UNIVERSITY OF CALIFORNIA SAN DIEGO

Capsular polysaccharide structure governs virulence in *Acinetobacter baumannii* LAC-4

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by

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ABSTRACT OF THE THESIS

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Acinetobacter baumannii is an emerging gram-negative pathogen that is listed by the center of disease control as an urgent threat and is part of a select group of pathogens known as the ESKAPE pathogens due to its ability to continuously escape the lethal action of antibiotics. *A. baumannii* infection is most common in the healthcare setting putting immunocompromised patients at increased risk of infection through disease such as ventilator associated pneumonia.

For pathogens such as *A. baumannii*, the mechanisms of antimicrobial resistance are well understood but the understanding of the determinants of virulence have yet to catch up. A virulence factor of particular interest in the scientific community is the capsular polysaccharide and how it can play a role in pathogenicity. Bacterial capsules, comprised of tightly packed polysaccharide units, have been shown to provide various strains of bacteria protection from environmental pressures such as host immune response and antimicrobial agents. However, capsule loci in *A. baumannii* are highly variable providing the question if capsular virulence is specific to certain capsule types or universal. This study generated an unmarked knockout mutant of LAC-4 in which the capsule locus has been deleted. The resulting mutant was evaluated with in vitro cell based assays and against an established mouse model of bacterial systemic infection. Capsule deficient mutants of LAC-4 were found to be significantly less virulent than the wildtype LAC-4 strain showing that the capsule gene cluster appears to contribute to LAC-4's hypervirulence.

Chapter 1

Introduction

Antibiotics have been used to treat infection for thousands of years; however, it was not until just over the last century where people began to discover bacteria as one of the causative agents of infection. It is documented that some of the earliest civilizations of human life used herbs, honey, and even animal feces to treat infections. In fact, trace amounts of tetracyclines have been detected in human skeletons excavated from the ancient Sudanese Nubia in 350-550CE and the Roman occupation of Egypt (Gould, 2016). The definitive discovery of antibiotics has been one of the most valuable medical discoveries in history. For most of human existence, infectious diseases have been the leading causes of morbidity and mortality (Aminov, 2010). Antibiotics have revolutionized medicine through the countless amounts of lives saved and increasing the average human lifespan, whereas the morbidity caused by infectious agents prior to this discovery were left mostly unchecked (Davies, 2010).

It was not until the early twentieth century that a Scientist named Paul Ehrlich proposed a "magic bullet" that selectively targets only disease-causing microbes and not the host through his observations of synthetic dyes staining specific microbes, but not others (Aminov). This small, but significant, observation would lead to what is now coined the "Modern era of antibiotics". Paul's observations may have proved to be insignificant to many during his time, but his observations lead to him performing a systematic screening program to find a therapeutic against the sexually transmitted disease Syphilis, caused by the bacterium *Treponema pallidium.* After screening 606 compounds, Paul Ehrlich, Alfred Bertheim, and Sahachiro Hata came across a

compound named Salvarsan that was able to cure syphilis-infected rabbits (Ehrlich & Hata, 1910).

Eighteen years after the discovery of Salvarsan, a bacteriologist named Alexander Fleming would come across a chance event in his London laboratory that has changed medicine for over a century. After returning to the laboratory from a vacation, Fleming would come across a zone around an invading fungus on an agar plate in which bacteria did not grow. He later coined the active agent of the mold Penicillin, stating that the compound had antimicrobial effects (Fleming, 1929; Gaynes, 2017). For over a decade Fleming made no progress in isolating penicillin, and even offered to send *Penicillium* mold to anyone who requested it in hopes that one would be able to isolate the therapeutic compound. The interest in the vision of a "magic bullet" began to wane until Oxford scientists Ernst Chain, Norman Heatley and Howard Florey were able to purify the compound from the mold in 1939 and thus penicillin was able to enter mass production (Chain, 1940).

The introduction of Penicillin on a large scale as a treatment for bacterial infections, the discovery of Streptomycin, and Fleming's methods of observing Zones of Inhibition led to what is known as the Golden age of antibiotic discovery. This era is coined the golden age as one-half of the drugs commonly used today were discovered in this period: 1950-1960 (Davies, 2006). At the time, the sudden discovery of many antimicrobial agents proved to be highly beneficial to humanity. Not only were these newly discovered antibiotics being used in clinical settings to eradicate disease in human, but they were also employed in livestock farming and agriculture. Antibiotics quickly entered the scene in livestock farming with the introduction of these antimicrobial agents being used to treat animal disease, sub-therapeutic levels in animal feed for growth promotion, and even to control bacterial disease in plants. Although the introduction of

antibiotics to agriculture and livestock has been beneficial in many ways, it does not go without the result of higher concentrations of antibiotics and resistance genes entering the environment. (Manyi-Loh, 2018; Stockwell 2012). With the rapid accumulation of antibiotics entering the environment, it is without a doubt that resistance to the agents has and will continue to confer. This presents a large dilemma to the current progress of innovation of new antibiotics. The golden age of antibiotics provided the introduction of more than 20 new classes of antibiotics, and since then successful production of antibiotics have come to near halt with just two new classes reaching the market since 1940-1962 (Coates 2011). Various analogues of currently existing classes have reached the market since the golden age; however, multi-drug resistant bacteria continue to rampart throughout the world causing nearly half of the deaths from clinical infections in Europe (Watson, 2008). With this information, it is absolutely necessary to further the knowledge of the scientific community on these super-bugs to not only better understand them and improve patient care, but to also provide new targets for antimicrobial agents.

The discovery of antibiotics has surely been one of the most valuable medical discoveries in mankind, but they have not come without posing a new set of problems. By 1942, penicillin resistance had already been documented in four *Staphylococcus aureus* strains in hospitalized patients through the production of penicillinase. Just twenty short years after penicillin entered the markets, more than 80 percent of community and hospital acquired strains of S. aureus were penicillin resistant (Lobanovska, 2017). Once an antibiotic was used widely, scientists were quick to pursue synthetic studies to chemically modify existing therapeutics. As with the previous trends, this would work briefly until a resistant strain would arise withing a few years of their use (Davies, 2010). In a study of 12 Ethiopian communities, researchers took nasopharyngeal swabs from randomly selected azithromycin treated children and as well as from

control children. At the start of the study 3.6% of the monitored children carried azithromycin-resistant *S. pneumonia* whereas at the end of the 12-month study period, 46.9% of the treated children were carrying azithromycin resistant *S. pneumonia* compared to 9.2% of the controlled group. Quickly, it became evident that under the selective pressure of an antibiotic, bacteria were able to undergo random changes in their DNA that would allow them to survive and outgrow nonresistance bacteria (Skalet, 2010). Questions then arose as to how bacterial populations were able to require resistance, and how was it possible to gain widespread resistance at remarkable speeds. It is now understood that bacteria can acquire antimicrobial resistance through several mechanisms such as limiting the uptake of the drug, modifying the drug target, inactivation of the drug, or active drug efflux. In some cases, a single mutation can confer resistance to an antibiotic as is seen with mutation in the ribosomal protein rpsL conferring resistance to streptomycin (Reygaert, 2018; Springer, 2001). Mutational based resistance or the prior existence of resistance genes in bacteria are observed to rapidly spread throughout a population through the means of DNA transposition and plasmid transfer, otherwise coined horizontal gene transfer. With an average mutation rate of 1 for every 10^6-10^9 cell divisions, mutations that aid in antimicrobial resistance have the potential to quickly spread within a population through the transfer of plasmids. This is especially seen in certain competent bacteria such as *Acinetobacter*, who are known to be capable of acquiring genetic material from the outside environment with relative ease (Raygaert, 2018). Although the discovery of antibiotics has been valuable to mankind, they also present a huge conundrum in that their continued use, even at sub-therapeutic levels, can lead to the selection of highly resistance strains in successive generations. Even worse, is that the continued use of antibiotics, especially without new innovation, can further fuel the propagation of more multidrug resistant organisms.

Through the use of antibiotics and the spread of resistance mediated through horizontal gene transfer, bacterial pathogens associated with epidemics of human disease have quickly evolved into a greater enemy: multidrug resistant bacteria. Multidrug resistant bacteria (MDR) occur when resistance emerges to at least one antimicrobial agent in at least three different classes of antibiotics (Magiorakos, 2012). Multidrug resistant bacteria are increasingly important to address as their prevalence is not new and their spread is continued in recent years. In fact, the majority of nosocomial infections are being caused by a group of six pathogens: *Enterococcus facecium, Pseudomonas aeruginosa, Klebsiella pneumonia*, *Staphylococcus aureus*, the *Enterobacter* species, and what the CDC has recognized as the top emerging threat: *Acinetobacter baumannii* (CDC AR Report, 2019). This group of pathogens have been coined as the ESKAPE pathogens as they have shown time and time again their ability to escape the lethal action of currently deployed antibiotics (Rice, 2008). Infections such as *Acinetobacter baumannii* are most common in the healthcare setting, putting those who are on ventilators at the biggest risk of infection as well as likely poor prognosis through ventilator associated pneumonia. *A. baumannii* infection is also a great risk in immunocompromised individuals who have devices such as catheters, open wounds from surgery, placed in Intensive care units, and in general prolonged hospital stays. Infection is consistent with serious and sometimes life-threatening prognosis through pneumonia, blood infection, meningitis, urinary tract infections, and skin/wound infection (CDC). Further, the ESKAPE superbugs have concurred antimicrobials inefficient against them due to various resistance mechanisms such as drug inactivation, modification of drug binding sites, changes in cell permeability, enzymes inactivating B-lactam antibiotics, and mutations. The preceding statement has resulted in several studies that have revealed an association between EKSAPE infections and negative patient outcomes such as

longer hospital stays, higher morbidity, and higher mortality, which when compounded with the need of these MDR pathogens having to be treated with more expensive/more toxic agents such as Colistin and daptomycin provide an increasing problem (Pandey, 2021). The emergence of MDR *Acinetobacter* has the potential to become an even more major threat in the near future and without innovation and/or control of the misuse and overuse of antimicrobial agents, the world may be left where it stood in the pre-antibiotic era. In a study that isolated ESKAPE pathogens for over 5 years in a Hungarian emergency department, they showed multi-drug resistance was present in 32.6% isolates of *K. pneumoniae,* 19.6% of MRSA, 17.7% of *Enterobacterales*, and most prevalent in *Acinetobacter baumannii* with 65.5% of isolates conferring multidrug resistance (Benko, 2020). A study ran in Iran showed that the prevalence of MDR among *Acinetobacter* species was 100*, which is far higher than the 65.5% in the Hungarian study, 74% reported by Saudi Arabia and 71.6% reported in Ethiopia. Just during the Iranian team's study period, multidrug resistant *Acinetobacter* species infections rose from 7.2% to 13.3% (Namdari). Data as such provides reason as to why the CDC has listed Carbapenem-resistant Acinetobacter as the top urgent threat in their 2019 AR threats report. Further from the impact on human health, multidrug resistant bacteria such as Methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococci,* extended-spectrum B-lactamase, carbapenem-resistant *Enterobacteriaceae,* carbapenem-resistant *Acinetobacter,* and multidrug resistant *Pseudomonas* prove to be a huge financial burden to not only patients, but the healthcare system in whole generating over $4.6 billion USD in associated healthcare costs (Nelson). Per the World Health Organization, drug-resistant disease could cause up to 10 million deaths each year by 2050 thus damaging the economy as well as possibly forcing up to 24 million people into extreme poverty by 2030 (https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-

avert-antimicrobial-resistance-crisis). With such a grave threat among us, it is of utmost importance to dedicate resources to comprehensive assessments of these pathogens as well as infection-control interventions.

Although antibiotic resistance has been shown to be highly prevalent in *Acinetobacter baumannii*, it is likely that the general public, some of the scientific community, and even some ER physicians are not familiar with the pathogen due to its lower prevalence. It is estimated that there are 45,000 cases of Acinetobacter infections per year in the United States, with 7,300 of those infections being Multidrug resistant. Of these infections, the mortality rates of patients with *A. baumannii* infection are reported to be between 26.0-55.7%, causing for the utmost importance to not only educate the general public on the pathogen, but the scientific community as a whole (Logan, 2019; Xiao, 2017). From this point on, this paper will discuss the opportunistic, gram negative, obligate aerobe, coccobacillus that is one of the most prevalent causes of nosocomial infections: most notably, ventilator associated pneumonia. Over the past 20 years, the threat of *A. baumannii* has significantly increased due to a lethal combination of pathogenicity and antimicrobial resistance plaguing the hospitals that they mostly reside in. As mentioned earlier in this introduction, *A.baumannii* is considered the CDC's and the WHO's number one critical priority pathogen for which new therapeutics are urgently required to an increasing concern that hospital acquired *A.baumannii* infections will soon be untreatable. With only a limited number of "traditional" virulence factors, which are not conserved across all strains, *A. baumannii's* success has proved to be an area of increasing interest to researchers to better be able to characterize virulence factors and their role in pathogenicity and host-immune response (Morris, 2019).

Virulence is a term used to describe the harmfulness of a pathogen to its host implying that an increase in virulence is synonymous to increasing harmfulness, although important to note pathogens must be able to maintain an intrinsic balance of virulence in order to survive and transmit to other hosts. The term virulence factors then refers to factors that they help to not only invade the host but to enable replication and dissemination within a host by subverting or eluding host defenses (Cross, 2008). These factors may be encoded on chromosomal DNA, bacteriophage DNA, plasmids, or transposons in either plasmids or the bacterial chromosome (Peterson). To be precise, the most commonly accepted roles of virulence factors are able to help bacteria invade the host, cause disease, and evade disease through traditional virulence factors such as adherence factors, invasion factors, endotoxins, exotoxins, siderophores, and of great interest bacterial capsules.

One of the most well characterized virulence factors in *A. baumannii* is its outer membrane protein A (OmpA). Conserved in most clinical isolates, it provides for a highly attractive target for vaccine development. *A. baumannii* is also host to other well established virulence factors such as phospholipase C and D, which target phosphatidylcholine on eukaryotic cell membranes. Further specific virulence factors include secretion systems, efflux systems, siderophores to aid in iron acquisition, quorum sensing genes, biofilm formation, lipopolysaccharide (LPS), lipooligosaccharide (LOS), and the bacterial capsule. LPS, LOS, and the capsule are synthesized in the cytoplasm where they are then exported to the outer portion of the cell envelop. The three components of LPS are lipid A, glycosylated oligosaccharides, and the O-antigen. *A. baumannii* actually does not produce the O-antigen, so instead *A. baumannii's* outer membrane is coated with LOS (Morris). Interestingly, *A. baumanni* is the third observed pathogen to be able to survive in the absence of lipid A, which may provide an explanation for

observed in-vitro colistin resistance of strains such as ATCC 19606 (Moffatt). Of particular interest, is the bacterial capsule of *A. baumannii*. Capsules are the outmost structure of bacterial cells and have the capacity to be able to protect bacteria from the mammalian immune system. By forming a protective layer on the extracellular surface of the bacteria, the capsule is able to mediate resistance to cationic antimicrobial peptides and host serum. The sheer presence of a capsule is not the determinant if a strain of *A. baumannii* will be virulent, rather the presence of a capsule may be a driver in the virulence of a strain through potentially driving innate immune evasion. Keeping in mind that current antibiotics target less than 40 of 200 or more essential genes that most bacterium possess, it is essential to further investigate alternative approaches to combating bacterial pathogens (Lange). An approach that draws interest is targeting virulence factors as disabling a virulence factor can potentially alter the entire course of infection. To be able to disable a virulence factor that may allow bacteria to overcome the host innate immune system may improve clinical prognosis, so it is essential to further research in investigating less characterized virulence factors of *A. baumanni* such as its capsular polysaccharide network. As bacterial capsules continue to show their critical role in advancing bacterial pathogenicity, they provide a promising target for an alternative approach to combating *A. baumannii* as more strains begin to display multidrug resistance.

The bacterial capsule is comprised of tightly packed polysaccharide units, which form a barrier around the bacterial cell well. Not only is it proposed that the capsule provides protection from environmental pressures such as host immune response, but it is also shown to confer resistance to several clinically relevant antimicrobial compounds. In *A. baumaniii*, capsule assembly and export occurs through a Wzy-dependent pathway and usually consists of 4-6 repeating sugars. The K unit of the capsule is synthesized on the lipid carrier molecule

undecaprenyl pyrophosphate providing the role as a scaffold for the growing polysaccharide chains (Woodward, 2016). Each subsequent K unit is then transferred to the periplasmic side of the inner membrane by the Wzx translocase and then polymerized by Wzy. After the capsular polysaccharide is synthesized, it is transported to the cell surface through the combination of Wza, Wzb, and Wzc. The synthesis of the capsular polysaccharide is part of a bifurcated pathway as K units are also used to decorate surface proteins via O-linked protein glycosylation, which is believed to contribute to biofilm formation through enhancing attachment and maturation of biofilms (Iwashkiw 2012; Harding 2015). The capsule locus of *A. baumannii* have great diversity with over 40 diverse K unit structures being elucidated using NMR spectroscopy and other chemical analysis (Singh, 2019). These variations are in strucutrual differences of sugar composition including addition of derivatives of common UDP-linked sugars or atypical sugars such as non-2-ulosonic acid. Variations between capsules can be as subtle as difference of linkage between two glycans up to more striking variations such as the incorporation of more rare sugars such as legionaminic acid as seen *in A. baumannii* LAC-4. LAC-4's K locus gene cluster contains a series of genes leg1-6/ABLAC_37030-37800, which encode for the enzymes necessary for the biosynthesis of legionaminic acid (Fig. 1).

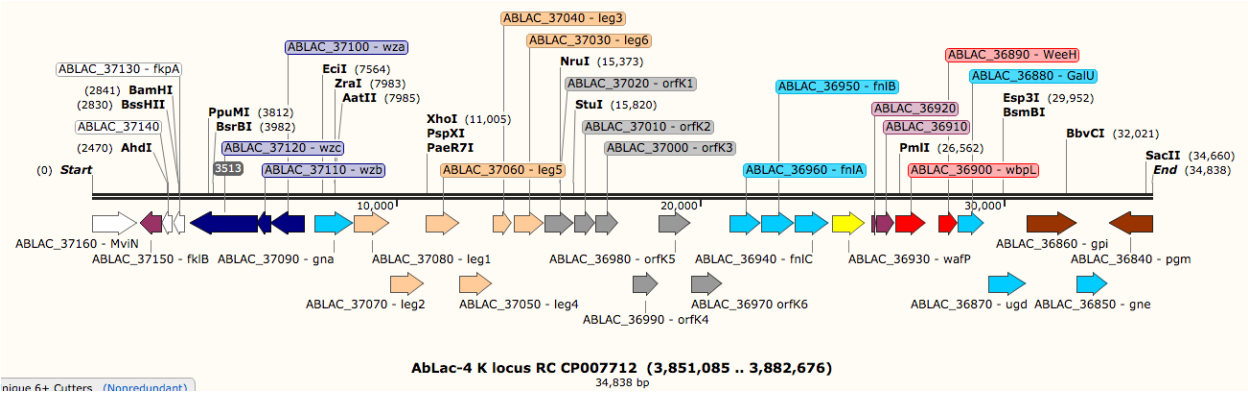

Figure 1) Schematic diagram of the K locus gene cluster in A. baumannii LAC-4.

This acid is the precursor to an uncommon sugar known as α-8-epi-legionaminic acid. Legionaminic acid structurally resembles sialic acid on mammalian host cell's surface glycoproteins. Sialic acid, a nine-carbon α-keto sugar is commonly found incorporated on mammalian cell surface glycoproteins and are responsible for intercellular reactions. Legionaminic acid shares the stereochemistry of sialic acid providing the possibility that LAC-4's increased virulence can be attributed to the pathogen utilizing this sugar to mimic host cell surfaces to escape from immune surveillance (Morrison 2014; Ou 2015).

It is highly likely that the presence of a capsular polysaccharide, and a unique one in LAC-4, is essential for the hypervirulence of *A. baumannii* LAC-4. Not only have capsular polysaccharides been linked to be important for resistance to antimicrobial compounds, but it is believed that capsular polysaccharides are important in limiting the interactions between immunogenic surface structures of the bacteria and the host defense system. Up-regulation of capsule production has been seen in strains of *A.baumanni* such as ATCC 17978, where increased serum resistance is observed. Further, growth of A. baumannii in subinhibitory levels of antimicrobials such as chloramphenicol positively influence the level of capsular polysaccharide protection (Geisinger 2015) as well as capsule deficient strains of *A. baumannii* being shown to have reduced survival in human serum. *A. baumannii*, notorious for its ability to persist for months on hospital surfaces, may also be able to do so due to capsule enhancing dessication tolerance by providing a physical barrier, which facilitates water retention (Roberts 1996; Webster 2015). The ability to enhance dessication tolerance is worrisome for a nosocomial infection as it provides a reservoir of source of transmission and infection for susceptible populations. As a result of this preceding information, it is important to conduct studies on more

strains of *A. baumannii* to potentiate if virulence through capsular polysaccharides is strain specific, capsule type specific, or universal.

Chapter 2

Materials and Methods

Bacterial strains, Plasmids, and Growth media

The bacterial strain *Acinetobacter baumannii* LAC-4 was obtained from an undisclosed Los Angeles County Hospital during an *A. baumannii* outbreak in 1997 (Valentine). It was then kindly donated to our lab by Dr. Wangxue Chen from the National research council of Canada. The suicide vector pMo130 was acquired from addgene (Fig. 2).

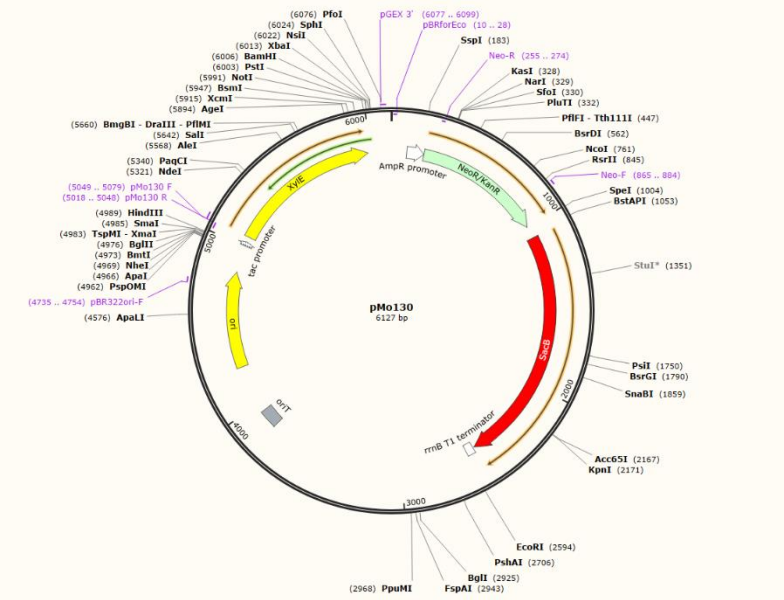

Figure 2) Diagram of the pMo130 suicide vector. Plasmid contains a kanamycin resistance gene, sacB gene for sucrose counter selection, and the xylE reporter gene. Restriction enzyme sites are also noted.

The recombinant plasmid pMo130 containing the knockout construct was developed in a cloning host, Escherichia coli DH5α. The pKo plasmid was also obtained from addgene and was harbored in E. coli DH5α to be used as a template for the apramycin resistance gene (Fig. 3).

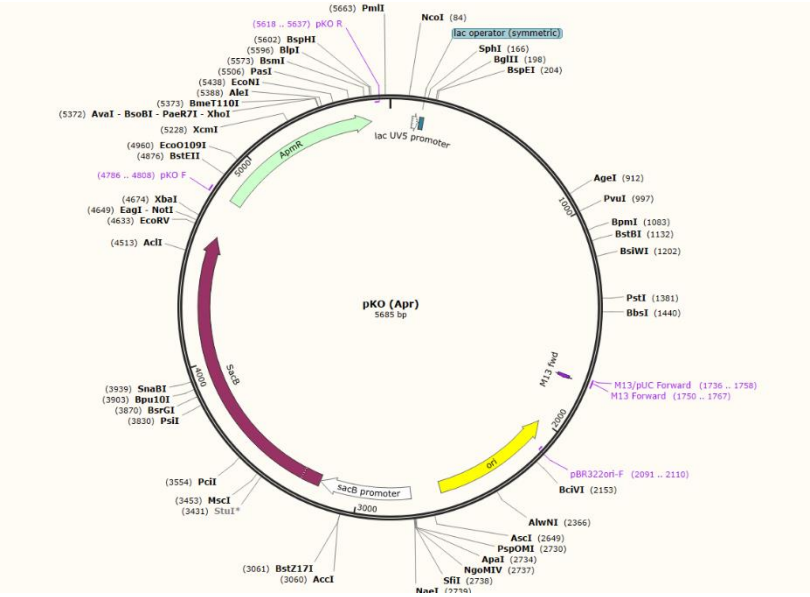

Figure 3) Diagram of the pKo plasmid. Plasmid contains the apramycin resistance gene, which was shuttled into pMo130 to allow for two antibiotic selection markers while working with the multi drug resistance A. baumannii LAC-4.

E. coli DH5α were grown shaking at 200rpm in difco luria bertani (LB) media at 37C. *A. baumannii* cells were grown shaking at 200rpm in tryptic soy broth (TSB) media at 37C. *A. baumannii* LAC-4 cells with the integrated knockout cassette and suicide vector were counter-selected using TSB media made from 15 g tryptic soy broth (Thermo Fisher) into 500 mL of Milli-Q purified water. E. coli DH5α with the pMo130 plasmid was grown in LB media supplemented with 15ug/mL of apramycin. For antibiotic selection, TSB medium was supplemented with 15ug/mL of apramycin and 15ug/mL of kanamycin to select E. coli DH5α with the recombinant pMo130 plasmid. For transformations, electroporated cells were grown in TSB with catabolite repression (SOC) medium (Invitrogen by Life Technologies). Single crossover transformants of *A. baumannii* LAC-4 were selected for using TSB media with 50ug/mL apramycin. YT media supplemented with 15% sucrose was used to counter-select for wildtype and seamless knockout double-crossover transformants.

Genomic and Plasmid DNA extraction

Genomic DNA was extracted using the DNEasy kit (Qiagen). Extracted genomic DNA was diluted to 100 ng/ul for use in subsequent PCR.

Recombinant plasmids containing the knockout construct were isolated and purified from liquid culture using the QIAprep Spin Miniprep kit by QIAGEN. Plasmid DNA used as a PCR template was diluted to 80 ng – 500 ng/uL for subsequent PCR.

Construct construction and PCR

All primers were designed to have melting temperatures of approximately 60ºC +/- 12ºC. Further, primers were designed to amplify regions of 400-1,000 base pairs (bp) upstream and downstream of the desired knockout target for use in manipulation of homologous recombination with the *A. baumanii* LAC-4 genome. Primers containing 15-24bp overlaps were designed to shuttle the apramycin resistance gene from the pKo plasmid into the pMo130 suicide vector (Table. 1) alongside Gibson assembly to stitch the amplified regions together.

Table 1) PCR primers used to shuttle apramycin resistance gene into pMo130.

Primer Name	Sequence 5'→3'
pKo F	5' gaatatcacagaaggtgatcgactgatgtcatcagcgg 3'
pKo R	5' catagtattctgtctttgcccctccaacgtcatct 3'
pMo130 F	5' gacgttggaggggcaacagacagaatactatgaacaaaggtgtaatgc 3'
pMo130 R	5' gacatcagtcgatcaccttctgtgatattccacacattatacgagc 3'

PCR reaction to shuttle the apramycin resistance contained 40uL of H20, 5uL of PfuUltra buffer (Agilent), 1.25uL of dNTPs, 1uL of DNA equal to 100ng, 2uL of primers, and 1uL of PfuUltra high fidelity polymerase (Agilent). Dpn1 digestion then followed to digest methylated DNA while preserving PCR products. PCR conditions were as follows.

Table 2) Thermal cycler conditions to shuttle the apramycin resistance gene from pKo into the pMo130 suicide vector.

Temperature	Time
95C	2 minutes
95C	20 seconds
60C (Primer Tm-5C)	20 seconds
72C	15C
	X35
72C	3 minutes
4C	Hold

Amplification of 550 base pairs upstream of *A. baumannii* LAC-4's capsule region with BamHI and NotI flanking restriction enzyme sites was done to create fragments of the upstream region. Primers were designed to have melting temperatures of 58C in a 50uL reaction containing 22uL of H20, 25uL of 2X PCR master mix, 2uL of F/R primers, and 1uL of DNA constituting 100ng. Reaction conditions were as followed (Table. 3).

Table 3) Thermal cycler conditions for amplification of Ab. LAC-4 upstream region with BamHI and NotI restriction enzyme flanking sites.

Temperature	Time
94C	2 minutes
94C	20 seconds
50-60C	20 seconds
68C	15C
	X30
68C	Hold

Product was run on a 1% agarose gel and product was then gel extracted using the Qiagen QiaQuick spin gel extraction kit according to manufacturer's protocol and then 2ug of gel extracted products were subject to double digest according to NEB's protocols with 1uL of NotI-HF (NEB) and 1uL BamHI-HF (NEB). Double digested products were then run on 1% agarose gel and extracted to be use in subsequent electro ligase. 100ng of the pMo130Apr vector, 25ng of the upstream insert of Ab. LAC-4, 3.11uL of H20, 5uL of electro ligase reaction buffer, and 1uL of electro ligase was incubated at 25ºC for 1 hour and then heat inactivated at 65ºC for 15 minutes. 5uL of this mixture was electroporated into 100uL of electrocompetent DH5a at 1600mV and then 950uL of SOE media was added to be incubated at 37ºC with aeration for 90 minutes. This was then plated onto LB agar supplemented with 15ug/mL of kanamycin and apramycin to create overnight liquid cultures in 5mL of LB and 15ug/mL of kanamycin and apramycin. Clones were miniprepped using QiaSpin Miniprep kit and a double digestion with

1uL of NdeI (NEB) and 1uL (SphI-HF) was done to confirm integration of the upstream fragment of *A. baumannii* LAC-4 into the pMo130Apr plasmid.

Amplification of 550 base pairs downstream of the *A. baumanii* LAC-4 capsule region with BamHI and NotI flanking restriction enzyme sites was done to create fragments of the downstream region. Primers were designed to have melting temperatures of 58C in a 50uL reaction containing 22uL of H20, 25uL of 2X PCR master mix, 2uL of F/R primers, and 1uL of DNA constituting 100ng. Thermal cycler conditions did not differ from amplification of the upstream region. Product was run on a 1% agarose gel and product was then gel extracted using the Qiagen QiaQuick spin gel extraction kit according to manufacturer's protocol. 1ug of gel extracted DNA was subject to double digest according to NEB's protocols with 3ug DNA, 1uL of SphI-HF (NEB) and 1uL of BamHI-HF (NEB). Double digest was subject to 1% agarose gel and then subsequently gel extracted for electro ligation. 100ng of the pMo130Apr vector, 25ng of the downstream insert of Ab. LAC-4, 3uL of H20, 5uL of electro ligase reaction buffer, and 1uL of electro ligase was incubated at 25C for 1 hour and then heat inactivated at 65C for 15 minutes. 5uL of this mixture was electroporated into 100uL of electrocompetent DH5a at 1600mV and then 950uL of SOE media was added to be incubated at 37C with aeration for 90 minutes. This was then plated onto LB agar supplemented with 15ug/mL of kanamycin and apramycin to create overnight liquid cultures in 5mL of LB and 15ug/mL of kanamycin and apramycin. Clones were miniprepped using QiaSpin Miniprep kit and a double digestion with 1uL of EcoRI (NEB) and 1uL SpeI (NEB) was done to confirm integration of the downstream fragment of *A. baumannii* LAC-4 into the pMo130Apr plasmid, which had the upstream region integrated to it in the previous step. 100ng of pMo130AprUpDn was electroporated into 75uL of electrocompetent *A. baumannii* LAC-4 with 2.5kV. 950uL of SOE media was added and then

incubated with aeration for 90 minutes to then be plated on LB agar supplemented with 50ug/mL of apramycin.

Transformation into *E. coli* DH5α & Isolation of plasmid DNA

The pMo130AprUp construct created with electroligase, BamHI/NotI digested fragments of *A. baumanii's* genome and pMo130-Apr were fused to create a recombinant plasmid to be transformed into *E. coli* DH5α. The recombinant pMo130AprUp as well as pMo130AprUpDn plasmid were transformed into *E. coli* DH5α cells using electroporation. 2 and 5 uL of ligation mixtures were electroporated into 50-100 uL of electrocompetent DH5α at 1,600 mV. 950 uL of SOE media was then added and mixtures were incubated at 37C with aeration for 90 minutes. Cells were then plated on to LB Agar plates containing 15 ug/mL of Kanamycin and 15 ug/mL Apramycin to then be grown overnight at 37C for 16-18 hours. Transformants were then inoculated into fresh LB media supplemented with 15 ug/mL of Kanamycin and 15 ug/mL of Apramycin to be grown overnight at 37C while shaking at 200rpm. Liquid culture was then pelleted down for plasmid isolation using QiaSpin's Miniprep kit with the manufacturers recommended protocol.

Verification of recombinant plasmid

Verification of successful ligation was accomplished through two means: sanger sequencing (Genewiz) and restriction digest. Double digest using the restriction enzymes EcoRI and SpeI was performed, yielding products of 6,473 bp and 1,590 bp if successful. Restriction digest was prepared with the following conditions for verification of recombinant plasmid: 1 ug of miniprepped plasmid DNA, 5 uL of CutSmart (NEB), 1 uL of EcoRI (NEB), 1 uL of SpeI

(NEB) and 38 uL of H20. Digestions were incubated at 37C for 30 minutes and then ran on a 1% agarose gel.

Preparation of *A. baumannii* LAC-4 electrocompetent cells

Electrocompetent A. baumannii Lac-4 cells were prepared by subculturing stationary phase Ab. Lac-4 into 100 mL fresh TSB in a 500 mL flask. Ab Lac-4 was grown to OD 600 0.4. The flask was transferred to ice and chilled for 15 minutes. The cultures were spun down at 4°C at 10,000 RPM for 5 minutes in a pre-chilled rotor. The pellets were resuspended on ice in 100 mL dd$H_2$0 and centrifuged again under the same conditions. The pellet was washed one additional and centrifuged. The final pellet was resuspended in 3-5 mL ice cold 10% glycerol. 100 µl aliquots were snap frozen in ethanol and dry ice and stored at -80°C until used.

Transformation of recombinant plasmids into *A. baumannii* LAC-4

Recombinant pMo130AprUpDn plasmids were electroporated into *A. baumannii* LAC-4 competent cells. Electrocompetent LAC-4 cells were thawed out and aliquoted into 75 uL aliquots. Either 100 ng or 1 ug of the recombinant pMo130-AprUpDn plasmid was added to each aliquot and then incubated on ice for 5 minutes. Contents were then transferred to pre-chilled 1mm electroporation cuvettes and then electroporated at either 2.2 kV or 2.5 kV using (Eppendorf). 950 uL of SOE media was added and then placed in a 37C incubator with aeration for 90 minutes. Electroporated *A. baumannii* LAC-4 cells were then plated onto LB agar supplemented with 50 ug/mL of Apramycin and then grown at 37C for 24 hours to select for single crossover mutants.

Single crossover screening

Successful transformants of A. baumanii LAC-4 would incorporate the apramycin resistance gene that was integrated into the recombinant plasmid into the LAC-4 genome using a RecBCD mediated single-crossover event as pMo130 is a suicide vector in A. baumannii (Fig. 4) (Dillingham 2008). Mutants that have integrated the plasmid containing the knockout cassette into their genome would also express the xylE reporter gene. The xylE reporter gene codes for catechol 2,3 dioxygenase, which in the presence of catechol or para-catechol, will cleave the catechol substrate into 2-hydroxymunoic semialdehyde. This reaction will then yield a bright yellow pigmentation in colonies expressing the xylE gene on agar plates or in liquid media (Saunders 1996; Lee1 1996). According to unpublished data from the lab of Howard Xu at CSU LA, Acinetobacter as a species is capable of producing its own catechol like substrate as is observed in my own experience with the species.

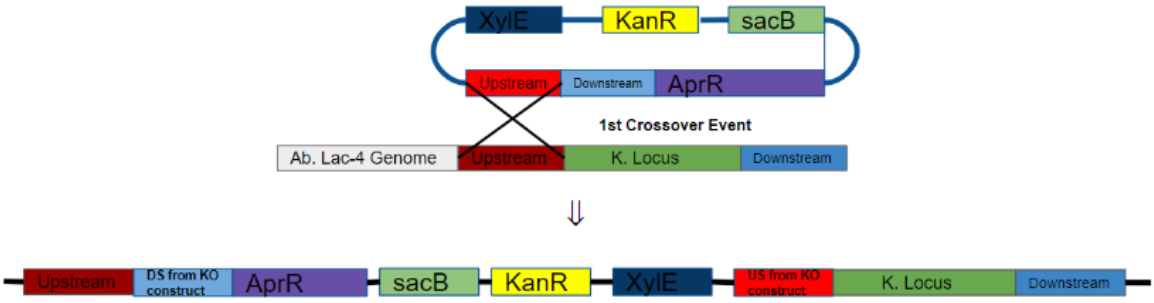

Figure 4) Co-integration of recombinant plasmid via a single-cross over event. Single crossover event occurs between homologous recombination of the upstream flanking region or downstream flanking region with the bacterial chromosome. As shown above, upstream recombination is depicted to illustrate how the plasmid inserts itself into the chromosome.

As a result, exposure of exogenous catechol was not needed when selecting for single crossover mutants in the engineering of the capsule deficient A. baumannii LAC-4 strain.

Colonies that grew on transformation plates appeared yellow, which indicates the integration of the recombinant plasmid and subsequent XylE plasmid. These colonies were then spot platted onto LB agar to grow for 24 hours as well as being simultaneously inoculated to grow shaking overnight at 37C in 2 mL of LB supplemented with 50 ug/mL of apramycin. Colonies on the spot plate and overnight cultures that appeared yellow have successfully incorporated the recombinant plasmid and were selected to undergo passaging to select for double crossover events.

Passaging and plating to select for double crossover event

As mentioned in the preceding section, single crossover mutants were identified by positive indication of the xylE reporter gene. To select for double crossover mutants, individual clones were passaged under sucrose stress to select for the loss of pMo130 plasmid containing the sacB gene. The single crossover mutants were grown in LB media overnight and then 10uL of culture was inoculated into 5mL of YT broth supplemented with 15% sucrose to be grown at 30C with aeration. The culture was then passaged to fresh YT broth with 15% sucrose after 24 hours. The passaging process was repeated three times with the final passage being serially diluted in PBS. 100uL of serially diluted culture was plated onto YT agar containing 10% sucrose to grow overnight at 30C.

Spot plating selection of double mutant crossover

Colonies with white pigmentation, instead of yellow pigmentation due to xylE expression, were indicative of a double crossover event resulting in complete knockout of the capsule locus or reversion to wildtype (Fig. 5)

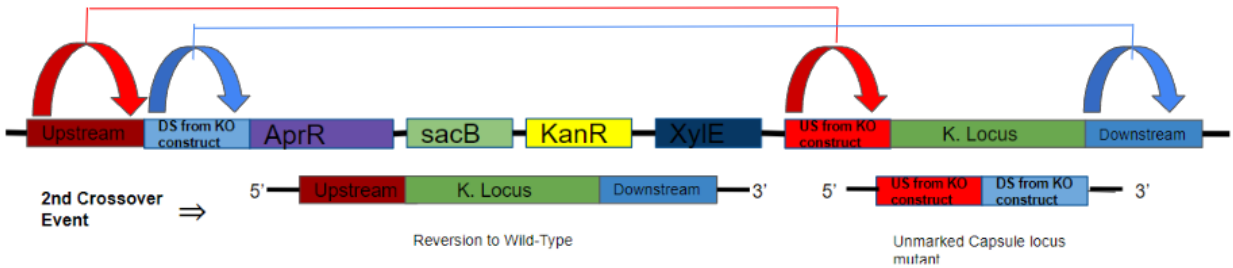

Figure 5) Double crossover event resulting in complete capsule knockout or wildtype reversion. After successful single crossover, the remaining flanking regions may undergo homologous recombination to excise the plasmid backbone and target locus resulting in knockout. Also, the original flanking regions that underwent single crossover can recombine again as illustrated in red will result in reversion to wildtype.

Due to the separation of colonies in plating, the catechol like substance may not have been produced in high enough of a concentration to produce yellow pigmentation, so to strengthen the screen, the passaged double crossover mutants were spot platted onto LB agar plates and LB agar plates supplemented with apramycin. Colonies that were able to grow on LB agar plates, but non on LB agar plates supplemented with apramycin, indicated that these colonies have flipped out the pMo130 plasmid due to sucrose counter selection. Loss of the pMo130 plasmid indicates a double crossover event in which the capsule region is knocked out or the bacteria have reverted back to the wildtype. Clones that only grew on the LB agar plates were then subject to further downstream processes to confirm knockout of the capsule locus.

Confirmation of knockouts

Centrifugation is the most common method for the separation of subcellular particles; however, it has become apparent that density gradient mediums such as percoll are well suited for the separation of fragile elements such as bacterial capsules. Specifically, percoll possesses the characteristics of low viscosity, low osmolarity, and no toxicity towards cells providing a useful tool to separate subcellular particles. (Pertoft, 2000). Density-based separations by gradient centrifugation are common with eukaryotic cell types, but rarely used in microbiological

research. It is now known that density-based separations provide use in observation of capsules through the reasoning that a highly capsulated bacteria will take more time to pellet by centrifugation and will then settle at the top of the percoll gradient (Feltwell, 2019). Nine percoll dilutions ranging from 90% percoll to 10% percoll in PBS were created in 50mL falcon tubes. 5mL of each dilution was carefully pipetted into a singular falcon tube as to not disturb the layers with the most concentrated layer at the bottom. Falcon tubes were then centrifuged at 8,000xG for 10 minutes to identify mutants with altered capsule production shown by migration of the bacteria to the bottom layer of the gradient. For further confirmation of a double crossover knockout event, 6 primer sets (Table. 5) were designed to amplify several regions withing the *A. baumannii* capsule locus. Genomic DNA was extracted from four clones suspected to have lost their capsule and were subjected to PCR. 25uL of Platinum II hot-start green PCR master mix (Invitrogen), 22uL of H_20, 2uL of forward and reverse primers, and 2uL of DNA from possible knockouts were used in PCR reactions. Further, a positive control in *A. baumanni* LAC-4 wildtype was assessed to confirm for the presence of the selected amplified regions in the wildtype encapsulated LAC-4. Thermal cycler conditions were as depicted (Table. 5).

Table 4) Primers used to confirm absence of selected regions within the capsule locus of A. baumannii LAC-4. F or R refers to directionality of each primer in a primer pair.

Primer name	Sequence 5'→3'
DKO_pgm_F	5'ATG CCG TAT ACG GTG GTG AAA TGA 3'
DO_gne_R	5'GAA ATG CCA ACA GGT ATG CCA AGT 3'
DKO_GalU_F	5'ACA GATAGG TTT GAT GGT GCA GCA 3'

Table 4 continued)

DKO_WeeH_R	5’TGA TGC ACA GGG TAA TCC ATT ACC T 3’
DKO_36950_F	5’GGC AAA AAG TTG CAA CTG CAG AG 3’
DKO_36960_R	5’TTA ATG CAA TGG GCA TTT CTA AAG CCA T 3’
DKO_37000_F	5’AAT ATA GCC GCC ACT AAA CGC G 3’
DKO_orfK6_R	5’TAA TGA CAA CAG CGA AAG GAG CTG T 3’
DKO_37100_F	5’TCA GGG TAT GAC ATT AAG CGA TGC A 3’
DKO_37120_R	5’TGT GAT AAA TCA CCT AAG AGT GCA GCA 3’
DKO_flkB_F	5’GCT CAA AAT AAG ACA GCA CGC CTA AC 3’
DKO_MuiN_R	5’AGG CAT TGT TCC AAC GCG G 3’

Table 5) Thermal cycler conditions used for confirmation of complete knockout while screening for double crossover mutants.

Temperature	Time
94C	2 minutes
94C	20 seconds
60C	20 seconds

Table 5 continued)

68C	30 seconds
	X 30
68C	2 minutes
4C	Hold

Antimicrobial susceptibility testing

A. baumanni LAC-4 and capsule deficient mutant clones 1 and 9 were grown shaking at 200 rpm overnight at 37C in 5mL of TSB media. Liquid overnight cultures were subsequently subcultured into 9.8mL of RPMI-10% TSB at a 1:50 ratio. Subcultures were grown up shaking at 200rpm at 37C to around OD600 = 0.40. Bacteria was then spun down and washed with RPMI-10%TSB two times and resuspended at a density of 400,000 cells per 180uL in each well of a 96 well plate. Chloramphenicol, streptomycin, colistin, ampicillin, sulbactam, and polymyxin B (Sigma-Aldrich) were diluted to 2.5mg/mL and then subsequently serially diluted two-fold down 7 wells on the antibiotic dilution preparation plate. Assay plate was then set up with 180uL of prepared bacteria and 20uL of the serially diluted antibiotic preparations from the antibiotic dilution plate. Antibiotic concentrations from 250 ug/mL to 1.95 ug/mL were used in these sets of susceptibility testing. Control wells containing just RPMI-10%TSB containing bacteria and the solvent the antibiotic was dissolved were prepared. Control wells containing just media and solvent were prepared to for normalization of the data. Round bottomed 96 well plates were incubated shaking at 200rpm at 37C for 24 hours. Plates were then read for OD_{600}nm using the EnSpire Alpha plate reader (Perkin Elmer) to then be compared to CLSI breakpoints. Data was

normalized to the media and to bacterial growth without addition of antibiotic to obtain MIC90 values.

In vitro whole blood and serum killing assays

A. baumannii LAC-4, capsule knockouts 1 and 9, as well as wildtype revertant clone 8 were grown shaking overnight at 37C in 10mL of TSB. LAC-4 and the wildtype revertant were subcultured in 10mL of TSB and capsule deficient mutants were subcultured in 20mL of TSB. Bacteria were grown to 0.40 OD_{600} and were washed one time in PBS. Bacteria were then washed for a second time in RPMI-2% FBS. Final bacterial preparation was prepared at a density of 2x10^5 CFUs/10uL or 200,000 cells per well in a 96 well assay plate. Healthy adult males and females in coordinance with a US board certified doctor provided whole blood for the purpose of these studies. 15mL of whole blood was collected in a green top heparin vacutainer (BD) and 15 mL of whole blood was collected in a tiger top tube (BD) to separate human serum via centrifugation at 2,500 rpm for 15 minutes at room temperature. A 96 well assay plate was set up with the following conditions: first column consisted of non-treated wells containing 200,000 bacterial cells of each respective strain in triplicates in 10 uL and 90 uL of RPMI-2%FBS. The second column consisted of 200,000 bacterial cells of each respective strain in triplicates in 10 uL, 20 uL of human serum, and 70 uL of RPMI-2%FBS. The third column consisted of 200,000 bacterial cells of each respective strain in triplicates with 90 uL of human whole blood. Bacteria was then incubated while rotating at 37C for two hours. Bacteria were plated out to 10^-7 fold at one hour and two hours. Bacterial CFUs were then counted and compared to the non-treated conditions to analyze for fold change of bacteria in response to whole blood and serum treatment.

In vitro neutrophil killing assay

A. baumannii LAC-4, capsule knockouts 1 and 9, as well as wildtype revertant clone 8 were grown shaking overnight at 37C in 10mL of TSB. LAC-4 and the wildtype revertant were subcultured in 10mL of TSB and capsule deficient mutants were subcultured in 20mL of TSB. Bacteria were grown to 0.40 OD, spun down, washed, and resuspended with RPMI-2%FBS. Blood draw was coordinated with healthy adult donors and a physician to collect 30 mL of venous blood with heparin in a heparin vacutainer. Blood is pooled into one container where 25 mL of collected blood is carefully and slowly added over 20 mL of polymorph prep. Container was then centrifuged in an Eppendorf 5810 swinging bucket centrifuge at 630 x g for 30 minutes with the brakes off. After removing the tubes form the centrifuge, the top layer will contain peripheral blood mononuclear cells (PMNCs) and the bottom layer contains polymorphonuclear leukocytes (PMNs), or in other terms, neutrophils. The top layer is aspirated and PMNs are carefully collected with a 10 mL serological pipette. Collected cells are placed in a 50 mL conical tube at no more than 15 mL in each tube. PBS is added to bring the volume of the tube to 50 mL and then spun down at 500 x g for 10 minutes with brakes set at 2. At this point, PMNs are pelleted at the bottom of the tube and supernatant is aspirated for the pellet to be resuspended by gentle flicking. To lyse any present red blood cells, 5 mL of sterile water is added and pipetted up and down four times over the course of 30 seconds and then PBS is promptly added to bring up the conical's volume to 50 mL to stop the lysing reaction. Conical is then spun down at 500 x g for 10 minutes with brake set at 1. The remaining supernatant is aspirated and the PMN pellet is gently resuspending via gentle flicking in around 70 uL of the remaining media. 930 uL of PBS is then added to bring the volume up to 1 mL. Cells are diluted 1:100 in PBS and counted using a hemacytometer. 200,000 cells are added in each well in a 96 well plate and

bacteria diluted in RPMI 1640 was added at a multiplicity of infection of 1:1. Cells are incubated standing at 37C with 5% CO2 for 2 hours. Control wells without neutrophils were used to determine baseline bacterial counts at the assay endpoint.

In vivo murine studies

In vivo assays of *A. baumannii's* virulence were conducted by me with assistance of post-doctoral mentor Elisabet Bjanes. Mice were inoculated intraperitoneally to model systemic infection with approximately 1 x 10^7 CFU of fresh culture prepared from frozen glycerol stocks of *A. baumannii* LAC-4 wildtype, revertant, and mutant strains. For survival studies, mice were monitored every 6 hours post infection for 48 hours with each group consisting of 5 mice. For bacterial burden, mice were sacrificed 4 hours post-inoculation. Cardiac puncture was performed to quantify bacterial burden in the blood and spleens were removed for bacterial quantification of a major secondary lymphoid organ.

Chapter 2 is coauthored with Bjanes, Elisabet and Nizet, Victor. The thesis author was the primary author of these chapters.

Chapter 3

Results

Construct construction, transformation into E. Coli DH5α, and ligation into suicide vector

To knockout the capsule gene locus in *A. baumannii* LAC-4, we began to construct knockout cassettes containing both flanking regions of the gene cluster fused together into the pMo130Apr plasmid backbone. Flanking regions are homologous to the identical regions in *A. baumannii* LAC-4 and as a result could be swapped with the existing regions in the genome via a RecBCD mediated homologous recombination pathway (Dillingham 2008). To begin, amplification of 550 base pairs upstream of the target region in *A. baumannii* LAC'4s was done with BamHI and NotI restriction enzyme flanking sites. The resulting fragment would be 550 base pairs and would be used to fuse to the pMo130Apr backbone (Fig. 6). Fragment alongside pMo130Apr were subject to double digest using NotI-HF and BamHI-HF to then be gel extracted to be used in electro ligated together to create a recombinant pMo130AprUp vector (Fig. 7). Recombinant pMo130AprUp was then transformed into cloning host cell line *E. coli*

DH5α to create large amounts of plasmid to be miniprepped and stored for use when ligating the downstream element of the capsule region of *A. baumannii* LAC-4.

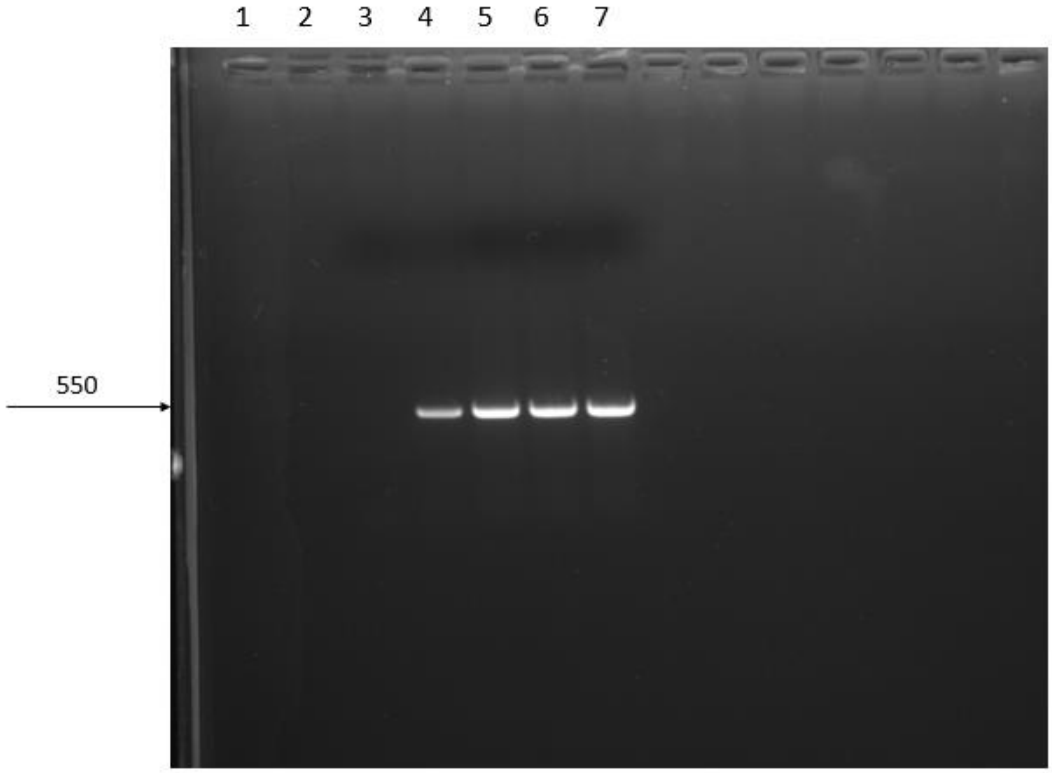

Figure 6) Confirmation of successful upstream fragment amplification. PCR samples were run on 1% agarose gel and visualized using SYBRR Safe stain and UV light. Ladder is not pictured. Lane1: Ladder (not added) Lane2: Blank Lane 3: H20 control. Lane 4: 56C annealing temperature. Lane5: 57C annealing temperature. Lane 6: 58C annealing temperature. Lane 7: 59C annealing temperature.

pMo130AprUp underwent double digestion once more with NdeI and SphI for the purpose to confirm successful ligation. Successful ligation would be represented by products of 6,277 base pairs and 1,228 base pairs (Fig. 8). Once again, amplification of 550 base pairs, but now downstream of the target region in *A. baumannii* LAC'4s was done with NdeI and SphI restriction enzyme flanking sites. The resulting fragment would be 550 base pairs and would be used to fuse to the pMo130AprUp vector (Fig. 9). The downstream fragment and pMo130AprUp were subjected to double digestion using the BamHI and SphI-HF restriction enzymes to then be run on a 1% agarose gel and subsequently be gel extracted for electro ligation of the digested

downstream fragment with the digested pMo130AprUp vector, for which the image was not saved.

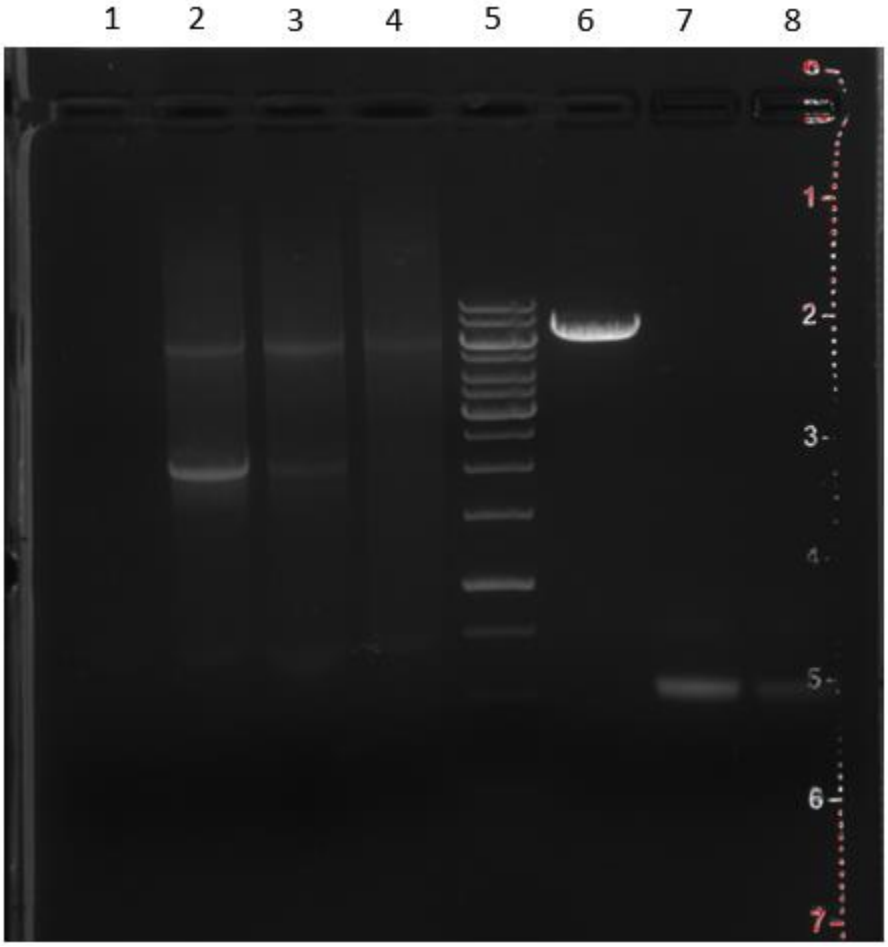

Figure 7) Double digestion results with BamHI/NotI. Lane 1: H20. Lane 2: pMo130 Gibson vector 53C. Lane 3: pMo130 Gibson vector 54C. Lane 4: pMo130 PCR Gibson vector 55C. Lane 5: GeneRuler 1kb DNA ladder (Thermo Scientific). Lane 6: pMo130Apr BamHI/NotI double digest. Lane 7: Ab. LAC-4 Up BamHI/NotI double digest. Lane 8: Ab. LAC-4 Up BamHI/NotI double digestion

The electro ligated product being pMo130AprUpDn was transformed into cloning host cell line *E. coli* DH5α to create large amounts of plasmid to be miniprepped and stored. pMo130AprUpDn then underwent sequencing and double digestion with EcoRI and SpeI to confirm successful ligation (Fig. 10). Successful products would be represented by products of 6,473 bp and 1590 bp. After confirmation of successful plasmid construct construction,

pMo130AprUpDn was transformed into electrocompetent A. baumannii LAC-4 to screen for successful single crossover mutants.

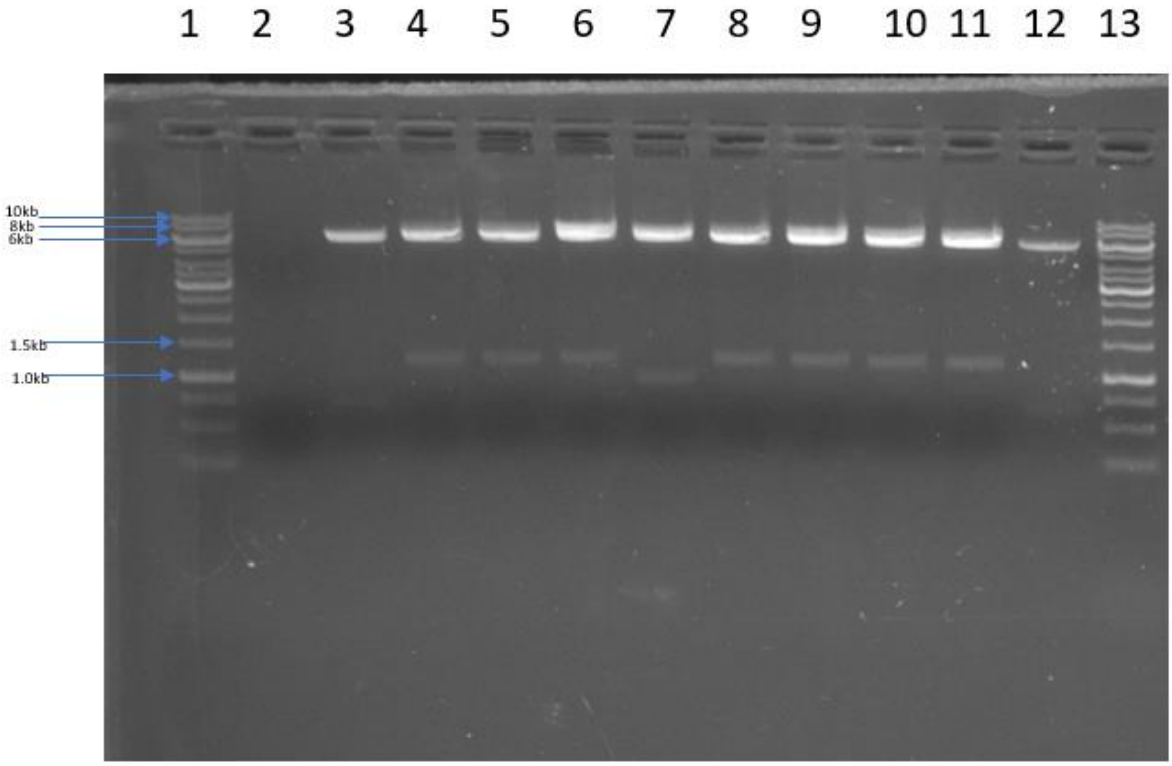

Figure 8) Double digestion confirmation of successful ligation of upstream region to pMo130Apr backbone. Lane 1: Generuler 1kb ladder. Lane 2: H20. Lane 3: pMo130Apr parent clone. Lane: 4-11: pMo130AprUp Cl.1-Cl.8 Lane 12: pMo130Apr parent clone. Lane 13: Generuler 1kb ladder. All clones besides clone 4, successfully have the expected digestion products of 6,277 base pairs and 1,228 base pairs suggesting successful ligation.

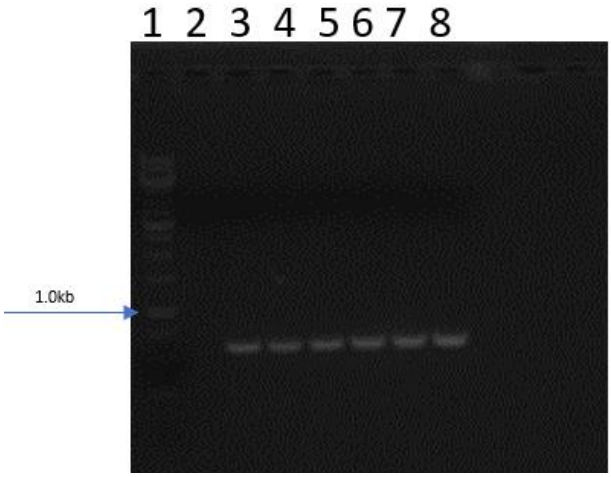

Figure 9) Confirmation of successful downstream fragment amplification. PCR samples were run on 1% agarose gel and visualized using SYBRR Safe stain and UV light. Lane 1: Generuler 1kb ladder. Lane 2: H20. Lane 3-8: Ab. LAC-4Dn 50C. 52C. 54C. 56C. 58C. 60C.

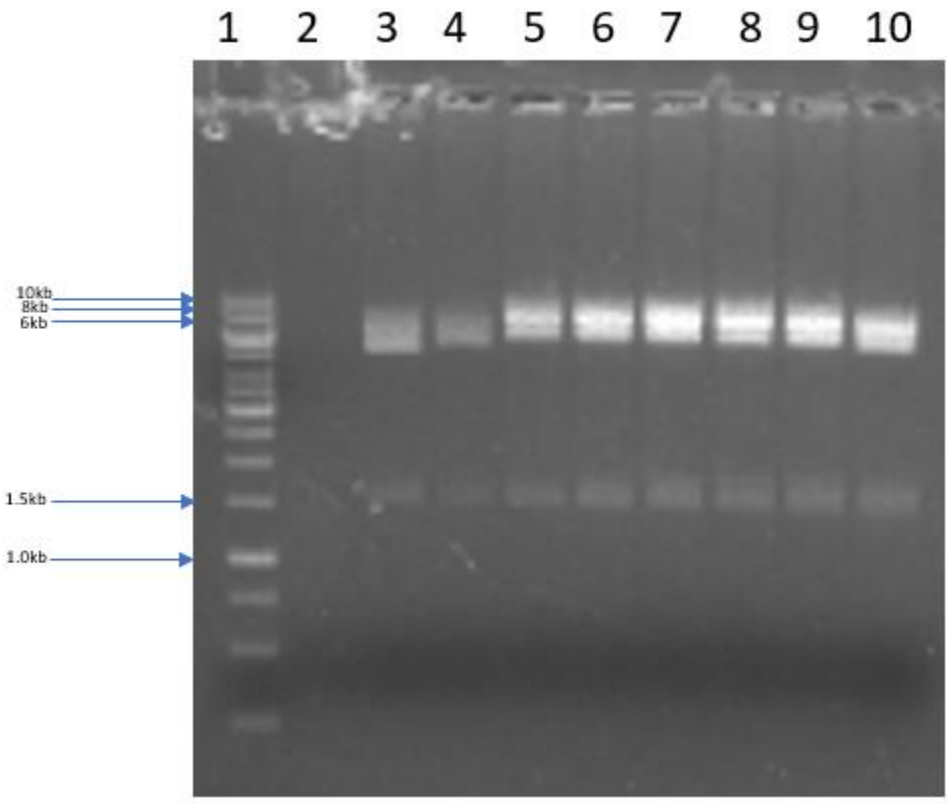

Figure 10) Double digestion confirmation of successful ligation of downstream region to pMo130AprUp backbone. Lane 1: Generuler 1kb ladder. Lane 2: H20. Lane 3: pMo130Apr parent clone. Lane 3: pMo130AprUp Cl. 1 Lane 4-10: pMo130AprUpDn Cl. 1-6. All clones successfully have the expected digestion products of 6,473 base pairs and 1,590 base pairs suggesting successful ligation

Transformation into electrocompetent *A. baumanni* LAC-4

In order to introduce the recombinant pMo130AprUpDn plasmid into *A. baumannii* LAC-4 for RecBCD mediated integration into the bacterial genome, the recombinant plasmid was electroporated at 2.5kV into electrocompetent *A. baumannii* LAC-4 (Fig. 11). Colonies that grew on the transformation plate consisting of LB agar supplemented with 50ug/mL of apramycin were selected to be spot plated on a grid as well as inoculated into liquid overnights of 2mL LB supplemented with 50ug/mL of apramycin.

Figure 11) Transformation of pMo130AprUpDn into electrocompetent A. baumannii LAC-4. Transformation was plated on LB agar supplemented with 50ug/mL of apramycin.

Verification of single crossover mutants

Incubation of the transformation plates and liquid cultures overnight revealed colonies of both white pigmentation as well as bright yellow pigmentation (Fig 12&13).

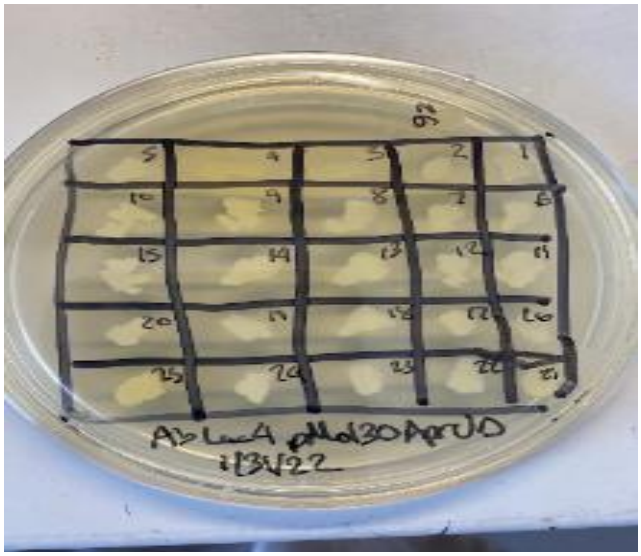

Figure 12) Spot plate transformants of Ab. LAC-4. Successful transformants expressing the xylE reporter gene appear yellow.

Figure 13) Liquid culture transformants of Ab. LAC-4. Successful transformants expressing the xylE reporter gene appear yellow.

The recombinant pMo130AprUpDn plasmid contains the reporter gene xylE, which acts upon catechol-like substrates. This reaction yields a bright yellow 2-hydroxymunoic semialdehyde compound (Lee, 1996). No catechol was added to the plates or media, suggesting that *A. baumannii* LAC-4 can produce its own endogenous catechol-like substrate that xylE can incorporate dioxygen into. This phenomenon allowed for the rapid identification of possible single crossover mutants expressing yellow pigmentation to then be streaked on LB agar supplemented with 50ug/mL of apramycin to be able to isolate single colonies for stress passaging (Fig. 14).

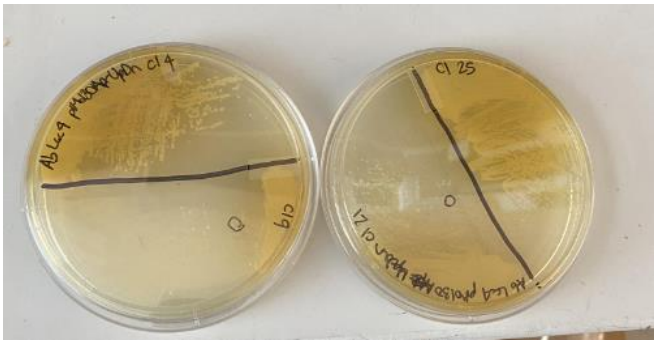

Figure 14) Single crossover transformants streaked for isolation of single colonies. Bright yellow pigmentation is due to the expression of xylE reporter gene.

Passaging and plating for double crossover mutants

A single colony from the streaked out single crossover plates in figure 14 were passaged under 15% sucrose stress in YT media before being plated on YT agar supplemented with 10% sucrose (Fig. 15). After 16 hours of incubation at 30C, some plates still had yellow pigmented colonies, but the majority of the passaged clones appeared to be white in pigmentation. Notice how in the plates that retain the yellow pigmentation are densely clustered whereas plates with good separation between each colony express white pigmentation. It is possible that denser groups of colonies may be able to express the bright yellow color as there is likely a higher concentration of catechol like substrate for xylE to act on.

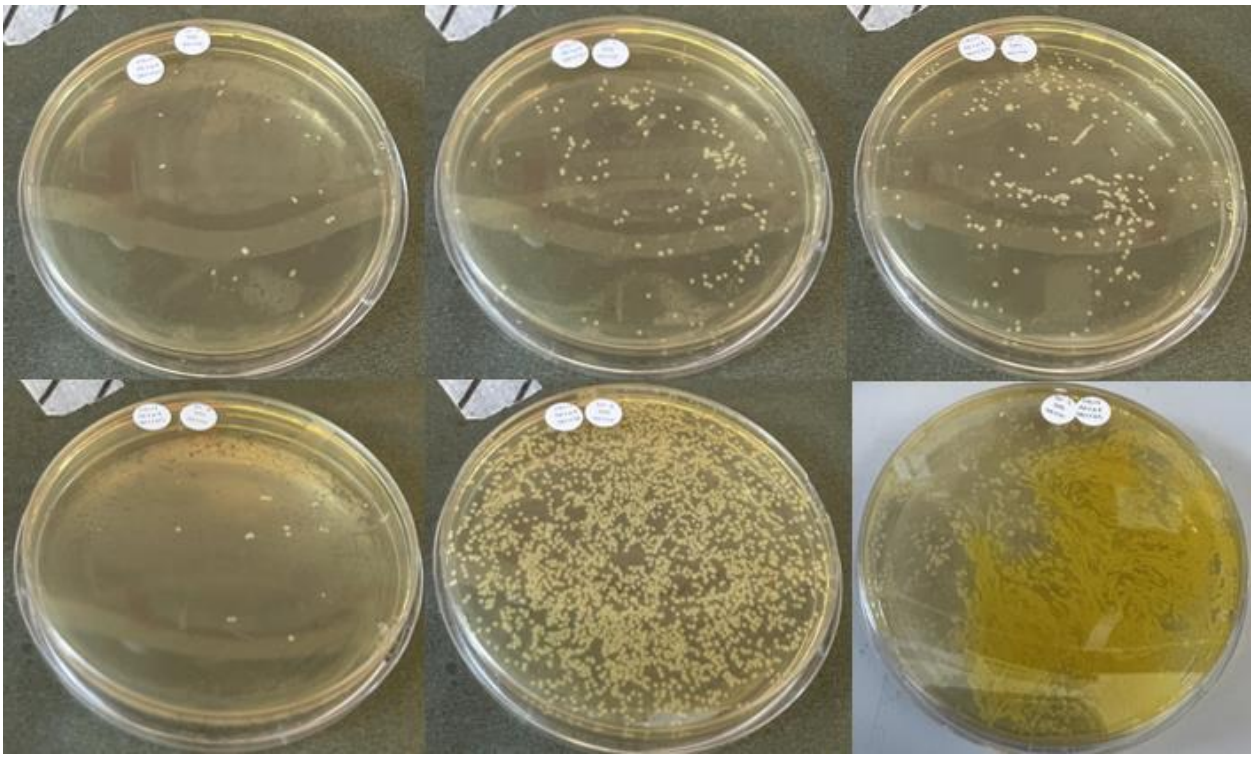

Figure 15) YT plates supplemented with 10% sucrose with double crossover transformants. Yellow pigmentation suggests retention of plasmid and unsuccessful double crossover event. White pigmentation suggest positive double crossover through loss of the target locus and plasmid or reversion to wildtype. Individual clones were spot platted to further confirm double crossover.

To account for this possibility, clones were spot plated on two separate plates. Clones were spot plated on LB agar plates as well as LB agar plates supplemented with 50ug/mL of apramycin as colonies that have reverted to wildtype or have lost the capsule locus alongside the plasmid will only grow on the LB agar plates (Fig. 16). Colonies that were white colored and grew on LB agar but failed to grow on LB agar supplemented with apramycin were identified as possible double crossover knockout mutants or wild type revertant. To further isolation of seamless capsule locus A. baumanni LAC-4 knockout mutants, clones that grew only on LB agar spot plates had their genomic DNA extracted to be subjected to PCR verification of the presence or absence of the capsule locus.

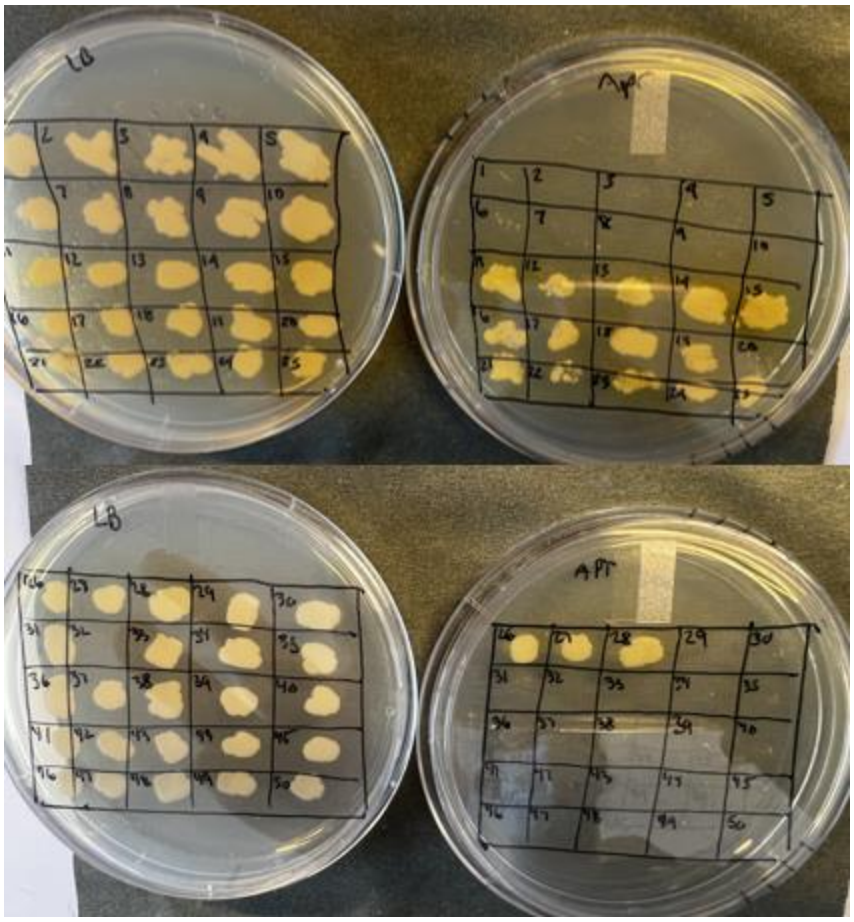

Figure 16) Spot plating of white pigmented colonies on LB agar and LB agar supplemented with 50ug/mL of apramycin. Plates on the left are LB agar medium. Plates on the right are selective LB agar medium. The absence of growth on the corresponding apramycin plate indicates the absence of the apramycin resistance selection marker suggesting reversion to wildtype or a double crossover knockout.

Confirmation of deletion of the capsule gene locus

Four clones were selected from the spot plate of white pigmented colonies to undergo PCR confirmation on if the clones had reverted to wildtype or had their capsule locus knocked out. 6 primer sets (Table. 5) were designed to amplify several regions spanning throughout the whole *A. baumannii* capsule locus. The clones were tested against a positive control in *A. baumannii* LAC-4 wildtype to determine loss of the capsule region. As the wildtype has not been genetically engineered in any fashion, positive PCR signal should be visualized with UV light on

an agarose gel. If reversion to wildtype had occurred, these positive signals would be identical to that of the wildtype whereas a knockout would be confirmed through the absence of signal, suggesting the loss of the capsule locus (Fig. 17).

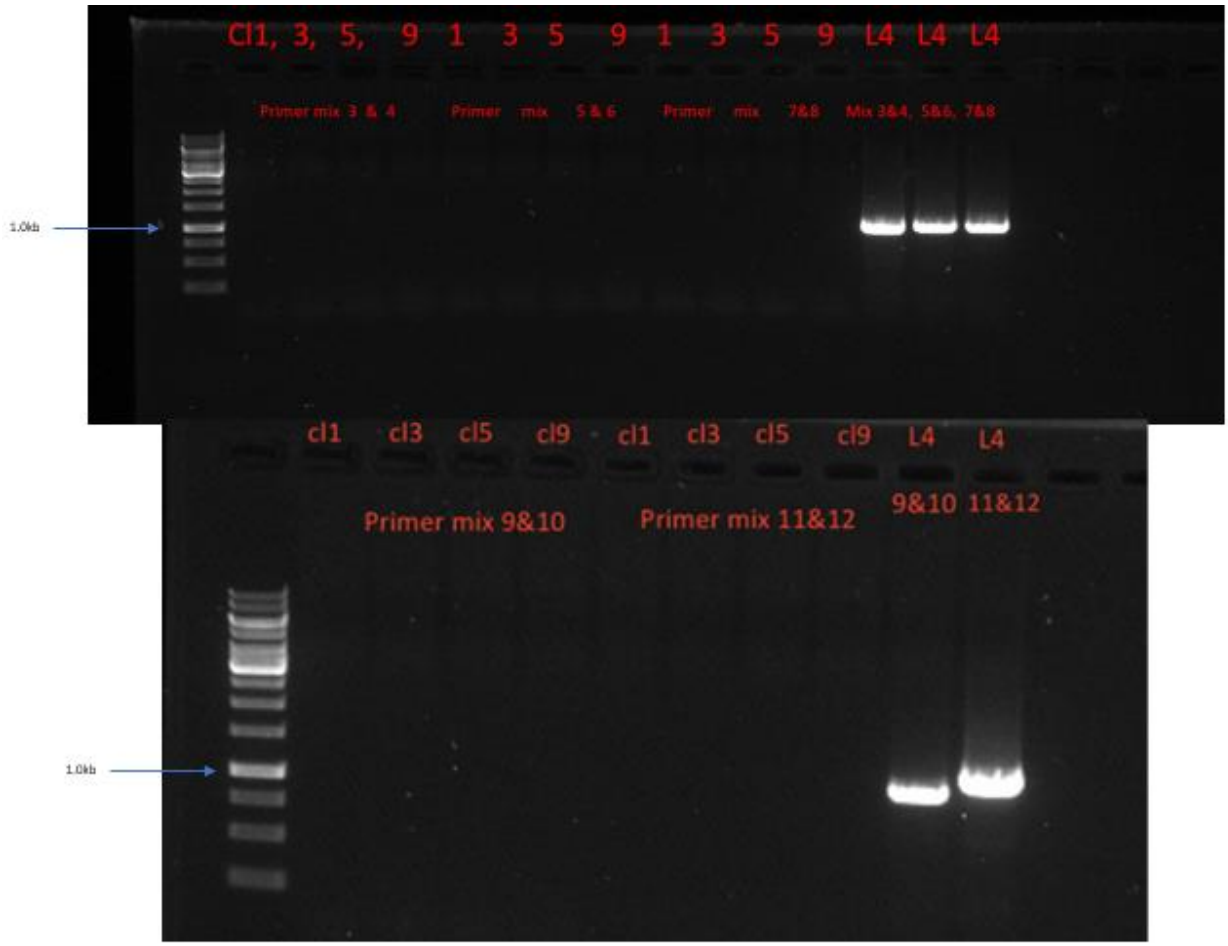

Figure 17) PCR confirmation of capsule knockout of clones 1, 3, 5, & 9 against a wildtype LAC-4 control. Primer sets are as seen in table 5. The absence of signal in the double crossover clones versus the positive signal from the wildtyle LAC-4 suggests the confirmation of complete capsule locus knockout as the primers were designed to span across the whole locus. Images from primer set 1&2 are not pictured.

Percoll density gradient

Nine percoll dilutions ranging from 90% percoll to 10% percoll were carefully created in a 50mL falcon tube and centrifuged 8,000xG for 10 minutes to identify mutants with altered capsular polysaccharide production (Fig. 18).

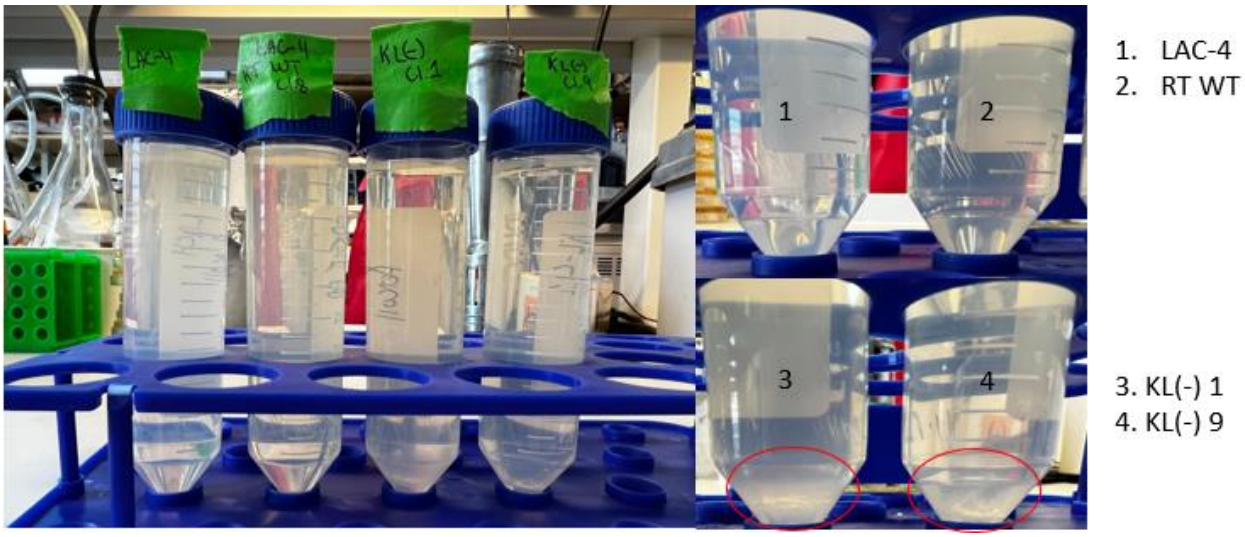

Figure 18) Percoll density gradient results of Ab. LAC-4, revertant wildtype RTWT, and capsule deficient strains KL(-) 1&9. As expected, capsule deficient strains settled at the bottom of the gradient as seen in the red circles.

Density-based separations such as percoll provide use in observation of capsules through the reasoning that a highly capsulated bacteria will take more time to pellet by centrifugation and will then settle at the top of the percoll gradient (Feltwell, 2019). Mutants with altered capsule production such as capsule deficient clones 1 & 9 are shown to be opaque at the bottom of the falcon tubes indicating migration of the bacteria to the bottom layer of the gradient, suggesting the loss of the capsule production. Wildtype LAC-4 and a revertant wildtype identified during double crossover screening coined RT WT do not settle at the bottom of the falcon tube, and instead settle at the top of falcon tube due to matter that the encapsulation of these clones caused them to not pellet at the bottom of the tube given the centrifugation time.

Antimicrobial susceptibility profiling

As *A. baumannii* LAC-4 is a multidrug resistant pathogen that has already gained resistance to commonly used antibiotics and is quickly gaining resistance to last line antibiotics such as the carbapenems (Akoolo 2022), it is important to be able to analyze the observable antimicrobial susceptibility of varying strains of A. baumannii to be able to optimize patient treatment and prognosis. As it has been shown that the capsule of A. baumannii aids in resistance to antibiotics and that mutations in genes responsible for capsule assembly can affect virulence in vivo, it is important to be able to identify how MIC90 values may change due to absence of the capsule in individual strains, especially hypervirulent strains such as LAC-4 (Fig. 19). Not only does this provide information for antimicrobial databanks, but if able to identify antibiotics that are better suited to kill the pathogen when it is unencapsulated, it may be a possibility to create a synergistic therapeutic where the capsule is targeted in conjunction with an antimicrobial agent that is better suited to target the bacteria when it is not encapsulated. Antibiotic concentrations from 250 ug/mL to 1.95 ug/mL were used in these sets of susceptibility testing and several observations were noted. Through loss of the capsular polysaccharide production, *A. baumannii*

MIC 90	Ab. LAC-4	KL (-) Cl. 1	KL (-) Cl. 9	
Chloramphenicol	31.25-62.5 ug/ml	3.90625-7.8125 ug/ml	3.90625ug/ml	⇩
Streptomycin	15.625-31.25 ug/ml	15.625-31.25 ug/ml	1.953125ug/ml	⇩
Colistin	<1.95ug/ml	15.625- 31.25 ug/ml	15.625- 31.25 ug/ml	⇧
Ampicillin	>250 ug/mL	<1.95 ug/mL	<1.95 ug/mL	⇩
Sulbactam	<1.95 ug/mL	31.25 - 62.5 ug/mL	31.25-62.5 ug/mL	⇧
Polymyxin B	125-250 ug/mL	7.8125 - 15.625 ug/mL	7.8125 - 15.625 ug/mL	⇩

Figure 19) MIC90 values of Ab. LAC-4 and capsule deficient strains. Susceptibility to chloramphenicol, streptomycin, ampicillin, and polymyxin B increased as a result of loss of the capsule. Resistance to colistin and sulbactam increased as a result of the loss of the capsule.

LAC-4's susceptibility increased to antibiotics such as chloramphenicol, streptomycin, ampicillin, and polymyxin B. This data suggests the possibility of better outcome of treating unencapsulated *A. baumannii* strains with the preceding antibiotics as well as the possibility of attenuating LAC-4's capsule before treatment with these antibiotics to provide for better prognosis. Interestingly, resistance to sulbactam and colistin increased as a result of the loss of the capsular polysaccharide probing the question between the relationship of the capsule region, LOS, and LPS which colistin is known to target (Sabnis 2021).

Whole blood and serum killing assays

Serum resistance is one of the major virulence contributors of gram-negative pathogens and K1 polysaccharides have been linked to contribute to serum resistance in certain strains of bacteria (Leying 1990). *Acinetobacter* as a species is linked to high serum resistance, with most isolates tested being able to survive a one-hour incubation in 30% serum (Magda, 2022). It is of utmost importance that further strains are tested in regard to whole blood and serum resistance to elucidate if these resistance mechanisms are universal of multiple capsule types or more specific to K1 capsules. A whole blood and serum killing assay was set up in order to investigate if the loss of the capsule in the hypervirulent *A. baumannii* LAC-4 causes a reduction in resistance to whole blood and serum killing (Fig. 20) As expected, wildtype LAC-4 as well as the revertant wildtype were highly resistant to both whole blood and serum killing implying that the capsular type of this strain also portrays similar resistance profile to whole blood and serum complement activity. Of note, keep in mind the axis of LAC-4 and RTWT assay as the fold change at the 2-hour mark are both approximately 1.0 relative to the non-treated samples meaning that there was not substantial killing of the bacteria due to whole blood or serum presence at the 2-hour mark. The opposite is seen when the capsule deficient strains are incubated alongside human whole

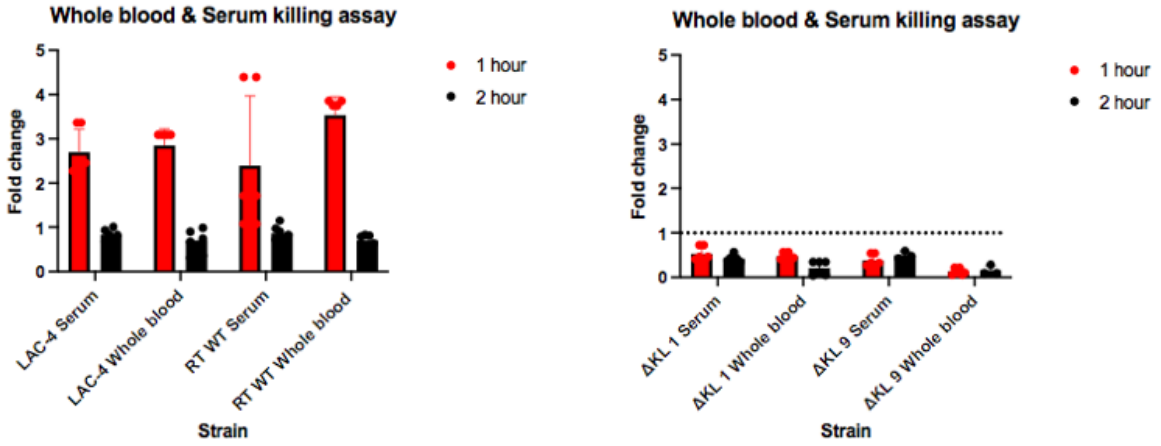

Figure 20) Whole blood & serum killing assay results of LAC-4 WT, RT WT, and capsule deficient strains. Axis is measured by fold change relative to non-treated bacteria. Keep note of the axis of both graphs. LAC-4 and RT WT exhibit high resistance to whole blood and serum killing due to the relative fold change being greater than 1.0 at the one-hour mark and approximately 1.0 at the 2-hour mark. Loss of capsule greatly reduced the resistance of A. baumanii to whole blood and serum as relative fold change compared to non-treated wells is significantly below 1.0 at all-time points.

blood or serum. Capsule mutant clone 1 exhibits a fold change relative to non-treated bacteria of around 0.5 at the one-hour mark and at around 0.4 at the 2-hour mark. After 2 hours of incubation, whole blood significantly reduced bacterial survival when compared to non-treated cells. The same results are seen in capsule mutant clone 9 where at all time points, bacterial survival is significantly under 0.5-fold relative to the non-treated cells.

Neutrophil killing assays

Neutrophils, also known as polymorphonuclear leukocytes, are the most abundant cell type in the human blood. They are one of the important effector cells in the innate immune system as they constantly patrol the blood and tissue looking for signs of microbial infection. When infection is detected, neutrophils quickly respond to kill invading pathogens through three main mechanisms: phagocytosis, degranulation and the release of condensed DNA known as neutrophil extracellular traps (Rosales, 2018). It is also elucidated that gram-negative pathogen such as *E. coli* chemotaxis is modulated by the capsule and the O-specific antigen (Russo, 2003).

To investigate whether the capsule locus of *A. baumannii* LAC-4 may affect neutrophil killing, a neutrophil killing assay was set up in which 200,000 bacteria were incubated with 200,000 neutrophils for 2 hours at 37C with 5% CO2 (Fig. 21). When in the presence of neutrophils,

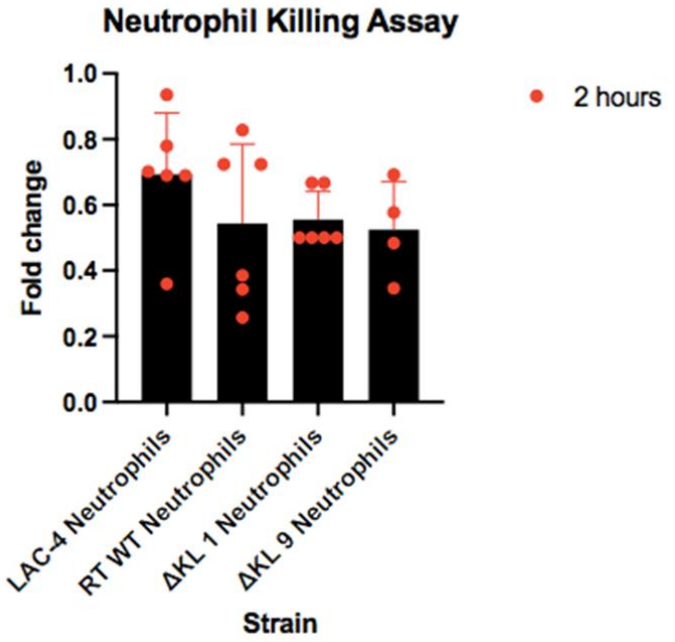

Figure 21) Neutrophil killing assay of LAC-4 WT, RT WT, and capsule deficient mutants. Mean fold change relative to non-treated cells were as follows. LAC-4: 0.69, RT WT: 0.54, KL(-) 1: 0.55, KL(-) 9: 0.52. Mean fold change reduced by .2-fold for the capsule mutants relative to the wildtype; however, the revertant mutant had a similar level of survival in presence of neutrophils when compared to the capsule mutants.

wildtype LAC-4 had a mean fold change of 0.69 relative to the non-treated bacterial cells. The capsule deficient mutant clones 1 & 9 exhibited 0.2-fold less survival relative to wildtype LAC-4. On the other hand, the revertant wildtype displayed similar relative fold change to the capsule knockouts, which may be in part due to a cluster of datapoints at around 0.2-0.4-fold change whereas the first replicate displayed similar activity to LAC-4 WT. This data may have to be replicated as well as further experiments to elucidate differences in neutrophil responses to capsule deficient *A. baumanni* LAC-4 as the differences displayed in these neutrophil killing

assays does not portray a significant change in neutrophil killing between the WT, RT WT, and mutant strains of *A. baumanii* LAC-4.

In vivo virulence assays of the capsule gene cluster knockout

Alongside my post-doctoral mentor Elisabet Bjanes, in vivo infection studies were done to investigate differences in virulence of the capsule deficient mutants when compared to wildtype *A. baumannii* LAC-4. Results showed that the capsule gene cluster mutants had significant reduction in in vivo virulence and bacterial burden in an intraperitoneal systemic infection model. Specifically, mice infected with capsule mutant clones 1&9 had 100% survival and no significant temperature reductions when infected with 1x10^7 CFUs of bacteria. On the other hand, mice infected with 1x10^7 CFUs of LAC-4 had a 10% survival rate and mice infected with 1x10^7 CFUs of the revertant wild type RT WT had 30% survival. Furthermore, mice infected with the wildtype LAC-4 and the revertant mutant, had consistent drop in rectal temperature consistent with *A.baumanii* LAC-4 systemic infection. Mice infected with capsule deficient

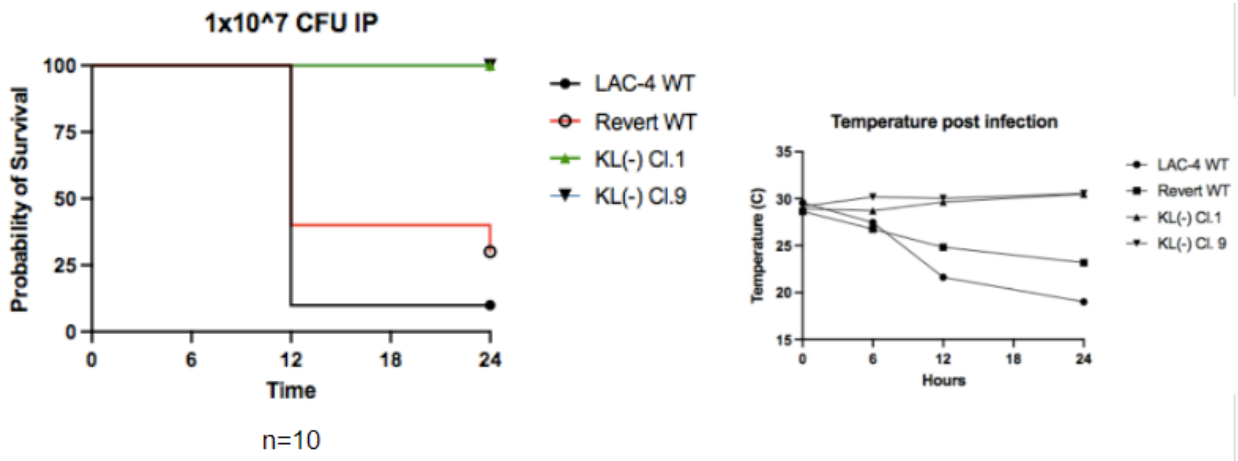

Figure 22) IP infection model with Ab. LAC-4 WT, RT WT, and capsule deficient mutant strains. Survival of mice when infected with 1x10^7 CFU of LAC-4 WT and RT WT were 10% and 30% respectively. There was 100% survival when mice were infected with 1x10^7 CFU of capsule gene locus mutant strains. Temperature of mice infected with LAC-4 WT and RT WT consistently dropped until death, which is consistent with Ab. LAC-4 systemic infection. Mice body temperature fluctuated normally when infected with capsule deficient mutants.

deficient mutant strains had normal fluctuations of body temperature suggesting a great decrease of the virulence of the mutants when in an in vivo systemic infection model. Further, spleen bacterial burden is decreased by 4 log fold in the capsule deficient mutant strains in comparison to the wildtype LAC-4 and revertant strain (Fig. 23). Bacterial burden in the blood is reduced by 5 log fold in the capsule deficient mutant strains when compared to the wildtype LAC-4 and revertant strain.

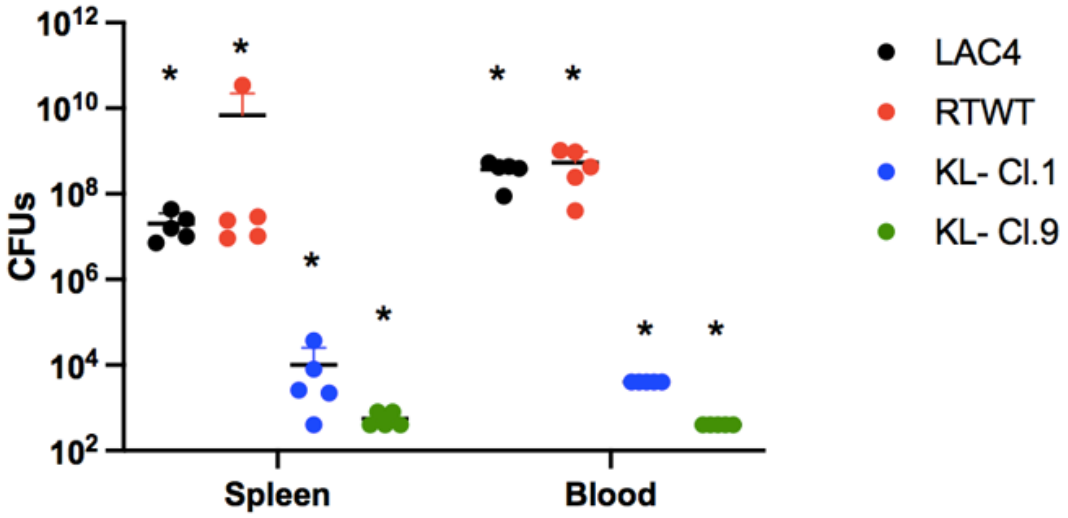

Figure 23) In vivo murine study comparing virulence between wildtype LAC-4 and revertant wildtype RT WT with capsule deficient mutant strains. Bacterial burden in blood and spleen are both significantly decreased with systemic infection of 1x10^7 CFUs of capsule deficient mutants vs 1x10^7 CFUs of WT and RT WT strains.

Chapter 3 is coauthored with Bjanes, Elisabet and Nizet, Victor. The thesis author was the primary author of these chapters.

Chapter 4

Discussion

Increasing prevalence of multidrug resistant pathogens alongside the lack of innovation in developing new antibiotics and therapeutic strategies threatens future generations with increasing difficulty to treat these MDR infections with modern day conventional antibiotics. The continuing rapid accumulation of antibiotics entering the environment will continue to contribute to the problem of antimicrobial resistance unless a change is initiated. Without change in the use of antibiotics and innovation, a large dilemma is left in the hands of the future of the world. The golden age of antibiotics provided the introduction of more than 20 new classes of antibiotics, and since then successful production of antibiotics have come to near halt with just two new classes reaching the market since 1940-1962 (Coates 2011). With this halt in new antibiotics and the hesitance of big pharmaceutical companies to deploy new antibiotics due to economics, it becomes increasingly important to consider non-conventional treatments for MDR organisms such as targeting virulence factors that may help the course of infection by allowing the host immune system to better fight off infection. Targeting virulence factors with a synergistic course of antibiotics may be a beneficial direction to head towards to improve patient prognosis in clearing infection as well as minimizing the introduction of antibiotics to the environment.

The development of ant virulence treatments has been addressed in the past. Specifically, there is conversation about anti-virulence therapeutic strategies targeting bacterial adhesion, bacterial toxins, and quorum sensing (Rasko, 2010). The problem with such strategies is that none of the strategies discussed directly address a bacteria's able to enter the bloodstream, successfully evade the immune system, and thus cause successful bacteremia. There is a

possibility that these strategies are not addressed due to insufficient understanding of bacterial virulence factors causing for evasion of host immune surveillance and even more so in less characterized bacteria like *A. baumanni*. While there is still much to learn, advancements in the understanding of capsular polysaccharides continues to advance providing hope for new antimicrobial targets. Further, the advancement of microbial genomics has proved to be beneficial in provided substantial information on a variety of microorganisms. Through the increased power of genomics and molecular techniques, the characterization of new targets as well as antimicrobial strategies for existing and new targets continue to advance (Pucci, 2006).

In this study, we have hypothesized that *A. baumanii* LAC-4's hypervirulence is partially driven by its unique capsular polysaccharide locus. To then test this hypothesis, we constructed a capsule locus gene locus knockout to assess the capsular polysaccharide's role in LAC-4's hypervirulence. The construct was constructed using a combination of molecular techniques including overlap PCR, Gibson assembly, and restriction enzyme digestion. Through linking an apramycin resistance gene outside of the knockout cassette, the mutant was able to be made unmarked (Oh, 2015). After successful double crossover events, the suicide plasmid containing the knockout cassette was electroporated into LAC-4. The use of the xylE reported gene also proved to be a quick method of distinguishing between successful integration through the means of *A. baumannii* producing its own catechol like substrate. Alongside sucrose-based counter-selection, optimization of selecting for knockouts was reliable and able to repeat. Through use of reporter genes and selective markers, massive colony PCR was able to avoid, thus significantly increasing the quicker identification of a capsule locus mutant.

Through the use of in vitro assays such as minimum inhibitory concentration testing, we were able to elucidate that the capsule polysaccharide of LAC-4 indeed does play a role in

conferring resistance to several antibiotics as well as possibly being a target of antibiotics such as colistin and sulbactam. In vitro whole blood, serum, and neutrophil killing assays showed results that clearly indicated the capsule locus's role in LAC-4's hypervirulence; although, there are likely other factors that also contribute to driving the hypervirulence of the strain. Survival of mice dramatically increased when infected with capsule deficient *A. baumannii* LAC-4 as well as the observation of the mice not appearing to get nearly as ill as those infected with the wildtype strain. It is indicative that without the capsule locus, which also includes several key metabolism genes, the bacteria may not be able to grow in high numbers in the spleen and blood before being detected and disseminated by the host immune system. Further, LAC-4's capsule locus contains a gene locus leg1-6 coding for enzymes responsible for the biosynthesis of a rare sugar legionaminic acid. We propose that legionaminic acid, due to its structural similarities to sialic acid, which sits on mammalian cell surface glycoprotein, allows for bacteria possessing this locus to effectively evade host immune surveillance through interacting with sialic acid-binding immunoglobulin-type lectin receptors at the host-pathogen interface thus causing down activation of innate immune cells such as macrophages (Chang & Nizet 2020).

While the preceding results do not provide a definitive mechanism for explaining the capsule locus's involvement in LAC-4's hypervirulence, the results do confirm that the capsule does play a pivotal role in contributing to LAC-4's virulence. The capsule deficient mutants of *A. baumannii* LAC-4 definitively show significant reduction in virulence and this research sets the groundwork for additional research in our lab and other labs to continue investigations to elucidate a mechanism as to how the capsule locus of LAC-4 contributes to hypervirulence. While focused on *A. baumannii* LAC-4, this research also provides framework for investigation on other multidrug resistant pathogens with homologous capsule loci or specific clusters within

the locus. The rise of multidrug resistance, but this framework provides a set foundation for future experimentation to help understand the mechanisms of virulence of highly pathogenic bacteria in hopes to develop new therapeutic strategies.

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