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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 CG43S3Δ*mrkA* (the type 3 fimbriae-deficient strain). The expression of type 1 fimbriae in CG43S3Δ*mrkA* was reduced by deleting the *fimK* gene, but was unaffected by removing the 3′ end of *fimK* encoding the C-terminal EIL domain (EIL_{fimK}). 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_{fimK} was shown to encode a functional phosphodiesterase (PDE) via enhancing motility in *Escherichia coli* JM109 and *in vitro* using PDE activity assays. Moreover, EIL_{fimK} 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_{fimK} or AIL_{fimK}, 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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INTRODUCTION

Klebsiella pneumoniae is an important nosocomial pathogen that causes suppurative lesions, septicaemia, 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 *fimAICDFGH* gene cluster, a unique gene, *fimK*, was found immediately downstream of the *fimH* gene in the *K. pneumoniae* genome. The *fimK* gene had also been shown to be transcribed as part of the

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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.

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

Strain or plasmid	Properties*	Reference or source
Strain		
K. pneumoniae		
CG43S3	Sm^r, CG43 derived strain	Lai *et al.* (2003)
CG43S3Δ*mrkA*	CG43S3 with deletion of *mrkA* gene	This study
CG43S3Δ*fimK*	CG43S3 with deletion of *fimK* gene	This study
CG43S3ΔEIL$_{fimK}$	CG43S3 with deletion of EIL$_{fimK}$ coding region	This study
CG43S3Δ*mrkA*Δ*fimK*	CG43S3 with deletion of *mrkA* and *fimK* gene	This study
CG43S3Δ*mrkA*Δ*HTH*$_{fimK}$	CG43S3 with deletion of *mrkA* and HTH$_{fimK}$ coding region	This study
CG43S3Δ*mrkA*ΔEIL$_{fimK}$	CG43S3 with deletion of *mrkA* and EIL$_{fimK}$ coding region	This study
CG43S3Δ*mrkA*Δ*lacZ*	CG43S3Δ*mrkA* with deletion of *lacZ* gene	This study
CG43S3Δ*mrkA*Δ*fimK*Δ*lacZ*	CG43S3Δ*mrkA*Δ*fimK* with deletion of *lacZ* gene	This study
CG43S3Δ*mrkA*ΔEIL$_{fimK}$Δ*lacZ*	CG43S3Δ*mrkA*ΔEIL$_{fimK}$ with deletion of *lacZ* gene	This study
E. coli		
JM109	Cloning host	Laboratory stock
NovaBlue BL21(DE3)	Recombinant protein overexpression host	Laboratory stock
Plasmid		
pETQ31	Km^r; expression vector	Wu *et al.* (2010)
pETQ33	Km^r; expression vector	Wu *et al.* (2010)
pKAS46	Km^r and Ap^r; suicide vector	Skorupski & Taylor (1996)
pRK415	Tc^r; broad-host-range vector	Keen *et al.* (1988)
pBAD33	Cm^r; expression vector	Guzman *et al.* (1995)
pET30a-*mrkJ*	Km^r; *mrkJ* coding region cloned into pET30a	This study
pQE31-*fimK*	Ap^r; *fimK* coding region cloned into pQE31	This study
pETQ31-EIL$_{fimK}$	Km^r; *fimK*-EIL domain region cloned into pETQ31	This study
pETQ31-AIL$_{fimK}$	Km^r; *fimK*-AIL domain region cloned into pETQ31	This study
pETQ33-*fimB*	Km^r; *fimB* coding region cloned into pETQ33	This study
pETQ33-*ydeH*	Km^r; *ydeH* coding region cloned into pETQ33	This study
pETQ33-EIL$_{fimK}$ 3	Km^r; *fimK*-EIL domain region cloned into pETQ3	This study
pETQ33-AIL$_{fimK}$	Km^r; *fimK*-AIL domain region cloned into pETQ33	This study
pETQ33-*mrkJ*	Km^r; *mrkJ* coding region cloned into pETQ33	This study
pRK415-*fimK*	Tc^r; *fimK* gene cloned into pRK415	This study
pRK415-*fimK*$_{E245A}$	Tc^r; *fimK* gene cloned into pRK415	This study
pRK415-HTH$_{fimK}$	Tc^r; *fimK* N-terminal HTH domain cloned into pRK415	This study
pRK415-EIL$_{fimK}$	Tc^r; EIL$_{fimK}$ coding region cloned into pRK415	This study
pBAD33-*mrkJ*	Cm^r; *mrkJ* coding region cloned into pBAD33	This study
pBAD33-EIL$_{fimK}$	Cm^r; *fimK*-EIL domain region cloned into pBAD33	This study
pBAD33-AIL$_{fimK}$	Cm^r; *fimK*-AIL domain region cloned into pBAD33	This study
pBAD33-*ydeH*	Cm^r; *ydeH* coding region cloned into pBAD33	This study

*Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance.

Table 2. Oligonucleotide primers used in this study

Purpose	Primer name	Sequence (5′→3′)
Gene deletions		
mrkA	SL0141	CGAGCTCAGCGTGATGTCTATCCAG
	SL0142	CGCGGATCCCGAATCAATGAGCACACT
	SL0143	CGCGGATCCACAATAATAAAGCGGCAAT
	SL0158	TGCTCTAGAGACTGCCGACAATAAAGC
fimK	K1	TCGCTTCCCGCTGCAGGCC
	K2	CTTCGCGGCGTTCAGCATC
	K3	AGATCTTCGTATTCGCGGGTG
	K4	CATGATCTGCGCGTCGAGG
EIL_{fimK}	WCC32	AGGCTACTCGACGAGGCCTTGC
	WCC33	GGATCCTTAAAACGCCGTCAGTGC
	WCC34	GGATCCGGCGGATCGTTGAGG
	WCC35	GGTACCCGGATGAAGTGGATGTCG
HTH_{fimK}	WCC127	ACCTCTAGACAGGCGGTGATTAACGTCACCTATAC
	WCC128	ATTAAGCTTCGAGCAGGGCGAGAGGATATAAT
	WCC133	ATTAAGCTTAACGCACTGACGGCGTTTGAA
	WCC130	ATTGAGCTCAAGATTATCCCTCTCTGCCCG
Gene expression		
$fimK/fimK_{E245A}$	WCC71	GATGACCGATTATATCCTCTCGCCC
	WCC74	GAATTCAACGATCCGCCGGATCG
	WCC81	ACCTCTAGAAGGAGATCAATGATGACCGATTATATCCT
	WCC84	ATTAAGCTTAACGATCCGCCGGATCGC
HTH_{fimK}	WCC72	GAATTCTCATTCAAACGCCGTCAGTGCGTT
	WCC82	ACCAAGCTTATTCCTGTTCAAACGCCGT
EIL_{fimK}/AIL_{fimK}	WCC73	GATGGCCCCCCGCTTTCC
	WCC83	ATTTCTAGAAGGAGGTGGAATGCATCCGCAT
ydeH	WCC75	GATGATCAAGAAGACAACGGAAATTGATG
	WCC76	GAATTCTTAAACTCGGTTAATCACATTTTGTTCGTC
	WCC79	ACCTCTAGAGTGAAAAAGGAGTGGCAATG
	WCC80	ACCAAGCTTTGAATGTTAAACGGAGCTTA
mrkJ	WCC77	GATGAACACTAAAATATTCGAAGACAACATTTTATCTC
	WCC78	GAATTCTTACATGGCAATATCATCGGCGAC
	WCC85	ACCTCTAGAAGGAGGGATAATGAACACTAAAATATT
	WCC86	ACCAAGCTTACATGGCAATATCATCGG
fimB	B1	GGATCCGAAAACCAAAAATATGC
	B2	CCTTGGATCAGCGACGATCGC
Site-directed mutation		
$fimK_{E245A}$	M1	CTATCGTCGACAGTCGCTCACAGCTACAGGGGGTGGCGATCCTG
	M2	CAGGATCGCCACCCCCTGTAGCTGTGAGCGACTGTCGACGATAG
EMSA		
	WCC89	GCCATATTTCCGCAAAAAAAAAT
	WCC90	GATCCGTCGCCAACGCC
	WCC91	CCACATAATACCAAGTTGACACAAAATA
	WCC93	biotin-TTGGGGCCAAACTGTTTATATCAT
	WCC94	TTGGGGCCAAACTGTTTATATCAT
	WCC95	biotin-TTGGGGCCATTTTGACTCG
	WCC96	TTGGGGCCATTTTGACTCG
	WCC98	GCCGGTGCAGCAGAGATATG
	WCC99	TTTTAAATTCTGCCAAATTTGGTTTT
	WCC121	biotin-AAGGAAAAGCGATGGCGTTGGCG
	WCC122	AAGGAAAAGCGATGGCGTTGGCG
	WCC123	AATGTTTTGACATATTTTGCAACTCACTGCGC
qRT-PCR		
fimA	fimA-F	GAACGATGTCGAAATAACGAACCGG
	fimA-R	AACAATGACCTGACGCAGGCTCG

Table 2. cont.

Purpose	Primer name	Sequence (5′→3′)
23S rRNA	23S-F	AGCGACTAAGCGTACACGGTGG
	23S-R	GATGTTTCAGTTTCAGTTCCCCCGGTTC
Promoter activity assay		
P_{fimA}-lacZ	pfimA3	TTGGATCCATTTTGACTCGTTG
	pfimA4	GGGGCCAAACTGTTTAGATCTT
P_{fimB}-lacZ	pfimB4	TGTCGGCGGGATTCCTCATGG
	pfimB5	CAAGATCTTGAGCATACCACAGC
P_{fimE}-lacZ	pfimE1	ACGGATGGCGTTGTATCGCG
	pfimE2	CCTGAACTTCTTTGGCGGTTAGAAA

structure may induce C-terminal PDE activity to decrease the intracellular c-di-GMP level.

METHODS

Bacterial strains, plasmid, primer and growth conditions. Table 1 lists 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 (10^8 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 His_6-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 His_6-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*, EIL_{fimK} and HTH_{fimK} 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/CC128 and WC133/WCC130, respectively. The amplified DNA fragments were individually cloned into suicide vector pKAS46 (Skorupski & Taylor, 1996). The resulting plasmid was transformed into *E. coli* S17-1λ*pir* 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 supplemented with kanamycin and propagated in 2 ml LB broth 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.

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 I 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 H_2O 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 $2^{-\Delta\Delta C_T}$ 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 rewashed, 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 (10^9 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

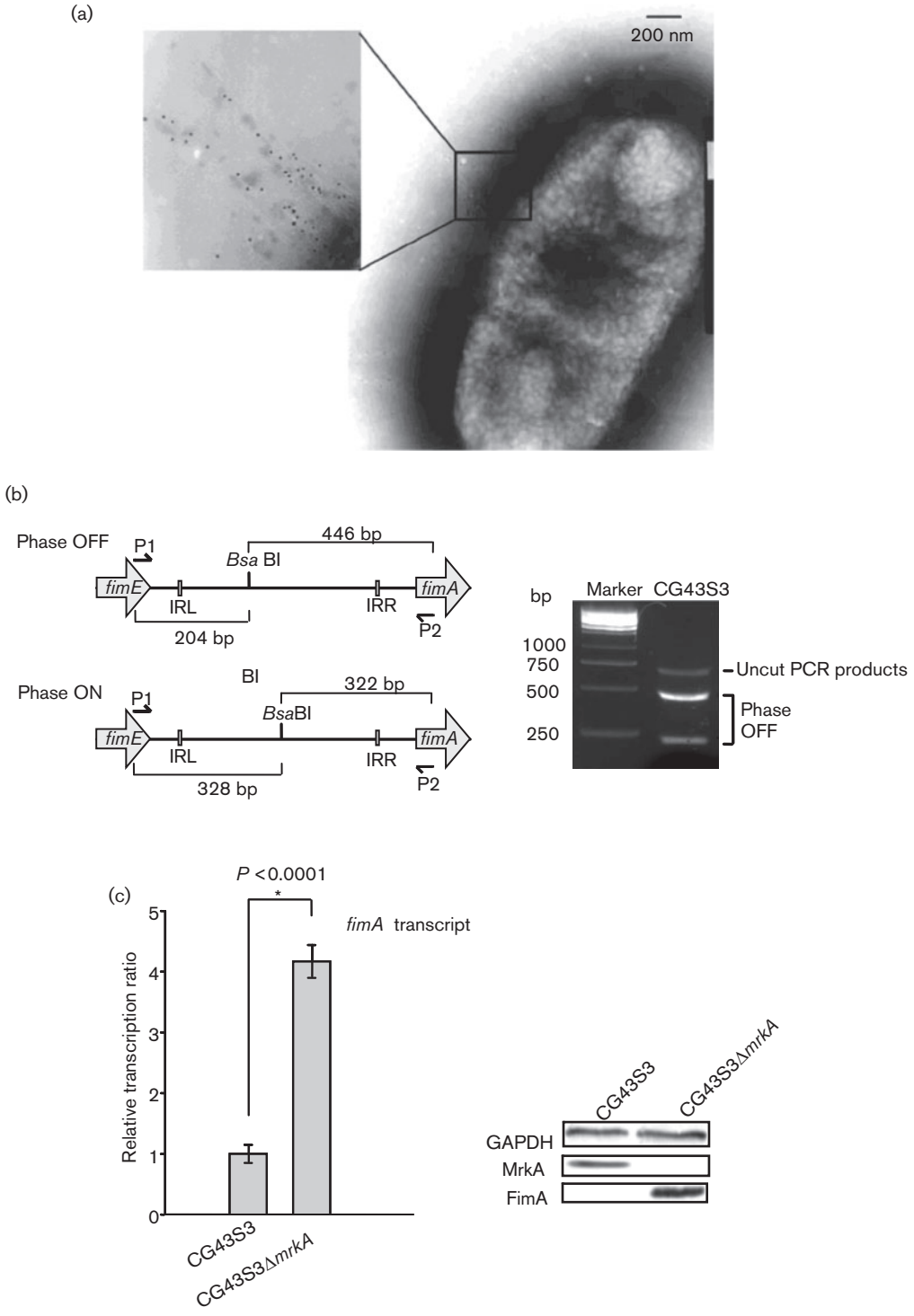

Fig. 1. Type 1 and type 3 fimbriae expression in *K. pneumoniae* CG43S3. (a) Transmission electron micrograph of *K. pneumoniae* CG43S3 labelled with anti-MrkA antibodies. (b) Diagrammatic representation of *fimS* promoter analysis (left). The primer pair P1 and P2 was used for amplifying the region 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.

distilled water. The stained biomass was dissolved in 1 % (w/v) SDS, and the OD_{595} was determined to assess biofilm-forming activity.

Plasmid construction. The coding region of *fimB*, *fimK*, 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 CG43S3 genome. The amplified DNA was individually cloned into cloning vector yT&A (Yeastern 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-HTH_{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-HTH_{fimK} and pRK415-EIL_{fimK} and overexpression clones pETQ33-*fimB*, pETQ31-EIL_{fimK}, pETQ31-AIL_{fimK}, pQE31-*fimK*, pETQ33-EIL_{fimK}, pETQ33-AIL_{fimK}, pETQ33-*mrkJ* 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_{fimA}-lacZ (locked on), P_{fimB}-lacZ and P_{fimE}-lacZ. The promoter–reporter plasmids were individually mobilized into *K. pneumoniae* CG43S3 strains by conjugation from *E. coli* S17-1λ*pir*. The bacteria were grown to the exponential phase in LB broth (OD_{600} of 0.7), and 100 µl of the culture was mixed with 900 µl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 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 Na_2CO_3 and the absorbance at OD_{420} 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* NovaBlue BL21(DE3), and recombinant His_6-MrkJ was induced with 0.5 mM IPTG for 5 h at 37 °C. The recombinant protein His_6-FimK 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/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 nM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM DTT,

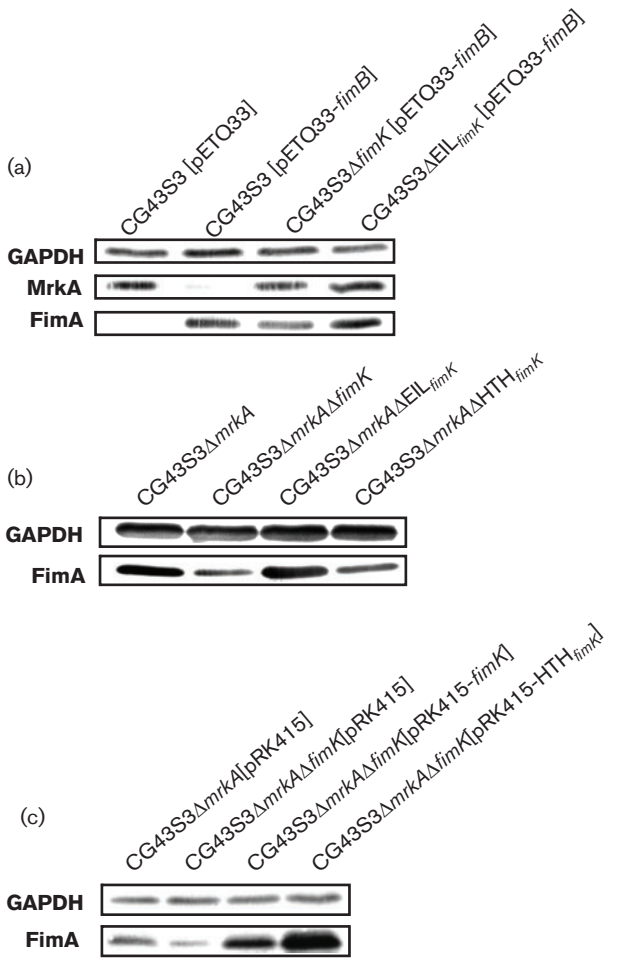

Fig. 2. Western blot analysis of *fimK* deletion effects on type 1 fimbriae expression. Expression of the type 1 fimbriae major pilin FimA was determined in (a) FimB recombinase overexpression bacteria CG43S3[pETQ33-*fimB*], and the derived strains CG43S3Δ*fimK*[pETQ33-*fimB*] and CG43S3ΔEIL_{fimK}[pETQ33-*fimB*] and (b) CG43S3Δ*mrkA*, CG43S3Δ*mrkA*Δ*fimK*, CG43S3Δ*mrkA*ΔEIL_{fimK} and CG43S3Δ*mrkA*ΔHTH_{fimK}. (c) Analysis of FimK complementation by comparing FimA production of CG43S3Δ*mrkA*[pRK415], CG43S3Δ*mrkA*Δ*fimK*[pRK415], CG43S3Δ*mrkA*Δ*fimK*[pRK415-*fimK*] and CG43S3Δ*mrkA*Δ*fimK*[pRK415-HTH_{fimK}]. 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.

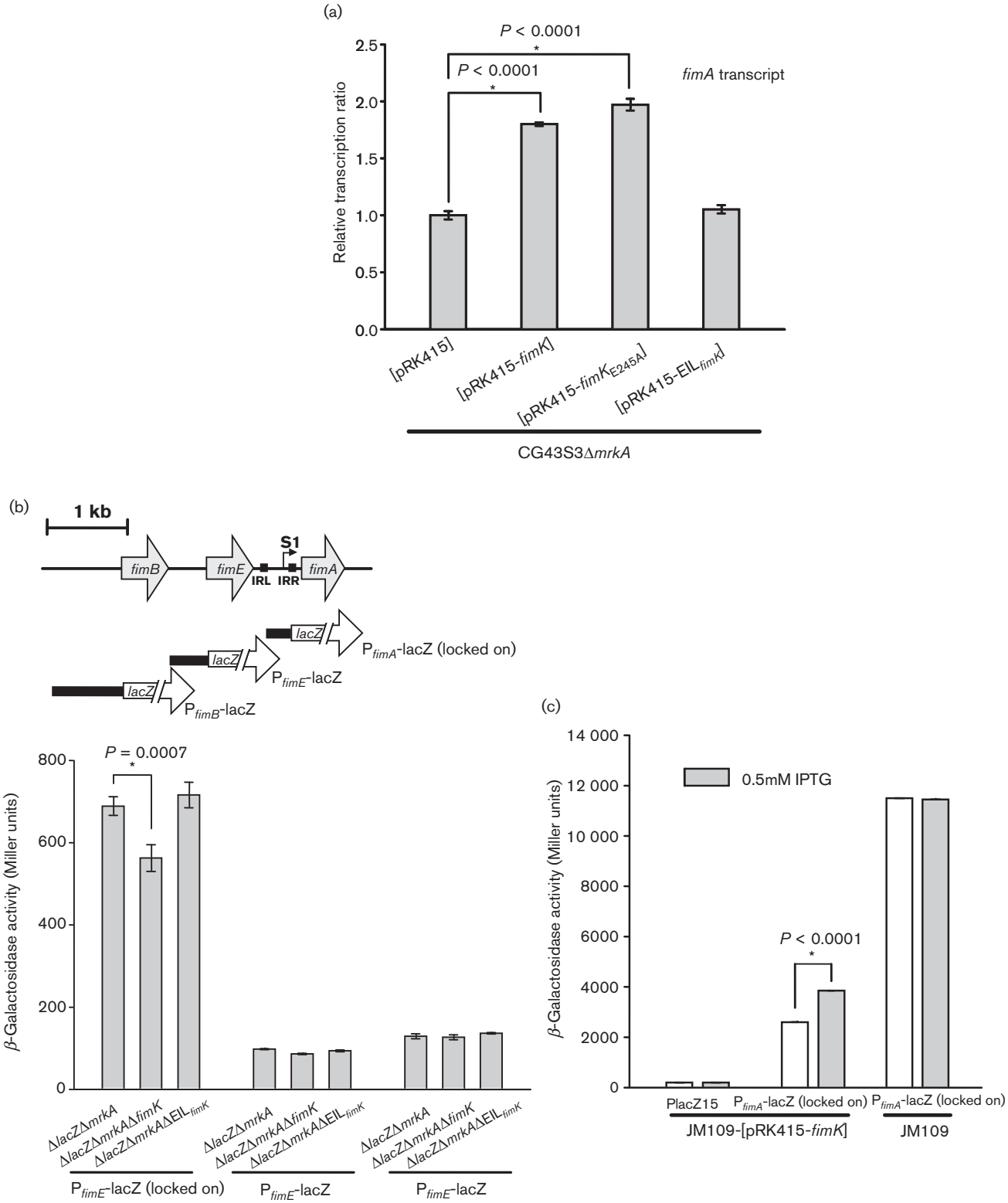

Fig. 3. FimK positively affects type 1 fimbriae transcript levels. (a) qRT-PCR analysis of the type 1 fimbriae (*fimA*) transcription. *K. pneumoniae* CG43S3Δ*mrkA* carrying pRK415 or the derivative plasmids was grown in LB broth supplemented with 0.01 mM IPTG at 37 °C for 20 h with agitation. RNA extraction 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 *fimS* and the transcription start site. Lower panel: the promoter activity of *fimA*, *fimB* and *fimE* as, respectively, assessed by expression of the β-galactosidase reporter plasmid P_{fimA}-lacZ (locked on), P_{fimB}-lacZ and P_{fimE}-lacZ, in *K. pneumoniae* CG43S3Δ*lacZ*Δ*mrkA* and its isogenic derivatives Δ*lacZ*Δ*mrkA*Δ*fimK* and Δ*lacZ*Δ*mrkA*ΔEIL_{fimK} were determined. (c) Two-plasmid heterologous expression analysis in *E. coli* JM109 for the regulation of FimK on the *fimA* promoter activity. Expression of *fimA* assessed via β-galactosidase activity of the strains JM109[P_{fimA}-*lacZ*][pRK415-*fimK*] and JM109[Plac*Z*15][pRK415-*fimK*], and also the control strain JM109[P_{fimA}-lacZ] with or without IPTG addition into the cultures. The bacteria were grown in LB broth at 37 °C and FimK expression was induced with 0.5 mM IPTG for 5 h. Data represent an average of three independent experiments.

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 *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-pNPP). 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 EIL_{fimK}, AIL_{fimK}, *mrkJ* and *ydeH* were amplified from pyT-*fimK*, pyT-$fimK_{E245A}$, pyT-*mrkJ* and 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 *E. coli* JM109. Swimming activity analysis for the recombinant bacteria was then conducted as described by Wood *et al.* (2006). Four microlitres of the bacteria grown overnight carrying pBAD33-EIL_{fimK}, pBAD33-AIL_{fimK}, pBAD33-*mrkJ* or pBAD33-*ydeH* was spotted onto a tryptone swimming plate (0.25 % Bacto agar, 0.25 % NaCl, 1 % tryptone, 35 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 *K. pneumoniae* CG43S3. Expression plasmids pETQ33, pETQ33-EIL_{FimK}, pETQ33-AIL_{FimK} or pETQ33-*ydeH* were individually transformed into *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 EIAab Science).

RESULTS

Inverse expression of type 1 and 3 fimbriae in *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 *K. pneumonaie* CG43S3 (Fig. 1a). Expression of the *fim* operon could be assessed by the restriction pattern of the *fimS* DNA amplified from the bacterial culture. Fig. 1(b) shows some incomplete digested amplicons and the *Bsa*BI restriction fragments of approximately 450 and 200 bp, corresponding to the phase 'OFF' *fimS* in 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 *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 *mrkA* gene increased type 1 fimbriae expression. The inverse expression pattern between the two types of fimbriae was further observed by introducing pETQ33-*fimB*, which overexpresses the FimB recombinase, into *K. pneumoniae* CG43S3. Fig. 2(a) shows that overexpression of the FimB recombinase turns on expression of FimA but depletes MrkA production.

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

As shown in Fig. 2(a), deleting *fimK* from CG43S3[pETQ33-*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 CG43S3[pETQ33-*fimB*] 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 were individually deleted in an in-frame fashion from CG43S3Δ*mrkA*. Compared with CG43S3Δ*mrkA*, CG-43S3Δ*mrkA*Δ*fimK* and CG43S3Δ*mrkA*ΔHTH_{fimK} reduced FimA production slightly while CG43S3Δ*mrkA*ΔEIL_{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-*fimK* or pRK415-HTH_{fimK}, which expressed an intact FimK or the FimK DNA-binding domain, into CG43S3Δ*mrkA*Δ*fimK* increased FimA expression levels. The FimK-dependent type 1 fimbriae activities were also observed using mannose-sensitive yeast agglutination assay. As shown in Fig. S2(a, b), deleting *fimK* from CG43S3[pETQ33-*fimB*] or CG43S3-Δ*mrkA* had no dramatic change on the mannose-sensitive agglutination activity. However, the complementation analysis clearly revealed an FimK- or HTH domain-dependent mannose-sensitive agglutination activity (Fig. S2c). These results suggest that FimK positively affects the type 1 fimbriae expression possibly through its N-terminal DNA-binding domain.

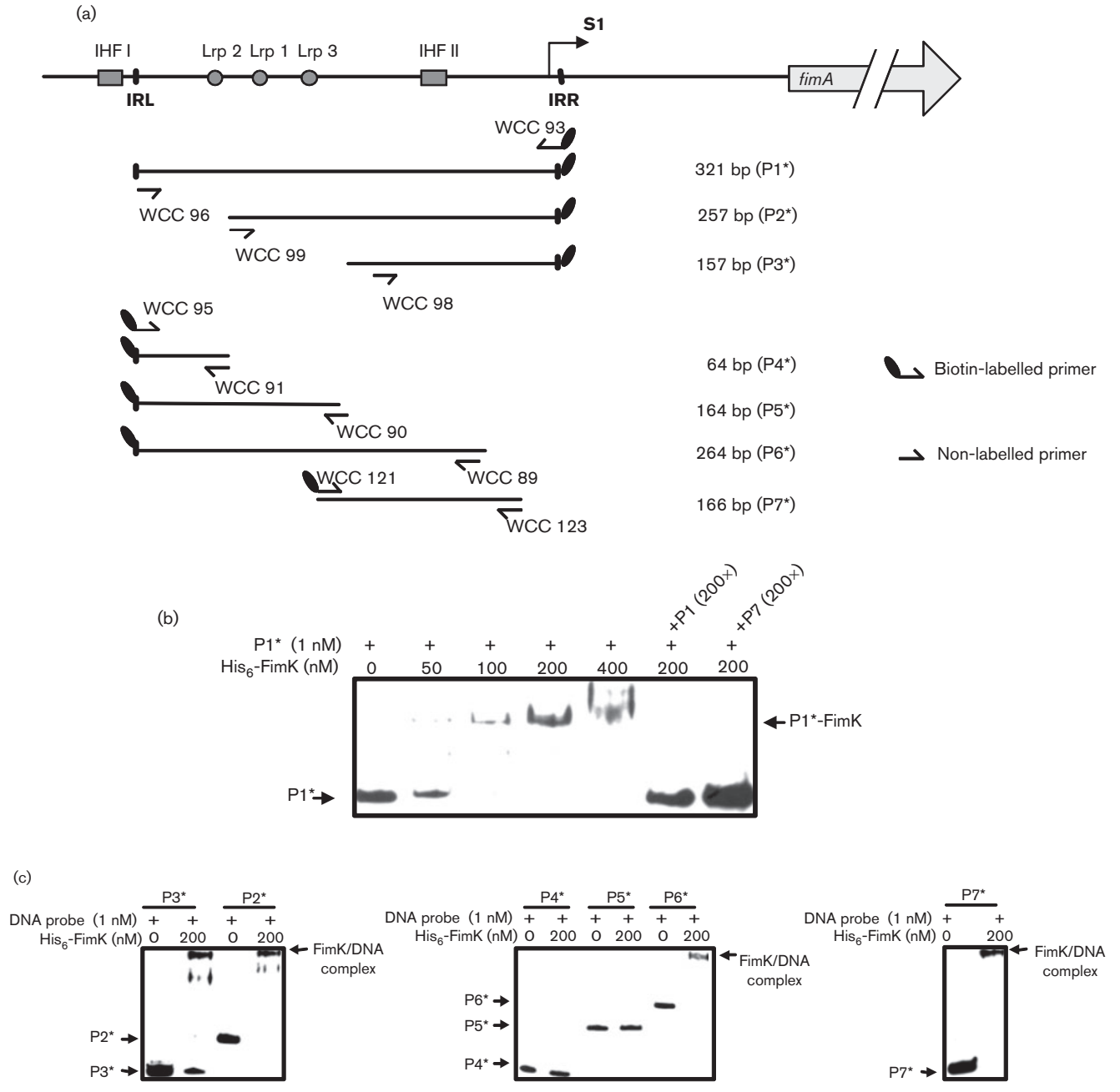

Fig. 4. EMSA of the interaction between His_6-FimK and *fimS*. (a) Schematic diagram of the DNA probes for the analysis. The relative positions of the primer sets used in PCR amplification of the DNA probes are indicated. Names and sizes of the biotin-labelled DNA probes are shown on the right. S1, the 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_6-FimK and the putative promoter DNA. The biotin-labelled P_{fimA} (P1*) 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*, P3*, P4*, P5*, P6* and P7* were applied for the specific binding region.

The N-terminal domain of FimK positively influences P_{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-$fimK_{E245A}$ into CG43S3Δ*mrkA* increased the *fimA* transcripts to approximately twice the value of CG43S3Δ*mrkA*[pRK415] or CG43S3Δ*mrkA*[pRK415-EIL_{fimK}]. This suggests that the *fimA* transcript-level change might not be directly influenced

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 CG43S3Δ*fimK* 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 CG43S3Δ*mrkA* 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 CG43S3Δ*mrkA*ΔEIL$_{fimK}$, 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 His_6-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 His_6-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 AIL$_{fimK}$ 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

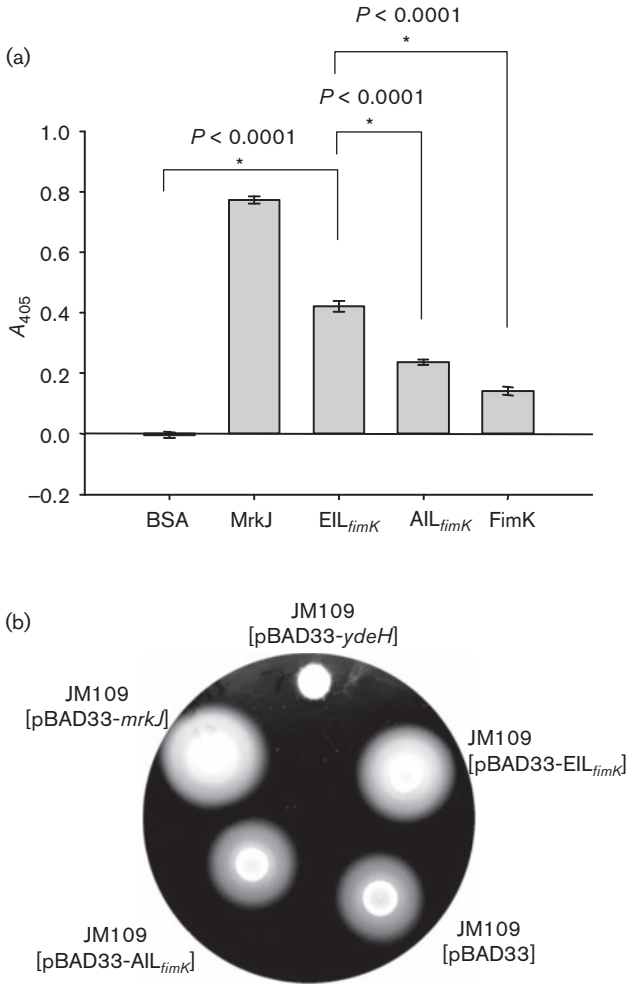

Fig. 5. The recombinant FimK protein exerts PDE activity. (a) The recombinant MrkJ, the EIL domain and AIL domain of FimK, and FimK were analysed for PDE activity. BSA was used as a negative control for activity determination. Approximately 300 nM of each of the purified proteins was incubated with the substrate bis-pNPP at 37 °C for 3 h. Release of *p*-nitrophenol was determined at 405 nm. Results shown are representative of three independent experiments. Error bars represent SD. (b) Motility assay for the recombinant *E. coli* JM109[pBAD33], JM109[pBAD33-*mrkJ*], JM109[pBAD33-EIL$_{fimK}$], JM109[pBAD33-AIL$_{fimK}$] and JM109 [pBAD33-*ydeH*].

EIL$_{fimK}$ exhibited a higher level of PDE activity than the recombinant FimK and AIL$_{fimK}$. 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-AIL_{fimK} 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-AIL_{fimK}]. 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-AIL_{fimK}] and CG43S3[pETQ33-*mrkJ*] 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-AIL_{fimK}] 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 σ^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 CG43S3Δ*mrkA*ΔEIL_{fimK}, suggesting that FimK regulates *fim* expression through the N-terminal region. Complementation analysis confirms that the HTH_{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 recombinase.

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 AIL_{fimK} expressed PDE activity, and only AIL_{fimK} 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 P_{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).

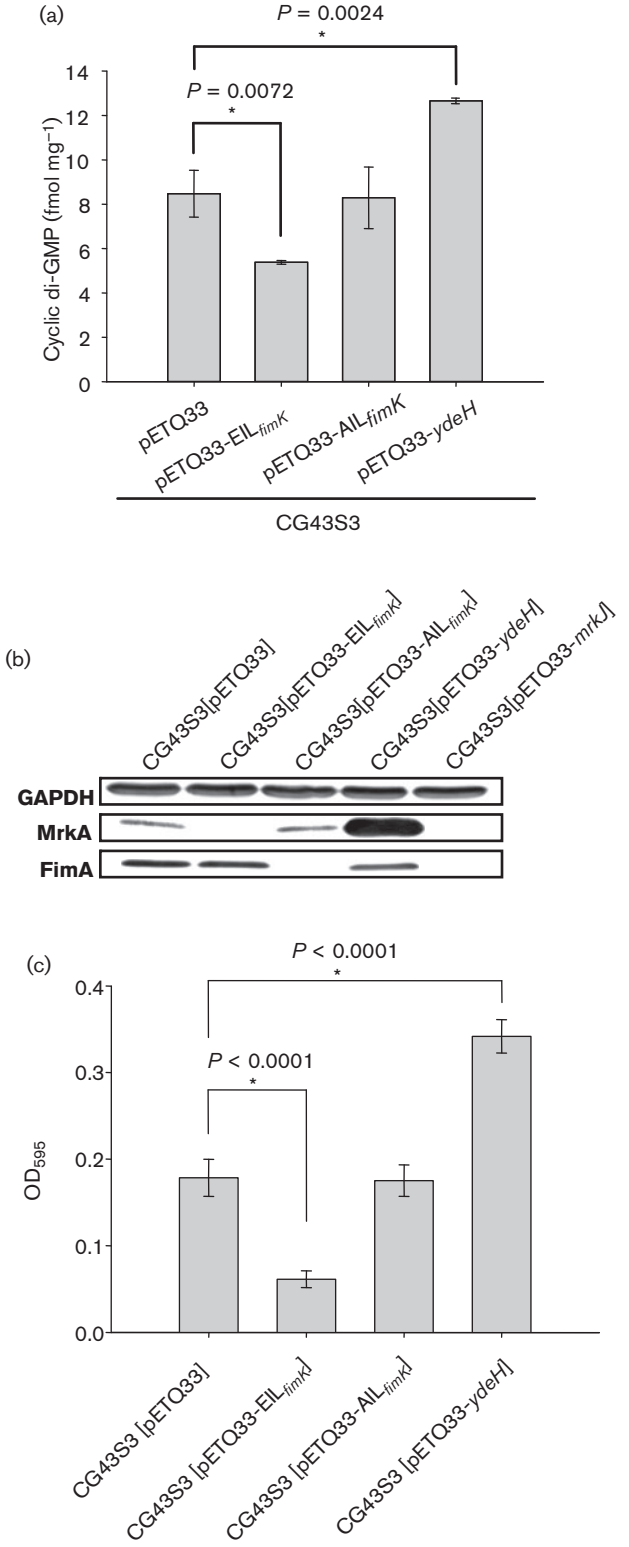

Fig. 6. c-di-GMP effects on the expression of type 1 fimbriae. (a) Quantification of c-di-GMP using ELISA according to the manual (Wuhan ElAab Science). Measurement of c-di-GMP was performed in three independent experiments. Error bars shown are SD, and asterisks indicate statistically significant differences. (b) Western blot analysis of FimA and MrkA production in *K. pneumoniae* CG43S3[pETQ33], CG43S3[pETQ33-EIL$_{fimK}$], CG43S3[pETQ33-AIL$_{fimK}$], CG43S3[pETQ33-*ydeH*] and CG43S3[pETQ33-*mrkJ*]. Bacteria carrying pETQ33 and other derivative plasmids were grown to mid-exponential phase and then supplemented with 0.5 mM IPTG, and the culture continued for 4 h at 37 °C. Thereafter, total proteins were isolated and resolved by SDS-PAGE. The proteins were transferred to PVDF membrane and hybridized with anti-FimA, anti-MrkA or anti-GAPDH. (c) 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.

ACKNOWLEDGEMENTS

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