



Functional Insights From KpfR, a New Transcriptional Regulator of Fimbrial Expression That Is Crucial for *Klebsiella pneumoniae* Pathogenicity

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Although originally known as an opportunistic pathogen, *Klebsiella pneumoniae* has been considered a worldwide health threat nowadays due to the emergence of hypervirulent and antibiotic-resistant strains capable of causing severe infections not only on immunocompromised patients but also on healthy individuals. Fimbriae is an essential virulence factor for *K. pneumoniae*, especially in urinary tract infections (UTIs), because it allows the pathogen to adhere and invade urothelial cells and to form biofilms on biotic and abiotic surfaces. The importance of fimbriae for *K. pneumoniae* pathogenicity is highlighted by the large number of fimbrial gene clusters on the bacterium genome, which requires a coordinated and finely adjusted system to control the synthesis of these structures. In this work, we describe KpfR as a new transcriptional repressor of fimbrial expression in *K. pneumoniae* and discuss its role in the bacterium pathogenicity. *K. pneumoniae* with disrupted *kpfR* gene exhibited a hyperfimbriated phenotype with enhanced biofilm formation and greater adhesion to and replication within epithelial host cells. Nonetheless, the mutant strain was attenuated for colonization of the bladder in a murine model of urinary tract infection. These results indicate that KpfR is an important transcriptional repressor that, by negatively controlling the expression of fimbriae, prevents *K. pneumoniae* from having a hyperfimbriated phenotype and from being recognized and eliminated by the host immune system.

Keywords: *Klebsiella pneumoniae*, transcriptional regulation, fimbriae, adherence, biofilms, coculture, urinary tract infection, host-microbe interactions

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative pathogen responsible for a wide range of healthcare-acquired infections in genitourinary, respiratory, and gastrointestinal tracts, mostly in immunocompromised patients (Podschun and Ullmann, 1998; Paczosa and Mecsas, 2016). Polysaccharides capsule, lipopolysaccharides, siderophore-mediated iron acquisition systems, and

fimbrial adhesins are among the virulence determinants of *K. pneumoniae* (Paczosa and Meccas, 2016). Currently, this bacterium has become a worldwide public health threat due to the emergence of hypervirulent and antibiotic-resistant strains of *K. pneumoniae* causing various types of invasive infections (Lee et al., 2017; Rodriguez-Villar et al., 2019; Choby et al., 2020). In addition, the increasing number of severe community-acquired infections in healthy individuals has emphasized the importance of studying the virulence mechanisms that determine the pathogenicity of *K. pneumoniae*.

Historically known for being responsible for pneumonia, *K. pneumoniae* has been considered an important causative agent of urinary tract infections (UTIs) (Paczosa and Meccas, 2016). The successful colonization of the urinary epithelium by *K. pneumoniae* relies on the expression of fimbriae. These structures mediate adherence to urothelial cells and are also essential for biofilm formation by promoting adhesion of the bacterium to abiotic surfaces, such as urinary catheters (Stahlhut et al., 2012). The establishment of a biofilm structure provides the bacterium more resistant to the host defense system and increases colonization of the host urinary tract (Rosen et al., 2007, 2008a,b; Struve et al., 2008; Hannan et al., 2012; Stahlhut et al., 2012; Murphy et al., 2013). Fimbriae are also required for uropathogens to adhere to specific receptors on urothelial cells and invade the urinary epithelium (Oelschlaeger and Tall, 1997; Rosen et al., 2008b; Struve et al., 2008; Hannan et al., 2012; Murphy et al., 2013). Once in the intracellular environment, the uropathogen replicates to form biofilm-like intracellular bacterial communities (IBCs) (Hunstad and Justice, 2010). Later maturation of IBCs leads to epithelium exfoliation with consequent spread of the bacteria to other sites, initiating a new cycle (Rosen et al., 2007; Hunstad and Justice, 2010). Thus, this intracellular niche allows uropathogens to hide from the host immune system and represents a quiescent intracellular reservoir of bacteria that leads to recurrent episodes of UTI (Rosen et al., 2007; Hunstad and Justice, 2010).

Several types of fimbriae have been identified in *K. pneumoniae*, with types 1 and 3 fimbriae being the main and best characterized adhesive structures (Paczosa and Meccas, 2016). Type 1 fimbriae are encoded by the *fim* gene cluster, composed of eight genes (*fimAICDFGHK*) (Rosen et al., 2008a,b; Struve et al., 2008). These fimbriae are made up of repeating subunits of the major fimbrial subunit protein FimA, with the adhesin molecule FimH located at the distal end of the structure (Rosen et al., 2008b). FimH has a binding affinity for mannose residues present on the bladder cells surface (Rosen et al., 2008b). Thus, type 1 fimbriae promote the adhesion and invasion of the pathogen in host cells and favor successful colonization (Rosen et al., 2008a,b; Struve et al., 2008). Type 3 fimbriae are encoded by the *mrkABCD* gene cluster. MrkA subunits make up the major structure of the type 3 fimbriae, with the MrkD adhesin located at the tip. Although the MrkD-specific cell surface receptor has not yet been identified, type 3 fimbriae promote adhesion to tracheal, lung, and kidney epithelial cells (Hornick et al., 1992), and are fundamental in the process of biofilm formation on biotic and abiotic surfaces (Struve et al., 2009). Both type 1 and type 3 fimbriae promote biofilm formation on urinary catheters and,

therefore, play a significant role in colonization and persistence in the bladder in catheter-associated UTI (Stahlhut et al., 2012; Murphy et al., 2013).

K. pneumoniae controls the expression of fimbrial genes through transcriptional regulators (Rosen et al., 2008a; Wilksch et al., 2011; Wu et al., 2012; Wang et al., 2013; Ares et al., 2016; Lin et al., 2017), utilizing various environmental stimuli (Schroll et al., 2010; Wu et al., 2012; Lin et al., 2016, 2018) and according to specific anatomic sites (Struve et al., 2008, 2009). Phase variation mediated by invertible DNA elements (Struve et al., 2008; Wu et al., 2010), intracellular levels of cyclic di-GMP (Johnson and Clegg, 2010), iron availability (Wu et al., 2012) and DNA binding regulators (Rosen et al., 2008a; Wilksch et al., 2011; Wu et al., 2012; Wang et al., 2013; Ares et al., 2016; Lin et al., 2017) are among the known mechanisms that regulate expression of fimbriae in *K. pneumoniae* (Clegg et al., 2011). For instance, the expression of *fim* gene cluster is regulated by phase variation mediated by an invertible promoter element, named *fimS* element, whose orientation is switched by FimE- and FimB-recombinases (Struve et al., 2008). Besides this mechanism, the expression of *K. pneumoniae* *fim* gene cluster is also regulated by the transcriptional regulator FimK encoded by *fimK*, a gene found only in *fim* gene cluster of *K. pneumoniae* and absent in *Escherichia coli* (Rosen et al., 2008a; Wang et al., 2013). On the other hand, the expression of type 3 fimbriae has been attributed to extracellular iron levels and ferric uptake regulator (Fur) (Wu et al., 2012). Fur is a transcriptional regulator that modulates gene expression by complexing with ferrous iron and binding to regulatory sequences, named boxes Fur, located in the promoter region of target genes (Escolar et al., 1999).

In its classical mechanism of action, Fur acts as a transcriptional repressor that utilizes ferrous iron as corepressor to bind on Fur box consensus sequence and to block RNA polymerase entry on the promoter region, thus leading to transcription repression of the target genes (Escolar et al., 1999). However, reports in the literature have indicated not only that the Fur-iron complex can also function as a transcriptional activator, but also that both repression and activation of transcription can be achieved by Fur in its holo or apo forms—i.e., complexed or not complexed with ferrous iron, respectively (Carpenter et al., 2009; Troxell and Hassan, 2013; Seo et al., 2014; Deng et al., 2015). This alternative mode of action has been described in some pathogenic bacteria, including *Helicobacter pylori* (Delany et al., 2001; Gilbreath et al., 2012), *Neisseria meningitidis* (Delany et al., 2004; Yu and Genco, 2012), *Salmonella enterica* (Teixidó et al., 2011), *Campylobacter jejuni* (Butcher et al., 2012), *Staphylococcus aureus* (Deng et al., 2012), and *Bacillus subtilis* (Pi and Helmann, 2017).

In addition to *fim* and *mrk*, the genome of *Klebsiella pneumoniae* presents at least seven other fimbrial gene clusters still poorly characterized (Wu et al., 2010; Khater et al., 2015). Recently, Alcántar-Curiel et al. (2013) identified and characterized an operon homologous to the *Escherichia coli* common pilus (ECP) in the *K. pneumoniae* genome. The fimbriae encoded by the *ecp* operon, which comprises the *ecpABCDE* genes, have been associated with epithelial cell infection *in vitro* and in biofilm-associated bacterial communities

(Alcántar-Curiel et al., 2013). A fimbrial gene cluster not yet characterized is *kpf* gene cluster, first described by Wu et al. (2010) on *K. pneumoniae* NTUH-K2044. The synthesis of this broad repertoire of fimbrial structures requires a precise and coordinated control that involves specific regulatory proteins. This control guarantees the production of these structures only when necessary, avoiding the uncontrolled expression of fimbriae and unnecessary energy expenditure by bacteria. Besides resulting in unnecessary consumption of energy, the uncontrolled expression of the fimbriae may result in bacterial clearance by the immune cells (Athamna et al., 1991; Przondo-Mordarska et al., 1991).

In the present study, we describe the transcriptional regulator of *kpf* gene cluster, a poorly characterized cluster of fimbrial genes encoding type 1-like fimbriae. We also demonstrate that KpfR regulator plays an important role in the pathogenicity of *K. pneumoniae* in murine urinary tract.

MATERIALS AND METHODS

Bacterial Strains, Eukaryotic Cell Lines, and Growth Conditions

For this study, we used *Klebsiella pneumoniae* strain #8 (UKP8), a clinical strain isolated from a human patient with urinary tract infection. PCR genotyping using primers described elsewhere (Turton et al., 2008) indicated that the strain UKP8 presents the capsular serotype K2 (data not shown). Bacterial cells were routinely grown in Lysogeny Broth (LB; BD, United States) at 37°C with shaking at 200 rpm, or on LB agar plates in static cultures. Bacterial growth was monitored by measuring the optical density of the cultures at a wavelength of 600 nm (O.D._{600 nm}), using the GeneQuant Spectrophotometer (GE Healthcare). For growth under iron-replete and iron-limiting conditions the LB medium was supplemented with FeSO₄ (Sigma-Aldrich) or the iron chelator Dipyriddy (Sigma-Aldrich), respectively.

For coculture assays, we used the human bladder epithelial cell line T24, purchased from the Bank of Cells of Rio de Janeiro (BCRJ, cell line with code number 0231). T24 cells were cultivated at 37°C in an atmosphere of 95% relative humidity at 5% CO₂, in McCoy's 5A medium (Thermo Fisher Scientific®) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific®) and subcultivated twice a week at a ratio of 1:8.

Identification of Regulatory Binding Sites on *kpfR* Gene Promoter

Bioinformatics analyses were performed to identify putative regulatory binding sites on *kpfR* promoter. These analyses were conducted on the genomic sequence of *kpfR* on *Klebsiella pneumoniae* strain ATCC 700721/MGH 78578 (GenBank accession number CP000647.1). To identify Fur-binding sites on *kpfR* gene we employed a theoretical approach (Ferraz et al., 2011) previously adapted to *K. pneumoniae* (Gomes et al., 2018). To identify the putative transcription start site and possible Sigma factor positions on the promoter region of *kpfR* gene we used

the web-based programs *Neural Network Promoter Prediction* (available at https://www.fruitfly.org/seq_tools/promoter.html) and *BPROM-Prediction of Bacterial Promoters* (available at <http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>; Solovyev and Solovyev, 2011), respectively. These predictive analyses were performed on 100 nucleotides upstream to the initial codon of *kpfR* gene.

To determine whether *kpfR* cotranscribe with the genes from *kpf* cluster, reverse-transcription-PCRs were performed using cDNAs synthesized from total RNA extracted from *K. pneumoniae* and primer pairs spanning *kpfR* to *kpfA* and *kpfA* to *kpfD* genes (see **Supplementary Table S1**). The resulting amplicons were analyzed by agarose gel electrophoresis. Primers were designed using *Primer3 version 0.4.0* web-program (available at <http://bioinfo.ut.ee/primer3-0.4.0/>; Rozen and Skaletsky, 2000).

Complementary oligonucleotides containing the sequences of the putative Fur boxes were annealed to form double-stranded DNA probes. These DNA probes were cloned into high copy number pGEM®-T Easy vector (Promega) and the recombinant vectors were used in Fur titration assay (FURTA) and DNA Electrophoretic Mobility Shift Assay (EMSA), in order to validate the Fur protein interactions with the putative Fur boxes.

FURTA was performed with *Escherichia coli* strain H1717, which carries the *lacZ* reporter gene under the control of the Fur-regulated *fhuF* gene promoter. When transformed with multicopy plasmids cloned with functional Fur box, H1717 strain will appear red on MacConkey lactose agar plates (Lac⁺ phenotype) because the high number of newly introduced Fur boxes will titrate the Fur repressor from *fhuF:lacZ* fusion, thus releasing the transcription of *lacZ*. On the other hand, if H1717 strain is transformed with a vector cloned with a non-functional Fur box, the Fur repressor will remain bound to the *fhuF* promoter region, and the *lacZ* reporter gene is not expressed, rendering colorless *E. coli* H1717 colonies on MacConkey lactose agar plates (Lac⁻ phenotype). FURTA was conducted as previously described (Johnson and Clegg, 2010). *E. coli* strain H1717 was transformed with pGEM®-T Easy vector cloned with the DNA probe containing the sequences of the putative Fur box identified on *kpfR* gene. The transformants were then plated onto MacConkey lactose agar containing 100 µg/mL ampicillin and 100 mM of iron sulfate (FeSO₄) (Sigma). After 18 h of incubation at 37°C, the functionality of the putative Fur box on *kpfR* was assessed according to the Lac ± phenotype (i.e., the color of the H1717 colonies). *E. coli* strain H1717 transformed with circular pGEM®-T Easy vector alone (i.e., with no insert) was used as negative control, whereas H1717 strain transformed with vector cloned with the previously validated Fur box of the *K. pneumoniae entC* gene (Carpenter et al., 2009) was used as a positive control.

EMSA was conducted essentially as described by Gomes et al. (2018). DNA probes for EMSA were obtained by PCR amplifying the pGEM®-T Easy vector cloned with the putative Fur identified on *kpfR* gene, using the universal M13 primers (Gomes et al., 2018). The resulting PCR product of 285 base pairs was then used as probes on the binding reactions, along with the previously purified His-tagged recombinant Fur

protein from *K. pneumoniae*. The negative control consisted of a 254 base pairs DNA fragment without Fur box sequence, obtained by PCR amplification of a pGEM[®]-T Easy vector without insert (i.e., vector not cloned with the putative Fur boxes) using M13 primers. Reactions were performed with 100, 250, and 500 nM of His-Fur protein, previously equilibrated for 10 min on ice in 10 µL reaction volume containing 1× binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), 0.5 mM MgCl₂, 0.5 mM MnSO₄ and 2.5% (v/v) glycerol. Next, 50 ng of the DNA probes was added and the mixture was incubated for 20 min on ice. EMSA was also performed under divalent cation-free conditions by adding EDTA to a final concentration of 2 mM in the above reaction mixture. Samples were loaded onto a 2% (w/v) agarose gel prepared with 1× Bis-Tris borate buffer containing 0.1 mM MnSO₄. After 30 min of electrophoresis, the gels were stained with ethidium bromide solution (0.5 µg/mL) for 15 min, and the DNA bands were visualized and recorded under a digital photodocumentation system (Biorad).

Construction of the *kpfR* Mutant Strain

Knockout of the *kpfR* gene from *K. pneumoniae* UKP8 was constructed using the *TargeTron Gene Knockout System* (Sigma-Aldrich), according to the manufacturer's instructions. This system is based on site-specific and not random disruption of the gene of interest by insertion of group II intron harboring a kanamycin resistance gene (*kan^R*).

Firstly, a computer algorithm at TargeTron Design website (Sigma-Aldrich) was used to identify potential target sites for intron insertion on the *kpfR* coding region. The most efficient target site was selected, based on the lowest *E-value* predicted by TargeTron Design algorithm (see **Supplementary Table S2**). The TargeTron Design algorithm also provided the primers used in PCR reactions to mutate (re-target) the RNA segment of the intron (see **Supplementary Table S3**). The 350 pb PCR product was digested and ligated into chloramphenicol-resistant pACD4K-C vector provided by the manufacturer (Sigma-Aldrich). The resulting recombinant pACD4K-C vector was transformed into *Escherichia coli* DH5a by heat shock, and clones of the recombinant vector were obtained using *Wizard Plus SV Minipreps DNA Purification kit* (Promega). The pACD4K-C vector contains a T7 promoter and, therefore, requires T7 RNA Polymerase to express the retargeted intron that will disrupt the target gene. Since *Klebsiella pneumoniae* does not express T7 RNA Polymerase, it was necessary to use the ampicillin-resistant pAR1219 vector (Sigma-Aldrich) in co-transformations because this vector contains the T7 RNA Polymerase gene under the control of the IPTG-inducible *lac* UV5 promoter. Chemically competent *K. pneumoniae* UKP8 cells were co-transformed with pAR1219 and the pACD4K-C recombinant vectors and cultured overnight in LB containing 100 µg/mL of ampicillin and 25 µg/mL of chloramphenicol. On the next day, the culture was diluted 1:50 in fresh LB containing ampicillin and chloramphenicol, and cell growth was monitored until they reached O.D._{600 nm} of 0.2. At this point, to induce intron expression and insertion, 1 mM (final concentration) of IPTG was added to the medium and the culture was incubated

at 30°C for 30 min. After induction, cells were harvested by centrifugation at 14,490 g for 1 min, resuspended in antibiotic-free LB broth, and incubated again for 1 h at 30°C. The cells were then plated on LB agar supplemented with 25 µg/mL kanamycin and grown at 30°C for 2–3 days. Knockout bacteria were selected from kanamycin-resistant colonies and the insertion of the intron RNA harboring *kan^R* gene was confirmed by PCR using primers flanking the target insertion site in the coding region of the *kpfR* gene (**Supplementary Table S1**). Since the knockout is based on the insertion of *kan^R* gene on *kpfR*, the *K. pneumoniae* UKP8 mutant strain was renamed *kpfR::kan^R*.

A complemented strain, named *kpfR::kan^R_{comp}*, was obtained by introducing the *kpfR* gene back into the mutant strain. For this, a DNA fragment comprising the entire coding region of *kpfR* plus approximately 550 bp of 3' and 5' flanking regions (**Supplementary Table S4**) was inserted on pCR2.1-TOPO vector (Invitrogen) previously cloned with erythromycin-resistance gene. Chemically competent *kpfR::kan^R* was transformed with the recombinant vector and plated on LB agar supplemented with 50 µg/mL erythromycin. Complemented strains were recovery by screening erythromycin-resistant colonies.

Growth Experiments and Phenotypic Assays

Initially, the growth of wild-type UKP8 and mutant *kpfR::kan^R* strains was assessed to investigate whether the lack of KpfR compromises bacterial growth. Strains were separately inoculated into LB medium and grown until saturation (overnight) at 37°C while shaking. The next day, the culture was diluted 1:200 in fresh LB and the bacterial growth was monitored every 15 min by measuring the O.D._{600 nm}. Growth curves were constructed by plotting the O.D._{600 nm} values against time.

Ten microliters of saturated cultures of UKP8, *kpfR::kan^R*, and *kpfR::kan^R_{comp}* were plated on blood agar (Trypticase Soy Agar plates supplemented with 5% sheep blood) for 18 h at 37°C to check for macroscopic differences in each strain's morphology.

Motility assays were carried out in triplicate for each *K. pneumoniae* strains, according to protocol described elsewhere with minor modifications (Losensky et al., 2015). In brief, 10 µL of bacterial cultures standardized at O.D._{600 nm} of 0.4 was inoculated in the center of soft LB agar plates (prepared with 0.8% bacteriological agar) and incubated at 37°C for 120 h. After this, the extension of the colonies diameter was measured in cm.

Transmission Electron Microscopy (TEM) was carried out for visualization of fimbriae production by the *K. pneumoniae* strains. Bacterial cells were grown statically on LB agar plates at 37°C for 18 h and resuspended in 500 µL of PBS buffer. A droplet of the cell suspension was spotted on Formvar coated carbon-reinforced copper grid (400 mesh) and incubated at room temperature for 2 min. Grids were negatively stained using a drop of 1.25% phosphotungstic acid pH 6.5 (Sigma-Aldrich) filter sterilized through a 0.22 µm pore-size filter membranes (Millipore). The staining lasted 15 s and then the grids were air-dried on a piece of filter paper. Samples were examined and electron micrographs were obtained with a Zeiss LEO 906 Transmission Electron Microscope operated at 60 kV.

Biofilm formation assays were conducted in polyvinylchloride (PVC) microplates, following a protocol described elsewhere with minor modifications (O'Toole and Kolter, 1998). This protocol is based on the ability of bacteria to form biofilm on PVC microplates and on the ability of crystal violet to stain bacterial cells but not PVC. Strains were grown until saturation at 37°C under shaking. Bacterial cells from saturated cultures were harvested by centrifugation and resuspended in LB broth to a final concentration of 10⁶ cells/mL. 5 µL of this suspension were added on flat-bottom 96-well PVC microplates containing 150 µL of LB and incubated at 37°C for 1, 5, and 10 h under static conditions. After incubation, the culture medium was removed and the wells were gently washed twice with sterile water to remove unattached cells. Next, 25 µL of 1% crystal violet was applied in the wells and the plates were incubated for 15 min at room temperature. After incubation, the excess of dye was removed and the wells were washed with deionized water. Then, the remaining crystal violet staining of the adherent cells was solubilized with absolute ethanol and the absorbance of the ethanol containing the eluted dye was measured at O.D._{600 nm} in a spectrophotometer. The biofilm formation assays were repeated at least three times.

Yeast agglutination assays were performed to investigate the expression of fimbriae by the *K. pneumoniae* strains. Assays were carried out in triplicate for each *K. pneumoniae* strain and were conducted on Kline concavity slides, according to protocol previously described (Roe et al., 2001; Schembri et al., 2004). Bacterial strains grown on LB agar plates up to 72 h were resuspended in PBS buffer and the concentration was standardized at O.D._{600 nm} of 0.6. Bacteria were then mixed with an equal volume of 5% (wt/vol) suspension of *Saccharomyces cerevisiae* cells (Sigma-Aldrich) prepared in PBS buffer. The time required for agglutination and its intensity were documented. The agglutination of the yeast cells is specifically mediated by type 1 fimbriae since these fimbriae have great affinity for mannose, a highly abundant residues on yeast cell-surface. Therefore, the assays were also performed in the presence of 5% D-(+)-Mannose (Sigma-Aldrich) to confirm if the agglutination was indeed mediated by the type 1 fimbriae.

To visualize capsular polysaccharide (CPS) produced by UKP8, *kpfR::kan^R*, and *kpfR::kan^R_{comp}*, strains were cultured under the same growth conditions, stained with India ink, and analyzed under optical microscopy. The presence of capsule is indicated by a negative staining area around the bacteria. CPS production was quantified by extracting and measuring glucuronic acid, a significant component of the *K. pneumoniae* capsule, using a colorimetric assay previously described (Lin et al., 2012; Ares et al., 2016). Briefly, 500 µL of *K. pneumoniae* cultures were mixed with 100 µL of 1% Zwittergent 3-14 detergent (Sigma-Aldrich) in 100 mM citric acid (pH 2.0) and then incubated for 20 min at 50°C. After centrifugation for 2 min at 14,490 g, 250 µL of the supernatants were transferred into new tubes, 1 mL of cold absolute ethanol was added, and the mixtures were incubated for 20 min at 4°C for CPS precipitation. After centrifugation at 14,490 g for 2 min, the supernatants were discarded and the pellets were allowed to dry. The dried pellets were dissolved in 200 µL of distilled water and then

1,200 mL of 12.5 mM tetraborate in concentrated H₂SO₄ were added. Next, the mixtures were vigorously vortexed, heated in a boiling-water bath for 5 min, and cooled down, before the addition of 20 µL of 0.15% 3-hydroxydiphenol (Sigma-Aldrich) prepared in 0.5% NaOH. Then, the absorbance was measured in a spectrophotometer at a wavelength of 520 nm. The glucuronic acid concentration in each sample was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed in micrograms/10⁹ CFU. Quantification of CPS production was performed from five independent cultures of the strains.

Epithelial Cell Adhesion, Invasion, and Intracellular Replication Assays

Coculture assays were performed as previously described (Elsinghorst, 1994; Oelschlaeger and Tall, 1997), with some modifications. Saturated overnight cultures of UKP8 and *kpfR::kan^R* were inoculated on fresh LB medium and incubated at 37°C with shaking until they reached the mid-logarithmic growth phase (O.D._{600 nm} of 0.4). Bacteria were added to an 80% confluent T24 cell monolayer seeded into 12-well plates at a multiplicity of infection (MOI) of 200 bacteria per host cell, and the infected monolayers were incubated at 37°C for three different periods, specific for each assay. For adhesion assay, infected T24 cells were incubated for 30 min, washed three times with phosphate-buffered saline (PBS buffer) to remove non-adhered bacteria, and lysed by adding 0.1% Triton X-100 diluted in PBS. Serial dilutions of the lysate were plated on blood agar and incubated overnight at 37°C for counting bacterial colony-forming units (CFUs). For invasion assay, infected T24 cells were initially incubated for 4 h to allow invasion of the bacterial strains into T24 cells. Plates were washed once with PBS buffer and incubated again for an additional 1 h in fresh culture medium containing the antibiotic gentamicin (25 µg/mL) to eliminate extracellular bacteria (both planktonic and adhered). The plasma membrane of host cells is impermeable to gentamicin. Therefore, after treatment with this antibiotic, only extracellular bacteria will be eliminated, while those present in the intracellular environment will be preserved. After treatment with gentamicin, the T24 cells were washed, lysed with Triton X-100 and serial dilutions of the lysate were plated on blood agar for counting of CFUs. For intracellular replication assay, infected T24 cells were initially incubated for 4 h to allow invasion of the bacterial strains. Plates were washed once with PBS buffer and incubated again for 24 h in fresh culture medium containing gentamicin at 10 µg/mL. Then, the T24 cells were washed, lysed with Triton X-100 and serial dilutions of the lysate were plated on blood agar for CFUs counting. The integrity of the T24 cells during the coculture assays was assessed by the standard exclusion protocol of trypan blue 0.4% (Thermo Fisher Scientific®).

Mouse Model UTI

For mouse urinary tract infection analyzes were used 6–8 weeks old female BALB/c mice (CEMIB, UNICAMP, Brazil). Infections were carried out by transurethral inoculation as previously described (Thai et al., 2010; Reis et al., 2011). UKP8 and *kpfR::kan^R* strains were grown on LB agar plates

at 37°C for 18 h and resuspended at a concentration of $\sim 5 \times 10^8$ CFU/mL. Fifty microliters of the bacterial suspension were inoculated by transurethral catheterization in mice anesthetized intraperitoneally with ketamine and xylazine. Mice were euthanized at the indicated times, and urine and bladders were aseptically collected and processed for histology and CFU titration. Serial dilutions of urine and homogenized bladders were plated on blood agar and incubated overnight at 37°C for counting of CFUs. Whole bladders were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 μ m-thick sections were stained with hematoxylin and eosin (H&E).

Mice experimental procedures were conducted in accordance with guidelines of the Brazilian College for Animal Experimentation (COBEA) and received prior approval by the Ethics Committee on the Use of Animals in Research of Universidade São Francisco (CIAEP/CONCEA No. 01.0226.2014, protocol number 004.04.2015).

RNA Extraction and RT-qPCR Analyses

Gene expression analysis was performed using Reverse Transcription Quantitative real-time Polymerase Chain Reaction (RT-qPCR). The expression of *kpfR* under iron-replete and iron-limiting conditions was investigated as previously described (Gomes et al., 2018). Briefly, *K. pneumoniae* strains were grown in LB broth at 37°C with shaking at 200 rpm until they reached the mid-logarithmic growth phase (O.D._{600 nm} of 0.4). At this point, ferrous iron (100 μ M of FeSO₄, final concentration) or an iron chelator (100 μ M of 2,2'-Dipyridyl, final concentration) were added and the cells were incubated for 1 h. After 1 h incubation, bacterial cells were harvested by centrifugation and the pellets were resuspended in *RNAprotect[®] Bacteria Reagent* for RNA stabilization. All culture conditions were performed at least twice. Control condition consisted of *K. pneumoniae* cells grown in LB medium without supplements.

The expression of fimbrial genes and *galF*, the first gene of the capsular polysaccharide synthesis (*cps*) gene cluster, were also assessed on UKP8 and *kpfR::kan^R* strains grown on blood agar plate. For this, bacterial colonies from each strain were individually collected from the blood agar plates, and the cell pellets were resuspended in *RNAprotect[®] Bacteria Reagent* until the moment of the RNA extraction. Total RNA from the cell pellets was extracted using *RNeasy Protect Bacteria Mini Kit* (Qiagen), following the manufacturer's protocol. An on-column DNase digestion with the RNase-free DNase Set (Qiagen) was performed to remove genomic DNA contamination in RNA samples.

To assess gene expression of the bacterial strains during coculture with T24 bladder epithelial cells, the adhesion and invasion assays were conducted as previously described in section "Materials and Methods," the only exception that the T24 cells were seeded in 6-well plates. After the incubation periods, extracellular bacteria were removed by washing with PBS buffer and T24 cells were collected for the extraction of bacterial total RNA. RNA was extracted using MaxTM Bacterial RNA Isolation kit (Ambion), following the manufacturer's instructions. MICROBEnrichTM kit (Ambion) was applied, in order to promote the selective removal of total RNA from T24

cells, preserving bacterial RNA. Total RNA extracted was further treated with DNase.

After treatment with DNase, 0.5–1 μ g of total bacterial RNA was used for cDNA synthesis by using the *ThermoScriptTM RT-PCR System for First-Strand cDNA Synthesis Kit* (Invitrogen) according to the manufacturer's instructions. The synthesized cDNAs were used in RT-qPCR reactions done in triplicates using the *Platinum[®] SYBR[®] Green qPCR SuperMix-UDG Kit* (Invitrogen) on 7300 *Real-Time PCR System* equipment (Applied Biosystems). Data were normalized using *rho* and *recA* as endogenous genes, which encode transcription termination factor and recombinase A, respectively (Song and Abraham, 2008). The relative expression levels of the selected genes were calculated by the comparative critical threshold ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). GraphPad Prims 7.00 (GraphPad Software, Inc.) was used for the statistical analyses. Differences on the expression levels were evaluated by Student's *t*-test, and differences with $p \leq 0.05$ were considered statistically significant. Primers used on RT-qPCR reactions are listed in **Supplementary Table S5** and were designed using *Primer3 version 4.1.0* web-program.

RESULTS

kpfR Is a Fur-Regulated Gene That Encodes the Transcriptional Regulator of *kpf* Gene Cluster

The *kpf* gene cluster comprises 4 genes designated as follow: *kpfA*, encoding the major pilin subunit, *kpfB*, encoding a chaperone, *kpfC*, encoding an usher, and *kpfD*, encoding an adhesin. The first gene adjacent to *kpf* cluster at 5' extremity encodes a putative helix-turn-helix transcriptional regulator. To determine whether this adjacent gene belongs to *kpf* cluster, reverse-transcription-PCRs were performed using cDNA and primer pairs spanning the entire cluster and confirmed that *kpf* cluster and the adjacent gene are co-transcribed as a single polycistronic transcript (**Figure 1A**). Since the adjacent gene encodes a putative helix-turn-helix transcriptional regulator, and following Wu et al. (2010) nomenclature, we designated it *kpfR* gene.

To better understand the expression regulation of *kpf*, bioinformatic analyses were employed to identify regulatory domains in the promoter region of the cluster. Thus, a putative Fur box sequence was identified 100 nucleotides downstream to start codon of the *kpfR* gene (**Figure 1B**). The long-distance between the Fur box and the promoter region of the *kpf* cluster led us to investigate additional regulatory elements on the adjacent sequences. As indicated in **Figure 1B**, a putative binding site of the DNA bending protein IHF (integration host factor) was identified at the intervening region between the promoter and the Fur box. This putative site closely resembles to the consensus recognition sequence of IHF, but its functionality was not validated in the present study.

The putative Fur box identified inside the coding region of *kpfR* was validated by Fur Titration Assay (FURTA) and DNA Electrophoretic Mobility Shift Assay (EMSA). **Figure 1C**

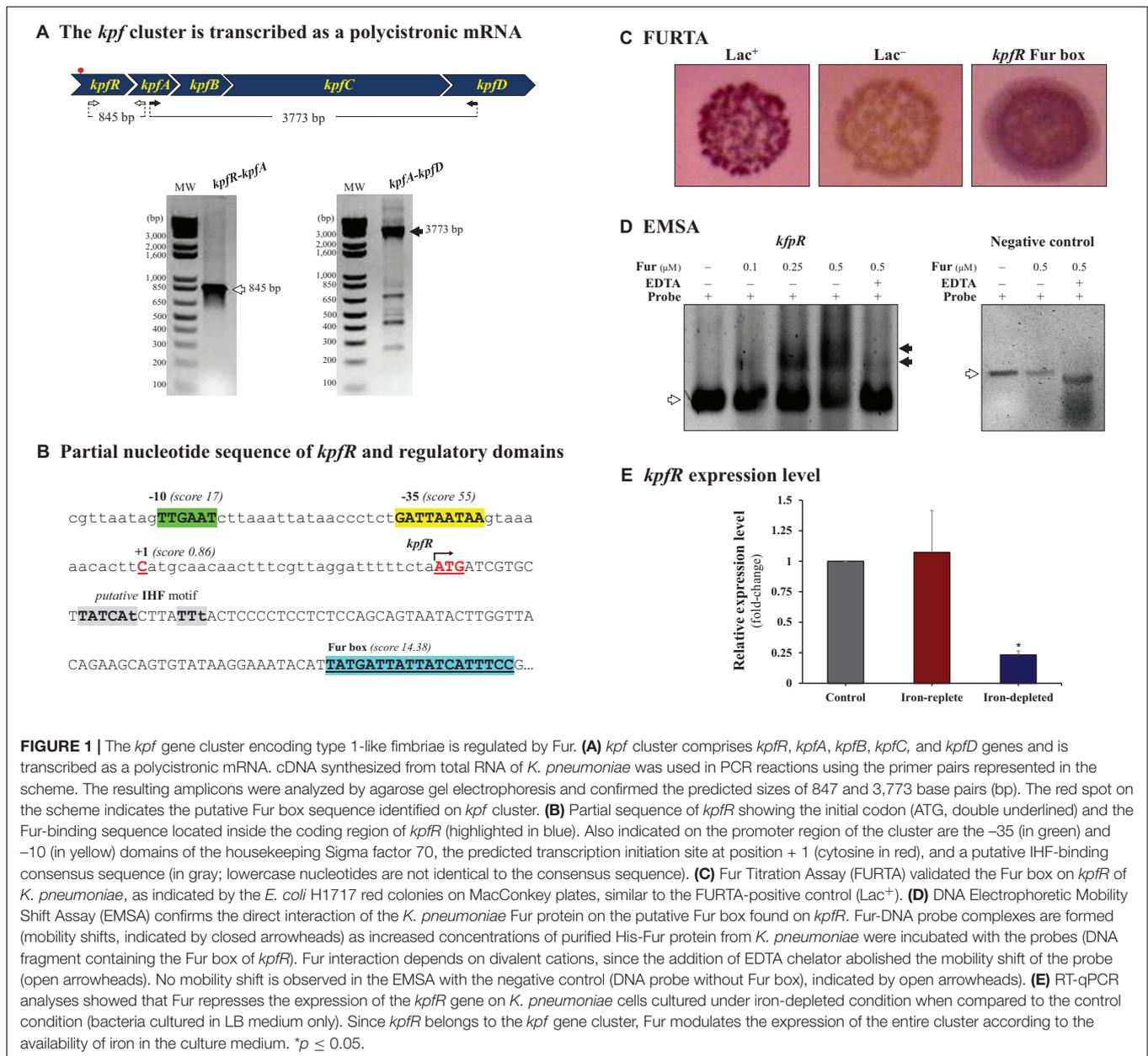


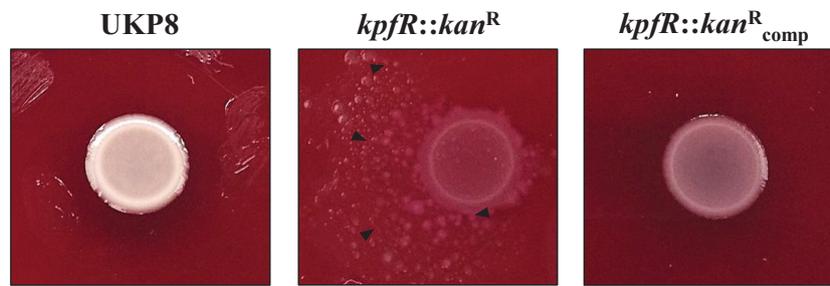
FIGURE 1 | The *kpf* gene cluster encoding type 1-like fimbriae is regulated by Fur. **(A)** *kpf* cluster comprises *kpfR*, *kpfA*, *kpfB*, *kpfC*, and *kpfD* genes and is transcribed as a polycistronic mRNA. cDNA synthesized from total RNA of *K. pneumoniae* was used in PCR reactions using the primer pairs represented in the scheme. The resulting amplicons were analyzed by agarose gel electrophoresis and confirmed the predicted sizes of 847 and 3,773 base pairs (bp). The red spot on the scheme indicates the putative Fur box sequence identified on *kpf* cluster. **(B)** Partial sequence of *kpfR* showing the initial codon (ATG, double underlined) and the Fur-binding sequence located inside the coding region of *kpfR* (highlighted in blue). Also indicated on the promoter region of the cluster are the -35 (in green) and -10 (in yellow) domains of the housekeeping Sigma factor 70, the predicted transcription initiation site at position + 1 (cytosine in red), and a putative IHF-binding consensus sequence (in gray; lowercase nucleotides are not identical to the consensus sequence). **(C)** Fur Titration Assay (FURTA) validated the Fur box on *kpfR* of *K. pneumoniae*, as indicated by the *E. coli* H1717 red colonies on MacConkey plates, similar to the FURTA-positive control (Lac⁺). **(D)** DNA Electrophoretic Mobility Shift Assay (EMSA) confirms the direct interaction of the *K. pneumoniae* Fur protein on the putative Fur box found on *kpfR*. Fur-DNA probe complexes are formed (mobility shifts, indicated by closed arrowheads) as increased concentrations of purified His-Fur protein from *K. pneumoniae* were incubated with the probes (DNA fragment containing the Fur box of *kpfR*). Fur interaction depends on divalent cations, since the addition of EDTA abolished the mobility shift of the probe (open arrowheads). No mobility shift is observed in the EMSA with the negative control (DNA probe without Fur box), indicated by open arrowheads). **(E)** RT-qPCR analyses showed that Fur represses the expression of the *kpfR* gene on *K. pneumoniae* cells cultured under iron-depleted condition when compared to the control condition (bacteria cultured in LB medium only). Since *kpfR* belongs to the *kpf* gene cluster, Fur modulates the expression of the entire cluster according to the availability of iron in the culture medium. * $p \leq 0.05$.

shows the FURTA-positive control (Lac⁺, red colonies), the FURTA-negative control (Lac⁻, colorless colonies) and the validation of the Fur box on *kpfR*, indicated by the red colonies. This result confirms that Fur regulator from *E. coli* H1717 was able to bind *in vivo* to the cloned putative Fur box from *kpfR* of *K. pneumoniae*, rendering the colonies with red color. Next, EMSA was performed to verify the direct interaction of *K. pneumoniae* Fur protein on the putative Fur box found on *kpfR*. As shown on **Figure 1D**, a mobility shift of the DNA probes containing the putative Fur box was observed when increasing concentrations of *K. pneumoniae* purified His-Fur protein was incubated with these probes. Besides, the addition of the divalent cations chelator EDTA abolished the mobility shift of the probe, indicating that divalent cations are required for Fur interaction.

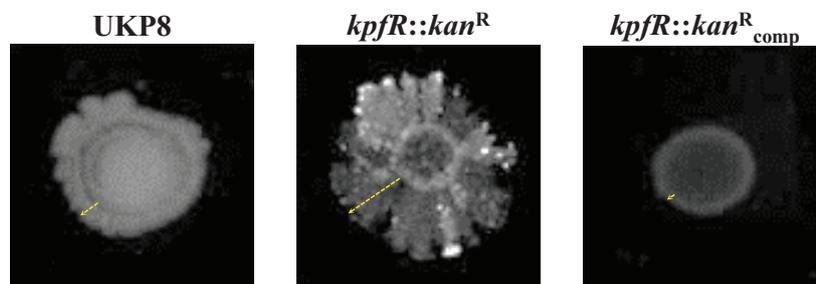
No mobility shift is observed when *K. pneumoniae* Fur protein is incubated with a DNA probe without Fur box sequence.

Taken together, our results confirm that Fur protein recognizes and binds to the Fur box on *kpfR* of *K. pneumoniae*, indicating that the expression of *kpfR* seems to be modulated by Fur transcriptional regulator in an iron-dependent manner. To further investigate how Fur modulates the expression of *kpfR*, RT-qPCR analyses were performed on *K. pneumoniae* cells kept under iron-replete and iron-limited conditions. As displayed on **Figure 1E**, the iron-replete condition presents no changes in the expression pattern of *kpfR* gene when compared to the control condition. On the other hand, *kpfR* is repressed under condition of iron scarcity. These results show that Fur modulates the expression of *kpfR* gene according to the availability of iron

A Macroscopic morphologies of on blood agar plates



B Motility assay on soft-agar plates



C Measurement of colonies spreading

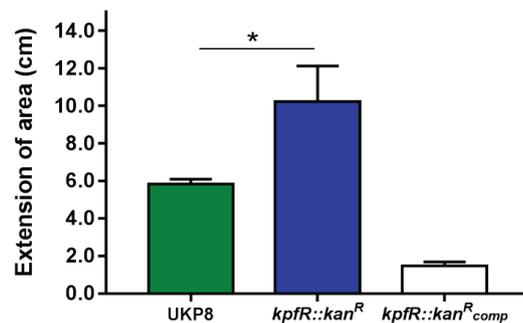


FIGURE 2 | Bacterial colonies of *K. pneumoniae* cells depleted of *kpfR* show altered macroscopic morphologies and rudimentary motility. **(A)** Colony morphology of wild-type (UKP8), mutant (*kpfR::kan^R*), and complemented (*kpfR::kan^R_{comp}*) strains were investigated on blood agar plates. The altered pattern on the mutant strain was fully restored in the complemented strain, which exhibited a pattern similar to the wild-type. **(B,C)** In soft agar plates, *kpfR::kan^R* cells exhibited a pronounced asymmetric dispersion on the surface of the plates when compared to UKP8, resembling a sort of rudimentary motility. Dispersion was only partially restored by complementation. The greater dispersion of the mutant colonies than the wild-type is statistically significant. * $p \leq 0.05$.

in the culture medium. Thus, *kpfR* belongs to the Fur regulon of *K. pneumoniae*.

kpfR Disruption Modified the Bacterial Morphology and Enabled a Rudimentary Motility

To evaluate the role of KpfR as regulator of morphology and virulence of *Klebsiella pneumoniae*, a mutant (*kpfR::kan^R*) was generated (as described in section “Materials and Methods”). The mutant strain exhibits prolonged growth at the lag phase, leading

to delayed entry into the logarithmic (log) phase of growth (see **Supplementary Figure S1**). Nonetheless, once reaching the log phase, the mutant strain displayed a growth rate similar to that observed in the wild-type strain. Slight differences in growth patterns were observed among the UKP8 wild-type strain and the complemented *kpfR::kan^R_{comp}* strain; i.e., the mutant strain transformed with a *kpfR*-carrying vector.

Macroscopic morphologies of wild-type UKP8 and mutant *kpfR::kan^R* strains were investigated on blood agar plates. As shown on **Figure 2A**, the wild-type strain exhibited circular shape, high elevation, entire margin, and smooth bright surface.

On the other hand, the mutant *kpfR::kan^R* strain presented irregular shape, flat elevation, lobed margin, and rough matte surface. In addition, *kpfR::kan^R* colonies exhibited a slight dispersion on the plates. The morphological features observed on the mutant strain can be attributed to the lack of KpfR, because the pattern observed on the wild-type strain was fully reestablished on the complemented mutant strain (*kpfR::kan^R_{comp}*).

The spreading behavior of *kpfR::kan^R* colonies on blood agar plates prompted us to perform motility assays on soft agar plates. As displayed on **Figure 2B**, the wild-type UKP8 strains presented a slight and linear radial migration on soft agar plates. On the other hand, *kpfR::kan^R* cells exhibited a more prominent and asymmetric dispersion on the surface of the plates. The complemented mutant strain was partially restored since it had a reduced migration extension when compared to both wild-type and mutant strains. The extent of the area in which the wild-type, mutant, and complemented strains migrated on the plates was measured, and confirmed that the mutant strain had a greater dispersion when compared to UKP8 and *kpfR::kan^R_{comp}* strains (**Figure 2C**). These results indicate that *kpfR* disruption provided a sort of rudimentary motility to the mutant bacteria.

Lack of KpfR Triggers the Production of Bacterial Surface Appendages, Enhances Yeast Agglutination and Biofilm Formation but Reduces Capsule Production

Since loss of *kpfR* rendered *K. pneumoniae* a prominent dispersion on agar plates, we evaluated the cell surface morphology of the wild-type, mutant, and complemented strains by Transmission Electron Microscopy (TEM). While UKP8 strain appears with no surface appendages, the cell surface of the *kpfR::kan^R* cells was covered by numerous appendages similar to fimbriae or pili that are long and flexible with a varied size larger than 1 μm (**Figure 3A**). These numerous appendages were no longer seen on the surface of the complemented mutant strain (*kpfR::kan^R_{comp}*).

The presence of numerous appendages on *kpfR::kan^R* cells led us to investigate the expression of fimbrial genes on UKP8 and *kpfR::kan^R* cells grown on blood agar plate. Disruption of *kpfR* resulted in up-regulation of *fimA*, from type 1 fimbrial gene cluster *fim*, and *kpfR* and *kpfA* genes, from *kpf* gene cluster (**Figure 3B**). *kpfR* and *kpfA* presented a similar expression pattern, confirming the polycistronic transcription of these genes, as previously shown. Moreover, the up-regulation of *kpfR* in the cells with disrupted *kpfR* suggests that KpfR autoregulates its own expression. On the other hand, lack of KpfR regulator resulted in down-regulation of *ecpA* gene, from the *ecp* cluster, whereas *mrkA* gene, from type 3 fimbrial gene cluster *mrk*, presented unchanged expression pattern between wild-type and mutant strains.

To investigate the phenotypic effects of increased type 1-like fimbriae production due to KpfR absence, biofilm formation,

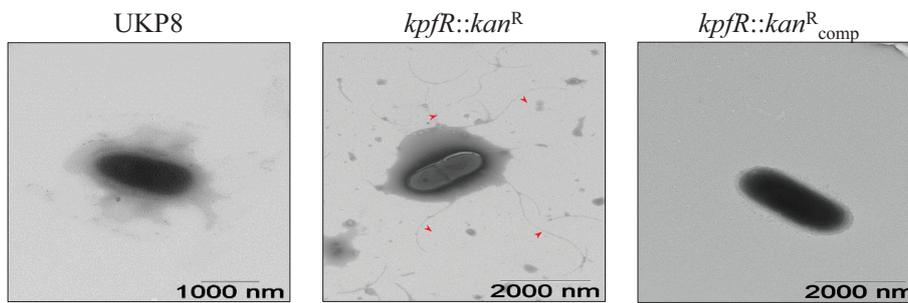
and yeast agglutination assays were performed with the UKP8, *kpfR::kan^R*, and *kpfR::kan^R_{comp}* strains. Biofilm formation by *kpfR::kan^R* cells was significantly superior than UKP8 after 3, 5, and 10 h of incubation (**Figure 3C**). The complemented *kpfR::kan^R_{comp}* strain failed to restore the biofilm formation presented by the wild-type UKP8, since exhibited a phenotype similar to the mutant *kpfR::kan^R* strain. Yeast agglutination assays allow specific detection of type 1 fimbriae because type 1 fimbrial adhesins have a great affinity for mannose-containing receptors on the yeast cell surface. UKP8 showed no yeast agglutination, whereas the *kpfR::kan^R* promptly agglutinated the yeast cells (**Figure 3D**). The lack of agglutination observed on UKP8 was fully restored on the complemented *kpfR::kan^R_{comp}* strain. To determine if the agglutination pattern exhibited by the mutant strain was due to type 1 fimbriae expression, the assay was also performed in the presence of mannose prior to the addition of the yeast cells. In the presence of mannose, the mutant strain loses the ability to agglutinate yeast cells. These results reveal that the observed agglutination of yeast cells was indeed mediated by type 1 fimbriae.

As the lack of KpfR regulator resulted in increased expression of type 1-like fimbriae, we decided to investigate the production of capsular polysaccharide, another important virulence factor of the bacterial cell surface. Negative staining with India ink revealed no visible capsule in the *kpfR::kan^R* mutant strain as compared to the UKP8 wild-type strain (**Figure 4A**), while this phenotype was partially restored on the complemented strain. In addition, quantification of glucuronic acid revealed that the mutant strain presents 10-fold decrease in capsule production compared to that in the wild-type strain (**Figure 4B**). Capsule production by the complemented strain was partially restored to the levels observed in the wild-type strain. We next investigated the expression of the *cps* gene cluster on the wild-type and the mutant strains by determining the expression levels of *galF* using RT-qPCR analysis. *galF* is the first gene of the *cps* cluster and encodes an enzyme dedicated to the synthesis of sugar nucleotides, which are the precursors that will compose the repeating-units of the capsular polysaccharide. Consistent with the less capsule production by the *kpfR::kan^R* mutant strain, RT-qPCR analysis revealed down-regulation of the capsule-producing gene *galF* on *kpfR::kan^R* in comparison with the expression on UKP8 wild-type strain (**Figure 4C**).

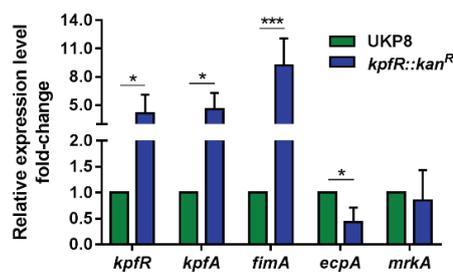
kpfR::kan^R Colonizes More Efficiently Bladder Epithelial Cells *in vitro* but Loses Resistance *in vivo* and Is Unable to Form IBC in Mouse Urinary Bladder

Considering fimbriae important mediators of bacterial adhesion and internalization into host cells, we investigated the role of *kpfR* gene in host-pathogen interactions by assessing the adhesion, invasion, and intracellular replication of UKP8 and *kpfR::kan^R* strains on human bladder epithelial cell line T24. The data presented on **Figure 5A** indicate that the *kpfR::kan^R* strain showed greater adhesion to T24 bladder cells than the wild-type UKP8 strain. Moreover, only the mutant strain was able to invade

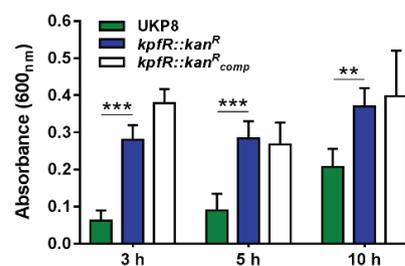
A Transmission Electron Microscopy



B Expression of fimbrial genes



C Biofilm formation



D Yeast agglutination assay

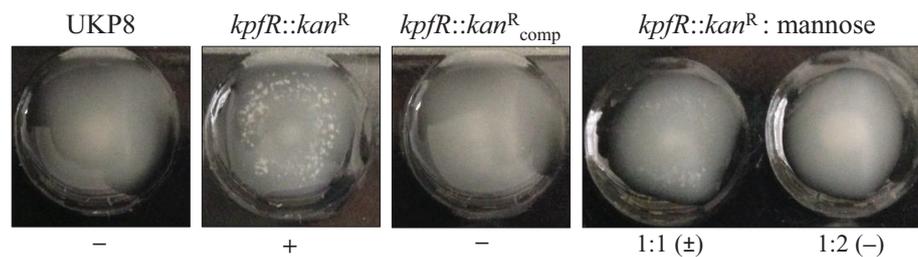
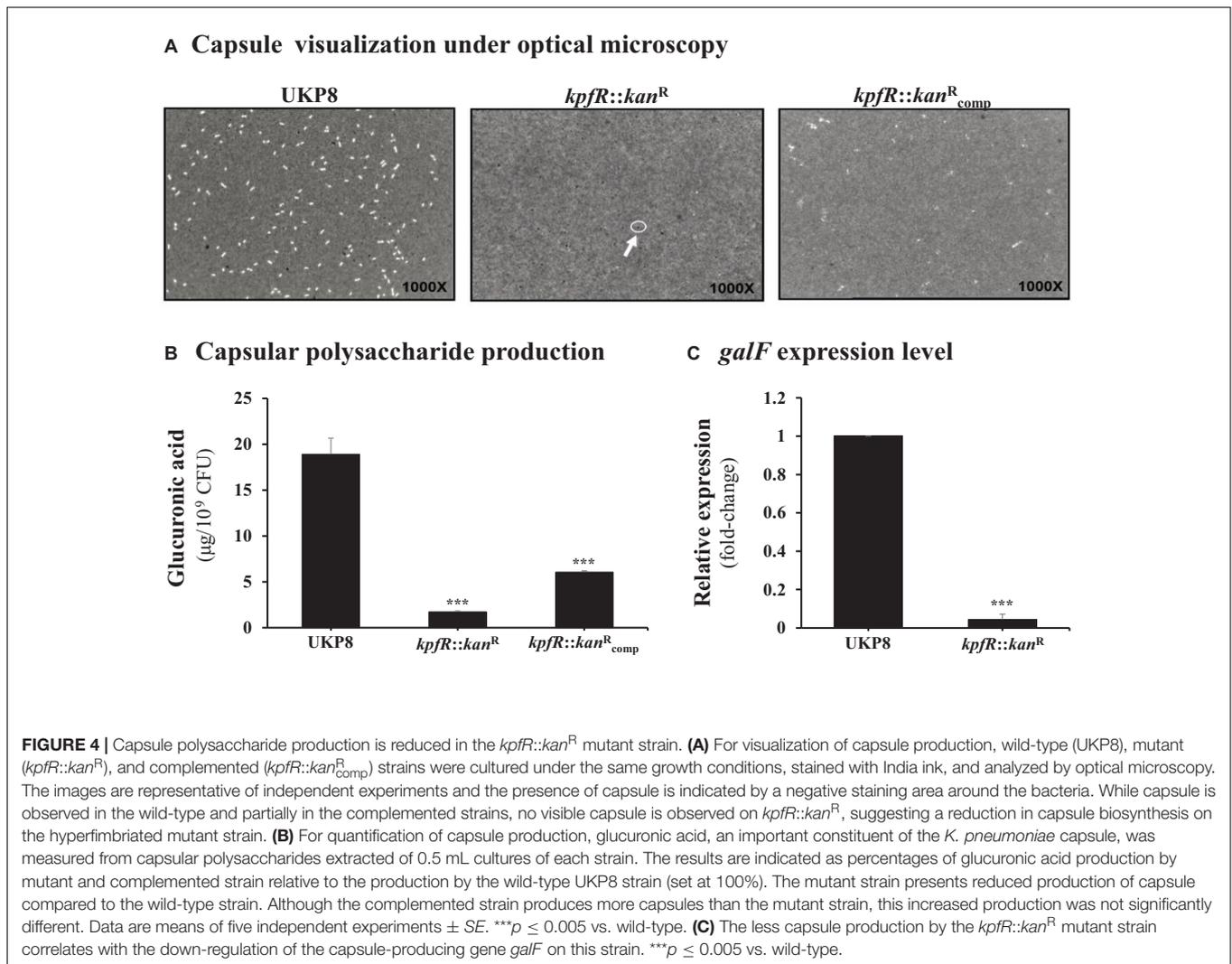


FIGURE 3 | Lack of KpfR triggers the expression of type 1-like fimbriae, increases biofilm formation and yeast cells agglutination, and reduces production of capsule polysaccharide. **(A)** TEM analyses revealed *kpfR::kan^R* mutant cells covered with numerous fimbriae-like appendages that were absent in wild-type and complemented *kpfR::kan^R_comp* cells. The images are representative of independent experiments. **(B)** This hyperfimbriated phenotype is corroborated by the fimbrial genes expression analyses. The *ecpA* gene, from *ecp* gene cluster, is downregulated, while *fimA* (*fim* gene cluster of type 1 fimbriae) and *kpfR* and *kpfA* genes (*kpf* gene cluster of type 1-like fimbriae) are up-regulated in the *kpfR::kan^R* mutant cells. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$. **(C, D)** Because of the hyperfimbriated phenotype, the mutant strain forms more biofilm than the wild-type and agglutinates yeast cells. This agglutination is mediated by type 1-like fimbriae because in the presence of mannose the mutant strain loses the ability to agglutinate yeast cells. Complementation failed to restore the biofilm formation exhibited by the wild-type strain, but fully restored the agglutination of yeast cells. The images are representative of independent experiments.

and replicate within T24 cells (Figure 5A). No significant changes in T24 cells viability were observed at a multiplicity of infection (MOI) of 200 bacteria:1 T24 cell and incubation of coculture for 24 h (data not shown). According to the results, the lack of KpfR repressor improved the ability of the mutant strain to adhere, invade and even replicate intracellularly in T24 bladder cells. We next evaluated the expression of *kpfR* and *kpfA* (cluster *kpf*) and *fimA* (cluster *fim*) in UKP8 and *kpfR::kan^R* strains during adhesion to the T24 cells. As shown on Figure 5B, all three genes

had the expression significantly induced in *kpfR::kan^R* compared to UKP8.

To further evaluate the *in vivo* role of KpfR repressor in *Klebsiella pneumoniae* virulence, a mouse urinary tract infection model was used to compare the ability of UKP8 and *kpfR::kan^R* strains to colonize the bladder of the animals (Figure 5C). At 6 hpi, there were no differences in UKP8 and *kpfR::kan^R* load in mice urine and bladder. At 24 hpi, UKP8 was recovered from bladder tissue and urine, while *kpfR::kan^R* was not present



on these samples. At 48 hpi, UKP8 and *kpfR::kan^R* were no longer found in urine, whereas only UKP8 was still present in mice bladder tissue.

Aliquots of urine were analyzed under an optical microscope (data not shown) and revealed red blood cells, leukocytes and scaly epithelium in urine of the animals infected with both UKP8 and *kpfR::kan^R* strains at 6 hpi. At 24 hpi, a larger population of leukocytes and bacteria were observed only in the urine samples of mice infected with the wild-type strain. Urine of mice infected with *kpfR::kan^R* during 24 h presented neither erythrocytes nor leukocytes.

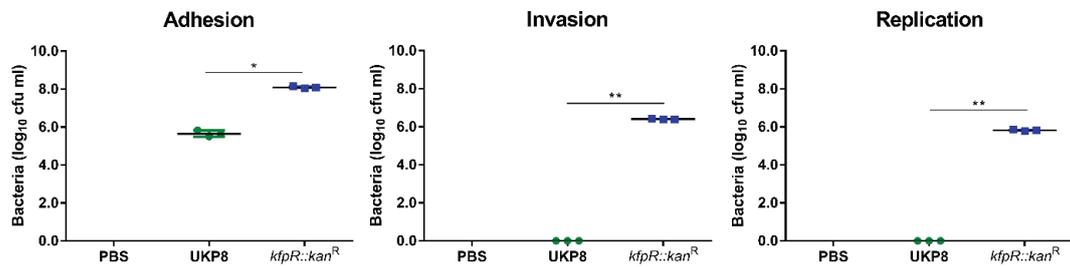
The colonization experiments suggested that the lack of KpfR repressor confers a disadvantage on *kpfR::kan^R* strain. To characterize these effects further, histological analyses were performed to obtain images of mouse bladders infected with the wild-type and *kpfR::kan^R* mutant strains. As shown on **Figure 5D**, an inflammatory infiltrate consisting mainly of neutrophils and hyperemia of the bladder blood vessels was observed 6 hpi with both strains. At 24 hpi, the hyperemia was more prominent in the bladder tissue infected with UKP8 than *kpfR::kan^R*, and an

abundant migration of inflammatory cells was noticed only with UKP8 at 48 hpi (**Figure 5D**). In addition, only wild-type strain was able to form intracellular bacterial communities (IBCs) in the urothelium at 24 and 48 hpi (**Figure 5E**). Taken together, the data show successful bladder colonization and formation of IBC in urothelial cells by the wild-type UKP8 strain, while the *kpfR::kan^R* mutant strain loses resistance and ability to survive in the host.

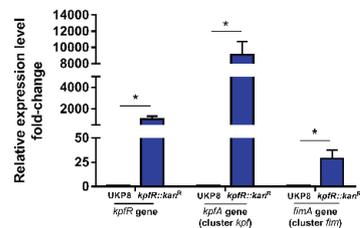
DISCUSSION

Fimbriae are important mediators of UTIs caused by *K. pneumoniae* (Tinker and Clegg, 2000; Yeh et al., 2002; Rosen et al., 2008a; Stahlhut et al., 2012). Considering the broad number of fimbrial gene clusters on the *K. pneumoniae* genome, the expression of this range of fimbrial structures demands a coordinated and finely adjusted control. In the current study, we describe the role of KpfR as a transcriptional regulator of fimbrial expression in *K. pneumoniae* and its role in the pathogenicity of this bacterium.

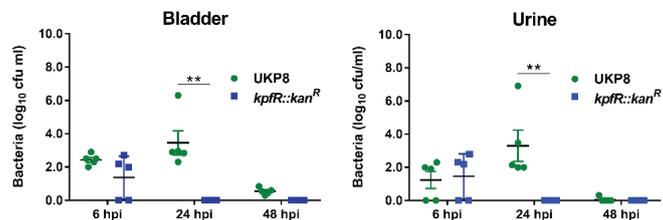
A Coculture assay of the bacterial strains with human bladder epithelial cell line T24



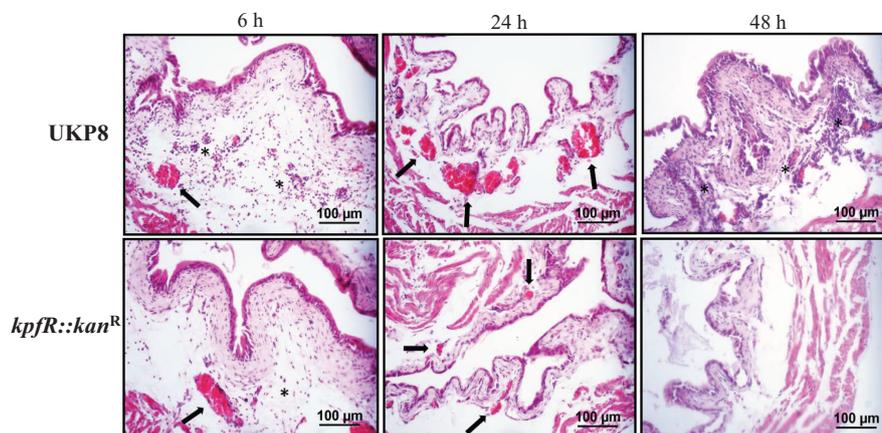
B Expression analysis during adhesion to T24 cell



C UKP8 and *kpfR::kan^R* load in mice



D Histological analyses of mouse bladders infected with UKP8 and *kpfR::kan^R* strains



E IBC formation by the wild-type UKP8 strain in the urothelium

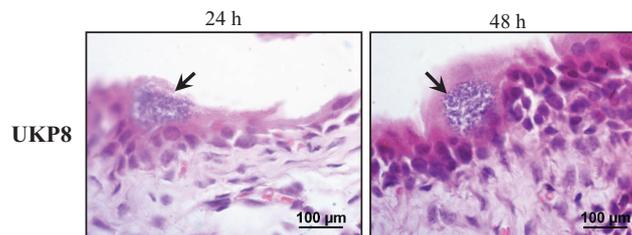


FIGURE 5 | *K. pneumoniae* mutant for *kpfR* adheres more efficiently and replicates within bladder epithelial cells, but loses resistance in the mouse model of urinary infection. For the coculture assays, human bladder epithelial cell line T24 were inoculated with the wild-type and *kpfR::kan^R* strains at an MOI of 200 to assess the adhesion, invasion, and intracellular replication of the strains on the host human bladder cell. The incubation periods are described in section “Materials and Methods.” **(A)** The mutant strain has greater adhesion to T24 bladder cells than the wild-type UKP8 strain and is the only strain able to invade and replicate within T24 cells. **(B)** During adhesion to T24 bladder cells, the *kpfR::kan^R* mutant strain have increased expression of *kpfR* and *kpfA* (cluster *kpf*) and *fimA* (cluster *fim*) than the wild-type UKP8 strain, which may explain the improved ability of the mutant strain to adhere the T24 cells. * $p \leq 0.05$; ** $p \leq 0.01$. For the mouse model of urinary (Continued)

FIGURE 5 | Continued

infection, animals were inoculated by transurethral catheterization with 5×10^8 CFU/mL of UKP8 and *kpfR::kan^R* strains. At the indicated times, the animals were euthanized, and urine and bladders were aseptically collected and processed for CFU enumeration and H&E staining. **(C)** Bacterial CFUs were counted on urine and bladder tissue after 6, 24, and 48 hpi with UKP8 and *kpfR::kan^R* strains. In mice urine, the wild-type strain is recovered at 6 and 24 hpi, while the mutant *kpfR::kan^R* strain is recovered only at 6 hpi. In the bladder tissue, the wild-type is present at 6, 24, and 48 hpi, whereas the mutant strain is also recovered only after 6 hpi. Mice are represented by symbols. Data were statistically analyzed by ANOVA test. $**p \leq 0.01$. **(D)** Histological analyses of bladders infected with the wild-type and *kpfR::kan^R* mutant strains show both strains triggering an inflammatory infiltrate consisting of neutrophils (asterisks) and a hyperemia (arrows) at 6 hpi. At 24 hpi, the hyperemia is more pronounced with the wild-type strain, and inflammatory cells migration is observed at 48 hpi only with UKP8, suggesting that at this period of infection the mutant strain has already been completely eliminated. Images are from an individual representative experiment. **(E)** Only the wild-type UKP8 is able to form biofilm-like intracellular bacterial communities (IBCs) in the urothelium of mice. Images from histological analyses of bladders infected with the wild-type at 24 and 48 hpi reveal IBCs (arrows) within superficial urothelial cells. Images are from an individual representative experiment.

KpfR is encoded by *kpfR*, a gene belonging to the *kpf* gene cluster that encodes type 1-like fimbriae (Wu et al., 2010). We showed that the Fur regulator binds to a Fur box identified on *kpfR* gene when complexed with iron and that iron scarcity downregulates *kpfR*. Since the genes of the *kpf* cluster are cotranscribed as an operon, we propose that Fur exerts a direct regulation of the *kpfR* gene and *kpf* gene cluster by acting as a transcriptional activator. This is not the first report of Fur activating the expression of transcriptional regulators of fimbriae. Wu et al. (2012) showed that the Fur controls the expression of type 3 fimbriae by positively regulating *mrkHI*, which is involved in the regulation of type 3 fimbriae *mrk* gene cluster.

The location of this Fur box inside the coding region of the *kpfR* gene is an intriguing finding. Interestingly, a putative IHF binding site that matches the IHF consensus binding sequence was identified at the intervening sequences between the promoter region of the *kpf* gene cluster and the Fur box inside *kpfR*. IHF is a nucleoid-associated protein involved in many cellular processes including site-specific recombination, transcription and DNA replication (Freundlich et al., 1992). IHF acts as a transcriptional coactivator that binds to a consensus sequence (WATCARNNNNTTR, where W is A or T, N is any nucleotide, and R is G or A; Freundlich et al., 1992) and bends the DNA into loops that approximate far distant regulatory elements (Clark et al., 2019). Even though the putative IHF binding site found on *kpfR* has not been functionally validated in the present study, we can hypothesize that IHF induces a bend in the DNA that brings the Fur regulator close to the regulatory elements on the promoter region of *kpf*, thus allowing Fur to exert its transcriptional activation on the cluster. Nonetheless, this hypothesis deserves a deeper investigation in future research to elucidate the exact mechanism of regulation played by Fur on the *kpf* cluster.

Historically described as a transcriptional repressor that utilizes ferrous iron as co-repressor to prevent transcription of target genes by binding on the promoter region of these genes and blocking the entry of RNA polymerase (Escobar et al., 1999), recent studies have shown that Fur can also function as an activator of transcription (Carpenter et al., 2009; Troxell and Hassan, 2013; Seo et al., 2014; Deng et al., 2015). According to Troxell and Hassan (2013), Fur may exert transcriptional activation by (i) indirectly regulating small regulatory RNAs, (ii) binding on regulatory elements that enhance recruitment of the RNA polymerase, or (iii) acting as an antirepressor, in which the binding of Fur-iron complexes on the promoter

elements blocks the binding of a transcription repressor (Troxell and Hassan, 2013). Fur activation through “RNAP recruitment” mechanism has been described in *H. pylori* (Delany et al., 2001) and *S. enterica* (Teixidó et al., 2011), while Fur activation through “antirepressor” mechanism has been described in *E. coli* (Nandal et al., 2010) and also in *H. pylori* (Gilbreath et al., 2012). For instance, the Fur-Fe²⁺ complex releases the transcription of the *ftnA* gene, which encodes ferritin in *E. coli*, by binding to the *ftnA* promoter and displacing the binding of the global repressor H-NS, resulting in the release of access of the RNA polymerase to the promoter and reverting the repression exerted by H-NS (Nandal et al., 2010). Interestingly, our results show Fur binding to the Fur box on *kpfR* only when complexed with iron, but this binding does not result in expression modulation of *kpfR*. On the other hand, Fur is no longer bound to the Fur box in conditions of iron scarcity, and the gene is downregulated. This unaltered expression pattern of *kpfR* under iron-rich conditions despite Fur-Fe complex binding to the *kpfR* Fur box suggests Fur exerting transcription activation of *kpfR* through an “antirepressor” mechanism, similarly to that observed in transcription of the *E. coli ftnA* gene. In this mode of regulation involving an “antirepressor” mechanism, Fur is not required for the transcription of *kpfR* gene, but it is required to relieve its repression by a transcriptional repressor, possibly the product of *kpfR* gene itself, the transcriptional repressor KpfR. This hypothesis is corroborated by the upregulation of *kpfR* and *kpfA* genes on the mutant strain *kpfR::kan^R* cells, which suggests that KpfR autoregulates its own expression. Additional experiments, such as fimbrial gene expression in a Fur null mutant strain and promoter-reporter gene fusion assays, are needed to better understand the role of Fur as a transcriptional activator of *kpfR*.

The *K. pneumoniae* strain mutant for *kpfR* gene exhibited phenotypic changes, among which macroscopic changes on colony morphology, a slight dispersion on blood agar plates, greater ability to form biofilm, and capacity to agglutinate yeast cells. Also, the mutant strain presented up-regulation of the type 1-like fimbriae gene clusters *fim* and *kpf* and exhibited an increased production of cell surface appendages similar to fimbriae, as revealed by TEM analysis. Unfortunately, in the present study we did not conduct further investigations to identify the nature of these appendages, whether they are type 1 or Kpf fimbriae. Nevertheless, given the up-regulation of both *fim* and *kpf* gene clusters by the mutant strain and its ability to agglutinate yeast cells, which specifically indicates the production of type 1-like fimbriae, we may infer that these structures revealed

by TEM analysis are probably type 1 and Kpf fimbriae. However, further studies are needed to fully and precisely identify the nature of those appendages.

Strikingly, the hyperfimbriated phenotype seems to provide the mutant strain a kind of rudimentary motility. Although *K. pneumoniae* is recognized as immobile, the finding of a rudimentary motility in this bacterium is not an unprecedented result. Carabarin-Lima and León-Izurietta (2016) reported the presence of flagellar genes on a *K. pneumoniae* strain isolated from nosocomial infections and described a swim-like motility phenotype mediated by flagella in these clinical isolates. Besides, the *K. pneumoniae* genome contains the *flk* gene that encodes a poorly characterized regulator of flagella biosynthesis. It remains to be clarified whether the rudimentary movement observed in the mutant strain is due to the excess of type 1-like fimbriae production, or if the KpfR regulator plays some role in regulating the expression of flagellar genes.

One of the phenotypic changes observed in the *kpfR* mutant strain was a drastic reduction in the production of capsular polysaccharide as opposed to the higher expression of fimbriae structures. Reports in the literature describe an inverse effect between the production of fimbriae and capsules, and also suggest that capsule production can inhibit the expression of fimbriae and interferes in fimbriae functionality (Matatov et al., 1999; Schembri et al., 2005; Paczosa and Meccas, 2016). In this respect, it is suggested that a possible coordinated regulation between fimbriae and capsules occurs through an environmental stimulus not yet known. For instance, Schwan et al. (2005) showed that adherence of uropathogenic *E. coli* to mannose receptors on bladder epithelial cells via type 1 fimbriae triggers a cross-talk that leads to the down-regulation of capsule production. Regarding *K. pneumoniae*, a possible cross-regulation between the capsule and fimbrial expression has been speculated, although not yet proven (Schembri et al., 2005; Struve et al., 2008; Schroll et al., 2010). A clue to this apparent cross-regulation of capsule and fimbriae expression in *K. pneumoniae* came from the work of Huang et al. (2014). These authors showed that the deletion of the *cps* gene cluster, responsible for capsular polysaccharide biosynthesis, inhibits the expression of genes encoding type 1 and type 3 fimbriae. In conformity to these reports, our findings indicate KpfR as a repressor of fimbriae expression which, when knocked out, leads to activation of fimbriae expression and inhibition of the production of capsules in *K. pneumoniae*.

Most of the phenotypic changes exhibited by the mutant strain were fully or partially restored on the complemented strain. However, complementation tests rendered unexpected results for capsule production and biofilm formation. Although the complemented strain presented a more than threefold increase in capsule production compared to the mutant strain, this increase was not significantly different, and the production did not reach the levels presented by the wild-type strain. Biofilm formation, which is closely related to capsule and fimbriae expression, also rendered unexpected results, since the complemented strain failed to recover the wild-type phenotype and, instead, presented similar capacity to form biofilm as exhibited by the mutant strain. This phenotype may be related to the capsular polysaccharide production by these strains. In fact, although

capsule plays a key role for *K. pneumoniae* biofilm establishment and maturation (Balestrino et al., 2008), it is well known that the exacerbated production of capsule shields short bacterial adhesins and inhibits fimbriae assembly and activity (Matatov et al., 1999; Schembri et al., 2004, 2005). Besides, unencapsulated *K. pneumoniae* mutant strains produce greater amounts and more organized biofilms compared to the wild-type strains (Dzul et al., 2011; Huang et al., 2014). Thus, the reduced production of capsule by the *kpfR* mutant strain and the complemented strain seems to render these strains a greater capacity to form biofilm. Another possible explanation for the biofilm formation by the complemented strain may be related to the construction of the complemented strains. For complementation tests, we cloned the *kpfR* gene into an ordinary cloning vector, not a simple-copy vector. Thus, some of the unexpected results may likely be due to enhanced expression of *kpfR* gene from the vector, probably above the native levels. The effects of this unbalanced expression would be further enhanced when the cloned gene under investigation encodes a transcriptional regulator. Finally, the complexity of the mechanisms that regulate the expression of fimbriae and capsular polysaccharides (CPS), the two main surface components of prokaryotic cells, may also explain the enhanced capacity of the complemented strain to form biofilm. In fact, the production of these structures demands a coordinated and tightly regulated expression of numerous gene clusters and it is under the control of a myriad of regulators, such as RmpA (Hsu et al., 2011), Fur (Lin et al., 2011), IscR (Wu et al., 2014), H-NS (Ares et al., 2016), RcsAB (Peng et al., 2018), KvrA and KvrB (Palacios et al., 2018), and RmpC (Walker et al., 2019) for capsule expression, and FimK (Rosen et al., 2008a), MrkH (Wilksch et al., 2011), Fur (Wu et al., 2012), H-NS (Ares et al., 2016), OmpR (Lin et al., 2018), and IscR (Lin et al., 2017) for fimbriae expression. A possible cross-regulation of these processes and the complexity of this fine-tuned regulation may explain the difficulty in recovering the wild-type phenotype in complemented strains. Regarding biofilm, both type 1 and type 3 fimbriae have been related to its formation (Rosen et al., 2008b; Struve et al., 2009; Stahlhut et al., 2012), but the type 3 are considered the predominant fimbriae in biofilm formation *in vitro*, especially on abiotic surfaces such as urinary catheters and medical devices (Struve et al., 2009). In addition to that, a cross-regulation of both type 1 and type 3 fimbriae during the biofilm formation by *K. pneumoniae* has been proposed, so that the lack of type 3 fimbriae is compensated by up-regulation of type 1 fimbriae (Schroll et al., 2010). While we cannot ensure that *kpfR* is being expressed stably in the complemented strain and, therefore, we cannot exclude the effects of this unbalanced expression, we can infer that the unexpected biofilm formation by the complemented strain may be due either to the type 3 fimbriae exerting a prominent role on this strain or that compensation of fimbriae expression may be occurring. Additionally, some authors also reported partial recovery of biofilm formation by *K. pneumoniae* complemented strains, especially in mutant strains for transcriptional regulators of type 1 fimbriae, such as FimK (Rosen et al., 2008a) or type 3 fimbriae, such as IscR (Lin et al., 2017) and MrkH (Wilksch et al., 2011). These reports illustrate how difficult complementation of the biofilm formation

by mutant strains can be, especially when involving mutant genes that encode transcription factors.

Although the hyperfimbriated phenotype yielded advantageous in *in vitro* coculture assays—as revealed by the greater efficiency of the mutant strain in adhering, invading, and replicating within eukaryotic host cells—the increased expression of fimbrial structures proved to be detrimental during *in vivo* colonization in the murine model of urinary tract infection. In fact, our *in vivo* experiments showed that the *kpfR* mutant strain was unable to form intracellular bacterial communities (IBCs), exhibited lower titers in the bladder, and was quickly eliminated by the host. Therefore, by negatively controlling the expression of fimbriae and preventing the bacteria from having a hyperfimbriated phenotype, the KpfR regulator protects *K. pneumoniae* from being recognized and eliminated by the host immune system.

The loss of resistance and ability of the *kpfR* mutant strain to survive in the host can be attributed to its hyperfimbriated phenotype, which has made it to be readily recognized by the host defenses and becomes more efficiently and rapidly eliminated *in vivo*. In fact, while adherence is a crucial step for the pathogen to establish contact with the mucosa and invade and colonize the host tissue, pathogen-host interaction activates host antimicrobial defense, and a key event in this process is the bladder epithelium stimulation following bacterial adherence, which results in cytokines production by the epithelial cells through activation of host Toll-like receptor 4 (Ferraz et al., 2011). As stated by Song and Abraham (2008), several Toll-like receptors have been identified on epithelial cells of the bladder which mediate a powerful immune response. Studies have shown that *K. pneumoniae* type 3 fimbriae can stimulate an oxidative response in neutrophils (Przondo-Mordarska et al., 1991) and that the expression of fimbriae by *K. pneumoniae* increases their binding to phagocytes and triggers the phagocytosis, thus leading to bacterial killing (Athamna et al., 1991).

Our results with KpfR repressor bear similarities, but also striking differences, with FimK, another transcriptional regulator of fimbriae well characterized in *K. pneumoniae* (Rosen et al., 2008a, 2015; Wang et al., 2013). Unlike FimK, which has an EAL domain in its carboxy-terminal region (Rosen et al., 2008a; Wang et al., 2013), KpfR does not have such a domain and, therefore, its mechanism of action should not involve the second messenger cyclic-di-GMP. The effects of KpfR and FimK on repressing the expression of type 1-like fimbriae emphasize the role of both regulators as inhibitory factors of biofilm formation. Also, both *kpfR* and *fimK* knockouts result in hyperfimbriated mutant bacteria (Rosen et al., 2008a) and reduced capsule production (Rosen et al., 2015). However, contrary to that observed in *fimK* mutant strain, the *kpfR* mutant strain did not have its virulence increased in murine UTI model. FimK attenuates virulence on the urinary tract since lack of *fimK* resulted in higher bacterial titers, increased numbers of IBCs, and enhanced virulence in the murine urinary tract (Rosen et al., 2008a). In contrast, KpfR seems to be essential for virulence in the urinary tract, because the loss of an active KpfR resulted in lower bladder titers, inability to form IBCs, and attenuated effect on urinary tract virulence. A probable explanation for this is that in the absence of KpfR

there is an induction of expression of two gene clusters of type 1-like fimbriae (*kpf* cluster and *fim* cluster), whereas FimK regulates only the *fim* gene cluster. Also, the detrimental effect of FimK is restricted to the urinary tract, since in the respiratory tract it has a potentiating effect on virulence (Rosen et al., 2015). Thus, both FimK and KpfR are repressors of fimbriae expression in *Klebsiella pneumoniae*. But, while FimK seems crucial for virulence in the respiratory tract, the KpfR revealed to be critical for UTI.

In summary, pathogens employ survival strategies when in hostile environments, and to accomplish that they control the expression of virulence factors as needed. The expression of fimbriae represents an important factor of bacterial pathogenicity and, in this sense, it is of fundamental importance to understand the mechanisms that regulate the expression of this virulence factor. Herein, we described the role KpfR, a new transcriptional repressor of fimbrial expression hitherto not characterized, in the *K. pneumoniae* pathogenicity. By negatively controlling the expression of fimbriae, KpfR prevents *K. pneumoniae* from having a hyperfimbriated phenotype and from being eliminated in a murine model of urinary tract infection. Further investigations of the *kpfR* mutant strain on a murine model of respiratory tract infection will help to further elucidate whether loss of KpfR has an opposite effect according to distinct host niches.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on the Use of Animals in Research of Universidade São Francisco (CIAEP/CONCEA No. 01.0226.2014, protocol number 004.04.2015).

AUTHOR CONTRIBUTIONS

LFCF conceived the experiments and supervised the execution of the study. AÉIG, TP, CSS, and JAP performed the experiments and data analysis. MD and MLR contributed with data analysis. LFCF, AÉIG, and TP wrote the manuscript. MD and MLR assisted with critical revision of the manuscript and LFCF coordinated its revision. All authors contributed to the manuscript revision and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.601921/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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