Predicting Toxin-Antitoxin Systems Responsible for Host-Induced Persistence of *Burkholderia pseudomallei*

The Dissertation Committee for Brittany Nicole Ross Certifies that this is the approved version of the following dissertation:

Predicting Toxin-Antitoxin Systems Responsible for Host induced Persistence of *Burkholderia pseudomallei*

Committee:

Dr. Alfredo G. Torres
Mentor

Dr. Sophie Helaine

Dr. Janice Endsley

Dr. Richard Pyles

Dr. Vladimir Motin

Predicting Toxin-Antitoxin Systems Responsible for Host-Induced Persistence of *Burkholderia pseudomallei*

by

Brittany Nicole Ross B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

October 2019

Dedication

"May there never develop in me the notion that my education is complete but give me the strength and leisure and zeal continually to enlarge my knowledge." –
Maimonides –

This document is dedicated to my husband Sergio, parents Richard and Terri, grandparents Mike and Millie, and brother Cody for their tolerance and for supporting me along my seemingly never-ending pursuit of knowledge.

Acknowledgements

My love of science all began in the high school science classroom of Mrs. Mitchell. You were the first person who saw my passion for science. You spent time fostered my curiosity of how life works and planted the first seed of a career in science. Without your initial guidance, I may not have found this career path for which I am immensely excited to continue pursing. Along my academic career, I have encountered several influential people, one of the most prominent being Dr. David Lo. Thank you, David for providing the first opportunity to conduct an independent project. I still remember our meetings debating about alternative hypothesis and wagering on who's was correct.

To my most recent and profound mentor, Dr. Alfredo Torres thank you for your guidance, patience, and trust. You took me on as a research associate and your enthusiasm and support made it impossible to choose another lab for my graduate studies. Dr. Torres, you saw my drive and continuously fanned the flame of curiosity even if the flame got a bit too big and unruly at times. You provided me the resources, time, tools, and learning opportunities that has made me the young scientist I am today. I am proud to say that I am your legacy and will try my best to continue to make you proud. To the past and present lab members of the Torres' lab, thank you for your company, support, and friendship throughout this journey.

This dissertation in not just the project of one mind but many. To my dissertation committee, Drs. Helaine, Pyles, Motin, and Endsley, thank you for your investment of time, knowledge, and guidance through this project. I have learned that science is best when a product of several minds working together. Your questions all originate from different lenses and have influenced the science presented in this document. You all have pushed

me to be a strong critically thinker, a necessary skill as a move forward in the pursuit of a career as academic scientist.

Lastly, I would like to thank my family, for without whom, I would not be defending this PhD. To my grandparents, Mike and Millie, thank you for investing in my education at such an early age and being a source of both silliness and wisdom in my life. To my mother and father, first off, thank you for bringing me into this world. You two stood by me when I was stubborn, though my innocently talk about science, and when I traveled across the country to pursue this degree. You taught me the meaning of hard work, for which has made me the successful and strong-willed person I am today. To my brother, you are the kindest soul I know. Thank you for being there to talk through life's ups and downs. Lastly, to my partner in life and science, Sergio. Thank you for choosing me to spend your life with. You are the yin to my yang. You have taught me how relax and enjoy the moment which has kept me sane through the roller coaster of graduate school. Life is sweeter with you and I look forward to the many adventures we will embark upon together.

Predicting Toxin-Antitoxin Systems Responsible for Host Induced Persistence of *Burkholderia pseudomallei*

Publication No._____

Brittany Nicole Ross, PhD

The University of Texas Medical Branch, 2019

Supervisor: Alfredo G. Torres

Burkholderia pseudomallei (BPM) is the infectious agent that causes the disease. Melioidosis. The mortality rate for Melioidosis is up to 40% regardless of treatment. Despite the ability of certain antibiotics to reduce mortality, infection relapse occurs in 15-23% of treated patients. The inability of antibiotics to clear a pathogen is partly due to persistence, a mechanism employed by bacteria to enter a dormant state and evade the effects of antibiotics and host defenses. Toxin-antitoxin systems (TAS) have been identified as important modulators of persistence and targeting them provides an avenue for new drug development to reduce chronic infection. When investigating TAS, the predominant issue is the presence of a large number of TAS in an organism. Currently, there is no predictive model to determine which conditions toxins respond to. Because the major reason to investigate TAS is to define their role in chronic infection, this study aimed to identified and predict which toxins are associated with host survival. In the genome of BPM, 103 putative toxins were identified. Toxins were associated to a stress condition using unsupervised bipartite network analysis on an existing expression dataset that exposes BPM to 82 different conditions. Thirty-two toxins showed association to host-like conditions of which, six toxins were further examined for their role in host persistence. Toxins BPSS0390, BPSS1584, and BPSS0395 were upregulated in host-like conditions, but only moderately conserved among BPM strains. Loss of function moderately reduces

persistence in macrophages and reduces chronic infection in a murine model of Melioidosis. Three novel toxins, BPSS0899, BPSSS1321, and BPSL1494 were found to be highly conserved among BPM strains and demonstrated to be functional but were constitutively expressed. These toxins played an active role in *in vitro* persistence and macrophage survival, but not macrophage or *in vivo* persistence, likely due to redundancy of toxin systems. These findings shift the paradigm of toxin-antitoxin (TA) research to use a data-driven approach and indicate the utility of investigating constitutively expressed toxins as highly conserved regulators of persistence.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Illustrations

List of Abbreviations

UTMB	University of Texas Medical Branch
CDC	Center of Disease Control
GSBS	Graduate School of Biomedical Science
TDC	Thesis and Dissertation Coordinator
BPM	*Burkholderia pseudomallei*
TA	Toxin-Antitoxin
TAS	Toxin-Antitoxin System
MNGC	Multinucleated Giant Cell
ITS	Internal Transcribed Spacer
RFLP	Restriction Fragment Length Polymorphism
MLST	Multi-locus Sequence Typing
MALDI-TOF	Matrix-assisted Laser Desorption/ Ionization-Time of Flight
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
T3SS	Type III Secretion System
Hcp1	Heme Carrier Protein 1
FliC	Flagellin (gene)
BimA	Burkholderia Intracellular Motility Protein A
IV	Intravenous
IP	Intraperitoneal
IN	Intranasal
SC	Subcutaneous

SMX-TMP	Sulfathiazole-trimethoprim
CDC	Center of Disease Control
Mbps	Megabase Pairs
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
MIC	Minimal Inhibitory Concentration
MBC	Minimal Bactericidal Concentration
LD_{50}	Lethal Dose 50
LPS	Lipopolysaccharide
CFU	Colony Forming Unit(s)
LB	Luria-Bertani
RPMI	Roswell Park Memorial Institute (medium)
h	Hour(s)
Min	Minutes
μg	Microgram
mg	Milligram
kg	Kilogram
ANOVA	Analysis of Variance
MOI	Multiplicity of Infection
MΦ	Macrophage
DC	Dendritic Cell
TssM	Type VI secretion system ATPase and inner membrane protein
NOD2	Nucleotide-binding oligomerization domain-containing protein 2

TLR	Tol Like Receptor
PAMPS	Pathogen-Associated Molecular Patterns
CPS	Capsular Polysaccharide

CHAPTER 1 INTRODUCTION TO THE PATHOGEN

BURKHOLDERIA PSEUDOMALLEI

Burkholderia pseudomallei (BPM) is a gram-negative bacterium that was discovered in Yagon city of Myanmar formally known as Rangoon, in 1911. British doctor Alfred Whitmore and his colleagues noticed that the organism, they found, mimicked another pathogenic *Burkholderia* species known as *Burkholderia mallei* [1]. Over the years, this pathogen has been renamed as *Bacterium* (or *Bacillus*) *whitmori, Malleomyces pseudomallei, Loefflerella pseudomallei, Pfeifferella whitmori, Pseudomonas pseudomallei,* but as of 1992, it was officially renamed to *Burkholderia pseudomallei* by the Center of Disease Control (CDC) [1]. The *Burkholderia* genus is composed of 122 species that are from rhizosphere organisms, plant pathogens, or animal and human pathogens (May 2019; http://www.bacterio.net/burkholderia.html) [2]. Within the genus, BPM, *B. mallei*, and *B. thailandensis* make up the clade designated 1b [3]. BPM is predominately an environmental organism that survives in the soil, surface water, and within amoebas, but can also infect humans and animals[1]. In humans, the bacterium is a facultatively intracellular organism that can lead to asymptomatic infection, acute disease, or chronic infection with a chance of relapse[1]. *B. mallei* is a mammalian restricted pathogen that evolved from BPM through reductive evolution[4,5]. Like BPM, *B. mallei* causes human infections with the same symptomatology. Due to the lack of environmental colonization, naturally acquired *B. mallei* cases are infrequent [6]. *B. thailandensis* is a BSL1 classified, soil organism. On rare occasion, *B. thailandensis* has caused infection in humans, however it is 1000 times less lethal than BPM [3,7,8]. Due to the conservation of 85% of core genes between BPM and *B. thailandensis*, it is often used a surrogate for BPM studies[3,7].

BPM is a 1-2 μm bacilli bacterium containing two high GC (68%) circular chromosomes that encoding for 2590 genes[1,9]. Chromosome 1 is 4.07 Mbps and primarily

encodes for core housekeeping genes involved in metabolism, cell growth, motility, and cell wall synthesis[10]. The smaller chromosome, chromosome 2, is 3.17 Mbps and encodes for accessory RNAs and genes involved in environmental adaption[10]. Among BPM strains, 86% percent of the genome is conserved, while the remaining 14% is highly plastic consisting mainly of genomic islands[1]. Sequence variability has been attributed to homoplastity, recombination, and deletions [4,11-13]. Sequential sampling shows that while in the host, BPM undergoes adaptive mutations and genomic reduction[14,15].Genome variation makes it difficult to use phylogenic approaches such as multi-locus sequence typing, restriction fragment length polymorphism (RFLP), and pulse-field gel electrophoresis [4,11-13]. Although whole-genome sequence can accurately distinguish multiple interspecies clades, it is cost-prohibitive. Alternatively, the stability of three 16S-23S ribosomal DNA internal transcribed spacer (ITS), has allowed for broad phylogenic classification. Use of ITS has allowed of classification into six phylogenic types: C, E, G, GE, CE, CG, which captures geographic segregation [11,12,16].

ENDEMICITY

For many decades BPM endemicity was of public health importance in Southeast Asia and Northern Australia. In recent years, renewed interest in the bacterium has led to identification of the organism in previously nonendemic areas (**Illustration 1**). Based on whole-genome sequencing, Australia is considered to be the earliest source of BPM. The organism was next transmitted to southeast Asia and repeated transmissions were observed to neighboring countries facilitating expansion into South Asia and then East Asia [17]. Phylogenetic data indicates that isolates from Asia were transmitted to Africa and from Africa the bacteria was transmitted to the Americas (Central and South) between 1650 and 1850, coinciding with the slave trade[17,18]. In a 2016 report, cases had been reported in 45 countries, and an additional 34 countries were predicted to be endemic for the bacterium

[19]. Most recently, the pathogen was confirmed in both Central, South America, and Africa, but it is significantly underreported [19-21]..

Importation and establishment of BPM into previously non-endemic countries is possible through transport and contamination of the environment (soil, water, animals) where it can persist. To date, one case of confirmed importation with environmental establishment was reported in Paris, France (1975) [19]. The importation led to an outbreak, killing two and resulting in soil persistence for up to six years [19]. More recently there was an unintentional release in Louisiana, USA at the Tulane National Primate Research Center. The result was a small outbreak in nonhuman primates and soil contamination was suspected but was not detected [22]. Environmental suitability predictions indicate that Florida, Louisiana, and regions of Texas are at risk of BPM establishment. The potential for US infections increases with recent reports of documented cases in Mexico [20]. As of 2019, there is evidence of BPM presence in Texas, USA. Two independent human cases have been reported and genomic analysis classifies them as an independent lineage (unpublished). Currently, there are ongoing studies being conducted to trying to identify the source of the Texas cases. Aside from the threat of natural introduction into non-endemic area and the increasing global burden of this disease, the closely-related pathogen *B. mallei* has been successfully used as a bio-weapon. The nefarious use has resulted in both BPM and *B. mallei* being declared Tier 1 Select Agents by the CDC. With the lack vaccines, treatment is the only mechanism of control. Treatment in itself is challenging indicating the urgent need for better countermeasures[23].

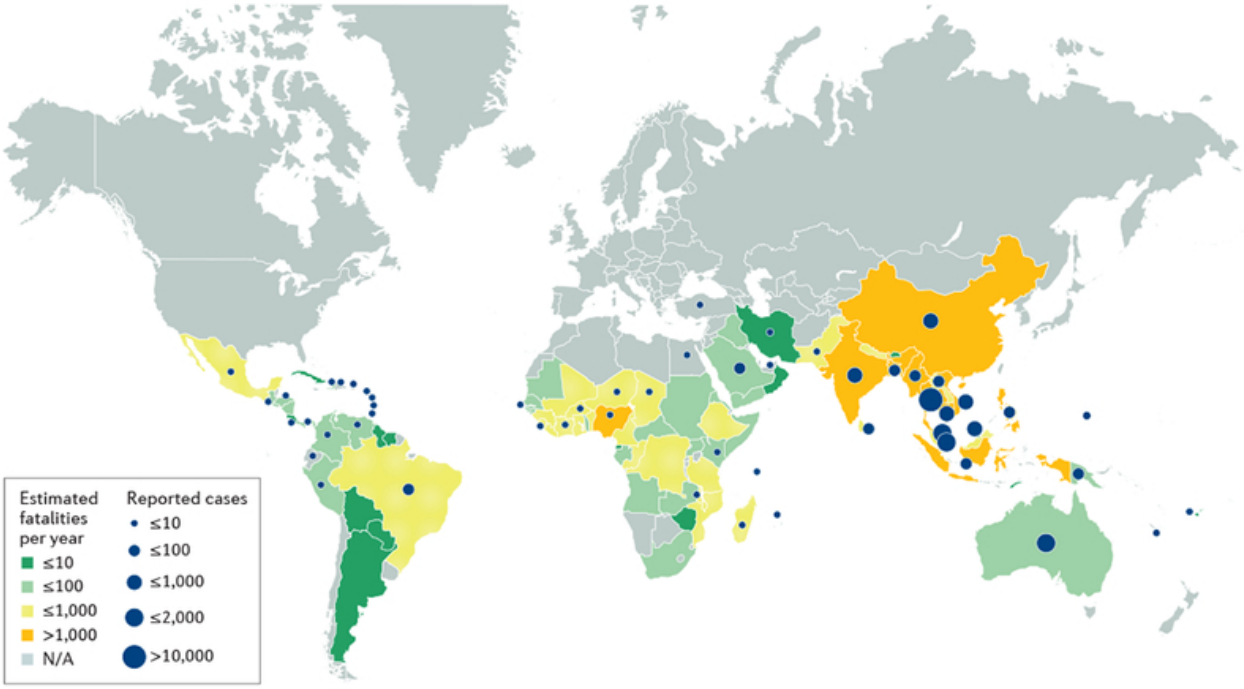


Illustration 1: Global Occurrence and Estimated Fatalities of Melioidosis

Distribution of BPM *reported cases (circles) and estimated fatalities (color coded) based on hospital data, national surveillance reports (Australia, Brunei, and Singapore), or predicted mortality rates per country. This figure highlights the burden however there are discrepancies between country-based reporting, hospital data, and prediction models. Discrepancies are due to under use of clinical microbiology laboratories, lack of awareness of Melioidosis, and poor disease reporting systems*[19]*. Used with the permission of Springer Nature Publishing Group 2019; License number 4654271189903.*

THE DISEASE: MELIOIDOSIS

It was not until 1921, 11 years after the discovery of BPM, that the disease was coined Melioidosis. Six years later, the first human case was reported in Sri Lanka[1]. Clinical manifestations vary widely, and the nonspecific symptoms overlap with many diseases which has led to the infection being termed "the great mimicker" [24]. Infection with BPM occurs through skin penetration, ingestion, or inhalation (**Illustration 2**). In endemic areas, seasonal rainfall and flooding leads to increased number of cases[25]. Based on seasonality data, the mean incubation period is nine days (range 1-21 days) [26].

Manifestations of Melioidosis can range from skin abscess, acute septicemia, neurological infection, recurring chronic disease, or asymptomatic chronic infection [27,28]. Skin infections generally lead to local ulceration and abscess, while ingestion causes mucosal ulceration and lymphadenopathy. An inhalational infection has the most severe manifestation and leads to pneumonia [29]. Regardless of the route, the bacterium is readily able to disseminate leading to acute detectable bacteremia (40-60% of all patients), septicemia (~20% of all cases), neurological infection, bone infections, and recurring chronic disease in 15-23% of treated patients[30-33]. Secondary organs most commonly colonized are the spleen, liver, prostate, and kidneys [1]. Melioidosis mortality ranges based on the route, dose, strain, and host risk factors. Symptomatic individuals are at risk of developing septicemia, which has as >90% mortality rate in the absence of treatment. When treatment is administered the mortality rate is 10-40%[1,34]. The most recent predictions of global disease incidence is 169,000 Melioidosis cases and 89,000 (54%) deaths occur worldwide per year [19]. To put this into perspective, the predicted number of deaths per year by BPM exceed dengue (12,500 deaths/year) and is comparable to the global mortality of measles (95,600 deaths/year) [19].

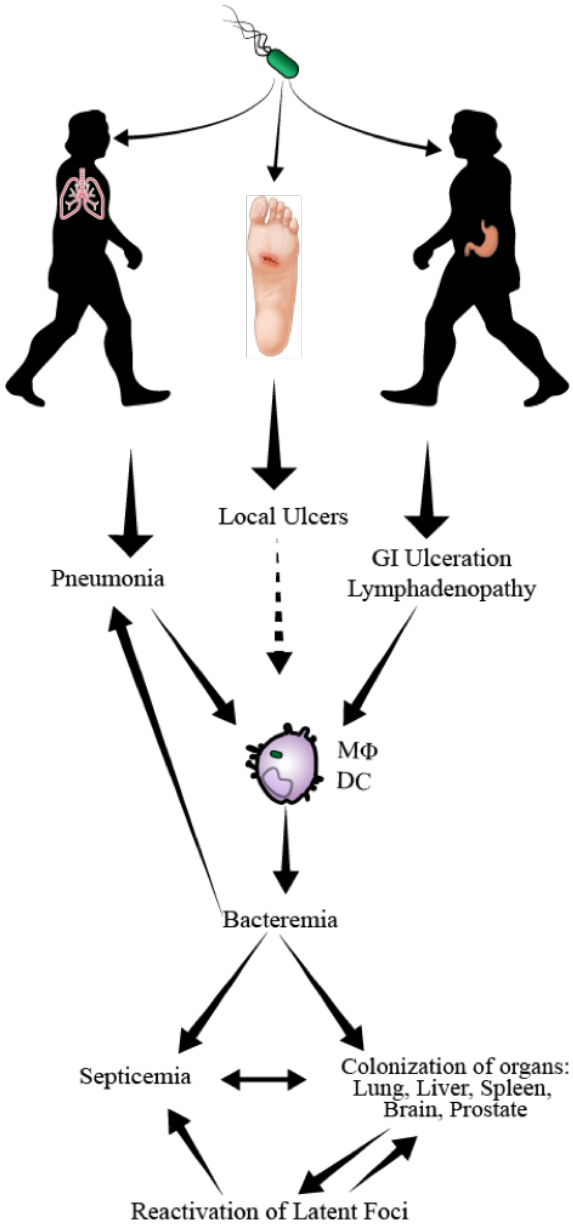


Illustration 2: Infection Progression

Melioidosis can be the result of inhalational, subcutaneous, or gastrointestinal exposure. Progression of infection is depicted here. Inoculation dependent symptoms are noted for each route of exposure. Evidence suggests that macrophages and dendritic cells facilitate dissemination within 24-48 h. Dissemination of the bacteria from one site of infection to another may lead to several symptomologies correlated with other route of infections, as is depicted[35,36]. All routes can lead to chronic infection in specific organs such as the spleen, liver, prostate and later lead to recrudescence[29,37].

People most at risk of having symptomatic Melioidosis are those with the following conditions: diabetes; thalassemia; age >45; excess alcohol consumption; liver disease; chronic lung disease; renal disease; use of immunosuppressants; being male; and participating in activities that increase their exposure, such as working in rice fields where BPM is prevalent [1,38]. Of all Melioidosis cases worldwide, 23-60% of infected persons have diabetes mellitus, making it the most common predisposing risk factor [1,39,40].. Although there are several risk factors, there is widespread evidence of asymptomatic infection. Epidemiological surveys have found varying rates of seroconversion in endemic countries. For example, 80% of people in Thailand seroconvert by age 4 [41]. In India, 29% of people have evidence of exposure [42,43] and in Australia 3% of people have antibody titers consistent with exposure[44]. Clinical reports have found that patients can carry the disease without any clinical manifestations confirming that asymptomatic cases do occur[14,38,45].

DIAGNOSTICS

Melioidosis is a disease with nonspecific symptoms, making accurate diagnosis difficult. Laboratory culturing is still the mainstream method of diagnosis. The bacterium can grow on most laboratory medium, but due to the slow growth rate, selective medium such as Ashdown or MacConkey, are superior. Both medias allow for improved identification because they highlight the phenotypic lack of lactose fermentation. Agar should be incubated for four days to allow for the wrinkled colony morphology to become visible; however, BPM can have several morphologies [1]. Appropriate samples for testing are blood, throat swabs, rectal swabs, pus, or urine (centrifuged) [1]. Repeated analysis is recommended due to low sensitivity of cultures samples[1]. Biochemical kits are not used for identification because they often lead to misidentification [46]. More accurate methods used to confirm BPM infection are antibody-antigen binding agglutination assays or antibiotic disc diffusion which can indicate BPM by its characteristic pattern of

susceptibility (regional resistance to gentamicin; resistance to colistin/polymyxin; susceptibility to amoxicillin-clavulanic acid) [28,46-48] . In countries with ample resources, identification is achievable with matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry, sequencing (16s rRNA), and target specific nucleic acid amplification (Type 3 Secretion System (T3SS) is the most promising)[1]. Serological approaches are not used due to the poor characterization of bacterial antigens and reports of false-negatives in septic patients [1,49,50]. Additionally, high rates of seroconversion and high baseline antibodies, due to prior exposure to BPM or closely related organisms in endemic areas, confound serological diagnosis [1,49,50].Novel, cost effective approaches are currently being developed to target endemic regions. One such assay currently being tested in clinics is a lateral flow immunoassay that detects the extracellular capsular polysaccharides [47]. Although diagnostic can be achieved, most Melioidosis cases occur in resource-poor environments where the presence of BPM may not be known, making diagnosis problematic.

INFECTION MANAGEMENT

The key to effective treatment of Melioidosis is early diagnosis and use of the correct antibiotics. In a state-of-the-art facility, which can manage sepsis, mortality rates are 10% while in resource-limited areas, as is the case in most endemic regions, mortality can exceed 40% [51]. Without an existing vaccine, the only way to combat a BPM infection is through a partially effective antibiotic treatment that results in a relapse of 15-23% of convalescent patients [30]. Although reinfection is possible, relapse in 75% of cases is due to recurrent infection [31]. BPM is intrinsically resistant to most antibiotics, (penicillin, ampicillin, first- and second-generation cephalosporins, gentamicin, tobramycin, streptomycin, macrolides, and polymyxins), but a handful of antibiotics are effective for which the emergence of newly resistant strains is rare [28,52-54].

Intravenous Therapy (10-14 days)		
Drug	Dose	Class; Action
Ceftazidime	50 mg/kg (max 2g/day)	Cephalosporins Bactericidal: Inhibits cell wall synthesis
Meropenem	25 mg/kg (max 1g/day)	Carbapenems Bactericidal: Inhibits cell wall synthesis
+/- Trimethoprim–sulfamethoxazole	Children: 6+30 mg/kg Adults: 320+1600 mg/kg	Sulfonamides Bacteriostatic: Inhibits dihydrofolic acid synthesis
Extended intravenous therapy (≥4–8 weeks) is recommended for complicated pneumonia, deep-seated infection (including prostatic abscesses), neurological melioidosis, osteomyelitis and septic arthritis		
Oral Eradication Therapy (≥3 months)		
Trimethoprim–sulfamethoxazole	Children: 6+30 mg/kg Adults: 320+1600 mg/kg	Sulfonamides Bacteriostatic: Folate synthesis inhibition
Extended eradication therapy (≥6 months) is recommended for osteomyelitis and neurological melioidosis		

Table 1: Melioidosis Recommended Therapy

Recommended treatment plan for Melioidosis infection is biphasic, including an initial intravenous therapy followed by oral therapy[1].

Treatment for Melioidosis lasts for several weeks to months consisting of a biphasic antibiotic regimen (**Table 1).** Patients first receive 10-14 days of intravenous therapy to promote survival. Ceftazidime and meropenem are used for intravenous therapy, either of which can be supplemented with trimethoprim–sulfamethoxazole for deep tissue infections, osteomyelitis, or abscesses[55]. Intravenous therapy is recommended to be extended to 4-8 weeks for unresolved infections, neurological Melioidosis, osteomyelitis, and septic arthritis[1,56]. Oral-eradication is the second stage of treatment, consisting of > 3 months of antibiotics. Standard of care is use of trimethoprim–sulfamethoxazole, but co-amoxiclav or doxycycline are also effective [55]. An extended eradication period of > 6

months is recommended for neurological Melioidosis and osteomyelitis [1]. During eradiation therapy, adverse effects occur in 40% of patients due to the long-term course of therapy [57]. Although ceftazidime, meropenem, and trimethoprim-sulfamethoxazole are the frontline drugs, BPM strains are also often susceptible to the bactericidal drugs imipenem, co-amoxiclav, ciprofloxacin, levofloxacin; and bacteriostatic drugs doxycycline, and chloramphenicol[1]. The bacteria are also susceptible to piperacillin, ceftriaxone, and cefotaxime *in vitro*, but these drugs lack clinical efficacy *in vivo*[1]. Several novel treatment options have also been tested *in vitro*; however, lack of *in vivo* efficacy highlights the difficulty of finding new therapies for this pathogen[58].

HOST-PATHOGEN INTERACTION

BPM has several virulence mechanisms that allow the bacterium to evade and survive in the mammalian hosts (**Illustration 3**). An extensive repertoire of quorum sensing molecules (N-acyl homoserine lactones) are believed to aid in coordinating an attack against the host [59,60]. This bacterium can infect nearly any cell type including epithelial, endothelial [61], fibroblasts [61], keratinocytes [61], neuronal [62], hepatocytes, and antigen-presenting cells *in vitro*. Limited work has been done to examine the preferential cell type in the host, but within 24-48 h after infection, regardless of the route (IV, IP, IN), spleen and liver are colonized, with the spleen often harboring more bacteria[37,63]. An investigation into the cells infected in the spleen, bone marrow, and blood showed that CD11b+ Ly6C+ monocytes predominately carry the bacteria[37]. Follow up studies showed that these cells are responsible for bacterial dissemination between 2-10 days post-infection [37,64,65]. Although Ly6G+ neutrophils are also permissive to infection, they represent a much smaller percentage of infected cells and harbor less bacteria compared to CD11b+ cells[66]. The short lifespan of neutrophils also prevents them from being long term reservoirs in the host[66].

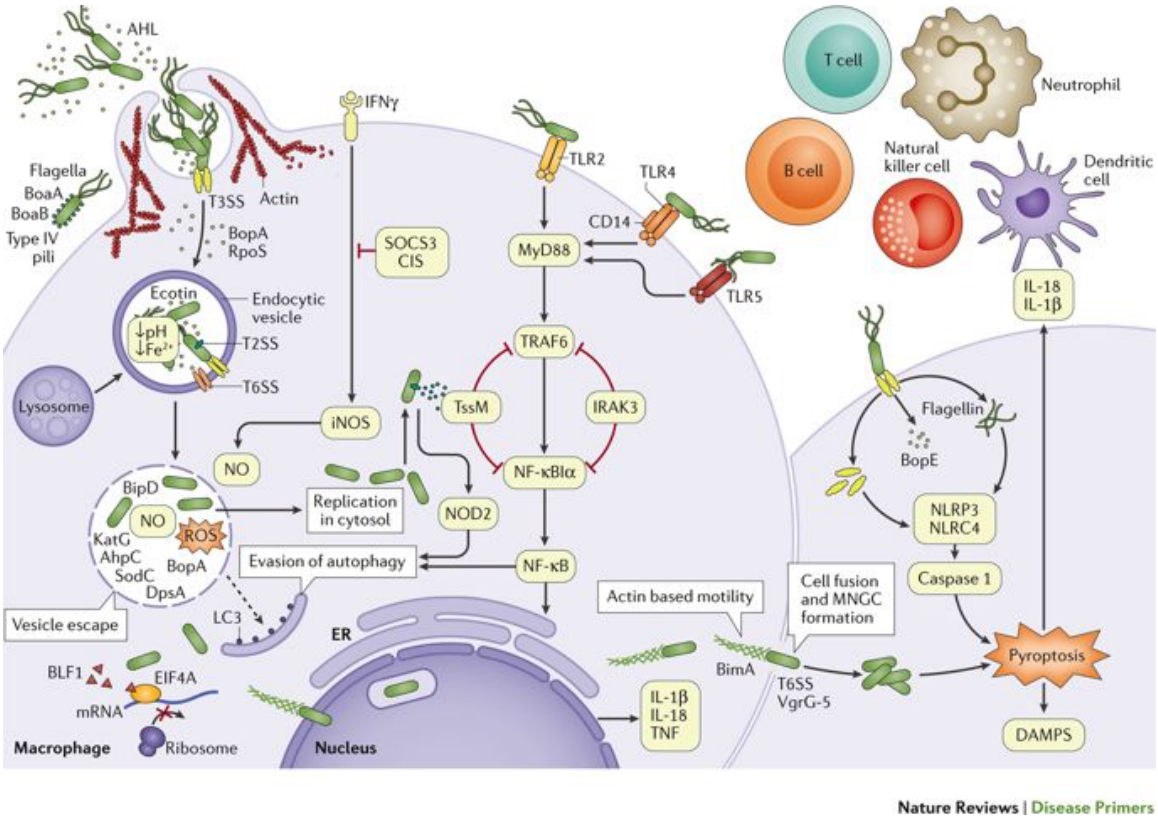


Illustration 3: Virulence Mechanism and Immune Evasion

Interaction of BPM *and the host cell. The bacteria readily infect several cell types but most notably the phagocytic cells. Once inside the cell, the bacterium readily escapes the endocytic pathways and prevents autophagy and immune activation, promoting a cytoplasmic lifestyle. Cell-to-cell spread is a notable mode of transmission to neighboring cells. Several of the factors important in the interaction with the host are depicted and described in the text below*[1]*. Used with the permission of Springer Nature Publishing Group 2019; License number 4654271189903.*

When the bacterium comes into contact with macrophages it can actively invades and can survive for long periods intracellularlly[67]. Adhesion relies on serval bacterial components, including the type IV pili, adhesins BoaA and BoaB, flagellin, and LPS (**Illustration 3**)[68-70]. Invasion is dependent on the presence of the T3SS and its effectors[71]. Once in the endosome, bacteria readily avoid phagolysosomal fusion through action of BipD which blocks microtubular trafficking of endocytic vesicles, disruption of the host membrane with BopA, and lysis of the endosome by *bsaQ*, *bsaZ*, and *bsaU* gene products which leads to egress from the endocytic pathways within 15 minutes of entry (**Illustration**

3)[1]. BPM *'s* metabolism is malleable, allowing it to adjust the carbon source available in the cell cytosol. Typically, when immune cells are infected they undergo metabolic reprograming where aerobic respiration is shutdown (oxidative phosphorylation and TCA cycle) [72]. Mitochondria transition from a bioenergic role to a biosynthetic role producing amino acids, nucleotides, and lipids. RNA analysis of BPM in U937 derived macrophages shows downregulation of genes associated with oxidative phosphorylation and TCA cycle enzymes similar to the macrophage[67]. The bacteria also upregulate anaerobic metabolism and lipid metabolism indication that it can use metabolites in the host as energy sourses[73]. Although replication of the bacteria can occur in both phagocytic and non-phagocytic cells *in vitro* the growth rate is significantly reduced (i.e. 6.2 h doubling time in U937 derived macrophages[67].

Cell surface recognition of the bacteria occurs through TLR2, TLR4, and TLR5; however, effector protein TssM inhibits immune signaling, preventing the complete activation of host cells (**Illustration 3)**[1]. Inside the macrophages, pathogen recognition and downstream innate activation are limited due to inhibition by the bacteria and reduction of pathogen-associated molecular patterns (PAMPS) exposure through down-regulation of LPS biosynthesis, CPS biosynthesis, and flagella [74]. When macrophages are activated, they have improved killing capacity; however, the bacteria can reduce its growth rate and survive using a persistence-like mechanism [67,75,76]. When in the host cell cytosol, activation of NOD2 by T3SS results in the upregulation of autophagy. To counter act this, the T3SS effector BopA is secreted inhibiting autophagy of the bacteria through an unknown mechanism (**Illustration 3)**[77]. Melioidosis susceptibility has been correlated to NOD2 polymorphisms which indicates the importance of autophagy in infection control[78,79]. Bacterial killing is mediated by reactive nitrogen intermediates and reactive oxygen species. To combat this, the bacteria produces enzymes that degrade superoxide and H_2O_2; inhibits iNOS, cytokine signaling three (SOD3), and cytokine-inducible SH2 containing protein (CIS) (**Illustration 3)** [1].

Disease symptoms is believed to result from lysis, and infection of neighboring cells. Alternatively, BPM is known for the ability to spread cell-to-cell by continuously polymerization host actin, using bacterial protein BimA, which propels the bacteria through the cell[80]. Force against the host cell membrane causes protrusions that lead to membrane fusion with neighboring cells, a process facilitated by T6SS and T4SS (**Illustration 3**). The fusion of three or more cells is a hallmark characteristic of infection, called multinucleated giant cells[81]. Depending on the strain host cell death can occur through apoptosis or pyroptosis [82,83]. Most fatal cases can be attributed to septicemia or bacteremic pneumonia, with organ fialure[38,84].

CHAPTER 2 INTRODUCTION TO BACTERIAL PERSISTENCE

ANTIBIOTIC PERSISTENCE VS. RESISTANCE VS. HETERORESISTANCE VS. TOLERANCE

In 1944, a scientist by the name of Dr. Gladys Hobby noticed that bactericidal antibiotics could not kill 100% of a bacterial population[85]. Two years later, Dr. Bigger published an article calling this small population of survivors "persisters"[86]. For nearly 60 years, persisters were ignored, but with the rising concern of antibiotics resistance, a renewed interest in persistence has led to resurgence in publications. Even with a reinvigorated field on this topic, the underlying mechanisms of persistence are still under debate. To unify the field, the leading researchers recently published guidelines for persistence research and described the difference between other mechanism of antibiotic evasion [87].

Antibiotic persistence is the ability of susceptible bacteria to survive antibiotic treatment. Antibiotic persister cells are represented by a culture that has biphasic killing kinetics. Early in antibiotic exposure, there is a sharp decrease in the population, then killing plateaus and slowly decreases over time but does not sterilize the culture fully indicating different rates of antibiotic killing of the clonal population [87-89]. When persisters are regrown, the population has identical survival kinetics compared to the original population, indicating a lack of inheritable changes. Repeated exposure leads to the same survival frequencies, and it is only weakly dependent on the antibiotic concentration above the minimal inhibitory concentration (MIC) [87]. This form of survival differs from antibiotic resistance in that resistant bacteria are not susceptible to the antibiotic at physiological relevant ranges (higher MIC)[87,90]. Resistant bacteria inherit gene(s) that actively combat antibiotics. Typical mechanism of resistance include degrading or modifying the antibiotic target, affecting antibiotic recognition/binding, or actively pumping the antibiotics out of the cell ultimately, permitting the bacteria can grow while in the presence of the antibiotic[90].

Resistance relies on a specific mechanism, which is drug-specific, while persistence is generally a phenotype across drug classes[91].

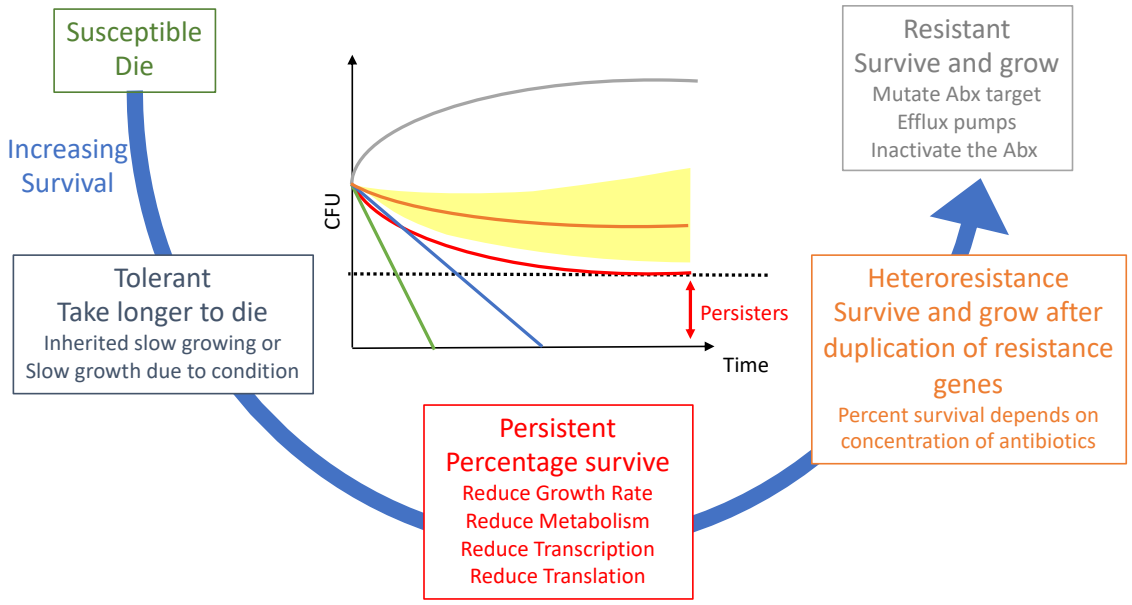


Illustration 4: Mechanisms of Treatment Failure

Antibiotic survival mechanisms are depicted in a graphical fashion showing bacteria exposed to antibiotics at a level above the MIC. The effect of antibiotics is depicted by the change of CFU over time. Each line represents a different outcome and description of the associated mechanism description. Heteroresistance depends on antibiotic concentration and is depicted with high variance (yellow)[92]. The persistence survival frequency is depicted in red and represents the fraction of surviving bacteria. Adapted from [87]

Heteroresistance, first described in 1970, is another mechanism that has also caused confusion. In a heteroresistant population, the subpopulations do not respond to the antibiotics the same way as antibiotic resistance where all bacteria have a survival advantage [93]. When exposed to antibiotics, only a subpopulation survives and expands. Different levels of the heteroresistance subpopulation affect the MIC, leading to a MIC range for a particular strain [93,94]. Conversely, the amount of antimicrobial agent affects the rate of survival[87,92]. The easiest way to determine if a strain has heteroresistance is looking

for colonies (not a lawn) within the cleared region of a disc diffusion assay. Persisters will survive in the cleared region of a disk diffusion assay but will not generate colonies until the antibiotic disk is removed or the antibiotic gets degraded [95,96].

Compared to persistence, tolerance is a similar phenomenon allowing for increased bacterial survival. Both phenomena rely on similar molecular mechanisms, such as reduced growth and metabolism; however, persistence relies on phenotypic population heterogeneity and is a feature of a subpopulation, while tolerance is a general population feature and it is not affected stochasticity [87,97,98]. Persistence and tolerance are often confused with each other because an increase in tolerance or persistence does not affect the MIC [87,99]. The easiest way to distinguish tolerance from persistence is by the lack of biphasic killing. Tolerant bacteria are killed at a slower rate compared to susceptible bacteria. Due to the slower rate of killing, tolerant bacteria have a higher minimum duration to killing 99% of the population (MDK_{99}) [99,100].

CLINICAL RELEVANCE

As the concern of antibiotic resistance comes to the forefront of international initiatives, persistence research is gaining momentum. Once curable infections are now difficult to treat, which has been attributed to not only antibiotic resistance but persistence as well. It was generally assumed that upon antibiotic treatment, most of the bacteria are eliminated and the immune system clears the remaining persister population. This may be true for immunocompetent people, but persons who are immunocompromised have higher rates of persistent infection and are more at risk of relapse[101]. Bacterial mechanism such as immune evasion or organisms hiding in immune-privileged niches or in biofilms also contribute to the lack of sterilization[102-104]. Persister cells have been associated with the lack of sterilization of chronic pathogens such as *Escherichia coli*, *Mycobacterium tuberculosis*,

Staphylococcus aureus, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and the fungal pathogen *Candida albicans*[105-107].

In the clinic, persistence can be discerned by the recurrence of infection after treatment and in the absence of antibiotic resistance based on laboratory testing. Because microbiological tests used clinically focus solely on antibiotic susceptibility, persister rates are not taken into account in patient care. Furthermore, persisters are favored in growth-limiting conditions which are not recapitulated in laboratory tests because nutritionally rich medium is used[108]. Evidence for the role of persisters in chronic infection is best shown in *P. aeruginosa* and *M. tuberculosis* infections[106,107,109].

P. aeruginosa is the leading cause of death in Cystic fibrosis (CF) patients. Phenotypic changes to a mucoid morphotype of the bacteria are correlated with the establishment of a permanent infection[110]. Despite antibiotic failure, resistance-conferring mutations are not always found suggesting alternative methods of survival. Mulcahy *et al.* (2010) hypothesized that with the lack of resistance development, higher persistence rates would be selected for. This hypothesis is supported by the 100-fold increase in persistence rates in longitudinal samples from CF patients[106]. *M. tuberculosis* is the most recognizable chronic infection and estimated to be latent in one of every three people. Of the third of the population infected, 5-10% will experience active infection due to the resuscitation of the bacteria[111] Treatment rarely results in clearance of the organism because a subpopulation can enter a reversible persister state known as "latency" [107,109]. Latent bacteria then can reactivate following external stimuli and lead to active infection[107,109].

More recently, resistance generation has been attributed to persistence populations. Aside from the fact that prolonged survival of persisters increases the chances of successful resistance generation, it is becoming clear that persistence is a state that promotes adaptive evolution [112]. An in-depth comparison shows that both resistance and persistence elicit the stringent response (SR) and SOS response[113]. Stringent response is activated in response to several cues and incuses through activation of SpoT and/or RelA and synthesis of

(p)ppGpp[114]. The alarmone, (p)ppGpp, has broad effects leading to changes in gene expression of several pathways that promote cell survival, including increased basal mutation in *E. coli*[115]. Antibiotic treatment of *E. coli* lacking the SR is unable to generate resistance compared to the wild type counterpart, suggesting that the SR elicited in persisters is required for the evolution of resistant strains[116]. The SOS response is induced in response to DNA damage and has been shown to occur during the induction and maintenance persistence. Lack of the SOS response leads to lower rates of persisters in *E. coli*[117]. The SOS response promotes resistance by expression of a mutagenic polymerase in *P. aeruginosa* and *S. aureus*[118] and increases horizontal gene transfer in *E. coli* and *V. cholerae*[119]. Evolution studies have shown that both persisters and tolerant bacteria are positively correlated with resistance generation[120,121]. Strains with higher persister rates developed resistance faster and when time was controlled for, persisters had a higher basal mutation rate in genes associated with resistance[121]. The questions that still remains to be answered is which enzymes are involved, whether the mutation rate effects the entire genome, and if the hypothesized stochiometric reawakening of persisters in antibiotics is instead lethal mutations made due to the higher mutation rate.

Treatment failure was estimated to have cost 2.2 billion dollars in the U.S. healthcare system. The CDC estimated that antibiotic failure leads to the death of 23,000 people per year today and it is expected to kill 50 million by 2050[122]. An overwhelming percent of data (88%) used to generate this data originates from cases not designated resistance but treatment failure[122]. Owing to the mounting evidence that persistence is a precursor mechanism, and currently, there are no clinical applications to survey and prevent persistence, the antibiotic resistance crisis will only get worse[122].

DISTINGUISHING BACTERIAL PERSISTENCE FROM PERSISTENT INFECTION

Although persistent bacteria are associated with persistent infection it is important to distinguish the difference between persister cells and persistent infection. The

persistence research field has focused mainly on antibiotic revealed persistence; however, the clinical relevance of persistence is the inability to clear the pathogen, both by antibiotics and the host immune system. In a recent publication, persistent infection was considered as one that occurs with or without treatment (**Illustration 5**) [87]. The persistent infection relies on several mechanisms including immune evasion, antigenic variation, reduced growth rate, immune modulation/suppression, and antibiotic promoted persistence may also contribute[87]. Bacterial persistence can occur in any environment and mechanisms specific to survival in the human host are not necessary.

Of the current antibiotic persister cell models, the most commonly studied is *E. coli*, which generates persisters at a frequency of 0.0001-1% of the entire population[97,123]. In the host, however, the bioavailability of antibiotics cannot reach the same levels used in persister assays, and as mentioned, the host environment enriches for persistence likely making treatment more complicated than what is seen *in vitro*[102,124]. Additionally, the antibiotics can function differently in the host, contributing to the inconsistency between *in vitro* and *in vivo* assays.

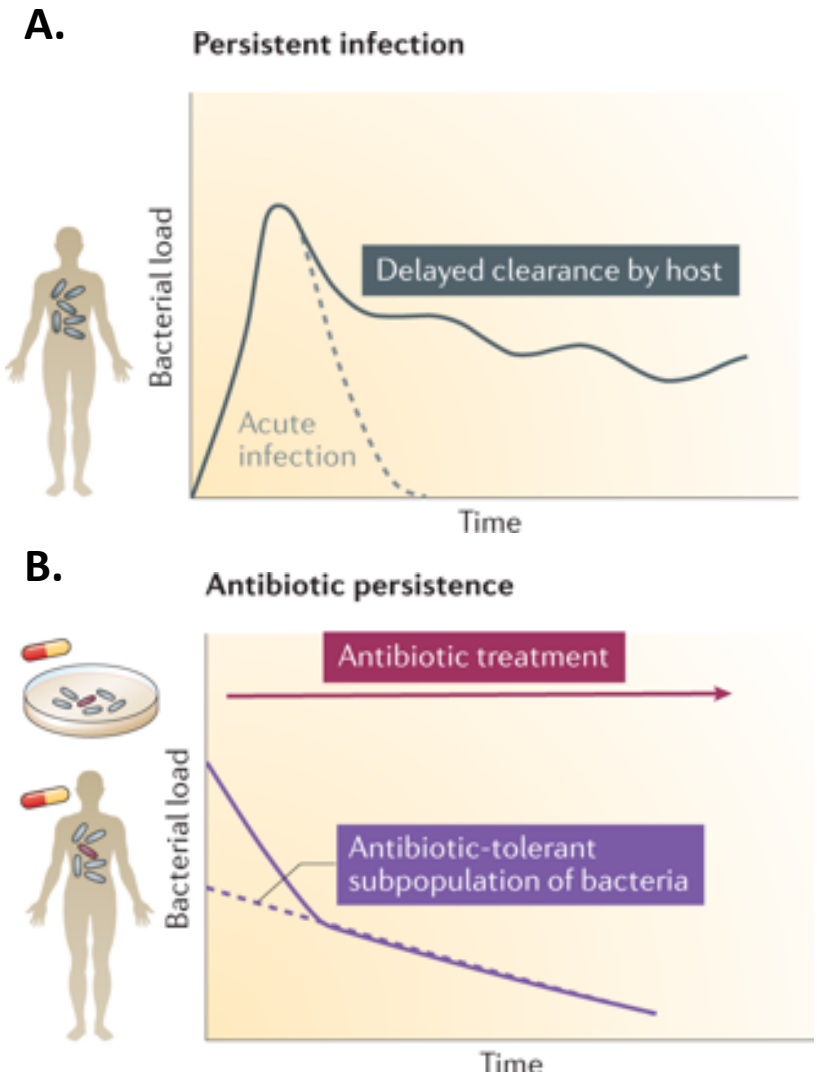


Illustration 5: Bacterial Persistence Compared to Persistent Infection

Persistent infections and persistent bacteria are considered two independent ideas, as depicted here. Persistent infection refers to the lack of cleared bacteria by the host. People who control the infection but do not fully clear the bacteria are susceptible episodic relapse. Factors that influence recurrent infection including immune state, pathogen immune evasion mechanism (antigenic variation, antigen mimicry, direct immune inhibition), and presences of antibiotic[87]. Antibiotic persistence is defined as the bacteria that are refractory to antibiotic killing specifically[87]. Used and modified under the Creative Commons Attribution 4.0 International License[125].

PHYSIOLOGY OF PERSISTENCE

Persistence is the ability of a microbe to be transiently refractory to killing by antibiotics and other environmental stresses[126]. The persister state is a physiologically dormant state that occurs by a global slowdown of processes, allowing the bacteria to remain in a suspended state until the stress is removed. When bacteria are exposed to bactericidal antibiotics, the compounds attack central cellular processes damaging the cell and leading to death[127]. However, in an attempt to prevent being killed by antibiotics, bacteria slow or shutdown the processes, which are traditionally targeted by antibiotics, rendering them ineffective. Persisters are transiently multi-drug tolerant because many of the process altered during persistence are targets of different antibiotic classes.

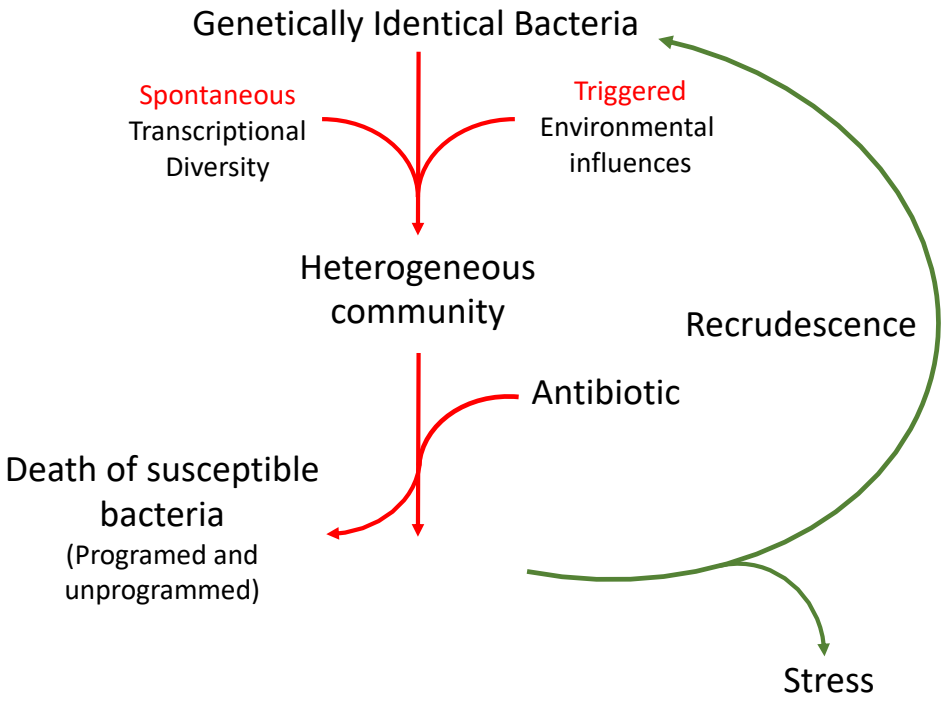


Illustration 6: Mechanism of Persistence Induction

Depicted is the consensus of persistence generation, which can arise from spontaneous or triggered mechanisms leading to a heterogeneous population. When exposed to antibiotics, a majority of the bacteria die by unprogrammed cell death or programmed. Programmed cell death being a mechanism of altruistic death aimed to help the survives[128]. Upon removal of the antibiotic stress, the surviving organisms can expand a genetically identical population compared to the starting bacteria [87].

There are two classes of persistence induction: triggered and spontaneous (**Illustration 6**). Triggered persistence occurs when the bacteria encounter a stress that increases persister rates in subsequent antibiotic exposure. Triggers of persistence include low dose antibiotics, starvation, high cell density, immune factors, acid stress, or exposure to stress when encountering host cells [87,89,102,129-131]. One of the more recent findings is the induction of persistence by macrophages. Inside a macrophage, bacteria encounter several stresses including nutrient limitation, low pH, zinc, and copper exposure in the endosome, reactive oxygen species (ROS), and nitric oxide (NO)[132]. Data with *Salmonella* show that in the macrophage, there is a higher rate of persistence when inside the host cell or after isolation from a macrophage (**Illustration 7**)[102].

Spontaneous persisters arise when the bacterium is not triggered, but rather stochastically arise in a culture regardless of growth state. This form of persistence strictly relies on population heterogeneity and resembles that seen in viral swarms [89]. As observed in viral a swarm, the offspring may not be the most fit (i.e. slow growth) but act as an insurance policy in case the culture encounters a bottleneck[133]. In the persister field, this is known as hedge-betting[134,135]. In the case of a substantial bottleneck, the arguably less fit population has higher rates of survival and can repopulate the environment once the stress is removed, or alternatively can generation resistance.

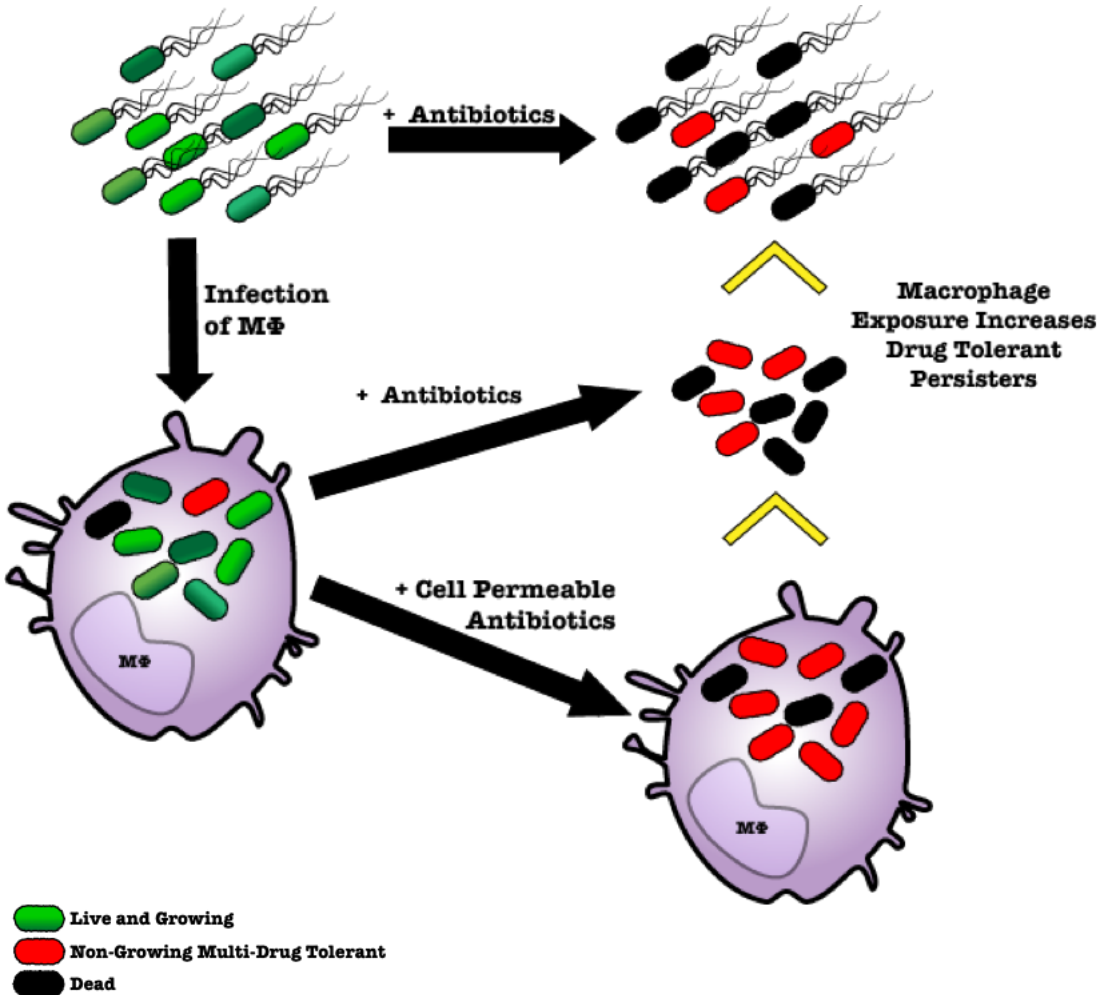


Illustration 7: Triggered Persistence by Macrophages

Exposure to macrophages triggers a higher rate of persister cells when in the macrophage environment or when isolated from macrophages and exposed to supralethal levels of antibiotics. Adapted from a lecture by Dr. Sophie Helaine.

Regardless of the route, when persistence is induced, the overt multi-drug tolerant phenotype is the same across bacteria. The underlying mechanisms/ traits are under debate. There is a consensus that there is reduced transcription, reduced translation, lack of replication, and metabolic remodeling (although different pathways)[136]. Other traits identified but not accepted across the field are shutdown of peptidoglycan synthesis[126], ROS detoxification[137], reduced energy production through the electron transport chain (ETC)[138], drug efflux pumps use[139], SOS response, stringent response[137], and quorum

sensing (QS) bacterial communication[89,101,140]. Although often considered dormant, persistent cells are a distinct population that is dormant-like. This was made evident by studies showing that not all dormant cells are drug-tolerant [141]. This is strengthened by the presence of several pathways that mediate the transition to persistence and particularly the presence of pathways such as (p)ppGpp signaling and TAS. Both of these pathways are common among nearly all bacteria. TAS sense environmental changes and lead to the induction of persistence[142]. Generation of (p)ppGpp is the result of TA activation and leads to metabolic reshaping[142].

TOXIN-ANTITOXIN SYSTEMS

TAS are found across bacteria, archaea, and fungi[143,144]. In 1983, the first persister gene described was the type II toxin system *ccdA*/*ccdB*. This TAS leads to conditional post-segregationally killing (PSK), promoting plasmid maintenance. The *ccdA*/*ccdB* TAS includes a long-lasting toxin and a short-lived antitoxin that are encoded on a plasmid. During replication the offspring that do not maintain a copy of the plasmid are killed because they can no longer replace the quickly degrading antitoxin that was encoded by the plasmid. The toxin, now uninhibited, leads to cell damage[145]. Daughter cells that had the plasmid could continually make the inhibitory protein which prevented the activity of the toxic protein, thus promoting survival [145]. Over the years, there have been an increasing number of TAS described with a wide range of functions. TAS have been identified as one of the molecular switches that sense stress and activate the persister phenotype by drastically altering the bacterial transcriptome, proteome, and metabolome[89]. In most cases, TAS are composed of two cistronic genes, one encoding an antitoxin followed by a toxin. During a favorable environment, the toxin is bound and neutralized by the antitoxin. Upon encountering a stressful stimulus, the toxin is freed from the repression by the antitoxin and induces persistence by selectively inhibiting cellular processes [146,147]. Not all

toxin are reversible, but those activated in persistence are, allowing for a dynamic population that is able to respond to the environment and recover when the stress is removed. TAS may be encoded in either plasmids, chromosomes or bacteriophages and may be horizontally obtained through mobile genetic elements or bacteriophages [148]. Due to the ability to move both vertically and horizontally, phylogenic analysis of TAS is difficult. To date, there are several TAS families that vary in function[149].

Types of Toxin-Antitoxin Systems

As of 2016, there are four classes of TAS with two additional systems proposed[150]. TAS are classified by the antitoxin's mode of toxin inhibition. Within each class of TAS, there are several families of toxins which have the same antitoxin regulation mechanism but differ in toxin enzymatic activities (**Illustration 8**)[150]. Antitoxins may be RNA or a protein and act by inhibiting toxin translation or the protein product directly. The antitoxin of type I and type III TAS is in the form of RNA and inhibits the translation of the toxin in type I, while inhibiting the protein activity of type III toxins[146,150]. The remaining TAS (II, IV, V, and VI) have protein antitoxins that inhibit the toxin in RNA form (type V) or protein form (II, IV, and VI) [146,150]. Type IV TAS differ in that the antitoxin does not inactivate the toxin but rather counteracts the enzymatic effect, stabilizing the cell[146,150]. Type II and VI TAS are very similar, consisting of toxin that is inhibited by a proteinaceous antitoxin directly in type II or indirectly in type VI. Type VI antitoxins counteract the toxin by acting on the toxin target, reverses ADP ribosylation, remove the toxin from the target, promote the degradation of the toxin by ClpPX proteases[146,150,151]. Activation of the systems is the same across all classes and occurs through a reduction of the number of antitoxins via degradation by cellular proteases, leading to release or translation of the functional toxin[152]. Although the six types of toxin classes have been described, the majority of TAS work has

been done on type II protein-protein systems due to the widespread presence among bacteria and will be the focus in this work moving forward.

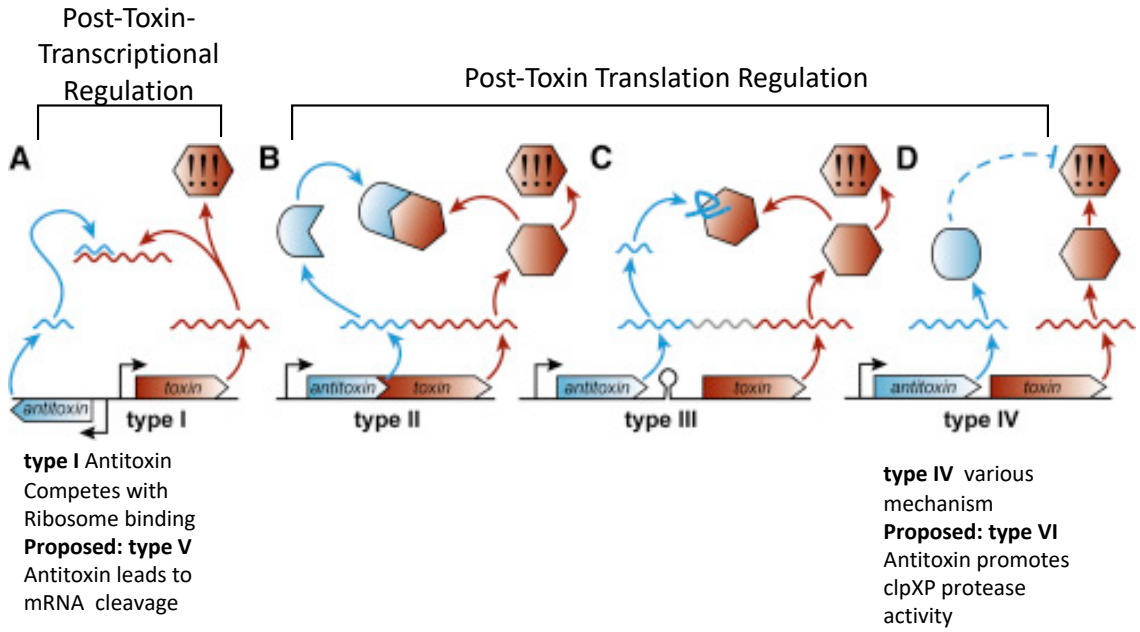


Illustration 8: Classes of Toxin-Antitoxin Systems

TAS are classified by the mechanism of antitoxin regulation. Currently, there are four classes and two additional classes that have been proposed. (A) Type I and proposed Type V toxins are regulated at the pre-translational level by binding of an RNA antitoxin. The remaining classes involve post-translational regulation. (B) Type II toxins are inhibited by a protein antitoxin. (C) Type II toxins are inhibited by binding of an RNA antitoxins. (D) Type IV and proposed VI toxins counteract the antitoxins through various mechanisms. [150] *Used with the permission of Elsevier Publishing Group 2019; License number 4657711181685*

Type II Toxin Functionality

Type II toxins are abundant in all prokaryotes [144]. Today studies mostly focus on type II, protein-protein, TA systems. In the past few decades, comparative genomics has improved the identification and accurate prediction of type II systems. Initially, there were 8-14 families; however, evidence of cross family TAS operons has come to light leading to an expansion to 13 operon-based toxin super-families, four solitary toxin super-families, and 20 antitoxin super-families across bacteria[153].

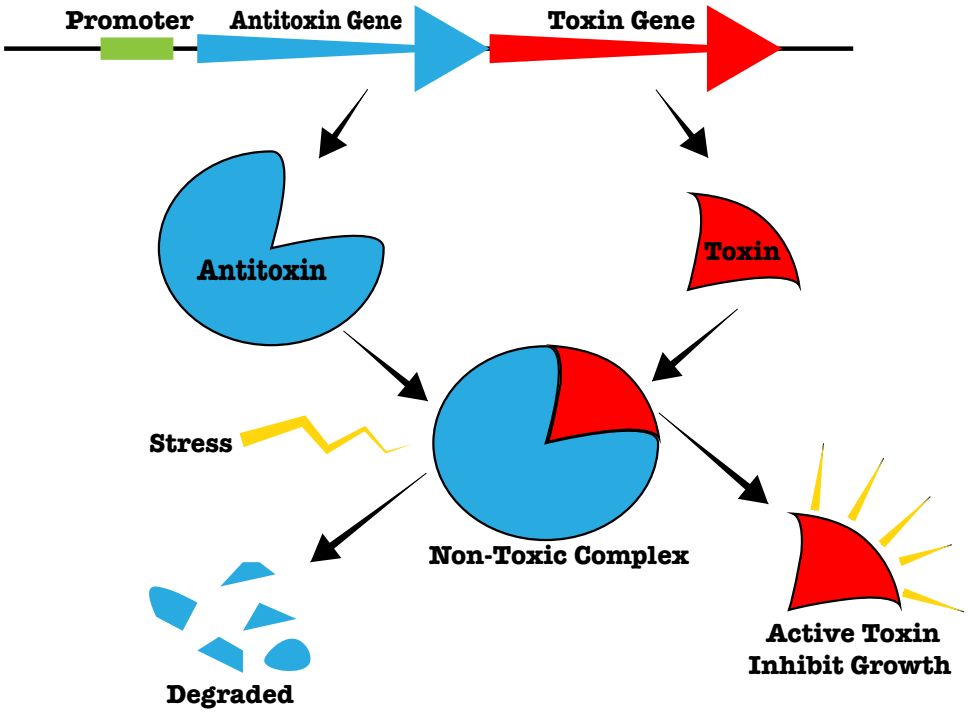


Illustration 9: Type II Toxin Antitoxin System

Adapted from Page et al., 2016[154].

The most common type II TAS is made of a single operon encoding for two proteinaceous genes. Generally, the antitoxin upstream of the toxin gene but that is not always the case. The antitoxin generally has two domains, an N-terminal DNA binding domain which is responsible for autoregulation of the operon and a C-terminal toxin binding domain[153]. Upon system activation, the antitoxin becomes unstable and is degraded by cellular proteases, liberating the active toxin. Relative stability of the antitoxin (half-life) has a critical role in the biological activity of a TAS system[155]. Toxins either modify, cleave, or degrade their target, which is achieved through several mechanisms (listed in **Table 2**)[153].

Family		Toxin Function
Toxin	Antitoxin	
ccdB	*ccdA*	inhibit FtsZ or MreB polymerization
doc	*phd*	kinase that targets elogation factor Ef-Tu
hicA	*hicB*	ribosome-independent mRNA endonuclease
higA	*higB*	mRNA endonuclease
hipA	*hipB*	phosporylates gultamyl-tRNA synthestase GltX deading to inhbition
mazF	*mazE*	ribosome-independent mRNA endonuclease
mqsR	*mqsA*	mRNA endonuclease
parE	*parD*	inhibits DNA gyrase and Topoisomerase IV
RelE	*relB*	ribosome-dependent mRNA endonuclease
vapC/pilT (PIN)	*yvapB*	cleave tRNA or rRNAs
yafO	*yafN*	mRNA endonuclease
yafQ	*dinJ*	mRNA endonuclease
yhaV	*prlF*	mRNA endonuclease
yoeB	*yafM*	mRNA endonuclease

Table 2: Mechanism of Type II Toxin Action

The toxins families listed have been identified and studies in several bacterial species.

METHODS OF TARGETING PERSISTER CELLS

Persister populations have been found in all significant pathogens and contribute to the resuscitation of chronic infections and the emergence of antibiotic resistance[156]. Effective targeting of persisters can reduce treatment duration and the associated adverse effects as well as reduce the emergence of new antibiotic resistant strains. To date, there are three main avenues of targeting persisters: reduce or interfere with the formation of persisters, directly kill persisters, or sensitize persisters to antibiotics by promoting resuscitation[103]. The first anti-persister strategy described was by Dr. Bigger in the 1940s[86]. He speculated that intermittent sterilization would eliminate persisters because once antibiotics are removed, the persisters resume growth and if treated again, would be eliminated[86]. This theory is supported by mathematical modeling and *in vitro* data but is dependent on the treatment window and may be more challenging to accomplish in a complex human enviroment[103].

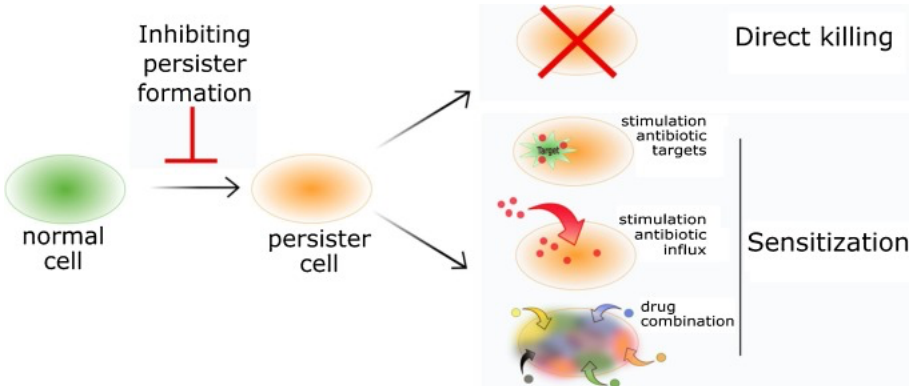


Illustration 10: Methods to Target Persister Cells

The three main avenues of targeting persistent bacteria are (1)Inhibiting formation of persisters (2) killing persisters directly, and (3) increasing the efficacy of antibiotics by making persisters more susceptible [103].

Direct killing of persister organisms can be accomplished by targeting pathways used, whether in persistence or not. Targets include the inhibition of enzymes, DNA cross-linking, depolarization or destruction of the membrane, and generation of ROS[103]. Several of these methods are found in cancer therapies, and even cancer drugs have been shown to have efficacy against persisters. That being said, many of these drugs may not be bacterial specific and have intolerable adverse effects for infection therapy[103].

Methods of antibiotic sensitization largely rely on permeabilizing the membrane to increase drug influx or stimulation of the metabolism. Several metabolites have been shown to be effective at sensitization by leading to the increase of membrane transport and/or jumpstarting the electron transport chain (ETC). One issue our lab came across is the broad effect metabolites have and the potential for inconsistent effects. In *S. aureus*, jumpstarting metabolism with nicotinamide (NAM) increased sensitivity to aminoglycosides[157,158]; however, when NAM was used in BPM treatment it exacerbated disease leading to more severe mortality in mice even without combination treatment with an antibiotic[159]. Additionally, metabolites have broad effects on the human immune

responses which may be a large bottle neck for metabolite incorporation into antibiotic therapy[160].

Targeting persister formation is the last alternative and, like the last two methods, has its own problems. Direct targeting of persisters is the most direct method of treatment intervention. Targeting persister formation can be done by inhibiting quorum sensing, preventing the stringent response by inhibiting (p)ppGpp accumulation, inhibiting the SOS, and oxidative stress responses. Several of these pathways have been shown to be induced by TAS which sense environmental stresses and push the bacteria into the persistence state. Although we still do not understand how TAS sense the environment, many studies have shown that inhibiting their action reduced persistence[102,161,162]. One issue when trying to target TAS is that many pathogens encode large numbers of these systems in their genome, e.g. *Mycobacterium tuberculosis*, has 88 TA systems[162]. Not all TAS respond to every stress, and instead respond to specific stresses[144,163,164]. For example, a subset of toxins may become active in the host environment while the same or others are activated to survive in the soil. At this time, a prediction model to identify what kind of stress a toxin responds to is not available. This project will test a novel prediction model that allows identification of TAS critical for host persistence and can provide a pipeline for fast and accurate identification of anti-persistence drug targets. Data generated can identify which TAS are essential in latent infections and during antibiotic survival, and which could be potentially targeted to improve the outcome of current treatment regimens.

PERSISTENCE AND TAS IN *BURKHOLDERIA* SPECIES

BPM is a highly adaptable bacterium able to respond to environmental stimuli to maintain a survival advantage. Studies have shown that BPM can survive in water for 16 years[165,166] and lead to latent infection 19-29 years after an initial exposure[167-169]. The particular mechanisms of persistent infection are still unclear; however, the presence of

particular genes encoding metabolic enzymes, TAS, and genomic reduction have been shown to be important. A previous publication showed that BPM has higher persister rates than several other bacteria, reaching up to 10-64% when stationary bacteria are exposed to antibiotics alone.[161,170,171]. This same group examined the functionality of TAS and found 4 to be functional when over expressed in *E. coli*[170]. Loss of the *hicAB* (BPSS390/BPSS0391) operon causes a reduction in persistence[161]. Deletion of the metabolic enzymatic gene *kynB* which encodes for kynurenine formamidase causes an increase in persistence to ciprofloxacin[59]. Isocitrate lyase has been associated with persistence on the bases that it is a key enzyme in the glyoxylate shunt which is utilized by for several pathogens during the persistent state, including BPM [172].

Several studies have examined how differential environmental states alter antibiotic susceptibilities. In an anaerobic state, 100% of a BPM culture can survive ceftazidime or trimethoprim-sulfamethoxazole treatment[173]. When stored in water for prolonged periods, BPM can survive cefotaxime at increasing rates. Following 34 h in water, exposure of antibiotic results in no bacterial killing[171]. When mimicking the low pH hypoxic condition seen in a host, the bacteria can survive for a year in a non-replicating state[173]. When replicating the innate antimicrobial generation of NO, there is an increase of persistence in imipenem by more than 3 logs[174]. Clearly BPM is capable of utilizing the persister phenomena to improve its survival however, compared to other bacteria, there is little known about how the bacteria initiates persistence, maintains persistence, and recovers from the dormant-like state.

AIMS OF THIS STUDY

The aim of this study is to examine the role of type II TAS in host-induced persistence of *B. pseudomallei*. Since many pathogens have a large number of toxins, a prediction model will be developed to provide a data-driven approach to select toxins to study. Focusing on toxins utilized while in the host, we will test selected toxins for their roles through over-expression and mutagenesis studies.

CHAPTER 3 MATERIALS AND METHODS

ETHICS STATEMENT

All manipulations with BPM were conducted in CDC/USDA-approved and registered biosafety level 3 (BSL3) facilities at the University of Texas Medical Branch (UTMB). Experiments with select agents were performed in accordance with BSL3 standard operating practices. The animal studies were carried out humanely in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The protocol (IACUC #0503014D) was approved by the Animal Care and Use Committee of the UTMB.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in **Table 3**. *B. pseudomallei* (BPM) strain K96243 was obtained from BEI Resources; Manassas, VA, USA. *E. coli* and BPM strains were stored in LB with glycerol at –80°C and prior to use. Strains were streaked onto Luria-Bertani (LB) agar and grown for 36-48 h at 37°C. For liquid cultures, 3-5 colonies were inoculated into LB broth, unless otherwise stated, and grown for 16 h at 37°C with agitation (200 rpm).

Table 3: Strains and Primers

Strains	
B. pseudomallei K96243	BEI Resources (Manassas, VA, USA)
B. pseudomallei K96243 ΔBPSS0390	This publication
B. pseudomallei K96243 ΔBPSS0395	This publication
B. pseudomallei K96243 ΔBPSS1584	This publication
B. pseudomallei K96243 ΔBPSS0899	This publication
B. pseudomallei K96243 ΔBPSS1321	This publication
B. pseudomallei K96243 ΔBPSL1494	This publication
E. coli S17 λ*pir* pMO130Δ0390	This publication
E. coli S17 λ*pir* pMO130Δ0395	This publication
E. coli S17 λ*pir* pMO130Δ1584	This publication
E. coli S17 λ*pir* pMO130Δ0899	This publication
E. coli S17 λ*pir* pMO130Δ1321	This publication
E. coli S17 λ*pir* pMO130Δ1494	This publication
E. coli DH10B pBAD Empty	This publication
E. coli DH10B pBAD BPSS0899	This publication
E. coli DH10B pBAD BPSS1321	This publication
E. coli DH10B pBAD BPSS2196	This publication
E. coli DH10B pBAD BPSL2775	This publication
E. coli DH10B pBAD BPSL1494	This publication
B. thailandensis E264	
B. thailandensis pScrhaB2 Empty	This publication

B. thailandensis pScrhaB2 BPSS0899	This publication
B. thailandensis pScrhaB2 BPSS1321	This publication
B. thailandensis pScrhaB2 BPSL1494	This publication
Primers	
pBAD BPSS099 Sac1 F	aaagagctcgatgagcacgatttccac
pBAD BPSS0899 Knp1 R	tttggtacctcatggtgtgcctcgcga
pBAD BPSS2775 Sac1 F	aaagagctcgatgcgcacgacgctgaaacgg
pBAD BPSS2775 Knp1 R	tttggtacctcatgctgcgctctgctcctt
pBAD BPSL1494 Sac1 F	aaagagctcgatgagcgatgccccgcccacc
pBAD BPSL1494 Knp1 R	tttggtacctcatgcgcttgtcccgcccgt
pBAD BPSS2196 Knp1 F	aaaggtaccatgtcattgcgagaccagatg
pBAD BPSS2196 HindIII R	tttaagcttttacgccacttcgaagcggcg
BPSS0899 pSCrhaB2 F	tgaaattcagcaggatcacatatgagcacgatttccacgacg
BPSS0899 pSCrhaB2 R	ctcatccgccaaaacagccaagctcatggtgtgcctcgcgatc
BPSS1321 pSCrhaB2 F	tgaaattcagcaggatcacatatgttgcgcatcgacagaatc
BPSS1321 pSCrhaB2 R	ctcatccgccaaaacagccaagctcaagcgctccccagaatc
BPSL1494 pSCrhaB2 F	tgaaattcagcaggatcacatatgagcgatgccccgccc
BPSL1494 pSCrhaB2 R	ctcatccgccaaaacagccaagctcatgcgcttgtcccgcc
pMO130Δ BPSS0899 1F	gagctgatatcagggccccgctagcgggcgaaagcttcgccca
pMO130Δ BPSS0899 1R	ctcatggtgtcgtcgtggaaatcgtgctcatc
pMO130Δ BPSS0899 2F	ttccacgacgacaccatgagcgagccgc
pMO130Δ BPSS0899 2R	cagctcaagcttcccgggaagatctgcgttttgcgccttcgcg

pMO130ΔBPSS1321 1F	gagctgatatcagggccccgctagcgcgaaatgggcgtccggc
pMO130Δ BPSS1321 1R	ctcaagcgctgattctgtcgatgcgcaatcgc
pMO130Δ BPSS1321 2F	cgacagaatcagcgcttgaggagatgttc
pMO130Δ BPSS1321 2R	gattaattgtcaacagctcaagcttgtacagatcgggaatcacc
pMO130Δ BPSL1494 1F	gagctgatatcagggccccgctagccttcggcgcggcgcgcaa
pMO130Δ BPSL1494 1R	ctcatgcgctggtgggcggggcatcgctc
pMO130Δ BPSL1494 2F	cccgcccaccagcgcatgagtgccgcgc
pMO130Δ BPSL1494 2R	gattaattgtcaacagctcaagcttgcgcccatcatggggccg
pMO130ΔBPSS0390 F1F	gatatcagggccccgctagcaagcaggccgtcttgtcgcc
pMO130ΔBPSS0390 F1R	catcacaggatcagcttcgatgagttcatagctcc
pMO130ΔBPSS0390 F2F	agctgatcctgtgatggccggtggtttttcgac
pMO130ΔBPSS0390 F2R	ttaattgtcaacagctcaagcttaatccagggcagcacgcgca
pMO130ΔBPSS0395 F1F	gatatcagggccccgaacgacaatccggctatc
pMO130ΔBPSS0395 F1R	tacgcttgacgacactccatgcttgg
pMO130ΔBPSS0395 F2F	agtgcgtcaagcgtaagatcgtcatg
pMO130ΔBPSS0395 F2R	ttaattgtcaacagctcaatcggtgtcaaccttgaac
pMO130ΔBPSS1584 F1F	gatatcagggccccgctagctggccatcctcatcgagcacgaga
pMO130ΔBPSS1584 F1R	gtcatggcgcgcgtgcgcggcgggcgct
pMO130ΔBPSS1584 F2F	ccgcgcacgcgcgccatgacgggatggc
pMO130ΔBPSS1584 F2R	ttgtcaacagctcaagcttggtcgagcgcgcgatcgt

Toxin Identification

All known toxins used by Ramage and colleague[162] to identify TAS in *M. tuberculosis* were used **(**presented in **Table 4)**. The protein family (Pfam) for each toxin was identified and all bacterial and archaeal sequences were downloaded from the EMBL-EBI Pfam database (http://pfam.xfam.org). Several Pfams within the same clan were also included if they represented a domain of a toxin (i.e. N-terminal or C-terminal domain). Each sequence was blasted against BPM K96243 (taxid:272560) using PSI-Blast with an expect cut-off 0.002. Expect value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database. Similar to a p-value, the lower the expect value, the more significant the match.

All identified genes were further processed by removing duplicates, keeping alignments with the lowest expect value. The results were sorted and, due to the high number of hits, the expect value was further reduced to 0.001. Although most toxins are around 210 amino acids, BPM carries the functional toxin, BPSS1584, that is 450 amino acids[175]. For this study, all toxins below 500 amino acids were included.

Known TA proteins	Toxin or Antitoxin	Gene Origin	Accession number	Pfam	Clan Grouping
CcdA	**Antitoxin**	plasmid F	CAP07666		Clan Member Duf1918 Included
CcdB	**Toxin**	Escherichia coli	YP_003829231.1 /CAP07667	**PF01845**	
MazE	**Antitoxin**	E. coli	NP_417263		
MazF/srkA/PemK	**Toxin**	E. coli	NP_417262	**PF02452**	
Duf1918	**Toxin Clan Member**	NA	NA	**PF08940**	
BrnA	**Antitoxin**	*Escherichia coli*	BNA		Clan
BrnT	**Toxin**	*Escherichia coli*	WP_001350372.1	**PF04365**	
DUF2281	**Toxin Clan Member**	NA	NA	**PF10047**	
Gp49	**Toxin Clan Member**	NA	NA	**PF05973**	
HigB-like toxin	**Toxin Clan Member**	NA	NA	**PF05015**	
mqsA -Antitoxin for MSqR	**Antitoxin**	Escherichia coli str. K-12 substr. MG1655	NP_417493.1		
mqsR	**Toxin**	Escherichia coli str. K-12 substr. MG1655	AAA69190.1	**PF15723**	
ParD	**Antitoxin**	*Escherichia coli*	WP_011205807.1		
ParE	**Toxin**	plasmid RK2	AAA92775	**PF05016**	
ParE-like	**Toxin Clan Member**	NA	NA	**PF15781**	
RelB	**Antitoxin**	E. coli	AP_002186		
RelE	**Toxin**	Escherichia coli str. K-12 substr. W3110	AP_002185	**PF06296**	
prlF	**Antitoxin**	*Escherichia coli (strain K12)*	WP_000615983.1		
YhaV	**Toxin**	*Escherichia coli (strain K12)*	WP_000347273.1	**PF11663**	
dinJ	**Antitoxin**	*Escherichia coli (strain K12)*			
YafQ	**Toxin**	*Escherichia coli (strain K12)*	WP_001307405.1	**PF15738**	
YefM	**Antitoxin**	E. coli	AP_002618		
YoeB (toxin, RelE family)	**Toxin**	substr. MG1655	YP_588458.1	**PF06769**	
Phd	**Antitoxin**	Enterobacteria phage P1	YP_006570		
Doc/fic (toxin)	**Toxin**	Enterobacteria phage P1	YP_006571	**PF02661**	Clan
	Toxin Clan Member	NA	NA	**PF13784**	
HicB	**Antitoxin**	B. pseudomallei k96243	YP_110414.1		
HicA	**Toxin**	Escherichia coli str. K-12 substr. MG1655	NP_415954.2	**PF07927**	
HigA	**Antitoxin**	plasmid Rts1 (E.coli)	AAC43983		
HigB	**Toxin**	Escherichia coli str. K-12 substr. MG1655	NP_417554.1	**PF09907**	
HipB	**Antitoxin**	E. coli	AP_002130		
HipA	**Toxin**	E. coli	AP_002129	**PF07804**	
	Toxin Clan Member	NA	NA	**PF13657**	
kis	**Antitoxin**	europaeus	NA		
kid	**Toxin**	Komagataeibacter europaeus	YP_009062441.1	**PF02452**	
LsoB	**Antitoxin**	pOSAK1	WP_000710826.1		
LsoA/RnlA	**Toxin**	E. coli O157:H7 plasmid pOSAK1	BAA31756.1	**PF15935**	
VapB	**Antitoxin**	Frankia sp.	ABD13456		
VapC	**Toxin**	Frankia sp.	ABD13457	**PF01850**	
StbC (VapB family)	**Antitoxin**	Pseudomonas syringae (pDC3000B)	AAO59110.1		
StbB (VapC family)	**Toxin**	Pseudomonas syringae(pDC3000B)	AAO59109.1	**PF01850**	
SpoIISB	**Antitoxin**	*Bacillus subtilis (strain 168)*	WP_003232646.1		
SpoIISA	**Toxin**	*Bacillus subtilis (strain 168)*	WP_003244695.1	**PF14171**	

Table 4: Toxin Families for Identification

Repurposing Existing Data

Gene expression of BPM following exposure to 82 independent conditions was obtained from the publication by Ooi and colleagues [176]. Median log10 raw reads of putative toxins and selected control genes were isolated form the datum. This datum was normalized on the expression of bacteria in LB at Stationary phase (LBS) and min-max normalized prior to being analyzed in Pajek V5.01. Both conditions and genes were clustered in Pajek with Manhattan distance metric. The datum was also visualized as a heat map, as an alternative method for visual interpretation using the web interface (http://www1.heatmapper.ca/expression/).

Gene expression of BPM K96243 following infection in U397 monocytic cell line differentiated to macrophages was obtained from NCBI OmniBus under the call number GSM681313 (https://www.ncbi.nlm.nih.gov/gds/?term=GSE27558[Accession) [73]. The datum was previously validated and published[73] prior to being uploaded on NCBI for public distribution. The expression datum was downloaded, log2 transformed, and experimental conditions (1, 2, 4, and 6 h post macrophage infection) normalized on gene expression of bacteria in RPMI. One-Way-ANOVA statistical analysis was done to examine significant fold changes in gene expression between 1, 2, 4, and 6 h post macrophage infection.

Sequence Conservation

The toxin gene sequences were run through BLASTN against for BPM (677), *B. mallei* (65), *B. thailandensis* (28), and *B. cenocepacia* (243) sequences available on the *Burkholderia* Genome Database (https://burkholderia.com). All genomes were included regardless of their completion. Genomes with homologous genes with an expect value of 0.0001 were considered positives homologs.

Over-Expression Assays

Selected toxin genes of interest were amplified with primers specified in **Table 3**. PCR products were cleaned with Qiagen PCR purification kit (Qiagen, Hilden, Germany) then ligated into either pBad-myc-his (Thermo Fisher Scientific, Massachusetts, USA) or pSCRba2 (Addagene.com), using Gibson kit (NEB, Massachusetts, USA). All constructs were transformed into *E. coli* DH5α competent cells (NEB, Massachusetts, USA). Constructs were confirmed with PCR and Sanger sequencing (UTMB Sequencing core). pBAD-myc-his constructs were transferred into *E. coli* DH10b cells which have a disrupted arabinose metabolism pathway (*araD139*; NEB, Massachusetts, USA). pSCRba2 constructs were transferred into *B. thailandensis E264*.

E. coli DH10b cells harboring pBAD-toxin constructs or empty plasmid were grown in M9 minimal medium overnight. The next day, the cultures were diluted to an OD_{600} of 0.01 and then grown at 37°C with agitation (200 rpm) until they reached an OD_{600} of 0.2. Bacterial were induced with 0.2% arabinose, repressed with 0.2% glucose, or not stimulated and growth monitored by optical density over time. *B. thailandensis* E264 harboring pSCRba2-toxin or empty plasmid were grown in M9 minimal medium overnight. The next day the cultures were diluted to an OD_{600} of 0.05 and then grown at 37°C with agitation (200 rpm) until they reached an OD_{600} of 0.2. Bacteria were induced with 0.2% rhamnose, repressed with 0.2% glucose, or not stimulated and growth monitored at 37°C with agitation (200 rpm). All experiments were carried out with antibiotics to maintain the plasmids.

Persister assays conducted on *B. thailandensis* E264 harboring pSCRba2-toxin were carried out by collected bacteria after 2 h of induction. Bacteria were normalized to the same quantity and exposed to either 5 or 10x MIC of levofloxacin. After 24 h of antibiotic exposure, bacteria were quantified and normalized on the input quantity. Data is

represented as a fold-change in persistence compared to non-induced bacteria. One-Way-ANOVA statistical analysis was done to examine significant difference between bacteria with empty vector and those expressing a toxin.

Mutagenesis Scheme

The BPM ΔBPSS0390, ΔBPSS0395, ΔBPSS1584, ΔBPSS0899, ΔBPSS1321, and ΔBPSL1494 mutants were constructed using a parental mating approach using pMo130 as previously described[177]. Briefly, Q5 polymerase (NEB, Massachusetts, USA) was used to amplify the following two fragments: 400-600 base pair upstream and the first 21 base pairs of the toxin gene, and the last 9 base pairs of the toxin gene accompanied by 400-600 bases pairs downstream (primers listed in **Table 3**). The fragments were inserted into linearized pMo130 using Gibson Assembly (NEB, Massachusetts, USA) and transformed into *E. coli* S17-1 λ*pir*. Bacteria harboring the pMo130Δ0390, pMo130Δ0395, pMo130Δ1584, pMo130Δ0899, pMo130Δ1321, and pMo130Δ1494 constructs were used to introduce to BPM K96243 via biparental mating. Merodiploids were selected using kanamycin containing LBG agar. then Plasmid loss was promoted by counter selecting on YT agar supplemented with 15% sucrose to obtain single deletion mutants. The mutations were then confirmed via PCR and Sanger sequencing (UTMB Sequencing core). All primer pairs can be found in **Table 3**.

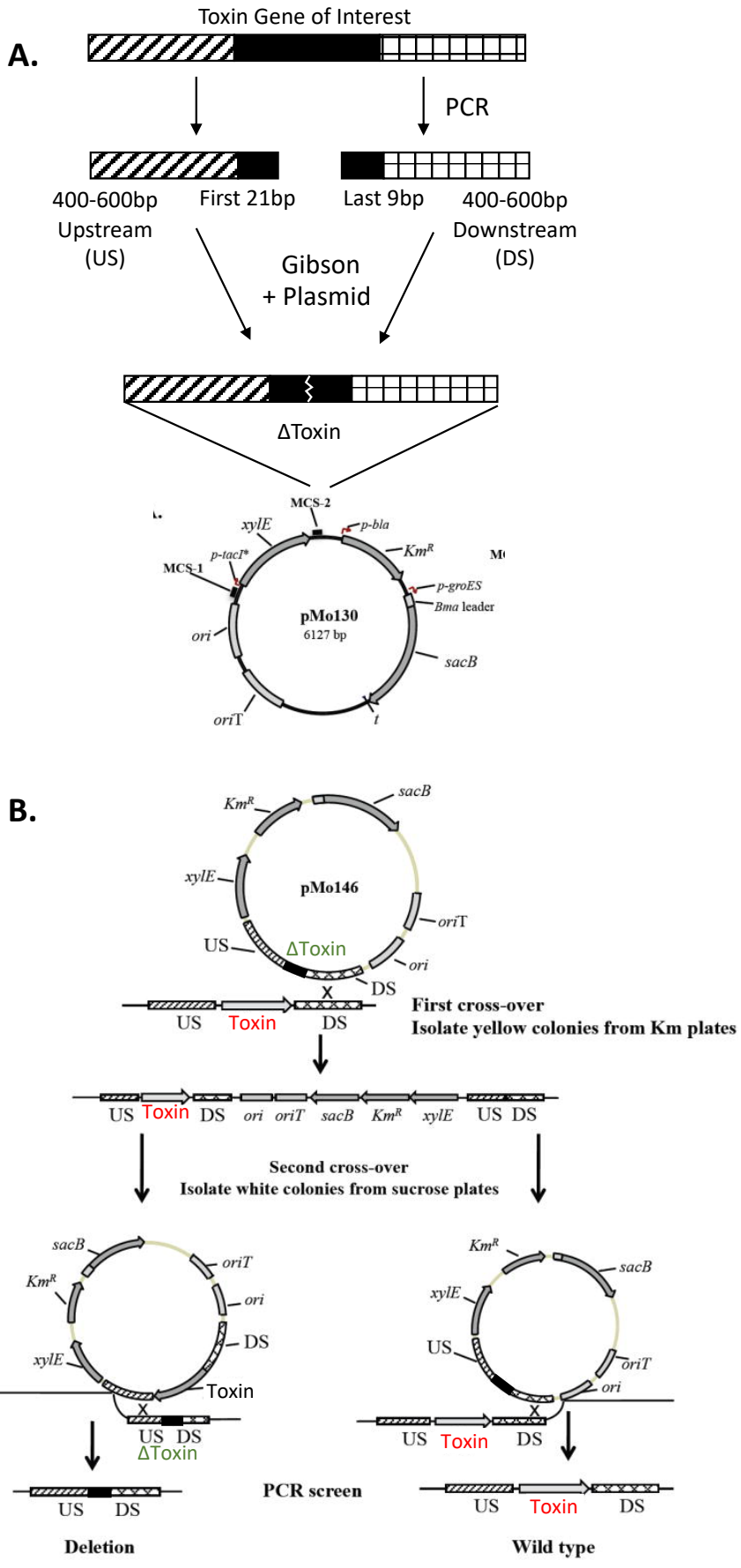

A.
Toxin Gene of Interest
PCR
400-600bp
Upstream
(US)
First 21bp
Last 9bp
400-600bp
Downstream
(DS)
Gibson
+ Plasmid
ΔToxin
MCS-2
xylE
p-bla
p-tacI*
Km^R
MCS-1
p-groES
Bma leader
pMo130
6127 bp
ori
sacB
oriT
t
B.
Km^R
sacB
pMo146
xylE
oriT
US
ΔToxin
ori
DS
X
US Toxin DS
First cross-over
Isolate yellow colonies from Km plates
US Toxin DS ori oriT sacB Km^R xylE US DS
Second cross-over
Isolate white colonies from sucrose plates
sacB
oriT
Km^R
ori
DS
xylE
Toxin
X
US DS
ΔToxin
US DS
Deletion
PCR screen
Km^R
sacB
xylE
US
oriT
ori
US Toxin DS
US Toxin DS
Wild type

Illustration 11: Mutagenesis Scheme

(A) The allelic exchange plasmid, Pmo130, was used to construct pMo130Δ0390, pMo130Δ0395, pMo130Δ1584, pMo130Δ0899, pMo130Δ1321, and pMo130Δ1494 by inserting 400-600 base pair upstream and the first 21 base pairs of the toxin gene, and the last 9 base pairs of the toxin gene accompanied by 400-600 bases pairs downstream. (B) Allelic exchange was conducted to mutate the toxin of interest in BPM *K96243. E. coli S17-1 λpir carrying a pMo130 plasmid was conjugated with* BPM. *Successful conjugation led to plasmid incorporation into* BPM *using the homologous upstream or downstream regions found in the plasmid and were selected for using the kanamycin (km) resistance cassette. These merodiploids then underwent counter selection by plating on sucrose plates. The sacB gene from the plasmid encodes for levansurase, which breaks down sucrose to levan which is toxin. Production of levan leads to recombination to remove the plasmid or bacterial death. The resulting colonies are either wild type or carry the mutated toxin gene, with no scars in the upstream or downstream regions. Colonies were screened by PCR followed by sequencing. Used with the permission of Elsevier Publishing Group 2019; License number* 4697861077256.

Growth Curves

Isolates were grown overnight in LB or RMPI with HEPES (Gibco, Thermo Fisher Scientific, Massachusetts, USA) and the following day, diluted to an OD_{600} of 0.1. Cultures were incubated at 37°C with agitation (200 rpm) and optical density read over 48 h.

Swarming Assays

Isolates were grown overnight in LB and the following day, diluted 1:10 and 2 µl was spotted in the center of semisolid nutrient agar plate (5 g/l bacto agar, 8 g/l nutrient broth N°2, with or without 0.5%(w/v) glucose). Plates where incubated for 24 h and the diameter of bacterial growth measured and presented as a percentage of the plates' total diameter. One-Way ANOVA with Kruskal-Wallis correction was used to determine if toxin mutants had significantly different swarming rates compared to wild-type.

Biofilm Assays

The protocol used was adapted from a prior publication [178]. Briefly, isolates were grown overnight in LB with 4% glycerol (LBG). The next day, cultures were diluted 1:100 into LBG supplemented with 20 mM glucose. Medium containing glycerol and glucose was used because the addition of promoted generation of biofilms (data not shown). Cultures were incubated aerobically at 37°C for 24 h, then sub-cultured by diluting 1:100 into LBG+ glucose in non-tissue culture polyvinyl chloride plates (BD, 353911) and incubated for 24 h. The plates were then washed 3x with PBS and stained with 1% crystal violet (Sigma (C3886), Sigma Missouri, USA) for 30 min. Wells were washed twice and dried for 24 h. To solubilize the bound crystal violet, 125 µl of 30% acetic acid was added to each well and incubated for 10 min. The biofilm biomass was determined at OD_{550}. All data was analyzed by using One-Way ANOVA followed Kruskal-Wallis correction when comparing more than two groups.

Traditional Persistence Assays

To determine the persister frequency, bacteria were grown for 16 h and adjusted to 1×10^8 CFU/ml in medium containing 100x MIC of levofloxacin (400 μM), ceftazidime (400 μM), ciprofloxacin (100 μM), meropenem (75 μM), or doxycycline (100 μM), in triplicate. Cultures were incubated for 24 h at 37°C without shaking, after which the surviving bacteria were quantified by serial dilution and plating on LB agar. Persistence was quantified by normalizing the surviving bacteria to the input concentrations and was expressed as percent survival. One-Way ANOVA with Kruskal-Wallis correction was used to determine if toxin mutants had significantly different persistence rates compared to wild-type.

Pre-Induced Persistence Assays

Bacteria were grown for 16 h in RPMI with HEPES and L-glutamine (Gibco, Thermo Fisher Scientific, Massachusetts, US). Bacteria were adjusted to approximately 5 × 10^7 CFU/ml in RPMI with 100 μM of the following: spermine NONOate as a NO donor (sNO, Sigma Missouri, USA), minimal medium supplemented with 2% glycerol and 1% casamino acids (M9; Becton Dickinson, New Jersey, USA), 100uM iron sulfate heptahydrate (Sigma Missouri, USA), 100uM copper sulfate heptahydrate (Sigma Missouri, USA). After 30 min of exposure at 37°C without shaking, the samples were diluted 1:1 with medium containing levofloxacin to yield a final concentration of 20x MIC (80 μg/ml) with or without the persistence inducer to maintain a their respective concentration. Bacteria were then incubated for 2 h at 37°C without shaking and then survival enumerated. Percent survival was determined by normalizing the input after the initial 30 min incubation. Statistical significates were determined using One-Way ANOVA with Kruskal-Wallis correction.

Macrophage Survival Assays

RAW 264.7 murine macrophages (ATCC, Virginia, USA) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Massachusetts, USA), 1 mM essential amino acids (Invitrogen, California, USA), 1 mM sodium pyruvate (Invitrogen, California, USA), and 1 mM penicillin streptomycin (Invitrogen, California, USA). RAW cells were seeded at 2 × 10^5 cells/well in 24-well trays (Nunc) and incubated overnight before infection.

Human U937 pleural monocytic cells (CRL-1593.2) were maintained in Modified RPMI-1640 Medium ATCC 30-2001 (ATCC, Virginia, USA) supplemented with 10% FBS (Gibco), 1 mM sodium pyruvate (Invitrogen, California, USA), and 1 mM penicillin-streptomycin (Invitrogen). For infection assays, 2 × 10^5 cells/well were seeded in 24-well cell culture plates and supplemented with 25 ng/ml phorbol myristate acetate (PMA;

Sigma, Missouri, USA) to induce macrophage differentiation 72 h prior to infection. Twenty-four h prior to infection, U937 cells were washed once with PBS and incubated an additional day without PMA to allow for further differentiation. For experiments where cells were differentiated to M1, medium was changes 16 h prior to infection with medium supplemented with 10 ng/ml of human INFγ (Peprotech).

For short term assays, cells were infected with bacteria at a multiplicity of infection (MOI) of 10. After 1 h at 37°C, extracellular bacteria were removed by washing with PBS. Fresh medium containing 250 μg/ml kanamycin was added to each plate and incubated for 1 h after which the medium was replaced with antibiotic-free medium. At 3-4 h post infection, cells were washed with PBS and lysed with 0.1% Triton-X100 (Sigma). Samples were collected, serial diluted in PBS, and plated on LB agar. Plates were incubated at 37°C for 48-72 h, then the colonies were counted.

For 24 h term assays, cells were infected with bacteria at a MOI of 10. After 30 minutes at 37°C with 5% CO_2, extracellular bacteria were removed by washing with PBS. Fresh medium containing 100 μg/ml kanamycin was added to each plate and incubated. At 24 h post infection, cells were washed with PBS and lysed with 0.1% Triton-X100 (Sigma). Samples were collected, serial diluted in PBS, and plated on LB agar. Plates were incubated at 37°C for 48-72 h, then the colonies were counted. For experiments in which iNOS was inhibited, cells were infected for 30 minutes then washed and 500 μM aminoguanidine (Sigma; Missouri, USA) was added concurrently with levofloxacin (20x MIC). All assays were analyzed with One-Way ANOVA with Kruskal-Wallis correction was used to determine if toxin mutants had significantly different invasion and survival rates compared to wild-type.

Macrophage Induced Persistence Assays

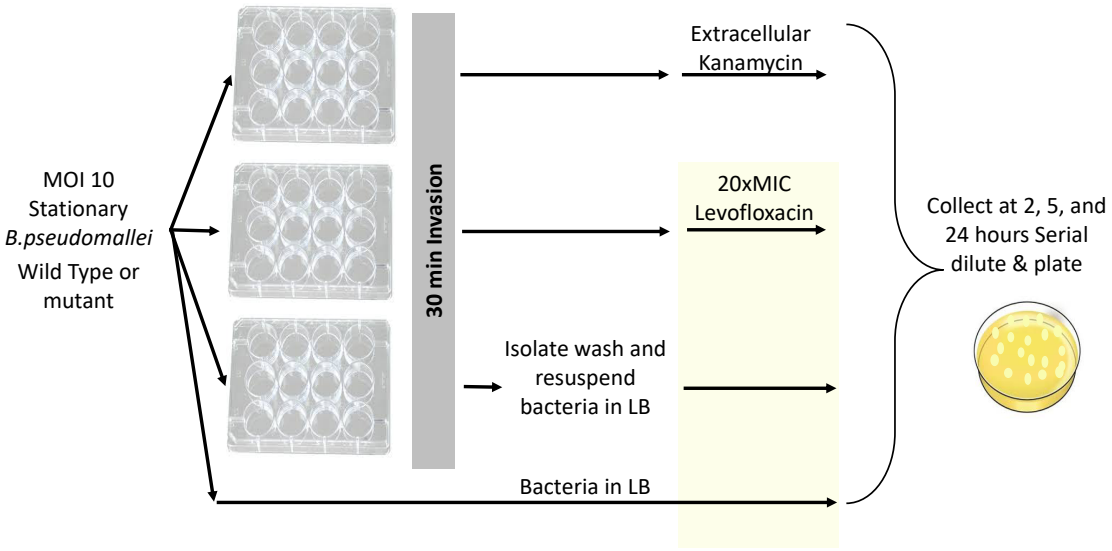


Illustration 12: Macrophage-Induced Persistence Assay Scheme

To test the ability of the bacteria to enter a persister state, the following protocol was used [102]. U937 pleural monocytic cells (ATCC, Virginia, USA) were differentiated to macrophages then were infected at a multiplicity of infection of 10 and incubated at 37°C with 5% CO_2 for 30 minutes to favor invasion. General macrophage survival was tested by washing the cells with PBS and adding medium with 100 µg/ml kanamycin. Alternatively, cells were washed with PBS and then 80 µg/ml of levofloxacin was added (20x MIC). An additional 15 wells were washed, and macrophages lysed with 0.1% Triton-X100 (Sigma; Missouri, USA). Samples were collected and re-suspended, seeded into 24 well plates and 80 µg/ml of levofloxacin added. For this condition, a sample was plated prior to levofloxacin addition to calculate the input. To compare macrophage-induced persistence to traditional antibiotics, bacteria were seeded at 1 x 10^6 CFU/ml and treated with 80 µg/ml of levofloxacin in LB. At 2, 5, and 24 h, samples from individual wells were collected in triplicate for every condition, serially diluted, and plated on LB agar for CFU enumeration. Bacterial survival was calculated as a ratio of the input. Macrophage-induced persistence was calculated as a ratio of bacteria exposed to macrophages and levofloxacin over levofloxacin persistence. Differences in survival were tested by student's t-test with

Tukey's correction for experiments with wild-type and a single mutant. To compare multiple mutants to wild type or multiple conditions for one strain, One-way ANOVA was used.

In vivo Bacterial Infection Model

Female, 6 to 8-week-old BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in microisolator cages under pathogen-free conditions and provided with rodent feed and water *ad libitum* and maintained on a 12 h light cycle. Before experiments, mice were given 5 days of acclimation. Anesthetized BALB/c mice (n = 5 per group) were inoculated intranasally (I.N.) with equivalent to 3.5 LD_{50} BPM K96243 wild-type, ΔBPSS0390, ΔBPSS0395, ΔBPSS1584, ΔBPSS0899, ΔBPSS1321, or ΔBPSL1494 diluted with PBS in a total volume of 50 μL (25 μL/ nare).

For attenuation studies, mice were not treated. For persistence studies, mice received daily intraperitoneal injections of levofloxacin (25 mg/kg/day in PBS) starting at 24 h post-infection and continuing for five days. Mice were monitored and weighed daily throughout the 20-day study. Humane endpoints were strictly observed, and time of death was recorded upon animals succumbing to infection or at the study's endpoint. Animals were observed closely throughout the study for clinical symptoms (immobility, dyspnea, paralysis) and moribund animals were humanely euthanized. Survival curves were generated and analyzed by using the Kaplan-Meier method. A significant difference ($p \leq 0.05$) in survival curves was ascertained via a log-rank test. For CFU enumeration, animals were euthanized, their lungs, liver, and spleen collected, and homogenized using Covidien Precision tissue grinders (Thermo Fisher Scientific, Massachusetts, USA).Tissue homogenates were serially diluted in PBS, plated, and incubated for 48-72 h at 37°C. Colonies were counted and normalized to organ weight (g) and significance determined

using T-test with a Mann-Whitney correction or One-way ANOVA depending on the number of groups.

CHAPTER 4 INVESTIGATING THE CAPACITY OF *B. PSEUDOMALLEI* TO ENTER PERSISTENCE

INTRODUCTION

Therapeutic eradication of BPM infection is particularly challenging although new antibiotic resistance is rare [28,52-54]. Bacterial persistence may explain the high rates of treatment failure seen in Melioidosis patients. Previous reports on BPM *in vitro* shows that 0.001-64% of a stationary population can enter persistence, which is several orders of magnitude above reported rates of persistence in other bacteria [161,171,174]. For the work in this chapter, I focused on further examining of the capacity of BPM to enter persistence with selected clinically-relevant antibiotics, the effect of intracellular survival in macrophages on persistence generation, and the survival in a murine host following antibiotic treatment[75].

***Data from this chapter is published in**
Ross, B. N., Micheva-Viteva, S., Hong-Geller, E. & Torres, A. G. Evaluating the role of Burkholderia pseudomallei K96243 toxins BPSS0390, BPSS0395, and BPSS1584 in persistent Infection. Cell Microbiol, e13096, doi:10.1111/cmi.13096 (2019). *Used with the permission of Elsevier Publishing Group 2019; License number* 4703721198313

RESULTS

Antibiotic Revealed Persistence

First, the antibiotic persistence capacity of wild-type BPM K96243 was determined. The rate of persistence generation was assessed in the presence of supra-lethal concentrations (100x MIC) of clinically relevant antibiotics meropenem, ceftazidime, sulfamethoxazole-trimethoprim, doxycycline, ciprofloxacin, and levofloxacin (**Figure 1A**). Although not all these antibiotics are the preferred frontline antibiotics, all have been used in the clinic. In the early stages of Melioidosis infection, meropenem and ceftazidime

are frontline antibiotics given intravenously to reduce human mortality. When tested *in vitro* at supra-lethal levels, meropenem and ceftazidime were the least effective at killing BPM, leaving 48.30 ± 18.67% and 16.84 ± 11.37% of the population surviving in a persister state, respectively (**Figure 1A**). Drugs typically used for the eradiation stage to prevent relapse, performed better than drugs administered to promote host survival. Among orally administered drugs, ciprofloxacin demonstrated the strongest potency leaving only 0.15 ± 0.10% of persisters, while levofloxacin and doxycycline treatment resulted in 0.48 ± 0.34% and 0.34 ± 0.15% of the bacteria surviving, respectively (**Figure 1A**). Sulfamethoxazole -trimethoprim (SMX-TMP) is the frontline drug that can be used to supplement intravenous (IV) therapy and used for oral eradication therapy even though it is considered bacteriostatic. When STX-TMP was tested, 100% of the bacteria survived. To confirm that the surviving population was indeed persisters, kinetics of bacterial death was examined. Consistent with the literature, all antibiotics led to a biphasic kill curve indicative of persistence (**Figure 1B;** STX-TMP was not tested).

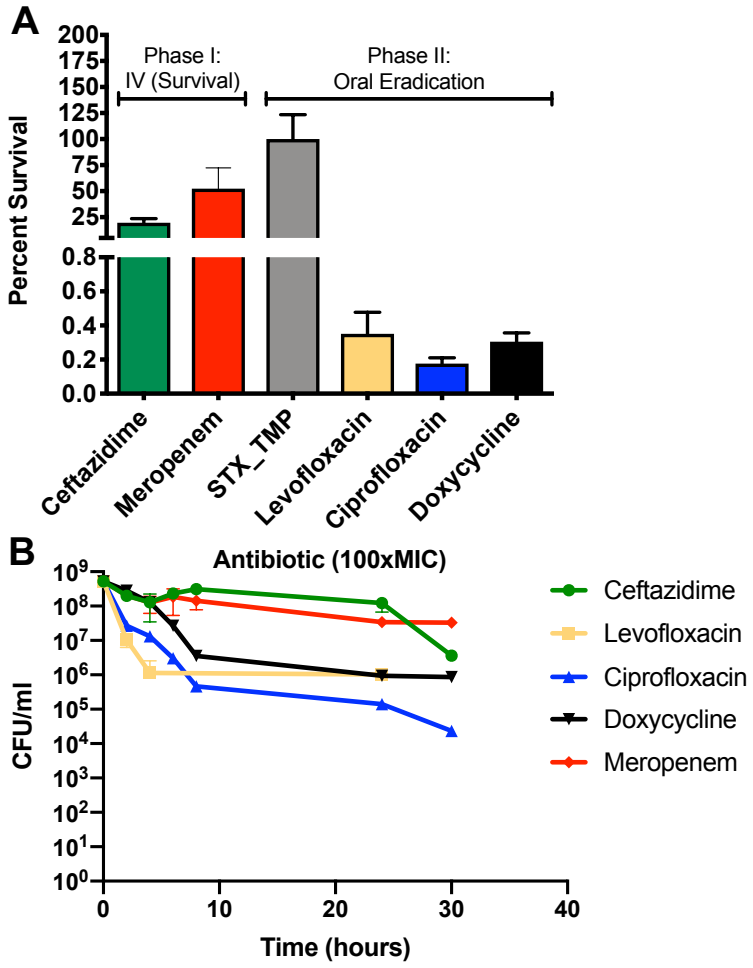


Figure 1: Antibiotic Persistence of BPM

(A) The persister frequency was determined following 24 h of treatment with clinically relevant antibiotics at 100 x the MIC. Bacterial survival was calculated as a ratio of the input. (B) Bacteria quantity was monitored for 30 h following exposed to 30 h to examine the kinetics of bacterial killing.

Macrophage-Induced Persistence

Antibiotics are not the only environmental stimuli that can trigger a persister phenotype. Previous studies with *Salmonella enterica* serovar Typhimurium have shown that stress from residing in macrophages can induce higher rates of persisters[102]. BPM are found in circulating CD11b+ macrophages, and because these cells have been shown to serve as a reservoir for the bacteria, it was hypothesized that the harsh environment in the macrophage may initiate a persister state [66,102,179]. Previously, it was shown that U937

derived macrophages could sustain BPM infection up to 36 h without killing of the macrophage [73]. To test this hypothesis, U937 derived macrophages were infected with BPM and then treated with the cell-permeable antibiotic levofloxacin (**Figure 2**). The control group (green line) was treated with extracellular kanamycin to ensure that bacteria were not in the culture medium and represents the overall intracellular bacteria (persisters and non-persisters). Exposure to the macrophage environment followed by cell-permeable antibiotics resulted in a 48.6-fold increase in persistence compared with exposure to antibiotics alone. These results indicated that in the presence of cell-permeable antibiotics, 2.44% of surviving intracellular bacteria are in a persister state at 24 h and are transiently refractile to host and antibiotic killing (**Figure 2**). Bacteria isolated from macrophages and treated with levofloxacin in LB medium also survive at a higher rate (0.20 ± 0.53%) than bacteria not exposed to macrophages (0.10 ± 0.005%).

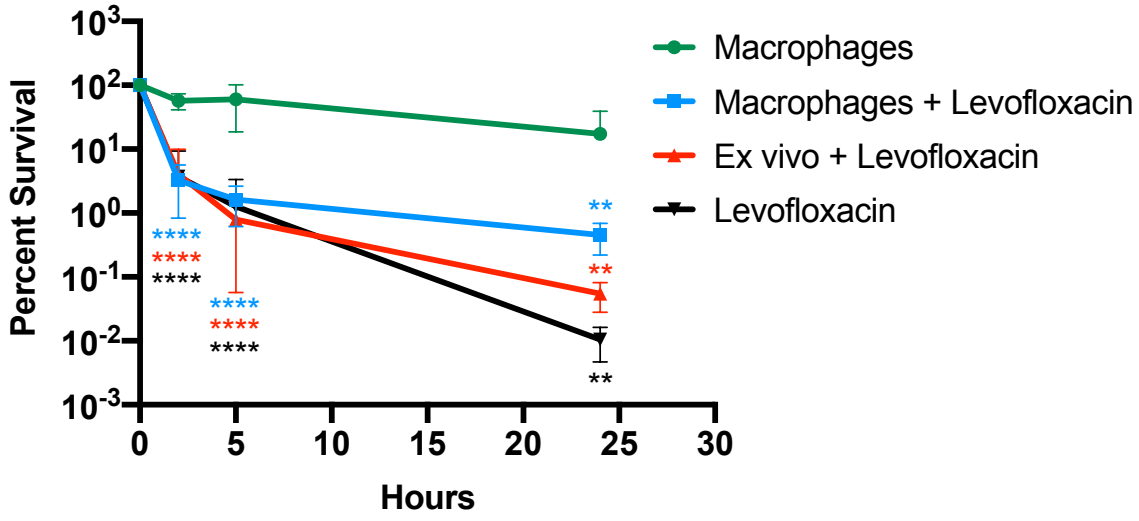


Figure 2: Macrophage-Induced Persistence

Using an existing protocol macrophage-induced persistence was determined[102]. U937 derived macrophages were infected with wild type BPM at a MOI of 10. To determine macrophage-induced persistence intracellular replicating bacteria were killed by adding levofloxacin (cell permeable; blue). This was compared to macrophages treated with extracellularly restricted kanamycin to show overall (persister and non-persister) survival (green) and bacteria treated with levofloxacin alone (black). Since the macrophage is a

*barrier that levofloxacin has to pass, a control was included where bacteria were isolated from macrophages and then treated with levofloxacin (red; ex vivo). Percent survival after 24 h was assayed by CFU enumeration. ** P<0.01, **** P<0.0001*

In vivo Antibiotic Persistence

Establishment of a Melioidosis persistent infection model was done in Balb/c mice to define the organ reservoir for bacterial persisters and to investigate the impact of antibiotic treatment on organ colonization. Our study was designed to mimic an acute infection that would be otherwise be lethal if left untreated. This was followed by treatment to select for non-replicating "persisters" which have the capacity to lead to relapse at later stages (**Figure 3**). Increasing the duration of antibiotic exposure improved clearance in the lung; however, did not affect bacterial colonization and survival in the liver or spleen when collected 21 days post-infection (**Figure 3**). Therefore, subsequent studies were conducted with 5 days of treatment.

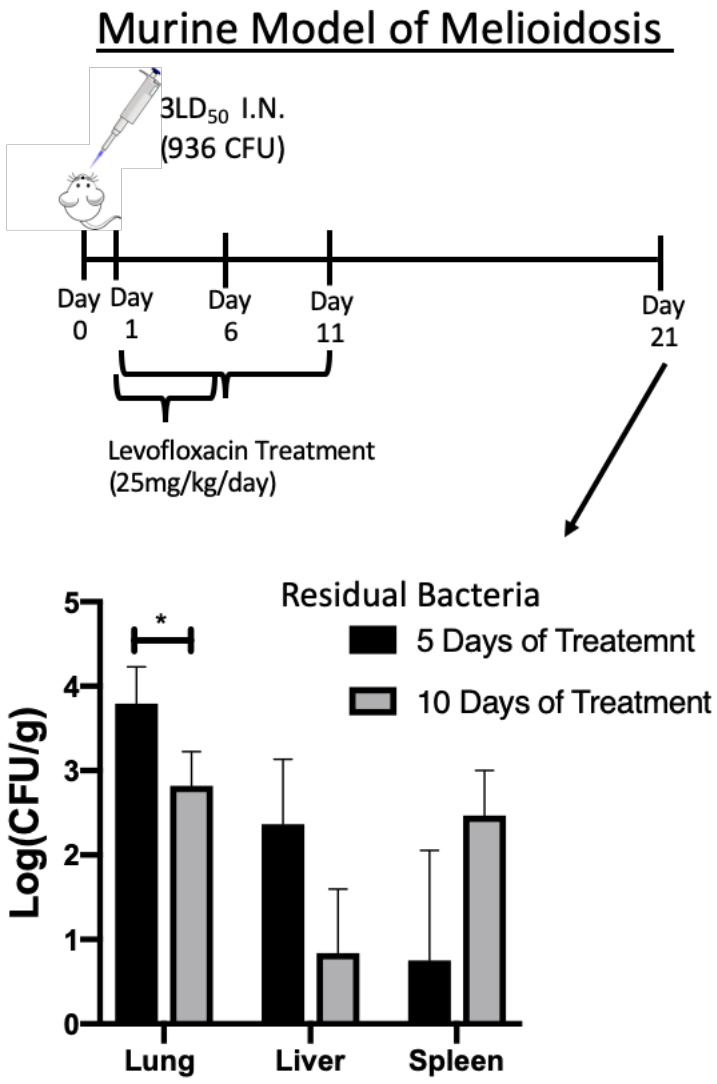


Figure 3: Melioidosis Persistent Infection Model

BALB/c mice (n=3) were infected intranasally with 2.9 LD_{50} of BPM *K96243 and levofloxacin (25 mg/kg/day) was given daily for 5- or 10-days treatment. Organs were collected at 21 days post-infection and CFUs enumerated. Assessment of the residual chronic infection showed that the lung, liver, and spleen were colonized, and duration of treatment only impacted the colonization of the lung. * P<0.05*

DISCUSSION

To improve treatment for Melioidosis patients, the mechanistic elements associated with the failures of treatment must be determined. Antibiotic resistance may serve as a mechanism for treatment failure; however, the rate of emergence of resistant strains is rare,

suggesting that other mechanisms are at play [28,52-54]. These studies demonstrate that BPM is adept at generating a reservoir of persister cells while residing in macrophages and decreases the efficacy of antibiotics.

Examination of the ability of BPM to generate persister cells revealed a range of persister frequencies when exposed to clinically relevant antibiotics. Although not widely used due to severe side effects during long treatment regimens, ciprofloxacin was able to kill bacteria to the greatest extent. In the clinic, ciprofloxacin has been replaced by the next generation quinolone levofloxacin [180-182]. Levofloxacin was not as effective against persistent bacteria, but it was superior to the frontline drugs ceftazidime and meropenem. BPM exposure to ceftazidime and meropenem led to persister frequencies several orders higher than persister rates recorded for other bacteria, in the literature [102,161,162]. Based on these findings, biphasic treatment plans used in the clinic, starting with antibiotics to promote survival, but do not kill the bacterium well, may pose a problem for bacterial eradication. If the antibiotics used early during treatment promote survival over killing, this puts the bacteria in a multi-drug tolerant state which is likely reducing the efficacy of eradication drugs ability to clear the bacteria.

An added complication in the treatment of BPM is its ability to survive in the macrophage. Our findings are similar to those with *Salmonella*, showing that macrophage internalization indeed leads to a subset of bacteria that stop growing and are not killed by subsequent antibiotic administration [102]. A trend was observed indicating that "*ex vivo*" antibiotic exposed cells had reduced persister frequency compared with bacteria maintained in macrophages. This could be due to cellular damage during centrifugation when isolated, or the need for continuous host cell stresses to maintain the phenotype. Alternatively, insufficient levels of levofloxacin entering the cells may explain the differences; however, I used 20x MIC and it has been shown that levofloxacin along with other quinolones are actively pumped into macrophages [183]. Transitioning into a murine model of Melioidosis, our preliminary animal study indicated that duration has limited

effect on colonization. Compared to 5 days, 10 days of treatment with a high dose of levofloxacin, reduced lung burden but did not impact the colonization of the bacteria in the liver or spleen. These findings provide strong evidence for further investigation of BPM persistence and the potential impact of TAS on persistence initiation.

CHAPTER 5 IDENTIFICATION OF B. PSEUDOMALLEI TYPE II TOXIN-ANTITOXIN SYSTEMS AND ENVIRONMENTAL FUNCTIONALITY PREDICTION

INTRODUCTION

Although in recent years the role of TAS in persistence induction has been questioned in *E. coli*, there is been strong evidence of these systems playing a role in more complex organisms such as *Salmonella*, *M. tuberculosis*, and BPM [102,161,162]. Novel types of TAS have been identified by experimental approaches such as shotgun cloning, or plasmid stabilization which test for TAS functionality across a genome[146]. The first method, shotgun cloning, involves fragmenting a genome and cloning the fragments into a permissive plasmid that is transforming into *E. coli* to test functionality based on growth alterations [184]. Plasmid stabilization testing relies on the same method of cloning DNA fragments, but instead the fragments are ligated into a highly unstable plasmid. After several rounds of replication the unstable plasmid is lost unless the plasmid encodes a toxin that promote plasmid maintenance [185]. Using these methods several TAS have been identified and have provided the foundation of TAS identification using *in silico* approaches.

In silico TAS identification replies on what is known about the sequence or structure of TAS. Genomic and protein architecture of TAS have been utilized in programs like RASTA[175]. Typically, TAS architecture consists of a small operon containing two genes, with less than 150 bp of separation, and that encode products ranging from 65-135 amino acids[175]. Sequence based information can be used to identify TAS in other bacteria by homology searches using NCBI BLAST, TBLASTN, or PSI-BLAST, which are the basis of toxin identification of programs like TADB and TASmania[186-188]. Following identification confirmation, validation is done through conditional over-expression in the same bacterium lacking the operon that is being investigated or in a surrogate bacterium

such as *E. coli* or *M. smegmatis* (an *M. tuberculosis* surrogate)[162,170]. When the toxin is not detoxified by the antitoxin, they predominately function by inhibiting growth or killing of the bacteria. When the expression is induced, functionality is validated based on whether the production of the putative toxin leads to the killing or senescence of growth in the induced population compared to a non-induced sample[162].

TAS identification methods often identify several genes, but the complexity in targeting toxins is that many organisms have numerous TAS and the stress cues that activate them are unknown. Similar to *M. tuberculosis*, which has 88 identified toxin-antitoxin systems, Butt and colleagues found eight BPM toxins using the Toxin-Antitoxin Database (TADB) and 62 toxins via the RASTA bioinformatics tool [189,190]. One of the predominant issues when investigating TAS functionality, is the large number of TAS indicating a potential for redundant mechanism of persistence induction. Surely the 88 toxins in *M. tuberculosis* and the 103 toxins identified here, in BPM, do not all function at the same time and respond to the same stress stimuli, so how do we define which toxins to study?

The significant reason for investigating toxins-antitoxin systems is to define their role in antibiotic tolerance and chronic infection, I argue that predicting toxins associated with host survival provides an avenue to direct TAS studies toward solving these persistence phenotypes pertinent in human health. Currently, the TAS field lacks a prediction model that would direct toxin research towards a specific stress condition. With the advances in transcriptome profiling of a population, TAS mRNA regulation can be used to identify toxins associated with a given condition, signaled by its upregulation. Additionally, the use of existing data sets allows for data-mining without the high cost of running RNAseq on bacteria in several environmental states. Here, I expanded the list of identified toxins using an architecture-independent method and used unsupervised bipartite network analysis on existing datum to identify toxins associated with antibiotic and host exposure.

RESULTS

Identification of Putative Toxins

To predict which toxins, play a role in host persistence, it is important to determine the toxins present in BPM. Traditional methods of toxin identification often rely on using known *E. coli* toxins to identifying homologous toxins within a genome of choice[162]. The *E. coli* strains predominately studied are nonpathogenic strains (i.e. K-12), bringing into question their relevance. When *E. coli* toxins were used to identify homologs in the prototypical strain BPM K96243, very few toxins were identified including known toxins, such as BPSS0390. Conversely, use of toxin sequences from pathogenic bacteria such as *Klebsiella pneumoniae* or Methicillin-resistant *Staphylococcus aureus* (MRSA) led to identification of additional TAS. Based on these findings, the method used to identify PIN domain-containing toxins was adopted. Previous publications identifying PIN domain-containing toxins relied on using all sequenced genes found in the PIN protein family (i.e. PFAM PF01850) regardless of their bacterial origin as a query for PSI-BLAST analysis. This method was used with 21 previously identified type II toxin families (**Table 3; scheme in Illustration 6)**. Using this method, 135 distinct BPM toxins were identified with an expect value of <0.001(**Illustration 6)**.

Toxins are generally smaller than 165-210 amino acids, with the exception of Zeta toxins [153,175]. In a previous study, the 450 amino acid product of BPSS1584 was shown to be a functional toxin in BPM [170]; therefore, for this study, a size cut-off of 500 amino acids was used. Of the BPM toxins identified, 103 toxins were 500 amino acids or smaller, of which 68% had an expect value lower than 0.0005 (**Figure 4A**). Examining the size distribution, 39.8% of toxins had less than 199 amino acids and 60.2% had 200 amino acids or more (**Figure 4B**). The predominant classes of toxins identified were PIN (a.k.a VapC), and Doc toxins, representing 17% and 22% of all toxins, respectively (**Figure 4C**). BPM

has two chromosomes, for which the larger chromosome 1, encodes core genes associated with central metabolism and growth, while the smaller chromosome 2, encodes accessory functions linked to adaptation and survival in different niches. Toxins identified were asymmetrically distributed with a heavier concentration in chromosome 1 (**Figure 4D**)

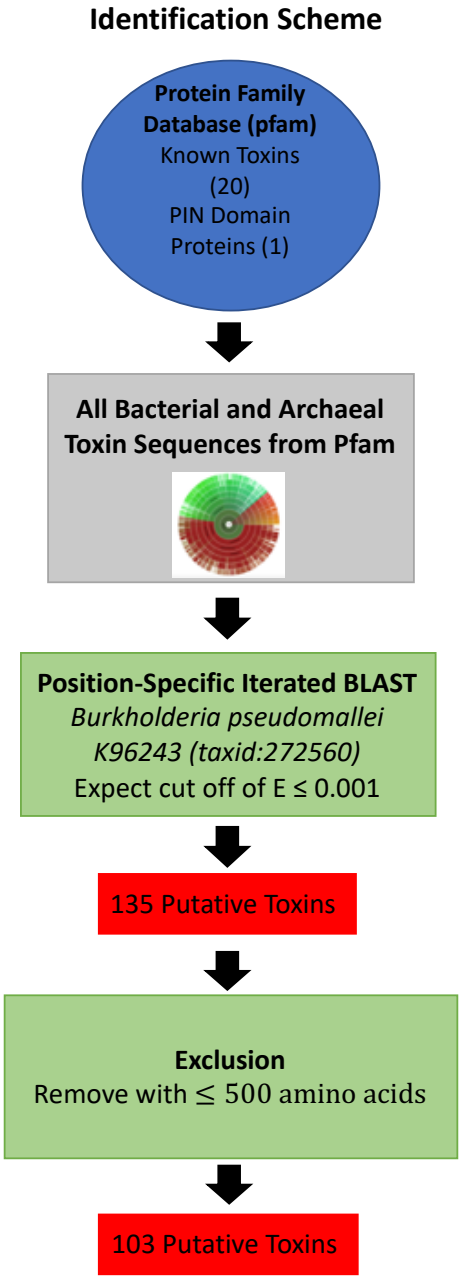


Illustration 13: Identification Scheme of Putative BPM Toxins

Toxins were identified by taking the pfam file containing all known toxins of a given family and blasting against BPM *K96243. Identified genes were excluded if they had an expect value above 0.001 and were greater than 500 amino acids.*

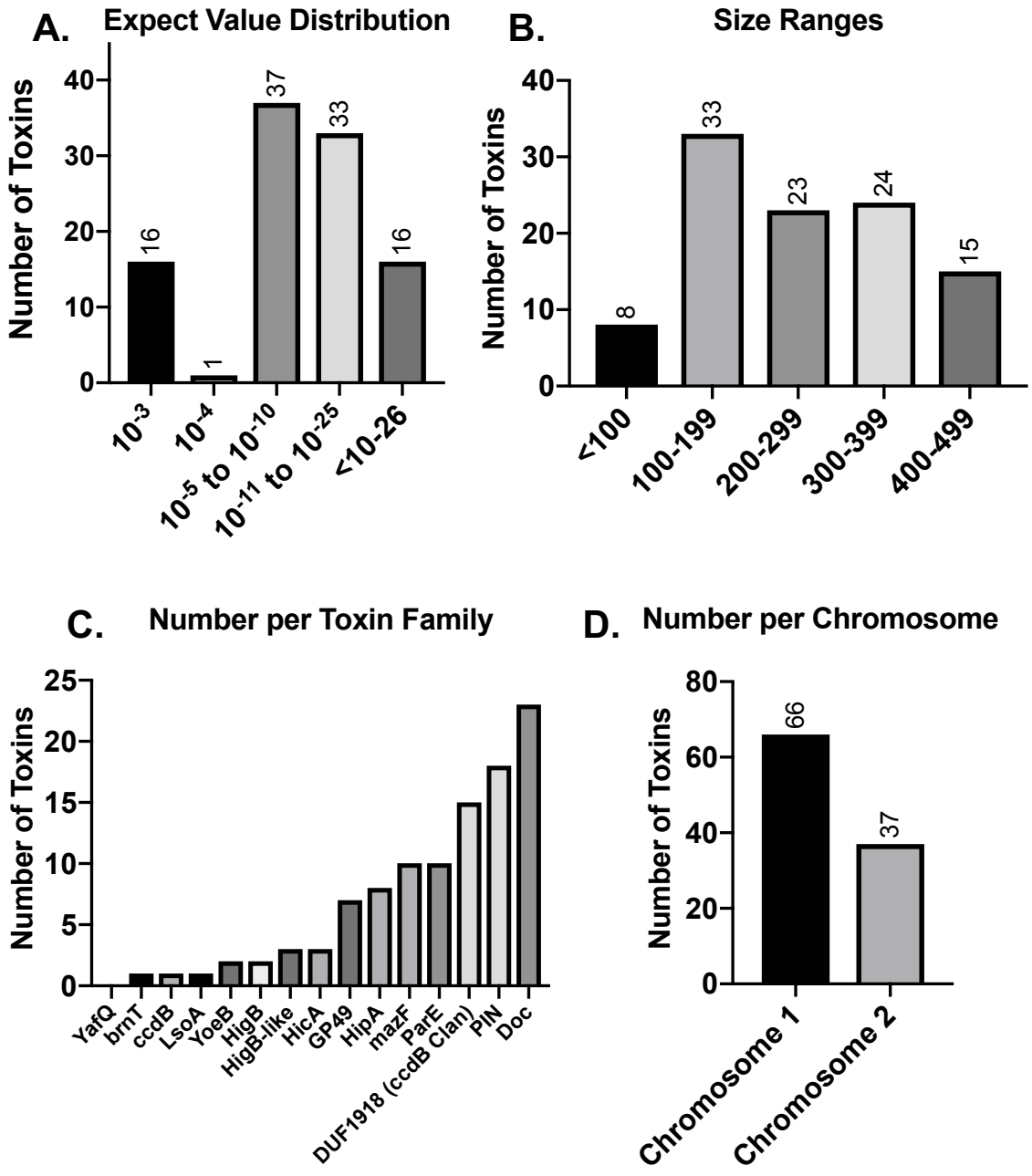


Figure 4: Putative Toxins Identified in *B. pseudomallei* K96243

Characteristics of putative toxins identified are presented as a factor of the (A) expect value, (B) number of amino acids, (C) the toxin family they belong to, and (D) the chromosomal location.

Overlap with Existing Programs

The toxins identified here were compared to the toxins identified by the available programs RASTA and TASmania. A previous RASTA search done by Butt *el al.*, 2011 identified 62 putative BPM TAS[170]. Rerunning the RASTA program using the same genome led to different results, likely due to program updates. Using the results obtained by Butt *el al.*, 2011 only 7 of 103 identified in this study were also found by RASTA (**Figure 5A**)[189]. RASTA is an algorithm built to annotate a genome and identify operons containing TAS based on architecture. Putative toxins are then classified using Pfam, Smart, COG, KOG, and CD alignment collections to determine the toxin family an operon belongs to. RASTA's approach relies on very stringent size and gene structure filters, limiting its identification capacity of larger toxins or structurally different toxins. A more recent platform, TASmania, relies on 369 Hidden Markov Models (HMM; generalized protein sequence) to identify toxins. TASmania identified 29 TAS with an expect value below 0.001, of which 10 (34.4%) were found in our study (**Figure 5B**).

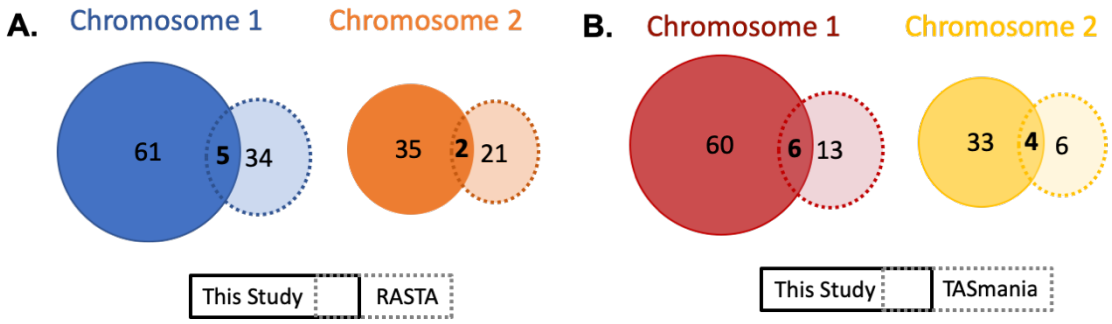


Figure 5: Toxin Identification Overlap Between Programs

The number of toxins found in this study was compared to putative toxins found by both (A) RASTA and (B) TASmania. The data is presented based on chromosomal location. Solid circles denote our study, while alternative programs are denoted with the hash marked circle.

Due to the low overlap between the approach used here and the approach used by RASTA and TASmania a subset of toxins was tested for functionality. Eight toxins were cloned into pBAD and transformed into *E. coli* DH10b. When over-expressed, 75% led to a reduction in growth indicating functionality (**Figure 6**).

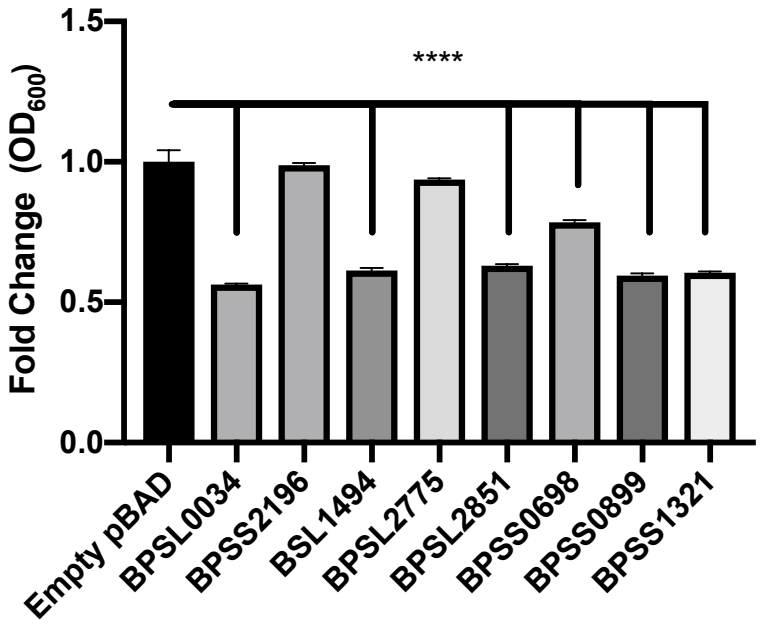


Figure 6: Over-Expression of Putative Toxins

E. coli DH10b *carrying empty pBAD or pBAD carrying a putative toxin gene was grown to an* OD_{600} *of 0.2 and induced with 0.2% arabinose. After 7 h the* OD_{600} *was taken and normalized to the* OD_{600} *of* E. coli *carrying the empty plasmid. ****p<0.0001*

Prediction of Toxins Important in the Host Using Existing Datasets

Toxins-antitoxin systems are investigated due to their role in antibiotic persistence and chronic infection. In order to find potential drug targets that could inhibit bacterial persistence, previous group have investigated each toxin individually through functional assays without knowing which environment they are important for. For organisms like

BPM, which has 103 toxins, this approach is impractical especially since many of the toxins probably play a role in survival in soil or water. It was hypothesized that predicting which toxins associated with host survival would provide more direct avenue to direct TAS studies with a goal of eliminating the persistence phenotypes important in human health.

To predict host-associated toxins, existing RNA expression data sets were mined and utilized. The datum selected was generated by Ooi et al., 2011 and consists of microarray expression datum of BPM exposed to 82 different conditions [176]. The experimental conditions range from general growth, stress conditions, infection, and available mutant expression profiles (**Table 5**). Datum for putative toxins was extracted along with three control genes BimA, Hcp1, FliC. The three control genes were selected based on the knowledge that BimA and Hcp1 are expressed during infection and important in cell-cells spread[191]. FliC encodes for flagellar subunit and not expressed in the host[1].

Table 5: Exposure Conditions in the Dataset

General Condition	Bacterial Strain	Specific Condition	Medium	Details	Condition name
GENERAL					
Growth (liquid medium)	BpK96243	Rich medium	Luria-Bertani broth (LB)	Stationary phase; Reference mRNA for ALL samples.	K9LBS
				Mid-Logarithmic phase	K9LBML
				Early Stationary phase	K9LBES
		Minimal medium	Chemically Defined	Mid-Logarithmic phase	K9CDMML
				Early Stationary phase	K9CDMES
	Bp22	Rich medium	LB	Mid-Logarithmic phase	Bp22LBML
				Early Stationary phase	Bp22LBES
		Minimal medium	CDM	Mid-Logarithmic phase	Bp22CDMML
				Early Stationary phase	Bp22CDMES
Growth (Plate culture)	BpK96243	Bacterial lawn, Solid-air interface biofilm	Luria-Bertani Agar (LA)	10^7 cfu/ml plated out on LA and incubated for 48hrs	K9Plate
Anaerobic condition	BpK96243	Aerobic culture, 1 week	LB	Normal incubation oven, 1 week	K9Aer1wk
		Anaerobic culture, 1 week	LB	Devoid of oxygen in Oxoid Anaerobic Jar, 1 week	K9Anaer1wk
		Aerobic culture, 2 weeks	LB	Normal incubation oven, 2 weeks	K9Aer2wks
		Anaerobic culture, 2 weeks	LB	Devoid of oxygen in Oxoid Anaerobic Jar, 2 weeks	K9Anaer2wks
Desiccation and Rehydration	BpK96243	Hydrated Bp pellet	LB	Pellet remains hydrated in dH2O.	K9DesRef
		Desiccated Bp pellet	LB	Air-dried and cracked Bp pellet.	K9Des24hrs
		Rehydrated Bp pellet	LB	Air-dried and cracked Bp pellet rehydrated and revived in dH2O.	K9Revived

General Condition	Bacterial Strain	Specific Condition	Medium	Details	Condition name
GENERAL					
Amino acid supplement (Taurine as a sulfur source)	BpK96243	250uM Na2SO4 (free sulfur source)	Modified M63**	Mid-Logarithmic phase	K9Na2SO4ML
			Modified M63	Early Stationary phase	K9Na2SO4ES
		250uM Taurine (amino acid sulfur	Modified M63	Mid-Logarithmic phase	K9TaurineML
			Modified M63	Early Stationary phase	K9TaurineES
Divalent cations supplement	BpK96243	Chelated medium (low cations content)	1XTSBDC***	Mid-Logarithmic phase	K91XTSBDCML
		200uM Ca2+	1XTSBDC	Mid-Logarithmic phase	K9Ca2+ML
		200uM Mg2+	1XTSBDC	Mid-Logarithmic phase	K9Mg2+ML
		200uM Cu2+	1XTSBDC	Mid-Logarithmic phase	K9Cu2+ML
		200uM Mn2+	1XTSBDC	Mid-Logarithmic phase	K9Mn2+ML
		200uM Zn2+	1XTSBDC	Mid-Logarithmic phase	K9Zn2+ML
Iron supplement	BpK96243	200uM Fe3+	1XTSBDC	Mid-Logarithmic phase	K9Fe3+ML
		200uM Fe3+	1XTSBDC	Stationary phase	K9Fe3+24hrs
Human factors supplement	BpK96243	Saline	1XDPBS****	Overnight incubation	K91XDPBS16hrs
		Insulin, 11U/ml	1XDPBS	Overnight incubation at human physiological concentration of insulin.	K9Insulin
		Normal Human Serum (NHS), 30%	1XDPBS	Overnight incubation at 30% NHS.	K930NHS
STRESSES					
PHYSICAL STRESS					
Temperature Stress	BpK96243	Cold stress	LB	Initial response to cold stress.	K904C30mins
			LB	Overnight incubation under cold stress.	K904C16hrs
		Ambient temp	LB	Initial response to ambient temp.	K928C30mins
			LB	Overnight incubation under ambient temp.	K928C16hrs
		Body temp	LB	Initial response to body temp.	K937C30mins
			LB	Overnight incubation under body temp.	K937C16hrs
		Heat stress	LB	Initial response to heat stress.	K942C30mins
			LB	Overnight incubation under heat stress.	K942C16hrs
Osmotic Stress	BpK96243	Normal osmolarity	LB	Normal growth medium osmolarity.	K9OsmRef
		High Salinity	LB	2M NaCl	K92MNaCl
		High Osmolarity	LB	2M Sorbitol	K92MSorbitol
UV Irradiation	BpK96243	Normal fluorescent light	LB	Normal white fluorescent light, 10mins	K9UV10minsref
			LB	Normal white fluorescent light, 1hr	K9UV1hrref
		UV irradiation	LB	114 uW/cm2, for 10 mins	K9UV10mins
			LB	114 uW/cm2, for 1hr	K9UV1hr
Nutrient Deprivation	BpK96243	Normal growth medium	LB	Usual nutrients in normal LB medium	K9H2O1hrref
			LB	Usual nutrients in normal LB medium	K9H2O24hrref
		Deionized water	Deionized water	Initial response to nutrient deprivation.	K9H2O1hr
			Deionized water	Response to nutrient deprivation.	K9H2O24hr

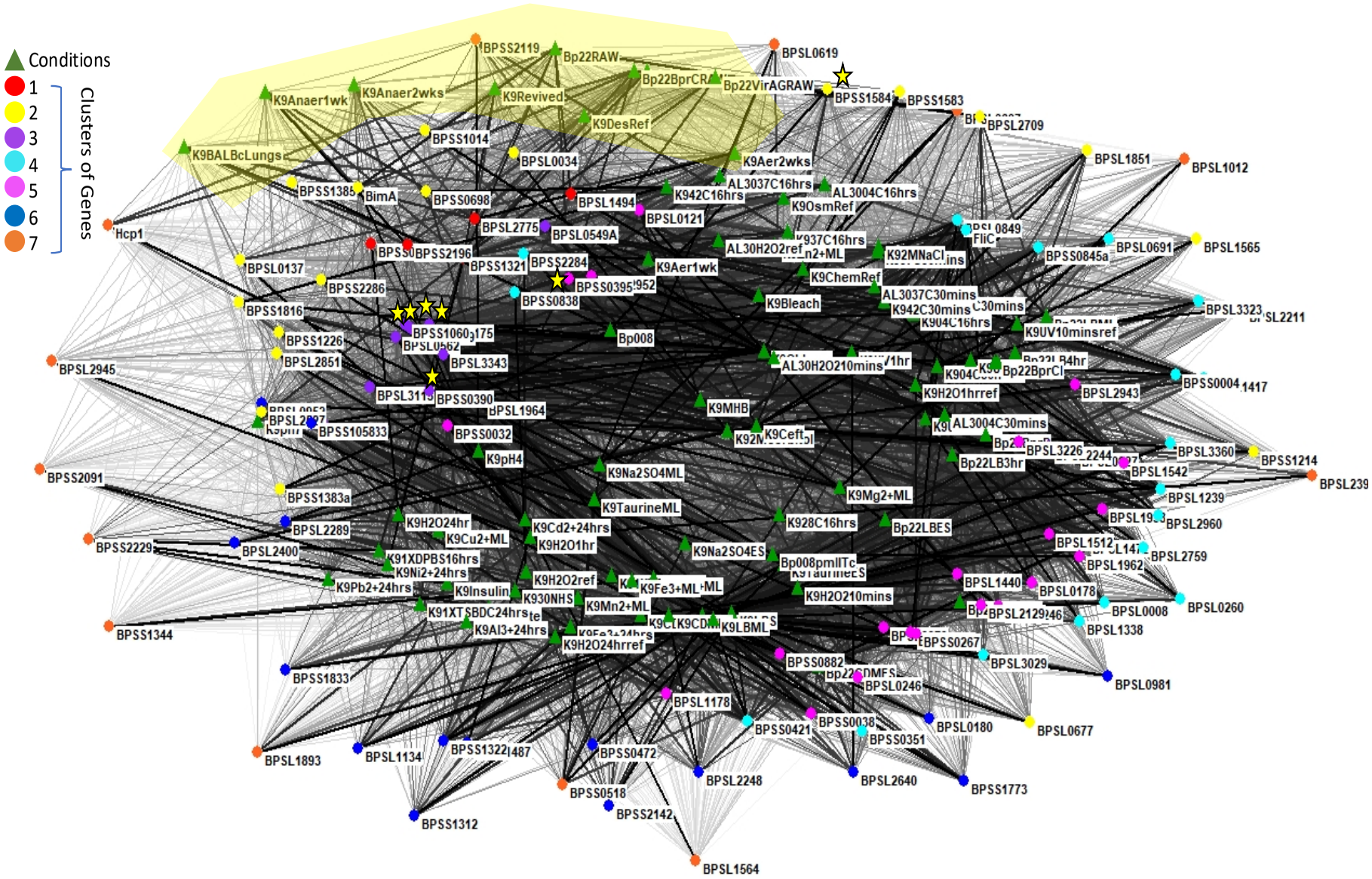
General Condition	Bacterial Strain	Specific Condition	Medium	Details	Condition name
CHEMICAL STRESS					
Antibiotics treatment	BpK96243	Normal growth medium without antibiotics	Mueller-Hinton Broth (MHB)	Usual MHB medium without antibiotics	K9MHB
		Bacteriocidal antibiotics	MHB	2ug/ml ceftazidime (1X MIC)	K9Ceft
		Bacteriostatic antibiotics	MHB	8ug/ml chloramphenicol (1X MIC)	K9Chlamp
Acid and Alkali#	BpK96243	Neutral solution	1XDPBS	pH7.0	K9pH7
		Acidic solution	1XDPBS	pH4.0 (HCl)	K9pH4
		Alkaline solution	1XDPBS	pH10.0 (NaOH)	-
Oxidative Stress	BpK96243	Normal growth medium without Hydrogen peroxide	LB	Reference condition without oxidative stress	K9H2O2ref
		Hydrogen peroxide	LB	100mM Hydrogen peroxide	K9H2O210mins
		Normal growth medium without Sodium hypochloride	LB	Reference condition without oxidative stress	K9ChemRef
		Sodium hypochloride	LB	0.01% Bleach	K9Bleach
Heavy Metals	BpK96243	Chelated medium (low cations content)	1XTSBDC	Stationary phase	K91XTSBDC24hrs
		200uM Cd2+	1XTSBDC	Stationary phase	K9Cd2+24hrs
		200uM Pb2+	1XTSBDC	Stationary phase	K9Pb2+24hrs
		200uM Ni2+	1XTSBDC	Stationary phase	K9Ni2+24hrs
		200uM Al3+	1XTSBDC	Stationary phase	K9Al3+24hrs
INFECTION					
In vitro	Bp22	Wild type	-	RAW 264.7 cell line (Mouse leukaemic monocyte macrophage), MOI 100:1	Bp22RAW
	Bp22 ΔbprC	T3SS3 mutant (BPSS1520)	-	RAW 264.7 cell line (Mouse leukaemic monocyte macrophage), MOI 100:1	Bp22BprCRAW
	Bp22 ΔvirAG	T6SS5 mutant (BPSS1495, BPSS1494)	-	RAW 264.7 cell line (Mouse leukaemic monocyte macrophage), MOI 100:1	Bp22VirAGRAW
In vivo	BpK96243	Infected BALB/c lungs (Acute Melioidosis)	-	Intranasal infection by log phase BpK96243, DOI 1000cfu	K9BALBcLungs
MUTANTS					
Sigma factor	BpAL30 (RpoE) (BPSL2436)	Cold stress	LB	Initial response to cold stress.	AL3004C30mins
			LB	Overnight incubation under cold stress.	AL3004C16hrs
		Body temp	LB	Initial response to body temp.	AL3037C30mins
			LB	Overnight incubation under body temp.	AL3037C16hrs
		Normal growth medium without Hydrogen peroxide	LB	Reference condition without oxidative stress	AL30H2O2ref
		Hydrogen peroxide	LB	100mM Hydrogen peroxide	AL30H2O210mins
Quorum sensing	Bp008	Wild type	LB	Stationary phase	Bp008
	Bp008::pmlI:Tc (BPSS0885)	Quorum sensing mutant	LB	Stationary phase	Bp008pmlITc
T3SS3	Bp 22	Wild type	LB	Wild type	Bp22LB3hr
		Wild type	LB	Wild type	Bp22LB4hr
	Bp 22 ΔbprP	TTSS3 mutant (BPSS1553)	LB	TTSS3 mutant	Bp22BprP
	Bp 22 ΔbsaN	TTSS3 mutant (BPSS1546)	LB	TTSS3 mutant	Bp22BsaN
	Bp22 ΔbprC	TTSS3 mutant (BPSS1520)	LB	TTSS3 mutant	Bp22BprC

The datum was processed by normalizing to stationary growth in LB (labeled LBS). Since the datum set contains 82 different conditions, it was hypothesized that each gene's expression range would be represented, so to place all genes on the same expression scale (equally weighted) the data was min-max normalized. Manhattan distance metric was used to cluster genes and conditions. Seven clusters of genes and seven clusters of conditions were identified. Unsupervised bipartite network analysis indicated to examine association of gene and condition clusters. As seen in **Figure 7**, the proximity and density of the edge (connecting line) between each gene and condition indicates the strength of association. The conditions associated with the host (i.e. *in vivo*, macrophages assay, pH 7, anaerobic conditions) are clustered in the yellow box. The three gene clusters colored in yellow, red, and purple are closest to the host conditions. Since proximity correlates to association, these clusters are predicted to be associated with host conditions.

To achieve a closer look at the genes associated with lung colonization, the network was temporary manipulated to pull out genes that have a dense edge (line connecting the gene to condition). The genes with the densest edges connected to Balb/c mouse lung were BPSS1321, BPSL1494, BPSS2775, BPSS2196, and Hcp1 (**Figure 8).** Hcp1 is part of the T6SS apparatus which is important in cell-cell spread through the generation of MNGCs in the host cells[192]. Here, Hcp1 functions as our positive control as it is known to be upregulated in bacteria during host infection[193]. The 8 toxins identified by TADB and previously functionality tested[170] are denoted with a yellow star, and found in the center of the network. These toxins are skewed to the left toward the host-associated conditions, but they have several dense lines attached suggesting a role in several conditions.

Visualizing the same data in a heatmap format has similar results with three clusters associated with host conditions (**Figure 9**). Using the heatmap, it is easier to see that 54% of the toxins are associated with most of the conditions, suggesting redundancy in toxin functions (**Figure 9;** right half of the heat map). In the network, the three clusters of toxins

(colored yellow, red, and purple) that were close in proximity to host condition are the same three clusters identified in the heatmap (**Figure 9;** denoted 1, 2, and 3).


Conditions
1
2
3
4
5
6
7
Clusters of Genes
BPSS2119
Bp22RAW
Bp22BprCRAW
Bp22VirAGRAW
K9Anaer1wk
K9Anaer2wks
K9Revived
K9DesRef
K9BALBcLungs
BPSS1014
BPSL0034
K9Aer2wks
BPSS1385
BimA
BPSS0698
BPSL1494
K942C16hrs
AL3037C16hrs
AL3004C16hrs
K9OsmRef
BPSL0121
BPSL2775
BPSL0549A
Hcp1
BPSS2196
BPSS1321
BPSS2284
K9Aer1wk
BPSS0838
K9ChemRef
BPSL0137
BPSS2286
BPSS1816
K9Bleach
AL3037C30mins
K942C30mins
K9UV10minsref
BPSS1060
BPSS1226
Bp008
BPSL2851
BPSL3343
BPSL2945
BPSL3119
BPSS0390
BPSL1964
K9MHB
AL30H2O210mins
K9H2O1hrref
BPSS105833
BPSS0032
K9pH4
K9Na2SO4ML
BPSS2091
BPSS1383a
K9TaurineML
K9Mg2+ML
BPSL2289
K9H2O24hr
K9Cd2+24hrs
K9Cu2+ML
K9H2O1hr
K928C16hrs
Bp22LBES
BPSS2229
BPSL2400
K9Na2SO4ES
Bp008pmolTc
K91XDPBS16hrs
K9Ni2+24hrs
K9Pb2+24hrs
K9H2O2ref
K9Fe3+ML
K9Insulin
K930NHS
K9H2O210mins
K9Mn2+ML
K91XTSBDC24hrs
K9Al3+24hrs
K9LBML
K9H2O24hrref
BPSS1344
BPSS1833
BPSS0882
BPSL0246
BPSL1178
BPSS0421
BPSS0038
BPSS0351
BPSL0180
BPSL0677
BPSL1893
BPSL1134
BPSS0472
BPSL2248
BPSL2640
BPSS1773
BPSS0518
BPSS2142
BPSS1312
BPSL1564
BPSL0619
BPSS1584
BPSS1583
BPSL2709
BPSL1851
BPSL1012
BPSL0849
FliC
BPSS0845a
BPSL0691
BPSL1565
BPSL3323
BPSL2211
BPSS0004
BPSL1417
BPSL2943
BPSL3226
BPSL3360
BPSS1214
BPSL239
BPSL1542
BPSL1239
BPSL2960
BPSL1512
BPSL2759
BPSL1962
BPSL1440
BPSL0178
BPSL0008
BPSL0260
BPSL1338
BPSL3029
BPSL0981

-Previous Page-

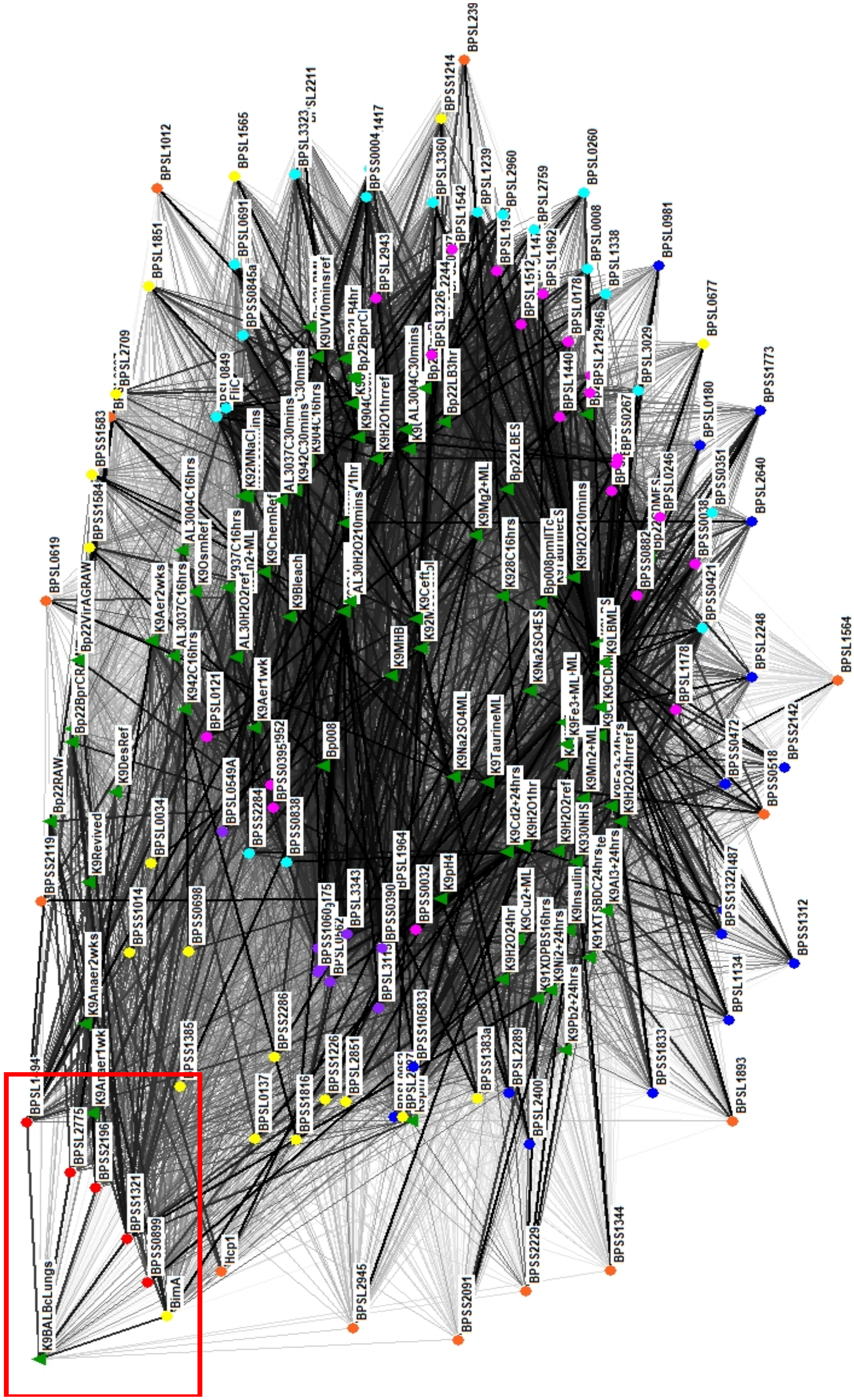
Figure 7: Unsupervised Bipartide Network of Toxin-Gene and Association Conditions

Expression data was extracted from an existing dataset exposing BPM *to 82 different conditions[176]. Only genes identified as toxins and control genes Hcp1, BimA, and FliC which have known expression profiles were included in the analysis. Toxin expression was normalized to expression of bacteria in LB growth medium to stationary phase followed by min-max normalization across each gene. The unsupervised bipartite network was generated using the software program Pajek. Conditions are depicted as green triangles. All genes are depicted as colored circles that are color coded based on higharcgical clustering. The condition cluster that contained host-like conditions are highlighted by the yellow box. The connecting lines link every gene to every condition and increasing density signifies increased association. Previously investigated toxins are denoted with yellow stars[170].*

-Next Page-

Figure 8: Network Manipulation to Examine High Associated Toxins in the Lung

To ease the interpretation of the network, nodes can be temporarily pulled out to more closely examine associations. Here all genes with dense edges (line) attached to the condition Balb/c lung were pulled to the top left (red box).



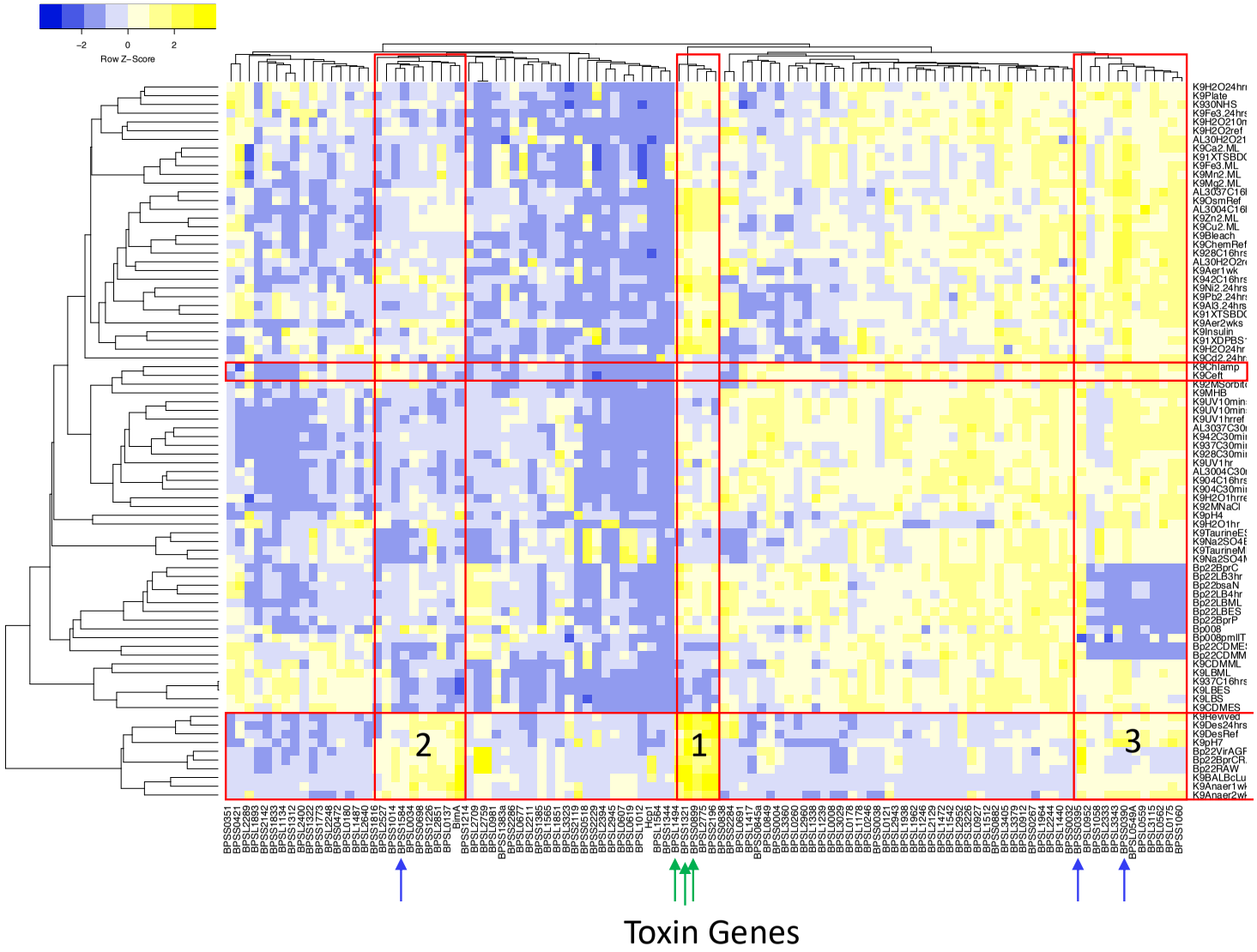

Row Z-Score
−2
0
2
2
1
3
Antibiotic Exposure
Host-Associated Conditions
Macrophages
Balb/c Lungs
Anaerobic
Toxin Genes

Figure 9: Heatmap of Toxin-Condition Association

As an alternative visualization, the same dataset is presented as a heat map. Toxin genes are presented on the horizontal axis and conditions on the vertical axis. Both genes and conditions are clustered as shown on the top and left. Red boxes highlight antibiotic exposure and exposure to host-like conditions, such as isolation from Raw 264.7 macrophages, isolation from Balb/c lung, pH 7, and exposure to anaerobic conditions. For ease, genes investigated in chapter 6 are denoted with blue arrows and genes investigated in chapter 7 denoted in green arrows.

Conservation of High Priority Toxins

The goal of this study is to find toxins that have the potential to be drug targets. The ideal drug target must be highly conserved among BPM strains to be a competitive candidate. Among genes in cluster one, 80% of toxins were conserved in all of the 677 BPM strains sequenced (**Table 6**). In cluster two, 77% of the toxins were conserved among 99% of the strains and none of the toxins in cluster three are completely conserved. Compared to the host restricted pathogen *B. mallei,* there were similar trends with higher conservation in cluster one and the least conservation in cluster three (one of twelve toxins) (**Table 6**). Three toxins in cluster one and one toxins in cluster three were found in 100% of *B. mallei* strains. *B. thailandensis* share 85% of its genes with BPM, but is an environmental restricted pathogen that has only caused a handful of illness in humans [7]. This organism has even fewer conserved toxins than BPM. Comparing conservation between *B. mallei* and *B. thailandensis,* three toxins, BPSS1321, BPSS1226, and BPSL2851 were highly conserved in *B. mallei,* but not found in *B. thailandensis*. Reciprocally, *B. thailandensis* carries BPSS2775, BPSS1816, and BPSS1058 while *B. mallei* did not. Conservation in a more distantly related *Burkholderia* species, *B. cenocepacia* was also investigate. *B. cenocepacia* is notorious for causing chronic, life threatening infections in patients with lung abnormalities such as CF[194]. *B. cenocepacia* strains share only three of the putative toxins.

	B. pseudomallei (677)	B. mallei (65)	B. thailandensis (28)	B. cenocepacia (243)
Cluster 1				
BPSL1494	100	383.08	100.00	26.34
BPSS1321	100	100.00	14.29	0.00
BPSS0899	100.590842	100.00	185.71	134.16
BPSL2775	100.295421	1.54	100.00	1.65
BPSS2196	366.026588	327.69	450.00	302.06
Cluster 2				
BPSS1816	100	0.00	100.00	0.00
BPSL2527	100.590842	100.00	232.14	102.06
BPSS1014	100.443131	100.00	100.00	59.67
BPSS1584	99.4091581	290.77	189.29	115.23
BPSL0034	101.033973	44.62	53.57	7.41
BPSS0698	200.443131	187.69	214.29	187.65
BPSS1226	99.8522895	95.38	0.00	0.41
BPSL2851	101.329394	98.46	28.57	4.12
BPSL0137	29.8375185	0.00	3.57	0.00
BimA	617.282127	332.31	328.57	134.16
Cluster 3				
BPSS0395	84.6381093	0.00	28.57	0.00
BPSL0952	13.5893648	0.00	7.14	0.00
BPSS1058	68.3899557	0.00	132.14	30.04
BPSL2333	99.4091581	0.00	60.71	0.00
BPSL3343	89.6602659	0.00	50.00	7.41
BPSS0390	5.76070901	0.00	35.71	0.00
BPSL0549A	10.0443131	0.00	0.00	0.00
BPSL0559	10.3397341	0.00	0.00	0.00
BPSL3115	18.9069424	0.00	0.00	0.41
BPSL0562	9.01033973	0.00	0.00	9.88
BPSL0175	48.7444609	38.46	32.14	1.23
BPSS1060	48.7444609	38.46	32.14	1.23

Expect Value=0.0001 Word size=11 Filter= On Current DB
Version: 8.1 (2018-04-30)

Conservation
>75%
25-75%
<25%
Broad Homology

Table 6: Conservation of Toxins from Cluster 1, 2, and 3

Nucleotide sequences for each putative toxin BLASTed against all genomes for BPM, B. mallei, B. cenocepacia, *and* B. thailandensis *available on the Burkholderia database. The cut-off value for homolog was an expect-value of 0.0001 and the number of hits was normalized on the total number of genomes for each species. The percent conservation is color coded based on high, moderate, and low conservation.*

Reactivity of High Priority Toxins

It was hypothesized that having such as wide conditional landscape, each gene's expression range would be captured. To normalize the range of expression, all genes were placed on the same scale by min-max normalizing the datum. Min-max normalization is a strategy that linearly transforms data so that the minimum value for each gene is 0 and the maximum value for each gene is 1. The use of min-max normalization places all toxins on the same scale but inflates the importance of consecutively active genes. Due to this, the standard deviation across all conditions was investigated for each gene in clusters 1-3 to was evaluate inflation and overall gene reactivity (**Table 7**).

Cluster one and two represented genes which did not change their expression, suggesting that they are constitutively active genes. Cluster three genes had high standard deviation indicating high reactivity. When comparing the standard deviation and conservation the expression data shows a correlation with high conservation and low standard deviation and vice versa (**Table 7**). Cluster one and two genes that have low standard deviation had high conservation with the exception of two genes in cluster two. In contrast, cluster three encompasses active genes and 66% are conserved in less than 50% of BPM strains (**Table 7**).

	Gene Standerd Deviation of Log10(raw reads)	Conservation
Cluster 1		
BPSL1494	0.240544453	677 (100%)
BPSS1321	0.252029408	677 (100%)
BPSS0899	0.25577242	681 (100.5%)
BPSL2775	0.274519372	679 (100.2%)
BPSS2196	0.22001332	2478
Cluster 2		
BPSS1816	0.762700688	677 (100%)
BPSL2527	0.382745491	681 (100.5%)
BPSS1014	0.275683074	680 (100.4%)
BPSS1584	0.492920867	673 (99.4%)
BPSL0034	0.211870864	684 (101%)
BPSS0698	0.246087812	1357
BPSS1226	0.241355944	676 (99.8)%
BPSL2851	0.303437519	686 (101.3%)
BPSL0137	0.501542119	202 (29.8%)
BimA	0.312204692	4179
Cluster 3		
BPSS0395	1.198412079	573 (84.6%)
BPSL0952	1.454056943	92 (13.6%)
BPSS1058	0.770638809	463 (68.4%)
BPSL2333	1.182388825	673 (99.4%)
BPSL3343	1.637633841	607 (89.7%)
BPSS0390	2.180786633	39 (5.8%)
BPSL0549A	1.185272557	68 (10.0%)
BPSL0559	1.155280715	70 (10.3%)
BPSL3115	1.688841514	128 (18.9%)
BPSL0562	1.454056943	61 (9.0%)
BPSL0175	1.65623805	330 (48.7%)
BPSS1060	1.677832124	330 (48.7%)
Expect Value=0.0001 Word size=11 Filter= On Current DB Version: 8.1 (2018-04-30)		

	High SD		High Conservation
	Moderate SD		Moderate Conservation
	Low SD		Low Conservation
			Broad Homology

Table 7: Association of Conservation and Standard Deviation of Expression

Toxins in cluster one, two and three were analyzed for the conservation among other BPM *using Blast against 677 genomes on the Burkholderia Database. Conservation is represented as a percentage of the total number of strains with the toxins. Genes that are homologous to broad families such as hydrolase or kinases are shown in gray. Color coded represents high, moderate, or low conservation and standard deviation.*

DISCUSSION

Up until this point there has not been a method of predicting toxins of clinical importance such as was attempted here. In this chapter, traditional TA discovery pathways were utilized to identify 103 putative toxins in prototypical strain BPM K96243. In the past, RNA profiles have been used as indicators of importance and here the use of existing expression datum made it possible to generate a prediction platform using unsupervised network analysis. The findings indicate that of the 103 toxins, half are associated with a wide variety of stress conditions indicating redundant induction of the persistent phenomenon. Nearly a half of the toxins associated were associated with antibiotic exposure, mimicking the great majority of other conditions. This is expected because antibiotics are a strong pressure encountered predominately in the environment through bacterial-bacterial competition. Most of the other conditions are also likely to be encountered due to environment changes or bacterial competition. Our findings suggest that the majority of the toxins may be responsible for environmental survival. Infection-simulating conditions had far fewer toxins associated and had a distinct toxin expression pattern. These findings are understandable because bacteria have been adapting to their environment and cohabitating bacteria for the past 7 billion years. Conversely, bacterial-human interaction has occurred for at most 3-4 million years which is a blink of an eye compared to the co-evolution in the environment or with other bacteria.

The prediction method used here proved to be an effective method of down-selecting toxins to study. Genes that reacted to host-like conditions were identified (cluster 3), however they had limited conservation among BPM strains. One obstacle faced was the limitations of the bioinformatic methods used. With any bioinformatic platform, there are strengths and weaknesses. Here, min-max normalization was used and resulted in over representation of constitutively active genes. This brought to light a reciprocal correlation

between gene expression activity and conservation. Constitutively expressed genes have not been the focus in TAS research, likely due to the strong reliance on RNA expression to constitute importance. Theoretically though, TAS that rely on upregulation of their genes, may not be as effective when trying to survive in an environment with an imposing stress. Instead, TAS that rely on post-translational activation have the potential to react faster and may induce persistence more readily. Being that there are few constitutively active genes (clusters 1 and 2) that are highly conserved, they offer a new avenue for TAS research. Moving forward, three highly reactive genes with moderate conservation and three constitutively expressed genes with 100% conservation were investigated for their importance in host-associated persistence.

CHAPTER 6 THE EFFECT OF MUTATING TOXIN WITH HIGH REACTIVITY AND MODERATE CONSERVATION

INTRODUCTION

In chapter 5, toxins associated with host infection were predicted for the first time. Of 103 putative toxins identified in BPM, three clusters consisting of 25% of the genes were associated to host-like conditions. All genes in clusters three and some of the genes in cluster two represented toxins that respond to conditional changes as depicted by their standard deviation. These toxins also tend to have low to moderate conservation compared to the constitutively active genes identified in cluster 1. Traditionally in persistence research, the focus has been on toxins that are upregulated in a given condition; therefore, a subset of toxins upregulated in host conditions from clusters 2 and 3 was investigated.

In a previous study investigating TAS in BPM, eight toxins were over-expressed to test functionality. Three toxins BPSS0390, BPSS0395, and BPSS1584 were found functional in the study and are present in cluster 2 and 3 from this study[170,189]. BPSS0390 is a HicA homolog which has been shown to induce persistence by ribosome-independent mRNA cleavage, ultimately leading to inhibition of translation[195]. BPSS1584 is a HipA homolog which functions a serine/threonine kinase that phosphorylates glutamyl-tRNA synthetase (GltX) leading to a build of uncharged glutamate tRNA [196]. BrnT homolog, BPSS0395, is a ribonuclease that has been shown to control bacteriostasis [197]. To analyze the role of these three toxins, BPM K96243 isogenic mutants were made and tested for persister rates, during antibiotic exposure, while in the intracellular environment, after host-stimulated stress, and *in vivo* by attenuation and colonization studies.

***Data from this chapter is published in**
Ross, B. N., Micheva-Viteva, S., Hong-Geller, E. & Torres, A. G. Evaluating the role of Burkholderia pseudomallei K96243 toxins BPSS0390, BPSS0395, and BPSS1584 in persistent Infection. Cell Microbiol, e13096, doi:10.1111/cmi.13096 (2019). *Used with the permission of Elsevier Publishing Group 2019; License number* 4703721198313

RESULTS

Selection of Toxins to Study

In a previous study, BPSS0390 and BPSS1584 were shown to be functional when over-expressed in *E. coli* [170]. Analysis for conservation among the 677 strains available on the *Burkholderia* Genome Database indicates that 39 (5.8%) and 673 (99.4%) strains possessed BPSS0390 and BPSS1584, respectively. BPSS0395 was not shown to be function when over-expressed in *E. coli* [170]. When examining the expression of the toxins when BPM is infected U937 derived macrophages, toxin BPSS0390 was not upregulated, while BPSS0395 and BPSS1584 were, warranting further investigation (**Figure 10A**)[73]. Conservation of BPSS0395 among the 677 available BPM strains showed that 573 (84.6%) possessed this gene. As an internal control, genes known to be upregulated or downregulated in the host were also analyzed. Hemolysin-co-regulated protein 1 (Hcp1) is a type 6 secretion system protein which forms part of the secretion machinery. During infection, Hcp1 is upregulated and is critical for multinucleated giant cell formation [198]. In the dataset, infection leads to a significant increase of Hcp1, starting at 2 h post-infection. Conversely, BPM is a flagellated bacteria and flagella is a strong potentiator of the host immune response. Interestingly during infection, the bacteria downregulates flagellum expression, including the flagellin subunit FliC, because it switches to actin-based motility as seen in the expression data (**Figure 10A**) [199]. The three genes BPSS0390, BPSS0395, and BPSS1584 (**Figure 10B**) were further studied through the generation of isogenic mutation (**Figure 10C)** and phenotypic characterization.

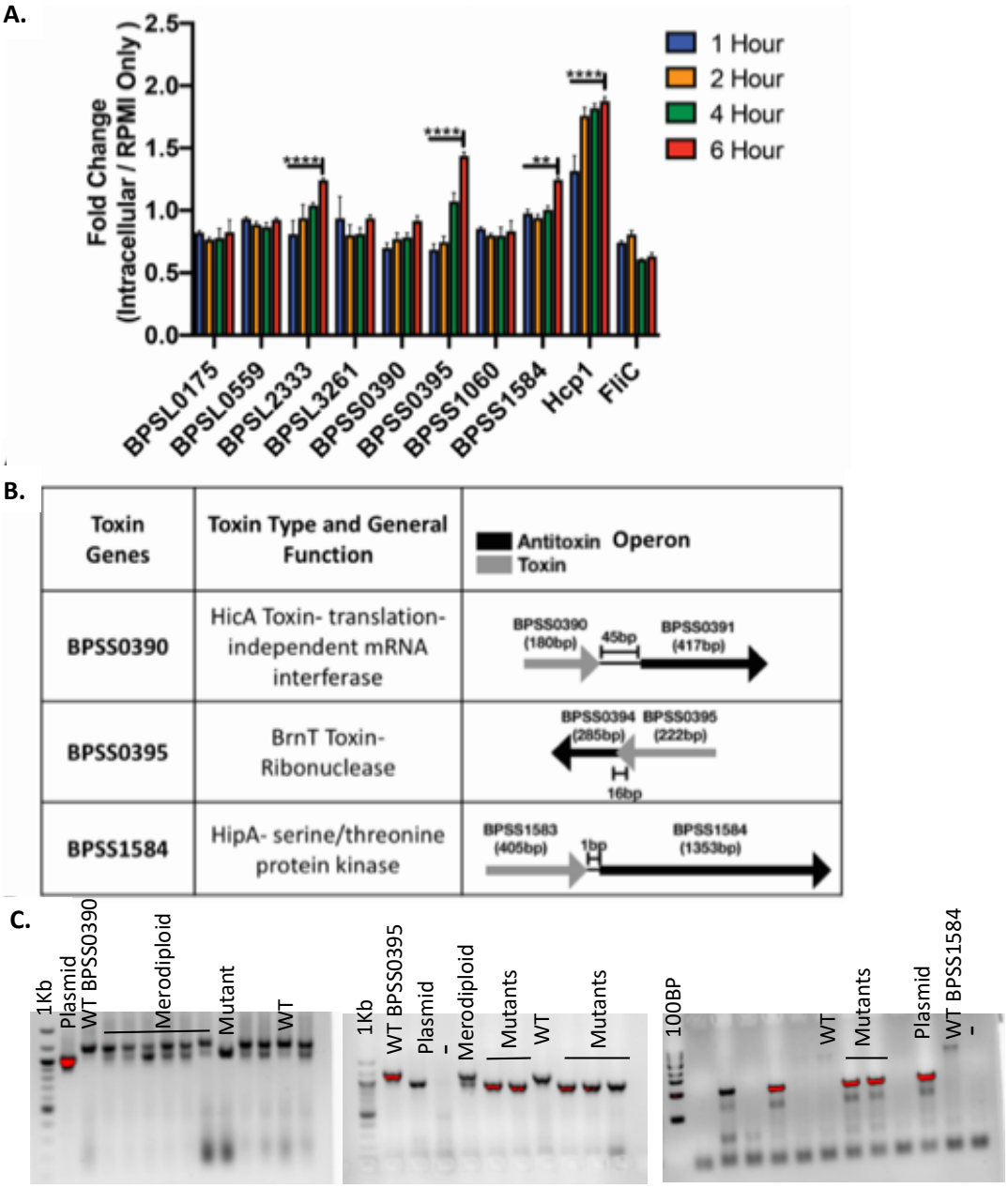


Figure 10: Functional Toxins Investigated

The toxins further examined in this publication are displayed in panel (A) along with their antitoxin and gene orientation. (B) Expression of previously identified toxins was examined by repurposing existing datum from NCBI OmniBus datum set GSM681313 of BPM *surviving in U937 pleural monocytic cells differentiated to macrophages. (C) Isogenic mutations were made for BPSS0390, BPSS0395, and BPSS1584. An intergenic deletion was made leaving approximately 30 bp to keep upstream and downstream genes intact. The PCR products here represent amplification of the gene and the surrounding1000bp. ** P<0.01, **** P<0.0001*

Testing Persistence Associated Phenotypes: Swarming, Biofilm, Antibiotic Persistence, and Growth

To examine the role of BPSS0390, BPSS0395, and BPSS1584 in persistence, toxin mutants were generated and evaluated for their role in persistence-associated phenotypes. Persister mutants have been shown to generate deficient biofilms and reduced ability to swarm compared to the wild-type counterpart [200,201]. Although BPM has not been shown to generate biofilms *in vivo*, the formation of biofilm is an indicator of the propensity of bacteria to generate persister cells that are a critical in the structural architecture of the biofilm, allowing for multi-drug tolerance [147,156]. Wild-ty BPM K96243 forms a low-density biofilm as seen in **Figure 11A**. Loss of only BPSS0390 resulted in a reduction of biofilm formation (**Figure 11B**). Although swarming is not intuitively linked to persistence, this phenotype relies on bacterial communication that leads to the differentiation of subsets of bacteria and increased drug tolerance [201-205] . BPM is adept at swarming to the extent that it can cover an agar plate completely within 18 h. Loss of any of the three toxins resulted in a significant reduction in swarming (**Figure 11C**).

The hallmark assay for examining persistence is exposure of a pathogen to supra-lethal concentrations of antibiotics. Upon exposure to 100x MIC of levofloxacin (**Figure 11D**) and ceftazidime (**Figure 11E**), there was no significant difference in the survival of ΔBPSS0390, ΔBPSS0395, and ΔBPSS1584 compared to wild-type. Lastly, the ability of the bacteria to reach high titers at stationary phase was tested. Stationary phase is a nutrient-limited environment that induces "triggered" persister populations until nutrients are replenished[87]; however, when grown in LB, all three toxins mutants showed no significant difference in growth compared to the wild-type strain (data not shown).

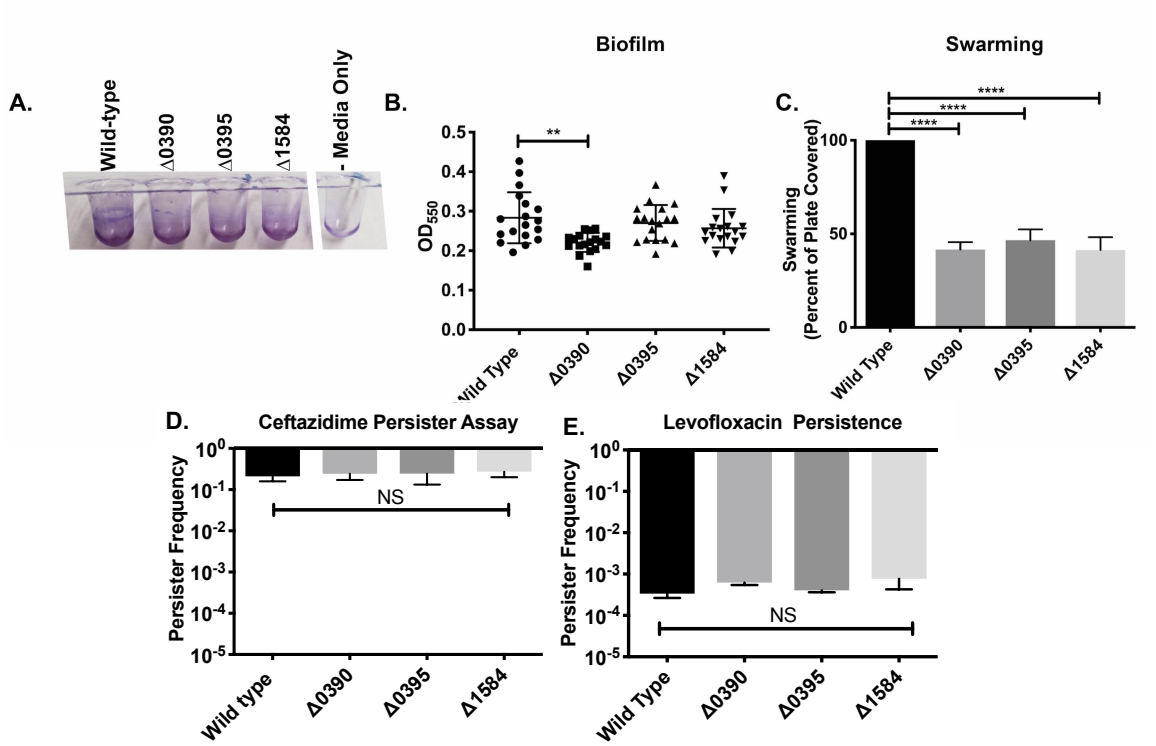


Figure 11: Effect of toxin mutations on Biofilm Formation, Swarming, Antibiotic Persistence. and Growth.

*The ability of the bacteria to form biofilm after sub-culturing in LBG. Biofilms were imaged (A) and crystal violet solubilized for quantification (B). Swarming was assayed by inoculation of bacteria on semisolid agar and allowing bacteria to grow for 24 h. The swarms diameter was measured and presented as a percentage of the size (C). Isogenic mutants were exposed to 100x MIC of levofloxacin or ceftazidime. Survival was assayed at 24 h to determine persister frequency (D and E). Each experiment was done in triplicate and repeated three times with the exception of the biofilms which were done in sextuplicate. * ** P<0.01, **** P<0.0001*

Survival and Persistence in Macrophages

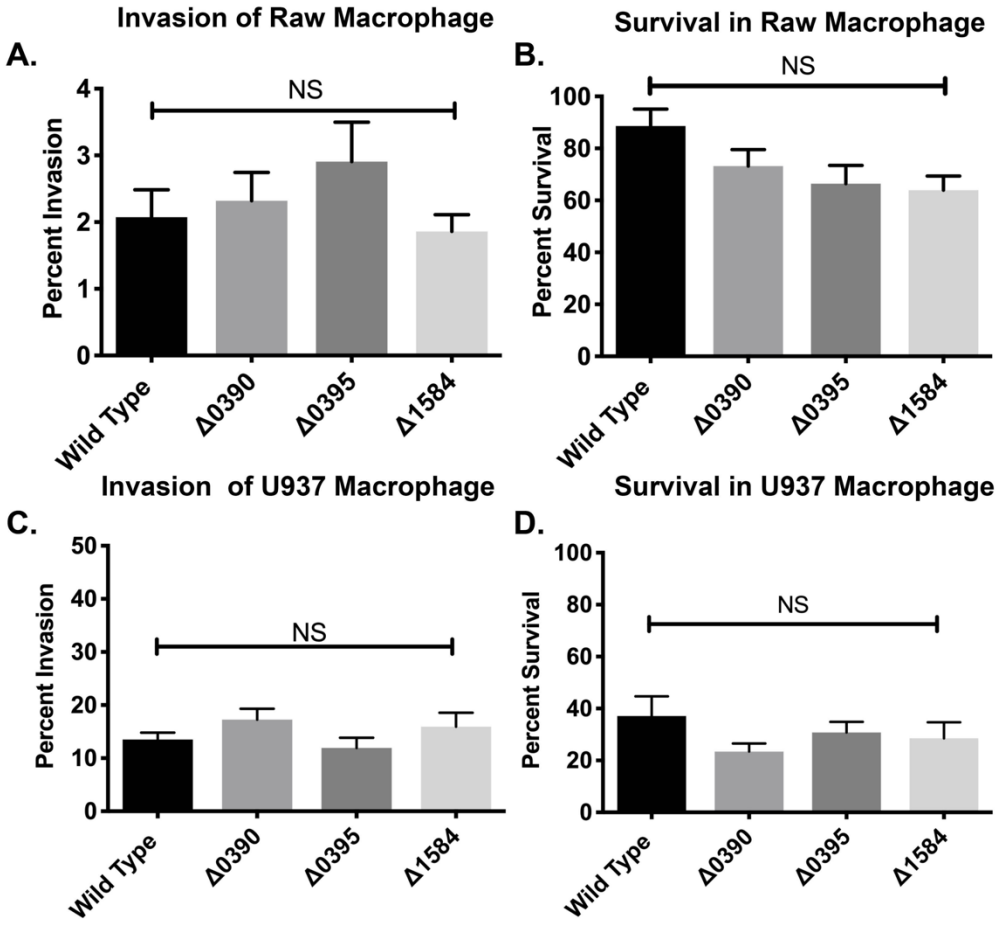


Figure 12: Role of toxin mutations on Macrophage Survival

Invasion and intracellular survival of BPM *strains was quantified in RAW 264.7 macrophages at 3 h (A and B) or U937 derived macrophages (C and D) at 4 h post invasion. Each experiment was done in triplicate and repeated three times. NS Not significant.*

In the host, BPM encounters many stressful stimuli that can lead to the persister phenotype; however, an extracellular bacterium that is dormant is vulnerable to detection by the immune system. BPM is a facultative intracellular pathogen that has been shown to utilize macrophages and dendritic cells to disseminate [66]. Macrophages are also suspected to participate in the establishment of chronic infection [66,81,206]. To test if the toxin mutants

had altered rates of survive in macrophages, RAW 264.7 murine macrophages (**Figure 12A and B**) and human U937 derived macrophages (**Figure 12C and D**) were infected and short-term survival assayed. Loss of each of the toxins did not affect uptake and survival in macrophages compared to the wild-type strain.

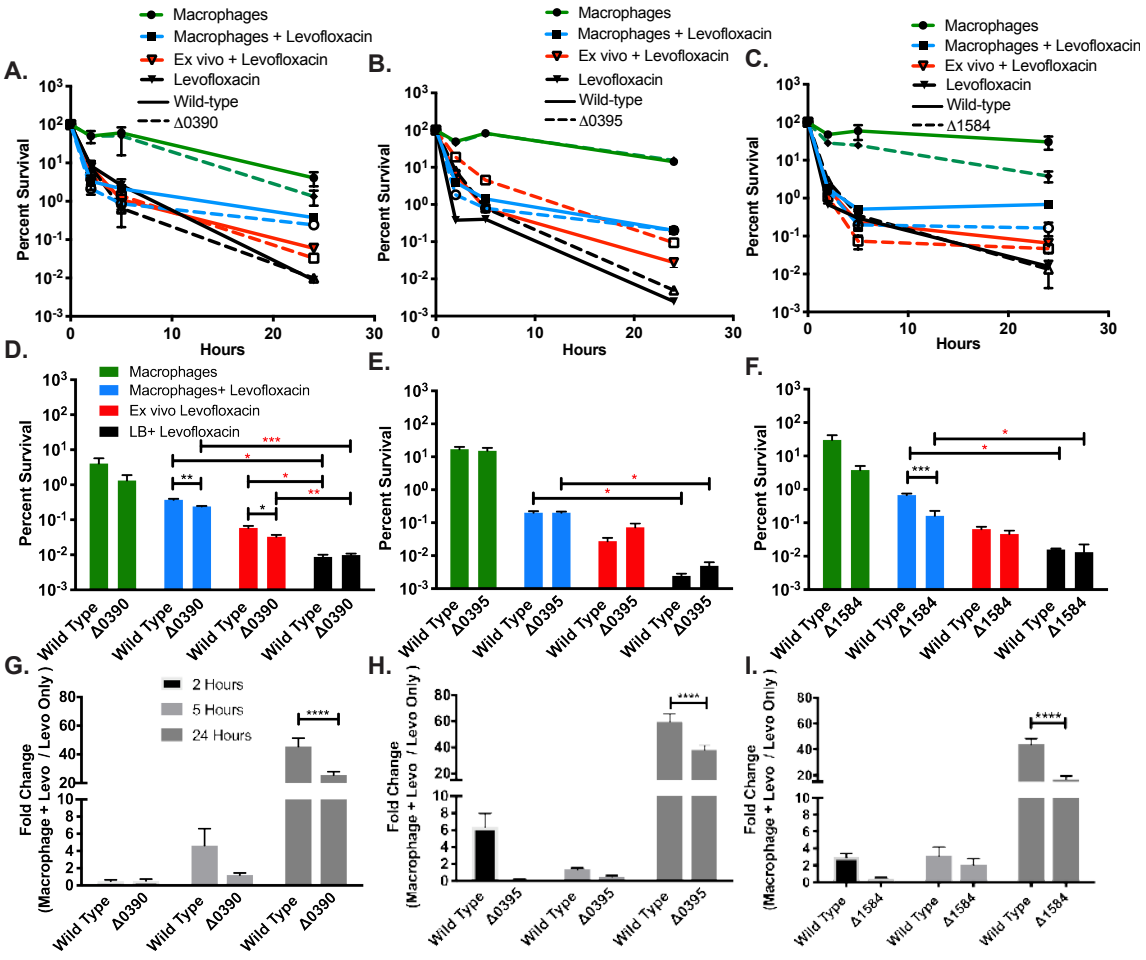


Figure 13: Macrophage-Induced Persistence.

*Persistence was assayed by infecting U937 derived macrophages and treating the macrophages directly with levofloxacin or isolated bacteria from macrophages then treating with levofloxacin (20x MIC). These groups were compared to bacteria only exposed to macrophage or naive bacteria treated with levofloxacin. Survival was monitored at 0, 2, 5, and 24 h post invasion/experimental start (A, B, C). Survival at 24 h is represented in (D, E, and F) and compared to wild-type. Macrophage-induced persistence was calculated as the ratio of macrophage infection + levofloxacin or bacteria only exposed to levofloxacin (G, H, and I). Each experiment was done in biological triplicate and repeated twice. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001*

Because exposure to macrophages confers a fitness advantage by either promoting or priming a larger population to enter a persister phenotype compared to levofloxacin treatment alone (**Figure 2 and 13**), toxin mutants were tested to see if they generate the same levels of macrophage-induced persisters as wild-type BPM. Bacterial survival of each strain was monitored for 24 h in four independent conditions: 1) exposed to macrophages and treated with kanamycin (**Figure 13** green lines; kanamycin is an extracellular active antibiotic; persisters and non-persisters represented here), 2) invasion into U937 derived macrophages followed by treatment with levofloxacin (**Figure 13**, blue lines, cell-permeable antibiotic that kills non-persisters), 3) invasion into macrophages followed by isolation and treatment with levofloxacin ("*ex vivo*", red lines), and lastly, 4) bacteria exposed to antibiotics only (black lines). All strains survived to the largest extent in macrophages exposed to extracellular-active antibiotic (green) while antibiotics alone were the most effective at killing the bacteria (black; **Figure 12 A-C**). In studies with *Salmonella* typhimurium, exposure to the stresses inside a macrophage before supra-lethal treatment of levofloxacin (20x MIC) led to higher rates of survival compared to only antibiotic treatment [102]. BPM also has a survival advantage, but ΔBPSS0390 or ΔBPSS1584 led to a significant reduction in survival when intracellular bacteria were exposed to levofloxacin, compared to wild-type (**Figure 13 D-F**, black asterisks**)**. After 24 h of exposure to macrophages and levofloxacin (total persister cells; blue), ΔBPSS0390, ΔBPSS395, and ΔBPSS1584 led to 0.03 ± 0.01%, 0.20 ± 0.05, and 0.1628 ± 0.028% of the population surviving via persistence, respectively, compared to wild-type. This resulted in an overall population entering persistence of 0.4557 ± 0.235% (**Figure 13 D-F**). Levofloxacin is actively pumped into macrophages (red) [183] and can kill non-persister bacteria. When bacteria were isolated from the macrophage and then treated, wild-type and ΔBPSS0390 maintained a survival advantage, but ΔBPSS0395 and ΔBPSS1584 did not (**Figure 13 D-E)** [183]. Finally, all strains did not have a significant difference in antibiotic-induced persistence rates (**Figure 13 A-F** black lines/bars).

To specifically compare rates of macrophage-induced persistence, all strains exposed to macrophages and then levofloxacin were normalized to the persistence rate of only antibiotic exposure and compared to wild-type (**Figure 13 G, H, and I**). Macrophage exposure led to 25.7 ± 5.7%, 38.0 ± 10.9%, 17.1 ± 6.2%, and 51.1 ± 16.5% persister cells for ΔBPSS0390, ΔBPSS395, ΔBPSS1584, and wild-type, respectively. Once again, exposure to levofloxacin could not eradicate the wild-type or toxin mutant strains. Although exposure to macrophages improved the ability of the bacteria to survive the antibiotic treatment in all strains, the three toxins had significantly lower macrophage-induced persister cells compared to wild-type.

In vivo Attenuation and Persistent Infection

Although infection with BPM can occur through many routes, inhalational infection is the most severe and results in dissemination of the bacterium as early as 24-48 h post-infection [63,66,179,207]. To assess the attenuation of the ΔBPSS0390, ΔBPSS0395, and ΔBPSS1584 mutants, mice were infected with approximately 1,000 CFU (equivalent to 3.5 LD_{50} of wild-type). Both ΔBPSS0395 and ΔBPSS1584 showed reduced lethality while ΔBPSS0390 led to 100% mortality, like the wild-type strain (**Figure 14A**). Weight datum was consistent with the survival findings showing that post-infection with wild-type and ΔBPSS0390, mice had consistent weight loss (**Figure 14B**). Assessment of organ burden of the surviving mice showed that ΔBPSS1584 had higher lung colonization while ΔBPSS0395 had a higher burden in the spleen (**Figure 14C and D**).

In the same experiment, another group of mice were treated with levofloxacin to select for non-replicating "persisters," early during infection, and to test if the toxin mutants had altered their ability to persist in the lungs or spleen of infected animals. As expected, all mice survived the infection and the groups had no significant weight differences throughout the study (**Figure 14E and F**). At 21-days post-infection, mice were

euthanized, and organ burdens determined. Although attenuated, loss of ΔBPSS1584 did not have a significant effect on organ burden following treatment. Loss of BPSS0390 significantly impacted the number of bacteria able to survive in the lungs (**Figure 14 G**), based on reduced organ burden, but the mutation did not affect splenic colonization (**Figure 14 H**). Loss of BPSS0395 had the biggest impact on bacterial survival, significantly reducing the bacteria's ability to survive in the lungs and reducing the colonization to less than 1 CFU/mg in 4 of the 5 spleens **(Figure 14G and 14H).** When comparing the untreated and treated mice, antibiotic addition did not affect splenic colonization of ΔBPSS1584. In contrast, treatment of ΔBPSS0395 infected mice reduced the burden of the bacteria in both lung and spleen as compared to untreated mice.

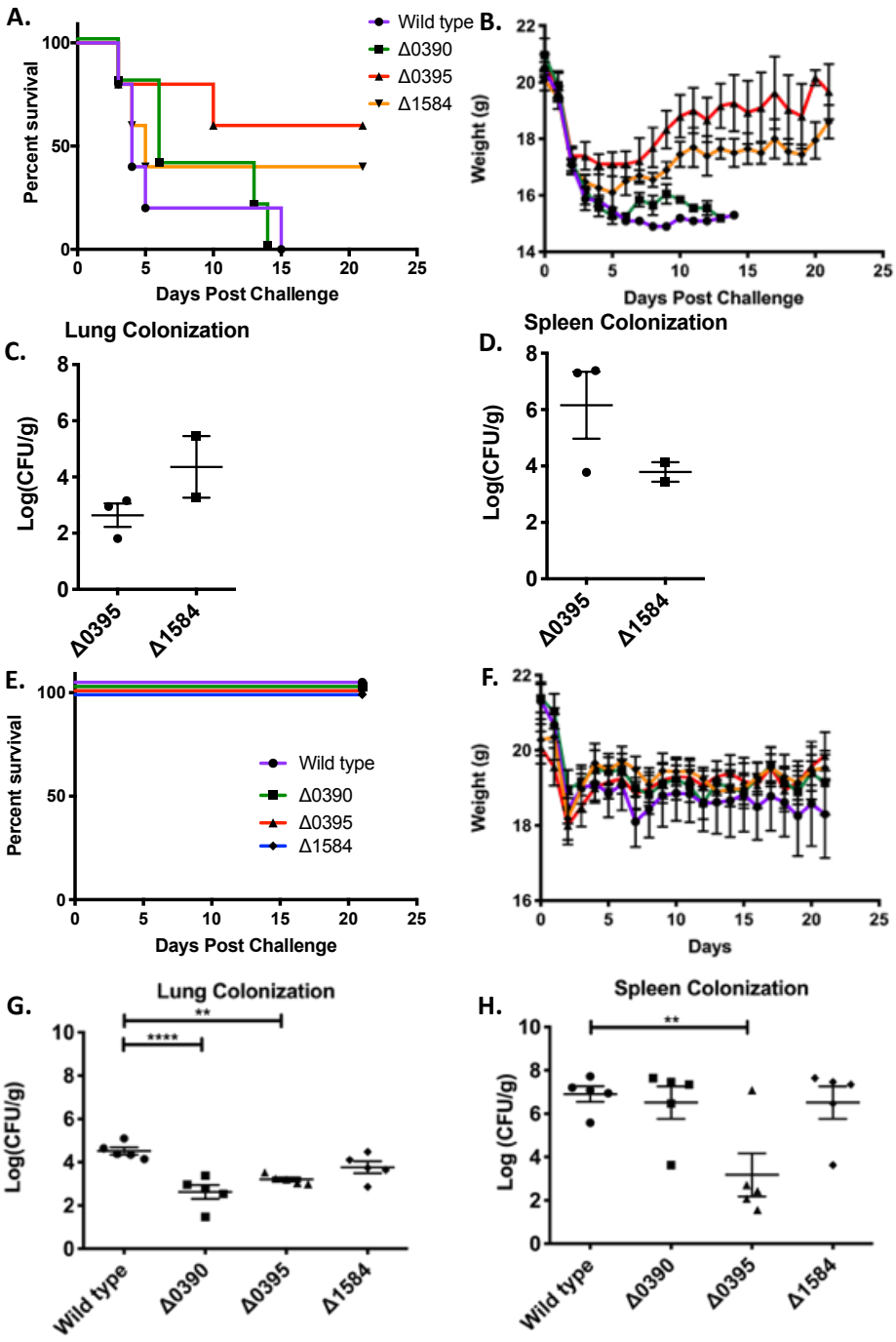


Figure 14: *In vivo* Attenuation and Persistent Infection

*BALB/c mice were challenge with approximately 1,000 cfu and monitored for survival (A) and weight change (B). At 21-days post-infection, surviving mice were euthanized and the bacterial burden determined in the lungs (C) and spleen (D). A second group of mice infected at the same time received 5 days of levofloxacin treatment starting at 24 h post-infection. Weight change was monitored (E) and at the end of the study, mice were euthanized and lung (F) and spleen (G) burden enumerated. ** P<0.01, **** P<0.0001*

Nitric Oxide and Nutrient Limitation Priming of Persistence

Exposure to the macrophage environment offers a fitness advantage which I speculate is through the induction of persistence by intracellular stress [208]. Several innate pathways may inadvertently lead to increased survival of bacteria by inducing or priming toward a persister state. Such pathways include the generation of reactive oxygen species, NO, nutrient deprivation, or other stimuli. The effect of NO and nutrient limitation were tested to see if they alter bacterial persistence through survival in a supra-lethal concentration of antibiotics.

For these studies, LB was not used because it is a rich media that lacks any resemblance to the host environment. Here bacteria were grown in RPMI to attempt to mimic the host environment. Bacteria were exposed to soluble nitric oxide (sNO) or vehicle for 30 minutes before antibiotic exposure. All strains that were exposed to sNO resulted in a significant increase in their survival (10^7 cfu/ml) when treated with 20x MIC of levofloxacin and compared to vehicle (10^4 cfu/ml) (**Figure 15A**). Compared to wild-type, ΔBPSS0390 could not achieve the same level of drug-tolerant persisters (34.6 ± 3.3% compared to 49 ± 6.7%). Loss of ΔBPSS0395 did not significantly reduce survival and did not reach the same levels of persistence as the wild-type strain. Finally, ΔBPSS1584 led to identical levels of persisters as the wild-type strain. To test the effect of NO in macrophages directly, nitric oxide synthase (iNOS) was inhibited with aminoguanidine then macrophages were infected. Upon treatment with levofloxacin and an iNOS inhibitor, I found no significant difference in bacterial survival between the toxin mutants and the wild-type strain (data not shown).

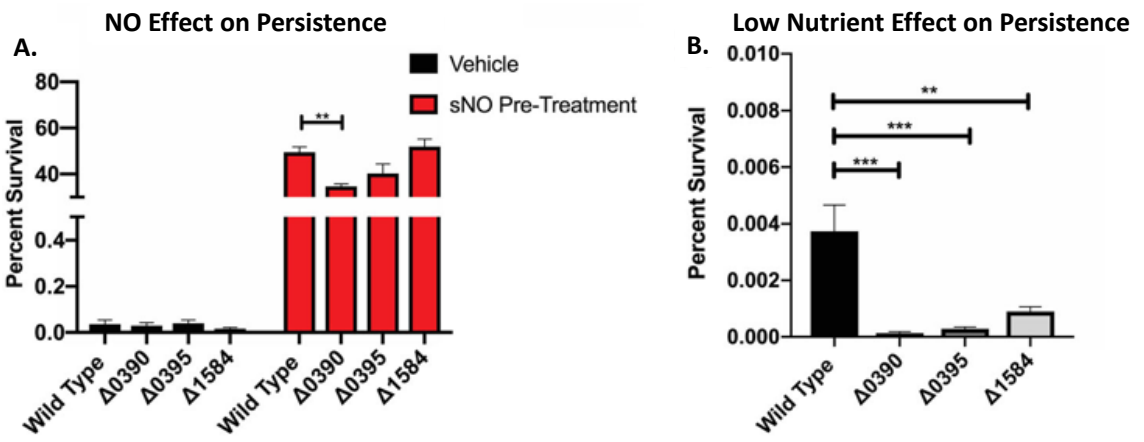


Figure 15: Nitric Oxide and Nutrient Starvation Induction of Persistence.

*(A) Bacteria were pretreated with sNO or vehicle control for 30 minutes prior to treatment with 20x MIC of levofloxacin after which survival was determined. (B) Bacteria were placed in M9 minimal medium for 30 minutes then treatment with 20x MIC of levofloxacin after which survival was determined. Each experiment was done in triplicate and repeated twice. ** P<0.01, *** P<0.001*

Next, the effect of nutrient depletion on persister priming was tested. In order to look at the response to limited nutrients without affecting the growth state, bacteria were grown in RPMI, transitioned to M9, and then treated with antibiotics. Priming the bacteria by nutrient limitation led to a significantly lower level of survival in all the toxin mutants compared to the wild-type strain (**Figure 15B).** Although not as efficient as inducing persistence when compared to NO, the survival of wild-type bacteria went from $>10^8$ to 5.5 x 10^3 cfu/ml and all the mutants survived at a rate of 3-8 x10^2 cfu/ml.

DISCUSSION

Investigation of three toxins, BPSS0390, BPSS0395 and BPSS1584 showed that none of the toxins influenced antibiotic-induced persistence. The three toxins also did not affect survival in macrophage, but following invasion into U937 derived macrophages, the ΔBPSS0390, ΔBPSS0395, and ΔBPSS1584 mutants had reduced rates of persister cells. This suggests that exposure to macrophages can trigger a subset of bacteria to enter a

persister state, which is maintained even if the cells are removed from being in contact with the macrophage. Similar findings have been shown with *Salmonella*, indicating that indeed macrophage internalization leads to a subset of bacteria that stop growing and are not killed by subsequent antibiotic administration [102].

Transitioning into a murine model of Melioidosis, all mutants displayed a colonization defect. Both ΔBPSS0395 and ΔBPSS1584 were attenuated and if treatment was administered, both ΔBPSS0390 and ΔBPSS0395 had reduced colonization. Loss of ΔBPSS0395 had the biggest effect on survival based on its attenuation and near clearance of the bacteria from spleens of treated mice. Upon infection, the bacteria have a preference for macrophages which are a harsh environment and may lead to induction of persistence. However, the exact stimuli that induce the switch to persistence are unknown.

Previously, several *in vitro* stimuli such as oxygen limitation, nutrient starvation, NO, and reactive oxygen species have been shown to increase drug tolerance [208-211]. Here, the loss of toxins led to reduced induction of persistence following nutrient limitation or NO. Indeed, ΔBPSS0390 could not generate the same level of persisters after sNO exposure, while all three toxins had a significant reduction in persistence due to nutrient starvation [212,213].

The data presented in this chapter clearly shows that toxins that are upregulated in response to stress, posed by a host, are important in persistence. Revisiting the inverse correlation between upregulation and conservation that was shown in the previous chapter, draws in to question the drugability of these toxins if not completely conserved. Both BPSS0390 (5%) and BPSS0395 (84%) lack complete conservation across all BPM strains and are completely absence in *B. mallei* strains. TAS have been shown to be encoded in and acquired by mobile elements such as plasmids, phages, and transposable elements. *B. mallei* originated from BPM after undergoing reductive evolution. This information prompts the hypothesis that the toxins, BPSS0390 was acquired more recently as a means to better survive in the host and BPSS0395 was lost and not necessary for fitness in *B.*

mallei. On the other hand, BPSS1584, is completely conserved among BPM and *B. mallei* strains, indicating that it was likely acquired prior to the origination of *B. mallei* and has been maintained.

As the persistence field has progressed, more and more families of toxins are being identified. If the upregulated toxins are to be the primarily studied as candidates for drug targets, they should only be done so only if the toxins are highly conserved. The next problem to face with targeting toxins is how to go about targeting the toxins. A benefit of toxins that are upregulated is potential to target them at a post-translational level or a mRNA level using novel RNA technology such as CRISPR. Although CRIPSR is not currently used to combat bacterial infection it does provide and avenue that eliminates the need to fully understand the protein biology. One simply needs to know that knocking out a gene drastically limits the bacteria's pathogenicity.

CHAPTER 7 INVESTIGATION OF HIGHLY CONSERVED AND CONSTITUTIVELY ACTIVE TOXINS IN HOST PERSISTENCE

INTRODUCTION

Up until now, toxin-antitoxin research has primarily focused on active genes that respond to an environmental stress and little consideration has been placed on genes that do not respond at an mRNA level. In our prediction model, it showed an association between the host environment and of toxins that do not respond to over 80 conditions, suggesting constitutive expression. These toxins were also found to be more highly conserved compared to toxins that are upregulated during stress. This brings up an interesting question of whether lack of upregulation across several conditions is a good indicator of a toxin with a broad-spectrum role. One can argue that the TAS are regulated at a post-transcriptional level and unless examined at a protein level, there is a lack of evidence to initiate investigate. However, from an energy conservation standpoint, continuous expression is expensive and from an evolutionary perspective, continuous presence of the TAS proteins lack dependence on mRNA transcription and provides a faster response time.

In this chapter, toxins from clusters one were examine for functionality and BPSS0899, BPSS1321, and BPSL1494 were further investigated through mutagenesis. Both BPSS0899 and BPSL1494 are putative Doc toxins. Doc toxins are involved in regulating bacterial replication and lead to reversible growth arrest by inhibiting protein translation machinery[214]. BPSS1321 is a putative PemK/MazF-like toxins, which are toxins that initiate persistence by cleaving mRNA in a ribosome-independent manner[215]. Both toxin types have been found in plasmid addiction which led to bacterial death of progeny

that do not have the plasmid[214-216]. The toxins also have been reported to lead to the induction of reversible cellular arrest which is focus here [214-216].

RESULTS

Over Expression of Toxins in Surrogate Organisms

All five toxins in cluster one were cloned into pBAD and over-expressed in *E. coli* DH10b (**Figure 16A**). When toxin expression was induced, 3 of 5 toxins, BPSS0899, BPSS1321, and BPSL1494 reduced the growth rate compared to induction of bacteria carrying an empty vector. Reduced growth indicated some form of functionality in growth arrest. Often, *E. coli* over-expression is called into question as being too much of an artificial system; therefore, expression of BPSS0899, BPSS1321, and BPSL1494 were investigated in the more suitable surrogate, *B. thailandensis*. *B. thailandensis* is a BSL1, nonpathogenic bacterium, that shares 85% of its genes with BPM [7]. Due to the high homology with BPM the functionality of toxins in this model would more accurately replicate the functionality that is expected to be seen in BPM. When over-expressed using the *Burkholderia* specific plasmid pSCRba2, only BPSL1494 showed a reduction in growth compared to induction of a strain carrying the empty plasmid (**Figure 16B**).

It is important to note that *B. thailandensis* carries orthologous operons for BPSS1321 and BPSS1494 which may have abrogated the over-expressed toxins' activity by binding of the orthologous antitoxin. When bacteria are stressed, antitoxins no longer repress toxin activity, usually through antitoxin degradation. To promote antitoxin degradation and test functionality of the three toxins, *B. thailandensis* was grown with or without the inducer and then exposed to 5 – 10x MIC (10 μg/ml) of levofloxacin and survival was assayed. After 24 h of antibiotic exposure, expression of BPSS1321 conferred a survival advantage (**Figure 16C**).

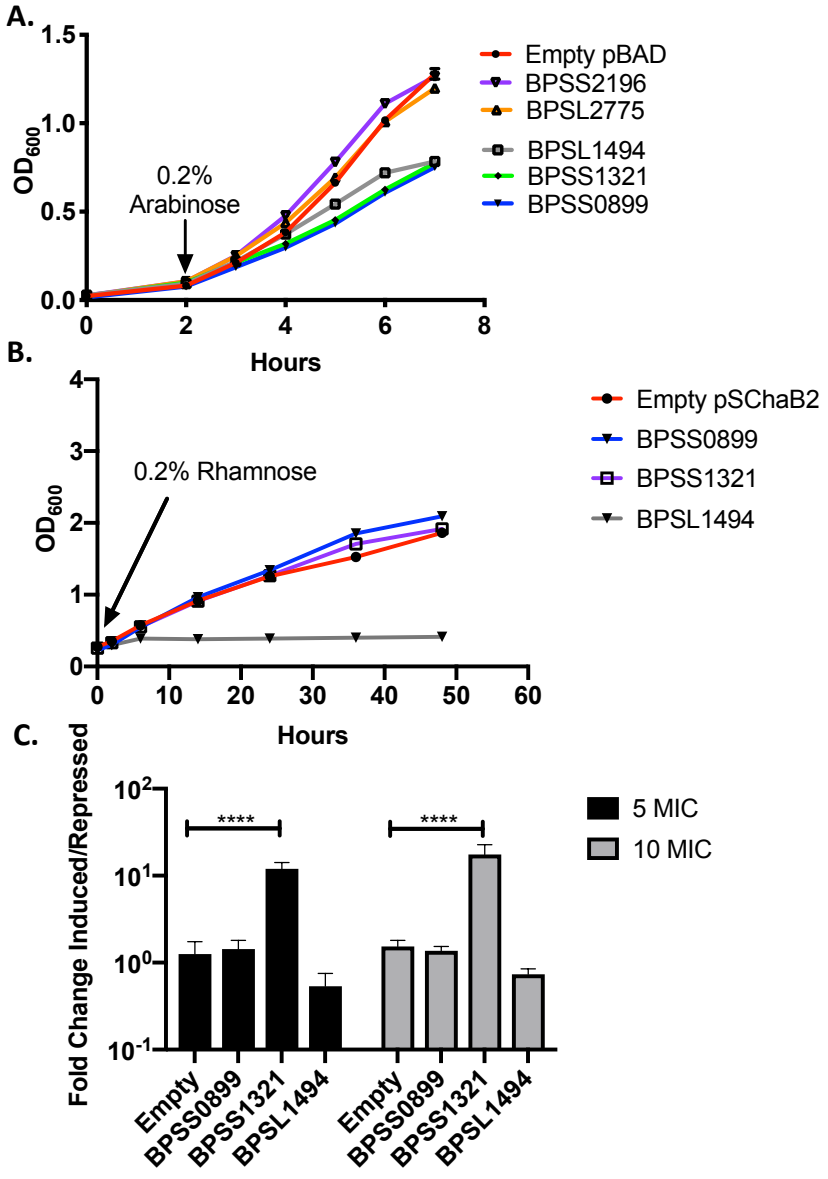


Figure 16: Overexpression of Cluster 1 Toxins

(A) Toxins were cloned into pBAD expression plasmid and transformed into E. coli *DH10b or into pSCRba2 and transformed into* B. thailandensis *(B). Bacteria were grown overnight in minimal medium (with glycerol and casamino acids). Bacteria were diluted and grown to an OD600 of 0.20 after which the cultures were split into 6 tubes and toxin expression induced with 0.2% arabinose (pBAD), 0.2% rhamnose (pSCRba2), or mock treated with PBS and growth monitored by optical density. (C) Induced and repressed* B. thailandensis *strains were treated with 5x MIC of levofloxacin to assay if toxin overexpression affected persistence. **** P<0.0001.*

Phenotypic Characterization of Toxin Mutants: Growth Rate, Swarming and Morphology

Isogenic mutations were made in the toxin genes BPSS0899, BPSS1321, and BPSL1494 (**Figure 17 A, B, and C**). Because persistence relies on growth rate, it was hypothesized that the lack of a functional toxin would prevent the bacteria from sensing nutrient limitation when grown to higher titers. To test this, growth curves were completed for the three toxin mutants and compare to wild type in both a basic laboratory medium (LB) and a host-like medium (RPMI.) In both environments all three toxin mutants grew to higher titers than the wild type strain at 48 h (**Figure 18A and B**). Reduces swarming has been found in bacteria that have lower persistence rates. The mutants were tested to see if toxin loss affected the ability to swarm[59,217]. All three strains had reduced capacity to swarm even when supplemented with a higher level of glucose (**Figure 18C**).

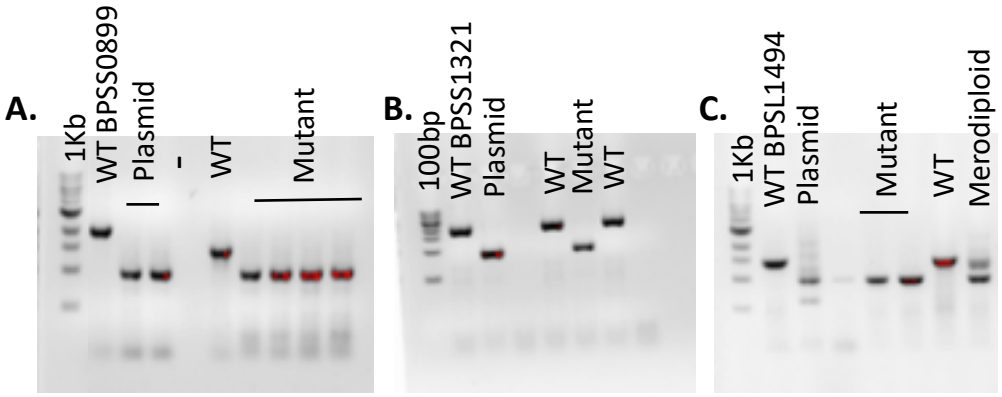


Figure 17: Confirmation of Isogenic Mutations

Isogenic mutations were made for BPSS0899 (A), BPSS1321 (B), and BPSL1494 (C). An intergenic deletion was made leaving approximately 30 bp to keep upstream and downstream genes intact. The PCR products here represent amplification of the gene and the surrounding1000 bp.

BPM can have several different colony morphologies. The BPM K96243 strain at UTMB has two predominant colony morphologies (**Figure 18D**; unpublished). Loss of BPSS0899 and BPSS1494 resulted in morphological changes leading to halo-like colonies when plated on LB agar (**Figure 18D**). Loss of BPSS1321 retained one of the two colony morphologies found in the wild type strain. Some morphology types have been attributed to severity of virulence or persistence, for example, small colony morphotypes are associated with persistence for several pathogens such as BPM, *L. monocytogenes*, and *S. aureus* [218-221]. When exposed to antibiotic stress, all strains presented with a small colony variant (not shown).

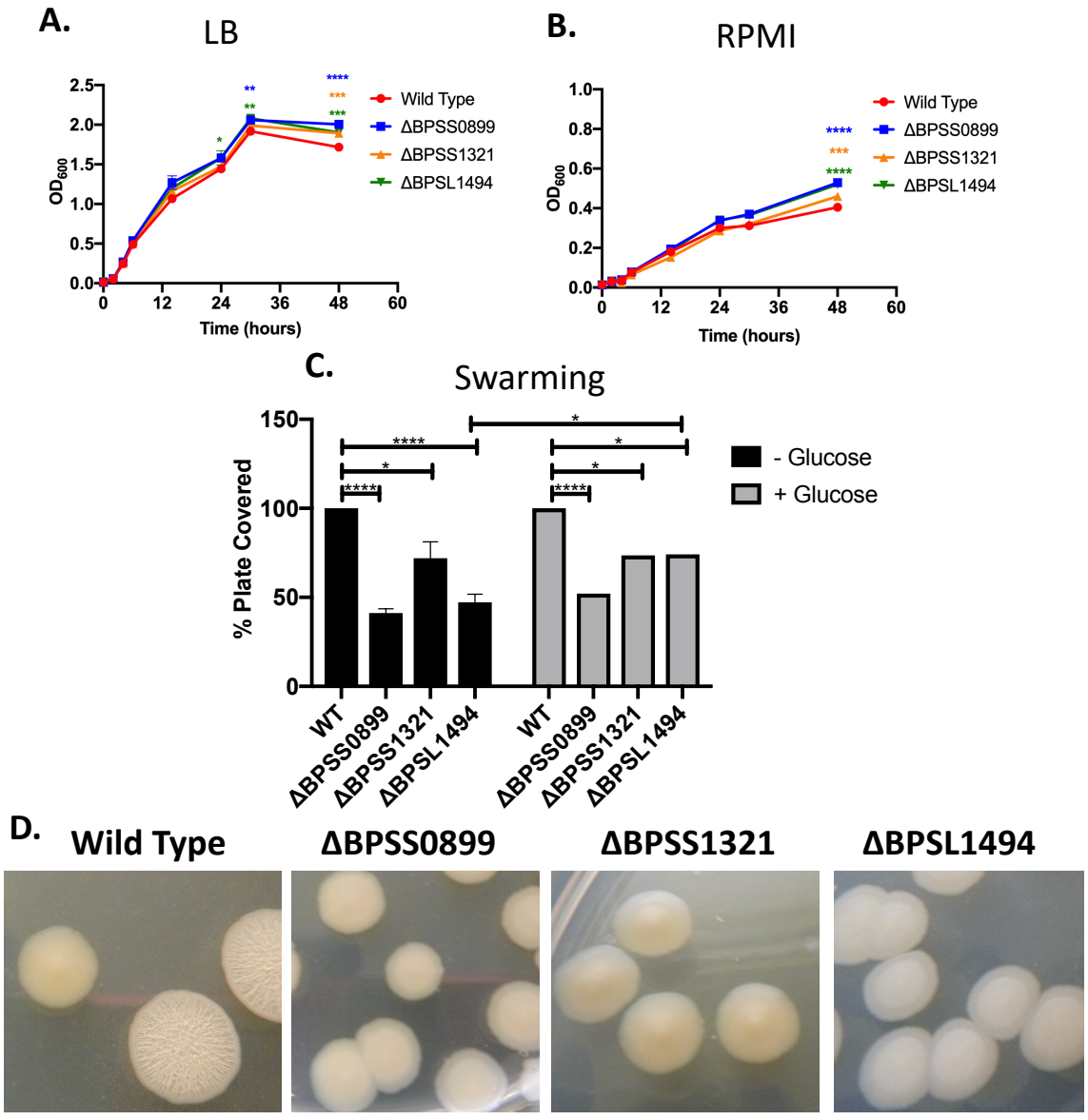


Figure 18: Phenotypic Characterization of Toxin Mutants

*(A and B) Bacteria were grown in either LB or RPMI overnight then diluted to and OD_{600} of 0.01 and growth monitored over 48 h. (C) Reduced swarming motility is linked to persistence. To test swarming, bacteria were spotted on the center of a semi-solid agar plate with or without glucose supplementation and diameter swarmed measured after 24 h of incubation at 37℃. (D) Morphology of each strain under investigation was examined 72h after plating on LB agar. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001*

Persistence

Although this study is focused on the role of toxins in host persistence, antibiotic persistence was tested *in vitro* to gleam if these toxins may respond to antibiotics. Traditional persister assays were conducted by exposing the bacteria to 20x MIC of levofloxacin. The experiments were done on bacteria in both LB medium (**Figure 19A**), which is the traditional medium used, and RPMI (**Figure 19B**) to try to mimic the human host more closely. LB led to higher persister frequency than RPMI. Upon treatment with the antibiotic, ΔBPSS0899 resulted in a significant increase of persistence in RPMI, but not LB (**Figure 19A and B**). In contrast to ΔBPSS0899, ΔBPSS1321 and ΔBPSL1494 had reduced persistence in LB only (**Figure 19A**). In RPMI, ΔBPSS1321 and ΔBPSL1494 had similar persister rates to wild type (**Figure 19B**).

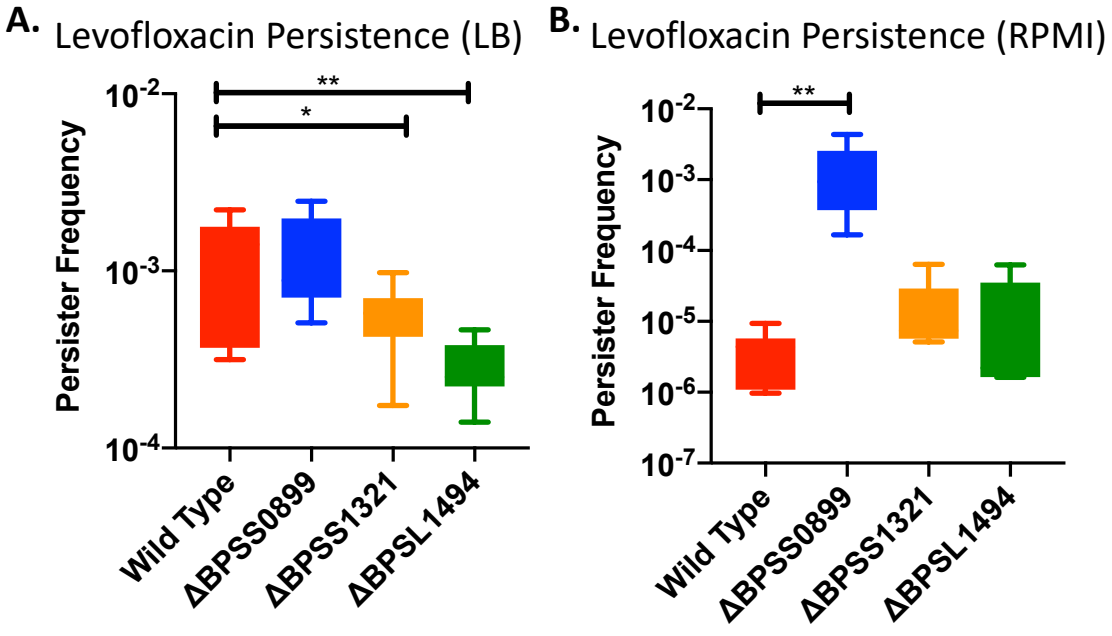


Figure 19: The effect of toxin mutants on Persistence

BPM *wild type and toxin mutants ΔBPSS0899, ΔBPSS1321, and ΔBPSL1494 were grown in either LB (A) or RPMI (B), and then exposed to 20x MIC of levofloxacin. * P<0.05, ** P<0.01.*

Macrophage Uptake, Survival, and Persistence

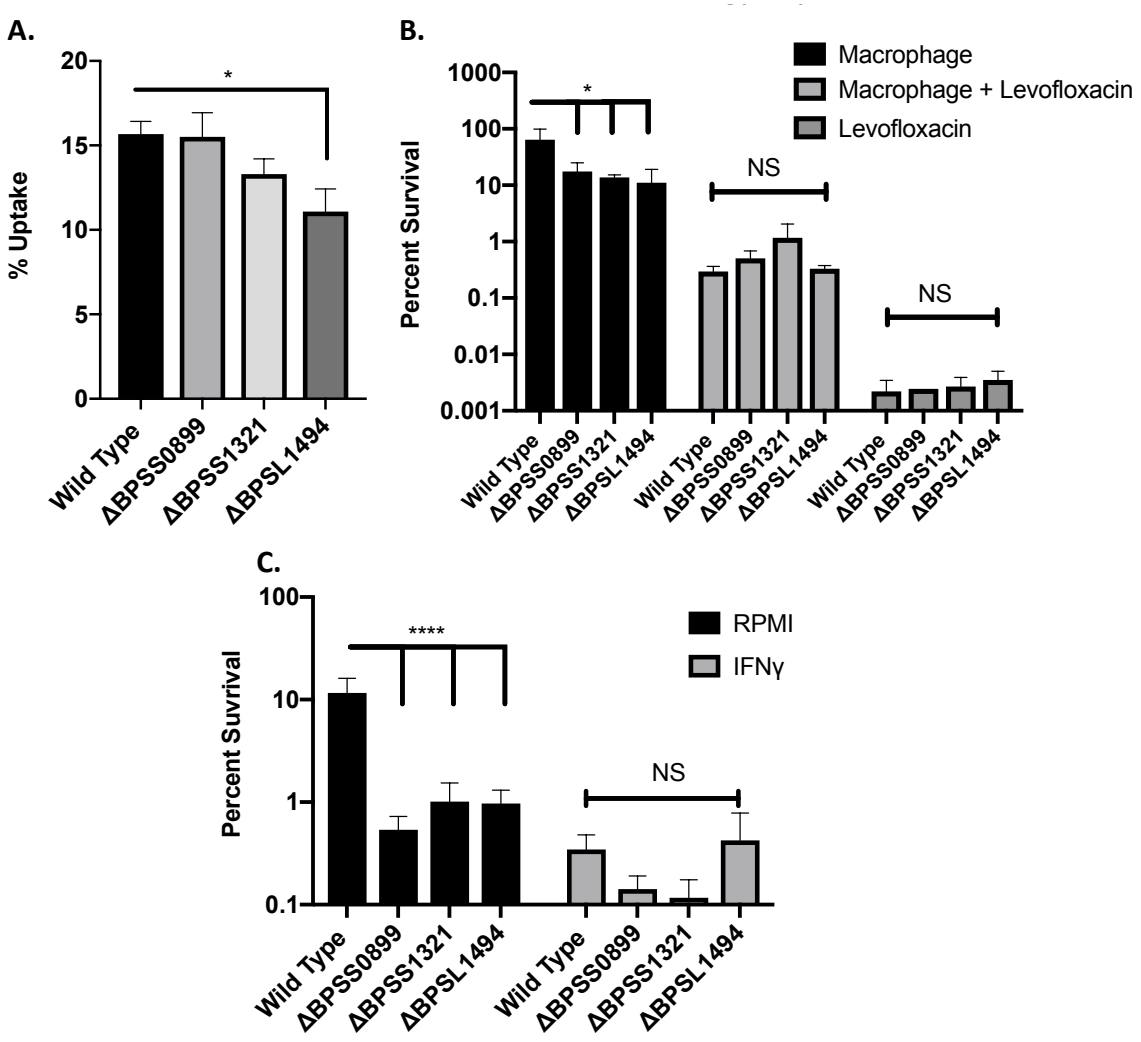


Figure 20: Invasion, Survival, and Persistence in Macrophages

*Macrophages were derived by incubating the U937 pleural monocytic cell line with PMA for 48 h. Invasion (A), survival (B) and persistence (B) was assayed after 30 minutes of uptake followed by a 24 h incubation with or without 20xMIC of levofloxacin. (C) To further investigate the role of macrophage pressure on survival, U937 derived macrophages were polarized to a M1phenotype by IFNγ 16 h prior to infection. One-way ANOVA was used to evaluate the difference with in a treatment group and * P<0.05, **** P<0.0001.*

Evidence shows that macrophages assist in dissemination of BPM and are hypothesized to be a chronic infection niche[37]. To assess the survival and persistence in a human host-like environment, U937 derived macrophages were infected then uptake, survival, and persistence were assayed. Following a 30-minute uptake, there was

significantly lower uptake of only ΔBPSL1494 compared to wild type (**Figure 20A**). General survival at 24 h post-uptake was obtained by incubating the infected macrophages with extracellularly restricted kanamycin. All three mutants had a significantly lower capacity to survive, suggesting a defect in intracellular adaptation (**Figure 20B**). To enrich for intracellular persistent bacteria, the cell permeable antibiotic levofloxacin was added to kill actively growing bacteria (**Figure 20B**). There was no significant difference in intracellular persistence between the mutants and wild type. Compare to levofloxacin treatment alone macrophage exposure increased persistence as previously shown (**Figure 20B**).

The reduced survival of ΔBPSS0899, ΔBPSS1321, and ΔBPSL1494 in macrophages indicated a potential increase in susceptibility to innate antimicrobial pathways in the macrophage. Previous studies show that M1 differentiated macrophages kill BPM more efficiently than MO macrophages through NO production[208]. U937 cells were differentiated with PMA then stimulated with IFNγ to promote an M1 phenotype prior to infection. Activated cells were more readily able to kill BPM; however, there was no significant difference between wild type or mutant strains (**Figure 20C**).

Effect of Toxin Loss on Persistence Induction Following Host-Like Stimuli

All three toxin mutants had a reduced capacity to survive in the macrophage but behaved the same as wild type when levofloxacin was added in the macrophage-induced persistence assays. This suggest that in the absence of levofloxacin, non-persister bacteria are dying, other TAS are being activated by the antibiotic, or a population is entering a viable but not culturable cells state which would be undetectable regardless of antibiotic presence. To answer this, wild type BPM and mutant strains were assessed for persistence induction after exposure to macrophage-like stimuli. Two predominate stimuli that would be found in a macrophages and that effect persistence are nutrient limitation and NO.

Nutrient limitation has been shown to be important in persister formation across many species of bacteria including *B. pseudomalle*[75,222,223]. NO is produced by infected macrophages as an antimicrobial [174]. BPM exposure to NO increases persistence to imipenem and levofloxacin by several logs[75,208].

To better mimic the human host for these assays, bacteria were grown in RPMI then exposed to the stress condition (NO or nutrient limitation) prior to antibiotic addition. For nutrient limitation, bacteria were also grown in RPMI and then diluted into M9 to examine their acute response to nutrient starvation (**Figure 21A**). When exposed to the nutrient limited environment, ΔBPSS0899 and ΔBPSS1494 has a significant defect in survival. Loss of BPSS1321 trended toward lower survival but was not statistically different from wild type. Exposure to NO for 30 minutes prior to levofloxacin treatment led to a reduction in survival of ΔBPSS1321 and ΔBPSL1494 (**Figure 21B**). Note that ΔBPSS0899 has a significant survival advantage in traditional RPMI persistence (**Figure 18**), but here it does not, suggesting a negative effect of NO on ΔBPSS0899 survival (**Figure 21B**).

To start teasing out additional stimuli that these three toxins might respond to, PaperBlast (http://papers.genomics.lbl.gov/cgi-bin/litSearch.cgi) was used to interrogate functions of homologous genes. BPSS1321 is a putative SAM dependent methyltransferase. BPSS1321 had a limited number of homologs for which their roles are largely unknown. BPSS0899 homology PA2949 in *P. aeruginosa* is upregulated in M9 and is down regulated in an escape mutant of the novel antimicrobial agent BAL30072, an iron chelating siderophore monosulfactam[224 225]. When the toxin mutants were exposed to iron prior to levofloxacin, not only did ΔBPSS0899 have reduced persisters, but all three mutants had reduced persister rates (**Figure 21C**). BPSS1494 homologs are highly induced in *S. aureus* and *B cenocepacia* under oxygen limation[226,227]. Our lab does not have the capacity to test the role of oxygen limitation in our BSL3 facility, so this was not tested. Several homologs to BPSL1494 have also been shown to be copper responsive transcriptional regulators[228,229]. When persistence following copper exposure was tested,

ΔBPSL1494 and ΔBPSS1321 had significantly lower rates of persistence compared to wild type.

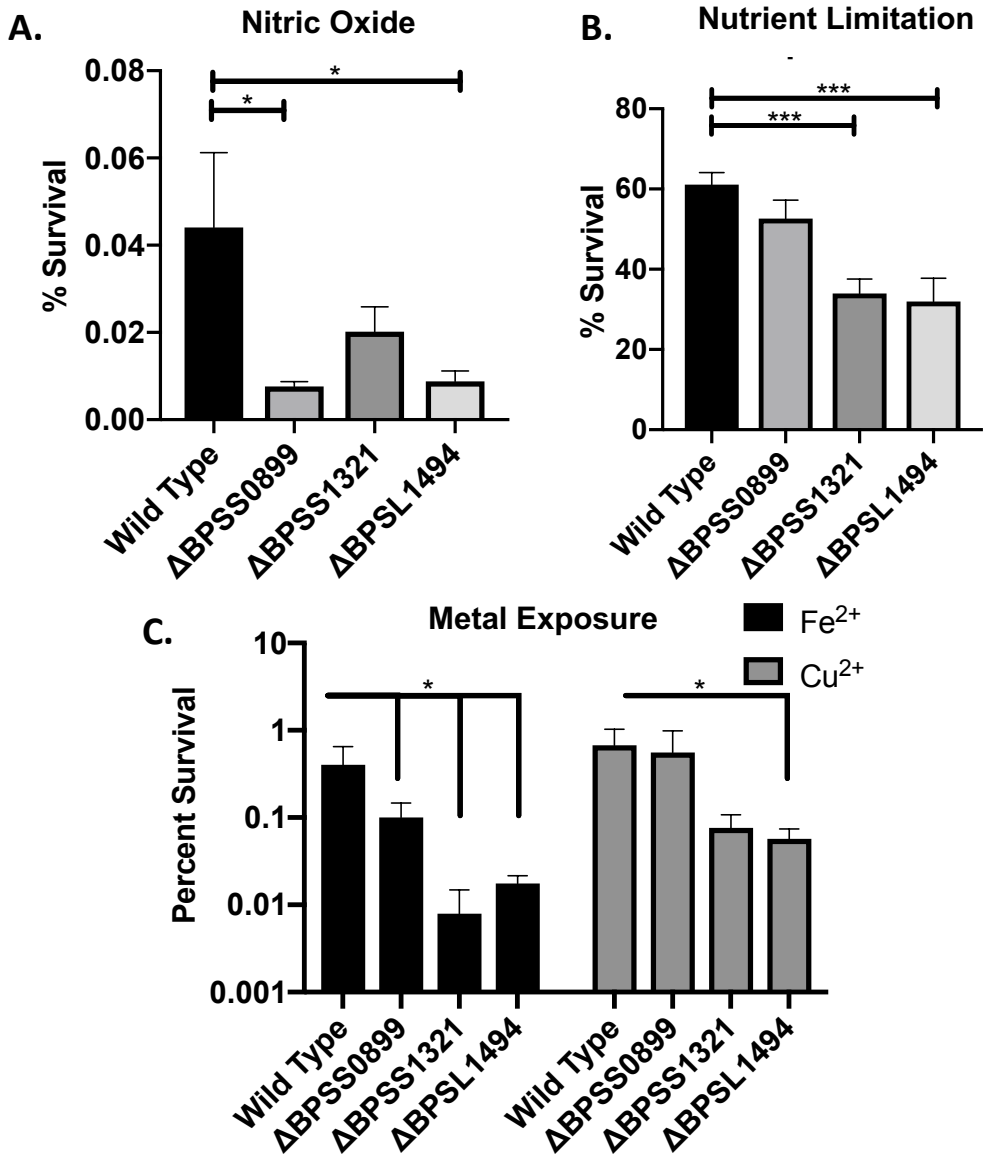


Figure 21: The Effect of Nutrient Limitation, NO, and Metal Exposure on Persistence

*Bacteria were grown overnight in RPMI then exposed to (A) nutrient limitation, (B) nitric oxide (NO), or (C) 100uM copper or iron, for 30 minutes prior to exposure to 20x MIC of levofloxacin. One-way ANOVA was done with in each conditional group or between conditions for one strain. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.*

***In vivo* Persistence**

In the chapter 6, ΔBPSS0395 and ΔBPSS0390 had reduced bacterial colonization after an acute infection and treatment with an antibiotic. The same experimental protocol was conducted with ΔBPSS0899, ΔBPSS1321, and ΔBPSS1494 with the exception that attenuation studies were not carried out (Scheme in **Figure 22A**). Throughout the experiment, the mice did not regain the weight they lost due to infection (**Figure 22B**). Mice infect with ΔBPSS0899 and ΔBPSL1494 had transiently higher weights on days 14-18. At 20 days post-infection, lung, liver, and spleen were collected. All three mutants had lower splenic weight (**Figure 22C**). Examination of gross pathology showed that spleens from mice infect with wild-type (**Figure 21D)** BPM had more severe splenic abscesses and ΔBPSS1321infected mice had either severe abscess or no visible pathology (**Figure 22D panel 3 and 4**). Infection with ΔBPSS0899 or ΔBPSL1494 resulted in mostly unremarkable spleens. Quantitation of bacterial burden showed no significant difference in bacteria in the spleens, lungs, or livers (**Figure 23A-C**).

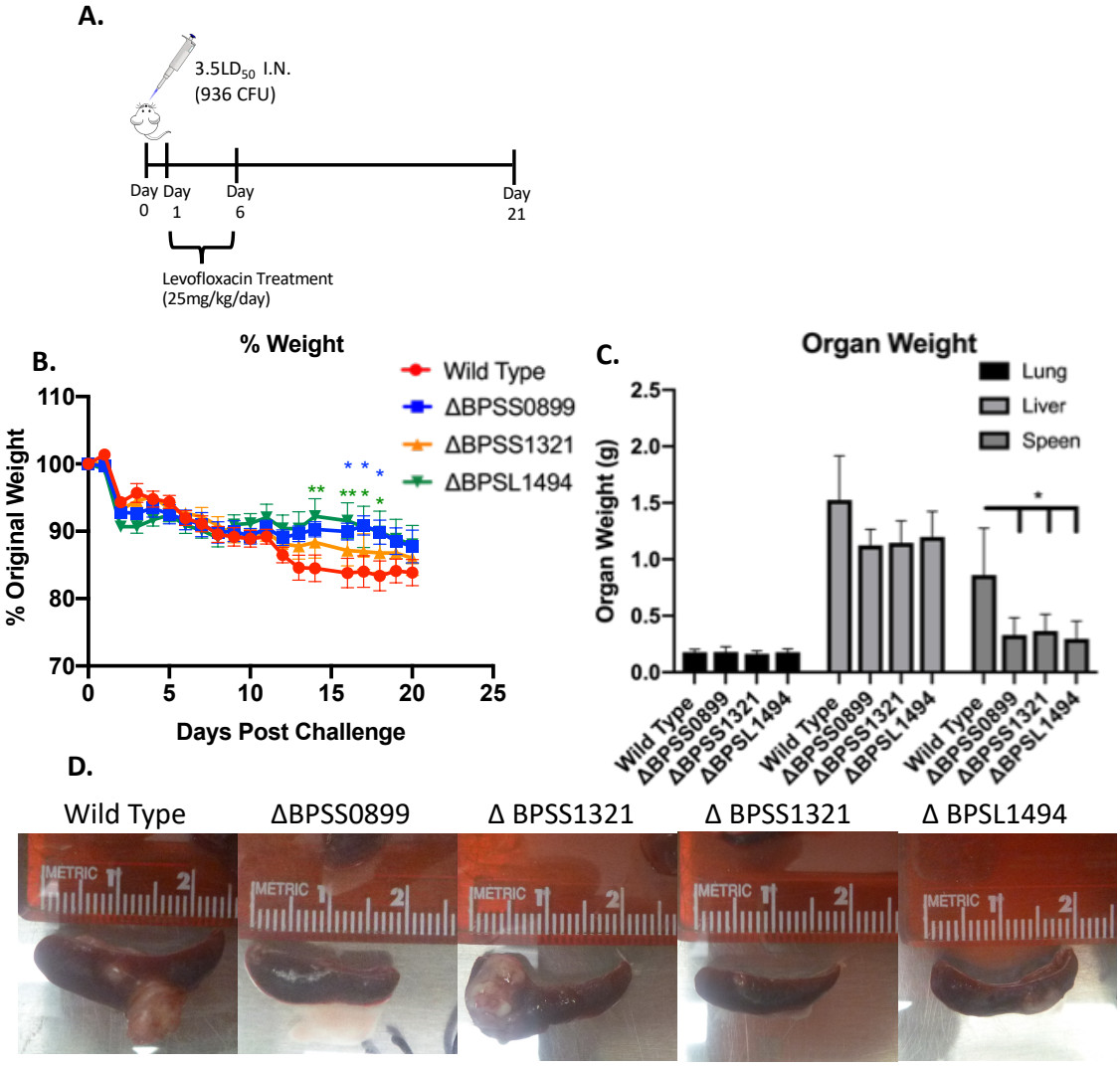


Figure 22: *In vivo* Persistence of ΔBPSS0899, ΔBPSS1321, and ΔBPSS1494

*(A) Mice were infected with 3.5 LD50 and treated for 5 days with 25 mg/kg/day of levofloxacin as depicted. (B) Weight was monitored for the 20-day period after which mice were euthanized and organs collected, (C) weighed, and (D) imaged. Representative images are displayed two images are displayed for BPSS1321 due to the wide variety pf pathology seen. Statistics were completed using Two-Way ANOVA in panel B and One-Way ANOVA in panel C. * equates to P<0.05 and ** P<0.01.*

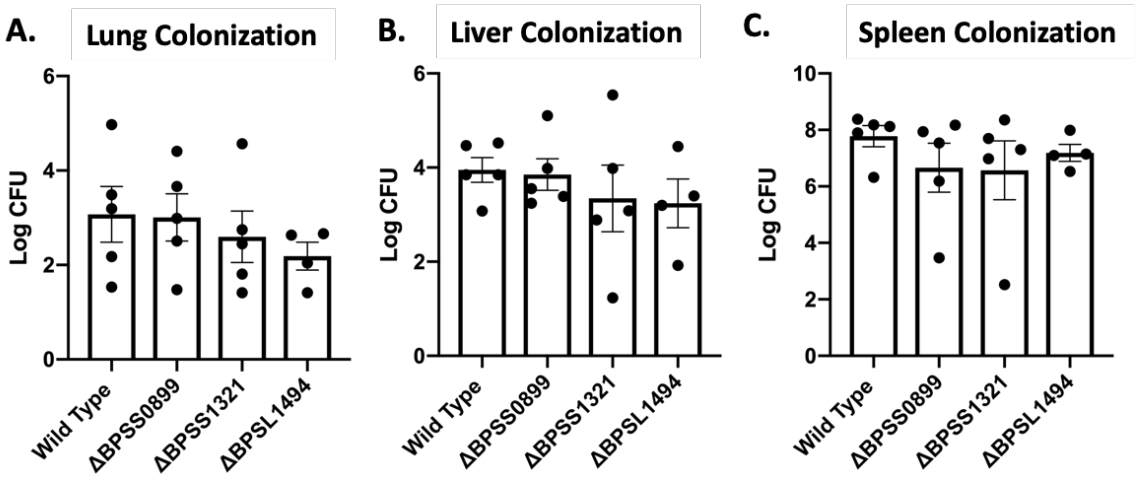


Figure 23: Organ Burden Following Infection and Treatment

Organs were homogenized, and bacteria cultured on agar to assess bacterial burden in the (A) lung, (B) liver, and (C) spleen. Statistics show no significant difference.

DISCUSSION

In this chapter, the toxins BPSL2775, BPSS2196, BPSS0899, BPSS1321, and BPSL1494 were investigated based on the predicted association with host persistence. It was uncovered that these genes are actually constitutively expressed and have high conservation. Our findings led us to the few whether the few constitutively expressed genes are important in host persistence which was tested here.

Functionality assays indicated that BPSS0899, BPSS1321, and BPSL1494 are functional and promote growth senesces in *E. coli*. Data from *B. thailandensis* over-expression assays shows that BPSL1494 was functional but BPSS1321 and BPSS0899 do not induce a growth reduction. This can be explained by expression of homologous or orthologous antitoxins by *B. thailandensis*. To test this, *B. thailandensis* expressing each of the three toxins were exposed to antibiotic to promote degradation of any homologous antitoxin. BPSS1321 was the only toxins that led to a survival advantage.

The toxins BPSS0899, BPSL1321, and BPSL1494 were further investigated using BPM genetic knock out strains. Phenotypic assays done on these mutants departed from

the data seen in chapter 6 in the following assays: growth kinetics, persistence and macrophage-induced persistence. All three mutants grew to significantly higher titers compared to wild type indicated a role in growth modulation. Overall, BPM grows faster in LB compared to RPMI. Persistence was also higher in LB compared to RPMI. Factors that may be contributing to the persistence difference between LB and RMPI include: the rate of replication (slower in RPMI), bacterial titer (LB>RMPI), a component of the media, or a combination of all three. It is well established that stationary cultures led to higher persister rates than logarithmic stage cultures. It is important to note that all assays use 16-hour cultures and LB cultures reach stationary phase closer to 30 h.

Examination of antibiotic persistence of ΔBPSS0899, ΔBPSS1321, and ΔBPSL1494 revealed a significant increase of persistence of ΔBPSS0899 in RPMI, but not LB. Irrelevant of the statistics, ΔBPSS0899 RPMI persistence rate is similar to the LB persistence rate, highlight. In both medias ΔBPSS0899 grows faster, than wildtype so the difference is likely due to a medium component or overcompensation by other toxins due to the loss of BPSS0899. Although mutation of BPSS1321 and BPSL1494 had higher growth titers at 48h, they had reduced persistence in LB only. This was not true in RMPI suggesting a dependence on growth rate or an influence of a medium component.

The increase in antibiotic persistence seen of ΔBPSS0899 did not translates to an increase on macrophage induced persistence, instead, all toxins behave similar to wild type. In the absence of cell permeable antibiotics, all three toxins have a significant reduction in survival. Based on these findings, it is suggested that the three toxins prevent killing by the host specifically, which would corroborate with our prediction that these toxins might play a role in host associated persistence. This was further supported by the effect innate antimicrobial stresses on antibiotic revealed persistence. Exposure to nutrient limitation BPSS0899 and BPSS1494 had persistence defects while nitric oxide pre-exposure reduced BPSS1321 and BPSS1494 persistence.

When tested in an *in vivo* model of murine inhalational Melioidosis, there was no effect on colonization following treatment. These findings are consistent with the results from the macrophage-induced persister assay where host stress and antibiotics are combined the mutants survived just as well as the wild type. If the *in vitro* assays with macrophages are true predictors of *in vivo* colonization it could be hypothesize that infection in the absence of antibiotics may result in difference; however, in this model, lack of treatment wild type BPM is a fully lethal infection to answer this question requires model development and therefore was not tested. One difference that was found was the reduced splenic pathology of BPSS0899 and BPSS1494. This indicates a potential role for these toxins in immunes system modulation or antigen expression.

The findings in this chapter clearly showed that constitutively expressed toxins play a role in bacterial survival within macrophages. When antibiotics were added the bacteria was as resilient as their wild type counterpart. This data, although interesting only applies to fluoroquinolone and interaction needs to be tested with different antibiotic classes. In the persistence field, the goal is to find an anti-persister compound that improves antibiotic efficacy. Here, the data suggest that targeting some toxins without antibiotics can increase bacterial clearance by the host. The next question warranting investigation is whether these toxins play a role in modulating the host immune response.

CHAPTER 8 DISCUSSION

Antibiotic failure is a growing problem worldwide; however, until the past two decades, the research field has focused on understanding the mutations behind particular resistances. This avenue of research has been fruitful in identifying methods of reversing resistance by subverting bacterial mechanisms, most notably with the discovery of beta-lactamase inhibitors that extend beta-lactam antibiotic usefulness. Formation of resistance is theoretically possible for all classes of antibiotics, as well as for disinfectants; however, the underlying mechanism that results in the generation of all forms of resistance or survival without resistance is rarely studied. Bacterial persistence is a mechanism that microbes have developed to become transiently refractory to antibiotics and host defenses [126]. Recently, growing evidence supports bacterial persistence as a precursor mechanism of resistance. Not only do the two phenomena share several mechanisms, but persisters have been shown to be hypermutable. Importantly, persistence prolongs survival of the bacteria and increases the chances of successful resistance generation[112,113,116,120]. Aside from the importance as a resistance precursor, persistence generation has been associated with nearly all bacteria that have the propensity to cause chronic infections[156]. The phenomenon is evident in several instances in which patients experience treatment failure without the isolation of a resistant organism[106].

Currently, there are three main anti-persister avenues under investigation: reduce or interfere with the formation of persisters, directly kill persisters, or sensitize persisters to antibiotics by promoting resuscitation[103]. All three avenues of persister targeting have both advantages and disadvantages as treatment options[103]. Because persistence is a phenomena seen in bacteria, fungi, archaea, and human cancer cells, finding a bacterial specific compound that does not have broad off-target effects is one of the more difficult challenges [144,230,231]. Restimulation of bacterial growth via metabolic supplementation has shown promise, but metabolites have broad effects on the immune system and other co-

colonizing bacteria, which may lead to undesirable side effects such as microbiota disregulation[157-159]. Similarly, trying to kill persisters directly has been shown to be a fruitful approach, as several drugs have been identified with anti-persister activity[103]. Several compounds identified are used as cancer therapies which have side effects unsuitable for general antibiotic treatment, however, can be used in a life-threatening situation. Lastly, preventing persister formation is largely done by the investigation and targeting of toxin-antitoxin systems, which initiate persistence. This method provides more bacterial specific targets; however, many pathogens harbor numerous systems suggesting redundancy[144,162]. In this dissertation, I investigated the role of toxin-antitoxin systems in BPM and successfully produce a method for rational selection of toxins to study (**Illustration 13** - **Figure 9**).

Over the past several decades, novel toxin-antitoxin systems have been identified and homologous systems have been found in a wide variety of organisms[144]. A seminal paper in the field examining 126 prokaryotes, identified a correlation between free-living organisms and increased toxins numbers, while host-restricted organisms had fewer toxins[144]. This is likely due to the greater number of bottlenecks faced in a changing environment compared to an organism whose niche is more stable. The capacity of an organism to cause epidemics is also associated with a higher number of toxins due to the increased ability to adapt and evade killing in a new environment, thus making bacteria more difficult to combat[232]. Although the role of TAS in persistence induction has been questioned, there has been strong evidence of these systems playing a role in more complex organisms, such as *Salmonella* typhimurium, *Mycobacterium tuberculosis*, and based on our research with BPM[233]. This lack of confidence in the role of toxins in persistence may stem from the investigation in non-pathogenic systems that lack evolutionary pressure to promote survival mechanisms. Alternatively, we know that the environment shapes a bacterial population's behavior, but we still use rich medium that lacks any resemblance to host environments[234]. This general practice may be leading to misleading data.

Additionally, persistence field has been limited itself to testing all toxins for survival in one condition and that is antibiotic exposure [234]. On this planet there are an infinite number of stresses that can be found; thus, a toxin may be important for something as abstract as surviving at 37°C when a bacterium normally lives in a thermophilic environment. This issue could confound data, leading to false negative results by testing toxin functionality in the wrong condition.

Here is where a challenge arises, of all the toxins being identified, how do predict which toxin(s) to focus our research on? Approaching the issue with a focus on persistent infection, I wanted to find a way to rationally select toxins to study using a data-driven approach. Prior to predicting a toxin's conditional functionality, an updated search of toxins was done that led to the identification of 103 putative toxins in BPM K96243 (**Figure 4**). These genes were subjected to unsupervised bipartite network analysis to examine their conditional associations. There was a dominate pattern indicating that most toxins were associated with a majority of conditions. This data complements the findings that free-living organisms have more toxins likely to promote adaptation to changing surroundings[144]. Because BPM is a soil and water dwelling organism, the network indicated a dependence on toxins for several environments. Infection-associated conditions had a different pattern, with fewer genes of importance found specifically in three distinct clusters. Further examination of the clusters showed an inverse correlation between expression and conservation. Nearly all the genes that did not change their expression across the 82 different conditions were conserved among all available BPM genomes (**Table 7**). These finding indicate an importance of constitutive genes, contrary to general trends of placing importance on upregulated genes.

To untangle the role of upregulated, moderately conserved genes compared to highly conserved, constitutively expressed genes, mutagenesis studies were conducted on three toxin genes selected from each class. Examination of loss of three moderately conserved, but upregulated toxins (BPSS0390, BPSS0395, and BPSS1584) showed no

effect on antibiotic persistence and macrophage survival. When the two conditions were combined; however, the mutants had a survival defect in macrophage induced persistence (**Chapter 6**). Because the stress produced in macrophages increases persistence of wild type bacteria but not in ΔBPSS0390, ΔBPSS0395, and ΔBPSS1584, the three toxins likely activated by stress stimuli in macrophages.

To the contrary, loss of BPSS0899, BPSS1321, and BPSS1494, which are highly conserved genes that are expressed at a steady state, had altered antibiotic persistence levels and reduced macrophage survival. The survival defect, however, was lost when macrophage induced persistence was assayed (**Chapter 7**). These findings indicate that the selective role of BPSS0899, BPS1321, and BPSS1494 in surviving the harsh environment of the host cell, but their loss is compensated by activation of other toxins when antibiotics are added.

Persistence has been shown to be dependent on growth; therefore it is important to take a closer look at the difference in persister rates of ΔBPSS0899, ΔBPSS1321, and ΔBPSL1494[234]. First, the discrepancy between the rate of persistence induced in LB versus RPMI may be due to the fact that BPM has a higher rate of replication in LB, bacterial titer (LB>RMPI), a component of the media, or a combination of all three (**Figure 19**). It is well established that stationary cultures led to higher persister rates than logarithmic stage cultures. It is important to note that all assays use 16-hour cultures and LB cultures reach stationary phase closer to 30 h. The lack of a difference in ΔBPSS0899 persistence when grown in LB and RPMI is may due to the ability of ΔBPSS0899 to grows faster, the difference medium component, or overcompensation by other toxins due to the loss of BPSS0899 (**Figure 18A and B**). When exposed to LB, both ΔBPSS1321 and ΔBPSS1494 had a persistence defect (**Figure 19**). During replication kinetics both ΔBPSS1321 and ΔBPSS1494 grew faster than wild type suggesting they would have a persistence advantage, which is not the case **(Figure 19**). This data suggests that both ΔBPSS1321

and ΔBPSL1494 are true functional toxins, confirming the utility of my data-driven approach to identify toxins to study.

In a murine model of Melioidosis, loss of BPSS0390 and BPSS0395 attenuated the bacteria. When antibiotic treatment was administered in an acute infection model, loss of BPSS0395 and BPSS1584 had rescued colonization, improving the outcome of infection (**Figure 14**). For the mutants BPSS0899, BPSS1321, and BPSL1494, we only studied an acute infection with treatment to examine bacterial dissemination and end-point burden. Similar to the macrophage-induced persister assay (**Figure 20**), that bacteria exposure to the host (mouse) and antibiotics had no effect on colonization (**Figure 23**). We speculated that this was because although the toxins were important for host survival, other toxin systems were responding to the antibiotic and promoting a persister state.

This study was focused on host-associated persistence, I investigated the potential triggers that lead to toxin activation. Host innate pathways constitute several stresses that bacteria are exposed to, including serum, acidification, ROS, NO, nutrient sequestration, etc. For this study, NO and nutrient limitation were tested and because these conditions do not necessarily lead to bacterial killing, we examined if these conditions altered antibiotic persistence. When exposed to nutrient limitation, all toxin mutants had a survival disadvantage (**Figure 15 and 21**). When exposed to NO ΔBPSS0390 and ΔBPSS1494 had reduced persistence, indicating stimuli selectivity of these toxins (**Figure 15 and 21**). Use of a protein-based literature search indicated that ΔBPSL1494 may sense and respond to copper, while data has shown that abrogation of iron acquisition alters expression of a BPSS0899 homolog. Evaluation of the influence of iron and copper influence on the induction of persistence identified that indeed iron exposure reduced ΔBPSS0899 persistence and copper exposure reduced ΔBPSL1494 persistence (**Figure 21).** Collectively these studies confirmed the utility of *in silico* approaches developed in this project.

At the completion of this dissertation, the evaluation of the BPM persistence phenotype perpetuates the idea that BPM is a difficult organism to control and much more work is needed to further decipher the mechanisms behind the persister cells that lead to chronic Melioidosis. Our findings indicated that the *in silico* prediction model accurately predicted toxins associated with the host and our mutagenesis work clearly shows there is a cluster specific phenotype when toxins are mutated. The two different phenotypes indicated that targeting the toxins can improve treatment in different ways. Targeting upregulated genes (BPSS0390, BPSS0395, and BPSS1584) can be a method for supplementing current antibiotics treatment, while targeting constitutively toxins (BPSS0899, BPSS132, and BPSL1494) function without antibiotic supplementation and identifies a new method for the development of anti-microbial agents. Future directions

Contrary to popular belief, toxins that were constitutively expressed were highly conserved and played a role in macrophage persistence without antibiotics. This is very important finding for the development since many toxins across bacterial species lack 100% conservation. The major issue that this work highlights, is the ability to test true host persistence in the absence of antibiotics. In Thailand, 80% of people seroconvert to BPM by age 4 and maintain high levels of antibodies throughout their life. This suggests that there is asymptomatic carriage of the organism and further implicated the ability of the host to promote persistence in the absence of antibiotics. To overcome this drawback in persistence research, future work should aim at identifying organism specific markers that correlate to growth states in the human host. Until then, the findings here shift the paradigm of toxin-antitoxin research to use a data-driven approach and indicate the utility of approaches that investigate constitutively expressed toxins as major regulators of the persistence phenotype.

REFERENCES

1 Wiersinga, W. J. *et al.* Melioidosis. *Nat Rev Dis Primers* **4**, 17107, doi:10.1038/nrdp.2017.107 (2018).

2 Yabuuchi, E. *et al.* Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251-1275 (1992).

3 Sawana, A., Adeolu, M. & Gupta, R. S. Molecular signatures and phylogenomic analysis of the genus Burkholderia: proposal for division of this genus into the emended genus Burkholderia containing pathogenic organisms and a new genus Paraburkholderia gen. nov. harboring environmental species. *Front Genet* **5**, 429, doi:10.3389/fgene.2014.00429 (2014).

4 Price, E. P. *et al.* Within-host evolution of Burkholderia pseudomallei in four cases of acute melioidosis. *PLoS Pathog* **6**, e1000725, doi:10.1371/journal.ppat.1000725 (2010).

5 Losada, L. *et al.* Continuing evolution of Burkholderia mallei through genome reduction and large-scale rearrangements. *Genome Biol Evol* **2**, 102-116, doi:10.1093/gbe/evq003 (2010).

6 Magill, A. J. & Hunter, G. W. *Hunter's tropical medicine and emerging infectious diseases*. 9th edn, (Saunders, 2013).

7 Majerczyk, C. D. *et al.* Cross-species comparison of the Burkholderia pseudomallei, Burkholderia thailandensis, and Burkholderia mallei quorum-sensing regulons. *J Bacteriol* **196**, 3862-3871, doi:10.1128/JB.01974-14 (2014).

8 Wiersinga, W. J. *et al.* Inflammation patterns induced by different Burkholderia species in mice. *Cell Microbiol* **10**, 81-87, doi:10.1111/j.1462-5822.2007.01016.x (2008).

9 Robertson, J., Levy, A., Sagripanti, J. L. & Inglis, T. J. The survival of Burkholderia pseudomallei in liquid media. *Am J Trop Med Hyg* **82**, 88-94, doi:10.4269/ajtmh.2010.09-0226 (2010).

10 Holden, M. T. *et al.* Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. *Proc Natl Acad Sci U S A* **101**, 14240-14245, doi:10.1073/pnas.0403302101 (2004).

11 Liguori, A. P. *et al.* Diversity of 16S-23S rDNA internal transcribed spacer (ITS) reveals phylogenetic relationships in Burkholderia pseudomallei and its near-neighbors. *PLoS One* **6**, e29323, doi:10.1371/journal.pone.0029323 (2011).

12 De Smet, B. *et al.* Whole-genome sequencing confirms that Burkholderia pseudomallei multilocus sequence types common to both Cambodia and Australia are due to homoplasy. *J Clin Microbiol* **53**, 323-326, doi:10.1128/JCM.02574-14 (2015).

13 Tsang, A. K. L., Lee, H. H., Yiu, S. M., Lau, S. K. P. & Woo, P. C. Y. Failure of phylogeny inferred from multilocus sequence typing to represent bacterial phylogeny. *Sci Rep* **7**, 4536, doi:10.1038/s41598-017-04707-4 (2017).

14 Price, E. P. *et al.* Within-host evolution of Burkholderia pseudomallei over a twelve-year chronic carriage infection. *MBio* **4**, doi:10.1128/mBio.00388-13 (2013).
15 Pearson, T. *et al.* Pathogen to commensal: longitudinal within-host population dynamics, evolution, and adaptation during a chronic >16-year Burkholderia pseudomallei infection. *bioRxiv*, 552109, doi:10.1101/552109 (2019).
16 Nandi, T. *et al.* Burkholderia pseudomallei sequencing identifies genomic clades with distinct recombination, accessory, and epigenetic profiles. *Genome Res* **25**, 608 (2015).
17 Chewapreecha, C. *et al.* Global and regional dissemination and evolution of Burkholderia pseudomallei. *Nat Microbiol* **2**, 16263, doi:10.1038/nmicrobiol.2016.263 (2017).
18 Sarovich, D. S. *et al.* Phylogenomic Analysis Reveals an Asian Origin for African Burkholderia pseudomallei and Further Supports Melioidosis Endemicity in Africa. *mSphere* **1**, doi:10.1128/mSphere.00089-15 (2016).
19 Limmathurotsakul, D. *et al.* Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol* **1**, 1–5 (2016).
20 Sanchez-Villamil, J. I. & Torres, A. G. Melioidosis in Mexico, Central America, and the Caribbean. *Trop Med Infect Dis* **3**, doi:10.3390/tropicalmed3010024 (2018).
21 Rolim, D. B. *et al.* Melioidosis in South America. *Trop Med Infect Dis* **3**, doi:10.3390/tropicalmed3020060 (2018).
22 Control, C. o. D. Centers for Disease Control and Prevention Conclusion of Select Agent Inquiry into Burkholderia pseudomallei release at Tulane National Primate Research Center (Center of Disease Control, 2015).
23 Perumal Samy, R., Stiles, B. G., Sethi, G. & Lim, L. H. K. Melioidosis: Clinical impact and public health threat in the tropics. *PLoS Negl Trop Dis* **11**, e0004738, doi:10.1371/journal.pntd.0004738 (2017).
24 Yee, K. C., Lee, M. K., Chua, C. T. & Puthucheary, S. D. Melioidosis, the great mimicker: a report of 10 cases from Malaysia. *J Trop Med Hyg* **91**, 249-254 (1988).
25 Merritt, A. J. & Inglis, T. J. J. The Role of Climate in the Epidemiology of Melioidosis. *Curr Trop Med Rep* **4**, 185-191, doi:10.1007/s40475-017-0124-4 (2017).
26 Currie, B. J., Fisher, D. A., Anstey, N. M. & Jacups, S. P. Melioidosis: acute and chronic disease, relapse and re-activation. *Trans R Soc Trop Med Hyg* **94**, 301-304, doi:10.1016/s0035-9203(00)90333-x (2000).
27 Fisher, D. A. & Harris, P. N. A. Melioidosis: Refining management of a tropical time bomb. *Lancet* **383**, 762-764 (2014).
28 Wiersinga, W. J. *et al.* Melioidosis. *Nat Rev Dis Prim* **4**, 17107 (2018).
29 Wiersinga, W. J., Currie, B. J. & Peacock, S. J. Melioidosis. *N Engl J Med* **367**, 1035-1044, doi:10.1056/NEJMra1204699 (2012).
30 Chaowagul, W. *et al.* Relapse in Melioidosis: Incidence and Risk Factors. *J Infect Dis* **168**, 1181–1185 (1993).

31 Maharjan, B. *et al.* Recurrent Melioidosis in Patients in Northeast Thailand Is Frequently Due to Reinfection Rather than Relapse. *J Clin Microbiol* **3**, 6032-6034 (2005).
32 Vadivelu, J., Puthucheary, S. D., Drasar, B. S., Dance, D. A. & Pitt, T. L. Stability of strain genotypes of Burkholderia pseudomallei from patients with single and recurrent episodes of melioidosis. *Trop Med Int Health* **3**, 518-521 (1998).
33 Desmarchelier, P. M. *et al.* Relationships among Pseudomonas pseudomallei isolates from patients with recurrent melioidosis. *J Clin Microbiol* **31**, 1592-1596 (1993).
34 Whitmore, A. An Account of a Glanders-like Disease occurring in Rangoon. *J Hyg (Lond)* **13**, 1-34.31, doi:10.1017/s0022172400005234 (1913).
35 Flemma, R. J., DiVincenti, F. C., Dotin, L. N. & Pruitt, B. A. Pulmonary melioidosis; a diagnostic dilemma and increasing threat. *Ann Thorac Surg* **7**, 491-499, doi:10.1016/s0003-4975(10)66386-2 (1969).
36 Currie, B. J. *et al.* The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Trop* **74**, 121-127 (2000).
37 Liu, P. J. *et al.* Induction of mouse melioidosis with meningitis by CD11b+ phagocytic cells harboring intracellular B. pseudomallei as a Trojan horse. *PLoS Negl Trop Dis* **7**, e2363, doi:10.1371/journal.pntd.0002363 (2013).
38 Cheng, A. C. & Currie, B. J. Melioidosis: epidemiology, pathophysiology, and management. *Clin Microbiol Rev* **18**, 383-416, doi:10.1128/CMR.18.2.383-416.2005 (2005).
39 Currie, B. J. *et al.* Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia. *Trop Med Int Health* **9**, 1167-1174, doi:10.1111/j.1365-3156.2004.01328.x (2004).
40 Limmathurotsakul, D. *et al.* Increasing incidence of human melioidosis in Northeast Thailand. *Am J Trop Med Hyg* **82**, 1113-1117, doi:10.4269/ajtmh.2010.10-0038 (2010).
41 Kanaphun, P. *et al.* Serology and carriage of Pseudomonas pseudomallei: a prospective study in 1000 hospitalized children in northeast Thailand. *J Infect Dis* **167**, 230-233, doi:10.1093/infdis/167.1.230 (1993).
42 Vandana, K. E. *et al.* Seroprevalence of Burkholderia pseudomallei among Adults in Coastal Areas in Southwestern India. *PLoS Negl Trop Dis* **10**, e0004610, doi:10.1371/journal.pntd.0004610 (2016).
43 Maude, R. R. *et al.* Seroepidemiological surveillance of Burkholderia pseudomallei in Bangladesh. *Trans R Soc Trop Med Hyg* **106**, 576-578, doi:10.1016/j.trstmh.2012.06.003 (2012).
44 James, G. L. *et al.* Surprisingly low seroprevalence of Burkholderia pseudomallei in exposed healthy adults in the Darwin region of tropical Australia where melioidosis is highly endemic. *Clin Vaccine Immunol* **20**, 759-760, doi:10.1128/CVI.00021-13 (2013).
45 Chow, T. K. *et al.* Incidental Splenic Granuloma Due to Burkholderia pseudomallei: A Case of Asymptomatic Latent Melioidosis? *Am J Trop Med Hyg* **94**, 522-524, doi:10.4269/ajtmh.15-0774 (2016).
46 Hoffmaster, A. R. *et al.* Melioidosis diagnostic workshop, 2013. *Emerg Infect Dis* **21**, doi:10.3201/eid2102.141045 (2015).

47 Houghton, R. L. *et al.* Development of a prototype lateral flow immunoassay (LFI) for the rapid diagnosis of melioidosis. *PLoS Negl Trop Dis* **8**, e2727, doi:10.1371/journal.pntd.0002727 (2014).
48 Duval, B. D. *et al.* Evaluation of a latex agglutination assay for the identification of Burkholderia pseudomallei and Burkholderia mallei. *Am J Trop Med Hyg* **90**, 1043-1046, doi:10.4269/ajtmh.14-0025 (2014).
49 Dharakul, T. *et al.* Diagnostic value of an antibody enzyme-linked immunosorbent assay using affinity-purified antigen in an area endemic for melioidosis. *Am J Trop Med Hyg* **56**, 418-423, doi:10.4269/ajtmh.1997.56.418 (1997).
50 Currie, B. & Anstey, N. *Melioidosis: Epidemiology, clinical manifestations, and diagnosis - UpToDate*, <https://www.uptodate.com/contents/melioidosis-epidemiology-clinical-manifestations-and-diagnosis#H16110041> (2019).
51 Currie, B. J. Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. *Semin Respir Crit Care Med* **36**, 111-125, doi:10.1055/s-0034-1398389 (2015).
52 Sarovich, D. S. *et al.* Development of ceftazidime resistance in an acute *Burkholderia pseudomallei* infection. *Infect Drug Resist* **5**, 129–132 (2012).
53 Schweizer, H. P. Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: Implications for treatment of melioidosis. *Future Microbiol* **7**, 1389-1399 (2012).
54 Wuthiekanun, V. *et al.* Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei. J Antimicrob Chemother* **55**, 1029-1031 (2005).
55 White, N. J. *et al.* Halving of mortality of severe melioidosis by ceftazidime. *Lancet* **2**, 697-701, doi:10.1016/s0140-6736(89)90768-x (1989).
56 Dance, D. Treatment and prophylaxis of melioidosis. *Int J Antimicrob Agents* **43**, 310-318, doi:10.1016/j.ijantimicag.2014.01.005 (2014).
57 Chetchotisakd, P. *et al.* Trimethoprim-sulfamethoxazole versus trimethoprim-sulfamethoxazole plus doxycycline as oral eradicative treatment for melioidosis (MERTH): a multicentre, double-blind, non-inferiority, randomised controlled trial. *Lancet* **383**, 807-814, doi:10.1016/S0140-6736(13)61951-0 (2014).
58 Ross, B. N., Myers, J. N., Muruato, L. A., Tapia, D. & Torres, A. G. Evaluating New Compounds to Treat. *Front Cell Infect Microbiol* **8**, 210, doi:10.3389/fcimb.2018.00210 (2018).
59 Butt, A. *et al.* Burkholderia pseudomallei kynB plays a role in AQ production, biofilm formation, bacterial swarming and persistence. *Res Microbiol* **167**, 159-167, doi:10.1016/j.resmic.2015.11.002 (2016).
60 Ulrich, R. L. *et al.* Role of quorum sensing in the pathogenicity of Burkholderia pseudomallei. *J Med Microbiol* **53**, 1053-1064, doi:10.1099/jmm.0.45661-0 (2004).
61 Whiteley, L. *et al.* Entry, Intracellular Survival, and Multinucleated-Giant-Cell-Forming Activity of Burkholderia pseudomallei in Human Primary Phagocytic and Nonphagocytic Cells. *Infect Immun* **85**, doi:10.1128/IAI.00468-17 (2017).

62 St John, J. A. *et al.* Burkholderia pseudomallei Rapidly Infects the Brain Stem and Spinal Cord via the Trigeminal Nerve after Intranasal Inoculation. *Infect Immun* **84**, 2681-2688, doi:10.1128/IAI.00361-16 (2016).
63 Bearss, J. J. *et al.* Characterization of pathogenesis of and immune response to *Burkholderia pseudomallei* K96243 using both inhalational and intraperitoneal infection models in BALB/c and C57BL/6 mice. *PLoS One* **12**, e0172627 (2017).
64 St John, J. A. Trojan horse L-selectin monocytes: A portal of Burkholderia pseudomallei entry into the brain. *Virulence* **8**, 611-612, doi:10.1080/21505594.2016.1250997 (2017).
65 Hsueh, P. T. *et al.* Burkholderia pseudomallei-loaded cells act as a Trojan horse to invade the brain during endotoxemia. *Sci Rep* **8**, 13632, doi:10.1038/s41598-018-31778-8 (2018).
66 Liu, P. *et al.* Induction of Mouse Melioidosis with Meningitis by CD11b + Phagocytic Cells Harboring Intracellular *B. pseudomallei* as a Trojan Horse 7. *PLoS Negl Trop Dis* **7**, e2363 (2013).
67 Chieng, S., Carreto, L. & Nathan, S. Burkholderia pseudomallei transcriptional adaptation in macrophages. *BMC Genomics* **13**, 328, doi:10.1186/1471-2164-13-328 (2012).
68 Essex-Lopresti, A. E. *et al.* A type IV pilin, PilA, Contributes To Adherence of Burkholderia pseudomallei and virulence in vivo. *Infect Immun* **73**, 1260-1264, doi:10.1128/IAI.73.2.1260-1264.2005 (2005).
69 Chua, K. L., Chan, Y. Y. & Gan, Y. H. Flagella are virulence determinants of Burkholderia pseudomallei. *Infect Immun* **71**, 1622-1629, doi:10.1128/iai.71.4.1622-1629.2003 (2003).
70 Balder, R. *et al.* Identification of Burkholderia mallei and Burkholderia pseudomallei adhesins for human respiratory epithelial cells. *BMC Microbiol* **10**, 250, doi:10.1186/1471-2180-10-250 (2010).
71 Sun, G. W. & Gan, Y. H. Unraveling type III secretion systems in the highly versatile Burkholderia pseudomallei. *Trends Microbiol* **18**, 561-568, doi:10.1016/j.tim.2010.09.002 (2010).
72 Escoll, P. & Buchrieser, C. Metabolic reprogramming of host cells upon bacterial infection: Why shift to a Warburg-like metabolism? *FEBS J* **285**, 2146-2160, doi:10.1111/febs.14446 (2018).
73 Chieng, S., Carreto, L. & Nathan, S. *Burkholderia pseudomallei* transcriptional adaptation in macrophages. *BMC Genomics* **13**, 1–13 (2012).
74 Sun, G. W. *et al.* Identification of a regulatory cascade controlling Type III Secretion System 3 gene expression in Burkholderia pseudomallei. *Mol Microbiol* **76**, 677-689, doi:10.1111/j.1365-2958.2010.07124.x (2010).
75 Ross, B. N., Micheva-Viteva, S., Hong-Geller, E. & Torres, A. G. Evaluating the role of Burkholderia pseudomallei K96243 toxins BPSS0390, BPSS0395, and BPSS1584 in persistent Infection. *Cell Microbiol*, e13096, doi:10.1111/cmi.13096 (2019).
76 Miyagi, K., Kawakami, K. & Saito, A. Role of reactive nitrogen and oxygen intermediates in gamma interferon-stimulated murine macrophage bactericidal activity against Burkholderia pseudomallei. *Infect Immun* **65**, 4108-4113 (1997).

77 Yu, D. *et al.* Evolution of. *Biomed Res Int* **2016**, 6745028, doi:10.1155/2016/6745028 (2016).
78 Cullinane, M. *et al.* Stimulation of autophagy suppresses the intracellular survival of Burkholderia pseudomallei in mammalian cell lines. *Autophagy* **4**, 744-753, doi:10.4161/auto.6246 (2008).
79 Myers, N. D. *et al.* The role of NOD2 in murine and human melioidosis. *J Immunol* **192**, 300-307, doi:10.4049/jimmunol.1301436 (2014).
80 Stevens, M. P. *et al.* Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. *Mol Microbiol* **56**, 40-53, doi:10.1111/j.1365-2958.2004.04528.x (2005).
81 Lazar Adler, N. R. *et al.* The molecular and cellular basis of pathogenesis in melioidosis: How does *Burkholderia pseudomallei* cause disease? . *FEMS Microbiol Rev* **33**, 1079-1099 (2009).
82 Bast, A. *et al.* Caspase-1-dependent and -independent cell death pathways in Burkholderia pseudomallei infection of macrophages. *PLoS Pathog* **10**, e1003986, doi:10.1371/journal.ppat.1003986 (2014).
83 Sun, G. W., Lu, J., Pervaiz, S., Cao, W. P. & Gan, Y. H. Caspase-1 dependent macrophage death induced by Burkholderia pseudomallei. *Cell Microbiol* **7**, 1447-1458, doi:10.1111/j.1462-5822.2005.00569.x (2005).
84 Chien, J. M., Saffari, S. E., Tan, A. L. & Tan, T. T. Factors affecting clinical outcomes in the management of melioidosis in Singapore: a 16-year case series. *BMC Infect Dis* **18**, 482, doi:10.1186/s12879-018-3393-1 (2018).
85 Hobby, G. L. Observations on the mechanism of action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine*, 281–285 (1942).
86 Bigger, J. W. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. **Volume 244**, 497-500, doi:10.1016/S0140-6736(00)74210-3 (1944).
87 Balaban, N. Q. *et al.* Definitions and guidelines for research on antibiotic persistence. *Nat Rev Microbiol*, doi:10.1038/s41579-019-0196-3 (2019).
88 Lewis, K. Persister cells. *Annu Rev Microbiol* **64**, 357-372, doi:10.1146/annurev.micro.112408.134306 (2010).
89 Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622-1625, doi:10.1126/science.1099390 (2004).
90 Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* **13**, 42-51, doi:10.1038/nrmicro3380 (2015).
91 Wolfson, J. S., Hooper, D. C., McHugh, G. L., Bozza, M. A. & Swartz, M. N. Mutants of Escherichia coli K-12 exhibiting reduced killing by both quinolone and beta-lactam antimicrobial agents. *Antimicrob Agents Chemother* **34**, 1938-1943, doi:10.1128/aac.34.10.1938 (1990).
92 Andersson, D. I., Nicoloff, H. & Hjort, K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat Rev Microbiol* **17**, 479-496, doi:10.1038/s41579-019-0218-1 (2019).

93 El-Halfawy, O. M. & Valvano, M. A. Antimicrobial heteroresistance: an emerging field in need of clarity. *Clin Microbiol Rev* **28**, 191-207, doi:10.1128/CMR.00058-14 (2015).
94 Nicoloff, H., Hjort, K., Levin, B. R. & Andersson, D. I. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. *Nat Microbiol* **4**, 504-514, doi:10.1038/s41564-018-0342-0 (2019).
95 Superti, S. V. *et al.* Indications of carbapenem resistance evolution through heteroresistance as an intermediate stage in Acinetobacter baumannii after carbapenem administration. *Rev Inst Med Trop Sao Paulo* **51**, 111-113 (2009).
96 Hughes, D. & Andersson, D. I. Environmental and genetic modulation of the phenotypic expression of antibiotic resistance. *FEMS Microbiol Rev* **41**, 374-391, doi:10.1093/femsre/fux004 (2017).
97 Lewis, K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48-56, doi:10.1038/nrmicro1557 (2007).
98 Rotem, E. *et al.* Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *Proc Natl Acad Sci U S A* **107**, 12541-12546, doi:10.1073/pnas.1004333107 (2010).
99 Handwerger, S. & Tomasz, A. Antibiotic tolerance among clinical isolates of bacteria. *Rev Infect Dis* **7**, 368-386 (1985).
100 Gefen, O., Chekol, B., Strahilevitz, J. & Balaban, N. Q. TDtest: easy detection of bacterial tolerance and persistence in clinical isolates by a modified disk-diffusion assay. *Sci Rep* **7**, 41284, doi:10.1038/srep41284 (2017).
101 Fisher, R. A., Gollan, B. & Helaine, S. Persistent bacterial infections and persister cells. *Nat Rev Microbiol* **15**, 453-464, doi:10.1038/nrmicro.2017.42 (2017).
102 Helaine, S. *et al.* Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. . *Science* **343**, 204-208 (2014).
103 Defraine, V., Fauvart, M. & Michiels, J. Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics. *Drug Resist Updat* **38**, 12-26, doi:10.1016/j.drup.2018.03.002 (2018).
104 Lewis, E. R. & Torres, A. G. The art of persistence-the secrets to Burkholderia chronic infections. *Pathog Dis* **74**, doi:10.1093/femspd/ftw070 (2016).
105 Gunn, J. S. *et al.* Salmonella chronic carriage: epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol* **22**, 648-655, doi:10.1016/j.tim.2014.06.007 (2014).
106 Mulcahy, L. R., Burns, J. L., Lory, S. & Lewis, K. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol* **192**, 6191-6199, doi:10.1128/JB.01651-09 (2010).
107 Fauvart, M., De Groote, V. N. & Michiels, J. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. *J Med Microbiol* **60**, 699-709, doi:10.1099/jmm.0.030932-0 (2011).
108 Fung, D. K., Chan, E. W., Chin, M. L. & Chan, R. C. Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. *Antimicrob Agents Chemother* **54**, 1082-1093, doi:10.1128/AAC.01218-09 (2010).
109 Wallis, R. S. *et al.* Drug tolerance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* **43**, 2600-2606 (1999).

110 Harrison, F. Microbial ecology of the cystic fibrosis lung. *Microbiology* **153**, 917-923, doi:10.1099/mic.0.2006/004077-0 (2007).
111 Bloom, B. R. & Murray, C. J. Tuberculosis: commentary on a reemergent killer. *Science* **257**, 1055-1064, doi:10.1126/science.257.5073.1055 (1992).
112 Michiels, J. E., Van den Bergh, B., Verstraeten, N. & Michiels, J. Molecular mechanisms and clinical implications of bacterial persistence. *Drug Resist Updat* **29**, 76-89, doi:10.1016/j.drup.2016.10.002 (2016).
113 Cohen, N. R., Lobritz, M. A. & Collins, J. J. Microbial persistence and the road to drug resistance. *Cell Host Microbe* **13**, 632-642, doi:10.1016/j.chom.2013.05.009 (2013).
114 Potrykus, K. & Cashel, M. (p)ppGpp: still magical? *Annu Rev Microbiol* **62**, 35-51, doi:10.1146/annurev.micro.62.081307.162903 (2008).
115 Petrosino, J. F., Galhardo, R. S., Morales, L. D. & Rosenberg, S. M. Stress-induced beta-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the Escherichia coli chromosome. *J Bacteriol* **191**, 5881-5889, doi:10.1128/JB.00732-09 (2009).
116 Nguyen, D. *et al.* Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* **334**, 982-986, doi:10.1126/science.1211037 (2011).
117 Dörr, T., Lewis, K. & Vulić, M. SOS response induces persistence to fluoroquinolones in Escherichia coli. *PLoS Genet* **5**, e1000760, doi:10.1371/journal.pgen.1000760 (2009).
118 Cirz, R. T. *et al.* Complete and SOS-mediated response of Staphylococcus aureus to the antibiotic ciprofloxacin. *J Bacteriol* **189**, 531-539, doi:10.1128/JB.01464-06 (2007).
119 Beaber, J. W., Hochhut, B. & Waldor, M. K. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**, 72-74, doi:10.1038/nature02241 (2004).
120 Levin-Reisman, I. *et al.* Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**, 826-830, doi:10.1126/science.aaj2191 (2017).
121 Windels, E. M. *et al.* Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *ISME J* **13**, 1239-1251, doi:10.1038/s41396-019-0344-9 (2019).
122 Thorpe, K. E., Joski, P. & Johnston, K. J. Antibiotic-Resistant Infection Treatment Costs Have Doubled Since 2002, Now Exceeding $2 Billion Annually. *Health Aff (Millwood)* **37**, 662-669, doi:10.1377/hlthaff.2017.1153 (2018).
123 Wood, T. K., Knabel, S. J. & Kwan, B. W. Bacterial persister cell formation and dormancy. *Appl Environ Microbiol* **79**, 7116-7121, doi:10.1128/AEM.02636-13 (2013).
124 Boidin, C., Moshiri, P., Dahyot-Fizelier, C., Goutelle, S. & Lefeuvre, S. Pharmacokinetic variability of beta-lactams in critically ill patients: a narrative review. *Anaesth Crit Care Pain Med*, doi:10.1016/j.accpm.2019.07.016 (2019).
125 Commons, C. *Attribution 4.0 International (CC BY 4.0)*, <https://creativecommons.org/licenses/by/4.0/> (2017).
126 Amato, S. M. *et al.* The role of metabolism in bacterial persistence. *Front Microbiol* **5**, 1-9 (2014).

127 Kohanski, M. A., Dwyer, D. J. & Collins, J. J. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* **8**, 423-435, doi:10.1038/nrmicro2333 (2010).
128 Tanouchi, Y., Lee, A. J., Meredith, H. & You, L. Programmed cell death in bacteria and implications for antibiotic therapy. *Trends Microbiol* **21**, 265-270, doi:10.1016/j.tim.2013.04.001 (2013).
129 Manina, G., Dhar, N. & McKinney, J. D. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* **17**, 32-46, doi:10.1016/j.chom.2014.11.016 (2015).
130 Vega, N. M., Allison, K. R., Khalil, A. S. & Collins, J. J. Signaling-mediated bacterial persister formation. *Nat Chem Biol* **8**, 431-433, doi:10.1038/nchembio.915 (2012).
131 Gutierrez, A. *et al.* Understanding and Sensitizing Density-Dependent Persistence to Quinolone Antibiotics. *Mol Cell* **68**, 1147-1154.e1143, doi:10.1016/j.molcel.2017.11.012 (2017).
132 Stocks, C. J., Schembri, M. A., Sweet, M. J. & Kapetanovic, R. For when bacterial infections persist: Toll-like receptor-inducible direct antimicrobial pathways in macrophages. *J Leukoc Biol* **103**, 35-51, doi:10.1002/JLB.4RI0917-358R (2018).
133 Andino, R. & Domingo, E. Viral quasispecies. *Virology* **479-480**, 46-51, doi:10.1016/j.virol.2015.03.022 (2015).
134 Kussell, E., Kishony, R., Balaban, N. Q. & Leibler, S. Bacterial persistence: a model of survival in changing environments. *Genetics* **169**, 1807-1814, doi:10.1534/genetics.104.035352 (2005).
135 Domingo, E., Sheldon, J. & Perales, C. Viral quasispecies evolution. *Microbiol Mol Biol Rev* **76**, 159-216, doi:10.1128/MMBR.05023-11 (2012).
136 Amato, S. M., Orman, M. A. & Brynildsen, M. P. Metabolic control of persister formation in Escherichia coli. *Mol Cell* **50**, 475-487, doi:10.1016/j.molcel.2013.04.002 (2013).
137 Khakimova, M., Ahlgren, H. G., Harrison, J. J., English, A. M. & Nguyen, D. The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. *J Bacteriol* **195**, 2011-2020, doi:10.1128/JB.02061-12 (2013).
138 Van den Bergh, B. *et al.* Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. *Nat Microbiol* **1**, 16020, doi:10.1038/nmicrobiol.2016.20 (2016).
139 Pu, Y. *et al.* Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Mol Cell* **62**, 284-294, doi:10.1016/j.molcel.2016.03.035 (2016).
140 Möker, N., Dean, C. R. & Tao, J. Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *J Bacteriol* **192**, 1946-1955, doi:10.1128/JB.01231-09 (2010).
141 Orman, M. A. & Brynildsen, M. P. Dormancy is not necessary or sufficient for bacterial persistence. *Antimicrob Agents Chemother* **57**, 3230-3239, doi:10.1128/AAC.00243-13 (2013).

142 Ronneau, S. & Hallez, R. Make and break the alarmone: regulation of (p)ppGpp synthetase/hydrolase enzymes in bacteria. *FEMS Microbiol Rev* **43**, 389-400, doi:10.1093/femsre/fuz009 (2019).
143 Salvioli di Fossalunga, A., Lipuma, J., Venice, F., Dupont, L. & Bonfante, P. The endobacterium of an arbuscular mycorrhizal fungus modulates the expression of its toxin-antitoxin systems during the life cycle of its host. *ISME J* **11**, 2394-2398, doi:10.1038/ismej.2017.84 (2017).
144 Pandey, D. P. & Gerdes, K. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res* **33**, 966-976, doi:10.1093/nar/gki201 (2005).
145 Ogura, T. & Hiraga, S. Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci U S A* **80**, 4784-4788, doi:10.1073/pnas.80.15.4784 (1983).
146 Unterholzner, S. J., Poppenberger, B. & Rozhon, W. Toxin-antitoxin systems. *Bioengineered* **5**, 1–13 (2014).
147 Wen, Y., Behiels, E. & Devreese, B. Toxin-Antitoxin systems: Their role in persistence, biofilm formation, and pathogenicity. *Pathog Dis* **70**, 240-249 (2014).
148 Hayes, F. & Van Melderen, L. Toxins-antitoxins: diversity, evolution and function. *Crit Rev Biochem Mol Biol* **46**, 386-408, doi:10.3109/10409238.2011.600437 (2011).
149 Leplae, R. *et al.* Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. *Nucleic Acids Res* **39**, 5513-5525, doi:10.1093/nar/gkr131 (2011).
150 Harms, A., Brodersen, D. E., Mitarai, N. & Gerdes, K. Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Mol Cell* **70**, 768-784, doi:10.1016/j.molcel.2018.01.003 (2018).
151 Aakre, C. D., Phung, T. N., Huang, D. & Laub, M. T. A bacterial toxin inhibits DNA replication elongation through a direct interaction with the β sliding clamp. *Mol Cell* **52**, 617-628, doi:10.1016/j.molcel.2013.10.014 (2013).
152 Deter, H. S., Jensen, R. V., Mather, W. H. & Butzin, N. C. Mechanisms for Differential Protein Production in Toxin-Antitoxin Systems. *Toxins (Basel)* **9**, doi:10.3390/toxins9070211 (2017).
153 Unterholzner, S. J., Poppenberger, B. & Rozhon, W. Toxin-antitoxin systems: Biology, identification, and application. *Mob Genet Elements* **3**, e26219, doi:10.4161/mge.26219 (2013).
154 Page, R. & Peti, W. Toxin-antitoxin systems in bacterial growth arrest and persistence. *Nat Chem Biol* **12**, 208-214, doi:10.1038/nchembio.2044 (2016).
155 Kang, S. M., Kim, D. H., Jin, C. & Lee, B. J. A Systematic Overview of Type II and III Toxin-Antitoxin Systems with a Focus on Druggability. *Toxins (Basel)* **10**, doi:10.3390/toxins10120515 (2018).
156 Fauvart, M., de Groote, V. N. & Michiels, J. Role of persister cells in chronic infections: Clinical relevance and perspectives on anti-persister therapies. *J Med Microbiol* **60**, 699-709 (2011).
157 Kyme, P. *et al.* C/EBPε mediates nicotinamide-enhanced clearance of Staphylococcus aureus in mice. *J Clin Invest* **122**, 3316-3329, doi:10.1172/JCI62070 (2012).

158 Bettenworth, D. *et al.* Nicotinamide treatment ameliorates the course of experimental colitis mediated by enhanced neutrophil-specific antibacterial clearance. *Mol Nutr Food Res* **58**, 1474-1490, doi:10.1002/mnfr.201300818 (2014).
159 Micheva-Viteva, S. N. *et al.* Increased Mortality in Mice following Immunoprophylaxis Therapy with High Dosage of Nicotinamide in. *Infect Immun* **87**, doi:10.1128/IAI.00592-18 (2019).
160 Ghaly, S. *et al.* High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis. *Sci Rep* **8**, 11511, doi:10.1038/s41598-018-29759-y (2018).
161 Butt, A. *et al.* The HicA toxin from *Burkholderia pseudomallei* has a role in persister cell formation. *Biochem J* **459**, 333–344 (2014).
162 Ramage, H. R., Connolly, L. E. & Cox, J. S. Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS Genet* **5**, e1000767, doi:10.1371/journal.pgen.1000767 (2009).
163 Muthuramalingam, M., White, J. C. & Bourne, C. R. Toxin-Antitoxin Modules Are Pliable Switches Activated by Multiple Protease Pathways. *Toxins (Basel)* **8**, doi:10.3390/toxins8070214 (2016).
164 Kolodkin-Gal, I., Verdiger, R., Shlosberg-Fedida, A. & Engelberg-Kulka, H. A differential effect of E. coli toxin-antitoxin systems on cell death in liquid media and biofilm formation. *PLoS One* **4**, e6785, doi:10.1371/journal.pone.0006785 (2009).
165 Pumpuang, A. *et al.* Survival of Burkholderia pseudomallei in distilled water for 16 years. *Trans R Soc Trop Med Hyg* **105**, 598-600, doi:10.1016/j.trstmh.2011.06.004 (2011).
166 Limmathurotsakul, D. *et al.* Melioidosis caused by Burkholderia pseudomallei in drinking water, Thailand, 2012. *Emerg Infect Dis* **20**, 265-268, doi:10.3201/eid2002.121891 (2014).
167 Chodimella, U., Hoppes, W. L., Whalen, S., Ognibene, A. J. & Rutecki, G. W. Septicemia and suppuration in a Vietnam veteran. *Hosp Pract (1995)* **32**, 219-221 (1997).
168 Gee, J. E. *et al.* Phylogeography of Burkholderia pseudomallei Isolates, Western Hemisphere. *Emerg Infect Dis* **23**, 1133-1138, doi:10.3201/eid2307.161978 (2017).
169 Gan, Y. H. Interaction between Burkholderia pseudomallei and the host immune response: sleeping with the enemy? *J Infect Dis* **192**, 1845-1850, doi:10.1086/497382 (2005).
170 Butt, A., Müller, C., Harmer, N. & Titball, R. W. Identification of type II toxin-antitoxin modules in *Burkholderia pseudomallei. FEMS Microbiol Lett* **338**, 86–94 (2013).
171 Nierman, W. C., Yu, Y. & Losada, L. The In vitro Antibiotic Tolerant Persister Population in Burkholderia pseudomallei is Altered by Environmental Factors. *Front Microbiol* **6**, 1338, doi:10.3389/fmicb.2015.01338 (2015).
172 van Schaik, E. J., Tom, M. & Woods, D. E. Burkholderia pseudomallei isocitrate lyase is a persistence factor in pulmonary melioidosis: implications for the

development of isocitrate lyase inhibitors as novel antimicrobials. *Infect Immun* **77**, 4275-4283, doi:10.1128/IAI.00609-09 (2009).
173 Hamad, M. A. *et al.* Adaptation and antibiotic tolerance of anaerobic Burkholderia pseudomallei. *Antimicrob Agents Chemother* **55**, 3313-3323, doi:10.1128/AAC.00953-10 (2011).
174 Jones-Carson, J. *et al.* Nitric oxide from IFNγ-primed macrophages modulates the antimicrobial activity of β-lactams against the intracellular pathogens Burkholderia pseudomallei and Nontyphoidal Salmonella. *PLoS Negl Trop Dis* **8**, e3079, doi:10.1371/journal.pntd.0003079 (2014).
175 Sevin, E. W. & Barloy-Hubler, F. RASTA-Bacteria: a web-based tool for identifying toxin-antitoxin loci in prokaryotes. *Genome Biol* **8**, R155, doi:10.1186/gb-2007-8-8-r155 (2007).
176 Ooi, W. F. *et al.* The condition-dependent transcriptional landscape of Burkholderia pseudomallei. *PLoS Genet* **9**, e1003795, doi:10.1371/journal.pgen.1003795 (2013).
177 Hamad, M. A., Zajdowicz, S. L., Holmes, R. K. & Voskuil, M. I. An allelic exchange system for compliant genetic manipulation of the select agents Burkholderia pseudomallei and Burkholderia mallei. *Gene* **430**, 123-131, doi:10.1016/j.gene.2008.10.011 (2009).
178 Ramli, N. S. K., Eng Guan, C., Nathan, S. & Vadivelu, J. The Effect of Environmental Conditions on Biofilm Formation of *Burkholderia pseudomallei* Clinical Isolates. *PLoS One* **7**, e44104 (2012).
179 Williams, N. L., Morris, J. L., Rush, C. M. & Ketheesan, N. Migration of dendritic cells facilitates systemic dissemination of *Burkholderia pseudomallei*. *Infect Immun* **82**, 4233-4240 (2014).
180 Cheng, J. W. *et al. Burkholderia pseudomallei* Infection in US Traveler Returning from Mexico, 2014. *Emerg Infect Dis* **21**, 1884-1885 (2015).
181 Dance, D. Treatment and prophylaxis of melioidosis. *Int J Antimicrob Agents* **43**, 310-318 (2014).
182 Estes, D. M., Dow, S. W., Schweizer, H. P. & Torres, A. G. Present and future therapeutic strategies for melioidosis and glanders. *Expert Rev Anti Infect Ther* **8**, 325-338 (2010).
183 Michot, J. M., Seral, C., Van Bambeke, F., Mingeot-Leclercq, M. P. & Tulkens, P. M. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob Agents Chemother* **49**, 2429-2437 (2005).
184 Sberro, H. *et al.* Discovery of functional toxin/antitoxin systems in bacteria by shotgun cloning. *Mol Cell* **50**, 136-148, doi:10.1016/j.molcel.2013.02.002 (2013).
185 Hayes, F. A family of stability determinants in pathogenic bacteria. *J Bacteriol* **180**, 6415-6418 (1998).
186 Akarsu, H. *et al.* TASmania: A bacterial Toxin-Antitoxin Systems database. *PLoS Comput Biol* **15**, e1006946, doi:10.1371/journal.pcbi.1006946 (2019).
187 Shao, Y. *et al.* TADB: a web-based resource for Type 2 toxin-antitoxin loci in bacteria and archaea. *Nucleic Acids Res* **39**, D606-611, doi:10.1093/nar/gkq908 (2011).

188 Xie, Y. *et al.* TADB 2.0: an updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Res* **46**, D749-D753, doi:10.1093/nar/gkx1033 (2018).
189 Butt, A. T. *Identification and characterisation of toxin-antitoxin systems (TA) in Burkholderia pseudomallei.* PhD thesis, University of Exeter, (2013).
190 Ramage, H. R., Connolly, L. E. & Cox, J. S. Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS Genet* **5**, e1000767 (2009).
191 Srinon, V., Chaiwattanarungruengpaisan, S., Korbsrisate, S. & Stevens, J. M. BimC Is Required for Actin-Based Motility, Intracellular Survival, and Virulence. *Front Cell Infect Microbiol* **9**, 63, doi:10.3389/fcimb.2019.00063 (2019).
192 Burtnick, M. N. *et al.* The cluster 1 type VI secretion system is a major virulence determinant in Burkholderia pseudomallei. *Infect Immun* **79**, 1512-1525, doi:10.1128/IAI.01218-10 (2011).
193 Burtnick, M. N. & Brett, P. J. Burkholderia mallei and Burkholderia pseudomallei cluster 1 type VI secretion system gene expression is negatively regulated by iron and zinc. *PLoS One* **8**, e76767, doi:10.1371/journal.pone.0076767 (2013).
194 Scoffone, V. C. *et al.* Infections in Cystic Fibrosis Patients: Drug Resistance and Therapeutic Approaches. *Front Microbiol* **8**, 1592, doi:10.3389/fmicb.2017.01592 (2017).
195 Jørgensen, M. G., Pandey, D. P., Jaskolska, M. & Gerdes, K. HicA of Escherichia coli defines a novel family of translation-independent mRNA interferases in bacteria and archaea. *J Bacteriol* **191**, 1191-1199, doi:10.1128/JB.01013-08 (2009).
196 Germain, E., Castro-Roa, D., Zenkin, N. & Gerdes, K. Molecular mechanism of bacterial persistence by HipA. *Mol Cell* **52**, 248-254, doi:10.1016/j.molcel.2013.08.045 (2013).
197 Heaton, B. E., Herrou, J., Blackwell, A. E., Wysocki, V. H. & Crosson, S. Molecular structure and function of the novel BrnT/BrnA toxin-antitoxin system of Brucella abortus. *J Biol Chem* **287**, 12098-12110, doi:10.1074/jbc.M111.332163 (2012).
198 Allwood, E. M., Devenish, R. J., Prescott, M., Adler, B. & Boyce, J. D. Strategies for intracellular survival of *Burkholderia pseudomallei*. *Front Microbiol* **2**, 170 (2011).
199 Willcocks, S. J., Denman, C. C., Atkins, H. S. & Wren, B. W. Intracellular replication of the well-armed pathogen *Burkholderia pseudomallei*. *Curr Opin Microbiol* **29**, 94–103 (2016).
200 Aguilar, C., Schmid, N., Lardi, M., Pessi, G. & Eberl, L. The IclR-family regulator BapR controls biofilm formation in *B. cenocepacia* H111. *PLoS One* **9**, e92920 (2014).
201 Butt, A. *et al. Burkholderia pseudomallei kynB* plays a role in AQ production, biofilm formation, bacterial swarming and persistence. *Res Microbiol* **167**, 159-167 (2016).
202 Butler, M. T., Wang, Q. & Harshey, R. M. Cell density and mobility protect swarming bacteria against antibiotics. *Proc Natl Acad Sci U S A.* **107**, 3776-3781 (2010).

203 Harshey, R. M. & Partridge, J. D. Shelter in a Swarm. *J Mol Biol* **427**, 3683-3694 (2015).
204 Kim, W., Killam, T., Sood, V. & Surette, M. G. Swarm-cell differentiation in *Salmonella enterica* serovar typhimurium results in elevated resistance to multiple antibiotics. *J Bacteriol* **185**, 3111-3117 (2003).
205 Lai, S., Tremblay, J. & Déziel, E. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* **11**, 126-136 (2009).
206 Brett, P. J. & Woods, D. E. Pathogenesis of and immunity to melioidosis. *Acta Trop* **74**, 201-210 (2000).
207 Lever, M. S., Nelson, M., Stagg, A. J., Beedham, R. J. & Simpson, A. J. H. Experimental acute respiratory *Burkholderia pseudomallei* infection in BALB/c mice. *Int J Exp Pathol.* **90**, 16-25 (2009).
208 Jones-Carson, J. *et al.* Nitric oxide from IFNγ-primed macrophages modulates the antimicrobial activity of β-lactams against the intracellular pathogens *Burkholderia pseudomallei* and Nontyphoidal *Salmonella*. *PLoS Negl Trop Dis* **8**, e3079 (2014).
209 Brown, D. R. Nitrogen Starvation Induces Persister Cell Formation in *Escherichia coli. J Bacteriol* **201**, pii: e00622-00618 (2019).
210 Hamad, M. A. *et al.* Adaptation and antibiotic tolerance of anaerobic *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **55**, 3313-3323 (2011).
211 Zhang, Y. Persisters, persistent infections and the Yin-Yang model. *Emerg Microbes Infect* **3**, e3 (2014).
212 Kolodkin-Gal, I., Verdiger, R., Shlosberg-Fedida, A. & Engelberg-Kulka, H. A differential effect of *E. coli* toxin-antitoxin systems on cell death in liquid media and biofilm formation. *PLoS One* **4**, e6785 (2009).
213 Muthuramalingam, M., White, J. C. & Bourne, C. R. Toxin-Antitoxin Modules Are Pliable Switches Activated by Multiple Protease Pathways. *Toxins (Basel)* **8**, pii: E214 (2016).
214 Liu, M., Zhang, Y., Inouye, M. & Woychik, N. A. Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. *Proc Natl Acad Sci U S A* **105**, 5885-5890, doi:10.1073/pnas.0711949105 (2008).
215 Zhang, Y., Zhang, J., Hara, H., Kato, I. & Inouye, M. Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase. *J Biol Chem* **280**, 3143-3150, doi:10.1074/jbc.M411811200 (2005).
216 Amitai, S., Yassin, Y. & Engelberg-Kulka, H. MazF-mediated cell death in Escherichia coli: a point of no return. *J Bacteriol* **186**, 8295-8300, doi:10.1128/JB.186.24.8295-8300.2004 (2004).
217 Trastoy, R. *et al.* Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments. *Clin Microbiol Rev* **31**, doi:10.1128/CMR.00023-18 (2018).
218 Curtis, T. D., Gram, L. & Knudsen, G. M. The Small Colony Variant of Listeria monocytogenes Is More Tolerant to Antibiotics and Has Altered Survival in RAW 264.7 Murine Macrophages. *Front Microbiol* **7**, 1056, doi:10.3389/fmicb.2016.01056 (2016).

219 Kim, N. H. *et al.* Small-Colony Variants in Persistent and Recurrent Staphylococcus aureus Bacteremia. *Microb Drug Resist* **22**, 538-544, doi:10.1089/mdr.2015.0262 (2016).
220 See, J. X. *et al.* Experimental Persistent Infection of BALB/c Mice with Small-Colony Variants of Burkholderia pseudomallei Leads to Concurrent Upregulation of PD-1 on T Cells and Skewed Th1 and Th17 Responses. *PLoS Negl Trop Dis* **10**, e0004503, doi:10.1371/journal.pntd.0004503 (2016).
221 Tuchscherr, L. *et al.* Staphylococcus aureus develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J Antimicrob Chemother* **71**, 438-448, doi:10.1093/jac/dkv371 (2016).
222 Anutrakunchai, C. *et al.* Impact of nutritional stress on drug susceptibility and biofilm structures of Burkholderia pseudomallei and Burkholderia thailandensis grown in static and microfluidic systems. *PLoS One* **13**, e0194946, doi:10.1371/journal.pone.0194946 (2018).
223 Cabral, D. J., Wurster, J. I. & Belenky, P. Antibiotic Persistence as a Metabolic Adaptation: Stress, Metabolism, the Host, and New Directions. *Pharmaceuticals (Basel)* **11**, doi:10.3390/ph11010014 (2018).
224 Son, M. S., Matthews, W. J., Kang, Y., Nguyen, D. T. & Hoang, T. T. In vivo evidence of Pseudomonas aeruginosa nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* **75**, 5313-5324, doi:10.1128/IAI.01807-06 (2007).
225 van Delden, C., Page, M. G. & Köhler, T. Involvement of Fe uptake systems and AmpC β-lactamase in susceptibility to the siderophore monosulfactam BAL30072 in Pseudomonas aeruginosa. *Antimicrob Agents Chemother* **57**, 2095-2102, doi:10.1128/AAC.02474-12 (2013).
226 Fuchs, S., Pané-Farré, J., Kohler, C., Hecker, M. & Engelmann, S. Anaerobic gene expression in Staphylococcus aureus. *J Bacteriol* **189**, 4275-4289, doi:10.1128/JB.00081-07 (2007).
227 Cullen, L. *et al.* The involvement of the low-oxygen-activated locus of Burkholderia cenocepacia in adaptation during cystic fibrosis infection. *Sci Rep* **8**, 13386, doi:10.1038/s41598-018-31556-6 (2018).
228 Li, Z., Ma, Z., Hao, X., Rensing, C. & Wei, G. Genes conferring copper resistance in Sinorhizobium meliloti CCNWSX0020 also promote the growth of Medicago lupulina in copper-contaminated soil. *Appl Environ Microbiol* **80**, 1961-1971, doi:10.1128/AEM.03381-13 (2014).
229 Williams, C. L. *et al.* Copper Resistance of the Emerging Pathogen Acinetobacter baumannii. *Appl Environ Microbiol* **82**, 6174-6188, doi:10.1128/AEM.01813-16 (2016).
230 Vallette, F. M. *et al.* Dormant, quiescent, tolerant and persister cells: Four synonyms for the same target in cancer. *Biochem Pharmacol* **162**, 169-176, doi:10.1016/j.bcp.2018.11.004 (2019).
231 Wuyts, J., Van Dijck, P. & Holtappels, M. Fungal persister cells: The basis for recalcitrant infections? *PLoS Pathog* **14**, e1007301, doi:10.1371/journal.ppat.1007301 (2018).

232 Georgiades, K. & Raoult, D. Genomes of the most dangerous epidemic bacteria have a virulence repertoire characterized by fewer genes but more toxin-antitoxin modules. *PLoS One* **6**, e17962, doi:10.1371/journal.pone.0017962 (2011).
233 Ronneau, S. & Helaine, S. Clarifying the Link between Toxin-Antitoxin Modules and Bacterial Persistence. *J Mol Biol* **431**, 3462-3471, doi:10.1016/j.jmb.2019.03.019 (2019).
234 Pontes, M. H. & Groisman, E. A. Slow growth determines nonheritable antibiotic resistance in. *Sci Signal* **12**, doi:10.1126/scisignal.aax3938 (2019).

Vita

Brittany Nicole Ross was born in Riverside, California to Richard and Terri Ross on December 10th, 1989. Although Brittany showed passion and intense interest in the sciences from an early age, it was not until high school where her curiously was fostered

by the young and influential science teacher, Mrs. Mitchell. From there, Brittany decided to pursue a career in Science. She attended the University of California Riverside (UCR) where she pursed a bachelor's in science. Choosing the only degree that permitted her to take all the classes that piqued her interest, Brittany completed her degree majoring in Biological Science with an emphasis on Microbiology. During her time at UCR, Brittany's path became clear after volunteering in a basic research lab. After graduation she took a position working as a research associate for Dr. David Lo. Allowing her to work on an independent project Dr. Lo fostered Brittany intellectually. The focus of her studies was working on understanding the transcytosis pathways of microbes such as *Salmonella enterica, E. coli, T. gondeii*, and *S. aureus* from the gut lumen to the underlying lamina propria. Contrary to popular belief, they found that microbes are released into the lamina propria in microvesicles where they are taken up by underlying dendritic cells. At this time Brittany found her true calling and strayed from medicine to pursue a life of continual learning as an academic scientist.

Transitioning to focus on the bacterial side of host-pathogen interaction, Brittany joined Dr. Alfredo Torres laboratory in University of Texas Medical Branch as a research associate, while applying to graduate school. There she worked on an independent project investigating the role of long polar fimbriae in intestinal adhesion of the outbreak strain of *E. coli*O104: H4 and assisted on a handful of other projects on *Burkholderia pseudomallei*. Upon entering graduate school at UTMB, Dr. Torres offered a gamete of projects that led Brittany to pursue a PhD under his guidance and produced the dissertation above. To date, Brittany has ten publications and three in preparation for submission.

Having a great appreciation for the ability of bacteria to overcome environmental stresses by going "persistent", Brittany plan to take a post-doctoral fellowship with Dr. Marvin Whiteley, whose lab is at the cutting edge of understanding population heterogeneity of bacterial growth in the lung of those with

cystic fibrosis lung. While in graduate school Brittany's personal life has also flourished. She fell in love with fellow science enthusiast, and graduate student, Sergio Rodriguez for whom she married in December 2018. They are the proud parents of two adorable Boston terriers, Watson and Crick.

Permanent address: 713 Church St, Galveston, Texas, USA 77550

This dissertation was typed by Brittany's Fingers

Figures used with permission as denoted in legends or are original works by Brittany.