Rules of Engagement: The Type VI Secretion System in *V. cholerae*

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Abstract

Microbial species often exist in complex communities where they must avoid predation and compete for favorable niches. The type VI secretion system (T6SS) is a contact-dependent bacterial weapon that allows for direct killing of competitors through the translocation of proteinaceous toxins. *Vibrio cholerae* is a Gram-negative pathogen that can use its T6SS during antagonistic interactions with neighboring prokaryotic and eukaryotic competitors. The T6SS not only promotes *V. cholerae*'s survival during its aquatic and host life cycles, but also influences its evolution by facilitating horizontal gene transfer. This review details the recent insights regarding the structure and function of the T6SS as well as the diverse signals and regulatory pathways that control its activation in *V. cholerae*.

Descriptive Key Terms

Type VI Secretion System (T6SS); *Vibrio cholerae*; structure; function; regulation

A Versatile Weapon for a Deadly Pathogen

The type VI secretion system (T6SS) is a contact-dependent contractile nanomachine used by bacteria to translocate a toxin-coated, membrane-puncturing device into neighboring cells [1–3]. Translocation of T6SS effector proteins is lethal unless target cells produce cognate immunity proteins that bind and sequester the incoming toxic effectors. Since its discovery, T6SS genes have been identified in over a quarter of sequenced Gram-negative bacteria [4,5]. This highly abundant system has been shown to mediate antagonistic interactions against a wide variety of prokaryotic and eukaryotic organisms[1,6–8]. One of the first

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bacterium shown to possess the T6SS was *Vibrio cholerae* and thus, much of our understanding of T6SS structure, function, and regulation has been developed from continued study of the T6SS in this pathogenic organism [1].

V. cholerae is a Gram-negative bacterium responsible for the diarrheal disease cholera. There have been seven recorded cholera pandemics in the past 200 years, with the seventh still ongoing [9]. Cholera infections continue to impact 1.4 to 4.3 million people globally and result in 21,000 to 143,000 deaths every year [9]. While over 200 serogroups of *V. cholerae* have been characterized, pandemics have only been attributed to the O1 serogroup [9]. *V. cholerae* exists primarily in the aquatic environment, where it can be transmitted to a human host through the ingestion of contaminated food or water. *V. cholerae* utilizes its T6SS to compete with the diverse prokaryotic and eukaryotic organisms that it encounters in both the aquatic environment and human host. Recent work on the activation of the T6SS suggests that it may contribute to the persistence and evolution of *V. cholerae* through direct antagonism of competing microbes [1,10,11]. Additionally, the *V. cholerae* T6SS is known to be active during infection, though its exact role in pathogenesis is not yet understood [12–16]. Indeed, there is still much to be uncovered about when and how the T6SS is deployed and the role it plays in environmental survival and infection. This review provides an update on the current knowledge of the structure, activity, and function of the T6SS, as well as the signals and regulatory networks important for its activation in *V. cholerae*.

The Structure of the T6SS

The T6SS is a multicomponent toxin delivery apparatus that has structural and functional homology to the T4 bacteriophage tail spike and tube [17,18]. Imaging studies suggest that translocation of T6SS effector proteins occurs through a contraction event that propels a membrane puncturing spike into neighboring cells (Figure 1) [19]. Assembly of the T6SS begins with the assembly of the membrane complex, followed by recruitment of baseplate proteins that anchor the outer sheath and the inner tube to the lipid membranes of the bacteria [20]. The membrane complex is comprised of the three proteins TssJLM (VasDFK in *V. cholerae*), which span the inner membrane and provide structural support to the system [20]. The cytoplasmic proteins that form the baseplate complex, TssEFGK (HsiF and VasABE in *V. cholerae*) and VgrG1-3, are then recruited to the membrane complex and are anchored to the inner membrane [20,21]. These baseplate subunits are required for proper formation of the tail complex, which is assembled onto the VgrG1-3 trimer that forms the tip of the T6SS apparatus [20]. Though not shown to be essential components of the baseplate, proteins containing repeating proline-alanine-alanine-arginine (PAAR) motifs cap the VgrG1-3 trimer and act to sharpen the T6SS spike complex [22]. The tail complex is composed of an inner tube formed by hemolysin-coregulated protein (Hcp) hexamers encased within an outer VipA/VipB tube complex [20,22,23]. The outer and inner tubes polymerize into the cytosolic space over the course of ~30 seconds and can remain fully extended for several minutes until an unknown signal triggers rapid contraction of the outer sheath (~5ms) and translocation of the inner tube into the extracellular space [19,24]. The signals and mechanisms that govern contraction remain elusive. After contraction and secretion occurs, the ClpV ATPase is recruited to disassemble and recycle the VipA/VipB tube components [25,26].

Analysis performed in a variety of species suggests that effectors can associate with Hcp, PAAR-motif proteins, and VgrG [27–31]. To date, all characterized *V. cholera* T6SS effectors are either loaded onto the VgrG tip or are part of the tip proteins themselves. 'Cargo effectors', are loaded directly onto the tip of the T6SS. It was recently determined that loading of the cargo effector TseL is facilitated by the chimeric protein Tap-1 (also called Tec-1), which contains a VgrG-binding N-terminal domain and a TseL-binding C-terminal domain [32,33]. Finally, the so-called PAAR-motif proteins, which assemble into a cone-like structure at the tip of the T6SS and form a sharp point that facilitates membrane puncture have also been shown to harbor C- or N-terminal effector domains or to bind and load additional effectors [22,34,35].

Activity and Function of the T6SS

V. cholerae encodes two T6SS effectors that target eukaryotic cells and can utilize these effectors as a means of escape from predatory amoeba in the environment. One of these effectors is the structural component VgrG-1, which contains a C-terminal effector extension that causes cytotoxic actin-crosslinking in the predatory amoeba *Dictyostelium discoideum* and J774 macrophages (Figure 2B) [1,3]. Additionally, VgrG-1 has been associated with intestinal inflammation and diarrheal symptoms in the infant rabbit, as well as efficient colonization of the infant mouse, suggesting a role for the T6SS during infection [13,16]. The cargo effector VasX targets both eukaryotic and prokaryotic cells by disrupting the cell membrane and has demonstrated activity against *D. discoideum* and *Escherichia coli* [36,37]. Efficient colonization of the infant mouse and rabbit intestinal tract is also significantly influenced by the presence of the peptidoglycan degrading effector VgrG-3 [15]. The host intestinal tract is colonized with commensal bacteria that act as a barrier between *V. cholerae* and its preferred niche within the lumen of the small intestine. It has been speculated that the antibacterial effector VgrG-3 may promote intestinal colonization through killing of the host microbiota (Figure 2C) [15,16].

The bactericidal activity of *V. cholerae*'s T6SS plays a role in inter- and intra-species competition and clonal segregation [6,38]. When the *V. cholerae* T6SS targets another bacterial cell, two distinct outcomes can occur. If the neighboring bacterium encodes the same immunity genes as the predator cell, the delivered effectors are deactivated and the cell is protected [10]. These two bacteria are said to be compatible. For VgrG-3, this effector-immunity interaction appears to occur at 1:2 ratios, as dimerization of the immunity protein is critical to its function [39]. Alternatively, if the target bacterium does not encode the immunity genes, it will be subject to cell lysis. Though the T6SS gene clusters are widely conserved among *V. cholerae* strains, the effectors and corresponding immunity proteins encoded in these clusters have been reported to be highly variable [10]. This diversity in effector-immunity pairs is thought to contribute to intra-species competition in various environmental niches, though it is currently unknown which factors contribute to one strain outcompeting another. Some hypotheses include the arsenal of effectors, potential differences in growth rate, rate of firing of the T6SS, or how the T6SS is regulated under specific conditions [38,40].

Four effectors enact T6SS-mediated bacterial killing by pandemic *V. cholerae*. The putative lipase, TseL, and the pore forming colicin VasX, both target the cell membrane. Additionally, two effectors target peptidoglycan; the lysozyme VgrG-3 and the amidase TseH (Table 1) [39,41]. It is thought that the T6SS is only active against Gram-negative bacteria, likely because Gram-positive bacteria are protected from T6SS activity by their thick peptidoglycan [42]. Over the past decade, a plethora of effector classes have been identified in Gram-negative bacteria and it is likely more will be discovered. The study of T6SS effectors has illuminated the versatility and limitations of *V. cholerae*'s T6SS during antagonistic interactions with competing microorganisms. The continued identification and characterization of T6SS effectors will remain an important means of enhancing our understanding of the role the T6SS plays in bacterial and host interactions.

T6SS Genetic Organization and Regulation

The *V. cholerae* T6SS genes are encoded in one large operon (VCA0105-VCA0124), known as the large or major cluster, and at least three smaller operons known as auxiliary clusters 1, 2, and 3 (VCA0017-VCA0021, VC1415-VC1419, and VCA0284-VCA0286) (Figure 3) [1,43,44]. The large cluster encodes the majority of the T6SS structural components, with the exception of the essential secreted inner tube component Hcp, two of the VgrG proteins, and one PAAR-motif protein (VCA0284) [1,44]. Auxiliary clusters 1 and 2 encode for the nearly identical *hcp1* (VC1417) and *hcp2* (VCA0017) genes, either of which is sufficient to produce Hcp and form the inner tube [44]. Auxiliary clusters 1 and 2 also encode for the VgrG-1 (VC1416) and VgrG-2 (VCA0018) tip components respectively, as well as unique effector sets and their cognate immunity proteins [41,44]. The recently identified third auxiliary cluster is predicted to encode for one PAAR motif protein (VCA0284) and a unique effector-immunity pair (VCA0285-86) [22,43]. In addition to the promoters found upstream of each operon, internal promoter activity has been identified within the large cluster and auxiliary clusters 1 and 2 that lie just upstream of the immunity genes [41,45]. The transcriptional regulators that drive expression from these internal promoters are not well understood; however it has been hypothesized that they may allow immunity proteins to be constitutively expressed, thus ensuring protection from neighboring kin cells even in the absence of an active T6SS [41].

Significant advancements have been made in discerning the complex regulatory networks that govern the transcription and activation of the T6SS in *V. cholerae*. The transcriptional regulator VasH, which is encoded in the large T6SS cluster (VCA0117), was among the first T6SS regulators to be identified [1,46]. VasH is a bacterial enhancer-binding protein that complexes with the alternative sigma factor RpoN and coordinates transcription from the upstream promoters of auxiliary clusters 1 and 2 (Figure 4) [1,47-49]. The genetic organization of the T6SS clusters provides a mechanism by which activation of the large cluster prompts transcription of auxiliary clusters 1 and 2 and the production of an assembled T6SS. Since the discovery of VasH, a number of additional regulators have been identified and diverse signalling pathways that feed into T6SS activation in response to various environmental cues have been characterized. The T6SS is now known to be controlled via the quorum sensing, catabolite repression, and nucleoside scavenging pathways [13,49,50]. These signalling cascades are additionally integrated into the chitin-

induced competency cascade, which coordinates co-expression of the T6SS and competency genes [11,51]. Finally, the T6SS is influenced by a number of additional environmental and host signals including, but not limited to, temperature, osmolarity, the secondary messenger cyclic dimeric ($3'{\rightarrow}5'$) GMP (c-di-GMP), mucin, and bile [45,52-54]. Together, these regulatory mechanisms provide insights into how *V. cholerae* coordinates its T6SS activity in both the aquatic and host environments.

Quorum Sensing

Quorum sensing (QS) is a form of bacterial communication that occurs through the production, secretion, and sensing of small molecules known as autoinducers [55,56]. This communication allows alterations in gene expression to occur across a population of bacteria in response to changing cell density, which is signaled by increasing levels of autoinducers. QS is known to regulate a variety of behaviors important for *V. cholerae*'s aquatic and intestinal life cycles including biofilm formation, motility, natural competency, and virulence factor production [51,57–59]. It is now recognized that QS also coordinates T6SS activation by repressing the T6SS at low cell density (LCD) and upregulating the T6SS at high cell density (HCD) (Figure 4) [60]. In *V. cholerae*, QS-mediated gene regulation occurs through a phosphorelay cascade modulated by four sensor histidine kinases, CqsS, LuxPQ, CqsR, and VpsS. CqsS and LuxPQ sense the levels of cholerae autoinducer 1 (CAI-1) and autoinducer 2 (AI-2), respectively, while the ligands for CqsR and VpsS have not been identified [61–63]. At LCD, these four histidine kinases phosphorylate the phosphotransfer protein LuxU, which in turn phosphorylates LuxO [61,62]. Phosphorylated LuxO activates the expression of four small RNAs known as Qrr1-4, which bind to and destabilize the mRNA transcripts of the large cluster of the T6SS and HapR. At HCD, however, LuxO is unphosphorylated, and transcription of *qrr1-4* is inactive, thus permitting the translation of the large T6SS cluster and HapR [60]. HapR positively regulates transcription of auxiliary clusters 1 and 2, likely via direct binding to HapR binding motifs [64].

Chitin-Induced Competency Pathway

V. cholerae spends a majority of its life cycle in the aquatic environment, where it is frequently found associated with chitinous surfaces, such as the exoskeletons of zooplankton. Mounting evidence suggests that growth on zooplankton facilitates *V. cholerae's* persistence, transmission, and virulence [65,66]. Thus, mechanisms and strategies that promote *V. cholerae's* successful colonization of chitinous surfaces are of great interest. *V. cholerae* has evolved several signaling cascades that are influenced by the presence of chitin, including the chitin utilization program, natural competency, and activation of the T6SS [11,67-69].

Chitin is an insoluble polymer consisting of repeating β-1,4-linked N-acetylglucosamine (GlcNAc) residues. Upon growth on chitin, the histidine kinase (HK) ChiS, senses GlcNac polymers and initiates a regulatory cascade that results in the expression of genes important for the transport, degradation, and utilization of chitin as a carbon source, as well as those required for natural competency, which allows bacteria to import extracellular DNA (eDNA) from the enviornment [68,69]. In *V. cholerae,* the signaling cascade initiated by ChiS couples natural competency to the induction of the T6SS via the transcriptional regulator

TfoX (Figure 4) [70-73]. Briefly, ChiS is locked in an inactive state through interaction with the periplasmic chitin binding protein, CBP [74]. Upon growth on chitin, the extracellular chitinases ChiA-1 and ChiA-2 degrade chitin into short GlcNAc oligomers, which are then able to enter the cell through chitoporins [69]. GlcNAc oligomers bind to CBP resulting in disassociation of CBP from ChiS. In the absence of CBP, ChiS activates the transmembrane protein TfoS, which in turn promotes transcription of the sRNA *tfoR* [71-73]. The sRNA *tfoR* is necessary for the translation of TfoX and the activation of the TfoX regulon [71]. The ability of TfoX to activate competency and T6SS genes is dependent upon the presence of the QS and TfoX-dependent regulator, QstR, which is required for the production of T6SS structural components [11,51]. Thus, QS signals also appear to feed into the chitin-induced competency pathway to initiate activation of the T6SS; however, the molecular mechanisms underlying this activation have not been characterized. Additionally, the nucleoside scavenging regulator, CytR, is essential for natural transformation and contributes to T6SS gene activation; however, the means by which it regulates the T6SS remain unclear (Figure 4) [50,75]. During chitin-induced co-activation of the T6SS and natural competency, *V. cholerae* uses its T6SS to kill incompatible bacterial cells, freeing eDNA that is then taken up by its competency machinery [11]. Thus, the T6SS may facilitate the genetic diversity and evolution of *V. cholerae* strains in the environment via the acquisition of new genetic information through horizontal gene transfer, in addition to promoting *V. cholerae*'s colonization and persistence on chitinous surfaces through targeted killing of competing microbes (Figure 2A).

Carbon Catabolite Repression

The T6SS is positively regulated by the small molecule cyclic adenosine monophosphate (cAMP) and global regulator cAMP receptor protein CRP (Figure 4) [49]. When preferred carbon sources are exhausted or unavailable, transcription of the adenylate cyclase gene, *cyaA*, is upregulated, which leads to increased levels of cAMP [76,77]. Free cAMP binds to CRP and the resulting complex acts as a transcriptional regulator, controlling the activation and repression of a number of essential *V. cholerae* pathways, including carbon uptake, QS, chitin utilization, chitin induced natural competency, and the T6SS [49,78–80]. Deletion of either *cyaA* or *crp* prevents production of Hcp, indicating that the cAMP-CRP complex is essential for T6SS production [49]. The mechanism by which cAMP-CRP regulates the T6SS is unclear; however, it is possible that cAMP-CRP influences T6SS production through its regulation of QS and chitin-induced competency [78,79]. Additional studies are needed to determine whether cAMP-CRP controls T6SS production through these pathways or through alternative regulatory mechanisms.

Influence of c-di-GMP

C-di-GMP is a secondary messenger that is capable of binding to a wide variety of targets and influencing transcriptional and enzymatic activities [81]. C-di-GMP was recently found to regulate the T6SS in *V. cholerae* through a protein homologous to TfoX, known as TfoY [45]. The 5'UTR of *tfoY* contains a c-di-GMP riboswitch that prevents translation of TfoY in the presence of high levels of c-di-GMP [45,82]. When c-di-GMP levels are decreased, T6SS-mediated killing is increased in a TfoY-dependent manner [45]. TfoY appears to upregulate both the large cluster and the effector immunity pairs in auxiliary clusters 1 and

2, though not auxiliary cluster 3 (Figure 4) [45]. Regulation of T6SS by TfoY is independent of TfoX's T6SS regulation through the natural competency regulon. Though further study is needed to fully elucidate the molecular mechanisms by which TfoY regulates the T6SS, it is speculated that TfoY may be involved in a danger sensing and defensive escape reaction based on the observation that effectors targeted to eukaryotic cells appear to be more highly expressed in a TfoY overexpression background [45,83].

Post-Translational Regulation

Little is known about the post-translational regulation of T6SS protein production, assembly, and activation in *V. cholerae*. However, the Lon protease was recently identified as a negative regulator of the T6SS (Figure 4) [84]. While a major role of the Lon protease is to degrade misfolded or otherwise aberrant proteins, it also has the ability to degrade specific protein targets [85]. This targeted degradation by Lon provides post-translational regulation of a wide array of processes in a variety of bacteria, including *E. coli*, *P. aeruginosa*, and *B. subtilis*, though *V. cholerae* is the only bacteria in which the Lon protease has been shown to regulate the T6SS. In the absence of Lon, transcription of *hcp1 and 2* are upregulated ~5-fold while ~2-fold increases are observed in transcripts from the main cluster. Additionally, production and secretion of Hcp is increased, and killing of *E. coli* prey is increased by ~2-fold in a standard killing assay [84]. Though this study demonstrated that Lon is a post-translational regulator of the T6SS, the mechanism by which Lon exerts this regulatory effect has not yet been determined. Given the global effect a Lon deletion has on T6SS transcription, translation, and activity, it is likely that Lon is responsible for directly degrading a key regulator or regulators of the T6SS, such as those discussed in this review [84].

Regulation of the T6SS in the Host

Infection by *V. cholerae* occurs through the ingestion of contaminated food or water. The invading bacteria navigate through the digestive system to the epithelial surface of the small intestine where *V. cholerae* produces virulence factors that promote colonization and disease onset. Recent studies suggests that the T6SS is active in the host and contributes to virulence and intestinal colonization [13,14,16]. The regulatory mechanisms that govern T6SS expression during pathogenesis remain poorly defined. It is likely that many of the regulators and signaling pathways mentioned earlier contribute to T6SS induction during pathogenesis; however, most *in vivo* assays have focused on the function of the T6SS within the host rather than its regulation. QS is known to play an essential role in coordinating *V. cholerae*'s virulence cascade and current data suggests that the T6SS remains under the control of HapR and LuxO during infection [13,49,58,60]. Additionally, the H-NS-like protein TsrA was shown to repress virulence factors, including the T6SS; however, its mechanism of action remains unknown [13]. A recently identified two component system (TCS), VxrAB, was demonstrated to positively regulate intestinal colonization in a T6SS-dependent manner; however, the mechanism by which it regulates *in vivo* T6SS activity and the signal that feeds into this TCS is unclear [16].

The *V. cholerae* T6SS, however, is known to respond to host signals, such as mucin, bile, and indole (Figure 4). Mucins, the main component of the mucus layer in the intestine, are

known to increase T6SS-mediated killing of bacterial prey, while the bile salt deoxycholic acid represses T6SS killing via inhibition of T6SS tube formation [54]. The production of deoxycholic acid is facilitated by the commensal bacterium *Bifidobacterium bifidium*, which is capable of metabolizing certain bile acids to deoxycholic acid [54]. Additionally, *in vitro* exposure to indole, a signaling molecule found in large concentrations in the mammalian intestinal tract, was shown to activate T6SS gene expression and may feed into *in vivo* control of the T6SS [86]. Given that efficient colonization of the intestinal tract is known to be significantly influenced by the peptidoglycan degrading effector VgrG-3, the *V. cholerae*'s T6SS may target commensal bacteria to facilitate intestinal colonization (Figure 2C) [15,16]. Signals produced by the microbiota may serve to inhibit or activate *V. cholerae*'s T6SS and may influence host susceptibility to disease. Indeed, host microbial communities are known to increase colonization resistance against many pathogens by preventing access to desirable niches, limiting nutrient availability, and producing inhibitory compounds [87– 90]. Thus, *V. cholerae* may overcome these obstacles through T6SS killing of the host's microbiota; however, additional work is required to characterize the influence of the microbial community on *V. cholerae* pathogenesis and T6SS activity. The important role the T6SS appears to play in pathogenesis prompts additional studies of T6SS regulation within the host, which may aid in our understanding of this system.

Influence of Environmental Signals on T6SS Regulation

The T6SS of *V. cholerae* is also influenced by a number of environmental signals encountered in the aquatic environment or the host, though many of the key regulators that mediate these signals have not yet been identified. Osmolarity is known to be an important signal that influences the T6SS through the osmoregulator OscR, which represses T6SS gene expression at low osmolarities (85mM NaCl). The repressive effect of OscR is relieved when *V. cholerae* is placed in high osmotic conditions (340mM NaCl) (Figure 4) [53,91]. Temperature additionally modulates the activity and expression of the T6SS, repressing T6SS gene expression at low temperatures (15°C) and activating T6SS gene expression at high temperatures (25°C – 37°C) (Figure 4) [52,53]. The activation of the T6SS in response to elevated temperature appears to be regulated in part by the cold shock protein CspV. Deletion of *cspV*, significantly decreases the transcription of *hcp*, resulting in less killing of bacterial prey at 25°C and 37°C [52]. While the mechanisms through which OscR and CspV regulate the T6SS remain unclear, some studies have shown that T6SS gene induction and activity is optimal at osmolarities of 340mM NaCl and temperatures of 25°C [52,53]. These conditions are similar to those found in the estuarine habitats where *V. cholerae* resides [92– 95]. Thus, the regulatory mechanisms described here may have adapted to facilitate competition and survival within this niche.

Differential Regulation of the T6SS in *V. cholerae*

V. cholerae has over 200 serogroups based on LPS antigen classification [9]. While all sequenced *V. cholerae* isolates encode for T6SS genes, not all strains regulate their T6SS identically [1,13,96]. Many environmental isolates have a constitutively active T6SS, while most studied pandemic strains exercise more controlled regulation of the T6SS [1,13,49,96]. These differences are most apparent within the QS pathway [13,49,60]. In constitutively

active strains, HapR has relatively little influence on T6SS gene expression and killing, while the influence of QS on strains that heavily regulate their T6SS differs, with some strains requiring the removal of LuxO for T6SS gene expression under standard laboratory conditions [13,60]. This diversity of regulatory strategies may be indicative of evolutionary adaptations that are advantageous to the specific isolate's niche.

Concluding Remarks

Over the past decade, significant advancements have been made on the structural and mechanistic properties of the T6SS, as well as the signals and regulatory pathways that govern its activation in *V. cholerae.* It is now understood that *V. cholerae* can use its T6SS on chitinous surfaces and co-regulates T6SS activation with natural competency pathways [11]. This provides a mechanism that likely increases the survival and persistence of *V. cholerae* in the aquatic environment through the direct killing of microbial competitors and may contribute to the evolution of *V. cholerae* through horizontal acquisition of new genetic information from killed cells (Figure 2A). In addition, *V. cholerae* may utilize its T6SS as a colonization and virulence determinant by targeting the host's epithelial and immune cells, as well as the commensal microbial population (Figure 2C). While numerous insights have been made into the structure, function, and regulation of this bacterial weapon, substantial gaps remain (see Outstanding Questions). For example, the extent to which the T6SS facilitates inter- and intra-species competition and the biologically relevant targets of the T6SS remain unclear. Furthermore, the regulatory mechanisms and pathways important for T6SS activation in the host are poorly characterized. Additional research is required to identify the activating signals and mechanisms through which most regulators function. Given the wealth of knowledge revealed about the T6SS in the past decade, it is exciting to consider what future study of the T6SS will yield as we continue to explore the complexities of the T6SS in *V. cholerae* and other important human pathogens.

Acknowledgments

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Outstanding Questions

- To what extent does the T6SS facilitates inter- and intra-species competition in the aquatic and host environments and which targets are biologically relevant?

- How does the T6SS contribute to fitness within the host? During infection, does *V. cholerae* use its T6SS for the killing of host commensal bacteria, escape from immune cells, attack of the epithelium or a combination of the three?

- How does *V. cholerae* coordinate loading of multiple effectors? Can different effectors be loaded onto the same tip? Or are mechanisms in place to ensure only a single effector type is present on one T6SS complex?

- What is the exact location of internal promoters responsible for constitutive production of the immunity genes? What are the regulatory mechanisms that govern activation from these promoter sites?

- What are the regulatory networks and signals important for modulating T6SS activity during pathogenesis? Which of the known regulators take part in T6SS activation in the host? Are there host specific regulatory networks in place?

- How do OscR, CspV, TfoY, TsrA, VxrB, Lon, and cAMP-CRP govern T6SS activation and functionality? Do any of these signaling pathways act in concert or with quorum sensing and natural competency pathways? If so, what regulators link these networks? Do the transcriptional regulators bind to and directly facilitate transcription from T6SS gene clusters? Or do they activate the T6SS indirectly through an as of yet uncharacterized pathway?

Trends Box

- The T6SS is a contact-dependent toxin delivery machine found in Gram-negative bacteria. Translocation of toxins occurs through a contraction event that propels a membrane puncturing spike, decorated with effector proteins, into neighboring cells.

- *V. cholerae* can use its T6SS against a variety of organisms found in its aquatic and host environments including neighboring Gram-negative bacteria, phagocytic amoeba and immune cells.

- In many *V. cholerae* isolates, T6SS induction is under the control of diverse regulatory mechanisms such as quorum sensing, carbon catabolite repression, and chitin induced competency pathways in addition to a variety of environmental signals found in the aquatic and host environments.

- The T6SS enhances *V. cholerae's* survival and evolution in the aquatic environment and acts as a virulence factor to promote intestinal colonization in the host.
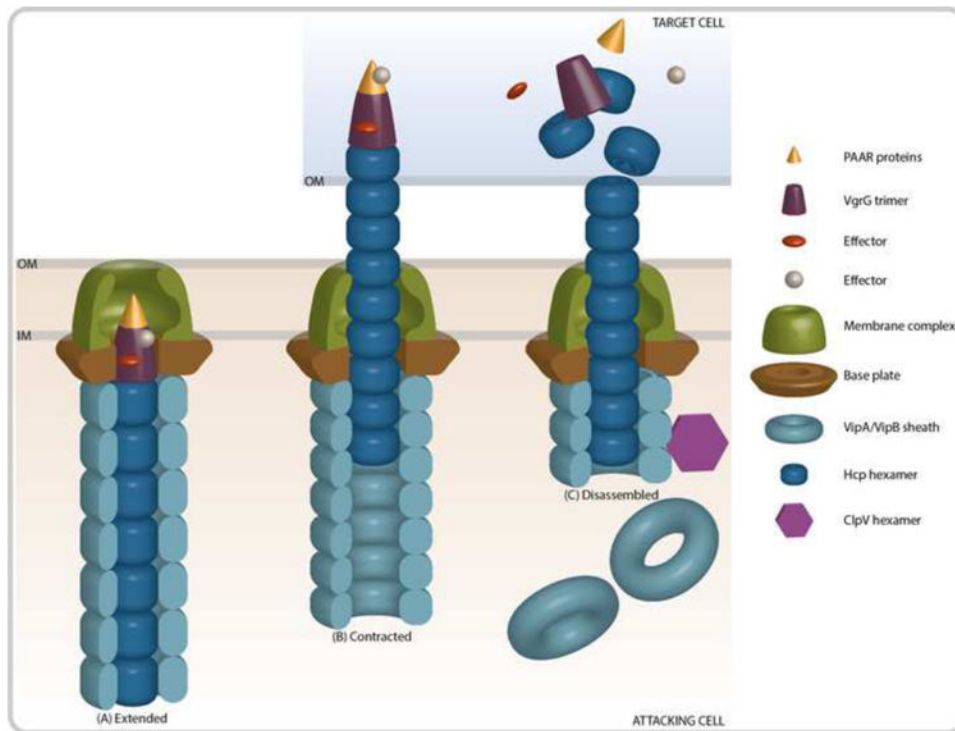
Figure 1. Contraction of the T6SS Results in the Translocation of Effector Proteins
(A) The membrane complex is comprised of both a baseplate structure TssEFGK (HsiF and VasABE in *V. cholerae*), as well as membrane anchoring components TssJLM (VasDFK in *V. cholerae*). The outer sheath (VipA/VipB) and inner tube (Hcp) proteins polymerize to form an extended tube that is assembled onto VgrG and PAAR-motif proteins at the cytoplasmic side of the membrane complex. Effector proteins are recruited to the PAAR or VgrG proteins. (B) After assembly, the T6SS complex remains stable until an unknown signal results in the contraction of the outer sheath and the propulsion of the inner tube into a neighboring cell. (C) Upon translocation of the inner tube, tip, and effector complex, the effector proteins can exhibit their toxic activity. Concurrently, the ATPase ClpV disassembles the outer sheath so that the components can be recycled.
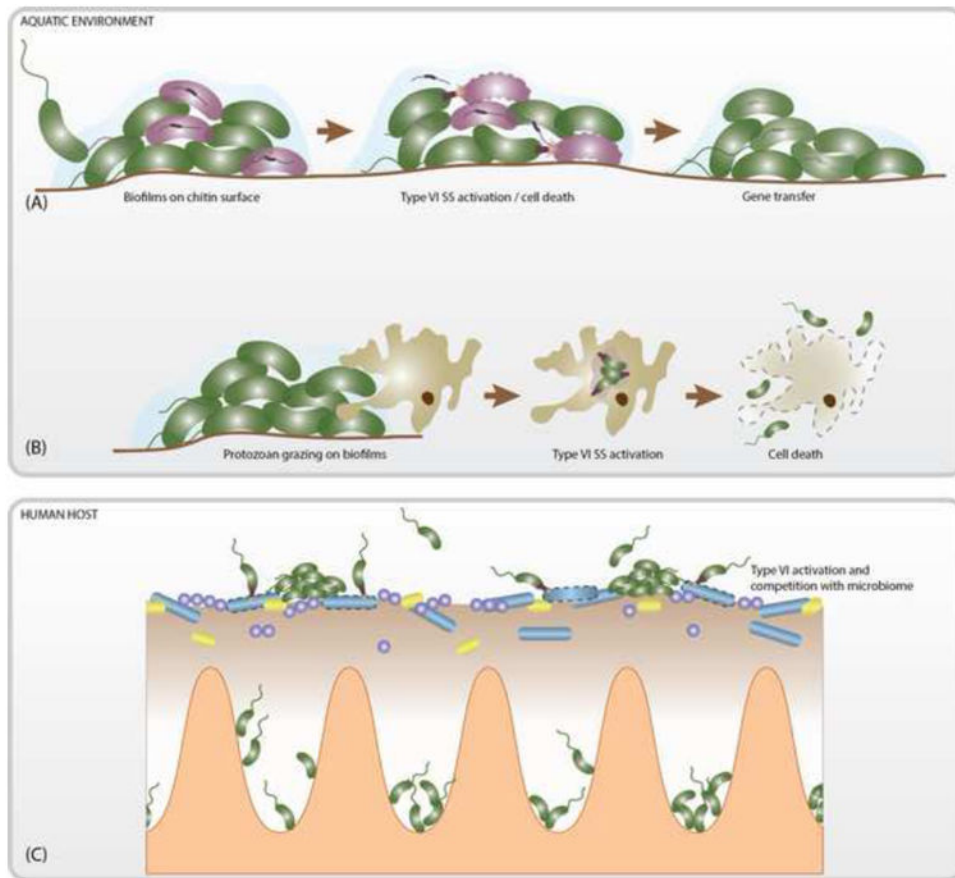
Figure 2. Proposed Functions of the T6SS in the Life Cycle of *V. cholerae*
(A) In the aquatic environment *V. cholerae* often forms biofilms on chitin surfaces. Chitin oligomers serve as a signal for the co-activation of natural competency and the T6SS, which allows for the killing of competing microbes and the acquisition of released DNA (represented as linear fragments inside of intact cells and outside of compromised cells). (B) *V. cholerae* can also utilize the T6SS to escape from predatory protozoans. (C) During infection the T6SS is believed facilitate *V. cholerae* colonization and virulence through the killing of host microbes. It may additionally target host macrophages or cause intestinal inflammation that can facilitate infection.
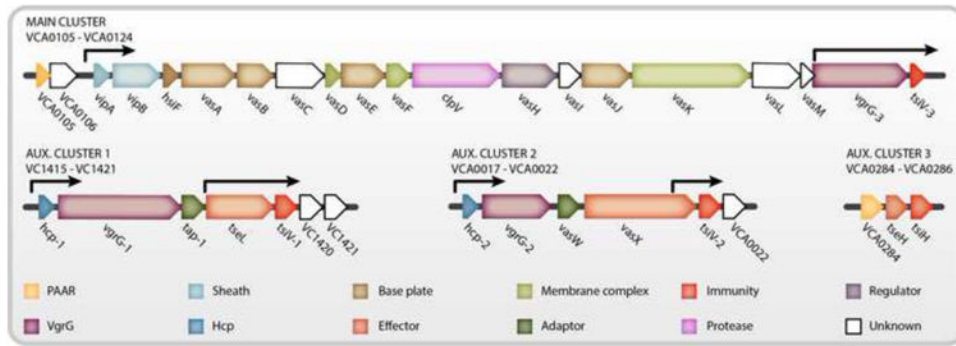
Figure 3. Genetic Loci of the *V. cholerae* T6SS

The genes of the T6SS are organized into one main cluster and at least 3 auxiliary clusters. Genes are color-coded based on predicted function and labeled with *V. cholerae* gene annotations. Black arrows denote known transcriptional start sites.
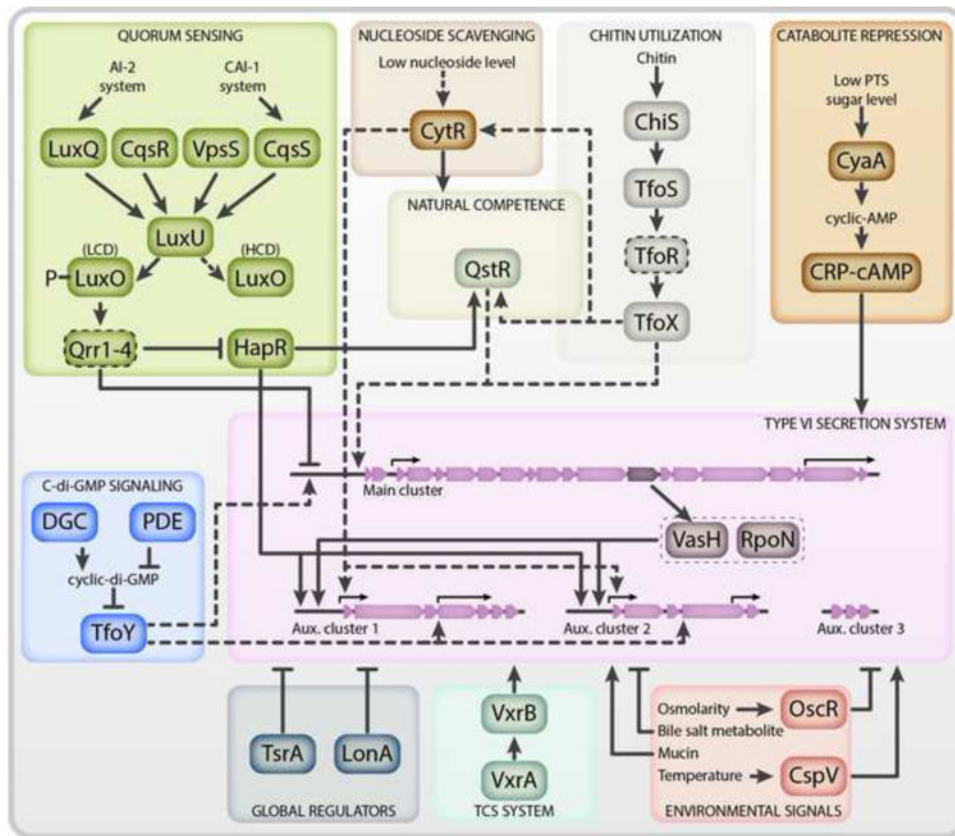
Figure 4. The Regulatory Network of the T6SS in *V. cholerae*
Activation is indicated by arrow-headed lines while inhibition is indicated by bar-headed lines. Lines that do not enter the Type VI Secretion System bubble represent regulation through an unknown mechanism. Regulators that directly bind to promoters are designated with solid lines while those that activate through as of yet unknown mechanisms have dashed lines. At low cell density, the quorum sensing small RNAs (sRNAs, denoted by a dashed outline) Qrr1-4 inhibit translation of *hapR* and the main T6SS cluster mRNAs. At high cell density, this inhibition is relieved. The chitin utilization cascade induces expression of TfoX, which acts in concert with HapR to activate QstR. TfoX and QstR facilitate transcription of the main cluster while HapR activates transcription of auxiliary clusters 1 and 2. When preferred carbohydrates are absent, CRP-cAMP accumulates and activates T6SS gene expression. At low nucleoside levels, CytR activates T6SS gene expression. Cyclic-di-GMP (c-di-GMP) is produced by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs). When c-di-GMP levels are low, TfoY is translated and activates expression of the main cluster and the effector and immunity pairs located within auxiliary clusters 1 and 2. Activation of auxiliary clusters 1 and 2 requires the VasH-RpoN complex. The VxrAB two component system (TCS) senses an unknown signal and activates T6SS gene expression. OscR represses the T6SS under conditions of low osmolarity. CspV is required for activation of the T6SS at temperatures between 25°C and 37°C. Mucins activate the T6SS while bile salt metabolites inhibit T6SS tube formation. The H-NS-like protein

TsrA and the LonA protease inhibit the T6SS. These regulatory cascades are described in detail within the main text.

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Table 1

T6SS Effector Proteins from Pandemic *V. cholerae* strains

Effector	Target Kingdom	Adaptor	Immunity	Effector activity	Effector Class	Reference
VgrG1	Eukaryotes	-	-	Actin Crosslinking	C-Terminal Extension	3
TseL	Prokaryotes	Tap-1	TsiV1	Putative Lipase	Cargo Effector	29,38
VasX	Prokaryotes Eukaryotes	VasW	TsiV3	Pore Forming	Cargo Effector	30,38
VgrG3	Prokaryotes	-	TsiV3	Peptidoglycan	C-Terminal Extension	19
TseH	Prokaryotes	-	TsiH	Peptidoglycan	Cargo Effector	36