REGULATION OF THE TYPE VI SECRETION SYSTEM IN ENVIRONMENTAL ISOLATES OF VIBRIO CHOLERAE

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Regulation of the Type VI Secretion System in Environmental Isolates of *Vibrio cholerae*

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LIST OF SYMBOLS, LIST OF ABBREVIATIONS

Δ: Delta, Signifies Deletion of a Gene

µ: Micro Sign

CDC: Centers for Disease Control and Prevention

DAP: Diaminopimelic Acid

E. coli: Escherichia coli

LB: Luria Broth

T6SS: Type-VI Secretion System

V. cholerae: Vibrio cholerae

WHO: World Health Organization
**SUMMARY**

*Vibrio cholerae* is a human pathogen that causes the severe diarrheal disease cholera, but can also inhabit aquatic environments. The type-VI secretion system (T6SS) is a macromolecular contractile machine that injects neighboring cells with cytotoxic effector proteins. Clinical strains of *V. cholerae* express the T6SS only when exposed to high cell density and starvation conditions in the presence of chitin, a process regulated by the master regulator QstR. The atypical clinical strain V52 expresses its T6SS constitutively, a trait shared by many *V. cholerae* strains isolated from the environment. Recently it was discovered that the TfoY regulator controls T6SS expression independent of QstR in V52. In examining strains from environmental sources, I found that one constitutive environmental strain is also under TfoY control. However, I also uncovered that T6SS-mediated constitutive killing in four additional environmental strains was unaffected by a tfoY deletion. Furthermore, I demonstrated that other known regulators (TfoX, QstR, OscR) also played no role in T6SS expression in these strains. For example, the environmental strain BGT69 remains capable of T6SS-mediated killing when these four known T6SS regulators were deleted. These results suggest the presence of a novel regulatory pathway(s) for type-VI secretion in this and other environmental strains of *V. cholerae*. 
CHAPTER 1
INTRODUCTION

_Vibrio cholerae_ is an aquatic gram-negative bacterium found in both aquatic and terrestrial environments. It is the causative agent of the diarrheal disease cholera that infects between 1.3—4 million people annually and results in an estimated 21,000—143,000 deaths each year, many of which are unreported (WHO 2016). Cholera outbreaks have reached pandemic proportions at least seven times since 1816 (Faruque et al 1998). The current outbreaks seen in Asia and other parts of the world are sometimes considered to be part of an eighth pandemic, which originated in Madras, India in 1992, with isolated cases being found as far afield as California (CDC 1993).

_V. cholerae_ strains are divided into several serogroups, based on the presence or absence of cell-surface antigens. All of the past seven cholera pandemics were caused by _V. cholerae_ strains of the O1 serotype (Faruque et al 1998). The current cholera pandemic differs from the previous pandemics in that the causative strain is from a different serogroup, O139 (Shimada et al 1994). Besides these two serotypes, the only other serotype that has been responsible for a major outbreak of cholera-like disease was O37, which resulted in 460 cases and 125 fatalities in Sudan in 1968 (Zinnaka and Carpenter 1972). Of the over 100 _V. cholerae_ serotypes that have been identified, only the three listed above (O1, O37 and O139) have been shown to cause outbreaks of the disease cholera. Naturally, most research has focused on the genetics and ecology of strains belonging to these pathogenic “clinical” serotypes. Less studied are the vast majority of the remaining classified _V. cholerae_ strains that are present throughout the environment, but which do not pose a direct risk to human health. Despite their lack of clinical
significance, it is still important to study strains that did not originate from patients since all but three *V. cholerae* serotypes, and therefore most of the strains, are not pathogenic. To better understand the ecology of *V. cholerae* as a whole it is necessary to take non-pathogenic strains into account as well. In initial studies to characterize “environmental” strains that were isolated from various non-clinical sources including water samples and shell fish, some very interesting phenotypic differences were observed when compared to strains isolated from patients.

One key difference is that while clinical strains require certain environmental stimuli to express the type VI secretion system (T6SS), the majority of sequenced environmental strains constitutively express the T6SS in lab conditions (Bernardy et al. 2016). The T6SS is a macromolecular contractile machine that fires a harpoon-like structure into neighboring cells in a contact-dependent manner. The apparatus itself is structurally similar to a phage-tail structure that has been modified to deliver toxic effector proteins instead of viral DNA (Ho et al. 2014). These effectors have various periplasmic and cytoplasmic targets in both bacterial and eukaryotic cells (Ho et al. 2014). Pukatzki and co-workers discovered the T6SS in *V. cholerae* (Pukatzki et al. 2006). Each *V. cholerae* strain sequenced to date contains a T6SS.

Early research into the apparatus suggested it played a role in evasion of eukaryotic predators like *Dictyostelium* spp. (Pukatzki et al. 2006). Later research into this system determined each strain typically encodes multiple toxic effector proteins that have both bacterial targets and eukaryotic targets (Schwarz et al. 2010, Unterweger et al. 2014, Thomas et al. 2017). It is now appreciated that the T6SS plays a role in both interbacterial competition and bacterial-eukaryotic interaction. One important discovery related to these T6SS effectors was that strains typically carry three T6 loci. The major cluster encodes the required structural genes, and two smaller auxiliary gene clusters (Aux1 and Aux2) encode different effectors (Dong et al. 2013).
Adjacent to each effector gene in these clusters is an immunity gene which confers resistance to
self-toxicity of the effector as well as defense against the T6 effectors of adjacent siblings.

Therefore, strains with the same effector/immunity pairs perceive each other as kin and can co-
exist together without killing each other. Unterweger and co-workers predicted based on
genomic sequencing that *V. cholerae* cells could exchange effector/immunity pairs with their
neighbors via horizontal gene transfer, potentially allowing the newly formed hybrid strain to
outcompete its parental strains (Unterweger et al. 2014). This horizontal transfer of
effector/immunity pairs was recently demonstrated by Thomas and co-workers (Thomas et al.
2017)

The Type VI secretion system apparatus consists of a membrane-bound complex and a
base plate structure that assembles the rest of the apparatus consisting of an outer sheath and an
inner spike (Figure 1, next page). The outer contractile sheath is made from 6 heterodimer
strands composed of VipA and VipB, which form a helical structure around the rigid inner spike
tube consisting of hexameric rings of HCP. The Hcp tube is capped by a VgrG protein at the tip,
and the bottom of the outer sheath is secured by the dodecameric TssA complex (Ho et al. 2014,
Zoued et al. 2016).

Most clinical *V. cholerae* strains control the expression of their T6SS and only produce
the apparatus under certain environmental conditions. In the presence of chitin, the TfoX
regulator upregulates T6SS expression in many clinical strains by controlling the downstream
regulator QstR, which is also activated by high cell density and starvation via HapR and CytR,
respectively (Figure 2, page 5) (Watve et al. 2015). In addition to regulating type-VI expression,
TfoX is also responsible for controlling horizontal gene transfer via natural transformation on
chitinous surfaces (Meibom et al. 2005).
Figure 1: Components of the Type VI Secretion System Apparatus. The T6SS machine is composed of a membrane complex that spans the periplasm and a base plate in the cytoplasm. The base plate recruits the T6 spike, composed of a VgrG trimer at the top and an HCP hexamer forming the tube, as well as the VipA/ VipB sheath around the HCP core. T6 effector molecules are loaded onto the VgrG tip and the tube is ejected from the cell into adjacent target cells when the outer sheath contracts.

Prior research by Metzger and co-workers has shown that in the clinical *V. cholerae* strain V52 of the O37 serotype, which constitutively expresses its T6SS apparatus, deleting *tfoX* did not have an impact on T6SS-activity (Metzger et al. 2016). However, when the recently discovered regulatory gene *tfoY* was deleted, they found that it was responsible for most of the type VI activity observed in this strain. From this result with clinical strain V52, it was hypothesized that all strains that do not require QstR to control T6SS use TfoY instead (Figure 2, next page).
Figure 2: The two known regulatory pathways of the Type VI Secretion System and a hypothesized third regulatory pathway. The QstR pathway, which is commonly found in clinical strains of *V. cholerae*, relies on three different environmental inputs to regulate T6SS expression. Only in the presence of chitin, quorum sensing signals from neighbors and in starvation conditions will these strains naturally express their T6SS genes by increasing the expression of *qstR* through three intermediate regulators, *TfoX*, *HapR* and *CytR*. To date, the TfoY pathway is only found in the atypical clinical strain V52 which constitutively expresses its T6SS in lab conditions.

I sought to determine whether or not this hypothesis held true for *V. cholerae* strains isolated from environmental rather than clinical sources. Since many sequenced environmental strains of *V. cholerae* constitutively express their T6SS in laboratory settings (Bernardy et al. 2016) my initial hypothesis was that deleting *tfoY* would eliminate most if not all of the type VI-mediated killing phenotype of these strains. To test this hypothesis, I selected several environmental strains that displayed robust T6 killing of target *E. coli*. I then deleted the *tfoY* gene and other known regulators of T6SS expression in four such strains. Understanding the
mechanisms by which the type-VI secretion system is regulated in these environmental strains will help us to better understand how *V. cholerae* competes with other organisms in the environment.
CHAPTER 2
METHODS

2.1 Culture Preparation: All bacterial cultures were grown in liquid Luria Broth (LB) broth made from 10g tryptone, 5g protein extract and 10g NaCl per liter of media, or on solid LB culture plates containing 1.5% agar. All *E. coli* and *V. cholerae* strains were incubated at 37°C statically for plates and in shaking incubators for liquid cultures.

Plates containing antibiotics were made using final concentrations of 100µg/mL kanamycin and 10µg/mL chloramphenicol. Diaminopimelic acid (DAP) was used where necessary at 50µg/mL. Sucrose plates were made using the above formula for LB agar plates but without NaCl, with a final concentration of 20% sucrose.

2.2 Cloning via Gibson Assembly: All plasmids used in these experiments were designed using the SnapGene software and created using Gibson assembly (Gibson et al. 2009). The plasmids contained a kanamycin resistance cassette and also carried the *sacB* gene conferring susceptibility to sucrose. Allelic exchange was made possible by incorporating 1kb long flanking regions found upstream and downstream of the gene to be deleted into the plasmid.

2.3 Gene Deletions: All gene deletions in this experiment were performed using allelic exchange with suicide vectors. The suicide vectors used in this experiment were propagated in an *E. coli* strain (S17-λpir ΔdapA) auxotrophic for diaminopimelic acid (DAP) and expressing the π protein permitting replication from the conditional plasmid origin of replication oriR6K (see Figure 3A for a plasmid map on the next page).
Figure 3: Vector maps of plasmids used for allelic exchange and complementation. (A) The vector map of the suicide vector used to perform allelic exchange with all strains of *V. cholerae* used in this experiment to delete *tfoY*. (Continued on next page)
Other deletions via allelic exchange were carried out using the same vector backbone. (B) The vector map of the plasmid carrying a functional copy of the *tfoY* gene under control of the IPTG-inducible ptac promoter was used to complement the deletion of *tfoY* in BGT64.

The donor *E. coli* cells were plated together with the recipient *V. cholerae* strains on LB agar containing DAP to allow the donor cells to transfer the plasmid to the recipient cells via conjugation. The mating plates were incubated at 37°C overnight. The cells were then re-streaked on LB agar plates containing the antibiotic kanamycin but lacking DAP to ensure selective growth of the recipient but not of the donor.

Plates containing appropriate antibiotics were incubated overnight at 37°C. The kanamycin-resistant cells were then re-streaked on 20% sucrose plates. The presence of the *sacB* sucrase susceptibility cassette ensured that only cells that lost the plasmid via recombination could grow on sucrose. All sucrose plates were incubated at room temperature for approximately 48 hours. Cells growing on sucrose were re-streaked on 20% sucrose plates for a second 48-hour incubation period before being re-streaked on both LB and kanamycin plates.

Gene deletions were verified by PCR-amplifying the region around the gene in question using primers that bound outside of the flanking regions used in the suicide vectors. A second round of PCR using primers specific for the *sacB* gene was used to confirm that the plasmid was absent from the chromosome. All gene deletions were further verified by Sanger sequencing.

**2.4 T6SS Killing-Assays:** The T6SS-mediated interbacterial killing potential of the various *V. cholerae* strains was assessed using killing-assays with a chloramphenicol resistant *E. coli* K-12 target strain, as described in (Watve et al. 2015). Cells were grown in liquid culture overnight at 37°C. Using a spectrophotometer, optical density (OD$_{600}$) of each culture was
measured and cultures were diluted to a uniform density. *V. cholerae* was mixed with the target 
*E. coli* at a ratio of 10:1. Then 50µL of this mixture was plated onto a piece of 0.2-micron filter 
paper on an LB plate and incubated at 37°C for three hours. Plates were made in triplicate. The 
filters were then removed and vigorously vortexed in 5mL LB. The resulting cell suspension was 
then serially diluted ten-fold to $10^{-7}$. A 100µL aliquot of each dilution was plated on 
chloramphenicol plates and mean CFUs/mL were calculated from the dilution plates.

**2.5 Statistical Analyses:** All p-values given in this paper were derived from Student’s t-
tests and were verified using single-factor ANOVAs followed by Tukey’s HSD.
3.1 Deleting *tfoY* in the clinical strain V52 severely impacted T6SS activity. To confirm prior results, I first deleted the *tfoY* gene of the constitutive clinical strain V52, since Metzger and co-workers demonstrated that a deletion of *tfoY* in this strain abolished T6 killing (Metzger et al. 2016). The pΔ*tfoY* suicide vector (Figure 3A) was propagated in an appropriate *E. coli* host and conjugated into the clinical strain V52. Allelic exchange was successfully carried out using kanamycin resistance and sucrose sensitivity as the selectable and counter-selectable markers, respectively. The deletion was verified by PCR and Sanger sequencing. The T6SS structural gene *vasK* was also deleted in V52 to act as a negative control. This gene has been used in the past to disable the type VI apparatus and has been shown to completely inhibit T6SS-mediated interbacterial killing (Pukatzki et al. 2006). To determine the effect of deleting *tfoY* on the expression of the T6SS, killing assays were performed with V52, V52Δ*vasK* and V52Δ*tfoY* (see Methods section for details). These mutants were competed against an antibiotic-resistant *E. coli* “target” strain. Cells were then plated on selective media and target *E. coli* colony forming units (CFUs) were counted.

Deletion of *tfoY* via allelic exchange in the clinical strain V52 caused a defect in T6SS-mediated interbacterial killing, increasing the number of surviving target cells by more than 1000-fold on average (p=0.031) compared to the wild-type (Figure 4, next page). The difference between the Δ*vasK* “no-killing” negative control and the Δ*tfoY* strain was not statistically significant (p=0.059). In the double deletion strain, in which both *tfoY* and another known T6SS regulator, *tfoX*, were deleted, the average increase in target survival was five-fold compared to the Δ*tfoY* strain. The difference seen between the Δ*tfoY* Δ*tfoX* deletion strain and the no-killing
ΔvasK control was not significant (p=0.827). Therefore, tfoY is the primary regulator of T6SS expression in the clinical strain V52. Using the same methods outlined above, ΔtfoY deletions mutants of the environmental strains BGT41, BGT61, BGT64, BGT65 and BGT69 were constructed along with ΔvasK mutants of BGT41, BGT64 and BGT69. The ΔvasK mutants of BGT62 and BGT65 are not complete yet.

Figure 4: The clinical strain V52 stopped killing when the T6SS regulator tfoY was deleted. Effect on target survival of deleting the T6SS regulators tfoY and tfoX during an inter-bacterial killing assay. VasK is a structural protein required for T6SS assembly and deletion of vasK results in a no-killing phenotype across all tested strains. Data come from killing assays between the above genotypes of V52 and a K-12 E. coli strain that acted as the target. CFUs per mL of surviving target E. coli were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.
3.2 Deleting *tfoY* in environmental strain BGT64 had a similar effect as in V52.

Expression of the type VI secretion system in the environmental strain BGT64 requires *tfoY*, as in the clinical strain V52 (Figure 5). The *tfoY* deletion mutant of BGT64 showed a marked increase in target survival, approximately 10,000-fold compared to the wild-type. However, the p-value was not significant (p=0.115), most likely due to the high amount of variability between biological replicates and not due to an actual lack of effect on type VI-mediated killing. This conclusion is supported by the fact that the Δ*tfoY*Δ*tfoX* mutant, which had very similar target survival to the Δ*tfoY* mutant, falling within five-fold of each other, was also not statistically significant (p=0.069) but the wild-type CFU counts compared to the double deletion mutant was highly significant with a p-value of 0.001. The differences between the Δ*vasK* no-killing control and the wild-type control as well as the single and double regulator deletion mutants were all significant. This suggests that some other, third T6SS regulator (or regulatory pathway) may be controlling the residual killing in this strain that explains the difference seen between the Δ*vasK* control mutant and the Δ*tfoY* Δ*tfoX* mutant. This may be explored at a later date.

3.3 Complementing the *tfoY* deletion in BGT64 restored the killing phenotype. To verify that deleting *tfoY* in BGT64 was the cause of the change in phenotype observed in this strain, a plasmid carrying a copy of *tfoY* (*ptac-*tfoY) under the control of an inducible promoter was introduced into the Δ*tfoY* mutant of BGT64 (see Figure 3B for a plasmid map on page 8). The *ptac* promoter is suppressed by the LacI<sup>+</sup> repressor whose gene is also encoded on the plasmid. In the presence of the allolactose mimic isopropyl β-D-1-thiogalactopyranoside (IPTG), which binds LacI<sup>+</sup>, the inhibitory protein falls off the *ptac* promoter and allows any downstream genes to be expressed (Hansen et al. 1998).
Figure 5: The environmental strain BGT64 also requires TfoY for killing of *E. coli*. Effect on target survival of deleting the T6SS regulators *tfoY* and *tfoX* during an inter-bacterial killing assay. VasK is a structural protein required for T6SS assembly and deletion of *vasK* results in a no-killing phenotype across all tested strains. Data come from killing assays between the above genotypes of BGT64 and a K-12 *E. coli* strain that acted as the target. CFUs per mL of surviving *E. coli* target cells were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.

The BGT64Δ*tfoY* mutant without a plasmid had over 1000-fold more target survival than the wild-type strain (p=0.002) (Figure 6, next page). By contrast, the BGT64Δ*tfoY* mutant carrying *ptac-tfoY* only had five-fold more target survival than the wild-type (p=0.374) while still having over 1000-fold less target survival than either the Δ*tfoY* mutant without any plasmid or the Δ*tfoY* mutant with a control vector. The control vector used the same plasmid backbone as the *ptac-tfoY* vector. This control was used instead of an un-induced version of the *ptac-tfoY* vector because even without IPTG induction, there was enough leaky expression of *tfoY* to restore most of the wild-type phenotype (data not shown).
Figure 6: The impaired killing phenotype of the BGT64 ΔtfoY mutant was restored when complemented with tfoY. Effect of deleting the T6SS regulator tfoY and complementing the deletion with a plasmid-borne copy of the gene on target survival during an inter-bacterial killing assay. VasK is a structural protein required for T6SS assembly and deletion of vasK results in a no-killing phenotype across all tested strains. The vector control shown used the same plasmid backbone as the complementation mutant but does not express TfoY. Data are from killing assays between the above genotypes of BGT64 and a K-12 E. coli strain that acted as target. CFUs per mL of surviving target E. coli were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.

3.4 Deleting tfoY in environmental strain BGT69 had no significant impact on killing. Next, a tfoY deletion mutant was constructed of the environmental strain BGT69. This strain was one of the most proficient killers of all environmental strains tested by Bernardy and co-workers that was amenable to conjugation (Bernardy et al. 2016). Only BGT70 was a better killer, but this strain proved difficult to conjugate (see Table 1 in the Discussion section for a
comparison of strains). The BGT69 ΔtfoY mutant was only modestly impaired for killing, with less than one-fold decrease in killing compared to the wild-type (Figure 7). This difference was not statistically significant (p=0.793). Target survival of a double regulator deletion of tfoY and tfoX was also not significantly different from either the wild-type (p=0.434) or the ΔtfoY mutant and resulted in only an average increase of target survival of 3-fold compared to the ΔtfoY mutant of BGT69. It is probable that the type VI secretion system of this strain is independent of both regulators. Based on this finding, BGT69 is constitutive for T6 killing, and requires neither of the known major T6 regulators QstR or TfoY.

Figure 7: Deleting the T6SS regulator tfoY in environmental strain BGT69 had no effect on the killing phenotype. Effect on target survival of deleting the T6SS regulators tfoY and tfoX during an inter-bacterial killing assay. VasK is a structural protein required for T6SS assembly and deletion of vasK results in a no-killing phenotype across all tested strains. Data come from killing assays between the above genotypes of BGT69 and a K-12 E. coli strain that acted as target. CFUs per mL of surviving target E. coli were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.
3.5 Deleting *tfoY* in environmental strain BGT41 had no significant impact on killing. Both *tfoY* and *tfoX* were deleted in another constitutive environmental strain of *V. cholerae*, BGT41, that was a robust killer like BGT69, to determine if other strains were also independent of the these two well-known T6SS regulators. The BGT41 Δ*vasK* no-killing control had significantly more target survival than the wild-type. The BGT41 Δ*tfoY* mutant had no discernible phenotype and was within a 2-fold difference compared to the wild-type (p=0.171, Figure 8, next page), similar to a *tfoY* deletion in BGT69. The Δ*tfoY* Δ*qstR* deletion mutant in BGT41 was also not significantly different from either the wild-type (p=0.242) or the Δ*tfoY* mutant, with a target strain survival around 3-fold greater than the wild-type and less than two-fold greater than the Δ*tfoY* mutant. Thus, neither TfoY nor QstR play a significant role in regulating the type VI expression of this environmental strain, similar to what was seen in BGT69. In both BGT69 and BGT64, the observed *E. coli* was T6SS-dependent, since Δ*vasK* mutants did not kill the target *E. coli* (see Figures 6 and 7).

3.6 Deleting other known T6SS regulators in environmental strain BGT69 still had no significant impact on killing. To determine how expression of the type VI apparatus was regulated in BGT69 and BGT41, other recently discovered regulators were deleted in sequence in BGT69 to determine whether Type VI-mediated killing would be compromised. QstR is a regulatory protein that is activated by TfoX as well as the quorum sensing regulator CytR and by HapR, which is related to the starvation response in cells (Watve et al. 2015). The T6SS regulator QstR was deleted first in the Δ*tfoY* Δ*tfoX* mutant of BGT69 as well as in the wild-type. OscR is an osmosis regulating protein that has been shown to enhance the interbacterial killing potential of the clinical *V. cholerae* strain A1552 against an *E. coli* target strain under high
osmolarity conditions (Ishikawa et al. 2012). Next the Type VI regulatory gene \textit{oscR} was deleted via allelic exchange in the \textit{ΔtfoY ΔtfoX ΔqstR} triple-deletion mutant.

Figure 8: A BGT41 \textit{ΔtfoY ΔqstR} mutant kills \textit{E. coli} as well as wild-type BGT41. Effect on target survival of deleting the T6SS regulators \textit{tfoY} and \textit{qstR} during an inter-bacterial killing assay. VasK is a structural protein required for T6SS assembly and deletion of \textit{vasK} results in a no-killing phenotype across all tested strains. Data come from killing assays between the above genotypes of BGT41 and an A K-12 \textit{E. coli} strain that acted as target. CFUs per mL of surviving target \textit{E. coli} were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p$<0.05$ compared to WT.

A killing assay was carried out with the wild-type strain of BGT69 as well as a \textit{ΔvasK} mutant, a \textit{ΔqstR} mutant, and triple and quadruple regulator deletion mutants (Figure 9, next page). The no-killing \textit{ΔvasK} control had over 10,000-fold more target survival than the wild-type
(p=0.037). The ΔqstR mutant showed a small increase in average target survival compared to the wild-type that was not significant (p=0.102). Surprisingly, the ΔtfoY ΔtfoX ΔqstR mutant of BGT69 was significantly different from the wild-type (p<0.005) even though the difference in target survival was less than ten-fold. This may again be due to the high intra-replicate variation seen in the wild-type compared to the much lower variability of the triple deletion mutant, an assumption which is corroborated by the fact that the ΔtfoY ΔtfoX ΔqstR ΔoscR mutant, which did not differ significantly from the triple deletion mutant with an increase in average target survival of only two-fold, was also not significantly different from the wild-type (p=0.124), even though the average target survival was over ten-fold greater than the wild-type. These results suggest that almost all of the Type VI-mediated killing seen in BGT69 is not regulated by either TfoY, TfoX, QstR or OscR.

![Graph showing mean surviving target E. coli CFUs/mL for different genotypes of BGT69](continued on next page)

**Figure 9:** Deleting other known T6SS regulators in environmental strain BGT69 also had no effect on killing. Effect on target survival of deleting the T6SS regulators tfoY, tfoX, qstR and oscR during an inter-bacterial killing assay. (Continued on next page)
VasK is a structural protein required for T6SS assembly and deletion of vasK results in a no-killing phenotype across all tested strains. Data come from killing assays between the above genotypes of BGT69 and a K-12 E. coli strain that acted as target. CFUs per mL of surviving target E. coli were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.

3.7 Deleting tfoY in environmental strains BGT65 and BGT62 had a similar effect as in BGT69. To determine how wide-spread TfoY-independence is among constitutive environmental strains of V. cholerae, the tfoY gene was deleted via allelic exchange in two more environmental strains, BGT65 and BGT62. A tfoY deletion in BGT65 resulted in a less than one-fold increase in target survival (Figure 10, next page). However, this difference was not statistically significant (p=0.795). Due to the lack of a ΔvasK control in this strain, the no-killing control used was the target strain by itself, mixed with LB in the same ratio as the one used for the wild-type and the ΔtfoY mutants. This target-only control had over 15,000-fold more target survival than either the wild-type or the ΔtfoY mutant in BGT65 (p<0.005 for both comparisons). This suggests that, similar to BGT69 and BGT41, TfoY has little to no control of type-VI expression in the BGT65 strain.

The tfoY deletion in BGT62 was not assessed by a CFU count due to time constraints. However, a preliminary spot-plating assay of different dilutions of the surviving target cultures showed that the ΔtfoY mutant of BGT62 had similar target survival to the wild-type (Figure 11, page 22). As with BGT65, a target-only control was used instead of a ΔvasK mutant. The control had on average 10,000-fold more target survival than either the wild-type or the ΔtfoY mutant. Based on the lack of difference between target survival in the wild-type and in the tfoY deletion mutant, TfoY most likely plays no role in regulating the T6SS of BGT62, although the
inaccurate nature of a spot-plating assay will necessitate a more thorough analysis of the strain in the future to make a more conclusive determination.

**Figure 10: Preliminary findings of deleting the T6SS regulator tfoY in environmental strain BGT65 show no effect on killing.** Effect on target survival of deleting the T6SS regulator tfoY during an inter-bacterial killing assay. Data come from killing assays between the above genotypes of BGT65 and a K-12 *E. coli* strain that acted as target. Due to the absence of a working no-killing phenotype mutant in this strain, the control used was the target *E. coli* by itself mixed with an amount of LB equal to the volume of predator added to the other conditions (see Methods). CFUs per mL of surviving target *E. coli* were extrapolated from CFU counts taken on chloramphenicol plates that only allowed survivors of the target strain to grow. Bars show mean CFU/mL of three biological replicates. Error bars represent standard deviation. Asterisks denote p<0.05 compared to WT.
Figure 11: Preliminary findings of deleting the T6SS regulator \textit{tfoY} in the environmental strain BGT62 also show no effect on killing. Spot-plating of serially-diluted strains of BGT62 mixed with a K-12 \textit{E. coli} target strain after a three-hour killing assay. Strains were plated on chloramphenicol plates that only allowed the target strain to grow. Each column corresponds to a different biological replicate and each row corresponds to a 10:1 dilution of the row below. The vertical direction indicates survival of the target \textit{E. coli} based on the dilution factor of culture at which single colonies of the target were observed. (A) Left: Three biological replicates of the control (the target \textit{E. coli} by itself mixed with an amount of LB equal to the amount of competitor strain culture added to the other conditions). Right: Three biological replicates of the wild-type strain of BGT62. (B) Left: Three biological replicates of a \textit{tfoY} deletion mutant of BGT62. Right: Three biological replicates (technical replicates of the biological replicates seen in (A)) of the target strain by itself.
CHAPTER 4
DISCUSSION

Metzger and co-workers hypothesized that the type-VI regulator TfoY, which controlled T6SS-expression in the clinical strain V52, would also control expression in all constitutively active environmental strains (Metzger et al. 2016). Of the five environmental strains tested here, only one strain, BGT64, showed a large dependence on TfoY for its T6SS-mediated interbacterial killing. Four other strains that I tested showed little to no effect to their killing efficacy when tfoY was deleted. Based on these findings, V. cholerae strains that constitutively express their T6SS can be roughly divided into two groups; those which are under the regulation of TfoY and those which are not (Figure 2). This repudiates the prior claim made by Metzger and co-workers.

Interestingly, two of the strains whose T6SS-mediated killing activity showed TfoY independence (BGT69 and BGT41) were also not affected by the deletion of a second T6SS regulator. A tfoX deletion in BGT69, which encodes a known type-VI regulator that controls expression of T6SS in the presence of chitin in some clinical V. cholerae strains such as C6706 and A1552, did not significantly impact T6SS-mediated killing. Neither did a qstR deletion in BGT41, which encodes a T6SS regulator that is partially controlled by TfoX. Both V52 and BGT64 were equally independent of TfoX, similar to BGT69, because deleting tfoX in the ΔtfoY background did not result in a further appreciable increase in target survival in these strains (Table 1, next page). This same pattern was maintained in BGT41 when qstR was deleted in a ΔtfoY background. One of these two TfoY-independent strains, BGT69, which was further tested
by deleting qstR as well another known T6SS-regulator, oscR, still showed similar levels of inter-bacterial killing as its wild-type (Figure 9, page 19).

**Table 1: Some constitutive strains of V. cholerae have a T6SS that is dependent on the regulator TfoY while others are independent of both TfoY and TfoX/QstR.** All values given are mean target *E. coli* survival normalized to the wild-type of each *V. cholerae* strain. The T6SS of the clinical strain V52 shows a clear dependence on the regulatory gene tfoY, with surviving target cfu counts similar to those of the no-killing ΔvasK control for both the ΔtfoY mutant and the ΔtfoY ΔtfoX mutant. The environmental strain BGT64 shows a similar dependence on TfoY, although there is slightly more residual killing activity than in V52. Both BGT41 and BGT69 show no appreciable decrease in T6SS expression when tfoY is deleted. The same holds true when a second regulator from the QstR pathway is deleted (QstR in BGT41 and TfoX in BGT69). This suggests that a third, unknown T6SS regulatory pathway may be important in these strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-Type</th>
<th>ΔvasK</th>
<th>ΔtfoY</th>
<th>ΔtfoY ΔqstR or ΔtfoY ΔtfoX</th>
</tr>
</thead>
<tbody>
<tr>
<td>V52</td>
<td>1</td>
<td>9,100</td>
<td>3,820</td>
<td>9,870</td>
</tr>
<tr>
<td>BGT64</td>
<td>1</td>
<td>230,000</td>
<td>9,220</td>
<td>22,500</td>
</tr>
<tr>
<td>BGT41</td>
<td>1</td>
<td>13,000</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>BGT69</td>
<td>1</td>
<td>15,800</td>
<td>1.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>

The question why some constitutive strains require TfoY to express their T6SS while others do not is an intriguing one. The serotype of the *V. cholerae* strain does not appear to be an indicator of TfoY-dependence since the five environmental strains tested are from the O1 serotype (Table 2, next page). Geographical location also does not appear to be significant, since BGT41, BGT62, BGT64 and BGT65 were all isolated from the South-Eastern United States but only BGT64 shows TfoY dependence. The other TfoY-dependent strain in the list, V52, was isolated from Sudan while BGT69, another TfoY-independent strain, came from Guam.
Table 2: The *V. cholerae* strains used in this experiment, including their reference names. All strains listed were isolated from environmental sources, except for V52 which was isolated from a patient. The country of origin where each strain was first isolated is also given, as well as the year the strains were first derived. All strains used in this experiment conform to the O1 serotype except for V52 which is an O37 strain.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Reference Name</th>
<th>Derived from</th>
<th>State/Country</th>
<th>Year Derived</th>
<th>Source</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>V52</td>
<td>V52</td>
<td>Patient</td>
<td>Sudan</td>
<td>1968</td>
<td>CDC</td>
<td>O37</td>
</tr>
<tr>
<td>BGT41</td>
<td>VC22</td>
<td>Oyster</td>
<td>FL, USA</td>
<td>1981</td>
<td>FDA</td>
<td>O1</td>
</tr>
<tr>
<td>BGT62</td>
<td>2512-86</td>
<td>Moore Swab</td>
<td>LA, USA</td>
<td>1986</td>
<td>CDC</td>
<td>O1</td>
</tr>
<tr>
<td>BGT64</td>
<td>692-79</td>
<td>Canal</td>
<td>LA, USA</td>
<td>1979</td>
<td>CDC</td>
<td>O1</td>
</tr>
<tr>
<td>BGT65</td>
<td>2479-86</td>
<td>Moore Swab</td>
<td>LA, USA</td>
<td>1986</td>
<td>CDC</td>
<td>O1</td>
</tr>
<tr>
<td>BGT69</td>
<td>3223-74</td>
<td>Storm Drain</td>
<td>Guam</td>
<td>1974</td>
<td>CDC</td>
<td>O1</td>
</tr>
</tbody>
</table>

One correlation that can be made between environmental strains to differentiate TfoY-dependent strains from the rest is their relative killing-efficacy against a common target *E. coli* strain. Bernardy and co-workers tested a large number of clinical and environmental strains under standard laboratory conditions (without specific induction of T6SS expression) and found that some environmental strains were much less effective killers than others (Bernardy et al. 2016). The environmental strains tested in this study were also included in that study, and when TfoY-dependence is correlated against target survival in that figure, we find that strains with greater interbacterial killing potential are independent of TfoY while BGT64, which did not kill as proficiently, requires TfoY for T6SS expression (see Figure 12, adapted from Bernardy et al. (2016)). To determine whether this trend holds true, *tfoY* deletions will have to be constructed in more environmental strains, from both ends of the killing-potency spectrum. This approach is complicated by the fact that some environmental strains cannot take up plasmids via conjugation and any deletions would have to be carried out via natural transformation. However, future
research could provide valuable insights into the regulation of constitutive T6SS-expression in *V. cholerae* Environmental strains.

**Figure 12:** Clinical and environmental strains of *V. cholerae* have varying rates of type VI-mediated interbacterial killing against target *E. coli*. The average CFUs/mL of surviving target *E. coli* after a three-hour killing assay between various *V. cholerae* strains and a target *E. coli* species. Top: Target survival of clinical *V. cholerae* strains. Bottom: Target survival of environmental *V. cholerae* strains. Bracketed strains were studied in this work. From left to right (low target survival to high target survival): BGT69, BGT65, BGT41, BGT62, BGT64. Figure adapted from (Bernardy et al. 2016).
Another interesting finding is that TfoY-independent strains such as BGT69 and BGT41 also appear to be unaffected in terms of interbacterial killing when other known type-VI regulators are deleted. In the case of BGT69, deleting four known T6SS regulators only increased target survival by about ten-fold compared to the wild-type, leaving over 1000-fold of killing compared to the ΔvasK no-killing control mutant that are as yet unexplained. One explanation of this strain’s apparent independence to known regulators is that the genes encoding the T6SS apparatus are controlled by constitutive promoters and that there are no regulators at work. This hypothesis is unlikely however because the T6SS apparatus is a large macromolecular structure that requires 1,600 molecules of ATP per contractile event (Basler 2015), which puts the organism at a significant disadvantage when the T6SS is not actually required. A second hypothesis is that there is another regulator (or regulatory pathway) at work that has not been tested yet. Two likely candidates are CspV, a cold-shock protein which has been shown to regulate genes involved in T6SS-expression as well as biofilm formation at low temperatures (Townsley et al. 2016) and VxrB, a protein involved in controlling T6SS genes during intestinal colonization (Cheng et al. 2015). Once these remaining known T6SS-regulators have been analyzed in BGT69, the next step will be to conduct a transposon mutagenesis to find other, novel regulators that could be responsible for the remaining T6SS-expression. By competing transposed V. cholerae colonies with fluorescently-labeled E. coli, it will be possible to pick bright colonies (where the E. coli were not killed off by the V. cholerae) and sequence the mutant V. cholerae to determine which gene was disrupted by the transposon insertion. The potential gene can then be deleted in the wild-type to determine what effect it has on T6SS-expression. If a new T6SS regulator is found, a novel regulatory scheme can be added to the two established pathways of T6SS regulation found in clinical and some environmental strains.
Currently *V. cholerae* strains tested to date can be classified into either the “QstR pathway,” such as most clinical strains including C6706 and A1552, or the “TfoY pathway,” which includes constitutive clinical strains like V52 and BGT64. The addition of a novel T6SS-regulation “pathway” would allow the classification of *V. cholerae* strains into discrete subgroups based on the manner in which their T6SS is regulated, including many environmental strains that do not fit into one of the current regulatory schemes.
REFERENCES


