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Fur regulation of the capsular polysaccharide biosynthesis and iron-acquisition systems in *Klebsiella pneumoniae* CG43

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The ferric uptake regulator Fur has been reported to repress the expression of *rmpA*, a regulatory gene for the mucoid phenotype, leading to decreased capsular polysaccharide (CPS) biosynthesis in *Klebsiella pneumoniae* CG43. Here, quantitative real-time PCR (qRT-PCR) analyses and electrophoretic mobility shift assays showed that Fur also repressed the expression of the CPS regulatory genes *rmpA2* and *rcsA*. Interestingly, deletion of *rmpA* or *rcsA* but not *rmpA2* from the Δ*fur* strain was able to suppress the deletion effect of Fur. The availability of extracellular iron affected the amount of CPS, suggesting that Fur regulates CPS biosynthesis in an Fe(II)-dependent manner. Increased production of siderophores was observed in the Δ*fur* strain, suggesting that uptake of extracellular iron in *K. pneumoniae* is regulated by Fur. Fur titration assays and qRT-PCR analyses demonstrated that at least six of the eight putative iron-acquisition systems, identified by a BLAST search in the contig database of *K. pneumoniae* CG43, were directly repressed by Fur. We conclude that Fur has a dual role in the regulation of CPS biosynthesis and iron acquisition in *K. pneumoniae*.

INTRODUCTION

Klebsiella pneumoniae is a rod-shaped Gram-negative bacterium that causes community-acquired diseases including pneumonia, bacteraemia, septicaemia, and urinary and respiratory tract infections, particularly in immunocompromised patients (Podschun & Ullmann, 1998). In Asian countries, especially in Taiwan and Korea, *K. pneumoniae* is the predominant pathogen responsible for pyogenic liver abscess in diabetic patients (Han, 1995; Lau *et al.*, 2000; Yang *et al.*, 2009). Among the virulence factors identified in *K. pneumoniae*, capsular polysaccharide (CPS) is considered as the major determinant for *K. pneumoniae* infections. Pyogenic liver abscess isolates often carry heavy CPS which could protect the bacteria from phagocytosis and killing by serum factors (Lin *et al.*, 2004; Sahly *et al.*, 2000). Apart from the anti-phagocytic function, *Klebsiella* CPS also helps bacterial colonization and biofilm formation at the infection sites (Boddicker *et al.*, 2006; Favre-Bonte *et al.*, 1999; Moranta *et al.*, 2010).

The Rcs system is a well-known two-component system (2CS) that regulates the expression of *cps* genes in bacteria (Stout, 1994). The transcription of *cps* genes is controlled by the response regulator RcsB in complex with the auxiliary regulatory protein RcsA (Gottesman & Stout, 1991; Majdalani & Gottesman, 2005). We have demonstrated that *cps* expression in *K. pneumoniae* CG43 is also affected by the coordinated action of the 2CSs KvgAS, KvhAS and KvhR, and in this case is independent of RcsB (Lin *et al.*, 2006). Besides RcsA, the regulators RmpA and RmpA2 also interact with RcsB for CPS biosynthesis regulation. Moreover, *rmpA* expression was repressed by Fur (ferric uptake regulator), the global regulator for the expression of iron-acquisition systems (Cheng *et al.*, 2010). Here we demonstrate that Fur also affects *rcsA* and *rmpA2* expression.

In *Escherichia coli*, under iron-replete conditions, dimeric Fur in complex with Fe(II) binds to a 19 bp consensus

Abbreviations: 2CS, two-component system; CPS, capsular polysaccharide; Dip, 2,2-dipyridyl; CAS, chrome azurol S; EMSA, electrophoretic mobility shift assay; FURTA, Fur titration assay; qRT-PCR, quantitative real-time PCR.

DNA sequence, the Fur box (GATAATGATwATCATT-ATC; w=A or T), in the promoters of the genes required for iron uptake, thereby preventing transcription from these genes (Griggs & Konisky, 1989). The regulation helps bacteria to avoid iron overload, which may lead to the formation of hydroxyl radicals. Multiple iron-acquisition systems are commonly present in bacteria for the uptake of iron in the environment (Andrews *et al.*, 2003). In an anaerobic environment, Fe(II) is prevalent and is imported into the bacterial cytoplasm via the Feo system (Hantke, 2003). However, in aerobic conditions and in mammalian tissues (*in vivo*), the majority of iron is found as Fe(III), and iron *in vivo* is almost entirely sequestered by iron-binding proteins (transferrin and lactoferrin) and haemo-proteins (haemoglobin and myoglobin) (Wandersman & Delepelaire, 2004).

Bacteria are generally equipped with iron/haem acquisition systems to transport iron directly from the exogenous iron/haem sources or release siderophore and haemophore compounds into the extracellular medium to scavenge iron/haem from various sources (Wandersman & Delepelaire, 2004). In *K. pneumoniae* NTUH-K2044, the expression of the ten putative iron-acquisition genes was highly upregulated in response to human serum, and bacterial virulence was decreased by the triple mutation of siderophore genes (Hsieh *et al.*, 2008). The siderophore genes *iucABCDiutA* and *iroNDCB* have also been reported to be the determinants of *K. pneumoniae*-caused liver abscess (Hsieh *et al.*, 2008; Koczura & Kaznowski, 2003; Tang *et al.*, 2010). Nevertheless, until now the regulation of iron-acquisition gene expression in *K. pneumoniae* has not been studied.

In this study, we investigated the regulatory roles of Fur on the expression of the *cps* regulators RmpA, RmpA2 and RcsA, and the expression of eight iron-acquisition systems in *K. pneumoniae* CG43.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured at 37 °C in Luria–Bertani (LB) medium or M9 minimal medium supplemented with appropriate antibiotics. The antibiotics used include ampicillin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), streptomycin (500 μg ml^{-1}) and tetracycline (12.5 μg ml^{-1}).

Construction of deletion mutants. Specific gene deletions were introduced into *K. pneumoniae* CG43 by using an allelic exchange strategy as described by Lai *et al.* (2003). The pKAS46 system was used in the selection of the mutants (Skorupski & Taylor, 1996), and the mutations were confirmed by PCR and Southern hybridization (data not shown).

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from bacterial cells grown to early exponential phase by using the RNeasy midi-column (Qiagen) according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. RNA (100 ng) was reverse-transcribed with the transcriptor first strand cDNA synthesis kit (Roche) by using random primers. qRT-PCR was performed in a Roche LightCycler 1.5 instrument by using LightCycler *Taq*Man master (Roche). Primers and probes were designed for selected target sequences by using the universal ProbeLibrary assay design center (Roche–Applied science) and are listed in Table 2. Data were analysed using the real-time PCR software of the Roche LightCycler 1.5 instrument. Relative gene expression was quantified by using the comparative threshold cycle $2^{-\Delta\Delta C_T}$ method with 23S rRNA as the endogenous reference.

Electrophoretic mobility shift assay (EMSA). Recombinant *K. pneumoniae* Fur protein was expressed in *E. coli* and purified as described previously (Cheng *et al.*, 2010). DNA fragments of the putative promoter regions of *rmpA*, *rmpA2* and *rcsA* were amplified by PCR using specific primer sets. The purified His_6-Fur was incubated with 10 ng DNA in a 15 μl solution containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 100 mM dithiothreitol, 200 μM $MnCl_2$ and 1 μg BSA μl^{-1} at room temperature for 20 min. The samples were then loaded onto 5 % native (non-denaturing) polyacrylamide gel containing 5 % glycerol in 0.5 × TB buffer (45 mM Tris/HCl, pH 8.0, 45 mM boric acid) and electrophoresed at 20 mA constant current at 4 °C for 2 h. The gel was stained with SYBR Green EMSA stain (Invitrogen), and was then visualized by using a Safe Imager blue-light transilluminator.

Extraction and quantification of CPS. CPS was extracted and quantified as described by Domenico *et al.* (1989). The glucuronic acid content, representing the amount of *K. pneumoniae* K2 CPS, was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and was expressed as μg $(10^9$ c.f.u.$)^{-1}$ (Blumenkrantz & Asboe-Hansen, 1973).

Identification of the iron-acquisition genes in *K. pneumoniae* CG43. The ten genes encoding different iron-acquisition systems in *K. pneumoniae* NTUH-K2044 (Hsieh *et al.*, 2008) were used as query sequences to search for homologues in the *K. pneumoniae* CG43 contig database (unpublished results from Dr S.-F. Tsai, National Health Research Institutes, Taiwan) as assessed by the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

Fur titration assay (FURTA). FURTA was performed according to the method described by Stojiljkovic *et al.* (1994). DNA sequences containing a putative Fur box were amplified by PCR with specific primer sets and then cloned into pT7-7. The resulting plasmids were introduced into the *E. coli* strain H1717, and the transformants were plated onto MacConkey-lactose plates containing 100 μg ampicillin ml^{-1} and 30 μM $Fe(NH_4)_2(SO_4)_2$. The indicator strain H1717 contained a chromosomal *fhuF*::*lacZ* fusion, and a low-affinity Fur box has been identified in the *fhuF* promoter. The introduction of pT7-7-derived plasmids carrying Fur-binding sequences could thus cause the removal of Fur from the *fhuF* Fur box (Hantke, 1987). H1717 harbouring pT7-7 was used as a negative control. Colony phenotype was observed after incubation at 37 °C for 10 h. A red colony colour (Lac+) denoted a FURTA-positive phenotype and indicated the binding of Fur to the DNA sequence cloned into the pT7-7 plasmid.

Chrome azurol S (CAS) assay. The CAS assay was performed according to the method described by Schwyn & Neilands (1987). Each of the bacterial strains was grown overnight in LB medium, and then 5 μl of culture was added onto a CAS agar plate. After 16 h incubation at 37 °C, effects of the bacterial siderophore production could be observed. Siderophore production was apparent as an orange halo around the colonies; absence of a halo indicated the inability to produce siderophores.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
K. pneumoniae		
CG43S3	CG43 Sm^r	Lai *et al.* (2001)
Δ*rmpA*	CG43S3Δ*rmpA*	Cheng *et al.* (2010)
Δ*rmpA2*	CG43S3Δ*rmpA2*	Lai *et al.* (2001)
Δ*fur*	CG43S3Δ*fur*	Cheng *et al.* (2010)
Δ*rcsA*	CG43S3Δ*rcsA*	This study
Δ*rmpA*Δ*rcsA*	CG43S3Δ*rmpA*Δ*rcsA*	This study
Δ*rmpA*Δ*rmpA2*Δ*rcsA*	CG43S3Δ*rmpA*Δ*rmpA2*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*	CG43S3Δ*fur*Δ*rmpA*	This study
Δ*fur*Δ*rmpA2*	CG43S3Δ*fur*Δ*rmpA2*	This study
Δ*fur*Δ*rcsA*	CG43S3Δ*fur*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*Δ*rcsA*	CG43S3Δ*fur*Δ*rmpA*Δ*rcsA*	This study
Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA*	CG43S3Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA*	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (*f80 lacZ*ΔM15) hsdR17 *recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
BL21-RIL	F^- *ompT* $hsdS_B$ [$r_B{}^- m_B{}^-$]*gal dcm* [DE3]	Laboratory stock
S17-1 λ *pir*	*hsdR recA pro* RP4-2 [Tc::Mu; Km::Tn*7*] [λ*pir*]	Skorupski & Taylor (1996)
H1717	*araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF*::λ *placMu*	Hantke (1987)
Plasmid		
pKAS46	Positive selection suicide vector, *rpsL* Ap^r Km^r	Skorupski & Taylor (1996)
pET30a-c	His-tagging protein expression vector, Km^r	Novagen
yT&A	TA cloning vector	Yeastern
pRK415	Broad-host-range IncP cloning vector, Tc^r	Keen *et al.* (1988)
pT7-7	Cloning vector, Ap^r	Tabor & Richardson (1985)
pfur03	1.7 kb fragment containing an internal 454 bp deletion in *fur*, cloned into pKAS46	Cheng *et al.* (2010)
prcsA03	2.0 kb fragment containing an internal 620 bp deletion in *rcsA*, cloned into pKAS46	This study
piroB_2	928 bp fragment containing the putative *iroBCD* promoter, cloned into pT7-7	This study
pentC_2	284 bp fragment containing the putative *entC* promoter, cloned into pT7-7	This study
piucA_2	700 bp fragment containing the putative *iucABCD* promoter, cloned into pT7-7	This study
phmuR_2	500 bp fragment containing the putative *hmuRSTUV* promoter, cloned into pT7-7	This study
pfeo_2	564 bp fragment containing the putative *feoABC* promoter, cloned into pT7-7	This study
pfec_2	296 bp fragment containing the putative *fecIRA* promoter, cloned into pT7-7	This study
pfhuA_2	313 bp fragment containing the putative *fhuA* promoter, cloned into pT7-7	This study
psitA_2	283 bp fragment containing the putative *sitABCD* promoter, cloned into pT7-7	This study
pFT01	0.5 kb fragment containing the putative *orf1-2* promoter, cloned into pT7-7	This study
pFT02	0.9 kb fragment containing the putative *orf3-15* promoter, cloned into pT7-7	This study
pFT03	0.3 kb fragment containing the putative *orf16-17* promoter, cloned into pT7-7	This study
pFT04	0.5 kb fragment containing the putative *rmpA* promoter, cloned into pT7-7	This study
pFT05	0.5 kb fragment containing the putative *rmpA2* promoter, cloned into pT7-7	This study
pFT06	0.5 kb fragment containing the putative *rcsA* promoter, cloned into pT7-7	This study

Statistical method. An unpaired *t*-test was used to determine the statistical significance and values of $P<0.001$ were considered significant. The results of CPS quantification and qRT-PCR analysis were derived from a single experiment representative of three independent experiments. Each sample was assayed in triplicate and the mean activity and standard deviation are presented.

RESULTS

Fur regulates the expression of RmpA, RmpA2 and RcsA

To investigate whether Fur affects the expression of the *cps* regulatory proteins RcsA, RcsB, RmpA2, KvgA and KvhR (Cheng *et al.*, 2010; Lai *et al.*, 2003; Lin *et al.*, 2006), in addition to RmpA (Cheng *et al.*, 2010), qRT-PCR analyses were performed to compare expression levels in *K. pneumoniae* CG43S3 and its isogenic Δ*fur* strain. As shown in Fig. 1(a), when the bacteria were grown in LB, the deletion of *fur* increased the expression of not only *rmpA* but also *rmpA2* and *rcsA*. By contrast, *fur* deletion appeared to have no effect on the expression of *rcsB*, *kvgA* or *kvhR*. Addition of the iron chelator 2,2-dipyridyl (Dip) to the growth medium also increased the expression of *rmpA* and *rcsA* in the wild-type strain, suggesting that a Fur–Fe(II) complex is involved in regulating the expression of *rmpA* and *rcsA*. However, *rmpA2* expression did not appear to change, suggesting a novel mechanism that requires further

Table 2. Primers used in this study

Primer	Sequence (5′→3′)*	Enzyme/*Taq*Man probe†	Target
For FURTA			
FA01	GAAGCTTGGAGCGCAGTTAGCGGAC	*Hin*dIII	P_{iroB}
FA02	CGGATCCGCCCATAGAGAGGAGGACC	*Bam*HI	
FA03	GAAGCTTCCTGGGCTGAGGTAATTCC	*Hin*dIII	P_{entC}
FA04	CGGATCCCTCAGCCAGTGACGTTTCC	*Bam*HI	
FA05	GGATCCAGAGGGTGATTTGCCAGCAT	*Bam*HI	P_{iucA}
FA06	AGATCTGGAAGCACTGAGCAGCCACA	*Bgl*II	
FA07	ACACCAAGCTTCTGACGGAG	*Hin*dIII	P_{hmuR}
FA08	CTCCGGGATCCAGACATCGC	*Bam*HI	
FA09	GGATCCCAACAGCGCGATGATGGAT	*Bam*HI	P_{feo}
FA10	AGATCTGCCAGCATGCCGAGGGAGA	*Bgl*II	
FA11	GAAGCTTGTCGCGGGCTGGATCAAG	*Hin*dIII	P_{fhuA}
FA12	CGGATCCCGCAGCGAGTGATTTGGC	*Bam*HI	
FA13	GAATTCGCAGCCTGATTGAC	*Eco*RI	P_{sitA}
FA14	GGTGTAGCATAGGATCCCTC	*Bam*HI	
For qRT-PCR			
GT56	ACCCCGCCAGCTTTAACTT	3	*entC*
GT57	TGTCCTTCTTTACGCAGCAG		
GT58	CAACCTGAACAGCGATTTCC	20	*fecA*
GT59	TCGGCGCTCTCTTTAACAGT		
GT62	CAGATGTCAGCGCAGATCC	20	*feoB*
GT63	CATAGGCCCGGCTGTAGA		
GT64	AAAGAGATTGGCCTCGAGTTT	20	*fepA*
GT65	TGTTGCGGTAGTCGTTGC		
GT66	AATAAACAGCTCGTTTCGTTAAAAG	160	*fepB*
GT67	GTATAGACCAGGGCGGTCAC		
GT68	GTTTGGTCGTATCGCCTGAC	3	*fhuA*
GT69	GGAAGGTGAAGTCAGTTTTATCG		
GT72	TGATGACCTACCTGCAGTACCA	20	*hmuR*
GT73	GAGCCGAGGTTCCAGGAG		
GT74	CGGAGGAACATTCGTCAAA	84	*iroB*
GT75	TTCGGAATCTAAGCCTGGTG		
GT78	TCTCCCGGCTTATTGTTGATA	67	*iucA*
GT79	GGAAGGTTTCGCAACTGGT		
GT82	GAAGATCCGTCAGACGATGG	20	*sitA*
GT83	TAGTCGCGGGCCAGATAG		
RT03	CGTCATCCAGACCAAAGAGC	83	*orf1*
RT04	CCGGTTTTTCAATAAACTCGAC		
RT05	CGATGACCGGCTTTTTAATG	83	*orf3*
RT06	CTAGCGGAGATTTGGTACTGC		
RT07	CAGTCCACCTTTATTCCGATTG	67	*orf16*
RT08	AGGTACGACCCCGACTGG		
RT11	GGTAGGGGAGCGTTCTGTAA	67	23S rRNA
RT12	TCAGCATTCGCACTTCTGAT		
RT17	TCAATAGCAATTAAGCACAAAAGAA	18	*rmpA*
RT18	TTGTACCCTCCCCATTTCC		
RT19	AAATCATTACCCACAACTAACAAAAA	80	*rmpA2*
RT20	TTAGACGGCTTTTTAATTCATGG		
GT25	AAAACAGAATCAAATATGCTGCAA	158	*rcsA*
GT26	CGTTGAGATTTGCGAAGTACC		
RT31	AAATTCACCCCGGAAAGC	120	*rcsB*
RT32	GCAGTACTTCGCTCTCTTTCG		
GT27	AAACCGTCCTGGAAAACCA	84	*kvgA*
GT28	CAACCAGCTGGATAGCATGA		

Table 2. cont.

Primer	Sequence (5′→3′)*	Enzyme/*Taq*Man probe†	Target
GT13 GT14	GTATTTTTATTCGCGATGTACTGC GCCTGAACAGCGGAGAGA	67	*kvhR*

*Underlining indicates the nucleotide sequence recognized by the restriction enzyme.
†Enzyme refers to the restriction enzyme used in FURTA; *Taq*Man probe refers to qRT-PCR.

study. Intriguingly, the expression of *rmpA*, *rmpA2* and *rcsA* in the Δ*fur* strain were all reduced by iron chelation, implying that the iron chelator Dip has a non-specific inhibitory effect on controlling the gene transcription.

As in P_{rmpA}, the promoter of *rmpA*, putative Fur box sequences could be found in the upstream regions of *rmpA2* and *rcsA* (Fig. 1b), although being less conserved in the *rmpA2* promoter. We performed an EMSA to determine whether Fur directly affects the expression of *rmpA2* and *rcsA*. As shown in Fig. 1(c), the purified recombinant His_6-Fur protein was able to bind to the upstream regions of *rmpA*, *rmpA2* and *rcsA*, but not to the P6 DNA, which did not contain a Fur box (Cheng *et al.*, 2010). Addition of 200 μM EDTA to the reaction mixture appeared to abolish the interactions (data not shown),

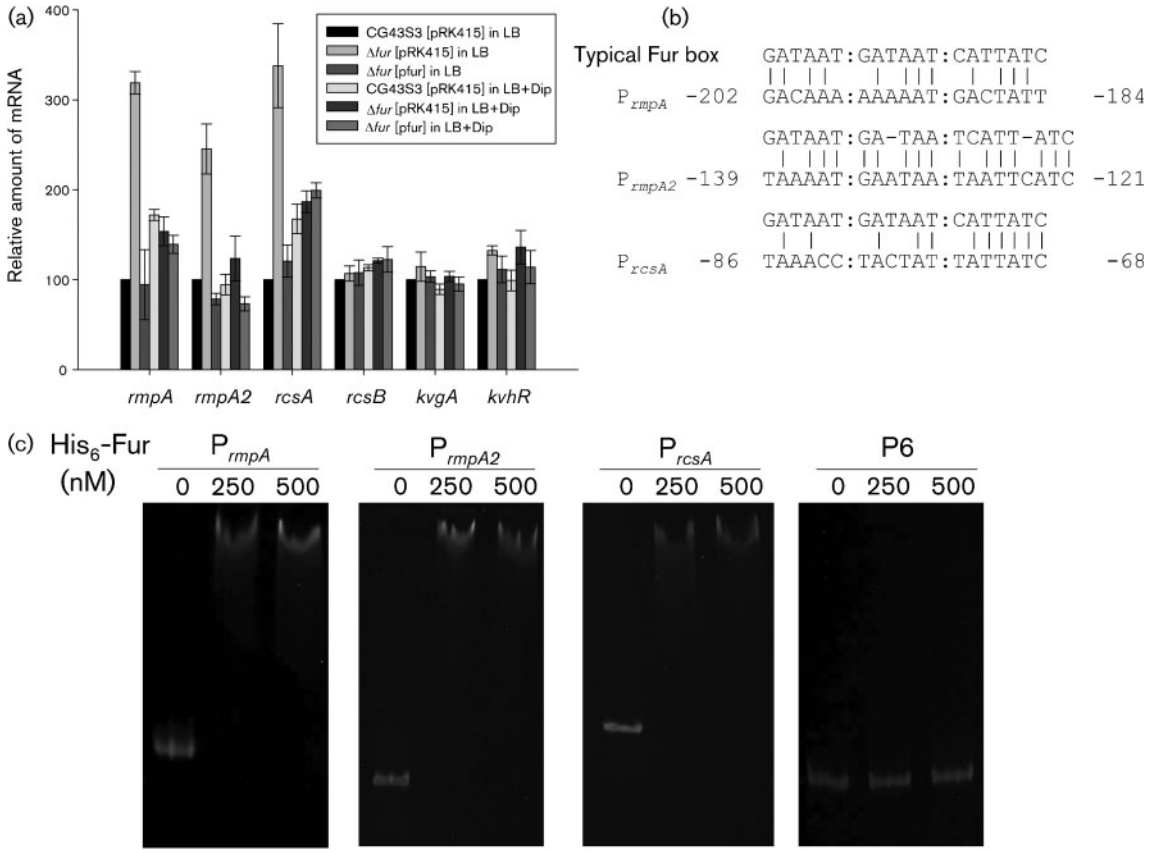


Fig. 1. Fur directly represses the expression of *rmpA*, *rmpA2* and *rcsA*. (a) qRT-PCR analysis. The *K. pneumoniae* CG43S3 [pRK415], Δ*fur* [pRK415] and Δ*fur* [pfur] strains were grown overnight in LB both with and without 200 μM Dip, and the relative expression of *rmpA*, *rmpA2*, *rcsA*, *rcsB*, *kvgA* and *kvhR* in bacteria was then measured by qRT-PCR analysis. Values are mean ± SD of three independent experiments. (b) DNA sequence alignment between the *E. coli* typical Fur box and the putative Fur boxes in the upstream regions of *rmpA*, *rmpA2* and *rcsA*. The relative positions to the translational start sites are indicated. (c) EMSA of the recombinant His_6-Fur and its target promoters. DNA of the upstream regions of *rmpA*, *rmpA2* and *rcsA* was incubated with an increasing amount of the His_6-Fur for 30 min and then loaded onto a 5 % non-denaturing polyacrylamide gel. The DNA fragment P6 was used as a negative control. The gel was stained with SYBR Green EMSA stain and photographed.

indicating that formation of the Fur–Fe(II) complex was required for the specific binding.

Fur represses CPS biosynthesis via RmpA and RcsA

To investigate how Fur differentially regulates the expression of the three CPS regulators, double mutants with a deletion of *rmpA*, *rmpA2* or *rcsA* from the Δ*fur* strain background were constructed, and the effects of the mutations on bacterial CPS biosynthesis were assessed. Consistent with previous reports (Cheng *et al.*, 2010; Ebel & Trempy, 1999; Lai *et al.*, 2003), deletion of *rmpA*, *rmpA2* or *rcsA* reduced the amount of bacterial CPS (Fig. 2). By contrast, a significant increase in the amount of CPS was found in the Δ*fur* strain. Interestingly, deletion of *rmpA* or *rcsA*, but not *rmpA2*, suppressed the *fur* deletion phenotype (Fig. 2). The results suggest that the activation of CPS biosynthesis in the Δ*fur* strain is mediated by RmpA or RcsA, but not RmpA2, under the assay conditions used.

It has been reported that the K2 *cps* gene cluster of *K. pneumoniae* Chedid contains 19 open reading frames (ORFs) organized into three transcription units, *orf1-2*, *orf3-15* and *orf16-17* (Arakawa *et al.*, 1995). Analysis of the *cps* promoters revealed no conserved Fur box, suggesting that Fur exerts indirect control over the transcription of *cps*. To investigate this possibility, transcripts of *orf1*, *orf3* and *orf16* in wild-type (CG43S3), Δ*fur*, Δ*rmpA*, Δ*rmpA2*, Δ*rcsA*, Δ*fur*Δ*rmpA*, Δ*fur*Δ*rmpA2*, Δ*fur*Δ*rcsA*, Δ*fur*Δ*rmpA*Δ*rcsA* and Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* strains were measured via qRT-PCR. As shown in Fig. 3, all three transcripts were differentially decreased in Δ*rmpA*, Δ*rmpA2* and Δ*rcsA* strains. Compared with either the *rmpA* or the *rcsA* deletions, the deletion of *rmpA2* had less effect on the transcription of *orf1*, *orf3* and *orf16*. Interestingly, deletion of *rmpA* had more profound reducing effects on the transcription of *orf1* and *orf16* than deletion of *rcsA*. Moreover, expression levels of *cps* in Δ*rmpA*, Δ*rmpA*Δ*rcsA* and Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar, suggesting a major regulatory role of RmpA in controlling *cps* expression. However, RcsA and RmpA2 may also play a major role in *cps* expression under conditions that have not been identified. Moreover, further study is needed to determine whether a regulatory interaction exists between RmpA, RmpA2 and RcsA.

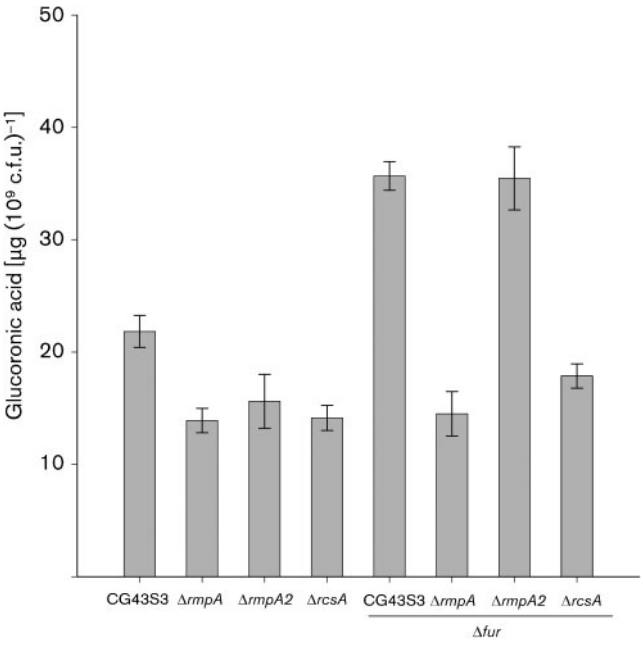


Fig. 2. Fur represses CPS biosynthesis via RmpA and RcsA. Bacterial strains were grown in LB medium at 37 °C with agitation. After 16 h growth, the bacterial glucuronic acid contents were determined. Values are mean ± SD of three independent experiments.

Consistent with the results shown in Fig. 2, the deletion effect of *fur* was eliminated in the Δ*fur*Δ*rmpA* or Δ*fur*Δ*rcsA* strains when the *orf1* and *orf16* transcripts were expressed (Fig. 3a, c). Deletion of *rmpA* from the Δ*fur* strain significantly decreased the level of all three *cps* transcripts. The quantities of the *cps* transcripts in Δ*fur*Δ*rmpA*Δ*rcsA* or Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar to that in the Δ*fur*Δ*rmpA* strain. These results further support the assumption that RmpA plays a major role in the Fur-mediated repression of *cps* transcription. By contrast, no apparent difference in *cps* expression was observed between Δ*fur* and Δ*fur*Δ*rmpA2*, indicating a minor role, if any, for RmpA2 in the Fur-mediated regulation of *cps* expression. Nevertheless, the much higher expression levels of *cps* that were observed in Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* than in strain Δ*rmpA*Δ*rmpA2*Δ*rcsA* suggest that an unknown regulator may be involved in the Fur-mediated control of *cps* expression.

Availability of iron affects CPS biosynthesis in *K. pneumoniae*

To determine whether Fur regulates gene expression in an Fe(II)-dependent manner (Andrews *et al.*, 2003; Escolar *et al.*, 1999), we analysed the effects of iron depletion and iron repletion on CPS biosynthesis. As shown in Fig. 4, the amount of CPS was increased in the Δ*fur* strain when the bacteria were grown in LB medium containing ~18 μM iron (Abdul-Tehrani *et al.*, 1999). The *fur* deletion effect was no longer observed in the *fur*-complemented strain, nor was it observed when Dip was added to the growth medium. In addition, the addition of 60 μM $FeSO_4$ to M9 medium caused an apparent decrease in the amount of CPS in the wild-type strain compared with that of wild-type strain grown only in M9 medium. The Δ*fur* strain grown in M9 medium both with and without $FeSO_4$ produced a higher amount of CPS than the wild-type strain, indicating that an iron level of approximately 2 μM in M9 medium (Abdul-Tehrani *et al.*, 1999) may be sufficient for Fur activity to repress CPS biosynthesis.

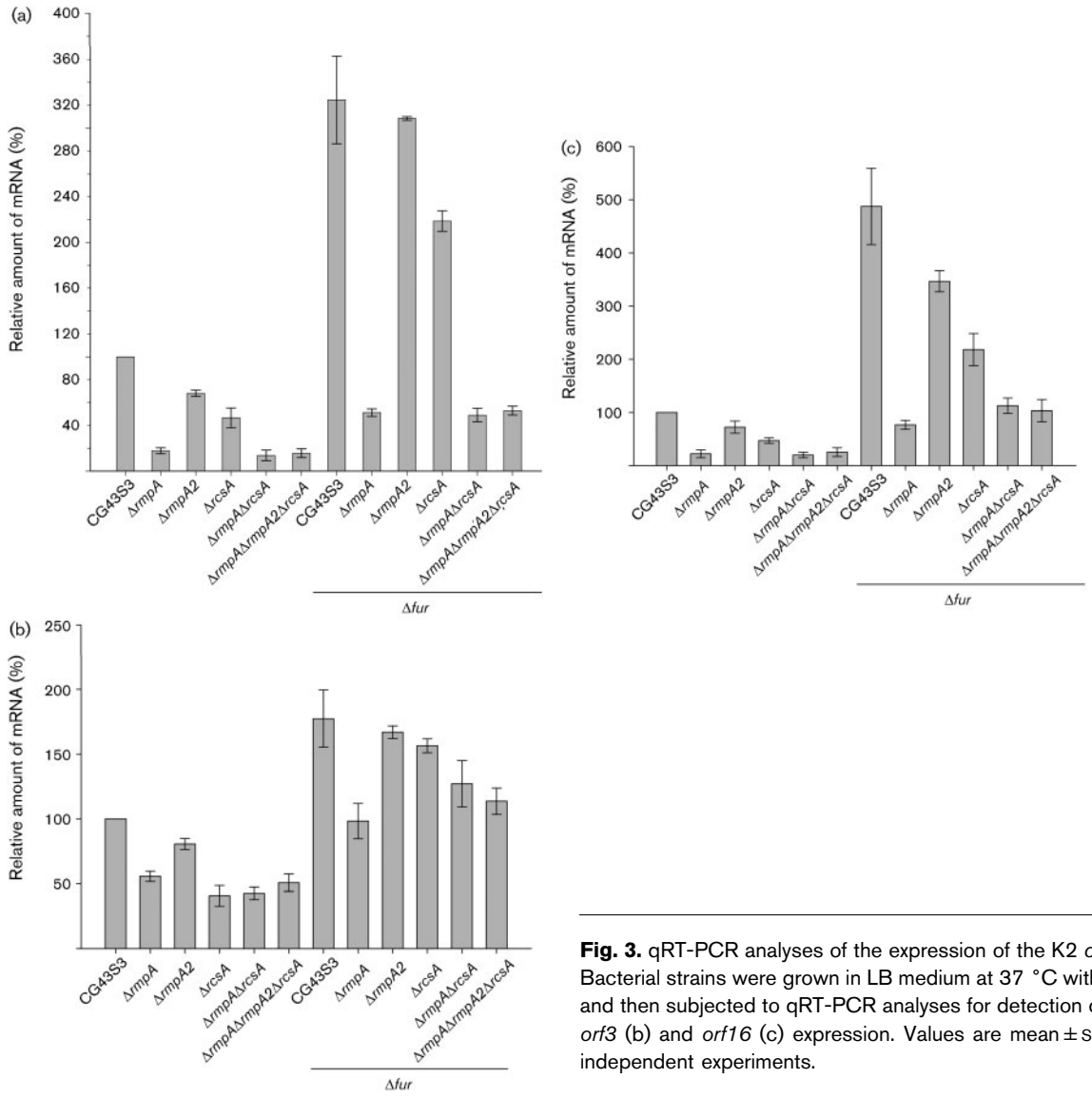


Fig. 3. qRT-PCR analyses of the expression of the K2 *cps* genes. Bacterial strains were grown in LB medium at 37 °C with agitation and then subjected to qRT-PCR analyses for detection of *orf1* (a), *orf3* (b) and *orf16* (c) expression. Values are mean ± SD of three independent experiments.

These results suggest that iron repletion increased Fur activity, thereby repressing the biosynthesis of CPS.

The regulatory role of Fur in iron-acquisition systems of *K. pneumoniae* CG43

To assess whether Fur affects iron acquisition in *K. pneumoniae* as in other bacteria, a CAS assay was performed to analyse siderophore secretion. As shown in Fig. 5(a), an orange halo around the colony of *K. pneumoniae* Δ*fur* strain grown on a blue CAS plate was observed. Introduction of the complement plasmid pfur into the Δ*fur* strain appeared to diminish the orange halo phenotype. A BLAST search with the DNA sequences of the iron-acquisition systems in *K. pneumoniae* NTUH-K2044 as templates (Hsieh *et al.*, 2008) for the homologues in the contig database of *K. pneumoniae* CG43 (unpublished results from Dr S.-F. Tsai) was subsequently performed. As shown in Table 3, eight putative iron-acquisition systems were identified. Expression of the genes (*iucA*, *fepA*, *fepB*, *entC*, *iroB*, *hmuR* and *feoB*), corresponding to five iron-acquisition systems assessed via qRT-PCR, were increased at least twofold in the Δ*fur* strain. Expression of the *fhuA*, *fecA*, *fecE* and *sitA* genes was also activated in the Δ*fur* strain, although with a less than twofold increase (Table 3).

As shown in Fig. 5(b), sequences with similarity to the *E. coli* Fur box (de Lorenzo *et al.*, 1987) could be identified in the putative promoters P_{iroB}, P_{entC}, P_{hmuR}, P_{feoA}, P_{fecA}, P_{fhuA} and P_{sitA}. A Fur box homologue was also found in the coding region of *iucA*, at position −4 to +15 relative to the start codon. These Fur-box-containing DNA fragments were then cloned into pT7-7, and the resulting plasmids were introduced individually into the *E. coli* indicator strain H1717. As shown in Fig. 5(c), *E. coli* H1717 harbouring a plasmid with P_{iucA}, P_{iroB}, P_{entC}, P_{hmuR}, P_{feoA} or P_{fecA} showed FURTA-positive phenotypes. However, the H1717 strains harbouring pT7-7 derivatives with the upstream regions of *fhuA* or *sitA* exhibited a FURTA-negative phenotype. The results suggest that Fur can bind to each of the predicted Fur

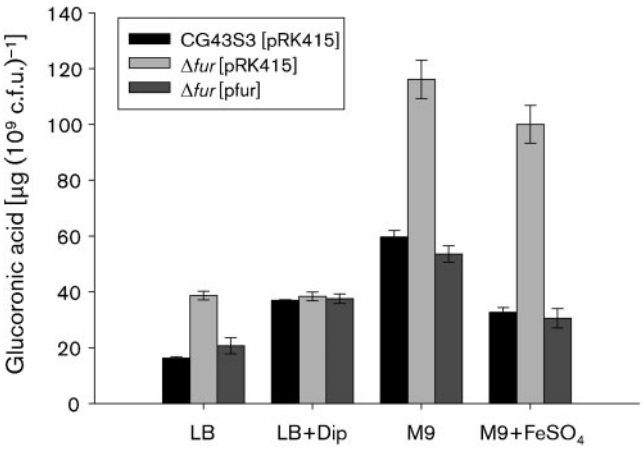


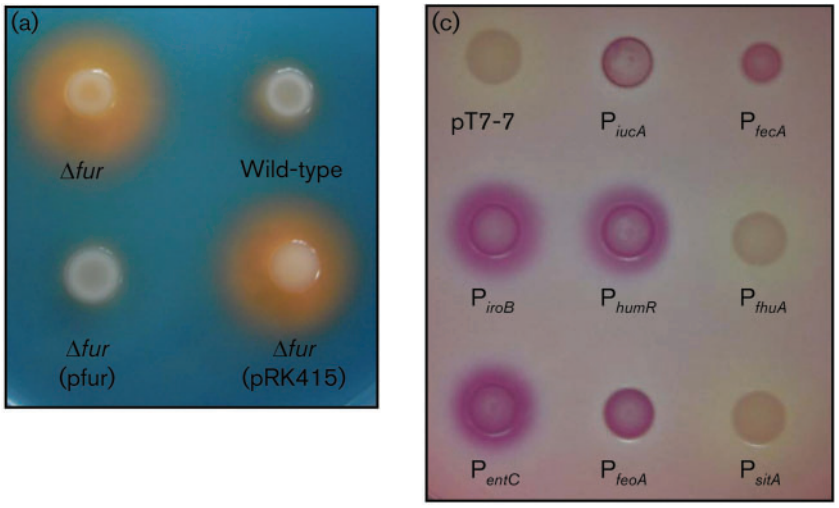
Fig. 4. Fur affects *K. pneumoniae* CPS biosynthesis in an Fe(II)-dependent manner. Bacteria were grown in media supplemented both with and without either 200 μM Dip or 60 μM $FeSO_4$ as indicated. After 16 h growth, the bacterial glucuronic acid contents were determined. Values are mean ± SD of three independent experiments.

box sequences on *iroB*, *entC*, *iucA*, *hmuR*, *feoB* and *fecA* to exert its regulatory function *in vivo*.

Extracellular Fe(II) has been demonstrated to be transported into bacteria via the iron-acquisition systems FeoABC and SitABCD (Cartron *et al.*, 2006; Sabri *et al.*, 2006). As shown in Fig. 5, expression of the *feo* gene but not the *sit* gene was affected by Fur. The *feoB* deletion mutant, which was predicted to have decreased the bacterial Fe(II) transport ability, was therefore generated to investigate whether the Fe(II)-dependent regulation of CPS biosynthesis is affected by the Feo system. However, no difference in the amount of CPS between the wild-type and Δ*feoB* strains, grown in both LB and M9 supplemented with various concentrations of Dip or $FeSO_4$, was found (data not shown). It is possible that SitABCD or other iron-acquisition systems are involved in the Fur–Fe(II)-dependent regulation of CPS biosynthesis, which may then compensate for the mutation effect of *feoB*.

DISCUSSION

We have demonstrated that Fur directly controls expression of the CPS regulators RmpA, RmpA2 and RcsA (Fig.



(b)

				Consensus
Typical Fur box		GATAAT:GATAAT:CATTATC		
P_{iroB}	-79	ACAAAT:GATAAT:CATTATC	-61	16/19
P_{entC}	-41	GATAAT:TATTCT:TAATATC	-23	15/19
P_{iucA}	-4	GATAAT:GGGAAT:CTTTATC	+15	16/19
P_{hmuR}	-124	GATAAT:ACCTAT:CATTACC	-106	15/19
P_{feoA}	-129	GATGAT:AAAAAC:CATTCTC	-111	15/19
P_{fecA}	-63	TGTAAT:GATAAC:CATTCTC	-45	15/19
P_{fhuA}	-198	CGTCAT:AATAAT:AATTCTC	-180	13/19
P_{sitA}	-99	GCAAAT:AAGAAT:TATTTTC	-81	13/19

Fig. 5. Fur regulation of iron acquisition in *K. pneumoniae* CG43. (a) Deletion of *fur* increases *K. pneumoniae* siderophore production, as assessed by using a CAS assay. Each bacterial strain assayed is indicated, and the orange haloes formed around the colonies correspond to the iron-chelating activity of siderophores in the bacteria. (b) DNA sequence alignment between the *E. coli* typical Fur box and the putative Fur boxes in the upstream regions of the eight iron-acquisition systems. Positions identical to the consensus sequences are underlined. (c) Assessment of the binding of Fur to the DNA sequences using FURTA. *E. coli* H1717 strains carrying the pT7-7 derivatives are indicated. Red colonies (Lac+) are denoted FURTA-positive phenotypes; pT7-7, FURTA-negative control.

Table 3. qRT-PCR analyses of the expression of iron-acquisition genes in *K. pneumoniae* wild-type and Δ*fur* strains

System	Gene	RNA expression ratio* (Δ*fur*: wild-type)	Reference
Fe^{3+}			
Ferrichrome	*fhuA*	1.73 ± 0.19	Ferguson *et al.* (1998)
Aerobactin	*iucA*	2.42 ± 0.18	Chen *et al.* (2004)
Enterobactin	*fepA*	2.11 ± 0.18	Nassif & Sansonetti (1986)
	fepB	2.25 ± 0.20	Nassif & Sansonetti (1986)
	entC	3.09 ± 0.15	Nassif & Sansonetti (1986)
Ferric citrate	*fecA*	1.61 ± 0.16	Braun & Mahren (2005)
	fecE	1.69 ± 0.26	Braun & Mahren (2005)
Salmochelin	*iroB*	6.28 ± 0.98	Chen *et al.* (2004)
Haem	*hmuR*	3.08 ± 0.65	Thompson *et al.* (1999)
Fe^{2+}			
Ferrous iron	*feoB*	4.08 ± 0.35	Cartron *et al.* (2006)
	sitA	1.97 ± 0.23	Sabri *et al.* (2006)

*Mean expression ratio (± SD) of *fur* mutant relative to wild-type parental strain CG43S3.

1). It has been reported previously that *fur* mutation does not produce an obvious change in *rmpA2* promoter activity, as assessed by the *lacZ* reporter system (Cheng *et al.*, 2010). By contrast, qRT-PCR analysis revealed that deletion of *fur* caused an approximately twofold increase in *rmpA2* mRNA (Fig. 1a). The discrepancy may be due to the dosage effect of the plasmid-based *lacZ* reporter system, which is known to overestimate β-galactosidase activity. The EMSA results shown in Fig. 1(c) also support the direct binding of Fur to the *rmpA2* promoter. The putative Fur box in the *rmpA2* promoter does not align as well as those in the *rmpA* and *rcsA* promoters with the *E. coli* Fur box (Fig. 1b), and hence it is possible that *K. pneumoniae* Fur exerts greater flexibility with respect to its recognition sequences and/or differences in its mode of action at the *rmpA2* promoter.

The two homologous genes *rmpA* and *rmpA2* are on plasmid pLVPK, and both encode CPS regulators for the activation of CPS biosynthesis (Chen *et al.*, 2004; Lai *et al.*, 2003). Compared with RmpA, RmpA2 has an extended N-terminal region and a different promoter sequence, implying that the two transcriptional factors are functionally different. As shown in Fig. 2, the deleting effect of *fur* was eliminated by the further deletion of *rmpA* or *rcsA*, but not of *rmpA2*, suggesting that these genes have different roles in the regulation of CPS biosynthesis. Further investigation is needed to clarify the roles of the two homologous regulators in *K. pneumoniae*.

Fur has been demonstrated to be a global regulator in many bacteria (Cornelis *et al.*, 2009; Mey *et al.*, 2005; Moore & Helmann, 2005). The deletion of *fur* in *Helicobacter pylori* was shown to reduce the expression of Lon protease (Choi *et al.*, 2009), which can affect the protein stability of RcsA and RmpA2 in *E. coli* and *K. pneumoniae* (Lai *et al.*, 2003; Trisler & Gottesman, 1984). However, *fur* deletion in *K. pneumoniae* CG43 has no obvious effect on the expression of *lon* (data not shown). The Fur protein sequences of *H. pylori* and *K. pneumoniae* have low identity (25.6 %), suggesting that the Fur regulatory circuit is different in the two bacteria.

The K2 *cps* gene cluster is predicted to encode proteins that are involved in the synthesis, transport, assembly and modification of CPS (Whitfield & Roberts, 1999). As shown in Fig. 3, the differential regulation exerted by RmpA, RmpA2 and RcsA on *cps* expression affects both the amount and the composition of CPS. Further studies are needed to investigate whether RmpA, RmpA2 and RcsA also affect CPS modifications, thus influencing the interactions between bacteria and host cells. The mutant Δ*fur*Δ*rmpA*Δ*rmpA2* Δ*rcsA* had a higher level of *cps* expression than the mutant Δ*rmpA*Δ*rmpA2*Δ*rcsA*, indicating that one or more unknown regulators besides RmpA, RmpA2 and RcsA may be involved in the Fur-mediated control of *cps* transcription. The complex regulation of *cps* expression in *K. pneumoniae* requires further exploration.

In *K. pneumoniae*, Fur regulates the expression of flavodoxin and CPS biosynthesis in addition to regulating its own expression (Achenbach & Genova, 1997; Achenbach & Yang, 1997; Cheng *et al.*, 2010). Here, we showed that Fur serves as a repressor in the regulation of at least eight iron-acquisition systems in *K. pneumoniae* CG43, although at different levels (Table 3). Analysis of the putative Fur boxes on *iroB*, *entC*, *hmuR*, *iucA*, *feo* and *fec* revealed high levels of identity to the consensus sequence (15–16 of 19 positions), whereas those of *fhuA* and *sitA* exhibited relatively lower identities (13 of 19 positions). This suggests that a highly conserved sequence of the 19 bp sequence is required for a positive FURTA phenotype. During infection, differential expression of the iron-acquisition system is anticipated to provide an adaptive advantage because of its flexibility in responding

to various environmental stimuli (Caza *et al.*, 2008; Valdebenito *et al.*, 2006). Therefore, it is suggested that the eight iron-acquisition systems in CG43 are coordinated differently. Whether CG43 harbours other iron-acquisition genes remains to be investigated.

In this study, we characterized the role of Fur in the CPS regulatory circuit of *K. pneumoniae* CG43, and found that RmpA, RcsA and RmpA2 are directly regulated by Fur. We also demonstrated that Fur regulates CPS biosynthesis via RcsA or RmpA, but not RmpA2, in an Fe(II)-dependent manner. Moreover, we report that a *fur* deletion affects the expression of the eight iron-acquisition systems identified in *K. pneumoniae* CG43.

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