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# Deciphering FUR-regulated gene networks in *Klebsiella pneumoniae* using FUR knockout mutant

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ABSTRACT

*Klebsiella pneumoniae* is a nosocomial pathogen causing life-threatening infections worldwide. It possesses many virulence properties and the ferric uptake regulator (FUR) is the transcription factor that regulates the expression of various genes in *K. pneumoniae*. FUR binds to a conserved 19 bp sequence, called the FUR box, for either positive or negative regulation of the gene. FUR alters gene expression according to iron availability and also participates in virulence, colonization & toxin secretion in *K. pneumoniae*. FUR deletion mutant is an interesting model to understand the virulence factors. In the present study, we identified the genes containing putative FUR boxes (>50 % sequence homology) from the genome of *K. pneumoniae* M33. FUR deletion mutant for *K. pneumoniae* M33 was created using lambda red recombinase system using the plasmids- pACBSR-hyg, pMDIAI, and pFLP-hyg. The mutant (M33ΔFUR) was characterized using various assays like CAS assay, string test, crystal violet assay to access siderophore production, capsule synthesis and biofilm formation respectively. FUR box was present upstream of 18 genes with distinct functions. In the FUR knockout mutant, M33ΔFUR an increase in expression of iron transport and siderophore related genes *feoC, fhuA, fepB and fes* was observed whereas genes *feoA, cirA, fecA, fepA* and *entC* were down-regulated. Genes related to biofilm (*fimA* and *mrkD*) were down-regulated whereas genes related to capsular polysaccharide (*rcsA* and *rcsB*) were upregulated upon FUR deletion. Among other genes *murG* and *sucA* were downregulated and *priB* and *srlB* were upregulated upon FUR deletion. This study shows that FUR regulates many genes involved in virulence, either positively or negatively.

## 1. Introduction

*Klebsiella pneumoniae* is a rod-shaped gram-negative bacterium belonging to Enterobacteriaceae family. It commonly causes nosocomial infections including- bacteremia, septicemia, urinary tract, and respiratory infections. *K. pneumoniae* strains that have acquired additional genetic traits and become either hypervirulent (HV) or antibiotic resistant. Four major virulence factors have been studied well in *K. pneumoniae*- capsule, lipopolysaccharide (LPS), siderophores and fimbriae (Paczosa and Mecsas, 2016). Ferric update regulator (FUR) has emerged as a regulator for most of these virulence factors and is, therefore, an important regulator with the potential to provide an insight in *K. pneumoniae's* pathogenic tendencies.

Transition metal, iron is a cofactor required for various metabolic reactions occurring within a bacterium at any given time. The ability of iron to transition between two oxidative states allows it to fulfill catalytic roles over a range of reactions. Each organism has devised its own strategy for iron acquisition, storage, and consumption. Bacteria possess different iron acquisition strategies, from using high-affinity chelators called siderophores like yersiniabactin, aerobactin, salmochelin, and others to direct uptake via the G-protein coupled transport – *feoABC* operon (Andrews et al., 2013). Iron homeostasis in prokaryotes is maintained by FUR. The FUR protein forms a homodimer and exhibits regulatory activity when bound to its co-repressor $Fe^{2+}$. Along with iron regulation FUR participates in virulence, colonization and toxin secretion. Under the presence of plentiful $Fe^{2+}$, FUR binds to iron, forms a dimer and binds to the FUR box, which is a 19-bp consensus DNA sequence -GATAATGATwATCATTATC; w=A or T (de Lorenzo et al., 1987), present in target promoters. This binding of FUR at the promoter prevents the binding of RNA polymerase, thereby preventing transcription from these genes. However, FUR has been reported to serve as both an activator and repressor of gene expression. The lambda red recombination system was initially reported in *E. coli* by Murphy, (1998) and was used for gene deletion in *K. pneumoniae* by Wei et al.

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**Table 1**
Bacterial strains and plasmids used in the study:

| Starin | Antibiotic susceptibility/selectable marker | Optimum growth temperature | Function in knock-out creation | Source | Ref. |
|---|---|---|---|---|---|
| Bacterial strains used | | | | | |
| M33 (accession no.-JAFFRD010000000) | Hygromycin sensitive, Apramycin Sensitive | 37 °C | Wild-type strain used for knockout creation | Clinical isolate from urine | (Shukla et al., 2023) |
| M33ΔFUR | Apramycin resistant, Hygromycin resistant (plasmid acquired) | 37 °C | The knock-out strain | This study | - |
| Plasmids used for knockout creation | | | | | |
| pACBSR-Hyg | Hygromycin | 30 °C | Expression of the lambda- red recombination system | Gift from Seeding labs, USA | (Huang et al., 2014) |
| pMDIAI | Ampicillin, Apramycin | 37 °C | Template for generation of knock-out cassette containing *apmR* gene and FRT sites | Procured from Addgene | (Yang et al., 2014) |
| pFLP-Hyg | Hygromycin | 30 °C | Expression of FLP recombinase | Gift from Seeding labs, USA | (Huang et al., 2014) |

| | | Location of FUR box wrt gene in M33 genome | % similarity wrt global seq. |
|---|---|---|---|
| **Global consensus sequence** | **G A T A A T G A T A/T A T C A T T A T C** | | |
| *feoA* | G A T G A T A A A A A C C A T T C T C | 114 bp upstream the gene | 73.60% |
| *feoC* | G G T A G T G T T G T T C A A C G T G | 120 bp upstream the gene | 52.60% |
| *fhuA* | C A T A A T A A T A A T T C T C G T T | 34 bp upstream the gene | 63.15% |
| *cirA* | G A T A A T C G T A A T C A T T A T C | 188 bp upstream the gene | 89.47% |
| *fecA* | G C G A A T A G T A A T C A T T A T T | 154 bp upstream the gene | 73.60% |
| *fepA* | C G T G G T G A A T A T C A T C A C C | 43 bp upstream the gene | 63.15% |
| *fepB* | G A T A T T A A G A A T A A T T A T C | 147 bp upstream the gene | 78.94% |
| *fes* | A A T A A T G A C A A T T A A T T A A | 33 bp upstream the gene | 63.15% |
| *entC* | A T A A A T G A T A A T T A T T C T T | 28 bp upstream the gene | 68.42% |
| *rcsA* | G G T C A T G T C A A C G A T G A T T | Present overlapping the gene | 63.15% |
| *rcsB* | T A T G A A C G T A A T T A T T G C C | Present with in the gene | 57.89% |
| *fimA* | G A C G G T A A T A T A C A T T A G T | 94 bp upstream the gene | 57.89% |
| *mrkD* | G A C G C T G T T A T T C A C T C T G | Present with in the gene | 57.89% |
| *sodA* | G G C A T T G A T A A G C A T T T T C | 81 bp upstream the gene | 73.60% |
| *murG* | T A T G T C G A C G G C C A T T A T G | 89 bp upstream the gene | 52.60% |
| *sucA* | A T T A A T G G C A G T T T T T A A A | 118 bp upstream the gene | 52.60% |
| *priB* | A G T A A T C C T G A T G A C C A A C | Present overlapping the gene | 52.60% |
| *srlB* | G G C G A C G G C G A G C A T T A T C | 142 bp upstream the gene | 57.89% |

A


B

**Fig. 1.** Alignment of putative FUR boxes extracted from M33 genome: (A) The sequence of extracted FUR box with its location and percentage similarity with respect to the global FUR box consensus (B) Consensus sequence of FUR boxes from M33 genome.

(2012)). in 2012 and Huang et al., (2014).

Recently, the transcriptional regulation of yersiniabactin was shown to be controlled by FUR (Yu et al., 2024). Earlier studies have shown that virulence factors like capsule synthesis and fimbriae are regulated by FUR (Cheng et al., 2010; Wu et al., 2012). It is very well known that FUR binds to a consensus sequence, the FUR box but detailed screening of FUR boxes in the *K. pneumoniae* is not attempted. In the present study, we screened the genome of a clinical strain of *K. pneumoniae* for the presence of FUR boxes, created a FUR knock-out, and analyzed the effect of FUR knock-out on the genes possessing the FUR box using qRT-PCR. We found some genes which were earlier not reported to be under the control of FUR.

## 2. Materials and methods

### 2.1. Strains used and their growth conditions

*K. pneumoniae* M33, used for creating FUR knockout, is a clinical strain isolated from urine and sequenced (accession no.-JAFFRD010000000). M33 was routinely maintained on McConkey agar plates. The plasmid bearing strains – pACBSR-hyg and pFLP-hyg were gifted to the lab by Seeding labs, USA and were routinely cultured on LA plates containing 100 μg/ml hygromycin. Strain bearing plasmid pMDIAI was procured from Add-gene and was cultured on 100 μg/ml ampicillin. Details regarding all strains used is mentioned in Table 1.

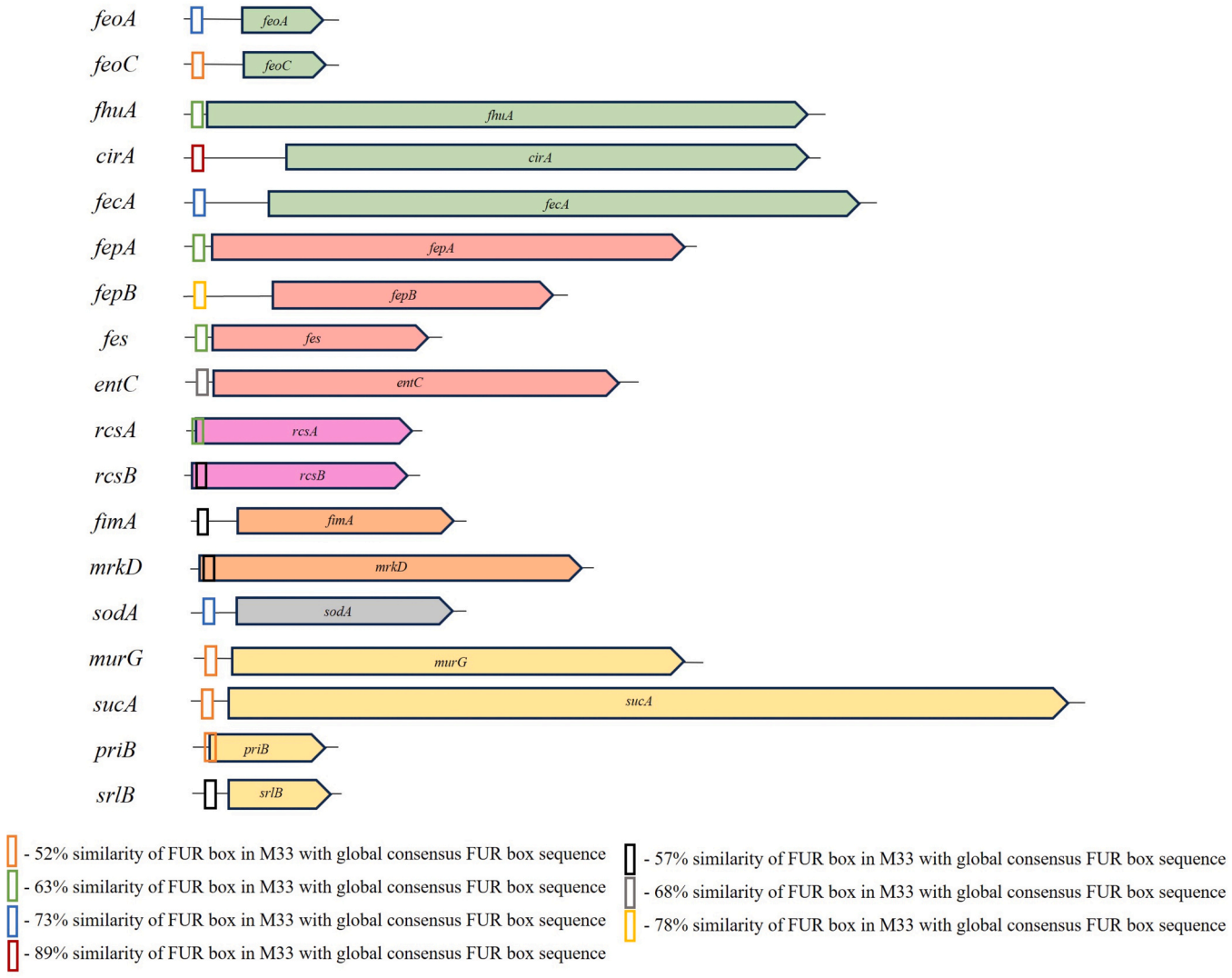


**Fig. 2.** Putative FUR boxes extracted from M33 genome: Location of putative FUR boxes with respect to genes in M33 genome, the legend shows %similarity of FUR box with global consensus sequence.

### 2.2. Generation of *K. pneumoniae (M33) fur* deletion strain

The basic strategy employed to replace *fur* gene in M33 (*K. pneumoniae* M33) with a selectable apramycin resistance gene was accomplished by lambda red-mediated recombination in the flanking homologies. The deletion strain was generated using the strategy described previously by Huang et al. (2014) and is indicated in detail in supplementary 1. In brief, a knockout cassette containing apramycin resistance gene (*apmR*), FRT (FLP recognition target) sites and *fur* overhangs was generated. PCR was performed using primers with priming (primer sequence in supplementary 2) for *apmR* and FRT sites on pMDIAI and additionally containing 61-nt extension (H1) in forward primer and 34-nt extension (H2) in reverse primer that are homologous to the region adjacent to the *fur* gene (Fig. S1). The PCR (PCR cycle in supplementary 1) was performed using Sapphire mastermix (Takara) and the product was purified using Thermo GeneJet Gel extraction kit. Wildtype M33 was electroporated and plasmid pACBSR-hyg was transformed, followed by the purified knockout cassette transformation to the selected transformants. After the transformation of knock-out cassette, the cells were supplemented with low salt LB, 1 M L-arabinose and 100 μg/ml hygromycin to facilitate homologous recombination and deletion of *fur* gene. The obtained transformants that had resistance to apramycin (50 μg/ml) were selected and named as M33ΔFUR.

### 2.3. Annotation, alignment and location of FUR boxes from *K. pneumoniae M33 genome*

de Lorenzo et al. (1987) in 1987 suggested the presence of a 19-bp consensus sequence, FUR box- GATAATGATwATCATTATC; w is A or T before FUR regulated genes. Geneious Prime Tool was used to find genes having putative FUR boxes with approximately 50 % similarity with the global consensus sequence and the location of the FUR box w.r.t the gene was also observed. The sequences of the FUR box were extracted and aligned.

### 2.4. Quantification of siderophores

Siderophores were quantified using Chrome Azurol S (CAS) Broth and Plate assays as described by Himpsl and Mobley, (2019).

CAS Plate Assay: Overnight cultures were streaked on CAS media plates and color change around the streaked cultures was observed the next day.

CAS Broth Assay: Briefly, 20 μL of M33ΔFUR and M33 strains were inoculated into 1 ml LB, with negative controls and in triplicates for 48 hours at 28°C. Cultures were then centrifuged and 50 μL of supernatant was mixed with 150 μL of CAS reagent in microplate wells and incubated for 15 min. Absorbance was measured at 630 nm and

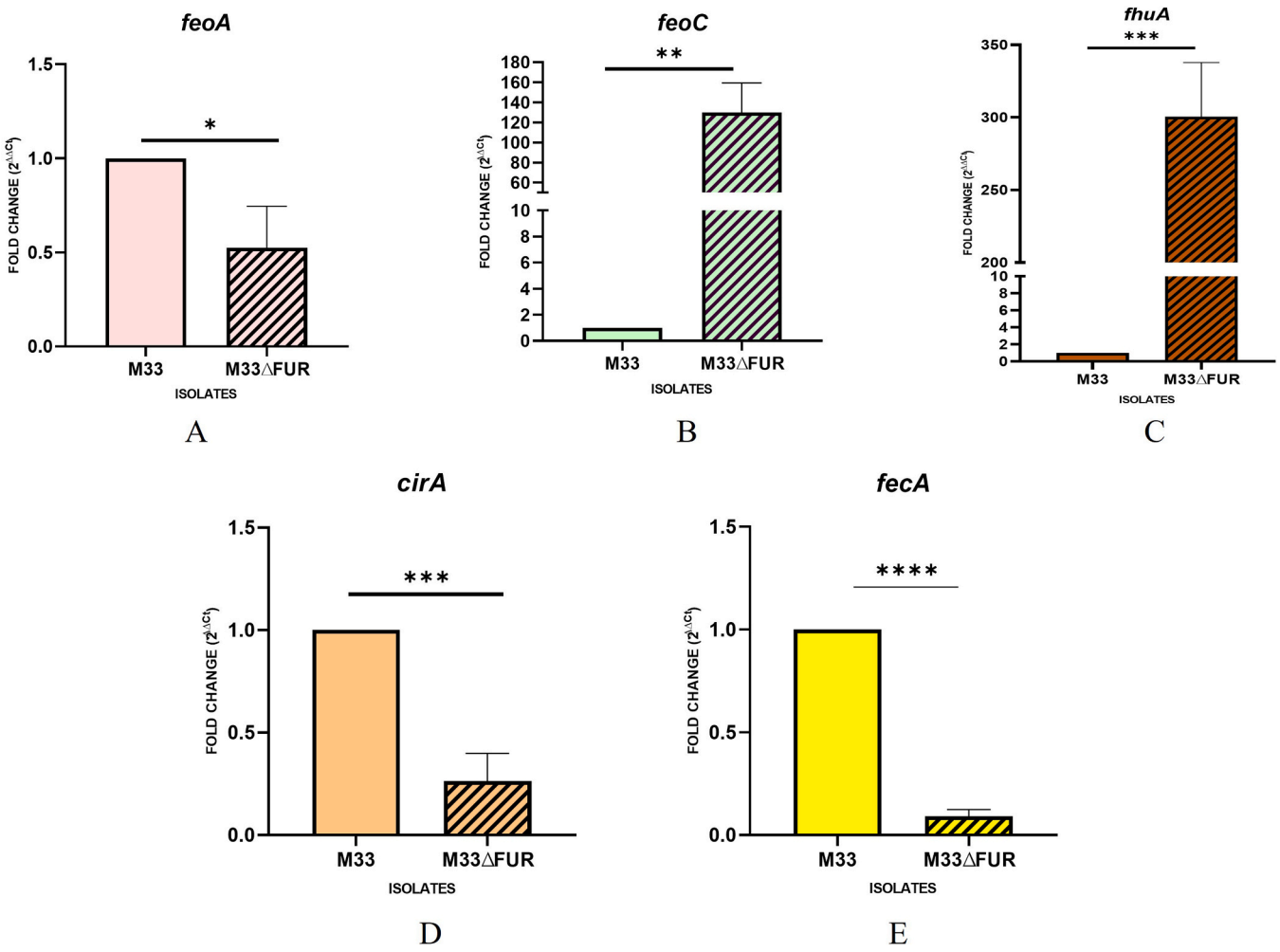


**Fig. 3.** Iron transporter genes: Quantitative real-time PCR (qRT-PCR) was employed to measure the relative expression of iron transporter genes in M33ΔFUR as compared to M33. Genes *feoC* (B) and *fhuA*(C) were upregulated in M33ΔFUR whereas genes *feoA*(A), *cirA*(D) and *fecA*(E) were downregulated in M33ΔFUR as compared to M33. Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

siderophore production (psu) was calculated using:

$$psu = [(A_r - A_s) \times 100] / A_s$$

where $A_r$ - Absorbance of control (uninoculated broth) and $A_s$ - Absorbance of sample

### 2.5. String test

The string test was performed to verify the capsule phenotype (Yu et al., 2007). Isolates were freshly sub-cultured and streaked onto sheep blood agar plates and incubated overnight at 37°C. The next day, a sterile loop was used to stretch the muco-viscous string from the colony. Strings >5 mm were considered positive.

### 2.6. Crystal violet assay

The biofilm formation assay was based on the method outlined by Stepanovic *et al.*in 2004 (Stepanović et al., 2004). Briefly, strains M33 and M33ΔFUR were incubated overnight in LB. The following day, the cultures were set to an $OD_{600\ nm}$ - 0.1 and then 20 µl of this culture was added to 180 µl sterile LB in a 96-well microtiter plate and was incubated at 37°C for 24 hours for biofilm formation. It was conducted in triplicate, with LB alone serving as the negative control. After 24 hours, the biofilm was washed twice with 0.85 % sterile saline to remove any non-adherent cells and fixed using methanol for 15 min. Later a saline wash was given and the biofilm was stained with 0.1 % crystal violet and incubated for 15 min. Later, unbound CV was rinsed with 0.85 % saline twice, air-dried and then treated with 33 % acetic acid to dissolve the stain for 15 mins. Optical density at 590 nm was quantified using a microtiter plate reader (Multiskan Go, Thermo Fisher Scientific, USA).

### 2.7. Quantification of gene expression using qRT-PCR

Total RNA was extracted from M33 and M33ΔFUR from early exponential phase culture using RNAiso reagent (Takara) as per manufacturer's instructions. The purity and concentration of RNA were assessed using Nanodrop (Multiskan Go, Thermo Fisher Scientific, USA) and then DNase treatment using DNaseA (Merck) manufacturer's instructions. Later, cDNA synthesis was carried out using Primescript 1st strand cDNA synthesis kit (Takara).

qRT-PCR was performed using TB Green Premix Ex *Taq*II (Takara) with gene-specific primers mentioned in Supplementary 2 (Table S1). Relative gene expression levels were quantified using comparative threshold cycle $2^{-\Delta\Delta CT}$ method, with *rpoB* serving as the housekeeping gene.

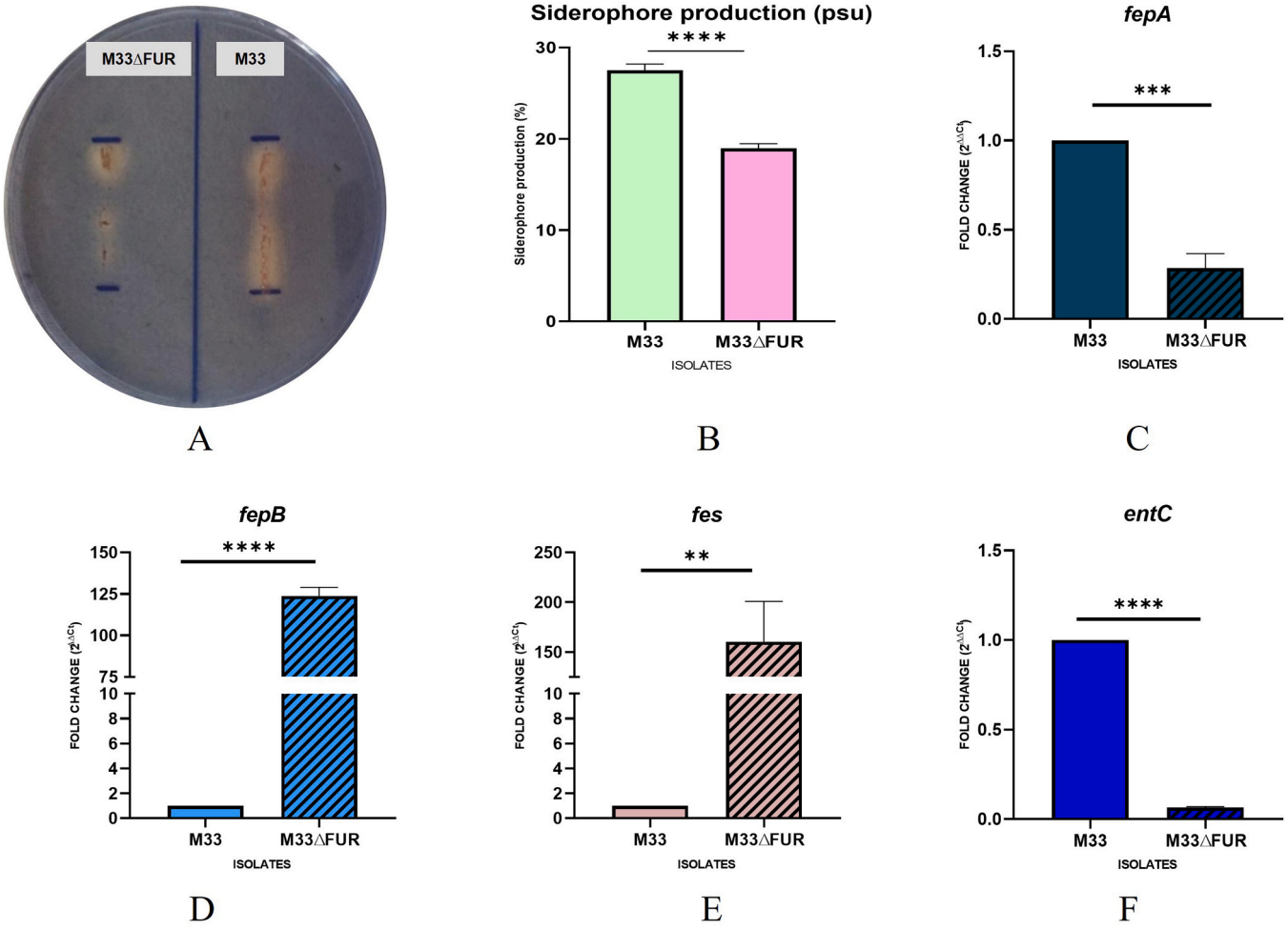


**Fig. 4.** Siderophore (ferric enterobactin) related genes: Siderophore production was evaluated through CAS plate assay -Left streak-wild type M33 and right streak-M33ΔFUR (A) and CAS Broth (B) indicating reduced siderophore production in M33ΔFUR. qRT-PCR analysis. qRT-PCR analysis unveiled heightened expression of *fepB*(D) and *fes*(E) in M33ΔFUR whereas *fepA*(C) and *entC*(F) show reduced expression. Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

## 3. Results

### 3.1. Annotation, alignment and location of FUR boxes from K. pneumoniae M33 genome

Using Geneious Prime Tool, 18 genes were identified to have putative FUR boxes in their promoter region in the clinical isolate M33, with percentage similarity ranging from 52 % to 89 % (Fig. 1A) with respect to the FUR box identified in *E. coli* by Lorenzo (de Lorenzo et al., 1987) in 1987 and the consensus of FUR box as per M33 isolate has been shown in Fig. 1B. The location of the putative FUR boxes from the gene is highly variable in the M33 genome. Fig. 2 gives the approximate location of putative FUR boxes from the genes. Among the genes having putative FUR boxes, most of these genes were related to iron uptake, siderophores (ferric enterobactin), capsule synthesis, fimbriae and some other genes with various functions in *K. pneumoniae*. Due to the presence of putative FUR boxes these genes might be regulated by FUR, either positively or negatively. It is interesting to note that a few genes have the putative FUR boxes with in the gene like *rcsB* and *mrkD* and a few genes have FUR boxes overlapping the gene with in initial sequences like *rcsA* and *priB*. We have analyzed various genomes of the clinical isolates for the presence and location of FUR boxes have obtained similar results (data not shown).

### 3.2. Effect of fur deletion on Iron transporter genes

qRT-PCR was done to analyse the relative expression of the genes harboring putative FUR boxes in M33ΔFUR as compared to wild type M33 strain. The expression of Iron transporter genes - *feoA*, *feoC*, *fhuA*, *cirA* and *fecA* were normalized to the housekeeping gene *rpoB*. Among the iron transporter genes, expression of *feoC* and *fhuA* in M33ΔFUR was upregulated to 130.05 and 300.47-fold respectively (Fig. 3A and C) as compared to M33. Expression of *feoA, cirA* and *fecA* were downregulated to 0.524, 0.263 and 0.091-fold respectively (Fig. 3C, D, E) in M33ΔFUR as compared to M33.

### 3.3. Effect of fur deletion on Siderophores

In the CAS broth assay, siderophore production was quantified and decreased siderophore production was observed in M33ΔFUR as compared to the M33 (Fig. 4A). Additionally, CAS media plate assays confirmed reduced siderophore production in M33ΔFUR compared to M33, which is evident by a large orange halo around colonies (Fig. 4B) in wildtype M33 after 48 hours of incubation. qRT-PCR of genes related to ferric enterobactin results indicate significant down-regulation in expression of *fepA* and *entC* to 0.285 and 0.064-fold respectively (Fig. 4C, F) in M33ΔFUR. On the contrary, genes *fepB* and *fes* show increased expression upto 123.71 and 160.30-fold respectively (Fig. 4 D, E) in M33ΔFUR.

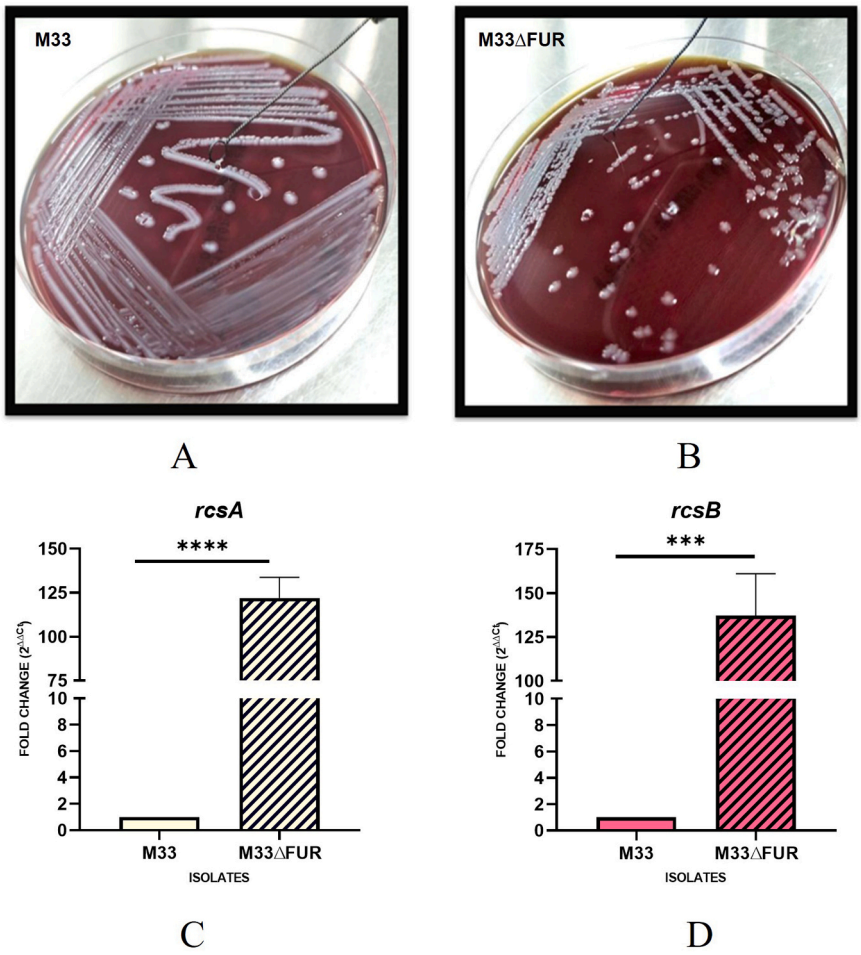


**Fig. 5.** Capsule related genes: String test results show a significant increase in capsule production in M33ΔFUR(B) as compared to M33(A). The qRT-PCR data showed upregulation of both *rcsA*(C) and *rcsB*(D) genes in M33ΔFUR. Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

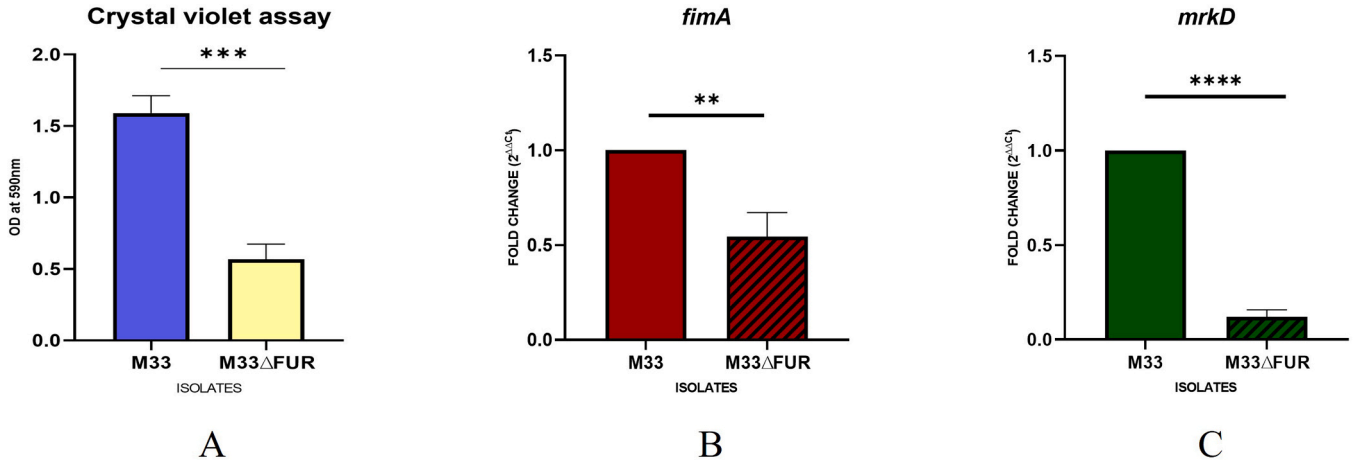


**Fig. 6.** Biofilm related genes: Crystal violet assay showed a reduction biomass in the knockout strain M33ΔFUR (A). qRT-PCR analysis also showed reduced expression of major fimbriae gene *fimA*(B) and *mrkD*(C). Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

### *3.4. Effect of fur deletion on capsule synthesis*

String test was conducted to see the effect of *fur* deletion on capsule synthesis. Interestingly, string test showed a significant difference in capsule production between the wild-type and knock-out strain. M33ΔFUR exhibited a positive result with a string length of about 9 mm (Fig. 5B), indicating pronounced hyper-mucoviscosity compared to M33 (Fig. 5A). Furthermore, the results on qRT-PCR collaborate with the results on string test. Upon deletion of *fur*, an increase in the expression of *rcsA* and *rcsB* genes to 122.02 and 137.22 fold respectively (Fig. 5C,

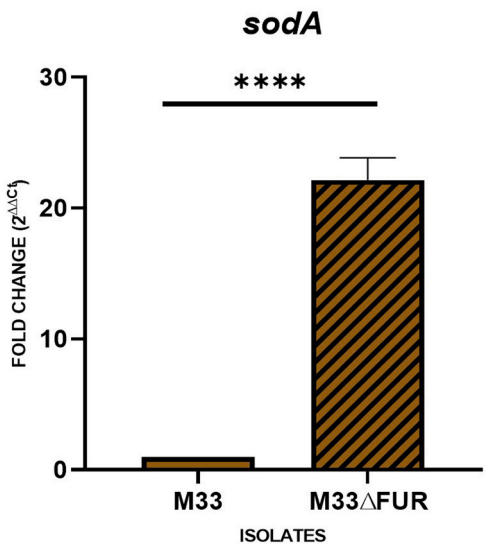


**Fig. 7.** *sodA* gene: qRT-PCR analysis also showed an increase in expression of *sodA* gene. Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

D).

### 3.5. Effect of fur deletion on biofilm formation

Crystal violet assay was used to quantify the biofilm biomass values for M33 and M33ΔFUR and the mean $OD_{590\ nm}$ was found to be 1.59 and 0.56 respectively (Fig. 6A), indicating decreased biofilm formation upon *fur* deletion. Additionally, qRT-PCR results also identical traits as both the major adhesins in *K. pneumoniae* were found to be down-regulated in the deletion strain. Expression of genes *fimA* and *mrkD* was reduced to 0.543 and 0.119-fold respectively (Fig. 6B, C) confirming the reduction in biofilm formation.

### 3.6. Effect of fur deletion on sodA gene

qRT-PCR analysis showed upregulation of the gene *sodA* to 22.15-fold (Fig. 7) upon *fur* deletion.

### 3.7. Effect of fur deletion on various miscellaneous genes

In addition to examining the expression of the gene of interest, we also investigated the expression of several other genes, including *murG*, *sucA*, *priB* and *srlB*, using qRT-PCR. The results, revealed an increase in expression of *priB* and *srlB* to approximately 71.58 and 434.47 folds (Fig. 8C, D), respectively. Conversely, the expression of *murG* and *sucA* decreased to approximately 0.070 and 0.172 folds (Fig. 8A, B) respectively.

## 4. Discussion

*K. pneumoniae* is a frequently encountered bacterial pathogen in hospital settings which mainly causes severe pulmonary and urinary tract infections (Han et al., 2024). The major virulence factors of *K. pneumoniae* include the capsule, fimbriae, siderophores and iron acquisition systems. Further, *K. pneumoniae's* pathogenic potential is influenced by presence of combination of these virulence properties that aid in its survival in the host by evading the immune system (Paczosa and Mecsas, 2016). The ferric uptake regulator (FUR) is a global regulator that influences the expression of virulence genes, and iron acquisition in many pathogens including *K. pneumoniae (*Troxell and Hassan, 2013*)*. In the classical model, FUR binds to the FUR box in the promoter region and impedes RNA polymerase from binding to the promoter causing repression of gene expression (Baichoo and Helmann, 2002). FUR is primarily known as a repressor; recent evidences highlight as an activator. FUR activation can occur through three distinct mechanisms (1) indirectly -via small RNAs (Massé and Gottesman, 2002), (2) direct binding at cis-regulatory elements to enhance recruitment of RNA polymerase holoenzyme (RNAP) (Delany et al., 2004; Teixidó et al., 2011), and (3) as an anti-repressor by removing or blocking DNA binding to transcription repressor (Massé and Gottesman, 2002). Though expression of many genes has been reported to be under the control of FUR in *K. pneumoniae*, an elaborate screening for FUR boxes has not been done. Hence, there was a need to examine the whole genome of *K. pneumoniae* for the presence of FUR boxes followed by identifying the genes under the control of FUR.

The present study was conducted to find the FUR boxes in the *K. pneumoniae* M33 genome and see the effect of FUR on the genes harboring FUR boxes. To do so, we first identified the FUR boxes based on sequence homology, we then created a partial FUR knockout strain using the λ- Red Recombinase method and lastly, we checked the expression of genes (having the FUR boxes) using qRT-PCR in wild type and FUR knockout (M33ΔFUR). In the *K. pneumoniae* genome (M33) we studied, the FUR boxes were present in 18 genes, which were Further divided into pathways for iron uptake, siderophore (enterobactin) synthesis, capsule synthesis, biofilm formation, oxidative stress response and other miscellaneous functions.

Most of the initial studies on sequence homology of the FUR box were reported in *E. coli* (de Lorenzo et al., 1987). Apart from *E. coli*, the bioinformatic analysis of FUR box has been done in *Bacillus subtilis* (Baichoo and Helmann, 2002) and *Helicobacter pylori* (Pich et al., 2012) where the sequence of FUR box was reported as a 7–1–7 motif (5′-TAATAATnATTATTA-3′). According to our results, FUR box identified upstream to *cirA* gene has maximum homology to the FUR box identified in *E. coli*. Gene *fepA* has a similarity of 78 % followed by genes *feoA, fecA* and *sodA* with 73 % sequence homology with the *E. coli* FUR box. It was observed that sequence similarity of FUR boxes varies amongst the genes, but the relative expression of genes has been significantly impacted upon *fur* deletion which is further discussed.

The *fur* gene from *Klebsiella pneumoniae* was first characterized by Achenbach and Yang in the year 1997. It regulates the expression of flavodoxin and CPS biosynthesis in addition to regulating its own expression (Achenbach and Yang, 1997). Iron is essential to many cellular processes and *K. pneumoniae* has many mechanisms for uptake of iron which include the ferrous iron transport (*feo*) system, catecholate siderophore receptor (*cirA*), *Klebsiella* ferric iron uptake (*kfu*) system, and ferric iron citrate receptors (*fecA*) (Daoud et al., 2022). We found FUR boxes in few genes from ferrous iron transport (*feo*) system, catecholate siderophore receptor (*cirA*), and ferric iron citrate receptors (*fecA*) but not in *Klebsiella* ferric iron uptake (*kfu*) system. The regulatory role of FUR in iron-acquisition systems in *K. pneumoniae* was first studied using the strain CG43 and putative iron-acquisition systems (*iucA, iroB, hmuR and feoB*) and siderophore genes (*fepA, fepB, entC*) were found to be under the influence of FUR, where the authors found more than 2-fold upregulation of most of these genes (Lin et al., 2011). Our results differ in terms of the number of genes and the fold changes in them. In case of iron-acquisition systems we found periplasmic proteins (*feoA, feoC*), ferrichrome iron receptor (*fhuA*), ferric iron citrate receptor (*fecA*) and catecholate siderophore receptor (*cirA*) under the regulation of FUR. In the knockout strain we found increased expression in *feoC, fhuA, fepB*, and *fes* while decreased expression in *feoA, cirA, fecA, fepA* and *entC*. A recent study suggested the involvement FUR in iron acquisition via the identified FUR box upstream of the yersiniabactin receptor gene, *fyuA* (Yu et al., 2024). The strain used in the present study did not carry the yersiniabactin gene and hence the receptor. In a study to validate housekeeping genes in *K. pneumoniae*, FUR boxes were identified on the upstream region of the genes *cirA, iroN* and *fiu*, which encode the catecholate-type siderophore receptors *cirA, fepA* and *fiu* (Gomes et al.,

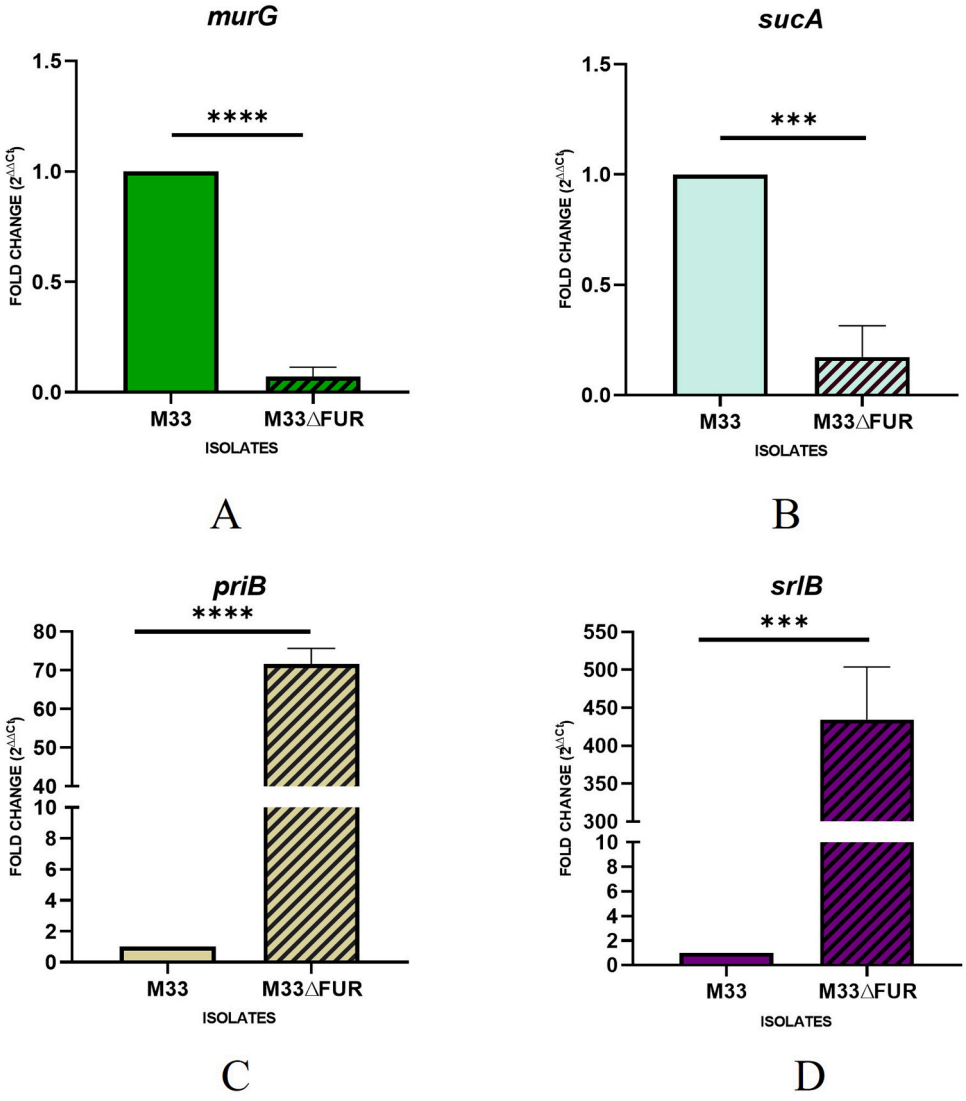


**Fig. 8.** qRT-PCR analysis of miscellaneous genes: Genes *priB*(C) and *srlB*(D)were upregulated in M33ΔFUR whereas genes *murG*(C) and *sucA*(D) were downregulated. Statistical analysis was done using GraphPad prism, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns indicates $p > 0.05$.

2018). Thus, based on the literature available it appears that FUR serves as a repressor in the regulation of at least five iron-acquisition systems in *K. pneumoniae*, although at different levels. However, on the other hand in the present study we showed decreased expression of genes from iron transport (*feoA, feoC, cirA, fecA, fepA*) and siderophores (*entC*) in the FUR deletion strain which indicates that FUR could act as activator for certain genes. Further experiments to prove the same are needed. Later, FUR deletion strains of CG43 and NTUHK2044 were created by a few researchers to elucidate the function of FUR in negatively regulating *rmpA*, *rcsA* of *K. pneumoniae* (Cheng et al., 2010; Lin et al., 2011). FUR indirectly controls the capsular polysaccharide synthesis via *rcsA* and *rmpA*. FUR positively regulates fimbrial genes like *mrkHI* (Type 3 fimbriae) (Wu et al., 2012). Results in the present study corroborate with these studies as we also found FUR negatively regulating *rcsA* and positively regulating the fimbriae gene cluster - *mrk*. Further, in addition to *rcsA*, for the first time we found FUR box in the promoter region of *rcsB* and in case of fimbriae we found FUR box in *fimA*. A direct regulation of the fimbrial regulatory (*kpfR*) gene and *kpf* gene cluster was shown by acting as a transcriptional activator by FUR (Gomes et al., 2021). However, we did not find the *kpfR* gene in the genome analyzed in the present study. Thus, it can be concluded that FUR positively regulates all fimbrial clusters (*fim, mrk, kpf*) and hence the FUR deletion strain shows low biofilm formation compared to the wild type. Later, it was shown that small non-coding *ryhB* participates in FUR regulon to modulate the bacterial CPS biosynthesis and iron acquisition systems in *K. pneumoniae (*Huang et al., 2014*)*.

In addition to the above, in the present study we found FUR boxes in miscellenous genes like *sodA, murG, sucA, priB* and *srlB*. Negative regulation of *sodA* and *sodB* by FUR is well understood in *E. coli* (Niederhoffer et al., 1990). We report the FUR box in *K. pneumoniae* for the first time in the promoter region of *sodA* but not *sodB*. We found increased expression of the gene *sodA* in case of FUR deletion. In our study, we have observed the presence of FUR box upstream *murG* and *priB* gene and a significant reduction and increase in their expression respectively was observed, but upon rigorous literature search we fail to obtain any published report and hence any correlation between FUR and these genes. The correlation of FUR with genes can be a good lead in future studies. FUR deletion mutant was created for *E. coli* by Zhang et al. (2005) and they observed decrease in expression of *sucA* in the mutant as compare to wildtype strain, and we have observed similar results in M33ΔFUR strain. *srlB* is a sorbitol phosphotransferase component of CpxAR two-component system (Element et al., 2023). *srlB* is indirectly involved in the expression of type 3 fimbriae and hence in biofilm formation. The CpxAR system negatively regulates the

expression of type 3 fimbriae by modulation iron levels in the bacterium which leads to the activation of *rhyB* gene that represses the expression of *mrkA* (type 3 fimbrial shaft) gene (Kuo et al., 2023). The repressed expression of *rhyB* gene leads to reduced expression of type 3 fimbriae and reduces biofilm formation. In our results, we have observed an increase in the expression of *srlB* in absence of FUR and have observed decrease in biofilm formation.

## 5. Conclusion

The present study's limitation is that we did not test the mutant strain for its pathogenicity and the actual binding of FUR to the putative boxes was not validated. Based on all earlier studies on *fur* gene deletion, the mutant strain should be less virulent. However, we showed that *K. pneumoniae* FUR acts as a repressor for some genes and an activator for another group of virulence genes. Hence, we conclude that the FUR regulating network comprises of many genes belonging to the iron transport, capsule synthesis, biofilm and other genes. Further studies are needed to support the findings of this study.

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## CRediT authorship contribution statement

**Purvi Joshi:** Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Data curation. **Twinkle Patel:** Writing – original draft, Investigation. **Devarshi Urvish Gajjar:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Reetu Maindolia:** Writing – original draft, Investigation, Formal analysis. **Dhanshree Dhakre:** Writing – original draft, Investigation.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Devarshi Gajjar reports financial support was provided by Gujarat State Biotechnology Mission. Devarshi Gajjar reports financial support was provided by India Ministry of Science & Technology Department of Biotechnology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2024.100201.

## Data Availability

Data will be made available on request.

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