ROLE OF *FIMK* IN MEDIATING HOST URINARY BLADDER EPITHELIAL CELL ASSOCIATION OF UROPATHOGENIC *KLEBSIELLA PNEUMONIAE* AND *QUASIPNEUMONIAE*

by

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Dedicated to my family

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by

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I thank the Almighty, Nature and all beings in the universe, on whose process of evolution I stand a chance to evolve and experience life.

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Klebsiella spp. commonly cause both uncomplicated urinary tract infection (UTI) and recurrent UTI (rUTI). *K. quasipneumoniae*, a relatively newly defined species of *Klebsiella,* has been shown to be metabolically distinct from *K. pneumoniae,* but its urovirulence mechanisms have not been defined. Type 1 and type 3 fimbriae, encoded by *fim* and *mrk* operons respectively, mediate attachment of *Klebsiella spp.* to host epithelial cells. *fimK* is a regulatory gene unique to the *Klebsiella fim* operon that encodes an N-terminal DNA binding domain and a C-terminal phosphodiesterase domain that has been hypothesized to cross-regulate type 3 fimbriae via modulation of cellular levels of cyclic di-GMP. Comparative genomic analysis between *K. pneumoniae* and *K. quasipneumoniae* revealed a conserved premature stop codon in *K. quasipneumoniae fimK* that results in loss of the C-terminal phosphodiesterase domain (PDE). We hypothesized that this truncation would ablate cross-regulation of type 3 fimbriae in *K. quasipneumoniae.* Here, we report that *K. quasipneumoniae* KqPF9 bladder epithelial cell association and invasion is dependent on type 3 but not type 1 fimbriae. Further, we

show that basal expression of both type 1 and type 3 fimbrial operons as well as bladder epithelial cell association are higher in KqPF9 than in *K. pneumoniae* TOP52. Interestingly, complementation of KqPF9Δ*fimK* with the TOP52 *fimK* allele markedly reduced type 3 fimbrial expression and bladder epithelial cell attachment, a phenotype that was rescued by mutation of the C-terminal PDE active site. Taken together these data suggest that C-terminal PDE of FimK modulates type 3 fimbrial expression in *K. pneumoniae* and its absence in *K. quasipneumoniae* leads to a loss of type 3 fimbrial cross-regulation.

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ANOVA	Analysis of variance
bp	Base pairs
c-di-GMP	Bis-(3’-5’)-cyclic dimeric guanosine monophosphate (cyclic di-GMP)
DNA	Deoxyribonucleic acid
FRT	Flippase recognition target
GC	Gene cluster
Kp	Klebsiella pneumoniae
Kq	Klebsiella quasipneumoniae
LB	Luria broth
OD	Optical density
PDE	Phosphodiesterase
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
rUTI	Recurrent urinary tract infection
UTR	Untranslated region
WT	Wild-type

CHAPTER 1
INTRODUCTION

1.1 Author contributions

This chapter was written by Sundharamani Venkitapathi (S.V.) and edited by Dr. Nicole J. DeNisco (N.J.D.).

1.2 Urinary tract infection

Urinary tract infection (UTI), an infection in any part of the urinary system including kidneys, ureters, urinary bladder and urethra is one of the most common bacterial infections affecting around 150 million people around the world annually (1, 2). UTI is caused by a diverse range of microbial organisms. Uropathogenic *Escherichia coli* (UPEC) is the most common etiologic agent implicated in UTI (1). UTI predominantly affects women with more than 50% women experiencing the illness at least once in their lifetime (3-5). Adult women are about 30 times more likely than men to develop uncomplicated UTI (2).

Recurrence of UTI including relapses or reinfections post-treatment is a common problem in women. Recurrent UTI (rUTI), defined as at least two episodes of UTI within a period of 6 months or 3 or more episodes of UTI within a period of 12 months, poses a major health issue (6, 7). About 50% of UTIs in postmenopausal women develop into and manifest as rUTI (8-10). The anatomic and functional changes associated with aging play a role in increased incidence of UTI in older women (11). Further, hormonal changes lead to a decrease in estrogen levels thereby contributing towards a decrease in urinary

glycoprotein and glycosaminoglycan secretions and the anti-bacterial activity associated with them (12-15). A decrease in the renal capacity to excrete acid and urea along with incomplete bladder voiding also allows for increased association of invading bacteria with bladder epithelial cells thereby leading to an increase in UTI susceptibility (11, 16). Previous studies have reported that antibiotics are effective against uncomplicated lower UTIs. Considering that uncomplicated UTIs respond better to antibiotic treatment, empirical use of antibiotics is common (17). Extensive use of antibiotics in the treatment of UTIs has led to antibiotic resistance by uropathogens (18, 19). The increase in antimicrobial resistance among uropathogenic bacteria leads to higher rates of chronic and recurrent UTIs, which greatly negatively affects the quality of life of affected women. Further, UTI places a substantial financial burden amounting to more than 6 billion dollars yearly in healthcare cost (20). An estimate of $3.5 billion per year in terms of societal costs associated with health care for UTIs is reported in USA alone (21). Recurrent UTIs also lead to serious consequences including sepsis, pyelonephritis and pre-term birth to go alongside adverse effects due to antibiotic usage and antibiotic resistance over a period of time (1).

1.3 Classification of UTIs

Clinically, UTIs can be classified as uncomplicated UTIs or complicated UTIs (1). UTIs occurring in individuals who are otherwise healthy without any underlying conditions are considered uncomplicated, while those infections in individuals who are immunocompromised, with structural and/or neurologic abnormalities, and presence of foreign bodies like indwelling catheters are categorized as complicated UTIs (22-25).

Catheter-associated UTI (CAUTI) is the most common form of complicated UTI (26). The female to male ratio of uncomplicated UTIs is about 5:1 to 8:1 (27-29). In contrast, the gender inequality in CAUTI is relatively narrow and is reported to be at a female to male ratio of 2:1 (1, 30, 31). Interestingly, men often experienced worse outcomes of CAUTI though it was not directly related to CAUTI (32). Anatomically, UTIs can be classified as upper UTIs corresponding to infections in the kidney and ureter or lower UTIs corresponding to infections in the urinary bladder or urethra (33-35). Based on the site of infection, UTIs can be classified as cystitis when inflammation and infection are observed within the urinary bladder or as pyelonephritis when infection and inflammation are localized to the kidneys (25, 36). Previous studies have shown that the urine of healthy individuals including both males and females is not sterile (37-39). The presence of urinary microbiome has complicated clinical efforts to distinguish between UTI and asymptomatic bacteriuria (ASB), especially in individuals who are unable to effectively communicate their symptoms (40-42). UTIs can also be classified as community acquired UTIs or nosocomial UTIs. Community acquired UTI is defined as an infection of the urinary system that takes place in a community setting or in hospital environment within 48 hours of admission (43). Nosocomial UTI is acquired within hospital settings and is usually defined as those urinary tract infections that occurs after 48 hours of hospital admission and were not present at the time of admission (44, 45).

1.4 Organisms implicated in UTIs

UTIs are caused by both Gram-positive and Gram-negative bacteria along with certain types of fungi (1). While UPEC accounts for 65% - 75% of rUTI's, it is followed in

prevalence by various organisms such as *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis,* group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida spp* (4, 26, 46-48). *E. coli* (80%) and *Staphylococcus saprophyticus* (10% to 15%) have been implicated as the most common causative organisms of uncomplicated community acquired UTIs, while other organisms such as *Klebsiella* and *Enterobacter* were incriminated at much lesser frequencies (22, 49). In complicated UTIs, *E. coli* is the most frequent organism accounting for ≈50% of infections (50). However, in case of complicated UTIs *E. coli* is followed in prevalence by *Enterococcus spp* and *Klebsiella spp* especially in patients with pre-existing conditions like diabetes (1, 51, 52). Further, in the context of rUTI, it has been reported that the genus *Klebsiella* accounts for about 15% - 17% of cases (53). *K. pneumoniae* has also been reported to account for up to 10% of all nosocomial bacterial infections including catheter associated UTIs (54-56). Apart from UTI, *K. pneumoniae* is also implicated in pneumonia, surgical wound infections, endocarditis, septicemia and accounts for about one-third of all Gram-negative infections (57, 58).

1.5 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a facultative anaerobe (59, 60). The members of *Klebsiella* genus are Gram-negative, encapsulated and non-motile (61, 62). The organism *Klebsiella* was first isolated from patients with respiratory tract infection (rhinoscleroma and pneumonia) by Anton von Frisch and Friedlander (63, 64). It was later named after German microbiologist Edwin Klebs (65). The organism resides in the mucosal surfaces of the gut in humans and other animals (55). Apart from being both human and animal

pathogens, *Klebsiella spp* are also widely pervasive and are found across diverse environments including water, soil and plants (55, 66-69). *K. pneumoniae* is gaining attention due to rise in the number of infections and the increasing number of strains that are resistant to antibiotic treatment. Among members of the family *Enterobacteriaceae*, *Klebsiella* genus have been identified as the most frequent members to harbor extended spectrum beta lactamase (ESBLs) conferring them resistance to beta – lactam antibiotics (70). Alongside ESBLs, *K. pneumoniae* strains have also been reported to encode carbapenemases (71), aminoglycoside-modifying enzymes like *aac(6')-Ib* and *aacA43* (53, 72) apart from mutations in *gyrA* and *parC* genes (73), efflux pumps and DNA gyrase protection systems all contributing towards resistance against a wide range of antibiotics (74, 75). *Klebsiella* species are also thought to be pivotal in the spread of antibiotic resistance genes to other Gram-negative bacteria resulting in an increased number of species becoming resistant to available antibiotics (76).

1.6 *Klebsiella* species - taxonomy

The genus *Klebsiella* has been reported to encompass 11 species namely, *K. pneumoniae*, *K. oxytoca*, *K. terrigena*, *K. planticola*, *K. ornithinolytica* (77, 78), *K. variicola* (79), *K. granulomatis* (80), *K. aerogenes* (81), *K. africanensis* (82), *K. grimontii* (83) and *K. quasipneumoniae* (84). Based on the phylogenetic analysis of nucleotide changes in *gyrA*, *parC* genes and until the recent updates on taxonomy of *Klebsiella* species in 2017 by Blin et al, the strains classified under *K. pneumoniae* could further be subdivided into phylogroups KpI, KpII and KpIII. Among the three phylogroups mentioned, it was reported that KpI corresponds to species *K. pneumoniae* while the relatively new species

K. variicola corresponded to the phylogroup KpIII (78, 79, 85-87). KpII was redefined as its own unique species, *K. quasipneumoniae,* and will be discussed further in the following section. Few other relatively newer species namely *K. singaporensis* and *K. michiganensis* were reported over the past two decades (79, 88, 89) and through phylogenetic analysis of the 16s rRNA (*rrs*) gene it was shown that *K. singaporensis* belonged to the *K. pneumoniae* clade while *K. michiganensis* branched close to *K. oxytoca* (84). Blin et al, in 2017 through their report updated the taxa of *Klebsiella* with two novel phylogroups KpV and KpVI based on phylogenetic analysis of concatenated alignment of the core genome involving 2738 orthologous genes of isolates used in the study (90). It was reported that KpVI corresponded to a phylogroup proposed to be named as *K. quasivariicola* (91).

1.7 *K. pneumoniae* phylogroup KpIIA and KpIIB – *K. quasipneumoniae*

The strains in KpII phylogroup are classified into KpIIA and KpIIB (84). To evaluate the strains grouped in KpII phylogroup, Brisse et al performed phylogenetic analysis of the 16s rRNA gene (*rrs*) of the strains in KpII group and compared it to strains in KpI and KpIII phylogroups. It was reported that the *rrs* gene of strains in phylogroups KpIIA and KpIIB differed from *K. pneumoniae* (KpI) and *K. variicola* (KpIII) at 6 and 8 sites, 4 and 6 sites, respectively. Also, phylogenetic analysis of the internal region of the *rpoB* gene encoding for the beta subunit of RNA polymerase showed that the KpIIA and KpIIB phylogroups were sister groups with a similarity of 97.8% between them despite being closely related to *K. pneumoniae* (84, 87).

To further understand the strains in KpII phylogroup, phylogenetic analysis of the internal portions of protein coding genes namely *fusA* (encoding Elongation factor G), *gapA* (encoding glyceraldehyde 3 phosphate dehydrogenase), *gyrA* (encoding DNA gyrase subunit A), *leuS* (encoding tRNA ligase) and *rpoB* was reported by Brisse et al, 2014 and a clear demarcation of the 4 phylogroups namely KpI, KpIIA, KpIIB and KpIII was described (84). Further, upon evaluation of the genome wide divergence of these groups it was reported that the average nucleotide identities (ANIs) between the strains in KpIIA and KpIIB group was 96.8% indicating that they belonged to the same species (92, 93). However, the ANIs in comparison with strains in KpI and KpIII groups were less than 94% indicating that strains in KpII group represented a novel species (84, 92). Apart from these genetic changes, the distinct metabolic phenotype of the strains in KpII group in using certain carbon sources like 3-phenylpropionate and tricarballylic acid further helped differentiate strains in KpII from *K. pneumoniae* and *K. variicola*. Considering the formation of two separate and distinct clusters based on phylogenetic analysis of 5 protein coding genes along with less than 94% ANIs upon comparison with KpI and KpIII groups, in addition to the metabolic differences, allowed for a separation of the strains in phylogroup KpII into a new species distinct from *K. pneumoniae* and *K. variicola*. This new species of strains in KpII group were named as *K. quasipneumoniae* in 2014 (84). There was greater than 96% ANIs between strains in KpIIA and KpIIB. Also, phylogenetic analysis of 5 protein coding genes showed smaller intergroup distance among strains in KpIIA and KpIIB in comparison to larger intergroup distance between strains in KpIIA/B with KpI and KpIII. These features demonstrate the relatedness between the two sister

groups KpIIA and KpIIB allowing them to be categorized as subspecies of *K. quasipneumoniae* with names *K. quasipneumoniae* subspecies *quasipneumoniae* (KpIIA) and *K. quasipneumoniae* subspecies *similipneumoniae* (KpIIB) (84). Because *K. quasipneumoniae* has just been recently established as a distinct *Klebsiella* species, there has been little work exploring phenotypic differences between *K. pneumoniae* and *K. quasipneumoniae,* especially in the context of UTIs.

1.8 Virulence factors of *Klebsiella*

Klebsiella spp. possess a range of virulence factors like capsular polysaccharides (94, 95), lipopolysaccharides (95, 96), siderophores that enable them to sequester iron from the host (97-100) and fimbriae (55, 101, 102). Fimbriae are filamentous projections located on bacterial cell surfaces. They are critical appendages that confer bacteria the ability to adhere to tissue surfaces in the host thereby facilitating initiation and progression of infection (101). Apart from attachment to host cells, fimbriae, also termed pili, enable biofilm formation, transfer of DNA and certain types of motilities. Depending on the type of pathway involved in secretion, these appendages can be divided into five types in Gram-negative bacteria, namely the chaperone–usher pili (CU pili), curli, type IV pili, the type III secretion needle and type IV secretion pili (103). Members of the *Enterobacteriaceae* family express different kinds of fimbriae and several types of fimbriae have been reported among *Klebsiella spp*. While some such as type 1 fimbriae are found in all members of *Enterobacteriaceae* (104), type 3 fimbriae, which are found in majority of *Klebsiella spp.*, are not as common among *Enterobacteriaceae* (105).

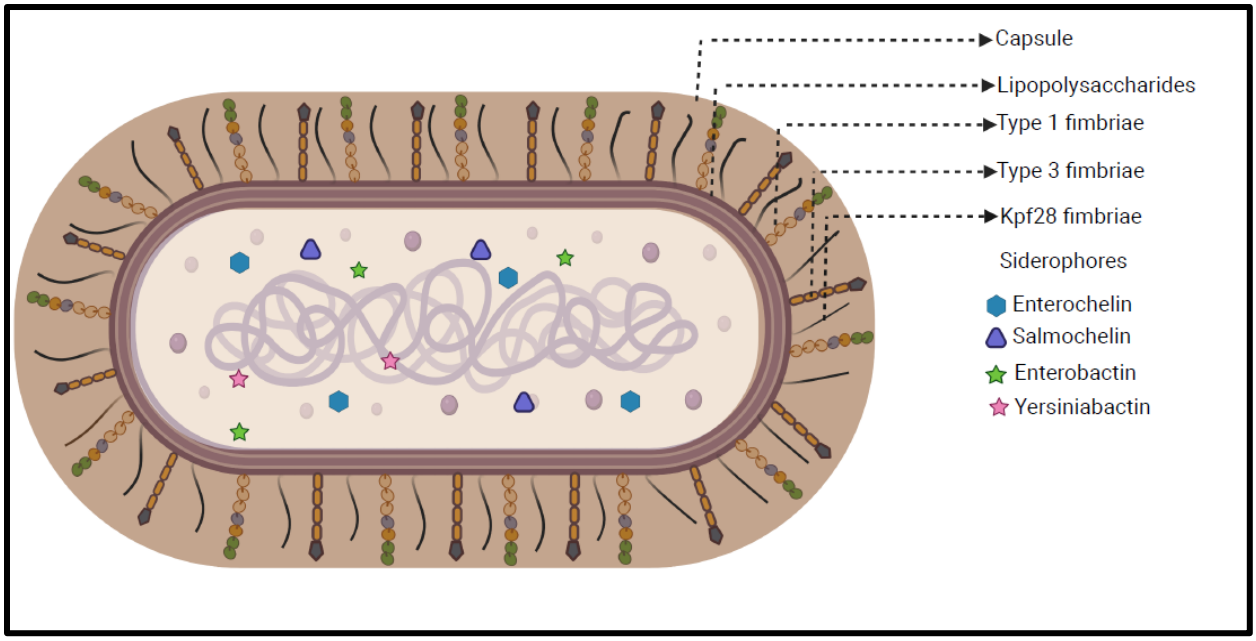

Figure 1.1. Virulence factors of *K. pneumoniae*. Capsule, lipopolysaccharides, fimbriae and siderophores are among a few well studied virulence factors of *K. pneumoniae*. Bacterial capsule is a polysaccharide matrix composed of repeating units of sugar and uronic acid subunits enveloping the bacteria. About 78 different capsular serotypes (K1-K78) of *Klebsiella* have been reported and among them K1 and K2 are reported to be more virulent (55, 58, 106, 107). Lipopolysaccharides (LPS) also called as endotoxin is a component of the outer cell membrane of Gram-negative bacteria. They are constituted by an 'O' antigen side chain, core oligosaccharide and lipid A molecule. Though 9 different 'O' antigens have been reported, O1 is the most common antigen (96, 108-110). They enable resistance to host complement mediated opsonization of bacteria (111). Fimbriae are organelles for bacterial attachment to host respiratory, urothelial, endothelial and intestinal cells. *K. pneumoniae* expresses type 1, type 3 and KPF28 fimbriae (102, 112-114). Siderophores are iron scavenging proteins that enable acquisition of iron from the host environment or from the host iron chelators. Four siderophores namely enterobactin, yersiniabactin, salmochelin and aerobactin with differing affinities to iron have been reported in *K. pneumoniae* (115-117). This figure was generated using biorender.

The type 1 and type 3 fimbriae of *Klebsiella spp.* are secreted through the chaperone usher system. Apart from type 1 and type 3 fimbriae, *K. pneumoniae* also expresses KPF28 fimbriae, Kpc fimbriae and an afimbrial adhesin CF29K which have also been reported to play in attachment to host intestinal cells (114, 118, 119).

1.9 Type 1 fimbriae

Almost all the family members of *Enterobacteriaceae* encode for type 1 fimbriae and it serves as an important appendage that enables attachment to host epithelial cells in the buccal mucosa, respiratory and urinary tracts (120-123). Type 1 fimbriae are encoded by *fim* operon. The type 1 fimbriae can range in length up to 1-2 μm and width of 7 nm (113, 124, 125). The type 1 fimbriae operon in *Klebsiella pneumoniae* encodes for *fimB*, *fimE*, *fimA*, *fimI*, *fimC*, *fimD*, *fimF*, *fimG*, *fimH* and *fimK* genes (Fig 1.2A, B) (101, 126-128). Each type 1 fimbria is composed of several structural subunits. The subunit FimA is the major structural subunit and is present in many copies per fimbria (129). The subunits FimF and FimG are minor structural subunits with FimH acting as the adhesin for type 1 fimbriae (127, 130, 131). Generally, one or two copies of FimH subunit is present at the tip of the fimbriae, while the subunits FimF and FimG connect the adhesin to the rest of the fimbria (130-132). The minor subunits FimF and FimG play a role in assembly thereby determining the length of the fimbriae (125). The *fimC* and *fimD* genes of type 1 fimbrial operon encode for the periplasmic chaperone (FimC) and β-barrel forming membrane usher protein (FimD) respectively (126, 133, 134). The *fimI* gene within the *fim* operon is thought to play a role in assembly of type1 fimbriae (135).

Type 1 fimbriae enable adherence of bacteria to mannose containing structures thereby facilitating attachment of bacteria to host epithelial cell surfaces. Type 1 fimbriae bind to mannosylated glycoproteins on the surfaces of epithelial cells due to the presence of mannose binding pocket within FimH, the type 1 fimbrial adhesin (127). Type 1 fimbriae agglutinate yeast and erythrocytes of guinea pig (136). This pattern of agglutination is mannose sensitive for the same reason as mentioned above (127). Type 1 fimbriae through their ability to attach to epithelial cells of the urinary tract and urinary bladder enable an important process for the bacteria towards establishing infection in the host (137, 138). The FimH adhesin is well characterized and is known to bind mannosylated glycoproteins on urothelial cells namely Uroplakin 1a (UP1a) and Integrin α3β1 followed by activation of downstream kinases (Src and FAK) thereby mediating invasion of bacterial cells (139). The assembly of type 1 fimbrial subunits follows a chaperon-usher (CU) secretion pathway (133).

A high degree of structural and genetic resemblance has been reported between the type 1 fimbriae of *E. coli* and *Klebsiella* except for the presence of phosphodiesterase domain containing *fimK* gene that is unique to *Klebsiella* (128, 137). The type 1 fimbriae have also been shown to be involved in the formation of intracellular bacterial communities (IBCs) in the bladder umbrella cells, contributing escape from immune responses thereby allowing re-colonization after antibiotic therapy (140, 141). Once inside the urothelial cells, these bacteria form intracellular communities (IBC's) that undergo different stages of maturation (early, middle and late) before fluxing out.

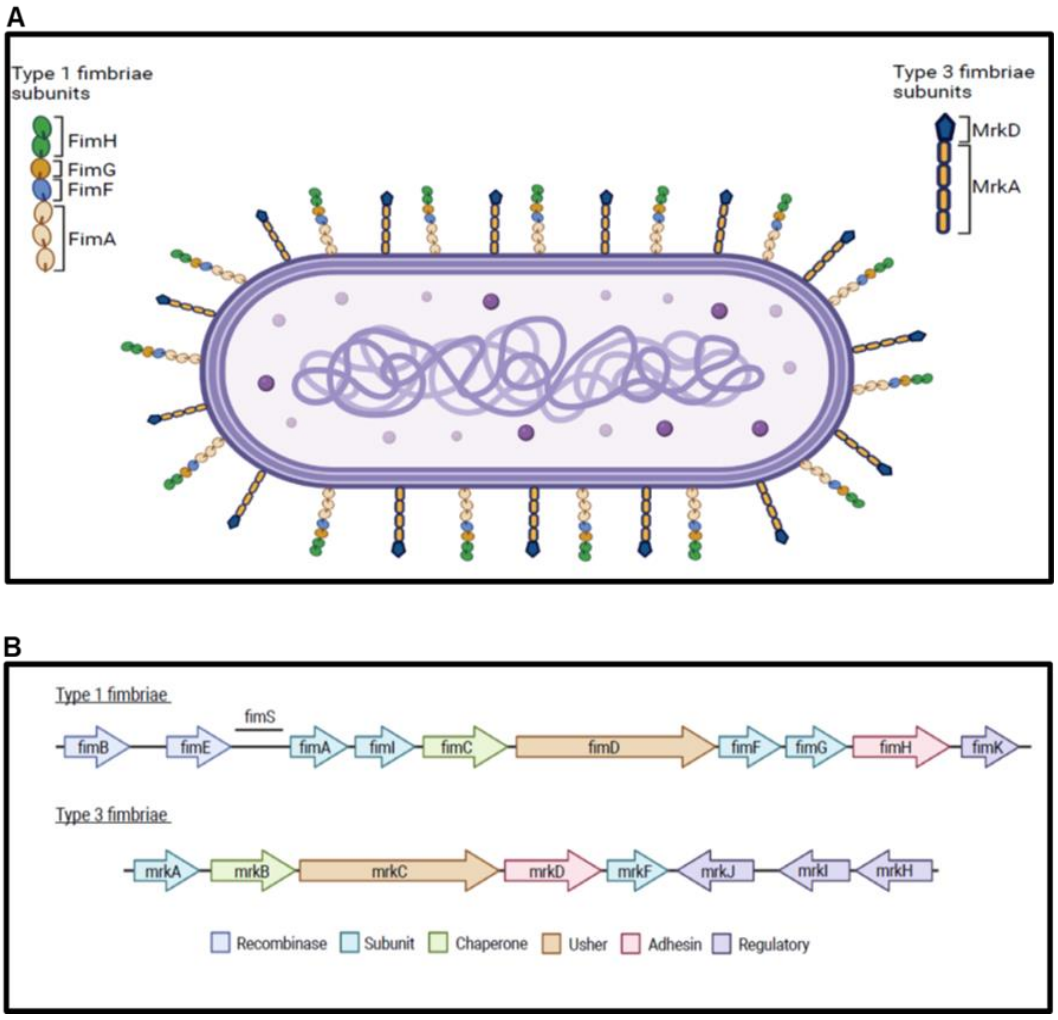

Figure1.2. *Klebsiella pneumoniae* expresses both type 1 and type 3 fimbriae. A. FimA, the major structural subunit; FimF and FimG the minor structural subunits alongside FimH the adhesin for type 1 fimbriae are shown. MrkA, the major structural subunit and MrkD adhesin of type 3 fimbriae are shown. B. The *fim* and *mrk* operons encoding for type 1 and type 3 fimbriae respectively are shown. The individual genetic constituents of the operons are shown in different colors indicating their respective function. This figure was produced using Biorender.

Some of these bacteria have a filamentous nature and are able to escape polymorphonuclear cell engulfment thereby leading to reinfection of other urothelial cells (141, 142). The ability of bacteria to form IBCs differ and it was shown that uropathogenic *K. pneumoniae* isolate TOP52 formed significantly fewer number of IBC's compared to UPEC UTI89 in a murine cystitis model (128).

1.10 Regulation of type 1 fimbriae expression

In *Klebsiella spp* and other members of *Enterobacteriaceae*, expression of genes encoding the type 1 fimbriae subunits is regulated by the promoter "*fimS*". Immediately upstream of *fimA* gene encoding for the major fimbrial subunit of type 1 fimbriae is located the promoter for *fimA*, a 314-bp invertible DNA element called *fimS* with a 9bp inverted repeat (IR) notated as inverted repeat left (IRL) and inverted repeat right (IRR) (133, 137, 143, 144). The promoter *fimS* is regulated by *fimB* and *fimE* recombinases encoded within the type 1 fimbrial operon (Fig 1.4A) (133, 145). The *fimS* region undergoes site-specific recombination, thereby allowing for positioning of the invertible element in either the phase-on (expression of type 1 fimbriae) or phase-off orientation (no expression of type 1 fimbriae). This mechanism enabling switching "on" or "off" of the *fimS* promoter is termed as phase variation. The process of phase switching is reversible. FimB binding to the *fimS* element results in a switch either from phase-on to phase-off or phase-off to phase-on (146-148). On the contrary, FimE typically binds to switch *fimS* from phase-on to phase-off (133). Apart from *fimB* and *fimE* recombinases, some global regulators namely, histone like nucleoid structuring protein (H-NS) alongside integration host factor

(IHF) and leucine responsive protein (LRP) also play a role in regulation of type 1 fimbriae expression (149-153).

1.11 FimK protein of *K. pneumoniae*

The *fimK* gene is transcribed as part of *fim* gene cluster encoding type 1 fimbriae in *K. pneumoniae*. The FimK protein of *Klebsiella* has two domains, a putative helix-turn-helix (HTH) DNA binding domain in the “N” terminus and EAL domain in the “C” terminus (154). The presence of “EAL” domain is characteristic of phosphodiesterase function known to hydrolyze cyclic di-GMP (c-di-GMP) (Fig 1.3A) (155, 156). Rosen et al, 2008; working on *K. pneumoniae* TOP52 uropathogenic isolate described that *fimK* isogenic mutants showed an increase in expression of type 1 fimbriae (128). Further, it was demonstrated that isogenic *fimK* mutants of TOP52 strain showed higher IBC formation in a murine cystitis model relative to their wild-type counterparts along with increased expression of type 1 fimbriae suggestive of a role for FimK in regulating type 1 fimbriae expression (128). Wang et al, 2013 working on *K. pneumoniae* liver isolate CG43S3 showed that FimK has a role in positive transcriptional regulation of type 1 fimbriae expression through the binding of HTH DNA binding domain (AA 1-218) to the *fimS* promoter region (154). The C-terminal domain (CTD) of FimK (AA 195-469) of isolate CG43S3 had an “EIL” motif instead of “EAL” motif characteristic of a phosphodiesterase. However, Wang et al demonstrated that the “EIL” motif in the C-terminal domain of FimK did play a role in regulating cyclic di-GMP levels demonstrating the phosphodiesterase activity of CTD of FimK (154). Though the reports suggest a contradicting function for *fimK* in uropathogenic

and liver isolates of *K. pneumoniae*, the results implicate a regulatory role for *fimK* in modulating type 1 fimbriae expression (128, 154).

1.12 Cyclic di-GMP signaling

Cyclic di-GMP is an intracellular second messenger that plays an important role in regulating an increasing number of cellular activities like cell cycle, motility, biofilm formation, differentiation, virulence among others (157-160). Cyclic di-GMP also known as Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) was originally identified in *Gluconacetobacter xylinus* as a component that was essential for activation of the enzyme cellulose synthase (161). Cyclic di-GMP levels in a cell are regulated by two classes of enzymes namely, digunalyate cyclases (DGCs) or cyclic di-GMP synthetases and phosphodiesterases (PDEs) (162, 163). Diguanylate cyclases are enzymes that have a conserved GGDEF motif, and they synthesize cyclic di-GMP from two molecules of GTP (162, 164, 165). On the contrary, enzymes known as phosphodiesterases containing "EAL" or "HD-GYP" domains hydrolyze cyclic di-GMP molecules resulting in two molecules of GMP (166-168). The phosphodiesterases utilize divalent cations like Mg^{2+} or Mn^{2+} to hydrolyze the ester bond of cyclic-di-GMP (156). The glutamate residue of the conserved "EAL" motif plays an important role in binding of divalent cation and hence mutation at this residue inactivates the phosphodiesterase activity (169). Alterations in cyclic-di-GMP levels are communicated to the cells by various effector molecules which then serves as an input to regulate the network of diguanylate cyclases and phosphodiesterases thereby regulating vital aspects of cellular function (170, 171).

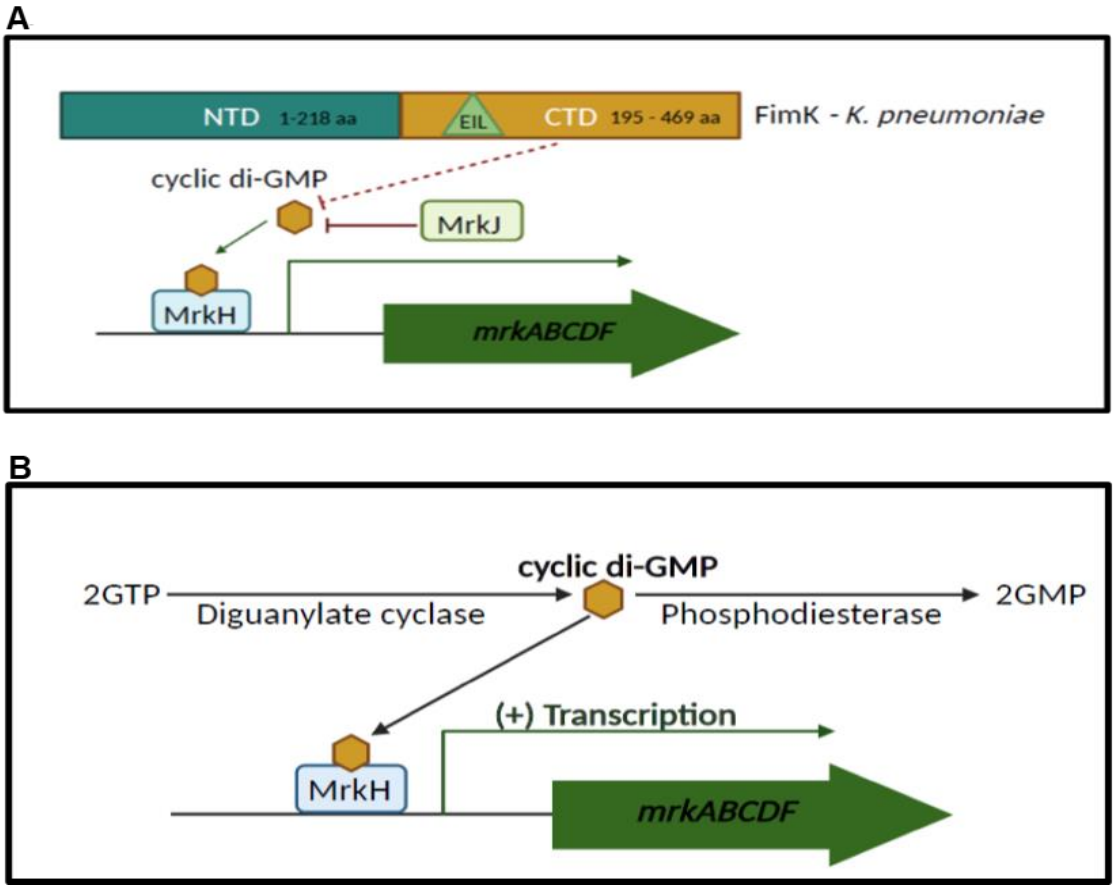

Figure 1.3. FimK protein of *K. pneumoniae* and cyclic di-GMP signaling. A. The FimK protein of *K. pneumoniae* encompasses an N-terminal and a C-terminal domain. The C-terminal domain of FimK has been reported to contain an EIL motif which confers the protein, phosphodiesterase activity, thereby regulating cyclic di-GMP levels (154). In the context of type1 and type 3 fimbriae, MrkJ is another phosphodiesterase that is encoded by *mrk* operon. B. The intracellular second messenger cyclic di-GMP is synthesized from GTP by diguanylate cyclase (DGC) and are hydrolyzed to GMP by phosphodiesterase (PDE) (163, 165). Cyclic di-GMP levels regulate type 3 fimbriae expression by modulating the activity of type 3 fimbriae activator protein MrkH. Phosphodiesterases with a conserved “EAL” or “HD-GYP” domain enable hydrolysis of cyclic di-GMP thereby decreasing the expression of type 3 fimbriae (155, 167, 168). This figure was made using biorender.

In the context of fimbriae, the synthesis of type 3 fimbriae is dependent on *mrk* operon expression regulated by MrkH, MrkI and MrkJ proteins which is influenced by cyclic di-GMP levels (Fig 1.3B) (155).

1.13 Type 3 fimbriae

The type 3 fimbriae, encoded by the *mrk* operon was initially identified in *K. pneumoniae* strains (172). Though first identified in *Klebsiella*, type 3 fimbriae are also present in other members of *Enterobacteriaceae* family including *Serratia spp*., and *Enterobacter spp*. They are also present in a few *E. coli* strains, encoded by a conjugative plasmid pOLA52 (173-175). The genetic constituents of type 3 operon encoding the type 3 fimbriae was first reported in the *K. pneumoniae* isolate IA565 (176). The Mrk proteins are encoded in the chromosome or in a plasmid by a gene cluster comprising the genes *mrkABCDF* which constitute the structural aspect of type 3 fimbriae in *Klebsiella* (Fig 1.2A, B) (101, 176, 177). The type 3 fimbriae can extend into filaments up to 2 µm in length and up to 4 nm in width (178). Type 3 fimbriae serve as organelles for attachment to epithelial cells of respiratory tract and urinary bladder (102, 179). But unlike type 1 fimbriae, the MrkD adhesin of type 3 fimbriae are mannose insensitive and can agglutinate yeast cells, sheep erythrocytes in a mannose insensitive manner (136). Analogous to type 1 and many other kinds of fimbriae, type 3 fimbriae are also secreted by the chaperone-usher pathway of protein translocation (180, 181). The structural components of type 3 fimbriae comprise of MrkA as the major fimbrial subunit, MrkB as the periplasmic chaperone, MrkC the usher translocase, MrkD as the adhesin at the tip of the fimbriae and MrKF acts a minor structural subunit (105, 176, 180, 182-184). Type 3 fimbriae have been demonstrated to

play a crucial role in biofilm formation especially on abiotic surfaces (185, 186). Further, it has been shown that type 3 fimbriae mediated biofilm formation is dependent on MrkA and not MrkD subunit (187). Apart from biofilm formation, type 3 fimbriae have also been reported to play a role in attachment to urinary bladder epithelial and endothelial cells (102).

1.14 Regulation of type 3 fimbriae expression

The expression of type 3 fimbriae is regulated by genes *mrkH*, *mrkI* and *mrkJ*. While MrkH and MrkI regulator proteins transcribed as part of the bicistronic operon *mrkHI* act as the major and minor transcriptional activators of *mrkABCDF* gene cluster respectively, the MrkJ protein transcribed by *mrkJ* is regulated by a distinct promoter and acts as a transcriptional repressor (155). Previous reports also indicate that global regulator protein H-NS differentially regulates type 3 fimbriae expression by acting as a repressor of *mrkH* and *mrkJ* while increasing *mrkA* expression (188). Further, MrkH is an autoinducer of itself apart from activating *mrkJ* expression (Fig 1.4B) (189). Also, MrkH is predicted to contain a “C” terminal PilZ domain (190, 191). The PilZ domain has been reported to be conserved and it contains amino acids that are essential for binding of cyclic di-GMP (192). An increase in DNA binding of MrkH has been reported in the presence of cyclic di-GMP suggestive of a role for cyclic di-GMP in regulating type 3 fimbriae expression through activation of MrkH (155, 191). The MrkI protein, a minor activator of type 3 fimbriae, contains a ‘C’ terminal helix turn helix (HTH) DNA binding domain and a receiver domain at the ‘N’ terminus while missing the cyclic di-GMP binding “PilZ” domain present

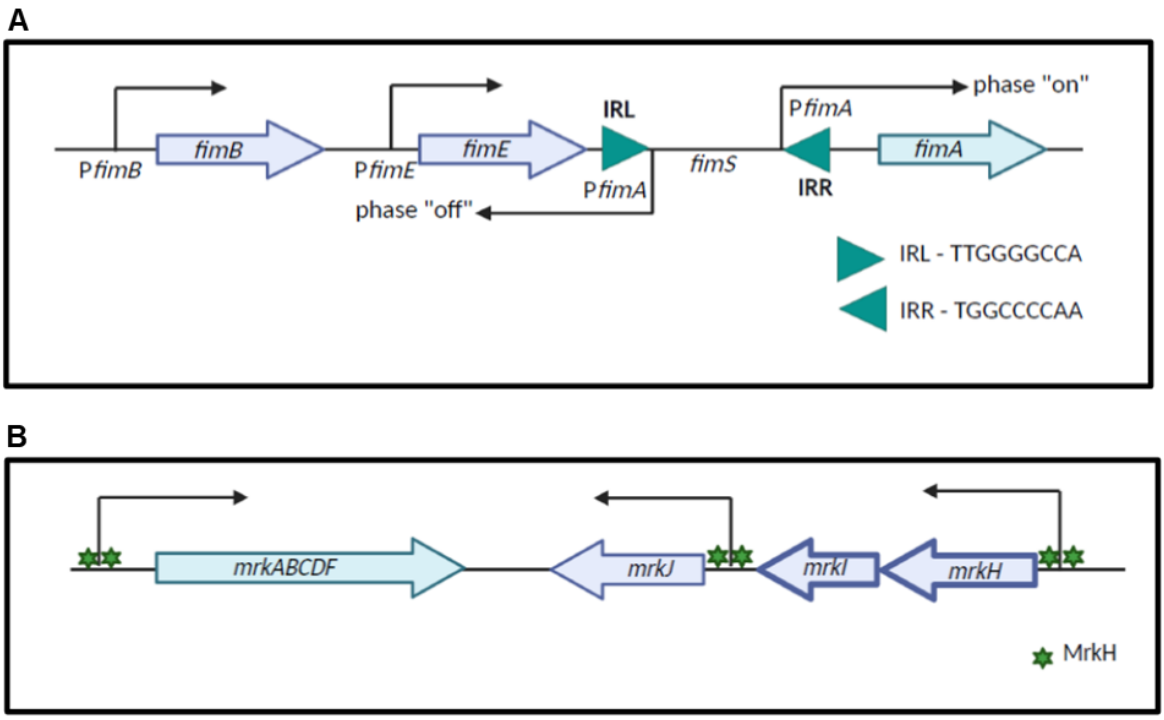

Figure 1.4. Regulation of *fim* and *mrk* operons encoding for type 1 and type 3 fimbriae respectively. A. *fimB* and *fimE* are site specific recombinases that enable phase transition of the *fimS* switch. The *fimS* region represents the 314base pair (bp) invertible element. The notations IRR and IRL represent the direction of the inverted repeat as inverted repeat right and inverted repeat left respectively. The phase “on” notation represents transcription of *fim* gene cluster (*fimAICDFGHK*) as indicated by transcription of *fimA* (145, 193). The phase “off” notation represents transcription in the other direction. B. MrkH protein acts as an autoinducer activating its own transcription (as part of the bicistronic operon *mrkHI*). It also acts as the major activator of type 3 fimbriae by activating transcription of *mrk* gene cluster along with serving as the activator of MrkJ, which is a repressor of type 3 fimbriae (155, 189). This figure was generated using biorender.

in MrkH (155, 194). The MrkJ protein has been reported to exhibit sequence homology with phosphodiesterases containing “EAL” domain (195). This protein in *K. pneumoniae* has been shown to contain an “ECL” domain retaining the phosphodiesterase activity (155). The MrkJ protein acts a repressor for *mrk* operon by hydrolysis of cyclic di-GMP thereby limiting its availability for MrkH binding (195). The dependence of type 3 fimbriae expression on the PilZ domain containing cyclic di-GMP binding MrkH transcriptional activator and cyclic di-GMP hydrolyzing PDE domain containing repressor is suggestive of the role of cyclic di-GMP in modulating type 3 fimbriae expression through the Mrk regulator proteins (155).

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CHAPTER 2
MATERIALS AND METHODS

2.1 Author contributions

This chapter was written by Sundharamani Venkitapathi (S.V.) and edited by Dr. Nicole J. DeNisco (N.J.D.).

2.2 Bacterial strains and culture conditions

Bacterial strains and plasmids used in the study are listed in Table 2.1. PF9, a *K. quasipneumoniae* strain isolated from the urine of a postmenopausal woman with rUTI (1), TOP52, a *K. pneumoniae* strain isolated from the urine of a woman with acute cystitis (2) and *UTI89* an *UPEC* cystitis isolate were used in the study (3). Bacteria were grown in Luria Bertani (LB) media overnight at 37°C in static conditions. For ribonucleic acid (RNA) extractions in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays, overnight cultures were sub-cultured at a starting density of OD_{600} = 0.01 and then incubated for 6 hours until reaching mid-log phase (OD_{600} = 0.4 – 0.6) induced with 0.2% L-arabinose (*mrkA* genotypes) or 0.0125% L-arabinose (*fimA*, *mrkH*, *mrkJ* genotypes) at 37°C in LB media in an incubator at static conditions. For negative staining electron microscopy experiments, bacteria were grown for 8 hours at 37°C in static conditions with 0.2% L-arabinose for induction of complementation plasmids in respective strains.

2.3 Bacterial cell association and gentamicin protection (invasion) assays

Cell association and invasion assays were performed as previously described (4). Urinary bladder epithelial (5637) cells (ATCC) were cultivated in Rosewell Park Memorial Institute (RPMI) 1640 medium (Sigma) supplemented with 10% FBS (ThermoFisher), 1% penicillin-streptomycin (Gibco), 1% sodium pyruvate (Gibco) and 1% glutamax (Gibco) overnight at 37°C and 5% CO_2. The next day, cells were washed with phosphate buffered saline (PBS) and incubated in RPMI medium (without antibiotics). Cells were infected at a multiplicity of infection (MOI) of 10. Bacterial contact with host cells was synchronized by centrifugation of plates at 600 g for 5 min. For association assays, two hours post-infection cells were washed in 1X PBS, then harvested and lysed with 0.3% Triton X-100. Cells in the input wells, representing total intra and extracellular bacteria were not washed and directly lysed with Triton X-100. For invasion assays, the medium was replaced with medium containing 100 µg/mL gentamicin two hours-post infection and allowed to incubate for an additional two hours before harvesting as described above. Association and invasion frequencies were calculated as a ratio of the number of bacteria recovered from washed lysates to the total number of bacteria present in the input (unwashed) wells. All cell association and invasion assays were performed at least in biological triplicate. For each biological replicate, a minimum of 3 technical replicates were evaluated.

2.4 Generation of *K. quasipneumoniae* and *K. pneumoniae* gene deletion mutants

Type 1 fimbriae knockout, type 3 fimbriae knockout or both type 1 and type 3 fimbriae knockout mutants of *K. quasipneumoniae* were generated by targeted gene deletion of

genes encoding the major fimbrial subunits, *fimA* and *mrkA* respectively (5-7). All gene deletions were performed using Lambda Red Recombinase system as previously described with respective primers as provided in Table 2.2 (8). Plasmid pKD4 served as template to amplify the kanamycin cassette and flanking FRT sites (9) to generate knockout cassettes respective to each gene. The Lambda Red Recombinase system was expressed from *pACBSR*-Hyg which carries *beta*, *gam*, and *exo* under the control of an arabinose inducible promoter. pFLP-Hyg, which expresses the FLP recombinase, was used to excise the kanamycin cassette via the FRT sites. PCR was performed using flanking primers (Table 2.2) to screen candidate colonies using Dreamtaq Master Mix (ThermoFisher). PCR products thus generated were analyzed by agarose gel electrophoresis and Sanger sequencing to confirm deletion of the respective gene.

2.5 Generation of complementation plasmids

For construction of complementation plasmids, genes were amplified from genomic DNA by PCR using Dreamtaq Mastermix (ThermoFisher) or PHUsion polymerase for PCR products >2,000bp (NEB) with respective primers (Table 2.3). For all plasmids NcoI and HindIII restriction sites were used except for plasmids encoding *mrk* and *fim* gene clusters where EcoRI and XhoI sites were used for ligation into pBAD33 (Kan^R) version A (10). For generation of the plasmid encoding the AIL mutant of $fimK_{E245A}$, site-directed mutagenesis was performed on $pfimK_{Kp}$ with the mutagenesis primers listed in Table 2.3 followed by digestion using DpnI enzyme. All plasmids were sequence verified by Sanger sequencing before transformation into respective isogenic mutant strains.

2.6 Bacterial transformation

Transformation of *Klebsiella* strains KqPF9 and TOP52 was performed by electroporation of respective electrocompetent cells in 0.1 cm gap-width cuvette, 2.5 kV using a micropulser electroporator (Bio-Rad) and selected on LB agar plates with respective antibiotic (kanamycin - 100µg/ml). For subcloning of complementation constructs, plasmids encoding PCR products of the respective coding regions were cloned into arabinose inducible pBAD33 vector - version A (10) followed by chemical transformation into *E. coli* DH5α cells.

2.7 RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from respective statically grown *K*qPF9 and TOP52 at mid-log phase OD_{600} = 0.5-0.6) using the Qiagen RNeasy Plus kit. RNA concentration and quality was analyzed by NanoDrop (Thermo). RNA with A260/280 ratios between 2.0-2.20 was used for cDNA synthesis using the Qscript cDNA kit (Quantabio). Following cDNA synthesis, qPCR was performed using SYBR green (Quantabio, SYBRMIX) and respective primer pairs (Table 2.4). The expression of gene of interest was normalized to the expression of *rho* (11). Relative expression was determined by the $\Delta\Delta$Cq method (12, 13). qRT-PCR was performed in biological and technical triplicates.

2.8 Static biofilm assays

Static biofilm assays were performed as described previously (14). Bacteria were grown overnight at 37°C under static conditions. Bacteria were normalized to an OD_{600} of 0.1 in LB broth and seeded into round bottom 96 well plates (Corning). 0.2% L-arabinose was

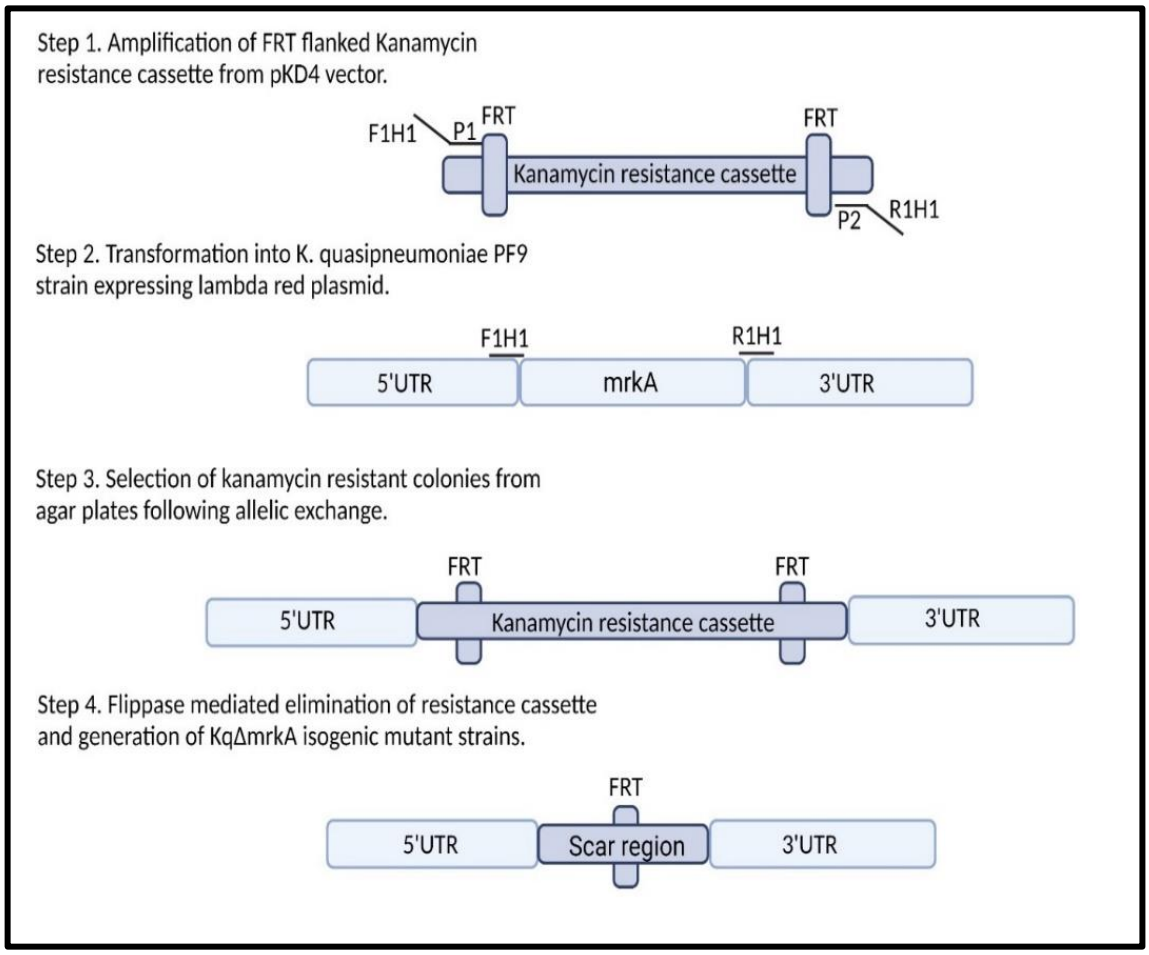

Figure 2.1. Generation of *K. quasipneumoniae* PF9Δ*mrkA* isogenic mutant strains. KqPF9 type 3 fimbriae mutant strains (KqPF9Δ*mrkA*) were generated by lambda red recombinase system. The knockout cassette includes 2 parts. The first part of primer is homologous to the respective 5' untranslated region (UTR) and 3' UTR of *mrkA* – namely F1H1 and R1H1. The second part of the primers represented as P1 and P2 constitute the part of knockout cassette with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (pACBSR) containing KqPF9 electrocompetent cells. The final isogenic mutant of KqPF9Δ*mrkA* had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

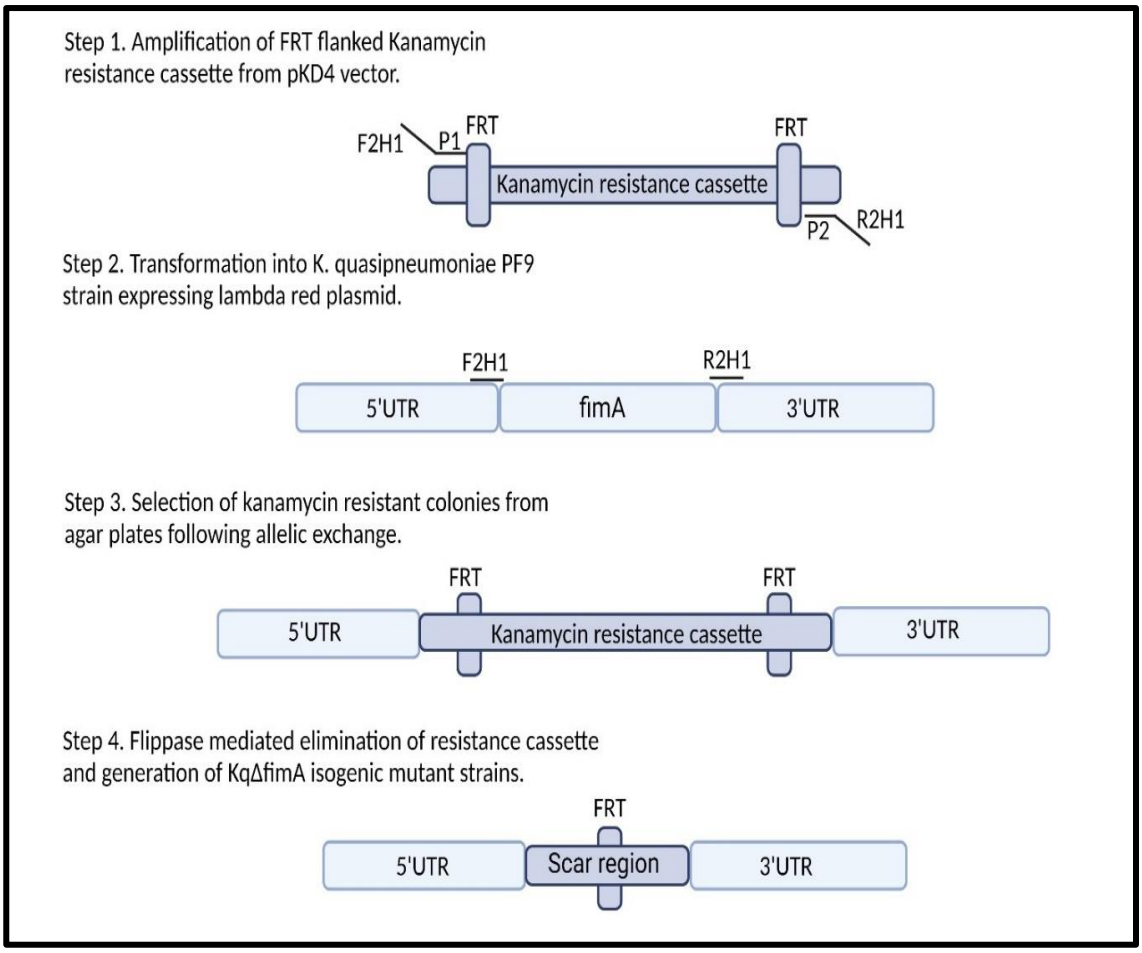

Figure 2.2. Generation of *K. quasipneumoniae* PF9Δ*fimA* isogenic mutant strains. The KqPF9 type 1 fimbriae mutant strains (KqPF9Δ*fimA***)** were generated by lambda red recombinase system. The knockout cassette includes 2 parts. The first part of primer is homologous to the respective 5' untranslated region (UTR) and 3' UTR of *fimA* – namely F2H1 and R2H1. The second part of the primers represented as P1 and P2 constitute the part of knockout cassette with sequence homology to plasmid *pKD4*.The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 electrocompetent cells. The final type 1 fimbriae mutant of KqPF9 had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

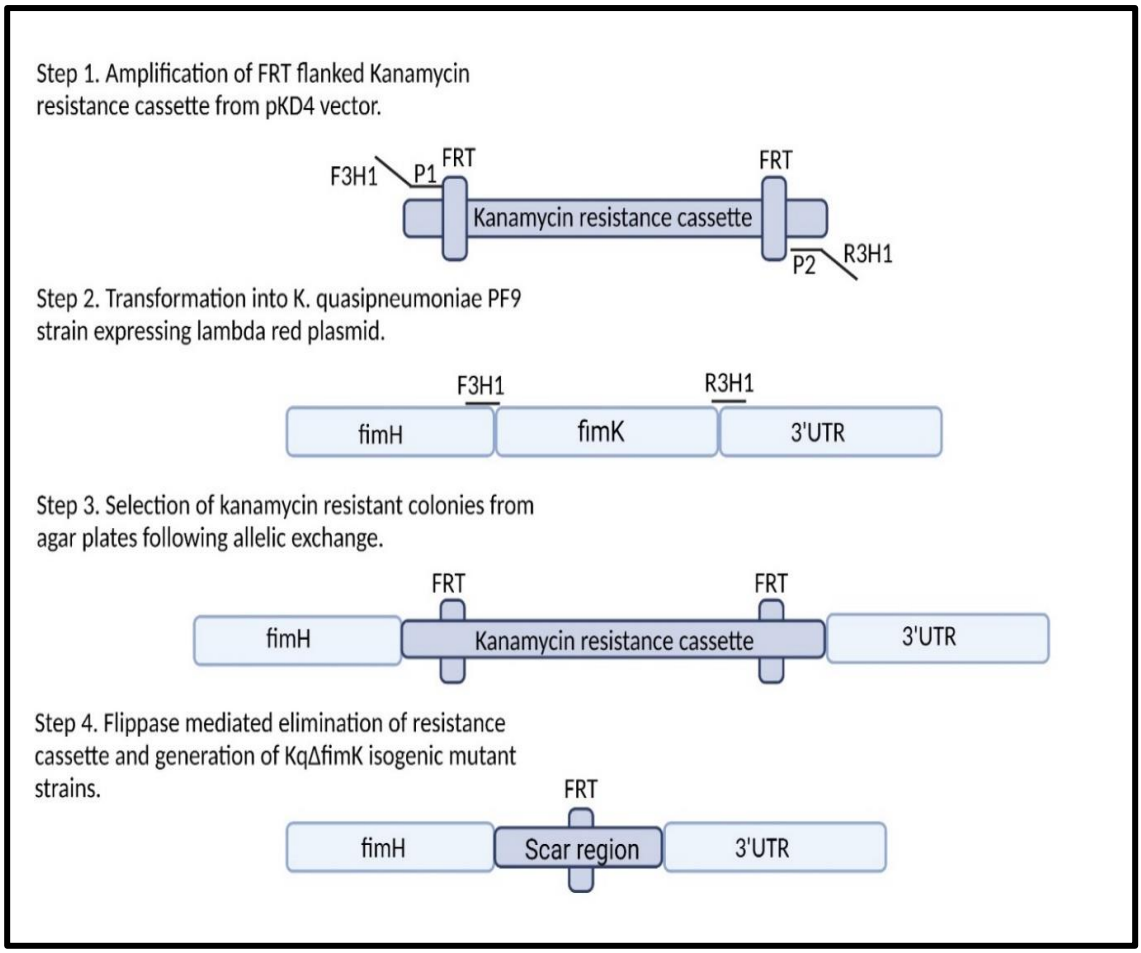

Figure 2.3. Generation of *K. quasipneumoniae* PF9Δ*fimK* isogenic mutant strains. The KqPF9Δ*fimK* strains were generated by lambda red recombinase system. The knockout cassette encompasses primer parts that are homologous to the 5' region of *fimK* encompassing *fimH* of *fim* operon and 3' UTR of *fimK* – named as F3H1 and R3H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 electrocompetent cells. The final isogenic *fimK* mutant of KqPF9 had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was generated using biorender.

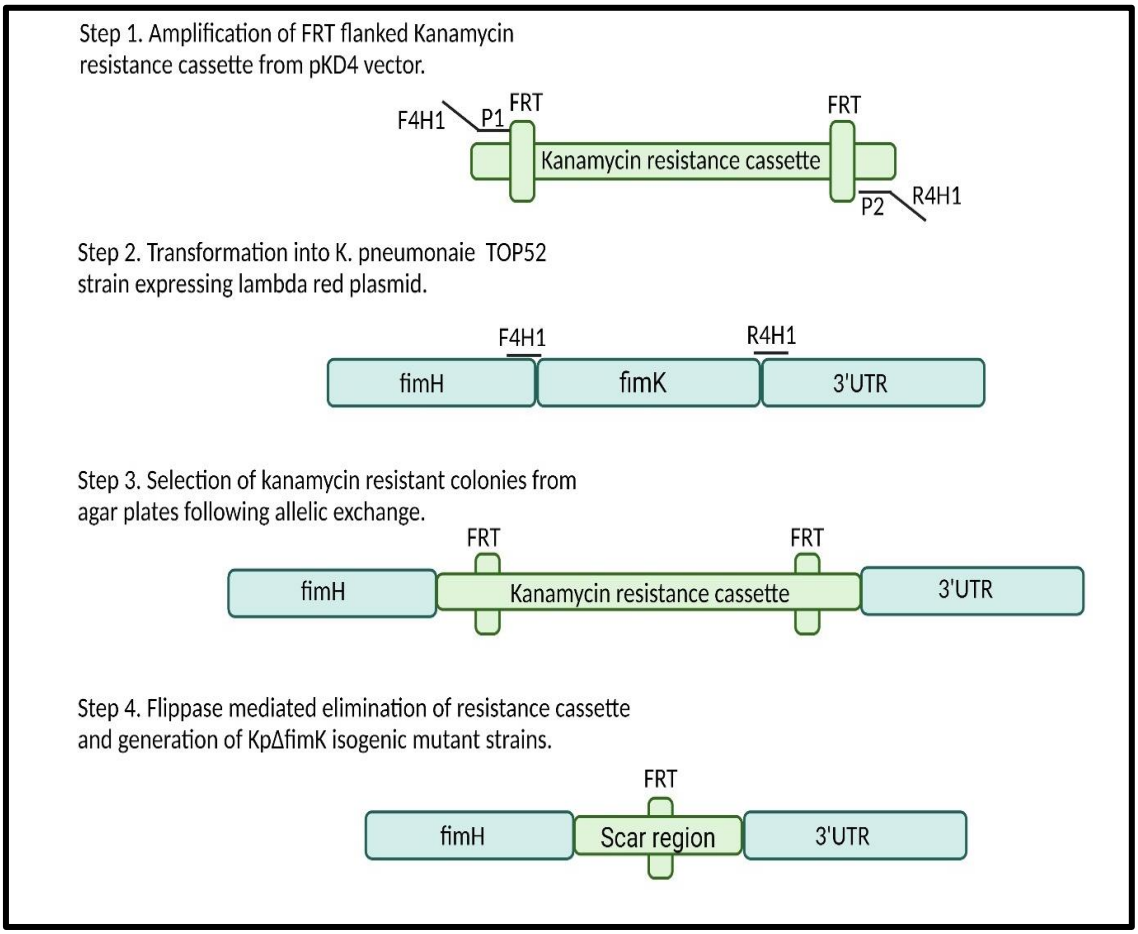

Figure 2.4. Generation of *K. pneumoniae* TOP52Δ*fimK* isogenic mutant strains. The TOP52Δ*fimK* strains of *K. pneumoniae* were generated by lambda red recombinase system. The knockout cassette encompasses primer parts that are homologous to the 5' region of *fimK* encompassing *fimH* of *fim* operon and 3' UTR of *fimK* – named as F4H1 and R4H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing TOP52 electrocompetent cells. The final isogenic *fimK* mutant of TOP52 had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

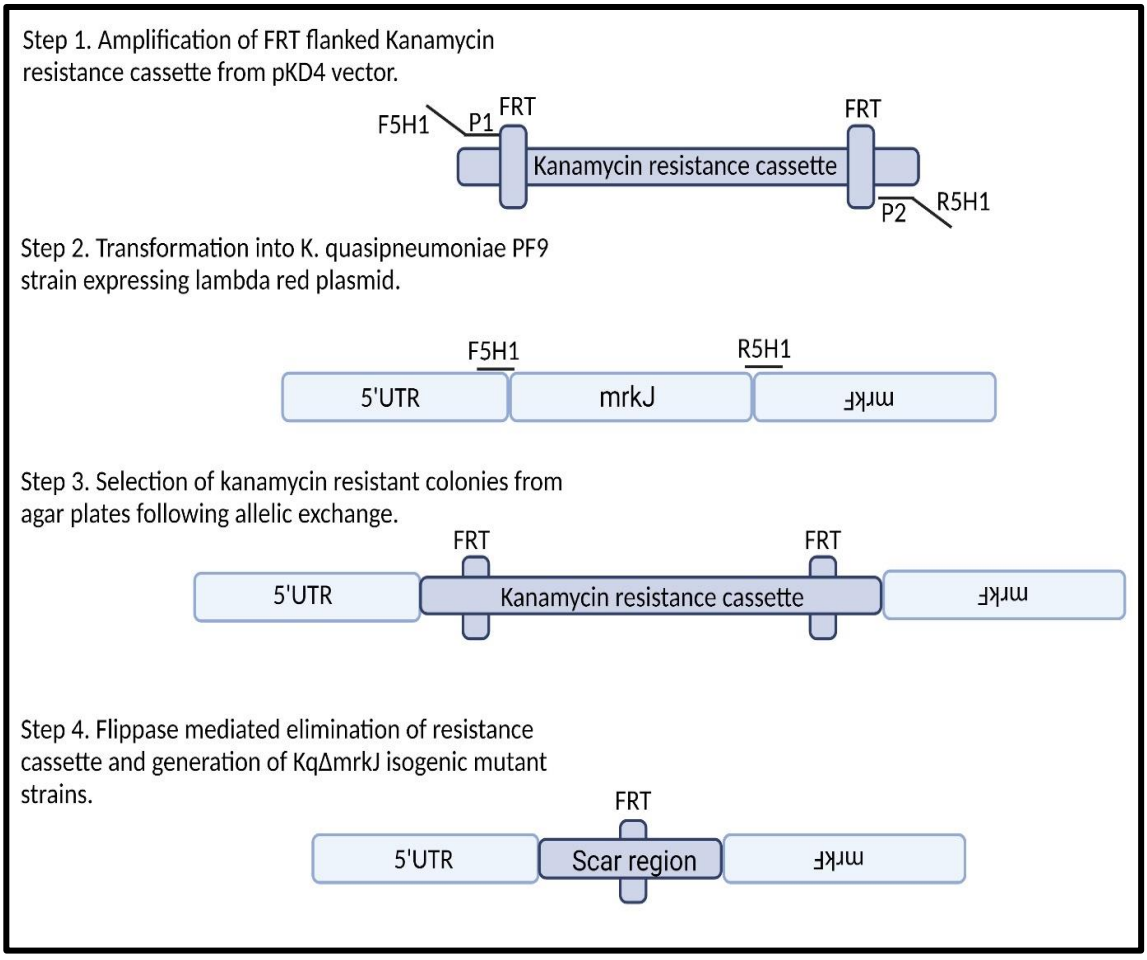

Figure 2.5. Generation of *K. quasipneumoniae* PF9Δ*mrkJ* isogenic mutant strains. The KqPF9Δ*mrkJ* strains were generated by lambda red recombinase system. The knockout cassette encompasses primer parts that are homologous to the 5' UTR of *mrkJ* regulator and 3' region of *mrkF* (*mrkABCDF* and *mrkJ* are transcribed in opposite directions) named as F5H1 and R5H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 electrocompetent cells. The final isogenic *fimK* mutant of KqPF9Δ*mrkJ* had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

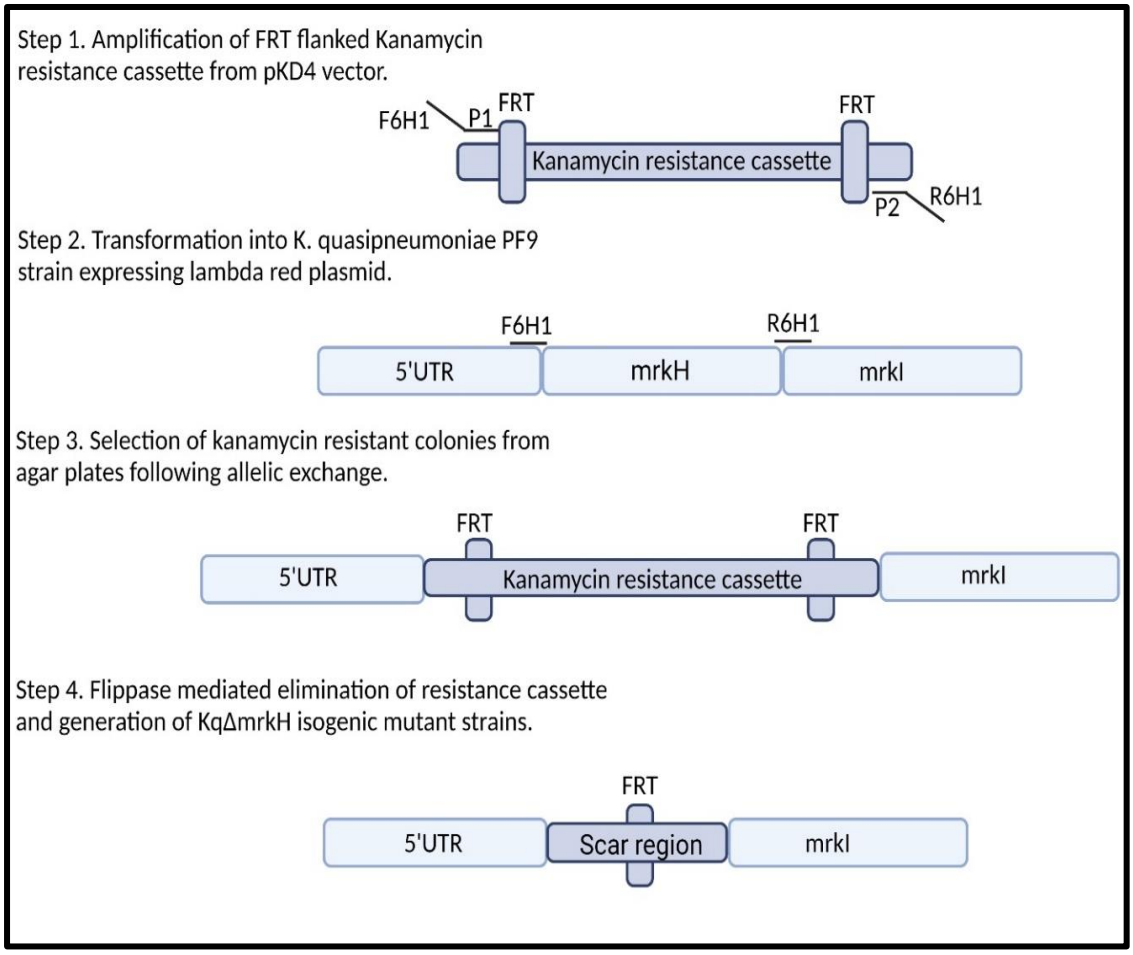

Figure 2.6. Generation of *K. quasipneumoniae* PF9Δ*mrkH* isogenic mutant strains. The KqPF9ΔmrkI strains of *K. quasipneumoniae* were generated by lambda red recombinase system. The knockout cassette encompasses primer parts that are homologous to the 5' UTR of *mrkH* regulator and 3' region of *mrkI* named as F5H1 and R5H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 electrocompetent cells. The final isogenic *fimK* mutant of KqPF9Δ*mrkH* had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was generated using biorender.

added for induction of complementation plasmids. Plates were incubated for 23 hours at 37°C in static conditions and then washed (2X) with sterile water and dried for 25 minutes. Biofilms were fixed with methanol stained with a 0.1% (w/v) crystal violet solution (Sigma) and washed (2X) with sterile water. The bound dye was solubilized with 30% acetic acid and the OD_{585} was measured. Each experiment was performed at least three times in technical triplicate.

2.9 Genome sequencing and assembly

Clean-catch midstream urine sample was obtained from three consenting subjects and plated onto CHROMagar Orientation (BD). Following overnight incubation at 37°C, single colonies were chosen for genus identification by Sanger sequencing of 16S rRNA gene and nucleotide (nr/nt) database query using megablast (BLAST v2.10.0) (15, 16). Species identification was performed by polymerase chain reaction (PCR) using primers specific for *K. quasipneumoniae* species targeting beta lactamase(*bla*) and deoxyribose regulator(*deoR)* genes (17). *K. quasipneumoniae* isolates were grown in Luria broth (LB) overnight at 37°C, and their genomic DNA (gDNA) extracted using gDNA extraction kit (BioBasic). The gDNA obtained was evaluated for 260/280nm absorbance ratio and assessed by agarose gel electrophoresis. The DNA samples were then subjected to sequencing using Illumina and Oxford Nanopore (ONT) technologies. For Illumina sequencing, library preparation was performed using Nextera DNA Flex library prep kit followed by sequencing using NextSeq 500 system to generate paired end reads (2x150bp). ONT libraries were prepared using ligation sequencing kit (SQK-LSK109) and barcode expansion kit 13-24 (EXP-NBD114); followed by sequencing

on MinION instrument using R9 FLO-MIN106 flow cells. Live fast base calling, demultiplexing, and barcode trimming were performed using ONT MinKNOW software. Illumina reads were quality assessed and trimmed using CLC Genomics Workbench v12.0.3 with cutoffs set at minimum Phred score of 20 and read length of 15bp. ONT reads were assessed for quality using NanoStats v1.2.0 (18) and trimmed using NanoFilt v2.6.0 (18) to retain reads with a Phred score >7 and length greater than 200bp. Illumina and ONT reads were used to construct hybrid assemblies of each strain using Unicycler v0.4.8 (SPAdes v3.13.0, Racon v1.4.10, and Pilon v1.23) at default parameters (19-22). Circular genomic sequences were rotated to the starting base of *dnaA* or *repA,* if found. The quality of hybrid assembly generated was evaluated using QUAST v5.0.2 and, completeness of genome was assessed using Bandage v0.8.1(23) and BUSCO v1(24) with bacteria ortholog set on gVolante server v1.2.1 (25). Upon completion of hybrid assembly, annotations of genomes were carried out using NCBI Prokaryotic Genome Annotation Pipeline v4.11. GC content and number of coding sequences were obtained using Geneious Prime v2020.0.5. Finally, sequence type and plasmid replicons of each strain were determined using MLST v2.0 (http://www.genomicepidemiology.org/) with *Klebsiella pneumoniae* configuration (26) and PlasmidFinder v2.1 (27, 28) with settings at 90% identity and 60% minimum coverage cutoffs.

2.10 Agglutination assay

Bacterial strains were grown overnight at 37°C in Luria Bertani (LB) media at static conditions. Cultures were normalized to an OD_{600} of 0.5 and centrifuged at 5,000 rpm for

5 minutes. The bacterial pellets were then resuspended in 1X sterile PBS or PBS + 2.5% D-Mannose solutions. *Saccharomyces cerevisiae* strain L40 was grown overnight at 27°C in Yeast Peptone Dextrose (YPD) media at 225 rpm. Yeast cells were normalized to an OD_{600} of 0.5, pelleted at 5,000 rpm and resuspended in 1X sterile PBS. Bacteria and yeast suspensions were then mixed in a 1:1 ratio on a glass slide and was gently rotated until agglutination was visible. The agglutination clumps were imaged under a light microscope (Zeiss) and images obtained using XCAM1080PHD and SEBA view software.

2.11 Multiple sequence alignment

The *fimK* genes of ten different *K. pneumoniae* and *K. quasipneumoniae* strains that were previously reported in NCBI database were compiled. The *fimK* sequences of three *K. quasipneumoniae* isolates with sequence IDs CP065841.1 (PF9), CP065838.1 (PF26) and CP065846.1 (PF42) whose whole genome sequencing was performed and previously reported were also used for alignment (1). Following collation of the translated *fimK* sequences, their amino acid sequence alignment was performed using Muscle software with default parameters (29, 30). The alignments were then exported to NCBI's multiple sequence aligner for further analysis of their amino acid sequences.

2.12 Phase assay of the *fimS* invertible element of *fim* operon

The *fimS* phase assays were performed as described previously (31). KqPF9 and TOP52 isolates were grown overnight at 37°C in static conditions, harvested by centrifugation and their genomic DNA extracted using the Easy-DNA kit (Invitrogen). PCR primers were designed (Table 2.5) to amplify nucleotide segments 5' and 3' to respective *fimS* regions.

The “on” phase generated a PCR product of size 950bp while the “off” phase generated a product of size 356bp. Based on size of the PCR products as analyzed by agarose gel electrophoresis, the corresponding “on” or “off” phase of the *fimS* switch was determined.

2.13 Negative-stain electron microscopy

Negative staining and electron microscopy were performed as previously described (32). Briefly, a formvar-coated carbon-reinforced copper grid was placed with the film side down, on a droplet of a bacterial suspension for 2 minutes. Filter paper was used to remove excess liquid and the grid was stained for 30 seconds on droplets of 1.25% phosphotungstic acid (pH 6.5). Electron microscopy was performed with a JEOL 1400+ transmission electron microscope using Gatan microscopy suite (GMS) software.

2.14 Statistical analyses

All statistical analysis was performed using GraphPad Prism (9.1.0). For pairwise comparisons, significance was evaluated by Paired two tailed Student’s ‘T’ test. For multiple comparisons, significance was evaluated using one way ANOVA with Dunnett’s multiple comparison test. For all tests, $p \leq 0.05$ was considered significant and represented as * while $p \leq 0.01$ was represented as **, $p \leq 0.001$ was represented as ***, $p \leq 0.0001$ was represented as ****.

Table 2.1. Table shows wild-type and respective mutant strains used as part of the study. It also shows various plasmids that were generated to complement respective mutant strains of KqPF9 and TOP52 isolates.

Strain and plasmids	**Description**	**Source**
PF9	*K. quasipneumoniae* cystitis isolate	This study
TOP52 1721	*K. pneumoniae* cystitis isolate	Rosen et al, 2008 (2)
UTI89	*E. coli* cystitis isolate	Mulvey et al, 2001 (3)
KqPF9Δ*mrkA*	Knockout of *mrkA* in PF9	This study
KqPF9Δ*fimA*	Knockout of *fimA* in PF9	This study
KqPF9Δ*mrkA*Δ*fimA*	Knockout of *mrkA* and *fimA* in PF9	This study
KqPF9Δ*mrkH*	Knockout of *mrkH* in PF9	This study
KqPF9Δ*mrkJ*	Knockout of *mrkJ* in PF9	This study
KqPF9Δ*fimK*	Knockout of *fimK* in PF9	This study
TOP52Δ*fimK*	Knockout of *fimK* in TOP52	This study
pBAD-versionA	Empty vector (EV), arabinose inducible, Kan^R	Guzman et al, 1995 (10)
pmrkgc	Plasmid expressing *mrk* gene cluster	This study
pfimgc	Plasmid expressing *fim* gene cluster	This study
pmrkH	Plasmid expressing *mrkH*	This study
pmrkJ	Plasmid expressing *mrkJ*	This study
$pfimK_{Kp}$	Plasmid expressing WT TOP52*fimK*	This study
$pfimK_{Kq}$	Plasmid expressing WT KqPF9 *fimK*	This study
$pfimK_{E245A}$	Plasmid expressing mutant TOP52 *fimK-AIL*	This study

Table 2.2. Table showing the primer sequences used to generate and verify respective isogenic mutant strains of KqPF9 and TOP52 strains.

Primer name	**Primer use**	**Primer Sequence**
PF9-*mrkA*-ko-f	Knockout cassette for *mrkA* deletion in PF9	TATGCGAATTCACAGTGTGCTCATTGATT CGTAATTCACTCTGACAAGGAAATGGCA ATGTGTGTAGGCTGGAGCTGCTTC
PF9-*mrkA*-ko-r	Knockout cassette for *mrkA* deletion in PF9	CGCTTTATTATTGTTATTAACTGCCCCAT CGCGGGGCAGTTTTATTTTCTGACGGAA TTAGCTGACATGGGAATTAGCCATGG
PF9-*fimA*-ko-f	Knockout cassette for *fimA* deletion in PF9	AGGCACAACGGCTGCCAATCCGGTTCG TTATTTCGACATCGTTCAAAGGAAAACA GTATGTGTGTAGGCTGGAGCTGCTTC
PF9-*fimA*-ko-r	Knockout cassette for *fimA* deletion in PF9	TGCAAAATTAAGCGCGGGCCCTCCTGG CCCGGATGGCTTCCTTGCCTGATGTTTG CCTTAGCTGACATGGGAATTAGCCATGG
PF9-*mrkH*-ko-f	Knockout cassette for *mrkH* deletion in PF9	TGTTGCTATTGCTATAAGAAAAATCAAAC GCCTCACGACAACTATTTACAAGGGATG CACTGTGTAGGCTGGAGCTGCTTC
PF9-*mrkH*-ko-r	Knockout cassette for *mrkH* deletion in PF9	GATAGATTGAGTGACCAATGAGATTATC ATTGGTGTACAGCAATATACTTTCCAAG GGTAGCTGACATGGGAATTAGCCATGG
PF9-*mrkJ*-ko-f	Knockout cassette for *mrkJ* deletion in PF9	GGTAGCCTATAATTAACCTATCCTCTGCT TATTGTCTGACTAACCTCGTGAAGAGGG ATATGTGTAGGCTGGAGCTGCTTC
PF9-*mrkJ*-ko-r	Knockout cassette for *mrkJ* deletion in PF9	GCAACGTGGGAATTAGTTTATAATTAAG CGGGACGAAAAAGCCGGGCAAGCCCGG CTTTTGCTGACATGGGAATTAGCCATGG

Table 2.2, continued

PF9-*fimK*-ko-f	Knockout cassette for *fimK* deletion in PF9	GGCCAGGTCACCGCCGGCAACGTGCAG TCGATCATCGGCATTACCTTTGTCTATCA ATGATGTGTAGGCTGGAGCTGCTTC
PF9-*fimK*-ko-r	Knockout cassette for *fimK* deletion in PF9	CGGCACCGGTGTAAACCGGTGCGCTTT TCTCTCGCCAGCGAATCCACGCCTTTAG TCACTCATCGCTTCCCCGCTGACATGGG AATTAGCCATGG
TOP52-*fimK*-ko-f	Knockout cassette for *fimK* deletion in TOP52	GGCCAGGTTACCGCCGGCAACGTGCAG TCGATCATCGGCATCACCTTTGTCTATC AATGATGTGTAGGCTGGAGCTGCTTC
TOP52-*fimK*-ko-r	Knockout cassette for *fimK* deletion in TOP52	GACGATATTCGCGCATGACGTACCGGC ACCGGTGCTAACCGGTGCGCTTTTCTCG CACCCGCTGACATGGGAATTAGCCATG G
mrkA-v1-f	Verify *mrkA* deletion	GCATTCTTTGACGCCGATAG
mrkA-v1-r	Verify *mrkA* deletion	CCTGGATAAATAAAGCGGGTA
fimA-v1-f	Verify *fimA* deletion	CCACATTAAACAGATTTTAATACCG
fimA-v1-r	Verify *fimA* deletion	CGTCGTGAAATCGGTTACTG
K1-rev	Kanamycin cassette	CAGTCATAGCCGAATAGCCT
K2-for	Kanamycin cassette	GGTGCCCTGAATGAACTGC
Kt-rev	Kanamycin cassette	GGCCACAGTCGATGAATC
mrkH-v1-f	Verify *mrkH* deletion	TCCCTCCTCAATATTTGCCTG

Table 2.2, continued

mrkH-v1-r	Verify *mrkH* deletion	GATTCTGATGGCAGAAATATCCT
mrkJ-v1-f	Verify *mrkJ* deletion	TTGCCGCCCTGCTCGG
mrkJ-v1-r	Verify *mrkJ* deletion	GTTTTTACTGACGGCGGTCG
PF9-*fimK*-v1-f	Verify *fimK* deletion	AACGCGATCTTCACTAAC
PF9-*fimK*-v1-f	Verify *fimK* deletion	TGGTGGAAAAAATGCGCC
TOP52-*fimK*-v1-f	Verify *fimK* deletion	AACAGCACGGTCTCGCT
TOP52-*fimK*-v1-r	Verify *fimK* deletion	GATGGAAATACTGGAAGGG

Table 2.3. Table shows the primer sequences used for generation of respective complements.

Primer name	**Primer use**	**Primer sequence**
mrkABCDF-XhoI-f	Generation of plasmid	GATCCTCGAGCATGAAAAAGGTTCTTCTCTCT GCA
mrkABCDF-EcoRI-r	Generation of plasmid	GATCGAATTCTTAATTATAAACTAATTCCCACG TTGC
fimAICDFGHK-XhoI-f	Generation of plasmid	GATCCTCGAGCATGAAAATCAAAACACTGGCA ATG
fimAICDFGHK-EcoRI-r	Generation of plasmid	GATCGAATTCTCATGCCCGGACAAACGC
PF9-*fimK*-NcoI-f	Generation of plasmid	GATCCCATGGCCGAGTACATCCTTTCT

Table 2.3, continued

PF9-*fimK*-HindIII-f	Generation of plasmid	GATCAAGCTTTCATGCCCGGACAAACGC
TOP52-*fimK*-NcoI-f	Generation of plasmid	GATCCCATG GCCGATTATATCCTCTCGC
TOP52-*fimK*-HindIII-r	Generation of plasmid	GATCAAGCTTTCAACGTTTCGCCGGATCGC
TOP52-*fimK*-AIL-f	Generation of plasmid	TACAGGGGGTGGCGATCCTGATCCG
TOP52-*fimK*-AIL-r	Generation of plasmid	CGGATCAGGATCGCCACCCCCTGTA
mrkH-NcoI-f	Generation of plasmid	GATCCCATGGCAGAGGGAACGATAAAGA
mrkH-HindIII-r	Generation of plasmid	GATCAAGCTTGATTCTCTTTTTTCGCTTGGCTT
mrkJ-NcoI-f	Generation of plasmid	GATCCCATGGACACTAAAATATTCGAAGACAA
mrkJ-HindIII-r	Generation of plasmid	GATCAAGCTTTATGCCAATATCGTCGGCAAC

Table 2.4. Table shows the primer sequences used for qRT-PCR assays of respective genes.

PF9-*mrkA*-f	qRT-PCR assay	GTTACCGATGTATCCTGTAC
PF9-*mrkA*-r	qRT-PCR assay	GGCAGTTAGAGACGTCAA

Table 2.4, continued

PF9-*fimA*-f	qRT-PCR assay	ATGATTGTTGTGTCAGCCCT
PF9-*fimA*-r	qRT-PCR assay	CCCAACTGGACGGTTTGA
PF9-*fimK*-r	qRT-PCR assay	GTGGTGAGCATCCACAG
TOP52-*fimK*-f	qRT-PCR assay	GGTTGAGCCAGCTGATG
TOP52-*fimK*-r	qRT-PCR assay	GTGGTGAGCATCCACAG
PF9-*mrkH*-f	qRT-PCR assay	ATAAAATTCGCTTTCTCCTGCAT
PF9-*mrkH*-r	qRT-PCR assay	TAAACGAAAGCGGGGATCG
TOP52-*mrkH*-f	qRT-PCR assay	ATAAAATTCGCTTTCTCCTGCAT
TOP52-*mrkH*-r	qRT-PCR assay	TAAACGAAAGCGGGGATCG
PF9-*rho*-f	qRT-PCR assay	AACTACGACAAGCCGGAAAA
PF9-*rho*-r	qRT-PCR assay	ACCGTTACCACGCTCCATA
TOP52-*rho*-f	qRT-PCR assay	AACTACGACAAGCCGGAAAA
TOP52-*rho*-r	qRT-PCR assay	ACCGTTACCACGCTCCATA

Table 2.5. Table shows the primer sequences used for determination of phase transition of KqPF9 and TOP52 strains.

PF9/TOP52-fimE-f	fimS orientation	GCAGGCGTATCGTATTATTCG
PF9/TOP52-fimS-f	fimS orientation	TGTTTTGACATATTTTGCAACTCAC
PF9/TOP52-fimA-r	fimS orientation	CGTAGTAACGTGCCTGGAAC

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CHAPTER 3

ROLE OF *FIMK* IN MEDIATING HOST URINARY BLADDER EPITHELIAL CELL ASSOCIATION OF UROPATHOGENIC *KLEBSIELLA PNEUMONIAE* AND *QUASIPNEUMONIAE*

3.1 Author contributions

This chapter was written by Sundharamani Venkitapathi (S.V.) and edited by Dr. Nicole J. DeNisco (N.J.D.).

3.2 Introduction

Klebsiella spp. including *K. pneumoniae, K. variicola, and K. quasipneumoniae* are common causes of both acute and recurrent urinary tract infections (1-5). Recurrent UTI (rUTI), defined as two UTI episodes within six months or three within twelve months, poses a major health issue with ~50% of UTIs in postmenopausal women estimated to develop into rUTI (6, 7). Uropathogenic *E. coli* (UPEC) is the most common organism implicated in rUTI, and is followed in prevalence by the genus *Klebsiella,* which accounts for 15-17% cases (8). *K. pneumoniae* has also been reported to be one of the most common causes of hospital acquired UTIs (9, 10). *K. pneumoniae* and closely related species *K. quasipneumoniae* and *K. variicola* are a growing clinical concern due to the prevalence of multi-drug resistant strains in patients with both community-acquired, and hospital-acquired UTIs (2, 3). Alongside extended spectrum beta-lactamases (ESBLs), *Klebsiella spp.* isolated from UTI patients often encode carbapenemases, aminoglycoside modifying enzymes, and fluoroquinolone resistance genes (11-15).

K. pneumoniae expresses both type 1 and type 3 fimbrial operons (16, 17). These fimbriae have been reported to play a role in attachment and invasion to bladder epithelial cells (18-20) in a mannose sensitive (18) and insensitive fashion (21), respectively. Type 1 fimbriae are encoded by the *fim* operon and are expressed among most *Enterobacteriaceae* family members (22, 23). The *fim* operon of *K. pneumoniae* shares a high degree of similarity with *E. coli* (24) except for the presence of *fimK* at the end of the operon (25). The expression of type 1 fimbriae is regulated by phase transition of the invertible *fimS* regulatory sequence (*fim* switch) (26). The phase variation is mediated by recombinases FimB and FimE that are also encoded as part of the *fim* operon (27). Type 3 fimbriae, though initially identified in *Klebsiella spp.*, have also been reported in other *Enterobacteriaceae* family members including *Serratia spp*., *Enterobacter spp.* and more recently encoded within plasmids in a few *E. coli* isolates (28-30). Type 3 fimbriae are encoded by *mrk* operon with *mrkA* encoding the major structural subunit and *mrkD* encoding the adhesin (31, 32). The expression of type 3 fimbriae is regulated by the *mrkHIJ* gene cluster (33, 34). MrkH is a PilZ domain containing protein that functions as the major activator of *mrkABCDF* transcription upon binding to the second messenger, cyclic di-GMP. MrkI has been reported to contain a LuxR type DNA binding domain and functions as a minor activator of *mrkABCDF* (34). MrkJ acts as a phosphodiesterase hydrolyzing cyclic di-GMP preventing its binding to MrkH and thereby repressing *mrkABCDF* expression (33).

K. quasipneumoniae was previously classified as part of *K. pneumoniae* phylogroups KpIIA and KpIIB, but were relatively recently distinguished from *K.*

pneumoniae as a new species with distinct metabolic phenotypes (35). *K. quasipneumoniae* isolates have been reported to be collected from patients with bloodstream infections without any pre-existing illnesses as well as from the urine of patients with uncomplicated UTI and rUTI (3, 5, 36). However, little is known about how *K. quasipneumoniae* interacts with the bladder environment. In this study, genomic analysis of the complete genomes of uropathogenic *K. quasipneumoniae* strains showed a difference in the structure of type 1 fimbrial regulatory gene *fimK* compared to *K. pneumoniae.* The *K. quasipneumoniae fimK* allele is truncated and lacks the C-terminal phosphodiesterase domain (PDE) conserved among *K. pneumoniae* isolates. This is important because the phosphodiesterase activity of *K. pneumoniae* FimK has been hypothesized to cross-regulate type 3 fimbriae by reducing cyclic-di-GMP levels necessary for MrkH activation (37). This study investigates the contribution of type 1 and type 3 fimbriae to *K. quasipneumoniae* bladder epithelial cell association and the role of *fimK* in regulation of type 3 fimbriae in uropathogenic *K. quasipneumoniae* and *K. pneumoniae*.

3.3 RESULTS

Mannose insensitive attachment and invasion of uropathogenic *Klebsiella quasipneumoniae* to cultured human bladder epithelial cells

Cell association and invasion phenotypes of *Klebsiella quasipneumoniae* have not been previously reported. We therefore first sought to use KqPF9, an uropathogenic *K. quasipneumoniae* strain that we recently isolated from a postmenopausal woman with rUTI to evaluate *K. quasipneumoniae* cell association and invasion. Because our whole genome assembly of KqPF9 revealed that the KqPF9 chromosome encodes both type 1 (*fim*) and type 3 (*mrk*) fimbrial operons, we first sought to determine if KqPF9 bladder epithelial cell association was mannose dependent, which would suggest that it was predominantly mediated by type 1 fimbriae (5). We measured the effect of D-mannose on KqPF9 association with bladder epithelial cell line 5637 (ATCC) via cell association assay using *K. pneumoniae* 78578 (KpMGH78578, ATCC) as a mannose insensitive control and uropathogenic *E. coli* UTI89 as a mannose sensitive control (38). While a significant 75.4% reduction was observed in association of UTI89, which expresses only type 1 fimbriae (39, 40), *K*qPF9 and KpMGH78578, which express both type 1 and type 3 fimbriae, showed no significant decrease in association with 5637 bladder epithelial cells in the presence of D-mannose (Figure 3.1A). Then, using gentamicin protection assays to measure bladder epithelial cell invasion, we observed that D-mannose treatment similarly reduced invasion frequencies of UTI89 (56.3%), while KqPF9 and KpMGH78578 invasion frequencies were not significantly reduced (Figure 3.1B). This pattern of invasion is consistent with that previously reported in uropathogenic *K. pneumoniae* isolate 3091

which expresses both type 1 and type 3 fimbriae (21). These results suggest that like in *K. pneumoniae*, adhesion and invasion of bladder epithelial cells by *K. quasipneumoniae* KqPF9 is mannose-independent and may not rely on type 1 fimbriae. To confirm these results, we also evaluated mannose sensitivity of KqPF9 cell association by yeast agglutination assay (41). Both UTI89 and KqPF9 were able to agglutinate *Saccharomyces cerevisiae* strain L40. However, while treatment with D-mannose abrogated agglutination of yeast by UTI89, we observed no effect on KqPF9-mediated yeast agglutination (Fig 3.7) (41). Together, these results suggest that association and invasion of bladder epithelial cells and yeast agglutination by *K*qPF9 is mannose insensitive and therefore may not rely on type 1 fimbriae.

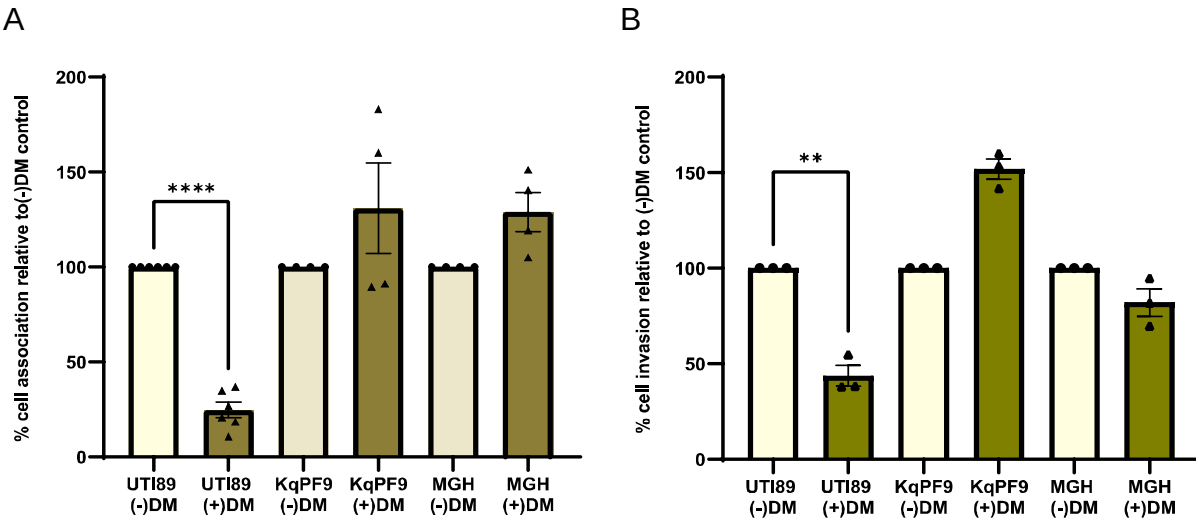


Figure 3.1. *Klebsiella quasipneumoniae* strain PF9 (KqPF9) shows a mannose insensitive pattern of urinary bladder epithelial cell association and invasion similar to that of *K. pneumoniae*. A, B. Cell association and invasion assay demonstrating the mannose insensitive association and invasion of KqPF9 isolate to urinary bladder epithelial cells is shown. UPEC UTI89 was used as negative control and *K. pneumoniae* type strain MGH78578 was used as a positive control. For each strain, the urinary bladder cell association of PBS control was evaluated as 100% and the influence of mannose was determined relative to respective PBS control. The experiments were performed as biological triplicate and at least three technical replicates for each biological sample. The error bars indicate standard error of mean. A two tailed, paired Student's T test was performed to evaluate the significance of the effect of mannose on bladder cell association and invasion. A p- value less than 0.05 was termed significant.

Type 3 but not type 1 fimbriae are required for *K. quasipneumoniae's* attachment to cultured bladder epithelial cells

To evaluate the role of type 1 and type 3 fimbriae of uropathogenic *K. quasipneumoniae* PF9 (KqPF9) in bladder epithelial cell association, knockout strains were generated by deletion of *fimA* and *mrkA,* which encode for the major fimbrial subunits of the type 1 and 3 fimbrial operons, respectively (32, 42). Deletion and complementation of each gene was verified by qRT-PCR (Figure 3.2A, 3.2B). The isogenic type 1 fimbriae mutant KqPF9Δ*fimA* strain showed no significant alteration in bladder epithelial cell association relative to wild-type; however, as has been previously reported in *K. pneumoniae,* complementation by overexpression of type 1 fimbrial gene cluster (*fimAICDFGHK)* significantly increased bladder epithelial cell association (43). A significant 92.4% decrease in cell association was observed in the double fimbriae mutant KqPF9Δ*mrkA*Δ*fimA*, suggesting that type 3 fimbriae may contribute to bladder epithelial cell association *in vitro* (Figure 3.2C). Complementation with type 1 fimbrial gene cluster (*fimAICDFGHK)* was able to rescue the cell association phenotype in KqPF9Δ*mrkA*Δ*fimA* strain, which can be attributed to overexpression of the operon (Figure 3.2A, 3.2C). In contrast to *KqPF9*Δ*fimA*, the type 3 fimbriae mutant *KqPF9*Δ*mrkA* showed a significant 71.6% reduction in bladder epithelial cell association, which was complemented by overexpression of the type 3 fimbrial gene cluster (*mrkABCDF*) (Figure 3.2D). The reduction of cell association in KqPF9Δ*mrkA*Δ*fimA* was also rescued by overexpression of the *mrkABCDF* gene cluster (Fig 3.2D). Taken together, these data suggest that *in vitro*

association of KqPF9 with bladder epithelial cells is dependent on type 3 but not type 1 fimbriae.

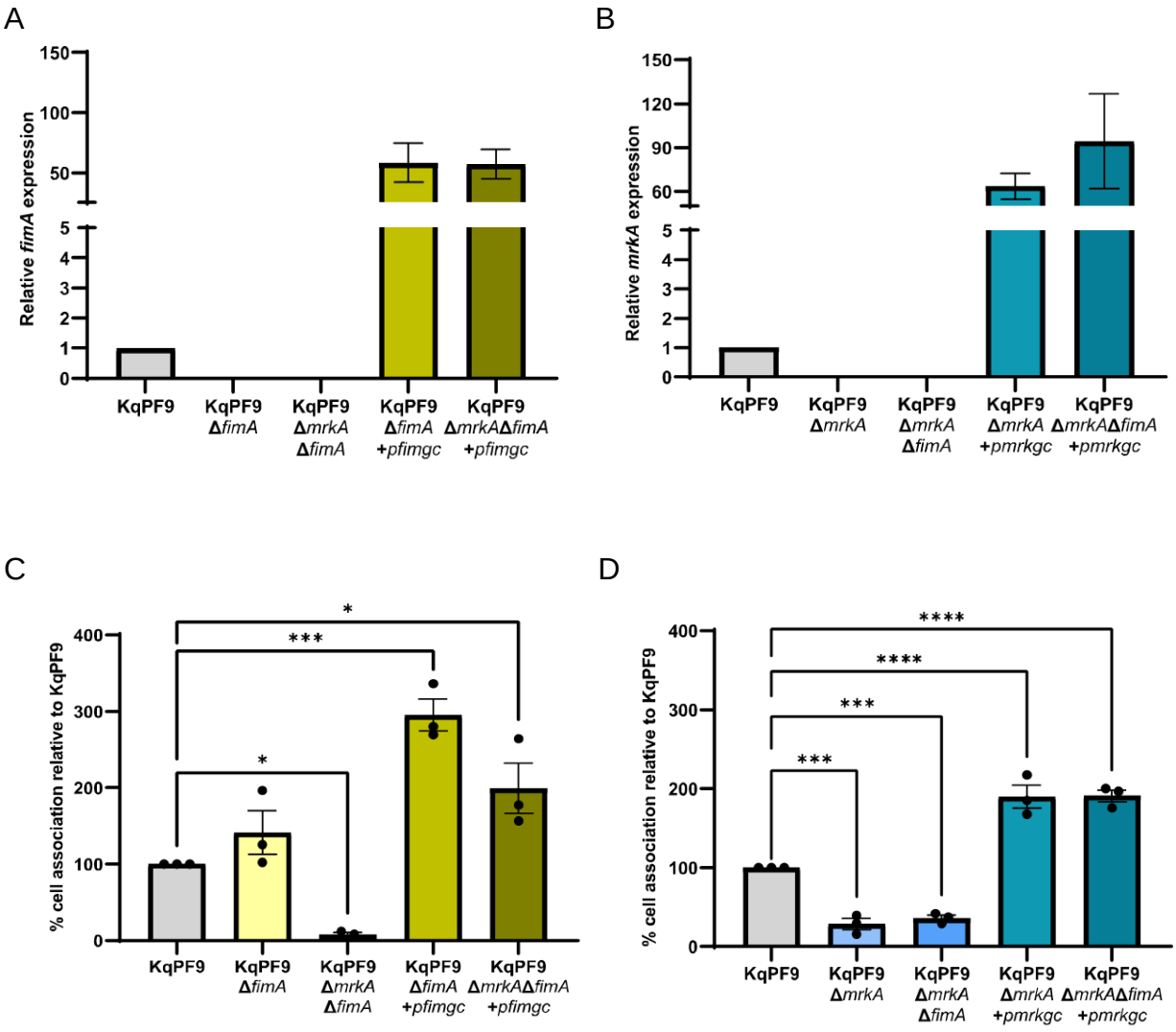


Figure 3.2. *K. quasipneumoniae's* association to cultured urinary bladder epithelial cells is dependent on type 3 fimbriae. A. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of type 1 fimbriae (*fimA*) transcription in KqPF9 wild-type and respective isogenic mutant strains carrying *pBAD* empty vector or *pBAD* vector expressing *fim* gene cluster (*fimAICDFGHK*) is shown. Samples were grown and RNA extraction, qRT-PCRs were performed as mentioned in the methods section. The error bars indicate standard error of mean. B. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of type 3 fimbriae (*mrkA*) transcription in KqPF9 wild-type and respective isogenic mutant strains

carrying *pBAD* empty vector or *pBAD* vector expressing *mrk* gene cluster (*mrkABCDF*) was performed. The expression value of each gene was normalized to expression of *rho* in triplicate and the average value was used to calculate the fold change. C, D. Cell association assay of KqPF9 strain, isogenic type 1 and type 3 fimbriae mutants and mutants expressing respective complementing plasmids was performed in vitro in urinary bladder derived 5637 cell-line. The urinary bladder cell association for KqPF9 strain was evaluated as 100% and the cell association of respective strains were evaluated relative to wild-type KqPF9 strain. The experiments were performed as biological triplicate and at least three technical replicates for each biological sample. The error bars indicate standard error of mean. Significant p-values were evaluated using one way ANOVA with Dunnett's multiple comparisons analysis. A p- value less than 0.05 was termed significant.

Elevated fimbrial expression and bladder epithelial cell association in KqPF9 compared to *K. pneumoniae* TOP52

Because variation in urovirulence phenotypes has been previously reported in *K. variicola,* which has also been recently speciated from *K. pneumoniae*, we next wanted to directly compare fimbrial expression and bladder epithelial cell association between KqPF9 and the well-studied uropathogenic *K. pneumoniae* strain TOP52 (2, 25). Quantitative RT-PCR analysis of *fimA* and *mrkA* expression in statically cultured TOP52 and KqPF9 showed a 1.5 fold higher *fimA* expression and 7.4 fold higher *mrkA* expression in KqPF9 than in TOP52 (Figure 3.3A, 3.3B). We then compared cell association frequencies between the two strains and observed 324.1% higher bladder epithelial association rates with KqPF9 than TOP52 (Figure 3.3C). Because we observed increased expression of the major type 3 fimbrial subunit *mrkA* in KqPF9, we also evaluated biofilm formation in each strain. In accordance with the elevated *mrkA* expression in KqPF9, we also observed a 744% increase in biofilm formation with respect to TOP52 (Figure 3.3D).

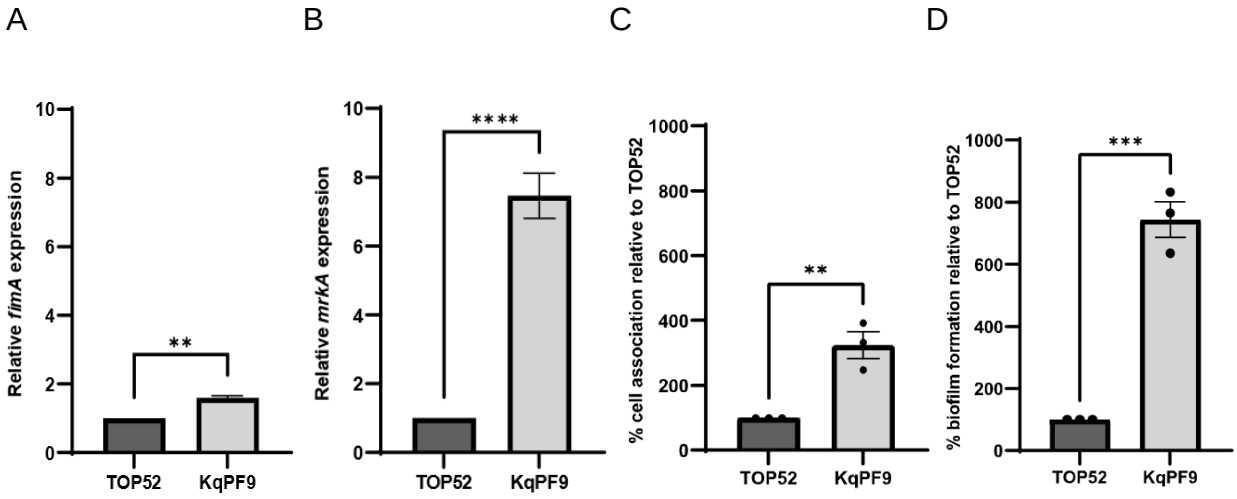
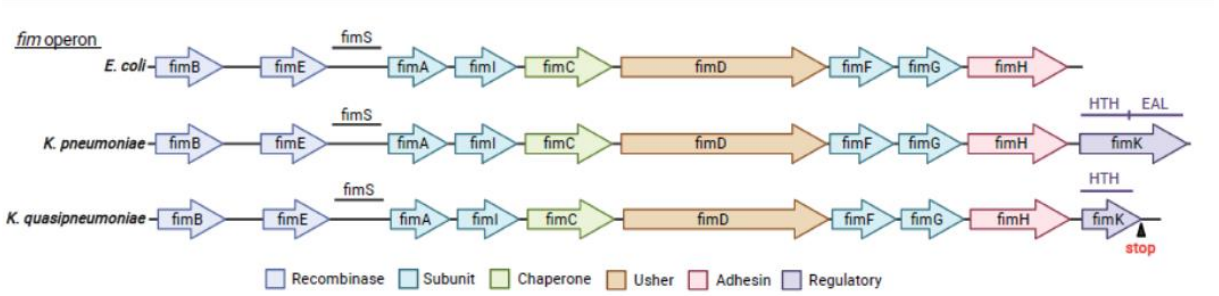


Figure 3.3. KqPF9 isolate shows higher expression of fimbriae and increased colonizing ability relative to TOP52. A, B. qRT-PCR analysis of type 1 fimbriae (*fimA*) and type 3 fimbriae (*mrkA*) transcriptions in TOP52 and KqPF9 isolates carrying *pBAD* empty vector is shown. The expression values of *fimA* and *mrkA* were normalized to *rho* and an average of three values for each biological sample was used to determine the fold change. C. Cell association assay to evaluate the associations of TOP52 and KqPF9 was performed in 5637 cell line. The experiment was performed three times with technical replicates for each biological sample. All error bars indicate standard error of mean. A two tailed, paired Student's T test was performed to evaluate the significance. D. Static biofilm assay evaluating the biofilm formation of strains TOP52 and KqPF9 was performed. The biofilm formation on strain TOP52 was evaluated as 100% and the biofilm formation of KqPF9 strain was determined relative to TOP52. The experiment was performed at least three different times with four technical replicates for each biological sample. All error bars indicate standard error of mean. A two tailed, paired Student's T test was performed to evaluate the significance. A p-value less than 0.05 was considered significant.

The type 1 fimbrial regulatory gene *fimK* is truncated in *K. quasipneumoniae*

We next used previously generated whole genome sequences of TOP52 and KqPF9 to identify genotypic differences explaining the increased expression of *mrkA* and rates of bladder epithelial cell association observed in KqPF9. While we observed no differences in *mrk* operon structure between the two *Klebsiella spp.,* we found that the type 1 fimbrial regulatory gene, *fimK* was truncated in KqPF9 (Figure 3.4A). Indeed, multiple sequence alignment of the FimK amino acid sequences extracted from 10 *K. pneumoniae* and *K. quasipneumoniae* genomes deposited in the NCBI database using MUSCLE software revealed that the premature stop codon observed in KqPF9 was conserved among *K. quasipneumoniae* isolates and resulted in a 218 aa protein as compared to the 470 aa protein observed in all *K. pneumoniae* isolates (Figure 3.4B, 3.4C) (44, 45). The *fimK* gene of *K. pneumoniae* encodes an N-terminal helix-turn-helix (HTH) DNA binding domain and a C-terminal phosphodiesterase domain (37). The C-terminal domain was shown to negatively regulate the expression of type 1 fimbriae possibly due to modulation of cyclic di-GMP levels by its phosphodiesterase activity (25, 37). Because cyclic di-GMP regulates the expression of type 3 fimbriae through MrkH (34), it has been hypothesized that the FimK phosphodiesterase domain may also negatively regulate type 3 fimbrial expression. We therefore hypothesized that the relative increase in *mrkA* expression and bladder epithelial cell association observed in KqPF9 may be due absence of the FimK C-terminal phosphodiesterase domain, which functions to negatively regulate type 3 fimbriae expression in *K. pneumoniae.*

A



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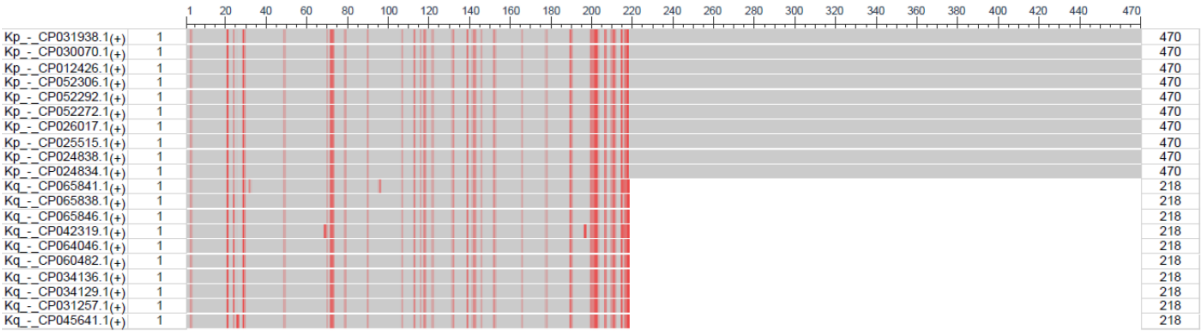

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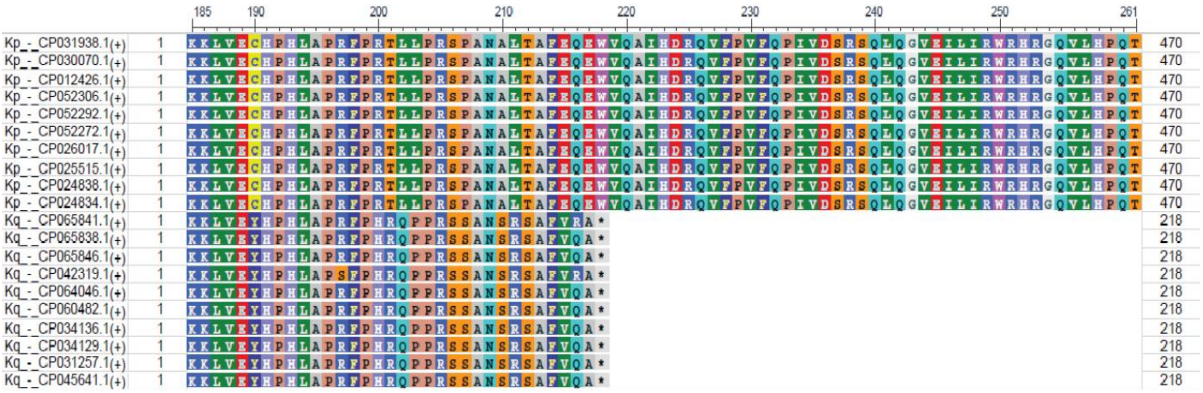

Figure 3.4. *Fim* operon of members of *Enterobacteriaceae*. A. A schematic representation of the *fim* operon encoding type 1 fimbriae among members of *Enterobacteriaceae*, *E. coli*, *K. pneumoniae* and *K. quasipneumoniae* are shown. The overall genetic composition

of the type 1 fimbriae is similar between *E. coli* and *Klebsiella* except for the presence of *fimK* in *Klebsiella spp.* Comparison of the *fimK* sequences between *K. pneumoniae* and *K. quasipneumoniae* shows that *K. quasipneumoniae* in contrast to *K. pneumoniae* encodes a truncated *fimK* encompassing only the putative helix-turn-helix (HTH) domain while missing the part encoding the “EAL” phosphodiesterase domain. B. An amino acid alignment of *fimK* sequences of various *K. pneumoniae* and *K. quasipneumoniae* strains deposited in NCBI database is shown with respective sequence IDs. The start and end positions indicate the protein lengths of FimK of respective strain. The coloring pattern shows amino acids that occur infrequently and those amino acid positions that show a mismatch in that respective column. C. A magnified version of the alignment in 3.4B encompassing amino acid region between 185 – 261 is shown. While FimK of *K. quasipneumoniae* terminates at amino acid position 218, the FimK of *K. pneumoniae* extends to a length of 470 amino acids. The coloring pattern is shown in Rasmol colors indicating amino acids with similar properties.

Role of *mrkH* and *mrkJ* of *K. quasipneumoniae* in type 3 fimbriae expression

Before investigating differences in FimK function between KqPF9 and TOP52, we first had to determine if known regulators of type 3 fimbrial expression, MrkH and MrkJ, functioned similarly in *K. quasipneumoniae* as in *K. pneumoniae.* MrkH and MrkJ function respectively as the major activator and repressor of the *mrkABCDF* gene cluster (33, 34). To verify the functions of MrkH and MrkJ in *K. quasipneumoniae*, we generated isogenic KqPF9Δ*mrkH* and KqPF9Δ*mrkJ* mutant strains. The deletion and complementation of respective genes were verified by qRT-PCR (Figure 3.5A, 3.5B). To evaluate the activation and repression of *mrk* gene cluster by *mrkH* and *mrkJ* respectively, we studied the expression of *mrkA*. In corroboration with the respective activator and repressor functions of *mrkH* and *mrkJ* previously reported in *K. pneumoniae*, *mrkA* expression was significantly decreased in the KqPF9Δ*mrkH* strain (2.9 fold) and increased in the KqPF9Δ*mrkJ* strain (2.5 fold) (Figure 3.5C) (34). In both cases, the respective complement strains, namely KqPF9Δ*mrkH*+*pmrkH* and KqPF9Δ*mrkJ*+*pmrkJ,* rescued *mrkA* expression levels (Figure 3.5C). Phenotypic analysis showed that bladder epithelial cell association followed a pattern analogous to *mrkA* expression. KqPF9Δ*mrkH* showed a 97.6% decrease in bladder epithelial cell association compared to wild-type KqPF9 and that was rescued in the complemented strain KqPF9Δ*mrkH*+*pmrkH,* which exhibited 124% higher cell association rates than KqPF9. Similarly, bladder epithelial cell association was 117% higher in KqPF9Δ*mrkJ* compared to wild-type and reduced by 80.9% in the complemented strain KqPF9Δ*mrkJ*+*pmrkJ* (Figure 3.5D). Because regulation of type 3 fimbriae by MrkH and MrkJ have been shown to play an important

role in biofilm formation in *K. pneumoniae,* we also assessed the role of these regulators in *K. quasipneumoniae* biofilm formation (34). The biofilm assay results followed a similar pattern as cell association (Figure 3.5E). Biofilm formation was significantly decreased in KqPF9Δ*mrkH* (79.4%) and increased in KqPF9Δ*mrkJ* (61%). These results mirror previously reported findings in uropathogenic *K. pneumoniae* strain AJ218 (34). Taken together, these results suggest that MrkH and MrkJ have similar functions in *K. quasipneumoniae* and *K. pneumoniae* with MrkH acting as an activator of type 3 fimbriae expression and MrkJ acting as a repressor.

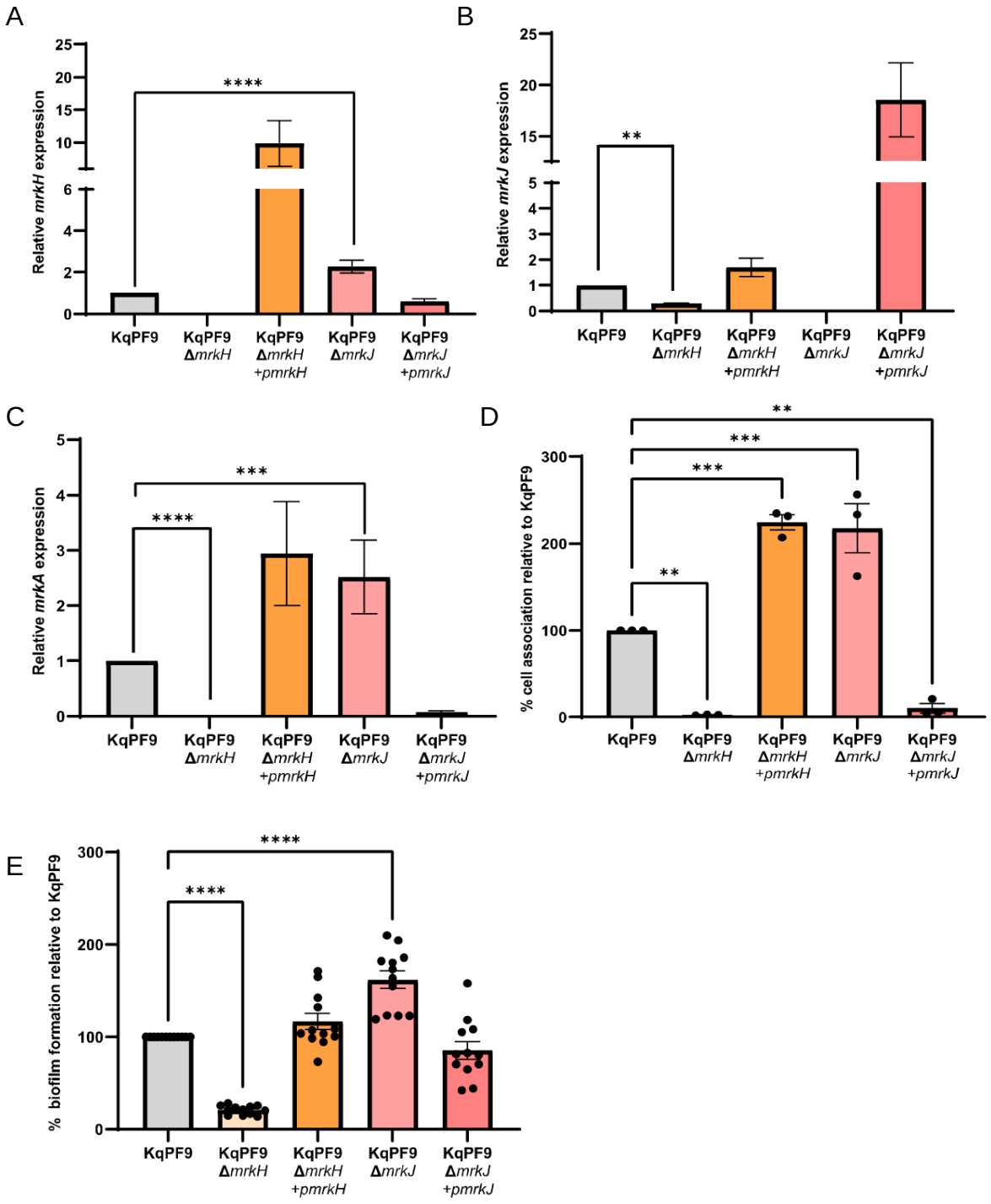

Figure 3.5. Role of *mrkH* and *mrkJ* in KqPF9 type 3 fimbriae expression. A, B, C. qRT-PCR analysis of *mrkH*, *mrkJ* and type 3 fimbriae (*mrkA*) transcription in KqPF9 isolate and respective isogenic mutant strains carrying *pBAD* empty vector or plasmid containing

the respective complementing gene is shown. The expression values of each gene were normalized to *rho* in triplicate and the average value was used to calculate the fold change. D. Cell association assay evaluating the role of *mrkH* and *mrkJ* genes in urinary bladder derived 5637 cell line association of KqPF9 in vitro is shown. The bladder epithelial cell association of KqPF9 strain was evaluated as 100% and the cell association of respective strains were evaluated relative to wild-type KqPF9 strain. The experiments were performed in biological and technical replicates. E. Static biofilm formation of strain KqPF9 along with isogenic mutants of respective genes, and strains expressing appropriate complementing plasmids is shown. The biofilm formation on strain KqPF9 was evaluated as 100% and the biofilm formation of rest of the strains were determined relative to KqPF9 strain. The experiment was performed at least three different times with four technical replicates for each biological sample. The error bars indicate standard error of mean. One way ANOVA with Dunnett's multiple comparisons analysis was used to determine the significance and a p-value less than 0.05 was termed significant.

Cross-regulation of type 3 fimbriae expression by TOP52 *fimK*

To evaluate the role of C-terminal phosphodiesterase domain of FimK in cross-regulation of type 3 fimbriae expression, we generated TOP52Δ*fimK* strains complemented with plasmids either encoding *pfimK*$_{Kp}$, *pfimK*$_{E245A}$ or *pfimK*$_{Kq}$ with *fimK*$_{Kp}$ encoding the full length TOP52 FimK*, fimK*$_{E245A}$ encoding a PDE dead allele of TOP52 *fimK,* and *fimK*$_{Kq}$ encoding KqPF9 FimK lacking the C-terminal PDE domain (37, 46, 47). Though no significant difference in *mrkH* or *mrkA* expression was observed in between TOP52Δ*fimK* and wild-type TOP52, complementation of TOP52Δ*fimK* with a plasmid encoding TOP52 *fimK* (*pfimK*$_{Kp}$*)* showed a significant reduction in *mrkH* (2.94 fold) and *mrkA* (6.25 fold) expression. Conversely, complementation of TOP52Δ*fimK* with *pfimK*$_{Kq}$ and *pfimK*$_{E245A}$, *mrkH* and *mrkA* expression remained at levels comparable to wild-type TOP52 (Fig 3.6A, 3.6B). Analogous to the changes in *mrkH* and *mrkA* expression, bladder epithelial cell association was significantly decreased (46.14%) in TOP52Δ*fimK*+*pfimK*$_{Kp}$ compared to wild-type TOP52 while TOP52Δ*fimK*+*pfimK*$_{Kq}$ and TOP52Δ*fimK*+*pfimK*$_{E245A}$ showed no significant difference in cell association (Fig 3.6C). Considering that only expression of a *fimK* allele containing an intact C-terminal PDE reduced type 3 fimbriae expression in TOP52, we hypothesized that its absence in KqPF9 FimK would translate to a lack of cross-regulation of type 3 fimbriae in *K. quasipneumoniae*. To investigate this hypothesis, we generated an isogenic Δ*fimK* mutant of KqPF9 along with complement strains containing either plasmids *pfimK*$_{Kq}$, *pfimK*$_{Kp}$ or *pfimK*$_{E245A}$. Similar to as observed in TOP52 (Figure 3.6A, 3.6B), significant decreases in *mrkH* (5.2 fold) and *mrkA* (20 fold) expression were observed in KqPF9Δ*fimK*+*pfimK*$_{Kp}$ compared to wild-type KqPF9 (Figure

3.6D, 3.6E). Complementation of KqPF9Δ*fimK* with the PDE dead TOP52 *fimK* allele (*fimK*$_{E245A}$) or KqPF9 *fimK* did not significantly alter *mrkA* or *mrkH* expression from wild-type levels (Figure 3.6D, 3.6E). Similarly, a significant decrease (88.8%) in bladder epithelial cell association was observed in KqPF9Δ*fimK*+*pfimK*$_{Kp}$ whereas no significant change from wild-type KqPF9 was observed in the association of KqPF9Δ*fimK*+*pfimK*$_{Kq}$ and KqPF9Δ*fimK*+*pfimK*$_{E245A}$ (Figure 3.6F). Taken together these results suggest that while the C-terminal PDE of FimK may function to co-regulate type 3 fimbriae expression in TOP52, truncation of this domain in KqPF9 FimK suggests that FimK does not co-regulate type 3 fimbrial expression in *K. quasipneumoniae.*

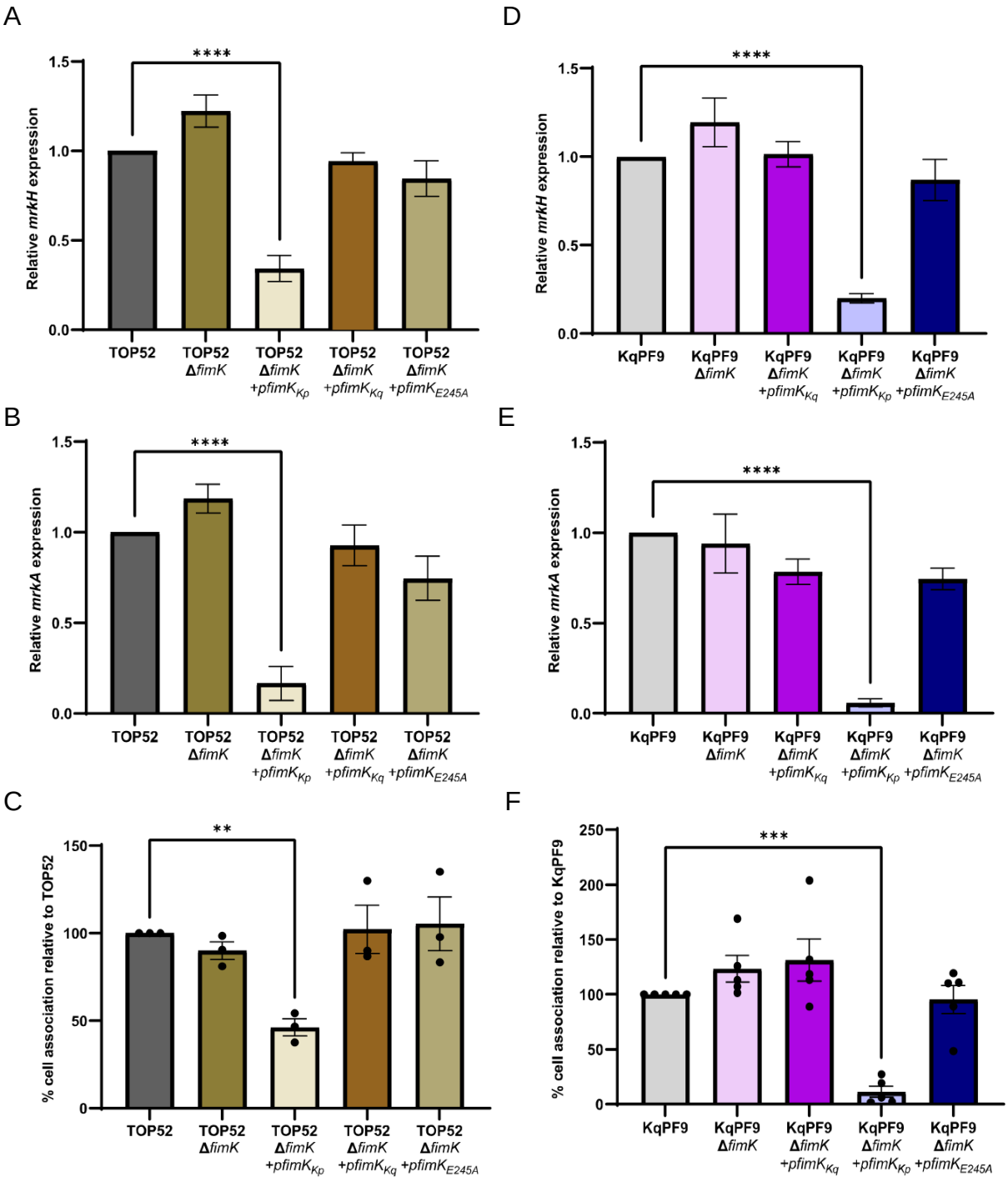

Figure 3.6. Role of *fimK* in bladder epithelial cell association of *K. pneumoniae* TOP52 strain. A, B. qRT-PCR analysis of *mrkH* and type 3 fimbriae (*mrkA*) transcription in wild-

type TOP52 and respective isogenic mutants carrying *pBAD* empty vector or plasmid expressing the respective complementing gene is shown. The expression values of each gene were normalized to *rho*. C. Bladder epithelial cell association assay evaluating the role of *fimK* in cell association of TOP52 strain is shown. D, E. qRT-PCR analysis evaluating the expressions of *mrkH* and type 3 fimbriae (*mrkA*) transcription in wild-type KqPF9 and respective isogenic mutants carrying *pBAD* empty vector or plasmid expressing the respective complementing gene is shown. The expression of respective gene was normalized to *rho*. F. Bladder epithelial cell association assay evaluating the role of *fimK* in cell association of KqPF9 strain is shown. The experiments were performed at least three times with three technical replicates for each biological sample. The error bars indicate standard error of mean. One way ANOVA with Dunnett's multiple comparisons analysis was used to determine the significance and a p-value less than 0.05 was termed significant.

3.4 Discussion

K. quasipneumoniae, previously designated as phylogroup KpII of *K. pneumoniae*, was recently classified as a distinct species of *Klebsiella* (35). Previous reports indicate misidentification of this species as *K. pneumoniae* due to lack of appropriate molecular biology tools in hospital settings (48). Considering that *K. quasipneumoniae* exhibits unique metabolic phenotypes, frequently harbors antibiotic resistance genes like ESBLs and carbapenemases, and is commonly isolated from patients with urinary tract infection, an understanding of *K. quasipneumoniae* virulence mechanisms involved in colonization in the bladder environment would enable design of targeted treatment strategies (2).

Both type 1 and type 3 fimbriae have been reported to play a crucial role in mediating host cell attachment and invasion by *K. pneumoniae* and are therefore crucial to infection (42, 49). However, no study had evaluated the expression of type 1 and type 3 fimbriae in *K. quasipneumoniae* and determined their role in bladder epithelial cell attachment. Using isogenic mutants lacking the major subunits of type 1 and type 3 fimbriae we show that *K. quasipneumoniae* strain KqPF9's attachment to cultured bladder epithelial cells is dependent on type 3 fimbriae (Fig 3.2). While bladder epithelial cell attachment was sharply reduced in KqPF9Δ*mrkA*, no significant difference was observed between KqPF9Δ*fimA* and wild-type KqPF9 (Fig 3.2). Previous reports indicate that type 1 and not type 3 fimbriae are important for *K. pneumoniae* bladder colonization in mice whereas type 3 fimbriae play a role in urinary bladder epithelial cell attachment in vitro (20, 43). This specificity could in part be due to differential expression of type 1 and type 3 fimbriae between the strains. Considering that type 1 and type 3 fimbriae are not usually

coincidentally expressed in *K. pneumoniae,* one possibility is that their expression is co-regulated by a single regulator or regulatory network (37).

Intracellular second messengers like cyclic di-GMP play a crucial role in responding to sensory input from extracellular stimuli and coupling them to physiologic changes (50-52). Diguanylate cyclases coordinate the synthesis of cyclic di-GMP while phosphodiesterases with conserved EAL domains are responsible for cyclic di-GMP hydrolysis (53-56). The type 3 fimbriae expression in *Klebsiella* is to a large extent dependent on transcriptional activator MrkH, a cyclic di-GMP binding protein and MrkJ which is a phosphodiesterase (34). The FimK protein of *K. pneumoniae* has two domains, a putative helix-turn-helix (HTH) DNA binding domain in the “N” terminus and an EAL phosphodiesterase domain in the “C” terminus known to hydrolyze cyclic di-GMP thereby regulating cyclic di-GMP levels (34, 37). It is possible that FimK through the C-terminal domain regulates type 3 fimbriae by modulating cyclic di-GMP levels.

The dependence of *K. quasipneumoniae’s* bladder epithelial cell association on type 3 fimbriae could be attributed to differences in expression due to differential regulation of type 1 and type 3 fimbriae between the two species. Indeed, we observed that basal expression of *mrkA* as well as rates of bladder epithelial cell association were significantly higher in KqPF9 than in *K. pneumoniae* TOP52 (Fig 3.3). Through comparative genomics of the type 1 fimbrial operons of several *K. pneumoniae* and *K. quasipneumoniae* strains, we discovered a pre-mature stop codon in KqPF9 *fimK* that results in truncation of the C-terminal phosphodiesterase domain (Fig 3.4). We discerned that this difference is conserved among all *K. quasipneumoniae* isolates and we

demonstrated the role of EIL phosphodiesterase domain of FimK in regulating type 3 fimbriae expression and urinary bladder epithelial cell attachment. Through this study we also demonstrated how the lack of EAL domain in *K. quasipneumoniae* leads to a loss of cross-regulation of type 3 fimbriae by FimK in this species (Fig 3.6). We also show that MrkH and MrkJ have conserved respective activator and repressor functions in the regulation of type 3 fimbriae in *K. quasipneumoniae* (Fig 3.5). However, it is possible that the presence of other diguanylate cyclases and phosphodiesterases in *Klebsiella quasipneumoniae* may contribute to co-regulation of type 1 and type 3 fimbriae in *K. quasipneumoniae* in different host environments (34).

The increased expression of type 1 (*fimA*) and type 3 (*mrkA*) fimbriae in KqPF9 strain explains the increased biofilm formation and bladder epithelial cell association of this strain relative to TOP52 indicative of stronger colonizing ability of *K. quasipneumoniae* (Fig 3.3). However, it is unclear at this point if the increased cell association of KqPF9 strain would enable it better fitness to colonize the urinary tract. It would be interesting to compare the expressions of type 1 and type 3 fimbriae of these strains in a murine cystitis model to further validate their cell association and the likely impact of differences in *fimK* in these strains.

In addition to local regulators of type 1 and type 3 fimbriae encoded within the *fim* and *mrk* operons respectively, global regulators like integration host factor (IHF), leucine responsive protein (LRP) and histone like nucleoid structuring protein (H-NS) also play an important role (57-60). Of the global regulators, H-NS is considered important as it has been reported to regulate both type 1 and type 3 fimbriae (57, 58). H-NS is a highly

abundant DNA binding protein that transcriptionally silences genes with AT-rich promoters (61). It regulates the phase variation of type 1 fimbriae by modulating the *fimS* region directly by binding to region adjacent to the invertible *fimS* element (62-64). H-NS also regulates type 1 fimbriae indirectly by repressing the transcription of *fimB* and *fimE* recombinases by binding to their promoters (65). In case of type 3 fimbriae, H-NS represses the expression of *mrkH* and *mrkI* (57), the major and minor activators of type 3 fimbriae (34), while activating *mrkA* expression (66). Interestingly, while cyclic di-GMP levels regulate various physiological changes in a cell, cyclic di-GMP associated biofilm formation has been reported to be coordinated by de-repression of genes repressed by H-NS that are involved in biofilm formation in Vibrio cholerae (67). Considering that *mrkA* plays a significant role in biofilm formation in *K. pneumoniae* (68) and in *K. quasipneumoniae* (Fig 3.8D) and that MrkH an activator of *mrk* gene cluster is dependent on cyclic di-GMP to bind to DNA (34) alongside *mrkH* being repressed by H-NS (66) it is possible that biofilm formation, type 3 fimbriae expression regulated by cyclic di-GMP are carried out in response to environmental changes that trigger expression of regulators like MrkH whose binding site overlaps with binding sites of H-NS thereby coordinating de-repression of genes involved in specific function that were originally repressed by H-NS (66, 69).

Taken together, the data presented here provide important insight not only into the co-regulation of type 1 and type 3 fimbriae by *fimK* in *K. pneumoniae* but also into important differences in bladder epithelial cell attachment and biofilm formation phenotypes between *K. pneumoniae* and *K. quasipneumoniae.*

3.5 Subsection of supplemental figures

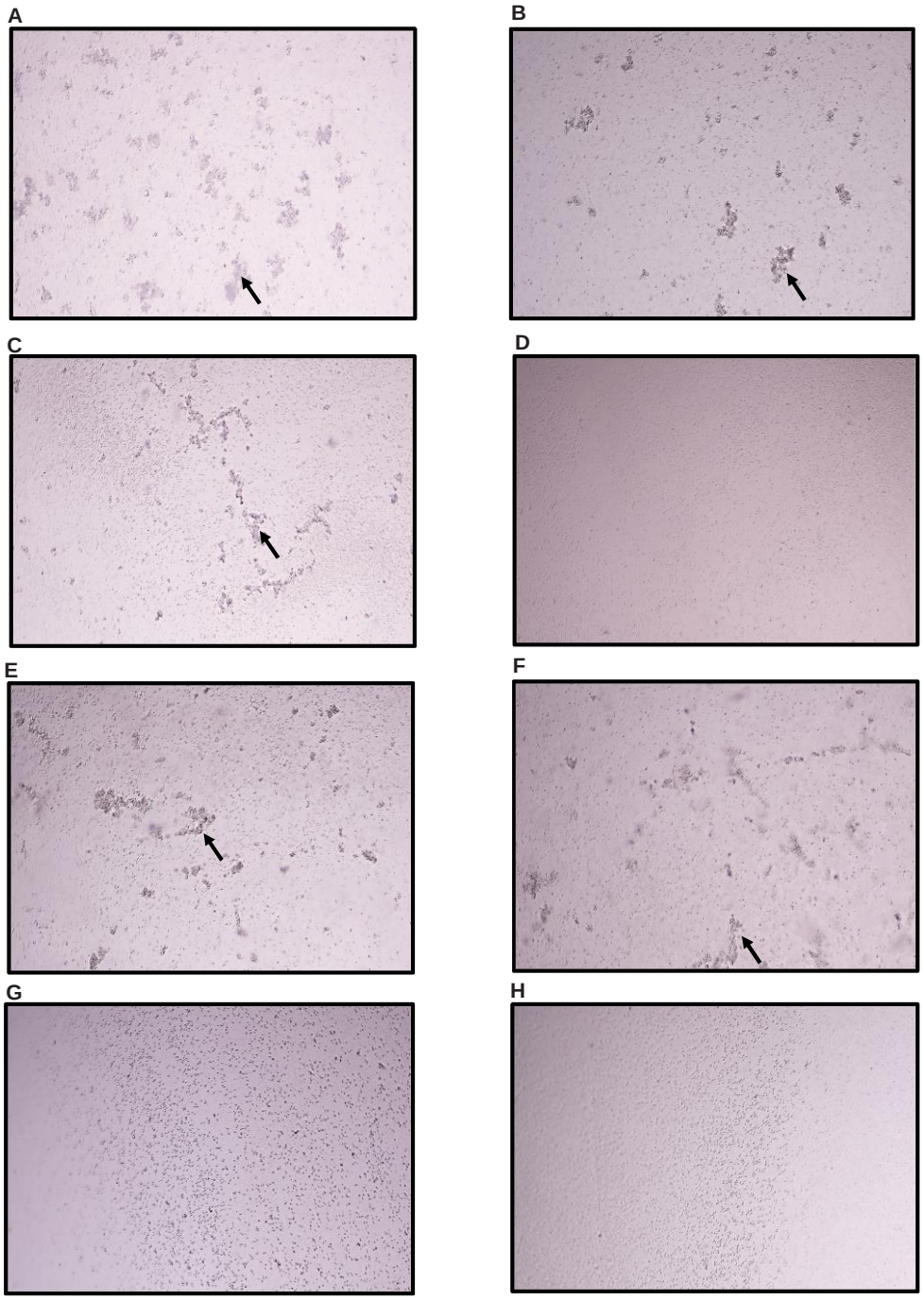

Figure 3.7. KqPF9 shows a mannose insensitive pattern of agglutination of yeast cells. Agglutination of yeast cells (as indicated by arrows) in the presence and absence of 2.5%

D-Mannose by KqPF9 isolate and its isogenic type 1 and type 3 fimbriae mutants are shown. A, B. Images show the agglutination of yeast cells by KqPF9 strain in the presence of control (PBS) or 2.5% D-Mannose respectively. C, D. Images show the agglutination pattern of isogenic type 3 fimbriae mutant KqPF9Δ*mrkA*. While KqPF9Δ*mrkA* strain is able to agglutinate yeast cells in the control sample, the presence of mannose was able to inhibit the agglutination of type 1 fimbriae showing that type 1 fimbriae are mannose sensitive. E, F. Images show the agglutination pattern of yeast cells by type 1 fimbriae mutant KqPF9Δ*fimA* in control or mannose treated samples respectively. Considering that KqPF9Δ*fimA* strain was able to agglutinate yeast cells in the presence or absence of mannose, it shows that type 3 fimbriae are mannose insensitive. G, H. Images show the agglutination pattern of yeast cells by KqPF9Δ*mrkA*Δ*fimA* strain in the presence and absence of mannose. As no agglutination was visible in control and mannose treated samples, it indicates that either type 1 or type 3 fimbriae are essential for agglutination of yeast cells by KqPF9 isolate.

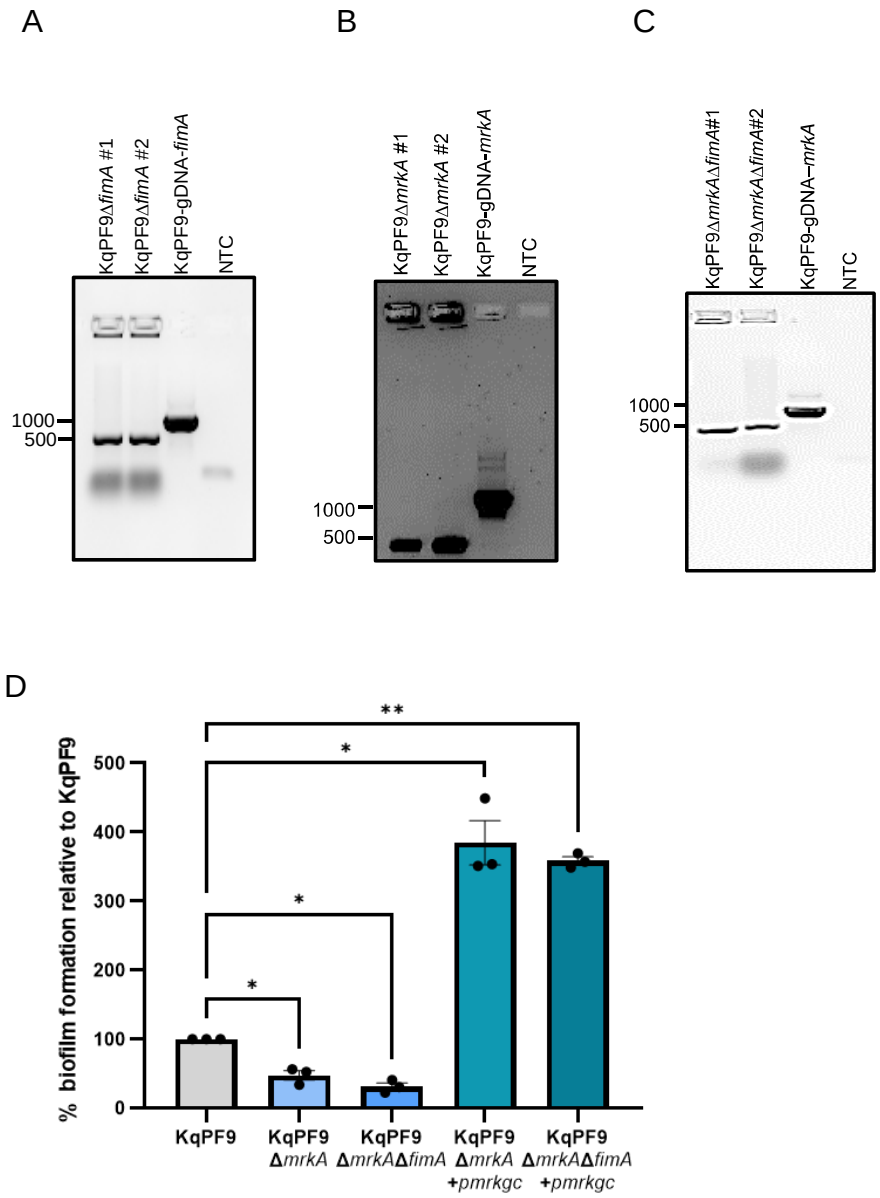

Figure 3.8. Generation of type 1 and type 3 fimbriae mutant strains of KqPF9. A, B**,** C. Images showing the PCR products of type 1 (KqPF9Δ*fimA*), type 3 (KqPF9Δ*mrkA*) or both type 1 and type 3 fimbriae (KqPF9Δ*mrkA*Δ*fimA*) isogenic mutants of KqPF9 as resolved on an 1% agarose gel. For the images discussed, genomic DNA of respective wild-type KqpF9 or TOP52 strains respectively were used as positive control to verify the size of PCR product. Also, a no template negative control (NTC) was used to verify the purity of reagents in PCR mix. D. Biofilm formation of KqPF9 strain, isogenic type 3, type 1 and

type 3 fimbriae mutant strains along with respective mutant strains expressing complementing plasmids is shown. The biofilm formation of strain KqPF9 was evaluated as 100% and the biofilm formation of other strains were determined relative to KqPF9 strain. The experiment was performed with biological and technical replicates.

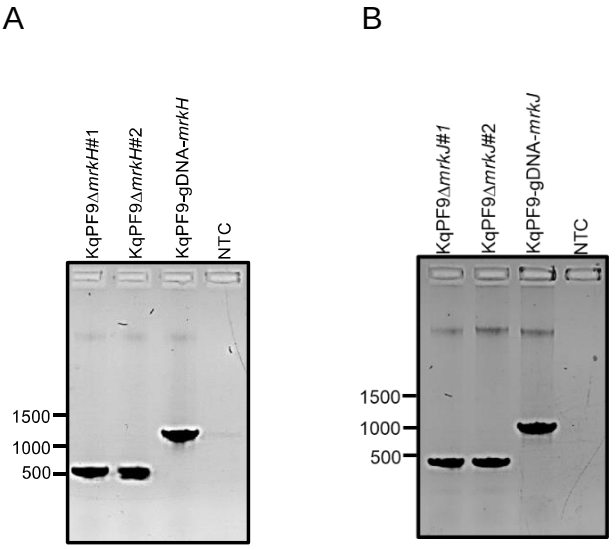

Figure 3.9. Generation of isogenic *mrkH* and *mrkJ* mutant strains of KqPF9. A, B. Images of the PCR products of isogenic *mrkH* and *mrkJ* mutants of KqPF9 strain resolved in an 1% agarose gel is shown. The genomic DNA of KqpF9 strain was used as positive control to verify the size of PCR product and a no template control (NTC) was used as negative control.

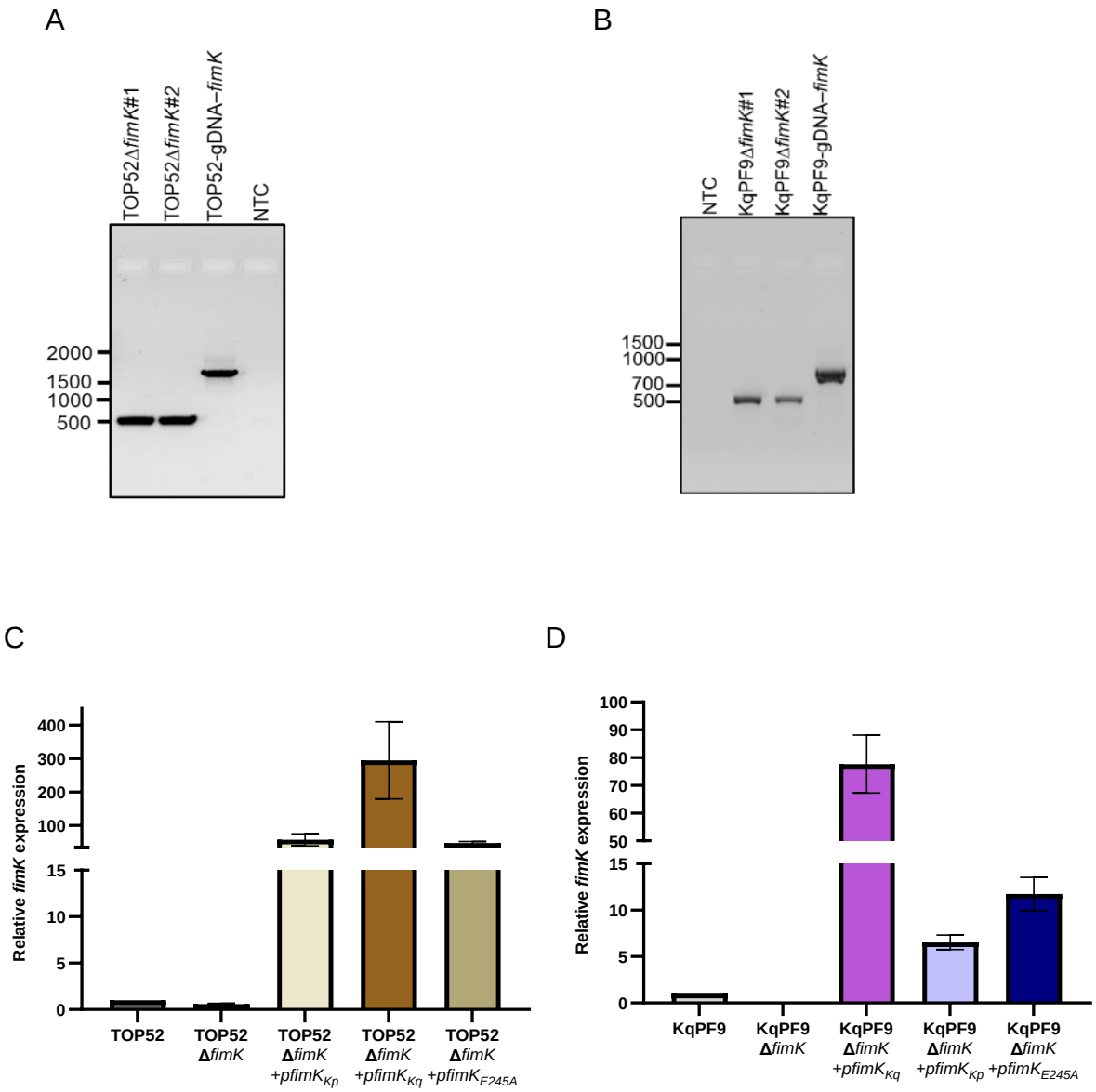

Figure 3.10. Generation of respective isogenic *fimK* mutants of TOP52 and KqPF9 strains. A, B. Images of PCR products of *fimK* isogenic mutants of TOP52 and KqPF9 resolved in an 1% agarose gel is shown. For the images discussed, genomic DNA of respective wild-type strains of TOP52 and KqPF9 were used as positive controls to verify the size of PCR product and a no template control (NTC) served as negative control. C, D. qRT-PCR analysis of *fimK* in TOP52 and KqPF9 isolates along with their respective

isogenic mutant strains carrying *pBAD* empty vector or plasmid expressing the respective complementing *fimK* gene is shown. The expression values of each gene were normalized to *rho* in triplicates and the average value was used to evaluate the fold change.

A

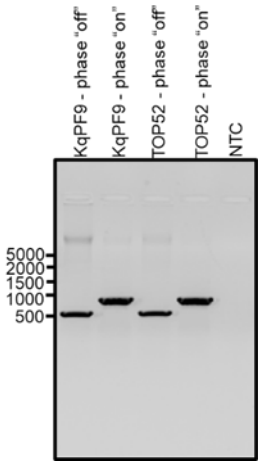

Figure 3.11. A. Phase assay determining the phase of KqPF9 and TOP52 static cultures. Image of PCR amplification products of genomic DNA extracted from overnight static cultures of wild-type KqPF9 and TOP52 strains resolved in an 1% agarose gel is shown. No template negative control (NTC) was used to verify the purity of reagents used in the PCR mix.

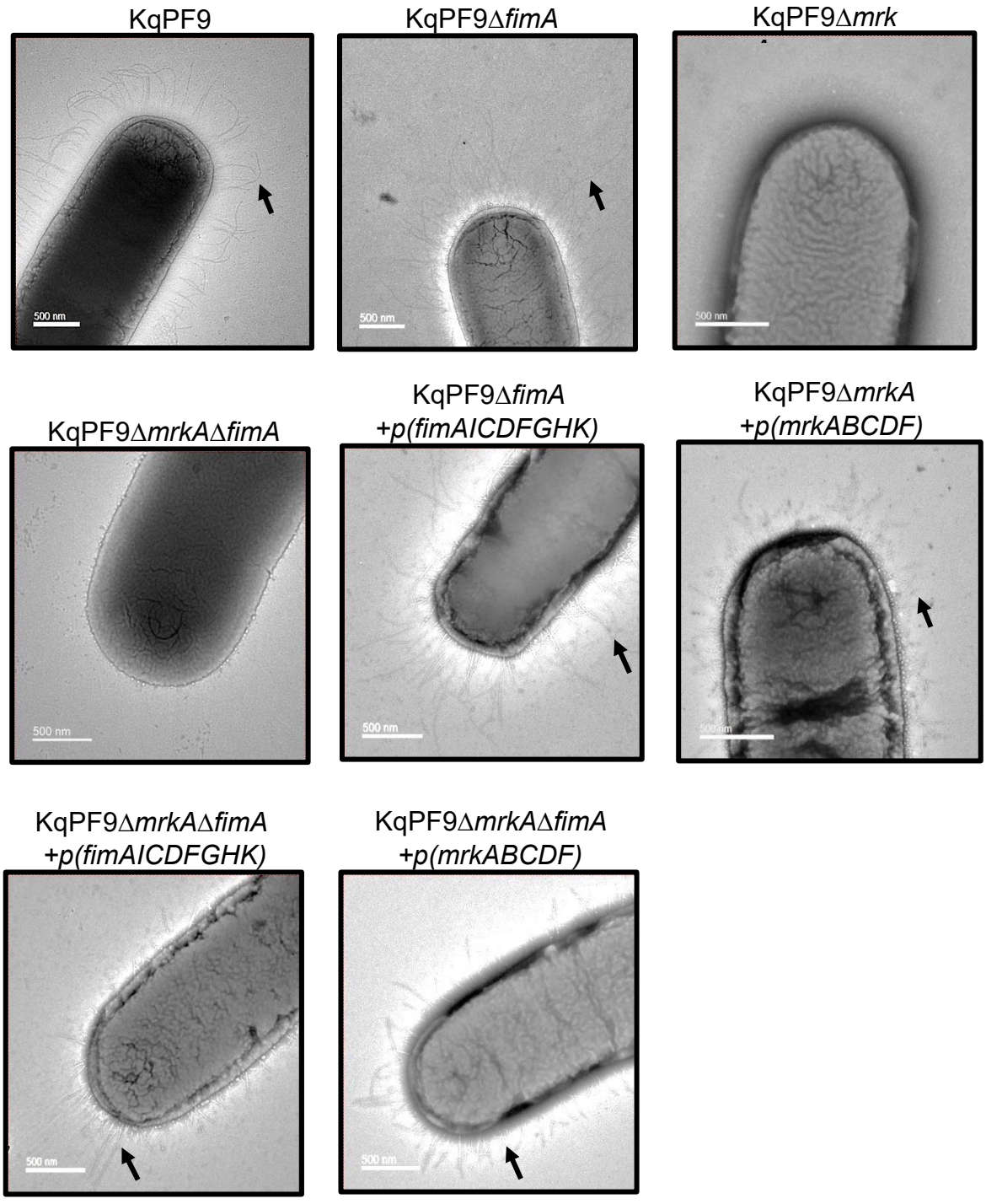

Figure 3.12. Visualization of fimbriae of KqPF9 wild-type, type 1, type 3 and both type 1, type 3 fimbriae mutant strains by negative stain electron microscopy. While KqPF9 wild-

type, type 1 fimbriae mutant and fimbriae mutants expressing respective complementation plasmids showed expression of respective fimbriae as indicated by arrows, type 3 and both type 1, type 3 fimbriae double mutants appeared bald suggestive of dependence of KqPF9 on type 3 fimbriae expression for attachment to host cells. The scale bar indicates a measure of 500 nm.

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CHAPTER 4

COMPLETE GENOME SEQUENCES OF THREE UROPATHOGENIC *KLEBSIELLA QUASIPNEUMONIAE* STRAINS ISOLATED FROM POSTMENOPAUSAL WOMEN WITH RECURRENT URINARY TRACT INFECTION

4.1 Author contributions

The original manuscript with the same title was published in Microbiology Research Announcement with authors Sundharamani Venkitapathi (S.V.) *, Belle M. Sharon (B.M.S.) *, Tahira A. Ratna (T.A.R.), Amanda P. Arute (A.P.A.), Philippe E. Zimmern (P.E.Z.), Nicole J. De Nisco (N.J.D.). * indicates equal contribution.

4.2 Abstract

Recurrent Urinary Tract Infection (rUTI) poses a major health issue, especially among postmenopausal women. We report complete genome sequences of three *Klebsiella quasipneumoniae* isolated from urine of postmenopausal women with rUTI. *K. quasipneumoniae* is a recently identified species of *Klebsiella* with clinical characteristics and virulence factors distinct from *K. pneumoniae*.

4.3 Introduction

Recurrent UTI (rUTI), defined as two UTI episodes within six months or three within twelve months, poses a major health issue with ~50% of UTIs in postmenopausal women

developing into rUTI (1, 2). The genus *Klebsiella* is the second leading cause of rUTI accounting for 15-17% cases (3). *K. quasipneumoniae* was originally thought to inhabit plants and other environmental niches, however, recent reports suggest that the organism is prevalent in human diseases as well (4). The lack of tests to distinguish *K. quasipneumoniae* from *K. pneumoniae* in clinical settings has led to fewer reports and hence an underestimation of the prevalence of *K. quasipneumoniae* (5).

The availability of complete genomes of uropathogenic *K. quasipneumoniae* isolates would allow for better understanding of virulence and metabolic traits specific to this species. We report the complete genomes of three *K. quasipneumoniae* strains (Table 1) isolated from urine of postmenopausal women meeting criteria for uncomplicated rUTI as part of an institutional review board (IRB)-approved study (STU032016-006, MR17-120) (6).

4.4 Materials and Methods

Clean-catch midstream urine sample was obtained from three consenting subjects and plated onto CHROMagar Orientation (BD). Following overnight incubation at 37°C, single colonies were chosen for genus identification by Sanger sequencing of 16S rRNA gene and nucleotide (nr/nt) database query using megablast (BLAST v2.10.0) (6, 7). Species identification was performed by polymerase chain reaction (PCR) using primers specific for *K. quasipneumoniae* species targeting beta lactamase(*bla*) and deoxyribose regulator(*deoR)* genes (8). *K. quasipneumoniae* isolates were grown in Luria broth (LB)

overnight at 37°C, and their genomic DNA (gDNA) extracted using gDNA extraction kit (BioBasic).

The gDNA obtained was evaluated for 260/280nm absorbance ratio and assessed by agarose gel electrophoresis. The DNA samples were then subjected to sequencing using Illumina and Oxford Nanopore (ONT) technologies. For Illumina sequencing, library preparation was performed using Nextera DNA Flex library prep kit followed by sequencing using NextSeq 500 system to generate paired end reads (2x150bp). ONT libraries were prepared using ligation sequencing kit (SQK-LSK109) and barcode expansion kit 13-24 (EXP-NBD114); followed by sequencing on MinION instrument using R9 FLO-MIN106 flow cells. Live fast base calling, demultiplexing, and barcode trimming were performed using ONT MinKNOW software. Illumina reads were quality assessed and trimmed using CLC Genomics Workbench v12.0.3 with cutoffs set at minimum Phred score of 20 and read length of 15bp. ONT reads were assessed for quality using NanoStats v1.2.0 (9) and trimmed using NanoFilt v2.6.0 (9) to retain reads with a Phred score >7 and length greater than 200bp (Table 4.1).

Illumina and ONT reads were used to construct hybrid assemblies of each strain using Unicycler v0.4.8 (SPAdes v3.13.0, Racon v1.4.10, and Pilon v1.23) at default parameters (10-13). Circular genomic sequences were rotated to the starting base of *dnaA* or *repA,* if found. The quality of hybrid assembly generated was evaluated using QUAST v5.0.2 and, completeness of genome was assessed using Bandage v0.8.1(14) and BUSCO v1 (15) with bacteria ortholog set on gVolante server v1.2.1 (16). Upon completion of hybrid assembly, annotations of genomes were carried out using

NCBI Prokaryotic Genome Annotation Pipeline v4.11. GC content and number of coding sequences were obtained using Geneious Prime v2020.0.5. Finally, sequence type and plasmid replicons of each strain were determined using MLST v2.0 (http://www.genomicepidemiology.org/) with *Klebsiella pneumoniae* configuration (17) and PlasmidFinder v2.1 (18, 19) with settings at 90% identity and 60% minimum coverage cutoffs.

4.5 Data availability

The complete sequences were submitted to GenBank under BioProject accession number PRJNA683049. The BioSample and SRA accession numbers for each isolate can be found in Table 4.1.

Table 4.1. Accession numbers, assembly parameters and isolate characteristics of three uropathogenic *Klebsiella quasipneumoniae* isolates from postmenopausal women with rUTI.

Strain	BioSample Accession No.	SRA Accession No (O: ONT, I: Illumina)	Total No. of Reads	N_{50} (bp)[a]	Read Depth (×)	MLST[b]	GenBank Accession No.	Type of Contig (circular)	Total Length (bp)[a]	GC content (%)	No. of CDSs[c]	Plasmid replicon(s)
KqPF9	SAMN17016746	SRX9774966 (O)	148,741	11,189	153	UN[e]	CP065841	Chromosome	5,272,842	58.1	5,138	NA[d]
		SRX9779643 (I)	16,068,400		359		CP065842	Plasmid	399,394	48.3	435	Col(pHAD28), Col440I, IncFIB
							CP065843	Plasmid	4,730	42.6	6	UN[e]
							CP065844	Plasmid	4,096	55.5	5	UN[e]
							CP065845	Plasmid	4,000	46.3	4	UN[e]
KqPF26	SAMN17016750	SRX9774961 (O)	26,344	13,446	39	3387	CP065838	Chromosome	5,242,686	58	5,039	NA[d]
		SRX9779644 (I)	11,492,042		271		CP065839	Plasmid	144,959	52.5	152	IncFIB(K), IncFII(K)
							CP065840	Plasmid	3,478	45.7	6	UN[e]
KqPF42	SAMN17016751	SRX9774962 (O)	124,662	11,819	147	1535	CP065846	Chromosome	5,278,208	58	5,092	NA[d]
		SRX9779645 (I)	17,989,506		420		CP065847	Plasmid	223,863	50.9	252	IncFIB(K)

[a] Base Pair
[b] Multilocus Sequence Type
[c] Coding Sequence
[d] Not Applicable
[e] Unknown

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CHAPTER 5
DISCUSSION AND SUMMARY

5.1 Author contributions

This chapter was written by Sundharamani Venkitapathi (S.V.) and edited by Dr. Nicole J. DeNisco (N.J.D.).

5.2 Discussion

K. quasipneumoniae, previously designated as phylogroup KpII of *K. pneumoniae*, was recently classified as a distinct species of *Klebsiella* (1). Previous reports indicate misidentification of this species as *K. pneumoniae* due to lack of appropriate molecular biology tools in hospital settings (2). Considering that *K. quasipneumoniae* exhibits unique metabolic phenotypes, frequently harbors antibiotic resistance genes like ESBLs and carbapenemases, and is commonly isolated from patients with urinary tract infection, an understanding of *K. quasipneumoniae* virulence mechanisms involved in colonization in the bladder environment would enable design of targeted treatment strategies (3).

Both type 1 and type 3 fimbriae have been reported to play a crucial role in mediating host cell attachment and invasion by *K. pneumoniae* and are therefore crucial to infection (4, 5). However, no study had evaluated the expression of type 1 and type 3 fimbriae in *K. quasipneumoniae* and determined their role in bladder epithelial cell attachment. Using isogenic mutants lacking the major subunits of type 1 and type 3 fimbriae we show that *K. quasipneumoniae* strain KqPF9's attachment to cultured bladder epithelial cells is dependent on type 3 fimbriae (Fig 3.2D). While bladder epithelial cell

attachment was sharply reduced in KqPF9Δ*mrkA*, no significant difference was observed between KqPF9Δ*fimA* and wild-type KqPF9 (Fig 3.2C). Previous reports indicate that type 1 and not type 3 fimbriae are important for *K. pneumoniae* bladder colonization in mice whereas type 3 fimbriae play a role in urinary bladder epithelial cell attachment in vitro (6, 7). This specificity could in part be due to differential expression of type 1 and type 3 fimbriae between the strains. Considering that type 1 and type 3 fimbriae are not usually coincidentally expressed in *K. pneumoniae,* one possibility is that their expression is co-regulated by a single regulator or regulatory network (8).

Intracellular second messengers like cyclic di-GMP play a crucial role in responding to sensory input from extracellular stimuli and coupling them to physiologic changes (9-11). Diguanylate cyclases coordinate the synthesis of cyclic di-GMP while phosphodiesterases with conserved EAL domains are responsible for cyclic di-GMP hydrolysis (12-15). The type 3 fimbriae expression in *Klebsiella* is to a large extent dependent on transcriptional activator MrkH, a cyclic-di-GMP binding protein and MrkJ which is a phosphodiesterase (16). The FimK protein of *K. pneumoniae* has two domains, a putative helix-turn-helix (HTH) DNA binding domain in the “N” terminus and an EAL phosphodiesterase domain in the “C” terminus known to hydrolyze cyclic di-GMP thereby regulating cyclic di-GMP levels (8, 16). It is possible that FimK through the C-terminal domain regulates type 3 fimbriae by modulating cyclic di-GMP levels.

The dependence of *K. quasipneumoniae’s* bladder epithelial cell association on type 3 fimbriae could be attributed to differences in expression due to differential regulation of type 1 and type 3 fimbriae between the two species (Fig 3.2D). Indeed, we

observed that basal expression of *mrkA* as well as rates of bladder epithelial cell association were significantly higher in KqPF9 than in *K. pneumoniae* TOP52 (Fig 3.3). Through comparative genomics of the type 1 fimbrial operons of several *K. pneumoniae* and *K. quasipneumoniae* strains, we discovered a pre-mature stop codon in KqPF9 *fimK* that results in truncation of the C-terminal phosphodiesterase domain (Fig 3.4). We discerned that this difference is conserved among all *K. quasipneumoniae* isolates and we demonstrated the role of EAL phosphodiesterase domain of FimK in regulating type 3 fimbriae expression and urinary bladder epithelial cell attachment (Fig 3.6). Through this study we also demonstrated how the lack of EAL domain in *K. quasipneumoniae* leads to a loss of cross-regulation of type 3 fimbriae by FimK in this species (Fig 3.6D, 3.6E, 3.6F). We also show that MrkH and MrkJ have conserved respective activator and repressor functions in the regulation of type 3 fimbriae in *K. quasipneumoiae* (Fig 3.5). However, it is possible that the presence of other diguanylate cyclases and phosphodiesterases in *Klebsiella quasipneumoniae* may contribute to co-regulation of type 1 and type 3 fimbriae in *K. quasipneumoniae* in different host environments (16).

The increased expression of type 1 (*fimA*) and type 3 (*mrkA*) fimbriae in KqPF9 strain explains the increased biofilm formation and bladder epithelial cell association of this strain relative to TOP52 (Fig 3.3) indicative of stronger colonizing ability of *K. quasipneumoniae*. However, it is unclear at this point if the increased cell association of KqPF9 strain would enable it better fitness to colonize the urinary tract. It would be interesting to compare the expressions of type 1 and type 3 fimbriae of these strains in a

mouse model to further validate their cell association and the likely impact of differences in *fimK* in these strains.

In addition to local regulators of type 1 and type 3 fimbriae encoded within the *fim* and *mrk* operons respectively, global regulators like integration host factor (IHF), leucine responsive protein (LRP) and histone like nucleoid structuring protein (H-NS) also play an important role (17-20). Of the global regulators, H-NS is considered important as it has been reported to regulate both type 1 and type 3 fimbriae (17, 18). H-NS is a highly abundant DNA binding protein that transcriptionally silences genes with AT-rich promoters (21). It regulates the phase variation of type 1 fimbriae by modulating the *fimS* region directly by binding to region adjacent to the invertible *fimS* element (22-24). H-NS also regulates type 1 fimbriae indirectly by repressing the transcription of *fimB* and *fimE* recombinases by binding to their promoters (25). In case of type 3 fimbriae, H-NS represses the expression of *mrkH* and *mrkI* (17), the major and minor activators of type 3 fimbriae expression (16), while activating *mrkA* expression (26). Interestingly, while cyclic di-GMP levels regulate various physiological changes in a cell, cyclic-di-GMP associated biofilm formation has been reported to be coordinated by de-repression of genes repressed by H-NS that are involved in biofilm formation in Vibrio cholerae (27). Considering that *mrkA* plays a significant role in biofilm formation in *K. pneumoniae* (28) and in *K. quasipneumoniae* (Fig S2D) and that MrkH an activator of *mrk* gene cluster is dependent on cyclic di-GMP to bind to DNA (16) alongside *mrkH* being repressed by H-NS (26) it is possible that biofilm formation, type 3 fimbriae expression regulated by cyclic di-GMP are carried out in response to environmental changes that trigger expression of

regulators like MrkH whose binding site overlaps with binding sites of H-NS thereby coordinating de-repression of genes involved in specific function that were originally repressed by H-NS (26, 29).

Taken together, the data presented here provide important insight not only into the co-regulation of type 1 and type 3 fimbriae by *fimK* in *K. pneumoniae* but also into important differences in bladder epithelial cell attachment and biofilm phenotypes between *K. pneumoniae* and *K. quasipneumoniae.*

In the second study (chapter 4), we report whole genome sequences of three *K. quasipneumoniae* strains isolated from the urine of post-menopausal women with rUTI (30). Considering the lack of information about this relatively new species of *Klebsiella*, the whole genome sequence of *K. quasipneumoniae* strains not only adds to the repository of information but also contributes towards an understanding of various antibiotic resistance genes, virulence factors and metabolic attributes that are specific to this species.

Evaluation of the hybrid assemblies of three *K. quasipneumoniae* isolates showed that all three isolates studied in the lab, contained plasmid replicons predicted by PlasmidFinder v2.1 (31, 32). In total, 4 different plasmid replicons were detected. KPF9 was predicted to contain one plasmid with col(pHAD28), col440I and IncFIB replicons and three plasmids with an unidentified replicon. KqPF26 was predicted to contain one plasmid with IncFIB(K) and IncFII(K) replicons and one unidentified replicon. KqPF42 contained one plasmid with a IncFIB(K) replicon. As a future objective it would be

interesting to study these plasmids for the presence of genes encoding conjugative transfer proteins, replication initiation and partitioning proteins, virulence factors, or other proteins that are known to be transferred horizontally. It would also be interesting to investigate the prevalence of these plasmids among sequenced *K. pneumoniae* and *K. quasipneumoniae* isolates to determine if there is any species-specificity or association with disease state.

All three *K. quasipneumoniae* strains contained antibiotic resistance genes encoding for beta lactamases (bla_{OKP}), efflux pumps (*oqxA* and *oqxB*) and glutathione S-transferase (*fosA*). Three groups of beta-lactamase gene namely, bla_{SHV}, bla_{OKP}, and bla_{LEN} evolving from a common ancestor alongside three *K. pneumoniae* phylogroups KpI, KpII and KpIII have been reported (33, 34). The bla_{OKP} group was reported to be present in *K. quasipneumoniae* previously categorized as KpII phylogroup (1, 34). Two bla_{OKP} subgroups, namely $bla_{OKP\text{-}A}$ and $bla_{OKP\text{-}B}$ have been reported (34, 35). All *K. quasipneumoniae* strains investigated in the lab encoded bla_{OKP} of B subgroup.

KqPF9 encoded the chromosomal beta-lactamase gene $bla_{OKP\text{-}B\text{-}15}$, KqPF26 showed the presence of a nucleotide region that had sequence homology to $bla_{OKP\text{-}B\text{-}5}$ and $bla_{OKP\text{-}B\text{-}16}$ while KqPF42 showed the presence of a nucleotide region that was homologous to $bla_{OKP\text{-}B\text{-}5}$, $bla_{OKP\text{-}B\text{-}16}$, $bla_{OKP\text{-}B\text{-}19}$, $bla_{OKP\text{-}B\text{-}3}$ variants all belonging to subgroup B of bla_{OKP}. Apart from beta lactamase, all three strains also contained *oqx*AB genes that encode for efflux pumps. Efflux pumps are present in most bacterial species and they play an important role in enabling antibiotic resistance to bacterial cells (36, 37). It has been reported that *oqx*AB in *K. pneumoniae* confers resistance to multiple drugs

namely, quinoxalines, quinolones, tigecycline, nitrofurantoin and chloramphenicol (38). In addition, all the three *K. quasipneumoniae* strains encoded for FosA protein which is a Manganese (Mn2+) and Potassium (K+)-dependent glutathione S-transferase. FosA confers fosfomycin resistance by facilitating conjugation of glutathione to the antibiotic Fosfomycin (39).

Multilocus sequence typing was performed using the hybrid assemblies of the respective strains to characterize the bacterial isolates through identification of the specific strain based on the sequences of internal regions of housekeeping genes (40). Sequence typing of *K. quasipneumoniae* isolates was performed using MLST V2.0 with *K. pneumoniae* configuration as a separate configuration of *K. quasipneumoniae* is not available yet. The internal regions of 7 housekeeping genes namely *gapA* (encoding glyceraldehyde 3 phosphate dehydrogenase), *infB* (encoding translation initiation factor 2), *mdh* (encoding malate dehydrogenase), pgi (encoding glucose 6 phosphate isomerase), *pho* (encoding phosphate regulon), *rpoB* (encoding beta subunit of the RNA polymerase), *tonB* (encoding TonB protein) were used to determine the sequence type (32, 41). Based on the combination of alleles of the house keeping genes analyzed, while the sequence type of KqPF9 couldn't be determined (unknown), the sequence type of KqPF26 and KqPF42 were determined to be 3387 and 1535 respectively.

5.3 Summary of the importance of this study

K. quasipneumoniae, previously designated as phylogroup KpII of *K. pneumoniae*, was recently classified as a distinct species of *Klebsiella* (1). Previous reports indicate

misidentification of these species as *K. pneumoniae* due to lack of appropriate molecular biology tools in hospital settings (2). Considering that *K. quasipneumoniae* harbors antibiotic resistance genes like extendend spectrum beta-lactamases (ESBLs), carbapenemases alongside being commonly isolated from urinary tract infections, an understanding of their virulence factors and mechanisms involved in bladder colonization would provide foundational knowledge necessary for designing effective treatment strategies (3).

This dissertation expands the field's understanding of *K. quasipneumoniae* through two major works. The first study provides insight into type 1 and type 3 fimbriae, which are important virulence factors in *Klebsiella spp.* (5, 7, 42). Fimbriae act as bacterial appendages for attachment to urinary bladder epithelial cells and enable a critical step that facilitates adhesion, invasion and biofilm formation thereby aiding in disease progression. An *in vitro* assessment of the role of type 1 and type 3 fimbriae in urinary bladder epithelial cell attachment showed that *K. quasipneumoniae* strain PF9 (KqPF9) is dependent on type 3 fimbriae. Evaluation of type 1 and type 3 fimbriae major subunit expression in KqPF9 and *K. pneumoniae* strain TOP52 showed a significant increase in the expression of both type 1 (*fimA*) and type 3 fimbriae (*mrkA*) in KqPF9. Interestingly, we observed that though *fimA* and *mrkA* expressions were higher in KqPF9, the fold change in *mrkA* expression (7.4 fold) between the two strains was higher than *fimA* expression (1.5 fold) probably indicating stronger dependence of KqPF9 on type 3 fimbriae. Further, the increase in type 1 and 3 fimbriae expression was coincident with a significant increase in cultured bladder epithelial cell attachment and biofilm formation of

KqPF9 relative to TOP52. These results provide an insight into the colonizing ability of *K. quasipneumoniae* relative to *K. pneumoniae* and the roles of type 1 and type 3 fimbriae in these species.

Also, as part of this study, we performed comparative genomics analysis of the type 1 fimbrial operons of *K. quasipneumoniae* and *K. pneumoniae* and discovered that the *fimK* gene of KqPF9 has a premature stop codon that is predicted to result in a shortened or truncated protein missing the C-terminal phosphodiesterase domain that is present in *K. pneumoniae*. This key difference in *fimK* architecture between *K. quasipneumoniae* and *K. pneumoniae* has not been previously reported. Because *fimK* is known to regulate the *K. pneumoniae fim* operon and predicted to also co-regulate the *mrk* operon, we hypothesized that this difference may have important phenotypic effects. We discerned that this difference is conserved among all *K. quasipneumoniae* isolates and we demonstrated the role of the *K. pneumoniae* phosphodiesterase EIL domain of FimK in co-regulating type 3 fimbriae expression and urinary bladder epithelial cell attachment. We also specifically show, using a E245A mutant of *fimK,* that phosphodiesterase activity is required for type 3 fimbrial co-regulation. Further through complementation studies, we demonstrated that the lack of EIL domain in *K. quasipneumoniae fimK* leads to a loss of cross-regulation of type 3 fimbriae by FimK. Although it had been postulated in the field that *fimK* may act to co-regulate type 3 fimbriae through its phosphodiesterase domain, no study had previously demonstrated this function. These findings allow us to understand how *fimK,* a regulatory component of type 1 fimbriae, also may coordinate the expression of type 3 fimbriae thereby

establishing a means of co-regulation between the expression of type 1 and type 3 fimbriae in *K. pneumoniae* but not in *K. quasipneumoniae*. Considering that FimK and MrkJ, through their respective EIL and ECL phosphodiesterase domains, are able to modulate cyclic di-GMP levels, along with MrkH being dependent on cyclic di-GMP for activating type 3 fimbrial expression, suggests that the second messenger cyclic di-GMP plays an important role in fimbrial cross-regulation in *K. pneumoniae* (8, 16, 43). An interesting question that results from this work is why *K. quasipneumoniae* has evolved to not rely on FimK for co-regulation of type 1 and type 3 fimbriae but *K. pneumoniae* has. *K. pneumoniae* is thought to utilize specific fimbriae for host cell attachment in different niches – for example type 3 in the lung and type 1 in the bladder, which is why co-regulation of the two fimbrial types may be important (6, 44, 45). Does *K. quasipneumoniae* co-regulate type 1 and type 3 fimbriae through another mechanism or is co-regulation of these two fimbrial types not important for the lifestyle or environmental niche of *K. quasipneumoniae* is yet unanswered.

5.4 Future directions

Comparative genomics of the *K. quasipneumoniae* and *K. pneumoniae* whole genome sequences of strains isolated from patients with rUTI

In the first study of this dissertation work, comparative genomics between the *K. quasipneumoniae* and *K. pneumoniae* in the context of type 1 fimbriae was performed. It would be interesting to perform a whole-genome comparative genomics analysis on a curated set of *K. pneumoniae* and *K. quasipneumoniae* complete genomes to identify

gene enrichments, insertions and deletions (INDELs), or single nucleotide polymorphisms (SNPs) that are characteristic to each species. Such a comparison would provide information on the basic genetic composition, evolution, plasmid content, antibiotic resistance, virulence factors and any potential metabolic differences between the strains of individual species or between different species. In the context of virulence factors, considering the role of iron in bacterial growth and replication, differences in iron scavenging system like siderophores could potentially affect the pathogenicity of these strains. Previous reports suggest that *K. quasipneumoniae* has lesser number of virulence genes associated with iron acquisition like those encoding for aerobactin (*iutA*), yersiniabactin (*ybtS*) and enterobactin (*entB*) (46-49). Also, differences in presence of genes involved in iron uptake system like *Kfu* have been reported between *K. pneumoniae*, *K. variicola* and *K. quasipneumoniae* (49). As it is still unclear if this difference in iron scavenging system gene enrichment affects the pathogenicity of *K. quasipneumoniae*, especially in the context of urinary tract infection, a study comparing their whole genomes and phenotypically evaluating their ability to colonize and infect a host (e.g. in a mouse bladder infection model) could offer better understanding of the importance of these virulence factors and would provide information on any differences in iron scavenging system that could potentially contribute towards alterations in the mechanisms of pathogenesis between the two species.

Phenotypic analysis of *K. quasipneumoniae* rUTI isolates KqPF26 and KqPF42

This work evaluated *in vitro* the role of type 1 and type 3 fimbriae of one specific *K. quasipneumoniae* isolate (KqPF9) in bladder epithelial cell attachment and invasion.

Though the nucleotide sequences of the *fim* and *mrk* operons encoding for respective fimbriae show a high degree of similarity between KqPF9 and other *K. quasipneumoniae* isolates investigated in the lab (KqPF26 and KqPF42), it would be interesting to study potential virulence mechanisms of these urinary *K. quasipneumoniae* strains through bladder epithelial cell association, cell invasion and biofilm formation assays. Comparison of these results with cell association and biofilm formation of KqPF9 strain and TOP52 *K. pneumoniae* strain would allow us to better relate these phenotypes and state with more conviction the colonizing ability of *K. quasipneumoniae* strains in comparison to *K. pneumoniae*. After evaluating *in vitro,* the urinary bladder epithelial cell association and biofilm formation of wild-type KqPF26 and KqPF42 strains, it would be interesting to generate isogenic type 1 and type 3 fimbriae mutants of KqPF26 and KqPF42 to evaluate the role of type 1 and type 3 fimbriae in urinary bladder epithelial cell association *in vitro*.

Investigate regulation of the *fimS* switch in different environmental conditions

Considering that type 1 fimbriae contribute towards cell association and biofilm formation (6, 42), it would be interesting to evaluate the phase transition of *fimS* promoter region in additional experimental conditions and in additional *K. quasipneumoniae* isolates. The strains KqPF9 and TOP52 used in the present study showed a mixed population of phase “off” and phase “on” colonies in static overnight cultures in corroboration with previous results (Fig S5) (5, 50). Considering that the urinary bladder environment is microaerophilic alongside *Klebsiella* species being facultative anaerobes (51, 52) it would be of importance to evaluate the phase transition in different conditions like during growth in anaerobic or microaerophilic environments, as well as during growth in media types

that better mimic the host environment like artificial urine media. It would also be important to evaluate the behavior of the *fim* switch in multiple *K. quasipneumoniae* and *K. pneumoniae* isolates to conclude that observed phenotypes are species-specific. These experiments would provide information on the phase regulation of type 1 fimbriae and the phenotypic behavior of these strains in colonizing host epithelial cells in different environments.

Determine the role of type 1 and type 3 fimbriae of *K. quasipneumoniae* in colonization of the mouse bladder

Considering that *K. quasipneumoniae* shows a significantly higher type 1 and type 3 fimbriae expression along with significantly higher bladder epithelial cell association and biofilm formation relative to TOP52, it would be interesting to evaluate *in vivo* the bladder colonizing abilities of KqPF9 versus TOP52 in a mouse model of bladder infection. If KqPF9 indeed is able to more effectively attach and invade bladder epithelial cells *in vivo,* we would expect that mice infected with KqPF9 would have higher bladder bacterial loads than those infected with *K. pneumoniae* TOP52. Because we also hypothesize based on our *in vitro* findings, that KqPF9 relies more on type 3 than type 1 fimbriae for bladder epithelial cell association, type 1 and type 3 fimbriae mutants of *K. quasipneumoniae* should be evaluated in a mouse bladder infection model. If type 3 fimbriae contribute to bladder colonization, then we would expect to observe lesser bacterial loads in the bladders of mice infected with isogenic mutants lacking *mrkA,* the major subunit of type 3 fimbriae compared to wild-type KqPF9. These experiments would allow us to clearly

define the contribution of type 1 versus type 3 fimbriae to bladder colonization and UTI progression *in vivo.*

In vivo evaluation of the role of *fimK* in *K. quasipneumoniae*

Previous studies have reported that isogenic *fimK* mutants of uropathogenic *K. pneumoniae* TOP52 strain showed significant increase in type 1 fimbriae expression and bladder epithelial colonization in vivo (50). In the present study we observed that KqPF9Δ*fimK* did not show any significant change in bladder cell association in vitro. In this context, it would be interesting to study in vivo the role of *fimK* of *K. quasipneumoniae* in bladder epithelial cell association.

Identify the regulatory role of C-terminal phosphodiesterase domain of FimK by evaluating differentially expressed genes through RNA seq

Considering the differences in *mrkH* and *mrkA* expression upon complementation with $pfimK_{Kp}$ in both TOP52Δ*fimK* or KqPF9Δ*fimK* strains that we observed in this work, it would be interesting and informative to evaluate genes that are upregulated and downregulated in these mutants and complemented strains by RNA-sequencing analysis. Considering that *mrkH* expression plays a crucial role in activation of type 3 fimbriae and knowing that overexpression of $pfimK_{Kp}$ relative to $pfimK_{Kq}$ causes a significant decrease in expression of *mrkH* and *mrkA*, investigation of differential expression of genes between the respective genotypes would provide information on genes and regulatory networks that are influenced by the C-terminal phosphodiesterase domain of FimK thereby allowing us to understand regulatory influences of phosphodiesterase activity of FimK in type 3

fimbriae regulation. Though RNA sequencing analysis of KqPF9, KqPF9Δ*fimK* and KqPF9Δ*fimK*+*pfimK*$_{Kq}$ did not reveal any significant difference in genes involved in regulation of type 3 fimbriae (Appendix table A.1, A.2), the results still corroborate with the phenotypic results indicating no significant difference in type 3 fimbriae expression and cell association phenotype among these genotypes. This could at least in part be attributed to the lack of C-terminal domain of FimK in *K. quasipneumoniae*. It is therefore probably more important to compare changes in gene expression between KqPF9Δ*fimK* and TOP52Δ*fimK* strains.

5.5 Concluding remarks of the study

K. quasipneumoniae is often indicated as the cause of many opportunistic infections including urinary tract infection (UTI), which affect >50% of women worldwide. However, the mechanism as to how *K. quasipneumoniae* colonizes the urinary bladder is unknown. This work evaluates the contribution of type 1 and type 3 fimbriae, which are critical colonization factors encoded by all *Klebsiella* species, to bladder epithelial cell attachment in *K. quasipneumoniae.* We show that *K. quasipneumoniae's* cell attachment is dependent on type 3 but not type 1 fimbriae*.* Importantly, we find that the *K. quasipneumoniae* fimbrial regulatory gene *fimK* lacks a C-terminal phosphodiesterase domain that is present in *K. pneumoniae*. We show that this domain down-regulates type 3 fimbriae when expressed in both *K. pneumoniae* and *K. quasipneumoniae,* but that *K. quasipneumoniae fimK,* which lacks this domain, does not affect type 3 fimbriae expression.

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APPENDIX

SUPPLEMENTARY FIGURES

K. pneumoniae strain PF25 follows a mannose insensitive pattern of urinary bladder epithelial cell association and invasion

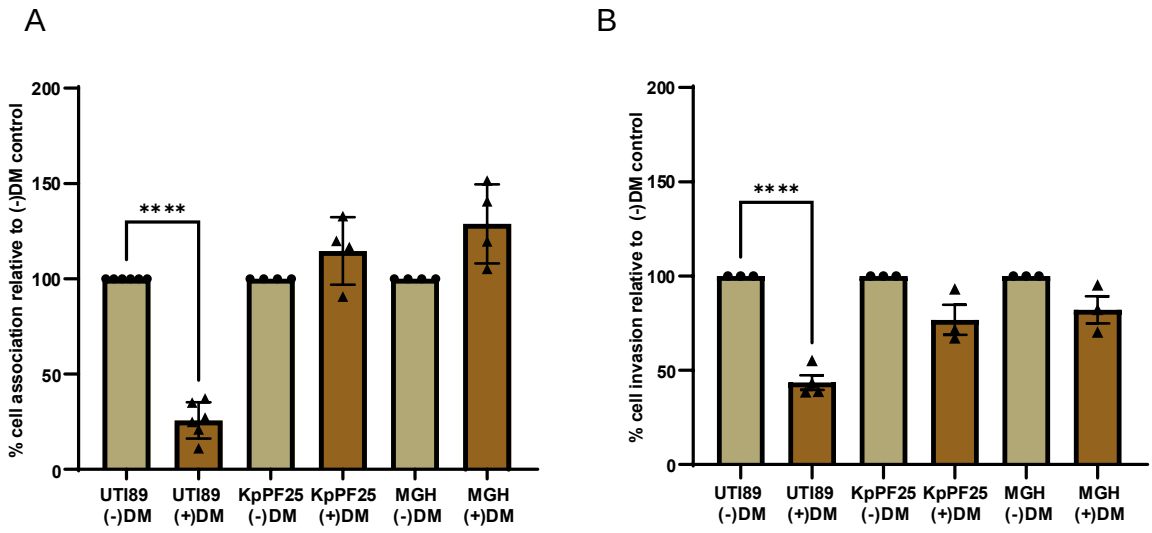

Figure A.1. *K. pneumoniae* uropathogenic isolate PF25 shows a mannose insensitive pattern of association and invasion to urinary bladder epithelial cells analogous to type strain MGH. A, B. Cell association and invasion assay demonstrating the mannose insensitive association and invasion of KpPF25 isolate to bladder epithelial cells, respectively. UPEC UTI89 was used as negative control and *K. pneumoniae* type strain MGH78578 was used as a positive control. For each strain, the cell association of PBS control was evaluated as 100% and the influence of mannose was determined relative to respective PBS control. The experiments were performed as biological triplicates and at least three technical replicates for each biological sample. The error bars indicate standard error of mean. A two tailed, paired Students T test was performed to evaluate the significance of effect of mannose on cell association and invasion. A p- value less than 0.05 was termed significant.

Generation of isogenic mutants of type 1 and type 3 fimbriae of KpPF25 strain

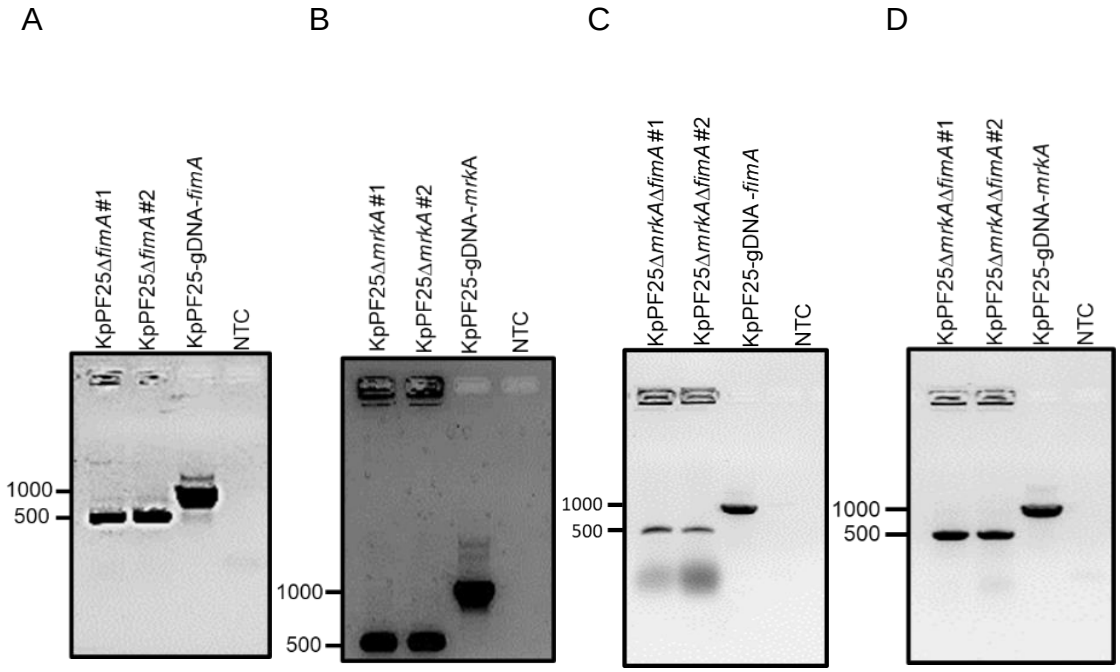

Figure A.2. Generation of isogenic type 1 and type 3 fimbriae mutants of KpPF25 uropathogenic strain. A, B, C, D. Images of PCR products of type 1 (KpPF25Δ*fimA*), type 3 (KpPF25Δ*mrkA*) or both type 1 and type 3 fimbriae (KpPF25Δ*mrkA*Δ*fimA*) isogenic mutants of KpPF25 resolved in a 1% agarose gel is shown. For the images discussed, genomic DNA of wild-type KpPF25 strain was used as positive control to verify the PCR product size. Also, a no template negative control (NTC) was used to verify the purity of reagents in PCR mix. The difference in PCR product sizes between the respective mutant and wild-type strain indicates the deletion of the gene of interest as amplified by respective verification primers.

Generation of isogenic mutants of *fim* and *mrk* gene clusters in KqPF9 and KpPF25 strains

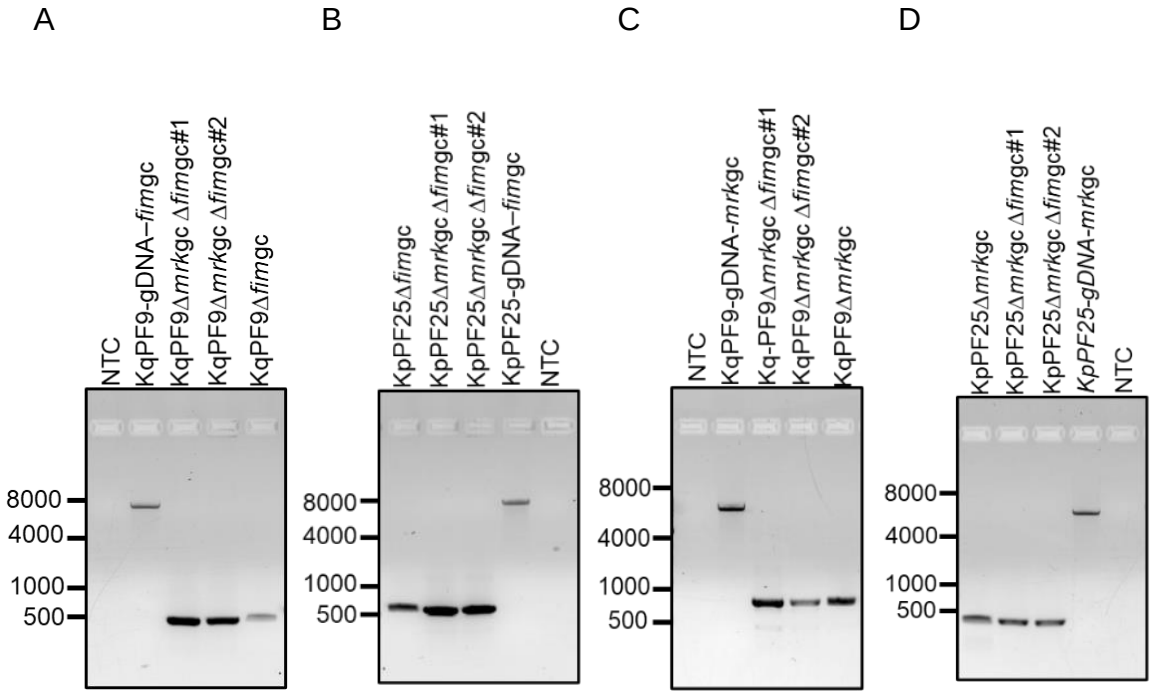

Figure A.3. Generation of respective isogenic type 1 and type 3 fimbriae mutants of KqPF9 and KpPF25 uropathogenic strains. A, B, C, D. Images of PCR products of type 1, type 1 and type 3 fimbrial gene clusters of KqPF9 and KpPF25 as resolved in a 1% agarose gel is shown. Genomic DNA of respective wild-type strain KqPF9 or KpPF25 were used as positive control to verify the PCR product size. Also, a no template negative control (NTC) was used to verify the purity of reagents in PCR mix. The difference in PCR product sizes between the respective mutant and wild-type strain indicates the deletion of the genes of interest as amplified by respective verification primers.

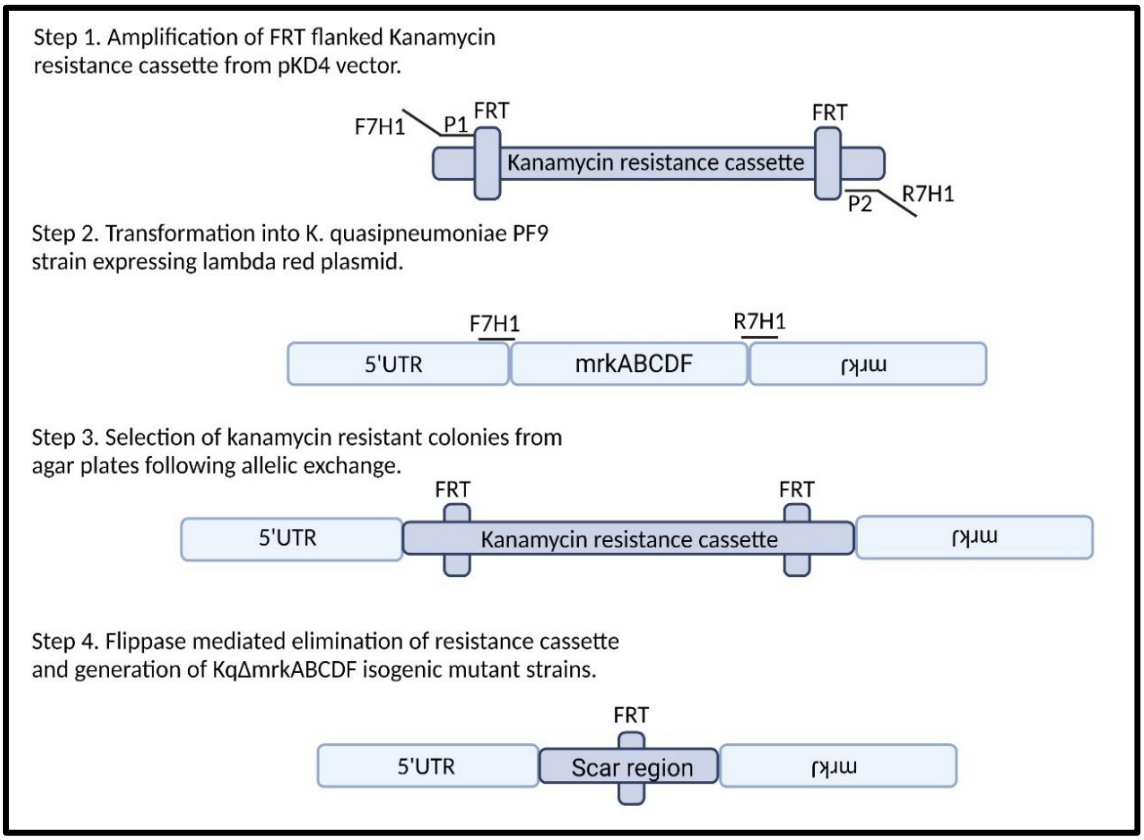

Figure A.4. Generation of KqPF9Δ*mrkABCDF* and KpPF25Δ*mrkABCDF* isogenic mutant strains. The respective *mrk* gene cluster deletions of KqPF9 and KpPF25 were generated by lambda red recombinase system. The individual knockout cassettes of KqPF9 and KpPF25 encompassing primer parts that are homologous to the 5' UTR of *mrkA* subunit and 3' region of *mrkJ* regulator (*mrkABCDF* gene cluster and *mrkJ* are transcribed in opposite directions) were named as F7H1 and R7H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flilppase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 and KpPF25 electrocompetent cells. The final isogenic type 3 fimbriae mutant clones of KqPF9Δ*mrkABCDF* and KpPF25Δ*mrkABCDF* had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

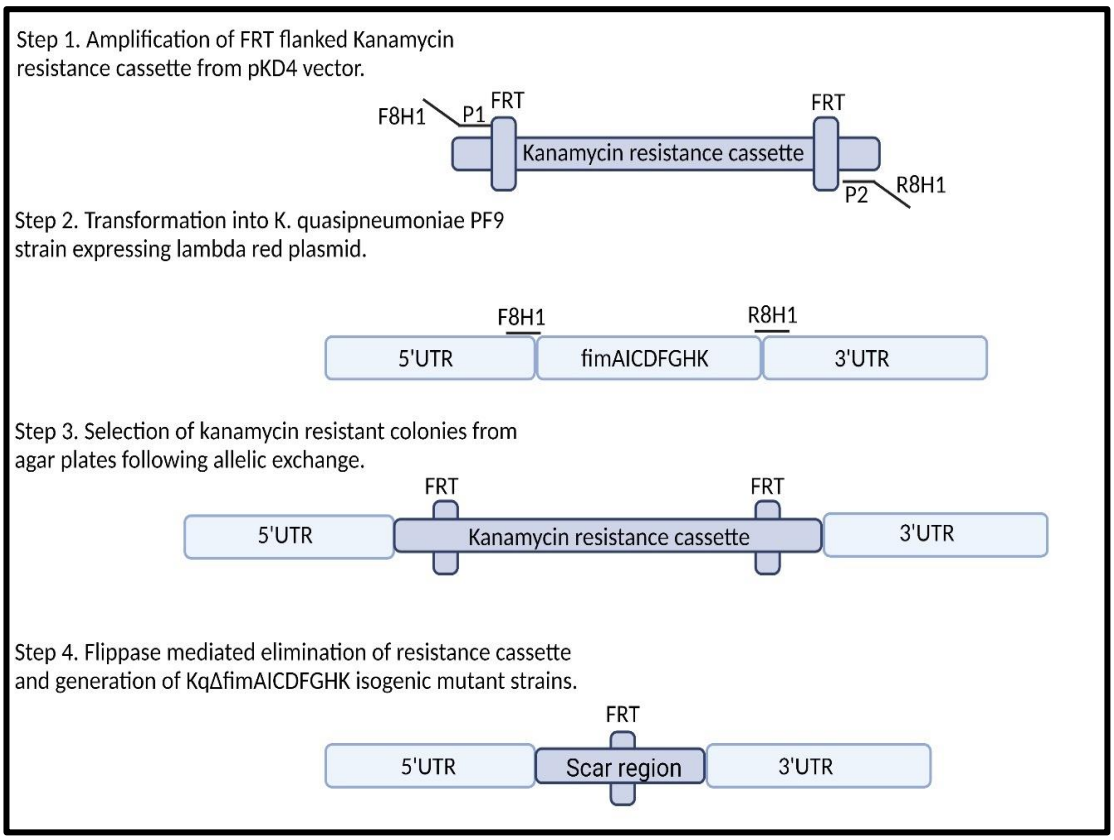

Figure A.5. Generation of KqPF9Δ*fimAICDFGHK* and KpPF25Δ*fimAICDFGHK* isogenic mutant strains. The respective *fim* gene cluster deletions of KqPF9 and KpPF25 were generated by lambda red recombinase system. The individual knockout cassettes of KqPF9 and KpPF25 encompassing primer parts that are homologous to the 5' UTR and 3'UTR were named as F8H1 and R8H1 respectively. The primers P1 and P2 represent the primer sequences with sequence homology to plasmid *pKD4*. The knockout cassette encompassing both parts enable amplification of the kanamycin cassette with the flanking Flippase Recognition Target (FRT) sites by PCR for transformation into lambda red plasmid (*pACBSR*) containing KqPF9 and KpPF25 electrocompetent cells. The final isogenic type 1 fimbriae mutant clones of KqPF9Δ*mrkABCDF* and KpPF25Δ*mrkABCDF* had a scar region (110 bp) containing a portion of kanamycin cassette and FRT site. This figure was made using biorender.

Verification of *mrk* gene cluster deletion and expression of respective complements in KqPF9 and KpPF25 strains

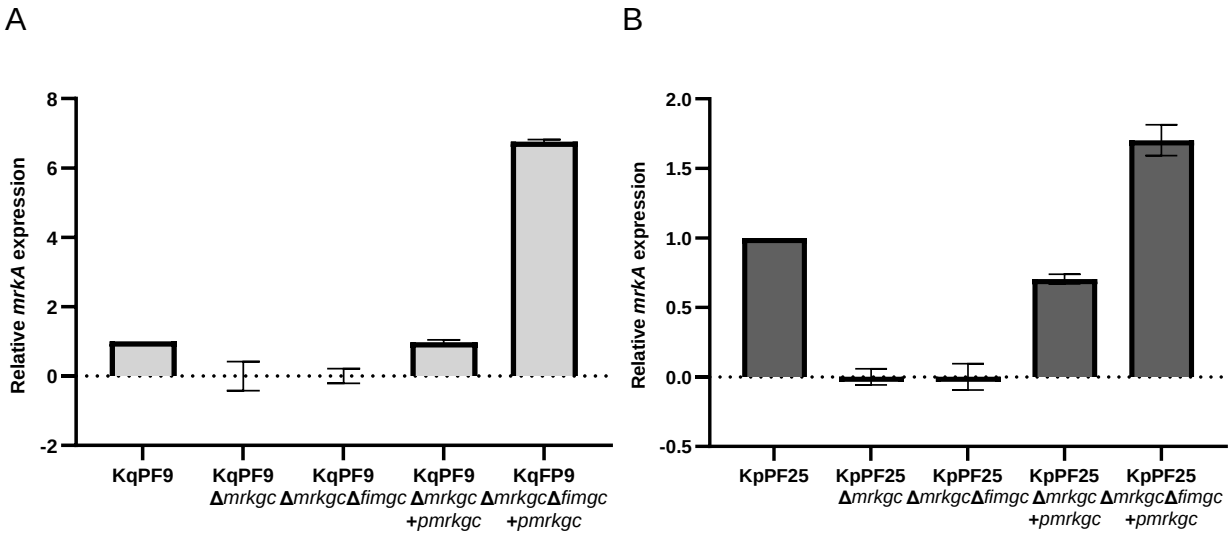

Figure A.6. Quantitation of *mrkA* expression in respective genotypes of KqPF9 and KpPF25 strains by qRT-PCR. A, B. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of type 3 fimbriae (*mrkA*) transcription in respective wild-type and isogenic mutant strains carrying *pBAD* empty vector or *pBAD* vector expressing *mrk* gene cluster (*mrkABCDF*) in KqPF9 and KpPF25 strains is shown. The error bars indicate standard error of mean.

Verification of *fim* gene cluster deletion and expression of respective complements in KqPF9 and KpPF25 strains

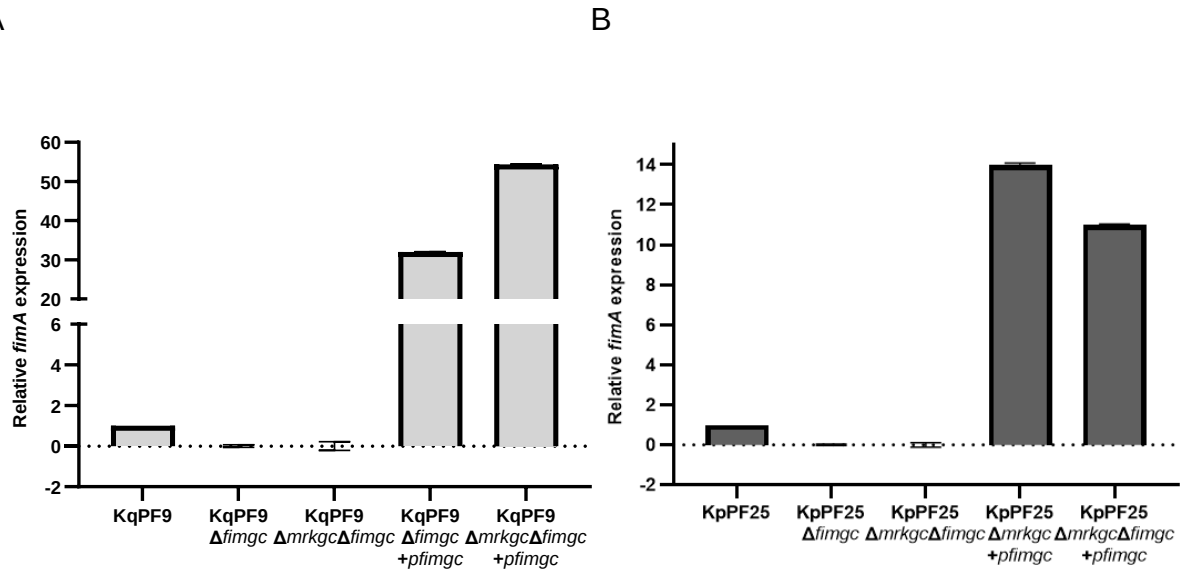

Figure A.7. Quantitation of *fimA* expression in respective genotypes of KqPF9 and KpPF25 strains by qRT-PCR. A, B. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of type 1 fimbriae (*fimA*) transcription in respective wild-type and isogenic mutant strains carrying *pBAD* empty vector or *pBAD* vector expressing *fim* gene cluster (*fimAICDFGHK*) in KqPF9 and KpPF25 strains is shown. The error bars indicate standard error of mean.

Role of type 3 fimbriae in bladder epithelial cell association of KpPF25 strain

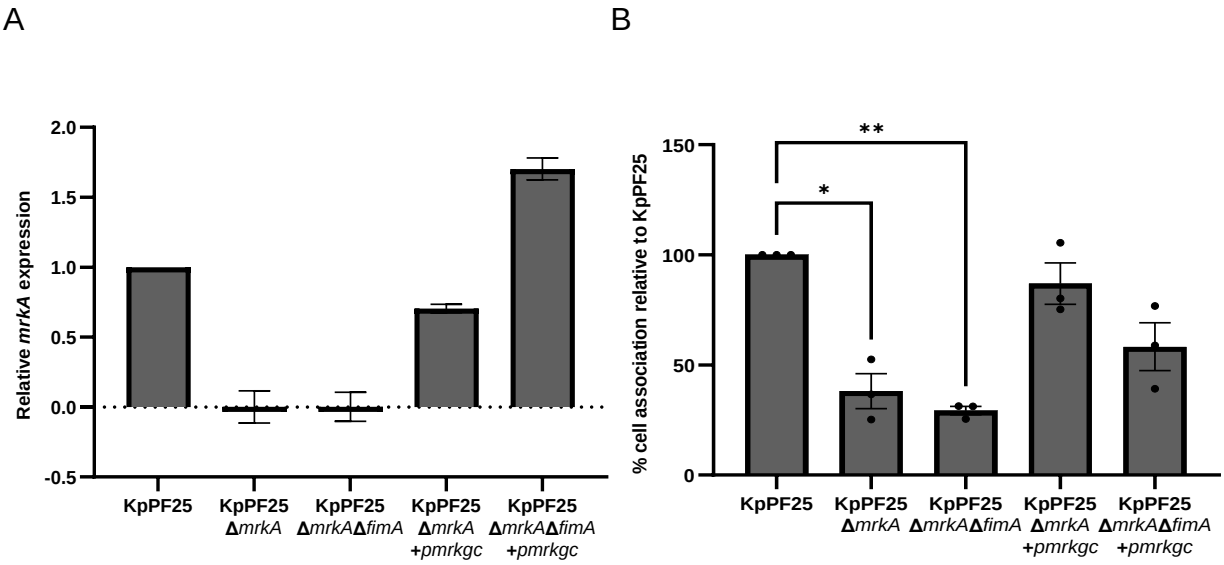

Figure A.8. The uropathogenic KpPF25 strain's urinary bladder cell association is dependent on type 3 fimbriae. A. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of type 3 fimbriae (*mrkA*) transcription in respective wild-type and isogenic fimbriae mutant strains carrying *pBAD* empty vector or *pBAD* vector expressing *mrk* gene cluster (*mrkABCDF*) in KpPF25 strain is shown. The error bars indicate standard error of mean. B. Urinary bladder cell association assay of KpPF25 strain, its isogenic fimbriae mutants along with respective mutants expressing complementing plasmids containing *mrk* gene cluster was performed in-vitro in urinary bladder derived 5637 cell-line. The urinary bladder epithelial cell association for KpPF25 strain was evaluated as 100% and the cell association of respective strains were evaluated relative to wild-type KpPF25 strain. The experiments were performed as biological triplicates and at least three technical replicates for each biological sample. The error bars indicate standard error of mean. Significant p-values were evaluated using one way ANOVA with Dunnett's multiple comparison analysis. A p- value less than 0.05 was termed significant.

Table A.1. Differential expression of genes following RNA sequencing between KqPF9 and KqPF9Δ*fimK* genotypes.

The genes were filtered for an absolute value of the fold change > 1.5 with a false discovery rate of 0.05 or less. The experiment was performed using triplicate samples for each genotype and an average of the triplicate value was used for analysis. Bacterial culture and RNA extraction was performed as mentioned earlier. RNA-sequencing was performed by Microbial genome sequencing (MIGS) and analysis of data was performed using CLC genomics workbench.

Name	**Max group mean**	**Fold change**	**FDR p-value**
Fimbrial adhesin	28.85	-2289.63	2.41E-03
Outer membrane porin KPN_04057/KPN_04724_2	69.07	-34.04	0
UPF0401 protein YkfF	256.37	-14.51	0
Single-stranded DNA-binding protein_1	81.65	-11.25	0
Outer membrane porin KPN_04057/KPN_04724_1	22.28	-11.1	0
COG1683: Uncharacterized conserved protein / FIG143828: Hypothetical protein YbgA	28.6	-10.44	0
Type-1 fimbrial protein, A chain	758.33	-9.74	0
Fimbrin-like protein FimI	329.25	-9.48	0
Chaperone protein FimC	162.88	-7.61	0
FIG002577: Putative lipoprotein precursor	12.29	-6.47	0
Outer membrane usher protein FimD	40.43	-6.18	0
Mobile element protein_15	141.53	-4.98	0
Antirestriction protein ArdA	4.33	-4.51	3.47E-06
Protein FimF (regulates length and adhesion of type 1 fimbriae)	14.32	-3.58	0
hypothetical protein_539	6.15	-3.56	0.05
Protein FimG (regulates length and adhesion of type 1 fimbriae)	24.02	-2.85	0
hypothetical protein_37	9.07	-2.76	7.20E-03
alpha-galactosidase (EC 3.2.1.22)_1	12.62	-2.76	0
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)_1	11.56	-2.69	0.01
Mobile element protein_13	1168.8	-2.58	0
hypothetical protein_139	12.7	-2.4	7.71E-03
Protein FimH (regulates length and adhesion of type 1 fimbriae, and mediates mannose binding)	26.49	-2.39	5.70E-14
Putative antirestriction protein	8.41	-2.36	4.90E-05
hypothetical protein_137	33.15	-2.34	1.60E-09
hypothetical protein_38	195.82	-2.26	0
putative plasmid stabilization protein	16.13	-2.24	0
Mobile element protein_12	108.36	-2.22	0
Mobile element protein_9	89.13	-2.16	0

Table A.1, continued

Mobile element protein_11	24.02	-2.03	0.02
Adenine-specific DNA methyltransferase	17.29	-1.89	2.50E-07
hypothetical protein_560	10.1	-1.87	1.15E-03
hypothetical protein_135	15.25	-1.86	2.84E-05
Retron-type RNA-directed DNA polymerase (EC 2.7.7.49)_2	439.55	-1.75	2.58E-13
Phage recombination protein Bet	29.61	-1.67	2.08E-04
Transcriptional regulator, GntR family_2	57.32	-1.67	0.02
Biofilm PGA outer membrane secretin PgaA	4.11	-1.67	1.33E-04
Phage Orf80 protein	19.6	-1.65	0.03
Phage DNA replication protein O_1	20.5	-1.54	2.33E-03
Methyltransferase type 11_2	27.58	-1.52	5.07E-03
hypothetical protein_461	129.76	-1.52	6.66E-03
hypothetical protein_182	104.76	-1.51	2.98E-06
Uncharacterized UPF0721 integral membrane protein	25.3	-1.51	0.01
Mhp operon transcriptional activator	19.49	-1.5	0.03
hypothetical protein_252	14.34	1.5	0.05
hypothetical protein_551	14.52	1.52	0.04
hypothetical protein_50	44.43	1.52	0.05
PduJ-like protein clustered with choline trimethylamine-lyase_1	44.99	1.53	1.60E-03
hypothetical protein_498	18.98	1.53	0.02
hypothetical protein_250	31.47	1.54	0.04
Zinc ABC transporter, permease protein ZnuB_1	13.7	1.54	0.01
Transcriptional regulator YbhD, LysR family	26.56	1.56	1.31E-05
Threonine dehydratase, catabolic (EC 4.3.1.19) @ L-serine dehydratase, (PLP)-dependent (EC 4.3.1.17)	306.68	1.57	2.77E-04
Stresses-induced protein Ves (HutD)	39.57	1.58	1.71E-05
hypothetical protein_291	72.9	1.59	3.20E-03
[NiFe] hydrogenase nickel incorporation protein HypA	56.2	1.6	1.71E-05
Metal-binding protein ZinT	117.28	1.6	4.30E-08
hypothetical protein_452	193.32	1.63	1.41E-06
Zinc ABC transporter, substrate-binding protein ZnuA_1	84.09	1.63	7.72E-09
hypothetical protein_284	5.32	1.64	1.47E-03
Uncharacterized MFS-type transporter_8	79.6	1.66	6.26E-10
Threonine catabolic operon transcriptional activator TdcA	204.56	1.69	3.83E-11
LSU ribosomal protein L31p @ LSU ribosomal protein L31p, zinc-independent	80.33	1.69	2.71E-07
hypothetical protein_353	73.44	1.71	3.41E-04
Manganese ABC transporter, ATP-binding protein SitB_2	22.65	1.75	2.74E-04

Table A.1, continued

hypothetical protein_546	18.05	1.75	0.02
Histidine ABC transporter, permease protein HisQ (TC 3.A.1.3.1)_2	32	1.79	3.21E-09
Methyltransferase type 11_3	12.73	1.79	4.54E-03
Chromate transport protein ChrA	11.57	1.88	2.65E-05
Histidine utilization repressor	313.21	1.88	0
LSU ribosomal protein L36p @ LSU ribosomal protein L36p, zinc-independent	47.76	2	1.10E-05
Transcriptional regulator, AraC family_4	7.66	2.04	1.62E-03
Formiminoglutamase (EC 3.5.3.8)	255.22	2.05	0
Histidine ABC transporter, substrate-binding protein HisJ (TC 3.A.1.3.1)_2	87.04	2.06	0
Imidazolonepropionase (EC 3.5.2.7)	364.92	2.08	0
Phage tail completion protein GpS_1	2.86	2.1	0.04
Histidine ammonia-lyase (EC 4.3.1.3)_1	46.27	2.14	0
Histidine ABC transporter, ATP-binding protein HisP (TC 3.A.1.3.1)_2	71.22	2.16	0
ABC transporter, permease protein 1 (cluster 5, nickel/peptides/opines)_4	1.17	2.19	0.04
Urocanate hydratase (EC 4.2.1.49)	1236.78	2.23	0
Histidine ammonia-lyase (EC 4.3.1.3)_2	970.14	2.24	0
Histidine ABC transporter, permease protein HisM (TC 3.A.1.3.1)_2	29.64	2.28	0
Histidine transport protein (permease)	278.13	2.32	0
Cytochrome c heme lyase subunit CcmL	2.13	2.71	0.02
Xyloside transporter XynT_1	1.93	2.98	3.33E-06
hypothetical protein_245	6.56	3.75	1.46E-03
hypothetical protein_208	6.18	3.84	9.76E-04

Table A.2. Differential expression of genes following RNA sequencicng between KqPF9 and KqPF9Δ*fimK*+*pfimK*$_{Kq}$ genotypes.

The genes were filtered for an absolute value of the fold change > 1.5 with a false discovery rate of 0.05 or less. The experiment was performed using triplicate samples for each genotype and an average of the triplicate value was used for analysis. Bacterial culture and RNA extraction was performed as mentioned earlier. RNA-sequencing was performed by Microbial genome sequencing (MIGS) and analysis of data was performed using CLC genomics workbench.

Name	**Max group mean**	**Fold change**	**FDR p-value**
Outer membrane porin KPN_04057/KPN_04724_2	69.07	-27.99	0
UPF0401 protein YkfF	256.37	-13.05	0
Fimbrin-like protein FimI	329.25	-10.14	0
Chaperone protein FimC	162.88	-9.7	0
Type-1 fimbrial protein, A chain	758.33	-9.64	0
Single-stranded DNA-binding protein_1	81.65	-9.18	0
Outer membrane porin KPN_04057/KPN_04724_1	22.28	-8.94	0
COG1683: Uncharacterized conserved protein / FIG143828: Hypothetical protein YbgA	28.6	-8.6	0
Outer membrane usher protein FimD	40.43	-7.69	0
FIG002577: Putative lipoprotein precursor	12.29	-4.8	0
Mobile element protein_15	141.53	-4.66	0
Protein FimF (regulates length and adhesion of type 1 fimbriae)	14.32	-4.51	0
hypothetical protein_260	7.97	-2.92	0.03
Protein FimG (regulates length and adhesion of type 1 fimbriae)	24.02	-2.75	0
Phage Orf80 protein	19.6	-2.67	7.49E-07
Protein FimH (regulates length and adhesion of type 1 fimbriae, and mediates mannose binding)	26.49	-2.67	0
Antirestriction protein ArdA	4.33	-2.62	4.33E-04
alpha-galactosidase (EC 3.2.1.22)_1	12.62	-2.55	0
Mobile element protein_13	1168.8	-2.54	0
putative plasmid stabilization protein	16.13	-2.49	0
hypothetical protein_14	6.4	-2.47	0.04
Phage activator protein cII_1	40.81	-2.46	0
hypothetical protein_37	9.07	-2.44	0.01
Mobile element protein_12	108.36	-2.38	0
hypothetical protein_187	14.35	-2.23	4.72E-04
Phage replication protein GpB	11.7	-2.19	3.54E-04

Table A.2, continued

Mobile element protein_9	89.13	-2.18	0
hypothetical protein_38	195.82	-2.17	0
hypothetical protein_66	64.35	-2.17	1.03E-09
FIL protein	8.13	-2.15	0.02
hypothetical protein_182	104.76	-2.13	0
Phage DNA adenine methylase (EC 2.1.1.72)	12.01	-2.1	5.05E-07
hypothetical protein_137	33.15	-2.07	1.54E-07
hypothetical protein_181	71.99	-2.02	2.16E-12
Biofilm PGA outer membrane secretin PgaA	4.11	-2	3.60E-08
hypothetical protein_146	6.63	-1.98	0.04
FIG00731545: hypothetical protein	6.78	-1.97	0.03
Phage DNA replication protein P	18.24	-1.93	4.24E-09
Transcriptional regulator, GntR family_2	57.32	-1.93	3.36E-04
hypothetical protein_135	15.25	-1.92	4.14E-06
Chaperone protein HtpG	475.52	-1.9	0
Phage TraR/YbiI family protein_1	14.1	-1.88	0.01
putative chromosome partitioning protein	48.94	-1.86	1.66E-11
16 kDa heat shock protein A	3443.62	-1.85	0
Phage lysozyme R (EC 3.2.1.17)_2	7.34	-1.85	7.75E-03
Putative antirestriction protein	8.41	-1.85	3.10E-03
Phage DNA replication protein O_2	41.41	-1.83	9.91E-12
hypothetical protein_560	10.1	-1.81	1.40E-03
hypothetical protein_183	56.52	-1.81	1.14E-06
hypothetical protein_143	10.51	-1.8	0.03
Phage recombination protein Bet	29.61	-1.8	4.63E-06
Adenine-specific DNA methyltransferase	17.29	-1.8	1.51E-06
16 kDa heat shock protein B	1478.24	-1.79	0
Retron-type RNA-directed DNA polymerase (EC 2.7.7.49)_2	439.55	-1.78	2.85E-14
Putative DNA methylase	23.05	-1.75	3.68E-06
Phage DNA replication protein O_1	20.5	-1.74	1.09E-05
Uncharacterized UPF0721 integral membrane protein	25.3	-1.71	1.50E-04
hypothetical protein_138	28.84	-1.71	0.03
hypothetical protein_184	17.43	-1.71	7.47E-07
hypothetical protein_461	129.76	-1.68	1.07E-04
Chaperone protein DnaK	1562.42	-1.67	1.54E-11
Chaperone protein ClpB (ATP-dependent unfoldase)	427.93	-1.66	3.82E-11
VirK_1	249.94	-1.62	2.10E-04

Table A.2, continued

ABC transporter, substrate-binding protein (cluster 10, nitrate/sulfonate/bicarbonate)_2	3.96	-1.62	0.03
hypothetical protein_173	23.92	-1.61	0.01
Small heat shock protein	377.56	-1.61	3.48E-09
Guanine deaminase (EC 3.5.4.3)_1	14.23	-1.6	3.24E-03
hypothetical protein_180	23.53	-1.59	0.01
Oligopeptide ABC transporter, substrate-binding protein OppA (TC 3.A.1.5.1)_2	3.89	-1.58	4.23E-03
lytic enzyme	8.47	-1.57	0.03
Mobile element protein_4	50.79	-1.56	5.41E-05
UPF0438 protein YifE	304.28	-1.55	6.96E-03
Heat shock protein 10 kDa family chaperone GroES	2596.76	-1.53	9.64E-08
FIG00732852: hypothetical protein	142.5	-1.53	1.82E-05
Putative prophage membrane protein_1	11.22	-1.51	0.04
major tail subunit	10.16	-1.51	7.83E-03
DNA-damage-inducible protein I_1	156.31	-1.5	4.72E-04
hypothetical protein_67	109.04	-1.5	5.44E-05
Autoinducer 2 (AI-2) ABC transporter, permease protein LsrD	60.74	1.51	3.91E-06
Acetoin dehydrogenase E1 component beta-subunit (EC 2.3.1.190)	148.91	1.51	1.52E-03
hypothetical protein_284	4.95	1.51	0.01
Sirohydrochlorin cobaltochelatase CbiK (EC 4.99.1.3) @ Sirohydrochlorin ferrochelatase activity of CbiK (EC 4.99.1.4)	18.47	1.51	3.50E-04
Zinc ABC transporter, permease protein ZnuB_1	13.45	1.52	0.01
Formate hydrogenlyase subunit 2	40.28	1.53	2.51E-05
Cobalt-precorrin-7 (C5)-methyltransferase (EC 2.1.1.289)	12.14	1.53	3.96E-03
hypothetical protein_570	127.53	1.54	2.96E-06
Oxidoreductase, short-chain dehydrogenase/reductase family_12	6.16	1.54	0.05
Autoinducer 2 (AI-2) ABC transporter, substrate-binding protein	104.49	1.55	2.35E-07
L-threonine transporter, anaerobically inducible	172.38	1.55	1.56E-03
Threonine catabolic operon transcriptional activator TdcA	194.26	1.55	7.45E-08
[NiFe] hydrogenase nickel incorporation-associated protein HypB	106.56	1.55	1.95E-07
Leader peptidase HopD	13.78	1.56	0.02
Manganese ABC transporter, ATP-binding protein SitB_2	20.03	1.56	6.37E-03

Table A.2, continued

Acetate kinase (EC 2.7.2.1) @ Propionate kinase (EC 2.7.2.15)	96.78	1.56	0.01
Metal-binding protein ZinT	116.06	1.57	1.48E-07
(4S)-4-hydroxy-5-phosphonooxypentane-2,3-dione isomerase (EC 5.3.1.32)	66.88	1.57	5.78E-03
Oxidoreductase, short-chain dehydrogenase/reductase family_7	8.85	1.57	0.03
Formate hydrogenlyase subunit 6	14.91	1.58	5.09E-03
ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17) @ ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17), ethanolamine utilization	8.5	1.58	5.14E-03
Dihydrolipoamide dehydrogenase of acetoin dehydrogenase (EC 1.8.1.4)	73.35	1.58	6.87E-04
Manganese ABC transporter, inner membrane permease protein SitC	24.09	1.59	3.04E-04
LSU ribosomal protein L31p @ LSU ribosomal protein L31p, zinc-independent	77.39	1.61	4.14E-06
Chromate resistance protein ChrB	22.66	1.62	1.72E-06
predicted branched-chain amino acid permease (azaleucine resistance)	159.2	1.62	2.67E-09
Putative inner membrane protein_7	159.34	1.63	5.55E-10
Ethanolamine utilization protein EutQ	11.24	1.65	0.01
Enterobactin esterase	6	1.66	1.21E-03
Pyruvate:H+ symporter BtsT	24.39	1.66	1.36E-07
Formate hydrogenlyase regulatory protein HycA	57.76	1.66	1.57E-07
Threonine dehydratase, catabolic (EC 4.3.1.19) @ L-serine dehydratase, (PLP)-dependent (EC 4.3.1.17)	325.6	1.66	1.08E-05
hypothetical protein_250	34.86	1.68	2.86E-03
hypothetical protein_291	78.18	1.68	2.17E-04
Formate hydrogenlyase subunit 7	18.86	1.7	2.72E-05
3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase (EC 2.3.1.245)	67.23	1.7	5.88E-10
RidA/YER057c/UK114 superfamily protein_2	135.99	1.7	5.54E-10
Methyltransferase type 11_3	12.32	1.71	8.21E-03
Cytochrome c-type biogenesis protein CcmE, heme chaperone	7.95	1.72	5.15E-03
Histidine ABC transporter, ATP-binding protein HisP (TC 3.A.1.3.1)_2	57.98	1.73	2.92E-10
MbtH-like NRPS chaperone => YbdZ	13.42	1.78	0.01
Histidine ABC transporter, permease protein HisQ (TC 3.A.1.3.1)_2	32.62	1.78	2.23E-09
Chromate transport protein ChrA	11.08	1.79	9.86E-05

Table A.2, continued

Hemin uptake protein HemP/HmuP	20.66	1.8	2.16E-04
Histidine utilization repressor	304.93	1.81	0
Formate hydrogenlyase subunit 3	23.29	1.83	5.61E-12
Formate hydrogenlyase subunit 5	23.56	1.83	6.63E-12
hypothetical protein_551	17.84	1.84	9.66E-05
Histidine ammonia-lyase (EC 4.3.1.3)_1	40.19	1.84	1.81E-13
Ferric enterobactin transport ATP-binding protein FepC (TC 3.A.1.14.2)	9.85	1.85	3.81E-04
LSU ribosomal protein L36p @ LSU ribosomal protein L36p, zinc-independent	44.86	1.85	1.28E-04
Stresses-induced protein Ves (HutD)	47.14	1.85	5.43E-11
Histidine ABC transporter, substrate-binding protein HisJ (TC 3.A.1.3.1)_2	80.02	1.87	2.85E-14
[NiFe] hydrogenase nickel incorporation protein HypA	67.99	1.91	1.57E-11
Transcriptional regulator, AraC family_4	7.32	1.93	3.74E-03
Ethanolamine ammonia-lyase light chain (EC 4.3.1.7)_1	17.36	1.94	7.43E-07
hypothetical protein_353	84.89	1.94	6.15E-07
Histidine ABC transporter, permease protein HisM (TC 3.A.1.3.1)_2	25.52	1.94	3.16E-11
Phosphonate ABC transporter permease protein PhnE2 (TC 3.A.1.9.1)	1.59	1.96	0.04
FIG002994: Putative transcriptional regulator	6.85	1.98	8.42E-04
Formate hydrogenlyase subunit 4	24.07	1.99	1.97E-09
Imidazolonepropionase (EC 3.5.2.7)	359.69	2.02	0
Phosphate acetyltransferase (EC 2.3.1.8), ethanolamine utilization-specific	4.81	2.03	2.88E-04
Ethanolamine utilization protein EutA	25.12	2.05	0
Ethanolamine permease_1	26.04	2.06	0
Ethanolamine ammonia-lyase heavy chain (EC 4.3.1.7)_1	32.57	2.06	1.90E-13
Ethanolamine utilization protein EutG	23.57	2.09	0
Formiminoglutamase (EC 3.5.3.8)	263.42	2.1	0
Urocanate hydratase (EC 4.2.1.49)	1181.04	2.11	0
FIG001571: Hypothetical protein	5.83	2.12	3.54E-04
Ethanolamine utilization polyhedral-body-like protein EutM_1	9.15	2.16	3.10E-04
Histidine ammonia-lyase (EC 4.3.1.3)_2	956.75	2.18	0
hypothetical protein_48	5.61	2.18	0.04
Histidine transport protein (permease)	281.58	2.32	0
hypothetical protein_220	11.21	2.4	5.41E-03

Table A.2, continued

Xyloside transporter XynT_1	1.6	2.43	4.67E-04
hypothetical protein_129	6.46	2.45	0.05
Ethanolamine utilization polyhedral-body-like protein EutN	31.29	2.52	2.26E-09
Acetaldehyde dehydrogenase (EC 1.2.1.10) @ Acetaldehyde dehydrogenase (EC 1.2.1.10), ethanolamine utilization cluster_1	23.32	2.71	0
Ethanolamine utilization protein EutJ	23.6	2.72	0
hypothetical protein_249	4.47	2.9	0.03
hypothetical protein_208	4.84	2.97	0.02
hypothetical protein_245	5.46	3.08	0.01
Cytochrome c-type biogenesis protein CcmD, interacts with CcmCE	3.01	3.29	0.01
hypothetical protein_306	1.68	3.99	0.05
hypothetical protein_351	2.84	7.76	0.04
Fimbrial adhesin	890.72	29.58	0

BIOGRAPHICAL SKETCH

Sundharamani Venkitapathi was born in Coimbatore, TamilNadu, India. Following graduation from high school, he pursued training in dental sciences and completed his bachelor's degree in dental surgery (2008) from Dr. MGR Medical University at Chennai, India. Following completion of the bachelor's program, he pursued Master of Science in Oral Biology (2016) at Boston University in Boston, Massachusetts. During this period, he was a part of many projects like evaluation of the role of EvC and EvC2 proteins in Hedgehog signaling and Ellis-van Creveld (EvC) syndrome, determination of the role of VwC protein in bone matrix mineralization, to name a few. Having associated himself with basic science research, he then wanted to understand how basic science knowledge could be applied to unearth information from genome sequences and how this information could be applied for biological sciences. To get acquainted with this branch of biology, he pursued Master of Science in Bioinformatics at Northeastern University in Boston, Massachusetts (2018). During this program he had an opportunity to be a part of Dr. Anand Asthagiri's lab to complete internship associated with the program. With guidance from Dr. Anand to fulfill his aspiration to obtain a PhD in biological sciences, he was accepted to the doctoral program in Molecular and Cell Biology at The University of Texas at Dallas where he was a member of Dr. DeNisco's lab while completing his dissertation on the role of *fimK* in urinary bladder epithelial cell association of *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae*.

CURRICULUM VITAE

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Educational Background	**Year**
PhD candidate in the Dept of Molecular and Cell Biology, School of Natural Sciences and Mathematics, The University of Texas at Dallas, PI. Dr. Nicole DeNisco	2018-present
Master of Science in Bioinformatics at Northeastern University	2019
Master of Science in Dentistry in Oral Biology division at Boston University (Boston, MA, USA)	2016
Bachelor of Dental Surgery at Dr. MGR medical university (Chennai, INDIA)	2008

Publications

1. Role of *fimK* in urinary bladder cell association of *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae*. Sundharamani Venkitapathi, Yalini Hansika Wijesundara, Jeremiah J. Gassensmith, Philippe E. Zimmern, Nicole J. De Nisco – manuscript in preparation.
2. Metal–Organic Framework Encapsulated Whole-Cell Vaccines Enhance Humoral Immunity against Bacterial Infection. Michael A. Luzuriaga, Fabian C. Herbert, Olivia R. Brohlin, Jashkaran Gadhvi, Thomas Howlett, Arezoo Shahrivarkevishahi, Yalini H. Wijesundara, Sundharamani Venkitapathi, Kavya Veera, Ryanne Ehrman, Candace E. Benjamin, Sarah Popal, Michael D. Burton,

Molly A. Ingersoll, Nicole J. De Nisco* and Jeremiah J. Gassensmith*. https://doi.org/10.1021/acsnano.1c03092.

3. Complete Genome Sequences of Three Uropathogenic Klebsiella quasipneumoniae Strains Isolated from Postmenopausal Women with Recurrent Urinary Tract Infection. Sundharamani Venkitapathi*, Belle M. Sharon*, Tahira A. Ratna, Amanda P. Arute, Philippe Zimmern, Nicole DeNisco. Microbiol Resour Announc. 2021 Mar 18;10(11). pii: 10/11/e00073-21. doi: 10.1128/MRA.00073-21. (* denotes equal contribution).
4. FAM20A binds to and regulates FAM20C localization. Ohyama Y, Lin JH, Govitvattana N, Lin IP, Venkitapathi S, Alamoudi A, Husein D, An C, Hotta H, Kaku M, Mochida Y. Sci Rep. 2016 Jun 13;6:27784. doi: 10.1038/srep27784, PMID: 27292199.
5. Expression of Evc2 in craniofacial tissues and craniofacial bone defects in Evc2 knockout mouse. Badri MK, Zhang H, Ohyama Y, Venkitapathi S, Alamoudi A, Kamiya N, Takeda H, Ray M, Scott G, Tsuji T, Kunieda T, Mishina Y, Mochida Y.Arch Oral Biol. 2016 Aug;68:142-52. doi: 10.1016/j.archoralbio.2016.05.002, PMID: 27164562.
6. Ellis Van Creveld2 is Required for Postnatal Craniofacial Bone Development. Badri MK, Zhang H, Ohyama Y, Venkitapathi S, Kamiya N, Takeda H, Ray M, Scott G, Tsuji T, Kunieda T, Mishina Y, Mochida Y. Anat Rec (Hoboken). 2016 Aug;299(8):1110-20. doi: 10.1002/ar.23353, PMID: 27090777.

Posters Presented

1. Role of type 3 fimbriae of *Klebsiella pneumoniae* in mediating attachment and invasion to host bladder epithelial cells.
   Sundharamani Venkitapathi, Belle M. Sharon, Tahira A. Ratna, Philippe Zimmern and Nicole DeNisco. American Society for Microbiolgy – 2021.

2. Role of type 3 fimbriae of *Klebsiella pneumoniae* in mediating attachment and invasion to host bladder epithelial cells.
   Sundharamani Venkitapathi, Belle M. Sharon, Tahira A. Ratna, Philippe Zimmern and Nicole DeNisco. Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction, 2020.

3. Ciliary colocalization and Interaction of EVC and EVC2 proteins. IADR #176254, 2013.

Posters co-presented

1. The role of EVC2 in Craniofacial Development. IADR #184007 in 2014.
2. Neural Crest-Specific Knockout of EVC2 Reduces Craniofacial Morphometrics IADR #184935, 2014.

Honors and awards

1. Best poster award at Society of Urodynamics, Female Pelvic Medicine and Urogenital Reconstruction, 2020 for the poster titled: Role of type 3 fimbriae of *Klebsiella pneumoniae* in mediating attachment and invasion to host bladder epithelial cells.
2. Henry I Russek's award (III place) – Boston university 2013.
3. PIERRE FAUCHARD ACADEMY Certificate of merit for academic excellence (2007).

Teaching experience

Fall 2018 semester	Teaching assistant for the course BIOL 3456 – Human anatomy and physiology II (UTD). Instructor: Dr. Yu Wen Ho.
Spring 2019 semester	Teaching assistant for the course BIOL 3456 – Human anatomy and physiology II (UTD). Instructor: Dr. Yu Wen Ho

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Spring 2021 semester	Teaching assistant for the course BIOL 3456 – Human anatomy and physiology II (UTD). Instructor: Dr. Yu Wen Ho.
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