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Carter Abbott April 19, 2021

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Abstract

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Antibiotic resistance is a major threat to healthcare. It is estimated that by 2050, ten million people will die per year due to infection by an antibiotic resistant pathogen. Due to this threat, the mechanisms behind antibiotic resistance must be studied in full. Heteroresistance, an understudied mechanism of antibiotic resistance, is the occurrence of a subpopulation of bacteria that are resistant to an antibiotic while the remaining population is susceptible. Antimicrobial susceptibility tests used in hospital clinics often are unable to detect resistant subpopulations, thus, heteroresistance is of unique interest. The inability to detect the resistant subpopulation may result in antibiotic treatment failure, complicating patient care. Of particular concern is tobramycin heteroresistance; tobramycin is an aminoglycoside that serves as a last line of defense antibiotic. In this study, we focus on the amplification of the aminoglycoside modifying enzyme, *aadB*, which results in tobramycin heteroresistance. We aim to investigate the prevalence and mechanisms surrounding the amplification of *aadB*. We report that 32% of the carbapenem-resistant *Acinetobacter baumannii* isolates contain *aadB* and that 56% of these isolates amplify the gene. Additionally, utilizing the tobramycin heteroresistant, carbapenem-resistant *Enterobacter cloacae* strain Mu1307, we establish that the inverted repeats that flank *aadB* are essential for its amplification. Finally, using a murine infection model, we demonstrate that treatment with tobramycin results in selection for *aadB* amplification, offering insight into why amplification of a resistance gene may result in treatment failure.

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Introduction:

Brief History of Antibiotics:

Until the mid-twentieth century, traditional medicine was the only treatment that used antimicrobial agents (1,2). However, it was not known that the plants and soil that were used for medicinal purposes in various cultures contained antimicrobial properties. Accordingly, effective treatments for infectious diseases were not developed; thus, pneumonia, tuberculosis, diarrhea, and diphtheria constituted the leading causes of death until the introduction of antibiotic use in healthcare (3). However, after Fleming's breakthrough discovery of penicillin in 1928 and its eventual introduction to patients by 1942, a sweeping changed occurred. A new age in medicinal care, labeled the "antibiotic era" was ushered in, and treatment for bacterial infections radically changed.

Before this major discovery, Paul Ehrlich believed that scientists could create a "magic bullet" that systematically targets and eliminates pathogenic bacteria (4). In 1909, Ehrlich accomplished his goal and created a drug that could treat patients infected with certain bacteria, including the bacteria that causes syphilis, thus curing syphilis, which had been untreatable at the time (4). Two decades after Ehrlich's discovery, Fleming made his well-known, accidental discovery of penicillin and its antimicrobial properties (5). Convinced that this drug could result in a revolutionary medicine to treat bacterial infections, he sought the aid of chemists to help purify and stabilize the drug. In 1940, Howard Florey and Ernest Chain created a methodology that enabled the mass manufacturing of penicillin (6).

Penicillin's unprecedented effectiveness against bacterial infections resulted in a great demand for antibiotics; thus, the golden age for antibiotic discovery began. During this 20-year span, 18 classes of antibiotics were discovered, forever changing the world of medicine. From

1936 to 1952, deaths due to bacterial illness decreased from over 270,000 to approximately 90,000 (7), life expectancy increased from 58.5 years old to 68.6, and the proportion of the older population increased to 13% from 4% (7,8). Although antibiotic therapy profoundly altered healthcare, Fleming issued a grave warning about the danger of misusing antibiotics: eventually, the bacteria will evolve and become resistant to the antibiotics.

The Public Health Crisis of Antibiotic Resistance:

When antibiotics first became readily available, they were viewed as miracle drugs. Infections that were once untreatable and had high mortality rates were easily treated with a course of antibiotics. Unfortunately, that is no longer the case, as Fleming's prediction has become reality. Bacteria have evolved over time, and these drugs that were once the key to treating bacterial infections are often no longer effective. Over the past few decades, bacteria have become increasingly resistant to antibiotics. In some instances, bacteria even exhibit resistance to antibiotics that have only been widely used for a few years. For example, carbapenems were first used in 1985 and resistance to these antibiotics were recorded as early as 1991 (9).

The rapid increase of antibiotic resistant bacteria has been deemed a public health crisis by both the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (10,11). In the United States, 2.8 million antibiotic resistant infections occur per year, resulting in over 35,000 deaths (10). Additionally, individuals infected by antibiotic resistant bacteria have a 24% longer hospital stay and have a substantially higher mortality rate than individuals infected by susceptible bacteria (12,13). By 2050, it is estimated that 10 million people per year will be killed by antibiotic resistant bacteria (14).

The sharp rise of antibiotic resistant bacteria is largely due to the misuse of antibiotics. In the United States, over 270 million antibiotics are prescribed per year from outpatient pharmacies (15), and almost 30% of these prescribed antimicrobial treatments are not needed (16). Additionally, antibiotics are often unnecessarily used as growth promoters and medicine for livestock, which constitutes 80% of American antibiotic consumption (17). The overuse of these drugs selects for the survival of resistant bacteria and the spread of antibiotic resistance genes (4).

Over time, pathogens have amassed a multitude of resistant traits and have become difficult to treat, even with the antibiotics considered to be the last line of defenses against antibiotic resistant infections (18). This category includes the aminoglycoside tobramycin, which is the drug utilized in the following investigation. Aminoglycosides function by targeting the bacterial ribosomal 30S subunit, preventing the bacteria from correctly producing protein, resulting in bacterial death (19). Due to the increase in multidrug-resistant bacteria, aminoglycosides, including tobramycin, have begun to be used more often. Given tobramycin's significance as a last line of defense antibiotic, it is important that we better understand the mechanisms that allow the bacteria to prevent tobramycin from interfering with protein assembly. While many of these resistant mechanisms have been well-studied, heteroresistance to tobramycin has not (20).

Heteroresistance:

It is typically assumed that a bacterial isolate that is resistant to an antibiotic is resistant on the population level (21). In other words, it is expected that the entire population of bacteria will demonstrate homogeneous resistance. However, this assumption does not hold true for

heteroresistance. Heteroresistance occurs when an isolate harbors a subpopulation of bacteria that has a greater resistance to a specific antibiotic than the remainder of the population (22).

Heteroresistance poses a unique diagnostic challenge in the hospital setting. The gold standard for identifying heteroresistant bacteria is the population analysis profile (PAP) (22). This technique involves plating various dilutions of bacteria on increasing concentrations of an antibiotic, in order to determine the frequency of resistant cells within a population (22). PAPs are labor-intensive and time-consuming; thus, hospital clinics utilize other methods to determine antimicrobial susceptibility, such as E-tests or disc diffusion tests. These methods rely on the expectation that if any bacterial isolates are resistant, the resistance is visible in the entire population. Thus, the disc diffusion test is frequently unable to detect the resistant subpopulation in a heteroresistant isolate (23,24). These undetected resistant subpopulations pose a risk because they can result in inappropriate antibiotic prescription, potentially resulting in the resistant bacteria surviving, causing treatment failure (25–28).

A retrospective study was recently performed to determine the frequency of heteroresistance to colistin in clinical isolates of Carbapenem-Resistant *Enterobacterales* (29). The results of the study showed that out of the 408 isolates that were screened through PAP, 41 (10.1%) were heteroresistant, while 29 (7.1%) were resistant to colistin. Out of the 41 isolates that were heteroresistant, 38 of them would have been classified as susceptible in the clinic. Furthermore, an additional study demonstrated that when mice were infected with colistin heteroresistant isolates and treated with colistin, the antibiotic treatment was unsuccessful (30). These studies demonstrate the risk of undetected heteroresistance in the clinical setting. Despite this threat, the mechanisms that allow bacteria to demonstrate heteroresistance to last line antibiotics, such as aminoglycosides, are understudied. However, it is understood that

amplification of an antibiotic resistance gene is one such mechanism that allows a subpopulation of bacteria to have increased resistance.

Gene Amplification in Heteroresistance:

Gene amplification, the increased copy number of a specific gene, occurs when DNA regions flanked by inverted repeat sequences experience recurrent duplication. This duplication occurs through homologous recombination, which is when identical regions of DNA interact with each other, forming an intersection. The formation of the intersection allows for a crossover between the two regions, resulting in a duplication of the region and increasing the copy number of the genes. The increased copy number results in the genes having a higher expression rate, but this increased copy number is highly unstable and produces a large fitness cost (31,32). Accordingly, the bacteria will revert to a lower copy number over time if there is not a selective pressure for a higher copy number.

Gene amplification can be a cause of heteroresistance when a region of a bacterial genome containing at least one antibiotic resistance gene is amplified in a subset of a bacterial population (33,34). The single copy of the antibiotic resistance gene does not cause the bacteria to be resistant to an antibiotic. However, when the antibiotic resistance gene is amplified, the bacteria's resistance is amplified also because the bacteria is able to increase the expression of said resistance gene in the population demonstrating amplification.

Gene amplification has been observed in heteroresistant bacteria since 1970 and occurs in important pathogens such as *Mycobacterium tuberculosis* and *Streptococcus pneumonia* (22). However, despite the clinical importance of heteroresistance, the importance of gene amplification in production of heteroresistance to aminoglycosides in important pathogens have not been well studied. A recent study has demonstrated that *aadB,* a gene that encodes an

aminoglycoside modifying enzyme, is amplified in *Acinetobacter baumannii* AB5075, resulting in aminoglycoside heteroresistance (35).

In this study, we aim to further the understanding of aminoglycoside heteroresistance in two Gram-negative pathogens classified as an urgent threat by the CDC. We acquired carbapenem-resistant *A. baumannii* (CRAB) isolates to determine the prevalence of aminoglycoside heteroresistance and examine amplification of *aadB.* Additionally, we studied a carbapenem-resistant *Enterobacterales* (CRE) isolate to further investigate the mechanisms relating to *aadB* amplification. Finally, we used a murine infection model to demonstrate that tobramycin treatment results in selection for amplification of *aadB.*

Materials and Methods:

Bacterial Strains and Growth:

The 108 carbapenem-resistant *A.baumannii* strains and the carbapenem-resistant *Enterobacter cloacae* strain Mu1307 were gathered by the Georgia Emerging Infections Program (EIP), Multisite Gram-Negative Surveillance Initiative (MuGSI) between 2013-2015 in and around Atlanta, Georgia, USA. All strains were stored at -80˚C in 15% glycerol and were plated on Muller-Hinton (MH) agar. MH broth was used to culture the isolates and tobramycin was supplemented in MH broth at various concentrations as indicated.

Genomic DNA isolation:

The genomic DNA (gDNA) was isolated from every bacterial strain using the Wizard Genomic DNA Purification Kit (Promega, Madison WI) (36). 1 mL of overnight culture was centrifuged and pellets were resuspended with nuclei lysis solution. Subsequently, the RNA was degraded with RNase solution, the protein was precipitated, and the gDNA was precipitated and

washed. Once the gDNA was washed, it was rehydrated overnight at -4 ˚C with DNA hydration solution.

PCR and Gel Electrophoresis:

The 108 *A. baumannii* strains were screened for the presence of *aadB.* PCR was performed using a Bio-Rad thermal cycler and primers were designed to amplify *aadB* (S. Fig. 1). To confirm the presence of *aadB*, the PCR product was loaded into a 1% agarose gel with 1x TAE as a running buffer and viewed using a ChemiDoc XRS.

Quantitative PCR:

The gDNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer and normalized to a concentration of 12.5 ng/µL. Primers specifically designed for the amplification of *aadB* for both *A. baumannii* and *E. cloacae* strains (S. Fig. 1, 2) and qPCR was performed on the StepOnePlus Real-Time PCR system with 100 ng of gDNA added per well. *clpX* and *rpoD* were used as reference genes for the *A. baumannii* and *E. cloacae* strains respectively. Fold change was determined for each isolate by dividing the *aadB* expression of the treated isolates by the untreated and normalized by the housekeeping gene using the expression $2^{-\Delta\Delta CT}$.

Mice Infection:

Overnight cultures of *E. cloacae* strain Mu1307 were diluted to an OD_{600} (optical density) of 1.0, which is approximately 1×10^8 CFU/mL. The diluted cultures were intraperitoneally injected into each mouse. Two hours post-infection, three of the mice were injected in the peritoneum with 25 mg/Kg of tobramycin and two mice were injected with PBS as a control every eight hours. 24 hours post-infection, the peritoneal fluid, spleen, and liver were

collected, homogenized, and plated on MH agar plates. The plates were incubated for 16 hours at 37 ˚C. After incubation, 5-6 colonies were collected six different times from each organ for every mouse and their gDNA isolated.

Results:

Prevalence of *aadB* Among Clinical Isolates:

To determine the prevalence of *aadB* in carbapenem-resistant *A. baumannii* isolates (CRAB), 108 isolates were obtained through the Georgia EIP, MuGSI collection (37). The isolates were screened for *aadB* through PCR using specific primers to amplify the *aadB* region. Out of 108 of the isolates, 34 of the isolates contained *aadB,* with 26 of these *aadB* confirmed isolates being heteroresistant. Surprisingly, 4 of the isolates that contained *aadB* were susceptible to tobramycin.

Table 1. Prevalence of *aadB* in CRAB Isolates

		Tobramycin Susceptibility		
	All Isolates	Heteroresistant	Resistant	Susceptible
Total	108	39	8	61
aadB	32% (34)	67% (26)	50% (4)	7% (4)

Table 2. Amplification of *aadB* in CRAB Isolates

		Tobramycin Susceptibility		
	All Isolates	Heteroresistant	Resistant	Susceptible
aadB isolates	34	26	4	4
aadB Amplification	56% (19)	62% (16)	75% (3)	0% (0)

Amplification of *aadB*:

The 34 isolates that were confirmed to have *aadB* were evaluated to determine if *aadB* amplification occurs. *aadB* amplification was assessed by calculating the fold change through qPCR. Isolates that had a fold change of 2 or greater were considered amplified. Out of the 34 *aadB* confirmed isolates, 19 of the isolates amplified *aadB*, with 3 of the 4 resistant isolates amplifying *aadB* (Table 2). For the 19 isolates that amplified *aadB*, the amount of amplification varied greatly, ranging from a fold change around 2 to over 100 (Figure 1).

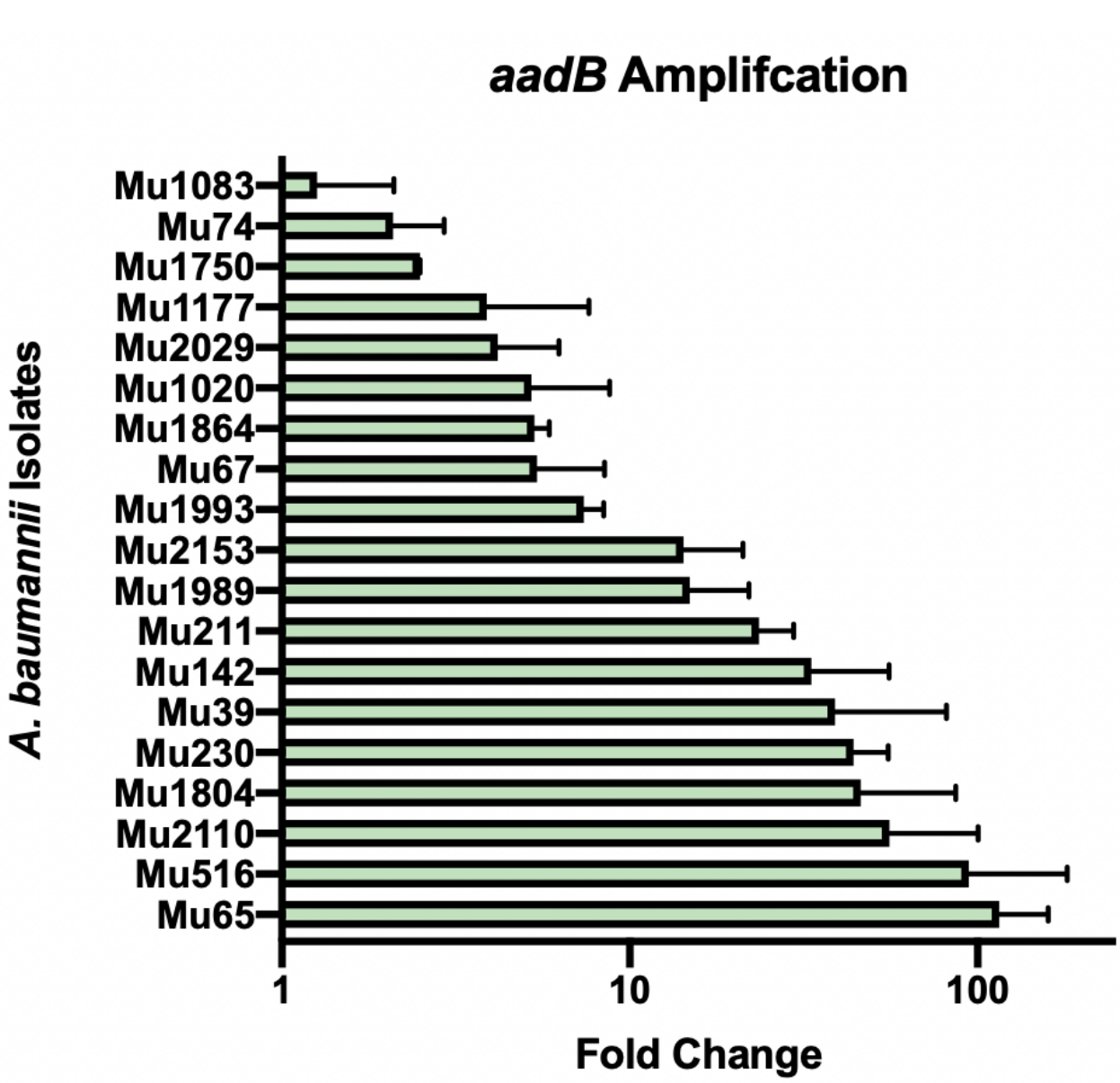


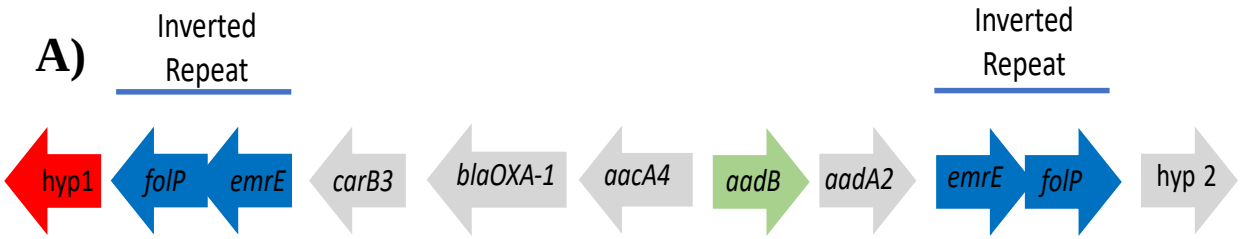
Figure 1. *aadB* Amplification in CRAB Isolates. Fold change was calculated by qPCR of gDNA extracted from the isolates. Each isolate was grown in treated and untreated Muller-Hilton (MH) broth for 18 hours. The treated MHB was supplemented with 32 µg/mL of tobramycin. Fold change was normalized by the expression of *clpX*. Data are the average of 2 biological replicates and the error bars represent standard deviation.

aadB Amplification in *Enterobacter cloacae* Strain Mu1307:

The tobramycin heteroresistant *E. cloacae* strain Mu1307 was utilized to study the mechanisms causing *aadB* amplification to occur. Illumina sequencing was performed on

Mu1307 to determine the DNA sequencing of the strain, confirming the presence of *aadB* in a region flanked by inverted repeats consisting of *folP* and *emrE*. This region also contains four additional antibiotic resistance genes: a chloramphenicol resistance gene (*carB3*), a carbapenemase gene (*bla*$_{OXA-1}$), and two additional aminoglycoside modifying enzyme genes (*aacA4*, *aadA2*) (Figure 2A).

To evaluate the importance of the inverted repeats in their role in homologous recombination, *aadB* as well as the region directly outside of the region that is flanked by the inverted repeats were investigated. This region adjacent to the inverted repeats was labeled hyp1. qPCR was performed on *aadB* and hyp1, showing that *aadB* was amplified while the hyp1 region was not (Figure 2B).



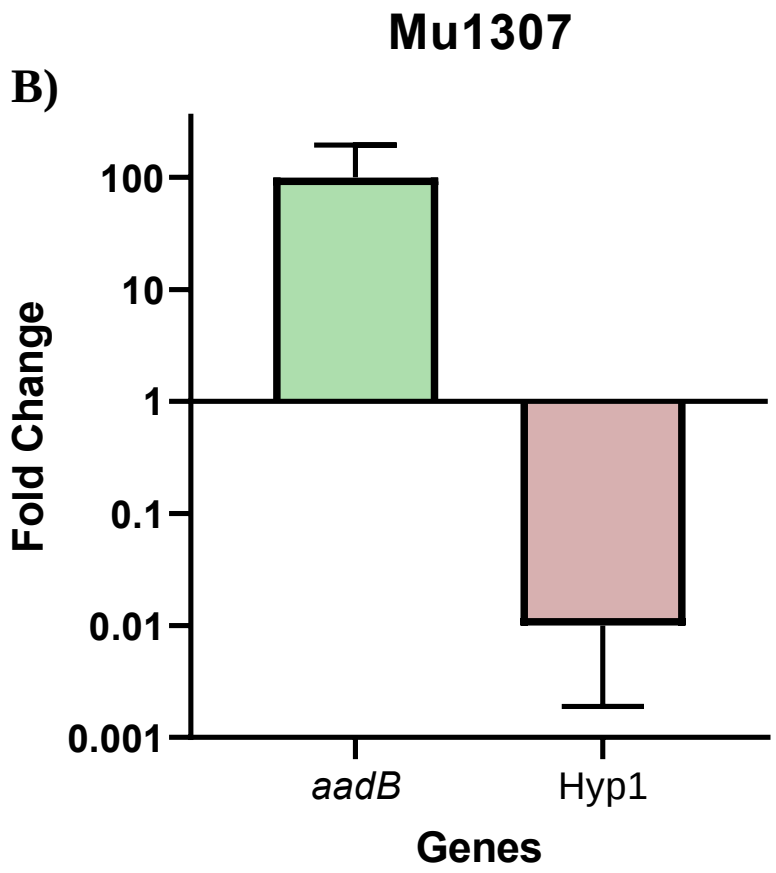


Figure 2. Amplification of Regions in Mu1307. A) Diagram of the region flanked by the inverted repeats, *folP* and *emrE,* as well as the area directly adjacent to the region. B) Fold change was calculated for *aadB* and hyp1 through qPCR of gDNA from the isolates. Each isolate was grown in treated and untreated MHB for 18 hours. The treated MHB was supplemented with 32 µg/mL of tobramycin. Fold change was calculated by comparing the expression in the treated and untreated and normalizing with the expression of *rpoD*. Data are the average of 2 biological replicates and the error bars represent standard deviation.

<u>*AadB* Amplification Dependent on RecA:</u>

To better understand the mechanisms related to *aadB* amplification, mutants of Mu1307 were generated. An Mu1307 *aadB* knockout (Δ*aadB*) was created to examine changes in *aadB* amplification. The results showed that amplification was decreased compared to the wildtype (Figure 3). To follow up, *aadB* was cloned in a plasmid and transformed back into Δ*aadB* (P*aadB*). The amplification of *aadB* in P*aadB* was increased when compared to Δ*aadB*, because the gene is again present, but decreased in comparison to the wildtype because the plasmid borne copy of *aadB* is not subject to amplification (Figure 3). A *recA* knockout strain (Δ*recA*) was also utilized to examine the role that homologous recombination has on *aadB* amplification due to RecA being an essential protein for recombination. The deletion of *recA* resulted in a decrease in the amplification of *aadB* when compared to the wildtype (Figure 3).

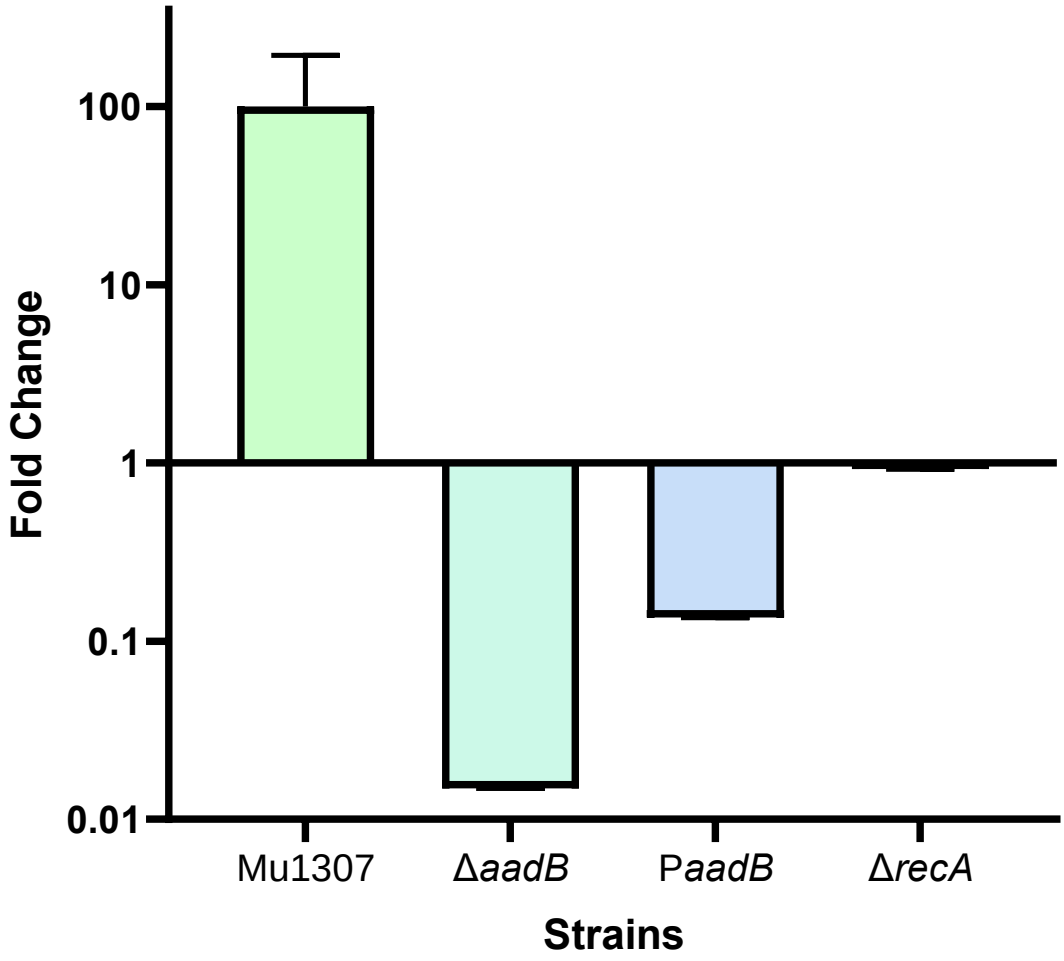


Figure 3. *aadB* and *recA* are Necessary for *aadB* Amplification. Fold change was calculated by qPCR of gDNA from the isolates. Each strain was grown in the highest concentration of tobramycin in MHB they could grow in as well as untreated MHB for 18 hours. Mu1307 was grown with 32 µg/mL of tobramycin, Δ*aadB* with 0 µg/mL, P*aadB* with 16 µg/mL, and Δ*recA* with 4 µg/mL. Fold change was calculated by comparing the expression of each strain to the expression of Mu1307 that was not supplemented with tobramycin and normalized by the expression of *rpoD*. Data are the average of 2 biological replicates and the error bars represent standard deviation.

Antibiotic Treatment Selects for *aadB* Amplification:

To determine if antibiotic treatment results in the selection of *aadB* amplification, an *in vivo* murine model was utilized. Mice were infected with the *E. cloacae* strain Mu1307 and treated with either tobramycin or a PBS control. The bacteria collected from the liver of the tobramycin treatment group showed a significant increase in *aadB* amplification when compared to mice that were injected with PBS (Figure 4). Bacteria collected from the peritoneum and spleen showed no significant difference in *aadB* amplification in the treated and untreated mice (Figure 4).

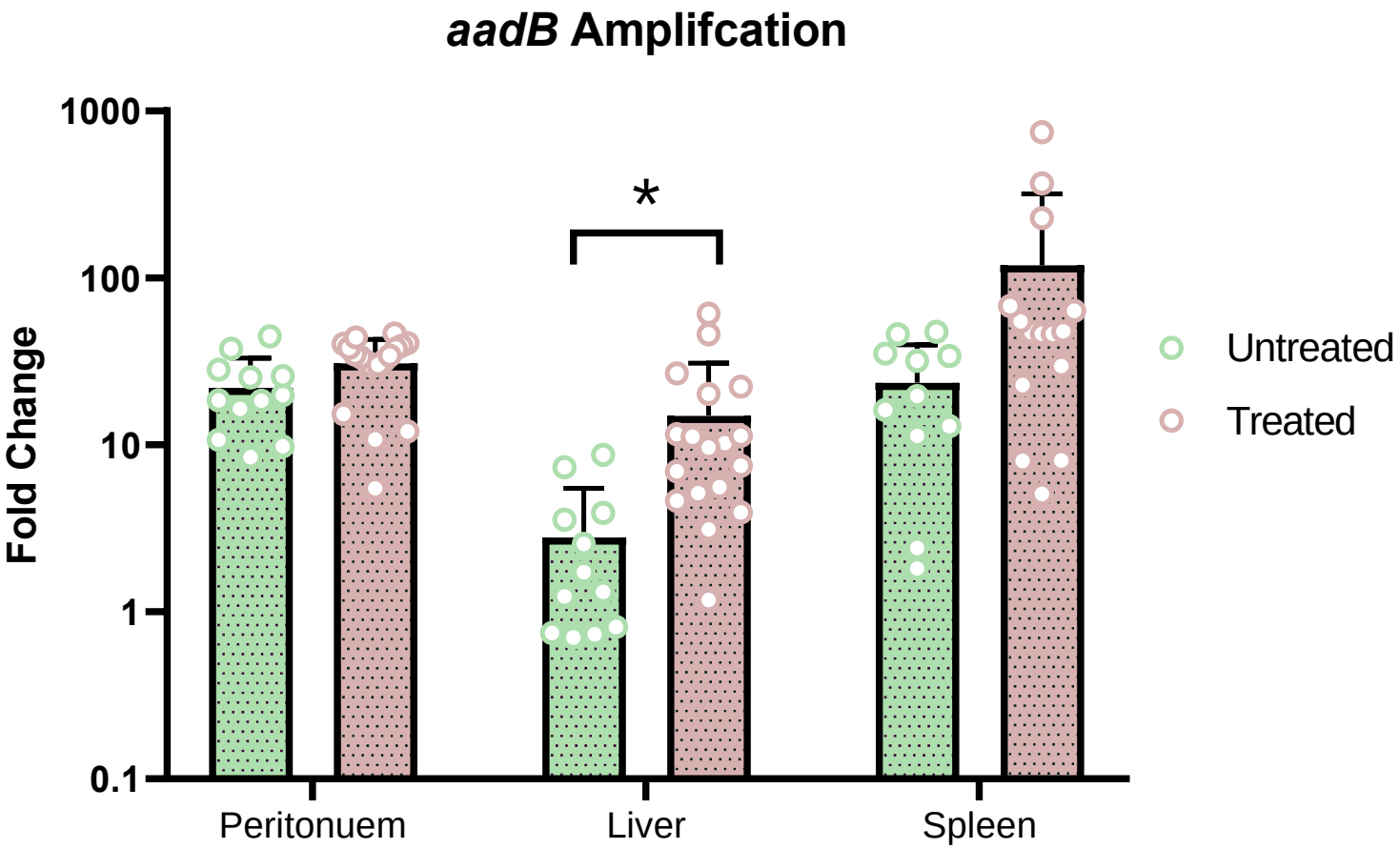


Figure 4. Tobramycin Treatment Selects for *aadB* Amplification. Five C57BL/6 mice were infected intraperitoneally with tobramycin heteroresistant *E. cloacae* strain Mu1307. The mice were then intraperitoneally injected with either 25 mg/Kg tobramycin (N=3) or PBS (N=2) every 8 hours, starting 2 hours post-infection. After 24 hours, the peritoneum, liver, and spleen were

collected, homogenized, and plated on MH agar. The plates were incubated for 16 hours at 37 ˚C and 5-6 colonies were collected six different times from the peritoneum, liver, and spleen for each mouse. These 5-6 colonies were grouped together for gDNA isolation and qPCR.Fold change was calculated by comparing the amplification of *aadB* in each group to *aadB* amplification in Mu1307 prior to the infection and then normalized with *rpoD* expression. Statistical analyses were performed using a two-sample unpaired *t*-test with Prism 8

Discussion:

Antibiotic resistance is an increasingly urgent threat to healthcare and threatens a shift to a post antibiotic era. A serious concern of antibiotic resistance is unexplained antibiotic treatment failure, the causes of which must be fully investigated. One potential reason for the occurrence of treatment failure is heteroresistance. The resistant subpopulations in heteroresistant pathogens often go undetected in the clinic, which can potentially result in the prescription of an ineffective antibiotic. One such class of antibiotics is aminoglycosides. Aminoglycosides serve an important role as last line of defense antibiotics and are often only used when a pathogen shows broad resistance to multiple classes of drug. Therefore, mechanisms of aminoglycoside heteroresistance must be well understood.

Our study first sought to determine the prevalence of the aminoglycoside modifying enzyme, *aadB*, in CRAB isolates. We determined that *aadB* was present in 67% of the heteroresistant isolates and that *aadB* was amplified in 58% of the heteroresistant isolates that contained *aadB* (Table 1, Table 2). The tobramycin heteroresistant isolates that did not amplify *aadB* may be heteroresistant due to the amplification of other aminoglycoside modifying enzymes such as *aacA4* and *aadA2*. Although these findings are limited due to only examining CRAB isolates, they enhance the characterization of resistant mechanisms in carbapenem-

resistant isolates. With over half the isolates containing *aadB*, we must improve our understanding of the mechanisms that result in *aadB* amplification.

In order to determine if *aadB* amplification is selected for with tobramycin treatment *in vivo*, a murine infection model was used. Our results showed that an increase of *aadB* amplification was selected for with treatment of tobramycin in bacteria that were collected from the liver and that the subpopulation of bacteria that amplified *aadB* substantially, survived antibiotic treatment (Figure 6). One possible reason why selection for bacteria that amplified *aadB* only occurred in the liver may be due to how tobramycin is metabolized differently in the liver than the kidney. These results potentially reflect why antibiotic treatment failure occurs in the clinical setting. The subpopulation of bacteria that are amplifying a resistance gene are resistant to the prescribed antibiotic and can survive in the host and continue to grow, causing the infection to continue.

To investigate this dependency, we used a strain of Mu1307 (P*aadb)* where *aadB* had been knocked out and inserted on a plasmid region, lacking inverted repeats. We observed that *aadB* amplification did not occur in the P*aadb* strain. We then investigated RecA, a protein essential for homologous recombination. When *recA* was knocked out of Mu1307 (Δ*recA*), *aadB* was no longer amplified (Figure 5). Based on these findings, it is probable that the inverted repeats play an essential role in *aadB* amplification, and therefore tobramycin heteroresistance, within Mu1307.

In order to determine if *aadB* amplification is selected for with tobramycin treatment *in vivo*, a murine infection model was used. Our results showed that an increase of *aadB* amplification was selected for with treatment of tobramycin and that the subpopulation of bacteria that amplified *aadB* substantially, survived antibiotic treatment (Figure 6). These

results potentially reflect why antibiotic treatment failure occurs in the clinical setting. The subpopulation of bacteria that are resistant to the prescribed antibiotic can survive in the host and continue to grow, causing the infection to continue.

In conclusion, our work improves our understanding of the mechanisms surrounding the amplification of resistance genes. Gaining a better understanding of these mechanisms is of major importance as heteroresistant pathogens pose a major problem in hospital clinics. In the future, we hope to examine how increased *aadB* amplification correlates to the minimum inhibitory concentration of tobramycin for each CRAB isolate. To avoid the looming threat of the loss of effective antibiotic treatments and the predicted 10 million deaths per year by 2050, we must continue to investigate heteroresistance in pathogens and establish effective ways to detect heteroresistance in the clinical setting.

Supplemental Figures:

Supplemental Figure 1. Primers used for CRAB Isolates

CRAB Isolates		
Gene	**PCR Primer Sequence**	**qPCR Primer Sequence**
aadB Fw	CTGCGGCAGATGAGCCAAATCT	TCCCCGATCTCCGCTAAGAA
aadB Rv	GCCGCATATCGCGACCTGAAA	CAGATGAGCGAAATCTGCCG
clpX Fw		GCGTTTGAAAGTCGGGCAAT
clpX Rv		CCATTGCAAACGGCACATCT

Supplemental Figure 2. Primers used for Mu1307

CRE Mu1307	
Regions	**qPCR Primer Sequence**
aadB Fw	ACTGGCCTACAAAGCACA
aadB Rv	ATATCGCGACCTGAAAGC
rpoD Fw	CCGGAAGACAAGATCCGTAAAG
rpoD Rv	CCTCGATGAAATCACCCAGATG
hyp1 Fw	TCA GAC GCC GGA TAG ATT C
hyp1 Rv	ATG GAC AGC GAG GAG CCT C

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