Insights into the Contribution of Type VI Secretion Towards AHPND Pathogenesis

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Abstract

The Type VI Secretion System (T6SS) is a bacterial organelle resembling a poison-tipped spear that can be deployed by a wide range of gram-negative bacteria to intoxicate prokaryotic and eukaryotic prey. In this capacity, Type VI Secretion Systems (T6SSs) play various roles in facilitating interbacterial competition and augmenting virulence towards animal and plant hosts. Prior reports have elucidated that many strains of *Vibrio parahaemolyticus* that cause Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp harbor two functional T6SSs (T6SS1 and T6SS2), leading to speculations about the contribution of these systems to disease progression. In the present study, we demonstrate the antibacterial functionality of T6SS1 and T6SS2 in a representative VP_{AHPND} strain, and provide direct evidence that T6SS1 augments the lethality of this isolate against whiteleg shrimp (*Litopenaeus vannamei*) postlarvae under conditions relevant to commercial aquaculture. Our findings provide early evidence that Type VI Secretion (T6S) plays a role in facilitating AHPND pathogenesis, and invites additional lines of inquiry aimed at uncovering the mechanisms responsible for this contribution.

Author Summary

Type VI Secretion Systems (T6SSs) have drawn interest as important determinants of virulence and fitness in the bacterial taxa that utilize them. Included among these species are members of the genus *Vibrio*, many of which have clinical and commercial relevance as pathogens or aquaculture pests. *Vibrio parahaemolyticus* isolates that harbor the *pirA/B* toxin genes encoded on pVA1-like plasmids have been identified as a causative agent of Acute Hepatopancreatic Necrosis Disease (AHPND), an illness which results in mass mortality events in shrimp aquaculture systems. It has previously been noted that, aside from the PirA/B binary toxin, many VP_{AHPND} strains harbor two T6SSs (T6SS1 and T6SS2), which they may rely on to outcompete and displace the native shrimp microbiome during colonization. In this study, we have directly assessed the effects of inactivating T6SS1 and T6SS2 on the lethality of a representative VP_{AHPND} isolate against *L. vannamei* postlarvae (PL) using a simple infection assay. Our findings indicate that T6SS1 putatively contributes to the pathogenicity of VP_{AHPND} against *L. vannamei* PL under warm, marine-like conditions. These initial results help to provide insight into the factors which enable AHPND pathogenesis, and may inform future efforts to develop countermeasures against this disease.

Introduction

Shrimp aquaculture constitutes one of the largest and fastest growing sectors of food production throughout the world and serves as one of the major sources for high-protein seafood in South America, China, Thailand, Malaysia, and Vietnam. Given the scale of the shrimp aquaculture industry, production losses caused by infectious diseases such as Acute Hepatopancreatic Necrosis Disease (AHPND; formerly known as Early Mortality Syndrome or EMS) can prove economically devastating [1, 2]. AHPND is responsible for mass mortality events in shrimp aquaculture systems and is caused primarily by strains of *Vibrio parahaemolyticus* which harbor a ~69kb plasmid that encodes the PirA/B binary toxins responsible for atrophy of the hepatopancreas in afflicted shrimp [1, 3]. Cumulative financial losses attributable to AHPND from the years 2010 to 2016 have been estimated at over $44 billion USD, and this figure likely constitutes a fraction of global losses that have occurred until the present [2]. AHPND has proven especially devastating to shrimp farmers throughout Mexico, the Philippines, and Southeast Asia. Indeed, the impacts of this disease also prove highly deleterious to the socio-economic standing of communities which lean heavily upon shrimp aquaculture for income and food production [2].

The economic impact of AHPND-associated *V. parahaemolyticus* (VP_{AHPND}) has prompted investigations into the mechanisms responsible for AHPND pathogenesis. While the *pirA/B* genes encoded on pVA1-like plasmids have

been cited as the main virulence genes responsible for necrosis of the shrimp hepatopancreas, it is also speculated that other genomic elements are necessary for survival, colonization, and pathogenesis within the shrimp host [4, 5]. Notably, the Type VI Secretion System (T6SS) has been identified as a potential virulence factor which may enable AHPND pathogenesis (5-7).

The Type VI Secretion System (T6SS) is a spear-like, membrane-anchored contractile injection system that shares evolutionary history with phage tail-associated protein complexes [8-10]. The T6SS apparatus consists of a central tube of stacking Hemolysin-coregulated protein (Hcp) monomers which terminate in a Valine-glycine repeat protein G (VgrG) spike. The VgrG spike protein, in turn, is adorned by a Proline-Alanine-Alanine-aRginine (PAAR) domain-containing protein which can serve to sharpen the tip of the "spear" and more effectively puncture into prey cells. The central tube of the T6SS is encompassed by an outer sheath which is anchored to the cell envelope by a baseplate complex [10]. Effector toxins can be loaded onto the T6SS apparatus in a variety of ways, and are subsequently delivered into prey cells by an injection mechanism facilitated by contraction of the tail tube/sheath complex (**Fig 1**) [9]. Assembly, firing, and disassembly of the T6SS organelle is an elaborate and energetically expensive process which is often governed by complex regulatory networks [11, 12].

A diverse arsenal of T6SS-associated effector toxins has been described to date [13-15]. The majority of effectors identified thus far are thought to be either specifically antibacterial or anti-eukaryotic, while others exhibit mechanisms that allow them to function as trans-kingdom effectors. Some antibacterial effectors, such as those harboring a DNase or RNase domain, exert toxicity through nuclease activity, while others disrupt the prokaryotic cell envelope through pore formation, lipase activity, or damage to the bacterial cell wall [16, 17]. Effectors that intoxicate through unique and sophisticated mechanisms have also been discovered. The *Pseudomonas aeruginosa* effector Tas1, for example, was shown to cause metabolic dysregulation and depletion of ATP in prey cells by rapidly synthesizing (p)ppApp [18].

Notably, antibacterial effector genes are almost always encoded next to a cognate immunity gene, which expresses an "antidote" protein that countervails effector toxicity by binding to and neutralizing the effector. The purpose of these immunity proteins is to prevent self-intoxication and limit kin-killing by sibling cells [9, 13].

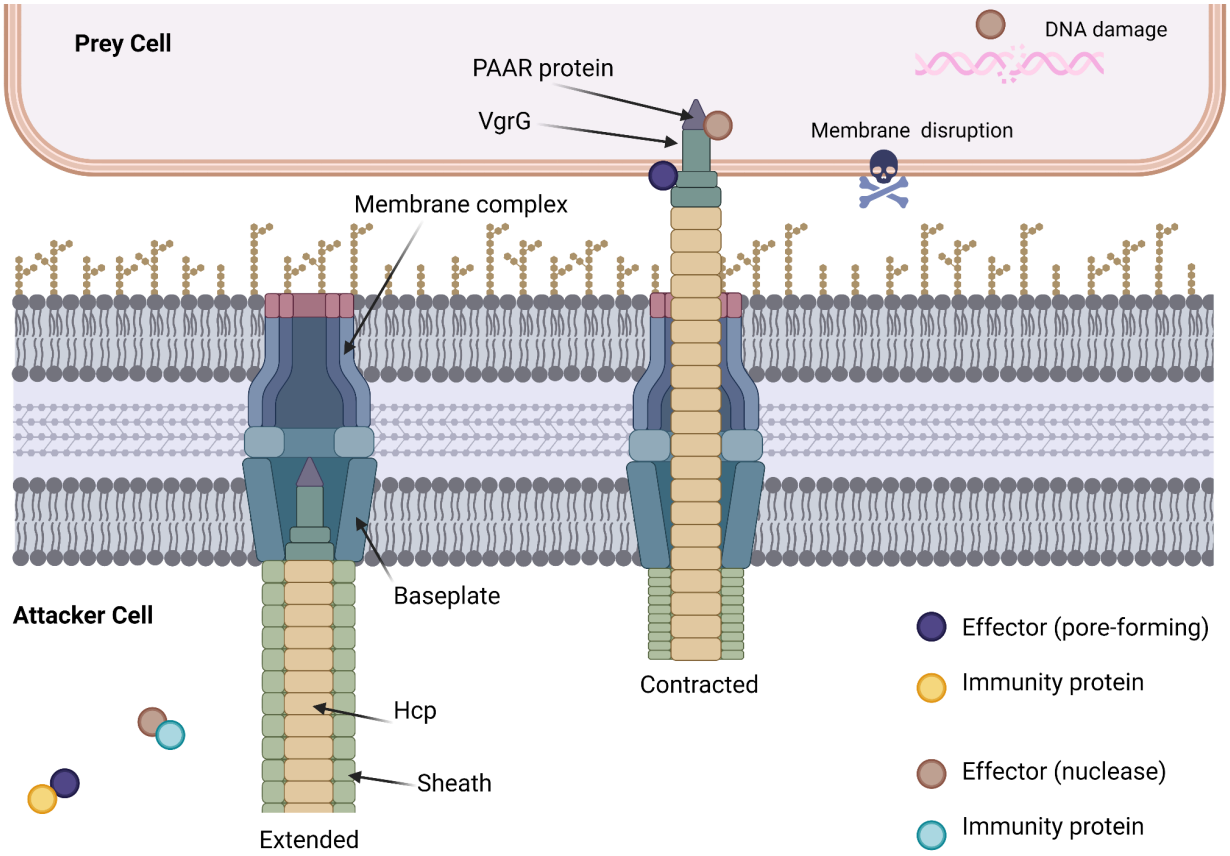

Figure 1. Structure and mechanism of the Type VI Secretion System (T6SS). The T6SS apparatus consists of a spiked tube which is surrounded by an outer sheath and anchored to the cell envelope of the possessing gram negative bacterium by the baseplate and membrane complex. Contraction (firing) of the device allows for delivery of multiple effectors with distinct mechanisms of action into an adjacent cell. Made in BioRender.

Type VI Secretion (T6S) has been implicated as a factor in the competitive fitness and virulence of a wide range of bacterial taxa, including many which belong to clinically significant genera such as *Yersinia*, *Pseudomonas*, and *Vibrio* [15, 19-22]. Notably, many species that utilize T6S harbor more than one functional T6SS, each of which may be regulated independently and employed in different scenarios [23]. Further, species possessing more than one T6SS may deliver a different payload of protein effectors with each [15, 23]. It has been demonstrated that some T6SSs are used primarily to deliver antibacterial effectors to antagonize non-kin bacterial competitors, while others facilitate virulence against eukaryotic targets such as host macrophages and other immune cells [9, 19].

It has previously been noted that, in addition to the canonical T6SS2 found in nearly all strains of *V. parahaemolyticus*, many AHPND-associated isolates also harbor a conserved T6SS1, leading to conjectures about the role of these systems, and particularly T6SS1, in AHPND pathogenesis [5, 6]. Prior work has demonstrated the antibacterial functionalities of T6SS1 and T6SS2 in *V. parahaemolyticus*, and additional investigations have elucidated the regulatory networks to which these systems are subject [6, 23-26].

Within this framework, it has been speculated that one or both of these T6SSs confer a fitness advantage that allows VP_{AHPND} to overcome and displace the native shrimp microbiota, thereby facilitating colonization of the host [5, 6]. In addition to enabling interbacterial antagonism, T6SS1 and T6SS2 may also play a role in adhesion to host cells by *V. parahaemolyticus* [27, 28]. Notably, it has been reported that inactivation of T6SS1 or T6SS2 via deletion of the corresponding *icmF* or *hcp* gene results in reduced adherence to cultured cell monolayers [27]. In

this study, we directly assess the extent to which T6SS1 and T6SS2 contribute to mortality in whiteleg shrimp (*Litopenaeus vannamei*) as caused by a representative VP_{AHPND} strain.

Results

T6SS1 and T6SS2 mediate antibacterial activity in *V. parahaemolyticus* 13-306/D4

To begin our investigation, we identified *V. parahaemolyticus* 13-306/D4 (also listed as 13-306D/4, and abbreviated D4) as a suitable VP_{AHPND} strain for future experiments. D4 was originally isolated from the hepatopancreas of a Mexican shrimp specimen afflicted with AHPND, and has been shown to cause high levels of mortality in commercially-relevant species such as *L. vannamei* [29]. Additionally, D4 harbors a functional T6SS1 and T6SS2, both of which are expressed under appropriate conditions [6]. Notably, early reports of in-vitro experiments suggest that T6SS1 is most active under warm, marine-like conditions (i.e. 30°C, 3% [wt/vol] NaCl), while T6SS2 is primarily expressed at lower temperatures and salinities (i.e. 23°C, 1% [wt/vol] NaCl) [6]. However, it has subsequently been shown that some strains of *V. parahaemolyticus* also exhibit T6SS2-mediated antibacterial activity under the warm, marine-like conditions that are conducive to T6SS1 [25, 30].

We first sought to evaluate how inactivation of T6SS1, T6SS2, or both systems would influence interbacterial killing by D4. To this end, we employed allelic exchange mutagenesis to delete the genes encoding Hcp1 and Hcp2 in D4, thus rendering one or both systems inoperable. The resulting D4 Δ*hcp1*, Δ*hcp2*, and Δ*hcp1/2* mutants were subsequently used as attacker strains in a T6SS competition assay in order to compare their killing efficiency against that of the parental D4 strain (**Fig 2, Dataset S1**). Growth experiments were additionally performed with PCR-confirmed mutants to ensure that the above mutations did not incur a growth defect (**Fig S1 and Fig S2, Dataset S2**).

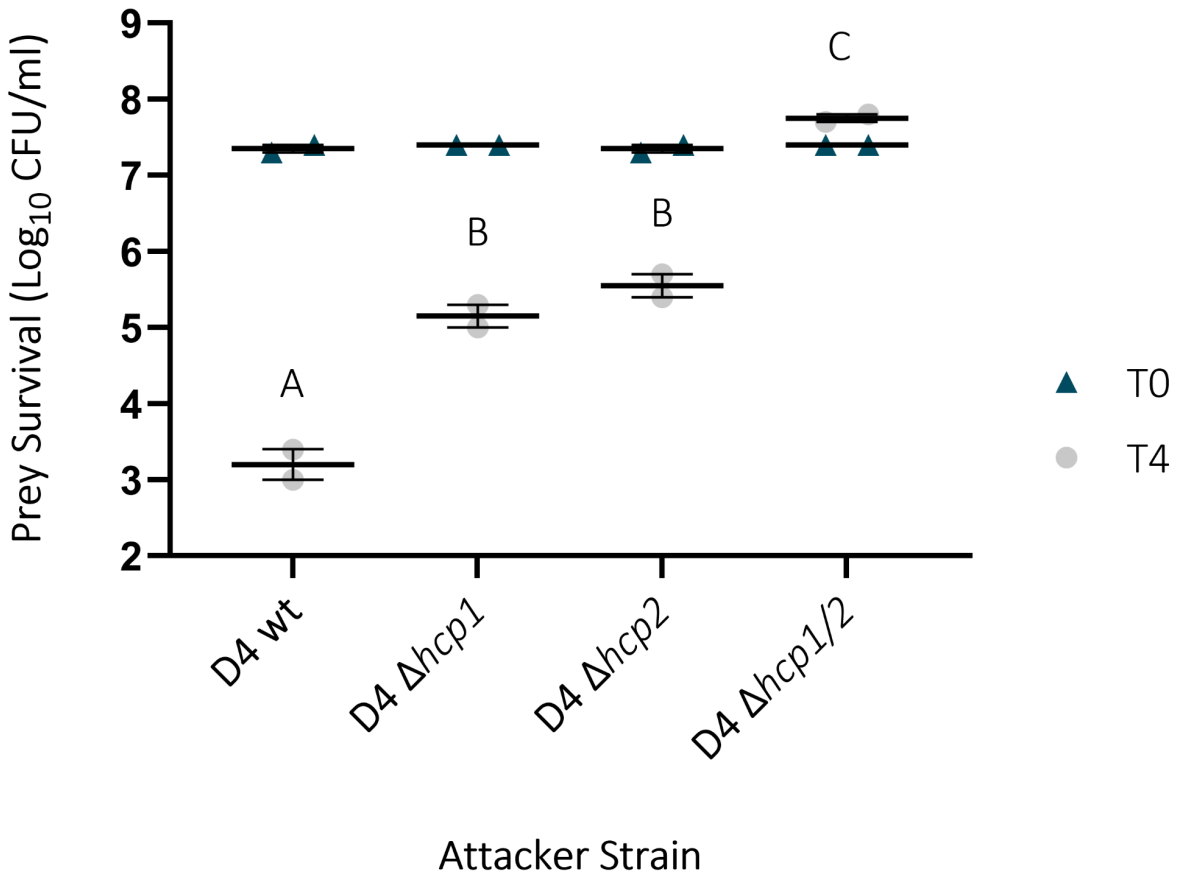

Figure 2. Antibacterial killing by *V. parahaemolyticus* 13-306/D4. Viability of *E. coli* DH5α harboring a pBAD33.1 plasmid (for selection on chloramphenicol) before (T0, triangles) and after (T4, circles) four-hour co-incubation with the indicated attacker strain on Marine Luria Bertani (MLB) at 30°C is shown. Data represent two independent experiments with three technical replicates each. Bars show mean ± SEM. T0 values are not statistically different (P>0.05). T4 values with different letters are significantly different from each other (P<0.0001). Data were analyzed using two-way ANOVA with Šídák's test for multiple comparisons.

Wild type D4 demonstrated efficient killing of *E. coli* prey under warm, marine-like conditions on a solid surface (i.e. agar media containing 3% [wt/vol] NaCl, with a 4-hour incubation at 30°C). Deletion of either *hcp1* or *hcp2* alone attenuated but did not abolish antibacterial activity, whereas deletion of both genes resulted in the inability to reduce prey CFU over the course of the assay. Remarkably, the individual contributions of T6SS1 and T6SS2 to antibacterial activity were similar, indicating that D4 likely utilizes both systems for competition in warm, marine-like environments.

Survey of effectors in *V. parahaemolyticus* 13-306/D4

In order to gain greater insight into the functionality of T6SS1 and T6SS2 in D4, we performed a survey of the effector landscape via genomic analysis. Homologs of several previously described effectors were identified, as summarized in **Table 1** (see **Dataset S3**).

Table 1. Predicted effectors identified in the *Vibrio parahaemolyticus* 13-306/D4 genome.

Predicted Effectors Identified in *Vibrio parahaemolyticus* 13-306/D4						
Associated T6SS	Predicted Activity or Domain	Literature homolog	Lit. Protein	D4 Protein	D4 Locus	Source
T6SS1	VP1390-like effector	VP1390	BAC59653.1	WP_085609853.1	B5C31_RS17700	31
	VP1388-like co-effector	VP1388	BAC59651.1	WP_024699427.1	B5C31_RS17690	31
	PAAR-like domain	VP1415	BAC59678.1	WP_139834351.1	B5C31_RS02640	32
	AHH nuclease domain	N/A	N/A	WP_025630797.1	B5C31_RS02635	6
T6SS2	Rhs repeats + PoNe*	T2Rhs-Nuc^{BB22OP}	WP_015296737.1	WP_085609714.1*	B5C31_RS12845	25
					B5C31_RS00500	
					B5C31_RS01355	
	Unknown	T2Unkwn^{BB22OP}	WP_015313171.1	WP_025550820.1	B5C31_RS25610	25
	Lipase	T2LipB^{BB22OP}	WP_015296300.1	WP_020840162.1	B5C31_RS22030	25
	α/β hydrolase	T2Hydro^{RIMD}	BAC61690.1#	WP_021451965.1#	B5C31_RS12315	25

*The gene encoding the T2Rhs-Nuc homolog found in D4 appears to be broken up onto multiple contigs, with the largest fragment encoding WP_085609714.1. Manual alignments putatively indicate that D4 encodes an identical protein to WP_191786262.1.

#The BAC61690.1 and WP_021451965.1 protein accessions contain a misannotated translation start site, as described previously [25].

Preliminary genomic analysis of D4 revealed putative effectors associated with T6SS1. Notably, a tricistronic operon which encodes homologous proteins corresponding to the VP1388-1390 effector module was identified at the end of the T6SS1 core gene cluster [31]. D4 additionally harbors two adjacent genes which encode a PAAR-like domain protein (homologous to the N-term of VP1415) and a nuclease domain protein, as previously described [6, 32].

Several T6SS2 effectors were also identified. D4 putatively encodes a T2Rhs-Nuc protein with a Polymorphic Nuclease (PoNe) -superfamily C-term domain [25]. The CDS for this protein is separated onto multiple contigs of the currently published assembly. Blast alignments and manual sequence inspection indicate that D4 likely encodes an identical protein to WP_191786262.1, although an updated assembly is required for confirmation. D4 also possesses homologs of the T2Unkwn, and T2Hydro effectors, as well as T2LipB, which serves as a T6SS2 substrate but remains unconfirmed as an effector [25].

T6SS1 contributes to lethality of *V. parahaemolyticus* 13-306/D4 against *L. vannamei* postlarvae under warm, marine-like conditions

We next sought to assess the indispensability of T6SS1 and T6SS2 within the context of shrimp infection by our representative VP_{AHPND} strain. To this end, we employed a small-scale challenge assay using the commercial shrimp species *L. vannamei*. Briefly, *L. vannamei* PL sourced from Miami Aquaculture were maintained in a filtered and aerated aquarium tank containing Artificial Seawater (ASW). Postlarvae (PL) between the PL20 and PL30 stage were allocated into plastic containers filled with ASW and maintained at a temperature of 30°C, with aeration provided by airstones. Assay containers were inoculated with a phosphate buffered saline (PBS) control or the

indicated D4 strain (to a final concentration of approximately 1E6 CFU/ml), and mortality of PL was determined at 12-hour intervals over the span of 2 days (**Fig 3 and Dataset S4**).

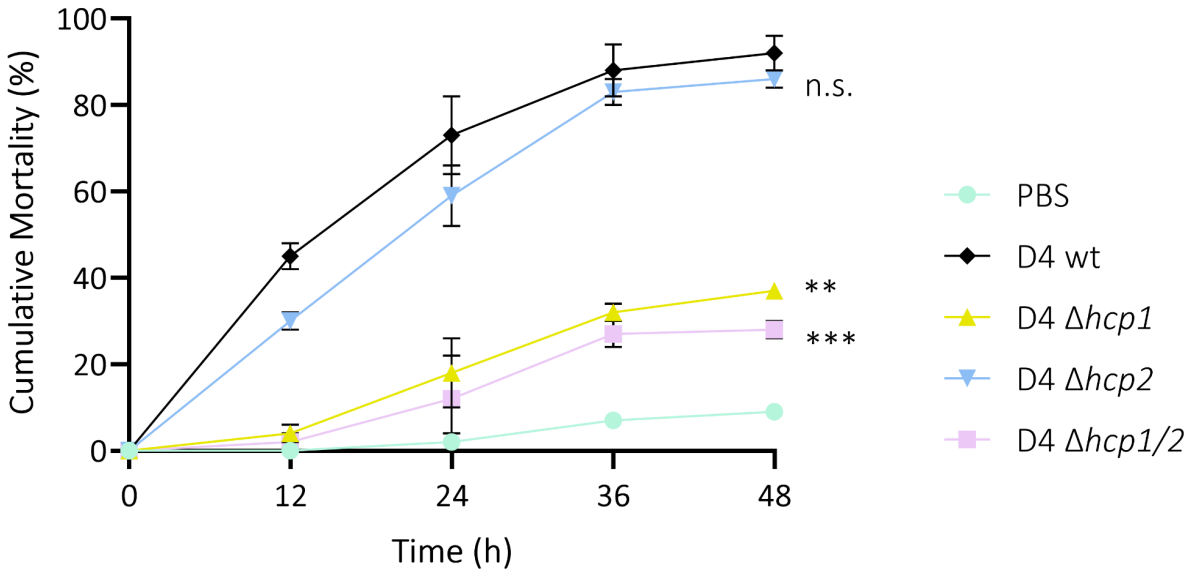

Figure 3. Cumulative mortality of *L. vannamei* PL following inoculation with the indicated D4 strain (for a final concentration of 1E6 CFU/ml) or a PBS control. Data represent two independent experiments with two technical replicates each. (n.s. = not significant, ** = p<0.01, *** = p<0.001). Error bars show SEM.

Exposure of *L. vannamei* PL to the wild type strain of D4 resulted in mortality approaching 100% after 48 hours, consistent with previous reports for this isolate. Remarkably, both D4 Δ*hcp1* and D4 Δ*hcp1/2* were considerably attenuated in their ability to induce mortality in *L. vannamei* when compared to the wild type (p<0.01 and p<0.001, respectively), while D4 Δ*hcp2* caused mortality at a level similar (n.s.) to that of the wild type strain.

The majority of dead or moribund PL in the non-control assay containers exhibited morphological and behavioral characteristics consistent with past reports of AHPND pathology, namely discoloration and atrophy of the hepatopancreas. Individuals that were putatively infected but still alive displayed lethargy punctuated by weak, “corkscrew” swimming. We note that deceased PL were in some cases scavenged by their living counterparts, and any missing PL were therefore presumed and defined as dead.

Discussion

Results of the *L. vannamei* challenge experiments indicate that the inability to utilize T6SS1 dramatically hampers the ability of D4 to cause mortality in PL under warm, high-salinity conditions. However, the extent to which T6SS2 facilitates virulence under these same conditions remains unclear. The finding that T6SS1 is putatively more essential to pathogenesis within *L. vannamei* PL lies in partial contrast to our observation that the T6SS1 and T6SS2 of D4 contribute similarly to in-vitro antibacterial activity against *E. coli*. We therefore speculate that the greater importance of T6SS1 during shrimp infection as seen in this study may result from the assay conditions we employed, or from differences in the susceptibility of native microbiota to the effector payload delivered by T6SS1 as opposed to T6SS2.

Notably, previous reports suggest that expression and secretion of T6SS2 by D4 is diminished under warm, marine-like conditions, as compared with lower temperature and salinity conditions. This suggests that additional stimuli applicable to the bacterial competition assay, such as cell-to-cell contact, may upregulate T6SS2 activation. It has also been observed that TfoX production, as occurs in the presence of chitin, induces T6SS2 activation in *V. parahaemolyticus* and enhances the antibacterial activity exerted by this system [26]. While our initial challenge experiments with D4 do not showcase the importance of T6SS2 during shrimp infection, we nonetheless speculate that the presence of chitin as a major constituent of shrimp shells may facilitate TfoX-dependent induction of T6SS2 during AHPND pathogenesis under certain conditions. We also propose that modifying environmental factors such as water temperature, salinity, stocking density, and age of PL may influence the extent to which T6SS1 and T6SS2 contribute to mortality caused by VP_{AHPND}. For instance, lower temperature and salinity conditions may diminish the impact of T6SS1 while simultaneously allowing VP_{AHPND} to employ T6SS2 to greater effect.

Here we note that the maintenance tank and challenge assay conditions (i.e. water temperature and salinity, aeration, stocking density) used in this study were calibrated based on 1) conditions that are applicable to *L. vannamei* aquaculture systems, and 2) conditions optimal for T6SS1 activity in *V. parahaemolyticus* as previously determined [6, 23, 33-35]. *L. vannamei* between PL20 and PL30 were used for infections to simulate the typical occurrence of AHPND outbreaks within 20-30 days after shrimp ponds are stocked with PL [36, 37]. While we have not attempted metagenomic characterizations of the *L. vannamei* PL used in this study, we cannot rule out the possibility that variations in prokaryotic communities between PL sourced from different settings will influence the extent to which VP_{AHPND} rely upon T6SS1 or T6SS2 during colonization. We anticipate that future efforts to elucidate the displacement of shrimp flora by VP_{AHPND} strains utilizing T6SS1 or T6SS2 exclusively may prove informative in this area.

In addition to the future directions outlined thus far, our initial findings may warrant larger-scale shrimp infection trials, as well as confirmatory experiments that utilize plasmid-complemented T6SS knockout strains. Repeating similar work with VP_{AHPND} isolates that differ from D4 in origin and effector repertoire may also prove useful in establishing whether our observations are widely applicable to other VP_{AHPND} strains. With regard to the broader impacts of this work, we highlight that the *V. parahaemolyticus* T6SS1 and T6SS2 are similar to T6SSs encoded by other members of the *Vibrio* pan-genome. Namely, the core gene cluster encoding T6SS2 in *V. parahaemolyticus* is similar in gene content and organization to the T6SS2 gene clusters found in *Vibrio vulnificus* and *Vibrio alginolyticus* [20, 38]. In a similar vein, the recently described T6SS1 of *Vibrio coralliilyticus* resembles the T6SS1 of *V. parahaemolyticus* [39]. Consequently, investigations that uncover the roles of T6SS1 and T6SS2 in the lifestyle and pathogenesis of VP_{AHPND} may contribute to a broader understanding of the functional significance of similar systems in clinically- and commercially- relevant *Vibrio* species. Finally, while D4 is not known to be a human-pathogenic strain of *V. parahaemolyticus*, other strains of this species cause seafood-borne illness in humans. Consequently, it is worth entertaining the possibility that T6S may be involved in some capacity during human infections by clinical isolates of *V. parahaemolyticus*, although minimal work in this area has been reported to date.

Materials and Methods

Bacterial Strains and Media Used

The AHPND-causing *V. parahaemolyticus* strain 13-306/D4 (abbreviated D4) and *E. coli* strain S17-1 (λ pir) were generous gifts from Kim Orth at the University of Texas Southwestern Medical Center and were used for various experiments reported here. *V. parahaemolyticus* strains were routinely grown at 30°C with continuous shaking (200 rpm) in 50 ml conical tubes containing marine Luria-Bertani (MLB) broth (LB adjusted to 3% [wt/vol]

NaCl) or on marine minimal medium (MMM) agar plates (1.5% [wt/vol] agar, 2% [wt/vol] NaCl, 0.4% [wt/vol] galactose, 5 mM MgSO4, 7 mM K2SO4, 77 mM K2HPO4, 35 mM KH2PO4, 2 mM NH4Cl), supplemented with antibiotics when appropriate [6].

E. coli strain S17-1 (*λ pir*) was used for maintenance and conjugation of pDM4 and derived plasmids. *E. coli* DH5α harboring an empty pBAD33.1 plasmid was used as a prey strain for competition assays. *E. coli* strains were cultured at 37°C (30°C for competition assays) in 50 ml conical tubes containing LB or on LB agar plates, supplemented with chloramphenicol (20 µg/ml) when appropriate for plasmid maintenance or selection. For a list of bacterial strains and plasmids used in this study, see **Table S1 and Table S2**.

Construction of In-frame Deletion Mutants

Mutants of *V. parahaemolyticus* harboring in-frame deletions of target genes were generated via allelic exchange using the Cmr OriR6K suicide plasmid pDM4 as previously reported, with minor modifications [6]. In brief, 1-kb regions directly upstream and downstream of the target gene were amplified from a genomic DNA template and inserted via Gibson Assembly into a pDM4 plasmid linearized with SacI-HF. The resulting constructs were first transformed into *E. coli* S17-1 (λ pir) and then subsequently conjugated into *V. parahaemolyticus*. Transconjugants were selected for using MMM containing 10 µg/ml chloramphenicol and were additionally screened for sucrose sensitivity. Transconjugants were streaked onto MMM without chloramphenicol for outgrowth, and then subsequently transferred to MMM containing 10% (wt/vol) sucrose for counterselection. Putative secondary recombinants were checked for chloramphenicol sensitivity, and PCR screens were carried out to identify and confirm allelic exchange mutants harboring the target deletion (**Fig S1**). For a list of primers used in this study, see **Table S3**.

Bacterial Competition Assays

T6SS competition assays were performed as described previously, with minor modifications [6]. Briefly, cultures of attacker and prey strains were grown overnight in MLB (*V. parahaemolyticus*) or LB (*E. coli*). Overnight cultures grown for 16 hours were pelleted, washed twice with phosphate buffered saline (PBS), and normalized to an optical density at 600 nm (OD_{600}) of 0.5 before being mixed at a 4:1 ratio (attacker-to-prey) and spotted in triplicate onto 12-well MLB agar plates. Competition plates were incubated for 4 hours at 30°C. Prey CFU/ml at the start of the assay (T0) was determined by spotting 10-fold serial dilutions of mixed cells, in triplicate, onto LB plates containing 20 µg/ml chloramphenicol for selection of *E. coli* prey harboring a pBAD33.1 plasmid. At the end of the assay (T4), competition spots were harvested, and surviving prey CFU/ml was determined by spotting 10-fold serial dilutions as above (see **Dataset S1**). Assays were repeated twice with similar results.

Acquisition and Maintenance of *L. vannamei*

Disease free *L. vannamei* postlarvae (PL) derived from a Specific Pathogen Free (SPF) broodstock were supplied by Miami Aquaculture (Boynton Beach, Florida, USA), and maintained in an aquarium tank filled with approximately 25L of Artificial Seawater (ASW; 30g Instant Ocean® per liter of deionized water) with filtration (Tetra Whisper EX30 Power Filter) and aeration with attached air stones. A gauze mesh was positioned around the strainer of the filtration unit to prevent loss of PL. Maintenance tank water was kept at a temperature of 30°C (Fluval Ultra-Slim Submersible Aquarium Heater) and salinity of 3%. Lighting was provided on a 12-hour schedule. Tank water was refreshed on a biweekly basis. PL were fed approximately 10% body weight per day of Zeigler PL Raceway Plus.

L. vannamei Infections

L. vannamei PL between the stage of PL20 and PL30 were counted and transferred to plastic containers with 250 ml sterile ASW and maintained under conditions similar to the maintenance tank (i.e. water temperature of 30°C, 3% salinity, with aeration provided by sterile air stones and lighting provided on a 12 hour cycle). Containers were labeled according to treatment, with duplicate containers for each condition. 25 PL were transferred to each container, after which time they were monitored for signs of stress or erratic behavior prior to the start of the challenge. PL were then inoculated with the appropriate *V. parahaemolyticus* 13-306/D4 strain (see Inocula Preparation) or PBS control.

During the challenge, PL were fed approximately 10% of their estimated body weight once per day, calculated based on the number of live PL at feeding time. Mortality of PL was determined at 12, 24, 36, and 48 hours post-inoculation (see **Dataset S4**). Live/dead determinations were made on the basis of morphological observations and movement or lack thereof. PL that displayed signs of atrophy and did not move for 5 minutes during observation were defined as non-viable.

Inocula Preparation

Inocula were prepared as follows. Colonies of D4 wild type and mutant strains were picked from MMM agar plates and inoculated into 50 ml conical tubes containing 10 ml of MLB. After 16 hours of growth at 30°C with continuous shaking (200 rpm), cells were pelleted, washed twice with PBS, and normalized to an OD_{600} of 0.5. Experiment containers were then inoculated with PBS (negative control) or an appropriate volume of the designated D4 strain to obtain a final concentration of approximately 1E6 CFU/ml. We determined the actual CFU/mL of each inoculum by plating serial 10-fold dilutions on Thiosulfate–citrate–bile salts–sucrose (TCBS) agar plates.

Growth experiments

Cultures of *V. parahaemolyticus* wild type and mutant strains were grown in MLB overnight at 30°C with continuous shaking (200 rpm). After 16 hours of growth, cultures were diluted 1:10, 1:100, and 1:1000 in fresh MLB in a 96-well plate (200 µl per well). Diluted cultures were then incubated in a BioTek 800 TS microplate reader at 30°C with continuous shaking. Growth was measured as OD_{600} in 20 minute intervals over the course of 8 hours. Growth experiments were repeated twice with similar results (see **Dataset S2**).

Genomic analysis and identification of effectors

The NCBI BLAST suite was employed to survey the *V. parahaemolyticus* 13-306/D4 genome (Genbank accession no. MYFH00000000.1) for homologs of previously described effectors, using protein accessions reported in literature as query sequences [40]. A protein FASTA file generated for D4 using Rapid Annotations using Subsystems Technology (RAST) was used as a subject for BLAST comparisons [41]. Protein accessions and locus information for predicted effectors was gathered from the NCBI database and summarized in **Table 1**. Default parameters were used for all alignments unless otherwise noted. For alignment data, see **Dataset S3**.

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