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Review

The Role of Type III Secretion System 2 in *Vibrio parahaemolyticus* Pathogenicity

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***Vibrio parahaemolyticus*, a Gram-negative marine bacterial pathogen, is emerging as a major cause of food-borne illnesses worldwide due to the consumption of raw seafood leading to diseases including gastroenteritis, wound infection, and septicemia. The bacteria utilize toxins and type III secretion system (T3SS) to trigger virulence. T3SS is a multi-subunit needle-like apparatus used to deliver bacterial proteins, termed effectors, into the host cytoplasm which then target various eukaryotic signaling pathways. *V. parahaemolyticus* carries two T3SSs in each of its two chromosomes, named T3SS1 and T3SS2, both of which play crucial yet distinct roles during infection: T3SS1 causes cytotoxicity whereas T3SS2 is mainly associated with enterotoxicity. Each T3SS secretes a unique set of effectors that contribute to virulence by acting on different host targets and serving different functions. Emerging studies on T3SS2 of *V. parahaemolyticus*, reveal its regulation, translocation, discovery, characterization of its effectors, and development of animal models to understand the enterotoxicity. This review on recent findings for T3SS2 of *V. parahaemolyticus* highlights a novel mechanism of invasion that appears to be conserved by other marine bacteria.**

***Keywords*:** *Vibrio parahaemolyticus*, type III secretion system, virulence, bacterial pathogenesis, gastroenteritis, enterotoxicity

Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that inhabits warm marine or estuarine environments. Many of the strains are strictly environmental, whereas some have acquired virulence mechanisms that account for *V. parahaemolyticus* being associated with seafood-borne illness worldwide (Joseph *et al*., 1982; McCarter, 1999). Consumption of raw or undercooked seafood, especially shellfish, contaminated by these pathogenic *V. parahaemolyticus* strains causes gastroenteritis, the symptom of which includes diarrhea, vomiting, nausea, abdominal cramping, and low-grade fever. Exposure to *V. parahaemolyticus* can also lead to wound infection and septicemia under certain medical conditions (Yeung and Boor, 2004; Su and Liu, 2007). This virulence attributes to various toxins and bacterial proteins termed effectors that are delivered into the host through type III secretion system (T3SS). T3SS utilizes a needle-like apparatus to translocate effectors into the host cells, which then target and hijack multiple eukaryotic signaling pathways. T3SS is a pivotal virulence machine used by numerous bacterial pathogens including *Yersinia*, *Salmonella*, *Shigella*, and pathogenic *Escherichia coli* spp.

The clinical isolates of *V. parahaemolyticus* have two major toxins - thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH). TDH has a β-hemolytic activity on a special blood media called Wagatsuma agar, the process of which is termed Kanagawa phenomenon (KP) (Nishibuchi and Kaper, 1995). KP has shown to be a useful indicator for pathogenic strains in seafood or patient samples. TDH also exhibits cytotoxic as well as enterotoxic effects, and is therefore speculated to be a major virulence factor of *V. parahaemolyticus* (Honda *et al*.,1990; Nishibuchi *et al*., 1992; Raimondi *et al*., 2000). However, the bacterial strains lacking functional TDH still manage to induce cytotoxicity and enterotoxicity during infection which indicates the presence of additional virulence factors. TRH shares high sequence homology (68%) with TDH and demonstrates similar hemolytic activity on red blood cells, although little is known about its attribution to pathogenicity (Ohnishi *et al*., 2011).

Whole-genome sequencing of *V. parahaemolyticus* RIMD 22106633, a pathogenic strain that contains two *tdh* genes, revealed two sets of T3SS gene clusters in each of the two circular chromosomes, hence named T3SS1 and T3SS2 respectively (Makino *et al*., 2003). The high sequence homology of T3SS1 genes with those of other *Vibrio* species suggests that these genes were ancestrally acquired and have been evolutionarily conserved. T3SS2 genes are found on a 80-kb pathogenicity island (Vp-PAI) in the second chromosome along with the two *tdh* genes (Fig. 1). Vp-PAI has a lower G+C content than the genomic average and contains higher proportions of genes unique to each *Vibrio* species, which is indicative of the recent acquisition of this DNA region through lateral gene transfer. Characterization of various effectors encoded from each secretion system demonstrates that T3SS1 induces cytotoxicity in cultured human cells

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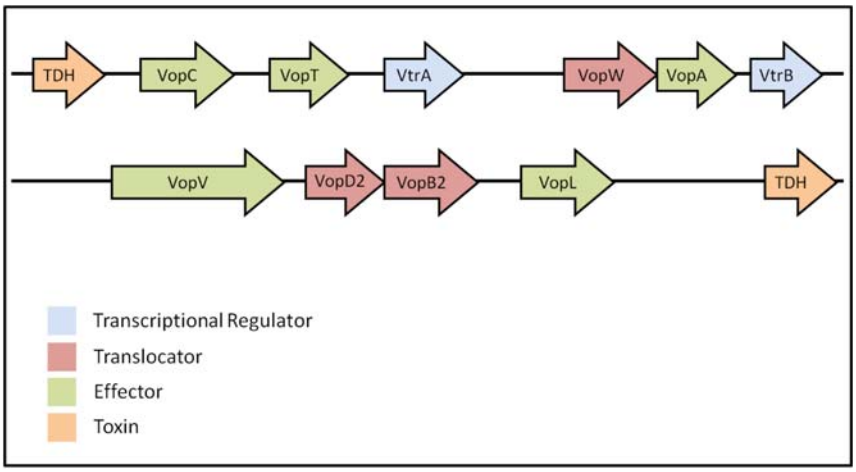

Fig. 1. Gene cluster of T3SS2 and related genes. Pathogenicity Island on the second choromosome of *V. parahaemolyticus* (Vp-PAI) harbors T3SS and related genes as well as *tdh* toxins.

whereas T3SS2 is associated with enterotoxicity in infected animal models and cytotoxicity in intestinal cell lines (Park *et al*., 2004).

This review will specifically focus on the recent findings on T3SS2; the transcriptional regulation of T3SS2 gene cluster, translocation of a repertoire of T3SS2 effectors into the host, how these effectors manipulate host systems in order to achieve successful infection, as well as development of animal models to study the effect of T3SS2 in enterotoxicity.

Transcriptional regulation of T3SS2

The expression of a number of proteins encoded from Vp-PAI including TDH toxins and T3SS2-related proteins are controlled by two major transcriptional regulators, VtrA (VPA1332) and VrtB (VPA1348), located in the same island (Kodama *et al*., 2010). VtrA and VtrB contain a Winged-Helix-Turn-Helix (WHTH) DNA-binding domain which is characteristic of the transcriptional regulator OmpR family proteins. Located upstream of VtrB, VtrA directly binds to the promoter region of VtrB and upregulates its transcription. Because VtrA and VtrB regulate the expression of many T3SS2-related genes and both *tdh* genes, they play a crucial role in T3SS2-mediated cytotoxicity as well as enterotoxicity. The genome-wide transcriptional analysis revealed that VtrA and VtrB target genes are mostly localized within the Vp-PAI region. However, the environmental cue for the activation of these master regulators and the circuit of virulence genes once the bacteria reach the intestines was unclear. Recently, Gotoh *et al*. (2010) showed that bile-acid is the host-derived inducer that triggers the expression of *tdh* genes and T3SS2-related genes via VtrA and VtrB activation. Bile acids are one of the components of crude bile present in the intestinal lumen and play an important role in the digestive process. They have an effect on virulence of other bacterial pathogens such as *Salmonella typhimurium*, *Shigella* spp., and *Vibrio cholearae* although their modes of action differ in each bacterial infection (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999; Prouty and Gunn, 2000; Begley *et al*., 2005; Olive *et al*., 2007). Bile salts can either repress (*S. typhimurium*, *V. cholerae*) or induce (*Shigella* spp., *V. parahaemolyticus*) the transcription of virulence factors in order to promote bacterial invasion or replication. Despite the diversified responses to bile acids during infection, all these bacteria have established an elaborate environment-sensing mechanism that contributes to their pathogenicity.

T3SS2-dependent translocation into host cell

Translocation of T3SS effectors is mediated by a specialized translocon complex. Once assembled, it forms a pore and translocation channel spanning the host cell membrane through which various effectors are delivered into the host cytoplasm. Many T3SS translocons are composed of three major translocator proteins: two of them are hydrophobic and constitute a pore structure across the membrane whereas a hydrophilic protein associates with the tip of the T3SS needle and bridges between the needle complex and the translocon (Buttner and Bonas, 2002; Mueller *et al*., 2008; Mattei *et al*., 2011). The hydrophobic translocon components specific for T3SS2 in *V. parahaemolyticus* are VopB2 (VPA1362) and VopD2 (VPA1361) (Kodama *et al*., 2008). Both proteins are secreted in T3SS2-dependent manner and are necessary for translocation of T3SS2 effectors. They also associate with the host cell membrane and form a pore, which is an essential feature for translocators. Importantly, VopB2 and VopD2 are required for T3SS2-mediated cytotoxicity in Caco-2 cells and enterotoxicity in rabbit ileal loop model. VopW, a hydrophilic component of the translocon complex has been recently identified (Zhou *et al*., 2012). Unlike the other translocator proteins, it is not encoded from the same operon with other translocon components. More interestingly, VopW appears to be translocated into the host cytoplasm which has not been observed in other translocator proteins. This suggests that VopW may act as an effector in addition to its role as a translocator, although further validation is needed to support this possibility.

T3SS2 effectors

In addition to toxins that are potent virulence factors, *V. parahaemolyticus* also delivers a repertoire of effectors via T3SSs that manipulate host signaling pathways to the pathogen's benefit. The effectors secreted from T3SS1 (VopQ, VopS, VopR, VPA0450) serve distinct functions that work

Table 1. List of T3SS2 effectors and their function

Effectors	Gene	Molecular activity	Host target	Biological activity
VopA/VopP	VPA1346	Acetyltransferase	MKKs	Inhibits MAPK pathway
VopL	VPA1370	Actin nucleation	actin	Induces stress fiber
VopT	VPA1327	ADP–ribosylation	Ras	Induces cytotoxicity/Inhibits yeast growth
VopV	VPA1357	F-actin binding/bundling	F-actin	Facilitates enterotoxicity
VopC	VPA1321	Deamidase	Rac, Cdc42	Promotes bacterial invasion

in harmony to cause cytotoxicity. VopQ (VepA, VP1680) induces autophagy in a PI3K-independent manner which, together with the other effectors, contributes to cell lysis (Burdette *et al*., 2009; Matsuda *et al*., 2012). VopS (VP1686) modifies host Rho family GTPases with adenosine monophosphate (AMP) and blocks their binding to downstream substrates regulating actin cytoskeleton, which leads to cell rounding (Yarbrough *et al*., 2009). VPA0450, a phosphatidylinositide phosphatase, hydrolyzes phosphatidylinositide (4,5)-bisphosphate ($PI(4,5)P_2$) located in the plasma membrane which disrupts the balance of the membrane and the associated actin-binding proteins, thus resulting in membrane blebbing (Broberg *et al*., 2010). The function and host target of VopR (VP1683) is unknown.

The T3SS2 effectors, on the other hand, are mostly associated with the enterotoxicity, though they have been shown to cause cytotoxicity in intestinal cell lines such as Caco2 cells and HCT cells. Animal studies have demonstrated that T3SS2 effectors cause fluid accumulation and inflammation in the intestinal tract as well as severe diarrhea, which mimicks the clinical symptoms of gasteroenteritis. Similar to the effect caused by T3SS1 effectors, this process is predicted to be an orchestration of various T3SS2 effectors serving distinct roles (Table 1).

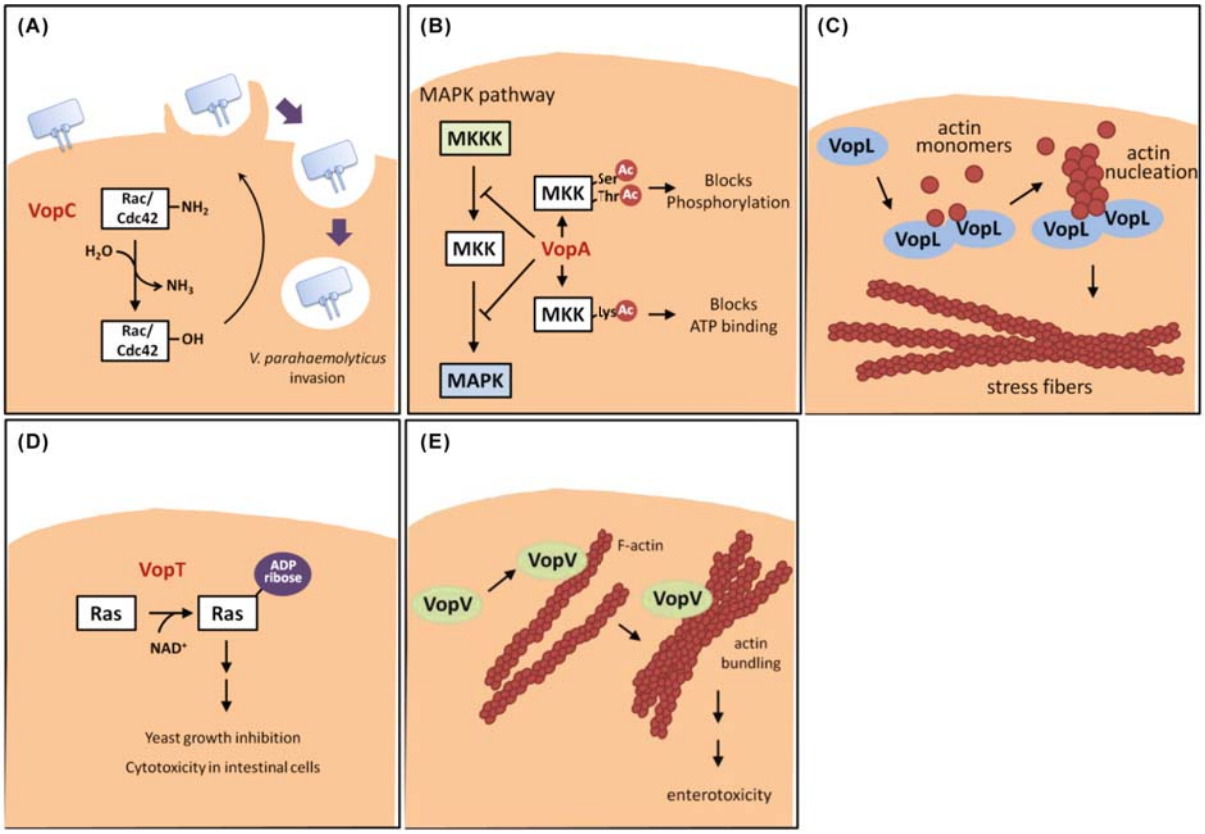

Fig. 2. Summary of T3SS2 effectors acting on various targets in the host cell. (A) VopC deamidates small GTPases such as Rac and Cdc42. This enzymatic activity of VopC is required for the invasion of *V. parahaemolyticus* during infection. (B) VopA acetylates Ser and The residues on the activation loop of MKKs that are critical phosphorylation sites, thereby blocking the activation of MKKs. It also acetylates Lys, which disrupts the ATP binding site of MKKs. Together, these modifications by VopA inhibit the MAPK pathway. (C) VopL dimerizes through its VCD domain and recruits actin monomers promoting actin nucleation. The massive actin nucleation mediated by VopL induces stress fibers throughout the cell. (D) Using NAD^+ as a substrate, VopT modifies small G protein Ras with ADP-ribose. Through unknown downstream events, this enzymatic activity of VopT triggers yeast growth inhibition and cytotoxicity in the intestinal cells. (E) VopV directly binds to F-actin through its long repeat (LR) and C-terminal domain. VopV also has actin bundling activity, forming thick bundles of actin filaments. VopV majorly contributes to enterotoxicity during infection which correlates with its actin binding activity.

***VopC*:** Recently, new evidence was reported which demonstrates that *V. parahaemolyticus*, which has been long thought to be an extracellular pathogen, invades the host cell (Zhang *et al*., 2012). Zhang *et al.* (2012) showed that *V. parahaemolyticus* enter the host cells and remain intracellular by a process of that is mediated by a T3SS2 effector VopC (VPA1321). VopC shares sequence homology to cytotoxic necrotizing factor (CNF) toxins found in *Yersinia* spp., *Bordetella* spp., and pathogenic *E. coli*. These toxins, once secreted into the host cell, target diverse eukaryotic factors to subvert host cell systems for the benefit of the pathogen, such as facilitating the invasion of bacteria into the host cell. Surprisingly, the authors observed, using gentamicin protection assays and cytotoxicity analyses, that the invasion of *V. parahaemolyticus* into non-phagocytic cells was dependent on the presence of VopC. The invasion is mediated by the enzymatic activity of VopC, a deamidase/transglutaminase activity shared by other CNF toxins with conserved catalytic residues. The deamidation of small Rho GTPases such as Rac and Cdc42 (on glumatine 61) by VopC renders them constitutively active and promotes actin cytoskeleton rearrangement of the infected cell so it can engulf the bacteria (Fig. 2A). The authors found that a VopC homolog in non-O1/non-O139 *V. cholerae* strains also mediates the bacterial invasion during infection. The authors predict that other marine pathogens containing a similar T3SS2 with a VopC-like gene will also be invasive. This new insight of the invasive nature of *V. parahaemolyticus* suggests new approaches for studying the pathogenesis of the bacteria. Furthermore, it makes it indispensable for re-evaluating the functions and mechanisms of the effectors previously characterized within the context of *V. parahaemolyticus* being thought of as an extracellular pathogen when it actually invades.

***VopA*:** VopA/P (VPA1346) is a homolog of YopJ, an effector from *Yersenia* spp. which inhibits mitogen-activated protein kinase (MAPK) and NFκB signaling pathway (Orth *et al*., 1999; Mukherjee *et al*., 2006; Mukherjee *et al*., 2007). These effectors share the same catalytic triad and both act as acetyltransferases targeting kinases. However, VopA/P only inhibits MAPK pathway and not NFκB signaling (Trosky *et al*., 2004). For *Yersenia* which is thought to be primarily an extracellular pathogen, YopJ is designed to target multiple pathways and block the host innate immune response. It also promotes cell death by blocking the NFκB survival pathway. Considering the invasive nature of *V. parahaemolyticus*, it is only natural that VopA/P does not target NFκB pathway in order to sustain the survival of the host cell which contains an intracellular niche for *V. parahaemolyticus*. Therefore, despite their conserved enzymatic activity, YopJ and VopA/P have evolved to have a different substrate specificity favoring their respective bacterial lifestyle. In addition, the mechanisms underlying the MAPK inhibition by YopJ and VopA/P are also different. YopJ acetylates the critical serine and threonine residues located on the activation loop of MAPK kinases and IKKβ blocking the phosphorylation sites that are necessary for the activationby upstream kinases. VopA/P also acetylates the same serine and threonine residues of MKKs thereby preventing kinase activation in a similar manner. Interestingly, VopA/P modifies an additional lysine also present in the catalytic loop that is essential for binding to the γ-phosphate of ATP (Trosky *et al*., 2007). The acetyl group masks the charge of lysine, which alters the change of the nucleotide binding pocket so it can no longer bind to ATP and modify the substrate (Fig. 2B). VopA/P uses a novel mechanism for inhibiting kinases, because it targets not only the inactive form but also the active form of the kinase.

***VopL*:** The actin cytoskeleton undergoes a highly dynamic process of assembly and disassembly in order to control cell shape and motility. Because its homeostasis is so crucial for cell survival, the actin cytoskeleton is one of the major targets of many bacterial effectors and is manipulated by diverse mechanisms. The assembly of actin filaments begins with the nucleation step involving three or more actin monomers where actin filament polymerization can occur. VopL (VPA 1370) contains three Wiskott-Aldrich homology 2 (WH2) domains that bind to actin monomers and promote actin nucleation (Liverman *et al*., 2007). Accordingly, VopL triggers an actin-related phenotype during infection which is to induce massive stress fibers throughout the cell (Fig. 2C). This process, unlike many other bacterial effectors that manipulate the actin cytoskeleton, does not involve small Rho family GTPases such as RhoA, Rac, and Cdc42 that govern the formation of stress fibers, lamellipodia, and filapodia, respectively (Takai *et al*., 2001). It is proposed that VopL serves as an actin nucleation factor promoting the assembly of actin filaments in a polarized manner. This is distinct from the action of eukaryotic actin-nucleator Arp2/3 complex which generates the branched network of actin filaments (Takai *et al*., 2001). The molecular mechanism of actin filament nucleation mediated by VopL was recently determined. VopL C-terminal domain (VCD) of VopL promotes dimerization of WH2 motifs as well as recruitment of actin subunits by binding of VCD to the pointed end of the actin nucleus, both of which is necessary for VopL-mediated nucleation activity (Namgoong *et al*., 2011; Yu *et al*., 2011). Considering that *V. parahaemolyticus* is an intracellular pathogen, the actin manipulation by VopL may potentially have a role in bacterial uptake into the host cell or membrane trafficking in order to form vacuoles where bacteria can replicate.

***VopT*:** VopT (VPA1327) contains a ADP-ribosyltransferase (ADPRT) domain that is found in ExoT and ExoS, the T3SS effectors from *Pseudomonas aeruginosa*. ExoS induces cell death which requires its ADPRT activity. ExoS modifies monomeric small G proteins including Ras, RalA, Rac1, Cdc42 and certain Rab proteins (Coburn and Gill, 1991; Bette-Bobillo *et al*., 1998; McGuffie *et al*., 1998; Kaufman *et al*., 2000; Fraylick *et al*., 2002). ExoT, on the other hand, ADP-ribosylates CT10 regulator of kinase (Crk) proteins and is likely to inhibit host cell phagocytosis while having no effect on cell viability (Sun and Barbieri, 2003; Barbieri and Sun, 2004). VopT exhibits cytotoxicity against the intestinal cell lines such as Caco-2 and HCT-8 as well as growth inhibition in yeast, which is all dependent on its ADPRT activity (Kodama *et al*., 2007). Similar to ExoS, VopT also ADP-ribosylates Ras both *in vivo* and *in vitro* (Fig. 2D). However, VopT has rather narrow substrate specificity and modifies only Ras among monomeric small G proteins. However, it is unclear if this modification activates or inactivates Ras. Whether or not this ADP-ribosylation of Ras is responsible

for VopT-mediated cytotoxicity remains to be determined. It is also possible that VopT may have another substrate(s) distinct from those of ExoS or ExoT that contribute to cytotoxic effects. Further analysis is required to understand the molecular mechanisms mediated by VopT ADP-ribosylation and to identify the key players in the pathway leading to cytotoxicity.

***VopV*:** VopV (VPA1357) was identified as a critical factor that contributes to T3SS2-mediated enterotoxicity in the rabbit ileal loop model (Hiyoshi *et al*., 2011). VopV directly binds to F-actin, a polymer form of actin, demonstrated by a high-speed cosedimentation assay. When Caco-2 cells were infected with *V. parahaemolyticus*, accumulation of F-actin filaments beneath bacterial microcolonies was observed in a VopV-dependent manner. VopV has a long repeat (LR) region between the N- and C-terminal domains that consists of three types of repeat sequence units. The LR and the C-terminal domain mediate the binding to F-actin and induce the F-actin accumulation observed *in vivo*. In addition to F-actin binding, VopV has F-actin bundling activity demonstrated by a low-speed cosedimentation assay where most of the F-actin was pelleted after a low-speed centrifugation in the presence of VopV. The assembly of F-actin filaments forming thick bundles was also observed using transmission electron microscopy (TEM). The authors determined the minimal region within LR that is responsible for F-actin binding, and revealed that multiple copies of this unit are required for F-actin bundling activity. The enterotoxicity caused by VopV was shown to correlate with the F-actin binding but not the bundling activity (Fig. 2E). How this F-actin binding activity of VopV is involved in causing enterotoxicity remains unknown. VopM, a VopV homolog found in non-O1/non-O-139 *V. cholearae* strains that carry T3SS2-like gene cluster has also shown to mediate enterotoxicity, underlining its crucial role during infection.

Models for studying T3SS2-mediated enterotoxicity

***In vitro* models:** One model system established to study enterotoxicity of *V. parahaemolyticus* is the rabbit ileal loop (Boutin *et al*., 1979). For this experiment, the ligated ileal loops of rabbits are injected with a known number of bacterial cells and the fluid accumulation is measured after bacterial colonization of the intestine. The fluid accumulation is thought to mimic the clinical symptoms of gasteroenteritis, albeit without a diarhetic flow. Histopathological analysis using hematoxylin-eosin staining is also used to look for signs of intestinal inflammation such as epithelial denudation, edema, and neutrophil infiltration in the lamina propria and submucosal area (Park *et al*., 2004). However, there are several caveats to be considered when interpreting data derived from these experiments: 1) This system does not allow a natural flow of contents through the intestinal cavity, but results in a stagnant pool of bacteria. 2) The incubations are extensive and much is missed within the 24 h incubation. 3) The tissue used may not be representative of the tissue targeted during the infection of the human host as damage and bleeding has been associated with other intestinal gastrointestinal tissue downstream of the small intestine.

Tissue culture cells have been extensively used to analyze molecular responses to bacterial virulence factors. While these have been extremely valuable for the molecular analysis of virulence factors that target evolutionarily conserved mechanism, there are some drawbacks from a pathological standpoint. For example, the tissue culture cells may or may not be a differentiated cell and more than likely they represent a uniform population of cells. *In vivo* models (see below) show damage through tissue that contains diverse layers of cell types. Another model extensively used to study the molecular mechanisms of T3SS effector is yeast, because the targets of many of these effectors is conserved and yeast genetics can be used for identifying specific effector targets. For example the yeast MAPK signaling pathway is conserved in yeast and was helpful in dissecting the mechanism used by VopA/P (Trosky *et al*., 2004).

***In vivo* models:** Animal models developed to study *V. parahaemolyticus* pathogenesis include orogastric, peritoneal, and oral infection in mice. A recent study by Piñeyro *et al*. (2010) presented two animal models as comparative systems to investigate the contribution of different T3SS effectors in the pathophysiology of gasteroenteritis and lethality. One is orogastric infection of piglets where multiple signs of gasterointestinal disease were evaluated, including the presence of acute diarrhea, vomiting, and fecal shedding of *V. parahaemolyticus*. Histological analysis revealed a mild to severe enema in cecal submucosa and serosa. The study demonstrated that such clinical symptoms correlate with the presence of T3SS2, indicating its crucial role in causing gesterointestinal disease. The other is a murine pulmonary model where the bacteria are inoculated into mouse lungs. The infected mice showed diffuse and severe pulmonary hemorrhage and mortality which was significantly attenuated by the absence of T3SS1, illustrating that two T3SSs serve distinct functions in pathogenesis for *V. paramaelyticus* infection.

More recently, Ritchie *et al*. (2012) developed another non-surgical animal model utilizing infant rabbits with orogastric infection where bacterial colonization and host responses to infection can be effectively monitored. The distal small intestine was observed to be a primary site of bacterial colonization where major tissue damages as well as inflammation occurred. However, the location of infections in this study could be due to the neutralization of the stomach of the rabbits prior to the bacterial inoculation resulting in a non-physiological condition for bacterial infection.

Concluding remark

A number of bacterial pathogens utilize toxins and T3SSs to subvert host signaling system as a strategy to promote their survival and replication during infection. The pathogenic strains of *V. parahaemolyticus* have acquired an additional T3SS in the second chromosome later during evolution to ensure successful infection in the human host. Various effectors secreted by T3SS2 target many host factors including MKKs, small Rho GTPases, and F-actin to manipulate critical signaling pathways and actin cytoskeleton organization. Although some effectors have been relatively well characterized, relevant host targets and detailed mechanism of actions of many other effectors still remain to be determined. It will be interesting to investigate how the distinct functions

of individual effectors work in an orchestrated manner to promote bacterial colonization of the intestine and trigger enterotoxicity. Importantly, the invasive nature of *V. parahaemolyticus* should be taken into account for all future studies dealing with the T3SS2 and pathogenicity of *V. parahaemolyticus*. In addition, transcriptional and post-transcriptional regulation of the T3SS2 genes and the mechanism of translocation are more open fields for future studies.

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