High-Throughput Identification and Characterization of *Klebsiella pneumoniae* Virulence Determinants in the Lungs

A thesis

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ABSTRACT

Klebsiella pneumoniae (Kp) is a gram-negative bacterial pathogen that causes a range of infections, including pneumonias, and has become an increasing concern due to the rise and spread of antibiotic resistant and hypervirulent strains. Despite the importance of this pathogen, its virulence determinants remain largely understudied. Therefore, to identify novel *Kp* virulence determinants needed to cause pneumonia, a high-throughput screen was performed in the lungs of WT mice using TnSeq with an arrayed library of more than 13,000 *Kp* transposon insertion mutants. Furthermore, *Kp* is classically an opportunistic pathogen that typically infects the immunosuppressed, such as those with conditions that result in defective neutrophil responses like diabetes and malignancies, and there remains much left to be discovered about *Kp's* interactions with the immune response of which neutrophils have been shown to play a strong role in the lungs. Therefore, the genes needed for *Kp* to infect neutropenic lungs were also assessed. A comparison of the two studies reveals genes *Kp* needs to protect against neutrophils, as well as genes that *Kp* requires in both immunocompetent and immunosuppressed hosts. The latter could encode factors that may potentially serve as therapeutic targets. Using this method, mutants with transposons in 166 genes were significantly less fit infecting lungs of WT mice. Of these, 51 genes were still significantly less fit in neutropenic mice, 52 were able to colonize neutropenic mice, and the differences in the fitness of the remainder in the absence versus the presence of neutrophils was modest. *In vitro* screens using a mini-library of *Kp* insertion mutants of genes with fitness defects in the initial screen identified a number of putative functions for a subset of these genes, such as in growth under different conditions, exopolysaccharide production, resistance to ROS and RNS, and *de novo*

synthesis of amino acids and metabolites. Further *in vivo* studies using single strain and mini-library competition lung infections confirmed roles in *Kp* virulence in the lungs for *dedA, dsbA, gntR, VK055_RS17445, VK055_26085, ycgE, yhjH, aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE,* and *tyrA,* all of which were assigned putative functions using the above-described *in vitro* assays. When *aroA* was assessed as a potential therapeutic target against *Kp,* treatment with the AroA-specific inhibitor glyphosate was found to significantly decrease *Kp* growth *in vitro* and bacterial loads in the lungs *in vivo.* Importantly, resistance to glyphosate occurred only rarely when assessing spontaneous resistance in the lab strain *Kp* ATCC 43816 and in clinical isolates, including ESBL and KPC expressing strains. Overall, this study provided evidence for *dedA, dsbA, gntR, VK055_RS17445, VK055_RS26085, ycgE, yhjH, aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* as novel *Kp* virulence factors in the lungs, uncovered putative functional roles in growth and protection against the neutrophil response for these factors, and identified *aroA* as a gene with strong therapeutic potential as a target of inhibitors.

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Paczosa MK, Mecsas J. 2016. *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev **80:**629-661.

LIST OF ABBREVIATIONS

$Apra^R$	Apramycin
BAL	Broncheoalveolar lavage
CFU	Colony forming units
CI	Competitive Index
gDNA	Genomic deoxyribonucleic acid
ESBL	Extended-spectrum beta lactamase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorite
$Hygro^R$	Hygromycin
Kan	Kanamycin
Kp	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharide
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
$Spec^R$	Spectinomycin resistant
Tn	Transposon
UTI	Urinary tract infection

CHAPTER 1: INTRODUCTION[1]

[1]Paczosa MK, Mecsas J. 2016. *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev **80:**629-661. Reprinted here with permission of publisher.

1.1 INTRODUCTION

Klebsiella pneumoniae (*Kp*) has recently gained notoriety as an infectious agent due to a rise in severe infections and the increasing scarcity of effective treatments. These concerning circumstances have arisen due to the emergence of *Kp* strains that have acquired additional genetic traits and become either hypervirulent (HV) or antibiotic-resistant. *Kp* was first isolated in the late nineteenth century and was initially known as Friedlander's bacterium (1, 2). It is a gram-negative, encapsulated, non-motile bacterium that resides in the environment, including in soil and surface waters and on medical devices (3, 4). Importantly, *Kp* readily colonizes human mucosal surfaces, including the gastrointestinal (GI) tract and oropharynx, where the effects of its colonization appear benign (3-5). From these sites, *Kp* strains can gain entry to other tissues and cause severe infections in humans. *Kp* is an extremely resilient bacterium, whose success as a pathogen seems to follow the model of "the best defense for a pathogen is a good defense" rather than "the best defense for a pathogen is a good offense." This is exemplified by the ability of these bacteria to evade and survive, rather than actively suppress, many components of the immune system and grow in many sites in hosts. This introduction will focus on *Kp* virulence factors that have been studied in-depth and are important in one or more types of infection, and on more recent searches for additional *Kp* virulence factors. To understand the roles of these factors in the context of *Kp* infections, the different types of *Kp* strains that are now causing significant disease will be reviewed, as well as the types of diseases caused by these *Kp* strains and the host factors that *Kp* encounters when establishing an infection.

1.2 CLASSICAL, ANTIBIOTIC RESISTANT, EMERGING AND HYPERVIRULENT STRAINS

Over the last few decades, there has been a concerning rise in the acquisition of resistance to a wide range of antibiotics by strains derived from "classical" *Kp*. As a consequence of this antibiotic resistance, simple infections, such as urinary tract infections (UTIs), have become recalcitrant to treatment, and more serious infections, such as pneumonias and bacteremias, have become increasingly life-threatening (6, 7). Two major types of antibiotic resistance have been commonly observed in *Kp*. One mechanism involves the expression of extended spectrum – β lactamases (ESBLs), which render bacteria resistant to cephalosporins, monobactams and penicillins. The other mechanism of resistance, which is even more troubling, is the expression of carbapenemases by *Kp*, which renders bacteria resistant to almost all available β -lactams including the carbapenems (8).

The first case of *Kp* expressing a carbapenemase was identified in North Carolina in 1996, and, thus, this type of carbapenemase is called KPC (9). Additional carbapenemases, such as MBL, NDM-1, IMP and VIM, have since been found in *Kp* strains (10). Notably, all of these carbapenemases, including KPC, have been found in other bacteria, and collectively, they contribute to the worldwide occurrence of carbapenem-resistant bacteria (see (11, 12) for recent reviews) (10). Regardless of the type of carbapenemase they carry, carbapenem-resistant *Kp* are termed CRE, for carbapenem-resistant Enterobacteriaceae. Due to a lack of available effective treatments, *Kp* infections caused by ESBL and carbapenem-resistant bacteria have significantly higher rates of morbidity and mortality compared to infections with non-resistant bacteria

(13). Work published by the CDC in 2013 demonstrates the frequency and severity of infections with these strains based on a 2011 survey of 183 hospitals in the United States (13). ESBL-producing strains caused 23% of nosocomial *Kp* infections, equaling 17,000 infections, and resulted in 1,100 deaths. Meanwhile, carbapenem-resistant *Kp* caused 11% of nosocomial *Kp* infections, equaling 7,900 infections, and resulted in 520 deaths.

The spread of multidrug resistant *Kp* strains has been an area of intensive investigation over the past decade (10, 12, 14-16). Analysis of the vast number of sequenced *Kp* strains in the past decade supports the idea that the *Kp* capsule encoding (*cps)* locus may frequently undergo genetic changes and that *Kp* strains undergo recombination with other strains giving rise to additional strains (14, 17). These recent findings point towards the idea that *Kp* strains are competent to change rapidly and acquire new traits. Furthermore, work implies that there is a reliance of *Kp* on different genetic traits to survive and that the disease caused likely varies among different strains.

Finally, it is important to note that neither the presence of KPC itself nor the specific form of KPC appear to correlate strongly with the virulence traits of *Kp* strains (18, 19). However, much needs to be done to confirm these findings and to understand whether the spread of certain antibiotic strains is due to their ability to persist better in the environment and/or in humans.

The dissemination and mechanisms of resistance by ESBLs and carbapenemases in *Kp* and other bacteria, as well as some of the major mechanisms of *Kp* drug resistance, will be briefly summarized (11, 20). There are four classes of carbapenemases (Class A-D) and KPCs fall into Class A. Sixteen variants of Class A KPCs (KPC2-17) have been identified and KPC-2 and KPC-3 are the best studied to date (21). These KPCs can be

encoded on transposons (14). One such KPC transposon has been found on a number of different types of plasmids in the KPC-expressing ST258 and closely related strains, and plasmids carrying these transposons can be transferred to other *Kp* strains (14, 22). To date, the spread of these plasmids and resistance cassettes to other types of bacteria has been infrequent; however, there are several documented cases (23-25), and it is prudent to expect that more cases will be found or that the transposon may hop into a more promiscuous plasmid. In addition to KPCs, *Kp* strains can carry other forms of carbapenemases, such as Class B metallo-β-lactamases (such as the New Delhi metallo-β-lactamase, NDM-1) and Class D OXA enzymes (20). The resistance of *Kp* to carbapenems depends on several factors. It requires the carriage of these carbapenem-resistance genes, but often also requires the carriage of other β -lactamases and/or mutations in outer membrane proteins (OMPs) that render the bacteria more resistant to β -lactams, particularly in combination with expression of a carbapenemase (10, 26). ESBLs, which are also found worldwide, can also be caused by a number of β-lactamases, the most common of which are TEM, SHV, CTX-M and OXA (11, 20).

Based on the recent emergence and worldwide distribution of certain KPC-expressing strains, it is clear that *Kp* strains can acquire DNA and traits that can change their ability to persist in the environment and human population. Furthermore, several virulence factors have been identified in *Kp* strains that are not widespread, indicating that a variety of factors can give contribute to the persistence and virulence of *Kp*. Combined, these observations indicate that *Kp* genomes and phenotypes can change rapidly, and, therefore, defining strains and strain types is a moving target. Nonetheless for the purposes of the remainder of this introduction, *Kp* strains that lack excessive

capsule (see below) and, therefore, rarely cause disease in otherwise healthy individuals (with the exception of UTIs), will be considered "classical" strains regardless of whether they are multi-drug resistant.

Typically, classical *Kp* strains cause serious infections, such as pneumonia, bacteremia or meningitis, when infecting immunocompromised individuals, including people suffering from diabetes or malignancies (27-29). The carriage and expression of drug-resistance does not enhance the virulence of *Kp* strains despite making them more difficult to treat. However, since the 1980s, strains of *Kp* that can cause serious infections in otherwise healthy individuals have also gained traction in the human population. These strains are considered hypervirulent (HV) compared to classical *Kp* strains due to their ability to infect both healthy and immunocompromised populations, and because of the increased tendency of these infections to be invasive; i.e. they can establish infection in the liver (30, 31). This additional virulence correlates with the acquisition of a 200-220 kb plasmid containing genes that enhance capsule production as well as encode for siderophores (32). These and other bacterial factors that contribute to the hypervirulence of these strains will be discussed in detail later in this review (33). An overview of the differences between classical and HV *Kp* strains can found in **Table 1.1**.

While currently infections caused by HV strains primarily occur in Taiwan and Southeast Asia versus the worldwide occurrence of infections with classical strains, unsurprisingly, there have been reports of the international spread of the HV strains, including into North America (33-35). In contrast to the majority of infections caused by classical *Kp* strains, many HV *Kp* infections originate in the community (36). HV strains are typically associated with pyogenic liver abscesses, although they can also cause

pneumonias, lung abscesses and other types of infections (36, 37). In fact, HV *Kp* has become the most commonly isolated pathogen in lung abscesses in Taiwan, with a report approximating *Kp* in lung abscesses in 21% of patients from 2000-2004, a shift from a previous report documenting 1985-1990, where *Kp* was associated with lung abscesses in only 4.6% of cases (38, 39). Troublingly, patients infected with these strains frequently become bacteremic, a condition that is correlated with a significantly worse prognosis (39). Of the people infected by HV *Kp*, approximately half of those who acquire infections in the community have underlying diabetes, while the other half are young, healthy, and have no underlying predisposing factors. Finally, although antibiotic resistance is primarily a phenomenon associated with classical *Kp*, there have been reports of HV *Kp* strains that carry ESBLs or carbapenemases (40, 41). One recent study in China found ESBLs in 56% of classical *Kp* strains compared to 17% of HV *Kp* strains (42).

Table 1- 1. Characteristics of classical and hypervirulent *Kp* strains

	Classical Strains	**Hypervirulent Strains**	**Refs**
Common causes of Infection	Pneumonia, UTI, bacteremia	Pyogenic liver abscess, bacteremia, lung, neck and kidney abscess, pneumonia, cellulitis, necrotizing fasciitis, myositis, meningitis, endophthalmitis	(32, 37)
Susceptible populations	Immunosuppressed (diabetics, patients with malignancies)	Diabetics, healthy people	(27, 29, 30, 38, 39)
Capsule Types	Capsule Serotypes K1-K78	Hypercapsule Serotypes K1 (93%) or K2	(33, 43-46)
Siderophores (% of strains expressing)	Enterobactin (100%) Yersiniabactin (17-46%) Salmochelin (2-4%) Aerobactin (6%)	Enterobactin (100%) Yersiniabactin (90%) Salmochelin (>90%) Aerobactin (93-100%)	(45, 47-51),
Geographical concentration	Worldwide	Primarily Taiwan and Southeast Asia	(33-36)
Primarily acquired infections	Nosocomial	Community-acquired	(36, 52, 53)
Reports of Antibiotic Resistance	Frequent: ESBL and carbapenemase producing	Infrequent	(40, 54, 55)

Abbreviations: HV=Hypervirulent, UTI=Urinary tract infection, ESBL=Extended-spectrum beta-lactamase

1.3 INFECTIONS CAUSED BY *KP*

Kp is the causative agent of a range of infections, including, but not limited to, pneumonia, sepsis, urinary tract infection (UTI), bacteremia, meningitis, and pyogenic liver abscesses. Here, a brief overview of some of the most frequent and/or deadly types of infection will be provided as a context in which to place the importance of various virulence factors that will be discussed. When considering these infections, it is important to recall that HV strains are far more likely to cause community-acquired and systemic infections in otherwise healthy individuals, but for the most part these strains are geographically limited to Taiwan and Southeast Asia. By contrast, classical strains of *Kp* typically cause serious nosocomial infections or UTIs and can be found worldwide (36, 52, 53, 56). While there is a broad overlap in the types of infections caused by classical versus HV strains, some diseases are far more likely to be caused by HV than classical strains.

Primary infections caused by classical *Kp* strains are usually pneumonias or UTIs. Classical *Kp* also cause very serious infections, such as bacteremia, and these can be either primary bacteremias or secondary bacteremias that arise from secondary spread from a primary infection in the lungs or bladder (57-59). *Kp* pneumonias can be split into two broad categories: community-acquired pneumonias (CAPs) and hospital-acquired pneumonias (HAPs). An overview of these two categories of *Kp* infection is presented in **Table 1.2**. *Kp* HAPs are far more prevalent than *Kp* CAPs and will be discussed first. The precise definitions of a HAP varies based on the publication, but it is generally defined as a pneumonia that presents at least 48 hours post-admission to the hospital in individuals with no symptoms of pneumonia prior to admission. Overall HAPs caused by

bacteria are one of the most frequent types of nosocomial infection and are the leading cause of mortality among nosocomial infections (56, 60-63). *Kp* is the underlying cause of approximately 11.8% of HAPs (56). *Kp* HAP presents similarly to other nosocomial pneumonias with respiratory symptoms that may include cough and unilateral pulmonary infiltrates, and systemic symptoms that include fever and leukocytosis (29). These HAPs occur in both ventilated and non-ventilated patients, and *Kp* is the causative agent in 8-12% and 7% of these cases, respectively (64-66). Worryingly, but not unexpectedly, there is a significantly higher risk of *Kp* being multi-drug resistant in nosocomial infections than in community-acquired infections because many patients have been treated with antibiotics and are carrying antibiotic-resistant flora (57).

While CAPS are fairly common, they are potentially serious infections that can progress rapidly and lead to hospitalization, intensive care unit (ICU) stays and high rates of morbidity and mortality (64). *Kp* is rarely the underlying cause of CAPs in North America, Europe, and Australia, as it is estimated as the causative agent in only ~3-5% of CAPs in these regions. It is, however, a more common etiologic agent of CAPs in Asia and Africa, where *Kp* is second only to *Streptococcus pneumoniae* as the underlying agent, causing about 15% of infections (27, 37, 66-68). While both classical and HV strains can cause CAPS, the comparatively increased prevalence of *Kp* as the etiologic agent of CAPs in Asia and Africa is likely due, at least in part, to the increased prevalence of hypervirulent strains in these areas (37). Significantly, regardless of geography, both types of *Kp* strains are over-represented as the etiologic agents of pneumonias in patients with severe CAPs. Reports estimate that *Kp* CAPs comprise 22-32% of cases requiring admission to the ICU with mortality rates in these ICU patients

ranging from 45-72% (67, 69, 70). In Asia, one report cites *Kp* CAPs as the most frequent causative agent of CAPs that required mechanical ventilation and another report estimated a mortality rate of 55% in hospitalized patients (71, 72). *Kp* CAPs usually present with symptoms typical of acute pneumonias, including cough, fever, leukocytosis and chest pain. These infections can also display the trademark *Kp* characteristic of "currant jelly sputum," which is the production of thick blood-tinged mucous resulting from high levels of inflammation and necrosis in the lungs (73, 74).

Depending on the patient population, classical *Kp* strains are the second or third most frequent cause of UTIs behind *Escherichia coli*, which causes the vast majority of UTIs (75-77). Typically, *Kp* accounts for 2-6% of nosocomial UTIs, and 4.3-7% of community acquired UTIs (78, 79). As with most UTIs, UTIs caused by *Kp* are thought to arise from seeding of *Kp* from the GI tract (80). The symptoms of these infections are similar to those caused by other bacterial pathogens, which include dysuria, increased frequency and urgency of voiding, and hematuria. While UTIs can typically be treated with antibiotics, concern is growing that there are more people carrying ESBL and CRE *Kp* strains in their GI tract. Seeding of these strains into the bladder results in UTIs that are resistant to treatment with many antibiotics, resulting in increased morbidity and prolonged treatment and hospital stays (81, 82).

An extremely serious consequence of *Kp* pneumonias and UTIs is their subsequent spread into the blood to cause bacteremia (39, 83, 84). Among gram-negative pathogens, *Kp* is second only to *E.coli* as the causative agent of both community- and nosocomial-associated bacteremias (43). One report concerning nosocomial *Kp* bacteremias found that 50% of them originated from primary infections in the lungs (83).

Alarmingly, *Kp* bacteremias have a high fatality rate. Mortality rates following *Kp* bacteremia ranged from 27.4-37% (28, 85-88). The higher risk of mortality due to *Kp* bacteremia is associated with a number of patient factors, including patients being admitted to the ICU, being more than 65 years old, having an underlying malignancy, presenting with pneumonia, requiring mechanical ventilation or urinary catheters, or being alcoholics (28, 85-89). Of grave concern to the medical community is the increase in antibiotic resistance that makes treating lung and bladder *Kp* infections more difficult and extends the length of time patients carry *Kp* at these sites, allowing more opportunities for *Kp* spread to the bloodstream and brain. Prolonged and difficult to treat infections could also increase the frequency of the already dire outcome of *Kp* bacteremia. Interestingly, there have been reports of using fecal microbiota transplantation to replace Kp in the GI tract (90, 91).

With the emergence of HV *Kp* strains in Taiwan, a different spectrum of *Kp* diseases has been observed clinically (32, 33). Many of these diseases are quite severe and can occur in otherwise healthy individuals, as well as in individuals with underlying diseases. Most notably, HV strains cause primary liver abscesses in patient populations that do not appear to have any underlying liver disease, unlike other pyogenic liver abscesses caused by polymicrobial sources (92, 93). These liver infections are likely initiated from a breach in host defenses in the GI tract that permit intestinal microbiota to seed tissue sites. In turn, liver abscesses can give rise to a number of other secondary infections as a result of hematogenous spread from the liver. For example, community-acquired *Kp* meningitis is rare in most parts of the world, but is observed as a secondary infection following community-acquired liver abscess in Taiwan (94-96), as well as in

nosocomial infections in Taiwan (97, 98). HV *Kp* infections can also lead to severe skin and soft tissue infections (e.g. cellulitis, necrotizing fasciitis and myositis), endophthalmitis, and abscesses in a number of other tissues (e.g. neck, lungs and kidneys) (94-96, 99, 100).

Table 1- 2. Characteristics of nosocomial and community-acquired *Kp* infections

	Nosocomial	**Community Acquired**	**Refs**
Types of Infection	Pneumonia, UTI, Bacteremia	Pyogenic liver abscess, UTI, meningitis	(27, 32, 33, 56, 95, 96, 101)
Frequency as Etiologic Agent of Pneumonia	11.8%	3-5% in North America, Europe and Australia, 15% in Asia and Africa	(37, 43, 56, 66, 68)
Frequency as Etiologic Agent of UTIs	2-6%	4.3-7%	(75-79)
Underlying conditions	Diabetes, Malignancies	Malignancies, Diabetes, Chronic obstructive pulmonary disease, Chronic alcoholism	(102)
Antibiotic Resistance	23% ESBL, 11% carbapenemase producing		(13)
Primarily classical or HV strains?	Classical	Classical and HV	(27, 36, 37, 52, 53, 66-68, 75)

Abbreviations: UTI=Urinary tract infection, ESBL=Extended-spectrum beta-lactamase,

HV=Hypervirulent

1.4 PATIENT RISK FACTORS

People with underlying forms of immunodeficiency are at much greater risk for infections with classical *Kp* strains than the general population. Risk factors for acquisition of *Kp* nosocomial bacteremia include malignancy, diabetes, chronic liver disease, solid organ transplantation, and dialysis (27, 28). Other risk factors for nosocomial infection are treatment with corticosteroids, chemotherapy (which can overlap with malignancy patients), transplantation, or other treatments or conditions, many of which result in neutropenia or neutrophil dysfunction (29). For both HAPs and CAPs, the primary route of bacterial lung infection is by aspiration of microbes colonizing the oropharyngeal tract or, less commonly, the GI tract (60, 103). Certain conditions increase the risk of aspiration of microbes, including alcohol intoxication, radiation therapy, and endotracheal intubation (104). Likewise, the chance one has of developing a CAP caused by *Kp* greatly rises if one has some form of defect in the defenses of the respiratory tract, such as a deficiency in mucociliary clearance or immunosuppression (105). CAP caused by *Kp* has historically been an infection associated with chronic alcoholism (102, 106, 107), which may be due, at least in part, to the suppression of the cough reflex, neutrophil functions and TNFα by alcohol consumption (108, 109). Other risk factors include chronic obstructive pulmonary disease and diabetes (102, 106, 107). Given the alarming rise of diabetics overall in the general population, one can anticipate that the numbers of CAPs caused by *Kp* strains will increase accordingly (110). Furthermore, because about half of community-acquired *Kp* bacteremias occur in diabetics, and 14% in patients with underlying malignancies, *Kp* bacteremias are also likely to increase with the concurrent increase in diabetes (27). In

addition, it is also important to realize that the appearance of the HV strains has changed the epidemiology of CAPs caused by *Kp* in Asia and could further change the epidemiology of *Kp* CAPs in the rest of the world as these strains become increasingly pandemic (37).

A common feature of diabetic patients, those with malignancies, and alcoholics is suppression of the innate immune system. Studies in rodent *Kp* lung infection models have identified prominent immune defects in neutrophil recruitment, phagocytosis, phagocyte production of cytokines, and antigen presentation that are caused by alcohol intoxication. These deficiencies lead to a defect in *Kp* clearance and increased morbidity and mortality (111-115). In patients with malignancies, populations of innate immune cells are often reduced, due to the side effects of cytotoxic therapies that kill rapidly dividing cells, including immune cells and malignant cells, resulting in conditions such as neutropenia (116). Finally, diabetics have impaired bacterial defenses, including altered chemokine and cytokine production, neutrophil responses, and phagocytic capabilities (117, 118). These defects arise in conjunction with many other alterations in the immune system that are all likely attributable to altered glucose metabolism and oxidative stress.

Other populations at risk for *Kp* infection are neonates and the elderly. Neonates, particularly those who are premature or in the ICU, are at risk due to immature immune defenses, a lack of established microbiota, and the relatively high permeability of the mucosa in the GI tract in these populations (43, 119). *Kp* is often the causative agent of sepsis in neonates, and is the leading cause of neonatal sepsis in certain developing countries (120). The elderly, the fastest growing segment of the population in developed countries, are more susceptible to many infections due to changes in their immune

responses over time that make it less efficacious at controlling pathogens (121, 122). The elderly are the patient cohort associated with the highest risk of mortality caused by *Kp*, with some estimates suggesting a mortality rate of 30% in these patients following hospitalization due to *Kp* CAPs (121, 123). Both HAPs and CAPs in the elderly are predominantly initiated through aspiration of oropharyngeal flora. Based on several studies where the mean patient age was at least 60 years old, *Kp* was the causative agent of 17.2% of all CAPs and 6.5-11.6% of all HAPs observed (123).

Finally, patients who undergo procedures with re-used scopes or other medical equipment or with inserted medical devices or implants have an open highway for *Kp* entry. In part due to their fimbriae (see below), some *Kp* strains are very sticky and attach to medical devices (124-127). One frequent route of acquisition of *Kp* pneumonia is through endotracheal intubation, which increases the risk of acquiring *Kp* ventilator-associated pneumonia (VAP), a form of nosocomial pneumonia that develops at least 48 hours post-intubation (11). This procedure most likely increases the risk of pneumonia by impairing respiratory tract clearance mechanisms, providing a substrate for the formation of bacteria containing biofilms and/or allowing accumulation of bacteria-laden oropharyngeal secretions around the endotracheal tube cuff (128). Insertion of a urinary catheter is another means of initiating *Kp* infection, where the implant allows a point of entry into the urinary tract, as well as a substrate on which *Kp* can form a biofilm (81, 129).

Carriage of *Kp* in the normal flora is directly correlated with *Kp* infection, indicating that the proximal source of most *Kp* infections originates from an individual's microbiota. For example, *Kp* colonization of the GI tract predisposes patients to infection

from both classical strains in hospital settings and abscesses caused by HV *Kp* strains (130, 131). Similarly, oropharyngeal *Kp* colonization is associated with an increased risk of *Kp* VAP (132). The frequency of *Kp* colonization at these sites depends on whether a person has been in the hospital or has undergone long-term antibiotic treatment. A lengthier hospital stay is associated with increased *Kp* carriage as *Kp* is often spread by the hands of healthcare workers or contaminated medical devices, while antibiotic treatment removes antibiotic-sensitive microbes from the gut microbiota, thus allowing an initially minor *Kp* population to significantly expand (131, 133-135). Strikingly, one study found only 10% of those patients who had a *Kp* infection during hospitalization were colonized with *Kp* prior to admission (135). Furthermore, 45% of patients who became colonized intestinally with *Kp* during hospitalization developed a fulminant *Kp* infection with a strain of the same serotype (135). In this study, approximately double the number of patients who carried *Kp* in their intestine acquired *Kp* infection within 3 weeks of admission to the hospital, compared to non-carriers. Strikingly, a 4-fold difference in the number of carriers to non-carriers acquiring HAP due to *Kp* was observed following a hospital stay of more than 3 weeks (135). As previously noted, antibiotic treatment amplifies the risk of *Kp* infection, and recent antibiotic use is an additional independent risk factor for colonization by and infections with ESBL and carbapenemase-producing *Kp* (136-139).

It is worth noting that since the vast majority of people with *Kp* infections have underlying medical conditions that have complex and multifactorial impacts on innate immune systems, healthy individuals likely have multiple or redundant mechanisms with which to withstand incidental infection with *Kp*. Nonetheless, the world-wide growing

diabetic epidemic and the aging of the population in many countries increase the number of people susceptible to *Kp* infections. Meanwhile, the spread of HV *Kp* strains, as well as the likely spread of antibiotic resistance among both classical and HV strains, may contribute to a rise in morbidity and mortality of *Kp* infections in both immunocompromised and healthy populations.

1.5 *KP* AND HOST IMMUNE DEFENSES

In order for *Kp* to cause infection, it has to overcome mechanical barriers, as well as humoral and cellular innate immune defenses (140). A number of *in vitro* studies and *in vivo* studies in rodent models have helped to delineate the role of these immune defenses in controlling *Kp* infection, as well as how *Kp* evades or counters these defenses. However, it is also critical to note that the contribution to bacterial clearance by different humoral defenses and cell types within the immune response varies based on *Kp* strain (141-144). Thus, different strains of *Kp* may express different factors that permit them to negate specific host factors. In this chapter, several well-studied virulence factors will be discussed that enable *Kp* to navigate, negate, and target host immune defenses. Some of the critical host defenses that restrict *Kp* during infection, including the role of neutrophils, will be detailed first.

Among the first defenses encountered by a pathogen in the respiratory tract is the mechanical mucociliary elevator, which consists of a blanket of mucus in the respiratory tract that traps particles and microbes and then shuttles them up and out using the ciliary lining. Likewise, in the genitourinary tract, the flow of urine is a strong mechanical force, which, in conjunction with the chemical defense of the low pH of the urine, removes *Kp* and other bacteria and prevents entry into the bladder (145, 146). To colonize the GI

tract, bacteria face a number of obstacles, both physical and chemical (146). Peristalsis pushes out non-adherent microbes, while the turnover of epithelial cells removes adherent microbes. Mucus, similarly to the respiratory tract, also prevents microbe binding to the epithelium and helps shuttle out non-adherent bacteria. Meanwhile, bile and digestive enzymes create a chemically harsh environment.

Once *Kp* passes these initial mechanical barriers, it must then overcome humoral and cellular innate defenses. Humoral defenses consist of a wide range of antimicrobial factors with many functions, including those that are opsonic, bactericidal, and bacteriostatic (140, 147). An example of a humoral defense is the complement system, which can mediate bacterial killing by several mechanisms. Activation of the complement cascade can lead to formation of membrane attack complexes, which insert as pores into bacterial surfaces, causing lysis (148). Additionally, activation of the complement cascade results in the release of proinflammatory mediators and chemoattractants for immune effector cells. Third, complement can bind to pathogens and serve as an opsonin for phagocytosis. There are also three different mechanisms by which the complement cascade can be activated and the cascade is divided into 3 pathways: classical, alternative and lectin pathways, depending on how it becomes activated (149). The classical pathway is activated by binding of complement to antibody/antigen complexes. The alternative pathway is activated following the spontaneous conversion of C3 into C3b that then binds to bacterial surfaces that lack the inhibitory molecule sialic acid. And finally, the lectin pathway is activated following the binding of mannose-binding lectin to mannose resides on bacterial surfaces (149).

Additional humoral defenses used to restrict bacterial infections include defensins, which are bactericidal factors in the lungs and GI tract that disrupt the bacterial membrane, and transferrin, which is a bacteriostatic factor that sequesters iron, a necessary growth factor for bacteria. Surfactants and immunoglobulins can also serve as opsonins for phagocytosis. In the lungs, surfactant protein (SP) A and a portion of the SP-B proprotein can act synergistically to enhance *Kp* killing and to promote neutrophil recruitment (150). As will be discussed in detail below, *Kp* has mechanisms in place that protect against many, but not all of these humoral defenses. For example, most *Kp* strains appear to be resistant to complement-mediated lysis, as well as opsonophagocytosis as evidenced by *in vitro* experiments and mouse infection models (151-157). In mouse infection models, no significant differences have been found in *Kp* bacterial load in the lungs between complement-depleted and control mice during lung infection (151). By contrast, there is much more strain-to-strain variability in the susceptibility of *Kp* to antimicrobial peptides and proteins, such as defensins and surfactants, depending upon the specific antimicrobial (152, 158-161). For example, human beta defensin (HBD)-1 and HBD-2 are not as efficient at killing *Kp* as HBD-3 (158). Interestingly, several studies have reported that ESBL *Kp* strains seem to be more susceptible than non-ESBL strains of *Kp* to HBDs (158, 160).

Innate cellular mediators act both synergistically with and independently of the humoral defenses to control *Kp* infection. In the lungs, the first innate cellular effectors encountered are resident alveolar macrophages, which have phagocytic capabilities, and mediate the amplification and/or resolution of immune responses through the production of chemokines and cytokines. When *Kp* establishes infection, neutrophils are recruited to

the infected tissues by chemokines and cytokines produced by macrophages during infection. These chemokines include interleukin-8 (IL-8), CXCL1, complement and leukotriene B4. Importantly, mouse models of lung infection have shown that alveolar macrophages help control *Kp* infection by recruiting neutrophils, as well as other mechanisms (141, 162).

In general, neutrophils are well-known first-line responders to bacterial infections, have a greater phagocytic capacity and killing capacity than alveolar macrophages, and are important for containment and clearance of *Kp* infection (163). Along these lines, there has been a notably large influx in neutrophils into the lungs during early *Kp* infection (164). Interestingly, it has been shown that optimal clearance of *Kp* from the lungs during the first 24 hours of infection occurs in the presence of neutrophils and/or CCR2+ monocytes, but the contribution of each cell type to the containment of *Kp* varies based upon *Kp* strain (141). For the commonly used lab strain, *Kp* ATCC 43816, neutrophils and CCR2+ monocytes are both necessary for efficient clearance of bacteria from the lungs (141). Once neutrophils are in the infected tissues, they employ the numerous effector functions in their arsenal to combat *Kp* infection. Studies using both human neutrophils and mouse models of *Kp* infection have noted neutrophilic use of a number of processes to contain *Kp* (141, 164-180). These include phagocytosis/opsonophagocytosis, production of inflammatory cytokines, and release of antimicrobial compounds and structures, such as reactive oxygen species, serine proteases (e.g. neutrophil elastase), lactoferrin, lipocalin-2, myeloperoxidase and neutrophil extracellular traps (NETs) to contain the bacteria and mediate clearance (141, 164-180). For example, neutrophils, as well as macrophages, can inhibit bacteria using

oxidative and nitrosative stress through release of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (181). ROS and RNS antimicrobial activities, such as inhibition of bacterial replication, DNA mutagenesis, and inhibition of metabolism, are mediated through a diverse range of targets, as both ROS and RNS can react with and modify metal centres, thiols and DNA (181) (182). The bacterial damage incurred by ROS comes mainly from direct DNA damage, while RNS is primarily via interference with bacterial respiration and replication of DNA by inhibiting zinc metalloproteins (182).These ROS include superoxide, H_2O_2 and HOCl, where superoxide anion is converted into H_2O_2 by NADPH oxidase (which is made up of several subunits, including gp91phox), and HOCl is subsequently generated by myeloperoxidase (MPO) from H_2O_2. RNS include NO, which is generated in phagocytes by inducible nitric oxide synthase (iNOS). Typically, neutrophils are considered larger generators of ROS than macrophages and monocytes, while the reverse is true of RNS production (183). Work has shown that *Kp* is inhibited by alveolar macrophage production of RNS *ex vivo,* and NO and HOCl *in vivo* (184) (185). Specifically, NO inhibition in mice intratracheally infected with *Kp* resulted in higher bacterial loads in the lungs and blood (184), while abrogation of HOCl production, but not H_2O_2, by immune cells using MPO-/- mice resulted delayed clearance of *Kp* from the lungs after intranasal infection (185). It has not yet been shown what effect abrogating H_2O_2 production by immune cells *in vitro* or *in vivo* has on *Kp* survival and infection. To date, few studies have been published characterizing *Kp* genes that contribute to bacterial resistance to oxidative and nitrosative stress. For H_2O_2, only genes encoding a transcriptional regulator with ties to oxidative stress resistance in a number of pathogens, *oxyR* (186, 187), a putative porin, *kpnO* (i.e.

ompC), a putative heat shock-induced serine protease, *htrA,* and a c-di-GMP phosphodiesterase, *yjcC,* have been demonstrated to increase *Kp* resistance (188-190). Of these, *kpnO* and *oxyR* are also the only two *Kp* genes shown to increase resistance to NO (188, 191). When tested in *in vivo* infection models, *Kp kpnO* and *oxyR* mutants were less virulent in a *C. elegans* infection model than WT (188, 192), and *Kp oxyR,* and *htrA* and *yjcC* mutants were less virulent in mouse GI tract colonization and IP infection models, respectively (189, 190, 192). For protection against HOCl, only a chlorite dismutase, *cld,* has been implicated in protecting *Kp,* likely through degradation of HOCl (193). However, the importance of this gene in *Kp* virulence in *in vivo* infection models has not been tested.

Dendritic cells (DCs) are also involved in containing and controlling *Kp* lung infections. In this site, their recruitment and maturation occurs during *Kp* infection in a TLR9 dependent manner, which may signal to the cell to optimize intracellular killing upon accumulation of bacteria within these cells (194). In fact, the role of TLR9 during *Kp* lung infection may be primarily in the context of DCs.

Studies on downstream TLR adaptors have highlighted the need for functional TLRs during *Kp* infection, where MyD88, TRIF and TIRAP are all necessary for pro-inflammatory cytokine production and controlling the bacterial population in the lungs. As with many bacterial infections, MyD88 plays a more significant role than TRIF (179, 195, 196). Furthermore, the protective role of MyD88 during infection seems to be through expression in both bone-marrow derived cells and non-bone marrow derived cells, while that of TRIF is only through expression in bone-marrow derived cells (197). Mice with defective TLR4 signaling have higher *Kp* CFU and greater mortality during

Kp pneumonia, indicating that TLR4 prevents mouse mortality and high bacterial loads (198-200). Signaling through this receptor is necessary for IL-23 and IL-17 production in the lungs during *Kp* infection (201).

IL-17 is an important cytokine for mediating effective immune responses during *Kp* infection in mice (166). This cytokine is produced during *Kp* pneumonia and assists in controlling infection, including through neutrophil recruitment to and activation at the site of infection (166, 202). Production of IL-17 in response to *Kp* infection requires IL-23 stimulation, which likely originates from alveolar macrophages and DCs (203). IL-12 also improves the response to infection by amplifying IL-17 expression through IFNγ production (203). Additionally, IL-23 and IL-17 both have roles very early in infection independent of production of IL-12 and IFNγ (203). Cell types expressing IL-17A during lung infection include γδ T cells, CD4 T cells, CD8 T cells and NK T cells, where IL-17A is predominantly produced by γδ T cells followed by Th17 cells (204, 205). However, work has shown that γδ T cells, but, interestingly, not αβ T cells, play protective roles during *Kp* pneumonia, suggesting that IL-17 from αβ T cells is not critical to control infection (206, 207). During *Kp* lung infection, γδ T cells are necessary for early upregulation of certain inflammatory cytokines, such as TNFα and IFNγ (206). IFNγ has varying importance in *Kp* infection depending on route of infection (208). During infection with the same strain of *Kp*, IFNγ is important for control of *Kp* primary infection of and dissemination from the lungs, however it is not required for protection from systemic infection following intravenous injection (208, 209).

IL-22, which is similar to (and often acts in concert with) IL-17 in that it is a Th17 cytokine and produced during *Kp* infection in an IL-23 dependent manner, is also critical

for control of *Kp* infection (202, 210, 211). IL-22 was shown to be important in both pneumonic and intraperitoneal models of infection. Specifically, depletion of IL-22 with a neutralizing antibody led to increased bacterial loads in the lungs and rates of mortality (210), while overexpression or the therapeutic administration of IL-22 ameliorated *Kp* peritonitis based on mouse mortality and *Kp* bacterial loads (211). One function of IL-22, as well as IL-17, is the induction of expression of lipocalin-2, a multifunctional molecule that interferes with bacterial iron acquisition by binding to the bacterial siderophore enterobactin (212), into the blood and at mucosal surfaces (202, 211). Both Th17 and Th22 cells were initially thought to be the origin of IL-22 during *Kp* infection (207). However, since recent work has shown αβ T cells are unnecessary in the control of infection (207), while NK cells both promote survival of mice during *Kp* pneumonia and produce IL-22 (213), it is now thought that NK cells are the major source of IL-22 and are critical for controlling Kp infections in lungs. Another innate lymphoid cell type, mucosal-associated invariant T (MAIT) cells, has also been suggested to play a non-redundant role in controlling infection, particularly in the *Kp* peritonitis model (207, 214, 215). In these experiments, mice lacking these MAIT cells had increased mortality, systemic spread and abnormal cytokine expression following IP infection compared to WT mice.

Another facet of the immune response involved in *Kp* control is the inflammasome. Specifically, deletion of NLRP3, NLRC4 or ASC in mice results in increased bacterial loads and mouse mortality in mouse models of *Kp* pneumonia (205, 216). This increased in mortality may be related, at least in part, to the resulting reduction in proinflammatory cytokine production, including IL-1β leading to fewer classically

activated macrophages, as well as decreased cell death through pyronecrosis (205, 216, 217).

1.6 *Kp* VIRULENCE FACTORS

Kp employs many strategies to grow and protect itself from the host immune response. Importantly, many *Kp* mutants are more rapidly cleared from the lungs of mice compared to wildtype *Kp* strains, suggesting that wildtype *Kp* employs various factors to circumvent early host responses (218). To date, there are four major classes of virulence factors that have been well-characterized in *Kp,* and these will be discussed in detail in this section (see Figure 1.1). These consist of capsule, including the production of hypercapsule in HV strains, lipopolysaccharide (LPS), siderophores, and fimbriae, also known as pili.

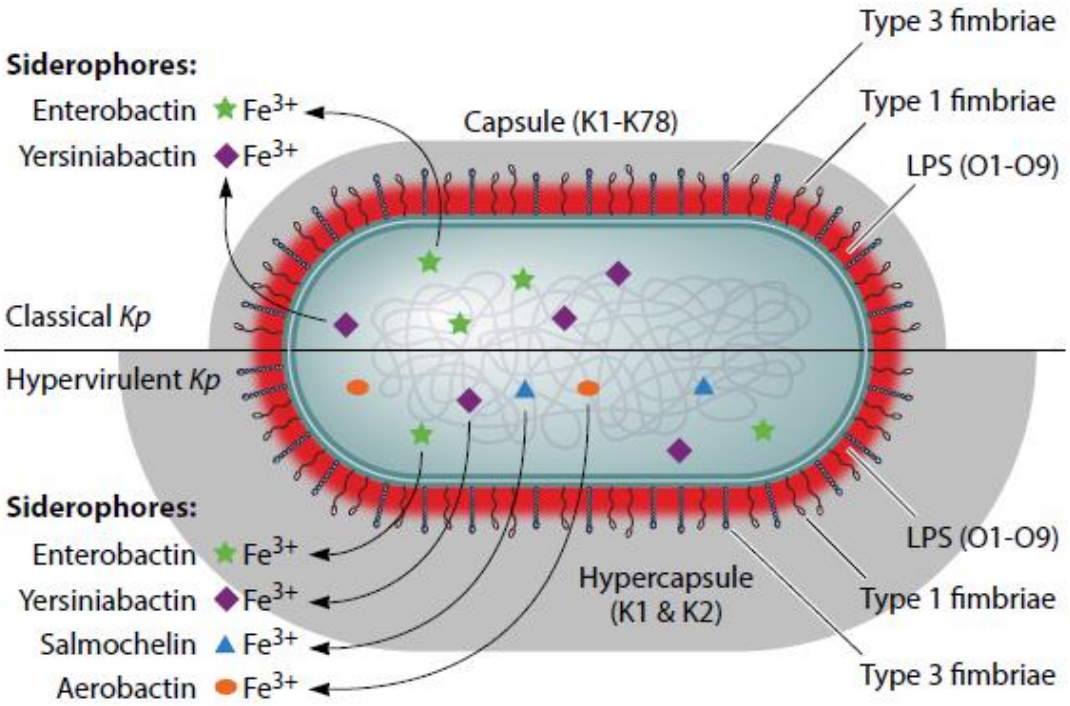
Several other factors have been recently identified as being important for *Kp* virulence. However, these factors are not yet thoroughly characterized and much work remains to fully understand their mechanisms of action and clinical significance. These virulence factors include outer membrane proteins (OMPs), porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism.

The virulence factors characterized for *Kp* play varying roles in different types of *Kp* infection and in different strains of *Kp*. In addition, several compelling recent studies are revealing a number of other factors that play critical roles in mammalian infection. Based on these known virulence factors, *Kp*'s *modus operandi* appears to be defensive rather than offensive in protecting itself against the host immune response. For example, pathogenic *Yersinia* species use type 3 secretion systems to inject toxins into attacking immune cells in order to inactivate the phagocytic capability of these cells (219). By

contrast, *Kp* appears to evade, rather than actively suppress, phagocytosis by using capsule to make it harder for the bacteria to be bound and taken up by phagocytes (220).

Figure 1- 1. Four well-characterized virulence factors in classical and hypervirulent *Kp* strains

There are four well-characterized virulence factors for pathogenic *Kp*: capsule, LPS, fimbriae (type 1 and type 3) and siderophores. Capsule is an extracellular polysaccharide matrix that envelops the bacteria. Classical *Kp* strains produce a capsule that can be any of the serotypes of K1-K78; K1 and K2 are associated with increased pathogenicity. HV strains make a hypercapsule, which is amplified production of capsular material resulting in a relatively larger capsule, and are predominantly of the K1 serotype while most of the remaining strains are K2. LPS, an integral part of the outer leaflet of the outer membrane, is produced by both classical and HV *Kp,* and can be O-antigen serotypes 1-9 (O1-9). Both types of *Kp* make the membrane-bound adhesive structures type 1 and type 3 fimbriae and secrete iron-scavenging siderophores. Of the siderophores, enterobactin is made by almost all strains, and yersiniabactin is made by approximately half of classical and almost all HV strains. Salmochelin and aerobactin are rarely produced by classical strains, but are typically secreted by HV strains with aerobactin being the most highly expressed of the siderophores.


Type 3 fimbriae
Siderophores:
Type 1 fimbriae
Enterobactin Fe3+
Capsule (K1-K78)
Yersiniabactin Fe3+
LPS (O1-O9)
Classical Kp
Hypervirulent Kp
Siderophores:
LPS (O1-O9)
Enterobactin Fe3+
Hypercapsule
(K1 & K2)
Yersiniabactin Fe3+
Type 1 fimbriae
Salmochelin Fe3+
Type 3 fimbriae
Aerobactin Fe3+

1.6.1 Capsule

Capsule, a polysaccharide matrix that coats the cell, is necessary for *Kp* virulence and is arguably the most thoroughly studied of *Kp*'s virulence factors (43, 221) (Figure 1.2). Acapsular *Kp* strains are dramatically less virulent than isogenic encapsulated strains in mouse models, based on decreased bacterial loads in the lungs, lower rates of mouse mortality, and an inability of the bacteria to spread systemically (177, 221-223). Furthermore, HV *Kp* strains produce a hypercapsule, also known as being hypermucoviscous, which consists of a mucoviscous exopolysaccharide bacterial coating that is more robust than typical capsule. This hypercapsule may contribute significantly to the pathogenicity of HV *Kp* (36, 224, 225).

Both classical capsule and HV hypercapsule are made up of strain-specific capsular polysaccharides, termed K antigens (i.e. K1, K2 up through K78) (226). The genes needed for both classical and HV *Kp* production of capsule are located on a chromosomal operon, *cps*, where both the organization and the sequence of the genes are conserved compared to *E. coli* (227, 228). The *cps* gene cluster encodes a number of genes involved in capsule production, including *manB, manC, wzi, wza, wzb, wzc, gnd, wca, cpsB, cpsG* and *galF* (229, 230). K antigens have been traditionally assigned using serological methods (226, 231, 232). Recently, however, K antigen typing is often performed by sequencing of the *wzi* locus (233-237). This locus is present in all capsular types of *Kp*, and different *wzi* loci sequences are strongly associated with specific K antigens. The gene *wzi* encodes for a surface protein involved with capsule attachment to the outer membrane, and loss of this protein results in essentially acapsular bacteria (238, 239). *Wzy*, also known as *orf4*, is involved in polymerization of capsular polysaccharides,

while *wza* and *wzc*, *orf5* and *orf6*, respectively, are involved in surface assembly (227, 240). Other genes, particularly those in the *cps* gene cluster, are involved in production of the capsule polymer, such as *cpsB* and *cpsG*, which encode a mannose-1 phosphate guanyltransferase and phosphomannomutase, respectively (227, 240), while *manB* and *manC* are involved in the synthesis of an intermediate, GDP-D-mannose, of the capsular component GDP-L-fucose (228). Like *E. coli, Kp* produces a variety of capsule types. The diversity in capsule types arises from the glycosyltransferase activities of *wbaP, wbaZ, wcaN, wcaJ* and *wcaO* (228, 240). Furthermore, comparative analysis of clinical isolates of *Kp* has shown a large amount of diversity in the *cps* gene cluster sequence between strains (228) and even within strain types (14, 18). For example, the *cps* gene cluster of a given strain will contain either, but not both, of the glycosyltransferases, *wbaP* and *wcaJ* (228).

In HV *Kp*, capsule production can be enhanced above basal levels in a number of different ways. Expression of two plasmid-borne transcriptional regulators, regulator of mucoid phenotype A (*rmpA*) and *rmpA2*, as well as expression of the chromosomal copy of *rmpA*, and the regulation of capsule synthesis A and B genes *(rcsA* and *B*), can all increase capsule production. Capsule synthesis can also be amplified in response to external cues. For example, increased glucose concentrations result in upregulation of capsule production though RmpA, while relatively high extracellular iron concentrations result in downregulation of capsule production. In fact, 55-100% of HV *Kp* strains express at least one copy of *rmpA* or *rmpA2*, compared to 7-20% of non-HV *Kp* strains (42, 241). These genes positively regulate the *cps* locus at the transcriptional level, resulting in the hypercapsule phenotype (227, 242-244). However, not all of these genes

must be upregulated or even present concurrently (241, 243, 245, 246). Hypercapsule production can be triggered in the absence of *rmpA/rmpA2* by the chromosomally encoded mucoviscosity-associated gene A (*magA*) (36). *magA* was discovered in 2004 during a search to identify genes necessary for the hypercapsule phenotype in *Kp* strains isolated from invasive liver disease; *magA* was found in 98% of invasive vs 29% of noninvasive strains (224, 247). Subsequent work determined that *magA* is actually specific to K1 strains and is a *wzy*-like polymerase that is necessary for capsule production. Later publications have redesignated *magA* as *wzy*_K1 (248-250), while other serotypes contain different alleles of *wzy*. For example, there is a *wzy* allele specific to K2 strains that has been suggested to be referred to as K_2A (251).

There may be an association between the K antigens produced by the infecting *Kp* strain and the severity of infection, especially in community-acquired infections. Of the 78 serotypes identified thus far, there is an overrepresentation of certain serotypes, as only 25 serotypes make up over 70% of the strains isolated from clinical samples (252). Based on the most frequently isolated serotypes collected from patients and results from mouse experiments, K1 and K2 strains are generally more virulent than other serotypes (44, 45, 71). K2 is the most prevalent type of *Kp* strain isolated clinically, followed by K1. There are several possible reasons for the increased virulence of K1 and K2 strains relative to other strains. One thought is that K1 and K2 serotypes may induce a lower amount of reactive oxygen species release by human neutrophils than other serotypes, and thus survive better in tissues (253). In addition, K1 and K2 serotypes are more resistant to phagocytosis and intracellular killing by alveolar macrophages and neutrophils than other strains, and this phenotype is independent of whether they are

hypercapsule producers (142, 143, 254). For K1 and perhaps K2, this reduced uptake may be in part due to the presence of sialic acid on their surfaces, which may mimic sialic acid typically produced by host cells and allow for evasion of host immune cells (142, 255, 256). Furthermore, functional studies have shown that K1/K2 strains may be more resistant to opsonophagocytic uptake by macrophages via mannose/lectin receptors than other serotypes (257, 258). Unlike other serotypes, K1 and K2 lack the specific mannose residue repeats that are recognized by two host factors: the mannose receptor on macrophages and the lung-secreted surfactant protein A (SP-A) (143, 259). Bacteria can either be bound directly by mannose receptors, or SP-A can attach to the bacteria and then be bound by mannose receptors; both forms of mannose receptor binding by macrophages can elicit anti-microbial responses. Therefore, the lack of mannose in the K1 and K2 serotypes prevents efficient lectinophagocytosis and subsequent proinflammatory signals that recruit neutrophils and monocytes, among other functions.

One study characterizing HV *Kp* strains isolated from 4 different continents found these strains were almost exclusively K1 (93%), while the remaining minority were K2 (46), a finding consistent with previous studies (17, 260-265). Interestingly, in one study 27/28 of the K1 HV *Kp* isolates belong to the same clonal complex (CC23), while the two K2 HV strains examined were genetically unrelated and arose from two distinct clonal lineages (ST25 and ST86) (46). The CC23 K1 HV Kp strains were significantly more closely related to each other than to other K1 strains, indicating that they have likely disseminated globally from a distinct variant (46). However, these CC23 K1 HV *Kp* were more closely related to the K1 reference strain used in this study than they were to other *Kp* strains (46). It has been suggested that the increased virulence associated with

K1 and K2 HV strains may be due to the concurrent carriage of several virulence-associated factors, namely hypercapsule and the siderophore aerobactin (see below), compared to their presence in other strains (45). For example, in a mouse liver abscess model, K1 and K2 strains that lacked a hypercapsule, *rmpA* and aerobactin were avirulent (45). In fact, HV CC23 strains all carried the genes for two siderophores as well as RmpA on a large plasmid as well as a genomic island with several differences from other *Kp* strains (46). On the other hand, some work suggests that the presence of K1/K2 serotypes themselves, rather than hypercapsule, is responsible for increased virulence, as typical capsule-producing K1/K2 strains are not significantly less virulent than hypercapsule-producing K1/K2 strains (254).

During *Kp* infection, capsule protects against the host immune response through multiple mechanisms, including inhibition of phagocytosis by immune cells, preventing activation of the early immune response, and by abrogating lysis by complement and antimicrobial peptides. Capsular *Kp* strains are significantly less likely than acapsular strains to be phagocytosed by innate immune cells, both in the presence and absence of opsonins (220). Capsule-mediated prevention of bacterial binding and internationalization by immune cells helps limit early inflammatory signals, resulting in a less robust induction of the immune response (266). Specifically, studies performed in *Kp*-infected lungs found that capsular strains induced lower levels of the proinflammatory cytokines TNFα and IL-6 and higher levels of the anti-inflammatory cytokine IL-10 than in acapsular infections (223, 267). Also, capsular strains of *Kp* dampen NF-κB through a mechanism dependent on NOD1 activation, which attenuates IL-1β-induced production of IL-8, a multipotent immune response protein important for

neutrophil recruitment and activation (268). Capsule is necessary, although not sufficient on its own to mediate this effect. Consistent with these cytokine responses, more immune cells are recruited to lungs infected with acapsular as opposed to capsular strains (223).

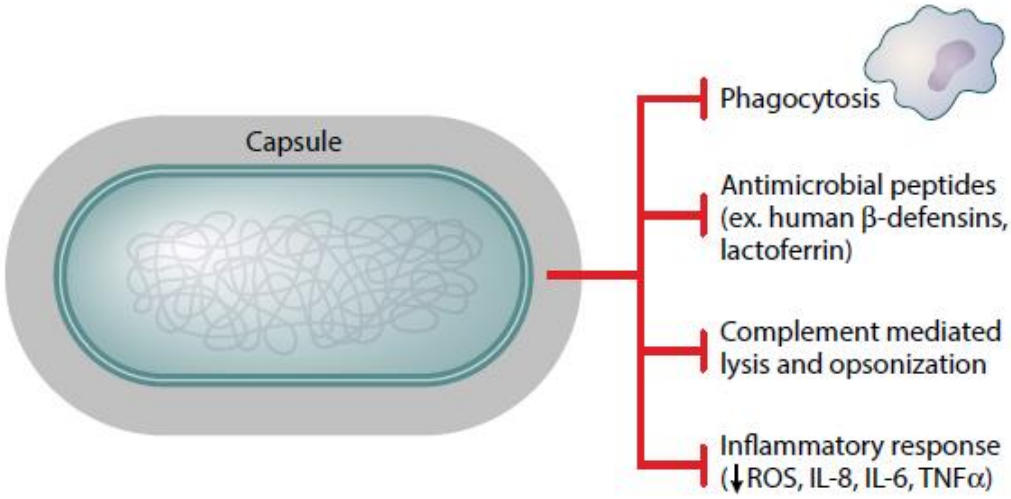
Capsule also contributes to resistance against complement, although work shows *Kp* strains vary in terms of whether capsule or LPS is the main deterrent to complement-mediated lysis (2, 154, 157, 224, 269, 270). In the absence of the O side chain, acapsular strains tend to be more commonly bound by C3, possibly due to the exposure of complement activators on *Kp*'s surface, which result in increased opsonophagocytosis and serum killing via the alternative complement pathway (155, 222). In some cases, capsule binds to antimicrobials produced by the host immune response and prevents the interaction of these molecules with the bacterial surface (152, 271). Capsule both blocks the bactericidal action of human beta-defensins (HBDs) and suppresses their production from airway epithelial cells (158). Interestingly, capsule may be upregulated in the presence of these antimicrobial molecules, as studies have found upregulation of capsule expression in the presence of antimicrobials lactoferrin and polymyxin B (272).

In general, the hypercapsule phenotype enhances resistance to a variety of humoral defenses, including complement killing, the defensins HBD 1-3, and other antimicrobial peptides, including human neutrophil protein-1 and lactoferrin (224). Even though capsule is not usually the most important defense against complement in classical strains, HV *Kp* is less sensitive to complement killing than classical strains (273). Furthermore, the hypercapsule has been correlated with an increased resistance to phagocytosis by human neutrophils and macrophages compared to a number of classical strains (254, 273, 274). Notably, the presence of fucose in the hyper capsule has been

implicated with evasion of the immune response, such as bacterial avoidance of phagocytosis (275, 276). The gene *wcaG* is involved in this fucose synthesis and, not surprisingly, is associated with *Kp* virulence (275, 276). One study found the presence of this gene in approximately 88% of clinical isolates with a range of serotypes, and was found in both HV and classical strains (277). Furthermore, work in an HV *Kp* strain noted that *wcaG* was necessary for hypercapsule production, but not LPS production, and virulence in a mouse IP injection model (275).

Figure 1- 2. Role of capsule in *Kp* virulence

A number of different functions for capsule have been delineated for *Kp* virulence. First, capsule prevents phagocytosis and opsonophagocytosis of the bacteria by immune cells. Second, it hinders the bactericidal action of antimicrobial peptides, such as human beta defensins 1-3 and lactoferrin, by binding these molecules distal from the outer membrane. Third, it blocks complement components, such as C3, from interacting with the membrane, thus preventing complement-mediated lysis and opsonization. Finally, it averts fulminant activation of the immune response, as measured by decreased ROS, IL-8, IL-6 and TNFα production, by assisting in activation of a NOD-dependent pathway and shielding LPS from recognition by immune cell receptors.



1.6.2 Lipopolysaccharide

Lipopolysaccharide (LPS), also known as endotoxin, is a major and necessary component of the outer leaflet of the cell membrane of all gram-negative bacteria (Figure 1.3). Although there is considerable variation in LPS structure among bacterial species, it is typically comprised of an O antigen, a core oligosaccharide, and lipid A. These components are encoded by genes in the *wb*, *waa* and *lpx* gene clusters, respectively (278-281). Unlike the 77 different K antigens that have been documented for *Kp* capsule, there have only been 9 different O antigen types identified in *Kp* isolates, and O1 is the most common (282). Based on work in both *Kp* and other gram-negative bacteria, the length of O-antigen is regulated, at least in part, by the *wzy* and *wzz* genes, such as *wzyE*, *wzzB* and *wzzE* (177, 283, 284).

LPS is both a benefit and a hindrance for *Kp* during infection, as it is an important virulence factor that protects against humoral defenses, but also can be a strong immune activator. The lipid portion of bacterial LPS, lipid A, is well-known for being a potent ligand of Toll-like Receptor 4 (TLR4), a pattern recognition receptor. TLR4 stimulation leads to the production of cytokines and chemokines that help recruit and activate cellular responses, including neutrophils and macrophages that clear *Kp* infection and control spread to other tissues. This has been demonstrated in mouse models of *Kp* infection, where mice lacking TLR4 or myeloid differentiation primary response gene 88 (MyD88), a signaling protein downstream of TLR4, are more susceptible to *Kp* pneumonia and systemic spread (179, 200, 285). In these more susceptible mice, there is impaired cytokine and chemokine production and recruitment of neutrophils. Certain *Kp* strains may use capsule to partially shield their LPS from detection by TLRs (2). Work

suggests that strains with the K1, K10 and K16 antigens can mask their LPS, while other strains, such as those expressing K2 antigens, cannot (2). A similar phenomenon occurs in HV *Kp* strains, where the hypercapsule dampens TLR4 signaling (286). Another method of preventing recognition by the immune response employed by some bacteria, such as *Yersinia pestis*, *Helicobacter pylori* and *Porphyromonas gingivalis*, is modification of the LPS to a form that is no longer recognizable by certain immune receptors (287-289). Recent evidence suggests that *Kp* may do the same (290). *Kp* demonstrates marked plasticity in its lipid A structure and in certain mammalian tissue sites, including the lungs, will switch to a 2-hydroxyacyl modification of its lipid A in a PhoPQ-regulated, LpxO-dependent manner. In a similar manner to other bacteria that have modified LPS, such as *Yersinia*, this modified lipid A does not activate the inflammatory response to the same degree as the native form of lipid A, effectively increasing the *in vivo* virulence of *Kp* (287, 290). Another modification that aids in *Kp* evasion of the immune response, particularly phagocytosis by alveolar macrophages and damage by antimicrobial peptides, is the decoration of lipid A with palmitate via PagP (291). The importance of this modification is supported by the decreased fitness of a Δ*pagP Kp* strain in the lungs (291). Furthermore, the lipid A portion of *Kp* LPS also plays a beneficial role in virulence, as a mutant strain of *Kp* with altered lipid A acylation was attenuated in a mouse model of pneumonia. Additionally, both *in vitro* and *in vivo* experiments have shown modified lipid A protects against some cationic antimicrobial peptides (161, 290).

Kp is recognized by and activates the classical, alternative, and lectin complement pathways, although there is heterogeneity among which pathways become activated

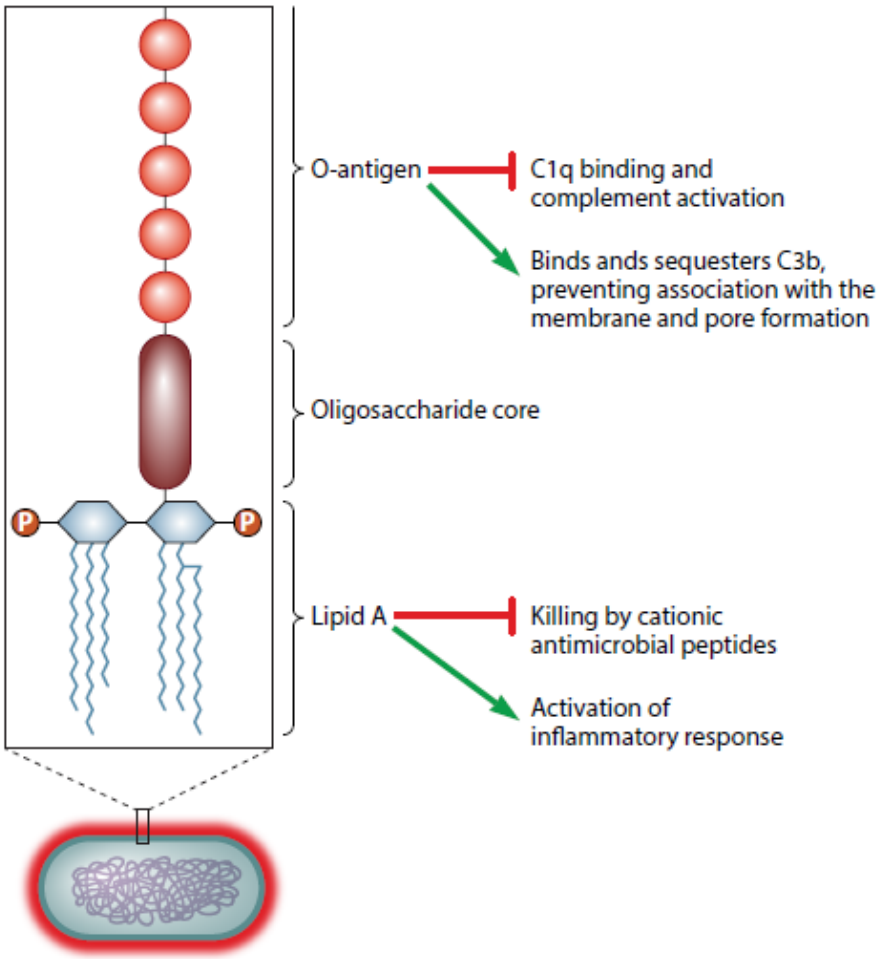
across *Kp* strains (156, 292, 293). LPS is the primary means of protection against complement (2). Strains that contain a full-length O antigen, or "smooth LPS," are resistant to complement-mediated killing, while those with truncated or absent O chains, or "rough LPS," are susceptible to complement-mediated killing, even in the presence of capsule (2). Specifically, complement-resistant strains activate the complement cascade but are not susceptible to killing due to the O antigen portion of LPS. The O antigen protects against C3 by binding C3b, a complement component that is both an opsonin and part of the pore-forming process, far away from the bacterial membrane and abrogating pore formation (2, 148, 153, 294). Furthermore, the absence of the O antigen renders the bacteria more susceptible to binding of C1q to the cell surface, resulting in activation of the classical pathway (154). In mouse pneumonic models, *Kp* lacking O antigen colonized the lungs to equal levels as the wild-type (WT) strain early after infection, but was incapable of spreading systemically (294). The authors of this study speculate that the lack of O antigen may render *Kp* sensitive to complement-mediated killing in the bloodstream, thus making the strain less virulent.

Supporting this role for LPS in virulence is work studying genes involved in proper LPS production including *uge*, which encodes a UDP galacturonate 4-epimerase, and *wabG*, which encodes a GalA transferase (295, 296). The gene *uge* is present in the majority of *Kp* isolates, including both disease-causing and commensal, and in the absence of this gene, *Kp* LPS produces rough LPS and is less capable of causing UTI, pneumonia and sepsis than WT strains (295, 297, 298). Meanwhile, *wabG* is likely present in almost all clinical isolates, as the majority of reports indicate it is encoded for in 88-100% of *Kp* isolates, although one report found *wabG* in only 5.3% of isolates

(296, 298-302). *Kp* lacking this gene is unable to generate the LPS outer core or to retain capsular antigen, and are attenuated in intraperitoneal, pneumonic and UTI rodent models of infection (303).

Figure 1- 3. Role of lipopolysaccharide in *Kp* virulence

Lipopolysaccharide (LPS) is composed of three major subunits: lipid A, an oligosaccharide core and O-antigen. Lipid A inserts into the bacterial membrane and is a potentially potent activator of inflammation. *Kp* may modify its lipid A to make it less inflammatory during infection and lipid A may also protect against the bactericidal action of cationic antimicrobial peptides. O-antigen is the outermost subunit of LPS. It has important roles in protecting against complement, including preventing C1q binding to the bacteria, which inhibits subsequent activation of the complement pathway, as well as binding C3b away from the outer bacterial membrane and, thus, abrogating bacterial lysis by the complement membrane attack complex.



1.6.3 Type 1 and 3 Fimbriae

Fimbriae represent another class of *Kp* virulence factors and are important mediators of *Kp* adhesion (Figures 1.4, 1.5). In *Kp*, type 1 and 3 fimbriae are the major adhesive structures that have been characterized as pathogenicity factors. Four other adhesive structures have been noted for *Kp*, including another fimbria called KPF-28, a nonfimbrial factor called CF29K, and a capsule-like material (304-306). These structures were found to confer binding of *Kp* to human carcinoma or intestinal cell lines, suggesting an involvement of these genes in GI tract colonization (304). One study found expression of KPF-28 in 30/78 nosocomial isolates and CF29K in 3/85 *Kp* nosocomial isolates, (17, 304, 305). However, little has since been done to characterize the structures of CF29K or KPF-28 or their functions in animal model systems. Therefore, this section will focus on type 1 and 3 fimbriae and their role in virulence during infection.

Type 1 fimbriae are thin, thread-like protrusions on the bacterial cell surface and are expressed in 90% of both clinical and environmental *Kp* isolates, as well as almost all members of *Enterobacteriaceae* (307, 308) (Figure 1.4). The *Kp* type 1 fimbrial gene cluster is homologous to that of *Escherichia coli* (124). The *fimA* gene encodes the subunit FimA, which makes up the majority of the structure, while the adhesive properties are imbued by the minor subunit FimH on the tip, which is encoded by *fimH* (124, 307, 309, 310). Other genes within the cluster include those encoding the minor structural subunits FimF and FimG, *fimC*, which encodes the fimbrial chaperone, *fimD*, which encodes an usher protein, and *fimI*, which encodes an uncharacterized product necessary for type 1 fimbriae. *Kp* also encodes a gene not encoded by *E.coli*¸ *fimK*, whose exact function is unknown. One theory is that FimK is involved in type 1 fimbrial

regulation, due to the loss of type 1 fimbriae expression upon deletion of *fimK* (124, 311). *Kp* type 1 fimbriae bind D-mannosylated glycoproteins and, therefore, binding by type 1 fimbriae is frequently termed “mannose-sensitive” binding (312, 313).

Type 3 fimbriae are helix-like filaments. In *Kp*, these fimbriae are encoded by the *mrkABCD* gene cluster (314). The bulk of the structure consists of MrkA subunits with the adhesin MrkD located at the tip (Figure 1.5). MrkB, C, and E are involved in assembly and regulation of expression, while MrkF is involved in surface stability of the fimbriae (315). In a manner similar to type 1 fimbriae, the type 3 fimbriae-encoding operon is found in and expressed in almost all *Kp* isolates. In contrast to type 1 fimbriae, type 3 fimbriae are “mannose-insensitive,” and therefore do not bind mannose. While a specific cell-surface receptor has not yet been identified for type 3 fimbriae, they have been shown to bind extracellular matrix proteins, such as type IV and V collagen (316).

Kp utilizes environmental cues to regulate expression of its type 1 fimbriae. For example, type 1 fimbriae genes are expressed in the urinary tract, but not in the GI tract or lungs (124, 317). This observation is in line with the fact that *Kp* type 1 fimbriae contribute to UTIs (317). In particular, type 1 fimbriae contribute to invasion of bladder cells by *Kp* and to biofilm formation in the bladder during a UTI in a mouse model system, although they are not needed for early colonization (124, 125). Based on the lack of expression of type 1 fimbriae in the GI tract and lungs, it is not surprising that this structure is superfluous for or even possibly detrimental during GI tract colonization by *Kp* and during *Kp*-induced pneumonia (124, 317). Notably, in a mouse lung model of infection, a *Kp* mutant lacking *fim* gene cluster was capable of not only colonizing the lungs, but also disseminating to the spleen and liver at WT levels (317). This suggests

that type 1 fimbriae expression may be detrimental for survival in these sites. Along these lines, experiments in a mouse model found that a *Kp* strain lacking *fimK,* which resulted in overexpression of type 1 fimbriae, was attenuated during lung infection (311). However, it should be noted that this phenotype could also be due to the associated decrease in capsule production that occurs in a *fimK* mutant (311).

Like their type 1 counterparts, type 3 fimbriae are not needed for GI tract colonization or for virulence in the lung. Type 3 fimbriae can bind to bladder epithelial cells grown in culture, but in mouse model systems they do not seem to contribute to UTIs (317-319). Moreover, in these organs, type 1 and 3 are not functionally redundant, i.e. a double knockout of the clusters encoding both type 1 and type 3 fimbriae was of equal virulence as a WT strain in the lungs (317). This finding was somewhat surprising, as earlier studies had found that type 3, but not type 1, fimbriae could mediate binding to tracheal cells, buccal cells, and lung sections *in vitro* via MrkD (320).

Arguably, the most important clinically significant roles for fimbriae may be in biofilm formation and in binding to abiotic surfaces, as the ability of *Kp* to bind to surfaces such as indwelling catheters or other devices provides it with an ability to seed vulnerable sites and to persist in patients (124, 317). Typically, a biofilm forms when planktonically grown bacteria come in contact with a surface, adhere, and then form a complex 3-dimensional structure. Bacteria within this structure often become resistant to chemicals to which planktonically grown bacteria may be susceptible (321). Biofilm structures can then give rise to planktonic bacteria that seed new sites of infection. In *Kp*, neither type of fimbriae is expressed while *Kp* is planktonic; however, type 3 fimbriae are expressed during biofilm formation on catheters, while expression of type 1 fimbriae is

controversial (322, 323). Therefore, one can speculate that these fimbriae may contribute to the occurrence of UTIs. In other *Kp* biofilm models, type 3 fimbriae in particular have been shown to be crucial for biofilm production (317, 323, 324). The precise role of type 1 fimbriae in the production of biofilms by *Kp* is not as clear. While one report found that type 1 fimbriae promoted *Kp* biofilm formation on abiotic surfaces in a role reminiscent of that played by the homologous FimH in biofilm production by *E. coli*, another study found that type 1 fimbriae were down-regulated in biofilm cells and did not influence biofilm formation (125, 323, 325, 326).

Type 3 and possibly type 1 fimbriae may also contribute to the delivery, entry, and persistence of *Kp* in VAPs. Endotracheal tubes, commonly used to ventilate patients, give bacteria access to the lungs, hinder patient mucociliary clearance, damage host tissues upon insertion, and provide a surface for biofilm formation (327, 328). Type 3 fimbriae assist *Kp* in the colonization of endotracheal tubes and, thus, lung infection, in two ways. First, MrkA binds directly to plastic surfaces, and second, MrkD facilitates binding to collagen or bronchial cell-derived extracellular matrix coated surfaces (324). This suggests that in VAP, MrkA may mediate binding of *Kp* to endotracheal tubes prior to or during initial insertion. Once these indwelling devices become coated with host-derived substances, such as collagen or other bronchial-derived extracellular matrix proteins that are released during damage to the tissues upon insertion of the tube, MrkD may facilitate the adherence of *Kp* to these tubes and/or to bronchial tissues themselves (329). Overall, this may lead to increased deposition and persistence of *Kp* in the lungs of ventilated patients. This idea is supported by work done in a catheter-associated UTI mouse model, where the presence of a catheter allowed for the persistence of *Kp* in the

bladder for at least 48 hpi. By contrast, clearance of *Kp* was observed in the absence of a catheter (330). In this model, colonization and persistence in the bladder and on the catheter was facilitated by type 1 and 3 fimbriae. Therefore, it is plausible that both fimbriae participate in increasing the likelihood of encountering *Kp* on abiotic and biotic surfaces.

In healthy individuals, the benefits of fimbriae to *Kp* conferred by increased adherence to biotic surfaces may be countered, in part, by their interactions with immune cells. Specifically, expression of fimbriae by *Kp* and by other organisms increases their binding to phagocytes (257). One consequence of bacterial binding to phagocytic cells is the triggering of phagocytosis, which often leads to bacterial internalization and killing. *Kp* type 1 fimbriae, as well as certain *Kp* capsule types, can trigger an opsonin-independent form of phagocytosis called lectinophagocytosis (257, 331, 332). Phagocytes that interact with pathogens via lectins include neutrophils and macrophages (258). Work in *E. coli*, which have homologous type 1 fimbriae, has shown that type 1 fimbriae are recognized by the opsonin-independent lectin binding integrin site on CR3 (CD11/CD18) (331). However, this has yet to be shown in *Kp*. Generally, an increase in internalization by phagocytes of extracellular bacteria during any infection leads to increased bacterial killing (259). This then initiates downstream inflammatory events that amplify cytokine production and the activation and recruitment of responding immune cells, such as neutrophils. For example, *Kp* binds to mast cells through FimH and activates these cells, which induces the release of cytokines that amplify neutrophil recruitment during infection (333). Interestingly, type 1 fimbriae were significantly less stimulatory to neutrophils, as measured by upregulation of the oxidative response, unless in the presence

of opsonins; this protection may be linked to masking by the capsule (220, 325, 334-336). In some strains, capsule may inhibit type 1 fimbriae expression and may sterically preclude its binding to phagocytic and epithelial cells. While the roles for type 3 fimbriae in phagocytosis are not as clear as with type 1, *in vitro* work has shown that *Kp* type 3 fimbriae can stimulate an oxidative response in neutrophils (334).

Figure 1- 4. Functions of Type 1 fimbriae during *Kp* infection and biofilm formation

Type 1 fimbriae are filamentous, membrane-bound adhesive structures primarily composed of FimA subunits with the FimH subunit on the tip. These fimbriae have a role in bladder cell invasion by *Kp*, as well as biofilm formation in the bladder and on abiotic surfaces. However, type 1 fimbriae may be a negative influence on *Kp* virulence *in vivo* in a couple ways. First, type 1 fimbriae amplify lectinophagocytosis of *Kp* by macrophages and neutrophils. Second, the FimH subunit increases binding to immune cells, such as mast cells, leading to increased immune cell activation and subsequent recruitment of neutrophils, which likely increases *Kp* clearance.

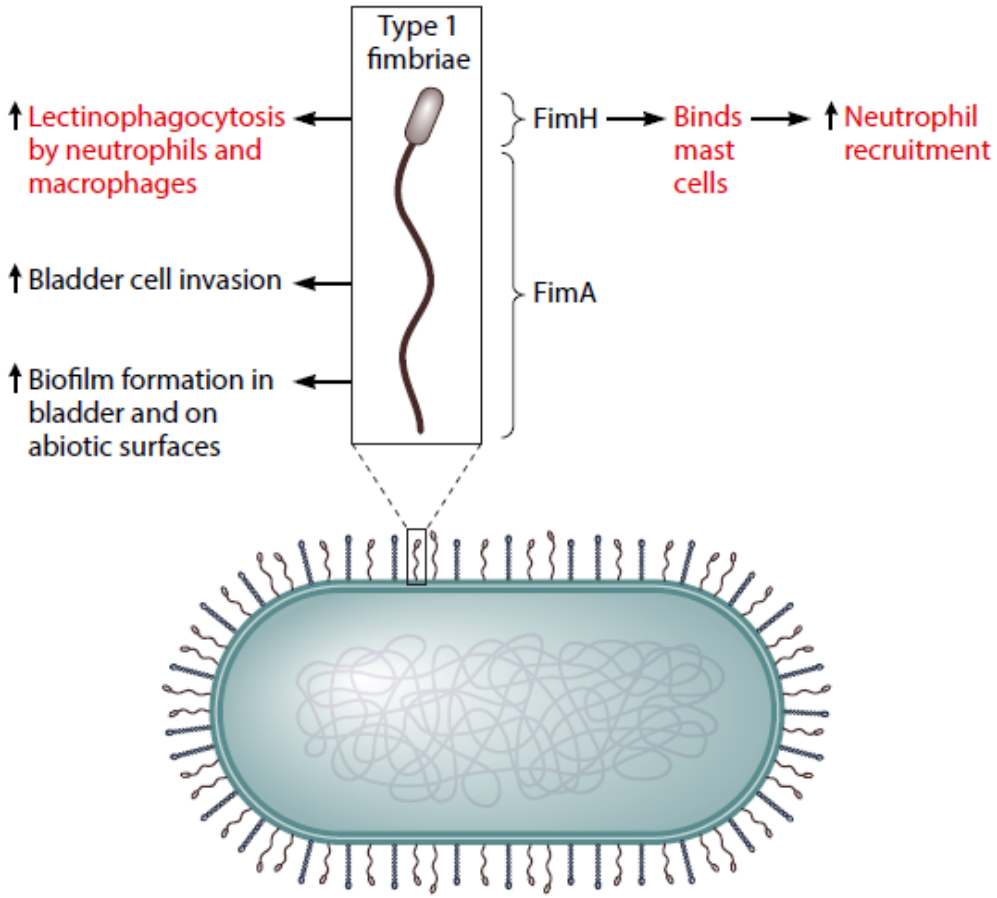
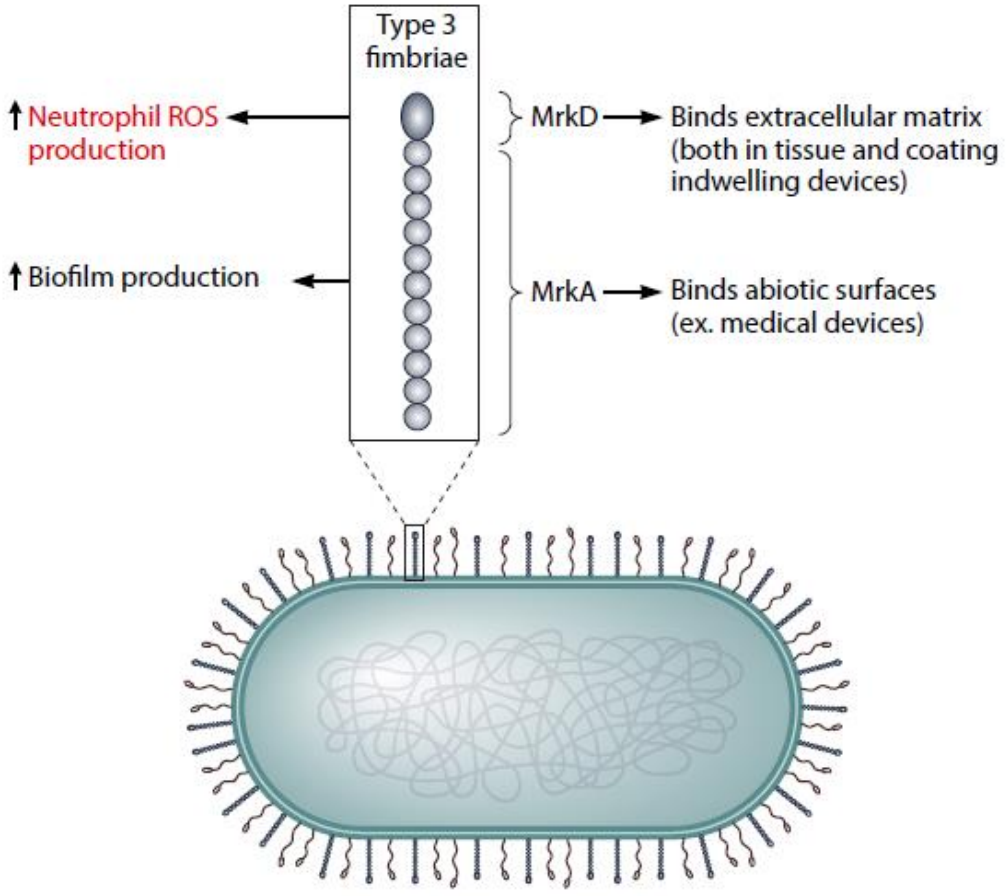


Figure 1- 5. Functions of Type 3 fimbriae during *Kp* infection and biofilm formation

Type 3 fimbriae are helix-like, membrane-bound adhesive structures on the surface of *Kp*. They are primarily composed of MrkA subunits with the MrkD subunit on the tip. Type 3 fimbriae have been found necessary for *Kp* biofilm production and binding to medical devices. MrkD has specifically been found to bind extracellular matrix, such as that exposed on damaged tissues and coating indwelling devices, while MrkA binds abiotic surfaces, such as on medical devices prior to insertion into patients or coating by host matrix after insertion. Type 3 fimbriae have been shown to have a possibly detrimental role, as their presence on *Kp* increases ROS production by neutrophils.



1.6.4 Siderophores

Iron is a limited resource that is required by *Kp* and must be acquired from the environment for *Kp* to thrive during infection. This metal is not readily available in the host during infection, primarily because, as part of the non-specific immune response, the host sequesters it to restrict growth of a number of possible pathogens (337, 338). Normally, there is little free iron in the host plasma**,** as it is bound by iron transport molecules, such as transferrin. Mammalian hosts can further reduce iron levels upon bacterial infection by shifting the binding of iron to lactoferrin, which is an innate defense protein present in bodily fluids (337, 339). Therefore, *Kp,* like many other bacterial pathogens, must employ tactics to acquire iron from the host in order to survive and propagate during mammalian infection.

The predominant tactic used by many pathogens, including *Kp,* to acquire iron is through the secretion of siderophores, which are molecules that possess a higher affinity for iron than host transport proteins. Siderophores can steal iron from host iron-chelating proteins or scavenge it from the environment (339). *Kp* strains encode several siderophores and the expression and contribution of each to virulence varies (Figure 1.6). The production of more than one siderophore by *Kp* may be a means to optimize successful colonization of different tissues and/or to avoid neutralization of one siderophore by the host (339, 340). Several siderophores are expressed in *Kp,* including enterobactin, yersiniabactin, salmochelin, and aerobactin, and the roles of these different siderophore molecules in *Kp* infection will be discussed in detail below. The affinity of these siderophores for iron ranges from aerobactin with the lowest to enterobactin with the highest (341, 342).

While expression of the other siderophores is less conserved, enterobactin expression is almost ubiquitous among both classical and HV *Kp* strains and is therefore considered to be the primary iron-uptake system utilized by *Kp* (48, 343-345). In *Kp*, the genes that are required for enterobactin biosynthesis are encoded on the chromosome by the *entABCDEF* gene cluster, while the *fepABCDG* gene cluster encodes the proteins that mediate its transport with *fepA* encoding specifically for the uptake receptor on the bacterial surface (49, 346). Previous work in other bacteria has delineated that FepB brings iron-bound enterobactin to a pore crossing the inner membrane formed by FepG and FepD, and the iron is released once in the cytoplasm (347). Importantly, while *fepA* expression has been shown to be upregulated during *Kp* infection, suggesting upregulation of enterobactin functionality, enterobactin is neutralized by the host-secreted molecule lipocalin-2 (51, 348). Lipocalin-2 is a multifunctional protein that has several antimicrobial capabilities and is released from many cell types, including neutrophils, during infection. It is expressed basally, but transcription of this factor is upregulated by the host in response to *Kp* infection in the respiratory tract (54, 55, 349)**.** Lipocalin-2 does not kill *Kp*, but rather inhibits its growth by eliminating the ability of *Kp* to scavenge iron from the host by binding to and neutralizing some of its secreted siderophores (350). Lipocalin-2 also has proinflammatory functions; an increase in its production by the host leads to a significant increase in neutrophil recruitment to the site of bacterial infection, most likely through the production of IL-8 (351, 352). Enterobactin, in the absence of lipocalin-2, assists in both colonization of and dissemination from the lungs (50). However, in the presence of lipocalin-2, *Kp* strains that only produce this siderophore are cleared (50).

Yersiniabactin was originally discovered in the gram-negative bacterial pathogen *Yersinia* as part of a *Yersinia* high pathogenicity island, but this siderophore has since been identified in other bacteria, including *Kp* (353). The proteins required for yersiniabactin synthesis are encoded by *irp* genes and it is predicted that the transporters required for secretion of this siderophore are encoded by the *ybt* and *fyu* genes, and the uptake receptor is encoded by *ybtQ*, although this remains to be thoroughly characterized in *Kp* (49, 51, 353). Interestingly, Yersiniabactin has been observed in approximately only 18% of classical but 90% of HV *Kp* clinical isolates (49, 50). However, in conjunction with enterobactin, it is over-represented in *Kp* isolates from the respiratory tract (50). Notably, Yersiniabactin is expressed during lung infection and its activity is not inhibited by lipocalin-2 *in vivo* during early lung infection, likely because its structure significantly differs from enterobactin (50, 51, 339). This allows *Kp* to grow to high bacterial loads in the lungs during infection (50). While Yersiniabactin seems to be impervious to lipocalin-2, it is unable to acquire iron required for *Kp* growth in the presence of the host protein transferrin (50). Thus, strains that only express the Yersiniabactin siderophore are not capable of disseminating from the lungs, likely because transferrin, which is concentrated in blood plasma, prevents *Kp* growth in blood. Therefore, immunocompetent individuals can likely withstand infection by *Kp* strains that only produce Yersiniabactin (50, 339). Work also suggests Yersiniabactin may play a role in HV *Kp* pathogenesis in IP mouse infections that serve as a model for liver abscesses, but not for HV *Kp* in a pneumonic model (49, 354).

Salmochelin is a c-glucosylated form of enterobactin (355, 356). This modification is carried out by genes found on either the chromosome or on a plasmid

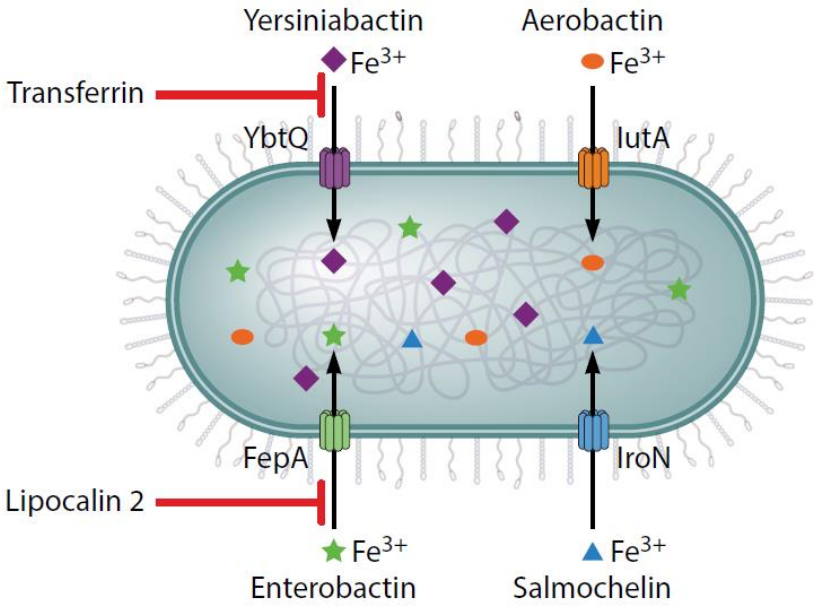
within the *iroA* gene cluster, *iroBCDE* (49). Transport of the iron-laden form is mediated by IroN (346, 357). Importantly, this modification prevents binding of salmochelin by lipocalin-2, thus preventing siderophore neutralization and lipocalin-2-dependent induction of inflammation (355). Therefore, it is not surprising that salmochelin enhances *Kp* colonization of the nasopharynx in a lipocalin-2 sufficient host (351). Because expression of salmochelin allows for nasopharyngeal colonization by *Kp* in hosts capable of producing lipocalin-2, one can predict that patient populations infected with salmochelin-positive strains may be less immunocompromised on average and that salmochelin producing-strains are more virulent. In line with this prediction, salmochelin is present in only about 2-4% of nosocomial *Kp* strains, but is much more prevalent in HV *Kp* strains, with one study reporting its presence in more than 90% of HV *Kp* strains associated with pyogenic liver abscess (48-50).

Aerobactin is a citrate-hydroxamate siderophore. It is rarely expressed by classical nosocomial *Kp* clinical isolates, as it is in only about 6% of classical strains, yet is present in 93-100% of HV *Kp* isolates (45, 47, 48, 343). The presence of aerobactin is always associated with a hypercapsule, although not all hypercapsulated strains possess this siderophore (45, 47, 339). This association is due to the fact that the aerobactin gene cluster, *iucABCD,* along with the aerobactin transporter *iutA* are carried on the same virulence plasmid that encodes *rmpA,* an enhancer of capsule production (49, 242, 358-360). Interestingly, while aerobactin is only rarely found in classical strains causing lung infection, it may be the predominant siderophore expressed in certain HV *Kp* strains causing lung infection (354). HV *Kp* strains have an increased ability to acquire iron through amplified siderophore production, and aerobactin makes up the vast majority of

total siderophore produced in at least one HV *Kp* strain (53). Furthermore, one study found aerobactin is needed for successful infection by HV *Kp* in pneumonic and subcutaneous mouse infection models, but not enterobactin, yersiniabactin and salmochelin (53, 354). In an intraperitoneal HV *Kp* infection mouse model, aerobactin plays a functionally redundant role with yersiniabactin and salmochelin, where only simultaneous deletion of all three siderophores attenuates the strain *in vivo* (49).

Figure 1- 6. Siderophore production and roles in virulence for *Kp*

The ability to acquire iron in an iron-poor environment during infection is necessary for *Kp* pathogenesis. Therefore, bacteria secrete proteins with a high affinity for iron, called siderophores. *Kp* strains have been found to produce one or more of the following siderophores: enterobactin, salmochelin, yersiniabactin and aerobactin. Enterobactin is the primary siderophore used by *Kp*, although it is inhibited by the host molecule lipocalin-2. Salmochelin is a c-glucosylated form of enterobactin that can no longer be inhibited by lipocalin-2. Yersiniabactin and aerobactin are structurally distinct from enterobactin and salmochelin. Neither can be inhibited by lipocalin-2, but yersiniabactin functionality is reduced in the presence of the host molecule transferrin. Production of a number of different siderophores may allow *Kp* to colonize and disseminate to a number of different sites within the host, with niche-specific roles for each siderophore. FepA, IroN, YbtQ and IutA serve as transporters specific to their corresponding siderophores of enterobactin, salmochelin, yersiniabactin and aerobactin, respectively.



1.6.5 OMPS

Several other factors have been recently identified as having roles in *Kp* virulence. However, these factors are not yet thoroughly characterized and much work remains to fully understand their mechanisms of action and clinical significance. These virulence factors include outer membrane proteins (OMPs), porins, efflux pumps, iron transport systems, and genes involved in allantoin metabolism.

Several OMPs have been noted to be important for *Kp* virulence, including outer membrane protein A (OmpA), peptidoglycan-associated lipoprotein (Pal) and murein lipoprotein (LppA), which are encoded by genes of the same name (361). OmpA aids in *Kp* virulence, at least in part, through protection against the innate immune response. However, studies using OmpA purified from *Kp* or, conversely, OmpA in the context of the whole *Kp* bacterium have yielded seemingly conflicting results. Use of purified OmpA has led to the observations that OmpA binds to bronchial epithelial cells, as well as DCs and macrophages, leading to enhanced cytokine production (362-364). This binding of OmpA to DCs and macrophages occurs through the scavenger receptor LOX-1, which then leads to TLR2 signaling (363, 364). Instillation of purified OmpA into lungs induces upregulation of cytokine and chemokine production, which leads to a subsequent transient neutrophil recruitment (362). On the other hand, OmpA in the context of the bacterium may inhibit cytokine production, as well as increase bacterial resistance to antimicrobial peptides, such as α-defensin (365). For example, a *Kp ompA* deletion mutant caused amplified bronchial epithelial cell IL-8 production *in vitro*, and increased TNFα and IL-6 levels in mouse lungs compared to an isogenic strain expressing OmpA (366). Furthermore, this *ompA* deletion mutant was attenuated in a

mouse model of pneumonia. The differences in outcomes in these studies highlight the importance of studying virulence factors in the context of the bacterium and indicate that results may vary between strains due to the presence of other bacterial antigens and virulence factors that modulate inflammation, such as LPS and capsule.

The contribution of the OMPs peptidoglycan-associated lipoprotein (Pal) and murein lipoprotein (LppA) to *Kp* virulence has only been minimally characterized. Work in a HV *Kp* strain using isogenic deletion mutants of these two genes in a mouse intraperitoneal infection model found that the loss of these genes resulted in a lower bacterial fitness (361). *In vitro* experiments with these strains indicate that the mechanisms behind the increased fitness these proteins confer may include protection against neutrophil phagocytosis, and killing by neutrophils and serum components (361). Furthermore, these proteins likely contribute to the integrity and selective impermeability of the cell membrane in an LPS- and capsule-independent manner. Along these lines, these proteins may also strengthen *Kp* against anionic detergents and certain antibiotics.

1.6.6 Porins

OmpK35 and OmpK36 are porins that, interestingly, are often poorly or not expressed in antibiotic-resistant *Kp* strains, including ESBL and carbapenem resistant strains (367-373). Downregulation of these porins appears to provide an advantage for these bacteria in the face of antibiotic selection, where perhaps the porins serve as a channel that allows entry of antibiotics into the bacteria (374). Restoration of expression of *ompK35* or *ompK36* in carbapenem resistant *Kp* strains significantly decreased antibiotic resistance, while deletion of *ompK36* from a K2 HV *Kp* strain increased resistance to certain antibiotics *in vitro* (373, 375). Furthermore, while deletion of

ompK35 did not change *Kp* susceptibility to certain antibiotics, concurrent deletion of both *ompK35* and *ompK36* lead to antibiotic resistance that was higher than even the *ompK36* single deletion mutant (375). However, porin downregulation may come at a fitness cost in terms of virulence (367). In an *in vivo* mouse intraperitoneal infection model, the presence of OmpK36 increased *Kp* virulence in the absence of OmpK35, but *Kp* expressing both OmpK35 and OmpK36 was even more virulent (375). Further studies showed that an *ompK36* deletion mutant can colonize the liver, but cannot persist, following intraperitoneal injection (367). Likewise, an *ompK36* deletion mutant could not persist in the lungs to WT levels in a pneumonic infection model (291). Further studies evaluating a classical *Kp* strain with and without *ompK36* showed that mice infected intraperitoneally or intranasally with this deletion mutant experienced significantly less mortality (291, 367). One mechanism by which OmpK36 may contribute to virulence *in vivo* is by preventing phagocytosis, as demonstrated by increased uptake of an *ompK36* deletion mutant by human neutrophils (367). This is possibly due to a change in bacterial binding to neutrophils conferred by the loss of OmpK36.

1.6.7 Pumps and Transporters

AcrAB is an efflux pump that has been implicated in both *Kp* virulence and resistance to antibiotics (376-378). This contribution to virulence was demonstrated in a pneumonic infection mouse model, where infection with an *acrB* deletion mutant in a K2 expressing *Kp* strain resulted in a decreased bacterial load in the lungs compared to the WT strain, demonstrating AcrB enhances bacterial fitness in the lungs (376). Furthermore, the *acrB* deletion mutant was more sensitive to exposure to human

bronchoalveolar lavage fluid and antimicrobial peptides, such as HBD-1 and HBD-2, indicating this protein is needed for protection against antimicrobial humoral components (376). The increased fitness imparted by AcrB is also supported by work in a *Caenorhabditis elegans* infection model (378). In terms of antibiotic resistance, an *acrB* deletion of *Kp* is more susceptible than WT to certain antibiotics, such as β-lactams (376). Along these lines, expression of AcrR in clinical strains is associated with antibiotic resistance (379). Overall, these AcrAB functions are likely mediated by export of detrimental host molecules or antibiotics out of the bacterial cell.

Kfu is an ABC iron transport system that is involved in *Kp* acquisition of iron and there is a strong association between expression of this factor and HV *Kp* strains (49, 94, 302). The relevance of this gene to virulence *in vivo* was established in a lethal mouse model of peritonitis with an HV *Kp* strain deletion strain, where an isogenic *kfu* deletion mutant failed to cause mortality (49). In this model, *kfu* was necessary for liver and brain abscess formation. In humans, *kfu* is more frequently found in invasive clinical strains, specifically strains that originate from a liver abscess to cause meningitis or endophthalmitis compared to non-invasive strains (380). This highlights the potential importance of Kfu to clinical infection and the importance of iron acquisition to *Kp* virulence.

1.6.8 Allantoin Metabolism

Metabolism of allantoin is a method by which bacteria can obtain carbon and nitrogen from their environment (381). An operon encoding genes involved in allantoin metabolism was identified when looking for *Kp* genes whose transcription was upregulated in HV *Kp* strains compared to classical strains (382). Further work *in vitro*

uncovered a dependence of HV *Kp* on this operon when using allantoin as the sole nitrogen source under aerobic conditions. HV *Kp* likely utilizes this operon for virulence *in vivo*, as deletion of *allS*, an activator of the operon involved in this process, resulted in an HV *Kp* strain with significantly reduced virulence based on LD_{50} in an intragastric model of infection (382). Along these lines, the presence of this allantoin operon is enriched in strains associated with liver abscess versus commensal strains (251, 383). For example, one study found that *allS* is present in 100% of HV Kp isolates from Taiwan, but 0% of K2 isolates and non-K1/K2 isolated from patient blood or liver samples (45), and another found an association between the presence of *allS* and the invasiveness of primary liver abscess strains (224).

1.7 NEW GENETIC SCREENS IDENTIFYING MORE PUTATIVE *KP* VIRULENCE FACTORS

As evidenced by the relatively few well-characterized virulence factors described above, there is much left to be uncovered about what *Kp* factors are required during infection. Two general types of studies have been executed to identify factors critical for *Kp* infection: those that involve high-throughput screening to identify mutations that render *Kp* avirulent in mouse models and those that involve comparative genomics of clinically isolated versus environmental strains. An overview of these screens is presented in **Table 1.3** and a summary of the hits categorized by function is presented in **Table 1.4**. Many of these hits were in genes that are involved in LPS, capsule and fimbriae. Others (metabolic and transcription factors) could potentially feed into the synthesis and/or production of previously identified virulence factors or these could reflect different aspects required for *Kp* to establish or maintain infection in different

niches. Therefore, we expect that further study of these factors will provide important and relevant additional insights into the disease sites infected by *Kp* as well as the physiology of *Kp* in these sites.

To our knowledge, there are five publications to date using high-throughput approaches in mouse models of infection that seek to identify *Kp* virulence determinants. Specifically, two screens were performed to identify *Kp* genes critical for survival in the lung, two for genes required for intestinal colonization (with one of those screens also looking at UTIs), and one screen was performed in a liver abscess model (177, 384-387). The majority of these screens were studying classical *Kp* strains, although one screen by Tu *et al* focused on an HV *Kp* strain (387). Another two studies used genomic comparisons between *Kp* strains, one study focusing on classical *Kp* and the other on HV *Kp,* to identify genes of interest and then characterized the role of specific *Kp* genes in a mouse lung infection model (178, 388). Finally, four large-scale screens, three in classical *Kp* and one in HV *Kp,* have examined *Kp* growth in *in vitro* assays and then assessed the importance of these genes in virulence in mouse models of infection (389-393). Specifically, in these studies, genes important for *in vitro* biofilm production, and genes contributing to inhibition of *in vitro* NFκB signaling were identified, followed by interrogation in pneumonic and intestinal colonization models to assess the contribution of these genes to the virulence of *Kp*.

The first published large-scale high-throughput study used to identify *Kp* virulence factors established a mouse model of pneumonia using a rifampicin-resistant derivative of *Kp* strain 43816. Infection with this strain resulted in systemic spread and significant mortality following intranasal infection. The authors evaluated the fitness of

4,800 signature-tagged *Kp* mutants in the lung and the spleen and identified 106 genes as essential for *Kp* fitness in this model (177). These genes encode products that are involved in a variety of processes including cellular metabolism, assembly of the outer membrane, cell-surface located proteins, regulators of transcription, transporters, and a number of hypothetical proteins. More recently, the largest high-throughput screen to date for *Kp* was published, and identified more than 300 genes necessary for successful lung infection by *Kp* in the first 24 hours (384). In this study, preliminary characterization of 6 of these genes found that they appear to be important for virulence by playing either a protective role against the immune response or in the acquisition of resources. Specifically, the authors found that *rfaH* promoted serum resistance and capsule production, *aroE* promoted serum resistance, *ilvC* and *ilvD* were important for branched chain amino acid synthesis, and *copA* promoted resistance to copper toxicity (384).

Using a different approach to identify putative virulence genes, Lau *et al.* utilized subtractive hybridization of genes to compare a nonvirulent strain, IA565, to the classical virulent *Kp* strain, 43816 (178). They identified nine genes putatively required for *Kp* virulence, *iroN, dcoB, orf350, kvgS, ybiT, Mrr,* and *sthB* and *fimD,* that were only present in the virulent strain. Based on homology to characterized genes in other bacteria, their functions include acquiring iron (*iroN*), producing and converting energy (*dcoB*), regulating transcription (*orf350*), signaling (*kvgS*), transporting substrates through the membrane (*ybiT*), restricting endonuclease activity (*Mrr*) and, as discussed above, adhesion (*fimD* and *sthB*).

In a similar vein, Lery *et al.* performed a comparative analysis of the newly sequenced HV *Kp* K2 genome Kp52.145 (the strain in which *rmpA* and the aerobactin

cluster were originally identified as virulence factors in *Kp*) against the low virulence K1 SB3193 and K2 SB2390 strains, as well as with the sequence of the well-characterized virulent reference K1 NTUH-2044 and K5 MGH78578 strains (242, 358, 388, 394). From this work, five genomic regions were highlighted with pathogenic features in the newly sequenced HV K2 strain. One region encoded yersiniabactin, colibactin, and a type IV secretion system, a second encoded a putative cytotoxic outer membrane protein, a third encoded proteins putatively involved in adhesion and evasion of the immune response, a fourth encoded a number of phage-related proteins that the authors speculated might play a role in immune evasion through an IL-17 related mechanism, and a fifth encoded phospholipase D family proteins (388). Follow-up experiments on one of the phospholipase D gene, *pld1,* found that it contributes to *Kp* virulence during murine lung infection by putatively altering cardiolipin metabolism, demonstrating a novel role for lipid metabolism in *Kp* virulence (388).

Based upon previous observations that *Kp* reduces NFκB signaling, Tomas *et al.* used a high-throughput screen to identify *Kp* genes that dampen NFκB signaling during infection of the human epithelial cell line A549 (393, 395). Using gain of NFκB signaling as a read-out, 5,320 transposon mutants were screened and 114 mutants that no longer prevented NFκB signaling were identified. These mutations were predominantly in genes related to metabolism and transport, but were also found in envelope and capsule-related genes. This latter finding is consistent with the hypothesis that *Kp* capsule may mask certain bacterial antigens from recognition by the host immune system. The authors of this study performed further characterizations of two of the factors identified in their screen, *waa,* which is known to be involved in O-antigen synthesis and promotes

virulence in other bacteria, and *pulA*, which is a component of a type II secretion system that had not previously been implicated in bacterial virulence. Supporting the relevance of these genes during infection and the overall approach, *Kp* mutants lacking these genes were attenuated in a pneumonic mouse model.

At least two studies have searched for *Kp* biofilm factors that play a role in a pneumonic mouse model (390, 391). In the first study, Lavender *et al.* screened 2,000 transposon mutants for the loss of the ability to form biofilms on abiotic plastic surfaces and/or human extracellular matrix (390). Four mutants were unable to make biofilm on at least one of the surfaces tested: *yciI, yadH, yhdH,* and *pduA.* When studied in an *in vivo* pneumonic model, only one mutant, *yciI*, was attenuated for virulence based on mouse lethality, indicating that some of the functions required for biofilm formation are important in a lung model of infection. In the second study, Boddicker *et al.* screened a transposon library of 1,175 signature-tagged *Kp* clones to identify mutants with defects in biofilm formation on a human extracellular matrix-coated plate in a flow-through continuous culture system (391). Interestingly, five of the eight confirmed mutants were attenuated in a pneumonic mouse model. Of these, three genes are homologues of genes involved in transcriptional regulation (*luxR, lysR* and *crp*), one gene is important for capsule synthesis, (*orf12*), and one gene (*fimA*) is important for fimbriae production. The other three mutants defective in biofilm formation retained full lethality in the mouse model. Two of these mutants are lacking genes encoding homologues of proteins involved in sugar transport (phosphotransferase EIIB and EIIC), and one mutant lacks a gene predicted to encode a protein of unknown function (YPTB1848). Combined, the results of this study and that by Lavender *et al.* suggest that biofilm production most

likely does not play an essential role during *Kp* pneumonia after instillation of the bacteria into the lungs (390, 391). Rather, the virulence defects observed in the pneumonic model with certain mutants with biofilm defects may be due to pleiotropic functions of these genes.

Two different studies have identified genes critical for intestinal colonization of *Kp* using signature-tagged mutagenesis (385, 386). One study used a *Kp* library of 2,200 signature-tagged transposon mutants in a mouse model of intestinal colonization and identified 44 mutants that, while present in the input, were not recovered from the colon after five days (385). The genes important for GI colonization had functions in regulation of transcription, adhesion, membrane transport, metabolism, DNA-related enzymatic activity, and several had unknown functions. A follow-up study confirmed the importance of a urease identified in the screen in colonization of the GI tract (396). The second signature-tagged mutagenesis screen tested the ability of 1,440 *Kp* signature-tagged transposon mutants to colonize the intestine and bladder. Thirteen genes were needed under both conditions, while six were needed only for establishing a bladder infection (386). Of the genes needed for both intestinal colonization and bladder infection, three were required for LPS production (*waaL, waaE* and *wbbO*), and three encoded cell membrane/surface associated proteins (*plsX*, *ompA* and *surA*). The other seven included *tufA*, which encodes a translation elongation factor, *hupA,* which encodes a DNA folding protein, *arcB*, which encodes a metabolic regulator , as well as two genes encoding hypothetical proteins, and two unknowns. Of those genes identified to be uniquely required for UTIs, two genes were involved in GDP L-fucose synthesis (*gmd* and *fcl*), which is thought to be necessary for proper capsule synthesis by some serotypes;

one gene, *fimB*, was involved in type 1 fimbriae production, one gene encoded a hypothetical protein and two genes were unknowns.

Another study specifically sought to first identify genes necessary for biofilm production and then, evaluated the essentiality of those genes during intestinal colonization (392). Wu *et al.* measured the ability of 2,500 transposon mutants in a HV *Kp* strain to form biofilms on plastic (392). From their initial screen, they identified 23 mutants that displayed decreased biofilm formation. These mutations were in four categories of genes: seven genes were involved in cellular processing and signaling, four genes encoded surface molecules, six were involved in carbohydrate transport or metabolism (including *treC*) and the remaining six encoded proteins with unknown functions. They also identified four mutations that resulted in an increase in biofilm formation. These mutations were in a multidrug-resistance gene (*sugE*), a cold-shock protein gene (*cspC*), as well as in two genes that had unknown functions. Following identification of biofilm-promoting genes, characterization of these mutants in an *in vivo* oral gavage mouse model demonstrated that *treC* was important for intestinal colonization. Again, as with the studies of biofilm-defective mutants that were tested in pneumonic models, these results suggest that functions required for biofilms are not generally required for infection of tissues.

Finally, a HV *Kp* strain liver abscess mouse model was used by Tu *et al*, to test the virulence of 2,880 signature-tagged *Kp* transposon mutants with insertions in non-capsular genes (387). In this model, 28 mutants failed to grow in the liver or spleen following oral infection. In a finding similar to studies of other tissue sites outlined above, these genes encoded metabolic proteins, cell surface proteins including type 1 and

type 3 fimbriae, transporters, transcriptional regulators, and proteins with unknown functions. One of these genes, *ymdF*, was further characterized as having a role in resistance to oxidative stress.

Table 1- 3. Overview of screens performed or confirmed *in vivo* to identify *Kp* virulence factors

Strain	Library Size/Type or Methods	Infection model	Tissues Studied	# of Hits	Genes of Note	Refs
Kp 43816 Rif^R (KPPR1)	4,800; STM	***In vivo* screen:** Intranasal, C57Bl/6 mice	Lungs, Spleen	106	*wecA, wzyE, rfbB*	(177)
Kp 43816 Rif^R (KPPR1)	25,000; Tn Mutagenesis	***In vivo* screen:** Retropharyngeal, C57Bl/6 mice	Lungs	>300	*rfaH, aroE, ilvC&D, copA*	(384)
Kp 43816, *Kp* IA565	N/A; Subtractive Hybridization	***In vivo* screen:** Intratracheal, C57Bl/6 mice	Lungs, Blood	9	*iroN, dcoB, orf350, kvgS, ybiT, mrr, fimD, sthB*	(178)
Kp 52145, *Kp* SB2390, *Kp* SB3193	N/A; Comparison of *Kp* genomes	***In silico analysis:*** genomic sequence analysis ***In vivo* follow-up:** Intranasal, BALB/c mice	N/A	N/A	*pld1, waa*	(388)
Kp 52145	5,320; Tn Mutagenesis	***In vitro* screen:** NFκB reporter A549 cell line ***In vivo* follow-up*:*** Intranasal, CD-1 mice	N/A	114	*waa,pulA*	(389)
Kp 43816	2,000; Tn Mutagenesis	***In vitro* screen:** Biofilm formation ***In vivo* follow-up**: Intranasal and intraperitoneal, mice	N/A	4	*yciI, yadH, yhdH, pduA*	(390)
Kp 43816	1,175; STM	***In vitro* screen:** Biofilm formation ***In vivo* follow-up:** Intranasal, BALB/c mice	N/A	8	*luxR, lysR, crp, orf12, fimA*	(391)
Kp LM21	2, 200; STM	***In vivo* screen:** Oral, IOPS mice ***In vitro* follow-up*:*** Intestinal cell line	Colon	29	*ntrC, fmdD, glgP, fucA-fucP, fruB/pftA,*	(385)

Kp 3091, *Kp* C3091 Sm^R Rif^R	1, 440; STM	***In vivo* screen*:*** Oral and transurethral, Ssc:CF1 mice	Feces, Bladder	19	*waaL, waaE, wbbO, plsX, ompA, surA, tufA, hupA, arcB, gmd, fcl, fimB*	(386)
NTUH-K2044 (HV)	2,500; Tn Mutagenesis	***In vitro* screen:** Biofilm formation ***In vivo* follow-up*:*** Intragastric, BALB/c mice	Colon	27	*treC, sugE, wza*	(392)
Kp CG43 (HV)	2,880; STM	***In vivo* screen*:*** Oral, BALB/c mice	Liver, Spleen	28	*ymdF*	(387)

Abbreviations: N/A=Not applicable, STM=Signature-tagged mutagenesis, Tn=Transposon *Kp=Klebsiella pneumoniae*, HV=Hypervirulent, Rif^R=Rifampicin resistant, Sm^R=Streptomycin resistant

Table 1- 4. *Kp* Genes Involved in Virulence Identified and/or Confirmed in *In Vivo* Mouse Models

Gene name	Infection Model	Strain	Putative function	Defect in *in vivo* screen	Defect in 1:1 Comp	Defect single strain	Ref
LPS							
waaL	GI and UTI	C3091	LPS core synthesis	GI and UTI	UTI	N/A	(386)
waaE	GI and UTI	C3091	LPS core synthesis	GI and UTI	UTI	N/A	(386)
wbbO	GI and UTI	C3091	O-antigen synthesis	GI and UTI	UTI	N/A	(386)
waaL	Pneumonia	52145	LPS core synthesis	N/A	N/A	Pneumonia	(389)
rfbB	Pneumonia	43816	O-antigen export	Pneumonia	N/A	Pneumonia	(177)
wecA	Pneumonia	43816	GlcNAc-1-phosphate transferase	Pneumonia	N/A	Pneumonia	(177)
Capsule							
gmd	UTI	C3091	Fucose synthesis	UTI	UTI	N/A	(386)
fcl	UTI	C3091	Fucose synthesis	UTI	UTI	N/A	(386)
ORF12	Pneumonia	43816	Capsule synthesis	N/A	N/A	Pneumonia	(391)
cpsB	Pneumonia	43816	Mannose-1-phosphate guanyltransferase	Pneumonia	N/A	Pneumonia	(177)
wzi	Pneumonia	43816	Capsule assembly Wzi family protein	Pneumonia	N/A	N/A	(384)
wcaJ	Pneumonia	43816	Undecaprenyl-phosphate glucose phosphotransferase	Pneumonia	N/A	N/A	(384)

rcsB	Pneumonia	43816	Transcriptional Regulator	Pneumonia	N/A	N/A	(384)
Adhesion							
hmw1A	GI	LM21	Surface protein	GI	GI	N/A	(385)
fimB	UTI	C3091	Type I fimbriae regulation	UTI	UTI	N/A	(386)
mrkC	Liver abscess	CG43	Usher protein of type III fimbriae	Liver abscess	N/A	Liver abscess *	(387)
fimC	Liver abscess	CG43	Chaperone protein of type I fimbriae	Liver abscess	N/A	Liver abscess *	(387)
fimA homologue	Pneumonia	43816	Fimbrial synthesis	N/A	N/A	Pneumonia	(391)
Metabolism							
arcB	GI and UTI	C3091	Regulation of aerobe-anaerobe metabolism	GI and UTI	UTI	N/A	(386)
ygjD	GI	LM21	*O*-sialoglycoprotein endopeptidase	GI	GI	N/A	(385)
lacI-lacZ	GI	LM21	Lactose metabolic enzyme	GI	GI	N/A	(385)
pheC	GI	LM21	Cyclohexadienyl dehydratase	GI	GI	N/A	(385)
glgP	GI	LM21	Alpha-glucan phosphorylase	GI	GI	N/A	(385)
PA2698	GI	LM21	Hydrolase	GI	GI	N/A	(385)

treC	GI	NTUH-K2044	trehalose-6-P hydrolase	N/A	GI	N/A	(392)
galK	Liver abscess	CG43	Galactokinase	Liver abscess	N/A	Liver abscess *	(387)
proV	Liver abscess	CG43	Glycine betaine/L-proline ABC transporter	Liver abscess	N/A	Liver abscess *	(387)
araF	Liver abscess	CG43	L-arabinose binding periplasmic protein	Liver abscess	N/A	Liver abscess *	(387)
rhaB	Liver abscess	CG43	Putative α-L-rhamnosidase	Liver abscess	N/A	Liver abscess *	(387)
gabD	Liver abscess	CG43	Succinate-semialdehyde dehydrogenase	Liver abscess	N/A	Liver abscess *	(387)
pgdH	Liver abscess	CG43	D-3 phosphoglycerate dehydrogenase	Liver abscess	N/A	Liver abscess *	(387)
galT	Liver abscess	CG43	Galactose-1-phosphate uridylyltransferase	Liver abscess	N/A	Liver abscess *	(387)
ahpC	Liver abscess	CG43	Alkyl hydroperoxide reductase	Liver abscess	N/A	Liver abscess *	(387)
lyxK	Liver abscess	CG43	Putative L-xylulokinase	Liver abscess	N/A	Liver abscess *	(387)
ilvC	Pneumonia	43816	Ketol-acid reductoisomerase	Pneumonia	N/A	Pneumonia	(384)

ilvD	Pneumonia	43816	Dihydroxy acid dehydratase	Pneumonia	N/A	Pneumonia	(384)
aroE	Pneumonia	43816	Dehydroshikimate reductase	Pneumonia	N/A	Pneumonia	(384)
argR	Pneumonia	43816	Arginine repressor	Pneumonia	N/A	N/A	(384)
ilvE	Pneumonia	43816	Branched-chain amino acid aminotransferase	Pneumonia	N/A	N/A	(384)
purF	Pneumonia	43816	Amidophosphoribosyltransferase	Pneumonia	N/A	N/A	(384)
purl	Pneumonia	43816	Phosphoribosylformylglycinamidine synthase	Pneumonia	N/A	N/A	(384)
trpD	Pneumonia	43816	Anthranilate synthase component II	Pneumonia	N/A	N/A	(384)
serA	Pneumonia	43816	D-3-Phosphoglycerate dehydrogenase	Pneumonia	N/A	N/A	(384)
leuC	Pneumonia	43816	3-Isopropylmalate isomerase subunit	Pneumonia	N/A	N/A	(384)
pheA	Pneumonia	43816	Bifunctional chorismate mutase/prephenate dehydratase	Pneumonia	N/A	N/A	(384)
purH	Pneumonia	43816	Phosphoribosylaminoi	Pneumonia	N/A	N/A	(384)

			midazole carboxamide formyltransferase				
Dgo	Pneumonia	43816	2-Oxo-3-deoxygalactonate 6-phosphate aldolase	Pneumonia	N/A	N/A	(384)
pld1	Pneumonia	52.145	Putative cardiolipin synthase	N/A	N/A	Pneumonia	(388)
pulA	Pneumonia	52145	Pullulan degrading glucanase	N/A	N/A	Pneumonia	(389)
Cell Surface							
ompA	GI and UTI	C3091	Outer-membrane protein	GI and UTI	UTI	N/A	(386)
surA	GI and UTI	C3091	Folding of outer-membrane proteins	GI and UTI	UTI	N/A	(386)
hgpA	Liver abscess	CG43	Hemoglobulin binding protein	Liver abscess	N/A	Liver abscess *	(387)
pls	Liver abscess	CG43	Surface large repetitive protein	Liver abscess	N/A	Liver abscess *	(387)
pteA	Liver abscess	CG43	Cellobiose-specific phosphotransferase IIA	Liver abscess	N/A	Liver abscess *	(387)
pteC	Liver abscess	CG43	Cellobiose-specific phosphotransferase IIC	Liver abscess	N/A	Liver abscess *	(387)
pagO	Liver abscess	CG43	PhoPQ-activated integral	Liver abscess	N/A	Liver abscess *	(387)

			membrane protein				
uraA	Liver abscess	CG43	Uracil permease	Liver abscess	N/A	Liver abscess *	(387)
Lipid Synthesis							
plsX	GI and UTI	C3091	Fatty acid and phospholipid synthesis	GI and UTI	UTI	N/A	(386)
Protein Synthesis							
tufA	GI and UTI	C3091	Protein synthesis-elongation factor	GI and UTI	UTI	N/A	(386)
Nucleic Acid-Related							
hupA	GI and UTI	C3091	DNA folding	GI and UTI	UTI	N/A	(386)
ECso303	GI	LM21	DNA primase	GI	GI	N/A	(385)
BAB36637	GI	LM21	Adenine-specific methylase	GI	GI	N/A	(385)
int	Liver abscess	CG43	Putative integrase	Liver abscess		Liver abscess *	(387)
Transporters							
AF315580	GI	LM21	Amide-urea-binding protein	GI	GI	N/A	(385)
hrcU	GI	LM21	Hairpin type III secretion system	GI	GI	N/A	(385)
copA	Pneumonia	43816	Copper-translocating P-type ATPase	Pneumonia	N/A	Pneumonia	(384)

tatC	Pneumonia	43816	Twin arginine-targeting protein translocase	Pneumonia	N/A	N/A	(384)
Regulators							
ntrC	GI	LM21	Nitrogen metabolism regulator	GI	GI	N/A	(385)
GCvR	GI	LM21	Glycine metabolism regulator	GI	GI	N/A	(385)
cbl	Liver abscess	CG43	HTH-type transcriptional regulator	Liver abscess	N/A	Liver abscess *	(387)
evgA	Liver abscess	CG43	Response regulator of two component system	Liver abscess	N/A	Liver abscess *	(387)
moaR	Liver abscess	CG43	Monoamine regulon positive regulator	Liver abscess	N/A	Liver abscess *	(387)
evgS	Liver abscess	CG43	Histidine kinase sensor	Liver abscess	N/A	Liver abscess *	(387)
esgD	Liver abscess	CG43	Response regulator for second curli	Liver abscess	N/A	Liver abscess *	(387)
kva15	Liver abscess	CG43	Putative LuxR family transcriptional regulator	Liver abscess	N/A	Liver abscess *	(387)
kva19	Liver abscess	CG43	Putative UphA family transcriptional regulator	Liver abscess	N/A	Liver abscess *	(387)

rfaH	Pneumonia	43816	Transcriptional activator	Pneumonia	N/A	Pneumonia	(384)
galF	Pneumonia	43816	Regulatory Protein	Pneumonia	N/A	N/A	(384)
phoR	Pneumonia	43816	Phosphate regulon sensor kinase	Pneumonia	N/A	N/A	(384)
VK055_4417	Pneumonia	43816	MarR family transcriptional regulator	Pneumonia	N/A	Pneumonia	(384)
luxR homologue	Pneumonia	43816	Transcriptional regulation	N/A	N/A	Pneumonia	(391)
lysR homologue	Pneumonia	43816	Transcriptional regulation	N/A	N/A	Pneumonia	(391)
CRP homologue	Pneumonia	43816	Transcriptional regulation	N/A	N/A	Pneumonia	(391)
Hypothetical							
ORF *b2512*	GI and UTI	C3091	Hypothetical protein	GI and UTI	UTI	N/A	(386)
ORF *b1631*	GI and UTI	C3091	Hypothetical protein	GI and UTI	UTI	N/A	(386)
ORF *ytfN*	UTI	C3091	Hypothetical protein	UTI	UTI	N/A	(386)
VK055_5096	Pneumonia	43816	Hypothetical protein	Pneumonia	N/A	N/A	(384)
VK055_5023	Pneumonia	43816	Hypothetical protein	Pneumonia	N/A	N/A	(384)
VK055_3515	Pneumonia	43816	Hypothetical protein	Pneumonia	N/A	N/A	(384)
kva7	Liver abscess	CG43	Hypothetical protein	Liver abscess	N/A	Liver abscess *	(387)
yfgG	Liver abscess	CG43	Hypothetical protein	Liver abscess	N/A	Liver abscess *	(387)
ymdF	Liver abscess	CG43	Hypothetical	Liver	N/A	Liver	(387)

			protein	abscess		abscess *	
Unknown							
yebE	GI	LM21	DUF533 family inner membrane protein	GI	GI	N/A	(385)
yciI	Pneumonia	43816	Putative DGPF domain-containing enzyme	N/A	N/A	Pneumonia	(390)

GI: Gastrointestinal colonization; UTI: Urinary tract infection; LPS: lipopolysaccharide; N/A=Not applicable

A number of other mutants were noted to be attenuated in the screen by (177), but no gene name was provided was given and so those genes were not included.

Reference (387) does not distinguish between whether mutants were confirmed by 1:1 competition or single strain infection

Only the top 25 genes identified in (384) were included in this table due to the large number hits.

1.8 CONCLUSIONS AND PERSPECTIVES

Kp is a medically important, yet understudied, pathogen. It causes infections in a variety of sites in humans, including the lungs, bladder, liver, brain, and bloodstream. The alarming increase in drug-resistant *Kp* infections that are challenging, if not impossible, to treat has recently raised our awareness of the fact that, though *Kp* was isolated over 100 years ago, only a few virulence factors are well-understood and likewise, critical host defenses for *Kp* infections were not a focus of intense study. This has recently changed in response to the rising interest and concern in *Kp*'s widespread antibiotic resistance, as well as in the appearance and spread of HV *Kp* strains that are serious pathogens in otherwise healthy individuals.

Overall, the discovery of *Kp* virulence determinants using a variety of genetic approaches has opened up many avenues of research to characterize these genes and understand how they function in different host environments and abiotic surfaces. However, much work remains to understand *Kp* physiology in tissues, to understand the virulence of newly emerging strains that have become pandemic within the last decade, and to develop methods to combat these drug-resistant or HV *Kp* strains. Of particular interest is whether the genes important for infection of one tissue site also have roles in other tissue sites. For example, certain genes needed for UTIs may not be required for pneumonia, and vice versa, which appears to be the case for type 1 fimbriae (124). Factors that are important in only one or a subset of tissues will teach us about the nutrient requirements and host defenses that are present and distinct to those tissues, but will restrict their usefulness as novel anti-infective targets for *Kp* infections.

It is also important to determine whether genes identified in screens that understandably use strains that are more virulent in mice than many human clinical isolates are critical for the majority of infections with classical *Kp* strains identified from patients, HV *Kp* strains, and multi-drug resistant *Kp* strains. Ideally, future investigations will reveal several classes of genes and pathways important across many types of infections and in many virulent strains, such as in the case of LPS (43). Such proteins and pathways would be excellent candidates for novel drug targets that may be effective against all types of *Kp* infections, particularly those that are caused by ESBL- or carbapenemase-producing *Kp* and/or HV *Kp* strains. Furthermore, it is important to remember that the majority of people presenting with *Kp* pneumonia and bacteremia are in hospitals or long-term care facilities, and are immunosuppressed in some manner. Many of the recent searches for virulence factors have focused on identifying virulence factors in "normal" mice; however, a subset of those factors may not be required in immunosuppressed environments (384). In fact, different genes may play a role in immunosuppressed patient subsets that have yet to be identified and characterized. Furthermore, differences in *Kp* infection based on patient subset may translate into different, and more optimal, approaches for treatment of these patients in the clinic. Therapeutics that restore or complement missing components of immunity in these patients may help prevent or combat *Kp* infections. Conversely, therapeutics that target a gene product that is not required when infecting an immunosuppressed host would not be ideal.

As mentioned previously, about half of patients infected with HV *Kp* are not notably immunosuppressed. Although a few studies aimed at determining what makes

these HV *Kp* strains more virulent than classical *Kp* strains have been published, there is still work to be done delineating their differences and at identifying "Achilles heels" of these HV *Kp* strains. In terms of therapeutics, conserved targets important for both HV and classical *Kp* strains would certainly be the most attractive, as the ability of clinicians to treat patients without waiting for strain diagnostics would likely decrease morbidity and mortality.

It is also important to understand how *Kp* escapes or protects itself against the immunological challenges it faces in primary sites of colonization, such as the GI tract and oropharynx, in addition to infection sites, such as the lungs, liver, blood, and bladder. In order to successfully cause an infection, *Kp* has to overcome the immunological defenses that normally contain it. Even in most patients with some form of immunosuppression, it is unlikely that all arms of the immune response against *Kp* have been abrogated. Therefore, it is of interest to investigate how *Kp* overcomes these remaining defenses to colonize tissues, replicate to high bacterial numbers at the primary site of infection, and then undergo systemic spread.

In summary, the recent emergence of a number of hard-to-treat *Kp* strains and infections is challenging the medical community to evaluate both host and bacterial factors critical during infection. Given the relatively new appreciation of the evolving diversity of clinical *Kp* strains, studies should be done in the many applicable mouse infection and colonization models, including pneumonia, UTI, liver abscess and GI tract colonization with a variety of different strains in order to best understand this pathogen, because previous studies have found that *Kp*'s virulence factors may only have a role in certain sites of infection. Fortunately, recently more and more studies have been

published using high-throughput approaches to identify virulence factors, and work is being done in a more directed manner to investigate specific virulence factors and innate immune defenses. Nonetheless, we still have an incomplete picture of *Kp*'s interactions with different components of the immune response in different tissues and how its virulence factors overcome host defenses and/or enable *Kp* to replicate and establish niches. Continued studies on these facets of *Kp* biology, physiology, and *Kp* interactions with host tissues should drive insights into how to combat *Kp* infections.

For these reasons, my thesis work has focused on utilizing high-throughput techniques to identify *Kp* virulence determinants needed to successfully cause infection, focusing on pneumonia, and has compared genes needed in healthy and neutropenic hosts as an immunosuppressed population. A number of genes were identified that are needed to protect against neutrophils, which gives clues to the stresses *Kp* faces in the lungs. Genes were also identified that are needed regardless of neutrophil status, which are potential therapeutic targets due to their more broadly applicable patient population. Furthermore, these genes were investigated to determine putative functions *in vitro,* both immune response-related and –unrelated, as well as to characterize their potential as therapeutic targets.

CHAPTER 2: MATERIALS AND METHODS

2.1 GENERATION OF *KP* STRAINS

A *Kp* WT strain (*Kp* ATCC 43816; MKP203) was made spectinomycin (Spec) resistant by introducing a transposon, mTn10, carrying a $Spec^R$ cassette through conjugation with a DAP auxotrophic *E.coli* strain MFDpir-pDL10987 (MKP214) (397). *Kp* $Spec^R$-derivatives of the WT strain were selected for on L+Spec (50 μg/mL) agar plates. Several *Kp* $Spec^R$ strains were verified to be equally fit compared to the parental *Kp* WT strain both for *in vitro* growth in L and *in vivo* in the lungs after intranasal mouse infection (data not shown). One strain, MKP220, was used as the WT *Kp* $Spec^R$ strain in all subsequent experiments.

Kp Δ*cpsB* was generated in the *Kp* $Spec^R$ strain (MKP220) using lambda red recombination as previously described (398). Briefly, pACBSR (398) containing a hygromycin (Hygro) resistance cassette and a recombinase were introduced into MKP220 by electroporation. The bacteria were plated on media containing Hygro to select for MKP220 carrying pACBSR. PCR was used to generate and amplify the FRT-flanked apramycin (Apra) resistance cassette of pJI773 using primers with homology to 60-bp of the upstream/start site and 60-bp of the downstream/stop codon of *cpsB*, MKP133 and MKP134, respectively. This PCR product was introduced by electroporation into MKP220+pACBSR that had been induced using L-arabinose and selection was performed by plating on L+Apra (50 μg/mL) agar plates. $Apra^R$ clones were confirmed for the correct replacement of the native *cpsB* gene with the apramycin cassette by PCR using primers MKP135 and MKP136, and these strains were passaged on L to remove pACBSR. Next, to excise the Apra cassette from the MKP220 Δ*cpsB*-$Apra^R$ $Hygro^S$ strains, a plasmid containing a Hygro resistance cassette and FRT-specific recombinase,

pFLP, was introduced into these strains by electroporation, and then selection was performed on L+Hygro (100 μg/mL) to isolate plasmid containing clones. The resulting Hygro^R clones were grown at 43°C to induce the FLP recombinase and splicing of the Apra cassette at the FRT sequences. Apra^S clones were passaged in L to promote loss of pFLP, and clones sensitive to both Hygro and Apra were confirmed to be Δ*cpsB* by PCR and sequencing with primers MKP135 and MKP136.

Kp Δ*aroA* (MKP453) was generated using lambda red recombination as previously described (398). Briefly, PCR was used to generate and amplify the FRT-flanked apramycin (Apra) resistance cassette of pJI773 with 60-bp of the upstream/start site and 60-bp of the downstream/stop codon of *aroA* using primers MKP189 and MKP190. Then, a WT *Kp* strain carrying the hygromycin (Hygro) resistant pACBSR was generated, incubated in media containing L-arabinose, transformed with this PCR product, and then the transformants were plated on L+Apra (50 μg/mL) agar plates. Apra^R clones were confirmed for the correct replacement of the native *aroA* gene with the Apra cassette by PCR using primers MKP191 and MKP192. These strains were passaged in the absence of Hygro to remove pACBSR, transformed with Hygro^R pFLP and plated on L+Hygro (100 μg/mL). The resulting Hygro^R clones were grown at 43°C to induce FLP expression and splicing of the Apra cassette at the FRT sequences. Apra^S clones were passaged to remove pFLP, and clones sensitive to both Hygro and Apra were confirmed to be Δ*aroA* by sequencing with primers MKP191 and MKP192. These were further verified by testing for their ability to grow on M9+gluc. *Kp* Δ*aroA* was complemented *in cis* to create *Kp* Δ*aroA::aroA* (MKP460), *Kp* Δ*aroA::aroAB3*, and *Kp* Δ*aroA::aroAB19* by transforming *Kp* Δ*aroA* pACBSR with a PCR product of the coding

and flanking regions of *aroA* amplified using primers MKP191 and MKP192 from WT, B3 and B19, *Kp* strains, respectively. These PCR products were transformed into *Kp* Δ*aroA* pABCSR and *aroA* expressing mutants were selected for by plating on M9+gluc. pACBSR was subsequently removed from *Kp* Δ*aroA::aroA*, *Kp* Δ*aroA::aroAB3*, and *Kp* Δ*aroA::aroAB19* by passaging these strains in the absence of Hygro. Each strain was confirmed to contain the correct *aroA* by sequencing PCR products with MKP191 and MKP192.

2.2 GENERATION AND ARRAYING OF *KP* TRANSPOSON INSERTION MUTANT LIBRARY

Kp insertion mutants were created by introducing a kanamycin resistant (Kan^R) *Himar1* mariner transposon by mating MKP220 (*Kp* WT $Spec^R$) and the *E. coli* DAP auxotrophic MFDpir pSC189 (399). *Kp* transposon mutants were selected for on L+Kan (100 μg/mL) +Spec (50 μg/mL) agar plates. Individual colonies were picked using a Genetix QPExpression and inoculated into L+Kan (100 μg/mL) and 15% glycerol containing wells of 384-well plates (Corning). The resulting arrayed library consisting of 34-384 well-plates was incubated at 37°C overnight and stored at -80°C until further use. A total of 13,056 *Kp* insertion mutants were generated and arrayed from 3 independent rounds of 3 separate conjugations. To locate insertion mutants of interest in the array, a previously published combinatorial pooling method was used (400). First, the arrayed library was thawed and duplicated into L+Kan using the Genetix QPExpression robot and incubated at 37°C overnight without shaking. Then, a Tecan Freedom Evo robot pipetted up an aliquot from each of 13,000 wells and combined them into a series of 24 predetermined

pools. gDNA was then isolated from these 24 pools, processed, barcoded, and deep sequenced as described below. The location of each insertion mutant identified in the pools was then mapped to specific wells based on which pools the mutant was present. *Kp* insertion mutants of interest were then retrieved and confirmed by PCR, where one primer complemented one end of the transposon (MKP030 or MKP079) and the other insertion mutant specific primer complemented a site 600-800 bp downstream of the expected insertion site. Any resulting PCR products of the correct size were then sequenced to further verify the site of transposon insertion. Using this method, there was an approximately 80% success rate of a well identified as the location of a specific insertion mutant being confirmed as correct.

2.3 Mouse Infections and Treatment with Glyphosate

Mouse infections were carried out as previously described (177, 384, 401, 402). Briefly, for single strain and 1:1 competition infections, *Kp* strains were cultured individually overnight at 37°C with aeration in L. From the overnight cultures, bacteria were serially diluted in sterile PBS. The infectious dose was quantified by serial dilutions on L-agar plates. For infections with the arrayed library of 13,056 *Kp* insertion mutants, the arrayed plates were duplicated into L+Kan, incubated overnight at 37°C without shaking, pooled, aliquoted and stored at -80°C until use. Prior to infection, an aliquot was thawed, and plated on L+Kan to obtain >100,000 colonies and grown overnight at 37°C. The resulting colonies were collected, pooled and used to start an overnight culture in L that was grown at 37°C with aeration. The following morning, this culture was diluted and 20,000 CFU in 50 μL was delivered intranasally into WT and PMN-depleted mice. Either 7-8 week old female Swiss Webster mice (NCI for the TnSeq screen in Fig. 1 and Supp. Table 1,

and Taconic for all remaining experiments) and 7-10 week old gp91phox-/- (Jackson) with age and sex matched C57Bl/6 mice (Jackson) were anesthetized with 3% isoflurane and infected with a 50 μL bolus of the indicated CFU of *Kp*. Infection was intranasal for Swiss Webster mice and retropharyngeal for C57Bl/6 and gp91phox-/- mice. In experiments where the mice were treated with glyphosate, the mice were anesthetized as described above at 6 and 25 hpi, and 50 μL PBS alone or containing 0.37 mg of glyphosate (Santa Cruz) was delivered intranasally. At the indicated times after infection, the mice were sacrificed by CO_2 asphyxiation. Tissues were harvested into sterile PBS, weighed, and homogenized by pushing tissue through a 70μM cell strainer. The homogenates were plated in serial dilutions to determine the CFU/g lung. For screening of the library, approximately 10,000 colonies from the lungs were plated on 10-150 mM plates to collect at least 100,000 CFU per sample for analysis by deep sequencing. All mice were handled in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Tufts University.

2.4 Neutrophil depletion and quantification

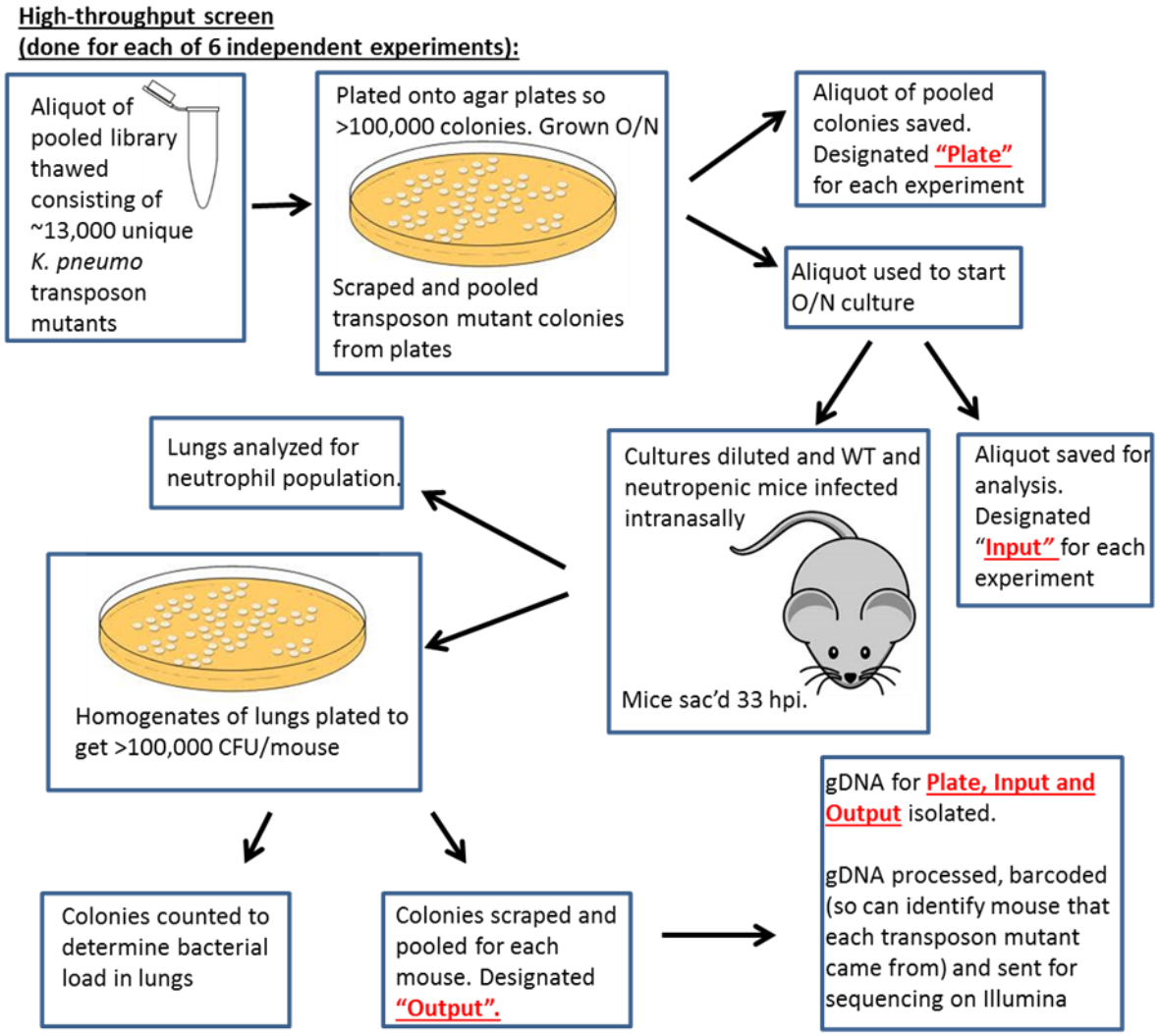
Mice were depleted of neutrophils as previously described (403) using an intraperitoneal injection of 200 μL αLy6G depletion antibody (0.5 mg/mL, IA8, Fisher Scientific) approximately 16 h prior to infection. Quantification of the neutrophil population in the lungs of both WT mice to measure neutrophil influx and PMN-depleted mice to confirm the efficacy of depletion was performed by staining lung homogenates with Ly6G PE-Cy7 (GR1, ebioscience) and CD11b-PE-Cy5 (ebioscience), quantifying on an LSRII (BD), and analyzing with FlowJo (version 10.0) by first gating on the live population and then the Ly6G(GR1)^{hi}CD11b^{+} population within the live population.

2.5 TnSeq screen in WT and PMN-depleted Swiss Webster mice

A schematic of the experimental set-up is presented in Figure 2-1. After infecting mice and collecting the bacteria from the lungs at 33 hpi as described above, the input bacteria and bacteria recovered from the lungs were lysed to obtain gDNA using a DNeasy Blood & Tissue kit (Qiagen). The obtained DNA was then processed as previously described (397). Briefly, the gDNA was sheared and C-tails were added to the 3' ends. PCR was used to amplify the fragments containing the transposon and immediately downstream using a primer on the 3' end of the transposon (MKP030) and a C-tail complementary primer (MKP028). A nested PCR reaction was then performed using a transposon specific primer (MKP036) and a primer with a different barcode for each sample. The concentration of DNA in in each of these completed PCR reactions was measured, and the samples were multiplexed in comparable concentrations. The samples were then deep sequenced (Illumina HiSeq), analyzed to obtain the frequency of each insertion mutant in each sample, aggregated by gene, and annotated against the *Kp* ATCC43816 genome (Accession No.: NZ_CP009208.1) using Galaxy (404). To obtain the fitness of each gene (i.e. insertion mutants aggregated by gene), the number of reads for all insertion mutants corresponding to each gene were combined and then normalized against the total reads for each sample. These values were then normalized against the size of the corresonding gene of interest (which roughly normalizes against the number of expected insertions per gene) compared to the rest of the genome to get a standardized read frequency for each gene using standardized read frequency=(# of reads for mutants with insertion in gene x/total # of reads in the sample)/size of gene x in bp/size of entire gene in bp) (397). The fitness was calculated for each gene within each sample using Fitness=$\text{Output}_{\text{Standardized read}}$

$_{\text{frequency of insertion mutants in a gene}}$/Input$_{\text{Standardized read frequency of insertion mutants in a gene}}$ and then the fitness values for each gene were averaged within each cohort to obtain the "WT Fitness Mean" and "PMN Dep Fitness Mean". A gene was considered to have a defect under in WT and PMN-depleted lungs if the "WT Fitness Mean" or "PMN Dep Fitness Mean" were less than the bottleneck in those mice (i.e. 0.27 and 0.43, respectively). To account for the different levels of variation observed in individual genes, the mean fitness of each gene in each cohort was adjusted using $\text{Mean}_{\text{Fitness of a Gene}} + t_{.99} * \text{SD}_{\text{Fitness of a Gene}} < \text{Mean}_{\text{Fitness of all Genes}}$ (i.e. "WT Fitness+2.43SD" and "PMN Dep Fitness+2.54SD"). The values for $t_{.99}$ of 2.43 and 2.54 for WT and PMN-depleted mice, respectively, were derived from a t-table based on the degrees of freedom (n-1, where n=38 for WT and n=20 for PMN-depleted mice). To account for the stochastic selection imposed by the bottleneck on the bacterial population, a gene was considered to have a statistically significant defect in each cohort only if the "WT Fitness+2.43SD" or "PMN Dep Fitness+2.54SD" was less than the corresponding bottleneck (i.e. $\text{Mean}_{\text{Fitness of all Genes}}$), which in WT and PMN-depleted mice was 0.27 and 0.43, respectively.

Figure 2- 1. Experimental Set-up of TnSeq Screen of *Kp* Transposon Insertion Mutant Library in WT and Neutropenic Lungs



2.6 Analysis of *Kp* insertion mutant mini-library *in vivo*

A mini-library made up of the indicated *Kp* insertion mutants **(Fig. 4-3C-D)** was assessed for fitness *in vivo.* The strains in this mini-library were cultured separately overnight in L at 37°C with shaking, then pooled so that 42% of the mini-library pool was comprised of 4 strains with transposon insertions at putative neutral sites (MKP330 and MKP437 each 15%, and MKP318 and MKP336 each 5.8% of the mini-library), and the other 58% comprised of the 10 indicated insertion mutants **(Fig. 4-3C-D)**. The mini-library was diluted in sterile PBS, and WT and PMN-depleted Swiss Webster mice were intranasally infected with 20,000 CFU. The inoculum was plated to obtain at least 10,000 colonies for analysis by deep sequencing and is referred to as the "input". At 33 hpi, the lungs were collected, the neutrophil population quantified, and the homogenate plated in serial dilutions to obtain CFU/g lung, as well as to collect >10,000 colonies/mouse for deep sequencing. To analyze the frequency of each specific insertion mutant in each experiment, colonies were collected and processed as described above to obtain gDNA and Illumina sequence. To obtain the fitness of each insertion mutant, the equation Fitness=Output frequency of insertion mutant X/Input frequency of insertion mutant X (Output/Input), where output values were compared to the input values from the same experiment.

2.7 Collection and Analysis of Broncheoalveolar Lavage Fluid by NMR

Swiss Webster mice were intranasally infected with 50 μL containing 20,000 CFU of *Kp* WT or 50 μL PBS as described above. At 32 hpi, the mice were sacrificed, and the

trachea was exposed and then an opening was made with a scalpel. Plastic tubing was inserted into the tracheal slit, and used to inject 1 mL of sterile saline into the lungs, which was then immediately collected. This was repeated 2 more times for a total of 3 mL. Methanol extraction was then performed on the BAL fluid to concentrate the sample and remove proteins. Briefly, a 2:1 volume of ice-cold methanol was added to each sample and the resulting dilution was incubated at 20°C for 20 min, and then centrifuged at 14k RPM for 4 min at 4°C to pellet the proteins. The supernatant was collected and then evaporated by spinning in a Speedvac overnight, leaving behind dried metabolites that were then resuspended in 600 μL D_2O. DSS, 1 μL, was then added to each sample to serve as a standard. All samples were analyzed by NMR (Bruker Spectrospin Ultrashield 600) and then peaks were assigned and the area of each peak quantified (Chenomx NMR Suite software 8.0).

2.8 Growth in M9+glucose, L, and Low-iron

To assess the ability *Kp* insertion mutants of interest to grow *in vitro*, the indicated *Kp* strains **(Fig. 3-3A-B** and **4-1A-B)** were cultured individually overnight in L at 37°C with aeration, and then pooled to create a mini-library. The mini-library was set up so that 50% was composed equally of mutants with insertions at neutral sites (MKP330, MKP332 and MKP354) and the other 50% was split by the remaining 59 insertion mutants listed in Table 1. The cultures were washed in M9+gluc, and then plated in serial dilutions to obtain DNA from inoculums, and also diluted 1:200 into 4 mL of M9+gluc, L or L containing 0.13 mM 2,2-bipyridyl (DIP; Sigma-Aldrich), an iron chelator *Kp* cannot use to acquire iron (405). The diluted samples were incubated for 5 hr at 37°C with aeration, and then plated in serial dilutions. The resulting colonies from the inoculums

and samples were collected for each sample (>10,000 colonies/sample), processed for gDNA, and deep sequenced as described above. Growth in M9+Gluc and growth in L were evaluated by comparing the frequency of each insertion mutant before ($Freq_{Inoc}$) and after ($Freq_{M9+gluc}$ or $Freq_{L}$) growth using Growth in M9+gluc=$Freq_{M9+gluc}/Freq_{Inoc}$ and Growth in L=$Freq_{L}/Freq_{Inoc}$. Growth in low iron was calculated by comparing the frequency of each insertion mutant after growth in L ($Freq_{-DIP}$) and in L with DIP ($Freq_{+DIP}$)=$Freq_{+DIP}/Freq_{-DIP}$. Several mutants were tested for growth in low iron in 1:1 competition assays against *Kp* WT $Spec^{R}$ (MKP220) paralleling the conditions of the screen above. Briefly, cultures were grown overnight in L at 37°C, which were then mixed at a 1:1 ratio by volume. The resulting 1:1 mixtures were then diluted into 4 mL L or L+0.26mM DIP, incubated at 37°C for 5 hr with shaking and then plated in serial dilutions on L and L+Kan plates to quantify bacteria. Growth in low iron was assessed by comparing the competitive index (CI) in L and L+DIP using Growth in Low Iron=((Kan^{R} CFU in L_{+DIP}/Kan^{S} CFU in L_{+DIP})/(Kan^{R} CFU in L_{-DIP} /Kan^{S} CFU in L_{-DIP})) and normalizing to the Neutral values.

2.9 GROWTH CURVES OF SINGLE STRAINS

To test the ability of single strains to grow in different conditions, each strain was grown overnight in L broth at 37°C with aeration and diluted into M9+glucose or L media. Amino acid and metabolites were added to the media at a final concentration of 10 mM from a stock solution of the indicated amino acids (Sigma) diluted in M9+gluc. Low iron concentrations were induced by adding the indicated final concentrations of the iron chelator 2,2-bipyridyl (DIP; Sigma-Aldrich) from a stock diluted in L. Glyphosate was dissolved in M9+gluc and added to obtain the indicated final concentrations. The samples

were transferred into a 96-well plate, incubated at 37°C for 20 hr with shaking, and the OD_{600} was measured every 15 min in a plate reader (Biotek Synergy HT). The resulting values were corrected for background and path-length.

2.10 Quantification of mucoviscosity

Mucoviscosity was quantified as previously described with modifications (384). The indicated *Kp* strains were grown overnight in L at 37°C with aeration. For each culture, 1 mL was transferred to an Eppendorf and centrifuged at 14k RPM for 4 min. The supernatant was removed and the pellets were resuspended in 1 mL PBS. The OD_{600} of each sample was measured (OD_{600} Total), then the samples were centrifuged at 1,000g for 5 min, and the OD_{600} of the supernatant was measured (OD_{600} Supernatant). Mucoviscosity=OD_{600} Total/OD_{600} Supernatant and was normalized to *Kp* WT for each experiment.

2.11 Evaluation of capsule by microscopy

For each of the indicated *Kp* strains, 0.5 mL of overnight culture was centrifuged at 14k RPM for 2 min. The supernatant was removed and the pellet was resuspended in 200 μL PBS. India ink, 10uL, was placed on a slide and was mixed with 5 uL of each bacterial suspension. The mixture was spread across the slide and sandwiched using a cover slip. Pressure was applied to remove extraneous liquid and bind bacteria between the slide and cover slip. The bacteria were visualized at 40X on bright-field with Hoffman's modulation for phase contrast (Nikon Eclipse TE2000-U microscope) and imaged using Nikon DS-LI software.

2.12 H_2O_2, HOCL AND NO RESISTANCE ASSAYS

The indicated *Kp* strains were cultured individually overnight in L at 37°C with aeration. When screening the mini-library, the overnight cultures were pooled by volume as described, diluted 1:50 in L, incubated for 2 hr at 37°C with aeration, washed with M9+gluc twice, and resuspended in an equivalent volume of M9+gluc. For 1:1 competition assays, the overnight cultures of the strains were individually diluted 1:50 in L, incubated for 2 hr at 37°C with aeration, washed with M9+gluc twice, resuspended in an equivalent volume of M9+gluc, and then pooled at a 1:1 ratio by volume. Two hundred μL of the resulting pools was added to 5 mL M9+gluc, M9+gluc with 1 mM H_2O_2 (CVS), 80 μM HOCl (Sigma-Aldrich) or 1 mM DETA-NONOate (Cayman chemical), incubated at 37°C with aeration for 1 hr, plated on L and/or L+Kan plates in serial dilutions, and then quantified. For the screens, greater than 10,000 colonies per sample were collected, processed for gDNA, and deep sequenced as described above. The H_2O_2, HOCl and NO resistance of each insertion mutant was calculated using H_2O_2 Resistance=$Freq_{+H2O2}/Freq_{-H2O2}$, HOCl Resistance=$(CI_{+HOCl})/(CI_{-HOCl})$ and NO Resistance=$(CI_{+NO})/(CI_{-NO})$, where CI=CFU of Kan^R insertion mutant/CFU of Kan^S *Kp* WT.

2.13 SPONTANEOUS RESISTANCE TO AND PLATING EFFICIENCY ON GLYPHOSATE

The frequency of spontaneous resistance to glyphosate and plating efficiency of the indicated strains was measured by culturing the WT $Spect^R$ *Kp* strain (MKP220) in 10 mL or 1 mL of L, respectively, overnight with shaking at 37°C. After 20-24 hr, the cultures were centrifuged at 14k RPM, washed twice in M9+gluc, resuspended in

M9+gluc, and plated in serial dilutions on M9+gluc or M9+gluc containing the indicated concentrations of glyphosate. The plates were incubated at 37°C overnight, and visible colonies were enumerated after approximately 24, 48 and 72 hr. The frequency of spontaneous resistance and plating efficiency were calculated using $CFU_{+glyphosate}$/ $CFU_{-glyphosate}$.

2.14 Statistical Analysis

Statistics were done for each experiment as described in the figure legends. All statistical analyses were performed using GraphPad Prism 7.01 (GraphPad Software; La Jolle, CA). A $p<0.05$ was considered significant, where ns=not significant, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, and ****$p<0.0001$.

Table 2- 1. Strain List

Strain	Description	Parental Strain	Source
E.coli			
MKP201	SM10λpir *Himar1* Tn #1		(399)
MKP214	MFDpir-pDL1098 mTn10-Spec^{R}		(397)
MKP215	MFDpir		(406)
MKP216	MFDpir+pSC189 *Himar1* Tn-Kan^{R}	MKP215	This study
MKP282	TOP10+pACBSR-Hygro^{R}		(398)
MKP284	TOP10+pFLP-Hygro^{R}		(398)
MKP286	TOP10+pIJ773-Apra^{R}		(398)
Klebsiella pneumoniae (Kp)			
MKP203	*Kp* WT		ATCC 43816
MKP220	*Kp* WT (Spec^{R})	MKP203	This study
MKP299	*Kp glpR*	MKP220	This study
MKP300	*Kp gltB*	MKP220	This study
MKP302	*Kp leuA*	MKP220	This study
MKP303	*Kp argO*	MKP220	This study
MKP304	*Kp adhE*	MKP220	This study
MKP306	*Kp* (Intergenic Tn)	MKP220	This study
MKP309	*Kp aqpZ*	MKP220	This study
MKP312	*Kp yigM*	MKP220	This study
MKP313	*Kp citF*	MKP220	This study
MKP315	*Kp leuB*	MKP220	This study
MKP317	*Kp trpS*	MKP220	This study
MKP318	*Kp cbpM*	MKP220	This study
MKP319	*Kp lysA*	MKP220	This study
MKP321	*Kp* (Intergenic Tn)	MKP220	This study
MKP322	*Kp VK055_RS17445*	MKP220	This study
MKP324	*Kp sdhB*	MKP220	This study
MKP325	*Kp yhjH*	MKP220	This study
MKP330	*Kp livG*	MKP220	This study
MKP330	*Kp livG*	MKP220	This study
MKP332	*Kp VK055_RS09515*	MKP220	This study
MKP332	*Kp VK055_RS09515*	MKP220	This study
MKP336	*Kp hisJ (upstream)*	MKP220	This study
MKP339	*Kp efeB*	MKP220	This study
MKP353	*Kp ubiH*	MKP220	This study

MKP354	*Kp livG*	MKP220	This study
MKP354	*Kp livG*	MKP220	This study
MKP359	*Kp tyrA*	MKP220	This study
MKP380	*Kp ΔcpsB*	MKP220	This study
MKP383	*Kp yajL*	MKP220	This study
MKP384	*Kp eptA*	MKP220	This study
MKP387	*Kp ybhU*	MKP220	This study
MKP388	*Kp VK055_RS25040*	MKP220	This study
MKP389	*Kp rcsD*	MKP220	This study
MKP390	*Kp nuoC*	MKP220	This study
MKP391	*Kp dedA*	MKP220	This study
MKP392	*Kp alaC*	MKP220	This study
MKP393	*Kp gntR*	MKP220	This study
MKP397	*Kp yaaA*	MKP220	This study
MKP399	*Kp yaeQ*	MKP220	This study
MKP400	*Kp rcsF*	MKP220	This study
MKP401	*Kp hlyD*	MKP220	This study
MKP402	*Kp seqA (upstream)*	MKP220	This study
MKP403	*Kp appC (upstream)*	MKP220	This study
MKP404	*Kp ycgE*	MKP220	This study
MKP405	*Kp aroA*	MKP220	This study
MKP406	*Kp gabD*	MKP220	This study
MKP407	*Kp putA*	MKP220	This study
MKP408	*Kp trpE*	MKP220	This study
MKP409	*Kp VK055_RS05435*	MKP220	This study
MKP411	*Kp VK055_RS26085*	MKP220	This study
MKP413	*Kp purR*	MKP220	This study
MKP414	*Kp rnfA*	MKP220	This study
MKP415	*Kp abgB*	MKP220	This study
MKP419	*Kp nadB*	MKP220	This study
MKP421	*Kp queF (upstream)*	MKP220	This study
MKP424	*Kp lsrR*	MKP220	This study
MKP427	*Kp outO*	MKP220	This study
MKP429	*Kp ptsG (upstream)*	MKP220	This study
MKP430	*Kp dsbA*	MKP220	This study
MKP431	*Kp araC (upstream)*	MKP220	This study
MKP432	*Kp menA (upstream)*	MKP220	This study
MKP435	*Kp metA*	MKP220	This study
MKP437	*Kp ppa (upstream)*	MKP220	This study
MKP438	*Kp celA (upstream)*	MKP220	This study

MKP439	*Kp serB*	MKP220	This study
MKP453	*Kp* Δ*aroA*	MKP203	This study
MKP460	*Kp* Δ*aroA ::aroA*	MKP203	This study
MKP466	*Kp* SR5	MKP220	This study
MKP467	*Kp* SR6	MKP220	This study
MKP468	*Kp* SR7	MKP220	This study
MKP469	*Kp* SR8	MKP220	This study
MKP470	*Kp* SR9	MKP220	This study
MKP471	*Kp* SR10	MKP220	This study
MKP472	*Kp* SR11	MKP220	This study
MKP473	*Kp* SR12	MKP220	This study
MKP474	*Kp* SR13	MKP220	This study
MKP475	*Kp* SR14	MKP220	This study
MKP476	*Kp* SR15	MKP220	This study
MKP477	*Kp* SR16	MKP220	This study
MKP478	*Kp* SR17	MKP220	This study
MKP479	*Kp* SR18	MKP220	This study
MKP480	*Kp* SR19	MKP220	This study
MKP481	*Kp* SR20	MKP220	This study
MKP482	*Kp* Δ*aroA*::*aroAB3*	MKP220	This study
MKP485	*Kp* Δ*aroA*::*aroAB19*	MKP220	This study
***Kp* Clinical Isolates**			
B1	Non-ESBL/KPC Blood Isolate		This study
B2	Non-ESBL/KPC Blood Isolate		This study
B3	Non-ESBL/KPC Blood Isolate		This study
B4	Non-ESBL/KPC Blood Isolate		This study
B5	Non-ESBL/KPC Blood Isolate		This study
B6	KPC Blood Isolate		This study
B7	Non-ESBL/KPC Blood Isolate		This study
B8	Non-ESBL/KPC Blood Isolate		This study
B9	Non-ESBL/KPC Blood Isolate		This study
B10	Non-ESBL/KPC Blood Isolate		This study
B11	Non-ESBL/KPC Blood Isolate		This study
B12	Non-ESBL/KPC Blood Isolate		This study
B13	Non-ESBL/KPC Blood Isolate		This study
B14	Non-ESBL/KPC Blood Isolate		This study
B15	Non-ESBL/KPC Blood Isolate		This study
B16	KPC Blood Isolate		This study
B17	ESBL Blood Isolate		This study
B18	KPC Blood Isolate		This study

B19	Non-ESBL/KPC Blood Isolate		This study
B20	ESBL Blood Isolate		This study
R1	ESBL Respiratory Isolate		This study
R2	KPC Respiratory Isolate		This study
R3	ESBL Respiratory Isolate		This study
R4	KPC Respiratory Isolate		This study
R5	KPC Respiratory Isolate		This study
R6	ESBL Respiratory Isolate		This study
R7	KPC Respiratory Isolate		This study
R8	ESBL Respiratory Isolate		This study
R9	ESBL Respiratory Isolate		This study
R10	KPC Respiratory Isolate		This study
R11	ESBL Respiratory Isolate		This study
R12	ESBL Respiratory Isolate		This study
R13	KPC Respiratory Isolate		This study
R14	KPC Respiratory Isolate		This study
R15	ESBL Respiratory Isolate		This study
R16	KPC Respiratory Isolate		This study
R17	KPC Respiratory Isolate		This study
R18	ESBL Respiratory Isolate		This study
R19	ESBL Respiratory Isolate		This study
R20	KPC Respiratory Isolate		This study

Table 2- 2. Primer List

Name	**Primer Sequence**	**Direction**	**Description**
MKP028	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG	rev/com	C-tail complementary primer for Illumina
MKP030	GAGCGGGACTCTGGGGTAC	for	*Himar1* transposon 3' end.
MKP036	AATGATACGGCGACCACCGAGATCTACACTCTTTGACCGGGGACTTATCATCCAACCTGTTA	for	Illumina Processing
MKP037	ACACTCTTTGACCGGGGACTTATCATCCAACCTGTTA	for	Illumina Sequencing primer *Himar1* transposon 5' end

MKP079	CTTCAAGCTTGTCATCGTCATC	rev/com	
MKP083	CACATTACCACCAGCCAG	rev/com	Confirmation primer for strain MKP315
MKP084	CTTACGTGATGGCGAACAAG	rev/com	Confirmation primer for strain MKP302
MKP085	CATAGAAGTGGCGGTCTTC	rev/com	Confirmation primer for strain MKP313
MKP089	GTCCATCTGCTGTGGATAG	rev/com	Confirmation primer for strain MKP332
MKP089	GTCCATCTGCTGTGGATAG	rev/com	Confirmation primer for strain MKP332
MKP090	TGAGGCATCCTTCGCCAG	rev/com	Confirmation primer for strain MKP332
MKP093	CTCCGGGATGGCGATTATC	rev/com	Confirmation primer for strain MKP309
MKP094	CAAGTGTAGGAGACTATCGGTC	rev/com	Confirmation primer for strain MKP339
MKP095	GTCGACATCTTTGACAGCGAC	rev/com	Confirmation primer for strain MKP325
MKP096	GACGCAGTTACACACCTG	rev/com	Confirmation primer for strain MKP304
MKP099	CAGCTTTCGCCGAGTAC	rev/com	Confirmation primer for strain MKP321
MKP103	GTAGTCCCAGACAAACTGCGAATAC	rev/com	Confirmation primer for strain MKP336
MKP109	GAAATGGCCTCTGGCCTG	rev/com	Confirmation primer for strain MKP359
MKP111	CTGTTTGATGGCGGCAAG	rev/com	Confirmation primer for strain MKP319
MKP113	CAGCCGTATGAGCAGATAG	rev/com	Confirmation primer for strain MKP353

MKP114	CTGTTCGCCAACCGTG	rev/com	Confirmation primer for strain MKP307
MKP115	CTGGCGAGTATGTCGAC	rev/com	Confirmation primer for strain MKP303
MKP117	GATGCTGTTCATCGCTTC	rev/com	Confirmation primer for strain MKP300
MKP120	GATTCGCGCAATGATATTCAG	rev/com	Confirmation primer for strain MKP317
MKP122	CATCGCGGTGTTCATC	rev/com	Confirmation primer for strain MKP299
MKP124	GAGATCGTCTCGCTGATC	rev/com	Confirmation primer for strain MKP330
MKP125	CACCAGCTGATATTCACTGTTG	rev/com	Confirmation primer for strain MKP322
MKP130	GATCGAAGTGGCGGTG	rev/com	Confirmation primer for strain MKP306
MKP132	GAAAGCGATCTGCGGTATC	rev/com	Confirmation primer for strain MKP312
MKP133	CTGGCCTCAGAATATATCTGGGTGTTTAATAACAGGGTTTTAATTAAAGGATATTAATATGATTCCGGGGATCCGTCGACC	for	*cpsB* lambda red recombination
MKP134	GATGTCATAAGCCTTAAAGCATGTTAACTGTGTCATTCTGCGATTTGTCCCGAAAAAATTATGTAGGCTGGAGCTGCTTC	rev/com	*cpsB* lambda red recombination
MKP135	GTATTCCATACCGAGTGGTTG	for	Confirmation primer for *cpsB*
MKP136	GTGGCGAAGTAAATCTCTTCCGTG	rev/com	Confirmation primer for *cpsB*
MKP137	CAGCTTGCTGTCTTTGATC	rev/com	Confirmation primer for strain MKP383

MKP138	GTCACCGACCTGAAGCTC	rev/com	Confirmation primer for strain MKP384
MKP141	CAAAGTCGTTAGCCGTG	rev/com	Confirmation primer for strain MKP387
MKP142	GTTAACCCACCCGCTG	rev/com	Confirmation primer for strain MKP388
MKP143	CAGCAGGCGAGAGACG	rev/com	Confirmation primer for strain MKP389
MKP144	GTACGACATCTATTCCGTCGTC	rev/com	Confirmation primer for strain MKP390
MKP145	GTATCTGGTGTCGGTGGAT	rev/com	Confirmation primer for strain MKP391
MKP146	GTTCGAAAGAGGGGCTG	rev/com	Confirmation primer for strain MKP392
MKP147	GACCATCAACGGTACCAAAG	rev/com	Confirmation primer for strain MKP393
MKP151	GTTAGCCTGCCTGTCGTC	rev/com	Confirmation primer for strain MKP397
MKP152	CTTTCGATGCCCGAGTC	rev/com	Confirmation primer for strain MKP399
MKP153	CTGGTGGCTGTCGGTAAC	rev/com	Confirmation primer for strain MKP400
MKP154	GACGACGGTGTTCTCCAG	rev/com	Confirmation primer for strain MKP401
MKP155	GATTGCCATTGCCTGATC	rev/com	Confirmation primer for strain MKP402
MKP156	CAGACCTACAAAGGTGGATTC	rev/com	Confirmation primer for strain MKP403
MKP157	CATTTATCAGATCCGGCTG	rev/com	Confirmation primer for strain MKP404

MKP158	GTCGGCGAAACGAATG	rev/com	Confirmation primer for strain MKP405
MKP159	GAAGAGCTGACTGACGATC	rev/com	Confirmation primer for strain MKP406
MKP160	CAGGTAGTGATCGACGTG	rev/com	Confirmation primer for strain MKP407
MKP161	GAAATCTCGACGCCGTTC	rev/com	Confirmation primer for strain MKP408
MKP162	CAAGGCTTAACTGAGCAAG	rev/com	Confirmation primer for strain MKP409
MKP163	CACGTTCTGGAAGCAATG	rev/com	Confirmation primer for strain MKP411
MKP164	CAGCAGCCCTGTAACTTAAC	rev/com	Confirmation primer for strain MKP413
MKP165	CTCATCACGGTATGCATG	rev/com	Confirmation primer for strain MKP414
MKP166	GTTTGGGCTGCAGGTG	rev/com	Confirmation primer for strain MKP415
MKP169	GTGCTGCTGGCTTTCTC	rev/com	Confirmation primer for strain MKP419
MKP171	CTCGCGGTCGATTTTC	rev/com	Confirmation primer for strain MKP421
MKP174	GTTGTCTCTTCCGTTACCAG	rev/com	Confirmation primer for strain MKP424
MKP176	CTGACGTGACTGGCAC	rev/com	Confirmation primer for strain MKP427
MKP178	CAGCTTCACCGGCATATAG	rev/com	Confirmation primer for strain MKP429
MKP179	GTACCCTTCATTGGCCTC	rev/com	Confirmation primer for strain MKP430
			Confirmation primer for

MKP180	GAAACGCTCCAGAACAAAG	rev/com	strain MKP431
MKP181	GTAAACGAAACCACCGAG	rev/com	Confirmation primer for strain MKP432
MKP183	GTCTCCGCGAGGATCTC	rev/com	Confirmation primer for strain MKP435
MKP185	CAATCAGCTGGCCTTC	rev/com	Confirmation primer for strain MKP437
MKP186	CTCCAGCCCTTTATGTTTC	rev/com	Confirmation primer for strain MKP438
MKP187	GTTTGTCGCGCAGATAC	rev/com	Confirmation primer for strain MKP439
MKP189	TCCTCCCCGCGGCCTGTCTGCGGG GTTTTTATTCTGTTTGTTTGAGAGT GAAGTTTGATGATTCCGGGGATCC GTCGACC	for	*aroA* lambda red recombination
MKP190	TTTGCGCAATTATAGCGGGCTGAC AGGACATCAGCCCGCGGGATGAC AAAGTTTTACTCATGTAGGCTGGA GCTGCTTC	rev/com	*aroA* lambda red recombination
MKP191	CTTCCATCTACAACGCCATG	for	Confirmation primer for *aroA*
MKP192	CAATACGCCGGAAAGTGAG	rev/com	Confirmation primer for *aroA*

Table 2- 3. Barcode Primer List

Name	Barcode	Primer sequence
BC33	ATCACG	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC34	CGATGT	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC35	TTAGGC	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT

BC36	TGACCA	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC37	ACAGTG	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC38	GCCAAT	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC39	CAGATC	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC40	ACTTGA	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC41	GATCAG	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC42	TAGCTT	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC43	GGCTAC	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC44	CTTGTA	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCT
BC45	AGTCAACA	CAAGCAGAAGACGGCATACGAGATTGTTGACTGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC46	AGTTCCGT	CAAGCAGAAGACGGCATACGAGATACGGAACTGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC47	ATGTCAGA	CAAGCAGAAGACGGCATACGAGATTCTGACATGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC48	CCGTCCCG	CAAGCAGAAGACGGCATACGAGATCGGGACGGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC49	GTCCGCAC	CAAGCAGAAGACGGCATACGAGATGTGCGGACGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC50	GTGAAACG	CAAGCAGAAGACGGCATACGAGATCGTTTCACGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC51	GTGGCCTT	CAAGCAGAAGACGGCATACGAGATAAGGCCACGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC52	GTTTCGGT	CAAGCAGAAGACGGCATACGAGATACCGAAACGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC53	CGTACGTA	CAAGCAGAAGACGGCATACGAGATTACGTACGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC54	GAGTGGAT	CAAGCAGAAGACGGCATACGAGATATCCACTCGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC55	ACTGATAT	CAAGCAGAAGACGGCATACGAGATATATCAGTGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT

BC56	ATTCCTTT	CAAGCAGAAGACGGCATACGAGATAAAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC57	CAACTA	CAAGCAGAAGACGGCATACGAGATTAGTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC58	CACCGG	CAAGCAGAAGACGGCATACGAGATCCGGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC59	CACGAT	CAAGCAGAAGACGGCATACGAGATATCGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC60	CACTCA	CAAGCAGAAGACGGCATACGAGATTGAGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC61	CAGGCG	CAAGCAGAAGACGGCATACGAGATCGCCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC62	CATGGC	CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC63	CGGAAT	CAAGCAGAAGACGGCATACGAGATATTCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC64	CTAGCT	CAAGCAGAAGACGGCATACGAGATAGCTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC65	CTATAC	CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC66	CTCAGA	CAAGCAGAAGACGGCATACGAGATTCTGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC67	TAATCG	CAAGCAGAAGACGGCATACGAGATCGATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC68	TACAGC	CAAGCAGAAGACGGCATACGAGATGCTGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC69	TCATTC	CAAGCAGAAGACGGCATACGAGATGAATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC70	TCCCGA	CAAGCAGAAGACGGCATACGAGATTCGGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC71	TCGAAG	CAAGCAGAAGACGGCATACGAGATCTTCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC72	TCGGCA	CAAGCAGAAGACGGCATACGAGATTGCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC73	TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC74	TAGATCGC	CAAGCAGAAGACGGCATACGAGATGCGATCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
BC75	CGTCTTAG	CAAGCAGAAGACGGCATACGAGATCTAAGACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

BC76	CTGACAGT	CAAGCAGAAGACGGCATACGAGATACTGTCAGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC77	AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC78	TATCCTCT	CAAGCAGAAGACGGCATACGAGATAGAGGATAGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC79	TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC80	AGAGTAGA	CAAGCAGAAGACGGCATACGAGATTCTACTCTGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC81	GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC82	GTAAGGAG	CAAGCAGAAGACGGCATACGAGATCTCCTTACGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC83	TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC84	ACTGCATA	CAAGCAGAAGACGGCATACGAGATTATGCAGTGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC85	CTCTCTAC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC86	AAGGAGTA	CAAGCAGAAGACGGCATACGAGATTACTCCTTGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC87	CAGAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC88	CTAAGCCT	CAAGCAGAAGACGGCATACGAGATAGGCTTAGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC89	GCTACGCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC90	CGAGGCTG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTGACT GGAGTTCAGACGTGTGCTCTTCCGATCT
BC91	AAGAGGCA	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT
BC92	GTAGAGGA	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTGACTG GAGTTCAGACGTGTGCTCTTCCGATCT

CHAPTER 3: HIGH-THROUGHPUT IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF *KLEBSIELLA PNEUMONIAE* VIRULENCE DETERMINANTS IN THE LUNGS

3.1 High-throughput identification of *Kp* genes necessary for virulence in the lungs of WT and neutropenic mice using TnSeq

In order to identify virulence determinants that *Kp* requires to colonize and persist during pneumonic infection, a high-throughput genetic approach called TnSeq was used (397, 399). A library of *Kp* transposon insertion mutants was generated by introducing the *Himar-1* mariner transposon into a $Spec^R$ derivative of the mouse virulent *Kp* strain ATCC 43816, which was verified to be as fit as the parental strain in both *in vitro* and *in vivo* in the lungs of WT mice (data not shown). The resulting 13,056 *Kp* transposon insertion mutants were arrayed, pooled, and then 20,000 CFU was intranasally delivered into Swiss Webster mice (177, 401, 402). This out-bred mouse strain was used because it is more genetically diverse than classic in-bred strains of mice and so attenuated mutants would be unlikely to have mouse strain specific defects. In addition, because *Kp* pneumonias are typically opportunistic infections that occur in hosts that are immunosuppressed, as well as the previously documented importance of neutrophils in controlling *Kp* in the lungs (29, 141, 165-167, 175), neutropenic Swiss Webster mice were also intranasally infected with 20,000 CFU of the *Kp* library concurrently with the WT mice.

At 33 hpi, the lungs were collected and analyzed for bacterial load and neutrophil population **(Fig. 3-1A-B).** A bacterial load of approximately 10^9 CFU/g lung was recovered from both cohorts **(Fig. 3-1B)** and a large neutrophil influx was observed in WT mice as neutrophils ($Ly6G(GR1)^{hi}CD11b^+$) made up ~60% of the cells in the lungs compared to ~5-15% in uninfected mice (**Fig. 3-1B** and data not shown). Neutrophil depletion using an anti-Ly6G(IA8) antibody (403) was effective, resulting in a

diminished population of approximately 7% of the lung cells **(Fig 3-1B)**. To limit confounding variables within cohorts, such as drastic differences in bacterial load or neutrophil population, cut-offs were set and the mice that were analyzed were those with $1x10^8$-$2x10^{10}$ CFU/g lung and more than 40% PMNs in the WT mice and less than 20% in neutropenic **(Fig 3-1A-B)**. Thirty-eight WT and 20 PMN-depleted mice fit within these cut-offs and were included in our analyses **(Fig. 3-1A-B)**. Deep-sequencing of the infection inoculums, "inputs", and bacteria recovered from the lungs following infection, "outputs", was performed and the identified insertion mutants were annotated using the sequenced genome for ATCC 43816, i.e. KPPR1, (384) a Rif^R version of ATCC 43816, and aggregated by gene. A comparison of the frequency of transposon insertion mutants by gene revealed that the libraries used in each experiment, where 4 of the 6 inputs used to infect WT mice were also used to infect PMN-depleted mice, were highly reproducible with a mean Spearman rho of 0.953 **(Fig. 3-1C)**. An average of 11,975 and 11,861 insertion mutants representing 3,722 and 3,691 genes were recovered from the WT and PMN-depleted inoculums, respectively **(Fig. 3-1D-E).** The annotated parental *Kp* strain used in this study has 5,247 genes, and, thus, the library used in this study covered approximately 71% of the *Kp* genome. An average of 3,276 and 5,147 insertion mutants representing 1,593 and 2,060 genes were recovered from the lungs of WT and PMN-depleted mice, respectively **(Fig. 3-1D-E)**. Based on previous observations that neutrophils control *Kp* infection and/or the outgrowth of the seeded bacteria (141, 165-167), this difference in the number of insertion mutants recovered between WT and PMN-depleted outputs was likely due to a more stringent bottleneck (i.e. the loss of infecting clones due to stochastic reasons) in the WT mice.

In order to identify which genes were significantly underrepresented in the output populations, the proportion of all insertion mutants corresponding to each gene combined in the outputs was compared to their proportion in the inputs to yield a fitness value for each gene. The average fitness values in WT and PMN-depleted mice were Log_2 transformed and plotted, which resulted in normal distributions with means of 0.27 and 0.43, respectively (**Fig. 3-1F-G).** The mean fitness of the entire population of genes evaluated under each condition is equivalent to the bottleneck. Therefore, with our infection parameters of a pool of 20,000 CFU consisting of 13,056 transposon mutants, on average 27% of clones colonized the lungs in each WT mouse, while the PMN-depleted mice had a less restrictive bottleneck that allowed 43% of clones to colonize mice at 33 hpi **(Fig. 3-1F-G).** To account for the selective pressure incurred by the bottlenecks, a neutral fitness value was considered 0.27 and 0.43 in WT and PMN-depleted mice, respectively, where a gene was considered underrepresented following infection if the fitness of insertion mutants in this gene was lower than this value. We identified genes that we were 99% confident corresponding insertion mutants had a defect in colonization by first taking into account the variation in fitness by gene using the $Mean_{Fitness\ of\ a\ Gene}+(t_{.99}*SD_{Fitness\ of\ a\ Gene}) < Mean_{Fitness\ of\ all\ Genes}$ **(Table 3-1),** and then considering the resulting values for each gene that fell below the previously described bottleneck values of 0.27 and 0.43 for WT and PMN-depleted mice, respectively, to be significant. Using this method, 166 genes were required for virulence in WT mice and 194 in PMN-depleted mice **(Fig. 3-1H-J, Table 3-1).** Of the genes defective for growth in WT mice, 51 were also clearly defective in PMN-depleted mice, while the virulence of 52 were clearly restored upon neutrophil depletion, suggesting a role in protecting *Kp*

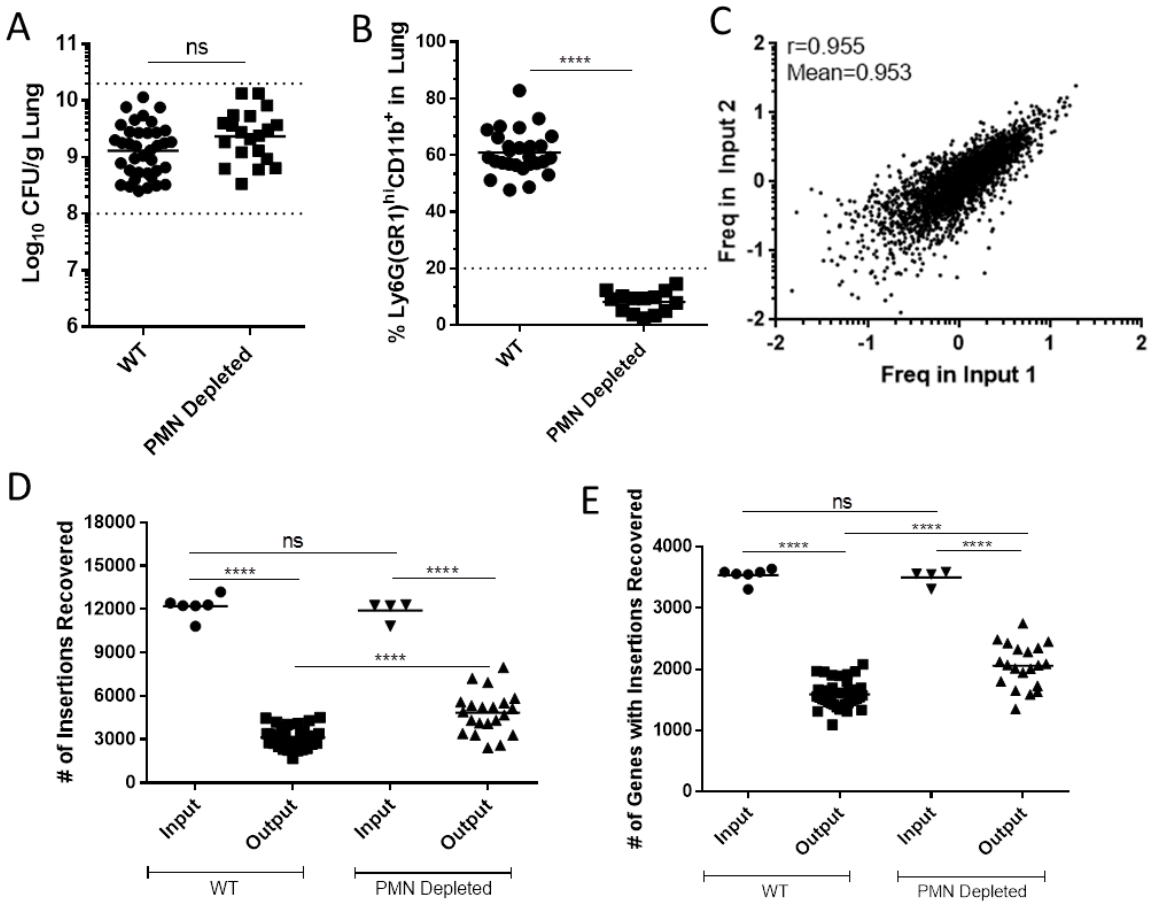
against neutrophils or a neutrophil-modified environment **(Table 3-1)**. The remaining 62 genes had a borderline fitness defect, where insertion mutants in these genes had a putative fitness defect based on a fitness <0.43, but were not statistically significant as the $Mean_{Fitness\ of\ a\ Gene}+(t_{.99}*SD_{Fitness\ of\ a\ Gene})>0.43$. Finally, 1 gene was not in the inoculum pools in the PMN-depleted mice so could not be evaluated. Importantly, several genes with functions critical for virulence factors identified in previous studies were identified here, including for capsule (e.g. *manC),* LPS (e.g. *pagP* and *wzzE*) and siderophores (e.g. *fepG*) **(Table 3-1)** (231, 272, 290, 340, 347).

To provide a broad overview of the putative functions of the genes necessary for *Kp* virulence in the lungs of WT mice, the protein products of genes with fitness defects in WT mice were matched to cluster of orthologous groups (COGs) **(Fig. 3-2).** The majority of these genes appear to have functions that are metabolic, such as in the transport and metabolism of amino acids, inorganic ions and carbohydrates, as well as those related to energy production and conversion (COGs E, G, P and C, respectively). A large number of genes were also involved in transcription and translation (COG J). The functional categories of the 52 genes needed to infect WT mice that were no longer necessary once neutrophils were depleted were also identified. Of these genes, a number were involved in carbohydrate metabolism (COG G), which includes genes needed for synthesizing capsule, such as *manC* (231). Interestingly, all 3 of the replication, recombination and repair genes (COG L) were required only in the presence of neutrophils, suggesting these genes are involved in the bacterial response to damage incurred by neutrophils.

Figure 3- 1. High-Throughput *in vivo* screen to identify *Kp* virulence determinants needed to infect the lungs of healthy and neutropenic hosts

(A-J) WT and PMN-depleted Swiss Webster female mice were intranasally infected with 20,000 CFU of a *Kp* library containing ~13,000 transposon insertion mutants. The mice were sacrificed at 33 hpi, and the lungs were collected for analysis. (A) The CFU/g Lung was quantified using serial dilutions on L-agar plates and (B) the neutrophil population (% $Ly6G^{hi}CD11b^{+}$ cells within the live population) was measured using flow-cytometry. (A-B) Dotted lines indicate the cut-offs for mice to be included for further analysis. The bacteria used to infect the mice, "inputs", and bacteria recovered from the lungs, "outputs" were enumerated and deep sequenced. (C) After deep sequencing, reproducibility between all inputs was assessed by calculating Spearman's correlation efficient, which averaged 0.934 and ranged from 0.9374 to 0.9657 with a $p<0.001$. A representative plot of inputs from 2 independent experiments is presented. (D-E) The number of *Kp* (D) individual insertion mutants and (E) genes represented by aggregated insertion mutants in the "inputs" and "outputs" of each cohort. (A-B, D-E) Statistical significance was determined using One-way ANOVA with Dunnet's post-test for the indicated comparisons. (F-G) Histograms of the distribution of the $\log_2$-transformed fitness of *Kp* insertion mutants by gene in (F) WT mice and (G) PMN depleted mice, where fitness was calculated for each gene using Fitness=Output frequency of insertion mutants by gene/Input frequency of insertion mutants by gene. (H-I) Genes were considered to have statistically significant fitness defects in (H) WT and (I) PMN depleted mice if a gene (i.e. all insertion mutants in a gene combined) had a $Mean_{\text{Fitness of a Gene}}+2.43\ SD_{\text{Fitness of a Gene}}<0.27$ (i.e. $Mean_{\text{Fitness of all Genes in WT}}$) or a $Mean_{\text{Fitness}}+2.54\ SD_{\text{Fitness}}<0.43$ (i.e. $Mean_{\text{Fitness of all Genes in PMN depleted}}$), respectively. The cut-off for a

significant fitness defect was considered the bottleneck in each condition and is indicated by a dotted line, where all genes with adjusted fitness values that fall to the left of this cut-off are considered to have a statistically significant defect under that condition. (J) Genes with a statistically significant defect in WT mice were categorized based on their fitness in PMN depleted mice, where "Defect in PMN Depleted" refers to a statistically significant defect as described above, "Borderline in PMN Depleted" refers to genes that had a mean fitness <0.43, but were not statistically significant, and "Rescued in PMN Depleted" refers to genes with a mean fitness of >0.43. "Unknown" refers to the gene represented in the WT inputs, but not the PMN depleted inputs.



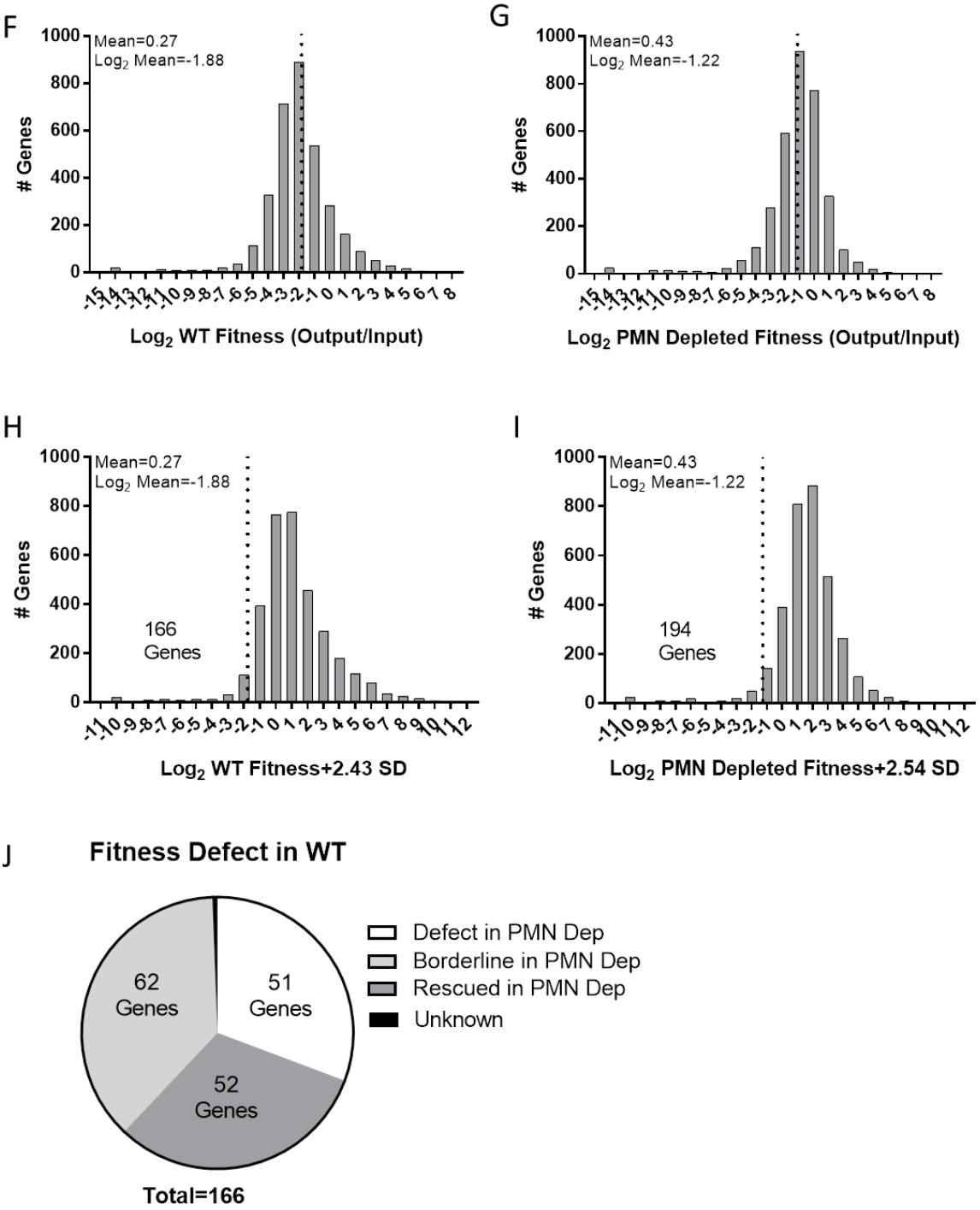

F
Mean=0.27
Log2 Mean=-1.88
Genes
Log2 WT Fitness (Output/Input)
G
Mean=0.43
Log2 Mean=-1.22
Genes
Log2 PMN Depleted Fitness (Output/Input)
H
Mean=0.27
Log2 Mean=-1.88
166
Genes
Genes
Log2 WT Fitness+2.43 SD
I
Mean=0.43
Log2 Mean=-1.22
194
Genes
Genes
Log2 PMN Depleted Fitness+2.54 SD
J
Fitness Defect in WT
62
Genes
51
Genes
52
Genes
Defect in PMN Dep
Borderline in PMN Dep
Rescued in PMN Dep
Unknown
Total=166

Table 3- 1A. *Kp* genes required to infect the lungs of WT mice

Fitness of *Kp* insertion mutants with significant defects in WT lungs by gene from screen presented in Figure 3-1.

Locus (VK055_RS)	Gene Name	Protein Description	WT Input Reads Mean[1]	WT Fitness Mean[2]	WT Fitness +2.43 SD[3]	PMN Dep Input Reads Mean[4]	PMN Dep Fitness Mean[5]	PMN Dep Fitness +2.54 SD[6]
24650	*glpC*	glycerol-3-phosphate dehydrogenase	248.50	0.00	0.00	179.63	0.00	0.00
23495	*hscA*	chaperone protein HscA	86.25	0.00	0.00	99.88	0.00	0.00
1735	*lysR*	LysR family transcriptional regulator	178.00	0.00	0.00	102.13	0.00	0.00
8925	*tolB*	translocation protein TolB	107.00	0.00	0.00	159.25	0.00	0.00
3245	*yciE*	hypothetical protein	44.92	0.00	0.00	50.63	0.00	0.00
24295	*yfcA*	membrane protein	259.33	0.00	0.00	163.13	0.00	0.00
9320	*nadD*	nicotinate-nucleotide adenylyltransferase	516.17	0.00	0.00	661.50	0.00	0.00
20990	*trxA*	copper-sensitivity suppressor protein D	136.75	0.00	0.00	110.88	0.00	0.02
8995	*sdhB*	succinate dehydrogenase iron-sulfur subunit	198.33	0.00	0.00	224.13	0.00	0.02
5395	*metQ*	methionine-binding protein	306.33	0.00	0.00	112.50	0.10	0.85
3935	*adhP*	alcohol dehydrogenase	196.83	0.00	0.00	129.63	0.10	1.21
120	*hisJ*	cystine transporter subunit	356.42	0.00	0.00	309.38	0.24	1.72
2055	*paaJ*	acetyl-CoA acetyltransferase	45.58	0.00	0.00	38.38	0.21	1.81
18825	*trpS*	tryptophanyl-tRNA synthetase	373.17	0.00	0.00	420.13	0.28	2.89
13600	*cusF*	copper-binding protein	283.17	0.00	0.00	232.13	0.49	4.41
9115	*seqA*	replication initiation regulator SeqA	249.33	0.00	0.00	300.00	0.67	6.18
14835		hypothetical protein	153.17	0.00	0.00	147.38	0.70	8.61
5785	*proP*	quinolone resistance protein	90.67	0.00	0.00	94.50	1.08	10.56
6655	*manC*	cupin	173.42	0.00	0.00	192.38	1.37	12.22
25975		tRNA-Asn	22.58	0.00	0.00	26.25	73.79	881.22
11140	*proC*	pyrroline-5-carboxylate reductase	21.58	0.00	0.00	17.00		

18650	*glpR*	transcriptional regulator	368.00	0.00	0.00	339.00	0.12	1.51
23330	*recO*	DNA repair protein RecO	295.17	0.00	0.00	330.00	0.53	4.11
1560	*acrR*	hypothetical protein	480.33	0.00	0.00	438.63	0.20	2.45
14060	*yigM*	N-acetyltransferase	196.00	0.00	0.00	248.50	0.00	0.00
7750	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	773.58	0.00	0.00	677.13	0.00	0.00
25470	*tagG*	ABC transporter permease	1084.42	0.00	0.00	1060.50	0.00	0.01
6810		hypothetical protein	549.33	0.00	0.00	353.13	0.03	0.33
17380		phophatase	447.42	0.00	0.00	354.38	0.13	1.58
23055	*pasT*	hypothetical protein	724.42	0.00	0.00	681.38	0.08	0.60
11825	*prfB*	hypothetical protein	505.75	0.00	0.00	546.50	0.00	0.00
5070	*adhE*	aldehyde dehydrogenase	895.17	0.00	0.00	747.13	0.13	1.15
8155		terminase	604.17	0.00	0.00	531.63	0.01	0.07
8990	*thiQ*	2-oxoglutarate dehydrogenase E1	249.92	0.00	0.00	262.13	0.01	0.05
14325	*thiQ*	hypothetical protein	909.58	0.00	0.01	783.88	1.49	13.55
25930	*araC*	AraC family transcriptional regulator	782.33	0.00	0.01	589.13	0.00	0.04
5115	*argD*	acetylornithine aminotransferase	748.00	0.00	0.01	833.50	0.10	1.22
16395	*metJ*	transcriptional regulator	460.67	0.00	0.01	529.25	0.02	0.26
8130		tail protein	169.08	0.00	0.01	187.50	1.32	13.31
2155	*pcbC*	2OG-Fe(II) oxygenase	572.67	0.00	0.01	502.75	0.00	0.00
1755	*tauA*	ABC transporter substrate-binding protein	671.58	0.00	0.01	651.00	0.24	2.91
11220	*tauC*	taurine ABC transporter permease	1444.67	0.00	0.01	1378.63	0.13	1.54
21055	*dsbG*	protein-disulfide isomerase	436.42	0.00	0.01	249.00	0.00	0.00
12895	*yaaA*	hypothetical protein	981.92	0.00	0.01	742.00	0.75	5.74
1595	*sufB*	cysteine desulfurase activator complex subunit SufD	878.67	0.00	0.01	924.13	0.52	4.31
8470	*rutF*	Asp/Glu/hydantoin racemase	501.00	0.00	0.01	459.88	0.55	3.94
4335	*pdxA*	4-hydroxythreonine-4-phosphate dehydrogenase	580.08	0.00	0.01	521.00	0.90	7.60
20935	*zapA*	Z-ring-associated protein	604.17	0.00	0.01	736.00	0.39	2.62
930		lipoprotein	746.83	0.00	0.01	573.50	0.02	0.27
8965	*appC*	cytochrome BD oxidase subunit I	179.83	0.00	0.01	208.75	0.55	6.74
8235		hypothetical protein	370.50	0.00	0.01	261.00	1.08	11.37

20950	*ubiH*	2-octaprenyl-6-methoxyphenyl hydroxylase	899.92	0.00	0.01	1025.25	0.13	1.31
2955	*gstA*	hypothetical protein	336.08	0.00	0.01	236.25	0.00	0.02
25970	*norM*	MATE family multidrug exporter	440.58	0.00	0.01	459.38	0.40	3.97
615	*ridA*	endoribonuclease L-PSP	733.75	0.00	0.02	569.25	0.07	0.79
5155	*tolC*	transporter	1277.92	0.00	0.02	665.75	0.01	0.08
1070	*sirB2*	hypothetical protein	572.25	0.00	0.02	418.63	0.02	0.15
16895	*pstA*	phosphate transporter permease subunit PtsA	139.75	0.00	0.02	92.13	0.00	0.00
5215	*caiD*	2,3-dehydroadipyl-CoA hydratase	277.00	0.00	0.02	204.38	0.07	0.84
9005	*sdhD*	succinate dehydrogenase cytochrome b556 small membrane subunit	374.08	0.00	0.02	448.13	0.00	0.02
6235	*yciW*	hypothetical protein	356.33	0.00	0.02	478.13	0.35	2.24
16690	*dsbA*	thiol-disulfide isomerase	208.58	0.00	0.02	131.00	0.03	0.38
24800	*yejL*	hypothetical protein	1080.67	0.00	0.02	836.50	0.04	0.49
16595	*fdoG*	formate dehydrogenase-N subunit gamma	461.42	0.00	0.03	327.38	0.10	1.20
8985	*aceF*	dihydrolipoamide succinyltransferase	84.08	0.00	0.03	70.63	0.00	0.01
2295	*glpR*	decarboxylase	729.33	0.00	0.04	643.88	0.02	0.25
7135	*dnaJ*	hypothetical protein	367.67	0.00	0.04	386.38	0.02	0.27
3215	*gloB*	beta-lactamase	571.67	0.00	0.04	692.88	0.47	3.33
2985	*GDB1*	sugar hydrolase	367.92	0.00	0.04	269.75	0.37	4.48
11095	*sbcC*	exonuclease SbcC	396.75	0.00	0.04	349.00	0.56	6.03
1570		hypothetical protein	262.33	0.00	0.04	157.75	1.02	12.25
25600	*cobT*	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase	281.50	0.00	0.04	232.00	0.10	0.89
23470	*iscR*	transcriptional regulator	220.50	0.00	0.04	171.50	0.97	11.50
10845	*sdhA*	3-oxosteroid 1-dehydrogenase	151.58	0.00	0.05	151.38	0.56	4.93
5470	*sciN*	hypothetical protein	548.25	0.00	0.05	518.50	0.10	0.86
22620	*fhlA*	transcriptional regulator	47.92	0.00	0.05	46.00	0.00	0.00
25780	*trbC*	TriB protein	90.92	0.00	0.05	72.00	0.71	7.85
24540	*nuoJ*	NADH:ubiquinone oxidoreductase subunit J	331.58	0.00	0.06	409.50	0.00	0.01

2585	*rnfA*	electron transport complex RsxE subunit	792.33	0.01	0.06	522.13	0.53	6.58
5995	*trpE*	anthranilate synthase component I	646.25	0.00	0.07	499.38	0.03	0.30
9400	*pagP*	phospholipid:lipid A palmitoyltransferase	170.67	0.00	0.07	171.38	0.06	0.78
11800	*rcsF*	membrane protein	704.58	0.01	0.07	574.13	0.19	1.69
20925	*serA*	3-phosphoglycerate dehydrogenase	612.17	0.01	0.07	629.38	0.01	0.09
375	*pykF*	pyruvate kinase	660.92	0.01	0.08	628.88	0.27	2.00
11355	*phnD*	phosphonate ABC transporter substrate-binding protein	615.00	0.01	0.08	418.13	9.99	122.16
5245	*maoC*	enoyl-CoA hydratase	708.08	0.01	0.09	539.50	0.24	2.08
24640	*uhpC*	MFS transporter	1085.25	0.01	0.09	1076.13	8.58	100.24
8755	*ycgE*	MerR family transcriptional regulator	95.42	0.01	0.10	28.25	0.00	0.02
580	*terC*	membrane protein	714.75	0.01	0.10	703.88	0.52	4.27
19770	*pnp*	polynucleotide phosphorylase/polyadenylase	652.67	0.01	0.10	723.38	0.06	0.46
795	*ilvA*	threonine dehydratase	249.92	0.01	0.10	217.25	0.00	0.01
14160	*celA*	PTS lactose transporter subunit IIB	360.08	0.01	0.10	398.50	0.09	1.04
395	*ilvD*	phosphogluconate dehydratase	962.00	0.01	0.11	970.00	0.35	3.79
8265	*pgpB*	UDP pyrophosphate phosphatase	449.50	0.01	0.11	499.75	0.09	0.66
1865	*ugpB*	iron ABC transporter substrate-binding protein	1302.58	0.01	0.12	1152.00	0.00	0.00
550	*rrmA*	23S rRNA methyltransferase	841.42	0.01	0.12	736.88	0.05	0.57
15660	*thiF*	molybdopterin biosynthesis protein MoeB	99.25	0.01	0.13	89.00	3.68	44.85
6315			912.17	0.01	0.13	917.38	0.02	0.14
24865	*yejB*	microcin C ABC transporter permease YejB	1587.17	0.01	0.14	1223.75	0.78	5.64
16485	*araC*	transcriptional regulator	432.92	0.01	0.14	356.75	0.00	0.00
7595	*ssuC*	sulfonate ABC transporter	248.67	0.01	0.14	208.38	0.00	0.03
2015	*rimL*	GNAT family acetyltraansferase	568.83	0.01	0.14	403.88	9.87	121.36
2010		isochorismatase-like hydrolase	1350.33	0.02	0.14	739.25	2.22	25.69

80	*rhaT*	EamA-like transporter family protein	2200.50	0.02	0.15	1735.50	0.22	1.37
14345	*msrA*	methionine sulfoxide reductase A	1205.25	0.02	0.16	986.88	0.14	1.69
2190	*acrA*	efflux transporter, RND family, MFP subunit	681.25	0.01	0.16	472.63	0.39	4.77
670	*mhpD*	hypothetical protein	761.67	0.01	0.16	614.00	0.44	3.97
16435	*menA*	1,4-dihydroxy-2-naphthoate prenyltransferase	2018.67	0.02	0.16	1720.38	1.41	8.70
16125	*wzzB*	lipopolysaccharide biosynthesis protein WzzE	1871.83	0.02	0.16	1613.50	1.20	7.38
8330	*iaaA*	isoaspartyl peptidase	672.08	0.01	0.16	518.38	6.54	79.92
985	*impG*	type VI secretion protein	1413.33	0.01	0.17	1496.38	0.12	1.15
7245	*uraA*	pyrimidine permease	1618.25	0.02	0.17	1202.88	0.03	0.33
19270	*prmA*	ribosomal protein L11 methyltransferase	544.17	0.01	0.17	387.25	14.01	162.79
1575	*narX*	signal peptide protein	926.33	0.02	0.17	744.63	0.26	1.81
15585	*purH*	purine biosynthesis protein purH	5082.25	0.02	0.17	5094.25	0.03	0.25
115	*amyA*	alpha-amylase	4632.00	0.03	0.17	4603.75	0.37	1.92
20900	*argO*	arginine exporter protein	1517.08	0.02	0.18	1299.50	1.32	11.54
14500	*ypfH*	esterase	923.17	0.01	0.18	849.88	1.53	10.04
20185	*yfdX*	hypothetical protein	1219.42	0.01	0.18	1238.13	0.95	7.37
7350	*gabD*	succinate-semialdehyde dehydrogenase	1666.25	0.02	0.18	1242.63	0.39	4.14
15550	*metA*	homoserine O-succinyltransferase	1820.58	0.02	0.19	1772.13	0.07	0.48
3655	*hisM*	polar amino acid ABC transporter permease	664.42	0.01	0.19	597.00	0.00	0.01
26165		hypothetical protein	1980.42	0.02	0.20	1311.63	0.21	1.83
12535	*lysR*	LysR family transcriptional regulator	1141.25	0.03	0.20	1034.25	3.55	29.84
23160	*tyrA*	chorismate mutase	5064.33	0.03	0.20	4593.88	0.12	0.69
26060		hypothetical protein	608.75	0.02	0.20	518.38	0.07	0.77
22675	*znuA*	metal ABC transporter substrate-binding protein	3865.25	0.03	0.20	4016.13	0.31	1.54
3560	*guaA1*	glutamine amidotransferase	1154.08	0.02	0.21	833.88	0.25	1.59
24240	*focA*	membrane protein	635.00	0.03	0.21	569.00	0.21	1.59

18995	*bfd*	bacterioferritin-associated ferredoxin	663.25	0.01	0.21	565.25	0.30	3.20
14320	*ppa*	inorganic pyrophosphatase	399.42	0.02	0.21	354.63	0.39	3.36
8855	*ybgS*	hypothetical protein	902.42	0.02	0.21	728.00	0.57	3.37
22145	*queF*	7-cyano-7-deazaguanine reductase	1437.92	0.01	0.21	1209.50	0.42	3.44
23540	*ndk*	nucleoside diphosphate kinase	810.58	0.01	0.21	769.25	0.10	0.75
85	*mutK*	hypothetical protein	1106.67	0.02	0.21	1050.88	0.37	2.63
23280	*nadB*	L-aspartate oxidase	6338.42	0.04	0.22	6292.25	0.07	0.51
12995	*serB*	phosphoserine phosphatase	806.42	0.01	0.22	789.88	0.00	0.01
3865	*purR*	cytochrome C peroxidase	813.83	0.01	0.22	901.63	0.00	0.00
18640	*glpE*	thiosulfate sulfurtransferase	3877.50	0.04	0.22	3474.38	1.08	9.78
17090	*ptsG*	PTS system alpha-glucoside-specific transporter subunit IICB	2191.25	0.03	0.22	1841.25	0.45	2.40
12495	*leuA*	2-isopropylmalate synthase	10182.75	0.04	0.22	9301.75	0.31	2.46
19495	*gltB*	glutamate synthase subunit alpha	11449.50	0.05	0.22	12086.63	0.08	0.41
1220	*rssA*	hypothetical protein	806.58	0.02	0.23	845.63	0.08	0.85
6620	*arsR*	ArsR family transcriptional regulator	520.50	0.02	0.23	418.75	53.16	653.73
1845	*abgB*	peptidase M20	1950.08	0.02	0.23	1848.75	0.11	0.97
18500	*livG*	amino acid ABC transporter ATP-binding protein	2284.75	0.04	0.23	2175.00	1.29	8.24
17445		hypothetical protein	988.25	0.02	0.23	998.75	0.29	2.25
6540	*yhjH*	diguanylate phosphodiesterase	1129.75	0.02	0.23	960.88	0.38	4.47
7200	*putA*	transcriptional regulator	2976.25	0.04	0.24	2417.88	0.13	0.83
7895	*aqpZ*	aquaporin Z	943.75	0.02	0.24	610.88	1.10	8.14
9810	*dppF*	peptide ABC transporter ATP-binding protein	3098.17	0.04	0.24	3267.50	0.45	2.38
1665	*fepC*	ABC transporter	772.17	0.02	0.24	590.00	0.00	0.01
15870	*tatB*	translocase	1154.67	0.02	0.24	1083.00	0.67	5.70
21045	*fldB*	flavodoxin	251.42	0.02	0.25	272.88	1.13	9.52
25375	*pgpB*	hypothetical protein	470.00	0.02	0.25	275.13	0.00	0.00
5435		2'-hydroxyisoflavone	1527.4	0.02	0.25	1411.7	0.33	2.85

		reductase	2			5		
10155	*hlyD*	membrane protein	3018.08	0.04	0.26	2828.00	0.76	5.46
7170	*efeB*	peroxidase	1414.17	0.03	0.26	1145.00	0.94	9.16
25905		salicyl-AMP ligase	2397.67	0.03	0.26	2168.75	0.08	0.52
24465		hypothetical protein	1469.58	0.02	0.26	1486.13	0.98	7.85
19600	*mlaE*	ABC transporter permease	3957.83	0.03	0.26	4382.75	0.04	0.30
6135		hypothetical protein	1630.33	0.03	0.26	1479.50	0.23	1.84
26015		tRNA-Asn	289.25	0.02	0.27	313.00	0.62	7.63
18795	*aroK*	shikimate kinase	1453.42	0.03	0.27	1415.13	0.02	0.23
19490		glutamate synthase subunit beta	1380.25	0.04	0.27	1472.25	0.05	0.39
14915	*cbpM*	chaperone-modulator protein CbpM	920.42	0.02	0.27	682.50	0.30	2.77

1. WT Input Reads Mean: Mean number of reads for all inputs used to infect WT mice.
2. WT Fitness Mean: Mean fitness of each gene across all mice tested in a cohort, where a defect is considered a fitness of <0.27.
3. WT Fitness+2.43SD: Assessing the fitness of genes in WT mice, where a gene was considered to have a statistically significant defect in WT mice if mutants with insertions in a gene had a $Mean_{Fitness\ of\ a\ Gene}$+2.43 $SD_{Fitness\ of\ a\ Gene}$ <0.27 (i.e. $Mean_{Fitness\ of\ all\ Genes\ in\ WT}$).
4. PMN Dep Input Reads Mean: Mean number of reads for all inputs used to infect PMN depleted mice.
5. PMN Dep Fitness Mean: Mean fitness of each gene across all mice tested in a cohort, where a defect is considered a fitness of <0.43.
6. PMN Dep Fitness+2.54SD: Assessing the fitness of genes in PMN Dep mice, where a gene was considered to have a statistically significant defect in PMN Dep mice if mutants with insertions in a gene had a $Mean_{Fitness\ of\ a\ Gene}$+2.54 $SD_{Fitness\ of\ a\ Gene}$ <0.43 (i.e. $Mean_{Fitness\ of\ all\ Genes\ in\ PMN\ Dep}$).

Table 3- 2B. *Kp* genes required to infect the lungs of PMN Dep mice

Fitness of *Kp* insertion mutants with significant defects in PMN Dep lungs by gene from screen presented in Figure 3-1.

Locus (VK055_RS)	Gene Name	Protein Description	WT Input Reads Mean[1]	WT Fitness Mean[2]	WT Fitness +2.43 SD[3]	PMN Dep Input Reads Mean[4]	PMN Dep Fitness Mean[5]	PMN Dep Fitness +2.54 SD[6]
24650	*glpC*	glycerol-3-phosphate dehydrogenase	248.50	0.00	0.00	179.63	0.00	0.00
23495	*hscA*	chaperone protein HscA	86.25	0.00	0.00	99.88	0.00	0.00
1735	*lysR*	LysR family transcriptional regulator	178.00	0.00	0.00	102.13	0.00	0.00
8925	*tolB*	translocation protein TolB	107.00	0.00	0.00	159.25	0.00	0.00
3245	*yciE*	hypothetical protein	44.92	0.00	0.00	50.63	0.00	0.00
24295	*yfcA*	membrane protein	259.33	0.00	0.00	163.13	0.00	0.00
11825	*prfB*	hypothetical protein	505.75	0.00	0.00	546.50	0.00	0.00
16895	*pstA*	phosphate transporter permease subunit PtsA	139.75	0.00	0.02	92.13	0.00	0.00
22620	*fhlA*	transcriptional regulator	47.92	0.00	0.05	46.00	0.00	0.00
16485	*araC*	transcriptional regulator	432.92	0.01	0.14	356.75	0.00	0.00
3865	*purR*	cytochrome C peroxidase	813.83	0.01	0.22	901.63	0.00	0.00
22525	*fixX*	formate hydrogenlyase subunit 2	289.00	0.04	0.62	164.63	0.00	0.00
4325	*ldh*	lactate dehydrogenase	466.50	0.08	0.81	382.88	0.00	0.00
2960	*pptA*	4-oxalocrotonate tautomerase	364.00	0.10	1.17	130.50	0.00	0.00
25520	*hisH*	imidazole glycerol phosphate synthase	394.00	0.12	1.38	451.75	0.00	0.00
12145	*dksA*	transcriptional regulator	143.08	0.11	1.72	168.75	0.00	0.00
21155	*fimD*	fimbrial assembly	44.75	0.22	2.01	65.50	0.00	0.00

		protein						
25515	*hisA*	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	613.25	0.31	3.25	607.88	0.00	0.00
6405	*lysR*	LysR family transcriptional regulator	48.33	0.29	3.37	71.50	0.00	0.00
3360	*araJ*	MFS transporter	250.08	0.66	7.95	238.00	0.00	0.00
17945	*glyQ*	glycyl-tRNA synthetase subunit alpha	86.42	0.68	10.09	83.13	0.00	0.00
25110	*yehW*	membrane protein	193.17	1.84	24.97	289.13	0.00	0.00
18375	*arnF*	4-amino-4-deoxy-L-arabinose-phospho-UDP flippase subunit F	138.00	1.66	26.44	60.25	0.00	0.00
9665	*fepC*	iron-enterobactin transporter ATP-binding protein	123.33			171.25	0.00	0.00
7750	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	773.58	0.00	0.00	677.13	0.00	0.00
21055	*dsbG*	protein-disulfide isomerase	436.42	0.00	0.01	249.00	0.00	0.00
8150		head completion protein	1014.33	0.06	0.60	991.00	0.00	0.00
14060	*yigM*	N-acetyltransferase	196.00	0.00	0.00	248.50	0.00	0.00
9320	*nadD*	nicotinate-nucleotide adenylyltransferase	516.17	0.00	0.00	661.50	0.00	0.00
14685	*emrE*	multidrug transporter	295.42	0.55	5.15	349.13	0.00	0.00
25375	*pgpB*	hypothetical protein	470.00	0.02	0.25	275.13	0.00	0.00
4045	*gntT*	major facilitator transporter	835.75	0.07	1.01	808.13	0.00	0.00
4520	*gntT*	transporter	248.58	0.02	0.34	113.25	0.00	0.00
2155	*pcbC*	2OG-Fe(II) oxygenase	572.67	0.00	0.01	502.75	0.00	0.00

25535	*hisD*	bifunctional histidinal dehydrogenase/ histidinol dehydrogenase	476.42	0.16	1.82	454.50	0.00	0.00
1865	*ugpB*	iron ABC transporter substrate-binding protein	1302.58	0.01	0.12	1152.00	0.00	0.00
2700	*tehA*	tellurite resistance protein TehA	498.92	0.25	3.92	688.13	0.00	0.00
16045		hypothetical protein	935.08	0.09	0.67	998.50	0.00	0.01
20960	*gcvT*	glycine cleavage system aminomethyltransferase T	768.08	0.06	0.88	589.88	0.00	0.01
3655	*hisM*	polar amino acid ABC transporter permease	664.42	0.01	0.19	597.00	0.00	0.01
6310	*ansA*	cytoplasmic asparaginase I	539.67	0.05	0.47	421.88	0.00	0.01
25470	*tagG*	ABC transporter permease	1084.42	0.00	0.00	1060.50	0.00	0.01
18440	*ftsX*	cell division protein FtsX	759.75	0.04	0.61	526.38	0.00	0.01
605	*pabB*	aminodeoxychorismate synthase	409.67	0.04	0.59	387.00	0.00	0.01
2415	*purR*	transcriptional regulator	1244.58	0.09	0.84	1130.00	0.00	0.01
11970	*degQ*	serine endoprotease	767.67	0.06	0.62	757.00	0.00	0.01
16420	*ftsN*	cell division protein FtsN	311.17	0.06	0.90	240.50	0.00	0.01
4060		hypothetical protein	354.25	0.10	1.45	267.38	0.00	0.01
8485		hypothetical protein	344.75	1.38	20.55	166.38	0.00	0.01
8985	*aceF*	dihydrolipoamide succinyltransferase	84.08	0.00	0.03	70.63	0.00	0.01
6200	*astB*	succinylarginine dihydrolase	597.92	1.33	19.88	457.00	0.00	0.01
24540	*nuoJ*	NADH:ubiquinone oxidoreductase subunit J	331.58	0.00	0.06	409.50	0.00	0.01
3160	*dppB*	peptide ABC transporter permease	881.67	0.09	0.82	958.38	0.00	0.01
1665	*fepC*	ABC transporter	772.17	0.02	0.24	590.00	0.00	0.01

4160	*nirD*	(2Fe-2S)-binding protein	575.92	0.06	0.56	418.75	0.00	0.01
5625		hypothetical protein	335.92	0.18	2.85	392.13	0.00	0.01
795	*ilvA*	threonine dehydratase	249.92	0.01	0.10	217.25	0.00	0.01
7150			422.33	0.34	5.26	397.38	0.00	0.01
12995	*serB*	phosphoserine phosphatase	806.42	0.01	0.22	789.88	0.00	0.01
20990	*trxA*	copper-sensitivity suppressor protein D	136.75	0.00	0.00	110.88	0.00	0.02
15745	*tufB*	elongation factor Tu	109.67	0.20	1.73	145.50	0.00	0.02
310	*yecE*	hypothetical protein	1468.25	0.08	0.73	1501.50	0.00	0.02
8995	*sdhB*	succinate dehydrogenase iron-sulfur subunit	198.33	0.00	0.00	224.13	0.00	0.02
9005	*sdhD*	succinate dehydrogenase cytochrome b556 small membrane subunit	374.08	0.00	0.02	448.13	0.00	0.02
19860	*rsmI*	16S rRNA methyltransferase	666.92	0.04	0.61	723.88	0.00	0.02
23460	*suhB*	inositol monophosphatase	855.08	0.13	1.77	668.63	0.00	0.02
2955	*gstA*	hypothetical protein	336.08	0.00	0.01	236.25	0.00	0.02
15815	*trkG*	potassium transporter	203.58	0.09	1.36	188.50	0.00	0.02
13825	*skfB*	radical SAM protein	122.50	0.08	0.87	155.63	0.00	0.02
23580		hypothetical protein	603.42	0.20	2.83	545.25	0.00	0.02
12970	*trpR*	Trp operon repressor	779.00	7.94	125.19	524.00	0.00	0.02
8755	*ycgE*	MerR family transcriptional regulator	95.42	0.01	0.10	28.25	0.00	0.02
7595	*ssuC*	sulfonate ABC transporter	248.67	0.01	0.14	208.38	0.00	0.03
13910		hypothetical protein	437.42	0.13	1.27	296.00	0.00	0.03
1990	*nrfG*	cytochrome C biogenesis protein	501.25	0.15	1.33	390.75	0.00	0.03

25930	*araC*	AraC family transcriptional regulator	782.33	0.00	0.01	589.13	0.00	0.04
9010	*sdhC*	succinate dehydrogenase cytochrome b556 large membrane subunit	194.25	0.88	10.72	225.13	0.01	0.04
6005	*trpC*	indole-3-glycerol phosphate synthase	2690.25	0.13	0.80	3102.75	0.00	0.05
13205	*agaB*	PTS sugar transporter	553.50	0.39	3.05	452.25	0.00	0.05
11245			1262.08	0.27	1.71	1408.50	0.00	0.05
17810	*cysE*	serine acetyltransferase	325.75	0.14	1.60	383.88	0.01	0.05
8990	*thiQ*	2-oxoglutarate dehydrogenase E1	249.92	0.00	0.00	262.13	0.01	0.05
18360	*ArnD*	4-deoxy-4-formamido-L-arabinose-phospho-UDP deformylase	740.58	0.02	0.31	695.00	0.00	0.06
980	*impG*	type VI secretion protein ImpG	393.42	0.42	5.27	406.38	0.00	0.06
26215		phage protein	1377.50	0.50	4.18	1215.38	0.01	0.06
8155		terminase	604.17	0.00	0.00	531.63	0.01	0.07
15840	*mcr1*	FMN reductase	1890.00	0.08	0.71	2059.75	0.01	0.08
5155	*tolC*	transporter	1277.92	0.00	0.02	665.75	0.01	0.08
25430	*wcaJ*	UDP-glucose lipid carrier transferase	1342.50	0.17	1.17	1696.38	0.01	0.09
10775	*clpX*	ATP-dependent protease	147.58	1.33	20.56	123.63	0.01	0.09
20925	*serA*	3-phosphoglycerate dehydrogenase	612.17	0.01	0.07	629.38	0.01	0.09
10335	*ybcJ*	ribosome-associated protein	1530.67	0.29	3.41	1582.38	0.01	0.10
19545	*rapZ*	glmZ(sRNA)-inactivating NTPase	1491.58	0.16	1.04	1714.00	0.01	0.11
19200	*aroE*	shikimate dehydrogenase	1779.92	0.05	0.46	1666.38	0.01	0.12
23390	*cheY*	response regulator GlrR	2013.92	0.22	1.92	1933.88	0.01	0.13
3670	*nifS*	aminotransferase V	1059.25	0.04	0.33	1056.13	0.01	0.13

12505	*leuC*	3-isopropylmalate dehydratase large subunit	4487.50	0.05	0.29	4416.75	0.02	0.13
4000	*glxK*	glycerate kinase	1637.08	0.10	0.57	1416.00	0.01	0.14
6315			912.17	0.01	0.13	917.38	0.02	0.14
840	*livF*	amino acid ABC transporter ATPase	1667.25	0.10	0.70	1746.88	0.01	0.14
8715	*uspA*	universal stress protein G	663.33	0.19	3.08	663.38	0.02	0.14
6435	*yeaL*	membrane protein	1451.08	0.10	1.09	1026.63	0.01	0.14
2775	*potD*	spermidine/putrescine ABC transporter substrate-binding protein	754.17	0.06	0.89	890.13	0.01	0.14
23725	*purM*	phosphoribosylaminoimidazole synthetase	2187.67	0.06	0.55	2326.25	0.02	0.15
1070	*sirB2*	hypothetical protein	572.25	0.00	0.02	418.63	0.02	0.15
21585	*maK*	sugar ABC transporter ATP-binding protein	958.08	0.11	0.86	761.88	0.03	0.15
25220	*deoR*	cytochrome C biogenesis protein CcdA	570.00	0.05	0.75	524.88	0.03	0.16
9480	*mglA*	sugar ABC transporter ATPase	354.17	0.64	7.47	309.88	0.02	0.16
1995	*araC*	AraC family transcriptional regulator	922.33	2.05	22.72	840.38	0.01	0.17
2565	*rnfG*	electron transport complex protein RsxA	1050.33	0.45	6.52	800.50	0.02	0.18
1895	*cse3*	CRISPR-associated protein Cse3	877.33	0.06	0.54	751.00	0.02	0.18
7960	*tctC*	arginine ABC transporter substrate-binding protein	3795.17	0.04	0.29	3380.00	0.03	0.19
355	*znuC*	zinc transporter	1177.42	1.26	11.70	864.88	0.02	0.20
6455	*ybbk*	hypothetical protein	552.17	20.92	333.56	409.75	0.02	0.20
12500	*leuB*	3-isopropylmalate dehydrogenase	12004.75	0.06	0.32	12027.38	0.04	0.20
6375	*btuE*	putative glutathione peroxidase	814.42	0.05	0.59	515.50	0.02	0.20

18795	*aroK*	shikimate kinase	1453.42	0.03	0.27	1415.13	0.02	0.23
16190	*lysR*	transcriptional regulator	4173.00	0.10	0.53	4530.63	0.05	0.23
23375	*purL2*	phosphoribosylformylglycinamidine synthase	7300.33	0.05	0.32	7910.13	0.03	0.23
3400	*skfB*	pyrroloquinoline quinone biosynthesis protein PqqE	647.92	0.26	3.02	668.50	0.02	0.23
4650	*fixX*	dimethyl sulfoxide reductase subunit B	797.58	0.17	1.65	665.38	0.02	0.23
21645	*purR*	transcriptional regulator	721.00	0.04	0.54	657.25	0.03	0.23
12705	*carB*	carbamoyl phosphate synthase large subunit	7204.33	0.07	0.37	7479.63	0.06	0.24
2295	*glpR*	decarboxylase	729.33	0.00	0.04	643.88	0.02	0.25
15585	*purH*	purine biosynthesis protein purH	5082.25	0.02	0.17	5094.25	0.03	0.25
7070	*ymdB*	RNase III inhibitor	2489.17	0.08	0.56	1856.13	0.03	0.25
735	*cirA*	ferrichrome-iron receptor	2924.08	0.06	0.53	3197.88	0.03	0.25
1255		hypothetical protein	279.42	0.15	2.39	273.63	0.03	0.26
19605	*mlaD*	phospholipid ABC transporter substrate-binding protein	3558.83	0.05	0.30	3557.25	0.04	0.26
4750	*accD*	malonate decarboxylase subunit beta	912.17	0.11	1.39	707.13	0.04	0.26
16395	*metJ*	transcriptional regulator	460.67	0.00	0.01	529.25	0.02	0.26
930		lipoprotein	746.83	0.00	0.01	573.50	0.02	0.27
5230	*ydbO*	phenylacetic acid degradation protein	1124.08	0.02	0.35	950.00	0.02	0.27
7135	*dnaJ*	hypothetical protein	367.67	0.00	0.04	386.38	0.02	0.27
2165	*hxlR*	hypothetical protein	576.67	0.16	2.31	306.13	0.02	0.27
2760	*appB*	ubiquinol oxidase subunit II	644.83	0.24	3.72	305.13	0.04	0.28
22580		hypothetical protein	732.67	7.16	76.63	873.25	0.02	0.28

8185		hypothetical protein	507.92	0.04	0.49	476.38	0.02	0.29
2510	*slyB*	outer membrane lipoprotein pcp	599.50	0.04	0.56	464.00	0.03	0.29
2505	*pleD*	diguanylate cyclase	1276.83	0.20	2.70	1277.63	0.03	0.29
19610	*mlaC*	ABC transporter substrate-binding protein	7650.83	0.12	0.49	9195.88	0.07	0.30
3425		peptidase	1183.17	0.05	0.46	969.88	0.04	0.30
6855	*lpoB*	penicillin-binding protein activator LpoB	762.25	0.05	0.55	607.63	0.03	0.30
19600	*mlaE*	ABC transporter permease	3957.83	0.03	0.26	4382.75	0.04	0.30
13055	*prfC*	peptide chain release factor 3	1965.75	0.05	0.44	2118.50	0.04	0.30
5995	*trpE*	anthranilate synthase component I	646.25	0.00	0.07	499.38	0.03	0.30
23805	*ypfJ*	hypothetical protein	337.50	0.11	0.99	223.75	0.03	0.30
18875	*hisH*	anthranilate synthase subunit II	5030.25	0.05	0.30	5018.38	0.06	0.31
1330	*tonB*	energy transducer TonB	915.33	0.02	0.34	962.38	0.04	0.31
8035	*nfsA*	membrane protein	160.08	0.07	0.94	127.63	0.03	0.32
9660	*FepG*	iron-enterobactin transporter permease	137.42	0.02	0.36	148.50	0.03	0.32
9790	*ddpA*	ABC transporter, periplasmic substrate-binding protein	683.33	0.03	0.42	619.25	0.04	0.32
7245	*rraA*	pyrimidine permease	1618.25	0.02	0.17	1202.88	0.03	0.33
6810		hypothetical protein	549.33	0.00	0.00	353.13	0.03	0.33
14565	*hflX*	GTPase HflX	1727.67	0.05	0.38	1966.88	0.04	0.33
19595	*mlaF*	organic solvent ABC transporter ATP-binding protein	2200.08	0.16	1.28	2379.63	0.05	0.33
7765	*ycaO*	hypothetical protein	2501.33	0.32	2.32	2601.00	0.04	0.33
25525	*hisB2*	imidazoleglycerol-phosphate dehydratase	2322.67	0.07	0.60	2277.25	0.04	0.34

14115	*pyrB*	aspartate carbamoyltransferase catalytic subunit	3543.67	0.09	0.43	3691.25	0.06	0.34
3475	*tauB*	ABC transporter ATPase	754.50	0.02	0.33	598.88	0.03	0.35
210	*rpiB*	hypothetical protein	2081.67	0.49	4.71	1706.38	0.04	0.35
25065	*yohC*	membrane protein	1486.50	0.54	6.27	1307.75	0.07	0.36
4125	*hipA*	serine/threonine protein kinase	622.42	0.05	0.59	508.25	0.04	0.36
5550			2907.08	0.16	1.08	2762.25	0.05	0.36
25075	*yncA*	acetyltransferase GCN5	707.83	0.03	0.32	680.00	0.03	0.36
24990	*rbsB*	methyl-galactoside ABC transporter substrate-binding protein	1364.75	3.10	45.49	1076.50	0.06	0.36
25370	*galU*	UTP--glucose-1-phosphate uridylyltransferase subunit GalU	2336.00	0.10	0.77	2077.63	0.06	0.37
3310	*YneE*	hypothetical protein	827.58	0.71	11.18	743.88	0.04	0.37
10			402.67	2.15	30.13	378.25	0.03	0.37
16200	*ilvA*	dihydroxy-acid dehydratase	4850.25	0.09	0.40	5625.38	0.05	0.38
16690	*dsbA*	thiol-disulfide isomerase	208.58	0.00	0.02	131.00	0.03	0.38
16235	*lysR*	transcriptional regulator	3396.75	0.08	0.50	3709.38	0.05	0.38
5750		membrane protein	953.83	0.21	1.41	992.00	0.04	0.39
875		LysR family transcriptional regulator	651.92	0.37	3.28	579.25	0.03	0.39
8015		putrescine/spermidine ABC transporter substrate-binding protein	2639.75	0.18	1.11	2714.75	0.07	0.39
19490		glutamate synthase subunit beta	1380.25	0.04	0.27	1472.25	0.05	0.39
26270		ArsR family transcriptional regulator	392.42	2.24	32.05	357.50	0.03	0.39
4240		hypothetical protein	553.00	0.37	5.68	555.00	0.03	0.39

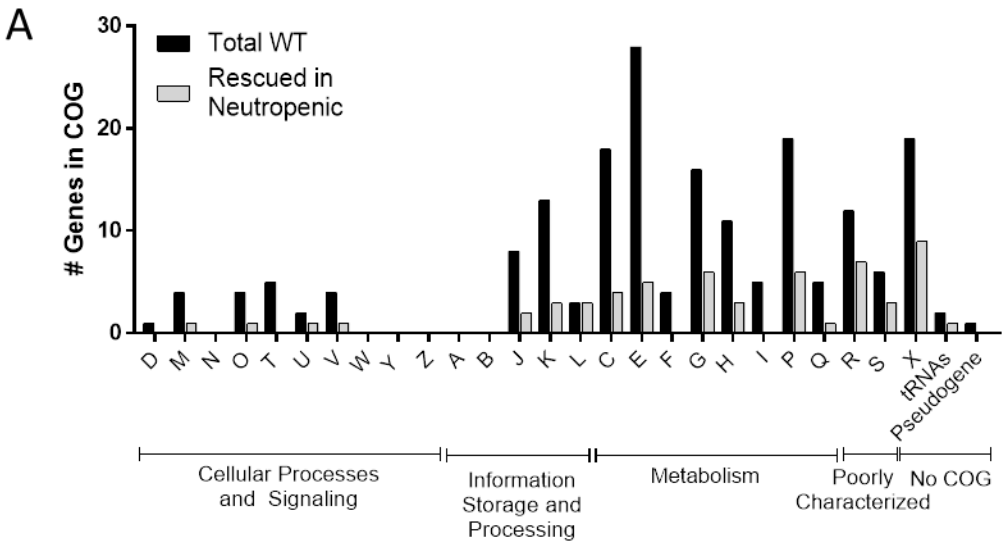
11320		hypothetical protein	170.92	0.51	7.71	145.63	0.04	0.39
24520		NADH dehydrogenase subunit F	1491.83	0.50	4.74	1413.63	0.06	0.40
7755		3-phosphoserine/phosphohydroxythreonine aminotransferase	1009.83	0.06	0.56	1119.00	0.03	0.40
24660		sn-glycerol-3-phosphate dehydrogenase subunit A	1398.67	0.14	1.21	1673.00	0.04	0.40
20370		outer membrane channel protein	3412.00	0.10	0.61	3510.13	0.06	0.40
25825		enterochelin esterase	549.50	0.18	2.84	377.88	0.04	0.41
23270		ATP-dependent RNA helicase SrmB	3101.67	0.09	0.61	2763.00	0.05	0.41
16205		branched-chain amino acid aminotransferase	6408.33	0.07	0.39	7343.88	0.07	0.41
19495	*gltB*	glutamate synthase subunit alpha	11449.50	0.05	0.22	12086.63	0.08	0.41
25915		oxidoreductase	3577.42	0.07	0.44	3831.63	0.09	0.41
24215		CMP deaminase	2729.50	0.13	0.69	2521.75	0.05	0.41
16120		UDP-N-acetylglucosamine 2-epimerase	951.33	0.89	9.60	1208.00	0.05	0.41
11080	*phoR*	phosphate regulon sensor protein	2032.25	0.08	0.60	2020.63	0.05	0.42
24245		membrane protein	1241.33	0.26	2.55	1194.88	0.05	0.42
9570		hypothetical protein	793.08	0.05	0.47	608.63	0.05	0.42

1. WT Input Reads Mean: Mean number of reads for all inputs used to infect WT mice.
2. WT Fitness Mean: Mean fitness of each gene across all mice tested in a cohort, where a defect is considered a fitness of <0.27.
3. WT Fitness+2.43SD: Assessing the fitness of genes in WT mice, where a gene was considered to have a statistically significant defect in WT mice if mutants with insertions in a gene had a $Mean_{\text{Fitness of a Gene}}+2.43\ SD_{\text{Fitness of a Gene}} <0.27$ (i.e. $Mean_{\text{Fitness of all Genes in WT}}$).
4. PMN Dep Input Reads Mean: Mean number of reads for all inputs used to infect PMN depleted mice.

5. PMN Dep Fitness Mean: Mean fitness of each gene across all mice tested in a cohort, where a defect is considered a fitness of <0.43.
6. PMN Dep Fitness+2.54SD: Assessing the fitness of genes in PMN Dep mice, where a gene was considered to have a statistically significant defect in PMN Dep mice if mutants with insertions in a gene had a $Mean_{Fitness\ of\ a\ Gene}+2.54\ SD_{Fitness\ of\ a\ Gene} <0.43$ (i.e. $Mean_{Fitness\ of\ all\ Genes\ in\ PMN\ Dep}$).

Figure 3- 2. Cluster of orthologous group (COG) categorization of *Kp* genes required to infect WT lungs

The protein products of genes identified as necessary for *Kp* virulence in WT mice, as well as genes needed in WT mice, but not in PMN depleted mice, were assigned into functional categories by matching them to Cluster of Orthologous Groups (COGs) using the COG database and the COGs assigned to proteins in *Kp* strain 352. (A) The number of genes in each cohort assigned to each COG. (B) Table of "Cluster of Orthologous Group Assignment" where there is a functional description of each COG, the "# of WT hits" (genes in each COG needed for *Kp* to infect WT mice) refers to the number of genes assigned into each COG, the "# of WT Resc in PMN Dep" (of the *Kp* genes needed in WT mice, the ones that were rescued in PMN depleted mice) refers to the number of genes that are assigned into each COG, and "% Needed against PMNs" refers to the proportion of genes in each COG category needed to assist *Kp* in the presence of neutrophils calculated as Proportion of genes in specified COG rescued in PMN depleted mice/Proportion of genes in specified COG needed in WT mice.


A
30
20
10
0
Genes in COG
Total WT
Rescued in Neutropenic
D M N O T U V W Y Z A B J K L C E F G H I P Q R S X
tRNAs
Pseudogene
Cellular Processes and Signaling
Information Storage and Processing
Metabolism
Poorly Characterized
No COG

B

Cluster of Orthologous Group Assignment	# of WT hits	# of WT Resc in PMN Dep	% Needed Against PMNs
Cellular Processes and Signaling			
[D] Cell cycle control, cell division, chromosome partitioning	1	0	0
[M] Cell wall/membrane/envelope biogenesis	4	1	25
[N] Cell motility	0	0	0
[O] Post-translational modification, protein turnover, and chaperones	4	1	25
[T] Signal transduction mechanisms	5	0	0
[U] Intracellular trafficking, secretion, and vesicular transport	2	1	50
[V] Defense mechanisms	4	1	25
[W] Extracellular structures	0	0	0
[Y] Nuclear structure	0	0	0
[Z] Cytoskeleton	0	0	0
Information Storage and Processing			
[A] RNA processing and modification	0	0	0
[B] Chromatin structure and dynamics	0	0	0
[J] Translation, ribosomal structure and biogenesis	8	2	25
[K] Transcription	13	3	23.1
[L] Replication, recombination and repair	3	3	100
Metabolism			
[C] Energy production and conversion	18	4	22.2
[E] Amino acid transport and metabolism	28	5	17.9
[F] Nucleotide transport and metabolism	4	0	0
[G] Carbohydrate transport and metabolism	16	6	37.5
[H] Coenzyme transport and metabolism	11	3	27.3
[I] Lipid transport and metabolism	5	0	0
[P] Inorganic ion transport and metabolism	19	6	31.6
[Q] Secondary metabolites biosynthesis, transport, and catabolism	5	1	20
POORLY CHARACTERIZED			
[R] General function prediction only	12	7	58.3
[S] Function unknown	6	3	50
[X] No COG assignment	19	9	47.4
tRNAs	2	1	50
Pseudogene	1	0	0
14 WT hits fall into 2 COGs, and 1 hit each into 3 and 4 COGs			
1 PMN rescued fell into 2 COGs and 1 into 4			

3.2 RETRIEVING *KP* TRANSPOSON INSERTION MUTANTS OF INTEREST FROM THE ARRAYED LIBRARY

One drawback of the classic approach to high-throughput bacterial transposon insertion and selection techniques, such as TnSeq, is that specific insertion mutants of interest are not available for study due to the pooled nature of the library. To overcome this and obtain insertion mutants of interest for further confirmation and functional studies, the 13,056 insertion mutants in the *Kp* library generated in this study were arrayed during library construction. A combinatorial pooling and deep sequencing approach (400) was utilized to locate specific insertion mutants, and PCR and sequencing was used to confirm the site of transposon insertion for the retrieved strains. Using this approach, we successfully assigned locations of specific insertions with high confidence for about 35% of the library. Sixty-two insertion mutants were retrieved using this method (**Table 3-2**), where 42 insertion mutants had a significant defect in WT mice in the TnSeq screen (**Tables 3-1, 3-2** and **4-1**) and 15 had defects that were not statistically significant but were included, in part, to ensure that our analysis of fitness cut-offs were adequately conservative. Of the 57 mutants with insertions in genes needed in WT mice, 13 also had a fitness defect in PMN-depleted mice, 16 appeared virulent and 28 had a modest fitness defect **(Tables 3-1, 3-2** and **4-1).** Notably, 10 of these mutants that had defects in WT mice and were defective or borderline in PMN-depleted had putative metabolic functions (**Table 4-1**). The results from these mutants will be presented in Chapter 4. Finally, 5 additional insertion mutants were retrieved for use as "neutral" fitness strains, where 3 were in genes with no defect in WT and PMN-depleted mice and 2 were in intergenic regions **(Tables 3-1, 3-2** and **4-1).**

Table 3- 3. Description and summary of phenotypes of *Kp* insertion mutants retrieved from array

The *Kp* transposon insertion mutants retrieved and confirmed from the arrayed library, and a summary of their phenotypes from the *in vivo* and *in vitro* assays presented in Chapter 3.

Strain Name (MKP)	**Locus (VK055_RS)**	**Gene Name**	**Tn Insertion site**	**TnSeq Screen: Defect in WT[1]**	**TnSeq Screen: Defect in PMN Dep[2]**	**M9+ gluc/ L/ Low Iron[3]**	**Mucoviscosity/ Capsule[4]**	**Res. to H_2O_2/ HOCl/ NO[5]**	**Defect *in vivo* as SS in WT mice[6]**
431	16485	*araC* (upstream)	3347394	Yes	Yes	=/=/=	=/=	=/=/=	N/D
430	16690	*dsbA*	3391100	Yes	Yes	-/-/=	=/=	=/=/=	Yes
413	03865	*purr*	768137	Yes	Yes	=/=/=	=/=	=/=/=	N/D
324	08995	*sdhB*	1808845	Yes	Yes	=/-/=	-/=	=/=/=	No
399	11830	*yaeQ*	2387939	Yes	Yes	=/=/=	=/=	=/=/=	N/D
404	08755	*ycgE*	1770023	Yes	Yes	-/-/=	-/=	+/=/=	Yes
312	14060	*yigM* (upstream)	2865305	Yes	Yes	=/=/=	=/=	=/=/=	N/D
415	01845	*abgB*	375394	Yes	NS	=/=/=	=/=	=/=/=	N/D
304	05070	*adhE*	999091	Yes	NS	=/=/=	=/=	+/+/=	N/D
318	14915	*cbpM*	3027402	Yes	NS	=/=/=	=/=	=/=/=	N/D
438	14160	*celA* (upstream)	2887842	Yes	NS	=/=/=	=/=	=/=/=	N/D
406	07350	*gabD*	1459252	Yes	NS	=/=/=	=/=	=/=/=	N/D
299	18650	*glpR*	3802954	Yes	NS	-/-/=	=/=	=/=/=	N/D
336	00120	*hisJ* (upstream)	25612	Yes	NS	=/=/=	=/=	=/=/=	N/D
437	14320	*ppa* (upstream)	2913962	Yes	NS	=/=/=	=/=	=/=/=	N/D
407	07200	*putA*	1431780	Yes	NS	=/=/=	-/=	=/=/=	N/D
421	22145	*queF* (upstream)	4482018	Yes	NS	=/=/=	=/=	=/=/=	N/D
400	11800	*rcsF*	2382506	Yes	NS	=/=/=	=/=	=/=/=	N/D
317	18825	*trpS*	3843551	Yes	NS	=/-/=	=/=	+/=/=	N/D
353	20950	*ubiH*	4240320	Yes	NS	=/=/=	=/=	=/=/=	N/D

325	06540	*yhjH*	1300667	Yes	NS	=/-/=	=/=	=/-/=	Yes
322	17445		3538671	Yes	NS	=/=/=	=/=	=/-/=	Yes
409	05435		1079672	Yes	NS	=/=/=	=/=	=/=/=	N/D
403	08965	*appC* (upstream)	1801672	Yes	No	=/=/-	=/=	=/=/=	No
309	07895	*aqpZ*	1592365	Yes	No	=/=/=	=/=	=/=/=	N/D
303	20895	*argO*	4230979	Yes	No	=/=/=	=/=	=/=/=	N/D
339	07170	*efeB*	1421698	Yes	No	=/=/=	=/=	=/=/=	N/D
401	10155	*hlyD*	2053919	Yes	No	=/=/=	=/=	=/=/=	N/D
432	16435	*menA*	3337664	Yes	No	=/=/=	=/=	=/=/=	N/D
429	17090	*ptsG* (upstream)	3471290	Yes	No	=/=/=	=/=	=/=/=	N/D
414	02585	*rnfA*	516764	Yes	No	=/=/=	=/=	=/=/=	N/D
402	09115	*seqA* (upstream)	1834794	Yes	No	=/=/=	=/=	=/=/=	N/D
397	12895	*yaaA*	2621821	Yes	No	=/=/=	=/=	-/=/=	No
313	12735	*citF*	2589604	NS	NS	=/=/=	=/=	=/=/=	N/D
391	24345	*dedA*	4930513	NS	NS	=/=/=	=/=	+/=/-	Yes
384	08615	*eptA*	1737112	NS	NS	=/=/=	=/=	=/=/=	N/D
319	21620	*lysA*	4374918	NS	NS	=/=/=	=/=	=/=/=	N/D
390	24510	*nuoC*	4959426	NS	NS	=/-/=	-/=	+/=/=	Yes
427	19000	*outO*	3874380	NS	NS	=/=/=	=/=	=/=/=	N/D
387	25280	*yhbU*	5143577	NS	NS	=/=/=	=/=	=/=/-	Yes
411	26085		5339145	NS	NS	=/=/=	=/=	+/-/=	Yes
392	24205	*alaC*	4901736	NS	No	=/=/=	=/=	=/=/=	N/D
393	18575	*gntR*	3785100	NS	No	=/=/=	=/=	-/=/-	Yes
424	20040	*lsrR*	4061575	NS	No	=/=/=	=/=	=/=/=	N/D
389	24735	*rcsD*	5015417	NS	No	=/=/=	=/=	=/=/=	N/D
383	10885	*yajL*	2203841	NS	No	-/=/=	=/=	=/=/=	N/D
388	25040		5088156	NS	No	=/=/=	=/=	=/=/=	N/D
332*	09515		1911182	No	NS	=/=/=	=/=	=/=/=	N/D
354*	18500	*livG* (3' end)	3772015	No	No	N/A	N/D	N/A	N/D
330*	18500	*livG* (3' end)	3772108	No	No	N/A	N/D	N/A	N/D
306*	N/A	intergenic	3028431	N/D	N/D	N/A	N/D	N/A	N/D
321*	N/A	intergenic	374581	N/D	N/D	N/A	N/D	N/A	N/D

1. TnSeq Screen: Defect in WT: Yes refers to genes with statistically significant defect, NS (not significant) to genes with defect that was not statistically significant, and No to genes with no defect in WT mice in TnSeq screen

2. TnSeq Screen: Defect in PMN Dep: Yes refers to genes with statistically significant defect, NS (not significant) to genes with defect that was not statistically significant, and No to genes with no defect in PMN-depleted mice in TnSeq screen

3. M9+gluc/L/Low Iron vs. WT: Growth in indicated condition, where = refers to equal, - refers to no defect and + refers to enhanced growth compared to WT

4. Mucoviscosity/Capsule vs. WT: = refers to equal, - refers to no defect and + refers to enhanced mucoviscosity or capsule expression compared to WT

5. Res. To H2O2/HOCl/NO: = refers to equal, - refers to no defect and + refers to enhanced resistance against the indicated stress compared to the neutral strains

6. Defect *in vivo* as SS in WT mice: SS=single strain, Yes refers to a defect and No refers to no defect during single strain infections of WT mice

N/D: Not determined

N/A: Not Applicable

*=Neutral strain

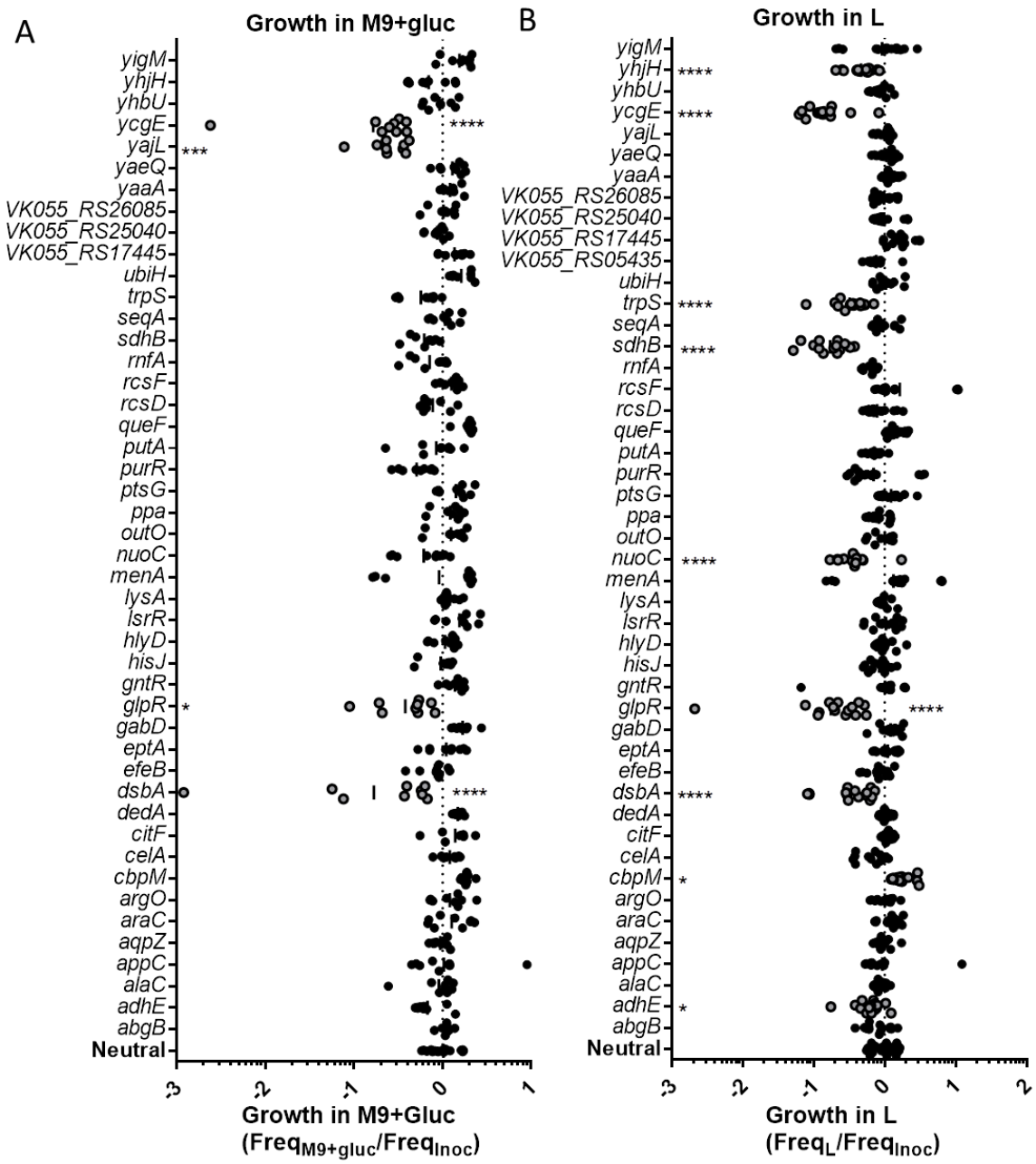
3.3 *Kp* REQUIRED *dsbA*, *yaeQ* AND *ycgE* FOR GROWTH IN NUTRIENT POOR CONDITIONS, WHILE *dsbA*, *ycgE*, *glpR*, *nuoC*, *sdhB*, *trpS*, AND *yhjH* MUTANTS WERE OUTCOMPETED IN NUTRIENT RICH CONDITIONS *IN VITRO*

To characterize the contribution to growth of the genes interrupted by our transposon mutants, the ability of the mutants to grow in nutrient-restrictive and non-restrictive conditions was assessed using minimal media containing glucose (M9+gluc) and low-salt Luria-Bertani broth (L), respectively **(Fig. 3-3)**. Each insertion mutant **(Table 3-2)** was cultured separately overnight in L and then pooled into a mini-library, where 50% of the pool consisted of equal amounts of the 3 neutral strains and the remaining 50% was divided equally by the remaining insertion mutants. This mini-library was used to inoculate M9+gluc and L, incubated at 37°C for 5 hrs with aeration, and then the bacteria from the samples were collected, processed and sequenced using Illumina. Growth in M9+gluc (**Fig. 3-3A)** and L **(Fig. 3-3B),** was calculated for each insertion mutant by comparing the frequency of each mutant after growth in the each media to its concentration in the inoculum. In nutrient-restricted conditions, *dsbA*, *yajL* and *ycgE* insertion mutants had a growth defect compared to the neutral mutants **(Fig. 3-3A).** All 3 of these mutants had defects in both WT and PMN-depleted mice in the TnSeq screen **(Tables 3-1** and **3-2).** Interestingly, *yajL* growth appeared normal in L broth, while *dsbA* and *ycgE* still grew slowly in L **(Fig. 3-3B),** as did mutants with no growth defect in M9+gluc with disruptions in *adhE, glpR, nuoC, sdhB, trpS,* and *yhjH* **(Fig. 3-3B).** Interestingly, none of the insertion mutants with defects in growth in M9+gluc or L when tested in competition in the mini-library **(Fig. 3-3A-B)** had defects in growth compared to *Kp* WT at 10 hr when grown as single strains under similar conditions (data not shown).

These results suggest the growth defects seen were a result of inter-strain competition rather than an inability of the mutants to grow under those conditions. Therefore, *dsbA, yajL, ycgE, adhE, glpR, nuoC, sdhB, trpS* and *yhjH* enhance *Kp* growth under certain conditions.

Figure 3- 3. *dsbA, glpR, nuoC, sdhB, trpS, yaeQ, ycgE* and *yhjH* enhance *Kp* growth *in vitro*

(A-B) A *Kp* mini-library of the insertion mutants listed in Table 3-2 consisting of 50% insertion mutants considered to have a neutral fitness in WT mice (MKP330, MKP332 and MKP354) and 50% of the remaining insertion mutants were grown overnight separately, pooled, and then used to inoculate (A) M9 minimal media+glucose (M9+gluc) and (B) L. These samples were incubated for 5 hrs at 37°C and then plated on L-Kan agar plates, as were the inoculums. Bacteria that grew on these plates were collected and processed to obtain gDNA. The gDNA was then sheared, barcoded and deep sequenced. The input inoculum was processed for gDNA and deep sequencing concurrently with the output bacteria. The growth in M9+Gluc and Growth in L were calculated by comparing the frequency of each insertion mutant before ($Freq_{Inoc}$) and after ($Freq_{M9+gluc}$ or $Freq_{L}$) infection using $Freq_{M9+gluc}/Freq_{Inoc}$ and $Freq_{L}/Freq_{Inoc}$, respectively. Each dot represents the fitness of a gene in a replicate and the geometric mean is indicated for each gene. Statistical significance was determined using the log-transformed values for each insertion mutant compared to the pooled Neutral strains values using One-way ANOVA with Dunnett's post-test. Data is representative of 2 independent experiments performed in triplicate.

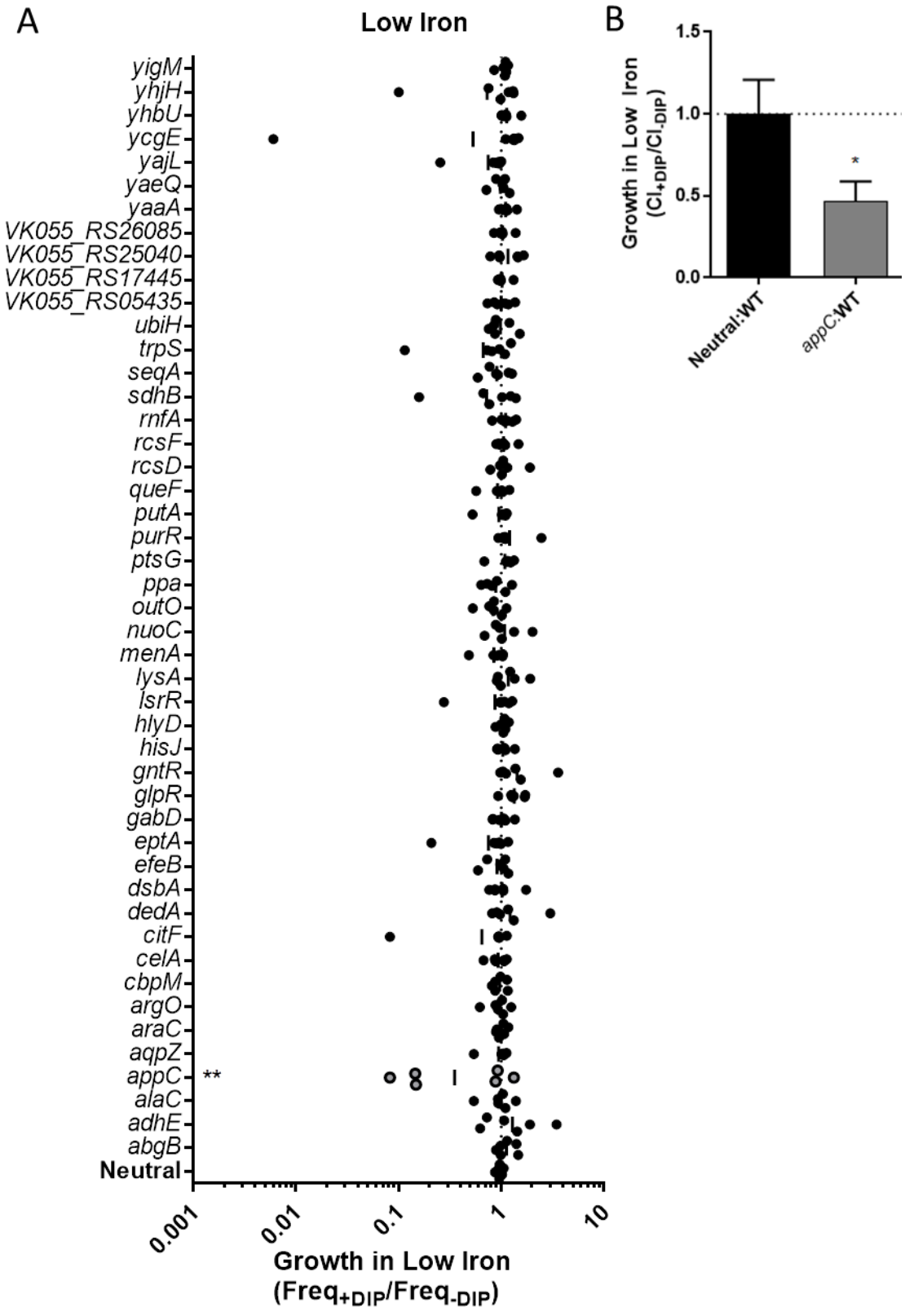

A
Growth in M9+gluc
yigM
yhjH
yhbU
ycgE
yajL
yaeQ
yaaA
VK055_RS26085
VK055_RS25040
VK055_RS17445
ubiH
trpS
seqA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC
menA
lysA
lsrR
hlyD
hisJ
gntR
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE
abgB
Neutral
***
****
*
****
-3
-2
-1
0
1
Growth in M9+Gluc
(Freq M9+gluc/Freq Inoc)
B
Growth in L
yigM
yhjH
yhbU
ycgE
yajL
yaeQ
yaaA
VK055_RS26085
VK055_RS25040
VK055_RS17445
VK055_RS05435
ubiH
trpS
seqA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC
menA
lysA
lsrR
hlyD
hisJ
gntR
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE
abgB
Neutral
****
****
****
****
****
****
****
*
*
-3
-2
-1
0
1
2
Growth in L
(Freq L/Freq Inoc)

3.4 *APPC* CONTRIBUTES TO *KP* GROWTH IN IRON-RESTRICTED CONDITIONS

Iron is an essential resource that bacteria, including *Kp*, must obtain from the environment in order to grow (339). However, during infection, iron availability is severely limited by the host, including through neutrophil-mediated means (54, 350, 351, 407), as a strategy to control pathogen growth (337-339). In order to determine if any of the genes in our mini-library aid *Kp* growth in an iron-restricted environment, an *in vitro* screen was performed, where the mini-library was concurrently grown for 5 hrs in L or L containing 0.13mM 2,2'-bipyridyl (DIP) (L+DIP), an iron chelator **(Fig. 3-4A)**. The samples were then deep sequenced and the ability of each insertion mutant to grow in iron-restricted conditions was assessed by comparing the frequency of each insertion mutant in L+DIP to the frequency of the same mutant in L alone. Of all the strains tested, only *appC* had a significant decrease in growth in low iron conditions compared to the neutral strains **(Fig. 3-4A).** To confirm this phenotype, *appC* was tested in 1:1 competition with WT *Kp* in the presence or absence of DIP and had a competitive index (CI) of 0.5 versus WT *Kp* **(Fig. 3-4B).** Combined, these findings support a role for *appC* in helping *Kp* thrive in iron-restricted conditions. Consistent with this, *appC* insertion mutants had a defect in WT mice that was restored upon removal of neutrophils in competition in the TnSeq screen **(Tables 3-1** and **3-2),** supporting the importance of PMN-dependent iron restriction in the lungs during *Kp* infection and the role of *appC* to enhance growth under these conditions.

Figure 3- 4. *appC* assists in *Kp* growth during iron restriction

(A) A *Kp* mini-library of the insertion mutants presented in Table 1 consisting of 50% neutral insertion mutants (MKP330, MKP332 and MKP354) and 50% of the remaining insertion mutants were grown overnight separately, pooled, and used to inoculate L in the absence or presence of 0.13mM iron chelator (DIP). These samples were incubated for 5 hrs at 37°C and then plated on L-Kan agar plates. Bacteria that grew on these plates were collected and processed to obtain gDNA. The gDNA was then sheared, barcoded and deep sequenced. Growth in Low Iron was calculated by comparing the frequency of each insertion mutant after growth in L ($Freq_{-DIP}$) and in L with DIP ($Freq_{+DIP}$) using $Freq_{+DIP}/Freq_{-DIP}$. Each dot represents a replicate and the geometric mean is indicated for each gene. Statistical significance was assessed for $Freq_{+DIP}/Freq_{-DIP}$ for each insertion mutant tested against the Neutral using One-way ANOVA on the log-transformed values with Dunnett's post-test. Data is representative of two independent experiments with each sample in triplicate. $Freq_{-DIP}$ values were also used in Figure 2 as $Freq_L$. **(B)** The indicated strains of *Kp* were grown overnight separately and used to inoculate L or L+0.26mM DIP in a 1:1 mixture of either the Kan^R *Kp* insertion mutant MKP330 (Neutral) or MKP403 (*Kp appC)*:KanS *Kp* WT. The samples were incubated for 5 hr at 37°C, and then plated on L and L-Kan to determine bacterial load of each strain in each sample. Growth in Low Iron was calculated using ((Kan^R CFU in L_{+DIP}/Kan^S CFU in L_{+DIP})/(Kan^R CFU in L_{-DIP} /Kan^S CFU in L_{-DIP})) and normalizing to the Neutral values. Statistical significance was calculated against the Neutral:WT values by One-way ANOVA followed by Dunnett's post-test. Data is from 3 independent experiments, where samples were tested at least in duplicate.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Low Iron
yigM
yhjH
yhbU
ycgE
yajL
yaeQ
yaaA
VK055_RS26085
VK055_RS25040
VK055_RS17445
VK055_RS05435
ubiH
trpS
seqA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC
menA
lysA
lsrR
hlyD
hisJ
gntR
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE
abgB
Neutral
**
0.001
0.01
0.1
1
10
Growth in Low Iron
(Freq+DIP/Freq-DIP)
B
Growth in Low Iron
(Cl+DIP/Cl-DIP)
1.5
1.0
0.5
0.0
*
Neutral:WT
appC:WT

3.5 *Kp nuoC, putA, sdhB* AND *ycgE* POSITIVELY AFFECT MUCOVISCOSITY

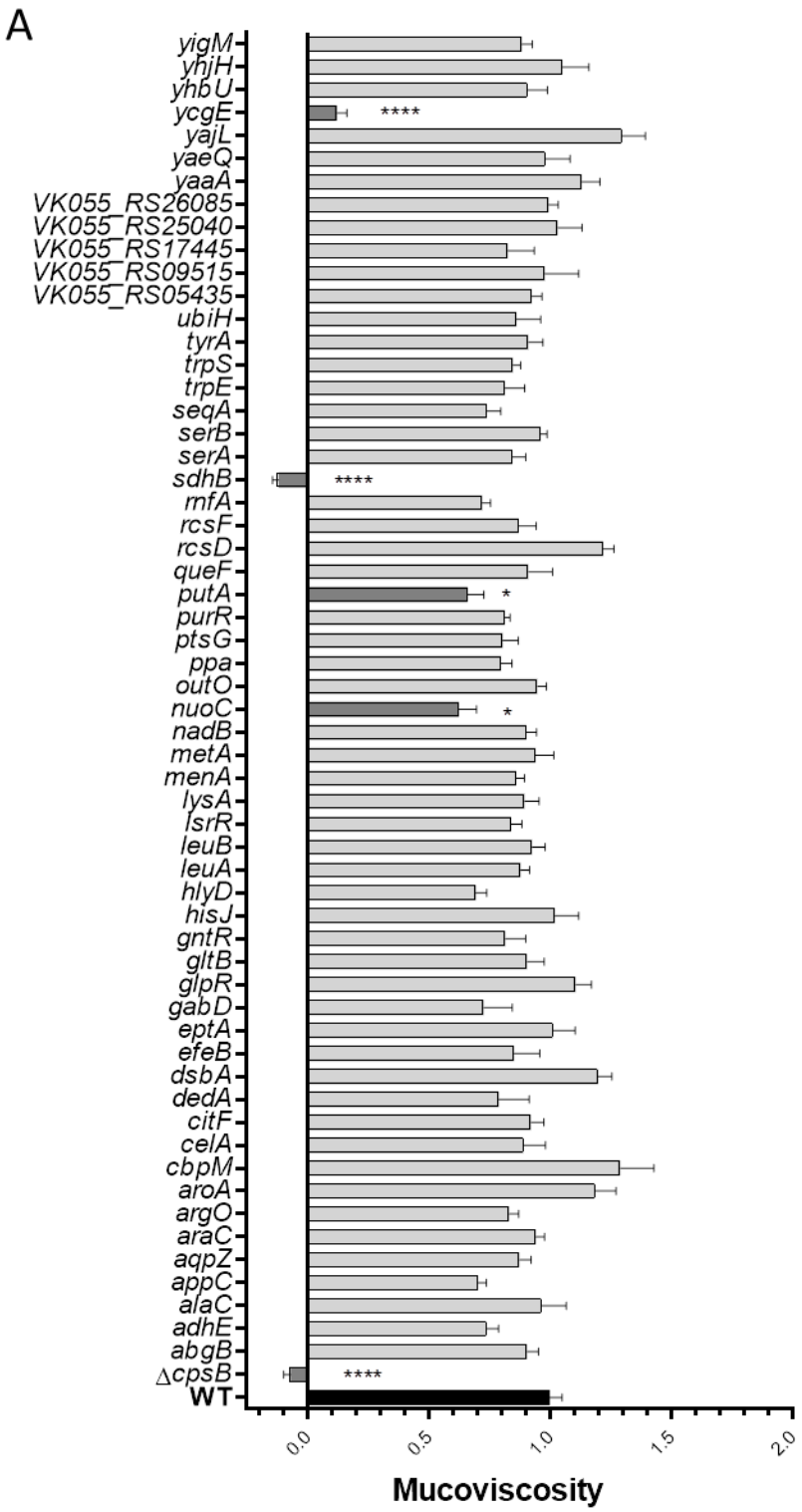
Capsule is a major virulence factor of *Kp* in the lungs, as well as other tissues (43, 177, 218, 408). Due to the general importance of capsule and capsular material in *Kp* infections and its role in increasing *Kp* fitness in the face of neutrophils (220, 224, 250), the amount of capsular and extracapsular polysaccharide produced, i.e. mucoviscosity, of the insertion mutants **(Table 3-2)** was measured using a previously published method of quantification (224, 384). First, the amount of released capsule material was measured **(Fig. 3-5A** and **see Materials and Methods).** Compared to WT *Kp*, disruption of *nuoC* and *putA* resulted in a moderate defect in mucoviscosity with an approximately 25% decrease compared to WT *Kp*. By contrast, disruption of *sdhB* and *ycgE* resulted in a much more dramatic loss of mucoviscosity of at least 90% compared to WT *Kp* **(Fig. 3-5A)**. As expected, an acapsular mutant, *Kp* Δ*cpsB*, had no mucoviscosity.

To determine if the decrease in mucoviscosity observed in the *nuoC, putA, sdhB* and *ycgE* insertion mutants were due to a lack of capsule synthesis, the presence of capsule associated with the bacteria for each of these strains was evaluated by microscopy using India ink. In this assay, the polysaccharide capsule excludes the India ink, forming a halo around the bacteria **(Fig. 3-5B).** There was a noticeable contrast between WT *Kp* and Δ*cpsB,* where WT *Kp* had a thick capsule around each bacterium, while the India ink appeared to abut the bacterial walls of the Δ*cpsB* strain **(Fig. 3-5B)**. Surprisingly, none of the insertion mutants tested lacked capsule, despite the significant decrease in mucoviscosity observed **(Fig. 3-5A-B)**. Taken together, *nuoC, putA, sdhB* and *ycgE* produce less extracapsular polysaccharide than WT *Kp*, which may reflect interference with capsule regulation or inability to obtain resources integral to exopolysaccharide

production. Because an increased capsule expression is correlated with an increase in *Kp* virulence (254, 335), the reduced release of capsule matter suggests that capsule production may be reduced under some conditions and thereby provide a link between these genes and *Kp* virulence *in vivo.* Alternatively, because *sdhB* and *ycgE* grew more slowly, the reduced mucoviscosity could be a reflection of a slower growth rate. Interestingly, none of these mutants had a clear restoration of fitness upon depletion of neutrophils in the TnSeq screen **(Tables 3-1** and **3-2),** supporting the idea that their defects in mucoviscosity reflect inefficiencies of growth regardless of the PMN-mediated immune environment.

Figure 3- 5. *nuoC, putA, sdhB* and *ycgE* enhance *Kp* mucoviscosity

(A-B) *Kp* WT, Δ*cpsB* and the indicated transposon insertion mutants were grown overnight in L. (A) The bacterial densities (OD_{600}) were then measured for each sample (i.e. OD_{600}Total), and the supernatant obtained by centrifuging each sample at 1,000 g for 5 min (i.e. OD_{600} Supernatant). The amount of capsule produced for each strain was calculated using (OD_{600} Supernatant)/(OD_{600} Total), and the values of each sample were normalized against *Kp* WT. Each sample was tested in triplicate in 2 independent experiments and Mean+SEM is presented. Statistical significance was determined using One-way ANOVA with Dunnet's post-test comparing each strain tested to *Kp* WT. (B) Each of the indicated strains was diluted in India ink and visualized by phase contrast-microscopy. Exclusion of India ink around a bacterium is indicative of capsule. A representative image is shown of each strain. Each strain was assessed in 2 independent experiments.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yaaA
VK055_RS26085
VK055_RS25040
VK055_RS17445
VK055_RS09515
VK055_RS05435
ubiH
tyrA
trpS
trpE
seqA
serB
serA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC
nadB
metA
menA
lysA
lsrR
leuB
leuA
hlyD
hisJ
gntR
gltB
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
aroA
argO
araC
aqpZ
appC
alaC
adhE
abgB
ΔcpsB
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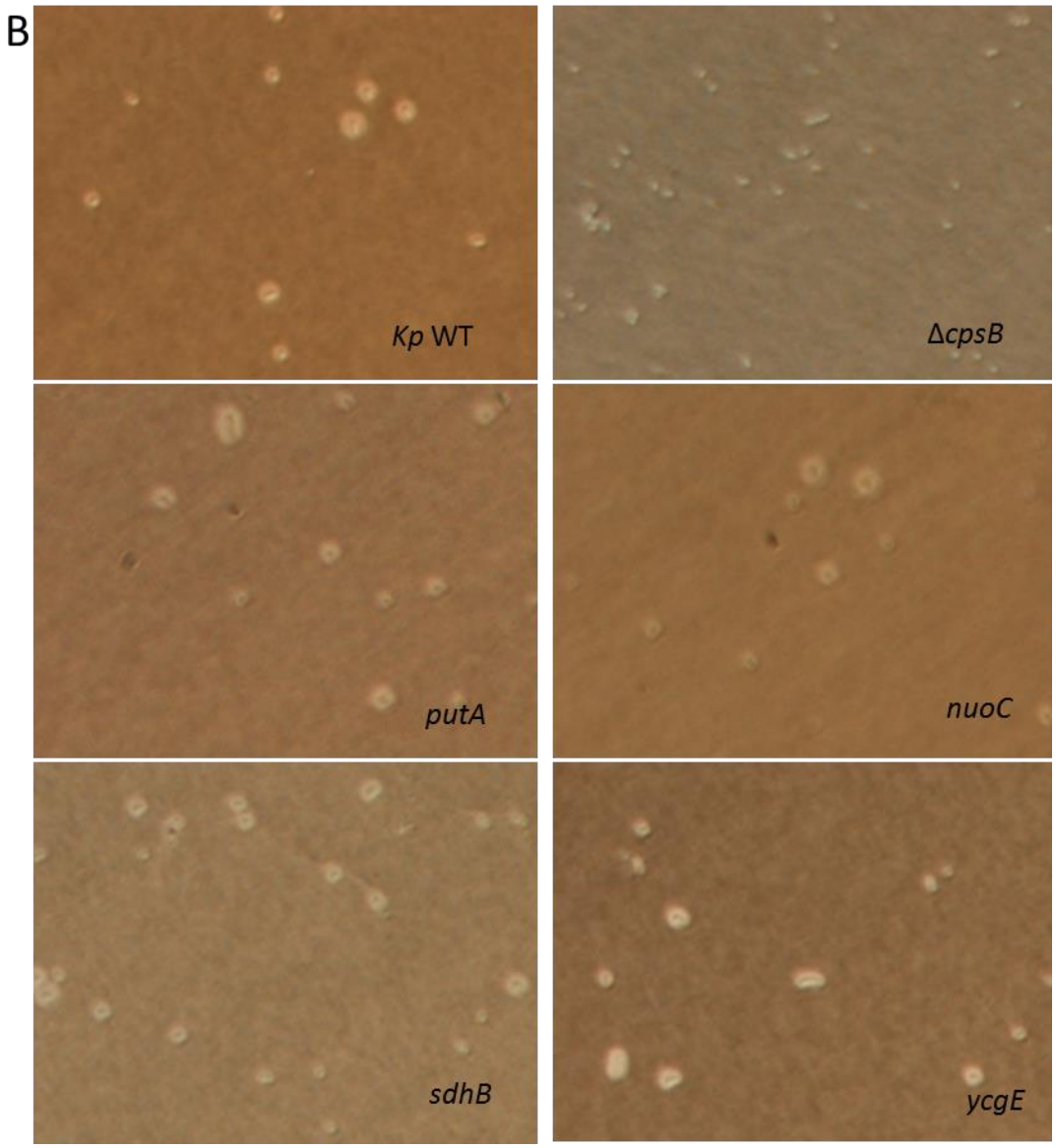
****
****
****
*
*
0.0
0.5
1.0
1.5
2.0
Mucoviscosity


B
Kp WT
ΔcpsB
putA
nuoC
sdhB
ycgE

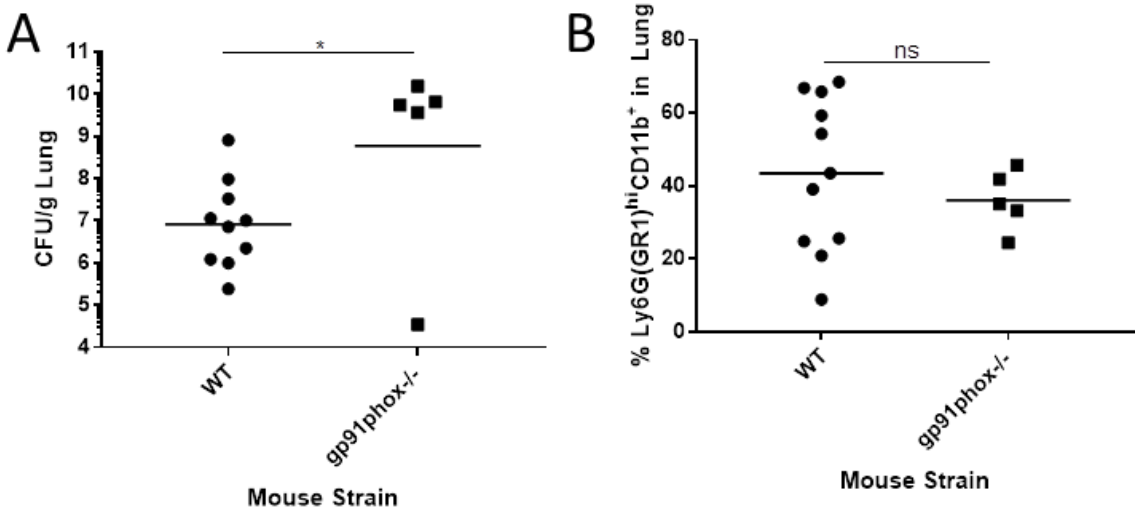
3.6 PHAGOCYTE DERIVED-REACTIVE OXYGEN SPECIES LIMIT *KP* LUNG INFECTION

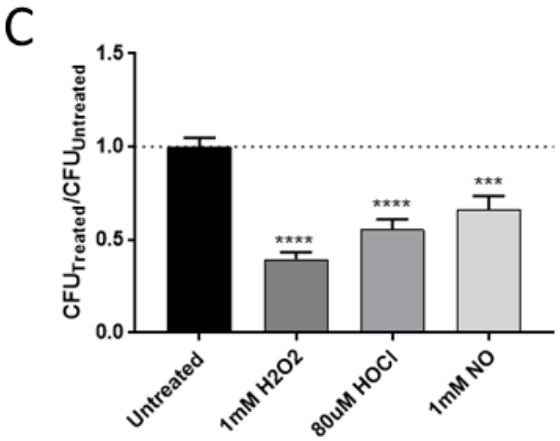
Neutrophils employ a number of methods to restrict pathogens, including the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (181, 409). ROS and RNS directly and indirectly damage and kill bacteria with efficiencies that varies by pathogen (410). To establish if *Kp* growth was limited in the lungs by ROS production, C57Bl/6 and gp91phox-/- mice, which lack phagocytic superoxide production due to a missing NADPH oxidase component (411) and, thus, fail to generate ROS (e.g. H_2O_2 and HOCl) (168), were retropharyngeally infected (384) with 1,500 CFU *Kp* WT. At 45 hpi, an average bacterial load of approximately 10^7 CFU/g lung was recovered in WT mice **(Fig. 3-6A).** Strikingly, the bacterial load in the gp91phox-/- mice was at least 100-fold higher, despite the presence of an equivalent neutrophil population in the lungs of both cohorts **(Fig. 3-6B).** This indicates that ROS production by phagocytes inhibits *Kp* lung infection, either directly or indirectly through its downstream effectors.

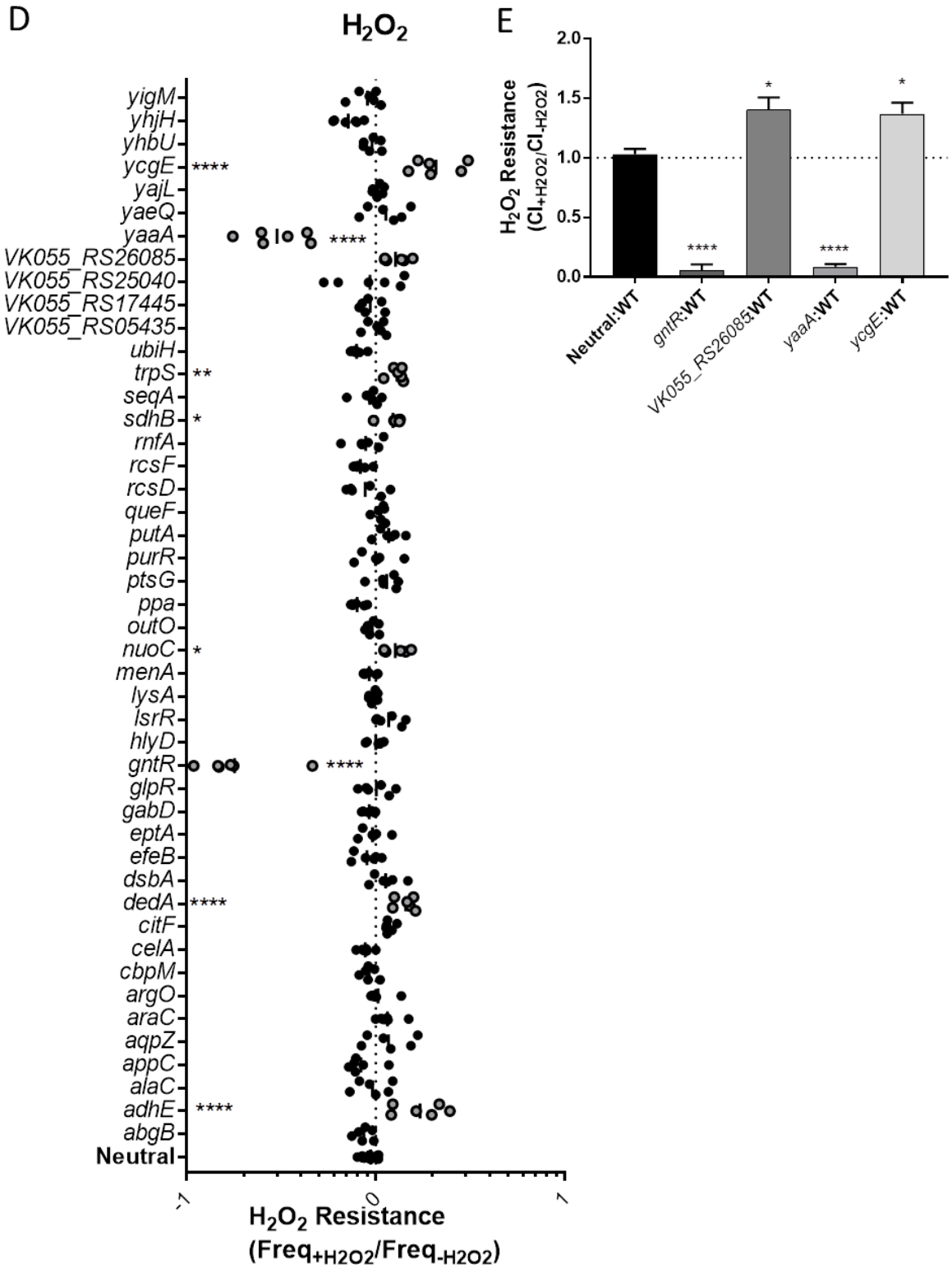
Figure 3- 6. *Kp* lung infection is limited by ROS production *in vivo,* and survival *in vitro* is promoted against H_2O_2 by *gntR* and *yaaA* and HOCl by *VK055_RS17445, VK055_RS26085* and *yhjH*

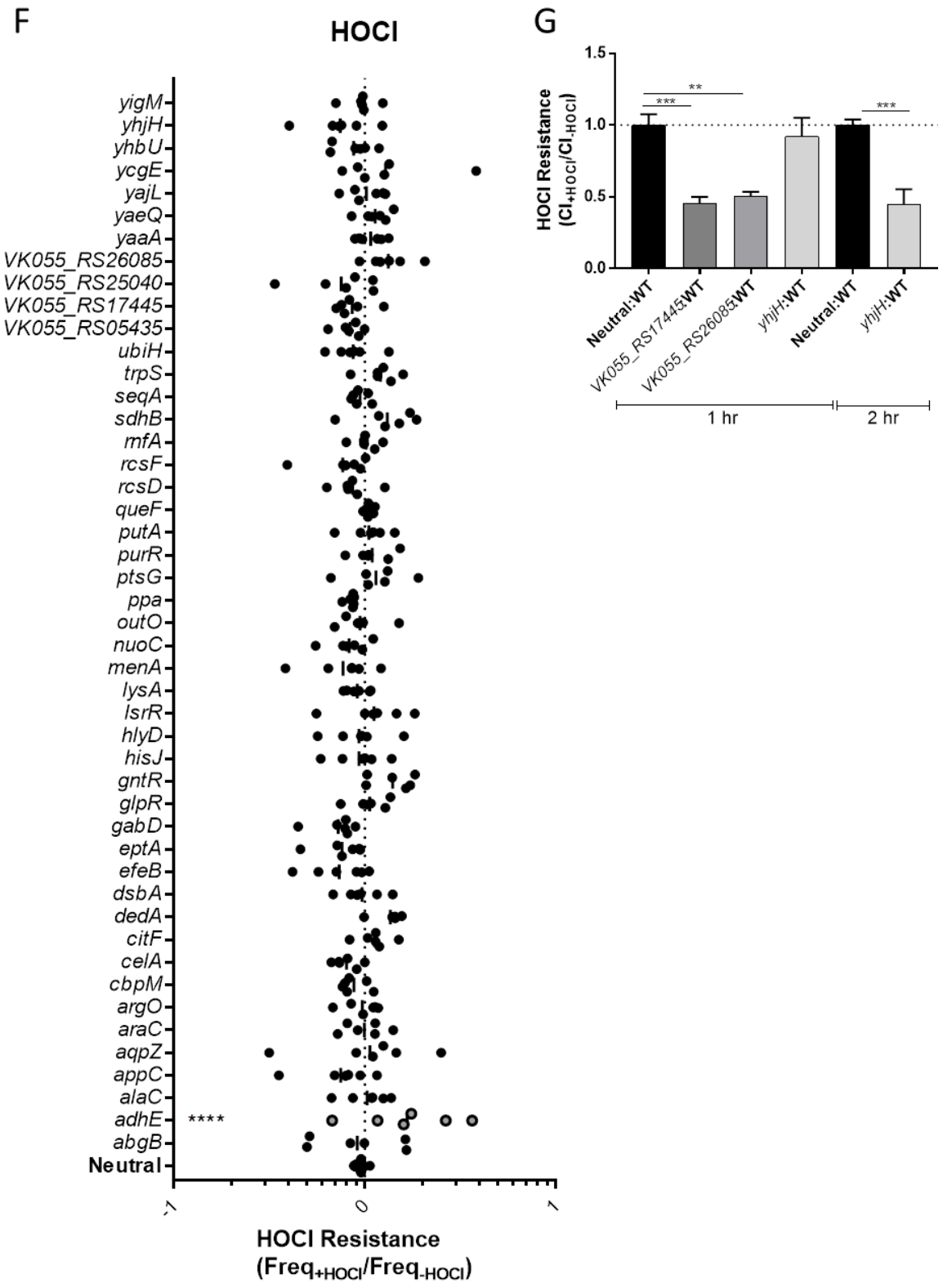
(A) C57Bl/6 and gp91phox-/- mice were intranasally infected with 3,000 CFU of 1:1 *Kp* WT (Kan^S) and a *Kp* transposon insertion mutant (Kan^R). At 45 hpi, the lungs were harvested, homogenized, (A) plated in serial dilutions to obtain the CFU/g Lung and (B) analyzed by flow cytometry to quantify the neutrophil population (% $Ly6G^{hi}CD11b^+$ within the live population). The *Kp* WT CFU (Kan^S) values are presented. (C, D+F) A *Kp* mini-library of the insertion mutants presented in Table 1 consisting of 50% neutral insertion mutants (MKP330, MKP332 and MKP354) and 50% of the remaining insertion mutants were grown overnight separately, pooled, diluted, grown in L until in mid-log phase, and used to inoculate (C, D+F) M9+gluc, (C+D) M9+gluc+ 1mM H_2O_2 and/or (C+F) M9+gluc+ 80 μM HOCl. Samples were incubated for 1 hr and then plated on L-Kan plates. Bacteria that grew on these plates were quantified, collected and processed to obtain gDNA that was subsequently deep sequenced. (C) Bacterial survival was calculated using $CFU_{Treated}/CFU_{Untreated}$ and then normalized against Untreated. Each sample was tested in triplicate in 2 independent experiments. Mean+SEM is presented and statistical significance was assessed using One-Way ANOVA with Dunnet's post-test comparing each condition to Untreated. (D+F) The resistance of each insertion mutant indicated in H_2O_2 and HOCl was assessed by comparing the proportion of each insertion mutant in (D) H_2O_2 ($Freq_{+H2O2}$) or (F) HOCl ($Freq_{+HOCl)}$ to the proportion in the (D+F) M9+gluc alone ($Freq_{-H2O2}$) or ($Freq_{-HOCl}$) using (D) H_2O_2 Resistance=$Freq_{+H2O2}/Freq_{-H2O2}$ and (F) HOCl Resistance= ($Freq_{+HOCl}/Freq_{-HOCl}$). Each dot represents 1 replicate, where the mini-library was tested in each condition in triplicate in 2 independent experiments.

Note the $Freq_{-H2O2}$ and $Freq_{-HOCl}$ values were the same and also used as $Freq_{-NO}$ in Figure 3-9. Statistically significant differences were determined by comparing each insertion mutants compared to the pooled Neutral insertion mutants using One-Way Anova with Dunnet's multiple-comparisons post-test on the log-transformed values. (E+G) *Kp* WT (Kan^S) and the indicated insertion mutants (Kan^R), where Neutral refers to MKP330, were mixed at a 1:1 ratio, incubated in M9+glucose in the presence or absence of (E) 1 mM H_2O_2 or (G) 80 μM HOCl for 1 hr or 2 hr, and then plated in serial dilutions on L and L-Kan. The CFU were quantified, the competitive index (CI) was calculated using CI=Kan^R CFU/Kan^S CFU, and H_2O_2 and HOCl resistance were calculated using (E) H_2O_2 Resistance=$(CI_{+H2O2})/(CI_{-H2O2})$ and (G) HOCl Resistance=$(CI_{+HOCl})/(CI_{-HOCl})$, respectively, and normalized against the Neutral. Each strain was tested at least in duplicate in 2 independent experiments, and statistical significance was determined using One-Way ANOVA with Sidak's multiple-comparison post-test against Neutral:WT.


A
CFU/g Lung
11
10
9
8
7
6
5
4
*
WT
gp91phox-/-
Mouse Strain
B
% Ly6G(GR1)hiCD11b+ in Lung
80
60
40
20
0
ns
WT
gp91phox-/-
Mouse Strain


C
CFUTreated/CFUUntreated
1.5
1.0
0.5
0.0
****
****
***
Untreated
1mM H2O2
80uM HOCl
1mM NO


D
H2O2
yigM
yhjH
yhbU
ycgE ****
yajL
yaeQ
yaaA ****
VK055_RS26085
VK055_RS25040
VK055_RS17445
VK055_RS05435
ubiH
trpS **
seqA
sdhB *
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC *
menA
lysA
lsrR
hlyD
gntR ****
glpR
gabD
eptA
efeB
dsbA
dedA ****
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE ****
abgB
Neutral
-1
0
1
H2O2 Resistance
(Freq+H2O2/Freq-H2O2)
E
H2O2 Resistance
(CI+H2O2/CI-H2O2)
2.0
1.5
1.0
0.5
0.0
****
*
****
*
Neutral:WT
gntR:WT
VK055_RS26085:WT
yaaA:WT
ycgE:WT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VK055_RS05435
ubiH
trpS
seqA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
ppa
outO
nuoC
menA
lysA
lsrR
hlyD
hisJ
gntR
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE
abgB
Neutral
****
-1
0
1
HOCl Resistance
(Freq+HOCl/Freq-HOCl)
G
HOCl Resistance
(Cl+HOCl/Cl-HOCl)
1.5
1.0
0.5
0.0
**
***
***
Neutral:WT
VK055_RS17445:WT
VK055_RS26085:WT
yhjH:WT
Neutral:WT
yhjH:WT
1 hr
2 hr

3.7 *Kp* GENES *gntR* AND *yaaA* PROTECT AGAINST H_2O_2, WHILE *VK055_RS17445*, *VK055_RS26085* AND *yhjH* ENHANCE FITNESS AGAINST HOCl

Since ROS inhibited *Kp* infection in our lung infection model, the *Kp* mini-library was screened *in vitro* for genes that are important for combatting H_2O_2 and HOCl, the major types of ROS produced by phagocytes (181, 409). The *Kp* mini-library was cultured overnight, pooled and incubated in M9+gluc alone or in the presence of H_2O_2 or HOCl for 1 hr, and deep sequenced. A decrease in the overall number of colonies in the treated samples compared to the untreated served as a positive control of the effectiveness of the H_2O_2 and HOCl **(Fig. 3-6C)**. Specifically, 1 mM H_2O_2 killed about 60% of the pooled *Kp* population, while 80 μM of HOCl killed 40% compared to the untreated samples **(Fig. 3-6C)**. The individual insertion mutants, *gntR* and *yaaA* mutants had survivals of only 10% and 25% in H_2O_2, respectively, compared to WT *Kp* **(Fig. 3-6D),** suggesting these factors protect *Kp* against H_2O_2. Interestingly, the disruption of a few genes increased *Kp* fitness in H_2O_2, including *adhE*, *dedA*, *nuoC*, *trpS*, *VK055_RS26085* and *ycgE* **(Fig. 3-6D)**. When the insertion mutants for *gntR, yaaA, VK055_RS26085* and *ycgE* were tested in a 1:1 competition with WT *Kp*, their phenotypes paralleled the *in vitro* H_2O_2 screen **(Fig. 3-6D-E).** Interestingly, the fitness defect noted in WT mice for both *gntR* and *yaaA* mutants was abrogated in PMN-depleted mice in the TnSeq screen **(Supp. Tables 3-1** and **3-2),** suggesting that *gntR* and *yaaA* be important *in vivo* to survive PMN-derived ROS and supporting the TnSeq screen results.

When the mini-*Kp* library was exposed to HOCl, no mutants had significant survival defects **(Fig. 3-6F).** However, several mutants had a modest defect, such as in *VK055_RS17445, VK055_26085* and *yhjH* **(Fig. 3-6F).** Interestingly, the *adhE* mutant

displayed a slightly, but significantly, enhanced *Kp* fitness in the presence of HOCl as it does in H_2O_2 **(Fig. 3-6D+F).** Evaluating 3 mutants with modest defects in a 1:1 competition with WT *Kp* in HOCl showed that *VK055_RS17445, VK055_RS26085* and *yhjH* mutants each had a 50% decrease in fitness, after 1 or 2 hrs of exposure to HOCl **(Fig. 3-6G).** The results parallel the moderate phenotype seen in competition for these strains in HOCl **(Fig. 3-6F).** All 3 of these mutants had borderline defects in PMN-depleted mice, therefore, it is unclear if their potential roles in HOCl resistance explains their potential defect in lung infections **(Tables 3-1** and **3-2).**

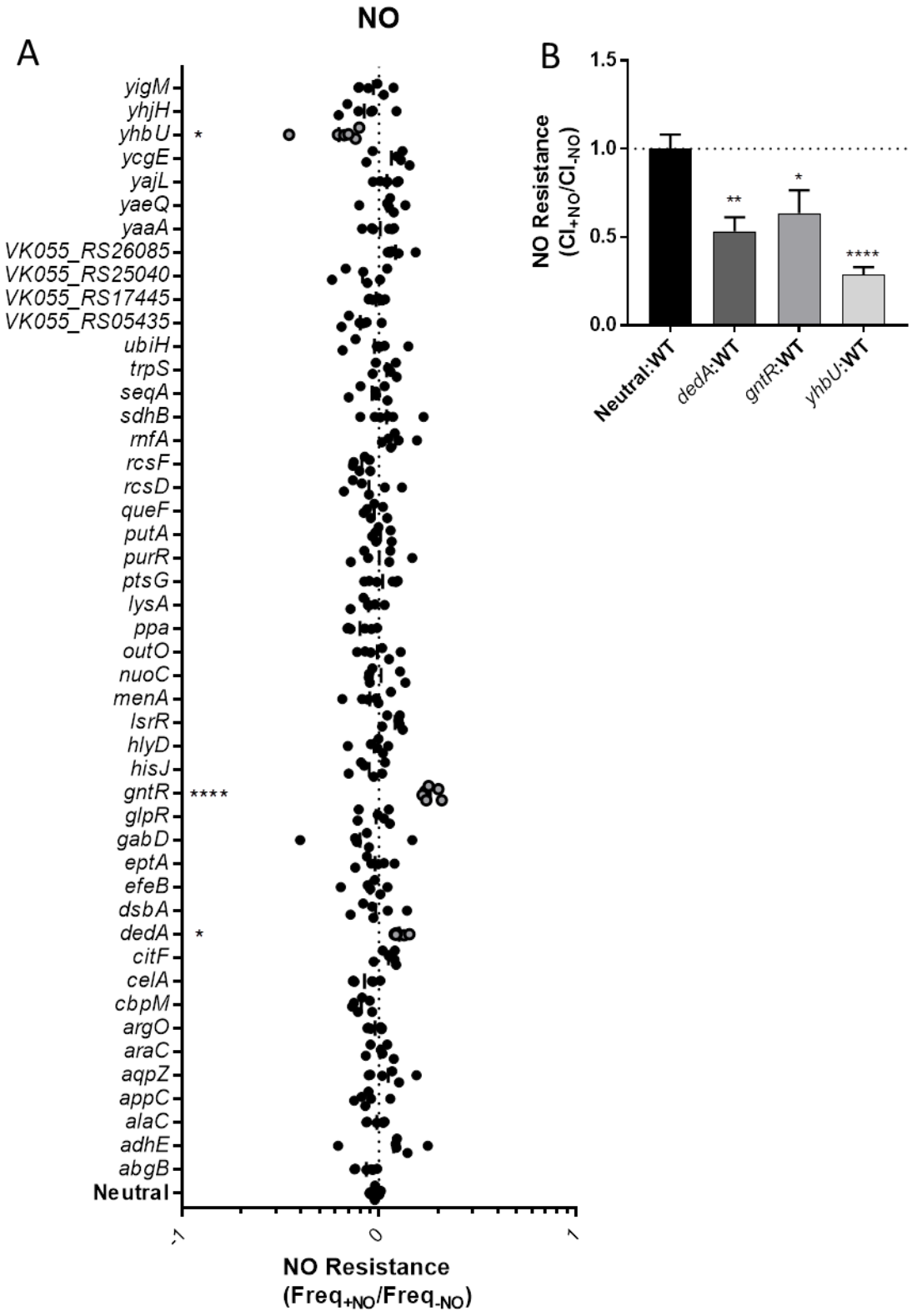
3.8 *YHBU,DEDA* AND *GNTR* ENHANCE *KP* RESISTANCE TO NO

To determine if any of the *Kp* mutants exhibited increased sensitivity to nitrosative stress, the mini-library was grown in M9 glucose in the presence of 1 mM DETA-NONOate, a NO donor, for 1 hr. Under these conditions, roughly 70% of the *Kp* population survived **(Fig. 3-6C).** . The *ybhU* insertion mutant was approximately 40% more sensitive to NO than the neutral mutants, while the *gntR* and *dedA* mutants were 10% and 5% more resistant, respectively **(Fig. 3-7A).** The phenotype of these insertion mutants was then analyzed in a 1:1 competition with WT *Kp* and the *yhbU* mutant was again sensitive to NO **(Fig. 3-7B).** However, surprisingly, both the *gntR* and *dedA* mutants had a decrease in fitness compared to the neutral mutant when exposed to NO, suggesting that when these mutants constitute a larger part of the bacterial population, the loss of these genes is detrimental to *Kp* fitness against RNS. Curiously, the *yhbU* mutant had a fitness defect in both WT and PMN-depleted mice in the TnSeq screen that was not statistically significant **(Tables 3-1** and **3-2).** This moderate defect of *yhbU* mutants *in vivo* and their

increased sensitivity to NO suggest that NO may not be a major PMN-derived selection in the lungs.

Figure 3- 7. *yhbU, dedA* and *gntR* play a role in protecting *Kp* against NO

(A) A *Kp* mini-library of the 62 insertions mutants presented in Table 1 consisting of 50% neutral insertion mutants (MKP330, MKP332 and MKP354) and 50% of the remaining insertion mutants were grown overnight separately, pooled, diluted, grown in L until in mid-log phase, and used to inoculate M9+gluc with or without 1mM DETA-NONOate. Samples were incubated for 1 hr and then plated on L-Kan plates. Bacteria that grew on these plates were collected and processed to obtain gDNA that was subsequently deep sequenced. The NO resistance of each insertion mutant indicated was calculated by comparing the proportion of each insertion mutant in the 1mM NO ($Freq_{+NO}$) to the proportion in the M9+gluc alone ($Freq_{-NO}$) using NO Resistance=$Freq_{+NO}/Freq_{-NO}$). Each mutant was tested in each condition in triplicate in 2 independent experiments. Note the $Freq_{-NO}$ values were also used as $Freq_{-H2O2}$ and $Freq_{-HOCl}$ in Figure 3-8D+F. Statistically significant differences were determined between each tested insertion mutant compared to the Neutral using One-Way Anova with Dunnet's multiple-comparisons post-test on the log-transformed values. (B) *Kp* WT (Kan^S) and the indicated insertion mutants (Kan^R), where Neutral refers to MKP330, were mixed at a 1:1 ratio, incubated in M9+glucose in the presence or absence of 1mM DETA-NONOate for 1 hr, and then plated in serial dilutions on L and L-Kan. The CFU were quantified, the competitive index (CI) was calculated using CI=Kan^R CFU/Kan^S CFU, and NO resistance was calculated using NO Resistance=(CI_{+NO})/(CI_{-NO}) and normalized against the Neutral. Each strain was tested at least in duplicate in 2 independent experiments, and statistical significance was determined using One-Way ANOVA with Sidak's multiple-comparison post-test against the Neutral:WT values.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yajL
yaeQ
yaaA
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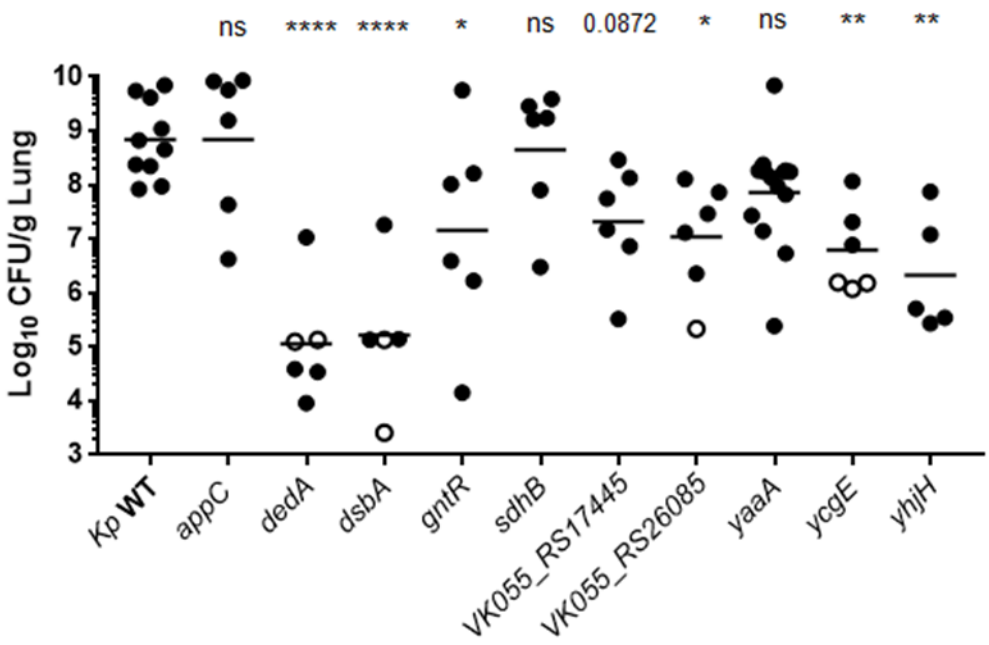
VK055_RS25040
VK055_RS17445
VK055_RS05435
ubiH
trpS
seqA
sdhB
rnfA
rcsF
rcsD
queF
putA
purR
ptsG
lysA
ppa
outO
nuoC
menA
lsrR
hlyD
hisJ
gntR
glpR
gabD
eptA
efeB
dsbA
dedA
citF
celA
cbpM
argO
araC
aqpZ
appC
alaC
adhE
abgB
Neutral
*
****
*
-1
0
1
NO Resistance
(Freq+NO/Freq-NO)
B
NO Resistance
(Cl+NO/Cl-NO)
1.5
1.0
0.5
0.0
**
*
****
Neutral:WT
dedA:WT
gntR:WT
yhbU:WT

3.9 *Kp* REQUIRES *dedA*, *dsbA*, *gntR*, *VK055_RS17445*, *VK055_RS26085*, *ycgE* AND *yhjH* *IN VIVO* IN THE LUNGS FOR VIRULENCE DURING SINGLE STRAIN INFECTIONS

To determine if mutants with *in vitro* virulence-related functional defects were impaired in colonizing lungs in single strain infections, Swiss-Webster mice were intranasally infected with 1,000 CFU of the indicated strains and the bacterial load in the lungs at 45 hpi was measured. The *dedA* and *dsbA* mutants had the most drastic defect with an approximately 10,000-fold decrease in bacterial load compared to the WT strain bacterial load of 10^9 CFU/g lung, while the *gntR, VK055_RS17445, VK055_26085, ycgE* and *yhjH* transposon mutants were approximately 100-fold lower than WT *Kp* **(Fig. 3-8)**. Interestingly, in the TnSeq screen, *dedA, gntR,* and *VK055_RS26085* mutants had fitness defects in WT mice that were not considered statistically significant **(Tables 3-1** and **3-2,** and **Fig. 3-8).** The *appC, sdhB* and *yaaA* mutants did not have defects in single strain infections **(Fig. 3-8)**, unlike in the TnSeq screen **(Tables 3-1** and **3-2)**, suggesting that, while these genes likely enhance *Kp* fitness, such as by enhancing growth in the case of *appC* and *sdhB* **(Fig. 3-3** and **3-4)** these genes are not needed to cause a successful infection of the lungs as single strains **(Tables 3-1** and **3-2).** Their observed defect in the TnSeq screen may have been due to an inability to compete as well for certain resources, such as iron in the case of the *appC* mutant **(Fig. 3-4A-B)**, compared to the other *Kp* mutants. Overall, this study provides evidence for *dedA, dsbA, gntR, VK055_RS17445, VK055_RS26085, ycgE* and *yhjH* as novel *Kp* virulence factors in the lungs, as well as suggests putative functional roles in growth and protection against the immune response for these factors.

Figure 3- 8. *Kp* requires *dedA, dsbA, gntR, VK055_RS17445, VK055_RS26085, ycgE* and *yhjH in vivo* in the lungs for virulence during single strain infections

Swiss Webster mice were intranasally infected with 1,000 CFU of *Kp* WT or the indicated transposon insertion mutant, and sacrificed at 45 hpi. The lungs were collected, homogenized and plated in serial dilutions to determine the CFU/g Lung. Each dot represents a mouse, and open dots represent the limit of detection where no CFU were recovered. Statistical significance was determined using One-Way ANOVA with Dunnet's post-test comparing each transposon insertion mutant to *Kp* WT.



CHAPTER 4: IDENTIFICATION AND CHARACTERIZATION OF *KLEBSIELLA PNEUMONIAE* METABOLIC GENES AS VIRULENCE DETERMINANTS IN THE LUNGS AND THEIR POTENTIAL AS THERAPEUTIC TARGETS

Experiments in Figure 4-4A were performed by Rebecca Silver

4.1 *Kp* REQUIRES *aroA*, *gltB*, *leuA*, *leuB*, *metA*, *nadB*, *serA*, *serB*, *trpE* AND *tyrA* FOR THE SYNTHESIS OF AMINO ACIDS AND METABOLITES

A number of *Kp* genes with putative roles in metabolism, *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA,* were identified as potential *Kp* virulence determinants for lung infection in a TnSeq screen of 13,056 transposon insertion mutants (Chapter 3 and **Table 4-1**). To evaluate the contribution of these genes to growth *in vitro*, strains carrying transposon insertions in these genes were assessed for their ability to grow under different conditions. First, they were assessed for their relative abilities to grow in nutrient poor and rich environments using minimal media containing glucose (M9+gluc) and low-salt Luria-Bertani broth (L), respectively in a competition experiment **(Fig. 4-1A-B)**. Compared to 3 *Kp* strains with transposon insertions in sites that are not predicted to have any role in metabolism, all of the potential metabolic mutants had a significant defect in growth in M9+gluc. The *aroA* mutant had the largest growth defect of approximately 750-fold lower than the neutral mutants, while the remaining mutants had defects ranging from 5-10-fold (*gltB* and *nadB*) to 50-100-fold (*leuA, leuB, metA, serA, serB, trpE* and *tyrA*) **(Fig. 4-1A).** Importantly, none of these mutants had growth defects in L broth, except for a mild defect seen in the *aroA* mutant **(Fig. 4-1B).**

To determine if these mutants were also defective for growth in M9+gluc when they were grown in isolation, each mutant was grown in L broth and then diluted into M9+gluc **(Fig. 4-1C)** or L **(Fig. 4-1D)**. Bacterial density (OD_{600}) was measured every 15 mins for 10 hr and the OD_{600} at 3 hr (when the WT *Kp* strain reached the end of exponential phase) and at 10 hr was assessed. In M9+gluc, all but one mutant, *gltB,* had growth defects during both early growth and the final time-point assessed at 3 and 10 hr,

respectively **(Fig. 4-1C).** The *gltB* mutant had a defect coming out of lag phase at 3 hr but recovered by 10 hr **(Fig. 4-1C).** That these genes are necessary to synthesize or acquire certain metabolites is supported by the finding that all the insertion mutants had no or extremely mild growth defects when grown in L broth as single strains compared to *Kp* WT in L **(Fig. 4-1D).**

Table 4- 1. *Kp* transposon insertion mutants in mini-library

Strain Name	Insertion site	Locus (VK055_RS)	Gene Name	TnSeq Screen: Defect in WT[1]	TnSeq Screen: Defect in PMN Dep[2]
MKP405	1554222	07750	*aroA*	Yes	Yes
MKP300	3960606	19495	*gltB*	Yes	Yes
MKP302	2533411	12495	*leuA*	Yes	NS
MKP315	2535057	12500	*leuB*	NS	Yes
MKP435	3157317	15550	*metA* (upstream)	Yes	NS
MKP419	4706048	23280	*nadB*	Yes	NS
MKP307	4234843	20925	*serA*	Yes	Yes
MKP439	2644463	12995	*serB*	Yes	Yes
MKP408	1197021	05995	*trpE*	Yes	Yes
MKP359	4678295	23160	*tyrA*	Yes	NS
MKP332*	1911182	09515		No	NS
MKP354*	3772015	18500	*livG* (3' end)	No	No
MKP330*	3772108	18500	*livG* (3' end)	No	No
MKP306*	3028431	N/A	intergenic	N/D	N/D
MKP321*	374581	N/A	intergenic	N/D	N/D

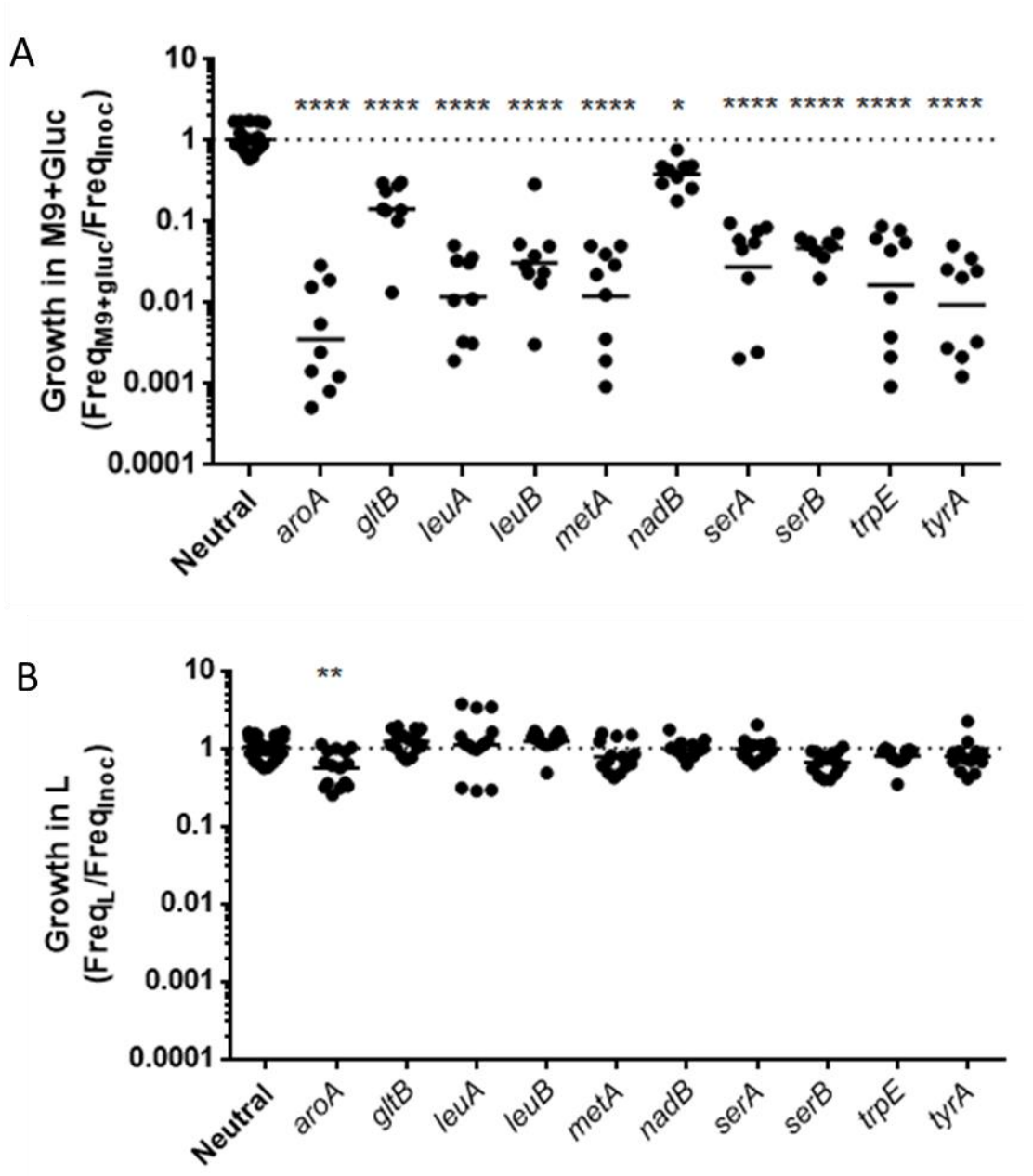
1. TnSeq Screen: Defect in WT: Yes refers to genes with statistically significant defect, NS (not significant) to genes with defect that was not statistically significant, and No to genes with no defect in WT mice in TnSeq screen

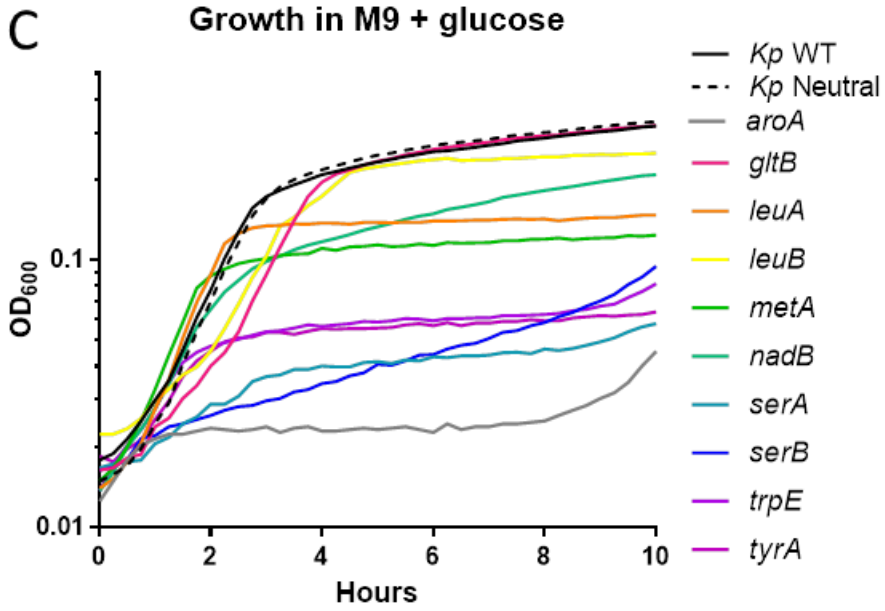
2. TnSeq Screen: Defect in PMN Dep: Yes refers to genes with statistically significant defect, NS (not significant) to genes with defect that was not statistically significant, and No to genes with no defect in PMN-depleted mice in TnSeq screen

*=Neutral strains

Figure 4- 1. *Kp* requires *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* for growth in nutrient restricted conditions *in vitro*

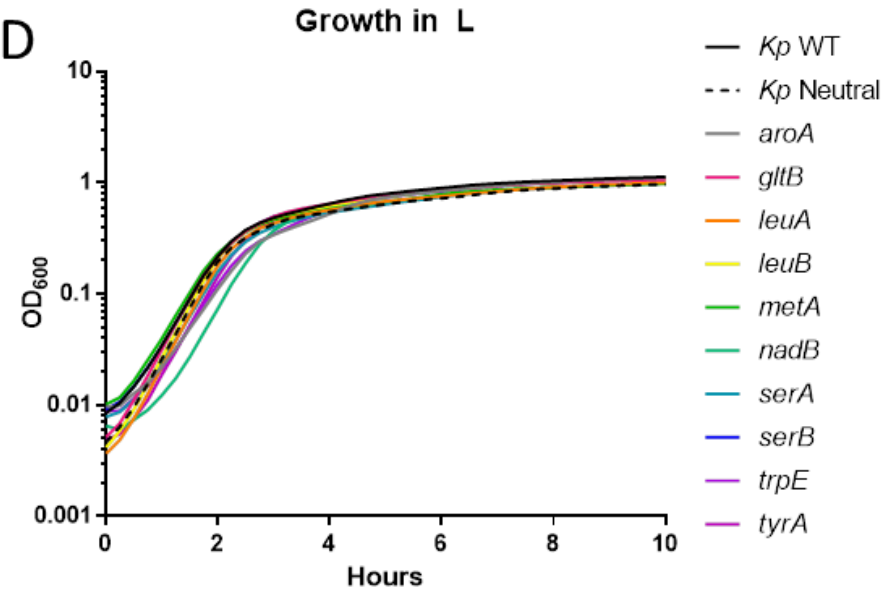
(A-B) A *Kp* mini-library was generated of 63 insertions mutants, where each strain was grown overnight separately and then pooled so 50% consisted of 3 insertion mutants considered to have a neutral fitness in WT mice (MKP330, MKP332 and MKP354) and 50% was split among the remaining insertion mutants, and used to inoculate (A) M9 minimal media+glucose (M9+gluc) and (B) L. These samples were incubated for 5 hr at 37°C and then plated on L-Kan agar plates, as were the inoculums. gDNA was isolated from the bacteria, barcoded and deep sequenced. Growth in M9+Gluc and Growth in L were calculated by comparing the frequency of each insertion mutant before ($Freq_{Inoc}$) and after ($Freq_{M9+gluc}$ or $Freq_{L}$) infection using $Freq_{M9+gluc}/Freq_{Inoc}$ and $Freq_{L}/Freq_{Inoc}$, respectively. Each dot represents the fitness of an insertion mutant in a sample and the geometric mean is indicated for each mutant. Statistical significance was determined for each insertion mutant compared to the pooled Neutral values using One-way ANOVA with Dunnet's post-test on the log-transformed values. Note the Neutral values have been included in another manuscript. Data is representative of 2 independent experiments performed in triplicate. (C-D) Separate dilutions were made from overnight cultures of the indicated *Kp* strains into (C) M9+glucose or (D) L media, which were incubated at 37°C and the OD_{600} was measured every 15 mins. Each sample was tested in at least 2 independent experiments in triplicate. Statistical significance was determined for the growth of each insertion mutant compared to *Kp* WT at 3 and 10 hrs post-inoculation using One-Way ANOVA with Dunnet's post-test.


A
Growth in M9+Gluc
(Freq$_{M9+gluc}$/Freq$_{Inoc}$)
10
1
0.1
0.01
0.001
0.0001
**** **** **** **** **** * **** **** **** ****
Neutral
aroA
gltB
leuA
leuB
metA
nadB
serA
serB
trpE
tyrA
B
Growth in L
(Freq$_{L}$/Freq$_{Inoc}$)
10
1
0.1
0.01
0.001
0.0001
**
Neutral
aroA
gltB
leuA
leuB
metA
nadB
serA
serB
trpE
tyrA



	Mean OD_{600} at 3 hrs	p value vs *Kp* WT at 3 hrs	Mean OD_{600} at 10 hrs	p value vs *Kp* WT at 10 hrs
Kp WT	0.173	N/A	0.317	N/A
Neutral	0.170	ns	0.329	ns
aroA	0.024	****	0.046	****
gltB	0.088	****	0.319	ns
leuA	0.134	*	0.147	****
leuB	0.115	****	0.177	****
metA	0.101	****	0.124	****
nadB	0.099	****	0.209	****
serA	0.037	****	0.058	****
serB	0.030	****	0.095	****
trpE	0.054	****	0.082	****
tyrA	0.054	****	0.064	****

D



	Mean OD_{600} at 3 hrs	p value vs *Kp* WT at 3 hrs	Mean OD_{600} at 10 hrs	p value vs *Kp* WT at 10 hrs
Kp WT	0.687	N/A	3.585	N/A
Neutral	0.619	ns	3.068	ns
aroA	0.351	ns	3.527	ns
gltB	0.684	ns	3.398	ns
leuA	0.564	ns	3.191	ns
leuB	0.652	ns	3.447	ns
metA	0.730	ns	3.126	ns
nadB	0.232	**	3.494	ns
serA	0.503	ns	3.13	ns
serB	0.669	ns	3.473	ns
trpE	0.389	ns	3.481	ns
tyrA	0.450	ns	3.453	ns

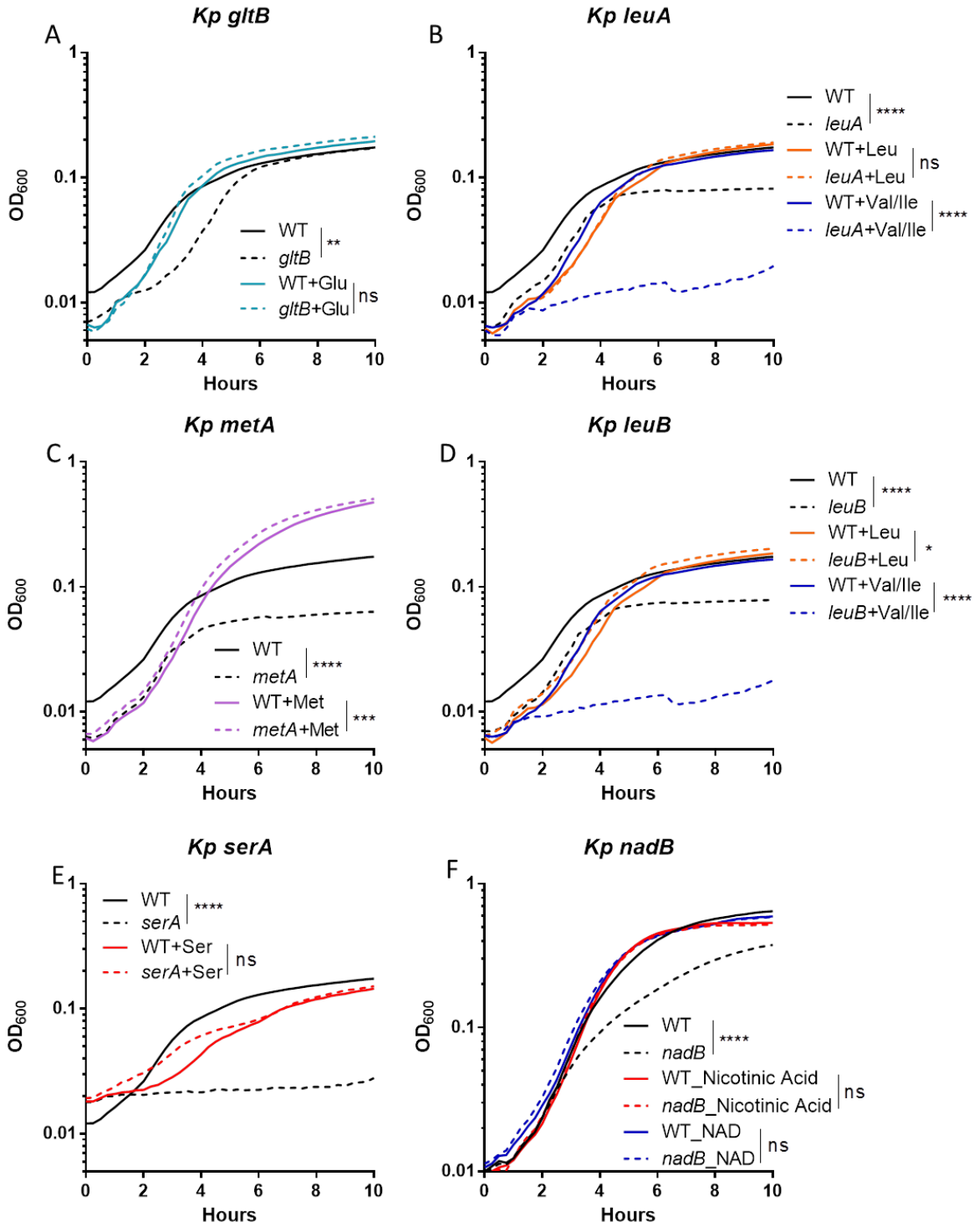
To determine whether the growth of *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* could be restored by addition of specific amino acids or metabolites, each strain was grown in M9+gluc with the addition of specific metabolites. Based on previous work in other bacterial species (412-421), we tested whether *aroA* is necessary for tryptophan (Trp), tyrosine (Tyr) and/or phenylalanine (Phe) synthesis, *gltB* for glutamate (Glu) synthesis, *leuA* and *leuB* for leucine (Leu) synthesis, *metA* for methionine (Met) synthesis, *nadB* for nicotinamide dinucleotide (NAD) and nicotinic acid (NA) synthesis, *serA* and *serB* for serine (Ser) synthesis, *trpE* for Trp synthesis, and *tyrA* for Tyr synthesis. With the exception of the *aroA* mutant, which still had a small growth defect in the presence of Trp, Tyr and Phe, the addition of these metabolites permitted the mutant strains to grow as well as or better than WT in the presence of these metabolites **(Fig. 4-2;** summarized in **Table 4-2).** This confirms that these genes are required for the synthesis of these amino acids**.**

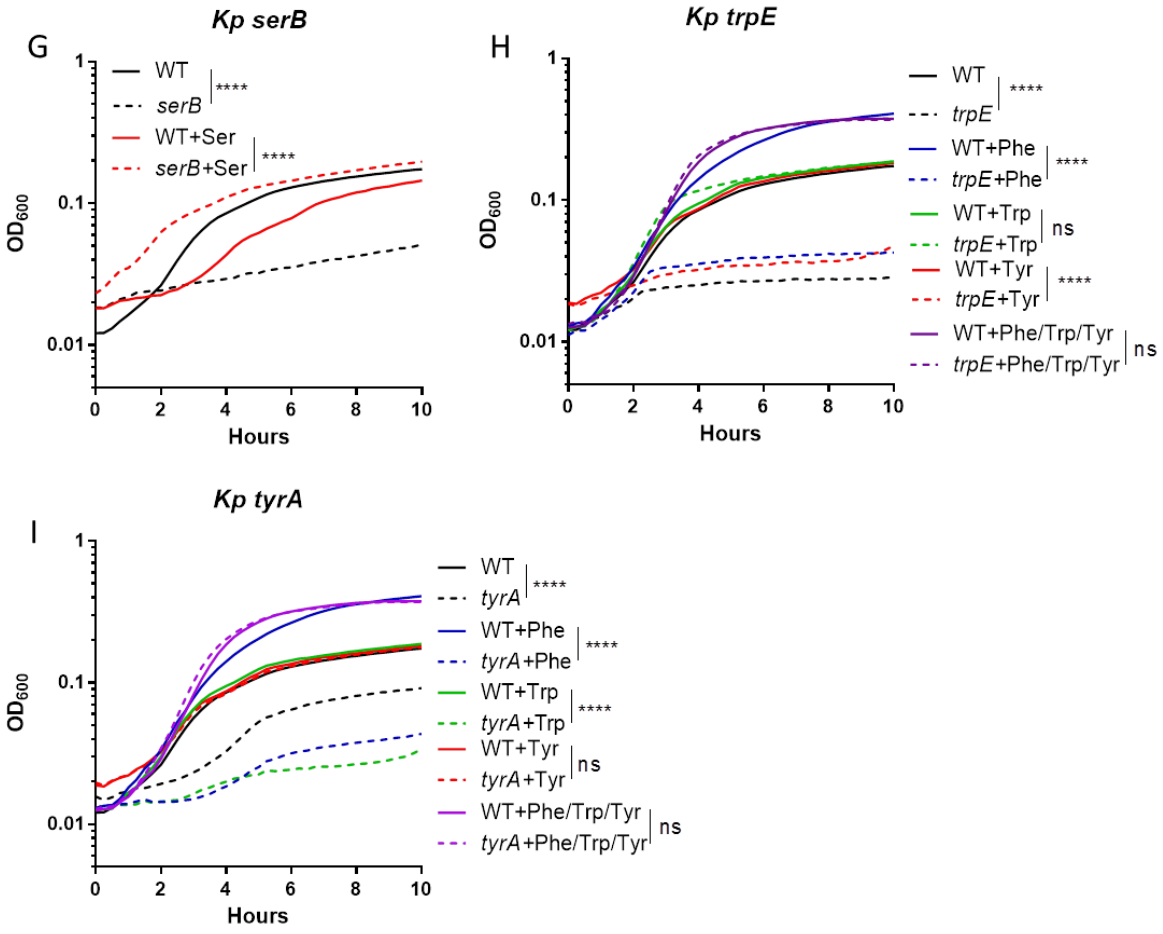
Importantly, while the *aroA* mutant had a small defect in growth compared to WT when Trp, Tyr, and Phe were all added back, growth remained severely attenuated when only one of these amino acids were supplemented, confirming that *aroA* functions upstream to mediate synthesis of all 3 aromatic acids **(Fig. 4-2E+H** and **Table 4-2)**. However, the severity of the growth defect of the *aroA* mutant **(Fig. 4-2)** and inability of added back amino acids to fully restore growth **(Fig. 4-2J** and **Table 4-2)** strongly indicate both the importance of this gene for *Kp* and the possibility of other functions in addition to amino acid synthesis. In other bacteria, *aroA* is also involved in the synthesis of certain siderophores (422, 423). To test whether *aroA* played a role in iron acquisition, WT *Kp* and the *aroA* mutant were inoculated into L with or without the iron chelator

2,2'-bipyridyl (DIP) and then growth was measured **(Fig. 4-2K** and **Table 4-2).** The *aroA* mutant was more susceptible to inhibition of growth in iron restricted conditions than *Kp* WT, as it grew more slowly in 0.25 mM DIP in contrast to having similar growth kinetics to *Kp* WT in L alone. Both strains were similarly inhibited by 1 mM DIP, demonstrating *Kp* WT is also limited by the amount of iron availability, but overall, the lack of *aroA* enhances *Kp* growth in low iron conditions. Based on these results and work on other bacteria (422, 423), *aroA* is likely to also play a role in siderophore production pathway in *Kp*. To confirm that the role for *aroA* in aromatic amino acid synthesis and iron acquisition was due to the transposon insertion in *aroA*, an in-frame Δ*aroA* strain was generated. This in-frame deletion was also restored with the WT *aroA* locus, thus generating Δ*aroA::aroA*. Importantly, the *aroA* transposon insertion mutant and in-frame Δ*aroA* behaved similarly, as did the *Kp* WT and *Kp* Δ*aroA::aroA* strains **(Fig. 4-2L+M** and **Table 4-2)**, confirming the phenotypes noted for the *Kp aroA* mutant are due to the disruption of *aroA* rather than changes at a different site in the *Kp* chromosome.

Figure 4- 2. ***Kp*** **requires** ***aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE*** **and** ***tyrA*** **for the synthesis of amino acids and metabolites**

(A-M) The indicated *Kp* transposon mutants with insertions in *gltB, leuA, leuB, serA, serB, metA, nadB, trpE, tyrA* and *nadB* and *Kp* WT, *Kp* Δ*aroA* and *Kp* Δ*aroA::aroA* were grown overnight and then diluted into (A-J and L) M9 minimal media supplemented with glucose (M9+gluc) alone or containing 10mM of the indicated amino acids, nicotinic acid (NA) or nicotinamide adenine dinucleotide (NAD), or (K+M) L alone or with 0.25 mM 2,2'-bipyridyl (DIP). Samples were then incubated at 37°C and the bacterial density (OD_{600}) of each well was measured every 15 mins. Data is from at least 2 independent experiments with each sample tested in triplicate. Statistically significant differences in the OD_{600} at (A) 3 hrs and (B-M) 10 hrs were determined between the indicated samples using One-way ANOVA with Sidak's post-test


A
Kp gltB
OD600
Hours
WT
gltB
WT+Glu
gltB+Glu
**
ns
B
Kp leuA
WT
leuA
WT+Leu
leuA+Leu
WT+Val/Ile
leuA+Val/Ile
****
ns
****
C
Kp metA
WT
metA
WT+Met
metA+Met
****
***
D
Kp leuB
WT
leuB
WT+Leu
leuB+Leu
WT+Val/Ile
leuB+Val/Ile
****
*
****
E
Kp serA
WT
serA
WT+Ser
serA+Ser
****
ns
F
Kp nadB
WT
nadB
WT_Nicotinic Acid
nadB_Nicotinic Acid
WT_NAD
nadB_NAD
****
ns
ns


G
Kp serB
WT
serB
WT+Ser
serB+Ser
****
****
OD600
Hours
H
Kp trpE
WT
trpE
WT+Phe
trpE+Phe
WT+Trp
trpE+Trp
WT+Tyr
trpE+Tyr
WT+Phe/Trp/Tyr
trpE+Phe/Trp/Tyr
****
****
ns
****
ns
I
Kp tyrA
WT
tyrA
WT+Phe
tyrA+Phe
WT+Trp
tyrA+Trp
WT+Tyr
tyrA+Tyr
WT+Phe/Trp/Tyr
tyrA+Phe/Trp/Tyr
****
****
****
ns
ns

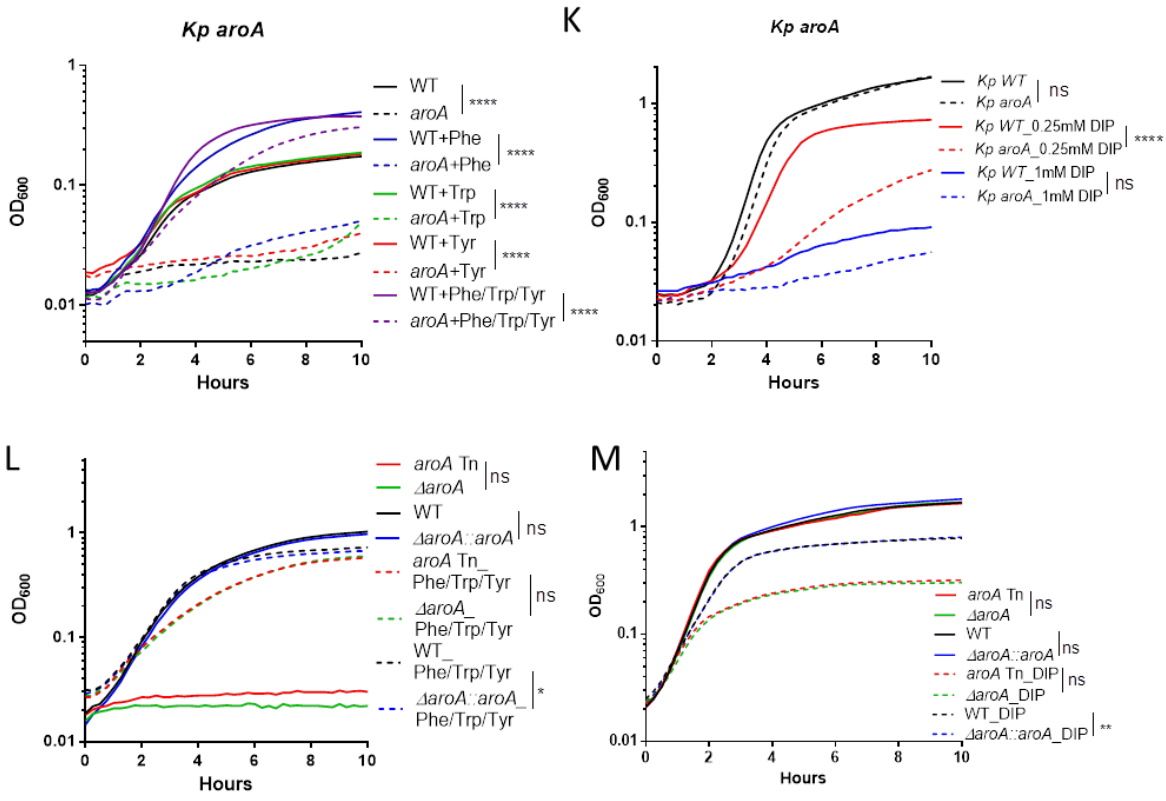

Kp aroA
OD600
Hours
WT
aroA
****
WT+Phe
aroA+Phe
****
WT+Trp
aroA+Trp
****
WT+Tyr
aroA+Tyr
****
WT+Phe/Trp/Tyr
aroA+Phe/Trp/Tyr
****
K
Kp aroA
Kp WT
Kp aroA
ns
Kp WT_0.25mM DIP
Kp aroA_0.25mM DIP
****
Kp WT_1mM DIP
Kp aroA_1mM DIP
ns
L
aroA Tn
ΔaroA
ns
WT
ΔaroA::aroA
ns
aroA Tn_ Phe/Trp/Tyr
ΔaroA_ Phe/Trp/Tyr
ns
WT_ Phe/Trp/Tyr
ΔaroA::aroA_ Phe/Trp/Tyr
*
M
aroA Tn
ΔaroA
ns
WT
ΔaroA::aroA
ns
aroA Tn_DIP
ΔaroA_DIP
ns
WT_DIP
ΔaroA::aroA_DIP
**

Table 4- 2. ***Kp* requires *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* for the synthesis of amino acids and metabolites**

Results from Figure 4-2. Cultures of the indicated *Kp* strains were diluted into the indicated media and treatment conditions, and bacterial growth was measured over time by measuring the $OD_{600.}$ Growth Defect refers to if a strain fails to grow as well as *Kp* WT under the same conditions, where "Yes" refers to a growth defect and "No" refers to a lack of a growth defect. Statistically significant differences in the OD_{600} at 3 hrs for *gltB* and 10 hrs for all other strains were determined between the indicated samples using One-way ANOVA with Sidak's post-test

Strain	Media	Treatment[1]	Growth Defect (vs indicated strain under same conditions)[2]	p value [3]
Kp gltB	M9+glucose	None	Yes, *Kp* WT (3 hr)	**
	M9+glucose	Glu	No, *Kp* WT (3 hr)	ns
Kp leuA	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Leu	No, *Kp* WT	ns
	M9+glucose	Ile/Val	Yes, *Kp* WT	****
Kp leuB	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Leu	No, *Kp* WT	ns
	M9+glucose	Ile/Val	Yes, *Kp* WT	****
Kp metA	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Met	No, *Kp* WT	ns
Kp serA	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Ser	No, *Kp* WT	ns
Kp serB	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Ser	No, *Kp* WT	ns
Kp trpE	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Phe	Yes, *Kp* WT	****
	M9+glucose	Trp	No, *Kp* WT	ns
	M9+glucose	Tyr	Yes, *Kp* WT	****
	M9+glucose	Phe/Trp/Tyr	No, *Kp* WT	ns
Kp tyrA	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Phe	Yes, *Kp* WT	****
	M9+glucose	Trp	Yes, *Kp* WT	****
	M9+glucose	Tyr	No, *Kp* WT	ns
	M9+glucose	Phe/Trp/Tyr	No, *Kp* WT	ns

Kp nadB	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Nicotinic Acid	No, *Kp* WT	ns
	M9+glucose	NAD	No, *Kp* WT	ns
Kp aroA	M9+glucose	None	Yes, *Kp* WT	****
	M9+glucose	Phe	Yes, *Kp* WT	****
	M9+glucose	Trp	Yes, *Kp* WT	****
	M9+glucose	Tyr	Yes, *Kp* WT	****
	M9+glucose	Phe/Trp/Tyr	Yes (Mild), *Kp* WT	****
	L	None	No, *Kp* WT	ns
	L	DIP	Yes, *Kp* WT	****
Kp* Δ*aroA	M9+glucose	None	No, *Kp aroA*	ns
	M9+glucose	Phe/Trp/Tyr	No, *Kp aroA*	ns
	L	None	No, *Kp aroA*	ns
	L	DIP	No, *Kp aroA*	ns
***Kp* Δ*aroA::*aroA**	M9+glucose	None	No, *Kp* WT	ns
	M9+glucose	Phe/Trp/Tyr	Yes (Mild), *Kp* WT	*
	L	None	No, *Kp* WT	ns
	L	DIP	Yes (Mild), *Kp* WT	**

Table is summary of results of Figure 4-2.

1. Treatment refers to the addition of 10 mM of the indicated amino acid/metabolite or 0.25 mM of DIP. NAD=Nicotinamide dinucleotide
2. Growth defect refers to bacterial density at 10 hr, unless indicated otherwise.
3. ns=not significant, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ and ****=$p<0.0001$

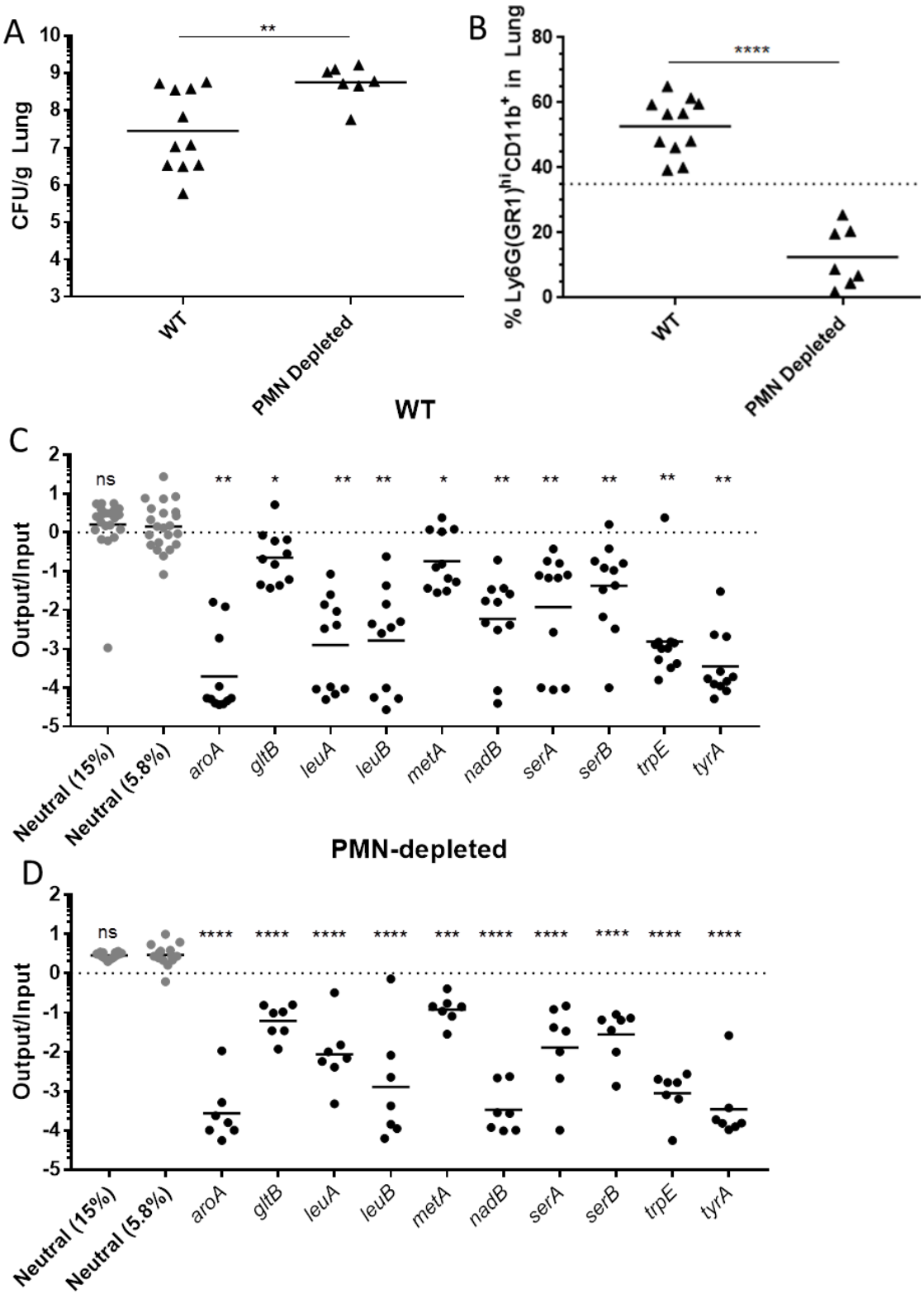
4.2 *Kp* REQUIRES THE ABILITY TO SYNTHESIZE GLUTAMATE, LEUCINE, SERINE, METHIONINE, NICOTINAMIDE DINUCLEOTIDE, TRYPTOPHAN, TYROSINE AND PHENYLALANINE TO INFECT THE RESOURCE LIMITED LUNGS

Because these genes required for glutamate, leucine, serine, methionine, nicotinamide dinucleotide, tryptophan, tyrosine and phenylalanine synthesis have not previously been assessed for their role in *Kp* virulence, the TnSeq screen results for the mutants with insertions in *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* were verified in a lung infection model. To determine if these genes were necessary in both immunocompetent and neutropenic mice, cohorts of Swiss Webster mice were intranasally infected with 20,000 CFU of a mini-library of these insertion mutants. This mini-library contained 4 mutants with transposon insertions at neutral sites (neutral), where 2 neutral mutants each comprised 15% of the pool and 2 others each comprised 5.8% of the pool, the same frequency as the mutants of interest to account for any issues with bottleneck effects. The remaining 58% of the pool consisted of the 10 mutants with insertions in *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* **(Fig. 4-3)**. At 33 hpi, the mice were sacrificed, and the bacterial load **(Fig. 4-3A)** and neutrophil population in the lungs **(Fig. 4-3B)** were quantified. The bacterial load differed significantly between the WT and PMN-depleted mice as on average, approximately $5x10^7$ and $5x10^9$ CFU g/lung were recovered, respectively (**Fig. 4-3A**). In the WT mice, a large influx of neutrophils was observed that resulted in neutrophils comprising 53% of the cells in the lungs **(Fig. 4-3B)**. By contrast, the lungs of the PMN-depleted mice had on average 13% neutrophils compared to the typical uninfected lung population of 5-10% in uninfected mice **(Fig. 4-3B** and data not shown). Combined, these results support previous findings by others that neutrophils limit this strain of *Kp* during lung infection

(141, 165, 166). While the neutral mutants had no fitness defects, all of the putative metabolic mutants tested had significant defects in WT mice **(Fig. 4-3C).** Notably, the *aroA* mutant had the strongest fitness defect of almost 8,000-fold, while the *leuA, leuB, nadB, tyrA,* and *trpE* mutants all had 500-1,000-fold defects. The *serA* and *serB* mutants had more than 10-fold defects, while insertions in *gltB* and *metA* had the mildest defects with a fitness approximately 8-fold lower than the neutral mutants **(Fig. 4-3C)**. Strikingly, all of the mutants had similar fitness defects in the PMN-depleted mice **(Fig. 4-3D)**. A comparison of the fitness of each insertion mutant in WT mice to its fitness in PMN-depleted mice showed no significant restoration of fitness in the PMN-depleted mice (One-way ANOVA with Sidak's post-test on log-transformed values, where all comparisons had a $p>0.05$). Combined, these results indicate that, while the total bacterial load increased upon depletion of neutrophils **(Fig. 4-3A)**, growth of all the mutants increased relatively equally and to the same degree as the neutral strains. Overall, this supports the idea that these genes do not contribute to protecting *Kp* against neutrophil attack and/or they have multiple functions that include neutrophil independent roles.

Figure 4- 3. ***Kp*** **genes** ***aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE*** **and** ***tyrA*** **are necessary for virulence in the lungs of WT and neutropenic mice**

(A-D) WT and neutrophil depleted Swiss Webster mice were intranasally infected with 20,000 CFU of a mini-library of *Kp* transposon insertion mutants, where 42% of each mini-library pool was made up of 4 mutants with transposon insertions at putative neutral sites (MKP330 and MKP437 each making up 15% and MKP318 and MKP336 each 5.8% of the mini-library), and the other 58% split by the 10 indicated insertion mutants. At 33 hpi, the mice were sacrificed, and their lungs were harvested, homogenized, plated in serial dilutions on L-Kan to obtain the (A) CFU/g Lung for each mouse and analyzed by flow cytometry to assess the (B) neutrophil population ($Ly6G^{hi}CD11b^{+}$) in the lungs. Plated bacteria were collected and processed to obtain gDNA, which was subsequently deep sequenced. The input and output sample gDNA was barcoded and deep sequenced. (C-D) The fitness of each insertion mutant was assessed using (C) WT Output/Input and (D) PMN Depleted Output/Input. (A-D) Each dot represents a mouse, and the (A, C-D) geometric mean and Mean+SEM (B) are indicated. (A-B) Statistical significance was determined by One-way Anova using Dunnet's post-test on (A) the log transformed and (B) non-transformed values. (C-D) Statistical significance was assessed using One-way ANOVA on the log-transformed values with Dunnet's post-test comparing each insertion mutant tested against the pooled Neutral (5.8%) mutants. To assess whether the insertion mutants had a statistically significant difference in fitness in the neutropenic mice compared to WT, the fitness of each mutant in the WT mice was compared to the fitness of the same mutant in the neutropenic mice using One-way ANOVA with Dunnet's post-test. None of the mutants had a significantly higher value in PMN depleted mice than WT.


A
CFU/g Lung
**
WT
PMN Depleted
B
% Ly6G(GR1)hiCD11b+ in Lung
****
WT
PMN Depleted
WT
C
Output/Input
ns
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*
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*
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**
**
**
**
Neutral (15%)
Neutral (5.8%)
aroA
gltB
leuA
leuB
metA
nadB
serA
serB
trpE
tyrA
PMN-depleted
D
Output/Input
ns
****
****
****
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***
****
****
****
****
****
Neutral (15%)
Neutral (5.8%)
aroA
gltB
leuA
leuB
metA
nadB
serA
serB
trpE
tyrA

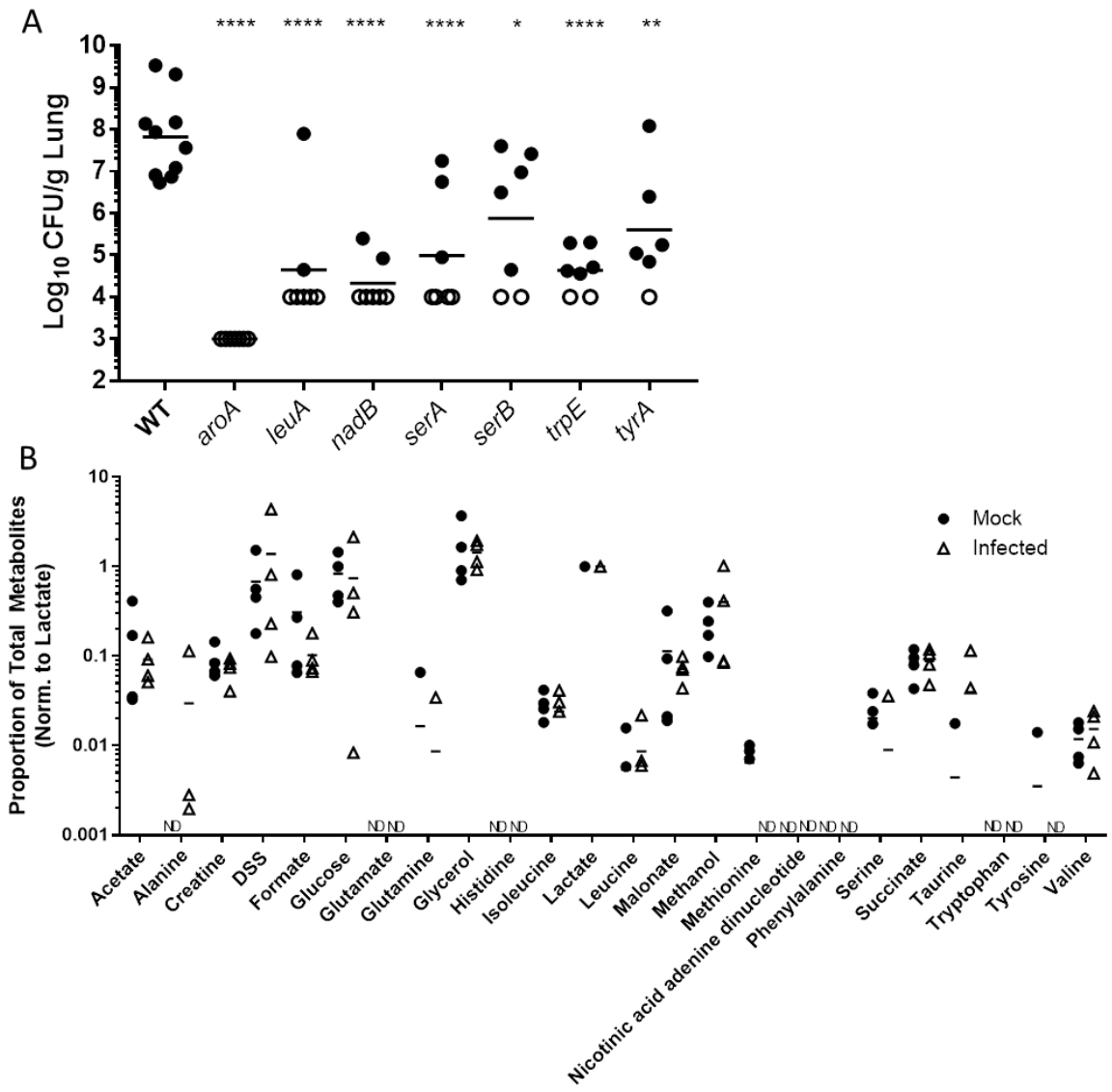
To determine whether these mutants are unable to grow in lungs in single strain infections or if they can scavenge these resources in the absence of competing WT Kp strains, Swiss Webster mice were infected with 1,000 CFU of a subset of the metabolic insertion mutants, *aroA, leuA, nadB, serA, serB, trpE* and *tyrA,* and the bacterial load was measured at 45 hpi **(Fig. 4-4A).** Strikingly, all of these mutants had a significant fitness defect in the lungs of WT mice during single strain infections compared to WT *Kp* **(Fig. 4-4A),** and in most cases, the defect was more pronounced in single strain infection than in competition **(Fig. 4-4C).** Once again, the *aroA* mutant had the largest defect compared to WT with a 100,000-fold defect in bacterial load, while *leuA, nadB, serA* and *trpE* had at least a 1,000-fold defect and the remainder, *serB* and *tyrA,* had at more than 100-fold defect **(Fig. 4-4A)**.

To evaluate the abundance of these metabolites in the lungs in the presence and absence of WT *Kp* infection, Swiss Webster mice were either infected intranasally with 20,000 CFU *Kp* WT or mock infected with an equivalent volume of PBS, and then broncheoalveolar lavage (BAL) fluid was collected and analyzed by nuclear magnetic resonance (NMR) **(Fig. 4-4B)**. Overall, no significant differences were noted for each metabolite assessed between the mock-infected versus the WT *Kp*-infected lungs, suggesting the host tightly controls the availability of these resources. Of the specific metabolites of interest, Phe, Trp, Tyr, Leu, Glu, Met, and NAD were not consistently present at detectable levels (**Fig. 4-4B)**, supporting the need for *de novo* synthesis of these metabolites by *Kp*. Ser was present in low levels, but the fitness defects of the *Kp serA* and *serB* insertion mutants *in vivo* **(Fig. 4-4A)** suggest the levels present are not

high enough to support and sustain *Kp* growth in the lungs. On the other hand, acetate, creatine, formate, glucose, malonate, and succinate were consistently detected at high levels **(Fig. 4-4B).** Lactate was detected because it is by-product of using CO_2 asphyxiation to sacrifice the mice (424), while glycerol and methanol were detected because of the BAL extraction methods used to collect the samples. Combined, these data indicate that *Kp* must synthesize a number of metabolites to grow within the lungs.

Figure 4- 4. *Kp* requires the ability to synthesize glutamate, leucine, serine, methionine, nicotinamide dinucleotide, tryptophan, tyrosine and phenylalanine to infect the resource limited lungs

(A) 7-10 week old Swiss-Webster mice were intranasally inoculated as with 1,000 CFU *Kp* WT and the indicated transposon insertion mutants. The mice were sacrificed at 45 hpi and the lungs were weighed, harvested, homogenized and plated in serial dilutions on L-agar plates to measure the CFU/g Lung. Each symbol represents an individual mouse, and an open symbol refers to the limit of detection when no bacteria were recovered. Each strain was tested in at least 2-4 mice per cohort in 2 independent experiments. Statistical significance was determined using One-way ANOVA on log-transformed values followed by Dunnet's post-test. (B) Mice were either mock-infected intranasally with PBS or infected with 20,000 CFU *Kp* WT, and then sacrificed and treated as described above. Prior to harvesting the lungs and plating to ensure *Kp* was present, the broncheoalveolar lavage (BAL) fluid of the lungs of the mice was collected using saline. The BAL was then analyzed by NMR, where all detectable metabolites and metabolites of interest are indicated. ND=Not detectable. Mean+SEM is presented. Data is from 2 independent experiments with 2 mice per cohort.


A
****
****
****
****
*
****
**
Log10 CFU/g Lung
10
9
8
7
6
5
4
3
2
WT
aroA
leuA
nadB
serA
serB
trpE
tyrA
B
Proportion of Total Metabolites (Norm. to Lactate)
Mock
Infected
10
1
0.1
0.01
0.001
ND
ND ND
ND ND
ND ND ND ND ND
ND ND
ND
Acetate
Alanine
Creatine
DSS
Formate
Glucose
Glutamate
Glutamine
Glycerol
Histidine
Isoleucine
Lactate
Leucine
Malonate
Methanol
Methionine
Nicotinic acid adenine dinucleotide
Phenylalanine
Serine
Succinate
Taurine
Tryptophan
Tyrosine
Valine

4.3 INHIBITION OF *AROA* EFFECTIVELY ATTENUATES *KP* GROWTH *IN VITRO* AND INFECTION OF THE LUNGS

Since *aroA* had the strongest phenotype in both *in vitro* and *in vivo* assays, we tested the in-frame deletion strain and the complemented strains, *Kp* Δ*aroA* and *Kp* Δ*aroA::aroA,* in single strain infections. Swiss Webster mice were intranasally infected with 20,000 CFU of the indicated strains and the bacterial load in the lungs was measured at 32 hpi. The *Kp* Δ*aroA* displayed a severe defect *in vivo* of approximately 100,000-fold compared to the WT strain, which, importantly, mimicked the defect seen in the *Kp aroA* insertion mutant **(Fig. 4-5A)**. Complementing *aroA (*Δ*aroA::aroA)* resulted in complete restoration of bacterial fitness to *Kp* WT levels **(Fig. 4-5A).**

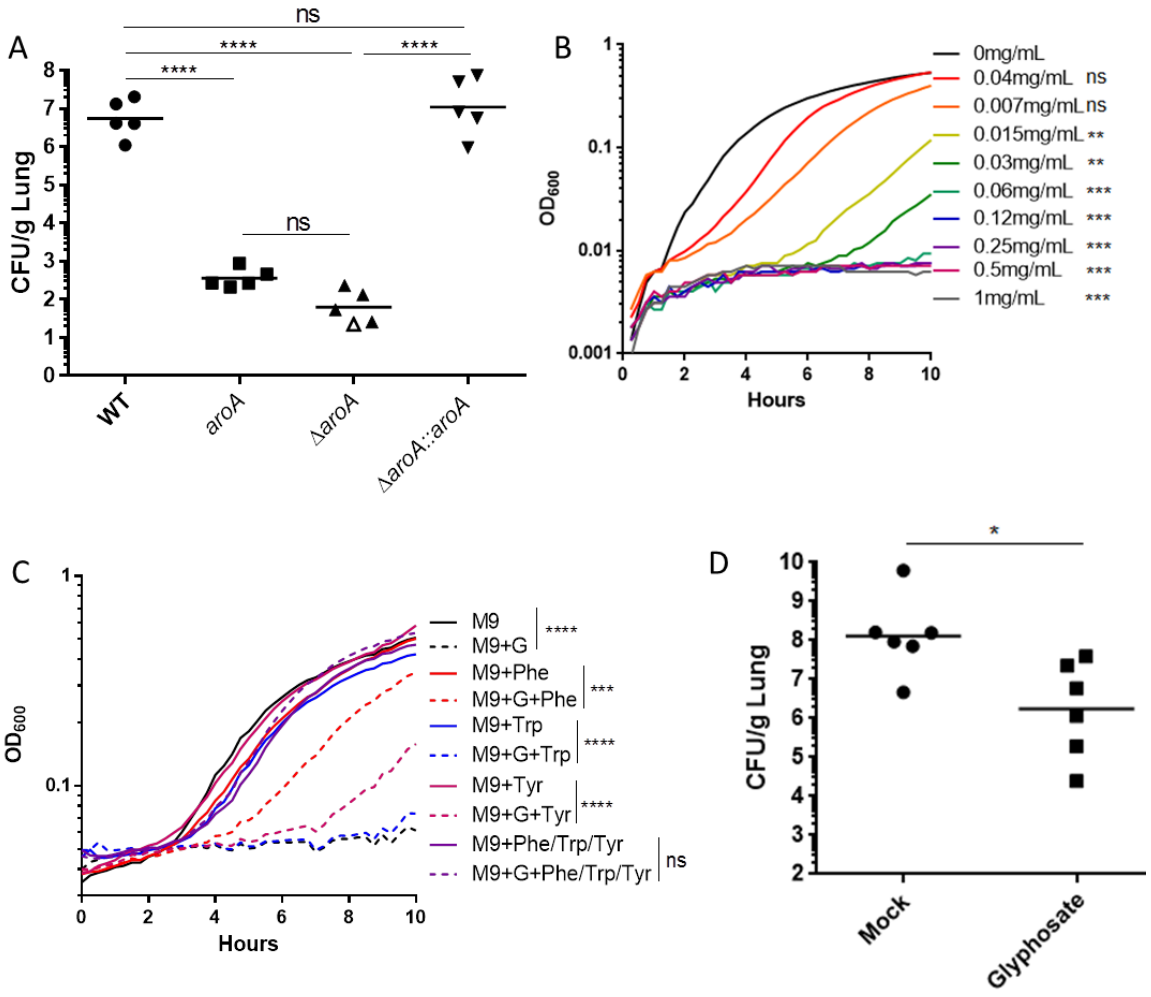
Due to the vital role *aroA* plays in *Kp* growth *in vitro,* the huge defect that disrupting *aroA* instills in *Kp* fitness in the lungs *in vivo,* and the absence of an *aroA* homolog in humans, we evaluated the *aroA* protein product, EPSP synthase or AroA, as a potential therapeutic target against *Kp*. First, the efficacy of an AroA specific inhibitor, glyphosate (425), was assessed as a proof-of-principle inhibitor of *Kp in vitro.* Glyphosate inhibited WT *Kp* growth in a dose-dependent manner, where concentrations of glyphosate equal to or greater than 0.06 mg/mL completely inhibited *Kp* growth when assessed after 10 hr **(Fig. 4-5B)**. This depended specifically on inhibition of *aroA* because adding back all 3 aromatic amino acids in combination, but not individually, restored bacterial growth in the presence of glyphosate to levels of growth in the absence of glyphosate **(Fig. 4-5C).**

Glyphosate was then tested as an inhibitor of *Kp* infection in the lungs *in vivo*. Swiss Webster mice were intranasally infected with 20,000 CFU of *Kp* WT, and mice

were either mock-treated with PBS or treated with 0.37 mg glyphosate intranasally at 6 and 25 hpi. At 32 hpi, the mice were sacrificed and the bacterial burden in the lungs was quantified. Remarkably, glyphosate inhibited *Kp in vivo* as the mock-treated mice had almost 100-fold more bacteria in the lungs than the glyphosate-treated mice (**Fig. 4-5D).** In total, this data suggests that inhibiting AroA effectively limits *Kp* infection and, thus, has strong potential as a therapeutic target against *Kp* infections.

Figure 4- 5. Inhibition of AroA Effectively attenuates *Kp in vitro* and *in vivo*

(A) 7-10 week old Swiss-Webster mice were intranasally infected with 20,000 CFU of the indicated *Kp* strains. The mice were sacrificed at 32 hpi and the lungs were, harvested, weighed, homogenized and plated in serial dilutions on L-agar plates to measure the CFU/g Lung. Each symbol represents an individual mouse and the geometric mean is presented. Open symbols indicate the limit of detection where no bacteria were recovered. Data is from 2 independent experiments with 2-3 mice per cohort. Statistical significance was determined by comparing the log-transformed values of the indicated cohorts using One-way ANOVA with Sidak's post-test. (B-C) *Kp* WT was grown overnight and then diluted into (B) M9 +gluc alone or with the indicated concentrations of glyphosate (i.e. G), and (C) M9+gluc alone or with 0.25mM glyphosate and 10 mM of the indicated amino acids. The samples were then incubated at 37°C and the OD_{600} was measured every 15 mins. The OD_{600} of each cohort after 10 hrs of growth was compared using One-way ANOVA with Dunnet's post-test. (D) 7-10 week old Swiss-Webster mice were intranasally infected with 20,000 CFU of *Kp* WT. At 6 and 25 hpi, the mice were treated intranasally with either PBS (Mock) or 0.37 mg glyphosate (Glyphosate). The mice were sacrificed at 32 hpi and the lungs were harvested, weighed, homogenized and plated in serial dilutions on L-agar plates to measure the CFU/g Lung. Each symbol represents an individual mouse and the geometric mean is presented. Data is from 2 independent experiments with 3 mice per cohort. Statistical significance was determined by comparing the indicated log-transformed values of the cohorts using One-way ANOVA with Sidak's post-test.


A
ns
****
****
****
ns
CFU/g Lung
WT
aroA
ΔaroA
ΔaroA::aroA
B
OD600
Hours
0mg/mL
0.04mg/mL ns
0.007mg/mL ns
0.015mg/mL **
0.03mg/mL **
0.06mg/mL ***
0.12mg/mL ***
0.25mg/mL ***
0.5mg/mL ***
1mg/mL ***
C
OD600
Hours
M9
M9+G ****
M9+Phe
M9+G+Phe ***
M9+Trp
M9+G+Trp ****
M9+Tyr
M9+G+Tyr ****
M9+Phe/Trp/Tyr
M9+G+Phe/Trp/Tyr ns
D
*
CFU/g Lung
Mock
Glyphosate

4.4 THE ACQUISITION OF SPONTANEOUS RESISTANCE TO GLYPHOSATE BY *KP* IS A NOTABLY RARE EVENT

One of the major reasons *Kp* is a concerning emerging human pathogen is the increasing spread of antibiotic resistant *Kp* strains. Previous work has shown that introducing a single amino acid substitution (G96A) can result in resistance to glyphosate (426, 427), but neither the rate of this occurring naturally in *Kp* strains nor the effect of these mutants on *Kp* growth have been evaluated. Therefore, the rate of spontaneous resistance of *Kp* to glyphosate was evaluated. WT *Kp* was grown overnight and then plated on M9+gluc with or without a high dose (1 mg/mL) of glyphosate. Strikingly, while colonies appeared on the M9+gluc alone media after 24 hours of growth, no discernable colonies were detected on the glyphosate plates **(Fig. 4-6A)**. Based on the number of *Kp* cells plated, the rate of resistance, where bacteria would be considered "resistant" when growth was completely unaffected by glyphosate, approximated less than 1 out of 9×10^{10} bacteria **(Fig. 4-6A)**. This very low late of resistance, despite literature identifying a 1 nucleotide substitution that would result in glyphosate resistance in *Kp*, implies that this substitution and others in *aroA* that provide resistance to glyphosate comes with a strong fitness cost (426, 427). By 48 h post-plating, colonies with an 'intermediate' resistance or increased tolerance to glyphosate were detected, where growth was still inhibited by glyphosate, but not completely abrogated **(Fig. 4-6A)**. These colonies appeared at a frequency of 1 in 5×10^8 bacteria **(Fig. 4-6A)**. While these colonies appeared to be more tolerant to the presence of glyphosate than the parental WT *Kp* strain, growth was still delayed compared to colonies grown on media lacking glyphosate.

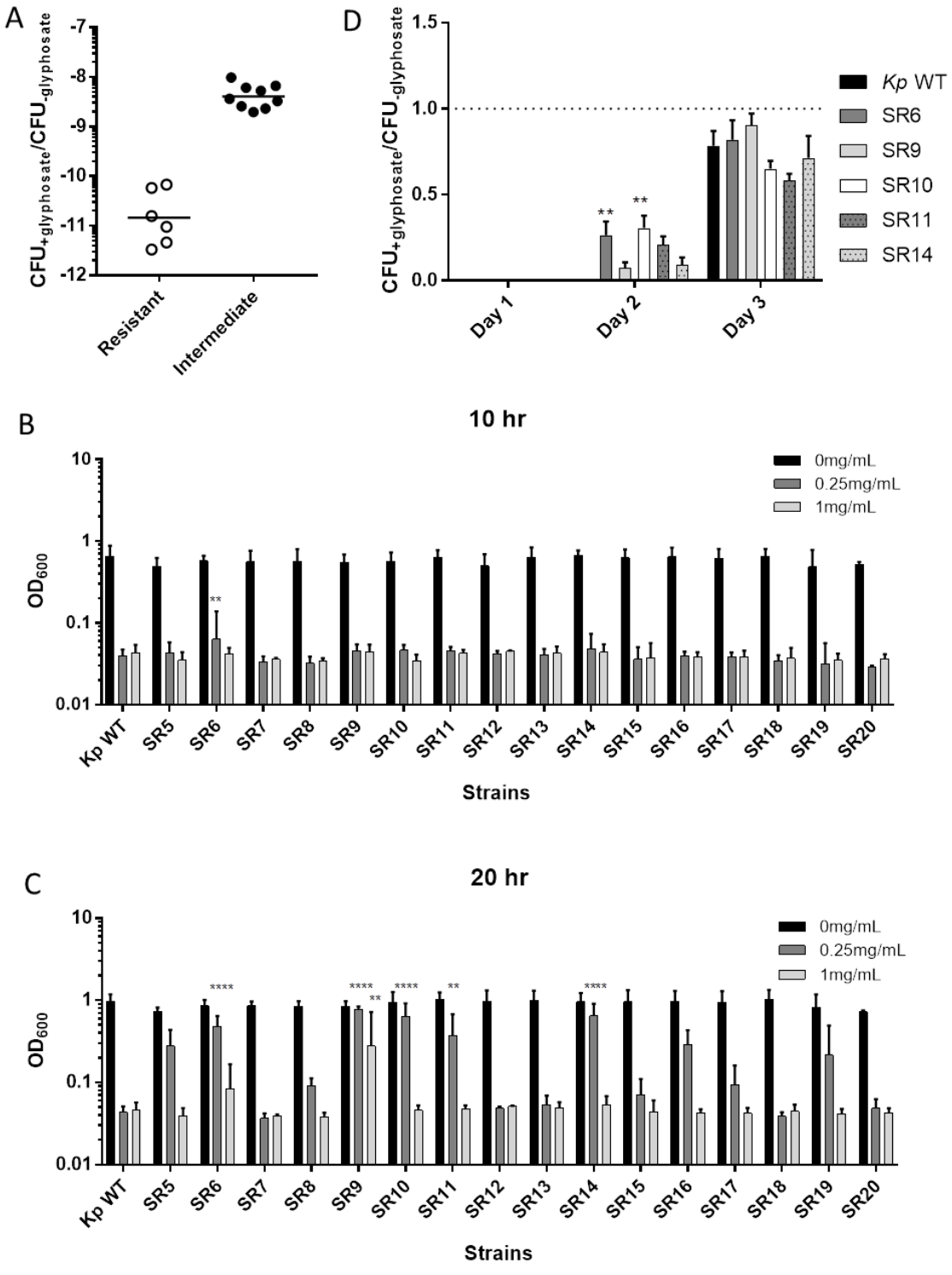
To investigate these strains further, several of these strains displaying increased glyphosate tolerance or "intermediate" resistance were isolated (designated SR5-20) and their growth kinetics over 20 hr were characterized in M9+gluc with or without 0.25 mg/mL or 1 mg/mL glyphosate **(Fig. 4-6B-C).** In the absence of glyphosate, the growth of the isolated "intermediate" strains in M9+gluc alone was similar to that of WT *Kp* after both 10 and 20 hours of growth **(Fig. 4-6B-C).** At 10 hours, the growth of all the intermediate-resistant strains tested was completely inhibited at both concentrations of glyphosate with the exception of SR6, which had a minor increase in growth in 0.25 mg/mL glyphosate compared to *Kp* WT **(Fig. 4-6B).** By 20 hr, several strains, SR6, SR9, SR10, SR11 and SR14, demonstrated more growth in 0.25 mg/mL glyphosate than *Kp* WT **(Fig. 4-6C).** However, only SR6 showed any growth in 1 mg/mL glyphosate **(Fig. 4-6C).**

To confirm this phenotype, the plating efficiency of the strains that displayed increased growth in 0.25 mg/mL glyphosate was measured by growing the indicated strains overnight in L, and then plating on M9+gluc with or without 1 mg/mL glyphosate **(Fig. 4-6D).** While at least 10^9 colonies grew on M9+gluc containing plates for each strain after incubating for 1 day (data not shown), no colonies were discernable on glyphosate **(Fig. 4-6D)**. By 48 hr, only 2 strains, SR6 and SR10, had significantly more colonies than *Kp* WT, where 26% and 30% of the colonies that grew on M9+gluc also grew in the presence of glyphosate **(Fig. 4-6D).** By 72 hpi, *Kp* WT had demonstrated significant growth on glyphosate, where 80% of colonies grew on glyphosate, and the plating efficiency of none of the mutants differed significantly from *Kp* WT **(Fig. 4-6D).** This demonstrates that, while these strains had a slight increase in resistance or tolerance

to the *aroA* inhibitor that was semi-heritable, they were still discernably inhibited by glyphosate. The coding region of *aroA* of all the intermediate strains (SR5-SR20) was sequenced, and no variations in sequence compared to the WT strain were found (data not shown), implying the increased tolerance to glyphosate was not due to a change in the primary sequence of *aroA*. Overall, these data support the idea that high levels of resistance to glyphosate is a rare event, and that, while some strains were recovered with an increased glyphosate tolerance, their growth was still significantly inhibited by this antibiotic.

Figure 4- 6. The acquisition of spontaneous resistance to glyphosate by *Kp* is a notably rare event

(A) *Kp* WT was incubated overnight in L, plated in serial dilutions on M9+gluc alone or with 1mg/mL glyphosate, and CFU were quantified at 24 and 48 hr. Several colonies were isolated from the glyphosate containing plates from each experiment after a 48 hr incubation for future analysis (designated SR5-20) (B-D). The frequency of resistance was calculated as $CFU_{+glyphosate}/CFU_{-glyphosate}$ after incubating for 1, 2 and 3 days. "Resistant" refers to the number of colonies that appeared on the glyphosate containing plates after 1 day, which is the same time colonies appeared on M9+gluc and "Intermediate" after 2 days. Data is from 2-3 independent experiments with cohorts performed in triplicate. Open circles represent the limit of detection when no CFU were recovered. (B-C) The intermediate strains acquired from (A) were grown overnight and then diluted into M9+gluc alone or containing 0.25mg/mL or 1mg/mL glyphosate. Samples were then incubated at 37°C and the OD_{600} of each well was measured every 15 mins and the bacterial densities after (B) 10 hr and (C) 20 hr of growth are displayed. Data is from at least 2 independent experiments performed in duplicate for each sample. (D) The plating efficiency of the indicated *Kp* strains was measured by plating overnight cultures grown in L on M9+gluc with or without 1 mg/mL glyphosate and determining CFU at 1, 2 and 3 days post-plating. Plating efficiency was measured by comparing the CFU that grew in the presence of glyphosate ($CFU_{+glyphosate}$) to the absence ($CFU_{-glyphosate}$) using plating efficiency=$CFU_{+glyphosate}$/ $CFU_{-glyphosate.}$ Statistical significance was determined by comparing each sample against the *Kp* WT values at the same (B-C) glyphosate concentration or (D) time-point using One-way ANOVA with Dunnet's post-test.


A
CFU+glyphosate/CFU-glyphosate
-7
-8
-9
-10
-11
-12
Resistant
Intermediate
D
CFU+glyphosate/CFU-glyphosate
1.5
1.0
0.5
0.0
**
**
Day 1
Day 2
Day 3
Kp WT
SR6
SR9
SR10
SR11
SR14
B
10 hr
OD600
10
1
0.1
0.01
0mg/mL
0.25mg/mL
1mg/mL
**
Kp WT
SR5
SR6
SR7
SR8
SR9
SR10
SR11
SR12
SR13
SR14
SR15
SR16
SR17
SR18
SR19
SR20
Strains
C
20 hr
OD600
10
1
0.1
0.01
0mg/mL
0.25mg/mL
1mg/mL
****
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Kp WT
SR5
SR6
SR7
SR8
SR9
SR10
SR11
SR12
SR13
SR14
SR15
SR16
SR17
SR18
SR19
SR20
Strains

4.5 Clinical Blood and Respiratory *Kp* Isolates, including ESBL and KPC producing, are Sensitive to AroA Inhibition

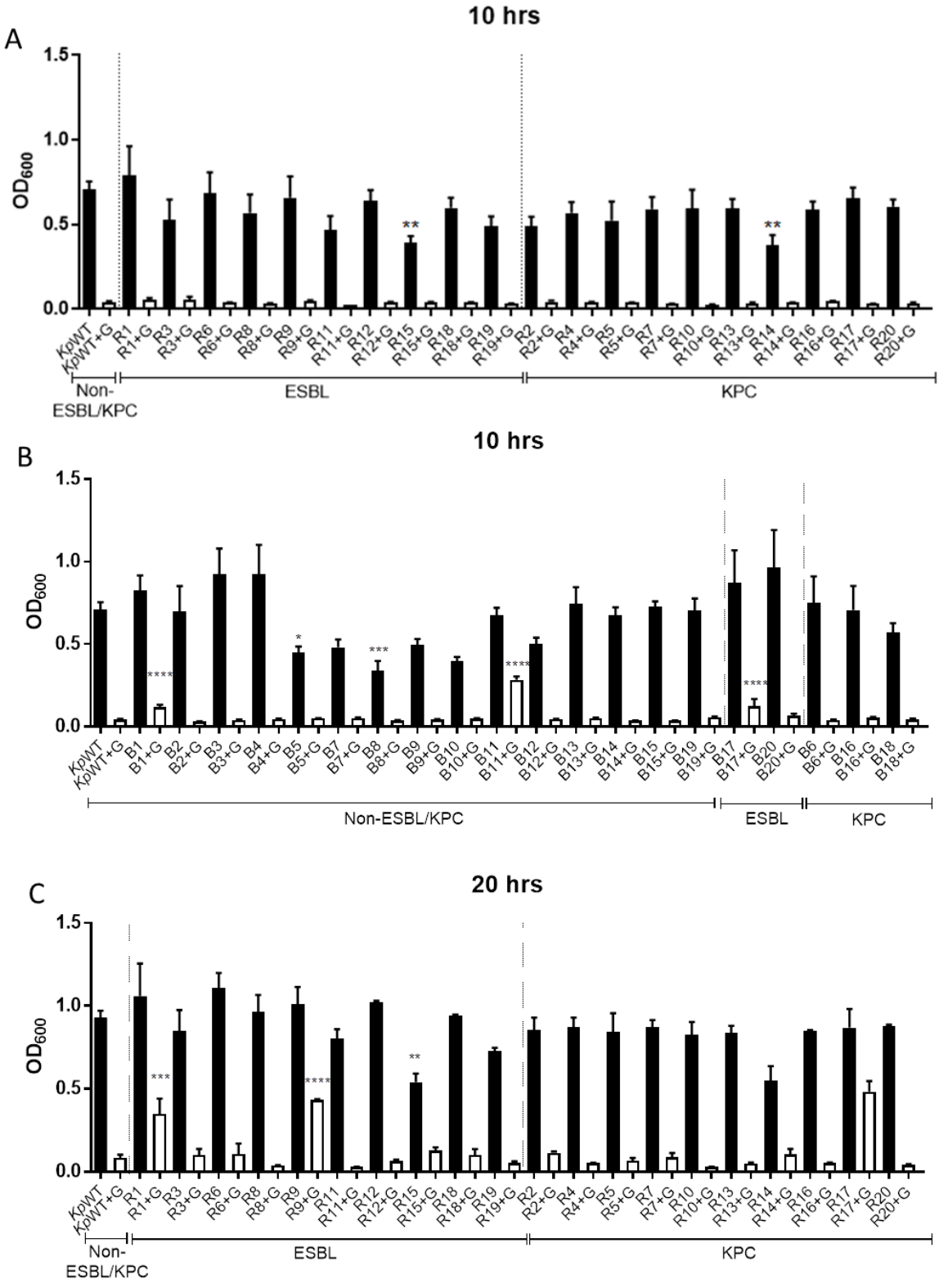
To determine if glyphosate was effective at inhibiting growth of clinical strains of *Kp*, 40 clinical isolates were collected from the blood and lungs of patients (designated B1-B20 and R1-R20, respectively). Importantly, 12 of these strains were ESBL- and 13 were KPC-producers. Each of these clinical isolates was cultured overnight in L, and then their growth in M9+gluc in the presence and absence of 0.25 mg/mL glyphosate was evaluated **(Fig. 4-7A-D).** In the absence of glyphosate, all the clinical strains grew to levels similar to WT *Kp* after 10 hours, with the exception of B5, B8, R14 and R15, which grew more slowly **(Fig. 4-7A-B**). By 20 hr, only R14 had a significant defect **(Fig. 4-7C-D).** In the presence of glyphosate, all of the clinical isolates were inhibited for growth at 10 hr, although B1, B11 and B17 were less inhibited than WT *Kp* **(Fig. 4-7A-B).** After 20 hr of exposure to glyphosate, several strains showed some growth. Five non-ESBL/KPC strains grew in glyphosate, B1, B3, B4, B11 and B19, but only B11 grew to the levels equal to the absence of glyphosate **(Fig. 4-7C-D).** Of the ESBL-producers, B17, B20, R1 and R17 grew in glyphosate by 20 hr, but none grew more than half the growth seen in the absence of glyphosate **(Fig. 4-7C-D).** A similar result was noted for the KPC-producing *Kp* strains, where B6, B16, B18 and R17 grew in glyphosate, but once again, to levels much lower than in the absence of glyphosate **(Fig. 4-7C-D).** To confirm the increased glyphosate tolerance or resistance displayed by some of the clinical isolates, the plating efficiency of a subset of these isolates, B1, B3, B11 and B19 **(Fig. 4-7E)** was determined on 1 mg/mL glyphosate**.** No colonies were discernable for any of the strains after 1 day when plated on glyphosate in contrast to the high plating efficiency in

its absence **(Fig. 4-7E)**. After 2 days, only B11, which averaged an 82% plating efficiency, had colonies on glyphosate **(Fig. 4-7E).** All of the strains, including WT *Kp*, grew on glyphosate by 3 days post-plating. The low plating efficiency on glyphosate on day 1 and 2 indicates that the clinical isolates have low to intermediate resistance/tolerance to glyphosate and are still inhibited by its presence. Overall, all of the clinical isolates tested had their growth inhibited by glyphosate.

To determine whether there were any differences in the *aroA* gene in these clinical strains more resistant or tolerant to glyphosate than *Kp* ATCC 43816, the *aroA* gene was sequenced in a subset of these strains, including B1, B2, B3, B11, B17, B19, R1, R9 and R17 **(Fig. 4-7F).** Only B3 (1256A>T) and B19 (661A>C) had any differences in their DNA sequence relative to our WT *Kp* strain. These changes resulted in Q419L and Q221K amino acid changes, respectively **(Fig. 4-7F)**. To determine if these changes in the *aroA* gene increased glyphosate resistance in B3 and B19, *aroA* was cloned from each of these strains and used to replace the native *aroA* in the WT *Kp* strain ATCC48316 to generate *Kp* Δ*aroA::aroAB3* and *Kp* Δ*aroA::aroAB19*. These strains were grown in M9+gluc in the presence or absence of glyphosate. Surprisingly, the *Kp* Δ*aroA::aroAB3* and *Kp* Δ*aroA::aroAB19* failed to grow in the presence of glyphosate **(Fig. 4-7G)**, suggesting that the sequence differences in *aroA* are not solely responsible for increased glyphosate resistance. Combined these results indicate that the increases in resistance or tolerance to glyphosate were not mediated by changes to *aroA* and, in some cases, may be due to epigenetic changes.

Figure 4- 7. Clinical Blood and Respiratory *Kp* Isolates, including ESBL- and KPC-producing strains, are Sensitive to AroA Inhibition

(A-D) Clinical *Kp* isolates were acquired from the blood and respiratory tract of patients, where strains are designated by their site of acquisition as B and R, respectively. The strains were grown overnight in L and then diluted in M9+gluc alone or containing 0.25 mg/mL glyphosate. Samples were then incubated at 37°C and the OD_{600} was measured every 15 min. The bacterial densities after (A-B) 10 hr and (C-D) 20 hr of growth are presented. Data is from at least 2 independent experiments performed in triplicate for each sample. Statistical significance was determined by comparing the OD_{600} for each sample against the *Kp* WT values in the same concentration glyphosate using One-way ANOVA with Dunnet's post-test. (E) The plating efficiency of the indicated *Kp* strains was measured by plating overnight cultures grown in L on M9+gluc alone or containing 1 mg/mL glyphosate and counting the number of colonies at 1, 2 and 3 days post-plating. Plating efficiency was measured by comparing the number of colonies that grew in the presence of glyphosate ($CFU_{+glyphosate}$) to the absence ($CFU_{-glyphosate}$) using plating efficiency=$CFU_{+glyphosate}$/ $CFU_{-glyphosate.}$ Statistical significance was determined by comparing each sample against the *Kp* WT values at the same time-point using One-way ANOVA with Dunnet's post-test. (F) The coding region of *aroA* was sequenced for the indicated strains, and any differences in the DNA or amino acid sequence compared to *Kp* WT are indicated. (G) Overnight cultures of the indicated strains were diluted into M9+gluc+/-0.25 mg/mL or 1mg/mL glyphosate. The samples were grown for 20 hrs at 37°C and the OD_{600} was measured. Each strain was tested in each condition in triplicate in at least 2 independent experiments.


A
10 hrs
OD600
1.5
1.0
0.5
0.0
KpWT
KpWT+G
R1
R1+G
R3
R3+G
R6
R6+G
R8
R8+G
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R10
R10+G
R13
R13+G
R14
R14+G
R16
R16+G
R17
R17+G
R20
R20+G
Non-ESBL/KPC
ESBL
KPC
B
10 hrs
OD600
1.5
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KpWT
KpWT+G
B1
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B2
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Non-ESBL/KPC
ESBL
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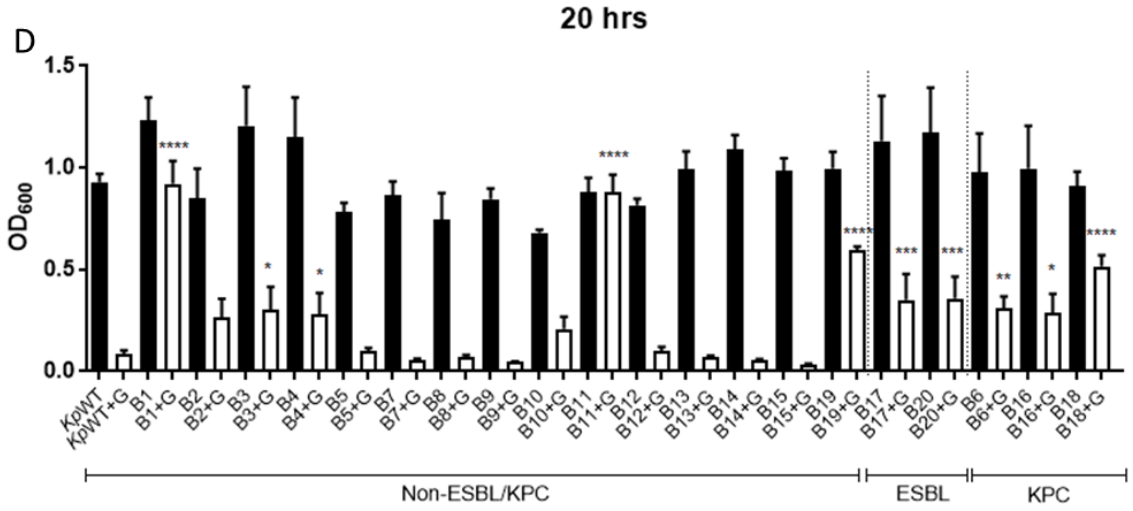
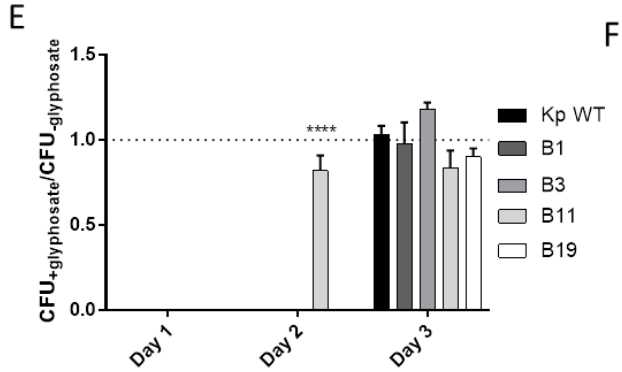
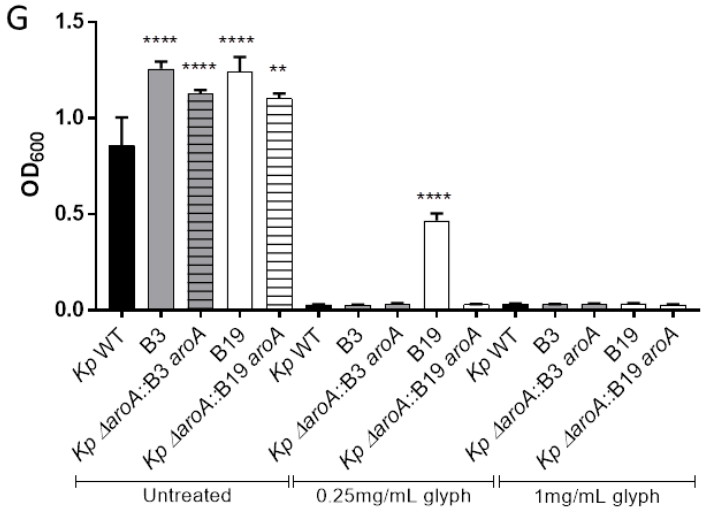
Non-ESBL/KPC
ESBL
KPC





F

Strain	gDNA Sequence Variation vs WT	AA Sequence Variation vs WT
B3	1256A>T	Q419L
B19	661C>A	Q221K
B1, B2, B11, B17, R1, R9, R17	None	None



CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Significance of This work

In this study, we sought to identify *Kp* genes that are required for growth in lungs of immunocompetent mice and immunodeficient mice. Studying the functions of the bacterial factors required to survive in mice provides direction into what immune effectors influence *Kp* infection, as well as the methods by which *Kp* evades or modifies these immune effectors. In addition, genes that are necessary in both WT and neutropenic mice have the potential to encode products that could be therapeutic targets, as a large proportion of the patient population is immunosuppressed in some manner, often involving neutropenia or neutrophil dysfunction (29), and effective therapeutics would target bacterial factors needed in a majority of the patient population. Using an arrayed library of 13,056 *Kp* transposon insertion mutants, 166 genes were found to be putatively required for *Kp* to successfully infect WT mice, of which 51 genes were also required in neutropenic mice, 52 were no longer needed upon removal of neutrophils. The fitness defect of the remainder in the absence of neutrophils was modest.

5.2 Identification and confirmation of novel virulence factors contributing to *Kp* virulence in both immunocompetent and neutropenic lungs

One challenge of using a high-throughput genomic screen of bacterial insertion mutants (in this study, TnSeq) is that there is not a standard method or even a predominant method of analysis to evaluate the outliers, i.e. potential hits (428). Our method of data analysis involved pooling the reads for all insertion mutants in a gene, normalizing to the gene size (and indirectly the number of insertion mutants per gene, as larger genes should theoretically be represented by more insertion mutants), and then

obtaining the frequency of the insertions for each gene in each sample. The fitness was determined by comparing the frequency of each "gene" after and before infection, as well as the standard deviation (SD) of the fitness of each gene independently. Genes were considered to have a statistically significant defect if their fitness was less than 2.43 and 2.54 SDs less than the mean fitness for WT and PMN-depleted mice, respectively, where the number of SDs added to the mean fitness was derived from a t-table as a $t_{.99}$ value based on the degrees of freedom (n-1, where WT=37 and PMN-dep=19). Here, the number of SDs added to the mean fitness based on experimental cohort took into account the size of the experimental population, as well as the variation of each individual gene as the SD was from the fitness of each individual gene, rather than the fitness of all the genes collectively covered by the screen. The benefits of this method are (1) it takes into account the individual variation of each gene by using a cut-off that takes into account the SD of each gene. This helps weed out some of the genes that appear to have fitness defects due to being represented at a low frequency in the input, because those genes with lower inputs often displayed more variation, i.e. had higher SDs. This reduced the number of genes whose putative defects *in vivo* were from not being abundant in the input and therefore having a less than average chance of seeding a lung (i.e. not overcoming the bottleneck) rather than a true fitness defect. This is because genes with a lower "fitness" due to the bottleneck generally have a larger SD because bottlenecks typically present as either having a severe defect or no defect at all. It also gives more weight to analysis of genes represented by more insertion mutants in the screen, as well as gives weight to using a larger experimental population, because the larger the experimental population, the lower the number of SDs is added to the mean fitness.

Another similar approach has been used in other studies (399), where a cut-off is somewhat arbitrarily chosen, such as 2+ $SD_{\text{Fitness all genes}}$ off the mean, where the $SD_{\text{Fitness all genes}}$ refers to the SD of the fitness of all the genes considered collectively. Both methods described above, when applied to our data, gave significance to genes whose input was in general less than the average input of the library; however, we noted that the second method enriched for significantly more insertion mutants that were not-well represented in the input. This enrichment for mutants not well-represented in the inputs is likely due to both real defects in the ability of these mutants to grow *in vitro* compared to the other mutants when prepping the library for infection of the mice, as well as being a "side-effect" of the way in which we do our analysis, suggesting there is room for optimization. Alternative methods of analysis include performing statistical tests, such as paired t-tests or rank tests, for each gene comparing before and after selection without adjusting for multiple-comparisons (397, 429) to determine significance. However, these methods seem more effective with smaller libraries or libraries with less stringent bottlenecks, as they are more sensitive to background "noise", as well as fail to address the false positives instilled by a large number of statistical comparisons. In addition, parametric tests, such as t-tests, assume the read counts follow a normal distribution, which is not typically the case, while nonparametric statistical tests, such as Mann-Whitney, are less likely to generate false positives due to background noise, but have a tendency to miss candidate genes of interest (428). Overall, we were concerned that we would disqualify a number of genes that were underrepresented in the library unless the bar of the decrease in frequency was low, and, should we use statistical tests and adjust for multiple comparisons to prevent false positives, the cut-off would have been $p<0.0001$. In some

cases, we may have been too conservative, as we also identified several genes that did not seem significantly attenuated in the library, but were in additional studies (discussed below). Furthermore, there were several genes that we would expect to be necessary for *Kp* virulence in the lungs, based on previously published studies or their direct role in pathways involving genes identified in our studies as necessary for *Kp* virulence, that were not. This includes genes related to the generation or functioning of known virulence factors, such as *entE* and *entF* in the synthesis of the siderophore enterobactin, and genes previously identified to be critical for virulence in the lungs, such as *aroE(384),* where *aroE* is also in the same pathway as the critical virulence factor *aroA* (412). These three genes were well-represented in our inputs, thus excluding the possibility they were not present in our library, and had defects in WT mice lungs in our TnSeq screen that were not statistically significant. One concern is that, despite optimization, our analysis is somewhat biased towards highlighting genes whose insertion mutants were present at a low-frequency in the input as having a defect, and, thus, biasing against those well-represented in the inputs. That this is the case for *aroE*, *entE* and *entF* is supported by there being a much higher number of input reads for these genes than the majority of genes identified as having a defect in WT mice in our screen. Overall, despite its caveats, the approach taken to analyze our TnSeq data yielded a number of novel *Kp* genes whose contribution to virulence in the lungs was confirmed *in vivo*.

The arraying of the TnSeq library of *Kp* insertion mutants followed by retrieval of a number of *Kp* mutants using combinatorial pooling was invaluable to this study. It provided a relatively rapid means of isolating a number of *Kp* mutants to screen in a high-

throughput manner in a number of assays, thereby highlighting genes that should be evaluated in further work. The presence of the same *Himar1* transposon in all the mutants of interest allowed the mutants to be tested in pools, as the mutants could be identified by deep sequencing the region flanking the insertion site of the transposon. Using this method, 61 insertion mutants were evaluated in a range of *in vitro* and *in vivo* assays, and the putative contribution to *Kp* virulence of a number of genes noted in the TnSeq screen was confirmed using both mini-libraries and single strain infections. It should be noted, however, that the use of this mini-library of *Kp* transposon insertion mutants comes with a few caveats. One is that, unlike with the use of clean deletion mutants, it is not necessarily clear if the phenotype seen in an insertion mutant is due to an interruption of the gene where the insertion is or genes further downstream in the same operon. Another is that, while the vast majority of insertion mutants evaluated had intragenic transposon insertions, several mutants in this library (*appC, araC, metA, ptsG, seqA* and *celA*) had insertions immediately upstream of the genes of interest, where the insertion was thought to be in the promoter region, where it theoretically interferes with expression; however, the promoters of these genes have not been confirmed. A lack of a phenotype with these insertion mutants under the conditions tested does not necessarily mean the corresponding gene does not play a role in those conditions. It is noteworthy though that the *appC* and *metA* insertion mutants had phenotypes in at least two assays in this study, suggesting upstream insertions did interfere with gene expression.

A number of mutants with significant or possible defects in WT lungs in the TnSeq screen were retrieved from the array and tested for their contribution to fitness under a number of *in vitro* conditions. In all, 27 out of 57 retrieved mutants with possible

or potential defects had *in vitro* or *in vivo* phenotypes in one or more assays (Tables 3-2 and 4-1 in Chapters 3 and 4, respectively). Notably, of our library, we were strongly confident in the lung infection model from the TnSeq screen of 42. Of these we tested 19 insertion mutants in single-strain and/or mini-library *in vivo* studies and found 16 (84%) of them were significantly defective, thus indicating that our cut-offs were very reasonable. Specifically, when *Kp* mutants with insertions in a subset of the genes with functions *in vitro* were tested *in vivo*, *dedA, dsbA, gntR, VK055_RS17445, VK055_26085, ycgE* and *yhjH*, were confirmed to contribute to *Kp* virulence in the lungs in single strain infections. These strains remain to be evaluated under neutropenic conditions using single strain or competition experiments. Meanwhile, *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA* were confirmed to enhance *Kp* fitness by competition in mini-library experiments in both WT and neutropenic mice, as well as in WT mice in single strain infections.

Surprisingly, a few insertion mutants expected to have defects in WT mice based on TnSeq data, *appC, sdhB,* and *yaaA,* did not have a defect during single strain infections in the lungs. There are several possible reasons why these mutants failed to show a defect in single strain experiments. First, there is the potential that the TnSeq analysis was performed in a manner that does not reflect the actual fitness of insertions mutants in these genes or our statistical analysis was not strict enough or that there are rare outliers. Second, we analyzed the data from our screen at the gene level, where all insertion mutants that were intragenic were included for analysis of fitness. However, when retrieving insertion mutants from our array, we isolated mutants with transposon insertions in the putative promoter region corresponding to certain genes as noted above.

In the case of the *appC* mutant, the transposon insertion was immediately upstream of the gene for experiments with the isolated mutant, such as the single strain mouse infections, whereas there were 3 transposon insertion mutants with intragenic insertions in *appC* in the TnSeq screen in the inputs. Thus, perhaps the expression of the isolated *appC* transposon insertion mutant is reduced enough to lead to the *in vitro* phenotype noted in low-iron, but not completely abrogated as it likely is in the intragenic mutants. Generating a clean deletion of *appC* and evaluating it both in the *in vitro* and *in vivo* assays utilized in this study would be important next steps when investigating this gene. Third, the library required the ability of transposon mutants to compete with other strains while the single strain infection experiments do not. There are many examples of some mutants performing better or worse under one type of experiment compared to the other. For example, for the *sdhB* mutant, the different phenotypes noted *in vivo* could be a reflection of the gene enhancing *Kp* fitness in a manner visible when observed in competition, but not in single strain. Specifically, the *sdhB* mutant has to compete for the resources, that perhaps other strains can generate *de novo* or more efficiently than the *sdhB* mutant, thus giving *sdhB* a relative disadvantage. This is reflected in the growth defect the *sdhB* mutant had for *in vitro* growth when evaluated in the mini-library, but not as a single strain under similar conditions. Fourth and finally, there may have been not enough data points, i.e. not enough power, for certain cohorts to obtain statistical significance. For example, the *yaaA* mutant had a fitness defect that was 10-fold lower than WT, but was not statistically significant. This is likely due to a lack of power for this comparison in our single strain analysis, because the *yaaA* defect was statistically significant compared to *Kp* WT when not corrected for multiple comparisons. On the

other hand, there was notable variation in the *yaaA* insertion mutant bacterial load between mice suggesting that the gene product may be critical at early or later in infection and our assay may be sampling the lungs on the cusp of when it is important.

Both the *Kp* transposon insertion mutant library of 13,056 mutants and the mini-library of the 62 isolated and identified *Kp* insertion mutants are great tools with which to study the role of *Kp* genes in a number of areas. In terms of pathogenesis, the library of 13,056 mutants could be used to determine which genes are necessary for infection in a number of models relevant to *Kp* epidemiology. For example, the libraries could be used to infect diabetic and alcohol-exposed mouse models, both of which are representative of at-risk populations for *Kp* infection. This is true for different types of *Kp* infection as well, such as for UTIs and bacteremias. Not only would the application of the mini-library in these models aid in identifying *Kp* genes necessary for virulence in these libraries, it would help in determining whether there are large differences in the *Kp* genes necessary for lung infection compared to other tissue sites, or in the absence of other immune effectors compared to neutropenia. Significant differences would be indicative of distinct conditions the bacteria are exposed to during infection. Using these assays, the genes needed to infect WT mice could be compared to those needed in other immune deficient conditions to help characterize what stresses *Kp* is encountering due to these immune facets, how these immune factors strive to limit *Kp* and what bacterial factors *Kp* utilizes to counteract these host defenses. For example, IL-17 is known to limit *Kp* infection. It would be of interest to determine if a distinct subset of genes is needed in IL-17 deficient mice versus neutropenic mice or if the same genes are critical against both host defenses.

5. 3 FUNCTIONAL CHARACTERIZATION OF PUTATIVE *KP* VIRULENCE DETERMINANTS

In this study, a number of genes were found to enhance *Kp* growth in nutrient restricted conditions, *glpR, dsbA, yajL, ycgE, aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE,* and *tyrA,* as well as a number of genes in nutrient rich conditions, *nuoC, glpR, dsbA, sdhB* and *yhjH*. These results suggest that the *in vivo* fitness defects noted for these genes in the TnSeq screen, as well as additional competition or single strain infections for several of these genes, may be due, at least in part, to a decreased ability of these mutants to grow rapidly, to adapt to different nutrient environments, and/or to grow in nutrient-restricted conditions. In support of this, none of these genes identified above had a clear restoration of fitness upon PMN-depletion in the TnSeq screen. Importantly, to our knowledge, these genes have not been previously characterized as factors necessary for *Kp* growth or virulence. A subset of these genes, *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA,* have metabolic functions which prevents their growth in minimal medium and are discussed below. While none of the remaining genes have been investigated in *Kp,* we can speculate on their functions based on work in other pathogens.

Interestingly, based on a previously published study in *E.coli, yajL* repairs certain glycated proteins. Therefore, the fitness defect in the *yajL* insertion mutant in M9+gluc may be due to decreased viability in the presence of glucose rather than less efficient growth, due to an inability to repair glycated proteins (430). Experiments could be performed to determine if replacing the glucose used to supplement M9 in these experiments with another carbon source restores growth of the *yajL* mutant. In addition, due to the hyperglycemia that occurs in patients with poorly controlled diabetes, it would

be interesting to determine if bacterial proteins, like host proteins, experience an increase in glycation in these hosts and if the absence of *yajL* would be additionally detrimental to *Kp* fitness in diabetic compared to healthy mice.

The gene *dsbA* was noted to have a growth defect in both M9+gluc and L. *Kp dsbA* is highly conserved to *E. coli dsbA* (431), which catalyzes disulfide bond formation during protein production in *E. coli* as well as other bacterial species (431, 432). Thus, the *Kp dsbA* mutant could be less fit due to an increase misfolding or instability of proteins translated during growth. Interestingly, while *dsbA* has not been previously evaluated in *Kp* virulence, it has been shown to contribute to virulence in mouse infections for several pathogens (433-435), and has been considered, along with other *dsb* family members, as a potential therapeutic target (436).

Based on *E.coli*, the *sdhB* and *nuoC* mutants, noted to contribute to growth in L, may fail to grow due a critical role in the aerobic oxidative TCA cycle (437-439). The succinate dehydrogenase encoded by *sdhB* has been shown to catalyze the production of fumarate from succinate (440), and it could be the limitation of the production of fumarate, a terminal electron acceptor that aids in ATP generation (441), that inhibits growth . This may be supported by our *in vivo* data, as well as provide an explanation for the growth defect seen for the *sdhB* mutant in competition *in vivo,* but not as a single strain, as our NMR data demonstrates there appears to be a significant quantity of succinate in the lungs, but no fumarate. As discussed above, in single strain infections, the *sdhB* mutant could be fit enough to cause an infection by scavenging the limited amounts of fumarate or downstream molecules or generating ATP through other, perhaps less efficient pathways, but is defective when put into competition against other *Kp*

bacteria. Interestingly, *sdhB* has been shown to contribute to virulence of *E.coli* in UTIs (441). *nuoC,* which encodes a subunit of the first complex in the TCA cycle NADH dehydrogenase and theoretically functions directly upstream of succinate dehydrogenase (442), may act in a similar manner.

Under low-iron conditions, both *aroA* and *appC* contributed to *Kp* growth. The generation of chorismate through the shikimate pathway, of which *aroA* is a member, is a critical step in the production of the siderophores enterobactin, salmochelin and yersiniabactin (443, 444). Therefore, it is likely the *Kp aroA* mutant is unable to produce these siderophores and, thus, has a decreased ability compared to the parental WT strain, which encodes for these siderophores (50), to acquire iron from an environment containing little free iron. Future work should include determining if there is a decrease in the amount of these siderophores being produced, as well as testing if the adding chorismate restores the growth of the *aroA* mutant in a low-iron environment. *appC* (i.e. *cydD*) putatively encodes a subunit of cytochrome bd-II, an ABC transporter (445). While work in *E.coli* has previously noted a defect in *cydD* mutants in the presence of iron chelators (445), it is still unclear what mediates this phenotype. Perhaps the *appC* mutant has lower intracellular iron levels than WT bacteria, maybe due to an inability transport in iron-bound siderophores.

While there were no mutants lacking capsule covalently bound to the bacterial surface found in our mini-library of *Kp* insertion mutants, there were a few that had decreased production of mucoviscosity/exopolysaccharide. These include mutants with insertions in *sdhB, nuoC, putA* and *ycgE.* These differences in mucoviscosity may be a reflection of a decrease in the production of capsular material, perhaps excreted or shed

from the surface, as capsule production is regulated in response to environmental cues and stresses. Interestingly, work in other pathogens suggest a functional TCA cycle is necessary for optimal capsule biosynthesis, likely through limitation of certain substrates generated by the TCA cycle used to create capsule (446). This is suggestive as to why a decrease in mucoviscosity was noted for the mutants lacking *nuoC* and *sdhB.* Meanwhile, *ycgE,* a putative transcriptional regulator, has been implicated in regulation of capsule production by *E.coli* that is in turn regulated in response to environmental cues (447), but how *ycgE* functions in *Kp* capsule production is unknown. Further studies are needed to determine the role of these genes in capsule production, such as by quantifying the components of the capsular material for each of these mutants, evaluating the potential regulatory roles these genes may play in the production of exopolysaccharides, and assessing whether mutants in these genes have decreased fitness when interacting with neutrophils or other phagocytes *in vitro*.

Several genes were found to play a role in the ability of *Kp* to withstand ROS and RNS. For H_2O_2, transposon insertions in *yaaA* and *gntR* mutants decreased *Kp* survival, while insertions in *VKO55_RS26085* and *ycgE* enhanced *Kp* survival. Likewise, transposon insertions in *VK055_RS17445, VK055_RS26085* and *yhjH* aided *Kp* survival in the presence of HOCl. A different set of genes appeared important for *Kp* survival of NO, where *dedA, gntR,* and *yhbU* contributed to NO resistance in 1:1 competition. To our knowledge at this time, none of these genes have been implicated in *Kp* resistance to ROS or RNS, and only *yaaA* has been published to protect against these stresses in other bacteria. In *E. coli, yaaA* protects against H_2O_2 by reducing iron levels and preventing damage by the Fenton reaction (448). Whether the same is true in *Kp* remains to be

determined. The identification of the roles of *VK055_RS17445* and *VK055_RS26085* in *Kp's* response to ROS is interesting because the proteins encoded by these genes are hypothetical. Further work remains to find potential homologs to these genes or conserved domains *in silico*, and evaluating the functions of these genes and the mechanisms behind these functions *in vitro*. However, our results demonstrating that there is a role for these genes in response to ROS assigns a putative function to these uncharacterized genes in *Kp* and aids in directing future studies. For all of the genes found to assist in *Kp's* response to ROS and RNS, a focus of future work should be to understand the function of these genes as virulence determinants *in vivo* and in the presence of neutrophils based on their role in dealing with these stresses. Both *in vitro* and *in vivo* studies would aid in characterizing these genes, using ROS and RNS deficient mice, such as gp91phox-/-, myeloperoxidase (MPO) -/-, and inducible nitric oxidase synthase (iNOS)-/- mice, and cells cultivated from these mice. In addition, the mechanisms of how these genes react to these stresses remains to be delineated, and may shed some light on some interesting phenotypes noted, such as the divergent roles of *VK055_26085* in H_2O_2 and HOCl, and the differences in fitness in *gntR* and *dedA* mutants in NO when evaluated in competition by mini-library versus 1:1.

5.4 Assessment of metabolic genes, particularly *aroA*, as targets of therapeutics against *Kp*

As previously discussed, antibiotic resistance in *Kp* is on the rise, and there is a critical need for novel antibiotics. Because of this, the identification of targets and development of anti-virulence drugs, while in its infancy, has been gaining popularity. These antimicrobial drugs inhibit bacterial virulence by targeting genes that bacteria

require specifically for infection rather than overall survival (*e.g. in vitro*). This, in theory, generates less selective pressure for the generation and spread of resistance to these drugs. These anti-virulence drugs are classically considered to target factors bacterial factors necessary to cause damage to tissues and cause disease, such as toxins and secretion systems, but can also include genes required for general survival *in vivo*, such as genes in metabolic pathways (449). These drugs could be used either as prophylactics that inhibit initial infection or treatments that slow the growth and spread of the infectious organism within the host enough for the immune response or another antibiotic to be effective. To-date, little work has been published on inhibitors of bacterial virulence factors and, of what has been published, has focused on virulence factors involved in quorum sensing/biofilm formation (*e.g. Salmonella typhimurium*), virulence associated secretion systems (*e.g.* pathogenic *Yersinia* and *Pseudomonas aeruginosa* type 3 secretion systems), toxin production/secretion (*e.g. Vibrio cholera* toxin) and adhesion (*e.g.* pili in *E.coli*)(449). Only a few *Kp* virulence factors have been previously published as potential targets of anti-virulence therapeutics, including DsbA (431). Importantly, only 1 study has demonstrated the effectiveness of an anti-virulence *Kp* inhibitor *in vivo* (450), where 2 inhibitors of capsule and LPS synthesis were found to attenuate *Kp* infection of mouse lungs. Overall, much work remains on identifying potential anti-virulence targets of *Kp*, as well as inhibitors of these targets, and assessing the efficacy of this approach.

Therefore, we set out to identify *Kp* gene products that could serve as novel targets of anti-virulence therapeutics, where the optimal gene products would have no homologs in humans, be necessary for *Kp* virulence *in vivo* in both immunocompetent

and immunosuppressed hosts, and would not readily become resistant due to fitness costs. In addition to the genes identified as necessary for *Kp* virulence discussed above, including *dedA* and *dsbA* (the latter of which has been previously discussed in the literature as belonging to a family of potential anti-virulence therapeutic targets (431, 451), but never demonstrated to be important for *Kp* virulence *in vivo),* we found a number involved in metabolic pathways. Specifically in this study, we found a number of genes involved in *de novo* synthesis of metabolites, *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE* and *tyrA,* to significantly contribute to *Kp* virulence in the lungs of both immunocompetent and neutropenic hosts. In particular, mutants with insertions in the genes involved in the aromatic amino acid synthesis pathway, *aroA, trpE,* and *tyrA,* had the most significant contributions *in vivo,* followed by those in the branched chain amino acid leucine biosynthesis, *leuA* and *leuB.* This is not surprising as the aromatic amino acids are the most resource expensive to synthesize followed by leucine (452). As humans and mice are unable to synthesize phenylalanine, tryptophan and leucine *de novo* and require phenylalanine to synthesize tyrosine, these pathways are promising bacterial targets of therapeutics. While this study continued to investigate targeting AroA as a means of inhibiting *Kp* growth, it will be worthwhile to assess the efficacy of inhibitors of components of the branched chain amino acid pathway, such as those that target LeuA and LeuB.

As a proof-of-principle, we found that inhibition of the *aroA* protein product, AroA or EPSP synthase, using the commercially available herbicide glyphosate is an efficient means by which to inhibit both *Kp* growth *in vitro* and during infection of the lungs. Due to the range of infections *Kp* is capable of causing, it will be interesting to

evaluate if *aroA* plays the same critical role in other infections, such as in UTIs and bacteremias, and, along those lines, if an AroA inhibitor would be broadly effective. Based on studies of other pathogens, it is in fact likely that *aroA* mutants will be attenuated in these sites (453-456). Not only did inhibition of AroA effectively limit *Kp* growth, but the frequency of spontaneous resistance to glyphosate was extremely rare and we did not isolate any mutants that were completely resistant to the compound in our study. A single base-pair mutation has been previously described that renders *Kp* AroA completely resistant to binding by glyphosate by changing the active site of the enzyme (426, 427), which is where glyphosate is thought to bind. However, the effect of this mutation on *Kp* viability and fitness has not been assessed. Given we never recovered any glyphosate resistant mutants in this study, particularly with resistance due to mutations in *aroA*, we speculate that this mutation and others like it that render AroA insensitive to glyphosate may come at a high fitness cost. Generation of a *Kp* mutant with this mutation would provide a means by which to evaluate these hypotheses, where we could compare fitness of the glyphosate resistant mutant to the parental strain for growth in minimal media *in vitro*, as well as virulence *in vivo*. Furthermore, it is interesting that, while we never recovered spontaneous *Kp* ATCC 43816 mutants or clinical isolates with complete resistance to glyphosate, there were several isolates with increased resistance or tolerance to glyphosate. As there were no mutations in *aroA* found that accounted for these phenotypes, the mechanisms of tolerance remain unknown and yet to be characterized. Possibilities include epigenetic changes, such as those that increase expression of *aroA*, and upregulation of transporters that export glyphosate and reduce

intracellular glyphosate levels, both of which have been noted means of glyphosate resistance in *E.coli* (457).

Overall, the results presented in this study demonstrate the importance of a number of metabolic genes and metabolite synthesis pathways for *Kp* virulence, such as *aroA* and the aromatic amino acid pathway as well as the branched chain amino acid, leucine. In addition, this work reveals the efficacy of targeting the protein product of one of these genes, *aroA*, for treatment of *Kp* infection.

5.5 Final Conclusions

In summary, using high-throughput approaches, several novel *Kp* virulence determinants needed for lung infection were discovered and confirmed, including *aroA, gltB, leuA, leuB, metA, nadB, serA, serB, trpE, tyrA, dedA, dsbA, gntR, VK055_RS17445, VK055_26085, ycgE* and *yhjH.* In addition, putative functions for these genes in metabolism, growth in nutrient limited, nutrient rich and low iron conditions, production of exopolysaccharide, and resistance to ROS and RNS were uncovered. Importantly, none of the genes identified in these assays have been implicated, to our knowledge, in having either the indicated functions or contributions to virulence in *Kp,* thus making these findings novel. However, much work remains further characterizing the roles of these genes both *in vitro* and *in vivo.* Furthermore, *aroA* was investigated as a potential therapeutic against *Kp* infection using an AroA specific inhibitor, glyphosate. Targeting AroA was found to be an effective means by which to limit *Kp* both *in vitro* and *in vivo.* Importantly, glyphosate resistance, both spontaneously in the lab strain used in this study and in clinical isolates, was a very rare event. Overall, this study revealed a number of putative *Kp* virulence determinants, uncovered both neutrophil-related and unrelated

functions for a number of these genes, and identified AroA as a strong potential therapeutic target that would likely be effective in both immunocompetent and immunosuppressed patients.

Furthermore, *aroA* was investigated as a potential therapeutic against *Kp* infection using an AroA specific inhibitor, glyphosate. Targeting AroA was found to be an effective means by which to limit *Kp* both *in vitro* and *in vivo.* Importantly, glyphosate resistance, both spontaneously in the lab strain used in this study and in clinical isolates, was a very rare event. Overall, this study revealed a number of putative *Kp* virulence determinants, uncovered both neutrophil-related and unrelated functions for a number of these genes, and identified AroA as a strong potential therapeutic target that would likely be effective in both immunocompetent and immunosuppressed patients.

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