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Fur-dependent MrkHI regulation of type 3 fimbriae in *Klebsiella pneumoniae* CG43

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Type 3 fimbriae play a crucial role in *Klebsiella pneumoniae* biofilm formation, but the mechanism of the regulation of the type 3 fimbrial operon is largely unknown. In *K. pneumoniae* CG43, three regulatory genes, *mrkH*, *mrkI* and *mrkJ*, are located downstream of the type 3 fimbrial genes *mrkABCDF*. The production of the major pilin MrkA is abolished by the deletion of *mrkH* or *mrkI* but slightly increased by the deletion of *mrkJ*. Additionally, quantitative RT-PCR and a promoter–reporter assay of *mrkHI* verified that the transcription of *mrkHI* was activated by MrkI, suggesting autoactivation of *mrkHI* transcription. In addition, sequence analysis of the *mrkH* promoter region revealed a putative ferric uptake regulator (Fur) box. Deletion of *fur* decreased the transcription of *mrkH*, *mrkI* and *mrkA*. The expression of type 3 fimbriae and bacterial biofilm formation were also reduced by the deletion of *fur*. Moreover, a recombinant Fur was found to be able to bind both promoters, with higher affinity for P_{mrkH} than P_{mrkA}, implying that Fur controls type 3 fimbriae expression via MrkHI. We also proved that iron availability can influence type 3 fimbriae activity.

INTRODUCTION

Klebsiella pneumoniae, an important nosocomial pathogen, causes a wide range of opportunistic infections, including pneumonia, bacteraemia, urinary tract infection and life-threatening septic shock (Keynan & Rubinstein, 2007; Podschun & Ullmann, 1998). The emergence of an invasive form of community-acquired *K. pneumoniae* infection, which presents as primary bacteraemic liver abscesses, endophthalmitis and meningitis, has been reported in Asia, especially Taiwan (Tsai *et al.*, 2008). Multiple-drug-resistant *K. pneumoniae*, including extended-spectrum β-lactamase- and carbapenemase-producing strains, have been increasingly observed (Hirsch & Tam, 2010; Nordmann *et al.*, 2009). Studies of the pathogenic mechanisms of this organism are therefore necessary to identify novel targets for antibacterial agents.

The reported virulence factors of *K. pneumoniae* include capsular polysaccharide (CPS), lipopolysaccharide, iron-acquisition systems and fimbriae (Brisse *et al.*, 2009; Podschun & Ullmann, 1998). Fimbriae (or pili) help bacterial pathogens adhere to host cells for successful infection. In *K. pneumoniae*, type 1 and type 3 fimbriae are well characterized in the chaperone–usher assembly class (Fronzes *et al.*, 2008). Type 1 fimbriae, found in virtually all members of the family *Enterobacteriaceae*, can bind to mannose-containing structures on host cells or the extracellular matrix (Hanson *et al.*, 1988; Klemm & Schembri, 2000). They have also been shown to play a critical role in urinary tract infection by mediating adhesion to the uroepithelium (Rosen *et al.*, 2008; Struve *et al.*, 2008). Type 3 fimbriae, originally characterized in *Klebsiella* strains, can cause mannose-resistant agglutination of tannic acid-treated human erythrocytes. The adhesion filaments encoded by the *mrkABCDF* operon (Huang *et al.*, 2009) can reportedly adhere to epithelial cells from the respiratory and urinary tracts as well as to the extracellular matrix proteins collagen IV and V (Hornick *et al.*, 1992; Jagnow & Clegg, 2003; Tarkkanen *et al.*, 1997). The role of type 3 fimbriae in biofilm formation of *K. pneumoniae* has also been repeatedly demonstrated (Di Martino *et al.*, 2003; Jagnow & Clegg, 2003; Schroll *et al.*, 2010; Struve *et al.*, 2009).

The analysis of available genome sequences of *K. pneumoniae* has revealed three regulatory genes (*mrkH*, *mrkI* and *mrkJ*) downstream of the type 3 fimbrial genes (Johnson & Clegg, 2010). MrkH, a PilZ domain protein, can bind to the

Abbreviations: c-di-GMP, bis-(3′-5′)-cyclic dimeric GMP; CPS, capsular polysaccharide; dipyridyl, 2,2′-dipyridyl; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDE, phosphodiesterase; qRT-PCR, quantitative RT-PCR; 5′-RACE, 5′-rapid amplification of cDNA ends.

Three supplementary figures and a supplementary table are available with the online version of this paper.

second messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) (Johnson *et al.*, 2011), and MrkI is predicted to be a LuxR-type transcriptional factor (Johnson & Clegg, 2010; Johnson *et al.*, 2011). Recent reports have shown that MrkH and MrkI positively regulate the expression of type 3 fimbriae (Johnson *et al.*, 2011), whereas MrkJ, a c-di-GMP phosphodiesterase (PDE), reduces that expression (Johnson & Clegg, 2010).

In many bacteria, Fur generally regulates genes responsible for the uptake and metabolism of iron. Not only is Fur involved in regulating iron homeostasis but it also participates in bacterial colonization, the oxidative stress response, toxin secretion and virulence (Carpenter *et al.*, 2009). Under iron-replete conditions, Fur binds to iron and the Fe^{2+}–Fur dimers bind to a 19 bp consensus DNA sequence, the Fur box (GATAATGATwATCATTATC; w=A or T), in target promoters (Baichoo & Helmann, 2002; Escolar *et al.*, 1999). The binding of Fur at the promoters impedes the binding of RNA polymerase, thereby preventing transcription from these genes. In some cases, however, Fur functions as an activator that may even regulate certain genes in the absence of iron (Carpenter *et al.*, 2009).

Using *K. pneumoniae* CG43, a heavy encapsulated K2 isolate (Chang *et al.*, 1996), we investigated the regulatory role of MrkH, MrkI and MrkJ by generating specific gene-deletion mutants and analysing their effects on type 3 fimbriae expression. We also studied whether Fur and extracellular iron level influence the expression of type 3 fimbriae.

METHODS

Plasmids, bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *K. pneumoniae* CG43 (Chang *et al.*, 1996) and its derivatives were propagated at 37 °C in Luria–Bertani (LB) broth or M9 minimal medium. The antibiotics used included ampicillin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), tetracycline (12.5 μg ml^{-1}) and streptomycin (500 μg ml^{-1}). Primers used in this study are listed in Table 2.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
***K. pneumoniae* strains**		
CG43S3	CG43 Sm^r	Lai *et al.* (2001)
Δ*mrkH*	CG43S3 Δ*mrkH*	This study
Δ*mrkI*	CG43S3 Δ*mrkI*	This study
Δ*mrkJ*	CG43S3 Δ*mrkJ*	This study
Δ*lacZ*	CG43S3 Δ*lacZ*	Lin *et al.* (2006)
Δ*lacZ*Δ*mrkH*	CG43S3 Δ*lacZ* Δ*mrkH*	This study
Δ*lacZ*Δ*mrkI*	CG43S3 Δ*lacZ* Δ*mrkI*	This study
Δ*mrkA*	CG43S3 Δ*mrkA*	This study
CCW40	Chromosomal integrated pWY28 in CG43S3 Δ*mrkI*, control strain	This study
CCW41	Chromosomal integrated pWY45 in CG43S3 Δ*mrkI*, *mrkI*-complemented strain	This study
***E. coli* strains**		
JM109	F′ (*traD36*, *proAB*$^+$ *lacI*^{q}, Δ(*lacZ*)M15) *endA1 recA1 hsdR17*(r_k^- m_k^+) *mcrA* *supE44* λ^- *gyrA96 relA1* Δ(*lacproAB*)*thi-1*	Laboratory stock
BL21(DE3)	F^- *ompT* $hsdS_B$(r_B^- m_B^+)*gal dcm trxB15*::*kan* BL21(DE3)	Novagen
S17-1 λ *pir*	*hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn*7*) (λ*pir*)	Skorupski & Taylor (1996)
Plasmids		
pKAS46	Ap^r Km^r, positive selection suicide vector, *rpsL*	Skorupski & Taylor (1996)
yT&A	Ap^r, TA cloning vector	Yeastern
pWY05	Ap^r, 2.5 kb fragment containing *mrkI* and its flanking regions cloned into yT&A	This study
pWY45	Ap^r Km^r, 2.5 kb fragment containing *mrkI* and its flanking regions cloned into pKAS46	This study
pWY28	Ap^r Km^r, 2 kb fragment containing the flanking regions of *mrkI* cloned into pKAS46	This study
pETQ	Km^r, protein expression vector	Keen *et al.* (1988)
pMrkH	Km^r, 711 bp fragment encoding full-length MrkH cloned into pETQ	This study
pMrkH*	Km^r, 711 bp fragment encoding full-length $MrkH_{R113D}$ cloned into pETQ	This study
pPilZ	Km^r, ~480 bp fragment encoding the PilZ domain of MrkH cloned into pETQ	This study
pPilZ*	Km^r, ~480 bp fragment encoding the PilZ domain of $MrkH_{R113D}$ cloned into pETQ	This study
$pMrkH_N$	Km^r, ~330 bp fragment encoding the N-terminal domain of MrkH cloned into pETQ	This study
pRK415	Tc^r, shuttle vector, *mob*$^+$	Lin *et al.* (2006)
pfur	Tc^r, 0.8 kb fragment containing a *fur* allele cloned into pRK415	Cheng *et al.* (2010)
placZ15	Cm^r, promoter selection vector, *lacZ*$^+$	Lin *et al.* (2006)
pmrkA-P2	Cm^r, 402 bp fragment containing the region upstream of *mrkA* cloned into placZ15	This study
pAW175	Cm^r, 407 bp fragment of the upstream region of *mrkH* cloned into placZ15	This study

Table 2. Primers used in this study

Primer	Sequence (5′→3′)
wc07	AGATCCTACAAATGGGGCGTGA
wc08	GGCCTGTTCACCTATTACGTTG
wc09	CTCTTTTTGCGCTTGGCTTCTA
wc10	TTCTCCCGGTAAATCAGTAGCG
GT29	TAAGCAAACTGGGCGTGAA
GT30	TAGCCCTGTTGTTTGCTGGT
GT31	GCGAGAAATCTCCGCTAACTC
GT32	CGTGGTGAAAGCTGTTTGC
GT46	GTTTAAGTTCCGCCATCTCG
GT47	TTGCGCTTGGCTTCTAAGAT
GT54	TTTCGAGGTAACCGAAAACG
GT55	GAGGTATCCTGTGGGCTCTG
RT11	GGGTAGGGGAGCGTTCTGTAA
RT12	TCAGCATTCGCACTTCTGAT
pcc212	GGATCCAAGGGATGCATATGACAGAGGG
pcc213	AAGCTTACTGTCCAAGGTTGTCAGATTCTC
pcc319	GGATCCAGACAAAATGGAGGGAACCCTATC
pcc320	GGATCCTTACTGGTCTTTATCGTTCCCTCTG
pcc323	GGATCCTGCATGCTGTTGCGGTCAC
pcc324	GGATCCGCGGTTGCCATTGCTGCAGAG
pcc331	GACTTTCGTTTACGCCATGAGCATG
pcc332	GGGATCGCGGCGACGCTG
pcc335	GGATCCATGCATGACAATAGCGGTGTCGATAAAGG
pcc336	AAGCTTGCTGCACTACCTGCAGGCATTC
wcc54	GAACTTATATTTTTAGGTTCGTTACCTGACGCC
wcc55	TCCTGTAATCAAGAGTTTCGAATTTTGGTC

Construction of gene-deletion mutants and complementation plasmids. Specific deletions were individually introduced into the chromosome of *K. pneumoniae* CG43S3 using an allelic exchange strategy (Lai *et al.*, 2003). The pKAS46 suicide plasmid was used in the selection of the mutants (Skorupski & Taylor, 1996), and the mutations were confirmed with PCR. The resulting mutant strains are listed in Table 1.

To obtain the *mrkI*-complementation plasmids, the DNA fragment containing *mrkI* and its flanking regions was PCR-amplified from *K. pneumoniae* CG43S3 genomic DNA using primers wc07 and wc08, and the amplicon was cloned into yT&A (Yeastern Biotech) to yield pWY05. The *mrkI* coding region carried on pWY05 was then removed with inverse PCR using primers wc09 and wc10. Subsequently, the DNA fragment containing the *mrkI* region or the *mrkI*-flanking regions only were subcloned into pKAS46 to yield pWY45 and pWY28, respectively. The plasmids were then individually introduced into *K. pneumoniae* CG43S3 Δ*mrkI* via conjugation. The resulting transconjugants, with pWY28 and pWY45 integrated into the chromosome via homologous recombination, were designated CCW40 and CCW41, respectively.

Construction and expression of the recombinant proteins. The DNA fragments containing the coding sequences of MrkH, $PilZ_{MrkH}$ or $MrkH_N$ were individually PCR-amplified with primer pairs pcc212/pcc213, pcc335/pcc213 or pcc212/pcc336 and cloned into the expression vector pETQ to generate pMrkH, pPilZ or $pMrkH_N$. The plasmids with site-directed mutations were constructed using inverse PCR. pMrkH was used as the PCR template to generate mutant allele-encoding $MrkH_{R113D}$ (MrkH*) recombinant proteins using primer pair pcc331/pcc332. The PCR product was resolved on an agarose gel, recovered, treated with *Dpn*I for 2 h, and subjected to T4 polynucleotide kinase treatment and self-ligation. The ligation product was transformed into *E. coli* JM109. The plasmid pMrkH*, carrying the mutant allele encoding $MrkH_{R113D}$, was prepared from the transformant colony and confirmed using sequence analysis. The DNA fragment encoding $PilZ_{MrkH}$ with the R113D mutation (PilZ*) was PCR-amplified with primer pair pcc335/pcc213, using pMrkH* as a template, and then cloned into pETQ to yield pPilZ*.

Western blot analysis. *K. pneumoniae* CG43S3 and its derived mutants were grown in LB broth with agitation at 37 °C. The bacterial cultures were collected by centrifugation and quantified with Bradford protein assays (Bio-Rad). The bacterial total protein, approximately 5 μg per lane, was then subjected to Western blot analysis using anti-MrkA antiserum. MrkA antiserum preparation and Western blot analysis were performed as described previously (Huang *et al.*, 2009). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was probed using specific antiserum (GeneTex) as a loading control for the total lysates.

Biofilm formation assay. The ability of bacteria to form biofilm was analysed as described previously, with a minor modification (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 48 h. Planktonic cells were removed, and the wells were washed once with distilled water to remove unattached cells. Crystal violet (0.1 %, w/v, Sigma) was used for 30 min to stain the attached cells. Unattached dye was removed by washing three times with distilled water, and stained biomass was solubilized in 1 % (w/v) SDS. A_{595} was determined and relative bacterial biofilm-forming activities were calculated.

Measurement of promoter activity. The promoter regions of *mrkA* and *mrkH* were PCR-amplified with primer pairs pcc323/pcc324 and pcc319/pcc320, and the amplicons were then cloned into placZ15 (Lin *et al.*, 2006) to generate pmrkA-P2 and pAW175, respectively. The promoter–reporter plasmids were individually mobilized into *K. pneumoniae* strains by conjugation from *E. coli* S17-1 *λpir*. The bacteria were grown to exponential phase in LB broth (OD_{600} 0.7) and the *β*-galactosidase activity was measured as described elsewhere (Lin *et al.*, 2006).

Quantitative RT-PCR (qRT-PCR). Total RNAs were isolated from early exponential phase *K. pneumoniae* CG43S3 cells using an RNeasy Midi column (Qiagen) according to the manufacturer's instructions. Purified RNA was DNase-treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. RNA (1 μg) was reverse transcribed with a Transcriptor First Strand cDNA Synthesis kit (Roche) using random primers in a reaction mixture of 20 μl. qRT-PCR was performed in a LightCycler 1.5 instrument (Roche). A LightCycler TaqMan Master kit (Roche) was used to detect *mrkA* (primers: GT29 and GT30; probe: 20), *mrkH* (primers: GT46 and GT47; probe: 120), *mrkI* (primers: GT31 and GT32; probe: 160), *mrkJ* (primers: GT54 and GT55; probe: 84) and 23S rRNA (primers: RT11 and RT12; probe: 67). These corresponding probes were selected using Universal Probe Library Assay Design Center (Roche Applied Science). Data were analysed using the software for the LightCycler 1.5 instrument. Relative gene expression was quantified using the comparative threshold cycle $2^{-\Delta\Delta C_T}$ method with 23S rRNA as the endogenous reference.

DNA electrophoretic mobility shift assay (EMSA). *K. pneumoniae* CG43S3 genomic DNA was used as a PCR template to amplify the P_{mrkA} and P_{mrkH} fragments F1, F2, F3 and F4 with primer pairs pcc323/pcc324, wcc54/pcc324, pcc319/pcc320 and pcc319/wcc55, respectively. The primers pcc323, pcc324, pcc319 and pcc320, each labelled with biotin at the 5′ end, were used to amplify the F1* and

F3* fragments. Purification of His_6::Fur and EMSA were performed as described previously (Lin *et al.*, 2011). The biotin-labelled DNA was detected with the LightShift chemiluminescent EMSA kit (Pierce).

Statistical methods. The results of the biofilm-forming and β-galactosidase activity assays were derived from a single experiment that represented three independent experiments. Each sample was assayed in triplicate and the data are presented as means (with SD). Differences between groups were evaluated with a two-tailed Student's *t* test. *P* values less than 0.001 were considered statistically significant.

RESULTS

MrkH and MrkI positively regulate the expression of type 3 fimbriae

As shown in Fig. 1(a), RT-PCR analysis revealed that in addition to the coding region of *mrkH*, *mrkI* and *mrkJ*, the amplicons also include the junction of *mrkH–mrkI* and *mrkI–mrkJ*, indicating that the three genes could be transcribed as a single transcription unit. Nevertheless, the presence of an additional promoter for *mrkJ* expression, located in the 143 bp intergenic region between *mrkI* and *mrkJ*, was also demonstrated (Fig. 1b). This implies that a different control is present for the expression of the c-di-GMP PDE activity of MrkJ, which modulates the c-di-GMP level, thereby differentially influencing type 3 fimbriae activity. This mechanism awaits further investigation.

Specific gene deletions of *mrkH*, *mrkI* and *mrkJ* in *K. pneumoniae* CG43, a highly virulent clinical isolate of the K2 serotype (Chang *et al.*, 1996), were constructed, and the derived mutants were analysed for the expression of type 3 fimbriae. As shown in Fig. 2(a), type 3 fimbriae major pilin MrkA production was abolished in Δ*mrkH* and Δ*mrkI* mutants. Slightly increased MrkA production was observed for Δ*mrkJ* (approximately 1.67-fold), which is consistent with another report (Johnson & Clegg, 2010). Moreover, the deletion effect of *mrkI* could be complemented, as MrkA production was restored in CCW41 carrying chromosomally integrated pKAS46-*mrkI* but not in CCW40, which carries a chromosomally integrated pKAS46 (Fig. 2a).

Sequence analysis revealed that MrkH carries an N-terminal sequence of unknown function and C-terminal PilZ domain. As shown in Fig. 2(b), by comparing this with the PilZ domain of YcgR (Ryjenkov *et al.*, 2006), the two conserved motifs and the critical residue for c-di-GMP binding could be identified. To investigate whether MrkH functions as a PilZ domain protein, the plasmids pMrkH*, carrying an R113D mutation on MrkH to impair c-di-GMP binding ability (Benach *et al.*, 2007; Johnson *et al.*, 2011; Ryjenkov *et al.*, 2006), $pMrkH_N$, carrying N-terminal coding DNA (residues 1–108), pPilZ, carrying the PilZ domain (residues 85–237), or pPilZ* ($PilZ_{MrkH}$ carrying an R113D mutation) were constructed. As shown in Fig. 2(c), the production of MrkA in Δ*mrkH* could also be restored by introducing pMrkH, which suggested a positive regulatory role for MrkH. The complementation effect was not observed after introduction of pMrkH*, $pMrkH_N$, pPilZ or pPilZ* into Δ*mrkH*. This result indicated that the N terminus, the PilZ domain and the R113 residue of MrkH are required for MrkH-mediated control of type 3 fimbriae expression. Nevertheless, the results suggest that MrkH and MrkI also play positive roles in determining type 3 fimbriae expression.

MrkI autoactivates the transcription of *mrkHI*

qRT-PCR analysis and promoter activity measurement were carried out to assess the effects of *mrkH* and *mrkI* deletion on the expression of type 3 fimbriae. As shown in Table 3, the *mrkA* mRNA level decreased approximately three- and eightfold in Δ*mrkH* and Δ*mrkI* strains, respectively, indicating that MrkH and MrkI regulate type 3 fimbriae expression at the transcriptional level. Additionally, the *mrkH* transcript was found to diminish by 11-fold in response to *mrkI* deletion. The *mrkI* deletion effect could be complemented, as the mRNA levels of *mrkA* and *mrkH* in CCW41 increased about 10-fold when compared with those in CCW40, suggesting that MrkI positively regulates the expression of *mrkHI* at the transcriptional level. As shown in Fig. 2(d), promoter activity measurement also revealed a decreased level of P_{mrkA} caused by the deletion of *mrkH* or *mrkI*. Moreover, the promoter activity of *mrkH* was apparently decreased by *mrkI* deletion (Fig. 2d), further supporting the possibility of auto-regulation by MrkI of the transcription of *mrkHI*. Conversely, the transcription of *mrkJ* was apparently unaffected by *mrkH* or *mrkI* deletion (Table 3).

Fur positively regulates the expression of *mrkHI*

Sequence analysis of the putative promoter region of *mrkH* or *mrkA* revealed a 19 bp Fur box (Fig. 3a). Although both Fur boxes show poor fits to the consensus (11/19), Fur-dependent regulation could not be excluded. To investigate this possibility, we performed qRT-PCR and promoter activity analyses. As shown in Table 3, *fur* deletion apparently decreased the mRNA level of *mrkH*, *mrkI* and *mrkA*, and the introduction of a *fur*-carrying plasmid, pfur (Cheng *et al.*, 2010), into Δ*fur* complemented the deletion effects. Reduced promoter activities of *mrkA* and *mrkH* were also observed in the Δ*fur* mutant (Fig. 3b). Moreover, MrkA production was abolished by the deletion of *fur* but could be restored by the introduction of pfur (Fig. 3c). Biofilm formation in Δ*fur* was reduced (by approximately 38 %) compared with that in the wild-type strain, and introduction of pfur into Δ*fur* complemented the deletion effect (Fig. 3d). These results indicate that Fur regulates *mrkA* and *mrkHI* expression and thereafter affects the activity of type 3 fimbriae.

Fur directly regulates the expression of *mrkHI*

EMSA was performed to analyse whether Fur directly affects the expression of *mrkH* or *mrkA* using the recombinant

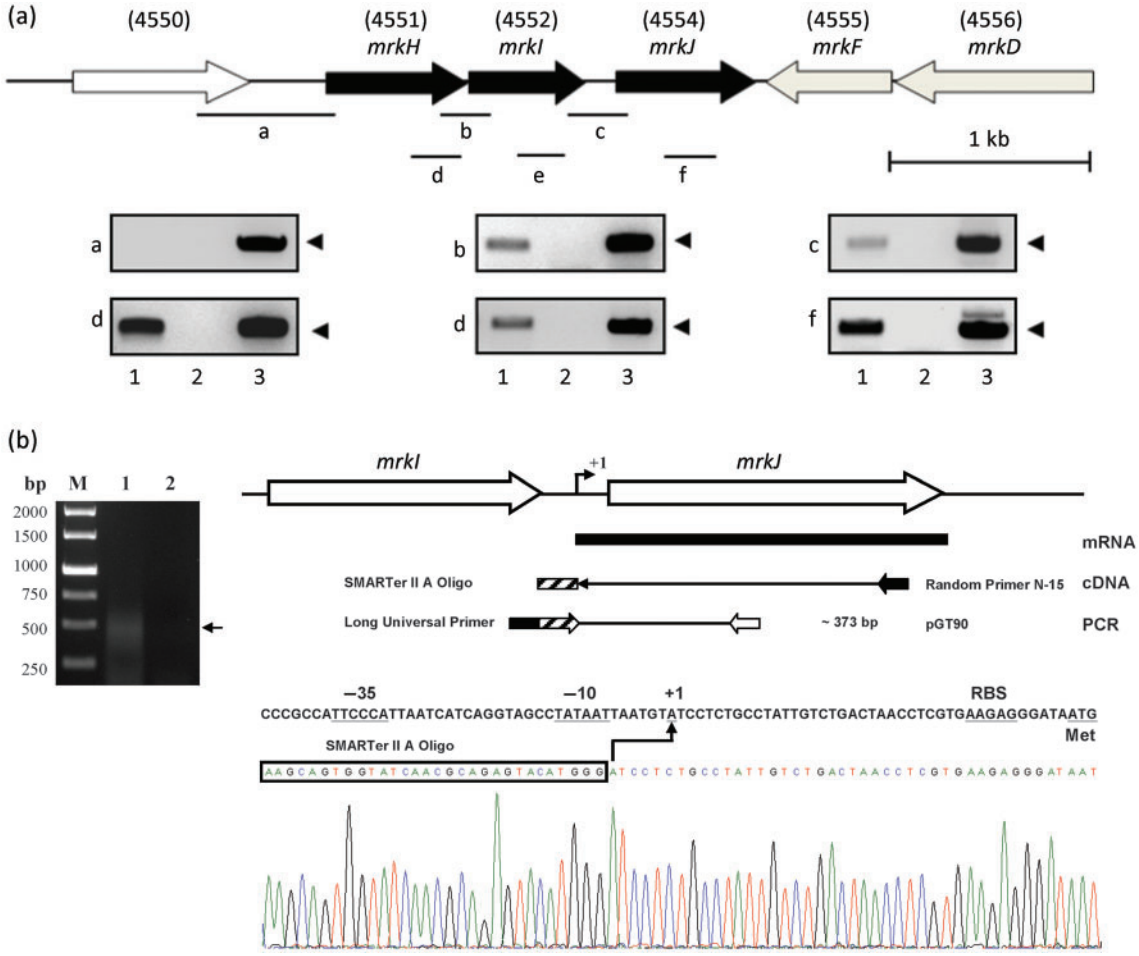

Fig. 1. (a) The transcription units of *mrkH*, *mrkI* and *mrkJ* defined using RT-PCR. The upper part of the panel shows the genetic organization of the downstream genes of the type 3 fimbrial gene cluster (*mrkABCDF*; only *mrkD* and *mrkF* are shown, as grey arrows) in *K. pneumoniae*. The designation and the locus tag (KP1_number) of the ORFs are indicated. The three ORFs encoding putative regulatory proteins are shown in black. The lower part of the panel shows RT-PCR results resolved in an ethidium-bromide-stained agarose gel. Panels a to f show the corresponding PCR products for primers (Supplementary Table S1) located at an ORF or the junction between ORFs. Lanes: 1, RT-PCR products; 2, PCR without reverse transcriptase, as a negative control; 3, PCR with genomic DNA as a template, as a positive control. Arrowheads indicate the expected sizes of RT-PCR products. (b) Identification of the *mrkJ* transcription start site by 5′-rapid amplification of cDNA ends (5′-RACE). Left panel: electrophoresis of 5′-RACE PCR products; M, DNA molecular size markers. The templates used in each PCR include cDNA from *K. pneumoniae* CG43S3 (lane 1) and a reverse-transcription reaction mixture without transcriptase, as a negative control (lane 2). The arrow indicates the expected size of the PCR products. Right panel: schematic representation of the *mrkJ* loci and the 5′-RACE experimental design. The large arrows represent the MrkI and MrkJ ORFs. Relative positions of the primer used and the expected size of the PCR product are indicated. The *mrkJ* transcriptional start site is marked as +1. The potential −10, −35, ribosomal binding and translational start sites are underlined. The pGT90 sequence is 5′-CAGGTCAGGGCGG-CGATTTTGGCTTTTAT-3′.

His_6::Fur protein and different DNA containing the putative promoter of *mrkA* and *mrkH* (Fig. 4a). As shown in Fig. 4(b, c), DNA–protein binding complexes were observed after the incubation of 250 nM purified His_6::Fur with 170 pM biotin-labelled P_{mrkA} (F1*) or P_{mrkH} (F3*). Both His_6::Fur-F1* and His_6::Fur-F3* bindings appeared to be specific, since their binding could only be competed by non-biotin-labelled F1 or F3 but not by a 200× concentration of F2 or F4 DNA that had the Fur box removed (Fig. 4a). Interestingly, F3, which contains a putative Fur-binding sequence, could readily outcompete the binding of His_6::Fur-F1* (Fig. 4b). By contrast, His_6::Fur-F3* binding could not be inhibited by the addition of a 50× concentration of F1 (Fig. 4c). This outcome indicated that Fur binds to the promoter of *mrkHI* with higher affinity than to the *mrkA* promoter.

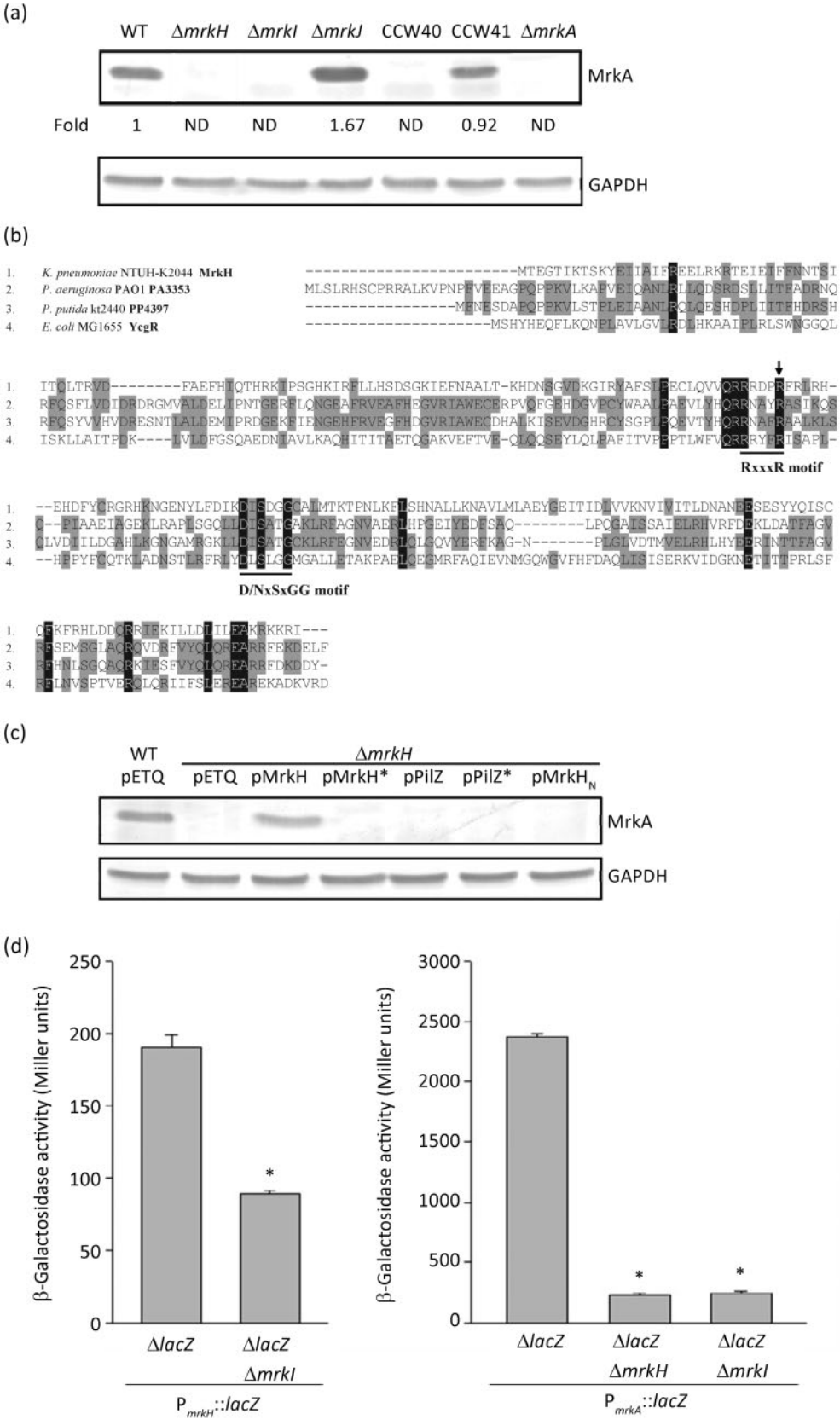
(a)
WT
ΔmrkH
ΔmrkI
ΔmrkJ
CCW40
CCW41
ΔmrkA
MrkA
Fold
1
ND
ND
1.67
ND
0.92
ND
GAPDH
(b)
1. K. pneumoniae NTUH-K2044 MrkH
2. P. aeruginosa PAO1 PA3353
3. P. putida kt2440 PP4397
4. E. coli MG1655 YcgR
RxxxR motif
D/NxSxGG motif
(c)
WT pETQ
ΔmrkH
pETQ
pMrkH
pMrkH*
pPilZ
pPilZ*
pMrkH_N
MrkA
GAPDH
(d)
β-Galactosidase activity (Miller units)
ΔlacZ
ΔlacZ ΔmrkI
P_mrkH::lacZ
ΔlacZ
ΔlacZ ΔmrkH
ΔlacZ ΔmrkI
P_mrkA::lacZ

Fig. 2. Deletion effects of *mrkH*, *mrkI* and *mrkJ* on the expression of type 3 fimbriae. (a) *K. pneumoniae* strains (indicated above the image; WT, wild-type) were grown overnight at 37 °C with agitation in LB broth and subjected to Western blot analysis using MrkA antiserum. The fold change of MrkA amount calculated using ImageJ software (Girish & Vijayalakshmi, 2004) is shown. GAPDH was probed as a protein loading control. (b) Amino acid sequence alignment of PilZ domain proteins. Sequences of the PilZ domain proteins, including MrkH, PA3353, PP4397 and YcgR, were aligned with Vector NTI software. The conserved RxxxR motif and the D/NxSxGG motif are underlined (x, any residue). The critical residue involving in the c-di-GMP binding activity of YcgR is indicated by an arrow. (c) *K. pneumoniae* strains carrying expression plasmids (shown above the image) were grown in LB broth at 37 °C with agitation. When the bacterial growth reached mid-exponential phase, expression of the recombinant protein was induced by the addition of 0.5 mM IPTG and an additional 3 h of incubation. Western blot analysis was performed as described in Methods. (d) β-Galactosidase activities of *K. pneumoniae* CG43S3Δ*lacZ* (Δ*lacZ*) and its isogenic *mrkH* or *mrkI* deletion mutants (Δ*lacZ*Δ*mrkH* or Δ*lacZ*Δ*mrkI*) carrying the reporter plasmid pmrkA-P2 for P_{mrkA} or pAW175 for P_{mrkH} were determined using exponential phase cultures grown in LB broth. *$P<0.001$ compared with Δ*lacZ*.

Extracellular iron availability affects the expression of type 3 fimbriae

To analyse the effects of iron level on type 3 fimbriae activity, we grew CG43S3 in LB broth supplemented with increasing amounts of the iron chelator 2,2′-dipyridyl (dipyridyl) and then analysed bacterial biofilm-forming activity. As shown in Fig. 5(a), increasing amounts of dipyridyl apparently reduced biofilm formation capability. Iron salts, $Fe(NH_4)_2(SO_4)_2$ and $FeCl_3$, but not magnesium chloride, appeared to restore biofilm formation partially (Fig. 5b), indicating that biofilm formation is influenced by iron concentration.

As shown in Fig. 5(c), MrkA production was gradually reduced as increasing amounts of dipyridyl were added to the medium. Moreover, addition of $Fe(NH_4)_2(SO_4)_2$ restored the production of MrkA in the iron-depleted medium (Fig. 5d). No MrkA production was detected in Δ*fur* under any of the growth conditions (data not shown), indicating that iron is a cue for the Fur-dependent expression of type 3 fimbriae.

DISCUSSION

The expression of fimbrial genes is usually controlled by adjacent genes encoding regulators, such as PapB/I for P fimbriae (Båga *et al.*, 1985; Forsman *et al.*, 1989; Hernday *et al.*, 2002; Hultdin *et al.*, 2010), FimW/Y/Z for *Salmonella* type 1 fimbriae (Saini *et al.*, 2009) and MrpJ for MR/P fimbriae (Lane *et al.*, 2009; Li *et al.*, 2001). Consistent with the findings of Johnson *et al.* (2011), we determined that type 3 fimbriae expression in *K. pneumoniae* CG43 is reduced by *mrkH* or *mrkI* deletion. We have also shown, however, that MrkI positively autoregulates the expression of *mrkHI*, which differs from the report on *K. pneumoniae* IApc35 (Johnson *et al.*, 2011). This discrepancy may result from differences in the bacterial strains or experimental methods used. Nevertheless, a negative role for *mrkJ*, which slightly increased MrkA expression in the Δ*mrkJ* mutant and the PDE activity of the recombinant His_6::MrkJ protein, was demonstrated (results not shown).

To investigate how MrkH affects the expression of type 3 fimbriae, we transformed Δ*mrkI* with pMrkH. As shown in Supplementary Fig. S2, the introduction of pMrkH into Δ*mrkI* restored MrkA production, suggesting that MrkI activates the expression of type 3 fimbriae via MrkH functioning. Moreover, the induced expression of *mrkH* in Δ*fur*, or Δ*fur*Δ*mrkI*, also restored the expression of type 3 fimbriae. These results imply a critical role for MrkH in this regulatory pathway.

In addition to the PilZ domain, which has recently been demonstrated to have c-di-GMP binding activity (Johnson *et al.*, 2011), the N-terminal region of unknown function was also required for MrkH regulatory activity. Previous reports have indicated that several PilZ domain proteins could bind to c-di-GMP and then perform their functions through protein–protein interactions (Boehm *et al.*, 2010; Hengge, 2009). In *Xanthomonas*, the binding among PilZ domain proteins, an ATPase (PilB) and an EAL domain protein

Table 3. qRT-PCR analysis of the expression of *mrk* genes

Deletion effects of *mrkH*, *mrkI* and *fur* on the expression of *mrkA*, *mrkH*, *mrkI* and *mrkJ*. ND, Not detected.

Gene	Expression ratio of mRNA (mean ± SD)				
	Δ*mrkH* : WT	Δ*mrkI* : WT	CCW41 : CCW40	Δ*fur* : WT	Δ*fur*[pfur] : Δ*fur*[pRK415]
mrkA	0.36 ± 0.01	0.13 ± 0.01	13.9 ± 3.6	0.15 ± 0.02	1.37 ± 0.03
mrkH	ND	0.09 ± 0.01	10.96 ± 0.98	0.05 ± 0.01	3.8 ± 0.21
mrkI	1.40 ± 0.43	ND	ND	0.11 ± 0.02	4.38 ± 0.17
mrkJ	1.42 ± 0.14	1.21 ± 0.15	0.77 ± 0.01	1.31 ± 0.07	0.71 ± 0.06

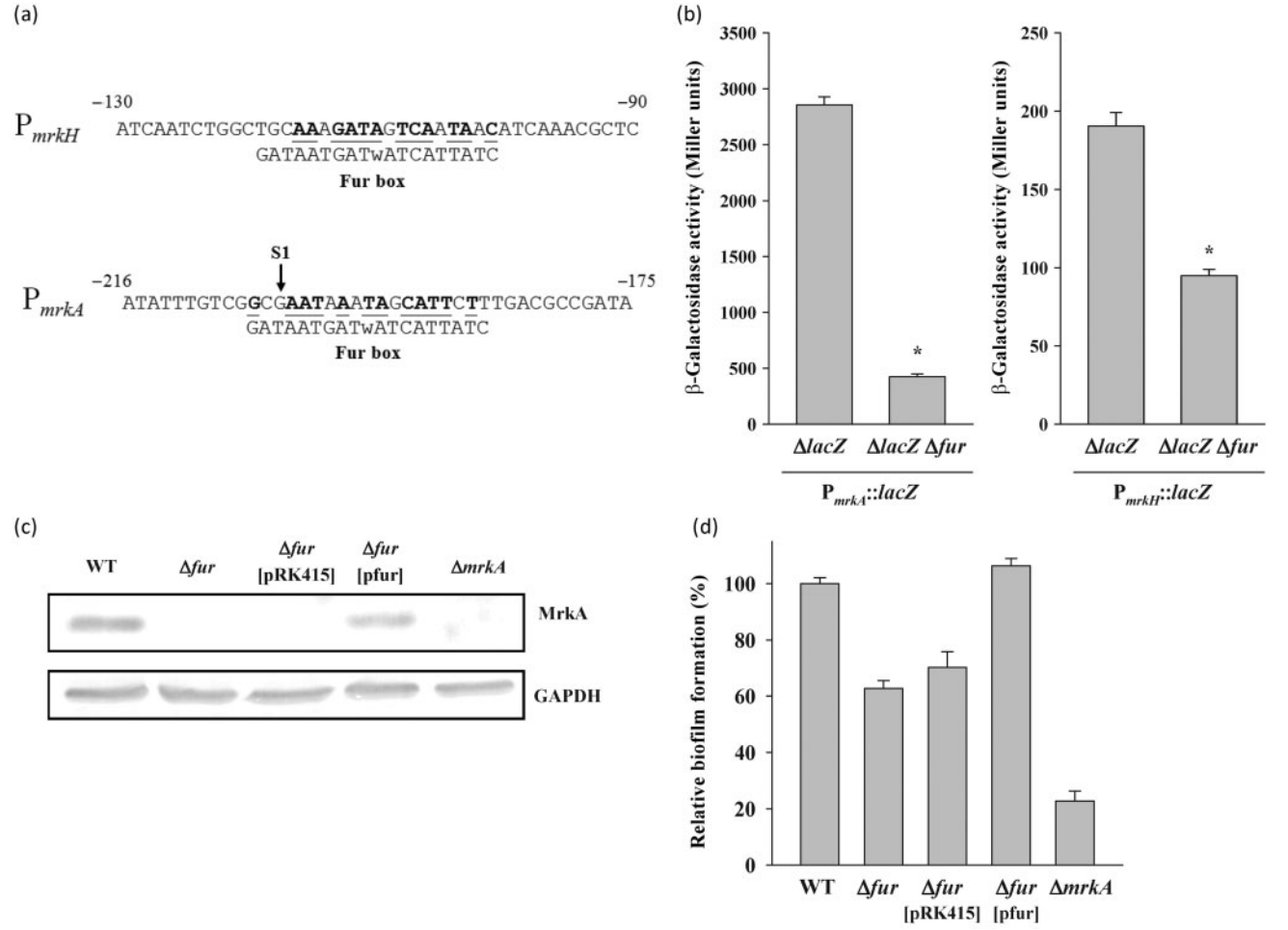

Fig. 3. Deletion of *fur* represses the expression of type 3 fimbriae and biofilm formation. (a) Predicted Fur-binding sequences on the putative promoters of *mrkH* and *mrkA*. The transcription start site of *mrkA* determined by 5′-RACE (Supplementary Fig. S1) is marked as S1. The sequence of the upstream region of *mrkH* or *mrkA* and the alignment with the 19 bp Fur box (w=A or T) are shown. (b) *β*-Galactosidase activities of *K. pneumoniae* CG43S3Δ*lacZ* and its isogenic *fur* deletion mutant (Δ*lacZ*Δ*fur*) carrying the reporter plasmid pmrkA-P2 for P_{mrkA} (left) or pAW175 for P_{mrkH} (right) were determined using exponential phase cultures grown in LB broth. *$P<0.001$ compared with Δ*lacZ*. (c) Anti-MrkA Western blot analysis. *K. pneumoniae* strains (indicated above the blot) were grown overnight at 37 °C with agitation in LB broth. Western blot analysis was performed as described in Methods. GAPDH was probed as a protein loading control. (d) Deletion of *fur* reduces bacterial biofilm formation. *K. pneumoniae* strains were grown at 37 °C for 48 h in LB, and bacterial biofilm formation was quantified as described in Methods. The mean of triplicate experiments is shown; error bars, SD.

(FimX) regulates type IV pilus biosynthesis (Guzzo *et al.*, 2009). Whether MrkH can interact with MrkI, MrkJ or other regulatory proteins via the N-terminal domain to influence type 3 fimbriae expression remains to be investigated.

Amino acid sequence analysis using the Pfam database (http://pfam.sanger.ac.uk/) revealed that MrkI is a LuxR-type transcriptional factor, and activation of LuxR-type regulators can be achieved via phosphorylation (response regulators of a two-component system) (Birck *et al.*, 2002; Maris *et al.*, 2002) or binding to ligands, including quorum-sensing molecules (Pappas *et al.*, 2004; Schlegel *et al.*, 2002). Besides the C-terminal DNA-binding domain, an uncharacterized N-terminal domain is contained in MrkI and is probably involved in receiving signals. Sequence alignment of MrkI and other response regulators also revealed a conserved aspartate residue, which may act as a phosphorylation target, in the N terminus of MrkI (Supplementary Fig. S3a). To determine whether D56 played a role in MrkI-mediated regulation, D56A and D56E site-directed MrkI mutants were created. As shown in Supplementary Fig. S3(b), a reduced production of MrkA in the D56A strain was observed. However, a slight decrease in MrkA was also found in the D56E strain. Hence, whether D56 phosphorylation of MrkI plays an important role in the regulation of type 3 fimbriae remains to be studied.

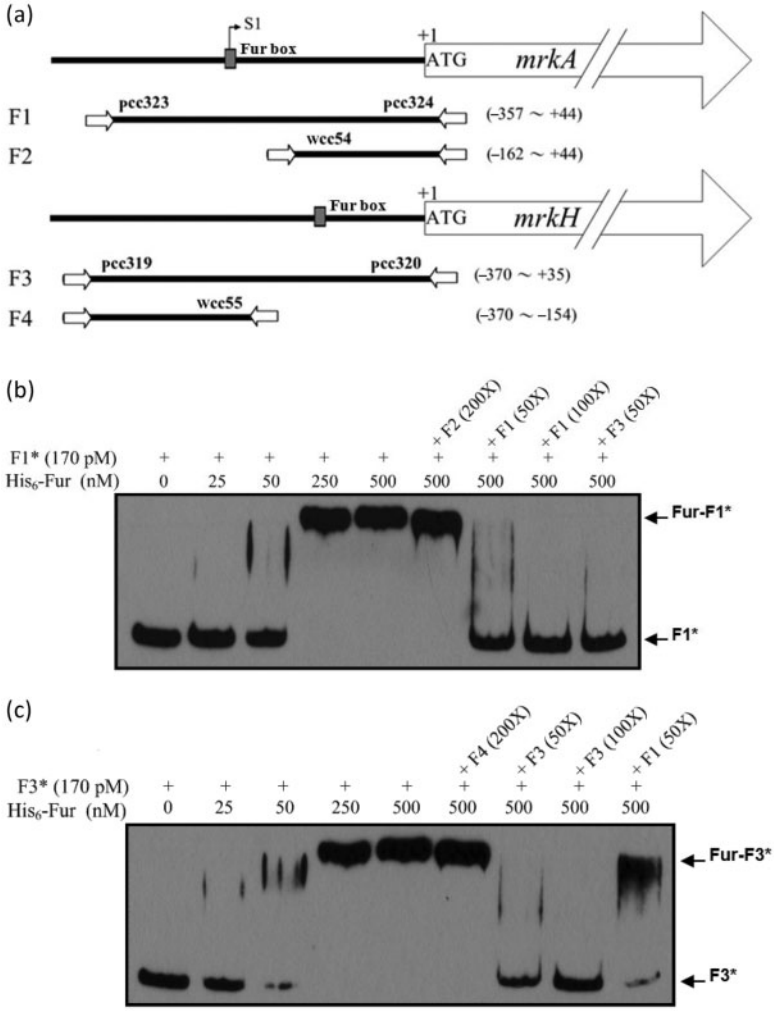

Fig. 4. Fur directly regulates MrkHI and MrkA for the expression of type 3 fimbriae. (a) Schematic illustration of the DNA fragments F1, F2, F3 and F4 used for EMSA analysis. (b) and (c) EMSA of recombinant Fur and the biotin-labelled promoters F1* and F3* of *mrkA* and *mrkH*, respectively. In addition, non-labelled F1, F2, F3 and F4 DNA was individually added in the competition assay. DNAs were incubated with increasing amounts of His_6-Fur for 30 min and then loaded onto a 5 % non-denaturing polyacrylamide gel. The biotin-labelled DNA was detected with the LightShift chemiluminescent EMSA kit (Pierce).

Finally, we have shown that in addition to autoregulatory control by MrkI, the expression of *mrkHI* is positively regulated by Fur. The subsequent EMSA analysis indicated that Fur as a transcriptional activator directly activates the transcription of *mrkHI*. Fur may activate gene transcription by competing with the binding of other repressors to the target promoter (Carpenter *et al.*, 2009; Nandal *et al.*, 2010). The possibility that another regulator(s) is involved in the Fur-activation of the *mrkH* promoter remains to be studied.

As shown in Fig. 3(a), the occlusion of the MrkA transcription start site with the Fur box precludes direct regulation by Fur. Moreover, a single transcription start site, also identified in the most recent report (Wilksch *et al.*, 2011), was mapped for the MrkA transcripts isolated from the bacteria grown in LB, LB supplemented with 500 μM dipyridyl, M9, or M9 supplemented with 60 μM $Fe(NH_4)_2(SO_4)_2$ (Supplementary Fig. S1). This implies that Fur exerts an indirect regulation of type 3 fimbriae expression. The finding that the recombinant Fur bound to the *mrkA* promoter with much lower affinity compared with the binding activity to the *mrkHI* promoter (Fig. 4c) further supports indirect control of the type 3 fimbriae by Fur. Here, we propose that Fur influences the expression of type 3 fimbriae by positively regulating MrkHI expression and concurrently exerting a negative regulation of an unknown MrkA repressor (Fig. 6).

Although the Δ*fur* mutant abolished MrkA production, we observed a relatively small reduction in biofilm formation in Δ*fur* compared with that in Δ*mrkA* (Fig. 3d). We have previously reported that *K. pneumoniae* CPS biosynthesis is activated in the Δ*fur* mutant (Cheng *et al.*, 2010). Thus, we hypothesized that increased CPS on the bacterial surface may influence biofilm formation. Biofilm formation of *E. coli* has also been reported to be repressed under iron limitation, which is regulated by the Fe–S metalloregulatory protein IscR through control of type 1 fimbriae expression in a Fur-independent manner (Wu & Outten, 2009). Although type 1 fimbriae have been shown not to participate in biofilm formation (Schroll *et al.*, 2010), whether IscR plays a role in *K. pneumoniae* biofilm formation remains to be studied.

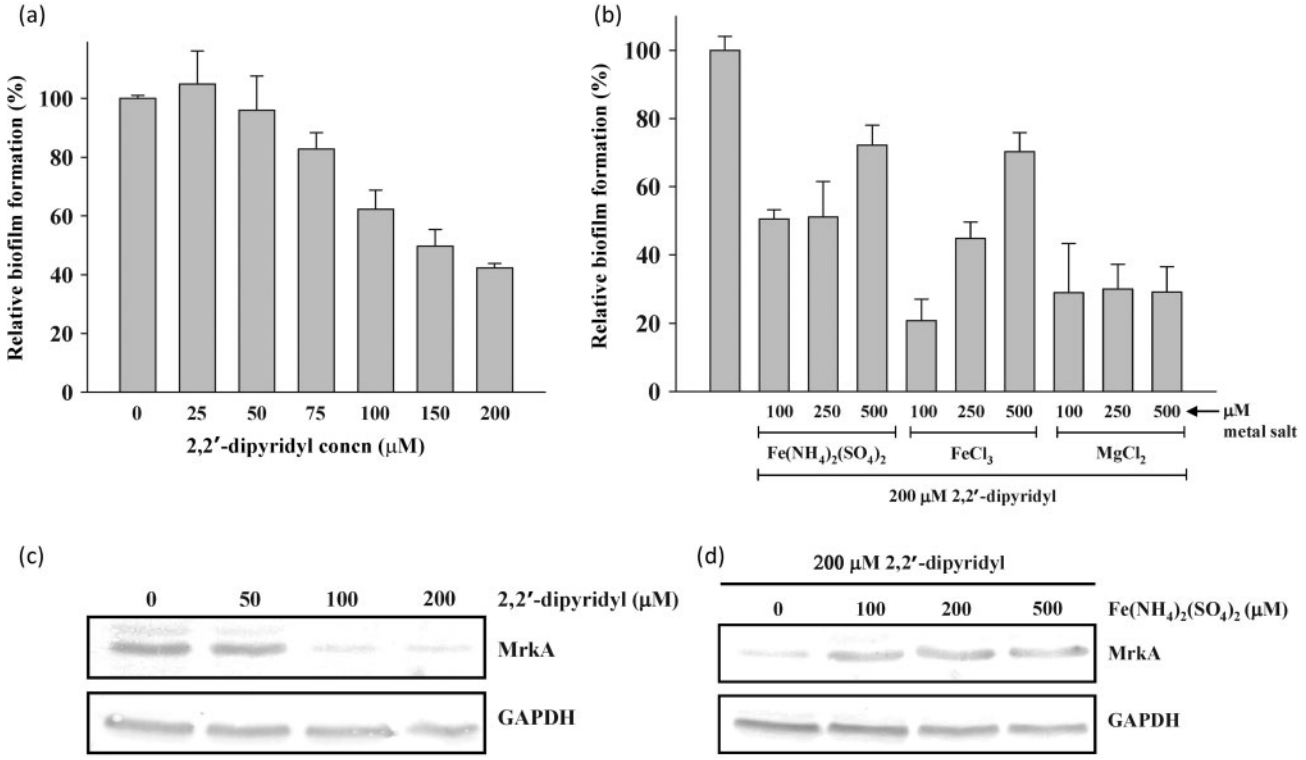

Fig. 5. Iron limitation reduces biofilm formation and the expression of type 3 fimbriae. (a) Inhibition of biofilm formation by the iron chelator dipyridyl. *K. pneumoniae* CG43S3 (wild-type) was grown in increasing amounts of dipyridyl. (b) *K. pneumoniae* CG43S3 was incubated in LB broth with 200 μM dipyridyl and increasing concentrations of iron or magnesium salts as indicated. Biofilm formation was analysed as described in Methods. The means of triplicate experiments are shown; error bars, SD. (c, d) *K. pneumoniae* CG43S3 was grown in LB broth supplemented with dipyridyl and iron salts, as indicated. Western blot analysis was performed as described in Methods. GAPDH was probed as a protein loading control.

Fur has been implicated in iron uptake and metabolism, the oxidative stress response, colonization and virulence in many bacteria (Carpenter *et al.*, 2009). Reports of activity in iron-uptake systems (Lin *et al.*, 2011) and CPS biosynthesis (Cheng *et al.*, 2010; Lin *et al.*, 2011) have been published, but this report is the first, to our

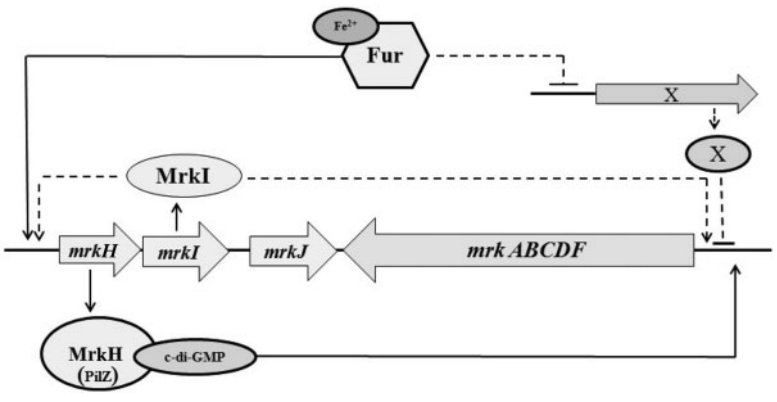

Fig. 6. Model of Fur control of type 3 fimbriae expression in *K. pneumoniae*. Upon ferric ion activation, Fur binds to the promoter region of *mrkHI* and the regulatory region of the not-yet-identified X repressor gene. The binding results in increasing expression of MrkH and MrkI, while reducing the X repressor expression. Subsequently, the increasing levels of MrkI act as a transcription activator and an autoactivator to activate the type 3 fimbriae-encoding operon *mrkABCDF* and *mrkHI* expression, while the reduced levels of X repressor release the suppression, which in turn increases type 3 fimbriae expression. In the presence of high MrkH and c-di-GMP levels, the MrkH–c-di-GMP complex exerts transcriptional activation of the *mrkABCDF* operon (Wilksch *et al.*, 2011).

knowledge, to demonstrate that Fur also participates in the regulation of type 3 fimbriae expression. In summary, the expression of type 3 fimbriae in *K. pneumoniae* CG43 is regulated by the availability of iron and is positively controlled by Fur. Because iron-uptake systems, CPS and type 3 fimbriae are well-known bacterial virulence factors, our findings substantiate the suggestion that Fur plays an important role in the regulation of *K. pneumoniae* pathogenicity.

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REFERENCES

Båga, M., Göransson, M., Normark, S. & Uhlin, B. E. (1985). Transcriptional activation of a Pap pilus virulence operon from uropathogenic *Escherichia coli*. *EMBO J* **4** (13B), 3887–3893.

Baichoo, N. & Helmann, J. D. (2002). Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. *J Bacteriol* **184**, 5826–5832.

Benach, J., Swaminathan, S. S., Tamayo, R., Handelman, S. K., Folta-Stogniew, E., Ramos, J. E., Forouhar, F., Neely, H., Seetharaman, J. & other authors (2007). The structural basis of cyclic diguanylate signal transduction by PilZ domains. *EMBO J* **26**, 5153–5166.

Birck, C., Malfois, M., Svergun, D. & Samama, J. (2002). Insights into signal transduction revealed by the low resolution structure of the FixJ response regulator. *J Mol Biol* **321**, 447–457.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C. A., Ackermann, M., Kaever, V., Sourjik, V., Roth, V. & Jenal, U. (2010). Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141**, 107–116.

Brisse, S., Fevre, C., Passet, V., Issenhuth-Jeanjean, S., Tournebize, R., Diancourt, L. & Grimont, P. (2009). Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS ONE* **4**, e4982.

Carpenter, B. M., Whitmire, J. M. & Merrell, D. S. (2009). This is not your mother's repressor: the complex role of fur in pathogenesis. *Infect Immun* **77**, 2590–2601.

Chang, H. Y., Lee, J. H., Deng, W. L., Fu, T. F. & Peng, H. L. (1996). Virulence and outer membrane properties of a *galU* mutant of *Klebsiella pneumoniae* CG43. *Microb Pathog* **20**, 255–261.

Cheng, H. Y., Chen, Y. S., Wu, C. Y., Chang, H. Y., Lai, Y. C. & Peng, H. L. (2010). RmpA regulation of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* CG43. *J Bacteriol* **192**, 3144–3158.

Di Martino, P., Cafferini, N., Joly, B. & Darfeuille-Michaud, A. (2003). *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. *Res Microbiol* **154**, 9–16.

Escolar, L., Pérez-Martín, J. & de Lorenzo, V. (1999). Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol* **181**, 6223–6229.

Forsman, K., Göransson, M. & Uhlin, B. E. (1989). Autoregulation and multiple DNA interactions by a transcriptional regulatory protein in *E. coli* pili biogenesis. *EMBO J* **8**, 1271–1277.

Fronzes, R., Remaut, H. & Waksman, G. (2008). Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. *EMBO J* **27**, 2271–2280.

Girish, V. & Vijayalakshmi, A. (2004). Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer* **41**, 47.

Guzzo, C. R., Salinas, R. K., Andrade, M. O. & Farah, C. S. (2009). PILZ protein structure and interactions with PILB and the FIMX EAL domain: implications for control of type IV pilus biogenesis. *J Mol Biol* **393**, 848–866.

Hanson, M. S., Hempel, J. & Brinton, C. C., Jr (1988). Purification of the *Escherichia coli* type 1 pilin and minor pilus proteins and partial characterization of the adhesin protein. *J Bacteriol* **170**, 3350–3358.

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**, 263–273.

Hernday, A., Krabbe, M., Braaten, B. & Low, D. (2002). Self-perpetuating epigenetic pili switches in bacteria. *Proc Natl Acad Sci U S A* **99** (Suppl. 4), 16470–16476.

Hirsch, E. B. & Tam, V. H. (2010). Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J Antimicrob Chemother* **65**, 1119–1125.

Hornick, D. B., Allen, B. L., Horn, M. A. & Clegg, S. (1992). Adherence to respiratory epithelia by recombinant *Escherichia coli* expressing *Klebsiella pneumoniae* type 3 fimbrial gene products. *Infect Immun* **60**, 1577–1588.

Huang, Y. J., Liao, H. W., Wu, C. C. & Peng, H. L. (2009). MrkF is a component of type 3 fimbriae in *Klebsiella pneumoniae*. *Res Microbiol* **160**, 71–79.

Hultdin, U. W., Lindberg, S., Grundström, C., Huang, S., Uhlin, B. E. & Sauer-Eriksson, A. E. (2010). Structure of FocB – a member of a family of transcription factors regulating fimbrial adhesin expression in uropathogenic *Escherichia coli*. *FEBS J* **277**, 3368–3381.

Jagnow, J. & Clegg, S. (2003). *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. *Microbiology* **149**, 2397–2405.

Johnson, J. G. & Clegg, S. (2010). Role of MrkJ, a phosphodiesterase, in type 3 fimbrial expression and biofilm formation in *Klebsiella pneumoniae*. *J Bacteriol* **192**, 3944–3950.

Johnson, J. G., Murphy, C. N., Sippy, J., Johnson, T. J. & Clegg, S. (2011). Type 3 fimbriae and biofilm formation are regulated by the transcriptional regulators MrkHI in *Klebsiella pneumoniae*. *J Bacteriol* **193**, 3453–3460.

Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 191–197.

Keynan, Y. & Rubinstein, E. (2007). The changing face of *Klebsiella pneumoniae* infections in the community. *Int J Antimicrob Agents* **30**, 385–389.

Klemm, P. & Schembri, M. A. (2000). Bacterial adhesins: function and structure. *Int J Med Microbiol* **290**, 27–35.

Lai, Y. C., Peng, H. L. & Chang, H. Y. (2001). Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. *Infect Immun* **69**, 7140–7145.

Lai, Y. C., Peng, H. L. & Chang, H. Y. (2003). RmpA2, an activator of capsule biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 *cps* gene expression at the transcriptional level. *J Bacteriol* **185**, 788–800.

Lane, M. C., Li, X., Pearson, M. M., Simms, A. N. & Mobley, H. L. (2009). Oxygen-limiting conditions enrich for fimbriate cells of uropathogenic *Proteus mirabilis* and *Escherichia coli*. *J Bacteriol* **191**, 1382–1392.

Li, X., Rasko, D. A., Lockatell, C. V., Johnson, D. E. & Mobley, H. L. (2001). Repression of bacterial motility by a novel fimbrial gene product. *EMBO J* **20**, 4854–4862.

Lin, C. T., Huang, T. Y., Liang, W. C. & Peng, H. L. (2006). Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner. *J Biochem* **140**, 429–438.

Lin, C. T., Wu, C. C., Chen, Y. S., Lai, Y. C., Chi, C., Lin, J. C., Chen, Y. & Peng, H. L. (2011). Fur regulation of the capsular polysaccharide biosynthesis and iron-acquisition systems in *Klebsiella pneumoniae* CG43. *Microbiology* **157**, 419–429.

Maris, A. E., Sawaya, M. R., Kaczor-Grzeskowiak, M., Jarvis, M. R., Bearson, S. M., Kopka, M. L., Schröder, I., Gunsalus, R. P. & Dickerson, R. E. (2002). Dimerization allows DNA target site recognition by the NarL response regulator. *Nat Struct Biol* **9**, 771–778.

Nandal, A., Huggins, C. C., Woodhall, M. R., McHugh, J., Rodríguez-Quiñones, F., Quail, M. A., Guest, J. R. & Andrews, S. C. (2010). Induction of the ferritin gene (*ftnA*) of *Escherichia coli* by Fe^{2+}–Fur is mediated by reversal of H-NS silencing and is RyhB independent. *Mol Microbiol* **75**, 637–657.

Nordmann, P., Cuzon, G. & Naas, T. (2009). The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* **9**, 228–236.

Pappas, K. M., Weingart, C. L. & Winans, S. C. (2004). Chemical communication in proteobacteria: biochemical and structural studies of signal synthases and receptors required for intercellular signalling. *Mol Microbiol* **53**, 755–769.

Podschun, R. & Ullmann, U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**, 589–603.

Rosen, D. A., Pinkner, J. S., Jones, J. M., Walker, J. N., Clegg, S. & Hultgren, S. J. (2008). Utilization of an intracellular bacterial community pathway in *Klebsiella pneumoniae* urinary tract infection and the effects of FimK on type 1 pilus expression. *Infect Immun* **76**, 3337–3345.

Ryjenkov, D. A., Simm, R., Römling, U. & Gomelsky, M. (2006). The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* **281**, 30310–30314.

Saini, S., Pearl, J. A. & Rao, C. V. (2009). Role of FimW, FimY, and FimZ in regulating the expression of type I fimbriae in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **191**, 3003–3010.

Schlegel, A., Böhm, A., Lee, S. J., Peist, R., Decker, K. & Boos, W. (2002). Network regulation of the *Escherichia coli* maltose system. *J Mol Microbiol Biotechnol* **4**, 301–307.

Schroll, C., Barken, K. B., Krogfelt, K. A. & Struve, C. (2010). Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. *BMC Microbiol* **10**, 179.

Skorupski, K. & Taylor, R. K. (1996). Positive selection vectors for allelic exchange. *Gene* **169**, 47–52.

Struve, C., Bojer, M. & Krogfelt, K. A. (2008). Characterization of *Klebsiella pneumoniae* type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence. *Infect Immun* **76**, 4055–4065.

Struve, C., Bojer, M. & Krogfelt, K. A. (2009). Identification of a conserved chromosomal region encoding *Klebsiella pneumoniae* type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity. *Infect Immun* **77**, 5016–5024.

Tarkkanen, A. M., Virkola, R., Clegg, S. & Korhonen, T. K. (1997). Binding of the type 3 fimbriae of *Klebsiella pneumoniae* to human endothelial and urinary bladder cells. *Infect Immun* **65**, 1546–1549.

Tsai, F. C., Huang, Y. T., Chang, L. Y. & Wang, J. T. (2008). Pyogenic liver abscess as endemic disease, Taiwan. *Emerg Infect Dis* **14**, 1592–1600.

Wilksch, J. J., Yang, J., Clements, A., Gabbe, J. L., Short, K. R., Cao, H., Cavaliere, R., James, C. E., Whitchurch, C. B. & other authors (2011). MrkH, a novel c-di-GMP-dependent transcriptional activator, controls *Klebsiella pneumoniae* biofilm formation by regulating type 3 fimbriae expression. *PLoS Pathog* **7**, e1002204.

Wu, Y. & Outten, F. W. (2009). IscR controls iron-dependent biofilm formation in *Escherichia coli* by regulating type I fimbria expression. *J Bacteriol* **191**, 1248–1257.

Wu, C. C., Huang, Y. J., Fung, C. P. & Peng, H. L. (2010). Regulation of the *Klebsiella pneumoniae* Kpc fimbriae by the site-specific recombinase KpcI. *Microbiology* **156**, 1983–1992.

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