Identification and characterization of toxtazins A and B, two small molecule inhibitors of virulence gene activation in *Vibrio cholerae*

by

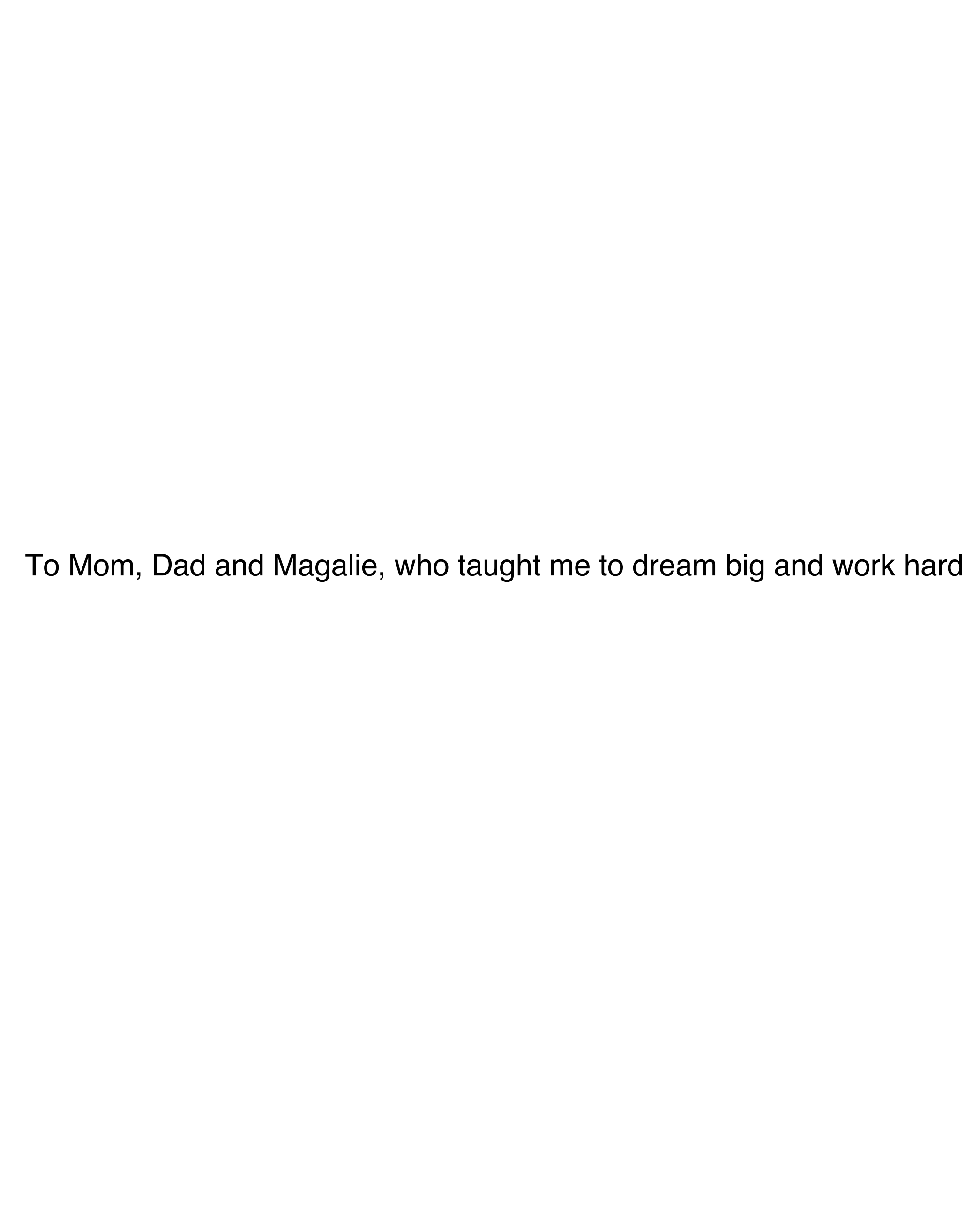
Rebecca Elizabeth Anthouard

**A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in the University of Michigan
2014**

Doctoral Committee:

Professor Victor DiRita, Chair
Professor Phillip Hanna
Professor Ursula H. Jakob
Associate Professor Eric Krukonis
Associate Professor Maria B. Sandkvist

© Rebecca Elizabeth Anthouard
2014



To Mom, Dad and Magalie, who taught me to dream big and work hard

Acknowledgements

People often say "No [wo]man is an island," and I am no exception. There are many people who were instrumental in helping me through graduate school. First, I would like to thank my advisor and mentor, Vic. Thank you for taking a chance on me, giving me a great thesis project, and molding me into the scientist I am today. You have not only taught me skills I use at the bench, but also how to give a great presentation and how to push through the disappointment that failed experiments can bring. I am forever changed because of your mentorship. I also thank my committee for all of their encouragement and suggestions, and for keeping our meetings productive and fun!

I could not have completed graduate school without the fellow members of the DiRita lab. Lindsay Gielda, thank you for all your help at the bench, for letting me vent, and for the wise advice you give me even today. Aimee, despite all of our differences I think I have learned the most from you. You are an example of a strong, determined woman who is smart and confident. Jeremy and Sam, our runs to Chipotle sustained me through a tough time, thanks. Jyl, thank you for all your advice on experiments and life as a scientist. You make the lab fun to be in, and we miss you. Mike and JJ, thank you for thinking so critically about my project and offering so many great suggestions. Thank you also for helping me out of my music shell! Wei Ping, thanks for being a great baymate. You keep things fun and interesting, and do amazing science. Keep it up! Jessica, thank you for being my source of comfort when experiments were not going well. Know that you can always count on me for the same. Travis, whether you join our lab or not, I know you will do great things as a scientist.

To all the friends I made in Michigan, fellow students, climbers, runners, and roommates, thank you for reminding me to enjoy life. In particular, thank you to Laura Jackson and Lauren Knapp for making our home a wonderful place to live. I don't know what I would have done without your support and encouragement, our impromptu singing, hiking, tubing, or froommate workdate clubbing. Thanks for keeping me sane during the last year of graduate school.

Most importantly, none of this could have happened without my family. From my earliest memories, my parents always encouraged me to ask questions and seek answers. They made learning fun and taught me that you can have anything you want if you work hard, so do not be afraid to dream big. They taught me that no man is an island, so don't be afraid to ask for help. They came to my first poster session with flowers, and even read my manuscript despite not being scientists. My sister, Magalie, cheered me up when I was down and reminded me that life is about loving and having fun. Thank you for all your calls and cards. Without my family's love and support, I would not be where I am today, so thank you.

Table of Contents

Dedication ii

Acknowledgements iii

List of Figures vii

List of Tables ix

List of Appendices x

Abstract xi

Chapter I Introduction 1

- Introduction 1
- Small molecules that regulate virulence gene expression 4
- Small molecules that affect host-pathogen interactions 16
- Small molecules that target structures 23
- Advantages and challenges of chemical genetics 32
- Vibrio cholerae pathogenesis 35
- References 38

Chapter II Small molecule inhibitors of *toxT* expression in *Vibrio cholerae* 47

- Summary 47
- Introduction 48
- Results 51
- Discussion 64
- Materials and Methods 71
- References 79

Chapter III Mechanism of action of toxtazin A 82

- Summary 82
- Introduction 82
- Results 84
- Discussion 106
- Materials and Methods 109

References 115

Chapter IV Mechanism of action of toxtazin B 118

Summary 118
Introduction 118
Results 120
Discussion 129
Materials and Methods 131
References 134

Chapter V Discussion 136

Appendices 146

List of Figures

1.1 Steps critical for bacterial pathogenesis 3

1.2 *Bacillus subtilis* regulates sigma B in response to stress 13

1.3 P pili and type 1 pili are structurally similar but encoded by different genes 30

2.1 Identification of inhibitors of the virulence cascade in *V. cholerae* 52

2.2 ToxTazin A, B, and B' inhibit the virulence cascade in *V. cholerae* 54

2.3 ToxTazin B reduces *V. cholerae* colonization in an infant mouse 59

2.4 Effects of toxtazin A and B on the virulence cascade 60

2.5 ToxTazins A and B do not affect AphA or AphB protein levels 63

S2.1 Ectopic expression of AphA and AphB in toxTazin B-treated cells 65

2.6 Model of the virulence cascade in *V. cholerae* and targets of the toxTazins 70

3.1 The toxtazins do not alter the membrane-localization of ToxR or TcpP 85

3.2 Toxtazin A does not inhibit ToxR from binding the *toxT* promoter 88

3.3 Toxtazin A causes a growth defect in M9 + NERS 90

3.4 Toxtazin A inhibits growth in LB + 3% ethanol 91

3.5 GO terms of proteins differentially expressed in the presence of toxtazin A 97

3.6 Toxtazin A treatment results in the overexpression of VC0731 99

3.7 VC0731 is activated by VC0732, not OxyR, and is induced at 37°C 101

3.8 VC0731 and VC0732 do not affect CT expression 102

3.9 FACS sorting of transposon mutants for resistance to toxtazins A and B 105

4.1 Colonization of S533 is not affected by toxtazin B 121

4.2 Toxtazin B does not stimulate TcpP degradation 123

4.3 Toxtazin B does not inhibit AphA or AphB binding to the *tcpP* promoter 126

4.4 CT expression of cells expressing the wild-type or C227S allele of AphB 128

A1 Procaine and lidocaine inhibit *toxT*-GFP in a dose-dependent manner 154

A2 Procaine inhibits *toxT* expression in a *tcpP*- and *aphB*-dependent manner 155

A3. Maltose inhibits *toxT*-GFP expression in LB pH 6.5 at 30°C 157

B1 OmpU and OmpT expression in the presence of various compounds 164

C1 Pharmacophore of toxtazin A and toxtazin B 177

C2 Chemical structure of txtz001 178

C3 Bioactivity of the txtz001 probe 179

List of Tables

1.1 Small molecules discussed in this introduction 5

2.1 Strains and plasmids used in this study 72

2.2 Primers used for qRT-PCR analysis 78

3.1 Proteins with 4-fold differential expression in toxtazin A-treated cells 93

3.2 Strains used in this study.

A1 Natural compound inhibitors of *toxT*-GFP in LB pH 6.5 at 30°C 150

A2 Natural compound inhibitors of *toxT*-GFP in M9 minimal media + NERS 152

B1 Small molecule inhibitors of both ToxR activity at the *ompU* promoter (ToxR screen) and of *toxT*-GFP expression (ToxGFP screen) 163

C1 SAR of toxtazin A analogs 172

C2 SAR of toxtazin B analogs 173

List of Appendices

A. Screening natural products for inhibitors of virulence gene expression in *Vibrio cholerae* 147

- Summary 147
- Introduction 147
- Results 148
- Discussion 156
- Materials and Methods 158
- References 159

B. Small molecule screen for inhibitors of ToxR activity 161

- Summary 161
- Results 162
- Materials and Methods 165
- References 168

C. Structure activity relationships of toxtazins A and B 169

- Summary 169
- Introduction 169
- Results 170
- Discussion 171
- Materials and Methods 181
- References 182

Abstract

Discovering how bacteria regulate their virulence mechanisms deepens our understanding of basic pathogenesis and allows us to uncover new potential targets for the treatment of disease. We applied a chemical genetics approach to probe the cellular requirements for virulence gene expression in *Vibrio cholerae* by targeting the main virulence gene regulator, ToxT. The screen revealed two novel classes of inhibitors, toxtazins A and B. Both reduce cholera toxin production and production of an important ToxT-regulated pilus, the toxin co-regulated pilus. We present evidence that toxtazin A works by inhibiting *toxT* transcription, and may do this by activating stress pathways in the cell. We also demonstrate that toxtazin B works by inhibiting *tcpP* transcription, and this may be due to a particular thiol switch in AphB, one of two transcriptional activators required to activate the *tcpP* promoter. Furthermore, treatment with toxtazin B resulted in a 100-fold reduction in colonization in an infant mouse model of infection. These results add to the growing body of literature indicating that small molecule inhibitors of virulence genes could be developed to treat infections and to learn more about the basic biological mechanisms required for virulence gene regulation.

Chapter I

Introduction

Introduction

Treatment of bacterial infections was revolutionized in the 1940s with the development of antibiotics (1). Despite the huge initial success of antibiotic therapy, their overuse and misuse has led to their increasingly limited effectiveness. Some antibiotics work by killing bacterial cells while others are bacteriostatic- both strategies that ensure resistant strains will have a strong selective advantage. It is not surprising then, though certainly alarming, that for every antibiotic known there exists at least one resistant strain of bacteria (2). Equally troubling is the fact that antibiotic therapy kills a large portion of the host microbiota as well as the offending pathogen. The resulting dysbiosis can lead to acute and chronic intestinal problems (3, 4) and is one of the leading causes of hospital-acquired *Clostridium difficile* (5).

The Center for Disease Control and Prevention recently estimated that 2,049,442 illness are caused by antibiotic resistant bacteria and fungi annually, which result in over 23,000 deaths, though this is likely an underestimation (6). In addition to the health burden, antibiotic resistance also presents a significant financial burden, with estimates ranging from as high as $20 billion in direct healthcare costs and $35 billion in loss of productivity (6, 7).

As more and more pathogens become resistant to our antibiotics, and as we become increasingly aware of the protective effects of the microbiota, researchers have started to look for alternative therapies for treating bacterial infections. Anti-infective drugs are attractive alternatives because they disarm pathogens rather than killing them, providing some advantages over antibiotic treatment. First, any resistance developed against anti-infective drugs man have a weaker selective pressure, thus resistance would take longer to develop, if it developed at all. Second, by targeting virulence traits, these anti-infectives will affect only the bacteria that possess those pathogenic traits- ideally leaving the microbiota relatively unaffected.

Anti-infective compounds have another important role in biomedical research because of their potential to uncover new virulence requirements and further our basic biological understanding of pathogenesis. There are several advantages to probing pathogenesis with small molecules at the bench: i) they act quickly, ii) they may be reversible or non-reversible, iii) they do not require manipulation of the genome, a quality that is especially advantageous in studying genetically intractable organisms, iv) the dose can be adjusted to fine-tune the effects, and v) they can be used across multiple bacterial species to determine how conserved a pathway is between different species or strains.

Pathogenic bacteria have evolved countless strategies for establishing infection and causing disease in their various hosts. For any given pathogen, there are numerous steps in the infectious process that can be inhibited to reduce the virulence potential and whose mechanisms are therefore of biological interest. Figure 1.1 is a

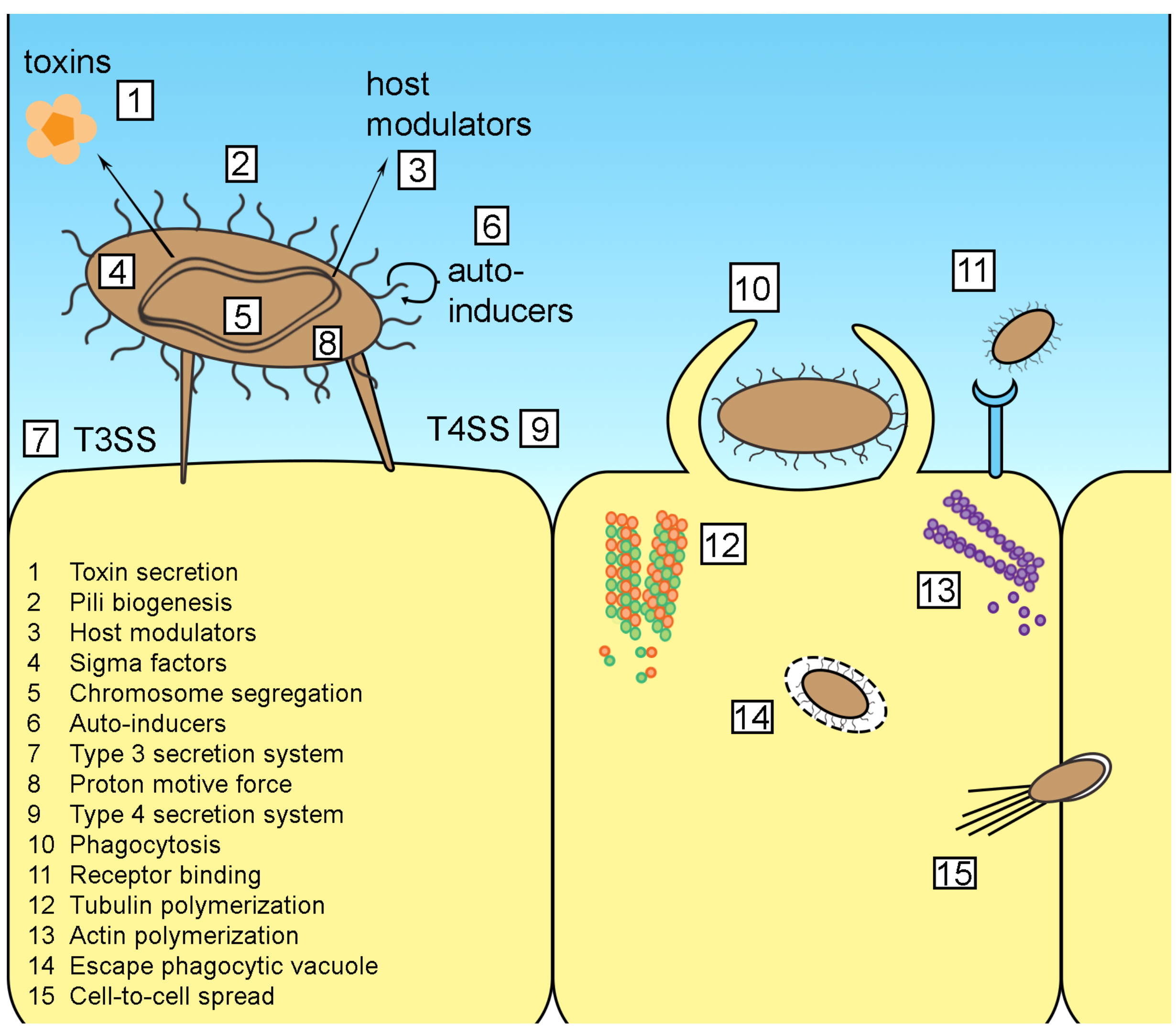


Figure 1.1 Steps critical for bacterial pathogenesis.

Presented here are fifteen events that are required for virulence in one or more pathogenic bacteria. Some of these events deal with bacterial gene regulation, others are involved in host-pathogen interactions, and others affect bacterial structures such as pili and the chromosomal segregation machinery.

generalized model of pathogenesis, combining aspects of pathogenesis that are sometimes unique to one organism (for example, actin-mediated cell-to-cell spread is used by *Listeria monocytogenes*) or conserved in all bacteria (such as chromosomal segregation). Figure 1.1 depicts the many facets of pathogenesis for which a small molecule inhibitor exists that is discussed here. This serves to illustrate that these “Achilles’ heels” of pathogenesis, many of which were discovered using chemical genetics, can be exploited to develop new treatment therapies. For simplicity, these small molecule inhibitors are grouped into three categories: those that regulate virulence genes, those that affect host-pathogen interactions, and those that alter the formation of important microbial structures. We present examples of small molecules (summarized in Table 1.1) that have deepened our understanding of the fundamental processes in each of these areas. We discuss how these discoveries have influenced our understanding of pathogenesis and how they have uncovered new potential drug targets. We explore the advantages and limitations of small molecule research, and conclude with some ways in which small molecule screens could further our understanding of pathogenesis.

Small molecules that regulate bacterial virulence gene expression

Many published chemical genetic screens have targeted bacterial virulence factors, an approach that boasts numerous advantages. First, because this treatment strategy uses anti-virulence compounds to disarm the pathogen rather than killing it, the host microbiota would presumably suffer less damage than it does with traditional.

Table 1.1 Small molecules discussed in this introduction.

Compound	Structure	Assay	IC50	Target	MOA	Refs	Organism Range
Virstatin		Live-cell, fluorescence	3-40 µM, depending on strain	ToxT	Inhibits ToxT dimerization	(8-10)	*Vibrio cholerae*
FPSS		Live cell, opuCA-gus reporter	3.0-3.5 µM	σB	Inhibits RsbV phosphorylation, freeing RsbW to sequester σB	(11, 12)	*Listeria monocytogenes, Bacillus subtilis*
CCG-2979		Live cell, ska-KanR reporter	< 31 µM	Strepto-kinase promoter	Unknown	(13-15)	Group A streptococcus
Pimozide		Bacterial-host system using the internalization of GFP-labeled bacteria as a readout for infection	Not reported	L. monocytogenes infection of bone marrow derived macrophages	Unknown	(16, 17)	*L. monocytogenes*
CCCP		Live-cell, LepA-BlaM reporter assay	About 1 µM	T4SS translocation	Known to affect the proton motive force (PMF)	(18)	*Legionella pneumophila*

Compound	Structure	Assay	IC50	Target	MOA	Refs	Organism Range
RWJ-60475		Live-cell, LepA-BlaM reporter assay	Not reported	T4SS translocation	Known to inhibit the receptor tyrosine phosphate phosphatase CD45	(18)	*L. pneumophila*
LED209		Live cell, LEE1-lacZ reporter	≤ 10 µM	QseC	Inhibition of QseC autophosphorylation	(19)	*Salmonella typhimurium, Francisella tularensis*
TTS29		Live cell, phospholipase secretion	Not reported	T3SS secretion	Inhibits formation or stability of the T3SS needle complex	(20)	*Yersinia enterocolitica, S. typhimurium, Pseudomonas aeruginosa, Francisella novicida, Pseudomonas syringae* pv tomato
A22		Low-throughput agar-based screen	Not reported	MreB	Inhibits ATP hydrolysis by MreB	(21-23)	*Escherichia coli, Caulobacter crescentus, Shigella flexneri, P. aeruginosa, V. cholerae, Leptospira biflexa, Myxococcus xanthus*
Pilicides		Rational design using PapD-PapG structure	Varies	Pilus biogenesis	Interferes with the chaperone-usher interaction	(24-27)	*E. coli*

antibiotics. Secondly, these molecules theoretically impose less of a selective pressure than traditional antibiotics, reducing the risk for the emergence of resistant strains. Third, small molecule inhibitors can be designed with varying species specificity depending on how conserved the target is, allowing a compound to target a single species or a range of species. Finally, anti-virulence molecules are also valuable as research tools, as they deepen our understanding of the molecular mechanisms required for virulence gene activation. One early example of such a molecule is virstatin.

Virstatin

Virstatin (see Table 1.1) is a small molecule that inhibits toxin production in *Vibrio cholerae* (#1 in Figure 1.1) (8). Virstatin was identified using a reporter strain of *V. cholerae* that has the cholera toxin promoter driving expression of the tetracycline resistance gene *tetA*. In the presence of a compound that inhibits expression of the cholera toxin genes, this strain cannot grow in tetracycline-containing media. The reporter strain was grown under standard toxin-inducing conditions with tetracycline and a library of compounds was added to each well. One hundred and nine compounds were identified that inhibit growth in this assay, 15 of which had low bacterial toxicity in the absence of tetracycline. One compound, ultimately termed virstatin, was chosen for follow-up studies.

Target identification is one of the largest challenges posed to researchers after performing a cell-based reporter screen because the compound could target any step prior to expression of the reporter. This is particular challenging because the target i)

may have an unspecified role in virulence; ii) may only be expressed under certain conditions, and/or iii) may not be a single protein but rather could be a protein complex or not a protein at all but rather DNA, RNA, lipids, or the redox state of the cell, to name a few.

In the case of virstatin, advantage was taken of the fact that the virulence regulatory cascade in *V. cholerae* is well defined (28-34). Expression profiles of genes expressed upstream of cholera toxin transcription in the regulatory cascade were examined and none were affected by virstatin. When ToxT, the transcriptional activator of the *ctx* promoter, was ectopically over-expressed in the presence of virstatin, cells were still sensitive to virstatin, indicating that virstatin inhibits the activity of ToxT rather than its transcription or translation (8).

Knowing that the target of virstatin was likely ToxT, the mechanism of action (MOA) of virstatin was investigated next. A library of *toxT* mutant alleles was screened and a virstatin-resistant allele, *toxT*^{L113P}, was identified (8). The protein expressed from this mutant allele resembled wild type ToxT in that it was found predominantly in the multimeric form; however, in the presence of virstatin, wild-type ToxT is largely found as monomers, while ToxT^{L113P} remains predominantly multimeric (9). This led to the discovery that ToxT activation at the *ctx* promoter requires ToxT dimerization. In fact, follow up work discovered that the oligomerization state of ToxT affects its activity at other ToxT-dependent promoters, with some promoters favoring the monomeric form and others favoring dimerized ToxT (35).

To assess its *in vivo* efficacy, virstatin was tested in an infant mouse model of *V. cholerae* colonization (8). Mice inoculated with wild type *V. cholerae* and given virstatin had a 4-log decrease in colonization relative to mice given the DMSO control. As a control, a strain of *V. cholerae* was used that colonizes the mouse in a ToxT-independent manner and should therefore be unaffected by virstatin. Whereas this strain colonized mice treated with the DMSO control or virstatin equally well, the more typical epidemic strain that requires ToxT for colonization colonized DMSO-treated mice much better than virstatin-treated mice, indicating that virstatin reduces colonization of *V.* cholerae by inhibiting ToxT activity *in vivo.*

The fact that virstatin can be used as an *in vivo* molecular probe to differentiate strains of *V. cholerae* that require ToxT for colonization from those that do not has proven very useful. In subsequent studies, virstatin was used to study the mechanisms by which non O1/non-O139 *V. cholerae* strains, which only cause sporadic disease, colonize their hosts (10). Although it remains to be directly tested, that ToxT-independent colonizing strains of *V. cholerae* are not affected by virstatin suggests that other bacteria lacking ToxT, including the host microbiota, would be unaffected by virstatin treatment, making virstatin an attractive therapeutic lead.

The virstatin work advanced the field of *V. cholerae* research by revealing the importance of the dimerization state of ToxT, a level of regulation that was previously only suggested. Furthermore, it provided a molecular probe that can be used *in vivo* to determine whether a *V. cholerae* isolate colonizes in a ToxT-dependent or independent manner. Finally, as a compound that significantly reduces colonization of a pathogen by

specifically inhibiting its virulence factors *in vivo*, it provides a proof-of-principle for the concept of anti-virulence drugs.

Fluoro-phenyl-styrene-sulfonamide (FPSS)

Unlike virstatin, which targets a species-specific virulence regulator and its regulon, fluoro-phenyl-styrene-sulfonamide (FPSS) targets a general regulatory cascade- the sigma B (σB) regulon (#4 in Figure 1.1) (11). Sigma factors are dissociable subunits of RNA polymerase (RNAP) that associate with RNAP in response to certain environmental signals and directly activate or repress a subset of genes, resulting in a rapid change in global transcription that is appropriate for the given environment. Different sigma factors respond to different sets of environmental signals, allowing bacteria to quickly respond to specific environments. In *Listeria monocytogenes*, σB responds to environmental stress (i.e. acidic conditions, ethanol, or high salt concentrations), energy stress, and growth at high or low temperatures (36). In response to these signals, σB activates or represses genes involved in central metabolism and activates PrfA, the master regulator of virulence (37).

Sigma factor inhibitors enable researchers to study the response of a specific sigma factor in isolation. This is critical because cellular responses to different environmental conditions are often complex. Different sigma factors can respond to the same signal, and different sigma factors can regulate the same genes in response to distinct signals, creating a complex web of regulation (38). The contributions of individual sigma factors and of different environmental signals can be assessed with

specific sigma inhibitors. This class of inhibitor may also have therapeutic potential because in some pathogens, sigma factors regulate virulence gene expression (39-41) (Reviewed in (42)).

FPSS was identified using a cell-based reporter assay (11). The screening strain contained a σB-dependent promoter, *opuCA*, fused to the gene for glucuronidase, *gus*, such that glucuronidase activity could be used as a readout for σB activity. Using this assay, approximately 57,000 compounds were screened, and FPSS was identified as the best inhibitor of σB activity in live *L. monocytogenes* cells.

FPSS inhibition of σB activity was confirmed by qRT-PCR, which showed that it inhibits *opuCA* transcription as well as another σB-dependent promoter, *gadA*, and does so in a dose-dependent manner. In fact, transcription of σB-dependent promoters was decreased to the level of a σB mutant, indicating that FPSS can completely inhibit σB activity at these promoters. Microarray analysis showed that FPSS-treated *L. monocytogenes* cells phenocopy a *L. monocytogenes* σB mutant, affecting 91% of genes identified as being σB-regulated in two or more previous studies (11). As an example of the power of small molecule probes in research, FPSS also affected 83 other genes, which excluding side effects, could potentially expanding the σB regulon. Gene set enrichment analysis (GSEA) determined that genes specifically regulated by σH or σL are not significantly enriched among genes differentially transcribed in FPSS-treated cells, suggesting that FPSS affects σB specifically, and not sigma factors in general.

Bacillus subtilis was used to determine the MOA by which FPSS inhibits σB activity because its σB activity is also inhibited by FPSS (11) and because its well-characterized σB regulon is highly conserved with the poorly understood σB regulon of *L. monocytogenes*. In *B. subtilis*, σB activity is regulated by three distinct branches (shown in Figure 1.2 and reviewed in (43)): one branch relays environmental stress (i.e. acidic conditions, ethanol, or high salt concentrations), another branch relays energy stress (i.e. limitation of glucose, ATP, GTP, of phosphate), and a third branch activates σB in response to growth at low temperatures.

In *B. subtilis*, σB is kept in an off state by interaction with an anti-sigma factor, RsbW. The ability of RsbW to sequester σB is controlled by a phosphorylated protein, RsbV. In the phosphorylated state RsbV cannot bind RsbW, so RsbW is free to sequester σB and the σB regulon is not expressed. When RsbV is unphosphorylated it binds RsbW, liberating σB, leading to activation of the σB regulon (44). RsbV phosphorylation is controlled by both environmental stress and energy stress (45). It was experimentally determined that FPSS inhibits σB activation by all three activation branches, indicating that it works on a factor common to all three branches (11). The possibility that FPSS could bind or interact with σB was ruled out with both *in vitro* and *in vivo* experiments (12). In addition, FPSS was shown to prevent RsbV phosphorylation (12). Taken together, these results support a model whereby FPSS targets the partner-switching mechanism between RNA polymerase, σB, and its anti-sigma factor, RsbW. FPSS affords a unique tool to study σB activity in multiple organisms. Microarray analysis of FPSS-treated cultures generated a list of 83

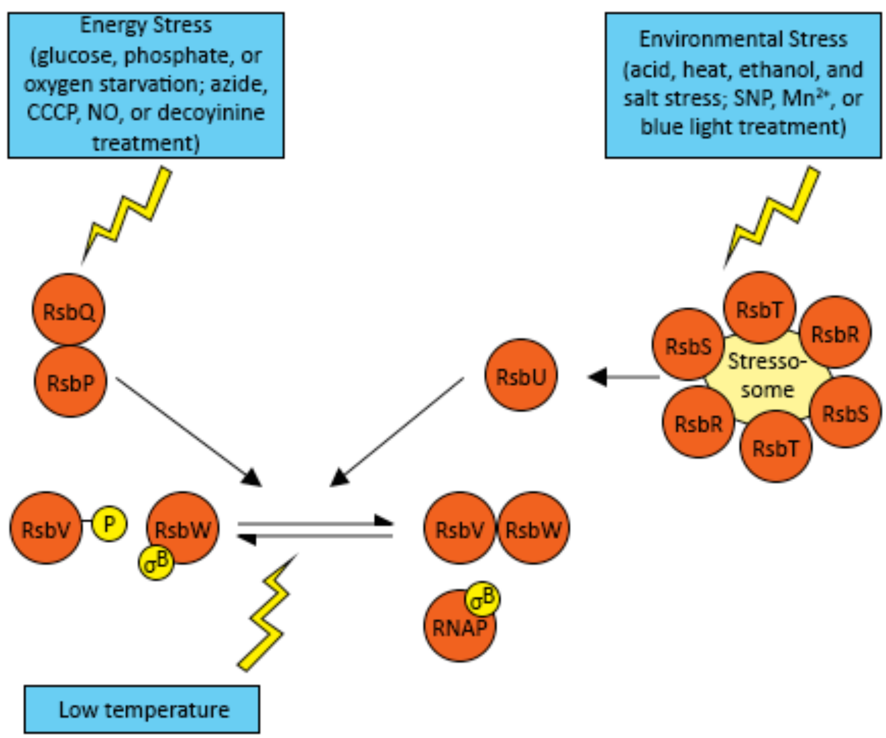


Figure 1.2 *Bacillus subtilis* regulates sigma B in response to stress.

Energy stress, environmental stress, and growth at low temperature are all signals that can activate σ^B activity in *B. subtilis*. Energy stress is relayed via RsbPQ, environmental stress is relayed via the stressosome and RsbU, and low temperatures are relayed via an unknown mechanism. All three stresses impinge upon the phosphorylation of RsbV, the anti-anti-sigma factor. When RsbV is phosphorylated, RsbW binds σ^B and the regulon is turned off. Sensing stress results in dephosphorylation of RsbV allowing it to bind RsbW and releasing σ^B, which interacts with RNAP and activates the σ^B regulon.

potentially σB-regulated genes (11), which merit further study as they could broaden understanding of σB activity. While animal studies with FPSS have not been reported, σB is an attractive therapeutic target because it is conserved in many pathogenic bacteria and is important in activation of virulence genes in several pathogenic bacteria (46-48). One potential drawback to the therapeutic potential of this molecule, however, is that σB is found in many bacteria including commensal strains of the microbiota, and may cause dysbiosis of the microbiota. This possibility remains to be determined.

CCG-2979

CCG-2979 (see Table 1.1 for structure) inhibits virulence by ultimately affecting the host response (See #3 in Figure 1.1). It targets the promoter of streptokinase (SK) (13), a host-specific virulence factor secreted by *Streptococcus pyogenes* and other group A streptococci (GAS), which activates the host zymogen plasminogen to form plasmin, the central protease of the fibrinolytic system critical for regulating blood clots (49, 50). The screen in which it was identified used a cell-based assay in which the SK gene promoter, *ska*, controlled expression of the kanamycin resistance gene; hits were considered those that decreased the growth of the reporter strain but not growth of a constitutive kanamycin-resistant strain. After screening 55,000 compounds, hits were triaged based on activity, commercial availability, bioavailability prediction, and low activity in other screens performed at that screening facility, and CCG-2979 was identified (13).

CCG-2979 reduces SK activity in a dose-dependent manner without inhibiting bacterial growth. Furthermore, GAS treated with five or 50 µM CCG-2979 were more susceptible to phagocytosis by host cells. To determine the effects of this molecule on bacterial gene expression, the effects of CCG-102487, a CCG-2979 analog, were investigated by microarray. The results showed that about 29% of GAS genes were altered by CCG-102487, including many virulence factors, genes involved in metabolism and energy production, as well as *ska* (13).

Because SK is host-specific, transgenic mice expressing the human plasminogen gene were used to determine the therapeutic potential of CCG-2979. Using this well-established model (50), mice were subcutaneously injected with GAS, given a day to establish an infection, then treated with compound intraperitoneally daily for five days. While CCG-102487 did not protect mice from GAS-induced mortality, CCG-2979-treated mice showed a statistically significant improvement in survival (13).

To determine the pharmacophore of CCG-2979, i.e. the chemical features essential for biological activity, its structure was altered and structure-activity relationship (SAR) studies were performed (14, 15). With this information, an analog (CCG-203592) was identified with a 35-fold increase in SK inhibition (14). *S. aureus* is also a major public health problem because it can form biofilms on implantable devices (51). CCG-203592 was found to inhibited biofilm formation of three different biofilm-producing GAS strains both in laboratory microtiter plates and on silicone, the most common surface used for implantable medical devices (14). These findings indicate that CCG-203592 may have therapeutic uses not only in clearing pre-formed GAS

infections, but also in preventing GAS biofilm formation on medical implants, making it an effective therapeutic lead.

Small Molecules that affect Host-Pathogen Interactions

Small molecules can provide valuable insight into underlying biological mechanisms in pathogenicity, and provide a relevant system for studying pathogens in the context of their hosts. Instead of screening compounds of unknown function for inhibition of pathogenesis, one can also screen compounds whose cellular effects have been previously characterized.

Pimozide

Pimozide (see table 1.1 for structure) was identified from a library of compounds with previously known cellular targets as one that inhibits *Listeria monocytogenes* infection (16). Pimozide is an FDA-approved antipsychotic molecule used to treat severe Tourette's syndrome and schizophrenia (52). Because the target is already known, such screens makes it easier to discover what processes are required for pathogenesis compared to a more open-ended screen with a large, diverse chemical library. *L. monocytogenes* infects macrophages (53) after which it escapes the phagocytic vacuole by producing a hemolysin, listeriolysin O (LLO) (#14 in Figure 1.1), and replicates in the cytosol before spreading to neighboring cells by polymerizing host cell actin to propel itself into adjacent cells (54, 55). While genetic and cellular biological

studies have uncovered much about *L. monocytogenes* pathogenicity, mechanisms regulating infection remain incompletely defined.

A chemical genetics approach was taken in which both bacteria and host cells were exposed to compounds (16). Primary bone marrow-derived macrophages (BMMs) were infected with *L. monocytogenes* constitutively expressing GFP to enable visualization of internalized bacteria. Four hundred and eighty compounds with diverse, known biological activities were screened, and 21 were found to alter *L. monocytogenes* infection in one of three ways, each of which could be monitored using GFP (16). The majority of inhibitory compounds inhibited BMM infection, as seen by a decrease in GFP fluorescence in BMMs relative to the DMSO control. Others enhanced bacterial uptake or intracellular replication, causing an increase in GFP fluorescence per BMM cell. Lastly, some compounds inhibited cell-to-cell spread (#15 in Figure 1.1), seen as an increase in GFP fluorescence per BMM, but only in very few cells.

Compounds that answered the screen fall into four categories based on the activities that they disrupt, and each category engenders testable hypotheses about conditions required for *L. monocytogenes* infection. The first category includes compounds that disrupt actin, which is required for phagocytosis of bacteria by BMMs and for cell-to-cell spread of *L. monocytogenes* (55); two compounds from the screen fall into this category. A second group of hits consists of four kinases/phosphatases. Such enzymes are important for actin rearrangement and were not further analyzed, though further study of these compounds could provide new insights on the role of specific kinases and phosphatases in *L. monocytogenes* infection. Six more

compounds were categorized together on the basis of lacking shared activities with other compounds from the screen; further studies on these may also yield new insights into *L. monocytogenes* infection. The fourth category, composed of nine compounds (43% of the hits), includes molecules that affect calcium pathways and probably play diverse roles in *Listeria* pathogenicity. Not only is calcium important in many cellular signaling pathways including phagocytosis (56), it is also released in response to protein kinase C (PKC) activation, which occurs during *L. monocytogenes* infection and modulates bacterial uptake and escape from the vacuole (57). Pimozide falls into this fourth category, and was chosen for further study.

Pimozide was shown to inhibit intracellular infection of BMMs by *L. monocytogenes* by one order of magnitude after a 10-hr treatment (16). While the exact mechanism for this is unclear, pimozide inhibited infection at three distinct steps. The most potent effect of pimozide was inhibition of macrophage phagocytosis of *L. monocytogenes*, as well as three other bacteria: *B. subtilis*, *Salmonella enterica* serovar Typhimurium, and *Escherichia coli* K12. Internalization by pimozide was inhibited by 99%, compared with by DMSO-treated BBM, and inhibition was calcium independent (16). Pimozide also reduced vacuolar escape of *L. monocytogenes* by 26%, although this was not due to inhibition of LLO. Finally, pimozide treatment decreased cell-to-cell spread by approximately 50% (16).

This screen generated many testable hypotheses regarding the cellular requirements of *L. monocytogenes* infection and revealed that pimozide can be used to probe the molecular mechanisms for BMM phagocytosis of bacteria in general, and of *L.*

monocytogenes specifically. This compound is an example of one that affects a particular host-pathogen system in multiple yet synergistic ways. Compounds like pimozide are particularly interesting as molecular probes because they illustrate the inter-relatedness and similarities of otherwise apparently different pathogenic mechanisms. Additionally, molecules like pimozide have increased therapeutic appeal because of the lowered probability of overcoming multiple effects by mutation.

Type 4 secretion inhibitors

Compounds with known biological activities have also been used to probe the type 4 secretion system (T4SS; also called the Icm/Dot Type IVB system) in *Legionella pneumophila*, which infects and replicates in lung alveolar macrophages and causes Legionnaires' disease (#9 in Figure 1.1) (58, 59). *L. pneumophila* avoids phagosome-lysosome fusion by using its T4SS to secrete effectors that interfere with vesicular trafficking, the host innate immune response, phosphoinositide metabolism, and ubiquitination (reviewed in (60)). Given its importance for intracellular survival and replication (61) the T4SS is an attractive target for drug development. The T4SS can be activated by contact with the host cell (62), but other signals that trigger secretion of effectors are not well understood.

Compounds with known biological targets were screened to probe more deeply the mechanisms of type 4 secretion in *Legionella* (18). An effector protein (LepA) was fused to the TEM-1-β-lactamase (BlaM), which can cleave a green substrate CCF4 to a blue product. Host cells were incubated with compounds for 24 hours, infected with the

L. pneumophila lepA-blaM reporter strain, and host-bacterial cell contact was initiated by a low-speed centrifugation step. One hour later, CCF4 was added and fluorescence was measured two hours later to quantify the amount of cleaved and uncleaved CFF4 (18).

Of 2,640 compounds screened, 86 tested positive in the screen, and 22 compounds remained after a triage step was performed to eliminate molecules that were toxic or inhibited the reporter itself (e.g. fluorescence quenchers). These 22 molecules inhibit translocation with efficiencies ranging from 63 to 100% and were categorized into groups based on known activity, including ionophore/protonophores, inhibitors of calmodulin, cytoskeleton dynamics, NF-κB, serine proteases, kinases or phosphatases, and others (18).

Ionophore/protonophores discharge membrane electric potential (Δψ) and collapse the proton gradient (pH), components of the PMF (#8 in Figure 1.1) (63, 64). All three identified ionophore/protonophores inhibited Icm/Dot-dependent lysis of red blood cells by *L. pneumophila* (18), indicating that translocation of effectors is at least partially dependent on the PMF. Furthermore, one ionophore, CCCP (shown in Table 1.1), inhibited LepA translocation in *L. pneumophila* in a dose-dependent and reversible manner (18). The PMF was not previously known to play a role in T4SS-mediated translocation, so this discovery points to the value of small molecule screening for uncovering previously unknown pathogenicity mechanisms.

The majority of the inhibitors identified in this screen are molecules previously known to affect host cytoskeleton dynamics, including molecules that affect tubulin (#12

in Figure 1.1), actin (#13 in Figure 1.1), and PI3K. They likely inhibit phagocytosis (#10 in Figure 1.1), a key function of macrophages. In fact, 19 of the 22 identified inhibitors were found to significantly affect the ability of macrophages to phagocytose other bacteria (18). To test whether phagocytosis is required for translocation of *L. pneumophila* Icm/Dot effector proteins, cytoskeleton inhibitors were used to inhibit coiling phagocytosis, the type of phagocytosis used by *L. pneumophila* to enter macrophages (65). Host-bacterial contact was instead initiated by opsonization, a mechanism quite different from coiling phagocytosis (66). Opsonization of *L. pneumophila* with *L. pneumophila*-specific antibodies restored effector translocation in the absence of phagocytosis. That is, when host cells and *L. pneumophila* were co-incubated with antibody, the cytoskeleton inhibitors did not increase the amount of bacterial uptake, but LepA and RalF were translocated to 90% of the level reached in DMSO- treated samples. In fact, Fc signaling is not required for triggering translocation, because when CHO cells expressing a non-signaling mutant of the FC receptor FcγRIIA (Y2F/Y3F) were used, opsonization still stimulated translocation (18). These results indicate that host-cell binding, and not phagocytosis, is required for translocation by the Icm/Dot system and supports a model where the T4SS in *L. pneumophila* is in a “locked and loaded” state, ready to inject effector proteins upon contact with a host cell (#11 in Figure 1.1).

Another important finding made from this study came by further analyzing the translocation inhibitor, RWJ-60475 (shown in Table 1.1), which was previously known to

inhibit a receptor tyrosine phosphate phosphatase (67). Work with this compound revealed that CD45 and CD148 are required to phagocytose *L. pneumophila* (18).

From screening just 2,500 small molecules, a wealth of new information about the T4SS in *L. pneumophila* was uncovered and many new testable hypotheses were generated. New information relating phagocytosis and effector translocation with PMF, host cell contact and CD45 or CD148 was uncovered, providing a good example of how chemical genetics can reveal pathogenicity mechanisms.

LED209

Pathogens generally initiate virulence gene expression in response to host environments. As examples, *E. coli*, *S. typhimurium*, and *Francisella tularensis*, express a sensor histidine kinase called QseC that detects host-derived adrenergic signals (epinephrine and norepinephrine) as well as the quorum-sensing autoinducer-3 (AI-3) (68, 69). In response to either of these signals, QseC autophosphorylates then phosphorylates the transcription factor QseB, leading to transcription of key virulence genes including the LEE1 operon in enterohaemorrhagic *E. coli* (EHEC) (#6 in Figure 1.1) (70).

A chemical library of 150,000 compounds was screened for those that block expression of a LEE1-*lacZ* reporter strain (19). Compounds were screened in an assay using spent media (which contains AI-3) to activate QseC and induce reporter gene expression. The most potent inhibitor identified was ultimately improved by SAR and named LED209 (see Table 1.1 for structure) (19).

As expected, LED209 did not inhibit bacterial growth, but rather selectively inhibited virulence gene expression by inhibiting QseC autophosphorylation (19). While LED209 was ineffective in an infant rabbit model of EHEC infection (perhaps due to rapid absorption from the gastrointestinal tract) (19), it significantly reduced mouse pathogenicity of both *S. typhimurium* and *F. tularensis*, which express QseC homologues of 87% and 57% similarity, respectively, to EHEC QseC (19). QseC is important for motility in *S. typhimurium* (71) and for systemic infection in *F. tularensis* (72).

Similar to virstatin, LED209 inhibits virulence by targeting a specific virulence regulator without affecting growth. It is a particularly attractive drug candidate because its target, QseC, is conserved in over 25 pathogenic bacteria but absent in mammals (19), giving this molecule a bacteria-specific broad spectrum of activity.

Small molecules that target structures

Another effective anti-virulence strategy is inhibiting the formation of structures required for virulence. Pathogens have multiple conserved mechanisms to deliver effector proteins into cells, such as the T4SS discussed above, and inhibiting the formation of structures critical for these mechanisms is an attractive anti-pathogen strategy. Several studies suggest this is a druggable target (73-76).

TSS29

In addition to type 4 secretion, the type 3 secretion system (T3SS) injectosome has been targeted to develop therapeutic leads given its broad conservation in Gram-negative pathogens (#7 in Figure 1.1). A screen to inhibit the *S. typhimurium* T3SS was developed by using a reporter strain that secretes phospholipase in a T3SS-dependent manner (20). Phospholipase activity of a culture was determined by adding the phospholipase substrate PED6, whose cleavage product is fluorescent and can be quantified on a plate reader. After screening 92,000 natural and synthetic small molecules, 89 compounds were identified and triaged to exclude those with no novelty or potential for drug development, those that inhibit bacterial translation, inhibit sec-dependent secretion and/or disulfide bond isomerization, or decrease expression of the T3SS components. One compound, a 2-imino-5-arylidene thiazolidinone, was investigated further (20). This compound, termed TTS29, inhibited effector secretion in a dose-dependent manner. Needle complexes from cultures grown with TTS29 displayed an overall reduction in protein levels, but the needle complex protein constituents were not reduced overall in whole cell lysates. This finding indicated that the proteins were being produced, but that their assembly into the needle complex was inhibited by TTS29 (20).

Because components of the T3SS are conserved in different bacteria, TTS29 has the potential to work against other T3S-encoding bacteria. In *Yersinia* species, there are two types of T3SS: the plasmid-encoded Ysc system in *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*, which secretes Yops (*Yersinia* outer

proteins) into the cytosol of target cells, and the chromosomally encoded Ysa system in *Y. enterocolitica*, which secrets Ysps (*Yersinia* secreted proteins) (77, 78). TSS29 inhibited secretion of both Yops and Ysps into *Y. enterocolitica* culture supernatants, indicating its potential utility as a broad inhibitor of T3SS (20). In contrast, TSS29 did not alter flagellar motility or decrease the levels of flagellar components in either *S. typhimurium* or *Pseudomonas aeruginosa*, bacteria that depend on a flagellar-specific T3SS for flagellar motility (20). This suggests that TSS29 targets a part of the T3SS that is not conserved with the evolutionarily related flagellar-specific T3SS (78).

However, as one component of the T3SS is conserved in the type 2 secretion system (T2SS) that delivers enzymes and other proteins across the Gram-negative envelope (79), TSS29 was tested for its ability to inhibit such systems to determine if its target is shared between the two secretion systems. Secretion of elastase (80) by the T2SS in *P. aeruginosa* was inhibited by TSS29, as was twitching motility, which is determined by the type four pilus (T4P) that has components similar to those of the T2SS (20). These results demonstrate that TSS29 is a broad inhibitor of secretion that affects multiple secretion systems in multiple bacterial species.

The *in vivo* effectiveness of TSS29 was supported by demonstrating its ability to reduce killing of bone marrow derived macrophages in a tissue culture model of infection (20). It also inhibits *Pseudomonas syringae pv tomato* DC3000 from inducing a hypersensitivity response in Tobacco plants (20). Thus, TSS29 has wide therapeutic potential because it can target a range of secretion systems in different bacterial species.

A22

A22 (shown in Table 1.1) has had a large impact on basic biology, having led the way to discoveries that unraveled the mystery behind bacterial replication, chromosome segregation, and cellular division (21, 22). Prior to discovery of A22, it was known that maintaining a proper rod shape was required for chromosomal partitioning in *E. coli* (81), although how the rod shape was maintained or why it was required was less understood. A22 played a major role in uncovering the function of MreB in bacterial chromosome replication and division (#5 in Figure 1.1), and was instrumental in eliminating a previously proposed model of chromosomal segregation that hypothesized that replication may provide the driving force for segregation.

This compound emerged in a screen for inhibitors of chromosome partitioning in *E. coli*; such molecules can induce formation of anucleate cells (21). Anucleate cells arise when cells divide before correctly segregating replicated chromosomes (81). Unlike mecillinam, an antibiotic that specifically inhibits penicillin-binding protein 2 (Pbp2) and thus blocks cell division, A22 bound none of the seven known PBPs *in vitro*, nor did it affect Pbp2 activity (21).

The compound was helpful for probing the molecular mechanism underlying chromosome segregation. Prior evidence suggested that MreB plays a role in chromosome segregation (82), but proving this was difficult because loss of *mreB* function is lethal and MreB depletion is slow and pleiotropic, disrupting processes unrelated to chromosome segregation such as cell shape, polar protein localization, and

cell division. Further analysis of A22 determined that it directly targets MreB (22). Colonies resistant to the compound had missense mutations in the *mreB* gene, some of which mapped to a predicted ATP binding pocket or to a helix that could contact ATP in that pocket (22). Biochemical experiments demonstrated MreB binds A22 with micromolar affinity in its nucleotide-binding pocket, which sterically inhibits ATP binding and prevents MreB polymerization (83).

Unlike in untreated cells where MreB localized in spirals along the length of the cell and in rings around the site of cellular division (84-86), in A22-treated cells MreB was dispersed evenly (22). Furthermore, when A22 was washed out of the cells, those that had MreB rings before A22 treatment recovered rings after A22 was washed out, cells that had spirals recovered spirals, and cells with partially compacted rings recovered partially compacted rings (22). This finding indicated that MreB localization is determined by other factors and not by MreB itself. Furthermore, the A22-resistant mutant (T158A) was unable to condense MreB into rings, suggesting that ATP hydrolysis is important for regulating MreB dynamics (22).

Chromosome segregation was monitored using a fluorescent repressor-operator system (FROS) in the presence and absence of A22, revealing that the compound prevented segregation, although the cells doubled in size (22). That A22 was simply inhibiting replication was ruled out by comparing incorporation of radiolabeled nucleotides in the presence of A22 or a known replication inhibitor hydroxyurea (HU) (22). Thus, by investigating the mechanism of A22 action a previously unknown role for

MreB activity in chromosome segregation was identified, and a previous hypothesis proposing that replication drives segregation was ruled out.

A22 has been a very useful tool for studying the mechanisms involved in cell shape maintenance and MreB function. It led to the discovery that MreB exhibits treadmilling *in vivo*, similar to actin treadmilling in eukaryotes (#13 in Figure 1.1) (87), and was instrumental in demonstrating the localization pattern of the MreC protein (88). A22 was used to show that MreB activity is important for effector protein secretion in *S. flexneri* (23), for motility in *Myxococcus xanthus* (89), and for tethering lipid II biosynthesis in *C. pneumoniae* (90). The fact that chromosome segregation is affected by A22 in many but not all MreB-encoding bacteria suggests that in some bacteria, alternate mechanisms exist for chromosome segregation (23).

Pilicides

While some microbes secrete pilus adherence structures through type 2-like secretion systems that can be inhibited by TSS29 as described above, others use pili assembled by pathways that rely on a periplasmic chaperone (91-93) and an outer membrane usher (94, 95) (see (96, 97) for reviews). The chaperone mediates the folding, stabilization, and transport of pili subunits, while the usher incorporates the subunits into the growing pilus (97).

Because this form of pilus assembly is so conserved in a wide range of pathogens (97), inhibitors that target the chaperone-usher systems are effective against a broad range of bacterial species. Substituted bicyclic 2-pyridones, called pilicides

(pharmacophore shown in Table 1.1), are a well-studied group of synthetic small molecule inhibitors that prevent the formation of pili in Uropathogenic *E. coli* (UPEC) (97).

UPEC produce two types of disease-associated pili- P pili (encoded by the *pap* genes) and Type 1 pili (encoded by the *fim* genes) shown in Figure 1.3 (97). P pili are made up of PapA (the major subunit), E, F, G, H, and K, and are assembled by the PapD chaperone and the PapC usher. Type 1 pili are made up of FimA (the major subunit), G, and H, and are assembled by the chaperon FimC and the usher FimD (97).

Whereas most small molecule inhibitors were discovered by screening compound libraries, pilicides were designed using a rational design approach (24). Knowing that pilus biogenesis requires the chaperone protein to bind its natural ligands (98), the solved crystal structure of the PapD-PapG complex was used to chemically design molecular mimetics that would bind within the active site of the periplasmic chaperones PapD and FimC (24). A library of pilicides was synthesized and subsequently shown to be effective at inhibiting pilus formation by electron microscopy (27).

After determining that pilicides do not affect cell growth or viability, pilicides were shown to inhibit pili-dependent phenotypes including type 1 pili-mediated mannose-sensitive hemagglutination (MSHA) and P pili-mediated hemagglutination (HA), indicating that pilicides affect pili-dependent phenomena *in vivo* (27). Pilicides also reduced biofilm formation and bacterial attachment to host cells, both of which are dependent on type 1 pili (27). Pilicide **2c** in particular reduced the ability of *E. coli*

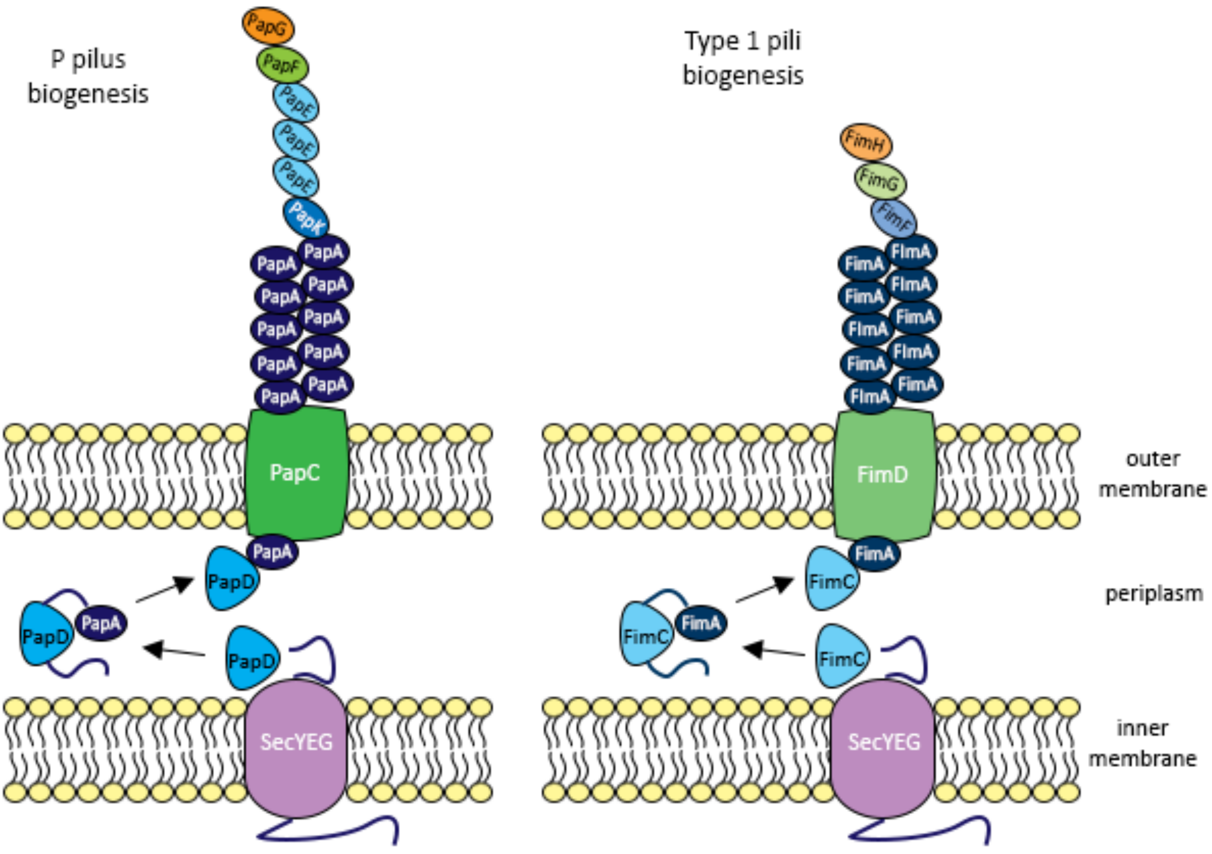


Figure 1.3 P pili and type 1 pili are structurally similar but encoded by different genes.

A diagram illustrating the similarities and differences in the P pilus (encoded by *papA-G*) and the Type 1 pili (encoded by *FimA-H*). Pili consist of several repeating subunits arranged in a helical structure. These subunits are translocated from the cytoplasm to the periplasm where a chaperone (PapD or FimC) folds the protein, stabilizes it, and transfers it to the usher protein (PapC or FimD), which secretes the protein and incorporates it into the pilus structure.

strains to adhere to cultured bladder cells by 90% (27). Taken together, these results strongly indicate that pilicides inhibit production of both P pili and type 1 fimbriae in *E. coli*.

To gain a deeper appreciation for the mechanism of pilicide inhibition, pilicide **2c** was co-crystalized with the PapD chaperone (27). The resulting crystal structure showed that pilicide **2c** forms close contacts with a hydrophobic patch that runs across the back of the F1-C1-D1 beta sheet on PapD, a region that is highly conserved in all pili periplasmic chaperones, and which may mediate interactions between the periplasmic chaperone and the N-terminal domain of the usher protein (99). The finding that pilicides bind to the site of chaperone-usher interaction suggests that pilicides work by preventing the interaction of the usher with the chaperone. Supporting this proposed mechanism is the finding that a point mutation (R58A) in the hydrophobic patch of PapD displays a marked reduction in pili production but is still able to bind, fold, and stabilize pilus subunits (27). Furthermore, pilicide **2c** was shown to inhibit FimC-FimH from binding to FimD using surface plasmon resonance (27), indicating that pilicide **2c** inhibits pili biogenesis by preventing the chaperone from passing the subunit to the usher.

Pilicides have uncovered new knowledge about the molecular mechanisms involved in pili biogenesis, and serve as an example of how crystallography can guide chemists to create a small molecule with a predictable activity, a technique called structure-based drug design (SBDD). SBDD is also useful for drug development

because resulting co-crystal structures provide information on compound-target binding interactions that can be modified to improve in the activity of the compound.

Advantages and Challenges of Chemical Genetics

Advantages

Chemical genetics allows scientists to probe biological pathways in new ways, giving us a new perspective on the requirements of pathogenesis. As biological probes, small molecule inhibitors are useful tools because they allow for the manipulation of processes that are genetically difficult to regulate. For example, genes that are essential for viability (such as *mreB*) are difficult to study with conventional genetics, but can be easily studied using these compounds. Genes whose deletion results in pleiotropic effects (such as sigma factors) can be more carefully studied with chemical inhibitors. In organisms where genetic manipulation is impossible or very difficult and time consuming, small molecule inhibitors provide an alternative avenue for studying conditions required for pathogenesis. In addition, small molecules act quickly, reversibly, and their effects can be titrated with dose.

From a therapeutic perspective, small molecule studies serve as a starting point for drug development, and give insight into the druggability of a particular protein or molecular target. Additionally, small molecules can be designed to target a certain species or a broad range of species by choosing a target that is specific to one organism or conserved among many species. For example, virstatin was designed with extreme specificity, acting only on *V. cholerae*. On the other hand, TSS29 inhibits the

type 3 secretion system, which is conserved across many species of bacteria, and therefore has a broader range of activity.

Challenges

Despite the wealth of information gained from chemical genetics, there are challenges inherent to small molecule screens. For one, compounds can have multiple targets and/or side effects (e.g. pimozide), which can confound the interpretation of results. Because of this, experiments with small molecules must be carefully controlled.

Possibly the largest hurdle in studying compounds identified in chemical screens is the precise identification of their molecular targets and elucidation of their mechanisms of action. New methods for target identification are constantly being developed and improved and have recently been the subject of a review (100). Screening molecules of known biological function helps avoid this issue altogether; however, some molecules have more than one biological activity, which can confound target identification and characterization.

One method of discovering the target of an inhibitor is to use a targeted "loss-of-target" approach. An example of this was demonstrated in uncovering the target of virstatin by testing known components of the toxin regulatory cascade to determine what was or was not affected by virstatin (8). After identifying ToxT as the target, the mechanism of virstatin action was investigated by screening for resistant mutants (8). As sequencing of entire genomes becomes more affordable, this method is becoming

increasingly useful. This loss-of-target approach was also used to identify targets of LED209 (19) and of TTS29 (20).

Another approach for target identification is to compare gene expression profiles of cells in the presence or absence of the inhibitor, which, combined with gene set enrichment analysis (GSEA), was used to determine the target of FPSS (11). Along the same vein, insight on the mechanism of a compounds activity can be gained by using technologies such as MUDpit (101) to analyze global changes in protein levels, or RNAseq (102) to look at global changes in transcript levels of both coding and non-coding RNAs.

In recent years, click-chemistry has also been adapted to uncover binding partner(s) of small molecules (103). In this approach, a small molecule inhibitor is modified such that it has two reactive handles. The first will “click” the small molecule to its target, covalently linking the compound to its target. The second handle can be biotinylated, allowing the compound-target complex to be purified on a column. After a series of washes, the eluate is analyzed by mass spectrometry to identify the protein(s) with which the small molecule interacts.

Understanding the mechanisms by which bacteria regulate virulence and cause disease has long been an area of active research. The use of chemical genetics has enriched this field by enabling us to deepen our knowledge of the basic molecular mechanisms underlying pathogenesis while simultaneously testing the druggability of these mechanisms. In many cases, chemical genetic studies have provided lead compounds with therapeutic potential that can be developed into treatment therapies.

Vibrio cholerae pathogenesis

For this thesis, a chemical genetics approach was used to probe the cellular requirements for virulence gene expression in *Vibrio cholerae*. *V. cholerae* is a Gram-negative rod that causes the human disease cholera (104). Found in coastal regions world-wide, it can live planktonically in fresh or brackish waters, or in association with copepods (105). *V. cholerae* is transmitted to humans either by the fecal-oral route, or by consumption of contaminated food or water allowing the bacteria access to the upper small intestine, where they secrete cholera toxin (CT; encoded by the genes *ctxAB*) (106). CT binds to and enters intestinal epithelial cells and increases cAMP production (106) causing a decrease in sodium uptake and an accompanying increase in chloride secretion, resulting in secretion of water into the lumen of the intestine and leading to severe diarrhea and dehydration (107).

The main method of treating cholera is oral rehydration therapy (ORT), in which a solution of glucose, sodium, potassium, and citrate is administered to counteract the severe dehydration caused by CT (108, 109). While antibiotics are effective at killing most *V. cholerae* strains, they do little to offset the severe diarrhea and dehydration caused by *V. cholerae*, and are therefore not sufficient as a monotherapy and are instead used to supplement ORT (104, 108).

The model in Figure 2.6 of Chapter 2 depicts the cascade of transcriptional regulators that control virulence in *V. cholerae* (110). ToxT, shown in orange, is the master regulator of virulence and activates over 20 genes, including CT and a

colonization factor, the toxin co-regulated pilus (TCP) (34). Transcription of *toxT* is regulated by a unique complex of inner membrane proteins, ToxR and its effector protein ToxS, and TcpP and its effector protein TcpH (31, 33, 110-113). The *tcpPH* operon is highly transcriptionally regulated, with AphA and AphB required to activate transcription of the *tcpP* promoter (114, 115), while HNS (116), cAMP-CRP (117), and PhoB (118) are known to repress *tcpP* transcription.

TcpP is also regulated at the post-translational level. When cells are shifted from growth under toxin-inducing to non-inducing conditions, TcpP is degraded by a two-step process. The site 1 protease, Tsp, cleaves TcpP in the inner membrane (Teoh, W.P. and DiRita, V.J., unpublished), producing the substrate for YaeL cleavage (119), and complete degradation of TcpP, resulting in the termination of the virulence cascade.

As noted above, a small molecule inhibitor of ToxT function, virstatin, was identified by screening a compound library for inhibitors of *ctx* gene expression (8). For my thesis research, a small molecule screen was performed to identify compounds that inhibit expression of *toxT*, and these would be predicted to decrease CT production in *V. cholerae*. Such a compound could theoretically reduce disease severity and duration, and would therefore be potentially useful in treating cholera in regions of the world where cholera is endemic, and would also have potential as a prophylactic in areas such as refugee camps or military bases that suffer frequent cholera outbreaks.

While the compounds identified in this small molecule screen would need decades of development before being potentially used for human health, their value as molecular probes can be exploited right away to deepen our understanding of the

cellular requirements for *V. cholerae* pathogenesis, and this is the focus of my dissertation.

This dissertation describes the identification and characterization of toxtazins A and B, small molecules that inhibit *toxT* gene expression leading to decreases in expression of genes encoding toxin and toxin-coregulated pilus. The two toxtazin molecules act at distinct steps in the regulatory pathway and one of them, toxtazin B, reduces the load of *V. cholerae* by two orders of magnitude in a mouse colonization model.

References

1. **Clardy J, Fischbach MA, Currie CR.** 2009. The natural history of antibiotics. Current biology : CB **19:**R437-441.
2. **D'Costa VM, McGrann KM, Hughes DW, Wright GD.** 2006. Sampling the antibiotic resistome. Science **311:**374-377.
3. **McFarland LV.** 2008. Antibiotic-associated diarrhea: epidemiology, trends and treatment. Future Microbiol **3:**563-578.
4. **Sekirov I, Russell SL, Antunes LC, Finlay BB.** 2010. Gut microbiota in health and disease. Physiol Rev **90:**859-904.
5. **Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH, Society for Healthcare Epidemiology of A, Infectious Diseases Society of A.** 2010. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America **31:**431-455.
6. **Center for Disease Control and Prevention.** 2013. Antibiotic resistance threats in the United States, 2013.
7. **Roberts RR, Hota B, Ahmad I, Scott RD, 2nd, Foster SD, Abbasi F, Schabowski S, Kampe LM, Ciavarella GG, Supino M, Naples J, Cordell R, Levy SB, Weinstein RA.** 2009. Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. Clin Infect Dis **49:**1175-1184.
8. **Hung DT, Shakhnovich EA, Pierson E, Mekalanos JJ.** 2005. Small-molecule inhibitor of Vibrio cholerae virulence and intestinal colonization. Science **310:**670-674.
9. **Shakhnovich EA, Hung DT, Pierson E, Lee K, Mekalanos JJ.** 2007. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proceedings of the National Academy of Sciences of the United States of America **104:**2372-2377.
10. **Shakhnovich EA, Sturtevant D, Mekalanos JJ.** 2007. Molecular mechanisms of virstatin resistance by non-O1/non-O139 strains of Vibrio cholerae. Molecular microbiology **66:**1331-1341.
11. **Palmer ME, Chaturongakul S, Wiedmann M, Boor KJ.** 2011. The Listeria monocytogenes sigmaB regulon and its virulence-associated functions are inhibited by a small molecule. mBio **2.**
12. **Ringus DL, Gaballa A, Helmann JD, Wiedmann M, Boor KJ.** 2013. Fluoro-phenyl-styrene-sulfonamide, a novel inhibitor of sigmaB activity, prevents the activation of sigmaB by environmental and energy stresses in Bacillus subtilis. Journal of bacteriology **195:**2509-2517.
13. **Sun H, Xu Y, Sitkiewicz I, Ma Y, Wang X, Yestrepsky BD, Huang Y, Lapadatescu MC, Larsen MJ, Larsen SD, Musser JM, Ginsburg D.** 2012. Inhibitor of streptokinase gene expression improves survival after group A streptococcus

infection in mice. Proceedings of the National Academy of Sciences of the United States of America **109:**3469-3474.

14. **Ma Y, Xu Y, Yestrepsky BD, Sorenson RJ, Chen M, Larsen SD, Sun H.** 2012. Novel inhibitors of Staphylococcus aureus virulence gene expression and biofilm formation. PloS one **7:**e47255.
15. **Yestrepsky BD, Xu Y, Breen ME, Li X, Rajeswaran WG, Ryu JG, Sorenson RJ, Tsume Y, Wilson MW, Zhang W, Sun D, Sun H, Larsen SD.** 2013. Novel inhibitors of bacterial virulence: development of 5,6-dihydrobenzo[h]quinazolin-4(3H)-ones for the inhibition of group A streptococcal streptokinase expression. Bioorganic & medicinal chemistry **21:**1880-1897.
16. **Lieberman LA, Higgins DE.** 2009. A small-molecule screen identifies the antipsychotic drug pimozide as an inhibitor of Listeria monocytogenes infection. Antimicrobial agents and chemotherapy **53:**756-764.
17. **Lieberman LA, Higgins DE.** 2010. Inhibition of Listeria monocytogenes infection by neurological drugs. International journal of antimicrobial agents **35:**292-296.
18. **Charpentier X, Gabay JE, Reyes M, Zhu JW, Weiss A, Shuman HA.** 2009. Chemical genetics reveals bacterial and host cell functions critical for type IV effector translocation by Legionella pneumophila. PLoS pathogens **5:**e1000501.
19. **Rasko DA, Moreira CG, Li de R, Reading NC, Ritchie JM, Waldor MK, Williams N, Taussig R, Wei S, Roth M, Hughes DT, Huntley JF, Fina MW, Falck JR, Sperandio V.** 2008. Targeting QseC signaling and virulence for antibiotic development. Science **321:**1078-1080.
20. **Felise HB, Nguyen HV, Pfuetzner RA, Barry KC, Jackson SR, Blanc MP, Bronstein PA, Kline T, Miller SI.** 2008. An inhibitor of gram-negative bacterial virulence protein secretion. Cell host & microbe **4:**325-336.
21. **Iwai N, Nagai K, Wachi M.** 2002. Novel S-benzylisothiourea compound that induces spherical cells in Escherichia coli probably by acting on a rod-shape-determining protein(s) other than penicillin-binding protein 2. Bioscience, biotechnology, and biochemistry **66:**2658-2662.
22. **Gitai Z, Dye NA, Reisenauer A, Wachi M, Shapiro L.** 2005. MreB actin-mediated segregation of a specific region of a bacterial chromosome. Cell **120:**329-341.
23. **Noguchi N, Yanagimoto K, Nakaminami H, Wakabayashi M, Iwai N, Wachi M, Sasatsu M.** 2008. Anti-infectious effect of S-benzylisothiourea compound A22, which inhibits the actin-like protein, MreB, in Shigella flexneri. Biological & pharmaceutical bulletin **31:**1327-1332.
24. **Svensson A, Fex T, Kihlberg J.** 2000. Preparation of fluorinated linkers: use of 19F NMR spectroscopy to establish conditions for solid-phase synthesis of pilicide libraries. Journal of combinatorial chemistry **2:**736-748.
25. **Hedenstrom M, Emtenas H, Pemberton N, Aberg V, Hultgren SJ, Pinkner JS, Tegman V, Almqvist F, Sethson I, Kihlberg J.** 2005. NMR studies of interactions between periplasmic chaperones from uropathogenic E. coli and pilicides that interfere with chaperone function and pilus assembly. Organic & biomolecular chemistry **3:**4193-4200.
26. **Aberg V, Hedenstrom M, Pinkner JS, Hultgren SJ, Almqvist F.** 2005. C-Terminal properties are important for ring-fused 2-pyridones that interfere with the

chaperone function in uropathogenic E. coli. Organic & biomolecular chemistry **3:**3886-3892.
27. **Pinkner JS, Remaut H, Buelens F, Miller E, Aberg V, Pemberton N, Hedenstrom M, Larsson A, Seed P, Waksman G, Hultgren SJ, Almqvist F.** 2006. Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. Proceedings of the National Academy of Sciences of the United States of America **103:**17897-17902.
28. **Matson JS, Withey JH, DiRita VJ.** 2007. Regulatory networks controlling Vibrio cholerae virulence gene expression. Infection and immunity **75:**5542-5549.
29. **Beck NA, Krukonis ES, DiRita VJ.** 2004. TcpH influences virulence gene expression in Vibrio cholerae by inhibiting degradation of the transcription activator TcpP. Journal of bacteriology **186:**8309-8316.
30. **Krukonis ES, DiRita VJ.** 2003. DNA binding and ToxR responsiveness by the wing domain of TcpP, an activator of virulence gene expression in Vibrio cholerae. Molecular cell **12:**157-165.
31. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.
32. **Yu RR, DiRita VJ.** 2002. Regulation of gene expression in Vibrio cholerae by ToxT involves both antirepression and RNA polymerase stimulation. Molecular microbiology **43:**119-134.
33. **DiRita VJ.** 1992. Co-ordinate expression of virulence genes by ToxR in Vibrio cholerae. Molecular microbiology **6:**451-458.
34. **DiRita VJ, Parsot C, Jander G, Mekalanos JJ.** 1991. Regulatory cascade controls virulence in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **88:**5403-5407.
35. **Hsiao A, Xu X, Kan B, Kulkarni RV, Zhu J.** 2009. Direct regulation by the Vibrio cholerae regulator ToxT to modulate colonization and anticolonization pilus expression. Infection and immunity **77:**1383-1388.
36. **Ferreira A, O'Byrne CP, Boor KJ.** 2001. Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in Listeria monocytogenes. Applied and environmental microbiology **67:**4454-4457.
37. **Xayarath B, Freitag NE.** 2012. Optimizing the balance between host and environmental survival skills: lessons learned from Listeria monocytogenes. Future Microbiol **7:**839-852.
38. **Chaturongakul S, Raengpradub S, Palmer ME, Bergholz TM, Orsi RH, Hu Y, Ollinger J, Wiedmann M, Boor KJ.** 2011. Transcriptomic and phenotypic analyses identify coregulated, overlapping regulons among PrfA, CtsR, HrcA, and the alternative sigma factors sigmaB, sigmaC, sigmaH, and sigmaL in Listeria monocytogenes. Applied and environmental microbiology **77:**187-200.
39. **Llamas MA, van der Sar A, Chu BC, Sparrius M, Vogel HJ, Bitter W.** 2009. A Novel extracytoplasmic function (ECF) sigma factor regulates virulence in Pseudomonas aeruginosa. PLoS pathogens **5:**e1000572.
40. **Raman S, Hazra R, Dascher CC, Husson RN.** 2004. Transcription regulation by the Mycobacterium tuberculosis alternative sigma factor SigD and its role in virulence. Journal of bacteriology **186:**6605-6616.

41. **Yin Y, Withers TR, Wang X, Yu HD.** 2013. Evidence for sigma factor competition in the regulation of alginate production by Pseudomonas aeruginosa. PloS one **8:**e72329.
42. **Kazmierczak MJ, Wiedmann M, Boor KJ.** 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiology and molecular biology reviews : MMBR **69:**527-543.
43. **Hecker M, Pane-Farre J, Volker U.** 2007. SigB-dependent general stress response in Bacillus subtilis and related gram-positive bacteria. Annual review of microbiology **61:**215-236.
44. **Benson AK, Haldenwang WG.** 1993. Bacillus subtilis sigma B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase. Proceedings of the National Academy of Sciences of the United States of America **90:**2330-2334.
45. **Hecker M, Volker U.** 2001. General stress response of Bacillus subtilis and other bacteria. Advances in microbial physiology **44:**35-91.
46. **Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M.** 2003. Listeria monocytogenes sigma B regulates stress response and virulence functions. Journal of bacteriology **185:**5722-5734.
47. **Depke M, Burian M, Schafer T, Broker BM, Ohlsen K, Volker U.** 2012. The alternative sigma factor B modulates virulence gene expression in a murine Staphylococcus aureus infection model but does not influence kidney gene expression pattern of the host. International journal of medical microbiology : IJMM **302:**33-39.
48. **Fouet A, Namy O, Lambert G.** 2000. Characterization of the operon encoding the alternative sigma(B) factor from Bacillus anthracis and its role in virulence. Journal of bacteriology **182:**5036-5045.
49. **Boxrud PD, Fay WP, Bock PE.** 2000. Streptokinase binds to human plasmin with high affinity, perturbs the plasmin active site, and induces expression of a substrate recognition exosite for plasminogen. The Journal of biological chemistry **275:**14579-14589.
50. **Sun H, Ringdahl U, Homeister JW, Fay WP, Engleberg NC, Yang AY, Rozek LS, Wang X, Sjobring U, Ginsburg D.** 2004. Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. Science **305:**1283-1286.
51. **Kokai-Kun JF, Chanturiya T, Mond JJ.** 2009. Lysostaphin eradicates established Staphylococcus aureus biofilms in jugular vein catheterized mice. The Journal of antimicrobial chemotherapy **64:**94-100.
52. **Tueth MJ, Cheong JA.** 1993. Clinical uses of pimozide. Southern medical journal **86:**344-349.
53. **Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P.** 1987. In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2. Infection and immunity **55:**2822-2829.
54. **Portnoy DA, Jacks PS, Hinrichs DJ.** 1988. Role of hemolysin for the intracellular growth of Listeria monocytogenes. The Journal of experimental medicine **167:**1459-1471.

55. **Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P.** 1992. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell **68:**521-531.
56. **TranVan Nhieu G, Clair C, Grompone G, Sansonetti P.** 2004. Calcium signalling during cell interactions with bacterial pathogens. Biology of the cell / under the auspices of the European Cell Biology Organization **96:**93-101.
57. **Wadsworth SJ, Goldfine H.** 2002. Mobilization of protein kinase C in macrophages induced by Listeria monocytogenes affects its internalization and escape from the phagosome. Infection and immunity **70:**4650-4660.
58. **Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brachman PS.** 1977. Legionnaires' disease: description of an epidemic of pneumonia. The New England journal of medicine **297:**1189-1197.
59. **McDade JE, Shepard CC, Fraser DW, Tsai TR, Redus MA, Dowdle WR.** 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. The New England journal of medicine **297:**1197-1203.
60. **Horwitz MA.** 1983. The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes. The Journal of experimental medicine **158:**2108-2126.
61. **Vogel JP, Andrews HL, Wong SK, Isberg RR.** 1998. Conjugative transfer by the virulence system of Legionella pneumophila. Science **279:**873-876.
62. **Chen J, Reyes M, Clarke M, Shuman HA.** 2007. Host cell-dependent secretion and translocation of the LepA and LepB effectors of Legionella pneumophila. Cellular microbiology **9:**1660-1671.
63. **Jana B, Panja S, Saha S, Basu T.** 2009. Mechanism of protonophores-mediated induction of heat-shock response in Escherichia coli. BMC microbiology **9:**20.
64. **Bakker EP, Mangerich WE.** 1981. Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. Journal of bacteriology **147:**820-826.
65. **Horwitz MA.** 1984. Phagocytosis of the Legionnaires' disease bacterium (Legionella pneumophila) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36:**27-33.
66. **Husmann LK, Johnson W.** 1992. Adherence of Legionella pneumophila to guinea pig peritoneal macrophages, J774 mouse macrophages, and undifferentiated U937 human monocytes: role of Fc and complement receptors. Infection and immunity **60:**5212-5218.
67. **Beers SA, Malloy EA, Wu W, Wachter MP, Gunnia U, Cavender D, Harris C, Davis J, Brosius R, Pellegrino-Gensey JL, Siekierka J.** 1997. Nitroarylhydroxymethylphosphonic acids as inhibitors of CD45. Bioorganic & medicinal chemistry **5:**2203-2211.
68. **Hughes DT, Sperandio V.** 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nature reviews. Microbiology **6:**111-120.
69. **Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB.** 2003. Bacteria-host communication: the language of hormones. Proceedings of the National Academy of Sciences of the United States of America **100:**8951-8956.

70. **Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V.** 2006. The QseC sensor kinase: a bacterial adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America **103:**10420-10425.
71. **Bearson BL, Bearson SM.** 2008. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of Salmonella enterica serovar Typhimurium. Microbial pathogenesis **44:**271-278.
72. **Weiss DS, Brotcke A, Henry T, Margolis JJ, Chan K, Monack DM.** 2007. In vivo negative selection screen identifies genes required for Francisella virulence. Proceedings of the National Academy of Sciences of the United States of America **104:**6037-6042.
73. **Gauthier A, Robertson ML, Lowden M, Ibarra JA, Puente JL, Finlay BB.** 2005. Transcriptional inhibitor of virulence factors in enteropathogenic Escherichia coli. Antimicrobial agents and chemotherapy **49:**4101-4109.
74. **Kauppi AM, Nordfelth R, Uvell H, Wolf-Watz H, Elofsson M.** 2003. Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. Chemistry & biology **10:**241-249.
75. **Li J, Lv C, Sun W, Li Z, Han X, Li Y, Shen Y.** 2013. Cytosporone B, an inhibitor of the type III secretion system of Salmonella enterica serovar Typhimurium. Antimicrobial agents and chemotherapy **57:**2191-2198.
76. **Iwatsuki M, Uchida R, Yoshijima H, Ui H, Shiomi K, Matsumoto A, Takahashi Y, Abe A, Tomoda H, Omura S.** 2008. Guadinomines, Type III secretion system inhibitors, produced by Streptomyces sp. K01-0509. I: taxonomy, fermentation, isolation and biological properties. The Journal of antibiotics **61:**222-229.
77. **Cornelis GR.** 2002. The Yersinia Ysc-Yop virulence apparatus. International journal of medical microbiology : IJMM **291:**455-462.
78. **Cornelis GR.** 2006. The type III secretion injectisome. Nature reviews. Microbiology **4:**811-825.
79. **Cianciotto NP.** 2005. Type II secretion: a protein secretion system for all seasons. Trends in microbiology **13:**581-588.
80. **Wretlind B, Pavlovskis OR.** 1983. Pseudomonas aeruginosa elastase and its role in pseudomonas infections. Reviews of infectious diseases **5 Suppl 5:**S998-1004.
81. **Hiraga S, Niki H, Ogura T, Ichinose C, Mori H, Ezaki B, Jaffe A.** 1989. Chromosome partitioning in Escherichia coli: novel mutants producing anucleate cells. Journal of bacteriology **171:**1496-1505.
82. **Gitai Z, Dye N, Shapiro L.** 2004. An actin-like gene can determine cell polarity in bacteria. Proceedings of the National Academy of Sciences of the United States of America **101:**8643-8648.
83. **Bean GJ, Flickinger ST, Westler WM, McCully ME, Sept D, Weibel DB, Amann KJ.** 2009. A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB. Biochemistry **48:**4852-4857.
84. **Jones LJ, Carballido-Lopez R, Errington J.** 2001. Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. Cell **104:**913-922.
85. **Figge RM, Divakaruni AV, Gober JW.** 2004. MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus. Molecular microbiology **51:**1321-1332.

86. **Shih YL, Le T, Rothfield L.** 2003. Division site selection in Escherichia coli involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. Proceedings of the National Academy of Sciences of the United States of America **100:**7865-7870.
87. **Kim SY, Gitai Z, Kinkhabwala A, Shapiro L, Moerner WE.** 2006. Single molecules of the bacterial actin MreB undergo directed treadmilling motion in Caulobacter crescentus. Proceedings of the National Academy of Sciences of the United States of America **103:**10929-10934.
88. **Dye NA, Pincus Z, Theriot JA, Shapiro L, Gitai Z.** 2005. Two independent spiral structures control cell shape in Caulobacter. Proceedings of the National Academy of Sciences of the United States of America **102:**18608-18613.
89. **Mauriello EM, Mouhamar F, Nan B, Ducret A, Dai D, Zusman DR, Mignot T.** 2010. Bacterial motility complexes require the actin-like protein, MreB and the Ras homologue, MglA. The EMBO journal **29:**315-326.
90. **Gaballah A, Kloeckner A, Otten C, Sahl HG, Henrichfreise B.** 2011. Functional analysis of the cytoskeleton protein MreB from Chlamydophila pneumoniae. PloS one **6:**e25129.
91. **Hultgren SJ, Abraham S, Caparon M, Falk P, St Geme JW, 3rd, Normark S.** 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell **73:**887-901.
92. **Klemm P.** 1992. FimC, a chaperone-like periplasmic protein of Escherichia coli involved in biogenesis of type 1 fimbriae. Research in microbiology **143:**831-838.
93. **Lindberg F, Tennent JM, Hultgren SJ, Lund B, Normark S.** 1989. PapD, a periplasmic transport protein in P-pilus biogenesis. Journal of bacteriology **171:**6052-6058.
94. **Nishiyama M, Vetsch M, Puorger C, Jelesarov I, Glockshuber R.** 2003. Identification and characterization of the chaperone-subunit complex-binding domain from the type 1 pilus assembly platform FimD. Journal of molecular biology **330:**513-525.
95. **Thanassi DG, Stathopoulos C, Dodson K, Geiger D, Hultgren SJ.** 2002. Bacterial outer membrane ushers contain distinct targeting and assembly domains for pilus biogenesis. Journal of bacteriology **184:**6260-6269.
96. **Geibel S, Waksman G.** 2013. The Molecular Dissection of the Chaperone-Usher Pathway. Biochimica et biophysica acta.
97. **Sauer FG, Remaut H, Hultgren SJ, Waksman G.** 2004. Fiber assembly by the chaperone-usher pathway. Biochimica et biophysica acta **1694:**259-267.
98. **Sauer FG, Futterer K, Pinkner JS, Dodson KW, Hultgren SJ, Waksman G.** 1999. Structural basis of chaperone function and pilus biogenesis. Science **285:**1058-1061.
99. **Nishiyama M, Horst R, Eidam O, Herrmann T, Ignatov O, Vetsch M, Bettendorff P, Jelesarov I, Grutter MG, Wuthrich K, Glockshuber R, Capitani G.** 2005. Structural basis of chaperone-subunit complex recognition by the type 1 pilus assembly platform FimD. The EMBO journal **24:**2075-2086.
100. **Schenone M, Dancik V, Wagner BK, Clemons PA.** 2013. Target identification and mechanism of action in chemical biology and drug discovery. Nature chemical biology **9:**232-240.

101. **Lomenick B, Olsen RW, Huang J.** 2011. Identification of direct protein targets of small molecules. ACS chemical biology **6:**34-46.
102. **Wacker SA, Houghtaling BR, Elemento O, Kapoor TM.** 2012. Using transcriptome sequencing to identify mechanisms of drug action and resistance. Nature chemical biology **8:**235-237.
103. **Kolb HC, Sharpless KB.** 2003. The growing impact of click chemistry on drug discovery. Drug discovery today **8:**1128-1137.
104. **Ayre J.** 1849. Cholera: its nature, symptoms, and treatment. Stringer & Townsend, New York,.
105. **Colwell RR.** 1996. Global climate and infectious disease: the cholera paradigm. Science **274:**2025-2031.
106. **Angelichio MJ, Spector J, Waldor MK, Camilli A.** 1999. Vibrio cholerae intestinal population dynamics in the suckling mouse model of infection. Infection and immunity **67:**3733-3739.
107. **Sanchez J, Holmgren J.** 2011. Cholera toxin - a foe & a friend. Indian J Med Res **133:**153-163.
108. **Guerrant RL, Carneiro-Filho BA, Dillingham RA.** 2003. Cholera, diarrhea, and oral rehydration therapy: triumph and indictment. Clin Infect Dis **37:**398-405.
109. **Hirschhorn N, Kinzie JL, Sachar DB, Northrup RS, Taylor JO, Ahmad SZ, Phillips RA.** 1968. Decrease in net stool output in cholera during intestinal perfusion with glucose-containing solutions. The New England journal of medicine **279:**176-181.
110. **Higgins DE, DiRita VJ.** 1994. Transcriptional control of toxT, a regulatory gene in the ToxR regulon of Vibrio cholerae. Molecular microbiology **14:**17-29.
111. **Hase CC, Mekalanos JJ.** 1998. TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **95:**730-734.
112. **Thomas S, Williams SG, Manning PA.** 1995. Regulation of tcp genes in classical and El Tor strains of Vibrio cholerae O1. Gene **166:**43-48.
113. **Krukonis ES, Yu RR, Dirita VJ.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Molecular microbiology **38:**67-84.
114. **Kovacikova G, Skorupski K.** 1999. A Vibrio cholerae LysR homolog, AphB, cooperates with AphA at the tcpPH promoter to activate expression of the ToxR virulence cascade. Journal of bacteriology **181:**4250-4256.
115. **Skorupski K, Taylor RK.** 1999. A new level in the Vibrio cholerae ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. Molecular microbiology **31:**763-771.
116. **Krishnan HH, Ghosh A, Paul K, Chowdhury R.** 2004. Effect of anaerobiosis on expression of virulence factors in Vibrio cholerae. Infection and immunity **72:**3961-3967.
117. **Kovacikova G, Skorupski K.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the Vibrio cholerae tcpPH promoter. Molecular microbiology **41:**393-407.
118. **Pratt JT, Ismail AM, Camilli A.** 2010. PhoB regulates both environmental and virulence gene expression in Vibrio cholerae. Molecular microbiology **77:**1595-1605.

119. **Matson JS, DiRita VJ.** 2005. Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **102:**16403-16408.

Chapter II

Small molecule inhibitors of *toxT* expression in *Vibrio cholerae*

Note: A modified version of this text has been published (Anthouard R, DiRita VJ. 2013. Small-molecule inhibitors of toxT expression in Vibrio cholerae. mBio 4).

Summary

Vibrio cholerae, a Gram-negative bacterium, infects humans and causes cholera, a severe disease characterized by vomiting and diarrhea. These symptoms are primarily caused by cholera toxin (CT), whose production by *V. cholerae* is tightly regulated by the virulence cascade. In this study, we designed and carried out a high-throughput chemical genetic screen to identify inhibitors of the virulence cascade. We identified three compounds, toxtazin A and toxtazin B and B', representing two novel classes of *toxT* transcription inhibitors. All three compounds reduce production of both CT and the toxin co-regulated pilus (TCP), an important colonization factor. We present evidence that toxtazin A works at the level of the *toxT* promoter, and that toxtazins B and B' work at the level of the *tcpP* promoter. Treatment with toxtazin B results in a 100-fold reduction in colonization in an infant mouse model of infection, though toxtazin A did not reduce colonization at the concentrations tested. These results add to the growing body of literature indicating that small molecule inhibitors of virulence genes

could be developed to treat infections, as alternatives to antibiotics become increasingly needed.

V. cholerae caused more than 580,000 infections worldwide in 2011 alone (1). Cholera is treated with an oral rehydration therapy consisting of water, glucose, and electrolytes. However, as *V. cholerae* is transmitted via contaminated water, treatment can be difficult for communities whose water source is contaminated. In this study, we address the need for new therapeutic approaches by targeting the production of the main virulence factor, CT. The high-throughput screen presented here led to the identification of two novel classes of inhibitors of the virulence cascade in *V. cholerae*, toxtazins A and toxtazins B and B'. We demonstrate that i) small molecules inhibitors of virulence gene production can be identified in a high-throughput screen ii), targeting virulence gene production is an effective therapeutic strategy, and iii) small molecule inhibitors can uncover unknown layers of gene regulation, even in well-studied regulatory cascades.

Introduction

Vibrio cholerae, a Gram-negative pathogen, colonizes the human intestine and causes cholera, an acute disease characterized by vomiting, profuse watery diarrhea and severe dehydration. The symptoms are caused by the secreted cholera toxin (CT) (2), which binds and enters intestinal epithelial cells and increases cAMP production. This leads to a decrease in sodium uptake and a concomitant increase in chloride

extrusion into the lumen of the intestine, resulting in water secretion thus causing diarrhea and dehydration (3).

Because CT is the major virulence factor produced by *V. cholerae*, much research has gone into understanding how its expression is regulated. Epidemic strains of *V. cholerae* are divided into two biotypes, classical and El Tor, both of which regulate the virulence cascade via the master virulence regulator, ToxT (refer to Figure 2.6). Transcription of the *ctxAB* operon, which encodes the two CT subunits, and the *tcp* operon, which encodes the genes for the toxin co-regulated pilus (TCP), is activated by ToxT (4, 5). Transcription of *toxT* is activated by a protein complex comprised of four inner membrane proteins ToxRS and TcpPH (6). The transcription of *tcpPH* is activated by two transcription activators, AphA and AphB, which respond to cell density, anaerobiosis, and other factors (7-9).

Currently, cholera is treated with oral rehydration therapy (ORT), which restores fluids to the patient and allows the immune system to clear the infection (10, 11). Antibiotics are sometimes administered as a second line of treatment, as they can reduce vomiting and diarrheal volume by 50% and shorten illness duration by 50%. However, antibiotics are not effective alone because patients are still at risk of severe dehydration caused by CT (10, 12). Because of this, investigating other treatment modalities could provide benefit for the treatment of cholera, and this is an area of active research (13-15). Previous work identified an inhibitor called virstatin, which inhibits ToxT dimerization and thereby alters its activity. Further experiments using virstatin revealed that ToxT dimerization affects its activity at various promoters (16).

Interestingly, when ToxT was crystalized, it was bound to a sixteen-carbon fatty acid *cis*-palmitoleate. This and other similar fatty acid ligands were shown to hold ToxT in a closed conformation, inhibiting its ability to bind and activate the *tcp* and *ctx* promoters (17).

Anti-virulence drugs are becoming an increasingly popular strategy in combating diseases. Unlike antibiotics, anti-virulence drugs aim to disarm a pathogen by eliminating its virulence potential and allowing the immune system to clear the infection, providing several advantages. First, targeting virulence genes imposes a weaker selective pressure than targeting growth, decreasing the potential for the emergence of resistant strains (18, 19). In fact, in some conditions, spontaneous non-toxigenic mutants of *V. cholerae* outcompete the pathogenic wild type parental strains (20). Additionally, targeting pathogens with anti-virulence drugs has minimal effects on endogenous microbiota, diminishing the risk of dysbiosis caused by antibiotics that can lead to acute and chronic intestinal problems (21, 22).

In addition to the potential therapeutic advantages of anti-virulence compounds, small molecules provide significant value as molecular probes for investigating the basic biology governing virulence. Because such compounds do not kill bacteria, they can be used experimentally to probe virulence traits without genetic manipulation of the organism. Compounds used as molecular probes act quickly, are often reversible, and their effects can be enhanced or diminished simply by changing the concentration.

In this study, we performed a high-throughput screen to identify small molecules that inhibit the expression of *toxT.* We identified three compounds- toxtazin A, and

toxtazin B and B'; the latter two are structural analogs of each other. All three compounds decrease CT and TCP levels. We determined that toxtazin A does not affect the protein or transcript levels of the ToxR, TcpP, AphA, or AphB, the genes upstream of *toxT* in the virulence cascade, indicating that it prevents *toxT* transcription. Toxtazins B and B' both inhibit virulence by decreasing TcpP protein and transcript levels but not AphA or AphB protein or transcript levels, indicating that these compounds function by inhibiting *tcpP* transcriptional activation.

Results

A GFP-based high-throughput screen identifies two novel classes of *toxT* expression inhibitors

To identify small molecule inhibitors of *toxT* transcription that do not affect general growth, a reporter strain was constructed consisting of wild type *V. cholerae* harboring a plasmid on which the *toxT* promoter drives the expression of GFP. Culturing the *toxT::gfp* reporter strain NB39 under toxin-inducing conditions resulted in high fluorescence intensity units in a wild type background and relatively low fluorescence intensity units in the isogenic *ΔtoxR* strain NB40 (Figure 2.1A). Molecules that decrease GFP expression in a wild type cell could be inhibiting any event in the virulence cascade prior to *toxT* transcription. Using NB39, approximately 63,000 diverse compounds were screened at the University of Michigan Center for Chemical

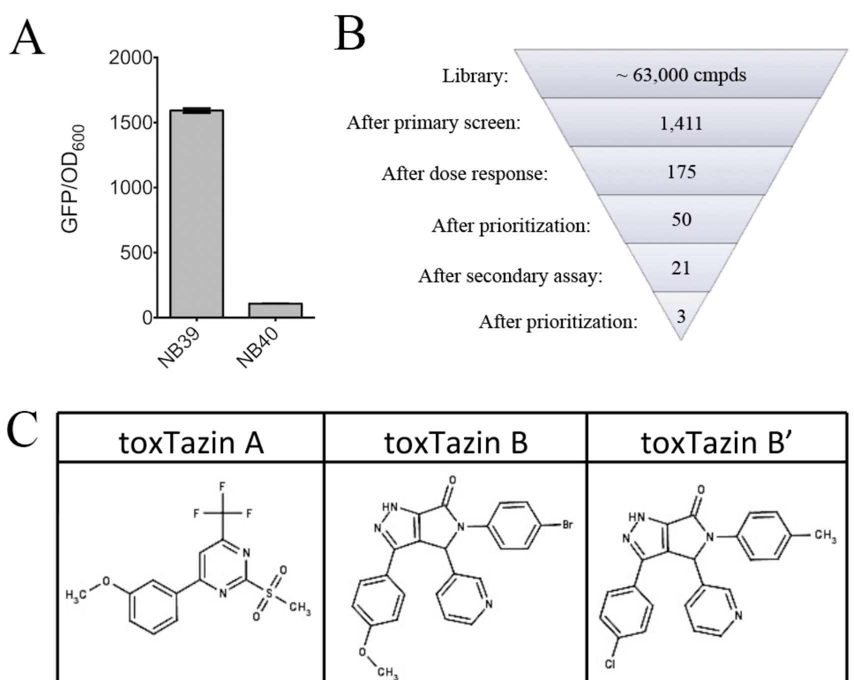


Figure 2.1 Identification of inhibitors of the virulence cascade in *V. cholerae.*

A. The wild type reporter strain NB39 has a high GFP/OD_{600} ratio when grown overnight under toxin-inducing conditions, while the isogenic *ΔtoxR* strain NB39 does not. B. Funnel figure illustrating the triage process used in the screen. C. Structure of the toxtazins.

Genomics. A compound was scored as active if it reduced GFP fluorescence to a level lower than six standard deviations from the mean of the untreated controls, without decreasing growth (measured as changes in OD_{600}) by greater than 10% of the untreated controls, indicating that the compound is not toxic at that dose. Of the 1,411 compounds that met these requirements, 175 exhibited dose-dependent inhibition of GFP fluorescence (Figure 2.1B). We prioritized these based on potency, and purchased the top 50 compounds for further characterization. We measured the levels of CT produced in cultures treated with the top 50 compounds by ELISA and selected the three compounds that resulted in the lowest level of toxin production for further characterization. We named these toxtazins A, B, and B', and their chemical structures are shown in Figure 2.1C.

Toxtazin A, B, and B' inhibit the virulence cascade

To determine an optimal concentration for our experiments, cultures of NB39 were inoculated overnight in toxin-inducing conditions, with various concentrations of compounds or the equivalent volume of DMSO. Addition of compounds had significant and dose-dependent effects on GFP fluorescence (Figure 2.2A) and the half maximal effective concentrations (EC_{50}) of toxtazins A, B, and B' were 24.5 µM, 2.7 µM, and 7.2 µM, respectively. All three compounds had a statistically significant effect on growth at concentrations of 25 µM and higher (Figure 2.2B) and a statistically significant effect on GFP fluorescence at concentrations of 5 µM and higher. Thus, subsequent *in vitro* experiments were performed at 10 µM unless otherwise indicated.

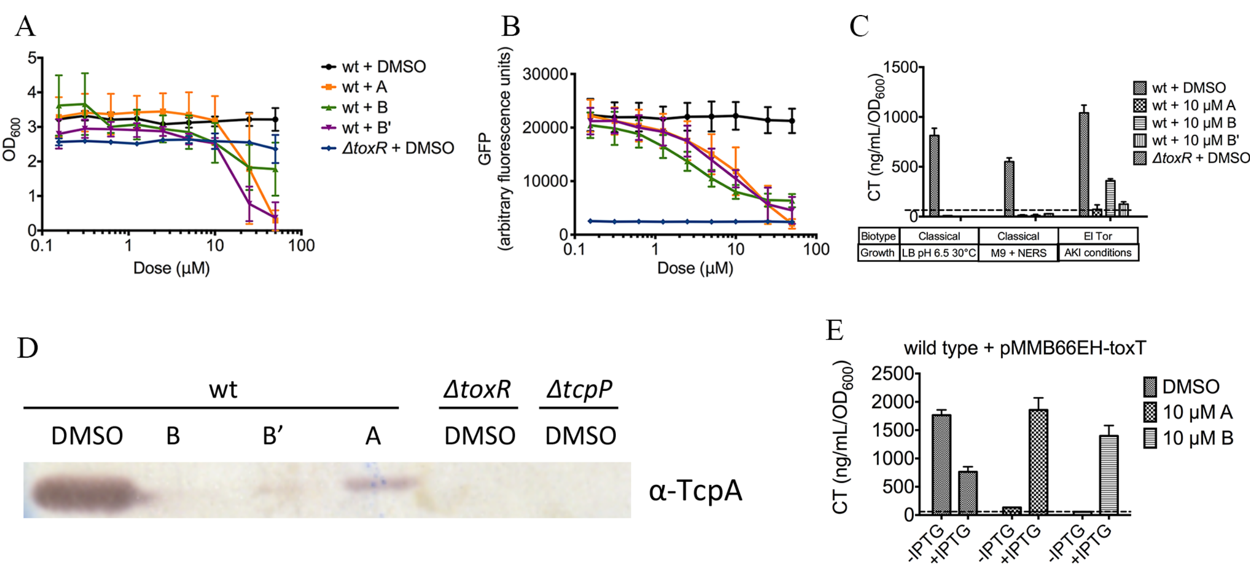


Figure 2.2 Toxtazin A, B, and B' inhibit the virulence cascade in *V. cholerae*.

A and B. GFP expression (2A) and terminal OD_{600} (2B) after 16 h in cultures grown overnight under toxin-inducing conditions with 0.16 μM to 50 μM of compounds. C. CT expression levels in wild type cultures grown overnight under various toxin-inducing conditions in the presence or absence of 10 μM compounds. The dotted line indicates the limit of detection. An isogenic *ΔtoxR* strains served as a control. D. Western blot of O395 cultures treated with 10 μM of compounds overnight in LB pH 6.5 at 30°C, using a TcpA antibody. E. CT levels in cultures of RA286 grown overnight under toxin-inducing conditions in the presence or absence of 10 μM compounds. Error bars represent the standard deviation of three biological replicates. The dotted line indicates the limit of detection.

If these compounds indeed inhibit *toxT* expression as opposed to altering GFP function, compound treatment should lead to decreased expression of ToxT-activated genes such as CT and TCP. Additionally, for these compounds to have therapeutic potential, they should work in both classical and El Tor biotypes of *V. cholerae*, and should work in various toxin-inducing growth conditions. CT levels were determined in both classical and El Tor biotypes under various *in vitro* conditions known to induce the ToxT system for each of the two biotypes. For the classical strain O395, cultures were incubated overnight with 10 µM of each compound in either LB pH 6.5 at 30°C, or in minimal media supplemented with amino acids asparagine, glutamate, arginine and serine (NERS). For the El Tor strain E7946, cultures were incubated with 10 µM of each compound under AKI conditions (Figure 2.2C). Toxtazins A, B, and B' significantly inhibited CT production in both biotypes and in all three tested growth conditions, validating the target of the compounds as *toxT* transcription and ruling out trivial effects, such as inhibition of GFP activity. These results also indicate that the compounds are not biotype- or condition-specific inhibitors, though toxtazin B is more effective against the classical biotype. We note that toxtazin A (but not toxtazin B or B') completely inhibited growth in M9 + NERS (data not shown), indicating that toxtazin A may affect a pathway required for growth under these conditions, and that toxtazin A and B/B' work by different mechanisms.

O395 cultures grown overnight in LB pH 6.5 at 30°C in the presence or absence of compounds were also analyzed for TcpA expression by immunoblot (Figure 2.2D). All three compounds decreased TcpA levels relative to the DMSO-treated control,

supporting the conclusion that these compounds affect the virulence cascade by altering *toxT* expression. To confirm that the compounds work on the cascade prior to *toxT* expression, we tested the effect of the toxtazins on bacteria expressing *toxT* ectopically, under control of an IPTG-inducible promoter. We predicted that if the compounds alter the cascade of gene regulation that leads to *toxT* expression, then removing *toxT* control from that cascade would confer resistance to the effects of the compounds. Strain RA286, wild type expressing an IPTG-inducible allele of *toxT,* was cultured overnight under toxin-inducing conditions in either DMSO, toxtazin A, or toxtazin B, and *toxT* was induced with 100 µM IPTG. The resulting supernatants were analyzed by ELISA to quantify CT levels (Figure 2.2E). Toxtazins A and B both led to decreased CT expression in bacteria cultured without IPTG, while CT levels were restored in bacteria cultured with IPTG to induce *toxT* transcription, indicating that both toxtazins A and B act upstream of *toxT* in the virulence cascade. We noted that over-expression of ToxT in the DMSO control led to a slight decrease in CT levels. While the mechanism for this is unclear, we have observed that overexpressing components of this regulatory cascade (including ToxR and TcpP) can have a slight inhibitory effect on CT, perhaps due to altered stoichiometry of activator complexes required for gene expression. This observation notwithstanding, that ectopic ToxT expression restores CT expression in the presence of the toxtazins indicates that they inhibit the virulence cascade prior to *toxT* transcription.

Toxtazin B, but not toxtazin A, decreases *V. cholerae* colonization in an infant mouse model

Host colonization by *V. cholerae* is dependent on TCP expression. Based on our *in vitro* findings that the toxtazins decrease TcpA levels, we hypothesized that they could decrease the colonization of *V. cholerae* in a mouse model of colonization. To test toxtazin A activity *in vivo*, 4-6 day old mice were orogastrically inoculated with 10^6 bacteria and either toxtazin A or DMSO, and received a boost of compound three hours post-infection. Because not all the compound administered will reach the desired location within the mouse (i.e. the small intestine), and because some of the compound may be processed within the mouse, we used higher concentrations of compounds in these experiments. To ensure that any observed decreases in colonization are due to inhibition of TcpA and not to toxicity against *V. cholerae,* three mL LB cultures were started using the same inoculum and boosted with compound three hours later, then cultured overnight. Administration of either 20 µg, 40 µg, or 60 µg toxtazin A to mice inoculated with *V. cholerae* did not decrease colonization levels relative to the DMSO-treated mice (Figure 2.3A). While toxtazin A did not reduce colonization even at the highest level tested, that concentration was toxic to *V. cholerae* grown *in vitro* (Figure 2.3B). We note that cultures grown in sub-lethal concentrations of toxtazin A routinely grow better in LB, and this was also seen in the *in vitro* experiment at 20 µg and 40 µg (Figure 2.3B). The mouse data indicated that toxtazin A was not toxic *in vivo* at 60 µg, but higher doses were not tested because it would be impossible to distinguish whether a decrease in colonization resulted from the ability of toxtazin A to inhibit ToxT activity or

from its antibacterial activity. The lack of *in vivo* killing by toxtazin A at 60 μg may reflect poor bioavailability of the compound in the infant mouse.

Because toxtazin B and B' are structural analogs and behave similarly in all assays thus far, and to reduce the number of animals used, we focused on toxtazin B in the following experiments. The same *in vivo* experiment performed with toxtazin A

was performed with toxtazin B. Administration of either 100 μg or 200 μg toxtazin B to mice inoculated with *V. cholerae* decreased colonization levels approximately 100-fold relative to the DMSO-treated mice (Figure 2.3C). This effect was not due to toxtazin B toxicity against *V. cholerae* since the three mL *in vitro* cultures treated with toxtazin B had no growth defect compared to the DMSO-treated cultures (Figure 2.3D). These results suggest that toxtazin B inhibits expression of the TcpA colonization determinant both *in vitro* and *in vivo*.

The toxtazins do not affect ToxR protein levels or activity.

To determine where in the regulatory cascade leading to *toxT* expression each compound works, a targeted approach was used. We first tested whether the compounds altered levels or activity of ToxR, one of the direct transcription activators of *toxT*. ToxR protein levels were unaffected by toxtazins A, B, and B' in cultures grown overnight under toxin-inducing conditions (Figure 2.4A). ToxR activity was investigated by measuring levels of OmpU and OmpT, outer membrane proteins whose transcription is activated or repressed, respectively, by ToxR. Inhibiting ToxR activity would decrease OmpU levels and elevate OmpT levels, similar to a *toxR* mutant. Cell lysates

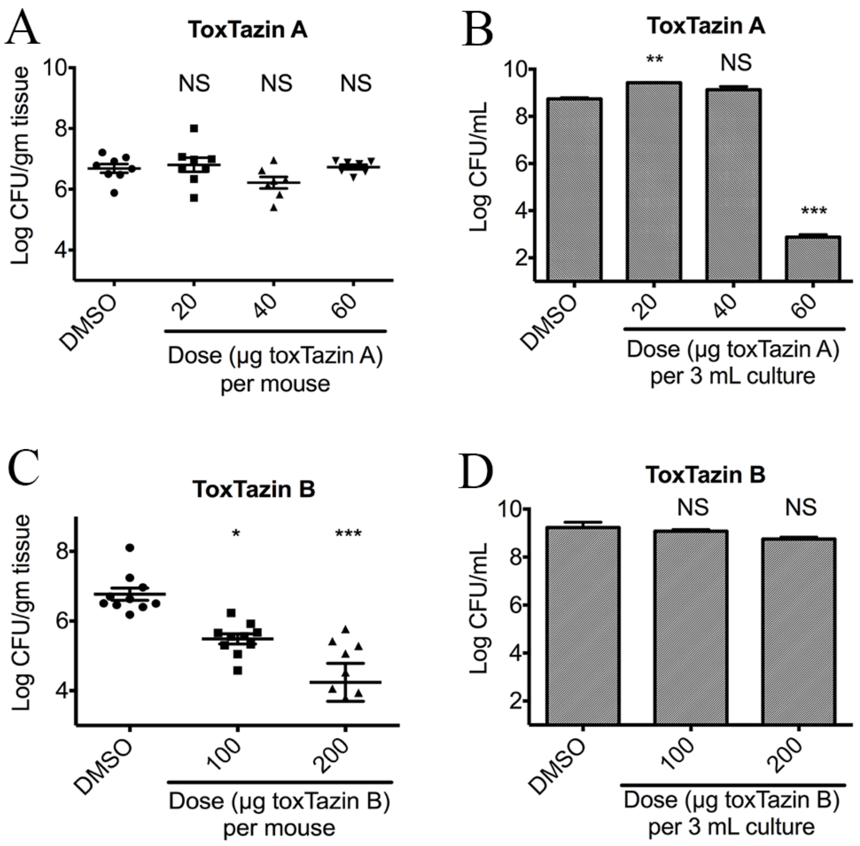


Figure 2.3 Toxtazin B reduces *V. cholerae* colonization in an infant mouse.

A. Number of *V. cholerae* recovered from mice orogastrically inoculated with 10^6 wild type *V. cholerae* and treated with either DMSO, 0, 20 µg, 40 µg, or 60 µg toxtazin A. B. Number of *V. cholerae* recovered from three mL cultures grown overnight with the same inocula and boosts as the mice. C. Number of *V. cholerae* recovered from mice orogastrically inoculated as in 3A and treated with DMSO, 100 µg or 200 µg toxtazin B. D. Number of *V.* cholerae recovered from three mL cultures grown overnight with the same inocula and boosts as the mice. Asterisks denote the statistical significance of a group relative to the DMSO control. NS = not significant, one asterisk = p-value < 0.05, two asterisks = p-value < 0.01, and three asterisks = p-value <0.001.

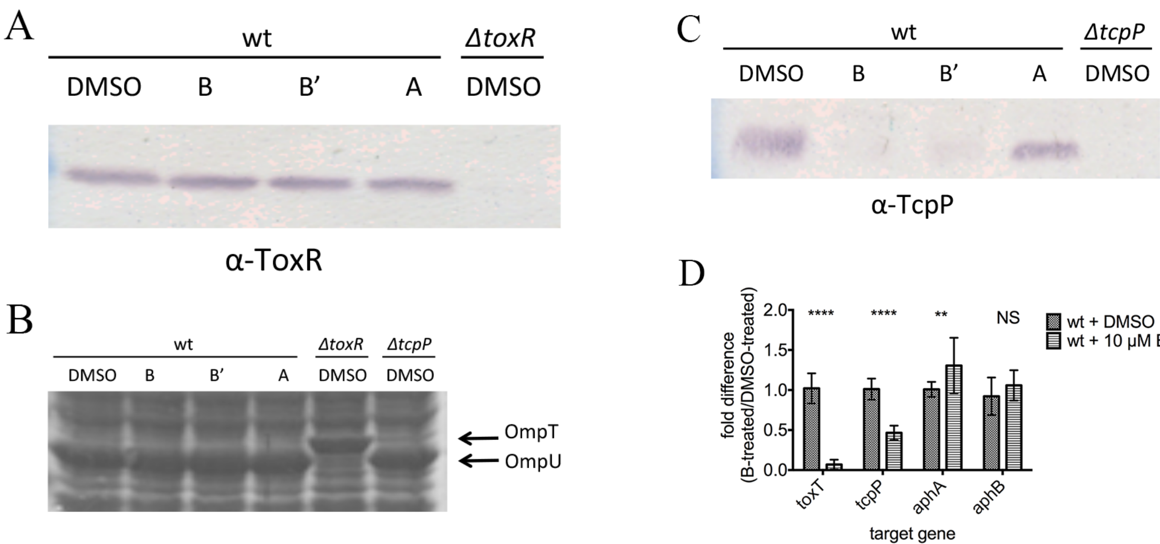


Figure 2.4 Effects of toxtazin A and B on the virulence cascade.

A. ToxR Western of cultures grown overnight under toxin-inducing conditions with or without 10 μM compounds. B. Coomassie showing OmpU and OmpT levels in cultures grown as in 4A. C. TcpP Western of cultures grown in the same way as in Figure 2.4A. D. Transcript levels of *toxT, tcpP, aphA,* and *aphB* were determined for cultures grown overnight under toxin-inducing conditions in the presence or absence of 10 μM toxtazin B. Significance was calculated by two-way ANOVA. NS = not significant, one asterisk = p-value < 0.05, two asterisks = p-value < 0.01, and four asterisks = p-value <0.0001.

from overnight cultures grown with or without compounds under toxin-inducing conditions were subjected to SDS-PAGE and stained with Coomassie Blue to visualize OmpU and OmpT (Figure 2.4B). Toxtazin-treated cultures have the same OmpU/OmpT profile as the DMSO-treated wild type culture, indicating that ToxR activity is not affected by toxtazin A, B, or B'.

<u>TcpP protein levels are differentially affected by toxtazin A and B/B'.</u>

Next, we analyzed the effect on TcpP protein levels, the other major transcription activator of the *toxT* promoter. Cultures were grown overnight under toxin-inducing conditions in the presence or absence of 10 µM toxtazin A, B, or B', and cell lysates were analyzed by Western blot with antiserum directed against TcpP. Toxtazin A did not alter TcpP protein levels, however toxtazins B and B' caused significant decreases in TcpP protein levels relative to the DMSO control (Figure 2.4C). These results confirm that toxtazin A and B/B' have different targets and mechanisms of action for inhibiting *toxT* transcription in *V. cholerae*. Toxtazin B and B' inhibit TcpP expression, while toxtazin A affects *toxT* expression without altering TcpP levels.

<u>Toxtazin B reduces transcript levels of *toxT* and *tcpP* but not of *aphA* or *aphB*.</u>

Because toxtazin B and B' behave similarly, we again focused on toxtazin B for the next set experiments. To determine where in the TcpP branch of the regulatory cascade toxtazin B acts, qRT-PCR was used to quantify the levels of *toxT, tcpP, aphA,* and *aphB* transcript in cultures treated overnight with 10 µM toxtazin B, incubated under

toxin-inducing conditions (Figure 2.4D). All results were normalized to 16s rRNA, and are reported as a ratio of the mRNA levels detected in toxtazin B-treated over DMSO-treated cultures. As expected, *toxT* expression was reduced by a factor of 10 in cultures treated with toxtazin B relative to the DMSO control. While *aphA* and *aphB* transcript levels were not affected by the compound, *tcpP* transcript levels were decreased 50% compared to the DMSO controls, consistent with the decrease observed in TcpP protein levels. These data indicate that toxtazin B targets *tcpP* transcription.

Neither toxtazin A nor B affects AphA or AphB levels.

Having determined that *aphA* and *aphB* mRNA levels are not altered by toxtazin B, we wanted to determine whether their protein levels might be affected. Cultures grown overnight under toxin-inducing conditions in the presence or absence of 10 µM toxtazin A or B were analyzed by Western blot (Figure 2.5). No decrease in AphA or AphB protein levels was observed in the presence of toxtazin B, though AphA levels increased in cells treated with toxtazin A. We do not know the reason for this, but speculate about it in the discussion. Nevertheless, our results indicate that the decrease in *tcpP* transcription caused by toxtazin B is not due to a decrease in AphA or AphB protein levels, pointing instead to the possibility that the activity of one of these proteins may be inhibited by this compound. Supporting this conclusion, ectopic expression of AphA or AphB did not make cells resistant to toxtazin B (supplemental

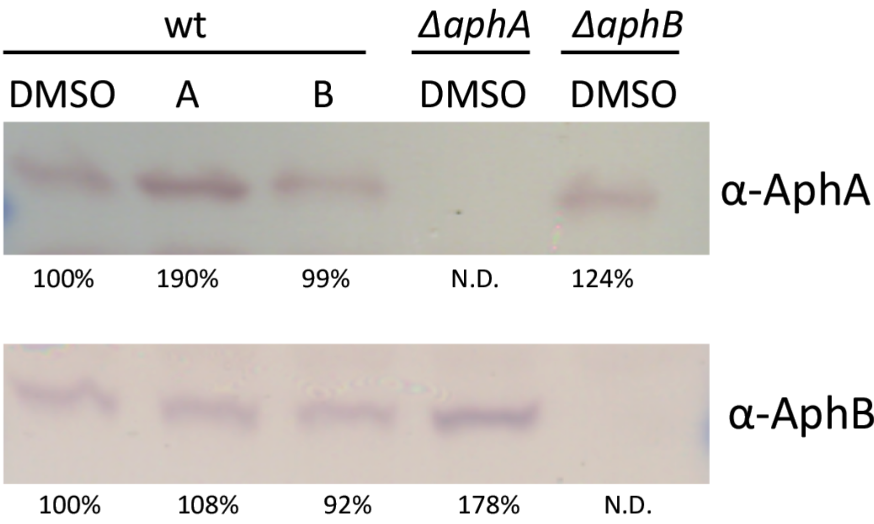


Figure 2.5 Toxtazins A and B do not affect AphA or AphB protein levels.

Western blot for AphA and AphB in samples grown overnight under toxin-inducing conditions in the presence or absence of 10 µM toxtazins A or B. Band intensities were quantified with Image J and normalized to the wild-type DMSO-treated samples.

Figure 2.1). As predicted, AphA and AphB levels were unaltered by growth in the presence of toxtazin A (Figure 2.5).

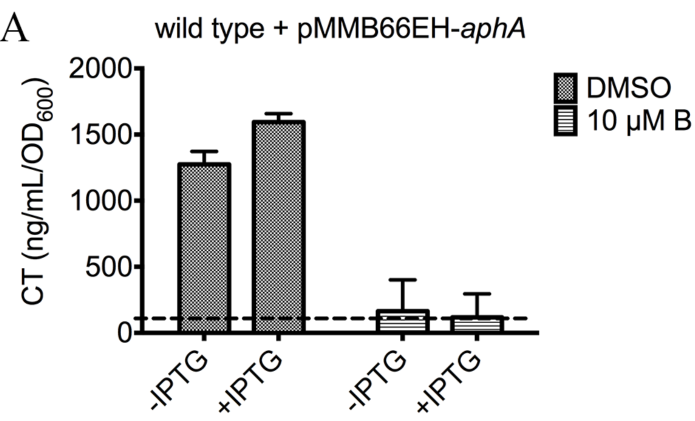
Discussion

Despite the use of antibiotics and oral rehydration therapy (ORT), *V. cholerae* remains a major public health concern in much of the developing world. The number of yearly cholera cases has steadily increased since 2007, and the number of fatalities has increased in the past year (1). New treatment therapies that target toxin production and colonization by *V. cholerae*, either alone or in combination with current therapies, could be very useful in reducing the global health burden caused by this pathogen.

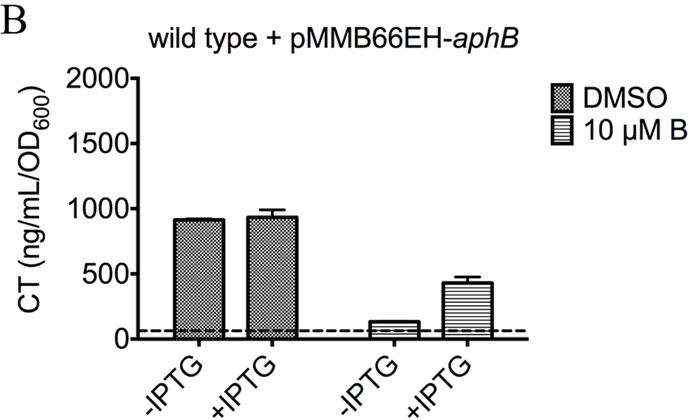
In this study, a bacterial cell-based, high-throughput screen was used to identify and characterize three small molecules, toxtazin A, B, and B'. All three reduce the virulence potential of *V. cholerae in vitro* and toxtazin B also reduced colonization *in vivo.* We show that all three compounds reduce CT production in both the classical and El Tor biotype, and reduce TcpA levels relative to a DMSO control.

The two classes of compounds identified here work by different mechanisms. Toxtazin A does not alter TcpP or ToxR protein levels, suggesting that it affects *toxT* transcription via a different mechanism. We have not ruled out the possibility that Toxtazin A inhibits ToxR or TcpP function, perhaps keeping one of them from physically binding to its site on the *toxT* promoter. Both ToxR and TcpP may sense environmental signals and transmit the information to the cytoplasm by activation of *toxT* transcription. Environmental signals such as temperature, pH, osmolarity, bile, oxygen, and amino

A



B



Supplemental Figure 2.1 Ectopic expression of AphA and AphB in toxtazin B-treated cells.

A. CT levels in a strain expressing AphA ectopically, grown overnight under toxin-inducing conditions in the presence or absence of 10 µM compound and in the presence or absence of one mM IPTG. In panels A and B, error bars represent the standard deviation of three biological replicates. The dotted line denotes the limit of detection. B. CT levels in a strain expressing AphB ectopically, grown as in A.

acids have all been shown to affect *toxT* activation *in vitro* (reviewed (23-25)). In fact, when ToxT was crystalized, a *cis*-palmitoleate was discovered in a solvent-inaccessible binding pocket. The authors of this study suggest that oleic acid, another C-9 monosaturated fatty acid, is the natural ligand for this binding pocket, as it also strongly regulates ToxT activity and is more abundant in bile than *cis*-palmitoleic acid. Another study identified the bile salt taurocholate as an *in vivo* signal that activates the virulence cascade of *V. cholerae* in mice. The stressed physiological state of the cell grown with toxtazin A revealed by proteomic analysis (data not shown) and the growth inhibitory phenotype we observed in minimal medium + NERS (Figure 2.2C) lead us to propose that toxtazin A induces a non-permissive physiological state in the cell which feeds back to shut off *toxT* transcription, and we are currently exploring this hypothesis. We investigated the potential for the toxtazins to act as general redox-active compounds by measuring PhoA activity both *in vitro* and in cells treated with the toxtazins and observed no effect on PhoA in either case, suggesting the toxtazins do not affect the general redox state of the cell. Furthermore, the compounds do not affect the BCA protein assay, indicating that, at the concentrations used in our experiments, these compounds do not act as general reducing agents.

The mechanism for toxtazin B/B' inhibition of virulence is more clear. Toxtazin B and B' decrease TcpP but not ToxR protein levels, and decrease the levels of *toxT* and *tcpP* transcript, but not those of *aphA* or *aphB*, nor do they decrease AphA or AphB protein levels.

We noticed that toxtazin A consistently caused an increase in AphA protein levels. While this does not explain the anti-virulence properties of toxtazin A, it may point to its mechanism of action. AphA is known to regulate 15 genes outside of the vibrio pathogenicity island, including genes required for acetoin biosynthesis, and two signal transduction proteins that influence motility and biofilm formation (26). It is possible that toxtazin A affects one of these pathways, and therefore induces AphA expression.

Taken together, the data suggest that toxtazin B inhibits the virulence cascade at the level of the *tcpP* promoter- downstream of AphA and AphB protein production, but upstream of *tcpP* transcription. Supporting this, ectopic expression of either AphA or AphB does not restore CT production (Figures 2.5B and 2.5C) in the presence of toxtazin B.

Toxtazin B inhibits virulence gene expression in the classical biotype more strongly than in the El Tor biotype. It is notable that *tcpP* transcription, the level at which our data suggest this compound works, is regulated differently in the two biotypes. A single A to G base pair difference in the *tcpP* promoters of the two biotypes disrupts the dyad symmetry of the AphB binding motif (27-29), thus AphB binds 10 times more strongly to the classical *tcpP* promoter than to the El Tor *tcpP* promoter (30, 31). We speculate that the natural differences in *tcpP* regulation in classical versus El Tor may be responsible for the different effectiveness of toxtazin B seen in the two biotypes in Figure 2.2C, but further study must be done to confirm this.

The *tcpP* promoter is a highly regulated feature of the complex regulatory cascade controlling *toxT* transcription, assimilating multiple signals including pH,

osmolarity, cAMP levels, and phosphate levels. For example, when cells are grown at the non-permissive pH, a protein called PepA partially inhibits *tcpP* transcription in the classical biotype (32). The *tcpP* promoter is also negatively regulated by the cAMP-CRP complex, whose binding site at the promoter overlaps the binding sites of AphA and AphB (29). Finally, the *tcpP* promoter is negatively regulated by PhoB, which binds at a site distinct of both the AphA and AphB binding sites (33). Toxtazin B may inhibit *tcpP* expression by altering the binding properties of any these proteins, or of the AphA and/or AphB proteins, at the *tcpP* promoter. Future work will aim to determine the precise mechanism by which toxtazin B inhibits gene expression required for colonization and pathogenicity.

In this study, the activity of the toxtazins *in vivo* was also determined using the infant mouse model of colonization. The ability of *V. cholerae* to colonize and replicate in this mammalian host is in large part dependent on expression of TCP, the major subunit of which is TcpA; wild type strains are significantly more competitive than *tcpA* mutants *in vivo* (34, 35). Our *in vitro* results (Figure 2.2B) demonstrate that toxtazin B leads to reduced TcpA levels, which we propose as the main reason for reduced colonization caused by toxtazin B. We note that a *tcpA* mutant colonizes more poorly than a toxtazin B-treated wild type, and take this to mean that the local compound concentration (that is, the amount of toxtazin B that actually reaches *V. cholerae* in the gut) is lower than what would be require for complete inhibition. Pharmacodynamic and pharmacokinetic studies might optimize the ability of toxtazin B to inhibit colonization.

Unlike toxtazin B, toxtazin A did not decrease colonization level of *V. cholerae* at the concentrations tested. Perhaps this compound does not inhibit colonization because it does not reduce TcpA levels as efficiently as toxtazin B, or perhaps not enough compound reached the bacteria in the gut. Structure-activity relationship (SAR) studies to identify an analog with increased potency and lower toxicity would be useful in future *in vivo* studies and for determining whether toxtazin A decreases colonization.

In summary, we identified two novel classes of *toxT* transcription inhibitors, toxtazin A, and toxtazin B and B'. These compounds are potent inhibitors of *V. cholerae* virulence and function at unique points in the virulence cascade (see Figure 2.6). Toxtazin A inhibits the virulence cascade by preventing *toxT* transcription late in the regulatory cascade, evidenced by the fact that ToxR and TcpP protein levels are equal to those of DMSO-treated cultures, and that mRNA levels of *tcpP, aphA,* and *aphB* are not affected. Toxtazin B works by altering *tcpP* transcription, shown by a decrease in both transcript and protein levels of *tcpP* but no decrease in the protein or transcript levels of AphA or AphB relative to the DMSO-treated controls. Toxtazin B also reduces colonization of *V. cholerae* in an infant mice model. We are currently working on better characterizing the mechanism of action of the toxtazins to gain deeper insight into the requirements of *V. cholerae* pathogenesis. In addition to providing new chemical probes for richer study of the virulence cascade in *V. cholerae*, the toxtazins add further proof-of-principle that potent small molecule inhibitors can be discovered by high throughput screening and can be used both as molecular probes for basic research and as a starting point for therapeutic development.

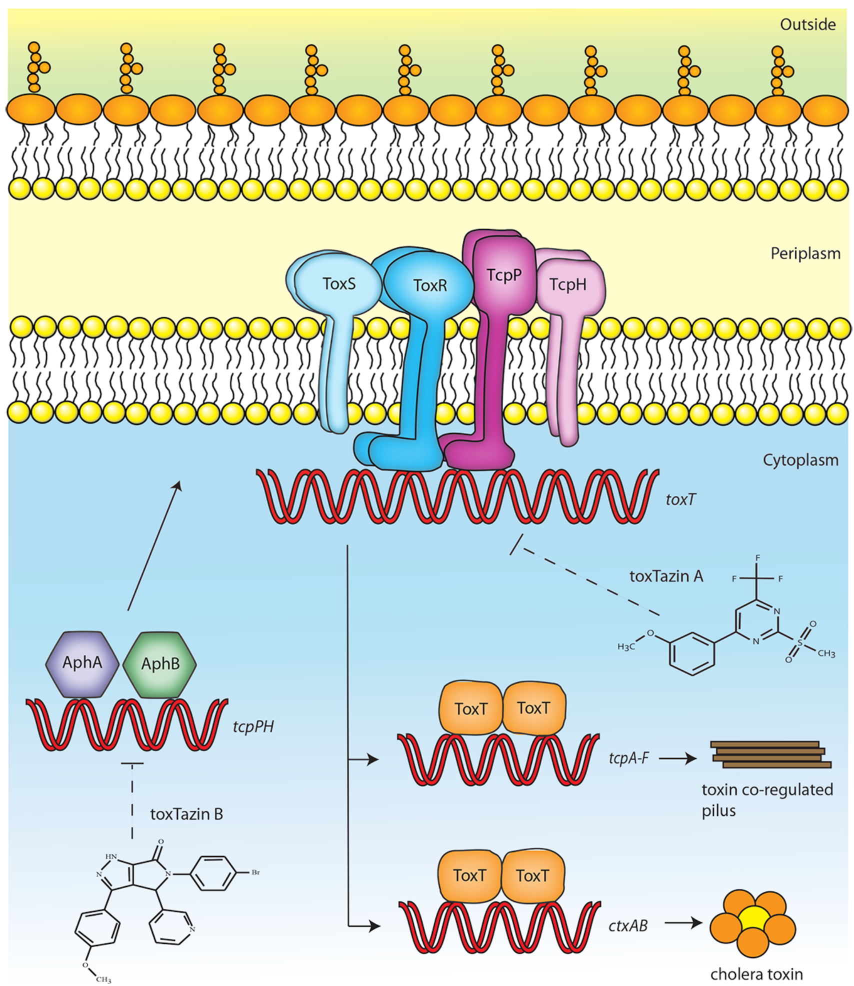


Figure 2.6 Model of the virulence cascade in *V. cholerae* and targets of the toxtazins.

The virulence cascade in *V. cholerae* is tightly regulated. AphA and AphB activate transcription of *tcpPH*. TcpPH form an inner membrane complex with ToxR and ToxS to activate transcription of *toxT.* ToxT activates the transcription of the *tcpA-F* operon, which encodes the toxin co-regulated pilus, and the *ctxAB* operon, which encodes the cholera toxin subunits. Based on our experiments, we propose that toxtazin A inhibits the *toxT* transcription, while toxtazin inhibits *tcpPH* transcription.

Acknowledgements

We thank Jun Zhu for the AphB antibody and the pWM91-Δ*aphA* and pWM91-Δ*aphB* constructs used in this study, Karen Skorupski for the AphA antibody, Eric Krukonis for the construction of the E7946 Δ*toxR* strain, and Nancy Beck for the construction of the reporter strains NB39 and NB40.

Materials and Methods

Bacterial strains, growth conditions, and chemical inhibitors

The strains and plasmids used in this study are outlined in Table 2.1. Unless otherwise noted, the *V. cholerae* classical biotype strain O395 was used in these studies. Strains were maintained at -80°C in Luria-Bertani broth (LB) containing 20% glycerol. Overnight cultures were grown in LB medium at 37°C. Growth of the O395 classical strain under toxin-inducing conditions consists of sub-culturing an overnight culture 1:100 in LB pH 6.5 and growing at 30°C shaking for 16-18 h, or as indicated in the text. Growth in minimal medium consists of sub-culturing an overnight culture 1:100 in M9 minimal medium + NERS (M9 salts, 2 mM MgSO4, 4% glycerol, 0.1 mM $CaCl_2$, and 5 mM each of asparagine, glutamic acid, arginine, and serine) and growing overnight at 37°C shaking (36). Growth in AKI conditions consist of diluting overnights of the El Tor strain E7946 1:1,000 in 10 mL of AKI medium and growing anaerobically

Table 2.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or phenotype	Source
	Vibrio cholerae	
O395	Classical Ogawa, Sm^R	Laboratory collection
E7946	El Tor, Sm^R	Laboratory collection
RA25	O395 Δ*toxR*	(4)
RA6	E7946 Δ*toxR*	This work
NB39	O395 + p*toxT-GFP*	Laboratory collection
NB40	O395 Δ*toxR* + p*toxT-GFP*	Laboratory collection
RA179	O395 Δ*toxT*	(37)
RA67	O395 Δ*tcpP*	(38)
RA305	O395 Δ*aphA*	This work
RA282	O395 Δ*aphB*	This work
RA286	O395 + pMT5	(39)
RA289	O395 + pMMB66EH-*aphA*	This work
RA290	O395 + pMMB66EH-*aphB*	This work
RA306	O395 Δ*aphA* + pMMB66EH	This work
RA307	O395 Δ*aphA* + pMMB66EH-*aphA*	This work
RA308	O395 Δ*aphB* + pMMB66EH	This work
RA309	O395 Δ*aphB* + pMMB66EH-*aphB*	This work
	Plasmids	
p*toxT*-GFP	pBH6119-*toxTpro-gfp*	This work
pMT5	pMMB66HE-*toxT*	(39)
pWM91-Δ*aphA*	pWM91-Δ*aphA*	(40)
pWM91-Δ*aphB*	pWM91-Δ*aphB*	(40)
pMMB66EH-*aphA*	pMMB66EH-*aphA* (O395 sequence)	This work
pMMB66EH-*aphA*	pMMB66EH-*aphA* (O395 sequence)	This work

for four hours statically at 37°C, then pouring the 10 mL cultures into 250 mL flasks and growing shaking for an additional 4 hours or longer (41, 42).

Toxtazin A (IUPAC: 2-methanesulfonyl-4-(3-methoxyphenyl)-6-(trifluoromethyl)pyrimidine), toxtazin B (IPUAC: 5-(4-bromophenyl)-3-(4-methoxyphenyl)-4-(pyridin-3-yl)-1H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-6-one), and toxtazin B' (IUPAC: 3-(4-chlorophenyl)-5-(4-methylphenyl)-4-(pyridin-3-yl)-1H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-6-one) were obtained from ChemDiv (San Diego, CA). Compound stocks were made to either 20 mM or 100 mM in DMSO and stored in the dark at -20°C. Cultures were treated with 10 µM unless otherwise specified in the text, and the controls always received an equal volume of DMSO. Streptomycin was used at 100 µg/mL, ampicillin at 100 µg/mL, and X-gal at 40 µg/mL.

The screening plasmid p*toxT-gfp* was created by digesting pTLI2 (43) with EcoRI and BamHI to generate the (-172) to (+45) *toxT* promoter fragment. This fragment was ligated into the promoterless GFP reported vector pBH6119 (44). The reporter plasmid was electroporated into wild type O395 and an isogenic Δ*toxR* mutant to generate the screening strains.

The E7946Δ*toxR* strain was constructed using the pKAS32-*ΔtoxR* suicide plasmid as done previously (6) using wild type E7946 as the recipient.

The clean deletions of *aphA* and *aphB* in O395 were generated by mating wild type O395 cells with SM10λpir cells containing either pWM91-Δ*aphA* or pWM91-Δ*aphB* (kindly provided by Jun Zhu) and selecting for sucrose-resistant colonies. Mutants were confirmed by DNA sequencing.

The pMMB66EH-AphA and AphB constructs were constructed by amplifying the AphA sequence using primers RAP186 (GCAACGAATTCATGTCATTACCA) and RAP 187 (GTCAAGCTTTTATGCCATCGC) and the AphB sequence using primers RAP218 (GCC GAATTCTTGCAACATAATGTGTCAGA) and RAP219 (CCGAAGCTTTTATTGCAGGTGGTAGCC) from O395. The resulting products were digested with EcoRI and HindIII and ligated into pMMB66EH. The resulting constructs (Table 2.1) were verified by DNA sequencing.

IC_{50} and EC_{50} determination

The concentration of compound that inhibits *toxT*-GFP activity to 50% of the DMSO-treated control (EC_{50}) was measured by inoculating two mL of LB with a 1:100 dilution of an overnight culture and monitoring GFP expression under toxin-inducing conditions with 0.16 µM to 50 µM of compounds. The OD_{600} of these cultures was also monitored to determine the IC_{50}, the concentration of compound that inhibits growth to 50% of the DMSO-treated control. EC_{50} and IC_{50} values were calculated in Graphpad Prism using the 4-parameter model (variable slope).

High-throughput screening for small molecule inhibitors of *toxT-gfp* production

The screening strain used (NB39) is an O395 classical strain harboring a plasmid with the *toxT* promoter driving expression of GFPmut3. An isogenic O395 Δ*toxR* strain with the *toxT*-GFP reporter (NB40) served as a control in the screen.

The primary screen, secondary screen, and dose-response studies were carried

out at the Center for Chemical Genomics (University of Michigan), where approximately 63,000 compounds and 11,000 natural products were tested. Overnight cultures of NB39 were diluted to a final OD_{600} of 0.02 in LB pH 6.5 + streptomycin and ampicillin, and transferred in a 40 µl volume to wells of a black, clear-bottom 384-well microtiter plate (Falcon 35-3948). The screening plates received 10 µM of compounds, which were pin-transferred from stock plates. Each test plate contained positive (strain NB40 + DMSO) and negative (strain NB39 + DMSO) controls, and all compounds were tested in at least duplicate. The plates were incubated at 30°C for 16-18 hours statically, after which the OD_{600} and GFP fluorescence (excitation λ = 385 nm, emission λ = 425 nm) were read for each well.

A compound was considered active if it met two criteria: i) it caused a decrease in GFP expression of more than six standard deviations from the DMSO control, and ii) the OD_{600} of cultures grown with the compound was within 10% of the DMSO control. A total of 1,411 compounds met both criteria and were retested for their effects on growth and *toxT-gfp* expression, and their dose responsiveness. Of the 175 compounds that retained activity and displayed dose-dependent activity, the 50 most potent (a low GFP/OD_{600} value) were ordered from ChemDiv and similarly retested for effects on growth and *toxT* transcription, and for effects on CT production. Of the 21 compounds that caused significantly reduced CT levels, we focused on toxtazins A, B, and B' because of their potency (Figure 2.1C).

Detection of cholera toxin by ELISA

Cultures of *V. cholerae* were grown under toxin-inducing conditions for 16-18 hours with 10 µM compound or DMSO. GM1 ganglioside enzyme-linked immunosorbent CT assays were performed as previously described (45) on equal volumes of the resulting supernatants. CT expression values were normalized to OD_{600} and are the average of samples grown in at least duplicate.

Western blot analysis of TcpA, ToxR, TcpP, AphB, and AphA

Cells were cultured under toxin-inducing conditions in the presence or absence of 10 µM compounds. Cell extracts were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (GE Water and Processes Technologies, Feasterville-Trevose, USA), probed with the appropriate antibody, and visualized by alkaline phosphatase. The TcpA antibody was used at a 1:10,000 dilution, the ToxR antibody at a 1:1,000 dilution, and the TcpP antibody at a 1:500 dilution. The AphB antibody was kindly provided by Dr. Jun Zhu. The AphA antibody (kindly provided by Dr. Karen Skorupski) was used at a 1:10,000 dilution. Band densities were determined with the Image J software (http://rsb.info.nih.gov/ij/) and normalized to the wild-type DMSO-treated samples.

qRT-PCR analysis of mRNA expression

Cells were cultured under toxin-inducing conditions in the presence or absence of 10 µM toxtazin B. RNA was harvested with TRIzol® (Invitrogen, Carlsbad, CA)

according to the manufacturer directions, and DNA was removed using TURBO DNase (Ambion, Austin, TX). The qRT-PCR experiments were performed using the Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) according the manufacturer manual. The qRT-PCR primers are shown in Table 2.2. Expression levels were normalized to 16s rRNA, and fold change was calculated using the $2^{-\Delta\Delta CT}$ method described in the Applied Biosystems User Bulletin No. 2 (P/N 4303859). Results are the average of three biological replicates with three replicates each. Significance was determined by two-way ANOVA.

Infant mouse colonization assays

Four to six day-old CD1 mice (Charles River, Wilmington, MA) were orogastrically inoculated with a 30 µl bolus containing 10^6 CFU of *V. cholerae* O395, Cremaphor EL (20% final volume to solubilize compounds), and either DMSO or compound (dose indicated in text). An additional 30 µl bolus lacking bacteria was delivered to each mouse three hours post-inoculation, and the mice were incubated at 30°C. Mice were euthanized 18-24 hours after inoculation, the intestines were isolated, weighed, and homogenized in PBS. Homogenates were serially diluted and plated on LB agar + X-gal + streptomycin to determine the number of CFU recovered. CFUs were normalized to the weight of the intestines and to the exact CFU of the initial inoculum. Significance was determined using one-way ANOVA.

Table 2.2 Primers used for qRT-PCR analysis.

Target	Forward Primer	Reverse Primer
toxT	CAGCGATTTTCTTTGACTTC	CTCTGAAACCATTTACCACTTC
tcpP	GCTTTGCTACCTGTGATTTGGTGG	CCCGGTAACCTTGCTAAATCTCGT
aphA	GAAGGCAAACCAGATCGCAAGGTT	AGACGGTAAGGTTCTGCCGATTGT
aphB	TCAAATGGCAACTGACCAACAGCC	AGCTCCAATCCGACAGCACTTGTA
16s RNA	GCATAACCTCGCAAGAGCAAAGCA	TGTCTCAGTTCCAGTGTGGCTGAT

References

1. **WHO.** 2012. Cholera, 2011. Wkly Epidemiol Rec **87:**289,Äì304.
2. **Angelichio MJ, Spector J, Waldor MK, Camilli A.** 1999. Vibrio cholerae intestinal population dynamics in the suckling mouse model of infection. Infection and immunity **67:**3733-3739.
3. **Sanchez J, Holmgren J.** 2011. Cholera toxin - a foe & a friend. Indian J Med Res **133:**153-163.
4. **Taylor RK, Miller VL, Furlong DB, Mekalanos JJ.** 1987. Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci U S A **84:**2833-2837.
5. **DiRita VJ, Parsot C, Jander G, Mekalanos JJ.** 1991. Regulatory cascade controls virulence in Vibrio cholerae. Proc Natl Acad Sci U S A **88:**5403-5407.
6. **Krukonis ES, Yu RR, Dirita VJ.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Molecular microbiology **38:**67-84.
7. **Skorupski K, Taylor RK.** 1999. A new level in the Vibrio cholerae ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. Molecular microbiology **31:**763-771.
8. **Kovacikova G, Skorupski K.** 1999. A Vibrio cholerae LysR homolog, AphB, cooperates with AphA at the tcpPH promoter to activate expression of the ToxR virulence cascade. Journal of bacteriology **181:**4250-4256.
9. **Liu Z, Yang M, Peterfreund GL, Tsou AM, Selamoglu N, Daldal F, Zhong Z, Kan B, Zhu J.** 2011. Vibrio cholerae anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. Proc Natl Acad Sci U S A **108:**810-815.
10. **Guerrant RL, Carneiro-Filho BA, Dillingham RA.** 2003. Cholera, diarrhea, and oral rehydration therapy: triumph and indictment. Clin Infect Dis **37:**398-405.
11. **Hirschhorn N, Kinzie JL, Sachar DB, Northrup RS, Taylor JO, Ahmad SZ, Phillips RA.** 1968. Decrease in net stool output in cholera during intestinal perfusion with glucose-containing solutions. The New England journal of medicine **279:**176-181.
12. **Ayre J.** 1849. Cholera: its nature, symptoms, and treatment. Stringer & Townsend, New York,.
13. **Yamasaki S, Asakura M, Neogi SB, Hinenoya A, Iwaoka E, Aoki S.** 2011. Inhibition of virulence potential of Vibrio cholerae by natural compounds. Indian J Med Res **133:**232-239.
14. **Fazil MH, Singh DV.** 2011. Vibrio cholerae infection, novel drug targets and phage therapy. Future Microbiol **6:**1199-1208.
15. **Hung DT, Shakhnovich EA, Pierson E, Mekalanos JJ.** 2005. Small-molecule inhibitor of Vibrio cholerae virulence and intestinal colonization. Science **310:**670-674.
16. **Shakhnovich EA, Hung DT, Pierson E, Lee K, Mekalanos JJ.** 2007. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proceedings of the National Academy of Sciences of the United States of America **104:**2372-2377.

17. **Lowden MJ, Skorupski K, Pellegrini M, Chiorazzo MG, Taylor RK, Kull FJ.** 2010. Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid regulation of virulence genes. Proc Natl Acad Sci U S A **107:**2860-2865.
18. **Clatworthy AE, Pierson E, Hung DT.** 2007. Targeting virulence: a new paradigm for antimicrobial therapy. Nature chemical biology **3:**541-548.
19. **Maeda T, Garcia-Contreras R, Pu M, Sheng L, Garcia LR, Tomas M, Wood TK.** 2012. Quorum quenching quandary: resistance to antivirulence compounds. The ISME journal **6:**493-501.
20. **Paul K, Ghosh A, Sengupta N, Chowdhury R.** 2004. Competitive growth advantage of nontoxigenic mutants in the stationary phase in archival cultures of pathogenic Vibrio cholerae strains. Infect Immun **72:**5478-5482.
21. **McFarland LV.** 2008. Antibiotic-associated diarrhea: epidemiology, trends and treatment. Future Microbiol **3:**563-578.
22. **Sekirov I, Russell SL, Antunes LC, Finlay BB.** 2010. Gut microbiota in health and disease. Physiol Rev **90:**859-904.
23. **Gardel CL, Mekalanos JJ.** 1994. Regulation of cholera toxin by temperature, pH, and osmolarity. Methods in enzymology **235:**517-526.
24. **Hung DT, Mekalanos JJ.** 2005. Bile acids induce cholera toxin expression in Vibrio cholerae in a ToxT-independent manner. Proceedings of the National Academy of Sciences of the United States of America **102:**3028-3033.
25. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. Journal of bacteriology **170:**2575-2583.
26. **Kovacikova G, Lin W, Skorupski K.** 2005. Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in Vibrio cholerae. Molecular microbiology **57:**420-433.
27. **Thomas S, Williams SG, Manning PA.** 1995. Regulation of tcp genes in classical and El Tor strains of Vibrio cholerae O1. Gene **166:**43-48.
28. **Ogierman MA, Voss E, Meaney C, Faast R, Attridge SR, Manning PA.** 1996. Comparison of the promoter proximal regions of the toxin-co-regulated tcp gene cluster in classical and El Tor strains of Vibrio cholerae O1. Gene **170:**9-16.
29. **Kovacikova G, Skorupski K.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the Vibrio cholerae tcpPH promoter. Molecular microbiology **41:**393-407.
30. **Kovacikova G, Skorupski K.** 2000. Differential activation of the tcpPH promoter by AphB determines biotype specificity of virulence gene expression in Vibrio cholerae. Journal of bacteriology **182:**3228-3238.
31. **Kovacikova G, Skorupski K.** 2002. Binding site requirements of the virulence gene regulator AphB: differential affinities for the Vibrio cholerae classical and El Tor tcpPH promoters. Mol Microbiol **44:**533-547.
32. **Behari J, Stagon L, Calderwood SB.** 2001. pepA, a gene mediating pH regulation of virulence genes in Vibrio cholerae. Journal of bacteriology **183:**178-188.
33. **Pratt JT, Ismail AM, Camilli A.** 2010. PhoB regulates both environmental and virulence gene expression in Vibrio cholerae. Molecular microbiology **77:**1595-1605.

34. **Merrell DS, Hava DL, Camilli A.** 2002. Identification of novel factors involved in colonization and acid tolerance of Vibrio cholerae. Molecular microbiology **43:**1471-1491.
35. **Chiang SL, Mekalanos JJ.** 1998. Use of signature-tagged transposon mutagenesis to identify Vibrio cholerae genes critical for colonization. Molecular microbiology **27:**797-805.
36. **Tischler AD, Camilli A.** 2005. Cyclic diguanylate regulates Vibrio cholerae virulence gene expression. Infect Immun **73:**5873-5882.
37. **Champion GA, Neely MN, Brennan MA, DiRita VJ.** 1997. A branch in the ToxR regulatory cascade of Vibrio cholerae revealed by characterization of toxT mutant strains. Molecular microbiology **23:**323-331.
38. **Yu RR, DiRita VJ.** 1999. Analysis of an autoregulatory loop controlling ToxT, cholera toxin, and toxin-coregulated pilus production in Vibrio cholerae. J Bacteriol **181:**2584-2592.
39. **DiRita VJ, Neely M, Taylor RK, Bruss PM.** 1996. Differential expression of the ToxR regulon in classical and E1 Tor biotypes of Vibrio cholerae is due to biotype-specific control over toxT expression. Proc Natl Acad Sci U S A **93:**7991-7995.
40. **Xu X, Stern AM, Liu Z, Kan B, Zhu J.** 2010. Virulence regulator AphB enhances toxR transcription in Vibrio cholerae. BMC Microbiol **10:**3.
41. **Iwanaga M, Yamamoto K, Higa N, Ichinose Y, Nakasone N, Tanabe M.** 1986. Culture conditions for stimulating cholera toxin production by Vibrio cholerae O1 El Tor. Microbiol Immunol **30:**1075-1083.
42. **Iwanaga M, Kuyyakanond T.** 1987. Large production of cholera toxin by Vibrio cholerae O1 in yeast extract peptone water. J Clin Microbiol **25:**2314-2316.
43. **Higgins DE, DiRita VJ.** 1994. Transcriptional control of toxT, a regulatory gene in the ToxR regulon of Vibrio cholerae. Molecular microbiology **14:**17-29.
44. **Hammer BK, Swanson MS.** 1999. Co-ordination of legionella pneumophila virulence with entry into stationary phase by ppGpp. Mol Microbiol **33:**721-731.
45. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.

Chapter III

Mechanism of Action of Toxtazin A

Summary

Target identification is a major challenge in research on small molecule inhibitors, and many reviews have been written describing various approaches that have successfully been used (1-3). To identify the mechanism by which toxtazin A inhibits *toxT* transcription, we combined a loss-of-target strategy, which generates and tests possible hypothetical mechanisms of action, with two screening approaches. The results of this research suggest that toxtazin A may affect *toxT* transcription by inducing envelope, oxidative, and nutrient stress responses in the cell.

Introduction

Vibrio cholerae causes the acute and sometimes fatal diarrheal disease cholera by producing and secreting cholera toxin (CT) into the lumen of the host (4). Transcription of CT is activated by ToxT (5), expression of which is activated by ToxR and TcpP, two inner membrane transcriptional activators (6-10). Two small molecule inhibitors of *toxT* transcription, toxtazin A and toxtazin B, were identified in a high-throughput screen, and toxtazin A was found to inhibit *toxT* transcription and CT expression without affecting ToxR or TcpP expression (11).

Experimental evidence presented here indicates that toxtazin A treatment causes cells to activate stress pathways, suggesting that stress signaling may be linked to virulence gene regulation in *V. cholerae.* While this has not been shown directly in *V. cholerae*, many bacterial pathogens alter their virulence programs in response to stress signals. For example, many pathogenic bacteria activate their virulence genes in response to temperature stress (reviewed in (12)). Bacteria also sense membrane perturbations, called envelope stress, and while the mechanisms for sensing and responding the envelope stress is bacteria-specific, it generally involves activating the alternate sigma factor E (σ^E), the Cpx two component system, and/or the BaeSR two component system, which in turn represses virulence factors (reviewed in (13)). In *V. cholerae*, σ^E is required for colonization of infant mice and for growth in 3% ethanol, though the mechanism behind this is not clear (14). Oxidative and nitrosative stress responses have also been shown to be inhibit virulence gene expression in a number of organisms (15-18). As is the case with envelope stress, mutants deficient in the oxidative or nitrosative stress responses are attenuated in animal models; however, the mechanism by which stresses affect pathogenesis is not fully understood. Additionally, growth under nutritional stress induces the stringent response, which affects the activation of virulence genes in many pathogens, including *V. cholerae* (19, 20).

Here, we present evidence that toxtazin A does not affect ToxR or TcpP localization to the inner membrane, nor does it inhibit ToxR binding to the *toxT* promoter. Toxtazin A causes a growth defect in M9 minimal media supplemented with amino acids asparagine, glutamate, arginine, and serine (NERS) and in LB supplemented with 3%

ethanol, suggesting that it can activate both nutrient and envelope stress responses in the cell. Furthermore, toxtazin A induces the expression of an oxidoreductase, suggesting toxtazin A induces an oxidative stress response. A comparative proteomic approach corroborates the above results. Based on these findings, we hypothesize that toxtazin A induces stress response(s), particularly redox stresses, which in turn signals the cell to shut off *toxT* transcription. Through this chemical biology approach, the work described expands our knowledge of the physiological control of virulence gene expression in *V. cholerae*.

Results

ToxR and TcpP localize correctly to the membrane

One mechanism by which toxtazin A could inhibit *toxT* transcription without altering ToxR or TcpP levels would be by altering the localization of either or both of these activators, rendering them incapable forming a complex at the inner membrane. To address this possibility, cultures were grown overnight under toxin-inducing conditions in the presence or absence of 10 µM toxtazin A before collecting the cells by centrifugation. Pellets were resuspended in a Tris buffer and lysed by French press before ultracentrifugation. The resulting supernatants, containing the cytoplasmic and periplasmic proteins, and pellets, containing membranes, were diluted or resuspended in sample buffer and boiled prior to electrophoresis and immunoblotting with TcpP or ToxR antisera (Figure 3.1). Toxtazins B and B’ were also analyzed by this approach. Anti-serum against EpsL, a well-characterized inner membrane protein from *V. cholerae*,

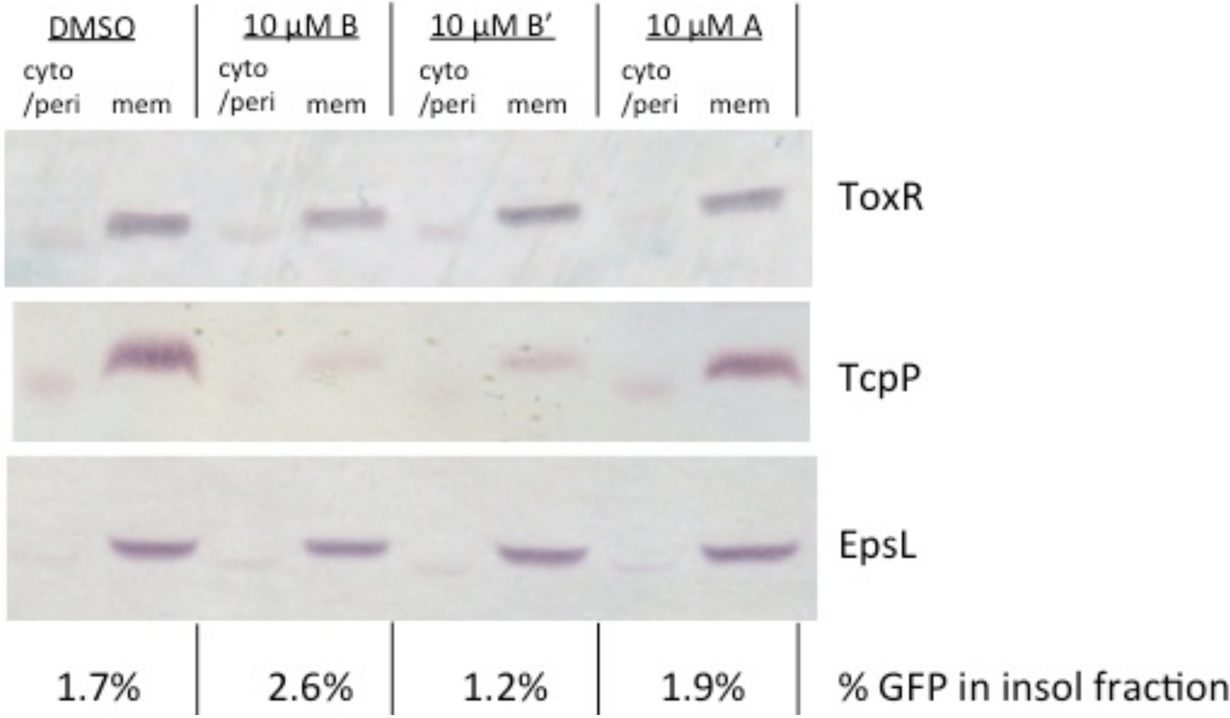


Figure 3.1 The toxtazins do not alter the membrane-localization of ToxR or TcpP

Cells treated overnight under toxin-inducing conditions in the presence or absence of 10 µM toxtazin A, B, or B' were lysed, and the membranes were separated from the cytoplasmic and periplasmic fractions by ultracentrifugation. The localization of ToxR and TcpP was determined by Western blot analysis. EpsL served as a control for the membrane fraction, and GFP fluorescence served as a control for the cytoplasmic fraction.

was used as a control for the membrane fraction, while expression of GFP was used as a control for the cytoplasmic fraction.

The GFP and EpsL results indicate that the fractionation protocol worked well, with 98% of GFP fluorescence identified in the soluble (cytoplasmic) fraction and all detectable EpsL identified in the pellet (membrane) fraction**.** Treatment with 10 µM of any of the toxtazins did not alter membrane localization of ToxR. Localization of TcpP was unaffected by toxtazin A or toxtazin B, but absolute levels of TcpP produced were severely decreased by toxtazin B treatment, just as previously described (Chapter 2; (11)). Taken together, these results rule out a mechanism by which the toxtazins affect *toxT* gene expression by reducing membrane localization of the *toxT* regulators ToxR or TcpP.

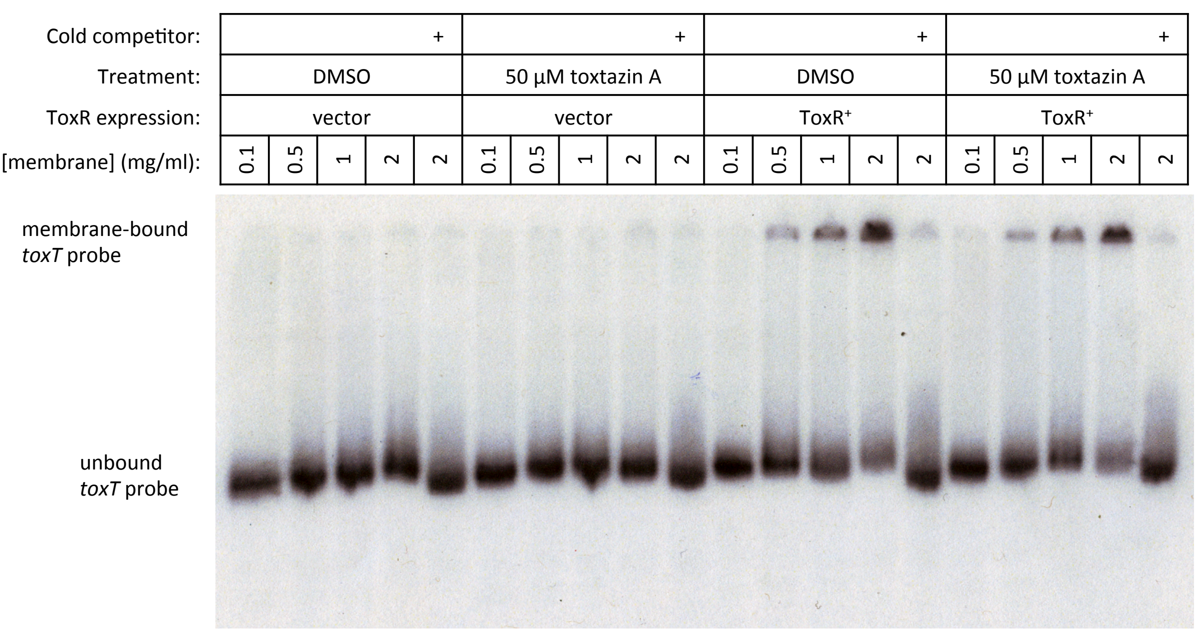
Toxtazin A does not inhibit ToxR from binding the *toxT* promoter

Having eliminated the possibility of toxtazin A inducing ToxR and/or TcpP mislocalization, we next tested the hypothesis that it may inhibit the ability of ToxR to bind the *toxT* promoter. Because ToxR and TcpP are single-pass inner membrane proteins, they are difficult to purify. Consequently, gel shifts have been performed using membrane fractions of cells over-expressing either ToxR or TcpP (10). These membrane fractions do not enter the gel, so complexes of protein and radiolabeled DNA are observed in the well, while non-complexed radiolabeled DNA is detectable within the gel proper.

Membrane fractions from cells lacking ToxR or with plasmid-expressed ToxR were isolated by ultracentrifugation. Varying amounts were incubated with radiolabelled *toxT* promoter and either 50µM toxtazin A in DMSO, or DMSO alone for 30 minutes at 30°C, after which the reaction mixture was subjected to electrophoresis through a 6% polyacrylamide gel. Radiolabeled *toxT* promoter was shifted by ToxR at membrane concentrations of 0.5 mg/ml or greater, but was not shifted by membranes from cells lacking ToxR. When 50 µM toxtazin A was added, ToxR still bound and shifted the *toxT* promoter, and the concentration at which this occurs was not altered, even at a concentration of toxtazin A five times greater than what we demonstrated is effective for reducing *toxT* expression (Figure 3.2). These results indicate that *in vitro*, toxtazin A does not inhibit the ability of ToxR to bind the *toxT* promoter, nor does it affect the amount of ToxR required for binding.

<u>Toxtazin A confers growth defects under some conditions</u>

Two observations suggest that toxtazin A may directly or indirectly affect central metabolism. One is that cultures grown at 30°C in M9 minimal media supplemented with asparagine, glutamate, arginine, and serine (NERS) – a condition known to stimulate toxin and pilus production – demonstrated a growth defect when toxtazin A was added (Figure 3.3). This was not observed when toxtazin B or B' was added, indicating that this phenomenon is specific to toxtazin A. This growth defect was more severe in the classical O395 strain than in the El Tor C6706 strain, indicating this effect may be either strain- or biotype-specific.



<u>Figure 3.2 Toxtazin A does not inhibit ToxR from binding the *toxT* promoter</u>

Membranes were purified from *V. cholerae* Δ*toxR* containing pSK empty vector or pSK-ToxR-HA. Varying concentrations of membranes were incubated with radiolabeled *toxT* probe in the presence or absence of toxtazin A, then run on a 6% polyacrylamide gel. An excess of cold *toxT* probe was added in the last lane of each group.

Envelope stress response and toxtazin A

Gram-negative bacteria respond to envelope stress by activating σ^E, encoded by the *rpoE* gene (13). RpoE is essential for responding to membrane stress and is important for virulence, as an *rpoE* mutant is 30-fold decreased for colonization of infant mice (14). It was previously shown that wild type *V. cholerae* can grow in LB + 3% ethanol, which induces envelope stress, while an *rpoE* mutant cannot (14). To determine if toxtazin A affects the ability of cells to sense and/or respond to envelope stress, cultures were grown in the presence and absence of 10 µM toxtazin A in LB + 3% ethanol at 37°C. An *rpoE* mutant served as a positive control. As shown in Figure 3.4, 10 µM toxtazin A prevented wild-type cells from growing in LB + 3% ethanol at 37°C to levels similar as that of an *rpoE* mutant. This was specific to toxtazin A, as toxtazin B showed only a mild growth defect.

It remains unclear why toxtazin A causes growth defects in some media (M9 minimal media + NERS and in LB + 3% ethanol), but not in others (LB and AKI medium). We hypothesize that cells grown in minimal media or in 3% ethanol undergo stress(es) that they do not encounter in rich media. Response to these stresses is required for wild type growth and we further hypothesize that toxtazin A blocks a key stress response mechanism. If this is true, it would suggest a link, whether direct or indirect, between cellular stress and virulence, and would uncover another level of regulation to the virulence cascade in *V. cholerae*.

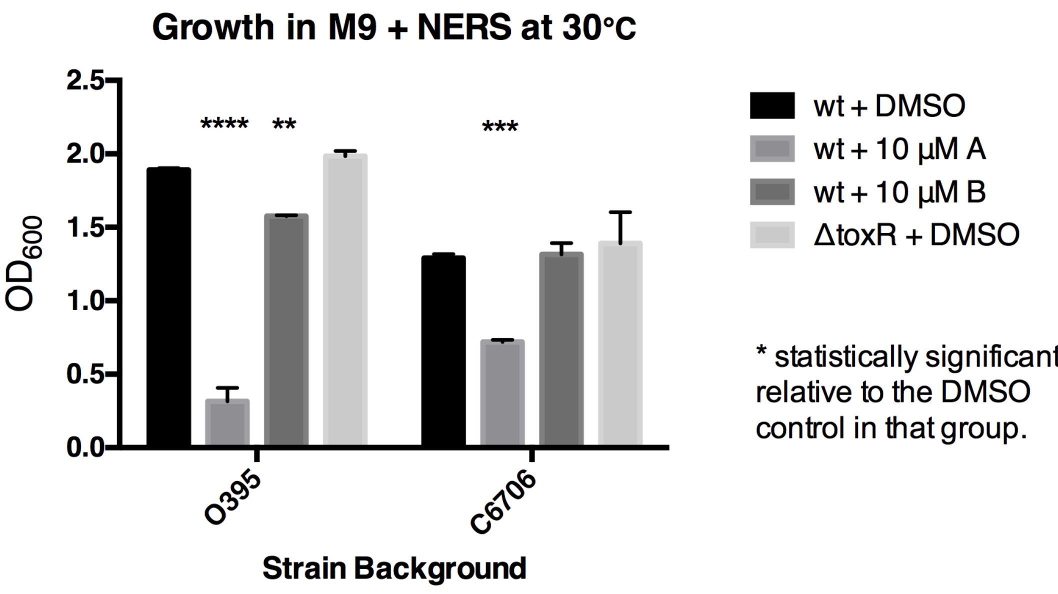


Figure 3.3 Toxtazin A causes a growth defect in M9 + NERS

Cultures of O395 or C6706 grown for 16 hours in M9 + NERS at 30°C are inhibited for growth by 10 µM toxtazin A, but not by 10 µM toxtazin B.

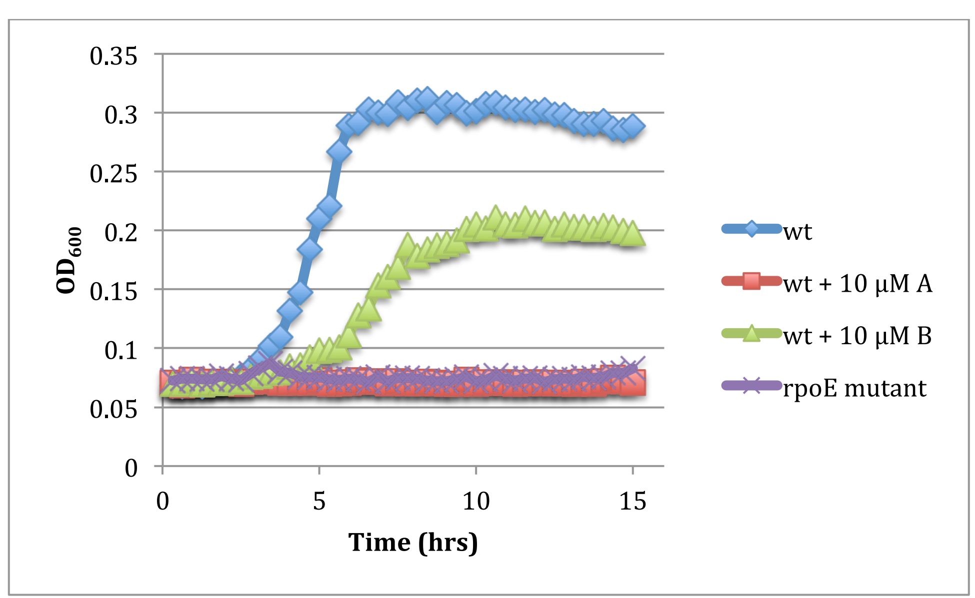


Figure 3.4. Toxtazin A inhibits growth in LB + 3% ethanol

Cultures of O395 were grown for 16 hours in LB + 3% ethanol in a 96-well plate at 37°C in the presence of either DMSO, 10 µM toxtazin A, or 10 µM toxtazin B, and the OD_{600} was read every fifteen minutes.

A proteomic approach toward MOA identification

In combination with the approaches described above, we sought to determine the mechanism of action of toxtazin A using an unbiased approach. To characterize effects of toxtazin A on O395 *V. cholerae* grown overnight under toxin-inducing conditions (LB pH 6.5 at 30°C shaking), we subjected protein extracts of these cells to gel LC-MS/MS. Proteins were collected from cell lysates by TCA precipitation and subjected to SDS-PAGE. Twenty sequential gel slices were digested with trypsin followed by analysis with tandem mass-spectrometry to generate a semi-quantitative list of proteins present in the sample.

We focused our initial analysis on proteins with a four-fold difference in quantity between extracts from DMSO- versus toxtazin A-treated samples (indicating the protein is more highly expressed in one sample than the other), excluding ~700 proteins that were detected 10 times or fewer in both samples to increase confidence (Table 3.1). Known ToxT-controlled proteins are marked by an asterisk in Table 3.1, and account for 19.4% of the proteins identified. These proteins were expected to be diminished by toxtazin A because of its effects on *toxT* transcription. In contrast, no AphA- or AphB-regulated proteins (21, 22) were differentially expressed in the presence of toxtazin A, corroborating the finding that AphA and AphB are not affected by toxtazin A. That ToxT-dependent proteins are so prevalent in these data while no AphA- or AphB-dependent proteins were identified suggests that this method of identifying proteins affected by toxtazin A is reliable.

Table 3.1 Proteins with 4-fold differential expression in toxtazin A-treated cells.

Proteins known to be controlled by ToxT are indicated by an asterisk.

Identified Protein	VC ID#	# Peptides (toxtazin A)	# Peptides (DMSO)	Fold difference (A/DMSO)	GO: Biological process	GO: molecular function
Malate synthase	VCA0957	13	0	#DIV/0!	Metabolic process	Malate synthase activity
Oye family NADH-dependent flavin oxidoreductase	VCA0998	115	0	#DIV/0!	Cell redox homeostasis	Oxidoreductase
Sulfite reductase (NADPH) flavoprotein alpha-component	VC_0384	14	0	#DIV/0!	Cysteine biosynthesis	Oxidoreductase
Sulfite reductase subunit beta	VC_0385	12	0	#DIV/0!	Cysteine biosynthesis	Oxidoreductase
Trehalose-6-phosphate hydrolase	VC0911	11	0	#DIV/0!	Carbohydrate metabolic process	alpha,alpha-phosphotrehalase activity
Superoxide dismutase, Cu-Zn	VC_1583	19	0	#DIV/0!	Cell redox homeostasis	Superoxide dismutase activity
Catalase PrtV	VC_1585	26	0	#DIV/0!	Cell redox homeostasis	Catalase activity
Ribosomal protein S12 methylthiotransferase	VC_2620	13	0	#DIV/0!	RNA processes	Transferase activity
Peroxiredoxin family protein/glutaredoxin	VC_2637	59	0	#DIV/0!	Cell redox homeostasis	Protein disulfide oxidoreductase activity
Dihydrolipoamide dehydrogenase	VC_2638	31	0	#DIV/0!	Cell redox homeostasis	Flavin adenine dinucleotide binding
Enterobactin receptor protein irgA	VC_0475	17	2	8.50	Pathogenesis	Transporter activity
Thiosulfate ABC transporter substrate-binding protein	VC_0538	26	4	6.50	Transport	Sulfate transmembrane-transporting ATPase activity
tRNA uridine 5-carboxymethyl-aminomethyl modification protein GidA	VC_2775	19	3	6.33	tRNA processing	Flavin adenine dinucleotide binding
Periplasmic alpha-amylase precursor	VC_A0860	12	2	6.00	Carbohydrate metabolic process	alpha-amylase activity

Identified Protein	VC ID#	# Peptides (toxtazin A)	# Peptides (DMSO)	Fold difference (A/DMSO)	GO: Biological process	GO: molecular function
tRNA-dihydrouridine synthase A dusA	VC_0379	11	2	5.50	tRNA processing	Flavin adenine dinucleotide binding
Protease	VC_0717	16	3	5.33	Proteolysis	Peptidase activity
Phosphopentomutase deoB	VC_2348	34	7	4.86	Deoxyribonucleotide catabolic process	Phosphopentomutase activity
Amino acid ABC transporter substrate-binding protein	VC_0010	55	12	4.58	Amino acid transport	Transporter activity
Dihydropteridine reductase	VC_A0637	49	11	4.45	Cell redox homeostasis	6,7-dihydropteridine reductase activity
Glutathione S-transferase	VC_A0585	24	6	4.00	Metabolic process	Glutathione transferase activity
Amino acid ABC transporter periplasmic amino acid-binding protein	VC_A0978	4	16	0.25	Amino acid transport	Transporter activity
Chemotaxis protein CheV	VC_2006	3	12	0.25	Chemotaxis	Signal transducer activity
Transcription-repair coupling factor	VC_1886	13	53	0.25	DNA processes	ATP-dependent helicase activity
Hypothetical protein VC1645	VC_1645	3	13	0.23	Metabolic process	Hydrolase activity
Septum site-determining protein MinD	VC_1960	3	13	0.23	Cytokinesis	ATPase activity
MarR family transcriptional regulator	VC_0649	3	15	0.20	Unknown	DNA binding transcription factor activity
* Accessory colonization factor AcfC	VC_0841	20	104	0.19	Pathogenesis	Pilus
Amino acid ABC transporter substrate-binding protein	VC_1362	2	11	0.18	Amino acid transport	Transporter activity
Zinc/cadmium/mercury/lead-transporting ATPase	VC_1033	29	173	0.17	Metal ion transport	Cation-transporting ATPase activity
Aldehyde dehydrogenase	VC_1819	8	48	0.17	Cell redox homeostasis	Oxidoreductase
Hypothetical protein VCA0271	VC_A0271	4	24	0.17	Unknown	DNA binding
Hypothetical protein VCA0981	VC_A0981	2	12	0.17	Unknown	Transporter activity

Identified Protein	VC ID#	# Peptides (toxtazin A)	# Peptides (DMSO)	Fold difference (A/DMSO)	GO: Biological process	GO: molecular function
ATP-dependent RNA helicase RhlB	VC_0305	2	12	0.17	RNA processes	ATP-dependent RNA helicase activity
Thiol peroxidase tpx, tagD	VC_0824	40	280	0.14	Cell redox homeostasis	Thioredoxin peroxidase activity
Hypothetical protein VC1249	VC_1249	12	85	0.14	Unknown	Unknown
Exonuclease V subunit gamma	VC_2322	3	23	0.13	DNA processes	Exodeoxyribonuclease V activity
Hypothetical protein VC1083	VC_1083	2	17	0.12	Unknown	Hydrolase activity
Sensory box sensor histidine kinase/response regulator VieS	VC1653	7	69	0.10	Phosphorelay signal transduction system	Phosphorelay sensor kinase activity
Sensory box sensor histidine kinase/response regulator	VC0622	2	21	0.10	Phosphorelay signal transduction system	Phosphorelay sensor kinase activity
DNA polymerase III subunit alpha DnaE	VC_2245	3	36	0.08	DNA processes	3'-5' exonuclease activity
Porin	VC_0972	2	29	0.07	Transport	Porin activity
* Cholera enterotoxin subunit A	VC_1457	5	115	0.04	Pathogenesis	Toxin
* Toxin co-regulated pilus biosynthesis protein S	VC_0834	0	58	0.00	Pathogenesis	Pilus assembly
* Accessory colonization factor AcfA	VC_0844	0	56	0.00	Pathogenesis	Colonization
* Toxin co-regulated pilus biosynthesis outer membrane protein C	VC_0831	0	46	0.00	Pathogenesis	Pilus assembly
* Toxin co-regulated pilus biosynthesis protein B	VC_0829	0	37	0.00	Pathogenesis	Pilus assembly
* ToxR-activated gene A protein TagA	VC_0820	0	36	0.00	Proteolysis	Metalloendopeptidase activity
* Toxin co-regulated pilus biosynthesis protein D	VC_0833	0	33	0.00	Pathogenesis	Pilus assembly
Aldehyde dehydrogenase	VC0819	0	26	0.00	Pathogenesis	Aldehyde dehydrogenase (NAD) activity
* Toxin co-regulated pilus biosynthesis protein Q	VC_0830	0	25	0.00	Pathogenesis	Pilus assembly

Identified Protein	VC ID#	# Peptides (toxtazin A)	# Peptides (DMSO)	Fold difference (A/DMSO)	GO: Biological process	GO: molecular function
* Accessory colonization factor AcfB	VC_0840	0	19	0.00	Pathogenesis	Signal transducer activity
Hypothetical protein VC0414	VC_0414	0	17	0.00	Unknown	Unknown
* Toxin co-regulated pilus biosynthesis protein E	VC_0836	0	17	0.00	Pathogenesis	Protein secretion
Protease	VC_A0223	0	16	0.00	Proteolysis	Metallopeptidase activity
Hypothetical protein VCA0574	VC_A0574	0	14	0.00	Unknown	N-acetyltransferase activity
TagA-related protein	VCA0148	0	13	0.00	Unknown	Metalloendopeptidase activity
Tryptophan synthase subunit alpha TrpA	VC_1169	0	13	0.00	Amino-acid biosynthesis	Tryptophan synthase activity
Cholera enterotoxin subunit B	VC_1456	0	12	0.00	Pathogenesis	Toxin
Hypothetical protein VC2507	VC_2507	0	12	0.00	Unknown	ATP binding
Cytochrome b561, putative	VC_A0249	0	11	0.00	Respiration	Electron carrier activity
Hypothetical protein VCA1024	VCA1024	0	11	0.00	Unknown	Unknown
Toxin co-regulated pilus biosynthesis protein P	VC_0826	0	11	0.00	Pathogenesis	DNA binding

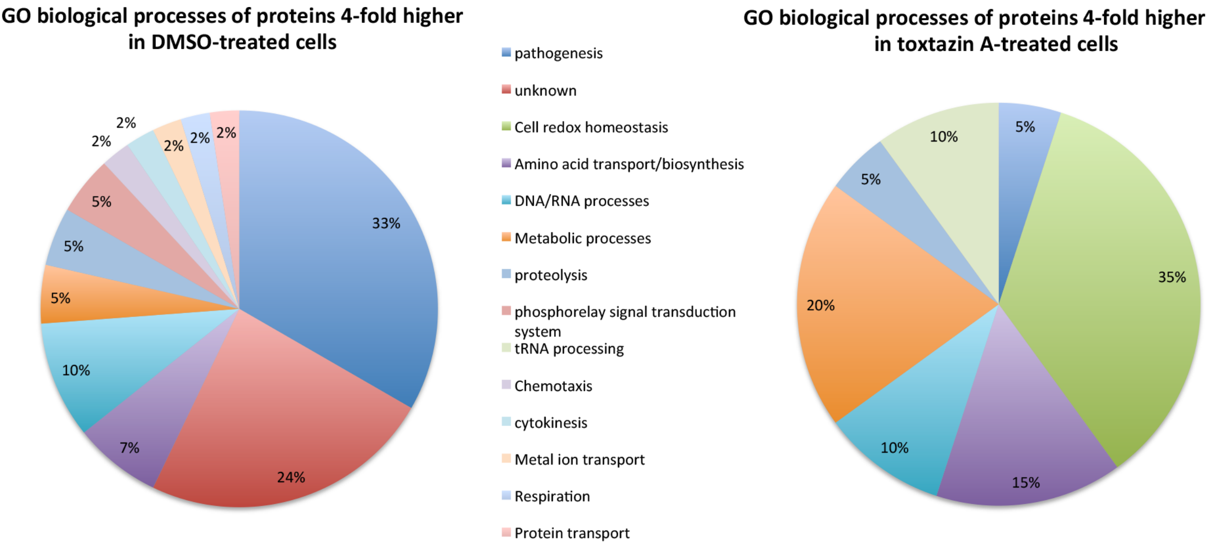


Figure 3.5 GO terms of proteins differentially expressed in the presence of toxtazin A.

Cultures of O395 *V. cholerae* were grown for 16 hours in the presence or absence of toxtazin A. Cells were pelleted, washed twice in PBS, and lysed. The proteins were separated by SDS-PAGE, trypsin digested, and the peptides were identified by liquid chromatography and tandem mass spectrometry (gel LC-MS/MS). The proteins that were four-fold differentially produced in the toxtazin A-treated cells, and detected at least 10 times or more in each sample. These pie charts indicate the GO terms that are differentially more highly produced in DMSO-treated cells (left pie chart) or in toxtazin A-treated cells (right pie chart).

Genome ontology (GO) terms were used to classify proteins altered by toxtazin A (Table 3.1; Figure 3.5). The largest category is pathogenesis, followed by proteins of unknown function. The next largest group represents proteins involved in cell redox homeostasis, which strengthens our hypothesis that toxtazin A-treated cells are experiencing and responding to stress. For example, the peroxiredoxin family protein PrxA (VC2637) was identified often (59 times) and exclusively in toxtazin A-treated cells. This protein is regulated by OxyR and is important for responding to H_2O_2 stress (23), and is required for infant mouse colonization by *V. cholerae* (24). That cells grown in the presence of toxtazin A activate OxyR-dependent genes suggests that they may be sensing and/or responding to oxidative stress or ROS.

Oxidoreductase VC0731 is induced by toxtazin A

The gel LC-MS/MS results indicated that six oxidoreductases (9.7% of the differentially expressed proteins) are differentially expressed in cells treated with toxtazin A. A seventh, VC0731, was also induced in toxtazin A-treated cells, though it was not recovered enough times to make the cutoff for confidence in the gel LC-MS/MS experiment. Rather, it was identified as a ~27 kDa band on coomassie-stained, one-dimensional SDS-PAGE gels that as consistently more expressed in toxtazin A-treated cells (Figure 3.6A). Mass spectrometry analysis determined this band to be VC0731, annotated as an AhpC-like oxidoreductase. Two toxtazin A analogs, toxtazins A2 and A3 that also reduced *toxT*-GFP levels, also induced VC0731 expression (Figure 3.6B), indicating that this phenotype is not an artifact but is specific to the toxtazin A

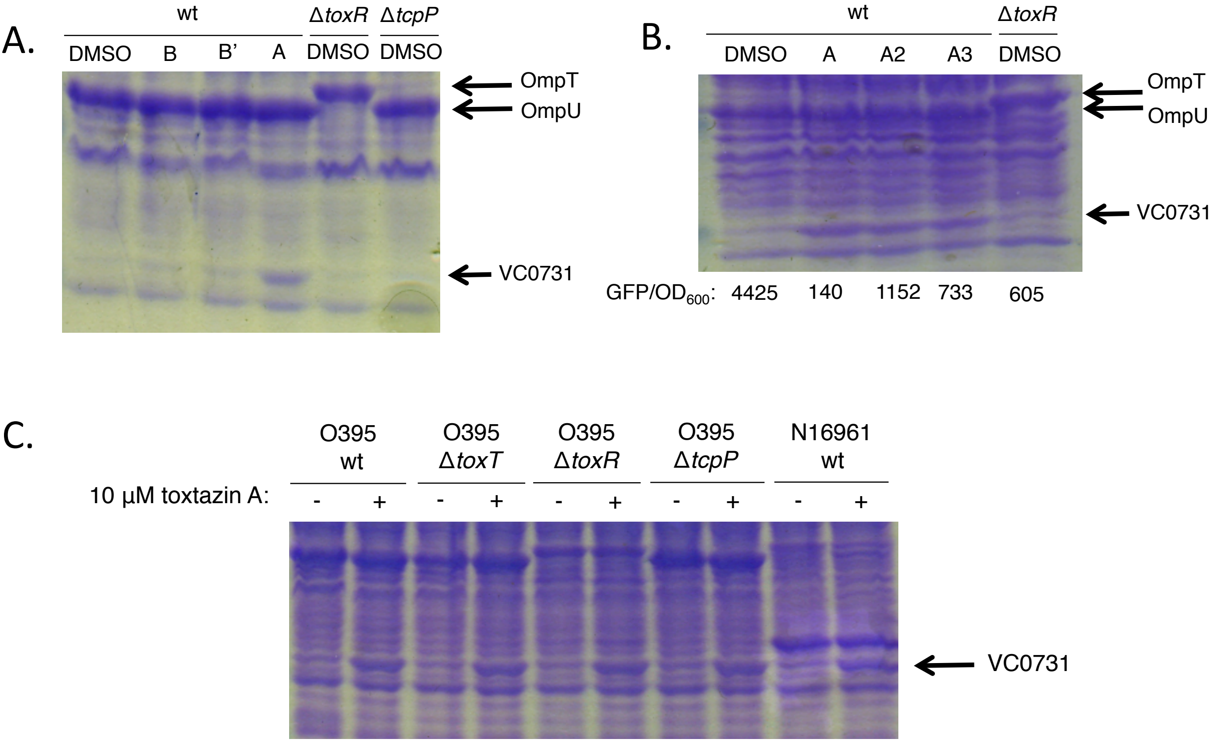


Figure 3.6 Toxtazin A treatment results in overexpression of VC0731.

Cultures were grown under toxin-inducing conditions (LB pH 6.5 at 30°C) for 16 hours in the presence or absence of 10 µM of various compounds. Cells were pelleted, lysed, and resuspended in sample buffer, normalizing for OD_{600}, then run on a 12.5% SDS-PAGE gel. The gel was stained with Coomassie to visualize VC0731. A. O395 cultures were grown with DMSO, toxtazins A, B, or B'. B. Reporter strain RA2 (O395 + p*toxT-gfp*) cultures were grown with toxtazin A, or analogs A2 and A3. C. Cultures of wild-type O395, Δ*toxT*, Δ*toxR*, Δ*tcpP*, or wild-type N16961 were grown with 10 µM toxtazin A.

pharmacocore. VC0731 was induced by toxtazin A in both classical (O395) and El Tor (N16961) biotypes and irrespective of *toxT*, *toxR*, and *tcpP* (Figure 3.6C).

Because AhpC is regulated by OxyR in *E. coli* (25), we sought to determine whether VC0731, an AhpC-like protein, might also be regulated by OxyR. We grew an *oxyR*::TnFGL3 insertion mutant (from a previously described ordered transposon library (26)) in the presence or absence of 10 µM toxtazin A overnight under toxin-inducing conditions. Extracts were analyzed by SDS-PAGE. The gene upstream of and divergently transcribed from VC0731, VC0732, is annotated as a LysR-family regulator. Because these regulators often control genes from which they are divergently transcribed (27), we also tested a strain carrying a transposon insertion in VC0732. The results indicate that expression of VC0731 requires VC0732, but not OxyR (Figure 3.7A). Further, its expression is elevated by growth at 37°C regardless of toxtazin A treatment (Figure 3.7B).

We hypothesized that over-expression of VC0731 caused by toxtazin A could inhibit *toxT* gene expression, thereby limiting toxin and pilus production. To test this, we ectopically expressed VC0731 on an arabinose-inducible plasmid in *V. cholerae* cells grown overnight under toxin-inducing conditions. Culture supernatants were then examined for CT levels by CT ELISA. Contrary to our hypothesis, overexpression of VC0731 per se does not lead to decreased CT expression (Figure 3.8A).

We also tested whether VC0732, the LysR-activator that controls VC0731 expression, regulates proteins that might inhibit CT expression. A mutant lacking *vc0732* was constructed and grown under toxin-inducing conditions. Toxin levels were

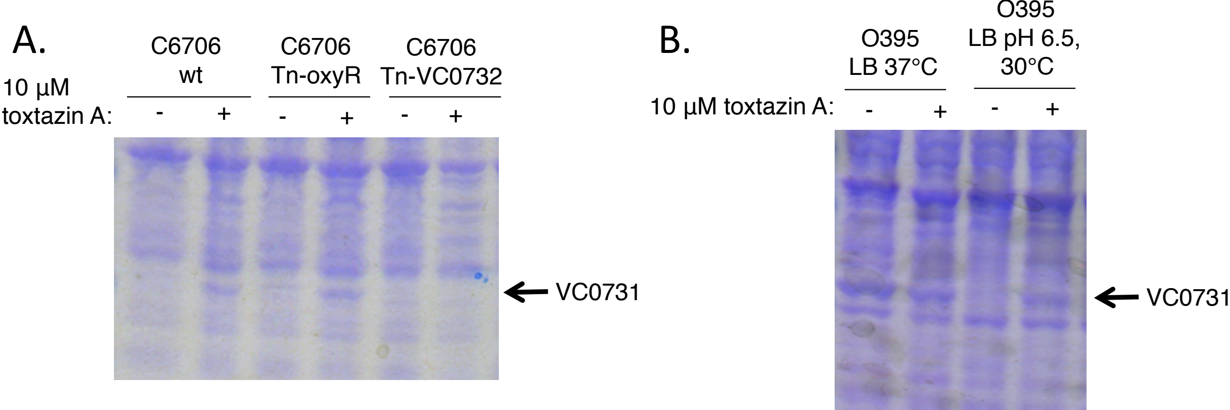


Figure 3.7 VC0731 is activated by VC0732, not OxyR, and is induced at 37°C.

A. Wild-type, Tn-*oxyR*, and Tn-*VC0732* C6706 strains were grown for 16 hours under AKI conditions, which induces virulence gene expression in the El Tor biotype. Cell extracts were run out on a 12.5% SDS-PAGE gel and VC0731 was visualized by Coomassie staining. B. Wild-type O395 cultures were grown in LB at 37°C or in LB pH 6.5 at 30°C, which induces virulence gene expression in the Classical biotype. VC0731 was visualized by coomassie staining.

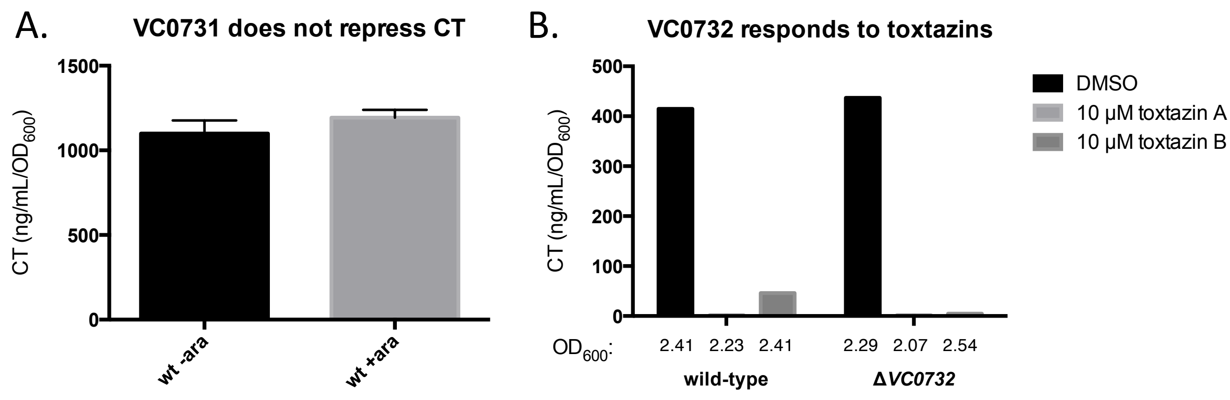


Figure 3.8. VC0731 and VC0732 do not affect CT expression.

A. Cultures of wild-type O395 containing arabinose-inducible pAD18-VC0731were grown for 16 hours under toxin-inducing conditions. Supernatants were collected and analyzed for CT levels by ELISA. B. Wild-type O395 and an isogenic Δ*VC0732* mutant were grown for 16 hours under toxin-inducing conditions (LB pH 6.5 at 30°C) in the presence or absence of 10 μM toxtazin A or B. CT levels were quantified by ELISA.

similar in wild type and *Δvc0732* cells, ruling out any action on toxin production by the toxtazins that works through a VC0732-dependent mechanism (Figure 3.8B).

These results indicate that toxtazin A does not reduce CT expression by controlling VC0731 or VC0732. However, they do clearly suggest that toxtazin A causes the cell to sense or respond to oxidative stress, which is associated with an inability of bacteria to activate *toxT* and leading to down-regulation of toxin and pilus production.

Screening for toxtazin A-resistant mutants

Using an open-ended approach to identify the mechanism of action of toxtazin A, we developed a screen for mutants that could no longer respond to toxtazin A. We reasoned that a mutation in the toxtazin A target would render strains non-responsive to the compound, resulting in elevated *toxT* expression in the presence of toxtazin A. Libraries of both transposon-induced mutants and potential spontaneous mutants generated by continuous cycles of growth over several generations were tested. For the transposon screen, a *mariner*-based transposon conferring kanamycin resistance was introduced into the original p$toxT_{pro}$-*gfp* reporter strain used to identify toxtazin A. Transposons were collected and pooled. Generation of spontaneous mutants was achieved by growing the reporter strain for 30 days with daily 1:100 dilutions.

To identify mutants resistant to either of the toxtazins, the transposon libraries were grown for 16 hours under toxin-inducing conditions (LB pH 6.5 at 30°C shaking) in the presence of DMSO, 50 µM toxtazin A, or 10 µM toxtazin B. The mutant libraries

grew similarly in the presence or absence of the toxtazins, and GFP levels decreased in the presence of either toxtazin A or B, indicating that the majority of mutants in the transposon libraries are sensitive to toxtazins A and B (Figure 3.9A and B). Cultures were then subjected to sorting by FACS (Figure 3.9C) to identify individual cells with elevated GFP levels in the presence of either toxtazin A or B. GFP levels of DMSO-treated cultures fit a single peak instead of displaying a bimodal distribution, indicating that the earlier GFP readings represent an average of GFP expression per cell, not an average of a population of cells expressing little GFP while and others expressing higher GFP. Treatment with either of the toxtazins followed this same trend but the GFP peak was shifted down, indicating that most of the mutants were still sensitive to treatment with either of the toxtazins, and any mutants resistant to toxtazin A or B treatment are rare, with no selective advantage in our growth conditions.

Toxtazin A-treated and toxtazin B-treated cells in the top 5% for GFP expression were sorted, and those that were in the top 1% for GFP expression were excluded to eliminate dead cells. The sorted cells, plated for isolation, were cultured in 96-well plates and frozen into glycerol stocks. Unfortunately, when the strains isolated as GFP-high in the presence of toxtazin A were retested in the presence of toxtazin A, they had low *toxT*-GFP levels indicating that were no longer resistant to toxtazin A. The same was true when the toxtazin B-resistant mutants were re-tested against toxtazin B. The reason for this remains unclear, but it is possible that the strains isolated by FACS sorting were GFP-high because they were not actively growing and dividing and thus

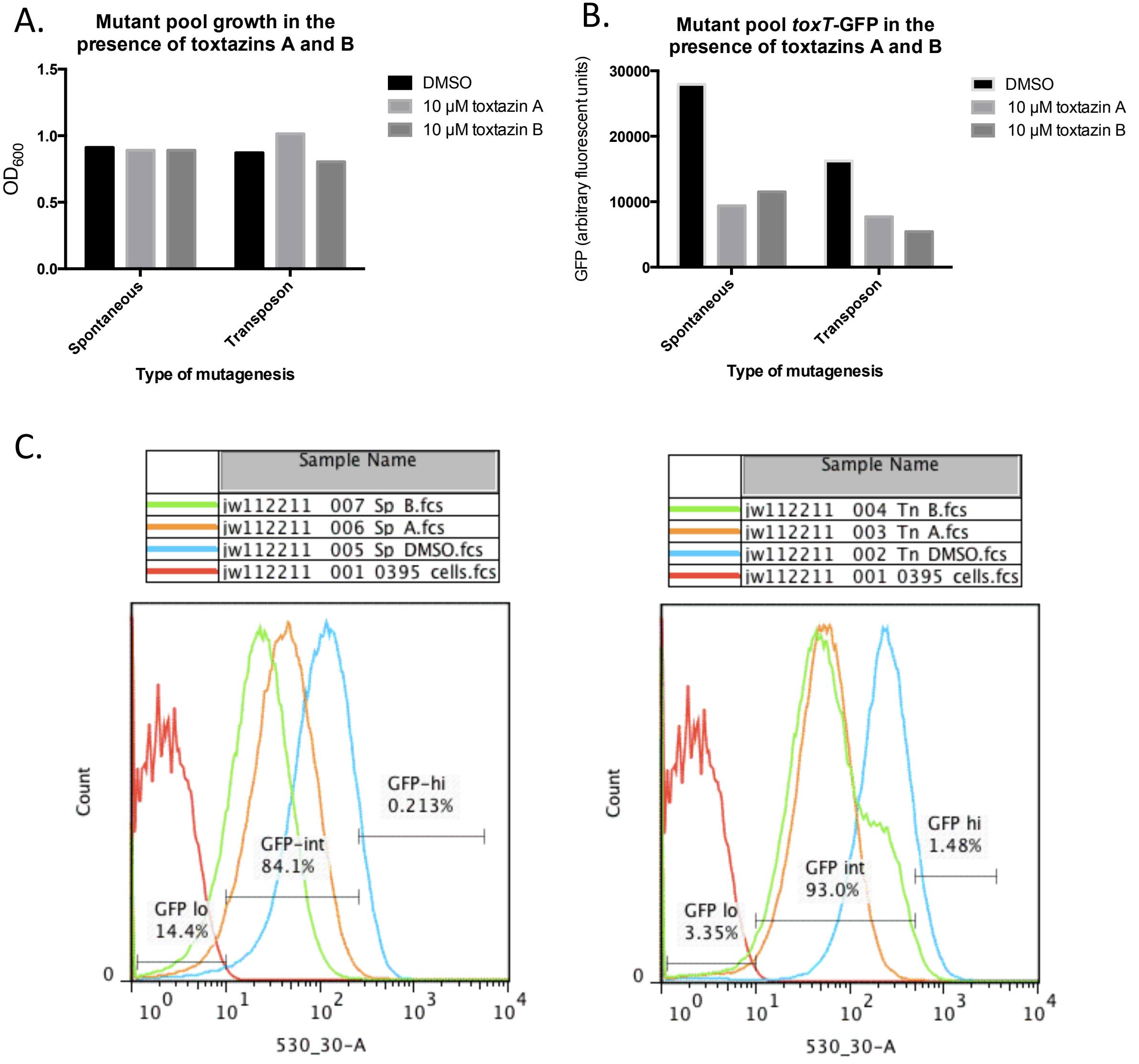


Figure 3.9 FACS sorting of transposon mutants for resistance to toxtazins A or B.

The OD_{600} (A) and GFP (B) levels of transposon mutant libraries grown overnight under toxin-inducing conditions in the presence or absence of the toxtazins. C. FACS analysis of the transposon libraries. Wild-type O395 was used to define the GFP-lo pool as it does not express GFP. The mutant strains contained the *toxT*-GFP reporter, and expressed GFP to varying levels.

accumulating GFP, rather than because *toxT-gfp* was more strongly expressed than in wild-type cells.

Discussion

The purpose of this work was to characterize the mechanism of action of toxtazin A, which was originally identified for its ability to inhibit transcription of *toxT* (11). Different hypotheses were examined including i) whether toxtazin A affects ToxR or TcpP, the transcriptional activators of *toxT*, and ii) whether cellular stresses, many of which are induced in the presence of toxtazin A, may regulate *toxT*.

Toxtazin A did not inhibit ToxR or TcpP from correctly localizing to the membrane, nor did it inhibit ToxR from binding the *toxT* promoter as shown by gel shift assays; however, this does not eliminate the possibility that a more complex mechanism may occur *in vivo.* For example, toxtazin A may stabilize or otherwise promote inhibition of ToxR binding through a molecule not present in these membrane fractions. This hypothesis could be tested by chromatin immunoprecipitation to determine *toxT* promoter occupancy by ToxR in cells treated or not treated with toxtazin A. Experiments also are being carried out to determine whether toxtazin A inhibits TcpP from binding the *toxT* promoter, and to determine whether ToxR-TcpP interaction is inhibited by toxtazin A treatment.

It was also found that toxtazin A-treated cells grew poorly or not at all under certain stress-inducing conditions (minimal media + NERS and LB + 3% ethanol)

suggests that toxtazin A affects one or more stress responses. Supporting this hypothesis is the observation that toxtazin A-treated cells up- or down-regulated expression of many proteins involved in stress pathways, including cell redox homeostasis and amino acid transport and biosynthesis. Experiments on VC0731 and its regulator VC0732, however, indicate that this pair of oxidoreductase and regulator does not inhibit the virulence cascade.

Identification of resistant mutants by FACS sorting has been successfully used by others (28, 29); however, we were not able to select toxtazin A- or B-resistant mutants with this technique. This approach could be improved by growing the GFP-high cells in the presence of toxtazin A or B and repeating the FACS sorting multiple times to enrich for resistant mutants. This enrichment step was necessary for the isolation of enzalutamide-resistant cells (30). Alternatively, a selective approach could be used to select for toxtazin A-resistant mutants using the growth defects in either LB + 3% ethanol or M9 minimal media supplemented with NERS. Mutants able to grow in the presence of toxtazin A under these stress conditions may be resistant to toxtazin A-dependent inhibition of *toxT*, and may therefore pinpoint the target of toxtazin A. One caveat to this experiment is that the growth arrest target may be different from the pathogenesis target.

Toxtazin A has been screened in 4 BioAssays in PubChem, and 29 toxtazin A analogs have been screened in BioAssays. In these BioAssays, micromolar levels of toxtazin A as well as toxtazin A analogs were found to inhibit the anti-apoptotic protein Bfl-1 (gene bcl2a1a of *Mus musculus*) (31). Other toxtazin A analogs were identified as

positive in 20 BioAssays reported in PubChem. Of those, the effects of the toxtazin A analogs were confirmed in secondary assays and shown to behave in a dose-dependent manner for the inhibition of NOD1 and NOD2, inhibition of peroxiredoxins in *Schistosoma mansoni*, and the inhibition of the mammalian selenoprotein thioredoxin reductase 1 (TrxR1).

The data presented here generate several new hypotheses about virulence gene regulation in *V. cholerae*. First, the observation that toxtazin A affects cellular stress pathways and reduces *toxT* expression prompted the hypothesis that one or more cellular stress responses can feedback to inhibit virulence gene expression. This hypothesis is exciting because it would be the first demonstration of cellular stress regulating virulence in *V. cholerae*. Furthermore, the fact that in the presence of toxtazin A, ToxR and TcpP are both present at wild-type levels and localized to the membrane yet do not activate *toxT* transcription is an exciting observation. This phenotype has also been observed in overnight cultures grown under non-inducing conditions (LB pH 8.5 at 37°C) and in LB at 37°C (Anthouard and DiRita, unpublished data). That ToxR and TcpP can be present without activating *toxT* transcription led to the hypothesis that ToxR and/or TcpP may be post-translationally regulated, and toxtazin A is a tool that can be used to induce and study this phenotype.

Discovering the mechanism of action of toxtazin A and following up on the two new hypotheses generated from toxtazin A research will be important future work as it will provide insight into a previously unknown layer of virulence regulation in this important pathogen.

Materials and Methods

Strains used in this study

The strains used in this study are outlined in Table 3.2, which were maintained at -80°C in Luria Bertani broth (LB) containing 20% glycerol. Cultures were grown overnight in LB at 37°C. Toxin-inducing conditions consist of growing cells in LB pH 6.5 at 30°C for 16 hours for Classical strain O395 (diluted 1:100), and growing in AKI medium as previously described (32) for El Tor strains N16961 and C6706 (diluted 1:1,000). Growth in minimal medium consists of diluting an overnight culture 1:100 in M9 minimal media supplemented with NERS (M9 salts, 2 mM MgSO4, 4% glycerol, 0.1 mM $CaCl_2$, and 5 mM each of asparagine, glutamic acid, arginine, and serine) at 37°C shaking (33).

Table 3.2. Strains used in this study.

Strain or plasmid	Relevant genotype or phenotype	Source
	Vibrio cholerae	
O395	Classical Ogawa, Sm^R	Laboratory collection
N16961	El Tor, Sm^R	Laboratory collection
C6706	El Tor, Sm^R	Laboratory collection
Tn::*oxyR*	C6706 Tn::*oxyR*	(26)
Tn::*VC0732*	C6706 Tn::*VC0732*	(26)
RA2	O395 + p*toxT-gfp*	(11)
RA218	O395 + pBAD18-VC0731	This work
RA247	O395 Δ*VC0732*	This work
EK1373	O395Δ*toxR*Δ*tcpP* + pSK-ToxR-HA	This work
EK1372	O395Δ*toxR*Δ*tcpP* + pSK	This work
RA246	SM10λpir + pKAS32-Δ*VC0732*	This work

Deletion of VC0732

The clean deletion of VC0732 in O395 was generated by mating O395 cells with SM10λpir cells containing the pKAS32-*ΔVC0732* plasmid. This plasmid was generated by PCR amplifying 500 bp upstream and 500 bp downstream of the VC0732 ORF with primers RAP182 (5'-GCAGATGATATCTCAACAGCAGTGTTA-3') and RAP183 (5'-GATGGAAGATGTGATTGGTCGTAGTGA-3'), and RAP184 (5'-TCACTACGACCAATCACATCTTCCATC-3') and RAP185 (5'-CTAGCTGAATTCTAAAGTCACTTAAAT-3'), respectively. The two fragments were sown together by overlap extension using primers RAP182 and RAP185, and cloned into pKAS32 by restriction digest using EcoRV and EcoRI.

Fractionation

Wild-type O395 cells were grown overnight under toxin-inducing conditions (LB pH 6.5 at 30°C shaking) in the presence or absence of 10 µM toxtazin A, B, or B'. Cultures were normalized for OD_{600}, and 5 ml were spun down at 4,000 rpm at 4°C for 15 minutes. The pellet was resuspended in 50 µl 0.2M Tris pH 8.0. To lyse the cells, the following were mixed into the sample in succession in this order: 100 µl 0.2M Tris pH 8.0 in 1 M sucrose, 10 µl 10 mM EDTA, 10 µl 10 mg/ml lysozyme, 300 µl ddH_2O, and 10 µl 50x protease inhibitor cocktail. The samples were incubated on ice for 10 minutes to allow cell lysis. Next, 5µl of Roche DNase and 500 µl of 50 mM Tris pH 8.0 were added to each sample. The cells were sonicated on ice for a total of 10 seconds to further lyse the cells. The membranes were separated from the cytoplasmic and

periplasmic fractions by ultracentrifugation at 65,000 rpm for 60 minutes at 4°C. The pellet was washed once with 500 µl of 50 mM Tris pH 8.0 to minimize contamination from the supernatant, and resuspended in 900 µl of 2% TritonX-100/50 mM Tris pH 8.0/10m M $MgCl_2$ using a 22 gauge needle. As controls for the efficiency of fractionation, expression of EpsL, a known inner membrane protein, was determined by Western blot analysis and GFP fluorescence, restricted to the cytoplasm, was determined by reading GFP fluorescence in a plate reader (excitation λ = 385 nm, emission λ = 425 nm).

DNA gel mobility shift assays

These were performed as previously described (10). First, 500 ml cultures of *V. cholerae* strain O395Δ*toxR*Δ*tcpP* + pSK-ToxR-HA (strain RA103) and the vector control strain O395Δ*toxR*Δ*tcpP* + pSK (strain RA104) were grown at 37°C to mid-log, induced with 1mM IPTG, and allowed to grow another 4 hrs. The cells were then pelleted, frozen overnight, resuspended in Lysis Buffer (100 mM Tris pH 6.8, 0.75 M sucrose, 2 mM EDTA, 60 µg/ml lysozyme, and Complete protease inhibitors), and incubated on ice for 20 minutes. The cells were further lysed by two rounds of french pressing at 12,000 psi. Unlysed cells and debris were pelleted by centrifugation at 4,000 rpm for 15 min at 4°C. The resulting lysate was ultracentrifuged at 35,000 rpm for 90 minutes at 4°C to separate the membranes from the cytoplasmic and periplasmic fractions. The membranes were frozen overnight, resuspended in a 5 mM EDTA /25% sucrose solution, and quantified using a Pierce BCA protein assay.

Next, radiolabeled *toxT* probe was prepared by PCR amplifying the (-172) to (+45) region of the *toxT* promoter from the pTLI2 plasmid (8) with primers RAP109 (5'-GTATAGCAAAGCATATTCAG-3') and RAP 110 (5'-AAATAAACGCAGAGAGC-3'). The PCR product was purified with a MinElute kit and 15 pmol of product was end-labeled with T4 polynucleotide kinase (NEB, Ipswich, MA). The radiolabelled probe was purified on an illustra ProbeQuant G-50 column (GE Healthcare, Amersham, UK).

Twenty microliter binding reactions were prepared in binding buffer consisting of 10 mM Tris pH 7.4, 1 mM EDTA, 50 µg/ml BSA, 5 mM NaCl, 50 mM KCl, 5% glycerol, with 100 µg/ml salmon sperm DNA, 3 µl of radiolabeled *toxT* probe (at 3,000 cpm/µl), and varying amounts of membrane preps. Compound was added at either 10 or 50 µM, and DMSO was added at an equivalent volume. Binding reactions were incubated at 30°C for 30 minutes before being run on a 6% polyacrylamide gel, which had been pre-run for 20 minutes with 5% thioglycolate. The gel was run for 720 Vhrs, dried on a vacuum dryer, and exposed to a piece of film or to a phosphor screen.

<u>Comparative proteomics</u>

Cells were grown overnight under toxin-inducing conditions (LB pH 6.5 at 30°C) in the presence or absence of 10 µM toxtazin A. Cells were pelleted, washed twice in PBS, and proteins were separated by SDS-PAGE. Proteins were in-gel digested then identified by liquid chromatography and tandem mass spectrometry (gel LC-MS/MS) at MS Bioworks in Ann Arbor, MI.

Detection of cholera toxin by ELISA

Cultures of *V. cholerae* were grown under toxin-inducing conditions for 16-18 hours with 10 µM compound or DMSO. GM1 ganglioside enzyme-linked immunosorbent CT assays were performed as previously described (34) on equal volumes of the resulting supernatants. CT expression values were normalized to OD600 and are the average of samples grown in triplicate.

Transposon mutagenesis

Overnight cultures of the parent strain O395 + p*toxT*-GFP (RA2) and the donor strain SM10λpir + pFD1 (RA150b) were grown in 100 µg/ml streptomycin and 100 µg/ml ampicillin, and 100 µg/ml ampicillin and 50 µg/ml kanamycin, respectively. Seven-hundred and fifty milliliters of each culture was spun down for five minutes at 12,000 rpm to pellet the cells, the cells were washed once in PBS, and both strains were combined in 50 µl LB. This mixture was spotted onto a thick LB agar plate and allowed to grow at 37°C for three hours, after which time they were resuspended in 10 ml LB + 1 mM IPTG and grown in a 50 ml flask at 37°C. After three hours of growth, the culture was plated on agar plates containing ampicillin, kanamycin, and streptomycin to select for *V. cholerae* with the transposon. The resulting colonies were pooled to generate the transposon library pool used in the FACS sorting.

FACS sorting

Mutants pools generated by transposon insertion of by continual growth for 30 days (labeled sp for spontaneous) were grown for 16 hours in LB pH 6.5 at 30°C with either 50 µM toxtazin A (note: This batch of toxtazin A was not toxic to cells at 50 µM), 10 µM toxtazin B, or an equivalent volume of DMSO. These cultures were then diluted 1:16 to be amenable to sorting, and run through the flow cytometer. The cells within the 2-5% highest GFP signal were sorted and plated on LB agar plate supplemented with streptomycin, ampicillin, and kanamycin. Colonies were transferred to a single well of a 96-well plate containing LB + 20% glycerol and frozen as stocks.

References

1. **Schenone M, Dancik V, Wagner BK, Clemons PA.** 2013. Target identification and mechanism of action in chemical biology and drug discovery. Nature chemical biology **9:**232-240.
2. **Sarker M, Talcott C, Galande AK.** 2013. In silico systems biology approaches for the identification of antimicrobial targets. Methods in molecular biology **993:**13-30.
3. **Ziegler S, Pries V, Hedberg C, Waldmann H.** 2013. Target identification for small bioactive molecules: finding the needle in the haystack. Angewandte Chemie **52:**2744-2792.
4. **Ayre J.** 1849. Cholera: its nature, symptoms, and treatment. Stringer & Townsend, New York,.
5. **DiRita VJ, Parsot C, Jander G, Mekalanos JJ.** 1991. Regulatory cascade controls virulence in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **88:**5403-5407.
6. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.
7. **DiRita VJ.** 1992. Co-ordinate expression of virulence genes by ToxR in Vibrio cholerae. Molecular microbiology **6:**451-458.
8. **Higgins DE, DiRita VJ.** 1994. Transcriptional control of toxT, a regulatory gene in the ToxR regulon of Vibrio cholerae. Molecular microbiology **14:**17-29.
9. **Hase CC, Mekalanos JJ.** 1998. TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **95:**730-734.
10. **Krukonis ES, Yu RR, Dirita VJ.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Molecular microbiology **38:**67-84.
11. **Anthouard R, DiRita VJ.** 2013. Small-molecule inhibitors of toxT expression in Vibrio cholerae. mBio **4**.
12. **Gophna U, Ron EZ.** 2003. Virulence and the heat shock response. International journal of medical microbiology : IJMM **292:**453-461.
13. **Raivio TL.** 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. Molecular microbiology **56:**1119-1128.
14. **Kovacikova G, Skorupski K.** 2002. The alternative sigma factor sigma(E) plays an important role in intestinal survival and virulence in Vibrio cholerae. Infection and immunity **70:**5355-5362.
15. **Richardson AR, Dunman PM, Fang FC.** 2006. The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. Molecular microbiology **61:**927-939.
16. **Saleh M, Bartual SG, Abdullah MR, Jensch I, Asmat TM, Petruschka L, Pribyl T, Gellert M, Lillig CH, Antelmann H, Hermoso JA, Hammerschmidt S.** 2013. Molecular architecture of Streptococcus pneumoniae surface thioredoxin-fold lipoproteins crucial for extracellular oxidative stress resistance and maintenance of virulence. EMBO molecular medicine.

17. **Huang CJ, Wang ZC, Huang HY, Huang HD, Peng HL.** 2013. YjcC, a c-di-GMP phosphodiesterase protein, regulates the oxidative stress response and virulence of Klebsiella pneumoniae CG43. PloS one **8:**e66740.
18. **Dons LE, Mosa A, Rottenberg ME, Rosenkrantz JT, Kristensson K, Olsen JE.** 2013. Role of the Listeria monocytogenes 2-Cys peroxiredoxin homologue in protection against oxidative and nitrosative stress and in virulence. Pathogens and disease.
19. **Pal RR, Das B, Dasgupta S, Bhadra RK.** 2011. Genetic components of stringent response in Vibrio cholerae. Indian J Med Res **133:**212-217.
20. **Potrykus K, Cashel M.** 2008. (p)ppGpp: still magical? Annual review of microbiology **62:**35-51.
21. **Kovacikova G, Lin W, Skorupski K.** 2005. Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in Vibrio cholerae. Molecular microbiology **57:**420-433.
22. **Kovacikova G, Lin W, Skorupski K.** 2010. The LysR-type virulence activator AphB regulates the expression of genes in Vibrio cholerae in response to low pH and anaerobiosis. Journal of bacteriology **192:**4181-4191.
23. **Stern AM, Hay AJ, Liu Z, Desland FA, Zhang J, Zhong Z, Zhu J.** 2012. The NorR regulon is critical for Vibrio cholerae resistance to nitric oxide and sustained colonization of the intestines. mBio **3:**e00013-00012.
24. **Davies BW, Bogard RW, Dupes NM, Gerstenfeld TA, Simmons LA, Mekalanos JJ.** 2011. DNA damage and reactive nitrogen species are barriers to Vibrio cholerae colonization of the infant mouse intestine. PLoS pathogens **7:**e1001295.
25. **Pomposiello PJ, Demple B.** 2001. Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends in biotechnology **19:**109-114.
26. **Cameron DE, Urbach JM, Mekalanos JJ.** 2008. A defined transposon mutant library and its use in identifying motility genes in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **105:**8736-8741.
27. **Schell MA.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annual review of microbiology **47:**597-626.
28. **Brun S, Berges T, Poupard P, Vauzelle-Moreau C, Renier G, Chabasse D, Bouchara JP.** 2004. Mechanisms of azole resistance in petite mutants of Candida glabrata. Antimicrobial agents and chemotherapy **48:**1788-1796.
29. **Laubacher ME, Melquist AL, Chandramohan L, Young KD.** 2013. Cell sorting enriches Escherichia coli mutants that rely on peptidoglycan endopeptidases to suppress highly aberrant morphologies. Journal of bacteriology **195:**855-866.
30. **Balbas MD, Evans MJ, Hosfield DJ, Wongvipat J, Arora VK, Watson PA, Chen Y, Greene GL, Shen Y, Sawyers CL.** 2013. Overcoming mutation-based resistance to antiandrogens with rational drug design. eLife **2:**e00499.
31. **Zhai D, Godoi P, Sergienko E, Dahl R, Chan X, Brown B, Rascon J, Hurder A, Su Y, Chung TD, Jin C, Diaz P, Reed JC.** 2012. High-throughput fluorescence polarization assay for chemical library screening against anti-apoptotic Bcl-2 family member Bfl-1. Journal of biomolecular screening **17:**350-360.
32. **Iwanaga M, Yamamoto K.** 1985. New medium for the production of cholera toxin by Vibrio cholerae O1 biotype El Tor. Journal of clinical microbiology **22:**405-408.

33. **Tischler AD, Camilli A.** 2005. Cyclic diguanylate regulates Vibrio cholerae virulence gene expression. Infection and immunity **73:**5873-5882.
34. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.

Chapter IV

Mechanism of Action of Toxtazin B

Summary

Similarly to toxtazin A, both a loss-of-target approach and two unbiased screening approaches were employed to determine the mechanism of action by which toxtazin B inhibits pathogenesis in *Vibrio cholerae*. Preliminary evidence suggests that toxtazin B may exert its effects on virulence gene regulation through AphB. While the exact mechanism of action has not yet been identified, many potential mechanisms have been ruled out. The results of this research are presented here, and implicate proteins involved in regulating the *tcpP* promoter.

Introduction

Vibrio cholerae produces and secretes cholera toxin (CT) into the lumen of its host, resulting in cramping and diarrhea (1). Production of CT is tightly regulated by the virulence cascade, which has multiple layers of regulation. Transcription of the *ctxAB* operon, which encodes CT, is activated by ToxT (2), which is activated by ToxRS and TcpPH, two inner membrane transcriptional activators and their respective effector proteins (3-7). Previously, two small molecule inhibitors of *toxT* transcription, toxtazin A

and toxtazin B, were identified, and toxtazin B was found to inhibit *tcpP* transcription but not AphA or AphB protein levels (8).

TcpP expression is activated by the transcriptional activator AphA (9), which is involved in quorum sensing (10), and AphB (11), which increases *tcpP* transcription in response to low pH (12) and to anaerobiosis growth conditions via a thiol switch at residue C227 (13).

The *tcpP* promoter assimilates multiple additional signals including the cAMP-CRP complex (14) and phosphate levels (15). In addition, in the absence of TcpH or upon sensing non-inducing conditions, pre-existing TcpP in the cell is removed by sequential degradation by the Tsp (Teoh, W.P. and DiRita, V.J., unpublished) and YaeL proteases (16). This complex system ensures TcpP is present in the cell only under the right conditions.

Through careful investigation of the multiple layers of TcpP regulation, we present evidence that toxtazin B does not promote Tsp/YaeL-mediated degradation of TcpP, nor does it prevent AphA or AphB from binding the *tcpP* promoter *in vitro*. Toxtazin B may inhibit *tcpP* transcription by mimicking an anaerobic state, by potentiating cAMP-CRP binding to the *tcpP* promoter, or potentiating PhoB inhibition at the *tcpP* promoter, and we are currently testing these hypotheses.

Results

Toxtazin B reduces Vibrio cholerae colonization of infant mice in a ToxT-dependent manner

We demonstrated that toxtazin B reduces colonization loads of *V. cholerae* in an infant mouse model in Chapter 2 (8). To rule out the possibility that toxtazin B acts as an antibiotic and kills *V. cholerae*, *V. cholerae*, strain S533 was tested. This strain colonizes the infant mouse through a mechanism that does not require *toxT*, *tcp*, or *ctxAB*, none of which is encoded in its genome, (17). The S533 strain was tested in infant mice following the same protocol used for the O395 strain in Chapter 2. Either 100 µg toxtazin B or an equal volume of DMSO were gavaged into mice along with 10^6 C.F.U. of S533, followed by a boost of either 100 µg toxtazin B or an equal volume of DMSO three hours later. After the boost, the infection was allowed to proceed for 16 hours, after which time mice were euthanized and the intestines removed. The number of S533 bacteria present in the intestine was enumerated by plating 10-fold dilutions on selective media. Unlike colonization by the classical strain *V. cholerae* O395 (Chapter 2), there was no significant difference in the colonization loads of strain S533 in mice treated or un-treated with toxtazin B (Figure 4.1). From this we conclude that toxtazin B targets the ToxR/TcpP/ToxT regulatory system both *in vivo* and *in vitro.*

Toxtazin B does not stimulate TcpP degradation

Toxtazin B has been shown to reduce *tcpP* transcript as well as TcpP levels (Chapter 2; (8)). To investigate the kinetics of TcpP inhibition, cultures were grown in

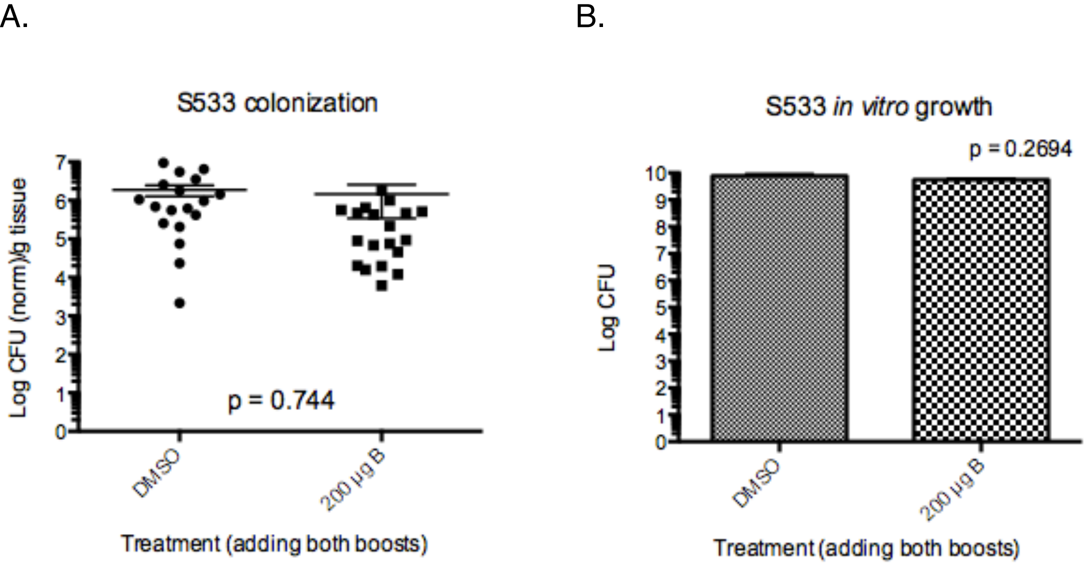


Figure 4.1 Colonization of S533 is not affected by toxtazin B.

A. Number of *V. cholerae* recovered from mice orogastrically inoculated with 10^6 of the S533 strain of *V. cholerae* treated with either DMSO or 200 µg toxtazin B. Number of S533 *V. cholerae* recovered from three mL cultures grown overnight with the same inocula and boosts as the mice in 4.1A. p-values were determined by a non-paired 2-tailed t-test.

the presence or absence of toxtazin B under toxin-inducing conditions (LB pH 6.5 at 30°C), and TcpP levels were measured every hour by running lysates on a 12.5% SDS-PAGE gel. Supernatants from each time point were analyzed for CT by ELISA. Where TcpP levels in DMSO-treated cultures stayed relatively steady during the 24-hour growth period, TcpP levels in toxtazin B-treated cells decreased over time (Figure 4.2A), consistent with our results in Chapter 2.

One curious result from this experiment is that, despite the fact that TcpP was present in cultures at early time points, CT production was not observed. We hypothesize a post-translational level of TcpP regulation in the virulence cascade that turns TcpP from an OFF to an ON state; however more work needs to be done to confirm this and uncover the nature of this regulation.

Previous work demonstrated that toxtazin B reduces *tcpP* transcript levels by 50% in cells (Chapter 2; (8)); is reasonable to hypothesize that TcpP levels decrease because of a reduction in *tcpP* transcription. However, it is also known that under conditions unfavorable for toxin expression, TcpP is degraded in a two-step proteolytic pathway, with Tsp acting first, and YaeL acting on the Tsp-cleaved TcpP* product (unpublished data and (16)). Therefore, toxtazin B may decrease TcpP steady-state levels by both inhibiting *tcpP* transcription and stimulating TcpP proteolysis. It is also possible that TcpP proteolysis could result in *tcpP* transcription inhibition by a feedback mechanism.

To rule in or out the possibility that toxtazin B may stimulate TcpP degradation in addition to inhibiting *tcpP* transcription, we used two different approaches. First, we

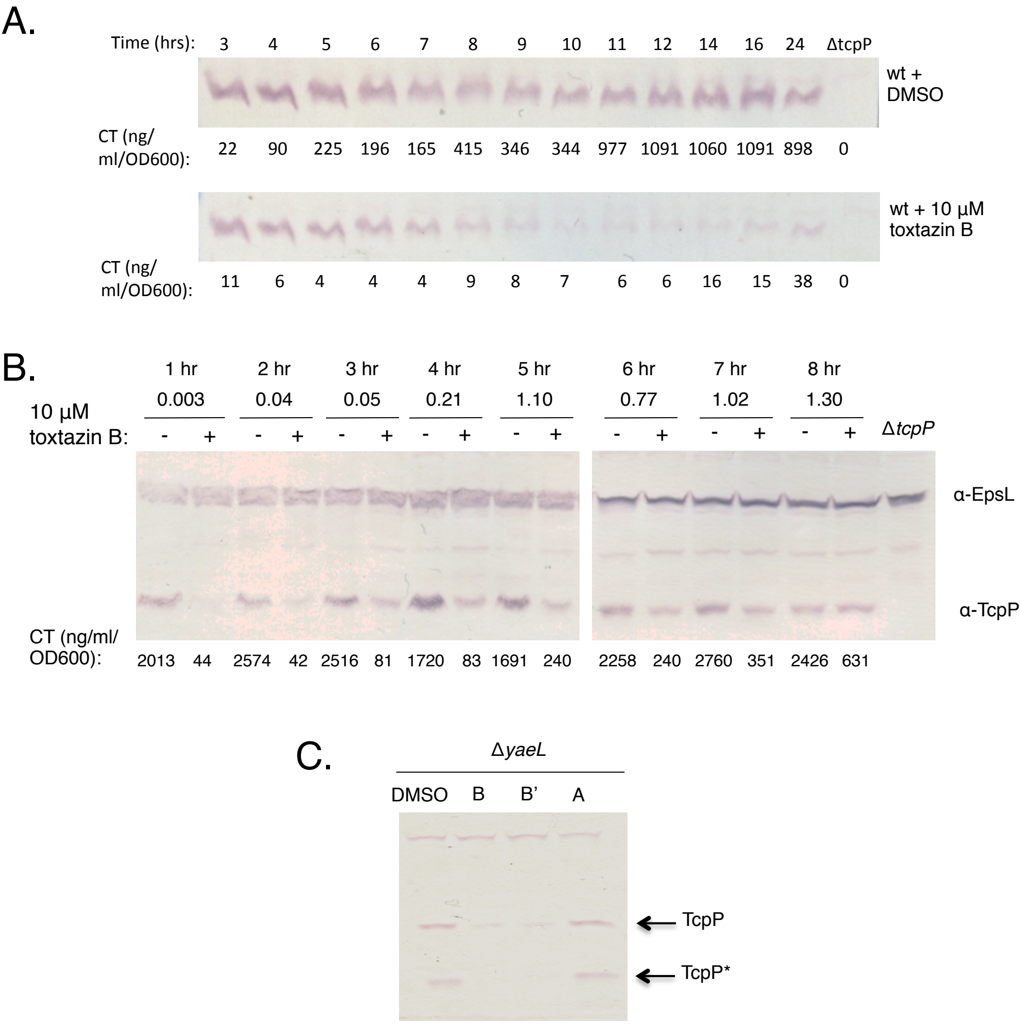


Figure 4.2 Toxtazin B does not stimulate TcpP degradation.

A. TcpP levels over time in cultures treated with DMSO or 10 µM toxtazin B. CT levels, determined by CT ELISA, are shown below each sample. B. TcpP steady-state levels in cultures grown overnight under toxin-inducing conditions with DMSO or 10 µM toxtazin B added at various times. EpsL was used as a loading control. CT levels, determined by ELISA, are indicated below each sample. C. TcpP Western blot of a *yaeL* mutant grown for 16 hours in the presence or absence of 10 µM toxtazins A, B, or B'.

added toxtazin B to cultures at varying stages of growth and monitored TcpP levels after 16 hours of exposure to toxtazin B. We reasoned that if toxtazin B induces TcpP degradation, TcpP levels that accumulate prior to addition of toxtazin B will decrease after its addition. Parallel cultures of wild-type cells were grown overnight in LB and diluted 1:100 in LB pH 6.5, followed by growth at 30°C to induce toxin expression. DMSO or toxtazin B was added to two tubes every hour, and the OD_{600} was noted. Cultures were then grown for 16 hours after toxtazin B addition such that all cells were exposed to toxtazin B for the same amount of time. After 16 hours of growth, cell pellets were analyzed for TcpP levels and supernatants were analyzed for CT expression (Figure 4.2B). Western blot analysis for TcpP shows that toxtazin B has a stronger effect on TcpP levels the earlier it is added to the culture. When toxtazin B was added to cultures after one hour of growth, TcpP levels are severely decreased after 16 hours. However, if the culture was allowed to grow for eight hours before toxtazin B was added, TcpP levels did not decrease, despite having been exposed to toxtazin B for the same amount of time. These results lead to the conclusion that toxtazin B affects the steady-state levels of TcpP protein in the cell by reducing the input of TcpP, not by increasing its turn-over.

Secondly, we tested whether toxtazin B induces Tsp-dependent cleavage of TcpP under toxin-inducing conditions. In a strain lacking YaeL grown under non-inducing conditions, TcpP is degraded by Tsp to yield a TcpP* fragment, but because YaeL is not present to degrade it further, the TcpP* fragment accumulates and can be detected by Western blot (16). We reasoned that if toxtazin B works by doubly inhibiting

tcpP transcription and by stimulating TcpP degradation, the TcpP present early in the culture (see Figure 4.2A) would be cleaved by Tsp, resulting in the accumulation of TcpP*. After 16 hours of growth under toxin-inducing conditions, TcpP is partially degraded by Tsp, as seen by the TcpP* band in DMSO-treated cells as well as in toxtazin A-treated cells (Figure 4.2C). In contrast, no TcpP or TcpP* is seen in cells treated with toxtazins B or B'. The lack of TcpP* accumulation suggests that TcpP is not degraded by the Tsp/YaeL pathway in the presence of toxtazin B. Taken together, our results indicate that toxtazin B inhibits *tcpP* transcription but not TcpP proteolysis by Tsp/YaeL.

<u>Toxtazin B does not inhibit AphA or AphB from binding the *tcpP* promoter</u>

Having eliminated the possibility that toxtazin B may promote the degradation of TcpP, we focused on the mechanism by which toxtazin B inhibits *tcpP* transcription (chapter 2, Figure 4). One possible mechanism is that toxtazin B may inhibit AphA or AphB from binding the *tcpP* promoter. To test this, AphA and AphB were purified and electrophoretic mobility shift experiments were performed with a radiolabeled *tcpP* probe as described previously (14). Either 10 µM or 50 µM toxtazin B or an equivalent volume of DMSO was added to the binding reactions, incubated for 30 minutes at 30°C, and the reactions were subjected to electrophoresis on a 6% polyacrylamide gel. Toxtazin B did not affect the ability of either AphA or AphB to bind the *tcpP* promoter, even at the higher concentration (50 µM) than typically used with this compound.

Cold competitor: + + +

Treatment: DMSO | 10 µM toxtazin B | 50 µM toxtazin B

[AphA] (ng): 0 5 10 20 40 40 | 0 5 10 20 40 40 | 0 5 10 20 40 40

AphA-bound *tcpP* probe

unbound *tcpP* probe

B.

Cold competitor: + + +

Treatment: DMSO | 10 µM toxtazin B | 50 µM toxtazin B

[AphB] (ng): 0 25 50 75 100 100 | 0 25 50 75 100 100 | 0 25 50 75 100 100

AphB-bound *tcpP* probe

unbound *tcpP* probe

Figure 4.3 Toxtazin B does not inhibit AphA or AphB binding to the *tcpP* promoter

AphA (panel A) and AphB (panel B) were purified and incubated with radiolabeled *tcpP* probe in the presence or absence of 10 µM or 50 µM toxtazin B, then run on a 6% polyacrylamide gel. An excess of cold *tcpP* probe was added in the last lane of each group.

The AphB thiol switch mutant C227S may be resistant to toxtazin B

Published evidence suggests that AphB induces *tcpP* transcription upon sensing anaerobic conditions via a thiol switch at residue C227, and that a C227S mutant is constitutively active (13). We hypothesized that toxtazin B may interfere with the thiol switch in AphB.

When Δ*aphB* cells complemented with an arabinose-inducible plasmid containing a wild-type AphB allele were grown overnight under toxin-inducing conditions (LB pH 6.5 at 30°C), CT expression was induced in an arabinose-dependent manner (Figure 4.4). Upon treating these cells with 10 µM toxtazin B, CT levels were reduced, as expected. However, if the experiment was repeated with Δ*aphB* cells complemented by an arabinose-inducible plasmid containing the C227S allele of *aphB*, CT production was resistant to toxtazin B treatment (Figure 4.4). While these results are preliminary, they suggest that the AphB – more specifically the putative thiol switch within AphB – may be a toxtazin B target. This experiment requires reconstitution of a chromosomally-encoded *aphBC227S* allele to determine whether this thiol switch is important for toxtazin B activity, to rule out a more trivial effect of protein expression levels.

A transposon mutagenesis screen for toxtazin B-resistant mutants

Just as for toxtazin A, attempting to identify a transposon or spontaneous mutant resistant to toxtazin B by FACS sorting was unsuccessful. See chapter 3 for a full description of this experiment and the results.

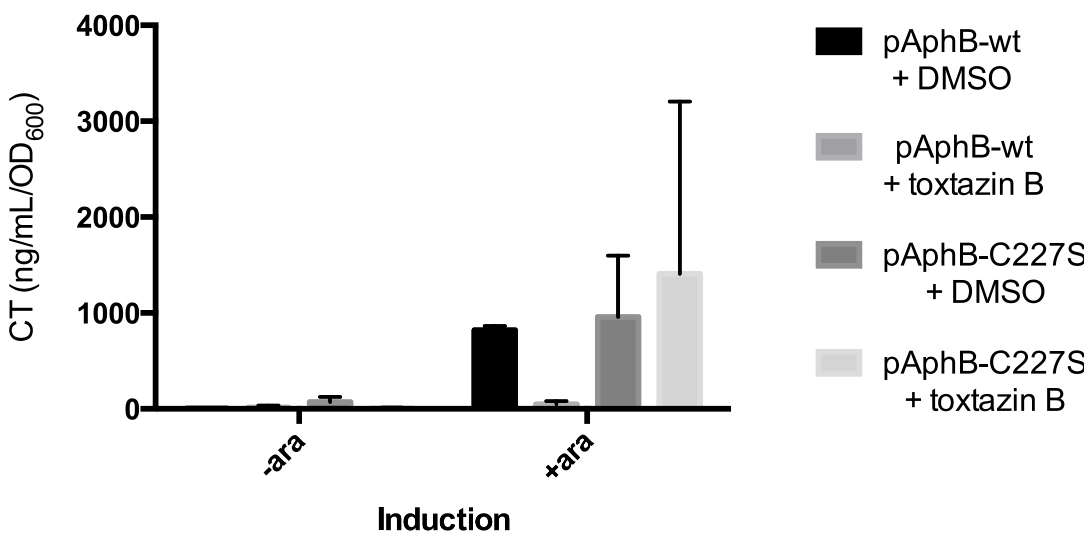


Figure 4.4. CT expression of cells expressing the wild-type or C227S allele of AphB

Cultures of Δ*aphB* cells complemented with arabinose-inducible wild-type or C227S alleles of AphB were grown overnight under toxin-inducing conditions in the presence or absence of arabinose and in the presence or absence of 10 µM toxtazin B. The resulting supernatants were analyzed to determine the amount of CT secreted. Error bars represent the standard deviation from three separate experiments.

Discussion

While the precise mechanism of action of toxtazin B is still unclear, here we tested several hypotheses and ruled some of them out, while generating new ones based on these findings. One specific point that was clarified in this work is that mouse colonization loads of *V. cholerae* in mice treated with toxtazin B are reduced likely as a result of the effect of toxtazin B on *toxT* expression, as opposed to a general anti-bacterial effect *in vivo*. We conclude this based on our observation that the S533 strain of *V. cholerae*, which colonizes through a mechanism that does not depend on ToxT, was unaffected for colonization when mice were treated with toxtazin B. This is in contrast to the effect of toxtazin B on colonization of the classic *V. cholerae* strain 0395 (Chapter 2).

Our original observation regarding toxtazin B was that cells exposed to this compound have reduced expression of *toxT* and that was associated with a similar decrease in TcpP protein and transcript levels in overnight cultures (Chapter 2; (8)). We speculated that, in addition to reducing *tcpP* gene expression, toxtazin B might also cause degradation of TcpP protein, which is understood to occur under particular growth conditions (16, 18). Here we explored this possibility further. Cells grown under toxin-inducing conditions without toxtazin B in the medium accumulated TcpP that remained stable after addition of the compound. However if toxtazin B were present at the start of the culture, TcpP did not accumulate, suggesting that it is not expressed in the presence of the compound. AphA and AphB, the transcriptional activators of the *tcpP* promoter, were still capable of binding the *tcpP* promoter at least *in vitro*, ruling out direct alteration

of their function by the compound. It remains possible that toxtazin B could disrupt the ability to bind DNA inside the cell, which could be examined further with an *in vivo* DNA binding assay such as chromatin immunoprecipitation.

A post-translational level of regulation may exist for either AphA or AphB, as has been suggested by another group studying AphB (13). In this model, AphB activity is regulated by the redox status of the cell, sensed by the reduction or oxidation of a key cysteine residue, C227. This redox sensor may be important in toxtazin B activity, as toxtazin B does not reduce CT expression in cells expressing AphB-C227S, though further analysis needs to be done to confirm this. Discovering the mechanism of action of toxtazin B is important, as it will likely provide new insight into the regulation of the *tcpP* promoter, an important step in virulence gene regulation in *V. cholerae*.

Materials and Methods

Infant mouse colonization assays

Four to six day-old CD1 mice (Charles River, Wilmington, MA) were orogastrically inoculated with a 30 µl bolus containing 10^6 CFU of *V. cholerae* S533, Cremaphor EL (20% final volume to solubilize compounds), and either DMSO or 200 µg toxtazin B. An additional 30 µl bolus lacking bacteria was delivered to each mouse three hours post-inoculation, and the mice were incubated at 30°C. Mice were euthanized 18-24 hours after inoculation, the intestines were isolated, weighed, and homogenized in PBS. Homogenates were serially diluted and plated on LB agar + X-gal + streptomycin to determine the number of CFU recovered. CFUs were normalized to the weight of the intestines and to the exact CFU of the initial inoculum. Significance was determined using one-way ANOVA. Three milliliter of LB was inoculated and treated as the mice were, to control for toxtazin toxicity.

Western blot analysis of TcpA, ToxR, TcpP, AphB, and AphA

Cells were cultured under toxin-inducing conditions in the presence or absence of 10 µM toxtazin B, and c. ell extracts were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (GE Water and Processes Technologies, Feasterville-Trevose, USA), probed with TcpP antiserum (diluted 1:500), and visualized by alkaline phosphatase. EpsL antibodies were used as a loading control, diluted 1:10,000.

DNA gel mobility shift assays

These were performed as previously described (11). AphA and AphB were purified from BL21 cells expressing pET32-AphA-His (strain RA304) or expressing pET32-AphB-His (strain RA313). One-hundred milliliters of culture was grown for two hours at 37°C shaking, then shifted to 16°C for one hour. Expression was induced with one mM IPTG, and the culture was allowed to grow for another 12-16 hours at 16°C. The cells from these cultures were pelleted and frozen at -80°C for 20 min or longer. Cells were lysed with eight ml Native Binding Buffer (50 mM NaH_2PO_4 pH 8.0, 2.5 M NaCl), one µl Benzonase and one Complete Mini Protease inhibitor tablet were added. Eight mg lysozyme was added and allowed to lyse the cells by incubating for 30 min on ice. Cells were then sonicated on ice for a total of 60 sec. The lysates were spun for 15 min at 3,000g at 4°C to pellet unlysed cells and debris. The clear lysate was applied to a 10 ml nickel column with 1.5 ml of resin, which had been pre-washed with ddH_2O and Native Binding Buffer. The His-tagged proteins were allowed to bind the nickel resin by rocking in the column for one hour at 4°C, and the resin was allowed to settle by gravity before letting the lysate flow through. Native Wash Buffer (50 mM NaH_2PO_4 pH 8.0, 2.5 M NaCl, 20 mM imidazole) was used to wash the column 3 times before eluting the purified His-tagged protein from the column with Native Elution Buffer (50 mM NaH_2PO_4 pH 8.0, 2.5 M NaCl, 250 mM imidazole). The resulting fractions 2 and 3 were dialyzed into 1X Native Purification Buffer overnight, then diluted 1:1 in glycerol and stored at -80°C until needed.

Next, radiolabeled *tcpP* probe was prepared by end-labeling 40 ng of the forward primer RAP222 (5'-GATCCGAATTCCTGTAACGAATATTGCTTCCG-3') with 4 µl of γ-^{32}P (10 mCi/ml) using T4 polynucleotide kinase (NEB, Ipswich, MA). This primer was ethanol precipitated and used to PCR amplify the *tcpP* promoter from a plasmid-derived sequence (in strain RA330) with the reverse primer RAP223 (5'-GATCGGGATCCTTTCTTAATCATAACGACCC-3'). The PCR product was ethanol precipitated and resuspended in 25 µl ddH_2O.

Thirty microliter binding reactions were prepared in binding buffer consisting of 10 mM Tris*OAc pH 7.4, 1 mM K*EDTA pH 7.0, 100 mM KCl, 1 mM DTT, 10% glycerol, 0.3 mg/ml BSA, and 100 µg/ml salmon sperm DNA mixed with 3 µl radio-labeled *tcpP* promoter probe, 10 µM or 50 µM toxtazin B, and varying concentrations of purified AphA or AphB. Binding reactions were incubated for 30 min at 30°C, then run on a 6% polyacrylamide gel for 600 Vhrs. The gels were dried on a vacuum dryer and exposed to film. Electromobility shift assays were repeated on three separate days.

<u>Detection of cholera toxin by ELISA</u>

Cultures of *V. cholerae* O395Δ*aphB* + pBAD-*aphB*^{wt} or O395Δ*aphB* + pBAD-*aphB*^{C227S} (kindly provided by Dr. Jun Zhu) were grown under toxin-inducing conditions for 16-18 hours with 10 µM compound or DMSO. GM1 ganglioside enzyme-linked immunosorbent CT assays were performed as previously described (19) on equal volumes of the resulting supernatants. CT expression values were normalized to OD600 and are the average of samples grown in triplicate.

References

1. **Ayre J.** 1849. Cholera: its nature, symptoms, and treatment. Stringer & Townsend, New York,.
2. **DiRita VJ, Parsot C, Jander G, Mekalanos JJ.** 1991. Regulatory cascade controls virulence in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **88:**5403-5407.
3. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.
4. **DiRita VJ.** 1992. Co-ordinate expression of virulence genes by ToxR in Vibrio cholerae. Molecular microbiology **6:**451-458.
5. **Higgins DE, DiRita VJ.** 1994. Transcriptional control of toxT, a regulatory gene in the ToxR regulon of Vibrio cholerae. Molecular microbiology **14:**17-29.
6. **Hase CC, Mekalanos JJ.** 1998. TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **95:**730-734.
7. **Krukonis ES, Yu RR, Dirita VJ.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Molecular microbiology **38:**67-84.
8. **Anthouard R, DiRita VJ.** 2013. Small-molecule inhibitors of toxT expression in Vibrio cholerae. mBio **4**.
9. **Skorupski K, Taylor RK.** 1999. A new level in the Vibrio cholerae ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. Molecular microbiology **31:**763-771.
10. **Kovacikova G, Lin W, Skorupski K.** 2003. The virulence activator AphA links quorum sensing to pathogenesis and physiology in Vibrio cholerae by repressing the expression of a penicillin amidase gene on the small chromosome. Journal of bacteriology **185:**4825-4836.
11. **Kovacikova G, Skorupski K.** 1999. A Vibrio cholerae LysR homolog, AphB, cooperates with AphA at the tcpPH promoter to activate expression of the ToxR virulence cascade. Journal of bacteriology **181:**4250-4256.
12. **Kovacikova G, Lin W, Skorupski K.** 2010. The LysR-type virulence activator AphB regulates the expression of genes in Vibrio cholerae in response to low pH and anaerobiosis. Journal of bacteriology **192:**4181-4191.
13. **Liu Z, Yang M, Peterfreund GL, Tsou AM, Selamoglu N, Daldal F, Zhong Z, Kan B, Zhu J.** 2011. Vibrio cholerae anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. Proceedings of the National Academy of Sciences of the United States of America **108:**810-815.
14. **Kovacikova G, Skorupski K.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the Vibrio cholerae tcpPH promoter. Molecular microbiology **41:**393-407.

15. **Pratt JT, Ismail AM, Camilli A.** 2010. PhoB regulates both environmental and virulence gene expression in Vibrio cholerae. Molecular microbiology **77:**1595-1605.
16. **Matson JS, DiRita VJ.** 2005. Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **102:**16403-16408.
17. **Hung DT, Shakhnovich EA, Pierson E, Mekalanos JJ.** 2005. Small-molecule inhibitor of Vibrio cholerae virulence and intestinal colonization. Science **310:**670-674.
18. **Beck NA, Krukonis ES, DiRita VJ.** 2004. TcpH influences virulence gene expression in Vibrio cholerae by inhibiting degradation of the transcription activator TcpP. Journal of bacteriology **186:**8309-8316.
19. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.

Chapter V

Discussion

This dissertation describes the identification of small molecules that inhibit the virulence cascade in *Vibrio cholerae*. We detail the *in vitro* and *in vivo* activities of two identified compounds, toxtazin A and toxtazin B, and present evidence that toxtazin A targets *toxT* transcription while toxtazin B targets *tcpP* transcription. Here, we summarize our findings, discuss possible mechanisms of action for toxtazins A and B, and consider the potential of each compound as molecular probes for scientific discovery and as therapeutic leads for the treatment of cholera. We conclude with a discussion on the impact of this work in the greater context of bacterial pathogenesis and drug discovery.

Toxtazins A and B inhibit virulence gene expression in *Vibrio cholerae*

Using a *toxT*-GFP reporter strain of *V. cholerae*, 63,000 small molecules were screened for those that reduce *toxT* transcription in live cells, leading to the discovery of toxtazins A and B. The reduction in GFP expression was due to a decrease in *toxT* transcription and not an effect on the GFP reporter because cholera toxin (CT) levels in cells were also reduced in cell treated with either toxtazin A or B. Additionally, toxtazin

A- or B-treated cells express less TcpA, the toxin co-regulated pilus, which is transcriptionally activated by ToxT.

In addition to their activity *in vitro*, these compounds were tested in an infant mouse model of colonization to assess their *in vivo* activity. While toxtazin A was toxic to bacteria at just 60 µg/ml and therefore not testable in this model, toxtazin B was not toxic to bacteria even at 200 µg/ml. Treating mice with 200 µg of toxtazin B significantly reduced colonization of the classical O395 strain of *V. cholerae*. However, the S533 strain of *V. cholerae*, which colonizes mice through a ToxT-independent mechanism, was not affected by the same dose of toxtazin B, indicating that toxtazin B reduces colonization in a ToxT-dependent manner.

Toxtazin A targets *toxT* transcription while toxtazin B targets *tcpP* transcription

Toxtazin A does not affect ToxR protein levels, ToxR activity at the *ompU* or *ompT* promoters, ToxR localization to the membrane, or the ability of ToxR to bind the *toxT* promoter. Furthermore, toxtazin A does not affect TcpP protein levels or its localization to the membrane. TcpP binding to the *toxT* promoter in the presence of toxtazin A is currently being evaluated. Taken together, our results indicate that toxtazin A targets *toxT* transcription.

Toxtazin B also does not affect ToxR protein levels, its activity at the *ompU* or *ompT* promoters, or it localization to the membrane. However, it reduces TcpP protein levels as well as *tcpP* transcript levels. Toxtazin B does not induce Tsp/YaeL-mediated proteolysis of TcpP, ruling out the possibility that toxtazin B causes the cells to respond

as though they are growing under non-inducing conditions. AphA and AphB, the transcriptional activators of *tcpPH*, are not inhibited by toxtazin B. Both are expressed at normal levels and both are able to bind the *tcpPH* promoter, suggesting that toxtazin B inhibits *tcpP* transcription independently of AphA or AphB.

Toxtazin A affects the envelope, nutrient, and redox stress responses

Toxtazin A-treated cells are unable to respond to envelope or nutrient stress, and seem to be responding to redox stress. *V. cholerae* responds to envelope stress, induced 3% ethanol, by activating an alternative sigma factor, σ^E, encoded by *rpoE* (1). In the presence of toxtazin A, cells cannot grow in 3% ethanol, suggesting they are unable to cope with envelope stress. It is possible that *rpoE* is important for virulence gene activation because an *rpoE* mutant is deficient for colonization of infant mice (1). Therefore, it is possible that toxtazin A inhibits RpoE activation, and that this could inhibit the virulence cascade. This hypothesis is currently under investigation.

Similarly, cells grown in M9 minimal media + 0.4% glycerol + NERS cannot grow in the presence of toxtazin A. Because cells treated with toxtazin B grow normally under these conditions, it is unlikely that the growth defect is due to inhibition of the virulence cascade, but is rather likely due to nutritional stress. Typically, nutritional stress in bacteria leads to changes in gene expression known as the stringent response, which is triggered in large part by the intracellular accumulation of guanosine 3'-diphosphate 5'-triphosphate and guanosine 3',5'-bis(diphosphate), collectively called (p)ppGpp (2). *V. cholerae* experiencing fatty acid or glucose starvation activate SpoT

and RelV proteins to synthesize (p)ppGpp, and cells under amino acid starvation activate RelA, which also activates and synthesizes (p)ppGpp. Together with DskA, (p)ppGpp responds to nutrient stress by activating motility, biofilm formation, and CT expression (3). A *relA* mutant has a severe growth defect in minimal media (4, 5), suggesting that a (p)ppGpp is required for virulence. Given that the stress response is important for virulence *in vivo*, it is possible that toxtazin A may inhibit the stringent response or prevent (p)ppGpp synthesis, which could in turn inhibit *toxT* transcription. This hypothesis is currently being tested.

Additionally, cells treated with toxtazin A behave as though they are responding to redox stress. Proteomic analysis revealed that cells treated with toxtazin A increase the expression of 20 proteins, seven (35%) of which are involved in redox homeostasis. The oxidative stress response in *V. cholerae* is not well characterized, but it is known to be mediated through OxyR (6) and that quorum sensing can activate production of RpoS, which responds to both oxidative stress (6, 7) and carbon starvation (8). While OxyR and RpoS are dispensable for colonization of infant mice (6, 8), this may be simply because OxyR and RpoS are not induced in this *in vivo* model. It remains possible that toxtazin A may induce an oxidative stress response, and that part of that response results in shutting off *toxT* transcription.

Toxtazin B may affect AphA or AphB activity post-translationally

We showed in chapter 2 that toxtazin B inhibits virulence by targeting *tcpP* transcription. The mechanism by which this is accomplished is still being experimentally

determined; however, a few key possibilities have been ruled out. First, the possibility that toxtazin B induces Tsp/YaeL-mediated cleavage of TcpP was ruled out. If toxtazin B promoted Tsp/YaeL-regulated proteolysis, a TcpP* band should accumulate in a Δ*yaeL* mutant treated with toxtazin B, but this was not observed. In addition, toxtazin B does not decrease TcpP expression when added to cells already expressing TcpP.

The possibility that AphA or AphB cannot bind the *tcpP* promoter in the presence of toxtazin B has also been ruled out. Gel shift experiments with purified AphA or AphB showed that toxtazin B does not affect the ability of either of these proteins to bind to the *tcpP* promoter, at least *in vitro*. Toxtazin B may affect post-translational regulation of either AphA or AphB, which could affect binding to the *tcpP* promoter *in vivo*. Supporting this hypothesis is the fact that cysteine 227 of AphB is post-translationally modified, and that this modification is important for its activity at the *tcpP* promoter (9). Distinct modifications to cysteine residues in proteins have been proposed to result in various protein activation states, leading to different phenotypic outcomes depending on the type of modification (10). It is possible that toxtazin B could inhibit AphB activity by affecting the modification at the C227 residue, a hypothesis supported by the finding that cells expressing AphB^{C227S} are resistant to toxtazin B (Figure 4.4). While AphA has not been shown to undergo post-translational modifications, it does contain two cysteine residues and thus has the potential to also be modified.

Small molecule screens as hypothesis-generating research

One key advantage of small molecule screening approaches is the testable hypotheses generated by this type of research. In the case of toxtazin A, my evidence suggests that ToxR and TcpP can be present in the inner membrane and yet do not activate *toxT*. Toxtazin A treatment is not the only condition that results in ToxR and TcpP being present in the membrane without activating *toxT* transcription- it is also seen in stationary cells grown in toxin non-inducing conditions (growth in LB pH 8.5 at 37°C) or grow at 37°C in LB (unpublished results, Anthouard and DiRita). Growth on malonate also inhibits *toxT* transcription as well as *tcpA* and *ctxAB* transcription, though ToxR and TcpP are not affected (11). These results suggest that ToxR and TcpP are necessary but not sufficient for *toxT* activation, and perhaps another protein or post-translational modifications to either ToxR and/or TcpP is required for activation of *toxT*. Toxtazin A will be a useful tool in testing this hypothesis.

Follow-up studies with toxtazin A also generated the hypothesis that cellular stress could feedback to the virulence cascade and shut it off by stopping transcription of *toxT*. Many pathogenic bacteria shut off virulence gene expression in response to stress, including *Staphylococcus aureus* (12), *Listeria monocytogenes* (13, 14), *Brucella melitensis* (15), *Pseudomonas aeruginosa* (16), and *Klebsiella pneumoniae* (17). While this has not been directly tested in *V. cholerae*, many genes involved in stress response are required for colonization *in vivo*, including those that respond to nitrosative stress (18, 19), nutrient stress (4), and extracytoplasmic stress (1). In addition, a recent study found that ethanol-induced extracytoplasmic stress, signaled by σ^E, resulted in the

transcriptional down-regulation of *toxT* and *ctxAB* but did not affect *tcpP* or *toxR* transcription (20). These findings suggest that stress responses may indeed regulate the virulence cascade in *V. cholerae*, but further work is needed to determine if this is the mechanism by which toxtazin A decreases *toxT* transcription.

Toxtazin B as a potential therapeutic lead

Small molecule screens often result in the identification of compounds that could theoretically have medicinal use in treating disease. For example, we have shown that toxtazin B reduces colonization of infant mice by 2 logs, indicating that it functions *in vivo* and thus could have potential therapeutic use. While the pharmacological characterization and drug development of toxtazin B still remains to be done, this molecule is attractive as a therapeutic lead because it affects toxin-expressing *V. cholerae* only. A strain of *V. cholerae* lacking the virulence genes *toxT*, *tcpA*, and *ctxAB* colonized mice equally whether the mice received toxtaxin B or DMSO carrier. This kind of specificity *in vivo* suggests that the compound would do minimal damage to other bacteria, theoretically leaving the microbiota unaffected by toxtazin B treatment.

Future Perspectives

While the mechanism of action for toxtazins A and B are still being worked out, new knowledge about the virulence cascade has already been generated from studying these molecules. For example, toxtazin A will serve as a tool to study the putative post-translational modifications to either ToxRS and/or TcpPH that are required for *toxT*

activation, mechanisms that have been previously proposed by others (21-23). Additionally, work with toxtazin A has led to the hypothesis that stress responses in *V. cholerae* may inhibit expression of *toxT.* Following up on these hypotheses in the future will further our understanding of how the virulence cascade in *V. cholerae* is regulated. It would also prove potentially insightful to perform an RNAseq experiment on cells treated with or without toxtazin A, to gain an understanding of what role(s) various stress signals play in regulating the virulence cascade.

In the case of toxtazin B, which targets transcriptional activation of the *tcpP* promoter, we have learned from our *in vivo* experiments that targeting *tcpP* transcription is a valid approach to designing new therapeutics for treating *V. cholerae* colonization. Performing some SAR, pharmacokinetic and pharmacodynamics studies would be the next step to developing this compound for potential clinical use. Additionally, follow-up work on the MOA of toxtazin B would also provide a deeper understand of how the virulence cascade in *V. cholerae* is regulated. Experiments looking at AphA and AphB post-translational modifications, in particular their disulfide-bonded state, are already underway and may corroborate with early findings about redox sensing by these proteins (9).

The work presented here serves as an example of the power of chemical genetics in both uncovering new biologically relevant information and in uncovering the “druggable” aspects of a pathogen’s virulence machinery for the development of therapeutic leads.

References

1. **Kovacikova G, Skorupski K.** 2002. The alternative sigma factor sigma(E) plays an important role in intestinal survival and virulence in Vibrio cholerae. Infection and immunity **70:**5355-5362.
2. **Potrykus K, Cashel M.** 2008. (p)ppGpp: still magical? Annual review of microbiology **62:**35-51.
3. **Pal RR, Bag S, Dasgupta S, Das B, Bhadra RK.** 2012. Functional characterization of the stringent response regulatory gene dksA of Vibrio cholerae and its role in modulation of virulence phenotypes. Journal of bacteriology **194:**5638-5648.
4. **Haralalka S, Nandi S, Bhadra RK.** 2003. Mutation in the relA gene of Vibrio cholerae affects in vitro and in vivo expression of virulence factors. Journal of bacteriology **185:**4672-4682.
5. **Silva AJ, Benitez JA.** 2006. A Vibrio cholerae relaxed (relA) mutant expresses major virulence factors, exhibits biofilm formation and motility, and colonizes the suckling mouse intestine. Journal of bacteriology **188:**794-800.
6. **Wang H, Chen S, Zhang J, Rothenbacher FP, Jiang T, Kan B, Zhong Z, Zhu J.** 2012. Catalases promote resistance of oxidative stress in Vibrio cholerae. PloS one **7:**e53383.
7. **Joelsson A, Kan B, Zhu J.** 2007. Quorum sensing enhances the stress response in Vibrio cholerae. Applied and environmental microbiology **73:**3742-3746.
8. **Yildiz FH, Schoolnik GK.** 1998. Role of rpoS in stress survival and virulence of Vibrio cholerae. Journal of bacteriology **180:**773-784.
9. **Liu Z, Yang M, Peterfreund GL, Tsou AM, Selamoglu N, Daldal F, Zhong Z, Kan B, Zhu J.** 2011. Vibrio cholerae anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. Proceedings of the National Academy of Sciences of the United States of America **108:**810-815.
10. **Paget MS, Buttner MJ.** 2003. Thiol-based regulatory switches. Annual review of genetics **37:**91-121.
11. **Minato Y, Fassio SR, Hase CC.** 2013. Malonate inhibits virulence gene expression in Vibrio cholerae. PloS one **8:**e63336.
12. **Richardson AR, Dunman PM, Fang FC.** 2006. The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. Molecular microbiology **61:**927-939.
13. **Dons LE, Mosa A, Rottenberg ME, Rosenkrantz JT, Kristensson K, Olsen JE.** 2013. Role of the Listeria monocytogenes 2-Cys peroxiredoxin homologue in protection against oxidative and nitrosative stress and in virulence. Pathogens and disease.
14. **Ferreira A, O'Byrne CP, Boor KJ.** 2001. Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in Listeria monocytogenes. Applied and environmental microbiology **67:**4454-4457.

15. **Delory M, Hallez R, Letesson JJ, De Bolle X.** 2006. An RpoH-like heat shock sigma factor is involved in stress response and virulence in Brucella melitensis 16M. Journal of bacteriology **188:**7707-7710.
16. **Deretic V, Schurr MJ, Boucher JC, Martin DW.** 1994. Conversion of Pseudomonas aeruginosa to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. Journal of bacteriology **176:**2773-2780.
17. **Huang CJ, Wang ZC, Huang HY, Huang HD, Peng HL.** 2013. YjcC, a c-di-GMP phosphodiesterase protein, regulates the oxidative stress response and virulence of Klebsiella pneumoniae CG43. PloS one **8:**e66740.
18. **Davies BW, Bogard RW, Dupes NM, Gerstenfeld TA, Simmons LA, Mekalanos JJ.** 2011. DNA damage and reactive nitrogen species are barriers to Vibrio cholerae colonization of the infant mouse intestine. PLoS pathogens **7:**e1001295.
19. **Stern AM, Hay AJ, Liu Z, Desland FA, Zhang J, Zhong Z, Zhu J.** 2012. The NorR regulon is critical for Vibrio cholerae resistance to nitric oxide and sustained colonization of the intestines. mBio **3:**e00013-00012.
20. **Chatterjee E, Chowdhury R.** 2013. Reduced virulence of the Vibrio cholerae fadD mutant is due to induction of the extracytoplasmic stress response. Infection and immunity **81:**3935-3941.
21. **Pfau JD, Taylor RK.** 1998. Mutations in toxR and toxS that separate transcriptional activation from DNA binding at the cholera toxin gene promoter. Journal of bacteriology **180:**4724-4733.
22. **DiRita VJ, Mekalanos JJ.** 1991. Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. Cell **64:**29-37.
23. **Fengler VH, Boritsch EC, Tutz S, Seper A, Ebner H, Roier S, Schild S, Reidl J.** 2012. Disulfide bond formation and ToxR activity in Vibrio cholerae. PloS one **7:**e47756.

Appendices

Appendix A

Screening natural products for inhibitors of virulence gene expression in *Vibrio cholerae*

Summary

While the toxtazins identified in the synthetic small molecule screen have provided insight into the cellular requirements for virulence gene regulation by *Vibrio cholerae*, they are not compounds naturally found in nature. Natural products were also screened for those that inhibit *Vibrio cholerae* pathogenesis using the *toxT-gfp* reporter strain NB39 used in Chapter 2. Two libraries were screened- a natural extract library from the Center for Chemical Genomics (CCG) at the University of Michigan, and a portion of the Biolog plates. Twenty-nine natural compounds inhibited *toxT* transcription, including procaine, lidocaine, and maltose, which were chosen for follow up studies presented here.

Introduction

There is an added benefit to screening naturally occurring molecules in that these may be biologically relevant molecules that *V. cholerae* may sense and respond to in its normal lifecycle. For example, *V. cholerae* senses cyclic di-guanosine monophosphate (c-di-GMP) through VpsT and VpsR and responds by inducing biofilm formation (1, 2). *V. cholerae* is also capable of sensing chitin and responding by becoming naturally competent (3). Thus, natural products could uncover ways in which a bacterium's

environment regulates gene expression. In addition, natural products tend to be larger, less molecularly flexible, more chemically diverse, have different polarity, aromaticity content, heteroatom content, and occupy different areas of chemical space (4, 5), making potential natural product hits different from the hits identified in synthetic small molecule screens. To broaden our search for inhibitors of *toxT* gene expression in *V. cholerae*, we screened two libraries of natural products, and discuss our findings here.

Results

Natural Extracts

The CCG collaborates with Dr. David Sherman to obtain natural product extracts from various marine ecosystems throughout the world including Papua New Guinea, Costa Rica, US Virgin Islands, Panama, Lake Erie, Lake Huron and Antarctica. We screened 11,121 extracts using our *toxT-gfp* reporter strain and identified 456 that reduced *toxT*-GFP expression by 6 standard deviations of the mean without affecting growth by more than 10%, a 4.1% hit rate.

The best natural product extract was chosen for follow up and the strain stock was cultured to reproduce the extract in a 3 L and 6 L culture size. Unfortunately, neither of these culturing methods produced extracts capable of reducing *toxT*-GFP activity. This illustrates some of the major issues in working with natural products- the results are not always reproducible, and recreating the same extract is often difficult (6). Because of these caveats, we prioritized the toxtazins for further analysis, as opposed

to the natural products. However, this and the other hits generated from screening the natural products library at the CCG could be of interest in future studies.

Biolog Screen

Biolog phenotypic microarrays have been successfully used in pathogenesis research to identify natural molecules that inhibit bacterial growth on beef (7), to identify substrates that persister cells use for respiration (8), and to further drug discovery by grouping compounds with similar mechanisms of action (9). To determine if any of the natural compounds in the Biolog phenotypic microarray could inhibit *toxT* transcription, the compounds in each Biolog plate was resuspended in toxin-inducing medium (LB pH 6.5), and each well was inoculated with a 1:100 dilution of an overnight culture of the *V. cholerae toxT*-GFP reporter strain and incubated at 30°C for 16-18 hours without shaking. GFP and OD_{600} levels were read to determine the level of growth and *toxT* activation in the presence of each compound, and the % inhibition was calculated as follows: $(FL/OD_{600})_{treated}/(FL/OD_{600})_{untreated}$ *100. Twenty-seven compounds were identified that significantly reduce *toxT*-GFP expression by 30% or more relative to untreated samples (see Table A1.)

In addition to the typical toxin-inducing conditions (LB pH 6.5 at 30°C), the compounds of one Biolog plate was also tested under minimal media toxin-inducing

Table A1 Natural compound inhibitors of *toxT*-GFP in LB pH 6.5 at 30°C.

Compound	% Inhibition
Iodonitro Tetrazolium violet	92.03
Chorpromazine	51.86
Sodium metavanadate	49.39
CCCP	49.21
Poly-L-lysine	46.47
2,3-Butanediol	45.1
Thioglycerol	41.58
Sodium bromate	40.31
Arg-Arg dipeptide	40
D-Tagatose	39.49
Procaine	39.07
γ-Amino butyric acid	38.54
Sodium azide	38.15
1,10-Phenanthroline	37.16
Sodium pyrophosphate decahydrate	35.77
Meadione	35.76
Butyric acid	35.44
L-arabitol	34.27
L-Leucine	33.55
Phenethicillin	32.25
Quinic acid	32.22
Sodium dichromate	31.7
Azathioprine	31.34
Arg-Lys dipeptide	31.15
Xylitol	30.88
Ala-Tyr dipeptide	30.18
Malonic Acid	30.11

condition- M9 minimal media with glycerol as the carbon source, supplemented with asparagine, glutamate, arginine, and serine (NERS) grown at 30°C. After resuspending the compounds in this medium, each well was inoculated with a 1:100 dilution of an overnight culture of the *V. cholerae toxT*-GFP reporter strain, and the cultures were grown at 30°C for 16-18 hours without shaking. The GFP and OD_{600} levels were read to determine the level of growth and GFP expression in the presence of each compound, and the % inhibition was calculated as follows: $(FL/OD_{600})_{treated}/(FL/OD_{600})_{untreated}$ *100. Twelve compounds that inhibit *toxT*-GFP expression by 30% or more were identified (Table A2).

Some of the molecules identified in the Biolog screen were already known to affect pathogenesis in *V. cholerae* and indicate that the screen worked. For example, chlorpromazine has previously been shown to reduce fluid loss and duration of symptoms in cholera patients (10). The protonophore CCCP collapses the proton motive force (PMF), reduces the transduction of the CTXΦ (11), and inhibits *V. cholerae* motility (12). Procaine (13) and malonic acid (14) have also been shown to reduce *toxT* transcription. Maltose and procaine, as well as a similar compound lidocaine, were chosen for follow up.

<u>Procaine and Lidocaine</u>

Procaine and lidocaine are both local anesthetics used to reduce pain by functioning as sodium channel blockers (15). Given the evidence in the literature that procaine can affect virulence gene regulation in *V. cholerae* (13, 16), we chose to

Table A2 Natural compound inhibitors of *toxT*-GFP in M9 minimal media + NERS.

Compound	% Inhibition
Maltose	95.33
D-Psicose	74.29
Phenylethylamine	50.69
L-Rhamnose	50.51
Glyoxylic acid	44.8
Propionic Acid	42.78
D-Xylose	42.15
L-arabinose	37.44
Tyramine	33.78
D-Fructose-6-phosphate	33.66
Maltotriose	32.46
D-Glucoronic acid	32.17

investigate the mechanism of action of this compound and a similar compound, lidocaine (Figure A1A). First, the *toxT*-GFP inhibitory activity of lidocaine and procaine was confirmed with fresh powders using the *toxT*-GFP reporter strain (Figures A1B and A1C).

To determine the mechanism of action for procaine-mediated *toxT*-GFP inhibition, RNA was harvested from cultures grown overnight under toxin-inducing conditions with 20 mM procaine and the levels of *toxT*, *tcpP*, aphA, and *aphB* transcripts were quantified by qRT-PCR (see chapter 2 for RNA preparation and qRT-PCR methods). Twenty-millimolar procaine was used in these experiments because that is the concentration used in previously published reports describing the effects of procaine on *V. cholerae* (13, 16).

The OD_{600} and fluorescence of these cultures are shown because there is a severe growth defect at this concentration both in a 96-well plate (Figure A2A) and a mild one in test tubes (A2B). Overnight growth in LB pH 6.5 at 30°C shaking resulted in high GFP expression, but addition of 20 mM procaine decreased *toxT, tcpP*, and *aphB* transcripts; *aphA* transcript was unaffected (Figure A2C).

Maltose

Maltose was identified as an inhibitor of *toxT*-GFP expression when cells were grown in a different toxin-inducing growth medium- M9 minimal medium with glycerol as the carbon source, supplemented with asparagine, glutamate, arginine, and serine (NERS). To determine if maltose also inhibits virulence gene expression in LB pH 6.5 at

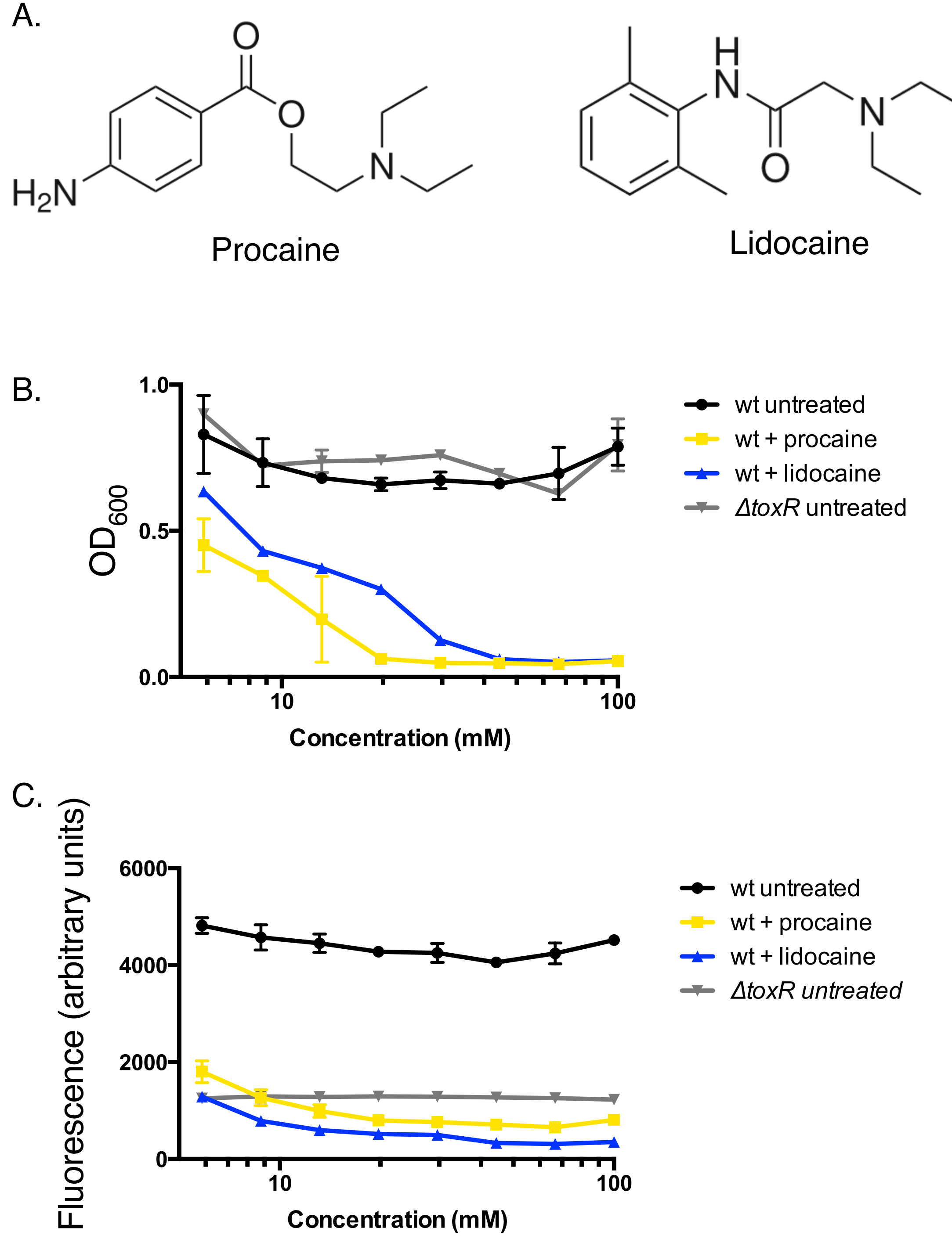


Figure A1 Procaine and lidocaine inhibit *toxT*-GFP in a dose-dependent manner.

A. Structure of lidocaine and procaine. B. Effects of lidocaine and procaine dose on OD_{600}. C. Effects of lidocaine and procaine dose on *toxT*-GFP fluorescence.

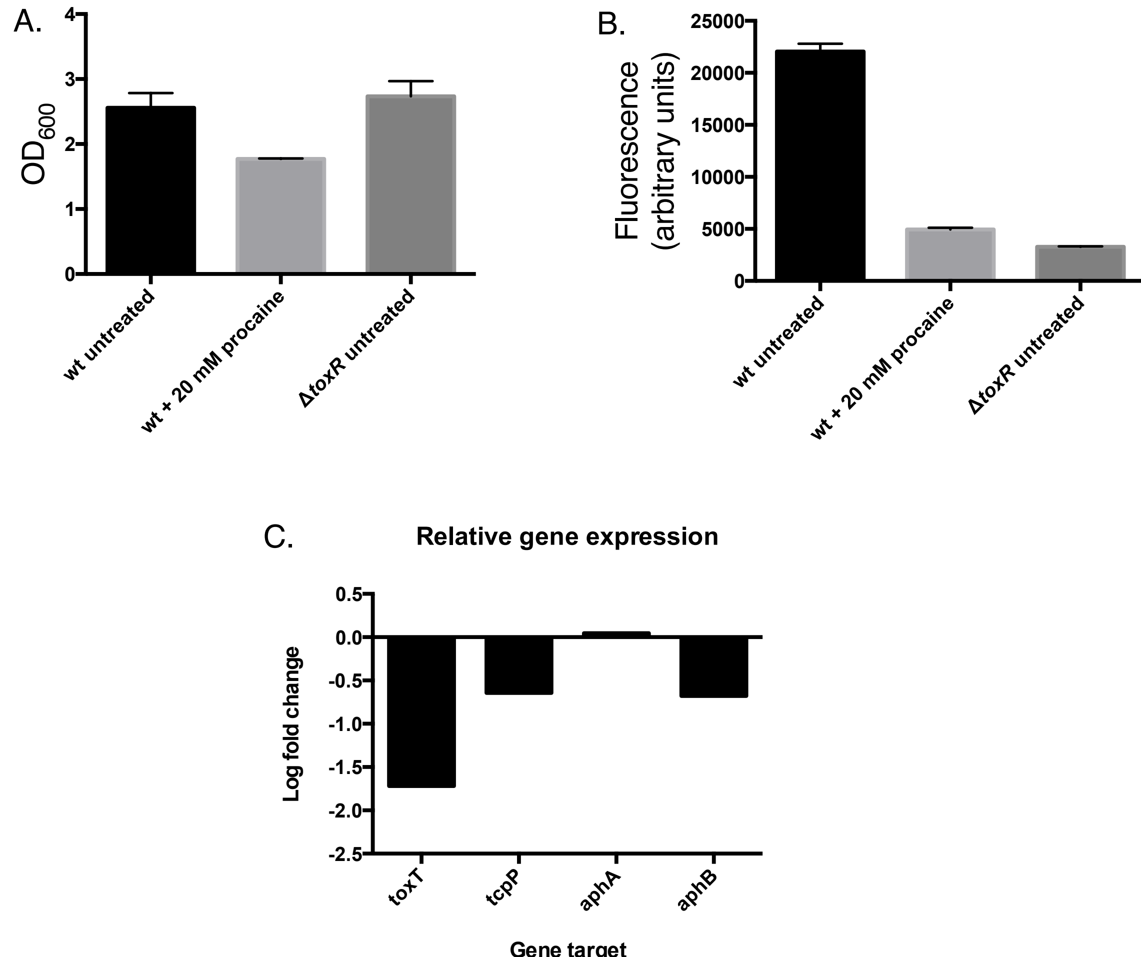


Figure A2 Procaine inhibits *toxT* expression in a *tcpP*- and *aphB*-dependent manner.

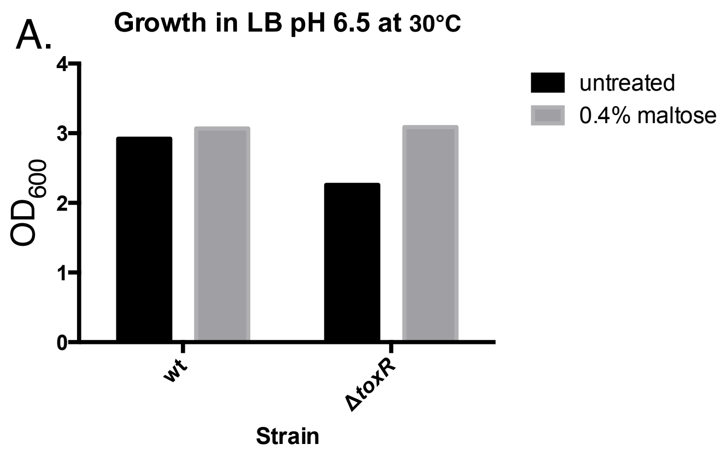
Cultures of *V. cholerae* O395 + p*toxT-gfp* or O395Δ*toxR* + p*toxT-gfp* were grown for 16 hours with or without 20 mM procaine, and the OD_{600} (A) and fluorescence (B) were measured. C. Relative gene expression of *toxT, tcpP*, *aphA* and *aphB* in the cultures was measured by qRT-PCR and expressed as a log fold change over untreated cells.

30°C, wild-type cells and an isogenic Δ*toxR* mutant were grown overnight under these conditions in the presence or absence of 0.4% maltose. As shown in figure A3A and A3B, the addition of maltose did not affect growth, however it reduced *toxT*-GFP expression to levels similar to those of a Δ*toxR* mutant, indicating that maltose inhibits virulence regardless of the growth conditions used for toxin induction. Maltose has already been shown to decrease CT production and secretion and *mal* mutants are less virulent in infant mouse colonization (17); thus, we did not follow up on this further.

Discussion

Appendix A describes the identification of 39 inhibitors of *toxT*-GFP expression in *V. cholerae.* These were identified by screening for natural product extracts collected from marine ecosystems (the CCG natural products collection) and from purified natural products (the Biolog collection). Some of the identified compounds were already known to inhibit virulence in *V. cholerae*, validating the methods used in our screen, but most have no known effect to pathogenesis in *V. cholerae*, and could be used in future studies as molecular probes, or for development for antimicrobial therapy.

Three hits were chosen for follow up studies. Maltose was found to inhibit *toxT*-GFP in two laboratory conditions used to induce *toxT* gene expression- LB pH 6.5 at 30°C, and M9 minimal media + NERS at 30°C. Lidocaine and procaine were characterized in more detail and found to inhibit *toxT* transcription by reducing *tcpP* and *aphB* transcription. Very little is known about transcriptional regulation of AphB, though a catalase (VC1585) and a response regulator CheV (VC1602) were identified in a



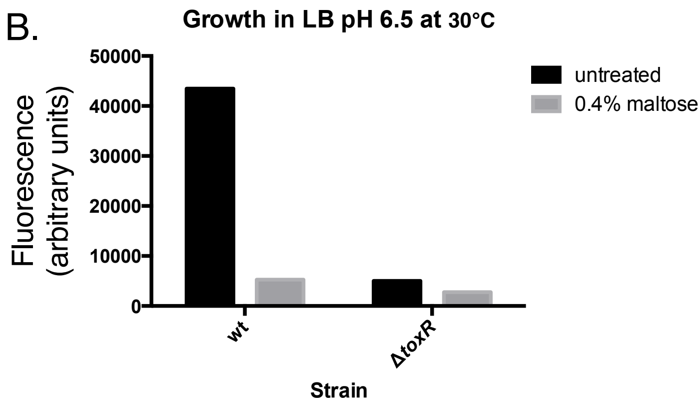


Figure A3. Maltose inhibits *toxT*-GFP expression in LB pH 6.5 at 30°C.

A. OD_{600} of the O395 + p*toxT-gfp* reporter strain grown in LB pH 6.5 at 30°C with 0.4% maltose. B. Fluorescence of the O395 + p*toxT-gfp* reporter strain grown in LB pH 6.5 at 30°C with 0.4% maltose.

transposon screen as mutants that no longer activate *aphB* transcription (18). Lidocaine and procaine may be beneficial in further exploring how AphB is transcriptionally regulated.

While our focus shifted to the toxtazins, the natural inhibitors identified in these screens remain interesting candidates for follow up in future studies. Further, despite there being some literature regarding both procaine and maltose as inhibitors of key *V. cholerae* virulence traits, the mechanisms underlying their effects remain unclear and merit further study.

Materials and Methods

The screens presented here were performed as described elsewhere (Chapter 2; (19)) using the natural products from the Center for Chemical Genomics (CCG) at the University of Michigan, Ann Arbor MI, USA and the Biolog plates were ordered from BIOLOG. The qRT-PCR analysis was performed as described in Chapter 2 and (19), using the same primer sets.

References

1. **Shanahan CA, Strobel SA.** 2012. The bacterial second messenger c-di-GMP: probing interactions with protein and RNA binding partners using cyclic dinucleotide analogs. Organic & biomolecular chemistry **10:**9113-9129.
2. **Krasteva PV, Fong JC, Shikuma NJ, Beyhan S, Navarro MV, Yildiz FH, Sondermann H.** 2010. Vibrio cholerae VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. Science **327:**866-868.
3. **Lo Scrudato M, Blokesch M.** 2012. The regulatory network of natural competence and transformation of Vibrio cholerae. PLoS genetics **8:**e1002778.
4. **Muigg PRJB, L.; Backlund, A.** 2013. In silico comparison of marine, terrestrial and synthetic compounds using ChemGPS-NP for navigating chemical space. Phytochemistry Reviews **12:**449-457.
5. **Bade R, Chan HF, Reynisson J.** 2010. Characteristics of known drug space. Natural products, their derivatives and synthetic drugs. European journal of medicinal chemistry **45:**5646-5652.
6. **Kingston DG.** 2011. Modern natural products drug discovery and its relevance to biodiversity conservation. Journal of natural products **74:**496-511.
7. **Lynnes T, Horne SM, Pruss BM.** 2013. ss-phenylethylamine as a novel nutrient treatment to reduce bacterial contamination due to Escherichia coli O157:H7 on beef meat. Meat science **96:**165-171.
8. **Orman MA, Brynildsen MP.** 2013. Establishment of a method to rapidly assay bacterial persister metabolism. Antimicrobial agents and chemotherapy **57:**4398-4409.
9. **Bourne CR, Wakeham N, Bunce RA, Nammalwar B, Berlin KD, Barrow WW.** 2012. Classifying compound mechanism of action for linking whole cell phenotypes to molecular targets. Journal of molecular recognition : JMR **25:**216-223.
10. **Rabbani GH, Greenough WB, 3rd, Holmgren J, Kirkwood B.** 1982. Controlled trial of chlorpromazine as antisecretory agent in patients with cholera hydrated intravenously. British medical journal **284:**1361-1364.
11. **Hase CC.** 2003. Ion motive force dependence of protease secretion and phage transduction in Vibrio cholerae and Pseudomonas aeruginosa. FEMS microbiology letters **227:**65-71.
12. **Gosink KK, Hase CC.** 2000. Requirements for conversion of the Na(+)-driven flagellar motor of Vibrio cholerae to the H(+)-driven motor of Escherichia coli. Journal of bacteriology **182:**4234-4240.
13. **Hase CC.** 2001. Analysis of the role of flagellar activity in virulence gene expression in Vibrio cholerae. Microbiology **147:**831-837.
14. **Minato Y, Fassio SR, Hase CC.** 2013. Malonate inhibits virulence gene expression in Vibrio cholerae. PloS one **8:**e63336.
15. **Gentry CL, Lukas RJ.** 2001. Local anesthetics noncompetitively inhibit function of four distinct nicotinic acetylcholine receptor subtypes. The Journal of pharmacology and experimental therapeutics **299:**1038-1048.
16. **Hase CC, Mekalanos JJ.** 1999. Effects of changes in membrane sodium flux on virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **96:**3183-3187.

17. **Lang H, Jonson G, Holmgren J, Palva ET.** 1994. The maltose regulon of Vibrio cholerae affects production and secretion of virulence factors. Infection and immunity **62:**4781-4788.
18. **Chen H, Zhu Z, Zhong Z, Zhu J, Kan B.** 2012. [Isolation and function of genes regulating aphB expression in Vibrio cholerae]. Wei sheng wu xue bao = Acta microbiologica Sinica **52:**256-261.
19. **Anthouard R, DiRita VJ.** 2013. Small-molecule inhibitors of toxT expression in Vibrio cholerae. mBio **4.**

Appendix B

Small molecule screen for inhibitors of ToxR activity

Summary

A screen more focused on identifying ToxR inhibitors was designed by using a reporter for the gene *ompU*. This gene is directly activated by ToxR and does not require TcpP, ToxT, or any other component of the *toxT* branch in the virulence regulatory pathway of *Vibrio cholerae* (1-3). Compounds that might be expected to arise in this type of screen include those that inhibit *toxR* transcription, about which little is known (4, 5), in contrast to the extensive amount of knowledge regarding *tcpP* transcription (6-13).

To probe the ToxR branch of the virulence cascade in *V. cholerae* using chemical biology, a small molecule screen was performed to identify molecules that inhibit ToxR activity using an *ompU::sacB* reporter strain such that activation of the *ompU* promoter by ToxR results in sacB expression, which is lethal in the presence of sucrose. Because ToxR expression is IPTG-inducible, it is possible to differentiate small molecule inhibitors that kill *V. cholerae* by inhibiting ToxR-dependent SacB expression (dead only in the presence of IPTG) from those molecules that are generally toxic to *V. cholerae* (dead in the presence or absence of IPTG). The results of this screen are presented here.

Results

After screening 52,396 compounds from 6 diverse libraries at the Center for Chemical Genomics (CCG) at the University of Michigan, 751 compounds were identified that reduced growth only when ToxR expression was induced. Of those, 192 retained activity in a secondary screen and behaved in a dose-dependent manner. Surprisingly, of the ~60,000 compounds screened in both the ToxR and ToxGFP small molecule screens (Chapter 2), only four compounds were found to inhibit both screens in a dose-dependent manner. The CCG numbers for these compounds and their pAC50 (the inverse log of the concentration required for 50% activity) are shown in Table B1.

To confirm the activity of the best ToxR inhibitors, ToxR activity was determined directly by comparing the protein levels of two ToxR-regulated outer membrane proteins, OmpU and OmpT, both of which are clearly visible on a coomassie gel. Lysates of the reporter strain (RA63) grown overnight under non-inducing conditions (LB pH 8.5 at 37°C) in the presence or absence of inhibitors were separated on 12.5% SDS-PAGE gels, and OmpU and OmpT were visualized by coomassie staining (Figure B1). The Δ*toxR* strain and the reporter strain grown in the absence of IPTG both express higher levels of OmpT than OmpU, while wild-type cells and the reporter strain grown in the presence of one millimolar IPTG express more OmpU than OmpT. When the reporter strain is grown with IPTG and one of the six identified ToxR inhibitors, the OmpU/OmpT ratio resembled a wild-type cell, indicating that despite having been identified as inhibitors of *ompU* expression, none of these compounds reduce OmpU levels, though.

CCG #	pAC50 ToxR	pAC50 ToxGFP
20343	4.26	5.08
44180	4.78	4.28
42440	4.63	4.41
41503	4.14	4.31

Table B1. Small molecule inhibitors of both ToxR activity at the *ompU* promoter (ToxR screen) and of *toxT*-GFP expression (ToxGFP screen).

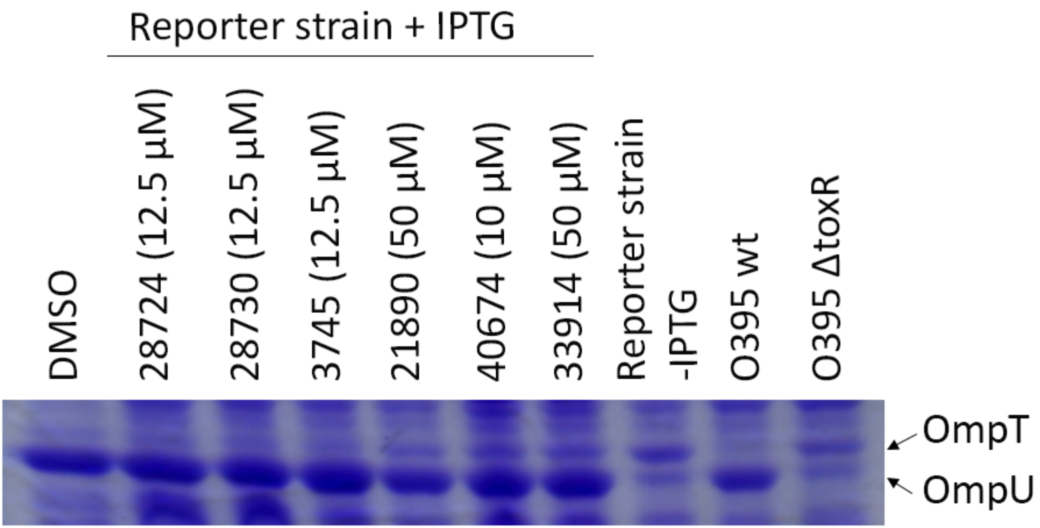


Figure B1 OmpU and OmpT expression in the presence of various compounds.

Cultures of RA63 were grown for 16 hours under toxin non-inducing conditions (LB pH 8.5 at 37°C) in the presence or absence of ToxR inhibitors 28724, 28730, 3745, 21890, 40674, or 33914 and 1mM IPTG to induce ToxR. Cell lysates were separated on SDS-PAGE and OmpU and OmpT were visualized by coomassie staining.

21890, 40674, and 33914 slightly increase OmpT expression.

To determine if our hits were false positives or if the OmpU/OmpT ratio was a poor reporter for ToxR activity, we used an alternative secondary screen that measure ToxR activity using an O395Δ*toxR ompU-lacZ* + pMMB66EH-*toxRS* strain (RA97). We tested 16 compounds with this assay (CCG numbers 2824, 3274, 3745, 5523, 12812, 12818, 12849, 20343, 20826, 21440, 21888, 21890, 21895, 21906, 21907, and 22437), but none of them showed inhibitory activity of the ToxR-dependent *ompU* promoter (Figure B2). Because the results of this screen were not confirmed in either of the secondary assays, we chose to focus on the ToxGFP screen results.

Material and Methods

High-throughput small molecule screen for inhibitors of ToxR activity

The screening strain (RA63) used was O395Δ*toxR* lacZ::*ompU-sacB* harboring the pMMB66EH-*toxRS* plasmid. In these cells, *ompU* activation results in SacB production, a protein that confers sucrose sensitivity. Thus, activation of the *ompU* promoter by ToxR results in sacB expression such that the cells will die in the presence of sucrose. As an additional control, the reporter strain was engineered to expressed ToxR from an IPTG-inducible vector, allowing for the differentiation of small molecule inhibitors that kill *V. cholerae* by inhibiting ToxR-dependent SacB expression (dead only in the presence of IPTG) from molecules that are generally toxic to *V. cholerae* (dead in the presence or absence of IPTG.)

The primary screen, secondary screen, and dose-response studies were carried

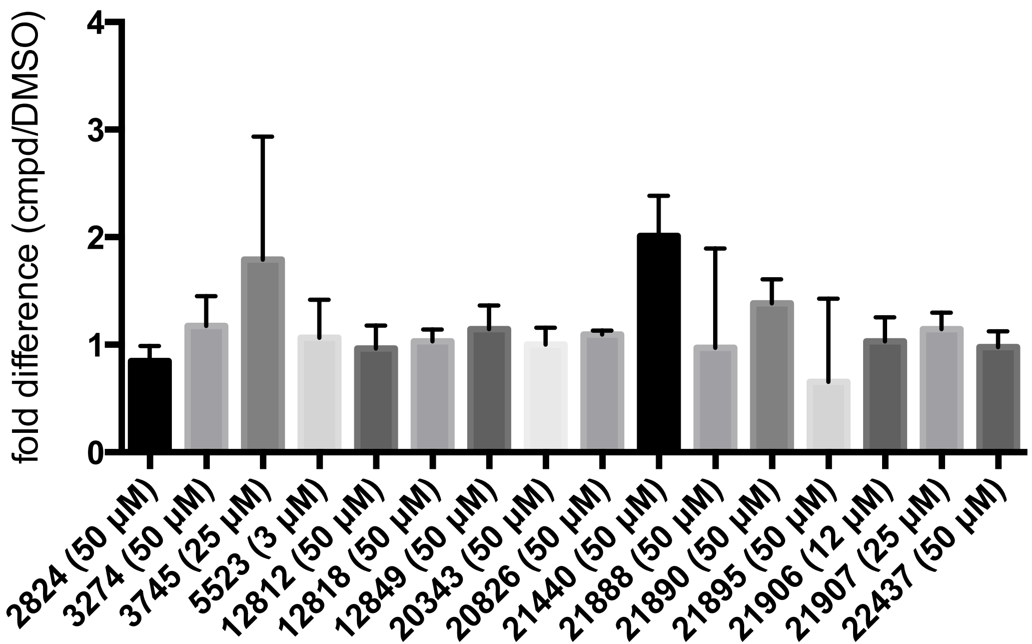


Figure B2.

Strain RA97 (O395Δ*toxR ompU-lacZ* + pMMB66EH-*toxRS*) was grown overnight with one millimolar IPTG and either DMSO or compounds at a range of doses. The culture with the highest concentration of compound that did not affect growth was used in a Miller assay to determine the activation at the *ompU* promoter. Error bars represent the standard deviation from two independent experiments.

out at the Center for Chemical Genomics (University of Michigan), where 52,396 compounds were tested. Overnight cultures of RA63 were diluted to a final OD_{600} of 0.04 into media (LB pH 8.5, 5% sucrose, 100 µg/ml streptomycin, 100 µg/ml ampicillin) and either induced with 100 µM IPTG, or left uninduced as a control. These cultures were added to 384-well plates containing various compounds, and allowed to grown for seven hours at 37°C. After this time, the OD_{600} of each well was read.

A compound was considered active if it caused a decrease in OD_{600} of 1 log or more compared to the –IPTG control. When the actives were retested to confirm their activity and to measure their dose responsiveness, 751 compounds remained. Of these, only four compounds were also identified in the *toxT*-GFP screen described in Chapter 2.

References

1. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. Journal of bacteriology **170:**2575-2583.
2. **Champion GA, Neely MN, Brennan MA, DiRita VJ.** 1997. A branch in the ToxR regulatory cascade of Vibrio cholerae revealed by characterization of toxT mutant strains. Molecular microbiology **23:**323-331.
3. **Matson JS, Withey JH, DiRita VJ.** 2007. Regulatory networks controlling Vibrio cholerae virulence gene expression. Infection and immunity **75:**5542-5549.
4. **Crawford JA, Krukonis ES, DiRita VJ.** 2003. Membrane localization of the ToxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. Molecular microbiology **47:**1459-1473.
5. **DiRita VJ.** 1992. Co-ordinate expression of virulence genes by ToxR in Vibrio cholerae. Molecular microbiology **6:**451-458.
6. **Hase CC, Mekalanos JJ.** 1998. TcpP protein is a positive regulator of virulence gene expression in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **95:**730-734.
7. **Kovacikova G, Skorupski K.** 1999. A Vibrio cholerae LysR homolog, AphB, cooperates with AphA at the tcpPH promoter to activate expression of the ToxR virulence cascade. Journal of bacteriology **181:**4250-4256.
8. **Skorupski K, Taylor RK.** 1999. A new level in the Vibrio cholerae ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. Molecular microbiology **31:**763-771.
9. **Krukonis ES, Yu RR, Dirita VJ.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Molecular microbiology **38:**67-84.
10. **Kovacikova G, Skorupski K.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the Vibrio cholerae tcpPH promoter. Molecular microbiology **41:**393-407.
11. **Krukonis ES, DiRita VJ.** 2003. DNA binding and ToxR responsiveness by the wing domain of TcpP, an activator of virulence gene expression in Vibrio cholerae. Molecular cell **12:**157-165.
12. **Beck NA, Krukonis ES, DiRita VJ.** 2004. TcpH influences virulence gene expression in Vibrio cholerae by inhibiting degradation of the transcription activator TcpP. Journal of bacteriology **186:**8309-8316.
13. **Matson JS, DiRita VJ.** 2005. Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America **102:**16403-16408.

Appendix C

Structure activity relationships of toxtazins A and B

Summary

Structural analogs of both toxtazin A and toxtazin B were tested for their ability to dose-dependently inhibit *toxT*-GFP expression of *V. cholerae* grown under toxin-inducing conditions. Although the determination of the structure activity relationship (SAR) did not result in the identification of more potent molecules, it did uncover the pharmacophore for each of the toxtazins, and provided the insight needed to develop a toxtazin B probe for click chemistry, a method used to pull down the target of a small molecule.

Introduction

The structure activity relationship (SAR) of a molecule is the relationship between that molecule's structure and its biological activity. Studying SAR allows for the determination of the chemical group(s) within a molecule required for producing a desired biological effect. The part of the molecule required for biological activity is called the pharmacophore. SAR data are useful for lead optimization, to decrease biodegradation, and increase bioavailability (1). SAR can also be used to define the

parts of a molecule that are dispensible for activity, and can therefore be modified for click chemistry.

Click chemistry is a method of pulling down a compounds biological target out of a cell or cell lysate (2, 3). One of the most common click chemistry reactions uses azide alkyne Huisgen cycloaddition using a copper catalyst (4) to covalently bind a small molecule to its target. The target can then be biotinylated, pulled out of the reaction on a streptavidin column, and identified by mass spectrometry. This method of target identification has proven successful for other groups (5-7).

Results

SAR

To determine which structural components of the toxtazins are important for their biological activity, structural analogs of each compound were tested for their ability to inhibit *toxT* transcription using the *toxT-gfp* reporter strains. Analogs available from the Center for Chemical Genomics were screened in 384-well plates containing cultures of the O395 strain of *Vibrio cholerae* growing under toxin-inducing conditions (LB pH 6.5 at 30°C) for 16-18 hrs. Analogs that decreased *toxT*-GFP in a dose-dependent manner without affecting the OD_{600} by more than 10% were considered active. The structure and activity of the toxtazin A analogs and the toxtazin B analogs are shown in Tables C1 and C2, respectively.

Based on the results of the SAR studies, pharmacophores for both toxtazin A and toxtazin B could be determined, shown in figure C1. The SO_2 moiety of toxtazin A is

absolutely required for activity, as all the analogs with modifications to the SO_2 group lost activity. The pharmacophore for toxtazin B is less well-defined, as modifications to each R group sometimes lost activity and sometimes retained activity, though the R3 ring could not be removed while the R1 ring could be changed to a methyl group without losing activity. It was suggested that perhaps toxtazin B analogs retain activity so long as one R group has an electron-accepting group and one has an electron-donating group, but that it did not matter which R group these are on.

Click Chemistry

In order to pull out the protein target of toxtazin B from a toxtazin B-treated cell lysate, we collaborated with Toni Kline, a chemist at the University of Washington, to develop photoaffinity probes. Using the results from the SAR studies, Txtz001 was designed (Figure C2) and tested for activity in the *toxT*-GFP reporter strain. Unfortunately, txtz001 did not inhibit *toxT*-GFP in our assay (Figure C3), and was not used in click chemistry because it lacked bioactivity. Alterations to the txtz probe could potentially result in a bioactive probe, and this merits further study.

Discussion

The SAR studies presented here resulted in a clear pharmacophore for toxtazin A, a 4-(difluoromethyl)-2-sulfonylpyrimidine. Toxtazin A was more potent than any of the analogs tested. The SO_2 moiety is absolutely required for function, while the R2 group seems to be dispensible. The R1 group can be small or rather bulky, suggesting

Table C1. SAR of toxtazin A analogs.

CCG number	Structure	Activity
27091		Active
27092		Active
27093		Active
27095		Active
27098		Active
117487		Active
120679		Active
27094 (toxtazin A)		Active
117477		Inactive
117478		Inactive
117479		Inactive
117584		Inactive
117585		Inactive
117586		Inactive

Table C2. SAR of toxtazin B analogs.

CCG number	Structure	Activity
28723		Active
28724 (toxtazin B)		Active
28730 (toxtazin B')		Active
131072		Active
131073		Active
131079		Active
131081		Active
131093		Active

CCG number	Structure	Activity
28725		Inactive
28726		Inactive
28727		Inactive
28728		Inactive
28729		Inactive
28731		Inactive
28732		Inactive
28733		Inactive

131074		Inactive
131075		Inactive
131076		Inactive
131078		Inactive
131080		Inactive
131082		Inactive
131084		Inactive
131086		Inactive
131089		Inactive
131094		Inactive
131123		Inactive
131135		Inactive
131137		Inactive
131140		Inactive
131141		Inactive
131142		Inactive

131145		Inactive
131146		Inactive
131147		Inactive
131150		Inactive
131152		Inactive
131153		Inactive
131159		Inactive
131161		Inactive
131162		Inactive
131163		Inactive
131164		Inactive
131165		Inactive
131166		Inactive
131167		Inactive
131172		Inactive
131173		Inactive

131178		Inactive
131180		Inactive
131181		Inactive
131182		Inactive

4-(difluoromethyl)-2-sulfonylpyrimidine 1H, 4H, 5H, 6H-pyrrolo[3,4-c]pyrazol-6-one

Figure C1. Pharmacophore of toxtazin A (left) and toxtazin B (right).

azide for click chemistry to biotin, fluor

photogenerted carbene
for protein insertion

TxTz001

Chemical Formula: $C_{27}H_{20}F_3N_9O_2$
Exact Mass: 559.17
Molecular Weight: 559.5

Figure C2. Chemical structure of txtz001.

The structure of txtz001 consists of the toxtazin B pharmacophore with a photoactivatable carbene on R1 and an azide on R3.

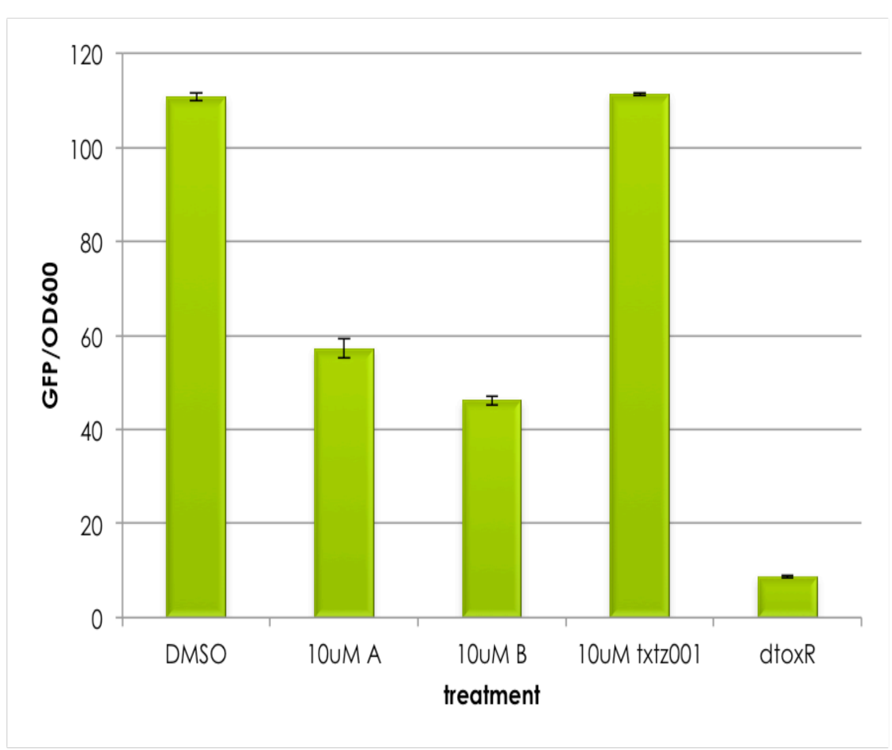


Figure C3. Bioactivity of the txtz001 probe.

The RA2 strain containing the p*toxT-gfp* reporter plasmid was grown for 16 hours under toxin-inducing conditions in the presence or absence of 10 µM toxtazins A, B, or txtz001. An isogenic Δ*toxR* strain was used as a control. The OD_{600} and GFP fluorescence were measured.

the SO_2 moiety activity is likely not due to it fitting into a small binding pocket. Based on these results, a photoaffinity probe could be made by putting the alkyne and carbene the R2 group, or possibly on the R2 group and in place of the trifuloromethyl group of the pyrimidine. This photoaffinity probe, if it retained its ability to inhibit *toxT* transcription, could be used for click chemistry to identify the molecular target(s) of toxtazin A in the cells.

Toxtazin B SAR studies did not present a clearly defined pharmacophore, so we conservatively define it as 1H, 4H, 5H, 6H-pyrrolo[3,4-c]pyrazol-6-one, though certainly not all molecules with this base structure are active. We investigated the possibility that the molecule retains activity if it has one electron-accepting group and one electron-donating group, and created the txtz001 probe. Unfortunately, this probe lost biological activity and was not used for click chemistry. In future iterations of photoaffinity probes, the R3 group should not be modified, as the SAR results show that this R group tolerates fewer deviations from the parent molecule's 4-bromophenzenyl group.

Overall, the SAR studies on toxtazin A and B provide information about what submolecular groups are important for biological function, and which are dispensable. This information could be used in the future to design a more potent analog, and to design a photoaffinity probe for click chemistry to pull down the *in vivo* target of these compounds.

Material and Methods

Analysis of toxtazin A and B analogs

The screening strain used was wild type O395 harboring a plasmid with the *toxT* promoter driving expression of GFPmut3 (strain RA2). Analogs were tested for dose-dependent activity by adding them to 384-well plates containing cultures diluted to a final OD_{600} of 0.02 in LB pH 6.5, and allowing cultures to grow for 16-18 hours at 30°C. The OD_{600} and GFP ((excitation λ = 385 nm, emission λ = 425 nm) levels were measured to monitor for growth and *toxT*-GFP expression. Analogs were considered active if they caused a dose-dependent decrease in GFP levels without affecting the OD_{600} by more than 10% of the DMSO controls.

References

1. **Guha R.** 2013. On exploring structure-activity relationships. Methods in molecular biology **993:**81-94.
2. **Kolb HC, Sharpless KB.** 2003. The growing impact of click chemistry on drug discovery. Drug discovery today **8:**1128-1137.
3. **Mackinnon AL, Taunton J.** 2009. Target Identification by Diazirine Photo-Cross-linking and Click Chemistry. Current protocols in chemical biology **1:**55-73.
4. **Rostovtsev VV, Green LG, Fokin VV, Sharpless KB.** 2002. A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angewandte Chemie **41:**2596-2599.
5. **Vila A, Tallman KA, Jacobs AT, Liebler DC, Porter NA, Marnett LJ.** 2008. Identification of protein targets of 4-hydroxynonenal using click chemistry for ex vivo biotinylation of azido and alkynyl derivatives. Chemical research in toxicology **21:**432-444.
6. **Lee K, Ban HS, Naik R, Hong YS, Son S, Kim BK, Xia Y, Song KB, Lee HS, Won M.** 2013. Identification of malate dehydrogenase 2 as a target protein of the HIF-1 inhibitor LW6 using chemical probes. Angewandte Chemie **52:**10286-10289.
7. **Staub I, Sieber SA.** 2009. Beta-lactam probes as selective chemical-proteomic tools for the identification and functional characterization of resistance associated enzymes in MRSA. Journal of the American Chemical Society **131:**6271-6276.