IDENTIFICATION OF COLISTIN RESISTANCE MECHANISMS IN *PMRC-*, *PMRA-*, AND *PMRB*-DEFICIENT *ACINETOBACTER BAUMANNII*

by

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ABSTRACT

Acinetobacter baumannii is a menacing nosocomial pathogen that readily develops resistance to antibiotics. Colistin, a polymyxin, is currently the last-line choice for treating infections caused by multidrug-resistant *A. baumannii*. This cationic peptide is attracted to the negatively charged lipopolysaccharide (LPS) on Gram-negative bacterial membranes and acts by destabilizing and permeabilizing it. Unfortunately, there is a disturbing trend of strains developing resistance to colistin in recent years. *A. baumannii* is known to modify its LPS in order to interrupt the initial charge interaction with colistin. The most common mechanism implicated in this resistance strategy is the upregulation of the *pmrAB* genes encoding a two-component regulatory system, which in turn upregulates *pmrC* that encodes phosphoethanolamine transferase. PmrAB have also been shown to be global regulators of cellular growth processes and virulence. Gain-of-function mutations in *pmrAB* are the most commonly reported colistin resistance mechanism. However, it is not known if and how *A. baumannii* strains deficient in any of the *pmrCAB* genes develop colistin resistance. We therefore aim to determine secondary mechanisms of colistin resistance in *pmrCAB*-deficient strains.

Using *A. baumannii* strain AB5075 and inactivation mutants of *pmrC, -A,* and *-B*, we generated genetically stable colistin-resistant mutants on agar containing increasing colistin concentrations. We conducted whole genome sequencing to identify genetic changes responsible for colistin resistance. We also characterized their phenotypes, including resistance profiles to Gram-positive antimicrobials, LPS structure, *in vitro* growth fitness, and *in vivo* virulence using a *Galleria mellonella* model.

Colistin-resistant mutants possessed mutations in *lpx* and *mla* genes, which are involved in lipid biosynthesis and membrane composition, respectively. Most mutants were susceptible to Gram-positive antimicrobial agents, likely resulting from severely compromised outer membranes. Furthermore, colistin-resistant mutants displayed reduced fitness *in vitro* and decreased virulence *in vivo*.

A. baumannii is adept at evading colistin by modifying its membrane structure, even outside of the control of *pmrCAB*. However, mutations that disrupt membrane integrity are costly and unlikely to persist in clinical settings. Therefore, colistin-resistant mutants with such mutations seemingly carry low public health significance. Even if these mutants do emerge in the clinic, various Gram-positive agents can be included as treatment options.

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PREFACE

This project would never have come to be without the help and support of many fantastic scientists. I would first and foremost like to thank my PI, Dr. Yohei Doi, for allowing me to complete this project in his lab in conjunction with our regular research. His expertise in antibiotic resistance and *Acinetobacter baumannii* were invaluable guides throughout this process. Next, I would like to thank my advisor, Dr. Robbie Mailliard, for always having an open door and being a wonderful educator. Additionally, I would like to thank Mrs. Christi McElheny, M.S., for teaching me how to be successful in the lab and Daniel Evans and Marissa Pacey for all of their genomics expertise. I also thank Dr. Vaughn Cooper's laboratory for the Illumina sequencing and Dr. Robert Ernst's laboratory for the MALDI-TOF spectra.

Last, but certainly not least, I would like to thank my loving parents, Susan Kushner and Ronald Mettus, for encouraging me, challenging me, and giving unending support in all my schooling over the course of my life.

1.0 INTRODUCTION

1.1 THE RISE AND FALL OF ANTIBIOTICS

1.1.1 Discovery of Antibiotics

The discovery of penicillin by Alexander Fleming, F.R.C.S, in the early 20th century was a happy accident that would forever alter the way physicians treated infectious diseases. He made this discovery by observing mold that grew on plates left on the bench for too long and produced a compound that inhibited growth of *Staphylococcus aureus*, and it was later given the name penicillin [1]. The next few decades saw the heyday of antibiotics; during these years, scientists isolated dozens of compounds produced naturally by environmental bacteria, especially those found in soil [2]. After this key discovery, antibiotics were heralded as a panacea for treating bacterial infections. While these drugs initially saved countless lives, their long-term success was limited. Unfortunately, with the debut of each successive class of antibiotics, resistant bacteria emerged within a few years of first clinical use, rendering the drugs useless [3].

1.1.2 Antibiotic resistance

Evolution and natural selection are the driving forces behind antibiotic resistance. Random genetic mutations naturally occur as bacteria replicate, and natural selective pressure from antibiotics favors mutations that confer resistance. Moreover, horizontal transfer of mobile elements that carry resistance genes also permit the spread of antibiotic resistance among bacteria.

As a result, resistant bacteria that survive in the presence of antibiotics grow into the dominant population [4]. As resistance becomes more common, infections that were once easily treated are reemerging, causing a significant public health crisis. In the United States alone, it is estimated that over 2 million people developed antibiotic-resistant bacterial infections in 2013, as documented in the CDC's most recent *Antibiotic Resistance Threats in the United States*. Overuse in clinical and agricultural settings are contributing to this alarming trend [4]. Moreover, multidrug-resistant (MDR) strains, defined as those resistant to at least three antibiotic classes, are responsible for a significant spike in the number of healthcare-associated infections.

These staggering statistics place a tremendous burden on the health care system and endanger patients' lives [5]. As antibiotic resistance spreads, critically ill patients with severe bacterial infections have few or no treatment options. There has not been a new class of antibiotics discovered in nearly 20 years [6]. Lawmakers are only now beginning to write legislation limiting their agricultural use, and hospitals are developing stewardship protocols that aim to mitigate careless overprescribing by healthcare providers. Nevertheless, experts warn that we may be headed toward a post-antibiotic era in which MDR "superbugs" cause infections that are not easily controlled [7]. Therefore, there is an urgent need to understand the mechanisms responsible for resistance in order to devise new treatment methods.

1.2 ACINETOBACTER BAUMANNII

One of the most threatening bacteria causing these MDR infections is *Acinetobacter baumannii*. This Gram-negative, non-motile, aerobic, coccobacillus is responsible primarily for opportunistic respiratory tract infections but has also been isolated from blood cultures, central lines, wounds,

and other sites [8-10]. *A. baumannii* is a naturally tenacious bacterium; it possesses many intrinsic resistance genes, including ones against penicillins, cephalosporins, and other β-lactams [11].

Furthermore, the cell membrane of *A. baumannii* is a crucial defense against antimicrobials. Normally, the lipid A moiety in the outer membrane is hexa-acylated. However, several varieties of this structure also exist. In particular, the hepta-acylated form of lipid A has been shown to provide extra protection against certain antibiotics, including colistin [12]. In hospital settings, *A. baumannii* is extremely difficult to eradicate from the environment. Thanks to its membrane, it can survive for extended periods of time on dry surfaces and is not susceptible to hospital disinfectants, facilitating nosocomial transmission [13]. Additionally, it has a propensity for acquiring resistance mechanisms through horizontal gene transfer and for evolving resistance through nonsynonymous mutations. Altogether, these factors make it a problematic organism in hospital and healthcare settings [9, 10].

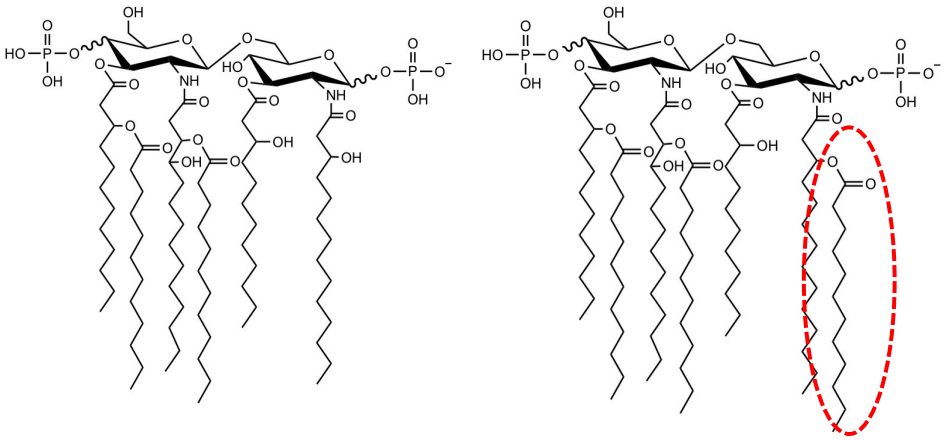

Figure 1. Hexa-acylated lipid A (left) and hepta-acylated lipid A (right)

The extra acyl group on the hepta-acylated lipid A is circled in red.

1.2.1 Colistin therapy

Nowadays, clinicians have fewer and fewer options for treating infections from MDR pathogens such as *A. baumannii* due to the emergence and spread of antibiotic resistance genes. For some time, physicians employed a highly potent β-lactam class called carbapenems against *A. baumannii* infections. While carbapenems initially seemed to be unaffected by resistance mechanisms that targeted other β-lactams, several globally distributed clones of *A. baumannii* developed carbapenem resistance in short order [14].

As a result, physicians have resorted to employing antibiotics that had been shelved decades ago, including colistin. Colistin, also known as polymyxin E, is a large, cationic polypeptide produced by the soil bacterium *Bacillus polymyxa.* The polymyxins were discovered in the 1940s. These large, amphipathic molecules interact with the negatively-charged lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria [15]. Colistin is a bactericidal agent that acts as a detergent, destabilizing the membrane and leading to catastrophic leakage and eventually death of the target cell [16]. It is administered to patients intravenously or by inhalation as a prodrug called colistimethate sodium. This prodrug in itself is inactive but is then hydrolyzed after administration to form the active colistin [17]. For nearly two decades, colistin was sidelined in clinical use due to high rates of nephrotoxicity and neurotoxicity [18]. In spite of these undesirable side effects, the use of colistin has increased in recent years for the treatment of severe MDR infections. Thus, it has become a drug of last resort when infections caused by highly resistant nosocomial pathogens, such as *A. baumannii* and *Pseudomonas aeruginosa*, do not respond to other therapies [18].

1.3 EMERGING COLISTIN RESISTANCE MECHANISMS IN *A. BAUMANNII*

Unfortunately, even the effectiveness of colistin is in peril. In fact, in the 2006-2009 SENTRY report from the Antimicrobial Surveillance Program, fewer than 2% of *A. baumannii* isolates tested were colistin-resistant [19]. Within a few years, the percentage of resistant strains rose to over 5% and continues to climb [20]. With *A. baumannii* infections that are already resistant to other antibiotics, emerging colistin resistance leads to treatment failure [21]. The demise of treatment options for MDR *A. baumannii* has earned this pathogen a spot on the World Health Organization's list of Priority Pathogens, those for which there is an urgent need to research and develop new treatments [22].

Several of the molecular bases of colistin resistance have been identified and studied in recent years. In addition to acquiring plasmid-mediated resistance genes, *A. baumannii* also readily develops colistin resistance via genetic mutations from selective pressure from *in vitro* and *in vivo* drug exposure [23, 24]. The genomes of certain *A. baumannii* strains are riddled with mobile genetic elements. Elements that contain genes in them other than transposase genes in them are called transposons, and ones that do not are called insertion sequences. The DNA sequences of insertion sequences can be spliced into existing genes in the bacterial chromosome to disrupt the functions of the genes which they interrupt [25]. Insertion sequences are often flanked by inverted repeats of DNA bases that facilitate random splicing into other genes. Oftentimes, *A. baumannii* insertion sequences, such as IS*Aba1* and IS*Aba11* interrupt genes that play crucial structural roles, and mutated forms of these genes result in structural variations [26]. On the other hand, non-synonymous single nucleotide polymorphisms (SNPs) in these key genes can also alter their functions.

1.3.1 Colistin-resistance mediated by the *pmrCAB* gene cluster

The most prominent, well-defined mechanism of colistin resistance in *A. baumannii* involves modification of colistin's target, the outer membrane [27]. The PmrAB two-component regulatory system, encoded by the *pmrCAB* gene cluster, is responsible for these changes; mutations in *pmrA* or more commonly *pmrB* have been implicated in colistin resistance in *A. baumannii* [28]. PmrB is a sensor histidine kinase that senses environmental conditions including pH, Fe^{3+} and Mg^{2+} concentrations, and the presence of certain antibiotics. Then, PmrB phosphorylates PmrA, a response regulator that controls transcription of *pmrC* [29]. Other literature indicates that PmrB is also suspected to be a global regulator of other cellular growth processes [30]. Certain gain-of-function mutations in *pmrA* and *pmrB* have been shown to increase expression levels of *pmrC*, which encodes a phosphoethanolamine transferase, PmrC, that adds phosphoethanolamine to the lipid A component of LPS [28, 31]. This lipid A modification disrupts the charge interaction between the negatively charged bacterial outer membrane and positively charged colistin and allows *A. baumannii* to survive despite the presence of high concentrations of colistin.

1.3.2 Colistin resistance mediated by *lpx* genes

Another strategy that *A. baumannii* uses to curtail the destructive effects of colistin is halting LPS production completely. Nine enzymes are involved in the biosynthesis of lipid A, the lipid component of LPS also known as endotoxin [32]. Deleterious mutations in any of the first three (LpxA, LpxC, or LpxD) have been associated with colistin resistance [27]. The enzyme UDP-*N*-acetylglucosamine acyltransferase is encoded by LpxA and catalyzes the first step in lipid A biosynthesis. If it is defective, the resulting mutant *A. baumannii* produces no lipid A and thus no

LPS in contrast to wild-type *lpxA* strains. As in the case of lipid A modification from elevated PmrC levels, the absence of LPS prohibits the charge interaction between colistin and the bacterial membrane, leading to colistin resistance [27].

1.3.3 Role of *mla* genes in Gram-negative membrane structure

In addition to mutating the outer membrane, *A. baumannii* has also been known to alter its inner membrane when challenged with colistin. Typically, LPS makes up the outer membrane while phospholipids exist only within the inner membrane. This balance is essential for proper barrier function in Gram-negative bacteria. Recently, a few groups have examined phospholipid synthesis pathways that may play a role in colistin resistance [33-35].

The Mla pathway prevents phospholipids from accumulating in the outer membrane. MlaA is an outer-membrane associated protein, and MlaC has been identified as the protein that shuttles stray phospholipids across the periplasmic space back to the inner membrane [33]. The functions of MlaB, -D, and -F are less clear, but mutated versions of these proteins have been linked to colistin resistance. Furthermore, when colistin-resistant strains emerge that lack LPS, the entire Mla system is upregulated to compensate for the faulty outer membrane [34]. This system may work independent of or in conjunction with the Lpx pathway to affect colistin resistance.

1.3.4 Fitness trade-offs associated with colistin resistance

Although *A. baumannii* possesses a wide variety of mechanisms that contribute to colistin resistance, deleterious mutations in membrane components often are accompanied by fitness-tradeoffs. Mutations in the PmrAB system confer a lower affinity for colistin, but these mutations

ultimately prove to be maladaptive. In *in vitro* growth experiments, the growth rates between wild-type *A. baumannii* and derivative *pmrB*-deficient strains do not differ substantially until around 72 hours. On the other hand, bacterial loads in mice show logarithmic differences within the first 24 hours with the wild-type strain displaying the higher tissue burden [36]. In other words, the deleterious effects on fitness of colistin resistance mutations in the PmrAB system are magnified *in vivo*. Furthermore, the virulence is greatly diminished in strains with *pmrB* mutations compared to wild-type strains. While the resulting mutant strains may be quickly eliminated by the host immune response at first, subsequent adaptive mutations may accumulate that compensate for the reduced fitness, allowing the infection to come to clinical fruition [36].

In contrast to clinical isolates recovered that possess mutations in the PmrAB system, mutations that directly compromise membrane integrity are related to lower fitness. Lpx mutants exhibit significantly lower growth rates when compared with wild-type strains within the first 24 hours of growth assays, unlike PmrAB mutants. Notably, however, both groups of mutants are outcompeted when grown in the same culture as their respective wild-type strains. Additionally, Lpx mutants are appreciably less virulent in *in vivo* experiments; infections with these strains rarely result in death in infected mice in comparison with wild-type infections [37]. There appears to be a much larger biological cost to mutations that affect the Lpx system than those that affect the PmrAB system. *A. baumannii* strains that have mutations in the Mla pathway also experience hindered growth and are less virulent than wild-type strains [38].

1.3.5 Colistin resistance in *pmrCAB*-deficient *A. baumannii*

Notwithstanding the large body of research that has been conducted on colistin resistance in *A. baumannii*, there is a dearth of information regarding the way that PmrCAB-deficient strains

respond when challenged with colistin. We report the results of generating colistin-resistant strains derived from *A. baumannii* strain AB5075 and three transposon-inactivated mutants with defective *pmrC, -A, or -B*. We used comparative genomic analyses to identify mutations in the *lpx* and *mla* genes that arose after exposure to colistin *in vitro*. We then characterized these strains phenotypically with growth curves, testing with antimicrobial agents other than colistin to elucidate the global impact of outer membrane perturbation on antimicrobial susceptibility, and analyzing the lipid structure of their membranes by MALDI-TOF mass spectrometry. Finally, consistent with the existing literature, these colistin-resistant mutants appeared to experience significant fitness costs due to their colistin resistance; they were less virulent than their colistin-susceptible parent strains. This study corroborates reports that colistin-resistant strains that have severe defects in their membrane components do not persist *in vivo* and are thus unlikely to present a clinical challenge. However, and importantly, we also demonstrate that the presence of the PmrCAB system is not a prerequisite for *A. baumannii* to develop colistin resistance. While PmrCAB may affect and regulate many cellular growth processes, colistin resistance may develop independently outside of its control.

2.0 STATEMENT OF THE PROJECT

Colistin-resistant *A. baumannii* strains have achieved global notoriety for the deadly, difficult-to-treat infections that they cause. The resistance mechanisms that confer colistin resistance are mechanistically complex. Resistance mediated by *pmrCAB* is the best-characterized mechanism among clinical strains in the literature, but it is known that mutations in *lpx* or *mla* genes may also play a role. Nevertheless, the associations between these three gene clusters has never been characterized. For this project, we obtained a wild-type *A. baumannii* strain and derivative transposon-inactivated mutants of *pmrC, pmrA,* or *pmrB* and evolved colistin-resistant mutant strains. In doing so, we determined that strains deficient in *pmrCAB* are able to develop colistin resistance. We identified the secondary genetic mechanisms responsible for resistance in those strains. We characterized the strains phenotypically, examining their fitness and virulence, in order to observe the other cellular processes that are affected by genotypic changes that confer colistin resistance.

2.1 AIM 1: GENERATE COLISTIN-RESISTANT MUTANTS FROM A. BAUMANNII 5075 AND THE DERIVATIVE PMRC, -A, AND -B-INACTIVATED STRAINS

This aim serves as the basis for the project and provided the bacterial strains used to carry out the study. Previous literature indicates that exposure to colistin drives evolution of resistant strains via genetic mutations, and thus we aimed to replicate this in the laboratory [24]. We generated resistant mutants both from the wild-type parent strain 5075 as well as from the mutants, 2238, 2241, and

2245, that have one of the three genes of *pmrCAB* disrupted. We demonstrated that having a functional *pmrCAB* is not necessary for developing colistin resistance in *A. baumannii*. We were able to compare genetic changes between the parent strain and the mutants to determine whether having *pmrCAB* intact has an effect on which mechanism allows the strains to prevail. Colistin-susceptible *A. baumannii* strains have minimum inhibitory concentrations (MICs) in the range of 0.25–2 μg/mL; therefore, we isolated resistant mutants with colistin MIC of ≥ 4 μg/mL [39].

2.2 AIM 2: CHARACTERIZE GENOTYPES AND PHENOTYPES OF RESULTING RESISTANT MUTANT STRAINS

Here we investigated the underlying genetic mechanisms responsible for colistin resistance in the mutant strains and also linked the genotypes with resulting phenotypic changes. The genotypic characterizations were addressed as follows in the sub-aims:

1. First, we conducted comparative genomic analyses on whole genomes to identify differences in the genomes of the paired susceptible and resistant strains. We compared non-synonymous SNPs and looked for insertions, deletions, and insertion sequences that may disrupt genes.
2. We looked for common loci where mutations occurred and investigated the cellular processes in which these genes are involved.

Once the sequences of these mutations were confirmed, we performed a thorough phenotypic characterization of the mutant strains and compared them with the strains from which they were derived. The below sub-aims describe the phenotypic characterization experiments:

1. Cell morphology was assessed by Gram stain. This indicates whether the structure of the cell is compromised as a result of mutations.
2. The strains' antibiogram, or their susceptibility profile to a panel of antibiotics, was tested. This demonstrated whether the mutations have effects on antibiotics other than colistin.
3. The strains were also subjected to MALDI-TOF mass spectrometry in order to define the composition of the lipids in their outer membrane. A peak at a *m/z* of 1728 represents normal hexa-acylated LPS, and that at *m/z* of 1911 indicates hexa-acylated LPS [40].

2.3 AIM 3: DEFINE TRADEOFFS THAT EXIST BETWEEN RESISTANCE, FITNESS, AND VIRULENCE IN THE COLISTIN-RESISTANT MUTANT STRAINS

Evolving colistin resistance typically involves structural modification in bacteria. Oftentimes, the resulting resistant phenotype can impose significant biological costs on the organism when it compromises vital parts of the cell. Therefore, resistance may come at a significant fitness cost that prevents the affected strains from establishing infection in a human host. Moreover, resulting resistance may be accompanied by "collateral susceptibility" to antibiotics that typically do not have any antimicrobial activity against Gram-negative bacteria due to their inability to penetrate the outer membrane [41].

For *A. baumannii* in particular, many of the known colistin resistance mechanisms involve modification of the lipid A component of LPS on the outer membrane. LPS is the endotoxin which is responsible for eliciting an immune response through its interaction with Toll-like receptor 4

[42]. *A. baumannii* strains with absent or compromised LPS are severely attenuated in many types of *in vivo* models [37]. We aim to model these fitness and virulence tradeoffs using *Galleria mellonella*, the caterpillar of the greater wax moth. This insect serves as an acceptable organism for modeling many aspects of human infections. It has both a cellular and humoral immune response; the former is mediated by at least six different types of hemocytes and the latter by opsonins, antimicrobial peptides, and a fibrous matrix that serves to trap bacteria [43]. We used *G. mellonella* because they are inexpensive and do not raise ethical concerns.

3.0 MATERIALS AND METHODS

3.1 STRAINS USED

We obtained *A. baumannii* strain AB5075-UW from the Manoil Laboratory at the University of Washington (UW). This well-characterized clinical strain was first isolated at Walter Reed Army Medical Center in 2004. It is a multidrug-resistant strain that accurately captures recent trends of resistance in clinical strains, including resistance to carbapenems; notably, however, it remains susceptible to tetracycline and hygromycin, making it suitable for genetic manipulations in laboratory settings [44]. AB5075-UW is also susceptible to colistin (MIC = 0.5 μg/mL). The Manoil Lab has curated a transposon-mutant library derived from AB5075-UW, and from it we also procured three mutants, each with a tetracycline-resistance (Tet-R) cassette disrupting and thus inactivating *pmrC, pmrA,* or *pmrB*, as shown in Figure 2 below [45]. The mutants were designated 2238 (*pmrC* disrupted), 2241 (*pmrA* disrupted), and 2245 (*pmrB* disrupted), and the parent was designated 5075.

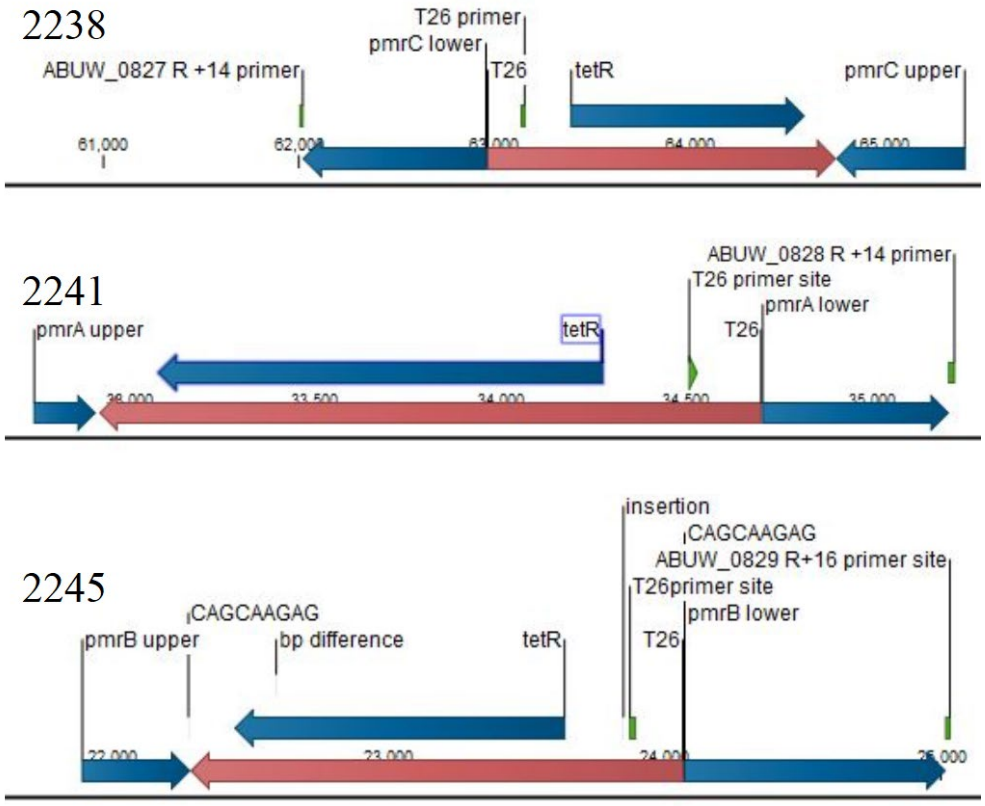

Figure 2 Transposon-inactivated mutants

The lower blue arrows represent *pmrC, pmrA,* and *pmrB.* They are split into upper and lower parts due to the transposon insertion. Red arrows represent T26 inserted into *pmrC* (2238), *pmrA* (2241), and *pmrB* (2245). The direction and position of the *tetR* gene are also indicated by the blue arrows above the genes.

3.1.1 Assessment of baseline colistin-susceptibility of University of Washington (UW) strains

These four strains were subjected to broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) standard methods to determine their baseline colistin MIC and to examine whether there was a dose-dependent inhibition by colistin. Briefly, a 0.5 McFarland solution of each strain was prepared in 0.85% NaCl. The solution was diluted 1000-fold into cation-adjusted Mueller Hinton broth. Colistin sulfate salt (Sigma-Aldrich Corp., St. Louis, MO,

USA) was prepared as a 10mg/mL solution in dH_2O and diluted in cation-adjusted Mueller Hinton broth. Then, 50 μL of colistin was serially diluted in a 96-well plate, and 50 μL of bacterial inoculum was added to each well. Plates were incubated with plastic lids overnight at 35°C and growth was observed the next day. The strains were tested in biological triplicate, and the OD_{600} measuring growth in each well was read using a Tecan Plate Reader Infinite 200 PRO (Tecan Life Sciences, Switzerland).

3.2 MUTANT GENERATION AND SELECTION

Colistin-resistant mutants were derived from 5075, 2238, 2241, and 2245, as diagramed in Figure 3 below. A culture of each was grown in lysogenic broth (LB) for approximately 4 hours until a density of 1.8 x 10^9 cfu/mL was achieved (Step 1). Then, 200μL of each cell suspension was plated on LB plates containing 4 μg/mL or 8 μg/mL of colistin (Step 2). The mutation rates for strains of *A. baumannii* have been shown to range from 2.6×10^{-9} (95% CI: 3.43×10^{-10} to 6.37×10^{-9}) for the wild-type ATCC 19606 (a susceptible control strain) to 2.1×10^{-6} (95% CI: 2×10^{-6} to 3×10^{-6}) for hypermutator MDR strains [46]. After overnight incubation at 35°C, colonies that grew were subcultured on an LB plate with the same concentration of colistin as the plate from which it was originally isolated (Step 3). In order to verify that mutants were genetically stable, the strains were then passaged 4 times on LB plates without colistin and grown overnight at 35°C (Step 4a). The resulting strains were then tested by broth microdilution according to CLSI standards (Step 5). Strains that had an MIC of 4 μg/mL or greater were saved and subjected to further testing, detailed in this project. The colonies originally isolated on plates with colistin 4

μg/mL and 8 μg/mL were also serially passaged on plates containing two-fold higher amounts of colistin until growth on a plate with 64 μg/mL was achieved (Step 4b). These strains were also then passaged four times on plain LB plates and their MICs were determined by broth microdilution (Step 5). Strains with MICs of ≥ 128 μg/mL were saved at -80°C and further investigated as well. Mutants were generated in biological triplicate. There were two pairs of isolates which initially had a colistin MIC of 4 or 8 μg/mL and then after serial passaged on colistin plates had an MIC of ≥ 128μg/mL, and they were included in the subsequent investigation.

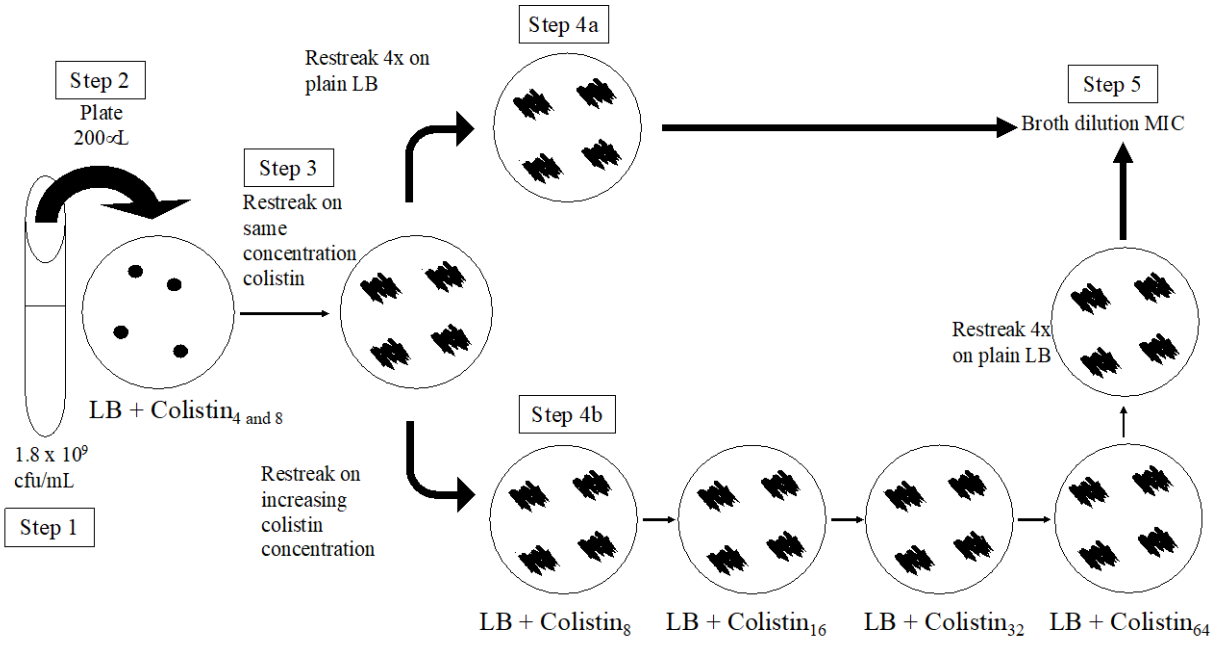

Figure 3. Diagram showing the process of colistin-resistant mutant generation

3.3 MUTANT CONFIRMATION BY PCR

In order to confirm that the resulting colistin-resistant mutant strains were *A. baumannii*, we performed polymerase chain reaction (PCR) with primers specific for β-lactamase gene $bla_{\text{OXA-51}}$ and Choice Blue Taq (Thomas Scientific, Swedesboro, NJ). [47]. This β-lactamase gene is intrinsic to the chromosome of *A. baumannii* species. Furthermore, in the University of Washington strains that had *pmrC, pmrA,* or *pmrB* disrupted by the transposon containing *tetR*, the presence of the transposon (T26 pgro-172) in the colistin-resistant mutants was verified using primers designed to amplify it as well as part of the gene that it disrupts. The primer binding sites are shown in Figure 2 above.

Table 1. Primers used for UW strain verification

	Primer name	Primer sequence (5′→3′)	Tm (°C)
	OXA51 U	AACAAGCGCTATTTTTATTTCAG	50.3
	OXA51 L	CCCATCCCCAACCACTTTT	55.4
	T26 Pgro-172	TGAGCTTTTTAGCTCGACTAATCCAT	56.2
(2238, *pmrC* disrupted)	ABUW_0827 R (+14)	GTTCTAGGCTCGCTTTAGTTTAC	53.3
(2241, *pmrA* disrupted)	ABUW_0828 R (+14)	CCCGAAATTTTAAATTATG	41.8
(2245, *pmrB* disrupted)	ABUW_0829 R (+16)	CAAGAGCTTACGTAATCACGC	53.8

3.4 COMPARATIVE GENOMIC ANALYSES

Genomic DNA from the four UW strains and the colistin-resistant mutant strains derived from them were extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD). Extracted gDNA was sequenced using Illumina MiSeq 250-bp paired-end sequencing. The mutants' genomes were mapped against the complete reference genome of *A. baumannii* AB5075-UW (Genbank accession no. CP008706.1). This genome was annotated with Prokka. SNPs were

determined using the Breseq mapping pipeline. The frequency threshold for identifying variants was set to 80% because a higher threshold was not able to identify any mutations in some of the strains (data not shown).

3.5 SANGER SEQUENCING

Sanger sequencing was used to confirm SNPs and small insertions or deletions in the mutant strains that were identified by BreSeq. The affected genes were amplified by PCR using the primers listed in Table 2 and then purified using the Qiagen PCR Purification Kit (Qiagen, Germantown, MD). Purified products were sent for Sanger sequencing (Genewiz, South Plainfield, NJ). The trace files were analyzed to confirm the nucleotide sequences.

Table 2. Primer sequences for *mla* and *lpx* genes

Target gene	Primer name	Primer sequence (5′→3′)	Tm (°C)
mlaA	AB_mlaA F	ATGAATTATTCTAATTTACTTTTGTCG	48.9
	AB_mlaA R	TTATTTTTCGGTTTTATCAG	43.6
mlaC	mlaC pro F KpnI	GCGGTACCTTGATGAAGATGCTTATATAA	56.6
	mlaC pro R SalI	GCGTCGACTTATTTTTGTTTATTCTGATT	54.8
mlaD	mlaD F seq	GCATGAAATCACGTACTAGTGAGCTGGCC	62.4
	mlaD R seq	CGCTCAACAAATGACGGCTGTGCA	62.5
mlaF	mlaF F seq	GCATGATTGCCATTATGAATAATAAAA	52.2
	mlaF R seq	GCTGGACGAACCTCGTTATC	55.5
lpxA	lpxA F seq	GCATGAGCAATCACGATTTAATCCATTC	56.9
	lpxA R external	CCAAAATCTGAAGAAGCAAAATTCTTTAACAAA	56.0
lpxC	ABUW_0152 F (-20)	CACCAAAAAACAGAGCAGGC	55.0
	ABUW_0152 R (-20)	GACAATGACTTATGTCAC	45.0
lpxD	lpxD F Seq	GCATGAAAGTGCAACAATATCGTT	54.7
	lpxD R Seq	GCTTTACGCAAATTAAAAGTTGATTC	52.3

3.6 GROWTH CURVES

Growth curves were generated over 6 hours for each mutant strain and compared with parent strains. Overnight cultures of each strain were grown in 5 mL of LB broth shaking at 150 rpm at 37°C. The next morning, starting OD_{600} were standardized to 0.1 for each culture. Strains were grown in 10 mL of LB broth shaking at 150 rpm at 37°C. OD_{600} of 1 mL of each culture was recorded every hour for 6 hours. Growth curves for each isolate were done in biological triplicate.

3.7 GRAM STAINING

The colistin-resistant mutant strains were stained in order to visualize their morphology and outer membrane composition. Briefly, a 10 μL loop of water was placed on a glass slide. A sterile toothpick was touched to the cell culture from an agar plate, and the cells were spread out in the loop of water. The slide was heat fixed. Crystal violet stain was applied to the slide and allowed to set for approximately 30 seconds. The slide was rinsed gently with water until the water ran clear. Next, Gram's iodine was added to the slide and allowed to set for approximately 30 seconds. The slide was rinsed as before. After that, the slide was destained by applying 90% ethanol just until the visible stain washed out. Finally, safranin was applied to the slide and allowed to set for approximately 30 seconds. The slide was gently rinsed and allowed to air dry. The slides were visualized under a light microscope and were imaged.

3.8 ANTIMICROBIAL SUSCEPTIBILITY TESTING WITH GRAM-POSITIVE AGENTS

Trek Sensititre™ GPN4F plates were used to test susceptibility of the strains against a panel of Gram-positive antimicrobial agents (Thermo Fisher Scientific, Waltham, MA). Plates were inoculated by diluting a McFarland 0.5 solution made in deionized sterile by a factor of 1000 in cation-adjusted Mueller-Hinton broth and adding 50 μL into each well with a multi-channel pipet. Plates were covered with adhesive stickers and incubated at 35°C for 18-24 hours. Visible pellets in wells were recorded as growth, and the lowest concentration without growth was designated the MIC.

3.9 LIPID A CHARACTERIZATION

Cell pellets of the mutant strains were prepared by growing a 1 μL loopful of cells in a 3-mL LB broth culture for approximately 2 hours. Cells were pelleted by centrifuging 1mL of culture at a time at 10,000 rpm for 5 minutes and removing the supernatant. Pellets were shipped on dry ice to the laboratory of Dr. Robert Ernst at the University of Maryland for lipid analysis.

3.9.1 Lipid A extraction

Lipid A from the bacterial outer membrane was isolated from whole cells using an isobutyric acid/ammonium hydroxide-based extraction procedure, as described by El Hamidi, et al. Cells from a broth culture were pelleted then resuspended in 70% isobutyric acid and 1M ammonium

hydroxide 5:3 and boiled at 100°C for 45 minutes. Samples were then cooled on ice and centrifuged for 5 minutes at 8000 x g. The supernatant was diluted 1:1 in endotoxin-free water in a new tube. Samples were flash-frozen using dry ice and were lyophilized overnight. The next day, the dry cells were washed twice with 1 mL of methanol, then lipid A was extracted in 100 mL of chloroform:methanol:water (3:1:0.25 vol:vol:vol). Samples of 1 μL of each were loaded onto a stainless steel MALDI target plate (Hudson Surface Technology, Fort Lee, NJ), and 1 μL of norharmane matrix (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mg/mL in 2:1 chloroform:methanol (vol:vol) was added. Spots were allowed to air dry before analysis [48].

3.9.2 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

The lipid A extracted as described above was analyzed in negative ion mode with reflectron mode on a Bruker microFlex (Bruker Daltonics, Billerica, MA) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. The data were collected with flexControl software and processed with flexAnalysis. Spectra were baseline-smoothed and then used to estimate lipid A structures based on molecular weight.

3.10 VIRULENCE OF PARENT STRAINS AND MUTANTS

The four UW strains and their derived mutants were injected into *G. mellonella* waxworm caterpillar larvae [49]. A 10 μL inoculum of 5 x 10^7 cfu/mL suspended in 10mM $MgSO_4$ was injected into the hemocoel of each larva via the last left proleg, giving an inoculum of 5 x 10^5

cfu/larva. Ten larvae were infected with each strain. The larvae were incubated in a dark incubator at 35°C and survival was recorded every 24 hours for 72 hours. This experiment was performed in biological triplicate.

4.0 RESULTS

4.1 AIM 1: MUTANT STRAIN GENERATION

4.1.1 Baseline colistin minimum inhibitory concentration (MIC)/dose response of UW strains 5075, 2238, 2241, and 2245

The colistin MICs of the four original UW strains were determined by broth microdilution as described in Chapter 3, and the resulting OD_{600} values were plotted on a $\log_2$ scale as below. The MICs of each strain were between 0.0625 µg/mL and 0.125 µg/mL of colistin. All of the strains were susceptible to colistin, based on CLSI standards, and there was no observable difference in the way these strains responded to colistin.

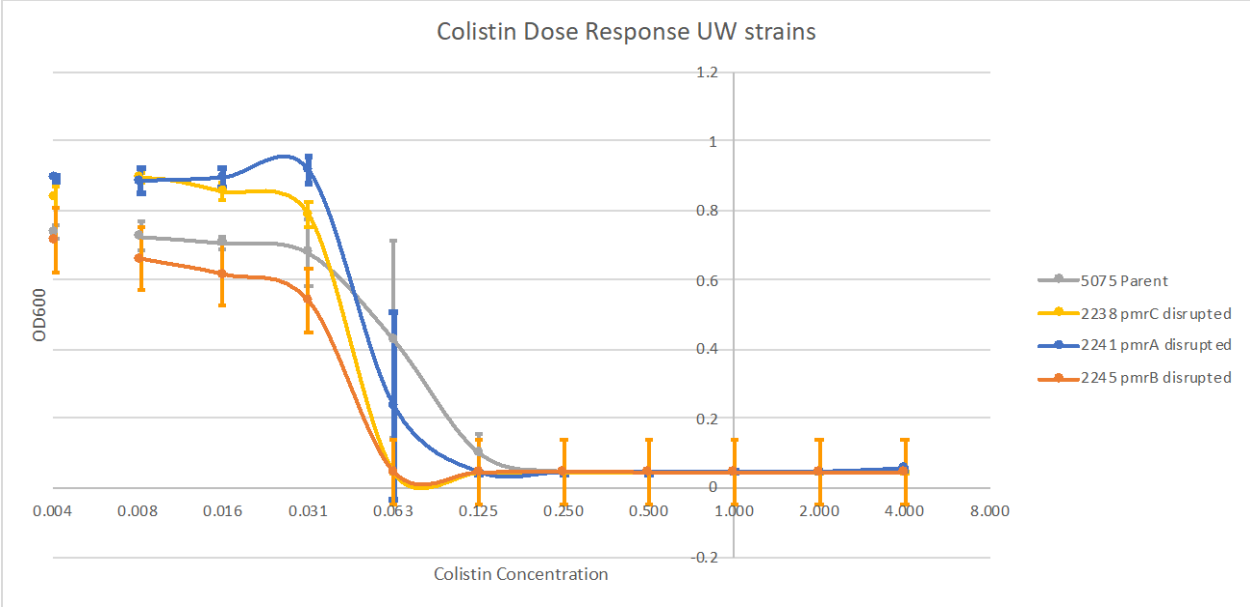

Figure 4. Graphical representation of colistin MIC in UW strains

Colistin MICs were determined to be 0.125 µg/mL for 5075, and 0.0625 µg/mL for 2238, 2241, and 2245. The CLSI clinical breakpoint for colistin susceptibility in *A. baumannii* is ≤ 2 µg/mL.

4.1.2 Generation of colistin-resistant mutant strains

Colistin-resistant mutant *A. baumannii* strains were derived from the four strains obtained from the Manoil Laboratory at the Universitiy of Washington, as described in Chapter 3. A total of 20 mutants were isolates and used for this study. Four different rounds of mutant generation were carried out so that each strain from UW produced at least one mutant in biological triplicate for the sake of reproducibility. The following Table 3 depicts the mutants generated from each round.

Table 3. Biological replicate rounds of mutant generation

Round	Parent 5075	2238 (*pmrC* mut)	2241 (*pmrA* mut)	2245 (*pmrB* mut)
1	5075B 5075F	2238M 2238O	X	2245G 2245L
2	X	2238EE	2241KK 2241MM 2241QQ	X
3	P1.4L P1.4H	X	A2.8	B2.4L
4	P6.4	C2.4 C7.8	A3.8L A3.8H	B7.8

Strains generated from 4 separate rounds of growing in the presence of colistin and the UW isolates from which they were derived. An X indicates that no mutants were obtained in that round.

4.1.3 Colistin broth microdilution on mutant strains

The four parent strains from UW and their respective mutant strains were tested by broth microdilution, as described in Chapter 3. The results are listed in Table 4 below.

Table 4. Colistin MIC of mutant strains from broth microdilution

	Strain	Colistin MIC (μg/mL)
5075 Parent Background	5075	0.125
	5075B	>128
	5075F	>128
	P1.4 H	>128
	P1.4 L	8
	P6.4	>128
pmrC-inactivated Background	2238	0.0625
	2238M	>128
	2238O	>128
	2238EE	>128
	C2.4	>128
	C7.8	>128
pmrA-inactivated Background	2241	0.0625
	2241KK	>128
	2241MM	128
	2241QQ	>128
	A2.8	128
	A3.8H	>128
	A3.8L	32
pmrB-inactivated Background	2245	0.0625
	2245 G	>128
	2245 L	>128
	B2.4L	8
	B7.8	>128

4.1.4 PCR confirming the presence of *bla*$_{\text{OXA-51}}$ and transposon T26

All of the mutants selected for this study, as well as the parent UW strains from which they were derived, were positive for *bla*$_{\text{OXA-51}}$ by PCR, indicating that they were *A. baumannii.* Strain 5075 and the colistin-resistant mutants derived from it did not have T26 and thus tested negative for it by PCR. The parent strains 2238, 2241, and 2245 and their respective colistin-resistant mutants tested positive for T26 when the primers listed previously were used. Therefore, we were able to conclude that these strains had *pmrC, pmrA,* or *pmrB* inactivated and thus unavailable to contribute to colistin resistance.

4.2 AIM 2: GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF MUTANTS

4.2.1 Comparative genomic analyses and Sanger sequencing results

Breseq identified many mutations in each strain. However, for this study, we elected to focus on mutations in *lpx* and *mla* genes, which are listed in the table below. These genes are well-characterized in the literature and have direct effects on compromising outer membrane integrity. A full list is available in the Appendix. Mutations were confirmed using the primers in Table 2, with the exception of C2.4 because the primer binding sites were likely too far away after the new junction rearrangement to give the expected PCR product.

Table 5. Mutations identified in *lpx* and *mla* genes of colistin-resistant mutant strains

	Strain	Gene	Mutation
5075 Background	5075B	*mlaA*	L12*
		lpxA	G68C
	5075F	*mlaF*	11bp del
	P1.4 H	*mlaD*	(A)7→6 frameshift
		lpxA	*263Y
	P1.4 L	*lpxA*	*263Y
	P6.4	*lpxC*	*ISAba1* +9bp
pmrC inactivated	2238M	*mlaC*	Frameshift, add C at 384/624 nt
	2238O	*lpxC*	H264Y
	2238EE	*lpxA*	New junction
	C2.4	*lpxA*	9 nt duplication**
	C7.8	*lpxC*	Gene broken and rearranged
pmrA inactivated	2241KK	*mlaC*	(GTTATTT) nt duplication
	2241MM	*mlaF*	19 nt deletion
	2241QQ	*lpxC*	P30L
		mlaD	(T) 7→6 frameshift
	A2.8	Unknown	-
	A3.8 H	Unknown	-
	A3.8 L	Unknown	-
pmrB inactivated	2245G	*lpxC*	L119*
		mlaA	23 nt duplication
	2245L	*mlaC*	1nt deletion frameshift
	B2.4 L	Unknown	-
	B7.8	*mlaF*	G50D
		lpxA	L253H

**The *lpxA* mutation in C2.4 was not confirmed by Sanger sequence.

4.2.2 Growth curves

Growth curves were generated for the UW strains and derivative colistin-resistant mutants as described in Chapter 3 in biological triplicate on different dates. The below graphs represent the ODs recorded at each time point, including error bars. For each set of mutants, the parent strain consistently exhibited higher rates of growth. The mean growth rate of the mutants varied, but the same general trends were observed each time for each strain relative to the rest of the group.

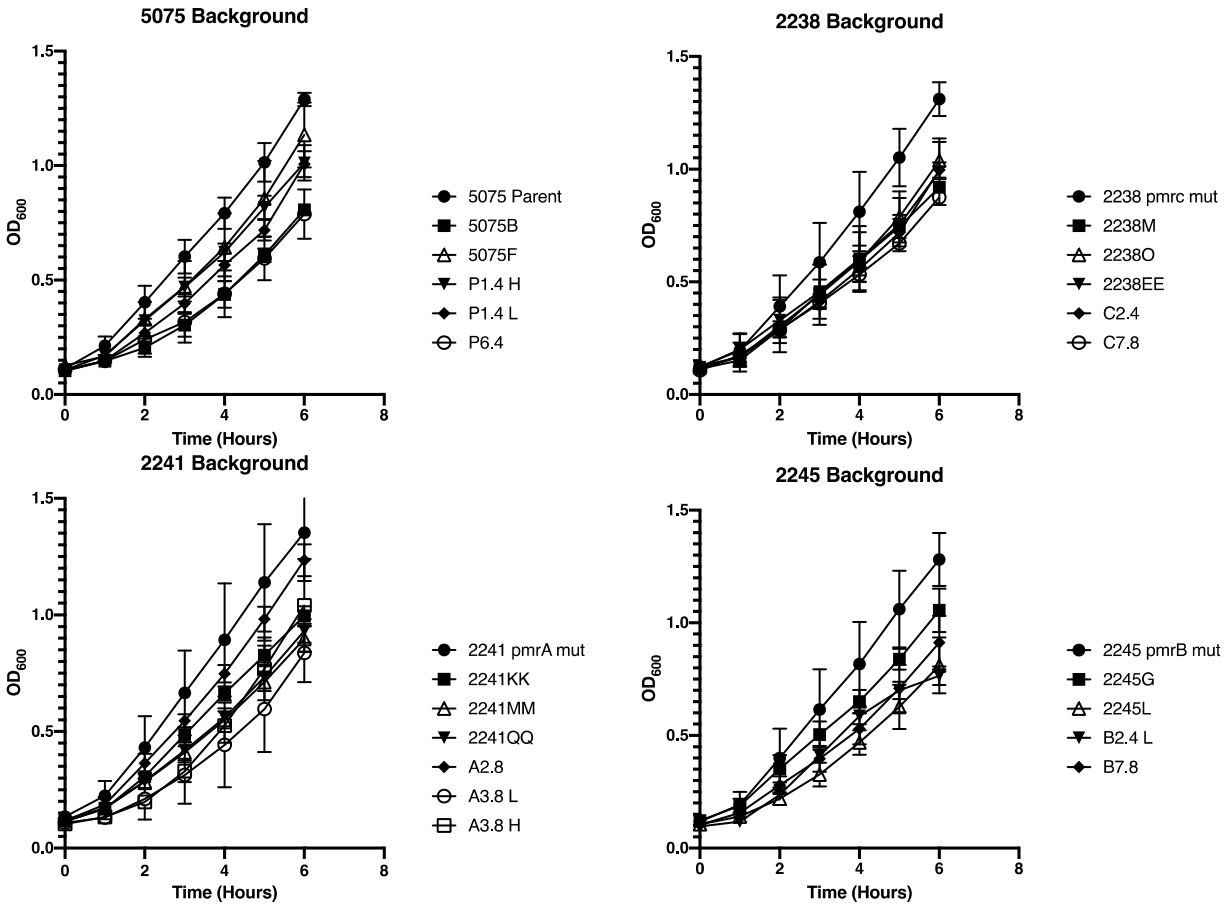

Figure 5. 6-hour growth curves of the UW strains and respective colistin-resistant mutants

4.2.3 Gram stain of colistin-resistant mutants

The four UW strains and their derivative colistin-resistant mutant strains were Gram-stained as described in Chapter 3. A control stain was prepared using a *Staphylococcus aureus* ATCC 25923 strain as a Gram-positive control (purple cocci in clusters) and an *Escherichia coli* ATCC 25922 strain as a Gram-negative control (pink bacilli) (Figure 6D). The wild-type 5075 strain showed Gram-negative, short bacilli (Figure 6A). The mutant strains exhibited varying results; some stained as Gram-negative coccobacilli (5075 B, Figure 6B), while others stained as Gram-positive cocci or had undefined shapes (2238 M, Figure 6C).

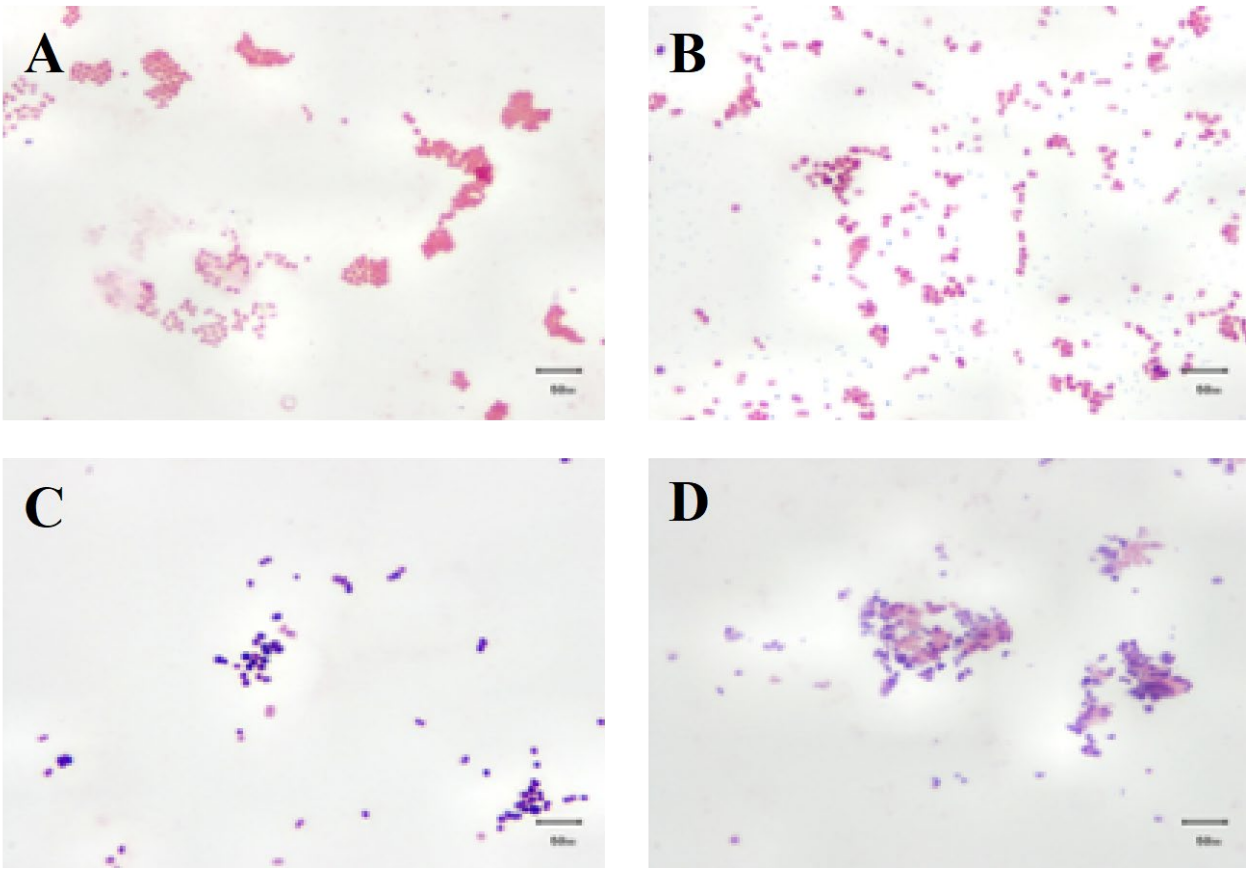

Figure 6. Gram Stains

5075 Parent (A) stained as Gram-negative coccobacilli, 5075 B (B) stained as Gram-negative coccobacilli, 2238M (C) stained as Gram-positive cocci, and positive and negative control strains (D) where *Staphylococcus aureus* stained as Gram-positive cocci and *Escherichia coli* stained as Gram-negative bacilli.

4.2.4 Antibiograms of strains

One Trek Sensititre ™ plate per isolates was inoculated as described in Chapter 3. The MIC of each strain is recorded in μg/mL in the table below. The strains from UW are highlighted in grey. Notably, the UW strains 2238, 2241, and 2245, which have the transposon containing the tetracycline resistance gene, have high tetracycline (TET) MICs.

For many of these Gram-positive agents, the mechanism of action involves inhibition of cell wall synthesis. Typically, the outer membrane of Gram-negative organisms prevents these antibiotics from breaching the membrane and entering the cell. However, without an outer membrane, the thin cell wall of these bacteria is exposed and thus is vulnerable to Gram-positive agents.

For drugs for which there is a range of MICs among the strains, factors other than an absent membrane may be contributing to the resulting MICs for certain drugs. For example, all of the strains derived from the *pmrA*-inactivated strain (2241) have very low daptomycin (DAP) MICs. Therefore, the underlying defect in *pmrA* may be affecting the organisms' ability to survive in the presence of daptomycin.

Finally, mutants A2.8 and B2.4 L have antibiograms that are identical to their respective parent UW strains. They may have reverted to the parent strain, or it is more likely that the strains saved in the -80° freezer contained a mixed culture of the parent UW strain and the colistin-resistant mutant. The mutant may be outcompeted when it is grown in co-culture with its parent UW strain because of the fitness cost of the mutations that confer colistin resistance.

Table 6 MIC of strains against a panel of Gram-positive antimicrobial agents. Concentration in µg/mL

		Antibiotics[1]																
		ERY	CLI	SYN	DAP	VAN	TET	AMP	GEN	LEVO	LZD	AXO	PEN	RIF	GAT	CIP	SXT	OXA+
	Strain																	
	5075	>4	>2	>4	>8	128	≤2	>16	>16	>8	>8	>64	>8	4	>8	>2	>0.5/9.5	>8
Parent Background	5075B	≤0.25	1	0.5	2	≤1	≤2	0.5	≤2	4	>8	≤8	0.5	≤0.5	2	>2	>0.5/9.5	≤0.25
	5075F	≤0.25	>2	1	>8	≤1	≤2	>16	>16	4	>8	16	>8	≤0.5	8	>2	>0.5/9.5	≤0.25
	P1.4 H	≤0.25	2	1	>8	2	≤2	>16	>16	8	>8	16	>8	≤0.5	4	>2	>0.5/9.5	>8
	P1.4L	≤0.25	>2	1	2	≤1	≤2	>16	8	8	>8	32	>8	≤0.5	4	>2	>0.5/9.5	>8
	P6.4	≤0.25	1	1	>8	≤1	≤2	8	16	4	>8	8	8	≤0.5	2	>2	>0.5/9.5	≤0.25
	2238	>4	>2	>4	>8	>128	16	>16	>16	8	>8	>64	>8	4	8	>2	>0.5/9.5	>8
pmrC inactivated	2238M	≤0.25	1	0.5	2	≤1	≤2	0.5	≤2	4	>8	≤8	2	≤0.5	2	>2	>0.5/9.5	≤0.25
	2238O	≤0.25	>2	1	>8	≤1	≤2	>16	>16	8	>8	16	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	2238EE	≤0.25	2	1	>8	≤1	≤2	8	>16	8	>8	8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	C2.4	≤0.25	1	1	>8	2	≤2	>16	>16	4	>8	≤8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	C7.8	≤0.25	2	1	0.5	≤1	≤2	2	8	2	>8	≤8	4	≤0.5	2	>2	>0.5/9.5	≤0.25
	2241	>4	>2	>4	>8	128	16	>16	>16	8	>8	>64	>8	4	4	>2	>0.5/9.5	>8
pmrA inactivated	2241KK	≤0.25	>2	1	0.5	≤1	≤2	16	>16	4	>8	8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	2241MM	≤0.25	>2	1	≤0.25	≤1	≤2	16	>16	8	>8	8	>8	≤0.5	4	>2	>0.5/9.5	0.5
	2241QQ	≤0.25	>2	1	≤0.25	≤1	≤2	16	16	8	>8	8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	A2.8	≤0.25	>2	>4	>8	128	16	>16	>16	8	>8	>64	>8	4	8	>2	>0.5/9.5	>8
	A3.8H	≤0.25	1	-.5	≤0.25	≤1	≤2	1	8	4	8	8	4	≤0.5	2	>2	>0.5/9.5	≤0.25
	A3.8L	≤0.25	2	0.5	≤0.25	≤1	≤2	1	8	4	>8	8	2	≤0.5	2	>2	>0.5/9.5	≤0.25
	2245	>4	>2	>4	>8	128	16	>16	>16	8	>8	>64	>8	4	8	>2	>0.5/9.5	>8
pmrB inactivated	2245 G	≤0.25	>2	0.25	4	2	≤2	>16	>16	4	8	8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25
	2245 L	≤0.25	2	0.5	8	≤1	≤2	1	4	2	>8	8	2	≤0.5	2	>2	>0.5/9.5	≤0.25
	B2.4L	≤0.25	>2	>4	>8	128	16	>16	>16	8	>8	>64	>8	4	8	>2	>0.5/9.5	>8
	B7.8	≤0.25	1	0.5	8	≤1	≤2	16	8	8	8	≤8	>8	≤0.5	4	>2	>0.5/9.5	≤0.25

[1]ERY, erythromycin; CLI, clindamycin; SYN, quinupristin-dalfopristin; DAP, daptomycin; VAN, vancomycin; TET, tetracycline; AMP, ampicillin; GEN, gentamicin; LEVO, levofloxacin; LZD, linezolid; AXO, ceftriaxone; PEN, penicillin; RIF, rifampin; GAT, gatifloxacin; CIP, ciprofloxacin; SXT, trimethoprim-sulfmethoxazole; OXA+, oxacillin + 2% NaCl.

4.2.5 MALDI-TOF Mass spectrometry

Lipid A was extracted from the four UW strains as described in Chapter 3. The lipid A was run on the MALDI-TOF mass spectrometer and produced mass spectra. Significant mass spectral peaks were identified. Representative spectra are shown below; the top panel represents a spectrum where no lipid A peaks were detected, and the bottom panel shows one that detected peaks at mass-to-charge ratios (*m/z*) of 1728 (hexa-acylated lipid A) and 1911 (hepta-acylated lipid A). Table 7 indicates whether peaks were detected at the aforementioned *m/z*.

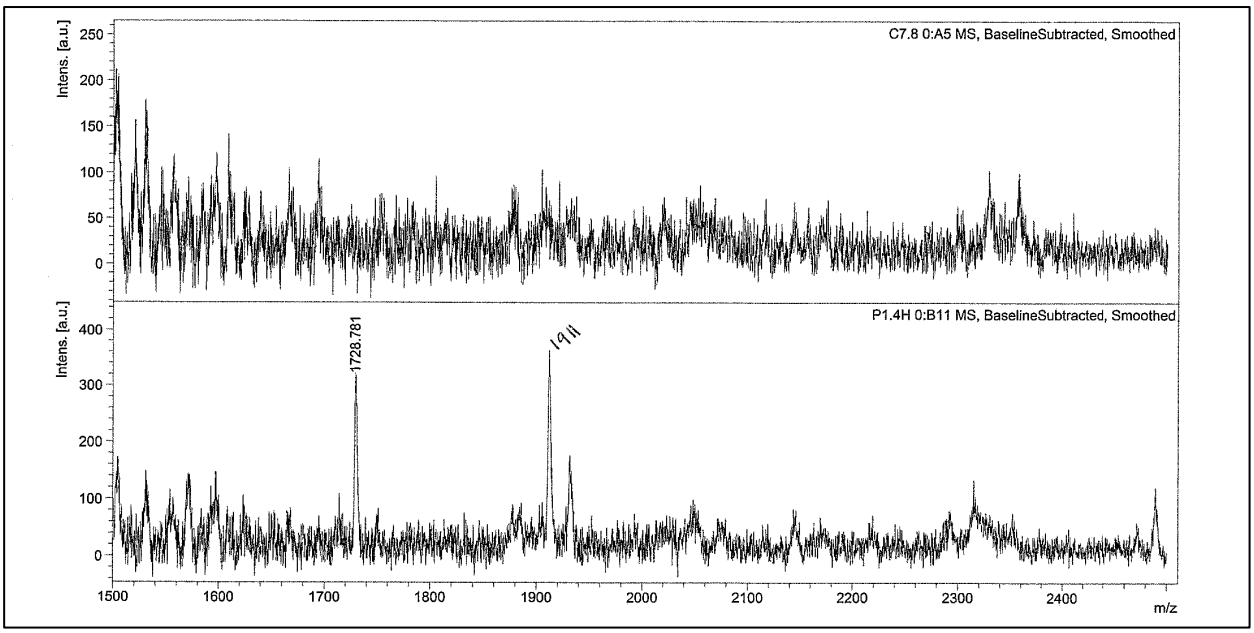

Figure 7. Example of spectra resulting from MALDI-TOF mass spectrometry

No peaks were detected in C7.8 (top), whereas peaks were detected at *m/z* of 1728 and 1911 in P1.4H (bottom).

Table 7. Presence or absence of hexa-acylated and hepta-acylated lipid A

	Peak	
Strain	1728	1911
5075	+	+
5075B	+	+
5075F	-	-
P1.4 H	+	+
P1.4 L	+	+
P6.4	-	-
2238	+	small
2238M	-	-
2238O	-	-
2238EE	-	-
C2.4	-	-
C7.8	-	-
2241	+	+
2241KK	-	-
2241MM	+	+
2241QQ	+	+
A2.8	+	+
A3.8 H	-	-
A3.8 L	-	-
2245	+	+
2245G	-	-
2245L	-	-
B2.4 L	+	-
B7.8	+	+

4.3 AIM 3: DEFINE RELATIONSHIP BETWEEN RESISTANCE, FITNESS, AND VIRULENCE IN COLISTIN-RESISTANT MUTANT STRAINS

4.3.1 Waxworm survival

G. mellonella caterpillars were infected as described in Chapter 3 and observed for 4 days. The infection model was carried out in biological triplicate, and the results were plotted in the below Kaplan-Meier survival curves. Significance was calculated with a Log-Rank test and based on $p \leq 0.05$.

Virulence varied between biological replicates of this experiment. The caterpillars typically weigh between 150 and 200 milligrams, but it is not possible to adjust the inoculum delivered to each worm based on its mass because the volume delivered is very small, which may affect the observed virulence.

Nonetheless, there were some general trends. The colistin-resistant mutants derived from UW strains 5075 and 2245 (*pmrB* inactivated) were less virulent than their respective parents. In contrast, no discernable trends were observed with the mutants derived from UW strains 2238 (*pmrC* inactivated) or 2241 (*pmrA* inactivated) because the parent strains themselves exhibited variability. As work continues on this project, we expect to gain a clearer picture of the roles these genes play in virulence.

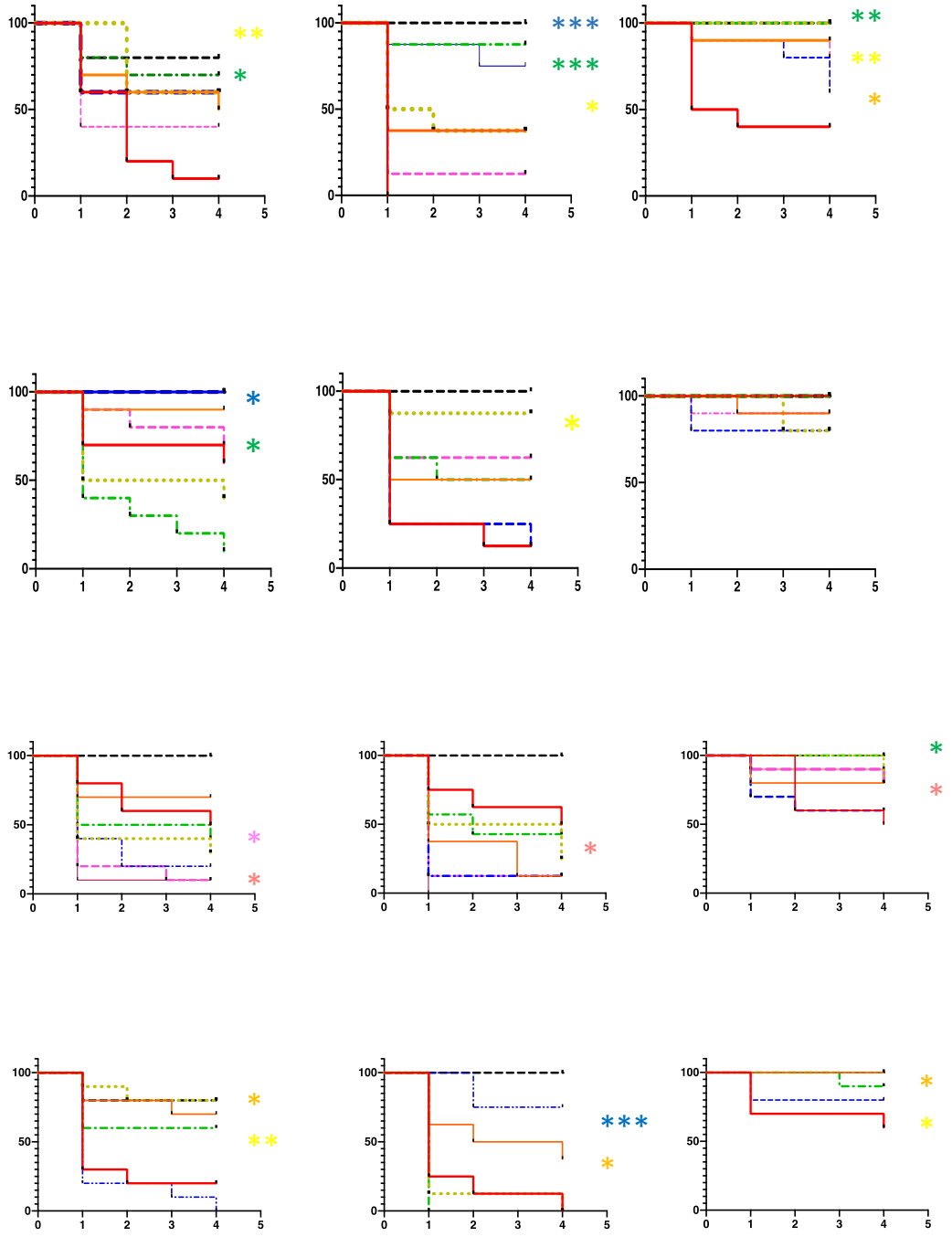

Figure 8, Kaplan-Meier survival curves of *G. mellonella* infected with *A. baumannii* strains

5.0 DISCUSSION

Since the first antibiotics were discovered nearly a century ago, physicians and researchers have been engaging in an arms race with the bacteria that are responsible for causing numerous infectious diseases. The collection of antibiotics available shows promise early on in treating new disease outbreaks, but small subsets of the original population that evolve resistance linger. When those few organisms expand in number, they become the dominant population. In hospital settings, especially those in which patients are already severely immunocompromised, these newly-minted resistant bacteria can wreak havoc as they cause infections that cannot be treated by typical antibiotic regimens [4].

Acinetobacter baumannii is a type of bacteria that is becoming particularly difficult to treat due to the development of antibiotic resistance. What first started out as an innocuous soil organism has evolved to become one of the World Health Organization's priority pathogens: those for which there is a desperate need to develop new treatment options [22]. Equipped with many intrinsic resistance genes, *A. baumannii* is able to persist when challenged with common antimicrobials. Furthermore, its genome is prone to genetic changes and rearrangements that confer resistance [9].

Because of its unique resilience, treatment options for *A. baumannii* are dwindling, and physicians are turning to colistin, a polymyxin antibiotic, as a last-resort. However, colistin resistance in *A. baumannii* is already making these infections even more problematic. Gene clusters including *pmrCAB, lpx,* and *mla* are all areas of interest to those studying resistance mechanisms in *A. baumannii* [35]. These genes are all related to the outer membrane of *A. baumannii*. Existing literature cites *pmrC* as the primary component responsible for membrane modifications that confer colistin resistance. The aim of this project was to investigate what

mechanisms other than *pmrC* are involved in colistin resistance. We employed mutants with inactivated *pmrC* and its two-component regulators, *pmrA* and *pmrB* to accomplish this goal [28, 31].

It was, in fact, possible to generate colistin-resistant mutants *in vitro* from the UW 5075 strain and each of its three transposon-inactivated mutants of *pmrCAB.* In total, 20 colistin-resistant mutants were generated from the four UW strains. Their colistin MICs were in the resistant range, according to the CLSI breakpoint, in contrast to the original strains, which were all colistin-susceptible and had no large differences in their MICs, nor did they exhibit a dose-dependent inhibition by colistin. The mutants were genetically stable, and the ones derived from the transposon-inactivated strains maintained the transposon even after developing colistin resistance, making it possible to compare the colistin-resistant mutants to their respective parent strains by variant calling between their whole genomes.

The Breseq analysis pipeline identified many SNPs and small insertions and deletions, but the most notable mutations were clustered in the *lpx* and *mla* loci. Therefore, we decided to pursue those genes for this study since they have been investigated in existing literature [33, 35]. All of the mutations were confirmed by Sanger sequencing, with the exception of C2.4. Breseq indicated that there was new junction evidence in this gene; therefore, it is possible that the gene was broken and spliced into two different locations in the genome, which prohibited the primers from amplifying the section between the fragments. Additionally, Breseq did not identify mutations in *lpx* or *mla* within the genomes of B2.4L, A2.8, A3.8H, or A3.8L, suggesting that additional, yet unknown pathways to colistin resistance may exist. These mutants had other candidate genes that may be related to colistin resistance, but analyzing them was beyond the scope of this project.

As future work, it would be beneficial to plot the mutated genes in a KEGG pathway in order to see which global processes they are a part of and whether said processes are tied to colistin resistance. Performing RNAseq would also be beneficial because we would be able to see the global impact on upregulation and downregulation of genes under the control of each component of *pmrCAB*. Alternatively, we could determine whether any components of *pmrCAB* were upregulated in these mutants using qRT-PCR.

Notably, none of the colistin-resistant mutant strains had mutations in *pmrCAB*, even the ones derived from the 5075 strain. However, this is consistent with previous literature which suggests that *pmrCAB* mutants are more likely to be recovered from clinical specimens [37]. Such mutations are more favorable for isolates that need to have an intact membrane in order to stand the challenges posed by the host's internal environment, whereas laboratory strains are able to survive with compromised membranes in the *in vitro* growth conditions used here. This discrepancy between resistance mechanisms seen in clinical isolates versus ones generated *in vitro* could be because mutations in *pmrCAB* lead to gain of function mutations, which are energetically costly to the bacteria. On the other hand, loss of function mutations such as the ones seen here are not as costly.

The phenotypic assessment of these mutants revealed that the mutant strains struggled more in the growth assay than the parent strains from which they were derived. Without an outer membrane, the bacteria may be more susceptible to changes in their environment, such as osmotic pressure or pH, limiting the robustness of their growth. The Gram stains offer visual corroboration of this hypothesis; the irregular shape of some of the cells suggests that they are not well contained and may be falling victim to changes caused by the environment. Furthermore, some strains stained Gram-positive. Without the LPS on their outer membrane, the crystal violet stain is able to

penetrate to the peptidoglycan in the cell wall much easier. The same is true for the Gram-positive antimicrobial agents. The decreased MICs to many of the drugs in the Sensititre™ plates indicate that the drugs are able to infiltrate the barrier that is left vulnerable due to the absence of the outer membrane.

The MALDI-TOF analysis gives molecular confirmation of the lack of lipid A in some strains. Particularly with the strains derived from the *pmrC* mutant, we observe that neither hexa-acylated nor hepta-acylated lipid A is present, suggesting perhaps a complete loss of the outer membrane. This may be due to the underlying disruption of *pmrC* in the UW strain. However, these strains had mutations only in *lpxA, lpxC,* and *mlaC*; these may be the most crucial genes for ensuring an intact outer membrane due to their pivotal roles in LPS synthesis and lipid asymmetry maintenance, respectively.

The survival data from the waxworms were inconsistent. However, a few strains exhibited consistently different virulence from their respective parent strains. For example, 5075F and P1.4L were consistently significantly less virulent than 5075, and 2245G was consistently significantly less virulent than 2245. The mutant strain A3.8 H was consistently significantly different from 2241, but twice it was less virulent and once it was more virulent. The waxworm model has many benefits, but it also has several limitations, including the variation in the viability of the worms.

Overall, for some of the mutant strains, we were able to obtain a clear picture of the mechanisms responsible for their colistin resistance and associated reduced fitness and/or virulence. However, for other strains, the data were either inconsistent or we would need further characterization to pinpoint the exact genetic changes that confer colistin resistance.

6.0 CONCLUSIONS

In conclusion, we were able to generate colistin-resistant *A. baumannii* strains in the laboratory, even when the components of *pmrCAB*, which are the primary source of colistin resistance in this species, were not functioning. We were able to characterize genetic mutations responsible for the defective outer membrane that allowed these strains to live unaffected by the presence of colistin.

Mutations in the *lpx* and *mla* genes were the focus of this study, as they have been well-characterized previously in the literature. It is important to note that these are distinct mechanisms that may serve as secondary colistin resistance mechanisms in situations where *pmrCAB* are defective. Variations in the *lpx* and *mla* genes had direct effects on the presence or integrity of the outer membrane and affected the bacteria's ability to grow and cause infection. These mutant strains also were easily killed by low concentrations of Gram-positive antibiotics, likely due to their cell wall being exposed without the protection of the outer membrane. The *in vivo* waxworm model gave varied results with regards to virulence. However, certain mutants which were consistently less virulent than their respective parent strains may not be viable.

Moving forward, other genes identified by the Breseq pipeline should be investigated for their role in colistin resistance. Additionally, the wild-type versions of the mutated *lpx* and *mla* genes can be complemented back into the colistin-resistant mutant strains in order to observe whether restoring those components also restores the phenotype of the wild-type strains when confronted with Gram-positive antibiotics.

7.0 PUBLIC HEALTH SIGNIFICANCE

Antibiotic resistance is a critical threat that is putting millions of people's lives in danger [4]. In recent years, antibiotic-resistant bacteria have repeatedly made headlines due to their public health significance. The bacteria responsible for these diseases are costly to the healthcare industry and patients alike and have been associated with poorer patient outcomes [50]. As multidrug-resistant bacterial infections spread, doctors and researchers clamor for new treatment options [6]. Tantamount to the urgent need for new antibiotics is the need to understand mechanisms of resistance to existing drugs. With understanding of the processes and key genes involved, researchers can develop strategies to exploit weaknesses in resistant bacteria.

This study presents a possible scenario in which *A baumannii* is not able to employ its primary mode of colistin resistance. We observed that it is possible for *A. baumannii* to develop additional but distinct mechanisms of resistance by mutating genes in other pathways. However, after a thorough characterization, it is evident that these colistin-resistant mutants experience significant fitness and virulence costs that make them unlikely to cause infection in human hosts and ascend to public health relevance. However, were they to thrive in a human host, these mutant strains experience a reversion of phenotype that renders them essentially Gram-positive organisms, and they could easily be treated with common Gram-positive agents. Nevertheless, it is important to have this knowledge as we develop best practices for treating *A. baumannii* infections in clinical settings.

APPENDIX SUPPLEMENTAL TABLE

Table 8 Other mutations detected

Strain	Gene	Function	Mutation
5075B	nhaP	Na+/H+ antiporter	+T frameshift
	rocC	Amino-acid permease	missing coverage evidence
	pepN_2	Aminopeptidase N	missing coverage evidence
	mdlY	mandelamide hydrolase	missing coverage evidence
	"intergenic"		4 deletions of 500-600bp
5075F	ABUW5075_02303	Putative phospholipase A1	(A)6->7
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	rnd_1	n/a	delete 3142bp
2238M	prc	Tail-specific protease	1bp deletion
	"intergenic"		deletions of 703bp and 524bp
	yqiJ	hypothetical inner membrane protein	missing coverage evidence
	xerD	tyrosine recombinase	unassigned new junction evidence
2238O	ABUW02238_01322	hypothetical protein	(CAGTT)duplication
	pepN_2	Aminopeptidase N	missing coverage evidence
	aadB	2"-aminoglycoside nucleotidyltransferase	missing coverage evidence
		Putative phospholipase A1	unassigned new junction evidence
	xerD	tyrosine recombinase	unassigned new junction evidence
2238EE		"intergenic"	703bp deletion
	nhaP	Na+/H+ antiporter	missing coverage evidence
	hvrA_2	Trans-acting regulatory protein	unassigned new junction evidence
	xerD	tyrosine recombinase	unassigned new junction evidence
2241KK	rocC	Amino-acid permease	T->A bp change intergenic
	copB_1	copper resistance protein B	A->T bp change intergenic
		Putative phospholipase A1	+G frameshift
	yxaF	yxaF_3 txn regulator	712bp deletion
	pepN_2	Aminopeptidase N	missing coverage evidence
	gdhA_2	glutamate dehydrogenase	missing coverage evidence
	smf-1_2	major fimbrial subunit SMF-1	missing coverage evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence

Table 8 continued

	yqiJ	hypothetical inner membrane protein	unassigned new junction evidence
	OXA-133	beta lactamase	unassigned new junction evidence
			994, 683, and 501bp intergenic deletions
2241MM	copB_1	copper resistance protein B	A->T bp change intergenic
	yxaF	yxaF_3 txn regulator	712bp deletion
	rocC	Amino-acid permease	missing coverage evidence
	mdlY/emrA_3	mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	pepN_2	Aminopeptidase N	missing coverage evidence
	gdhA_2	glutamate dehydrogenase	missing coverage evidence
	nhaP	Na+/H+ antiporter	missing coverage evidence
			994, 683, and 501bp intergenic deletions
2241QQ	yxaF	yxaF_3 txn regulator	712bp deletion
	rocC_1	Amino-acid permease	missing coverage evidence
	mdlY/emrA_3	mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	pepN_2	Aminopeptidase N	missing coverage evidence
	gdhA_2	glutamate dehydrogenase	missing coverage evidence
	nhaP	Na+/H+ antiporter	missing coverage evidence
		Putative phospholipase A1	unassigned new junction evidence
			683bp deletion intergenic
2245G	mexB_2	multidrug resistance protein MexB	S79*
	xerD	tyrosine recombinase	A217E, V151L, R148S
		putative HTH-type transcriptional regulator	missing coverage evidence
		Putative phospholipase A1	unassigned new junction evidence
			657, 583, and 507bp intergenic deletions
2245L	xerD	tyrosine recombinase	A217E, V151L, R148S
	mdlY/emrA_3	mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	cpdA_2	cyclic adenosine monophosphate phosphodiesterase	missing coverage evidence
	yveA	Aspartate-proton symporter	missing coverage evidence
			583bp intergenic deletion
	OXA-133	beta lactamase	unassigned new junction evidence
A2.8		hydroxycinnamoyl-CoA hydratase/lyase	3 and 4 bp

Table 8 continued

	pyrB	aspartate carbamoyltransferase	T167T (just nucleotide variation)
	wecB_2	UDP-N-acetylglucosamine 2-epimerase	C315R
	aacA4	Aminoglycoside N(6')-acetyltransferase type 1	C->A bp change intergenic
	pepN_2	Aminopeptidase N	missing coverage evidence
	gltC_5	HTH-type transcriptional regulator	missing coverage evidence
	mdlY	Mandelamine hydrolase	missing coverage evidence
	qseB_2	transcriptional regulatory protein QseB	missing coverage evidence
	anoR	transcriptional activator prtoein	missing coverage evidence
	fadD	long-chain-fatty-acid-AMP ligase FadD32	missing coverage evidence
	smf-1_2	major fimbrial subunit SMF-1	missing coverage evidence
	aacA4	Aminoglycoside N(6')-acetyltransferase type 1	missing coverage evidence
	yfcG_1	Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	tuf1	elongation factor Tu	unassigned new junction evidence
	repE	replication initiation protein	unassigned new junction evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	sdaA	L-serine dehydratase 1	unassigned new junction evidence
	fusA	elongation factor G	unassigned new junction evidence
	rpoN	RNA polymerase sigma-54 factor	unassigned new junction evidence
	aac	aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	qseC_2	sensor protein QseC	unassigned new junction evidence
	sixA	phosphohistidine phosphatase SixA	unassigned new junction evidence
	copA_2	copper resistance protein B	unassigned new junction evidence
	ppiA	peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	pdhD	dihydrolipoyl dehydrogenase	unassigned new junction evidence
	dnaB_2	replicative DNA helicase	unassigned new junction evidence
	higA1	antitoxin HigA1	unassigned new junction evidence
A3.8H		hydroxycinnamoyl-CoA hydratase/lyase	3 and 4 bp

Table 8 continued

	aacA4	Aminoglycoside N(6')-acetyltransferase type 1	C->A bp change intergenic
	pepN_2	Aminopeptidase N	missing coverage evidence
	gltC_5	HTH-type transcriptional regulator	missing coverage evidence
	emrA_3	multidrug export protein EmrA	missing coverage evidence
	copB_1	copper resistance protein b	unassigned new junction evidence
	copA_1	copper resistance protein a	unassigned new junction evidence
	smf-1_2	major fimbrial subunit SMF-1	missing coverage evidence
	yfcG_1	Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	tufl	elongation factor Tu	unassigned new junction evidence
	repE	replication initiation protein	unassigned new junction evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	sdaA	L-serine dehydratase 1	unassigned new junction evidence
	fusA	elongation factor G	unassigned new junction evidence
	rpoN	RNA polymerase sigma-54 factor	unassigned new junction evidence
	aac	aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	sixA	phosphohistidine phosphatase SixA	unassigned new junction evidence
	copA_2	copper resistance protein A	unassigned new junction evidence
	ppiA	peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	pdhD	dihydrolipoyl dehydrogenase	unassigned new junction evidence
	higA1	antitoxin HigA1	unassigned new junction evidence
		hydroxycinnamoyl-CoA hydratase/lyase	3 and 4 bp
A3.8L		hydroxycinnamoyl-CoA hydratase/lyase	3 and 4 bp
	aacA4	Aminoglycoside N(6')-acetyltransferase type 1	C->A bp change intergenic
		ABUW_03858	533bp del
	pepN_2	Aminopeptidase N	missing coverage evidence
	emrA_3	multidrug export protein EmrA	missing coverage evidence
	smf-1_2	major fimbrial subunit SMF-1	missing coverage evidence
	yfcG_1	Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	tufl	elongation factor Tu	unassigned new junction evidence

Table 8 continued

	repE	replication initiation protein	unassigned new junction evidence
	copA_2	copper resistance protein A	unassigned new junction evidence
	copB_1	copper resistance protein b	unassigned new junction evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	sdaA	L-serine dehydratase 1	unassigned new junction evidence
	fusA	elongation factor G	unassigned new junction evidence
	rpoN	RNA polymerase sigma-54 factor	unassigned new junction evidence
	aac	aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	sixA	phosphohistidine phosphatase SixA	unassigned new junction evidence
	ppiA	peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	pdhD	dihydrolipoyl dehydrogenase	unassigned new junction evidence
	dnaB_2	replicative DNA helicase	unassigned new junction evidence
	higA1	antitoxin HigA1	unassigned new junction evidence
B2.4L	pyrB	aspartate carbamoyltransferase	1bp deletion 1044 and 775bp deletions
	gdhA_2/rocC_1	glutamate dehydrogenase/amino-acid permease RocC	missing coverage evidence
	alaA/gltT	glutamate-pyruvate aminotransferase/proton/sodium-glutamate symport protein	
	fabG_2/glpE_1	3-oxoacyl-reductase/Thiosulfate sulfurtransferase GlpE	missing coverage evidence
	mdlY/emrA_2	mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	wecH	O-acetyltransferaseWecH	missing coverage evidence
	hfq	RNA-binding protein hfq	missing coverage evidence
	dnaX_2	DNA polymerase III subunit tau	missing coverage evidence
	nhaP	Na+/H+ antiporter	missing coverage evidence
	tuf1	elongation factor Tu	unassigned new junction evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	yfcG_1	Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	copA_1	copper resistance protein A	unassigned new junction evidence
	sdaA	L-serine dehydratase 1	unassigned new junction evidence
	repE	replication initiation protein	unassigned new junction evidence

Table 8 continued

	fusA	elongation factor G	unassigned new junction evidence
	aac	aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	hchA_3	protein-nucleic acid deglycase	unassigned new junction evidence
	dnaB_2	replicative DNA helicase	unassigned new junction evidence
	sixA	phosphohistidine phosphatase SixA	unassigned new junction evidence
	ppiA	peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	pdhD	dihydrolipoyl dehydrogenase	unassigned new junction evidence
	higA1	antitoxin HigA1	unassigned new junction evidence
B7.8	hfq	RNA-binding protein hfq	A->T bp change intergenic
	gltC_2	HTH-type transcriptional regulator	missing coverage evidence
	yxaF_3		missing coverage evidence
	mdlY/emrA	mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	rpoN	RNA polymerase sigma-54 factor	unassigned new junction evidence
	tuf1	elongation factor Tu	unassigned new junction evidence
	yveA	Aspartate-proton symporter	unassigned new junction evidence
	yfcG_1	Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	sdaA	L-serine dehydratase 1	unassigned new junction evidence
	repE	replication initiation protein	unassigned new junction evidence
	fusA	elongation factor G	unassigned new junction evidence
	esiB_4	secretory immunoglobulin A-binding protein	unassigned new junction evidence
	sixA	phosphohistidine phosphatase SixA	unassigned new junction evidence
	ppiA	peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	higA1	antitoxin HigA1	unassigned new junction evidence
C2.4	pepN_2	Aminopeptidase N	T->A bp change intergenic
	smf-1_1	major fimbrial subunit SMF-1	missing coverage evidence
	rocC_2/gdhA_2	Amino-acid permease/Glutamate dehydrogenase	missing coverage evidence
	_01978	Extracellular serine proteinase	missing coverage evidence
	nhaP	Na+/H+ antiporter	missing coverage evidence
	anoR	transcriptional activator prtoein	missing coverage evidence

Table 8 continued

	aac		aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	tuf1		elongation factor Tu	unassigned new junction evidence
	yfcG_1		Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	sdaA		L-serine dehydratase 1	unassigned new junction evidence
	fusA		elongation factor G	unassigned new junction evidence
	dnaB_2		replicative DNA helicase	unassigned new junction evidence
	sixA		phosphohistidine phosphatase SixA	unassigned new junction evidence
	ppiA		peptidyl-prolyl cis-trans isomerase A	unassigned new junction evidence
	higA1		antitoxin HigA1	unassigned new junction evidence
C7.8		2491	hypothetical protein	Q11*
	queH		Epoxyqueuosine reductase	missing coverage evidence
	mdlY/emrA		mandelamide hydrolase/multidrug export protein EmrA	missing coverage evidence
	nhaP		Na+/H+ antiporter	missing coverage evidence
	tuf1		elongation factor Tu	unassigned new junction evidence
	yfcG_1		Disulfide-bond oxidoreductase YfcG	unassigned new junction evidence
	sdaA		L-serine dehydratase 1	unassigned new junction evidence
	copA_1		copper resistance protein A	unassigned new junction evidence
	rpoN		RNA polymerase sigma-54 factor	unassigned new junction evidence
	fusA		elongation factor G	unassigned new junction evidence
	yveA		Aspartate-proton symporter	unassigned new junction evidence
	aac		aminoglycoside 2'-N-acetyltransferase	unassigned new junction evidence
	dnaB_2		replicative DNA helicase	unassigned new junction evidence
	sixA		phosphohistidine phosphatase SixA	unassigned new junction evidence
	higA1		antitoxin HigA1	unassigned new junction evidence
	hvrA_2		Trans-acting regulatory protein	unassigned new junction evidence
P1.4H	dnaX		DNA polymerase III subunit tau	missing coverage evidence
	_1904		antibiotic biosynthesis monooxygenase	missing coverage evidence
	_1948/1949		indoleacetamide hydrolase/RNDfamily drug transporter	missing coverage evidence
	_1982		outer membrane protein E	unassigned new junction evidence
P1.4L	rnd		ribonuclease D	R353S
	hfq		host factor Hfq	missing coverage evidence

Table 8 continued

	_0684	coproporphyrinogen III oxidase	missing coverage evidence
	_3773/3774	acyl-coA synthetase/AMP-acid ligase/R transcriptional regulator	missing coverage evidence
	_1982	outer membrane protein E	unassigned new junction evidence
P6.4		putative Na+/H+ antiporter	ISAba1 + 9bp
	_0884	putative Na+/H+ antiporter	missing coverage evidence
	_)885	biofilm-associated protein	missing coverage evidence
	pepN	peptidase M1, alanyl aminopeptidase	missing coverage evidence
	dnaX	DNA polymerase III subunit tau	missing coverage evidence
	_1904	antibiotic biosynthesis monooxygenase	missing coverage evidence
	filE	filE	missing coverage evidence
		ISAba13	unassigned new junction evidence
	_1802	peptidase M16 domain protein	unassigned new junction evidence
	_1982	outer membrane protein E	unassigned new junction evidence

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