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Investigating Putative Toxin-Immunity Pairs from a New Class of RHS Proteins in Pseudomonas Aeruginosa

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INVESTIGATING PUTATIVE TOXIN-IMMUNITY PAIRS FROM A NEW CLASS OF RHS PROTEINS IN *PSEUDOMONAS AERUGINOSA*

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LIST OF ABBREVIATIONS

MDR	Multidrug-resistant
T/I	Toxin/Immunity
LB	Luria Broth
PCR	Polymerase Chain Reaction
RPM	Revolutions Per Minute
RHS	Rearrangement Hot Spot
HA-tag	Hemagglutinin tag
His-tag	Histidine tag
DTT	Dithiothreitol
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
NBT	Nitro Blue Tetrazolium
BCIP	5-bromo-4-chloro-3-indoyl phosphate
ICU	Intensive Care Unit
VAP	Ventilator-Associated Pneumonia

CAUTI	Catheter-Associated Urinary Tract Infections
SSI	Surgical Site Infection
SM	Specialized Metabolite
CDI	Contact Dependent growth Inhibition
T6SS	Type 6 Secretion System
T5SS	Type 5 Secretion System
ORF	Open Reading Frame
WCL	Whole Cell Lysate
Co-IP	Co-immunoprecipitation

ABSTRACT

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that is found in ubiquity throughout nature. This is due, in part, to the arsenal of toxins and proteins encoded in the genome of the organism which aid in molding its environment to suit its needs. Based on bioinformatic analysis, we may have identified a novel locus in the *P. aeruginosa* genome that may encode a toxin/immunity system that mediates interbacterial competition. This thesis takes biochemical approaches to assess the interactions between the putative toxin and immunity proteins which are expected to occur amongst this class of proteins.

Bioinformatic analysis performed by Dr. Allen suggests that the locus encodes a set of proteins that are enriched in the rearrangement hotspot (RHS) motif. The locus is composed of three ORFs which we ad-interim termed *rhsB*, *rhsC*, and *rhsI*. Structural analysis by Alpha Fold suggests that *rhsBC* encodes two halves of a protein, forming a hollow shell-like structure. The shell encapsulates a putative effector domain present at the 3' end of *rhsC* which covaries with *rhsI*. We hypothesize that the 3' end of *rhsC* and *rhsI* encode toxin/immunity pairs that mediate interbacterial competition. A current lab member, Abigail Banas, has performed growth curves in *E. coli* carrying the putative toxin/immunity alleles on an inducible promoter. These growth curves have revealed variable toxicity dependent on the expressed allele. The growth defect induced by a toxin is relieved in the presence of the corresponding immunity factor. These data led me to hypothesize that the immunity factor physically interacts with the toxin to neutralize its activity which consequently recovers the growth defect. To determine if this was

true, I performed Co-IPs of whole cell lysates from cell cultures expressing His-tagged toxin and HA-tagged immunity proteins. During these experiments, I had to troubleshoot the immunoblots due to a weak signal for the His-tagged toxin. To resolve this issue, I performed a series of experiments to establish a protocol that consistently detects the toxin. Overall, these experiments revealed the co-elution of the toxin with the immunity factor when using beads conjugated with an HA antibody.

In conjunction with the Co-IPs, I performed a series of experiments to establish a protocol for the purification of the tagged Toxin and Immunity proteins. Although I made significant progress in this endeavor, I was unable to successfully purify the proteins. This was due to the detection of proteins at higher molecular weights (MW) than expected. A denaturing purification of the WCLs provided data suggesting that homodimers of the proteins and/or interactions amongst the toxin and immunity proteins were responsible for the high MW bands. A western blot screen of the cell pellet and WCLs of empty BL21 cells and empty pMCSG53 BL21 cells detected no background signal. These data suggest that in the absence of a strong denaturant, the toxin and immunity protein may readily bind to each other or themselves making it difficult to successfully purify them.

To summarize, this thesis provides evidence of physical interaction between the toxin and immunity proteins. In the process of producing these data, this thesis establishes multiple protocols for the consistent detection of the toxins and the purification of the toxin and immunity proteins. Together, this work provides further evidence that the rhs locus in *P. aeruginosa* encodes functional toxin and immunity proteins alongside establishing protocols for further assessment of the locus.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Pseudomonas aeruginosa Is a Leading Opportunistic Pathogen

The prevalence of antibiotic resistance in bacteria has led to an ever-increasing burden on the healthcare system worldwide. One of the many bacteria that is a cause for concern is the gram-negative, motile, rod-shaped, heterotrophic, facultative aerobe *Pseudomonas aeruginosa* [1]. *P. aeruginosa* is found in ubiquity throughout nature, from soil to water, and is especially prevalent in human/animal-impacted environments. It is a serious concern for individuals with recently experienced trauma, those with indwelling devices, immunocompromised individuals, or those experiencing a structural lung disease such as Cystic Fibrosis or Bronchiectasis [2]. Amongst nosocomial infections, *P. aeruginosa* is a major contributor to infections through a respiratory source in Intensive Care Unit (ICU) patients [3]. In the case of ventilator-associated pneumonia (VAP), *P. aeruginosa* accounts for 10-20% of the infections [4] and has a mortality rate that ranges from 32-42.8% [5-7]. Alongside these, *P. aeruginosa* is the cause of 10% of all catheter-associated urinary tract infections (CAUTIs) and 16% of all UTIs in ICU patients [8, 9]. *P. aeruginosa* is also a concern during surgical procedures as it can produce tenacious surgical site infections (SSIs). A large study conducted from 2000-13 in England found that *P. aeruginosa* is responsible for 4.3%-6.5% of all SSIs, and another study in a hospital over seven years noted an increased mortality rate upon *P. aeruginosa* infection following cardiac surgeries [10, 11]. Amongst the healthy population, *P. aeruginosa* is a common culprit of corneal infections being isolated at a frequency of 6.8% to 55%, depending on the country, with poor

clinical outcomes [12, 13]. Furthermore, *P. aeruginosa* infections are particularly prevalent in patients with Cystic Fibrosis (CF) and are an indicator of increased morbidity and mortality [14]. It is believed that *P. aeruginosa* is adept at colonizing the CF lung due to its ability to undergo mutations and genetic changes to survive in an anaerobic environment. Because of this, most treatments against *P. aeruginosa* focus on early eradication to prevent a chronic infection, as once it is established, it becomes exceedingly difficult to clear the infection [15].

An additional layer of difficulty in treating *P. aeruginosa* infections arises when the particular infecting strain acquires resistance to a broad range of antibiotics, from ß-lactams to polycationic antimicrobials [16]. Additionally, individuals infected with multiple different strains of *P. aeruginosa*, (*i.e.,* mixed strain populations) are at risk for rapid evolution of multi-drug resistant (MDR) strains within the host [17]. With the increase in multi-drug resistant (MDR) *P. aeruginosa* infections throughout the world, it is becoming increasingly important to research new avenues to discover therapeutics against MDR *P. aeruginosa*. In recent years, phage therapy has proved to be an effective tool for reducing bacterial loads of MDR bacteria in animal models [18-25] as well as being shown to restore antibiotic sensitivity in *P. aeruginosa* [26]. Although there are shortcomings with phage therapy as well, such as the development of bacterial resistance against phages and immune response against phages resulting in the neutralization of phage activity [27]. Thus, it is important to continue the search for alternative means by which we can deal with MDR *P. aeruginosa*. One avenue which may yield potential results is studying how *P. aeruginosa* engages in interbacterial competition. As the systems used by *P. aeruginosa* to kill its neighbors may not only provide us with new insights into how it establishes an infection but also may provide us with new therapies against *P. aeruginosa* itself.

Interbacterial Competition Is Categorized as Either Exploitative or Interference

In any given ecological niche, bacteria are constantly secreting a veritable arsenal of enzymes, scavenging molecules, and signaling molecules that promote their growth and survival [28-31]. A major purpose of these secretions is to help secure limited nutrients from the surrounding environment. Incidentally, as one bacterium secures the nutrients, another bacterium loses it. This manipulation of a shared limited resource is commonly characterized as exploitative competition [32]. For example, siderophores are utilized by bacteria to acquire external iron in the environment, which helps the producing cell survive while reducing the availability of iron to competitors reducing their competitive fitness [33]. Although exploitative competition is not limited to just resource manipulation, it can also arise from the buildup of toxic waste products or the activity of a specialized metabolite (SM) [34].

Interference competition involves the secretion and delivery of SMs, enzymes, multifunctional metabolites, and more into surrounding cells to antagonize and disrupt them, thereby imparting competitive fitness to the producing cell. An example of this type of competition includes the premature sporulation and secretion of prodigiosin by *Streptomyces coelicolor* in the presence of jadomycin B at subinhibitory concentrations [35]. Therefore, it seems to be that the difference between exploitative and interference competition is that exploitative competition may occur as a result of the normal functions of the cell, or may occur indirectly, while interference competition involves the deliberate secretion of molecules that disrupt the fitness of target cells [36]. More importantly, it must be understood that there is much that is unknown about how bacteria compete with one another and the overarching purpose of interbacterial competition.

Interference Competition Is Separated into Contact-Dependent and Independent Forms.

Bacteria utilize diverse strategies to engage in interference competition, some of which require direct contact while others rely on passive diffusion. Contact-dependent antagonism utilizes various cell-anchored protein delivery platforms to usher toxic effector domains into a neighboring bacterium using various secretion mechanisms including type V secretion (T5SS), type VI secretion (T6SS), or outer membrane exchange (OME)[37]. Of these examples, the T6SS is the most researched in terms of its structure and function. The T6SS is a large protein complex that contains a needle-like filament encased in contractile sheath proteins anchored to the membrane through a baseplate/membrane-spanning complex. Upon activation, the outer sheath proteins function like a molecular syringe to eject the "needle", which punctures the membrane of a neighboring cell and delivers the toxic cargo directly into the periplasm of the prey bacterium [38-41]. Toxin-producing cells are protected because they encode specific immunity factors that neutralize the respective toxic effectors. The toxic effectors delivered by the T6SS target critical biological processes [42-44]. For example, the *P. aeruginosa* effectors Tse1 and 3 cleave peptidoglycan and cause cell lysis while Tse2 induces cell stasis [45-47]. The Type VI lipase effector (Tle) 1-5 are a family of effectors that target the cell membrane of intoxicated cells [48]. Additionally, the effector Tas1 from *P. aeruginosa* strain PA14 rapidly depletes cellular ATP to produce (p)ppApp resulting in cell death from widespread dysregulation of essential metabolic pathways [49].

In another form of contact-dependent competition, bacteria utilize a large surface-bound protein (CdiA) to release and translocate a toxic effector domain into a neighboring bacterium in a process termed contact-dependent growth inhibition (CDI)[50]. This competition system is encoded in the *cdiBAI* gene cluster, where CdiB helps in the secretion and surface anchoring of

CdiA using a type V secretion mechanism. The specifics of CdiA-mediated effector delivery are ill-defined; however, CdiA-dependent intoxication requires recognition of "receptors" on the target bacterium, which aid in the translocation of the toxic effector domain across the outer and inner membranes of the prey [51-58]. As with many antagonistic toxin systems, the *cdiI* gene encodes a specific immunity factor that protects the toxin-producing cell [59, 60].

Outer Membrane Exchange is another form of interbacterial competition which has primarily been noted in Myxobacteria and has been studied most extensively in *Myxococcus Xanthus*, so far. The OME process begins when two cells carrying similar TraA and TraB receptors glide past one another, allowing the proteins to coalesce and form foci that resemble eukaryotic gap junctions. Following this event, the cells engage in a bidirectional exchange of OM components, including endogenous lipoproteins as well as SitA toxins [61-64]. This exchange of OM components serves two purposes: the first goal is to ensure the homogeneity and fitness of the population as the exchange can help damaged cells survive, while the second goal is for the discrimination against nonkin to ensure the population remains clonal [65]. Kin discrimination is accomplished through the exchange of SitA toxins, most of whom are believed to target nucleic acids. During this exchange, kin are protected from the toxins since they carry the cognate immunity factor, SitI. The SitA toxins belong to six distinct families which share domain organization and delivery mechanism but contain unique domains responsible for the delivery of the toxin into the target cell's cytoplasm [64]. Altogether, the OME mechanism of Myxobacteria serves to not only help ensure the survival and overall health of the community but also helps them engage in intraspecies competition to improve the survival odds of their kin [66].

On the other hand, interference competition through contact-independent modes occurs through a variety of mediums like antibiotics, metabolic by-products, enzymes, and more [67,

68]. Among these, the bacteriocins have gained increasing attention in recent times due to their presence in almost all bacterial species identified to date [69]. Bacteriocins are a highly diverse and heterogeneous group of molecules loosely defined as ribosomally-synthesized peptides secreted by bacteria to kill other bacteria [70]. Within the bacteriocin family, the colicins of *E. coli* have been studied extensively. Colicins contain several modular domains that function to help recognize target prey and translocate and release a toxic effector domain into a susceptible bacterium. Colicin-producing bacteria also encode an immunity factor that neutralizes the effector domain to protect kin. The effector domains of different colicins have diverse functions such as pore formation or nuclease activity against different nucleic acids [71-77]. These general characteristics appear conserved across all bacteriocins [70].

Overall, protein toxins involved in interference competition share a similar framework. These toxin systems tend to be encoded in a single locus with multiple ORFs, which express multidomain proteins required for secretion, prey recognition, toxin delivery, and effector function. They also encode immunity factors that neutralize the toxic effector domain to protect toxin-producing kin [78]. In this thesis, I will be investigating a locus in *P. aeruginosa* which we hypothesize to encode a novel interference competition system described below.

RHS Toxins Are Delivered through Contact-Dependent and Independent Systems

Rearrangement hotspot (RHS) elements are a repeating peptide sequence with a YDxxGRL(I/T) consensus motif found in several different proteins from bacteria to mammals [79-81] [82]. A misnomer of its early discovery in a hypervariable region of the *E. coli* genome [83], RHS elements actually form an antiparallel beta-sheet that wraps around itself to form a cocoon or shell (Fig. 1). These elements are commonly found as part of different modular proteins, in which the RHS domain can be attached to different N- and C-terminal domains [80].

Figure 1. Structure of an RHS Shell. Depicted is a rainbow shading of the RHS shell from *P. protegens* RhsA (PDB:7Q97).

Two major types of RHS-containing proteins have been characterized in bacteria. These include the T6SS effectors RhsA of *Pseudomonas protegens* [84]and Rhs1 of *Photorhabdus laumondii*, as well as the insecticidal toxin complex proteins TcB and TcC of *Photorhabdus luminescens*, *Xenorhabdus nematophila*, and *Yersinia entomophila* [85]. Whereas the RHS shell of the T6SS effectors is a single protein, the Tc toxins have split the shell into two separate open reading frames (ORFs) with TcB containing the "bottom" half of the shell and TcC containing the "top" half (Fig. 2).

In both examples, a toxic effector domain is encapsulated within a complete RHS shell. This effector domain is located at the C-terminus of the T6SS RhsA/Rhs1 protein or the corresponding C-terminus of the TcC protein. In both instances, the effector domain is demarcated by a highly conserved aspartic protease motif that functions to free the toxic effector domain from the rest of the protein. It is unclear at what specific time this autocatalytic event happens, but it is required for effector delivery. For the T6SS effectors RhsA/Rhs1, the "bottom" half of the shell is connected to a PAAR domain located at the N-terminus of the protein. This

domain facilitates anchoring to the T6SS VGR spike and eventual delivery into a prey bacterium. In contrast, TcB contains a TcA-binding b-propellor domain that facilitates binding to a pentamer of TcA, the 3rd protein of the insecticidal toxin complex. In this example, a pentamer of TcA forms a channel in which the released effector domain of TcC can snake through to enter and intoxicate epithelial cells of the insect gut. Thus, in both examples, one end of the holotoxin functions in the delivery of an effector domain located at the opposite end of the protein that is encased within an RHS shell.

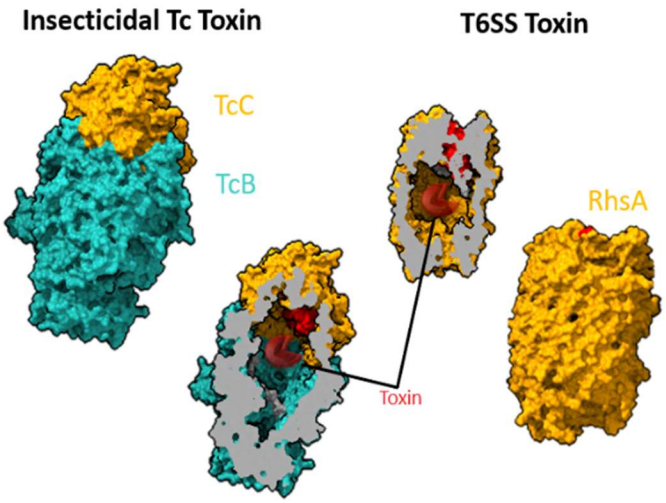

Figure 2. Structural Comparison of Different RHS Proteins. The RHS protein family has been shown to produce a large hollow shell which holds the effector domain. This figure depicts the RHS shell from two different organisms which utilize separate delivery mechanisms. (Models designed by Abigail B. and Dr. Allen)

Bioinformatic studies suggest that the toxic effector domains of many RHS proteins are exchanged through some undefined recombination process to produce new toxin variants [86]. In many instances, a small downstream open reading frame is also exchanged, suggesting that it may encode for a toxin-neutralizing immunity factor. This type of genetic linkage is common for effectors that target bacteria, whereas effectors that target eukaryotes do not contain a linked immunity gene. Altogether, the RHS motif appears to be part of a toxin delivery system with the capability of encapsulating a diverse array of toxic effector domains.

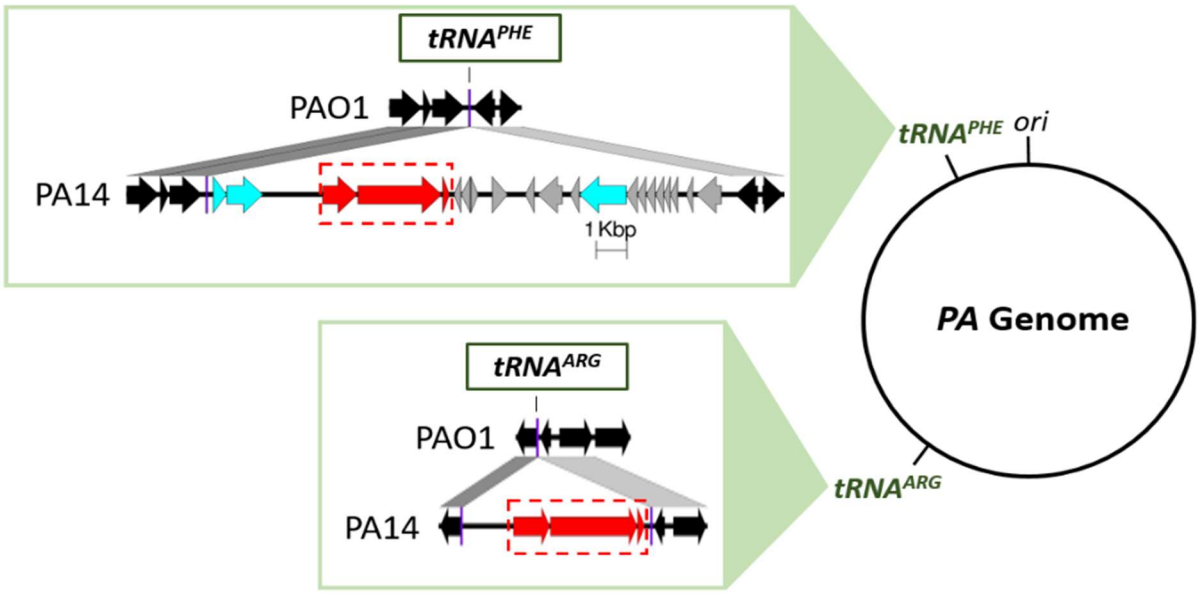

Figure 3. An Uninvestigated Locus of *Pseudomonas aeruginosa.* This figure depicts the gene clusters, in Red, that Dr. Allen identified in PA01 and PA14, strains of *P. aeruginosa*, present at the tRNA genetic recombination sites. (Graphic designed by Abigail B. and Dr. Allen)

A Previously Unidentified Locus in *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous bacterium, found in soil and water, which is known for its ability to intoxicate human and bacterial cells alike. It has a veritable arsenal at its disposable like the Type 6 secretion system (T6SS) [87], contact dependent growth inhibition (CDI) [88], bacteriocins (pyocins) [89], and rhamnolipids [90]. Over time the number of processes by which *P. aeruginosa* engages in interbacterial competition as well as host cell attack has only increased. Thus, it should come as no surprise to say that we may have discovered another locus in the *P. aeruginosa* genome which encodes for a protein system that mediates interbacterial competition. This locus was first noted by Dr. Jonathan Allen during his post-doctoral work in Dr. Alan Hauser's Lab at Northwestern when they were performing genomic analysis of *P. aeruginosa* to identify its core and accessory genomes [91].

During this study, Dr. Allen noticed the gene cluster depicted in Figure 3, which was found at two separate tRNA insertion sites in strain PA14 but absent within strain PAO1. This

suggested that the unstudied operon was part of a mobile genetic element and may have moved around through horizontal gene transfer. This locus is organized into three ORFs, we ad-interim term *rhsB*, *rhsC*, and *rhsI*, where both *rhsB* and *rhsC* contain RHS elements at their 3' and 5' end, respectively (Fig. 4). Although we have not resolved the structure of the protein encoded by *rhsB* and *rhsC*, Alpha Fold predictions indicate that it forms a hollow shell-like structure (Fig. 5) similar to other RHS-element-containing proteins like RhsA of *P. protegens* [84]. Notably, the sequence of *rhsC* is nearly identical across all strains of *P. aeruginosa*, except for the hypervariable 3' end of the gene that encompasses the toxic effector domain (Fig. 4). Additionally, there is a concomitant change of the downstream *rhsI* gene with each *rhsC* allele (Fig. 4). This co-variance is quite common in toxin-immunity pairs of interference competition systems, where the immunity specifically neutralizes its toxin to avoid autointoxication as well as the intoxication of nearby siblings. There is also an independent variance of the 5' end of *rhsB,* whose significance we are still unsure of but that we believe functions in toxin delivery. Finally, we hypothesize this is a secreted holotoxin because of the presence of strong N-terminal secretion signals for the SEC translocon on both RhsB and RhsC.

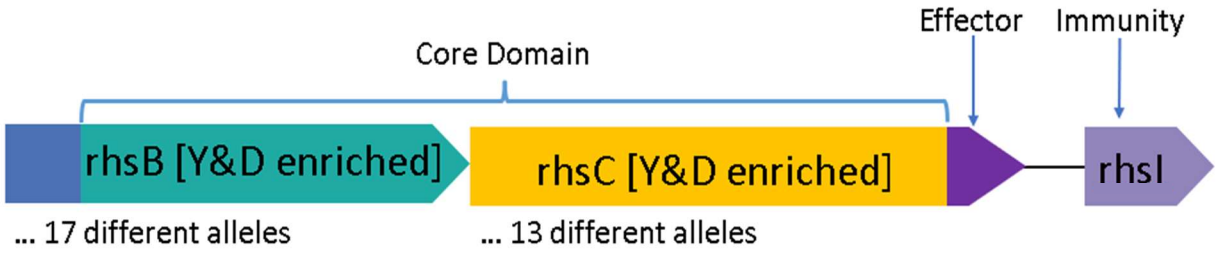

Figure 4. Genetic Organization of the Locus. This is a depiction of the genetic organization of a novel rhs locus in the *P. aeruginosa* genome

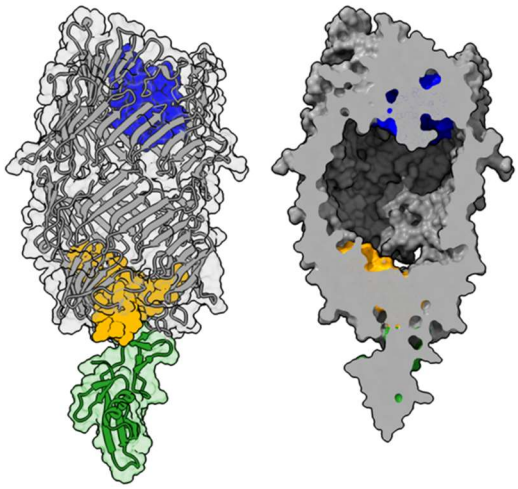

Figure 5. Model of the RhsBC Holotoxin in *P. aeruginosa*. The variable N-terminus of RhsB is highlighted in Green. Orange represents the plug domain of RhsB, and blue represents the autoprotease domain of RhsC.

Overall, we hypothesize that this uncharacterized locus in the *P. aeruginosa* genome encodes toxin-immunity pairs that mediate interbacterial competition. Our current working model is depicted in Figure 6. We propose that the RhsBC holotoxin is assembled in the periplasm and secreted using the Type 2 secretion system, where it can diffuse away from the bacterium to intoxicate cells at a distance. Target cell recognition and effector translocation mechanisms are unclear but ultimately result in the delivery of the toxic effector domain of RhsC into the prey bacterium. Toxin-producing bacteria are protected because of the neutralizing properties of the specific RhsI immunity factors.

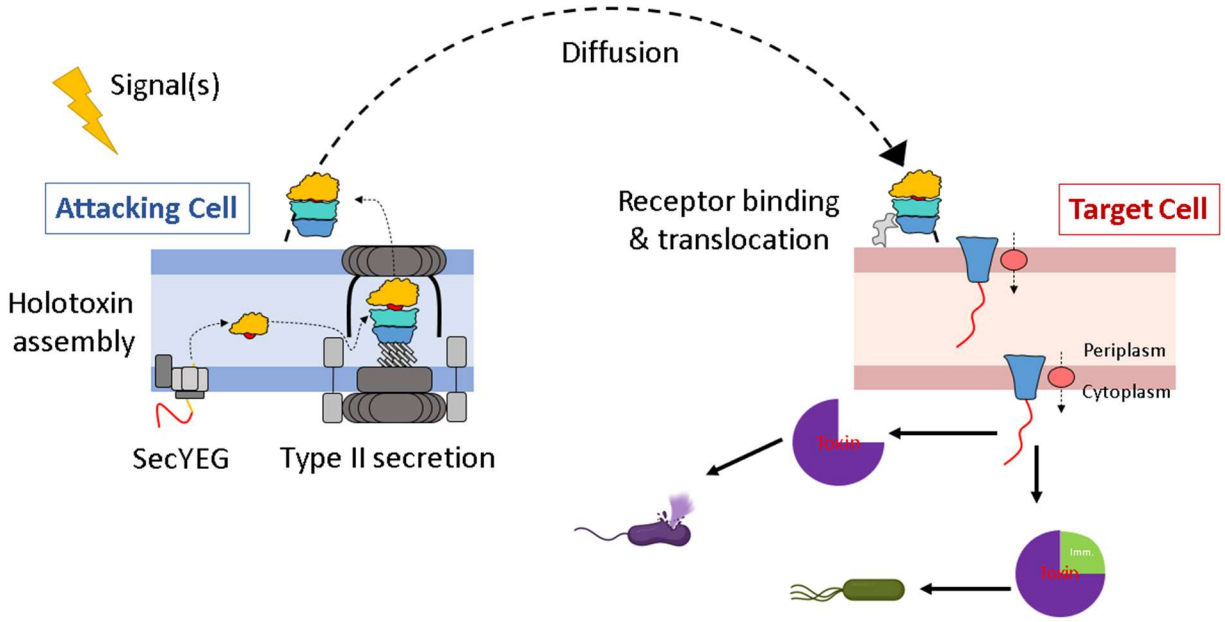

Figure 6. Proposed Model of RhsBC Secretion and Intoxication. This is the overall model of how we believe the system to be functioning to intoxicate neighboring cell imparting competitive fitness to *P. aeruginosa* (Modified version of model designed by Abigail B. and Dr. Allen)

Concluding Remarks.

Pseudomonas aeruginosa is a ubiquitous, gram-negative bacteria of significant concern for human health due to its ability to persist in any given environment. It is very well known that it encodes an arsenal of antimicrobial proteins, which aid in its ability to outcompete other bacteria within its immediate environment. We believe that we may have identified another locus in the organism's genome that encodes an antagonistic toxin that mediates interbacterial competition. By understanding how this system functions, we can potentially repurpose it to deliver therapeutics that target *P. aeruginosa*. This may provide us with another avenue of attack against this particularly resilient bacterial species.

In this thesis, I intended to provide substantial evidence to support our hypothesis that the effector domain of RhsC is neutralized by RhsI through physical binding to each other. To accomplish this, I focused on biochemical approaches to prove that the different alleles of *rhsC*

encode specific toxin-immunity pairs. Co-immunoprecipitation (Co-IP) experiments revealed the physical interaction of the unique effectors with their respective immunity proteins. I also established a reliable protocol for protein expression and cell lysis that lays the foundation for further purification work. the conclusions of this study have aided in obtaining evidence that supports our current hypothesis. Alongside that, the protocols that I have been able to establish will be helpful to future lab members in further investigating the *rhs* locus of *Pseudomonas aeruginosa*.

CHAPTER TWO

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions.

The bacterial strains listed in Table 1 were grown under the following conditions. *Escherichia coli* was grown in Luria Broth (LB) (ThermoFisher), cultures were grown overnight at 37ºC at 275 rpm (revolutions per minute) at ~45º angle unless noted otherwise. Antibiotics and nutrients were supplemented depending on the strain at the following concentrations: ampicillin (10 mg/mL), gentamycin (50 mg/mL), rhamnose (0.5 mg/mL), arabinose (10%), chloramphenicol (10 µg/mL).

Table 1. List of Bacterial Strains.

Designation	Description	Strain
NEBα	*E. coli* strain used for heterologous expression	NEBα
BL21 DE3	*E. coli* strain used for heterologous expression	BL21 DE3
Lemo21	BL21 DE3 *E. coli* strain used for tunable expression, deficient in Lon and OmpT proteases containing a pMCSG53 plasmid with a mutated Toxin/Immunity 6 allele	Lemo21(DE3) Competent *E. coli*
LysY/I^q	BL21 DE3 *E. coli* strain used for expression control, deficient in Lon and OmpT proteases containing a pMCSG53 plasmid with a mutated Toxin/Immunity 6 allele	T7 Express *lysY/I^q* Competent *E. coli* (High Efficiency)
pTES2	NEBα *E. coli* strain carrying the pTES2 plasmid capable of differential tuning	pTES2 NEBα
pMCSG53	NEBα *E. coli* strain containing an empty pMCSG53 plasmid to serve as a control	pMCSG53 Empty V1

pMCSG53 T/I 1	BL21 DE3 *E. coli* strain carrying pMCSG53 plasmid with Toxin and Immunity 1 cloned in	pMCSG53 RHS Tox/Imm. 1 V1
pMMB67 T/I 6	NEBα *E. coli* strain carrying pMMB67 plasmid with Toxin and Immunity 6 cloned in	pMMB67 pTAC RHS Toxin/Immunity 6 V1
pMMB67 T/I 7	NEBα *E. coli* strain carrying pMMB67 plasmid with Toxin and Immunity 7 cloned in	pMMB67 pTAC RHS Toxin/Immunity 7 V2
17 CdiA [HA] 3238HB	BL21 DE3 *E.coli* strain carrying pMCSG53 plasmid with an attenuated CdiA at amino acid 3238 with an HA and Hibit tag, created by Dr. Jonathan Allen	pMCSG53 17 CdiA(HA) 3238(HA)-HB #1 F7
BL21 p53 T/I 1	BL21DE3 *E. coli* strain carrying pMCSG53 plasmid with Toxin and Immunity 1 cloned in	pMCSG53 RHS Tox/Imm. 1 V1
T/I 1	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 1 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 1 V1
T/I 2	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 2 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 1 V2
T/I 3	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 3 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 3 V1
T/I 4	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 4 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 4 CTG V1
T/I 5	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 5 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 5 V1
T/I 6	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 6 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 6 V1
T/I 7	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 7 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 7 V1
T/I 8	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 8 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 8 V2
T/I 9	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 9 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 9 V2

T/I 10	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 10 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 10 V1
T/I 11	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 11 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 11 V1
T/I 12	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 12 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 12 CTG V1
T/I 13	NEBα *E. coli* strain carrying pSB109 plasmid with Toxin and Immunity 13 cloned in, created by Abigail B.	pSB109 RHS Toxin/Immunity 13 V1

Molecular Genetic Technique

Genomic DNA was isolated from a 5 mL culture grown overnight in LB supplemented with respective antibiotics. The ZymoPURE Plasmid Miniprep Kit or QIAprep Spin Miniprep Kit was used to extract plasmids using their respective protocols. The 5 mL culture was centrifuged at 1,400 rcf for 15 minutes to pellet the bacteria. Following this, the supernatant was discarded, and the supplied protocol of the respective kit was used to isolate plasmid DNA. For Polymerase Chain Reaction (PCR), GoTaq Polymerase (Promega) or Phusion High-Fidelity DNA polymerase (New England Biolabs) was used. The oligonucleotide primers used for amplification reactions were ordered from Integrated DNA Technologies (Table 2). DNA digestion was conducted using SspI, XmaI, BamHI, and Kpn2I (Thermo Fisher Scientific), depending on plasmid and insert. DNA separation by electrophoresis was conducted using a 1.2% solidified agarose (VWR). DNA was extracted from agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo) and the supplied protocol. Plasmid assembly was carried out using Gibson Assembly Protocol supplied by New England Biolabs using Gibson Assembly Master Mix (2X) (New England Biolabs).

Table 2. List of Primer Oligonucleotides.

Primer	Sequence (5' – 3')
P53_RhsC1ToxIm_F	ACCTGTACTTCCAATCCAATCTAAACAACCCTGAAATGACATT
P53_RhsC6ToxIm_F	ACCTGTACTTCCAATCCAATTTGAATACCTATGGGTATGGGTATGTAATG
P53_RhsI-HA_R	ATCCGTTATCCACTTCCAATTTAAGCGTAATCTGGAACATCGTAT
pTES2_RhsI6_F	AATTTATTATGCAAATCTCCACTTGGTTCGTTGTTAGCATTGCATC
pTES2_RhsI6_R	CCATCGTCATCGTCCTTTCCTTCTTCTGAATCATCAACGCATTTTAAGAG
pTES2_RhsI13_F	AATTTATTATGCAAATCTCCACCACCAGTGACGGAATAACAGATAG
pTES2_RhsI13_R	CCATCGTCATCGTCCTTTCCCTGATTGCTAGGGTTTGGTTGATTAAG
pTES2_RhsI1_F	AATTTATTATGCAAATCTCCAGTAGCCCCTACAACATAATAGCAG
pTES2_RhsI1_R	CCATCGTCATCGTCCTTTCCGTTTTTTATTGTAAACTGTAAAATCATC
pTES2_RhsC6tox_F	ACCTGTACTTCCAATCCAATAGCATCGCGATAGTAGAAGCGCTTG
pTES2_RhsC6tox_R	ATCCGTTATCCACTTCCAATTTATTGGAACCTATTTTTGCCGTCG
pTES2_RhsC3tox_R	ATCCGTTATCCACTTCCAATTTAGTTGCATTGCTCACCGCAAACC
pTES2_RhsC3tox_F	ACCTGTACTTCCAATCCAATAGTCCTACAGCTACTGCAGGCGCAG
pTES_pBAD-SeqF	TGTAACAAAGCGGGACCA
pTES_pBAD_SeqR	TCGCCAATTGTAGAAACGCA
pTES2_RhsC4Tox_F	CGAGAACCTGTACTTCCAATCCAATATGCAGGATATTGGATGTAT
pTES2_RhsC4Tox_R	TTCGGATCCGTTATCCACTTCCAATCTATTTCTGTCCATATCTAATTTTT
pTES2 RhsI4 F	GGAGGAATTTATTATGCAAATCTCCCAGGAACTACTTGAGCC
pTES2 RhsI4 R	ATCCTCCATCGTCATCGTCCTTTCCATTATAGATGTACGAAACTTGGG
pTES2_RhsC5Tox_F	CGAGAACCTGTACTTCCAATCCAATCACCCAGGCGATAAACTATTCGG
pTES2_RhsC5Tox_R	TTCGGATCCGTTATCCACTTCCAATTTAGCACATGGGTATTTTGGGCT

pTES2_RhsI5_F	GGAGGAATTTATTATGCAAATCTCCTCATTTGAGTGC ACTTTAGAAATAA
pTES2_RhsI5_R	ATCCTCCATCGTCATCGTCCTTTCCGCTCTCGCTGGAG CAT
pTES2_RhsC9Tox_F	CGAGAACCTGTACTTCCAATCCAATFCAGCAATAGAG ATTGATATTCCT
pTES2_RhsC9Tox_R	TTCGGATCCGTTATCCACTTCCAATTCAGTTTATAAAC GGCTCTACTGGA
pTES2_RhsI9_F	GGAGGAATTTATTATGCAAATCTCCAGATATTCAAAT GGAAAAAAAGTAG
pTES2_RhsI9_R	ATCCTCCATCGTCATCGTCCTTTCCCTTGGCTTTTCTG CTTATTAGTTTTATC
pTES2_RhsC13Tox_F	CGAGAACCTGTACTTCCAATCCAATTCAAAAACCAAT GGAAAG
pTES2_RhsC13Tox_R	TTCGGATCCGTTATCCACTTCCAATTTATAGCTGGCC AAGA
pTES2_RhsI13_F	GGAGGAATTTATTATGCAAATCTCCACCACCAGTGAC GGAATAACA
pTES2_RhsI13_R	ATCCTCCATCGTCATCGTCCTTTCCTGATTGCTAGGGT TTGGTTGA
pMMB67_seqF	TTGCGCCGACATCATAACGGT
pMMB67_seqR	GTGGGACCACCGCGCTACTG

E. coli Heat Transformation.

Plasmid transformation into *E. coli* was carried out by adding 5 µL of purified plasmid or Gibson Assembly product to 50 µL of competent *E. coli* cells in a microcentrifuge tube. The tube was flicked 2-3 times to mix the added product with the bacterial cells following which the cells were incubated on ice for 30 mins. Then, the cells were heat shocked for 30 seconds in a 42ºC water bath and 450 µL of SOC medium was added to the tube. Following this, the cells were left to recover in the shaking incubator at 37ºC, 250 rpm for at least 2 hours. Following incubation, the cells were plated on LB with the respective antibiotics to select transformed cells.

Generation of Plasmids Carrying Toxin and Immunity Alleles

Gene blocks (Table 3) were designed carrying C-tagged putative Toxin (6X His Tag) and Immunity (HA tag) Alleles to be cloned into either pSB109, pMMB67, pMCSG53, or pTES2 by using their respective primers to amplify appropriate gene fragments (Table 2). Each plasmid was digested using the respective digestion enzyme following which the digested product was Gibson assembled with a tagged Toxin/Immunity allele. This was done to clone all 13 alleles of the putative toxin with its respective putative immunity allele to generate plasmids carrying the toxin and immunity genes (Table 4). Following the generation of each plasmid, it was transformed into NEBα *E. coli* cells and the primers listed in Table 2 were used to verify correct transformants. The sequence for each plasmid was verified by sending minipreps to Plasmidasaurus.

Table 3. List of Gene Blocks.

Name	Sequence (5' – 3')
pTES1_insert	ATTTTTTAAATACCCGCGAGAAATAGAGTTGATCGTCAAA ACCAACATTGCGACCGACGGTGGCGATAGGCATCCGGGT GGTGCTCAAAAGCAGCTTCGCCTGGCTGATACGTTGGTCC TCGCGCCAGCTTAAGACGCTAATCCCTAACTGCTGGCGGA AAAGATGTGACAGACGCGACGGCGACAAGCAAACATGCT GTGCGACGCTGGCGATATCAAAATTGCTGTCTGCCAGGTG ATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCC ATCGGTGGATGGAGCGACTCGTTAATCGCTTCCATGCGCC GCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCAGCTC CGAATAGCGCCCTTCCCCTTGCCCGGCGTTAATGATTTGCC CAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGG GCGAAAGAACCCCGTATTGGCAAAGATTGACGGCCAGTT AAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGTAAAC CCACTGGTGATACCATTCGCGAGCCTCCGGATGACGACCG TAGTGATGAATCTCTCCTGGCGGGAACAGCAAAATATCAC CCGGTCGGCAAACAAATTCTCGTCCCTGATTTTTCACCACC CCCTGACCGCGAATGGTGAGATTGAGAATATAACCTTTCA TTCCCAGCGGTCGGTCGATAAAAAAATCGAGATAACCGTT GGCCTCAATCGGCGTTAAACCCGCCACCAGATGGGCATTA

	AACGAGTATCCCGGCAGCAGGGGATCATTTTGCGCTTCAG CCATACTTTTCATACTCCCGCCATTCAGAGAAGAAACCAA TTGTCCATATTGCATCAGACATTGCCGTCACTGCGTCTTTT ACTGGCTCTTCTCGCTAACCAAACCGGTAACCCCGCTTATT AAAAGCATTCTGTAACAAAGCGGGACCAAAGCCATGACA AAAACGCGTAACAAAAGTGTCTATAATCACGGCAGAAAA GTCCACATTGATTATTTGCACGGCGTCACACTTTGCTATGC CATAGCATTTTTATCCATAAGATTAGCGGATCCTACCTGA CGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTT TTTGGGCTAACAGGAGGAATTTATTATGCAAATCTCTTAC CCGTACGACGTGCCGGACTACGCCGGAGGAGACGATGAC GATAAGGGCCCGGGGATGATCTCCCCATGCGAGAGTAGG GAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGA AAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAAC GCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGA ACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGAC GCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGG CCATCCTGACGGATGGCCTTTTTGCGTTTCTACAATTGGCG AATGGGACGCGCC
pTES2_insert	GCTGAAAGGAGGAACTATATTTATGACAACTTGACGGCTA CATCATTCACTTTTTCTTCACAACCGGCACGGAACTCGCTC GGGCTGGCCCCGGTGCATTTTTTAAATACCCGCGAGAAAT AGAGTTGATCGTCAAAACCAACATTGCGACCGACGGTGGC GATAGGCATCCGGGTGGTGCTCAAAAGCAGCTTCGCCTGG CTGATACGTTGGTCCTCGCGCCAGCTTAAGACGCTAATCC CTAACTGCTGGCGGAAAAGATGTGACAGACGCGACGGCG ACAAGCAAACATGCTGTGCGACGCTGGCGATATCAAAATT GCTGTCTGCCAGGTGATCGCTGATGTACTGACAAGCCTCG CGTACCCGATTATCCATCGGTGGATGGAGCGACTCGTTAA TCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGCAGATT TATCGCCAGCAGCTCCGAATAGCGCCCTTCCCCTTGCCCG GCGTTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCT GGTGCGCTTCATCCGGGCGAAAGAACCCCGTATTGGCAAA GATTGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGC GGACGAAAGTAAACCCACTGGTGATACCATTCGCGAGCCT CCGGATGACGACCGTAGTGATGAATCTCTCCTGGCGGGAA CAGCAAAATATCACCCGGTCGGCAAACAAATTCTCGTCCC TGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGA GAATATAACCTTTCATTCCCAGCGGTCGGTCGATAAAAAA ATCGAGATAACCGTTGGCCTCAATCGGCGTTAAACCCGCC ACCAGATGGGCATTAAACGAGTATCCCGGCAGCAGGGGA TCATTTTGCGCTTCAGCCATACTTTTCATACTCCCGCCATT CAGAGAAGAAACCAATTGTCCATATTGCATCAGACATTGC CGTCACTGCGTCTTTTACTGGCTCTTCTCGCTAACCAAACC GGTAACCCCGCTTATTAAAAGCATTCTGTAACAAAGCGGG ACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTAT

	AATCACGGCAGAAAAGTCCACATTGATTATTTGCACGGCG TCACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTA GCGGATCCTACCTGACGCTTTTTATCGCAACTCTCTACTGT TTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTTATT ATGCAAATCTCCCCGGGGAAAGGACGATGACGATGGAGG ATACCCGTACGACGTGCCGGACTACGCCTGATCTCCCCAT GCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAA GGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTT TGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGG AGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTG GCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAAT TAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTT CTACAATTGGCGAATGGGACGCGCC
pSB109RhsC1HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTAAACAACCCTG AAATGACATTTTTAAATTCTGGCGAGAGCATGCTGCAAGC CACTCCGTATTGGGAACATGGATTCACGCCGAATCATAAC TACACATACTCCGATAACAATCCAACTGCAAAAAGCGACA AGCATGGTCTTTCACCAAACCCTACGGACAACTTGATCTA CACACCTGACACCAACTGCACATGTACATTAGAGTGCAAG AAAAAATTTACTGGAAATGGAAAGTCTTTCCTTGTGGGAG CTCTATGTAGCAAAGCCACCACCCCGTTCTTTGGGGGAGT AGTATGCAATAGCACAATAGTAATGATCTGCGGAGCATCT TGCAGCCAAGAGTGCAATAGAGCACCAAGCTGCGAAAAA GAAGGAAGCGGCAATGAGTAGCCCCTACAACATAATAGC AGTTCTGTGCGCCATCACACTGGTAGTATTAGAGCTAAAA AGAACAACATTCATAAGAACTTTAAAGAAAGAAGAGCCA AACGCCTGGGAGAAGCTTGGCAGGCCATCAGGGTATTTCT TATCCTATCTAGTAAAAATAGACGGATTCAAGCTTGAGAA ATTTATATTCCGAAAGCAATACAATGCCTTGGAAAATAAC GAAATCAGAAAAATAGGGAGGCAACTCCTCTACCTGCAA TCAACTTTCCTCACCTTAATTATTGCACTCATCGCCTTGAT GATTTTACAGTTTACAATAAAAAACGGTGGTTCTTACCCA TACGATGTTCCAGATTACGCTTAAGGCAGCAGCCATCATC ATCATCATCACAGCAGCG
pSB109RhsC2HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTTTAAATACATATGG ATATGTAGAGGGAAATCCACTCGGACTTTCAGATCCACTA GGACTGGCTCCAGGGGATCTGTTTGCAACAGAGGCTGCGG CACGAGCAGATGCTCTTGCTTATCAGGAGTCTTTGAACAG TAGCATTGATAGATGGCTATGGGGGAATATGGTCTATGGC TTCCGTGTGTTTAAAACCTCCGATTGCCTTTGGACCTACGA AGTACAGACCCCAGTCTTGGGAATTGCGCCTCCACTTGGC CCCAAAGGTCCTTGGAAGGTCAACAAGCCAGGAGTTTCAG GAAAGGCAGGAGCTAAAGACGTTCCAAGCTGGGCAAAAG GGGATAGGCCATATCAAGGCGAATCAGGGAAAGACTTTG

	CCAAACGCATCATGGATCAAAAATATGGCAAAGGTAACT GGAAAGACGGACCAGGCTCTGAATACAACCAGATCAAAA AGTGGGGTGACAGATCGTTCATAGACCCCAAGTAATCAAA CTCCTAAAAAAATGGAGGCACTGATGGATAACGTATTCAT ACTCCACCATACATACGGAGACTCTGAAAGTGAAAGCTAT AAGCTTCTAGGAGTATTCAGCACAGAGGATAGGGCAAAC TCAGAAATCCCCAAATACCTAAAGCTCCCAGGGTTCAAAG AGTTCCCTGACGGATTTACTGTAACCAAGTACTCCCTTGAT GAACCACATTGGTTAAGCGGTTTCGAAGGAGCAGAGTCTA GCGTTATAGAAGATAAGGGTGGTTCTTACCCATACGATGT TCCAGATTACGCTTAAGGCAGCAGCCATCATCATCATCAT CACAGCAGCG
pSB109RhsC3HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTGAATACTTACGC CTATGTTGGGTCCAATCCGGTAGGGCTGGTTGACCCTAAC GGACTGAGTCCTACAGCTACTGCAGGCGCAGGAGCTGGTT TTGTTGTCGGTGGGCCTCCTGGTGCAGTGGTTGGGGGGCT TATTGGTCTTGGGTTGGGCGTCTGGGGAGCAAATGCTGCT TGGGATGCCTATCACGACGAGGCTAGTGAGGGTGGCAGT GCATCAGCTGGCACTAGCCCAGGAACTGCGGCAGAAAAG GCTAGGGAGCGGAAGGAATACAGCCGGATTTGCAAGACT CCCATACCGCCGACAGGGGACCAATGCAAAGACGCTAAG GCTAACTTAGAGAGGCTTCAGCAGTGTTTGGCCTTGCGTG AGAACTTCAGCAAGAAATGGTTTAACGATAATGATGCAG GCCACGTTACAGAGATTAATAACACTAGGCAGGCCATTGA AAATCTGAAGGACTTCCTGCGGAGGGTTTGCGGTGAGCAA TGCAACTAATCAAGAGAAAGTTTCTCTACTGCTCGCAGAG TATTCGACTCTTCCCGAGTTCAGCGGGCTGGAGTGCGCAG ATGTTAACAGCCTTAGTTTATTCGGGGATAGACCAATCCA CATTGCCGCAACGCGCGGTGACATTGATGAAATTCAGCTG ATATTGGGTCATGGAGCTGACATCAACTGCAAAGGAGAA CATGGATATACAGCCCTGCACGATGCAGTCGAGCAGGGG CATAGCGCAGCTGTCGAATACCTTCTAAATCAGGGTGCAG ACCCCGAATCACTAAATGATGATGGAGTCTCGCCTGCCGA ACTTGCCAAGCTGCTCGATGAGGGTGAAATCCTTCACTTA TTTGAGCGAGGAGCCAGTGGTGGTTCTTACCCATACGATG TTCCAGATTACGCTTAAGGCAGCAGCCATCATCATCATCA TCACAGCAGCG
pSB109RhsC4HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTGAATACCTATGG ATACGTAGAAGCAAATCCAGTACTACGGACAGACTCCCTA GGGCTTATGCAGGATATTGGATGTATTACGCCACCAAGAT CAACTGCAGAGTTTTGGTTTCCATGCAAACCAGGTGGAGG AGCTGGAGCATCCGCTAGCGGTGCAACTGGGTTAGGCCTT TGGTGCGTTCTAAACGGAACGTGTGCTGCCAATAACGCAG ACGAAGAGTGTCCTGTACCGGGTGCAACCCCAGGCGAGA

	AAACACGAGGGCCTTCTCAGGTTTGGGAGAAACCAGGTG ACAGCAATACCGCTGATGATGATTTTGATAATCTATCGCC AACGGATGTACAGGAAATACCCGTAGGACGTAAAGGAAC ACTTCCAGACGGGCGGACTGTAGTAGTCAGACCTGTCAGC AGCGACGGCCGACCAACCATTGAGATTCAAGACGGTAGA AACAGGATAAAAATTAGATATGGACAGAAATAGCCATAA CATGCAGGAACTACTTGAGCCGGCACCAATCGAGCCCCTA AAAGGGAAAAAATTGCATCTGGATAAAATTATCGATTCAG TAGAGGGGCTCAAAATCTATCTAGCGGAGTCACTGGATGA AAAGAAAAGGATAGTTCTTTCATTCACACTTAACGAAATA GTTGGCTACTCCGTAACAAATGACAGCTACACCTGGAAAA GAACGAAAGAGCGAAAATGCGAACCAGGCCACTCCATAT ACATAGTAAGTAATTCCCGGGAAATATTAAAGTTCCAAGA AGAAACCTACAGCACAACATGTTTCGAAGAGGCTGAGCA CTACTGCATAATACTAGATGAAGAAGAGATAGACATCGTG ACATTCGCCCCACCCCAAGTTTCGTACATCTATAATGGTG GTTCTTACCCATACGATGTTCCAGATTACGCTTAAGGCAG CAGCCATCATCATCATCATCACAGCAGCG
pSB109RhsC5HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTTTTAACACATTCGC CTATGTAGGAGGAAACCCTCTTTCTTTTGCGGATACTCTTG GGCTCCACCCAGGCGATAAACTATTCGGTTTGCCTAAAGC TTTCTGGAAATGGTTCCACAAACAGGGAGACATGAACGAC CTGAAAGGACCTAATGGACAAGTATCCAAAGAAATAGCC GAAGACTACCATCGAGAATGGCTAAATAATGGTAAGCCA AGCCCTGACAGCAAGGGGCGATCTAGCCCAATTAATGAA ATTTTAGATTCAATACCGCCTCTAATACTCCCTCCTGGCTT TGAGGAAGCGTGCGAAATTAACCCTACAATGTTTGGCTGC CCAAAGCCCAAAATACCCATGTGCTAAAATCCACAGGAA AGAAGACAATGTCATTTGAGTGCACTTTAGAAATAACCGC AAACTCTAGCCAAAACTTCAGTTCATCTAAATTTTATGTA AGCCTACCTAAACAATATGCATACAAAGAAAATCAAAAG AATGGAAATATTTCAGTGTCTTCAAACGACTTCAGCTTCTC AAGCTGGCCAGAGTTTGAAAAGAACACTTACGATTTCCTT GAAAATCTAAATTTTATGAGCAGTGAAAAAGAGCTTGATG CTCTGGCTTTAAGAATTGCCATTTACTATGACATCGAGGA AACCGTCATATTTCCTTTCTGCTTTTCTAACGGCTTAATTA AAAAAGCTTACGAAATGAAACTATCTCTGGAAATCAATGG CTATCCATGCTCCAGCGAGAGCGGTGGTTCTTACCCATAC GATGTTCCAGATTACGCTTAAGGCAGCAGCCATCATCATC ATCATCACAGCAGC
pSB109RhsC6HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTTTGAATACCTATGG GTATGTAATGGGAAACCCTCTCCGGTATATTGACCCCACA GGAGAAAGCATCGCGATAGTAGAAGCGCTTGTAGTGGGG GCGGTAATTGTCGGCGGCGCAATGATTATTAACAGCTTGG

	GGAATCCTGCTGGCCAAGACTCGCAAGGTGGAGATAACT ATGGCGTCATACCTGACTGGCATAACCCTGACTACACTGG GCCTATTGCGCCGGAAGCGCCATCTGAAATGGCAAAAGG CGGAAAGCAAAACATTGATAATGAGTATGTTCGAGATGTT CTGGCTCAAGGGAAAAACTGCAACCCTTGCGAGTACTTAA GAAACCTCTATCAGAATGAGAGGAACGCAGTAGAGAGAC AGAAAATCAAGCAAGCGATGAAGCGCTTTAACTGCGACG GCAAAAATAGGTTCCAATAATGACTTGGTTCGTTGTTAGC ATTGCATCAACAATAGAGCTCTTGGCCCCCCCCCAAGAAG AGTATCCAATTTATGAGGACTTCTACCTATTCGAGGCGCA CTCTGAGGTTGAGCTTCAAAAAAAAATAAAATCCACGATG CAGATTATTGATAGCGCAGGAGACTGCACTTACCAAGGAA GCCCCGCTAAGCAGAAATGCTTAGGAACTCGAAAGGTAA GATCCATATACAACCCTCCGCCTTCAGATCTGGATTCATC GCCGCCCTCGGACGGAACAGAGCTTAGTCATTCGTTCTTC ATCGCAAGCTCATTGAAGGACGCTGAGGATTTTGCCCAAG GGAAGTCCGTACTCTTAAAATGCGTTGATGATTCAGAAGA AGGTGGTTCTTACCCATACGATGTTCCAGATTACGCTTAA GGCAGCAGCCATCATCATCATCACAGCAGCG
pSB109RhsC7HTlHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTATCAACACTTATGG TTATGTGAATGCAAATCCGATCAATCAAACAGATTGGCGA GGAACAGTCCCCGATCAAATCTGTTTAGCTGCATGCATTA CCGTAGGTACAGTGACGGGTGGTTATGTAGGTACTGTGGT CGGTGGATTAGCAGGGGGGGCAGGAGGTACGCTGGTATT GCCTATAGGTGGGACGATTTCCGGCGGTGTTGCTGGTGCA TCCGCAGGTGGTGCAATTGGAGCTATTGGCGGTGGTTTGG CAGGGAATGCTGCAGGAAACGCTTTTTGCCCAGAAGACG AATGTGATCCACCGGCAGGCACACAGTGTTATGAGTACAA TACTGGCCACAAACACAAAGGGATGGATCCCCATTACCAT ATATGGCAAATGAACAAATCCCCTAATGGGTGCTTCTGGA ATAAAAAAAGAGGACTGGCAGACACTCACCCCGTACAAC CCAGCGGACTTTCGTCCTGCTCATCGTACTCAAGTTGGACT AGTCAAAACGGAGGTTAAGCTATGGAGTTCAAATGCATCG GGTTCGATATTAGGCTCCCCGAAAGTCTTGATAATATTTC AGCCGACGCCACAGCCTGGCCGCAAGACACTCAACTTTAT GAGAGGGCACTTAATAGTCTAAAACTTCAAGAAAACGAG TTACAGCTAATCCAGATAGATACGCAAGAACAACTAGCG GAGCTTCAAGGCCTTATCAAGGCAGAAGGGAATGGCTCTG TTCTACTATCAATGGAAGTATACAGCGAAGTAATTGACGC ACTAAAGCTCTCTCGATTTCGTGATTTCAACTCAACTCAGG CCAGCATAGCCAGCTGGACCGAACTGGGTCTGGATGTATG CGATATAAACGGATACTTCTCTATACTTAAAATGGAGACT TCAATCTCTCAAGAAATATTAATACCGCCAAAAGAGATTG AGAAAGCTCACGCAATTTGCGAAGCAGCCAACATCCTCAT CAAAGAACACGCCCCCTTCGTTACTATAAAGTTGCGACAC

	TATTCAGGCTCTGCAGGTGGTTCTTACCCATACGATGTTCC AGATTACGCTTAAGGCAGCAGCCATCATCATCATCATCAC AGCAGCG
pSB109RhsC8HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTTTGAATACGCTTGG ATATGCCGGCCAAAATCCTGTAATGCACCAAGATCCTGAT GGCTTGGCATTTATGCTTTTAGCTCTCCCCTTTGCAGGTGG AACCAGCCTAGGCTCTGCGCTCACATCAGCTACAGCATTA GGAATAGGGGCTTGGGGGCTAACCACCCTTGGTGACTCCC CCGTGCAAATGGCTGCTCCAGGGAACGTAGCTGATTCGCA AATTGTTCGAGATTACGGCGAAGACGCATCTCAAGCACGC CAGTGTGGAGGTAAAGCCCCTGATCGATGTGAATGGCTTG AGCAAAACAAAAATAACTATCGCCCTGACCAGGTTAAGG CAACCCAAAAAGCATGGGGATGTCGTAGAAGTAGGCATG GTCGTTAAACTTAAATAGTGGAGTACGAAATGATTGACGA CCTTGACGAGTCATCCTCAATACTTAGGCTGCTCGTTGCA GACTCACTTAAAAGATGGACAGATAATGCTCTTCTTGGTC TGCTAAGTGAACAGGATGCTATTGTTAGAACCGCCGCAGC TCGAGAGCTACAGATGAGAGGGGGGCGTGACATATTTGA GAAAGTTCAGCATCTATCTGGAAACGAGAATCCCGAGACT CGGGAGATTGCAGCTTTCATTCTTGGGCAAATTGGAACAC CTAAAATGCCTTTTAAGGATGAATCGCTTCCGACCCTGTT ATCGCTTGCAGATGATGAGGATGCGGGGGTTCGATCAGCC ACTGCTGCTGCATTTGGACATTTGTGCTATGAAAGTATGC CTCTCAATGTAGAGAAATGCTTAATTAAGCTTTGCTCTGA CAGTAATGAAAGTGTCAGGGCATGTGCAGCATATGCACTA GGTAACTCTTCTGGAGGAAAAGAAGTTCGAATTTTACTTG AAAAGCTTTTGGCTCAAGAAGGAGTTGGCGAATATGCCGA GCTTGGGCTTGAAATTCTTGAGGCCAAAAAAAATAAAGGT GGTTCTTACCCATACGATGTTCCAGATTACGCTTAAGGCA GCAGCCATCATCATCATCATCACAGCAGCG
pSB109RhsC9HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTGTGAACACATACG GATATGTACAGGGAGCCCCCCTCAACCGTATTGATCCTTT AGGTCTAGCAGCAATAGAGATTGATATTCCTAAATCAGCA TACGATTGGATACCCGGAAATATTCGATTACCTGCGGGAA GACTGTTAGGGGGAGTATTGCTGGTGGCTAGTATTTCCGG AGCTACGCCACAAGCTGACAGCGATACAAAAGAGCAAAA CTGCCCAAAAGACTGCCCGCCGTGCAAGACAATTAGCGGC AGGATCATTCCTGTTGGAACCTTGGGCTATAGACCATTAG ACATTATTCCAGACGACGAAATGCAACACGGGGTTTATGG ATCTCATCACAACATGTTTGTTGCCAATCAGAACCCCAAC AATTGCCGATGCTTCTGGCAGAAGAAAAACTACGTATTAA AACCTGAGCAGCTACCGAAAAACGCAGTTCCAGTAGAGC CGTTTATAAACTGAGGTTACTCATGAGATATTCAAATGGA AAAAAAGTAGAGCTGGGAGACAAAATTGATCTTGGCGAC

	GGAGATACAGCTATAATTGTCGGAATCATTGATGAGAATC TCTACTCAGAAGAATACCCCAAAAGTGATTGGGAATACTT AAAATCAGGACTATTGATGCTCACAAGAGACTCAACACTA CTCCACTACCCAAAAATAGAAGACGAGATAAAACTAATA AGCAGAAAAGCCAAGGGTGGTTCTTACCCATACGATGTTC CAGATTACGCTTAAGGCAGCAGCCATCATCATCATCATCA CAGCAGCG
pSB109RhsC10HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTTAATACATTCGG GTATGTAACTGGAAATCCAATAAATTACTTTGACCCCAAC GGGCTTTGTGGCGTGGGTAGTCACATGGAGGTTTATTGGG ACAGATATAATGGACTAAAAACACGTTGCATTCTGAATCC ACCACCACCTAACCCTGCTGCAAAGTGCCTGACTGCAGAG TGCGTAATGAGGAATGGGGATGGTCGTGACACCAGAGTTC CATCCAATTGCCGGATGGTTTGCAGAGCAACTAGCGTCCC ATGCGGCATTCTTGGGGGTCCGCTAACGCCTGCTGGTGCG GCTTGTAGGGCCGCAGTAAGCAGCACGGCATGTTATATGC TGTGTTCTCCATATGACCCACCCTCGGAAGATGCACCCAA ACAGTGTGAGTAGTGATTATAATGAGAATCTTAAATTTGG CCTGTCCGAAATGTGGAAACAGCATTTCTTTTATTAGGCTC GGCTCTGACGGAAGAGCAACTTGCAAAAAGTGCAAAACA ACACTAATTGCAAGAAACTACCATAGAGACTTAGGAGTA GCGAATGCAGTCTTCTTTCTTGCCGCACTTCCAATTAATGC ACTACTATTCAAGGGCTGGATATTCTGGATGATGGATTTTT TGTCAGCACTTATTATTACCTCTATATTTTTACGAGGGGTC GAGTTGGAGATTGATCACGATCTAGTTGATGGTGGTTCTT ACCCATACGATGTTCCAGATTACGCTTAAGGCAGCAGCCA TCATCATCATCATCACAGCAGCG
pSB109RhsC11HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTTTGAACACCTATGG CTACGTCTATGGGAACCCTCTGACGTATTCAGACCCCAAA GGCTTAACCCCTGCGGCTGCTGGCTTATGCTTCGTCCCCGG TGTTGGCTGGGTTGGATGCGGTGCCGTCGCTGTCGGCGCT GGCATTGGAGGGTTGGCTTGCTACCTCACTGGCACCTGTC AGCGGTTTGCAGAGGCCTGTAGCAATGTTTGGAACGAGGT AGCTGGTGGTGACGATGCGGGTGGTGGTGAAGGCAACAC CAACCCGTATGCCGGGCCTGTCGATGAACCGGTAATCGTT GTTGATGAGAACGGCAACGCGATTCCCGTTGAACCTGGGC AGAGCGTGAACAGCTCACCGAATGGCGACTACCAACAAG TCGTTGGTGCAGACGGTAAACCAACGGGGGATCGACTTGA TCGAGGAGGACATCGGAATCAGTCCGATCCTCGCGCTCAG GGCCCTCATGGACATCGGCCAGGAGTGACTACTCCTGATG GCAACCCCCACCTACCGATATATTAACTATGCCTAAATTT AACTACGACGACATCGTTACAGCTACCAGCGCCGCGCCAT TAAATGCACGCCCAGGGAATAAGGCTTGGGTTGTTGGGGT CTTTGAAACTCGAAGAGGCGATTTTTTGAAGTCTTTCCCG

	GAAGGAGTTGTGTATGTAATCGAGTTTGAGGATGGCGCTT CAGTGGAAGTAGCTGAGAACCACTTAGAGCTTTACATCGA CCCTACAAGTGGTGGTTCTTACCCATACGATGTTCCAGATT ACGCTTAAGGCAGCAGCCATCATCATCATCATCACAGCAG CG
pSB109RhsC12HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTGAACACATTTAG TTATGTGTATGGGAATCCAGTAAATCTTATTGATCCTAATG GATTGGCAGGTACGACAGTCGATGCTTACTGCCTGAGATA TGGACCACAAGCATGTGCTGATGTAACCGGCGGGTCAGCC ACATCAGGATCAATCAATGCAACAGGAATCAGTCTATGGT GCTTGCTTACTAAAACCTGTTCTGCAAATGAAAGCTCCGC CCAAAATGAAAAAAAACCAAAAGACTGTCCACCTGGAAC GAAAGATATCGATAAAGCCAAGAATGACTATGGCTGGGA CAAAGATACACTTCATGGAATAAAGTCGGGAGCCCATGG CGGGATGAGCAATGGAAAGTCCTGGACAGGAATAACACC AGACGGAGAGGTTGGAATAAATGAAGGTGGAAAGTGGCA ACCTCAAGGACACTGGGAGGATCTATTGTGAATAGCTCTA CATACCGGCTTACCTTTCATATACAACACCCCACTATACCC GCCTCTGAAATAGAAGCATCTCTCAGCTTTCCCACACGAA TTTCTCAATCTTCAGGAGCGCGTAGAAAGACAAAATCCGG AAAGATTTTAGAGGGAACCTACGCTTGCACTAGTATTATT TTTCTTTTACATAGAACACCTTTAAAATTCGAAGAAACCC CCATAGAAAAAAAAATAGAAGAATCCATAGATAAATTAG ATACCGACTACCTGAAAACCCTTGTCAACTCAGGTGGAAA ATGCAGTTTTATTGCAGGAGTCTATTCCGATCAAAACATT GCATTCTCATTAGACCTGAGAATCATTGAGAGACTGGCTG CCGATAAAATAGGTGTAAAATTCGACTTCTATGGAGGTCC AGAGGGTGGTTCTTACCCATACGATGTTCCAGATTACGCT TAAGGCAGCAGCCATCATCATCATCATCACAGCAGCG
pSB109RhsC13HTIHA	CCATACCCGTTTTTTTGGGCTAGCGAAGGAGATATACATG CACCATCATCATCATCATGGTGGTTCTCTTAATACATATGT ATATGTCGAAGGAAACCCCCTTGCCTATGTAGATGAATGG GGGCATTCAAAAACCAATGGAAAGCATGCAAACAAATCA AGCCCAATGACAGTGGGCGACTTTAACAAGAACTCTGACC CTGCCAAGATACAAGAAGCAATCAGAGAGGCCGAAACCA ATGGGAAAAACGCACATGCAAAAGCACTCAAGGGACTAC TGAAAGTAATTAAACGTACTAAAAGCCTAACCCCGCATGG TGTTATAGAGGACATCCTGGAAAATCTATGTGAATCCCAT CCTGAGGAACCGGCCTGCAAAATATTTACTCCACCTCCCG CTATAGTTTGGTGCAAAGAAGGAGAAACTTCGCCTCCCTT ACCCCCTATGTCCAGTTTAAGTTCCCCGGACCGACCAGAA ATTATAGATCTTGGCCAGCTATAAGGATCGCCATATGACC ACCAGTGACGGAATAACAGATAGCGACTGGGAAAGCATT ATCATTTCTGCAGAGGAGATTGCCGAGTTGACAGGCAGGG AGATAGATGCTCGCTTTGCTCAAAAAAAGATACTTTTCCA

	GCTAGATAGATTGGAGAAAAAATATGGCCGCCTTCCAACC ATACTAAGCACAAAAGCGGACTATATCGACTCTACTGACG AGCGGCTATCTTTACTCAAAGAGGCATATATAACGGCTGA CGAAATTCAGGACAAAAAAAACAAGGCATTTATCAGCAG TTCCATCATAGAAGTCTATCTAGAATTGCCAGAAAAAAAA TCCTTTGCCCTGTACTGGTTAGAGAAATTCGAGAGCGACC TGAAAGACTATCCCAAGGACGAATATCTTCTCGATCTACA CGTGCAGTTCAATAAAAAACTTAATCAACCAAACCCTAGC AATCAGGGTGGTTCTTACCCATACGATGTTCCAGATTACG CTTAAGGCAGCAGCCATCATCATCATCATCACAGCAGCG

Table 4. List of Plasmids.

Plasmid	Primers/gene fragments used	Source
pSB109		[92]
pTES1	pTES1_insert	This study
pTES2	pTES2_insert	This study
pMCSG53		This study
pMMB67		This study
pMMB67 T/I 6	pSB109RhsC6HTIHA, P53RhsC6Tox/Imm_F, P53RhsI-HA_R	This study
pMCSG53 T/I 1	pSB109RhsC1HTIHA, P53RhsC1Tox/Imm_F, P53RhsI-HA_R	This study

Generation of Induced Cell Pellets

E. coli strains carrying the putative toxin and immunity alleles (Table 1) were grown overnight at 37ºC, 250 rpm supplemented with the appropriate antibiotic. The overnight cultures were subcultured the following day into 500mLs of sterilized LB medium supplemented with the appropriate antibiotic. Once the subcultures reached an OD_{600nm} of ~0.7, the culture was induced with 0.1% IPTG and/or 1.0% Arabinose and grown at 37ºC, 250 rpm for at least 3½ hours. Cell Pellets were made by centrifuging the induced cell culture at 3,214 rcf at 4ºC for 40 minutes or until the supernatant appeared clear. The supernatant was discarded following which the induced cell pellets were frozen at -80ºC for later use for purification or Co-IPs.

Generation of Whole Cell Lysates (WCLs)

The frozen cell pellet was thawed at room temperature for 20 minutes, then Lysis Buffer (50 mM Sodium Phosphate (pH 7.6), 300 mM NaCl, 10 mM Imidazole, 200 µM DTT) was added to the cells. The cells were resuspended in the lysis buffer using a mixture of inversions and vortex mixers. Once resuspended, the mixture was added into a 50 mL plastic cup to be sonicated. For sonication, the tip was submerged into the mixture slightly above the bottom of the cup and then an amplitude of 45% was used to sonicate for 10 seconds at a time. Following each 10-second sonication burst, the culture was incubated in ice for 1 minute or until cooled. In total, the mixture was sonicated for 3:00 minutes unless noted otherwise. Then the mixture was added to a 50-mL conical tube and centrifuged at 3,214 rcf at 4ºC for 40 minutes. Following centrifugation, the supernatant was filtered through 0.22 µm filters until at least 15 mL of filtered whole-cell lysates were obtained which were used for Co-IPs. The leftover unfiltered WCLs were saved at -80ºC. In the case of protein purifications, all the supernatant was filtered through 0.22 µm filters before column loading unless noted otherwise.

Generation of WCLs with Lysozyme

The frozen cell pellet was thawed at room temperature for 20 minutes, then Lysis Buffer (50 mM Sodium Phosphate (pH 7.6), 300 mM NaCl, 10 mM Imidazole, 200 µM DTT) with Lysozyme (1mg/mL) was added to the cells. The cells were resuspended in the lysis buffer by inverting the tube repeatedly until a homogenous mixture was produced. Once resuspended, the mixture was incubated for 1 hour in a 37ºC water bath. Following incubation, the mixture was poured into a 50 mL plastic cup to be sonicated. Refer to previous section for the remaining methodology.

Purification of Toxin and Immunity Alleles

To obtain purified fractions of the putative toxin and immunity factors, filtered whole-cell lysates prepared on the day of purification were loaded onto 2 mL of HisPur™ Ni-NTA Resin (ThermoFisher). The WCLs were allowed to slowly flow through the column until only all of it had flown through. Then, the column was treated with 100 mL of Wash Buffer 1 (50 mM Sodium Phosphate (pH 7.6), 300 mM NaCl, 20 mM Imidazole, 200 µM DTT), 100 mL of Wash Buffer 2 (50 mM Sodium Phosphate (pH 7.6), 300 mM NaCl, 50 mM Imidazole, 200 µM DTT), and 100 mL of Wash Buffer 3 (50 mM Sodium Phosphate (pH 7.6), 300 mM NaCl, 100 mM Imidazole, 200 µM DTT). Following each of these steps, 1 mL of flowthrough was collected as a representative sample to be used during Western Blots. Once the last wash buffer had completely flown through, the Elution Buffer (50 mM Sodium Phosphate (pH 7.6), 500 mM NaCl, 20 mM Imidazole, 200 µM DTT) was added and the flow through was collected in 1 mL fractions until complete.

Co-IP of Toxin and Immunity Factor

Filtered whole cell lysates were prepared on the day of the Co-IP and kept on ice throughout the Co-IP process. To perform the Co-IPs magnetic Pierce™ G protein beads (ThermoFisher) were used. The protocol provided by the manufacturer was used to perform the Co-IP using the filtered WCLs. The beads were incubated with either 6 µL of Rabbit HA IgG antibody or with no antibody to serve as the control. Samples from each step of the Co-IP were saved in microcentrifuge tubes and stored at -20°C to Western Blot at a later point.

Immunoblot of Purification and Co-IP Samples

Samples for Western Blot were prepared by mixing 60 µL of each sample with 40 µL of 4X Laemmli SDS buffer unless noted otherwise. The samples were then loaded onto a 12% polyacrylamide gel, and proteins were separated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) at 105 V through the stacking and resolving gel unless noted otherwise. The separated proteins were transferred from the gel to a nitrocellulose blotting membrane (Amersham™ Protran™) with a 0.45 µm pore size at 105 V for 1 hour and 15 minutes. Membranes were blocked for 1 hour with a mixture made with 1X TBS (pH 7.6) and Non-Fat Powdered Milk (0.05 mg/mL). Then, the membranes were incubated with 10 mL of the Milk in TBS mixture with 1:8000 dilution of α-HA (Abcam ab18184) antibody or 1:2000 µL of α-His (Sigma Aldrich H6908) antibody overnight at 4°C. The following day, the membranes were washed with Milk in a TBS mixture 3 times for at least 5 minutes each. After the washes, the membrane was once incubated with a secondary Goat anti-Rabbit IgG (H+L) antibody conjugated with alkaline phosphatase (Invitrogen), at 1:8000 dilution, or secondary Goat anti-mouse IgG (H+L) conjugated with alkaline phosphatase (Invitrogen), 1:4000 dilution, diluted with Milk in TBS for at least 3 hours. Following the incubation, the membranes were once more washed with Milk in TBS three times for at least 5 minutes each wash and one time with TBS alone for at least 5 minutes. Finally, the membranes were incubated with 264 µL of nitro blue tetrazolium (NBT) (50 mg NBT in 1 mL 70% dimethylformamide (DMF)/30% H2O) and 132 µL of 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (50 mg BCIP in 1 mL DMF) mixed in 40 mL of alkaline phosphatase buffer (AP) (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM, $MgCl_2$) until a signal showed up or overnight. Finally, the membranes were washed with water and dried at room temperature. Pictures of the blot were taken with a Mobile Phone.

CHAPTER THREE

EXPERIMENTAL RESULTS: PUTATIVE TOXIN AND IMMUNITY PROTEIN INTERACTIONS

Growth Curves in *E. coli* Suggest 3' of *rhsC* and *rhsI* Are Toxin and Immunity Pairs

Our bioinformatic analysis of the *rhs* locus in *P. aeruginosa* supported a hypothesis that the 3' end of *rhsC* encodes a bactericidal toxin domain and *rhsI* encodes an immunity factor to neutralize the toxin. To investigate this hypothesis, we started by expressing the putative toxin and immunity alleles in a heterologous system to study their effects. This work was carried out by a current lab member, Abigail Banas who is a fourth-year PhD candidate. The RhsC-toxin domain (immediately downstream from the predicted protease cleavage site) was cloned with or without its cognate immunity gene into a low-copy plasmid backbone, pSB109. Following this she performed an overnight growth curve, where she either induced the toxin alone or the toxin in the presence of the immunity factor in *E. coli*. The growth curves provided us with results that suggested that the putative toxin allele does encode a toxic protein whose toxicity was neutralized in the presence of the immunity protein (Figure 7). She has performed this growth curve experiment with the 13 different alleles of the toxin and immunity factors and observed similar results (data not shown). These results support our hypothesis that the C-terminal domain of RhsC functions as a toxin and RhsI a cognate immunity factor.

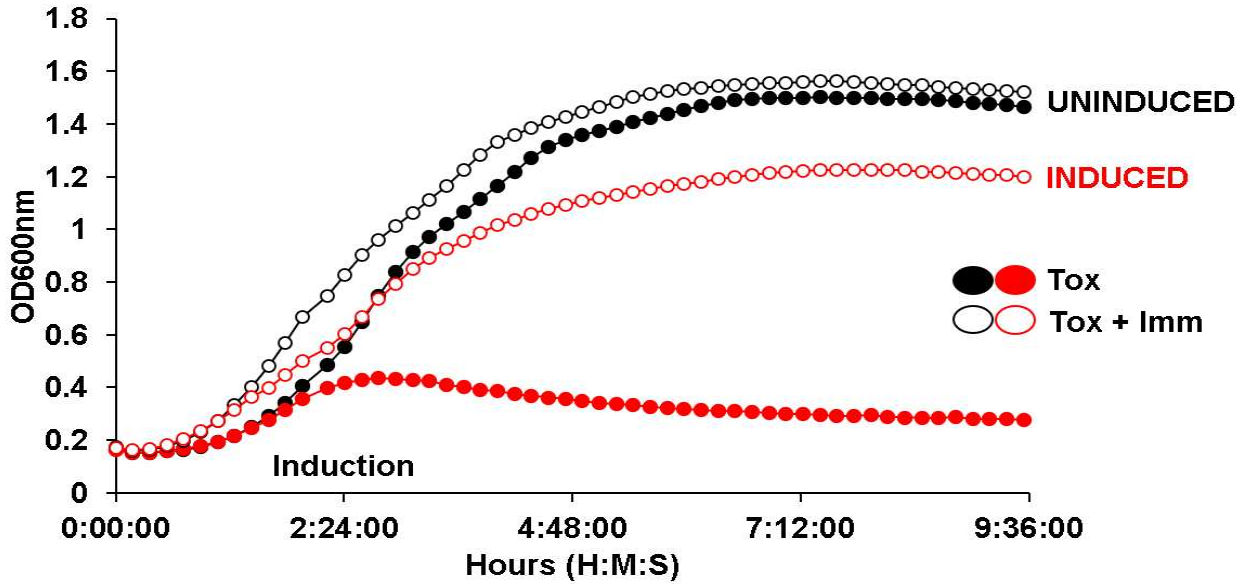

Figure 7. The RhsC-Toxin Domain and RhsI Function as Toxin-Immunity Pairs. A growth curve of *E. coli* expressing a toxin and immunity allele upon induction with 0.1% arabinose , generated by Abigail B., which shows the toxicity of the putative toxin allele in the absence of its immunity factor (red filled circle). The apparent toxicity is reduced in the presence of the putative immunity factor (red unfilled circle). This growth defect is not present when arabinose is not added (black filled and unfilled circles).

Generation of pMMB67 Plasmid Carrying Toxin and Immunity Alleles

The growth curves provided evidence that suggested that each immunity factor was responsible for the neutralization of their respective RhsC-toxin domain. This neutralization effect has been shown to occur via direct binding and occlusion of the active site in other toxin/immunity systems [86]. We hypothesized that RhsI neutralizes the C-terminal toxin domain of RhsC in the same way. We began by performing Co-IPs to test whether the RhsC-toxin domain and respective RhsI physically bind to each other. I began by cloning all of the toxin and immunity alleles into the pMMB67 plasmid using their appropriate primers and gene blocks (Table 2,3). I chose this plasmid because we could easily add an N-terminal 6x-HIS tag to the RhsC-toxin domain and a C-terminal HA tag to RhsI. The plasmid carries the pTAC promoter and the $lacI^q$ element, which allow for much higher expression efficiency and strong

repression in the absence of the inducer Isopropyl ß-D-1-thiogalactopyranoside (IPTG), respectively [93]. We hoped that by using a plasmid with these properties, we would overcome previous cloning difficulties like the toxin being mutated, and immunity factor being mutated following cloning of toxin. I made this construct by Gibson assembling the gene blocks listed in Table 2 with a digested pMMB67 plasmid product. Following the Gibson assembly, I transformed NEBα *E. coli* cells with the Gibson product and obtained correct transformants through PCR verification. To ensure that we were working with a plasmid containing the correct toxin and immunity alleles, all transformants were saved and sequenced by Sanger Sequencing and MUSCLE alignment. Overall, I was able to generate NEBα *E. coli* transformants with pMMB67 carrying the toxin and immunity alleles that I used to perform my initial Co-IPs.

Co-immunoprecipitation of Toxin with Immunity Factor

To test our hypothesis, I first subcultured *E. coli* containing the pMMB67 Toxin/Immunity 6 construct in 100 mL of LB medium until it reached an OD_{600nm} of 0.7. Then, I induced the culture with 0.1 mM IPTG and harvested the cells by centrifugation after 3½ hours. I sonicated the cultures for a total of 3:00 minutes to obtain WCLs which I used to perform a Co-IP. In these experiments, HA-tagged RhsI was used as prey to pull down the RhsC-toxin domain. Upon western blotting of the resulting sample fractions, I observed that the toxin domain was co-eluting with the immunity factor, lane 8, but only in the presence of beads conjugated with antibody against the HA-tag (Fig. 8). These results suggest that the RhsC-toxin domain and RhsI physically bind to each other. This further suggests that RhsI binding may be responsible for the recovery of the RhsC-toxin domain-dependent growth defect (Fig. 7).

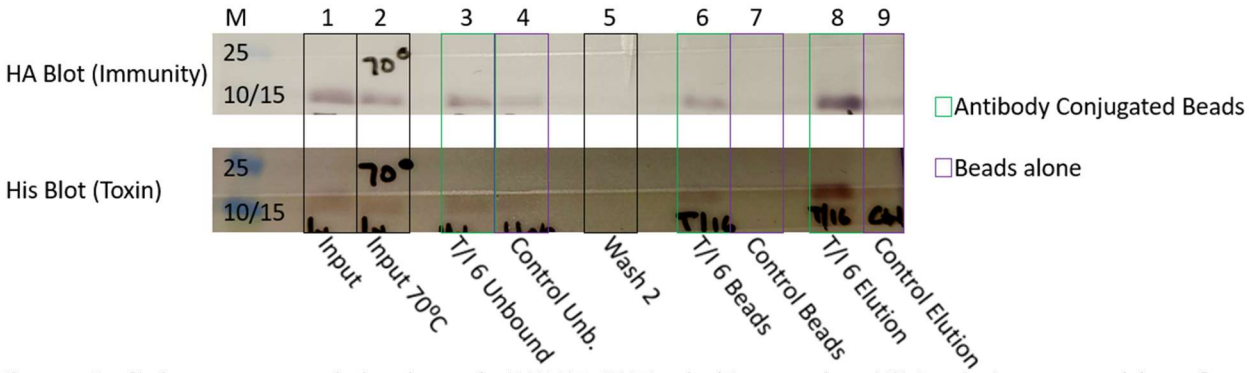

Figure 8. Coimmunoprecipitation of pMMB67 Toxin/Immunity Allele 6. A western blot of samples from a Co-IP of Toxin/Immunity 6, the top blot is immunoblotting for the HA tag while the bottom is immunoblotting for the His tag. (Lanes 1) Samples of the whole cell lysates of T/I 6. (Lane 2) Sample of whole cell lysate of T/I 6 that was heated to 70ºC. (Lane 3) Sample of the whole cell lysates following incubation with beads conjugated with α-HA antibody. (Lane 4) Sample of the whole cell lysates following incubation with beads conjugated with no α-HA antibody. (Lane 5) Sample of the wash step following incubation of beads with WCLs. (Lane 6) Sample of the beads conjugated with α-HA antibody. (Lane 7) Sample of the beads that were not conjugated with any antibody. (Lane 8) Sample of elution from beads that were conjugated with α-HA beads. (Lane 9) Sample of elution from beads that were not conjugated with α-HA beads. The toxin blot has increased contrast to improve visibility.

Optimization of Co-IP Protocol to Improve His-Western Blots

As I performed the Co-IPs on different Toxin/Immunity (T/I) alleles, there was a recurring issue. Although I was able to get a bright signal of the immunity factors, the toxin was extremely difficult to see regardless of exposure duration or amount of sample that was loaded. I performed a series of experiments to parse out why exactly the blotting issue may be occurring. First, I performed an experiment to confirm that our anti-His antibody was functioning properly. For this experiment, I used a purified sample of the His-tagged protein, C68A obtained from the Ulijasz Lab, which blotted extremely well (Fig. 9). I diluted the protein with 4X Laemmli SDS buffer to the concentration of 4.5, 2.5, 1.25, and 0.05 mg/mL and then incubated the blot membranes overnight in either 2.5 µg or 5.0 µg of primary antibody. This experiment confirmed

that the primary and secondary antibodies were functioning appropriately but regardless of antibody concentration, it was unable to detect the protein at a concentration of 0.05 mg/mL (Figure 9-lane 4,8). These data suggested that either my cultures were not induced properly or that the protein concentration in the culture was insufficient for detection by the antibody.

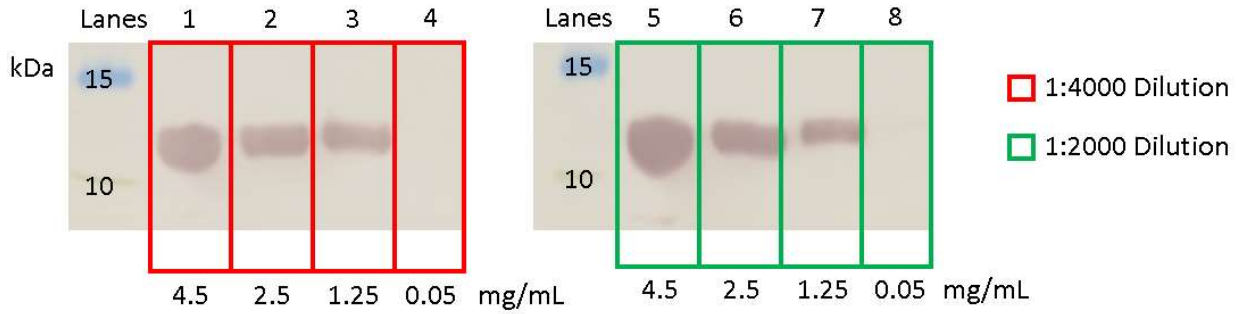

Figure 9. Protein & Antibody Concentration for Improved Detection. The left western blot is immunoblotting for the His-tag with an antibody dilution of 1:4000 while the right one is also immunoblotting for the His-tag but with an antibody dilution of 1:2000. In each case the protein, C68A, was diluted down from 5 mg/mL to 4.5, 2.5, 1.25, and 0.05 mg/mL, respectively (Lanes 1-8).

Next, I performed several experiments to assess the time needed for the proper induction and detection of the toxin. To start, I subcultured 1 mL of each overnight culture of T/I 5 and T/I 11 into 5 mL of fresh LB media. Once the cultures reached an OD_{600nm} of 0.7, I induced them with 0.1% Arabinose and pulled 1 mL of culture every hour to check induction using Western Blot. As shown in Figure 10, the concentration of T/I 4 increased in a time-dependent manner with the highest concentration appearing to be at 4 hours; however, toxin 11 was undetectable regardless of time. As 4 hours of induction were not enough to detect the presence of the toxin in T/I 11, I next tried an overnight induction at a lower temperature and a lower rpm in the shaking incubator. For this experiment, I also increased my culture volumes to 500 mL instead of 100 mL which I had been using up till this point. So, I subcultured 5 mL of overnight culture of T/I 5 and T/I 11 into 500 mL of fresh LB supplemented with the appropriate antibiotics. Then, I let the culture grow at 37°C, 250 rpm until it reached an OD_{600nm} of 0.7 following which I induced the

cultures with 0.1% arabinose. After induction, I lowered the temperature and rpm to 18°C at 150 rpm and incubated the cultures overnight. The following day, I harvested the cells via centrifugation at 3,214 rcf for 40 minutes and sonicated the samples as usual. As a result of this experiment, I saw that there was an increase in the detection of toxin 5 in the overnight induction compared to 4 hours of induction (Figure 11). Although it was still difficult to detect toxin 11 compared to toxin 5 (Fig. 11), I did see a faint signal in both 4 hours of induction and overnight induction (Fig. 11). Hence, I decided to proceed with another Co-IP with cultures from an overnight induction of T/I 11.

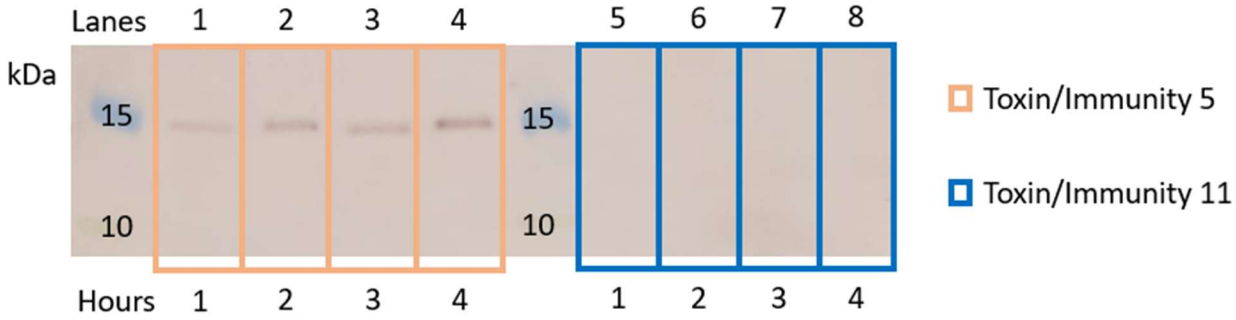

Figure 10. Time-Dependent Induction of Toxin. A western blot of cell pellets that were collected every hour following a 0.1 mM IPTG induction. (Lanes 1,5) Samples collected after 1 hour of induction. (Lanes 2,6) Samples collected after 2 hours of induction. (Lanes 3,7) Samples collected after 3 hours of induction. (Lanes 4,8) Samples collected after 4 hours of induction.

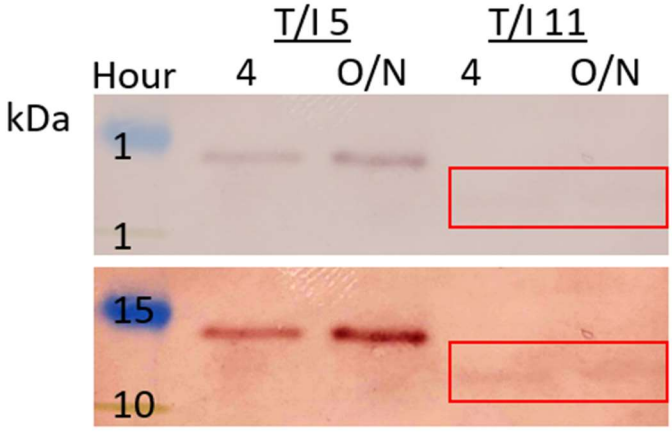

Figure 11. Overnight Induction vs. 4 hours induction of toxin. A western blot of cell pellets obtained from cultures that were either induced for 4 hours or overnight at 18°C, 150 rpm. The red box indicates where the faint bands for Toxin 11 were visible on the physical blot. Both blots are the same, the bottom one has increased contrast for improved visibility.

Performing Co-IPs Using the Updated Protocol

Using the updated expression protocol, I performed Co-IPs with T/I 11. I also included an additional centrifugation between the sonication and filtration step of my whole cell lysate to remove as much cellular debris as possible. When I performed the Co-IP with the WCLs of T/I 11 following this updated protocol, I saw a clear signal in my His immunoblots (Figure 12B) which had not been present up while using the old protocol (Figure 12A). The blot also shows that the toxin was present in the elution fraction even though the beads were conjugated with an antibody for the immunity factor (Fig. 12B). Therefore not only was my updated protocol successful in showing a clear His-tagged toxin signal but also clearly showed that the Toxin was co-eluting with the Immunity factor.

Following this successful Co-IP immunoblot, I proceeded to perform the Co-IP with six other alleles of the toxin and immunity factor. Here, I have shown toxin and immunity factor alleles 2, 3, 4, 5, 11, and 12 (Fig. 13-18). Overall, these data indicate that RhsC-toxin domains physically bind their respective RhsI immunity factors.

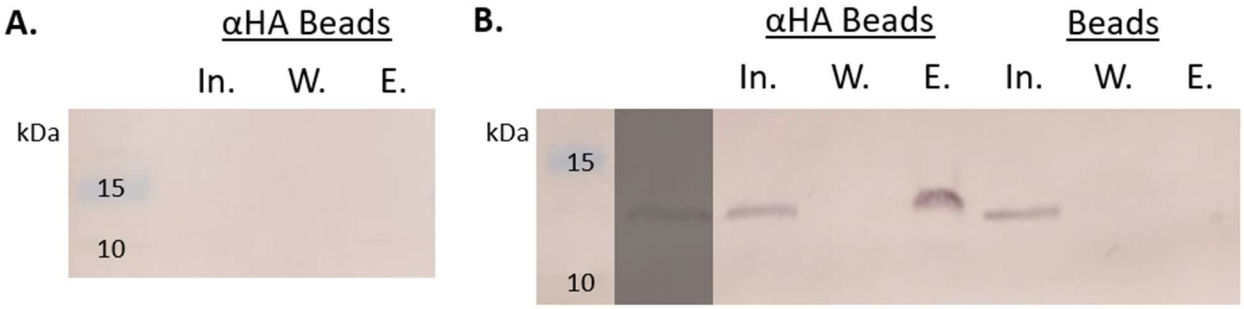

Figure 12. Co-IP of Toxin/Immunity 11. (In.) This lane contains a sample of the whole cell lysates which were loaded on the gel. (W.) Lane containing a sample of the buffer that the beads were washed with during Co-IP. (E.) Sample obtained following elution of the beads with SDS. (A) This is a His western blot of samples from an old Co-IP where the cell culture preparation as well as immunoblot preparation was carried out using an already existing protocol in the lab. (B) This is a His western blot of samples obtained from a Co-IP of T/I 11 where the cell culture preparation and immunoblot was carried out using my updated protocol. The grayed-out area of the blot contains the sample as In. lane.

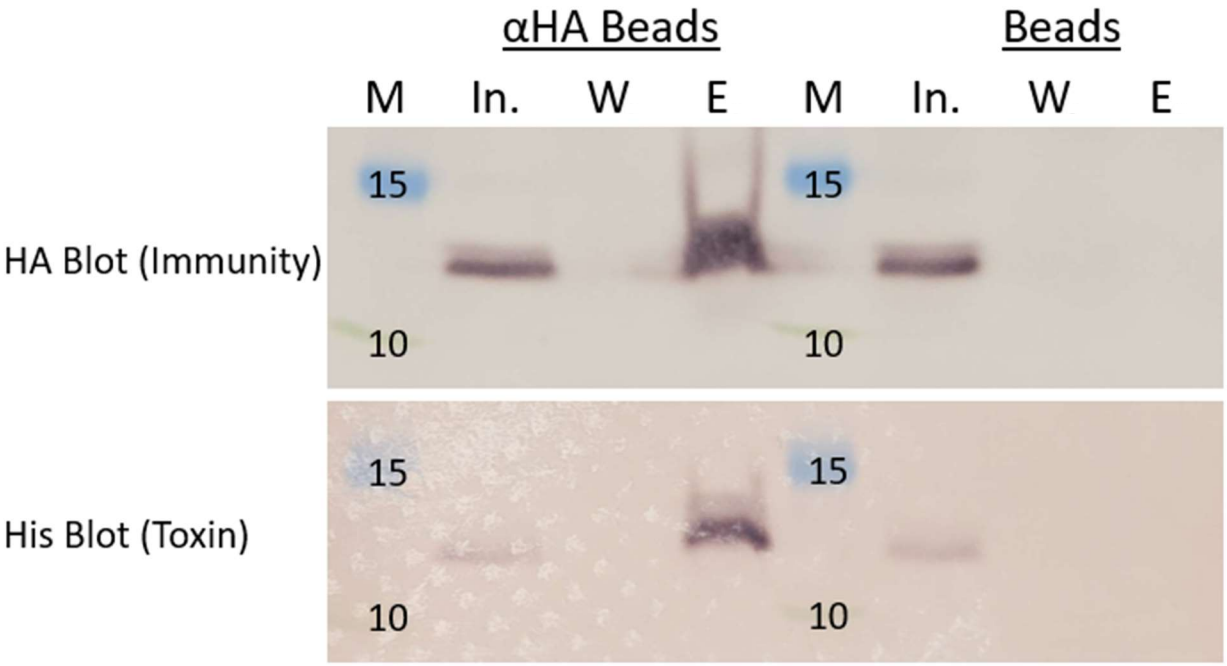

Figure 13. Co-IP of T/I 2. This is a western blot of samples from a Co-IP of T/I 2. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor.

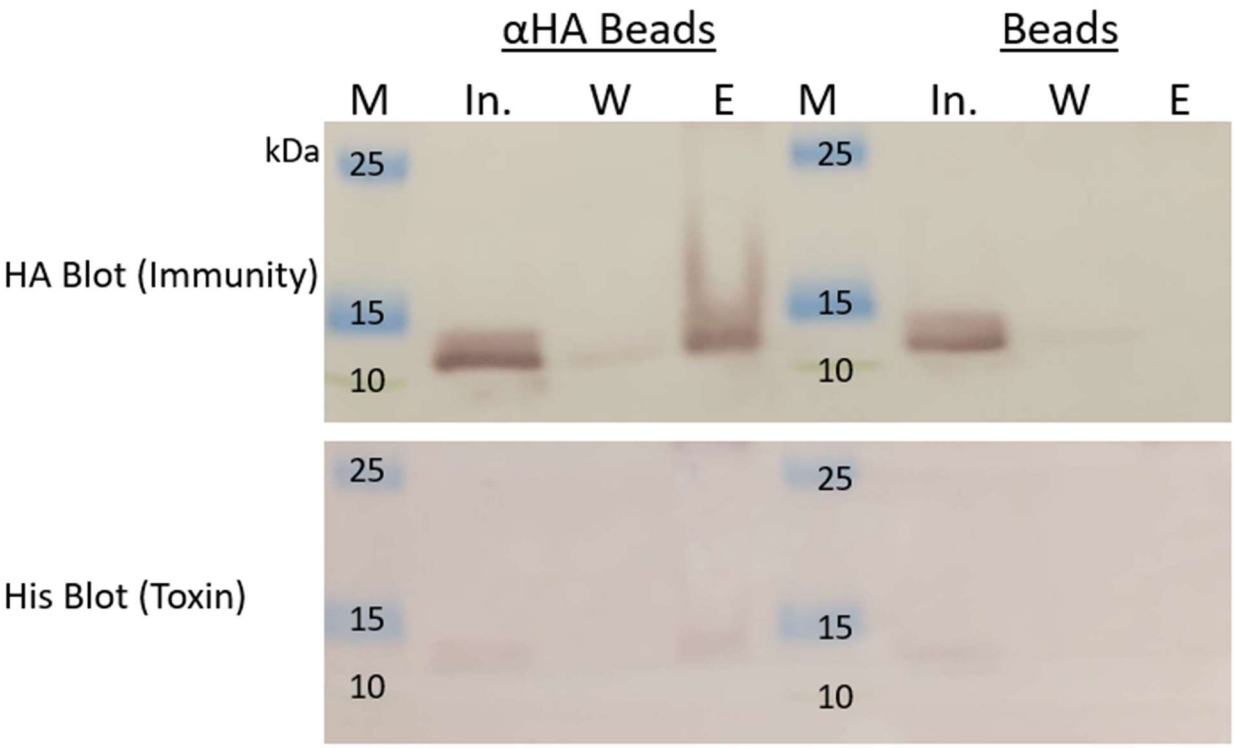

Figure 14. Co-IP of T/I 3. This is a western blot of samples from a Co-IP of T/I 3. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor.

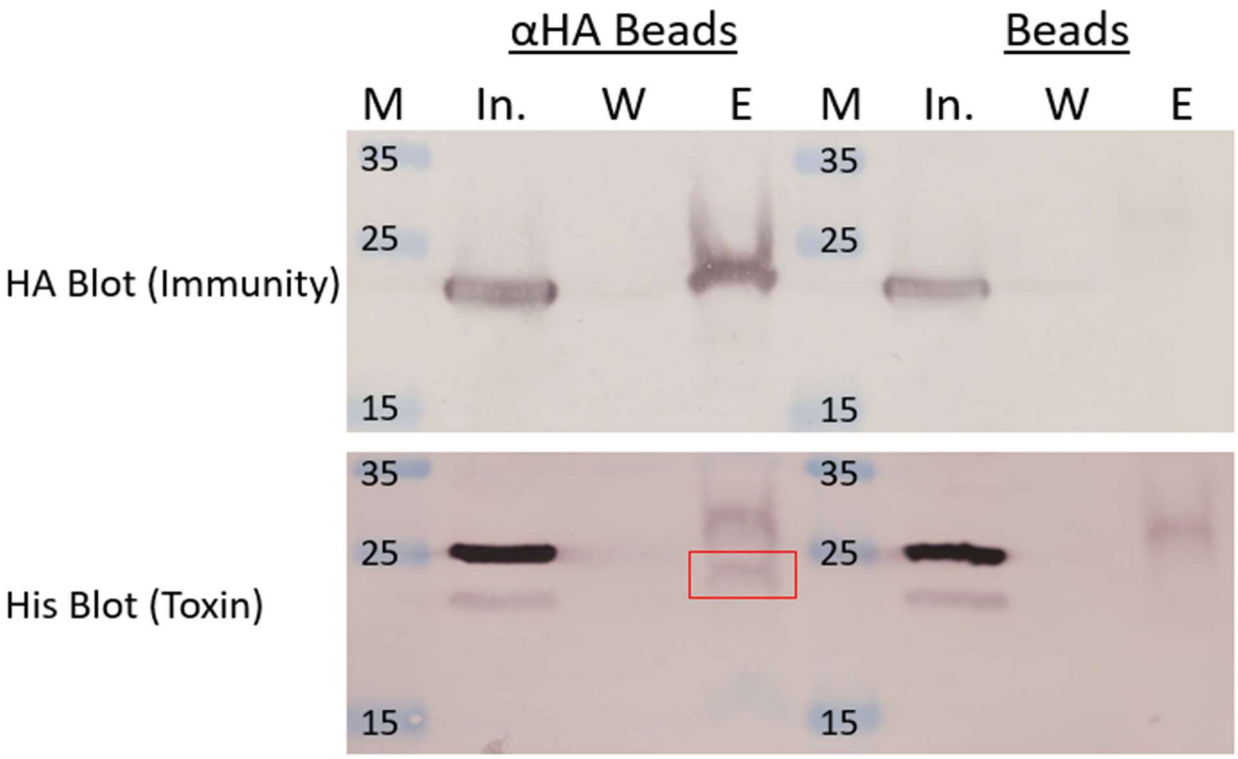

Figure 15. Co-IP of T/I 4. This is a western blot of samples from a Co-IP of T/I 4. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor (highlighted in red).

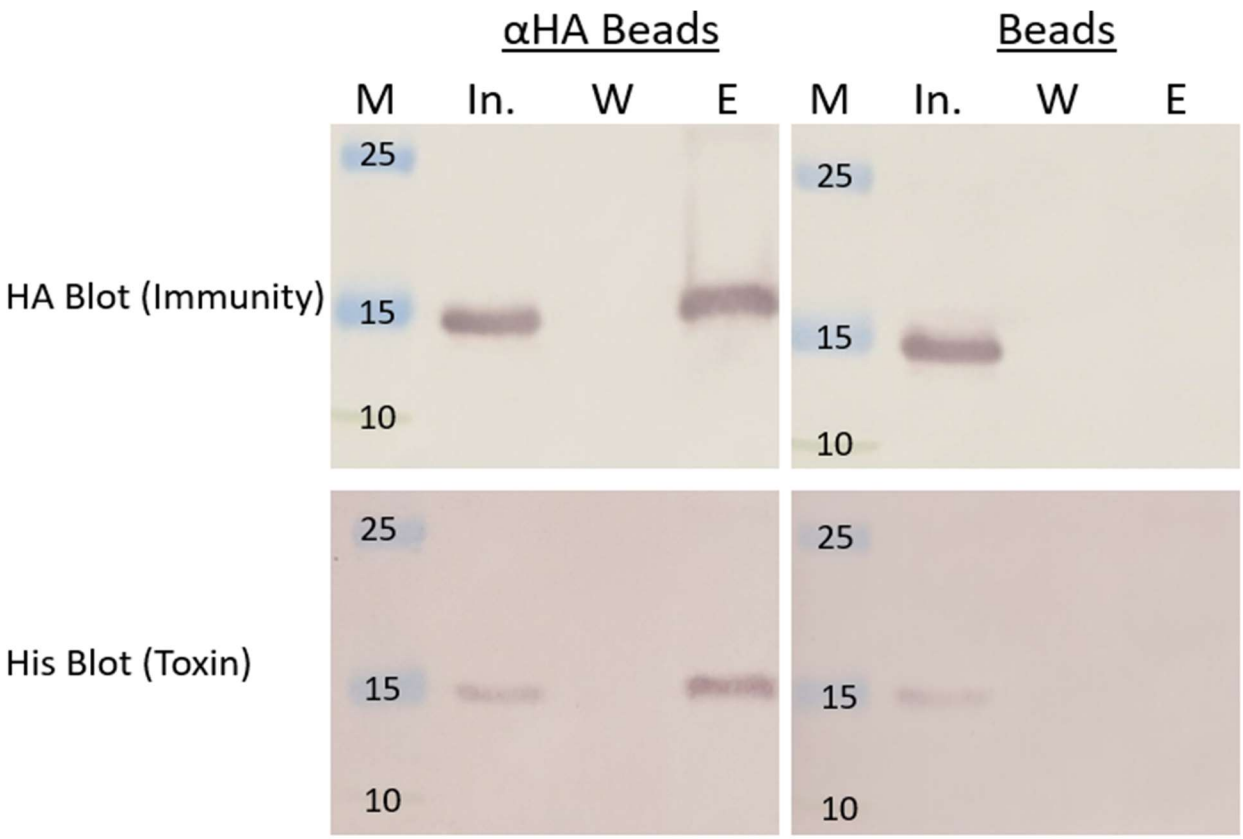

Figure 16. Co-IP of T/I 5. This is a western blot of samples from a Co-IP of T/I 5. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor.

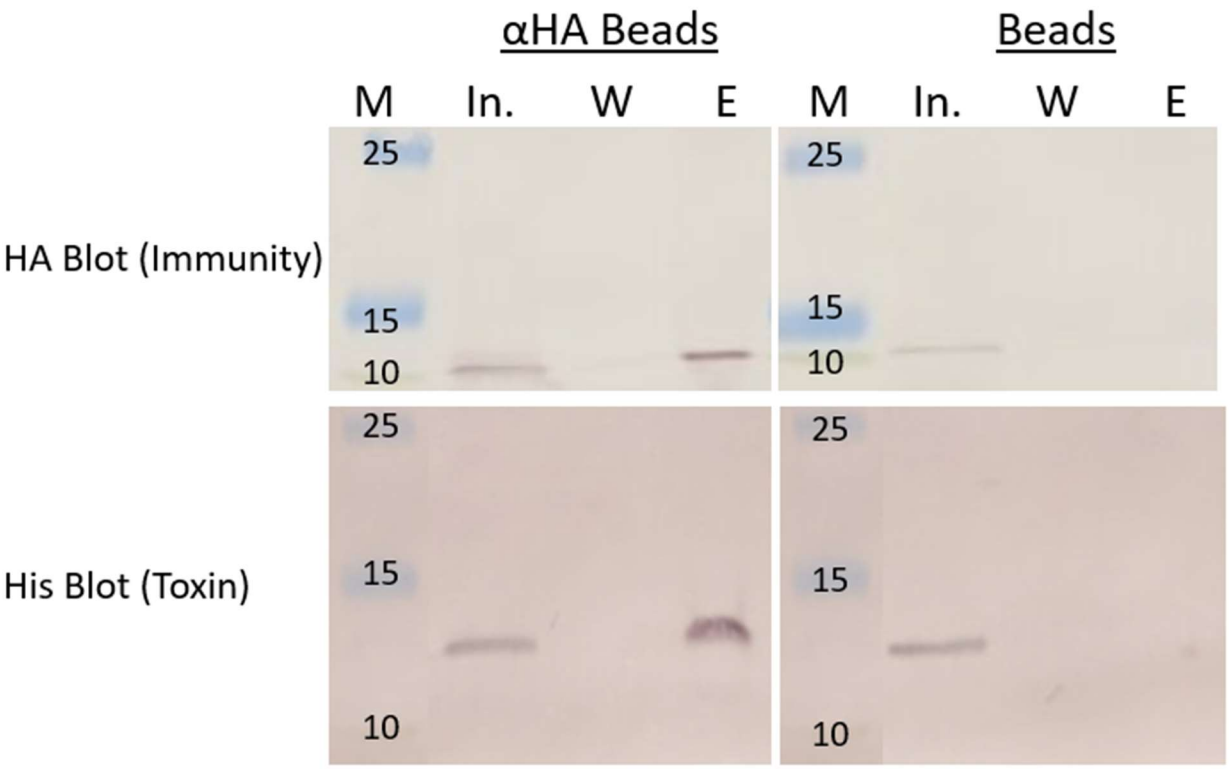

Figure 17. Co-IP of T/I 11. This is a western blot of samples from a Co-IP of T/I 11. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor. The his blot of the toxin is a cropped version of the blot shown in figure 12B.

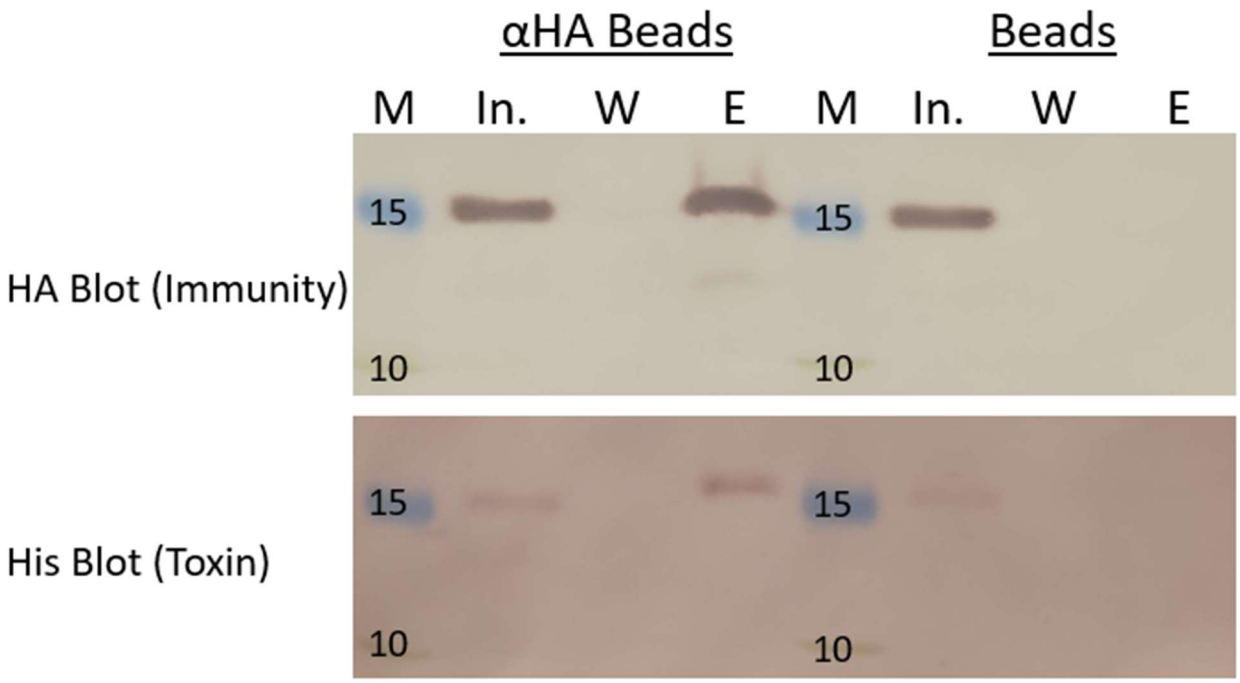

Figure 18. Co-IP of T/I 12. This is a western blot of samples from a Co-IP of T/I 12. Sample of whole cell lysates is denoted by In. Sample of the buffer used to wash the beads is denoted by W. Sample of the elution at the end of the Co-IP is denoted by E. The blot shows the co-elution of the toxin with the immunity factor.

Purification of Toxin and Immunity Proteins for Further Analysis

The successful co-elution of the toxin with the immunity factor during Co-IPs suggests that the toxin and immunity factor interact with each other, but it tells us nothing about the binding specificity and strength of these proteins. Understanding the binding specificity and strength across the different alleles of the toxin and immunity factors would provide us with further evidence that these alleles encode toxin and immunity pairs. The first hurdle to overcome to test these aspects of the protein lifecycle was that I needed the proteins in extremely high concentrations. As noted previously, this was a substantial problem as I must express toxins that target bacteria inside of bacteria. This meant that I had to utilize cloning strategies which involved either a very tight control over the expression of the toxin or the constitutive expression of the immunity factor to minimize the side effects of a leaky promoter for the toxin. As noted in

the previous sections, I was able to generate strains carrying all the toxin/immunity alleles which I used to perform Co-IPs as I worked on designing a working protocol to start performing protein purifications of the toxin and immunity factors.

Generating a Protocol for Producing WCLs

To optimize a general purification protocol for 6xHIS-tagged proteins, I started working with a construct of a catalytically inactive toxin domain from CdiA^{PABL017} (pMCSG53 17 CdiA [HA] 3238-Hibit). This was done because the protein has been purified before, immunoblots extremely well, and is not toxic to *E.coli*. My first protein purification resulted in the finding that a majority of the protein was stuck in the insoluble fraction following sonication (Figure 19, Lane 1). Alongside that, any protein that did remain in the lysate (Fig. 19, Lane 2) was either not bound to the beads or was lost during the wash steps (Fig. 19, Lanes 3-7) which meant that there was no detectable protein present in the elution step (Fig. 19, Lane 9). Hence, I hypothesized that the protein was most likely getting stuck in inclusion bodies that were being generated during sonication which led to the protein being stuck in the insoluble fraction.

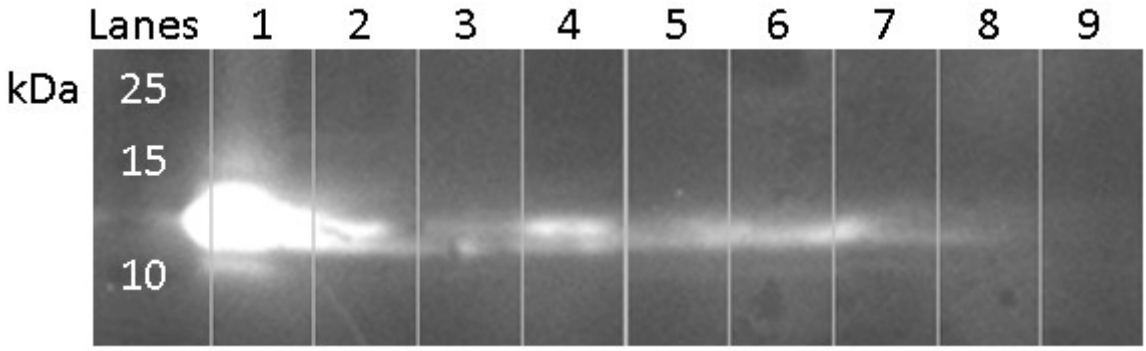

Figure 19. Protein Purification of 17 CdiA [HA]3238-Hb. This is an image of western blot of samples obtained from a protein purification of 17 CdiA[HA]3238-Hb. (Lane 1) This is a sample of the insoluble fraction produced following sonication. (Lane 2) This is a sample of the WCL produced via sonication of the cell pellet. (Lane 3,4) This is a sample of the flowthrough following loading of the resin with WCLs. (Lane 5,6,7,8) These are samples of the flowthrough following the loading of the resin with the different wash buffers. (Lane 9) This is a sample of the flowthrough following loading of the resin with Elution Buffer.

To address this issue, I performed a literature review and found a paper on viscotoxins in which the authors incubated their cell pellets in a lysis buffer containing lysozyme and DNase I before sonication [94]. Hence, I conducted two experiments to assess the effectiveness of lysozyme treatment for improved lysis. In one experiment I added lysozyme (1 mg/mL) to my lysis buffer and incubated the cell pellets for either 30 mins or 60 mins at 37ºC. For the second experiment, I only sonicated the pellets for 2 minutes or 3 minutes. As a result of these experiments, I found that the best conditions to improve the release of the protein into the soluble fraction was 60 minutes of incubation with lysozyme at 37ºC (Fig. 20A) followed by sonication for 3 minutes (Fig. 20B). Overall, I used the CdiA [HA] 3238-Hb protein to improve the original protocol to include an incubation step with lysozyme, a longer sonication of 3:00 minutes instead of the original 2:00 minutes and reduced the number of washes to 3 from 4.

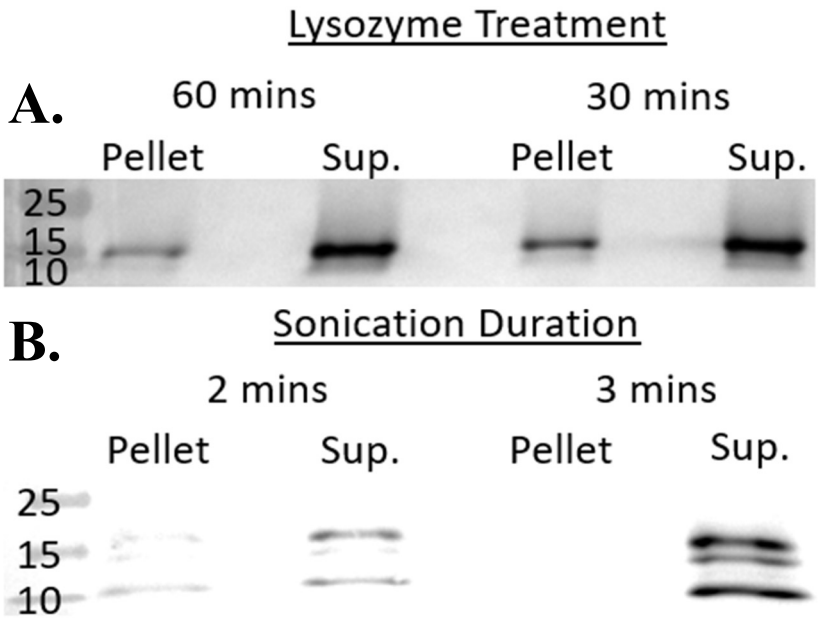

Figure 20. Optimization of WCL Preparation. These are western blots of CdiA[HA]3238-Hb construct samples obtained following different WCL preparation protocols. (A) A western blot of samples obtained from the lysozyme treatment of a cell pellet. (B) Cell pellets were sonicated for different durations and western blotted to assess the quality of sonication. Pellet was obtained by centrifugation of whole cell lysates following each treatment.

Protein Purification of pMMB67 Toxin/Immunity 7

Once I had produced a protocol for the preparation of the WCLs using the CdiA[HA] 3238-Hb construct, I performed a protein purification with an RhsC Toxin/Immunity 7 construct. This was selected as our first construct because it expressed well as determined by the Western Blot of cell pellets. For this protein purification, I harvested cells from a 100 mL induced culture of T/I 7, following which I incubated the cell pellet in Lysis Buffer with lysozyme before sonication. To purify the toxin and immunity protein, I loaded the WCLs on 2 mL of Ni-NTA resin and washed it three times with 100 mL each of wash buffers with increasing concentrations of imidazole (20 mM, 50 mM, and 100 mM) followed by elution with a buffer containing 300 mM imidazole. In this purification, I found that although the protein was present in the WCLs, all of the protein was flowing through the column instead of binding to the beads (Fig. 21, WCL & FT Lanes). Importantly, there appeared to be very little Toxin protein present in the WCLs to begin with and this problem appeared in other attempts at protein purification with other alleles as well (data not shown). These repeated failures at protein purification led me to work with a different plasmid, pMCG53, in the hopes that it will help overcome the low protein concentration issue and allow for better detection of the toxin via immunoblotting.

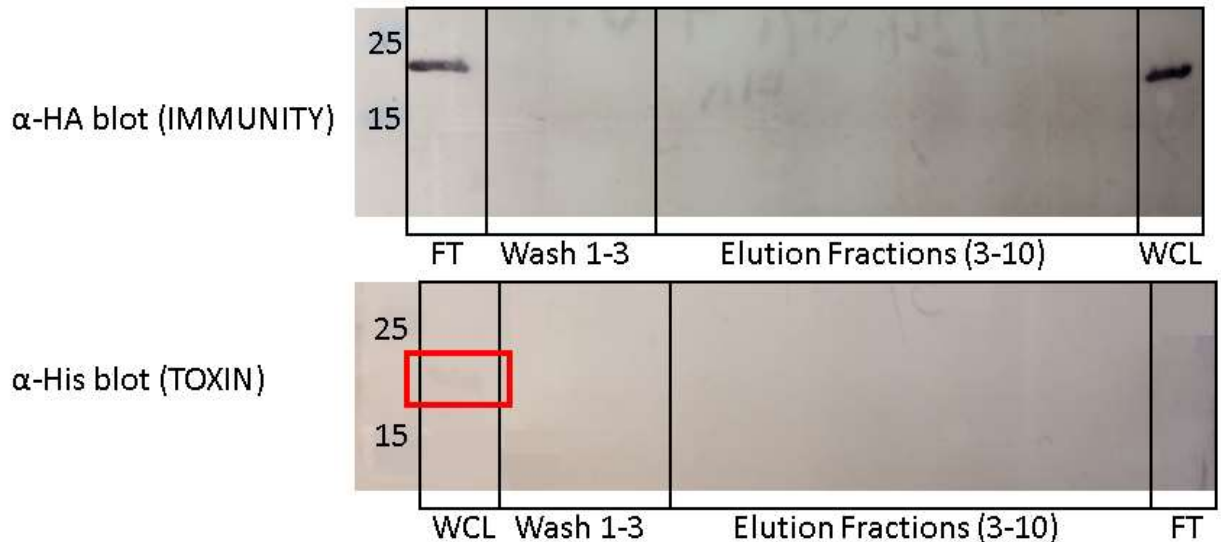

Figure 21. Protein purification of pMMB67 Toxin/Immunity 7. This is a western blot of samples obtained from a protein purification of T/I 7. (WCL) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (Wash 1-3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Elution Fractions (3-10)) These are the different fractions collected from the column following loading of the elution buffer. (FT) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. The red box is to highlight where the Toxin blotted due to the band being exceptionally light.

Generation of pMCSG53 Plasmid Carrying Toxin and Immunity Genes

The high-copy plasmid, pMCSG53, is routinely used to generate recombinant proteins in high concentrations. The plasmid provides high expression alongside strong repression through the use of the T7 promoter. This promoter is only recognized by the bacteriophage T7 RNA polymerase which is present in specific *E. coli* cell lines like BL21 DE3. This allows for the cloning of highly toxic proteins in alternate cell lines which do not have the bacteriophage T7 RNA polymerase as they should not be able to express the target protein. Thus, I started by cloning Toxin/Immunity 1 and 6 due to the low toxicity of allele 1 and the high toxicity of allele 6. To do this I utilized primers P53_RhsC6ToxIm_F for allele 6, P53_RhsC1ToxIm_F for allele 1, and P53_RhsI-HA_R which anneal to the gene block pSB109RhsC6HTIHA, and

pSB109RhsC1HTIHA, respectively, to amplify Toxin/Immunity fragments. These fragments were then Gibson assembled with a SspI digested pMCSG53 product to produce two pMCSG53 plasmids carrying toxin/immunity 6 and 1, respectively. Following transformation, minipreps of each NEBα strain carrying T/I 1 and 6 were sequenced. Unfortunately, I was unable to recover any transformants carrying an unmutated toxin 6 but I recovered multiple transformants carrying an unmutated Toxin/Immunity 1. Thus, I proceeded with the transformation of BL21 DE3 with pMCSG53 Toxin/Immunity 1 which expressed a detectable amount of the Toxin and Immunity factor during western blots (Fig. 22).

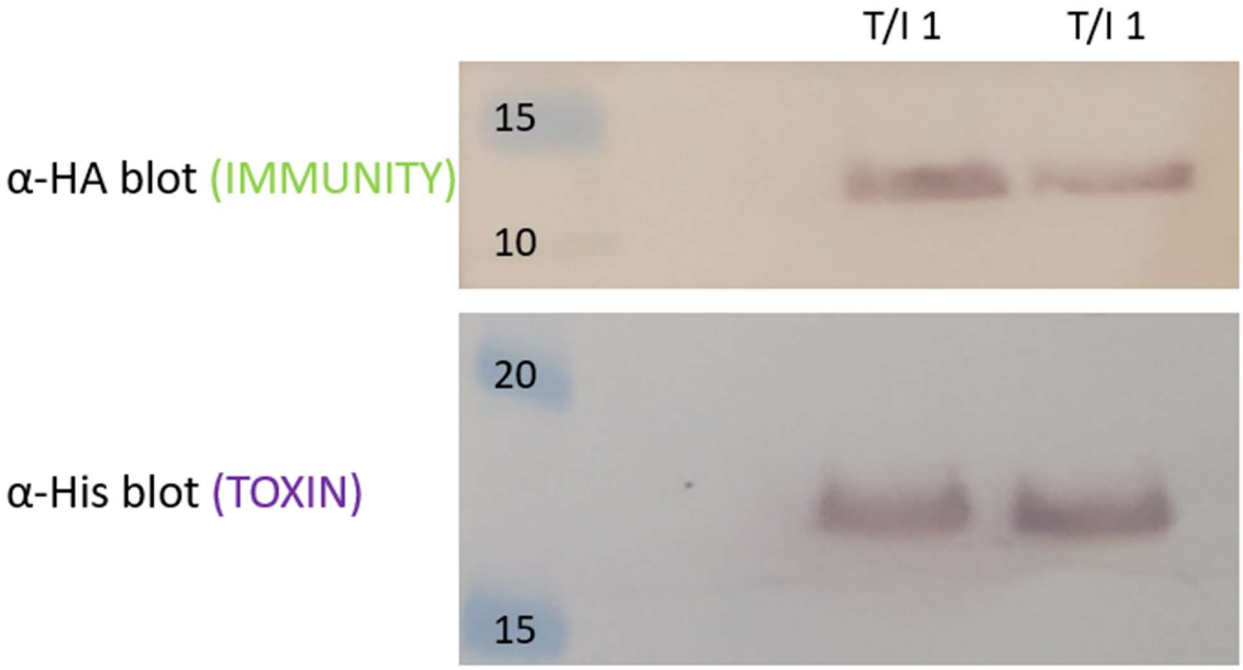

Figure 22. Western Blot of pMCSG53 T/I 1 Cell Pellets. This is a western blot of induced cell pellet harvested from 1 mL of induced cultures from replicates.

Troubleshooting Protein Purification of pMCSG53 Toxin/Immunity 1

The expression of p53 T/I 1 appeared to be high enough for clear detection (Fig. 22) and it sequenced correctly as well. Therefore, I proceeded with a protein purification using a 100 mL induced culture of BL21 p53 T/I 1. I performed this purification exactly as noted under the section "Protein purification of pMMB67 Toxin/Immunity 7" and once again found that my

target proteins appeared to be flowing through the column suggesting a lack of binding to the Ni-NTA resin. Even more peculiar was the finding that the toxin appeared to not be present at all in the immunoblot for the His tag (Fig. 23). These data suggested two distinct possibilities to me. First, the proteins did not have enough time to bind to the resin. Second, the toxin was being degraded; consequently, the immunity factor flows through and the toxin is not detected during the immunoblot. The toxin degradation hypothesis appeared to be the more viable of the two possibilities as it has been noted to be the case in many toxin/anti-toxin systems in bacteria [95].

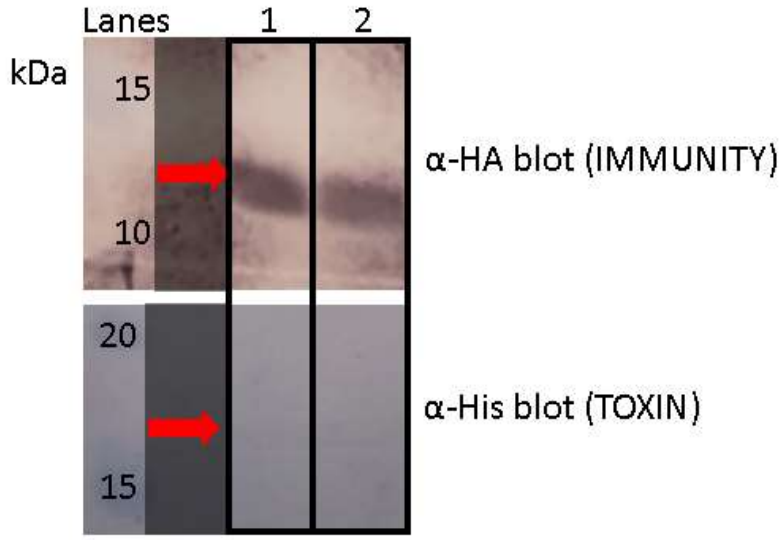

Figure 23. Protein Purification of pMCSG53 T/I 1. This is a western blot of samples obtained during the protein purification of T/I 1. The red arrows indicate the area where the proteins are expected to blot. (Lane 1) This is a sample of the WCLs obtained following the sonication of the induced cell pellet. (Lane 2) This is a sample of the flowthrough obtained following loading of the resin with WCLs.

I decided to perform some experiments which would allow me to test for both possibilities as this system appears to be unique based on bioinformatic analysis hence, either occurrence was possible. To test the first possibility, I performed an experiment where I incubated the resin with the WCLs overnight at 4°C while keeping every other aspect of the protein purification the same. A western blot of the resulting samples showed a distinct banding

pattern that I had not seen yet, but the issue of the proteins not binding to the resin was unchanged (Fig. 24).

Figure 24. Incubation of WCLs with resin overnight. These are western blots of samples obtained from a protein purification of T/I 1 after incubation of resin with WCLs overnight. The red boxes are to highlight the new bands alongside the expected bands. (WCL) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (FT) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. (Wash 1-3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Elution Fractions) These are the different fractions collected from the column following loading of the elution buffer.

Therefore, I proceeded with a second experiment to test the possibility of the toxin being degraded during the lysate preparation and purification. During this experiment, I did not change anything except for the addition of phenylmethylsulfonyl fluoride (PMSF), a non-specific protease inhibitor, at a concentration of 1 µL/mL, in all the buffers used during protein purification. This resulted in the clear detection of the toxin in the samples obtained during the purification, highlighted in red squares (Fig. 25). Overall, these data suggest that the toxin was being degraded, but that alone appeared not to be responsible for a lack of binding of the proteins to the resin.

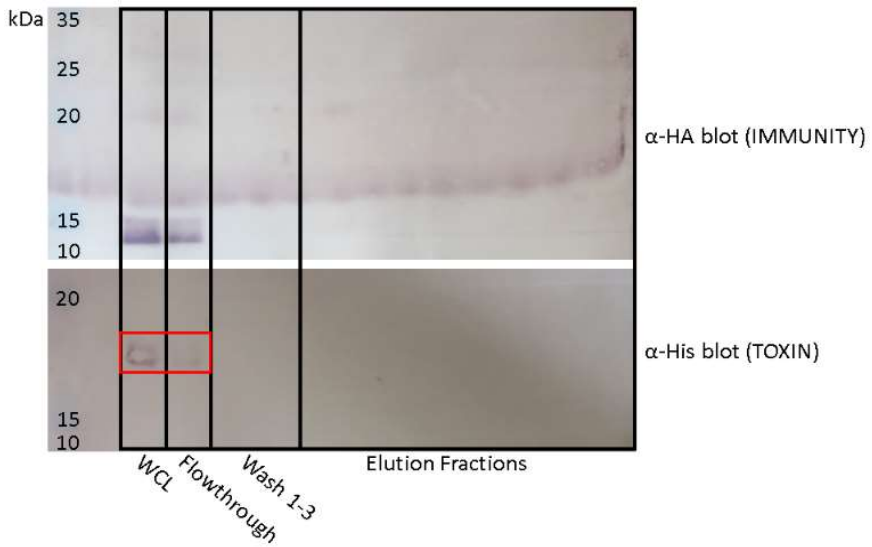

Figure 25. Protein purification of pMCSG53T/I 1 with PMSF. These are western blots of the samples obtained during the protein purification of T/I 1 with PMSF. The red box is to highlight the presence of the Toxin bands that were previously not blotting clearly. (WCL) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (Flowthrough) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. (Wash 1-3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Elution Fractions) These are the different fractions collected from the column following loading of the elution buffer.

These experiments provided some answers, but they were unsuccessful in resolving the failed protein purifications. To further assess why this might be occurring, I hypothesized that the toxin might be targeted by a variety of proteases, that PMSF could not inhibit, which were released during the lysozyme treatment and sonication to produce WCL. To test this possibility, I designed one experiment where I added PMSF and a broad spectrum protease inhibitor cocktail, Roche's cOmplete protease inhibitor mini tablets. In another experiment, I added PMSF and the inhibitor cocktail but did not perform the lysozyme treatment. In this experiment, I also increased the sonication time to 4:00 minutes to compensate for any lost lysis efficiency. These two experiments served as a critical step towards my first successful purification as I found that, unexpectedly, the addition of the cOmplete tablets and PMSF did not resolve the binding issue (Fig. 26A, Lanes 1,2). Meanwhile, in the second experiment in which I skipped the lysozyme

treatment, the toxin blotted extremely well with the toxin and immunity factor not blotting in the flowthrough sample (Fig. 26B, Lanes 1,2). This suggested that the lysozyme treatment resulted in a negative effect on the toxin in such a way that it was unable to bind the resin which resulted in it flowing through. Yet, the proteins were not detected in the elution fractions (Fig. 26B, Lane 4) which suggested that the proteins were still stuck on the beads. These data led me to hypothesize that the elution buffer did not have a high enough concentration of imidazole to disrupt the binding of the toxin to the resin.

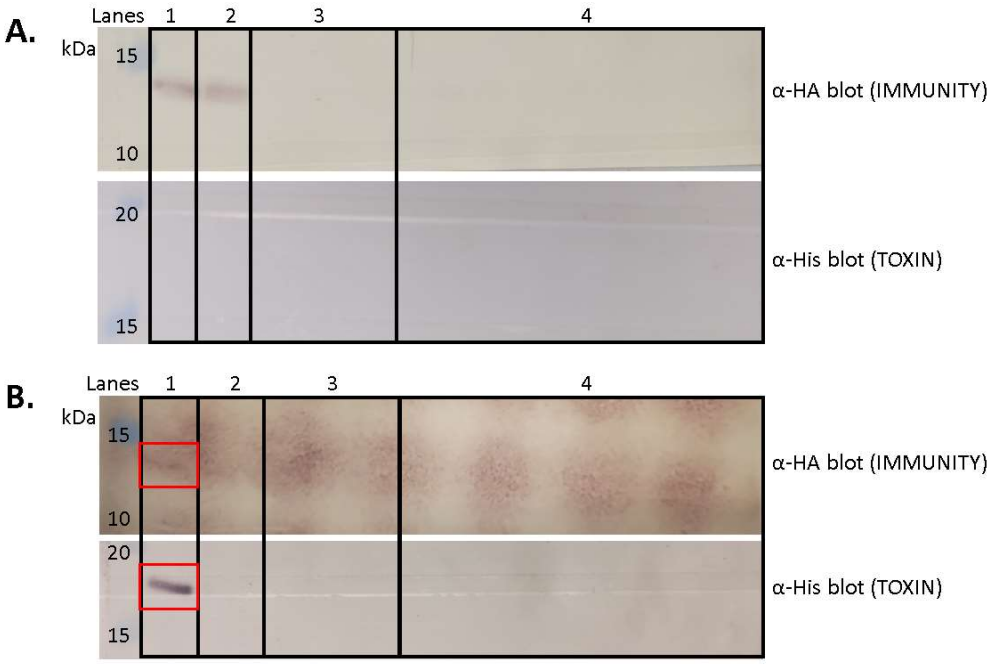

Figure 26. Troubleshooting cell pellet lysis protocol using T/I 1. These are western blots of samples obtained during the protein purification of T/I 1 following alternate cell lysis protocols. The red square is to highlight where the expected bands for the Toxin and Immunity factor are present. (A) These are samples obtained from the column that was loaded with WCLs which were prepared as usual with the addition of PMSF and cOmplete mini tablets. (B) Western Blots of samples obtained from the column that was loaded with WCLs where were not treated with lysozyme for an out but did include treatment with PMSG and cOmplete mini tablets. (Lane 1) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (Lane 2) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. (Lane 3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Lane 4) These are the different fractions collected from the column following loading of the elution buffer.

Therefore for my next protein purification of pMCSG53 T/I 1, I increased the concentration of imidazole in the elution buffer from 300 mM to 500 mM. Altogether, the following protein purification experiment included the following changes: no lysozyme treatment of the cell pellet before sonication, the addition of PMSF (1µL/mL) with cOmplete mini tablets (1 tablet/10 mL), increased concentration of imidazole in the elution buffer, and collection of a sample of the resin for western blot. The western blot of the samples from this experiment showed that the toxin and immunity factors were detected in the elution fractions (Fig. 27, Lane 5). Interestingly, I found that while the Immunity factor blotted at its expected weight, signified by the red arrow, the toxin was primarily detected at a much higher molecular weight. This higher band was also detected in the WCLs as well but a band at the expected weight was also present (Fig. 27, Lane 1). A faint band at the expected size appears in the resin sample as well as the first elution fraction but these samples are predominantly composed of a very high molecular weight protein (Fig. 27, Lane 4,5). Meanwhile, a high molecular weight protein was also detected in the immunity factor immunoblot but a majority of the sample was composed of a protein that blots at the expected weight of the immunity factor (Fig. 27, Lane 5).

Together, it is unclear what this protein purification is suggesting as the high molecular weight proteins may be dimers and trimers of the toxin. It is also possible that the higher molecular weight protein in the toxin immunoblot is the toxin interacting with the immunity factor and the SDS-PAGE is not enough to disrupt this interaction. Alongside these, it could be that the higher molecular weight proteins are some *E. coli* proteins that are being detected by the antibody. Hence, I next set out to perform a series of experiments to test these possibilities.

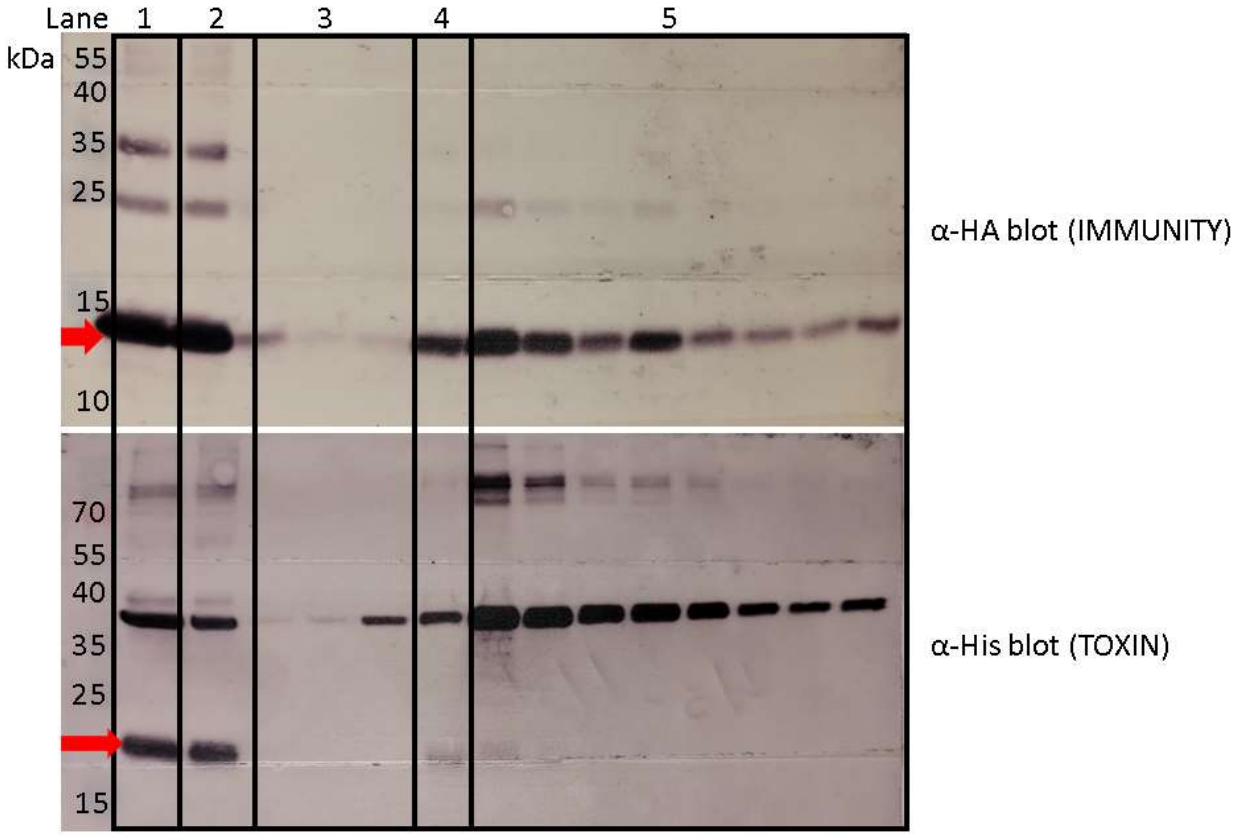

Figure 27. Protein Purification of T/I 1 with updated protocol. Western blots of samples from the protein purification of T/I 1 following the updated lysis protocol with increased imidazole concentration in the elution buffer. The red arrows indicate the proteins are expected to blot. (Lane 1) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (Lane 2) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. (Lane 3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Lane 4) This is a sample of the resin which was obtained at the end of the protein purification. (Lane 5) These are the different fractions collected from the column following loading of the elution buffer.

Checking Empty Plasmid Inductions and Denaturing Purification of Toxin/Immunity 1

There were several possibilities for the bands that were present in the protein purification of Toxin/Immunity 1, as noted in the previous section. I started by verifying that the bands that were present in Figure 27, were not due to an *E. coli* protein. To test this, I produced WCLs of induced cell cultures of *E. coli* BL21 DE3 and BL21 DE3 pMCSG53 according to the protocol which I used to prepare Toxin/Immunity 1 WCLs. If the higher bands were due to BL21 DE3

cell proteins then I expected to see a detection of these proteins in a western blot of the cell pellet and as well as the WCLs. In the western blot, I found that regardless of cell pellet or WCLs no proteins were detected by the antibodies in the absence of the toxin and immunity alleles (Fig. 28 A/B, Lanes 1-4). This suggests that the high molecular weight bands were not due to the non-specific detection of *E. coli* proteins rather they are specific to Toxin/Immunity 1.

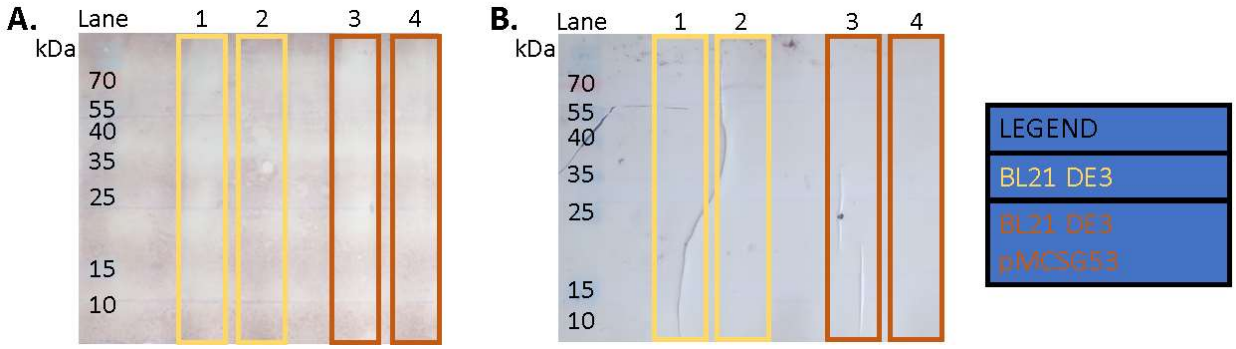

Figure 28. Control Experiments to assess non-specific detection of *E. coli* proteins. Western blots of cell pellets and WCLs to assess any background signal due to the cell line and plasmid that is being used. (A) α-HA Blot of cell pellets and WCLs. (B) α-His Blot of cell pellets and WCLs. (Lane 1) This is a sample of an induced cell pellet of BL21 DE3 prior to sonication. (Lane 2) This is a sample of the WCLs produced by sonication of the cell pellet of BL21 DE3. (Lane 3) This is a sample of an induced cell pellet of pMCSG53 BL21 DE3 prior to sonication. (Lane 4) This is a sample of the WCLs produced by sonication of the cell pellet of pMCSG53 BL21 DE3.

Next, I set out to perform a denaturing protein purification of T/I 1 to test whether any specific interactions between or among the toxin and immunity proteins are responsible for the high molecular weight bands. To test this, I followed the usual protein purification protocol but used buffers that also contained 6M Guanidine, a very strong denaturant. A western blot of the purification samples (Fig. 29) presented a banding pattern that is much closer to what I was expecting to see. The toxin blotted at its expected molecular weight in the elution fractions (Fig. 29B, Lane 5), which is also the case for the immunity factor as well (Fig. 29A, Lane 5). Although I still saw the high molecular weight bands, the intensity of the bands appeared to be significantly lower than what I saw previously (Fig. 27, Lane 5). Additionally, I found that a

significant amount of the immunity factor had flown through the resin (Fig. 29A, Lane 2) while the toxin appeared to not leak at all (Fig. 29B, Lane 2). This suggests that guanidine may have disrupted the interaction between the two proteins resulting in the immunity factor flowing through as it is unable to bind the resin on its own. So, it appears that the guanidine treatment is not only able to disrupt the interaction that was responsible for the high molecular weight bands but it was also able to disrupt the interaction between the toxin and the immunity factor. Unfortunately, I did not have more time to perform more denaturing purifications to parse out the details of what may be responsible for these unexpected bands which continued to be present following severe denaturing conditions. Instead, I spent a significant amount of time and effort in obtaining clones that carried alleles beside T/I 1 to assess if these banding patterns would be present in those too.

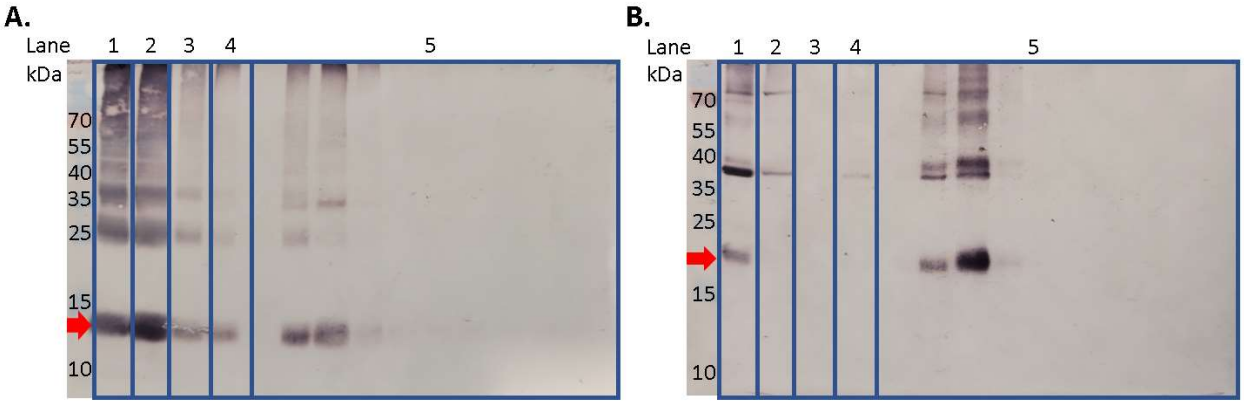

Figure 29. Denaturing Protein Purification of T/I 1. Western Blots of samples obtained during the denaturing protein purification of Toxin/Immunity 1. (A) α-HA Blot of samples from the denaturing protein purification. (B) α-His Blot of samples from the denaturing protein purification. (Lane 1) This is a sample of the whole cell lysates supernatant following sonication which was loaded on the resin. (Lane 2) This is a sample of the flowthrough that was collected following the loading of the resin with WCLs. (Lane 3) These are samples of the flowthrough obtained following the loading of the resin with different wash buffers. (Lane 4) This is a sample of the resin which was obtained at the end of the protein purification. (Lane 5) These are the different fractions collected from the column following loading of the elution buffer.

Alternate Cell Lines and Plasmids to Clone Other Toxin and Immunity Alleles

As protein purifications of Toxin and Immunity 1 provided us with conflicting results, I set out to clone different Toxin and Immunity alleles into high-copy plasmids so that I could obtain cell cultures carrying significant amounts of protein for further analysis. At first, I tried to work with new cell lines of *E. coli* which had sophisticated plasmid systems which allow for extremely tight repression of the promoter for the target genes. The cell lines that I worked with were the BL21 DE3 T7 Express LysY/I^q and Lemo21 cells. The benefit of the LysY/I^q cell line is that it constitutively expresses the LysY lysozyme which represses the T7 RNA polymerase which is required for the expression of the gene of interest. Meanwhile, the benefit of the Lemo21 cell line is the same, the main difference is that the expression of LysY is tied to a rhamnose inducible promoter allowing for tuneable expression of LysY which in turn allows for tuneable repression of the T7 RNA Polymerase. Using these strains I was able to clone toxin and immunity allele # 6, I chose this allele because it had an extremely toxic phenotype. Although I was able to successfully clone the toxin and immunity allele into the cell lines, I was never able to recover an unmutated clone which is extremely unfortunate as the toxin and immunity were highly expressed (data not shown).

Since I was unable to use cell lines that showed promise for the cloning of bacterial toxins into bacteria, we decided that the best next possible step would be to make our plasmid system express these alleles. In this plasmid we made two major changes, we split the toxin and immunity factor to separate inducible promoters and we added cleavage sites ahead of the HA and His tag that were recognized by different enzymes. To make this plasmid, we ordered Gene blocks pTES1 and pTES2, TES being an acronym for Toxin Expression System, which carried a version *araC* gene and promoter which another group designed to have an extremely high

expression [96]. The main difference between pTES1 and pTES2 is the placement of the HA tag, the pTES1 plasmid carries an N-terminal HA tag while pTES2 has it on the C-terminus. We did this in case the placement of the tag causes problems in the binding affinity of the toxin and immunity factor, as we have not resolved the structure of these proteins due to which the binding mechanism is currently unknown. Our cloning strategy was as follows: First, I used the pTES1 and pTES2 gene blocks to Gibson assemble with a Kpn2I digested pMCSG53 plasmid to create the base vector. Second, I digested the base vector with XmaI to Gibson assemble with the immunity fragment ahead of P_{araC}. I did this so that the immunity factor can be constantly expressed, with arabinose, while the toxin is being cloned into the cell line to avoid the possibility of autointoxication due to the leakiness of the promoter. Once the immunity factor was cloned in, which I verified using PCR, I sent a miniprep sample to be sequenced while I verified the expression of the immunity factor. If the expression was intact and the clone contained the correct sequence, I digested the plasmid with SspI to clone the toxin fragment ahead of the T7 promoter. Unfortunately, no matter what I tried I was unable to clone an unmutated toxin allele into the plasmid. During the cloning of the toxin, I tried a longer recovery period following heat shock, changing the amount of SOC added following heat shock, plating and allowing for growth at 37^{o}C or room temperature over two days, changing the amount of Gibson assembly product added at the start of transformation, and changing the amount of arabinose added during transformation. Regardless of any of these changes, I failed to recover a single clone that had an unmutated toxin or plasmid. Even more odd was the finding that the immunity factor would be cloned in correctly but would be mutated following the attempt at cloning the toxin. I have tried to clone toxin and immunity alleles # 3,4,5,6,9,12, and 13, all of whom show variable toxicity during Abigail's growth curves. So, it appears that regardless of

toxicity, plasmid system, and cell line, the *E. coli* cells are unable to uptake the toxin-containing plasmid and survive if the plasmid is unmutated.

Conclusions

Here I have shown that there is an interaction between the immunity factor and toxin, at least in the case of T/I 2, 3, 4, 5, 6, 11, and 12. This suggests that the two proteins do interact with each other which is a defining characteristic of toxin and immunity pairs. In support of this, Abigail's growth curves also show that the putative toxins induce a growth defect in *E. coli* cells which is recovered in the presence of the immunity factor. Together, these data strongly support our hypothesis that the rhs locus of *P. aeruginosa* encodes a toxin/immunity system. Importantly, I was able to troubleshoot and organize a protocol from the ground up which will help future lab members to further inquire about the interactions amongst the remaining toxin and immunity alleles. The initial protocol, when I first joined the lab, resulted in very mixed pieces of data which were incredibly difficult to analyze as they were unreliable due to the lack of toxin detection in the immunoblots. Now, we have a protocol that appears to work on all alleles that I have been able to test and it reliably detects the presence of the toxin in the immunoblots.

Alongside this, I was able to perform a protein purification of Toxin/Immunity 1 which contained unique bands of extremely high molecular weight. I performed control experiments with an empty BL21 DE3 cell line as well as BL21 DE3 containing an empty plasmid and found no bands of similar molecular weight. This suggests that an *E. coli* protein is not responsible for the high molecular weight bands shown in Figure 27. I also performed a protein purification in denaturing conditions via the addition of 6M guanidine, which showed substantial changes in the blotting profile of the elution fractions. I found that following the denaturing conditions some of the high molecular bands were no longer being detected and found more protein at its

appropriate molecular weight. This suggests that there were interactions among the Toxin and Immunity proteins which were responsible for the higher molecular bands. To further verify this, I attempted to clone other toxin and immunity alleles into different cell lines as well as a self-designed plasmid. Regardless of the different strategies for cloning the *toxin* and *immunity* genes, I was never able to recover an unmutated clone.

Altogether, the work detailed here should lay the foundation for not only further testing but also will help future lab members ask more in-depth questions regarding these toxin and immunity allelic variants encoded in the rhs locus of *Pseudomonas aeruginosa* among its numerous strains.

CHAPTER FOUR

DISCUSSION

Introduction

Pseudomonas aeruginosa is ubiquitous in the environment due, in part, to the arsenal of toxins and proteins encoded within its genome. Over the past few decades, an ever-increasing list has been compiled of these toxins and proteins that *P. aeruginosa* utilizes to mold its environment to suit its needs. Through bioinformatic analysis, we believe that we may have identified a previously uninvestigated locus in the bacteria's genome that encodes for a toxin/immunity system that may play a role in interbacterial competition. According to our analysis, the locus consists of 3 ORFs where the first and second ORF encodes a protein that has been shown to form two halves of a hollow shell-like structure. This is followed by an ORF further downstream which appears to co-vary with the 3' end of the second ORF. Further analysis has led us to find that the first two ORFs consist of a repeating element called rearrangement hotspot (rhs), due to which we have termed them *rhsB* and *rhsC*, respectively. The 3' end of *rhsC* is also hypervariable compared to the 5' end of *rhsB* and *rhsC* which suggests, to us, that the 3' end of *rhsC* encodes an effector. We have also identified 13 different alleles of this effector which co-varies with the third ORF which suggests that it is an immunity factor for the effector due to which we refer to it as *rhsI*. We have also found significant variation of the 5' end of *rhsB* which we believe to be responsible for providing target specificity to the overall protein and we, so far, have noted 17 distinct alleles. These bioinformatic analyses

were the foundation for the work that I have detailed in this thesis. Although I set out at first to accomplish biochemical work that not only showed the interactions between the putative toxin and immunity factor but also provided some mechanistic insight into a subset of the toxin alleles. Unfortunately, the work I needed to do for the first goal of my thesis took a lot more effort and time than we expected due to which I was unable to do any work related to the latter.

In this thesis, I have shown that the toxin and immunity factors do bind to each other as would be expected of toxin-immunity pairs. In the process of showing this interaction through western blots of Co-IPs, I have built a protocol that reliably detects the toxins which had been a consistent problem with the different alleles, previously. Another sub-aim of this part of my thesis was to purify the toxin and immunity factor to further assess their binding affinity and specificity. I was able to make considerable progress in creating a protocol that allows for the purification of these proteins but due to cloning issues and unpredictable results, I was unable to entirely accomplish this goal. Altogether, I have been able to lay down the foundation upon which future lab members can build and potentially address questions that would allow us to utilize this system as a tool for the targeted delivery of therapeutics against *P. aeruginosa*.

Physical Interactions Occur between Toxin and Immunity Factor

To start, I performed biochemical assays to investigate the interactions between the toxin and immunity factor encoded in the rhs locus of *P. aeruginosa*. Data from another lab member, Abigail Banas, showed that the expression of each toxin gene induces a variable growth defect which is recovered in the presence of its immunity factor. Generally, the neutralization of the toxic effect in the presence of the immunity factor is believed to occur through a physical interaction between the toxin and the immunity factor [78]. To determine if this physical interaction was responsible for the recovery of the growth defect that Abigail saw in her

experiments, I performed Co-IPs where I used an antibody against the HA tag of the immunity factor as bait. When I performed the initial Co-IPs, I did see an interaction between the toxin and immunity factor albeit the signal for the toxin was barely visible. This was a problem that I needed to address as the detection of the toxin was of utmost importance since, we were using an antibody against the immunity factor. One solution was to use an antibody against the His-tag of the toxin, but it was difficult to assess if the toxin was not blotting due to a subpar antibody or if the His-tag was difficult to access for the antibody. Therefore, I decided to first troubleshoot the current protocol and assess the His antibody to see if that was enough to address the problem.

This led me to perform a series of experiments to optimize the protocol for not only the cell preparation but also the western blot itself. Fortunately, I was able to optimize the protocol for the whole cell lysate preparation as well as the western blot which helped me clearly show that there is an interaction between the toxin and immunity pairs. Although I only showed this to be the case only for a subset of the toxin and immunity alleles due to time limitations, Toxin/Immunity 2,3,4,5,6,11, and 12. Alongside this, I found that even with the new protocol some toxin/immunity alleles did not blot well, Toxin/Immunity 1 and 6. If I had more time, I would re-perform these Co-IPs but with a 1-L culture instead of a 500-mL culture as it is possible that there was insufficient protein available for detection. I would also induce with 1.0% Arabinose instead of 0.1% Arabinose as the latter may have been insufficient for the production of a substantial amount of toxin and immunity protein. These two changes may be able to resolve the problem as the strains used in these experiments carry the toxin and immunity genes on a low-copy plasmid, pSB109. Altogether, 6 of the 13 alleles encoded in the rhs locus appear to bind to each other which is a hallmark of toxin and immunity pairs encoded in protein systems that mediate interbacterial competition.

Purification of Toxin and Immunity Factor

In combination with showing the physical interaction between the toxin and immunity factor, my other sub-goal was to successfully purify the proteins. The motivation behind accomplishing this goal was that these proteins have never been studied before due to which there were more questions than answers. This has been a significant roadblock in our ability to further assess the rhs locus of *P. aeruginosa*. If we can successfully obtain purified fractions or even a protocol for the purification of these proteins, we would be able to resolve the structure of the proteins through a structural biologist. This is of critical importance as it would allow us to assess the function of the different toxins as they have variable toxicity in Abigail's growth curves. Besides this, we would be able to perform quantitative assays to assess the activity of the purified toxins. Purified fractions would also allow us to utilize isothermal calorimetry to assess the binding specificity and strength of the immunity factor and the toxin. This will help us assess if the toxins have their cognate immunity factor or if the immunity factors impart general immunity against the toxins. All of these are important questions that we need to answer to be able to confidently say that the rhs locus is encoding toxin/immunity pairs which are mediating interbacterial competition and assessing their potential role in pathogenesis.

When I first started in the lab there were two hurdles to overcome: I needed to establish a protocol that was usable for our proteins, and I needed to clone the alleles into a high-copy plasmid to have sufficient protein concentration for purification. I started by using the CdiA[HA]3238-Hb construct to start teasing out the protocol for WCL preparation and found that the cell pellet had to be treated with lysozyme for an hour alongside sonication. This allowed for the maximal release of the proteins into the supernatant for use in protein purification. Oddly, this protocol seemed to fail when I started using it for the preparation of WCLs of *E. coli* cells

carrying the Toxin/Immunity alleles. I found that, consistently, the toxin would not blot following sonication, and/or the proteins were unable to bind to the resin and would flow through the column. At first, I hypothesized that the proteins did not have sufficient time to bind to the resin, so I decided to incubate the WCLs overnight with the resin. When I performed the purification with this protocol, I still found that the proteins were flowing through the column, although the toxin was detected during the western blot, albeit with a faint band. This led me to hypothesize that the toxin may be sensitive to degradation and the long incubation was contributing to it being degraded by a protease, or other cellular elements, which in turn caused the toxin to be unable to bind to the resin, resulting in the proteins flowing through the column. This seemed like the most likely possibility as toxins are known to be sensitive to degradation by a variety of enzymes and in some cases been found to be more prone to degradation following association with the immunity factor [95]. To assess this, I added the protease inhibitor PMSF to my buffers during lysozyme treatment and purification. Finally, I saw a clear signal of the toxin during my blots, but the proteins were still unable to bind to the resin. This suggested that the degradation may be the primary cause of the lack of detection as well as the proteins flowing through the column. Therefore, I decided to conduct two experiments to assess this further. In these experiments, I either performed the lysozyme treatment or skipped it in the presence of PMSF and another broad-spectrum protease inhibitor, Roche cOmplete. As a result, I found that the lysozyme treatment was contributing to the toxin being unable to bind the resin since in the experiment where I skipped the lysozyme treatment, the toxin and immunity factor blotted only in the Input sample and nowhere else. These data strongly suggest that the toxin was prone to degradation or some other manipulation during the lysozyme treatment which was hindering its ability to bind the resin. However, this presented a new issue, albeit easy to resolve, the proteins

were bound to the resin and were not eluting off the resin. To resolve this, I increased the imidazole concentration from 300 mM to 500 mM in my elution buffer. This led to my first successful protein purification of Toxin and Immunity 1, but the bands in the elution fractions were confounding. Unexpectedly, the toxin in the elution fractions was blotting at a much higher molecular weight (MW) while the immunity factor was blotting at its appropriate MW. Some reasons for this include the toxin being bound to an immunity factor, toxin being bound to an *E. coli* protein, toxin forming dimers and trimer, and non-specific detection of an *E. coli* protein. Hence, I set out to check for background signals by performing a western blot of induced cell pellets and WCLs of BL21 DE3 and pMCSG53 BL21 DE3 strains using the His antibody. As a result of this experiment, I found that no *E. coli* protein was non-specifically detected by the His-antibody which suggests that the bands were specific to the toxin proteins. Therefore, the other possibility was that proteins were interacting with the Toxin protein resulting in it blotting at a much higher kDa than expected.

Next, I performed a denaturing protein purification by adding 6M guanidine to all of my buffers used during the WCL preparation and protein purification. The western blot of the samples obtained from this experiment detected bands for the Toxin at its appropriate size, but the higher bands were still present. It is noteworthy that the blot for the immunity factor detected the immunity factor in the flow-through sample, but the toxin blot did not detect the toxin in the flow-through. Alongside this, the immunity factor is eluting during the wash step as well which further suggests that the guanidine was able to interfere with the interaction between the toxin and immunity factor. Therefore, the change in the kDa of the detected band maybe not only because it is interfering with the toxin associating with itself or other *E. coli* proteins but also because it is interfering with the interaction between the toxin and the immunity factor. This

suggests that it may be possible to use a denaturing protein purification to separate the immunity factor and toxin in their fractions. Furthermore, this clarifies that the high kDa bands that were detected during the non-denaturing protein purification were due to a physical interaction amongst these proteins, but denaturation through guanidine is not sufficient to fully disrupt the interactions.

If I had more time, I would re-perform this denaturing purification with the updated protocol which I had formed while troubleshooting Co-IP western blots. I would produce a 1-L culture from an overnight induction of the cell line at 18ºC 150 rpm and then prepare the WCLs as usual with a buffer containing 6M guanidine. Here I would make another change and incubate the WCLs for 2 hours at 4ºC before centrifugation, filtering, and performing the protein purification. The reason for this is that guanidine disrupted the interactions between the proteins so, an incubation period may help in the resolution of the high kDa bands entirely. Since the rhs Toxin and Immunity factors are unique, another strategy would be to utilize urea instead of guanidine as the denaturant during the protein purification. This might also result in the resolution of the high molecular weight bands as the mode of action of guanidine and urea as a denaturant is quite different. Alternatively, I would also perform size exclusion chromatography (SEC) on these samples to see if the unexpected banding pattern was an artifact of an SDS-PAGE due to it denaturing the proteins. Overall, if these experiments resulted in the toxin and immunity factors being separated and/or blotting at their appropriate weight, we would be able to pursue further analysis of the proteins via isothermal calorimetry, structure resolution, and microscopy.

Cloning of Toxin and Immunity Factors Using Alternate Strategies

While performing Co-IPs and protein purifications, I spent a significant amount of time trying to clone the different toxin and immunity alleles into alternate cell lines and plasmids. The purpose of doing this was to be able to perform protein purifications on additional alleles to assess if their western blots detected aberrant banding patterns or not. One of the first strategies that I utilized was using the LysY/I^q and Lemo21 cell lines to clone the toxin and immunity factors on the pMCSG53 plasmid. Unfortunately, I was unable to recover an unmutated clone even with the sophisticated control of the T7 promoter with these cell lines. As it appeared that we could not clone the toxin and immunity factor in their native conformation into a high-copy plasmid regardless of the sophisticated nature of the control of the promoter, we set out to design a plasmid for our system specifically. To do this Dr. Allen designed the Toxin Expression System plasmid, pTES, where he separated the toxin and immunity factor to different promoters. This allowed me to clone the immunity factor on a highly inducible promoter before cloning the toxin. Oddly, I was able to obtain unmutated clones carrying the immunity factor which showed strong expression of the immunity factor (data not shown) but the attempt at cloning the toxin led to the recovery of transformants with not only hypermutated toxins but also hypermutated immunity factors. In some attempts, the base plasmid was entirely mutated which suggests that the cells find the toxin to be extremely intolerable. In these cloning attempts, I have tried a pTES plasmid carrying the HA tag on the N-terminus as well as the C-terminus, and I have tried allele numbers 1, 3, 4, 5, 6, 9, and 13. Regardless of the allele, I was unable to recover an unmutated clone even though I was using the NEBα cell line, which is deficient in the T7 RNA polymerase, and the cells were expressing the immunity factor throughout the transformation process. Interestingly, when I attempted using the pTES1 plasmid, which carries the HA tag on the N-

terminus of the immunity factor, I repeatedly failed to recover the clone which carries the immunity factor alone. Unfortunately, I did not have the time to redo the assembly of the pTES1 base plasmid before re-attempting the cloning on the immunity factor to assess if the base plasmid had degraded somehow. Regardless, it appears that utilizing plasmids such as pTES or cell lines with extremely tight control of the promoter is insufficient for the cloning of the toxin. Therefore, the best possible action would be to utilize an alternative promoter which has been noted to be much more tightly controlled than the T7 promoter for further cloning attempts.

Concluding Remarks

The data that I have presented in this thesis shows that the Toxin and Immunity factors encoded in the rhs locus of *P. aeruginosa* physically interact with each other. This provides further evidence that the proteins encoded in *rhsC* and *rhsI* encode toxin and immunity pairs in addition to the growth curves conducted by Abigail Banas. I have also shown the work that I conducted to troubleshoot the unclear detection of the toxin in the initial Co-IPs. Now, we have a reliable protocol for the detection of the toxin in Co-IPs which clearly shows the co-elution of the toxin with its immunity factor. Using this protocol, I have shown the physical interaction between the proteins to be true for Toxin and Immunity allele numbers 2,3,4,5,6,11, and 12. Alongside this, I have produced a protocol that allows us to purify toxin and immunity proteins. Altogether, future studies should be able to use these protocols to not only resolve the structure of the proteins but also to assess the physical interactions between the toxins and immunity factors and address questions related to their binding specificity as well as the mode of action of the different toxins. This may one day allow future lab members to harness the rhs protein system of *Pseudomonas aeruginosa* for the targeted delivery of therapeutics against the bacteria.

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VITA

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