Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Edgar Sherman Date

Aminoglycoside Heteroresistance: Gene Amplification and Antibiotic Treatment Failure in Gram-Negative Pathogens

By

Edgar Sherman
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Microbiology and Molecular Genetics

David S. Weiss, Ph.D.
Advisor

William M. Shafer, Ph.D.
Committee Member

Charles P. Moran, Ph.D.
Committee Member

Philip N. Rather, Ph.D.
Committee Member

Graeme L. Conn, Ph.D.
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

Aminoglycoside Heteroresistance: Gene Amplification and Antibiotic Treatment Failure in Gram-Negative Pathogens

By

Edgar Sherman
B.S., The University of Texas at San Antonio, 2014

Advisor: David S. Weiss, PhD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Microbiology and Molecular Genetics
2020

Abstract

Aminoglycoside Heteroresistance: Gene Amplification and Antibiotic Treatment Failure in Gram-Negative Pathogens

By Edgar Sherman

Antibiotic resistance is a global challenge that threatens public healthcare by reducing our ability to prevent and treat bacterial infections. Antibiotic resistance results in approximately 2.8 million infections and 35,000 deaths annually in the United States. Further challenging our limited treatment strategies is unexplained antibiotic treatment failure, which may be due to heteroresistance. Heteroresistance occurs when a bacterial strain harbors a majority susceptible population as well as a minority resistant subpopulation. The resistant subpopulations can go undetected by clinical diagnostic techniques but nonetheless prevent antibiotic therapy from clearing an infection. In this report, we describe heteroresistance to aminoglycosides. Many Gram-negative pathogens have acquired widespread resistance to multiple classes of antibiotics but remain susceptible to aminoglycosides, leading to a revived interest in their clinical use. Here, we report results of a surveillance study for aminoglycoside heteroresistance in the Gram-negative pathogens carbapenem-resistant -*Acinetobacter baumannii* (CRAB) and -*Enterobacteriaceae* (CRE; *Klebsiella pneumoniae, Escherichia coli,* and *Enterobacter cloacae*). We find that in sampling of multidrug resistant clinical isolates, heteroresistance was observed to the drugs tobramycin, gentamicin, and amikacin. Interestingly, we report the presence and amplification of an aminoglycoside modifying enzyme *aadB* in over one-third of the tobramycin heteroresistant CRAB isolates. Additionally, in CRE we compared our detection rate of aminoglycoside heteroresistance to clinical diagnostic data and report the frequency of inaccurate diagnostic testing. To explore the mechanisms facilitating heteroresistance, we use a genetic approach to analyze a tobramycin heteroresistant strain of *E. cloacae*, Mu1307. We find that Mu1307 exhibits amplification of the aminoglycoside modifying enzyme *aadB*. We describe amplifications that are found in distinct populations and that amplifications are unstable and reverted in the absence of the antibiotic. Furthermore, we show that tobramycin heteroresistance is mediated by *aadB* utilizing a knockout strain of Mu1307. Finally, we show that tobramycin heteroresistance causes treatment failure. These findings demonstrate the *in vivo* impact of aminoglycoside heteroresistance and highlight the clinical challenges that may be posed by this form of antibiotic resistance.

I dedicate this dissertation first and foremost to my mother, Patricia Sherman for all her hard work in making me the person that I am today who without this work would not have been possible. Also, to my brothers, Adrian sherman and Osvaldo Lara, with love.

I would also like to dedicate this dissertation to Elda A. Trevino, and Lorena S. Trevino. Thank you for providing your love and emotional support as well as encouragement throughout my graduate school career, and all the amazing happy memories and laughs together.

I would like to acknowledge the special people in my life including [DRC] (Pete, Rene, Danny, George, Kevin, Sammy, and FG), RaulRoberto, Amy, Kassie, Aly, Paris, Party people and the Fedora bois! (Luis, Seyi, Caro, Roxana, Jessie, Jackie, Lisa). They have seen me at my best and worst and celebrated and commiserated. To all the friends I have made here at Emory and throughout my graduate career, thank you all for everything.

I want to thank Dr. Karl E. Klose, Dr. Gail P Taylor, and Darrell Balderrama and the everyone at the Emory Microbiology and Molecular Genetics Program, and IMSD for showing me the value of hard work, preparing me for research, supporting my education as well as countless others and believing in me.

Lastly, this dissertation is dedicated to all my family and friends, new and old that have been there and helped me along the way; and for the people of Del Rio, to show that anyone can do anything regardless of background or circumstance. SI SE PUEDE!

I would like to thank my advisor David S. Weiss and my committee members for all the valuable input and training they have provided me over the course of my graduate education. I would also like to thank Dr. William M. Shafer for my inclusion as a trainee in the Antimicrobial Resistance and Therapeutic Development Training Grant which was instrumental to my development as a graduate student.

Aminoglycoside Heteroresistance: Gene Amplification and Antibiotic Treatment Failure in Gram-Negative Pathogens

By

Edgar Sherman
B.S., The University of Texas at San Antonio, 2014

Advisor: David S. Weiss, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Microbiology and Molecular Genetics
2020

Table of Contents

Chapter 1: Introduction

Antibiotic Resistance

The discovery and purification of antibiotics has allowed for significant advances in modern medicine and reduction in patient mortality [1,2]. Before antibiotic therapy was widely available, treatment of infections relied on the antimicrobial properties of traditional medicines based on plant and mold extracts [3,4]. Before the early 20th century, it was not only common to die from bacterial infections, but surgical interventions posed a severe health risk due to development of post-procedure infections. With the advent of antibiotic therapy, patients not only recovered from bacterial infections, but could more safely undergo complex surgical procedures with minimal risk of infection, undergo organ transplants that require lifelong immune suppressants, or tolerate chemotherapy that impairs the immune system. Though these advanced treatment options and medical techniques are possible now, there may come a time in the near future when we are no longer able to safely treat or prevent bacterial infections due to the rise of antibiotic resistance.

Shortly after the discovery of the first isolated antibiotic, penicillin in 1928 by Alexander Fleming, he also noted that it was possible to generate penicillin resistant mutants in the laboratory [5]. Fleming even went on to warn about the dangers of antibiotic resistance during his Nobel Prize acceptance speech stating that the misuse of penicillin could generate mutants that would be resistant [6]. Many new classes of antibiotics have since been discovered, the vast majority of which were isolated from natural sources during the golden age of antibiotic discovery between the years 1950 and 1970 [7]. However, resistance has been observed to each of these antibiotic classes, sometimes shortly after their clinical introduction. This is due to the fact that bacteria have been encountering these threats since long before humans ever utilized them for their antibacterial

properties. Bacteria have been in a constant battle against other organisms for resources and that includes adapting and evolving methods to circumvent these natural antibiotic compounds found in their environment [8,9]. Now we are entering a period where antibiotic resistance has ignited global concern due to the danger to public health.

As of 2019, antibiotic resistant bacteria are estimated to cause over 2.8 million infections in the United States, which results in at least 35,000 deaths annually [10]. It is estimated that deaths caused by antibiotic resistance will surge dramatically if new antibiotic classes are not discovered, and by 2050, over 10 million people will die worldwide as a result of antibiotic resistant infections every year [11]. These increased deaths will not only be the result of direct bacterial infection but also the loss of our ability to safely perform complex medical procedures that require antibiotics to prevent infections. It is crucial that we address the causes of antibiotic resistance as well as ways to prevent the misuse and overuse of antibiotics. This includes the development of newer antibiotics, antibiotic stewardship, and regulating the use of antibiotics in agriculture. Finally, we must understand the mechanisms that cause antibiotic resistance in order to develop effective treatment strategies to improve patient care and health.

Aside from the resistance mechanisms that are known and studied extensively, another cause for concern is when the underlying basis for instances of antibiotic treatment failure are unclear. When a patient presents with a bacterial infection, the bacteria are cultured, identified, and then tested for susceptibility to antibiotics. The attending physicians will then treat the patient with antibiotics that are expected to resolve the infection. However, the patient’s condition may not change or continue to worsen as the infection progresses. The cause of this antibiotic treatment failure is often unclear.

It is crucial that we study the mechanisms of antibiotic resistance since this phenomenon is a looming threat to modern healthcare. We must direct our focus on preventing further decline of our antibiotic arsenal to remain effective against bacterial infections. In this report, we study an understudied resistance phenomenon to aminoglycosides, an important class of antibiotics that is seeing a resurgence in use due to multidrug resistance.

Aminoglycosides and Resistance Mechanisms

Aminoglycosides are a class of highly polycationic antimicrobials that include natural or semisynthetic drugs. They share a common cyclic structure with either a streptidine aminocyclitol ring found in streptomycin or a 2-deoxystreptamine moiety present in all other aminoglycosides. These rings contain substitutions on either the 4,5 or 4,6 regions that impart specific pharmacokinetic binding properties [12,13]. Natural aminoglycosides are isolated from the Gram-positive *Actinomycetes* species. The first aminoglycoside, streptomycin, was discovered and isolated in 1943 from *Streptomyces griseus*. Streptomycin was recognized for its potent bactericidal activity and was the first antibacterial to cure tuberculosis [14]. While aminoglycosides have broad bactericidal activity against Gram-negatives, use of these drugs was limited due to concerning side effects in patients, specifically nephrotoxicity and ototoxicity [15]. However, due to the increase in antibiotic resistance, there is a resurgence in the use of aminoglycosides to treat infections caused by multidrug resistant pathogens [16,17].

Aminoglycosides enter the cell through active transport by the electron transport chain. This active transport requirement makes aminoglycosides less effective against anaerobic bacteria [18,19]. Once inside the cell, the aminoglycosides target the bacterial 30S ribosomal subunit [20].

Specifically, they bind to the A site on 16S ribosomal RNA of the 30S subunit and diminish the accuracy of proofreading the encoded mRNA message. The different substitution groups found on aminoglycosides determine their affinity for a particular region of the A site [21,22]. This interaction results in incorrect amino acid placement during protein elongation which can result in misfolded or truncated proteins [23,24]. Though the exact mechanism of aminoglycoside killing is unclear, it is commonly thought that disrupted membrane proteins assemble into the cell membrane, thereby compromising the integrity of the bacterial structure resulting in lysis [25]. Some aminoglycosides have also been shown to block elongation or directly inhibit protein translation instead of affecting mRNA proofreading fidelity [12]. While aminoglycosides retain activity against most Gram-negative bacteria, several resistance mechanisms have been characterized against this class of antibiotics.

Aminoglycoside Resistance Mechanisms

Aminoglycoside resistance can occur through a variety of different mechanisms which are outlined in **Figure 1.1** [26]. Aminoglycoside resistance can occur through modification of the outer LPS membrane to prevent entry into the cells or through mutations that affect the rate of transfer through the electron transport chain and arrest growth, thereby reducing self-promoted uptake through porin channels that was first described by Hancock *et al.* [27-31]. Active transport of aminoglycosides out of the cell through efflux pumps has been reported [32,33]. Mutations to the 16S rRNA that affect aminoglycoside binding can occur, though this mutation is not very common as this can be lethal to the cell. The mutants A1408G, C1402T, and G1484T

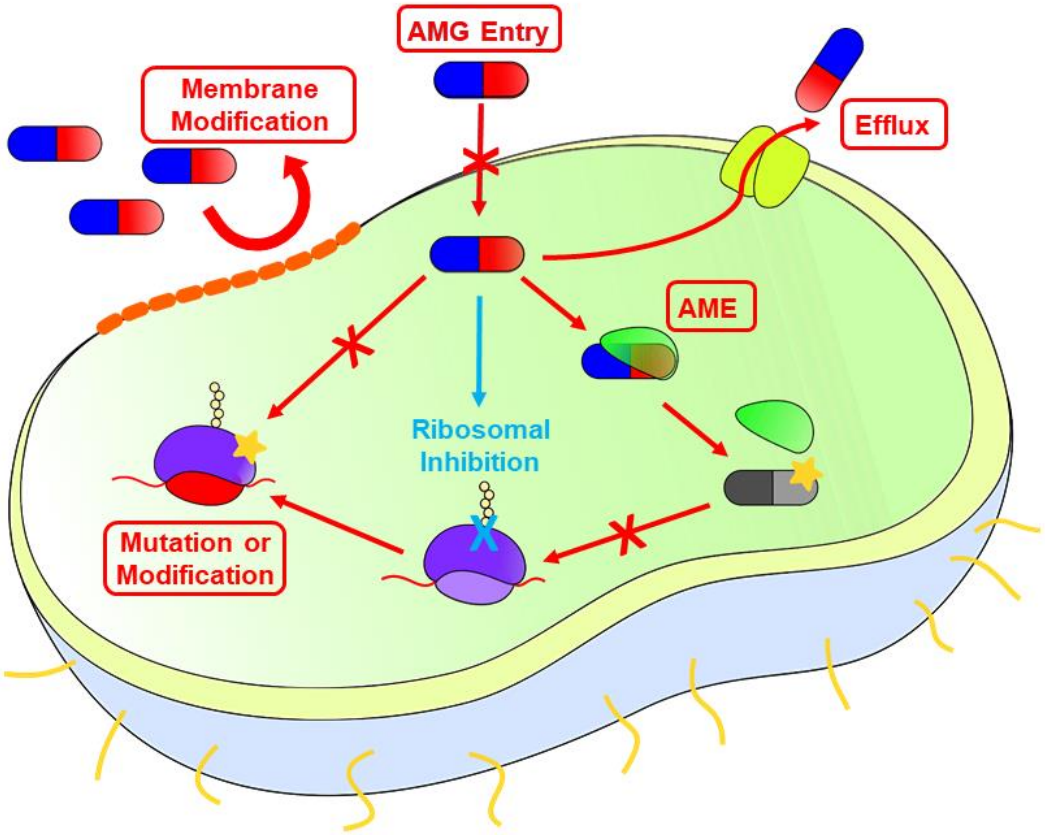


Figure 1.1. Examples of Aminoglycoside Resistance Mechanisms. Aminoglycosides enter the cell by active transport through the electron transport chain. Once in the cytosol, aminoglycosides bind to the 30S bacterial ribosomal subunit and diminish the ribosomal proofreading precision of the mRNA. The lack of proofreading results in misfolding or truncated proteins that cause cellular lysis. Several resistance mechanisms to aminoglycosides have been reported. These mechanisms include 1) decreased transport through the bacterial membrane. 2) Mutation or modification of the ribosome to prevent aminoglycoside binding. 3) Efflux through multidrug transporters. 4) Inactivation by aminoglycoside modifying enzymes (AMEs). AMEs are the most common and abundant mechanism of aminoglycoside resistance.

have been observed and studied in *Mycobacterium tuberculosis* [34]. A more common mechanism of ribosomal resistance is modification of the aminoglycoside binding site by RNA methyltransferase enzymes, which likely originated from aminoglycoside-producing *Actinomycetes* species to methylate their own ribosomes to protect against aminoglycoside self-intoxication [35]. One of the most common mechanisms of aminoglycoside resistance is the acquisition of aminoglycoside modifying enzymes, which this report will focus on.

Aminoglycoside modifying enzymes (AMEs) are proteins that bind to aminoglycosides and chemically modify the drugs. This modification inactivates the drug, usually by addition of a chemical group that prevents the aminoglycoside from binding to the 30S ribosomal subunit. AMEs are divided into three classes depending on the modification that occurs to their respective aminoglycoside substrate. These classes are N-Acetyltransferases, which comprise the largest subclass and transfer an acetyl group; O-nucleotidyltransferases, the second largest class that transfer an adenyl group; and finally, O-phosphotransferases, which add a phosphate group. AMEs target aminoglycosides based on specific chemical groups present in their structure. Thus, if aminoglycosides share the same chemical group targeted by a given AME, multiple aminoglycosides can be affected by the same enzyme. Over 100 AMEs have been characterized to date [36-38]. Semi-synthetic aminoglycosides have been developed since the 1970s to circumvent AMEs by substituting the targeted chemical group with another group that allows the aminoglycoside to retain binding and activity against the bacterial ribosome. The most commonly used semisynthetic aminoglycoside is amikacin, and the most recently developed being plazomicin [39,40].

If the use of aminoglycosides is to be revived for the treatment of multidrug resistant pathogens, it is imperative that we fully appreciate resistance mechanisms. One area that requires

further investigation is aminoglycoside heteroresistance, as the study of this phenomenon are lacking.

Heteroresistance

Antibiotic resistance is assumed to affect an entire bacterial population that evolves or acquires additional resistance mechanisms [41,42]. Antibiotic resistance caused by phenotypic variation within one bacterial isolate has not been extensively reported. However, phenotypic variation may appear more frequently than previously observed among cell subpopulations.

Heteroresistance is Distinct from Homogenous Resistance

Heteroresistance occurs when an antibiotic resistant subpopulation is present in a bacterial isolate that harbors a majority of susceptible cells. The resistant subpopulation shows a higher level of antibiotic resistance but can be difficult to detect through routine diagnostic testing if it is present at a very low frequency (>1 in 10,000 cells) [42,43]. The most accurate methods of detecting these resistant subpopulations has been through population analysis profiles, in which the population of bacteria is subjected to increased concentrations of antibiotic on agar plates and the colony-forming units (CFUs) are enumerated at each concentration, providing an overall outline of the resistant subpopulation [44,45]. The resistant subpopulation is able to replicate in the presence of antibiotics, which distinguishes it from metabolically dormant persister cells, another class of resistant subpopulation. The frequency of the resistant subpopulation in heteroresistance is often unstable, increasing to nearly 100% as the susceptible population is killed in the presence of drug, but often decreasing to the baseline frequency as the antibiotic is removed from the environment and the susceptible population expands. This instability reveals that these subpopulations are not the result of spontaneous stable mutations.

Heteroresistance was first observed in 1947 after subpopulations resistant to the aminoglycoside streptomycin were discovered in *Haemophilus influenzae* [46]. Since the initial discovery of these resistant subpopulations, heteroresistance has been observed to numerous classes of antibiotics (including polymyxins, beta-lactams, trimethoprim, and aminoglycosides) and in distinct bacterial species such as *Acinetobacter*, *Klebsiella*, *Pseudomonas*, and *Escherichia* [42,47,48]. While heteroresistance has been observed in select isolates, studies examining the prevalence and mechanisms underlying this phenotype have been sparse. Heteroresistance has also been observed in other domains of life beyond prokaryotes. Resistant subpopulations have been reported in eukaryotes such as *Candida* spp. to antifungals, *Trypanosoma* spp. to antiparasitics, and in cancer cells to anticancer therapies[49-53]. Investigating the factors that mediate this phenomenon would therefore have a broader impact not just for antibiotic treatment in bacteria, but other important fields as well.

Heteroresistance Mediates Antibiotic Treatment Failure In Vivo

The clinical relevance of heteroresistance on patient outcome has been in dispute for some time. This is exemplified by several reports with vancomycin heteroresistance in *Staphylococcus aureus* (hVISA). These reports contain conflicting evidence that the resistant subpopulations either alter treatment outcomes or have no significant impact [54-56]. This discrepancy may in part be due to the instability and variable frequency of the resistant subpopulations. Resistant subpopulations can be observed between orders of magnitude of 10^{1} (~10%) to as low as 10^{-4} (0.0001%) within a bacterial population, though it is unclear how low the rates of these populations can exist due to the accuracy of the detection methods used. These undetectable resistant subpopulations can result in the incorrect administration of antibiotics that select for the resistant

subpopulation instead of treating the bacterial infection. This lack of detection may be one reason that antibiotic therapy can fail in patients.

A recent study by Band *et al.* has shown that heteroresistance to the last line antibiotic colistin does in fact mediate *in vivo* treatment failure using a murine infection model [41,57]. It was shown that in two strains of *Enterobacter cloacae* with resistant subpopulations to colistin, occurring at frequencies of 1/10 or 1/10,000 were sufficient to render colistin treatment ineffective in mice. These studies suggest that the antimicrobial resistant subpopulations do indeed have an *in vivo* impact. While these studies highlight that heteroresistance can cause treatment failure, studies of the clinical relevance to other important classes of antibiotics are still lacking, including aminoglycosides. A potential mechanism that has been observed for generating resistant subpopulations in heteroresistance is gene amplification [48,58,59].

Homologous Recombination and Gene Amplification

Homologous recombination is a widely conserved biological process across all three domains of life, allowing for the repair of double stranded DNA breaks and genetic rearrangement of the genome [60-62]. This process occurs when regions of DNA that share repeated sequences interact creating junctions where cross-over events occur between these areas. In bacteria, this process is mainly facilitated by the RecBCD pathway. Briefly, the RecBCD enzyme complex initiates recombination by binding to a double stranded break in the DNA strand. The next step is unwinding of the double stranded DNA segment using helicase activity by both RecB and RecD and digestion of one strand by the RecB nuclease domain. Once RecBCD encounters a Chi site composed of a specific nucleotide sequence, the enzyme undergoes a conformational change and causes RecBCD to cleave the DNA strand containing the Chi site. RecA proteins are then loaded onto the single stranded DNA sequence to prevent degradation and this protein-DNA complex

interacts with regions of DNA with similar homology. Once a homologous region is encountered by the RecBCD complex, this interaction facilitates a conformational change in the protein-DNA complex to form a Holliday junction with the DNA strand. There are several outcomes that can result from homologous recombination of a Holliday junction: gene deletion, or duplication [63-65].

Gene amplifications occur when regions of DNA undergo repeated cycles of gene duplication between regions flanked by repeat sequences. As the genes within the repeat sequences increase in copy number, this increases expression of these genes as well. Gene amplification has been described as a mechanism for antibiotic resistance in bacteria since the 1970s. In *Proteus mirabilis* strain NR1, amplifications were detected within a plasmid-borne region containing three resistance genes for chloramphenicol, streptomycin, and sulphonamide. When NR1 was grown in a low concentration of drug, plasmid size was shown to increase as well as the level of resistance. This was confirmed to be a direct result of gene amplification and the genes encoding resistance to these antibiotics were found to be encoded in the same region. This region was flanked by direct repeats of insertion sequence 1 (*IS*1) [66,67]. Other studies have shown amplification for other classes of antibiotics including tetracyclines, beta-lactams, and macrolides [59,68-71]. Similar to heteroresistance, gene amplification has been implicated in resistance to antimicrobials in eukaryotic organisms such as anticancer therapeutic resistance, indicating a much broader relevance for investigating this phenomenon [72,73].

In a recent study by Nicoloff *et al.*, it was observed that gene amplifications were seen in the majority of heteroresistant isolates that were examined. The amplifications occurred in genes facilitating resistance to beta-lactams, trimethoprim, and aminoglycosides [48]. It has been reported that gene amplification of the AME *aadB* is required for tobramycin heteroresistance in

A baumannii strain AB5075 [58]. An AB5075 *recA* mutant showed increased susceptibility and a sharp decrease in the resistant subpopulations, though a small subset of RecA-independent colonies were observed and will be discussed at the end of this report.

As the threat of antibiotic resistance continues to grow, it is important that we fully understand the mechanisms facilitating resistance to maintain and increase the efficacy of our current antimicrobial treatment options. Heteroresistance is a phenomenon that may be one reason that antibiotic treatments sometimes fail unexpectedly. This failure is due in part to the presence of resistant subpopulations that go undetected by routine clinical diagnostic testing. Therefore, it is crucial that we determine the full scope of heteroresistance, especially against antibiotics that are being revived for treatment of multidrug resistant pathogens, such as aminoglycosides, to prepare strategies that will allow us to adapt when faced with this phenomenon in a clinical environment.

In this study, we examine the prevalence of aminoglycoside heteroresistance among two distinct groups of Gram-negative pathogens labeled as urgent threats by the Centers for Disease Control and Prevention due to their high rates of multidrug resistance and limited treatment options. These groups are carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Enterobacteriaceae* (CRE; *Enterobacter* spp., *Escherichia* spp., and *Klebsiella* spp.) We characterize heteroresistance in these pathogens to the aminoglycosides tobramycin, gentamicin, and amikacin. We further investigate the role of gene amplification of the aminoglycoside modifying enzyme *aadB* in tobramycin heteroresistance for both CRAB and CRE. Finally, we determine if tobramycin heteroresistance can mediate treatment failure using an *in vivo* model of a heteroresistant infection in a mouse model. These results provide new insight of the clinical impact of aminoglycoside heteroresistance.

Chapter 2:

Aminoglycoside Modifying Enzyme *aadB* in Heteroresistant Carbapenem-Resistant *Acinetobacter baumannii* Clinical isolates mediates treatment failure.

Edgar X. Sherman, Sarah E. Anderson, Chui-Yoke Chin, Carter Abbott, Sarah W. Satola, Eileen M. Burd, Philip N. Rather, David S. Weiss.

The work in this chapter will also be submitted for publication in a peer-reviewed journal.

Abstract

As antibiotic resistance continues to increase, we must expand our understanding of the underlying genetic mechanisms that bacteria utilize and devise new approaches to combat them. Heteroresistance is a phenomenon in which a resistant subpopulation of cells can rapidly replicate in the presence of a given antibiotic and co-exist with a majority susceptible bacterial population. Heteroresistance can lead to antibiotic treatment failure and pose a challenge in treating highly resistant infections such as carbapenem-resistant *Acinetobacter baumannii* (CRAB). Recently, aminoglycoside heteroresistance was described in *A. baumannii* but its prevalence and relevance are unclear. Here, we report the widespread prevalence of heteroresistance to tobramycin, gentamicin, and amikacin among 108 CRAB clinical isolates. Heteroresistance was observed more frequently than conventional homogenous resistance, in which all the cells in a population exhibit resistance. Further, many of these heteroresistant isolates exhibited amplifications of the resistance gene *aadB* (17 of the 26 isolates encoding the gene). We report a positive correlation between higher MIC values and increased *aadB* amplification among CRAB isolates. Finally, we examined the *in vivo* significance of *aadB* in a pathogenic strain of *A. baumannii* and show that the amplified region harboring *aadB* mediates tobramycin treatment failure in a murine infection model. These data suggest that amplifications of aminoglycoside modifying enzymes may appear frequently in resistant subpopulations, and that aminoglycoside heteroresistance is prevalent among CRAB and can mediate treatment failure.

Keywords

Acinetobacter baumannii, Heteroresistance, Aminoglycosides, Gene Amplification, Population Analysis Profile

Introduction

Antibiotic resistance continues to remain an inevitable danger to modern healthcare as antibiotic susceptibility decreases and antibiotic treatments become limited [10]. Annual deaths as a result of antibiotic resistance are projected to surpass 10 million globally by 2050 [11]. Further expanding our knowledge of the antibiotic resistance mechanisms used by bacteria will be critical to preventing further risk to public health. Heteroresistance is the phenomenon where antimicrobial resistance subpopulations co-exist within a susceptible population of genetically susceptible bacteria [42]. These populations can actively grow in the presence of antibiotic unlike persistor cells, which remain dormant until the antibiotic is cleared [74,75]. The frequency of these resistance subpopulations can fluctuate during and after antibiotic treatment, making this resistance unstable and unlikely the result of a spontaneous mutation. The underlying concern with heteroresistance is that the unstable variation in resistant population frequency enables these subpopulations to go undetected by routine clinical examination [76]. These heterogenous resistant populations complicate treatment as only a fraction of the population is resistant to a particular antibiotic and may be difficult to eliminate, resulting in antibiotic treatment failure [41,57]. This is in contrast to homogenous resistance in which the entire bacterial population is resistant due to either a mutation, expressed resistant gene, or modification that is present throughout the entire population.

Heteroresistance was first identified in 1947 and since then has been reported to several important classes of antimicrobials such as β-lactams, glycopeptides, antimicrobial peptides, and aminoglycosides [42,46,54,58,77-79]. Heteroresistance has been observed in several pathogenic bacterial species, including *Acinetobacter baumannii*. Carbapenem-resistant *A. baumannii* (CRAB) is a Gram-negative nosocomial pathogen that is classified as an urgent threat by the CDC

due to widespread multidrug resistance, and a critical priority pathogen for the development of novel therapeutic interventions by the world health organization [10,80,81]. Mechanisms of *A. baumannii* antibiotic resistance include decreased membrane permeability, increased expression of efflux pumps, target site alterations, acquisition of β-lactamases, and aminoglycoside modifying enzymes. Resistant subpopulations to colistin and aminoglycosides have been reported in *A. baumannii* [58,82].

Like conventional antibiotic resistance, there is a pressing need to determine the genetic mechanisms that lead to heteroresistance to find novel strategies to combat this distinctive threat to global health. While the exact mechanisms have not yet been well characterized, amplifications of various resistance genes have been described to several classes of antibiotics including aminoglycosides. Recently, it was reported that subpopulations resistant to the aminoglycosides tobramycin and gentamicin were present in the model pathogenic strain of *A. baumannii* AB5075 [58]. These resistant subpopulations fluctuated during antibiotic treatment and an amplification event was found in these populations of an aminoglycoside modifying enzyme, *aadB*. Aminoglycoside modifying enzymes are among the most common resistance mechanism to this class of drugs and new data have shown that heteroresistance to aminoglycoside occurs through amplification of aminoglycoside modifying enzymes [16,36,48].

While these findings may give an indication that gene amplifications may play a role in heteroresistance, surveillance data for the occurrence of heteroresistance in pathogenic bacteria is lacking to conduct any thorough investigation, including for aminoglycosides. These data can help guide our decisions on antibiotic use to prevent unwanted treatment failures. Here, we report the rate of aminoglycoside heteroresistance among clinical isolates of CRAB. We used population analysis profiles (PAP) to determine the rate of heteroresistance to the aminoglycoside tobramycin

in CRAB isolates which occurred at 35.8%. Additionally, we tested for heteroresistance to another aminoglycoside targeted by *aadB*, gentamicin and found that 44.4% CRAB isolates were heteroresistant. We also tested CRAB isolates for amikacin heteroresistance (21.3%), an aminoglycoside which is unaffected by this enzyme. We screened the tobramycin heteroresistant CRAB isolates for the presence of *aadB* and detected it in 66.7% of these strains. We then used qPCR and found that aadB is amplified in 64.4% of the tobramycin heteroresistance aadB-positive isolates. Finally, we show that tobramycin heteroresistance mediates treatment failure in a murine infection model using the *A. baumannii* pathogenic laboratory strain AB5075 and that that aadB is required.

Results

Prevalence of aminoglycoside heteroresistance among clinical isolates

Following the report that tobramycin heteroresistance was found in the *A. baumannii* strain, AB5075, we first set out to determine if heteroresistance was also present in additional isolates of *A. baumannii*. For the surveillance network, we sampled strains of carbapenem-resistant *A. baumannii* (CRAB) which were obtained from the Georgia Emerging Infections Program, Multi-site Gram-Negative Surveillance Initiative (MuGSI) collection [83]. This program collects carbapenem resistant- *Enterobacteriaceae* (CRE), *Acinetobacter baumannii* (CRAB), and *Pseudomonas aeruginosa* (CRPA) isolates to provide routine surveillance data for emerging antibiotic resistance trends along with data provided by clinicians. From this collection, we surveyed 108 CRAB isolates collected in Georgia between 2014-2015.

We performed population analysis profiles to determine if these isolates contained resistant subpopulations to the aminoglycoside tobramycin [45]. Briefly, isolates were selected from single colonies and grown overnight in broth. Cultures were diluted by serial dilution with PBS to a factor

of 1:10^6, and 10 μL spots were plated on agar plates with and without antibiotic and grown overnight as described in Sherman *et al.* [45]. Antibiotic concentrations were calculated using doubling dilutions above and below the antibiotic breakpoint (1X). Survival was calculated by dividing the cells that grew on the plates with antibiotic from the control plate without antibiotic. Isolates were either classified as susceptible if the colonies did not survive above 2X the breakpoint, resistant if the colonies survived above the breakpoint and remained above 50% survival, or heteroresistant if the surviving population was below 50% at 2-4X above the breakpoint (**Figure. 2.1a**).

From the population analysis profiles (PAP), we report that heteroresistance was found for tobramycin at a rate of 35.8% in the CRAB isolates (**Table 2.1**). Since the aminoglycoside modifying enzyme *aadB* effects more than one aminoglycoside, we tested the CRAB isolates for heteroresistance to a second *aadB*-effected aminoglycoside, gentamicin. When tested by PAP, gentamicin had the highest reported rate of heteroresistance at 44.4% and appeared at a similar rate to tobramycin heteroresistance. We tested another aminoglycoside, amikacin, that is unaffected by *aadB,* and the amikacin heteroresistance rate occurred at a rate of 21.3% among the CRAB isolates (**Table 2.1**) and was below the levels detected for tobramycin and gentamicin. We observed that heteroresistance rates in these isolates appear to occur more frequently than conventional homogenous resistance, appearing at almost 3-5X more frequently than the conventionally resistant isolates (**Figure 2.1b)**. We compared these results to the rates at which these strains were identified in the clinic and saw that when heteroresistant and homogenous resistant isolates were combined, their values generally aligned with what is being seen in the clinic (**Table 2.1**). These data show that heteroresistance is occurring in CRAB isolates and that

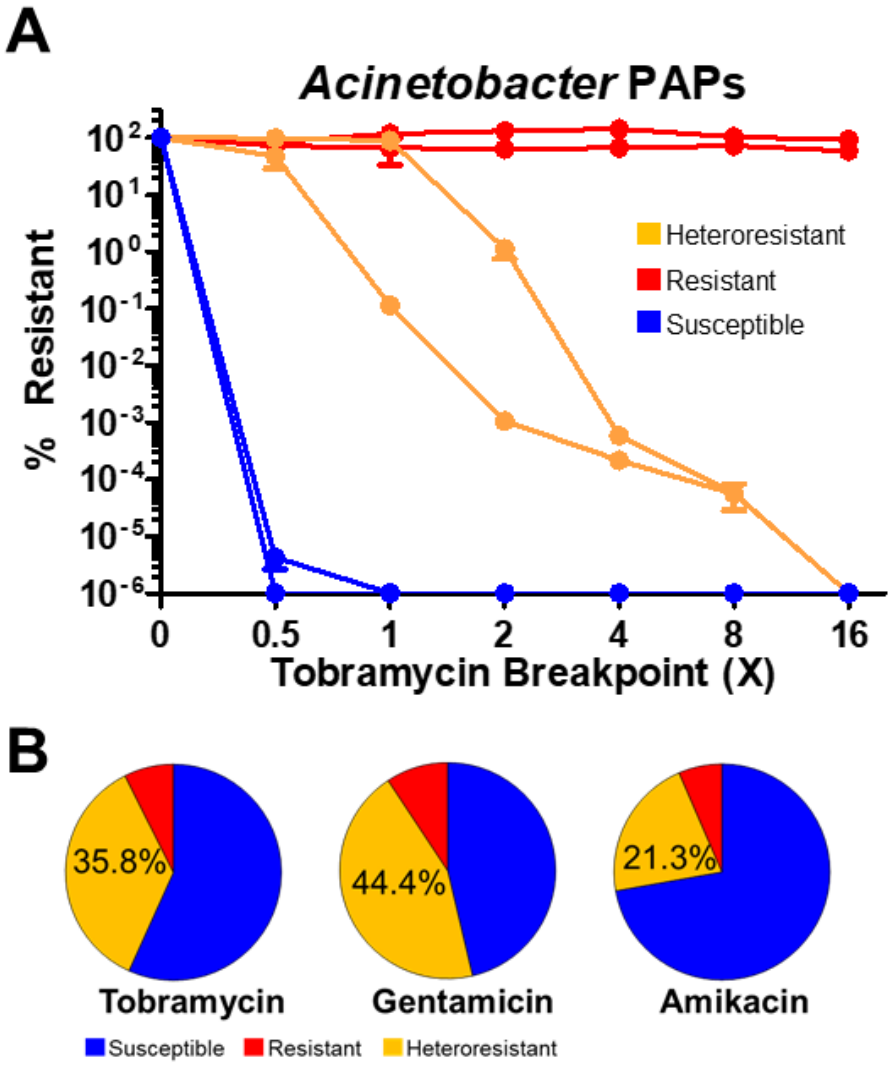


Figure 2.1. Heteroresistance Incidence Rates in Carbapenem-Resistant *Acinetobacter baumannii* (CRAB). A) An example of a tobramycin population analysis profile with two resistant strains (Mu1812, Mu1864), two susceptible strains (Mu693, Mu700), and two heteroresistant strains (Mu32, Mu230). B) Distribution of aminoglycoside heteroresistance among CRAB isolates. Strains were identified as heteroresistant if the surviving population was below 50% and at 2-4X above the breakpoint for each drug tested by population analysis profile.

	Clinical Determination		PAP Results		
Drug	**Susceptible**	**Resistant**	**Heteroresistant**	**Susceptible**	**Resistant**
TOB	60.2% (65)	39.8% (43)	35.8% (39)	56.5% (61)	7.4% (8)
GEN	33.3% (36)	66.7% (72)	44.4% (48)	46.3% (50)	9.3% (10)
AMK	58.4% (63)	41.6% (45)	21.3% (23)	72.2% (78)	6.5% (7)

Table 2.1. Clinical Summary of Vs. Rates of Heteroresistance in CRAB Isolates

the frequency varies based on the drug. The frequency at which these colonies appear also varies within each strain and for each aminoglycoside (**Supplementary Figure 2.1**).

Presence and amplification of *aadB* among CRAB clinical isolates

In the tobramycin resistant subpopulation of the laboratory *A. baumannii* strain AB5075, amplification of the aminoglycoside modifying enzyme *aadB* was detected and associated with heteroresistance. *aadB* is a 2”-aminoglycoside nucleotidyltransferase (ANT(2”)-Ia) gene which adenylates the 2-deoxystreptamine aminoglycosides at the hydroxyl group at position 2" with an adenylmonophosphate group [84]. This enzyme can be encoded in plasmids or within integrons and has been found in *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Morganella morganii*, *Escherichia coli*, *Salmonella typhimurium*, *Citrobacter freundii* and *A. baumannii* [84-87]. The aminoglycosides targeted by *aadB* are tobramycin, gentamicin, and kanamycin[88]. In AB5075, this gene was discovered on the plasmid p1AB5075 contained in an integron region along with 4 other resistance genes which were all likely amplified along with *aadB*.

We screened for the presence of *aadB* in all the CRAB isolates by PCR as previously described [58]. We found *aadB* in all three tobramycin susceptibility categories of the 108 CRAB isolates (**Table 2.2**). Among the 39 tobramycin heteroresistant strains, *aadB* was detected in 26 isolates (65.8%). While in the 8 tobramycin resistant strains, *aadB* was detected in 4 (50.0%) and in only 4 out of the 61 tobramycin susceptible strains (7.4%). Most of the strains that contained *aadB* were also heteroresistant to gentamicin (**Table 2.3**), which was expected as *aadB* targets this aminoglycoside as well. Tobramycin heteroresistance was not associated with amikacin heteroresistance, as this drug would not be affected by *aadB.*

		Tobramycin Determination		
	All Isolates	**Heteroresistant**	**Resistant**	**Susceptible**
Total	108	39	8	61
aadB	31.5% (34)	66.7% (26)	50.0% (4)	6.6% (4)

Table 2.2. Presence of *aadB* in CRAB Isolates.

Acinetobacter baumannii Complex MuGSI Strains	MIC (μg/ml)			PAP Analysis			*aadB* Amplification
	TOB	GEN	AMK	TOB	GEN	AMK	
AB5075	96	>256	>256	HR	HR	R	+
Mu32	4	8	>256	HR	HR	HR	-
Mu39	>8	>8	≤16	HR	HR	S	+
Mu54	>8	8	>32	HR	S	S	-
Mu65	>8	>8	>32	HR	HR	S	+
Mu67	>8	>8	>32	HR	HR	S	+
Mu74	>8	>8	32	HR	HR	S	+
Mu142	8	>8	≤16	HR	HR	S	+
Mu211	4	4	128	HR	HR	S	+
Mu230	>8	>8	32	HR	HR	S	+
Mu516	8	>8	≤16	HR	HR	S	+
Mu1020	8	≥16	16	HR	HR	S	+
Mu1051	≤1	≤1	2	S	S	S	-
Mu1083	≤4	>8	≤16	HR	HR	S	+
Mu1177	>8	>8	>32	HR	HR	S	+
Mu1188	≥16	≥16	>256	R	R	R	-
Mu1264	>8	>8	>32	HR	S	S	-
Mu1381	8	≥16	6	HR	HR	S	-
Mu1480	>8	>8	≤16	HR	S	S	-
Mu1676	>8	>8	>32	HR	HR	S	+
Mu1734	>8	>8	>32	HR	HR	S	-
Mu1737	>8	>8	>32	HR	HR	S	-
Mu1750	2	8	64	HR	HR	HR	+
Mu1792	>8	>8	32	HR	HR	HR	-
Mu1804	≥16	≥16	>256	R	R	R	+
Mu1857	≤1	≥16	4	S	S	S	-
Mu1864	≥16	≥16	>256	R	R	R	+
Mu1988	≤1	2	48	S	S	S	-
Mu1989	≥16	≥16	32	HR	HR	S	+
Mu1993	≥16	≥16	>256	R	R	R	+
Mu2029	>8	>8	≤16	HR	HR	S	+
Mu2034	≤1	≤1	2	S	S	S	-
Mu2043	8	≥16	>256	HR	HR	HR	-
Mu2110	8	≥16	256	HR	R	S	+
Mu2153	8	≥16	>256	HR	R	S	+

Table 2.3. MIC Values and PAP Results for Tobramycin Heteroresistant CRAB Isolates Encoding *aadB*.

Amplification of *aadB* among CRAB clinical isolates

From the 26 *aadB*-positive isolates, we checked for amplifications by qPCR. We examined fold changes by comparing *aadB* activity in the treated strains and dividing by the untreated control and normalizing using the *clpX* housekeeping gene. We noted any strains that had a fold change of two or greater as a positive result. In the 26 CRAB isolates, 17 showed amplification of *aadB* compared to the untreated control at a rate of 65.4% (**Figure 2.2**). We also report amplifications in 3 of the 4 resistant strains that contained *aadB* but did not detect any amplifications in the susceptible strains. The level of amplifications varied by strain, with some as low as 2X differences in copy number and other strains showing over 100X changes in copy number.

Based on this difference in copy number, we wanted to determine if there was a correlation between increased amplification level and MIC. When comparing the amplifications and the MIC of each strain using linear regression, we see that there is a positive correlation between strains that have higher levels of amplifications and higher MIC values, with an R^2 value of 0.79 (**Figure 2.3**). This indicates that amplifications may influence the overall resistance of the population. The exact mechanisms of how amplifications are controlled for heteroresistance is being investigated.

In vivo* Treatment Failure in Tobramycin Heteroresistant AB5075 is Mediated by *aadB

Lastly, we wanted to investigate the role of *aadB* using a murine infection model as the *in vivo* relevance of tobramycin heteroresistance has remained unclear. We choose to use the

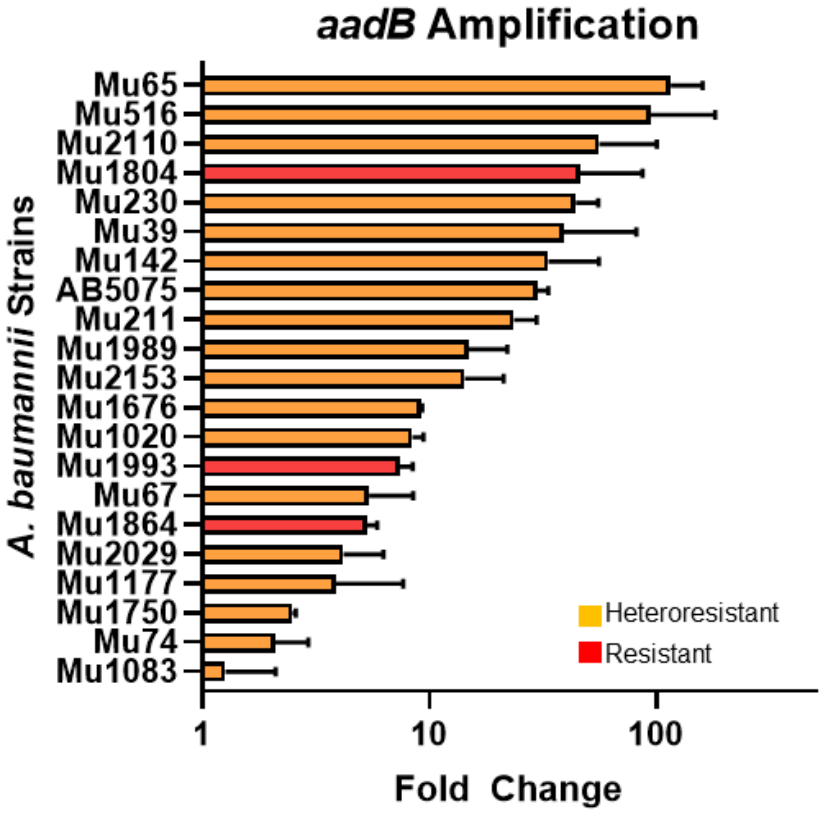


Figure 2.2. Amplification of *aadB* in CRAB Clinical Isolates. Amplifications were measured by qPCR of gDNA isolated from CRAB isolates grown in MH media supplemented with 32 μg/ml of tobramycin for 24 hours and comparing the fold change of qPCR from untreated strains grown in MH media alone for 24 hours. Data are presented as the averages of three independent biological replicates. Error bars represent the standard error of mean (S.E.M.)

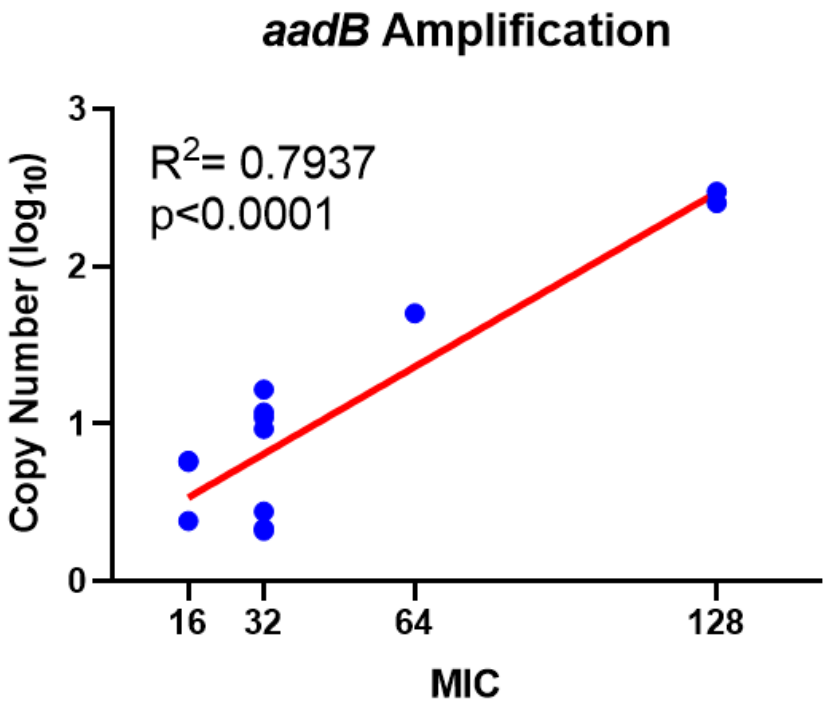


Figure 2.3. Increase in *aadB* Copy Number Correlates with Higher MIC Value in CRAB. Fold changes in *aadB* shown in previous figure were compared with the MIC values measured by broth microdilution for each of the CRAB isolates then analyzed by fitting the data using a simple linear regression analysis in Prism 8 (GraphPad).

laboratory strain AB5075 for our infection model as that was the strain where *aadB* amplifications were initially detected and the infection model is well established to limit the variance that may come from using clinical isolates. When we systemically infected mice with a tobramycin susceptible strain of *A. baumannii* (Mu1649) and treated with tobramycin, there was a significant decrease in the CFUs in the spleen, liver, and peritoneum. There was no significant CFU decrease when AB5075 was treated with tobramycin, showing that treatment is ineffective in this strain (**Figure 2.4**).

To assess the role of *aadB*, we used a strain of AB5075 (R38) that lacked *aadB* as well as four other resistance genes that were initially discovered in the integron region that was being amplified in the p1AB5075 plasmid, though none of those genes target tobramycin. Systemic infections were repeated as in the first experiment. When R38 was treated with tobramycin, there was a significant decrease in the CFUs compared to the wild type (**Figure 2.5**). R38-infected mice were also rescued during survival experiments when treated with tobramycin, whereas all the mice died early regardless of tobramycin intervention in AB5075-infected mice. To conclude if *aadB* was involved for the tobramycin resistance in R38, we complemented *aadB* alone on a plasmid and observed that there was a restoration of resistance to tobramycin (**Supplementary Figure 2.3**). From these data, we see that tobramycin heteroresistance mediates *in vivo* treatment failure in mice.

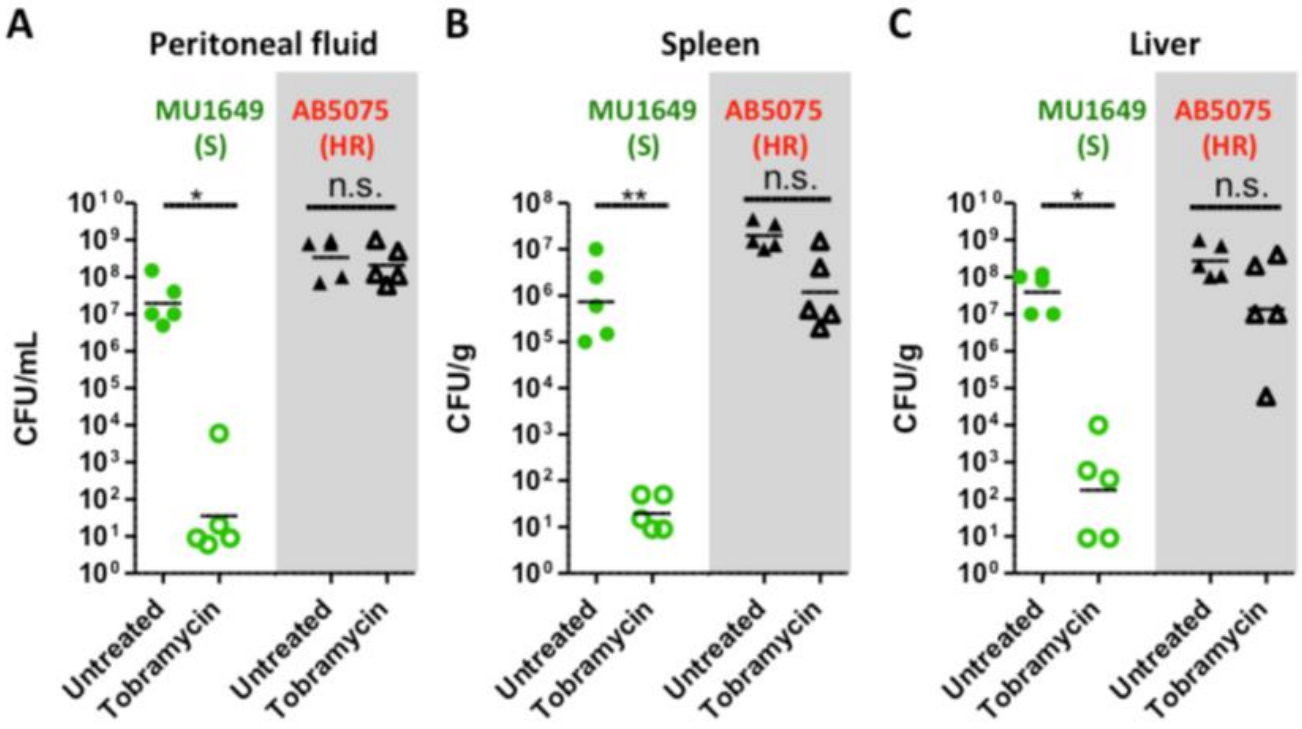


Figure 2.4. Tobramycin Heteroresistance Causes Tobramycin Treatment Failure in AB5075. Groups of 5 female C57BL/6 mice were infected via intraperitoneal injection with approximately $1x10^8$ CFUs of the tobramycin-susceptible *A. baumannii* isolate Mu1649 or heteroresistant isolate AB5075. Subsequently, they were treated with either PBS vehicle control (untreated) or tobramycin 5 mg/kg every 8 hours starting at 1-hour post-infection. Organs were harvested 24 hours later and CFUs were quantified. Bacterial loads from the (A) peritoneal fluid, (B) spleen, (C) liver were calculated at 24 hours post-infection.

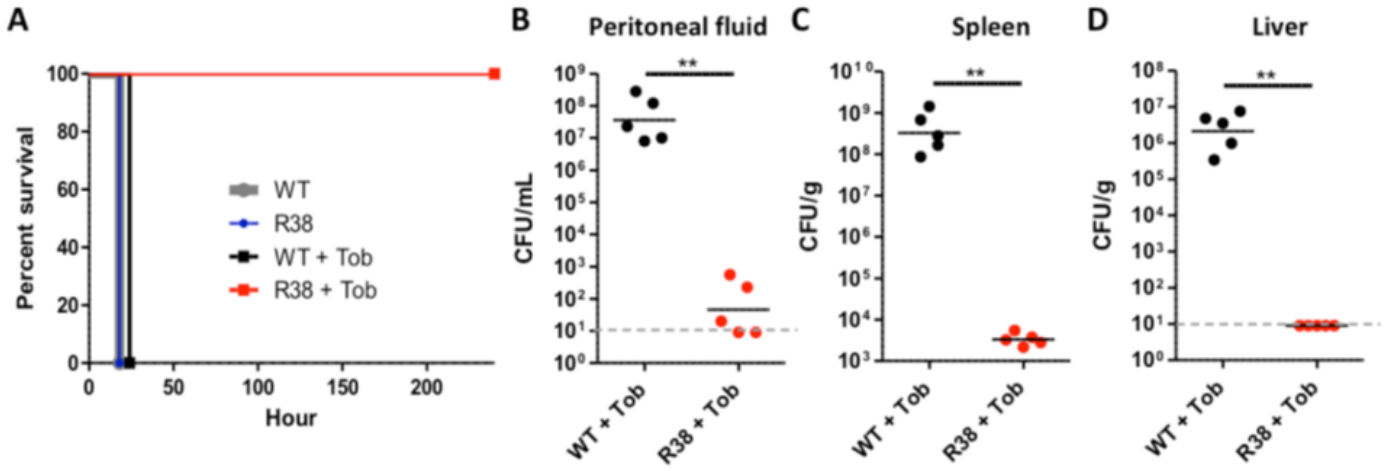


Figure 2.5. *AadB* Mediates Tobramycin Treatment Failure in Heteroresistant Isolate AB5075. C57BL/6 mice were infected intraperitoneally with tobramycin heteroresistant strain AB5075 (WT) or the isogenic R38 mutant in which the *aadB*-containing amplified region was deleted and were treated with PBS or 5mg/kg tobramycin every 8 hours starting at 1-hour post-infection. (A) Mice were monitored for signs of illness and euthanized if they appeared sick or if their weight dropped below 80% of starting weight. (B-D) AB5075 (WT) and R38 infected mice were euthanized at 20 hours post-infection following treatment as in (A), and the (B) peritoneal fluid, (C) spleen, and (D) liver were collected and plated for enumeration of CFU. $p<0.005$ (**).

Discussion

The results of this study show that heteroresistance occurs to aminoglycosides and appears relatively common among carbapenem-resistant *A. baumannii* clinical isolates. This collection of clinical isolates can begin to help determine the extent of the underlying resistance mechanisms that may be occurring throughout the healthcare environment. These resistant subpopulations occurred at varying rates for each drug shown, even with drugs affected by the same resistance gene. We found that the aminoglycoside modifying enzyme *aadB* was present in over half of the tobramycin heteroresistant isolates and that amplification not only occurs in AB5075, but also in the CRAB clinical isolates as well. This study also highlights the importance of *aadB* in an infection model and demonstrate that heteroresistance can mediate treatment failure.

For the surveillance work, we choose two drugs that were affected by *aadB* and one that was not. Both tobramycin and gentamicin are modified by *aadB*, while amikacin is unaffected by this enzyme. We observed that heteroresistance rates in these isolates appear to occur more frequently than homogenous resistance, which may suggest that these resistant subpopulations may be more common than expected, appearing at almost 3-5X more frequently than the conventionally resistant isolates (**Table 2.1**). The values of the strains that were identified as homogenous resistant isolates was combined with the strains that were detected as heteroresistant by population analysis profiles and approximately align with what was found through clinical diagnostic testing. While this may be true for aminoglycoside heteroresistance in CRAB isolates, there have been reported examples where heteroresistant isolates to other classes of antibiotics were missed by routine clinical diagnostic testing and may account in some part for instances of unexpected antibiotic treatment failure. We also show that strains that appeared to have higher measured MIC also had higher amplification fold changes. This may signal that higher

amplifications have an effect on resistance, though this is not a perfect correlation as there are additional factors that can determine MIC, and this cannot be based solely on the amount of *aadB* copy number.

If gene amplifications play a role in heteroresistance, then the RecA-dependent homologous recombination system may also factor in determining heteroresistance [60,89]. It has been shown that a mutation in *recA* resulted in a decrease in the tobramycin resistant subpopulation in AB5075. While we noted that over half of the tobramycin heteroresistant strains contained *aadB* and that this may be more common among aminoglycoside heteroresistant isolates, this does not account for the other *aadB*-positive strains that did not show amplifications or *aadB*-negative tobramycin heteroresistant isolates that were also found by PAP. A cause for *aadB* independent heteroresistance in these isolates could be the result of amplification of another aminoglycoside resistance gene that also targets tobramycin which was shown in Nicoloff *et al* [48]. There may also be other mechanisms generate resistant subpopulations independent of gene amplification. Persister cells formation or mutations that result in aminoglycoside resistance through the electron transport chain have been associated with heteroresistance in Gram-negative bacteria [29,75,90,91]. Regulation of two component systems has been shown to result in colistin heteroresistance. Mutations that affect efflux pumps, or the *pmrAB* two-component signaling system in *A. baumannii* may also generate resistant subpopulations similar to other bacterial species observed so a similar mechanism may exist for aminoglycosides [92,93].

The data presented here show that aminoglycoside heteroresistance is common among CRAB isolates and in *A. baumannii* species in general, though more studies are necessary to determine how common this resistance phenotype is. This study highlights the clinical relevance of aminoglycoside heteroresistance in an established experimental model and that other strains

found in healthcare settings may also possess these resistance mechanisms, furthering the need to explore how to combat these elusive bacterial tactics. Aminoglycoside heteroresistance may cause antibiotic treatment that selects for an increase in resistance instead of elimination of the isolate and result in treatment failure. The exact caused that leads to gene amplification mediated heteroresistance still remains unsolved and further studies are being conducted to elucidate the mechanisms that facilitate this resistance.

Materials and Methods

Strains collection and growth conditions

Liquid cultures were prepared in sterile Mueller-Hinton (MH) broth, supplemented with tobramycin (Spectrum, New Brunswick, NJ) at the concentrations indicated. Plates were supplemented with tobramycin as indicated. Strains of *A. baumannii* were collected between 2013 and 2015 by the Georgia EIP MuGSI from patients at Atlanta area hospitals. MuGSI collects isolates in 8 states, including Georgia, representing a surveillance population of over fifteen million patients. Strains of *A. baumannii* were maintained at −80°C in 15% glycerol.

Population analysis profiles

Population analysis profile (PAP) analysis was performed by growing bacteria overnight to stationary phase and then plating serial dilutions on MH agar alone or with various concentrations of tobramycin mentioned (Spectrum, New Brunswick, NJ). Plates were then incubated at 37°C, and CFU were enumerated after 24 hours. Percent tobramycin resistance was calculated as the number of bacterial colonies that grew on tobramycin plates divided by the number of bacteria that grew on MHA alone without tobramycin.

Clinical susceptibility testing

All isolates were tested for their clinical MIC and resistance designation in the clinical microbiology laboratory at Emory University Hospital. Gentamicin and Tobramycin were tested by automated Vitek 2 (Biomerieux, Marcy l'Etoile, France) using the GN74 susceptibility card. Amikacin susceptibility was tested using the Etest gradient strips (Biomerieux, Marcy l'Etoile, France). Susceptibility results were interpreted by a licensed clinical microbiologist.

Genomic DNA (gDNA) isolation

Genomic DNA was prepared using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Briefly, 1 ml of overnight culture were spun down and resuspended in 600µl of nuclei lysis solution. Cells were lysed and gDNA was precipitated using isopropanol and washed with ethanol as described in the protocol. Following gDNA precipitation, the DNA pellet was resuspended in dH_20 overnight at 4ºC and diluted to a final concentration of 100 ng/µL.

qPCR

Concentrations of gDNA samples were determined using a NanoDrop ND-1000 spectrophotometer. Samples of gDNA were added to a total concentration of 100 ng/well, and qPCR was performed as outlined above for gDNA samples. Standard curves using 10-fold serial dilutions of wild-type gDNA were used to ensure that primers exhibited similar efficiencies. Relative gene copy numbers normalized to *clpX* and were determined using the equation $2^{-\Delta CT}$ [94].

Mice

Female WT C57BL/6J mice were purchased from Jackson Laboratories and used at age 8–10 weeks; all experiments used age- and sex-matched mice. Mice were housed under specific pathogen-free conditions at Yerkes National Primate Center, Emory University. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee. Mouse sample size was determined based on previous studies that generated highly statistically significant results by the Mann–Whitney test ($n \geq 4$), while also minimizing the number of animals used (5 mice per group for the majority of experiments).

Mouse infection model

Approximately 1×10^8 CFU were administered per mouse for infections to quantify the bacterial load and administered for survival experiments. For mouse infections, overnight standing bacterial cultures at room temperature were sub-cultured in LB broth and grown at 37°C with shaking, washed and re-suspended in phosphate buffered saline and diluted to a concentration of 1×10^9 CFU/ml. 100 μL of bacterial inocula were inoculated intraperitoneally (i.p.) to each mouse. At each time point, the mice were sacrificed, and the peritoneal fluid, spleen and liver were harvested, homogenized, plated for CFU on LB plates.

Statistical analysis

All data presented are from experiments that were repeated at least twice to ensure the reproducibility of the results. The significance of the mouse experiments was determined with the Mann–Whitney test, as not all data were normally distributed, and all in vitro experiments were analyzed using the two-tailed student's t-test (for data with a normal distribution). Error bars represent the standard error of the mean of the biological replicates. Statistical analyses were performed with Prism 8 (GraphPad Software, Inc., La Jolla, CA).

Supplemental Material

Primer	Sequence	Use
AadB qPCR Fw	TCCCCGATCTCCGCTAAGAA	*aadB* qPCR
AadB qPCR Rv	CAGATGAGCGAAATCTGCCG	*aadB* qPCR
ClpX Fw	GCGTTTGAAAGTCGGGCAAT	*clpX* qPCR
ClpX Rv	CCATTGCAAACGGCACATCT	*clpX* qPCR

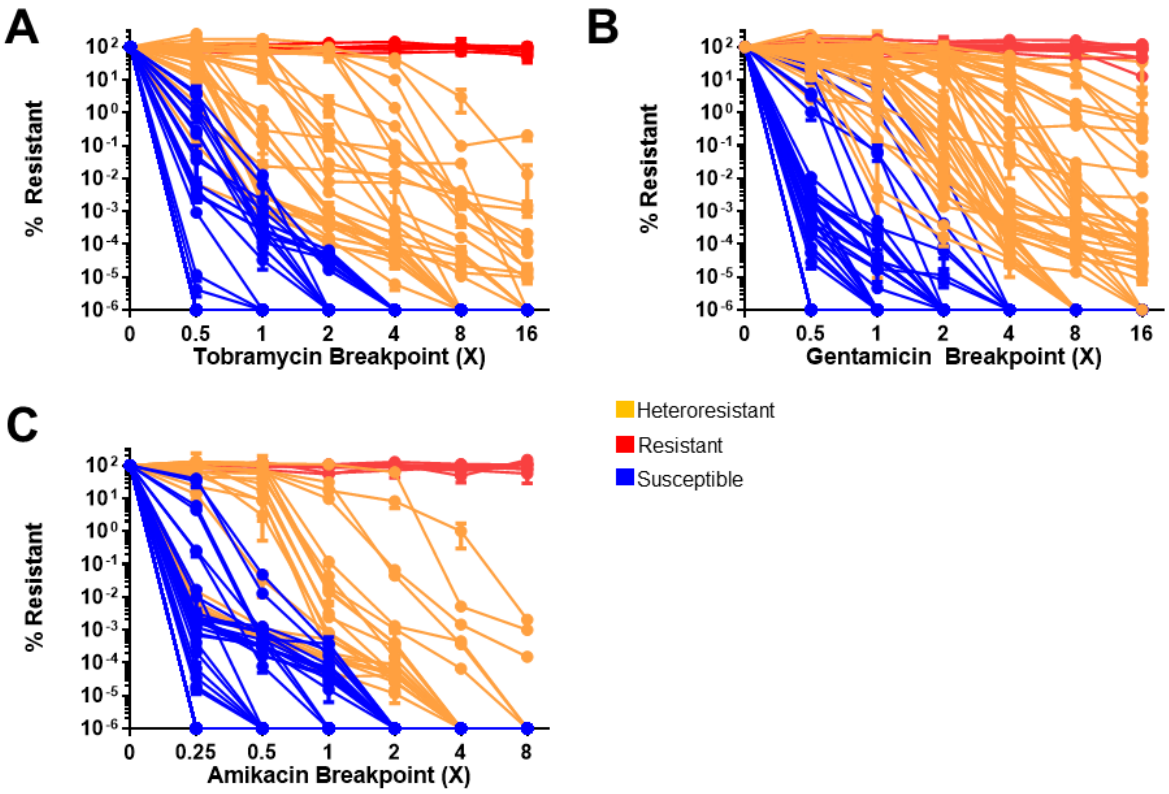
Supplementary Table 1. Primers Used in This Study

Acinetobacter baumannii Complex MuGSI Strains	MIC (μg/ml)			PAP Analysis			*aadB* Confirmed	*aadB* Amplification
	AMK	GEN	TOB	AMK	GEN	TOB		
AB5075	>256	>256	96	R	HR	HR	+	+
Mu32	>256	8	4	HR	HR	HR	+	+
Mu39	≤16	>8	>8	S	HR	HR	+	-
Mu42	192	≤1	≤1	S	S	S	-	-
Mu54	>32	8	>8	S	S	HR	+	+
Mu65	>32	>8	>8	S	HR	HR	+	-
Mu67	>32	>8	>8	S	HR	HR	+	+
Mu74	32	>8	>8	S	HR	HR	+	-
Mu105	>32	>8	>8	S	HR	R	-	-
Mu138	≤16	≤4	8	S	S	HR	-	-
Mu142	≤16	>8	8	S	HR	HR	+	-
Mu211	128	4	4	S	HR	HR	+	-
Mu215	32	4	≤1	S	S	S	-	-
Mu229	8	2	≤1	S	S	S	-	-
Mu230	32	>8	>8	S	HR	HR	+	-
Mu259	8	4	≤1	S	S	S	-	-
Mu268	≤16	>8	>8	S	HR	HR	-	-
Mu286	32	4	≤1	S	S	S	-	-
Mu312	32	>8	>8	S	HR	HR	-	-
Mu316	≤16	>8	>8	S	HR	HR	-	-
Mu341	≤16	>8	8	S	HR	HR	-	-
Mu461	32	2	≤1	S	HR	HR	-	-
Mu516	≤16	>8	8	S	HR	HR	+	-
Mu666				S	HR	HR	-	-
Mu693	2	≤1	≤1	S	S	S	-	-
Mu700	≤16	8	≤48	S	S	S	-	-
Mu701	≤16	>8	>8	S	HR	HR	+	+
Mu737	>32	>8	>8	S	S	S	-	-
Mu768	≥256	≥16	8	S	HR	HR	+	+
Mu797	12	2	≤1	S	S	S	-	-
Mu872	≤16	≤4	≤4	S	S	S	-	-
Mu877	3	≤1	≤1	S	S	S	-	-
Mu891	12	2	≤1	S	S	S	-	-
Mu949	12	4	≤1	S	S	S	-	-
Mu1020	16	≥16	8	S	HR	HR	+	+
Mu1051	2	≤1	≤1	S	S	S	-	-
Mu1083	≤16	>8	≤4	S	HR	HR	+	+
Mu1100	≤16	>8	≤4	S	HR	S	-	-
Mu1177	>32	>8	>8	S	HR	HR	+	+
Mu1188	>256	≥16	≥16	R	R	R	-	-
Mu1264	>32	>8	>8	S	S	HR	+	+
Mu1281	≤ 16	8	≤4	S	S	S	-	-
Mu1376	8	≤1	≤1	S	S	S	-	-
Mu1381	6	≥16	8	S	HR	HR	-	-
Mu1426	≤16	>8	≤4	S	HR	S	-	-
Mu1480	≤16	>8	>8	S	S	HR	-	-
Mu1485	8	≤1	≤1	S	S	S	-	-
Mu1545	32	>8	>8	S	S	S	-	-
Mu1564	≤16	8	≤4	S	S	S	-	-
Mu1572	≤16	>8	≤4	S	HR	S	-	-
Mu1590	≤16	8	≤4	S	S	S	-	-

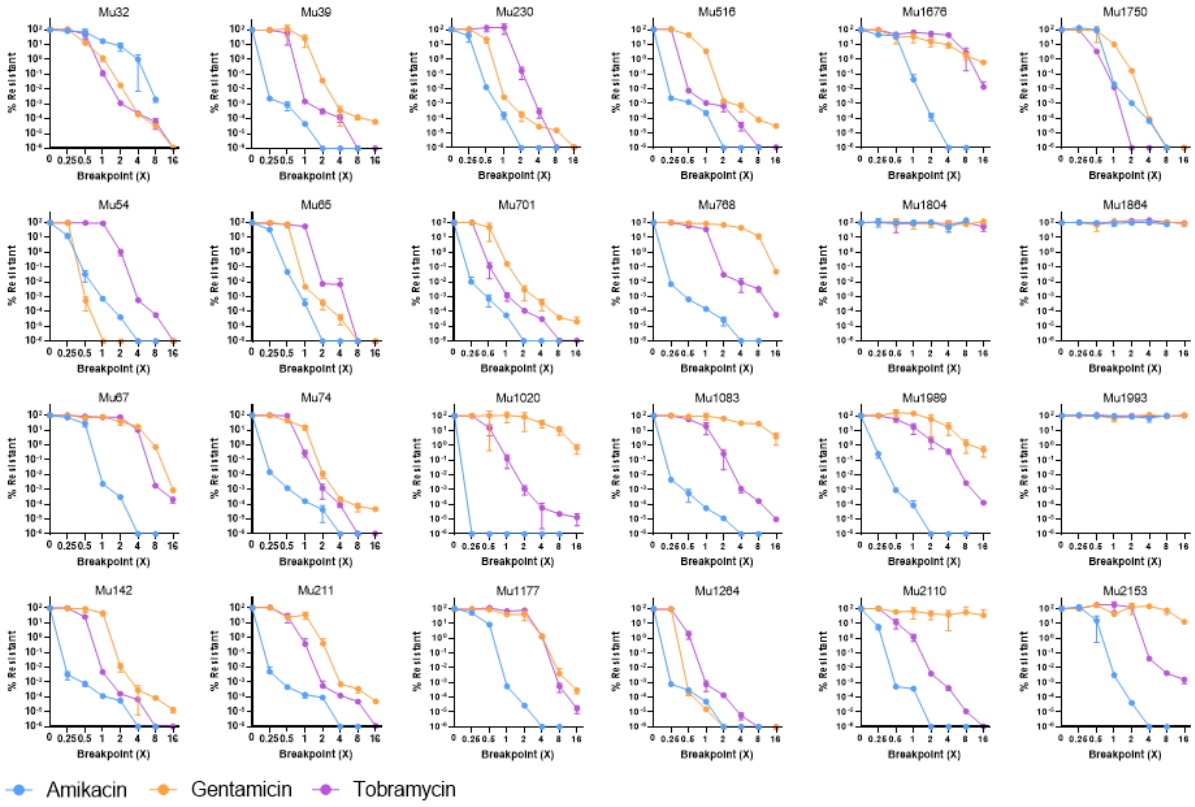
Continued on next page.

Mu1639	8	4	≤1	S	S	S	-	-
Mu1646	≤16	>8	≤4	S	HR	S	-	-
Mu1648	≤16	≤4	≤4	S	S	S	-	-
Mu1649	≤16	8	≤4	S	S	S	-	-
Mu1659	≤16	>8	≤4	S	HR	S	-	-
Mu1676	>32	>8	>8	S	HR	HR	+	-
Mu1697	≤16	>8	≤4	S	HR	S	-	-
Mu1700	8	≤1	≤1	S	S	S	-	-
Mu1716	8	≤1	≤1	S	S	S	-	-
Mu1718	>256	≥16	≥16	S	HR	HR	-	-
Mu1720	12	8	≤1	S	HR	S	-	-
Mu1721	1	≤1	≤1	S	S	S	-	-
Mu1726	256	8	≤1	S	S	S	-	-
Mu1731	3	≥16	≤1	S	HR	S	-	-
Mu1734	>32	>8	>8	S	HR	HR	-	-
Mu1737	>32	>8	>8	S	HR	HR	-	-
Mu1750	64	8	2	HR	HR	S	+	-
Mu1781	4	≤1	≤1	S	S	S	-	-
Mu1787				R	R	R	-	-
Mu1792	32	>8	>8	HR	HR	HR	-	-
Mu1800	≤16	>8	≤4	S	HR	S	-	-
Mu1804	>256	≥16	≥16	R	R	R	+	-
Mu1812	>256	≥16	≥16	R	R	R	-	-
Mu1824	≤16	>8	≤4	S	HR	S	-	-
Mu1832	≤16	>8	≤4	S	HR	S	-	-
Mu1857	4	≥16	≤1	S	S	S	-	-
Mu1864	>256	≥16	≥16	R	R	R	+	-
Mu1873	>32	>8	>8	HR	HR	HR	-	-
Mu1905	>32	>8	>8	S	S	S	-	-
Mu1915	8	4	≤1	S	S	S	-	-
Mu1916	4	4	≤1	S	S	S	-	-
Mu1924	32	>8	≤4	S	S	S	-	-
Mu1925	≤16	8	≤4	S	S	S	-	-
Mu1944	≤16	>8	≤4	S	HR	S	-	-
Mu1956	≤16	>8	≤4	S	HR	S	-	-
Mu1963	≤16	>8	≤4	S	HR	S	-	-
Mu1984	≤16	>8	≤4	S	HR	S	-	-
Mu1988	48	2	≤1	S	S	S	-	-
Mu1989	32	≥16	≥16	S	HR	HR	+	+
Mu1993	>256	≥16	≥16	R	R	R	+	-
Mu2005	≤16	8	≤4	S	S	S	-	-
Mu2007	≤16	8	≤4	S	S	S	-	-
Mu2008	4	≤1	≤1	S	S	S	-	-
Mu2029	≤16	>8	>8	S	HR	HR	-	-
Mu2034	2	≤1	≤1	S	S	S	-	-
Mu2043	>256	≥16	8	HR	HR	HR	-	-
Mu2050	64	≤1	≤1	S	S	S	-	-
Mu2060	8 S	4 S	≤1	S	S	S	-	-
Mu2071	1	≤1	≤1	S	S	S	-	-
Mu2104	>256	≥16	≥16	R	R	R	-	-
Mu2110	256	≥16	8	S	R	HR	+	-
Mu2135	6	≤1	≤1	S	S	S	-	-
Mu2136	>32	>8	>8	HR	R	HR	-	-
Mu2137	>32	>8	>8	S	HR	HR	-	-
Mu2143	8	2	≤1	S	S	S	-	-
Mu2146	>256	≤1	≤1	HR	S	S	-	-
Mu2150	24	2	≤1	S	S	S	-	-
Mu2153	>256	≥16	8	S	R	HR	+	-

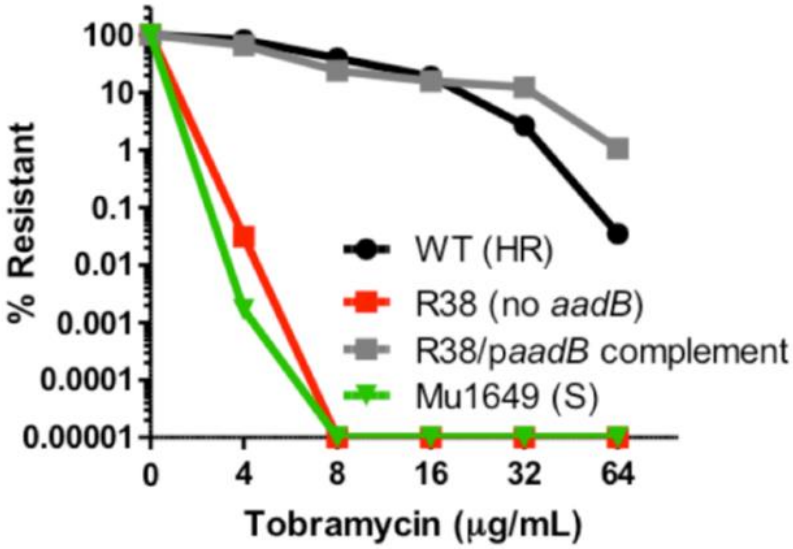
Supplementary Table 2. Clinical Data and Population Analysis Profile Data for All Strains Used in This study



Supplementary Figure 1. Aminoglycoside Population Analysis Profile Distribution Among CRAB Isolates. Strains were identified as heteroresistant if the surviving population was below 50% and at 2-4X above the breakpoint for each drug tested (a, tobramycin; b, gentamicin, and c, amikacin) by population analysis profile and compared to the clinical breakpoint for tobramycin (16 μg/ml), gentamicin (16 μg/ml), and amikacin (64 μg/ml).



Supplementary Figure 2. Population Analysis Profiles of *aadB*-positive CRAB Isolates. Population Analysis Profiles for each CRAB isolate that was confirmed to contain *aadB*.



Supplementary Figure 3. AadB is Required for Tobramycin Heteroresistance in *A. baumannii* AB5075. Population analysis profile (PAP) of WT AB5075 (WT), the R38 isogenic mutant lacking the amplified region that contains *aadB*, the R38 mutant complemented with *aadB*, and Mu1649 which is susceptible to tobramycin. Each strain was plated on increasing concentrations of tobramycin and the % resistant bacteria plotted for each dose.

Chapter 3:

Antibiotic Treatment Failure and Gene Amplifications detected in Aminoglycoside Heteroresistant *Enterobacter cloacae*

Edgar X. Sherman, Carter Abbott, Robert A. Petit, Sarah W. Satola, Eileen M. Burd, David S. Weiss.

The work in this chapter will also be submitted for publication in a peer-reviewed journal.

Abstract

Antibiotic resistance remains a growing threat to public health and there is an urgent need to elucidate the underlying mechanisms. One of the mechanisms that remains understudied is heteroresistance. Heteroresistance occurs when a small subpopulation of bacteria achieves a higher level of antibiotic resistance than the majority susceptible population, and we have found that this phenomenon can lead to antibiotic treatment failure. Here we describe heteroresistance to the aminoglycosides tobramycin, gentamicin, and amikacin in carbapenem-resistant (CRE) *Enterobacteriaceae* spp. and find strains where these resistant subpopulations went undetected by routine clinical diagnostics. *Enterobacter* strain Mu1307 exhibits amplification of the aminoglycoside modifying enzyme, *aadB*, which is required for tobramycin heteroresistance. The modifying enzyme is found in a 21kb region that is flanked by inverted repeats. We show that *aadB* amplification can be selected for in distinct populations in increasing concentrations of tobramycin. Further, we demonstrate that amplification of *aadB* is initially selected for during tobramycin therapy but begins to revert after tobramycin treatment has subsided. Finally, we report that tobramycin heteroresistance mediates treatment failure *in vivo* using a murine animal model, and that *aadB* facilitates treatment failure in Mu1307.

Importance

Carbapenem-resistant *Enterobacteriaceae* (CRE) remain an important collection of pathogens that makes treating infections difficult due to increased resistance and higher rates of mortality in hospitalized patients. This has led the Centers for Disease Control and Prevention to classify CRE an urgent threat to public healthcare if nothing is done to address the issues of antibiotic resistance in these species. We report amplification of an aminoglycoside modifying enzyme *aadB*, which is an aminoglycoside adenylytransferase that affects tobramycin, gentamicin, and kanamycin. This study demonstrates for the first time the clinical impact of aminoglycoside heteroresistance by modeling tobramycin treatment failure in a murine model and show that amplification of *aadB* can be selected for *in vivo.* We find that deletion of *aadB* increases susceptibility and removes the resistant subpopulation *in vitro* and allows for successful tobramycin therapy. This study highlights the need to understand the mechanism underlying heteroresistance to effectively combat this understudied phenomenon.

Introduction

Antibiotic resistance continues to threaten modern healthcare and hinder our ability to treat bacterial infections [10]. It is estimated that antibiotic resistance results in approximately 2.8 million infections per year and causes 35,000 deaths in the US [11]. Further complicating our limited antibiotic treatment regimens is when antibiotic treatment failure occurs, which results in extended patient stay, higher healthcare costs, and increased mortality [95,96]. The causes for antibiotic treatment failure are largely unknown but may be due to mechanisms of resistance that occur in bacterial populations at frequencies too low to be detected by routine clinical diagnostic techniques.

Carbapenem-resistant *Enterobacteriaceae* (CRE; which includes *Enterobacter* spp., *Klebsiella* spp., and *Escherichia* spp.) have become a cause for concern due to their increased multi-drug resistance, with several species developing pan-drug resistance which has led the Centers for Disease Control and Prevention to label CRE as an urgent threat to public health [10,97-99]. Due to the emergence of CRE, aminoglycosides have been proposed as a frontline therapy to CRE with limited treatment options as aminoglycosides still show *in vitro* efficacy [16,100,101]. Although CRE isolates with MICs at or approaching clinical breakpoints for aminoglycosides have been observed [102]. Mechanisms of aminoglycoside resistance found in CRE include decreased entry with mutations in the electron transport chain, efflux, ribosomal mutations, and acquisition, increased expression, or increased copy number of aminoglycosides modifying enzymes [36,103-105]. Thus, it is crucial to evaluate the efficacy of these drugs in CRE in preparation to adapt and respond to changes in resistance trends to allow for successful treatment in patients with limited treatment options.

Due to the complications from antibiotic treatment failure, it is imperative we elucidate the factors surrounding antibiotic failure and the mechanisms in bacteria that facilitate this phenomenon. One of the potential causes for antibiotic treatment failure is heteroresistance. Heteroresistance occurs when a small subpopulation of bacteria is resistant to an antibiotic while the majority of the population remains susceptible [41,42,77]. Due to the wide variety in frequency of the resistant subpopulation among bacterial isolates, some of these resistant subpopulations may go undetected by clinical diagnostic testing which may result in improper antibiotic therapy [76].

Heteroresistance was first identified in a study in 1947 that reported the phenomenon in *Haemophilus influenzae* with a small population of cells that showed increased resistance to streptomycin [46]. Since this study, heteroresistance has been reported to several important classes

of antimicrobials such as β-lactams, glycopeptides, antimicrobial peptides, and aminoglycosides [54,77-79]. Heteroresistant has been observed beyond the scope of pathogenic bacteria in eukaryotes such as *Candida* spp. to antifungals, *Trypanosoma* spp., to antiparasitics and anticancer therapies in cancer cells [49-53]. While heteroresistance has been reported, few studies have evaluated the exact mechanisms that result in antibiotic resistant subpopulations or its clinical relevance in patients, though recent evidence suggests that resistance subpopulations can mediate treatment failure [41,57,106]. Newer studies investigating the genetic determinants and clinical impact in select models exist such as colistin heteroresistance, and tobramycin heteroresistance in *A. baumannii* [48,58]. It is crucial that we understand the mechanisms leading to heteroresistance in order to prevent further antibiotic treatment failure.

One of the mechanisms that has been shown to generate these resistant subpopulations is DNA duplications/amplifications that result in increased gene copy number. Gene amplifications are widely conserved across all domains of life, are involved in DNA repair, and are one of the most frequent types of mutational event that occur [73,107]. These amplifications can occur by homologous recombination in regions of DNA flanked by sequence repeats. Gene amplifications have been implicated in increased bacterial resistance to several antibiotic classes and recently have been shown to occur in heteroresistant isolates [48,59,68,108]. It was recently reported that an aminoglycoside modifying enzyme, *aadB*, resulted in tobramycin and gentamicin resistant subpopulations in the *Acinetobacter baumannii* strain AB5075 [58]. Nicoloff *et al.* reported that in a majority of heteroresistant isolates analyzed by whole genome sequencing contained gene amplifications of known resistance genes, including aminoglycoside modifying enzymes [48].

In this study we evaluate heteroresistance to the aminoglycoside, tobramycin as well as gentamicin and amikacin in CRE. We test a collection of CRE strains consisting of *Enterobacter*

spp., *Klebsiella* spp., and *Escherichia* spp. that were collected through routine surveillance of carbapenem-resistant clinical isolates by the Georgia Emerging Infections Program. We evaluated heteroresistance using population analysis profiles (PAP) and find that aminoglycoside heteroresistance occurs for each of the drugs tested for each species. We then compared our results by PAP with clinical diagnostic data for each isolate and determined that heteroresistance went undetected in several of these isolates. Following our surveillance work, we investigated heteroresistance to tobramycin in a CRE *Enterobacter cloacae* strain, Mu1307. We report an amplification event of the aminoglycoside modifying enzymes *aadB* as well as *aadA2*. We show that deletion of *aadB* results in decreased tobramycin susceptibility and no resistant subpopulation in Mu1307. We determined that *aadB* was located in a 21kb region in Mu1307 that was flanked by inverted repeats along with 4 additional resistance genes. Finally, we show that heteroresistance mediates treatment failure and that *aadB* is necessary for tobramycin treatment failure to occur in Mu1307.

Results

Aminoglycoside Heteroresistance in Carbapenem-Resistant *Enterobacteriaceae* (CRE)

Reports detailing the prevalence of heteroresistance to antibiotics are lacking, including for aminoglycosides. Here we report the prevalence of heteroresistance to the aminoglycosides tobramycin as well as gentamicin and amikacin. To determine if aminoglycoside heteroresistance was present in the CRE clinical isolates we first performed population analysis profiles (PAPs) on clinical isolates obtained through the Georgia Emerging Infections Program [83]. We tested several strains from *Enterobacter* spp., *Escherichia* spp., and *Klebsiella* spp. In total, 116 CRE isolates were evaluated for heteroresistance to tobramycin, gentamicin, and amikacin

(**Supplementary Table 3.1**). Briefly, strains were grown overnight to stationary phase and then plated on MH agar with or without the specified concentrations of antibiotic indicated. Percent resistance was then measured by dividing the enumerated CFUs on plates with drug by the control plates without antibiotic. Strains were identified as heteroresistant if subpopulations were detected below 50% survival at 2-4X above the clinical breakpoint. After evaluating the CRE isolates for resistant subpopulations we found heteroresistance in each of the species screens for each aminoglycoside tested (**Table 3.1**). For each aminoglycosides tobramycin, gentamicin, and amikacin, the highest rates of heteroresistance found for each of these drugs in CRE was *Klebsiella* (42.9%), *Escherichia* (35.3%), and *Klebsiella* (41.4%) spps. respectively.

Undetected Aminoglycoside Heteroresistance in CRE

Using clinical data that was provided along with the CRE isolates, we wanted to determine if we could identify potential strains that might fail antibiotic treatment. We compared CRE strains that were identified as susceptible using clinical diagnostic data then compared the PAP data that were obtained for these isolates to find heteroresistant strains that were identified as susceptible. We refer to these heteroresistant CRE isolates as ‘undetected heteroresistance’ (**Table 3.1**). Using the undetected heteroresistant strains, we calculated the total resistance trends that may more accurately reflect what is being seen in clinical settings by adding the undetected heteroresistant strains to the resistance that was initially reported to find the total resistance (**Figure 3.1**).

Among the CRE isolates, every species tested contained heteroresistant strains that were identified as susceptible. The highest rates of undetected heteroresistance that occurred for each

	Tobramycin						
	PAP Results			Clinical Determination		Undetected	**Total**
Species	HR	S	R	S	R	HR	**Resistance**
Enterobacter spp. (29)	17.2% (5)	62.1% (18)	20.7% (6)	55.2% (16)	44.8% (13)	10.3% (3)	55.1% (16)
Escherichia spp. (17)	29.4% (5)	47.1% (8)	23.5% (4)	47.1% (8)	52.9% (9)	5.9% (1)	58.8% (10)
Klebsiella spp. (70)	42.9% (30)	17.1% (12)	40.0% (28)	22.9% (16)	77.1% (54)	2.9% (2)	80.0% (56)

	Gentamicin						
	PAP Results			Clinical Determination		Undetected	**Total**
Species	HR	S	R	S	R	HR	**Resistance**
Enterobacter spp. (29)	13.8% (4)	72.4% (21)	13.8% (4)	72.4% (21)	27.6% (8)	10.3% (3)	37.9% (11)
Escherichia spp. (17)	35.3% (6)	47.1% (8)	17.6% (3)	58.8% (10)	41.2% (7)	17.6% (3)	58.8% (10)
Klebsiella spp. (70)	15.7% (11)	54.3% (38)	30.0% (21)	67.1% (47)	32.9% (23)	10.0% (7)	42.9% (30)

	Amikacin						
	PAP Results			Clinical Determination		Undetected	**Total**
Species	HR	S	R	S	R	HR	**Resistance**
Enterobacter spp. (29)	13.8% (4)	86.2% (25)	0.0% (0)	96.6% (28)	3.4%(1)	13.8% (4)	17.2% (5)
Escherichia spp. (17)	41.2% (7)	52.9% (9)	5.9% (1)	82.4% (14)	17.6% (3)	29.4% (5)	47.0% (8)
Klebsiella spp. (70)	41.4% (29)	45.7% (32)	12.9% (9)	55.7% (39)	44.3% (31)	11.4% (8)	55.7% (39)

Table 3.1. Aminoglycoside Heteroresistance and Undetected Heteroresistance Among CRE Isolates.

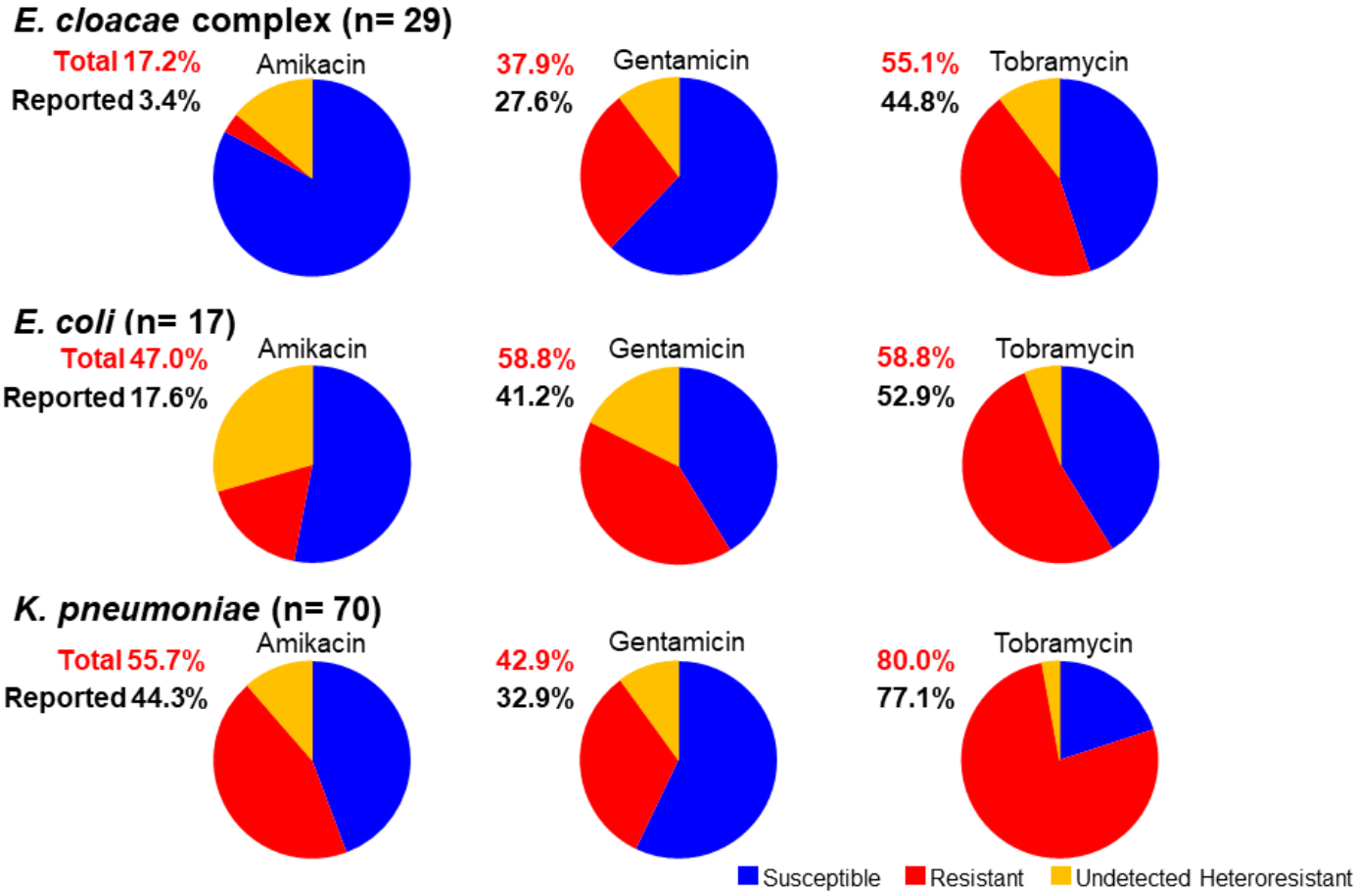


Figure 3.1. Rates of Undetected Heteroresistance Among Carbapenem-Resistant Enterobacteriaceae (CRE) Isolates. Rates of undetected Heteroresistance were found for all three species of CRE isolates tested to amikacin, gentamicin, and tobramycin. The total resistance was calculated by adding the heteroresistant strains that were identified as susceptible (undetected heteroresistance) and adding that value to the reported resistance value.

antibiotic tested was 10.3% for tobramycin in *Enterobacter* spp., 17.6% for gentamicin in *Escherichia* spp., and 29.4% for amikacin also in *Escherichia* spp. The lowest rate of undetected heteroresistance was 2.9% for tobramycin in *Klebsiella* spp. (**Table 3.1**). These results indicate that heteroresistant strains may go undetected by routine clinical diagnostic testing.

Amplification of Aminoglycoside Modifying Enzymes in CRE *Enterobacter cloacae* Strain Mu1307

To investigate the mechanisms of tobramycin heteroresistance, we choose to use the CRE *Enterobacter cloacae* strain Mu1307. This strain was isolated from the urine of a patient and showed resistance to multiple classes of antibiotics (**Supplementary Figure 3.1**) and contained a resistant subpopulation to tobramycin that was identified by both PAP and Etest (**Figure 3.2**). Mu1307 is heteroresistant to gentamicin as well, but that data is not shown.

Aminoglycoside Modifying Enzyme Amplification in Mu1307

Mu1307 was selected for as a model strain since it was determined that this CRE isolate contained the aminoglycoside modifying gene, *aadB* through analysis of Illumina sequencing data and confirmed by PCR. The *aadB* gene is located on a 21kb region that is likely carried on a 97kb plasmid in Mu1307 (**Figure 3.3a**). This region is flanked by inverted repeats consisting of two genes, *folP* and *emrE*, which are involved in the folate synthesis pathway and a multidrug transporter, respectively. Notably, five resistance genes are contained within this 21kb region, three aminoglycoside modifying enzymes (*aadB, aacA4*, and *aadA2*), a chloramphenicol resistance gene (*catB3)*, and a carbapenemase (bla_{OXA-1}). *aadB* is a 2”-aminoglycoside adenylytransferase (ANT(2”)-Ia) gene which results in adenylation of 2-deoxystreptamine

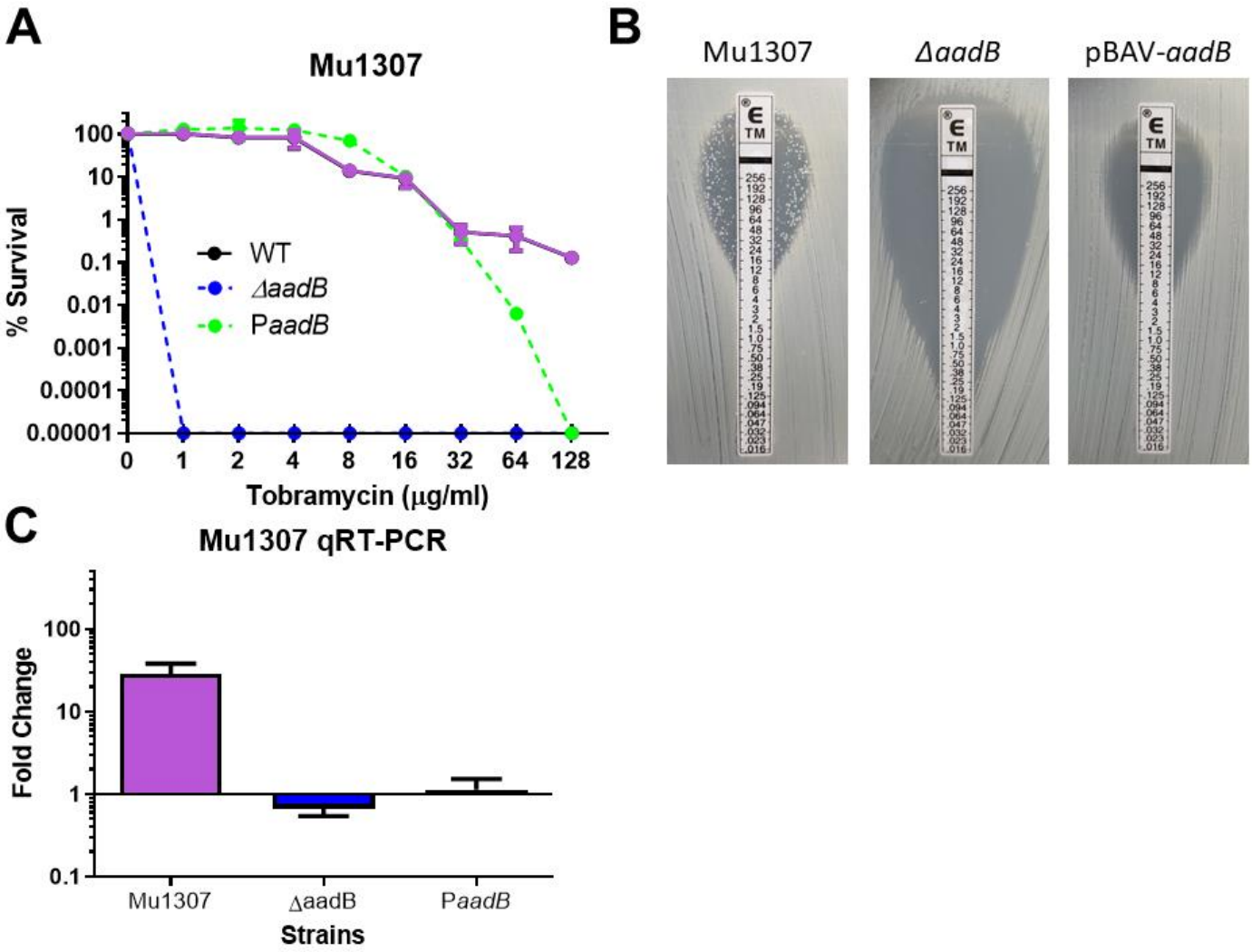


Figure 3.2. *aadB* is Required for Tobramycin Resistance and the Presence of Resistant Subpopulations. Tobramycin population analysis profiles (A) and Etest strips (B) for Mu1307 (WT), a deletion strain lacking *aadB*, and the complemented strain. Complementation increases tobramycin resistance but does not restore the resistant subpopulation, likely due to the lack of *aadB* amplification in the plasmid background.

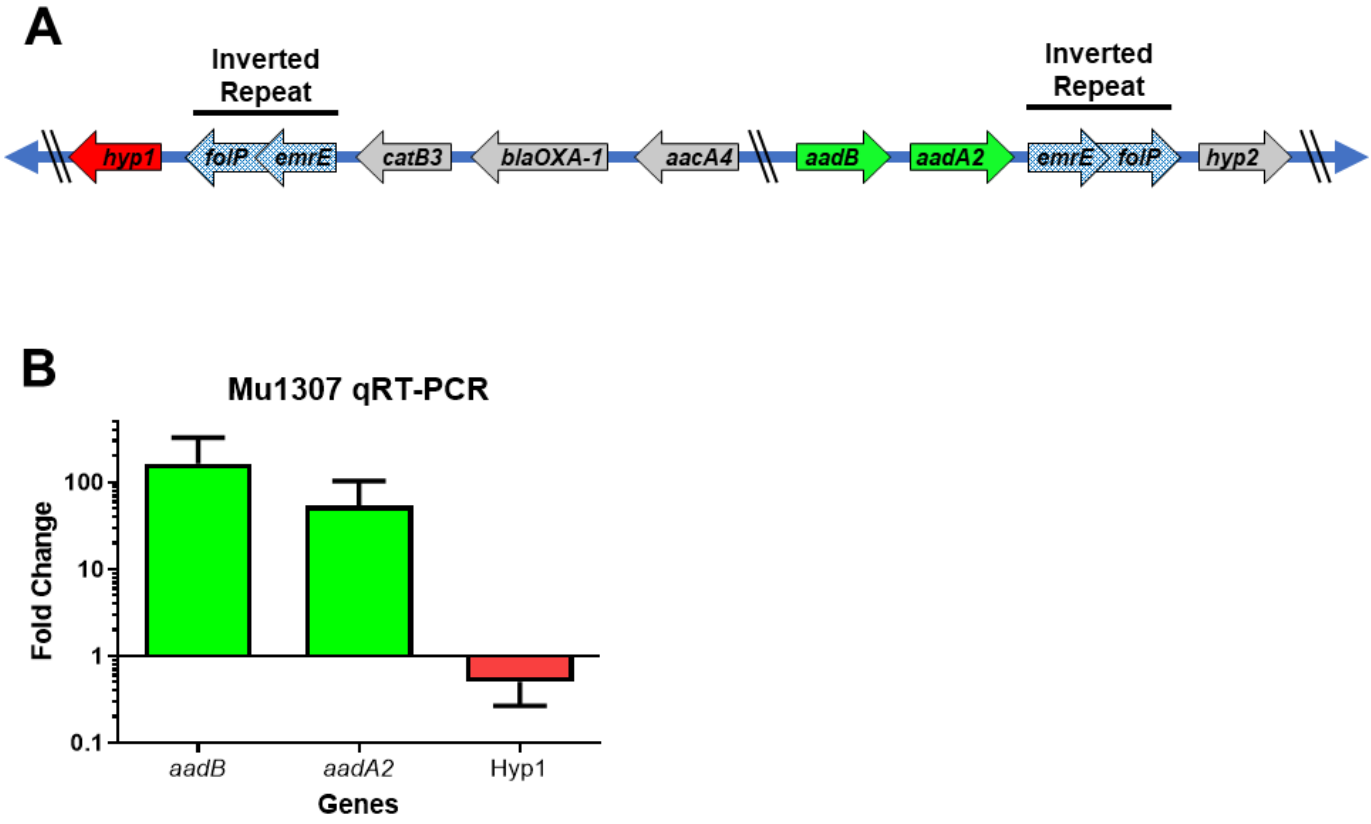


Figure 3.3. Amplification of Resistance Genes in Mu1307. A) Diagram of the 21Kb region found in Mu1307 by Illumina and PACBIO Sequencing. The region is flanked by inverted repeats (blue) of the genes *folP* (Folate synthesis) and *emrE* (Efflux pump). Contained within this region are five antibiotic resistance genes for chloramphenicol (*catB3*), carbapenems (bla_{OXA-1}), and aminoglycosides (*aacA4*, *aadA2*, *and aadB*). B) Amplifications of genes inside and outside the region flanked by inverted repeats. Amplifications were measured by qPCR of gDNA from isolates grown in MH media supplemented with 32 μg/ml of tobramycin for 24 hours and comparing the fold change of qPCR from untreated strains grown in MH media alone for 24 hours. Genes *aadB* and *aadA2* show fold changes when treated with tobramycin (green), while a hypothetical gene outside the inverted repeat region did not show increased amplifications (red). Genes that have not been tested are grey.

aminoglycosides at the hydroxyl group at the 2” position[109]. The aminoglycosides targeted by *aadB* are tobramycin, gentamicin, and kanamycin [84].

Amplifications of *aadB* in a tobramycin resistant subpopulation was first reported by Anderson *et al*. in the pathogenic *Acinetobacter baumannii* strain AB5075 [58]. Recently, it was also shown that amplifications were present in a majority of heteroresistant clinical isolates, including aminoglycoside modifying enzymes [48]. Due to the presence of *aadB* in Mu1307, and no reports of aminoglycoside modifying enzyme amplifications present in heteroresistant *E. cloacae*, we investigated if an amplification event was also occurring in the tobramycin resistant subpopulation.

Since the five resistance genes mentioned previously are contained within a region flanked by inverted repeats, this may suggest that an amplification may be occurring within this region of DNA as repeats sequences allow homologous recombination to occur which can lead to amplification events [110]. We find that when examining isolated gDNA of Mu1307 treated with tobramycin and untreated by real-time quantitative PCR (RT-qPCR), *aadB* amplification is higher in the treated samples than untreated (**Figure 3.3b**). We examined amplifications of another gene contained within this 21kb region, *aadA2* and find that amplification is occurring at around the same levels as *aadB* when treated with tobramycin. Finally, we examined amplification of a hypothetical protein that was found directly outside the flanking repeats in Mu1307 named *hyp1*. We show that no amplification was detected in this gene suggesting that amplification is only occurring within the 21kb region in Mu1307.

Increased Amplification of *aadB* Occurs During Tobramycin Treatment and is Reduced Upon drug-free passage

When performing tobramycin population analysis profiles, we observe several distinct surviving subpopulations that occur at various frequencies. This may indicate that these subpopulations contain different levels of *aadB* amplification that are preexisting and selected for during antibiotic treatment. To test this hypothesis, we treated Mu1307 with increasing concentrations of tobramycin, and measured the survival. As expected, as the concentration of tobramycin in increased, survival is reduced (**Figure 3.4a, left axis**). Interestingly, when gDNA was isolated from the surviving subpopulations at each drug concentration tested for *aadB* amplifications by RT-qPCR, increased amplification of *aadB* were also detected (Figure 4a, right axis). This indicates that the subpopulations that survive at higher doses of antibiotic also contain increased levels of gene amplifications in Mu1307.

Genetic amplification events are unstable as the increased copy number and expression of resistance mechanisms can have an associated fitness cost [111]. It has been shown previously and observed throughout this study that aminoglycoside heteroresistance is unstable (data not shown). When treated with tobramycin, the resistance percentage increases to approximately 100% as the susceptible population is eliminated. However, once the antibiotic has been cleared from the environment or isolates are sub-cultured in fresh media without antibiotic the resistance percentage decreases as the susceptible population returns. Thus, we wanted to investigate whether *aadB* amplifications also decay as the same rate as the resistance percentage.

We first measured survival of the resistant subpopulation in overnight culture of Mu1307 grown in media without antibiotic. Then we sub-cultured the isolate into media containing tobramycin for 24 hours and measured survival of the resistant population. Following treatment

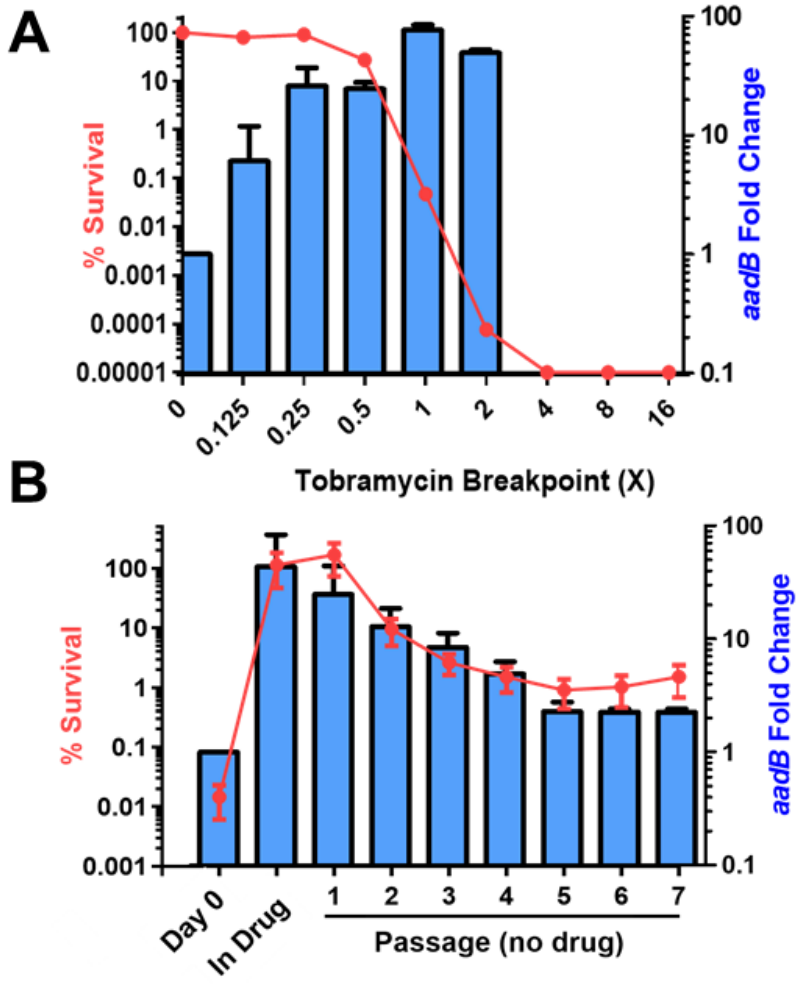


Figure 3.4. Fluctuation of aadB Amplification During and After Tobramycin Selection. Survival was measured by counting CFUs in MH agar plates supplemented with specified tobramycin concentrations relative to the breakpoint (16 μg/ml) and dividing by CFUs plated on MH agar alone. A) As the concentration of tobramycin was increased, survival of the population was shown to decrease in Mu1307. However, the amplification of *aadB* was shown to increase at higher tobramycin concentrations. B) When plating initial Mu1307 overnight culture without any tobramycin treatment on plates containing 32 μg/ml, the resistant subpopulation and *aadB* amplifications are low. When sub-cultured to MH broth supplemented with 32 μg/ml of tobramycin overnight and plating on tobramycin after 24 hours, the resistant subpopulation was selected for, as was increased *aadB* amplification. Following passage without tobramycin, the resistant subpopulation decreased and *aadB* amplifications were also reduced in Mu1307.

with tobramycin, we passaged the strain for seven days in media without tobramycin and measured survival. As previously observed, the resistance of the population increases to nearly 100% following tobramycin treatment but begins to decrease following serial passage in antibiotic free media (**Figure 3.4b, left axis**). When *aadB* amplification was measured from the gDNA of the surviving populations and compared to the untreated control a similar trend was observed that corresponded with the measured survival. Measured amplification of *aadB* increased following tobramycin exposure but began to decrease as Mu1307 was passaged in media without tobramycin (**Figure 3.4b, right axis**).

aadB and *recA* are Required for Tobramycin Heteroresistance

To assess the role of *aadB* in tobramycin heteroresistance, an *aadB* deletion was generated in Mu1307. When tested by PAP, the resistant subpopulation was eliminated in the *aadB* knockout and the susceptibility to tobramycin was increased (**Figure 3.1a**). These results were also confirmed by Etest (**Figure 3.1b**). When *aadB* was inserted onto a plasmid and complemented back in the *aadB* mutant, resistance was restored by PAP, but resistant subpopulations were not observed at higher levels as seen in the wild type. Tobramycin Etest results also confirmed this result, showing a zone of clearing similar to the wild type, but lacking the presence of resistant colonies in the zone of clearing.

To confirm that the gene amplification event is dependent on homologous recombination, we also created a strain of Mu1307 with a deletion in *recA*. When examined by tobramycin PAP and Etest, the Mu1307 *recA* mutant showed decreased susceptibility (**Figure 3.5a**). However, colonies were observed along the outer edge of the zone of clearing (**Figure 3.5b**), though these colonies did not appear to be resistant as they did not grow past the clinical breakpoint in the Etest and the populations were not detected by PAP. We then measured *aadB* amplification by RT-

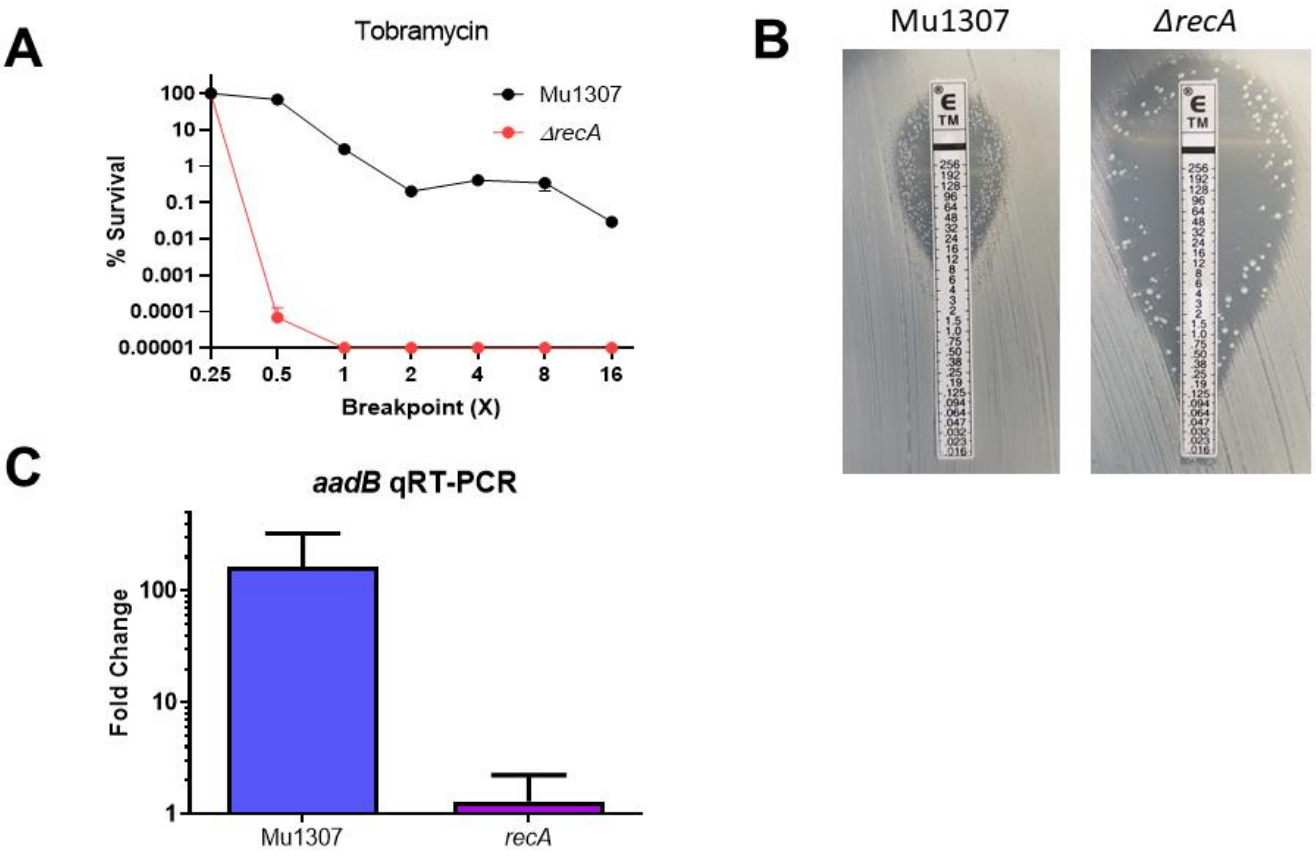


Figure 3.5. Resistant Subpopulation is Decreased in a recA Mutant. A) Tobramycin population analysis profile with Mu1307 (WT) and a *recA* deletion shows a loss of resistant subpopulation B) Tobramycin Etest shows and an increase in susceptibility by. C) When measuring *aadB* amplification by qPCR as previously described, the *recA* deletion shows no amplification compared to the WT strain.

qPCR and found that the *recA* deletion strain showed no amplifications when compared to the wild type strain grown in tobramycin (**Figure 3.5c**). These data show that *recA* is important for both resistance and heteroresistance at higher concentrations of tobramycin though *recA* independent subpopulations are present in the absence of *recA*.

Bioinformatic Analysis Suggests Repeat Region Undergoes Amplification in Mu1307

To determine the exact region where gene amplifications were occurring in Mu1307, we used bioinformatic analysis using the assembled Mu1307 genome. The Illumina sequencing reads, were prepared by the Georgia Emerging Infections program and uploaded to NCBI (accession no. DHQP1604376). Briefly, Illumina reads were aligned to the assembled 97kb region in Mu1307. A coverage plot was then generated showing the alignment of the Illumina reads. If amplification was occurring within a specific region of the 97kb region, then more Illumina reads would be generated during Illumina sequencing and would show higher coverage in amplified regions. The coverage plot shows an increase in peaks in a 22kb region that spans approximately from the 60kb-82kb position (**Figure 3.6**). When aligned with the annotated outline, this 22kb region aligns approximately where the inverted repeats begin and end in Mu1307. This suggests that increased amplification is occurring from this region in Mu1307.

Antibiotic Treatment Failure Mediated by *aadB* in Tobramycin Heteroresistant Mu1307

To determine the impact of tobramycin heteroresistance on clinical outcomes, we used an in an *in vivo* murine model. We infected mice intraperitoneally with the tobramycin susceptible control strain Mu819, and the tobramycin heteroresistant strain Mu1307. Both strains caused a high bacterial burden in the peritoneum, spleen, and liver (**Figure 3.7**). When treated with tobramycin, the susceptible strain showed a significant decrease in CFU burden across all three

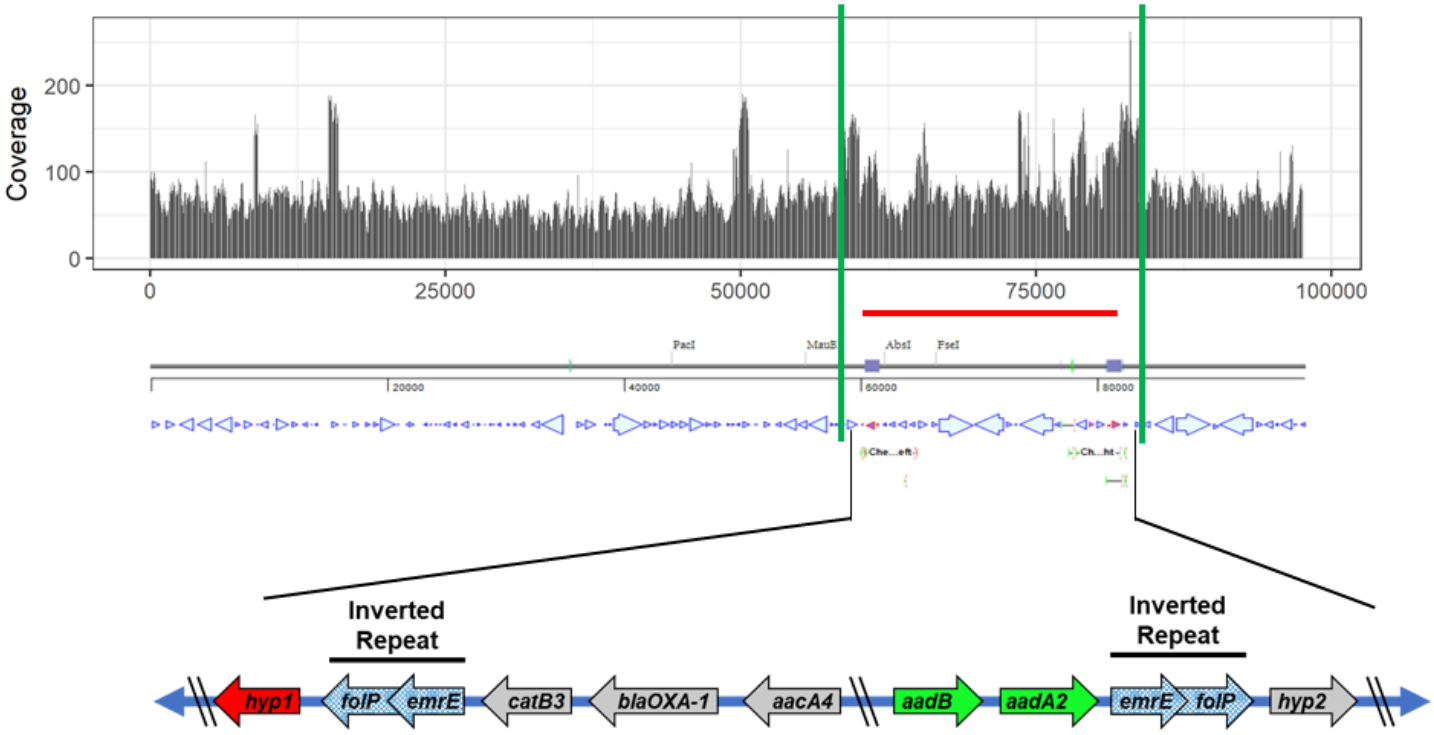


Figure 3.6. Alignment of Illumina Data Suggests Amplified 21KB Region in Mu1307. Following Assembly of the 97KB region found in Mu130, raw Illumina reads were aligned to the region using bwa and samtools and a per-base coverage plot was generated and plotted in R. When the annotated sequence in Mu1307 is aligned to the plot, the sequence containing the higher number of peaks is located approximately where the 21KB region is flanked by the inverted repeats *folP* and *emrE*.

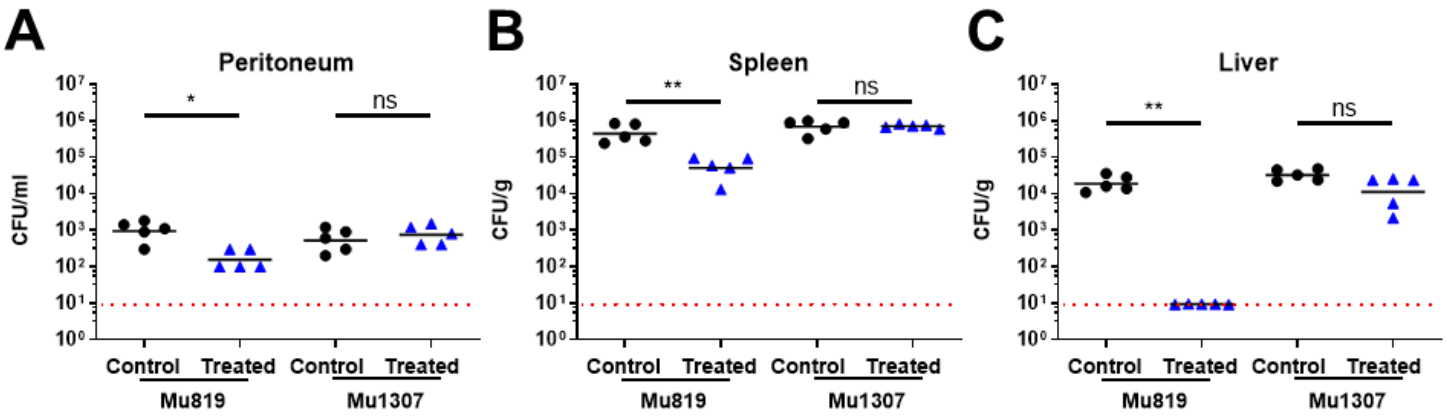


Figure 3.7. Tobramycin Heteroresistance Mediates Treatment Failure *In Vivo*. C57BL/6 Mice were infected intraperitoneally with either the tobramycin heteroresistant strain *E. cloacae* strain Mu1307 or susceptible strain Mu819. Then mice were treated with either PBS (control) or 25 mg/kg tobramycin every 8 hours at two hours post-infection. Bacterial CFU burden from the peritoneal fluid (A), spleen (B), and liver (C) were quantified 24 hours post-infection.

organs. However, the heteroresistant strain was not significantly impacted by tobramycin therapy. These data show that tobramycin heteroresistance impacts antibiotic therapy *in vivo*. Finally, we wanted to investigate the role of *aadB* in tobramycin treatment failure. Mice were infected as described above with the parental Mu1307 and the *aadB* deletion strain. In the *aadB* mutant, CFU burden was significantly reduced when treated with tobramycin compared to the wild type strain (**Figure 3.8**). This data suggests that *aadB* mediates tobramycin treatment failure.

Discussion

In this study we report the rates at which heteroresistance occurs in three different carbapenem-resistant *Enterobacteriaceae* species. By comparing clinical data with our population analysis profiles, we find strains that were identified as susceptible in the clinical lab contain resistant subpopulations that may impact treatment outcome. In the carbapenem-resistant *Enterobacter cloacae* strain Mu1307, we show that the aminoglycoside adenylating enzyme, *aadB,* is located on a 21kb region flanked by inverted repeats that is amplified, which leads to a tobramycin resistant subpopulation that is RecA-dependent. The amplifications in the population can be selected for during tobramycin treatment and the resistant subpopulations found at higher concentrations of antibiotic show an increase in *aadB* amplification. Tobramycin heteroresistance is unstable, and in the absence of selective pressure, the resistant subpopulation begins to revert corresponding with a decrease in *aadB* amplification. Since the clinical relevance of aminoglycoside heteroresistance in CRE was unclear before this study, we used an *in vivo* mouse model to show that tobramycin heteroresistance leads to treatment failure that is mediated by *aadB* in Mu1307.

We identified potential strains of CRE that may fail aminoglycoside antibiotic treatment due to the fact that they were identified as susceptible by diagnostic testing but found to contain

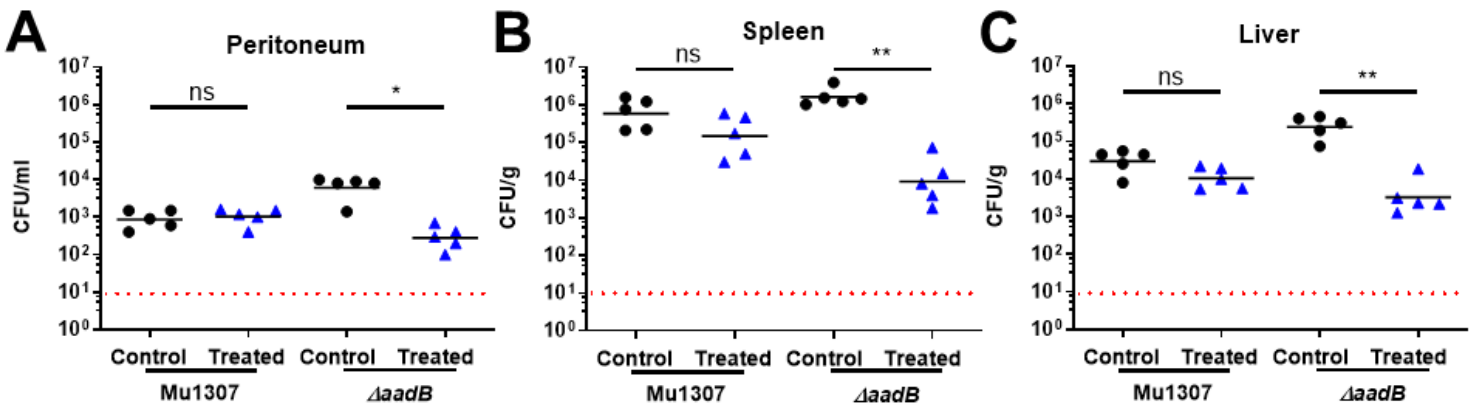


Figure 3.8. *AadB* Mediates Treatment Failure in Heteroresistant Strain Mu1307. C57BL/6 mice were infected intraperitoneally with either the tobramycin heteroresistant strain *E. cloacae* strain Mu1307 or the genetically derived *aadB* deletion. Then mice were treated with either PBS (control) or 25 mg/kg tobramycin every 8 hours at two hours post-infection. Bacterial CFU burden from the peritoneal fluid (A), spleen (B), and liver (C) were quantified 24 hours post-infection.

resistant subpopulations by PAP. There are some limitations to this study, the first is the sample size of the CRE isolates used, while 70 isolates were used for *Klebsiella*, only 29 isolates were used for *Enterobacter* spp., and 17 for *Escherichia* spp. The second limitation is that these isolates were only collected in Georgia. While this limited sample size and location sampling may not accurately reflect nationwide trends in aminoglycoside heteroresistance, it is clear that these resistant subpopulations lead to misidentification. It is also important to determine if all the misidentified strains that contain resistant subpopulations mediate treatment failure. While resistant subpopulations may be present, the frequency at which they appear may be easily neutralized by the hosts immune system and not impact the clinical outcome of the patient. It has been shown that a heteroresistant isolate with resistant subpopulations appearing at a frequency of 0.001% was sufficient to cause treatment failure in colistin heteroresistance.

Strains containing resistant subpopulations may go undetected during routine diagnostic testing due to the sensitivity of the methods used to test these strains in the clinic. Due to the variable frequency at which these subpopulations appear, they may be too small to measure accurately, which leads to incorrect diagnoses and improper antibiotic treatment. Other methods such as Etests and molecular genetic screening methods can be used but these methods also have issues with specificity and accuracy when detecting subpopulations that occur at lower frequencies. More accurate analyses such as the PAP, are too laborious or difficult to implement practically in a clinical environment. Understanding the mechanisms that lead to heteroresistance can allow for more accurate detection when efforts are focused on specific resistance mechanisms, such as testing for amplification of *aadB* for tobramycin, gentamicin, and kanamycin resistance.

The aminoglycoside modifying enzyme *aadB* was found in a 21kb region flanked by inverted repeats consisting of the genes *folP* and *emrE* in Mu1307. Contained within this 21kb

region are five resistance genes, the carbapenemase *bla*$_{OXA-1}$, chloramphenicol resistance gene *catB3*, and three aminoglycoside modifying enzymes (*aacA4*, *aadA2*, and *aadB*). Amplifications were found in both resistance genes tested in this region (*aadA2* and *aadB*) but were not found the hypothetical gene that was tested outside of this 21kb region (*hyp1)*. Our data suggests that amplifications are occurring within the region flanked by inverted repeats. These data are supported by bioinformatic analysis of the entire 97kb region found in Mu1307 showing that the 21kb region has increased sequence coverage, likely due to increased amplifications present during sample preparation. Further experiments are needed to conclude the role that the inverted repeat regions found in Mu1307 have in tobramycin heteroresistance.

The *aadB* deletion in Mu1307 showed increased susceptibility and no resistant subpopulations by both PAP and Etest. When the *aadB* deletion was complemented, there was a restoration of resistance but there was no indication of resistant subpopulations, most notable in the tobramycin Etest. This result is likely due to the *aadB* complementation occurring through a separate plasmid and not in the original region, so it is unlikely that *aadB* amplification is occurring. This was confirmed through RT-qPCR analysis of the *aadB* mutant and complementation strain where no amplifications were detected, in contrast to the parental wild type Mu1307 (**Figure 3.2c**). These results show that amplification of *aadB* is required for subpopulations with higher resistance to tobramycin to appear when compared to having only the gene being expressed, which only provides a certain level of resistance to the entire population.

A strain of Mu1307 that contained a deletion in *recA* showed no amplifications of *aadB* when tested by RT-qPCR and decreased tobramycin susceptibility by PAP and Etest, indicating that homologous recombination is important for tobramycin heteroresistance [89,112]. There were, however, colonies that appeared around the outer edge of the zone of clearing on a

tobramycin Etest. These colonies appeared to have slightly decreased susceptibility and did not grow past the tobramycin clinical breakpoint level. This suggest an additional *recA*-independent mechanism present in Mu1307 that may result in subpopulations, the exact mechanism and relevance of these populations will need to be investigated further but may be due to mutations that result in persistor formation or mutations that arrest growth in the electron transport chain pathway [29,74,75].

Our data show that as the dose of the antibiotic in increased, the subpopulations that survive decrease, but the amount of amplifications found in the remaining populations increase. These amplifications are likely present before treatment is occurring and selected for during antibiotic therapy with cells containing fewer amplifications being eliminated at lower doses of antibiotics. Amplification events occur stochastically during DNA replication, with cells that contain higher levels of duplication events occurring less frequently than cells with fewer amplification events present occurring at a higher percentage. This phenomenon may explain why different levels of survival are observed at various concentrations of antibiotics, with cells that survive at higher concentrations of drug showing the highest level of amplifications but making up the smallest population. We show that the amplifications in Mu1307 are unstable, with the both the resistance percentage and amplifications increasing during antibiotic therapy and decreasing once the antibiotic has been cleared from the environment. Both of these events are due to the restoration of the susceptible population decreasing both the resistance and the average of the amplification events occurring.

In conclusion, while aminoglycoside heteroresistance appears to be common among CRE pathogens, more studies are needed to determine the full scale of aminoglycoside heteroresistance and how it impacts the clinical environment and patient outcome. This is the first study to show

the clinical impact of tobramycin heteroresistance in CRE. Aminoglycoside heteroresistance is cause for concern since these heteroresistant strains may go undetected through clinical diagnostic methods due to the variety in the frequency of the resistant subpopulations, and studies that show the prevalence or *in vivo* relevance are lacking. Amplification of aminoglycoside modifying enzymes appears to be key in mediating both resistance and the appearance of resistant subpopulations. It is crucial that we fully evaluate the mechanisms leading to aminoglycoside heteroresistance to better combat this phenomenon and also increase the accuracy at which we can detect even low percentages of resistant subpopulations if the use of aminoglycosides is considered to treat multi-drug resistant infections.

Materials and Methods

Strains collection and growth conditions

Liquid cultures were prepared in sterile Mueller-Hinton (MH) broth, supplemented with tobramycin (Spectrum, New Brunswick, NJ) at the concentrations indicated. Plates were supplemented with tobramycin as indicated. Strains of carbapenem-resistant *Enterobacteriaceae* (CRE) were collected between 2013 and 2015 by the Georgia EIP MuGSI from patients at Atlanta area hospitals. Strains of CRE were maintained at −80°C in 15% glycerol.

Population analysis profiles

Population analysis profile (PAP) analysis was performed by growing bacteria overnight to stationary phase and then plating serial dilutions on MH agar alone or with stated concentrations of tobramycin mentioned relative to the clinical breakpoint (Spectrum, New Brunswick, NJ). Plates were then incubated at 37°C, and CFU were enumerated after 24 hours. Percent tobramycin

resistance was calculated as the number of bacterial colonies that grew on tobramycin plates divided by the number of bacteria that grew on MHA alone without antibiotic [45].

Antibiotic susceptibility testing

All isolates were tested for their clinical MIC and resistance designation in the clinical microbiology laboratory at Emory University Hospital. Gentamicin and Tobramycin were tested by automated Vitek 2 (Biomerieux, Marcy l'Etoile, France) using the GN74 susceptibility card. Amikacin susceptibility was tested using the Etest gradient strips (Biomerieux, Marcy l'Etoile, France). Susceptibility results were interpreted by a licensed clinical microbiologist. The tobramycin susceptibility of Mu1307 and related strains was determined using the Etest method. Briefly, the inoculum was prepared from colonies grown on MH agar plates for 24 hours. Several colonies were suspended in phosphate buffered saline solution and adjusted to a concentration equivalent to a 0.5 McFarland turbidity standard. The suspension was used to streak a 100-mm-diameter MH agar plate and the Etest strip (Biomerieux) was placed on it. The plate was incubated at 37°C for 20 h and the minimum inhibitory concentration (MIC) was read where inhibition of growth intersected the Etest strip.

Bacterial genetics

A linear DNA fragment OMP-Zeo-FRT was created fusing the *Franciscella* OMP26 promoter from to the zeocin resistance cassette [113] from pCMV/Zeo flanked by FRT sites[114] using the primers FRT-OMP Fw, OMP-Zeo SOE Rv, Zeo-OMP SOE Fw, and Zeocin-FRT Rv using splicing by overlap extension (SOE) PCR[115] (Supplementary Table 1). The linear fragment along with the plasmid pEX100T were linearized with NcoI and BamHI (New England Biolabs) to create the plasmid pEXES1. To generate the *ΔaadB* strain, 50 bp upstream and downstream

fragments of the genomic region surrounding *aadB* were PCR amplified from the OMP-Zeo-FRT DNA from pEXES1 with primers *aadB*::ZeoRS Fw and *aadB*::ZeoRS Rv, respectively. The linear fragment was then electroporated into Mu1307 competent cells that had been transformed with plasmid pKD46-Tet after inducing expression of the lambda-red recombination system by growing in 20% arabinose. To generate the Δ*recA* strain, 50 bp upstream and downstream fragments of the genomic region surrounding *aadB* were PCR amplified from the OMP-Zeo-FRT DNA from pEXES1 with primers *recA*::ZeoRS Fw and recA::ZeoRS Rv, respectively and steps were repeated as outlined above to generate the *ΔrecA* strain. The OMP-Zeo-FRT fragment was removed using FLP-recombination by transforming the mutant strains with the pFLP-Tet plasmid. Complement strains were generated by fusing the OMP26 promoter with the respective gene for complementation using SOE PCE and adding the flanking restriction sites NcoI and BamHI for insertion into the plasmid pBAV1k-T5-GFP [116].

Genomic DNA (gDNA) isolation

Genomic DNA was prepared using Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Briefly, 1 ml of overnight culture were spun down and resuspended in 600µl of nuclei lysis solution. Cells were lysed and gDNA was precipitated using isopropanol and washed with ethanol as described in the protocol. Following gDNA precipitation, the DNA pellet was resuspended in dH_20 overnight at 4ºC and diluted to a final concentration of 100 ng/µl.

qPCR

Concentrations of gDNA samples were determined using a NanoDrop ND-1000 spectrophotometer. Samples of gDNA were added to a total concentration of 100 ng/well, and qPCR was performed as outlined above for gDNA samples. Standard curves using 10-fold serial

dilutions of wild-type gDNA were used to ensure that primers exhibited similar efficiencies. Relative gene copy numbers were normalized to *rpoD* and were determined using the equation $2^{-\Delta CT}$ [94].

***De Novo* genome assembly and sequence analysis.**

Bactopia (v.14.0 [117]) was used to process both Illumina and PacBio data. The Illumina reads were cleaned and error corrected using BBDuk (v38.86 [118]) and Lighter (v1.1.2 [119]). The processed Illumina reads were then combined with the PacBio reads to create a hybrid assembly with Unicycler (v 0.4.8 [120]). The hybrid assembly was annotated with Prokka (v1.14.6 [121]). BLAST+ (v2.9.0 [122]) was used to identify which contig of the hybrid assembly contained the *aadB* gene (accession no. DHQP1604376). The Illumina reads were mapped back to the aadB containing contig using BWA (v0.7.17 [123])) and the per-base coverage was extracted with genomeBedCoverage (v2.29.2 [124]).

Mice

Female WT C57BL/6J mice were purchased from Jackson Laboratories and used at age 8–10 weeks; all experiments used age- and sex-matched mice. Mice were housed under specific pathogen-free conditions at Yerkes National Primate Center, Emory University. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee. Mouse sample size was determined based on previous studies that generated highly statistically significant results by the Mann–Whitney test ($n \geq 4$), while also minimizing the number of animals used (5 mice per group for the majority of experiments).

Mouse infection model

Approximately 1×10^8 CFU were administered per mouse for infections to quantify the bacterial load and select for quantitative PCR analysis. For mouse infections, overnight standing bacterial cultures at room temperature were sub-cultured in LB broth and grown at 37°C with shaking, washed and re-suspended in PBS. 50 μL of bacterial inocula were inoculated intraperitoneally (i.p.) to each mouse. At 2 hours post-infection, mice were treated with 100μL of 25mg/mL of tobramycin (125 mg/Kg/8Hrs). After 24 hours, the mice were sacrificed, and the peritoneal fluid, spleen and liver were harvested, homogenized, plated for CFU on MHA plates.

Statistical analysis

All data presented are from experiments that were repeated at least twice to ensure the reproducibility of the results. The significance of the mouse experiments was determined with the Mann–Whitney test, as not all data were normally distributed, and all in vitro experiments were analyzed using the two-tailed student's t-test (for data with a normal distribution). Error bars represent the standard error of the mean of the biological replicates. Statistical analyses were performed with Prism 8 (GraphPad Software, Inc., La Jolla, CA).

Supplemental Material

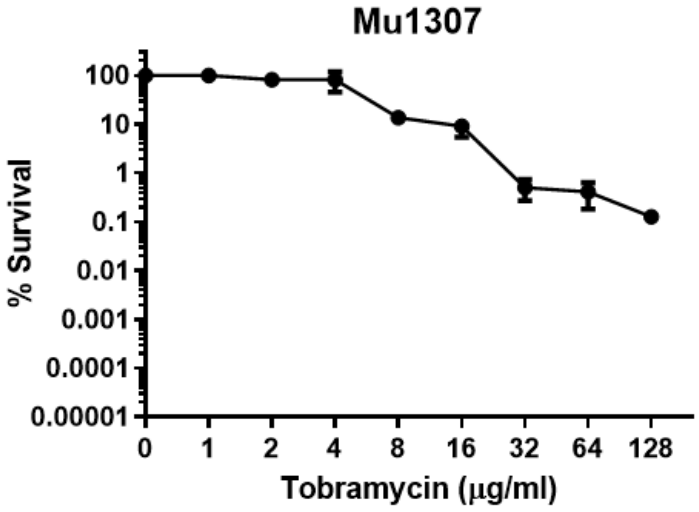
	Total	%
Genus		
Enterobacter	29	25.0%
Escherichia	17	14.7%
Klebsiella	70	60.3%
Culture Source*		
Urine	96	83.5%
Blood	16	13.9%
Peritoneal Fluid	3	2.6%
Sex		
Male	53	45.7%
Female	63	54.3%
Age Groups		
0-9	2	1.7%
10-19	1	0.8%
20-29	3	2.6%
30-39	5	4.3%
40-49	7	6.0%
50-59	22	19.0%
60-69	35	30.2%
70-79	22	19.0%
80+	19	16.4%
* Data missing in 1/116 Isolates		
Data previously shown in Band *Et. Al.* 2019 Nature Microbiology		

Supplementary Table 3.1. Carbapenem-Resistant *Enterobacteriaceae* (CRE) Isolate Patient Demographics

Primer	Sequence	Use
aadB Fw	ACTGGCCTACAAAGCACA	*aadB* qPCR
aadB Rv	ATATCGCGACCTGAAAGC	*aadB* qPCR
aadA2 Fw		
aadA2 Rv		
rpoD Fw	CCGGAAGACAAGATCCGTAAAG	*rpoD* qPCR
rpoD Rv	CCTCGATGAAATCACCCAGATG	*rpoD* qPCR
FRT-OMP Fw	GAGGTCGACGGTATCGATAA	Amplify OMP26 promoter fused with FRT site
OMP-Zeo SOE Rv	CACCGGAACGGCACTGGTCAACTTGGCCATAACACCCCTTAAGAT TGTCGCTATAACCCT	Splicing overlap extension fragment to fuse OMP-FRT fragment with Zeocin Resistance cassette
Zeo-OMP SOE Fw	AGGGTTATAGCGACAATCTTAAGGGGTGTTATGGCCAAGTTGACC AGTGC	Splicing overlap extension fragment to fuse OMP-FRT fragment with Zeocin Resistance cassette
Zeocin-FRT Rv	GCATAGCTGCAGGATCGATATCGAAGTTCCTATACTTTCTAGAGA ATAGGAACTTCGGAATAGGAACTTCACAACCAATTAACCAATTCT GACTAGTCCTGCTCTTCGGCCA	Amplify Zeocin resistance cassette and fuse FRT site
aadB::ZeoRS Fw	GCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTA GGCCGCGAGGTCGACGGTATCGATAA	Amplify *aadB::Zeo* Redswap fragment
aadB::ZeoRS Rv	AACACCTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGGAC GAATTGGCATAGCTGCAGGATCGATA	Amplify *aadB::Zeo* Redswap fragment
recA::ZeoRS Fw	GCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTA GGCCGCGAGGTCGACGGTATCGATAA	Amplify *recA::Zeo* Redswap fragment
recA::ZeoRS Rv	AACACCTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGGAC GAATTGGCATAGCTGCAGGATCGATA	Amplify *recA::Zeo* Redswap fragment
OMP Fw +NcoI	GCTACCATGGTTTGGGTTGTCACTCATCGTATTTGG	Amplify OMP26 promoter fused with NcoI restriction site for insertion into pBAV1K-T5-GFP
OMP-aadB SOE Rv	GTGTATCAATGTGACCTGCGTTGTGTCCATAACACCCCTTAAGAT TGTCGCTATAACCC	Splicing overlap extension fragment to fuse OMP promoter with *aadB*
aadB-OMP SOE Fw	AGGGTTATAGCGACAATCTTAAGGGGTGTTATGGACACAACGCA GGTCACATTGATA	Splicing overlap extension fragment to fuse OMP promoter with *aadB*
aadB Rv +BamHI	CGTAGGATCCTTAGGCCGCATATCGCGA	Amplify *aadB* with BamHI restriction site for insertion into pBAV1K-T5-GFP
OMP-recA SOE Rv	CGCTTTCTGTTTGTTTTCGTCGATAGCCATAACACCCCTTAAGATT GTCGCTATA	Splicing overlap extension fragment to fuse OMP promoter with *recA*
recA-OMP SOE Fw	AGGGTTATAGCGACAATCTTAAGGGGTGTTATGGCTATCGACGAA AACAAACAG	Splicing overlap extension fragment to fuse OMP promoter with *recA*
recA Rv +BamHI	CGTAGGATCCTTAAAAGTCTTCGTTGGTTTCTTCAGC	Amplify *recA* with BamHI restriction site for insertion into pBAV1K-T5-GFP

Supplementary Table 3.2. Primers Used in This Study

A



B

Antimicrobial agent	MIC	Interpretation
Pip/Tazo	≥128	R
Cefazolin	≥64	R
Cefoxitin	≥64	R
Ceftazidime	≥64	R
Ceftriaxone	32	R
Cefepime	8	R
Aztreonam	≥64	R
Ertapenem	4	R
Meropenem	≥64	R
Amikacin	≤2	S
Gentamicin	8	I
Tobramycin	8	I
Levofloxacin	≥8	R
Tetracycline	4	S
Tigecycline	2	S
Nitrofurantoin	256	R
Bactrim	80	R

Supplementary Figure 3.1. Mu1307 Antibiogram. A) Tobramycin population analysis profile of Mu1307 showing resistant subpopulation appearing at 8 μg/ml of tobramycin and surviving at the highest concentration tested (128 μg/ml). B) Antibiogram of Mu1307. Antibiotic susceptibility was tested by automated Vitek 2 (Biomerieux) using the GN74 susceptibility card. Susceptibility results were interpreted by a licensed clinical microbiologist at Emory Hospital.

Chapter 4: Discussion and Relevance

Antibiotic resistance remains a global crisis that threatens the advances of modern healthcare due to our decreasing capacity to prevent and treat bacterial infections. Adding to this challenge are instances of unexplained antibiotic treatment failure. One explanation for why treatment failure may occur is heteroresistance, in which the antibiotic resistant subpopulations are undetected in the clinic but nonetheless negatively impact clinical outcomes. Aminoglycosides remain an important class of antibiotics in treating multidrug resistant pathogens, thus it is critical that we investigate the mechanisms resulting in resistance. The findings presented in this report highlight the need to further study heteroresistance not just to aminoglycosides, but to other important classes of antibiotics used in treating multidrug resistant pathogens. The results of this report provide insight into the concerns regarding aminoglycoside heteroresistance.

The first key finding of this work is that heteroresistance occurs extensively among priority Gram-negative pathogens. We surveyed carbapenem-resistant *Acinetobacter baumannii* (CRAB) and three species of carbapenem-resistant *Enterobacteriaceae* (CRE; *Enterobacter* spp., *Escherichia* spp., and *Klebsiella* spp.) for heteroresistance to the aminoglycosides tobramycin, gentamicin, and amikacin. Resistant subpopulations for each drug were found for all four species tested by population analysis profiles (PAP). In *A. baumannii* the rates of heteroresistance surpassed the rates of homogenous resistance detected (**Table 2.1)**. Even more alarming were instances of resistant subpopulations identified in our PAPs that were undetected by clinical diagnostic methods (**Figure 3.1**). This incorrect diagnosis may be one of the causes of antibiotic treatment failure.

We show that the cause of tobramycin heteroresistance is amplification of the 2″-aminoglycoside nucleotidyltransferase gene *aadB.* Amplification of *aadB* was first described in

the *A. baumannii* pathogenic laboratory strain AB5075 [58]. Following our surveillance work in CRAB for aminoglycoside heteroresistance, we identified *aadB* amplification occurring in 17 out of the 26 tobramycin heteroresistant strains that were observed to contain *aadB* (65.4%) (**Figure 2.2**). We then found *aadB* amplification in the CRE *Enterobacter cloacae* strain Mu1307 (**Figure 3.2**). We report that *aadB* amplification can be selected for during tobramycin therapy and is unstable in Mu1307 (**Figure 3.4**). The instability of these amplification events increases the difficultly in detecting resistant subpopulations which can impact antibiotic treatment. Finally, we demonstrated in both CRAB and CRE that tobramycin heteroresistance mediates treatment failure *in vivo* (**Figure 2.4** and **Figure 3.7**, respectively). Our reports are the first findings to present evidence of aminoglycoside heteroresistance leading to *in vivo* treatment failure. Our study sheds light on the clinical importance of this phenotype during antibiotic therapy which will be discussed below. This work reveals an urgent need to continue to study the mechanisms that facilitate aminoglycoside heteroresistance.

Gene Amplification Mediated by Homologous Recombination

The mechanism observed for tobramycin heteroresistance was amplification of the aminoglycoside modifying enzyme *aadB* in both CRAB and CRE isolates. Amplification of the aminoglycoside modifying enzyme *aphA1* has previously been described in *A. baumannii*, causing unstable tobramycin resistance [108].

The role of *aadB* in tobramycin heteroresistance was confirmed by genetic analysis of an *aadB* deletion mutant in Mu1307. This deletion strain lacked the resistant subpopulation of cells as assayed by Etest an PAP (**Figure 3.2)**. In Mu1307, *aadB* was located on a 21kb region that was flanked by inverted repeats consisting of the genes *folP* and *emrE*, involved in the folate synthesis pathway and a multidrug transporter, respectively (**Figure 3.3)**. Four additional resistance genes

were also found within this region by sequencing analysis: two aminoglycoside modifying enzymes (*aacA4* and *aadA2*), the carbapenemase bla_{OXA-1}, and the chloramphenicol resistance gene *catB3*.

Amplification of *aadA2* was also observed when measured by real-time quantitative PCR (RT-qPCR) (**Figure 3.2)**. We tested a gene encoding a hypothetical protein that was found directly outside the inverted repeats and found no amplification events at this location. To determine if homologous recombination mediates tobramycin heteroresistance, a deletion of the single stranded binding protein RecA was created in Mu1307. When tested by PAP and Etest, the *recA* mutant showed increased susceptibility and no tobramycin resistant subpopulations (**Figure 3.5)**. Colonies in the *recA* mutant Etest were observed along the outer edges of the zone of clearing and are discussed further below.

Based on these data, it is likely that 21kb region flanked by inverted repeats is being amplified in Mu1307. Amplification of this region would not only provide increased resistance to tobramycin, but potentially to other antibiotics due to the additional resistance genes present in this region as well. In AB5075, *aadB* was found in a region containing four other resistance genes flanked by the repeated sequences of the integrase genes *intL*. Amplification was observed in the region flanked by *intL* but not outside this region similar to the findings we present here. Together, these data suggest that heteroresistance by gene amplification may be more complex than initially thought.

Aminoglycoside Heteroresistance Dynamics

One topic that requires more investigation is determining the genetic dynamics involved in generating resistant subpopulations. There are several factors which make studying and detecting

heteroresistance quite difficult. The variability in resistant subpopulation frequency and the level of the resistance generated throughout these populations make it difficult to determine the sensitivity needed to identify these populations. This information is crucial in order to create more accurate methods that could be simplified and applied to routine clinical diagnostics. It has been shown that resistant subpopulations with low frequencies (~1/10,000) can cause colistin treatment failure in mice [57]. However, these populations were able to tolerate high levels of colistin (>500 μg/ml). The clinical relevance of subpopulations that occur at lower frequencies and with levels of resistance that occur closer to the antibiotic breakpoint remain unclear.

The mechanisms that result in unstable heteroresistance, such as gene amplifications or mutations that produce small colony variants, make tackling this issue difficult [48]. This instability results in complications when trying to study these resistant subpopulations as it is unclear if the resistant subpopulation will be present once the strain has been passaged, either in the clinic or the lab. Thus, heteroresistance requires more laborious methods to study and detect the resistant subpopulations. In studying the prevalence rates of *aadB* in CRAB, we observed 1) that the strains tested had varying frequencies of subpopulations occurring when tested by PAP and 2) that in heteroresistant strains that harbored *aadB* amplifications, these amplifications occurred at varying levels, with some strains showing a fold change of just over 2, and others 100-fold changes in copy number when treated with tobramycin. It is unclear what factors cause this variation in amplification level, but one explanation may be the result of homology length present in the repeat sequences, which will be explored below. Further complicating the issues with heteroresistance are that the preexisting resistant subpopulations can be selected for in increasing concentrations of antibiotics, but their presence may affect resistance phenotype interpretations when trying to examine them in an academic setting, similar to the phase variation seen in *A.*

baumannii which results in multiple opacity phenotypes that can affect antibiotic resistance and virulence [125].

If heteroresistance is mediated by homologous recombination, mutations that affect the rate of homologous recombination may offer some insight into the factors that determine the frequencies at which resistant subpopulations can appear. It has been shown that total homology length is a factor in homologous recombination. In the study by Inbar *et al.*, it was shown that when there was more homology present between DNA strands, higher numbers of cross-over events occurred [126]. If this principle were applied to heteroresistant strains where amplifications were observed, it would be possible to either decrease or increase the length of homology and measure the effects on amplification and resistance. This may even allow for fine tuning expression of not only resistance genes but other genes and being able to select populations with desired expression levels, if simply expressing the gene constitutively alone was not possible in the entire population. This may provide another rationale to study heteroresistance.

Amplification-Independent Heteroresistance Mechanisms

As mentioned above, when the *recA* mutant was examined by Etest in Mu1307, susceptibility to tobramycin was increased, and resistant subpopulations were not detected. However, colonies were observed along the outer edges of the zone of clearing on the Etest, though the *recA* mutant did not display any *aadB* amplifications (**Figure 3.5)**. These colonies did not appear to be resistant as they were only observed below the clinical breakpoint. A similar result was shown for the *recA* mutant previously mentioned in AB5075. These colonies likely arise through additional means of antibiotic tolerance and are *recA*-independent.

We note that *aadB*-independent mechanisms may be present in tobramycin heteroresistant CRAB isolates. In chapter 2, out of the 39 tobramycin heteroresistant *A. baumannii* strains that were tested, 17 had confirmed *aadB* amplification. This suggests that in over half of the remaining heteroresistant strains, there are other mechanisms responsible for the generation of resistant subpopulations. The most probable explanation is the amplification of another tobramycin resistance gene, though this has not been confirmed yet in these isolates. There may be other possible causes which will be outlined below.

Mutations affecting electron transport can lead to the formation of small colony variants (SCVs) in the presence of aminoglycosides [29,90]. Mutations that generate SCVs would show increased tolerance to aminoglycosides due to slower rates of growth and increased aminoglycoside resistance. Mutations found in known resistance genes have been shown to generate heteroresistance, such as *fadB* in *A. baumannii* [127]. Persister formation, which results in the formation of cells that are metabolically dormant is another type of subpopulation that shows increased resistance to antibiotics [75]. Mutations in similar genes or pathways could arise during tobramycin treatment and may explain the subpopulations seen with increased tolerance to tobramycin not mediated by *aadB* or recA-dependent homologous recombination. The relevance of these tolerant colonies in Mu1307 has yet to be explored but may be important to discover how multiple subpopulations can occur through different pathways.

Altogether, the data presented in this study demonstrate that aminoglycoside heteroresistance is present among multidrug resistant Gram-negative pathogens and mediates treatment failure *in vivo* (**Figure 4.1**). It is crucial that we investigate the mechanisms that facilitate heteroresistance, including gene amplification, to develop more accurate methods to detect and study these resistant subpopulations. Due to the fact that aminoglycoside heteroresistance can

cause treatment failure, it is necessary for these resistant subpopulations to be appreciated by clinicians and considered when deciding on antibiotic therapies to prevent further antibiotic treatment failure.

Several studies have shown gene amplification is not only important for antibiotic resistance in various other bacterial species, but also resistance to antimicrobials in eukaryotic organisms, and even anticancer drug resistance, indicating a broader application for examining the mechanisms [59,68,72,73,128]. In recent years, two important studies have provided detailed insight into mechanisms and treatment strategies for tackling antibiotic heteroresistance [47,48]. Both studies present surveillance data detecting heteroresistance to multiple species of bacteria to multiple classes of antibiotics. Nicoloff *et al.* went on to show that a majority of the heteroresistance observed was caused by gene amplifications [48]. Band *et al.* reported that by targeting distinct heteroresistance pathways (where subpopulations do not share resistance to the same antibiotics), heteroresistance to multiple drugs can be exploited by combination therapy targeting individual subpopulations [47]. Heteroresistance is a mechanism that needs more precise scrutiny as it may lead to the development of newer treatment options and expand our knowledge of the antibiotic resistance landscape.

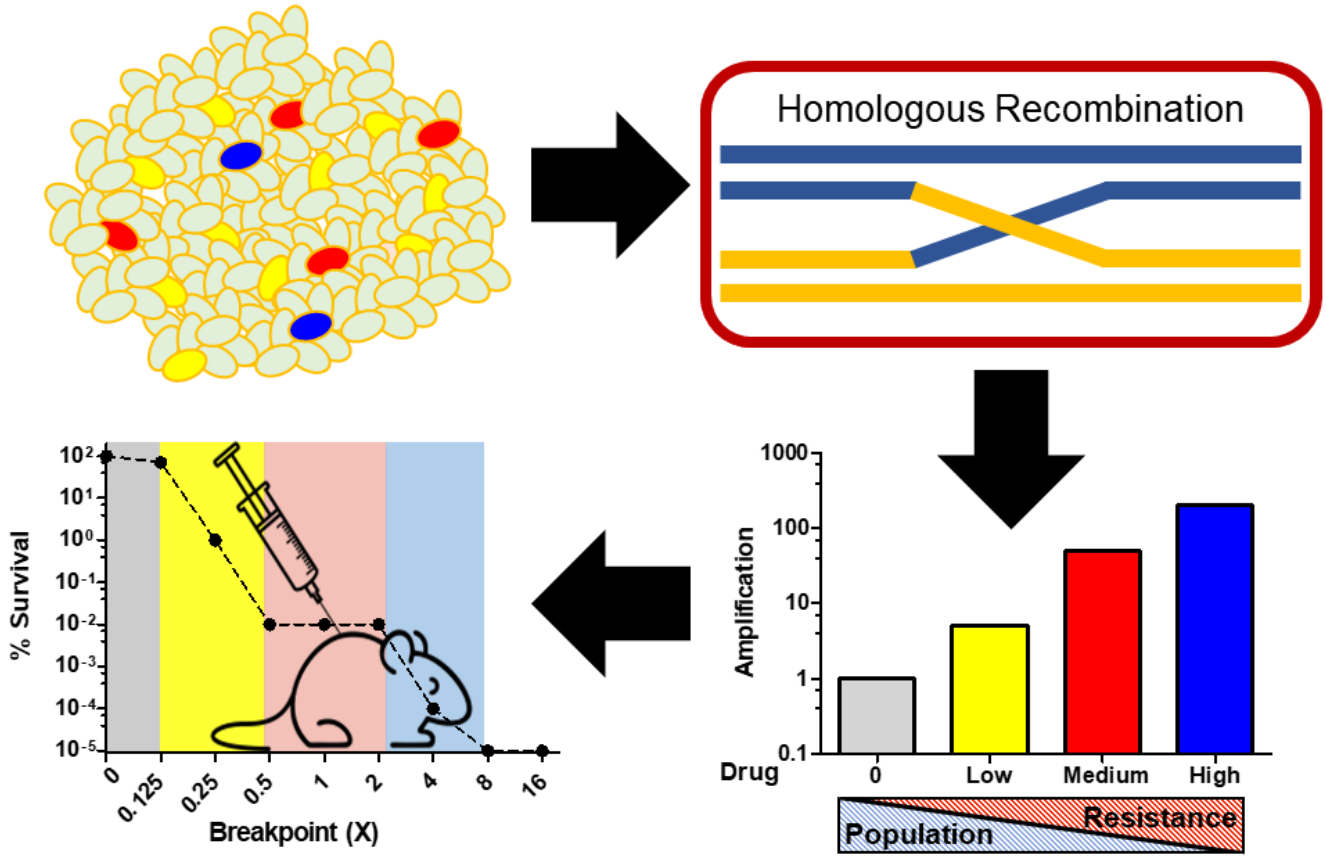


Figure 4.1. Summary of Work Presented in This Study. Heteroresistance creates resistant subpopulations within a susceptible population of bacteria. These subpopulations are mediated by homologous recombination. Homologous recombination facilitates gene amplifications that occur at various levels in the resistant subpopulations, with the highest amplifications present in the smallest subpopulations. These subpopulations are detected though population analysis profiles (PAP) as cells with decreased survival but higher resistance relative to the antibiotic breakpoint. These resistant subpopulations mediate antibiotic treatment failure in a mouse model.

Chapter 5: Appendix

5A: Aminoglycoside heteroresistance in *Acinetobacter baumannii* AB5075

Sarah E. Anderson,[a,b] **Edgar X. Sherman**,[a,b,c] David S. Weiss,[a,b,c,d,e] Philip N. Rather[a,b,e]#

Originally published in mSphere 2018 Jul-Aug; 3(4): e00271-18.

[a]Department of Microbiology and Immunology, Emory University, Atlanta, Georgia, USA

[b]Emory Antibiotic Resistance Center, Emory University, Atlanta, Georgia, USA

[c]Emory Vaccine Center, Emory University, Atlanta, Georgia, USA

[d]Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, Georgia, USA

[e]Research Service, Atlanta VA Medical Center, Decatur, Georgia, USA

Keywords: Heteroresistance, *Acinetobacter baumannii*, gene amplification, aminoglycoside

#Address correspondence to Philip N. Rather, prather@emory.edu

Abstract

Heteroresistance is a phenomenon where a subpopulation of cells exhibits higher levels of antibiotic resistance than the general population. Analysis of tobramycin resistance in *Acinetobacter baumannii* AB5075 using Etest strips demonstrated that colonies with increased resistance arose at high frequency within the zone of growth inhibition. The presence of a resistant subpopulation was confirmed by population analysis profiling (PAP). The tobramycin-resistant subpopulation was cross resistant to gentamicin but not amikacin. The increased tobramycin resistance phenotype was highly unstable, and cells reverted to a less resistant population at frequencies of 60 to 90% after growth on nonselective media. Furthermore, the frequency of the resistant subpopulation was not increased by preincubation with subinhibitory concentrations of tobramycin. The tobramycin-resistant subpopulation was shown to replicate during the course of antibiotic treatment, demonstrating that these were not persister cells. In *A. baumannii* AB5075, a large plasmid (p1AB5075) carries *aadB*, a 2″-nucleotidyltransferase that confers resistance to both tobramycin and gentamicin but not amikacin. The *aadB* gene is part of an integron and is carried adjacent to four additional resistance genes that are all flanked by copies of an integrase gene. In isolates with increased resistance, this region was highly amplified in a RecA-dependent manner. However, in a *recA* mutant, colonies with unstable tobramycin resistance arose by a mechanism that did not involve amplification of this region. These data indicate that tobramycin heteroresistance occurs by at least two mechanisms in *A. baumannii*, and future studies to determine its effect on patient outcomes are warranted.

Importance. *Acinetobacter baumannii* has become an important pathogen in hospitals worldwide, where the incidence of these infections has been increasing. *A. baumannii* infections have become exceedingly difficult to treat due to a rapid increase in the frequency of multidrug- and pan-resistant isolates. This has prompted the World Health Organization to list *A. baumannii* as the top priority for the research and development of new antibiotics. This study reports for the first time a detailed analysis of aminoglycoside heteroresistance in *A. baumannii*. We define the mechanistic basis for heteroresistance, where the *aadB(ant2")Ia* gene encoding an aminoglycoside adenylyltransferase becomes highly amplified in a RecA-dependent manner. Remarkably, this amplification of 20 to 40 copies occurs stochastically in 1/200 cells in the absence of antibiotic selection. In addition, we provide evidence for a second RecA-independent mechanism for aminoglycoside heteroresistance. This study reveals that aminoglycoside resistance in *A. baumannii* is far more complex than previously realized and has important implications for the use of aminoglycosides in treating *A. baumannii* infections.

Introduction

Antibiotic heteroresistance occurs when subpopulations of an isogenic bacterial strain exhibit decreased susceptibility to a particular antibiotic [42]. Although the clinical significance of heteroresistance has been the subject of debate, resistant subpopulations of otherwise susceptible strains have been demonstrated to mediate treatment failure in animal models [57], and have been associated with treatment failure in human patients [129-132]. Heteroresistance has been reported to a wide variety of antibiotics, including β-lactams, glycopeptides, and antimicrobial peptides [42,54,77,133]. However, reports of aminoglycoside heteroresistance are uncommon. In 1947, it was reported that populations of type B *Haemophilus influenzae* contained rare cells with increased streptomycin resistance, although the mechanism responsible for the formation of these cells was not investigated [46]. More recently, decreased expression of the porin gene *ompC* was associated with nonmutational kanamycin resistant subpopulations in *Salmonella enterica* [134]. However, to our knowledge, heteroresistance to tobramycin or gentamicin has not been previously reported.

Acinetobacter baumannii is a Gram-negative, nosocomial, opportunistic pathogen [135-137]. Widespread antibiotic resistance in this species recently led the World Health Organization to name carbapenem-resistant *A. baumannii* as its most critical priority pathogen for research and development of new interventions [138]. Aminoglycoside resistance in *A. baumannii* has been associated with the acquisition, increased expression, and/or gene amplification of aminoglycoside-modifying enzymes and efflux pumps [136,139,140]. The multidrug-resistant isolate AB5075 carries a number of antibiotic resistance genes, many of which are carried on the large plasmid p1AB5075. This plasmid includes an integron-like structure encoding four aminoglycoside-modifying enzymes, including the tobramycin resistance gene *aadB*, and a

chloramphenicol resistance transporter [141]. The plasmid also carries an additional tobramycin-modifying gene, *aacA4* [127,141]. A recent study of loci required for tobramycin resistance in AB5075 showed that in addition to *aadB* and *aacA4*, 34 chromosomal genes also contribute to resistance to this drug [127].

AB5075 is resistant to tobramycin and gentamicin, meaning that its MIC values for these drugs are above the CLSI breakpoints [127,142]. Here, we report that AB5075 also exhibits tobramycin and gentamicin heteroresistance, as it produces subpopulations of cells that grow at concentrations of these drugs that are higher than the MIC for the general population. The subpopulations with increased resistance were shown to be unstable. We demonstrated that the integron-like structure of five adjacent antibiotic resistance genes, including *aadB*, becomes amplified to 20 to 40 copies in this resistant subpopulation. While this amplification was RecA dependent, colonies with increased tobramycin resistance could also be selected in a *recA::Tc* mutant. These resistant isolates did not contain amplifications of the region containing *aadB*, indicating that tobramycin heteroresistance can occur by at least two distinct mechanisms.

Results

An AB5075 subpopulation exhibits increased tobramycin and gentamicin resistance. When performing tobramycin Etest assays with the *A. baumannii* strain AB5075, we observed colonies arising at a high frequency within the zone of inhibition, consistent with a phenomenon termed heteroresistance (**Fig. 5A.1A**). Heteroresistance was not observed during Etest assays with colistin, rifampin, or tetracycline (data not shown). In order to characterize the population with increased tobramycin resistance, colonies representative of this subpopulation were isolated by plating AB5075 on agar plates with various inhibitory concentrations of tobramycin. In general, colonies representing the resistant subpopulation were heterogenous in size on tobramycin plates, but exhibited normal size on medium without drug. The colony size differences in the presence of tobramycin likely reflect differences in the levels of resistance. The frequency of resistant colonies decreased with increasing drug concentrations, so we chose a concentration twice the baseline MIC to determine the frequency. In three independent experiments, colonies with increased resistance arose at an average frequency of 0.52% $\pm$ 0.24%. Among the resistant subpopulation, both virulent opaque (VIR-O) and avirulent translucent (AV-T) colony variants were observed, as described previously [125,143]. Because VIR-O and AV-T variants were previously reported to exhibit subtle differences in tobramycin resistance [125,144], both VIR-O and AV-T tobramycin-resistant colonies were selected for further characterization. These resistant isolates were designated hetR-O2, hetR-O3, hetR-T1, and hetR-T4.

The subpopulation with increased tobramycin resistance was analyzed for cross-resistance to other antimicrobials. Etest assays were used to compare antimicrobial susceptibilities of the tobramycin-resistant isolates to those wild-type VIR-O and AV-T variants with baseline levels of resistance. Etest assays for a variety of antimicrobials were conducted, revealing that hetR-O2,

hetR-O3, hetR-T1, and hetR-T4 all exhibited cross-resistance to gentamicin but not amikacin (**Table 5A.1**). In addition, cross-resistance to nonaminoglycosides was not observed (**Table 5A.1**).

The tobramycin-resistant subpopulation is not induced by tobramycin and is not composed of persister cells. Further phenotypic characterization of the tobramycin-resistant subpopulation was performed. The ability of subinhibitory concentrations of tobramycin to induce the formation of the tobramycin-resistant subpopulation was first investigated. Population analysis profile (PAP) results for treated and untreated cultures of AB5075 were identical (**Fig. 5A.2A**), suggesting that the formation of the tobramycin resistant subpopulation was not increased by the presence of tobramycin. To confirm that the tobramycin-resistant subpopulation is capable of growing in the presence of antibiotic, a killing assay with AB5075 was conducted. This assay clearly demonstrated that the tobramycin-resistant subpopulation was capable of growing in high tobramycin concentrations, as the CFU counts increased during treatment (**Fig. 5A.2B**). These data show that AB5075 forms a subpopulation with increased resistance to tobramycin that is distinct from persister cells, which are antibiotic tolerant at the expense of metabolic activity and active growth [145]. Although exposure to a subinhibitory concentration of tobramycin does not increase the frequency of the resistant subpopulation, selection with a high level of drug kills off the majority of susceptible cells, allowing the resistant subpopulation to dominate the culture.

Increased tobramycin resistance is an unstable phenotype. The stability of the increased tobramycin resistance phenotype was first tested in liquid culture. Cultures containing a mix of VIR-O and AV-T cells were grown, treated with tobramycin, and then subcultured without drug. Serial dilutions of cultures at each step indicated that following subculture without drug, the

population reverted from being almost entirely tobramycin resistant to containing only about 10% resistant cells (**Fig. 5A.2C**). To determine whether VIR-O and AV-T representatives of the tobramycin-resistant subpopulation behave similarly in terms of their resistance stability, the hetR-O2 and hetR-T1 stocks were examined for loss of resistance on agar plates. Colonies of hetR-O2 and hetR-T1 were passaged from plates containing tobramycin onto plates with and without drug. After 24 h and 48 h of growth, individual colonies were resuspended and serially diluted onto plates with and without tobramycin to determine the percent resistance within individual colonies (**Fig. 5A.2D, E**). Interestingly, hetR-O2 and hetR-T1 exhibited differing levels of resistance stability. At 24 h, the percent resistance values of hetR-T1 were similar for colonies grown with and without tobramycin, with about half of the cells from each condition exhibiting resistance. In contrast, hetR-O2 colonies grown without tobramycin for 24 h lost their increased resistance, whereas the majority of cells taken from colonies grown with tobramycin remained resistant (**Fig. 5A.2D**). In colonies at 48 h, the frequency of resistant cells for both hetR-O2 and hetR-T1 continued to drop (**Fig. 5A.2E**). Taken together, these results show that increased tobramycin resistance in AB5075 is an unstable phenotype, although the level of instability varies within the tobramycin-resistant subpopulation.

Tobramycin-resistant subpopulations exhibit increased expression of *aadB*. The mechanism of tobramycin heteroresistance in AB5075 was investigated by performing quantitative reverse transcriptase PCR (qRT-PCR) on hetR-O2 and hetR-T1, as well as the parental AB5075 VIR-O and AV-T variants not exposed to tobramycin. Two genes, *aadB* and *aacA4,* known to confer tobramycin resistance were selected for gene expression analysis [127]. Differences in *aadB* or *aacA4* expression between the wild-type VIR-O and AV-T variants were not observed (**Fig.**

5SA.3A and B). However, hetR-O2 and hetR-T1 exhibited 5- and 15-fold increased expression of *aadB*, respectively, compared to VIR-O (Fig. 3A). No differences in expression were observed for *aacA4* (**Fig. 5A.3B**). These results are consistent with the aminoglycoside resistance profiles of hetR-O2 and hetR-T1, as *aadB* is associated with resistance to both tobramycin and gentamicin but not amikacin [88].

Tobramycin-resistant subpopulations contain a highly amplified region that includes *aadB*. To determine whether the preexisting tobramycin-resistant subpopulation in AB5075 could be due to gene amplification, we measured gene copy number of the region surrounding *aadB*. The *aadB* gene is found on the large plasmid p1AB5075 and is carried adjacent to four other resistance genes that are all flanked by two copies of an integrase (*intI*) gene in the same orientation (**Fig. 5A.4**). We hypothesized that if an amplification event were occurring, it would be within the interval flanked by the *intI* genes, possibly facilitated by recombination between the *intI* genes on adjacent plasmids during DNA replication. Gene copy number was measured by quantitative PCR (qPCR) using genomic DNA (gDNA) from the hetR-O2 and hetR-T1 isolates, as well as gDNA generated from VIR-O cells not exposed to tobramycin. The relative copy numbers of the *strB* and *aadB* genes (located immediately inside the interval flanked by *intI*) and the *ABUW_4052* and *ABUW_RS19335* genes (located immediately outside the *intI* region) were normalized to levels of *aacA4*, which is carried outside this region and served as a control for changes in plasmid copy number. In both the hetR-O2 and hetR-T1 isolates, the *strB* and *aadB* genes were highly amplified, whereas the *ABUW_4052* and *ABUW_RS19335* genes were not amplified (**Table 5A.3**). As presented previously in Fig 2D and E, cells lost tobramycin resistance when grown in the absence of antibiotic. Consistent with this loss of resistance, the levels of *aadB* amplification were reduced

in colonies at 24 h in the absence of selection (**Table 5A.3**). These results strongly suggest that tobramycin heteroresistance in AB5075 is due to the amplification of *aadB*.

To confirm the role of *aadB* in heteroresistance, we obtained three independent *aadB::T26* transposon insertion mutants from the University of Washington library. However, PCR analysis of the mutants revealed that all three had two copies of *aadB*, a wild-type copy and a T26-disrupted copy (data not shown). Similarly, in our wild-type VIR-O AB5075 parent strain, qPCR analysis indicated that there were two copies of *strB* and *aadB* relative to the *aacA4* gene (**Table 5A.2**), which is in contrast to the published genome sequence, where a single copy of each gene is present [141]. Southern blot analysis confirmed that the region between the *intI* genes was duplicated in our AB5075 parental strain (data not shown). Due to the duplication of *aadB* in our parental strain and the presence of a wild-type copy of *aadB* in the University of Washington library mutants, we have been unable to construct and test a defined *aadB* mutant.

Tobramycin heteroresistance can occur by RecA-dependent and -independent mechanisms. To determine if the above amplification event between duplicated copies of the *intI* gene required homologous recombination, tobramycin heteroresistance was examined in a *recA::Tc* mutant. The *recA::Tc* mutant exhibited intrinsic levels of tobramycin resistance that were lower than the wild-type parent (**Fig. 5A.1B and Table 5A.3**). Although this strain still appeared heteroresistant, the frequency of tobramycin-resistant colonies arising in the zone of clearing was lower than in wild-type cells (**Fig. 5A.1B**). Introduction of the wild-type *recA* gene into the *recA::Tc* mutant partially restored heteroresistance (**See Fig 5A.S1** in the supplementary material). Interestingly, when six tobramycin-resistant isolates from the *recA::Tc* mutant were tested for amplification of *aadB*, the copy number was similar to the *recA::Tc* parent strain (**Table 5A.4**). This indicates that increased

resistance arose in these isolates by a mechanism that did not involve *aadB* amplification. When three of these resistant isolates, 1-2, 1-4, and 1-10 were cultured for approximately 30 generations in the absence of tobramycin, the frequency of cells retaining tobramycin resistance was 11%, 23%, and 35%, respectively, demonstrating that these isolates are not stable mutants. The resistant subpopulation in the *recA::Tc* mutant consisted of both VIR-O and AV-T cells (data not shown).

To confirm that the tobramycin-resistant subpopulation in the *recA::Tc* mutant was capable of growing in the presence of antibiotic, a tobramycin killing assay was conducted (**Fig. 5A.S2A**). This indicated that the tobramycin-resistant subpopulation was capable of growing in the presence of drug and was not composed of persister cells. In addition, PAP analysis demonstrated that pretreatment with tobramycin did not alter the frequency of cells that became resistant to tobramycin in the *recA::Tc* background (**Fig. 5A.S2B**).

Discussion

This study demonstrates that subpopulations of *A. baumannii* AB5075 exhibit heterogeneous levels of resistance to aminoglycosides. The subpopulation with increased tobramycin resistance can be visualized using both an Etest MIC assay and PAP analysis. Application of selective concentrations of tobramycin resulted in the outgrowth of the tobramycin-resistant subpopulation, allowing it to dominate the surviving culture. However, once selective pressure was removed, the majority of the subpopulation returned to baseline levels of resistance. Independent isolates from the resistant subpopulation were found to exhibit increased expression and amplification of the 2″-aminoglycoside nucleotidyltransferase gene *aadB.* This gene is likely involved in heteroresistance as the substrate profile of this enzyme matches the resistance profile observed, i.e., resistance to tobramycin and gentamicin but not amikacin.

The role of phenotypic heterogeneity in the formation of subpopulations that are able to survive antibiotic treatment is well appreciated. This has been best studied in the case of dormant persister cells and slow-growing small-colony variants (SCVs), both of which exhibit increased tolerance to antibiotics, including aminoglycosides [75,146-148]. Both persistence and SCV formation confer antibiotic tolerance at the expense of normal growth. In contrast, heteroresistance involves the formation of a subpopulation of cells with increased antibiotic resistance, which maintain the ability to actively grow during antibiotic exposure [42]. Although heteroresistance has been reported in many species, including *A. baumannii* [82,149], reports of aminoglycoside heteroresistance are exceedingly rare. This study is the first report of aminoglycoside heteroresistance in *A. baumannii*, and to our knowledge only the second definitive report of this phenomenon in any species. In *Salmonella enterica*, aminoglycoside heteroresistance has been shown to occur when a subpopulation of cells expresses decreased levels of the porin gene *ompC*. This limits uptake of kanamycin into the cells, causing increased resistance [134].

This study demonstrates that tobramycin heteroresistance can arise by the extensive amplification (20-40 copies) of a region carried on the large p1AB5075 plasmid that includes five resistance genes in tandem, including *aadB,* flanked by copies of an integrase (*intI*) gene. The mechanism by which the *aadB*-containing region gets extensively amplified is unclear, but our work has established that RecA is required. Duplications can occur by nonequal recombination between directly repeated regions on adjacent replicons [59]. Gene amplification of the aminoglycoside-modifying enzyme gene *aphA1* has previously been reported to cause unstable tobramycin resistance in *A. baumannii* AB0057 and a clinical isolate [139]. However, in that study, amplifications were dependent on prior exposure to tobramycin and were likely selected for by the antibiotic. Heteroresistance resulting from gene amplification of a chromosomal locus has also been reported in *Salmonella enterica*, where amplification of a region containing *pmrD* conferred colistin heteroresistance [150]. Our data suggest that extensive amplifications preexist in 1/200 cells (i.e., the frequency of the resistant subpopulation) in the absence of any selective tobramycin pressure. As extensive gene amplifications typically require growth in the presence of selective pressures, this suggests that additional mechanisms, such as the rolling-circle-dependent generation of tandem arrays, may contribute to amplification of this region in *A. baumannii* [59]. In a *recA::Tc* mutant, cells with increased resistance still arose, but none of the isolates examined contained duplications of the *aadB* gene (**Table 5A.3**). Therefore, heteroresistance can occur by at least one additional mechanism. A recent study by Gallagher *et al.* demonstrated at least 32 chromosomally carried genes in AB5075 function to maintain intrinsic tobramycin resistance [127]. In principle, amplification of any of these genes could potentially lead to increased tobramycin resistance and may account for the resistant subpopulation that does not contain

amplification of *aadB*. However, if this amplification is occurring, it does not appear to require RecA.

When the stability of the tobramycin-resistant subpopulation was examined, contrasting results were found for the virulent opaque (VIR-O) and avirulent translucent (AV-T) isolates (**Fig. 5A.2D**). As long as selection was maintained, the majority of hetR-O2 cells remained tobramycin resistant; however, resistance was lost in approximately 90% of the population in colonies grown without drug for 24 h. In contrast, cells of hetR-T1 lost their increased resistance at roughly the same rate regardless of whether selection was maintained, with approximately 50% of the population in a 24-h colony maintaining the increased resistance state. However, in both isolates the loss of heteroresistance at high rates is consistent with the unstable nature of extensive duplications [59]. The increased stability of heteroresistance in hetR-T1 in the absence of selection may be due to the larger number of tandem repeats that includes *aadB* (**Table 5A.2**).

The clinical relevance of the aminoglycoside heteroresistance phenomenon described here remains to be determined. The plasmid-borne *aadB* gene is common in *A. baumannii* and strains carrying this gene should exhibit clinically relevant resistance to tobramycin and gentamicin [151-154]. Further research is needed to determine whether *A. baumannii* strains identified as being aminoglycoside sensitive also exhibit heteroresistance, which could pose problems for appropriately treating these infections. It is possible that strains lacking *aadB* could still exhibit heteroresistance by an *aadB*-independent mechanism, similarly to the *recA::Tc* mutant discussed in this work. The data presented here illustrate that the full picture of antibiotic resistance in *A. baumannii* is more complicated than has been traditionally recognized, with both the acquisition of resistance determinants and phenotypic heterogeneity contributing to resistance.

Materials and Methods

Bacterial strains and growth conditions. Strains of *A. baumannii* were maintained at -80ºC in 15% glycerol. Pure stocks of opaque and translucent variants were prepared as previously described [144]. Liquid cultures were prepared in sterile LB broth, supplemented as needed with tobramycin (Sigma-Aldrich, St. Louis, MO) at the concentrations indicated. Resistance stability experiments and experiments to select isolates with increased resistance were performed using 0.5x LB supplemented with 0.8% agar. All other experiments were performed using regular LB supplemented with 1.5% agar. Plates were supplemented with tobramycin as indicated.

A T26 insertion mutant in *recA* was obtained from the AB5075 transposon mutant library maintained at the University of Washington [141]. A culture of this strain was grown overnight at 37ºC with shaking and used to prepared genomic DNA (gDNA) as outlined below. A culture of VIR-O AB5075 was grown at 37ºC with shaking to late log phase and used to prepare electrocompetent cells by washing three times with 10% glycerol. The *recA::T26* mutant DNA was electroporated into these cells, and transformants were selected on LB supplemented with 10 μg/ml tetracycline. A single colony was isolated and designated the *recA::Tc* strain. The presence of the *recA* mutation was confirmed by PCR.

Isolation of tobramycin-resistant subpopulations was conducted by plating serial dilutions of an early-log-phase culture of the wild-type or *recA::Tc* VIR-O variant on 0.5X LB and 0.5X LB containing tobramycin (Sigma-Aldrich, St. Louis, MO) at 2.5, 5, 10, 15, 20, 25, and 30 μg/ml. Colonies exhibiting increased resistance were apparent at 15 μg/ml tobramycin for wild type and at 5 μg/ml for the *recA:;Tc* mutant. Resistant colonies were restreaked on 0.5X LB with

tobramycin and examined under a stereomicroscope with oblique lighting to determine whether they were opaque or translucent variants.

Oligonucleotides. All oligonucleotides used in this study are listed in **Table 5A.5**

Complementation of the *recA* mutant. The wild-type *recA* gene was amplified by PCR using the primers oSA77 5'-GCTCATCGTTTCGTTTGAAC-3' and oSA78 5'- GAATAAAAACGTC GAGTTGTG-3' (Table 5). This fragment was then cloned into the SmaI site of pQF1266Blue, a derivative of pQF50 [155] where a hygromycin resistance gene has been cloned into the *bla* gene encoding β-lactamase. In addition, this plasmid contains an origin of replication from pWH1266 [156] cloned into the NcoI site. The resulting plasmid was designated precA.

MIC Assays. MICs of different antibiotics were measured using Etest strips (bioMérieux, Marcy-l'Étoile, France). For the tobramycin MICs used to visualize heteroresistance, AB5075 wild-type or *recA::Tc* VIR-O cells were inoculated into LB broth and grown at 37^{o} with shaking to a concentration of 1.1 to 1.3 x 10^7 CFU/ml. Lawns were inoculated by spreading 100 µl of culture onto an LB plate, followed by application of the Etest strip. The plate was photographed following incubation for 16 h at 37^{o}C.

For MIC experiments reported in Table 1, strains were inoculated into LB broth, grown overnight statically at room temperature, grown at 37^{o}C with shaking to an optical density at 600nm (OD_{600}) of 0.1, and stored at 4^{o}C for use later in the day. Etest strips were placed on LB plates, and strains were inoculated next to each strip by spotting 10 µl of culture next to the bottom of the strip, tilting the plate so that the culture spread up the side of the strip, and removing excess

culture at the top of the strip by pipetting. Two strains were inoculated on each side of each strip to facilitate a direct comparison of susceptibility. MIC values were recorded after incubation for 5 h or 16 h at 37ºC, as noted. MIC experiments were performed two independent times to confirm the reproducibility of trends.

For the *recA::Tc* mutant, colonies that were growing in the zone of tobramycin inhibition were placed into a small vial of 20% glycerol and stored at -80ºC. MIC experiments were performed by growing cells from the -80ºC glycerol stock for several hours in LB and performing Etest assays as outlined above for the data presented in Table 1.

Population analysis profile (PAP). Population analysis profile (PAP) was performed by growing bacteria overnight to stationary phase and then plating serial dilutions on LB agar with or without various concentrations of tobramycin (Spectrum, New Brunswick, NJ). Plates were then incubated at 37°C and CFU were enumerated after 24 h. Percent tobramycin resistance was calculated as the number of bacterial colonies that grew on tobramycin plates divided by the number of bacteria that grew on LB alone without drug.

Tobramycin killing assays. Briefly, AB5075 was grown overnight to stationary phase in LB media and serially diluted to $1x10^6$ CFU/ml. Tobramycin (Spectrum) was added at a concentration of 64 µg/ml. One-hundred-microliter aliquots were taken at desired time points, serially diluted, and plated on LB media alone (to quantify total CFU) or LB plates containing 64 µg/ml tobramycin (to quantify resistant CFU).

Stability measurements of the resistant subpopulation. For experiments conducted with broth cultures, AB5075 was grown overnight to stationary phase in LB medium. The bacteria were then serially diluted and plated on LB agar plates with and without 64 µg/ml tobramycin (Spectrum) to enumerate total and resistant CFU for the pretreatment group (day 1). A subculture (1:1,000) was then grown overnight in LB supplemented with 64 µg/ml tobramycin, serially diluted, and plated on LB agar with or without 64 µg/ml tobramycin to enumerate total and resistant CFU for the treatment group (day 2). This process was repeated in LB broth without antibiotics (day 3), and dilutions were plated on agar plates with and without 64 µg/ml tobramycin to enumerate total and resistant CFU.

For experiments with hetR-O2 and hetR-T1 colonies, cells from the two subpopulations were struck from freezer stocks onto 0.5x LB supplemented with 20 µg/ml tobramycin (Sigma-Aldrich). Plates were incubated overnight at 37^{o}C, and single colonies were resuspended in 1 ml LB and struck onto 0.5x LB agar with and without 40 µg/ml tobramycin. After 24 h or 48 h of incubation at 37^{o}C, individual colonies were resuspended in 1 ml LB and serial dilutions were plated in duplicate on 0.5x LB with and without tobramycin 40 µg/ml. Plates were incubated for up to 48 h at 37^{o}C, and colonies were enumerated to determine the percent tobramycin resistance by comparing the CFUs on plates with and without tobramycin.

RNA isolation. Cultures of different *A. baumannii* AB5075 subpopulations were grown in LB medium at 37^{o}C with shaking to an OD_{600} of 0.5. Cells were pelleted by centrifugation and RNA was isolated using the MasterPure RNA purification kit according to the manufacturer's protocol (Epicentre, Madison, WI). Contaminating DNA was degraded by two treatments with TURBO DNA-free according to the manufacturer's protocol (Invitrogen, Waltham, MA). DNA

contamination was evaluated by PCR with purified RNA as template, and RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer.

Quantitative reverse transcriptase PCR. Total RNA (1 µg) was used to prepare cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) with random primers and either iScript or SuperScript III (Invitrogen, Waltham, MA) reverse transcriptase. Reactions with mixtures lacking reverse transcriptase were also performed as a control for the presence of contaminating DNA. Incubation conditions for cDNA synthesis were 25°C for 5 min, 42°C for 45 min, and 85°C for 5 min. cDNA reaction mixtures and controls were then diluted 1:10 with sterile water and used as a template for reverse transcriptase quantitative PCR (qRT-PCR). Oligonucleotide primer pairs for qRT-PCR were designed to amplify approximately 150-bp fragments from each gene of interest and were generated using the Primer-BLAST program available at www.ncbi.nlm.nih.gov/tools/primer-blast. qRT-PCR was performed using iQ Sybr green Supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX Connect cycler. Cycle parameters were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. Melt curve data were then collected to confirm the specificity of the oligonucleotide primer pairs. Data were generated using cDNA prepared from three independent RNA isolations, and qRT-PCRs were performed in technical triplicate to ensure accuracy. Fold changes in gene expression relative to the control strain (VIR-O) and a control gene (*clpX*) were determined using the threshold cycle ($2^{-\Delta\Delta Ct}$) method [94].

Genomic DNA isolation. For experiments presented in Table 2, cultures of different AB5075 subpopulations were grown in LB medium with shaking at 37°C to an OD_{600} of 0.5. For

experiments presented in Table 3, colonies were grown for 24 h on 0.5x LB with or without 40 µg/ml tobramycin; individual colonies were then resuspended in 1 ml of LB. In both cases, cells were pelleted by centrifugation and resuspended in Tris-EDTA (TE). Cells were lysed by incubation with 0.5% SDS and 400 µg/ml proteinase K for 1 h at 37^{o}C. Following lysis, NaCl was added to a final concentration of 0.7 M, and DNA was extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol. DNA was precipitated by mixing with 1.5 volumes of 95% ethanol until a precipitate formed. DNA pellets were collected by centrifugation, washed twice with 75% ethanol, dried, and resuspended in molecular grade water.

Quantitative PCR (qPCR). Concentrations of gDNA samples were determined using a NanoDrop ND-100 spectrophotometer. Samples were diluted to a concentration of 15 µg/ml and qPCR was performed as outlined above for cDNA samples. Standard curves using 10-fold serial dilutions of wild-type gDNA were used to ensure that primers exhibited similar efficiencies. Relative gene copy numbers normalized to *aacA4* were determined using the equation $2^{-\Delta Ct}$ [94].

Statistical analyses. Statistical analyses were performed with Prism 7 (GraphPad Software, Inc. La Jolla, CA).

Table 5A.1. The tobramycin resistant subpopulation exhibits increased cross-resistance to gentamicin but not other antimicrobials.

Direct comparison of strains	MIC (μg/ml) of drug[a]:						
	TOB	CST	RIF	TET	AMK	CHL	GEN
Comparison 1							
VIR-O	48	1	4	3	192	256	96
hetR-O2	128	1	4	3	192	>256	192
Comparison 2							
VIR-O	64	1	4	3	96	96	32
hetR-O3	192	1	4	3	96	128	128
Comparison 3							
AV-T	48	1	4	2	128	256	96
hetR-T1	256	1	4	2	128	192	>256
Comparison 4							
AV-T	32	1	4	3	64	96	32
hetR-T4	384	1	4	3	64	96	128

[a]Abbreviations: TOB, tobramycin; CST, colistin; RIF, rifampin; TET, tetracycline; AMK, amikacin; CHL, chloramphenicol; GEN, gentamicin. MICs were measured after 16 (TOB, CST, RIF, and TET) or 5 (AMK, CHL, and GEN) h of growth at 37°C. MICs were determined using Etest strips. Direct comparisons between isolates were performed by inoculating two cultures at an optical density A_{600} of 0.1 along the same Etest strip. Two independent experiments were performed for each comparison to assess reproducibility; data from a single representative experiment are shown.

Table 5A.2. HetR-O2 and hetR-T1 exhibit gene amplifications that include *aadB*.

	Relative copy no. (mean ± SD) of gene[a]:			
Strain	***ABUW_4052***	***strB***	***aadB***	***ABUW_RS19335***
VIR-O	1.31 ± 0.09	2.94 ± 0.07	2.45 ± 0.06	0.72 ± 0.01
hetR-O2	1.80 ± 0.53	20.26 ± 6.18*	17.23 ± 6.80*	0.83 ± 0.31
hetR-T1	1.44 ± 0.04	55.62 ± 9.27*	44.07 ± 8.26*	0.64 ± 0.03

[a]Relative copy number of genes surrounding *aadB* measured in three biological replicates. Relative copy numbers were measured by qPCR using normalization to *aacA4*, a presumed single-copy gene located on the same plasmid. Copy numbers of *aadB* and *strB* were significantly increased in hetR-O2 and hetR-T1 relative to VIR-O not exposed to tobramycin (*, $P < 0.001$ relative to VIR-O by two-way analysis of variance with Dunnett's posttest).

Table 5A.3. *aadB* copy number in the presence and absence of antibiotic selection.

Strain	Copy no. (mean ± SD) of gene[a]:	
	aadB	*ABUW_RS19335*
hetR-O2 + Tob	40.35 ± 0.62	0.72 ± 0.16
hetR-O2 − Tob	16.75 ± 0.16*	0.71 ± 0.03
hetR-T1 + Tob	55.12 ± 4.35	0.72 ± 0.05
hetR-T1 − Tob	40.38 ± 10.71	0.77 ± 0.08

[a]Copy numbers of *aadB* and *ABUW_RS19335* relative to *aacA4* in hetR-O2 and hetR-T1 colonies grown with (+) or without (−) tobramycin for 24 h. The means and standard deviations of two biological replicates are shown. The copy number of *aadB* was significantly decreased in hetR-O2 grown without tobramycin compared to this strain grown in the presence of drug (*, $P < 0.05$ by paired two-tailed *t* test).

Table 5A.4. Relative copy number of *aadB* in *recA::Tc* isolates with increased tobramycin resistance.

Strain	*aadB* relative copy no.[b]	MIC (μg/ml)
recA::*Tc*	2.49[a]	8
hetR-*recA*-1-1	3.34	16
hetR-*recA*-1-2	3.15	64
hetR-*recA*-1-3	2.85	16
hetR-*recA*-1-4	2.64	32
hetR-*recA*-1-5	2.63	16
hetR-*recA*-1-10	2.72	96

[a]The *recA*::*Tc* control sample was used as a control in three independent experiments; the value shown represents the average relative copy number across the three experiments.

[b]Relative copy number of *aadB* normalized to *aacA4* was measured using gDNA isolated from a *recA* transposon mutant and 6 independently isolated derivatives with increased tobramycin resistance. Data shown are from a single sample for each strain. None of the tobramycin-resistant *recA*::*Tc* isolates exhibit increased copy number of *aadB*, suggesting that gene amplification of *aadB* in wild-type cells is RecA dependent.

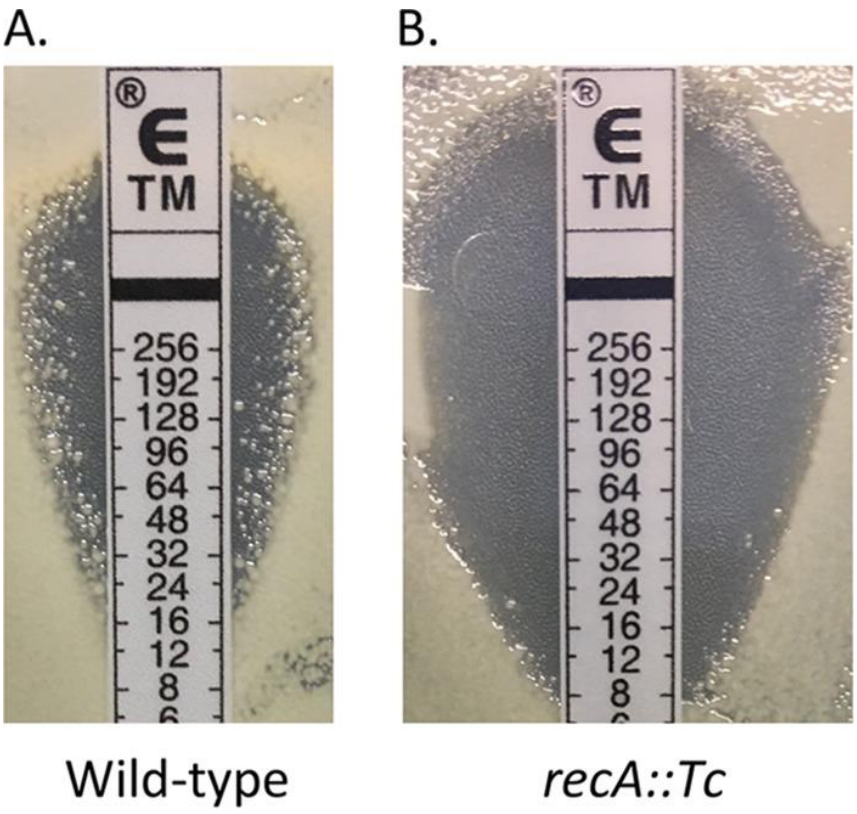
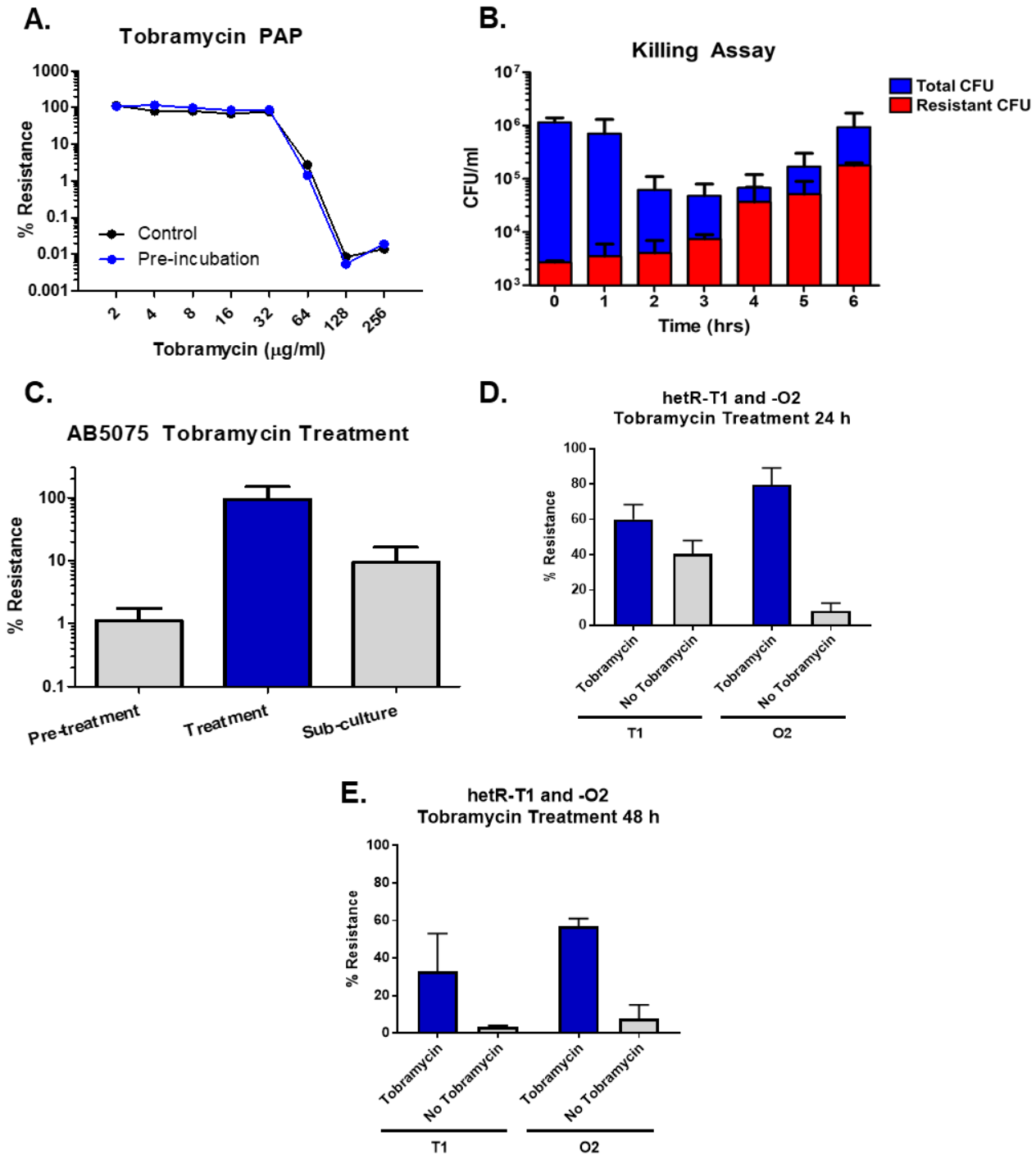


Figure 5A.1. AB5075 produces a subpopulation of cells with increased resistance to tobramycin. Virulent opaque (VIR-O) wild-type (A) or *recA::Tc* (B) cells of AB5075 were grown to 1 × 107 CFU/ml, and 100 µl was plated on LB agar to obtain a lawn of growth. After plating, a tobramycin Etest strip was added, and the plate was incubated for 16 h at 37°C.



Continued on next page

Figure 5A.2. Characterization of tobramycin heteroresistance in AB5075. (A) Preincubation of AB5075 with a subinhibitory concentration of tobramycin had no effect on the population analysis profile (PAP) for this strain, indicating that increased resistance is not induced by exposure to this drug. **(B)** Incubation of AB5075 in liquid culture with 64 μg/ml of tobramycin resulted in killing of the majority of the population, while the CFU of the tobramycin-resistant subpopulation continued to increase. This indicates that the tobramycin-resistant subpopulation is distinct from persisters, which would be unable to replicate in the presence of drug. **(C)** Increased tobramycin resistance is unstable in liquid cultures. Following exposure of AB5075 to 64 μg/ml tobramycin, the majority of the surviving population exhibited increased tobramycin resistance. However, when tobramycin selection was removed, most of the cells in the population lost their increased resistance. **(D)** Twenty-four-hour stability of the tobramycin resistance phenotype in colonies was examined using stocks of hetR-O2 and hetR-T1. Colonies were plated from stock onto plates supplemented with tobramycin. Individual colonies were picked and passaged onto plates with 40 μg/ml tobramycin or with no tobramycin. After 24 h of growth, individual colonies were resuspended and the percentages of resistant cells within each colony were determined. Data represent the means and standard errors of means for four (hetR-T1) or three (hetR-O2) independent replicates. **(E)** Forty-eight-hour stability of the tobramycin resistance phenotype was assessed for stocks of hetR-O2 and hetR-T1. The experiment was performed as described for panel D but with colonies incubated on plates with and without 40 μg/ml of tobramycin for 48 h. Data represent the means and standard errors of means for two independent replicates.

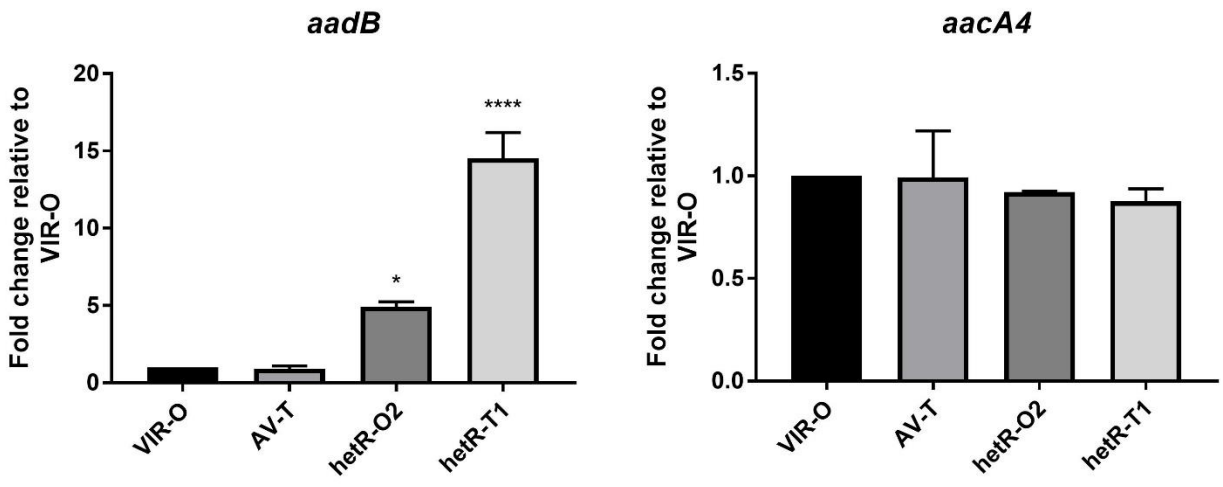


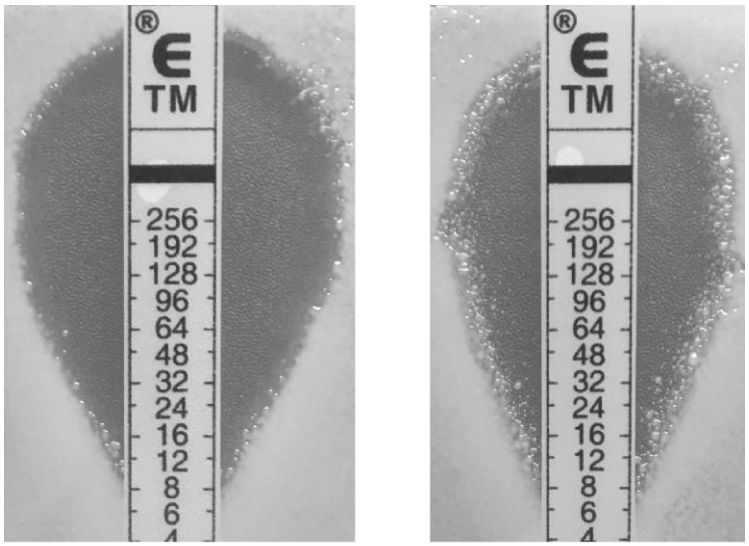
Figure 5A.3. Increased expression of *aadB* in cells with increased tobramycin resistance. Expression of aminoglycoside resistance genes aadB **(A)** and aacA4 **(B)** was quantified by qRT-PCR. Data are presented as the averages and standard errors of means of three independent biological replicates (*, $P < 0.05$, and ****, $P < 0.0001$, relative to AB5075 VIR-O by one-way analysis of variance with Dunnett's posttest).

Figure 5A.4. Amplified region in p1AB5075 that includes *aadB*. The genes surrounding *aadB* on p1AB5075 are shown. Gene annotations are based on the most recent sequence annotation of p1AB5075 available on NCBI (accession NZ_CP008707.1); some small annotated open reading frames are not shown. Based on qPCR results, open reading frames annotated in green were amplified in the tobramycin-resistant subpopulation, whereas open reading frames annotated in blue were not changed. The two integrase genes annotated in gray were not examined by qPCR.

Supplementary Figures

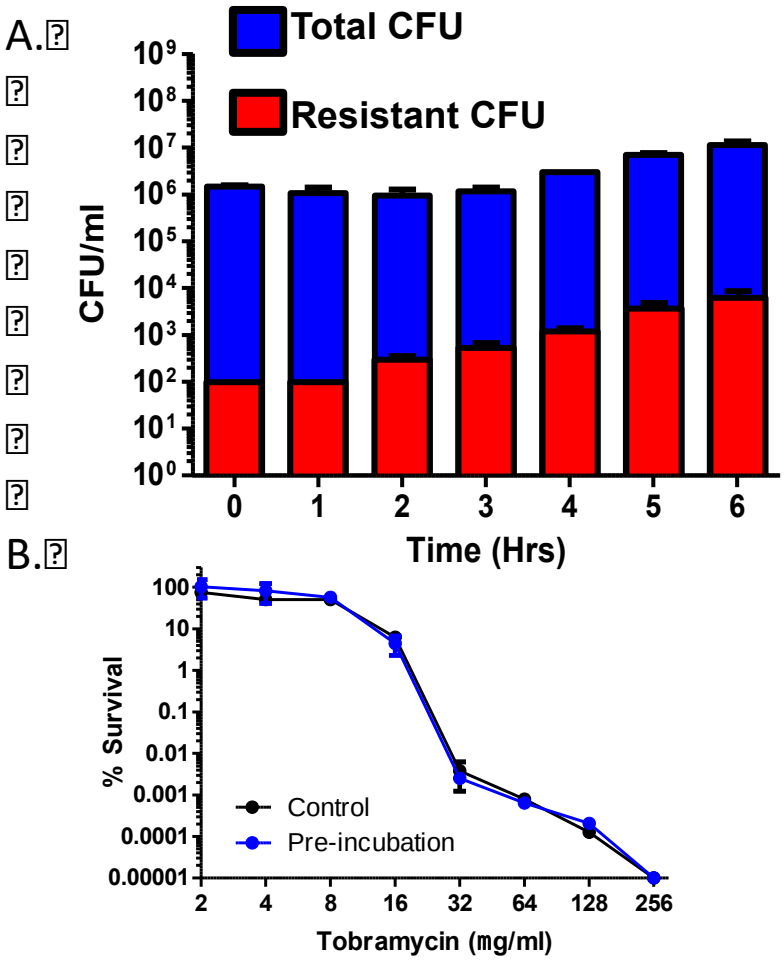
Supplementary Table 5A.1. Oligonucleotides used in this study.

Primer	Sequence	Use
oSA61	ACTTGCCAAGCGTTTTAGCG	*aacA4* qPCR
oSA62	CTTGGTTCCCAAGCCTTTGC	*aacA4* qPCR
oSA69	TCCCCGATCTCCGCTAAGAA	*aadB* qPCR
oSA70	CAGATGAGCGAAATCTGCCG	*aadB* qPCR
oSA77	GCTCATCGTTTCGTTTGAAC	*recA* complementation
oSA78	GAATAAAAACGTCGAGTTGTG	*recA* complementation
oSA84	AGCCACAGTACGTGCCATAG	*ABUW_4052* qPCR
oSA85	GGTGCAGCTTAGCGACAATG	*ABUW_4052* qPCR
oSA86	TAAGCGTCAGGCAGACAAG	*ABUW_RS19335* qPCR
oSA87	TTTTCCACTCTGCTGAAGG	*ABUW_RS19335* qPCR
oSA94	TCGGCAACGATGTGAGAGAG	*strB* qPCR
oSA95	ACTCCTGCAATCGTCAAGGG	*strB* qPCR
clpX qRT-For	GCGTTTGAAAGTCGGGCAAT	*clpX* qPCR
clpX qRT-Rev	CCATTGCAAACGGCACATCT	*clpX* qPCR



recA::Tc/vector *recA::Tc*/precA

Supplemental Fig. 5A.S1. Complementation of *recA* leads to increased heteroresistance in the *recA::Tc* mutant. The *recA::Tc* strain was complemented by introducing the recA gene on a multicopy plasmid. Lawns were inoculated by spreading 2 × 106 CFU of each strain on an LB plate. Lawns were overlaid with an Etest strip, incubated at 37°C for 16 h, and photographed.



Supplemental Fig. 5A.S2. Characterization of tobramycin heteroresistance in a *recA* mutant. (A) Incubation of the *recA::Tc* mutant in liquid culture with 16 µg/ml of tobramycin resulted in growth of the resistant subpopulation over time. **(B)** Population analysis profile (PAP) of the *recA::Tc* mutant with or without preincubation (3 h) in tobramycin.

5B: *mcr-1* Confers Cross-Resistance to the Cationic Host Antimicrobial Lysozyme

Edgar X. Sherman,[a,b,c,d] -B.Sc, David A. Hufnagel, Ph.D [b,c,d] , and David S. Weiss Ph.D [b,c,d,e*]

Originally Published in Lancet Infectious Disease, November 16, 2016, Vol. 16 Issue 11

[a]Department of Microbiology and Immunology, Emory University, Atlanta, Georgia 30329, USA. [b]Emory Vaccine Center, Atlanta, Georgia 30329, USA. [c]Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, USA. [d]Emory Antibiotic Resistance Center, Atlanta, Georgia 30329, USA. [e]Research Service, Atlanta VA Medical Center, Decatur, Georgia 30033, USA.

mcr-1 has become an increasing concern due to the fear of rapid transferable resistance to our last resort antibiotics [157]. While *mcr-1* confers resistance to the polymyxin antibiotic colistin, it remains unclear whether this gene also confers resistance to other antimicrobials. Here we report that *mcr-1* confers cross-resistance to the cationic host antimicrobial, lysozyme.

mcr-1 positive *Escherichia coli* isolates, CDF-1 and IHD86_4 (patient isolates from Switzerland [158]and Cambodia [159], respectively), were cured of their *mcr-1* carrying plasmid by serial passage in Lysogeny Broth (LB). Curing of the *mcr-1* gene was confirmed using colony PCR comparing parental *mcr-1* positive strains vs. *mcr-1* cured strains (**Fig. 5B.1A**). Colony PCR using *E. coli* specific *uspA* primers was performed as a positive control (**Fig. 5B.1B**). When tested by broth microdilution in 25% LB, both cured strains exhibited a four and two-fold increase in susceptibility to colistin and polymyxin B, respectively.

The *mcr-1* encoded phosphoethanolamine transferase adds a positively charged phosphoethanolamine moiety to the lipid A portion of the bacterial outer membrane component lipopolysaccharide [157]. This reduces the overall net negative surface charge of the bacteria, likely leading to repulsion of the cationic antibiotic, colistin. The host's innate immune system employs multiple positively charged antimicrobials to combat bacterial infection. Therefore, we sought to determine if *mcr-1* was capable of providing cross-resistance to the cationic host protein lysozyme. We measured survival rates between *mcr-1* positive isolates and cured strains in the presence of lysozyme in 25% LB. Strains lacking *mcr-1* were killed in the presence of lysozyme while the parental strains were able to grow (Fig. 1C). Specifically, *mcr-1* negative strains were 5 to 20-fold more susceptible to multiple concentrations of lysozyme when comparing percent survival.

To our knowledge, this is the first report of *mcr-1* conferring cross-resistance to a host antimicrobial. Resistance to the host's innate immune defenses through *mcr-1* could drive plasmid maintenance in strains carrying *mcr-1, mcr-2,* or other transferable resistance plasmids leading to propagation of colistin resistance [160,161]. Therefore, mammals may currently or in the future serve as reservoirs for bacteria harboring transmissible colistin resistance regardless of polymyxin exposure. More studies are needed to determine the broader effect of mobilized colistin resistance and resistance to cationic antimicrobials within the context of the host.

Declaration of Interests

The authors declare no competing financial interests.

Author Contributions

Experiments were conducted by EXS. Data collection and analysis was performed by EXS. Data interpretation was conducted by EXS, DAH, and DSW. The manuscript was prepared by EXS, DAH and DSW. Study was planned and directed by DSW.

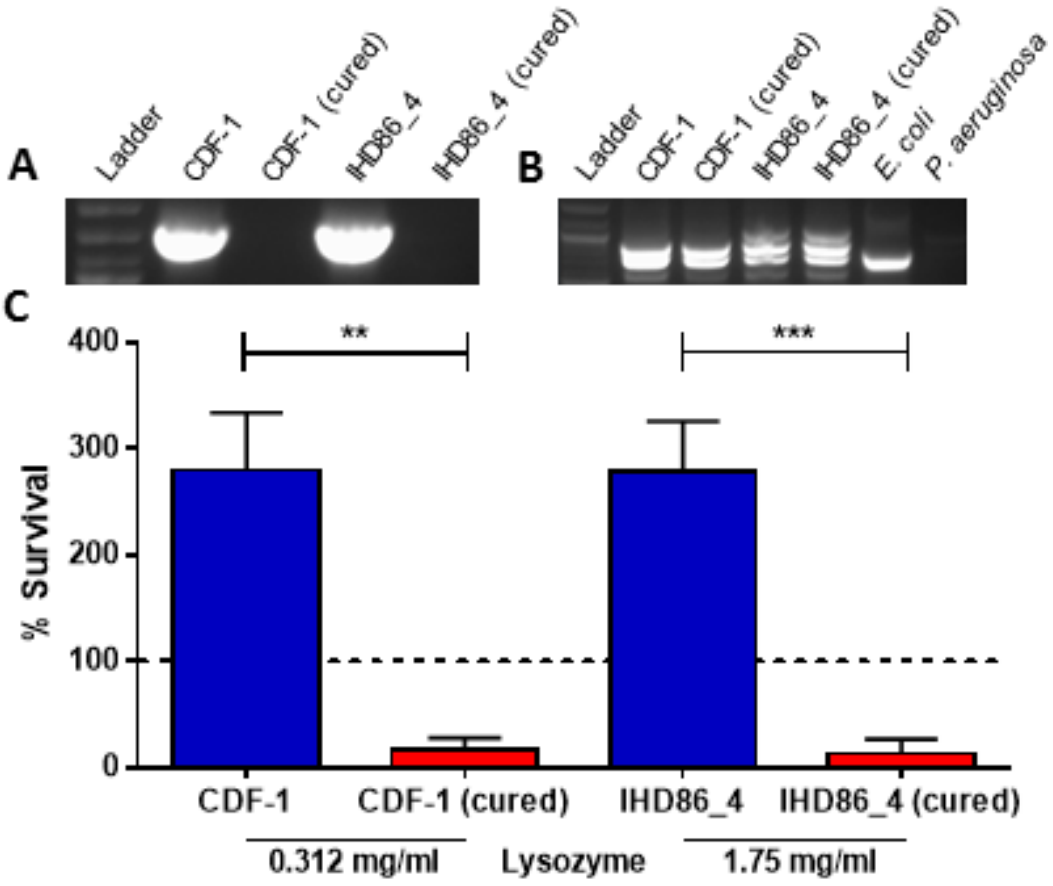


Figure 5B.1. mcr-1 mediates resistance to lysozyme. A, B, Colony PCR using **(A)** *mcr-1* specific or **(B)** *uspA* specific primers on *mcr-1* positive isolates CDF-1 and IHD86_4 and their corresponding cured, *mcr-1* negative derivatives. A molecular weight ladder was included in each figure, and *E. coli* strain NCM3722 was used as a positive control and *Pseudomonas aeruginosa* strain PAO1 was used as a negative control in Figure 1B. **C**, Percent survival of *mcr-1* positive isolates and their corresponding, cured *mcr-1* negative derivatives, calculated by dividing the surviving CFUs after three-hour incubation with lysozyme by the initial inoculum (represented by dashed line, $1x10^6$ CFU/ml). Data shown are representative of three biological replicates. Error bars represent mean ± standard deviation, ***P*=0·0012, ****P*=0·0008.

5C: Methods to Evaluate Colistin Heteroresistance in *Acinetobacter baumannii*

Edgar X. Sherman,[a,b,c,d#] , Jessie E. Wozniak [a,b,c,d#], and David S. Weiss [b,c,d,e*]

Originally Published in Methods in Molecular Biology, February 24, 2019, Volume 1946

[a]Department of Microbiology and Immunology, Emory University, Atlanta, Georgia 30329, USA. [b]Emory Vaccine Center, Atlanta, Georgia 30329, USA. [c]Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, USA. [d]Emory Antibiotic Resistance Center, Atlanta, Georgia 30329, USA. [e]Research Service, Atlanta VA Medical Center, Decatur, Georgia 30033, USA.

#These authors contributed equally to this work

Abstract

The nosocomial pathogen *Acinetobacter baumannii* is a growing threat to public health due to its increasing resistance to antibiotics including the last-line polymyxin, colistin. Heteroresistance to colistin has been described in *A. baumannii*, wherein a resistant subpopulation of cells co-existing with a majority susceptible subpopulation actively grows in the presence of antibiotic and can cause treatment failure. The shortcomings of diagnostic tests in detecting colistin heteroresistance are especially worrisome as they may lead to clinicians unknowingly prescribing an ineffective antibiotic, leading to increased patient morbidity and mortality.

Several techniques can be used to detect heteroresistance and the purpose of this chapter is to outline effective methods for identifying, quantifying, and analyzing heteroresistance to colistin in *A. baumannii*. We will highlight the advantages and disadvantages of techniques including population analysis profile (PAP), Etest, and disc diffusion, as well as additional methods to distinguish heteroresistance from other forms of resistance. While the scope of this chapter will focus on colistin heteroresistance in *A. baumannii*, these techniques can be adapted for the study of heteroresistance to other antibiotics and in other bacteria with slight modifications.

Keywords

Heteroresistance; resistant subpopulations; population analysis profile; antibiotic susceptibility testing; phenotypic antibiotic resistance; polymyxin; colistin.

Introduction

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen causing multiple types of infections including ventilator-associated pneumonia (VAP) and bacteremia,

primarily in immunocompromised patients [80,162]. This pathogen is a growing global health concern due to its increasing resistance to antibiotics. The Centers for Disease Control and Prevention have categorized multidrug resistant *A. baumannii* as a serious threat to public health and the World Health Organization listed *A. baumannii* as the top priority antibiotic resistant pathogen for new drug development [163,164]. Unfortunately, isolates resistant to all available antibiotics have emerged, making this organism a significant obstacle to treat in the clinic.

To combat the growing threat of antibiotic resistance, we must address remaining gaps in our understanding of resistance mechanisms. Heteroresistance is an underappreciated and understudied phenomenon wherein a small subpopulation of bacterial cells resist an antibiotic, while the remainder of a clonal population is susceptible [42]. This is in contrast to "conventional" resistance in which the entire population is resistant to the antibiotic. During drug treatment of a heteroresistant isolate, the susceptible population is killed while the resistant cells survive and can rapidly expand. This rapid expansion makes this phenomenon distinct from persistence, in which a metabolically quiescent population of cells remains dormant and thereby resists antibiotics [74]. Heteroresistance was first described in 1947 and has since been identified in both Gram-negative and Gram-positive organisms against multiple classes of antibiotics [46,77,165-170].

The frequency of the resistant subpopulation in heteroresistant strains can vary, and when it is exceedingly low (less than ~1 in 10,000 cells), this form of resistance is often undetected by current diagnostic tests. Recently, Band *et al* described strains of *Enterobacter cloacae* exhibiting heteroresistance (either detected or undetected) to the last-line polymyxin antibiotic colistin that caused colistin treatment failure and subsequent lethal infections in mice [57]. These results clearly demonstrated that heteroresistance can mediate failure of antibiotics *in vivo*. The shortcomings of diagnostic tests in detecting colistin heteroresistance is especially worrisome as this may lead to

clinicians unknowingly prescribing ineffective antibiotics, causing increased patient morbidity and mortality.

Heteroresistance to colistin has been reported in *A. baumannii* [82,171-175]. The purpose of this chapter is to detail effective methods of detecting, quantifying, and analyzing heteroresistance to colistin in *A. baumannii*.

1. Materials

Lysogeny Broth (LB) - *Approximate formula per liter: Tryptone (10.0g), Yeast Extract (5.0g), Sodium Chloride (10.0g)*

Lysogeny Broth (LB) Agar - *Approximate formula per liter: Tryptone (10.0g), Yeast Extract (5.0g), Sodium Chloride (10.0g), Agar (15.0g)*

Sterile 100mm x 15mm polystyrene petri dishes

LB Agar plates

Sterile phosphate-buffered saline (PBS)

Sterile 5 mL polystyrene tubes

Sterile 96-well polystyrene plates

Blank 6 mm filter paper discs

Colistin Sulfate (*Sigma-Aldrich*)

Sterile 1 µL inoculating loops

Colistin Etest Strips (*Biomerieux*)

Bacterial strains

2. Methods

Unless otherwise mentioned, all steps are to be performed at room temperature and in a biosafety cabinet. Working stocks of colistin should be prepared fresh for each experiment and diluted in sterile water.

3.1 Population analysis profile (PAP)

Population analysis profile (PAP) is the gold standard method for detecting heteroresistance. Briefly, this technique involves the quantification of the proportion of resistant cells existing within a culture at a variety of antibiotic concentrations. PAP is the only method for identifying heteroresistance that also quantitatively determines the frequency of the resistant subpopulation of cells. While it is the most reproducible and reliable method for detecting heteroresistance, it is also more time-consuming and requires more materials than other methods [176].

Day 1

1. Streak out isolates to be tested from freezer stocks on Lysogeny Broth (LB) agar plates and incubate overnight at 37°C.

   Prepare Lysogeny Broth agar plates as described:

   a) Prepare LB agar in sterile H_2O on a magnetic stir-plate.

   b) Autoclave LB agar.

   c) Cool LB agar in a water bath (60°C) for 1 hour.

   d) Remove agar from the water bath and place on a spinning magnetic stir plate.

e) When agar has cooled (hot, but not uncomfortable to the touch), add colistin.

f) Final concentrations of colistin in LB agar should be 0, 1, 2, 4, 8, 16, 32 or 128µg/ml.

g) Pour agar into petri dishes and let stand overnight or until plates harden.

h) Store plates at 4°C - Do not use plates that are more than 10 days old.

   i. Agar plates should have a depth of 4mm (approximately 31 mL in a 100mm circular plate).

   ii. Plates should be dried before use with no visible drops of water on the agar surface or lid[5].

   iii. Plates may be stored at 4˚C but must be properly dried before use to remove excess moisture.

Day 2

1. Pick an isolated colony with a sterile loop from the LB plates and inoculate into 2mL sterile Lysogeny Broth and grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Dilute 10µL of the overnight culture into 90µL sterile PBS in a sterile 96-well polystyrene plate. Make 10x serial dilutions ranging in concentration from 10^{-1} to 10^{-7}.
2. Plate dilutions ranging from 10^{-2} to 10^{-8} in 10µL drops on agar plates (**Fig. 5C.1**) of each colistin concentration,0-128µg/mL (*see* **Note 1**).
3. Allow plates to dry with covers removed for 15 minutes or until no liquid is visible on plates (*see* **Note 2**).
4. Incubate plates at 37°C for 16 hours.

Day 4

Analysis of data should be as follows:

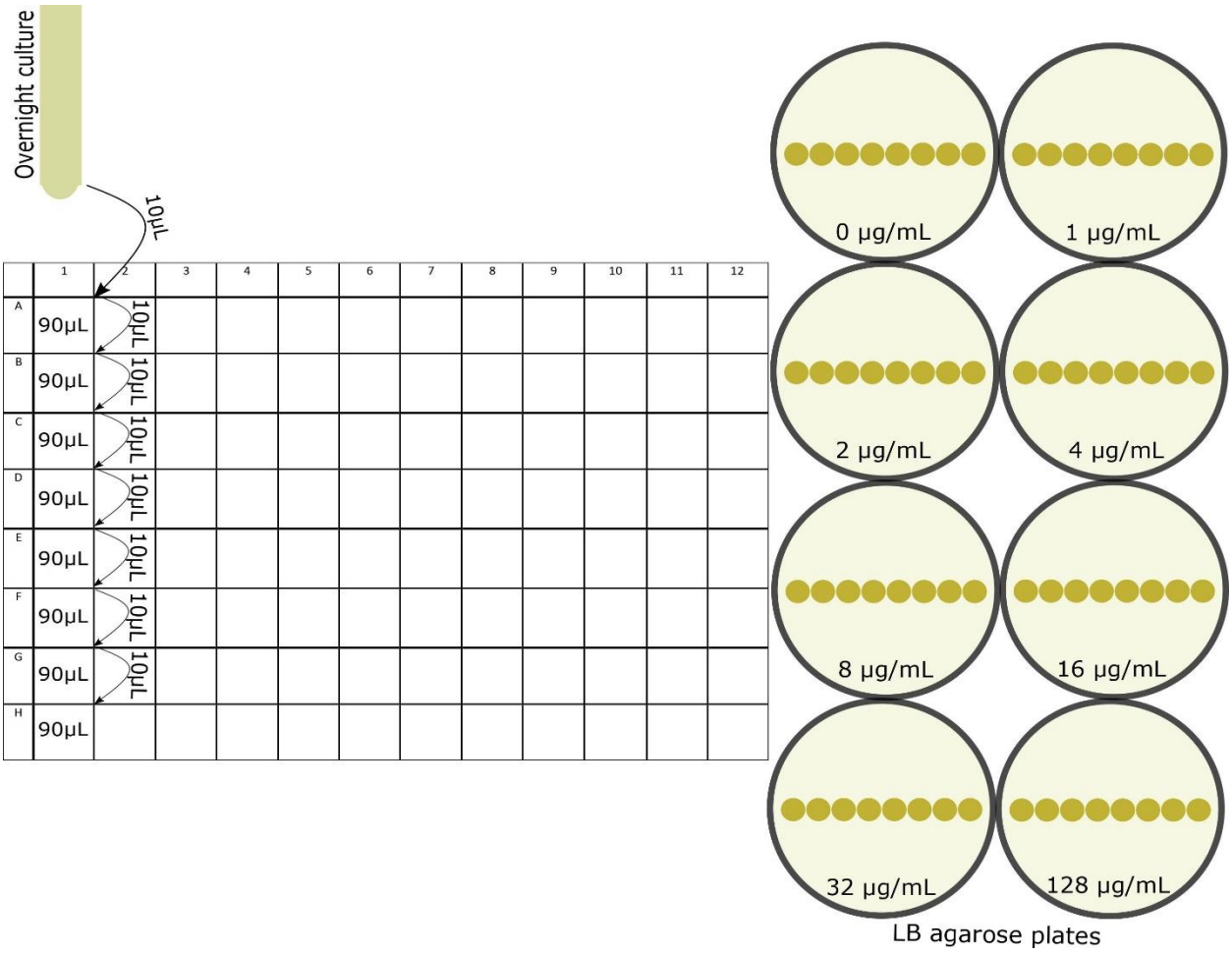


Figure 5C.1. Sample population analysis profile (PAP) procedure using 96-well plate. Note- while the authors prefer to plate eight serial dilutions in 10µL drops per plate, there are multiple ways to achieve these results (see **Note 1**). Up to 6 rows can be spot plated on a 100mm circular agar petri dish plate.

Count the number of colonies at the highest dilution factor at which there is growth (*see* **Note 3**). For statistical interpretation, only count dilutions wherein the countable colonies are between 10 and 100. If it appears there are fewer than 10 colonies at your lowest dilution factor, we recommend plating a sample from this dilution in a higher volume on a single plate. With these data, the proportion of colistin resistant cells at each concentration can be quantified using the following formula:

$$\frac{(number\ of\ colonies\ on\ colistin\ plate\ \times\ dilution\ factor)}{(number\ of\ colonies\ on\ antibiotic\ free\ plate\ \times\ dilution\ factor)}$$

Interpret results as follows:

Based on our laboratory's experience performing this assay, as well as commentary in the literature, we recommend the following interpretation of population analysis profile results (*see* **Note 4**). [42,57]:

Susceptible: Proportion of surviving bacteria at 2µg/mL-128µg/mL is below 10^{-7} (the limit of detection).

Resistant: Proportion of surviving bacteria at a concentration above 2µg/mL is above 50%.

Heteroresistant: Proportion of surviving bacteria at a concentration at least four-fold above the antibiotic's breakpoint is between 10^{-7} and 50% (**Fig. 5C.2**).

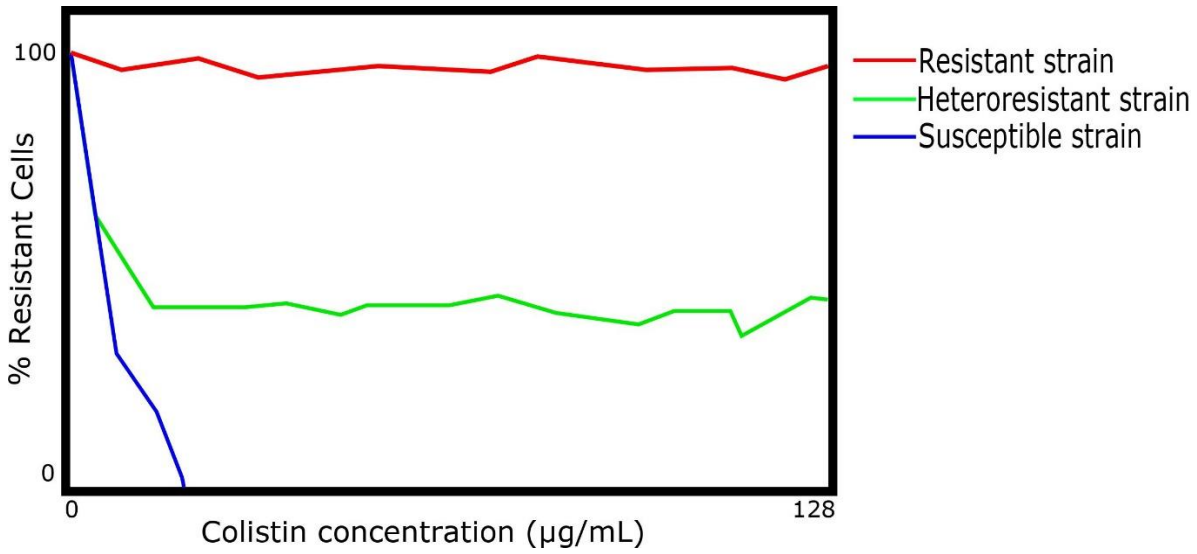


Figure 5C.2. Example graph of a typical population analysis profile with three strains of varying susceptibility.

a) Alternatively, dilutions can be plated with one sample per agar plate instead of the spot method. To do this, serial dilutions can be made by diluting 100 µL of overnight culture into 900µL sterile PBS in 5mL culture tubes, making 10x serial dilutions ranging in concentration from 10^{-1} to 10^{-7}. Once prepared, spread 100µL of each dilution onto an individual agar plate. To enumerate CFU, use the dilution that allows between 30-300 colonies to be counted and quantify using the methods mentioned above.

3.2 Etest

An alternative to PAP that can be utilized for heteroresistance testing are Etests (**Fig. 5C.3**). Etests are plastic strips manufactured to contain a gradient of antibiotic concentrations from top (highest concentration) to bottom (lowest concentration). These strips are applied to an agar plate on which bacteria have been streaked, and after incubation, a "zone of clearing" appears in which no bacterial growth is observed. The Minimum Inhibitory Concentration (MIC) for Etest is interpreted as the lowest concentration where the zone of clearing meets the Etest strip along the bottom edge. When heteroresistant isolates are plated with an Etest strip, colonies can be observed within the zone of clearing. Compared to PAP, Etests are a non-quantitative method to observe heteroresistance as the appearance of colonies within the zone of clearing will depend on the frequency of the resistant subpopulation of cells (for heteroresistant isolates with a low frequency resistant subpopulation, colonies will not appear in the zone of clearing and thus these strains will not be classified as heteroresistant using Etest). Another shortcoming is that Etest strips can be expensive and may not be ideal to screen numerous strains for heteroresistance. This method has

been adapted and modified from the agar disc diffusion method standard described by CLSI [177] and is outlined below:

Day 1

1. Streak out bacterial strains from freezer stocks on Lysogeny Broth agar plates and grow overnight in a 37°C incubator.

Day 2

1. Pick an isolated colony with a sterile inoculating loop from the LB plates and transfer into 2mL sterile Lysogeny Broth. Subsequently grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Prepare LB Agar plates for inoculation (see instructions and notes in the *population analysis profile* (PAP) section. LB agar plates used for Etest should not contain antibiotics).
2. Remove Etest strips from 4˚C and allow them to reach room temperature before application (this takes about 30 minutes).
3. Dilute culture to be tested to between $1x10^8$-$2x10^8$ CFU/ml (~0.5 McFarland Standard) (*see* **Note 5**) and use the suspension within 15 min (*see* **Note 6**).
4. Dip a sterile swab in the diluted culture tubes and rub off excess culture along the side of the tube. Swab along the surface of a dry petri dish (no visible water spots), rotate the plate 60 degrees, and then repeat these steps two more times. Allow excess moisture to dry before Etest strip application but do not exceed 15 min after inoculation (*see* **Note 7**).
5. Apply Etest strip to the surface of the agar plate with the label side up. Make sure the strip does not shift during placement.
6. Within 15 min of Etest strip application, invert plates and incubate at 37°C for 16-20 hours.

Day 4

Examine plates for zones of clearing and then examine zones for any resistant colonies that may have appeared. Colonies can appear uniformly throughout the zone or close to the edge of the zone. Colonies may be potential spontaneous mutants rather than the resistant subpopulation of a heteroresistant strain and should thus be tested through the methods described in the "*Further distinction between heteroresistance and resistance*" section below.

3.3 Disc Diffusion

Disc diffusion is used to determine an organism's MIC to an antibiotic using discs concentrated with the drug (**Fig. 3**). Similar to Etest, this method is non-quantitative but can detect heteroresistant isolates that harbor a resistant subpopulation present at a high frequency. Unlike Etest which contains a gradient of antibiotic concentrations, in disc diffusion, each disc is loaded with a specific concentration. The discs are placed on agar plates after bacteria have been spread out in a lawn. The antimicrobial diffuses radially from the disc creating gradients of antibiotic concentrations. After overnight incubation, the MIC for disc diffusion is interpreted by comparing the diameter of the zone of clearing and is drug and dose dependent. This method can be adapted to detect resistant subpopulations by observing colonies that may grow within the zone of clearing. While this method is not as sensitive as PAP, it can be useful to screen multiple strains for heteroresistance. Discs preloaded with antibiotic can be purchased commercially but can also be prepared in a laboratory. Preparing discs can be more cost effective when testing multiple isolates and can be used to work within a specific range of antibiotic concentrations for which commercial discs are not available. Results can vary if disc preparation or application is not carefully controlled. This method has been adapted and modified from the agar disc diffusion method standard described by EUCAST [178] and is outlined below:

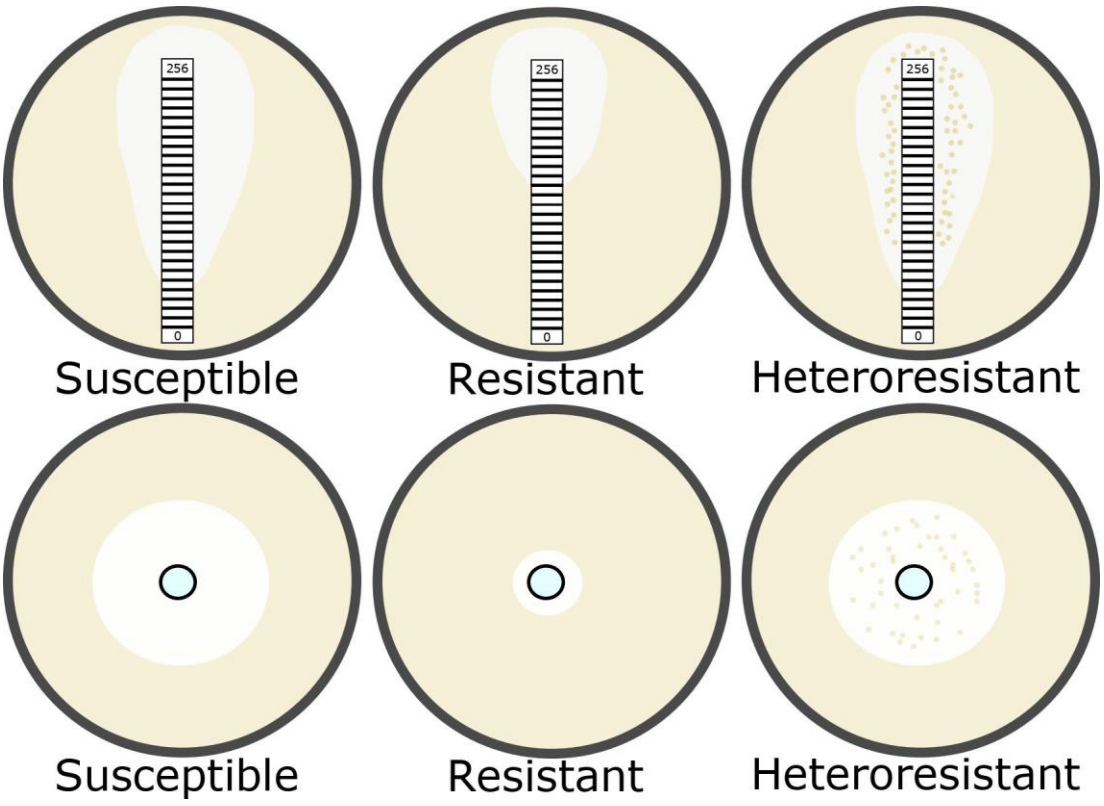


Figure 5C.3. Example of Etest and disc diffusion assay results showing three different susceptibility profiles. The Minimum Inhibitory Concentration (MIC) for Etest (top row) is interpreted as the lowest concentration where the zone of clearing meets the Etest strip along the bottom edge. The MIC for disc diffusion (bottom row) is interpreted by comparing the diameter of the zone of clearing and is drug and dose dependent and can be accessed through EUCAST guidelines [178].

Day 1

1. Streak out bacterial strains from freezer stocks on Lysogeny Broth agar plates (see instructions and notes in the *population analysis profile* (PAP) section. LB agar plates used for disc diffusion should not contain antibiotics) and grow overnight in a 37°C shaking incubator.

Day 2

1. Pick an isolated colony with a sterile inoculating loop from the LB plates and transfer into 2mL sterile Lysogeny Broth, and then grow at 37°C shaking at 225 rpm overnight (~16 hours).

Day 3

1. Prepare antibiotic discs (or antibiotic discs can be purchased from a manufacturer).
    a. Prepare colistin sulfate stock solution (concentrations are based on total µg/disc so calculate accordingly, ex. 128µg disc would require 12.8µL of 10mg/mL stock solution).
    b. Add 5-20µL of antibiotic/solution to blank filter paper discs (*see* **Note 8**).
    c. Allow discs to air-dry for at least 15-20 minutes before application to plates.
2. Dilute culture to be tested to between $1x10^8$-$2x10^8$ CFU/ml (~0.5 McFarland Standard) (*see* **Note 5**) and use the suspension within 15 min (*see* **Note 6**).
3. Dip a sterile swab into the diluted culture tubes, rub off excess culture along the side of the tube, and swab along the surface of a dry petri dish (no visible water spots). Rotate the plate 60 degrees and repeat, and then rotate the plate an additional 60 degrees and repeat. Allow excess moisture to dry before application of the disc, but do not exceed 15 min after inoculation (*see* **Note 7**).
4. Apply disc to the surface of the agar plate with the label side up. Make sure the strip does not shift during placement.
5. Within 15 min. of disc application, invert plates and incubate at 37°C for 16-20 hours.

Day 4

1. Examine plates for zones of clearing and then examine zones for any resistant colonies. Colonies can appear uniformly throughout the zone or close to the edge of the zone. These colonies can be examined for potential heteroresistance with the methods described below.

3.4 Further distinction between heteroresistance and resistance

To verify that heteroresistance observed by PAP, Etest, or disc diffusion is not due to a stable mutation in a small proportion of the total population or due to persister cells, further examination is necessary. Two methods can be utilized to distinguish between heteroresistance and these other forms of resistance [57].

3.4a Time-Kill Assay

The purpose of this assay is to distinguish the resistant subpopulation in heteroresistance from persisters. Canonically, persisters are a subset of the population of a strain of bacteria that resist antibiotics by entering a state of quiescence with no or very limited growth. Heteroresistance differs from persistence in that the resistant subpopulation of a heteroresistant strain is able to grow and rapidly expand in the presence of antibiotics.

Day 1

2. Streak out isolates from freezer stocks on Lysogeny Broth agar plates and grow overnight in a 37°C incubator.

Day 2

1. Inoculate 2mL LB with a single colony from overnight plates and grow at 37°C shaking at 225rpm.

Day 3

2. Dilute 10µL overnight culture into 10mL LB without colistin, and 10mL LB with colistin (10µg/mL).
3. Serially dilute and plate these cultures on LB agar plates with (10µg/mL) and without colistin. This is time zero.
4. Every hour for eight hours, serially dilute and plate both cultures on LB agar plates with (10µg/mL) and without colistin overnight (~16 hours).

Day 4

Analysis of data should be as follows

Colony forming units counted from LB agar plates without colistin (Total CFU) and Colony forming units counted from LB agar plates with colistin (Resistant CFU) should be plotted on a graph over the eight-hour duration of this experiment. An increase of Resistant CFU is expected to be observed over time in colistin heteroresistant strains of *Acinetobacter baumannii.*

3.4b Resistant Colony Restreak

A single colony picked from a population analysis profile plate or from within the zone of clearing on an Etest or disc diffusion assay plate inoculation (*see* **Note 9**) can be grown overnight in broth in the absence of colistin (several days of subculture if necessary) and the methods used to detect heteroresistance can be repeated to determine whether the cells maintain or lose their resistance phenotype inoculation (*see* **Note 10**). If the strains are heteroresistant, there should be a decrease in the frequency of the resistant subpopulation after culture in the absence of colistin. If the colonies now exhibit stable resistance, the frequency of the resistant subpopulation should not decrease after just a few days of subculture in media without colistin.

4. Notes

1. Lysogeny Broth agar plates containing colistin result in difficulty spot plating. If this becomes a problem during population analysis profile assays, we recommend pouring thinner plates than you may normally pour (15mL or less) and allowing plates to dry in the biosafety cabinet for 10-15 minutes prior to plating. While it is our recommendation to plate 10µL per dilution so as to conserve both time and plates, it is possible to use one LB plate per dilution.
2. Plates can be dried in a biosafety cabinet for 30 min with the lid removed, 20-25˚C overnight, or at 35˚C with the lid removed for 15 min.
3. For all assays, we recommend counting colonies at 24 hours after plating, and leaving plates at 37°C for an additional 24 hours to observe slow growing colonies within the bacterial population.
4. To control for the variance that appears by creating population analysis profile plates, it is advised to use a control strain where percent survival can be verified for each set of PAP plates that are prepared to ensure consistency with results.
5. Alternatively, a direct colony suspension method can be used in which morphologically similar colonies are picked directly from a plate and resuspended in saline and adjusted to a turbidity resulting in a 1-2 x 10^8 CFU/ml suspension.
6. Do not allow the suspension to sit for more than 60 min to prevent overgrowth and incorrect interpretation if MIC is also desired.
7. If inoculated plates are left sitting for prolonged periods of time before the disc is applied, the bacteria may begin growing in the absence of antibiotic, leading to erroneous results.
8. An empty sterile petri dish may be used to prepare discs.

9. If colonies are present.
10. It may require several passages before the subpopulation frequency begins to lower, if after several passages the percent resistance does not decrease then the increase may be due to conventionally resistant bacteria.

Acknowledgements

Chapter 2

The Authors would like to thank C. Bower and the GA EIP/MuGSI staff for providing CRE isolates. Philip Rather and Sarah Anderson for providing *Acinetobacter baumannii* strain R38. Chui-Yoke Chin for providing assistance in animal studies. E.X.S. is supported by T32 training grant AI106699 from the National Institutes of Health (NIH). D.S.W. is supported by a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award and NIH grant AI141883. The GA EIP is funded by the Centers for Disease Control and Prevention. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the CDC.

Chapter 3

The Authors would like to thank C. Bower and the GA EIP/MuGSI staff for providing CRE isolates and *Enterobacter cloacae* strain Mu1307. The Duke University Center for Genomic and Computational Biology for PACBIO sequencing of Mu1307. Robert Petit for bioinformatic analysis. E.X.S. is supported by T32 training grant AI106699 from the National Institutes of Health (NIH). D.S.W. is supported by a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award and NIH grant AI141883. The GA EIP is funded by the Centers for Disease Control and Prevention. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the CDC.

Chapter 5

This study was supported by a VA Merit Award I01 BX001725 and a Research Career Scientist Award from the Department of Veterans Affairs to P.N.R. D.S.W. is supported by a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award, VA Merit award I01 BX002788 and National Institutes of Health (NIH) grant AI098800. S.E.A. and E.X.S. were both supported by a T32 training grant AI106699 from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Department of Veterans Affairs. The authors have no conflicts of interest to disclose.

D.S.W. is supported by a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award, VA Merit Award I01 BX002788, and NIH grant AI098800. D.S.W. had full access to all data presented in the study with final responsibility to submit for publication.

References

1 Zaffiri, L., Gardner, J. & Toledo-Pereyra, L. H. History of antibiotics: from fluoroquinolones to daptomycin (Part 2). *J Invest Surg* **26**, 167-179, doi:10.3109/08941939.2013.808461 (2013).

2 Zaffiri, L., Gardner, J. & Toledo-Pereyra, L. H. History of antibiotics. From salvarsan to cephalosporins. *J Invest Surg* **25**, 67-77, doi:10.3109/08941939.2012.664099 (2012).

3 Holliday, C. B. Moulds, fungi, and medicine. *Nurs Mirror Midwives J* **84**, 371 (1947).

4 Cui, L. & Su, X. Z. Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev Anti Infect Ther* **7**, 999-1013, doi:10.1586/eri.09.68 (2009).

5 Frere, J. M., Sauvage, E. & Kerff, F. From "An Enzyme Able to Destroy Penicillin" to Carbapenemases: 70 Years of Beta-lactamase Misbehaviour. *Curr Drug Targets* **17**, 974-982, doi:10.2174/1389450116666151001112859 (2016).

6 Lobanovska, M. & Pilla, G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *Yale J Biol Med* **90**, 135-145 (2017).

7 Aminov, R. I. A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol* **1**, 134, doi:10.3389/fmicb.2010.00134 (2010).

8 Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**, 417-433, doi:10.1128/MMBR.00016-10 (2010).

9 Martinez, J. L. General principles of antibiotic resistance in bacteria. *Drug Discov Today Technol* **11**, 33-39, doi:10.1016/j.ddtec.2014.02.001 (2014).

10 CDC, U. Antibiotic Resistant Threats in the United States, 2019. (2019).

11 Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. *Review on Antimicrobial Resistance* (2014).

12 Krause, K. M., Serio, A. W., Kane, T. R. & Connolly, L. E. Aminoglycosides: An Overview. *Cold Spring Harb Perspect Med* **6**, doi:10.1101/cshperspect.a027029 (2016).

13 Vakulenko, S. B. & Mobashery, S. Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* **16**, 430-450, doi:10.1128/cmr.16.3.430-450.2003 (2003).

14 Schatz, A., Bugie, E. & Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944. *Clin Orthop Relat Res*, 3-6, doi:10.1097/01.blo.0000175887.98112.fe (2005).

15 Prayle, A., Watson, A., Fortnum, H. & Smyth, A. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. *Thorax* **65**, 654-658, doi:10.1136/thx.2009.131532 (2010).

16 Becker, B. & Cooper, M. A. Aminoglycoside antibiotics in the 21st century. *ACS Chem Biol* **8**, 105-115, doi:10.1021/cb3005116 (2013).

17 Durante-Mangoni, E., Grammatikos, A., Utili, R. & Falagas, M. E. Do we still need the aminoglycosides? *Int J Antimicrob Agents* **33**, 201-205, doi:10.1016/j.ijantimicag.2008.09.001 (2009).

18 Le Goffic, F., Capmau, M. L., Tangy, F. & Baillarge, M. Mechanism of action of aminoglycoside antibiotics. Binding studies of tobramycin and its 6'-N-acetyl derivative to the bacterial ribosome and its subunits. *Eur J Biochem* **102**, 73-81, doi:10.1111/j.1432-1033.1979.tb06264.x (1979).

19 Mingeot-Leclercq, M. P., Glupczynski, Y. & Tulkens, P. M. Aminoglycosides: activity and resistance. *Antimicrob Agents Chemother* **43**, 727-737, doi:10.1128/AAC.43.4.727 (1999).

20 Kotra, L. P., Haddad, J. & Mobashery, S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob Agents Chemother* **44**, 3249-3256, doi:10.1128/aac.44.12.3249-3256.2000 (2000).

21 Lin, J., Zhou, D., Steitz, T. A., Polikanov, Y. S. & Gagnon, M. G. Ribosome-Targeting Antibiotics: Modes of Action, Mechanisms of Resistance, and Implications for Drug Design. *Annu Rev Biochem* **87**, 451-478, doi:10.1146/annurev-biochem-062917-011942 (2018).

22 Wilson, D. N. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nat Rev Microbiol* **12**, 35-48, doi:10.1038/nrmicro3155 (2014).

23 Jana, S. & Deb, J. K. Molecular understanding of aminoglycoside action and resistance. *Appl Microbiol Biotechnol* **70**, 140-150, doi:10.1007/s00253-005-0279-0 (2006).

24 Trylska, J. & Kulik, M. Interactions of aminoglycoside antibiotics with rRNA. *Biochem Soc Trans* **44**, 987-993, doi:10.1042/BST20160087 (2016).

25 Davis, B. D. Mechanism of bactericidal action of aminoglycosides. *Microbiol Rev* **51**, 341-350 (1987).

26 Garneau-Tsodikova, S. & Labby, K. J. Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives. *Medchemcomm* **7**, 11-27, doi:10.1039/C5MD00344J (2016).

27 Macfarlane, E. L. A., Kwasnicka, A. & Hancock, R. E. W. Role of Pseudomonas aeruginosa PhoP-phoQ in resistance to antimicrobial cationic peptides and aminoglycosides. *Microbiology (Reading)* **146 (Pt 10)**, 2543-2554, doi:10.1099/00221287-146-10-2543 (2000).

28 Kwon, D. H. & Lu, C. D. Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in Pseudomonas aeruginosa PAO1. *Antimicrob Agents Chemother* **50**, 1615-1622, doi:10.1128/AAC.50.5.1615-1622.2006 (2006).

29 Paradise, M. R., Cook, G., Poole, R. K. & Rather, P. N. Mutations in aarE, the ubiA homolog of Providencia stuartii, result in high-level aminoglycoside resistance and reduced expression of the chromosomal aminoglycoside 2'-N-acetyltransferase. *Antimicrob Agents Chemother* **42**, 959-962, doi:10.1128/AAC.42.4.959 (1998).

30 Du Toit, A. Bacterial physiology: Persisters are under the pump. *Nat Rev Microbiol* **14**, 332-333, doi:10.1038/nrmicro.2016.72 (2016).

31 Hancock, R. E. & Bell, A. Antibiotic uptake into gram-negative bacteria. *Eur J Clin Microbiol Infect Dis* **7**, 713-720, doi:10.1007/BF01975036 (1988).

32 Poole, K. Aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **49**, 479-487, doi:10.1128/AAC.49.2.479-487.2005 (2005).

33 Poonsuk, K. & Chuanchuen, R. Contribution of the MexXY multidrug efflux pump and other chromosomal mechanisms on aminoglycoside resistance in Pseudomonas aeruginosa isolates from canine and feline infections. *J Vet Med Sci* **74**, 1575-1582, doi:10.1292/jvms.12-0239 (2012).

34 Maus, C. E., Plikaytis, B. B. & Shinnick, T. M. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* **49**, 3192-3197, doi:10.1128/AAC.49.8.3192-3197.2005 (2005).

35 Wachino, J. & Arakawa, Y. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. *Drug Resist Updat* **15**, 133-148, doi:10.1016/j.drup.2012.05.001 (2012).

36 Ramirez, M. S. & Tolmasky, M. E. Aminoglycoside modifying enzymes. *Drug Resist Updat* **13**, 151-171, doi:10.1016/j.drup.2010.08.003 (2010).

37 Salimizand, H. *et al.* Diversity of aminoglycoside modifying enzymes and 16S rRNA methylases in Acinetobacter baumannii and Acinetobacter nosocomialis species in Iran; wide distribution of aadA1 and armA. *Infect Genet Evol* **66**, 195-199, doi:10.1016/j.meegid.2018.09.028 (2018).

38 Lalitha Aishwarya, K. V., Venkataramana Geetha, P., Eswaran, S., Mariappan, S. & Sekar, U. Spectrum of Aminoglycoside Modifying Enzymes in Gram-Negative Bacteria Causing Human Infections. *J Lab Physicians* **12**, 27-31, doi:10.1055/s-0040-1713687 (2020).

39 Ramirez, M. S. & Tolmasky, M. E. Amikacin: Uses, Resistance, and Prospects for Inhibition. *Molecules* **22**, doi:10.3390/molecules22122267 (2017).

40 Clark, J. A. & Burgess, D. S. Plazomicin: a new aminoglycoside in the fight against antimicrobial resistance. *Ther Adv Infect Dis* **7**, 2049936120952604, doi:10.1177/2049936120952604 (2020).

41 Band, V. I. & Weiss, D. S. Heteroresistance: A cause of unexplained antibiotic treatment failure? *PLoS Pathog* **15**, e1007726, doi:10.1371/journal.ppat.1007726 (2019).

42 El-Halfawy, O. M. & Valvano, M. A. Antimicrobial heteroresistance: an emerging field in need of clarity. *Clinical microbiology reviews* **28**, 191-207, doi:10.1128/cmr.00058-14 (2015).

43 Dewachter, L., Fauvart, M. & Michiels, J. Bacterial Heterogeneity and Antibiotic Survival: Understanding and Combatting Persistence and Heteroresistance. *Mol Cell* **76**, 255-267, doi:10.1016/j.molcel.2019.09.028 (2019).

44 Wootton, M. *et al.* A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. *J Antimicrob Chemother* **47**, 399-403, doi:10.1093/jac/47.4.399 (2001).

45 Sherman, E. X., Wozniak, J. E. & Weiss, D. S. Methods to Evaluate Colistin Heteroresistance in Acinetobacter baumannii. *Methods Mol Biol* **1946**, 39-50, doi:10.1007/978-1-4939-9118-1_4 (2019).

46 Alexander, H. E. & Leidy, G. MODE OF ACTION OF STREPTOMYCIN ON TYPE b HEMOPHILUS INFLUENZAE : II. NATURE OF RESISTANT VARIANTS. *J Exp Med* **85**, 607-621, doi:10.1084/jem.85.6.607 (1947).

47 Band, V. I. *et al.* Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. *Nat Microbiol* **4**, 1627-1635, doi:10.1038/s41564-019-0480-z (2019).

48 Nicoloff, H., Hjort, K., Levin, B. R. & Andersson, D. I. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. *Nat Microbiol* **4**, 504-514, doi:10.1038/s41564-018-0342-0 (2019).

49 Claudino, A. L. *et al.* Mutants with heteroresistance to amphotericin B and fluconazole in Candida. *Braz J Microbiol* **40**, 943-951, doi:10.1590/S1517-83822009000400028 (2009).

50 Ferreira, G. F. *et al.* Heteroresistance to Itraconazole Alters the Morphology and Increases the Virulence of Cryptococcus gattii. *Antimicrob Agents Chemother* **59**, 4600-4609, doi:10.1128/AAC.00466-15 (2015).

51 Xong, H. V. *et al.* A VSG expression site-associated gene confers resistance to human serum in Trypanosoma rhodesiense. *Cell* **95**, 839-846, doi:10.1016/s0092-8674(00)81706-7 (1998).

52 Turner, N. C. & Reis-Filho, J. S. Genetic heterogeneity and cancer drug resistance. *Lancet Oncol* **13**, e178-185, doi:10.1016/S1470-2045(11)70335-7 (2012).

53 Haven, B. *et al.* Registered report: A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Elife* **5**, doi:10.7554/eLife.09462 (2016).

54 Hiramatsu, K. *et al.* Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. *Lancet* **350**, 1670-1673, doi:10.1016/S0140-6736(97)07324-8 (1997).

55 Moore, M. R., Perdreau-Remington, F. & Chambers, H. F. Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate Staphylococcus aureus in a patient with endocarditis and in the rabbit model of endocarditis. *Antimicrob Agents Chemother* **47**, 1262-1266, doi:10.1128/aac.47.4.1262-1266.2003 (2003).

56 Khatib, R. *et al.* Relevance of vancomycin-intermediate susceptibility and heteroresistance in methicillin-resistant Staphylococcus aureus bacteraemia. *J Antimicrob Chemother* **66**, 1594-1599, doi:10.1093/jac/dkr169 (2011).

57 Band, V. I. *et al.* Antibiotic failure mediated by a resistant subpopulation in Enterobacter cloacae. *Nat Microbiol* **1**, 16053, doi:10.1038/nmicrobiol.2016.53 (2016).

58 Anderson, S. E., Sherman, E. X., Weiss, D. S. & Rather, P. N. Aminoglycoside Heteroresistance in Acinetobacter baumannii AB5075. *mSphere* **3**, doi:10.1128/mSphere.00271-18 (2018).

59 Andersson, D. I. & Hughes, D. Gene amplification and adaptive evolution in bacteria. *Annu Rev Genet* **43**, 167-195, doi:10.1146/annurev-genet-102108-134805 (2009).

60 Chen, Z., Yang, H. & Pavletich, N. P. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature* **453**, 489-484, doi:10.1038/nature06971 (2008).

61 Liu, X. *et al.* Homologous recombination as a resistance mechanism to replication-induced double-strand breaks caused by the antileukemia agent CNDAC. *Blood* **116**, 1737-1746, doi:10.1182/blood-2009-05-220376 (2010).

62 Hiom, K. Homologous recombination: how RecA finds the perfect partner. *Curr Biol* **22**, R275-278, doi:10.1016/j.cub.2012.03.002 (2012).

63 Taylor, A. F. & Smith, G. R. RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* **423**, 889-893, doi:10.1038/nature01674 (2003).

64 Courcelle, J., Wendel, B. M., Livingstone, D. D. & Courcelle, C. T. RecBCD is required to complete chromosomal replication: Implications for double-strand break frequencies and repair mechanisms. *DNA Repair (Amst)* **32**, 86-95, doi:10.1016/j.dnarep.2015.04.018 (2015).

65 Smith, G. R. How RecBCD enzyme and Chi promote DNA break repair and recombination: a molecular biologist's view. *Microbiol Mol Biol Rev* **76**, 217-228, doi:10.1128/MMBR.05026-11 (2012).

66 Rownd, R. & Mickel, S. Dissociation and reassociation of RTF and r-determinants of the R-factor NR1 in Proteus mirabilis. *Nat New Biol* **234**, 40-43, doi:10.1038/newbio234040a0 (1971).

67 Rownd, R., Kasamatsu, H. & Mickel, S. The molecular nature and replication of drug resistance factors of the Enterobacteriaceae. *Ann N Y Acad Sci* **182**, 188-206, doi:10.1111/j.1749-6632.1971.tb30656.x (1971).

68 Sandegren, L. & Andersson, D. I. Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nat Rev Microbiol* **7**, 578-588, doi:10.1038/nrmicro2174 (2009).

69 Bertini, A. *et al.* Multicopy blaOXA-58 gene as a source of high-level resistance to carbapenems in Acinetobacter baumannii. *Antimicrob Agents Chemother* **51**, 2324-2328, doi:10.1128/AAC.01502-06 (2007).

70 Mattes, R., Burkardt, H. J. & Schmitt, R. Repetition of tetracycline resistance determinant genes on R plasmid pRSD1 in Escherichia coli. *Mol Gen Genet* **168**, 173-184, doi:10.1007/BF00431443 (1979).

71 Musher, D. M. *et al.* Emergence of macrolide resistance during treatment of pneumococcal pneumonia. *N Engl J Med* **346**, 630-631, doi:10.1056/NEJM200202213460820 (2002).

72 Verhaak, R. G. W., Bafna, V. & Mischel, P. S. Extrachromosomal oncogene amplification in tumour pathogenesis and evolution. *Nat Rev Cancer* **19**, 283-288, doi:10.1038/s41568-019-0128-6 (2019).

73 Matsui, A., Ihara, T., Suda, H., Mikami, H. & Semba, K. Gene amplification: mechanisms and involvement in cancer. *Biomol Concepts* **4**, 567-582, doi:10.1515/bmc-2013-0026 (2013).

74 Maisonneuve, E. & Gerdes, K. Molecular mechanisms underlying bacterial persisters. *Cell* **157**, 539-548, doi:10.1016/j.cell.2014.02.050 (2014).

75 Shan, Y., Lazinski, D., Rowe, S., Camilli, A. & Lewis, K. Genetic basis of persister tolerance to aminoglycosides in Escherichia coli. *mBio* **6**, doi:10.1128/mBio.00078-15 (2015).

76 Kuper, K. M., Boles, D. M., Mohr, J. F. & Wanger, A. Antimicrobial susceptibility testing: a primer for clinicians. *Pharmacotherapy* **29**, 1326-1343, doi:10.1592/phco.29.11.1326 (2009).

77 Napier, B. A., Band, V., Burd, E. M. & Weiss, D. S. Colistin heteroresistance in Enterobacter cloacae is associated with cross-resistance to the host antimicrobial lysozyme. *Antimicrob Agents Chemother* **58**, 5594-5597, doi:10.1128/AAC.02432-14 (2014).

78 Hung, K. H., Wang, M. C., Huang, A. H., Yan, J. J. & Wu, J. J. Heteroresistance to cephalosporins and penicillins in Acinetobacter baumannii. *J Clin Microbiol* **50**, 721-726, doi:10.1128/JCM.05085-11 (2012).

79 Ryffel, C., Strassle, A., Kayser, F. H. & Berger-Bachi, B. Mechanisms of heteroresistance in methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* **38**, 724-728, doi:10.1128/aac.38.4.724 (1994).

80 Antunes, L. C., Visca, P. & Towner, K. J. Acinetobacter baumannii: evolution of a global pathogen. *Pathog Dis* **71**, 292-301, doi:10.1111/2049-632X.12125 (2014).

81 Govindaraj Vaithinathan, A. & Vanitha, A. WHO global priority pathogens list on antibiotic resistance: an urgent need for action to integrate One Health data. *Perspect Public Health* **138**, 87-88, doi:10.1177/1757913917743881 (2018).

82 Li, J. *et al.* Heteroresistance to colistin in multidrug-resistant Acinetobacter baumannii. *Antimicrob Agents Chemother* **50**, 2946-2950, doi:10.1128/AAC.00103-06 (2006).

83 CDC, U. Multi-site Gram-negative Surveillance Initiative. (2016).

84 Jones, L. A., McIver, C. J., Kim, M. J., Rawlinson, W. D. & White, P. A. The aadB gene cassette is associated with blaSHV genes in Klebsiella species producing extended-spectrum beta-lactamases. *Antimicrob Agents Chemother* **49**, 794-797, doi:10.1128/AAC.49.2.794-797.2005 (2005).

85 Segal, H. & Elisha, B. G. Identification and characterization of an aadB gene cassette at a secondary site in a plasmid from Acinetobacter. *FEMS Microbiol Lett* **153**, 321-326, doi:10.1111/j.1574-6968.1997.tb12591.x (1997).

86 Segal, H. & Elisha, B. G. Characterization of the Acinetobacter plasmid, pRAY, and the identification of regulatory sequences upstream of an aadB gene cassette on this plasmid. *Plasmid* **42**, 60-66, doi:10.1006/plas.1999.1403 (1999).

87 Syrmis, M. *et al.* High prevalence of a class 1 integron-associated aadB gene cassette in Pseudomonas aeruginosa isolates from an Australian cystic fibrosis patient population. *Pathology* **40**, 524-525, doi:10.1080/00313020802197921 (2008).

88 Shaw, K. J., Rather, P. N., Hare, R. S. & Miller, G. H. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological reviews* **57**, 138-163 (1993).

89 Cassuto, E., West, S. C., Podell, J. & Howard-Flanders, P. Genetic recombination: recA protein promotes homologous pairing between duplex DNA molecules without strand unwinding. *Nucleic Acids Res* **9**, 4201-4210, doi:10.1093/nar/9.16.4201 (1981).

90 Ramiro, R. S., Costa, H. & Gordo, I. Macrophage adaptation leads to parallel evolution of genetically diverse Escherichia coli small-colony variants with increased fitness in vivo and antibiotic collateral sensitivity. *Evol Appl* **9**, 994-1004, doi:10.1111/eva.12397 (2016).

91 Massey, R. C., Buckling, A. & Peacock, S. J. Phenotypic switching of antibiotic resistance circumvents permanent costs in Staphylococcus aureus. *Curr Biol* **11**, 1810-1814, doi:10.1016/s0960-9822(01)00507-3 (2001).

92 Machado, D. *et al.* Contribution of efflux to colistin heteroresistance in a multidrug resistant Acinetobacter baumannii clinical isolate. *J Med Microbiol* **67**, 740-749, doi:10.1099/jmm.0.000741 (2018).

93 Charretier, Y. *et al.* Colistin Heteroresistance and Involvement of the PmrAB Regulatory System in Acinetobacter baumannii. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.00788-18 (2018).

94 Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* **3**, 1101-1108 (2008).

95 Tomson, G. & Vlad, I. The need to look at antibiotic resistance from a health systems perspective. *Ups J Med Sci* **119**, 117-124, doi:10.3109/03009734.2014.902879 (2014).

96 Watkins, R. R. & Bonomo, R. A. Overview: Global and Local Impact of Antibiotic Resistance. *Infect Dis Clin North Am* **30**, 313-322, doi:10.1016/j.idc.2016.02.001 (2016).

97 Davin-Regli, A. & Pages, J. M. Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment. *Front Microbiol* **6**, 392, doi:10.3389/fmicb.2015.00392 (2015).

98 Sanders, W. E., Jr. & Sanders, C. C. Enterobacter spp.: pathogens poised to flourish at the turn of the century. *Clin Microbiol Rev* **10**, 220-241, doi:10.1128/CMR.10.2.220-241.1997 (1997).

99 Livermore, D. M. *et al.* What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline,

nitrofurantoin, temocillin and tigecycline. *Int J Antimicrob Agents* **37**, 415-419, doi:10.1016/j.ijantimicag.2011.01.012 (2011).

100 Sheu, C. C., Chang, Y. T., Lin, S. Y., Chen, Y. H. & Hsueh, P. R. Infections Caused by Carbapenem-Resistant Enterobacteriaceae: An Update on Therapeutic Options. *Front Microbiol* **10**, 80, doi:10.3389/fmicb.2019.00080 (2019).

101 Livermore, D. M. *et al.* Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. *J Antimicrob Chemother* **66**, 48-53, doi:10.1093/jac/dkq408 (2011).

102 Zavascki, A. P., Klee, B. O. & Bulitta, J. B. Aminoglycosides against carbapenem-resistant Enterobacteriaceae in the critically ill: the pitfalls of aminoglycoside susceptibility. *Expert Rev Anti Infect Ther* **15**, 519-526, doi:10.1080/14787210.2017.1316193 (2017).

103 Krahn, T. *et al.* Determinants of intrinsic aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **56**, 5591-5602, doi:10.1128/AAC.01446-12 (2012).

104 Pitiriga, V., Dimitroulia, E., Saroglou, G. & Tsakris, A. The challenge of curbing aminoglycoside resistance: can antimicrobial stewardship programs play a critical role? *Expert Rev Anti Infect Ther* **15**, 947-954, doi:10.1080/14787210.2017.1382355 (2017).

105 Serio, A. W., Keepers, T., Andrews, L. & Krause, K. M. Aminoglycoside Revival: Review of a Historically Important Class of Antimicrobials Undergoing Rejuvenation. *EcoSal Plus* **8**, doi:10.1128/ecosalplus.ESP-0002-2018 (2018).

106 Fusco, D. N. *et al.* Clinical failure of vancomycin in a dialysis patient with methicillin-susceptible vancomycin-heteroresistant S. aureus. *Diagn Microbiol Infect Dis* **65**, 180-183, doi:10.1016/j.diagmicrobio.2009.05.017 (2009).

107 Reams, A. B. & Roth, J. R. Mechanisms of gene duplication and amplification. *Cold Spring Harb Perspect Biol* **7**, a016592, doi:10.1101/cshperspect.a016592 (2015).

108 McGann, P. *et al.* Amplification of aminoglycoside resistance gene aphA1 in Acinetobacter baumannii results in tobramycin therapy failure. *MBio* **5**, e00915, doi:10.1128/mBio.00915-14 (2014).

109 Vanhoof, R., Content, J., Van Bossuyt, E., Dewit, L. & Hannecart-Pokorni, E. Identification of the aadB gene coding for the aminoglycoside-2"-O-nucleotidyltransferase, ANT(2"), by means of the polymerase chain reaction. *J Antimicrob Chemother* **29**, 365-374, doi:10.1093/jac/29.4.365 (1992).

110 Cardenas, P. P. *et al.* RecX facilitates homologous recombination by modulating RecA activities. *PLoS Genet* **8**, e1003126, doi:10.1371/journal.pgen.1003126 (2012).

111 Hjort, K., Nicoloff, H. & Andersson, D. I. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in Salmonella enterica. *Mol Microbiol* **102**, 274-289, doi:10.1111/mmi.13459 (2016).

112 Cunningham, R. P., DasGupta, C., Shibata, T. & Radding, C. M. Homologous pairing in genetic recombination: recA protein makes joint molecules of gapped circular DNA and closed circular DNA. *Cell* **20**, 223-235, doi:10.1016/0092-8674(80)90250-0 (1980).

113 Hu, B., Joshua, M. N., Dong, C. & Qi, Y. Development of a novel recombinant adenovirus containing gfp-zeocin fusion expression cassette for conditional replication in p53-deficient human tumor cells. *J Virol Methods* **117**, 129-136, doi:10.1016/j.jviromet.2004.01.018 (2004).

114 Ishikawa, M. & Hori, K. A new simple method for introducing an unmarked mutation into a large gene of non-competent Gram-negative bacteria by FLP/FRT recombination. *BMC Microbiol* **13**, 86, doi:10.1186/1471-2180-13-86 (2013).

115 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61-68, doi:10.1016/0378-1119(89)90359-4 (1989).

116 Bryksin, A. V. & Matsumura, I. Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. *PLoS One* **5**, e13244, doi:10.1371/journal.pone.0013244 (2010).

117 Petit, R. A., 3rd & Read, T. D. Bactopia: a Flexible Pipeline for Complete Analysis of Bacterial Genomes. *mSystems* **5**, doi:10.1128/mSystems.00190-20 (2020).

118 Bushnell, B. BBMap short read aligner. *University of California, Berkeley, California* (2016).

119 Song, L., Florea, L. & Langmead, B. Lighter: fast and memory-efficient sequencing error correction without counting. *Genome Biol* **15**, 509, doi:10.1186/s13059-014-0509-9 (2014).

120 Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* **13**, e1005595, doi:10.1371/journal.pcbi.1005595 (2017).

121 Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069, doi:10.1093/bioinformatics/btu153 (2014).

122 Camacho, C. *et al.* BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421, doi:10.1186/1471-2105-10-421 (2009).

123 Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-595, doi:10.1093/bioinformatics/btp698 (2010).

124 Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841-842, doi:10.1093/bioinformatics/btq033 (2010).

125 Tipton, K. A., Dimitrova, D. & Rather, P. N. Phase-Variable Control of Multiple Phenotypes in Acinetobacter baumannii Strain AB5075. *Journal of bacteriology* **197**, 2593-2599, doi:10.1128/jb.00188-15 (2015).

126 Inbar, O., Liefshitz, B., Bitan, G. & Kupiec, M. The relationship between homology length and crossing over during the repair of a broken chromosome. *J Biol Chem* **275**, 30833-30838, doi:10.1074/jbc.C000133200 (2000).

127 Gallagher, L. A., Lee, S. A. & Manoil, C. Importance of Core Genome Functions for an Extreme Antibiotic Resistance Trait. *mBio* **8**, doi:10.1128/mBio.01655-17 (2017).

128 Haber, J. E. & Debatisse, M. Gene amplification: yeast takes a turn. *Cell* **125**, 1237-1240, doi:10.1016/j.cell.2006.06.012 (2006).

129 Fusco, D. N. *et al.* Clinical failure of vancomycin in a dialysis patient with methicillin-susceptible vancomycin-heteroresistant S. aureus. *Diagnostic Microbiology and Infectious Disease* **65**, 180-183, doi:10.1016/j.diagmicrobio.2009.05.017 (2009).

130 Lin, S.-Y. *et al.* Molecular epidemiology and clinical characteristics of hetero-resistant vancomycin intermediate Staphylococcus aureus bacteremia in a Taiwan Medical Center. *Journal of Microbiology, Immunology and Infection* **45**, 435-441, doi:10.1016/j.jmii.2012.05.004 (2012).

131 Sola, C. *et al.* Heterogeneous vancomycin-intermediate susceptibility in a community-associated methicillin-resistant Staphylococcus aureus epidemic clone, in a case of

Infective Endocarditis in Argentina. *Annals of Clinical Microbiology and Antimicrobials* **10**, 15, doi:10.1186/1476-0711-10-15 (2011).

132 Charles, P. G. P., Ward, P. B., Johnson, P. D. R., Howden, B. P. & Grayson, M. L. Clinical Features Associated with Bacteremia Due to Heterogeneous Vancomycin-Intermediate Staphylococcus aureus. *Clinical Infectious Diseases* **38**, 448-451, doi:10.1086/381093 (2004).

133 Hung, K.-H., Wang, M.-C., Huang, A.-H., Yan, J.-J. & Wu, J.-J. Heteroresistance to Cephalosporins and Penicillins in Acinetobacter baumannii. *Journal of clinical microbiology* **50**, 721-726, doi:10.1128/jcm.05085-11 (2012).

134 Sanchez-Romero, M. A. & Casadesus, J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proc Natl Acad Sci U S A* **111**, 355-360, doi:10.1073/pnas.1316084111 (2014).

135 Peleg, A. Y., Seifert, H. & Paterson, D. L. Acinetobacter baumannii: emergence of a successful pathogen. *Clin Microbiol Rev* **21**, 538-582, doi:10.1128/CMR.00058-07 (2008).

136 Lee, C. R. *et al.* Biology of Acinetobacter baumannii: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options. *Front Cell Infect Microbiol* **7**, 55, doi:10.3389/fcimb.2017.00055 (2017).

137 Baumann, P. Isolation of Acinetobacter from soil and water. *Journal of bacteriology* **96**, 39-42 (1968).

138 *WHO Priority Pathogens List for R&D of New Antibiotics*, <http://who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf> (2017).

139 McGann, P. *et al.* Amplification of Aminoglycoside Resistance Gene aphA1 in Acinetobacter baumannii Results in Tobramycin Therapy Failure. *mBio* **5**, doi:10.1128/mBio.00915-14 (2014).

140 Yoon, E. J. *et al.* Contribution of the Ade Resistance-Nodulation-Cell Division-Type Efflux Pumps to Fitness and Pathogenesis of Acinetobacter baumannii. *mBio* **7**, doi:10.1128/mBio.00697-16 (2016).

141 Gallagher, L. A. *et al.* Resources for Genetic and Genomic Analysis of Emerging Pathogen Acinetobacter baumannii. *Journal of bacteriology* **197**, 2027-2035, doi:10.1128/jb.00131-15 (2015).

142 Jacobs, A. C. *et al.* AB5075, a Highly Virulent Isolate of Acinetobacter baumannii, as a Model Strain for the Evaluation of Pathogenesis and Antimicrobial Treatments. *MBio* **5**, e01076-01014, doi:10.1128/mBio.01076-14 (2014).

143 Chin, C. Y. *et al.* A high-frequency phenotypic switch links bacterial virulence and environmental survival in Acinetobacter baumannii. *Nat Microbiol* **3**, 563-569, doi:10.1038/s41564-018-0151-5 (2018).

144 Tipton, K. A., Farokhyfar, M. & Rather, P. N. Multiple roles for a novel RND-type efflux system in Acinetobacter baumannii AB5075. *MicrobiologyOpen*, doi:10.1002/mbo3.418 (2016).

145 Shah, D. *et al.* Persisters: a distinct physiological state of E. coli. *BMC Microbiol* **6**, 53, doi:10.1186/1471-2180-6-53 (2006).

146 Massey, R. C., Buckling, A. & Peacock, S. J. Phenotypic switching of antibiotic resistance circumvents permanent costs in Staphylococcus aureus. *Current Biology* **11**, 1810-1814, doi:10.1016/s0960-9822(01)00507-3 (2001).

147 Edwards, A. M. Phenotype Switching Is a Natural Consequence of Staphylococcus aureus Replication. *Journal of bacteriology* **194**, 5404-5412, doi:10.1128/jb.00948-12 (2012).

148 Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. *J Bacteriol* **186**, 8172-8180, doi:10.1128/JB.186.24.8172-8180.2004 (2004).

149 Li, P. *et al.* Isolation and Whole-genome Sequence Analysis of the Imipenem Heteroresistant Acinetobacter baumannii Clinical Isolate HRAB-85. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **62**, 94-101, doi:10.1016/j.ijid.2017.07.005 (2017).

150 Hjort, K., Nicoloff, H. & Andersson, D. I. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in Salmonella enterica. *Molecular microbiology* **102**, 274-289, doi:10.1111/mmi.13459 (2016).

151 Karah, N. *et al.* Novel Aminoglycoside Resistance Transposons and Transposon-Derived Circular Forms Detected in Carbapenem-Resistant Acinetobacter baumannii Clinical Isolates. *Antimicrobial agents and chemotherapy* **60**, 1801-1818, doi:10.1128/aac.02143-15 (2016).

152 Aliakbarzade, K., Farajnia, S., Karimi Nik, A., Zarei, F. & Tanomand, A. Prevalence of Aminoglycoside Resistance Genes in Acinetobacter baumannii Isolates. *Jundishapur journal of microbiology* **7**, e11924, doi:10.5812/jjm.11924 (2014).

153 Hamidian, M., Nigro, S. J. & Hall, R. M. Variants of the gentamicin and tobramycin resistance plasmid pRAY are widely distributed in Acinetobacter. *J Antimicrob Chemother* **67**, 2833-2836, doi:10.1093/jac/dks318 (2012).

154 Nigro, S. J. & Hall, R. M. Loss and gain of aminoglycoside resistance in global clone 2 Acinetobacter baumannii in Australia via modification of genomic resistance islands and acquisition of plasmids. *The Journal of antimicrobial chemotherapy* **71**, 2432-2440, doi:10.1093/jac/dkw176 (2016).

155 Farinha, M. A. & Kropinski, A. M. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *Journal of bacteriology* **172**, 3496-3499 (1990).

156 Hunger, M., Schmucker, R., Kishan, V. & Hillen, W. Analysis and nucleotide sequence of an origin of DNA replication in Acinetobacter calcoaceticus and its use for Escherichia coli shuttle plasmids. *Gene* **87**, 45-51 (1990).

157 Liu, Y.-Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases*, doi:10.1016/s1473-3099(15)00424-7 (2015).

158 Nordmann, P., Lienhard, R., Kieffer, N., Clerc, O. & Poirel, L. Plasmid-Mediated Colistin-Resistant Escherichia coli in Bacteremia in Switzerland. *Clin Infect Dis* **62**, 1322-1323, doi:10.1093/cid/ciw124 (2016).

159 Stoesser, N., Mathers, A. J., Moore, C. E., Day, N. P. J. & Crook, D. W. Colistin resistance gene mcr-1 and pHNSHP45 plasmid in human isolates of Escherichia coli and Klebsiella pneumoniae. *The Lancet Infectious Diseases* **16**, 285-286, doi:10.1016/s1473-3099(16)00010-4 (2016).

160 Nicolet, S., Goldenberger, D., Schwede, T., Page, M. & Creus, M. Plasmid-mediated colistin resistance in a patient infected with Klebsiella pneumoniae. *The Lancet Infectious Diseases* **16**, 998-999, doi:10.1016/s1473-3099(16)30197-9 (2016).

161 Xavier, B. B. *et al.* Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. *Euro Surveill* **21**, doi:10.2807/1560-7917.ES.2016.21.27.30280 (2016).

162 Dijkshoorn, L., Nemec, A. & Seifert, H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. *Nat Rev Microbiol* **5**, 939-951, doi:10.1038/nrmicro1789 (2007).

163 Badave, G. K. & Kulkarni, D. Biofilm Producing Multidrug Resistant Acinetobacter baumannii: An Emerging Challenge. *J Clin Diagn Res* **9**, DC08-10, doi:10.7860/JCDR/2015/11014.5398 (2015).

164 Bergogne-Berezin, E. & Towner, K. J. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin Microbiol Rev* **9**, 148-165 (1996).

165 Bianco, C. *et al.* First report of spondylodiscitis due to vancomycin heteroresistant Staphylococcus capitis in immunocompetent host. *J Infect Chemother* **20**, 639-642, doi:10.1016/j.jiac.2014.04.004 (2014).

166 Chen, Y. *et al.* Efflux Pump Overexpression Contributes to Tigecycline Heteroresistance in Salmonella enterica serovar Typhimurium. *Front Cell Infect Microbiol* **7**, 37, doi:10.3389/fcimb.2017.00037 (2017).

167 Cherkaoui, A. *et al.* Imipenem heteroresistance in nontypeable Haemophilus influenzae is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation. *Clin Microbiol Infect* **23**, 118 e119-118 e119, doi:10.1016/j.cmi.2016.10.009 (2017).

168 Gosciniak, G., Biernat, M. M., Binkowska, A., Kus, A. & Iwanczak, B. Frequency of infection with Helicobacter pylori isolates of different antimicrobial profiles in children and adolescents: A preliminary study. *Adv Clin Exp Med* **26**, 263-268 (2017).

169 Sun, J. D. *et al.* Impact of carbapenem heteroresistance among clinical isolates of invasive Escherichia coli in Chongqing, southwestern China. *Clin Microbiol Infect* **21**, 469 e461-410, doi:10.1016/j.cmi.2014.12.013 (2015).

170 Zheng, C. *et al.* Mixed Infections and Rifampin Heteroresistance among Mycobacterium tuberculosis Clinical Isolates. *J Clin Microbiol* **53**, 2138-2147, doi:10.1128/jcm.03507-14 (2015).

171 Hawley, J. S., Murray, C. K. & Jorgensen, J. H. Colistin heteroresistance in acinetobacter and its association with previous colistin therapy. *Antimicrob Agents Chemother* **52**, 351-352, doi:10.1128/AAC.00766-07 (2008).

172 Moosavian, M. *et al.* Post Neurosurgical Meningitis due to Colistin Heteroresistant Acinetobacter baumannii. *Jundishapur J Microbiol* **7**, e12287, doi:10.5812/jjm.12287 (2014).

173 Rodriguez, C. H. *et al.* Selection of colistin-resistant Acinetobacter baumannii isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. *Diagn Microbiol Infect Dis* **65**, 188-191, doi:10.1016/j.diagmicrobio.2009.05.019 (2009).

174 Rodriguez, C. H. *et al.* Trends in the resistance profiles of Acinetobacter baumannii endemic clones in a university hospital of Argentina. *J Chemother* **28**, 25-27, doi:10.1179/1973947814y.0000000213 (2016).

175 Yau, W. *et al.* Colistin hetero-resistance in multidrug-resistant Acinetobacter baumannii clinical isolates from the Western Pacific region in the SENTRY antimicrobial surveillance programme. *J Infect* **58**, 138-144, doi:10.1016/j.jinf.2008.11.002 (2009).

176 Satola, S. W., Farley, M. M., Anderson, K. F. & Patel, J. B. Comparison of detection methods for heteroresistant vancomycin-intermediate Staphylococcus aureus, with the population analysis profile method as the reference method. *J Clin Microbiol* **49**, 177-183, doi:10.1128/JCM.01128-10 (2011).

177 224 (Clinical and Laboratory Standards Institute (CLSI), 2017).

178 Matuschek, E., Brown, D. F. & Kahlmeter, G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect* **20**, O255-266, doi:10.1111/1469-0691.12373 (2014).