SUITABILITY OF RELBE AND MAZEF TOXIN-ANTITOXIN SYSTEMS AS ANTIBACTERIAL TARGETS

BY

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DISSERTATION

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ABSTRACT

Toxin–antitoxin (TA) systems are unique modules that effect plasmid stabilization via post-segregational killing of the bacterial host. The genes encoding TA systems also exist on bacterial chromosomes, and it has been speculated that these are involved in a variety of cellular processes. Interest in TA systems has increased dramatically over the past five years as the ubiquitous nature of TA genes on bacterial genomes has been revealed. The exploitation of TA systems as an antibacterial strategy via artificial activation of the toxin has been proposed and has considerable potential; however, efforts in this area remain in the early stages and several major questions remain. This thesis research investigated the tractability of targeting the TA systems RelBE and MazEF and these systems were found to be ubiquitous in clinical isolates of

*Pseudomonas aeruginosa* and *Staphylococcus aureus*. A high-throughput *in vitro* screen to identify small molecule disruptors of RelBE was developed using the photonic crystal biosensor technology. Peptide activators of the toxin RelE were sought by screening phage-displayed peptide against the RelB antitoxin. Additionally, a kinetic assay using a fluorogenic substrate was designed for the ribonuclease toxin MazF\textsubscript{Sa} and applied in a screen for peptide enhancers of MazF\textsubscript{Sa} enzymatic activity. A highly sensitive radiometric gel-based assay allowed for the first detection of endogenous MazF\textsubscript{Sa} activity in *S. aureus* lysate, which will enable further studies to identify both artificial and natural activators of the MazF\textsubscript{Sa} toxin. Further defining the prevalence of TA systems in clinical isolates and developing a variety of assays for assessment of MazF\textsubscript{Sa} enzymatic activity have advanced our progress towards the goal of identifying TA system activators for use as an antibacterial therapy.
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# TABLE OF CONTENTS

## CHAPTER 1: ARTIFICIAL ACTIVATION OF TOXIN-ANTITOXIN SYSTEMS AS AN ANTIBACTERIAL STRATEGY ................................................................. 1

### 1.1 THE PROBLEM OF ANTIBIOTIC RESISTANCE ................................................................. 1

### 1.2 CLINICAL SIGNIFICANCE OF DRUG-RESISTANT BACTERIAL PATHOGENS ................................................................. 3

#### 1.2.1 Methicillin-resistant *S. aureus* ................................................................. 3

#### 1.2.2 Multi-drug resistant *P. aeruginosa* ................................................................. 4

#### 1.2.3 Multi-drug resistant *A. baumannii* ................................................................. 5

### 1.3 TOXIN-ANTITOXIN SYSTEMS ................................................................. 5

#### 1.3.1 Role of proteic TA systems on plasmids ................................................................. 5

#### 1.3.2 Proposed role of TA systems on bacterial chromosomes ................................................................. 6

#### 1.3.3 Overview of classes of TA systems ................................................................. 7

#### 1.3.4 Regulation of TA systems ................................................................. 8

### 1.4 TA SYSTEMS AS AN ANTIBACTERIAL TARGET ................................................................. 9

#### 1.4.1 Strategies for toxin activation ................................................................. 10

#### 1.4.2 Which protein should be targeted? ................................................................. 11

#### 1.4.3 Which TA systems should be targeted? ................................................................. 13

##### 1.4.3.1 Bioinformatics studies reveal widespread nature of TA systems ................................................................. 13

##### 1.4.3.2 Epidemiology in enterococci ................................................................. 14

##### 1.4.3.3 Further defining prevalence and functionality in clinically significant bacteria ................................................................. 15

##### 1.4.3.4 Toxin mode of action/Potential for resistance ................................................................. 16

##### 1.4.3.5 TA system involvement in persistence ................................................................. 16

#### 1.4.4 Previous work toward discovering toxin activators ................................................................. 17

##### 1.4.4.1 Extracellular death factor and MazEF<sub>Ec</sub> ................................................................. 17

##### 1.4.4.2 Inhibitors of the PemI-PemK/MoxX-MoxT interaction ................................................................. 19

##### 1.4.4.3 Disruptors of ε-ζ ................................................................. 19

### 1.5 STRATEGIES TO TARGET TA SYSTEM PROTEINS ................................................................. 20

#### 1.5.1 Protein-protein interaction inhibition ................................................................. 20

#### 1.5.2 Enzyme activators ................................................................. 23

### 1.6 CHALLENGES FOR THE DISCOVERY OF TOXIN ACTIVATORS ................................................................. 23

### 1.7 REFERENCES ................................................................. 26
CHAPTER 2: PREVALENCE AND TRANSCRIPTION OF TOXIN-ANTITOXIN SYSTEMS IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA AND ACINETOBACTER BAUMANNII

2.1 INTRODUCTION

2.2 INVESTIGATION OF TOXIN-ANTITOXIN SYSTEMS IN PSEUDOMONAS AERUGINOSA

2.2.1 Assessment of P. aeruginosa clinical isolate diversity

2.2.2 Presence of TA systems in P. aeruginosa genomic DNA

2.2.3 TA gene location assessment

2.2.4 Detection of TA transcripts

2.3 INVESTIGATION OF TA GENES IN ACINETOBACTER BAUMANNII

2.3.1 Presence of TA genes in A. baumannii genomic DNA

2.3.2 RT-PCR analysis

2.4 CONCLUSIONS

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Sources of clinical isolates

2.5.2 MLVA

2.5.3 DNA isolation

2.5.4 RNA isolation

2.5.5 PCR analysis

2.5.6 RT-PCR analysis

2.6 TABLES AND FIGURES

2.7 REFERENCES

CHAPTER 3: TARGETING R\text{elBE} AS AN ANTIBACTERIAL STRATEGY

3.1 INTRODUCTION

3.2 HIGH THROUGHPUT SCREEN FOR R\text{elBE} DISRUPTORS USING PHOTONIC-CRYSTAL BIOSENSOR

3.2.1 Development of PC biosensor for use in a high-throughput screen

3.2.2 Dose-dependent binding of RelBE to the PC biosensor

3.2.3 Quantification of (His)\text{6}RelBE bound to the biosensor surface

3.2.4 High-throughput screen preparation

3.2.5 High-throughput screen results
3.3 REL/E PEPTIDE FRAGMENT ANALYSIS ................................................................. 74
  3.3.1 Assessment of RelE peptide fragment binding to RelB(His)_6 ...................... 74
  3.3.2 Quantification of binding of RelE36-65 derivatives by SPR ...................... 75
3.4 PHAGE-DISPLAYED PEPTIDE LIBRARY SCREEN ........................................... 76
  3.4.1 T7 415-1b Library X_9.1 ........................................................................ 79
    3.4.1.1 Competitive biopan of hit phage ...................................................... 80
    3.4.1.2 ELISA of phage displaying consensus peptides ............................... 81
    3.4.1.3 PC biosensor and SPR analysis of synthetic peptides ....................... 81
  3.4.2 T7 415-1 Library X_9.2 ........................................................................ 82
  3.4.3 T7 10-3 libraries against RelB(His)_6 ....................................................... 82
    3.4.3.1 Biopan with 9-amino acid linear peptide library X_9.3 ....................... 83
3.5 EFFORTS TOWARDS PURIFICATION OF REL/E .............................................. 84
  3.5.1 Purification of untagged RelE using the pTWIN2 vector ............................. 84
  3.5.2 RelE triple mutant: R81A, R83A, Y87A ............................................... 85
  3.5.3 pMBP fusion with TEV cleavage .......................................................... 85
  3.5.4 RelB trypsinolysis .............................................................................. 86
3.6 CONCLUSIONS ......................................................................................... 86
3.7 EXPERIMENTAL PROCEDURES ...................................................................... 88
  3.7.1 Construction of plasmids ...................................................................... 88
  3.7.2 Purification of RelBE(His)_6 and RelB(His)_6 under native conditions ....... 89
  3.7.3 Expression and purification of RelE from pTWIN2 .................................. 90
  3.7.4 Expression and purification of MBP-RelE(His)_6 ...................................... 91
  3.7.5 Set-up for PC biosensor assay ............................................................... 91
  3.7.6 Quantification of RelBE(His)_6 eluted from PC biosensor surface ............. 92
  3.7.7 High-throughput screening protocol ..................................................... 92
  3.7.8 Detection of RelE or peptide binding to RelB(His)_6 on PC biosensor .......... 93
  3.7.9 Peptide purification ............................................................................ 93
  3.7.10 Surface plasmon resonance assay ...................................................... 93
  3.7.11 Biopanning ....................................................................................... 94
  3.7.12 Competitive biopan .......................................................................... 95
  3.7.13 ELISA ......................................................................................... 95
3.8 REFERENCES .......................................................................................... 95
CHAPTER 4: INVESTIGATION INTO THE ACTIVATION OF MazF<sub>SA</sub> BY SYNTHETIC PEPTIDES AND IN RESPONSE TO ENVIRONMENTAL STRESS .......... 99
4.1 INTRODUCTION ........................................................................................................... 99
4.2 CLONING, EXPRESSION AND PURIFICATION OF MazF<sub>SA</sub> ........................................... 103
4.3 CHARACTERIZATION OF MazF<sub>SA</sub> AND MazE<sub>SA</sub> ................................................... 104
4.4 KINETIC ASSAY FOR DETERMINATION OF MazF<sub>SA</sub> KINETIC PARAMETERS ........ 105
4.5 GEL-BASED ASSAY FOR MazF<sub>SA</sub> ENDORIBONUCLEASE ACTIVITY ................... 106
4.6 MazE<sub>SA</sub> PEPTIDE FRAGMENT ANALYSIS .................................................................. 108
  4.6.1 Assessment of peptides in kinetic assay ................................................................. 108
  4.6.2 Assessment of peptides 5 and 6 in gel based assay .................................................. 108
  4.6.3 Derivatives of peptides 5 and 6 ............................................................................. 111
  4.6.4 Derivatives of LNLSL and LNLSLAN ................................................................... 112
  4.6.5 Peptide 5 non-specifically prevents protein adsorption to assay tube ................. 113
4.7 DETECTION OF MazF<sub>SA</sub> ACTIVITY IN S. AUREUS CELL LYSATE ......................... 114
  4.7.1 Development of a radiolabeled substrate for MazF<sub>sa</sub> activity ............................. 114
  4.7.2 Detection of endogenous MazF<sub>sa</sub> activity in S. aureus cell lysate ...................... 115
4.8 CONCLUSIONS .......................................................................................................... 115
4.9 EXPERIMENTAL PROCEDURES .................................................................................. 117
  4.9.1 Materials ............................................................................................................. 117
  4.9.2 Cloning ............................................................................................................... 118
  4.9.3 Protein Expression and purification ..................................................................... 118
  4.9.4 HPLC analysis of oligonucleotide cleavage products ......................................... 119
  4.9.5 Fluorometric oligonucleotide cleavage assay ...................................................... 120
  4.9.6 In vitro transcription of RNA substrate ................................................................ 120
  4.9.7 MazF<sub>sa</sub> kinetic assays in the presence of peptide ............................................. 121
  4.9.8 Effect of peptide 6 on RNase A activity in fluorogenic assay ............................... 122
  4.9.9 Effect of peptide on MazF<sub>sa</sub> activity in gel-based assay ........................................ 122
  4.9.10 Detection of protein adsorption to tube .............................................................. 122
  4.9.11 Effect of peptides on MazF<sub>sa</sub> activity in the presence of BSA or CHAPS ........... 123
  4.9.12 Synthesis of radiolabeled RNA transcript .......................................................... 123
  4.9.13 Gel-based RNA cleavage assay ......................................................................... 123
  4.9.14 Detection of MazF<sub>sa</sub> activity in S. aureus cell lysate ....................................... 123
4.10 FIGURES ..................................................................................................................... 124
4.11 REFERENCES .......................................................................................................... 125
CHAPTER 1
ARTIFICIAL ACTIVATION OF TOXIN-ANTITOXIN SYSTEMS AS AN ANTIBACTERIAL STRATEGY


1.1 THE PROBLEM OF ANTIBIOTIC RESISTANCE

The discovery of penicillin by Alexander Fleming in 1928 marked the beginning of an era that would see the reduction in deaths and sustained illness due to bacterial infections. The “Golden Age” of antibiotics saw the discovery of various natural products and synthetic small molecules capable of curing once untreatable ailments. The successful use of antibiotics was a breakthrough in medicine and saved many lives. Unfortunately, application of antibiotics in the clinic had the adverse consequence of selecting for bacteria that evade their lethal effect, and introduction of each antibiotic was inevitably and eventually followed by identification of infections resistant to those that drug (Figure 1.1) [87, 177]. Nonetheless, the plentiful stockpile of new antibiotics ensured successful treatment of infections, so although the problem was recognized, it did not appear to pose a major threat to human health.

Eventually the arsenal of antibiotics dwindled, no novel drugs were introduced into the clinic from 1970-2000 (Figure 1.1) and drug-resistant infections became increasingly common with limited treatment options. The rise in antibiotic resistance continues to impede our ability to treat bacterial infections; the emergence of strains resistant to nearly all classes of antibiotics threatens a reversion to the “pre-antibiotic era” [54]. Resistance can be due to one or more of a variety of mechanisms, including inherent tolerance due to cell wall impenetrability, upregulation of efflux pumps, mutation in the cellular target, or drug modification. Many of the new therapeutics are derivatives of existing drugs, thus bacterial resistance is observed sooner, especially if it is achieved by a general mechanism, such as efflux pumps. Recently, novel classes of antibiotics have been introduced, including the oxazolidinones, lipopeptides, glycyclyclines and pleuromutilins, which are useful in treating some infections resistant to older drugs. However, not surprisingly, bacteria have evolved resistance to these compounds as well. One limitation is that all antibiotics target one of five essential cell functions—DNA replication, transcription, translation, cell-wall biosynthesis and folic acid biosynthesis (Figure 1.1). Thus, there is a need to identify novel drug scaffolds that hit new targets within the cell to keep pace with ever-evolving bacteria.
The introduction of the major classes of antibiotic into the clinic and the subsequent observed resistance. The “Golden Age” of antibiotics throughout 1940-1960 was followed by a 30-year gap in the discovery of a novel antibiotic. Resistance to nearly every class of antibiotics has been observed in the clinic, severely limiting treatment options.

In addition to identifying novel targets and drugs, development of narrow-spectrum antibiotics may result in prolonged efficacy. Broad-spectrum drugs that kill commensal as well as pathogenic bacteria expand the effect of natural selection by orders of magnitude, providing a competitive advantage for bacteria to harbor genes that confer resistance, thereby increases the pool of drug-resistant genes that bacteria can trade via horizontal gene transfer [146]. Furthermore, complications arise from prolonged use of antibiotics, such as increased susceptibility to chronic infections (e.g., *Clostridium difficile*-associated disease). However, the success of narrow-spectrum drugs requires the concurrent implementation of improved diagnostic methods to facilitate proper prescription.

History has proven that bacteria will develop resistance to any antibacterial compound, thus the concept of a “magic bullet,” as antibiotics were once perceived, is not realistic. However, staying one step ahead of bacterial evolution by replenishing the stockpile of antibiotics is a feasible goal. With fewer large pharmaceutical companies investing in antibacterial discovery and development [87, 123], there is a great need for novel efforts to maintain a continued supply into the pipeline of antibacterial drugs and avoid increased morbidity and mortality associated with the looming or realized “post-antibiotic era [4, 8].”

This document describes progress towards the development of toxin-antitoxin (TA) systems as an antibacterial target. The efforts of this dissertation focused on validating TA
systems as a viable antibacterial target and identifying a compound that artificially activates the toxin TA systems for use as a therapeutic against drug-resistant infections. Discussed in Chapter 1 of this thesis is the biological role of TA systems, their prevalence in bacteria, our strategy for targeting them, other efforts in the field to target TA systems, and the precedence for the types of compounds we pursued, namely protein-protein interaction inhibitors and enzyme activators. The inherent toxicity of TA systems makes them an attractive candidate for antibacterial development; however success has yet to be realized for this strategy. One requirement for the success of this strategy is determining the prevalence of TA systems, and although bioinformatics studies have demonstrated their widespread nature across bacterial species, only a few studies have confirmed these data by detecting the presence of TA systems in clinical isolates. Additionally, there are few examples targeting TA systems and the success of those efforts has been limited. The background information discussed in Chapter 1 sets the stage for efforts towards investigating TA systems as an antibacterial target, including the epidemiological data on TA systems present in clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, presented in Chapter 2, work towards identifying a disruptor of the RelBE TA system in Chapter 3 and efforts towards the activation of the *Staphylococcus aureus* toxin MazF<sub>Sa</sub> presented in Chapter 4.

1.2 CLINICAL SIGNIFICANCE OF DRUG-RESISTANT BACTERIAL PATHOGENS

The proportion of hospital-associated infections caused by resistant bacteria is rising [63]. The most commonly encountered drug-resistant bacteria in the clinic have been called the “ESKAPE” pathogens and include *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species [19, 128, 136]. These problematic pathogens are noteworthy because they are responsible for the majority of hospital-acquired infections and some strains are resistant to nearly every available antibiotic. The work presented in this thesis focused primarily on *S. aureus*, *P. aeruginosa* and *A. baumannii*; the others will not be further discussed.

1.2.1 Methicillin-resistant *S. aureus*

*S. aureus* is an invasive Gram-positive bacterium that causes a range of acute and chronic infections, from boils and pneumonia to meningitis and septicemia [95]. Hospital-associated MRSA (HA-MRSA) is a major cause of nosocomial infections, and community-associated MRSA (CA-MRSA) has emerged as a leading cause of skin and soft tissue infections in otherwise healthy individuals who had no healthcare contact [23, 72]. HA-MRSA usually infects hospitalized elderly, immunocompromised patients who have a history of antibiotic therapy and
indwelling devices whereas CA-MRSA affects younger, healthy individuals [110]. Recently, hospital-associated outbreaks caused by the endemic CA-MRSA clone USA300, which produces the Panton-Valentine leukocidin virulence factor, have been reported [119].

Methicillin resistance is conferred via acquisition of a genomic island called staphylococcal chromosome cassette mec. SCCmec harbors mecA, which encodes the penicillin-binding protein PBP2a, and due to its reduced affinity for β-lactam antibiotics allows cell wall synthesis to occur in the presence of methicillin [116]. Previously, vancomycin was the drug of choice to control MRSA infections, but now vancomycin non-susceptibility and vancomycin-intermediate resistance is becoming increasingly common [47, 141, 153]. Fortunately, MRSA remains largely susceptible to newer antibiotics, such as daptomycin, linezolid, tigecycline, quinupristin-dalfopristin, and ceftaroline [11, 22, 68, 102, 166]; however, MRSA is notorious in its ability to acquire resistance to antibiotics [23] and some strains resistant to linezolid have been identified [56].

1.2.2 Multi-drug resistant \textit{P. aeruginosa}

\textit{P. aeruginosa}, a Gram-negative opportunistic pathogen, causes infections of the lungs, burn wounds and eyes and is a common nosocomial pathogen in the United States [49, 52]. Acute \textit{P. aeruginosa} infection occurs in immunocompromised, burned, or neutropenic patients or those undergoing mechanical respiration [99]. Chronic \textit{P. aeruginosa} infection of the lung occurs in 80% of cystic fibrosis patients and is the leading cause of death associated with CF, despite intensive antibiotic treatment [40, 51].

\textit{P. aeruginosa} utilizes a variety of mechanism to evade antibacterials. The impenetrability of many antibiotics due to the largely impassable Gram-negative cell membrane contributes to the difficulty in treatment [7, 154]. Additionally, the formation of hardy biofilms confers resistance to many antibiotics, including pencillins, carbapenems and aminoglycosides [39, 51, 107]. Intrinsic mechanisms impart resistance to β-lactam antibiotics [5]. \textit{P. aeruginosa} can also acquire laterally-transferred resistance determinants; however, its ability to develop resistance during the course of treatment is most troubling. This is achieved primarily by the production of cephalosporinase AmpC, the outer membrane porin OmpD and multidrug efflux pumps [92]. \textit{P. aeruginosa} infections are sometimes treated with potentially synergistic combinations of antibiotics including fluoroquinolones, aminoglycosides and β-lactams, as well as colistin, sometimes supplemented with rifampicin [92, 115, 160].
1.2.3 Multi-drug resistant *A. baumannii*

*A. baumannii*, another Gram-negative opportunistic pathogen, primarily causes infections in patients in intensive care units [1, 71, 127]. Hospital- and community-acquired *A. baumannii* infections result in bacteremia, ventilator-associated pneumonia, surgical-site infections, urinary tract infections and skin and soft-tissue infections [64, 65, 104]. Wound infections are typically contracted by soldiers in Iraq and Afghanistan [149, 165].

Pan-resistant strains refractory to all antimicrobial agents have been identified and resistance genes are typically clustered into large “resistance islands” [1, 46, 172]. Resistance is achieved by intrinsic mechanisms or by acquisition of mobile genetic elements carrying resistance determinants, including efflux pumps, porins, extended-spectrum β-lactamases, and other drug-inactivating enzymes [46, 71, 127]. Similar to *P. aeruginosa*, chromosomally-encoded cephalosporinase AmpC and multidrug efflux pumps play a major role in resistance [112]. Carbapenems are the drug of choice for treating *A. baumannii* infections and tigecycline, aminoglycosides, polymyxins, sulbactam, and piperacillin/tazobactam and are also typically effective [104, 112]

1.3 TOXIN-ANTITOXIN SYSTEMS

Toxin-antitoxin (TA) systems are unique genetic modules that effect plasmid stabilization via post-segregational killing of the bacterial host. The genes encoding TA systems also exist on bacterial chromosomes, where they are speculated to be involved in a variety of cellular processes. Interest in TA systems has increased dramatically over the past five years as the ubiquitous nature of TA genes on bacterial genomes has been revealed. The exploitation of TA systems as an antibacterial strategy via artificial activation of the toxin has been proposed and has considerable potential; however, efforts in this area remain in the early stages, and several major questions remain. The following section will investigate the tractability of targeting TA systems to kill bacteria, including fundamental requirements for success, recent advances, and challenges associated with artificial toxin activation.

1.3.1 Role of proteic TA systems on plasmids

Type II toxin-antitoxin (TA) systems consist of an antitoxin protein that binds to and inactivates a toxin protein. In relative terms, the toxin is considerably more stable (*i.e.*, longer cellular half-life) than the antitoxin, as the less ordered structure of the antitoxin makes it more susceptible to proteolytic degradation. The TA functionality capitalizes on this differential stability between the two proteins (Figure 1.2). TA systems were discovered in 1983 to confer
plasmid stabilization via toxin-induced post-segregational killing (PSK) [117]. If the plasmid encoding the TA system is not inherited by a daughter cell, the antitoxin is degraded by cellular proteases and not replenished, liberating the latent toxin to kill the cell and thereby diminishing the population of plasmid-free cells [25, 50, 164]. Many such “addiction” modules stabilize plasmids that carry drug-resistance determinants in important pathogens, notably the \( \text{axe-xte} \) and \( \varepsilon-\zeta \) TA systems commonly found on \( \text{vanA} \) plasmids in vancomycin-resistant enterococci (VRE) [105, 138].

**Figure 1.2** The role of toxin-antitoxin (TA) systems. Plasmid-encoded TA systems (left panel) stabilize plasmids via a post-segregational killing mechanism. The co-expressed antitoxin (A) binds to and inhibits the toxin (T), preventing its toxic effect on the cell. Plasmid loss is concomitant with antitoxin degradation, releasing the toxin to kill the cell, removing plasmid-free cells from the population. Chromosomally-encoded TA systems (right panel) have been implicated in a number of functions. In one model, cell stress modulates the expression at the promoter as well as upregulating the activity of proteases. The result is increased antitoxin degradation, freeing the toxin to inhibit growth or kill the cell.

TA systems are found on both plasmids and chromosomes. The role of plasmid-encoded TA systems is clear: they function as post-segregational killing systems (PSK) (**Figure 1.2**) [50, 66, 117]. Proteic TA systems produce a stable toxic protein and a labile antitoxin protein and are used by plasmids to ensure that only those daughter cells that inherit the plasmid survive after cell division. When both proteins are present, the antitoxin binds to the toxin, inhibiting its toxic activity. However, if a plasmid-free daughter cell arises, the labile antitoxin is quickly degraded (and not replenished), freeing the toxin to induce cell death. Because of this indelible link between plasmid maintenance and bacterial life, TA systems have been termed ‘plasmid addiction systems’ [83].

**1.3.2 Proposed role of TA systems on bacterial chromosomes**

Although the role of plasmid-encoded TA systems is clear, there is no such consensus for chromosomal TA genes, and in fact there are at least ten proposed biological roles for such systems [78, 96, 169]. Once regarded as superfluous genetic material with ambiguous benefit to
the bacterial cell [163], chromosomal TA systems have recently been proposed to be involved in numerous cellular pathways including starvation-induced cell stasis [32, 125], stress response [41, 61], genetic stabilization [181, 183], programmed cell death [111, 144], biofilm formation [75, 175], quorum sensing [78], antiphage protection [45, 60], virulence [20], persistence [76, 97], and gene regulation [6].

A straightforward model to understand chromosomally-encoded TA systems is based on data from several studies that indicate that these systems function to halt bacterial growth during times of stress (Figure 1.2) [32, 125]. For example, the relBE TA system modulates the stringent response induced by amino acid starvation [30, 32]. In this scenario, amino acid starvation triggers transcription of relBE as well as increased activity of Lon protease, which degrades the antitoxin RelB, freeing RelE [31, 89, 121]. RelE inhibits translation by cleaving mRNA in the ribosomal A site, resulting in a stalled ribosome [126]. Similarly, the mazEF TA system has been described as a suicide module that causes programmed cell death (PCD) in response to extreme amino acid starvation. In this scenario, relA synthesizes the stringent response molecule guanosine 3',5'-bispyrophosphate (ppGpp), inhibiting mazEF transcription, activating MazF, and ultimately leading to cell death [3, 33, 44]. Ultimately, the cell response to stress modulates transcription at the TA promoter which skews the ratio of toxin to antitoxin towards free toxin, which exerts its toxic effect and inhibits cell growth.

1.3.3 Overview of classes of TA systems

There are three known types of TA systems; this thesis work focuses exclusively on the Type II proteic modules. The 10 toxin families within the Type II proteic systems are depicted in Figure 1.3, categorized by general toxin mode of action [59, 108, 169]. The toxins of the CcdAB [9, 14, 36] and ParDE [67] families inhibit DNA replication by locking gyrase and DNA in an inactive complex (Figure 1.3A). The ζ toxin from the ω-ε-ζ TA system is the only known toxin to inhibit cell wall biosynthesis by blocking peptidoglycan synthesis [109] (Figure 1.3B). The ribosome-independent ribonucleases MazF [185, 186] and HicA [69, 98] cleave free mRNA (Figure 1.3C). There are two classes of ribosome-dependent toxins. The RelE [29, 126] and HigB [21, 28] family of toxins cleave mRNA in a ribosome-dependent manner and thereby inhibit translation (Figure 1.3D). The remaining toxins HipA [35, 80, 148], Doc [48, 93] and VapC [38, 180] inhibit translation by phosphorylating the elongation factor Ef-Tu, binding the 30S ribosome and cleaving tRNA^{fMet}, respectively (Figure 1.3E).
Figure 1.3 The 10 major classes of toxins, grouped by mode of action. (A) The CcdB and ParE family toxins inhibit replication by locking DNA gyrase in an inactive complex on the DNA. (B) The ζ toxin phosphorylates UDP-Glc-Nac (uridine diphosphate-N-acetylglucosamine) resulting in inhibition of peptidoglycan biosynthesis. (C) The MazF and HicA toxin families comprise the ribosome-independent ribonucleases that cleave free mRNA. (D) RelE and HigB families cleave mRNA in a ribosome-dependent manner. (E) The last group is characterized by ribosome inhibition mediated by a mechanism other than mRNA cleavage. HipA phosphorylates the elongation factor EF-Tu, Doc binds the 30S ribosomal subunit and VapC cleaves tRNAfmet.

1.3.4 Regulation of TA systems

The toxin and antitoxin are encoded in an operon and are co-transcribed in a polycistronic message, ensuring that toxin is produced only in the context of the antitoxin (Figure 1.4A). Transcriptional regulation is accomplished by the ability of the antitoxin to bind upstream operator sequences and inhibit transcription (Figure 1.4B). Furthermore, the TA complex binds the operator with higher affinity and suppresses transcription to greater levels, enabling the cell to sense the level of toxin and fine-tune transcription to maintain manageable levels in the cell (Figure 1.4B). Additionally, in many TA operons, the start codon of the toxin overlaps with the stop codon of the antitoxin (Figure 1.4A), achieving translational regulation that ensures higher levels of antitoxin compared to toxin. Indeed, a quantitative Western blot showed that the RelB antitoxin exists in a 10-fold excess over the toxin RelE is actively growing cells (Figure 1.4C) [120]. The higher level of antitoxin is required however, to account for the constant turnover of the antitoxin in the TA complex due its increased susceptibility to proteolysis. The half-life of the antitoxin alone is approximately 15-50 minutes [3, 24, 32, 42] (Figure 1.4D); however when bound to the toxin, the half-life increases by approximately 50% [26, 114, 170]. The toxin alone is stable on the order of hours and its stability is not affected the presence of the antitoxin [3, 32, 42, 170].
Figure 1.4 Regulation of TA systems. (A) Co-transcription ensures production of the toxin only in the context of the antitoxin. (B) Transcription is autoregulated by the antitoxin and to a further extent by the TA complex. (C) Translational coupling achieved by a single Shine-Dalgarno sequence and overlapping start and stop codons ensures excess of antitoxin over toxin to maintain inhibition of the toxin. (D) The inherent instability of the antitoxin increases its susceptibility to proteases, resulting in turnover within the complex. (E) Extensive electrostatic and hydrophobic interactions impart a low nanomolar affinity between the toxin and antitoxin.

In addition to transcriptional and translational regulation, the toxin activity is harnessed by binding with high affinity to the antitoxin (Figure 1.4E). The affinity of the TA complex has been measured by SPR and NMR experiments to be on the order of 0.1-10 nM [70, 90, 120] and separation of the toxin and antitoxin requires proteolytic degradation of the antitoxin or harsh denaturing conditions in vitro. Although in vitro the two proteins exist as a stable complex with no apparent “off-rate,” in the cell, the proteolytic susceptibility of the antitoxin is central to the function of the TA systems. The activity of the toxin is unleashed in conditions under which the antitoxin is degraded and not replenished, such as plasmid loss or environmental stress (Figure 1.2).

1.4 TA SYSTEMS AS AN ANTIBACTERIAL TARGET

Inspired by their lethal effect in the plasmid-directed post-segregational killing mechanism, we set out to exploit the inherent toxicity of TA systems as a therapeutic strategy. TA genes have no human homologs and appear to be present in the most important bacterial
pathogens (as described below); thus, toxin proteins could serve as ideal targets for novel antibacterial drugs via one of the mechanisms depicted in Figure 1.5 and described below.

1.4.1 Strategies for toxin activation

Activation of the toxin could be achieved by either an indirect means of freeing the toxin from the antitoxin or directly interacting with the TA proteins. The goal of indirect approach is expedited proteolytic degradation of the antitoxin (Figure 1.5). A molecule that binds promoter DNA and inhibits transcription at the TA locus would prevent replenishment of the antitoxin; there is considerable precedent for such sequence-specific DNA binders [135]. Degradation of the existing antitoxin by Lon or Clp proteases would release the toxin, allowing it to kill the cell. It has also been suggested that toxin activation in response to cellular stress requires increased expression or activity of Lon or Clp, responsible for degrading the antitoxins [32]. Thus, activation of Lon or Clp could serve as an indirect mechanism for toxin activation within the cell, and there is recent precedent for the identification of such compounds in other systems [85, 143]. Although this strategy may be generally toxic to the cell, there is evidence that Lon overproduction specifically activated the toxin YoeB from its complex with the YefM antitoxin, resulting in mRNA cleavage and cell lethality [31].

Although the indirect approaches have promise, this thesis work focused on methods to directly activate the toxin. In the most straightforward approach, a drug would directly target the TA system proteins and relieve antitoxin inhibition of the toxin. This could be achieved by disruption of the TA complex or prevention of complex formation, as shown in Figure 1.5. Complete disruption may be required for activation of a toxin such as the ribosome-dependent ribonuclease RelE, as the RelB-RelE complex is likely too large to access the ribosomal A-site [158]. In contrast, activation of a toxin such as MazF, which cleaves free mRNA, may not require full disruption of the complex and instead may be achieved by vacating MazE from the MazF active site or by allosteric activation of MazF in complex with MazE. Thus, direct toxin activation may be accomplished by targeting the complex, the antitoxin, or the toxin.
1.4.2 Which protein should be targeted?

When designing experiments to identify a direct toxin activator, one must decide which protein to target: the complex, the antitoxin or the toxin (Figure 1.6). There are advantages and disadvantages to each approach. Identification of a compound that activates the toxin by directly disrupting the interaction between the toxin and antitoxin would be the ultimate triumph, as the complex constitutes the most physiologically relevant form. The limitation of this approach is overcoming the incredibly high affinity complex formed between the toxin and antitoxin. As mentioned previously, the TA interaction is on the order of sub- to low double digit nanomolar affinity and has no apparent “off-rate” in vitro. It is likely that the static nature of the in vitro complex does not accurately reflect the situation in the cell, where proteases are involved in constant turnover of the antitoxin in the complex. However, faithfully recapitulating the conditions in the cell to take advantage of the turnover of the antitoxin in vitro presents its own challenges.
Binding the antitoxin to compete it away from the toxin and thus prevent complex formation is an intuitive strategy for toxin activation. The major advantage of a molecule that targets the antitoxin is to remove its inhibition on the toxin. Additionally, a compound that targets the antitoxin may interfere with the antitoxin binding to the operator DNA, alleviating the transcriptional repression and resulting in higher amounts of toxin available to mediate cell killing. Of course, the levels of the antitoxin would increase as well, which highlights the major disadvantage of targeting the antitoxin: the antitoxin exists in abundance over the toxin, so high concentrations of compound may be required for full effect. One experimental limitation with targeting the antitoxin is its inherently unstructured nature, which makes it less suitable for in vitro binding assays.

A compound that binds the toxin could act by either preventing the binding of the antitoxin to the toxin or by enhancing the enzymatic activity of the toxin, or both. One potential limitation of this strategy is that since the antitoxin usually inhibits the toxin by occupying its active site, a molecule that competes for binding with the antitoxin may also inhibit the toxin. However, there are many points of contact between the toxin and antitoxin so it is possible that preventing any of these contacts would effectively preclude antitoxin inhibition of the toxin. A compound that enhances the enzymatic activity may or may not modulate the interaction between the toxin and the antitoxin, but rather amplify the potency of the toxin by increasing its catalytic efficiency. Although this strategy could potentially be applied to any enzymatic toxin, the feasibility of in vitro assays currently limits it to the ribosome-independent ribonucleases. One physiologically relevant limitation of this strategy with ribosome-dependent ribonucleases is the

![Diagram](image-url)
potential allosteric change or increase in size that might prevent the toxin from binding the ribosome due to steric hindrance.

Each of these strategies was employed throughout efforts towards identifying TA systems activators described herein. Disruption of the RelBE TA system by targeting either the complex or the antitoxin, as discussed in Chapter 3, were tenable strategies as RelE is a ribosome-dependent ribonuclease that may require complete disruption from RelB to gain access to the ribosome. Furthermore, analysis of RelE activity requires actively translating ribosomes, which is not amenable for high throughput experiments. After discovering that the antitoxin is a poor target for \textit{in vitro} studies, the focus switched to the MazEF TA system. The advantage of working with the ribosome-independent ribonuclease MazF toxin was that its robust \textit{in vitro} activity enabled a meaningful assessment of the compound’s effect on the toxin activity, as presented in Chapter 4 and Appendix 2.

1.4.3 Which TA systems should be targeted?

Although the strategies described above are mechanistically distinct, their common goal is to artificially activate the toxin from the inert TA complex to kill the bacterial cell. There are two main requirements for successful application of this target-based strategy; the first is an understanding of which TA pairs would serve as ideal targets of an artificial activator. Once that is achieved, the search for molecules that activate the toxin and lead to toxin-mediated cell death can commence. Considerations for an ideal target include prevalence, toxicity, and potential for resistance.

1.4.3.1 Bioinformatics studies reveal widespread nature of TA systems

Recent genome sequencing and bioinformatic studies have revealed a plethora of TA systems across bacterial species. In 2005, Pandey and Gerdes performed an exhaustive search of 126 sequenced prokaryotic genomes and reported that genes predicted to encode TA systems are highly abundant in free-living bacteria but are absent from the genomes of host-associated bacteria [122]. Shao and co-workers expanded on existing datasets to identify 10,753 putative TA pairs in 1240 sequenced genomes representing 962 bacterial and archaeal strains [152]. More recently, Leplae and co-workers revealed 7034 toxins and 10,829 antitoxins in a search for Type II TA systems in 2181 prokaryotic genomes [57, 84]. From this work novel toxins and antitoxins were discovered, some of which were experimentally validated using a host killing and rescue assay in \textit{E. coli} [84]. In addition to discovering a multitude of TA systems and advancing our
understanding of the evolutionary relationships between them, these bioinformatics studies serve as a starting point for more detailed analyses of TA systems within their respective hosts.

Genes for TA systems have been identified in nearly all bacterial pathogens, contributing to their attractiveness as potential antibacterial targets, but which ones will make the best targets? Since many TA systems exist on plasmids or are closely linked with mobile genetic elements, their presence within a given bacterial species is likely to be heterogeneous. Thus, studying TA systems within actual clinical isolates is a necessary and complementary approach to bioinformatics studies. The crucial steps in investigating the tractability of TA systems as antibacterial targets are to determine (i) if TA systems are present in drug-resistant bacterial pathogens, (ii) which TA system are most prevalent, and (iii) whether the TA systems are functional.

1.4.3.2 Epidemiology in enterococci

In 2007, an examination of TA genes within total genomic DNA from clinical isolates of VRE was reported. Using a PCR-based screen with gene-specific primers for individual TA systems, certain TA genes were found to be widespread across the collection of 75 VRE isolates, namely mazEF (100%), axe-txe (75%), relBE (47%), and ω-ε-ζ (44%) [105] (Table 1.1). Many of these TA systems were present on plasmids carrying the vanA gene cassette. Reverse transcription PCR (RT-PCR) analysis showed that the ubiquitous TA system, mazEF, was transcribed in VRE. Furthermore, the mazEF genes, cloned with their native promoter from a VRE isolate, stabilized the unstable enterococcal plasmid pAM401, demonstrating the functionality of this TA system [105]. This epidemiological survey was the first to define which TA systems are most prevalent in clinical isolates of pathogenic bacteria, suggesting these as a viable target for exploitation. Further examination of six axe-txe-positive VRE strains from this study revealed that axe-txe was transcribed in all cases, and physical linkage to the VanA resistance determinant was confirmed by DNA sequencing [58]. Another survey of plasmid DNA isolated from a collection of 93 geographically and epidemiologically diverse Enterococcus faecium strains revealed that 42 (45%) and 18 (19%) harbor genes for axe-txe and ω-ε-ζ, respectively [138] (Table 1.1). A smaller study of VRE strains carrying VanB-type vancomycin resistance genes, each from different pulse-field gel electrophoresis (PFGE) types, showed that axe-txe was physically linked to the plasmid encoding vanB in eight of nine strains [15].
Table 1.1 Prevalence of TA systems determined by PCR screen and BLASTN homology search

<table>
<thead>
<tr>
<th>TA system</th>
<th>Organism</th>
<th>Location</th>
<th>PCR resultsa</th>
<th>Referenceb</th>
<th>presence on published sequence from specific organism (blastn)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>mazEF</td>
<td>enterococci</td>
<td>plasmid</td>
<td>75/75</td>
<td>[105]</td>
<td>0 genomes</td>
</tr>
<tr>
<td>axe-txe</td>
<td>enterococci</td>
<td>plasmid</td>
<td>56/75</td>
<td>[105]</td>
<td>6 plasmids</td>
</tr>
<tr>
<td>relBE</td>
<td>enterococci</td>
<td>plasmid</td>
<td>42/75</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>ω-ε-ζ</td>
<td>enterococci</td>
<td>plasmid</td>
<td>33/75</td>
<td>[105]</td>
<td>7 plasmids w/ ≥90% identity</td>
</tr>
<tr>
<td>mazEF_{sa}</td>
<td>S. aureus</td>
<td>chromosome</td>
<td>78/78</td>
<td>[178]</td>
<td>44 chromosomes</td>
</tr>
<tr>
<td>parDE_{pa}</td>
<td>P. aeruginosa</td>
<td>chromosome</td>
<td>13/42</td>
<td>[178]</td>
<td>2 chromosomes</td>
</tr>
<tr>
<td>relBE_{pa}</td>
<td>P. aeruginosa</td>
<td>chromosome</td>
<td>42/42</td>
<td>[178]</td>
<td>9 chromosomes</td>
</tr>
<tr>
<td>higBA_{pa}</td>
<td>P. aeruginosa</td>
<td>chromosome</td>
<td>42/42</td>
<td>[178]</td>
<td>9 chromosomes</td>
</tr>
<tr>
<td>relBE_{spn}</td>
<td>S. pneumoniae</td>
<td>chromosome</td>
<td>70/70</td>
<td>[113]</td>
<td>30 chromosomes [113]</td>
</tr>
</tbody>
</table>

aPCR using gene-specific primers to screen for presence of TA systems in collections of pathogenic bacteria
bReference refers to manuscript in which PCR result was published
cNumber of hits in blastn search and location (plasmid or chromosome) of TA genes. Blast searches were performed as part of this study unless otherwise indicated.

1.4.3.3 Further defining prevalence and functionality in clinically significant bacteria

Additionally, the prevalence of TA systems was studied in methicillin-resistant Staphylococcus aureus (MRSA) and P. aeruginosa. This survey demonstrated the ubiquity of mazEF_{sa} in 78 MRSA clinical isolates, and higBA_{pa} and relBE_{pa} in 42 P. aeruginosa clinical isolates [178] (Table 1.1). It was also shown that these TA systems are transcribed by their respective hosts, suggesting that they are functional units. Importantly, the PCR-based screen revealed that the parDE_{pa} TA system was present in only 30% of the clinical isolates. Inspection of the three sequenced genomes of P. aeruginosa clinical isolates shows that parDE_{pa} is present in PAO1 and PA7, but not PA14. Furthermore, genotyping of P. aeruginosa isolates using multi-locus variable number tandem repeat analysis (MLVA) revealed that the presence of parDE_{pa} did not correlate with genome relatedness. Thus, the inconsistent presence of parDE_{pa} suggests that activation of ParDE_{pa} would not be a good candidate for a TA-based therapeutic strategy versus P. aeruginosa. Some of these results, along with a survey of A. baumannii, are presented in more detail in Chapter 2 of this thesis. A similar study revealed the conservation of relBE2Spn in 70 clinical isolates and 30 sequenced strains of Streptococcus pneumoniae [113] (Table 1.1).

A combination of factors will determine whether a given TA system is a good antibacterial target. Prevalence and functionality within clinical isolates are absolutely required. The aforementioned epidemiological studies showed that the genes for TA systems were present...
and transcribed; however, Western blot analysis using antibodies raised to the specific TA systems would lend further support for these protein targets. While more data must be collected, based on prevalence, functionality, and mode-of-action, MazEF, RelBE, and HigBA appear to be reasonable targets for artificial toxin activation that could lead to bacterial cell death [105, 178]. Additional points to consider revolve around the activity of the toxin itself and will be discussed below.

1.4.3.4 Toxin mode of action/Potential for resistance

The toxin mode of action could influence both the toxicity of the toxin and the propensity for resistance to toxin-activating molecules to arise, leading to reduced efficacy of the strategy. For example, resistance to the toxin CcdB is conferred by a single point mutation within its target, DNA gyrase [13], suggesting limitations with CcdB activation strategy. However, it is more difficult to envision how resistance would arise to a toxin like MazF, which cleaves single-stranded mRNA [186]. Mutational inactivation of the toxin could occur, but presumably the cell would incur a fitness cost associated with such a mutation.

1.4.3.5 TA system involvement in persistence

Persistor cells are defined as a small fraction of a bacterial population that tolerate antibiotics not by mutation or acquisition of resistance determinants but by entering a state of dormancy [86]. Further culturing of these dormant cells restores normal growth, and subsequent application of antibiotic selects for a new sub-population of persistor cells [74]. One model proposes that persisters arise when a small fraction of cells in a mid-exponential phase experience stochastic changes in gene expression, producing individual dormant cells that are recalcitrant to subsequent antibiotic treatment [10, 150]. This phenotypic switch has been shown to be induced by activation of chromosomally-encoded toxin genes [97]. Additionally, upregulation of the transcripts for TA genes have been observed in persistor cells [150], and the occurrence of persistor cells progressively diminished with successive deletion of the ten ribonuclease-encoding toxin genes in E. coli [97]. A separate model proposes that the SOS response induces persistor cell formation via the Type I TA system tisAB/istR. Treatment with fluoroquinolones induces the SOS response, causing transcription of the LexA-controlled tisB, which encodes a membrane acting toxin [43]. TisB decreases the proton motive force, which leads to decreased ATP levels and a state of dormancy [167]. Although additional work is required to describe the exact mechanism of persistor cell formation and resuscitation, it will be important to determine whether
artificial toxin activators induce persister cell formation as this may contribute to chronic infections [86].

1.4.4 Previous work toward discovering toxin activators

The exploitation of TA systems as an antibacterial strategy has been proposed by many groups as a viable tactic, however success remains elusive. There have been efforts towards identification of peptides that modulate the activity of TA systems; these peptides have been shown to enhance the toxin enzymatic activity, preclude the inhibition of the toxin by the antitoxin, reduce the degree of complex formation or disrupt the TA complex. Although these works have advanced our understanding of TA systems and how to target them, they are not without issues, limitations or drawbacks, which are discussed below.

1.4.4.1 Extracellular death factor and MazEF<sub>Ec</sub>

*E. coli* mazEF is one of the best characterized TA systems and has been implicated in cell stress responses and programmed cell death. A variety of stressors cause MazF-induced cell death, including short-term exposure to antibiotics that target transcription or translation [144], DNA damage due to thymine starvation [145], overproduction of ppGpp [3], and exposure to DNA damaging agents such as mitomycin C or nalidixic acid [61].

The Engelberg-Kulka group has published a series of papers in which they claim to have identified an endogenous peptidic activator of the MazF [12, 77-79]. If confirmed, this would be a significant discovery and would lend considerable support to the notion that TA systems are exploitable antibacterial targets. However, as described below, this work is controversial and awaits independent validation. These studies began with the observation that the ability of mazEF to mediate cell death in response to stress was dependent on population size. Brief treatment of cells with rifampicin triggered mazEF-mediated cell death at densities of 3 x 10<sup>8</sup> or 3 x 10<sup>7</sup> cells/mL, but not when the same culture was diluted to 3 x 10<sup>5</sup> or 3 x 10<sup>4</sup> cells/mL [78]. Furthermore, transfer of supernatant from a dense culture to a dilute culture followed by a short treatment with rifampicin, chloramphenicol, or trimethoprim resulted in mazEF-dependent cell death. These results suggest that mazEF-dependent cell death requires an “extracellular death factor” (EDF). Subsequent isolation experiments identified EDF as a linear pentapeptide quorum-sensing molecule with the sequence NNWNN. Synthetic NNWNN also induced mazEF-mediated cell death in response to antibiotic stress [78].

The effect of EDF on MazF activity *in vitro* was assessed using a continuous fluorometric assay for MazF, and in this experiment EDF significantly enhanced the endoribonucleolytic
activity of MazF in both a concentration- and sequence-dependent manner [12]. The derivatives NNGNN and NWN gave no enhancement of MazF activity, whereas other residue substitutions, additions, and deletions were well-tolerated. Furthermore, when increasing concentrations of EDF were mixed with MazE and then added to MazF and fluorogenic substrate, the ability of MazE to inhibit MazF activity was diminished, indicating that EDF prevented the inhibition of MazF by MazE in vitro. A structural model suggests that EDF directly competes with the MazE71-75 sequence IDWGE by placing the EDF Trp3 in the hydrophobic MazF pocket that is typically occupied by MazE Trp73.

**Figure 1.7** A possible model for mazEF-dependent cell death induced by extracellular death factor (EDF). EDF production requires primarily the protein Zwf and the protease ClpXP. Preliminary investigation suggests that EDF freely diffuses in and out of the cell. Treatment with an antibiotic triggers the stress response, which inhibits transcription of mazEF. EDF binds to and enhances MazF ribonuclease activity in vitro, although it is not known whether EDF binds to free MazF or to the MazEF complex (as it is depicted here). MazF cleaves single-stranded mRNA in a sequence-specific manner, leading to cell death. It should be noted that there is controversy about EDF, and the role of EDF has not been independently confirmed by multiple laboratories. Figure taken from [179].

Given the experimental results, the authors proposed that the endoribonuclease MazF serves as a cytoplasmic sensor of EDF [12, 77, 78] and that EDF is required for mazEF-dependent cell death. A possible model for this phenomenon is shown in **Figure 1.7** and summarized in **Table 1.2**. It is important to note that several laboratories have tried to replicate critical elements of the EDF experiments, but have been unsuccessful [57, 59, 169]. As such, it is too early to classify EDF as a bona fide TA system activator; a rigorous validation of the
NNWNN peptide in various bacterial strains by multiple research groups will be required for the EDF phenomena to be widely accepted.

1.4.4.2 Inhibitors of the PemI-PemK/MoxX-MoxT interaction

PemK, of the *B. anthracis* PemIK TA module, cleaves single-stranded RNAs and is inhibited by the binding of antitoxin PemI [2]. Analysis of PemI deletion variants indicated that the C-terminus is required to bind to PemK. Based on this information, six hepta- and octa-peptides, representing fragments of the antitoxin located in a predicted helical region within the TA binding interface, were analyzed for their ability to inhibit the PemI-PemK interaction [2]. ELISA results revealed that each designed peptide was capable of preventing the PemI-PemK interaction to a certain extent (Table 1.2), whereas nonspecific 15- and 9-residue peptides based on the N-terminus of PemI did not affect the PemI-PemK interaction [2].

The authors then examined the effect of the peptides on PemK ribonuclease activity using a fluorogenic chimeric DNA-RNA substrate or a fluorogenic rC substrate [2]. The two peptides that prevented the PemI-PemK interaction to the greatest extent, LLFQHLTE (35% prevention) and RRGYIEMG (30% prevention), inhibited the PemK ribonuclease activity *in vitro* (Table 1.2), while the remaining four peptides did not inhibit PemK ribonuclease activity. This result is not surprising, as one might expect a peptide fragment of the antitoxin to reduce the activity of the toxin. Recently, this *B. anthracis* TA system was re-named MoxXT [27]. A rationally designed octapeptide, SKIGAWAS, which has potential to form an α-helix and is predicted to occupy the binding interface between MoxX and MoxT, was shown to prevent the MoxXT complex formation *in vitro* by 42% (Table 1.2). However, this peptide also partially inhibited the ribonuclease activity of MoxT [27]. These experiments are encouraging in that they demonstrate that TA interactions can be prevented by peptides; the next step is to design and identify peptides that prevent the protein-protein interaction without inhibiting the toxin enzymatic activity.

1.4.4.3 Disruptors of ε-ζ

A publication disclosed the results from a screen of peptides for disruption of the *Streptococcus pyogenes* plasmid-derived TA system called ε-ζ [91]. This screen utilized Luc-ε and ζ-GFP fusion proteins in a bioluminescence resonance energy transfer (BRET) assay. An extensive collection of various peptide libraries, including over $4.95 \times 10^7$ 6-residue peptides, $2.74 \times 10^4$ 14-residue β-sheet peptides, and $2.74 \times 10^4$ 17-residue α-helix peptides were evaluated for their ability to disrupt the interaction between Luc-ε and ζK46A-GFP in a cell-free extract. Peptides were tested at both 0.6 µM and 7 µM. Hits were selected based on their ability to
decrease the BRET signal relative to untreated controls. No hits were observed at either concentration with the 6- and 14-residue peptide libraries; however, two wells containing members of the 17-residue library decreased the BRET signal [91] (Table 1.2). These peptide mixtures were not tested for their ability to activate or inhibit the ζ toxin. When the number of peptides from the sub-libraries that contained the positives tested was reduced, the decrease in BRET signal was lost [91]. Thus, the disruption of the Luc-ε-ζK46A-GFP complex was possibly due to more than one peptide with weak activity. While this could mean that the ε-ζ interaction can be disrupted, more investigation is required to confirm this finding and to determine whether the peptide(s) binds to the antitoxin or to the toxin and if toxin activity is affected in the process.

<table>
<thead>
<tr>
<th>Target</th>
<th>Peptide</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MazEF&lt;sub&gt;E&lt;/sub&gt;</td>
<td>NNWNN</td>
<td>Isolated from <em>E. coli</em> supernatant; referred to as Extracellular Death Factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces MazF-dependent death in response to rifampicin treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increases MazF activity in the presence of MazE by ~60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enhances enzymatic activity of MazF by ~60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Model suggests direct competition with MazE71-75 sequence IDWGE</td>
</tr>
<tr>
<td>MoxXT</td>
<td>LLFQHLTE</td>
<td>Represents fragment of antitoxin within TA binding interface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevents MoxX-MoxT complex formation by 35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits MoxT ribonuclease activity by ~30%</td>
</tr>
<tr>
<td></td>
<td>RRGYIEMG</td>
<td>Represents fragment of antitoxin within TA binding interface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevents MoxX-MoxT complex formation by 30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits MoxT ribonuclease activity by ~30%</td>
</tr>
<tr>
<td></td>
<td>SKIGAWAS</td>
<td>Designed based on modeled structure of MoxXT complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prevents MoxX-MoxT complex formation by 42%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibits MoxT ribonuclease activity by ~38%</td>
</tr>
<tr>
<td>ε-ζ</td>
<td>17-residue α-helix library</td>
<td>2 wells containing mixtures of peptide library appeared to disrupt ε-ζ interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduction in number of peptides caused a loss in disruption effect</td>
</tr>
</tbody>
</table>

1.5 STRATEGIES TO TARGET TA SYSTEM PROTEINS

The strategies employed to target TA system proteins are disruption of the protein-protein interaction, as noted in each case discussed above, and enhancement of the enzymatic activity of the toxin. There have been considerable efforts towards both of these strategies in other drug discovery endeavors, which will be discussed now.

1.5.1 Protein-protein interaction inhibition

Interactions between some globular proteins are mediated by relatively large, flat and featureless interfaces, which are typically more challenging to target. The interaction between the
toxin and the antitoxin is typically mediated by extensive electrostatic and hydrophobic interacts and the contact surface between the two proteins is significant. In MazEF\textsubscript{Ec}, the C-terminus of the antitoxin MazE resembles a long, relatively unstructured polypeptide that binds between within the toxin MazF dimer to occupy its active site [70]. In RelBE\textsubscript{Ec}, antitoxin RelB\textsubscript{Ec} appears to wrap around the toxin RelE\textsubscript{Ec} to inactivate it [17, 90]. The limited amount of crystallographic data for TA proteins impedes our ability to understand the intricate interactions between the two proteins. Furthermore, although we understand that the protein-protein interface is extensive, there is limited information on residues important for binding between the two proteins or if binding “hot spots” exist. “Hot spots,” are short interfaces that facilitate high affinity binding [16, 34]. Alanine-scanning mutagenesis is typically used to reveal such hot-spots [37], which may help guide the development of protein-protein interaction (PPI) inhibitors.

Site-directed mutagenesis of MazE\textsubscript{Ec} revealed that residues L55 and L58 were required for binding to MazF\textsubscript{Ec} [184]. Additionally, a RelB\textsubscript{Ec} A39T mutant exhibited decreased stability and was degraded faster than WT RelB\textsubscript{Ec}, leading to hyperactivity of RelE\textsubscript{Ec} [30]. Unfortunately, it was never fully elucidated if the increased proteolytic susceptibility of RelB\textsubscript{Ec} A39T was due to improved recognition or cleavage by Lon or because its affinity for RelE\textsubscript{Ec} was lower thus reducing the protection gained by being in the TA complex, so the contribution of this residue to the interaction remains to be determined. Crystallographic or NMR structural data are invaluable to the design of PPIs; indeed, such data exist for nearly all PPIs that have been successfully disrupted [168].

Targeting the interfaces between protein binding partners is an attractive therapeutic strategy and considerable progress has been made in identification of PPI antagonists for antiviral, antibacterial and anticancer targets [176]. The peptide-based HIV gp41 fusion inhibitor T20, sold as enfuvirtide or Fuzeon, was approved for the treatment of resistant HIV infections in 2003 [82, 137]; however, limited potency and poor pharmacokinetic properties motivate continuous searches for improved HIV fusion inhibitors [182]. The most extensive target studied for antibacterial application is the ZipA-FtsZ interaction. Inhibitors of ZipA-FtsZ have been identified by a variety of methods, including a fluorescence-polarization assay to screen 250,000 small molecules, [73], structure-based design combining two weak fragments to form a more potent inhibitor [157], NMR screening of 825 fragments, the hits of which were evaluated in a co-crystal structure [161], and a shape-directed lead-hopping approach facilitated by Rapid Overlay of Chemical Structures [140]. Although hits have been evaluated, a PPI with sufficient potency remains to be identified [161].
The widely studied p53-MDM2 system has become the model system for the inhibition of PPIs, and identification of such inhibitors has been tackled by a variety of approaches. The crystal structure MDM2-p53 revealed a deep hydrophobic p53-binding pocket on MDM2; p53 binds with a $K_D$ of 420nM [81]. Inhibition of MDM2-p53 has been accomplished using both peptides and small molecules. Isothermal titration calorimetry revealed a 10-mer peptide based on the p53 sequence with a $K_D$ value of 46 nM [147]. Additionally, a peptide with a $K_D$ value of 3.3 nM for MDM2 was selected from a phage displayed peptide library [124] and structure-based design guided modifications which improved the potency 5-fold [133]. The potent small molecule antagonist discovered by a screening method, Nutlin-3, has been extensively studied and optimized to yield a $K_i$ value of 36 nM [151, 171]. All peptides and small molecules able to disrupt MDM2-p53 bind in the hydrophobic cleft of MDM2 [124, 147, 151, 171].

Other notable drug targets for which potent PPI inhibitors have been identified include two anti-apoptotic proteins, Bcl-X$_L$ and XIAP. A 25-mer peptide that binds Bcl-X$_L$ and prevents its interaction with pro-apoptotic molecule, BAD, was shown to have a $K_D$ of 0.2nM [132]. Additionally, a potent small molecule inhibitor with a $K_i$ value of 0.6 nM was developed using NMR to carry out fragment-based design [131]. A high throughput “SAR by NMR” strategy was employed to discover ABT-737, a potent inhibitor of the Bcl-XI-BAD interaction with a $K_i \leq 1$ nM [118]. Poor bioavailability prompted the development of a similarly potent derivative, ABT-263 [162]. It functions as a single-agent therapeutic to cause regression of tumor growth by activating pro-apoptotic proteins and has undergone evaluation in Phase II clinical trials [139].

The anti-apoptotic protein XIAP directly inhibits caspase-3 and -7; inhibition is relieved by binding of Smac to XIAP, mediated by the four N-terminal amino acids, AVPI [155]. Fluorescence polarization was used to identify a potent non-peptide mimetic of Smac, SM-164, which showed an IC$_{50}$ value of 1.39 nM [94, 155]. A number of molecules targeting inhibitors of apoptosis proteins (IAP inhibitors) have been tested in clinical trials [53, 156].

Strategies employed to identify PPI inhibitors include peptide fragment analysis, chemical library screening, phage display of peptide libraries and alanine screening mutagenesis [106, 176]. Peptide fragment analysis provides useful information regarding the key regions for binding and the minimal sequence required to disrupt, which can then be guide the design of peptidomimetic or small molecule disruptors. Screening libraries of diverse chemical structures has also successfully identified small molecule PPI inhibitors.
1.5.2 Enzyme activators

RNase L is an endoribonuclease that is involved in antiviral innate immunity of higher vertebrates to viral infections. Its activity is stimulated by 2’-5’oligoadenylate (2-5A), which induces self-association of RNase L, driving formation of the catalytically-active high-order homo-oligomers that cleave dsRNA and prevent expression of viral proteins [173]. Two lead compounds that activate RNase L were identified from a high throughput screen of 32,000 compounds [159]. Unfortunately, although it was shown that these 2 hits activate by the same mechanism as the natural activator, the activators exhibited an EC$_{50}$ of ~20 µM, approximately 5 orders of magnitude higher concentration than 2-5A, which has an EC$_{50}$ of 0.5 nM.

Restoration of a function apoptotic pathway by activation of the precursor to executioner caspase-3, procaspase-3, has been developed as a targeted anticancer strategy. A high throughput screen of 20,500 compounds revealed the procaspase-activating compound PAC-1 which promotes maturation of the zymogen procaspase-3 to active caspase-3 with an EC$_{50}$ of 0.22 µM [134]. PAC-1 functions by chelating inhibitory zinc from procaspase-3, enhancing autocleavage to form active caspase-3 [129]. The non-neurotoxic derivative sulfonamide-PAC-1 also displays potent anticancer activity and has shown success in a phase I pet dog clinical trial [130].

The activation of glucokinase is a promising antidiabetic strategy [100]. The role of glucokinase (GK) as a glucose sensor in pancreatic cells stimulates glucose-dependent insulin production and release; it also directs the ability of the liver to convert glucose to glycogen. Evaluation of 120,000 small molecules revealed one GK activator, which led to the development of RO0281575 [55]. This compound enhances the enzymatic activity of GK by increasing both its affinity for glucose and its maximal catalytic rate and in mice it was shown to increase insulin release from the pancreas and stimulate glucose usage in the liver [55]. However, toxicity in preclinical trials prompted the development of safer derivatives, including the compound RO4389620 or Piragliatin [142], which was shown to lower glucose levels in Type II Diabetes patients in a Phase II clinical trial [18].

1.6 CHALLENGES FOR THE DISCOVERY OF TOXIN ACTIVATORS

Artificial activation of TA systems is a potentially powerful antibacterial strategy. However, the three examples discussed in 1.4.4 are the state-of-the art for TA activation with a drug-like compound, thus as of yet there is no molecule convincingly capable of modulating the TA interaction. Such compounds are needed to fully explore the potential of TA disruption and toxin activation as an antibacterial strategy. Five key questions regarding the discovery of an artificial toxin activator are discussed below.
First, will the strength of the TA interaction preclude disruption with a peptide or small molecule? Most toxin-antitoxin pairs have strong affinities, mediated by extensive electrostatic and hydrophobic interactions [70], resulting in $K_D$ values on the order of 1 nM [88, 120]. In most cases, the antitoxin wraps around the toxin to form the inactive complex. In contrast, interactions between other protein-protein pairs that have been successfully inhibited are characterized by long, shallow pockets that are accessible to small molecules [62, 176]. Perhaps not surprisingly, in all the three examples presented above, peptides were evaluated as toxin-antitoxin disruptors; a small molecule TA modulator has yet to be discovered.

Confounding this issue is the relatively limited information on the specifics governing the TA interaction. Crystal structures have been solved for some TA systems including *S. pyogenes* ε2-ζ2 [101], and *E. coli* MazEF [70], and there is a solution structure of *E. coli* RelBE [90]. However, although there is considerable homology between toxins of the same family and even of different families, the sequence and secondary structure of antitoxins are much more divergent; thus, more structural data is needed. Additionally, minimal data is available regarding the amino acid residues that define the ‘hotspots’ between toxin and antitoxin. Defining these hotspots and minimal TA binding regions through mutational analysis will facilitate the design of molecules specifically targeting these interactions.

Second, can the limitations in current assays for toxin activity be overcome? The process of searching for toxin activators is hindered by significant limitations in current *in vitro* enzymatic assays for toxin activity. Efficient screening of potential activators requires a robust assay, such as the continuous fluorometric assay developed to monitor MazF ribonuclease activity [174]. This assay, utilized in two separate examples presented above, allows for high-throughput analysis of molecules that modulate the enzymatic activity of toxins that cleave free mRNA, such as MazF and PemK. In contrast, toxins such as the ribosome-dependent ribonuclease RelE must be evaluated by reconstituting actively translating ribosomes, which requires a fairly complicated *in vitro* assay not easily amenable to high-throughput screening. The development of high-throughput methods of assessing ribosome-dependent ribonuclease activity would facilitate the discovery of activators of RelE and HigB.

Additionally, *in vitro* assays lack key components that exist within the cell, namely the proteases that naturally relieve antitoxin inhibition of the toxin. Thus, current *in vitro* assays are unable to discover compounds that work through an indirect mechanism. For example, a molecule could increase the proteolytic susceptibility of the antitoxin or modulate TA expression. Discovery of a molecule that acts by one of these mechanisms would best be achieved using cell-based assays.
Third, should the toxin or the antitoxin serve as the target? The examples presented above do not give a unified answer. On one hand, EDF (suggested to bind MazF by mimicking a region of the MazE antitoxin) is claimed to both enhance MazF activity and prevent MazE inhibition by directly binding to MazF [12]. On the other hand, peptide fragments of the antitoxin MoxX bind to and inhibit the ribonuclease activity of the toxin MoxT [2, 27]. Different antitoxin fragments can have varying effects on the toxin; obviously, if binders to the toxin are developed, they should activate, rather than inhibit. Conceptually, a molecule that binds the antitoxin and modulates its interaction with the toxin is desirable; however, the intrinsically disordered structure of the antitoxin makes it a difficult target for \textit{in vitro} screens. Nevertheless, a promising new class of molecules that specifically target intrinsically disordered peptides is being developed [103]. A number of small molecules that target the oncoprotein c-Myc have been discovered via high throughput screening efforts [103]. Many of these compounds bind to highly unstructured c-Myc monomers, precluding their dimerization as well as complex formation with its binding partner, Max [103].

On a related note, is full TA disruption is necessary, or is activation of the toxin sufficient for an antibacterial effect? EDF is purported to prevent TA complex formation, but was not shown to activate MazF from the pre-formed MazE-MazF complex [12]. Similarly, the peptide fragments of MoxX prevented complex formation between MoxX and MoxT but were not shown to disrupt the interaction between MoxX and MoxT in a pre-formed complex [2, 27]. Is this significant in the cell where the antitoxin is subject to metabolic turnover?

Fourth, will a toxin-activating compound kill bacteria as a single-entity agent? Toxin-mediated cell death is typically studied in response to some outside stimulus, such as amino acid starvation or treatment with an antibiotic. Despite the ability of EDF to enhance MazF activity \textit{in vitro}, it is unable to induce MazF-dependent cell death on its own, and requires prior activation of the \textit{mazEF} module via an antibiotic such as rifampicin or chloramphenicol [77, 78].

Finally, how does the location of the TA genes (plasmid or chromosomal) influence the TA-targeting strategy? It seems clear that artificial activation of TA proteins encoded on plasmids would kill the cell in a manner analogous to antitoxin degradation, toxin activation, and cell death in post-segregational killing though TA plasmid stabilization mechanisms. When the TA genes are chromosomally-encoded, however, the copy number of resulting TA proteins may not be high enough to induce death after toxin activation. Furthermore, chromosomal TA systems have been implicated in the formation of persister cells, which some believe may contribute to chronic infection. There is concern that artificial activation of chromosomally-encoded toxins could potentially induce persister cell formation, an obviously undesirable effect.
TA systems present exciting opportunities for the development of novel antibacterial agents. The first requirement, demonstration of the ubiquity and functionality of TA systems in clinical isolates, has been satisfied for key pathogens, including VRE, MRSA and P. aeruginosa and S. pneumoniae. Additional epidemiological surveys and biochemical analyses of toxins will add to the catalog of potential TA systems to target. Although potentially significant, the Extracellular Death Factor story needs further clarification and independent validation. Preliminary work on the disruption of the ε-ζ complex and prevention of the MoxXT complex suggests that peptides can indeed be used to modulate the TA system interaction. Development of a toxin activator and extension of the TA activation strategy to in vivo studies are required to fully assess the potential of this strategy.

1.7 REFERENCES


CHAPTER 2
PREVALENCE AND TRANSCRIPTION OF TOXIN-ANTITOXIN SYSTEMS IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA AND ACINETOBACTER BAUMANNII


2.1 INTRODUCTION

Genes encoding toxin-antitoxin (TA) systems were found to be widespread in Enterococcus [9, 15, 20, 22], Staphylococcus aureus [25] and Streptococcus pneumoniae [18], however information regarding the prevalence of TA systems in Gram-negative bacteria is lacking. As discussed in Chapter 1, infections caused by the Gram-negative pathogens Pseudomonas aeruginosa and Acinetobacter baumannii are difficult to treat due to their intrinsic resistance and ability to acquire resistance to nearly all available antibiotics. Novel treatment strategies, such as TA system activation, are required to combat these hardy pathogens. Thus, we sought to define the prevalence of TA systems in a collection of clinical isolates of P. aeruginosa (PA) and A. baumannii (AB).

Previously, a bioinformatics survey of 126 prokaryotic genomes identified three TA loci, relBE, parDE, and higBA, on PA strain PAO1 [19]. Although no additional work has been published on the TA systems in PA, functional homologs have been described in other pathogenic bacteria, including RelBE in Streptococcus pneumoniae [17], Yersinia pestis [8] and Mycobacterium tuberculosis [27]; ParDE in Vibrio cholerae [28]; and HigBA in V. cholerae [3, 4], Proteus vulgaris [10] and Y. pestis [8]. Five TA loci belonging to the relBE gene family and one solitary toxin belonging to the ζ gene family were identified in the published sequences of one AB chromosome and five AB plasmids [11, 23]. Until recently, no information regarding TA systems in AB was available; however, a bioinformatics study of AB genomes identified at least 15 TA pairs, five of which were shown to be functional ribonucleases [12].

While the analysis of sequenced genomes has been informative, defining the presence and identity of TA loci in collections of clinical isolates is a preliminary requirement for the success of the TA targeting strategy. Thus, we surveyed 42 PA and 39 AB clinical isolates for the existence of the specific TA systems identified by bioinformatics analysis on PA [19] and on the sequenced genomes of AB [11, 23]. We found that relBE and higBA are ubiquitous in PA
clinical isolates and that \( relBE-I_{Ab} \) and the lone \( \zeta \) toxin were present in approximately half of the AB clinical isolates. Establishing the prevalence of specific TA systems in important pathogens dictates their potential for success as an antibacterial target.

2.2 INVESTIGATION OF TOXIN-ANTITOXIN SYSTEMS IN \textit{PSEUDOMONAS AERUGINOSA}

2.2.1 Assessment of \textit{P. aeruginosa} clinical isolate diversity

Twenty PA clinical isolates from cystic fibrosis patients were obtained from Carle Foundation Hospital (Urbana, IL) [16] and an additional twenty-two PA strains isolated from various acute infections were a gracious gift from Cubist Pharmaceuticals, Inc (Lexington, MA); these strains were collected from eight geographically distinct locations. These isolates were analyzed by the DNA-based typing method multi-locus variable number tandem repeat analysis (MLVA) to assess intra-species relatedness. The experimental variation between duplicate experiments was determined for the MLVA profile using five PA isolates and applied to establish a cutoff value of 97\% for typing strains with identical DNA banding patterns. For PA, using the 97\% cutoff value, 43 MLVA banding patterns were formed out of the 44 strains. When a cutoff value of 75\% was applied, 10 clusters were generated comprising 28 strains, and 26 MLVA banding patterns were discerned (Figure 2.1). Strains that group according to these two cutoff values are in a variety of clusters, demonstrating that the isolates studied were not clonal.

2.2.2 Presence of TA systems in \textit{P. aeruginosa} genomic DNA

Armed with the knowledge that the collection of PA clinical isolates were sufficiently diverse, an effort was made to define the prevalence of TA genes in the strains. The PA isolates were probed for homologs of the \( higBA \), \( parDE \) and \( relBE \) systems identified in PA strain PAO1 [19]. The oligonucleotide sequences of all PCR primers used to amplify TA genes are listed in Table 2.1 and the homologous regions are represented in Figure 2.2.

Total DNA preparations from each of the 42 PA strains were analyzed by PCR, and results were designated positive if a distinct band was observed at the expected size on an agarose gel. For the 42 PA isolates, \( relBE_{Pa} \) (42/42, 100\%) and \( higBA_{Pa} \) (42/42, 100\%) were ubiquitous, whereas \( parDE_{Pa} \) (13/42, 30\%) was less prevalent (Figure 2.3). Table 2.2 contains a complete list of all PA isolates and the TA genes detected by PCR. Comparison of the MLVA genotypes of PA strains that carry \( parDE_{Pa} \) showed that these strains are dispersed throughout the
dendrogram, indicating that there is no correlation between genome relatedness and carriage of \(parDE_{Pa}\).

Figure 2.1 Multi-locus variable number tandem repeat analysis of \(P.\ aeruginosa\) clinical isolates. The 97% clonal cutoff value and 75% similarity cutoff value are indicated by solid and dashed vertical lines, respectively. The clusters generated are shown in corresponding solid and dashed brackets alongside the dendrogram.
**Figure 2.2** Locations of primary homology for primers used in PCR screen, RT-PCR and flanking region PCR. (A-C) Primer sequences were based off the *P. aeruginosa* PAO1 genome. (A) The same internal primers were used to amplify a region of *parDE* for both the PCR-based screen and RT-PCR. Flanking *parDE* are genes encoding an integrase, a tRNA and a transferase. Primers were designed to amplify the sequence between the integrase gene and *parD* and between *parE* and the transferase gene. (B) Separate sets of internal primers were used to amplify regions of *relBE* for the PCR-based screen and for RT-PCR. Flanking *relBE* are genes encoding a transcriptional regulator and a hypothetical protein. Primers were designed to amplify the sequence from the transcriptional regulator gene to *relB* and from *relE* to the hypothetical protein gene. (C) Separate sets of internal primers were used to amplify regions of *higBA* for the PCR-based screen and for RT-PCR. Flanking *higBA* are genes encoding a hypothetical protein and a Ton-B dependent receptor. Primers were designed to amplify the sequence from the hypothetical protein gene to *higA* and from *higB* to the Ton-B dependent receptor gene. (D) In *P. aeruginosa* PA7, *higBA* is flanked by genes encoding a restriction endonuclease and a hypothetical protein. Primers were designed to amplify the sequence from the restriction endonuclease gene to *higA* and from *higB* to the hypothetical protein gene.
Figure 2.3 PCR analysis of PA isolates. The prevalence of genes encoding TA systems as assessed by PCR from the total genomic DNA of 42 clinical *P. aeruginosa* isolates.

The identity of approximately 10% of the positive PCR products was confirmed by sequencing. Sequenced PCR products revealed strong sequence identity (97.8 – 100%) to the reference TA system sequences (*higBA*<sub>Pa</sub>, 99.4% average identity; *parDE*<sub>Pa</sub>, 99.6% average identity; and *relBE*<sub>Pa</sub>, 98.7% average identity); alignments are shown in Figures 2.7-2.9).

2.2.3 TA gene location assessment

It was next investigated whether the TA genes were located on a plasmid or the chromosome of the PA isolates. The sequences directly upstream and downstream of the *relBE*<sub>Pa</sub> TA genes are highly conserved among the *P. aeruginosa* genomes in the National Center for Biotechnology Information (NCBI) Genome database, whereas the flanking regions of *parDE*<sub>Pa</sub> and *higBA*<sub>Pa</sub> are conserved in *P. aeruginosa* PAO1, LESB85 and UCBPP-PA14, but are different in strain PA7. Primers were designed (Table 2.1 and Figure 2.2) to amplify the sequences flanking the TA genes based on the conserved sequence in *P. aeruginosa* strains PAO1 and PA7. In this experiment the presence of a PCR product would suggest chromosomal location of the TA systems.

PCR analysis revealed that in the PA isolates, both flanking regions of the *parDE*<sub>Pa</sub> genes in all isolates (13/13, 100%) were amplified using primers homologous to the PAO1 reference sequence. The flanking regions of nearly all *relBE*<sub>Pa</sub> genes (41/42, 97%) were amplified, except for strain 1284, for which no flanking region could be amplified. Amplification was observed for the downstream sequence of every *higBA*<sub>Pa</sub> loci (42/42, 100%) as well as for the region upstream of *higBA*<sub>Pa</sub> except for in 10 strains (32/42, 76%). For these 10 strains, PCR was performed with various primers designed based on the PAO1 reference sequence, as well as primers designed to
probe the upstream sequence of higBA<sub>Pa</sub> observed in <i>P. aeruginosa</i> PA7; however, no product was amplified in any of these cases. All results from the flanking region PCR are listed in Table 2.3.

DNA sequencing was performed on >10% of the PCR products to confirm the identity of the amplified sequence. The flanking region PCR products of parDE<sub>Pa</sub> (92.6 – 98.2%), relBE<sub>Pa</sub> (96.2 – 99.4%) and higBA<sub>Pa</sub> (91.8 – 99.4%) also showed strong sequence identity to the reference <i>P. aeruginosa</i> PAO1 sequence (Figures 2.10-2.12).

2.2.4 Detection of TA transcripts

To determine if the TA systems were transcribed by the clinical isolates, RT-PCR was performed with total RNA isolated from >10% of strains shown by PCR to contain the genes for each TA system. The oligonucleotide sequences of all primers used for RT-PCR are listed in Table 2.1 and Figure 2.2 depicts the regions of homology. The transcripts for relBE<sub>Pa</sub> (6/6), higBA<sub>Pa</sub> (5/5) and parDE<sub>Pa</sub> (3/3) transcripts were detected in all PA strains probed by RT-PCR (Figure 2.4). For all samples, no amplification products were observed in the absence of reverse transcriptase, confirming that the products seen by RT-PCR were due to the presence of the TA transcript in the clinical isolates and not DNA contamination (Figures 2.4).

![Figure 2.4](image)

**Figure 2.4** Detection of TA system transcripts. RT-PCR with primers complementary to the genes encoding each TA system indicates that transcripts are produced in the clinical isolates of <i>P. aeruginosa</i>, (+) RT row. Controls for DNA contamination, in which the reverse transcriptase is omitted from the reaction mix, yield no product, (-) RT row. Clinical isolates analyzed are indicated by the strain number above each column.
2.3 INVESTIGATION OF TA GENES IN *ACINETOBACTER BAUMANNII*

2.3.1 Presence of TA genes in *A. baumannii* genomic DNA

Thirty-nine *A. baumannii* (AB) clinical isolates obtained from Prof. John Quale were gathered from citywide surveillance studies in Brooklyn, NY from 2001 to 2006 [2]. The following homologs identified in the specified AB chromosomes or plasmids were probed: relBE-1<sub>Ab</sub> from strain ACICU [11], relBE-2<sub>Ab</sub> from plasmid pACICU [11], a solitary ζ<sub>Ab</sub> toxin from plasmid pACICU2 [11, 23], relBE-3<sub>Ab</sub> from p1ABAYE [23], relBE-4<sub>Ab</sub> from p3ABAYE [23], relBE-5<sub>Ab</sub> from p2ABSDF [23]. The oligonucleotide sequences of all PCR primers used to amplify TA genes are listed in Table 2.1. For AB, relBE-1<sub>Ab</sub> (21/39, 53%) and the solitary ζ<sub>Ab</sub> toxin (18/39, 46%) were each observed in approximately half of the 39 AB strains (Figure 2.5). For those AB strains that harbored no detectable TA systems, PCR performed with primers to amplify recA [13] confirmed the presence of DNA (data not shown). Sequence analysis confirmed the identity of approximately 10% of the positive PCR products (relBE-1<sub>Ab</sub>, 100% identity, ζ<sub>Ab</sub>, 100% identity; alignments are shown in Figures 2.13 and 2.14).

![Figure 2.5](image)

**Figure 2.5** PCR analysis of *A. baumannii* (AB) isolates. The prevalence of genes encoding TA systems as assessed by PCR from the total genomic DNA of 39 clinical AB isolates.

2.3.2 RT-PCR analysis

To determine if the TA systems were transcribed by the clinical isolates, RT-PCR was performed with total RNA isolated from >10% of strains shown by PCR to contain the genes for each TA system. In contrast, the transcripts for relBE-1<sub>Ab</sub> (0/3) and ζ<sub>Ab</sub> (0/2) were not observed in any of the AB isolates tested (Figure 2.6). However, transcripts were detected for recA in the same AB RNA samples, confirming the presence of RNA (data not shown). Although the genes encoding relBE-1<sub>Ab</sub> and solitary ζ<sub>Ab</sub> toxin were present in approximately half of the AB isolates, the transcripts were unable to be detected in the isolates probed.
2.4 CONCLUSIONS

Bioinformatics analyses of published prokaryotic genomes have demonstrated the pervasive nature of TA loci [14]; however, little effort has been made to survey large collections of clinical bacterial strains for the presence and functionality of TA systems. Herein we used PCR to determine that higBA<sub>Pa</sub> and relBE<sub>Pa</sub> are ubiquitous in a collection of P. aeruginosa clinical isolates, whereas parDE<sub>Pa</sub> is less commonly observed. The genes encoding relBE-1<sub>Ab</sub> and a lone ζ<sub>Ab</sub> toxin were found in approximately half of the A. baumannii clinical isolates. This PCR method is complementary to the whole genome sequencing that has previously been used to examine the presence of TA systems in PA and AB, and the results reveal the value of inspecting large numbers of clinical isolates in this manner. For example, of the three sequenced PA clinical isolates that have been analyzed, PA14 does not have the genes for parDE<sub>Pa</sub>, whereas PAO1 and PA7 do [14]. However, the results presented herein show that PA clinical isolates that cluster with PA14 (via MLVA) are just as likely to have the genes for parDE<sub>Pa</sub> as those PA strains that do not cluster with PA14. The results with AB demonstrate the heterogeneous nature of the distribution of TA systems in this important pathogen. One recent study has identified at least five functional TA systems in AB, belonging to the relBE, higBA, hicAB, splTA and cheTA families [12].

Assessment of the flanking sequence of the TA systems in PA revealed that the chromosomal location was conserved across all strains carrying parDE<sub>Pa</sub>, in nearly all strains for relBE<sub>Pa</sub> and in the majority of strains for higBA<sub>Pa</sub>. The inability to amplify the upstream sequence of higBA<sub>Pa</sub> in 10 strains suggests that the upstream sequence has diverged or that the higBA loci of these 10 strains is located elsewhere; however, the conservation of the downstream sequence implies that higBA<sub>Pa</sub> is chromosomally encoded.
Defining the identity of TA systems in clinical isolates satisfies the first requirement in validating TA systems as a viable antibacterial target. However, it is imperative to establish which TA systems are transcribed in clinical isolates. Thus RT-PCR analysis was performed to determine if the TA systems were transcribed. Importantly, it was shown by RT-PCR that higBA\textsubscript{Pa}, relBE\textsubscript{Pa}, and parDE\textsubscript{Pa} were transcribed in strains that carried the genes. In AB, despite a positive RT-PCR result for recA, no TA transcript was detected, suggesting that the TA systems are not functional in AB or that the TA systems are not expressed under the specific laboratory conditions used. Collectively, the results presented herein indicate that the TA genes detected in PA strains reside on the chromosome and are active TA modules, whereas further investigation will be required to fully characterize the TA systems in AB.

It has been suggested that activation of TA systems could be an attractive antimicrobial strategy, as the freed toxin would kill the host bacterial cell [1, 5-7, 26]. While the presence of TA systems in sequenced prokaryotic genomes has been established, prior to this work the prevalence of TA systems in clinical isolates PA and AB was unknown. In addition, this is the first time that the higBA\textsubscript{Pa}, parDE\textsubscript{Pa}, and relBE\textsubscript{Pa} transcripts have been shown to be produced in these bacteria, and one of the few examples of demonstrated transcription of any TA genes from a clinical isolate. Given the results of a previous study showing that TA systems are ubiquitous and transcribed in VRE [9, 15] and MRSA [25], and the current survey showing that TA systems are also highly prevalent and transcribed in PA, it appears that these problematic bacterial pathogens would indeed be susceptible to TA-based antibacterial strategies. Specifically, activation of RelE\textsubscript{Pa} or HigBA\textsubscript{Pa} appear to be attractive strategies against \textit{Pseudomonas aeruginosa}.

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Sources of clinical isolates

The clinical isolates of PA designated CI01-CI20 were obtained from the sputum of 20 different cystic fibrosis patients at Carle Foundation Hospital, as described previously [16]. The remaining 22 PA clinical isolates were a kind gift from Cubist Pharmaceuticals, Inc. (Lexington, MA) and had been obtained from various acute infections over eight geographically diverse clinical sites in the US. Thirty-nine \textit{A. baumannii} clinical isolates were a generous gift from Prof. John Quale and were gathered from citywide surveillance studies in Brooklyn, NY from 2001 to 2006 [2].
2.5.2 MLVA

To assess the clonality of the clinical PA isolates, basic molecular typing was performed by PCR-based multiple-locus variable number of tandem repeats analysis (MLVA) previously described [21, 24]. For PA, 10 of the 15 minisatelites described by Vu-Thien and co-workers were analyzed (ms142, ms211, ms 212, ms213, ms214, ms215, ms216, ms217, ms222, ms223) and 1 µL of PCR products was electrophoresed in 2.0% Molecular Biology Grade agarose (Fisher) at 10 V/cm. JPEG files of the MLVA gel images were visually evaluated with BioNumerics software (Applied Maths) and a dendrogram of banding patterns was constructed using the Dice or Pearson coefficients, respectively, and the unweighted-pair group method using average linkages (UPGMA).

2.5.3 DNA isolation

Total DNA from PA and AB strains was prepared using a modified genomic DNA extraction method from Qiagen (Valencia, CA). Cells were resuspended in 1 mL Buffer B1 containing 40 µg/mL RNase A, 20 mg/mL lysozyme and 50 µg/mL pronase and incubated at 37°C for 30 minutes. Next, Buffer B2 was added and the mixture was incubated at 50°C for 30 minutes, followed by phenol-chloroform-isoamyl alcohol extraction. The total DNA was precipitated with isopropanol and pelleted by centrifugation. The DNA pellet was washed with 70% ethanol and resuspended in 10 mM Tris-HCl (pH 8.0).

2.5.4 RNA isolation

PA and AB cultures were grown in TSB and harvested at mid-log phase, when approximately 1 x 10^9 CFU/mL were present, at which point 0.25 mL aliquots were harvested by centrifugation and frozen at -80°C. Total RNA was extracted using TRIzol reagent (Invitrogen) following a protocol adapted from the manufacturer’s instructions. The frozen bacterial pellets were thawed and resuspended in 0.25 mL TRIzol reagent and sonicated using the Ultrasonic Processor (Heat System Ultrasonics, Inc.) for 10 seconds without pulse with the microtip at the half-maximal power (setting 5, 50% duty cycle). Following sonication, an additional 0.75 mL TRIzol reagent was added and the cell suspension was incubated at room temperature for 5 minutes. Next, 0.2 mL chloroform was added and the tubes were shaken vigorously by hand for 15 seconds. The phases were separated by centrifugation at maximum speed for 15 minutes at 4°C. To further purify the RNA from the upper aqueous phase, an equal volume of acidified phenol-chloroform (5:1) (pH 4.7) (Sigma) was added and the solution was vortexed at maximum speed for 15 seconds and centrifuged at maximum speed for 10 minutes at 4°C. This extraction
was repeated on the resultant upper aqueous phase, after which the RNA was precipitated by mixing with an equal volume of isopropanol and centrifuging at maximum speed for five minutes at room temperature. The pellet was washed with 1 mL ice cold 70% ethanol, centrifuged 5 minutes at 4°C, allowed to dry at room temperature for 5 minutes and resuspended in 100 µL RNase-free dH₂O.

To remove genomic DNA contamination, 20 µL of total RNA was treated with 50 units of Turbo DNase (Ambion) 37°C for at least 24 hours, followed by treatment with RNase-free DNase (Qiagen) according to the protocol for RNA clean-up with the on-column DNase digestion. The RNA was eluted with 50 µL RNase-free dH₂O. The yield of DNA-free RNA from PA was typically low, ranging from 5-35 ng/µL.

2.5.5 PCR analysis

For all PA and AB strains, PCR amplification was performed from purified total DNA. Gene-specific internal primers were used to amplify the \textit{relBE}_{Pa}, \textit{parDE}_{Pa}, and \textit{higBA}_{Pa} \textit{relBE-1}_{Ab}, \textit{relBE-2}_{Ab}, \textit{relBE-3}_{Ab}, \textit{relBE-4}_{Ab} and \zeta_{Ab}. TA genes and separate intergenic primers were used to amplify the upstream and downstream flanking regions. The oligonucleotide sequences of the primers are listed in Table 2.1, and Figure 2.2 depicts the homologous region of the primers for the PCR-based screen and the flanking region primers. PCR amplification was carried out in a DNA thermal cycler (PTC-200, MJ Research, Inc.) under reaction conditions as described previously [15] with a lowering of the annealing temperature to 49°C for most primers. PCR amplified products were analyzed by agarose gel electrophoresis in 1% agarose and stained with ethidium bromide.

2.5.6 RT-PCR analysis

RT-PCR was performed by using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen). The primers used to amplify the \textit{parDE}_{Pa}, \textit{relBE-1}_{Ab}, \zeta_{Ab}, and \textit{recA} sequence for RT-PCR are the same as those designed for PCR analysis, whereas RT-PCR for \textit{higBA}_{Pa} and \textit{relBE}_{Pa} was performed with separate intragenic primers designed from the \textit{P. aeruginosa} PAO1 sequence. The sequences of all primers used in RT-PCR are listed in Table 2.1 and the homologous regions are depicted in Figure 2.2. The extracted total RNA (up to 40 ng) was used in RT-PCR, as well as PCRs with Platinum Taq DNA polymerase (Invitrogen) to detect DNA contamination. Reverse transcription and PCR amplification were carried out in a DNA thermal cycler (PTC-200, MJ Research, Inc.) under reaction conditions as described previously [15], with modifications made to the PCR annealing temperature as follows: 58°C for \textit{relBE}_{Pa}, 50.8°C for
both higBA<sub>Pa</sub> and parDE<sub>Pa</sub>, and 49°C for relBE-1<sub>Ab</sub>, ζ<sub>Ab</sub>, and recA. RT-PCR amplification products were analyzed by agarose gel electrophoresis in 1% agarose and stained with ethidium bromide.

### 2.6 TABLES AND FIGURES

**Table 2.1** Primers used in this study, (+), sense primer; (-), antisense primer

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Figure 2.7 Alignment of parDE<sub>PA01</sub> sequences. Three of the 13 clinical <i>P. aeruginosa</i> strains identified by PCR to contain the parDE<sub>PA01</sub> genes were submitted for DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference parDE<sub>PA01</sub> sequence from the <i>P. aeruginosa</i> PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the parDE<sub>PA01</sub> genes and bases highlighted in bold indicate non-conserved bases.

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**Figure 2.8 Alignment of relBE<sub>PA01</sub> sequences.** Four of the 42 clinical <i>P. aeruginosa</i> strains identified by PCR to contain the relBE<sub>PA01</sub> genes were submitted for DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference relBE<sub>PA01</sub> sequence from the <i>P. aeruginosa</i> PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the relBE<sub>PA01</sub> genes and bases highlighted in bold indicate non-conserved bases.

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**Figure 2.9:** Alignment of the DNA sequences from the strains identified by PCR to contain the parDE<sub>PA01</sub> and relBE<sub>PA01</sub> genes. The sequences were aligned using ClustalW Multiple Alignment (BLOSUM62 Matrix) and the base identities were determined using the ClustalW Multiple Alignment (BLOSUM62 Matrix). The sequences were then aligned with the reference DNA sequences from the <i>P. aeruginosa</i> PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the parDE<sub>PA01</sub> and relBE<sub>PA01</sub> genes and bases highlighted in bold indicate non-conserved bases.
Figure 2.9 Alignment of higBA<sub>P</sub> sequences. Four of the 42 clinical P. aeruginosa strains identified by PCR to contain the higBA<sub>P</sub> genes were submitted for DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference higBA<sub>P</sub> sequence from the P. aeruginosa PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the higBA<sub>P</sub> genes and bases highlighted in bold indicate non-conserved bases.

Figure 2.10 Alignment of parDE<sub>P</sub> upstream (A) and downstream (B) flanking sequences. The upstream and downstream flanking regions of parDE<sub>P</sub> were analyzed by PCR amplification and subsequent DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference upstream and downstream sequences from the P. aeruginosa PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the region between the integrase and parDE<sub>P</sub> genes (upstream region) and parDE<sub>P</sub> and transferase genes (downstream region) and bases highlighted in bold indicate non-conserved bases.
**Figure 2.10**

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**Figure 2.11**

Alignment of relBE<sub>pa</sub> upstream (A) and downstream (B) flanking sequences. The upstream and downstream flanking regions of relBE<sub>pa</sub> were analyzed by PCR amplification and subsequent DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference upstream and downstream sequences from the *P. aeruginosa* PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the region between the transcriptional regulator and relBE<sub>pa</sub> genes (upstream region) and relBE<sub>pa</sub> and hypothetical protein genes (downstream region) and bases highlighted in bold indicate non-conserved bases.
Figure 2.12 Alignment of \textit{higBA}\textsubscript{P} upstream (A) and downstream (B) flanking sequences. The upstream and downstream flanking regions of \textit{higBA}\textsubscript{P} were analyzed by PCR amplification and subsequent DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference upstream and downstream sequences from the \textit{P. aeruginosa} PA01 genome. Bases underlined on the reference sequence indicate the location of the primers used to PCR for the region between the hypothetical protein and \textit{higBA}\textsubscript{P} genes (upstream region) and \textit{higBA}\textsubscript{P} and Ton-B dependent receptor genes (downstream region) and bases highlighted in bold indicate non-conserved bases.
Figure 2.12 (cont.)

Table 2.12

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| A. baumannii 1284 | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1000 | 1100 |

Figure 2.13 Alignment of relBE-1ab sequences. Three of the 21 clinical A. baumannii strains identified by PCR to contain the relBE-1ab genes were submitted for DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62 Matrix) the sequences were aligned with the reference relBE-1ab sequence from the A. baumannii ACICU genome. Bases highlighted in bold on the reference sequence indicate the location of the primers used to PCR for the relBE-1ab genes.
Figure 2.14 Alignment of ζ_{4d} sequences. Two of the 18 clinical A. baumannii strains identified by PCR to contain the ζ_{4d} gene were submitted for DNA sequencing. Using a ClustalW Multiple Alignment (BLOSUM62) matrix the sequences were aligned with the reference ζ_{4d} sequence from the A. baumannii pACICU plasmid. Bases highlighted in bold on the reference sequence indicate the location of the primers used to PCR for the ζ_{4d} gene.

2.7 REFERENCES


CHAPTER 3
TARGETING RELBE AS AN ANTIBACTERIAL STRATEGY

3.1 INTRODUCTION

The RelBE toxin-antitoxin system was first discovered in Escherichia coli and homologues of it have been identified on many other bacterial chromosomes and plasmids [5, 13, 18, 20, 23, 26, 32, 33]. The relB gene encodes a 9.07 kDa labile antitoxin protein that inhibits the toxic activity of the relE-encoded metabolically-stable 11.2 kDa toxin by direct protein-protein interaction [1, 13, 27]. The RelB antitoxin is susceptible to proteolysis by the Lon protease, with a half-life of ~15 min [8]. The relBE operon structure follows the TA system paradigm, in which the relB stop codon and relE start codon overlap, as described in Section 1.3.4. Additionally, transcriptional repression is autoregulated by the antitoxin RelB and the toxin RelE acts as a co-repressor by increasing the DNA binding affinity of the antitoxin [13, 16, 25].

The highly sensitive transcriptional regulation phenomenon employed by the relBE system is called “conditional cooperativity,” which describes the cooperative binding of TA complexes to the operator DNA that can only occur under proper stoichiometric relationships between the toxin and antitoxin [3, 25]. Various levels of transcriptional autoregulation are achieved, dependent upon the ratio of proteins present. If RelB forms a dimer, the RelB$_2$ complex will repress transcription; however if RelE is present in sufficient concentrations to bind the RelB dimer, the RelB$_2$RelE complex will bind tightly and cooperatively to the operator to further repress transcription [25]. Moreover, if RelE levels greatly exceed RelB, binding of RelE to the RelB$_2$RelE complex forms RelB$_2$RelE$_2$, which is destabilized from the promoter, allowing transcription to proceed [25]. Modeling of the crystal structure of the fully RelE-saturated RelB$_2$RelE$_2$ complex with the operator DNA showed that the distance between the two operator sites would not allow the RelB$_2$RelE$_2$ complex to bind, providing further evidence for the conditional cooperativity phenomenon [2]. The sensitivity of this transcriptional control system ensures low level of toxin under normal conditions and also allows for fast response to amino acid starvation [3].

During steady state growth, the relBE promoter is severely repressed, but onset of amino acid starvation results in a rapid and significant increase in relBE transcription [3, 8, 13, 25]. As diagrammed in Figure 3.1, Lon protease-mediated degradation of RelB is required for both induction of relBE transcription and for activation of the ribonuclease activity of RelE by relieving the inhibition by RelB [7, 8, 24]. Interestingly, Lon protease is also activated by the stringent response [31], and it has been proposed that Lon is specifically activated to degrade
antitoxins involved in the stringent response [12]. Upon release from its inactive complex, RelE associates with the ribosome and mediates sequence-specific cleavage of mRNAs in the ribosomal A site (Figure 3.1); thus, RelE is a ribosome-dependent mRNA interferase, preferentially cleaving stop codons (UAG > UAA > UGA) and the sense codons UCG and CAG [6, 11, 28]. Translation arrest is reversed by RelB neutralization of RelE and rescue of stalled ribosomes by tmRNA, which tags polypeptides for degradation, increasing the supply of amino acids.

The X-ray crystal structure of *E. coli* RelBE (Figure 3.2C) is a heterotetramer, in which two RelB molecules dimerize via an N-terminal leucine-zipper like motif. The C-terminus of RelB wraps around the surface of RelE, forming extensive intermolecular electrostatic and hydrophobic interactions [2, 17, 30]. Examination of the interactions of *E. coli* RelE and a C-terminal region of RelB, RelB$_C$ (Lys$^{47}$-Leu$^{79}$) using an intrinsic tryptophan fluorescence assay to determine the $K_D$ of RelB$_C$ for wild-type RelE to be 154 nM [17]. Additionally, the NMR structure of a reduced-activity mutant RelE$^{R81A/R83A}$ and its complex with RelB$_C$ were solved (Figures 3.2A and B). In the free RelE$^{R81A/R83A}$ structure, the C-terminal $\alpha$4-helix is in a closed position, forming contacts with the $\beta$-strand core domain [17]. A highly conserved cluster of positively charged residues adjacent to this interface contains the putative mRNA binding site. Binding of RelB$_C$ to RelE$^{R81A/R83A}$ displaces the RelE$^{R81A/R83A}$$\alpha$4-helix, creating an open conformation, which is expected to be responsible for RelE inhibition[17]. In addition to occupying the active site, the binding of RelB to RelE may also preclude entry of RelE into the ribosomal A site [17].
Although RelE contains an RNA binding site, the canonical catalytic triad observed in the well characterized RNase SA is not conserved in RelE [16]. However, RelE displays the similar microbial RNase fold [16]. Interestingly, RelE is unable to cleave free mRNA, but RelE-mediated cleavage occurs when both mRNA and RelE are associated with the ribosome [28]. Indeed, crystal structures of *E. coli* RelE and *Thermus thermophilus* 70S ribosomes before and after mRNA cleavage showed that both RelE and the ribosome are required for mRNA cleavage (Figure 3.2D) [22]. The crystal structure revealed that RelE interacts closely with conserved regions of the 16S rRNA and that conserved basic side chains on RelE interact with the phosphate backbone of the mRNA, inducing a conformational change in the mRNA that is stabilized by stacking with bases in the 16S rRNA [22]. Thus, the 16S rRNA supplies the missing interactions necessary to properly orient the mRNA for cleavage by RelE [22].
The well-studied RelBE-mediated stringent response has been shown to induce a temporary RelE-dependent metabolically-dormant state until the supply of amino acids is replenished, at which point the cells recover due to inhibition of RelE by RelB. This phenomenon has become the paradigm for the cell stasis model. However, the inherent toxicity of RelBE has been shown by its ability to stabilize an unstable plasmid in bacterial culture [13]. Furthermore, overexpression of RelE results in a drastic and significant inhibition of protein synthesis and cell viability (Figure 3.3) [8, 27]. Thus, the artificial activation of RelBE would likely result in cell death and thus is a viable antibacterial strategy.

![Figure 3.3](image)

**Figure 3.3** RelE inhibits protein synthesis and cell growth. (A) Induction of RelE alone on a low-copy number plasmid under control of the lac promoter rapidly inhibits protein synthesis, but has minimal effect on DNA and RNA synthesis (figure from [8]). (B) Expression of RelE alone, but not the inactive mutant RelE<sub>R81A</sub>, from a low-copy number plasmid under control of the arabinose promoter causes a significant decrease in viable cell counts (figure from [27]).

The prevalence of relBE in clinical isolates further supports this strategy. The relBE locus was found in 35/75 VRE clinical isolates [20], a homologue relBE<sub>p</sub> was identified in 42/42 P. aeruginosa isolates [32], and another homologue, relBE-1<sub>Ab</sub>, was detected in 21/39 A. baumannii clinical samples (Section 2.2.1) (Figure 3.4). The epidemiological data combined with the extensive knowledge of RelBE role in the cell and structure lends great support for its exploitation as an antibacterial target. This chapter describes efforts towards identifying a molecule capable of modulating the interaction between RelB and RelE, employing the strategies of high-throughput screening, peptide fragment analysis and phage-displayed peptide library screens.
3.2 HIGH THROUGHPUT SCREEN FOR RELBE DISRUPTORS USING PHOTONIC-CRYSTAL BIOSENSOR

Given that RelE is a ribosome-dependent ribonuclease, we reasoned that full disruption from RelB was required for activation of RelE. Thus, a high-throughput screen of 170,000 small molecules was undertaken to identify compounds capable of disrupting the RelB-RelE complex. To perform the screen in vitro, a sensitive, high-throughput assay was employed using the photonic crystal (PC) biosensor.

3.2.1 Development of PC biosensor for use in a high-throughput screen

PC biosensors are a recently developed technology that enables the highly sensitive detection of biomolecules binding to its surface [4, 9, 10]. The PC biosensor is formed by the periodic grating of a subwavelength surface structure comprised of dielectric low and high refractive index material. When illuminated with white light, the subwavelength structure allows for the coupling of discrete wavelengths of light, resulting in the formation of a standing wave from which constructive interference results in reflected light at the resonant wavelength (Figure 3.5). The reflected light is reported as a peak wavelength value (PWV) shift. Reflectance is modulated by the composition of the media above the biosensor, thus binding of biomolecules to the surface of the biosensor causes an increase in the PWV of the reflected light. The biosensor is functionalized with streptavidin and a biotin-tris-nitrilotriacetic acid (BTN) linker that can be charged with Ni^{2+}, facilitating the binding of His-tagged proteins. Stripping of the Ni^{2+} with EDTA removes the His-tagged protein, allowing for the reuse of the PC biosensor. This design, when used in 384-well microplate format, greatly facilitates the applicability of this technology to high-throughput screening [4, 14].

Figure 3.4 Prevalence of relBE TA system in three collections of clinical isolates. The relBE TA system was detected by PCR in 100% of P. aeruginosa [32], 53% of A. baumannii and 47% of vancomycin-resistant enterococci (VRE) [20].

![relBE Abundance](image)
All assays performed using the PC biosensor plate follow the same general protocol [4, 14]. An initial baseline PWV shift is measured (Figure 3.6A). Following each step described hereafter, the PWV shift is measured. Assay buffer containing Ni$^{2+}$ is added to charge the BTN (Figure 3.6B), and the plate is washed. Protein is then added to the plate and incubated for 30 minutes (Figure 3.6C) to achieve maximal binding and excess protein is removed in subsequent washing. The BTN surface is regenerated by addition of buffer containing EDTA to remove the His6-tagged protein (Figure 3.6D).

3.2.2 Dose-dependent binding of RelBE to the PC biosensor

The RelBE complex is expressed and purified as a His6 fusion protein in *E. coli*, with the His-tag on the C-terminus of RelE. To assess the PWV shift in response to RelBE binding of the sensor, concentrations ranging from 100μg/mL to 2mg/mL were tested. The absence of binding in control wells lacking Ni$^{2+}$ confirms that RelBE binding is specific to Ni$^{2+}$-NTA (Figure 3.6). Monitoring the PWV shift every 30 seconds revealed that binding plateaus approximately 25-30 minutes after addition of RelBE, as indicated by a leveling off of the PWV shift. Dose dependent binding of RelBE to the biosensor was observed, with a minimal PWV shift of 0 nm for 100 μg/mL RelBE and 1.28nm for the highest concentration, 2mg/mL (Figure 3.6). A PWV shift of ~1 nm is desired to give a robust signal without saturating the biosensor surface; thus, subsequent experiments will be carried out using 0.05mg/mL RelBE, which resulted in a 1.10 nm shift.
3.2.3 Quantification of \((\text{His})_6\text{RelBE}\) bound to the biosensor surface

Although approximately 2 μg (40 μL of 0.5 mg/mL) of \(\text{RelBE}(\text{His})_6\) was added to the biosensor plate, only a small fraction bound the BTN-Ni\(^{2+}\) on the surface. To quantify the amount of \(\text{RelBE}(\text{His})_6\) bound to the chip, 0.05 mg/mL \(\text{RelBE}(\text{His})_6\) was added and allowed to bind for at least 30 minutes. After the wells were washed, EDTA stripping buffer was added and the plate was incubated overnight. The protein eluted from the BTN was collected and concentrated. Various concentrations of eluted \(\text{RelBE}(\text{His})_6\) were subjected to tris-tricine polyacrylamide gel electrophoresis and visualized by both post-staining with Sypro Ruby protein stain.

Figure 3.6 Schematic and graphical data representation of the PC biosensor assay. A PC biosensor surface coated with biotin (purple spheres) is functionalized with a biotin-tris-NTA linker. Measurement of this surface establishes the baseline, as shown on the graph. Addition of Ni\(^{2+}\) causes a slight bulk increase in the PWF shift, which is restored to baseline after a wash (B). Addition of 0.001 – 2 mg/mL \(\text{RelBE}(\text{His})_6\) results in a dose-dependent increase in PWV shift as the protein immobilizes (C) and binding stabilizes within approximately 35 minutes. A wash step removes excess protein and reveals that binding is saturated at approximately 0.1 mg/mL. The optimal PWV shift for the protein surface is 1 nm, so 0.05 mg/mL \(\text{RelBE}(\text{His})_6\) is optimal. Addition of EDTA chelates the Ni\(^{2+}\) and strips the protein (E) restoring the PWV shift to baseline.

Figure 3.7 Quantification of \(\text{RelBE}(\text{His})_6\) deposited on PC biosensor. 2 μM (500 μg/mL) \(\text{RelBE}(\text{His})_6\) was added to the charged biosensor plate and eluted with EDTA stripping buffer. Eluted protein was analyzed as 1X, 5X, 10X solutions by SDS-PAGE alongside known amounts of \(\text{RelBE}(\text{His})_6\) and visualized by Western blot (A) or Sypro Ruby Stain (B). Densitometry analysis using Image J software revealed that approximately 150 ng \(\text{RelBE}(\text{His})_6\) binds the PC biosensor (~170 nM in a standard 40 μL well volume).
dye and a gradient of RelBE(His)$_6$ ranging from 1 to 750 ng was included for reference. ImageJ was used to perform densitometry analysis of known amounts of RelBE(His)$_6$ and the protein eluted from the BTN revealed that approximately 150ng of the RelBE added binds to the sensor (Figure 3.7), which is 0.75% of the total amount loaded.

3.2.4 High-throughput screen preparation

The screen was performed using 384-well format PC biosensor plates. The High-Throughput Screening Facility on campus houses the Chembridge, HTSF and Marvel libraries, which contain 150,000, 4700, and 10,000 compounds, respectively. Compounds were tested at 100 μM and were multiplexed at 10 compounds per well. RelBE(His)$_6$ was added to the plate at a concentration of 2 μM (approximately 0.05 mg/mL) of which 200 nM (approximately 150 ng) binds the plate, thus compounds will be in ~500-fold excess over RelBE. The plates were incubated at room temperature for at least 12 hours and then washed to remove compound and protein. Finally, the PWV shift was determined to assess disruption. To determine a hit compound, two values were compared: the PWV shift observed after the excess RelBE has been washed away, and the shift observed after the final wash step following the 14-hour incubation (after removal of compound and disrupted RelB). A hit compound was expected to result in a decreased PWV shift measured after the final wash.

To assess the stability of RelBE(His)$_6$ at room temperature and the variability across the plate, 2 μM RelBE(His)$_6$ was added to each of the 384 wells and incubated in assay buffer for 14 hours at room temperature. Figure 3.8A shows the average PWV shift observed across the plate at each step in the procedure, with the error bars reporting the standard deviation. The average PWV shift observed after washing away excess RelBE(His)$_6$ was 1.22 ± 0.046 nm, and for the wash step following the 14-hour incubation was 1.09 ± 0.046 nm. Thus there was a small decrease in the amount of RelBE(His)$_6$ bound to the sensor; however, no further decrease was observed in the subsequent wash step.

The compounds in the library are dissolved in DMSO. Since 10 compounds were tested per well, the final concentration of DMSO in the assay buffer was 10%, thus the effect of 10% DMSO was also determined across an entire 384-plate. Again, 2 μM RelBE(His)$_6$ was added to each well and after removal of excess protein, the plate was incubated for 14 hours at room temperature in assay buffer containing 10% DMSO. Figure 3.8B shows the average PWV shift for each step of the procedure, with the standard deviation reported. The average PWV shift after washing away excess RelBE(His)$_6$ was 1.25 ± 0.033 nm and following the final wash was 1.33 ± 0.059 nm. A significant increase in PWV shift is observed in response to the DMSO, but the
lower pre-DMSO PWV shift was mostly restored after washing. Incubation with DMSO does not affect the stability of RelBE(His)$_6$; however, additional wash steps were performed during the screen to further restore the PWV shift to the RelBE(His)$_6$ baseline value.

To test the stability of RelBE(His)$_6$ on the PC biosensor in the presence of compound, one plate comprising compounds from Marvel Library plates 1-9 was tested. In this case, 9 compounds were included in each well, so the final DMSO concentration was 9%. The PWV shift was measured for each step and the averages, along with standard deviations, are reported in Figure 3.8C. The average PWV after washing away excess RelBE was 1.26 ± 0.044 nm and after the final wash was 1.50 ± 0.148 nm. The PWV shift observed after the 14 hour incubation and wash step increased measurably. This confirmed that extensive washing is necessary to properly measure any hit compounds.

3.2.5 High-throughput screen results

The screen was performed as described above. Briefly, a streptavidin-coated PC biosensor plate was functionalized with BTN and charged with Ni$^{2+}$. After RelBE(His)$_6$ was immobilized and the PWV shift was measured, compounds were added at 100 μM and incubated at room temperature for 14 hours. The wells were washed extensively the final PWV shift was determined. The change in PWV shift was plotted and “hit” compounds were those that caused a
PWV shift three standard deviations below the average of the DMSO control wells, as shown in Figure 3.9.

Figure 3.9 Representative screening plate. The data are reported as the change in peak wavelength value (PWV) after ~12 hours incubation with the compound. The red dashed line represents three standard deviations from the mean of the RelBE + DMSO control wells; any point below that line represents a “hit” compound. The points with highly positive PWV shifts likely represent wells with precipitated compound.

From the 170,000 compounds tested in the 10 compound/well multiplex, 110 multiplexed compounds resulted in a decreased PWV and were scored as hits, 59 of which were confirmed in the re-test. Deconvolution of the multiplexed wells revealed 66 active compounds (Figure 3.10B), which all contained a common metal-binding motif; representative compounds are shown in Figure 3.10A. We hypothesized that these compounds acted by chelating the Ni$^{2+}$ required to immobilize the His-tagged proteins to the BTN. Thus, the 66 compounds were tested against an unrelated His-tagged protein (FKBP) and also caused a decrease in the PWV shift (Figure 3.10C), confirming them as chelators. Thus, all compounds were eliminated in the counterscreen.

Figure 3.10 Results of HTS for RelBE disruptors. (A) Representative hits from the screen, all of which contain a common metal-chelating motif. (B) Graph representing approximately half of the deconvoluted hits, which caused a PWV shift. (C) Counterscreen of the hit compounds against an unrelated His-tagged protein also caused a decrease in the PWV shift, confirming them as non-specific metal-chelators that strip the His-tagged protein from the PC biosensor surface by chelating the Ni$^{2+}$.
3.3 RelE Peptide Fragment Analysis

The RelBE interaction is characterized by extensive electrostatic interactions and many contact surface areas (Figure 3.2). However, the importance of each region of interaction is not well understood, so we employed peptide fragment analysis to further define the interactions between RelB and RelE and identify a peptide able to modulate that interaction. Peptide fragments of RelE were analyzed because they may have the capacity to bind the antitoxin RelB, preventing or inhibiting its interaction with the toxin RelE, allowing RelE to bind the ribosome and inhibit translation.

3.3.1 Assessment of RelE Peptide Fragment Binding to RelB(His)₆

To perform the peptide fragment analysis, 30-amino acid fragments overlapping by 10 amino acids and spanning the entire primary sequence of RelE (Figure 3.11) were obtained from Genscript as crude preparations and purified by HPLC. Some peptides were insoluble and thus unable to be purified and tested: peptide 4, 7, and 9-14. Peptides 1, 2, 3, 5, 6, and 8 were soluble and purified to ~90% purity as assessed by MALDI. The purified peptides were tested for binding to RelB(His)₆ using the PC biosensor. In this assay, RelB(His)₆ was immobilized to the PC biosensor surface and peptides that bound to RelB(His)₆ caused an increase in the PWV shift.

Each pure peptide fragment was tested from 0-500 μM and the results are summarized in Figure 3.12. As a positive control, pure RelE (0-18 μM) was bound to immobilized RelB(His)₆ (Figure 3.12); however, the interaction between RelE and RelB(His)₆ was much tighter, as noted by the much lower concentration of RelE required to give comparable increases in the PWV as the peptides. Peptide fragment 8 corresponding to RelE sequence 36-65 was the strongest binding peptide, so derivatives of it were obtained analyzed.
Two truncations, corresponding to the first and last 25 amino acids of RelE36-65 (RelE36-60 and RelE41-65, respectively) were ordered, along with an extended peptide, RelE31-70, including the 5 amino acids on either side of RelE36-65. The longer peptide, RelE31-70, was insoluble and unable to be tested. The truncated derivatives bound similarly to RelE36-65, with RelE41-65 showing slightly higher binding (Figure 3.12). Thus, two additional truncations were obtained, RelE42-57 and RelE50-63 to identify a minimal peptide required for binding to RelB(His)$_6$. As shown in Figure 3.12B, the 14-mer RelE50-63 appeared to bind RelB(His)$_6$ the best, whereas the 16-mer RelE42-57 did not bind as well.

3.3.2 Quantification of binding of RelE36-65 derivatives by SPR

The PC biosensor assay is useful as an initial assay to provide a binary assessment of binding; however, it does not provide quantitative data since it is a static assay. Thus, surface-plasmon resonance was used to quantify the binding of the peptides to RelB(His)$_6$. RelB(His)$_6$ was immobilized to 900-1000 RU (arbitrary response units) on a NTA chip. Increasing concentrations of each peptide were tested for their binding to and dissociation from RelB(His)$_6$. As shown in Figure 3.13, saturated binding was never achieved, even with very high concentrations of the peptides. Furthermore, the rapid dissociation suggested a low affinity of the peptide for RelB(His)$_6$. These SPR profiles are suggestive of non-specific binding [21, 29]. Additionally, 4 peptide derivatives, RelE45-61, RelE52-61, RelE50-59 and RelE54-63, showed no binding to RelB(His)$_6$ by SPR. Thus the RelE peptide fragments were no long pursued. A full summary of the RelE peptide fragments and their binding to RelB(His)$_6$ is included in Table 3.1.
3.4 PHAGE-DISPLAYED PEPTIDE LIBRARY SCREEN

Since the RelE peptide fragments suffered from minimal or nonspecific binding to RelB(His)_6, we set out to identify peptides that could bind RelB with high affinity and specificity. The powerful tool of phage display offers a robust screening method to identify high-affinity peptides that bind specifically to the bait protein, which was RelB(His)_6 in this case. Peptides of
a specified length displayed on the T7 phage capsid are incubated with the bait protein, subjected to stringent washes, and then eluted from the bait protein. Captured phages are propagated in *E. coli* and the resultant lysate (T7 is a lytic phage) is applied to the bait protein in the subsequent round, constituting the iterative process called biopanning (Figure 3.14). After 4 biopan rounds, the eluted phage are propagated in *E. coli* on agar plates and plaques are analyzed by PCR and DNA sequencing to reveal the sequence of the displayed peptide. Typically, the appearance of a consensus sequence indicates specific binding of the phage-displayed peptides to the bait protein and synthetic peptides based on this consensus sequence can be tested in a variety of “off-phage” assays.

A variety of phage libraries were constructed by Amy Larson in the Hergenrother Lab. The first two libraries constructed were using phage that displayed 415 copies of the 9 amino acid recombinant peptide on their capsid. Library X9.1 contained 2.4x10^6 primary recombinants and Library X9.2 contained 1.8x10^7 primary recombinants (Table 3.2), so offered 10-fold more diversity than the first library. Although these libraries resulted in consensus peptides, the peptides did not appear to have high affinity for the bait protein (discussed in more detail in the following sections) and there was concern of a possible avidity effect due to the high density of peptides on the phage capsid. Thus, to increase the chances of capturing high affinity peptides, we constructed four libraries displaying only 10 peptides on the phage surface, one displaying 9 linear amino acids (Library X9.3) with 3.05x10^6 primary recombinants, and three displaying amino acids that are cyclic under oxidizing conditions due to terminal cysteine residues: CX7C with 2.85x10^6 primary recombinants, CX8C with 1.1x10^6 primary recombinants, CX10C with 1.0x10^6 primary recombinants (Table 3.2).
Table 3.2 Characteristics of phage libraries and consensus sequence recovered.

<table>
<thead>
<tr>
<th>Library</th>
<th>Copies displayed</th>
<th>Motif displayed</th>
<th>Primary recombinants</th>
<th># Biopans performed</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₀,1</td>
<td>415</td>
<td>9 aa, linear</td>
<td>2.4 x 10⁶</td>
<td>9</td>
<td>X₀PWX(V/L)</td>
</tr>
<tr>
<td>X₀,2</td>
<td>415</td>
<td>9 aa, linear</td>
<td>1.8 x 10⁷</td>
<td>5</td>
<td>X₀VCF</td>
</tr>
<tr>
<td>X₀,3</td>
<td>10</td>
<td>9 aa, linear</td>
<td>3.05 x 10⁶</td>
<td>1</td>
<td>TPYWLFSL</td>
</tr>
<tr>
<td>CX₀,1</td>
<td>10</td>
<td>7 aa, cyclic</td>
<td>2.85 x 10⁶</td>
<td>3</td>
<td>no consensus</td>
</tr>
<tr>
<td>CX₀,2</td>
<td>10</td>
<td>9 aa, cyclic</td>
<td>1.1 x 10⁶</td>
<td>3</td>
<td>no consensus</td>
</tr>
<tr>
<td>CX₀,3</td>
<td>10</td>
<td>10 aa, cyclic</td>
<td>1.0 x 10⁶</td>
<td>3</td>
<td>CWWRSAGVWVAC</td>
</tr>
</tbody>
</table>

Throughout the biopanning experiments performed with Library X₀,1, a variety of conditions were tested with the main goal being high stringency to increase the likelihood of recovering phage that specifically interacted with RelB(His)₆ with a high affinity. A basic high stringency protocol is summarized in Table 3.3, with variations summarized listed in Table 3.4. For some of the biopan experiments, the phage solutions were first subjected to a “pre-clear” involving just phage and solid support to remove any phage that bound to the solid support. Also, the solid support and the blocking solution were switched between each round to select against phage that bound the solid support or the blocking agent. With each subsequent round, decreasing amounts of immobilized RelB(His)₆ was used and the incubation time between phage and RelB(His)₆ was decreased. Lastly, the number of washes and sometimes the concentration of Tween-20 were increased with each round. Following four rounds of biopanning, the eluted phage were propagated in E. coli on agar plates and 15 plaques from each biopan were analyzed. The recombinant region in the phage genome was amplified by PCR, which was then sequenced to reveal the specific peptide that was displayed on the phage capsid. Importantly, analogous biopan experiments lacking RelB(His)₆ were always performed alongside to provide a benchmark for the specificity of the recovered peptides.

Table 3.3 Standard biopan protocol.

<table>
<thead>
<tr>
<th>Round</th>
<th>solid support</th>
<th>Pre-clear solid support</th>
<th>vol phage</th>
<th>vol solid support for RelB(His)₆ immobilization</th>
<th>Blocking agent</th>
<th>Phage incubation</th>
<th>Washes</th>
<th>% Tween-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round 1</td>
<td>Ni-NTA resin</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>10 μL</td>
<td>5% milk</td>
<td>25 min</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>Round 2</td>
<td>MagneHis beads</td>
<td>0.05 mL</td>
<td>0.2 mL</td>
<td>6 μL</td>
<td>5% BSA</td>
<td>22 min</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>Round 3</td>
<td>Ni-NTA resin</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>5 μL</td>
<td>5% milk</td>
<td>18 min</td>
<td>7</td>
<td>0.8</td>
</tr>
<tr>
<td>Round 4</td>
<td>MagneHis beads</td>
<td>0.05 mL</td>
<td>0.2 mL</td>
<td>3 μL</td>
<td>5% BSA</td>
<td>15 min</td>
<td>8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

78
### Table 3.4 Variations to the standard biopan protocol

<table>
<thead>
<tr>
<th>Library, biopan number</th>
<th>Pre-clear</th>
<th>Washes</th>
<th>elution solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₀.1 #3</td>
<td>yes</td>
<td>5, 6, 7, 8</td>
<td>0.2</td>
</tr>
<tr>
<td>X₀.1 #4</td>
<td>no</td>
<td>5, 6, 7, 8</td>
<td>0.2</td>
</tr>
<tr>
<td>X₀.1 #5</td>
<td>yes</td>
<td>5, 6, 7, 8</td>
<td>0.2</td>
</tr>
<tr>
<td>X₀.1 #7</td>
<td>no</td>
<td>5, 6, 7, 8</td>
<td>0.2</td>
</tr>
<tr>
<td>X₀.1 #8</td>
<td>yes</td>
<td>15, 6, 20, 14</td>
<td>0.2</td>
</tr>
<tr>
<td>X₀.1 #9-X₀.2 all</td>
<td>no</td>
<td>15, 6, 20, 14</td>
<td>0.2, 0.5, 0.8, 1.1</td>
</tr>
<tr>
<td>X₀.3, CXC all</td>
<td>no</td>
<td>10, 11, 12, 13 mL</td>
<td>0.2, 0.5, 0.8, 1.1</td>
</tr>
</tbody>
</table>

#### 3.4.1 T7 415-1b Library X₀.1

As described above, this library displayed 415 copies of 9-amino acid recombinant peptides on its surface. Nine biopan experiments were performed with this library; biopans 1, 2 and 6 resulted in peptides that appeared to bind non-specifically as they were very similar to the peptides recovered from the (-)RelB(His)₆ biopan. The protocol for each biopan varied slightly and the details can be found in Experimental Procedures section. For each biopan, the phage DNA from 15 plaques was analyzed and the results giving rise to the consensus sequence X₃PWX(V/L) from biopans 3-5 and 7-9 are summarized in Figure 3.15. Three peptides, AESESPWWRV, NGKGHPWSL and DKPPSPWTV were recovered from multiple biopans. Many other peptides containing the PWX(V/L) motif were only recovered from a single plaque from one biopan. Some of the PWX(V/L)-containing phage were assessed, along with other recovered phage, in a competitive biopan.

![Figure 3.15 Biopanning from Library X₀.1 resulted in the consensus sequence X₃PWX(V/L).](image-url)

(A) Dominant peptides that arose from Biopans 3-9 are shown on the right, with the PW-V/L alignment highlighted in red. In the left column, the large number refers to the biopan number and the superscript refers to the number of plaques (out of 15) from which the sequence was recovered. (B) A WebLogo plot displaying the consensus sequence of the dominant peptides. The majority of the peptides recovered had the P in the 6th position, thus this became the consensus sequence.
3.4.1.1 Competitive biopan of hit phage

The purpose of the competitive biopan was to “rediscover” the hit phage. The hit phage was mixed with WT phage in approximately the expected starting ratio from the initial experiment, based on the number of primary recombinants. Since there were ~1x10⁶ primary recombinants in Library X₀₁, the hit phage was mixed with WT phage in a ratio of 1:1x10⁶. The biopan was carried out according to the protocol used for the initial discovery biopan and the plaques were screened after each round to determine when the hit phage was in high enough numbers to be detected by PCR. The phage subjected to the competitive biopan displayed the following peptide sequences: DSCSSVCVE, AESESPWRV, NGKGHPWSL, DKPPSPWTV and FPSKNPWQV. The peptide DSCSSVCVE was recovered from one plaque in biopan 3, whereas AESESPWRV was recovered from 5 plaques in Biopan 3, plus from Biopans 5, 7 and 9. However, both recombinant phage were selected for by Round 4 in the (+)RelB(His)₆ competitive biopan and were not selected by the (-)RelB(His)₆, as shown in Figure 3.16. Recovering the recombinant phage in 4 rounds (the number of rounds used in the original biopan) suggests that the phage are specifically binding RelB(His)₆. Unfortunately the PCR results for the competitive biopans with the remaining three phages were ambiguous as the PCR products were of unexpected sizes (data not shown).

![Figure 3.16](image-url)  
**Figure 3.16** Competitive biopan to assess the specificity of phage for RelB(His)₆. Recombinant phage 3-1 (A) or 3-5 (B) were mixed with WT phage in a 1:1x10⁶ ratio and carried through the biopan protocol (+)RelB(His)₆ (left) or (-)RelB(His)₆ (right). Plaques were analyzed by PCR and separated on a high-resolution agarose to distinguish the bands corresponding to wild-type (350 bp) and recombinant (386 bp) phage. M, DNA marker. WT, wild-type. WT/3-1 or 3-5, starting ratio 1:1x10⁶. 3-1 and 3-5 correspond to the phage displaying the sequence DSCSSVCVE and AESESPWRV, respectively.
3.4.1.2 ELISA of phage displaying consensus peptides

The affinity of the phage for RelB(His)\textsubscript{6} was evaluated by ELISA. Briefly, RelB(His)\textsubscript{6} was adsorbed to a Maxisorp immunoplate (Nunc) plate, blocked with 5% milk, incubated for 1 hour with phage lysate. An Anti-T7 HRP-conjugated antibody was added and developed with colorimetric substrate ABTS, the absorbance of which was measured at 405 nm. The results of the ELISA with select phage recovered from Library X\textsubscript{9.1} (listed in Figure 3.17A) are summarized in Figure 3.17B. From this graph, 4-9 appears to bind the best, followed by 4-6 and 8-9, then 4-5 and 3-5 and finally 3-1, 4-3, 9-6, 9-10 and 9-15. Phage clone 3-12 was included as a negative control; although it was recovered from a (+)RelB(His)\textsubscript{6} biopan, its sequence was similar to those recovered from (-)RelB(His)\textsubscript{6} biopans. Three phage clones were shown to have dose-response binding to RelB(His)\textsubscript{6} as shown in Figure 3.17C, with 3-5 performing much better in this assay than the previous ELISA.

Based on the number of times they were recovered from the biopan experiments and the dose-response binding to RelB(His)\textsubscript{6}, the following peptides (with corresponding phage clone name) were ordered from Genscript: AESESPWVRV (3-5), DKPPSPWVT (4-6) and FPSKNPWQV (4-9). Additionally, an alanine-substituted derivative of 4-9, FPSKNAQQV, was purchased to assess the importance of the PW motif characteristic of the consensus sequence. These peptides were evaluated for binding to RelB(His)\textsubscript{6} by the PC biosensor and SPR.

3.4.1.3 PC biosensor and SPR analysis of synthetic peptides

To evaluate the binding of the synthetic peptides, the PC biosensor assay was used, with the RelE50-63 derivative peptide (described in Section 3.3.1) as a positive control. A range of concentrations of the peptides were tested against immobilized RelB(His)\textsubscript{6} and as shown in Figure 3.18, although RelE50-63 bound as expected, the “off-phage” synthetic peptides showed...
minimal, if any, binding to RelB(His)$_6$. To confirm this result, the peptides were also tested for binding to RelB(His)$_6$ by SPR and not surprisingly, the peptides showed no binding (data not shown). Thus, these peptides were no longer pursued.

**Figure 3.18** Assessment of synthetic “off-phage” peptides. (A) Sequence and attributes of the synthetic peptides. (B) Peptide binding to RelB(His)$_6$ evaluated by the PC biosensor.

### 3.4.2 T7 415-1 Library X$_9$.2

To increase the chances of identifying a peptide that could specifically bind to RelB(His)$_6$, biopanning with the second generation linear 9-amino acid library, X$_9$.2, was performed. Five biopan experiments were performed following the standard biopan protocol, omitting the pre-clear of the lysate and eluting the phage using MBP-RelE(His)$_6$, (MBP, maltose-binding protein) which may have specifically competed for binding with RelB(His)$_6$ or nonspecifically competed for binding to the NTA solid surface. The peptides recovered along with the frequency of their recovery throughout the five biopans are shown in Figure 3.19A. The peptides displaying the X$_6$VCF consensus did not constitute the majority of the peptides recovered from each biopan; however, they did form the only pattern observed amongst the phage-displayed peptides. Interestingly, the peptides shared some homology with the RelE43-51 amino acids (Figure 3.19B). Nonetheless, the binding of these phage to RelB(His)$_6$ assessed by ELISA was minimal. Recall that the hit phage from X$_9$.1 gave absorbance readings of 2-4 (Figure 3.17B) in the ELISA, whereas clone 5-2 is best with an absorbance of 0.45 (Figure 3.19C). Since these phage appeared to bind RelB(His)$_6$ with lower affinity than those recovered from Library X$_9$.1, the phage from X$_9$.2 were no longer pursued.

### 3.4.3 T7 10-3 libraries against RelB(His)$_6$

Although the phage recovered from the 415-copy library series resulted in consensus sequences, they did not perform well enough once the corresponding synthetic peptides were tested. We hypothesized this was due to an avidity effect and thus focused our efforts on libraries in which only 10 copies of the peptide were displayed on the phage capsid. Four libraries were tested: X$_9$.3, CX$_7$C, CX$_9$C, CX$_{10}$C, as well as mixture containing the three cyclic libraries.
Figure 3.19 Biopanning from Library X₉.2 resulted in the consensus sequence X₉VCF. (A) Dominant peptides that arose from Biopans 1-5 are shown on the right, with the VCF alignment highlighted in red. In the left column, the large number refers to the biopan number and the superscript refers to the number of plaques (out of 15) from which the sequence was recovered. (B) Alignment of the RelE₄₃-₅₁ sequence with the 5 VCF peptides. Residues highlighted in purple, green and blue indicate low, moderate and high homology, respectively. (C) ELISA of 1x10⁹ phage/well against adsorbed RelB(His)₆.

3.4.3.1 Biopan with 9-amino acid linear peptide library X₉.3

One biopan was performed with the X₉.3 library. Ten plaques were analyzed, seven of which had the sequence CLNAQVGRR and three of which were TPYWLFSL (Figure 3.20A). The presence of the proline and aromatic amino acids in the latter peptide made it more structurally interesting, whereas the former peptide contained mostly uncharged or hydrophobic residues. Three biopans each were performed with the CX₇C and CX₉C libraries; however, no real consensus sequence emerged from these. Although phage displaying certain peptides appeared in multiple plaques, it was unclear if these were specifically binding RelB(His)₆ since no pattern was observed.

Three biopans were also performed with the CX₁₀C libraries and one clone with the sequence CWWRSAVGWVAC was present in 13 out of 15 plaques analyzed. Interestingly, this same clone was also recovered from 6 out of 15 plaques from the mixed cyclic peptide biopan.

The phage displaying TPYWLFSL and CWWRSAVGWVAC were tested in an ELISA using the X₉.1 clone 4-9 as a benchmark (Figure 3.20). Although the X₉.10 and CX₁₀C-1 phage did not bind with the same affinity as 4-9, their corresponding synthetic peptides were ordered from Genscript and tested for binding to RelB(His)₆ by SPR. Unfortunately the TPYWLFSL showed no binding and the CWWRSAVGWVAC was insoluble in water, acetonitrile and DMF and thus could not be tested.
### Figure 3.20 Results of the biopans with X\textsubscript{9.3} and CX\textsubscript{10C}.

(A) Dominant peptides that arose from biopans with the third generation linear 9-amino acid library X\textsubscript{9.3} and the cyclic 10-amino acid CX\textsubscript{10C} are shown in the right column. In the left column, the large number refers to the biopan number and the superscript refers to the number of plaques (out of 10 or 15) from which the sequence was recovered. (B) ELISA of 1x10\textsuperscript{9} phage/well against adsorbed RelB(His)\textsubscript{6}.

<table>
<thead>
<tr>
<th>Biopan\textsuperscript{#}plaques</th>
<th>Sequence</th>
<th>Absorbance 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–9\textsuperscript{2}</td>
<td>FPSKNPwQV</td>
<td>WT</td>
</tr>
<tr>
<td>X\textsubscript{9}-10\textsuperscript{3}</td>
<td>TPYWLFSL</td>
<td>4-9</td>
</tr>
<tr>
<td>CX\textsubscript{10C}-1\textsuperscript{6,9}</td>
<td>CWRASGVWVAC</td>
<td>X9-10 CX10C-1</td>
</tr>
</tbody>
</table>

Considering the vast success using phage display in the literature, we were surprised by our results of no peptides that bound RelB with high affinity. We hypothesized that the nature of the bait protein must be negatively influencing the outcome of the biopan experiments but confirmed that the protein remained stable during the biopan rounds and was not degraded by proteases released into the phage lysate. Thus, it seemed likely that the antitoxin, due to its unfolded nature, served as a poor target for in vitro binding studies. Overcoming this inherent pitfall, however, required conquering the challenge of purifying sufficient quantities of stable toxin protein.

### 3.5 EFFORTS TOWARDS PURIFICATION OF REL\textsubscript{E}

#### 3.5.1 Purification of untagged RelE using the pTWIN2 vector

The pTWIN2 vector series enables expression of a recombinant protein fused to an intein, which will catalyze its own cleavage under the proper conditions. Our hypothesis was that expression of a large fusion protein containing RelE would abrogate its toxicity and allow sufficient quantities to accumulate in the cell. The intein is fused to a chitin binding domain (CBD), allowing for affinity purification using chitin beads. Thus the construct CBD-intein-RelE or (Figure 3.21A) was expressed in *E. coli* and purified under native conditions. Cleavage between the intein and RelE was induced by decreasing the pH from 8.5 to 6.0 and liberated RelE was collected off the column (Figure 3.21B). Although sometimes the CBD-intein co-purified, a second size-exclusion chromatography (Figure 3.21B) separated the two proteins. This method worked brilliantly a handful of times and facilitated performance of the experiments using untagged RelE as described in Section 3.2; however, for unknown reasons after a certain time, cleavage never proceeded to sufficient levels again.
Figure 3.21  Purification of untagged RelE from CBD-intein fusion.  (A) Schematic of the T7-promoter controlled CBD-intein-RelE construct showing the intein autocleavage site.  (B) Purification of RelE from the intein resulted in an elution fraction containing a mixture of the fusion, the free intein and RelE.  Subsequent purification size exclusion chromatography separated RelE from the intein.

3.5.2  RelE triple mutant:  R81A, R83A, Y87A

The pET-28a-relBE construct had been mutated to carry the RelE$^{R81A}$ mutant, which was shown to be less toxic than the WT protein [27].  The relE$^{R81A}$ mutant was sub-cloned by itself, but could not be overexpressed.  Thus, a second mutation, R83A, was introduced using the quickchange (QC) primers listed in Table 3.5.  However, the RelE$^{R81A/R83A}$ double mutant could also not be expressed on its own.  As a last effort, a third mutation, Y87A, was introduced as it was found that RelE$^{R81A/Y87A}$ double mutant had no activity [22].  Unfortunately, the triple mutant was also unable to be expressed.

3.5.3  pMBP fusion with TEV cleavage

The pMBP vector carries maltose binding protein (MBP) and a multiple cloning site juxtaposed by a TEV protease cleavage site, allowing for expression of RelE as a non-toxic MBP fusion and subsequent release of RelE from the MBP, as diagrammed in Figure 3.22A.

Construction and expression of the pMBP-RelE(His)$_6$ proceeded as expected and the fusion was purified to homogeneity on amylose resin (Figure 3.22B).  The addition of the MBP-fused TEV protease liberated RelE(His)$_6$ from the fusion, although only ~50% cleavage was ever achieved.  A final affinity purification step over an amylose resin was expected to retain the liberated MBP and MBP-TEV constructs, allowing pure RelE(His)$_6$ to flow through.  Unexpectedly, RelE(His)$_6$ was never collected in the flow through.  The mixture was subjected to purification by size exclusion chromatography, ion exchange chromatography, further amylose affinity chromatography, and Ni-NTA affinity chromatography, but despite these extensive efforts, the liberated RelE(His)$_6$ was never captured and thus this method abandoned.
3.5.4 RelB trypsinolysis

One clever strategy was to take advantage of the increased proteolytic susceptibility of RelB over RelE and use trypsin to selectively degrade RelB, leaving free RelE. A similar method had been reported in the literature, wherein the VapBC TA system was used to degrade VapB, leaving active VapC [19]. To establish more controlled conditions, sequencing-grade trypsin was added to affinity-purified RelBE(His)<sub>6</sub> in a range of concentrations and allowed to incubate for at 4, 25 and 37°C for various time points; however, although it appeared that some degradation of RelB occurred, complete degradation was never achieved. Perhaps success would have been realized if Lon, a specific RelBE-specific protease was used, however, this was never pursued.

3.6 CONCLUSIONS

The work with RelBE, although it did not lead to a disruptor was highly informative, revealing that the antitoxin is not the best target for two reasons: experimentally, due to its unstructured nature and theoretically, because merely binding the antitoxin is at least one step away from the major goal of the project which is to artificially activate the toxin. The high-throughput screen for RelBE disruptors gave perhaps the best chances to achieve our goal; however, although it was satisfying that the technology worked (PWV shift decreased when protein was removed from the surface) the screen only revealed chelators. There were many factors contributing to the low chance for success with the HTS, primarily including the subnanomolar $K_d$ between RelB and RelE in the absence of a protease and working in a static system which puts the small molecules in constant competition with the high affinity binding partners. Recapitulating the in vivo effect of a specific protease in vitro is potentially possible and could be pursued in the future. Additionally, current technology limits the throughput of compounds tested in flow cells so this particular challenge could not be readily overcome.
A peptide capable of binding RelB could have worked by directly preventing or inhibiting the interaction between RelB and RelE or have an indirect effect by competing with RelE for binding to RelB, weakening the interaction between RelB and RelE, and effectively making RelB more available for proteolysis in the cell. Either strategy would require a peptide with extraordinary affinity for RelB to overcome the subnanomolar affinity between RelB and RelE. The peptide fragment analysis may not have taken into account the complex, multi-faceted interactions that exist between the globular RelE and RelB. It is possible that a peptide based on RelB would bind RelE, as the crystal structure shows that RelB resembles an extended polypeptide throughout its contact with RelE. An argument against this strategy is that a peptide of RelB may actually inhibit RelE, either by occupying its active site, by obstructing RelE entrance into the ribosomal A site due to increased size, or by masking some of the RelE residues required for stable interaction with the ribosome to carry out mRNA cleavage.

The possibilities for the lack of success with the phage display seem endless. As there is plentiful literature precedent for success and since analogous biopanning experiments against the antitoxin YefM-Sa1 [15] were also unsuccessful, the inherent unfolded nature of the antitoxin proteins may explain some of the challenges. A high-affinity peptide may have been discovered using a larger library, biopanning under different conditions, or using the M15 phage system which may remove some selection placed on the phage in culture due to its slower infection time than T7 phage. Nonetheless, despite approximately 25 biopan experiments, the peptides identified to bind RelB in the context of the phage failed to do so when tested on their own.

Recovering peptides with certain sequences from multiple biopans, specifically the X_5PWXV/L and X_6VCF motifs, suggests that these peptides did confer binding to immobilized RelB(His)_6. The fact that these peptides were not recovered in the control biopan experiments lacking RelB(His)_6 further supports the notion that the peptides specifically interacted with RelB(His)_6 and not the other materials used in the biopan. The high titer of phage required for interaction as detected by ELISA suggests that the peptides bound RelB(His)_6 with low affinity or due to an avidity effect. The multiple conformations adopted by an unfolded protein may also explain the apparent low affinity interaction between the phage-displayed peptide and RelB. The inability of the synthetic peptides to bind RelB(His)_6 may indicate that in the context of the phage capsid protein, the peptides adopted a certain secondary structure that was required for binding to RelB(His)_6. Recovering fewer peptides with consensus sequences in the phage libraries displaying only 10 copies of the peptide on the capsid protein further suggests that the unfolded nature of RelB precluded high affinity interactions with the peptides. It was predicted that phage-displayed peptides recovered from the libraries displaying 10 copies would have higher affinity.
for RelB(His)$_6$ than the phage displaying 450 peptides, but were in fact shown to have lower affinity by ELISA. This further implicates avidity effects for the initial results observed with the seemingly promising $X_5$PWXV/L and $X_6$VCF motifs.

The inability to reliably purify RelE presented another set of challenges for this project. Despite attempting literature-reported expression and purification methods and previously successful methods in the lab, an established protocol to consistently obtain pure RelE remained elusive. Additional issues with RelE center around its ribosome-dependent ribonuclease activity which complicates the ability to perform in vitro activity assays on any large scale. The advantage of a ribosome-independent ribonuclease is the facile nature of testing its in vitro enzymatic activity, which could allow for direct toxin activation experiments. The culmination of the above-mentioned factors provided the motivation to turn to another TA system, MazEF$_{sa}$ which is discussed in Chapter 4.

3.7 EXPERIMENTAL PROCEDURES

3.7.1 Construction of plasmids

All primers used to construct expression vectors are listed in Table 3.5. Standard cloning procedures were followed. The pET28a-relBE(His)$_6$, pET-28a-relB(His)$_6$ and pET-21a-relE(His)$_6$ plasmids were obtained from Elizabeth Moritz.

To construct pTWIN2-RelE, relE was amplified from pET28a-relBE(His)$_6$ using the primers RelE-NcoI-For and RelE-PstI-Rev. pTWIN2-relE produces a CBD-SspB-RelE fusion upon induction with IPTG in E. coli BL21(DE3).

To construct pMBP-RelE, the RelE-BamHI F and RelE-XhoI R primers were used to amplify relE from pET-21a-relE(His)$_6$. pMBP-relE produces a MPB-RelE fusion protein upon induction with IPTG in E. coli BL21(DE3).

To construct pMBP-RelE(His)$_6$, RelE-BamHI F and His6-stop-XhoI R primers were used to amplify relE(his)$_6$ from pET-21a-relE(His)$_6$. pMBP-relE(His)$_6$ produces a MPB-RelE fusion protein upon induction with IPTG in E. coli BL21(DE3).
3.7.2 Purification of RelBE(His)$_6$ and RelB(His)$_6$ under native conditions

Clones were obtained from Elizabeth Moritz (see thesis for details). The plasmids pET-28a/RelBE(His)$_6$ or pET-28a/RelB(His)$_6$ were transformed by electroporation into E. coli BL21(DE3) and transformants were selected on LB agar containing 50 µg/mL kanamycin. LB/Kan$^{50}$ broth was inoculated with a single colony for an overnight culture which was used to seed 2 L in a 100-fold dilution. The culture was grown at 37°C until the OD$_{600}$ reached 0.4-0.6, at which point expression was induced with 1 mM IPTG (final concentration) for 4 hours at 37°C. Pellets were stored at -20°C.

RelBE(His)$_6$ and RelB(His)$_6$ was purified under native conditions and all steps were performed on ice or at 4°C. A pellet corresponding to 2 L culture was thawed for 5-10 min and resuspended in cold binding buffer (20 mM Tris, pH 7.9, 0.5 M NaCl). The cells were lysed by 5 min sonication with 1 s pulse at 50% amplitude. The lysate was cleared by 30 min centrifugation at 35000g at 4°C and the supernatant was batch loaded with 1.0 mL 1:1 Ni-NTA resin slurry (Qiagen) for 30 min at 4°C with inversion. RelBE(His)$_6$ and RelB(His)$_6$ were eluted with 5 mL binding buffer containing 0.5 M imidazole, concentrated to ~0.5 mL and dialyzed against assay buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 20 µM EDTA, 0.005% Tween-20). Purity and concentration were assessed using SDS-PAGE (Figure 3.23) and the BCA Assay following manufacturer’s instructions (Pierce).
3.7.3 Expression and purification of RelE from pTWIN2

Overnight cultures started from single colonies of *E. coli* BL21(DE3) pTWIN2-RelE were used to seed 1.5 L cultures in LB + 100 μg/mL ampicillin. The cultures were induced with 1 mM IPTG (final concentration) at $\text{OD}_{600} = 0.4$-$0.6$ and harvested after 3 hours growth at 37°C with shaking.

The pellet was resuspended in cold 10 mL Buffer B1 (20 mM Tris, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.1% Tween-20) and sonicated on ice for two cycles of 3 min with 1 sec pulse, with 3 min rest in between. A 5 mL bed volume of chitin resin was equilibrated with 10 column volumes of Buffer B1 and 23 gauge 1” needle was affixed to the column to control the flow rate. The lysate was centrifuged at 35000 g for 30 min at 4°C, brought up to 50 mL with Buffer B1 and applied to the column. For the wash step, a 22 gauge 1” needle was used to slightly increase the flow rate. The column was washed with 10 column volumes of cold Buffer B1 and then the needed was removed to allow a quick flush of 3 column volumes of cold Buffer B2 (20 mM Tris, pH 6.0, 0.5 M NaCl, 1 mM EDTA, 0.1% Tween-20) to induce intein autocleavage. The column was capped to retain sufficient buffer to cover the resin and then incubated at room temperature overnight. To elute liberated RelE, the column was washed with 5 column volumes of room temperature Buffer B2 which was collected multiple fractions.

Because the intein typically co-eluted with RelE, a gel filtration step was used to separate RelE from the intein. Bio-Gel P-100 was packed to a 9 cm bed height and equilibrated with binding buffer (20 mM Tris, pH 7.9, 0.5 M NaCl). The elution fraction from the chitin column was concentrated to 0.5 mL, slowly applied to the column and binding buffer was added in 0.5-1
mL aliquots until the A$_{220}$ returned to baseline. The flow rate was ~ 1 mL/hr and 0.5 mL fractions were collected. Protein purity was assessed by SDS-PAGE gel and stained with Sypro Ruby.

3.7.4 Expression and purification of MBP-RelE(His)$_6$

The plasmids pMBP-RelE(His)$_6$ was transformed by electroporation into *E. coli* BL21(DE3) and transformants were selected on LB agar containing 50 µg/mL kanamycin. LB/Kan$_{50}$ broth was inoculated with a single colony for an overnight culture which was used to seed 2 L in a 100-fold dilution. The culture was grown at 37°C until the OD$_{600}$ reached 0.4-0.6, at which point expression was induced with 1 mM IPTG (final concentration) for 3 hours at 37°C. Pellets were stored at -20°C.

For purification, a 1 L pellet was resuspended in 10 mL cold column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4) and sonicated for 5 min with a 1 sec pulse at 35-40% amplitude. The cell debris was centrifuged at 35000 g for 30 min at 4°C. Cold column buffer was added to bring the volume of the lysate to 150 mL, which was then passed over equilibrated amylose resin (Invitrogen) in a column affixed with a 22 gauge 1” needle to control flow rate. The resin was washed with 50 mL cold column buffer and the protein was eluted with 30 mL 10 mM maltose, collected in 10 equal fractions. Purity was assessed by SDS-PAGE gel, stained with Coommassie Brilliant Blue.

3.7.5 Set-up for PC biosensor assay

To prepare the 384-well PC biosensor plate for His$_6$-tagged protein binding, wells were washed using an automatic plate washer 3-4 times with 100 µL assay buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 20 µM EDTA, 0.005% Tween-20). Wells were then incubated with 5 µM D-biotin-tris-NTA hybrid compound (BTN) in assay buffer overnight at 4°C. The next day, the wells were washed as before and charged with Ni$^{2+}$ by incubating with 40 µL charging buffer (assay buffer containing 0.5 mM NiCl$_2$ ) for at least 5 minutes at room temperature to allow for His$_6$-tagged protein binding. Wells were washed with assay buffer three times before adding either 40 µL 0.05 mg/mL RelBE(His)$_6$ or RelB(His)$_6$ in assay buffer or assay buffer alone and incubating at room temperature ~30 minutes or until PWV shift reached 1.0-1.1 nm. Wells were again washed with assay buffer three times and the PWV shift measured with a BIND plate-based reader (SRU Biosystems).
3.7.6 Quantification of RelBE(His)$_6$ eluted from PC biosensor surface

The protocol for the PC biosensor assay was followed to bind RelBE(His)$_6$ to the surface. The protein was eluted from multiple wells using stripping buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.005% Tween-20) and concentrated by speed vacuum to 5X and 10X. The 1X, 5X and 10X eluted samples were analyzed on a 4-20% 15 μL tris-tricine gel (BioRad) alongside known amounts of RelBE(His)$_6$. At 100 V for 1 hr 45 min, with cathode buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3) in the inner chamber and anode buffer (200 mM Tris, pH 8.9) in the outer chamber. Protein in identical gels was visualized either by staining with Sypro Ruby (Invitrogen) for 16 hours and or by α-His tag Western blot.

For the Western blot, protein was transferred to PVDF membrane in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) at 60V for 2 hrs at 4°C. The membrane was blocked in PBS + 1% BSA for 1 hour at room temperature then washed 3 times in PBS + 0.01% Tween-20. The membrane was probed with 1:20,000 dilution of anti-His$_6$ antibody in PBS + 0.01% Tween-20 for 1 hour at room temperature, followed with three washes. The blot was incubated for 5 min with 3 mL each luminol and hydrogen peroxide solutions (BioRad) and developed by exposing to film for various times. The bands in the gel and Western blot were quantified by using the Image J program.

3.7.7 High-throughput screening protocol

PC biosensor plates were prepared with RelBE(His)$_6$ as described above. Compound plates were prepared by multiplexing 10 compounds per well in assay buffer + DMSO and 40 μL compound was added to give 100 μM of each compound and 10% DMSO at the final concentration. Plates were sealed with foil seals and covered with a plate filled with water to prevent condensation. The plates were incubated at room temperature for ~12 hours, then washed 3 times with 100 μL assay buffer in the automatic plate washer. To take the final read, 40 μL assay buffer was added. Finally, the plate was stripped by adding stripping buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.005% Tween-20) for 10 min and washing 5 times with 100 μL assay buffer using the automatic plate washer.

The data were plotted as the change in PWV value (PWV shift of RelBE baseline – PWV shift of the final wash) to reveal any compounds that caused a decrease in the PWV shift, indicating that protein had been removed from the PC biosensor surface. A hit was determined as any well that had a PWV shift three standard deviations below the average of the four RelBE(His)$_6$ + DMSO control wells.
3.7.8 Detection of RelE or peptide binding to RelB(His)$_6$ on PC biosensor

RelB(His)$_6$ was bound to the PC biosensor as described above in the basic set-up protocol, except that 50 mM sodium phosphate, pH 7.0 buffer was used. RelE (0-18 μM) or peptide (0-500 μM) were added and incubated for 12 hours at 4°C. The wells were washed 3 times with sodium phosphate buffer and the final PWV shift was measured.

3.7.9 Peptide purification

Reverse-phase HPLC was performed on a Beckman analytical n-butyl (C4) column housed in the van der Donk laboratory to purify the RelE peptides. The “A” line of the HPLC contained dH$_2$O and 0.1% trifluoroacetic acid (TFA) and the “B” line contained 80% acetonitrile and 0.086% TFA. Before subjecting a peptide to HPLC-purification, the A and B pumps were first primed with their respective solutions. Next, 100% of the B solution was flowed through the C4 column at a flow-rate of 1 mL/min for 5 minutes, followed by 2% B/98% A for at least 5 minutes at 1 mL/min to equilibrate the column. A “blank” was then run to examine the baseline absorbance at 220 nm. The blank program used an acetonitrile gradient with the following steps: Minutes 1-21 increase from 2% B/98% A to 100% B, minutes 21-26 hold at 100% B, minutes 26-31 decrease from 100% B to 2% B/98% A. After the blank was run, the column was again equilibrated with 2% B/98% A for 8-10 minutes at 1 mL/minute.

Approximately 1 mg of crude peptide was resuspended in 1 mL dH$_2$O and 500 – 700 μL was injected onto the HPLC. The program for an acetonitrile gradient on the C4 column consisted of the following steps: Minutes 1-46 increase from 2% B/98% A to 100% B, minutes 46-51 hold at 100% B, minutes 51-56 decrease from 100% B to 2% B/98% A, minutes 56-66 hold at 2% B/98% A. After running peptide over the column, the blank program was repeated, followed by flowing a 50% acetonitrile solution through the column for 15 minutes at 1 mL/minute and priming pumps A and B again.

MALDI-TOF mass spectrometry analysis was performed by the UIUC SCS mass spec facility to confirm the identity and purity of each purified peptide.

3.7.10 Surface plasmon resonance assay

The NTA chip (Biacore) was prepared by washing with 10 μL eluent buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 EDTA, 0.005% surfactant p-20) at a flow rate of 20 μL/min, then 30 μL regeneration/stripping buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.005% surfactant P-20) 10 μL/min. The surface was charged with 20 μL of charging buffer Eluent buffer containing 0.5 M NiCl$_2$) at 20 μL/min, then washed with eluent buffer again.
To immobilize protein, 1 μg/mL RelB(His)_6 was injected to only one flow cell until the RU reached ~800-1000. Various concentrations of the peptides were injected at 10 μL/min to both the reference cell (no immobilized protein) and the RelB(His)_6-containing flow cell, with 120 sec contact time and 120 sec dissociation time. All buffers used in SPR were filter-sterilized and degassed.

3.7.11 Biopanning

The basic protocol is outlined in Table 3.3 and the variations between each individual biopan are highlighted in Table 3.4. For Biopans X₀.1-3 and X₀.1-5, the phage solutions were first subjected to a “pre-clear” involving just phage and solid support to remove any phage that bound to the solid support. To pre-clear the phage with Ni-NTA resin, 0.5 mL of phage and 0.5 mL of Ni-NTA resin were incubated for 30 min at room temperature. The pre-clear the phage with the MagneHis magnetic Ni-NTA beads, 50 μL beads was incubated with 200 μL phage lysate for 30 min. The “cleared” phage lysate was then applied to the immobilized protein. To immobilize RelB(His)_6, 100 or 200 μL of 1.0 mg/mL was incubated with decreasing volumes of the solid support throughout each biopan round: 20 or 10 μL Ni-NTA resin for Rounds 1 and 3 and 6 μL MagneHis beads for Rounds 2 and 4. The incubation proceeded for 30 min at room temperature. The blocking solution was 5% BSA for Rounds 1 and 3 and 5% milk for Rounds 2 and 4. The incubation time for the phage and the immobilized protein was 25, 22, 18 and 15 min for Rounds 1, 2, 3, and 4, respectively. The washes were as follows: for Biopans X₀.1-3, 4, 5, and 7, TBS+0.2% Tween was used in 5, 6, 7, and 8 washes for Rounds 1, 2, 3, and 4 respectively. For Biopan X₀.1-8, TBS+0.2% Tween was used for 15, 6, 20 and 14 washes for Rounds 2 and 4. The solution used for elution of the phage varied between 500 mM imidazole, 1% SDS or 1.6 mg/mL MBP-RelE(His)_6 according to Table 3.4. Eluted phage were propagated in an E. coli culture at OD_{600} = 0.8. Following four rounds of biopanning, the eluted phage were propagated in E. coli on agar plates and 15 plaques from each biopan were analyzed.

Plaque analysis was performed by scraping along the plaque with a sterile 200 μL filter tip and resuspending in 100 μL filter-sterilized 10 mM Tris, pH 7.9. The solution was vortexed for 5 sec, heated to 65°C for 10 min and centrifuged 3 min at full speed. For the PCR, 2 μL of
this solution was used along with T7-21346-Up primer (5’-GTAAAGCTGCGTGACTTGGC-3’) and T7-21696-Dn primer (5’-GTAGCGTCACCTCCAGCG-3’). Purified PCR products were sequenced using T7-21346-Up primer.

3.7.12 Competitive biopan

The recombinant phage and WT phage were in a 1:1x10^6 ratio and the exact protocol used in the initial biopan was repeated. Plaques were analyzed as described above. To separate the amplicons generated from the WT and recombinant phage, a 2% gels was prepared with the high resolution MetaPhor agarose (Lonza), according to manufacturer’s instructions.

3.7.13 ELISA

To prepare the plates, 100 μL of 0.1 mg/mL RelB(His)_6 was adsorbed to wells of a MaxiSorp plate (Nunc) for 3-4 hours at room temperature or overnight at 4°C. The wells were washed 5 times with 300 μL binding buffer, blocked with 200 μL 5% milk in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.5), then washed 5 times with TBS. Phage lysate was added to the wells in 100 μL volumes and incubated for 1 hour at room temperature. The wells were washed 10 times with 300 μL TBS + 0.2% Tween-20 (TBST) and 100 μL of a 1:2000 dilution of the anti-T7-HRP conjugated antibody (Novagen) in TBST was incubated for 1 hour. Following 5 washes with 300 μL TBST, 100 μL of 1 mg/mL of freshly-prepared ABTS (Sigma) in 100 mM phosphate-citrate buffer, pH 4.0 mixed with 0.03% H₂O₂ (final concentration) was added and incubated for 30 min in the dark. The absorbance was read at 405 nm.

3.8 REFERENCES


CHAPTER 4
INVESTIGATION INTO THE ACTIVATION OF MAZFS A BY SYNTHETIC PEPTIDES
AND IN RESPONSE TO ENVIRONMENTAL STRESS

Sections from Chapter 4 have been published in “Detection of endogenous MazF enzymatic activity in *Staphylococcus aureus*,” Williams JJ and Hergenrother PJ. (2013) *Anal Biochem*, in press.

4.1 INTRODUCTION

As discussed in Chapter 1, the *mazEF*Sa toxin-antitoxin (TA) system was detected in 100% (78 of 78) clinical isolates of MRSA, and the *mazEF*Sa transcript was also detected in all of the 8 isolates analyzed [39]. Additionally, a BLAST search revealed the *mazEF*Sa genes in 44 *S. aureus* chromosomes (Table 1.1). The *mazEF*Sa genes are organized similarly to other TA systems, wherein the toxin start codon overlaps the antitoxin start codon; however, unlike many other TA systems, MazEFSa does not autoregulate its transcription.

The *mazEF*Sa genes are located in the *sigB* operon (Figure 4.1) which encodes for the stress-activated alternative sigma factor, $\sigma^B$, which allows for rapid response to environmental insults. Full *sigB* activity is dependent on $P_{mazE}$, which is upstream of *sigB* [10, 14]. The $P_{mazE}$ promoter is induced in response to sub-MIC levels of certain antibiotics and other environmental stressors such as heat shock [10, 34] and it was found that the SarA protein binds to the DNA upstream *mazEF*Sa [10]. A negative feedback loop appears to require *sigB*; however it is unknown how transcription is downregulated [10].

![Figure 4.1](image)

**Figure 4.1** Genetic organization of *mazEF*Sa in the *sigB* operon. Transcription from the $P_{mazE}$ promoter is upregulated in response to heat shock and subinhibitory levels of antibiotics. The transcriptional activator SarA is thought to bind the -35 element in $P_{mazE}$. Transcription from $P_{mazE}$ significantly contributes to the expression levels of *sigB*, which encodes for the stress-induced sigma factor $\sigma^B$, and full $\sigma^B$ activity is dependent on $P_{mazE}$. Repression of transcription at $P_{mazE}$ is dependent on *sigB*, likely through an intermediary factor. Figure from [10]

Canonical antitoxin proteins contain an N-terminal leucine-zipper domain to facilitate homodimerization and binding to operator DNA. However, as mentioned above, *mazEF*Sa is unique in that it does not autoregulate its transcription; indeed alignment with the *B. anthracis*
MoxX antitoxin reveals the absence of the N-terminal dimerization domain in MazE<sub>Sa</sub> (Figure 4.2A). Secondary structure prediction revealed a conserved RHH domain at the N-terminus of MoxX that was required for repression of the moxXT promoter [6]. Modeling of MoxX with the crystal structure of the MoxT dimer revealed an extended polypeptide structure of MoxX that binds the MoxT dimer interface (Figure 4.2B) [6]. This structure closely resembles that of the E. coli MazEF crystal structure (Figure 4.2C) [23].

In <i>S. aureus</i>, the ClpPC protease was demonstrated to specifically cleave MazE<sub>Sa</sub>. Upon treatment with rifampicin to halt transcription, the ectopically overexpressed MazE<sub>Sa</sub> was rapidly degraded, with a half-life of approximately 18-20 min, whereas levels of endogenous MazF<sub>Sa</sub> remained stable [11]. Indeed, the <i>S. aureus</i> clpPC transcript is also upregulated in response to various stressors, including heat shock [13]. Thus, exposure of <i>S. aureus</i> to certain stressors may result in the activation of MazF<sub>Sa</sub>.

The toxin MazF<sub>Sa</sub> is a sequence-specific endoribonuclease that inhibits the growth of <i>S. aureus</i> and <i>Escherichia coli</i> upon ectopic overexpression [14, 15] (Figure 4.3A). Overexpression in <i>S. aureus</i> decreased cell viability by 2-log CFU/mL after 60 min of induction; however, there was only ~27% difference in cell death at the 60 minute time point, suggesting that MazF<sub>Sa</sub> induces stasis and not cell death (Figure 4.3B) [15]. MazF<sub>Sa</sub> preferentially cleaves RNA at U↓ACAU sites which are abundant in certain transcripts, including those that code for
virulence factors such as the serine-rich pathogen adhesion factor SraP [43]. Overexpression of MazF$_{Sa}$ in $S$. aureus results in time-dependent cleavage of other virulence transcripts, including hla and spa, whereas essential housekeeping transcripts recA and gyrB were not cleaved [15]. The sraP transcript has a total of 43 UACAU cleavage sites which makes it a likely target for MazF$_{Sa}$ degradation in the cell [43]. Indeed it was shown that MazF$_{Sa}$ degrades a portion of the sraP transcript containing nine cleavage sites faster than a region of sraP containing five cleavage sites [43].

Figure 4.3 Reduction in cell viability by ectopic overexpression of MazF$_{Sa}$ in $S$. aureus. (A) Induction of MazF$_{Sa}$ resulted in a 2-log decrease in CFU counts over 60 min, whereas uninduced cultures continued to grow. (B) Live/dead staining revealed a 27% decrease in cell viability over 60 min. Figure from [15].

The ten transcripts most susceptible to MazF$_{Sa}$ cleavage, based on number of UACAU sites, are shown in Table 4.1. It is unknown if cleavage at these sites is responsible for the observed cell growth inhibition upon overexpression of MazF$_{Sa}$. It has been shown that MazF$_{Sa}$ preferentially cleaves only single-stranded mRNA and secondary structure can prevent access to cleavage sites [43]. Thus, for example, although the 16S rRNA contains a UACAU site, its extensive secondary structure likely protects it from MazF$_{Sa}$ cleavage and indeed 16S rRNA isolated from cells overexpressing MazF$_{Sa}$ is intact [10, 15].

Table 4.1 Top 10 MazF$_{Sa}$-susceptible transcripts. Table adapted from [43].

<table>
<thead>
<tr>
<th>gene length</th>
<th>UACAU count expected</th>
<th>UACAU count actual</th>
<th>fold increase*</th>
<th>locus</th>
<th>gene product</th>
</tr>
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<tbody>
<tr>
<td>6816</td>
<td>11.42</td>
<td>43</td>
<td>3.77</td>
<td>SA2447</td>
<td>Serine-rich adhesin protein SraP</td>
</tr>
<tr>
<td>1215</td>
<td>2.51</td>
<td>9</td>
<td>3.59</td>
<td>SA0794</td>
<td>D-alanine transport protein DltB</td>
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<tr>
<td>1557</td>
<td>2.33</td>
<td>8</td>
<td>3.43</td>
<td>SA1626</td>
<td>Type I restriction modification protein on pathogenicity island</td>
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<td>1026</td>
<td>1.71</td>
<td>7</td>
<td>4.09</td>
<td>SA1466</td>
<td>5'-adenosylmethionine tRNA ribosyltransferase isomerase QueA</td>
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<td>6</td>
<td>4.69</td>
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<td>oligopeptide transporter protein Opp-1D</td>
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<tr>
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<td>6.33</td>
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</tr>
<tr>
<td>297</td>
<td>0.61</td>
<td>4</td>
<td>6.56</td>
<td>SA1222</td>
<td>truncated transposase</td>
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<tr>
<td>390</td>
<td>0.65</td>
<td>4</td>
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<td>SA0830</td>
<td>Hypothetical protein (integral membrane protein)</td>
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<tr>
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<td>21.43</td>
<td>SA0930</td>
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<tr>
<td>327</td>
<td>0.46</td>
<td>3</td>
<td>6.52</td>
<td>SA0456</td>
<td>Regulatory protein SpoVG</td>
</tr>
</tbody>
</table>

*Fold increase of actual UACAU count over expected UACAU count for specific transcript
The activation of MazF<sub>sa</sub> may serve as an anti-virulence strategy by enhancing its ability to cleave virulence-factor encoding transcripts. We set out to identify peptides capable of enhancing the endoribonucleolytic activity of MazF<sub>sa</sub>. Given the difficulty experienced with targeting the high-affinity TA complex or the intrinsically disordered antitoxin (discussed in Chapter 3 and Appendix II), we elected to target the toxin. Furthermore, the MazF toxin is conserved across Gram-positive positive bacteria, including the problematic pathogens <i>Listeria monocytogenes</i> and <i>Bacillus anthracis</i> (Figure 4.4) [29]. Thus, an activator of the MazF<sub>sa</sub> toxin may also activate the MazF homologues in these pathogens. Additionally, the ribosome-independent activity of MazF facilitates facile analysis of activators for their effect on such analogs.

### Figure 4.4

ClustalW alignment of MazF homologues from <i>S. aureus</i>, <i>B. anthracis</i> and <i>L. monocytogenes</i>, which are 120, 116 and 115 amino acids, respectively. The three proteins share 60% identity and 26% similarity. Blue *, strong identity; green ., strong similarity and purple ., weak similarity.

<table>
<thead>
<tr>
<th></th>
<th>S. aureus</th>
<th>B. anthracis</th>
<th>L. monocytogenes</th>
</tr>
</thead>
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<td>MIVKRGDVFADLSPVQSEQGGVRPVVIIQNDIGNRFSPTVIIVAATIQKAKLPTHV</td>
<td>MMVIRGQVYADLSPVQSEQGGIRPVVIIQNDIGNRFSPTVIIVAATIQKAKLPTHV</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>B. anthracis</td>
<td>L. Monocytogenes</td>
</tr>
<tr>
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<td>EIDA!KKG!FERDSVILLEQIRTDK!QRLTDDKTHLDEVMIRVEALQ!SLG!QO</td>
<td>EATRKDFERDSVILLEQIRTDKQRLTDDKTHLDEVMIRVEALQ!SLG!QO</td>
</tr>
</tbody>
</table>

To identify peptides that increased the activity of MazF<sub>sa</sub>, peptide fragment analysis of the antitoxin MazE<sub>sa</sub> was performed using a kinetic assay with a short fluorogenic chimeric oligonucleotide substrate and a gel-based assay that employed a more relevant transcript substrate. Although peptide-mediated enhanced ribonuclease activity was not achieved, the assays developed to detect MazF<sub>sa</sub> activity led to the first detection of endogenous MazF<sub>sa</sub> activity in <i>S. aureus</i> lysate. All studies characterizing the growth inhibitory effect of MazF<sub>sa</sub> and cleavage of specific transcripts have relied on overexpression systems [14, 15, 43], which likely do not faithfully recapitulate the effect of endogenous MazF<sub>sa</sub> on mRNA cleavage and growth inhibition. Additionally, the role of MazF<sub>sa</sub> in <i>S. aureus</i> stress response has been limited to the identification of conditions that upregulate transcription of <i>mazEF</i><sub>sa</sub>, such as exposure to antibiotics [10, 14], without determining the effect of increased transcription such as increased MazF<sub>sa</sub> activity. Thus it remains unknown if MazF<sub>sa</sub> is involved in stress response and conditions that result in its activation are undefined. The robust, sensitive assays described in this chapter provide a toolkit for the identification, analysis, and validation of molecules or conditions that...
induce MazF<sub>sa</sub> activity and should assist in the discovery of both artificial and natural activators of the MazEF<sub>sa</sub> TA system.

4.2 CLONING, EXPRESSION AND PURIFICATION OF MAZEF<sub>SA</sub>

The mazEF<sub>sa</sub> genes were cloned from the MRSA isolate C2, which was previously shown to carry mazEF<sub>sa</sub> [39], into the T7 promoter-controlled expression vector pET-28a; expression of this clone results in the C-terminal His6-tagged protein complex, MazF<sub>sa</sub>(His)<sub>6</sub>. Additionally, mazE<sub>sa</sub> alone was cloned from MRSA C2 into pET-28a to facilitate expression of (His)<sub>6</sub>MazE<sub>sa</sub>.

Initial expression and purification efforts revealed that the MazEF<sub>sa</sub> proteins were not expressed to high levels in E. coli BL21(DE3), thus purification by Ni<sup>2+</sup>-NTA affinity chromatography resulted in the co-purification of common contaminants (data not shown). Thus, the expression vectors were introduced into E. coli NiCo21, a derivative of strain BL21(DE3) engineered to reduce the co-purification of proteins that have high intrinsic affinity for Ni<sup>2+</sup>-NTA resin [32]. The common contaminants were fused to chitin binding domains in the engineered strain [32], thus chitin-bead affinity chromatography removes these proteins.

The MazF<sub>sa</sub> toxin was obtained by expression of the MazEF<sub>sa</sub>(His)<sub>6</sub> complex in E. coli NiCo21 and subsequent denaturing purification on Ni<sup>2+</sup>-NTA resin to disrupt the interaction between MazE<sub>sa</sub> and MazF<sub>sa</sub>(His)<sub>6</sub>. On-column refolding by stepwise reduction of the urea concentration from 8 M to 0 M and subsequent purification over chitin resin resulted in highly pure MazF<sub>sa</sub>(His)<sub>6</sub> (Figure 4.5). The size and identity of MazF<sub>sa</sub> were confirmed by SDS-PAGE, mass spectrometry and trypsin digest.

The MazE<sub>sa</sub> protein was obtained from cultures of E. coli NiCo21 expressing (His)<sub>6</sub>MazE<sub>sa</sub> by Ni<sup>2+</sup>-NTA affinity chromatography under native conditions. Mass spectrometry revealed the susceptibility of MazE<sub>sa</sub> to degradation by cellular proteases during the purification. The addition of protease inhibitors to the buffers used in purification allowed for purification of
intact MazE<sub>sa</sub>, which was confirmed by SDS-PAGE (Figure 4.5) and mass spectrometry. The MazE<sub>sa</sub> sequence contains only two arginines and one lysine and is thus not suitable for identification by tryptic digestion.

4.3 CHARACTERIZATION OF MazF<sub>sa</sub> AND MazE<sub>sa</sub>

MazF<sub>sa</sub> optimally cleaves at the sequence U↓ACAU in single-stranded RNA and was also shown to cleave the synthetic RNA substrate 5’-AAGUCUACAUCA-3’ [43]. Based on this substrate, a chimeric DNA-RNA substrate consisting of DNA bases at all positions except for the U and the A surrounding the cleavage site was designed. The DNA bases offer greater stability than RNA bases as well as protection from general ribonucleases. The synthetic chimeric substrate 5’-AAGUCrUrACATCAG-3’ (Figure 4.6A) was subjected to cleavage by MazF<sub>sa</sub> and analyzed by HPLC [41]. The HPLC trace in Figure 4.6B shows the cleavage of the chimeric substrate by MazF<sub>sa</sub> [41]. The mass of each product separated by HPLC was determined by MALDI mass spectrometry and the expected products were observed (data not shown). Furthermore, the addition of (His)<sub>6</sub>MazE<sub>sa</sub> inhibited the ribonuclease activity of MazF<sub>sa</sub>(His)<sub>6</sub> (Figure 4.6C) [41].

![Figure 4.6](image)

**Figure 4.6** Substrate cleavage by MazF<sub>sa</sub>(His)<sub>6</sub>. (A) Non-fluorogenic substrate design, “r” denotes RNA. (B) Products from 16 hour incubation of oligonucleotide (32 μM) in the absence (black) or presence (blue) of 16 μM MazF<sub>sa</sub>(His)<sub>6</sub> were separated by HPLC and analyzed by MALDI mass spectrometry. The peaks at the longest retention times correspond to intact oligonucleotide (expected molecular weight [MW] = 3960.6). The peaks at shorter retention times correspond to 5’ and 3’ fragments generated from MazF<sub>sa</sub>(His)<sub>6</sub> cleavage; expected MWs = 1854.68 and 2105.4, respectively. (C) 34 μM MazE<sub>sa</sub> and 5 μM MazF<sub>sa</sub> were incubated separately (black and blue line, respectively) or together (green line) with 12 μM substrate at 37°C for 12 hours and the products were analyzed by HPLC.

The interaction between MazE<sub>sa</sub> and MazF<sub>sa</sub> was demonstrated by native gel electrophoresis (Figure 4.7). (His)<sub>6</sub>MazE<sub>sa</sub> and MazF<sub>sa</sub>(His)<sub>6</sub> were mixed in 2:1, 2:2 or 1:2 and
analyzed on a native gel. Complex formation was observed in all ratios, but no free antitoxin band was detected in the 1:2 MazE:MazF ratio. Although the crystal structure for MazEF \textit{Sa} has not been solved and there is no definitive data describing the ratio between the two proteins in complex, the \textit{E. coli} MazE:MazF complex forms in a 1:2 ratio \cite{23}, suggesting the MazEF \textit{Sa} complex may be similar.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4_7.png}
\caption{Interaction of MazE\textsubscript{Sa} and MazF\textsubscript{Sa} detected by native gel electrophoresis. Various ratios of (His\textsubscript{6})\textsubscript{MazE\textsubscript{Sa}}:(His\textsubscript{6})\textsubscript{MazF\textsubscript{Sa}} were analyzed by native gel and stained with Sypro Ruby dye. (His\textsubscript{6})\textsubscript{MazE\textsubscript{Sa}}, with a theoretical pI of 5.63, is able to enter the pH 8.0 gel whereas the theoretical pI of MazF\textsubscript{Sa} is 9.55, precluding its entrance into the gel.}
\end{figure}

4.4 KINETIC ASSAY FOR DETERMINATION OF MAZF\textsubscript{SA} KINETIC PARAMETERS

The continuous fluorometric assay developed for MazF\textsubscript{Ec} \cite{37} was modified for kinetic evaluation of MazF\textsubscript{Sa}. The chimeric substrate was created with a 6-FAM fluorescein on the 5’ end and a quencher (Black Hole Quencher, BHQ) on the 3’ end (Figure 4.9A). Analysis of MazF\textsubscript{Sa} cleavage in sodium phosphate buffer from pH 6.0-8.0 revealed that the optimal pH was \textasciitilde6.5, with activity declining as the pH of the buffer increased (Figure 4.8A). Additionally, the fluorescence of the intact substrate (Figure 4.8B) and cleavage products (Figure 4.8C) increased with higher pH. Thus, it was determined that a pH of 6.5 was optimal for MazF\textsubscript{Sa} activity as well as reduction of background fluorescence in the assay.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4_8.png}
\caption{pH dependence on fluorogenic assay. (A) Initial velocity of 0.8 \textmu M MazF\textsubscript{Sa} cleavage reaction with 0.25 \textmu M fluorogenic substrate in phosphate citrate buffer, pH 6.4-7.0 and phosphate buffer, pH 7.1-8.0. Fluorescence of intact substrate (B) and cleavage products (C) in phosphate buffer from pH 6.0-8.0.}
\end{figure}
Cleavage of this fluorogenic substrate by MazF$_{Sa}$ resulted in increased fluorescence over time, allowing progress curves to be generated at various substrate concentrations (Figure 4.9B) [41]. The continuous nature of the fluorometric assay enabled the characterization of MazF$_{Sa}$(His)$_6$ enzyme kinetics ($V_{\text{max}} = 0.12$ pmol/min, $K_M = 0.56$ μM, $k_{\text{cat}}/K_M = 0.0079$ M$^{-1}$s$^{-1}$) (Figure 4.9C) [41]. As has been noted previously, the absolute rate of processing of the fluorogenic substrate by recombinantly expressed and purified toxins is low and thus requires higher concentrations of enzyme than normally used in standard assays [5, 37]. Thus, although the Michaelis-Menten equation was fit to the data, the enzymatic parameters observed for MazF$_{Sa}$(His)$_6$ do not follow standard Michaelis-Menten kinetics.

![Figure 4.9](image)

**Figure 4.9** Substrate cleavage by MazF$_{Sa}$(His)$_6$. (A) Fluorogenic substrate design, “r” denotes RNA. (B) Representative set of progress curves showing cleavage of the fluorogenic substrate (5′-6-FAM-AAGTCrUrACATCAG-BHQ-3′) by MazF$_{Sa}$(His)$_6$. Enzyme (0.8 μM) was added to the indicated concentration of substrate and the fluorescence was monitored over time in 384 well plates. (C) Slopes from progress curves were fit to the Michaelis–Menten equation (solid line). Error bars represent standard deviation, n=3.

### 4.5 GEL-BASED ASSAY FOR MAZF$_{Sa}$ ENORIBONUCLEASE ACTIVITY

Although the fluorogenic substrate is useful for kinetic assessment, MazF$_{Sa}$ naturally cleaves mRNA transcripts. Thus, a method for the detection of MazF$_{Sa}$(His)$_6$ endoribonuclease versus mRNA was developed. An RNA substrate was generated by *in vitro* transcription as described in the Materials and Methods. The resultant transcript from this template contained three MazF$_{Sa}$ cleavage sites, including one preferentially cleaved site (site 1, UACAU), a sub-optimal site at which cleavage was not detected (site 2, UACGU) and a secondarily-cleaved sub-optimal site (site 3, UACAU) (Figure 4.10, left). The primary cleavage site was 86 bases from the 5′ end of the full length transcript, complicating analysis of the cleavage products by gel electrophoresis (Figure 4.10, Left) [41]. To improve the resolution of the cleavage products on gels, the original template was engineered by site-directed mutagenesis by removing sites 1 and 2 and optimizing site 3, such that the new substrate contained only one cleavage site of the optimal sequence UACAU (Figure 4.10, Right) [41]. Cleavage of this substrate by MazF$_{Sa}$ resulted in easily distinguishable products (Figure 4.10, Right).
Figure 4.10 Development of transcript substrates for MazF<sub>Sa</sub>(His)<sub>6</sub> generated by <em>in vitro</em> transcription. Left: Cleavage of the 3-site substrate (50 nM) produces 4 cleavage products within 240 s incubation with MazF<sub>Sa</sub>(His)<sub>6</sub> (50 nM). Right: Cleavage of the 1-site substrate results in two well-separated products. RNA was separated on a denaturing 3.5% polyacrylamide gel and stained with ethidium bromide.

The sensitivity of this substrate for MazF<sub>Sa</sub>(His)<sub>6</sub> activity was tested with lower and perhaps more physiologically relevant concentrations of MazF<sub>Sa</sub>. A time course experiment with 1 nM MazF<sub>Sa</sub>(His)<sub>6</sub> and 25 nM RNA resulted rapid cleavage, with approximately 50% processing of the substrate in 5 minutes (Figure 4.11) [41]. To our knowledge, this is the most sensitive assay for <em>in vitro</em> assessment of the ribonuclease activity of purified MazF<sub>Sa</sub>. Furthermore, MazF<sub>Sa</sub>(His)<sub>6</sub> activity was inhibited by (His)<sub>6</sub>MazE<sub>Sa</sub>, demonstrating the specificity of the cleavage of this substrate (Figure 4.11) [41].

Figure 4.11 Timecourse of MazF<sub>Sa</sub> endoribonuclease activity. MazF<sub>Sa</sub>(His)<sub>6</sub> (1 nM) was incubated at room temperature with 25 nM single site substrate. Activity is detected within 10 s and >50% processing is achieved by 5 min. To inhibit MazF<sub>Sa</sub>(His)<sub>6</sub>(His)<sub>6</sub>MazE<sub>Sa</sub> was added prior to reaction with RNA for 60 seconds (+E lane). RNA was separated on a denaturing 5% polyacrylamide gel and stained with ethidium bromide.
4.6 MazE<sub>Sa</sub> Peptide Fragment Analysis

Peptide fragment analysis of the antitoxin MazE<sub>Sa</sub> was performed to identify peptides that increased the activity of MazF<sub>Sa</sub>. Ten peptides of 10 or 11 amino acids covering the MazE<sub>Sa</sub> sequence and overlapping by 5 amino acids (Figure 4.12A) were obtained in ~90% purity from Genscript (Table 4.2). The effect of these peptides on MazF<sub>Sa</sub> activity was assessed in both the kinetic and gel-based assays.

**Figure 4.12** Peptide fragment analysis of MazE<sub>Sa</sub>. (A) A collection of ten-amino acid peptide fragments overlapping by five amino acids spanning the entire 56-residue MazE<sub>Sa</sub> were investigated. (B) Each peptide 1-10 was incubated at 60 μM for 3 hours with 0.8 μM MazF<sub>Sa</sub> prior to incubation with 0.25 μM fluorogenic substrate. The activity of MazF<sub>Sa</sub> with no peptide is represented by the dotted line at 100% and the activity of MazF<sub>Sa</sub> in the presence of each peptide is plotted.

4.6.1 Assessment of peptides in kinetic assay

For an initial screen, each peptide was tested at 60 μM for its effect on MazF<sub>Sa</sub> activity and as shown in Figure 4.12B, peptide 6 appeared to enhance the activity of MazF<sub>Sa</sub> by 30%, whereas the others had minimal to no effect on the activity.

To further examine its effects on MazF<sub>Sa</sub> enzymatic activity, peptide 6 was tested at three concentrations, 5 μM, 25 μM and 100 μM, with three substrate concentrations corresponding to 0.089<sub>Km</sub>, 0.45<sub>Km</sub>, and 3.6<sub>Km</sub>. As shown in Figure 4.13A, peptide 6 enhances MazF<sub>Sa</sub> enzymatic activity in a dose-dependent manner across the three substrate concentrations. Kinetic data was obtained for MazF<sub>Sa</sub> in the presence or absence of 100 μM peptide 6 and it was shown that peptide 6 enhances the catalytic efficiency of MazF<sub>Sa</sub> 1.7-fold (Figure 4.13C). To assess the specificity of peptide 6 enhancement, it was tested with MazF<sub>Sa</sub> and RNaseA. As shown in Figure 4.13B, peptide 6 does not enhance the activity of RNaseA, thus suggesting that peptide 6 specifically enhances MazF<sub>Sa</sub> enzymatic activity.

4.6.2 Assessment of peptides 5 and 6 in gel based assay

The peptide fragments of were assessed for their effect on MazF<sub>Sa</sub> ribonuclease activity. Each of the 10 peptides was incubated at 100 μM with 100 nM MazF<sub>Sa</sub> for 3 hours prior to incubation for 1 min at room temperature with the single-site RNA substrate. As shown in
Figure 4.14A, peptides 5 and 6 enhance the ribonuclease activity of MazF$_{Sa}$, in the absence of peptide, MazF$_{Sa}$ activity is not detectable at this timepoint in the assay.

Figure 4.13 The effect of peptide 6 on MazF$_{Sa}$ and RNaseA activity. (A) MazF$_{Sa}$ was treated with 0, 5, 25 or 100 μM peptide 6 prior to addition of 0.05, 0.25 or 2 μM fluorogenic substrate. MazF$_{Sa}$ activity in the absence of peptide was set at 1.0 and the relative increase in initial velocity is shown for each concentration of peptide 6. Error bars indicate standard deviation; $P$, statistical significance between designated groups. (B) Increasing concentrations of peptide 6 was added to MazF$_{Sa}$ or to RNaseA. The initial velocity of each enzyme in the absence of peptide 6 was set to 1.0 and the velocity of the enzyme in the presence of 25 or 100 μM peptide 6 was plotted as a fold-change over enzyme alone. (C) The effect of 100 μM peptide 6 on the kinetic parameters of 0.8 μM MazF$_{Sa}$ with increasing concentrations of fluorogenic substrate (0.025-4 μM) was determined as described in Figure 4.9C. Relative $V_{max}$, $K_M$ and $k_{cat}/K_M$ values were determined from the Lineweaver-Burk plot of the kinetic (data not shown).

Peptide 5 and 6 showed a dose-dependent response on enhancement of MazF$_{Sa}$ activity. Peptide 5 was tested from 5-200 μM and showed optimal activity as low as 20 μM (Figure 4.14B). Peptide 6 was tested from 10-200 μM and showed optimal activity at 100 μM. Both peptides were tested at 100 μM in the absence of MazF$_{Sa}$ and had no effect on the RNA substrate (Figure 4.14B).

Figure 4.14 MazF$_{Sa}$ peptide fragment analysis assessed by gel-based assay. (A) MazF$_{Sa}$ peptide fragments were tested at 100 μM for their effect on the endoribonuclease activity of 100 nM MazF$_{Sa}$ using the single-site substrate in the absence (-) or presence (1-10) of peptide was incubated with 100 nM RNA for 1 min at room temperature. (B) Dose-response of peptides 5 and 6. Peptides 5 and 6 were tested from 0-200 μM. Both peptides were tested with RNA in the absence of MazF$_{Sa}$ (P5, P6) and did not affect the RNA. (C) Time-course assay with peptides 5 and 6. 200 nM MazF$_{Sa}$ was incubated in the absence of (-) or presence of 100 μM peptide 5 or 6 (5, 6), followed by incubation with 100 nM of the three-site RNA substrate for 0-8 min at 37°C.
Peptides 5 and 6 were tested in a time-course assay with MazF<sub>sa</sub> using the 3-site substrate. As shown in Figure 4.14C, quantitative cleavage of Site 1 is achieved by approximately 30 seconds and 3 minutes in the presence of peptide 5 and 6, respectively, whereas MazF<sub>sa</sub> alone shows minimal activity at 7 min.

Table 4.2  Sequence and characteristics of peptides tested for MazF<sub>sa</sub> activity enhancement

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<thead>
<tr>
<th>peptide #</th>
<th>location</th>
<th>Sequence</th>
<th>purity (%)</th>
<th>attributes</th>
<th>% hydrophobic residues</th>
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<td>4.37</td>
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<tr>
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<td>3.8</td>
<td>1091.24  -1  40</td>
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<td>5.52</td>
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<td>97.4</td>
<td>5.52</td>
<td>707.77   0  57.14</td>
</tr>
<tr>
<td>26-32 A7</td>
<td>26-32</td>
<td>LNLSLLA</td>
<td>96.7</td>
<td>5.52</td>
<td>700.83   0  71.43</td>
</tr>
<tr>
<td>Ac26-32</td>
<td>26-34</td>
<td>LNLSLAN</td>
<td>90.3</td>
<td>5.52*</td>
<td>785.87   -1  57.14</td>
</tr>
<tr>
<td>26-32Am</td>
<td>26-35</td>
<td>LNLSLAN</td>
<td>91.8</td>
<td>5.52*</td>
<td>742.87   1  57.14</td>
</tr>
</tbody>
</table>
4.6.3 Derivatives of peptides 5 and 6

Peptides 5 and 6 overlap by five amino acid residues, so the sequence they share in common became the building block for the second generation of peptides. To identify the shortest active peptide, single amino acid truncations from either the N-terminus of peptide 5 or the C-terminus of peptide 6 were obtained (Figure 4.15A). Additionally, a new preparation of peptide 5 was obtained to rule out a non-specific effect of the original stock. The old and new preparations of peptide 5 along with the second generation derivatives were assessed at 20 μM with 100 nM MazF<sub>Sa</sub>. As shown in Figure 4.15B, both the original and a new preparation of peptide 5 are active. Moreover, the truncated derivatives LNLSL and LNLSLAN enhanced MazF<sub>Sa</sub> activity to a greater extent than peptide 5 (Figure 4.15B).

Many of the experiments described were performed at one minute points with a concentration of MazF<sub>Sa</sub> that does not cleave significant RNA in that timeframe to more clearly differentiate the most potently active peptides. To show enhancement over an active MazF<sub>Sa</sub> baseline, the peptides were assessed at 10 μM with 200 nM MazF<sub>Sa</sub>, which cleaves detectable RNA at the minute timepoint, as shown in Figure 4.15C. Although all of the peptide 5 and 6 derivatives enhance MazF<sub>Sa</sub>, enhanced processing of the RNA by MazF<sub>Sa</sub> is observed in the presence of LNLSL and LNLSLAN (Figure 4.15C). The QMADLNLSL and MADLNLSL derivatives also enhanced MazF<sub>Sa</sub> activity, but given the results observed in the previous assay, the shorter derivatives LNLSL and LNLSLAN were further investigated.

![Figure 4.15](image-url)
The *E. coli* Extracellular Death Factor (EDF) is a pentapeptide with the sequence NNWNN and has been shown to enhance the enzymatic activity of MazF<sub>Ec</sub>. It was proposed that EDF binds at the interface of MazF<sub>Ec</sub> dimers and increases its catalytic efficiency by decreasing the affinity of MazF<sub>Ec</sub> for the RNA substrate. EDF was tested from 6.25-200 μM and shown to have no effect on MazF<sub>Sa</sub> activity, whereas the active 5-amino acid derivative LNLSL was active at 12.5 μM and showed optimal activity at 25 μM (Figure 4.15D).

4.6.4 Derivatives of LNLSL and LNLSLAN

Based on the enhancement observed with the peptide LNLSL, three- and four-amino acid derivatives (Figure 4.16A) were assessed at 10 and 20 μM with 100 nM MazF<sub>Sa</sub>. As shown in Figure 4.16B, none of the three- and four-amino acid peptide derivatives enhanced MazF<sub>Sa</sub> activity. Thus, LNLSL was the shortest active peptide.

![Figure 4.16 Derivatives of LNLSL and LNLSLAN.](Image)

To determine the important amino acid residues that contribute to the enhancement effect of peptide LNLSLAN, alanine-substituted derivatives were obtained (Figure 4.16A). Additionally, derivatives modified with an N-terminal acetylation or C-terminal amidation were obtained. These peptides were assessed at 10 and 20 μM with 100 nM MazF<sub>Sa</sub>. As shown in Figure 4.16B, the A1, A3, A5 and C-terminal amidated peptides showed no enhancement of MazF<sub>Sa</sub> activity of these peptides was completely abolished. However, the A2 derivative showed increased activity over the parent, the N-acetylated derivative had comparable activity to the parent peptide, the A4 derivative showed slightly reduced activity compared to the parent and the A7 derivative showed a further reduction in activity (Figure 4.16B). The results are summarized...
in Figure 4.16C, with the number of stars indicating the ranked level of activity of each peptide. These rankings were used to determine the contribution of each feature of the parent LNLSLAN peptide, depicted in Figure 4.16D. In summary, each Leu residue and the carboxylic functionality are required for activity. The C-terminal Asn residue also contributed to activity, as its removal reduced the enhancement effect on MazF<sub>sa</sub>. The N-terminal functionality appears to be inconsequential to the activity. Improved activity was observed when the Asn2 was replaced with Ala and activity appeared slightly diminished at 10 μM when the Ser4 residue was replaced with Ala.

4.6.5 Peptide 5 non-specifically prevents protein adsorption to assay tube

Time course experiments were performed to determine the length of incubation between peptide and MazF<sub>sa</sub>(His)<sub>6</sub> required for activity enhancement. Unexpectedly, these experiments revealed that MazF<sub>sa</sub> activity diminished over the course of the experiment. The addition of BSA or CHAPS to the buffer solution prevented the loss of activity of 100 nM MazF<sub>sa</sub>; also, higher concentrations of MazF<sub>sa</sub> maintained activity for a longer period of time. The peptides were tested under these improved conditions and found to have no effect (Figure 4.17). Thus, peptides 5 and 6 did not enhance MazF<sub>sa</sub> when its activity remained stable throughout the experiment.

![Figure 4.17](image1)

Figure 4.17 Peptides were tested for their effect on MazF<sub>sa</sub> activity in the presence of (A) 0.01% BSA or (B) 0.1% CHAPS. 10 nM MazF<sub>sa</sub> was incubated for 1 hour in the absence (-) or presence (1-10) of 100 μM peptide prior to incubation with 100 nM RNA for 45 sec at room temperature. RNA was separated on a denaturing polyacrylamide gel and stained with ethidium bromide.

It was determined that in the absence of BSA or CHAPS, MazF<sub>sa</sub> adsorbed to the polypropylene microcentrifuge tube, causing the enzyme to lose activity over time, especially at the relatively low concentrations used in the peptide experiments (100 nM). Four unrelated His<sub>6</sub>-tagged proteins, MazF<sub>sa</sub>, MBNL, Procaspsae-3 and Thi4p were shown to adsorb to the tubes and peptide 5 prevented adsorption of each protein to a large degree (Figure 4.18). Thus, peptide 5 did not actually enhance MazF<sub>sa</sub> activity; rather it kept a high percentage of MazF<sub>sa</sub> in solution throughout the three hour incubation that was typically employed to test the activity of the peptides. Since no other peptide enhanced MazF<sub>sa</sub> activity under optimal conditions (Figure 4.17), the MazE<sub>sa</sub> peptide fragments were no longer pursued.
Peptide 5 prevents adsorption of proteins to tubes. Four unrelated His6-tagged proteins (each ~100 μM) were assessed for their time-dependent adsorption to polypropylene microcentrifuge tubes. Proteins were incubated in a tube for 5, 30, 60 or 180 min and at each time point, the solution (S) was transferred to another tube and SDS loading dye was added to keep the protein in solution. Addition of buffer containing SDS resolubilized the protein from the walls of the original tube (E). Proteins were visualized by α-His6 tag Western blot. In the absence of peptide (top row) the protein is nearly all adsorbed to the tube, whereas peptide 5 (bottom row) prevents some of the adsorption.

4.7 DETECTION OF MAZF<sub>SA</sub> ACTIVITY IN S. AUREUS CELL LYSATE

4.7.1 Development of a radiolabeled substrate for MazF<sub>SA</sub> activity

The next step for the assessment of the peptides was to test their effect on endogenous MazF<sub>SA</sub> in S. aureus cell lysate. Thus, a radiolabeled version of the single site substrate was prepared. Even though the peptides were no longer being tested, the detection of endogenous MazF<sub>SA</sub> activity was unprecedented but potentially had many important applications. Primarily, a sensitive assay allowing the specific detection of MazF<sub>SA</sub> activity could enable studies to determine the effect, if any, of MazF<sub>SA</sub> activity in response to stressors such as antibiotics.

As a prelude to experiments with endogenous MazF<sub>SA</sub> from S. aureus lysate, the activity of MazF<sub>SA</sub>(His)<sub>6</sub> was assessed with a <sup>32</sup>P-labeled version of the single site substrate in a time course experiment. As shown in Figure 4.19, the sensitivity of the assay allows for clear differentiation in the activity of increasing concentrations of MazF<sub>SA</sub> [41]. In this experiment, minimal cleavage is detected by 1 nM MazF<sub>SA</sub>(His)<sub>6</sub> after 11 min incubation, moderate processing is observed by 5 nM MazF<sub>SA</sub>(His)<sub>6</sub>, and significant processing by 10 nM MazF<sub>SA</sub>(His)<sub>6</sub> (Figure 4.19) [41].

![Radiometric assay for MazF<sub>SA</sub>](image)

**Figure 4.19** Radiometric assay for MazF<sub>SA</sub>. MazF<sub>SA</sub>(His)<sub>6</sub> was incubated at room temperature with <sup>32</sup>P- labeled single-site substrate (100 nM) and activity visualized by phosphorimaging.
4.7.2 Detection of endogenous MazF<sub>sa</sub> activity in <i>S. aureus</i> cell lysate

The highly sensitive and specific processing of the radiolabeled substrate allowed for detection of endogenous MazF<sub>sa</sub> activity in <i>S. aureus</i> cell lysate. Lysate was prepared from the <i>S. aureus</i> strain NRS26, in which the <i>mazEF</i><sub>sa</sub> genetic loci and transcripts were previously detected [39]. The radiolabeled substrate was added to the lysate 7, 15, 35 and 65 min post-lysis. As shown in Figure 4.20, cleavage of the RNA substrate increased with longer post-lysis incubation times [41]. This result suggests that MazE<sub>sa</sub> is susceptible to proteolysis in the lysate, releasing active MazF<sub>sa</sub>. Addition of recombinant (His)<sub>6</sub>MazE<sub>sa</sub> to lysate 65 min post-lysis inhibited MazF<sub>sa</sub> activity, demonstrating the specificity of the RNA cleavage by endogenous MazF<sub>sa</sub>. This marks the first assessment of the activity of endogenous MazF<sub>sa</sub> [41].

![Figure 4.20 Detection of endogenous MazF<sub>sa</sub> activity in S. aureus cell lysate. Radiolabeled RNA transcript (100 nM) was added to of S. aureus lysate at 7, 15, 35 or 65 min post-lysis and incubated at room temperature for 2, 5 or 10 min. Far Right: The endogenous MazF<sub>sa</sub> present in lysate 65 min. Controls of RNA only (R) and RNA processed in vitro by MazF<sub>sa</sub>(His)<sub>6</sub>(R+F) were also analyzed. post-lysis was inhibited by a 10 min incubation with (His)<sub>6</sub>MazE<sub>sa</sub> prior to reaction with radiolabeled RNA for 10 (65+E). Products were separated by gel electrophoresis and visualized by phosphorimaging.](image)

4.8 CONCLUSIONS

The enhancement of toxin activity by a peptide fragment based on the antitoxin is not an intuitive strategy, but one for which there is precedent. As discussed in Section 1.4.4.1, the <i>E. coli</i> EDF peptide NNWNN was demonstrated to both enhance the kinetic activity of <i>E. coli</i> MazF<sub>Ec</sub>, as well as allow MazF<sub>Ec</sub> to remain active in the presence of MazE<sub>Ec</sub> [3]. It seems counterintuitive that a peptide fragment that apparently competes for the most intimate interaction [3], between MazE<sub>Ec</sub> and MazF<sub>Ec</sub>, would not inhibit the ribonuclease activity of MazF<sub>Ec</sub>. However, this observation can be explained by analysis of the MazEF<sub>Ec</sub> crystal structure, which revealed that the binding sites for mRNA and MazE<sub>Ec</sub> overlap [22]. MazE actually occupies only one of the possible two MazE<sub>Ec</sub> binding sites within a MazF<sub>Ec</sub> homodimer; furthermore, only one of the mRNA binding sites is ever occupied by mRNA in a MazF<sub>Ec</sub> homodimer [22, 25]. Binding of MazE<sub>Ec</sub> to one site in the MazF<sub>Ec</sub> homodimer perturbs mRNA binding in the other site [20, 25]. It was proposed that EDF competitively inhibits the MazE<sub>Ec</sub>-MazF<sub>Ec</sub> interaction, while still allowing mRNA to bind in the second catalytic site [3, 20]. Additionally, the affinity of MazF<sub>Ec</sub> for mRNA is reduced in the presence of EDF, which likely explains the enhancement of MazF<sub>Ec</sub>.
ribonucleolytic activity [3, 20]. Although the EDF story remains controversial [18, 21, 36], it provides rationale for a provocative TA system-targeting strategy.

Without a crystal structure of the MazEF<sub>sa</sub> complex, it is difficult to speculate why none of the MazE<sub>sa</sub> peptide fragments enhanced MazF<sub>sa</sub> activity. There is a structure for the <i>B. subtilis</i> MazF homologue [17], which shares both high sequence homology with MazF<sub>sa</sub>, as shown in Figure 4.4 (<i>B. subtilis</i> and <i>B. anthracis</i> MazF proteins are identical), and structural similarities to <i>E. coli</i> MazF<sub>ec</sub> [17]. However, the mRNA binding sites of the Gram-positive MazF proteins have not been extensively characterized and so it remains unknown if there are dual MazE and substrate binding sites in these toxins, as in <i>E. coli</i> MazF<sub>ec</sub> [25]. Additionally, modeling studies suggested that the interaction between EDF (NNWNN) and <i>E. coli</i> MazF<sub>ec</sub> is mediated largely by the tryptophan residue that competes with Trp73 of MazE<sub>ec</sub> [3], which occupies a hydrophobic pocket of MazF<sub>ec</sub> forming the most intimate interaction between MazE<sub>ec</sub> and MazF<sub>ec</sub> [23]. MazE<sub>sa</sub> does not contain an analogous tryptophan residue. Additionally, the crystal structure of MazEF<sub>ec</sub> showed that the most extensive contact interface is mediated by conserved aromatic amino acids Tyr39, Tyr58 and Phe60 of MazF [23]; however, MazF<sub>sa</sub> does not have any analogous aromatic amino acids. A more in depth understanding of the interaction between MazE<sub>sa</sub> and MazF<sub>sa</sub> would be informative and highly useful. The difficulty with this project further highlights the need for additional crystal structures of TA systems, as discussed in Chapter 1.

Although the peptide fragment analysis did not facilitate the discovery of a toxin activator, it led to a set of key assays that have provided a tool to probe the effect of stress on toxin activation in the cell. In <i>S. aureus</i>, exposure to heat or subinhibitory concentrations of certain antibiotics results in increased transcription of the TA genes. However, the consequence of the transcriptional upregulation, if any, is unknown. The organization of TA genes ensures that the toxin is only transcribed in the context of the antitoxin and that less toxin than antitoxin will be translated, as discussed in Section 1.3.4. Indeed, the natural activation of the toxin is dependent on the specific proteolysis of the antitoxin [7]. In <i>S. aureus</i>, the ATP-dependent ClpPC protease degrades MazE<sub>sa</sub> [11] and is also upregulated in response to stress, namely, heat shock [13].

Many other type II TA systems have been implicated in stress management [16, 38]. <i>E. coli</i> RelBE is involved in the stringent response induced by amino acid starvation, inhibiting translation and causing bacteriostasis [7, 31]. MazEF in <i>E. coli</i> has been postulated to induce cell death under stressful conditions [2] or to modulate translation such that the cells become bacteriostatic until conditions improve [8, 31].
The location of the mazEFsa TA system in the sigB operon [14], transcriptional response to antibiotics and heat shock [10], and proteolytic regulation by a stress-induced protease [11] certainly implicates the role of MazFsa as a modulator of the stress response in S. aureus. However, an increase in MazFsa activity in response to stress has not been demonstrated. The sensitive and specific assay we have developed for the detection of endogenous MazFsa activity in S. aureus cell lysate will allow the effect of stressors on the activation of MazFsa to be unambiguously measured.

Toxin-antitoxin systems are an ingenious method to maintain a plasmid in a bacterial host. Although their role on the bacterial chromosome is unclear, at the biochemical Type II TA systems operate via the same mechanism whether plasmid or chromosomal, that is, a stable toxin protein is inhibited by a proteolytically labile (and co-transcribed) antitoxin protein. Important work has shown that the genes for TA systems are present in a variety of pathogenic bacteria [4, 19, 27, 28, 33, 39]. Fewer publications have reported on the detection of TA transcripts in such bacteria [19, 27, 39], and fewer still on the detection of the actual TA proteins [10, 14]. While the presence of the DNA, mRNA, and proteins is clearly informative, it does not tell the whole story, especially for TA systems. Specifically, transcription and translation does not mean that the protein toxin is enzymatically active, given the co-transcription and (presumably) co-translation of the proteic antitoxin.

Herein described is an assay that allows, for the first time, for the detection of MazFsa enzymatic activity from its endogenous source. The principles applied for the design of this substrate and this assay should be readily transferable to the study of other toxins from TA pairs, especially those that are ribonucleases. There are many questions about the roles of TA systems in basic bacteriology [21, 24, 26, 35, 36, 42], their function in response to stress [16, 38] and their potential as novel antibacterial targets [1, 9, 12, 30, 40]. The tools described herein for the direct detection of endogenous toxin activity should significantly facilitate the answering of those questions.

4.9 EXPERIMENTAL PROCEDURES

4.9.1 Materials

Primers and oligonucleotides were synthesized by IDT. Pepstatin A, leupeptin, aprotinin, phenylmethanesulfonyl fluoride (PMSF) and lysozyme were purchased from Sigma. Restriction enzymes, BSA, Low-Range ssRNA Ladder, RNase Inhibitor—Human Placenta, and E. coli strains DH5α and NiCo21 were purchased from New England Biolabs (NEB). Subcloning
Efficiency DH5α Chemically Competent *E. coli* was purchased from Invitrogen. IPTG and kanamycin were purchased from GoldBio. Lysing Matrix B was obtained from MP Biomedicals. \([\alpha-^{32}P-UTP]\) was purchased from Perkin-Elmer. Sypro RED protein stain was purchased from VWR.

4.9.2 Cloning

*E. coli* DH5α and NiCo21(DE3) were used for cloning and protein expression, respectively. The \(mazEF_{Sa}\) gene cassette was amplified by PCR using primers \(mazEF_{Sa}-NcoI-F\) (5’-ACACCCATGGATAATGGTTATCTTTTAGTCAAAATAG-3’) and \(mazEF_{Sa}-XhoI-R\) (5’-CACACTCGAGATTTTCTGGTGAGCTAC-3’) (Figure 4.21) from the total DNA of the MRSA strain C2 [39]. The amplicon was inserted into the corresponding restriction sites of pET-28a to create resulting in pET-28a-\(mazEF_{Sa}\), which encodes for MazEF\(_{Sa}\) containing a C-terminal histidine-6 tag on MazF\(_{Sa}\). The \(mazE_{Sa}\) antitoxin gene was amplified from the same strain using primers \(mazE_{Sa}-NdeI-F\) (5’-ACACCCATGGATAATGGTTATCTTTTAGTCAAAATAG-3’) and \(mazE_{Sa}-XhoI-stop-R\) (5’-ACACCTCGAGGTATCTTTTGTTAATTAGGAATTAGAA-3’) and cloned into pET-28a, resulting in pET-28a-\(mazE_{Sa}\), which encodes for an N-terminal histidine-6 tagged MazE\(_{Sa}\) (Figure 4.22). The correct sequence of all clones was confirmed by sequencing. *E. coli* carrying the recombinant plasmids was cultured in LB containing 50 µg/mL kanamycin.

4.9.3 Protein Expression and purification

To express MazEF\(_{Sa}\)(His)\(_{6}\), an overnight culture inoculated with a single colony of *E. coli* NiCo21 freshly transformed with pET-28a-\(mazEF_{Sa}\) was diluted 100-fold into 2 L of LB+Kan. The culture was grown at 37°C until the OD\(_{600}\) reached 0.6-0.8, at which point expression was induced with 1 mM IPTG (final concentration) for 4 hours at 37°C. Expression of (His)\(_{6}\)MazE\(_{Sa}\) was performed the same, except IPTG was added when the OD\(_{600}\) reached 0.4-0.6. The 2 L cultures were harvested by centrifugation at 4°C (8,000 g for 500 mL bottles and 10,000 g for 50 mL conical tubes) and stored at -20°C.

MazF\(_{Sa}\)(His)\(_{6}\) was purified from the complex under denaturing conditions. A pellet corresponding to 2 L culture was thawed in a room temperature water bath for 10 minutes. The pellet was resuspended in 20 mL binding buffer (10 mM Tris, pH 7.9, 500 mM NaCl, 10 mM imidazole) containing 8 M urea and cell lysis was achieved by 30 minutes inversion at room temperature. Cell debris was pelleted by centrifugation at 40,000 g at room temperature for 15 minutes. The clarified lysate was mixed with 1 mL 1:1 Ni-NTA resin slurry (Qiagen) and batch loaded for 30 min at room temperature with inversion. The slurry was applied to a gravimetric
flow column and the resin was washed with 50 mL binding buffer with 8M urea to fully disrupt the MazE<sub>Sa</sub>-MazF<sub>Sa</sub> complex. On-column refolding of MazF was achieved with seven washes of 10 mL urea/binding buffer decreasing the concentration of urea by 1 M with each wash. Wash steps containing more than 4 M urea were performed at room temperature; all subsequent wash steps were performed at 4°C. Refolding was followed with 10 mL washes of binding buffer (containing no urea) and binding buffer containing 60 mM imidazole. MazF<sub>Sa</sub>(His)<sub>6</sub> was eluted with 5 mL binding buffer containing 250 mM imidazole. To remove common contaminants retained on Ni-NTA resin, the eluate was diluted with 5 mL binding buffer and applied to a 5 mL bed volume of chitin resin (NEB) followed by washing with 8 mL binding buffer. The flow through and wash fractions were combined and concentrated to ~1 mL using an Amicon Ultra-15 3 kDa molecular-weight cutoff spin concentrator (Millipore) at 4°C. After overnight dialysis in PBS, pH 6.5, the MazF<sub>Sa</sub> purity and concentration was assessed by SDS-PAGE using 4-20% TGX Mini-Protean gels (Bio-Rad). Concentration was determined using densitometry of the SDS-PAGE and by BCA Assay (Pierce) using lysozyme (molecular weight 14.3 kDa) as the standard for both quantification assays.

(His)<sub>6</sub>MazE<sub>Sa</sub> was purified under native conditions and all steps were performed on ice or at 4°C. A pellet corresponding to 2L culture was thawed for 5-10 min and resuspended in cold binding buffer containing protease inhibitors (2 µg/mL pepstatin A, 1 µg/mL leupeptin, 1 µg/mL aprotinin and 1 mM PMSF). The cells were lysed by 5 min sonication with 1 s pulse at 50% amplitude. The lysate was cleared by 15 min centrifugation at 40,000 g at 4°C and the supernatant was batch loaded with 0.5 mL 1:1 Ni-NTA resin slurry (Qiagen) for 30 min at 4°C with inversion. Protease inhibitors were included in the wash and elution buffers. The resin was washed with 20 mL binding buffer, followed with 25 mL binding buffer containing 60 mM imidazole. (His)<sub>6</sub>MazE<sub>Sa</sub> was eluted with 5 mL binding buffer containing 250 mM imidazole, concentrated to ~0.5 mL and dialyzed against 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1 mM DTT. Purity and concentration were assessed using the same method described for MazF<sub>Sa</sub>(His)<sub>6</sub>.

4.9.4 HPLC analysis of oligonucleotide cleavage products

A 10 µL solution of 16 µM MazF<sub>Sa</sub>(His)<sub>6</sub> and 32 µM 5-AAGTCrUrACATCAG-3’ (“r” denotes RNA base) in 10 mM Tris pH 7.9, 500 mM imidazole, 20% glycerol was incubated overnight at 37°C. The reaction was mixed with 20 µL 0.1 M triethylammonium acetate (TEAA), pH 7.0, and 10 µL was analyzed by high performance liquid chromatography using an Alliance HPLC System (e2965 Separations Module, Waters) with detection at 260 nm (2489
UV/Visible Detector, Waters). The full length oligonucleotide was separated from the cleavage products on a YMCbasic S5 column (4.6 x 150 mm, 5 µm, Waters) with a linear gradient of 100 mM TEAA to 50 mM TEAA/50% acetonitrile over 25 min. Fractions were analyzed by MALDI mass spectrometry.

4.9.5 Fluorometric oligonucleotide cleavage assay

The fluorogenic substrate 5’-6FAM-AGTCrUrACATCAG-BHQ-3’ (6-FAM, 6-carboxyfluorescein, BHQ, black hole quencher; “r” denotes RNA base) was diluted in phosphate-citrate (PC) buffer (71 mM phosphate, 14.5 mM citrate, pH 6.5). Wells of a 384-well sterile black tissue-culture plate (ThermoFisher) were filled with 15 µL of each dilution (0.025-4 µM final concentration). After substrate equilibrated for 30 min at room temperature, 15 µL of MazF<sub>sa</sub>(His)<sub>6</sub> was added to the wells containing intact substrate. The fluorescence of the plate was measured every 30 s for 45 min using a Criterion Analyst AD (Molecular Devices) with 485 ± 15 nm excitation and 530 ± 15 nm emission filters and a 505 nm cutoff dichroic mirror. The fluorophore was excited with a 1000 W continuous lamp with 10 reads per well. The Z-height was set to 1 nm.

A calibration curve of the independently synthesized substrate halves corresponding to the cleavage products, 5’-6-FAM-AGTCG and ACATCAG-BHQ-3’ [37], was constructed to quantify the amount of cleavage product formed based on the RFU value. Wells containing 0.0625-2 µM of the cleavage fragments were prepared alongside the intact oligonucleotide following the same protocol, except PC buffer was added instead of MazF<sub>sa</sub>(His)<sub>6</sub>. The calibration curve was constructed by plotting the average RFU measured over the 45 min experiment against amount of oligonucleotide in the well.

4.9.6 In vitro transcription of RNA substrate

Quickchange site-directed mutagenesis was performed to modify the MazF<sub>sa</sub> cleavage sites in pET200-mazEF recombinant plasmid pKm6EF [37]. MazF<sub>sa</sub> optimal cleavage site 1 was removed by changing TACAT to GG CAT using the following primers Site1-QC-F (5’-GTTTAACTTTAAGAAGGAGATA GG CATATGCGGGTTCTCATC ATC and Site1-QC-R GATGATGAGAACCCCGCATATG GCCGGGATCC-3’), suboptimal cleavage site 2 was removed by changing TACGT to CACAT using Site2-QC-F (5’-GGATCCGGCCACGTATGCAATGA GGATCC-3’) and Site2-QC-R (5’-GATTGAGCGCCTGCATATACGTG GCGG ATCC-3’) and the suboptimal cleavage site 3 was optimized by changing from TACGT to TACAT using Site3-QC-F (5’-
GGTAATGGTAAGCCGATAC and Site3-QC-R (5’-CCATATCGGGTATGTATCGGCTTACCATTACC-3’). The quickchange PCR was performed following the guidelines in the QuikChange manual (Stratagene) with annealing temperatures of 58°C for Site1 and Site2 PCRs and 61°C for Site 3 PCR. The PCR product was purified and digested with DpnI and transformed into Sub-cloning Efficiency CaCl2-treated E. coli following the manufacturer’s instructions except the cells were heat shocked for 30 s. The transformation was plated on LB containing 50 µg/mL kanamycin. Each mutation was confirmed by sequencing of both strands. The plasmid containing the optimized substrate is referred to as pET200-mazEF-1Δ2Δ3opt.

To prepare the RNA substrate, the pET200-1Δ2Δ3opt plasmid was digested with HindIII for 3 hours at 37°C. The digested plasmid was purified using the QiaPrep Spin Column (Qiagen) and eluted with 30 µL DEPC-treated water. The digested plasmid (0.5-1 µg) served as the template for in vitro transcription using the T7 High Yield RNA Synthesis Kit (NEB) according to the manufacturer’s instructions. Each in vitro transcription reaction was divided into two equal portions and purified according to the RNeasy Mini handbook (Qiagen) RNA Cleanup protocol using reagents from the Total RNA Kit I (Omega Bio-Tek). RNA was eluted twice with 30 µL 0.1 mM EDTA prepared in DEPC-treated water and all elution fractions from both columns were pooled, quantified by A260 and checked for integrity by gel electrophoresis on a 5% acrylamide 8M urea 1X TBE gel post-stained for 15 min with 0.05 µg/mL ethidium bromide.

4.9.7 MazF<sub>sa</sub> kinetic assays in the presence of peptide

For the peptide initial screen, 0.8 µM MazF<sub>sa</sub> was incubated alone or with 60 µM each peptide for 3 hours at room temperature prior to addition of 0.25 µM fluorogenic substrate. Fluorescence was monitored for 80 minutes and the rate of cleavage was determined by the increase in fluorescence over time.

The effect of peptide 6 on MazF<sub>sa</sub> and RNaseA activity was determined by incubating 0.8 µM MazF<sub>sa</sub> (final concentration) with treated with 0, 5, 25 or 100 µM peptide 6 for 3 hours prior to addition of 0.05, 0.25 or 2 µM fluorogenic substrate. Fluorescence was monitored for 1 hour.

The effect of peptide 6 on MazF<sub>sa</sub> kinetics was determined by treating 0.8 µM MazF<sub>sa</sub> with 100 µM peptide 6 for 3 hours prior to adding the fluorogenic substrate at 0.025-4 µM. Fluorescence was monitored for 1 hour and the relative $V_{max}$, $K_M$ and $k_{cat}/K_M$ values were derived from the Lineweaver-Burk plot of the kinetic.
4.9.8 Effect of peptide 6 on RNase A activity in fluorogenic assay

RNaseA was diluted to $3 \times 10^{-7}$ U/mL, a concentration that resulted in a similar initial velocity as 0.8 μM MazF<sub>Sa</sub> when incubated with 2 μM fluorogenic substrate, and treated with 0, 25 or 100 μM peptide 6 for 3 hours prior to addition of 2 μM fluorogenic substrate. Fluorescence was monitored for 1 hour and the initial velocity was plotted.

4.9.9 Effect of peptide on MazF<sub>Sa</sub> activity in gel-based assay

To screen the peptides in the gel-based assay, 100 nM MazF<sub>Sa</sub> was incubated alone or in the presence of 100 μM each peptide for 3 hours prior to reaction with 100 nM RNA (single-site substrate) for 1 min at room temperature.

For the dose-response experiments, peptides 5, 6, LNLSL or NNWNN were tested from 0-200 μM with 100 nM MazF<sub>Sa</sub>. After 3 hours incubation, 100 nM of the single site substrate was added and incubated for 1 min at room temperature. Peptides 5 and 6 were also incubated at 100 μM with 100 nM RNA in the absence of MazF<sub>Sa</sub>

For the time-course assay 200 nM MazF<sub>Sa</sub> was incubated in the absence of (-) or presence of 100 μM peptide 5 or 6, followed by incubation with 100 nM of the 3-site RNA substrate for 0-8 min at 37°C.

To test the peptide 5, 6 and LNLSLAN derivatives, the peptides were incubated at 10 or 20 μM for 3 hours with 100 nM MazF<sub>Sa</sub> prior to addition of 100 nM RNA and incubation at room temperature for 1 min.

To observe increased MazF<sub>Sa</sub> activity in the gel-based assay, the concentration of MazF<sub>Sa</sub> was increased to 200 nM and the peptides were tested at 10 μM. This mixture was reacted with 100 nM RNA for 1 min at room temperature.

4.9.10 Detection of protein adsorption to tube

Pure solutions of three His-tagged proteins, MBNL, Procaspease-3 and Thi4p (obtained from lab members) were tested alongside MazF<sub>Sa</sub>(His)<sub>6</sub> at 100 μM for adsorption to the microcentrifuge tube. A 25 μL solution was aliquoted to 4 tubes and after 5, 30, 60 or 180 min, the solution was transferred to another tube. This solution is referred to as “S” to denote the “soluble” fraction, which was kept soluble by the addition of 5 μL of Laemmeli SDS loading dye. The protein adsorbed to the original tube was resolubilized using 5% SDS. “E” denotes the “eluted” fraction. This assay was performed in this presence and absence of 100 μM peptide 5. The protein solutions were detected by α-His Western blot.
4.9.11 Effect of peptides on MazF<sub>Sa</sub> activity in the presence of BSA or CHAPS

The addition of 0.01% BSA or 0.1% CHAPS to 10 nM MazF<sub>Sa</sub> allowed maintained the activity of MazF<sub>Sa</sub> over at least a 3 hour period (data not shown). Following incubation of 10 μM MazF<sub>Sa</sub> for 1 hour in the absence or presence of 100 μM each of the MazE<sub>Sa</sub> peptide fragments 1-10, 100 nM single-site RNA was incubated for 45 sec at room temperature.

4.9.12 Synthesis of radiolabeled RNA transcript

Synthesis and purification of the radiolabeled RNA substrate followed the same protocol as for the unlabeled transcript, except that 3.87 μCi of [α-<sup>32</sup>P]-UTP was added in addition to the standard amounts of unlabeled NTPs.

4.9.13 Gel-based RNA cleavage assay

To assess the cleavage of the 3-site and 1-site substrates, 50 nM MazF<sub>Sa</sub>(His)<sub>6</sub> was incubated with 50 nM RNA in buffer (71 mM sodium phosphate, 14.5 mM citrate, 137 mM NaCl, 2 mM KCl, pH 6.5) at room temperature. To stop the reaction, a 10 μL aliquot was added to 10 μL SDS-formamide loading dye (87% formamide, 2.75% SDS, 16 mM EDTA, 0.025% bromophenol blue). Samples were heated to 95°C for at least 5 min prior to analysis on a 3.5% acrylamide 8 M urea 1X TBE gel electrophoresed in 1X TBE running buffer, pH 8.3 and post-stained in 0.05 μg/mL ethidium bromide.

For the gel-based RNA cleavage time course assay, the same conditions were followed, except 1 nM MazF<sub>Sa</sub>(His)<sub>6</sub> and 25 nM RNA was prepared in assay buffer containing 0.01% BSA and the products were separated on a 3.5% acrylamide 8 M urea 1X TBE gel.

Activity against the radiolabeled substrate was assessed following the same conditions, except 1, 5 or 10 nM MazF<sub>Sa</sub>(His)<sub>6</sub> was mixed with 100 nM radiolabeled RNA substrate in assay buffer containing 0.05% BSA. The products were separated on 5% acrylamide denaturing gels which were exposed to an Imaging Screen-K (BioRad) for 10 min prior to visualization.

4.9.14 Detection of MazF<sub>Sa</sub> activity in <i>S. aureus</i> cell lysate

A single colony of MRSA strain NRS26 grown on tryptic-soy agar was inoculated into 10 mL tryptic-soy broth (TSB) and grown overnight at 37°C with aeration. A fresh 22 mL culture was seeded with a 1:100 dilution of the overnight culture and grown to an OD<sub>600</sub> of ~1.3 at which point 10 mL portions were harvested by centrifugation at 4°C, flash frozen in liquid nitrogen and stored at -80°C. The pellet was resuspended in 0.3 mL assay buffer and lysed by vortexing for 4 min with 0.15 g Lysing Matrix B silica beads (MP Biomedical). The lysate was
cleared by 30 s full speed centrifugation at room temperature and mixed with RNase inhibitor. After 7, 15, 35, and 65 min post-lysis, the radiolabeled RNA substrate was added to 100 nM (final concentration) and the samples were heated at 95°C for 1 min. After 2, 5 or 10 min reaction at room temperature with the RNA, 10 µL aliquots were quenched with SDS-formamide loading dye, electrophoresed and imaged as described above. To inhibit endogenous MazF<sub>Sa</sub> activity in the most active lysate, 200 nM purified (His)<sub>6</sub>MazE<sub>Sa</sub> was incubated with lysate 65 min post-lysis for 10 min prior to addition of RNA for a 10 min reaction. For negative and positive controls, 100 nM radiolabeled RNA was incubated without or with 10 nM MazF<sub>Sa</sub>(His)<sub>6</sub> in assay buffer with 0.01% BSA at 95°C for 1 min followed by room temperature for 1 min.

4.10 FIGURES

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**Figure 4.21** Nucleotide sequence of clone pET-28a-mazEF<sub>Sa</sub>(his)<sub>6</sub>. The MazEF<sub>Sa</sub> sequence was cloned into pET-28a between the NcoI and XhoI restriction enzyme sites to facilitate expression of a C-terminal His<sub>6</sub>-tag on MazF<sub>Sa</sub>.

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**Figure 4.22** Nucleotide sequence of clone pET-28a-(his)<sub>6</sub>mazE<sub>Sa</sub>. The MazE<sub>Sa</sub> sequence was cloned into pET-28a between the NdeI and XhoI restriction enzyme sites to facilitate expression of an N-terminal His<sub>6</sub>-tag on MazE<sub>Sa</sub>.
4.11 REFERENCES


CHAPTER 5
CONCLUSIONS AND FUTURE OUTLOOK FOR TARGETING TOXIN-ANTITOXIN SYSTEMS

5.1 CONCLUSIONS

The occurrence of drug-resistant bacterial infections continues to rise at an alarming rate and remains a world health crisis. The inability to effectively treat bacterial infections results in increased morbidity, mortality and healthcare costs. As discussed in Chapter 1, the majority of antibiotics used in the clinic exploit one of a handful of biological targets, including DNA gyrase, the ribosome, or macromolecules that make up the cell wall. Many of these antibiotics are derivatives of existing scaffolds that were discovered during the “Golden Age” of antibiotics. Thus, there is a true need for drugs with novel targets and mechanisms of action in the cell.

One intriguing novel antibacterial strategy is the exploitation of proteic toxin-antitoxin (TA) systems. The activation of TA systems as an antibacterial strategy was first proposed in 2004 [16] and has become a highly discussed topic in the field [3, 12, 19, 30, 33, 37, 48, 49]. Success of this strategy remains elusive, likely due to the inherent challenges associated with targeting TA systems. Chapter 5 provides insights into specific challenges experienced with the TA targeting strategy and evaluates factors to consider when planning experiments designed to target TA systems as an antibacterial strategy. Table 5.1 summarizes the general points discussed including the advantages and disadvantages of: (i) performing in vitro versus cell-based high-throughput screens, (ii) targeting ribosome-independent versus ribosome-dependent ribonucleases and, (iii) targeting the TA complex, antitoxin, or toxin protein.

Perhaps the most significant challenge associated with targeting TA systems is the incredibly tight interaction between the toxin and antitoxin proteins. The extensive electrostatic interactions between the toxin and antitoxin keep the toxin inactive in the complex. In the laboratory, there appears to be little to no dissociation between the antitoxin and toxin [35, 45]. In the cell, specific proteases that degrade the antitoxin are required for full activation of the toxin by freeing the toxin from the inactive complex. As discussed in Chapter 1, the antitoxin is more susceptible to proteolysis than the toxin [2, 5, 10, 13], and free antitoxin is degraded at a faster rate than toxin-bound antitoxin [6, 34, 44].

One major disadvantage of the two high-throughput screens reported in this thesis was the fact that they were performed in vitro and thus did not take advantage of the proteases that degrade the antitoxin. A molecule capable of disrupting the TA complex in vitro would require
| Screening Assay: | in vitro | + | simple, robust assays less likely to yield false positives | + | rapid secondary assays validate hits or false positives | + | hit compound is likely to have same effect on TA proteins in cell | - | protein expression and purification may be difficult | - | requires robust, high-throughput assay; enzymatic assay is ideal | - | one step away from ultimate goal of cell death |
| | in cell | + | pathways up- or down-stream of TA system are intact | + | antitoxin-specific proteolysis and turnover | + | enables assessment of TA system without protein purification | + | most direct assay to assess end goal of cell death | - | many potential off target effects of compounds | - | hit compound may have no effect on TA proteins in vitro, complicating MOA studies |
| Ribonuclease Selection: | ribosome-independent | + | rapid, straightforward enzymatic assays are available | + | toxin purification, refolding and activity validation are feasible | + | full disruption not necessarily required for toxin activation | - | toxin activity may not be sufficiently toxic to kill cell |
| | ribosome-dependent | + | toxin activity appears to be highly toxic to cell | + | toxin purification, refolding, and activity validation may be challenging | + | rapid, high-throughput activity assays are lacking | - | high-throughput disruption assay is two steps away from ultimate goal of cell death | - | full disruption required for toxin activation |
| Target Protein Selection for in vitro Assays: | TA complex | + | more faithfully mimics natural state of proteins in cell | + | complex is straightforward to express and purify | + | assessment of complex disruption or toxin activation is feasible | - | incredibly tight interaction |
| | antitoxin | + | binding the antitoxin would likely result in toxin activation | + | generally straightforward to express and purify antitoxin | + | excess of antitoxin over toxin in cell would require higher compound concentration | - | inherently unfolded nature of antitoxin complicates in vitro assays |
| | toxin | + | tertiary structure facilitates discovery of a compound that binds tightly | + | enhancement of toxin enzymatic activity can be assessed | + | binding to toxin may inhibit its activity | - | expression and purification may be challenging | - | high-throughput assays do not exist for every toxin |
an incredibly high affinity to compete for binding to either protein. However, in the cell, a molecule that bound to either TA protein with a low affinity could be effective by reducing the affinity between the toxin and antitoxin, resulting in increased degradation of the antitoxin and releasing the toxin. Furthermore, the constant turnover of the antitoxin from the complex would provide more opportunities for the small molecule to bind to either the toxin or antitoxin.

Although a cell-based screen for the discovery of toxin activators seems more ideal than an in vitro screen, two cell based screens performed by other members of the Hergenrother lab failed to identify any molecules that induced TA-system dependent death. These screens provided more “hits” that appeared to cause TA-dependent death; however, all the compounds were ruled out as false positives after performing many rigorous secondary assays [30, M. Rodriguez, personal communication]. This highlights one advantage of working in an in vitro system: the results of the assays are generally more straightforward to interpret than cell-based assays. In the high throughput screens discussed in Chapters 3 and Appendix B, elimination of the “hit” compounds as false positives was achieved relatively early, before spending valuable time characterizing the effect of the compounds. The value of straightforward, meaningful secondary assays cannot be underestimated in high-throughput screening endeavors.

Certainly, performing a cell-based screen and following up with meaningful secondary assays in vitro seems ideal; however, only molecules that act directly on the TA system proteins would be validated in this experimental set-up. Small molecules identified in a cell-based screen could work by many other mechanisms, including modulating the transcription of the TA systems, activating proteases, or activating some physiological response mechanism that naturally activates the TA system. All of these pathways could potentially be equally successful in the end goal of activating the toxin, but would be overlooked due to the challenge of accurately recapitulating complex biological systems in vitro.

Furthermore, the in vitro follow up assays require use of straightforward and potentially high-throughput enzymatic assays. Such assays are available to assess the CcdB-mediated gyrase inhibition using the a gel-based assay with supercoiled plasmid DNA and to assess the cleavage of free mRNA using the fluorogenic or gel-based assays described in Chapter 4 and Appendix B. However, evaluation of ribosome-dependent ribonucleases such as RelE requires reconstituting an actively-translating ribosome. Although these complex systems are commercially available, they are potentially cost-prohibitive for many laboratories.

An additional requirement for in vitro assays is the need for pure and active toxin protein. This has posed a major challenge in some endeavors to study TA systems. Difficulty was experienced in expression of recombinant RelE alone, even enzymatically inactive mutants,
perhaps due to some residual binding of RelE in the ribosomal A site. Although the toxicity can be overcome by expression of the RelBE complex, and pure toxin can be obtained by denaturing purification, proper refolding of denatured RelE also proved difficult. In contrast, pure MazF<sub>Ec</sub> and MazF<sub>Sa</sub> were readily obtained by denaturing purification from their respective complexes, and the facile <i>in vitro</i> assays to assess their activity confirmed their properly folded and active state.

Other members of the Hergenrother lab have experienced similar difficulties with obtaining pure and active toxin proteins. One clever solution to this problem employed expression of a non-toxic form of the toxin YoeB, made inactive by replacement of an active-site tyrosine residue with a non-natural photocaged tyrosine derivative [29]. Exposure of the protein to light released the photocage, restoring the activity to the toxin [29]. This creative solution could be applied to other toxins, but also requires knowledge of the active site residues and the proper system to introduce the appropriate photocaged amino acids. However, this type of advancement is a key step in the progress towards employing a variety of TA systems whose toxicity could be exploited as an antibacterial target.

The difficulty of working with certain proteins <i>in vitro</i> emphasizes another advantage of cell-based screens, which enables the probing of TA systems for which there are not robust, high-throughput assays available. The fluorogenic kinetic assay developed in the Hergenrother lab [46] and described in Chapter 4 [50] and Appendix B is suitable for toxins that exhibit ribonuclease activity independent of the ribosome, but no analogous high-throughput assays have been described for ribosome-dependent toxins, gyrase inhibitors, or toxins that target cell wall biosynthesis. Thus there is a need to expand our repertoire of facile, straightforward, robust assays that can be applied to high-throughput screens.

The development of the photonic crystal (PC) biosensor technology [4, 24] was certainly significant to the studies with RelBE, as discussed in Chapter 3. Prior to its development, a method to screen a small molecule library for the disruption of a protein complex for which there is no enzymatic assay did not exist. The modulation of the PC biosensor technology to a high-throughput platform facilitated rapid assessment of 170,000 compounds for RelBE disruption as well as investigation of a number of peptides for their ability to bind RelB. This technology allowed for expediting the process of working through experiments and applying the knowledge towards the next strategy.

The PC biosensor experiments involving RelB were expected to reveal a peptide, either based on a RelE fragment or discovered from phage display, which could bind RelB. Although it seemed clear that interactions between RelB and the RelE fragments were indeed observed, it was
later determined by SPR that the interactions were likely nonspecific and possibly due to aggregation of the peptide. The phage recovered from biopanning also appeared to interact with RelB in a manner that was dependent on the specific displayed peptide, suggesting a specific interaction; however, the “off-phage” peptides suffered from low affinity to RelB. The inability to identify any peptides with high affinity for RelB may be due to the unstructured nature of RelB, which makes antitoxins less suitable targets for in vitro studies.

The MazF_{Sa} peptide fragments were never tested for their affinity to MazF_{Sa}, but rather for their ability to modulate the activity of MazF_{Sa}. Although some peptides appeared to enhance MazF_{Sa}, their effect was nonspecific. It was surprising that none of these peptides appeared to inhibit the activity of MazF_{Sa}, as other short antitoxin peptide fragments have been shown to inhibit their cognate toxin [1, 7]. Given the structure of the MazEF_{Ec} complex (shown in Figure 4.2), it seems likely that one of the peptides would have some effect on MazF_{Sa} activity. However, it remains unknown if the peptides tested would actually bind MazF_{Sa} or were simply tested under conditions that precluded binding. The effect of the peptides on MazEF_{Sa} complex was not pursued extensively, due in part to the fact that they did not seem to interact with MazF_{Sa} in any measurable way. Given the assays established for measuring MazF_{Sa} activity, this is a potential avenue for future studies, but would require investigation into conditions that are more suitable for peptide binding.

The feasibility of working with the MazEF_{Ec} or MazEF_{Sa} TA systems underlines their importance in establishing a blueprint for the identification of artificial toxin activators. The antitoxin and toxin proteins can be purified in their active forms. Their activity can be demonstrated in a variety of assays, such as the continuous fluorometric assay, the definitive HPLC assay and the gel-based assay employing a more physiologically relevant transcript substrate. Development and optimization of these assays have contributed to the progress towards identifying activators of TA system toxins, both synthetic activators for the purpose of an antibacterial strategy, and natural activators that are involved in the stress response.

The gel-based radiometric assay designed for the detection of endogenous MazF_{Sa} activity in S. aureus lysate could allow for the assessment of compounds or conditions that activate MazF_{Sa}, or similar ribonucleases, in the cell [50]. To the best of our knowledge, every study performed on TA systems in the cell has relied on ectopically overexpressed toxin or TA complex. Although this has enabled a wealth of information regarding the biological effects of TA systems, there is a gap in the understanding of what TA systems do in their natural context. Testing a variety of stressful conditions in this assay may reveal a natural mechanism for the activation of the endogenous TA system, and that condition can be applied to assess the effect of
such endogenous toxin activation. This may help to answer a long-standing question: are the levels of endogenously-expressed artificially-activated toxin great enough to cause growth inhibition or cell death?

The role of TA systems as plasmid addiction systems functioning by a post-segregational killing mechanism indicates that, at least when plasmid-encoded, sufficient toxin is present to kill the cell. The post-segregational nature of the killing suggests that half of the toxin molecules in any given cell are sufficient to cause cell death. Although their active role in plasmid maintenance suggests that plasmid-encoded TA proteins may be expressed to higher levels, the relative expression levels between plasmid- and chromosomally-encoded TA systems has not been studied, to our knowledge.

Thus, answering the long-standing question of toxin levels required to kill the cell may reveal which TA system is in fact the most suitable for an antibacterial strategy. Although there are many factors to consider when deciding which TA system to target, certainly the ability to activate the endogenous toxin and observe a significant toxic effect due to the activation is paramount. Indeed, the strategy of artificial activation of the toxin is inspired by the evident toxic effect of plasmid-encoded TA systems, thus it seems obvious to target plasmid-encoded TA systems. However, the heterogeneity of plasmids in important pathogens and of TA systems on plasmids, as demonstrated by the survey of 75 VRE isolates [32], suggests that plasmid-encoded TA systems may not be a reliable target. The conservation of many chromosomally-encoded TA systems, as demonstrated in 78 MRSA and 42 P. aeruginosa isolates [29, 47] lends support for targeting of these TA systems.

Certainly, the prevalence of the target is the most important factor when considering which TA system to target. Determining that $mazEF_{Sa}$, $higBA_{Pa}$, and $relBE_{Pa}$ TA systems were ubiquitous in their respective hosts was satisfies at least the first requirement for targeting these systems for antibacterial development [47]. Furthermore, discovering that the $parDE_{Pa}$ and $relBE-1_{Ab}$ TA systems were not present in all clinical isolates tested rules these TA systems out as potential targets [47].

Once ubiquity of a TA system has been established, the success of the strategy in the clinic is primarily dependent on the toxicity of the toxin protein. Some toxin proteins certainly seem more toxic than others, as evidenced by the inability to recover a recombinant clone of a vector containing the toxin gene or the inability to express the toxin on its own. In the Hergenrother lab, the toxins that have given the most trouble are RelE, Txe [31] and YoeB-Sa1 [29]. In contrast, wild-type MazF$_{Sa}$ can be ectopically overexpressed on its own [13, 18, 52]. This highlights the potential difference in effects based on toxin activity: RelE, Txe and YoeB-
Sal are all ribosome-dependent ribonucleases, which typically recognize conserved features of mRNA, such as start and stop codons [20, 38], whereas MazFsa is a ribosome-independent ribonuclease that cleaves at a five-nucleotide sequence, UACAU [52]. Indeed, it seems that the effect of ectopic overexpression of MazFsa on the cell is not as detrimental as that of RelE, Txe or YoeB-Sal [8, 9, 13, 18, 20, 29, 38, 52]. The differential toxicity of the toxins must be considered for the widespread, reliable application of this antibacterial strategy.

However, it has been shown that the UACAU motif is overabundant in some transcripts encoding virulence factors [52], thus the activation of MazFsa may better serve as an anti-virulence strategy as opposed to an antibacterial strategy. Many toxins have been shown to cleave at more specific sequences [36, 39, 42, 43, 52], thus possibly implicating their role in a more specific process than other ribonucleases which cleave at more sequences, such as MazFec, which cleaves at ACA [51], and has been implicated in numerous cellular pathways [2, 11, 14, 15, 21-23, 25-28, 40, 41]. Furthermore, ectopic overexpression of MazFec was shown to cause a 4-log reduction in colony forming units (CFU)/mL in 1 hour [2], whereas ectopic overexpression of MazFsa reduced the CFU/mL by only 2 orders of magnitude [17], suggesting that MazFec is more toxic than MazFsa.

Determining the mode of action of the toxins is crucial to their development and application as antibacterial or antivirulence targets. Additionally, detailed understanding of the interactions between the toxin and antitoxin, as determined by crystal structures or peptide mapping, may inform the design of, or provide insight into the types of, molecules that could modulate the interaction. Testing these molecules using robust, unambiguous assays that use the TA system complex would likely provide the most useful results. Specifically, assays that test for toxin-mediated cell death, toxin activation from the complex, or disruption of the toxin-antitoxin interaction more directly probe the feasibility of the strategy. Although there is no clear blueprint to discover toxin activators, the work presented herein highlights major considerations for the strategy and will hopefully serve as a guide for future endeavors.

5.2 REFERENCES


136


APPENDIX A
SEQUENCE ANALYSIS OF PLASMID PS177 ISOLATED FROM VANCOMYCIN-RESISTANT ENTEROCOCCUS FAECIUM STRAIN S177


A.1 INTRODUCTION

Enterococci such as Enterococcus faecium and E. faecalis are frequently implicated in nosocomial infections and are responsible for a large number of surgical site infections, bloodstream and urinary tract infections. Despite their intrinsic resistance to cephalosporin and aminoglycoside antibiotics, enterococcal infections were once considered easily treatable. However, acquisition of mobile genetic elements, such as plasmids, conferring resistance to multiple antibiotics, particularly vancomycin, has severely limited treatment options. Of over 7000 enterococcal bloodstream infections tested, approximately 28% were vancomycin resistant [2] and another survey revealed that up to 33% of the enterococcal isolates in hospitals were resistant to vancomycin [10]. Of bloodstream infections caused by E. faecium, the rate of vancomycin resistance increased from 57% in 2000 to 81% in 2010 [5]. Furthermore, 76% of 597 E. faecium isolates in North America were vancomycin resistant [12]. Thus, vancomycin, which was once considered a last resort antibiotic, is no longer a reliable treatment option. To evaluate the tractability of TA systems as an antibiotic target in VRE, the prevalence of TA system was determined [11].

The genes for TA systems were found to be ubiquitous on plasmids from 75 VRE strains studied [11]. It was shown that 56 of the 75 VRE isolates contained the genes for axe-txe, as determined by PCR analysis [11]. Of those 56 axe-txe-positive isolates, gel extractions and conjugative matings suggested a physical linkage between the vancomycin resistance genes and the axe-txe genes in 44 isolates [11]. Axe-Txe was originally discovered on the 24.8 kb nonconjugative plasmid pRUM and was one of the first proteic TA systems identified in Gram-positive bacteria [6]. pRUM was isolated from a multi-drug resistant E. faecium isolate and confers resistance to chloramphenicol, erythromycin, streptomycin and streptothricin. Interestingly, a 60 kb conjugative vancomycin resistance plasmid coexisted in the same E. faecium strain [6].

To further investigate the PCR results suggesting a physical linkage between axe-txe and the vanA resistance gene [11], we determined the DNA sequence of one plasmid, pS177, which
was positive by PCR for both *axe-txe* and *vanA*. Plasmid pS177 is a 39 kb non-conjugative plasmid isolated from the vancomycin-resistant *E. faecium* clinical isolate S177. pS177 confers resistance to kanamycin, streptothricin, streptomycin, erythromycin, and vancomycin and harbors the genes for *axe-txe* and a *relBE* homolog, *relBE*<sub>Ef</sub>.

A.2 SEQUENCE ANALYSIS OF pS177

The nucleotide sequence of plasmid pS177 was determined by shotgun cloning and sequencing, with average coverage of 12.46. pS177 is a 39,032 bp non-conjugative plasmid isolated from *E. faecium* clinical strain S177. Its DNA G+C content is 35.5%, consistent with that of other enterococcal plasmids and genomes [17]. Nucleotide BLAST analysis of pS177 revealed that 37,358 bp (95.7%) shared significant (99–100%) similarity with sequences
deposited in the GenBank database. Forty ORFs were identified and annotated based on these known sequences. For ORFs annotated in the database as hypothetical proteins, BLASTP searches were performed to assign a putative function for that potential protein based on amino acid similarity. pS177 has a mosaic structure comprising a pRUM backbone, two resistance gene cassettes and five insertion elements (Figure A.1, Table A.1). Interestingly, pS177 has 12 748 bp (32.6 %) and 18 930 bp (48.4 %) in common with plasmids p5753cA (GenBank accession no. GQ900435) and p5753cB (GenBank accession no. GQ900487), respectively, both isolated from E. faecium.

Plasmid pS177 shares 12 341 bp (31.6 %) of its sequence with pRUM and contains 75% of the total pRUM plasmid (GenBank accession no. AF50797). It harbors a pRUM-like replicon consisting of the putative RepA replication protein observed in various enterococcal plasmids [6, 13]. In addition to genes encoding stability mechanisms such as plasmid replication and partitioning, pS177 carries the TA system axe–txe [6]. The axe–txe genes and upstream promoter region share 100% homology with the pRUM sequence, suggesting that this TA system is functional. Plasmid pS177 harbors a gene cassette from Staphylococcus intermedius (GenBank accession no. AF299292), which confers resistance to streptothricin, streptomycin, kanamycin and erythromycin [3]. pRUM carries a nearly identical gene cassette in which the aphA-3 gene encoding kanamycin resistance is truncated [6]. The full-length S. intermedius resistance gene cassette is also carried on the enterococcal plasmids pRE25 [15] and p5753cB.

Additionally, vancomycin resistance is encoded by the VanA-type glycopeptide resistance determinant Tn1546, observed on many plasmids identified in VRE, including plasmids pVEF3 [16], 2008), p5753cA and plIP816 (GenBank accession no. AM932524). However, the Tn1546 cassette on pS177 contains the insertion element IS1251 between vanS and vanH. This unusual vanA cluster type has been observed previously [4, 8, 9] and is also present on the enterococcal plasmid p5753cA, which carries a truncated vanA-type gene cassette with IS1251 in the same position as observed in pS177.

Plasmid pS177 shares 12,748 bp (32.6 %) with p5753cA, including the putative RelBE TA system homologue from E. faecium, which we designate relBE_{E.f}. The ORFs encoding RelB_{E.f} and RelE_{E.f} were identified based on their homology with p5753cA, in which their putative gene products were annotated as hypothetical proteins. A BLASTP search of the amino acids encoded by relE_{E.f} showed up to 100% identity with a variety of E. faecium hypothetical proteins, as well
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<tr>
<td>6c</td>
<td>tse</td>
<td>4963</td>
<td>5220</td>
<td>pRUM Tse, toxin of TA system (AAO52832)</td>
<td>86%</td>
</tr>
<tr>
<td>7c</td>
<td>axe</td>
<td>5213</td>
<td>5482</td>
<td>pRUM Axe, antitoxin of TA system (AAO52833)</td>
<td>90%</td>
</tr>
<tr>
<td>8</td>
<td>orf8</td>
<td>5824</td>
<td>6126</td>
<td>pRUM conserved hypothetical protein (AAO52834)</td>
<td>101%</td>
</tr>
<tr>
<td>9</td>
<td>orf9</td>
<td>6169</td>
<td>6387</td>
<td>pRUM conserved hypothetical protein (AAO52835)</td>
<td>73%</td>
</tr>
<tr>
<td>10</td>
<td>tnp (IS66)</td>
<td>6559</td>
<td>7245</td>
<td>pRUM IS6 transposase (AAO52848)</td>
<td>229%</td>
</tr>
<tr>
<td>11c</td>
<td>corA</td>
<td>7536</td>
<td>8924</td>
<td><em>E. faecium</em> Mg/Ni/Co transporter CorA (ZP_05660433)</td>
<td>463%</td>
</tr>
<tr>
<td>12c</td>
<td>int</td>
<td>8989</td>
<td>9942</td>
<td><em>E. faecium</em> integrase, catalytic region (ZP_00603158)</td>
<td>318%</td>
</tr>
<tr>
<td>13c</td>
<td>orf13</td>
<td>10026</td>
<td>10156</td>
<td>p5753Ea hypothetical protein (ADA62235)</td>
<td>43%</td>
</tr>
<tr>
<td>14</td>
<td>res</td>
<td>10585</td>
<td>11148</td>
<td>p5753Ea resolvase, N-terminal domain (ADA62236)</td>
<td>188%</td>
</tr>
<tr>
<td>15</td>
<td>tnp (IS1678)</td>
<td>11336</td>
<td>12655</td>
<td><em>E. faecium</em> transposase, IS1678 (AAW32124)</td>
<td>440%</td>
</tr>
<tr>
<td>16c</td>
<td>relE</td>
<td>13111</td>
<td>13398</td>
<td>E. faecalis Abr-family antitoxin component of TA system (ZP_05742775)</td>
<td>96%</td>
</tr>
<tr>
<td>17c</td>
<td>relBEF</td>
<td>13388</td>
<td>13717</td>
<td>p5753Ea cadmium efflux system accessory protein (ADA62239)</td>
<td>110%</td>
</tr>
<tr>
<td>18</td>
<td>cadE</td>
<td>14022</td>
<td>14351</td>
<td>p5753Ea cadmium efflux system accessory protein (ADA62239)</td>
<td>100%</td>
</tr>
<tr>
<td>19c</td>
<td>vanZ</td>
<td>14638</td>
<td>15123</td>
<td>Tn5456 VanZ, teicoplanin resistance protein (AAA65959)</td>
<td>162%</td>
</tr>
<tr>
<td>20c</td>
<td>vanY</td>
<td>15276</td>
<td>16187</td>
<td>Tn5456 VanY, truncated carboxypeptidase (AAA65958)</td>
<td>304%</td>
</tr>
<tr>
<td>21c</td>
<td>vanX</td>
<td>16615</td>
<td>17223</td>
<td>Tn5456 VanX, D-Ala-D-Ala dipeptidase (AAA65957)</td>
<td>203%</td>
</tr>
<tr>
<td>22c</td>
<td>vanA</td>
<td>17229</td>
<td>18260</td>
<td>Tn5456 VanA, D-Ala-D-Lac ligase (AAA65956)</td>
<td>344%</td>
</tr>
<tr>
<td>23c</td>
<td>vanH</td>
<td>18253</td>
<td>19221</td>
<td>Tn5456 VanH, Pyruvate dehydrogenase (AAA65955)</td>
<td>323%</td>
</tr>
<tr>
<td>24</td>
<td>tnp (IS1251)</td>
<td>19553</td>
<td>20845</td>
<td><em>E. faecium</em> IS1251-like transposase (AAAF31111)</td>
<td>431%</td>
</tr>
<tr>
<td>25c</td>
<td>vanS</td>
<td>20943</td>
<td>22097</td>
<td>Tn5456 VanS, Truncated histidine kinase (AAA65954)</td>
<td>232%</td>
</tr>
<tr>
<td>26c</td>
<td>vanR</td>
<td>22075</td>
<td>22770</td>
<td>Tn5456 VanR, Regulator of two-component regulatory system (AAA65953)</td>
<td>232%</td>
</tr>
<tr>
<td>27c</td>
<td>res</td>
<td>22984</td>
<td>23559</td>
<td>Tn5456 resolvase (AAA65952)</td>
<td>192%</td>
</tr>
<tr>
<td>28</td>
<td>tnp (Tn1546)</td>
<td>23705</td>
<td>25856</td>
<td>Tn5456 transposase (AAA65951)</td>
<td>717%</td>
</tr>
<tr>
<td>29</td>
<td>tnp (IS66)</td>
<td>26661</td>
<td>27347</td>
<td>pRUM IS6 transposase (AAO52854)</td>
<td>229%</td>
</tr>
<tr>
<td>30c</td>
<td>aph-3</td>
<td>28195</td>
<td>28980</td>
<td>S. intermedius Aph-3, aminoglycoside phosphotransferase (AAG42234)</td>
<td>262%</td>
</tr>
<tr>
<td>31c</td>
<td>sat4</td>
<td>29082</td>
<td>29612</td>
<td>S. intermedius Sat4, streptothricin acetyltransferase (AAG42233)</td>
<td>177%</td>
</tr>
<tr>
<td>32c</td>
<td>aadE</td>
<td>29621</td>
<td>30529</td>
<td>S. intermedius AadE, streptomycin adenyltransferase (AAG42232)</td>
<td>303%</td>
</tr>
<tr>
<td>33c</td>
<td>MTase</td>
<td>30562</td>
<td>31296</td>
<td><em>E. faecium</em> methyltransferase (ZP_00603123)</td>
<td>245%</td>
</tr>
<tr>
<td>34c</td>
<td>dnaP</td>
<td>31277</td>
<td>32146</td>
<td><em>E. faecium</em> DNA polymerase, beta-like region (ZP_00603124)</td>
<td>290%</td>
</tr>
<tr>
<td>35c</td>
<td>tnp (IS1182)</td>
<td>32521</td>
<td>33195</td>
<td>S. intermedius transposase, IS1182 (AAG42229)</td>
<td>225%</td>
</tr>
<tr>
<td>36c</td>
<td>orf36</td>
<td>33652</td>
<td>33783</td>
<td>S. intermedius orf3 (AAG44228)</td>
<td>44%</td>
</tr>
<tr>
<td>37c</td>
<td>ermB</td>
<td>33788</td>
<td>34525</td>
<td>S. intermedius ErmB, erythromycin resistance methylase (AAG42227)</td>
<td>246%</td>
</tr>
<tr>
<td>38</td>
<td>tnp (IS6)</td>
<td>34873</td>
<td>35559</td>
<td>pRUM IS6 transposase (AAO52854)</td>
<td>229%</td>
</tr>
<tr>
<td>39c</td>
<td>repA</td>
<td>36175</td>
<td>37215</td>
<td>pRUM putative RepA replication protein (AAO52855)</td>
<td>347%</td>
</tr>
<tr>
<td>40c</td>
<td>orf40</td>
<td>37564</td>
<td>37890</td>
<td>pRUM hypothetical protein (AAO52856)</td>
<td>109%</td>
</tr>
<tr>
<td>41c</td>
<td>soj</td>
<td>37877</td>
<td>38680</td>
<td>pRUM Soj partitioning protein (AAO52857)</td>
<td>268%</td>
</tr>
</tbody>
</table>

*ORFs predicted to be transcribed on the complementary strand are denoted with 'c'.
as 53% amino acid identity with the RelE protein from *Lactobacillus antri*. The BLASTP search of the amino acids encoded by *relBE* resulted in hits sharing up to 100% homology with other *E faecium* hypothetical proteins, as well as 79% amino acid identity with the *E. faecalis* AbrB family antitoxin component.

A.3 CONCLUSIONS

The complete nucleotide sequence of the 39 kb non-conjugative plasmid pS177 was determined annotated. BLAST analysis revealed extensive homology to known sequences, including the enterococcal plasmid pRUM, the *vanA*-type glycopeptide resistance determinant Tn1546 and the *S. intermedius* resistance gene cassette. Thus, pS177 appears to have arisen from multiple recombination events between smaller plasmids and mobile genetic elements. Similarly, sequence analysis of pRUM suggested it also arose from a variety of mobile genetic elements, recombination events, and smaller plasmids [6]. Thus, it seems that multiple mobile genetic elements commonly coexist in Gram-positive bacteria, allowing for recombination events and the creation of new plasmids.

This report marked the first report, to our knowledge, of a completely sequenced VRE plasmid that harbors a full-length *vanA* cassette containing the insertion element IS1251 between *vanS* and *vanH*. Additionally, plasmid pS177 confers resistance to vancomycin, kanamycin, streptomycin, streptothricin and erythromycin and, indeed, VRE strain S177 was resistant to gentamicin, erythromycin and vancomycin [11]. The presence of TA systems on pS177, including *axe–txe* and *relBE*,

Axe–Txe, probably enhances plasmid stability and enables the persistence of this multidrug-resistant plasmid in clinical isolates of VRE. This is also the first report of a completely sequenced plasmid carrying both the *vanA*-type resistance determinant and *axe–txe*. Additionally, it was shown here for the first time to our knowledge that the *axe–txe* transcript is produced in VRE clinical isolates, indicating that the *axe–txe* operon is functional [7].

The conclusive link between the pRUM-like replicon, *axe–txe*, the *vanA*-type resistance determinant (as demonstrated from sequencing), the widespread prevalence of *axe–txe* genes and the presence of *axe–txe* transcripts in VRE clinical isolates further supports the importance of *axe–txe* in the maintenance of plasmids coding for multidrug resistance, and bolsters the notion of targeting Axe-Txe for antimicrobial development [7, 11, 13].
A.4 EXPERIMENTAL PROCEDURES

A.4.1 Plasmid DNA isolation

Plasmid DNA was isolated from *E. faecium* using a modified alkaline lysis midiprep protocol [14]. A 50 mL bacterial culture grown in BHI broth was harvested after 12-14 hrs growth and the pellet resuspended in 2 mL solution I (25 mM Tris, pH 8.0, 50 mM glucose, 10 mM EDTA) and 200 µL lysozyme (100 mg/mL in 25 mM Tris, pH 8.0). The suspension was incubated at 37°C for 1 hr. Next, 3 mL solution II (0.2 N NaOH, 1% SDS) was added and the tube was inverted gently 6 times, followed by 4.5 min incubation on ice. Finally, 3 mL solution III (5M potassium acetate, 11.5% glacial acetic acid) was added and the tube inverted 8 times, followed by 5 min incubation on ice. The cell debris was collected by centrifuging for 20 min at 20,000 x g at 4°C. To extract the nucleic acid, 7 mL supernatant was transferred to a new tube and an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1, w/v/v) was added and the tube shaken vigorously, followed by centrifuging as described previously. Nucleic acid was precipitated from 6 mL of the aqueous layer by adding an equal volume of isopropanol and incubating at room temperature for 2 min, followed by centrifuging for 30 min at 27,000 x g. The nucleic acid pellet was washed with 5 mL 70% ethanol and centrifuged for 10 min at 27,000 x g at 4°C. All ethanol was removed and the pellet was allowed to air dry, then dissolved in 100 µL 10 mM Tris, pH 8.0 overnight at 4°C. The RNA was digested by incubation with 5 µL RNase A (10 mg/mL) for 30 min at 37°C.

Plasmid DNA isolated from *E. faecium* S177 was electrophoresed in a 0.65% agarose gel, containing 0.5 µg/mL ethidium bromide. The dominant supercoiled plasmid band was excised from the gel and the DNA recovered by electroelution, following a modified protocol [18]. Dialysis tubing with a 12-14 kDa nominal molecular weight cutoff (Spectra/Por) was prepared by boiling in 25 mM EDTA, pH 8.0, for 15 min, rinsed thoroughly in dH₂O, and rinsed in 1× TAE at 4°C. The gel slice and approximately 2 mL 1× TAE were secured in the dialysis tubing and electrophoresed in 1× TAE at 70 V for 2 hrs, at which point the polarity was reversed for 2 min. Without removing the gel slice, the solution was subjected to dialysis against 10 mM Tris, pH 8.0, overnight at 4°C. The electroeluted plasmid DNA was concentrated by centrifuging at 500 x g in a 0.5 mL 30 kDa molecular weight cutoff tube (Microcon) until the volume was reduced to 100 µL.

A.4.2 Plasmid DNA sequencing

The shotgun cloning, sequencing and assembly of plasmid pS177 was performed by the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. Briefly, DNA was sheared with a nebulizer, end-repaired and dephosphorylated. The DNA was electrophoresed
in a 0.8% low-melting-point agarose gel, from which DNA ranging from 1.5 to 5.0 kb was purified and cloned into the pSMART-HCKan vector (Lucigen) according to the manufacturer’s instructions. Sequencing was performed on 480 subclones from both the 5’ and 3’ ends of the insert using ABI Big-Dye terminator chemistry (Applied Biosystems). Custom primers were used in PCRs and sequencing reactions to close gaps and to ensure at least 3x coverage at each nucleotide (average coverage was 12.4×). The sequence data were assembled using the Phrap (http://www.phrap.org) and Sequencher (GeneCodes) software programs. BLAST [1] analysis of the assembled plasmid was used to determine sequence similarity and annotations were made accordingly. The nucleotide sequence of plasmid pS177 was deposited to the GenBank database under accession number HQ115078.

A.5 REFERENCES


APPENDIX B
HIGH-THROUGHPUT SCREEN FOR ACTIVATORS OF MazEFEc PERFORMED IN COLLABORATION WITH THE NIH MLPCN

B.1 INTRODUCTION

The TA system mazEF was found in 75 out of 75 clinical VRE isolates and shown to be functional by stabilizing an unstable plasmid in E. faecium [7]. In E. coli, mazE and mazF are overlapping genes located within the rel operon, downstream of relA. Expression is controlled by the P2 promoter which is regulated by the stringent response molecule 3′-5′-bispyrophosphate (ppGpp) [1]. The alarmone ppGpp is synthesized by the RelA protein under amino acid starvation conditions [6]. The MazEEc-MazFEc complex efficiently negatively autoregulate expression at the transcriptional level by binding to an alternating palindrome in the promoter sequence [6]. MazF Ec exists as a dimer and binds MazE Ec in a 2:1 ratio [10]; the crystal structure revealed an extended heterohexamer consisting of alternating homodimers, resulting in the formation MazF2-MazE2-MazF2 (see Figure 4.2) [3]. The mazEF Ec genes encodes the labile antitoxin MazE Ec, which is degraded by the ClpA serine protease [1] and the stable toxin MazF Ec, which is a ribonuclease that inhibits bacterial growth by specifically cleaving at the sequence ACA, thereby prohibiting protein synthesis [11]. The toxic effect of MazF Ec can be recovered by MazE Ec; however, a ‘point of no return’ exists after which cells cannot recover [2, 4, 8]. Thus, small molecule activation MazF Ec could result in bacterial cell death.

A fluorogenic assay was developed to detect the ribonuclease activity of MazF Ec [9]. This enzymatic assay uses a chimeric DNA-RNA oligonucleotide containing a 5′ fluoroscein (6-FAM) and a 3′ black hole quencher (BHQ), giving the sequence 5′-6-FAM-AAGTCrGACATCAG-BHQ-3′. The fluorescence of the intact substrate is dampened due to the proximity between the quencher and fluorophore. Incubation of the substrate with an RNase, such as MazF Ec, releases the fluorophore from the quencher; thus, MazF Ec activity can be measured by monitoring the fluorescence over time [9].

This robust, straightforward assay was applied to a high throughput screen to identify small molecules that activate the MazF Ec toxin from the MazEF Ec complex. The MazEF Ec complex was incubated with compound and substrate, thus allowing for any MazF Ec activity to be detected. Full disruption between MazE Ec and MazF Ec is not necessarily required for the success of this strategy; a molecule that perturbs MazE Ec binding or induces an allosteric change in MazF Ec may allow access of the substrate to the active site.
B.2 HIGH THROUGHPUT SCREEN RESULTS

The NIH Molecular Libraries Probes Centers Network (MLPCN) performed the high throughput screen of 350,000 small molecules. The basic protocol described previously [9] was followed, with slight modifications. Whereas 3 μM MazF<sub>Ec</sub>(His)<sub>6</sub> was used previously as a positive control in the assay, it was determined that sufficient fluorescent signal could be detected using 0.3 μM MazF<sub>Ec</sub>(His)<sub>6</sub>. To increase the probability that any activation of MazF<sub>Ec</sub> from the complex was detected, 0.75 μM (His)<sub>6</sub>MazEF<sub>Ec</sub> was employed in the high throughput screen. This improvement reduced the amount of protein to perform the screen 4-fold. The original protocol called for 12.5 nM substrate, and as the substrate is the most expensive reagent for the screen, it was not altered. To ensure that a robust signal would be obtained by active MazF<sub>Ec</sub>, the incubation time was increased from 2.5 hour to 5 hours.

From the ~350,000 compounds screened at 20 μM, 1104 were scored as initial hits, giving a hit rate of 0.3%. From these, 464 were confirmed in the re-test and were tested in a dose-response assay, which revealed 23 compounds that showed a dose-response. We received 20 of these compounds and the associated EC<sub>50</sub> data, which is summarized in Figure B.1.

![Figure B.1](image)

**Figure B.1** EC<sub>50</sub> of 20 lead compounds on MazF<sub>Ec</sub> activity from the MazEF<sub>Ec</sub> complex. Dose-response assays for the activation of MazF<sub>Ec</sub> resulted in EC50 values ranging from 1.8-21 μM.

B.3 INVESTIGATION OF HITS

The HPLC assay developed for the detection of MazF<sub>Ec</sub> activity [9] was employed to definitively assess the effect of the compounds on MazF<sub>Ec</sub> activity. (His)<sub>6</sub>MazEF<sub>Ec</sub> was incubated for 16 hours with 100 μM compound and the non-fluorogenic substrate 5'-AAGTCrGACATCAG-3’. A portion of this reaction was analyzed by HPLC for each of the 20 compounds. **Figure B.2** shows HPLC traces of intact and cleaved substrate and the two overlaid for direct comparison. Cleaved substrate was not observed from any of the reactions containing (His)<sub>6</sub>MazEF<sub>Ec</sub> and compound; thus the compounds were ruled as false positives. Some of the compounds had intrinsic fluorescence and others demonstrated MazF<sub>Ec</sub> and oligo-dependent
increases in fluorescence. The cause of the false positive result was not determined for every compound, given the definitive result of no effect on MazF<sub>Ec</sub> activity.

**Figure B.2** HPLC assay. HPLC corresponding to intact oligo (A), cleavage products (B) and the two overlaid (C) demonstrate the definitive assessment of MazF activity using this assay.

**B.4 CONCLUSIONS**

The failure to identify small molecule activators of the MazEF<sub>Ec</sub> TA system emphasizes the inherent challenges associated with the TA-system targeting strategy, namely, overcoming the incredibly tight interaction between the two proteins. The affinity of the MazEF<sub>Ec</sub> complex has been estimated at below 100 nM [5]. Testing compound in approximately 30-fold excess over the MazEF<sub>Ec</sub> complex provided conditions that would likely identify even a weak modulator of the MazEF<sub>Ec</sub> interaction. The facile nature of the screen enabled the examination of ~350,000 compounds, and we were fortunate to take advantage of the collaboration with the MLPCN. Although the hits initially seemed promising, they were all deemed as false positives using the definitive HPLC assay. Some of the hit compounds resembled fluorophores and were not expected to perform well in the HPLC assay. Other compounds were shown to increase fluorescence in a MazF<sub>Ec</sub>- and fluorophore-dependent manner but did not induce cleavage of the non-fluorogenic substrate. The explanation for this phenomenon remains elusive; however, these compounds were ruled as false positives as well. The definitive nature of the HPLC assay serves as a highly useful secondary assay to evaluate potential hit compounds.

**B.5 EXPERIMENTAL PROCEDURES**

**B.5.1 Protein expression and purification**

Expression of (His)<sub>6</sub>MazEF<sub>Ec</sub> or MazEF<sub>Ec</sub>(His)<sub>6</sub> was achieved by inducing cultures of <i>E. coli</i> BL21(DE3) carrying the plasmid pKm6EF or pKmEF6 with IPTG, as previously described [9]. (His)<sub>6</sub>MazEF<sub>Ec</sub> was purified under native conditions as previously described [9].
MazF\textsubscript{Ec}(His\textsubscript{6}) was obtained by denaturing purification from MazEF\textsubscript{Ec}(His\textsubscript{6}). A pellet corresponding to 1 L culture was thawed in a room temperature water bath for 10 minutes. The pellet was resuspended in 5 mL binding buffer (10 mM Tris, pH 7.9, 500 mM NaCl, 10 mM imidazole) containing 8 M urea and cell lysis was achieved by 1 hour incubation at room temperature with inversion. Cell debris was pelleted by centrifugation at 35,000 \textit{g} at 4°C for 30 minutes. The clarified lysate was mixed with 1.5 mL 1:1 Ni\textsuperscript{2+}-NTA resin slurry (Qiagen) and batch loaded for 60 min at room temperature with inversion. The slurry was applied to a gravimetric flow column and the resin was washed with 20 mL binding buffer with 8 M urea to fully disrupt the MazE\textsubscript{Ec}-MazF\textsubscript{Ec} complex. On-column refolding of MazF\textsubscript{Ec} was performed with seven washes of 10 mL urea(binding buffer decreasing the concentration of urea by 1 M with each wash. Wash steps containing more than 4 M urea were performed at room temperature; all subsequent wash steps were performed at 4°C. Refolding was followed with 10 mL washes of binding buffer (containing no urea) and binding buffer containing 60 mM imidazole. MazF\textsubscript{Ec} was eluted with 5 mL binding buffer containing 250 mM imidazole.

B.5.2 Fluorometric assay for MazF activity

The fluorogenic substrate 5'-6FAM-AGTCTACACATCAG-BHQ-3’ (6-FAM, 6-carboxyfluorescein, BHQ, black hole quencher; “r” denotes RNA base, synthesized by IDT) was diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Wells of a 384-well sterile black tissue-culture plate (ThermoFisher) were filled with 30 \textmu{}L of 0.3 \mu{}M MazF(His\textsubscript{6}), (final concentration) diluted in assay buffer (10 mM Tris, pH 7.9, 500 mM NaCl) and 12.5 nM substrate (final concentration). The fluorescence of the plate was measured immediately and after 5 hours using Criterion Analyst AD (Molecular Devices) with 485 ± 15 nm excitation and 530 ± 15 nm emission filters and a 505 nm cutoff dichroic mirror. The fluorophore was excited with a 1000 W continuous lamp with 10 reads per well. The Z-height was set to 1 nm.

B.5.3 High throughput screen

The screen followed the same general conditions for the MazF\textsubscript{Ec} fluorometric assay. A 30 \mu{}L volume of 0.75 \mu{}M (His\textsubscript{6})\textsubscript{MazEF\textsubscript{Ec}} with 12.5 nM fluorogenic substrate and 20 \mu{}M of each compound were incubated at room temperature for 5 hours. Buffer alone and substrate alone were used as negative and positive controls, respectively. The fluorescence was read immediately after adding all components and after 5 hours incubation.
B.5.4 HPLC assay

The HPLC assay was performed using the non-fluorogenic substrate 5'-AAGTCrGACATCAG-3' (IDT). Briefly, 3 μM (His)_6 MazEF_Ec was incubated with 100 μM compound and 24 μM substrate for 16 hours at room temperature. RNaseI was used as a positive control for cleavage. A 10 μL portion of the reaction was analyzed by HPLC, as described previously [9].

B.6 REFERENCES


