lin et al., 2006; Wu et al., 2010), bacteria diluted 1 : 100 in LB broth supplemented with appropriate antibiotics were inoculated into each well of a 96-well microtitre dish (Orange Scientific) and statically incubated at 37 °C for 20 h. After removing planktonic cells, the wells were washed once with distilled water to remove unattached cells. Crystal violet (0.1 % w/v; Sigma-Aldrich) was used to stain the attached cells for 30 min. Unattached dye was removed by washing three times with overnight. A small aliquot of the culture was plated on LB agar containing 500 µg streptomycin ml⁻¹. Colonies susceptible to kanamycin were isolated, and the specific gene deletions were verified by PCR analysis.

### Table 2. cont.

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<td></td>
<td>pfimE2</td>
<td>CCTGAACTTCTTTTGCGGTAGAAA</td>
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**Fig. 1.** Type 1 and type 3 fimbriae expression in *K. pneumoniae* CG43S3. (a) Transmission electron micrograph of *K. pneumoniae* CG43S3 labelled with anti-MrkA antibodies. (b) Diagrammatic representation of *fimS* promoter analysis (left). The primer pair P1 and P2 was used for amplifying the region containing *fimS*. The DNA pattern corresponding to incomplete digested amplicons or phase OFF of type 1 fimbriae is shown (right). (c) Analysis of FimA expression using qRT-PCR (left panel) and Western blot analysis (right panel). GAPDH was probed as a protein loading control. Values are means of three independent experiments. Error bars shown are SD, and asterisks denote statistically significant differences.
Plasmid construction. The coding region of *fimB*, *fimE*, the N-terminal regions of the HTH domain (1–218 aa) or EIL domain (195–469 aa) of *fimK*, and *mrkJ* were PCR-amplified with primer pairs B1/B2, WCC71/WCC74, WCC71/WCC72, WCC73/WCC74 and WCC77/WCC78, respectively, from the CG4353 genome. The amplified DNA was cloned into cloning vector yT&A (Yeasen Biotech). Site-directed mutation plasmid pyT-*fimK*E245A was generated by substituting the glutamic acid at FimK position 245 with alanine using plasmid pyT-*fimK* as a template with overlapping primers M1 or M2 by PCR-based mutagenesis. Primer pair WCC73/WCC74 was then used to amplify the AIL*-fimk* coding region using pyT-*fimK*E245A as a template.

For complementation analysis or protein overexpression, the coding region from pyT-*fimB*, pyT-*fimK*, pyT-*fimK*E245A, pyT-THH*-fimk*, pyT-EIL*-fimk*, pyT-AIL*-fimk* or pyT-*mrkJ* was subcloned into the broad-host-range vector pRK415 (Keen et al., 1988) or expression vectors pETQ31, pETQ33 (Wu et al., 2010), pQE31 (Qiagen) or pET30a (Novagen). This resulted in low-copy-number complementation plasmids pRK415-*fimK*, pRK415-*fimK*E245A, pRK415-THH*-fimk* and pRK415-EIL*-fimk* and overexpression clones pETQ33-*fimB*, pETQ31-AIL*-fimK*, pETQ31-AIL*-fimK*, pETQ33-AIL*-fimK*, pETQ33-AIL*-fimK* and pET30a-*mrkJ*. The coding region of gene *ydeH* (Jonas et al., 2008) was PCR-amplified with primer pair WCC75/WCC76 from the *E. coli* MG1655 genome and cloned into yT&A to generate plasmid pyT-*ydeH*. The *ydeH* coding DNA was then isolated from pyT-*ydeH* by restriction enzyme digestion and then cloned into pETQ33 to yield YdeH expression plasmid pETQ33-*ydeH*.

Promoter activity measurement. The putative promoter regions of *fimA*, *fimB*, and *fimE* were PCR-amplified with primers pfimA3/pfimA4, pfimB4/pfimB5 and pfimE1/pfimE2, respectively. The amplicons were then cloned into placZ15 (Lin et al., 2006) to generate P*planA*-lacZ (locked on), P*planA*-lacZ, and P*planA*-lacZ. The promoter–reporter plasmids were individually mobilized into *K. pneumoniae* CG4353 strains by conjugation from *E. coli* S17-1pir. The bacteria were grown to the exponential phase in LB broth (OD600 of 0.7), and 100 μl of the culture was mixed with 900 μl of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol), 17 μl of 0.1% SDS and 35 μl chloroform, followed by vigorous shaking. After incubation at 30 °C for 5 min, 200 μl of a 4 mg ml⁻¹ concentration of ONPG (Sigma-Aldrich) was added to the mixture to initiate the reaction. When the mixture became yellow, the reaction was stopped by adding 500 μl 1 M Na2CO3 and the absorbance at OD405 was recorded. The promoter activity was expressed as Miller units. Each sample was assayed in triplicate and at least three independent experiments were conducted. The data were calculated from one representative experiment and are shown as the means and SD from three samples.

Expression and purification of the recombinant proteins. Recombinant plasmids pETQ31-EIL*-fimk* and pETQ31-AIL*-fimk* were transformed into *E. coli* JM109, and protein production was induced with 0.5 mM IPTG for 12 h at 22 °C. The expression plasmid pET30a-*mrkJ* was introduced into *E. coli* NovBlue BL21(DE3), and recombinant His₆-MrkK was induced with 0.5 mM IPTG for 5 h at 37 °C. The recombinant protein His₆-MrkK was induced in *E. coli* SG13009 [pREP4] with 0.01 mM IPTG for 24 h at 15 °C. All recombinant proteins were then purified from the soluble fraction of the cell lysate by affinity chromatography using His-Bind resin according to the QiAexpress expression system protocol (Qiagen). The purified proteins were dialysed against Tris-buffered saline (pH 7.4) containing 10% (v/v) glycerol at 4 °C overnight, followed by condensation with PEG 20 000. Protein purity was determined by SDS-PAGE.

DNA electrophoretic mobility shift assay (EMSA). The variant truncated putative promoter of *fimA* was PCR-amplified using biotin-labelled primer pairs WCC93/WCC96, WCC93/WCC98, WCC93/WCC99, WCC93/WCC98, WCC95/WCC91, WCC95/WCC90, WCC95/WCC89 and WCC121/WCC123 or non-labelled primer pairs WCC94/WCC96 and WCC122/WCC123. The DNA-binding reaction was performed in 20 μl binding buffer [100 mM MnCl₂, 1 mM MgCl₂, 0.5 mM DTT, 50 mM bicine, 10 mM DTT, 0.5% Nonidet N-40, 1 mg/ml BSA, 10 μg/ml each of plasmids pETQ33-*fimB* and pETQ33-*fimE*, 1 μg/ml of the target DNA fragment of *fimA*-lacZ and *fimE*-lacZ and 1 μg/ml of the target DNA fragment of *fimA*-lacZ. The promoter activity was expressed as Miller units. Each sample was assayed in triplicate and at least three independent experiments were conducted. The data were calculated from one representative experiment and are shown as the means and SD from three samples.

Western blot analysis of FimK regulation of *K. pneumoniae* type 1 fimbriae expression. Expression of the type 1 fimbriae major pilin FimA was determined in (a) FimB recombinase overexpression bacteria CG4353[pETQ33-*fimB*], and the derived strains CG4353Δ*fimK* [pETQ33-*fimB*] and CG4353Δ*eil*-fimk [pETQ33-*fimB*] and (b) CG4353Δ*mrkJ*, CG4353Δ*mrkJ*Δ*eil* and CG4353Δ*mrkJ*Δ*eil*Δ*thm* and CG4353Δ*mrkJ*Δ*eil*Δ*thm* (c) Analysis of FimK complementation by comparing FimA production of CG4353Δ*mrkJ*Δ*eil* [pRK415], CG4353Δ*mrkJ*Δ*eil*Δ*thm* and CG4353Δ*mrkJ*Δ*eil*Δ*thm*. The bacteria carrying pETQ33 or pETQ33-*fimB* plasmid were grown at 37 °C for 2 h, and subsequently grown in the presence of 0.5 mM IPTG for 4 h. The bacteria carrying pRK415 or the derivative plasmids were grown in LB broth supplemented with 0.01 mM IPTG for 20 h at 37 °C.

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Fig. 2. Western blot analysis of *fimK* deletion effects on type 1 fimbriae expression. Expression of the type 1 fimbriae major pilin FimA was determined in (a) FimB recombinase overexpression bacteria CG4353[pETQ33-*fimB*], and the derived strains CG4353Δ*fimK* [pETQ33-*fimB*] and CG4353Δ*eil*-fimk [pETQ33-*fimB*] and (b) CG4353Δ*mrkJ*, CG4353Δ*mrkJ*Δ*eil* and CG4353Δ*mrkJ*Δ*eil*Δ*thm* and CG4353Δ*mrkJ*Δ*eil*Δ*thm* (c) Analysis of FimK complementation by comparing FimA production of CG4353Δ*mrkJ*Δ*eil* [pRK415], CG4353Δ*mrkJ*Δ*eil*Δ*thm* and CG4353Δ*mrkJ*Δ*eil*Δ*thm*. The bacteria carrying pETQ33 or pETQ33-*fimB* plasmid were grown at 37 °C for 2 h, and subsequently grown in the presence of 0.5 mM IPTG for 4 h. The bacteria carrying pRK415 or the derivative plasmids were grown in LB broth supplemented with 0.01 mM IPTG for 20 h at 37 °C.
Fig. 3. FimK positively affects type 1 fimbriae transcript levels. (a) qRT-PCR analysis of the type 1 fimbriae (fimA) transcription. *K. pneumoniae* CG43S3ΔmrkA carrying pRK415 or the derivative plasmids was grown in LB broth supplemented with 0.01 mM IPTG at 37 °C for 20 h with agitation. RNA extraction and qRT-PCR detection were performed as described in Methods. qRT-PCR assays were conducted in triplicate. The error bars indicate the variations of three replicates from the mean. 

(b) The upper panel depicts the promoterless lacZ fusion to the putative promoter of fimA, fimB and fimE. IRR, IRL and S1 are,
respectively, the inverted repeat right and left of \textit{fimS} and the transcription start site. Lower panel: the promoter activity of \textit{fimA}, \textit{fimB} and \textit{fimE} as, respectively, assessed by expression of the β-galactosidase reporter plasmid \textit{P}_{\text{fimA-lacZ}} (locked on), \textit{P}_{\text{fimB-lacZ}} and \textit{P}_{\text{fimE-lacZ}}, in \textit{K. pneumoniae CG43S3} for the regulation

50 mM KCl, 10 mM Tris/HCl (pH 7.5), 0.05 mg BSA ml$^{-1}$ and 4 % (v/v) glycerol. In the reaction, sheared salmon sperm DNA was added at 0.05 mg ml$^{-1}$ to prevent non-specific DNA binding. After transferring to a Biodyne B nylon membrane, biotin-labelled DNA was detected using a LightShift chemiluminescent EMSA kit (Pierce).

**PDE activity measurement.** An \textit{in vitro} PDE activity assay was performed as described by Johnson & Clegg (2010) with 300 nM of the purified recombinant proteins or a non-phosphodiesterase control, BSA (Sigma-Aldrich), in assay buffer [50 mM Tris/HCl, 1 mM MnCl$_2$ (pH 8.5)] supplemented with 5 mM bis-p-nitrophenol phosphate (bis-p-NPP). Reaction mixtures were incubated at 37 °C for 3 h, and the release of p-nitrophenol was quantified at 405 nm.

**Swimming activity analysis.** The coding regions of \textit{EII}$_{\text{fimK}}$, \textit{AIL}$_{\text{fimK}}$, \textit{mrk}$_{\text{E}}$ and \textit{ydeH} were amplified from \textit{pyT-fimK}, \textit{pyT-fimK}_{CG43S3} and \textit{pyT-mrk}$_{\text{E}}$ and \textit{pyT-ydeH}, respectively, with primer pairs WCC83/WCC84, WCC85/WCC86 and WCC79/WCC80 (Table 2). The amplified DNA products were individually cloned into expression vector pBAD33 (Guzman et al., 1995), and the resulting plasmids were transformed into \textit{E. coli} JM109. Swimming activity analysis for the recombinant bacteria was then conducted as described by Wood et al. (2006). Four microcultures of the bacteria grown overnight carrying \textit{pBAD33-EII}$_{\text{fimK}}$, \textit{pBAD33-AIL}$_{\text{fimK}}$, \textit{pBAD33-mrk}$_{\text{E}}$ or \textit{pBAD33-ydeH} was spotted onto a tryptone swimming plate (0.25 % Bacto agar, 0.25 % NaCl, 1 % tryptone, 5 μg chloramphenicol ml$^{-1}$ and 0.1 % arabinose), and the plate was incubated for 12 h at 37 °C.

**Measuring c-di-GMP level in \textit{K. pneumoniae CG43S3}**. Expression plasmids pETQ33, pETQ33-\textit{AIL}$_{\text{fimK}}$, pETQ33-\textit{EII}$_{\text{fimK}}$ or pETQ33-\textit{ydeH} were individually transformed into \textit{K. pneumoniae CG43S3}, and overexpression of the recombinant proteins was induced with 0.5 mM IPTG for 4 h at 37 °C. To measure the c-di-GMP content, cellular extracts were prepared as described by Simm et al. (2004). The IPTG-induced bacteria were collected and treated with formaldehyde (0.19 % final concentration), and then pelleted by centrifugation. The pellet was suspended in de-ionized water and heated to 95 °C for 10 min before nucleotides were extracted using 65 % ethanol. The lyophilized samples were resuspended in de-ionized water, and the suspension was used to detect c-di-GMP with the cyclic diguanylate ELISA kit (Wuhan ELIab Science).

**RESULTS**

**Inverse expression of type 1 and 3 fimbriae in \textit{K. pneumoniae CG43S3}**

Transmission electron microscopy detection with an immunogold-labelled antibody against the major pilin, MrkA, was used to demonstrate the synthesis of type 3 fimbriae on the surface of \textit{K. pneumoniae CG43S3} (Fig. 1a). Expression of the \textit{fim} operon could be assessed by the restriction pattern of the \textit{fimS} DNA amplified from the bacterial culture. Fig. 1(b) shows some incomplete digested amplicons and the BsaBI restriction fragments of approximately 450 and 200 bp, corresponding to the phase ‘OFF’ \textit{fimS} in \textit{CG43S3}. This restriction pattern reflects no detectable expression of the type 1 fimbriae. The expression of type 1 fimbriae pilin FimA was only evident in the \textit{mrkA} deletion mutant, as shown by qRT-PCR and Western blot analysis (Fig. 1c). The results show that deleting the predominant type 3 fimbriae pilin \textit{mrkA} gene increased type 1 fimbriae expression. The inverse expression pattern between the two types of fimbriae was further observed by introducing pETQ33-\textit{fimB}, which overexpresses the FimB recombinase, into \textit{K. pneumoniae CG43S3}. Fig. 2(a) shows that overexpression of the FimB recombinase turned on expression of FimA but depleted MrkA production.

**FimK plays a positive regulatory role in the expression of type 1 fimbriae**

As shown in Fig. 2(a), deleting \textit{fimK} from \textit{CG43S3}[pETQ33-\textit{fimB}] slightly decreased the expression of FimA but greatly increased the expression of MrkA as judged by immunoblot analysis targeting MrkA and FimA. However, deleting only the DNA coding for the FimK EIL domain (195–469 aa) in an in-frame fashion from \textit{CG43S3} had no apparent effect on FimA production. To further confirm the regulatory role of FimK on the expression of type 1 fimbriae, the FimK-coding DNA and the DNA coding for the HTH domain of FimK were individually deleted in an in-frame fashion from \textit{CG43S3}. Compared with \textit{CG43S3}Δ\textit{mrkA}, \textit{CG43S3}Δ\textit{mrkA}Δ\textit{fimK} and \textit{CG43S3}Δ\textit{mrkA}Δ\textit{fimK} reduced FimA production slightly while \textit{CG43S3}Δ\textit{mrkA}Δ\textit{fimK} had no apparent effect on FimA expression (Fig. 2b). The results suggest that the FimK regulation of type 1 fimbriae is dependent on the N-terminal DNA-binding domain. As shown in Fig. 2(c), introducing plasmid pRK415-\textit{fimK} or pRK415-\textit{HTH}Δ\textit{fimK}, which expressed an intact FimK or the FimK DNA-binding domain, into \textit{CG43S3}Δ\textit{mrkA}Δ\textit{fimK} increased FimA expression levels. The FimK-dependent type 1 fimbriae activities were also observed using mannosensitive yeast agglutination assay. As shown in Fig. S2(a, b), deleting \textit{fimK} from \textit{CG43S3} or \textit{CG43S3}Δ\textit{fimK} had no dramatic change on the mannosensitive agglutination activity. However, the complementation analysis clearly revealed an FimK- or HTH domain-dependent mannosensitive agglutination activity (Fig. S2c). These results suggest that FimK positively affects the type 1 fimbriae expression possibly through its N-terminal DNA-binding domain.
The N-terminal domain of FimK positively influences P\textsubscript{fimA} activity

To investigate whether the fimK gene affects type 1 fimbriae biosynthesis at a transcriptional level, the fimA transcript level was determined using qPCR. As shown in Fig. 3(a), introducing either plasmid pRK415-fimK or pRK415-fimKE\textsubscript{245A} into CG43S3ΔmrkA increased the fimA transcripts to approximately twice the value of CG43S3ΔmrkA[pRK415] or CG43S3ΔmrkA[pRK415-EIL\textsubscript{fimk}]. This suggests that the fimA transcript-level change might not be directly influenced

**Fig. 4.** EMSA of the interaction between His\textsubscript{6}-FimK and fimS. (a) Schematic diagram of the DNA probes for the analysis. The relative positions of the primer sets used in PCR amplification of the DNA probes are indicated. Names and sizes of the biotin-labelled DNA probes are shown on the right. S1, the transcription start site; IHF I and IHF II, the binding sites for integration host factor IHF; Lrp 1, Lrp 2 and Lrp 3, the leucine-responsive regulatory protein (Lrp) binding sites. (b) Interaction of the His\textsubscript{6}-FimK and the putative promoter DNA. The biotin-labelled P\textsubscript{fimA} (P1\textsuperscript{*}) was incubated with increasing amount of recombinant FimK protein. Binding specificity was investigated by adding 200-fold unlabelled specific competitor DNA fragments (P1 or P7). (c) The biotin-labelled DNA probes P2\textsuperscript{*}, P3\textsuperscript{*}, P4\textsuperscript{*}, P5\textsuperscript{*}, P6\textsuperscript{*} and P7\textsuperscript{*} were applied for the specific binding region.
by the C-terminal EIL domain. To investigate whether the N-terminal DNA-binding domain of FimK enhanced type 1 fimbriae expression by regulating fimB or fimE promoter activity or directly affected fimA promoter activity, a LacZ reporter assay was performed. Fig. 1(b) shows that the fimS is mostly OFF-phased in CG43S3, and a locked ON fimA promoter was therefore used. These promoter activities in CG43S3ΔfimK appeared to be at similar levels to those in CG43S3 (data not shown). We reason that fimK is transcribed as part of the fimAICDFGH operon (Rosen et al., 2008) and expression of type 1 fimbriae in CG43S3 is not detected in the culture condition. Therefore, the promoter activities of fimA, fimB and fimE were measured in CG43S3ΔmrkA to examine the fimK gene deletion effect. As shown in Fig. 3(b), the promoter activity of fimA, but not of fimB or fimE, was reduced by deleting fimK. This suggests that FimK affects type 1 fimbriae expression by directly influencing the fimA promoter. The negative effect was not observed for P fimA in CG43S3ΔmrkAΔEil-fimK implying that the N-terminal region of FimK plays a regulatory role. As shown in Fig. 3(c), P fimA Activity in E. coli JM109 was not changed with IPTG addition, while heterologous expression of FimK by IPTG induction increased the expression levels of fimA, further supporting a direct regulation of FimK on fimA.

**Recombinant FimK exhibits a specific DNA-binding activity with P fimA**

The DNA fragments encompassing the full-length fimS (P1) and the truncated forms P2*, P3*, P4*, P5*, P6* and P7* as depicted in Fig. 4(a) were isolated and biotin-labelled for the analysis. As shown in Fig. 4(b), formation of the P1*/FimK complex could be observed as the amount of His6-FimK increased, and the binding specificity was demonstrated as the complex diminished in the presence of excess non-labelled P1 or P7 acting as specific competitor DNA. The sheared salmon sperm DNA was added as non-specific competitor reagent. Fig. 4(c) shows that the purified recombinant His6-FimK protein was able to bind to the DNA probes P2, P3, P6 and P7, but not to P4 or P5. The results support the conclusion that the recombinant FimK protein could specifically interact with fimS DNA and also suggest that the FimK-binding site is located within P7.

**The recombinant FimK protein exerts PDE activity**

Some PDE domain proteins do not exhibit PDE-specific enzyme activity (Römling, 2009), so we studied whether FimK possesses PDE activity. As shown in Fig. 5(a), the purified recombinant FimK exhibited considerably less bis-PNPP catalytic activity than the recombinant MrkJ, for which PDE activity has been reported (Johnson et al., 2011). Recombinant clones expressing Eil-fimK and Ali-fimK containing the C-terminal EIL domain of FimK and the domain with critical E245 residue replaced with alanine, respectively, were also generated and the corresponding overexpressed proteins were purified. The recombinant Eil-fimK exhibited a higher level of PDE activity than the recombinant FimK and Ali-fimK. The FimK protein exhibited the lowest PDE activity of all the tested proteins, except the negative control BSA.

The second-messenger c-di-GMP levels determine whether E. coli and many other bacteria are in a motile or sessile state (Jonas et al., 2009). As assessed using swimming activity analysis shown in Fig. 5(b), the heterologous expression in E. coli JM109 of the EIL domain of FimK or the intact MrkJ increased the swimming zone compared with that of JM109[pBAD33] or JM109[pBAD33-ydeH].
which expresses diguanylate cyclase activity (Jonas et al., 2008). The result supports that the FimK EIL domain exerts PDE activity, which is able to lower the intracellular c-di-GMP content, thereby increasing motility of the recombinant E. coli.

**FimA production is not directly dependent on c-di-GMP levels**

To further confirm EIL-fimK PDE activity, the c-di-GMP levels in *K. pneumoniae* CG43S3 transformed with pETQ33, pETQ33-ydeH, pETQ33-EIL-fimK and pETQ33-AIlfimK were measured. As shown in Fig. 6(a), the c-di-GMP concentration was approximately 12.7 fmol mg\(^{-1}\) in CG43S3[pETQ33-ydeH], which was higher than that in CG43S3[pETQ33] or CG43S3[pETQ33-AIlfimK]. The CG43S3[pETQ33-EIL-fimK] contained the lowest level at 5.3 fmol mg\(^{-1}\), also confirming c-di-GMP PDE activity of EIL-fimK.

Comparative analysis of the bacteria which carry different c-di-GMP levels was conducted to determine if the second-messenger levels regulate the expression of type 1 fimbriae. As shown in Fig. 6(b), FimA production was observed in CG43S3[pETQ33-EIL-fimK], CG43S3[pETQ33-AIlfimK] and CG43S3[pETQ33-mlrkA] but not in CG43S3[pETQ33] or CG43S3[pETQ33-ydeH], reflecting MrkA expression. Approximately the same level of c-di-GMP was detected in CG43S3[pETQ33-AIlfimK] and CG43S3[pETQ33], indicating that FimA production is not directly affected by c-di-GMP level. Despite this, the c-di-GMP level change altered MrkA production and biofilm formation (Fig. 6c).

**DISCUSSION**

Research shows that cross-talk regulation may occur between different fimbriae (Snyder et al., 2005; Sjöström et al., 2009). In *E. coli*, during coordinate regulation for type 1 and type P fimbriae, PapB, a regulator for type P fimbriae expression, inhibits FimB-promoted recombination, thereby affecting the expression of type 1 fimbriae (Xia et al., 2000). Analysing *K. pneumoniae* CG43 genome sequences revealed at least ten fimbriae-coding gene clusters (unpublished data). It is possible that the expression of individual adhesins must be cooperatively regulated in the bacteria. Fimbriated and non-fimbriated planktonic cells display different outer-membrane protein patterns (Otto et al., 2001). Decreased expression of type 1 fimbriae was reported for the *E. coli* K1 ompA deletion mutant (Teng et al., 2006). Misfolding of the P fimbriae subunit triggered the 2CS Cpx and \(\sigma^E\) regulatory pathways (Jones et al., 1997). These studies suggest that deleting *mrkA* may change the outer-membrane protein pattern or trigger an envelope stress system, leading to the expression of type 1 fimbriae in *K. pneumoniae* CG43S3.

As shown in Fig. 2(b), deleting fimK from the *mrkA* deletion mutant reduced FimA production. These reduction effects were not observed in CG43S3AmrkAAAEL-fimKs, suggesting that FimK regulates fim expression through the N-terminal region. Complementation analysis confirms that the HTH\(\text{fimK}\) domain, but not the C-terminal EIL domain, positively regulates type 1 fimbriae expression via increasing fimA transcription (Figs 2c and 3a). Our LacZ reporter assay and EMSA data indicated that FimK may bind to an fimS region upstream of the fimA transcriptional start site and then activate fimA promoter activity. In *E. coli*, IHF and Lrp could specifically bind on and bent fimS DNA, enabling proper positioning of the inverted repeat sequences to facilitate recombination (Schwan, 2011). EMSA data indicate that FimK binds to P7* DNA. Regional sequence analysis identified a putative IHF binding sequence, 5′-TNYAANNNR-TTGAT-3′, where Y is pyrimidine and R is purine (Eisenstein et al., 1987). In *Salmonella enterica* serovar Typhimurium, the fimA promoter is activated when FimZ forms a complex with FimY protein but repressed by the FimZ–FimW complex (McFarland et al., 2008). Hence, FimK may regulate fim expression by cooperating with IHF or Lrp or an unidentified protein such as FimZ in *S. Typhimurium*. Nevertheless, FimK-mediated regulation may be also achieved by changing the structure of on-phase DNA, thereby improving transcription, or by facilitating fimS switch from off to on through altering the interaction between DNA and FimB or FimE recombinate.

As shown in Fig. S1, the FimK EIL domain includes the critical residues involved in Mg\(^{2+}\) substrate binding and Loop 6 required for signal transduction (Rao et al., 2008, 2009; Römling, 2009). This suggests PDE activity for FimK. Several studies have shown that PDE activity was inactivated when critical residue E from the EAL domain was replaced with alanine (Kuchma et al., 2007; Bassis & Visick, 2010). However, recombinant proteins EIL-fimK and AIIfimK expressed PDE activity, and only AIlfimK exhibited lower enzyme activity levels. When responding to different internal and external signals, the sensory modules, such as PAS, GAF, HAMP, REC and HTH domains, commonly present with the GGDEF, EAL and HD-GYP domains, activate diguanylate cyclase or PDE activity (Ho et al., 2000; Galperin et al., 2001; Christen et al., 2005; Tamayo et al., 2007; Cruz et al., 2012). FimK exerted higher PDE activity when the N-terminal DNA-binding domain was removed, suggesting that PDE activity may be activated after the N-terminal domain is stimulated by a specific signal.

This study provides novel insight into the function and mechanism of FimK regulating type 1 fimbriation in *K. pneumoniae*. The data demonstrate that FimK positively affects the expression of type 1 fimbriae at the transcriptional level by modulating fimA promoter activity after binding to fimS. When interaction between the N-terminal domain and DNA occurs, FimK PDE activity may be activated to reduce intracellular c-di-GMP levels, thus negatively affecting the expression of other surface structures, such as type 3 fimbriae (Wilksch et al., 2011).
Formation in three independent experiments. Error bars shown are SD, (Wuhan EIAab Science). Measurement of c-di-GMP was performed as described in Methods. Biofilm formation activity. Bacteria were inoculated into each well of a 96-well microtitre dish and statically incubated at 37 °C for 20 h, and then the biofilm-forming activity measurement was performed as described in Methods.

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