1 Original Article

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3 # Fur regulation on the capsular polysaccharide biosynthesis and

4 # iron-acquisition systems in *Klebsiella pneumoniae* CG43

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33

## ABSTRACT

Ferric uptake regulator (Fur) has been reported to repress the expression of *rmpA*, a regulatory gene for the mucoid phenotype, leading to the decrease of capsular polysaccharide (CPS) biosynthesis in *Klebsiella pneumoniae* CG43. Here, quantitative real-time polymerase chain reaction (qRT-PCR) analyses and electrophoretic mobility shift assay 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 could suppress the deletion effect of Fur. The availability of extracellular iron affected the CPS amount suggesting that Fur regulates CPS biosynthesis in an Fe(II)-dependent manner. Increased production of siderophores was observed in the Δ*fur* strain suggesting the uptake of extracellular iron in *K. pneumoniae* is regulated by Fur. Fur titration assay 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. Thus, 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, bacteremia, septicemia, and urinary and respiratory tract infections, occurring particularly in immune-compromised 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. The pyogenic liver abscess isolates often carry heavy CPS that could protect the bacteria from phagocytosis and killing by serum factors (Lin *et al.*, 2004; Sahly *et al.*, 2000). Apart from the antiphagocytic 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).

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). Recently, we demonstrated that *cps* expression in *K. pneumoniae* CG43 is affected by the

1 coordinated action of the 2CSs KvgAS, KvhAS, and KvhR, whereas gene regulation
2 is independent of RcsB. (Lin *et al.*, 2006). Besides RcsA, the regulators RmpA and
3 RmpA2 also interact with RcsB for CPS biosynthesis regulation. Moreover, *rmpA*
4 expression was repressed by Fur, the global regulator for the expression of
5 iron-acquisition systems (Cheng *et al.*, 2010). Whether Fur affects RcsA or RmpA2 is
6 yet to be investigated.
7
8 Under iron-repletion conditions, dimeric Fur in complex with Fe(II) binds to a 19-bp
9 consensus DNA sequence, the Fur box (GATAATGATwATCATTATC; w=A or T), in
10 the promoters of the genes required for iron uptake, thereby preventing transcription
11 from these genes (Griggs & Konisky, 1989). The regulation helps bacteria to avoid
12 iron overload, which may lead to the formation of hydroxyl radicals. Multiple
13 iron-acquisition systems are commonly present in bacteria for the uptake of iron in the
14 environment (Andrews *et al.*, 2003). In an anaerobic environment, Fe(II) is prevalent
15 and is imported into the bacterial cytoplasm via the Feo system (Hantke, 2003).
16 However, in aerobic conditions and in mammalian tissues (*in vivo*), the majority of
17 iron is found as Fe(III), and iron *in vivo* is almost entirely sequestered by iron-binding
18 proteins (transferrin and lactoferrin) and hemoproteins (hemoglobin and myoglobin)
19 (Wandersman & Delepelaire, 2004).

1

2 Bacteria are generally equipped with iron/heme acquisition systems to directly
3 transport iron from the exogenous iron/heme sources or release siderophore and
4 hemophore compounds into the extracellular medium to scavenge iron/heme from
5 various sources (Wandersman & Delepelaire, 2004). In *K. pneumoniae* NTUH-K2044,
6 the expression of the ten putative iron-acquisition genes was highly up-regulated in
7 response to human serum, and bacterial virulence was decreased by the triple
8 mutation of siderophore genes (Hsieh *et al.*, 2008). The siderophore genes
9 *iucABCDiutA* and *iroNDCB* also have been reported to be the determinants of *K.*
10 *pneumoniae*-caused liver abscess (KLA) (Hsieh *et al.*, 2008; Koczura & Kaznowski,
11 2003; Tang *et al.*, 2010). Nevertheless, the regulation of iron-acquisition gene
12 expression in *K. pneumoniae* has not yet been studied.

13

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

17

## MATERIAL AND 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), kanamycin (25 μg/ml), streptomycin (500 μg/ml), and tetracycline (12.5 μg/ml).

**Construction of deletion mutants.** Specific gene deletions were introduced into *K. pneumoniae* CG43 using an allelic exchange strategy as previously described (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 polymerase chain reaction (qRT-PCR).** Total RNAs were isolated from bacteria cells grown to early exponential phase 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. Hundred nanogram of RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using random primers. qRT-PCR was performed in a Roche LightCycler® 1.5 Instrument using LightCycler TaqMan Master (Roche). Primers and probes were designed for selected target sequences using Universal

ProbeLibrary Assay Design Center (Roche-applied science) and are listed in Table 2. Data were analyzed using the real time PCR software of Roche LightCycler® 1.5 Instrument. Relative gene expressions were quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ 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 previously described (Cheng *et al.*, 2010). DNA fragments of the putative promoter regions of *rmpA*, *rmpA2*, and *rcsA* were PCR amplified 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/μl BSA at room temperature for 20 min. The samples were then loaded onto 5% native (nondenaturing) 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℃ for 2 hr. The gel was stained with SYBR Green EMSA stain (Invitrogen), and then visualized using the Safe Imager™ blue-light transilluminator.

**Extraction and quantification of CPS.** CPS was extracted and quantified as previously described (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 expressed as micrograms per $10^9$ CFU

1 (Blumenkrantz & Asboe-Hansen, 1973).

2 **Identification of the iron acquisition genes in *K. pneumoniae* CG43.** The ten genes

3 encoding different iron acquisition systems in *K. pneumoniae* NTUH-K2044 (Hsieh *et*

4 *al.*, 2008) were used as query sequences to search for homologs in *K. pneumoniae*

5 CG43 contig database (unpublished results from Dr. S.-F. Tsai, National Health

6 Research Institutes, Taiwan) as assessed by the BLAST search program

7 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

8 **Fur titration assay (FURTA).** FURTA was performed according to the method

9 described by Stojiljkovic *et al* (Stojiljkovic *et al.*, 1994). DNA sequences containing a

10 putative Fur box were PCR amplified with specific primer sets and then cloned into

11 pT7-7. The resulting plasmids were introduced into the *E. coli* strain H1717, and the

12 transformants were plated onto MacConkey-lactose plates containing 100 μg/ml

13 ampicillin and 30 μM $Fe(NH_4)_2(SO_4)_2$. The indicator strain H1717 contained a

14 chromosomal *fhuF::lacZ* fusion, and a low affinity Fur box has been demonstrated in

15 the *fhuF* promoter. The introduction of pT7-7 derived plasmids carrying Fur-binding

16 sequences could thus cause the removal of Fur from the *fhuF* Fur box (Hantke, 1987).

17 H1717 harboring pT7-7 was used as a negative control. Colony phenotype was

18 observed after incubation at 37°C for 10 h. Red colony (Lac+) denoted a

19 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 and Neilands (Schwyn & Neilands, 1987). Each of the bacterial strain was grown overnight in LB medium, and then 5 μl of culture was added onto a CAS agar plate. After 16 hr incubation at 37℃, 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.

**Statistical method.** 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 which is representative of three independent experiments. Each sample was assayed in triplicate and the average 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 the expression levels in *K. pneumoniae* CG43S3 and its isogenic Δ*fur* strain. As shown in Fig. 1A, 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*. Inclusion of the iron chelator 2, 2-dipyridyl (Dip) in the growth medium eliminated the effects caused by *fur* deletion, suggesting that a Fur-Fe(II) complex is involved in regulating the expression of *rmpA*, *rmpA2*, and *rcsA*. Although the expression of both *rmpA* and *rcsA* increased upon adding 200 μM Dip, *rmpA2* expression did not appear to change, suggesting a novel mechanism that requires further study.

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). We performed an EMSA to determine whether Fur directly affects the expression of *rmpA2* and *rcsA*. As shown in Fig. 1C,

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 ethylenediaminetetraacetic acid (EDTA) to the reaction mixture appeared to abolish the interactions (data not shown), indicating that the formation of Fur-Fe(II) complex was required for the specific binding.

Fur repressed 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* caused a reduction in the amount of bacterial CPS (Fig. 2). By contrast, a significant increase in CPS amount 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.

It has been reported that the K2 *cps* gene cluster of *K. pneumoniae* Chedid contains 19

1 open reading frames (ORFs) organized into three transcription units, *orf1-2*, *orf3-15*,

2 and *orf16-17* (Arakawa *et al.*, 1995). Analysis of the *cps* promoters revealed no

3 conserved Fur box, suggesting that Fur exerts indirect control over the transcription of

4 *cps*. To investigate this possibility, transcripts of *orf1*, *orf3*, and *orf16* in wild-type

5 (CG43S3), Δ*fur*, Δ*rmpA*, Δ*rmpA2*, Δ*rcsA*, Δ*fur*Δ*rmpA*, Δ*fur*Δ*rmpA2*, Δ*fur*Δ*rcsA*,

6 Δ*fur*Δ*rmpA*Δ*rcsA*, and Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* strains were measured using

7 qRT-PCR. As shown in Fig. 3A–C, all three transcripts were differentially decreased

8 in Δ*rmpA*, Δ*rmpA2*, and Δ*rcsA* strains. Compared to either the *rmpA* or *rcsA* deletions,

9 the deletion of *rmpA2* had less effect on the transcription of *orf1*, *orf3*, and *orf16.*

10 Interestingly, *rmpA* deletion had more profound reducing effects on the transcription

11 of *orf1* and *orf16* than *rcsA* deletion. Moreover, the *cps* expression levels in Δ*rmpA*,

12 Δ*rmpA*Δ*rcsA*, and Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar, suggesting a major regulatory

13 role of RmpA for controlling *cps* expression. However, RcsA and RmpA2 may also

14 play a major role in *cps* expression under conditions that have not been identified.

15 Moreover, further study is needed to determine whether a regulatory interaction exists

16 between RmpA, RmpA2, and RcsA.

17

18 Consistent with the results shown in Fig. 2, the deletion effect of *fur* was eliminated in

19 the Δ*fur*Δ*rmpA* or Δ*fur*Δ*rcsA* strains when the *orf1* and *orf16* transcripts were

1 expressed (Fig. 3A and C). Deletion of *rmpA* from the Δ*fur* strain significantly
2 decreased the level of all three *cps* transcripts. The quantities of the *cps* transcripts in
3 Δ*fur*Δ*rmpA*Δ*rcsA* or Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA* were similar to that of the Δ*fur*Δ*rmpA*
4 strain. These results further support the assumption that RmpA plays a major role in
5 the Fur-mediated repression of *cps* transcription. By contrast, no apparent difference
6 in *cps* expression was observed between Δ*fur* and Δ*fur*Δ*rmpA2*, indicating that a
7 minor role, if any, in the Fur-mediated regulation of *cps* expression. Nevertheless, the
8 much higher expression levels of *cps* that were observed in Δ*fur*Δ*rmpA*Δ*rmpA2*Δ*rcsA*
9 than the strain Δ*rmpA*Δ*rmpA2*Δ*rcsA* suggest that an unknown regulator may be
10 involved in the Fur-mediated control of *cps* expression.
11
12 Availability of iron affects CPS biosynthesis in *K. pneumoniae*
13 To determine whether Fur regulates gene expression in an Fe(II)-dependent manner
14 (Andrews *et al.*, 2003; Escolar *et al.*, 1999), we analyzed the effects of iron depletion
15 and iron repletion on CPS biosynthesis. As shown in Fig. 4, the CPS amount was
16 increased in the Δ*fur* strain when the bacteria were grown in LB medium containing
17 ~18 μM iron (Abdul-Tehrani *et al.*, 1999). The *fur* deletion effect was no longer
18 observed in the *fur*-complement strain, nor was it observed when Dip was added to
19 the growth medium. In addition, the addition of 60 μM $FeSO_4$ in M9 medium caused

an apparent decrease in the amount of CPS in the wild-type strain compared to 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. 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 analyze the activity of siderophore secreted. As shown in Fig. 5A, 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 using the DNA sequences of the iron-acquisition systems in *K. pneumoniae* NTUH-K2044 as templates (Hsieh *et al.*, 2008) for the homologs in the contig database of *K. pneumoniae* CG43 (unpublished results from Dr. S.-F. Tsai, National Health Research Institutes, Taiwan) was subsequently performed. As shown in Table 3, eight putative iron-acquisition systems were identified. Expression of the genes (*iucA*, *fepA*, *fepB*,

1 *entC*, *iroB*, *hmuR*, and *feoB*), corresponding to five iron-acquisition systems assessed
2 using qRT-PCR, were increased at least two-fold in Δ*fur* strain. Expression of *fhuA*,
3 *fecA*, *fecE*, and *sitA* genes was also activated in Δ*fur* strain, although with less than
4 two-fold increase (Table 3).
5
6 As shown in Fig. 5B, homologous sequences of the Fur box (de Lorenzo *et al.*,
7 1987) could be identified in the putative promoters $P_{iroB}$, $P_{entC}$, $P_{hmuR}$, $P_{feo}$, $P_{fec}$, $P_{fhu}$ and
8 $P_{sit}$. A Fur box homolog was also found in the coding region of *iucA*, at the position -4
9 to +15 relative to the start codon. These Fur box-containing DNA fragments were then
10 cloned into pT7-7, and the resulting plasmids were introduced individually into the *E.*
11 *coli* indicator strain H1717. As shown in Fig. 5C, the *E. coli* H1717 harboring the
12 plasmid with $P_{iucA}$, $P_{iroB}$, $P_{entC}$, $P_{hmuR}$, $P_{feo}$, or $P_{fec}$, showed FURTA-positive phenotypes.
13 While the H1717 strains harboring pT7-7 derivatives with the upstream regions of
14 *fhuA* or *sitA* exhibited a FURTA-negative phenotype. The results suggest that Fur can
15 bind to each of the predicted Fur box sequences on *iroB*, *entC*, *iucA, hmuR*, *feoB*, and
16 *fecA* to exert its regulatory function *in vivo*.
17
18 Extracellular Fe(II) has been demonstrated to be transported into bacteria via the iron
19 acquisition systems FeoABC and SitABCD (Cartron *et al.*, 2006; Sabri *et al.*, 2006).

1 As shown in Fig. 5, expression of the *feo* but not the *sit* genes was affected by Fur.
2 The *feoB* deletion mutant, which was predicted to decrease the bacterial
3 Fe(II)-transport ability, was therefore generated to investigate if the Fe(II)-dependent
4 regulation of CPS biosynthesis is affected by the Feo system. However, no difference
5 in CPS amount between the wild-type and Δ*feoB* strains, grown in both LB and M9
6 supplemented with various concentrations of Dip or $FeSO_4$, was found (data not
7 shown). It is possible that the SitABCD or other iron acquisition systems are involved
8 in the Fur-Fe(II)-dependent regulation on CPS biosynthesis, which may then
9 compensate the mutation effect of *feoB*.
10

1 **DISCUSSION**

2

3 In this study, we demonstrated that Fur direct controls the expression of the CPS
4 regulators RmpA, RmpA2, and RcsA (Fig. 1). It has been reported previously that *fur*
5 mutation does not produce an obvious change in *rmpA2* promoter activity, as assessed
6 by the *lacZ* reporter system (Cheng *et al.*, 2010). By contrast, qRT-PCR analysis
7 revealed that deletion of *fur* caused an approximately two-fold increase in *rmpA2*
8 mRNA (Fig. 1A). The discrepancy may be due to the dosage effect of the
9 plasmid-based *lacZ* reporter system, which is known to over-estimate β-galactosidase
10 activity. The EMSA results shown in Fig. 1C also support the direct binding of Fur to
11 the *rmpA2* promoter. Because the *rmpA2* promoter does not fit well with the Fur of *E.*
12 *coli*, it remains to be investigated whether *K. pneumoniae* Fur exerts less rigid
13 recognition sequences.

14

15 The two homologous genes *rmpA* and *rmpA2* are on pLVPK, and both encode CPS
16 regulators for the activation of CPS biosynthesis (Chen *et al.*, 2004; Lai *et al.*, 2003).
17 Compared to RmpA, RmpA2 has an extended N-terminal region and a different
18 promoter sequence, which implied that the two transcriptional factors are functionally
19 different. As shown in Fig. 2, the deleting effect of *fur* was eliminated by the further
20 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). Recently, 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 reveals 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 regulations exerted by RmpA, RmpA2, and RcsA on *cps* expression affect both the amount and composition of CPS. Further studies should 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

1 Δ*rmpA*Δ*rmpA2*Δ*rcsA*, indicating that one or more unknown regulators besides RmpA,
2 RmpA2, and RcsA may be involved in the Fur-mediated control of *cps* transcription.
3 The complex regulation of *cps* expression in *K. pneumoniae* requires further
4 exploration.
5
6 In *K. pneumoniae*, Fur regulates the expression of flavodoxin and CPS biosynthesis,
7 in addition to regulating its own expression (Achenbach & Genova, 1997; Achenbach
8 & Yang, 1997; Cheng *et al.*, 2010). Here, we showed that Fur serves as a repressor in
9 the regulation of at least eight iron-acquisition systems in *K. pneumoniae* CG43,
10 although at different levels (Table 3). Analysis of the putative Fur boxes on *iroB*, *entC*,
11 *hmuR*, *iucA*, *feo*, and *fec* revealed high identities to the consensus sequence (15-16 of
12 19 positions), whereas those of *fhuA* and *sitA* exhibited relatively lower identities (13
13 of 19 positions). This suggests that a highly conserved sequence of the nineteen base
14 pairs sequence is required for a positive FURTA phenotype. During infection,
15 differential expression of the iron-acquisition system is anticipated to provide an
16 adaptive advantage because of its flexibility in responding to various environmental
17 stimuli (Caza *et al.*, 2008; Valdebenito *et al.*, 2006). Therefore, it is predicated that the
18 eight iron-acquisition systems in CG43 are coordinated differently. Whether CG43
19 harbors other iron-acquisition genes remains to be further 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 a *fur* deletion effect on the expression of the eight iron-acquisition systems identified in *K. pneumoniae* CG43.

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## FIGURE LEGENDS

**Figure 1. Fur directly repressed 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 2, 2-dipyridyl (Dip), then the relative expression of *rmpA*, *rmpA2*, *rcsA*, *rcsB*, *kvgA*, and *kvhR* in bacteria were measured by qRT-PCR analysis. (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. DNAs of the upstream regions of *rmpA*, *rmpA2*, and *rcsA* were 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 imaged.

**Figure 2. Fur represses CPS biosynthesis via RmpA and RcsA.** Bacteria strains, as indicated in the margin, were grown in LB medium at 37℃ with agitation. After 16 hr growth, the bacterial glucuronic acid contents were determined. Values are mean ± standard error of three independent experiments.

**Figure 3. qRT-PCR analyses of the expression of the K2 *cps* genes.** Bacteria strains, as indicated in the margin, were grown in LB medium at 37℃ with agitation and then subjected to qRT-PCR analyses for the detection of *orf1* (A), *orf3* (B), and *orf16* (C) expression.

**Figure 4. Fur affects the *K. pneumoniae* CPS biosynthesis in a 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 hr growth, the bacterial glucuronic acid contents were determined. Values are mean ± standard error of three independent experiments.

**Figure 5. Fur regulation on iron acquisition in *K. pneumoniae* CG43.** (A) Deletion of *fur* increases the *K. pneumoniae* siderophore production assessed using CAS assay. Each bacterial strain assayed is indicated, and the orange halos formed around the colonies correspond to iron-chelating activity of siderophore in 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+) denoted FURTA-positive phenotypes. pT7-7, the FURTA-negative control.

## Table 1. Bacterial strains and plasmids used in this study

| Strains or plasmids | Descriptions | 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*] [*λpir* | (Skorupski & Taylor, 1996) |
| H1717 | *araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF::λ placMu* | (Hantke, 1987) |
| Plasmids | | |
| 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, | This study |

| | | |
|---|---|---|
| | cloned into pT7-7 | |
| pFT06 | 0.5 kb fragment containing the putative *rcsA* promoter, cloned into pT7-7 | This study |

2

## Table 2. Primers used in this study

| Primer | Sequence (5’→3’) | Enzyme cleaved | 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 | Sequence (5’→3’) | TaqMan probes | Target |
| 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 | | |
| GT13 | GTATTTTTATTCGCGATGTACTGC | 67 | *kvhR* |
| GT14 | GCCTGAACAGCGGAGAGA | | |

1 **Table 3. qRT-PCR analyses of the expression of iron acquisition genes in *K.***
2 ***pneumoniae*** **wild-type and Δ*fur* strains**
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| Systems | Gene | RNA expression ratio[a] 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) |
| Heme | *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) |

4 a. Mean expression ratio of *fur* mutant relative to wild-type parental strain CG43S3
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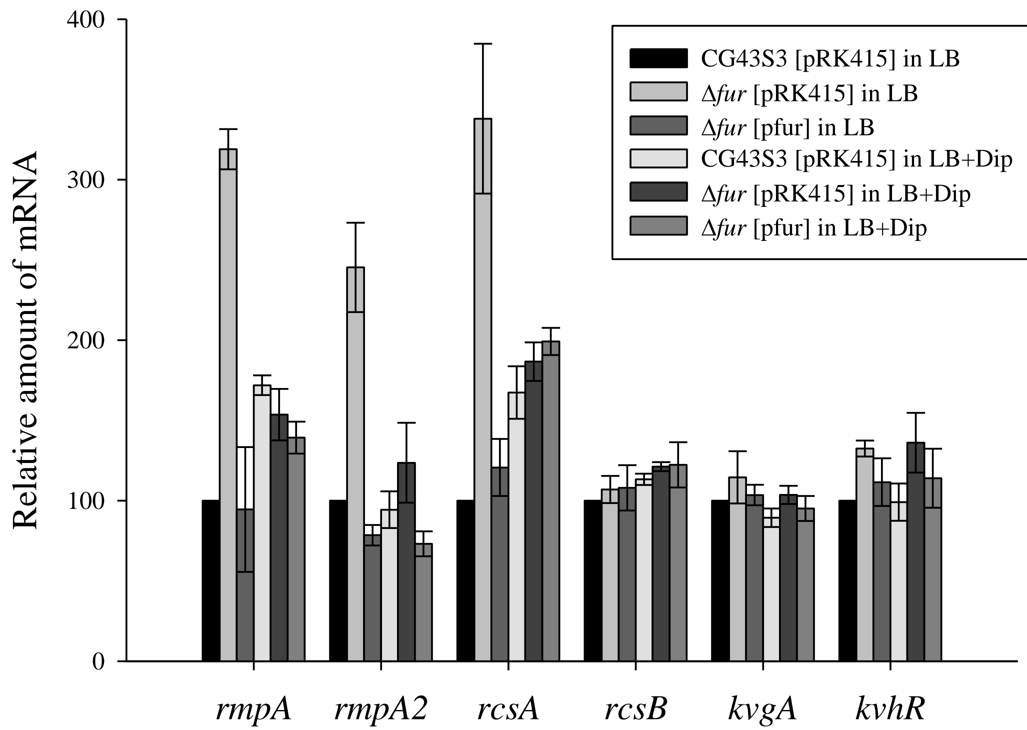
1 Fig. 1

2 (A)




3

4

(B)

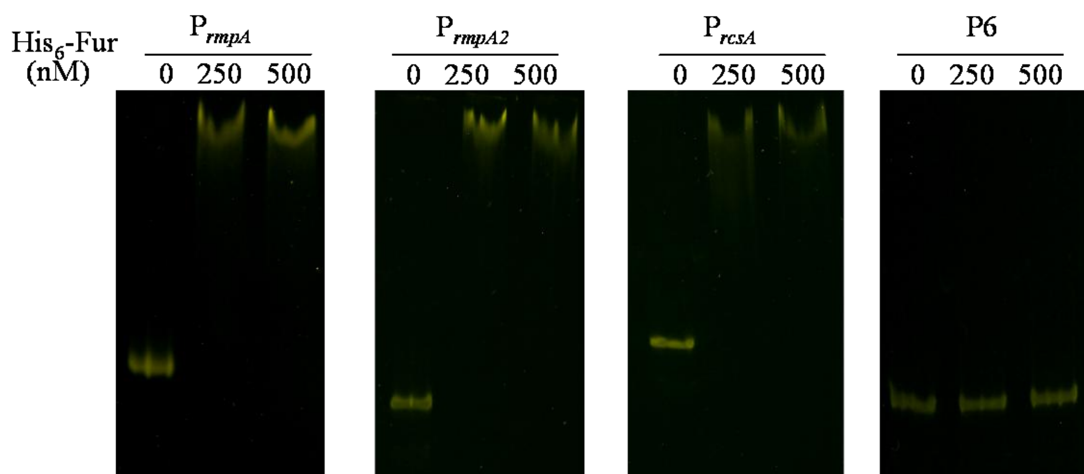
**Typical Fur box**

```
                   GATAAT:GATAAW:CATTATC
                   ||  ||    | |||   | |||
P rmpA     -202    GACAAA:AAAAAT:GACTATT        -184

                   GATAAT:GA-TAA:WCATT-ATC
                    |  |||  || |||  |  ||| |||
P rmpA2    -139    TAAAAT:GAATAA:TAATTCATC      -121

                   GATAAT:GATAAW:CATTATC
                    |  |     |   ||   ||||||
P rcsA      -86    TAAACC:TACTAT:TATTATC         -68
```

(C)



1 Fig. 2
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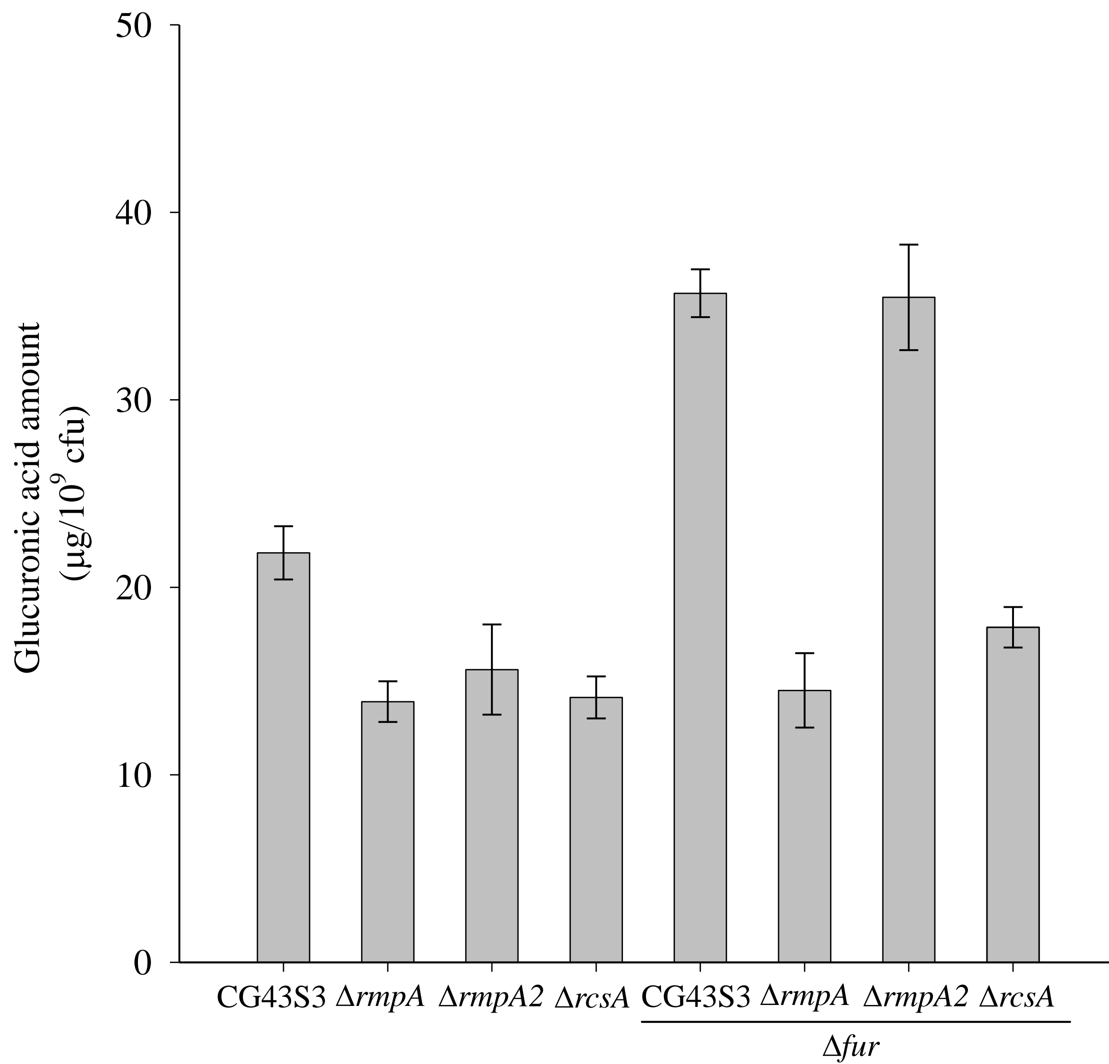
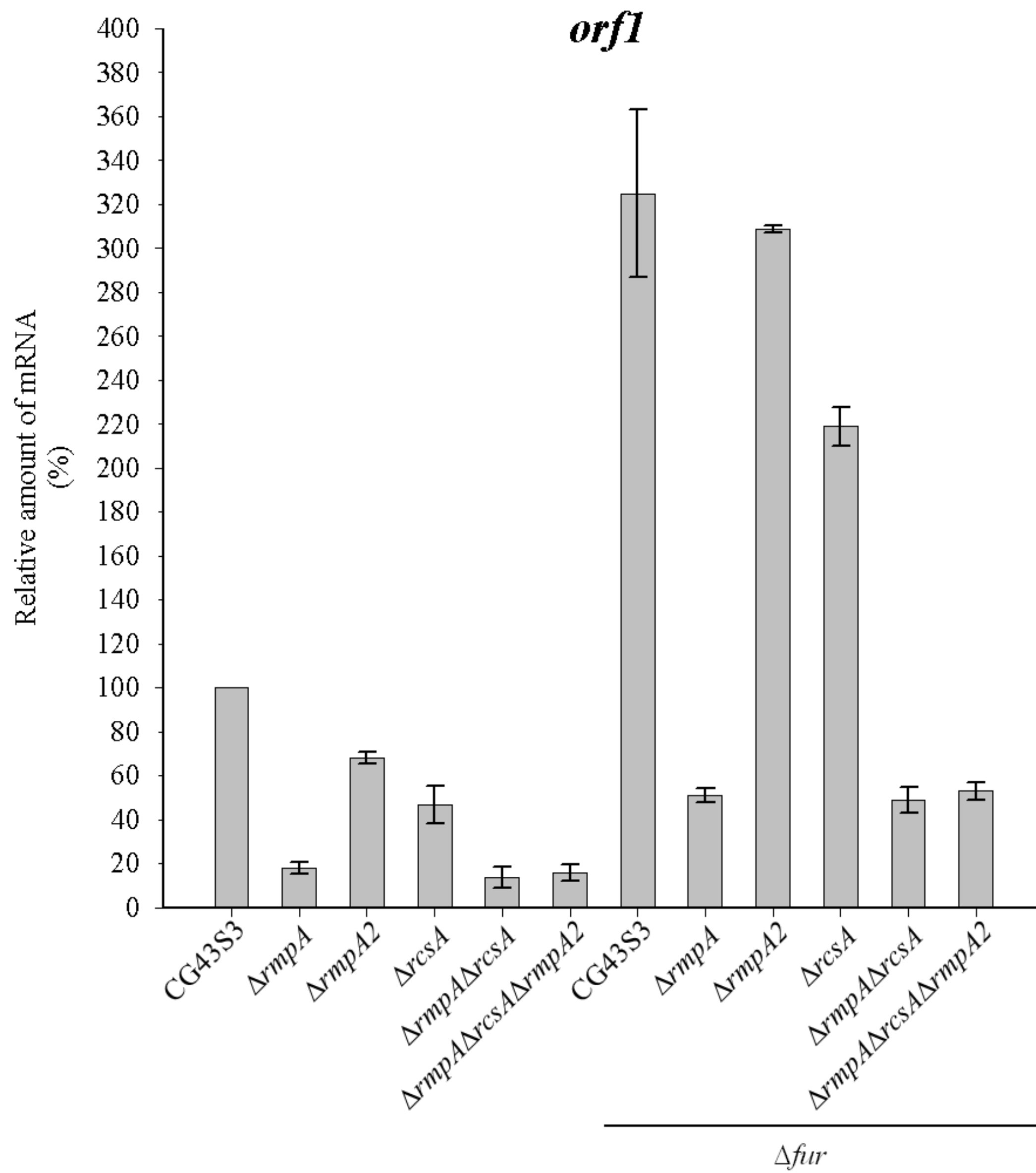


1 Fig. 3

2 (A)

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1 (B)

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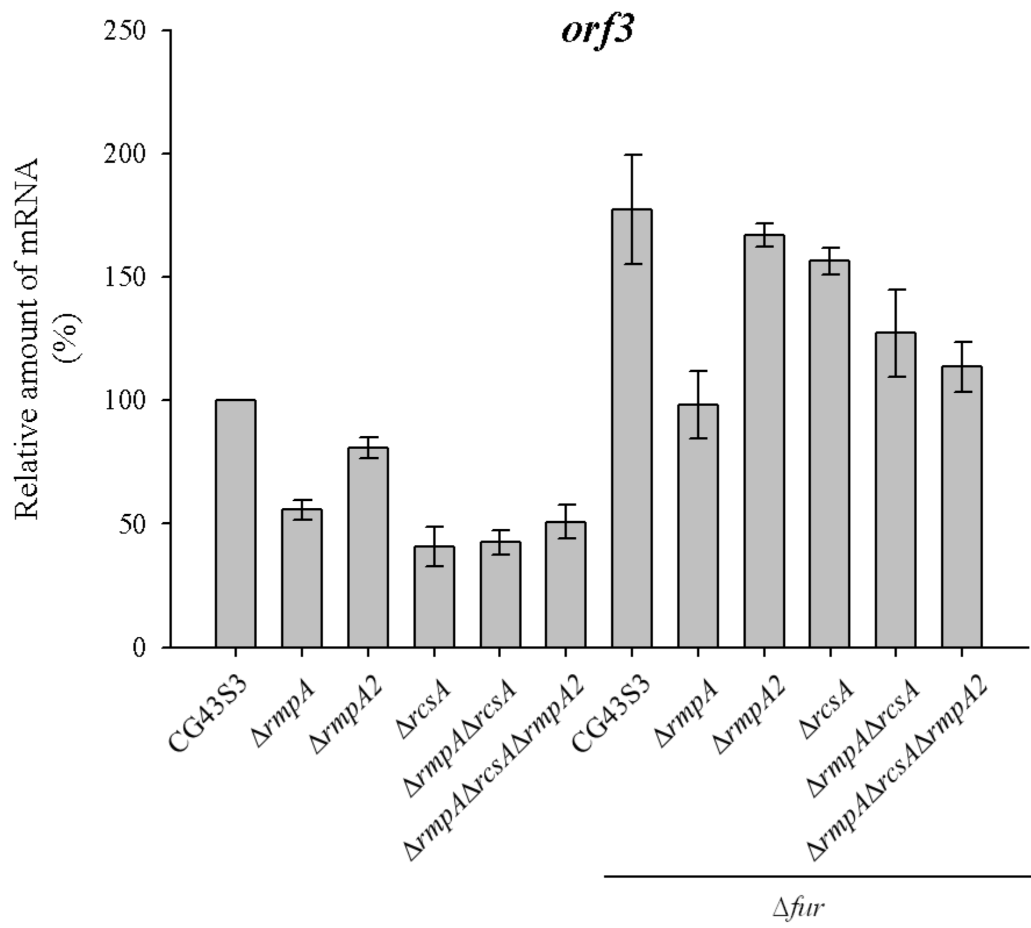


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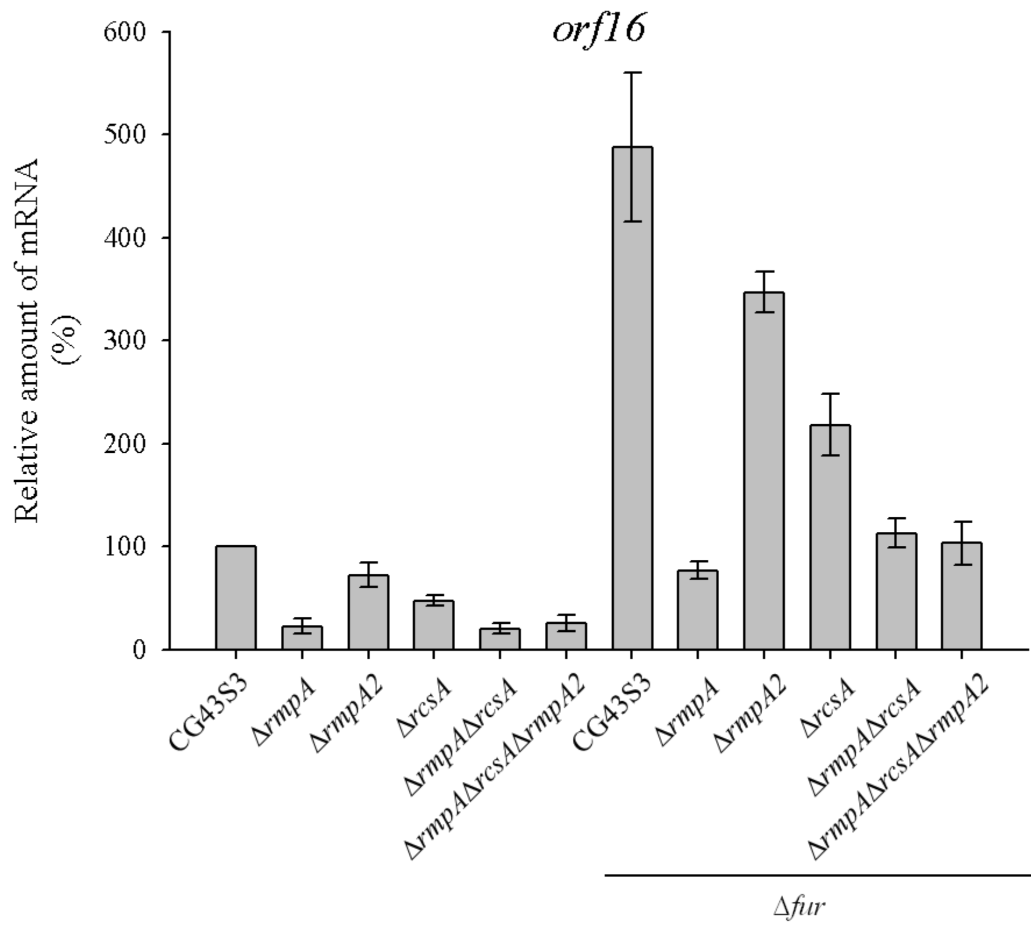
1 (C)

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1 Fig. 4
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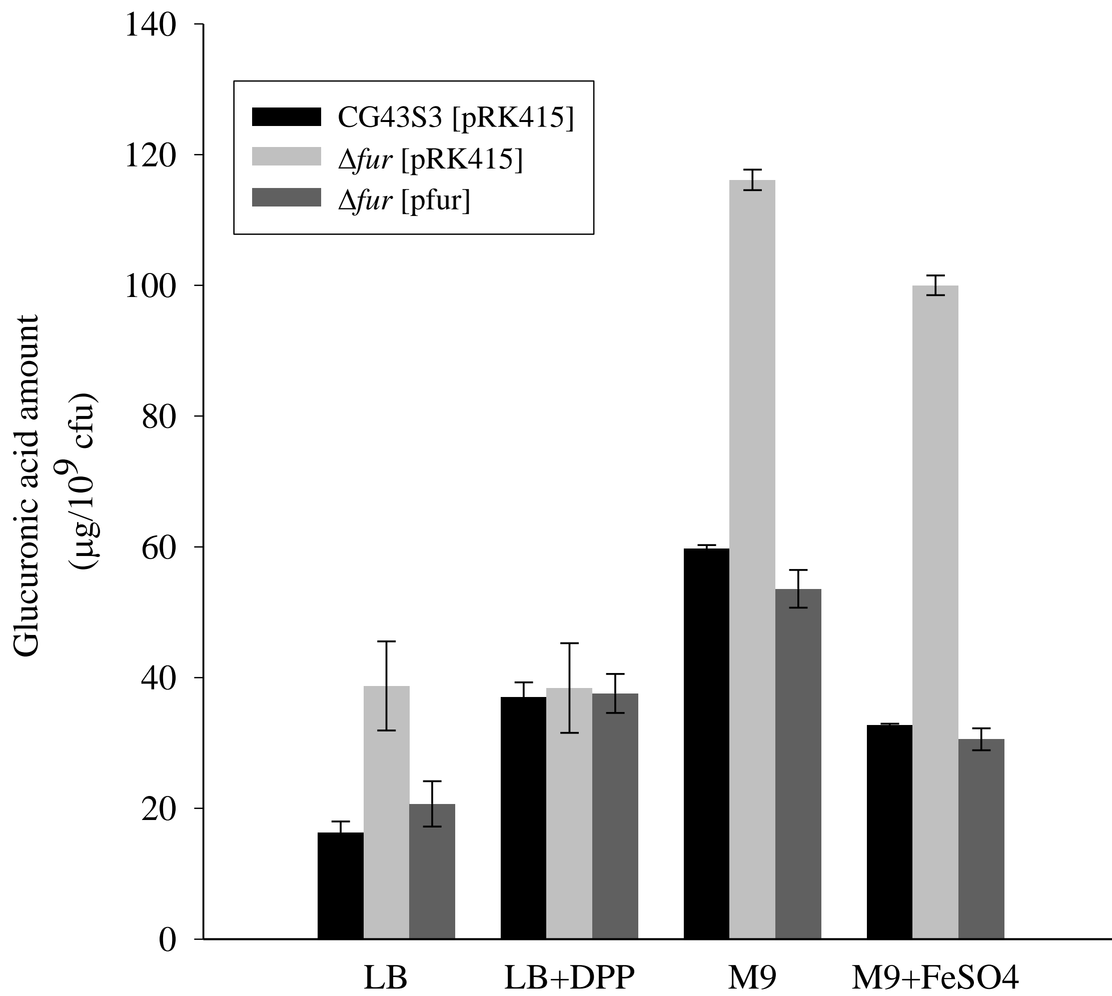
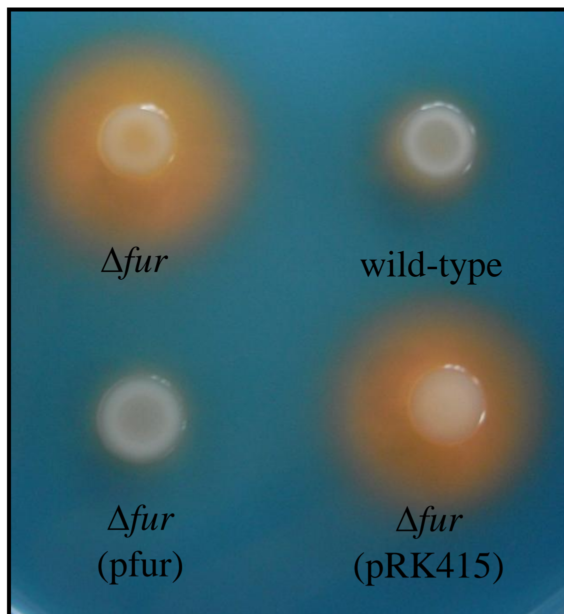
4



1 Fig. 5

2 (A)




3

4

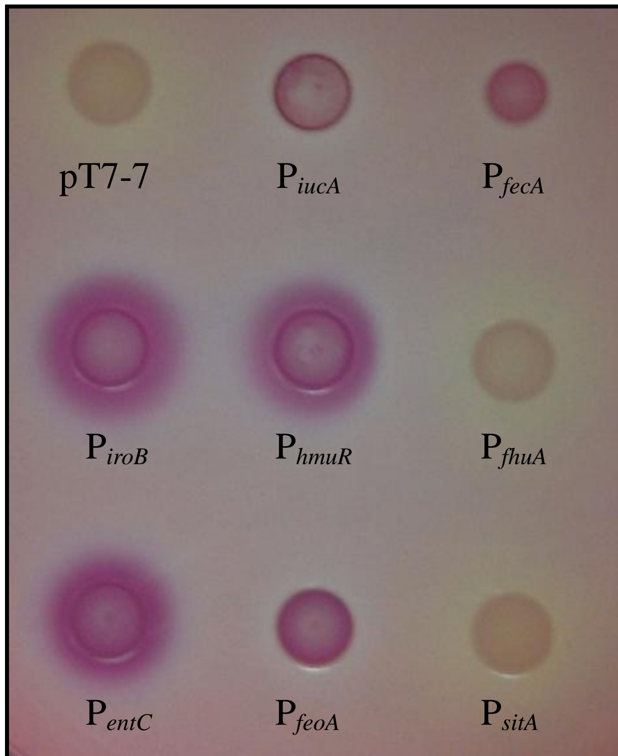
5 (B)

| | | Typical Fur box | | Consensus |
|---|---|---|---|---|
| | | 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 |

6

7

1 (C)




2

3