IDENTIFICATION OF CONSERVED *Klebsiella pneumoniae* VIRULENCE FACTORS

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ABSTRACT

Michelle Palacios: Identification of conserved *Klebsiella pneumoniae* virulence factors
(Under the direction of Virginia L. Miller)

Klebsiella pneumoniae is an urgent threat to public health due to nosocomial outbreaks of multi-drug resistant strains and the emergence of hypervirulent community acquired infections. Only in recent years have we gained a better understanding of the population structure of emergent clones and insight on the high level of strain-to-strain genomic variation *K. pneumoniae* strains display. While capsule and siderophores are considered primary virulence factors of *K. pneumoniae*, the number of siderophore types encoded and the capsule type can vary significantly from strain-to-strain. Thus, identifying highly conserved virulence mechanisms is essential for therapeutic development. This work focused on the use of a murine pneumonia model of infection and a sputum-derived isolate of *K. pneumoniae* to identify previously uncharacterized virulence factors. In Chapter 2, we describe how a serendipitous mutation led us to identify the periplasmic importer of enterobactin, FepB, as being a unique contributor to virulence. Interestingly, the contribution of FepB to virulence was greater than the contribution of enterobactin to virulence suggesting a greater role than enterobactin transport. Given that FepB is present in other pathogenic *Enterobacteriaceae*, our findings may be applicable to other pathogens. In an attempt to expand our knowledge of the repertoire of *K.*

pneumoniae virulence factors, we also conducted an *in vivo* screen of the contribution of MarR-like transcriptional regulators to virulence in Chapter 3. We identified two previously unidentified regulators of virulence in *K. pneumoniae*, and have named these KvrA and KvrB. KvrA and KvrB are highly conserved across *K. pneumoniae* species. Both regulators regulated capsule expression and production, and this regulation appeared to be conserved in an isolate of a different capsule type. Interestingly, while KvrA and KvrB regulate capsule in a similar fashion, the *in vivo* kinetics of infection of the *kvrA* and *kvrB* mutants were quite distinct suggesting that the regulons of KvrA and KvrB are unique from each other. Because our studies focused on highly conserved virulence factors, our work has implications for the development of novel anti-*Klebsiella* therapeutics.

To my family, for their unconditional love and support

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BMDM- bone marrow derived macrophage

CFU- colony forming units

CPS- capsule

CRE- carbapenem-resistant *Enterobacteriaceae*

DNA- deoxyribonucleic acid

EE- endogenous endopthalmitis

FBS- fetal bovine serum

FFPE- formalin fixed paraffin embedded

GI- gastrointestinal

H_2O_2- hydrogen peroxide

H&E- hematoxylin & eosin

HAI- hospital-acquired infections

HAP- hospital-acquired pneumonias

HPI- hours post inoculation

HMV- hypermucoviscous

HV- hypervirulent

IRB- Institutional Review Board

KAN- kanamycin

KAS- *Klebsiella*-associated syndrome

KP- *Klebiella pneumoniae*

LPS- lipopolysaccharide

NCBI- National Center for Biotechnology Information

O_2- oxygen molecule

OD- optical density

PBP- periplasmic binding protein

PBS- phosphate buffered saline

RNA- ribonucleic acid

UTI- urinary tract infections

WHO- World Health Organization

WT- wild-type

CHAPTER 1: INTRODUCTION*

1.1 Overview of *Klebsiella*

The first observation of bacteria in the lung occurred in 1875 by the pathologist, Edwin Klebs (1, 2). Later in 1882, Carl Friedlander recognized that nearly all persons dying from pneumonia had bacteria in their lungs, although it is unknown if these infections were due to *Klebsiella* or other bacterial species (3). First identified as *Friedlander's bacterium* in 1882, *Klebsiella* was later renamed from *F. bacterium* to *Klebsiella* in honor of Edwin Klebs (4, 5).

1.1.1 *Klebsiella pneumoniae*

Klebsiella are Gram-negative, non-motile bacteria found in the soil, surface water, and the mucosal surfaces of mammals (6-8). Of the many species within the genus *Klebsiella*, *Klebsiella pneumoniae* is considered the most medically important *Klebsiella* species, causing 75% to 86% of clinical *Klebsiella* infections, with the next most commonly recovered species being *K. oxytoca*, which accounts for 13% to 25% of infections (9-11). In an environmental study of the incidence of *Klebsiella* species in surface waters, it was found that in over half of the samples tested, *K. pneumoniae* was the predominant species. The same group determined that the virulence capabilities (as measured by siderophore production, serum resistance,

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and fimbriae presence) of the “environmental” *K. pneumoniae* isolates appear to be similar to those of “clinical” *K. pneumoniae* isolates (8). Of notable difference was the relative absence of characterized virulence factors in the less medically prevalent, or “environmental”, species: *K. planticola* and *K. terrigena*. These two “environmental” species were recently renamed *Raoultella planticola* and *R. terrigena*, respectively (12).

Interestingly, similar *Klebsiella* species distribution is seen in clinical isolates of *Klebsiella* from animals where *K. pneumoniae* is the predominant species (13). As in humans, infections of the respiratory tract, urinary tract, sepsis, and abscesses are the most common infection types (14, 15). *K. pneumoniae* is also of particular concern in dairy farms as it causes mastitis and leads to loss of milk production and death of affected livestock (16-18). Additionally, drug-resistant strains and evidence of horizontal transfer of resistance genes have been reported in strains isolated from animals (19, 20). The ability to horizontally transfer resistance genes is of concern for the agriculture industry and for domestic animal owners as extended spectrum β-lactamase (ESBL)-producing strains have been isolated in companion animals and may serve as a reservoir for drug-resistant strains that then colonize and cause disease in humans (21-23).

1.1.2 Phylogenetic Classification

Historically, *Klebsiella* species were classified according to their biochemical properties (24). To improve outbreak outcome through better tracking, several methods have emerged and evolved to type *Klebsiella*. Some of these methods, such as serotyping, are particularly problematic because of the time needed to

perform the test and subjective interpretation due to weak reactions and cross-reactions. Furthermore, the high number of capsule types complicates matters, as sera for all capsule types are not readily available. In 2005, advances in sequencing and analysis of genomic data led to the classification of *K. pneumoniae* strains by their sequence types (STs), which refer to their nucleotide sequences at 7 loci (*mdh, infB, tonB, gapA, phoE, pgi,* and *rpoB*) (25). Genetically related STs were referred to as clonal complexes (CC) (25). In 2014, the MLST typing approach was extended to a core gene MLST (cgMLST) targeting 694 core genes and this modified approach results in a higher resolution classification of strains into clonal groups (CG) (26). Many *K. pneumoniae* STs include multiple capsule (CPS) types (27-29).

1.1.3 Classical and hypervirulent strains

Classical strains of *K. pneumoniae* can cause both community-acquired and nosocomial infections and have a worldwide distribution (30-33). The most common primary infection types caused by classical *K. pneumoniae* strains are pneumonias, UTIs, and bacteremias (24, 30). Classical *K. pneumoniae* strains are defined by their ability to cause potentially fatal infections when infecting immunocompromised hosts, including diabetics, cancer patients, or individuals suffering from chronic liver disease (30, 34). While *K. pneumoniae* can cause community-acquired pneumonia, hospital acquired pneumonias (HAPs) are much more common (35, 36). HAPs are one of the most common type of nosocomial infection and are particularly worrisome as they carry a high mortality rate (31). It is estimated that *K. pneumoniae* causes ~11.8% of HAPs and can be either ventilator or non-ventilator associated (31). Especially problematic is that these strains are more likely to be multidrug resistant

and the incidence of carbapenem resistance in *K. pneumoniae* increased in the United States from 0.1% to 4.5% between 2002 and 2010 (37). In these carbapenem resistant strains, drug resistance does not enhance the virulence of these strains although they become more difficult to treat (32).

The past three decades have ushered in a new *Klebsiella*-associated syndrome (KAS)— Pyogenic Liver Abscesses (PLAs)—and, likely owing to the dissemination of bacteria from PLA infection sites, other metastatic complications (for example, endophthalmitis, meningitis, and necrotizing meningitis) have emerged (38). Unique cases of liver abscesses, sometimes referred to as *Klebsiella* invasive syndrome, were reported in the early 1980s in Taiwan (39, 40); this syndrome includes hepatic infection with *K. pneumoniae* along with dissemination to other sites. *K. pneumoniae* is now the primary cause of PLA in Hong Kong, Singapore, Korea, and Taiwan (39). PLA is no longer limited to Asian geographic regions nor is it restricted to individuals of Asian descent as cases have been reported in non-Asian countries (41-44). Although the reasons for the higher prevalence of *Klebsiella*-associated liver abscesses in Asian countries are unclear, studies suggest that environmental factors in the Southeast Asia region play a role in colonization of the gastrointestinal (GI) tract by *K. pneumoniae*, which is likely associated with disease (45). Clinical isolates from KAS affected individuals have a hypermucoviscous phenotype compared with environmental and other clinical isolates. These strains, often referred to as “hypervirulent” because of their increased likelihood to cause invasive infection (i.e. establish infection of the liver followed by dissemination), have a hypermucoviscous colony phenotype that has

been attributed to the presence of RmpA/RmpA2 (**r**egulator of **m**ucoviscous **p**henotype) (46, 47). The hypermucoviscosity is due to an increase in the production of capsule. This phenotype appears to be required to cause liver abscesses in a mouse model for this disease (47). Besides the presence of RmpA, there are other characteristics that distinguish hypervirulent strains from classical strains. The presence of additional siderophores, namely yersiniabactin, is associated with hypervirulent strains (48). More detail on virulence factors of *K. pneumoniae* can be found in section 1.3.

Pneumonia and septicemia due to *K. pneumoniae* in developed countries are now most commonly associated with nosocomial infections and with long-term nursing facilities (24, 49-52). Furthermore, there have been reports of drug-resistant *K. pneumoniae* outbreaks in these facilities (53). Whereas only 5% of acute-care hospitals reported at least one carbapenem-resistant *Enterobacteriaceae* (CRE) case, 18% of long-term acute care facilities reported CRE infections in 2012 (54). Additionally, both the carriage and the incidence of *Klebsiella* infections increase with age (45, 55). With an increase in the average age of our populations, we can expect an increase in these types of infections if we do not implement changes in our ability to control *Klebsiella* spp. in the environment, or if we do not develop improved treatments.

1.1.4 Emerging diseases

Endogenous endophthalmitis (EE) secondary to *K. pneumoniae* liver infection (KPEE) is also an increasing concern (56, 57). The prevalence of KPEE among EE cases appears to be higher in Asian countries than in non-Asian countries (58).

Whether this is because a liver abscess is the source for the majority of cases in Asia (whereas it is endocarditis in the United States [US]) or there are other factors is still unknown (59). As with liver abscesses, the primary risk factors associated with the development of KPEE are diabetes mellitus and liver disease (60). Given that the total number of people with diabetes is projected to rise to nearly 366 million by the year 2030, it is reasonable to expect the number of *K. pneumoniae* cases to reflect this increase (61).

Although there have been several studies highlighting and investigating *Klebsiella* liver abscesses and resulting complications, an understudied yet severely affected population consists of burn injury patients (62). Infection is a common cause of death in these patients (63). Bacterial identification records at the US Army Institute of Surgical Research Burn Center indicate that *K. pneumoniae* is one of the most common isolates recovered post-admission. Importantly, this timing suggests nosocomial transmission and calls attention to the need for improved healthcare setting practices (64).

1.1.5 Antibiotic Resistance

In recent years, antibiotic resistant *K. pneumoniae* strains have acquired a variety of genes resulting in the production of drug inactivating enzymes generally classified under the following categories: extended-spectrum beta-lactamase (ESBL), which render strains resistant to cephalosporins and monobactins, and carbapenamase, which results in resistance to almost all beta-lactam antibiotics including carbapenems (32). Carbapenems are used as a last resort to treat Gram-negative infections. *K. pneumoniae* strains expressing carbapenamases, have

spread rapidly in the United States and globally (65). Collectively, there are four classes of carbapenamases (A-D) based on Ambler classification (66). These enzymes are categorized based on their amino acid sequence and active site. Class A, C, and D comprise serine β-lactamases while class B enzymes are metallo-β-lactamases that typically require a bivalent metal ion (66). The first case of a *K. pneumoniae* carbapenamase (KPC) expressing isolate was documented in North Carolina in 1996 (67). Other carbapenamases such as MBL (metallo-beta-lactamase), NDM-1 (New Delhi metallo-beta-lactamase), IMP (Imipenemase metallo-beta-lactamase), and VIM (Verona integrin-encoded metallo-beta-lactamase) have also been identified in *K. pneumoniae*, as well as in other bacterial strains (68). There were over 9,000 Carbapenem-resistant *Enterobacteriaciae*, or CRE, related deaths in 2011 (69). Of those, 8,000 were attributed to *Klebsiella* species.

K. pneumoniae is considered a source and reservoir for AMR genes and can transfer antibiotic resistance plasmids (70-72). This is concerning as the plasmid can be transferred to members of the normal gut flora, such as *E. coli* and other *Enterobacteriaceae*, which can ultimately lead to an increase in the pool of potential drug resistant pathogens (71, 73). In a nosocomial setting, *K. pneumoniae* infections often originate from strains colonizing the patient's own gut (74-76).

1.2 Host Response to *K. pneumoniae*

The current understanding of how *K. pneumoniae* causes disease suggests it "evades and survives" rather than actively suppress the host immune system (32). Rather than produce factors to actively dampen the host response, *K. pneumoniae*

produces a polysaccharide capsule to shield itself from host immune mechanisms. This capsule aids in reducing adherence to phagocytic cells, reducing uptake by phagocytic cells, and resisting complement-mediated lysis (77-81). In the context of a lung infection, *K. pneumoniae* will first encounter alveolar macrophages. Alveolar macrophages have been shown to be important for fighting *K. pneumoniae* infections in a mouse model (82). Once *K. pneumoniae* establishes an infection in the lung, neutrophils are recruited to the site of infection (83). In a murine pneumonia study, alveolar macrophage depletion led to decreased mouse survival and increased bacterial burden in the lung, as well as an increase in neutrophil infiltration (82).

K. pneumoniae clearance from the host lung requires TNF and IL-17A (84-87). Inflammatory monocytes are recruited to the lungs of *K. pneumoniae* infected mice and subsequently produce TNF, which results in an increase of IL-17 producing type 3 innate lymphoid cells (ILC3s) (85). This production of IL-17 by ILC3s enhances monocyte antimicrobial activity and contributes to bacterial clearance in the lung (85). Monocyte depletion in mice leads to impaired resolution of pneumonia (85). Thus, identifying therapies that enhance monocyte activity may contribute to combating *K. pneumoniae* infections.

1.3 Virulence factors

1.3.1 Genetic Screens

Several genetic screens have been conducted to identify bacterial factors important for *Klebsiella* virulence. One of the first large scale mutagenesis screen in *K. pneumoniae* (KPPR1; Rifampicin derivative of strain ATCC43816) used

transposon insertion mutants to identify bacterial factors important for a pneumonic infection and dissemination to the spleen (88). Not surprisingly, one of the primary virulence determinants identified from that screen was capsule, as demonstrated by the virulence defect of a capsule (*cpsB)* mutant (89). Follow-up studies also led to the characterization of the siderophores, enterobactin and yersiniabactin (90). A more saturated transposon screen with over 25,000 transposon insertion mutants was recently performed also using KPPR1 (91). This study expanded the virulence repertoire by identifying outer membrane components and amino acid synthesis clusters as important for virulence in the lung.

K. pneumoniae is also a common cause of PLA and attempts to understand virulence in an animal model have also been attempted. One screen focused on the genetic requirements for infection in a murine liver abscess model (92). This screen used an oral inoculation model, since colonization of the GI precedes *K. pneumoniae* infections (75). Transposon mutants appearing to affect capsule were excluded in this screen since the requirement for capsule in virulence had already been established. Components of type I and type III fimbriae, along with a few putative transcriptional regulators were identified in this screen.

In the context of HAI, *K. pneumoniae* infections are frequently associated with biofilm growth on indwelling devices (93). The first biofilm screen using transposon mutagenesis was performed in 2004 screening Tn*5* insertion mutants *in vitro* using a microtiter plate assay followed by subsequent studies in mice (94). This study identified type III fimbriae (*mrk*) as being necessary for biofilm formation. A later study used a flow through culture system and identified additional players in biofilm

formation, such as *cps* genes (95). An additional study focused on different stages of biofilm development differentiated genes needed for initial biofilm formation versus biofilm maturation (96). Genes necessary for capsule and LPS production were important for early adherence of cells whereas proteins involved in DNA metabolism and membrane transport were important for unique to later stages. Further screens have focused on identifying players in biofilm formation in non-lung pathogenic strains such as liver isolates (97). SugE (inner membrane protein) and TreC (involved in trehalose uptake and phosphorylation) were found to affect biofilm production through modulating CPS production in the NTUH-K2044 strain (97).

Klebsiella can also colonize the gastrointestinal (GI) tract of humans, thus screens to identify bacterial factors important for GI colonization have also been attempted (98). Proteins involved in metabolic pathways and an adhesion factor (homologous to HMW in *H. influenza*) were identified to be important for GI colonization. Interestingly, no gene associated with capsule production was identified in this study. However, it is worth mentioning, the mutagenesis was not saturating as only 2,200 mutants were screened. Another study was the first published use of a genomic library as a positive-selection based screening model where a *K. pneumoniae* genomic library was expressed in *E. coli* and screened in a GI mouse model (99). The response regulator ArcA and GalET of the galactose operon, along with two membrane-associated proteins of unknown function were found to be important for colonization in *E. coli*. Although polysaccharide production was not directly examined, *K. pneumoniae* GalET reduced sensitivity of *E. coli* to bile salts. Because the *galETKM* operon has been demonstrated to play a role in

modifying galactose for incorporation into LPS (100), GalET may be important for gut colonization due to a role in LPS modification.

When comparing the results from these screens, there is a general lack of overlap between the findings from each study. One consideration is that most of the screens were not saturating and thus only a limited number of genes were identified. Furthermore, the screens were done in different strains and using different infection models, thus complicating comparisons. One interesting perspective is that *K. pneumoniae*, as a population, has over 30,000 genes (48). In a comparative genomics study of over 300 isolates, it was determined that while each *Klebsiella* strain has about 5,500 genes, less than 2,000 are considered “core” genes that are common to all *K. pneumoniae* (101). Given the high number of *Klebsiella* subspecies and resulting disease manifestations, current studies are moving toward large-scale comparative genomic analyses to identify bacterial factors associated with particular infection sites and antibiotic resistant strains. The developments in sequencing technology have shed light on new associations between previously identified virulence factors and clinical outcomes.

1.3.2 Fimbriae

The two types of fimbriae that have been characterized in *K. pneumoniae* are type 1 (*fim*) and type 3 (*mrk*) fimbriae. Type 1 fimbriae have been associated with adherence to uroepithelial cells and to plants, and *fim* genes are expressed in the UTI murine infection models but appear to be dispensable for GI colonization and lung infections (102-104). The type 1 filamentous fimbriae are composed of FimA subunits with the adhesive subunit, FimH, on the tip. A *fim* cluster mutant was able

to colonize the lung and colonize the spleen, suggesting type 1 fimbriae are not important for lung colonization or dissemination (104). Like type 1 fimbriae, type 3 fimbriae are not needed for GI colonization or virulence in the lung (104). Interestingly, type 3 fimbriae are not needed for colonization of the urinary tract. However, type 3 fimbriae are expressed during biofilm formation on catheters and endotracheal tubes, suggesting their contribution may be more important in a nosocomial setting where bacteria can colonize indwelling medical devices and serve as a source for infections of other sites (105, 106).

Associations between surface exposed polysaccharides and fimbriae content have been found. The O1 serotype is the most common O serotype of *K. pneumoniae* and O1 strains are more likely to contain type 1 fimbriae compared to any other O antigen type (27). K1 and K2 are the two most common isolated K serotypes (27). K2 strains are more likely to have type 1 fimbriae than any other K serotype, whereas no K1 strain was found to contain type 1 fimbriae genes (27). The biological significance of this virulence factor association with O and K- types is unclear and may warrant further study.

1.3.3 Siderophores

Siderophores are small molecules secreted by bacteria that aid in iron acquisition (107-109). They are produced intracellularly and then transported in and out of the bacterial cell in a controlled manner using siderophore specific receptors and transport proteins (Figure 1.1, Figure 1.2) (107, 109). Siderophores are produced and secreted in response to iron limitation. The transcriptional regulator, Fur (ferric uptake regulation), regulates iron uptake in many Gram-negative bacteria,

including *Klebsiella* (110). In iron-replete conditions, Fe(II) complexes with dimerized Fur which then binds *fur* box consensus sequences, thus inhibiting transcription of downstream genes such as those involved in siderophore production for iron acquisition (111). In iron-deplete conditions, the lack of Fe(II) prevents the dimerization of Fur and subsequent binding to Fur boxes, thereby alleviating transcriptional repression and increasing production of iron regulated systems, including siderophores.

1.3.3.1 Siderophore distribution in *Klebsiella*

Siderophores are divided into five major classes: catecholates, phenolates, hydroxyamates, α-hydroxy-carboxylates, and mixed-type siderophores (109). The best characterized and most conserved siderophore system in *Klebsiella* is the catecholate siderophore, enterobactin (8, 48). *Klebsiella* species produce enterobactin, with some strains producing one or more additional siderophores (i.e. salmochelin, yersiniabactin, and/or aerobactin). Studies in Chapter 2 and Chapter 3 used strain KPPR1S and this strain encodes enterobactin, salmochelin, and yersiniabactin, but not aerobactin.

In a hypervirulent *K. pneumoniae* isolate (belonging to ST86) that encodes all four siderophores, aerobactin was the only siderophore to be required in murine infection models, both in a subcutaneous and pulmonary challenge (112, 113). Yet, it was also found that in a set of 12 hypervirulent strains, siderophores are produced at higher levels than in classical *K. pneumoniae* strains (6 to 9.6 times higher) and that aerobactin accounts for 90% of the siderophores produced in these strains which may explain the *in vivo* phenotypes (113). However, a different study with the

hypervirulent NTUH-K2044 strain (that also encodes the four siderophores) found that an aerobactin mutant was not attenuated in an IP or IG murine infection model (114). Differences in murine models or variations of siderophore production in the strains tested could have accounted for this discrepancy. However, second to enterobactin, yersiniabactin is the next most common siderophore and is present in nearly 1/3 of *K. pneumoniae* isolates whereas aerobactin is less commonly seen (48). Thus, findings pertaining to aerobactin may only be applicable to a small subset of clinical infections.

1.3.3.2 Siderophore synthesis and transport

Enterobactin is synthesized from chorismate through a series of chemical modifications by proteins encoded by the *entABCDEF* genes (Fig. 1.1). Once synthesized in the cytoplasm, enterobactin is exported through the inner membrane via EntS and through the outer membrane via TolC (115). Secreted enterobactin can bind ferric iron and be imported back into the bacterial cell via the outer membrane receptor, FepA (116). The periplasmic protein FepB shuttles the iron bound siderophore to the inner membrane complex FepDG (117-119), where the siderophore can be imported and the iron ultimately released by the esterase, Fes. Salmochelin has transport mechanism similar to enterobactin (Fig. 1.1). The synthesis of salmochelin is dependent on the synthesis of enterobactin, as salmochelin is a glycosylated version of enterobactin (120). Once synthesized, salmochelin is exported via IroC and an unknown outer membrane protein. Once bound to ferric iron, salmochelin can be imported via the IroN receptor, and from

there, salmochelin import converges to the enterobactin import pathway (121, 122). However, IroD, rather than Fes, is the esterase required for iron release (123).

Yersiniabactin utilization is much less well understood than enterobactin or salmochelin utilization (Fig. 1.2) (124). Yersiniabactin is a phenolate-type siderophore synthesized from chorismate to the yersiniabactin precursor, salicylate (124, 125). Once produced, it is exported via an unknown pathway. Ferric bound yersiniabactin can then bind FyuA, where it is ultimately transported through the inner membrane via YbtPQ (126). No esterase (Fes/IroD-like protein) has been identified for this system.

Aerobactin is the least common siderophore encoded by *K. pneumoniae* strains (8, 48). It is classified as a mixed-hydroxymate type siderophore synthesized by proteins encoded by the *iucABCD* locus (109, 127, 128). Export of aerobactin occurs via an unknown pathway. The aerobactin surface receptor, IutA, allows entry of ferric-bound aerobactin into the periplasmic space (129). The periplasmic protein, FhuD, then mediates transport to the inner membrane proteins FhuBC (130-132). No esterase (Fes/IroD-like) protein has been identified for this system. Aerobactin is absent from KPPR1S.

More recent studies have begun to identify unique characteristics of the enterobactin system. The structure of ferric-enterobactin bound FepB has been determined (133). Interestingly, FepB was shown to be unlike other PBP (periplasmic binding proteins) in that it can form a trimer by binding four siderophore iron-bound enterobactin molecules (133). Because excess ferric-enterobactin can exit the periplasmic space via TolC and re-bind FepA to once again enter the

bacterial cell, it has been speculated that the oligomerized FepB may serve to reduce this energy expenditure. One proposed mechanism is that the trimerization of FepB may allow iron to be stored in the periplasmic space as a means of conserving energy by preventing the re-exportation of excess iron-bound enterobactin. It is thought that the symmetrical structure of enterobactin trimer facilitates the formation of this complex (134).

1.3.3.3 Non-iron roles for siderophores

Non-iron roles for siderophores have recently begun to be characterized in other bacteria. While the role of enterobactin and salmochelin beyond iron sequestration has been expanded to include resistance to oxidative stress, the role of yersiniabactin includes not only the former, but also a role in cell signaling, and zinc and copper binding in *Yersinia pestis* (135). *In vitro* studies in *E. coli* have found yersiniabactin to have a role in minimizing copper toxicity to bacterial cells by binding to host copper (II) and preventing its reduction to copper (I) by catecholate siderophores such as enterobactin (136).

Recent research suggests that siderophores may have a much greater contribution to virulence than just sequestration of host iron or other metals. *In vitro* and *in vivo* studies show that siderophores deplete epithelial cell iron, induce cytokine secretion, and activate the vascular permeability transcriptional regulator, HIF-1α (137). It is unknown if all siderophores are capable of inducing these conditions or if these characteristics are limited to a particular class of siderophores. In *K. pneumoniae,* a *tonB* mutant that is able to secrete siderophores but is unable

to utilize them, siderophores were found to contribute to dissemination and lung inflammation (137).

1.3.4 LPS and Capsule

Historically, *Klebsiella* isolates were classified into serotypes based on the recognition of the surface exposed polysaccharides, O-antigens (the outermost part of LPS) and K-antigens (capsule, CPS) by specific antibodies (24, 138, 139). Capsule is necessary for *K. pneumoniae* virulence as acapsular strains are less virulent than encapsulated strains in mouse models, and display decreased bacterial loads and lower mouse mortality rates (80, 89, 140, 141). For years, the number of different serotypes had been estimated to 8 for O-antigen and 77 for K-antigen (24, 138, 139, 142, 143). With the advancements in whole genome sequencing, Wyres et al. analyzed over 2500 *K. pneumoniae* isolates to investigate the diversity of *Klebsiella* capsule types and found 134 distinct K-loci (101). Historically, capsule type has been associated with particular infection sites, however recent studies using larger data sets found no correlation between K type and sample site (blood, sputum, or urine) (24, 27). A correlation was found between K2 capsule and invasive infection (144). Interestingly, no association between LPS core type and infection type, LPS and infection site, or LPS and acquisition mode has been detected (27).

1.3.4.1 LPS

Compared to other *Enterobacteriaceae*, *K. pneumoniae* has a surprisingly low number of O antigens. Seroepidemiology studies have shown that for human host associated isolates, the 3 most common O serotypes are O1, O2, and O3 (145, 146). In a study examining 573 isolates, 296 isolates (52%) were O1, followed by O2

(91, 16%), O3 (86, 15%), O5 (33, 6%), OL101 (26, 5%), O4 (18, 3%), and O12 (9, 2%) (27).

The O antigen biosynthesis enzymes are encoded by the *rfb* locus (147). The LPS synthesis pathway is an ABC-transporter dependent pathway broken into three functional steps: 1) synthesis of nucleotide activated sugars, 2) polysaccharide repeat unit synthesis, and 3) assembly of repeat units and transport across the membrane (27, 147). Both the O1 and O2 polysaccharide chains are based on a repeat-unit designated as D-galactan I, where O1 is capped by a distal galactan II unit and O2 is not (27).

1.3.4.2 Capsule Synthesis and Export

Studies on LPS O-antigen synthesis in Gram-negative bacteria have provided much of the groundwork for understanding the synthesis of capsule and exopolysaccharides, as capsule assembly closely resembles synthesis of LPS. Capsule assembly is classified into four major categories, depending on the nature of the polymerases and transporters involved (148). Like the group I CPS pathway of *E. coli, Klebsiella* capsule synthesis occurs via Wzy-dependent polymerization (149). Capsule synthesis involves the activation of sugars (the addition of a nucleotide to form a nucleotide diphosphate sugar), the initiation of synthesis and formation of a repeat unit, transport of this unit to the outer surface of the inner membrane, and polymerization of this unit into polymers (150-152). The acidic polysaccharide capsule of *Klebsiella* is comprised of three to six repeating sugar monomers. The capsule assembly process in *Klebsiella* (and some *E. coli* strains) is unique from other bacteria due to the presence of Wzi (153, 154). Wzi is a surface protein

involved in capsule attachment to the outer membrane. In the absence of *wzi*, bacteria retain their hypermucoviscous colony phenotype but have a reduction in cell-associated capsule (153, 154). However, even non-hypermucoviscous *K. pneumoniae* strains have *wzi*, so the contribution to mucoviscosity may not be its only role.

1.3.4.3 Capsule Regulation

The *cps* locus of *K. pneumoniae* encodes the genes necessary for the capsule production process. Expression of the *cps* locus is believed to be under the control of at least three promoters located upstream of *galF, wzi,* and *cpsB* in *K. pneumoniae* ATCC43816 (and the Rif and Rif/Strep derivatives, KPPR1 and KPPR1S) (155-157). The first gene in *cps* loci across all strains analyzed is *galF*, followed by a cluster of conserved translocation and surface assembly genes, including *wzi,* then glycosyltransferases involved in the production of the capsule sugar monomer precursors (27, 158). Typically at the terminal end of the locus is the gene encoding UDP-glucose-6-dehydrgenase (*ugd*) (27) (Fig. 2A).

RcsB and RmpA regulate transcription of the capsule loci in some *K. pneumoniae* strains, including KPPR1S (Fig. 2B) (156). Much of what is known about RcsB regulation comes from studies of the RcsD/RcsC/RcsB two-component system in *E. coli* (159), where RcsC is the sensor and RcsB is the response regulator. In *E. coli* and *K. pneumoniae*, RcsB can bind the capsule locus with the accessory positive regulator, RcsA (159, 160). In *K. pneumoniae* CG43*,* an RcsAB box is located upstream of the *galF* and *cpsB* promoters (156). RcsB was found to regulate expression of the *galF* and *cpsB* promoters, capsule production, and

mucoviscosity in this strain (156). In KPPR1S, no RcsAB box could be located in the *galF* promoter region (unpublished data), highlighting the complexity of capsule regulation and the strain-to-strain genomic variation in *K. pnemoniae*.

The contribution of RmpA to capsule regulation is filled with ambiguity. For instance, not all strains encode RmpA and those that do can have anywhere from one to three variants (156, 157, 161). The contribution of each RmpA variant has varied based on the strain examined. RmpA can be encoded on the chromosome, or plasmid, or both (157). A second variant, RmpA2 also exists as a fully functional protein in some strains or as a non-functional truncated gene in others. In the K2 strain, CG43, RmpA2 was found to bind at the *galF* promoter and the *wzi* promoter (161). In the K1, NTUH-K2044 strain, the plasmid encoded pRmpA2 was dispensable for capsule regulation and production and the plasmid encoded RmpA contributed to *galF* regulation and capsule production (157). KPPR1S, the strain used in the studies described in Chapters 2 and 3, encodes only one RmpA, a chromosomally encoded RmpA. RmpA contributes to capsule production and virulence in KPPR1S although its mechanism of regulation is currently being determined (unpublished data).

1.4 Research Objectives

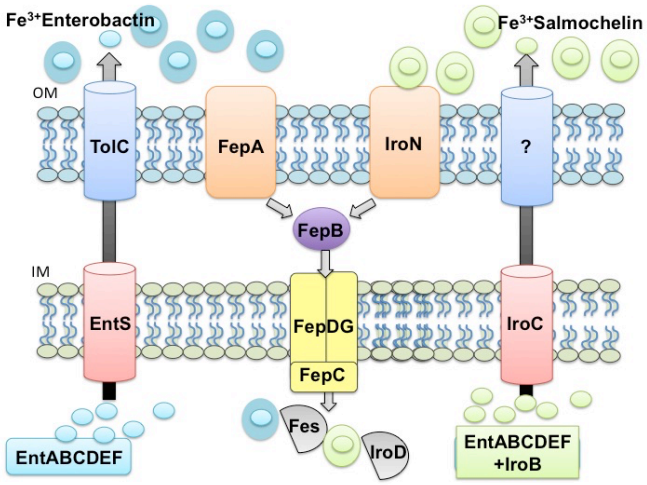
While there have been many attempts to expand our understanding of the virulence repertoire of *Klebisiella*, these studies have not identified and characterized a significant number of virulence determinants beyond previously known factors (LPS, capsule, siderophores, fimbriae) (32, 162). The average *K. pneumoniae* genome has approximately 5,500 genes, however, fewer than 2,000

are highly conserved between isolates and considered part of the core genome (48). The high variability in the genomic content from strain to strain brings to light the importance of understanding highly conserved systems and how they contribute to virulence.

Siderophores have been extensively studied due to their ability to sequester and transport host-derived iron into the bacterial cell. Chapter 2 provides evidence of a novel role in *K. pneumoniae* virulence for a periplasmic siderophore binding protein. Like siderophores, capsule synthesis and regulation has been widely studied. In Chapter 3 we demonstrate there are additional transcriptional regulators that control capsule production and virulence. Importantly, the work presented here identified and characterizes conserved factors that impact *K. pneumoniae* virulence.

1.5 Figures

A



B

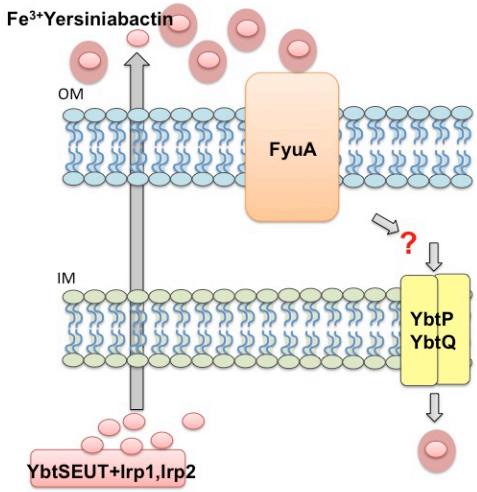


Figure 1.1 Siderophore transport. (A) The catecholate siderophore enterobactin is synthesized in the cytoplasm. Export of enterobactin is mediated by the inner membrane protein, EntS. The TolC outer membrane protein is required for secretion. Once bound to ferric-iron, FepA mediates import through the outer membrane in a TonB dependent manner. Periplasmic transport is mediated by FepB to the FepDGC transport system which translocates the ferric-enterobactin complex to the bacterial cytoplasm. The esterase, Fes, is required for iron release from enterobactin in the cytoplasm. Salmochelin utilization is similar to enterobactin utilization except export is mediated via IroC and import via IroN. **(B)** Yersiniabactin synthesis occurs in the cytoplasm and export is via an unknown pathway. Import occurs through the FyuA receptor and YbtPQ proteins. Several black boxes in yersiniabactin transport remain. Figure modified from (109).

A

B

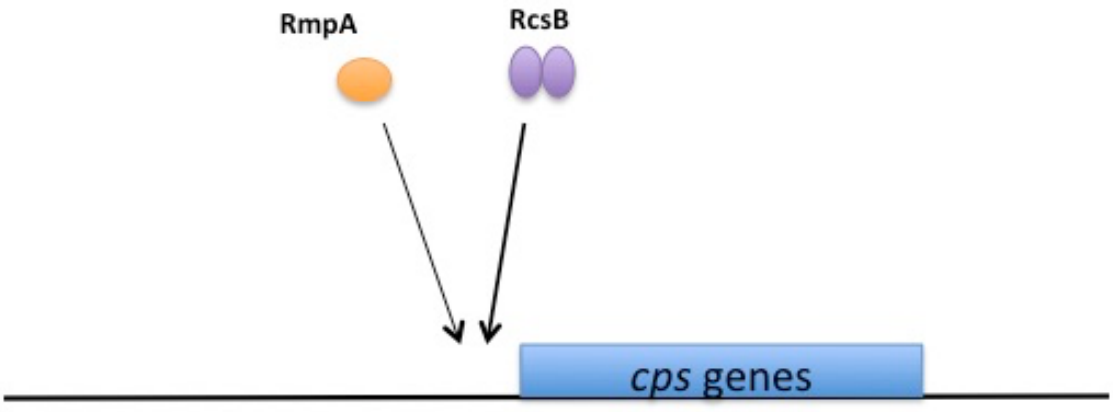


Figure 1.2 KPPR1S capsule locus. (A) Genomic organization of the capsule locus in KPPR1S. Arrows indicate characterized promoters. Genes in blue depicts highly conserved genes (including genes for capsule core assembly) across different K types. Genes in orange encode glycosyltransferases. The gene encoding the flippase is in red. The capsule-repeat unit polymerase is in aqua. Genes encoding sugar-processing genes are in purple. The acetyltransferase-encoding gene is depicted in green. **(B)** Characterized transcriptional regulators of capsule in KPPR1S.

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CHAPTER 2: SERENDIPITOUS MUTATION REVEALS SEVERE VIRULENCE DEFECT OF A *Klebsiella pneumoniae fepB* MUTANT*

2.1 Overview

Klebsiella pneumoniae is considered a significant public health threat because of the emergence of multidrug-resistant strains and the challenge associated with treating life- threatening infections. Capsule, siderophores and adhesins have been implicated as virulence determinants of *K. pneumoniae*, yet we lack a clear understanding of how this pathogen causes disease. In a previous screen for virulence genes, we identified a potential new virulence locus and constructed a mutant (*smr*) deleted for this locus. In this study, we characterize the *smr* mutant and show this mutation renders *K. pneumoniae* avirulent in a pneumonia model of infection. The *smr* mutant was expected to have a deletion of three genes but subsequent genome sequencing indicated a much larger deletion had occurred. Further analysis of the deleted region indicated the virulence defect of the *smr* mutant could be attributed to the loss of FepB, a periplasmic protein required for import of the siderophore enterobactin. Interestingly, a Δ*fepB* mutant was more

* I constructed most of the previously unpublished mutants that were made to characterize the original *smr* mutant. I conducted all of the animal work (inoculations, organ harvests, and bacterial enumeration). I performed the *in vitro* growth curves cross-feeding assays, and capsule production experiments. I did the majority of the writing and created figures and tables.

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attenuated than a mutant unable to synthesize enterobactin, suggesting that additional processes are affected. As FepB is highly conserved among the *Enterobacteriaceae*, targeting FepB therapeutically may be useful for treatment of *Klebsiella* and other bacterial infections.

2.2 Introduction

Klebsiella pneumoniae is a Gram-negative bacterium commonly classified as an opportunistic, nosocomial pathogen capable of causing a variety of infections including urinary tract infections, pneumonia and sepsis (1-5). It is often found as a commensal resident of the gastrointestinal tract and this is believed to be a primary source of infection (2, 6-8). Recently *K. pneumoniae* also has been shown to be capable of causing community acquired diseases such as pyogenic liver abscesses, meningitis, and endopthalmitis (9-11). The increasing prevalence of antibiotic resistant strains only serves to compound the clinical importance of *K. pneumoniae* and the difficulty of treating those infected with extended spectrum β-lactamase-resistant strains (ESBLs) or carbapenem-resistant strains (12-16). Carbapenem resistance is of particular concern as it is used as a drug of last resort to treat Gram-negative infections (12, 17). Carbapenem resistance was first identified in *Klebsiella* and is typically encoded on mobile genetic elements that can be transferred from *Klebsiella* to other species (14, 17, 18).

During infection, sequestration of iron by the host limits the availability of free iron and as a result, bacteria produce their own chelators to scavenge iron. Iron acquisition is an essential component of most bacterial pathogens as iron is required for cellular and metabolic activities (19). Siderophores are small secreted molecules

with a high affinity for ferric iron; these are classified based on the chemical nature of the Fe^{3+} coordination (20). Among *K. pneumoniae* strains, enterobactin, a catecholate type siderophore, is produced by almost all strains (21, 22). However, community acquired isolates of *K. pneumoniae* and those that cause invasive disease typically encode additional siderophore systems (salmochelin, yersiniabactin, aerobactin) (23). Salmochelin is a C-glucosylated enterobactin produced by some isolates of *Salmonella*, *E. coli*, and *Klebsiella* and its synthesis is dependent on enterobactin. Mutants unable to produce enterobactin are also unable to produce salmochelin (24, 25). The *iroA* locus encodes enzymes necessary to modify enterobactin as well as proteins required for transport of salmochelin (26). The yersiniabactin locus is found in many invasive *K. pneumoniae* isolates and encodes a phenolate type siderophore that was first identified as part of a pathogenicity island in *Yersinia* (27). Interestingly, in a genome wide association study of a broad range of *K. pneumoniae* isolates, yersiniabactin was found to be the most prevalent virulence associated locus and was found to be a predictor of infection versus carriage (23). Aerobactin is yet another siderophore produced by a smaller fraction of *K. pneumoniae* strains than either enterobactin or yersiniabactin (23). Although aerobactin has a lower affinity for Fe^{3+} than enterobactin or yersiniabactin, it is frequently produced by isolates from pyogenic liver abscesses (28).

To date, identified virulence factors of *K. pneumoniae* primarily include capsule, lipopolysaccharide (LPS), fimbriae, and siderophores, and these factors also have been identified as virulence factors for the strain used for the studies

presented here (4, 29-35). Several high-throughput studies have been done in mouse models to identify additional bacterial virulence factors (35-41). Two of these screens were signature tagged mutagenesis (STM) screens for factors affecting gastrointestinal colonization and/or infection of the urinary tract (37, 38). These studies identified adhesins, LPS and capsule. Another screen for gain of function when expressed in *E. coli* identified a regulator and LPS (41). A screen for *in vivo* expressed genes during septicemia identified genes involved in use of siderophores (aerobactin and enterobactin) (40). An STM screen in a model for liver abscess formation identified adhesins and regulators (39). Two of these studies focused on the identification of bacterial genes needed for bacterial survival in the lung; one approach used STM and the other TnSeq (35, 36). These screens identified capsule, LPS, siderophores and transcriptional regulators. All of these screens also identified genes predicted to contribute generally to growth as well as genes of unknown function.

Overall, there has been a lack of overlap in identified genes amongst the different screens conducted in lung, urinary tract, liver infection, and gastrointestinal colonization models. This may be due to the fact that none of the screens were saturating, or this could be indicative of mechanisms that compensate for loss of individual genes. These findings are further complicated by the use of different infection models and different strain backgrounds, both pathogen and host. While typically focused on the goal of identifying previously unknown bacterial factors contributing to disease, these screens primarily identified known virulence factors of *K. pneumoniae* as well as metabolic functions generally contributing to growth.

We previously conducted a STM screen of *K. pneumoniae* in an intranasal model of pneumonia to identify virulence genes (35). From this screen, yersiniabactin was identified as important for our strain to colonize the lung and to cause disseminated infection (34). In addition, a number of mutants with insertions in or near *ramA* were identified (35). RamA has been implicated in virulence and multidrug-resistance in other pathogenic bacteria, and mutations in *ramA* have been associated with fluoroquinolone resistance in *K. pneumoniae* (42-45). Furthermore, a recent study reported that overexpression of RamA affects virulence and results in modified LPS (46).Thus, we sought to determine if RamA is a virulence determinant for a highly virulent *K. pneumoniae* strain. These studies found no role for *ramA* or nearby genes for virulence in a pneumonia model of infection. However, a serendipitous secondary mutation was identified and further analysis of this mutation implicates FepB, a periplasmic protein required for transport of enterobactin and salmochelin, as essential for virulence. Surprisingly, there were interesting differences in virulence between enterobactin synthesis mutants and an enterobactin transport mutant.

2.3 Materials and Methods

2.3.1 Ethical statement. Mouse experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of North Carolina-Chapel Hill (Protocol 14-110). All efforts were made to minimize suffering. Animals were

monitored daily following inoculation and were euthanized upon exhibiting signs of morbidity.

2.3.2 Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. Wild-type parental strains used in this study are KPPR1, Rif^R derivative of ATCC 43816 (35), and KPPR1S, a $Strep^R$ derivative of KPPR1. KPPR1and KPPR1S have identical growth characteristics *in vitro* and *in vivo* (data not shown). *K. pneumoniae* strains were grown aerobically in Luria-Bertani (LB) medium or M9 media supplemented with 0.4% glucose and 0.2% casamino acids (M9-CAA) overnight at 37^{o}C. Where indicated 100 µM 2-2'dipyridyl was added to the media to deplete available iron. Antibiotics were added to the media as appropriate at the following concentrations: kanamycin (50 µg/mL), rifampin (30 µg/mL) and streptomycin (500 µg/mL). Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

2.3.3 Construction of bacterial mutants. Isogenic mutants of KPPR1S (Δ*ramA*, Δ*orf82,* Δ*orf82*Δ*ramA*Δ*romA,* Δ*entS*, Δ*ybdB2entABEC* (locus encoding enterobactin synthesis)*,* Δ*fes,* Δ*fepB,* Δ*smr_A*, Δ*smr_B*, and Δ*smr_C* were generated using allelic exchange as described using the pKAS46 vector (47). pKAS46 is a suicide vector that allows the use of streptomycin as selection for the loss of the vector (47, 48). For allelic exchange to generate unmarked mutations, ~500 bp DNA fragments were generated by PCR using the primer sets indicated in Table 2 and cloned into pKAS46. Constructs were confirmed by sequence analysis.

Overnight cultures of *K. pneumoniae* KPPR1S and *E. coli* S17-λ*pir* (49) carrying a derivative of pKAS46 were mixed, collected by centrifugation, plated on LB agar (no antibiotics), and grown overnight at 37°C. Transconjugants were selected by plating on LB agar with $Rif_{30}Kan_{50}$. Rifampin selects against the donor strain and kanamycin selects for the plasmid integrants. Several $Rif^{R}Kan^{R}$ colonies were grown for 5-6 hours in LB (no antibiotics), then plated on LB agar with $Strep_{500}$ to select for transconjugants that had excised the plasmid. KanS clones were screened by PCR to verify loss of the targeted gene(s).

An insertional disruption of the *fepDCG* operon was constructed in KPPR1S (*fepD::pKAS46).* This mutant was made by plasmid integration into the *fepD* gene. The appropriate DNA fragment, generated by PCR using primers MP313 and MP314 indicated in Table 2, was cloned into pKAS46. The resulting plasmid, pKAS46_*fepD::kan* was mated into KPPR1S using *E. coli* S17-λ*pir* carrying pKAS46_*fepD::kan* as the donor. *K. pneumoniae* with an integration of the plasmid on the chromosome were identified by plating on LB agar with $Rif_{30}Kan_{50}$ as described above.

Isogenic mutants in KPPR1 (Δ*romA*, Δ*smr)* were generated using allelic exchange with the pKO3 vector as previously described (34). pKO3 is a vector that allows the use of sucrose as a positive selection for the loss of the vector. The appropriate DNA fragments generated by PCR using the primer sets indicated in Table 2 were cloned into pKO3.

2.3.4 Whole genome sequencing of the* smr *mutant. Total DNA from the *smr* mutant, VK82, was isolated using a genomic DNA purification kit (Qiagen) and the sample was submitted to the High Throughput Sequencing Facility (HTSF) at the University of North Carolina (UNC) for sequencing. An Illumina HiSeq 2000 instrument generated 2 x 75bp pair-end reads. A mapped genome assembly was produced using the "Map Reads to Reference" tool in CLC Genomic Workbench v7.5.1 with the published *K. pneumoniae* KPPR1 genome as the template (50). The *smr* and KPPR1 parent strain genomes were then compared using the "Basic Variant Detection" tool in CLC Genomic Workbench to identify mutations in the *smr* strain. Mutations were identified by aligning these genomes using Mauve (51).

2.3.5 Murine model of pneumonia. Five-to-eight week old, female C57BL/6 mice (Jackson Laboratories) were anesthetized by intraperitoneal (i.p.) injection with a mixture containing ketamine (8 mg/mL) and xylazine (1.6 mg/mL). Overnight bacterial cultures were diluted in phosphate-buffered saline and a 20 µL suspension was inoculated intranasally (i.n.) in two 10 µL aliquots for a total of ~$2x10^4$ colony forming units (CFU)/mouse as described (35). Total CFUs for each inoculum were confirmed by plating dilutions onto LB agar. After 24, 48, or 72 hours post inoculation (hpi), mice were euthanized by a lethal injection of 200 µL sodium pentobarbital (150mg/kg). Organs were removed and homogenized in 500 µL phosphate-buffered saline (PBS). Homogenized organs were serially diluted and plated to quantify CFU/g tissue.

2.3.6 Low speed centrifugation capsule assay. The mucoviscosity was determined as previously described (52). Briefly, overnight cultures were grown in LB and back diluted to an optical density at 600 nm (OD_{600}) of 0.2 in fresh media and grown at 37 °C. After 6 hours, 1.0 OD/mL was centrifuged for 5 minutes at 1000 x *g* and a post-centrifugation OD reading was measured.

2.3.7 Extraction and quantification of capsule. Uronic acid content was extracted and quantified as previously described (29). Briefly, overnight cultures were grown in LB and back diluted to an OD_{600} of 0.2 in fresh media and grown at 37 °C. After 6 hours, 500 µL of culture were added to 100 µL of Zwittergent and incubated at 50 °C for 20 minutes. Following incubation, 300 µL of the supernatant was added to 1.2 mL of absolute ethanol and incubated at 4 °C. The mixture was centrifuged for 5 minutes at maximum speed. The pellet was resuspended in 200 µL of water and added to 1.2 mL of tetraborate and incubated for 5 minutes at 100 °C. 20µL of phenolphenol was added and the absorbance read at 520nm. The glucuronic acid content was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micrograms per OD.

2.3.8 Measurement of promoter activity. To examine yersiniabactin expression *in* vitro, a previously reported transcriptional fusion containing 500bp upstream of the *ybtA* promoter cloned into the pPROBE vector was used (34, 53).The reporter plasmids were individually mobilized into *K. pneumoniae* strains by electroporation. The bacteria were grown overnight at 37°C in LB and subcultured to an OD600 of

0.2 and grown for 6 hours, with and without the presence of 200 μM 2,2'-dipyridyl (Sigma Chemical, St. Louis, MO). All strains were assayed in triplicate. GFP measurements were taken (Synergy HT microplate reader, BioTek Instruments, Winooski, VT) and readings were normalized to OD_{600}.

2.3.9 In vitro growth curves. To monitor bacterial growth *in vitro,* bacterial strains were grown overnight in M9-CAA at 37°C, back diluted to an OD_{600} of 0.05 in fresh media, placed into 250 mL flasks and shaken for 6 h at 37°C. Values for OD_{600} of each sample were recorded at the indicated intervals. Medium was supplemented with 100 μM 2,2'-dipyridyl (Sigma Chemical, St. Louis, MO) to examine bacterial growth under iron-limiting conditions.

2.3.10 Cross-feeding assay. This method is based on a previously described method with minor modifications (54). Bacteria were grown overnight at 37°C M9-CAA overnight at 37°C. Approximately $1x10^5$ CFU of each test strain was spread onto M9-CAA agar plates containing 100 μM 2,2'-dipyridyl. Feeder strains were then spotted (2.5 μL of the overnight culture) to test for growth complementation of the test strains previously spread on the plate.

To determine if purified yersiniabactin can allow growth of test strains, test strains were spread on M9-CAA as described above. Iron-free yersiniabactin (EMC Microcollections, Germany) was resuspended in ethanol and 10 μL of either 1 mM or 100 μM of yersiniabactin (diluted in water) was spotted onto filter disks on the plate to assess yersiniabactin-dependent growth complementation.

2.3.11 Statistical analysis. Statistical analyses were performed using GraphPad Prism, version 6.0 (San Diego, CA).

2.4 Results

2.4.1 The smr mutant is severely attenuated in a mouse model of pneumonia.

A previously conducted screen of transposon mutants of *K. pneumoniae* strain KPPR1 identified genes required for colonization and survival in the lungs of infected mice (35). Thirteen mutants containing disruptions within *ramA* or an adjacent gene *orf82* failed to be recovered in the lungs and spleen of infected mice. RamA is a transcriptional regulator previously linked to survival of *Salmonella* in RAW 264.7 macrophages and virulence in BALB/C ByJ mice (42, 43). This led us to hypothesize that the *ramA* locus is important for the ability of *K. pneumoniae* to infect the lung. To test this, we constructed the *smr* mutant, where *ramA* and the two flanking genes (*orf82* and *romA*) were targeted for deletion, and tested this strain in a mouse model of pneumonia (Fig. 1). The *smr* mutant had a slight decrease in bacterial burden at 24 hpi compared to KPPR1 (wild-type, hereafter referred to as WT). At 72 hpi, nearly 5-logs fewer CFU were recovered from mice infected with the *smr* mutant compared to WT-infected mice. The spleens of mice infected with the WT strain had nearly 10^7 CFU/g of tissue while the *smr* mutant was rarely detectable in the spleen at 72 hpi, reflecting a dissemination or systemic survival defect. Together these data indicated the *smr* mutant is essentially avirulent in this infection model.

2.4.2 Deletions of individual genes in the targeted smr locus do not recapitulate the phenotype of the smr mutant. To identify the gene(s) responsible for the phenotype of the *smr* mutant, we made in-frame deletions of each of the three genes (Δ*ramA*, Δ*romA*, Δ*orf82*) in the *smr* locus and tested them in our pneumonia model (Fig. 2A). All three mutant strains displayed phenotypes like the WT strain, suggesting that loss of a single gene was not sufficient to affect virulence (Fig. 2B). We concluded that neither *ramA, orf82,* nor *romA*, individually contributed to virulence in this model and were not responsible for the phenotype of the *smr* mutant.

In examining the region more closely, we noted that an RND (resistant/nodulation/division superfamily) efflux pump system was encoded just upstream of *orf82*, and that the *smr* deletion could have impacted the promoter driving expression of this locus (Fig. 2A). RND efflux systems have been shown to play roles ranging from resistance to human antimicrobial peptides in *Pseudomonas* to flagellar motility in *Burkholderia* (55). To determine if this RND system contributed to the *smr* phenotype, we constructed two additional mutants, one deleting the *rnd* genes, and the other deleting *orf82*, *ramA* and *romA* (the original three genes targeted in the *smr* mutant) but leaving the putative *rnd* promoter intact (Δ*rnd and* Δ*orf82ramAromA*). When tested in mice, the Δ*rnd* mutant colonized mice as efficiently as the WT strain (Fig. 2B). Intriguingly, the second mutant (Δ*orf82ramAromA*) lacking the same three genes as the *smr* mutant also had no virulence defect.

2.4.3 Resequencing of the smr mutant reveals a large deletion. As targeted genetic mutations of the *smr* locus failed to recapitulate the *smr* phenotype, we hypothesized that a secondary mutation was acquired during construction of the *smr* mutant. Therefore, we sequenced the genome of the *smr* strain to identify potential genetic changes that could have contributed to the virulence defect. The sequence data was mapped and compared to the parental strain genome, and it was revealed that the deletion in the *smr* mutant was larger than intended. Instead of the targeted deletion of *orf82, ramA* and *romA*, the *smr* mutant had a deletion of 87,290 base pairs spanning 78 putative open reading frames.

2.4.4 A component of the enterobactin transport system contributes to virulence. To identify the factor(s) responsible for the virulence defect of the *smr* mutant, we divided the region deleted in the *smr* mutant into three segments (Fig. 3A), and constructed three smaller deletion mutants (Δ*smr_A*, Δ*smr_B*, Δ*smr_C*) each deleted for approximately one third of the genes that were deleted in the *smr* mutant. The putative *orfs* deleted in each of these segments are listed in Table 3. These mutants were tested in the mouse pneumonia model to assess their effect on virulence (Fig. 3B). At 24 and 72 hpi both the Δ*smr_A* mutant and the Δ*smr_B* mutant had bacterial burdens comparable to mice infected with the WT strain. However, the Δ*smr_C* mutant had over one log fewer CFU/g at 24 hpi and nearly 6-logs fewer CFU/g at 72 hpi when compared to mice infected with the WT strain. Thus, the Δ*smr_C* mutant recapitulated the phenotype of the *smr* mutant whereas Δ*smr_A* and Δ*smr_B* behaved like the WT strain.

Encoded within the region deleted in Δ*smr_C* are genes necessary for synthesis, export and import of the siderophore enterobactin. Based on the attenuation of the Δ*smr_C* mutant, we hypothesized that a component of the enterobactin transport system was responsible for the virulence defect of the *smr* mutant. We did not believe the siderophore itself was responsible, as an Δ*entB* mutant that is unable to synthesize enterobactin and salmochelin is only modestly attenuated in this mouse pneumonia model (34). The enterobactin receptor, FepA, also was not implicated as FepA is encoded within the region deleted in the Δ*smr_B* mutant.

Siderophore transport involves several membrane proteins. For enterobactin, EntS and TolC are required for export, whereas FepA, FepDGC, and Fes are required for import. In addition, the periplasmic protein FepB is required for both enterobactin and salmochelin import. Because previous studies had implicated siderophore transport components in virulence (56), we targeted specific components of the enterobactin siderophore transport system and tested loss of function mutants (Δ*entS*, Δ*fes,* Δ*fepB, fepD::*pKAS46) in the mouse pneumonia model (Fig. 4A). We also tested a different enterobactin synthesis mutant (Δ*entsyn*) to confirm our previous findings seen with the Δ*entB* mutant (34). We found that only the Δ*fepB* mutant recapitulated the phenotype of the *smr* mutant as demonstrated by the attenuation in the lungs and the lack of dissemination at 24 and 72 hpi (Fig. 4B). Consistent with previously studies, neither the Δ*entsyn* mutant (Fig. 4B) nor the Δ*entB* mutant (Fig. 5) recapitulated the *smr* phenotype (34). Thus, the periplasmic transport protein, FepB, contributes to virulence in a manner distinct from

enterobactin and salmochelin uptake alone. How FepB affects virulence is not clear but it does not affect the amount of capsule produced (Fig. 5A) or the mucoviscosity of the capsule, as the uronic acid content and sedimentation of the Δ*fepB* mutant was comparable to WT (Fig. 5B). Similarly, loss of *fepB* did not affect expression of the yersiniabactin system, consistent with previously published results of an enterobactin mutant (Fig. 5C)(34). A variety of different approaches were used to complement the Δ*fepB* mutant, but all were unsuccessful. Problems with *fepB* complementation are not unprecedented and were also reported for a *fepB* mutant of *Salmonella* (56). To ensure the phenotype observed with Δ*fepB* was not a consequence of secondary mutations, a second *fepB* mutant was constructed and found to recapitulate the virulence and growth phenotype of the original *fepB* mutant (data not shown). We were able to sequence across the deletion junction of both of these *fepB* mutants and obtained the expected sequence, suggesting a larger deletion of the region surrounding *fepB* had not occurred. Thus, we can conclude that deletion of *fepB* alone resulted in the observed attenuation.

2.4.5 A fepB mutant resembles a Δentb Δybts double mutant. A previous study in our lab tested a Δ*entB*Δ*ybtS* mutant that is deficient for all siderophore production and found that it was severely attenuated in the lung and failed to cause a systemic infection (34). In comparing the defect of the Δ*fepB* strain to our previous work with siderophore mutants, we noticed that the phenotype of the Δ*fepB* mutant was similar to the Δ*entB*Δ*ybtS* mutant. Because the attenuation of the Δ*fepB* mutant in mice was much greater than the attenuation seen with the Δ*entB* mutant, we hypothesized that

the role of FepB was not limited to enterobactin import and that it might be involved in an additional iron acquisition system. We tested the Δ*fepB* mutant together with the Δ*entB*Δ*ybtS* mutant to determine if its virulence defect resembles a Δ*entB*Δ*ybtS* mutant *in vivo*. We included an enterobactin/salmochelin synthesis mutant, Δ*entB* as a control (Fig. 5). The Δ*fepB* and Δ*entB*Δ*ybtS* mutants had similar attenuation levels, which differed from Δ*entB*. This finding raises the question of whether FepB may be required for acquisition of iron via systems other than enterobactin and salmochelin.

To address the role of FepB in iron uptake, and to determine if the virulence defect could be due to reduced iron acquisition, we turned to an *in vitro* growth model. The Δ*fepB,* Δ*entB,* and the Δ*entB*Δ*ybtS* mutants were grown in defined media with our without iron-chelating agent, 2-2' dipyridyl (DP). No difference in the growth rate of the mutants compared to WT was observed when strains were grown in the absence of DP, indicating that the mutants grow normally when iron levels are sufficient (Fig. 7A). However, in the presence of 100 mM DP, growth of the Δ*fepB* and Δ*entB*Δ*ybtS* mutants was severely restricted (Fig. 7B). Interestingly, growth of the Δ*entB* mutant (required for synthesis of enterobactin/salmochelin), was restricted compared to the WT strain, but the triple siderophore mutant (Δ*entB*Δ*ybtS*) as well as the Δ*fepB* mutant, showed an even greater attenuation in growth than the Δ*entB* mutant. These data suggest that FepB contributes to growth in an iron-dependent manner that is distinct from its known role in enterobactin and salmochelin uptake.

2.4.6 Import of yersiniabactin is unaffected in a fepB mutant. The Δ*fepB* mutant had a stronger phenotype than an enterobactin/salmochelin synthesis mutant and resembled that of a triple siderophore mutant for both virulence and growth under iron limitation. Yersiniabactin is the only known siderophore produced by the Δ*entB* mutant but not the Δ*entB*Δ*ybtS* mutant. Thus, we wanted to assess whether or not the Δ*fepB* mutant is defective for utilization of yersiniabactin. To test this, we performed a cross-feeding experiment to determine if the growth defect of the Δ*fepB* mutant in iron limited conditions could be restored in the presence of yersiniabactin by co-culturing Δ*fepB* with a yersiniabactin-producing strain. If FepB is required for import of yersiniabactin, we would predict that a feeder strain producing yersiniabactin would be unable to restore growth of the Δ*fepB* mutant. In this assay, test strains are spread onto M9-CAA agar containing 100 μM DP, and feeder strains are then spotted on the surface of the plates. The WT, Δ*entB,* Δ*entB*Δ*ybtS,* and Δ*fepB* were used as test strains, and WT, Δ*entB* (capable of producing yersiniabactin), and Δ*ybtS* (does not produce yersiniabactin) were used as feeder strains. As expected, the Δ*ybtS* mutant was not able to complement the growth defect of the Δ*fepB* mutant, as the Δ*fepB* mutant should not be able to use the enterobactin produced by this strain (Fig. 8A). The WT strain and the Δ*ybtS* mutant were able to complement the growth of the Δ*entB*Δ*ybtS* mutant strain, as expected (Fig. 8B). Importantly, the Δ*entB* mutant and the WT strain were able to restore growth of the Δ*entB* mutant (as expected), as well as the Δ*fepB* mutant. This data suggests yersiniabactin can still be imported by a Δ*fepB* mutant strain.

To determine if the complementation of the Δ*fepB* growth defect by a yersniabactin-producing strains in the cross-feeding experiment was due production of yersiniabactin rather than due to the production of other secreted bacterial products, we performed a similar experiment by spotting purified apo-yersiniabactin instead of feeder strains. As above, test strains (WT strain, Δ*fepB* and Δ*entB*Δ*ybtS* mutants*)* were spread onto M9-CAA agar containing 100 μM DP. Various concentrations of apo-yersiniabactin were applied to paper discs that were placed on the agar plate to test for growth restoration, and thus, the ability to utilize yersiniabactin (Fig. 7C). The WT strain was able to grow even without yersiniabactin supplementation. The Δ*entB*Δ*ybtS* and Δ*fepB* mutants had a growth defect and did not grow around the vehicle control disc (dH_2O). However, upon addition of yersiniabactin, the growth defect of the Δ*entB*Δ*ybtS* mutant was restored in a concentration dependent manner; this is an expected result because this strain is still able to import exogenous yersiniabactin. Addition of apo-yersiniabactin also restored growth of the Δ*fepB* mutant (Fig. 8C). Together, these data suggest that FepB is not required for yersiniabactin import, and the virulence defect of the Δ*fepB* mutant is due to a mechanism unrelated to yersiniabactin import.

2.5 Discussion

The repertoire of confirmed virulence factors for *K. pneumoniae* has changed little during the past two decades (2, 4). Although a number of large screens for virulence determinants of *K. pneumoniae* have been performed (35-41), unfortunately there have been few follow up analyses of the results from these

screens. In a screen of signature tagged mutants in a pneumonia model of infection, we identified a locus that included *ramA* as potentially important for virulence (35), and a recent study suggested that over expression of *ramA* affects virulence and leads to modifications of LPS (46). In this study, we constructed a mutant (*smr*) that deleted this locus and found it was cleared from the lung following intranasal inoculation and that it was unable to spread systemically. Why deletion of *ramA* or the surrounding genes did not result in a virulence defect in the lung and/or spleen when eleven insertions in this region were identified in the signature tagged mutagenesis (STM) screen is not clear (35). One possibility is that in the STM screen, each insertion mutant was screened essentially in competition with 95 other mutants, most of which will behave like the WT strain. Therefore, a *ramA* mutant may have a competitive disadvantage when at a ratio of ~1:100 with WT *K. pneumoniae* but will not exhibit a defect when inoculated on its own. RamA has been implicated as regulating pathways important for multi-drug resistance (44, 45) and thus it may still be important in the context of antibiotic treatment, or in a strain background that is not hyper-virulent.

Subsequent analysis of the *smr* mutant indicated the virulence defect was not due to deletion of the *ramA* locus but rather to deletion of *fepB,* a gene encoding a protein required for enterobactin and salmochelin import (57-59). The *fepB* mutant had a more severe growth defect in iron-limited media and a more severe *in vivo* defect compared to an enterobactin synthesis mutant (Δ*entB*); the Δ*entB* mutant would also be deficient in production of salmochelin. The contribution of the siderophores enterobactin, salmochelin, and yersiniabactin to *Klebsiella* virulence

previously have been examined and individually were found to contribute minimally to infection (33, 34, 60). Data presented here reveal that while enterobactin/salmochelin may be dispensable for virulence in a lung model of *K. pneumoniae* infection by a strain also able to produce yersiniabactin, the enterobactin/salmochelin importer, FepB, is necessary to establish infection. Furthermore, in both *in vitro* and *in vivo* conditions, the Δ*fepB* mutant resembles a Δ*entB*Δ*ybtS* mutant, which is unable to produce any of the three siderophores encoded by this strain (enterobactin/salmochelin/yersiniabactin). Together these observations suggest that FepB contributes to virulence and growth under iron limitation in an unanticipated way.

Siderophores are synthesized in the cytoplasm and require machinery for export and subsequent import following iron sequestration. Enterobactin is synthesized by EntABCDEF, and is exported to the periplasm via the inner membrane protein, EntS, and subsequently through the outer membrane via the membrane channel protein, TolC (61). Once bound to ferric iron, enterobactin-Fe^{3+} binds the outer membrane siderophore receptor, FepA, and is translocated into the periplasm by a TonB-dependent mechanism. In the periplasm, enterobactin-Fe^{3+} then binds the periplasmic chaperone FepB and is shuttled to the inner membrane where it interacts with the inner membrane transport complex, FepDGC, and is ultimately released into the cytoplasm (58, 62, 63). Salmochelin utilizes a similar export apparatus but is imported via the bacterial outer membrane receptor, IroN and FepB (64). Export and import of yersiniabactin appears to be similar, although several steps in yersiniabactin transport remain to be elucidated (61). Specifically,

no periplasmic protein (FepB equivalent) has been identified for the yersiniabactin import system. Because of the similarities in the phenotypes of the Δ*fepB* and triple siderophore mutants, and because no FepB equivalent has been identified for the yersiniabactin system, we initially hypothesized FepB may be involved in yersiniabactin import. However, our results show that a Δ*fepB* mutant can still utilize yersiniabactin for growth, and thus the role of FepB in growth under iron limitation and virulence remains unclear. A recent crystal structure of FepB indicates FepB can form a trimer (65) and thus possibly could co-ordinante another a target other than Fe-enterobactin, but this has yet to be demonstrated.

A contribution of the periplasmic enterobactin transporter, FepB, to pathogenesis also was observed for *Salmonella enterica* (56). *Salmonella* produces both enterobactin and salmochelin and both siderophores require FepB for import (26). However, Nagy et al. found that a *fepB* mutant had lower colonization levels in mice than a *fepA-iroN* double mutant (encoding the outer membrane receptors for enterobactin and salmochelin) in a gastric model of infection (56, 66). This is comparable to our results in *K. pneumoniae* and suggests that the role of FepB in virulence extends beyond siderophore transport. The fact that this phenomenon has been reported for two Gram-negative pathogens hints that this may be a conserved mechanism in other bacterial species. One possible explanation for this observation is that in a Δ*fepB* mutant, enterobactin is not recycled properly, accumulates extracellularly and perhaps this is detrimental to the bacteria given that enterobactin can enhance copper toxicity (67). However, in this scenario, the Δ*smr_C* mutant (which is a Δ*entB*Δ*fepB* double mutant as well as other deleted genes [see Table 3

for list]) should relieve this phenotype, as it would be unable to produce enterobactin. However the data presented here suggests this is not the case as the Δ*smr_C* mutant has a virulence defect comparable to a Δ*fepB* mutant.

Interestingly, recent studies have noted that the complement of siderophore systems produced by an individual strain of *K. pneumoniae* has a significant impact on its ability to colonize versus cause an infection, or its ability to cause invasive disease associated with the hypervirulent phenotype (23). In an analysis of a broad sampling of over 300 strains, Holt et al. found that only 33% of an individual strain's genome is part of the core *Klebsiella* genome, and the remaining 67% is composed of 'accessory' genes that vary significantly from strain to strain (23). Until recently, the gene profiles necessary to cause the different types of infections associated with *K. pneumoniae* were not clear. However, the recent bioinformatics analyses of large strain collections, combined with information on the type of infection, has revealed that some specific gene profiles are associated with colonization vs. infection vs. invasive disease. For example, the presence of *rmpA* (a regulator of capsule) as well as the genes required for production and use of the siderophores aerobactin, salmochelin, and yersiniabactin were highly associated with strains isolated from infections versus carriage alone (23). Interestingly, an additional five loci were associated with invasive infections (vs. non-invasive infections or carriage), including *fepB*. This is consistent with the requirement we observed for *fepB* to cause disseminated infection in mice, and what has been observed for *Salmonella* (56).

With antibiotic resistance on the rise, the development of new therapeutics to combat infection by multi-drug resistant bacteria is an urgent need (68). Siderophore

systems present an attractive target for drug development due to the conservation of these systems amongst Gram-negative pathogens (69). Immunization with the yersiniabactin receptor, FyuA, or the siderophores themselves (yersiniabactin and aerobactin) was protective when tested in a murine model of *E. coli* urinary tract infection (70-72). FepB may be an especially attractive target to consider for drug development as it is required for disseminated infections and is found in a wide variety of bacteria. In addition to being potential targets for drug development, siderophores represent an attractive system to exploit as a drug delivery mechanism to overcome the permeability barrier of the outer membrane. In essence, the siderophore can be used as a "Trojan horse" to target a siderophore-drug conjugate to the siderophore-iron transport systems (69). This would allow delivery of drugs to the periplasm and potentially to the cytoplasm. From the work presented here and with *Salmonella*, one such periplasmic target could be FepB itself. Drug-siderophore conjugates have been developed and a catechol-cephalosporin conjugate, Cefiderocol (S-649266), was found to have lower MIC_{90} values than the antibiotics cefepime, piperacillin/tazobactam, and merepenem, when tested against several Gram-negative bacteria including MDR and carbapenem-resistant strains (73-75). Cefiderocol displayed antibacterial properties when tested *in vivo* and is currently being tested in a Phase 3 clinical trial against carbapenem resistant Gram-negative infections in humans (74, 76). Thus, investigations probing the mechanisms of siderophore transport can provide the basis for promising new therapeutics.

2.6 Figures and tables

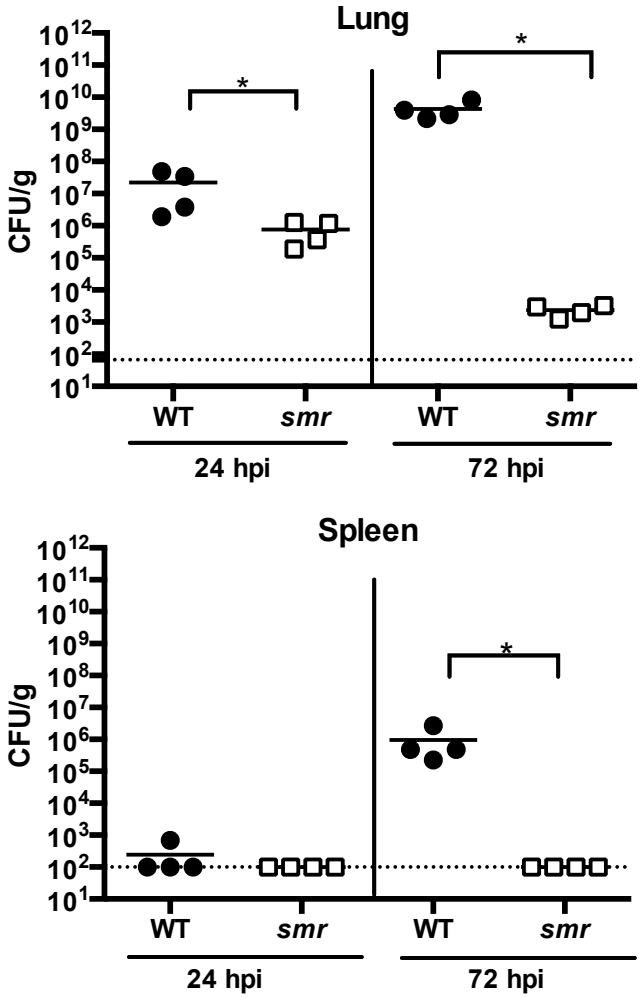


Figure 2.1. The *smr* mutant is attenuated in a mouse model of pneumonia. Mice were inoculated i.n. with $2x10^4$ CFUs of either the wild-type strain (KPPR1S; black circles) or the *smr* mutant (VK82; white squares). At 24 or 72 hours post inoculation (hpi), mice were euthanized and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. Data are from an individual representative experiment. Mann-Whitney tests were performed for statistical analysis; *=p<0.05.

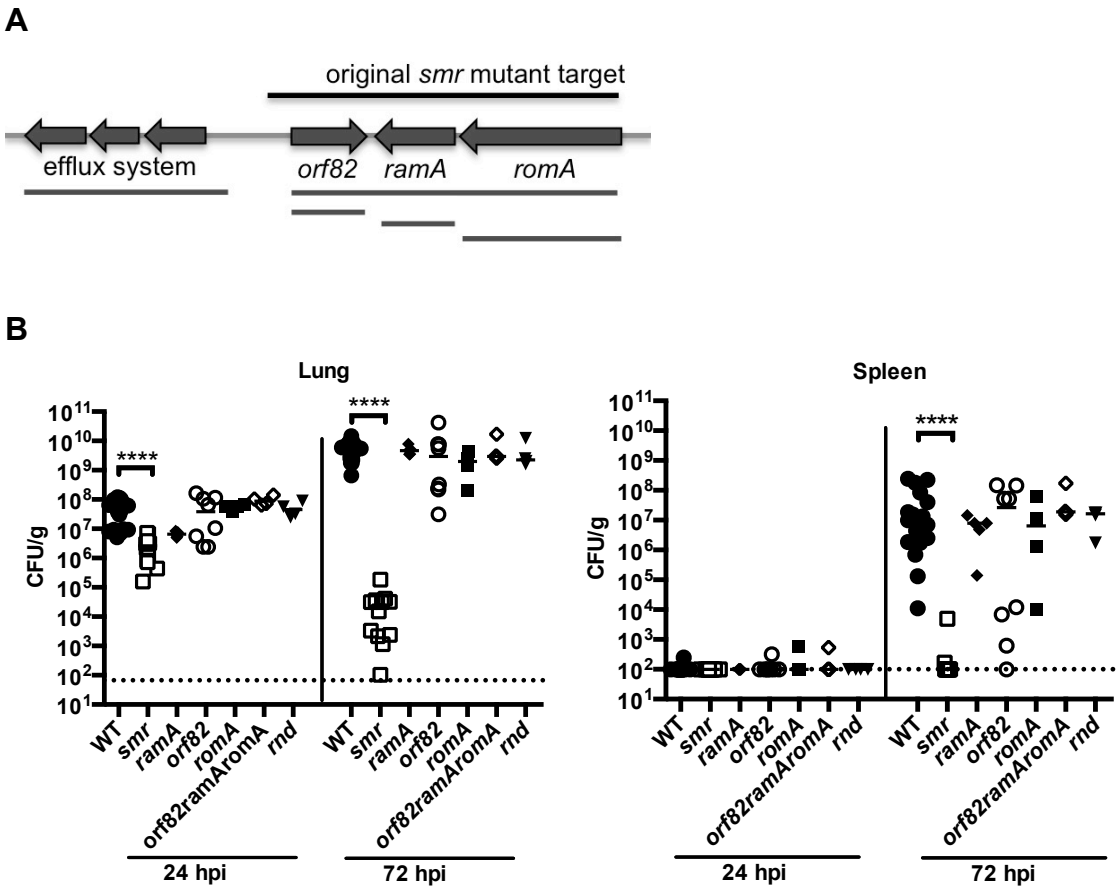


Figure 2.2. Schematic of *smr* targeted region and *in vivo* phenotype of mutants. (A) Schematic depicting open reading frames within or adjacent to the *smr* target region (not to scale). Lines indicate regions deleted in the indicated mutants. (B) Mice were inoculated i.n. with $2x10^4$ CFUs of the wild-type strain (KPPR1S; black circles) or Δ*smr* (VK082; white squares), Δ*ramA* (VK174; black diamonds), Δ*orf82* (VK270; white circles), Δ*romA* mutants (VK131; black squares), Δ*orf82ramAromA* (VK266; white diamonds), or Δ*rnd* (VK269; upside down triangles) mutants. After 24 or 72 hpi, mice were sacrificed, and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate that CFU were below the limit of detection. These data were compiled from several independent experiments. Mann-Whitney tests were performed for statistical analysis; ****=p<0.0001.

A

B

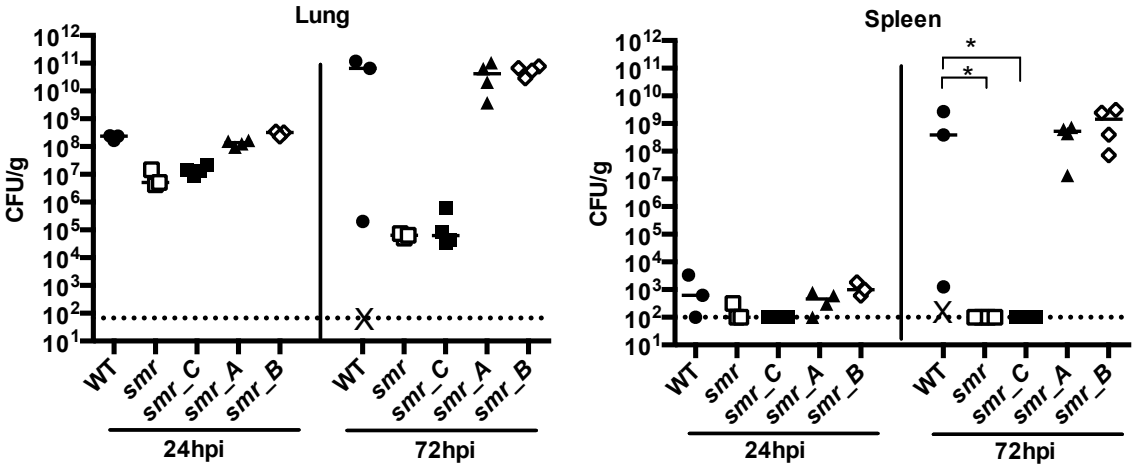


Figure 2.3. The *smr* mutant phenotype is recapitulated by a smaller targeted deletion. (A) Schematic depicting targeted sub-regions of the *smr* mutant (not to scale). (B) Mice were inoculated i.n. with $2x10^4$ CFUs of WT (KPPR1S; black circles), Δ*smr* (VK082; open squares),or Δ*smr_A* (VK274; black triangles), Δ*smr_B* (VK275; open diamonds), or Δ*smr_C* (VK276; black squares). After 24 or 72 hpi, mice were sacrificed, and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicates CFU were below the limit of detection. X indicates a mouse that succumbed to infection prior to 72 hpi. These data are from an individual representative experiment. Mann-Whitney tests were performed for statistical analysis; *=p<0.05.

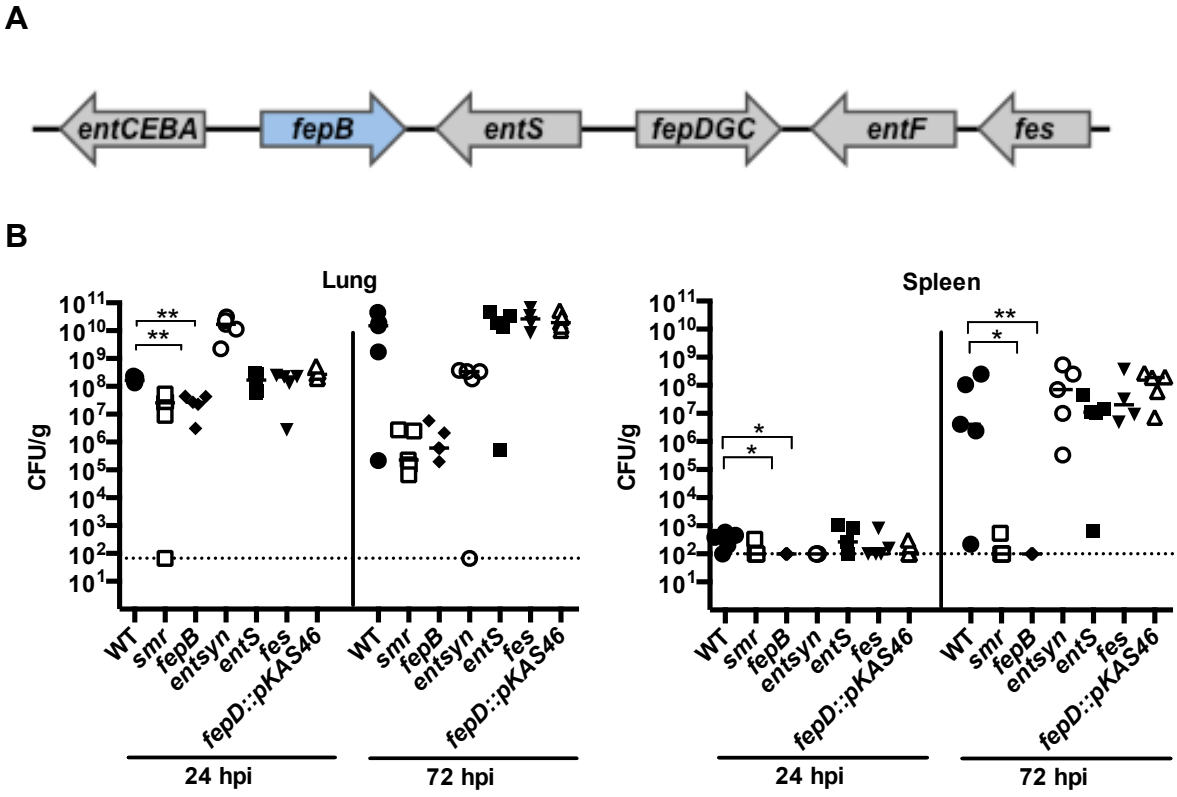


Figure 2.4. FepB is responsible for *smr* phenotype. (A). Schematic of the enterobactin genes encoded within the Δ*smr_C* region. (B) Mice were inoculated i.n. with 2x10^4 CFUs of the WT (KPPR1S; black circles), *smr* mutant (VK082; open squares), Δ*fepB* mutant (VK412; black diamonds), Δ*entsyn* (VK321; open circles), Δ*entS* mutant (VK411; black squares), Δ*fes* mutant (VK320; black upside down triangles), or *fepD::kan* mutant (VK413; open triangles). After 24 or 72 hpi, mice were sacrificed, and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. The data are from an individual representative experiment. Mann-Whitney tests were performed for statistical analysis; *=p<0.05. **=p<0.01.

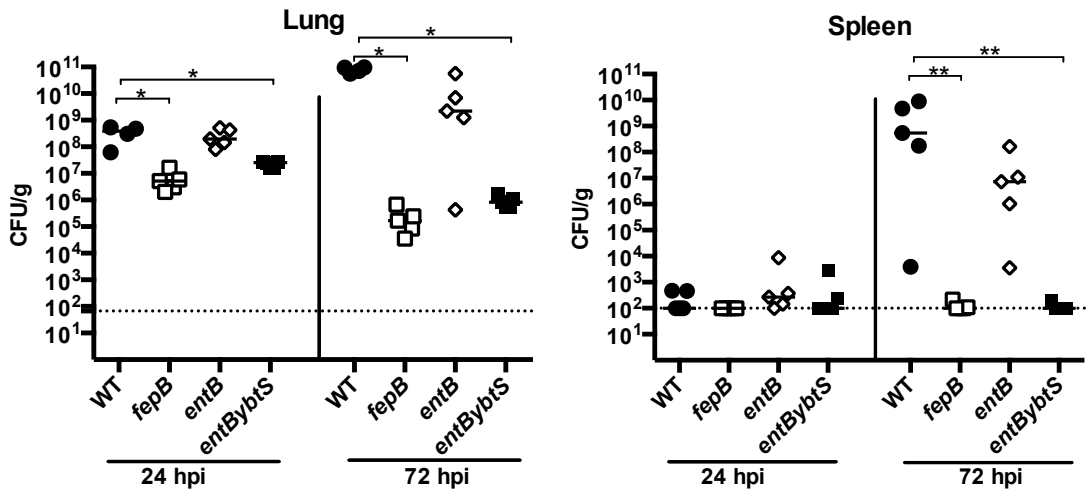


Figure 2.5. A Δ*fepB* mutant resembles a triple siderophore mutant *in vivo*. Mice were inoculated i.n. with $2x10^4$ CFUs of the WT (KPPR1S; black circles), Δ*fepB* mutant (VK412; open squares), Δ*entB* mutant (VK087; open diamonds), or Δ*entBybtS* mutant (VK089; black squares). After 24 or 72 hpi, mice were sacrificed, and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. The data are from an individual representative experiment. Mann-Whitney tests were performed for statistical analysis; *=p<0.05, **=p<0.01.

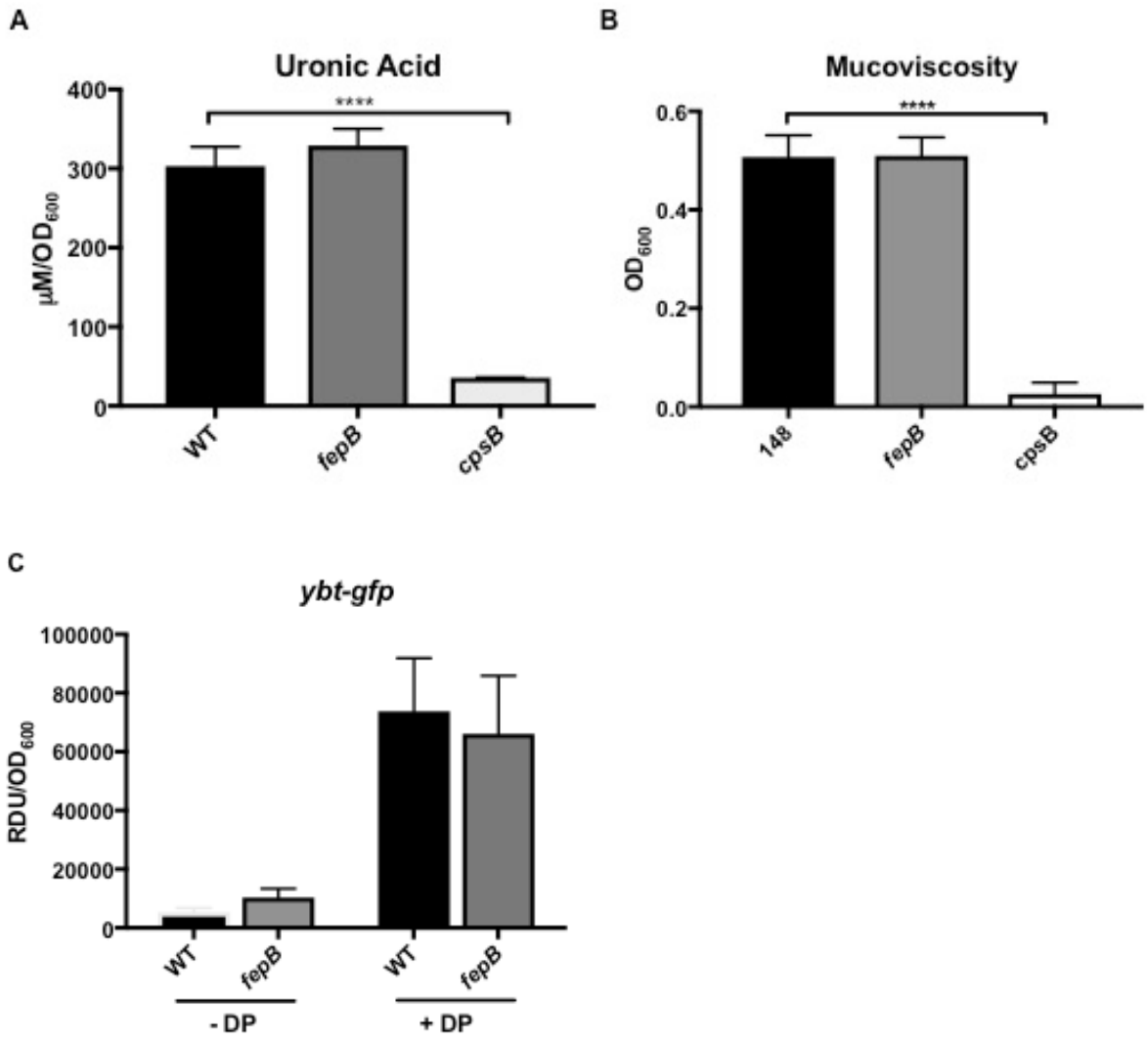


Figure 2.6. Capsule production and yersiniabactin expression is unchanged in the Δ*fepB* mutant. Overnight cultures of the WT strain, Δ*fepB* mutant, and a capsule mutant (*cpsB::kan*) were backdiluted to an OD_{600}=0.2 and grown in LB for 6 hours and total capsule production was measured using the (A) uronic acid assay and the (B) low-speed centrifugation assay to measure mucoviscosity. The WT strain and Δ*fepB* mutant containing the yersiniabactin synthesis gene promoter, *ybtA*, cloned into the pPROBE *gfp* reporter construct, were grown overnight and backdiluted to an OD_{600}=0.2 and grown in LB for 6 hours with (+DP) and without (-DP) 200uM 2,2'-dipyridyl. These data are from strains grown in triplicate from an individual experiment. Student t-tests were performed for statistical analysis; ****=p<0.0001.

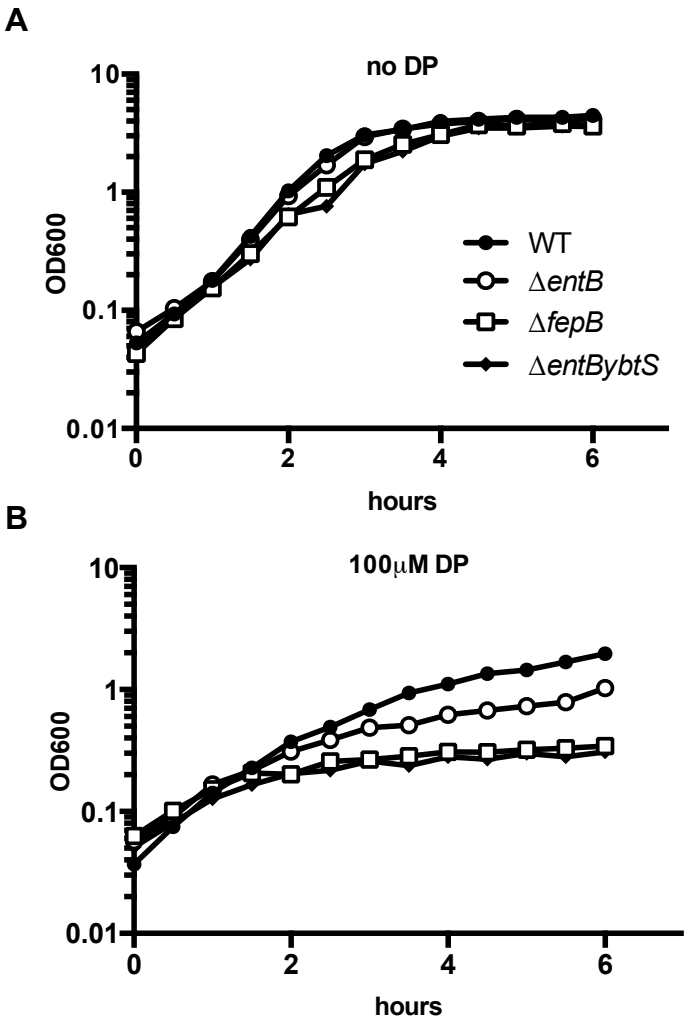
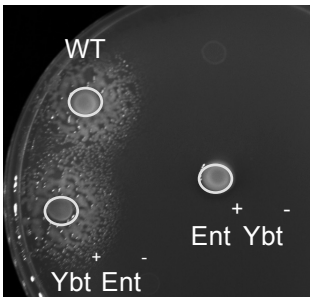


Figure 2.7. The Δ*fepB* mutant has a significant growth defect in iron limited conditions. The WT strain (KPPR1S; black circles), Δ*fepB* mutant (VK412; open squares), Δ*entB* mutant (VK087; open circles), and Δ*entBybtS* mutant (VK089; black diamonds) were grown in M9-CAA media (A) or M9 media supplemented with 100uM 2,2'-dipyridyl (B). The optical density (OD) at 600nm was monitored for 6 hours. The data are from an individual representative experiment.

A



B

	feeder strain		
Test strain	**Wild-type**	**Ent⁻Ybt⁺**	**Ent⁺Ybt⁻**
WT	+	+	+
Δ*fepB*	+	+	-
Δ*entB*	+	+	+
Δ*entB*Δ*ybtS*	+	+	+

C

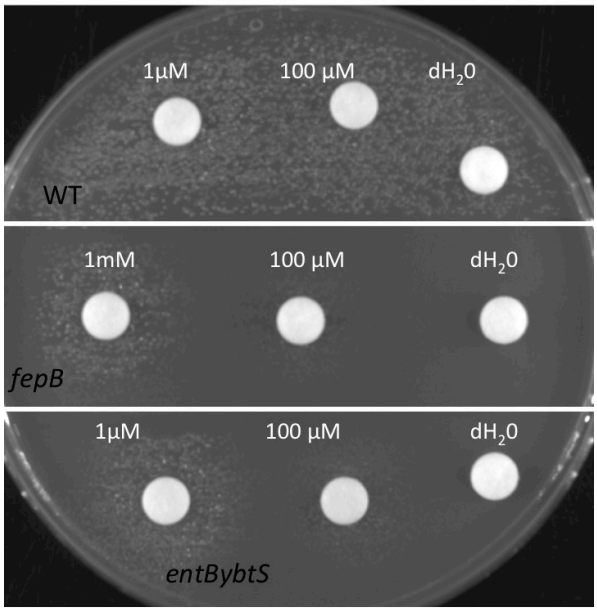


Figure 2.8. Addition of yersiniabactin restores the growth defect of the Δ *fepB* mutant in iron-limited conditions. Test strains were grown in M9-CAA media and spread plate onto M9-CAA agar containing 100µM DP. (A) Plate testing the Δ*fepB* mutant (spread plated) is shown. Feeder strains (WT, Δ*entB*, Δ*ybtS)* were then spot plated to test for complementation (growth restoration around feeder spot). (B) Summary of results represented as "+" for growth and "–" for no growth of the WT strain, Δ*fepB* mutant, Δ*entB* mutant, or the Δ*entB*Δ*ybtS* mutant. (C) Addition of purified yersiniabactin (1mM or 100µM) or water vehicle to the wild-type strain, Δ*fepB* mutant, or the Δ*entB*Δ*ybtS* mutant. Shown are data from an individual experiment and is representative of data obtained from several independent experiments.

Table 2.1
Primers used in this study

Primer	Sequence [a] (5' to 3')	Description
MP66	TGACTA**GATATC**GCTGATTACCGAAGCGGACTG	5' flank forward Δ*ramA*
MP67	TGCATA**TCTAGA**GGAAATCGTCATATGCTCTCT	5' flank reverse Δ*ramA*
MP68	TGCATA**TCTAGA**CACTGAGGCGCGCCTCTCCTG	3' flank forward Δ*ramA*
MP69	TCGATA**GCGGCCGC**CGACTGGCGCTGTACATCGCG	3' flank reverse Δ*ramA*
MP114	TGACTA**GATATC**TCGCCCGAGGGCGTCGTAAAC	5' flank forward Δ*orf82*
MP71	TGCATA**TCTAGA**CTCGAGCGGTAAACCAGGAGA	5' flank reverse Δ*orf82*
MP72	TGCATA**TCTAGA**CAGTGGATGTTTCATGTCATG	3' flank forward Δ*orf82*
MP115	TCGATA**GCGGCCGC**GGGATGAACCGTATCAACGGC	3' flank reverse Δ*orf82*
MP124	TGACTA**GATATC**CGATTTTGCCTGCTATGCGCA	5' flank forward Δ*rnd*
MP125	TGCATA**TCTAGA**CATCGGCGGGGGTAAGCGCGG	5' flank reverse Δ*rnd*
MP126	TGCATA**TCTAGA**GTTCACCCGGTCGCCCAGCGG	3' flank forward Δ*rnd*
MP127	TCGATA**GCGGCCGC**GCCACGGCAGGTCTGGCAGCA	3' flank reverse Δ*rnd*
MP103	TGACTA**GATATC**GGCGTCGTAAACTTTGGGTTA	5' for Δ*orf82ramAromA*
MP104	TGCATA**TCTAGA**TTCCAGTGGATGTTTCATGTC	5' rev Δ*orf82ramAromA*
MP105	TGCATA**TCTAGA**CTGACCAGACAAAAGCCCCCA	3' for Δ*orf82ramAromA*
MP106	TCGATA**GCGGCCGC**CGACAGCTGGCACATTTCGTT	3' rev Δ*orf82ramAromA*
MP171	TCGATA**GCGGCCGC**CTGTGCGCTCCCTGCGCCATG	5' flank forward *smr*ΔA
MP172	TGCATA**TCTAGA**CTGGCGAAGTAGGGGAGGGGG	5' flank reverse *smr*ΔA
MP173	TGCATA**TCTAGA**ACCGAGATTTAATCTCTCCAC	3' flank forward *smr*ΔA
MP174	TGACTA**GATATC**TCCAACTTTTGGGGTGCAGTC	3' flank reverse *smr*ΔA
MP175	TGACTA**GATATC**CCATGCGCTTGCGCGGGCCTA	5' flank forward *smr*ΔB
MP176	TGCATA**TCTAGA**GCTTACGATATTTCCAATCCG	5' flank reverse *smr*ΔB
MP177	TGCATA**TCTAGA**TGCGCCTCATTAAGCGGGTCC	3' flank forward *smr*ΔB
MP178	TCGATA**GCGGCCGC**AATGACAGAATGTTAAGGACA	3' flank reverse *smr*ΔB
MP179	TGACTA**GATATC**TGCGCCTCATTAAGCGGGTCC	5' flank forward *smr*ΔC
MP180	TGCATA**TCTAGA**AGTCACGCTATACATAGGGTT	5' flank reverse *smr*ΔC
MP181	TGCATA**TCTAGA**GCGCACCCTGGCGGAGCCACT	3' flank forward *smr*ΔC
MP182	TCGATA**GCGGCCGC**ATTAACGACAGGTTGCGCGAA	3' flank reverse *smr*ΔC
MP282	TGACTA**GATATC**AGAATTTAACAACACCGAAAC	5' flank forward Δ*ybdB2entABEC*
MP192	TGCATA**TCTAGA**ACCGCGGTGCTGGGCTAAGAA	5' flank reverse Δ*ybdB2entABEC*
MP193	TGCATA**TCTAGA**AGCCAGTGACGTTTCCATATC	3' flank forward Δ*ybdB2entABEC*
MP194	TCGATA**GCGGCCGC**GCAACCTCGCTCCACTGGCGC	3' flank reverse Δ*ybdB2entABEC*
MP195	TGACTA**GATATC**GGATATAGAGCTCGGAAGGCT	5' flank forward Δ*fepB*
MP196	TGCATA**TCTAGA**GAAGTTCACGTCATCGCATCC	5' flank reverse Δ*fepB*
MP197	TGCATA**TCTAGA**CTGTTCGGCTAACGCGGGCTG	3' flank forward Δ*fepB*
MP198	TCGATA**GCGGCCGC**CGCTGGCGCTTGTCGGCGTGC	3' flank reverse Δ*fepB*
MP199	TGACTA**GATATC**GCGCTCTGCTGGTGCTCCAGC	5' flank forward Δ*entS*
MP200	TGCATA**TCTAGA**ATTGTCAACGAAAGTTAAGTA	5' flank reverse Δ*entS*
MP201	TGCATA**TCTAGA**GGATTGTCGGTTCATTACAGC	3' flank forward Δ*entS*
MP202	TCGATA**GCGGCCGC**CGGGTGAGCGTCTGCATCAGC	3' flank reverse Δ*entS*
MP207	TGACTA**GATATC**GCGCGGCAACCAGCGGTAAAC	5' flank forward Δ*fes*
MP208	TGCATA**TCTAGA**GGCCAACGCGAACCGATTATT	5' flank reverse Δ*fes*

MP244	TGCATA**TCTAGA**TGCGCCTCATTAAGCGGGTCC	3' flank forward Δ*fes*
MP231	TCGATA**GCGGCCGC**AATGACAGAATGTTAAGGACA	3' flank reverse Δ*fes*
MP313	TGACTA**GATATC**CCTTAGCCGCCGCGCTTA	5' forward *fepD::kan*
MP314	TGCATA**TCTAGA**TTGCGGGTGAGCGTCTGC	3' reverse *fepD::kan*
ramKOA5'INsmaI	TCC**CCCGGG**ACCGCTTTGACGGTCAT	5' flank forward *smr*
ramKOA3'IN2	CGCGGTAGATTCCAAACATA	5' flank reverse *smr*
ramKOB5'IN	ATCCTGACCAGACAAAAGCCCCATCC	3' flank forward *smr*
ramKOB3'INSma	TCC**CCCGGG**GACAGCTGGCACATTTC	3' flank reverse *smr*
romA5'inXba	GC**TCTAGA**GCCAGTCCGCTTCGGTAA	5' flank forward Δ*romA*
romA5'in	CGACTTTCATCGCTTTCCTAATA	5' flank reverse Δ*romA*
romA3'in	CGTCATATGCTCTCTCCTCTGAT	3' flank forward Δ*romA*
romA3'inXbaI	GC**TCTAGA**GCACAGCTTAGCCAGGTG	3' flank reverse Δ*romA*

[a] Restriction sites are in bold

Table 2.2
Bacterial Strains and Plasmids used in this work

Strain	Description	Reference
E. coli		
DH5α	F$^-$ ϕ80*lacZ*ΔM15Δ(*lacZYA-argF) U169deoPrecA1endA1hsdR17* ($r_k^- m_k^-$)	Invitrogen
S17-1λ*pir*	Tp^{r}Str^r*recA thi pro hsdR hsdM*$^+$ RP4-2-Tc::Mu::Km Tn7 λ*pir* (lysogen)	49
K. pneumoniae		
KPPR1	Rif^R derivative of ATCC 43816	35
KPPR1S	Strep^R derivative of KPPR1	This work
VK060	KPPR1 *cpsB::Tn5Kn2*	29
VK082	*smr* mutant	This work
VK087	KPPR1 Δ*entB*	34
VK088	KPPR1 Δ*ybtS*	34
VK089	KPPR1 Δ*entB*Δ*ybtS*	34
VK131	KPPR1 Δ*romA*	This work
VK174	KPPR1S Δ*ramA*	This work
VK266	KPPR1S Δ*orf82*Δ*ramA*Δ*romA*	This work
VK269	KPPR1S Δ*rnd*	This work
VK270	KPPR1S Δ*orf82*	This work
VK274	KPPR1S Δ*smr*_A	This work
VK275	KPPR1S Δ*smr*_B	This work
VK276	KPPR1S Δ*smr*_C	This work
VK320	KPPR1S Δ*fes*	This work
VK321	KPPR1S Δ*ybdB2entABEC* (Δ*entsyn)*	This work
VK411	KPPR1S Δ*entS*	This work
VK412	KPPR1S Δ*fepB*	This work
VK413	KPPR1S *fepD::pKAS46*	This work

Plasmid	Description	Reference
pKAS46 vector	Kanamycin resistant, suicide vector, *rpsL*$^+$	47
pK03 vector	*sacB*, temperature sensitive origin of replication	34
pPROBE vector	Km^R, *gfp*	53
pKO3Δ*romA*	*romA* flanking region in pKO3	This work
pKO3Δ*ram*KO	*smr* flanking region in pKO3	This work
pKAS46Δ*ramA*	*ramA* flanking region in pKAS46	This work
pKAS46Δ*orf82*	*orf82* flanking region in pKAS46	This work
pKAS46Δ*orf82ramAromA*	*orf82ramAromA* flanking region in pKAS46	This work
pKAS46Δ*rnd*	*rnd* flanking region in pKAS46	This work
pKAS46Δ*fepB*	*fepB* flanking region in pKAS46	This work
pKAS46Δ*smr*_A	*smr*_A flanking region in pKAS46	This work
pKAS46Δ*smr*_B	*smr*_B flanking region in pKAS46	This work
pKAS46Δ*smr*_C	*smr*_C flanking region in pKAS46	This work
pKAS46Δ*fes*	*fes* flanking region in pKAS46	This work
pKAS46Δ*entS*	*entS* flanking region in pKAS46	This work
pKAS46Δ*ybdB2entABEC*	*ybdB2entABEC* (Δ*entsyn*) flanking region in pKAS46	This work
p*fepD::pKAS46*	disruption of *fepD*	This work
pY4	*ybtA* promoter-GFP in pPROBE	34

Table 2.3
List of genes deleted in breakdown mutants.

Mutant	Locus Tag	Annotated gene product
Delta A		
	VK055_1987	Oxygen-insensitive NADPH nitroreductase
	VK055_1986	Hypothetical protein
	VK055_1985	Bacterial transcriptional regulator, TetR family
	VK055_1984	Metallo-beta-lactamase superfamily protein (RomA)
	VK055_1983	Bacterial regulatory helix-turn-helix, AraC family protein (RamA)
	VK055_1982	Hypothetical protein (Orf82)
	VK055_1981	Putative aldo/keto reductase
	VK055_1980	HAD ATPase, P-type, IC family protein
	VK055_1979	Efflux transporter, RND family, MFP subunit
	VK055_1978	Efflux pump membrane transporter, BepE
	VK055_1977	Hypothetical protein
	VK055_1976	Gamma-glutamyl cysteine ligase YbdK
	VK055_1975	Hypothetical protein
	VK055_1974	Bacterial extracellular solute-binding protein
	VK055_1973	Binding-protein-dependent transport system inner membrane component
	VK055_1972	Binding-protein-dependent transport system inner membrane component
	VK055_1971	Oligopeptide/dipeptide ABC transporter, ATP-binding
	VK055_1970	Oligopeptide/dipeptide ABC transporter, ATP-binding
	VK055_1969	Amidase. Hydatoinase/carbamoylase family protein
	VK055_1968	EamA-like transporter family protein
	VK055_1967	Bacterial transcriptional regulator, GntR family protein
	VK055_1966	Bacterial transcriptional regulator, GntR family protein
	VK055_1965	Bacterial extracellular solute-binding
	VK055_1964	ABC transporter, permease
	VK055_1963	ABC type amino acid transport system, permease
	VK055_1962	ABC transporter family protein
	VK055_1961	Serine 3-dehydrogenase
	VK055_1960	Aminotransferase class-III family protein
Delta B		
	VK055_1959	ABC transporter family protein
	VK055_1958	ABC transporter family protein
	VK055_1957	Oligopeptide transport permease family protein
	VK055_1956	binding-protein-dependent transport system inner membrane component family protein
	VK055_1955	bacterial extracellular solute-binding protein
	VK055_1954	acetyltransferase family protein
	VK055_1953	choline dehydrogenase
	VK055_1952	betaine aldehyde dehydrogenase
	VK055_1951	transcriptional repressor BetI
	VK055_1950	transporter, betaine/carnitine/choline transporter family protein
	VK055_1949	*ykfE*, inhibitor of vertebrate C-type lysozyme
	VK055_1948	bacterial regulatory helix-turn-helix , LysR family protein
	VK055_1947	mechanosensitive ion channel family protein
	VK055_1946	Hypothetical kinase
	VK055_1945	Glycerol kinase
	VK055_1944	L-fucose isomerase, C-terminal domain protein
	VK055_1943	transketolase, pyrimidine binding domain protein
	VK055_1942	thiamine pyrophosphate enzyme, C-terminal TPP binding domain

		protein
	VK055_1941	Hypothetical protein
	VK055_1940	Putative transcriptional regulator
	VK055_1939	branched-chain amino acid transport system/permease component family protein
	VK055_1938	heme ABC exporter, ATP-binding protein CcmA
	VK055_1937	Hypothetical protein
	VK055_1936	periplasmic binding and sugar binding domain of LacI family protein
	VK055_1935	4'-phosphopantetheinyl transferase superfamily protein, EntD
	VK055_1934	tonB dependent siderophore receptor family protein, FepA
Delta C		
	VK055_1933	Fes
	VK055_1932	MbtH-like family protein
	VK055_1931	EntF
	VK055_1930	FepC
	VK055_1929	FepG
	VK055_1928	FepD
	VK055_1927	EntS
	VK055_1926	FepB
	VK055_1925	EntC
	VK055_1924	EntE
	VK055_1923	EntB
	VK055_1922	EntA
	VK055_1921	proofreading thioesterase in enterobactin biosynthesis, YbdB2
	VK055_1920	carbon starvation CstA family protein
	VK055_1919	helix-turn-helix family protein
	VK055_1918	hypothetical protein
	VK055_1917	plasmid stabilization system family protein
	VK055_1916	short chain dehydrogenase family protein
	VK055_1915	iron-containing alcohol dehydrogenase family protein
	VK055_1914	ABC transporter family protein
	VK055_1913	branched-chain amino acid transport system/permease component family protein
	VK055_1912	periplasmic binding and sugar binding domain of LacI family protein
	VK055_1911	LVIVD repeat family protein

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CHAPTER 3: IDENTIFICATION OF TWO CONSERVED NOVEL REGULATORS OF VIRULENCE IN *Klebsiella pneumoniae*[*]

3.1 Overview

Klebsiella pneumoniae has gained widespread recognition as a pathogen with a propensity for acquiring antibiotic resistance. It is capable of causing a range of hospital-acquired infections including UTIs, pneumonia, and sepsis, and some strains are able to cause community acquired invasive infections such as liver abscesses. The genetic heterogeneity of *K. pneumoniae* isolates complicates our ability to understand virulence of *K. pneumoniae* strains in general and for specific disease syndromes. Characterization of both conserved and strain specific factors will improve our understanding of this important pathogen. Our lab uses a mouse model system that recapitulates human infection by establishing bacterial colonization of the respiratory tract followed by development of a systemic infection. The MarR (*m*ultiple *a*ntibiotic *r*esistance *r*egulator) family of regulatory proteins is widely distributed in bacteria and regulates a number of bacterial cellular processes such as antibiotic resistance and the expression of virulence factors. *Klebsiella* was

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[*] I constructed the plasmids to create the mutants and the mutants tested. I performed all of the animal work for bacterial enumeration and for histology. I assisted with the animal experiments where immune cell analysis was performed. I constructed reporter constructs for all of the known capsule promoters (and constructed and tested others not shown). I performed the uronic acid and sedimentation assays. I did the majority of the writing as well as created all tables and most of the figures.

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found to encode a higher than average number of MarR-like proteins, and it is likely these regulators contribute to the ability of *K. pneumoniae* to respond to, and survive in, a wide variety of environmental conditions such as the human body. To test this we constructed loss of function mutations in several *marR* homologues and found that while most of the mutations did not affect virulence in our murine pneumonia model, mutations in genes encoding two previously uncharacterized regulators (*kvrA* and *kvrB*) of *K. pneumoniae* significantly impacted virulence. Mutations in *kvrA* and *kvrB* resulted in several phenotypes associated with reduced production of capsule and reduced mucoviscosity, suggesting they may affect capsule production. Analysis of transcriptional fusions of promoters from the capsule synthesis and assembly locus (*cps*) indicated KvrA and KvrB are involved in regulation of *cpsB* expression. Additionally, when testing the corresponding *cpsB* promoter of a 'hypervirulent (*hv*)' strain (liver-abscess derived), *cpsB* expression also was regulated by KvrA and KvrB in this *hv* strain, suggesting a conserved role for these regulators among pathogenic *K. pneumoniae* strains. Together our data indicate KvrA and KvrB have distinct effects on virulence, and impact expression of capsule promoters and the hypermucoviscosity phenotype.

3.2 Introduction

Klebsiella pneumoniae is a Gram-negative bacterium capable of causing a wide range of infections such as urinary tract infections, wound infections, sepsis, liver abscesses, and pneumonia (1-6). *K. pneumoniae* infections have become more difficult to control due to increasing antibiotic resistance. Capsule, LPS, adhesion factors, and siderophores frequently emerge as the primary *K. pneumoniae*

virulence determinants, with capsule being the most studied. There are now 134 identified capsule types (7). One trait largely associated with virulence in *Klebsiella* is the overproduction of capsule leading to a hypermucoviscous phenotype (*hmv*) (5, 8). Colonies of strains that are *hmv* are “string test” positive” and tend to be hypervirulent (8, 9). Our relatively limited knowledge of conserved virulence determinants, and the high diversity of surface polysaccharides poses a challenge to vaccine and therapeutic development (10-12).

While first characterized for its role in antibiotic resistance in *E. coli* (13, 14), the MarR family of transcriptional regulators regulate the expression of genes encoding proteins involved metabolic pathways, stress responses, and virulence factors (14-20). These proteins are characterized by a winged helix-turn-helix DNA binding domain and can both positively and negatively affect gene expression (21, 22). Typically, regulation of expression by these proteins often results in modifications of the bacterial cell surface (23). Several MarR family members have been linked to virulence in the *Enterobacteriaceae.* RovA, a member of the MarR/SlyA family, regulates expression of *inv* (an adhesion and invasion factor) in the enteric pathogens, *Yersinia enterocolitica* and *Y. pseudotuberculosis* (24, 25). RovA also regulates expression of the *psa* locus encoding fimbrial genes necessary for virulence of *Y. pestis,* the causative agent of bubonic and pneumonic plague (26). In *Salmonella,* SlyA reglulates *Salmonella* pathogenicity island-2 (SPI-2) genes and contributes to resistance to oxidative stress, bacterial survival within macrophages, and bacterial survival in a murine model of infection (17, 18, 23, 27-29).

An *in silico* comparative study found the copy number of *marR*-like genes to range from two to eleven in the *Enterobacteriaceae*, with the average copy number of *marR* genes in *Enterobacteriaceae* being 5.9 (22). This same study identified some *K. pneumoniae* strains that have as many as eleven copies of *marR* homologues (22). Our lab utilizes a lung model of infection and we found the strain used in our studies (a derivative of ATCC 43816) encodes nine *marR*-like genes (30). We hypothesize that this high number of *marR*-like genes contributes to the ability of *K. pneumoniae* to survive in a wide variety of environments, including the human host, and that a subset of these genes contribute to virulence.

Here we describe the contribution of the MarR family to *K. pneumoniae* virulence in the lung. To study the contribution of *marR* homologues, we constructed insertion disruption mutations in each of the nine putative *marR* homologues of our strain and tested them in our murine model of pneumonia. Two of these genes, *kvrA* and *kvrB*, affected virulence. Their impact on virulence is likely due at least in part to their effect on expression of capsule genes and the hypermucoviscosity (Hmv) phenotype. Importantly, these roles were conserved in a hypervirulent (*hv*) *K. pnemoniae* strain that produces a different capsule type.

3.3 Materials and Methods

3.3.1 Ethical statement. Mouse experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill

(Protocol 14-110). All efforts were made to minimize suffering. Animals were monitored daily following inoculation and were euthanized upon exhibiting signs of morbidity.

3.3.2 Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. Our wild-type *K. pneumoniae* strain KPPR1S, a $Strep^R$ derivative of KPPR1 [a Rif^R derivative of ATCC 43816 (30)], as well as mutants of KPPR1S, were grown aerobically in Luria-Bertani (LB) medium overnight at 37^{o}C. Antibiotics were added to the media as appropriate at the following concentrations: kanamycin (50 µg/mL), rifampin (30 µg/mL) and streptomycin (500 µg/mL). Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

3.3.3 Construction of bacterial mutants. Isogenic mutants (KPPR1SΔ*kvrA* and KPPR1SΔ*kvrB*, KPPR1SΔ*0870)* were generated using allelic exchange as described using the pKAS46 vector (31). pKAS46 is a suicide vector that allows for positive selection with streptomycin for the loss of vector following integration onto the chromosome (31, 32). For allelic exchange to generate unmarked mutations, ~500 bp DNA fragments were generated by PCR using the primer sets indicated in Table 2 and cloned into pKAS46. Constructs were confirmed by sequence analysis. Overnight cultures of *K. pneumoniae* KPPR1S and *E. coli* S17-λ*pir* (33) carrying a derivative of pKAS46 were mixed, collected by centrifugation, plated on LB agar (no antibiotics), and grown overnight at 37°C. Transconjugants were selected by plating

on LB agar with $Rif_{30}Kan_{50}$. Rifampin selects against the donor strain and kanamycin selects for the plasmid integrants. Several Rif/Kan-resistant colonies were grown for 5-6 hours in LB (no antibiotics), then plated on LB agar with $Strep_{500}$ to select for transconjugants that had excised the plasmid. Kan-sensitive clones were screened by PCR to verify loss of the targeted gene(s).

Insertional disruption mutants (KPPR1S *0496::pKAS46,* KPPR1S *4504::pKAS46*, KPPR1S *4417::pKAS46,* KPPR1S *4238::pKAS46,* KPPR1S *1202::pKAS46,* KPPR1S *2679::pKAS46,* KPPR1S *4504::pKAS46,* KPPR1S *1021::pKAS46,* KPPR1S *1682::pKAS46*) were constructed in KPPR1S. NTUH *kvrA::pKAS46* and NTUH *kvrB::pKAS46* were constructed in NTUH-K2044. These mutants were made by plasmid integration at the gene of interest. A DNA fragment containing a portion of the gene of interest, generated by PCR using primers listed in Table 2, was cloned into pKAS46. The resulting plasmids were mated into KPPR1S (or NTUH-K2044) using *E. coli* S17-λ*pir* carrying the plasmids as the donor. *K. pneumoniae* with integration of the plasmids on the chromosome were selected for by plating on LB agar with $Rif_{30}Kan_{50}$ as described above.

3.3.4 Murine model of pneumonia. Five-to-eight week old, female C57BL/6 mice (Jackson Laboratories) were anesthetized by intraperitoneal (i.p.) injection with a mixture containing ketamine (8 mg/mL) and xylazine (1.6 mg/mL). Overnight bacterial cultures were diluted in phosphate-buffered saline and a 20 µL suspension was inoculated intranasally (i.n.) in two 10 µL aliquots for a total of ~2×10^4 colony forming units (CFU)/mouse as described (34). Total CFUs for each inoculum were

confirmed by plating dilutions onto LB agar. After 24, 48, or 72 hours post inoculation (hpi), mice were euthanized by a lethal injection of 200 µL sodium pentobarbital (150mg/kg). Organs were removed and homogenized in 500 µL phosphate-buffered saline (PBS). Homogenized organs were serially diluted and plated to quantify CFU/g tissue.

3.3.5 Histopathology*.* Groups of two to three mice were inoculated intranasally as described above. The mice were sacrificed at 72 hpi and lungs were inflated with 1 mL of 10% neutral buffered formalin for a minimum of 24 hours. Lungs were then washed once in PBS for 2 hours, immersed in 70% ethyl alcohol (EtOH), and embedded in paraffin. Three 5-micrometer sections 200 µm apart per lung were stained with hematoxylin-eosin (H&E) for examination. Histology services provided by the Histology Facility in the Department of Cell and Molecular Physiology.

3.3.6 Flow cytometry and antibodies*.* The lungs were processed into fine pieces by mincing with two surgical steel razor blades. After processing, the lung was digested in 5.66 mg Collagenase II (Gibco, Carlsbad, CA) in 4 mL PBS/5% serum for one hour at 37ºC. Following digestion, the lung homogenate was triturated with an 18G needle three times and then passed through a 100 micron filter.

For flow cytometry staining, approximately 10,000,000 cells were counted from RBC lysed lung samples. Each sample was incubated with Tonbo Biosciences (San Diego, CA) anti-CD16/32 (Fc Block) antibody cocktail for 5 minutes at room temperature. Following blocking, surface labeling of cellular markers was performed

by adding 1:200 dilution for 20 minutes at 4ºC. The following antibodies/dyes were used to immunophenotype cellular infiltrates: anti-CD103:<BV510> (clone 2E7), anti-CD4:<BV605> (clone RM4.5), anti-CD8:<BV711> (clone 53-6.7), Ly-6C:<BV786> (clone HK1.4), and anti-CD64:<PE> (clone X54-5/7.1) from BioLegend (San Diego, CA, USA); anti-CD45:<vf450> (clone 30-F11), anti-CD11b:<FITC> (clone M1/70), anti-CD11c:<PerCP-Cy5.5> (clone N418), anti-F4/80:<PE/Cy7> (clone BM8.1), and anti-CD19:<rf710> (clone 1D3) from Tonbo Biosciences; anti-Ly-6G:<ef660> (clone RB6-8C5) and ef780 fixable viability dye (used at 1:100 dilution) from Affymetrix ebioscience (San Diego, CA).

3.3.7 Adherence and internalization assay. Femurs of C57BL/6 mice were harvested and rinsed thoroughly with DMEM (Gibco, Carlsbad, CA) media to extract immature bone marrow cells. The marrow cells were then strained through a 70-micron filter and collected. For each mouse used, the marrow was resuspended to 60 mL of DMEM supplemented with 10% serum and 10% L cell supernatant. The 60 mL cell suspension was then divided into 6 untreated 100 mm petri dishes with 10 mL of cell suspension in each plate. The cells were allowed to differentiate for 6-7 days with feeding occurring every 72 hours. At endpoint, the cells were analyzed for F4/80 and CD11b expression greater than or equal to 90% by FACS analysis.

Bone marrow derived macrophages (BMDM) were seeded into 24-well plates at a density of 500,000 cells per well. The cells were allowed to adhere and stabilize over night at 37ºC, 5% CO_2. Bacterial cultures were grown in 2 mL LB broth + 30 µg/mL rifampicin for 16-17 hours at 37ºC. To infect macrophages and initiate the

time point, the macrophages were rinsed and replaced with DMEM/10% FBS. For adherence assay, the wells were initially pretreated for one hour with 2 μM cytochalasin D (Sigma, St. Louis, MO) to halt any internalization of bacteria; this step was omitted for internalization assays. The macrophages were then inoculated with 50 MOI of *K. pneumoniae*. After one hour the wells were gently rinsed with sterile phosphate-buffered saline (PBS) three times and then lysed with 0.5% saponin for plating on LB agar to determine the number of CFU.

For the internalization assay, following a one-hour incubation with an MOI of 50 *K. pneumoniae,* the wells were rinsed with sterile PBS three times, and then the wells were treated with a high dose of gentamycin (Gibco) (200 μg/mL) in DMEM/10%FBS for 30 minutes to kill extracellular bacteria. Following the gentamicin treatment, the wells were lysed with 0.5% saponin and plated on LB agar to determine the number of CFU.

3.3.8 Construction of reporter fusions. DNA fragments containing the promoter region of *cpsB, galF,* and *wzi* were amplified by PCR using Pfu Turbo and cloned into the pPROBE (tagless) vector (35). This vector contains a promoterless *gfp* gene. Plasmids in which *gfp* was under the control of the *Klebsiella* promoters were identified by restriction digest analysis and confirmed by sequencing. All primers used are listed in Table S1 in the *Supplementary material.*

3.3.9 Measurement of promoter activity. To examine capsule locus expression *in vitro*, the *galF*, *wzi,* and *cpsB* promoters of KPPR1S, or *cpsB* from NTUH-K2044,

were cloned into the promoterless pPROBE *gfp* reporter vector (35).The reporter plasmids were individually mobilized into *K. pneumoniae* strains by electroporation. The bacteria were grown overnight at 37°C in LB and subcultured to an OD_{600} of 0.2 and grown for 6 hours. All strains were assayed in triplicate. Measurements or relative light units were taken (Synergy HT microplate reader, BioTek Instruments, Winooski, VT) and normalized to OD_{600}.

3.3.10 Mucoviscosity Assay. The mucoviscosity of the capsule was determined using the sedimentation assay as previously described (36). Briefly, overnight cultures were grown in LB and back diluted to an OD_{600} of 0.2 in fresh media and grown at 37 °C. After 6 hours, 1.0 OD/mL was centrifuged for 5 minutes at 1000 x *g* and a post-centrifugation OD reading was measured.

3.3.11 Extraction and quantification of capsule. Uronic acid content was extracted and quantified as previously described (37). Briefly, overnight cultures were grown in LB and back diluted to an OD_{600} of 0.2 in fresh media and grown at 37 °C. After 6 hours, 500 µL of culture were added to 100 µL of Zwittergent and incubated at 50 °C for 20 minutes. Following incubation, 300 µL of the supernatant was added to 1.2 mL of absolute ethanol and incubated at 4 °C. The mixture was centrifuged for 5 minutes at maximum speed. The pellet was resuspended in 200 µL of water and added to 1.2 mL of tetraborate and incubated for 5 minutes at 100 °C. 20µL of diphenol was added and the absorbance read at 520nm. The glucuronic

acid content was determined from a standard curve of glucuronic acid (Sigma-Aldrich) and expressed as micromolars per OD_{600}.

3.3.12 Statistical analysis. Statistical analyses were performed using GraphPad Prism, version 7.0 (San Diego, CA).

3.4 Results

3.4.1 A subset of MarR family proteins contribute to *K. pneumoniae* virulence in a mouse model of pneumonia

The MarR family of transcriptional regulators has been previously implicated in virulence in other members of the *Enterobacteriaceae* (17, 21, 26, 27, 38, 39). *Klebsiella* species have a higher copy number than average of *marR*-like genes, and we hypothesize some members of this family could be important for bacterial adaptation in the host. To test the contribution of the MarR family of proteins to a pneumonic infection, we screened the genome of KPPR1S for putative *marR* genes and constructed loss-of-function mutants for each of the nine *marR* homologues. None of the mutants displayed a growth defect *in vitro* in LB (data not shown). To assess effects on virulence, mice were infected with KPPR1S and each mutant strain individually and sacrificed at 48 hpi for bacterial enumeration. Two mutants, VK055_0496 and VK055_4504 displayed a decrease in bacterial burden in the lungs of infected mice compared to KPPR1S (wild-type, hereafter referred to as WT) infected mice at 48 hpi (Fig. 3.1A). The spleens of WT infected mice had nearly 10^6 CFU/g of tissue while the VK055_0496 mutant was barely detectable (Fig. 3.1A).

Based on our initial screen, we identified two regulators, VK055_0496 and VK055_4504, to be important for infection of the lung. We named these regulators, KvrA and KvrB for *Klebsiella* virulence regulators, respectively. Further kinetics experiments using in-frame deletion mutants of *kvrA* (VK277) and *kvrB* (VK410) indicated that at 24 hpi the Δ*kvrA* mutant was barely detectable in the lung while the Δ*kvrB* mutant had ~1 log fewer CFU compared to WT. By 72 hpi the bacterial burdens of mice infected with WT had increased several logs, the Δ*kvrA* mutant had been cleared, and bacterial burden of the Δ*kvrB* mutant, while reduced compared to WT, remained comparable to 24 hpi (Fig. 3.1B). The spleens of Δ*kvrA* infected mice had few recoverable CFU at either 24 or 72 hpi, while mice infected with the Δ*kvrB* mutant had splenic burdens more than 2 logs lower than WT at 72 hpi (Fig. 3.1B). To test if the survival defect of the mutants was due to an inability to reach the lung, mice were infected with the WT, Δ*kvrA,* or Δ*kvrB* mutants and the lungs were harvested 90 minutes post inoculation. The WT, Δ*kvrA,* and Δ*kvrB* mutants all had comparable bacterial burdens in the lungs suggesting the ability of the mutants to colonize the lungs was not impacted (Fig. 3.2). Subsequent analysis confirmed the virulence defect of the Δ*kvrA* and Δ*kvrB* mutants could be attributed to the loss of KvrA and KvrB, as demonstrated by chromosomal complementation of the Δ*kvrA* and Δ*kvrB* mutants (Fig. 3.1B). Together these results demonstrate that KvrA and KvrB contribute to *K. pneumoniae* virulence in a lung model of infection.

3.4.2 The Δ*kvrA* and Δ*kvrB* mutants induce less inflammation in the lung and Δ*kvrA* mutant infected mice have an increase in the proportion of inflammatory monocytes

Infection with wild-type *K. pneumoniae* is known to induce a significant inflammatory response marked by lung lesions and neutrophil infiltration (37, 40, 41). In the lung, *K. pneumoniae* first encounters alveolar macrophages (42). Once an infection is established, neutrophils contribute to bacterial clearance of some *K. pneuomoniae* strains, but not others (43, 44). Neutrophils are considered important for clearance of ATCC43816 (i.e. KPPR1S), however, their high numbers may also be detrimental to host health (44). Inflammatory monocytes are considered a primary defensive cell type against *K. pneumoniae* (43). Consistent with the reduced number of CFU in the lung, infection with the Δ*kvrA* and Δ*kvrB* mutants resulted in less inflammation compared to WT infected mice at 72 hpi (Fig. 3.3A). To assess changes in response of host cell types associated with increased bacterial clearance, the lungs of mice infected with WT or the Δ*kvrA* or Δ*kvrB* mutants were processed at 24 hpi for flow cytometric analysis. A significant increase over mock-infected mice of neutrophils was observed for WT and the two mutants (Fig. 3.3B), although this increase was less for the two mutants. We also observed three times the proportion of inflammatory monocytes in mice infected with the Δ*kvrA* mutant compared to mice infected with WT; there was no significant difference between WT and the mutants in the proportion of alveolar macrophages (Fig. 3.3B). The reduced proportion of alveolar macrophages compared to mock infected mice is likely due to

the influx of neutrophils in the infected mice. Together, these results suggest inflammatory monocytes contribute to KvrA and KvrB clearance.

3.4.3 The Δ*kvrA* and Δ*kvrB* mutants are more adherent and more likely to be internalized in murine BMDM than WT

K. pneumoniae is known to produce a capsule that interferes with adherence to epithelial cells and reduces internalization by phagocytic cells (45, 46). Because of the reduced bacterial burden of the Δ*kvrA* and Δ*kvrB* mutants in mice, we wanted to determine if the mutants had altered interactions with murine macrophages. The Δ*kvrA* and Δ*kvrB* mutants were added to murine BMDM, and after 1 hour, adherent bacteria were enumerated. Consistent with previous reports, a capsule mutant, Δ*cpsB* (VK60), was nearly ten times more adherent to BMDM than the WT strain (Fig. 3.4A). The Δ*kvrA* and Δ*kvrB* mutants were 4 to 6-fold more adherent than the WT strain, reflective of an intermediate phenotype (Fig. 3.4A).

In addition to its anti-adhesive properties, capsule also has been shown to play a role in inhibiting internalization by phagocytic cells (47-49). The Δ*kvrA* and Δ*kvrB* mutants were added to murine BMDM and after 1 hour, gentamycin was added to kill extracellular bacteria. BMDM were then lysed and bacteria were enumerated to calculate internalized bacteria. The Δ*kvrA* and Δ*kvrB* mutants had a higher number of internalized CFU than the WT strain (Fig. 3.4B).

3.4.4 KvrA and KvrB contribute to capsule production

The *Klebsiella* capsule is a polysaccharide matrix that can vary in composition based on the glycosyl transferases it encodes (7, 50, 51). Our WT strain produces a K2 capsule composed of glucuronic acid, glucose, and mannose. Measurement of the uronic acid content is frequently used to quantify capsule production (52, 53). During mutant construction, we observed that colonies of the Δ*kvrA* and Δ*kvrB* mutants appeared to be less mucoid and less hypermucoviscous (i.e. were string test negative) than WT. Thus, we hypothesized that capsule production would be decreased in the mutants. We found that the Δ*kvrA* and Δ*kvrB* mutants produced 25% less uronic acid compared to WT, but the levels remained higher than a Δ*cpsB* mutant (Fig. 3.5A).

Mucoviscosity can be quantified using a sedimentation assay (54, 55). Hmv strains such as KPPR1S do not form tight pellets and this can be quantified by measuring the OD_{600} of the supernatant following low speed centrifugation. When compared to the WT strain, the Δ*kvrA* and Δ*kvrB* mutants had a reduction in OD_{600} of the supernatant indicating reduced hypermucoviscosity (Fig. 3.5B). A capsule mutant (Δ*cpsB*) that produces no capsule and is not hypermucoviscous also had a significant decrease in OD_{600} of the supernatant (Fig. 3.5B). Together, these data suggest capsule production is reduced in the Δ*kvrA* and Δ*kvrB* strains. These findings are consistent with the adherence and internalization data as capsule is considered to inhibit adherence to, and internalization by, host cells (56).

3.4.5 KvrA and KvrB positively regulate capsule gene expression

We hypothesize that expression of the capsule locus (*cps* locus) is decreased in the Δ*kvrA* and Δ*kvrB* mutant strains because the Δ*kvrA* and Δ*kvrB* mutants are less hypermucoviscous and produce less uronic acid than WT. To test if expression of the *cps* locus was affected, we constructed strains where the known capsule promoters upstream of *galF, wzi,* and *cpsB* (57) were cloned into a *gfp* reporter construct and these constructs were transformed into our test strains (Fig. 3.6A). After 6 hours, the *wzi* promoter appeared minimally affected by the loss of KvrA or KvrB (Fig. 3.6C), whereas *cpsB* promoter activity was significantly decreased in both the Δ*kvrA* and Δ*kvrB* mutants (Fig. 3.6D). Interestingly, expression from the *galF* promoter decreased only in the Δ*kvrB* mutant (Fig. 3.6B) suggesting that although both KvrA and KvrB affect capsule production, they do so in distinct ways.

KvrA and KvrB homologues exist in most other *K. pneumoniae* strains, thus we wanted to determine if KvrA and KvrB affect expression of the *cpsB* promoter of another K serotype. Because of the high prevalence of the K1 serotype among hypervirulent isolates associated with community acquired liver abscess infections (5, 58), we cloned the *cpsB* promoter ($cpsB_{NTUH}$) of the NTUH-K2044 strain [liver abscess derived; (59)] into our *gfp* reporter construct and transformed this plasmid into the Δ*kvrA* and Δ*kvrB* mutants. We found that in both the Δ*kvrA* and Δ*kvrB* mutants, $cpsB_{NTUH}$ activity was reduced compared to our WT strain (Fig. 3.6E), suggesting that KvrA and KvrB can regulate the *cpsB* promoter from a K1 capsule locus as well as from the K2 capsule locus.

3.4.6 KvrA and KvrB contribute to capsule production and virulence of a K1 liver abscess derived strain

K. pneumoniae strains display significant genetic heterogeneity and not every identified virulence factor is conserved between strains . However, KvrA and KvrB homologues are conserved in *K. pneumoniae* strains and thus, could have a conserved role in regulating *cps* locus expression and virulence. The contribution of virulence factors can be highly dependent on strain background. Therefore, to assess if KvrA and KvrB contribute to virulence in a *K. pneumoniae* isolate with a different capsular type we constructed *kvrA* and *kvrB* loss of function mutants in the NTUH-K2044 strain (K1 capsule) (60). Analysis of expression from the $cpsB_{NTUH}$ promoter in Δ*kvrA* and Δ*kvrB* mutants of our K2 strain suggested KvrA and KvrB may have a conserved role in regulating capsule gene expression. We found that uronic acid content was significantly reduced in both the NTUH-K2044 *kvrA* (VK559) and *kvrB* (VK560) mutants (Fig. 3.5A). Similarly, in the sedimentation assay, both *kvrA::*pKAS46 and *kvrB::*pKAS46 were reduced in hypermucoviscosity (Fig. 3.5B). It is interesting to note though that the NTUH-K2044 *kvrB* mutant does not have as strong of an effect on either uronic acid or mucoviscosity as it does in KPPR1S, suggesting some strain specific factor may influence KvrB function. Together though these data suggest the role of KvrA and KvrB in capsule production is conserved.

To assess if KvrA and KvrB contribute to virulence of a *K. pneumoniae* isolate of a different capsular type we tested the *kvrA* and *kvrB* loss of function mutants in the NTUH-K2044 background *in vivo* using our murine lung infection model. At 48 hpi, mice were euthanized and bacterial burden was assessed in the lungs and

spleens. As for the KPPR1S strain, the *kvrA* mutant in the NTUH-K2044 background was strongly attenuated in our lung model of infection (Fig. 3.7). Unlike the KPPR1S strain, the *kvrB* mutant in the NTUH-K2044 background displayed no attenuation at 48 hpi (Fig. 3.7). Similar trends were observed in the spleens of infected mice. These results suggest that the role of KvrA in virulence in the lung is conserved across *K. pneumoniae* strains.

3.5 Discussion

The MarR family of transcriptional regulators has been implicated in regulation of virulence genes in several members of the *Enterobacteriaceae* (17, 18, 21, 27, 29, 39, 61). Our work examines the contribution of the MarR family to *K. pneumoniae* virulence. We identified and screened loss-of-function mutations in nine *marR*-like genes and found two uncharacterized transcriptional regulators, KvrA and KvrB, are important for *K. pneumoniae* infection of the lung. Furthermore, the *kvrA* mutant is associated with a significant increase in the proportion of inflammatory monocytes in the lung, suggesting that the severe attenuation of this mutant could be due to an increase in monocyte-mediated clearance.

We also found that both KvrA and KvrB can regulate capsule gene expression and capsule production, and can do so in distinct ways where KvrB regulates both the *cpsB* and *galF* promoters while KvrA only regulates the *cpsB* promoter. Whether or not these effects on expression of the *cpsB* and *galF* promoters are direct or indirect is not known at this time. Consistent with the known role of capsule as a shield from the host immune system (47), we found that both

kvrA and *kvrB* are important for inhibiting adherence to and uptake by murine BMDM. We also showed that KvrA and KvrB can regulate the *cpsB* promoter and capsule production of a hypervirulent, community acquired *K. pneumoniae* isolate of a different K type (NTUH-K2044) suggesting a conserved role for these regulators, thus expanding the potential application of these findings to additional *K. pneumoniae* capsule types. Interestingly, as in the KPPR1S background, the *kvrA* mutant in the NTUH-K2044 background was strongly attenuated in a pneumonia infection model.

The contribution of the MarR family to virulence may be strain dependent as *K. pneumoniae* strains have a high percentage of accessory, or non-conserved, genes (62). *K. pneumoniae* encodes more species-specific *marR* genes than any other *Enterobacteriaceae* (22). Previously, the role of a MarR family protein, PecS, was shown to be important for repressing the type 1 fimbriae gene locus (encoded by *fim*) in *K. pneumoniae* CG43 (63). Type 1 fimbriae has been implicated in binding mannose containing structures and are important for causing urinary tract infections (64). The *pecS* homologue in our strain (VK323) was tested but was not attenuated in the pneumonia infection model. This is consistent with a previous study where *mrk* (type 3 fimbriae) and *mrkfim* mutants were not attenuated in a lung model of *Klebsiella* infection (65). PecS belongs the urate-responsive subfamily of MarR, and while our study demonstrates it is dispensable in the lung, it may serve an important role in other infection sites such as the urinary tract. A high throughput genetic screen for virulence factors important for a pneumonic infection found geneID *4417*, encoding a MarR family protein, to be only modestly important in a 1:1 WT:mutant

co-infection experiment (66). The same gene was targeted in our initial screen and upon an independent infection with the *4417* mutant (VK322), *4417* was found to be dispensible for virulence in the lung. Thus, some of our findings regarding dispensable MarR proteins in the lung are consistent with previously published studies.

The bacterial capsule serves a variety of functions, including but not limited to, protection from the immune response of the host (47, 67-69). Capsule synthesis in *K. pneumoniae* is homologous to assembly of the Wzy-dependent assembly of Group 1 capsules of *E. coli* (70). Capsule synthesis involves synthesis of sugar precursors, the activation of sugars (the addition of a nucleotide to form a nucleotide diphosphate sugar), the initiation of synthesis and formation of a repeat unit, transport of this unit to the outer surface of the inner membrane, and polymerization of this unit into polymers (70, 71). Following the polymerization, the polysaccharide chains are transported across the outer membrane and anchored to the surface via the lectin, Wzi (70, 72, 73). Interestingly, Wzi proteins are found in *E. coli* and *K. pneumoniae*, but few other organisms from the Gammaproteobacteria class (73). It has been observed that organisms that produce polymers in the mostly secreted form (such as colonic acid in *E. coli.*), lack a *wzi* homologue (73). While it is proposed that Wzi acts as a lectin to bind nascent exopolysaccharide as it is secreted, it is unknown what determines attachment versus release of secreted polysaccharide.

Our study has revealed a role for KvrA and KvrB in regulation of sugar production as depicted by a reduction in expression of the *cpsB* promoter and a

reduction in uronic acid levels. Furthermore, hypermucoviscosity, a property associated with highly virulent strains and generally associated with a reduction in capsule production in the literature, was significantly reduced in both the *kvrA* and *kvrB* mutants. However, it is unclear whether cell associated capsule, free extracellular polysaccharide, or both are responsible for changes in hypermucoviscosity. The difference between surface-associated versus free capsule polysaccharide may reflect a difference in function. For instance, surface associated capsule can inhibit complement deposits and evasion of the MAC complex and inhibition of attachment to phagocytic cells (74), whereas free capsule may act as a decoy to sequester antimicrobial peptides produced by the host (75). Therefore it is possible that the reduction in uronic acid and mucoviscosity we observe may reflect a change in the ratio between free and bacterial surface-associated saccharides. Based on previous studies conducted in *E. coli* (73), this change may be mediated by Wzi, as we observe a modest decrease in *wzi* promoter activity in the Δ*kvrB* mutant. However, it could also be due in part to the reduction in synthesis of precursor sugars via reduced *cpsB* expression, or altered expression of other genes that influence mucoviscosity.

Here, we demonstrate that KvrA and KvrB are involved in regulation of capsule biosynthesis. One of the best-studied regulators of capsule synthesis in *E. coli*, RcsB, is also present and functional in *K. pneumoniae* (76, 77). In *E. coli*, the RcsBCD hybrid two component system, is a key regulator of group 1 capsule biosynthesis (78, 79). Following environmental stimuli, RcsC and RcsD in the inner membrane relay a phosphate to the cytoplasmic response regulator RcsB, resulting

in its activation (79). Phosphorylated RcsB interacts with RcsA to bind the *cps* promoters as a heterodimer, resulting in increased colonic acid synthesis. In *K. pneumoniae,* RcsB has been shown to interact with a unique regulator, RmpA (77). RcsA, RcsB, and RmpA belong to the LuxR family of transcriptional regulators (77, 78). RmpA is unique in that it has only been identified in *K. pneumoniae* strains associated with a hypermucoviscous phenotype and no other bacteria (77). Whether or not RmpA regulates capsule synthesis by binding the *cps* locus directly, or via intermediate players such as KvrA or KvrB, is currently under investigation.

The primary sequences of the MarR/SlyA/RovA proteins are highly conserved among different bacteria, however, the identity between MarR-family homologues can vary. For instance while KvrA shares 84% identity with SlyA of *S. enterica* and KvrB shares 93% identity with EmrR of *E. coli,* KvrA and KvrB share only 31% identity to each other. No consensus sequence exists for binding to promoters for many of these family members and regulation appears largely to be mediated by de-repression of H-NS transcriptional silencing (80). H-NS is believed to be influence the expression of nearly 5% of all genes in *E. coli* by interacting with curved DNA rather than by recognizing particular sequences, although H-NS is associated with binding of A-T-rich regions. (81). H-NS regulates horizontally acquired genes, and in many instances this regulation has been shown to be counteracted by regulators of the MarR family (24, 80). Thus, the regulons of MarR regulators can vary between closely related bacterial species and even between strains of the same species. Although KvrA and KvrB both affect capsule production they have distinct effects on capsule promoter expression and distinct *in vivo* virulence profiles. We also

observed overlapping but distinct phenotypes of KvrA and KvrB mutants in two different *K. pneumoniae* strains, suggesting the regulons between strains could have both conserved and unique components. Current studies are underway to characterize the regulons of the Δ*kvrA* and the Δ*kvrB* mutants to gain a fuller understanding of how they influence capsule production and virulence.

In summary, we found that a subset of regulators of the MarR family are important for virulence of *K. pneumoniae* in the lung. We identified KvrA and KvrB to be necessary for capsule expression and given the comparable capsule production levels yet different disease outcomes, we believe that KvrA and KvrB may be regulating other genes and warrant further study. Given that less than 40% of genes are conserved across *K. pneumoniae* strains (62), identifying highly conserved targets affecting growth and survival of *K. pneumoniae* in the host is essential to overcoming strain heterogeneity for new therapeutic developments.

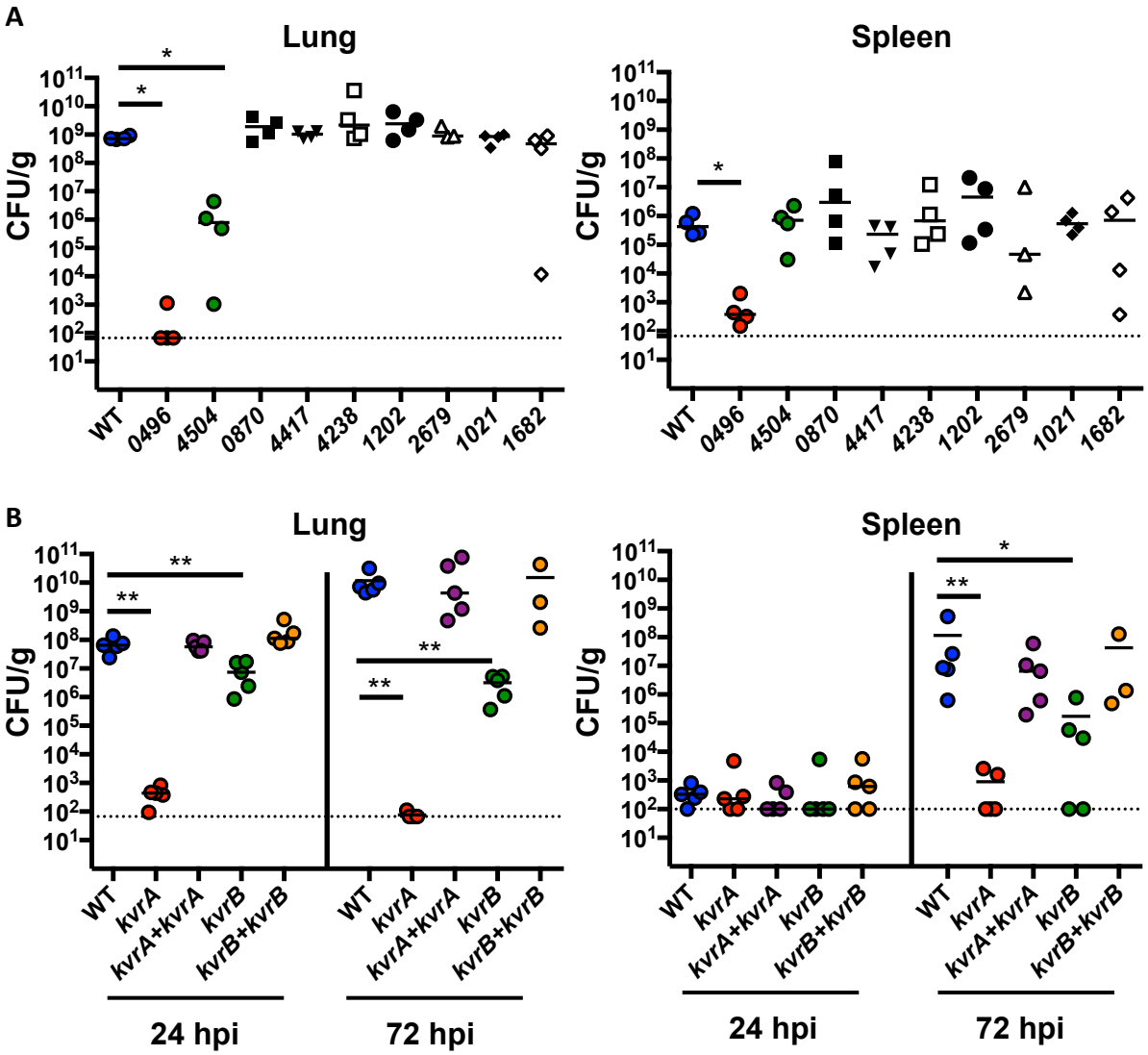

Figure 3.1. Members of the *marR* family contribute to virulence in a mouse model of pneumonia. Mice were inoculated i.n. with $2x10^4$ CFUs of either the wild-type strain or mutant strains. (A) At 48 hpi, mice were euthanized and the lungs and spleens were homogenized and plated for bacterial enumeration. (B) Mice were inoculated i.n. with $2x10^4$ CFUs of either the wild-type, the Δ*kvrA* or Δ*kvrB* mutant, or the complemented strains. At 24 or 72 hpi, mice were euthanized and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. Data are from an individual representative experiment. Mann-Whitney tests were performed for statistical analysis; *=p<0.05, **=p<0.01.

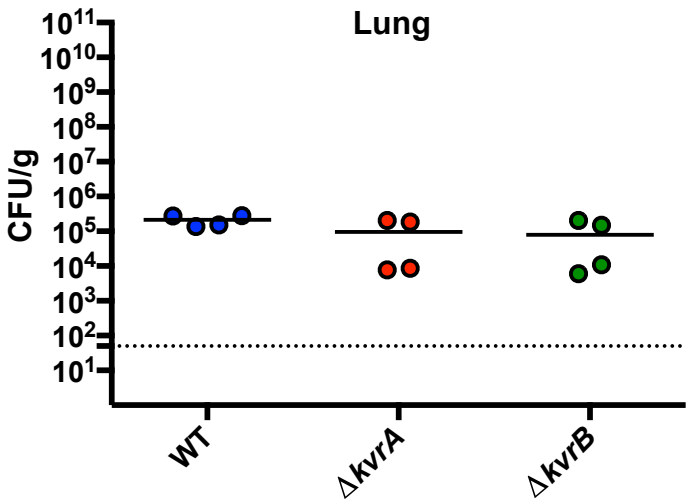

Figure 3.2. The *kvrA* and *kvrB* mutants are not altered in their ability to reach the lung. Mice were inoculated i.n. with $2x10^4$ CFUs of either the wild-type strain (KPPR1S) or mutant strains (VK277, VK410). 90 minutes post inoculation, mice were euthanized and the lungs were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. Data are from an individual experiment.

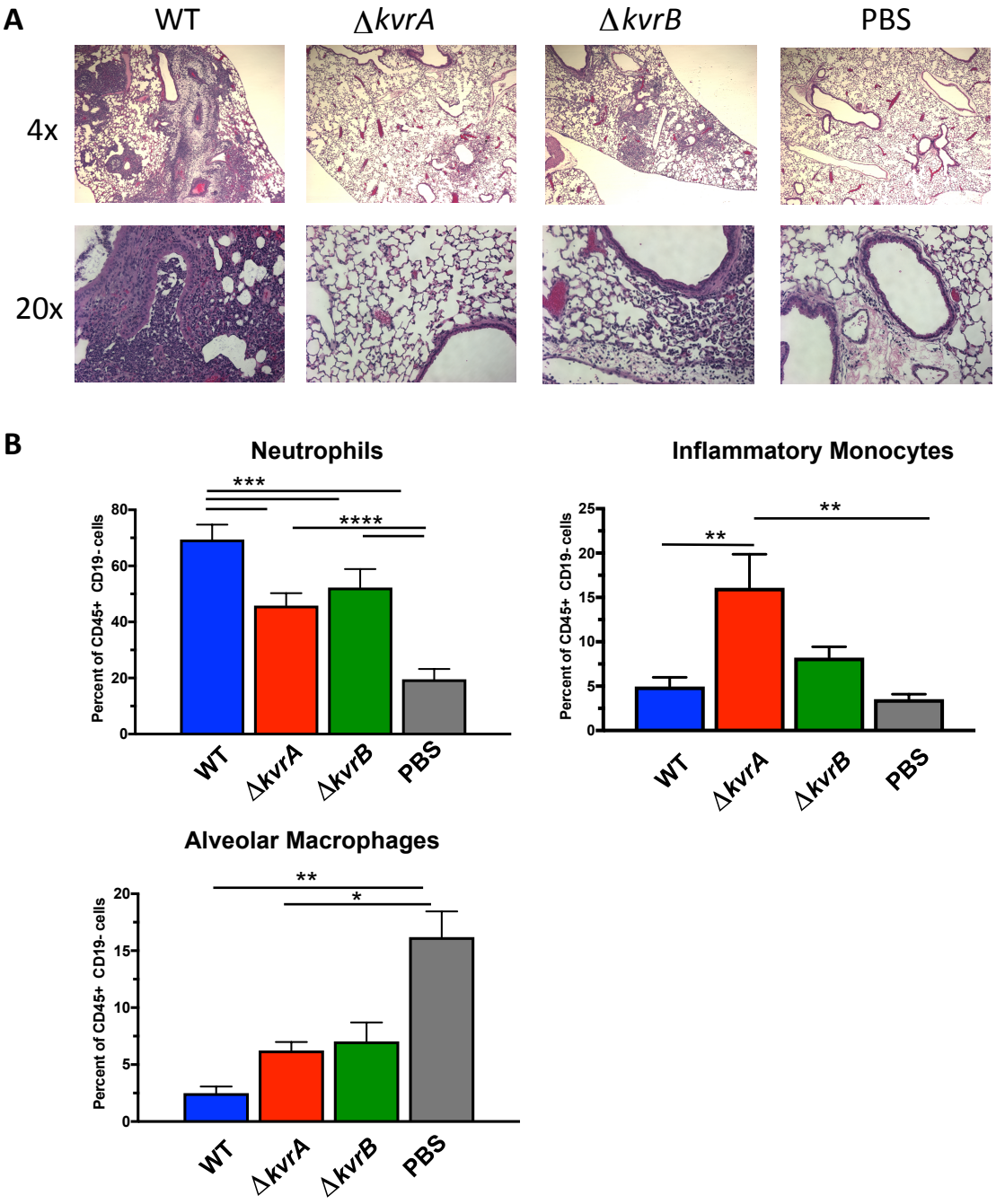

Figure 3.3. Innate immune cell infiltration during *K. pneumoniae* infection. **(**A**)** H&E staining of mouse lungs inoculated with either the wild-type strain, the Δ*kvrA* or Δ*kvrB* mutant, or PBS at 72 hpi. (B) Lungs were processed and evaluated by flow cytometry to identify innate immune cell populations. $CD45^+$ cells in lung homogenates were gated to analyze $Ly6G^+CD11b^+$ neutrophils, $Ly6C^+CD11b^+CD64^+$ inflammatory monocytes, and $CD11c^+F4/80^+$ alveolar macrophages. Each group represents three to five mice. Error bars represent standard error of the mean. Data are from an individual representative experiment, repeated three times. Two-way ANOVA tests followed by Tukey's test were performed for statistical analysis; **=p<0.01, ***=p<0.001.

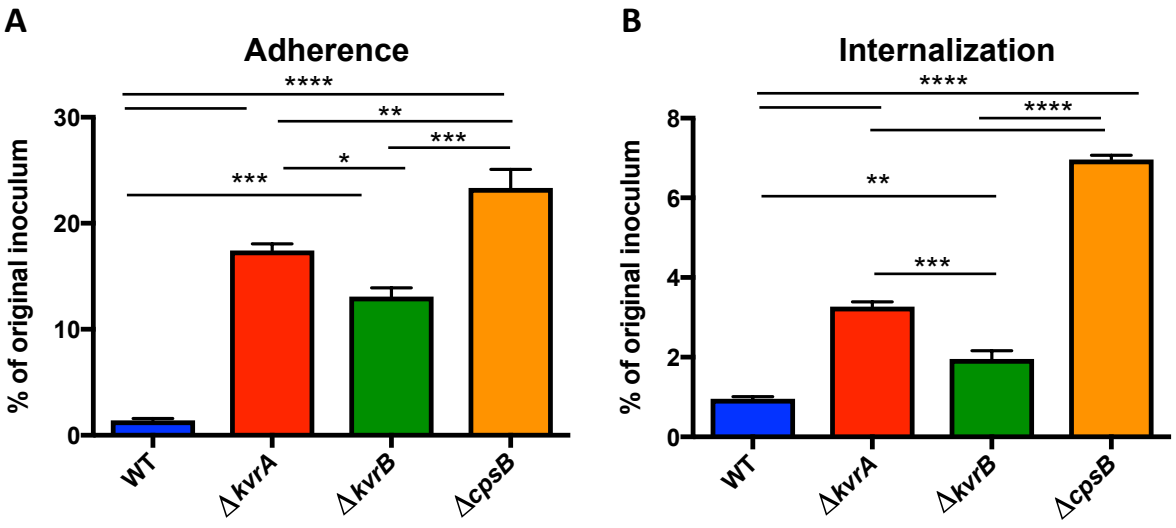

Figure 3.4. Adherence and bacterial uptake of *kvrA* and *kvrB* mutants by macrophages. BMDMs were inoculated with *K. pneumoniae* strains at an MOI of 50 as described in materials and methods. (A) After one hour of incubation the monolayers were washed to remove non-adherent bacteria and then lysed for plating to measure adherent CFU. (B) To measure intracellular survival following one hour incubation, the monolayers were washed to remove non-adherent bacteria and treated with gentamycin as described in materials and methods. After 30 minutes of incubation with gentamycin, the cells were lysed for plating to measure intracellular CFU. One-way ANOVA tests followed by Dunnett's test were performed for statistical analysis; *=p<0.05, **=*p*<0.01, ***=*p*<0.001, ****=*p*<0.0001.

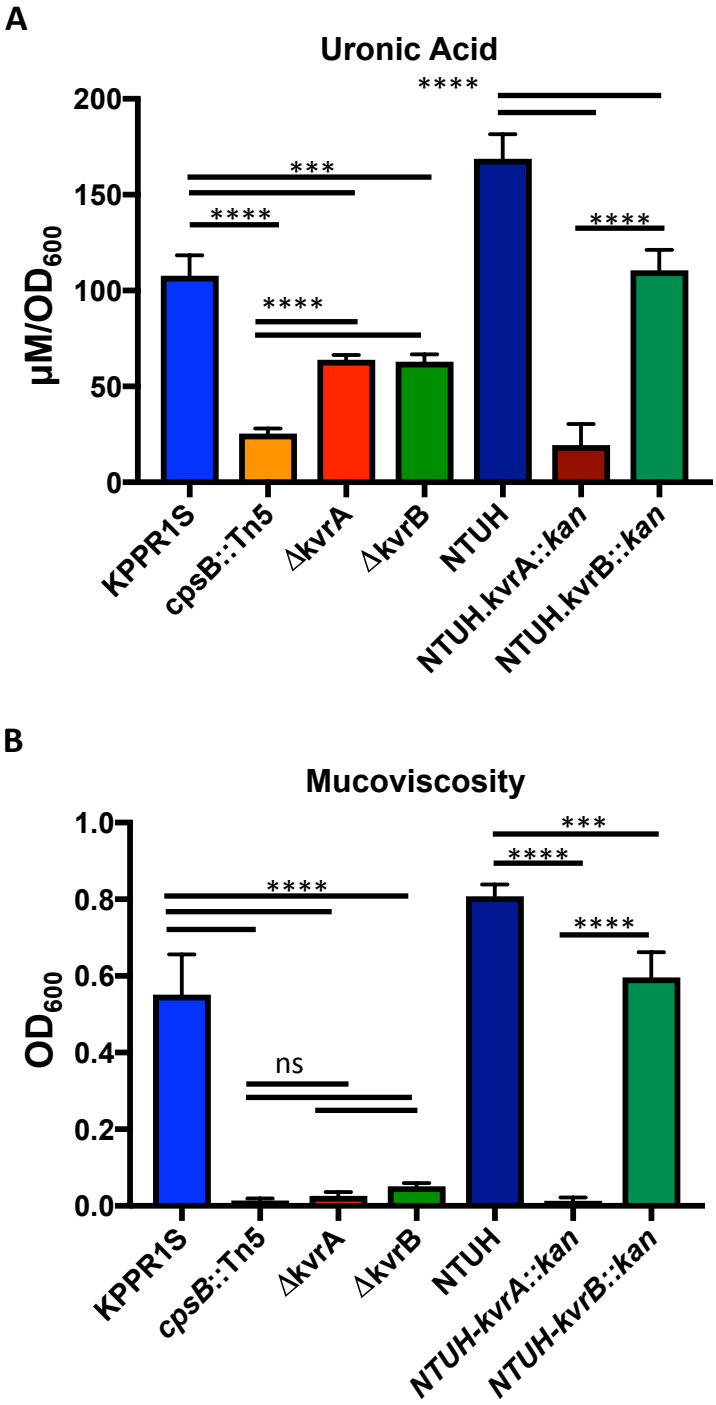

Figure 3.5. Capsule production and mucoviscosity is reduced in the *kvrA* and *kvrB* mutants. (A) Uronic acid quantification of cultures grown in LB for 6 hours. **(**B) Mucoviscosity of cultures grown in LB for 6 hours was determined as described in materials and methods. Each strain was tested in triplicate. Data is representative of an individual experiment. One-way ANOVA tests followed by Dunnett's test were performed were performed for statistical analysis; ***=p<0.001, ****=p<0.0001

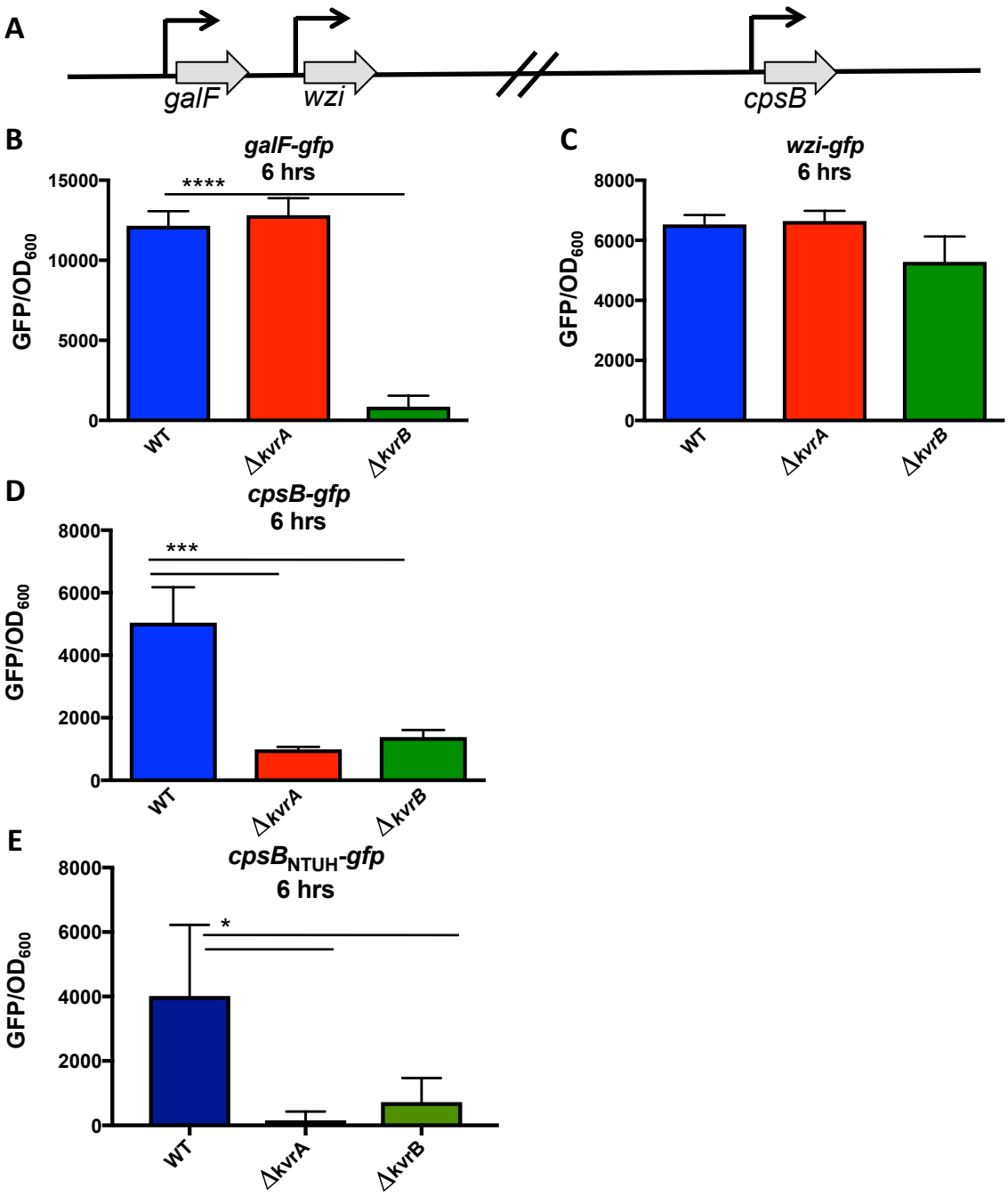

Figure 3.6. KvrA and KvrB regulate capsule gene expression. (A) A schematic of the *cps* locus is shown, indicating the location of previously identified promoters. The promoter regions upstream of *galF, wzi*, and *cpsB* were cloned into the pPROBE *gfp* vector. (B, C, D) Cultures of strains containing the *gfp* reporter constructs to the *galF, wzi,* and *cpsB* promoters of KPPR1S were grown in LB for 6 hours and fluorescence was measured and normalized to an OD_{600}. (E) Strains containing the pPROBE construct with the *cpsB* promoter region from NTUH-K2044 fused to *gfp were* grown in LB for 6 hours and fluorescence was measured and normalized to an OD_{600}. Each strain tested in triplicate. Data is representative of an individual experiment. One-way ANOVA tests followed by Dunnett's test were performed for statistical analysis; ***=p<0.001, ****=p<0.0001

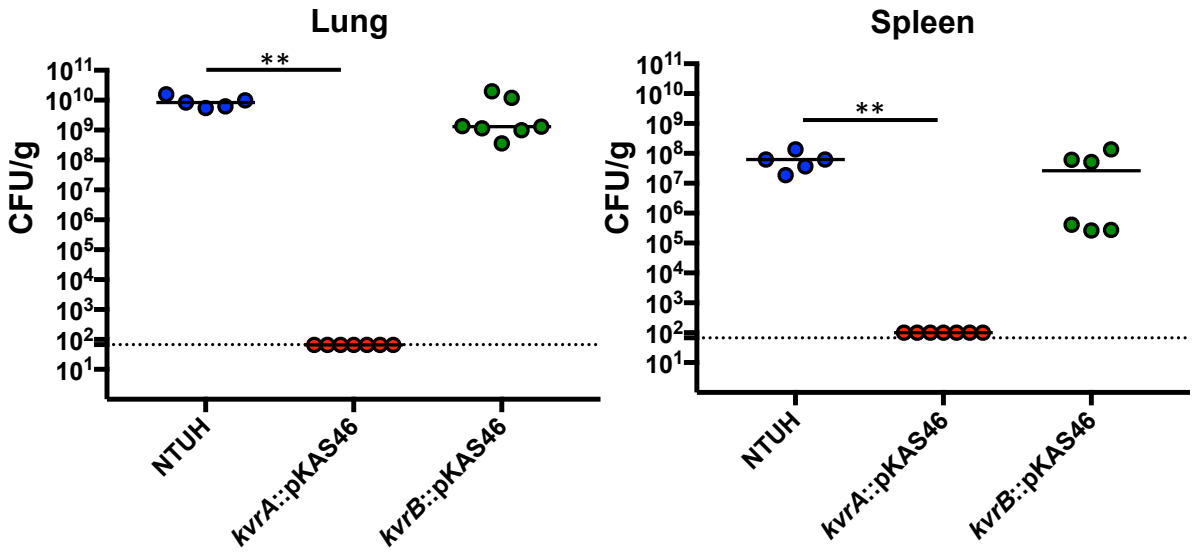

Figure 3.7. The *kvrA* mutant is attenuated in the NTUH-K2044 strain background. Mice were inoculated i.n. with $2x10^4$ CFUs of either the wild-type strain (NTUH-K2044) or *the kvrA* or *kvrB* mutant strains (VK559, VK560). 48 hpi, mice were euthanized and the lungs and spleens were homogenized and plated for bacterial enumeration. Each symbol represents one mouse. The dotted line indicates the limit of detection and symbols on the dotted line indicate CFU were below the limit of detection. Data are from an individual experiment. Mann-Whitney tests were performed for statistical analysis, **=p<0.01.

Table 3.1
Primers used in this study

Primer	Sequence [a] (5' to 3')	Description
MP159	TGACTA**GATATC**CGACACTCTTAACCAACAGCT	5' forward Δ*0496*
MP160	TGCATA**TCTAGA**CTCCAGGCTAAAGATTAATTC	5' reverse Δ*0496*
MP161	TGCATA**TCTAGA**TGGCGATTCCAATTTCATCTC	3' forward Δ*0496*
MP162	TCGATA**GCGGCCGC**CATGCGGCAATCAGGGCGACG	3' reverse Δ*0496*
MP228	TGCATA**TCTAGA**GTGCGCACACCTATAAGCGTA	5' forward for *cpsB* promoters
MP229	CAGTAC**GAATTC**GCTCGCGAGACATCGGCCAGA	3' reverse for *cpsB* promoters
MP232	CAGTAC**GAATTC**TGGGCTCCCAGGGAGGAAAGC	5' forward for *wzi* promoter
MP233	TGCATA**TCTAGA**CTGTACGACTGCGGTATGTGT	3' reverse for *wzi* promoter
MP234	TGCATA**TCTAGA**CTGTACGACTGCGGTATGTGT	5' forward for *galF* promoter
MP235	CAGTAC**GAATTC**GCATATGCTGCCGGCCACAAA	3' reverse for *galF* promoter
MP251	TGACTA**GATATC**CACGACACCATCAGGATGGCG	5' forward Δ*0870*
MP252	TGCATA**TCTAGA**GCTGGTACTTTTCATAATGGT	5' reverse Δ*0870*
MP253	TGCATA**TCTAGA**CTCAAGAAGGTCCTGCCGTAA	3' forward Δ*0870*
MP254	TCGATA**GCGGCCGCC**AAGAGTACGATAGCTGCGGC	3' reverse Δ*0870*
MP256	TGACTA**GATATC**GGAACGGTTAAGCAACGCCTC	5' forward *4417::kan*
MP257	TGCATA**TCTAGA**CTGACTAAACCTCAGTATGCG	3' reverse *4417::kan*
MP258	TGACTA**GATATC**CTCCAGCGCCTTCAGACGATG	5' forward *4238::kan*
MP259	TGCATA**TCTAGA**GCGGCCAGACCTCGACTGCAG	3' reverse *4238::kan*
MP260	TGACTA**GATATC**GCTGACGGTGGTGTAATCGCG	5' forward *1202::kan*
MP261	TGCATA**TCTAGA**TCAGCGTAATGAACCAGCCGC	3' reverse *1202::kan*
MP262	TGACTA**GATATC**CGGCGGTCAGCGTCAGCAGGC	3' forward *2679::kan*
MP263	TGCATA**TCTAGA**GCCAGTGGCGCGCCATTATTG	3' reverse *2679::kan*
MP274	TGACTA**GATATC**TCAGCGATACGGGTGGCGTTG	5' forward *4504::kan*
MP275	TGCATA**TCTAGA**CCGCCATGAAGATTTCCCGTT	3' reverse *4504::kan*
MP276	TGACTA**GATATC**GGCGGTCAATCGTCGGCTGAA	5' forward *1021::kan*
MP277	TGCATA**TCTAGA**GCAGTCGCTTACGTTTGTCGC	3' reverse *1021::kan*
MP280	TGACTA**GATATC**GGATCTGTTCAATAAGACCGA	5' forward *1682::kan*
MP281	TGCATA**TCTAGA**AAGAGCACGTGGAAGGGTTTC	3' reverse *1682::kan*
MP285	TGACTA**GATATC**CCGATCGAGCGTGACCAGCGG	5' forward Δ*4504*
MP286	TGCATA**TCTAGA**GAAGGCGTGCTAGCGGACTCT	5' reverse Δ*4504*
MP287	TGCATA**TCTAGA**AAACGAACTATCCATTTGGGT	3' forward Δ*4504*
MP288	TCGATA**GCGGCCGC**TCTGCGGGTGTCTGACGGCGC	3' reverse Δ*4504*

[a] Restriction sites are in bold

Table 3.2
Bacterial Strains and Plasmids used in this work

Strain	Description	Reference
E. coli		
DH5α	F⁻ ϕ80*lacZ*ΔM15Δ(*lacZYA-argF)U169deoPrecA1endA1hsdR17* ($r_k^-m_k^-$)	Invitrogen
S17-1λ*pir*	Tp^{r}Str^r*recA thi pro hsdR hsdM*$^+$ RP4-2-Tc::Mu::Km Tn7 λ*pir* (lysogen)	34
K. pneumoniae		
KPPR1	Rif^R derivative of ATCC 43816	35
KPPR1S	Strep^R derivative of KPPR1	This work
VK60	KPPR1 *cpsB*::Tn*5*	38
VK280	KPPR1S *4504::pKAS46*	This work
VK277	KPPR1S Δ*kvrA (*VK055*_0496)*	This work
VK410	KPPR1S Δ*kvrB* (VK055*_4504)*	This work
VK278	KPPR1S *kvrAcomplemented*	This work
VK417	KPPR1S *kvrBcomplemented*	This work
VK322	KPPR1S *4417::pKAS46*	This work
VK323	KPPR1S *4238::pKAS46*	This work
VK324	KPPR1S *1202::pKAS46*	This work
VK325	KPPR1S *2679::pKAS46*	This work
VK326	KPPR1S *4504::pKAS46*	This work
VK327	KPPR1S *1021::pKAS46*	This work
VK328	KPPR1S *1682::pKAS46*	This work
VK332	KPPR1S Δ*0870*	This work
VK427	NTUH-K2044	61
VK559	NTUH-K2044 p*0496::pKAS46*	This work
VK560	NTUH-K2044 p*4504::pKAS46*	This work

Plasmid	Description	Reference
pKAS46 vector	Kanamycin resistant, suicide vector, *rpsL*$^+$	32
p*0496::pKAS46*	disruption of *0496*	
p*4417::pKAS46*	disruption of *4417*	This work
p*4238::pKAS46*	disruption of *4238*	This work
p*1202::pKAS46*	disruption of *1202*	This work
p*2679::pKAS46*	disruption of *2679*	This work
p*4504::pKAS46*	disruption of *4504*	This work
p*1021::pKAS46*	disruption of *1021*	This work
p*1682::pKAS46*	disruption of *1682*	This work
pKAS46Δ*marR*	*0870* flanking region in pKAS46	This work
pKAS46Δ*kvrA*	*kvrA* flanking region in pKAS46	This work
pKAS46Δ*kvrB*	*kvrB* flanking region in pKAS46	This work
pKAS46 *kvrA*comp	*kvrA* flanking region and gene in pKAS46	This work
pKAS46 *kvrB*comp	*kvrB* flanking region and gene in pKAS46	This work
pPROBE vector	pPROBE tagless *gfp* reporter	36
pPROBE*_galF*	*galF* promoter of KPPR1S in pPROBE	This work
pPROBE*_wzi*	*wzi* promoter of KPPR1S in pPROBE	This work
pPROBE*_cpsB*$_{KPPR1S}$	*cpsB* promoter of KPPR1S in pPROBE	This work
pPROBE*_cpsB*$_{NTUH}$	*cpsB* promoter of NTUH-K2044 in pPROBE	This work

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CHAPTER 4: DISCUSSION

4.1 Summary of Main Findings

The contribution of capsule and siderophore systems to virulence of *K. pneumoniae* has been widely studied, however, translating *in vitro, in vivo,* and *in silico* findings to therapeutic development is challenging due to the high variability between clinical strains. Thus, identifying and focusing on highly conserved virulence mechanisms among *K. pneumoniae* strains is especially important for *K. pneumoniae* research. Prior to these studies, research suggested that while enterobactin is the most widely conserved siderophore in *K. pneumoniae*, enterobactin only modestly contributed to *K. pneumoniae* virulence in the lung (1). While previous studies have examined the contribution of siderophores to virulence, few studies have examined the role of siderophore transport proteins to virulence. In Chapter 2, we described how a serendipitous mutation led us to focus on enterobactin utilization proteins. From this work, we show that the enterobactin transport protein, FepB, is important for *K. pneumoniae* virulence in the lung and that this contribution appears to be independent of its role in enterobactin import. Importantly, unlike other siderophore systems, the enterobactin system, along with FepB, is present in all *K. pneumoniae* strains. Not only is enterobactin present in *K. pneumoniae*, it is also present in other *Enterobacteriaceae* therefore our findings may be relevant to other pathogenic bacteria.

Previous studies on transcriptional regulators have focused on capsule production but most work, with the exception of RmpA studies, has been confined to *in vitro* assays. Additionally, individual studies of capsule regulation focus on an individual strain and fail to extend findings to other strains. Our work was the first to characterize the contribution of the MarR family of regulatory proteins to *K. pneumoniae* virulence. Results from these studies reveal that while *K. pneumoniae* contains several *marR*-like genes, not all are important for a pneumonic infection, and those that are, contribute to disease in distinct ways. Work discussed in Chapter 3 describes the characterization of two newly identified conserved regulators of *K. pneumoniae* virulence, KvrA and KvrB. We show *kvrA*- and *kvrB*- deficient strains are attenuated for virulence in the lung and that the kinetics of infection with these two mutants are distinct, suggesting differences in their regulons. Furthermore, we demonstrate that KvrA and KvrB both affect capsule production and that loss of *kvrA* or *kvrB* results in increased adherence and uptake by murine BMDM. Importantly, we demonstrate that the effect of KvrA and KvrB on capsule is at least in part due to a conserved regulatory mechanism, as *cpsB* promoter expression and capsule phenotypes are also affected by these regulators in a liver abscess-derived K1-type isolate. Thus, our findings are applicable to other *K. pneumoniae* clinical isolates.

4.2 Gaps in knowledge: Future directions

4.2.1 The contribution of FepB to virulence

Enterobactin is widely conserved amongst *K. pneumoniae* and *Enterobacteriaceae* (2-4). Like our studies in Chapter 2, one other study in *S. enterica* demonstrated a contribution of FepB to virulence (5). Both studies found the

contribution of FepB to virulence to be greater than that of the enterobactin/salmochelin systems alone. One caveat to our Chapter 2 study is that we were unable to complement the *fepB* mutant. Plasmid-based complementation approaches failed to restore growth in iron-deleted conditions despite constitutive expression of *fepB.* Problems with *fepB* complementation are not unprecedented and also were reported for a *fepB* mutant in *Salmonella* (5). However, to ensure the phenotype was not a consequence of secondary mutations, a second *fepB* mutant was constructed and found to recapitulate the *in vitro* growth phenotypes and virulence defect of the original *fepB* mutant. qRT-PCR analysis revealed that deletion of the *fepB* gene resulted in loss of expression of the adjacent genes, *entC* and *entS*. EntC and EntS may be needed for growth *in vitro* in iron limited conditions, and their lack of expression could explain the failed complementation in our *in vitro assays*. However, this alone cannot explain the attenuation *in vivo* as an Δ*entS* mutant was not attenuated and an Δ*entC* mutant (enterobactin synthesis) had a more modest attenuation level than the Δ*fepB* mutant. Thus, we believe that deletion of *fepB* results in a phenotype distinct from other enterobactin system mutants. One potential future experiment would be to express *fepB* and its adjacent genes (i.e. *entS* and *entC*) in the *fepB* mutant and test if this complementation approach restores the *in vitro* growth defect and *in vivo* phenotype.

Our studies focused on the role of FepB in siderophore import since there is already an established role for FepB in enterobactin import. Because the Δ*fepB* mutant resembles a Δ*entB*Δ*ybtS* mutant in *in vitro* and *in vivo* assays, we hypothesized FepB may be playing a role in yersiniabactin import, as no

perisplasmic import protein has been identified for this system (Fig. 4.1). However, our results did not support a role for FepB in yersiniabactin import. We did not explore the possibility that FepB may be involved in siderophore export as there is no precedent for this role. We did, however, test a mutant deficient in enterobactin export, Δ*entS,* and did not find this mutant to be attenuated in our mouse model. However, we did not examine a possible role for *fepB* in yersiniabactin export. Unfortunately, because of the growth defect seen in the Δ*fepB* mutant, results from testing this hypothesis in our cross-feeding assay would be difficult to interpret. In this assay, we assess the ability of feeder strains (strains secreting one or more siderophores) to restore the growth defect of siderophore deficient strains in low-iron conditions. It would be difficult to differentiate a lack of siderophore secretion from poor strain growth of the feeder strain (the Δ*fepB* mutant). Furthermore, we do not have an *in vitro* phenotype for the yersiniabactin synthesis mutant, thus there is no growth phenotype to complement as a control for these experiments. More sensitive analysis of siderophore secretion of the Δ*fepB* mutant, such as mass spectrometry, may be a more suitable approach to measure any differences in the export of siderophores in the Δ*fepB* mutant.

The role of siderophore systems in virulence has been expanded beyond iron acquisition. Enterobactin has been shown to enhance copper toxicity on the bacterial cell surface (6). One possible explanation is that in the *fepB* mutant, enterobactin accumulates extracellularly and perhaps this is detrimental to the bacteria. However, in this scenario, the Δ*smr_C* mutant (which is a Δ*entB*Δ*fepB* double mutant and also has other genes deleted) should relieve this phenotype, as it would be unable to

produce enterobactin. However, the data presented in Chapter 2 suggests this is not the case as the Δ*smr_C* mutant resembles the Δ*fepB* mutant.

Unlike some *Enterobacteriaceae*, *Yersiniae* do not encode enterobactin. However, *fepB* is present in *Y. pestis* (unpublished data). This raises the question of why *Y. pestis* encodes *fepB*. Genomic context provides little information on a potential role, as it is not in an operon and not located adjacent to iron acquisition genes. Perhaps this is further evidence that FepB may have a role outside of enterobactin import. Although we do not know what other function FepB has in the cell, we believe its high conservation among *Enterobacteriaceae* suggests an important contribution to virulence. Therefore, FepB could serve as a possible therapeutic target.

4.2.2 Capsule diversity and pathogenesis

Prior to 2015, specific K-types were believed to be associated with particular infection sites (7). K1 and K2 isolates were believed to be the most common with K2 isolates most frequently associated with UTIs, pneumonia, and bacteremia and K1 isolates associated with the development of liver abscesses (7, 8). Since 2015, several epidemiological studies have focused on capsule classification using whole genome sequences and have provided much insight on capsule diversity and the associations with clinical outcomes (3, 9, 10). While it remains true that K1 and K2 capsule types are the most prevalent capsule types, the five most common K-types only account for about 20% of all K-types (11). Contrary to older, more limited studies using serotyping, recent bioinformatics data suggest that no single K-type is associated with a given infection site (9). These trends pose a challenge for vaccine

development as no capsule types can be singled out to target. It does, however, emphasize that most strains do produce capsule and that conserved aspects of capsule production could serve as therapeutic targets.

Interestingly, only two associations between capsule type, specifically K1 and K2, and isolate type have been identified. The K1 capsule type is mostly restricted to community-acquired isolates (9). Pyogenic liver abscesses are most prevalent in Southeast Asia (12, 13). A search for genes overrepresented in K1 strains versus the other K-type isolates identified a gene encoding an alginate lyase isozyme (9). This lyase was absent in nearly all the non-K1 strains examined. An alginate lyase allows alginate to be used as a carbon source. Alginate is a polyuronic saccharide present in the cell walls of brown seaweed species (14). Furthermore, the likelihood of carrying a K1 isolate in the gut is higher in Koreans living in Korea versus individuals of Korean ethnicity living in other countries (15). This suggests that the incidence of carrying a K1 strain is driven more by environmental factors, such as diet, than host genetics. Incidentally, residents in southeast Asian countries consume the greatest proportion of seaweed (16). Thus, diet may be contributing the emergence of K1 liver abscess in Southeast Asia. K2 isolates are more likely to be associated with invasive infections. Although the reason for this association is unclear, both K1 and K2 are more likely than any other K type to have additional virulence factors (such as RmpA, aerobactin, salmochelin, and yersiniabactin) which may also account for the identified pathogenic associations mentioned above (9).

4.2.3 Conserved aspects of capsule production

The capsule locus contains genes involved in sugar precursor synthesis, chain assembly and translocation. The genes involved in the synthesis of sugar precursor molecules can vary from strain. These include genes for enzymes involved in mannose (*manCB*) and rhamnose (*rmlBADC*) synthesis. Interestingly, 43.1% of isolates from a 2016 study of over 2500 genomes, contained the *manCB* genes (*manC* is an alternate name for *cpsB*) whereas 30% of isolates contained *rmlBADC* genes, and 10.2% of genomes contained both sets of sugar synthesis genes (11). This suggests, while capsule type is highly variable, capsule production is dependent on production of mannose and/or rhamnose and that altering the available pool of these precursors by targeting regulators needed for their production may be a therapeutic approach.

The work described in Chapter 3 demonstrates that KvrA and KvrB regulate capsule expression and production of both K1 and K2 capsule. Both of these cps loci contain *manCB,* as the K2 capsule contains mannose and the K1 capsule contains fucose (that requires mannose for its synthesis). Thus, the contribution of KvrA and KvrB to capsule regulation may extend beyond K1 and K2 capsule types, to other *manCB* containing strains. Furthermore, while most studies have focused on the conservation of genes within the locus, no study has focused on studying the conservation of the promoter sequences. It is possible that the same regulators that contribute to *manCB* expression, also contribute to *rmlBADC* expression although this has not been tested. As described below, this may be the case if KvrA or KvrB regulation of capsule gene expression is via H-NS displacement rather than by direct

activation of transcription. Genome wide association studies provide information on the absence or presence of a gene, but not information on expression level or regulatory pathways. Thus, regulation studies may reveal previously unidentified conserved virulence pathways.

4.2.4 Regulatory network

Previous studies on transcriptional regulators in *K. pneumoniae* have focused on capsule production but have been conducted in different strains and most studies do not link *in vitro* regulation studies with *in vivo* phenotypes. Regulation of capsule in *K. pneumoniae* has previously been linked to Fur, CRP, IscR, RcsB, RmpA, and RmpA2 (17-23). Work in our lab has begun to characterize the capsule regulatory network in order to identify conserved aspects of capsule production. Our lab has begun to characterize the regulation of the *galF*, *wzi*, and *cpsB* promoters by KvrA, KvrB, RmpA, RcsB, and a newly identified regulator, RmpC (unpublished data, Fig 4.2). Consistent with previous studies, we have demonstrated regulation of the *cpsB* promoter by RmpA and RcsB (unpublished data, (17)). Contrary to previously published studies we did not find RmpA or RcsB to regulate the *galF* promoter (unpublished data, (17)). Unfortunately, because there is so much variation between strains, including variation in the contribution of RmpA to virulence, it is difficult to compare capsule regulation across strains. Therefore, future studies in our lab hope to fill-in important gaps in the literature and develop a clearer picture not only of capsule gene expression but also virulence regulatory networks.

The histone-like nucleoid-structuring protein (H-NS) is a DNA-binding protein found in enteric pathogens such as *E. coli, S. typhimurium, Y. pestis,* and *K.*

pneumoniae (24) (25, 26). In *K. pneumoniae*, H-NS was found to repress CPS production and hypermucoviscosity (24). H-NS was found to reduce expression of *galF, wzi,* and *cpsB (manC)* as determined by qRT-PCR (24). The *cps* locus of the KPPR1S strain used in Chapter 3 contains AT-rich regions, a common trait of H-NS regulated regions (27). Several regulators have been shown to function by derepressing H-NS (25, 27). Of these, the KvrA homologues, RovA and SlyA, have been shown to lead to transcriptional activation by displacing H-NS, thus inhibiting H-NS repression (27, 28). If KvrA and KvrB regulate *cps* gene expression by binding directly or through interfering with H-NS mediated repression remains to be elucidated. If capsule regulation by KvrA and KvrB is less dependent on binding a consensus sequence and functioning via H-NS displacement, then the findings of our studies may have broader implications in *K. pnuemoniae* virulence as H-NS is a global regulator in bacteria (24, 27).

4.2.4.1 MarR family regulon

The MarR family of transcriptional regulators is widely conserved in bacteria (29). Members of the MarR family of transcriptional regulators characterized in other bacteria have typically been shown to regulate multiple genes. In *S. enterica,* SlyA was demonstrated to regulate dozens of genes, including some important for virulence and resistance to antimicrobial peptides (30). In *Y. pestis*, the MarR-like regulator RovA also served as a global regulator of virulence determinants in a subcutaneous infection model (28). The work in Chapter 3 focused on the regulation of capsule by KvrA and KvrB. Because both KvrA and KvrB affect capsule production to a similar degree but display different *in vivo* infection profiles, we

hypothesize their regulons differ and plan to conduct RNA-seq experiments to examine transcriptional changes.

4.2.5 Summary of Future Questions

Capsule Regulatory Network

Do KvrA and KvrB bind the capsule promoters directly? Compete with H-NS for binding?

Do KvrA and KvrB regulate other previously identified capsule regulators?

Can the kvrB *mutant serve as a vaccine?*

What other promoters exist within the capsule locus?

Do KvrA and KvrB regulate other virulence factors?

Are kvrA *and* kvrB *important for other K. pneumoniae infection models?*

FepB

Is fepB *important for virulence in pathogens that do not encode enterobactin?*

Is fepB *important for other K. pneumoniae infection models?*

4.3 Implications for the study of *Klebsiella*

Multi-valent protein-conjugate polysaccharide vaccines have been effective against bacterial pathogens such as *Streotococcus pneumoniae*, however, the high diversity of K-antigens in *K. pneumoniae* has proved to be challenge in vaccine development. Initial attempts at developing *Klebsiella* vaccines focused on targeting K-antigens. One vaccine included capsule from 24 *Klebsiella* types and LPS from 8 *Pseudomonas aeruginosa* O-types, but was ineffective as there was no significant reduction in the incidence of contracting *Klebsiella* infections in patients that

received the vaccine (31, 32). Currently, the only ongoing clinical trial is testing a multicomponent vaccine (containing antigens from *K. pneumoniae*, *Proteus vulgaris, E. coli,* and *Staphylococcus aureus*), Immunovac, being tested on patients with asthma and COPD (clinicaltrials.gov search terms: "*Klebsiella* vaccine", (33)).

Whole genome comparative analyses reveal the diversity of the *K. pneumoniae* genome and how less than 40% of its genome is conserved within the species (3). Given how the best characterized virulence determinant is capsule, and that 134 distinct K-loci have been identified (9), vaccine development and therapeutic studies must account for this variability when targeting surface components. Thus, highly conserved systems, such as the regulators KvrA and KvrB, involved in capsule production are potential targets for drug design.

The *kvrB* mutant characterized in Chapter 3 was attenuated but not immediately killed. While a few mice cleared the infection and others succumbed to it, kinetics experiments determined that the *kvrB* mutant could be detected in the lung 7 days post inoculation. If mice infected with the *kvrB* mutant are protected from subsequent infection from the same or different *K. pneumoniae* strain was not investigated. Additionally, if the reduction of capsule or mucosviscosity resulted in a change of surface exposed antigens, such as an increase in exposed LPS is unknown.

If there is any change within the O-antigen (LPS) in any of the capsule regulatory mutants has not been examined. The *rfb* locus responsible for the production of LPS is located adjacent to the *cps* locus. It is possible that one or multiple of the characterized regulators affect expression of the *rfb* locus.

Furthermore, it is unknown if changes in the capsule structure or sugar precursor pool affects LPS. Interestingly, in comparison to other *Enterobacteriaceae* such as *E. coli* (161 O-types), *Klebsiella* has significantly less O-antigen diversity (9). Constitutive expression of capsule *in vivo* has not been examined therefore it is unknown if there is a stage during infection when capsule is not produced and LPS is exposed.

4.4 Conclusions/Perspective

K. pneumoniae is considered an urgent threat to public health due to nosocomial multi-drug resistant strain outbreaks and the emergence of hypervirulent strains capable of causing community acquired infections. Recent studies highlight the increase in incidence of this pathogen and its increased propensity to develop antimicrobial resistance. It has been only in recent years that we gained a better understanding of the population structure of emergent strains. Our studies attempt to overcome the known challenge of strain-to-strain variation and provide the groundwork for potential conserved therapeutic targets.

4.5 Figures and Tables

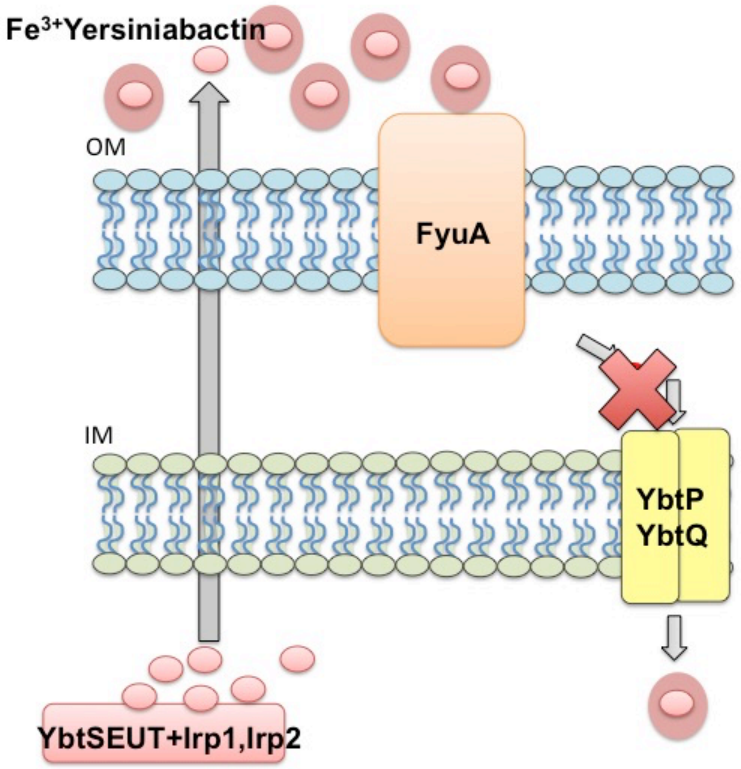

Figure 4.1. **Yersiniabactin transport**. Yersiniabactin synthesis occurs in the cytoplasm and export is via an unknown pathway. Import occurs through the FyuA receptor and YbtPQ proteins, but does not appear to involved FepB5. Several black boxes in yersiniabactin transport remain.

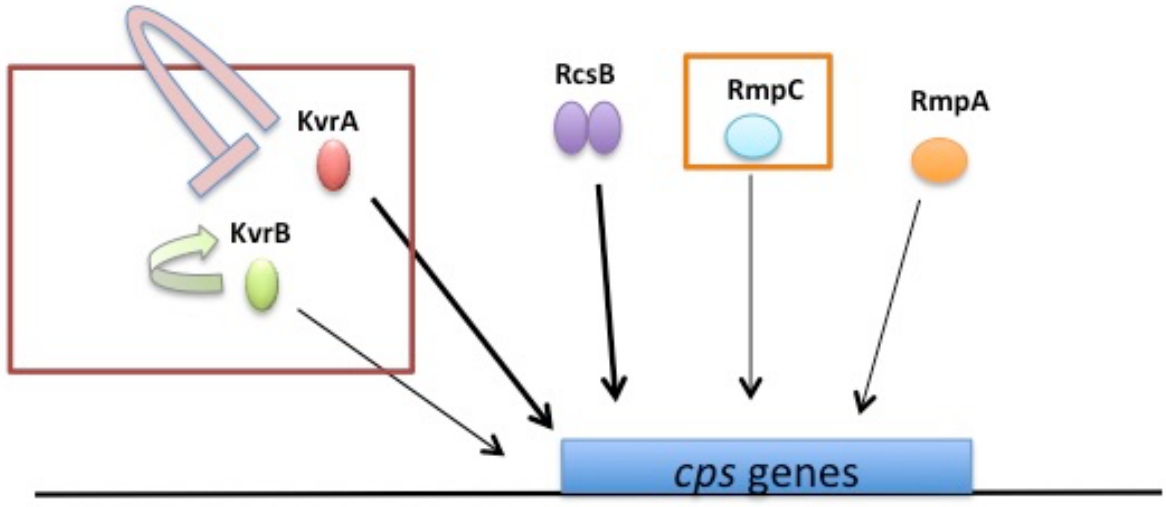

Figure 4.2. **Schematic of regulators currently under investigation in KPPR1S**. KvrA and KvrB (red box) were the focus of the study in Chapter 3. KvrA has since been shown to repress KvrB activity and KvrB has been shown to autoregulate (Walker, unpublished data). RmpC (orange box) is a newly identified regulator by the lab. RcsB and RmpA are regulators previously characterized in other *K. pneumoniae* strains, but their contribution to capsule production and virulence has been confirmed in our strain.

Regulator (Reference)	Strain	K-type	Effect on capsule?	*in vivo* study?
KvrA this work	KPPR1S, NTUH-K2044	K2, K1	KvrA regulates capsule expression (*cpsB* promoter in KPPR1S and NTUH-K2022) and production	yes
KvrB this work	KPPR1S, NTUH-K2044	K2, K1	KvrB regulates capsule expression (*galF, cpsB* promoters in KPPR1S, *cpsB* in NTUH-K2022) and production	yes
RmpA unpublished work, (13), (19)	KPPR1S, CG43, NTUH-K2044	K2, K1	RmpA regulates capsule expression (*cpsB* promoter in KPPR1S, *galF* and *cpsB* in CG43) and production; RmpA did not contribute to capsule in NTUH-K2044	yes
RcsB unpublished work, (13)	KPPR1S, CG43	K2	RcsB regulates capsule expression (*cpsB* promoter, *galF* and *cpsB* in CG43) and production	yes
RmpC unpublished work	KPPR1S	K2	RmpC regulates capsule expression (*cpsB* promoter) and production	yes
IscR (15)	CG43	K2	In iron limited conditions, less IscR binds to the capsule promoters and less capsule is produced	no
Fur (13)	CG43	K2	In iron replete conditions, Fur inhibits capsule synthesis through inhibiting *rmpA* expression	no
CRP (18)	123/01	K39	In low glucose conditions, CRP inhibits CPS production	no
H-NS (19)	123/01	K39	H-NS repressed CPS production and mucoviscosity	no

Table 4.1 Table of transcriptional regulators of the capsule locus in *K. pneumoniae*. Studies of the regulators shaded in light blue have begun in our strain. Other regulators that have not been analyzed in KPPR1S are shown below the blue shaded area.

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