Role of the small RNA RyhB in the Fur regulon in mediating the capsular polysaccharide biosynthesis and iron acquisition systems in Klebsiella pneumoniae

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Abstract

Background: The capsular polysaccharide (CPS) and iron acquisition systems are important determinants of Klebsiella pneumoniae infections, and we have previously reported that the ferric uptake repressor (Fur) can play dual role in iron acquisition and CPS biosynthesis. In many bacteria, Fur negatively controls the transcription of the small non-coding RNA RyhB to modulate cellular functions and virulence. However, in K. pneumoniae, the role played by RyhB in the Fur regulon has not been characterised. This study investigated Fur regulation of ryhB transcription and the functional role of RyhB in K. pneumoniae.

Results: Deletion of fur from K. pneumoniae increased the transcription of ryhB; the electric mobility shift assay and the Fur-titration assay revealed that Fur could bind to the promoter region of ryhB, suggesting that Fur directly represses ryhB transcription. Additionally, in a Δfur strain with elevated CPS production, deletion of ryhB obviously reduced CPS production. The following promoter-reporter assay and quantitative real-time PCR of cps genes verified that RyhB activated orf1 and orf16 transcription to elevate CPS production. However, deletion of ryhB did not affect the mRNA levels of rcsA, rmpA, or rmpA2. These results imply that Fur represses the transcription of ryhB to mediate the biosynthesis of CPS, which is independent of RcsA, RmpA, and RmpA2. In addition, the Δfur strain’s high level of serum resistance was attenuated by the deletion of ryhB, indicating that RyhB plays a positive role in protecting the bacterium from serum killing. Finally, deletion of ryhB in Δfur reduced the expression of several genes corresponding to 3 iron acquisition systems in K. pneumoniae, and resulted in reduced siderophore production.

Conclusions: The regulation and functional role of RyhB in K. pneumoniae is characterized in this study. RyhB participates in Fur regulon to modulate the bacterial CPS biosynthesis and iron acquisition systems in K. pneumoniae.

Keywords: RyhB, Fur, Capsular polysaccharide, Iron acquisition system, Klebsiella pneumoniae
Background

*Klebsiella pneumoniae*, a member of *Enterobacteriaceae*, is a rod-shaped gram-negative opportunistic pathogen. A common cause of nosocomial infection, it is also found in various community-acquired infections, including bacteremia, septicemia, and urinary tract and respiratory infections, particularly in immunocompromised patients [1-4]. In Asian countries, especially Taiwan and Korea, *K. pneumoniae* is the predominant pathogen found in pyogenic liver abscess in diabetic patients [2,3,5]. The rapid development of antimicrobial resistance in *K. pneumoniae* has further troubled the clinical choices for treatments [6,7]. Studies of the pathogenic mechanisms of *K. pneumoniae* are, therefore, essential in identifying new targets for the development of antibacterial agents.

Multiple virulence factors have been identified to be involved in *K. pneumoniae* infection, which include capsular polysaccharide (CPS), lipopolysaccharides, fimbriae, iron-acquisition system, and antibiotic resistance. Among these factors, CPS is probably considered the major determinants of pathogenesis. The pyogenic liver abscess isolates often carry heavy CPS that could protect the bacteria from phagocytosis and killing by serum factors [8,9]. Apart from the antiphagocytic function, *Klebsiella* CPS also helps the bacterial colonization and biofilm formation at the infection sites [10-12]. The capsular serotypes of *K. pneumoniae* have been classified as more than 77 recognized capsular antigens [13,14]. In Taiwan, a high prevalence of K1 and K2 serotypes of *K. pneumoniae* was documented in liver abscess of diabetes mellitus patients [15]. The *cps* gene clusters that are responsible for the synthesis of different serotypes of CPS have been determined [16]. The K2 *cps* gene cluster of *K. pneumoniae* Chedid contains a total number of 19 open reading frames (ORFs) organized into three transcription units, *orf1*-2, *orf3*-15, and *orf16*-17 [16]. In the previous studies, numerous regulatory systems were demonstrated to control the biosynthesis of CPS via regulating the *cps* transcriptions in *K. pneumoniae*, such as the Rcs system, RmpA, RmpA2, KvhR, KvgAS, and KvhAS [17-20]. Among these, ferric uptake regulator (Fur) represses the gene expression of *rcsA*, *rmpA*, and *rmpA2* to decrease CPS biosynthesis [21,22]. Therefore, overlapping regulons governed the regulation of these assorted virulence genes in response to numerous stress conditions.

Bacterial cells are constantly challenged by various environmental stresses from their natural habitats. Similar to many gastrointestinal (GI) pathogens, *K. pneumoniae* faces several challenges during infection and colonisation of the human body. These include gastric acid, the immune system, and a limited supply of oxygen and nutrients [23,24]. Among these, the concentration of iron in the environment is critical for the control of cellular metabolism. Limitation of iron abolishes bacterial growth, but high intracellular concentrations of iron may damage bacteria because of the formation of undesired reactive oxygen species (ROS). Iron homeostasis maintained by the transport, storage, and metabolism of iron is tightly controlled by Fur in many gram-negative bacteria [25-27]. To regulate gene transcription, Fur protein functions as a dimer with Fe^{2+} as a cofactor to bind to a 19-bp consensus sequence, called the Fur box (GATAATGATwATTCATTAC; w = A or T), in the promoters of downstream genes [28]. In several gram-negative pathogens, Fur represses the expression of genes involved in iron homeostasis and in the regulation of multiple cellular functions such as oxidative stress, energy metabolism, acid tolerance, and virulence gene production [29-32]. In *K. pneumoniae*, Fur plays a dual role in controlling CPS biosynthesis and iron acquisition [21]. Recently, we also found that type 3 fimbriae expression and bacterial biofilm formation were also controlled by Fur and iron availability [33]. Therefore, the regulatory mechanism of Fur in control of multiple cellular function and virulence factors in *K. pneumoniae* needs to be further investigated.

Although Fur typically acts as a repressor, it also functions as a transcriptional activator for the gene expression such as *acnA*, *fumA*, and *sdhCDAB* (tricarboxylic acid [TCA] cycle enzymes), *bfr* and *ftnA* (iron storage), and *sodB* (iron superoxide dismutase [FeSOD]) [34-38]. However, positive regulation by Fur is often indirect, mediated by Fur-dependent repression of a small non-coding RNA (sRNA), RyhB [39]. RyhB negatively regulates gene expression by base pairing with mRNAs to trigger their degradation via RNAse E and RNAse III [40]. In many bacteria, RyhB participates in Fur-mediated positive regulation of various important cellular functions, including TCA cycle activity, resistance to oxidative stress, and iron homeostasis in *Escherichia coli* and *Vibrio cholerae* [35,39,41-43]; biofilm formation in *V. cholerae* [44]; and virulence in *Shigella dysenteriae* [45]. In *E. coli*, RyhB has been demonstrated to directly regulate more than 18 transcripts, encoding a total of 56 proteins, most of them involved in iron metabolism [35]. Although the significance of RyhB has been demonstrated in different species, to date, the regulatory relationship of RyhB and Fur, and functionality of RyhB in *K. pneumoniae* has not been studied.

In this study, the regulatory role of Fur in *ryhB* expression in *K. pneumoniae* was investigated. A *ryhB*-deletion mutant in wild type (WT) and Δfur strains and the induced expression of *ryhB* in WT were generated to demonstrate the role of RyhB in mediating CPS biosynthesis and iron acquisition systems.
Results

Fur directly represses ryhB expression in K. pneumoniae

To determine whether K. pneumoniae ryhB is regulated by Fur, a LacZ reporter system was used. The ryhB promoter was cloned into the upstream region of a promoterless lacZ gene in placZ15. The resulting plasmid pRyhB15 was then introduced into K. pneumoniae CG43S3 ΔlacZ and ΔlacZΔfur. The bacterial β-galactosidase activity was measured to assess the expression level of ryhB. As shown in Figure 1A, the expression of ryhB was higher in ΔlacZΔfur than ΔlacZ. Introduction of the complement plasmid pfur, but not the empty vector control (pRK415), into ΔlacZΔfur restored the Fur-deletion effect. Moreover, addition of the iron chelator 2, 2-dipyridyl (Dip) to the growth medium increased ryhB promoter activity, suggesting that a Fur-Fe(II) complex influences ryhB expression. To verify that Fur directly regulates the expression of ryhB, an electrophoretic mobility shift assay (EMSA) was performed. As shown in Figure 1B, purified recombinant His6::Fur protein was able to bind the upstream region of ryhB (P

Figure 1 Fur directly represses the expression of ryhB. (A) The β-galactosidase activities of the K. pneumoniae CG43S3ΔlacZ strain and the isogenic fur deletion mutant carrying pRyhB15 (P

DNA was incubated with an increasing amount of His6::Fur for 30 min, and then loaded onto a 5% non-denaturing polyacrylamide gel. The gel was stained with SYBR Green EMSA stain and photographed. P

(C) Assessment of the binding of Fur to the ryhB promoter by using the FURTA. E. coli H1717 strains carrying the vector control, pT7-7, or the P1 region harboured on pT7-7 are indicated. A red colony (Lac+) is considered to have a FURTA-positive phenotype.
when harbouring a plasmid containing *K. pneumoniae* P$_{ryhB}$ also showed a Fur titration assay (FURTA)-positive phenotype (Figure 1C). The results suggest that, in an iron dependent manner, Fur suppresses *ryhB* promoter activity in *K. pneumoniae* by direct interaction with the Fur-box region upstream of *ryhB*.

**RyhB activates CPS biosynthesis**

In *K. pneumoniae* CG43, we found that the deletion of *fur* resulted in elevated CPS production [21,22]. To investigate if RyhB participates in Fur-regulated CPS biosynthesis, the CPS amount was assessed using measuring glucuronic acid content, which served as an indicator for *Klebsiella* glucuronic acid content, which served as an indicator for investigate if RyhB participates in Fur-regulated CPS biosynthesis. To investigate whether RyhB affects the expression of RyhB increased the transcriptional level of the K2 CPS strain CG43 increased CPS production, which confirms that RyhB positively regulates CPS biosynthesis. In previous studies, *K. pneumoniae* Fur was found to repress the expression of genes encoding the *cps* gene clusters. To further investigate whether RyhB acts as a transcriptional activator for the promoter activity of *orf1*, *orf3*, and *orf16*, the reporter plasmids pOrf12 (P$_{orf1-2}$::lacZ), pOrf315 (P$_{orf3-15}$::lacZ), and pOrf1617 (P$_{orf16-17}$::lacZ), each carrying a lacZ reporter gene transcriptionally fused to the putative promoter region of the K2 *cps* gene cluster [17], were used to transform the *K. pneumoniae* strains CG43S3ΔlacZΔfur and ΔlacZΔfurΔryhB. The promoter activity measurements shown in Figure 3C revealed that the deletion of *ryhB* in ΔlacZΔfur reduced activity of P$_{orf1-2}$::lacZ by at least 50%, while no obvious change was detected in the activity of P$_{orf3-15}$::lacZ. The activity of P$_{orf16-17}$::lacZ was reduced by more than 75% in ΔlacZΔfurΔryhB as compared to the ΔlacZΔfur strain. These results imply that RyhB enhances CPS biosynthesis in *K. pneumoniae* by boosting the transcriptional level of the *orf1* and *orf16* gene clusters.

**RyhB increased the transcriptional level of the K2 cps gene cluster**

To investigate whether RyhB affects the expression of the three *cps* gene clusters, the mRNA levels of *orf1*, *orf3*, and *orf16* in Δfur and ΔfurΔryhB strains were measured by quantitative real-time PCR (qRT-PCR). As shown in Figure 3A, compared to the mRNA levels in the Δfur strain, the mRNA levels of *orf1* and *orf16* were apparent decreased in the ΔfurΔryhB strain, and that of *orf3* also had a slight reduction in the ΔfurΔryhB strain. The result suggests that overexpression of RyhB activated the *cps* gene expression. To confirm our hypothesis, the effect of *ryhB* induction on the mRNA levels of *orf1*, *orf3*, and *orf16* was tested using an IPTG-inducible vector, pETQ. As shown in Figure 3B, the mRNA levels of *orf1* and *orf16* were higher in the pETQ-ryhB strain with IPTG induction than the pETQ mock strain, while no significant difference in *orf3* expression was observed.

**RyhB does not affect the rcsA, rmpA2, and rmpA mRNA expression level**

In previous studies, *K. pneumoniae* Fur was found to repress the expression of genes encoding the *cps*

![Figure 2](http://www.biomedcentral.com/1471-2180/12/148)
regulatory proteins RcsA, RmpA, and RmpA2 [21,22]. To investigate whether RyhB affects the expression of rcsA, rmpA, and rmpA2 to increase the orf1 and orf16 transcripts, the mRNA levels were measured by qRT-PCR after inducing the expression of ryhB in WT. However, qRT-PCR results did not show a significant effect of ryhB on the mRNA levels of rmpA, rmpA2, and rcsA (Data not shown), suggesting that the activation of RyhB on the orf1 and orf16 expression is not via RmpA, RmpA2, and RcsA.

Deletion of ryhB attenuated the higher serum resistance in Δfur strain

In addition to the roles played by RyhB and Fur in regulating the CPS amount, we suggest that RyhB and Fur may also affect the ability of the strain to resist the bactericidal effects of serum. In a human serum resistance assay, we found that the deletion of fur in WT increased the survival rate in treatment with 75% normal human serum from 63.3% to 87.9% (Figure 4). However, the deletion of ryhB in WT had no apparent effect on the survival rate under the growth condition that ryhB is poorly expressed. Our results suggest that in the regulation of iron-acquisition systems, RyhB plays a role downstream of Fur in K. pneumoniae.

The regulatory role of RyhB in iron-acquisition systems

To assess whether RyhB affects iron-acquisition in K. pneumoniae, the Chrome azurol S (CAS) assay was used to measure siderophore secretions in Δfur and ΔfurΔryhB strains (Figure 5). When bacteria were grown in M9 minimal medium (~2 μM iron) to mimic iron-limited condition, the deletion of ryhB in Δfur reduced the formation of the orange halo. However, this change was not observed when bacteria were grown in LB medium (~18 μM iron). Compared to M9 minimal medium contains ~2 μM iron, LB medium is considered an iron-repletion medium. Under iron-repletion, Fur is able to exert its repression on ryhB transcription. Thus, ryhB-deletion effect is difficult to observed under the growth condition that ryhB is poorly expressed. Our results suggest that in the regulation of iron-acquisition systems, RyhB plays a role downstream of Fur in K. pneumoniae under iron-limiting conditions.

To investigate the effects on downstream targets of RyhB in iron-acquisition regulons, the expression of genes corresponding to the eight putative iron-acquisition systems in K. pneumoniae CG43 was measured in Δfur and ΔfurΔryhB by qRT-PCR (Table 1).

Figure 3 RyhB activates the transcriptional level of the orf1 and orf16. (A) qRT-PCR analyses of the expression of the K2 cps genes (orf1, orf3, and orf16) were measured in Δfur and ΔfurΔryhB strains. (B) WT strain carrying the IPTG inducible vector pETQ and pETQ-ryhB in response to 100 μM IPTG. (C) The β-galactosidase activities of K. pneumoniae CG43ΔlacZΔfur and ΔlacZΔfurΔryhB carrying the reporter plasmid pOrf12 (Porf1-2::lacZ), pOrf15 (Porf15-15::lacZ) or pOrf1617 (Porf1617-17::lacZ) were determined using log-phased cultures grown in LB broth. The results shown are an average of triplicate samples. Error bars indicate standard deviations.

Figure 4 Effect of Fur and RyhB on susceptibility to normal human serum. Survival percentage of WT, ΔryhB, Δfur, ΔfurΔryhB, and ΔgalU (negative control) strains on treatment with 75% healthy human serum was determined, respectively. The results shown are an average of triplicate samples. Error bars indicate standard deviations.
that RyhB activates the expression of $iucA$, $fepA$, $fepB$, $entC$, $fecA$, and $fecE$, but represses the expression of $fhuA$ and $sitA$.

### Discussion

In this study, we provide an initial characterisation of *K. pneumoniae* RyhB. In *K. pneumoniae*, sequence comparison indicated that the nucleotide sequence of the $ryhB$ gene (91 bp) is 92.3% identical to the *E. coli* version (90 bp). However, the promoter sequence of *K. pneumoniae* $ryhB$ is only 72.4% identical to that of *E. coli*. In this study, we found that the expression of $ryhB$ in *K. pneumoniae* is directly repressed by Fur-Fe(II), as is the case in *E. coli* (Figure 1).

In addition, structure of the genomic neighbourhood of $ryhB$ differs between the 2 species. In the *E. coli* genome, $ryhB$ is found between $yhhX$ and $yhhY$. In the *K. pneumoniae* genome, $ryhB$ is flanked by $yhlY$ and a hypothetical ORF. By Pfam search, the hypothetical ORF was found to contain a bactofilin domain (E-value = 3.7e-24), which belongs to a new class of polymer-forming proteins that serve as versatile molecular scaffolds in a variety of cellular pathways [47]. Even though the function of this hypothetical protein in *K. pneumoniae* has not yet been investigated, we found that RyhB could strongly repress the expression of this hypothetical protein (unpublished data). This result suggests that RyhB could participate in a variety of cellular pathways in *K. pneumoniae*.

We previously showed in *K. pneumoniae*, Fur represses CPS biosynthesis via regulation of RmpA, RmpA2, and RcsA. In addition to these 3 regulators, one or more regulators may be involved in the Fur-mediated control of *cps* transcription [21]. In this study, we found that RyhB also participates in Fur-regulated CPS biosynthesis via activation of *orf1* and *orf16* transcription and is independent of the 3 regulators, RmpA, RmpA2, and RcsA (Figure 2 and 3). We want to further analyse whether any potential transcriptional regulator-binding motifs exist in the promoter sequences of *orf1* and *orf16*. We noted that a binding site typical of IscR, a transcriptional repressor that controls Fe–S biosynthesis [48], was located 172 bp upstream of the translation start site of GalF (encoded by *orf1*, 5′-ATAACCTGAAACGAAAAAATAGATTAT-3′). The predication indicated that IscR could participate in control of *orf1* expression. Furthermore, a previous study reported that RyhB promotes the degradation of *iscSUA* transcripts, resulting in an increase in the ratio of apo-IscR/holo-IscR [48]. Whether RyhB activates CPS biosynthesis via regulation of the ratio of apo-IscR/holo-IscR in *K. pneumoniae* awaits further analysis. However, the regulatory mechanism of *cps* transcription is more complex than expected; whether another unknown transcriptional regulator is involved in

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**Table 1** qRT-PCR analyses of the expression of iron-acquisition genes in *K. pneumoniae* Δfur ΔryhB and Δfur strains

| Systems         | Gene     | RNA expression ratio $^a$ ΔfurΔryhB/Δfur |
|-----------------|----------|-----------------------------------------|
| Fe$^{3+}$       | $fhuA$   | 2.62 ± 0.07                             |
| Ferrichrome     | $iucA$   | 0.19 ± 0.06                             |
| Aerobactin      | $fepA$   | 0.36 ± 0.01                             |
| Enterobactin    | $fepB$   | 0.33 ± 0.05                             |
| EntC            | $entC$   | 0.46 ± 0.02                             |
| Ferric citrate  | $fecA$   | 0.19 ± 0.02                             |
| $fecE$          |          | 0.34 ± 0.03                             |
| Salmochelin     | $iroB$   | 0.52 ± 0.05                             |
| Heme            | $hmuR$   | 0.69 ± 0.01                             |
| Fe$^{2+}$       | $fcoB$   | 0.55 ± 0.18                             |
|                 | $sitA$   | 2.81 ± 0.08                             |

$^a$ Mean expression ratio (±SD) of ΔfurΔryhB relative to Δfur.
activation of RyhB’s effect on orf16 transcription needs to be investigated. In addition, CPS is considered the major determinant that can protect the bacteria from phagocytosis and killing by serum factors [8,9]. In this study, higher serum resistance was found in Δfur, but this higher serum resistance was attenuated by further deletion of ryhB (Figure 4). We suggest the protective role of RyhB against serum killing is due to the activation of CPS biosynthesis.

In E. coli, RyhB plays a positive role in control of the intracellular iron concentration via the degradation of nonessential iron-using proteins or an increase in siderophore production [49-51]. In this study, we also found the deletion of ryhB in Δfur decreased siderophore production on the CAS plate under iron-limiting condition (Figure 5). Consistent with E. coli [51], RyhB in K. pneumoniae regulates siderophore production by activating the expression of enterobactin system genes (entC, fepA, and fepB). In addition, we found that RyhB may activate iucA and fecA expression. Since sRNA may positively regulate its target mRNAs via an anti-antisense mechanism to disrupt an intrinsic inhibitory structure in the 5’ mRNA region that sequesters the ribosome-binding site and the first translation codon [52,53], the 5’-untranslated regions of the iuc and fec operons were analysed for sequences complementary to RyhB by prediction with the bioinformatics application RNAhybrid [54] (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). However, no apparent base pairing was found in the 5’-untranslated region of the iuc or fec operons, suggesting that the activation of iucA and fecA by RyhB is not a result of direct interaction. Furthermore, RyhB was found to repress the expression of fluA and sitA in K. pneumoniae. In E. coli, RyhB represses the expression of fluA, which also corresponds to our results [35]. A possible paring between RyhB with the adjacent sequence of translational start site of fluA and sitA was also predicted by the RNAhybrid algorithm. Alignment of the protected residues suggests that RyhB forms a 7 + 4 + 4 bp RNA duplex with the sitA mRNA (Additional file 1: Figure S1), but no apparent base pairing was found between RyhB and fluA. However, the direct interaction of RyhB with the sitA mRNA remains to be confirmed. In E. coli, RyhB has been shown to repress several genes that are involved in iron-binding, which may increase the intracellular iron concentration, thereby allowing a better usage of iron and more complete Fur repression of these genes [35,55]. Nevertheless, this possibility in K. pneumoniae needs to be proven by careful experiments. In this study, the coordinated action of Fur and RyhB was found to regulate the expression of the iron acquisition systems for maintaining intracellular iron homeostasis in K. pneumoniae.

Conclusions
In this study, we provide an initial characterisation of K. pneumoniae RyhB. Our results suggest that RyhB plays an important role in the Fur regulon, which modulates the CPS biosynthesis and iron acquisition systems in K. pneumoniae, both of which contribute to the infectivity and survival of the bacterium.

Methods
Bacterial strains, plasmids, and media
Bacterial strains and plasmids used in this study are listed in Table 2. Primers used in this study are list in Additional file 2: Table S1. Bacterial 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 the gene-deletion mutants
Specific gene deletion was introduced into K. pneumoniae CG43S3 using an allelic exchange strategy as previously described [57]. The pKAS46 system was used in the selection of the mutants [59], and the mutations were respectively confirmed by PCR and Southern hybridization (data not shown).

Measurement of promoter activity
The promoter region of ryhB was PCR-amplified with primer pair pGT44/pGT45, and the amplicons were then cloned into placZ15 [63]. The promoter-reporter plasmids, pRyhB15, pOrf12, pOrf315, and pOrf1617, were individually mobilized into K. pneumoniae strains by conjugation from E. coli S17-1 λpir. The bacteria were grown to logarithmic phase in LB broth with or without 200 μM Dip (OD600 of 0.7), and the β-galactosidase activity was measured as previously described [63].

EMSA
Recombinant K. pneumoniae Fur protein was expressed in E. coli and purified as previously described [22]. DNA fragment of the putative promoter region of ryhB was respectively PCR amplified by using specific primer sets (Table 2). The purified His6-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 MnCl2, and 1 μg/μl BSA at room temperature for 20 min. The samples were then loaded onto a native gel of 5% nondenaturing polyacrylamide containing 5% glycerol in 0.5x TB buffer (45 mM Tris–HCl, pH 8.0, 45 mM boric acid). Gels were electrophoresed with a 20-mA current at 4°C and then stained with SABR safe Gel stain (Invitrogen).
FURTA was performed according to the method described by Stojiljkovic et al. [64]. DNA sequences containing a putative Fur box were PCR amplified 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/ml ampicillin and 30 μM Fe(NO₃)₂(SO₄)₂. The indicator strain H1717 contained a chromosomal fhuF::lacZ fusion, and a low affinity Fur box has been demonstrated 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 [60]. H1717 harboring pT7-7 was used as a negative control. Colony phenotype was observed after incubation at 37°C for 10 h. Red colony (Lac+) denoted a FURTA-positive phenotype and indicated the binding of Fur to the DNA sequence cloned into the pT7-7 plasmid.

Extraction and quantification of CPS
CPS was extracted and quantified as previously described [65]. The glucuronic acid content, represents the amount of K. pneumoniae K2 CPS, was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micrograms per 10⁹ CFU [46].

qRT-PCR
Total RNAs were isolated from early-exponential-phase grown bacteria cells by use of the RNeasy midi-column
(QIAGEN) according to the manufacturer’s instructions. RNA was DNase-treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. RNA of 100 ng 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 listed in Additional file 2: Table S1. 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^{ΔΔCt} method with 23S rRNA as the endogenous reference.

Bacterial survival in serum
Normal human serum, pooled from healthy volunteers, was divided into equal volumes and stored at −70°C before use. Bacterial survival in serum was determined with minor modifications [57]. First, The bacteria were grown to log phase in LB broth and the viable bacterial concentration was adjusted to 1 × 10⁶ colony forming units/ml. 1 ml of the cultures was washed twice by using phosphate-buffered saline (PBS) and resuspended in 1 ml PBS. The mixture containing 250 μl of the cell suspension and 750 μl of pooled human serum was incubated at 37°C for 60 min. The number of viable bacteria was then determined by plate counting. The survival rate was expressed as the number of viable bacteria treated with human serum compared to the number of pre-treatment. The assay was performed triple, each with triplicate samples. The data from one of the representative experiments are shown and expressed as the mean and standard deviation from the three samples. The 0% survival of *K. pneumoniae* CG43S3ΔgalU served as a negative control.

CAS assay
The CAS assay was performed according to the method described by Schwyn and Neilands [66]. Each of the bacterial strain was grown overnight in M9 minimal medium, and then 5 μl of culture was added onto a CAS agar plate. After 24 hr 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.

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.

**Additional files**

**Additional file 1:** Figure S1. RyhB pairs with sitA. The file contains supplemental figure S1 that the potential base pairing in RyhB/sitA mRNA in this study.

**Additional file 2:** Table S1. Primers used in this study. The file contains supplemental Table S1 that the detailed information of primer sets used in this study.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

SHH, CKW, HLP, and CTL made substantial contributions to design and conduct the experiments. YMH performed qRT-PCR and growth experiments. SHH and CKW performed the bioinformatics analyses and interpretation of data. CCW, YTC, and HLP contributed to the writing and editing of the manuscript. CTL coordinated the study and performed manuscript editing. All authors have read and approved this work.

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