A Novel *Vibrio cholerae* Type VI Secretion System Gene Cluster is Involved in Interbacterial Competition

Kenneth Williams

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First Reader: Dr. Brian Hammer

Second Reader: Dr. Thomas DiChristina

Abstract

The waterborne pathogen *Vibrio cholerae* inhabits microbiomes on chitinous crustaceans in marine environments and within the gastrointestinal tracts of animals, including humans, after ingestion of contaminated water. All sequenced isolates of *V. cholerae* contain genes encoding for a Type Six Secretion System (T6SS), a harpoon-like apparatus capable of delivering toxic effectors into neighboring cells and conferring a competitive advantage in dense microbial communities. The T6SS has been primarily studied in host-derived *V. cholerae* isolates, which have four T6 loci: a main cluster encoding structural and regulatory components, and four auxiliary (Aux1-3) clusters that each encode an effector toxin and cognate immunity protein to avoid kin or self-intoxication. We applied a predictive algorithm we developed to the genome sequences from a set of environmental isolates and identified in two isolates a novel Aux cluster which we named Aux5. Aux5 was predicted to encode a T6 effector with homology to T6-secreted lipase of *Pseudomonas aeruginosa*. Due to limited genetic tractability of the environmental isolate, the Aux5 cluster was horizontally transferred onto the chromosome of a host-derived, reference *V. cholerae* strain that is naturally transformable on chitin. The transformed strain killed the parental reference strain in a T6SS-dependent manner. Disruption of the Aux5 effector gene (Tse5) abolished the ability of the transformed strain to kill its parent. These results demonstrate 1) the *V. cholerae* T6 arsenal includes a novel Aux5 effector, and 2) non-native auxiliary clusters can be acquired by natural transformation and utilized for T6-mediated competition. We predict that horizontal exchange of active T6 loci allows rapid adaptation of bacteria in host and environmental microbiomes.

Introduction

Vibrio cholerae, known for causing the human diarrheal disease cholera, is a Gram-negative bacterium capable of living a dualistic lifestyle in natural marine environments or as a human pathogen following the ingestion of contaminated water. Competition is a driving force which allows this microbe and others to better survive and evolve to their ever-changing environments.[1,2] The ability to fight competitors, especially in conditions with limited resources, ensures the propagation of a species' genes through successive generations. A mechanism recently discovered to facilitate this competition in *V. cholerae* and other *Proteobacteria* is known as the Type Six Secretion System (T6SS).[3,4]

The T6SS apparatus can be envisioned as a molecular harpoon capable of delivering toxic effector proteins into neighboring bacterial and eukaryotic cells. The major structural components of the T6SS consist of a transmembrane complex that forms a pore in the killer cell.[5] The baseplate of the apparatus allows for the attachment of the tip of the nanomachine made up of a trimer of valine-glycine repeat (VgrG) proteins and a proline-alanine-alanine-arginine (PAAR) domain-containing protein to sharpen the tip.[6,7] From the tip complex, hemolysin-coregulated proteins (Hcp) polymerize to form the needle shaft that is surrounded by a sheath of ClpV-interacting proteins A and B (VipA and VipB).[8,9] The toxic effectors proteins are loaded onto the apparatus, which can be facilitated using T6 adaptor protein (Tap) chaperones in *V.*

cholerae. In a single, dynamic event, the sheath can contract to propel the loaded needle into a target cell and deliver the payload of effectors.[11] These effectors can then have a variety of killing mechanisms that include an actin crosslinking domain as a eukaryotic antagonist, hydrolases to degrade cell wall components, nucleases for DNA degradation, lipases to attack the phospholipid membrane, and pore-forming complexes to facilitate the disruption of membrane integrity.[12,13,14] Killing occurs if the target cell lacks the cognate immunity proteins for these effectors, which are also used to avoid self-intoxication or the killing of kin.[14] In previously studied *V. cholerae* genomes, the T6 genes are organized into 4 loci: the main cluster encoding for structural and regulatory components and three auxiliary clusters (Aux1-3).[3] The Aux clusters diversify the arsenal of the T6SS by encoding a variety of effector-immunity pairs in addition to structural components.[14] While these clusters have been well-characterized and studied in a few reference clinical isolates like C6706, a broad search to find and characterize additional Aux clusters in the genomes of various environmentally-acquired isolates has not been performed.

Considering life in its aquatic environment, *V. cholerae* can degrade chitin as a carbon source on various surfaces like zooplankton and crab shells using secreted chitinases.[27] In turn the $(GlcNAc)_n$ oligosaccharides that are released upregulate genes for natural transformation[26] and a T6SS.[16,25] It was shown that all sequenced strains of *V. cholerae* have a T6SS.[15] In addition, studies with a clinical reference strain (A1552) demonstrated that T6 mediated target cell lysis releases DNA and facilitates Horizontal Gene Transfer (HGT) through natural transformation.[16] HGT has been further theorized and modeled to increase diversity in effector-immunity genes in *Vibrios.*[14,17,18] Our lab confirmed this theory in *V. cholerae* by showing that when cocultured on a chitinous crab shell in artificial sea water, C6706 was able to kill an environmental isolate with a different effector-immunity pair and swap its own Aux cluster with that from its target.[19] Mathematical modeling also showed that a strain with inferior effectors can undergo higher rates of HGT to acquire effectors of its competitor that may be more potent.[19] However, this swapping requires the loss of the killer's original Aux cluster since this HGT event is achieved by homologous recombination.[19]

In this study, we developed a predictive algorithm and found a novel Aux cluster, which we called Aux5, in two recently sequenced environmental isolates.[20] Through the acquisition and expression of this cluster onto the chromosome of a clinical reference isolate using chitin-induced HGT, this study shows that Aux5 is an active, T6-dependent cluster and that an entire novel Aux clusters can be obtained through natural transformation. Supporting prior models that HGT increasing diversity,[14,17,18] HGT is therefore predicted to be a mechanism for rapid diversification of the T6SS arsenal through acquisition of novel Aux clusters in host and environmental microbiomes for *V. cholerae*.

Methods and Materials

Bacterial Strains and Culturing Conditions *V. cholerae* strains used were derivatives of El Tor C6706 *str-2*[20] or the environmental strain VC56[20]. Strains were grown with constant

shaking in lysogeny broth (LB) at 37°C, or statically on LB agar. Growth media was supplemented with the following where appropriate: kanamycin (50 g/ml), ampicillin (100 g/ml), streptomycin (5 mg/ml), spectinomycin (100 g/ml), and diaminopimelic acid (50 g/ml).

Recombinant DNA techniques and Deletion Mutants DNA manipulation was performed using standard molecular biology-based methods. Restriction nucleases (Promega and New England Biolabs), Gibson assembly mixes (New England Biolabs), and Phusion and OneTaq polymerases (New England Biolabs) were used according to manufacturer's protocols. Constructs were tested and verified using PCR and Sanger-sequencing (Eurofins). Allelic exchange was used as previously described to make an in-frame deletion of *vasK* using the suicide vector pKAS32 digested at EcoRI and XbaI with 500 bp flanking regions from the sequence upstream and downstream of *vasK*.[21]

Natural Transformation Assay of *V. cholerae* Chitin-induced natural transformation assays were performed on both C6706 and VC56 as previously described.[22] Briefly, cells were back diluted from an overnight culture at 37°C with shaking to an optical density at 600 nm (OD_{600}) of 0.3. Cells were washed with artificial sea water (ASW), diluted to an OD_{600} of 0.15, and statically grown in the presence of a sterilized crab shell fragment in 2 mL of artificial sea water (ASW) at 30°C. Each crab shell fragment with the attached bacteria was moved into 2 mL of fresh ASW, the extracellular DNA (eDNA) of interest with an antibiotic resistance cassette was added, and the cells were incubated statically for 24 hours at 30°C. The transformed strains were selected for on antibiotic plates and confirmed using PCR and Sanger-sequencing.

In VC56, natural transformation was used to introduce a selectable marker downstream of Aux5 cluster. The eDNA for this assay was a pUC18 vector digested at EcoRI and BamHI. The construct included a kanamycin resistance (Kan^r) cassette flanked by 500 basepairs corresponding to the two halves of the 1000 basepair region downstream of *tse5*. This location was chosen to avoid possible mutations in the cluster introduced by cloning. A C6706 mutant that constitutively expressed a positive regulator of the T6SS, qstR*, (T6+) was then transformed using the genomic DNA from the Kan^r VC56 to acquire the Aux5 cluster on its chromosome. This strain was named the "Killer Strain" and carries an additional T6 locus than its C6706 parent. To construct a Tse5 mutant of the Killer Strain, pUC18 was digested at EcoRI and BamHI to introduce an ampicillin resistance (Amp^r) cassette flanked by 857 basepairs on either side corresponding to the first and second half of *tse5*. This construct was used as eDNA in a natural transformation assay with the Killer Strain. Amp^r transformants were confirmed by PCR and Sanger sequencing to create an insertional inactivation of *tse5*.

Killing Assay T6SS killing assays were performed as previously described.[23] Briefly, the killer and target strains were grown in LB at 37°C in overnight culture, centrifuged, and resuspended in fresh LB to an OD_{600} of 1. The strains were mixed in 10:1 ratio of killer to target, and a 50 µL suspension was plated on a 3 by 3 square grid (3.1mm by 3.1mm) of a 0.2 µm disk filter paper placed on a solid agar LB plate. The filters were incubated statically for 3 hours at 37°C before they were vortexed in 5 mL of liquid LB broth and the dislodged cells were serial diluted and plated on an antibiotic selective agar LB plate to assess target survival through colony forming units (CFUs). The target strain in this study was a C6706 mutant with a

spectinomycin-resistance cassette integrated on the chromosome at *lacZ*. Killing was determined to be a reduction in the target's survival by greater than one log.[24] Reduction less than one log was considered non-T6-mediated competition between strains.

Statistical Analysis All statistics were calculated using Minitab 18. The data underwent a $\log_{10}$ transformation. ANOVA was then used to assess the presence of a significant difference in the data. A Tukey Simultaneous Test for Difference of Means was used for post-hoc analysis with an alpha value of 0.05 to determine which pairwise comparisons were statistically different.

Results

Aux5 is a novel gene cluster found is some *V. cholerae* isolates

While many studies have verified the presence and activity of 3 Aux clusters in various clinical strains, little work has been published regarding the Aux clusters of environmental isolates. To search our library of environmental isolate genomes for novel Aux clusters, a Hidden Markov Model was created and tested by lab members Aroon Chande and Samit Watve to look for *hcp*, *vgrG*, and *tap* in sequence. These three are found together in the two known Aux clusters of C6706 (Figure 1).[20] From their work and further sequencing and analysis done by myself, Cristian Crisan, and Vishnu Raghuram, the organization of the Aux5 cluster in VC56 was elucidated (Figure 1). This cluster differs from those of C6706 in that the immunity protein (Tsi5), which has been shown to inactivate the effector when both are co-expressed in E. coli by lab member Cristian Crisan, is found upstream of the effector (Tse5) (Figure 1). Also, there is what appears to be another putative immunity gene followed by a putative, truncated effector upstream of *tsi5*. These genes do have various single nucleotide polymorphisms (SNPs) in comparison to *tsi5* and *tse5*. Similar to C6706's Aux cluster, there is a putative promotor upstream to drive *tsi5* in the *tap* in addition to the putative promotor before the cluster. From studying other clusters, this is additional promotor is thought to ensure the immunity protein is being expressed to protect from Tse5 attacks by neighboring cells without the need for expressing the entire cluster.

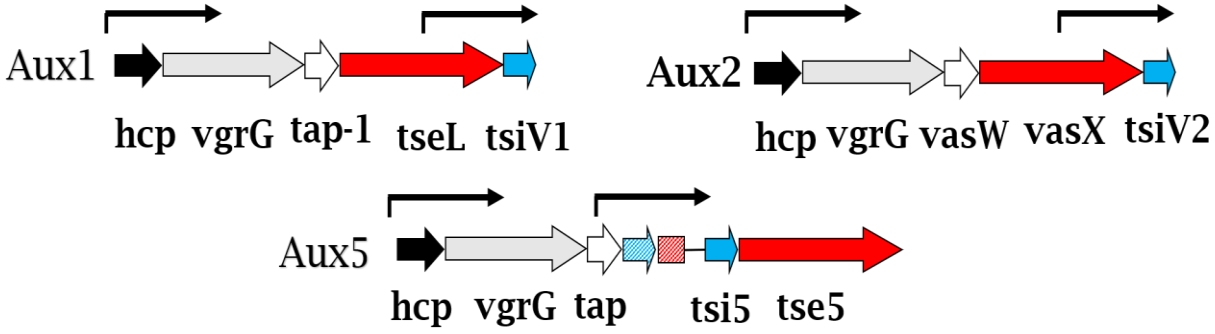

Figure 1. *Organization of Aux5 locus in VC56 is different in comparison to Aux1 and 2 in C6706. The cluster was found using a Hidden Markov Model and the organization of the genes was further studied using PCR, Sanger Sequencing, and Whole Genome Sequencing (PacBio). The first three genes in each cluster are functionally the same and code for the tubule subunits, the tip, and the effector loading chaperone respectively. The effector of the cluster is shown in red, and the cognate immunity is shown in blue. However, Aux5 encodes a putative immunity*

(striped blue) and putative truncated effector (striped red) in addition to its immunity protein gene (tsi5) which appears before the effector gene (tse5).

HGT can be used by *V. Cholerae* to acquire functional Aux Clusters

From previous experiments, VC56 was shown to have limited genetic tractability. Most significantly, we were unable to have VC56 successfully take in plasmids through conjugation. Consequently, further study of this cluster benefitted from its transfer and expression in C6706 T6+ by natural transformation. A selectable marker (Kan^r) downstream of the Aux5 cluster in VC56 allowed for the screening of C6706 cells which had acquired the Aux5 cluster from the eDNA of Kan^r VC56. The acquisition of this cluster occurred with the induction of chitin, a polymer whose monomers act as a signal molecule to upregulate the T6SS and competence in V. cholerae.[23] This strain now carried an additional Aux cluster which was not found in the parental C6707 strain while maintaining its Aux1 and 2 clusters (Figure 2).

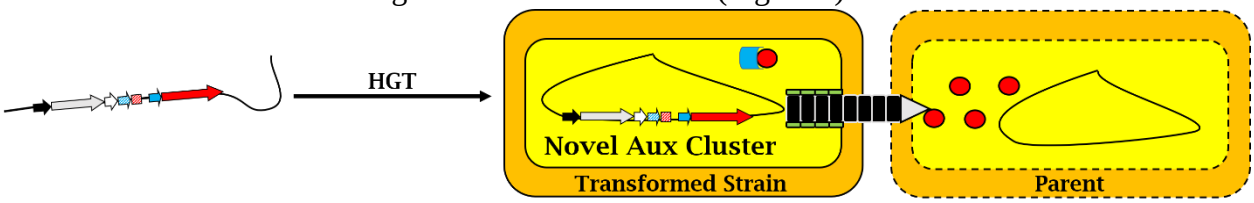

Figure 2. *Novel Aux clusters can be obtained by HGT. This study used a Kan^r marked Aux5 cluster in VC56 as eDNA in a natural transformation assay with C6707 T6+. The movement of this cluster onto the chromosome of C6707 allowed for genetic investigation of the cluster which was not as readily possible in VC56. After acquisition, an active, T6-dependent, novel effector (red circle) can be loaded and used by the transformed strain to kill its parental strain lacking this cluster. The transformed strain also receives immunity (blue cap) from the acquired effector to protect against self-intoxication and future attacks.*

Aux5 is an active, T6-dependent gene cluster

To assess the efficacy of Aux5, killing assays were performed using the Killer Strain and its various mutants against the WT parental C6706 (Figure 3). When pitted against the Killer Strain, the parent strain's survival dropped by about 4 logs compared to competition with C6706 T6+ without the Aux5 cluster (p=0.000, ANOVA and Tukey) (Figure 3). Deleting a structural gene which left the Killer Strain unable to fire its T6SS removed its ability to kill the parental strain (p=0.774, ANOVA and Tukey) (Figure 3). Furthermore, a disruption of *tse5* in the Killer Strain also removes its ability to kill the parental strain (p=0.835, ANOVA and Tukey) (Figure 3).

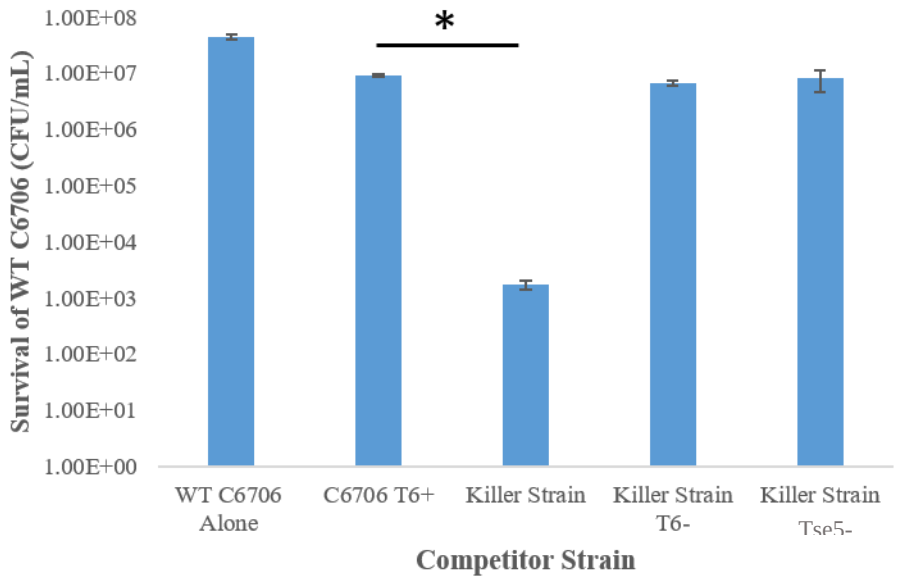

Figure 3. *Aux5 allows the Killer Strain to kill parental C6707 in a T6-dependent manner. Killing assays assessed the ability of the Killer Strain, as well as mutants defective in firing the T6SS and Tse5, to kill its parent strain. The bars denote mean parental survival ± standard error of the mean with n = 3 biological replicates. The Killer Strain showed a significantly greater killing ability compared to the background C6706 T6+ strain (p-value < 0.05, ANOVA and Tukey). Conversely, T6- and Tse- Killer strain mutants lost their ability to kill the parental strain (p-value > 0.05, ANOVA and Tukey).*

Discussion

From this study, Aux5 was shown to require an active T6SS to deliver its toxic Tse5 effector in *V. cholerae.* This adds another verified Aux cluster to the arsenal of some *V. cholerae*'s T6SS. The mechanism by which this effector kills is still not well understood. Homology based on folding predictions was found to Tle1, a lipase used in the T6SS of *P. aeruginosa*. However, amino acid substitutions of the putative catalytic triad to alanine by lab member Cristian Crisan did not appear to affect the potency of the effector. Also, within the cluster, the function of the putative immunity and putative truncated effector is still unknown and should be further tested to see if the putative immunity can also protect the cell from intoxication by Tse5.

Through whole genome sequencing (PacBio), 4 copies of the Aux5 cluster with the same organization were found on the genome of VC56. Further testing is required to determine if all copies are functional and delivered in a T6-dependent manner. Since there are various SNPs found throughout the effector and immunity genes, studies can be performed to determine if there is specificity in the protection of immunity proteins to the various effectors, assuming they are all active.

In addition, novel Aux clusters can be acquired through HGT. In the environment, this phenomenon can be envisioned to take place on chitinous surfaces in the environment or in a host where conditions may induce competence. As strains of *V. cholerae* battle with their T6SS

and potentially cause target cell lysis or otherwise encounter eDNA, novel Aux clusters can be incorporated to diversify its T6 arsenal (Figure 2). Moreover, the transformed strain has also acquired immunity to another effector which will increase its fitness in its environment (Figure 2). In general, the work has shown that HGT gene transfer provides an avenue for *V. cholerae* to quickly expand its T6 arsenal with novel Aux clusters, like Aux5, to rapidly adapt in its environment.

Given the diversity of environmental strains as previously shown by this lab in comparison to the extensively studied clinical isolates, further work should be done to better understand the effect of *V. cholerae*'s dualistic lifestyle on the genome of various isolates and the ecological implications of this diversity.[24]

References

1. Paterson S., et al. (2010) Antagonistic coevolution accelerates molecular evolution. *Nature.* **464**:275-287
2. Pekkonen M., et al. (2013) Resource Availability and Competition Shape the Evolution of Survival and Growth Ability in a Bacterial Community. *PLoS ONE* 8(9):e76471
3. Pukatzki S., et al. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proc Natl Acad Sci USA* **103**:1528–1533.
4. Mougous J.D., et al. (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526–1530.
5. Durand E., et al. (2015) Biogenesis and structure of a type VI secretion membrane core complex. *Nature* **523:**555–560.
6. Leiman P. G., et al. (2009) Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc. Natl. Acad. Sci. U. S. A.* **106**:4154–4159.
7. Shneider M.M., et al. (2013) PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* **500**:350–353.
8. Kudryashev M., et al. (2015) Structure of the type VI secretion system contractile sheath. *Cell* **160**:952–962.
9. Zoued A., et al. (2016) Priming and polymerization of a bacterial contractile tail structure. *Nature* **531**:59–63
10. Unterweger D., et al (2017) Adaptor proteins of type VI secretion system effectors. *Trends Microbiol* **25**:8–10
11. Basler M., et al. (2012) Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature* **483**:182–186.
12. Russell A. B., et al. (2014) Type VI secretion system effectors: poisons with a purpose. *Nat. Rev. Microbiol.* **12,** 137–48.
13. Hachani A., et al. (2016). Type VI secretion and anti-host effectors. *Curr. Opin. Microbiol.* **29,** 81–93
14. Unterweger D., et al. (2014) The Vibrio cholerae type VI secretion system employs diverse effector modules for intraspecific competition. *Nature communications.* **5**:3549
15. Boyer F., Fichant G., Berthod J., Vandenbrouck Y., Attree I. (2009) Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics* **10**:104.

16. Borgeaud S., Metzger L.C., Scrignari T., Blokesch M. (2015) The type VI secretion system of Vibrio cholerae fosters horizontal gene transfer. *Science* **347**:63–67.
17. Kirchberger P.C., et al. (2017) Sequential displacement of type VI secretion system effector genes leads to evolution of diverse immunity gene arrays in *Vibrio cholerae*. *Sci Rep* **7**:45133.
18. Salomon D., et al. (2015) Type VI Secretion System Toxins Horizontally Shared between Marine Bacteria. *PLoS Pathog.* **11:**1–20.
19. Thomas J., et al, (2017) Horizontal Gene Transfer of Functional Type VI Killing Genes by Natural Transformation. *mBIO* **8**(4):e00654-17.
20. Watve S.S., et. al (2016) Whole-genome sequences of 26 *Vibrio cholerae* isolates. *Genome Announc*, **4**(6):e01396-16.
21. Skorupski K., Taylor R.K. (1996) Positive selection vectors for allelic exchange. *Gene.* **169**:47–52.
22. Watve S. S., Bernardy E. .E, Hammer B. K. (2014) Vibrio cholerae: measuring natural transformation frequency. *Curr Protoc Microbiol* **35**:6A.4.1– 6A.412.
23. Watve, S. S., Thomas, J. & Hammer, B. K. (2015). CytR Is a Global Positive Regulator of Competence, Type VI Secretion, and Chitinases in Vibrio cholerae. *PLoS One* **10,** 1–18
24. Bernardy E. E., Turnsek M. A., Wilson S. K., Tarr C. L., Hammer B.K. (2016) Diversity of Clinical and Environmental Isolates of Vibrio cholerae in Natural Transformation and Contact-Dependent Bacterial Killing Indicative of Type VI Secretion System Activity. *Appl. Environ. Microbiol* **82**(9):2833-2842.