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Genetic characterization of the K2 serotype capsule of Klebsiella pneumoniae ATCC 43816 and the development of a bioluminescent strain.

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GENETIC CHARACTERIZATION OF THE K2 SEROTYPE CAPSULE OF *KLEBSIELLA PNEUMONIAE* ATCC 43816 AND THE DEVELOPMENT OF A BIOLUMINESCENT STRAIN

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A Thesis Approved on

April 23, 2013

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Dr. Jonathan Warawa (Chairman)

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Dr. David A. Scott

DEDICATION

This thesis is dedicated to my family

Mrs. Jamie Nelson Scott,

Mr. James Stanley Scott,

Mrs. Paula Scott,

Mr. Ian James Scott,

Mr. JC Huggins,

Mrs. Joanne Huggins,

James Virgil Nelson,

Karen Susie Nelson,

Andrew James Nelson,

and

Leo

who helped me through this Master's Degree.

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ABSTRACT

GENETIC CHARACTERIZATION OF THE K2 SEROTYPE CAPSULE OF *KLEBSIELLA PNEUMONIAE* ATCC 43816 AND THE DEVELOPMENT OF A BIOLUMINESCENT STRAIN

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April 23, 2013

OBJECTIVE: *Klebsiella pneumoniae* is a Gram-negative enterobacterium that is a major cause of community-acquired and nosocomial infections. We performed a genetic characterization of *K. pneumoniae* strain ATCC 43816, which is a well-studied strain with a K2 serotype capsular polysaccharide. To provide the ability to monitor bacteria within a host, we will develop a bioluminescence strain of *K. pneumoniae* by integrating the *lux* operon into the chromosome. **METHODS**: The ATCC 43816 capsule cluster sequencing was performed by closing gaps in the Next Generation Sequencing by long range PCR for template and primer walking. We constructed a bioluminescent *K. pneumoniae* by using allelic exchange strategies and subsequently characterized the bioluminescent strain JSKP001 in a macrophage infection model. **RESULTS**: We have sequenced and annotated the CPS cluster as a 23,804-base pair sequence and identified eight homologous genes conserved between all serotypes of *Klebsiella*. Using our

bioluminescent strain JSKP001, we demonstrate that *K. pneumoniae* may enter a reduced metabolic state within macrophages. **CONCLUSION**: We have successfully sequenced and annotated a K2 serotype CPS cluster with high homology to other K2 serotypes *K. pneumoniae* strains. We observed that *K. pneumoniae* is internalized by macrophages, but proliferates at a slow rate. We tested our bioluminescent strain in numerous growth conditions and found that rich growth media supports higher metabolic activity while the lowest activity was detected in *K. pneumoniae* internalized within macrophages. This suggests that macrophages may only provide a minor role as an intracellular replicative niche *in vivo,* but might be a potential niche for chronic persistence in sublethal infections.

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CHAPTER I

INTRODUCTION

1.1 History

It has been less than 140 years since our understanding of the ability of bacteria to cause lethal pneumonia was first established from the combined efforts of Edwin Klebs and Carl Friedländer in the late 19th century [1, 2]. It was in Friedländer's laboratory where Hans Christian Gram developed the "Gram stain" technique to detect bacteria in histological sections of the mammalian lung [3]. This technique stains bacteria one of two colors: Gram-positive (purple/blue) and Gram-negative (pink/red), of which *Klebsiella pneumoniae* was the first identified as a Gram-negative organism, distinguished from the previously observed Gram-positive *Streptococcus pneumoniae* [4].

1.2 Epidemiology

K. pneumoniae subsp. *pneumoniae* (*K. pneumoniae)* is a Gram-negative, non-motile, encapsulated, rod-shaped bacterial pathogen that is ubiquitously found in nature as well as mucosal surfaces of animals [5]. *K. pneumoniae* is an opportunistic pathogen that primarily infects immunocompromised individuals with underlying or pre-existing health conditions such as diabetes mellitus, alcoholism, and chronic pulmonary obstructions. As an opportunistic pathogen, *K. pneumoniae* is responsible for causing a wide-range of infections from urinary

tract infections (UTI's), septicemia, intestinal infections, wound infections and pneumonia [6, 7]. Furthermore, antibiotic resistant *K. pneumoniae* is a significant contributor to nosocomial and community-acquired infections worldwide [8, 9].

1.2.1 Nosocomial Infections

K. pneumoniae has been described as the 7th most frequently cultured clinical isolate overall in U.S. hospitals [10] and the 4th overall in European hospitals [11]. The two most common presentations of *K. pneumoniae* in hospitals are in the airway and urinary tract infections, frequently associated with the use of contaminated medical devices such as endotracheal tubes or catheters (Frank et al., 2009; Jones, 2010). Since the 1970's, *K. pneumoniae* nosocomial infections in westernized hospitals have become increasingly associated with resistance to aminoglycoside antibiotics allowing for increased persistence within the health care setting [12]. However, in recent years *K. pneumoniae* strains have acquired plasmid-based extended-spectrum β-lactamases (ESBLs) [13], which convey resistance against newer cephalosporins. Currently, carbapenem antibiotics (imipenem and meropenem) are the most effective antibiotics against Gram-negative, respiratory-associated infections [14]. Consistent with the ability of *K. pneumoniae* to acquire antibiotic resistance, the pathogen has recently developed resistance to carbapenem antibiotics leaving limited treatment options and causing an increase in concern over the spread of carbapenemases expressing *K. pneumoniae* (KPC) strains which are associated with mortality rates of bacteremia ranging from 24% to 70% [15]. Current treatment options for KPC infections include the use of polymyxin

combination therapies (73% success rate) or tigecycline (71% success rate) [12]. While *K. pneumoniae* nosocomial infections are primarily found in western countries, in developing countries community-acquired *K. pneumoniae* infections are more prevalent [16-18].

1.2.2 Community-Acquired Infections

In Southeast Asia and Africa, *K. pneumoniae* is among the most frequently isolated causative agents of community-acquired pneumonia (CAP) (15.8%). This geographical representation of CAP could be due to differences in bacterial strains, host defense, or socioeconomic factors (e.g. environmental sanitation and primary healthcare) [19, 20]. In a worldwide study, 5% of patients in Taiwan and South Africa received antibiotic treatment prior to hospitalization compared to 23% of patients in westernized countries [19]. Additionally, 1/5 of patients admitted to hospitals with community-acquired *K. pneumoniae* presented bacteremia and required intensive care with mortality rates raising up to 55% [21-23]. *K. pneumoniae* CAP infections are associated with alcoholism in South Africa and Taiwan as 18% of the patients were alcoholics unlike westernized countries where alcoholism is not prevalent in patients [19].

In Taiwan, the manifestation of soft tissue infection caused by community-acquired *K. pneumoniae* was originally characterized as a liver abscess and the patients who were diagnosed with this disease had an overall mortality rate of 23% [24, 25]. Liver abscesses associated with *K. pneumoniae* are associated with K1 or K2 serotypes and have been predominately found in Southeast Asia with the infections often complicated with meningitis, which results in a mortality

rate of 30% to 40% [24, 26, 27]. This prominence of *K. pneumoniae* in both nosocomial and community-acquired infections has highlighted the importance of identifying mechanisms of virulence for this pathogen.

1.3 Virulence Factors:

1.3.1 Siderophores

Iron is a critical co-factor for *K. pneumoniae* virulence in animal models as it functions as a redox catalyst in proteins participating in oxygen and electron transport processes [28, 29]. *K. pneumoniae* secures iron from hosts by secreting siderophores such as yersiniabactin that competitively take up iron bound to host proteins [30-34]. More virulent strains of *K. pneumoniae* have been found to express three siderophores, yersiniabactin, enterobactin and salmochelin, suggesting that these three siderophores may play a key role in enhancing the virulence of the pathogen in an intranasal mouse model [30, 33, 35].

1.3.2.1 Fimbrial Adhesion

The ability of *K. pneumoniae* to adhere to mucosal and epithelial cell surfaces is mediated in part through fimbriae adhesions, a key component to colonization and infection [36]. *K. pneumoniae* possesses 2 types of fimbriae: i) Type 1 fimbriae which facilitates attachment to bladder epithelium [37, 38] and ii) Type 3 fimbriae which mediates biofilm formation in endothelial cells, as well as adhesion to the basement membrane of lung tissue [39-41].

1.3.2.2 Biofilm Formation

Fimbriae are required for biofilm formation of *K. pneumoniae* and facilitate the colonization of various ecological niches [40]. Biofilm formation allows *K. pneumoniae* to colonize surfaces like catheters in hospitalized patients and has led to the spread of nosocomial infections [39]. The biofilm is linked to enhanced bacterial resistance to antibiotic therapies and host defenses against *K. pneumoniae* [42, 43].

1.3.3 Lipopolysaccharide

The lipopolysaccharide (LPS) of *K. pneumoniae* is a major component of the outer membrane which consists of lipid A, a core polysaccharide, and the side chain O-antigen polysaccharide. LPS is critical for *K. pneumoniae* to resist complement-mediated killing during infection [44]. The structure of the oligosaccharide-repeating units of the O-antigen of *K. pneumoniae*'s LPS serves as a site for attachment of surfactant protein D, which decreases the adhesion to lung epithelial cells [45]. Surfactant protein D plays an important role in the regulation of innate immune responses in the lungs and causes agglutination and enhanced macrophage-dependent killing of *K. pneumoniae* [46]. *Klebsiella* strains that bind and agglutinate surfactant protein D by their mannose-rich O antigens are less able to infect the lungs compared to strains expressing galactose-rich O antigens [47]. *K. pneumoniae* that expresses O antigen recognized by surfactant protein D triggers the induction of cytokine response, which sets the stage for the innate immunity components [47]. The LPS of *K. pneumoniae* is considered to be a major virulence factor, which helps the

bacteria evade host immune responses and promote the persistence of infection in various hosts.

1.3.4 Capsular Polysaccharide

The capsular polysaccharide (CPS) of *K. pneumoniae* covers the bacterial surface and protects the bacterium from the host's inflammatory response, from opsonization, and subsequent phagocytosis [48]. *K. pneumoniae* produces a large hydrophilic capsule (160nm) consists of repeating subunits of 4 to 6 sugars (glucose, galactose, mannose, fucose and rhamnose) with numerous configurations leading to a diverse capsule library of 80 different capsular antigens which has been described for *Klebsiella* [49].

1.3.4.1 CPS Regulation

The mucoid phenotype found in liver abscess isolates is thought to be regulated by the *rmpA* gene [50]. The serotypes K1 and K2 are significantly more prevalent in strains that cause liver abscess than in the strains causing bacteremia alone [51]. In the K2 serotype strains, the *rmpA* has an isoform *rmp2A* gene that has shown to regulate CPS biosynthesis [52]. The *rmpA* and *rmp2A* gene belongs to the UhpA-LuxR family of transcription factors, which also includes *rcsA* and *rcsB* that are also known as regulatory genes for capsule synthesis [53]. The RscA, RmpA or RmpA2 proteins have all been demonstrated to interact with the RcsB protein to form a heterodimer in order to bind specifically to the CPS promoter for transcription initiation [52, 54-56]. This regulation of the CPS by the RscA, RmpA or RmpA2 proteins has been demonstrated to be associated with temperature as the production of the CPS is

at maximum levels when bacterial growth rate is low [57]. Since the biosynthesis of capsule in *K. pneumoniae* is controlled by a two-component regulatory system [54, 58], it is likely that multiple accessory factors may be used in order to increase the CPS biosynthesis in response to different environmental stimuli [52, 55].

1.3.4.2 Complement Cascade Defense

The CPS of *K. pneumoniae* resists the nonspecific host defense mechanism such as the complement cascade, which plays a crucial role in the early host defense against invading pathogens [59]. *In vitro* studies have shown that CPS inhibits the deposit of the C3 complement component onto the bacteria's surface and reduces the formation of the membrane attack complex [60, 61]. *K. pneumoniae* strains that express low amounts of CPS can cause an increase of complement activators which increases deposit of C3 and both complement-mediated and opsonophagocytic killing [60].

1.3.4.3 Antimicrobial Peptide Defense

Antimicrobial peptides pose a threat to the survival of *K. pneumoniae* during infection and the CPS acts as a countermeasure that can limit antimicrobial peptides interaction with the bacterial cell. The CPS mimics human cells by expressing polysialic acids in the capsule causing *K. pneumoniae* to be poorly immunogenic [62]. *In vitro* findings show that low concentrations of antimicrobial peptides (APs) up-regulate transcription of CPS, increasing the amount of capsule particulates shed by *K. pneumoniae* from its cell surface [63].

Additionally, free anionic CPS released from *K. pneumoniae* naturally attracts AP neutralizing the killing ability of APs [64].

1.3.4.4 CPS Cluster Organization

CPS gene clusters are found in a single chromosomal locus, which allows the coordinate regulation of a large number of genes involved in the biosynthesis and export of the CPS [65]. Sequenced *Klebsiella* strains have 8 genes (*galF*, *acid PPC*, *wzi*, *wza*, *wzb*, *wzc*, *gnd* and *ugd*) that are highly conserved in the CPS clusters [66]. The JUMPstart (for "just upstream of many polysaccharide starts") [67] element is part of transcriptional antitermination of the CPS cluster and is found upstream of the gene *wzi*. Genes *wza*, *wzb* and *wzc* are a common translocation-surface assembly pathway for cell surface CPS [66]. The regions described above are called "Region 1" and encode for the proteins necessary for translocation of the polysaccharide through the periplasm and across the outer membrane [68]. The region following these genes is serotype-specific and is responsible for biosynthesis and polymerization of oligosaccharide repeating units [68].

1.3.4.5 *magA*

The *magA* gene is located in the CPS cluster of *K. pneumoniae* of K1 serotype has been identified as a virulence gene, responsible for the hypermucoviscosity phenotype. The *magA* gene has been reported with a high prevalence of 98.1% for *K. pneumoniae* isolates from patients with liver abscesses [50]. The disruption of the *magA* gene has resulted in the complete loss of resistance to serum and phagocytosis with greater than a 10^5-fold

increase in LD_{50} for mice [69]. More recent studies, indicate that *magA* is a good tool for molecular typing rather than a major virulence determinant for *K. pneumoniae* strains that cause liver abscesses [50]. *MagA* and *rmpA* have been proposed as good markers for the rapid diagnosis of liver abscess [70, 71].

1.4 Animal Models

The use of animal models aids our understanding of the pathogenesis of infectious diseases, as well as, the development of therapeutics and vaccines. Given that *K. pneumoniae* is capable of colonizing a wide-range of host niches several surrogate animal models have been developed to study the pathogenesis of this bacterium. *In vivo* experiments involving *K. pneumoniae* in the mouse model use a number of routes of infection: UTI, intramuscular, intravenous, intraperitoneal, oral inoculation, intranasal, surgical intratracheal and/or non-surgical intratracheal infection [72-78]. In the mouse model, there are three models that have been used to study pulmonary infection and they are: intranasal, surgical intratracheal and/or non-surgical intratracheal infection. All three models are designed to inoculate the lung to model human respiratory disease caused by both community-acquired pneumonia as well as nosocomial (e.g. ventilator associated pneumonia disease). The lung possesses several forms of innate defenses against invading organisms including physical barrier, the ciliary clearance of mucus from the lungs, and the rapid epithelial cell signaling of immunoprotection, such as the activation of resident alveolar macrophages [79].

1.4.1 Intranasal Model

The *in vivo* intranasal route of infection is used to target the lungs, which *K. pneumoniae* readily colonizes and is found to disseminates to both the liver and the spleen following intranasal infection [72]. Infections established by intranasal delivery using strain ATCC 43816 resulted in rapid fatal disease of mice with a mortality rate at 2 to 4 day and LD_{50} values of 3.0×10^3 [72, 80]. Even though rapid morality occurs in the intranasal infection model, the lower respiratory tract is only one of three sites the inoculum is able to infect. When the inoculum is deposited in the nares, it is able to infect both the upper and lower respiratory track along with the digestive track [81]. Although *K. pneumoniae* is known to infect all of these three locations, the target organ when using the intranasal infection model is the lungs [81].

1.4.2 Surgical Intratracheal Model

The benefit of the intratracheal model is the direct delivery of *K. pneumoniae* to the lungs and the avoidance of the infection in the upper respiratory track. The surgical method involves the an incision to the ventral neck skin to expose the trachea, injecting the bacterial suspension by needle through the trachea, and then stitching up the incision [82]. The intratracheal infection using strain ATCC 4386 closely mimics intranasal infection rapid mortality rate with similar LD_{50} values (2×10^3) in ICR strain mice [83]. However, this method is supposed to enhance delivery specifically to the lungs, but the trauma caused by surgery has the potential to initiate a secondary infection.

1.4.3 Non-surgical Intratracheal Model

In our laboratory, we developed a non-surgical, intubation-mediated intratracheal model (IMIT) for *K. pneumoniae* respiratory disease studies. This method involves intubation of mice followed by injection of the inoculum directly into the lungs [84]. The non-surgical intratracheal model removes infection from the upper respiratory track while also eliminating the trauma caused by the surgical method and mimics contaminated ventilation catheters of hospitals in the US. This mode of infection ensures complete delivery of the inoculum to the lungs to simulate bacterial pneumonia in humans. We have identified an LD_{50} of 4.71×10^1 for *K. pneumoniae* ATCC 43816 by the IMIT model in BALB/c mice [84] and $10^{3.6}$ CFU in female C57BL/6 mice (unpublished findings). This IMIT model demonstrates a lung-specific infection model which mimics the contaminated ventilation catheters which is the major mode of infection for hospitals acquired *K. pneumoniae* pneumonia infections in the US.

1.5 Hypothesis

Capsule is an important virulence determinant for *K. pneumoniae*, however, recent Next Generation Sequencing efforts conducted by our laboratory failed to fully sequence the K2 CPS cluster from strain ATCC 43816, a commonly used *K. pneumoniae* strain to study respiratory disease. We predicted that K2 CPS clusters would be highly conserved with previously sequenced K2 CPS clusters, and that the sequence of the ATCC 43816 K2 CPS cluster would allow us to preform subsequent studies to investigate the role of CPS in *K. pneumoniae* virulence. We also aimed to develop a novel bioluminescent strain

of *K. pneumoniae* through stable chromosomal introduction of the *lux* operon, allowing for the monitoring of the disease process in cell infection assays and living animals. Finally, combining the use of these new tools (CPS sequence and bioluminescent *K. pneumoniae*), we intended to monitor the role of *K. pneumoniae* CPS in *in vivo* studies.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial Strains and Media

All bacteria were stored in -80°C as 25% glycerol stocks and cultured in Luria broth (LB; Lennox, 1955) overnight at 37°C with shaking at 200 rpm unless otherwise stated. All *Klebsiella* strains were then subcultured (1:25, vol/vol) into 2.5 ml trypticase soy broth (dialyzed and chelated) (TSBDC; Brett et al., 1997) containing fresh monosodium glutamate (2.5 M MSG stock) at a 1:50 concentration and grown at 37°C for 3 hours unless otherwise noted. *E. coli* strain DH10B was used for cloning and *E. coli* strain S17-1 was used for conjugation with *K. pneumoniae*. When appropriate, antibiotics were used at the following concentrations: kanamycin (Km: 25 µg/ml), gentamicin (Gm: 20 µg/ml for NTUH and ATCC, 1000 µg/ml for MGH), carbenicillin (Cb: 100 µg/ml), and zeocin (Zeo: 25 µg/ml or 100 µg/ml). Details of strains and plasmids used in this study are shown in Table 2.1.

2.2 Molecular Biology

2.2.1 PCR Reactions

PCR amplification of cloning fragments or for PCR sequencing of fragments smaller than 4 Kb was performed with Phusion High-Fidelity DNA polymerase using the following protocol: initial denaturation at 98°C for 30 seconds followed

Table 2.1

Bacterial Strains and Vectors

Strain	**Genotype/description**	Source
DH10B	Electrocompetent *E. coli* cloning strain	Invitrogen
S17-1 λ pir	*E. coli* strain for conjugation	[85]
ATCC 43816	*K. pneumoniae*	[86]
NTUH-K2044	*K. pneumoniae*	[9]
MGH 78578	*K. pneumoniae*	[72]
ATCC43816::pJMW106 -*galF*	*K. pneumoniae*::pJMW106-*galF*	This Study
ATCC43816::pJMW106 -*uge*	*K. pneumoniae*::pJMW106-*uge*	This Study
WKP001	*K. pneumoniae*::pKSVS2-Kp ATCC-*nif*-P*rplU-lux*-floxZeo	This Study
JSKP001	*K. pneumoniae*::Kp ATCC-*nif*-P*rplU-lux*	This Study
Plasmid		
pJET1.2	Blunt cloning vector	Fermentas
pCR4	TOPO-TA Cloning vector for sequencing	Invitrogen
pCR4-*galF*	TOPO-TA Cloning vector harboring *galF* fragment	This Study
pCR4-*uge*	TOPO-TA Cloning vector harboring *uge* fragment	This Study
pJMW106-*galF*	Suicide vector harboring *galF* fragment	This Study
pJMW106-*uge*	Suicide vector harboring *uge* fragment	This Study

Table 2.1 continued

Bacterial Strains and Vectors

Plasmid		
pSK	pBluescript SK (+)	Stratagene
pSK-Δcap-Zeo	pBluescript SK (+) vector harboring Δcap-Zeo	This Study
pSK-Δcap	pBluescript SK (+) vector harboring Δcap	This Study
pJMW106	Suicide vector	Unpublished lab vector
pJMW106-Δcap-Zeo	Suicide vector harboring Δcap-Zeo	This Study
pJMW106-Δcap	Suicide vector harboring Δcap	This Study
pGSVS	Suicide vector	This Study
pKSVS2	Suicide vector	This Study
pKSVS2-Kp ATCC-*nif*-P*rplU-lux*-floxZeo	Suicide vector harboring P*rplU-lux*-floxZeo	This Study
pKSVS2-Kp ATCC-*nif*-P*rplU-lux*	Suicide vector harboring P*rplU-lux*	This Study
pKSVS-P*tolC-cre*	Suicide vector harboring P*tolC-cre*	This Study
pKSVS-P*tolC*-cre-*galF*	Suicide vector harboring P*tolC-cre-galF*	This Study

by 30 cycles based on the following: 98°C for 10 seconds, 68°C for 30 seconds and 72°C for 60 seconds for 1-2 Kb fragment, 120 seconds for 2-4 Kb fragments and the final extension of fragment at 72°C for 10 minutes. Long Range PCR amplification was performed with Phusion High-Fidelity DNA polymerase for fragments larger than 4 Kb. The following protocol is used for long-range PCRs: initial denaturation at 98°C for 30 seconds followed by 30 cycles based on the following: 98°C for 10 seconds, 68°C for 30 seconds and 72°C for 300 seconds for 4-8 Kb fragment or 600 seconds for 20 Kb fragments, and the final extension of fragment at 72°C for 10 minutes. All primers used are listed in Table 2.2.

2.2.2 Bacterial Enumerations

Viable cell count was performed by serial dilution of the original sample in PBS, plating aliquots of the dilutions in triplicate onto an LB plates and then incubating at 37° for 8 hours or at room temperature for 24 hours. After incubation, the colonies were enumerated and the number of viable cells was calculated. Serial dilution was conducted in a 96 well plate using 6 fold serial dilutions in eight wells. A multichannel pipettor was subsequently used to replica plate the samples on an LB agar plate for enumeration (Figure 2.1).

2.3 Sequencing Capsular Polysaccharide Cluster

Next Generation Sequencing (NGS) was performed on ATCC 43816 which yielded five contigs of DNA spanning the capsule cluster. The five contigs were bridged using either genomic DNA capture or primer walking techniques. Similar methods were used in order to close gaps in contig level sequencing efforts and obtain complete genome sequence for KCTC 2242 (AN: CP002910),

Table 2.2

Oligonucleotides

Primer Number	Oligonucleotide	Sequence (5' → 3')
1	galF SacI (+)	GCGAGCTCCAGGCGCAGCCGCTGGGCCTGG GCCACTC
2	galF XbaI (-)	GTTCTAGACCGGCCCAGATATCCGCCGACAG CACGTAG
3	uge SacI (+)	GCGAGCTCCGGGCTGCGCTTCTTTACAGTGT ATGGTCCGTG
4	uge XbaI (-)	GCTCTAGAGTCCAGCACATCTCCCGGCTGGA TCGGCATCATATTCTTCTG
5	43816cap8-1 (+)	GCTTCCAGCTCGTAGGAGGTGTCGAAGTGG
6	43816cap8-2 (-)	ACAGCATAATTAAATCGCGGGCGCTAAAGCTG AAGTCG
7	43816cap4-1 (+)	GTGATGGTTTCCTGTAGCATGGAGTTCTGCCC G
8	43816cap4-2 (-)	CAGTTCCTGCAAAAGATCACTGATGCTTATGC GCAAAACG
9	43816cap3-1 (-)	TGGATCCGCGTATCGGCAACCATTATAACAAC CCG
10	43816cap3-2 (+)	GCGGGTGTAAACCTTGTCCGCCACATCTGC
11	43816cap3-3 (-)	GGAATGGAAGCGCAGAAGAATATGATGCC
12	43816cap3-4 (+)	GTTCTTCAACTCCCGCGTCGTGCG
13	43816cap5-1 (+)	CTTCAGCAATCAGCTGCATGTCACCATACTCA ATGCC
14	43816cap7-2 (-)	ATGCTGATGACCGGCGAAAGCTACGACTGCG
15	43816wzi (+)	CCAACGTCATCGCTGTCGCTGTTGTTAGCG
16	43816wzi (-)	TCGACATGGCCGCTAAGCCAGGAAGAGATCG
17	43816 wzc (+)	GCGACAATACACCTGTATCTTTTCCAGTATCT GCGACAG
18	43816 wzc (-)	TATTAATGGGGAGTCGCATTGAAAAGTTCCTT GAGTGGGC
19	43816cap7-3 (+)	GTACAACTGGGCTGGAGCGGTGACG
20	43816cap7-4 (+)	ATCACCCGGAGCTGACAACACCTGC
21	43816cap7-5 (+)	GGCAATGCTGAAGGTATGTCGCAAGCG
22	43816cap7-6 (+)	CAACATTGCACCCGAAGCACGAGG
23	43816cap7-7 (+)	GGGCAAGGTTAAGTGGAGAGCACCAGGG
24	43816cap7-8 (-)	GAGTCTCAGGCATTGTTGCAACACGC
25	43816cap7-9 (-)	GCAAGATCCGCAGGATTTTCTGTAGCAAGTAA
26	galF-wzi (+)	AAAGAAGGCGCCAAATTCCGCGAAAGTATTAA AAAACTGC

Table 2.2 continued

Oligonucleotides

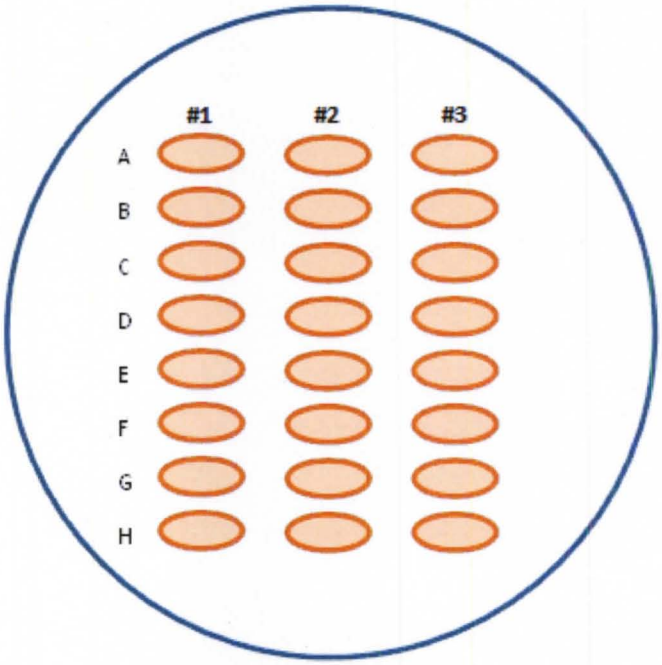
Primer Number	Oligonucleotide	Sequence (5' → 3')
27	43816wza-wzc(+)	CAGCGACGCTACCGCCCCTGGC
28	wzi-wzc (+)	GTGTCTGACCAAACCCCTGCGGAGTGC
29	43816wzi-wza (+)	CGCGTATTACACCAGCGCCTGTCTGCC
30	43816wzi2 (-)	CGTCATCGCTGTCGCTGTTGTTAGC
31	Chedid Orf 9 (+)	GTTTCAACTGATTGCCCATATCCTGCC
32	Chedid Orf 9 (-)	GCAAATAGCACTTCGTCATTGG
33	43816wzx (+)	CTATCTCGTATTCTGGAGCCATTTGAATTCGG TGTTTTGTC
34	43816wzx (-)	GACGAAATAAGCCTTTCATCTCAGCAAGTAAC TTATTAGGGC
35	43816Cap6-3 (+)	ACTATGAGTATAAATGTGCTGTAGATGCAGG
36	43816Cap6-4 (+)	CATCTAATAAATTCAGGAAAAGCGATATGG
37	43816Cap6-5 (+)	CGATTGCGGTATCATTATGGTCGC
38	43816cap5-3 (-)	GTGGTCACTGCGACACGTTCGCAGC
39	43816cap5-4 (-)	GCGCAAGGTTACGCCCCATCACAGCC
40	43816cap5-5 (-)	CGGACATCTGACCAAAAAGTACGTTAAAGAA CTGCGG
41	43816cap5-6 (-)	CCTCAAACCCTAACCAAGGCTGC
42	43816cap5-7 (-)	GGAAGCATTTGCATTCGAGCG
43	43816cap5-8 (-)	CGTGATTAACATTTGACAACCCG
44	43816cap5-9 (-)	CGCGCCTGGAGTGAAACACCATC
45	43816cap5-10 (-)	CAGTATAACTCCGATTATCAGCAAAC
46	43816cap5-11 (-)	GAGGTAAGCATTCGTTGTGTGCCGC
47	43816cap5-12 (-)	GCTTCTTGGCCACTAATAAAAACACACTCACC
48	43816cap5-13 (+)	CGCTCGGTATTGCTGGCCATGAGGC
49	43816cap5-14 (+)	CGTTAGTACAAGCATTGGAAGTGAGGGCACT GGAAC
50	43816cap5-15 (-)	GACAAAACACCGAATTCAAATGGCTCCAG
51	43816cap5-16 (-)	CCCCTTGTTGCCACGGATGAATATGTGTCTAT ATCACTTGCAAGTGC
52	galF EcoRI (+)	GAGAATTCGTGAAAAACAATCAGCGGTTTGG TCAGGGTG
53	galf XhoI (-)	ATCTCGAGTCCTGCGACCGGAATAACCGC
54	ugd XhoI (+)	CCCTCGAGCTGTTTGGTAACGATTAATTCTGC CTG
55	ugd KpnI (-)	ACGGTACCGCTGTATGACCTGGTGGGC
56	5' Kpin2 EcoRI(+)	GAAGAATTCCGCGCCGGGACGGTCCATCTTG TTGACGAAGGCCAGCC

Table 2.2 continued

Oligonucleotides

Primer Number	Oligonucleotide	Sequence (5' → 3')
57	5' Kpin2 NheI(-) int1	CAGCAGCGTATCCAGCAGGCCGGGAAAACGG GCGTCCAGGTCTTCCCG
58	3' Kpin2 KpnI(-) new	GTCGGTACCGTGATTTATGGCGTGTTTGAAGG CGAGGG
59	3' Kpin2 NheI(+) new2	GCGGCTAGCCCTGACCCGGCCTACGGCGGC CGCCCGTCAGCTTATGTCC
60	floxZeo BspEI(+)	CTTCCGGATGGCTCGAGTTTTTCAGCAAGATG TCCGGATCATAACTTCG
61	floxZeo NotI(-)	GATCTTCTAGAAAGATGCGGCCGCCATAACTT CG

Figure 2.1



Plating Scheme for Bacterial Enumerations

To enumerate bacteria, samples were diluted through serial dilutions on a 96 well plate, and a row of eight samples was replica plated in 10 µl aliquots on an LB agar plate. The spots were plated in oval shape to avoid merging of independent spots.

NTUH-K2044 (AN: AB198423), and MGH 78578 (AN: CP000647).

2.3.1 Genomic Capture

The genomic capture process was performed by using insertional mutagenesis to insert the vector pJMW106 into the *galF* and *uge* genes located in or near the capsule cluster. Approximately 400 bp fragments containing portions of the genes *galF* and *uge* were PCR amplified using primer pairs 1/2 and 3/4 (Table 2.2). Each fragment was cloned into pCR4 vector generating the vectors pCR4-*galf* and pCR4-*uge* respectively. The *galF* and *uge* fragments were then cloned into the pJMW106 vector using the SacI/XbaI restriction sites to generate the plasmids pJMW106-*galF* and pJMW106-*uge* respectively. S17-1 was used to conjugate the pJMW106-*galF* and pJMW106-*uge* constructs into *K. pneumoniae* ATCC 43816 and integration of the constructs were selected on carbenicillin and kanamycin LB plates. Insertions were confirmed by PCR and named ATCC43816::pJMW106-*galF* and ATCC43816::pJMW106-*uge,* respectively.

ATCC43816::pJMW106-*galF* and ATCC43816::pJMW106-*uge* genomic DNA was isolated using the Promega Wizard Genomic DNA purification Kit. Genomic DNA was digested using one of the following enzymes: EcoRI, SpeI, NotI or SacI. The digested genomic DNA was then ligated and electroporated into DH10B and grown on Km25 plates. Single colonies were chosen and grown in LB broth containing kanamycin at 37°C with shaking. Plasmid DNA was isolated by miniprep and digested using an appropriate restriction enzyme (listed above). The digestion mixture was then placed into agarose gel for gel

electrophoresis to show fragment captured along with the pJMW106 vector. Sequencing efforts were conducted to characterize the captured chromosomal fragments.

2.3.2 Primer Walking

PCR was used to close gaps between contigs that were missing from the NGS of ATCC 43816 genome. Long range PCR was used to acquire template to be used for primer walking. The first step was to extend the boundary of known sequence into the unknown region of template by using PCR base primer extension. Primers were then designed based off of the newly acquired sequence and used to extend boundary of known sequence into unknown region in an iterative process. All oligonucleotides used are listed in Table 2.

2.3.3 Sequencing of CPS Cluster

NGS returned five contigs of DNA spanning the capsule cluster leaving 4 gaps to close. Using ATCC 43816 genomic DNA, a 1.2 Kb fragment was PCR amplified and sequenced using primer pair 5/6 (Table 2.2 and Figure 2.2) to sequence the gap adjacent to gene *galF*. Subsequently, a 673-bp fragment was PCR amplified and sequenced using primer pair 7/8 (Table 2.2 and Figure 2.2) to sequence the gap between genes *gnd* and *manC.* A 1.5-Kb fragment was PCR amplified using primer pair 9/10 (Table 2.2 and Figure 2.2) for template to sequence the region from genes *ugd* to *uge*. Primers 9, 10, 11, and 12 (Table 2.2 and Figure 2.2) were used to sequence the 1.5-Kb fragment connecting sequence of genes *ugd* and *uge*.

Using long range PCR, a 20-Kb fragment was amplified using the primer pair 13/14 (Table 2.2 and Figure 2.2). This 20-Kb fragment was used for template to sequence remaining region between genes *galF* and *gnd.* Primers were designed based off Chedid and NTUH-K2044 sequence and the following two primer pairs 15/16 and 17/18 (Table 2.2 and Figure 2.2) were used to acquire two sequencing islands at 5' end of CPS cluster. This gave us two sequencing gaps between genes *galF* and *wzc* and using the primer walking technique made use of primers 19-30 to complete closing of the gap (Table 2.2 and Figure 2.2).

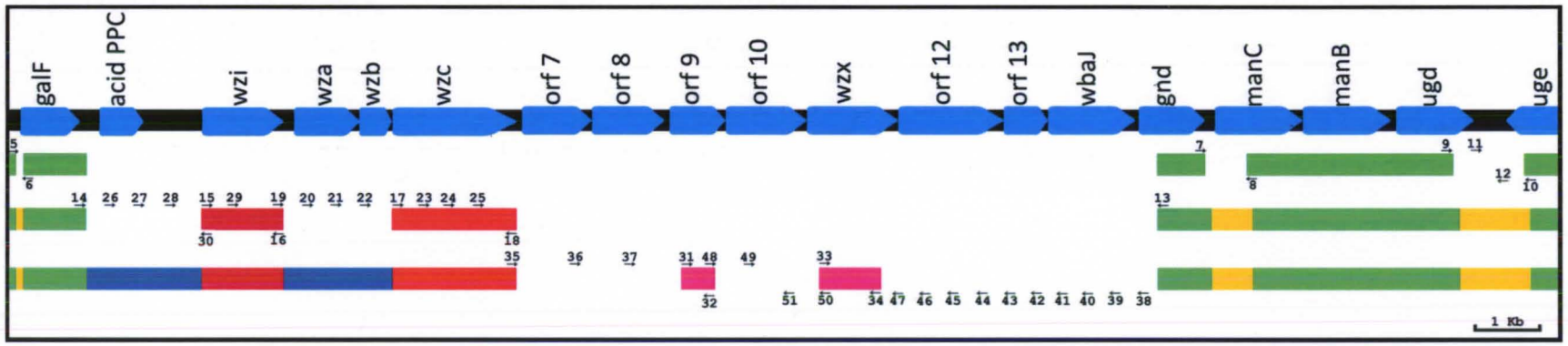
Primers were designed based off Chedid sequence, and the two primer pairs 31/32 and 33/34 (Table 2.2 and Figure 2.2) were used to acquire two sequencing islands in the central region of the CPS cluster. The remaining sequence was acquired using the primer walking technique primers 35-51 (Table 2.2 and Figure 2.2). The sequence was then manually aligned in Microsoft 2010 Word.

2.4 CPS Cluster Annotations

The sequenced capsule cluster was analyzed using Acaclone pDraw32 DNA analysis software to predict open reading frames (ORFs) encoding for putative proteins greater than 35 amino acids. Homology searches of putative proteins predicted by pDraw32 were conducted using BLASTP Genbank (NCBI) database. The gene name, description, and probable function of each predicted gene was assigned according to the results of BLASTP (identity 70% and results of BLASTP (identity 70% and matched length 80%) against NCBI *K. pneumoniae* strains. The BLAST and/or ClusterW2 algorithm was used to compare the

Figure 2.2

Sequencing of CPS Cluster



homology of putative proteins identified by pDraw32 to all published *K. pneumoniae* sequenced CPS clusters as of 12/1/12. The *K. pneumoniae* strains used with accession number (AN) were: serotype K1 (NTUH-K2044 AN: AP006725 and DTS AN: AY762939), K2 (CHEDID AN: D21242 and VGH-535 AN: AB371296), K5 (NTUH-K9534 AN: AB289646 and VGH404 AN: AB371292), K9 (VGH484 AN: AB371293), K14 (VGH916 AN: 371294), K20 (NTUH-KP13 AN: AB289648 and 889/50 AN: AB289647), K52 (MGH 78578 AN: CP000647), K54 (NTUH-KP35 AN: AB289650) K57 (A1142 AN: AB334776), K62 (VGH698 AN: AB371295), non-typeable (NT) strain (A1517 AN: AB334777) and not determined (ND) serotypes (NK29 AN: AB371290, NK8 AN: AB371289, NK245 AN: AB371291, KCTC 2242 AN: CP002910, HS11286 AN: CP003200, Kp13 AN: 377737, and 1084 AN: CP003785).

2.5 Mutagenesis and Construction of the *K. pneumoniae* Capsule Cluster Δcap Mutant

Allelic exchange mutagenesis was performed in *K. pneumoniae* ATCC 43816 using the vector pJMW106. 1-Kb fragment containing a portion of the gene *galF* was PCR amplified using the primer pair 52/53 (Table 2.2) to make a 1-Kb fragment containing a portion of the gene a *ugd* was PCR amplified using primer pair 54/55 (Table 2.2). The two fragments were cloned separately into pJET1.2 and the two PCR fragments were then subsequently assembled together using the common XhoI restriction site into the pSK vector creating the vector pSK-Δcap. A 500-bp fragment containing the zeocin antibiotic marker *Sh ble* gene was PCR amplified using primer pairs 60/61 (Table 2.2).The fragment

was cloned into the common XhoI site between fragments *galF* and *ugd* in the vector pSK-Δcap creating vector pSK Δcap-Zeo. The assembled 2.5-Kb Δcap-Zeo fragment was cloned into pJMW106 using EcoRI/KpnI restrictions sites to generate the allelic exchange plasmid pJMW106-Δcap-Zeo. S17-1 was used to conjugate the pJMW106-Δcap-Zeo construct into *K. pneumoniae* ATCC 43816 for the two-stage allelic exchange procedure, with the first stage selecting for integration of the construct on LB agar plates containing carbenicillin and kanamycin to isolate a merodiploid colonies. Single colonies were chosen and grown in LB broth containing carbenicillin, kanamycin and zeocin. The second stage of the allelic exchange procedure was done to select for the excision of the vector from the genome while also removing the CPS cluster. 20 µl of LB broth containing merodiploid colony grown in carbenicillin, kanamycin and zeocin was transferred to LB broth contain 15% sucrose and grown at room temperature overnight. Subsequently, the next day 10 µl of LB broth was transferred to LB agar plates containing 15% sucrose and grown at room temperature overnight. The following day single colonies were transferred to a LB agar plate containing kanamycin followed by the transformation to an LB agar plate to confirm the loss of the vector and the 25,000-bp fragment.

2.6 Infection of Cultured Macrophages

J774A.1 cells were maintained in DMEM (Gibco 11995) medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and grown at 37°C with 5% CO_2 and 100% humidity. For infection studies, monolayers of J774A.1 cells were seeded at a concentration of $7.5x10^4$ cells per

well (100 µl) in a white 96-well plate (Greiner Bio-One) one day prior to infection. *K. pneumoniae* strains were grown as described above and bacterial concentrations were estimated using spectrophotometry. The bacterial suspensions were diluted in PBS to reach an MOI of 10, 3 and 1 and 5 µl aliquot was inoculated into each well containing J774A.1 cells. One hour post-inoculation, gentamicin was added to each well to kill the extracellular bacteria. At 3, 4.5, 6, 9 and 12 hour post-inoculation, DMEM media was removed and 100 µl of 0.1% Trition was added to lyse cells. The solution was then transferred to 96 well U-bottom plate for bacterial enumeration (2.2.2).

2.7 Chromosomal Introduction of *luxCDABE* Operon

We designed *lux* knock-in mutation using allelic exchange strategy to insert the *lux* operon between genes *fusA* and *yeeF*. A 407-bp fragment was synthesized containing the stem loop terminator from the *K. pneumoniae* genome and the promoter gene P*rplU* to regulate *lux* production. The two PCR fragments from the *K. pneumoniae* genome [primer pair 57/58 (Table 2.2) and 59/60] were cloned separately into pSK at EcoRI/ApaI (1192-bp) and NotI/KpnI (1074-bp) fragments (Figure 2.3). The synthesized fragment was assembled together with 5' fragment using the common BsiWI site engineered into both the promoter and the 5' fragments were added to the pSK 5' Kpin2 vector. The two fragments (5' Kpin2-P*rplU* and 3' Kpin2) were then assembled in pGSVS on either end of the lux operon to yield pGSVS-Kpin-P*rplU-lux* (Figure 2.3).

The zeocin antibiotic marker *Sh ble* gene was PCR amplified using primers 60/61. The fragment was cloned into pSK creating the vector pSK

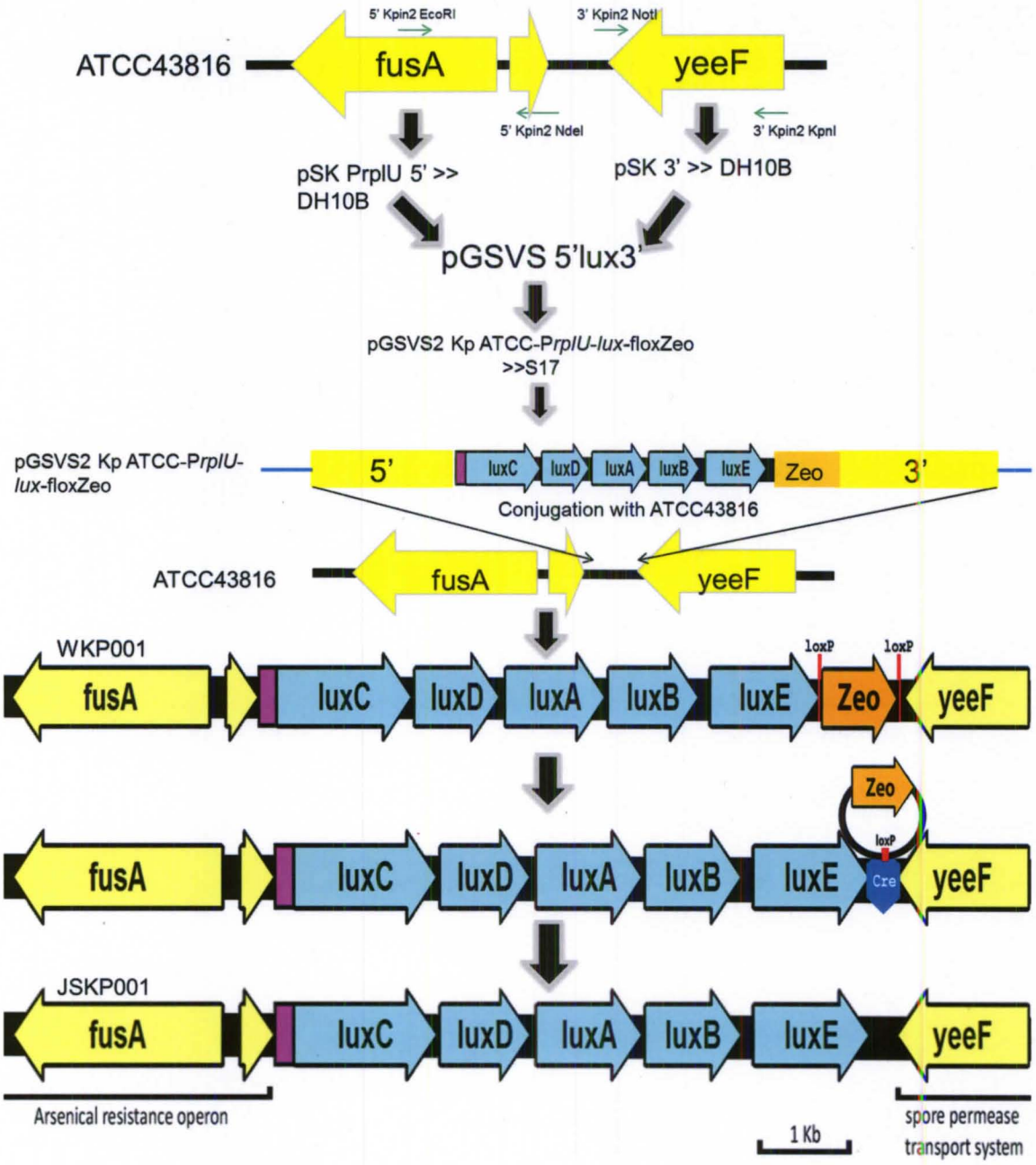
floxZeo. The floxZeo fragment was then cloned between 3' fragment and *luxC* gene of the *lux* operon in the plasmid pGSVS-Kpin-P*rplU-lux*. This created the plasmid called pGSVS2-Kp ATCC-P*rplU-lux*-floxZeo (Figure 2.3). The assembled pGSVS2-Kp ATCC-P*rplU-lux*-floxZeo plasmid was electroporated into S17-1 and then conjugated into *K. pneumoniae* ATCC 43816 for a two-stage allelic exchange procedure described above. The ATCC 43816::Kp ATCC-P*rplU-lux*-floxZeo strain was named WKP001 (Figure 2.3).

2.8 Removal of Zeocin Antibiotic Marker from Strain WKP001

A 1-Kb fragment containing a portion of the gene *galF* was PCR amplified using primer pairs 1/2 and inserted into pSK creating pSK-*galF*. The 1-Kb fragment was then cloned into pKSVS-P*tolC-cre* at SpeI/NotI sites resulting in the vector pKSVS-P*tolC-cre-galF*. Strain WKP001 and S17-1 pKSVS-P*tolC-cre-galF* were grown separately in 4 ml of LB broth shaken overnight at 37°C. On the following day, 1 ml of S17-1 pKSVS-P*tolC-cre-galF* was centrifuged for 1 minute at 15,000g. The pellet was washed with 10 mM $MgSO_4$ and centrifuged again. The pellet was re-suspended in 100 µL of WKP001 LB overnight culture and then transferred to a LB plate and grown at 37°C. Bacterial conjugation mixture was streaked for single colony isolation on a Cb100/Km25 LB plate and grown at 37°C. Three single colonies were cultured overnight in 5 ml of LB broth with 15% sucrose at 37°C with shaking. Subsequently, 10 µl of the overnight culture was streaked for single colonies on an LB plate and cultured at 37°C. The ten colonies were replica plated on both Km25 and Cb100 LB plates. Colonies which were Cb^R/Km^S were confirmed to be

Figure 2.3

Chromosomal Introduction of *luxCDABE* Operon and Removal of Zeo Antibiotic Marker



also Zeo^S in LB Zeo100 broth and the strain was named JSKP001 (Figure 2.3).

2.9 Investigation of the Light Production by Strain JSKP001

K. pneumoniae strain JSKP001 was grown overnight in LB in triplicate and the cultures were subcultured in TSBDC broth in the 37°C incubator. Every 30 minutes for 4 hours, 100 µl was taken from each triplicate and bioluminescent measurements of plate were taken with Synergy HT plate reader (BioTek, Winooski, VT) (1 sec read, sensitivity of 135). Additionally, at each 30 minute time point, 100 µl was taken from each subculture processed for bacterial enumerations as described above.

2.10 Chloramphenicol Spike of Strain JSKP001

Sub-lethal concentrations of chloramphenicol (150 µg/ml) were added 1 hour post-subculture to restrict new protein synthesis and subsequently restrict bacterial growth. Chloramphenicol was added at a final concentration of 150 µg/ml to JSKP001 cultures. Bacterial growth, bioluminescent measurements, and enumerations were performed as described above.

2.11 Infection of Cultured Cells with Bioluminescent Strain JSKP001

J774A.1 cells were infected in a black 96-well plate using strain JSKP001 at an MOI of 10 to determine the correlation between bioluminescent readings and bacterial numbers of *K. pneumoniae*. The luminescent measurements (counts per second, cps) were taken with Synergy HT plate reader (BioTek, Winooski, VT) immediately before harvesting samples for bacterial enumerations at the 3, 4.5, 6, 9 and 12 hour post-infection time point, then immediately bacterial enumeration was conducted as described above.

2.12 Data Presentation and Statistical Analysis

Unless otherwise stated, Graph Pad Prism 5 was used to plot data and conduct statistical analysis by unpaired Student's *t*-test.

CHAPTER III

RESULTS

GENOMIC ORGANIZATION OF K2 SEROTYPE *K. PNEUMONIAE* CPS

3.1 Introduction

K. pneumoniae ATCC 43816 is a representative K2 capsular serotype strain that has been shown to be virulent in murine respiratory disease models. Despite its relevance in the pathogenesis of respiratory disease, the genomic sequence had not been previously determined. Therefore, our laboratory performed Next Generation Sequencing (NGS) to acquire the sequence for the complete genome of the ATCC 43816 strain. The NGS gave us an estimated ~80% coverage at the contig level based on previously sequenced genomes of other *K. pneumoniae* stains [84]. This coverage is comparable to other NGS sequencing efforts [87, 88] and other historically used methods such as the shotgun approach and genome fragmentation [9, 89]. However, only 22.12% sequence coverage of the CPS cluster was obtained from our NGS sequencing efforts. Given this underrepresentation and the importance of the CPS cluster for virulence, we decided to manually sequence and assemble the remainder of the cluster. This type of gap closure is historically done by assembling contigs using a large-insert library to determine orientation of contigs [9, 89], however, more recently orientation is based on homology to previously sequenced strains and

gaps are closed by sequencing PCR products [87, 88]. We used genomic capture, primer walking and long-range PCR to close the gaps in the sequence of the CPS cluster.

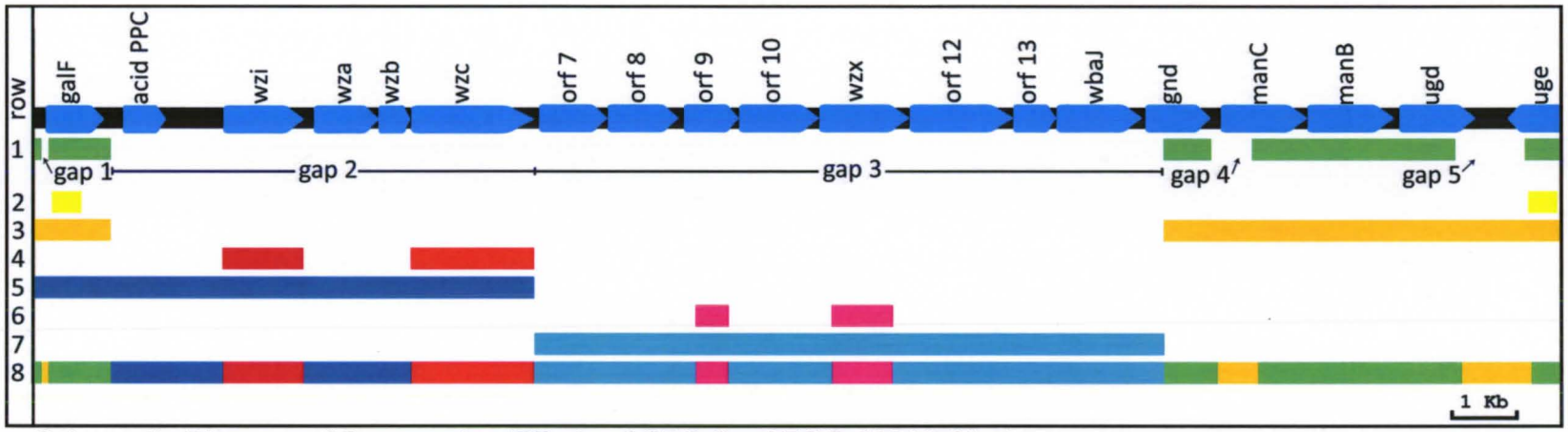
3.2 Genomic Capture

One approach we used to sequence the capsular polysaccharide cluster was the genomic capture technique by using insertional mutagenesis of the vectors pJMW106-*galF* and pJMW106-*uge*, which flanked the CPS cluster returned by NGS (Figure 3.1, yellow bars). Homologous portions of *galF* and *uge* genes were independently cloned into pJMW106, which is a non-replicating vector in *K. pneumoniae*. The vectors were named pJMW106-*galF* and pJMW106-*uge* respectively and were conjugated into *K. pneumoniae* genome. Growth in LB broth containing kanamycin was used to select for insertion of the vector and the strains were named Kp ATCC::pJMW106-*galF* and Kp ATCC::pJMW106-*uge*. Purified genomic DNA of Kp ATCC::pJMW106-*galF* and Kp ATCC::pJMW106-*uge* was digested using a selection of enzymes available that would retain the vector backbone and capture out a large portion of the genomic DNA of the capsule cluster which was then ligated and amplified in *E. coli* S17-1. Initial efforts did result in capture of genomic DNA, however, other efforts described below yielded sequence more rapidly and, this technique was discontinued.

3.3 Primer Walking and Long Range PCR

We used primer walking and long range PCR to sequence the remaining regions of the capsular polysaccharide cluster. NGS returned five contigs in the

Figure 3.1



Schematic Diagram of Sequencing Efforts of CPS the ATCC 43816 Cluster

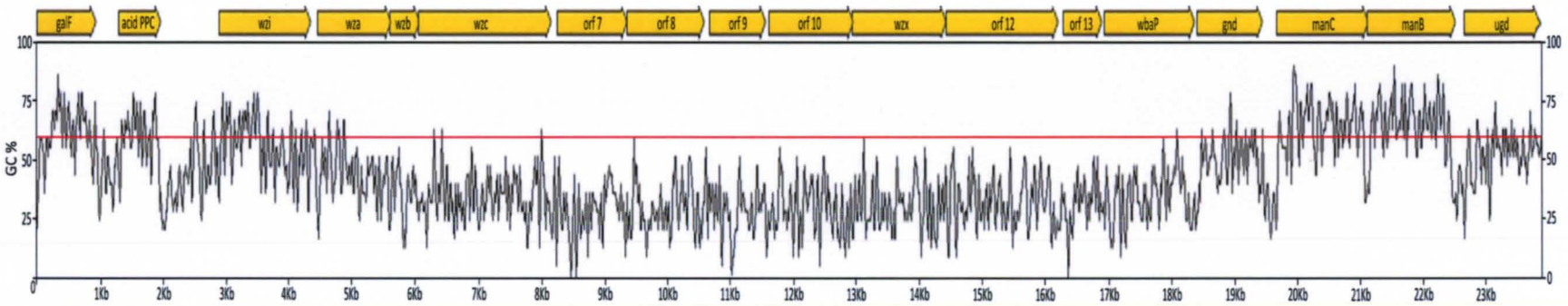
Scale representation of the CPS cluster illustrating the progression of closing sequencing gaps using genomic capture and primer walking. The conserved CPS cluster (*galF* to *ugd*) with gene identifiers indicated above each open reading frame represented by blue arrows. Missing gaps named (1, 2, 3, 4 and 5) in ascending order of the direction of the ORFs. Row 1 (green bars) - contig level of NGS coverage; Row 2 (yellow bars) – sites for genomic capture by insertional mutants; Row 3 (orange bars) – closure of gaps 1, 4 and 5; Row 4 (Red bars) - sequence obtained for genes *wzi* and *wzc*; Row 5 (blue bars) – finishing gaps 2; Row 6 (purple bars) – generating intermediate islands with genes *wzx* and *ORF 9*; Row 7 (teal bars) - gap 3 completed; Row 8, summary of gap closure efforts to sequence the CPS cluster.

CPS cluster (Figure 3.1, green bars) and the remaining unsequenced region was broken up into five gaps. The small gaps 1, 4 and 5 were amplified by PCR and sequenced using genomic DNA, leaving one large gap located between genes *galF* and *gnd* (Figure 3.1, orange bars). Using long range PCR, a 20-Kb fragment was amplified and served as the template for sequencing the remaining region of the CPS cluster. Next, the sequence for the genes *wzi* and *wzc* was obtained using primer walking technique with the 20-Kb fragment as template (Figure 3.1, red bars) and provided two intermediate sequencing islands to extend the sequence from the *galF* gene. A 7-Kb *galF-wzc* fragment was amplified by PCR and this fragment was used as a template to close the gaps in sequence between *galF* and *wzc* (Figure 3.1, dark blue bar). The sequence from *ORF 9* to *wzx* was determined by using previously sequence K2 serotype *K. pneumoniae* strains (Figure 3.1, pink bars) which providing two intermediate sequencing islands to connect the sequence of genes *wzc* to *gnd* (Figure 3.1, teal bar). This completed the sequencing of the CPS cluster for the K2 serotype *K. pneumoniae* strain ATCC 43816.

3.4 GC content of *K. pneumoniae* ATCC 43816 K2 CPS cluster

We investigated the GC content of the CPS cluster of *K. pneumoniae* ATCC 43816 as this provides insight into the acquisition of the CPS cluster. The CPS gene cluster of ATCC 43816 is 23,804 bp in length and contains 18 open reading frames (ORFs) from *galF* to *ugd* (Figure 3.2). The average GC content of the capsular polysaccharide cluster is 42.02%. Interestingly, we observed a central region of seven genes, between gene *wzc* and *gnd*, to have a much lower GC

Figure 3.2



Organization of CPS Cluster for *K. pneumoniae* ATCC 43816

The CPS spans from *galF* to *ugd* and the open reading frames are represented by arrows. A plot of the GC content for the region using 30-bp sliding window is shown below the open reading frames. The red horizontal line represents the mean GC content (59.53%) of the Next Generation Sequencing which covered ~80% of the genome.

GC content of 31.8% (Figure 3.2 and Table 3.1). By comparison, both the average CPS cluster and central region are well below the average GC content of the entire ATCC 43816 genome (59.5%)[84]. This observation of low GC content in the central region is consistent with 14 other *K. pneumoniae* CPS serotype clusters [90]. This suggests that there is a recent acquisition of the capsule cluster and perhaps rapid substitution of the core genetic element, which may impact plasticity of capsular serotype.

3.5 Identification of K2-specific genes

We analyzed the sequence of the K2 CPS cluster of *K. pneumoniae* strain ATCC 43816 against 23 other sequenced strains to identify the genes conserved between the K2 serotype (Figure 3.3). The open reading frames (ORFs) of ATCC 43816 were annotated and given a probable function based on their alignment to previously sequenced *K. pneumoniae* strains (Table 3.1). When additional flanking sequence is available for sequenced *K. pneumoniae* CPS clusters, the cluster is always located between genes *yegH* and *uge*. At the 5' end of the CPS cluster there are six genes (*galF*, *acid PPC*, *wzi*, *wza*, *wzb* and *wzc*) that are well conserved among all *K. pneumoniae* strains and have been described as a common translocation-surface assembly pathway for cell surface CPS [66]. A central region located between genes *wzc* and *gnd* appears to be the variable region for the CPS clusters; however, a high homology is observed between *K. pneumoniae* strains containing the same serotypes suggesting the variable region contains serotype specific genes (Figure 3.3). Amongst the sequenced K2 isolates (n=3), we observed high homology at both the genetic and amino acid

Table 3.1

General Features of the 18 Genes Identified in ATCC 43816 CPS Gene Cluster

Putative Function	Gene	Gene Name/Annotation	Best BLAST Hit	% GC	Amino-acid Length	Amino-acid Identity*	Species (Serotype/Strain Name)
D-galactose synthesis	galF	UTP-glucose-1-phosphate uridylyltransferase	galF	59.1	298	100%	Kp (NTUH-K2044, K2, NTUH-K9534, K9, K14, K52, K62, NK245,Nk29, 1084)
Membrane-associated lipid phosphatase	AcidPPC	Acid phosphate homolog	AcidPPC	58.6	209	99%	Kp (K2)
Polymerization and surface assembly	wzi	Outer membrane protein	wzi	54.2	477	99%	Kp (K2)
Polymerization and surface assembly	wza	Multimeric putative translocation channel	wza	43.8	378	96%	Kp (K2, KCTC 2242)
Polymerization and surface assembly	wzb	Protein tyrosine phosphatase	wzb	36.3	144	97%	Kp (K2, KCTC 2242)
Polymerization and surface assembly	wzc	Inner membrane tyrosine autokinase	wzc	35.6	686	97%	Kp (K2, KCTC 2242)
Catalyze the transfer of sugar	orf 7	Group 1 glycosyl transferase	orf 7	30.9	358	99%	Kp (K2, KCTC 2242)
Catalyze the transfer of sugar	orf 8	Group 1 glycosyl transferase	orf 8	30.7	400	100%	Kp (K2, KCTC 2242)
Catalyze the transfer of sugar	orf 9	Group 1 glycosyl transferase	orf 9	30.1	286	96%	Kp (K2)
Unknown	orf 10	Hypothetical protein	orf 10	31.1	438	100%	Kp (K2, KCTC 2242)
Polymerization and surface assembly	wzx	Putative flippase wzx	wzx	31.9	485	99%	Kp (K2)
Unknown	orf 12	Hypothetical protein	orf 12	33.6	579	99%	Kp (K2, KCTC 2242)
Unknown	orf 13	Acetyltransferase	orf 13	33.2	192	98%	Kp (K2, KCTC 2242)
UDP-glucose lipid carrier transferase	wcaJ	Undecaprenyl-phosphate glucose phosphotransferase	wcaJ	35.1	465	99%	Kp (K2, KCTC 2242)
Unknown	gnd	Gluconate-6-phospate dehydrogenase	gnd	49.8	333	91%	Kp (K1, K5, K9, K14, K54, K62, KCTC 2242, NK29, 1084)
Synthesis of GDP-mannose from mannose-6-phosphate	manC	GDP-mannose phosphorylase	manC	61.4	471	100%	Kp (K1, 1084)
Synthesis of mannose-1-phosphate from mannose-6-phosphate	manB	Phosphomannomutase	manB	62.5	394	100%	Kp (NTUH-K2044, KCTC 2242, NK245, 1084)
Synthesis of UDP-glucuronic acid from UDP-glucose	ugd	UDP-glucose 6-dehydrogenase	ugd	50.6	388	100%	Kp (K9, KCTC 2242, 1084)

* ATCC 43816 amino-acid identity percentage to species (serotype or strain) listed in adjacent column.

Figure 3.3


K2: ATCC 43816
K2: Chedid (AN: D21242)
K2: VGH525 (AN: AB371296)
ND: KCTC 2242 (AN: CP002910)*
K1: NTUH-K2044 (AN: AP006725)
K1: DTS (AN: AY762939)
ND: 1084 (AN: CP003785)*
K5: NTUH-K9534 (AN: AB289646)
K5: VGH404 (AN: AB371292)
K9 VGH484 (AN: AB371293)
K14: VGH916 (AN: AB371294)
K20: NTUH-KP13 (AN: AB289648)
K20: 889/50 (AN: AB289647)
K52: MGH 78578 (AN: CP000647)
K54: NTUH-KP35 (AN: AB289650)
K57: A1142 (AN: AB334776)
K62: VGH689 (AN: AB371295)
K62: NTUH-KP53 (AN: AB290716)
NT: A1517 (AN: AB334777)
ND: NK245 (AN: AB371291)
ND: NK29 (AN: AB371290)
ND: NK8 (AN: AB371289)
ND: Kp13 (AN: JN 377737)
ND: HS11286 (AN: CP003200)*

Figure 3.3

Comparison of Sequenced *K. pneumoniae* CPS Clusters

The *K. pneumoniae* ATCC 43816 CPS gene clusters compared to the CPS loci to ten *K. pneumoniae* serotypes (K1, K2, K5, K9, K14, K20, K52, K54, K57, and K62), one new serotype (NT) and seven undetermined serotypes (ND). The arrows with dotted lines belong to flanking genes of the CPS cluster. Blue arrows represent highly conserved genes present in all CPS regions. Orange arrows correspond to genes of the highly conserved sequence, but presence and or absence depends on serotype. ATCC 43816 K2 serotype genes with high homology with other K2 serotype *K. pneumoniae* are represented by teal arrows. Black arrows correspond to gene *wzx*, which are highly variable at the sequence level, but have an amino acid identity ≥ 11%. Red outline arrow represents pseudogenes. In ATCC 43816, the suggested names for the ORFs are based on amino-acid homology greater than 70%, with at least one annotated gene from another CPS region. For the previously published CPS regions, names indicated above each gene correspond to annotations found in public databases, whereas names in italic under the genes are suggestions based on pairwise comparisons.

* Re-annotation of NCBI data P: Promoter

Image adapted from Ferve, et al. [91]

levels (Table 2). At the 3' end of the CPS cluster there are two genes (*gnd* and *ugd*) highly conserved between all sequenced strains with the gene *ugd* which is considered the last gene of the CPS cluster [91]. This data suggests that there is both a conserved and variable region of the CPS cluster. The conserved is seen in all serotype suggesting that the conserved genes are necessary for capsule production, while the variable region is serotype specific.

3.6 Generating a capsular polysaccharide mutant

Capsular polysaccharide has been demonstrated to be a critical factor in the virulence of *K. pneumoniae* in *in vivo* and *in vitro* models [72, 92]. From this sequence data, we intended to generate a capsule mutant strain which removed the capsular polysaccharide from genes *galF* to *ugd*. The development for the capsule mutant was designed to create an in-frame mutant, which fused the *galF* start codon to the *ugd* stop codon deleting 23.7-Kb of DNA. These two fragments were assembled flanking the zeocin antibiotic marker (*Sh ble* gene) containing the *loxP* sites on either side. The *loxP* sites are recognized by Cre recombinase, which catalyzes the recombination of DNA between two *loxP* sites. The pJMW106-Δcap-floxZeo plasmid was then conjugated into *K. pneumoniae* ATCC 43816 for two-stage allelic exchange mutagenesis. During the conjugation, a merodiploid *K. pneumoniae* strain was successfully generated; however, we had difficulty in generating a Zeo^R clone that lacked the capsule cluster. Future efforts will be required to identify whether essential genes are present within the CPS cluster, whether other technical issues prevented the generation of this 23.7 Kb

region of DNA or the development of smaller multiple gene mutant or single gene mutant.

3.7 Conclusion

The CPS cluster investigated in this study has high homology to other sequenced K2 serotypes suggesting that genetic information could be used in the future to predict serotype. The observation of a variable region located in the central genes between *wzc* and *gnd* appears to be unique for each serotype.

CHAPTER IV

RESULTS

J774A.1 MACROPHAGE INVOLVEMENT IN *K. PNEUMONIAE* INFECTIONS

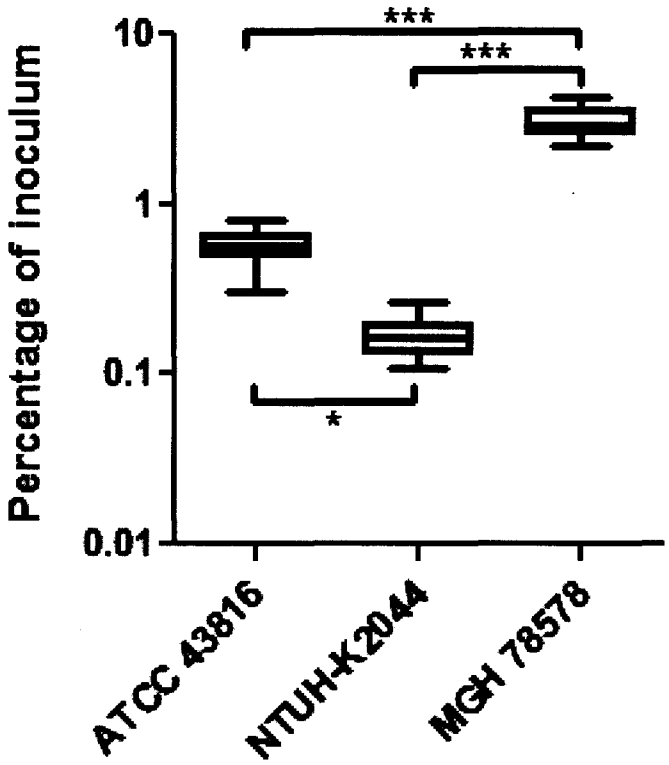
4.1 Introduction

Successful bacterial pathogens must be capable of circumventing their hosts' innate defenses, traffic to their preferred replicative niche and ultimately persist within their hosts. *K. pneumoniae* is widely considered to be an extracellular pathogen, though evidence for this characterization is circumstantial [93, 94]. However, recent studies have demonstrated its ability to be internalized by epithelial cells and macrophages [47, 48, 61]. *K. pneumoniae* is known to cause a wide range of infections and is commonly isolated from the lungs. Pulmonary infections have been shown to result in dissemination from the pulmonary airspace to the bloodstream. Alveolar macrophages reside in the interphase between air and lung tissue and provide the first line of cellular defense against microbes [95]. Previous research has demonstrated that alveolar macrophages contribute to the defense against *K. pneumoniae* infection in the lungs [75]. Considering the fact that macrophages are the primary responders against pneumonia, we decided to study the relationship between *K. pneumoniae* and J774A.1 murine macrophages.

4.2 Internalization of *K. pneumoniae*

To investigate host-pathogen interaction of *K. pneumoniae* ATCC 43816, NTUH-K2044 and MGH 78578 we infected J774A.1 murine macrophage monolayers. To determine internalization of *K. pneumoniae* by J774A.1 cells, we used gentamicin protection assays (1 hour post-infection). The cells were challenged with an MOI of 10 for each strain of *K. pneumoniae* and 3 hours post-infection cells were lysed and enumerations were taken. The macrophages were observed to be viable by using immunofluorescence microscopy (data not shown) and our results demonstrated that all three strains of *K. pneumoniae* were able to be internalized by J774A.1 cells (Figure 4.1). For comparison, the uptake efficiencies of all three strains were assessed by percentage of inoculum internalized by macrophage (i.e. survived gentamicin treatment by being internalized by macrophages). We observed the internalization of strain MGH 78578 has the highest percentage inoculum uptake of 3.09%, with much lower rates of uptake observed for both ATCC 43816 (0.57%) and NTUH-K2044 (0.17%). This data suggests that strain MGH 78578 is more readily phagocytized by macrophages when compared to strains ATCC 43816 and NTUH-K2044 ($P<0.001$, ***). These findings are consistent with the hypothesis that *K. pneumoniae* is an extracellular pathogen which represses phagocytosis to increase its virulence, as we have demonstrated that NTUH-K2044 and ATCC 43816 are virulent in our respiratory murine disease model, while MGH 78578 is relatively avirulent [84].

Figure 4.1



Internalization of Three *K. pneumoniae* Strains

Percentage of bacterial inoculum internalized by J774A.1 cells in four independent (n=12) experiments represented by a whisker box plot . 7.5×10^4 cells per well murine macrophages were infected with *K. pneumoniae* at an MOI of 10 with strains ATCC 43816, NTUH-K2044, and MGH 78578. Gentamicin was added one hour post-infection to kill extracellular bacteria and bacteria uptake was assessed at 3 hours post-inoculation. Cells were lysed with 0.1% Triton X-100 and enumerated by plate counting and percentage of inoculum was calculated. Statistical analysis was carried out by one way ANOVA and Tukey post test ($P<0.05$, *; $P<0.01$, **; $P<0.001$;***) (Limit of detection 0.0013% of inoculum).

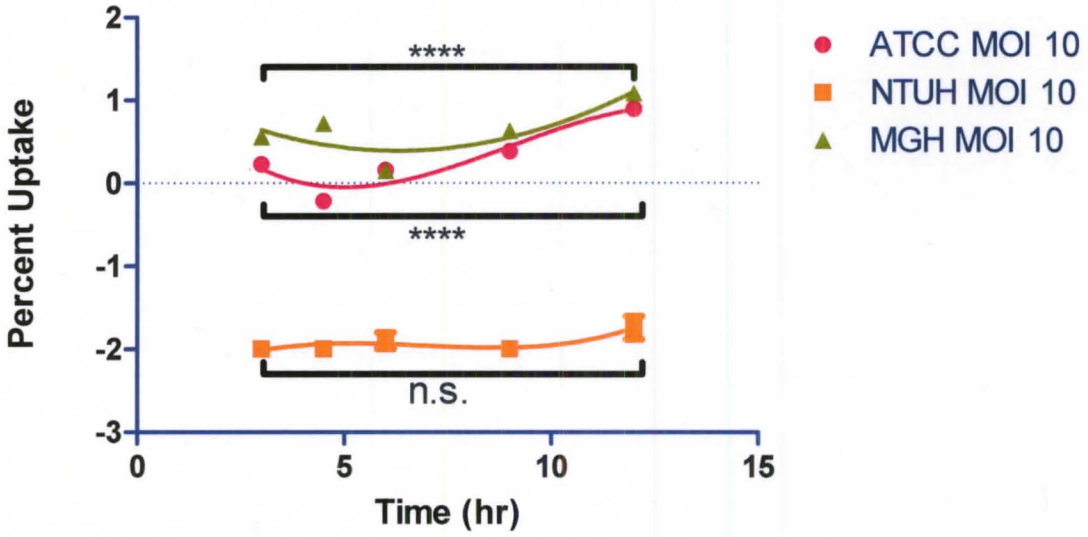
4.3 Proliferation of *K. pneumoniae*

We observed that *K. pneumoniae* strains could be internalized by J774A.1 macrophages at different levels, and we decided to investigate whether the three *K. pneumoniae* strains exhibited different rates of proliferation within macrophages. We infected replicate sets of J774A.1 cells with the three *K. pneumoniae* strains at an MOI of 10 and evaluated the ability of *K. pneumoniae* to proliferate within J774A.1 cells with enumerations taken at 3, 4.5, 6, 9 and 12 hours post-infection. Our results show that all three strains can be internalized by 3 hours by the J774A.1 macrophage as similar numbers were observed (Figure 4.1 and Figure 4.2). However, both MGH 78578 and ATCC 43816 showed an initial decrease in bacterial numbers followed by proliferation over the course of the infection ($P<0.001$, ***). The strain NTUH-K2044 was internalized as shown previously, but exhibited little growth over the course of the experiment (No significance). We also observed that ATCC 43816 was taken up at a lower bacterial number than MGH, however at later time points, ATCC 43816 proliferated to similar bacterial numbers as MGH 78578. The low internalization and proliferation of all three strains suggests that macrophages may not be the preferred replicative niche for *K. pneumoniae.*

4.4 Conclusion

This work demonstrates that all three strains of *K. pneumoniae* can be internalized and persist within macrophages for prolong periods of time. The higher rate of internalization of the MGH 78578 strain could account for some

Figure 4.2



Growth Curve of *K. pneumoniae* in Cultured Macrophage

Growth of internalized *K. pneumoniae* in J774A.1 cells over 12 hours. *K. pneumoniae* strains ATCC 43816, NTUH-K2044, and MGH 78578 were used to infect $7.5x10^4$ cells per well in triplicate at an MOI of 10. After 1 hour, extracellular bacteria were killed with gentamicin. At time points of 3, 4.5, 6, 9 and 12 hours post-inoculation, samples were lysed with 0.1% Triton X-100 and enumerated by plate counting. This figure is a representative of three independent trails with all displaying similar results. The data is representative of at least three replicate trials with plots of mean and standard error for each data point. Statistical analysis was carried out by one way T-test ($P<0.05$, *; $P<0.01$, **; $P<0.001$;***, $P<0.0001$;****) (Limit of detection 0.0013% of inoculum).

lack of *in vivo* pathogenicity in the pulmonary infection model [84], whereas both ATCC 43816 and NTUH-K2044 may control their internalization by phagocytes.

CHAPTER V

RESULTS

ENGINEERING OF A LUMINESCENT *K. PNEUMONIAE* STRAIN

5.1 Introduction

Bioluminescence reporter systems have been engineered into many species of bacteria to allow for the production of light to be used to monitor disease processes in living systems [96-104]. The engineering of this bioreporter into *K. pneumoniae* ATCC 43816 would enable us the ability to perform real-time quantification of viable bacteria within a host. One of the most frequently used bioluminescent reporter systems used in bacteria is the *luxCDABE* operon of *Photorhabdus luminescens* which is a bacterial specific system with many advantages over eukaryotic luciferases as the luxCDABE operon produces both the luciferase enzyme and the substrates required for light production, eliminating the need to provide substrate exogenously to the assay [105].

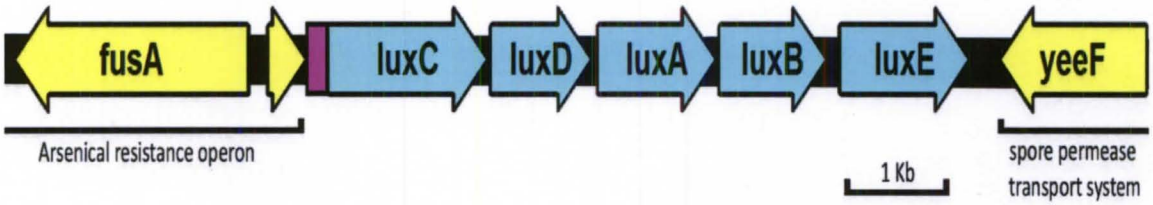
The single transcriptional *luxCDABE* unit contains *luxA* and *luxB* genes coding for the α- and β-subunits that form a heterodimer luciferase [106]. The *luxCDE* products work as a multienzyme fatty acid reductase complex required for regeneration of the long-chain fatty aldehydes [105]. The long-chain fatty aldehydes are subsequently oxidized by the luciferase heterodimer, with energy supplied by flavin mononucleotide ($FMNH_2$), to produce a photon of blue/green

light (~500nm)[105]. Unlike eukaryotic luciferases, bacterial luciferase system requires no additional exogenous substrates to generate bioluminescence during both *in vitro* and *in vivo* assays. This makes the *lux* operon an ideal system for real time monitoring of bacteria.

5.2 Generating a *lux+ K. pneumoniae* strain

ATCC 43816 strain is a well-studied *K. pneumoniae* strain, capable of causing respiratory disease in animal models. We engineered the *luxCDABE* operon into this strain for use in both *in vitro* and *in vivo* studies. The ATCC 43816 strain was engineered to produce constant, stable luminescence from the *luxCDABE* operon and we used allelic exchange mutagenesis to insert the operon into the chromosome. The insertion site of the *lux* operon was selected based on the observation that a related *Klebsiella* strain possesses a nitrogen fixation operon located between the arsenical resistance operon (*fusA*) and the spore permease transport system (*yeeF*) which was found to be a conserved region in other strains of *K. pneumoniae* (Figure 5.1). Therefore, this site was selected for the insertion of the *lux* operon to minimize the impact on other genetic systems or virulence. We cloned homologous portions of DNA to facilitate allelic exchange including a portion of *fusA* and the neighboring hypothetical gene (Figure 5.1, left side) and part of the *yeeF* (Figure 5.1, right side). These two fragments were assembled to flank the *lux* operon in pGSVS along with the *rplU* promoter, which was used to constitutively express the *lux* operon. The *rplU* gene is a subunit of the 50S ribosomal subunit of protein L21 which catalysis mRNA-directed protein synthesis. We predicted that this

Figure 5.1



Schematic Diagram of the Genetic Organization of JSKP001

Scale representation of JSKP001 genetic organization. Nucleotide sequence of the *P. luminescens lux* operon is given in GenBank (AN: M90093). *lux* operon is flanked at the 5' end Gene *fusA* and a hypothetical gene (arsenical resistance operon) and at the 3' end *yeeF* (spore permease transport system). The *rplU* promoter was cloned upstream of the luxCDABE operon (purple rectangle).

ribosomal protein promoter will allow the *lux* operon to be constitutively transcribed whenever the bacteria are metabolically active. The zeocin antibiotic marker (*Sh ble* gene) was cloned at the end of the *luxE* gene with flanking *loxP* (flox) sites. These sites, known as the *loxP* sequences, contain specific binding sites for Cre that surrounds the two directional core *loxP* sequences and allowing for excision by recombination. The pGSVS2-Kp ATCC-P*rplU-lux*-floxZeo plasmid was then conjugated into *K. pneumoniae* ATCC 43816 for two-stage allelic exchange mutagenesis, producing the luminescent strain WKP001. The removal of the zeocin antibiotic marker was performed done by introducing the vector pKSVS2-P*tolC-cre-galF* into WKP001. Cre recombinase was transiently expressed to excise the antibiotic marker, while leaving the P*rplU-lux* construct in the chromosome. The *sacB*-marked Cre expression vector was selected against using sucrose, and the resulting strain was named JSKP001.

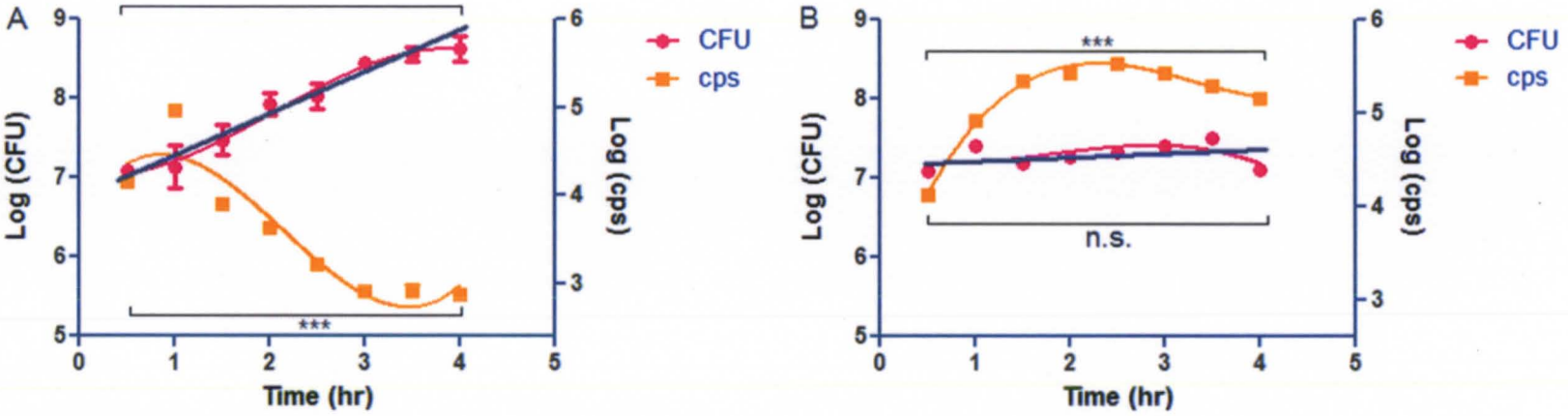
5.3 *In vitro* bioluminescence of *K. pneumoniae* JSKP001

To investigate whether the light production from JSKP001 correlates with bacterial numbers, we cultured JSKP001 in TSBDC broth media and examined both light production and bacterial numbers at multiple time points. The bacteria were grown with shaking in LB overnight and subcultured into TSBDC for four hours (Figure 5.2A). TSBDC is a metal-depleted media which we routinely use to mimic the metal-limited environment of the host. We observed statistically significant exponential growth for the first three and a half hours of the broth growth with evidence of stationary phase beginning at four hours. A trend line was plotted through the first 3.5 hours of growth and we calculated a doubling

time of 52.9 minutes. This doubling time is greatly reduced from *K. pneumoniae* grown in LB which has a double time of 13 minutes [30], suggesting that the metabolic state of the bacteria may be significantly different in LB and TSBDC. During the time course of the TSBDC growth, we also measured bioluminescence (counts per second (cps)) bacterial number's. The cps/CFU ratio decreased after the first hour with statistically significant a decrease in light emitted per bacteria over the course of the experiment. At three hours, the bioluminescence resumed an altered correlation for the last hour (Figure 5.2A). This suggests that JSKP001 grown in LB may have been more metabolically active than in TSBDC and that a transition to an altered luminescent activity after three hours growth in TSBDC reflects a change in the metabolic activity of the bacteria. Alternatively, it is possible that the P*rplU* promoter may not be constitutive in TSBDC.

Because the metabolic activity may impact the magnitude of light production per bacteria, we hypothesized that altering the competition for energy within the bacteria could impact light production. Therefore, we investigated how arresting new protein synthesis with sub-lethal concentrations of chloramphenicol [107] affects bioluminescence of JSKP001. We grew JSKP001 in LB overnight and then subcultured into TSBDC, for one hour before adding chloramphenicol. As expected, bacterial growth was repressed by chloramphenicol and bacterial numbers remained unchanged throughout the experiment (Figure 5.2B). Unlike previous trials in which we had a rapid decrease in the emitted light per bacteria (Figure 5.2A), we observed that the cps/CFU dramatically increased in a

Figure 5.2



Broth Growth of Lux-Expressing *K. pneumoniae*

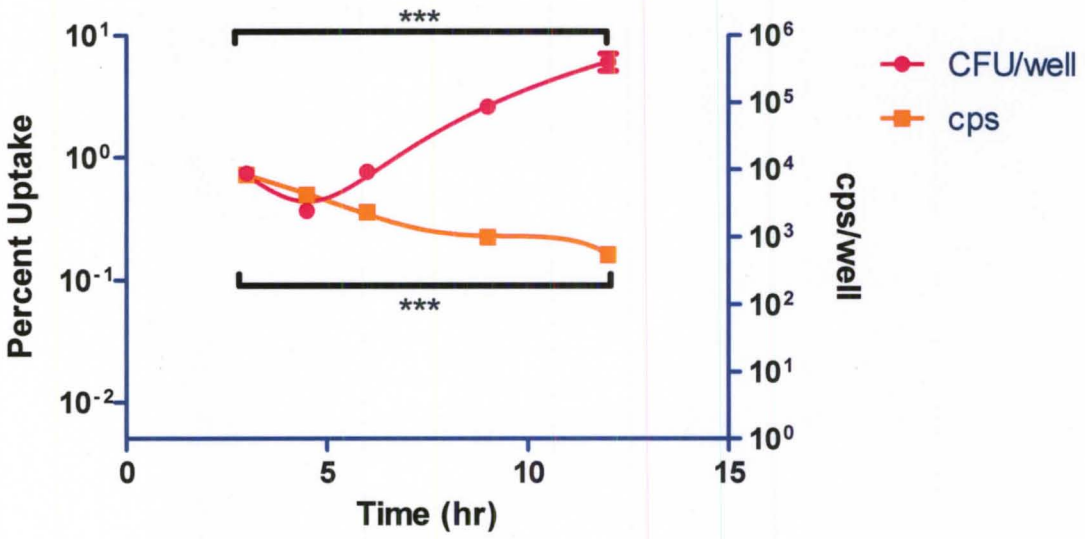
K. pneumoniae strain JSKP001 was grown in TSBDC broth for 4 hours. Every 30 minutes, a 100 µl volume was drawn from the bacterial culture for a bioluminescent reading and bacterial enumeration. The exponential growth is indicated with a trend line and used to determine doubling time (A). In a separate experiment, chloramphenicol antibiotic was added to the TSBDC broth 1 hour after initiating the experiment. A trend line was plotted to show bacterial growth was repressed (B). Both experiments were performed in triplicate. Each data point is the mean and standard error. Statistical analysis was carried out by one way T-test from the first to last data points of the growth study ($P<0.05$, *; $P<0.01$, **; $P<0.001$;***). (Limit of detection 100 CFU of inoculum; Limit of detection 355 cps).

statistically significant manner after the addition of chloramphenicol (Figure 5.2B). This level of light production remained high throughout the course of the experiment and only began to decrease a later time points. This data suggests that most energy-requiring bacterial proteins have relatively short half-lives which break down and leave excess energy for the Lux proteins. Conversely, the data suggests that the Lux proteins remain stable for the period of observation with high level production of light for four hours. The elevated cps/CFU suggests that typically the metabolic activity in bacterial cells leaves little energy available for the luciferase activity. However, we hypothesize that blockage of protein synthesis led to rapid turnover of many host proteins, decreasing the competitive demand for energy of the relatively stable Lux proteins.

5.4 Intracellular Survival of *lux+ K. pneumoniae*

We decided to evaluate the ability of *K. pneumoniae* strain JSKP001 to produce light in a potentially important host-pathogen interaction model. To investigate whether the light production from JSKP001 correlates with bacterial numbers in an intracellular niche, JSKP001 was examined using our previous gentamicin protection assay of cultured macrophages. We infected replicate sets of J774A.1 cells with JSKP001 at an MOI of 10 and bioluminescence was measured by the plate reader immediately before harvesting the samples for bacterial enumeration at 3, 4.5, 6, 9 and 12 hours post-infection (Figure 5.3). As previously observed (Figure 4.1), approximately 0.7% of the *K. pneumoniae* JSKP001 (ATCC 43816 derivant) inoculum was taken up by the J774A.1 macrophages, with statistically significant increase in bacterial numbers over the

Figure 5.3



Intracellular Survival of *K. pneumoniae* JSKP001 in J774A.1 Cell Line

K. pneumoniae strain JSKP001 was used to infect murine J774A.1 cell line ($7.5x10^4$ cells/well) in triplicate at an MOI of 10. After 1 hour, extracellular bacteria were killed with gentamicin. At time points of 3, 4.5, 6, 9 and 12 hour post-inoculation, the light production of the sample sets as measured. The cells were washed, then lysed using 0.1% Triton X-100, and the bacteria were enumerated by plate counting. CFU/well was plotted as a function of time for JSKP001. Bioluminescence was measured prior to cell lysing and cps/well was plotted as a function of time. Each data point is the mean and standard error. Statistical analysis was carried out by one way T-test comparing the first and last data points ($P<0.05$, *; $P<0.01$, **; $P<0.001$;***) (Limit of detection 0.0013% of inoculum; Limit of detection 345 cps).

course of the experiment (Figure 5.3). Initially, the JSKP001 strain produced light consistent with bacterial numbers, however after four and half hours the luminescence per bacteria decreased in a statically significant manner over the course of the experiment. These findings suggest that JSKP001 produces light during host-pathogen interaction in the macrophage infection model; however, the decrease in cps readings suggests that Lux proteins have a reduced availability of energy for bacteria which persist in intracellular niches.

5.5 Conclusion

In this study, we developed a bioluminescent strain of *K. pneumoniae* by engineering the *luxCDABE* operon into the chromosome. Lux proteins may be more stable than other bacterial proteins, but appear to compete for the same energy source as host bacterial proteins, with the potential for significant variation in the availability of energy from one environment to another. Future studies will be required to confirm by Western blot whether the Lux proteins indeed possess longer half-lives than most host proteins. This study provided evidence that *K. pneumoniae* might enter a dormant stage of reduced metabolic activity when internalized by macrophages, or that the *rplU* promoter may be significantly repressed within macrophages.

CHAPTER VI

DISCUSSION

The CPS is one of the best characterized virulence factors of *K. pneumoniae* as it facilitates resistance to complement-mediated bacteriolysis and phagocytosis [48]. Studies have indicated that capsule-negative strains are highly susceptible to rapid clearance in *in vitro* and *in vivo* assays which indicates that CPS has a major impact on disease [61, 75, 92, 108]. Because the CPS of *K. pneumoniae* is an important virulence factor, one of our objectives was to completely sequence and preform bioinformatics analysis of the ATCC 43816 capsule biosynthetic cluster. Our results added to a growing list of genetically characterized capsular clusters and revealed high sequence homology among the K2 serotypes. Similar trends of high sequence homology are observed in four additional serotypes that have multiple sequenced strains [91]. This information suggests that if a sufficient number of sequenced and well characterized CPS biosynthetic clusters were available, that genetic data could be used to predict the serotype for *Klebsiella*. For example, the sequenced strain KCTC 2242 has not yet been serotyped; however, the strain has high genetic homology to the sequenced CPS clusters of K2 serotype and strongly suggests from genetics that KCTC 2242 is a K2 serotype strain, which could be demonstrated in future studies. Additionally, serotyping may encounter technical issues, as in the

instance where serotyping efforts of the *K. pneumoniae* strain KpL1 were not previously successful in spite of the fact that it possesses the *magA*, *gmd* and *wcaG* genes similar to the K1 capsular clusters [109].

Our data is adding to the growing set of evidence that every sequenced strain from the *Klebsiella* genus has a highly conserved set of CPS genes suggesting that capsule is a critical structure present in all *Klebsiella*. The capsule biosynthetic cluster has 15 to 25 genes that are required for capsule synthesis and of these, eight are conserved with high sequence homology amongst *K. pneumoniae* subspecies *pneumoniae*, *rhinoscleroma*, *oxytoca* and *K. variicola* [91]. Six of these genes cluster together in what appears to be a transcriptional cluster at the 5' end of the CPS cluster and are involved in polymerization control. They are thought to form a membrane pore through which translocation of the CPS product is moved from the inner membrane to the cell surface for capsule assay [110]. Single gene mutants within these genes lead to acapsular phenotypes with loss of high-molecular weight extracellular polymer with the accumulation of the CPS precursors in the periplasm of the bacteria [111]. Two additional conserved genes (*gnd* and *ugd*) are located downstream of the variable region of the CPS biosynthetic cluster. The *gnd* gene encoding an NADP-dependent 6-phosphogluconate dehydrogenase is involved in the pentose phosphate pathway which converts 6-phosphogluconate into ribulose 5-phosphate and produces NADPH for reductive biosynthesis and nucleotide biosynthesis [112]. The *ugd* gene encodes UDP-glucose dehydrogenase, which converts UDP-glucose into UDP-glucuronic acid, the substrate for capsule

synthesis [113]. The high degree of conservation of the core set of eight genes amongst members of the *Klebsiella* genus suggests a common ancestry of these genes and conserved mechanism of capsule assembly. Homologues of these eight conserved genes are also found in a variety of intestinal flora bacteria, such as *Enterobacter aerogenes*, *Salmonella enterica*, *Escherichia coli*, *Serratia marcescens* and *Shigella boydii* and potentially amongst other members of the *Enterobacteriaceae* family [114-118].

The genetic arrangement of the CPS cluster has a genetically variable CPS locus which is thought to contribute to the diversity of the capsular polysaccharide serotype of *K. pneumoniae* and is flanked by conserved genes. Because of this conservation found within the *Enterobacteriaceae* family there may be exchange of the CPS using homologous recombination in the intestine. The intestine has been previously demonstrated to be a potential site for interchange of genetic material as *K. pneumoniae* has been observed to transfer antibiotic resistant genes to *E. coli* in the mouse intestine [119]. While homologous recombination of large segments of the CPS cluster might happen at very low frequency, there is evidence for horizontal gene transfer of capsule genes amongst members of the *Enterobacteriaceae* family as observed by a high genetic similarity of the capsule clusters of *E. coli* K30 and *K. pneumoniae* K20 suggesting recent exchange of CPS clusters between these two species [120, 121]. Additionally, the capsule of *E. coli* K42 was observed to have a serologically identical capsule as a *K. pneumoniae* K63 strain [113, 122],

supporting the evidence that members of the *Enterobacteriaceae* family may exchange CPS gene clusters, potentially in the intestine.

The CPS of *K. pneumoniae* may be one factor in directing host niche specificity for different *K. pneumoniae* strains. It has been shown that multiple serotypes infect different niches. For example, serotypes 21, 33 and 69 are predominately found causing infections in the urinary tract, while serotypes 30 and 31 predominately are found causing wound infection [123]. However, of the 80 different serotypes identified, most reports indicate that K1 and K2 serotypes are amongst the most common isolated from patients and additionally are the most invasive strains [123-127]. It has been suggested that the degree of virulence conferred by a particular K antigen of *K. pneumoniae* might be connected to the mannose content of the CPS. Capsular types associated with low virulence, such as the K7 or K21a antigen, contain sequences of mannose-α-2/3-mannose or L-rhamnose-α-2/3-L-rhamnose which are recognized by a surface lectin of macrophages [128-130]. Macrophages with the mannose-α-2/3-mannose-specific lectin or mannose receptor recognize, ingest, and subsequently kill *K. pneumoniae CPS* serotypes containing mannose. In contrast, strains with theK2 serotype, which lack the mannose repeating sequence, are not phagocytized [131]. Thus, *K. pneumoniae* CPS may play an important role in directing the preferred host niche, in part mediated by immunomodulation.

Macrophages are considered major effector cells of host defense against respiratory tract infections by virtue of their potent phagocytic properties. In

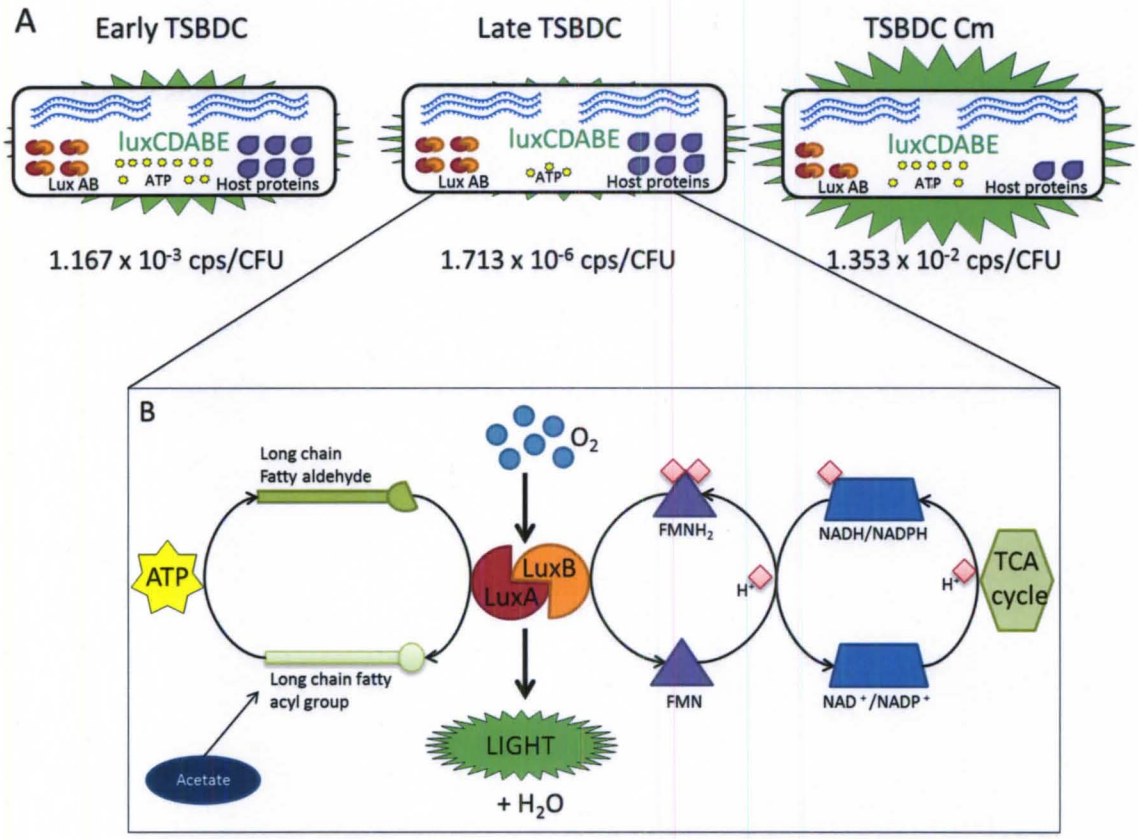
addition, macrophages may regulate the host inflammatory response to infection by the production of cytokines [132]. Alveolar macrophages are important for the rapid elimination of *K. pneumoniae* from respiratory tract and by orchestrating the inflammatory response against this pathogen [133]. We analyzed the interplay between *K. pneumoniae* strains and J774A.1 macrophage cells to get a better understanding of the host response to *K. pneumoniae* infections. Our results demonstrated that *K. pneumoniae* can be internalized by macrophages at low levels and survive for prolonged periods of time. However, some strains of *K. pneumoniae* may not proliferate in macrophages at high levels typically observed for intracellular bacterial pathogens, which suggests *K. pneumoniae* may excel as an extracellular pathogen. However, macrophages might be a potential niche for chronic persistence in sublethal infections. There is clinical evidence that *K. pneumoniae* is capable of causing chronic disease with frequent and recurrent manifestation in the liver up to ten year post infection [134, 135]. Recurrent infection is also seen in other bacteria such as *Mycobacterium tuberculosis,* which colonize macrophages, enter a latent phase, and then causes a subsequent acute disease [136].

We engineered a bioreporter into *K. pneumoniae* ATCC 43816 to enable us the ability to perform real-time quantification of viable bacteria. While investigating how light production from JSKP001 correlated to bacterial numbers, we first observed that bacteria cultured in TSBDC had a relatively high level of light emitted per bacteria (1.167×10^{-3} cps/CFU), but after about three hours, the bacteria transitioned to a much reduced light emission per bacteria (1.713×10^{-6}

cps/CFU) (Figure 6.1A). This brought up several questions for us. Was the transcriptional regulation of the *rplU* promoter causing reduced expression of Lux proteins or was the efficiency of the enzymes negatively impacted by the metabolic state of the bacteria? Because the metabolic activity may impact the magnitude of light production per bacteria, we altered the competition for energy within the bacteria by stopping new protein synthesis. We observed that the Lux proteins were competing for the same energy source as host bacterial proteins (ATP) (Figure 6.1), but also observed that the Lux proteins appear to have a longer half-life than most host proteins and are able to access additional energy for light production in the absence of competition. When examining the products needed for light production, ATP is used to regenerate long chain fatty aldehydes and the TCA cycle to regenerate $FMNH_2$ (Figure 6.1B). Both products are oxidized by the LuxAB dimer in the presence of oxygen to product light (Figure 6.1B). Because the Lux proteins appear to be competing for the same energy source there is a potential for variation in the availability of energy when *K. pneumoniae* goes from one environment to another causing an alteration in metabolism within the bacteria. We have not addressed the possibility that the *rplU* promoter may not be constitutively expressed in macrophages which we could investigate using RT-PCR studies.

An original aim of this thesis project was to bridge the two major aims by developing a capsule mutant and use the luminescent tool to monitor disease progression for the capsule mutant in the respiratory disease model. We were unsuccessful in generating a capsule mutant, thus future experiments will identify

Figure 6.1



Summation of Figure 5.2

Schematic diagram of *K. pneumoniae* cells in TSBDC during different time points and with or without chloramphenicol. Blue wavy lines represent mRNA transcript of the *luxCDABE* operon. The red/orange circles represent relative numbers of LuxAB protein dimer and purple shape represent relative numbers of bacterial host proteins. ATP is represented at yellow stars and lime green around cells is relative production of light produced by LuxAB proteins (A). The mechanism of light production is a complex series of energy requiring oxidation/reduction reactions where the LuxAB protein dimer requires energy for the production of light in the form of both ATP and NADH/NADPH from the TCA cycle (B).

whether essential genes are present within the CPS cluster or whether other technical issues prevented the generation of this 23.7 Kb region of DNA. Future studies will be needed to demonstrate that the transfer of capsule biosynthetic clusters can be shared between the *Enterobacteriaceae* family. Additionally, future studies will also be required to characterize the role of macrophages in recurrent *K. pneumoniae* disease. We are also interested in examining whether bioluminescence can be used to monitor disease progression in animal models. Some of these future directions are already being performed within the lab as the animal studies are currently on going in the laboratory and raise good evidence that the there is a correlation between luminescence and bacterial numbers within animal tissues.

In conclusion, we have genetically characterized the K2 serotype capsular polysaccharide cluster from ATCC 43816. Comparative genomics indicate that there are both conserved and variable regions within all sequenced capsular polysaccharide cluster of *K. pneumoniae* which could provide exchange of the serotype specific genes through the mechanism of horizontal gene transfer. Our understanding from our macrophage studies that *K. pneumoniae* prefers to be an extracellular pathogen as it enters a novel latent phase within the macrophages. Furthermore, we validated a diagnostic imaging tool for *K. pneumoniae* to support macrophage internalization studies and the tools developed in these studies is currently being used to support animal respiratory disease model. Plus, additional future studies will be needed to demonstrate that the transfer of

capsule biosynthetic clusters can be shared between the *Enterobacteriaceae* family.

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