

Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

Copper Resistance in the Emerging Pathogen *Acinetobacter baumannii*

by

Caitlin Lee Williams

Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2019



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

SCHOOL OF MEDICINE GRADUATE PROGRAMS

Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814



APPROVAL OF THE DOCTORAL DISSERTATION IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: “Copper Resistance in the Emerging Pathogen *Acinetobacter baumannii*”

Name of Candidate: Caitlin Williams
Doctor of Philosophy Degree
August 23, 2019

DISSERTATION AND ABSTRACT APPROVED:

DATE:

Dr. Ann E. Jerse
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Chairperson

Dr. D. Scott Merrell
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Dissertation Advisor

Dr. Edward Mitre
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Member

Dr. Kristi L. Frank
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Member

Dr. Daniel V. Zurawski
WRAIR
Committee Member

ACKNOWLEDGMENTS

To my advisor, Scotty: Thank you for allowing me to join your lab and for being an excellent mentor. I've learned so much from you and I am forever grateful.

To my thesis committee: Thank you for your guidance throughout this process. I am a better scientist because of your advice and support.

To my labmates: I've enjoyed these years together and will miss you all. I appreciate all the answered questions and brainstorming sessions. Equally important, I have felt at home among friends in the lab, and that is invaluable during the tough parts of graduate school. Thank you.

To my classmates: Graduate school definitely wasn't easy, and it would have been much more difficult and a lot less fun without you. Thank you for being there, both as colleagues and as friends.

To my family: I'm finally done being a student! Your unconditional support means so much, even when you had no idea what I was actually doing. I love you all.

Most importantly, to my husband, Grant: Thank you for being there to love and support me in so many ways. I wouldn't have made it otherwise. I love you forever.

DEDICATION

To all the women in science who inspire me to keep going.

“Never give up. Never let anybody tell you that you can’t do something.”

- Cher

“Being a goddess warrior isn’t not being scared; it is being shit scared and facing it anyway. And the strength to do that – to run your own show – that’s gotta come from you. Because God knows if a girl who’s always been shy and afraid of people can do it, so can you.”

- Star (Cher), *The Cher Show*

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: “Copper Resistance in the Emerging Pathogen *Acinetobacter baumannii*” is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Caitlin Williams

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

ABSTRACT

Copper Resistance in the Emerging Pathogen *Acinetobacter baumannii*

Caitlin L. Williams, Doctor of Philosophy, 2019

Thesis directed by: D. Scott Merrell, Ph.D.

Professor, Department of Microbiology and Immunology

While most *Acinetobacter* species are environmental soil bacteria, *Acinetobacter baumannii* has recently emerged as a prominent nosocomial pathogen. The ability to survive in harsh, dry environments likely aided in the establishment of *A. baumannii* in the healthcare setting. In the past few decades, *A. baumannii* has rapidly acquired numerous mechanisms of antibiotic resistance, and extensive drug resistance is now common. Lack of efficacious treatments leads to increased patient morbidity and mortality. Indeed, both the CDC and WHO have classified multidrug-resistant *A. baumannii* as a significant threat to human health. Clearly, novel therapeutic options are needed to treat patients infected with drug-resistant *A. baumannii*.

Copper resistance has been shown to contribute to virulence of some bacterial pathogens, likely by helping to combat host copper-mediated immune mechanisms. However, very little is known about the copper resistance mechanisms of *A. baumannii* or whether copper resistance plays a role in virulence. A more complete understanding of the copper resistance mechanisms of *A. baumannii* and their contribution to virulence

may lead to the identification of novel drug targets in multidrug-resistant organisms. The work presented in this dissertation identifies numerous genes that mediate copper resistance; these genes are present in multiple *A. baumannii* clinical isolates.

Furthermore, a particular set of genes that is found in some clinical strains is implicated in the highest levels of copper resistance. Our findings suggest that copper resistance is multifactorial in *A. baumannii* and that the range in levels of copper resistance seen among strains is dependent on the presence of certain copper-related genes.

Furthermore, in a model clinical isolate, we utilize transposon mutant strains to assess the roles of individual proteins in *in vitro* copper resistance and *in vivo* virulence in two infection models. We determine that many genes, most from the group previously predicted to convey a high degree of copper resistance, contribute individually to copper resistance; strains bearing mutations in these genes demonstrate reduced growth in the presence of copper. Furthermore, many of the genes contribute to virulence; strains bearing certain mutations are attenuated *in vivo*. *En masse*, our data suggest that copper resistance contributes to virulence of *A. baumannii* and is mediated by a number of genes; we believe that these factors have potential as novel drug targets in multidrug-resistant *A. baumannii*.

TABLE OF CONTENTS

LIST OF TABLES	xii
LIST OF FIGURES	xiii
CHAPTER 1: Introduction	1
Organism	1
History	1
Epidemiology and Disease	3
Antibiotic Resistance	7
Environmental Persistence and Virulence Factors	10
Metal Homeostasis and the Role of Metals During Infection	12
Importance of Metals During Infection	12
Copper-mediated Immune Mechanisms	13
Copper Resistance Mechanisms of Bacteria	16
Copper ATPases	17
Copper Oxidases	21
Other Transporters and Pumps	21
Chaperones	22
Regulatory Proteins	23
Other Copper Resistance Mechanisms	24
<i>Acinetobacter</i> Copper Resistance	25
Contribution of Copper Resistance to Virulence	28
Linking Virulence Phenotypes to Copper	32
Harnessing the Antimicrobial Power of Copper	34
Copper Surfaces for Decontamination	34
Advanced Copper Technology	36
Goals and Specific Aims	37
CHAPTER 2: Copper Resistance of the Emerging Pathogen <i>Acinetobacter baumannii</i> ..	40
Abstract	40
Introduction	42
Materials and Methods	45
Bacterial Strains and Growth Conditions	45
Determination of <i>A. baumannii</i> Sensitivity to Copper in Liquid Culture	49
Determination of <i>A. baumannii</i> Copper Sensitivity on Solid Metal Coupons	49
Quantification of Copper Ions via ICP-MS	50
Assessing Copper Sensitivity of <i>A. baumannii</i> Biofilms	51
Identification of Putative Copper-related Genes in <i>A. baumannii</i>	51
RNA Extraction, cDNA Synthesis, and qRT-PCR	52
Confirmation of Operonic Structure by Junction PCR	53
Data Analysis and Statistics	54

Results	55
Copper Sensitivity of <i>A. baumannii</i> Strains in Liquid Culture	55
Survival of <i>A. baumannii</i> Isolates on Metal Surfaces	59
Killing of <i>Acinetobacter</i> Biofilms by Copper	65
Copper Tolerance Response	65
Putative Copper-related Genes	69
Expression of Putative Copper-related Genes	75
Discussion	77
Acknowledgements	83
CHAPTER 3: Characterization of <i>Acinetobacter baumannii</i> Copper Resistance Reveals a Role in Virulence	85
Abstract	85
Background	87
Materials and Methods	93
Bacterial Strains and Growth Conditions	93
AB5075 Mutant Strains	94
Generation of Complemented Strains	101
Cloning of Region D from AB5075 into Other <i>A. baumannii</i> Strain Backgrounds	102
Determination of <i>A. baumannii</i> Sensitivity to Copper in Liquid Culture	103
Assessing Copper Sensitivity of <i>A. baumannii</i> Biofilm	103
Quantification of Intracellular Copper via ICP-MS	104
Infection of <i>Galleria mellonella</i> Caterpillars	106
Mouse Pneumonia Infection Model	108
Data Analysis and Statistics	108
Results	109
Mutant Strains of AB5075 Exhibit Copper Sensitivity	109
Region D Enhances Copper Resistance in Other <i>A. baumannii</i> Strains	114
Killing of Biofilms by Copper	114
Copper Sensitive Mutant Strains Accumulate More Intracellular Copper	117
Copper Resistance is Important for Virulence in <i>Galleria mellonella</i> Larvae	120
CusR and CopD are Important for Virulence in a Murine Pneumonia Infection Model	126
Discussion	128
Acknowledgements	135
CHAPTER 4: Discussion	137
Summary of Findings and Relevance	137
Copper Homeostasis in <i>A. baumannii</i>	139
Copper Resistance Genes as Virulence Factors	142
Targeting Copper Resistance with Therapeutics	145
Conservation of Copper-related Proteins	149
Potential Uses of Copper Resistance-targeted Therapeutics	153
Evolution of Copper Resistance	154
Future of Treatment of Infections with Drug-resistant <i>A. baumannii</i>	156
Conclusion	158

REFERENCES	159
------------------	-----

LIST OF TABLES

Table 1. Names of copper-related proteins in <i>E. coli</i>	18
Table 2. Homologs of known copper-related proteins identified in the genomes of <i>Acinetobacter</i> isolates by Hernandez-Montes <i>et. al.</i> in 2012	27
Table 3. Copper-related proteins with demonstrated roles in virulence	29
Table 4. Strains used in this study	46
Table 5. Oligonucleotides used in this study	47
Table 6. Adjusted <i>P</i> values for AB5075 growth following copper priming	68
Table 7. Putative copper-related genes/proteins in AB5075	70
Table 8. Strains used in this study	95
Table 9. Oligonucleotides used in this study	98
Table 10. Conservation of copper-related proteins of <i>A. baumannii</i> AB5075	150

LIST OF FIGURES

Figure 1. Mechanisms of copper-mediated damage.	14
Figure 2. Copper resistance mechanisms of <i>E. coli</i>	19
Figure 3. Copper sensitivity of <i>A. baumannii</i> strains in liquid culture.	56
Figure 4. Survival of <i>A. baumannii</i> isolates on metal surfaces.	60
Figure 5. Concentrations of copper that accumulated in droplets on metal surfaces.	64
Figure 6. Killing of <i>Acinetobacter</i> biofilms and planktonic cells by copper.	66
Figure 7. Preexposure to a low concentration of copper induces a copper tolerance response.	67
Figure 8. Genomic organization of putative copper-related genes.	73
Figure 9. Junctional PCR confirms the operonic structure of putative copper-related genes.	74
Figure 10. Expression of putative copper-related genes in response to copper exposure.	76
Figure 11. Organization of regions A - D in the AB5075 chromosome.	90
Figure 12. Growth of AB5075 mutant strains that displayed no change in copper sensitivity.	110
Figure 13. Growth of highly copper sensitive AB5075 mutant and complementation strains.	111
Figure 14. Growth of moderately copper sensitive AB5075 mutant and complementation strains.	112
Figure 15. Growth of AB4857 and AB5711 carrying Region D.	115
Figure 16. Copper-dependent killing of AB5075 mutant strain biofilms and planktonic cells.	116
Figure 17. Accumulation of intracellular copper.	119
Figure 18. Survival of <i>Galleria</i> infected with AB5075 mutant strains.	121
Figure 19. Survival of <i>Galleria</i> infected with AB5075 mutant and complemented strains.	122
Figure 20. Survival of <i>Galleria</i> infected with a high dose of AB5075 mutant and complemented strains.	123
Figure 21. Survival of <i>Galleria</i> infected with Tn7-containing strains.	124
Figure 22. Assessment of virulence of AB5075 mutant and complemented strains in a murine pneumonia model.	127
Figure 23. Model of putative copper resistance mechanisms in AB5075.	130
Figure 24. Potential action of a novel copper-targeting therapeutic.	146

CHAPTER 1: Introduction

ORGANISM

History

The genus *Acinetobacter* contains a large number of species, most of which are environmental soil bacteria. *Acinetobacter* spp. are aerobic, Gram-negative, coccobacilli. Based on unique DNA-DNA hybridization patterns, twelve species names were assigned within this genus in 1986 (156). In terms of pathogenesis, *A. baumannii* and its close relatives are responsible for the disease attributed to the *Acinetobacter* genus, and these microbes are often referred to as the “*A. baumannii* complex” or the “*A. baumannii-calcoaceticus* complex” because they are very difficult to distinguish by phenotypic properties (156; 200). *Acinetobacter* began to be recognized as a nosocomial pathogen in the early 1970s; at the time, the isolates were sensitive to common antibiotics, and thus were not of great concern (200). However, over the ensuing decades *A. baumannii* rapidly acquired antibiotic resistance and has evolved into a global pathogen of serious concern.

As there was a large increase in infections in military personnel at that time, *A. baumannii* gained notoriety in the military healthcare system in the early 2000s during the wars in Iraq and Afghanistan (156). This connection earned *A. baumannii* the nickname “Iraqibacter” (82). It was originally hypothesized that the large number of *A. baumannii* infections among injured soldiers was due to exposure to the organism in the soil or due to its establishment as part of the skin flora. However, upon investigation, *A. baumannii* was infrequently isolated from the skin or soil; instead, the bacterium was

isolated from the treatment areas in 7 out of 7 field hospitals, suggesting that the *A. baumannii* outbreak was largely due to contamination of the field hospitals and subsequent transmission within the health care setting (139; 156; 177). Notably, *A. baumannii* is now considered a true nosocomial pathogen without a significant infection-associated environmental reservoir outside the healthcare setting; the nature of this change from being soil associated to being solely healthcare associated is not well understood (200).

On a genetic level, *A. baumannii* isolates are diverse. There are approximately 2,200 coding sequences in the core genome, which accounts for 60% of the genetic material of an average strain (10). The other 40% of the genome, the accessory genome, is often dissimilar among strains. Thus, the total pan genome of *A. baumannii* is very large and is continuously expanding as more isolates are sequenced (10). For example, genomic analyses of approximately 250 genomes in 2015 revealed that the pan genome encodes nearly 12,000 sets of proteins, with paralogs counted once as a set (26). Despite the large amount of diversity among isolates, a few international clone types are dominant worldwide; international clones I and II, which are both multidrug-resistant, are most common (101; 233). In thinking about the evolution of *A. baumannii* as it relates to pathogenesis, it seems surprising that gene functions that are predicted to be important for virulence are found as part of the core genome; some interpret this to mean that accumulation of new virulence genes is not the reason for the recent increase in *A. baumannii* prevalence (10). Instead, antibiotic resistance, which is mediated by both core and accessory genome determinants, is thought to have been most important for the spread of the clonal lineages (10; 90). In keeping with *A. baumannii*'s ability to persist in

many environments, drug-resistant strains have rapidly evolved and spread in the hospital environment.

Epidemiology and Disease

A. baumannii is capable of causing a variety of types of infection, including pneumonia, bloodstream infection (BSI), wound infection, urinary tract infection (UTI), and meningitis (156). In the vast majority of cases, *A. baumannii* is an opportunistic, nosocomial pathogen that infects patients with pre-existing co-morbidities, often in intensive care units (ICU) (45; 128; 159). The mortality rate among *A. baumannii*-infected patients is very high (>60%) (111). However, it is difficult to determine the direct effect of *A. baumannii* infection on morbidity and mortality because these patients are already very ill. Despite this issue, several studies that were able to control for severity of disease revealed that i) increased mortality is attributable to *A. baumannii* in infected patients as compared to non-*A. baumannii*-infected patients, and ii) increased mortality is attributable to drug-resistant *A. baumannii* in infected patients as compared to those infected with drug-susceptible strains (156). Controlled or matched studies suggested that mortality attributable to *A. baumannii* was 7.8 – 23% in the hospital, and 10 – 43% in the ICU (50). Overall, it is clear that drug-resistant *A. baumannii* contributes to patient morbidity and mortality.

Rates of *A. baumannii* infection vary greatly by continent, county, city, and individual healthcare institution, and thus are difficult to generalize. However, some insight on prevalence was gained by the Extended Prevalence of Infection in Intensive Care (EPIC) II study. In this study, more than 14,000 patients were recruited from >1,200

ICUs in 75 countries (208). Because all of the patients were recruited on the same day (May 8, 2007), the data provide a snapshot of ICU infections at that time. *Acinetobacter* was identified in 8.8% of cultures worldwide; the highest rates were seen in Asia, Eastern Europe, Africa, and Central/South America (19.2%, 17.1%, 14.8%, and 13.8%, respectively), and the lowest rates were seen in North America, Oceania, and Western Europe (3.7%, 4.4%, and 5.6%, respectively) (208). Interestingly, rates of *A. baumannii* infection varied more by geographical region than those of any other organism in the study (208). This finding led the authors to suggest that regional infection control measures may be responsible for these differences. Regional differences in infection have similarly been found by other studies. A review of studies from 2010-2012 showed that the rates of nosocomial *A. baumannii* infections were highest in South America; additionally, rates of infection were higher in the ICU (198). It is important to note that the rise in *A. baumannii* infections is relatively recent. The first EPIC study, which analyzed data from ICU patients across Western Europe in 1992, did not even mention *Acinetobacter* species (207).

Though rates of infection tend to be lower in North America, the burden of *A. baumannii* infection is still substantial in the United States. Analysis of the epidemiology of Gram-negative nosocomial infections using the U.S.'s National Nosocomial Infection Surveillance (NNIS) system data from 1986 – 2003 revealed that *A. baumannii* accounted for 0.5% of surgical site infections, 0.6% of UTIs, 1.5% of pneumonia, and 1.8% of bloodstream infections hospital wide (61). Within the ICU, however, *A. baumannii* was responsible for 2.1% of surgical site infections, 1.6% of UTIs, 6.9% of pneumonia, and 2.4% of BSIs (61). Higher rates of *A. baumannii* infection in the ICU is reflective of the

opportunistic nature of the pathogen and the highly susceptible nature of these patients; in these scenarios the bacterium often gains access to an infection site through a break in an anatomical barrier (e.g. via a catheter, central line, or ventilator), leading to severe complications in these critically ill patients (74). A more recent 2014 multi-state surveillance study of 183 hospitals across the United States revealed that *A. baumannii* infections represented 1.6% of all healthcare associated infections, 3.6% of pneumonia, and 1.8% of surgical site infections (120); this data set includes ICU patients, which account for about 15% of the total patient population. While the percentage of total healthcare associated infections attributable to *A. baumannii* may seem small, healthcare associated infections are incredibly common; therefore, the number of associated infections is significant. Indeed, *A. baumannii* is estimated to infect 41,400 – 83,000 people annually in the United States alone; rates of infection are much higher in many other parts of the world, and the global estimate is 600,000 - 1,400,000 cases annually (190).

A. baumannii infection is also common among military patients. In a study of combat wounds from wounded soldiers in the capitol area (presumably, most at Walter Reed National Military Medical Center), *A. baumannii* was the most commonly identified organism, present in 23% of samples (17). The apparent increased risk of infection in the military population may be due to higher rates of severe wounds and other injuries as compared to civilians, as well as deployment to locations with higher rates of *A. baumannii* infection than seen in the United States. Indeed, it has been shown that among military patients, *A. baumannii* infection is more common among the deployed (103). Unfortunately, likely because the rates of drug resistance are higher in

the location of deployment, risk of infection with a multidrug-resistant strain is also increased (78).

It is widely believed that, in conjunction with its rapid acquisition of antibiotic resistance, the ability of *A. baumannii* to survive in the environment is largely responsible for its success as a nosocomial pathogen (165). In the lab, *A. baumannii* has been shown to survive on dry surfaces for up to 5 months (106). Within the hospital environment, *A. baumannii* can colonize many abiotic surfaces, including ventilators, bronchoscopes, bed frames, and sinks (200). Because of this ability to survive and colonize diverse landscapes, infection control measures have focused on enhanced staff hygiene and on cleaning protocols that seek to reduce environmental contamination and subsequent bacterial spread via fomites. The importance of these approaches is exemplified by several outbreak cases. For example, in one *A. baumannii* outbreak in a neurosurgical ICU, the number of environmental isolates observed during screening positively correlated with the number of infected individuals (38). Furthermore, reduction of environmental contamination through enhanced cleaning measures reduced patient colonization (38). Similarly, in another outbreak, 17 patients were infected in a surgical ICU before the outbreak was ended by strict control measures, including thorough cleaning (51). Finally, in a large hospital outbreak in Birmingham, UK, a real-time outbreak investigation traced *A. baumannii* transmission to a particular burn operating theater and deep cleaning of the burn theater temporarily stopped the outbreak (73). Unfortunately, six weeks later, another patient was infected, leading to the subsequent infection of more than a dozen new patients and re-contamination of the burn theater. This second wave of the outbreak was traced to a specialized burn care bed, and thorough

cleaning of all of the beds and the burn theater (again) finally ended the outbreak for good (73). These cases clearly highlight the importance of fomite transmission of *A. baumannii* in the healthcare setting as well as the importance of decontamination strategies in infection prevention.

Though certainly not as common as hospital-associated infections, it is worth mentioning that reports of community-acquired *A. baumannii* are accumulating; to date, these seem to be limited to patients with co-morbidities in tropical or subtropical climates (156). More research will be required to understand the transmission and biological mechanisms of this unique presentation.

Antibiotic Resistance

It is widely believed that the rapid acquisition of antibiotic resistance is largely responsible for the success of *A. baumannii* as a nosocomial pathogen (165). Furthermore, the prevalence of multidrug resistance, extensive drug resistance, and pan drug resistance have all risen in recent years. While there is heterogeneity in the literature concerning the definitions of these terms, the definitions agreed upon in 2012 are as follows: multidrug-resistant (MDR) bacteria are resistant to an agent of at least three different classes of antibiotics; extensively drug-resistant (XDR) bacteria are susceptible to only one or two antibiotic classes; pandrug-resistant (PDR) strains are resistant to all classes of antibiotics (121). The following nine antibiotic classes are considered for *Acinetobacter* strains: aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins + β -lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins + β -lactamase inhibitors,

polymyxins, and tetracyclines (121). Importantly, incorrect empirical therapy has repeatedly been shown to contribute to patient mortality (45; 128; 159; 194).

Likely in part due to differences in antimicrobial stewardship, the rates of drug resistance vary greatly with time and geographic location. However, trends toward high and increasing rates of drug resistance are observed worldwide. From 1986 to 2003 in the United States, rates of resistance to amikacin, imipenem, and ceftazidime all significantly increased over time (61). For example, less than 10% of *Acinetobacter* isolates were resistance to amikacin or imipenem in 1986. However, by 2003 more than 20% of isolates were resistant. More dramatically, rates of resistance to ceftazidime rose from ~25% in 1986 to ~65% in 2003 (61). Notably, the rates of resistance to ceftazidime were much higher in *Acinetobacter* isolates than in *Pseudomonas aeruginosa* isolates, which rose from 10% in 1986 to ~22% in 2003 (61).

In 2008, the EUROBACT International Cohort Study characterized isolates from ICU bloodstream infections in 162 ICUs across the globe (194). *A. baumannii* was the most commonly identified organism, found in 12.2% of cases. Remarkably, 91.9% of isolates were MDR, 71.3% were XDR, and 0.6% (a single case) were PDR. Indeed, *A. baumannii* showed the highest rates of drug resistance of all the bacterial and fungal pathogens identified. Another recent study looked more broadly at clinical isolates from hospital patients around the world; though 13% of all Gram-negative isolates collected from 2004 to 2014 were MDR, 44.3% of the *A. baumannii* isolates were MDR, the highest of all identified species (62). Furthermore, the global rate of MDR in *A. baumannii* increased from 23% to 63% over the 10 year period. Once again, geographic disparities were evident: the highest rates of drug resistance among *A. baumannii* isolates

were observed in Latin America, Africa, and the Middle East, and the lowest rates were observed in North America and Europe (62). *A. baumannii* is repeatedly shown to have the highest rates of drug resistance among nosocomial pathogens, highlighting the seriousness of this global pathogen.

A. baumannii utilizes many of the classic strategies that are involved in antibiotic resistance: altering the antibiotic compound, reducing antibiotic uptake, increasing antibiotic efflux, and modifying the antibiotic target. Indeed, *A. baumannii* has rapidly acquired genes that encode for factors that impart these specific mechanisms of antibiotic resistance (110). For example, *A. baumannii* possesses enzymes that deactivate antibiotic compounds; these include β -lactamases and aminoglycoside-modifying enzymes such as acetyltransferases, adenyltransferases, and phosphotransferases. Indeed, all four classes of β -lactamases (A – D), which inactivate β -lactam antibiotics, are found in *A. baumannii*. The prevalence and extent of these components is exemplified by a recent clinical isolate named AB5075 that was obtained from a patient at Walter Reed Military Medical Center. Of note, AB5075 has recently become popular as a model strain for currently circulating MDR isolates and is the strain used for the studies described herein (93). AB5075 carries fifteen β -lactamase genes, covering all four classes (56) as well as acetyltransferases, adenyltransferases, and phosphotransferases that modify aminoglycoside antibiotics (56).

In terms of other common mechanisms of resistance, *A. baumannii* can reduce uptake of antibiotic compounds via changes in outer membrane porin proteins or in surface charge. For example, changes in lipooligosaccharide (LOS) can provide colistin resistance (110). *A. baumannii* also utilizes many different families of efflux pumps to

remove antibiotic compounds from the cell; many pumps have been conclusively linked to increased resistance to a number of classes of antibiotics (110). In AB5075, more than 80 loci encode putative efflux pump proteins that may have roles in drug resistance (56). Finally, *A. baumannii* also gains antibiotic resistance via modification of drug targets. Classic examples include mutations in gyrase (GyrA) and topoisomerase (ParC), which confer quinolone resistance; alterations in dihydrofolate reductase, which confers trimethoprim resistance; and changes in dihydropteroate synthase, which confers sulfonamide resistance. AB5075 carries all of these mutations (56).

Many of these acquired antibiotic resistance genes are found in resistance islands, which are believed to have been transferred horizontally. Indeed, the ability of *A. baumannii* to take up foreign DNA is thought to contribute to its rapid acquisition of a vast number of antibiotic resistance elements (110). In support of this, a recent study that used *Acinetobacter baylyi* as a model demonstrated that the type VI secretion system could be used to kill “prey” *Escherichia coli* cells. *A. baylyi* was subsequently able to “steal” and express antibiotic resistance genes from the lysed *E. coli* cells. This type VI-dependent mechanism increased cross-species horizontal gene transfer by orders of magnitude and is likely important for the ability of *Acinetobacter* to rapidly acquire resistance (33).

Environmental Persistence and Virulence Factors

A. baumannii virulence is not yet well understood. A large percentage of the genome is made up of hypothetical proteins, and many of these genes are found on plasmids or in areas of horizontal gene transfer. It is certainly possible that *A. baumannii*

contains many yet unidentified virulence genes. To date, *A. baumannii*'s ability to persist in the environment and antibiotic resistance are thought to be the main contributors to its success (74). The biological mechanisms that underlie the ability of *A. baumannii* to persist in the environment are not yet well understood. However, it is clear that *A. baumannii* possesses significant desiccation resistance and remains viable for long periods of time when dry; the polysaccharide capsule may contribute to this desiccation resistance (74). Additionally, *A. baumannii* utilizes various mechanisms to resist the DNA damage and oxidative stress that accompany desiccation (74). Beyond desiccation, biofilm formation is thought to be important for the ability of *A. baumannii* to persist in the environment as well as to cause disease. The bacteria can form protective three-dimensional biofilm structures during infection in wounds, on wound dressings, and on medical implants. These biofilm structures enhance antimicrobial resistance and immune evasion (74). The genetic factors that contribute to biofilm formation seem to be diverse among strains; however, pili, regulatory proteins, capsule, and other surface proteins appear to be important (74).

A few important elements have been identified in the interaction between *A. baumannii* and the host. For example, the polysaccharide capsule is the main component required for resistance to complement-mediated killing (74; 110). *A. baumannii* encodes type I, II, IV, V, and VI secretion systems. For the virulence-associated secretion systems, a few effector proteins have been identified that aid in adherence, colonization, and/or dissemination (74). The phospholipase enzyme has been shown to contribute to host cell invasion (110). Several outer membrane proteins, including the most abundant porin in the outer membrane, OmpA, have roles in attachments and host cell cytotoxicity

(110). Finally, iron, zinc, and manganese acquisition systems have been identified, which help the bacteria acquire essential micronutrients from the host (74; 110).

METAL HOMEOSTASIS AND THE ROLE OF METALS DURING INFECTION

Importance of Metals During Infection

Transition metals are essential micronutrients for all forms of life. Indeed, metal ions are required for the function of many proteins, and almost 60% of known enzymes require a metal ion (8). Metalloproteins serve numerous functions because the redox potential of transition metals is useful in catalysis. However, for this same reason, unbound metal ions cause toxicity (153). In bacteria, the main mechanism of toxicity induced by many metal ions is thought to be production of reactive oxygen species by Fenton chemistry (181).

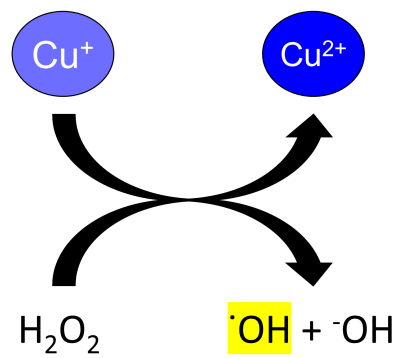
Because transition metals are both essential and toxic in excess, complex homeostasis systems are important to maintain the proper balance between having enough metal and having too much. Primary homeostasis systems include mechanisms for both metal uptake and efflux. The balance between these opposing functions likely reflects the availability of the metal and the quantity required by the cell; for example, many uptake systems are typically present for a scarce metal, like iron, which is required in large quantities for many essential functions.

Given the importance of transition metals, the human immune system has evolved to take advantage of both the need for metals as micronutrients and their inherent toxicity. In the context of infection, transition metals are strategically relocated by host

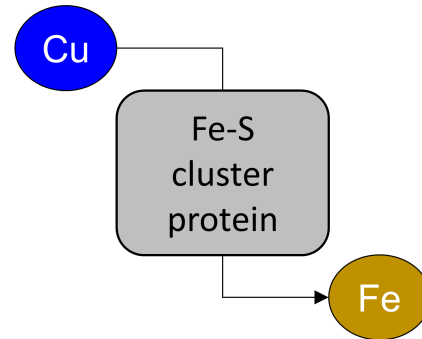
immune mechanisms (153). Some metals, e.g. iron, are sequestered from invading pathogens to hinder their growth; this process is termed “nutritional immunity.” Other metals, e.g. copper, are mobilized toward the site of infection to intoxicate the pathogen. Because of these host strategies, pathogens have in turn evolved sophisticated methods to acquire scarce metals and to resist intoxication by others. Indeed, despite nutritional immunity, bacterial pathogens have evolved ways to acquire essential metals, including systems to import, capture, and steal metals. To date, iron acquisition systems have been the most thoroughly studied, likely because iron is the most used metal and is required for many essential bacterial processes (153). Standard iron import proteins, which serve to transport free iron into the bacterial cell, are insufficient in the iron-limited host environment. Thus, bacterial pathogens secrete high-affinity molecules called siderophores that capture scarce metal ions from the surrounding environments. Additionally, bacteria have evolved “piracy” methods that allow them to steal iron from the host by taking up metal-bound host proteins, like transferrin or hemoglobin (153). Many of these iron acquisition systems have been shown to be important virulence factors for bacterial pathogens, including *A. baumannii* (53; 153). Similar bacterial systems have been identified for zinc and manganese acquisition, though less is known about the role of nutritional immunity for these and other metals (153).

Copper-mediated Immune Mechanisms

As mentioned above, the immune system also uses transition metals as ammunition for intoxication. Among these metals, the role of copper in immunity has been studied, but zinc and other metals may also be used as intoxicants (181). In bacteria,



Redox Reactions



Mismetallation

Figure 1. Mechanisms of copper-mediated damage.

Two of the most important mechanisms through which copper causes damage to bacteria are redox reactions and mismetallation. (Left) Copper ions react with hydrogen peroxide through Fenton chemistry to produce hydroxy radicals; these reactive oxygen species can damage important biomolecules, such as DNA and proteins. (Right) Copper ions can displace iron atoms from iron-sulfur clusters and inhibit the function of the protein.

the main mechanisms of toxicity induced by copper ions are thought to be production of reactive oxygen species by Fenton chemistry and mismetallation, which is binding of the incorrect metal ion in a metalloprotein, e.g. the displacement of iron from iron-sulfur clusters (Fig. 1) (181). Because excess copper ions are also damaging to the host cell, high concentrations are strategically localized to the phagosomal compartment, where the pathogen will be found after engulfment. The host mechanisms responsible for the copper burst in the phagosome have been characterized (reviewed by (122)). Briefly, in response to interferon- γ , expression of the copper importer CTP1 is upregulated in macrophages, increasing the supply of copper ions within the cell (220). Within the macrophage cytoplasm, copper ions are bound and protected from reactivity by the protein ATOX1. ATOX1 shuttles the copper ions to the phagosome, where they are moved across the phagosomal membrane by the copper ATPase ATP7A. Pro-inflammatory signals and/or exposure to bacterial LPS induces expression of ATP7A and the subsequent trafficking of this protein to the phagosomal membrane; this results in copper accumulation in the phagosome (2; 220). Concentrations of copper surpassing 500 μ M have been measured in phagosomes containing *Mycobacterium tuberculosis* (213).

The importance of copper to immunity has been demonstrated experimentally in several ways. First, dietary copper has been shown to contribute to immunity in animal models. Copper-deficient rats are more susceptible to infection with *Salmonella* (142), and, conversely, supplementation of the diet of guinea pigs with additional copper lowers the burden of *M. tuberculosis* in the lung (225). Similarly, macrophages that have been pre-treated with copper have been shown to kill more *E. coli* and *A. baumannii* than

untreated controls (77; 220); conversely, macrophages that have been treated with a chelator to reduce copper kill fewer *Salmonella* than untreated controls (2).

Second, bacterial killing has been directly linked to the function of the copper ATPase ATP7A, which is responsible for pumping copper ions into the phagosome; ATP7A-deficient macrophages have been demonstrated to be less efficient at killing of bacterial pathogens (108; 220). Furthermore, ATP7A-deficient mice have been utilized to demonstrate the importance of this pump in immunity to bacterial infection. To this end, copper sensitive *Salmonella* are less virulent than wild-type bacteria, but only if ATP7A is functional in the mouse host. Thus, the loss of virulence in the copper sensitive strain is dependent on the ability of the host to properly mobilize copper ions as ammunition against the infection (108). A similar phenomenon was observed using copper sensitive *E. coli* in ATP7A-deficient macrophages; the killing of copper sensitive *E. coli* is attenuated in ATP7A-deficient macrophages (220).

Copper Resistance Mechanisms of Bacteria

Whether in the context of infection or other environments, copper resistance mechanisms are important for the ability of bacteria to prevent copper overload. Copper resistance in bacteria was first noticed in response to the utilization of copper to control bacterial growth in agricultural settings. In the 1980s, plasmid-based copper resistance determinants were identified in *E. coli* that were isolated from pigs that had been fed copper-supplemented diets to enhance their growth (196). Similar plasmid-mediated resistance was also found in the plant pathogen *Pseudomonas syringae* following widespread use of copper to prevent bacterial and fungal growth on crops (18).

Agricultural use of copper led to contamination of soil, and copper resistant organisms were also isolated from the soil. Indeed, a copper resistant isolate of *Pseudomonas fluorescens* that was acquired from the soil of a citrus grove was shown to be more competitive than a copper sensitive derivative in sterile, copper-contaminated soil (230). However, in non-sterile soil with a low copper concentration, the copper resistant strain was also more competitive, indicating that copper resistance may play a role in overall competitive fitness (230).

Identified copper resistance genes have various functions, including efflux, oxidation, chaperoning, and regulation. Individual copper resistance proteins are highly conserved among bacteria; however, there is diversity in the combinations and organization of the genes present in a given species or strain (79). Unfortunately, the naming of copper resistance genes in bacteria is not well standardized; as a result, functionally homologous proteins have many names. Table 1 and Figure 2 describe the *E. coli* proteins that will be discussed below, which are among the first identified and characterized.

Copper ATPases

Copper ATPases are inner membrane proteins that use energy from ATP to push copper ions out of the cytoplasm into the periplasm. These ATPases are utilized for copper transport in organisms ranging from humans to bacteria to plants. The genes for these ATPases are the most frequently encoded copper resistance gene in bacteria and are arguably the most widely studied (79). Within the P_{1B-1}-type ATPases, there are two subtypes that are classified by their copper binding sequence motif. The “classical” P_{1B-1}.

Table 1. Names of copper-related proteins in *E. coli*

	Chromosome ^a	Plasmid ^b
Copper ATPase	CopA	
Copper Oxidase	CueO	PcoA
Outer Membrane Protein	CopB	PcoB
Chaperone	CopC, CusF	PcoC, PcoE
Inner Membrane Protein	CopD	PcoD
Efflux Pump	CusCBA	
Transcription Factor	CueR	
Two-Component System	CusRS	PcoRS

^aEncoded by genes found in the chromosome

^bEncoded by genes found on a plasmid

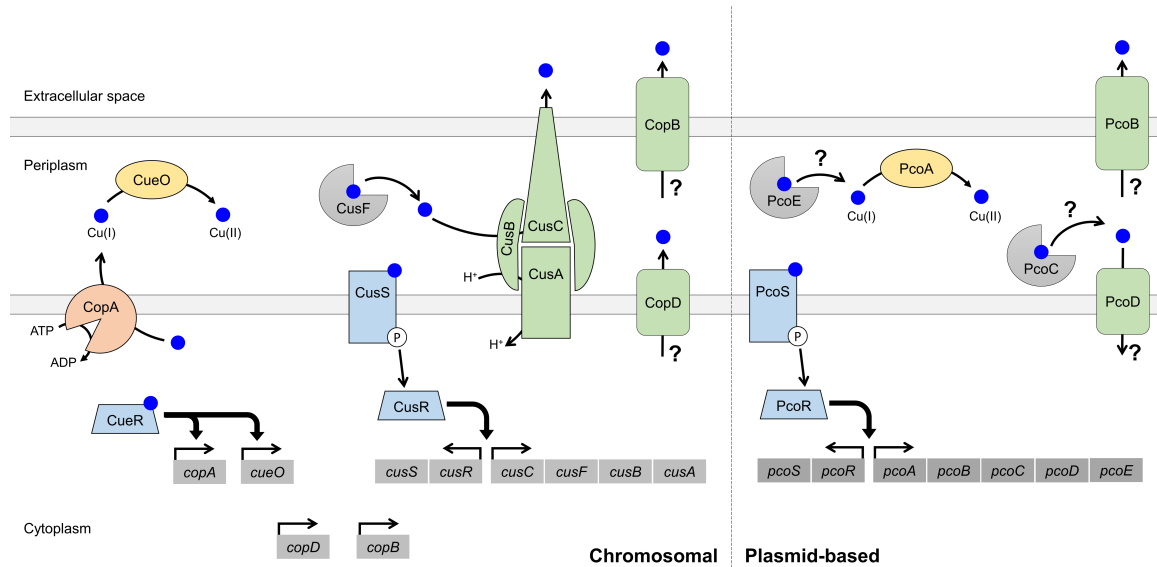


Figure 2. Copper resistance mechanisms of *E. coli*.

Copper resistance in *E. coli* is mediated by both chromosomally-encoded (left) and plasmid-based systems (right). Cytoplasmic copper is sensed by the regulator CueR; when bound to copper, CueR upregulates expression of CopA and CueO. CopA is a copper ATPase that uses the energy of ATP to pump copper ions across the inner membrane into the periplasm. CueO is a periplasmic copper oxidase that oxidizes the copper ions to the their less damaging form. Periplasmic copper is sensed by the CusRS two-component system; CusRS upregulates expression of the CusABC pump, which moves periplasmic copper ions out of the cell. CusF is a chaperone protein that transfers copper ions between the two pumps (CopA to CusB), enhancing movement of ions across both membranes. CopB and CopD are also membrane proteins with putative roles in copper efflux. The plasmid-based *pco* system is less well characterized; it encodes homologs of CusRS (PcoRS), CueO (PcoA), CopB (PcoB), and CopD (PcoD), as well as two chaperone proteins, PcoC and PcoE.

ATPases utilize multiple CxxC binding motifs, while the “histidine-rich” proteins instead use a single CxxC and many histidine residues (7).

Bacterial copper ATPases were first identified in *Enterococcus hirae* in the early 1990s (146). Uniquely, *E. hirae* uses two separate copper ATPases for opposing import and efflux functions, CopA and CopB (note that the CopB annotation is not currently used for copper ATPases) (187). The *E. hirae* CopB is associated with copper efflux and resistance, while CopA is associated with copper import. CopA is a classical P_{1B-1} ATPase, with two CxxC motifs; conversely, CopB is a “histidine-rich” P_{1B-1} ATPase, encoding one CxxC and a histidine rich stretch (130).

The bacterial copper ATPase CopA was identified in *E. coli* in 2000 (164). The protein was first recognized by its homology to the human protein ATP7A, which was identified in the early 1990s by its connection to copper-deficiency disease (211). The function of CopA was confirmed by measuring ATP-dependent movement of ⁶⁴Cu across vesicle membranes. Furthermore, it was demonstrated that an *E. coli* *copA* mutant strain is more sensitive to copper than the wild-type strain, and that expression of *copA* is induced by copper exposure (164).

P. aeruginosa, a bacterial pathogen that is more closely related to *A. baumannii* than *E. coli*, has two copper ATPases, CopA1 and CopA2; despite their structural homology, each have a distinct functional role (64). CopA1 is more similar to CopA of *E. coli* and is a classical type with multiple CxxC copper binding motifs; *copA1* can complement a *copA* mutation in *E. coli*. CopA1 similarly provides copper tolerance to *P. aeruginosa*. The function of CopA2, however, is linked to cytochrome oxidase, an essential copper-containing protein (64).

Copper Oxidases

The copper oxidases are also common copper resistance determinants in bacteria. Initial studies of the *cop* operon of *P. syringae*, which includes a copper oxidase CopA, revealed that CopA was a periplasmic protein (note that the annotation CopA typically applies to a copper ATPase, not a copper oxidase) (24). Next, a putative copper oxidase was identified in *E. coli* due to the presence of a potential CueR binding site in the promoter region of the gene; the protein was subsequently named CueO (151). When the protein was functionally characterized, it was demonstrated that CueO protects *E. coli* from periplasmic stress during copper exposure, and that the purified protein demonstrates multi-copper oxidase function (66). Additionally, it was shown that the presence of *cueO* increases bacterial growth in the presence of copper (66).

PcoA is the homolog of CueO in the *pco* system, which encodes the plasmid-based copper resistance determinants of *E. coli*. Loss of *pcoA* decreases copper resistance, and *pcoA* was demonstrated to confer copper resistance to a Δ *cueO* strain (112). It has been suggested that PcoA and CueO share a common ancestor and have, following duplication, independently evolved to form specialized functions (79).

Other Transporters and Pumps

In combination with the copper ATPases, there are additional pumps that move copper ions across either the inner or outer membrane. The CusCFBA system of *E. coli* is a resistance-nodulation-division (RND) family efflux pump; deletion of any one gene or the whole operon encoding these components leads to copper sensitivity (55). CusC, CusB, and CusA are believed to comprise the main tripartite structure of the pump, while

CusF is a periplasmic copper-binding protein (55). Additionally, there are other copper-related genes that encode membrane proteins that are thought to be involved in transport; however, the functions of these proteins have not been clearly defined. For example, CopB and CopD, which are also part of the *cop* operon of *P. syringae*, may be copper transport proteins. CopD is a predicted inner membrane protein; expression of CopCD leads to copper hypersensitivity, thus leading to the prediction that these proteins function in copper uptake (25). CopB is an outer membrane protein that possibly plays a role in copper efflux (24).

Chaperones

Copper chaperone proteins facilitate movement of copper ions among other copper-binding proteins, which leads to more efficient copper mobilization and likely more efficient copper efflux in the presence of dangerous excess ions. These functions are difficult to demonstrate biochemically, and thus remain less understood as compared to other copper resistance mechanisms. However, some examples include CopC, PcoC, CusF and CopZ. CopC is encoded as part of the *cop* operon of *P. syringae*, and is a periplasmic protein (25). While the absolute function of CopC is uncertain, expression of *copC* or *copCD* leads to copper hypersensitivity, leading to the suggestion that CopC may play a role in copper uptake rather than copper resistance in *P. syringae* (25). In *E. coli*, PcoC, a homolog of CopC, has also been demonstrated to be a periplasmic protein that binds one equivalent of copper (112). Furthermore, mutation of *pcoC* in *E. coli* leads to copper sensitivity, indicating a role in copper resistance (112). CusF, encoded alongside the CusCBA efflux pump in *E. coli*, is a periplasmic chaperone protein that receives

copper from the copper ATPase CopA and supplies it to CusB, the periplasmic portion of the CusCBA pump (15; 152). Finally, another intriguing copper chaperone protein was very recently recognized in *E. coli*, CopZ. CopZ was not previously identified because it is encoded by the same gene as the copper ATPase, CopA, but in a different frame (129). CopZ contributes to copper resistance independently of CopA (44). Overall, copper chaperones play an important role in copper resistance by binding ions to prevent reactivity and by facilitating movement of these ions to efflux proteins.

Regulatory Proteins

Copper-responsive gene regulation is complex and has been studied in many bacteria (163). Eight unique classes of regulatory proteins have been characterized, and four are copper-responsive transcriptional activators. Two of these four, classes one and two, have been well studied in *E. coli* and are routinely identified in many bacteria. CueR, a class one regulator, is a cytoplasmic regulatory protein that directly binds copper ions. CusRS, a class two regulator, is a two-component system that senses periplasmic copper. While class one regulators like CueR seem to be limited to proteobacteria, copper-responsive two-component systems have been identified in multiple bacterial phyla (163).

Some of the earliest understanding of copper-dependent regulation came with the discovery of CopRS in *P. syringae* in 1993; the genes encoding these proteins are found in the plasmid-encoded *cop* operon (133). The *copRS* genes were demonstrated to be necessary for the copper-dependent induction of the *cop* operon and were predicted to encode a two-component system (133). Soon after, the PcoRS and CusRS systems were

identified in *E. coli* as well (138; 166); PcoRS is a plasmid-based homolog, while CusRS is chromosomally encoded. The PcoRS and CusRS systems appear to operate independently (138).

In addition to regulation by two-component systems, bacteria classically upregulate copper-related genes with CueR, a cytoplasmic transcription factor that activates expression when it is copper bound. In *E. coli*, CueR directly upregulates expression of *copA* and *cueO* (151; 164; 192). A consensus binding sequence for CueR was identified in *E. coli* via alignment of regulated promoter sequences (138). Nearly 200 CueR binding sites have been identified in the *E. coli* genome; though these interactions have not been confirmed biochemically, these results suggest that CueR may be a more global regulator (228).

Other Copper Resistance Mechanisms

It is important to note that species-specific copper resistance proteins have been identified that have no obvious homologs in *A. baumannii*. While these will not be discussed in detail, it is worth noting that these unique copper resistance genes may have unexpected roles with broad implications among pathogenic bacteria. For example, one novel mechanism that was recently discovered is particularly interesting because it broadly affects metal homeostasis during infection and may be more universal than currently recognized. Siderophores, high-affinity iron capturing molecules, are used to acquire iron. These molecules are known to be important during infection because the host sequesters essential iron from pathogens (153). While not previously known to be related to metals other than iron, it was recently recognized that yersiniabactin, a

siderophore of uropathogenic *E. coli*, can also bind copper and protect against copper toxicity (28). Importantly, copper-bound siderophore was detected in the urine of human patients and experimentally infected mice (28). A copper-dependent role for yersiniabactin in virulence was also identified due to the fact that yersiniabactin-expressing strains survived better in copper-loaded macrophages than mutant strains (29). This novel mechanism of copper resistance warrants further research as it may also be an important virulence component of other bacterial pathogens that encode siderophores. Furthermore, it is intriguing to speculate that siderophore regulation may extend beyond the iron-dependent Fur regulation that typifies many of the characterized systems.

***Acinetobacter* Copper Resistance**

Previous studies have assessed copper resistance of various *Acinetobacter* isolates. For example, a panel of *Acinetobacter* isolates examined in the early 1990s was assessed for copper resistance using minimal inhibitory concentration (MIC) assays; 74% (29/39) of the isolates grew on the highest concentration of copper tested (10 mM), and the other 26% (10/39) of isolates were inhibited by the next highest concentration tested, 1 mM (39). To compare the copper resistance of *Acinetobacter* to other pathogens, it would be very useful to compare these results to those of other studies; however, this is virtually impossible since differences in study methodology have significant impact on the results. For example, the utilized growth medium can greatly impact the obtained results; copper toxicity is dependent on free, unbound copper ions, and therefore the complexity of the medium and the presence and concentration of copper-binding components in the medium is very important. The MIC of copper for a pathogenic strain

of *P. aeruginosa*, PAO1, increases from 100 μ M in minimal medium to 6 mM in rich medium (195). This large change is presumably due to the fact that only a small percentage of copper ions that are added to a complex medium remain unbound and reactive. To this end, it is important to note that the above-mentioned *Acinetobacter* MIC assays were carried out using the agar dilution method in GC agar containing 5% yeast extract; the complexity of the medium is likely why the MICs are in the millimolar range. MICs in the millimolar range have been reported for other pathogens when complex medium is used, for example: 5 or 16 mM, *P. aeruginosa* PAO1 (176; 195); 6 mM, *E. coli* clinical isolates (40); 4 mM, *E. coli* K-12 W3110 (112); >2.8 mM, *Salmonella* Typhimurium SL1344 (3). In contrast, inhibition of growth often occurs in the micromolar range when minimal media are used (70; 149; 195; 216).

In terms of copper-related proteins, previous studies have used the protein sequences of known copper-related factors to search for homologous proteins in other bacterial genomes, including *Acinetobacter*. For example, a robust 2012 study used the amino acids sequences of many *E. coli* proteins (CopA, CueO, CusABCF, PcoABCDE, YebZ, CutF) as the query to search 268 gammaproteobacterial genomes (79). Seven *Acinetobacter* genomes were included in the study; six were *A. baumannii* strains and one was a commonly used *A. baylyi* isolate. Intriguingly, there was observable heterogeneity in the membership of the copper-related protein homologs identified in the strains (summarized in Table 2). All of the chosen isolates carry a homolog of the copper ATPase CopA and the RND family efflux proteins CusABC. Most strains encoded the copper oxidase PcoA and the putative transporter PcoB. However, only a few of the strains encoded homologs of the chaperone CusF, the putative chaperone PcoC, and the

Table 2. Homologs of known copper-related proteins identified in the genomes of *Acinetobacter* isolates by Hernandez-Montes *et. al.* in 2012

	<i>A. baumannii</i> ^a						<i>A. baylyi</i>
	AB0057 ^b	AB307-0294	ACICU	17978	AYE	SDF	ADP1
CopA	+	+	+	+	+	+	+
CueO							
CusA	+	+	+	+	+	+	+
CusB	+	+	+	+	+	+	+
CusC	+	+	+	+	+	+	+
CusF				+			
CueP							
PcoA	+	+	+	+	+		+
PcoB	+	+	+	+	+		+
PcoC	+			+	+		
PcoD							
PcoE							
YebZ							
CutF						+	

^aTwo *Acinetobacter* species were analyzed: *A. baumannii* and *A. baylyi*.

^bSix total strains were analyzed, five *A. baumannii* and one *A. baylyi*.

^cA “+” indicates that a homolog was identified in the genome of the indicated strain using the Bidirectional Best Hit criterion; a blank cell indicates that there was no hit.

putative outer membrane protein CutF. The authors noted that these membership patterns were similar among the Pseudomonadales, which includes *Acinetobacter* and *Pseudomonas*, and the Xanthomonadales, which together represent a diverse group of marine and soil bacteria. The core copper-related proteins in these organisms include CopA, PcoAB, and CusABC (79). Beyond the studies described in this thesis, the only other molecular work on *A. baumannii* copper resistance genes was published this year and focused specifically on the copper ATPases in strain AB5075 (7). AB5075 has two P_{1B-1} ATPases, which were referred to as CopA and CueA in the published study but are referred to CopA1 and CopA2 within this thesis; CopA is subtyped as classical with two CxxC motifs, while CueA has one CxxC and seven histidine residues. In the published study, only CopA contributed to *in vitro* copper resistance of AB5075; the mutant strain demonstrated reduced growth in the presence of 1 mM copper sulfate (7). Based on homology to proteins of *E. coli* or *P. aeruginosa*, the researchers also identified additional putative copper resistance factors in AB5075, including copper oxidases (CueO, PcoA), chaperones (CopZ, CopC), transporters (CopB, PcoB, CzcX, CopD), and regulators (CueR, CopRS) (7). The roles of these additional proteins in copper resistance were not investigated. Clearly, much more work is needed to understand the mechanisms of copper resistance in *A. baumannii*.

Contribution of Copper Resistance to Virulence

Copper resistance genes have been shown to contribute to virulence of many bacterial and fungal pathogens; these studies are summarized in Table 3. While the

Table 3. Copper-related proteins with demonstrated roles in virulence

Protein	Organism	Model	Phenotype ^a	Year	Reference
Copper ATPases					
CueA/CopA1	<i>Pseudomonas aeruginosa</i>	Mouse, IP injection	Reduced LD ₅₀ and competitive index	2005	(176)
		Leaf infection	Reduced CFU/leaf punch	2010	(64)
CopA2	<i>P. aeruginosa</i>	Leaf infection	Reduced CFU/leaf punch	2010	(64)
CopA	<i>Streptococcus pneumoniae</i>	Mouse, intratracheal (systemic)	Increased survival of mice, decreased burden in lungs and blood	2011	(97; 180)
		Lung epithelial cells Macrophages	Reduced adherence and invasion Reduced survival		
		Mouse, intranasal	Increased survival of mice, reduced burden in nasopharynx	2015	(97)
CopA	<i>Neisseria gonorrhoeae</i>	Cervical epithelial cells	Attenuated intracellular replication	2012	(42)
CopA	<i>E. coli</i>	Macrophage	Reduced intracellular survival	2009	(220)
CopA/ CopA1	<i>A. baumannii</i>	Mouse, intranasal	Reduced CFU in nasal wash and lung	2019	(7)
CopA	<i>Campylobacter jejuni</i>	Chicken, oral	Slightly reduced CFU in cecal samples	2018	(60)
CueA	<i>Pseudomonas fluorescens</i> SBW25	Sugar beet root	Reduced competitiveness	2007	(234)
CopA & GolT (double mutant)	<i>Salmonella enterica</i> sv. Typhimurium	Macrophage	Reduced intracellular survival	2010	(149)
		Mouse, oral	No difference in liver or spleen burden		
CtpA	<i>Listeria monocytogenes</i>	Mouse, IV infection	Reduced liver burden	1997	(54)
CopX	<i>Staphylococcus aureus</i>	Macrophage	Reduced intracellular survival	2018	(162)
CopB	<i>S. aureus</i>	Macrophage	Decreased intracellular survival	2018	(162) (232)
		Human blood	Decreased survival	2018	(232)
CtpV	<i>Mycobacterium tuberculosis</i>	Guinea pig, aerosol	Decreased bacterial burden and lung pathology	2010	(216)
		Mouse, aerosol	Increased survival of mice; no change in burden, reduced lung pathology		

CrpA	<i>Aspergillus fumigatus</i>	Macrophage Mouse, intranasal	Reduced intracellular survival Increased survival of mice; reduced bacterial burden in lung	2017	(221)
Other Efflux Proteins					
MctB	<i>M. tuberculosis</i>	Mouse, aerosol Guinea pig, aerosol	Lower bacterial burden in lung Lower bacterial burden in lung and lymph node; fewer lesions	2011	(225)
CusSRCFBA	Uropathogenic <i>E. coli</i>	Mouse, ascending UTI	Reduced competitive index (decreased CFU) in the bladder and kidney	2014	(193)
Copper Oxidases					
CueO	<i>S. Typhimurium</i>	Mouse, oral Macrophage	Lower bacterial burden in liver and spleen No difference in survival	2010	(3)
CueO	Uropathogenic <i>E. coli</i>	Mouse, UTI Mouse, systemic	Increased bacterial burden in the bladder and urine No differences	2008	(203)
CueO	<i>C. jejuni</i>	Chicken, oral	Reduced CFU in cecal samples	2018	(60)
Mco	<i>S. aureus</i>	Macrophage Human blood	Decreased intracellular survival Decreased survival	2018	(232)
CopA	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	Cabbage leaves	Reduced leaf pathology	2011	(83)
Chaperones					
CopZ	<i>Streptococcus mutans</i>	<i>In vitro</i> competition	Decreased ability to inhibit growth of commensal streptococci	2016	(57)
Transcription Factors					
AceA	<i>A. fumigatus</i>	Macrophage Mouse, intranasal	Reduced survival of spores; reduced intracellular survival Increased survival; reduced bacterial burden in lung	2017	(221)
AfMac1	<i>A. fumigatus</i>	Mouse, intranasal Macrophage- and	Increased survival of mice, reduced lung pathology Decreased intracellular survival	2017	(154)

		neutrophil-like cells			
RicR	<i>M. tuberculosis</i>	Mouse, aerosol	Reduced bacterial burden in lung	2014	(182)
CopR	<i>P. fluorescens</i>	Japanese flounder, IP	Reduced CFU in liver	2009	(84)
CopRS	<i>Xanthomonas citri</i> subsp. <i>citri</i>	Grapefruit leaves	Reduced disease in leaves; reduced CFU/leaf punch	2011	(229)
Other or Unknown Functions					
CopL	<i>S. aureus</i>	Macrophage	Reduced intracellular survival	2018	(162) (232)
Ybt (siderophore)	Uropathogenic <i>E. coli</i>	Macrophage	Reduced intracellular survival	2014	(29)
Ton-B Cu receptor	<i>A. baumannii</i> 19606	Human serum Human alveolar epithelial cells Mouse, intranasal	Reduced survival Reduced adherence Increased survival of mice, reduced bacterial burden in lung and spleen	2018	(1)

^aDescriptions provided refer to the characteristics of the mutant strain relative to the parent strain. Additional publications report no attenuation of virulence in strains bearing mutations of copper-related genes in the chosen infection model: *Listeria monocytogenes* copper ATPase (34), *S. aureus* copper ATPase (162) (232), *Vibrio cholerae* copper ATPase (124), *S. pneumonia* copper chaperone (97), *V. cholerae* CopG and Cot (unknown functions) (124).

majority of the studies looked at the role of a copper ATPase protein, other efflux proteins, oxidases, chaperones, and transcription factors have also been studied.

While the importance of copper resistance to pathogenesis has been recognized in many bacterial pathogens, until the above referenced 2019 *A. baumannii* study, nothing was known about this topic in *A. baumannii*; the study showed that a CopA1 (copper ATPase) mutant strain of *A. baumannii* demonstrated reduced colonization in a murine model of respiratory infection (7). Bacterial burden in nasal washes, bronchoalveolar lavage, and in lung tissue was reduced ~2 logs as compared to wild-type levels (7). In addition, one other recent study reported the importance of a TonB-dependent copper receptor protein for adherence to host cells; given the adherence phenotype, any potential role in virulence is likely not metal resistance-related (1). Clearly, more work is required to determine which other *A. baumannii* copper-related proteins contribute to copper resistance and/or to virulence and to determine if these proteins can be targeted therapeutically.

Linking Virulence Phenotypes to Copper

In all the studies listed in Table 3, attenuation of a strain bearing a mutation in a particular copper-related gene was demonstrated. Given the *in vitro* role of these genes in copper resistance, it is presumed that these genes are important for virulence because they contribute to evasion of host copper-mediated immune mechanisms *in vivo*. Importantly, a few studies have performed additional experiments to confirm that the *in vivo* attenuation was actually dependent on copper-mediated immune mechanisms. For example, two studies found that copper sensitive mutant strains become more attenuated

when the infected animals are given additional dietary copper. Specifically, a *C. jejuni* *cueO* mutant strain displayed ~100x reduced CFU/gram in cecal samples relative to the wild-type strain, and recovery was reduced another 10x when chickens were fed feed supplemented with copper (60). Similarly, a copper efflux (*mctB*) mutant of *M. tuberculosis* demonstrated reduced bacterial burden in the lungs of infected mice relative to the wild-type strain; a ~1 log reduction in CFU was sustained throughout 100 days of infection (225). However, when mice were given water supplemented with copper, the attenuation was exacerbated, and the difference in CFU between the mutant and wild-type strains increased to 2 logs (225). Interestingly, this study also showed that infected granulomatous tissue from the lungs has higher concentrations of copper than the surrounding tissue, demonstrating that, on a larger scale, copper is mobilized to the site of infection (225). These important studies confirm that the level of copper in the host contributes to the degree of importance of copper resistance seen in particular pathogens.

The opposite approach, reducing copper-mediated killing in the host, has also been utilized to directly link attenuated virulence to copper sensitivity. As mentioned previously, host immune cells use their own copper ATPase, ATP7A, to mobilize copper to the phagosomal compartment to kill engulfed pathogens. A few studies have used cell lines or animals lacking functional ATP7A to demonstrate that copper resistance of the pathogen is important for evasion of host copper-mediated immunity, and thus for virulence overall. For example, an *E. coli* *copA* mutant strain is more sensitive to copper *in vitro* and more sensitive to killing by macrophages than the wild-type strain. However, in macrophages where ATP7A has been knocked down with RNAi, survival of *copA* mutant bacteria is the same as wild-type (220). In a more recent study by the same

research group, the same phenomenon was reported for *Salmonella* Typhimurium (108): in macrophages lacking ATP7A, bacterial survival was increased. Fluorescence microscopy with a copper probe showed that copper loading of the phagosomes was impaired in the ATP7A-deficient cells. Furthermore, when the genes encoding the bacterial copper ATPases CopA and GolT were deleted, bacterial survival was reduced in wild-type macrophages, but not in ATP7A-deficient macrophages (108). Taken together, these experiments convincingly demonstrate that copper resistance is important for bacterial pathogens to survive in macrophages because of copper trafficking to the phagosome.

Finally, ATP7A knockout mice were also used to demonstrate that the attenuation of the copper sensitive strain of *Salmonella* was ATP7A dependent. In competitive infections of the *copA/golT* deletion mutant of *Salmonella*, the copper sensitive mutant is outcompeted ~2:1 by wild-type bacteria in both the liver and spleen in wild-type mice. However, in the ATP7A knockout mice, the competitive defect is lost, indicating that *copA* and *golT* are not important for the ability of *Salmonella* to cause infection in mice that are not able to mobilize copper properly (108). These very important studies demonstrate that when the host is utilizing ATP7A to mobilize copper to the phagosome as a means to kill the engulfed pathogen, copper resistance is important for pathogenesis.

HARNESSING THE ANTIMICROBIAL POWER OF COPPER

Copper Surfaces for Decontamination

Many hospital-acquired infections are caused by environmental contamination. *A. baumannii* and other multidrug-resistant, nosocomial pathogens, including *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* spp. (VRE), persist on dry surfaces for long periods of time. This ability provides the opportunity for transmission of these pathogens among patients via contact with fomites. Thus, enhanced decontamination strategies are important for reducing the microbial burden of the environment. Excitingly, copper provides a continuously self-cleaning option and solid, dry, copper alloys have been shown to efficiently eliminate many bacteria, fungi, and viruses, including pathogenic *A. baumannii* (47; 126; 189; 209). Indeed, the use of copper surfaces has been demonstrated many times to be an effective strategy for limiting environmental contamination of healthcare facilities, including walk-in clinics, ICUs, and long-term care facilities (23; 31; 102; 123; 131; 171-173).

A few studies have demonstrated that, in addition to decreasing environmental contamination, the use of copper-containing surfaces also reduces the rate of nosocomial infection (137). In an important randomized controlled trial, alternating rooms in the ICUs of three hospitals were outfitted with copper coatings on many high-touch surfaces, including bed rails, IV poles, tray tables, and guest chair rails (168). Excitingly, the number of healthcare-acquired infections was reduced in the copper-containing rooms by 58% as compared to the adjacent control rooms; importantly, a strong correlation between microbial burden and infection rate was observed (168). In a follow-up study in a pediatric care unit, reduction in infections was also observed, though to a lesser degree

(19%); this difference may be due to shorter stays and lower baseline infection rates (210).

Advanced Copper Technology

In addition to the use of solid copper alloys, there has been a great deal of recent work investigating the effectiveness of copper-impregnated materials (145; 209). These technologies expand the possibilities for the use of copper-mediated bactericidal activity beyond metal-coated surfaces to plastics, textiles, and other molded copper-composite hard surfaces. For example, the efficacy of copper-impregnated surfaces and textiles in reducing environmental contamination and microbial burden was recently investigated in a large hospital in Norfolk, VA (185). Upon the construction of a new hospital wing, a variety of copper-impregnated composite hard surfaces were installed, including sinks, vanities, desks, and nurses' workstations. In addition, copper-impregnated linens were deployed, including patient gowns, bed sheets, bath towels, and blankets. The new wing observed 68% fewer infections from multidrug-resistant organisms and 83% fewer cases of *C. difficile* infection (185); the rates of multidrug-resistant *A. baumannii* infection in this hospital were very low (<1%) throughout the study period. It is worth noting that copper-containing textiles have been demonstrated to be effective against multidrug-resistant *A. baumannii* strains (92). Furthermore, copper-containing wound dressings are being developed and have been shown to enhance wound healing (21) and to reduce biofilm formation by *S. aureus* and *P. aeruginosa* in wounds (5). Further work will be needed to determine if copper-containing wound dressings could aid in healing of *A. baumannii*-infected wounds.

GOALS AND SPECIFIC AIMS

A. baumannii is an important emerging, nosocomial pathogen that is known for its extensive drug resistance. Novel therapeutics are urgently needed to treat patients infected with multidrug-resistant *A. baumannii*. Additionally, novel treatments must take current antibiotic resistance into account. Thus, identification of virulence factors in *A. baumannii* may reveal novel, less conventional drug targets. Furthermore, accumulating evidence suggests that copper resistance contributes to virulence of many bacterial pathogens. The goal of this dissertational work was to identify and analyze copper resistance mechanisms in *A. baumannii* and to determine if they contribute to virulence. To begin to understand these processes we (1) characterized copper resistance in a panel of *A. baumannii* clinical isolates, and (2) assessed the contribution of individual proteins to *in vitro* copper resistance and to virulence. To address aim one, we obtained a panel of recent clinical isolates from patients at Walter Reed National Military Medical Center. We assessed the ability of each strain to grow in the presence of increasing concentrations of copper; we found that there was diversity among strains in the degree of resistance to copper. Intriguingly, when we searched the genomes of these isolates for putative copper-related genes, we found that the highly resistant isolates possess more genes with putative copper resistance functions. Furthermore, we demonstrated that expression of the majority of the identified copper-related genes increases upon copper exposure, strongly suggesting a role in the response to copper. Importantly, we also demonstrated that all of the isolates were rapidly killed on copper-containing metal surfaces, and that copper ions are also bactericidal against *A. baumannii* biofilms.

To address aim two, we utilized an arrayed transposon mutant library in the modern clinical isolate AB5075. We obtained strains with insertions in each of the identified putative copper-related genes, and we performed similar growth experiments to assess their ability to grow in the presence of copper. We found eight strains that displayed a copper sensitive phenotype. We built complemented derivatives of each of the eight strains and observed restoration of growth in copper for most strains. Furthermore, we were able to increase copper resistance of other clinical isolates through plasmid-based expression of additional copper-related genes from AB5075. Through measurements of intracellular copper levels, we found that many mutant strains accumulated more copper than the wild-type strains, suggesting defects in copper efflux. Crucially, we demonstrated that copper resistance contributes to virulence in two infection models. We found that all eight mutant strains were attenuated for virulence in a *Galleria mellonella* caterpillar infection model. Subsequently, we tested two mutant strains in a murine pneumonia model, and we observed that both were also attenuated in this mammalian model.

In the following chapters, we present the characterization of copper resistance in a panel of *A. baumannii* clinical isolates and detail the identification of putative copper-related genes (chapter 2). Furthermore, we show that individual copper resistance genes contribute to both *in vitro* copper resistance and to *in vivo* virulence (chapter 3). Although other studies have measured growth of *A. baumannii* isolates in the presence of copper or have searched for homologs of copper-related proteins, this is the first comprehensive analysis of copper resistance in *A. baumannii*; also, this is the first work to demonstrate a role in virulence in any bacterium for a number of copper-related proteins. Given the

need for novel therapeutic targets in multidrug-resistant *A. baumannii*, work such as the studies presented in this dissertation will provide a better understanding of the biology of *Acinetobacter* infection, and may aid in the development of new treatments for patients infected with drug-resistant “superbugs.”

CHAPTER 2: Copper Resistance of the Emerging Pathogen *Acinetobacter baumannii*

Published as: **Caitlin L. Williams, Heather M. Neu, Jeremy J. Gilbreath, Sarah L. J. Michel, Daniel V. Zurawski, and D. Scott Merrell. 2016.** Copper Resistance of the Emerging Pathogen *Acinetobacter baumannii*. *Appl Environ Microbiol* **82**: 6174-6188.

The work presented in this chapter is the sole work of C. L. Williams with the following exception: H. M. Neu and S. L. J. Michel conducted the ICP-MS measurements.

ABSTRACT

Acinetobacter baumannii is an important emerging pathogen that is capable of causing many types of severe infection, especially in immunocompromised hosts. Since *A. baumannii* can rapidly acquire antibiotic resistance genes, many infections are on the verge of being untreatable, and novel therapies are desperately needed. To investigate the potential utility of copper-based antibacterial strategies against *Acinetobacter* infections, we characterized copper resistance in a panel of recent clinical *A. baumannii* isolates. Exposure to increasing concentrations of copper in liquid culture and on solid surfaces resulted in dose-dependent and strain-dependent effects; levels of copper resistance varied broadly across isolates, possibly resulting from identified genotypic variation among strains. Examination of the growth-phase-dependent effect of copper on *A. baumannii* revealed that resistance to copper increased dramatically in stationary phase. Moreover, *A. baumannii* biofilms were more resistant to copper than planktonic cells but were still susceptible to copper toxicity. Exposure of bacteria to subinhibitory

concentrations of copper allowed them to better adapt to and grow in high concentrations of copper; this copper tolerance response is likely achieved via increased expression of copper resistance mechanisms. Indeed, genomic analysis revealed numerous putative copper resistance proteins that share amino acid homology to known proteins in *Escherichia coli* and *Pseudomonas aeruginosa*. Transcriptional analysis revealed significant upregulation of these putative copper resistance genes following brief copper exposure. Future characterization of copper resistance mechanisms may aid in the search for novel antibiotics against *Acinetobacter* and other highly antibiotic-resistant pathogens.

Importance

Acinetobacter baumannii causes many types of severe nosocomial infections; unfortunately, some isolates have acquired resistance to almost every available antibiotic, and treatment options are incredibly limited. Copper is an essential nutrient but becomes toxic at high concentrations. The inherent antimicrobial properties of copper give it potential for use in novel therapeutics against drug-resistant pathogens. We show that *A. baumannii* clinical isolates are sensitive to copper *in vitro*, both in liquid and on solid metal surfaces. Since bacterial resistance to copper is mediated through mechanisms of efflux and detoxification, we identified genes encoding putative copper-related proteins in *A. baumannii* and showed that expression of some of these genes is regulated by the copper concentration. We propose that the antimicrobial effects of copper may be beneficial in the development of future therapeutics that target multidrug-resistant bacteria.

INTRODUCTION

Acinetobacter baumannii, a Gram-negative aerobe, is an important emerging pathogen in the United States and worldwide. Most *Acinetobacter* species are ubiquitous environmental bacteria, and the ability to survive in harsh, dry environments likely aided establishment of *A. baumannii* as a prominent nosocomial pathogen (156). Today *A. baumannii* is also nearly ubiquitous in hospital environments, especially in intensive care units (ICUs). *A. baumannii* is capable of causing many types of infections, including pneumonia, urinary tract infections, bacteremia, skin and soft tissue infections, osteomyelitis, and even meningitis (156). Typically *A. baumannii* causes serious disease only in immunocompromised individuals. However, the lack of a suitable treatment against antibiotic-resistant strains can lead to more severe disease and higher mortality (128; 159; 194). Because of the high prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, the CDC has classified *A. baumannii* as a “serious” threat (160).

Despite its relatively recent introduction into hospitals in the United States and countries in South America and Europe, *A. baumannii* is responsible for a significant proportion of hospital-acquired infections. A recent meta-analysis of studies carried out worldwide reported that *A. baumannii* consistently causes 5 to 10% of hospital-acquired infections and an even greater proportion of ICU-acquired infections (198). However, depending on patient demographics and the strains present, the proportion of certain infections caused by *A. baumannii* can be much larger than 5 to 10%. For example, a retrospective study of ICU-acquired infections in the ICU of the National Cancer Institute of Mexico reported that *A. baumannii* was the causative agent of 6% of total hospital-acquired infections but disproportionately represented 60% of pneumonia and 25% of

bloodstream infections (35). *A. baumannii* has rapidly evolved into a difficult-to-treat pathogen through acquisition, mostly via integrons and transposons, of multiple drug resistance genes that supplement the already high level of intrinsic drug resistance (157). A recent study analyzing more than 1,300 globally collected bloodstream isolates included over 150 *A. baumannii* isolates (194). Of these clinical strains, 91.9% were MDR, 71.3% were XDR, and 1% were pandrug-resistant (PDR; resistant to all antibiotic treatments). Worldwide, XDR and PDR strains limit the treatment options for physicians, which can lead to empirical therapy and complications. Not surprisingly, the mortality rate of infection correlates with the level of drug resistance of the infecting strain and whether or not the patient was given appropriate antimicrobial therapy (128; 159; 194). The extensive drug resistance in *A. baumannii* and other “superbugs” has prompted calls for more atypical antibacterial approaches that take into account the present resistance to most classes of antibiotics.

Most antibiotics target essential functions for bacterial growth and/or survival. Metal homeostasis is a vital process that provides an extensive list of potential new antibiotic targets. Copper is required for cellular function, e.g., for redox balance and as an enzyme cofactor. However, copper ions become toxic at high concentrations; these ions participate in Fenton chemistry to produce hydroxyl radicals that react with and damage essential biomolecules (114). Additionally, copper ions damage iron-sulfur cluster proteins by binding with sulfur and displacing iron (119). Recent studies in *E. coli* and *Salmonella* spp. showed that when bacteria are placed on copper surfaces, outer membrane integrity is compromised, hydroxyl radicals are produced, respiration is inhibited, and DNA is degraded (217). Because of the need for new therapeutics to treat

highly antibiotic-resistant pathogens, research into the use of copper as an antimicrobial has recently increased. Various pathogens have been assessed for their levels of copper sensitivity *in vitro*; unfortunately, these values are difficult to compare due to protocol differences, namely, the growth medium used. However, it is evident that among the pathogens tested, the concentration of copper required for successful inhibition or elimination in minimal medium is typically within the micromolar range (70; 149; 195; 216). Clinical isolates of *A. baumannii* have been assessed for their overall levels of copper resistance; however, previous experiments used a rich medium and reported MICs in the millimolar range, similar to those for other pathogens tested in rich medium (3; 39; 55; 195). Thus, these MICs may be influenced by medium choice.

Variation in the inhibitory concentration of copper ions among pathogens and across individual strains is likely due to differences in the proteins present in the organisms that sense or modulate copper levels and/or their regulation. The details of copper homeostasis mechanisms that are critical to copper resistance have been investigated in some bacterial species. Among these, the *E. coli* copper resistance system is likely the most fully characterized. Generally, copper resistance in bacteria is facilitated by detoxification and export systems (11). For example, in *E. coli*, the copper ATPase CopA effluxes copper ions from the cytosol to the periplasm, where they are oxidized to the less toxic Cu²⁺ form by the copper oxidase, CueO (66; 164). Expression of both *copA* and *cueO* is induced by the transcriptional regulator CueR when it is loaded with copper (151). In addition, a two-component system, CusRS, senses copper ions in the periplasm and directly upregulates expression of an RND family efflux pump that removes copper ions from the cell (55; 70; 104). *E. coli* also encodes the Pco system,

whose contributions to copper resistance are less clear; the system appears to help in the handling of periplasmic copper (112). Although there is extensive knowledge concerning copper homeostasis in *E. coli* and a few other pathogens, little is known about the overall level of copper resistance in *A. baumannii*. Protein sequences of *E. coli* copper-related genes have been used to identify putative homologous proteins in many bacteria, including *A. baumannii* (79), but the importance of these genes to copper resistance has not been investigated experimentally. The goal of this study was to assess the overall level of copper resistance in *A. baumannii* clinical isolates and to begin to investigate potential mechanisms of copper homeostasis in this important pathogen.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains used in this study are listed in Table 4, and primer sequences are shown in Table 5. Bacterial strains were grown overnight at 37°C on 1.5% lysogeny broth (LB) agar plates (MoBio, Carlsbad, CA). Liquid cultures of *A. baumannii* were grown in M9 minimal medium (170) supplemented with 0.1% Casamino Acids (Difco, Franklin Lakes, NJ) at 37°C with shaking at 190 rpm; the amino acid-supplemented medium is referred to throughout the text as simply “M9 medium.” Overnight cultures were started from a single colony and grown for 16 to 19 h. *A. baumannii* strains were maintained as frozen stocks at –80°C in M9 or LB medium supplemented with 40% glycerol.

Table 4. Strains used in this study

Strain	Description, yr of isolation	Source or reference
<i>A. baumannii</i> strains		
ATCC 17978	Common laboratory strain	ATCC
AB0057	Clinical isolate, 2004	(93)
AB4857	Clinical isolate, 2008	(93)
AB5075	Clinical isolate, 2008	(93)
AB5256	Clinical isolate, 2009	(93)
AB5711	Clinical isolate, 2009	(93)
<i>E. coli</i> strains		
K-12 MG1655	Common laboratory strain	(72)

Table 5. Oligonucleotides used in this study

Oligonucleotide use and name	Sequence (5'-3')
Quantitative Real-time PCR	
actP2/copA1 RT up	CCAGATATGAACTCGCTGGTC
actP2/copA1 RT dw	GACCGTGCCTTGTGGTAATA
ABUW_2708 RT up	GTGACTGCAACGATTCAAGATG
ABUW_2708 RT dw	ATCAACGCTTTCCTGCTTTC
ABUW_3322 RT up	AACTCTATTCCGTCGTAGCTTAAC
ABUW_3322 RT dw	GCATCAGCAGCCCAAGA
copR/cusR RT up	GACTGGGTTACAGATGGCTTATC
copR/cusR RT dw	CAGCCATCTAACTTCGGTAACA
actP1/copA2 RT up	AGATGGAGGCACAGTCAAATAG
actP1/copA2 RT dw	GGCAGTTACCGGGATGATTT
copC RT up	GTAGGTGCGACTATGGTTGTAG
copC RT dw	AGATGCATTAATAGCCGGAGTAG
ABUW_0265 RT up	CATGTCCGTCTTATTCTGTTTGC
ABUW_0265 RT dw	CATTTAAAGCTGCATGATGACCA
ABUW_3230 RT up	AATGCGATTGACCCAGTGTAT
ABUW_3230 RT dw	AGCGAGCGACTTTAGCATAAT
ABUW 16S RT up	GGACGGGTGAGTAATGCTTAG
ABUW 16S RT dw	CGTAGGACGTATGCGGTATTAG
Junction PCR	
ABUW_2705 / cueR jxn up	CACCCTGTACCATCTCTAAATCG
ABUW_2705 / cueR jxn dw	TGATTGCTGTGGTGACCAG
cueR / actP2 jxn up	CGCCGAAATACCCGAATGT
cueR / actP2 jxn dw	CAGCAGGTGCGCTATATCC
copB / copA jxn up	TCCATGCTTTGAACATCCGTATC
copB / copA jxn dw	GATGAGTTAGGTGAATGGGCTATTC
copA / ABUW_3322 jxn up	CTGCCATAACCCAAGTCGA
copA / ABUW_3322 jxn dw	CATGGACCATTCTAAGATGACGG
copR / copS jxn up	CGCGGAATGGGGTATGTCTTAG
copR / copS jxn dw	CATGATGGCCAACTAAAGCATC
ABUW_0265 / czcC jxn up	CTTACCAGTCTCCACATCTTAATGC
ABUW_0265 / czcC jxn dw	CATTTGCCATTTGTCCCGC
czcC / czcB jxn up	GTTTACGTCTAGGGCTAGAGC
czcC / czcB jxn dw	TCCCTCATCATGGTGTTCTTC
czcB / czcA jxn up	CAGCTAGGCCAACGTTCTAAAG
czcB / czcA jxn dw	GGAACGGCATCAATAGGGAG
czcA / czcD jxn up	CGATGGATGAATGAAGACAAGACG
czcA / czcD jxn dw	ACCTCAACAATTAAGAATGTCGTGG
copC / copD jxn up	CCACAATGGGTGCTGATGG
copC / copD jxn dw	CAGCCACGTTGCATATATCCAAG
ABUW_3230 / 3229 jxn up	CAAGAAGGTGCATTACAGGTG
ABUW_3230 / 3229 jxn dw	ATGCTGCATAGGTGTCTGAG
ABUW_3229 / pcoA jxn up	CAGCAAGCTGTACCAGATATTG

ABUW_3229 / pcoA jxn dw	CTTAATTGCCGCGAGAGTC
pcoA / pcoB jxn up	CACATGAGTGCTGGAATGATG
pcoA / pcoB jxn dw	CCTGTCCACCATGTTCGC
pcoB / ABUW_3226 jxn up	CAAACCGCTTGGCAAACCTG
pcoB / ABUW_3226 jxn dw	CACCCACAGCTAGAACAGC

Determination of *A. baumannii* Sensitivity to Copper in Liquid Culture

To assess the effect of copper on *A. baumannii* growth, subcultures were started from overnight cultures at an optical density at 600 nm (OD₆₀₀) of 0.05 in 10 mL of M9 medium containing 100 μ M to 1.5 mM CuSO₄ (Sigma-Aldrich, St. Louis, MO). The OD₆₀₀ of the cultures was measured every hour for 6 h. At 0, 2, 4, and 6 h, an aliquot of each culture was serially diluted and plated to enumerate CFU.

Determination of *A. baumannii* Copper Sensitivity on Solid Metal Coupons

The metals tested were stainless steel 304, copper 110 H02 (99.9% Cu), and cartridge brass C260 (~70% Cu) (Online Metals, Seattle, WA). Metal sheets were cut into coupons (1 × 1 × 0.04 to 0.05 cm) and degreased by vortexing in approximately 20 mL of acetone for 30 s. Coupons were stored immersed in absolute ethanol. Prior to use, coupons were flamed and placed in sterile petri dishes. Bacterial subcultures were grown as described above. At 2.5 h (log phase), 6 h (early stationary phase), and 24 h (stationary phase), 5- μ L samples were taken for (i) serial dilution and plating to determine the inoculum (T0) and (ii) spotting onto metal coupons. Dishes containing coupons were incubated on the lab bench at room temperature for up to 75 min. At various times, coupons were transferred to 5-mL screw-cap tubes (Axygen, Corning, NY) containing 3 mL of phosphate-buffered saline (PBS) and mixed in a Bullet Storm 5 blender (Next Advance, Averill Park, NY) on level 1 for 1 min. The samples were serially diluted and plated, and surviving CFU were enumerated. The limit of detection was 3 CFU per coupon.

Quantification of Copper Ions via ICP-MS

To measure the concentration of copper ions found in medium exposed to metal surfaces, droplets were collected and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Similar to the setup of the copper sensitivity assay described above, two 5- μ L droplets of M9 medium were placed on a 1- by 1.5-cm metal coupon for 0, 15, 45, or 75 min. The droplets were collected by running an additional 100 μ L of M9 medium across the surface into a 1.5-mL Eppendorf tube; the total volume was run across the surface two additional times to collect any precipitated ions. The collected sample was immediately vortexed, and 10- to 20- μ L aliquots were removed to separate tubes and frozen at -20°C for later analysis. Aliquots of each preparation of M9 medium were also analyzed. Samples were thawed at room temperature and diluted 1:100 in 2% HNO_3 (trace metal grade). The metal content of the samples was quantitated by ICP-MS. Copper concentrations were determined by injecting diluted samples into an Agilent 7700X ICP-MS instrument (Agilent Technologies, Santa Clara, CA). Copper levels were detected using an octopole reaction system (ORS) cell in He mode to remove interference. The ICP-MS parameters used for the analysis were as follows: a radio frequency (RF) power of 1,550 W, an argon carrier gas flow of 1.01 liters/min, an argon make-up gas flow of 0.1 liter/min, a helium gas flow of 4.3 mL/min, an octopole RF of 200 V, and an OctP bias of -18 V. Samples were directly infused using the model 7700X peristaltic pump with a speed of 0.1 rotation per s (rps) and a micromist nebulizer. Copper concentrations in samples were derived from a calibration curve generated by a series of dilutions of a copper atomic absorption standard (Fluka Analytical, St. Louis,

MO) prepared in the same matrix as the samples. Data analysis was performed using Agilent's Mass Hunter software.

Assessing Copper Sensitivity of *A. baumannii* Biofilms

Biofilms of *A. baumannii* strain AB5711 were grown in 1 mL of LB broth in 24-well tissue culture-treated plates (Corning, Corning, NY). Cultures were started at an OD₆₀₀ of 0.05 and incubated statically at 37°C for 18 h. Afterwards, the LB broth was gently removed and replaced with 1.5 mL of M9 medium containing up to 1.5 mM CuSO₄. The biofilms were incubated for another 6 to 24 h. At each time point (0, 6, and 24 h), the M9 medium was transferred to a 1.5-mL Eppendorf tube and vortexed for approximately 5 s to break up any clumped planktonic bacteria in the supernatant. Additionally, the biofilm was scraped from the sides and bottom of the well by use of a pipette tip and resuspended in 1 mL of PBS. Serial dilutions of both planktonic cells (supernatant) and biofilms were plated and CFU were enumerated. The wells were stained with crystal violet following processing to ensure that the biofilms were recovered equally from all wells.

Identification of Putative Copper-related Genes in *A. baumannii*

Initially, amino acid sequences from copper-related proteins in *E. coli* and *Pseudomonas aeruginosa* were used as queries for Protein BLASTp (NCBI) searches to identify putative copper-related genes in *A. baumannii* AB5075-UW. To determine the ultimate sequence similarity and identity between proteins in *A. baumannii*, *E. coli*, and *P. aeruginosa*, AB5075-UW (accession no. NZ_CP008706.1) proteins were compared to

those encoded in the reference genomes of *E. coli* and *P. aeruginosa*, namely *E. coli* K-12 MG1655 (accession no. NC_000913.3) and *P. aeruginosa* PAO1 (accession no. NC_002516.2). The identified amino acid sequences encoded by putative copper-related genes from *A. baumannii* AB5075-UW were entered as queries for BLASTp searches, and *E. coli* K-12 MG1655 (taxid 511145) and *P. aeruginosa* PAO1 (taxid 208964) were chosen individually as the search set. The following parameters were used: word size, 6; maximum number of matches in a query range, 0; BLOSUM62 matrix with gap costs of 11 for existence and 1 for extension; composition-based statistics; and automatic adjustments for short input sequences. To determine if each putative copper-related gene was present in the other *Acinetobacter* strains of the panel, the amino acid sequences from AB5075-UW were again used as queries, and each isolate was chosen as the search set. The same search parameters were used. Note that there are two locus numbers for each gene of AB5075-UW. In addition to the original annotation, there was a subsequent reannotation by GenBank that occurred very recently. Both identifiers are given in Table 7 and are available under accession no. NZ_CP008706.1. Based on the timing of the reannotation, we used the original annotation throughout this study.

RNA Extraction, cDNA Synthesis, and qRT-PCR

A large subculture of *A. baumannii* cells was grown to exponential phase (2.5 h) as described above and then aliquoted in 10-mL volumes into 25-mL flasks. Various concentrations of CuSO₄ (2.5 to 500 μ M) were added directly to the cultures, and the cells were incubated with shaking for an additional 30 min. Cells were harvested by centrifugation at room temperature, and RNA was isolated using TRIzol reagent

(Invitrogen, Carlsbad, CA) as described previously (175), with the exceptions that the aqueous phase was used directly in an RNeasy cleanup protocol (Qiagen, Hilden, Germany) and a 60-min DNase (Qiagen) digestion was performed during the cleanup. cDNA was synthesized using a Quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions and as previously described (63). Briefly, quantitative reverse transcriptase PCR (qRT-PCR) was performed using a Roto-gene Q instrument (Qiagen), 1× SYBR green RT-PCR master mix (Qiagen), 3 pmol each of the forward and reverse primers, and 1 µL of cDNA. A reaction mixture in which no reverse transcriptase enzyme was added was included as a no-RT control. Cycling conditions were as follows: an initial activation step for 5 min at 95°C and 35 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 10 s. SYBR green fluorescence was measured during each extension step. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, using the 16S rRNA gene as the internal reference.

Confirmation of Operonic Structure by Junction PCR

To detect each of the operon gene junctions, qualitative RT-PCR was conducted using GoTaq master mix (Promega, Madison, WI) per the manufacturer's instructions. Junctional PCRs were performed using 0.5 µL of cDNA, synthesized as described above, as the template. Chromosomal DNA was used as a positive control (0.5 µL; ~100 ng), and the original RNA sample was used as a negative control (0.5 µL; 150 to 200 ng). Each 25-µL reaction mixture was incubated for 5 min at 95°C, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. PCR products were separated in 1% agarose gels and visualized by staining with

ethidium bromide. Because the genomic DNA (gDNA) template produced more PCR product, a smaller volume was loaded on the gel in order to visualize all bands well. The gel was loaded with the following amounts of PCR products: gDNA, 1 μ l; RNA, 20 μ l; and cDNA, 1 to 20 μ l.

Data Analysis and Statistics

All graphs and statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Brown-Forsythe and Bartlett's tests were used to test for equality of variances, and graphical methods were used to assess normality. Welch's correction was used in the case of unequal variances, and nonparametric methods were used in the case of nonnormal data. Two-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to evaluate differences in growth for various copper treatments, using log-transformed data. Data are presented as geometric means plus standard errors of the means (SEM). The Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare surviving CFU on the coupons at each time point; data show numbers of CFU per coupon or percent survival and are presented as arithmetic means and interquartile ranges. One-way ANOVA with Welch's correction followed by linear contrasts with Bonferroni adjustment was used to compare biofilm and planktonic cell percentages of survival at each concentration and time point. Data are presented as arithmetic means and ranges. For all tests, a two-sided α level set at 0.05 determined significance. Copper concentrations measured by ICP-MS of droplets on all steel coupons (0 to 75 min) are presented as arithmetic means $\pm$ standard deviations.

RESULTS

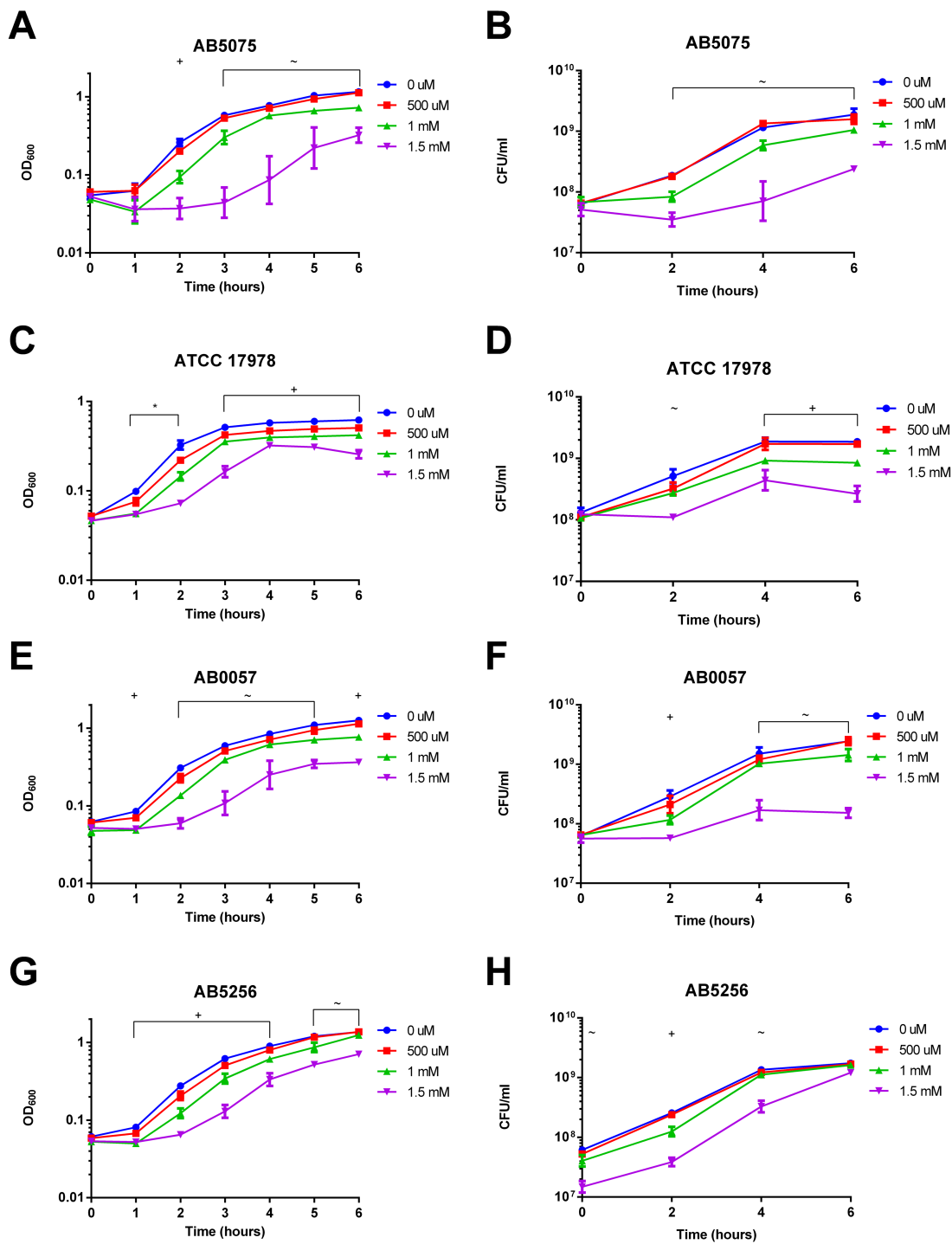
Copper Sensitivity of *A. baumannii* Strains in Liquid Culture

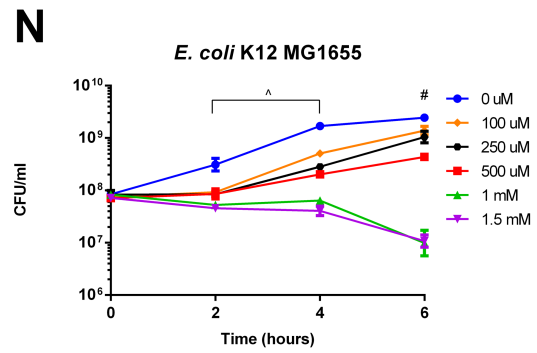
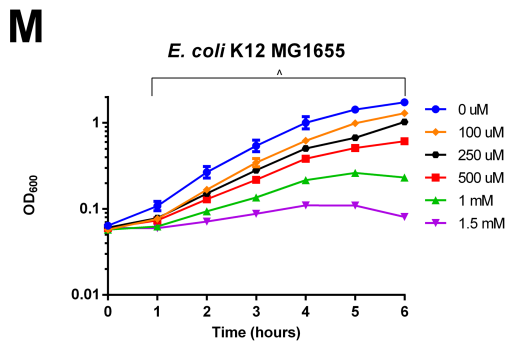
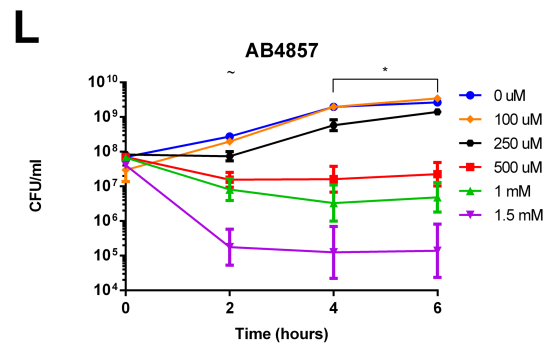
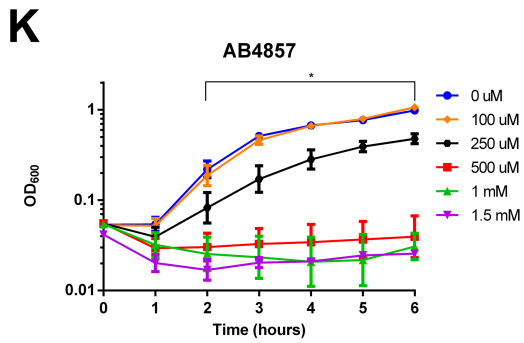
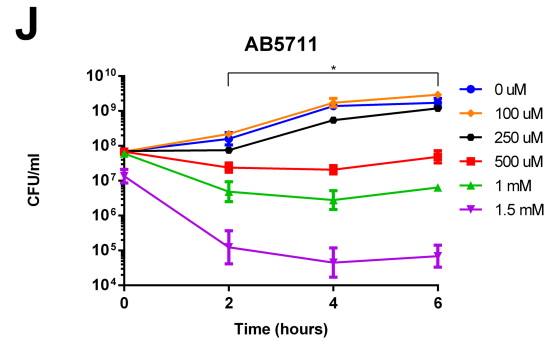
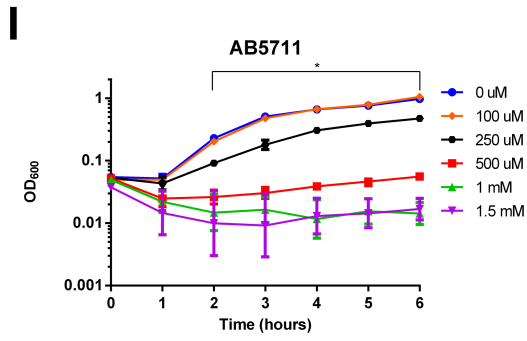
Few published studies have investigated copper resistance levels in *A. baumannii*, and most prior studies examined environmental isolates of *Acinetobacter* spp. (41; 85; 134; 183; 227). A single study examined clinical isolates of *A. baumannii*, and it found that MICs for copper in rich medium were in the millimolar range (39). Thus, we first sought to determine the resistance levels of various *A. baumannii* isolates in minimal medium for a panel of recent clinical and laboratory strains (Table 4). We supplemented liquid cultures of *A. baumannii* with 100 μ M to 1.5 mM CuSO₄ and measured bacterial growth (Fig. 3). We found that the *A. baumannii* isolates grouped into the following two levels of copper resistance: highly resistant and sensitive. The more resistant *A. baumannii* strains (AB5075, ATCC 17978, AB0057, and AB5256) grew similarly to control cultures in up to 500 μ M CuSO₄ (Fig. 3A to H). For these strains, 1 mM copper minimally inhibited growth, while 1.5 mM copper significantly delayed growth. However, no cell death was observed, and each of these strains was able to grow in 1.5 mM CuSO₄ following a 1- to 3-h delay. Unfortunately, we were unable to test the effects of higher copper concentrations in this assay, since the addition of higher concentrations of copper resulted in visible precipitation of copper from the solution.

The panel of tested strains included two *A. baumannii* isolates that were drastically more copper sensitive than the others: AB5711 and AB4857 (Fig. 3I to L). These strains were able to grow normally in 100 μ M copper, but growth was delayed by as little as 250 μ M copper. Furthermore, these strains were partially killed by copper concentrations of 500 μ M or higher. A common lab strain of *E. coli*, K-12 substrain

Figure 3. Copper sensitivity of *A. baumannii* strains in liquid culture.

Strains were grown with shaking at 37°C for 6 h in M9 medium supplemented with the indicated concentrations of CuSO₄. Bacterial growth was measured by OD₆₀₀ measurement (A, C, E, G, I, K, and M) and colony enumeration (CFU per milliliter) (B, D, F, H, J, L, and N). The data are presented as geometric means and SEM for three biologically independent experiments. *E. coli* K-12 MG1655 was included as a positive control (M and N). At each time point, growth in the presence of each copper concentration was compared to that of the 0 µM control by two-way ANOVA with Tukey's adjustment for multiple comparisons. The symbols above the graphs indicate which copper treatments were statistically significantly different ($P < 0.05$) from the 0 µM control, as follows: ~, growth in 1.5 mM CuSO₄ was different from the control; +, growth in 1 mM and 1.5 mM CuSO₄ was different from the control; *, growth in 500 µM, 1 mM, and 1.5 mM CuSO₄ was different from the control; #, growth in 250 µM, 500 µM, 1 mM, and 1.5 mM CuSO₄ was different from the control; and Λ, growth in 100 µM, 250 µM, 500 µM, 1 mM, and 1.5 mM CuSO₄ was different from the control.





MG1655, was tested as a positive control for copper sensitivity (Fig. 3M and N); K-12-derived strains of *E. coli* have been shown to be inhibited significantly by 250 μM CuSO_4 in modified M9 broth as well as on copper coupons (48; 70). Overall, we observed that the *A. baumannii* strains tested had various levels of copper resistance; some strains were significantly affected by low micromolar concentrations of copper, while others continued to grow in media with millimolar concentrations of copper.

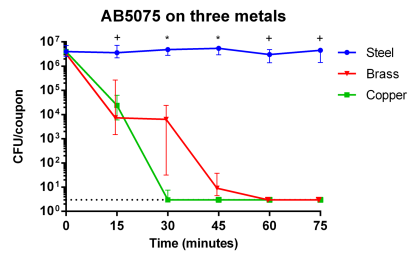
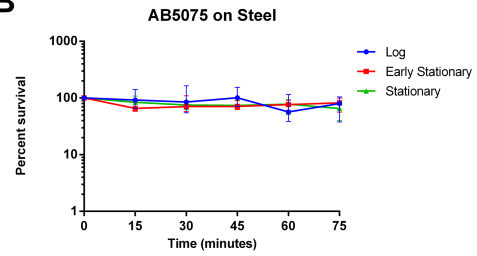
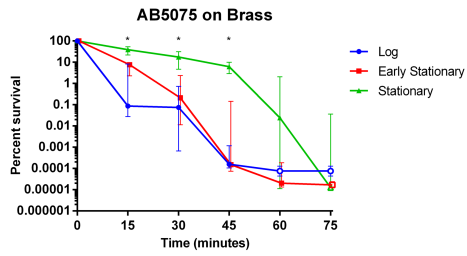
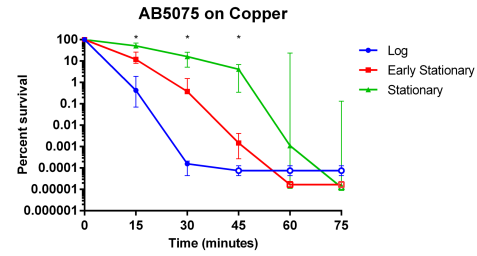
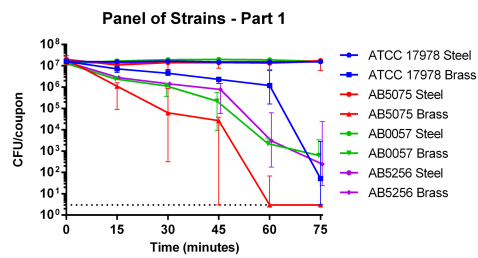
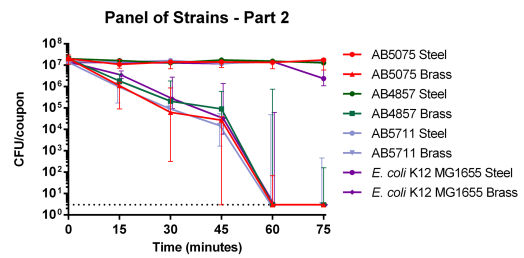
Survival of *A. baumannii* Isolates on Metal Surfaces

Given the recent deployment of copper as an environmental decontamination strategy (171), we additionally investigated the survival of *A. baumannii* isolates on metal surfaces. First, we assessed the effects of various metals on survival of strain AB5075. We chose to focus on this strain because it is a recent clinical isolate that is both highly copper resistant (Fig. 3A and B) and virulent in animal models of lung and wound infections (93; 197). To test survival on copper-containing surfaces, coupons of copper, cartridge brass, and stainless steel (negative control) were inoculated with AB5075 cells grown to log phase (2.5 h), and bacterial survival was determined at 15-min increments. As expected, AB5075 showed no loss of viability on stainless steel over the 75-min test period (Fig. 4A). Conversely, AB5075 was rapidly killed on the copper surface; the number of CFU dropped approximately 6 log and reached the limit of detection (3 CFU per coupon) in 30 min. On cartridge brass, which is approximately 70% copper, AB5075 was killed more slowly, but the limit of detection was still reached within 60 min.

Numerous studies have suggested that bacterial stress responses are affected by the growth phase, including in *A. baumannii* (52). Thus, we next investigated whether the

Figure 4. Survival of *A. baumannii* isolates on metal surfaces.

(A) Log-phase (2.5 h) bacterial cultures of AB5075 were spotted onto stainless steel 304, copper 110 H02 (99.9% copper), and cartridge brass C260 (~70% copper) coupons. Bacteria were recovered after the indicated times and plated for enumeration of surviving CFU. The data are presented as numbers of CFU per coupon for five biologically independent experiments; medians and interquartile ranges are shown. The dashed line indicates the limit of detection (3 CFU/coupon). At each time point, numbers of CFU per milliliter were compared among all three metals by the Kruskal-Wallis test with Dunn's correction for multiple comparisons. The symbols indicate statistically significant comparisons ($P < 0.05$), as follows: +, brass and copper different from steel; and *, copper different from steel. (B to D) Similar to the experiments in panel A, droplets of AB5075 were spotted onto steel, brass, and copper coupons. Bacteria were taken from log-phase (2.5 h), early-stationary-phase (6 h), and stationary-phase (24 h) cultures. Due to differences in the concentration of cells over the time course, data are presented as percent survival compared to that of the inoculum; open symbols indicate values at or below the limit of detection for all replicates. Data represent five biologically independent experiments; medians and interquartile ranges are shown. *, log- and stationary-phase cultures were significantly different from each other ($P < 0.05$) at the indicated time point. (E and F) Early-stationary-phase (6 h) cultures of each indicated strain were spotted onto steel and brass coupons. The results for the panel of strains were separated into two graphs for clarity, with AB5075 shown in both panels for comparison. The data are presented as numbers of CFU per coupon for 3 to 5 biologically independent experiments; medians and ranges are shown.

A**B****C****D****E****F**

growth phase of the culture affected survival on copper-containing surfaces. This was accomplished by testing temporal samples from an AB5075 culture, i.e., samples taken during log phase (2.5 h), early stationary phase (6 h), and stationary phase (24 h). As in Fig. 4A, stainless steel caused no change in viability; this was true regardless of the growth phase of the cells (Fig. 4B). However, copper resistance positively correlated with the age of the culture on both brass and copper coupons; AB5075 was most copper sensitive in log phase and most resistant in stationary phase (Fig. 4C and D). Indeed, the percentages of survival of stationary- and log-phase cells on brass and copper were significantly different from each other at 15, 30, and 45 min ($P < 0.05$).

Since the panel of *A. baumannii* isolates showed various levels of copper resistance in the liquid culture assay, we also individually assessed the full panel by using the coupon assay. In order to best discern differences among strains, we chose to focus on early-stationary-phase cultures and cartridge brass coupons; under these conditions, AB5075 died evenly and gradually, but its level still reached the limit of detection within 75 min. Stainless steel coupons and the *E. coli* strain were included as controls. While the differences were not statistically significant, ATCC 17978, AB5256, and AB0057 appeared to survive better on the brass surface than did AB5075, AB5711, AB4857, and *E. coli* K-12 MG1655 (Fig. 4E and F). Despite these differences in sensitivity, all strains were reduced approximately 7 log, to the limit of detection, during the 75-min test period. This finding highlights the ability of copper-containing surfaces to effectively eliminate *A. baumannii*.

Because we saw very limited killing in the liquid culture experiments (Fig. 3A to L), but saw dramatic killing of *A. baumannii* on the brass and copper surfaces, we

hypothesized that the concentration of copper experienced by bacteria in the droplets of medium on the metal coupons must be much higher than the maximum copper concentration (1.5 mM) achievable in growth assays. To test this hypothesis, we used ICP-MS to measure the concentration of copper that accumulated in the droplets (Fig. 5). As a control, we also assessed the copper content of the M9 growth medium. The M9 growth medium routinely contained approximately 1 to 2 μM copper. The droplets exposed to stainless steel accumulated relatively low concentrations of copper, which were insufficient to kill any of the bacterial strains ($131 \pm 86 \mu\text{M}$). The observed increase in copper ions following exposure to steel is presumably due to trace metal contamination in the stainless steel sheet preparation; the company specification sheet listed 0.54% Cu for the product, and the presence of copper in the steel coupons was confirmed by ICP-MS analysis of a steel sample treated with concentrated nitric acid (data not shown). While the concentrations of copper from steel were not sufficient to kill *A. baumannii*, the concentrations of copper in the droplets incubated on brass or copper surfaces were extremely high. The copper concentration in the droplets incubated on brass temporally increased to as high as 2 mM over the 75-min test period. Similarly, the copper concentration in the droplets incubated on pure copper temporally increased from $>2 \text{ mM}$ at 15 min to approximately 8 mM at 75 min. Thus, on these two metal surfaces, substantial concentrations of copper are released into the liquid environment, likely explaining why the bacteria were killed so rapidly under these conditions.

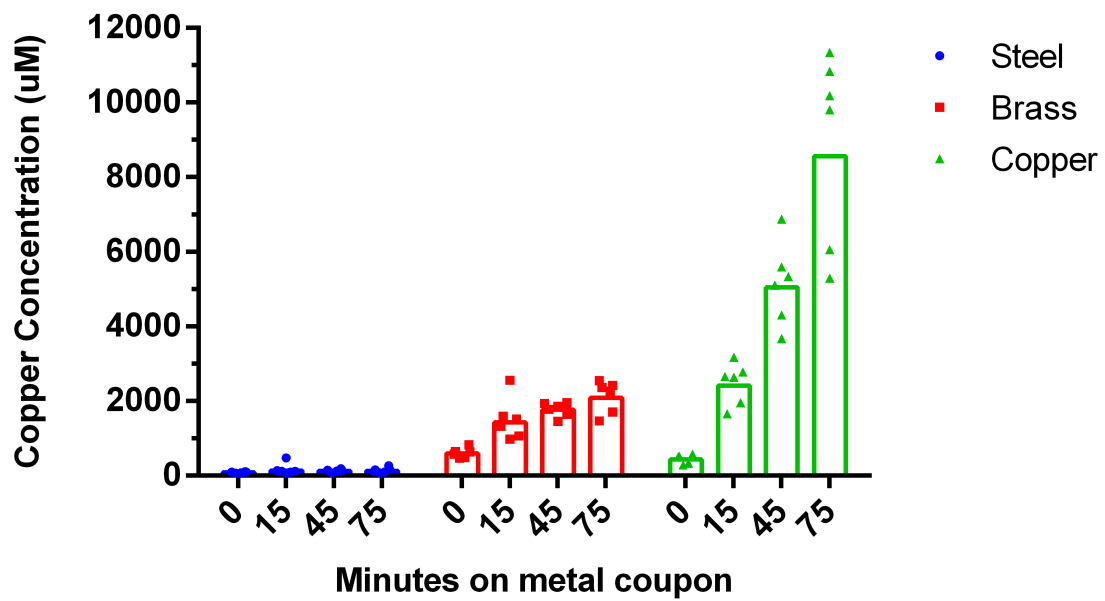


Figure 5. Concentrations of copper that accumulated in droplets on metal surfaces. Five-microliter droplets of M9 minimal medium were placed onto stainless steel, cartridge brass (~70% Cu), or 99.9% copper coupons for the indicated times. Droplets were recovered by washing an additional 100 μ L of M9 medium across the surface. Copper content was then measured by ICP-MS. Six biologically independent replicates were completed with six individual preparations of medium. Values for each replicate are shown as individual points, and the geometric means are represented by bars.

Killing of *Acinetobacter* Biofilms by Copper

Since *A. baumannii* forms biofilms on biotic and abiotic surfaces (118), and because bacteria found in biofilms are often more resistant to environmental stress (76; 147), we next sought to determine whether copper was efficacious against *A. baumannii* cells found within a biofilm. We tested the effects of copper on preformed biofilms of AB5711 because this strain formed substantial biofilms *in vitro* and was relatively sensitive to the copper concentration that is achievable in M9 medium (Fig. 3I and J). We observed dose-dependent killing of both planktonic and biofilm cells (Fig. 6). Bacteria within biofilms were reduced approximately 2 log over the 24-h period when the biofilms were treated with 1 or 1.5 mM copper. The planktonic cells were significantly more sensitive and were reduced approximately 5.5 log over the 24-h test period. Therefore, although biofilms did show increased copper resistance relative to that of planktonic cells, copper was still able to kill AB5711 within a biofilm.

Copper Tolerance Response

In the liquid assay, we observed that growth of strain AB5075 was delayed by the addition of 1.5 mM copper, but later continued at an ample rate (Fig. 3A). This pattern suggested that AB5075 was able to adapt to the copper stress. Tolerance responses following priming have been shown for other environmental stresses, e.g., the acid tolerance response (127). Therefore, we tested if copper sensitivity was altered by preexposure of the bacteria to a subinhibitory concentration of copper. To this end, we preexposed AB5075 to increasing subinhibitory concentrations of copper (1 to 100 μ M) before adding copper to a final inhibitory concentration of 1.5 mM. Preexposure of cells

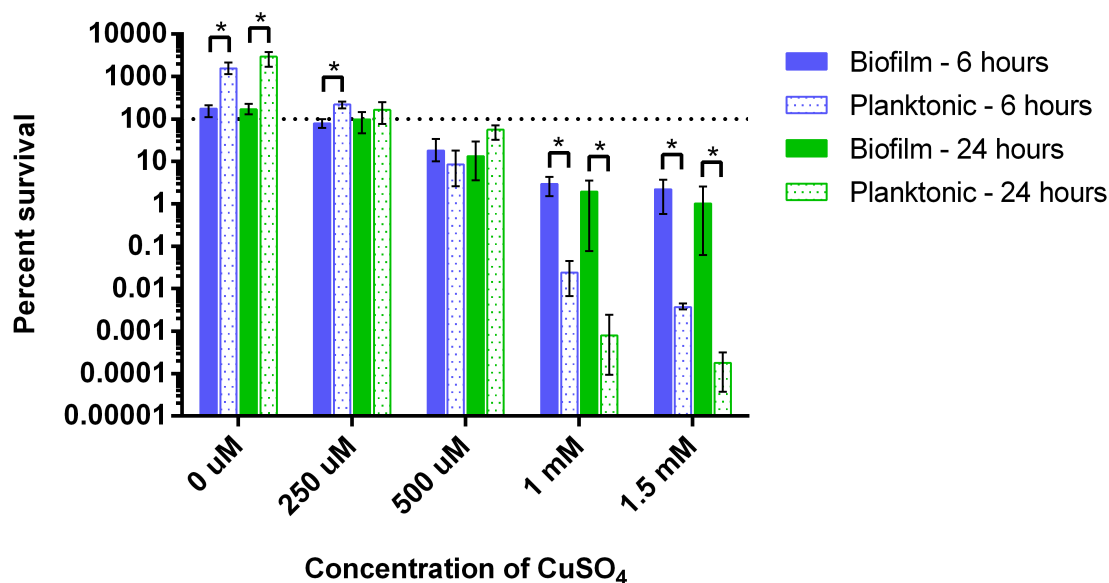


Figure 6. Killing of *Acinetobacter* biofilms and planktonic cells by copper.

AB5711 cultures were incubated statically overnight at 37°C in a 24-well tissue culture-treated plate to establish biofilms. The LB broth was removed and replaced with M9 medium containing the indicated concentration of CuSO₄. After 0, 6, or 24 h of additional incubation, bacteria from both the supernatant (planktonic) and the biofilm were serially diluted and plated for enumeration of CFU. Percent survival was calculated as the percentage of the bacteria present at time zero under each condition. Data are presented as mean and ranges for four biologically independent replicates. One-way ANOVA with Welch's correction followed by linear contrasts with Bonferroni adjustment was used to compare biofilm and planktonic cell percentages of survival at each concentration and time point. Statistically significant comparisons ($P < 0.01$) are indicated with asterisks.

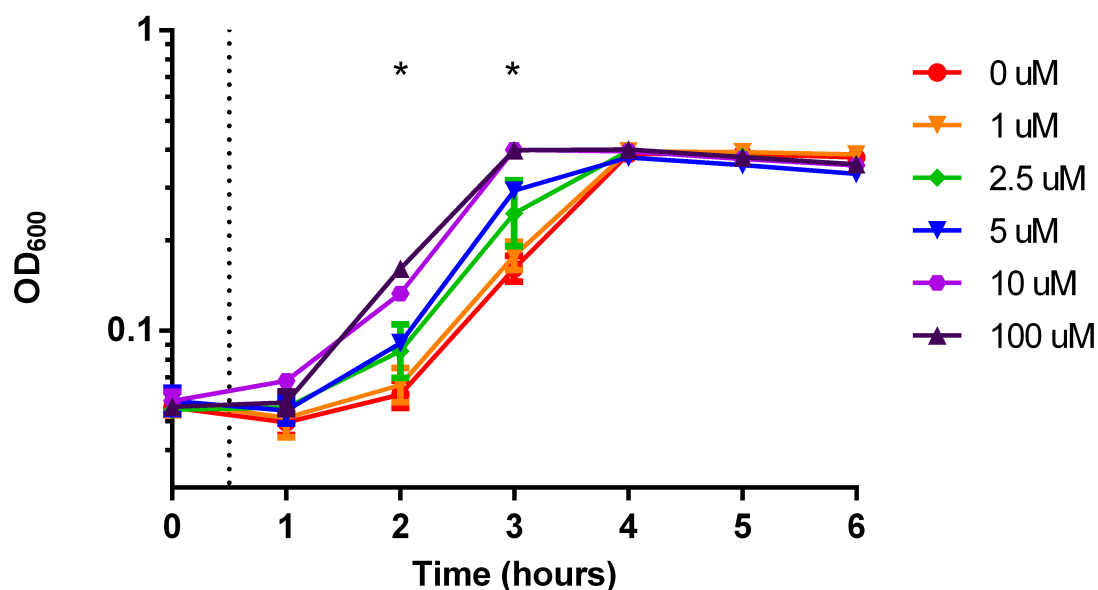


Figure 7. Preexposure to a low concentration of copper induces a copper tolerance response.

AB5075 was subcultured at an OD_{600} of 0.05 in M9 medium containing the indicated concentration of $CuSO_4$. After 30 min of incubation (dashed line), copper was added to a final concentration of 1.5 mM. Culture density was then measured by determining the OD_{600} at the indicated time points. The data are presented as geometric means and standard errors of the means for three biologically independent experiments. For each time point, growth under each priming condition was compared by two-way ANOVA with Tukey's adjustment for multiple comparisons. Significance ($P < 0.05$) was found at hours 2 and 3; P values are shown in Table 6.

Table 6. Adjusted *P* values for AB5075 growth following copper priming

Copper priming concn (μM) comparison	<i>P</i> value ^a	
	2 h	3 h
0 vs. 1	0.9884	0.9481
0 vs. 2.5	0.0452	0.0038
0 vs. 5	0.0096	< 0.0001
0 vs. 10	< 0.0001	< 0.0001
0 vs. 100	< 0.0001	< 0.0001
1 vs. 2.5	0.1961	0.0527
1 vs. 5	0.0571	0.0004
1 vs. 10	< 0.0001	< 0.0001
1 vs. 100	< 0.0001	< 0.0001
2.5 vs. 5	0.9943	0.6264
2.5 vs. 10	0.0023	0.0006
2.5 vs. 100	< 0.0001	0.0006
5 vs. 10	0.0131	0.0737
5 vs. 100	< 0.0001	0.0784
10 vs. 100	0.5352	> 0.9999

^a Differences in growth were evaluated among priming conditions for each time point by using two-way ANOVA with Tukey's correction for multiple comparisons. No significance was found at 0, 4, 5, or 6 h. *P* values <0.05 are highlighted in bold.

to very small amounts of copper allowed the bacteria to initiate growth in the presence of 1.5 mM copper much sooner than if they were not preexposed (Fig. 7; Table 6).

Furthermore, the pattern showed a classic dose-response relationship. As little as 2.5 μ M copper significantly reduced the lag in growth seen in the presence of 1.5 mM copper ($P < 0.05$), and preexposure to higher concentrations of copper further decreased the lag in growth; this trend was represented by a decrease in P values (Table 6). However, priming reached the maximum effect at approximately 10 μ M during the preexposure stage.

Overall, preexposure of AB5075 cells to very low concentrations of copper shortened the time required to initiate growth with a high concentration of copper, which further suggests that AB5075 is able to mount a tolerance response to copper stress.

Putative Copper-related Genes

Copper resistance in bacteria is mediated by a variety of copper homeostasis mechanisms, mainly copper detoxification and efflux (11). The ability of AB5075 to adapt to growth in a high concentration of copper suggests that this bacterium likely encodes copper resistance mechanisms that are induced by copper exposure. To identify putative copper resistance mechanisms in AB5075, we used the amino acid sequences encoded by known copper-related genes from *E. coli* and *P. aeruginosa* to search the AB5075 genome (11; 79); many of the identified targets were already annotated with copper-related functions. The levels of homology of each of the identified AB5075 proteins to *E. coli* K-12 MG1655 and *P. aeruginosa* PAO1 proteins are presented in Table 7. Given the closer relationship of the two, most *A. baumannii* proteins were more similar to those of *P. aeruginosa* than to those of *E. coli*. Overall, the level of homology

Table 7. Putative copper-related genes/proteins in AB5075

Annotated <i>A. baumannii</i> protein name/new name ^a	Locus no. (old/new) ^b	Putative Function	<i>E. coli</i> K-12 MG1655 (taxid 511145) homologue				<i>P. aeruginosa</i> PAO1 (taxid 208964) homologue					
			Protein name, locus	% identity/ % similarity	Bit score	Expect value	% query coverage	Protein name, locus	% identity/ % similarity	Bit score	Expect value	% query coverage
ABUW_0265 ^{c,e}	ABUW_0265/ ABUW_RS01300		None					None				
CzcC ^e	ABUW_0266/ ABUW_RS01305	RND family efflux pump	None					None				
CzcB	ABUW_0267/ ABUW_RS01310	RND family efflux pump	CusB, b0574	25/44	69.3	1E-13	80	CzcB, PA2521	34/53	182	5E-52	85
CzcA	ABUW_0268/ ABUW_RS01315	RND family efflux pump	CusA, b0575	34/56	582	0E+00	97	CzcA, PA2520	62/78	1,227	0E+00	97
CzcD	ABUW_0269/ ABUW_RS01320	RND family efflux pump	ZitB (zinc efflux system), b0752	40/59	155	9E-45	86	Cation efflux system protein, PA0397	56/73	286	2E-95	90
ABUW_1334	ABUW_1334/ ABUW_RS06510	Cation efflux	CusC, b0572	31/50	181	2E-51	96	Hypothetical protein, PA2525	37/56	283	5E-89	95
ABUW_2705 ^c	ABUW_2705/ ABUW_RS13125		Hypothetical protein ^d	28/45	57.8	8E-10	85	Hypothetical protein, PA2706	34/51	87.8	9E-23	91
CueR	ABUW_2706/ ABUW_RS13130	Transcriptional regulator	CueR, b0487	41/66	106	3E-30	94	CueR, PA4778	49/68	127	6E-38	96
ActP2/CopA1	ABUW_2707/ ABUW_RS13135	Copper-transporting P-type ATPase	CopA, b0484	41/61	561	0E+00	98	Metal transporting P-type ATPase, PA3920	46/64	577	0E+00	98
ABUW_2708	ABUW_2708/ ABUW_RS13140	Copper chaperone	None					Hypothetical protein, PA3520	46/58	54.3	1E-11	87
ABUW_3226 ^c	ABUW_3226/ ABUW_RS15660	Cation efflux	Sodium/proton antiporter ^d	51/69	286	3E-94	90	Sodium/proton antiporter ^d	52/69	290	2E-96	90
PcoB	ABUW_3227/ ABUW_RS15665		CopB ^d	30/50	83.6	3E-17	79	Copper resistance protein B, PA2064	27/52	77.4	5E-17	65
PcoA	ABUW_3228/ ABUW_RS15670	Copper oxidase	CueO, b0123	23/35	65.1	7E-12	91	Copper resistance protein A, PA2065	46/60	473	8E-159	92
ABUW_3229 ^{c,e}	ABUW_3229/ ABUW_RS15675		None					None				
ABUW_3230 ^c	ABUW_3230/ ABUW_RS15680		Sugar phosphatase YbiV, b0822	41/62	214	1E-68	99	None				
CopB	ABUW_3320/ ABUW_RS16130		Copper resistance protein B ^d	27/46	84.7	3E-17	67	Copper resistance protein B, PA2064	31/46	94	6E-26	65
CopA/CueO	ABUW_3321/ ABUW_RS16130	Copper oxidase	CueO, b0123	24/38	53.5	4E-08	71	Copper resistance	45/59	479	3E-160	93

	ABUW_RS16135		protein A, PA2065									
ABUW_3322 ^c	ABUW_3322/ ABUW_RS16140		Copper oxidase ^d	52/59	44.7	1E-04	80	Copper oxidase ^d	62/68	60.1	2E-10	53
CopR/CusR	ABUW_3323/ ABUW_RS16145	Response regulator	CusR, b0571	62/81	286	8E-98	99	CopR, PA2809	63/80	279	7E-95	99
CopS/CusS	ABUW_3324/ ABUW_RS16150	Sensor kinase	CusS, b0570	34/54	265	6E-83	94	CopS, PA2810	34/53	256	1E-79	98
ActP1/CopA2	ABUW_3325/ ABUW_RS16155	Copper-transporting P-type ATPase	CopA, b0484	46/65	507	8E-168	83	Metal transporting P- type ATPase, PA3920	43/63	471	6E-154	79
CopC	ABUW_3326/ ABUW_RS16160	Copper chaperone	CopC family protein YobA, b1841	33/43	57.4	4E-12	90	CopC precursor ^d	39/53	58.9	7E-10	82
CopD ^e	ABUW_3327/ ABUW_RS16165		None					None				

^a Some of the proteins identified in AB5075 were annotated with names commonly used for different copper-related proteins. To avoid confusion in discussing *A. baumannii* systems alongside those of other bacteria, we referred to these proteins by new names that match those used elsewhere. In these cases, we first provide the current annotated name of the protein followed by the new name used in this publication.

^b Two locus numbers are given for each protein in AB5075-UW: one from the original annotation and one from the subsequent reannotation by GenBank. Both are available under accession no. NZ_CP008706.1.

^c Genes located in operons with identified copper-related genes were included even if they were not also identified as being copper related.

^d No homologue was found in the selected *E. coli* or *P. aeruginosa* strain, but significant homology was found with a different strain when the species *E. coli* (taxid 562) or *P. aeruginosa* (taxid 287) was chosen as the search set. The ABUW_2705 homologue was found in *E. coli* KTE51, with the locus tag A1SA_00101. The ABUW_3226 homologues were identified in multiple *E. coli* strains (accession no. WP_004364974.1) and multiple *P. aeruginosa* strains (accession no. WP_031693306.1). The PcoB homologue was identified in multiple *E. coli* strains (accession no. WP_020996127.1). The CopB homologue was found in *E. coli* strain HM46, with the locus tag HM46_RS23285. The ABUW_3322 homologue was found in *E. coli* HM46, with the locus tag HM46_RS23290, and was identified in multiple *P. aeruginosa* strains (accession no. WP_043096474.1). The CopC homologue was found in *P. aeruginosa* strain E15_London_28_01_14, with the locus tag PAERUG_E15_London_28_01_14_00104.

^e Four proteins included here do not have homologues in *E. coli* or *P. aeruginosa*, but there is homology to proteins found in other organisms, e.g., *Bacillus* spp., *Moraxella* spp., *Shewanella* spp., when BLASTp searches are used to search all protein sequences.

to copper-related proteins in both organisms was high, suggesting that these copper resistance mechanisms are well conserved.

In total, 23 putative proteins of AB5075 were analyzed via comparison to *E. coli* and *P. aeruginosa* (Table 7). The most well-known copper resistance systems were present and well conserved in AB5075. The CopA (copper ATPase), CueO (copper oxidase), and CueR (regulator) system of detoxification was complete (ActP2/CopA1, CopA/CueO, and CueR). Additionally, AB5075 appeared to encode a second copper ATPase (ActP2/CopA1) and copper oxidase (PcoA). The copper-sensing two-component system, CopRS/CusRS, was also present and highly conserved. However, the presumed target of CopRS/CusRS regulation, the RND family efflux pump encoded by *czcABCD*, appeared to be less well conserved. Homologues of CzcA, CzcB, and CzcD were clearly present, but the CzcC protein did not match any proteins in *E. coli* or *P. aeruginosa*. However, a distally located gene, ABUW_1334, showed clear homology to the genes encoding CusC/CzcC of *E. coli* and an outer membrane efflux protein of *P. aeruginosa*. In addition, many homologues of copper-related genes with less-well-characterized functions were also conserved in AB5075: PcoB, CopB, and CopC.

We noted that the putative copper-related proteins were encoded by genes that were located in only four areas of the AB5075 chromosome; we arbitrarily named these regions A through D (Fig. 8). The genes were arranged as 6 apparent operons with 2 adjacent, monocistronic genes. The organization and operonic structure were confirmed experimentally using junctional PCR analysis of cDNA (Fig. 9).

To determine if the other *A. baumannii* isolates in the panel also carry the identified putative copper-related genes, we next used the amino acid sequences of

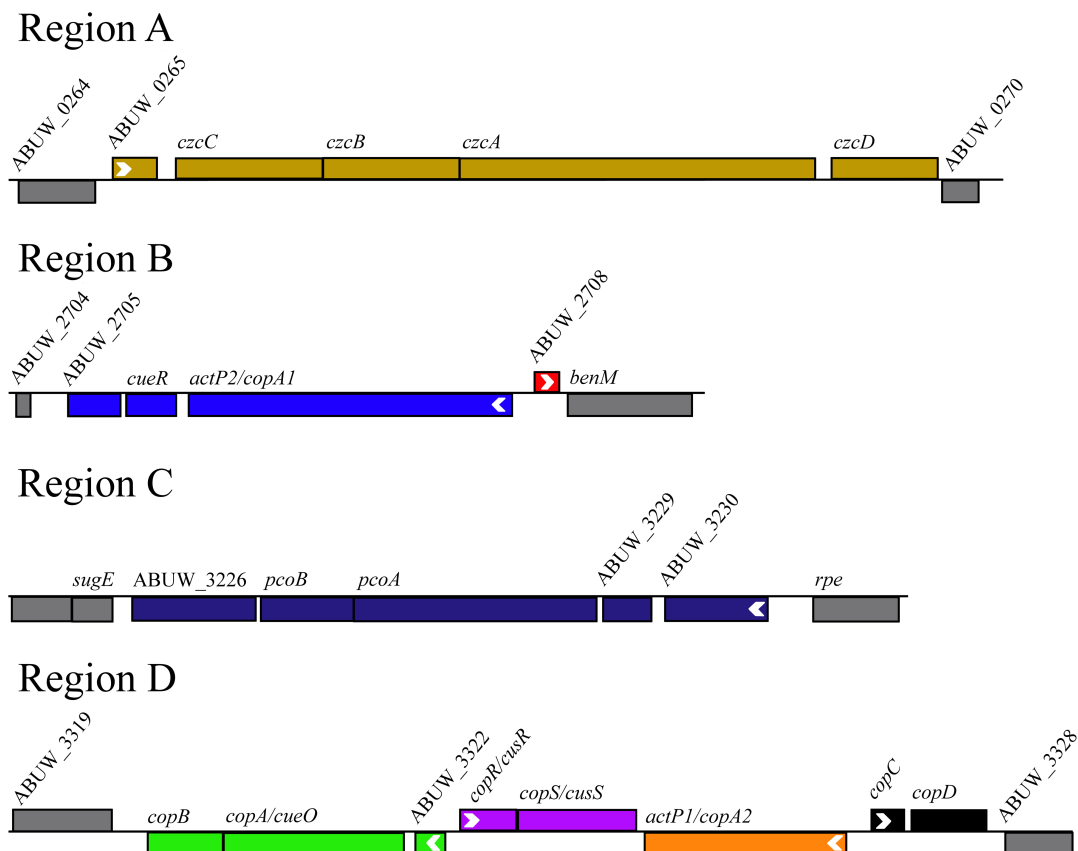


Figure 8. Genomic organization of putative copper-related genes.
 Putative copper-related genes that were identified in AB5075 are located in four regions of the chromosome, arbitrarily named regions A, B, C, and D. Colors represent contiguous mRNA molecules and match the colors used in Fig. 10. The direction of transcription is indicated by a white arrowhead at the N terminus of each individual gene or initial gene of an operon. Locus identification numbers are given for genes without annotated names. The annotation of five genes does not match the names used for other organisms, and thus the new gene names are included following the original name, as follows: *actP2/copA1*, *actP1/copA2*, *copA/cueO*, *copR/cusR*, and *copS/cusS*. This image was adapted from the AB5075-UW transposon browser (http://tools.uwgenomics.org/tn_mutants).

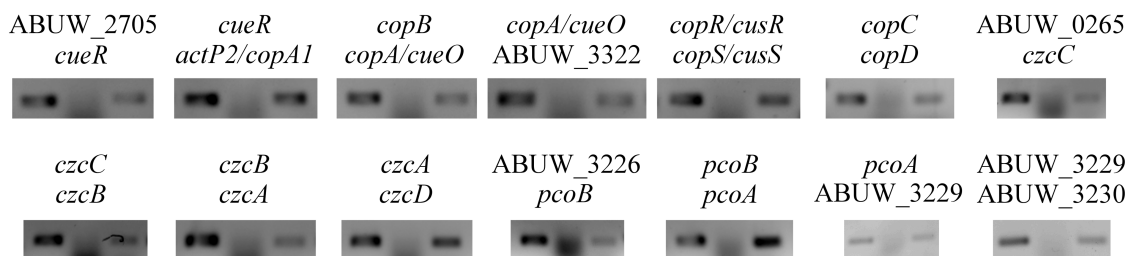


Figure 9. Junctional PCR confirms the operonic structure of putative copper-related genes.

To detect each of the gene junctions between genes of a putative operon, qualitative reverse transcription-PCR was conducted. PCRs were performed using genomic DNA, RNA, or cDNA as the template. Each reaction mixture was incubated for 5 min at 95°C, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. PCR products were separated in 1% agarose gels and visualized by staining with ethidium bromide. Each PCR was performed three times using biologically independent cDNA samples.

AB5075 proteins to search the other genomes. Genomes were searchable by BLASTp searches for all strains except AB4857. In most of the *A. baumannii* strains, we identified all of the putative copper-related genes identified in AB5075. However, AB5711 did not appear to carry all of the genes found in the other strains. AB5711 lacked all of the genes found in region D in AB5075, i.e., *copB*, *copA/cueO*, ABUW_3322, *copR/cusR*, *copS*, *actP1/copA2*, *copC*, and *copD*.

Expression of Putative Copper-related Genes

Finally, to determine if expression of the putative copper-related genes in AB5075 was regulated by copper, we used qRT-PCR to measure changes in transcript levels following 30 min of exposure to subinhibitory concentrations of copper (Fig. 10). For genes located in regions B and D, we saw significant increases (≥ 2 -fold) in expression in response to copper. In many cases, a dose-dependent increase in expression was observed as the copper concentration increased. Note that the observed expression patterns were most similar for genes located in close proximity on the chromosome. For example, genes located in region B were upregulated by as little as 2.5 μM CuSO_4 and showed a steady increase in expression as more copper was added. Conversely, all four gene sets in region D required addition of ≥ 10 μM CuSO_4 to induce increased expression, which then peaked at 250 μM . Expression of the operons in regions A and C did not increase in response to copper. Overall, we observed that many of the putative copper-related genes were induced by exposure to copper, suggesting that the corresponding proteins may indeed have functions related to copper resistance.

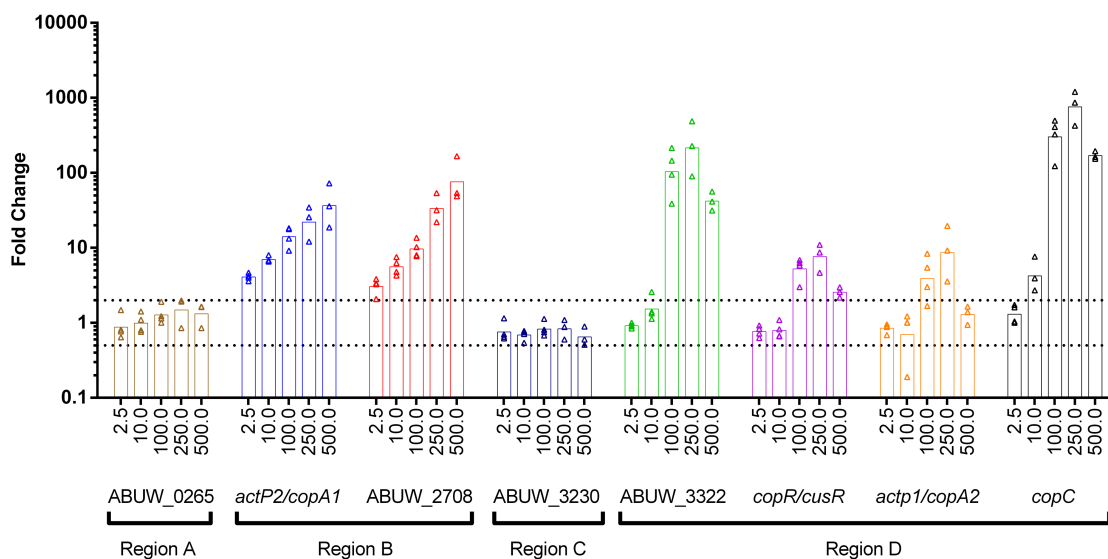


Figure 10. Expression of putative copper-related genes in response to copper exposure. Expression of putative copper-related genes was measured by qRT-PCR, using primers specific to the initial gene of each operon. RNA was isolated from AB5075 cells exposed for 30 min to the indicated increasing concentrations (micromolar) of CuSO₄. Generated cDNA was used as template DNA for qRT-PCR. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method, using the 16S rRNA gene as the internal reference. Three or four biologically independent replicates of each experiment were performed. Values for each replicate are shown as individual points, and the geometric means are represented by bars. Dashed lines represent significant (2-fold) up- and downregulation.

DISCUSSION

A. baumannii causes multiple types of severe nosocomial infections, for which treatment options are limited by antibiotic resistance found in MDR, XDR, and PDR strains. The intrinsic antimicrobial properties of copper give it unique potential for use in novel therapeutics. Thus, we sought to perform an initial characterization of copper resistance in recent clinical isolates of *A. baumannii*. In this study, we demonstrated that copper sensitivities varied among the *A. baumannii* isolates (Fig. 3). However, all of the tested *A. baumannii* strains were killed rapidly on copper-containing surfaces (Fig. 4). This was likely due in part to the high concentration of copper ions that accumulated in the droplets of medium (Fig. 5). While *A. baumannii* found in a biofilm was more resistant to copper toxicity than planktonic cells, biofilm cells were also susceptible to copper (Fig. 6). Additionally, we identified many putative proteins that may be involved in copper resistance and showed that expression of many of the genes that encode these proteins is upregulated following exposure to copper (Table 7; Fig. 10). Increased expression of these genes in response to copper likely mediates improved adaptation of AB5075 to copper shock when the culture is pretreated with subinhibitory concentrations of copper (Fig. 7). To our knowledge, this is the first comprehensive study characterizing copper resistance in *A. baumannii*.

Due to the increase in antibiotic resistance seen across an extensive array of bacterial pathogens, there is a pressing need to identify new means to treat infections caused by these organisms. The potential to use copper as a novel therapeutic to treat infections has led to a number of studies that have sought to determine the sensitivity of

various pathogens to this metal. Unfortunately, it becomes very difficult to compare these experiments to one another because the chosen growth medium can drastically affect the amount of copper required to inhibit bacterial growth. The MIC of soluble copper ions is dramatically increased in rich medium; this is presumably due to binding of copper ions by proteins in the medium, which effectively reduces the concentration of free copper ions able to impose damage. For example, the MIC of copper for *P. aeruginosa* PAO1 increases from 100 μ M in minimal medium to 6 mM in rich medium (195). While we recognize the potential limitations of comparing our results to those of experiments with different protocols, we note that the concentration of copper required to inhibit bacterial growth of *A. baumannii* appears to be strikingly different from that for other pathogens. Similar experiments conducted in minimal media found that the MIC of copper is 500 μ M or less for *Mycobacterium tuberculosis* strain H37Rv, *E. coli* strain BW25113, *P. aeruginosa* strain PAO1, and *Salmonella enterica* serovar Typhimurium strain SL1344 (70; 149; 195; 216). Conversely, we found that growth of most of the *A. baumannii* isolates was only slightly delayed by 1.5 mM CuSO_4 in minimal medium, and we were unable to kill these strains with soluble copper. Thus, it appears that *A. baumannii* may be more copper resistant than other bacterial pathogens that have been tested. The high levels of copper resistance may be related to the fact that the progenitor *A. baumannii* strain may have been a soil organism, as the levels of copper in soil may be significantly higher than those typically encountered by other bacterial species (206). Since copper resistance may also play a role in survival in the human host or hospital environment, the genes were retained. Additionally, *A. baumannii* is an exceptional example of a pathogen that rapidly obtains genetic information from other organisms via horizontal gene

transfer; indeed, copper resistance gene clusters have been identified in mobile genetic elements in *A. baumannii*, alongside its many antibiotic resistance genes (4).

Ultimately, the levels of copper resistance are no doubt directly influenced by the repertoire of copper-related genes carried by the organism. Thus, we identified a large set of putative copper-related genes in strain AB5075 (Table 7). While the functions of the putative proteins encoded by these genes remain to be elucidated for *A. baumannii*, the functions of some of the orthologous proteins have been well characterized for *E. coli*. For example, CopA, CueO, and CueR make up a canonical copper detoxification system, consisting of a copper ATPase to efflux copper ions to the periplasm, a periplasmic copper oxidase to detoxify the copper ions, and a transcriptional regulator to activate expression of the system in response to cytoplasmic copper ions. While homologues of all three of these proteins are present in AB5075, they are not encoded together (ActP2/CopA1, CopA/CueO, and CueR). Additionally, AB5075 appears to encode a second copper ATPase and copper oxidase (AcP1/CopA2 and PcoA, respectively) in its genome. In addition to the detoxification system, homologues of a copper efflux pump (CzcCBAD) and a potential regulator of this system (CopRS/CusRS) were also identified in AB5075. We note that while expression of the two-component system encoded by *copRS/cusRS* was increased upon copper exposure, expression of the RND family efflux pump encoded by *czcCBAD* did not increase (Fig. 10). Thus, it is possible that the pump is regulated differently in *A. baumannii* than in *E. coli*, that the pump is constitutively active, or that this pump is not actually used for copper. AB5075 encodes many putative efflux pumps, and thus *czcCBAD* may have been misannotated as a copper pump. Indeed,

future functional analysis will be required to conclusively determine the function of any of the putative proteins encoded by these copper-related genes.

Two of the *A. baumannii* clinical isolates tested, AB4857 and AB5711, were significantly more sensitive to copper than the rest of the strains of the panel (Fig. 3). Unfortunately, the genome of AB4857 is available only as a draft and is not searchable by BLASTp for comparison. However, AB5711 lacks a large group of copper-related genes that are present in AB5075 region D. These include *copB*, *copA/cueO*, ABUW_3322, *copR/cusR*, *copS*, *actP1/copA2*, *copC*, and *copD*. Thus, since the level of copper resistance of AB5711 is significantly lower than that of the other *A. baumannii* strains, it is tempting to speculate that the proteins encoded by the genes carried in region D are crucial to the higher level of copper resistance seen in the other *A. baumannii* isolates. Future mutational analysis will be required to define the role of each of these proteins in copper resistance.

Our analysis showed copper-dependent regulation of genes found in regions B and D of the chromosome; genes in region B were exquisitely sensitive to the copper concentration (Fig. 10). Indeed, expression from the two divergently transcribed genes/operons in region B increased significantly in response to the addition of as little as 2.5 μM copper sulfate, and it continued to increase with additional copper. Conversely, increased expression from region D, which carries adjacent pairs of divergently transcribed genes/operons, required the addition of ≥ 10 μM copper. We note that the threshold for upregulation of copper-related genes fits well with the copper tolerance response we observed (Fig. 7). Indeed, pretreatment with as little as 2.5 μM CuSO_4 significantly reduced the lag in growth seen with extreme copper shock, and pretreatment

with up to 10 μM CuSO_4 further shortened the lag in growth. Thus, perhaps the increased expression of copper-related genes found in regions B and D is responsible for the identified copper tolerance response. Future analysis of strains containing mutations in genes located in these regions will help to define the importance of these genes to the adaptation response.

In addition to the possible utilization of copper in novel therapeutics, copper is also being tested for use in environmental decontamination. Recent clinical trials showed that covering high-touch surfaces in hospital rooms with copper can significantly decrease the bacterial burden and can reduce the number of hospital-acquired infections by more than 50% (168; 171-173; 210). Because *A. baumannii* fomite-associated hospital outbreaks have been documented, specifically from a contaminated hospital bed and burn theater, evidence that copper effectively eliminates environmental *A. baumannii* is important (73). Indeed, we found that all tested strains of *A. baumannii* were reduced to near or below the limit of detection in only 60 to 75 min when exposed to brass and copper surfaces (Fig. 4). *A. baumannii* clinical isolates have previously been shown to be sensitive to copper-containing metal coupons (47; 126; 189). The data cannot be compared directly due to protocol differences, but similar trends were observed, with full survival on control surfaces, significant reductions on copper-containing surfaces, and more efficient killing by alloys with a higher copper content. In this study, we additionally showed the growth phase-dependent effects on survival on copper-containing surfaces and the variation in copper sensitivity among isolates of *A. baumannii*. Overall, these results demonstrate that copper has great potential to reduce *A. baumannii* in the environment.

Because we saw dramatic killing of *A. baumannii* on the copper coupons but only limited killing of some strains in the liquid assay, we used ICP-MS to measure the concentration of copper that accumulated over time in the 5- μ L droplets of medium on the metal surface. On the copper coupons, we saw a rapid accumulation of millimolar concentrations of copper ions, which aids in explaining the dramatic killing of bacteria under these conditions. A similar experiment was performed previously, in which millimolar concentrations of copper were reported to accumulate in a 25- μ L droplet of Tris-Cl on a copper coupon (125). However, the rates at which ions were taken up into the droplets were very different between the experiments. This is likely due to the difference in the droplet size between the two experiments; this minor protocol difference likely dramatically affects the resulting data.

Previously, biofilm formation by environmental *Acinetobacter* spp. was shown to be inhibited by increasing concentrations of copper (134). In the same vein, we found that copper was effective at killing modern clinical isolates of *A. baumannii* found within a preformed biofilm structure. Not surprisingly, the biofilm was partially protective, and cells in the biofilm were significantly more resilient than planktonic cells; however, even the biofilm was effectively reduced by high concentrations of copper (Fig. 6). We speculate that *A. baumannii* biofilms may be able to be eliminated completely by copper concentrations higher than those achievable in M9 medium. Our results demonstrate that copper-based therapies have the potential to be effective treatments, even against *A. baumannii* strains that have formed biofilms.

In summary, due to its intrinsic antimicrobial properties, copper has the potential to be used in new therapeutics for *A. baumannii* infection. Furthermore, if particular

protein functions can be identified that are critical for copper resistance, novel antibiotics may be able to be designed to target those functions as a means to enhance the inherent antibacterial properties of copper. Such a strategy may make a very limited amount of copper very effective against infection. The human immune system utilizes copper in various ways to fight infection, and concentrations upwards of 500 μM have been measured in macrophage phagosomes (80; 191; 213). Furthermore, methods of increasing the concentration of copper in the body to enhance its antimicrobial effects are being investigated (13; 22; 105; 225). Additional targeting and blocking of copper resistance systems may make these strategies increasingly efficacious; indeed, many copper resistance systems have been shown to be critical for full virulence in various pathogens (43). We hope that future identification of mechanisms that are critical for copper resistance and virulence in *A. baumannii* and other bacterial pathogens will open doors for development of novel copper-based therapeutics.

ACKNOWLEDGEMENTS

C.L.W., J.J.G., S.L.J.M., D.V.Z., and D.S.M. conceived and designed the experiments. C.L.W. and H.M.N. performed the experiments. D.V.Z. contributed reagents/materials. C.L.W. and D.S.M. wrote the paper.

We thank Cara Olsen for help with statistics. We thank G.W. Liechti and A.T. Maurelli for providing *E. coli* K-12 MG1655. C.L.W. is grateful to the members of the Merrell and Zurawski labs for their microbiology expertise.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense,

the Uniformed Services University of the Health Sciences, the Department of the Army, or any other agency of the U.S. Government. This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication.

Funding Statement

Research in the laboratory of DSM is supported by funding from the National Institutes of Health (NIH) and the Department of Defense (DoD). S.L.J.M. thanks the National Science Foundation (NSF) (grant CHE1306208). Research in the laboratory of D.V.Z. is supported by grants from the Military Infectious Diseases Research Program.

CHAPTER 3: Characterization of *Acinetobacter baumannii* Copper Resistance Reveals a Role in Virulence

Submitted for publication as: **Caitlin L. Williams, Heather M. Neu, Yonas A.**

Alamneh, Ryan M. Reddinger, Anna C. Jacobs, Shweta Singh, Rania Abu-Taleb,

Sarah L. J. Michel, Daniel V. Zurawski, and D. Scott Merrell. 2019. Characterization of *Acinetobacter baumannii* Copper Resistance Reveals a Role in Virulence.

The work presented in this chapter is the sole work of C. L. Williams with the following exceptions: H. M. Neu and S. L. J. Michel conducted the ICP-MS measurements; Y. A. Alamneh, R. M. Reddinger, A. C. Jacobs, S. Singh, R. Abu-Taleb, and D. V. Zurawski conducted the mouse infection experiments; and A. C. Jacobs made the pAJ100 vector.

ABSTRACT

Acinetobacter baumannii is often highly drug-resistant and causes severe infections in compromised patients. These infections can be life threatening due to limited treatment options. Copper is inherently antimicrobial and increasing evidence indicates that copper-containing formulations may serve as nontraditional therapeutics against multidrug-resistant bacteria. We previously reported that *A. baumannii* is sensitive to high concentrations of copper. To understand *A. baumannii* copper resistance at the molecular level, herein we identified putative copper resistance components and characterized 21 strains bearing mutations in these genes. Eight of the strains displayed a copper sensitive phenotype (*pcoA*, *pcoB*, *copB*, *copA/cueO*, *copR/cusR*, *copS/cusS*, *copC*,

copD); the putative functions of these proteins include copper transport, oxidation, sequestration, and regulation. Importantly, many of these mutant strains still showed increased sensitivity to copper while in a biofilm. Inductively coupled plasma mass spectrometry revealed that many of these strains had defects in copper mobilization, as the mutant strains accumulated more intracellular copper than the wild-type strain. Given the crucial antimicrobial role of copper-mediated killing employed by the immune system, virulence of these mutant strains was investigated in *Galleria mellonella*; many of the mutant strains were attenuated. Finally, the *cusR* and *copD* strains were also investigated in the murine pneumonia model; both were found to be important for full virulence. Thus, copper possesses antimicrobial activity against multidrug-resistant *A. baumannii*, and copper sensitivity is further increased when copper homeostasis mechanisms are interrupted. Importantly, these proteins are crucial for full virulence of *A. baumannii* and may represent novel drug targets.

Importance

Multidrug-resistant *A. baumannii* is a serious global concern; antibiotic resistance rates continue to rise, and extensively drug-resistant isolates are common. Antibiotic resistance severely limits treatment options, leading to increased patient morbidity and mortality. Clearly, novel therapeutic options are desperately needed to combat *A. baumannii* and other “superbugs.” Copper-based therapies and targeting of bacterial copper homeostasis mechanisms represent a promising new approach for the treatment of multidrug-resistant infections. Thus, we sought to define the genetic factors that contribute to copper resistance in *A. baumannii* as well as to define their potential role in

virulence. Marked copper sensitivity was observed in numerous mutant strains. Moreover, identified copper resistance genes contributed to virulence, likely by facilitating evasion of copper-mediated immune responses. Thus, copper resistance genes may represent novel therapeutic targets for the treatment of *A. baumannii* infection. Moreover, since these mechanisms are conserved in other bacterial species, novel copper-based therapies may have broad-spectrum activity.

BACKGROUND

Acinetobacter baumannii is responsible for a significant proportion of nosocomial infections worldwide, and an even greater number of ICU-acquired infections; co-morbidities are an important risk factor for *A. baumannii* infection (45; 115; 208; 226). Associated types of infections are diverse, and include pneumonia, urinary tract infections, bacteremia, skin and soft tissue infections, osteomyelitis, and meningitis (156; 226). Extensive drug-resistance is common among *A. baumannii* strains, and resistance limits treatment options and leads to higher morbidity and mortality (10; 45; 62). Indeed, multidrug-resistant *A. baumannii* has been named a “serious” threat by the CDC since 2013, and in 2017, carbapenem-resistant *A. baumannii* topped the World Health Organization's Priority Pathogen's List as a “Level 1: Critical priority” pathogen (148; 160). Clearly, new therapeutic options are desperately needed to treat multidrug-resistant *A. baumannii* infections.

Antibiotics target essential functions for bacterial growth and/or survival. Metal homeostasis is a vital process that provides an extensive list of potential new antimicrobial targets. Copper is required for cellular function, e.g. for redox balance and

as an enzyme cofactor. However, copper ions become toxic at high concentrations; thus, it is important that intracellular copper levels be tightly controlled. Copper ions cause damage by participating in Fenton chemistry to produce hydroxyl radicals that react with and damage essential biomolecules (114) and also by displacing iron from crucial iron-sulfur cluster proteins (119). Studies in *Escherichia coli* and *Salmonella* spp. have shown that when bacteria are placed on copper surfaces, outer membrane integrity is compromised, hydroxyl radicals are produced, respiration is inhibited, and DNA is degraded (217). Fenton chemistry-based killing of pathogens also occurs in the host via host-generated reactive oxygen species. Because of the need for new therapeutics to treat antibiotic-resistant pathogens, research into the use of copper as an antimicrobial has lately increased. For example, the use of copper-containing surfaces in hospitals has been shown to greatly reduce environmental contamination with nosocomial pathogens and to reduce rates of health care-acquired infections (168; 185; 210). Additionally, copper-containing wound dressings are in development to aid in healing of infected wounds (5; 21).

The damaging effects of copper have been harnessed by the host immune system. Indeed, phagocytic immune cells employ a copper burst within the phagosome to kill pathogens (181). Concentrations of copper upwards of 0.5 mM have been measured in macrophage phagosomes (213). Additionally, host mobilization of copper also occurs in response to infection: increased concentrations of copper have been measured in serum and in wound exudate (98; 132). Consequently, pathogens with mutations in crucial copper resistance genes have been found to have impaired intracellular survival, colonization, and/or virulence (43). Furthermore, it was recently shown that a copper

sensitive mutant strain of *A. baumannii* demonstrated reduced colonization of the respiratory tract of mice (7).

In bacterial species where copper homeostasis has been well characterized, a variety of proteins are utilized to facilitate copper homeostasis. Though this process remains poorly understood in *A. baumannii*, recent studies have identified many putative copper resistance gene homologs in *A. baumannii* clinical isolates (7; 79; 223). For example, in the model clinical multidrug-resistant isolate, AB5075, copper resistance genes exist in four distinct chromosomal regions named A - D (Fig. 11) (223). These regions contain genes predicted to encode homologs of the most important copper resistance proteins of bacteria, e.g. copper oxidases, copper ATPases, copper transporters, copper chaperones, and regulatory proteins (223).

Region A includes genes for a large RND family efflux pump, *czcCBAD*, and a hypothetical protein ABUW_0265, which is located at the start of the operon. A similar type of pump, CusCFBA, has been shown to contribute to copper resistance in *E. coli* (55). While the genes in this operon are annotated as copper-related in AB5075, expression does not increase in response to copper exposure (223) and it has been suggested that *A. baumannii* in fact does not encode *cus* homologs (7), thus the role of this operon in copper resistance is very tentative.

Region B encodes four genes, including homologs of two known copper resistance proteins. CueR, CopA1, and the hypothetical protein ABUW_2705 are encoded as an operon; divergently transcribed from that operon is a putative copper chaperone, ABUW_2708, recently named CopZ (7). CueR has been well studied in *E. coli* and is a cytoplasmic transcription factor that binds copper ions with exquisite

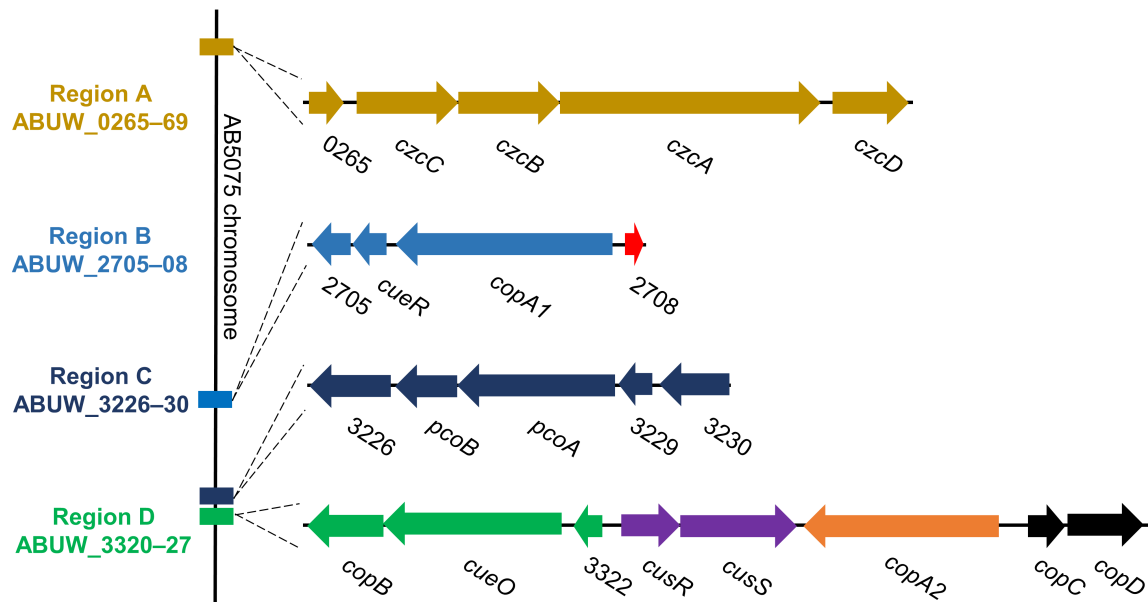


Figure 11. Organization of regions A - D in the AB5075 chromosome.

We previously identified 22 putative copper-related genes in the genome of AB5075 (223). These genes are located in four chromosomal regions named A - D. The relative location of each region on the AB5075 chromosome is shown on the left (not to scale), and the genes and regions are depicted to scale relative to one another. Each operon is colored uniquely, and the same colors are maintained in Figure 23. Gene annotations are shown when available; otherwise, ORF numbers are used.

sensitivity (27). Upon copper binding, CueR positively regulates transcription of copper resistance genes. In *E. coli*, CueR is known to regulate the copper ATPase CopA and the copper oxidase CueO (151; 228). The CueR regulon in *A. baumannii* is not yet characterized; however, a putative CueR binding site has been identified in the intergenic space of region B, suggesting that all of the genes in region B may be regulated by CueR (7). Appropriately, the genes of region B are all upregulated in response to copper in a similar manner (223). A CopA1 homolog, also encoded in region B, is a putative copper ATPase, which is another important and well-characterized copper resistance protein. Copper ATPases use the energy of ATP to move copper ions into the periplasmic space from the cytoplasm and are known to contribute to copper resistance in a number of bacteria (164). Very recently, the copper ATPase in region B was demonstrated to contribute to copper resistance of *A. baumannii* AB5075 *in vitro* and to contribute to colonization in a mouse respiratory infection model (7).

Region C encodes a single operon of five genes, ABUW_3226 – 3230. Two of the genes, ABUW_3227 and ABUW_3228, are annotated as *pcoB* and *pcoA*, respectively. In *E. coli*, the *pco* system is plasmid-based; while the *pco* proteins seem to be homologous to known chromosomal copper resistance genes, their functions are less well studied and understood (112). PcoB is homologous to CopB, an outer membrane protein that may function as a copper transporter (25). PcoA is homologous to CueO, a periplasmic copper oxidase that detoxifies by oxidizing copper ions to their less damaging form (66). Surprisingly, expression of the genes in region C does not change in response to copper exposure (223); however, that does not preclude the possibility that these genes are indeed involved in copper resistance.

Region D is the largest of the identified regions; it contains a total of eight genes arranged as two pairs of divergently transcribed operons. Among these eight genes are seven homologs of characterized copper resistance proteins: CopB, CueO, CusR, CusS, CopA2, CopC, and CopD. The functions of some of these copper-related proteins have been previously elucidated in other bacteria. CopB and CopD are membrane proteins and putative copper transporters, though not yet well characterized (25). CueO is a periplasmic copper oxidase, which detoxifies by oxidizing cuprous copper ions (Cu^{1+}) to their less damaging cupric (Cu^{2+}) form (66). CopA2 is a second copper ATPase, the other being CopA1 of region B; previous work did not reveal a role for this gene in copper resistance in AB5075 (7). CopC is a periplasmic copper chaperone protein that likely contributes to copper resistance by binding copper ions as a means to prevent reactivity and/or by shuttling ions between other copper resistance proteins (12; 152; 161). CusR and CusS form a two-component system that senses periplasmic copper ions and activates the CusR regulon in response (70). In *E. coli*, CusR is known to directly regulate expression of the *cusRS* operon and the divergently transcribed *cusCFBA* operon (70; 138; 228). It is not yet known which genes comprise the *A. baumannii* CusR regulon; however, putative CusR binding sites were identified in the intergenic regions upstream of the four operons in region D, suggesting that all of region D may be regulated by CusR (7).

Notably, not all clinical isolates of *A. baumannii* carry all the copper-related genes found in AB5075. Indeed, isolates show marked differences in copper sensitivity that directly correlates with the presence of the region D genes; clinical isolates lacking region D demonstrate significantly less copper resistance than isolates that encode the

region D genes (223). Moreover, expression of the genes in region D dramatically increases in response to copper exposure (223). These results strongly suggest that the region D genes are crucial for extensive copper homeostasis capability in *A. baumannii*.

Given the growing interest in targeting copper homeostasis as a novel antimicrobial strategy and the lack of understanding of which factors contribute to copper resistance in *A. baumannii*, we sought to identify genes that were important for copper resistance in this important pathogen. Herein we describe a detailed analysis of 21 mutant strains, each bearing insertions in the genes carried in regions A - D (Fig. 11). Marked copper sensitivity of a subset of the mutant strains, as well as defects in virulence, are described.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains are listed in Table 8. Strains were routinely streaked from freezer stocks and were grown overnight at 37°C on 1.5% lysogeny broth (LB) agar plates (MoBio, Carlsbad, CA). Liquid cultures of *A. baumannii* were grown at 37°C shaking at 190 rpm in LB or in M9 minimal medium (170) supplemented with 0.1% casamino acids (Difco, Franklin Lakes, NJ); the M9 medium was always supplemented with amino acids, but for simplicity is referred to as “M9 medium” throughout the manuscript. Importantly, overnight cultures were consistently started from a single opaque colony, as phase variation has been shown to affect growth and virulence (199). Overnight growth was

restricted to a period of 16 - 19 hours. *A. baumannii* strains freezer stocks were maintained at -80°C in medium supplemented with 40% glycerol.

AB5075 Mutant Strains

Transposon mutant strains were purchased from the comprehensive, ordered transposon library engineered by Dr. Colin Manoil's group at the University of Washington (56). The isolates were received as stabs, which were streaked onto LB agar plates. The following day, a swab of the growth was used to inoculate an overnight culture in LB medium, which was subsequently frozen as a master stock for each mutant strain. To verify the desired mutations, each master stock was streaked on LB agar containing 10 $\mu\text{g/mL}$ tetracycline to select for cells containing the Tn5 insertion that characterizes the transposon library strains. For each, a single opaque colony was picked and restreaked onto LB agar containing tetracycline. The following day, a single opaque colony was picked and used to inoculate an overnight culture in LB medium containing 10 $\mu\text{g/mL}$ tetracycline; the resulting cultures were frozen as working stocks for each mutant strain and were used for all future experiments. The desired transposon insertion was confirmed by PCR using primers that flank the proper ORF; primers are listed in Table 9. Throughout the manuscript, strains are always organized by ascending gene number, as in Figure 11. A few genes' annotated names differ from the conventional names for the given protein; these genes' annotated name and conventional name are both provided here, but the conventional names are used throughout for consistency and clarity: *actP2/copA1*, *copA/cueO*, *copR/cusR*, *copS/cusS*, *actP1/copA2*.

Table 8. Strains used in this study

Transposon Mutant Strains					
Strain Name	Shorthand name¹	Locus Tag	Strain Name in Transposon Library²	Lab Designation	Reference
AB5075	Wild-type			DSM1866	(93)
AB5075 <i>pcoB</i> 140::T26	<i>pcoB</i> ::T26	ABUW_3227	tnab1_kr121204p07q140	DSM1833	(56)
AB5075 <i>pcoA</i> 104::T26	<i>pcoA</i> ::T26	ABUW_3228	tnab1_kr121203p02q104	DSM1823	(56)
AB5075 <i>copB</i> 156::T26	<i>copB</i> ::T26	ABUW_3320	tnab1_kr121203p05q156	DSM1837	(56)
AB5075 <i>copA/cueO</i> 118::T26	<i>cueO</i> ::T26	ABUW_3321	tnab1_kr121128p03q118	DSM1840	(56)
AB5075 <i>copR/cusR</i> 106::T26	<i>cusR</i> ::T26	ABUW_3323	tnab1_kr121128p07q106	DSM1825	(56)
AB5075 <i>copS/cusS</i> 128::T26	<i>cusS</i> ::T26	ABUW_3324	tnab1_kr121205p01q128	DSM1832	(56)
AB5075 <i>copC</i> 160::T26	<i>copC</i> ::T26	ABUW_3326	tnab1_kr121119p04q160	DSM1844	(56)
AB5075 <i>copD</i> 117::T26	<i>copD</i> ::T26	ABUW_3327	tnab1_kr121204p06q117	DSM1830	(56)
AB5075 ABUW_0265-193::T26		ABUW_0265	tnab1_kr130913p10q193	DSM1856	(56)
AB5075 <i>czcC</i> 151::T26		ABUW_0266	tnab1_kr121127p01q151	DSM1849	(56)
AB5075 <i>czcB</i> 187::T26		ABUW_0267	tnab1_kr121205p01q187	DSM1848	(56)
AB5075 <i>czcA</i> 162::T26		ABUW_0268	tnab1_kr121204p04q162	DSM1846	(56)
AB5075 <i>czcD</i> 174::T26		ABUW_0269	tnab1_kr121205p05q174	DSM1851	(56)
AB5075 ABUW_2705-139::T26		ABUW_2705	tnab1_kr130903p04q139	DSM1857	(56)
AB5075 <i>cueR</i> 139::T101		ABUW_2706	tnab1_jr130919p01q139	DSM1858	(56)
AB5075 <i>actP2/copA</i> 1-181::T26		ABUW_2707	tnab1_kr121128p08q181	DSM1822	(56)
AB5075 ABUW_3226-176::T26		ABUW_3226	tnab1_kr121211p04q176	DSM1859	(56)
AB5075 ABUW_3229-182::T26		ABUW_3229	tnab1_kr130913p04q182	DSM1860	(56)
AB5075 ABUW_3230-184::T26		ABUW_3230	tnab1_kr130904p01q184	DSM1861	(56)
AB5075 ABUW_3322-131::T26		ABUW_3322	tnab1_kr130904p04q131	DSM1862	(56)
AB5075 <i>actP1/copA</i> 2-159::T26		ABUW_3325	tnab1_kr121203p05q159	DSM1828	(56)
Complemented Mutant Strains³					
Proper Name	Shorthand Name¹			Lab Designation	Reference

AB5075 <i>pcoB</i> 140::T26 <i>attTn7-hph-pcoB</i>	<i>pcoB</i> ::T26 ^C	DSM1904	This study
AB5075 <i>pcoA</i> 104::T26 <i>attTn7-hph-pcoA</i>	<i>pcoA</i> ::T26 ^C	DSM1905	This study
AB5075 <i>copB</i> 156::T26 <i>attTn7-hph-copB</i>	<i>copB</i> ::T26 ^C	DSM1901	This study
AB5075 <i>copA/cueO</i> 118::T26 <i>attTn7-hph-cueO</i>	<i>cueO</i> ::T26 ^C	DSM1906	This study
AB5075 <i>copR/cusR</i> 106::T26 <i>attTn7-hph-cusR</i>	<i>cusR</i> ::T26 ^C	DSM1890	This study
AB5075 <i>copS/cusS</i> 128::T26 <i>attTn7-hph-cusS</i>	<i>cusS</i> ::T26 ^C	DSM1902	This study
AB5075 <i>copC</i> 160::T26 <i>attTn7-hph-copC</i>	<i>copC</i> ::Tn5 ^C	DSM1897	This study
AB5075 <i>copD</i> 117::T26 <i>attTn7-hph-copD</i>	<i>copD</i> ::Tn5 ^C	DSM1898	This study
Mating Strains			
Strain Name	Description	Lab Designation	Reference
HB101/pRK2013	Mating helper strain (<i>oriT</i> helper)	DSM1881	(107)
DH5α λpir/pTNS2	Mating helper strain (transposase)	DSM1880	(107)
DH5α/pUC18T mini-Tn7- <i>hph</i>	Tn7 with hygromycin resistance gene, <i>hph</i> , and MCS	DSM1883	(93)
DH5α/pUC18T mini-Tn7- <i>hph-pcoA</i>	Tn7 with <i>hph</i> and <i>pcoA</i> with native promoter	DSM1894	This study
DH5α/pUC18T mini-Tn7- <i>hph-pcoB</i>	Tn7 with <i>hph</i> and <i>pcoB</i> with native promoter	DSM1895	This study
DH5α/pUC18T mini-Tn7- <i>hph-copB</i>	Tn7 with <i>hph</i> and <i>copB</i> with native promoter	DSM1899	This study
DH5α/pUC18T mini-Tn7- <i>hph-cueO</i>	Tn7 with <i>hph</i> and <i>cueO</i> with native promoter	DSM1903	This study
DH5α/pUC18T mini-Tn7- <i>hph-cusR</i>	Tn7 with <i>hph</i> and <i>cusR</i> with native promoter	DSM1889	This study
DH5α/pUC18T mini-Tn7- <i>hph-cusS</i>	Tn7 with <i>hph</i> and <i>cusS</i> with native promoter	DSM1900	This study
DH5α/pUC18T mini-Tn7- <i>hph-copC</i>	Tn7 with <i>hph</i> and <i>copC</i> with native promoter	DSM1893	This study
DH5α/pUC18T mini-Tn7- <i>hph-copD</i>	Tn7 with <i>hph</i> and <i>copD</i> with native promoter	DSM1896	This study
Additional Strains			
Strain Name	Description	Lab Designation	Reference
AB4857/pAJ100	AB4857 with empty vector, pAJ100	DSM1908	This study
AB4857/pAJ100 – Region D	AB4857 with pAJ100 carrying Region D	DSM1910	This study
AB5711/pAJ100	AB5711 with empty vector, pAJ100	DSM1909	This study
AB5711/pAJ100 – Region D	AB5711 with pAJ100 carrying Region D	DSM1911	This study

<i>E. coli</i> /pAJ100		DSM1884	This study
<i>E. coli</i> Top 10/pAJ100 – Region D		DSM1907	This study
AB5075 <i>attTn7-hph</i>	AB5075 with a hygromycin resistance gene in the <i>attTn7</i> site	DSM1926	This study
AB5075 <i>copR/cusR106::T26 attTn7-hph</i>	The <i>cusR</i> mutant strain with a hygromycin resistance gene in the <i>attTn7</i> site	DSM1927	This study
AB5075 <i>copC160::T26 attTn7-hph</i>	The <i>copC</i> mutant strain with a hygromycin resistance gene in the <i>attTn7</i> site	DSM1928	This study

¹For simplicity, mutant strains that were found to have increased copper sensitivity and were included in multiple assays were given a shorthand name.

²The mutant strains in the transposon library were created using a Tn5 transposon carrying a tetracycline resistance cassette (T26) or hygromycin resistance cassette (T101) (56). Individual strains were purchased from the ordered, annotated library.

³Complemented strains were created by tri-parental mating of each transposon mutant strain with *E. coli* DH5 α carrying the appropriate ORF and native promoter on the plasmid pUC18T mini-Tn7-*hph*, as well as with the two helper strains of *E. coli* that aid in the mating and transposition processes. The Tn7 from the pUC18T mini-Tn7-*hph* plasmid, containing a hygromycin resistance gene and the desired complementation ORF and promoter, inserts into the AB5075 chromosome at the *attTn7* site located in an intergenic region downstream of the *glmS* locus.

Table 9. Oligonucleotides used in this study

T26 Insertion Confirmation Primers		
Flanked Gene Annotation¹	Primer Name²	Sequence (5'-3')
ABUW_0265	ABUW_0265-up ABUW_0265-dw	GTGCAATTTCTAACAGCCATG CAATAAATAAAAGATGCGGTGGG
ABUW_0266 <i>czcC</i>	czcC-up czcC-dw	TGGCAGCAATTACTCCAACC GCCATCCCGACTTATTGAGCT
ABUW_0267 <i>czcB</i>	czcB-up czcB-dw	CACCAATCCACGCCAGAAC TGGCGAAAGATGCCTTAATGC
ABUW_0268 <i>czcA</i>	czcDA-up czcA-dw	GCAACAGTAGTCGCATTTGTGG GCTTCTTACTGAAGTCCGAATTGG
ABUW_0269 <i>czcD</i>	czcDA-up czcD-dw	GCAACAGTAGTCGCATTTGTGG GGCAACAGTGGTTATTGGTGG
ABUW_0611	ABUW_0611-up ABUW_0611-dw	GGTTTCTAGGAGCGCTGCC GCACCTGCAAAGTATGGCTCTAC
ABUW_2705	ABUW_2705-up ABUW_2705-dw	CATGACAGCAGGTCTTAAACC CCTGTGGATCGAGGAGATATTAC
ABUW_2706 <i>cueR</i>	cueR-up-NEW cueR-dw-NEW	GAGGTGTGAGATGAATATCGGTCAG GTACTTGAGTGATGGCCGC
ABUW_2707 <i>copA1</i>	CopA2-up CopA2-dw	GCTTGACCTTCCCATGATGG CTGACCGATATTCATCTCACACCTC
ABUW_3226	ABUW_3226-up ABUW_3226-dw	GTATTATGGAGCTGGGTAAACAC GTTCTCAGTATGTGAAGAC
ABUW_3227 <i>pcoB</i>	CopB2-up CopB2-dw	CTGAAAATGAGAAAGGAGCTAG CATAAGCTTACTTTTTGTTCGGC
ABUW_3228 <i>pcoA</i>	CopA2-up CopA2-dw	CGTCCACCAAAGCTTAATCC GAGAAAGCACCGCAACATATC
ABUW_3229	ABUW_3229-up ABUW_3229-dw	CCAAGATAAATAGATTGCTCAAGGC CGAGGCTGCTAATCGTTTTATG
ABUW_3230	ABUW_3230-up ABUW_3230-dw	GGATAACTGTGTTGATAACCATATGG CCATCTGAGTGGATTGTTGTTAAG
ABUW_3220 <i>copB</i>	CopB1-up CopB1-dw	CCTAATACTAATGATAAGGGGG CTTTATGAATAGTAGCTTCGGC
ABUW_3221 <i>cueO</i>	ABUW_3322-up CopA1-dw	CCGGTTCCAACCTATAAGTTAG GCAAAAGCTAGACCTGATAATCC
ABUW_3322	ABUW_3322-up ABUW_3322-dw	CCGGTTCCAACCTATAAGTTAG GTTAATTGAGGGCTAAGCAGG
ABUW_3323 <i>cusR</i>	CusR-up CusR-dw	CTTAATATGCGATACGGGGC GCAATACGGAACTGATCGC
ABUW_3324 <i>cusS</i>	CusS-up Cus-dw	GGAATGGGGTATGTCTTAGAGG GAAAGGATCATTCTCTGACTACTC
ABUW_3325 <i>copA2</i>	CopA1-up_NEW CopA1-dw_NEW	GCCGGAGTAGCACTTTCAAG GAGGAAGGCACAGGTTTAGG
ABUW_3326	CopC-up	GGAATTTCACTGGGTCATTAC

<i>copC</i>	CopC-dw	CTGCTGGCATATAGACTAC
ABUW_3327	CopD-up	GCATATTACTATCGCCGG
<i>copD</i>	CopD-dw	GAAAGCATTGCTACTACTCC
Complementation Cloning Primers		
Fragment	Primer Name	Sequence (5'-3') ³
<i>pcoAB</i> promoter	pcoAB promoter up	CCC <u>GGTACC</u> cggtagtgtagacgcttaa
	pcoAB promoter dw-A	GGCTGCTAATCGTTTTATGAGACATtaatgatctaggtctctactaaaa
	pcoAB promoter dw-B	AGAAAATAACTTAGTGATGCGCATtaatgatctaggtctctactaaaa
<i>pcoA</i> ORF	pcoA complement up	TTTtagTAAGAGACCTAGATCATTAAatgtctcataaaa
	pcoA complement dw	CCC <u>GGATCC</u> gagaaagcaccgcaacatc
<i>pcoB</i> ORF	pcoB complement up	TTTtagTAAGAGACCTAGATCATTAAatgcgcatacctaagttatttct
	pcoB complement dw	CCC <u>GGATCC</u> catctttgcaccataactgacac
<i>copAB</i> promoter	copAB promoter up	CCC <u>GGTACC</u> aagagcttgatgtttacctg
	copAB promoter dw	cattgattgctccaaaaataattttatataactaac
<i>cueO</i> ORF	NEW copA/cueO complement up	AAATTTATTTTTGGAGCAATCAatgtctagaaaattaagtc
	copA/cueO complement dw	CCC <u>GGATCC</u> ccatgctttgaacatccgtatc
<i>copB</i> ORF	NEW copB complement up	AAATTTATTTTTGGAGCAATCAatgcgcaccactaaaaa
	copB complement dw	CCC <u>GGATCC</u> gatttgaacgcttttaagccc
<i>cusRS</i> promoter and <i>cusR</i> ORF	cusR complement up	CCC <u>GGTACC</u> cccaacacacataaaagcag
	cusR complement dw	CCC <u>GGATCC</u> cttctagctgagtacggc
<i>cusRS</i> promoter	cusR complement up	CCC <u>GGTACC</u> cccaacacacataaaagcag
	NEW <i>cusRS</i> promoter dw – <i>cusS</i> SOE	GATCGCATTAACAGTTTATGAGTcatttattaagccccgtatcgcatattaag
<i>cusS</i> ORF	cusS complement up	AATATGCGATACGGGGCTTAATAAatgactcataaactgttaatgcgac
	cusS	CCC <u>GGATCC</u> gaaaggatcattctctgactactc

	complement dw	
<i>copCD</i> promoter and <i>copC</i> ORF	copCD promoter up	CCCGGTACCccatgccacagacaggatc
	copC complement dw	CCCGGATCCcgteccagtagaaaatagctaaagg
<i>copCD</i> promoter	copCD promoter up	CCCGGTACCccatgccacagacaggatc
	copCD promoter dw	catttcacacctttttaaattagtacttgc
<i>copD</i> ORF	copD complement up	GTACTAATTTTAAAAAGGTGATGAAatgaatcctgaa acttggatatatgcaac
	NEW copD comp dw	CCCGGATCCgacgtggtccttttatgcagc
Primers used in the creation of pAJ100		
pWHori NcoI For		GATTACACCATGGgatcgtagaaatatctatgattatcttgaa
pWHori NcoI Rev		GATTACACCATGGggattttaacattttgcgttggtccaa

¹Gene names are given where available. For those whose annotated name is different from the name traditionally given to that protein, the conventional name is listed.

²The primer name may not match the gene name.

³Capital letters signify added primer tail sequences for SOE or restriction site. Underlined sequences are restriction enzyme cut sites.

Generation of Complemented Strains

The previously described Tn7-based strategy of complementation was used to complement the transposon mutant strains (93; 94; 107). The wild-type ORF and native promoter were amplified using the primers listed in Table 9. If the ORF was not located directly downstream of the promoter due to operonic structure, the two DNA fragments were fused by SOE PCR. The full construct was amplified with Phusion Hot Start Polymerase (Thermo Fisher Scientific) and then cloned into pUC18T mini-Tn7-*hph* using the added restriction enzyme sites (Table 9). The resulting plasmids were transformed into electrocompetent DH5 α *E. coli* and transformants were selected for on LB agar with 100 μ g/mL ampicillin. Correct plasmid construction was confirmed by digestion and then the resulting *E. coli* strains were mated with the AB5075 transposon mutant strains using the tri-parental protocol described by Jacobs *et. al.* (94). Briefly, 100 μ L of four overnight cultures (*E. coli* carrying the complementation construct in pUC18T-mini Tn7-*hph*, two helper strains, and the AB5075 mutant strain) grown in LB medium with the appropriate antibiotics were mixed, washed twice, and spotted onto plain LB agar. The mating was allowed to proceed for 1 to 24 hours. The cells in the spot were collected with a sterile loop and plated on LB agar with 25 μ g/mL chloramphenicol to kill the *E. coli* strains and 250 μ g/mL hygromycin to select for *A. baumannii* with the Tn7 insertion. Resulting colonies were screened for Tn7 insertion using colony PCR with the previously described *att*Tn7 primers (93). Single colonies were restreaked onto LB agar with 10 μ g/mL tetracycline and 250 μ g/mL hygromycin. A single opaque colony was used to inoculate an overnight culture in LB with tetracycline and hygromycin, which was

ultimately used to create a freezer stock of the strain. The correct insertion at the *attTn7* site was confirmed by PCR and sequencing.

Cloning of Region D from AB5075 into Other *A. baumannii* Strain Backgrounds

Creation of pAJ100: An *E. coli* – *A. baumannii* shuttle vector with a hygromycin resistance gene was created to move genes into multidrug-resistant *A. baumannii*. To generate this vector, pMQ300 (100) was digested with BspHI to remove the 1.7 kb region that contained yeast replication genes URA3 and CEN6/ARSH4. The *A. baumannii* origin of replication of pWH1266 (89) was amplified using the pWHori NcoI For and pWHori NcoI Rev primers described in Table 9. This insert was digested with NcoI and then ligated to the remaining 4.7 kb of pMQ300. The resulting 6 kb plasmid was transformed into *E. coli* and plated on LB agar with 250 µg/mL hygromycin. The resulting vector was named pAJ100. This vector has a *lacZ* insertion site for genes that contains cut sites for the following enzymes: SmaI, BamHI, HindIII, KpnI, PstI, and PvuI.

Creation of pAJ100 – Region D: Region D was amplified from AB5075 chromosomal DNA with Phusion Hot Start Polymerase (Thermo Fisher Scientific) using the copB complement dw and copD complement dw primers described in Table 9. The fragment was cloned into pAJ100 using the added BamHI restriction enzyme sites. The resulting pAJ100 – Region D plasmid was electroporated into *E. coli* Top 10 cells, which were plated on 250 µg/mL hygromycin, 40 µg/mL X-gal, and 1mM IPTG. A white colony was restreaked for isolation, and a single colony was used to inoculate an overnight culture of LB medium with 250 µg/mL hygromycin. Plasmid was purified and

then electroporated into AB4857 and AB5711, which had been made electrocompetent using the protocol described by Jacobs *et. al.* (94). Transformants were selected for on LB with 250 µg/mL hygromycin. Double-purified single, opaque colonies were used to inoculate overnight cultures of LB medium with 250 µg/mL hygromycin, which were then used to create freezer stocks of the strains. Correct plasmid construction was confirmed by digestion and PCR.

Determination of *A. baumannii* Sensitivity to Copper in Liquid Culture

To assess the effect of copper on *A. baumannii* growth, bacterial strains were grown in the presence of increasing concentrations of copper sulfate as previously described (222; 223). Briefly, bacteria from overnight cultures were subcultured at an optical density (OD₆₀₀) of 0.05 in 10 mL of M9 medium containing 0.1 – 1.5 mM CuSO₄ (Aldrich, St. Louis, MO). Growth was measured every hour for six hours by optical density (OD₆₀₀) and by enumerating CFU every other hour. Three biologically independent experiments were completed for each strain showing noticeable copper sensitivity (Fig. 13 and 14); if no difference from the wild-type was observed, two biologically independent experiments were completed (Fig. 12).

Assessing Copper Sensitivity of *A. baumannii* Biofilm

Biofilms were grown, exposed to copper, and assessed for survival as previously described with minor adjustments to the incubation time and growth media (223). Briefly, overnight cultures of *A. baumannii* strains were grown in CFA medium (49), and bacteria were diluted to an OD₆₀₀ of 0.05 in 24-well tissue-culture treated plates (Corning,

Corning, NY) in 1 mL of CFA medium. Plates were incubated statically at 37°C for 24 hours. To expose the pre-formed biofilms to copper, the broth was removed and was replaced with 1.5 mL of M9 medium containing 1.5 mM CuSO₄; biofilms were incubated for another 6 or 24 hours. To determine the number of planktonic cells, at each timepoint (0, 6, and 24 hours) the medium was transferred to a 1.5 mL Eppendorf tube and vortexed for approximately 5 seconds to disrupt any clumped planktonic bacteria in the supernatant. To determine the number of biofilm cells, the biofilm was scraped from the sides and bottom of the well with a pipette tip and was resuspended in 1 mL of PBS. Samples of both planktonic cells and biofilm cells were plated to enumerate CFU. Three biologically independent experiments were performed.

Quantification of Intracellular Copper via ICP-MS

Sample Collection: To measure the intracellular copper concentration, dry cell samples were collected and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Overnight cultures were subcultured to an OD₆₀₀ of 0.05 in a 100 mL volume. The subcultures were grown for 2.5 hours to reach log phase, and a time zero (T0) sample was collected. The sample collection procedure was as follows: 6 mL of culture was pelleted in three 2-mL tubes using a 4°C table top centrifuge at max speed for 30 seconds; the supernatant was aspirated. The three pellets were resuspended in 1 mL of cold wash solution (PBS with 0.5 mM EDTA) in a single tube and pelleted again; the supernatant was aspirated. The wash steps were repeated for a total of three washes. After the final aspiration, the tube was left open to begin air drying. A 0.5 mL sample of the culture was separately collected in a cuvette to measure the OD₆₀₀ of the culture. The cell

pellets were fully dried in an 80°C SpeedVac vacuum concentrator for 1 hour and stored at -20°C. After collection of the T0 sample, 0.25 mM copper sulfate was added directly to the large culture, swirled immediately, and returned to the shaking incubator.

Additional samples were collected along the time course. In experiment two, with the complete set of mutant strains showing a copper sensitive phenotype, the final wash step was conducted in a 15-mL conical tube and spun for 3 minutes at 4150 rpm / 3,700 x g, and the cell pellets were dried overnight in a 37°C incubator. This eliminated the need for transfer of many samples to conical tubes before ICP-MS analysis.

After the copper shock portion of the experiment was completed, the remaining culture was washed and placed into fresh growth medium for recovery. Briefly, the remaining culture was placed in a 50-mL conical tube and spun for 15 minutes at maximum speed (4150 rpm / 3,700 x g) to pellet the cells. The cells were resuspended in 2 mL of wash solution and were transferred to two 2-mL tubes. The cells were pelleted by spinning for 1 minute at max speed in a tabletop centrifuge; the supernatants were aspirated. The wash steps were repeated for a total of three washes. The cells were resuspended in 50 mL of pre-warmed growth medium without added copper. Samples were collected in the same way as described above across the recovery time course.

Three biologically independent replicates were performed for both experiments.

Copper Measurement: The dry cell pellets were thawed at room temperature and dissolved in 200 µL of concentrated HNO₃ (trace metal grade) overnight at 80°C. The following day, the samples were cooled to room temperature and diluted with 2 mL of MilliQ water, for a final HNO₃ concentration of 6%. Where necessary, the samples were transferred to metal-free 15-mL conical tubes for ICP-MS analysis. The metal content of

the samples was then analyzed by quantitative ICP-MS. Copper concentrations were determined by injecting diluted samples into an Agilent 7700x ICP-MS (Agilent Technologies, Santa Clara, CA). Copper levels were detected using an Octopole Reaction System cell (ORS) in He mode. The ICP-MS parameters used for the analysis were: an RF power of 1550 W, an argon carrier gas flow of 0.99 L/min, helium gas flow of 4.3 mL/min, octopole RF of 200 V, and OctP bias of -18 V. Samples were directly infused using the 7700X peristaltic pump with a speed of 0.1 rps and a micromist nebulizer. Copper concentrations in samples were derived from a calibration curve generated by a series of dilutions of a copper atomic absorption standard (Fluka Analytical, St. Louis, MO) prepared in the same matrix as the samples. Data analysis was performed using Agilent's Mass Hunter software (4.4 version).

Calculations: The value originally obtained from ICP-MS (ppb, $\mu\text{g/L}$) was divided by the molecular weight of copper (63.546 g/mol) to determine the concentration of copper in the diluted sample (μM). The sample concentration was multiplied by the total volume (2.2 mL) to determine the quantity of copper in the sample (nmol). The total amount of copper was divided by the sample volume (6 mL) to determine nmol/mL of cells and divided by the OD_{600} to determine the nmol/ODU of cells. The data were plotted as nmol/ODU vs. time. This protocol was recently described in more detail as a methods chapter (224).

Infection of *Galleria mellonella* Caterpillars

A. baumannii strains were grown overnight in LB medium with the appropriate antibiotics: tetracycline for transposon mutant strains, and both tetracycline and

hygromycin for complemented strains. Overnight cultures were diluted to an OD₆₀₀ of 0.05 and were grown 3 hours to log phase. The cells were adjusted in PBS to a specific OD₆₀₀ to achieve the desired CFU/mL, and were then diluted 1:10 in PBS with 0.01% bromophenol blue dye for visibility. For a dose of 5×10^4 CFU, bacteria were adjusted to an OD₆₀₀ of 0.2 – 0.3, which corresponded to 10^7 CFU/mL; for a dose of 5×10^5 , bacteria were adjusted to an OD₆₀₀ of 2 – 3, or 10^8 CFU/mL. The exact number of bacterial cells in the inoculum was determined by serial dilution and plating for enumeration of CFU. The actual doses ranged from $3.53 - 9.63 \times 10^4$ and $3.38 - 9.60 \times 10^5$ CFU.

Galleria mellonella larvae (Vanderhorst Wholesale Inc, Saint Marys, OH) were utilized within 4 days of receipt. Larvae weighing 200 to 300 mg were used. The injections were carried out as described previously (93) with minor changes. Briefly, 5 µL of the sample was injected into the last left proleg using a 10-µL glass syringe (Hamilton, Reno, NV) fitted with a 31G needle. Each experiment included control groups of non-injected larvae and PBS-injected larvae. All larvae were incubated at 37°C, and death was assessed at 24 hour intervals for 5 days. Larvae were considered dead if they didn't respond to physical stimulus. All larvae that progressed to the pupation stage of the life cycle were excluded. For consistency, wild-type, mutant strain, and complemented strain infected groups that are plotted together were injected in the same experiment. Experiments were repeated using two or three different orders of larvae with 12 to 18 larvae per experimental group; the total n ranged from 25 to 48 larvae per strain. A single data set was excluded in which the wild-type strain killed only 30% of the caterpillars; no data from this set of infections were used.

Mouse Pneumonia Infection Model

Mice were infected with *A. baumannii* as described previously (93). Briefly, six-week-old female BALB-C mice were first rendered temporarily neutropenic by intraperitoneal injection with cyclophosphamide on days -4 and -1 before infection. On day 0, mice were anesthetized and infected intranasally with approximately 5×10^6 CFU of *A. baumannii* in a total volume of 25 - 50 μ L PBS. Animals were monitored for morbidity and mortality for 7 days and humanely euthanized according to protocol when necessary. All mouse studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (9), and procedures were approved by the Institutional Animal Care and Use Committee at the Walter Reed Army Institute of Research. Experiments with the *cusR* mutant strain and complemented derivative were performed in four biological replicates with 5 mice per group ($n = 20$ per strain), spaced two and two over time to ensure reproducibility; two biological replicates were completed with the *copD* mutant strain and complemented derivative ($n = 10$ mice per strain).

Data Analysis and Statistics

All graphs and statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). A two-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to evaluate differences in growth in various copper treatments. Data are presented as the geometric mean and standard error of the mean. A two-way ANOVA with Sidak's or Dunnett's correction was used to compare intracellular copper concentrations. Data are presented as arithmetic mean and range. An ordinary one-way ANOVA with Dunnett's correction followed by linear

contrasts with a Bonferroni adjustment was used to compare percent survival of biofilm and planktonic cells to the wild-type strain at each concentration and time point. Data are presented as arithmetic mean and range. Survival curves were compared using the Mantel-Cox log rank test with Holm's correction for multiple comparisons. For all tests, a two-sided α level set at 0.05 determined significance.

RESULTS

Mutant Strains of AB5075 Exhibit Copper Sensitivity

Many putative copper-related genes have been identified in *A. baumannii*; however, their predicted functions are based solely on protein homology, and the role of these proteins in copper resistance is largely unknown. To investigate the contribution of individual genes to copper resistance in AB5075, we used the available arrayed transposon mutant strain library (56) to initiate our studies. To this end, we obtained 21 mutant strains, each of which contained a T26 transposon insertion in a putative copper resistance gene. Specifically, we targeted the 22 genes found in regions A - D that we previously identified as putative copper resistance genes in AB5075 (223); only one small ORF, ABUW_2708, was not represented in the available mutant strain library. To assess the role of the remaining 21 genes in copper resistance, each of the mutant strains was individually grown in M9 medium containing increasing concentrations of CuSO₄. Thirteen of the mutant strains did not differ from the wild-type in their ability to grow in the presence of copper, indicating that the mutated gene was not individually contributing to copper resistance in the tested conditions (Fig. 12). Conversely, eight of the mutant

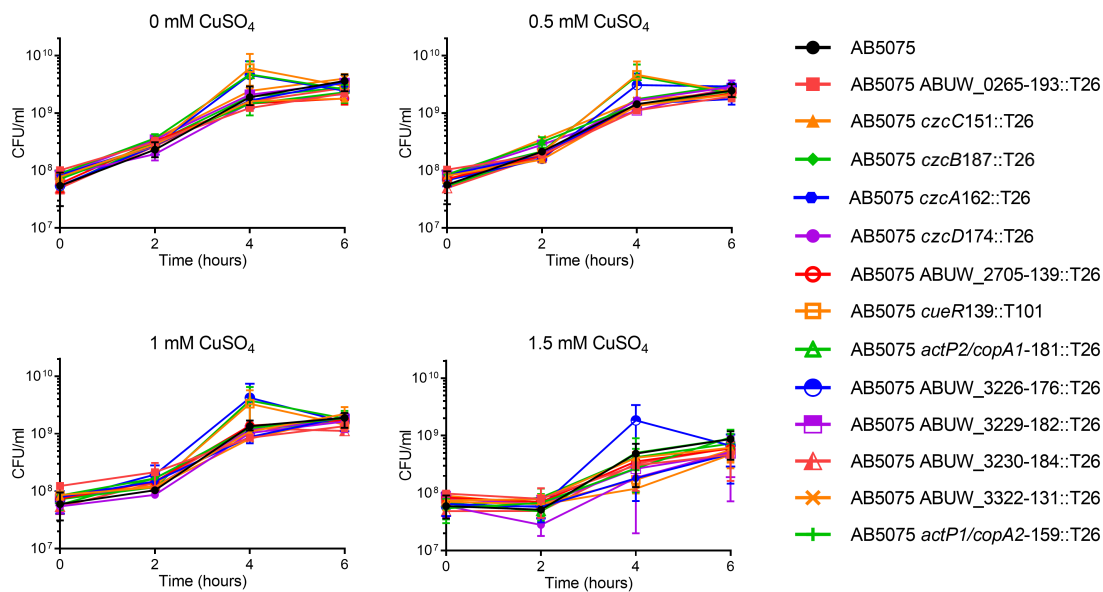


Figure 12. Growth of AB5075 mutant strains that displayed no change in copper sensitivity.

Strains were grown for 6 hours at 37°C in M9 medium supplemented with the concentration of CuSO_4 indicated in the legend. Growth was measured both by turbidity (OD_{600} , data not shown) and enumeration of viable colonies (CFU/mL). Two biologically independent experiments were completed, and the data are grouped by copper concentration and are presented as mean and range.

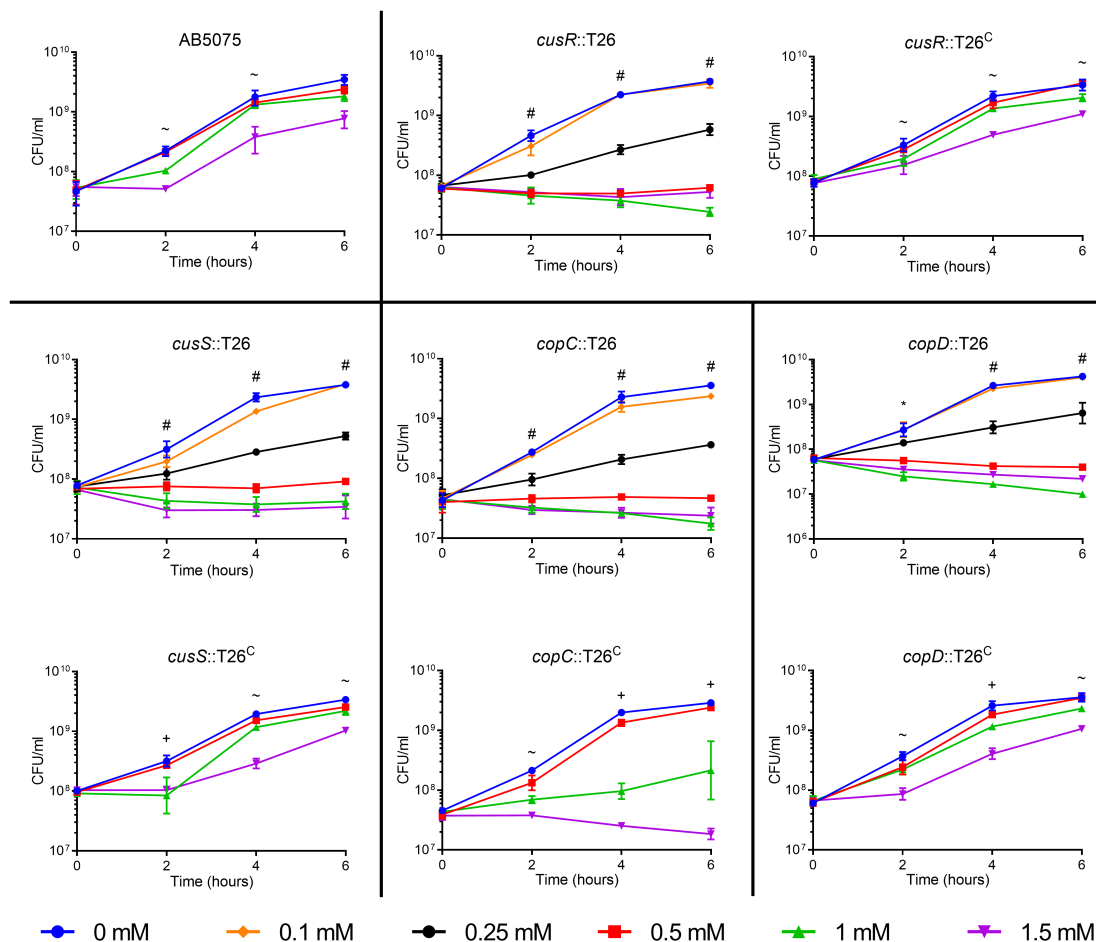


Figure 13. Growth of highly copper sensitive AB5075 mutant and complementation strains.

Strains were grown for 6 hours at 37°C in M9 medium supplemented with the concentration of CuSO_4 indicated in the legend. Growth was measured both by turbidity (OD_{600} , data not shown) and enumeration of viable colonies (CFU/mL). Black lines are used to group the mutant strain with the corresponding complemented derivative. The data are presented as geometric mean and SEM of three biologically independent experiments. Two-way ANOVA with Tukey's adjustment for multiple comparisons was used to compare growth in each copper concentration at each timepoint. The symbols indicate which copper treatments were statistically different ($P < 0.05$) from the 0 mM control, as follows: ~, growth in 1.5 mM CuSO_4 was different from the control; +, growth in 1 mM and 1.5 mM CuSO_4 was different from the control; *, growth in 0.5, 1, and 1.5 mM CuSO_4 was different from the control; and #, growth in 0.25, 0.5, 1, and 1.5 mM CuSO_4 was different from the control.

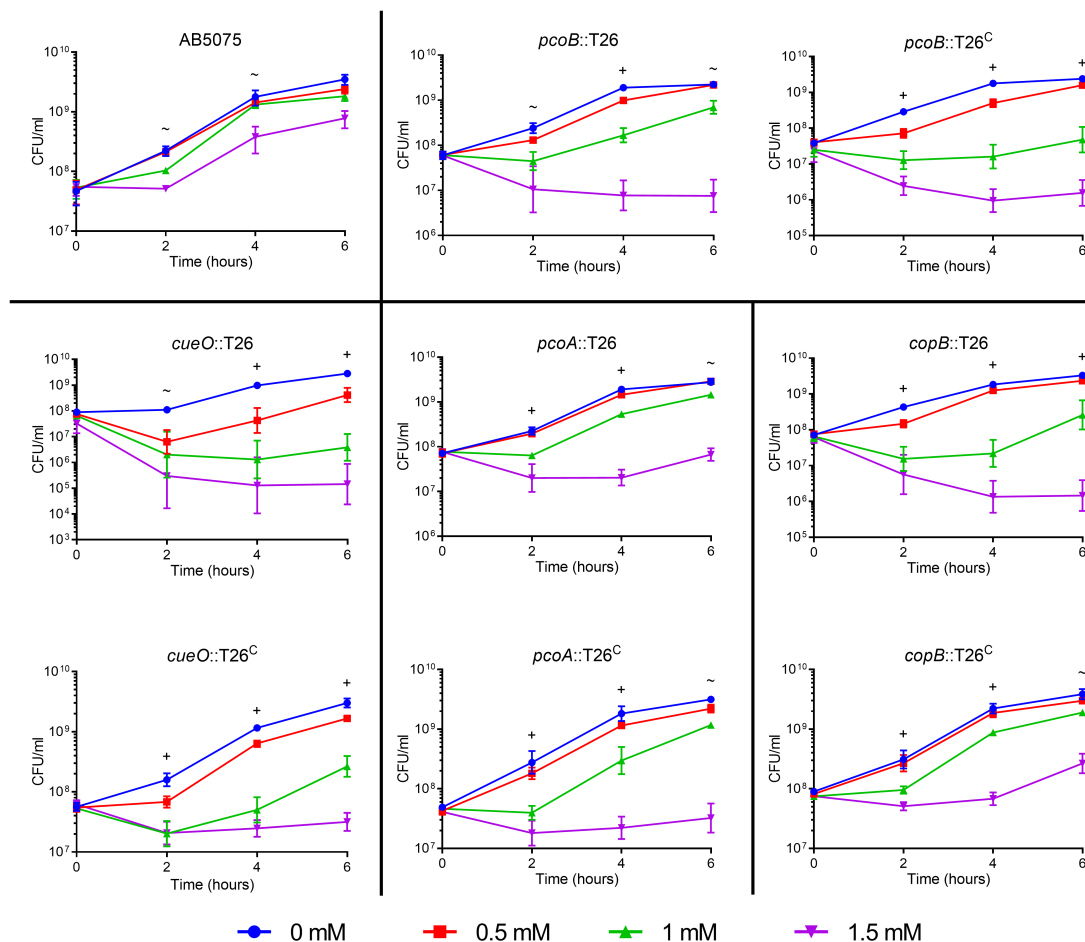


Figure 14. Growth of moderately copper sensitive AB5075 mutant and complementation strains.

Strains were grown for 6 hours at 37°C in M9 medium supplemented with the concentration of CuSO₄ indicated in the legend. Growth was measured both by turbidity (OD₆₀₀, data not shown) and enumeration of viable colonies (CFU/mL). Black lines are used to group the mutant strain with the corresponding complemented derivative. The data are presented as geometric mean and SEM of three biologically independent experiments. Two-way ANOVA with Tukey's adjustment for multiple comparisons was used to compare growth in each copper concentration at each timepoint. The symbols indicate which copper treatments were statistically different ($P < 0.05$) from the 0 mM control, as follows: ~, growth in 1.5 mM CuSO₄ was different from the control; +, growth in 1 mM and 1.5 mM CuSO₄ was different from the control.

strains showed significant changes in copper sensitivity relative to the wild-type strain. The phenotypes of these eight strains fell into two categories: moderately and highly sensitive. Strains bearing mutations in *cusR*, *cusS*, *copC* and *copD* were all highly sensitive to copper and showed significant growth defects in as little as 0.25 mM CuSO₄ (Fig. 13). In contrast, strains bearing mutations in *pcoB*, *pcoA*, *copB*, and *cueO* were moderately copper sensitive and only displayed increased sensitivity relative to the wild-type strain in the presence of at least 1 mM CuSO₄ (Fig. 14). Of note, nearly all the mutant strains with copper sensitive phenotypes contained mutations in genes carried in region D (Fig. 11), and these strains displayed the most substantial defects in copper resistance.

To confirm that the copper sensitive phenotype was due to the T26 transposon insertion, we constructed a complemented derivative of each of the eight mutant strains of interest. Complementation was achieved by insertion of a wild-type copy of the ORF and its native promoter in an *attTn7* site downstream of the *glmS* locus (107). Each of the complemented strains was individually tested in the same growth experiment. Complete restoration of copper resistance to wild-type levels was observed in the *cusR*, *cusS*, and *copD* complemented strains (Fig. 13). Partial restoration of copper resistance was observed in the *copB*, *cueO*, and *copC* complemented strains (Fig. 13, 14). No functional complementation was observed in the *pcoA* and *pcoB* complemented strains (Fig. 14), perhaps suggesting that the identified phenotypic changes are not purely due to the transposon insertion in *pcoA* and *pcoB*, or that this complementation strategy was insufficient for these two particular genes. Taken together, our mutational analyses indicate that many of the identified putative copper resistance genes indeed contribute to

copper resistance in *A. baumannii*. Moreover, the results suggest that the genes found in region D are crucial for the highest levels of copper resistance.

Region D Enhances Copper Resistance in Other *A. baumannii* Strains

Our previous analysis of multiple clinical isolates of *A. baumannii* identified two isolates, AB4857 and AB5711, that both showed inherently reduced copper resistance as compared to AB5075. Notably, neither of these strains carried the genes from region D in their genomes (223). Given our finding that mutations in the genes located in region D caused the greatest copper sensitivity in AB5075 (Fig. 13), we hypothesized that the transfer of region D to AB5711 and AB4857 would increase their overall level of copper resistance. Indeed, when region D from AB5075 was carried on the plasmid pAJ100 in these strains, pAJ100-Region D conferred increased copper resistance comparable to the level seen in AB5075; the empty pAJ100 vector did not affect resistance (Fig. 15). Thus, these results also indicate that the genes in region D are important for high levels of copper resistance in *A. baumannii*.

Killing of Biofilms by Copper

Biofilm formation is an important component of the *A. baumannii* lifecycle; the bacterium is known to create biofilms on both biotic and abiotic surfaces, which aides in fomite-based transmission and in virulence (118; 235). Moreover, biofilms are notorious for their ability to decrease the effectiveness of antibiotic treatment (81). Because any future copper-based therapeutics would likely need to be effective against *A. baumannii* found within a biofilm structure, we next tested the copper sensitivity of biofilms formed

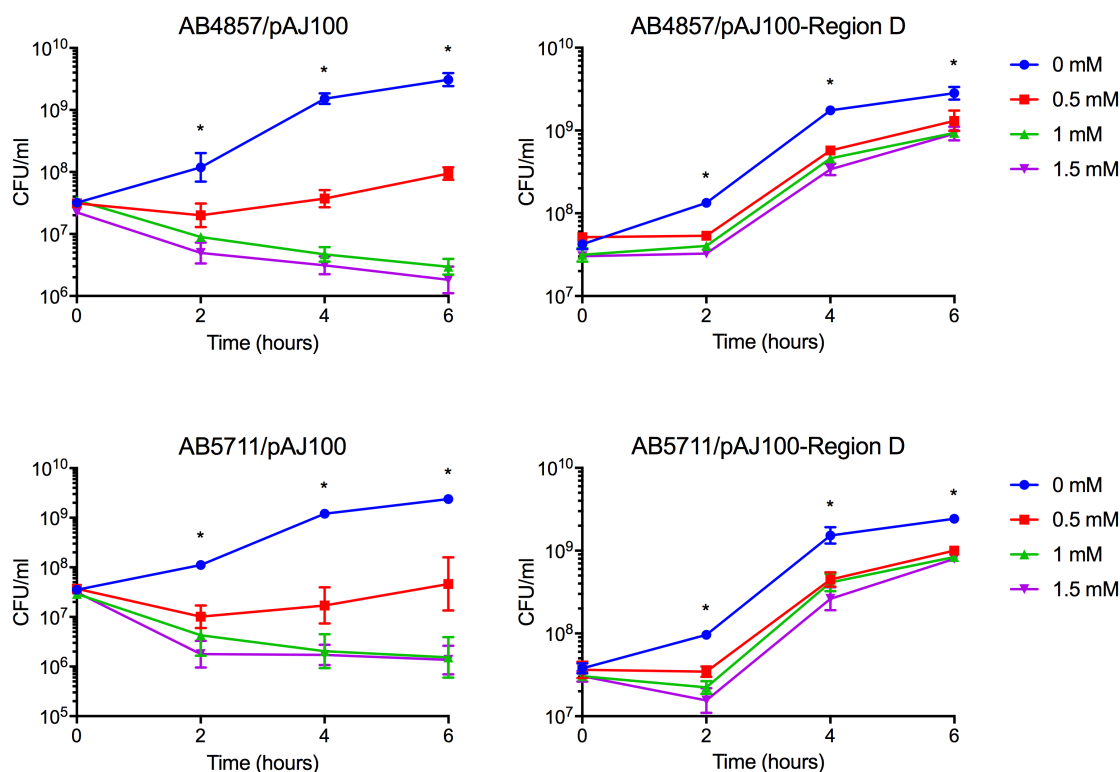


Figure 15. Growth of AB4857 and AB5711 carrying Region D.

Strains carrying the empty vector, pAJ100, were used as a control (left); region D from AB5075 was cloned into pAJ100 and transformed into AB4857 and AB5711, which do not naturally encode the region D genes (right). Strains were grown for 6 hours at 37°C in M9 medium supplemented with the concentration of CuSO₄ indicated in the legend. Growth was measured both by turbidity (OD₆₀₀, data not shown) and enumeration of viable colonies (CFU/mL). The data are presented as geometric mean and SEM of three biologically independent experiments. Two-way ANOVA with Tukey's adjustment for multiple comparisons was used to compare growth in each copper concentration at each timepoint. The symbols indicate which copper treatments were statistically different ($P < 0.05$) from the 0 mM control: *, growth in 0.5, 1, and 1.5 mM CuSO₄ was different from the control.

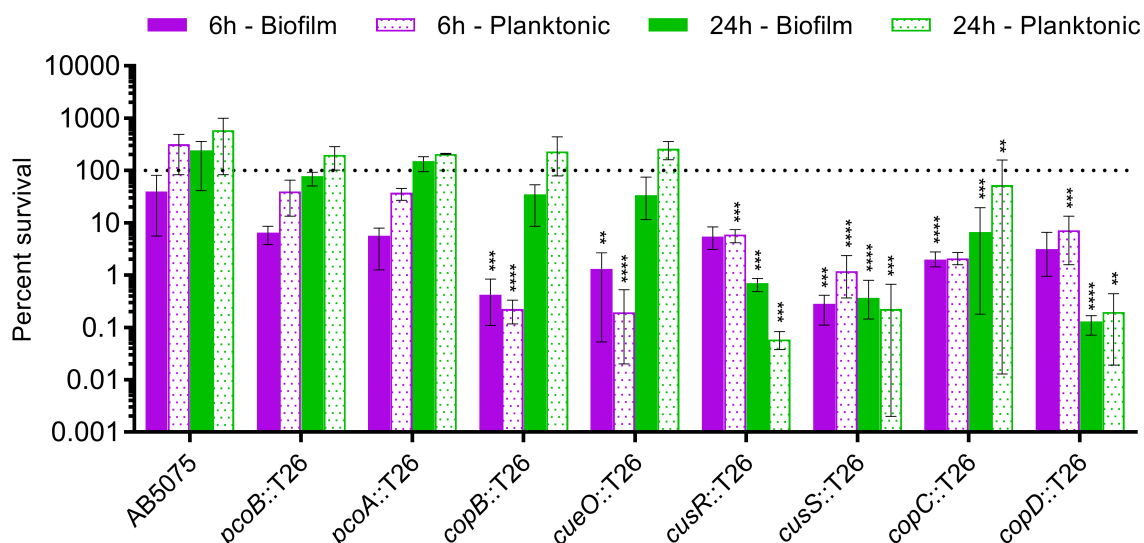


Figure 16. Copper-dependent killing of AB5075 mutant strain biofilms and planktonic cells.

Cultures were statically incubated in a 24-well tissue culture-treated plate at 37°C to establish biofilms. After 24 hours, the broth was replaced with M9 medium containing 1.5 mM CuSO₄. After an additional 0, 6, or 24 hours of incubation, bacteria from both the supernatant (planktonic) and the biofilm were plated for enumeration of CFU. Percent survival was calculated as the percentage of bacteria present at time zero under each condition. Data are presented as mean and range for three biologically independent experiments. One-way ANOVA with Dunnett's correction was used to compare each mutant strain to the wild-type at each indicated condition. Statistically significant comparisons are indicated with the asterisks, as follows: **, $P < 0.01$; *** $P < 0.001$; ****, $P < 0.0001$.

by the copper sensitive mutant strains as compared to wild-type AB5075. Biofilms were established in complex media for 24 hours before switching the medium to M9 medium supplemented with 1.5 mM CuSO₄. CFU were enumerated from both the biofilm and supernatant at 0, 6, and 24 hours post-copper shock, and data were expressed as percent survival relative to T0. For the wild-type AB5075, a slight decrease in biofilm CFU was observed at 6 hours, but no obvious sensitivity to copper was observed in the planktonic population at this timepoint or in the biofilm or planktonic populations at 24 hours (Fig. 16). While the *pcoA* and *pcoB* mutant strains of region B did not demonstrate survival differing from the wild-type strain in these biofilm conditions, the strains bearing mutations in region D (*copB*, *cueO*, *cusR*, *cusS*, *copC*, *copD*) each displayed statistically significant decreases in survival of both biofilm and planktonic cells relative to the wild-type strain; a 1-3 log loss in recoverable CFU was observed. The *copB* and *cueO* mutant strains displayed an early defect but recovered to wild-type levels by 24 hours. Conversely, the *cusR*, *cusS*, *copC*, and *copD* mutant strains did not recover. These data demonstrate that most mutant strains retain copper sensitivity relative to the wild-type strain even within a biofilm structure, suggesting that future copper resistance-targeting therapeutics would likely not be hindered by any inherent copper resistance of a biofilm.

Copper Sensitive Mutant Strains Accumulate More Intracellular Copper

To begin to understand the ability of the wild-type strain to adapt to copper stress as well as the nature of the copper homeostasis defects in the copper sensitive mutant strains, we utilized ICP-MS to temporally measure copper accumulation in strains exposed to a copper shock (0.25 mM). Previous studies in other bacteria have

demonstrated that intracellular copper accumulates in mutant strains bearing mutations in copper-related proteins, including *pcoB*, *pcoA*, *copA*, *cusR*, and *cusS* (64; 70; 112; 150). To optimize the assay, we focused our initial efforts on wild-type AB5075 and the *cusR* mutant strain. In wild-type AB5075, the intracellular copper concentration increased ~10x 1 minute following copper exposure. However, the level reduced to ~5x in approximately 15 minutes and fully recovered to baseline levels 60 minutes following removal of excess copper from the media (Fig. 17). Thus, in the wild-type strain, the cells appear to respond to copper stress via the deployment of efflux mechanisms that reduce the level of intracellular copper. In comparison, for the copper sensitive *cusR* mutant strain, intracellular copper levels immediately increased ~10x, but showed no reduction while copper was present. However, after copper removal, the level similarly recovered to baseline within 60 minutes (Fig. 17). These data suggest that in the presence of excess copper, the *cusR* mutant cells are unable to control the intracellular copper concentration and cannot efflux copper ions as well as the wild-type cells.

Based on the above results, we modified the sampled timepoints and collection strategy in order to simultaneously test the wild-type and all eight of the mutant strains that previously demonstrated increased copper sensitivity (Fig. 13, 14). Of note, the revised collection strategy resulted in a longer period of copper exposure, as the washing steps took ~20 minutes longer to collect the cells. Using this procedure, the *copB* and *cueO* mutant strains each showed significantly higher accumulation of copper relative to the wild-type strain during the 30 minute copper shock. In addition, the *cusR*, *cusS*, and *copD* mutant strain accumulated a higher concentration of intracellular copper within the additional time of copper exposure found with the modified washing steps. All strains

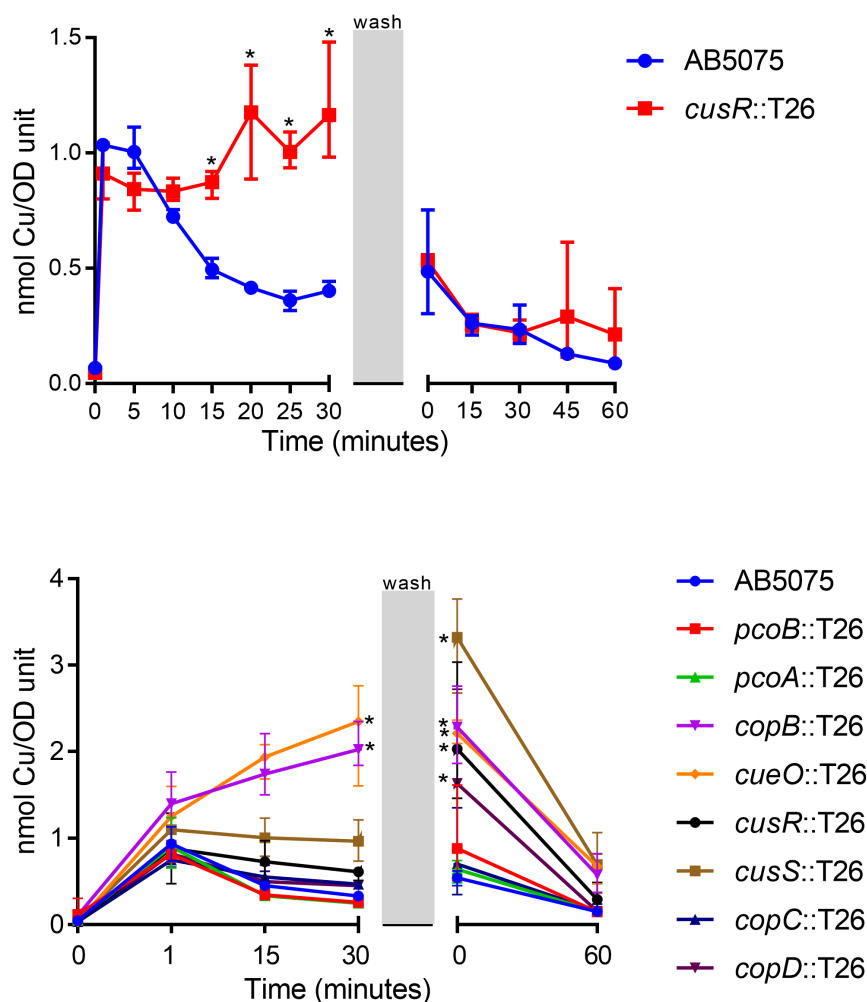


Figure 17. Accumulation of intracellular copper.

Log phase cultures were treated with 0.25 mM CuSO₄ and samples were collected at the indicated timepoints. During the wash phase, the remaining culture volume was washed and resuspended in fresh M9 medium without supplemented copper. Cell samples that were collected at each timepoint were washed and dried, and copper content was measured by ICP-MS. Data are presented as the mean and range for three biologically independent experiments. Two-way ANOVA with Sidak's correction was used to compare the *cusR* mutant strain to the wild-type at all timepoints (top); two-way ANOVA with Dunnett's correction was used to compare all the mutant strains to wild type at all timepoints (bottom). Statistically significant comparisons are indicated with an asterisk ($P < 0.01$). At 0 minutes post-wash, five strains were significantly different from wild-type: *copB*, *cueO*, *cusR*, *cusS*, and *copD* (bottom).

demonstrated recovery following the removal of the copper stress (Fig. 17). Taken together, these results indicate that many of the mutant strains have defects in copper homeostasis and copper efflux, which likely contribute to their copper sensitivity.

Copper Resistance is Important for Virulence in *Galleria mellonella* Larvae

Given that copper is an important component of immune defense, we hypothesized that copper resistance is an important trait that affects virulence of *A. baumannii*. To begin to test this possibility, we assessed the ability of the copper sensitive mutant strains to kill *Galleria mellonella* caterpillars. This model was chosen because: 1) *Galleria* have been established as an inexpensive and simple infection model for many pathogenic bacteria, including *A. baumannii*, 2) these caterpillars can be maintained at 37°C, 3) these caterpillars have both humoral and cellular immune response pathways, and 4) this invertebrate model has been used for identification of bacterial virulence factors and virulence results often correlate with those obtained in mammalian models (93; 155; 204). Groups of *Galleria* were individually infected with each strain of *A. baumannii*, incubated at 37°C, and observed for 5 days. Consistent with previous experiments with AB5075 (93), at a dose of approximately 5.0×10^4 CFU, wild-type AB5075 killed ~85% of the *Galleria*, with most death occurring in the first two days post-infection (Fig. 18). In contrast, the copper sensitive mutant strains were all attenuated in this model and killed significantly less *Galleria* than the wild-type strain (Fig. 18). The *pcoB*, *pcoA*, *cueO*, *cusS*, and *copD* mutant strains were the most attenuated and killed less than 30% of the *Galleria*. The *copB* and *cusR* mutant strains were

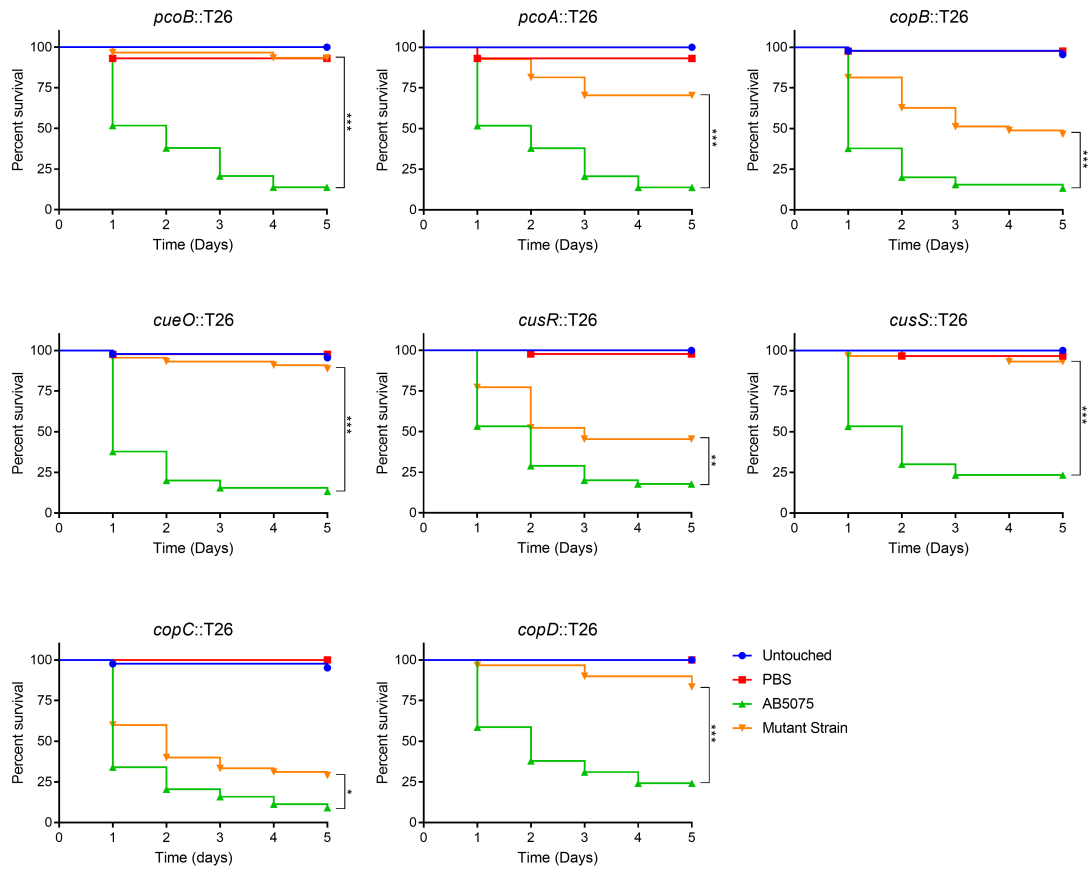


Figure 18. Survival of *Galleria* infected with AB5075 mutant strains.

Galleria were injected with approximately 5.0×10^4 CFU of the indicated strain and survival was monitored for 5 days. The gene interrupted in each mutant strain is indicated in the graph title. Experiments were repeated using two or three different orders of larvae with 12 – 15 larvae per experimental group; the total n ranged from 25 to 45 larvae per strain. Kaplan-Meier survival curves were compared (excluding Untouched and PBS controls) using the Mantel-Cox test with Holm's correction for multiple comparisons. Statistically significant comparisons of the mutant strain to the wild-type strain are indicated by bars with asterisks signifying the *P* value as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

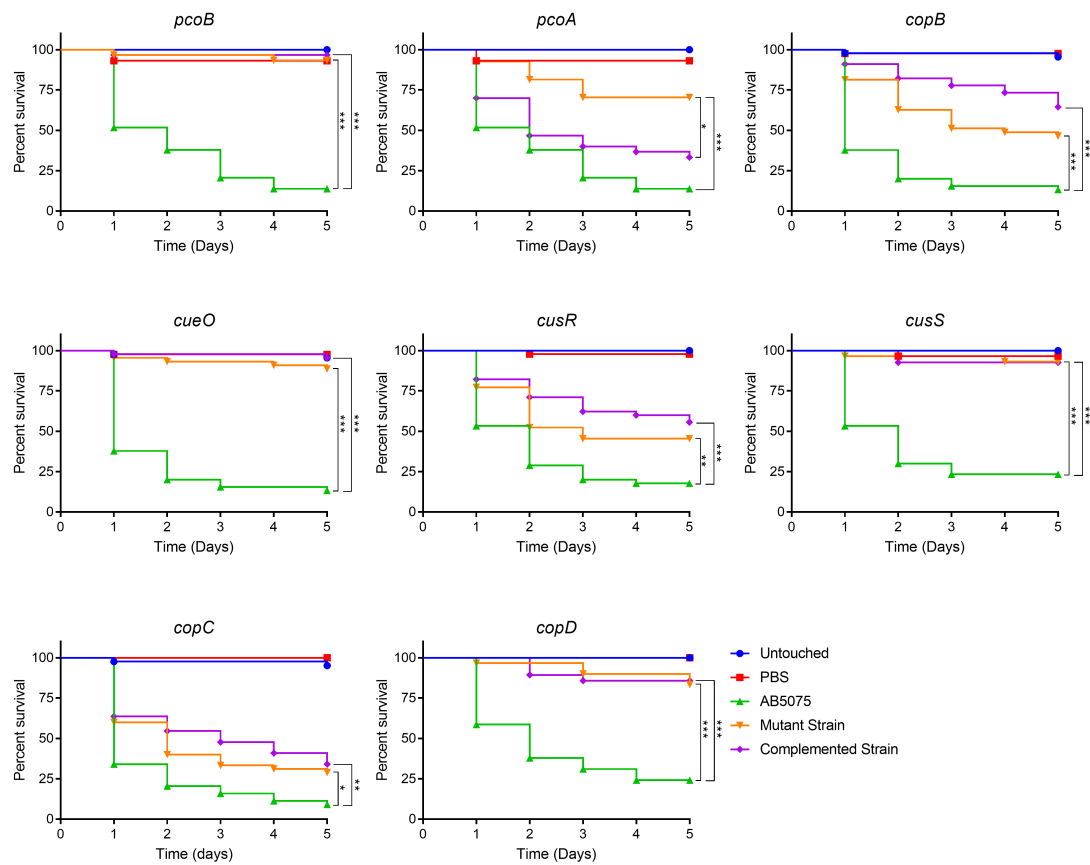


Figure 19. Survival of *Galleria* infected with AB5075 mutant and complemented strains. *Galleria* were injected with approximately 5.0×10^4 CFU of the indicated strain and survival was monitored for 5 days. The gene interrupted in each mutant strain and restored in the complemented derivative is indicated in the graph title. Experiments were repeated using two or three different orders of larvae with 12 – 15 larvae per experimental group; the total n ranged from 25 to 45 larvae per strain. Kaplan-Meier survival curves were compared (excluding Untouched and PBS controls) using the Mantel-Cox test with Holm's correction for multiple comparisons. Statistically significant comparisons (mutant strain vs. wild-type strain, mutant strain vs. complemented derivative, complemented derivative vs. wild-type strain) are indicated by bars with asterisks signifying the *P* value as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

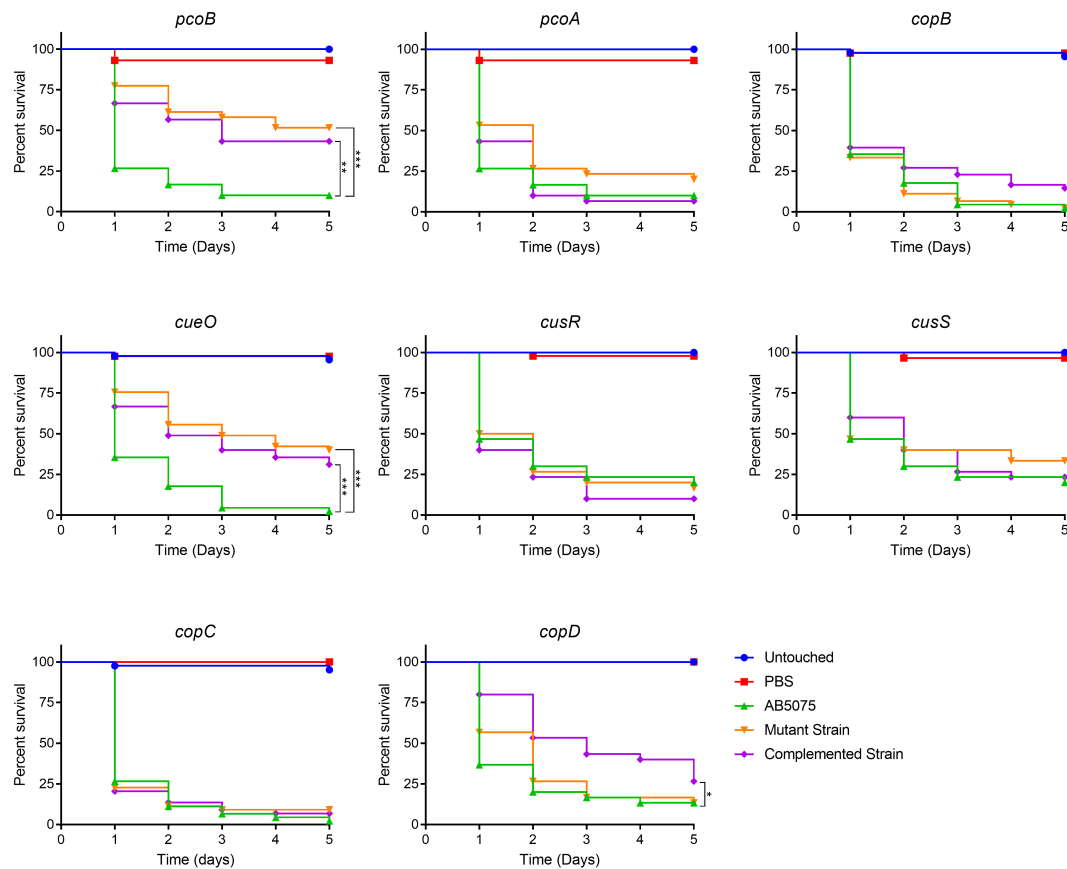


Figure 20. Survival of *Galleria* infected with a high dose of AB5075 mutant and complemented strains.

Galleria were injected with approximately 5.0×10^5 CFU of the indicated strain and survival was monitored for 5 days. The gene interrupted in each mutant strain and restored in the complemented derivative is indicated in the graph title. Experiments were repeated using two different orders of larvae with 12 – 18 larvae per experimental group; the total n ranged from 25 to 48 larvae per strain. Kaplan-Meier survival curves were compared (excluding Untouched and PBS controls) using the Mantel-Cox test with Holm's correction for multiple comparisons. Statistically significant comparisons (mutant strain vs. wild-type strain, complemented derivative vs. wild-type strain) are indicated by bars with asterisks signifying the *P* value as follows: **, *P* < 0.01; ***, *P* < 0.001.

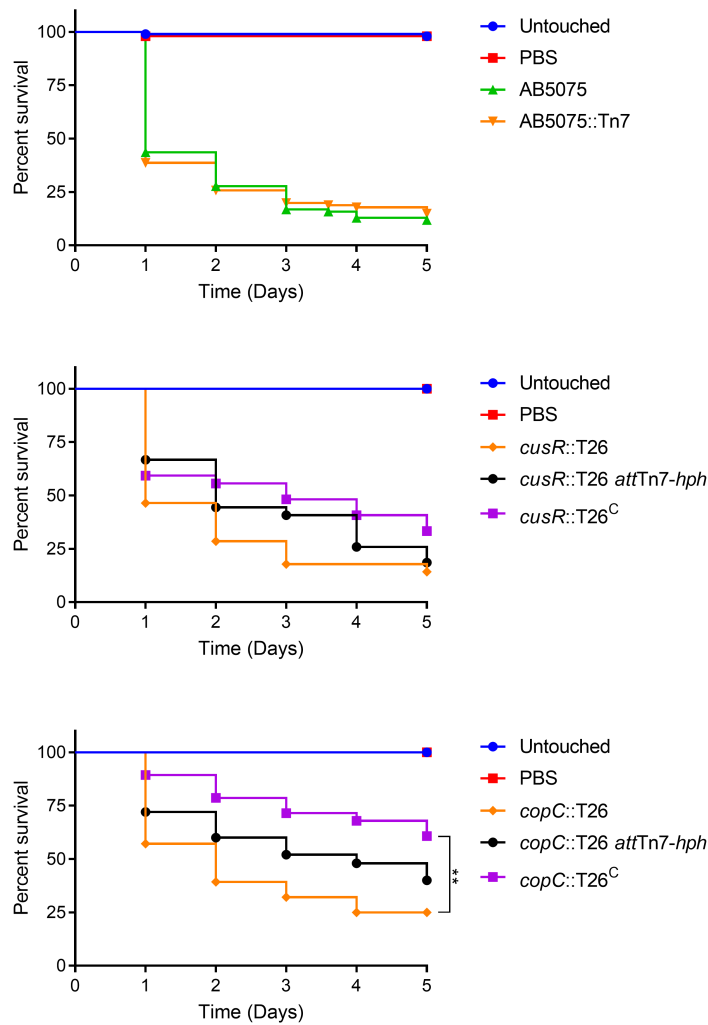


Figure 21. Survival of *Galleria* infected with Tn7-containing strains.

Galleria were injected with approximately 1.0×10^5 CFU (actual doses ranged from 6.99×10^4 – 1.4×10^5 CFU) of the indicated strain and survival was monitored for 5 days. For the comparison of wild-type AB5075 to AB5075::Tn7 (top), six biologically independent experiments were conducted using six separate batches of caterpillars; the combined data are presented, with 96 - 101 total caterpillars per group. For the strains derived from the *cusR* mutant strain (middle) and *copC* mutant strain (bottom), two biologically independent experiments were conducted using two separate batches of caterpillars; the combined data are presented, with 25 - 28 caterpillars total per group. Kaplan-Meier survival curves were compared (excluding Untouched and PBS controls) using the Mantel-Cox test with Holm's correction for multiple comparisons. Statistically significant comparisons (mutant strain vs. complemented derivative) are indicated by bars with asterisks signifying the *P* value: **, $P < 0.01$.

moderately attenuated and killed ~55% of *Galleria*. The *copC* mutant strain was very mildly attenuated and killed ~70% of *Galleria*.

As with the *in vitro* phenotypes, we also sought to functionally complement the virulence defects seen in the *Galleria* model. However, infection with the complemented strains resulted in similar rates of *Galleria* death as those infected with the corresponding mutant strain; in some cases, the complemented strains appeared slightly more attenuated (Fig. 19, 20). Based on these data, we speculated that the insertion at the *attTn7* site was causing unintended attenuation in this model; to our knowledge, no published studies have shown functional complementation using the Tn7-based strategy in assays of *Galleria* survival. To test this possibility, a strain carrying an *attTn7* insertion of only the hygromycin resistance gene was created and then compared in virulence to the wild-type background strain. Attenuation of the AB5075::Tn7 strain relative to AB5075 was not consistently observed (Fig. 21). We reasoned that perhaps the effect was only evident in strains already carrying the T26 transposon insertion and that the size of the *attTn7* insertion might be important. Thus, derivatives of the *cusR* and *copC* mutant strains were engineered to carry an *attTn7* insertion of only the hygromycin resistance gene and were then compared to the parental mutant strain as well as the complemented derivatives, which each carried much larger *attTn7* insertions. These hygromycin-only insertion strains were both slightly more attenuated than the parental strains (Fig. 21). Furthermore, the complemented strains carrying the larger *attTn7* insertion were even further attenuated (Fig. 21). These results suggest that the inability to functionally complement the mutant virulence phenotypes is due to the fact that insertion at the *attTn7* site is attenuating in the *Galleria* survival model. Overall, the data strongly suggest that

copper resistance, and many individual proteins in regions B and D, are needed for full virulence of *A. baumannii* in the *Galleria* model; however, these conclusions would clearly be further strengthened by functional complementation in this model.

CusR and CopD are Important for Virulence in a Murine Pneumonia Infection Model

Given the attenuated virulence observed in the *Galleria* model, we next wished to determine if *A. baumannii* copper resistance is also an important virulence determinant during infection of a mammalian host. To this end, we selected two of the mutant strains and their complemented derivatives to test in a murine pneumonia model; the *cusR* and *copD* mutant strains were selected due to their high level of copper sensitivity and successful *in vitro* complementation (Fig. 13), as well as their phenotypic differences from the wild-type strain in all other assays, including virulence in *Galleria* (Fig. 16, 17, 18). The murine pneumonia model was chosen because lung infections are among the most common *A. baumannii* infections, and AB5075 virulence has been characterized in this model (93). As shown in Figure 22, the wild-type strain killed 85% of mice by 7 days post-infection. However, the *cusR* mutant strain was attenuated in this model; death was delayed relative to wild-type and 35% of the mice survived. Virulence of the *cusR* mutant strain was restored by complementation of the *cusR* gene *in trans*; 85% of the mice were killed by the complemented strain by 7 days post-infection. The *copD* mutant strain showed an even more dramatic virulence defect and only killed 10% of mice as compared to 90% and 60% by the wild-type and complemented strains, respectively. The ability to functionally complement both the *copD* and *cusR* mutant strain phenotypes in the pneumonia model further supports our conclusions concerning failure to achieve

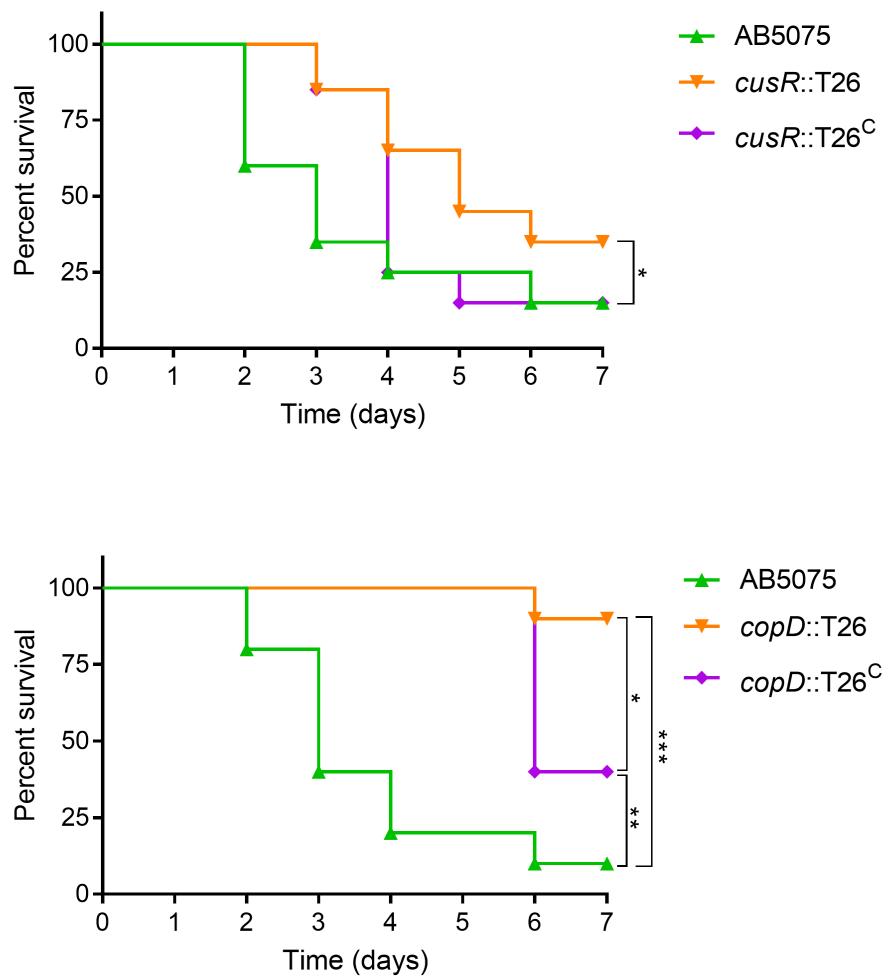


Figure 22. Assessment of virulence of AB5075 mutant and complemented strains in a murine pneumonia model.

Mice were infected intranasally with approximately 5×10^6 CFU of the indicated strain and survival was monitored for 5 days. Experiments with the *cusR* mutant strain and complemented derivative were performed in four biological replicates with 5 mice per group ($n = 20$ per strain), spaced two and two over time to ensure reproducibility; two biological replicates were completed with the *copD* mutant strain and complemented derivative ($n = 10$ mice per strain). Kaplan-Meier survival curves were compared using the Mantel-Cox test with Holm's correction for multiple comparisons. Statistically significant comparisons are indicated by bars with asterisks signifying the *P* value as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The only significant comparison in the top panel is wild-type AB5075 vs. the *cusR* mutant strain.

complementation in the *Galleria* model. In summary, both CusR and CopD individually contribute to virulence of *A. baumannii* in the murine pneumonia model.

DISCUSSION

Novel therapeutics are desperately needed to treat infections with drug-resistant *A. baumannii* and other “superbugs.” Because copper homeostasis is an essential process and copper ions themselves have potent toxicity, we sought to identify important copper resistance determinants in *A. baumannii*; we theorize that these could potentially be targeted with future therapeutics. Using a focused approach, we demonstrated that 8 of 21 tested genes individually contributed to *in vitro* copper resistance of AB5075; mutant strains displayed significant copper sensitive phenotypes during planktonic growth (Fig. 13, 14). Mutation of genes carried in region D caused the largest increase in copper sensitivity, indicating that this region is crucial for high levels of resistance. Furthermore, when a plasmid-borne copy of region D was introduced into *A. baumannii* strains that naturally lacked region D, copper resistance was dramatically increased (Fig. 15). Moreover, strains bearing mutations of genes in region D showed enhanced copper-dependent killing even when found in a biofilm structure (Fig. 16). The copper sensitivity is likely due to the fact that many of the mutant strains (*copB*, *cueO*, *cusR*, *cusS*, *copD*) demonstrated markedly increased accumulation of intracellular copper as compared to wild-type AB5075, which was able to efficiently efflux copper ions (Fig. 17). Finally, when tested *in vivo* in *Galleria mellonella* and a murine pneumonia model, the copper sensitive mutant strains showed distinct attenuation in virulence. In the *Galleria* model,

the mutant strains killed less *Galleria* than the wild-type strain, with the strongest attenuation observed with the *pcoB*, *cueO*, *cusS*, and *copD* mutant strains (Fig. 18). Similarly, in the murine mouse model, the *cusR* and *copD* mutant strains were also attenuated and the attenuation phenotype was able to be complemented (Fig. 22). *En masse*, our data indicate that copper resistance is mediated by many genes in AB5075 and show that copper resistance contributes to virulence.

Though the absolute functions of the proteins encoded by each of the genes showing homology to copper related systems remains to be determined, based on predicted functions, a model for the mechanisms of copper resistance in AB5075 was created (Fig. 23). When thinking about this model, it is important to keep in mind that based on the mutational analysis, proteins putatively involved in transport (*PcoB*, *CopB*, *CopD*), oxidation (*PcoA*, *CueO*), chaperoning (*CopC*), and regulation (*CusR*, *CusS*) individually contributed to copper resistance in AB5075 in our tested *in vitro* conditions. However, this does not negate a possible role for the other predicted copper-related factors within other environments. Also, it should be noted that the model proposed by another group for copper resistance proteins in AB5075 differs slightly from ours (7).

We utilized multiple assays to analyze *in vitro* copper resistance and to assess virulence. In many cases, we observed corroborating results across multiple assays. For instance, we observed similar patterns of sensitivity in our planktonic and biofilm growth assays. The four strains with the greatest increase in copper sensitivity during planktonic growth (*cusR*, *cusS*, *copC*, *copD*) also demonstrated sustained, increased copper sensitivity in the context of biofilm growth. The four additional strains that showed more moderate increases in copper sensitivity in the planktonic growth assay displayed

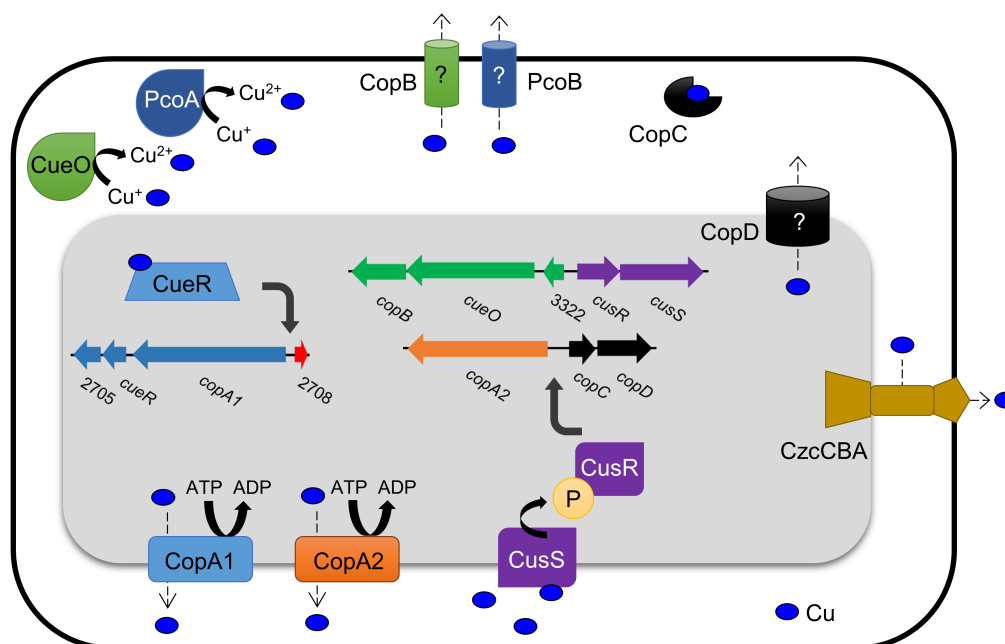


Figure 23. Model of putative copper resistance mechanisms in AB5075.

Predicted protein identifications were determined by amino acid sequence homology to known proteins from other bacteria, and proteins with putative copper resistance functions are indicated. In the mutational analysis described herein, proteins putatively involved in transport (PcoB, CopB, CopD), oxidation (PcoA, CueO), chaperoning (CopC), and regulation (CusR, CusS) individually contributed to copper resistance in AB5075. Copper ATPases are highly conserved inner-membrane proteins that efflux copper ions out of the cytoplasm; AB5075 appears to have two copper ATPases: CopA1 and CopA2. The RND family efflux pump CzcCBA may efflux copper from the periplasm to the extracellular milieu. Three other putative membrane proteins in AB5075 may also have roles in copper transport, though the function of these proteins is not yet well defined in any bacteria (CopB, PcoB, and CopD). Periplasmic copper oxidases reduce damage by converting Cu¹⁺ to the less toxic Cu²⁺; CueO and PcoA are both predicted copper oxidases. CopC is a highly conserved copper chaperone that is found in the periplasm; it prevents damage by binding and sequestering copper ions and may facilitate movement of copper ions between other copper-related proteins, i.e. delivering copper ions to an efflux pump. Two well-conserved putative regulatory systems are also present in AB5075: CueR senses cytoplasmic copper levels and then upregulates copper resistance genes in its copper-bound form, and CusRS is a two-component system that is thought to be activated by periplasmic copper. The regulons of CueR and CusR have not yet been defined in AB5075, though putative binding sites have been identified in regions B and D, respectively, and these putative target genes are indicated in the figure.

temporarily increased (*copB*, *cueO*) or no change (*pcoA*, *pcoB*) in sensitivity relative to the wild-type background in the context of biofilm growth. However, we did not observe a direct correlation between *in vitro* copper sensitivity and attenuation of virulence in the *Galleria* infection model. For example, the two most attenuated mutant strains in the *Galleria* model (*pcoB* and *cueO*) had only moderate copper sensitivity in the planktonic growth and biofilm survival assays, while the *cusR* and *copC* mutant strains demonstrated strong phenotypes in *in vitro* assays but were only mildly attenuated for virulence in *Galleria*. These differences among assays highlight the importance of the methods chosen to assess genes of interest; the roles of individual genes may vary depending on environmental conditions, and this will affect the experimental outcomes and the conclusions drawn. Indeed, experimental differences may account for contrasting results in this work and those published recently by another group (7). Under the conditions we tested, we did not observe increased copper sensitivity of either of the copper ATPase mutant strains; we originally hypothesized that perhaps CopA1 and CopA2 were functionally redundant, resulting in an inability to observe a copper sensitive phenotype. However, a recently published study of the copper ATPases in AB5075 found that the gene referred to as *copA1* in our publication (ABUW_2707, region B), but not *copA2* (ABUW_3325, region D), contributed to copper resistance (7); those results suggest that the CopA1 and CopA2 proteins are not functionally redundant. While the exact reason for the difference in study results is not clear, it is worth noting that the experimental methods used were different and that the various mutant strains utilized differed in their site of transposon insertion. Given the disparity in the results of the studies, it is currently unclear what the individual roles of these copper ATPases are in *A. baumannii*, and

additional studies will be required to clearly define the role of these factors in copper resistance.

Our studies identified many copper resistance proteins that individually contributed to virulence in the chosen models; these proteins have a variety of putative functions including copper efflux, oxidation, chaperoning, and regulation. Copper-related genes with similar functions have been shown to be important for virulence in many bacterial and fungal pathogens including *P. aeruginosa*, *Pseudomonas fluorescens*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas citri* subsp. *citri*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Salmonella* Typhimurium, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Listeria monocytogenes*, *E. coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Aspergillus fumigatus*, and *A. baumannii* (3; 7; 29; 34; 42; 54; 57; 60; 64; 83; 84; 97; 124; 149; 154; 162; 176; 180; 182; 193; 216; 220; 221; 225; 229; 232; 234). Of note, three types of copper-related proteins that have been previously shown to contribute to virulence of other bacteria were found to also contribute to virulence of *A. baumannii*: copper ATPases, copper oxidases, and copper chaperones. In the majority of the previous studies, the mutated copper resistance gene of interest was a copper ATPase, homologous to *copA1* and *copA2* of AB5075. Though we did not observe copper sensitivity in our copper ATPase mutant strains or assess their virulence, a role for CopA1 in virulence of *A. baumannii* was recently found (7). Copper oxidases have been shown to be important for virulence of *S. pneumoniae*, *C. jejuni*, and *S. aureus* (3; 60; 232). Here, we demonstrated that both copper oxidases of AB5075, PcoA and CueO, contribute to virulence of *A. baumannii* as well. Similarly, chaperone proteins have been previously linked to virulence in *S. pneumoniae* and *S. mutans* (57; 97), and

we found that the putative chaperone CopC contributes in *A. baumannii*. Though their functions may be similar, it is unclear how related CopC of *A. baumannii* is to the chaperones of other bacteria, as these chaperone proteins are small and have minimal sequence identity or similarity. While these three types of proteins are interesting as putative drug targets, an important consideration in identifying novel drug targets is assessment of homology with host proteins and potential toxicity from off-target activity. Copper ATPase and oxidase proteins have amino acid homology to human proteins, and the copper ATPases are also functionally similar. Thus, designing therapeutics that target these functions may be complicated by homology to the essential human proteins, e.g. human copper ATPases ATP7A and ATP7B. While not insurmountable, homology to host proteins certainly makes design of novel therapeutics more challenging.

Of particular interest, we identified a number of copper-associated proteins that have not previously been shown to contribute to virulence in other bacterial species. To our knowledge, this is the first publication to report attenuated virulence in any bacterial species of strains lacking the putative transporters PcoB, CopB, or CopD (193; 225). We also observed attenuated virulence of the strains bearing mutations in either portion of the two-component system CusRS. Again, to our knowledge, this is the first report that this copper-sensing two-component system contributes to virulence in any bacterial species. Additionally, none of these proteins share amino acid sequence homology with human proteins, and therefore may be targeted more readily.

While the focus of this work was copper resistance in *A. baumannii*, we note that copper homeostasis mechanisms are well conserved across bacterial species; the open reading frames studied here were originally identified in AB5075 due to their conserved

predicted protein sequence. Therefore, we expect that any novel therapeutics that are targeted against these proteins may also have broad-spectrum antibacterial activity. Despite the fact that individual proteins are well-conserved, the presence of these proteins or their homologs varies across bacterial species and even amongst strains (79). Indeed, we observed that not all the same copper resistance proteins are present in all clinical isolates of *A. baumannii*. Thus, these differences will be an important consideration when choosing which copper resistance genes may be the best therapeutic targets. Given all of these possible targets, copper homeostasis components could provide an attractive starting place for the development of future antimicrobial therapies.

The immune system has harnessed the antimicrobial power of copper for use against pathogens and copper-mediated immune mechanisms are known to play a role in clearance of bacterial pathogens (181). Because the innate immune response of phagocytic cells is crucial for clearance of *A. baumannii* infection, we hypothesize that copper resistance contributes to virulence of this pathogen by enhancing immune evasion and survival *in vivo*. Therefore, we predict that novel therapeutics targeting copper resistance of bacterial pathogens would enhance immune clearance by reducing bacterial survival in the copper-rich phagosomal compartment. The role of copper in the immune system has been demonstrated by prior studies that utilized copper-replete and copper-deficient cell lines and animal models. For example, macrophages that are pre-incubated with copper are able to kill more internalized bacteria than controls (220). Conversely, copper-deficient macrophages or macrophages that are unable to mobilize copper to the phagosomal compartment due to loss of function of ATP7A are less efficient at killing bacterial pathogens (2; 108; 220). The importance of copper in immunity has also been

demonstrated on an organismal level. Animals with copper-deficient diets are more susceptible to infection, while those with copper-rich diets more efficiently clear infection (60; 142; 225). Furthermore, mice with a knockout of ATP7A, the copper ATPase that pumps toxic copper ions into the macrophage phagosome, are more susceptible to infection with *Salmonella* Typhimurium (108). Comparable future studies using *A. baumannii* will help to shed light on whether copper resistance of this organism aids in virulence by enabling evasion of host copper-mediated immune strategies.

In summary, we identified several additional copper resistance proteins that may serve as potential therapeutic targets, including copper transporters PcoB, CopB, and CopD, chaperone CopC, oxidases CueO and PcoA, and regulatory proteins CusR and CusS. Future work using targeted antibodies and small molecules will seek to determine the efficacy of targeting these copper resistance proteins as a successful treatment strategy. Given the relative conservation of these proteins and pathways among bacteria, we believe several are viable therapeutic targets to treat *A. baumannii* infection as well as other multidrug-resistant bacterial pathogens, for which novel treatments are urgently needed.

ACKNOWLEDGEMENTS

C.L.W., S.L.J.M., D.V.Z., and D.S.M. conceived and designed the experiments. C.L.W., H.M.N., Y.A.A., R.M.R., A.C.J., S.S., and R.A. performed the experiments. C.L.W. and D.S.M. wrote the paper.

We thank Cara Olsen for guidance with statistics. We thank Colin Manoil's group for the construction of the transposon mutant library in AB5075. C.L.W. is thankful to members of the Merrell and Zurawski labs for sharing their time and knowledge.

Material has been reviewed by the Walter Reed Army Institute of Research (WRAIR). There is no objection to its presentation and/or publication. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense, the Uniformed Services University of the Health Sciences, the Department of the Army, or any other agency of the U.S. Government. Research was conducted in an AAALAC-accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

Funding Statement

Research in the Merrell laboratory is funded by the NIH and DoD. S.J.L.M. is grateful to the NSF (CHE 1708732). Dr. Zurawski's lab is funded by the Military Infectious Diseases Research Program and the Defense Health Agency.

CHAPTER 4: Discussion

SUMMARY OF FINDINGS AND RELEVANCE

A. baumannii is one of the most drug-resistant nosocomial pathogens, thus infections are notoriously difficult to treat. Unfortunately, rates of drug resistance continue to rise, and treatment options are increasingly limited. Clearly, novel therapeutics are needed to treat patients infected with multidrug-resistant organisms. We hypothesized that copper homeostasis mechanisms are essential functions for *A. baumannii* pathogenesis, and therefore that individual copper-related proteins would contribute to virulence. Furthermore, we predict that any identified factors could subsequently be targeted therapeutically as a novel approach to treat drug-resistant infections.

As described in Chapter 2, we began our studies by first characterizing overall levels of copper resistance in a panel of *A. baumannii* clinical isolates. We found that, as evidenced by their ability to grow in the presence of numerous concentrations of exogenous copper, the degree of *in vitro* copper resistance varied among clinical isolates. In contrast, we found that all isolates were rapidly killed on dry, copper-containing metal surfaces. Additionally, we observed that copper was still bactericidal against *A. baumannii* biofilms. During liquid exposure to copper, we observed an adaptation-like response, where growth was delayed but not permanently halted. We found that this copper tolerance response could be enhanced by preexposure to subinhibitory concentrations of copper, shortening the delay in growth. When we searched the genomes

of the *A. baumannii* isolates for homologs of known copper-related proteins, many were identified; expression of many of these genes increased in response to copper exposure. Intriguingly, the more copper sensitive isolates did not have as many copper-related genes and were missing the genes found as a group, called region D, in the more copper resistant isolates.

As described in Chapter 3, we continued our investigation into *A. baumannii* copper homeostasis by assessing the contribution of each of the identified proteins to *in vitro* copper resistance and to virulence. Using strains from a transposon mutant library, we found eight genes that individually contributed to copper resistance; the strains bearing mutations in these genes were more sensitive to copper than the parent strain, AB5075. Excitingly, six of eight of the genes were located in region D, which we previously hypothesized was important for the highest levels of copper resistance. We were able to introduce region D to the more copper sensitive *A. baumannii* isolates and to increase their level of copper resistance, confirming our hypothesis. Importantly, we observed that mutant strains bearing mutations in region D retained their copper sensitive phenotype while in a biofilm. We observed increased intracellular copper accumulation in a number of mutant strains as well, indicating a defect in copper efflux that likely contributes to their copper sensitivity. Crucially, when we assessed the virulence of the mutant strains, we found that all were attenuated for virulence in a caterpillar infection model. Moreover, two mutant strains tested in a murine pneumonia model were similarly attenuated.

Through this dissertation work, we have begun to characterize the copper resistance mechanisms of *A. baumannii* and have identified a number of individual genes

that contribute to virulence. Importantly, these genes may represent novel drug targets in this important multidrug-resistant organism, for which novel therapeutic options are desperately needed.

COPPER HOMEOSTASIS IN *A. BAUMANNII*

In this work, we conducted some of the first studies to characterize copper homeostasis in *A. baumannii*; clearly much remains to be discovered and characterized to achieve a complete understanding of this process. In our work, we used a targeted approach and identified proteins in *A. baumannii* that are homologous to known copper-related proteins. While this approach revealed a large collection of putative copper-related genes, we readily acknowledge that the biased approach has likely resulted in an incomplete list of important copper-related proteins. Thus, utilization of multiple unbiased approaches would likely lead to identification of additional copper-related genes in *A. baumannii*. Arguably, if such genes are present, these novel copper resistance mechanisms would be of great interest; they may represent previously unrecognized, but important, resistance mechanisms with broader implications among bacteria. To this end, the complete AB5075 transposon mutant library could be screened for copper sensitivity using a Tn-seq based approach. This analysis would reveal any additional single-gene factors that contribute to copper resistance *in vitro*. Of course, the limitations inherent in utilizing transposon mutant libraries still apply; most importantly, if another gene functionally complements the loss of the mutated gene, then the phenotype will be masked.

Analysis of global changes in gene expression in response to copper exposure using RNA-seq may also identify additional and/or novel copper resistance mechanisms in *A. baumannii*. Indeed, regulation of copper resistance in this organism remains largely uninvestigated and is an interesting path for future study. In this vein, we demonstrated that expression from regions B and D increased in response to copper exposure in strain AB5075 (Fig. 10). Interestingly, expression patterns of the operons of each region were very similar, perhaps suggesting shared regulatory mechanisms for the genes in each region. Indeed, the two homologs of known copper-responsive regulators are located in regions B and D, and putative regulatory bindings sites were identified in the same regions (7; 223). A homolog of *E. coli* CueR is carried in region B, and a putative CueR binding site is located in the intergenic promoter region of region B. The two-component system CusRS is encoded in region D, and putative CusR binding sites are located in both putative promoter regions in region D. Because the genes potentially regulated by both CueR and CusRS are different in *A. baumannii* than in previously studied bacteria, it would be very interesting to confirm these predicted interactions and to fully define the CueR and CusR regulons. Purification of CueR and CusR and subsequent electrophoretic mobility shift assays (EMSAs) or DNase footprinting would confirm direct binding of each promoter of interest; this approach has been used successfully with both CueR and CusR of *E. coli* (192; 205; 228). Additionally, analysis of changes in expression of the identified copper-related genes in response to copper shock in the wild-type and regulatory mutant strains could provide interesting insight into the potential effects of therapeutically targeting these regulatory proteins.

In order to analyze changes in expression globally, and to potentially identify new copper resistance mechanisms, RNA-seq technology could be employed to measure changes in transcription across the genome in response to copper exposure. This analysis would shed light on whether copper shock induces a response specific to copper or a more global stress response. Indeed, a recently published study analyzed the response to copper exposure in a newly isolated *Acinetobacter* sp. using comparative proteomics; in addition to upregulation of the expected copper resistance proteins, there were changes in metabolism, cellular processing, and signaling (65).

It is important to also analyze expression of these copper-related genes in the context of infection, as this determines whether the target of interest is present at the time a novel therapeutic would be utilized. Based on our results demonstrating importance of these genes for virulence, we would assume that these genes are expressed by *A. baumannii* during infection. Furthermore, expression changes in response to growth phases, biofilm formation, phase variation, and exposure to other heavy metals may alter expression of copper-related genes *in vivo*, and thus their availability for targeting during infection. Additionally, it would be interesting to measure expression of copper-related genes by the native microbiota during infection. Copper is mobilized to and utilized by the host at the site of infection; therefore, it is possible that copper resistance proteins are only important at this site. If so, a therapeutic targeting copper resistance may have very minimal damaging side effects on the host microbiome.

Interaction between the identified copper-related genes and other metals is another interesting area for further study. Copper and silver are structurally similar and are both in group 11 of the periodic table; therefore, silver binding by copper-related

proteins has been observed. Previous *in vitro* work in *E. coli* has shown that CueR can bind silver as well as copper, and expression of *copA* increases in response to either copper or silver, but not to zinc (164; 192). In *E. coli*, *cusS* confers silver resistance as well as copper resistance (70), and has been shown to directly bind silver ions (71). While there is currently no known natural role of silver in humans, silver-based therapeutics are becoming popular, thus interactions with silver become important.

The interplay between metals in the biology of *A. baumannii*, especially in the context of infection, is an important topic for further investigation. The role of zinc during infection is complex and not yet fully understood; intriguingly, it may be both sequestered as part of nutritional immunity and also be mobilized to the phagosome for intoxication (117). Zinc uptake has recently been shown to be important for *A. baumannii* virulence, suggesting that the host sequesters zinc as part of nutritional immunity (116). Excess zinc does not cause damage in the same way as copper, as zinc is not redox active; toxicity is dependent on displacing metals from key metalloproteins (181). Recently, an *A. baumannii* study established a connection between copper and zinc: exposure to high concentrations of zinc depletes intracellular copper stores (77); understanding this mechanism warrants further investigation.

COPPER RESISTANCE GENES AS VIRULENCE FACTORS

In our study, we were able to observe attenuation of virulence for many of the mutant strains bearing mutations in copper-related genes in two infection models: *Galleria mellonella* caterpillars and murine pneumonia. These phenotypes are significant

and crucial to the relevance of our study, but they do not provide information about the mechanism of attenuated virulence. Understanding of the molecular mechanisms behind attenuated virulence in the copper sensitive strains is important for the use of any future therapeutics that render *A. baumannii* sensitive to copper. For example, if the mutant *A. baumannii* strains are attenuated for virulence because they are more susceptible to killing by immune cells, then these therapeutics will only be effective in patients whose immune systems properly function in that way. Effectiveness could be greatly reduced in the context of immunodeficiencies; this is especially important to consider given the opportunistic nature of *A. baumannii* and the frequency of underlying conditions in infected patients.

Additional work is needed to investigate the role of copper resistance in virulence of *A. baumannii*. As previous studies with other bacterial pathogens have reported reductions in intracellular survival in macrophages in strains bearing mutations in copper resistance genes, we hypothesize that copper resistance enhances evasion of *A. baumannii* killing by immune cells. In fact, I spent a significant amount of time working on such an assay (data not shown). Unfortunately, we were unable to overcome the technical hurdles caused by the extensive antibiotic resistance of our model clinical isolate, AB5075. Specifically, in order to measure the number of surviving intracellular bacteria, the bacteria outside the macrophage cells must be killed by an antibiotic. Therefore, the chosen antibiotic must be effective against the utilized *A. baumannii* strain and must not penetrate the macrophage cell membrane. Traditionally, gentamicin is used, but AB5075 is gentamicin resistant. We put significant effort into optimizing the assay for use with polymyxin B; unfortunately, we were unable to find conditions that reliably

eliminated external bacteria without compromising the integrity of the macrophage cell membrane. Alternative future methods could include testing of additional antibiotics, or a less traditional approach using enzymes or phage. If necessary, the mutations in copper resistance genes could be made in another *A. baumannii* strain that is sensitive to gentamicin, such as ATCC 17978; however, we note that this approach would sacrifice the relevance of using a modern clinical isolate.

Another way to link the observed attenuation of virulence more specifically to the role of copper is to manipulate the presence of copper in the host. Previous studies have shown that increasing dietary copper in the infected animal can combat infection, and that this approach is more effective against copper sensitive organisms (60; 225). We would hypothesize that in a murine pneumonia experiment, the *A. baumannii* strains bearing mutations in copper-related genes would be more greatly impacted by the addition of copper to the drinking water, further increasing the attenuation relative to wild-type. Host copper-mediated immunity can also be blocked by impairment of ATP7A, the host copper ATPase that moves copper ions into the macrophage phagosome. It has been previously shown that ATP7A-deficient macrophages are less efficient at killing *E. coli* and *Salmonella* and that the attenuation of bacterial survival in macrophages that is conferred by mutation of the bacterial copper ATPase is dependent on ATP7A function (108; 220). Concordantly, the attenuated virulence of copper susceptible *Salmonella* in wild-type mice is not present in ATP7A knockout mice (108). Thus, attenuation of virulence in *E. coli* and *Salmonella* Typhimurium bearing mutations in their copper ATPases is due to increased susceptibility to killing by copper in the immune cell phagosomes. Similar studies using ATP7A knockout macrophages and mice could

definitively link the attenuation of virulence in the *A. baumannii* mutant strains to copper-mediated immune mechanisms of the host.

A. baumannii causes a variety of infection types, including wound infections, which are unfortunately common among soldiers. Thus, a wound infection model could offer exciting opportunities for study. Our collaborators have developed porcine and murine *A. baumannii* wound infection models (197; 235). As copper is known to be mobilized in response to inflammation and since high concentrations have been measured in wound exudate, it would be very interesting to see if the copper sensitive mutants of AB5075 are attenuated in these models, (98; 132). A wound model would also be conducive to testing the effects of added copper in a number of ways: copper-supplemented drinking water, copper solution at the wound site, or copper-impregnated wound dressings. Topical formulations of copper as well as a copper-impregnated wound dressings have demonstrated antibacterial activity against other bacterial species (14; 22; 140; 214); additionally, topical silver has shown efficacy against many bacterial pathogens infecting wounds, including *A. baumannii* (179; 201).

TARGETING COPPER RESISTANCE WITH THERAPEUTICS

The long-term goal of this work is to support the development of therapeutics that target copper-related proteins (Fig. 24). Clearly, there are many possible approaches and steps to the process of developing a novel therapeutic. One way to begin could be to purify target proteins and to raise antibodies against those proteins. Antibodies could then be tested *in vitro* for their ability to hinder copper resistance and *in vivo* as treatments.

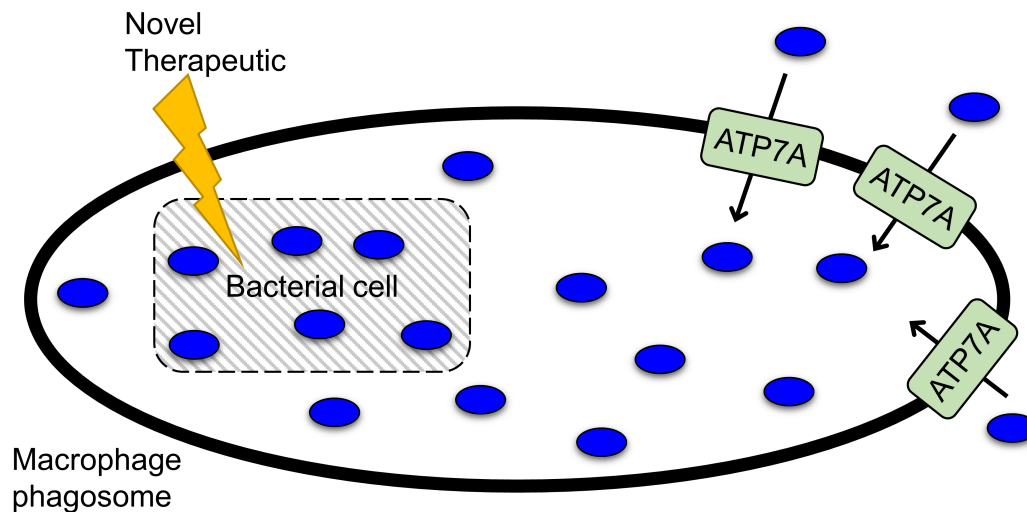
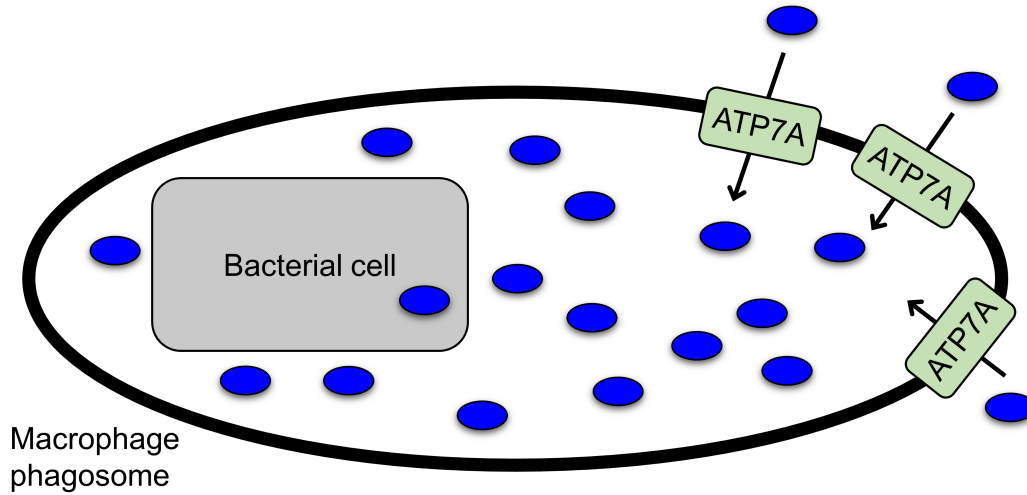


Figure 24. Potential action of a novel copper-targeting therapeutic. (Top) Macrophages utilize the copper ATPase ATP7A in the phagosome membrane to pump copper ions into the phagosome compartment. Excess copper can be toxic to the trapped bacterium; however, if the bacterium possesses copper resistance mechanisms, copper can be efficiently effluxed, allowing the bacterial cell to persist. (Bottom) A novel therapeutic that targets copper resistance would prevent the bacterial cell from persisting in the presence of excess copper.

Importantly, antibodies targeting important virulence factors of *A. baumannii* have been shown to be effective therapies in animal models of infection (86-88; 95; 113; 143; 167; 186; 231). The effectiveness of antibodies *in vivo* indicates that they could also be useful for proof of concept studies targeting copper resistance components.

Compared to antibodies, small molecules are typically easier to produce and administer to human patients. Small molecule inhibitors could be developed via screening strategies, either for the ability to prevent *in vitro* growth in the presence of copper or for prevention of a specific protein function. If small molecules that inhibit bacterial growth in copper are identified, their mechanism of action could then be further investigated. In order to screen compounds for inhibition of a specific protein's function, a defined biochemical assay would need to be developed. Of note, screening strategies have been used to identify small molecules with efficacy against *A. baumannii*. For example, an important *A. baumannii* drug efflux pump, AbeM, was cloned into drug-sensitive *E. coli*, and that *E. coli* strain was used to identify small molecules that prevented growth in the presence of ciprofloxacin (19). Additionally, a biochemical assay that measured compound binding was used to screen for compounds that bind BasE, an enzyme in the biosynthesis pathway of *A. baumannii*'s siderophore acinetobactin; the identified compounds inhibit bacterial growth in iron-limited conditions (141). Also, small molecules inhibiting lipase, a type 2 secreted effector protein, were identified by high-throughput screening in a biochemical assay; the assay was also optimized for identification of inhibitors of type 2 secretion, though no high-quality candidates were found (212). To combat pre-existing drug resistance, inhibitors of a common aminoglycoside-modifying enzyme were identified through small molecule

library screening (202). Finally, our collaborators identified a compound that blocks colistin resistance by interfering with regulation of the lipid A modification that confers resistance (75). Thus, small molecule approaches represent exciting possibilities for targeting of copper-related components in *A. baumannii*.

Beyond screening existing compounds, small molecules can also be designed from scratch using computer-based modeling strategies that predict compound binding; this strategy would require high resolution protein structures. For example, *in silico* modeling successfully identified compounds that targeted to an *A. baumannii* outer membrane protein, OmpW2; these compounds are bactericidal against many isolates (188). Modeling also aided in successful design of broad-spectrum compounds that target transcription in *A. baumannii* (36). Additionally, compounds targeted to diguanylate cyclases were identified by *in silico* screening, and efficacy was demonstrated against *A. baumannii* AB5711 biofilms (169).

To our knowledge, no therapeutics targeting bacterial copper homeostasis proteins have yet been investigated. However, similar types of bacterial proteins have been targeted successfully. Two-component regulatory systems, like CusRS, have been recognized in *A. baumannii* as potential drug targets; two different two-component systems in *A. baumannii* have been successfully inhibited by small molecules (37). Additionally, two-component systems have previously been targeted in other pathogens, including other ESKAPE pathogens like *P. aeruginosa* and *S. aureus* (37).

While it may seem like a bit of an aside, it is worth noting that copper homeostasis has been targeted in eukaryotic cells as a possible method to combat cancer (20; 219). While compounds that bind the universal CxxC copper-binding motif exist,

these are non-specific and thus have limited potential for clinical use (219). Recently, using computational and biochemical methods, compounds that specifically target the human copper chaperone proteins ATOX1 and CCS were identified; the compounds are predicted to prevent chaperone function by sterically blocking both protein-protein interaction and transfer of copper to other proteins (215). This success demonstrates that highly specific compounds can be developed against copper-related proteins.

CONSERVATION OF COPPER-RELATED PROTEINS

Because copper-related proteins are well conserved among bacteria, we hypothesize that any therapeutics that are eventually developed could potentially have broad-spectrum activity. We originally identified copper-related proteins in *A. baumannii* by homology with proteins in *E. coli* and *P. aeruginosa*; we found high levels of amino acid homology (Table 10). Nearly all of the proteins that we found to be important for copper resistance in AB5075 have homologs in both *E. coli* and *P. aeruginosa* (PcoB, PcoA, CopB, CueO, CusR, CusS, CopC, and CopD). Of these proteins, CusR is the most highly conserved and is well conserved in both other bacteria. PcoA and CueO, the copper oxidases, are well conserved in the more closely related *P. aeruginosa*, but less so when compared to *E. coli*. PcoB and CopB, homologous outer membrane proteins, are moderately well conserved in both organisms; the same is true for CusS and CopC. Further investigation into the nature of conservation of binding sites or important protein domains would enhance our understanding, but overall, these copper-related proteins are well conserved among these various bacterial species. This conservation would need to

Table 10. Conservation of copper-related proteins of *A. baumannii* AB5075

Genomic Region	Protein	Function	Homology (% identity / % similarity)	
			<i>E. coli</i>	<i>P. aeruginosa</i>
A	CzcC	Efflux?	-/-	-/-
	CzcB	Efflux?	25/44	34/53
	CzcA	Efflux?	35/56	62/78
	CzcD	Efflux?	40/59	56/73
B	CueR	Regulation	41/66	49/68
	CopA1	Efflux	41/61	46/64
C	PcoB	Efflux?	30/50	27/52
	PcoA	Oxidase	23/35	46/60
D	CopB	Efflux?	27/46	31/46
	CueO	Oxidase	24/38	45/59
	CusR	Regulation	62/81	63/80
	CusS	Regulation	34/54	34/53
	CopA2	Efflux	46/65	43/63
	CopC	Chaperone	34/43	39/53
	CopD	Efflux?	-/-	-/-

be kept in mind during the design of any future therapeutics.

While conservation of individual copper-related proteins lends itself to design of a broad-spectrum therapeutic, membership of the copper-related genes across bacterial species must also be considered because copper-related proteins are not found in bacteria at the same frequency. For example, while the copper ATPase CopA is by far the most abundant among gammaproteobacteria and is found in 85% of genomes, only ~35% have copper oxidases (CueO, PcoA) (79). Even fewer carry homologs of putative transport proteins PcoB (25%) or PcoD (4%). Clearly, these differences greatly impact the broadness of a therapeutic targeted at these proteins. More investigation is needed to fully understand the variation in copper-related genes among pathogenic bacteria and the potential effect this may have on therapeutics.

When developing any future copper-targeting therapeutic, it is also worth noting that some copper-related genes are also present and conserved in humans. This becomes a notable problem as activity against human proteins could lead to toxicity and side effects from treatment. Indeed, mutations impairing the function of human copper ATPases are associated with severe disease (reviewed in (158) and (99)). The human copper ATPases, ATP7A and ATP7B, are widely expressed throughout the body. They are important for trafficking of essential copper into metalloproteins, and thus are found in the Golgi membrane. Also, they are important for copper homeostasis within cells and can relocate to the outer membrane in vesicles to extrude copper; this is crucial for uptake of dietary copper through enterocytes by ATP7A and hepatocyte-dependent removal of excess copper into bile by ATP7B. Because ATP7A plays an important role in absorption of dietary copper, mutations in ATP7A result in copper deficiency, called Menkes disease.

Symptoms result from impairment of copper-requiring enzymes, and thus are diverse and very serious. Even with treatment with early copper replacement, patients typically do not live more than three years, unless the mutation in ATP7A only partially affects function; milder versions of Menkes with less severe symptoms have other classifications, including occipital horn syndrome and distal hereditary motor neuropathy. Because ATP7B is crucial for release of excess copper into bile, mutations in ATP7B are associated with copper overload in the body, called Wilson's disease. Copper accumulates first in the liver, leading to liver pathology, and later in the brain, leading to neurological and psychiatric symptoms. Copper also accumulates in the eye, and a golden ring in the iris is a pathognomonic sign of Wilson's disease. Treatment with a copper chelator is very successful for both prevention and treatment of pathology, though it must be continued throughout life. Clearly, novel therapeutics targeting copper ATPases will likely be toxic unless they can be designed to show specificity for the bacterium or can be delivered as a prodrug that is only metabolized within the bacteria.

In this same vein, the copper oxidases also have homology to human proteins, which poses concern for off-target side effects. While the entire protein is not conserved as with copper ATPases, there is ~25% identity across about 30% of the protein to the human protein ceruloplasmin, as well as to other hypothetical proteins (data not shown). Ceruloplasmin binds copper in the blood stream for transport, and thus is crucial for copper homeostasis in the host. Therefore, any putative therapeutics targeting CueO must be screened for effects on the function of ceruloplasmin.

Excitingly, the other copper-related proteins identified as virulence factors in AB5075 (PcoB, CopB, CusR, CusS, CopC, and CopD) do not have amino acid homology

with human proteins. Thus, these are exciting possibilities for novel treatments with reduced potential for human toxicity.

POTENTIAL USES OF COPPER RESISTANCE-TARGETED THERAPEUTICS

Novel therapeutics that target copper resistance have potential to be used to treat various types of infections. Copper-mediated immune mechanisms, through which we believe copper resistance becomes important for bacterial virulence, likely take place mostly inside phagocytic immune cells. Neutrophils are known to be important for clearance of *A. baumannii* from the lung, blood, and skin (58; 69). Thus, we believe that systemic administration of therapeutics that target copper resistance could be efficacious against multiple infection types. Additionally, because *A. baumannii* wound infections are common, especially among the military population, topical use of novel therapeutics is also an exciting possibility.

To enhance efficacy of any therapeutic targeting copper resistance, host copper stores could be increased, and we would expect that additional copper would have a synergistic effect with such a therapeutic. Host copper can be enhanced by increasing the amount of copper in the host diet or by targeted administration of the metal to the site of infection. Copper is an essential nutrient, and dietary copper could be increased in the infected individual within a safe range. A recommended daily intake for adults is about 1 mg and the typical intake from food is similar; however, the upper limit for intake is 8 – 10 mg per day, thus there is a reasonable range in which to increase dietary copper (91). To treat wound infections, a therapeutic could be used in combination with dietary copper

and/or copper-impregnated bandages. Clearly, the therapeutic window for exogenous copper is likely higher in the context of a superficial wound relative to dietary copper.

EVOLUTION OF COPPER RESISTANCE

While novel therapeutics targeting copper resistance and the use of copper for decontamination are exciting advances, the potential for evolution of resistance to these technologies is always present. Resistance to antibiotics often develops by alterations to the drug target, whether that be changes that prevent binding to the target or loss of the target altogether. This is, of course, possible for any therapeutic targeted at copper-related proteins as well. Bacteria evolve quickly and *A. baumannii* is famous for its ability to rapidly develop antibiotic resistance, though this ability is thought to be tied to horizontal gene transfer; therefore, resistance to a novel therapeutic targeting an atypical pathway hopefully should not be obtained as quickly.

It has been suggested that use of copper in hospitals may increase selective pressure and expedite the evolution of resistance. However, it is important to note that copper surfaces, as employed in hospital settings for decontamination, may kill bacterial pathogens in a unique way that is unaffected by the discussed copper resistance pathways. Indeed, on dry copper-containing surfaces we observed no difference in survival of our copper sensitive mutant strains relative to the wild-type parent strain (data not shown) and no statistically significant differences among the tested clinical isolates (Fig. 4). All isolates placed on copper-containing metal surfaces were killed to the point of sterilization in approximately 1.5 hours or less. Previous studies also report rapid

killing on copper-containing metals and very small differences resulting from mutations in similar copper-related genes (46; 48). The mechanisms by which copper-containing surfaces kill bacteria is unclear, and there is dispute in the literature about what mechanisms are most important. For example, work by Warnes *et al.* showed that cells are damaged on copper surfaces in many ways: i) membranes are rapidly depolarized, ii) respiration is inhibited, iii) reactive oxygen species (ROS) are produced, and iv) DNA is damaged (217; 218). A protective effect was observed with the addition of either copper chelating- or hydroxyl radical-chelating compounds; thus, it was proposed that damage is mediated by the generated hydroxyl radicals (217). However, there is discordance over whether damage is mediated by ROS or via another mechanism. In discrepancy with this hypothesis, contact-dependence of bacterial killing on copper surfaces has been shown; polymer structures on the surfaces of copper surfaces, which prevent cell contact with the surface, dramatically reduce killing of *Enterococcus hirae* even though copper ions are still released (125). Additionally, other studies have not observed less killing in conditions that reduce the production of reactive oxygen species (16; 48). Thus, it has been suggested that killing independent of ROS production may occur via non-specific opening of cation channels and rapid membrane depolarization (16). If true, the effectiveness of killing by copper surfaces would be independent of the discussed copper resistance mechanisms. The mechanism of copper-mediated killing clearly warrants further investigation as it will help to determine whether use of copper surfaces will place selective pressure on the copper resistance proteins of interest.

In thinking about the evolution of resistance, it is worth noting that some studies have investigated *in vitro* evolution of metal resistance in order to predict potential

methods by which bacteria might gain higher levels of copper resistance. Within the context of natural evolution of copper resistant plant pathogens, resistance genes are plasmid-based; resistance is passed to other bacteria via horizontal gene transfer and this has been conclusively observed *in vitro* (32). This type of evolution is based on gene sharing within a microbial community, which is possible in the hospital setting as well. However, it is important to remember that basic resistance to a stress is likely much easier to acquire via the acquisition of genes than is increased resistance to a particular therapeutic targeting a specific copper-related gene. One caveat to this would be if the additional genes made the drug target dispensable. Conversely, evolution of the targeted genes would more abruptly diminish the utility of a therapeutic. For example, one study observed a 5-fold increase in copper resistance in *Mycobacterium smegmatis* following *in vitro* exposure to gradually increasing concentrations of copper; the phenotype was mediated by increased expression of a copper ATPase (30). The exact mechanism by which expression of the efflux pump increased was not addressed. Similarly, evolution of resistance to silver *in vitro* has been assessed in *E. coli*, notably resulting in mutations in CusS, as it also binds silver (67). These results suggest that evolution and the potential for resistance must be taken into account in the design of copper-based technologies (68).

FUTURE OF TREATMENT OF INFECTIONS WITH DRUG-RESISTANT *A. BAUMANNII*

The current first line of treatment for *A. baumannii* infection is a broad-spectrum cephalosporin, a beta-lactam and beta-lactamase inhibitor, a carbapenem, or a combination of antibiotics. Unfortunately, treatment failure is common as rates of drug

resistance are very high. In cases of drug resistance, combination therapy, colistin, and/or tigecycline may be used (184).

In addition to our work and that of others identifying virulence components that may represent future drug targets, other strategies currently in development to combat drug-resistant *A. baumannii* include phage therapy, antibodies, vaccines, and nanoparticles. Phage therapy is an exciting possibility that has recently been used compassionately in a few human cases of serious infection with great success (109; 144; 174). However, evolution of resistance and personalization of treatment are still obstacles to widespread use of phage therapy against multidrug-resistant pathogens. Immunotherapy with antibodies is another area of interest for treatment of *A. baumannii*. Antibodies targeting important virulence factors of *A. baumannii* have been shown to be effective therapies in animal models of infection (86-88; 95; 113; 143; 167; 186; 231). However, antigenic heterogeneity is a major challenge to antibody therapy (135). Many of these antibody experiments originated from studies aiming to find an effective vaccine for *A. baumannii*. Much work has gone into identifying and testing vaccine candidates, ranging from individual proteins to whole cell vaccines (59).

Nanoparticles (diameter <100 nm) affect cell viability through an unknown mechanism that may be related to membrane stability; additionally, nanoparticles containing metals like silver and copper, have increased efficacy through increased surface area to volume ratio (178). Silver nanoparticles have been extensively studied and have efficacy both *in vitro* and *in vivo* (136). Very recently, *in vitro* efficacy of copper-containing nanoparticles against *A. baumannii* was demonstrated (6; 96). These studies

provide support for the use of metal-based therapeutics, and these nanoparticles could be used in combination with copper resistance-targeting therapeutics.

CONCLUSION

A. baumannii has recently evolved into a highly drug-resistant pathogen and is responsible for a significant number of infections and deaths in the United States and worldwide. Countless scientists have come together to further our understanding of *A. baumannii* biology and epidemiology in order to fight back against ever increasing rates of infection and drug resistance. Many novel infection control and treatment strategies are in development as a means to reduce the number of infections and the resulting patient morbidity and mortality. It is our hope that this thesis work contributes to the fight against drug resistance in *A. baumannii* and other important bacterial pathogens.

REFERENCES

1. Abdollahi S, Rasooli I, Mousavi Gargari SL. 2018. The role of TonB-dependent copper receptor in virulence of *Acinetobacter baumannii*. *Infect Genet Evol* 60:181-90
2. Achard ME, Stafford SL, Bokil NJ, Chartres J, Bernhardt PV, et al. 2012. Copper redistribution in murine macrophages in response to *Salmonella* infection. *Biochem J* 444:51-7
3. Achard ME, Tree JJ, Holden JA, Simpfendorfer KR, Wijburg OL, et al. 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. *Infect Immun* 78:2312-9
4. Adams MD, Goglin K, Molyneaux N, Hujer KM, Lavender H, et al. 2008. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J Bacteriol* 190:8053-64
5. Ahire JJ, Hattings M, Neveling DP, Dicks LM. 2016. Copper-Containing Anti-Biofilm Nanofiber Scaffolds as a Wound Dressing Material. *PLoS One* 11:e0152755
6. Alavi M, Karimi N. 2019. Biosynthesis of Ag and Cu NPs by secondary metabolites of usnic acid and thymol with biological macromolecules aggregation and antibacterial activities against multi drug resistant (MDR) bacteria. *Int J Biol Macromol* 128:893-901
7. Alquethamy SF, Khorvash M, Pederick VG, Whittall JJ, Paton JC, et al. 2019. The Role of the CopA Copper Efflux System in *Acinetobacter baumannii* Virulence. *Int J Mol Sci* 20
8. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM. 2008. Metal ions in biological catalysis: from enzyme databases to general principles. *J Biol Inorg Chem* 13:1205-18
9. Committee for the Update of the Guide for the Care and Use of Laboratory Animals. 2011. In *Guide for the Care and Use of Laboratory Animals*. Washington, DC: The National Academies Press.
10. Antunes LC, Visca P, Towner KJ. 2014. *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog Dis* 71:292-301
11. Arguello JM, Raimunda D, Padilla-Benavides T. 2013. Mechanisms of copper homeostasis in bacteria. *Front Cell Infect Microbiol* 3:73
12. Arnesano F, Banci L, Bertini I, Mangani S, Thompson AR. 2003. A redox switch in CopC: an intriguing copper trafficking protein that binds copper(I) and copper(II) at different sites. *Proc Natl Acad Sci U S A* 100:3814-9
13. Ashfaq M, Verma N, Khan S. 2016. Copper/zinc bimetal nanoparticles-dispersed carbon nanofibers: A novel potential antibiotic material. *Mater Sci Eng C Mater Biol Appl* 59:938-47
14. Ashfaq M, Verma N, Khan S. 2017. Highly effective Cu/Zn-carbon micro/nanofiber-polymer nanocomposite-based wound dressing biomaterial against the *P. aeruginosa* multi- and extensively drug-resistant strains. *Mater Sci Eng C Mater Biol Appl* 77:630-41

15. Bagai I, Rensing C, Blackburn NJ, McEvoy MM. 2008. Direct metal transfer between periplasmic proteins identifies a bacterial copper chaperone. *Biochemistry* 47:11408-14
16. Ballo MK, Rtimi S, Mancini S, Kiwi J, Pulgarin C, et al. 2016. Bactericidal activity and mechanism of action of copper-sputtered flexible surfaces against multidrug-resistant pathogens. *Appl Microbiol Biotechnol* 100:5945-53
17. Be NA, Allen JE, Brown TS, Gardner SN, McLoughlin KS, et al. 2014. Microbial profiling of combat wound infection through detection microarray and next-generation sequencing. *J Clin Microbiol* 52:2583-94
18. Bender CL, Cooksey DA. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. *J Bacteriol* 165:534-41
19. Bhattacharyya T, Sharma A, Akhter J, Pathania R. 2017. The small molecule IITR08027 restores the antibacterial activity of fluoroquinolones against multidrug-resistant *Acinetobacter baumannii* by efflux inhibition. *Int J Antimicrob Agents* 50:219-26
20. Blockhuys S, Wittung-Stafshede P. 2017. Roles of Copper-Binding Proteins in Breast Cancer. *Int J Mol Sci* 18
21. Borkow G, Gabbay J, Dardik R, Eidelman AI, Lavie Y, et al. 2010. Molecular mechanisms of enhanced wound healing by copper oxide-impregnated dressings. *Wound Repair Regen* 18:266-75
22. Borkow G, Okon-Levy N, Gabbay J. 2010. Copper oxide impregnated wound dressing: biocidal and safety studies. *Wounds* 22:301-10
23. Casey AL, Adams D, Karpanen TJ, Lambert PA, Cookson BD, et al. 2010. Role of copper in reducing hospital environment contamination. *J Hosp Infect* 74:72-7
24. Cha JS, Cooksey DA. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci U S A* 88:8915-9
25. Cha JS, Cooksey DA. 1993. Copper Hypersensitivity and Uptake in *Pseudomonas syringae* Containing Cloned Components of the Copper Resistance Operon. *Appl Environ Microbiol* 59:1671-4
26. Chan AP, Sutton G, DePew J, Krishnakumar R, Choi Y, et al. 2015. A novel method of consensus pan-chromosome assembly and large-scale comparative analysis reveal the highly flexible pan-genome of *Acinetobacter baumannii*. *Genome Biol* 16:143
27. Changela A, Chen K, Xue Y, Holschen J, Outten CE, et al. 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* 301:1383-7
28. Chaturvedi KS, Hung CS, Crowley JR, Stapleton AE, Henderson JP. 2012. The siderophore yersiniabactin binds copper to protect pathogens during infection. *Nat Chem Biol* 8:731-6
29. Chaturvedi KS, Hung CS, Giblin DE, Urushidani S, Austin AM, et al. 2014. Cupric yersiniabactin is a virulence-associated superoxide dismutase mimic. *ACS Chem Biol* 9:551-61
30. Chen Y, Yang F, Sun Z, Wang Q, Mi K, Deng H. 2015. Proteomic Analysis of Drug-Resistant Mycobacteria: Co-Evolution of Copper and INH Resistance. *PLoS One* 10:e0127788

31. Colin M, Klingelschmitt F, Charpentier E, Josse J, Kanagaratnam L, et al. 2018. Copper Alloy Touch Surfaces in Healthcare Facilities: An Effective Solution to Prevent Bacterial Spreading. *Materials (Basel)* 11
32. Colombi E, Straub C, Kunzel S, Templeton MD, McCann HC, Rainey PB. 2017. Evolution of copper resistance in the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* through acquisition of integrative conjugative elements and plasmids. *Environ Microbiol* 19:819-32
33. Cooper RM, Tsimring L, Hasty J. 2017. Inter-species population dynamics enhance microbial horizontal gene transfer and spread of antibiotic resistance. *Elife* 6
34. Corbett D, Schuler S, Glenn S, Andrew PW, Cavet JS, Roberts IS. 2011. The combined actions of the copper-responsive repressor CsoR and copper-metallochaperone CopZ modulate CopA-mediated copper efflux in the intracellular pathogen *Listeria monocytogenes*. *Mol Microbiol* 81:457-72
35. Cornejo-Juarez P, Vilar-Compte D, Perez-Jimenez C, Namendys-Silva SA, Sandoval-Hernandez S, Volkow-Fernandez P. 2015. The impact of hospital-acquired infections with multidrug-resistant bacteria in an oncology intensive care unit. *Int J Infect Dis* 31:31-4
36. Cossar PJ, Abdel-Hamid MK, Ma C, Sakoff JA, Trinh TN, et al. 2017. Small-Molecule Inhibitors of the NusB-NusE Protein-Protein Interaction with Antibiotic Activity. *ACS Omega* 2:3839-57
37. De Silva PM, Kumar A. 2019. Signal Transduction Proteins in *Acinetobacter baumannii*: Role in Antibiotic Resistance, Virulence, and Potential as Drug Targets. *Front Microbiol* 10:49
38. Denton M, Wilcox MH, Parnell P, Green D, Keer V, et al. 2004. Role of environmental cleaning in controlling an outbreak of *Acinetobacter baumannii* on a neurosurgical intensive care unit. *J Hosp Infect* 56:106-10
39. Deshpande LM, Kapadnis BP, Chopade BA. 1993. Metal resistance in *Acinetobacter* and its relation to beta-lactamase production. *Biometals* 6:55-9
40. Deus D, Krischek C, Pfeifer Y, Sharifi AR, Fiegen U, et al. 2017. Comparative analysis of the susceptibility to biocides and heavy metals of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates of human and avian origin, Germany. *Diagn Microbiol Infect Dis* 88:88-92
41. Dhakephalkar PK, Chopade BA. 1994. High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *Biometals* 7:67-74
42. Djoko KY, Franiek JA, Edwards JL, Falsetta ML, Kidd SP, et al. 2012. Phenotypic characterization of a *copA* mutant of *Neisseria gonorrhoeae* identifies a link between copper and nitrosative stress. *Infect Immun* 80:1065-71
43. Djoko KY, Ong CL, Walker MJ, McEwan AG. 2015. The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens. *J Biol Chem* 290:18954-61
44. Drees SL, Klinkert B, Helling S, Beyer DF, Marcus K, et al. 2017. One gene, two proteins: coordinated production of a copper chaperone by differential transcript formation and translational frameshifting in *Escherichia coli*. *Mol Microbiol* 106:635-45

45. Du X, Xu X, Yao J, Deng K, Chen S, et al. 2019. Predictors of mortality in patients infected with carbapenem-resistant *Acinetobacter baumannii*: A systematic review and meta-analysis. *Am J Infect Control*
46. Elguindi J, Wagner J, Rensing C. 2009. Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. *J Appl Microbiol* 106:1448-55
47. Eser OK, Ergin A, Hascelik G. 2015. Antimicrobial Activity of Copper Alloys Against Invasive Multidrug-Resistant Nosocomial Pathogens. *Curr Microbiol* 71:291-5
48. Espirito Santo C, Taudte N, Nies DH, Grass G. 2008. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. *Appl Environ Microbiol* 74:977-86
49. Evans DG, Evans DJ, Jr., Tjoa W. 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. *Infect Immun* 18:330-7
50. Falagas ME, Bliziotis IA, Siempos, II. 2006. Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Crit Care* 10:R48
51. Fierobe L, Lucet JC, Decre D, Muller-Serieys C, Deleuze A, et al. 2001. An outbreak of imipenem-resistant *Acinetobacter baumannii* in critically ill surgical patients. *Infect Control Hosp Epidemiol* 22:35-40
52. Fiester SE, Actis LA. 2013. Stress responses in the opportunistic pathogen *Acinetobacter baumannii*. *Future Microbiol* 8:353-65
53. Fleming ID, Krezalek MA, Belogortseva N, Zaborin A, Defazio J, et al. 2017. Modeling *Acinetobacter baumannii* wound infections: The critical role of iron. *J Trauma Acute Care Surg* 82:557-65
54. Francis MS, Thomas CJ. 1997. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. *Microb Pathog* 22:67-78
55. Franke S, Grass G, Rensing C, Nies DH. 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* 185:3804-12
56. Gallagher LA, Ramage E, Weiss EJ, Radey M, Hayden HS, et al. 2015. Resources for Genetic and Genomic Analysis of Emerging Pathogen *Acinetobacter baumannii*. *J Bacteriol* 197:2027-35
57. Garcia SS, Du Q, Wu H. 2016. *Streptococcus mutans* copper chaperone, CopZ, is critical for biofilm formation and competitiveness. *Mol Oral Microbiol* 31:515-25
58. Garcia-Patino MG, Garcia-Contreras R, Licona-Limon P. 2017. The Immune Response against *Acinetobacter baumannii*, an Emerging Pathogen in Nosocomial Infections. *Front Immunol* 8:441
59. Garcia-Quintanilla M, Pulido MR, McConnell MJ. 2013. First steps towards a vaccine against *Acinetobacter baumannii*. *Curr Pharm Biotechnol* 14:897-902
60. Gardner SP, Olson JW. 2018. Interaction of Copper Toxicity and Oxidative Stress in *Campylobacter jejuni*. *J Bacteriol* 200
61. Gaynes R, Edwards JR, National Nosocomial Infections Surveillance S. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* 41:848-54

62. Giammanco A, Cala C, Fasciana T, Dowzicky MJ. 2017. Global Assessment of the Activity of Tigecycline against Multidrug-Resistant Gram-Negative Pathogens between 2004 and 2014 as Part of the Tigecycline Evaluation and Surveillance Trial. *mSphere* 2
63. Gilbreath JJ, West AL, Pich OQ, Carpenter BM, Michel S, Merrell DS. 2012. Fur activates expression of the 2-oxoglutarate oxidoreductase genes (*oorDABC*) in *Helicobacter pylori*. *J Bacteriol* 194:6490-7
64. Gonzalez-Guerrero M, Raimunda D, Cheng X, Arguello JM. 2010. Distinct functional roles of homologous Cu⁺ efflux ATPases in *Pseudomonas aeruginosa*. *Mol Microbiol* 78:1246-58
65. Gracioso LH, Baltazar MPG, Avanzi IR, Karolski B, Oller Nascimento CA, Perpetuo EA. 2019. Analysis of copper response in *Acinetobacter* sp. by comparative proteomics. *Metallomics* 11:949-58
66. Grass G, Rensing C. 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem Biophys Res Commun* 286:902-8
67. Graves JL, Jr., Tajkarimi M, Cunningham Q, Campbell A, Nonga H, et al. 2015. Rapid evolution of silver nanoparticle resistance in *Escherichia coli*. *Front Genet* 6:42
68. Graves JL, Thomas M, Ewunkem JA. 2017. Antimicrobial Nanomaterials: Why Evolution Matters. *Nanomaterials (Basel)* 7
69. Grguric-Smith LM, Lee HH, Gandhi JA, Brennan MB, DeLeon-Rodriguez CM, et al. 2015. Neutropenia exacerbates infection by *Acinetobacter baumannii* clinical isolates in a murine wound model. *Front Microbiol* 6:1134
70. Gudipaty SA, Larsen AS, Rensing C, McEvoy MM. 2012. Regulation of Cu(I)/Ag(I) efflux genes in *Escherichia coli* by the sensor kinase CusS. *FEMS Microbiol Lett* 330:30-7
71. Gudipaty SA, McEvoy MM. 2014. The histidine kinase CusS senses silver ions through direct binding by its sensor domain. *Biochim Biophys Acta* 1844:1656-61
72. Guyer MS, Reed RR, Steitz JA, Low KB. 1981. Identification of a sex-factor-affinity site in *E. coli* as gamma delta. *Cold Spring Harb Symp Quant Biol* 45 Pt 1:135-40
73. Halachev MR, Chan JZ, Constantinidou CI, Cumley N, Bradley C, et al. 2014. Genomic epidemiology of a protracted hospital outbreak caused by multidrug-resistant *Acinetobacter baumannii* in Birmingham, England. *Genome Med* 6:70
74. Harding CM, Hennon SW, Feldman MF. 2018. Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat Rev Microbiol* 16:91-102
75. Harris TL, Worthington RJ, Hittle LE, Zurawski DV, Ernst RK, Melander C. 2014. Small molecule downregulation of PmrAB reverses lipid A modification and breaks colistin resistance. *ACS Chem Biol* 9:122-7
76. Harrison JJ, Ceri H, Turner RJ. 2007. Multimetal resistance and tolerance in microbial biofilms. *Nat Rev Microbiol* 5:928-38
77. Hassan KA, Pederick VG, Elbourne LD, Paulsen IT, Paton JC, et al. 2017. Zinc stress induces copper depletion in *Acinetobacter baumannii*. *BMC Microbiol* 17:59

78. Hawley JS, Murray CK, Griffith ME, McElmeel ML, Fulcher LC, et al. 2007. Susceptibility of *Acinetobacter* strains isolated from deployed U.S. military personnel. *Antimicrob Agents Chemother* 51:376-8
79. Hernandez-Montes G, Arguello JM, Valderrama B. 2012. Evolution and diversity of periplasmic proteins involved in copper homeostasis in gamma proteobacteria. *BMC Microbiol* 12:249
80. Hodgkinson V, Petris MJ. 2012. Copper homeostasis at the host-pathogen interface. *J Biol Chem* 287:13549-55
81. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35:322-32
82. Howard A, O'Donoghue M, Feeney A, Sleator RD. 2012. *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 3:243-50
83. Hsiao YM, Liu YF, Lee PY, Hsu PC, Tseng SY, Pan YC. 2011. Functional characterization of *copA* gene encoding multicopper oxidase in *Xanthomonas campestris* pv. *campestris*. *J Agric Food Chem* 59:9290-302
84. Hu YH, Wang HL, Zhang M, Sun L. 2009. Molecular analysis of the copper-responsive CopRSCD of a pathogenic *Pseudomonas fluorescens* strain. *J Microbiol* 47:277-86
85. Huang HI, Shih HY, Lee CM, Yang TC, Lay JJ, Lin YE. 2008. *In vitro* efficacy of copper and silver ions in eradicating *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*: implications for on-site disinfection for hospital infection control. *Water Res* 42:73-80
86. Huang W, Wang S, Yao Y, Xia Y, Yang X, et al. 2015. OmpW is a potential target for eliciting protective immunity against *Acinetobacter baumannii* infections. *Vaccine* 33:4479-85
87. Huang W, Yao Y, Long Q, Yang X, Sun W, et al. 2014. Immunization against multidrug-resistant *Acinetobacter baumannii* effectively protects mice in both pneumonia and sepsis models. *PLoS One* 9:e100727
88. Huang W, Yao Y, Wang S, Xia Y, Yang X, et al. 2016. Immunization with a 22-kDa outer membrane protein elicits protective immunity to multidrug-resistant *Acinetobacter baumannii*. *Sci Rep* 6:20724
89. Hunger M, Schmucker R, Kishan V, Hillen W. 1990. 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
90. Imperi F, Antunes LC, Blom J, Villa L, Iacono M, et al. 2011. The genomics of *Acinetobacter baumannii*: insights into genome plasticity, antimicrobial resistance and pathogenicity. *IUBMB Life* 63:1068-74
91. Institute of Medicine (U.S.). Panel on Micronutrients. 2001. *DRI : dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc : a report of the Panel on Micronutrients ... and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine*. Washington, D.C.: National Academy Press. xxii, 773 p. pp.
92. Irene G, Georgios P, Ioannis C, Anastasios T, Diamantis P, et al. 2016. Copper-coated textiles: armor against MDR nosocomial pathogens. *Diagn Microbiol Infect Dis* 85:205-9

93. Jacobs AC, Thompson MG, Black CC, Kessler JL, Clark LP, et al. 2014. AB5075, a Highly Virulent Isolate of *Acinetobacter baumannii*, as a Model Strain for the Evaluation of Pathogenesis and Antimicrobial Treatments. *MBio* 5:e01076-14
94. Jacobs AC, Thompson MG, Gebhardt M, Corey BW, Yildirim S, et al. 2014. Genetic Manipulation of *Acinetobacter baumannii*. *Curr Protoc Microbiol* 35:6G 2 1-6G 2 11
95. Jahangiri A, Owlia P, Rasooli I, Salimian J, Derakhshanifar E, et al. 2019. Specific egg yolk antibodies (IgY) confer protection against *Acinetobacter baumannii* in a murine pneumonia model. *J Appl Microbiol* 126:624-32
96. Jaramillo AF, Riquelme SA, Sanchez-Sanhueza G, Medina C, Solis-Pomar F, et al. 2019. Comparative Study of the Antimicrobial Effect of Nanocomposites and Composite Based on Poly(butylene adipate-co-terephthalate) Using Cu and Cu/Cu₂O Nanoparticles and CuSO₄. *Nanoscale Res Lett* 14:158
97. Johnson MD, Kehl-Fie TE, Klein R, Kelly J, Burnham C, et al. 2015. Role of copper efflux in pneumococcal pathogenesis and resistance to macrophage-mediated immune clearance. *Infect Immun* 83:1684-94
98. Jones PW, Taylor DM, Williams DR, Finney M, Iorwerth A, et al. 2001. Using wound fluid analyses to identify trace element requirements for efficient healing. *J Wound Care* 10:205-8
99. Kaler SG. 2013. Inborn errors of copper metabolism. *Handb Clin Neurol* 113:1745-54
100. Kalivoda EJ, Horzempa J, Stella NA, Sadaf A, Kowalski RP, et al. 2011. New vector tools with a hygromycin resistance marker for use with opportunistic pathogens. *Mol Biotechnol* 48:7-14
101. Karah N, Sundsfjord A, Towner K, Samuelsen O. 2012. Insights into the global molecular epidemiology of carbapenem non-susceptible clones of *Acinetobacter baumannii*. *Drug Resist Updat* 15:237-47
102. Karpanen TJ, Casey AL, Lambert PA, Cookson BD, Nightingale P, et al. 2012. The antimicrobial efficacy of copper alloy furnishing in the clinical environment: a crossover study. *Infect Control Hosp Epidemiol* 33:3-9
103. Keen EF, 3rd, Murray CK, Robinson BJ, Hospenthal DR, Co EM, Aldous WK. 2010. Changes in the incidences of multidrug-resistant and extensively drug-resistant organisms isolated in a military medical center. *Infect Control Hosp Epidemiol* 31:728-32
104. Kim EH, Nies DH, McEvoy MM, Rensing C. 2011. Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. *J Bacteriol* 193:2381-7
105. Klinkajon W, Supaphol P. 2014. Novel copper (II) alginate hydrogels and their potential for use as anti-bacterial wound dressings. *Biomed Mater* 9:045008
106. Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 6:130
107. Kumar A, Dalton C, Cortez-Cordova J, Schweizer HP. 2010. Mini-Tn7 vectors as genetic tools for single copy gene cloning in *Acinetobacter baumannii*. *J Microbiol Methods* 82:296-300

108. Ladomersky E, Khan A, Shanbhag V, Cavet JS, Chan J, et al. 2017. Host and Pathogen Copper-Transporting P-Type ATPases Function Antagonistically during *Salmonella* Infection. *Infect Immun* 85
109. LaVergne S, Hamilton T, Biswas B, Kumaraswamy M, Schooley RT, Wooten D. 2018. Phage Therapy for a Multidrug-Resistant *Acinetobacter baumannii* Craniectomy Site Infection. *Open Forum Infect Dis* 5:ofy064
110. Lee CR, Lee JH, Park M, Park KS, Bae IK, et al. 2017. Biology of *Acinetobacter baumannii*: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options. *Front Cell Infect Microbiol* 7:55
111. Lee HY, Chen CL, Wu SR, Huang CW, Chiu CH. 2014. Risk factors and outcome analysis of *Acinetobacter baumannii* complex bacteremia in critical patients. *Crit Care Med* 42:1081-8
112. Lee SM, Grass G, Rensing C, Barrett SR, Yates CJ, et al. 2002. The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. *Biochem Biophys Res Commun* 295:616-20
113. Li H, Tan H, Hu Y, Pan P, Su X, Hu C. 2017. Small protein A and phospholipase D immunization serves a protective role in a mouse pneumonia model of *Acinetobacter baumannii* infection. *Mol Med Rep* 16:1071-8
114. Liochev SI, Fridovich I. 2002. The Haber-Weiss cycle -- 70 years later: an alternative view. *Redox Rep* 7:55-7; author reply 9-60
115. Lob SH, Hoban DJ, Sahm DF, Badal RE. 2016. Regional differences and trends in antimicrobial susceptibility of *Acinetobacter baumannii*. *Int J Antimicrob Agents* 47:317-23
116. Lonergan ZR, Nairn BL, Wang J, Hsu YP, Hesse LE, et al. 2019. An *Acinetobacter baumannii*, Zinc-Regulated Peptidase Maintains Cell Wall Integrity during Immune-Mediated Nutrient Sequestration. *Cell Rep* 26:2009-18 e6
117. Lonergan ZR, Skaar EP. 2019. Nutrient Zinc at the Host-Pathogen Interface. *Trends Biochem Sci*
118. Longo F, Vuotto C, Donelli G. 2014. Biofilm formation in *Acinetobacter baumannii*. *New Microbiol* 37:119-27
119. Macomber L, Imlay JA. 2009. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc Natl Acad Sci U S A* 106:8344-9
120. Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, et al. 2014. Multistate point-prevalence survey of health care-associated infections. *N Engl J Med* 370:1198-208
121. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, et al. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 18:268-81
122. Magistrato A, Pavlin M, Qasem Z, Ruthstein S. 2019. Copper trafficking in eukaryotic systems: current knowledge from experimental and computational efforts. *Curr Opin Struct Biol* 58:26-33
123. Marais F, Mehtar S, Chalkley L. 2010. Antimicrobial efficacy of copper touch surfaces in reducing environmental bioburden in a South African community healthcare facility. *J Hosp Infect* 74:80-2

124. Marrero K, Sanchez A, Gonzalez LJ, Ledon T, Rodriguez-Ulloa A, et al. 2012. Periplasmic proteins encoded by VCA0261-0260 and VC2216 genes together with *copA* and *cueR* products are required for copper tolerance but not for virulence in *Vibrio cholerae*. *Microbiology* 158:2005-16
125. Mathews S, Hans M, Mucklich F, Solioz M. 2013. Contact killing of bacteria on copper is suppressed if bacterial-metal contact is prevented and is induced on iron by copper ions. *Appl Environ Microbiol* 79:2605-11
126. Mehtar S, Wiid I, Todorov SD. 2008. The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare facilities in the Western Cape: an *in-vitro* study. *J Hosp Infect* 68:45-51
127. Merrell DS, Camilli A. 2002. Acid tolerance of gastrointestinal pathogens. *Curr Opin Microbiol* 5:51-5
128. Metan G, Sariguzel F, Sumerkan B. 2009. Factors influencing survival in patients with multi-drug-resistant *Acinetobacter* bacteraemia. *Eur J Intern Med* 20:540-4
129. Meydan S, Klepacki D, Karthikeyan S, Margus T, Thomas P, et al. 2017. Programmed Ribosomal Frameshifting Generates a Copper Transporter and a Copper Chaperone from the Same Gene. *Mol Cell* 65:207-19
130. Migocka M. 2015. Copper-transporting ATPases: The evolutionarily conserved machineries for balancing copper in living systems. *IUBMB Life* 67:737-45
131. Mikolay A, Huggett S, Tikana L, Grass G, Braun J, Nies DH. 2010. Survival of bacteria on metallic copper surfaces in a hospital trial. *Appl Microbiol Biotechnol* 87:1875-9
132. Milanino R, Marrella M, Gasperini R, Pasqualicchio M, Velo G. 1993. Copper and zinc body levels in inflammation: an overview of the data obtained from animal and human studies. *Agents Actions* 39:195-209
133. Mills SD, Jasalavich CA, Cooksey DA. 1993. A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J Bacteriol* 175:1656-64
134. Mogilnaya OA, Lobova TI, Kargatova TV, Popova LY. 2005. Biofilm formation by bacterial associations under various salinities and copper ion stress. *Biofouling* 21:247-55
135. Motley MP, Fries BC. 2017. A New Take on an Old Remedy: Generating Antibodies against Multidrug-Resistant Gram-Negative Bacteria in a Postantibiotic World. *mSphere* 2
136. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. 2019. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front Microbiol* 10:539
137. Muller MP, MacDougall C, Lim M, Ontario Agency for Health P, Promotion Public Health O, et al. 2016. Antimicrobial surfaces to prevent healthcare-associated infections: a systematic review. *J Hosp Infect* 92:7-13
138. Munson GP, Lam DL, Outten FW, O'Halloran TV. 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J Bacteriol* 182:5864-71
139. Murray CK, Roop SA, Hospenthal DR, Dooley DP, Wenner K, et al. 2006. Bacteriology of war wounds at the time of injury. *Mil Med* 171:826-9

140. Muwaffak Z, Goyanes A, Clark V, Basit AW, Hilton ST, Gaisford S. 2017. Patient-specific 3D scanned and 3D printed antimicrobial polycaprolactone wound dressings. *Int J Pharm* 527:161-70
141. Neres J, Engelhart CA, Drake EJ, Wilson DJ, Fu P, et al. 2013. Non-nucleoside inhibitors of BasE, an adenylating enzyme in the siderophore biosynthetic pathway of the opportunistic pathogen *Acinetobacter baumannii*. *J Med Chem* 56:2385-405
142. Newberne PM, Hunt CE, Young VR. 1968. The role of diet and the reticuloendothelial system in the response of rats to *Salmonella typhimurium* infection. *Br J Exp Pathol* 49:448-57
143. Nielsen TB, Pantapalangkoor P, Luna BM, Bruhn KW, Yan J, et al. 2017. Monoclonal Antibody Protects Against *Acinetobacter baumannii* Infection by Enhancing Bacterial Clearance and Evading Sepsis. *J Infect Dis* 216:489-501
144. Nir-Paz R, Gelman D, Khouri A, Sisson BM, Fackler J, et al. 2019. Successful treatment of antibiotic resistant poly-microbial bone infection with bacteriophages and antibiotics combination. *Clin Infect Dis*
145. O'Gorman J, Humphreys H. 2012. Application of copper to prevent and control infection. Where are we now? *J Hosp Infect* 81:217-23
146. Odermatt A, Suter H, Krapf R, Solioz M. 1992. An ATPase operon involved in copper resistance by *Enterococcus hirae*. *Ann N Y Acad Sci* 671:484-6
147. Olsen I. 2015. Biofilm-specific antibiotic tolerance and resistance. *Eur J Clin Microbiol Infect Dis* 34:877-86
148. World Health Organization. 2017. Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics, WHO
149. Osman D, Waldron KJ, Denton H, Taylor CM, Grant AJ, et al. 2010. Copper homeostasis in *Salmonella* is atypical and copper-CueP is a major periplasmic metal complex. *J Biol Chem* 285:25259-68
150. Outten FW, Huffman DL, Hale JA, O'Halloran TV. 2001. The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* 276:30670-7
151. Outten FW, Outten CE, Hale J, O'Halloran TV. 2000. Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, *cueR*. *J Biol Chem* 275:31024-9
152. Padilla-Benavides T, George Thompson AM, McEvoy MM, Arguello JM. 2014. Mechanism of ATPase-mediated Cu⁺ export and delivery to periplasmic chaperones: the interaction of *Escherichia coli* CopA and CusF. *J Biol Chem* 289:20492-501
153. Palmer LD, Skaar EP. 2016. Transition Metals and Virulence in Bacteria. *Annu Rev Genet* 50:67-91
154. Park YS, Kim TH, Yun CW. 2017. Functional characterization of the copper transcription factor AfMac1 from *Aspergillus fumigatus*. *Biochem J* 474:2365-78
155. Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC, Jr., Mylonakis E. 2009. *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrob Agents Chemother* 53:2605-9

156. Peleg AY, Seifert H, Paterson DL. 2008. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* 21:538-82
157. Poirel L, Bonnin RA, Nordmann P. 2011. Genetic basis of antibiotic resistance in pathogenic *Acinetobacter* species. *IUBMB Life* 63:1061-7
158. Polishchuk R, Lutsenko S. 2013. Golgi in copper homeostasis: a view from the membrane trafficking field. *Histochem Cell Biol* 140:285-95
159. Prata-Rocha ML, Gontijo-Filho PP, Melo GB. 2012. Factors influencing survival in patients with multidrug-resistant *Acinetobacter baumannii* infection. *Braz J Infect Dis* 16:237-41
160. Centers for Disease Control and Prevention. 2013. Antibiotic Resistance Threats in the United States, 2013, CDC, Atlanta, GA
161. Puig S, Rees EM, Thiele DJ. 2002. The ABCDs of periplasmic copper trafficking. *Structure* 10:1292-5
162. Purves J, Thomas J, Riboldi GP, Zapotoczna M, Tarrant E, et al. 2018. A horizontally gene transferred copper resistance locus confers hyper-resistance to antibacterial copper toxicity and enables survival of community acquired methicillin resistant *Staphylococcus aureus* USA300 in macrophages. *Environ Microbiol* 20:1576-89
163. Rademacher C, Masepohl B. 2012. Copper-responsive gene regulation in bacteria. *Microbiology* 158:2451-64
164. Rensing C, Fan B, Sharma R, Mitra B, Rosen BP. 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proc Natl Acad Sci U S A* 97:652-6
165. Roca I, Espinal P, Vila-Farres X, Vila J. 2012. The *Acinetobacter baumannii* Oxymoron: Commensal Hospital Dweller Turned Pan-Drug-Resistant Menace. *Front Microbiol* 3:148
166. Rouch DA, Brown NL. 1997. Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant *pco*. *Microbiology* 143 (Pt 4):1191-202
167. Russo TA, Beanan JM, Olson R, MacDonald U, Cox AD, et al. 2013. The K1 capsular polysaccharide from *Acinetobacter baumannii* is a potential therapeutic target via passive immunization. *Infect Immun* 81:915-22
168. Salgado CD, Sepkowitz KA, John JF, Cantey JR, Attaway HH, et al. 2013. Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. *Infect Control Hosp Epidemiol* 34:479-86
169. Sambanthamoorthy K, Luo C, Pattabiraman N, Feng X, Koestler B, et al. 2014. Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development. *Biofouling* 30:17-28
170. Sambrook J, Russell DW. 2001. *Molecular cloning : a laboratory manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press
171. Schmidt MG, Attaway HH, Sharpe PA, John J, Jr., Sepkowitz KA, et al. 2012. Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *J Clin Microbiol* 50:2217-23
172. Schmidt MG, Attaway iii HH, Fairey SE, Steed LL, Michels HT, Salgado CD. 2013. Copper continuously limits the concentration of bacteria resident on bed rails within the intensive care unit. *Infect Control Hosp Epidemiol* 34:530-3

173. Schmidt MG, von Dessauer B, Benavente C, Benadof D, Cifuentes P, et al. 2016. Copper surfaces are associated with significantly lower concentrations of bacteria on selected surfaces within a pediatric intensive care unit. *Am J Infect Control* 44:203-9
174. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, et al. 2017. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant *Acinetobacter baumannii* Infection. *Antimicrob Agents Chemother* 61
175. Schoolnik GK, Voskuil MI, Schnappinger D, Yildiz FH, Meibom K, et al. 2001. Whole genome DNA microarray expression analysis of biofilm development by *Vibrio cholerae* O1 E1 Tor. *Methods Enzymol* 336:3-18
176. Schwan WR, Warren P, Keunz E, Stover CK, Folger KR. 2005. Mutations in the *cueA* gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of *Pseudomonas aeruginosa* in mice. *Int J Med Microbiol* 295:237-42
177. Scott P, Deye G, Srinivasan A, Murray C, Moran K, et al. 2007. An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. *Clin Infect Dis* 44:1577-84
178. Seil JT, Webster TJ. 2012. Antimicrobial applications of nanotechnology: methods and literature. *Int J Nanomedicine* 7:2767-81
179. Selcuk CT, Durgun M, Ozalp B, Tekin A, Tekin R, et al. 2012. Comparison of the antibacterial effect of silver sulfadiazine 1%, mupirocin 2%, Acticoat and octenidine dihydrochloride in a full-thickness rat burn model contaminated with multi drug resistant *Acinetobacter baumannii*. *Burns* 38:1204-9
180. Shafeeq S, Yesilkaya H, Kloosterman TG, Narayanan G, Wandel M, et al. 2011. The *cop* operon is required for copper homeostasis and contributes to virulence in *Streptococcus pneumoniae*. *Mol Microbiol* 81:1255-70
181. Sheldon JR, Skaar EP. 2019. Metals as phagocyte antimicrobial effectors. *Curr Opin Immunol* 60:1-9
182. Shi X, Festa RA, Ioerger TR, Butler-Wu S, Sacchettini JC, et al. 2014. The copper-responsive RicR regulon contributes to *Mycobacterium tuberculosis* virulence. *MBio* 5
183. Shih HY, Lin YE. 2010. Efficacy of copper-silver ionization in controlling biofilm- and plankton-associated waterborne pathogens. *Appl Environ Microbiol* 76:2032-5
184. Shin B, Park W. 2017. Antibiotic resistance of pathogenic *Acinetobacter* species and emerging combination therapy. *J Microbiol* 55:837-49
185. Sifri CD, Burke GH, Enfield KB. 2016. Reduced health care-associated infections in an acute care community hospital using a combination of self-disinfecting copper-impregnated composite hard surfaces and linens. *Am J Infect Control* 44:1565-71
186. Singh R, Capalash N, Sharma P. 2017. Immunoprotective potential of BamA, the outer membrane protein assembly factor, against MDR *Acinetobacter baumannii*. *Sci Rep* 7:12411

187. Solioz M, Odermatt A. 1995. Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. *J Biol Chem* 270:9217-21
188. Soojhawon I, Pattabiraman N, Tsang A, Roth AL, Kang E, Noble SM. 2017. Discovery of novel inhibitors of multidrug-resistant *Acinetobacter baumannii*. *Bioorg Med Chem* 25:5477-82
189. Souli M, Galani I, Plachouras D, Panagea T, Armaganidis A, et al. 2013. Antimicrobial activity of copper surfaces against carbapenemase-producing contemporary Gram-negative clinical isolates. *J Antimicrob Chemother* 68:852-7
190. Spellberg B, Rex JH. 2013. The value of single-pathogen antibacterial agents. *Nat Rev Drug Discov* 12:963
191. Stafford SL, Bokil NJ, Achard ME, Kapetanovic R, Schembri MA, et al. 2013. Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper. *Biosci Rep* 33
192. Stoyanov JV, Hobman JL, Brown NL. 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Mol Microbiol* 39:502-11
193. Subashchandrabose S, Hazen TH, Brumbaugh AR, Himpsl SD, Smith SN, et al. 2014. Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection. *Proc Natl Acad Sci U S A* 111:18327-32
194. Tabah A, Koulenti D, Laupland K, Misset B, Valles J, et al. 2012. Characteristics and determinants of outcome of hospital-acquired bloodstream infections in intensive care units: the EUROBACT International Cohort Study. *Intensive Care Med* 38:1930-45
195. Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR. 2006. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *J Bacteriol* 188:7242-56
196. Tetaz TJ, Luke RK. 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. *J Bacteriol* 154:1263-8
197. Thompson MG, Black CC, Pavlicek RL, Honnold CL, Wise MC, et al. 2014. Validation of a novel murine wound model of *Acinetobacter baumannii* infection. *Antimicrob Agents Chemother* 58:1332-42
198. Timsit JF, Soubirou JF, Voiriot G, Chemam S, Neuville M, et al. 2014. Treatment of bloodstream infections in ICUs. *BMC Infect Dis* 14:489
199. Tipton KA, Dimitrova D, Rather PN. 2015. Phase-Variable Control of Multiple Phenotypes in *Acinetobacter baumannii* Strain AB5075. *J Bacteriol* 197:2593-9
200. Towner KJ. 2009. *Acinetobacter*: an old friend, but a new enemy. *J Hosp Infect* 73:355-63
201. Tran PL, Huynh E, Hamood AN, de Souza A, Mehta D, et al. 2017. The ability of a colloidal silver gel wound dressing to kill bacteria *in vitro* and *in vivo*. *J Wound Care* 26:S16-S24
202. Tran T, Chiem K, Jani S, Arivett BA, Lin DL, et al. 2018. Identification of a small molecule inhibitor of the aminoglycoside 6'-N-acetyltransferase type Ib [AAC(6')-Ib] using mixture-based combinatorial libraries. *Int J Antimicrob Agents* 51:752-61
203. Tree JJ, Ulett GC, Ong CL, Trott DJ, McEwan AG, Schembri MA. 2008. Trade-off between iron uptake and protection against oxidative stress: deletion of *cueO*

- promotes uropathogenic *Escherichia coli* virulence in a mouse model of urinary tract infection. *J Bacteriol* 190:6909-12
204. Tsai CJ, Loh JM, Proft T. 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7:214-29
 205. Urano H, Umezawa Y, Yamamoto K, Ishihama A, Ogasawara H. 2015. Cooperative regulation of the common target genes between H(2)O(2)-sensing YedVW and Cu(2)(+)-sensing CusSR in *Escherichia coli*. *Microbiology* 161:729-38
 206. Vallenet D, Nordmann P, Barbe V, Poirel L, Mangenot S, et al. 2008. Comparative analysis of *Acinetobacters*: three genomes for three lifestyles. *PLoS One* 3:e1805
 207. Vincent JL, Bihari DJ, Suter PM, Bruining HA, White J, et al. 1995. The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care (EPIC) Study. EPIC International Advisory Committee. *JAMA* 274:639-44
 208. Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, et al. 2009. International study of the prevalence and outcomes of infection in intensive care units. *JAMA* 302:2323-9
 209. Vincent M, Hartemann P, Engels-Deutsch M. 2016. Antimicrobial applications of copper. *Int J Hyg Environ Health* 219:585-91
 210. von Dessauer B, Navarrete MS, Benadof D, Benavente C, Schmidt MG. 2016. Potential effectiveness of copper surfaces in reducing health care-associated infection rates in a pediatric intensive and intermediate care unit: A nonrandomized controlled trial. *Am J Infect Control* 44:e133-9
 211. Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. 1993. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nat Genet* 3:7-13
 212. Waack U, Johnson TL, Chedid K, Xi C, Simmons LA, et al. 2017. Targeting the Type II Secretion System: Development, Optimization, and Validation of a High-Throughput Screen for the Identification of Small Molecule Inhibitors. *Front Cell Infect Microbiol* 7:380
 213. Wagner D, Maser J, Lai B, Cai Z, Barry CE, 3rd, et al. 2005. Elemental analysis of *Mycobacterium avium*-, *Mycobacterium tuberculosis*-, and *Mycobacterium smegmatis*-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. *J Immunol* 174:1491-500
 214. Wahid F, Zhong C, Wang HS, Hu XH, Chu LQ. 2017. Recent Advances in Antimicrobial Hydrogels Containing Metal Ions and Metals/Metal Oxide Nanoparticles. *Polymers (Basel)* 9
 215. Wang J, Luo C, Shan C, You Q, Lu J, et al. 2015. Inhibition of human copper trafficking by a small molecule significantly attenuates cancer cell proliferation. *Nat Chem* 7:968-79
 216. Ward SK, Abomoelak B, Hoyer EA, Steinberg H, Talaat AM. 2010. CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* 77:1096-110

217. Warnes SL, Caves V, Keevil CW. 2012. Mechanism of copper surface toxicity in *Escherichia coli* O157:H7 and *Salmonella* involves immediate membrane depolarization followed by slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. *Environ Microbiol* 14:1730-43
218. Warnes SL, Green SM, Michels HT, Keevil CW. 2010. Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. *Appl Environ Microbiol* 76:5390-401
219. Weekley CM, He C. 2017. Developing drugs targeting transition metal homeostasis. *Curr Opin Chem Biol* 37:26-32
220. White C, Lee J, Kambe T, Fritsche K, Petris MJ. 2009. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Biol Chem* 284:33949-56
221. Wiemann P, Perevitsky A, Lim FY, Shadkchan Y, Knox BP, et al. 2017. *Aspergillus fumigatus* Copper Export Machinery and Reactive Oxygen Intermediate Defense Counter Host Copper-Mediated Oxidative Antimicrobial Offense. *Cell Rep* 19:1008-21
222. Williams CL, Merrell DS. 2019. Testing Metal Sensitivity of *A. baumannii* Strains: Survival in Copper-Supplemented Liquid Media and on Copper-Containing Surfaces. *Methods Mol Biol* 1946:51-60
223. Williams CL, Neu HM, Gilbreath JJ, Michel SL, Zurawski DV, Merrell DS. 2016. Copper Resistance of the Emerging Pathogen *Acinetobacter baumannii*. *Appl Environ Microbiol* 82:6174-88
224. Williams CL, Neu HM, Michel SLJ, Merrell DS. 2019. Measuring Intracellular Metal Concentration via ICP-MS Following Copper Exposure. *Methods Mol Biol* 1946:195-205
225. Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, et al. 2011. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 108:1621-6
226. Wong D, Nielsen TB, Bonomo RA, Pantapalangkoor P, Luna B, Spellberg B. 2017. Clinical and Pathophysiological Overview of *Acinetobacter* Infections: a Century of Challenges. *Clin Microbiol Rev* 30:409-47
227. Yadav KK, Mandal AK, Chakraborty R. 2013. Copper susceptibility in *Acinetobacter junii* BB1A is related to the production of extracellular polymeric substances. *Antonie Van Leeuwenhoek* 104:261-9
228. Yamamoto K, Ishihama A. 2005. Transcriptional response of *Escherichia coli* to external copper. *Mol Microbiol* 56:215-27
229. Yan Q, Wang N. 2011. The ColR/ColS two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. *J Bacteriol* 193:1590-9
230. Yang CH, Menge JA, Cooksey DA. 1993. Role of copper resistance in competitive survival of *Pseudomonas fluorescens* in soil. *Appl Environ Microbiol* 59:580-4
231. Yang FL, Lou TC, Kuo SC, Wu WL, Chern J, et al. 2017. A medically relevant capsular polysaccharide in *Acinetobacter baumannii* is a potential vaccine candidate. *Vaccine* 35:1440-7

- 232. Zapotoczna M, Riboldi GP, Moustafa AM, Dickson E, Narechania A, et al. 2018. Mobile-Genetic-Element-Encoded Hypertolerance to Copper Protects *Staphylococcus aureus* from Killing by Host Phagocytes. *MBio* 9
- 233. Zarrilli R, Pournaras S, Giannouli M, Tsakris A. 2013. Global evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages. *Int J Antimicrob Agents* 41:11-9
- 234. Zhang XX, Rainey PB. 2007. The role of a P1-type ATPase from *Pseudomonas fluorescens* SBW25 in copper homeostasis and plant colonization. *Mol Plant Microbe Interact* 20:581-8
- 235. Zurawski DV, Black CC, Alamneh YA, Biggemann L, Banerjee J, et al. 2019. A Porcine Wound Model of *Acinetobacter baumannii* Infection. *Adv Wound Care (New Rochelle)* 8:14-27