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***Vibrio* Variations on a Type Three Theme**

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Abstract

Mounting evidence suggests that Type 3 Secretions Systems (T3SS) are widespread among *Vibrio* species, and are present in strains isolated from diverse sources such as human clinical infections, environmental reservoirs, and diseased marine life. Experiments evaluating *V. parahaemolyticus* and *V. cholerae* T3SS mediated virulence suggest that *Vibrio* T3SS pathogenicity islands have a tripartite composition. A conserved “core” region encodes functions essential for colonization and disease *in vivo*, including modulation of innate immune signaling pathways and actin dynamics, whereas regions flanking core sequences are variable among strains and encode effector proteins performing a diverse array of activities. Characterizing novel functions associated with *Vibrio*-specific effectors is therefore essential for understanding how vibrios employ T3SS mechanisms to cause disease in a broad range of hosts and how T3SS island composition potentially defines species specific disease.

INTRODUCTION

***Vibrio* T3SS identification.**

Despite widespread identification in many bacterial genera that began in the mid-1980's with studies on pathogenic *Yersinia* species, Type 3 Secretion Systems (T3SSs) were not recognized as virulence mechanisms in pathogenic *Vibrio* species until the completed genome sequence of an O3:K6 serotype *V. parahaemolyticus* strain, RIMD 2210633, was reported in 2003 [1]. Rapid identification of T3SSs in other *Vibrio* species followed, and in 2005, genomic sequencing identified the first *V. cholerae* T3SS in AM-19226, a clinically isolated non-O1/non-O139 serogroup, cholera toxin negative strain that causes non-epidemic cholera [2]. More recently, molecular methods combined with annotation of genomic sequence data expanded the list of T3SS-positive *Vibrio* species [3-10]. T3SS association with pathogenic *Vibrio* species is easily explained within the context of a virulence

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mechanism, but knowing that *Vibrio* species often interact with multiple hosts (and not always as pathogens) raises the question of whether T3SSs can promote a more symbiotic relationship or alternatively, an advantage in the environmental niche. In either case, researchers now face the challenge of identifying and characterizing novel, often *Vibrio*-specific effector proteins in an effort to mechanistically understand T3SS mediated interactions with a wide variety of eukaryotic hosts.

Two T3SSs.

Most *Vibrio* spp. encode one T3SS, but *V. parahaemolyticus* strains can carry one on each chromosome, respectively termed T3SS1 and T3SS2 [1]. T3SS1 and 2 gene organization and content differs; each T3SS is assembled from distinct proteins and functions independently [3]. T3SS1 is nearly ubiquitous among *V. parahaemolyticus* strains, is most similar to the *Yersinia* Ysc T3SS in sequence and synteny, and is associated with mammalian cell cytotoxicity *in vitro* [1]. Multiple lines of evidence support an ancestral origin, and *Vibrio* species pathogenic for non-human hosts (*e.g.* *V. alginolyticus*, *V. campbellii*, *V. caribbenthicus*, *V. harveyi*, and *V. tubiashii*) typically encode T3SS1 [1,3,11,12].

In contrast, *V. parahaemolyticus* T3SS2 is encoded on a genomic pathogenicity island and appears restricted to pandemic O3:K6 serotype isolates and related, pathogenic serovariant strains. Historically, pathogenic strains were identified by a hemolytic property known as the Kanagawa phenomenon, encoded by *tdh* or *trh* genes. We now know that the *tdh* and *trh* loci are typically found within the T3SS2 genomic island, although the protein products are secreted by another mechanism and are not T3SS substrates [1,3,13].

A subset of *V. cholerae* non-O1/non-O139 serogroup strains, which can cause sporadic cholera but do not cause epidemic disease, encode T3SS2. The vast majority of T3SS-positive strains lack the major, canonical virulence factors associated with epidemic strains (*i.e.* toxin co-regulated pilus and cholera toxin), employing the T3SS mediated pathogenic mechanisms instead [2,14-16]. For both *V. cholerae* and *V. parahaemolyticus*, experimental evidence using animal models of infection indicates that T3SS2 is required for colonization and disease [17-20].

V. mimicus and *V. anguillarum* strains pathogenic for humans can also encode a T3SS2 [6,7]. Regarding other species, the use of increasingly sophisticated phylogenetic methods to redefine evolutionary relationships combined with our expanding knowledge of pangenomes results in a fluid understanding of pathogenic mechanisms associated with a particular species [10,21-23]. What follows next therefore summarizes our current knowledge of T3SS effector protein functions and species associations, based largely on experiments in *V. parahaemolyticus* and *V. cholerae*.

TYPE THREE SECRETION SYSTEM 1 (T3SS1)

Based on genetic content and organization, the ~40 kb *V. parahaemolyticus* T3SS1 locus is similar to that found in other *Vibrio* species. Effector proteins characterized thus far appear to functionally converge at the level of the host membrane, and in some cases, with multiple

activities attributed to single effectors. For example, VopQ (also known as VepA) reported activities include induction of autophagy *in vitro*, activation of the p38, JNK, and ERK Mitogen-Activated Protein Kinase (MAPK) pathways, and host cell lysosome rupture via interaction with the V₀ domain of the V-ATPase that forms gated channels [24-26]. VopS encodes a bacterial phosphoinositide-binding (PIB) domain, and PIP₂ ligand binding directs effector folding and targeting to the host plasma membrane where VopS mediates actin reorganization by AMPylation of Rho family GTPases, resulting in cytoskeletal collapse and cell rounding [27-29]. VopR also encodes a PIB domain and is localized to the plasma membrane, although its functions remain to be fully elucidated [29]. Interestingly, an effector encoded outside the T3SS1 island displays phosphatidylinositol phosphatase activity, leading Orth and colleagues to propose that other effector activities are influenced by depleting PIP₂ from the host cell membrane [30,31].

Although primarily a pathogen of marine life, *V. alginolyticus* causes T3SS1 dependent cytotoxicity in both fish and mammalian cell lines [32,33]. Interestingly, apoptotic features were present in fish cell lines, whereas mammalian cells appeared to undergo autophagy. Two identified effectors, Val1686 and Val1680, are VopS and VopQ orthologues. Like VopS, Val1686 induces cell rounding, but is also sufficient to trigger apoptosis in infected fish cells. Unlike VopQ, Val1680 does not induce autophagy in fish cells, but it does contribute to T3SS-induced LDH release by an unidentified mechanism [34].

Speculation that T3SS1 is important for survival in the aquatic environment is supported by its presence in both environmental and clinical strains of *V. parahaemolyticus* and evidence indicating an ancestral origin, consistent with the theory proposed by Zhang *et al.* that T3SS1-mediated cytotoxicity provides a mechanism to supply nutrients in a nutrient-poor environmental reservoir [35]. Additionally, T3SS1 is not ubiquitously found in *V. harveyi* strains, and T3SS1 presence was not associated with pathogenicity in a shrimp model even though *V. harveyi* is documented as a significant marine pathogen, particularly of shrimp [10,36]. A definitive association between T3SS1-mediated phenotypes and human/marine-life infection thus awaits additional molecular characterizations in model systems.

TYPE THREE SECRETION SYSTEM 2 (T3SS2)

T3SS2 clade classification and defining the core region.

The *V. cholerae* and *V. parahaemolyticus* T3SS2 gene clusters are more similar to each other in content and synteny than they are to T3SSs from other species. In addition, the *Vibrio* T3SS structural machinery components do not collectively align with a single T3SS family classification [37]. Rather, structural protein orthologues from each of the three families (Inv-Mxi-Spa, Ysc, and Ssa-Esc) are represented in the *Vibrio* gene clusters, although some components await definitive identification (Table 1). Given that the T3SS2 has been experimentally shown to function in both *V. cholerae* and *V. parahaemolyticus*, the prevailing opinion is that such proteins exist, but are encoded by novel sequences [2,3,17,23].

Despite lacking sequence similarity to other known T3SS hydrophilic translocators, VopW was experimentally identified by Zhou *et al.* as a third translocon component essential for

effector translocation in *V. parahaemolyticus* [38,39]. *vopW* sequences are present in all *V. cholerae* and *V. parahaemolyticus* T3SS2 islands, although gene location, number, and sequence identity are variable. Somewhat paradoxically, VopW was identified as a translocated effector protein in both *V. parahaemolyticus* and *V. cholerae* [38,40]. However, the collective results are consistent with reports from other systems of effector proteins having dual structural/effector function or T3SS-independent entry [41,42].

T3SS2 has been categorized into two clades, alpha (α) and beta (β), based on the sequences of genes encoding structural components and collective observations suggesting independent acquisition events by ancestral clones [23,43,44]. T3SS2 sequence comparisons that also combine genomic island organization indicate that *V. parahaemolyticus* and *V. cholerae* T3SS2 α (e.g. RIMD 2210633 and AM-19226) are more similar to each other than T3SS2 α and T3SS2 β of the same species (e.g. *V. cholerae* strains AM-19226 and 1587). *V. anguillarum* and *V. mimicus* T3SS2 clade classifications have remained elusive, largely due to insufficient sequence data and/or hybrid characteristics [6].

Seven proteins identified as *V. cholerae* effectors are encoded within and immediately adjacent to the cluster of operons encoding structural apparatus proteins (Vops, Figure 1). Based on sequence similarity, synteny and/or evidence of translocation, each has a *V. parahaemolyticus* ortholog. For both species, experiments demonstrated that effectors encoded within the structural apparatus cluster are essential *in vivo* for colonization or disease related phenotypes (described below). Effector proteins are also encoded within mosaic “flanking regions” that lie 5’ and 3’ adjacent to the structural gene operon cluster, but associated phenotypes are less dramatic or remain unknown.

We thus conclude that a *Vibrio*-specific “core” region can be defined within the T3SS pathogenicity island, having the following properties: 1) gene content and position well conserved between species, 2) encoding proteins essential for T3SS structural apparatus function, 3) including effector functions necessary and potentially sufficient for pathogenic mechanisms, such as colonization, and 4) encoding transcriptional regulatory proteins required for T3SS expression. Based on such criteria, the core region (using strain AM-19226 as a reference) is bounded 5’ by *vttRA*, encoding one of two essential, ToxR-like transmembrane transcriptional regulators, and 3’ by *vopF* [45]. Shared features between *V. cholerae* and *V. parahaemolyticus* thus raises the interesting possibility of a common T3SS mediated mechanism of *Vibrio* colonization orchestrated by orthologous, core encoded effector proteins.

The clinical spectrum of *Vibrio* T3SS2-associated human disease

V. parahaemolyticus and *V. cholerae* primarily cause gastroenteritis, though clinical manifestations of disease are host variable and are also influenced by species and serogroup differences: *V. parahaemolyticus* typically induces an inflammatory diarrhea, whereas epidemic, O1/O139 serogroup *V. cholerae* infection is characterized by secretory diarrhea with no damage to the intestinal epithelium [46,47]. Infection by cholera toxin negative, non-O1/non-O139 serogroup *V. cholerae* strains is historically considered clinically indistinguishable from epidemic strains, but a subset of cases presents with a mild inflammatory component [47,48]. *V. mimicus* can cause acute gastroenteritis and otitis

media after exposure to seawater or contaminated seafood. *V. anguillarum* is largely a pathogen of crustaceans and bivalves, but along with *V. hollisae*, is associated with wound infections and can cause severe illness in immunocompromised individuals [21].

It is therefore interesting to note that the inflammatory component of *Vibrio* spp. associated gastroenteritis has been linked to T3SS2 presence and the causality borne out by experiments recapitulating disease using an orogastrically inoculated infant rabbit model [18]. The intestinal epithelium remains intact during infection by cholera toxin-positive O1 serogroup strains, but infection by T3SS2 α -positive strains results in both diarrhea and an altered epithelial cell architecture, with *V. parahaemolyticus* infection causing increased inflammation and disruption compared to less dramatic damage observed by T3SS-positive *V. cholerae* strain infection [18,49,50]. One interpretation of the differing T3SS-related pathologies is that common effector proteins and mechanisms are used to colonize and establish an infection, but that clinical variations result from a combination of host factors and species-specific effector proteins or effector alleles.

Core encoded effectors.

The prototype T3SS2 α *V. cholerae* strain, AM-19226, encodes at least 13 translocated proteins. Seven are found within the core region of *V. cholerae* and are shared with *V. parahaemolyticus* (differing nomenclature is indicated in parentheses): VopZZ(VopZ), VopH, VopA, VopM (VopV), VopI, VopW, and VopF, (VopN/ VopL) (Figure 1) [40] (Dziejman laboratory, unpublished data). Variable amino acid sequence conservation (28-49% identity and 42-65% similarity) suggests that Vops have strain/species specific attributes while retaining structural conservation to carry out a subset of conserved functions. Whether all proteins detected as present in host cells and translocated in a T3SS-dependent manner function solely as effectors remains to be determined, since bioinformatic and experimental data suggest functions consistent with “missing” secretory apparatus components [19,51].

VopF/N/L all possess Wiscott-Aldrich homology 2 (WH2) domains, which promote mammalian cell actin rearrangement [17,52-55]. All three proteins nucleate actin *in vitro*, but the phenotypes associated with infection or transfection of mammalian cells differ in that VopF induces actin-rich protrusions, whereas VopN and VopL form actin stress fibers/non-functional actin filaments. *V. cholerae vopF* is required for wild-type levels of colonization in the infant mouse model, consistent with a role for modulating host cell cytoskeletal dynamics during the early stages of infection. A recent report documented an association between VopL actin dysregulation and limited ROS production resulting from halted assembly of the NADPH oxidase complex at the plasma membrane during *V. parahaemolyticus* infection [56].

VopV/VopM also display actin reorganization activities, and are essential for *in vivo* colonization. Biophysical analyses and *in vivo* results demonstrate that VopV binds actin via ~400bp repeated sequences and interacts with filamin via C-terminal sequences. The resulting cytoskeletal rearrangements play a critical role in brush border effacement and remodel the epithelial cell surface to promote attachment [57-59]. Thus, modulation of actin dynamics clearly plays an important role in *Vibrio* T3SS pathogenesis, although it is often challenging to determine the pathogenic outcome of cytoskeletal alterations and direct

activities vs. global effects resulting from rearrangements, and the precise mechanisms of bacterial adherence to host tissues remain unclear.

The *V. parahaemolyticus* VopZ effector protein (not to be confused with the *V. cholerae* effector VopZ, function unknown) is a bifunctional protein important for colonization and intestinal fluid accumulation in the infant rabbit model [60]. Investigators also identified N-terminal domain sequences required for inhibiting TAK1 kinase activation and thus interfering with the NF κ B and MAPK signaling pathways. The results thus suggest that like other T3SS positive bacteria, vibrios can modulate the immune response during infection. In *V. cholerae* strain AM-19226, the VopZZ effector (a VopZ homolog) is absolutely required to cause cytotoxicity *in vitro* and colonization *in vivo*, although molecular activities remain to be uncovered and are difficult to reliably predict based on sequence similarity (Dziejman laboratory, unpublished data).

Regions flanking the structural core are mosaic and encode diverse proteins.

Notable T3SS2 genomic island diversity in terms of size (~47kb~100kb) and genetic content is conferred by 5' and 3' flanking regions, which carry sequence remnants consistent with lateral acquisition events [1,2]. The *V. cholerae* 5' genomic island flanking sequences encode the VopE and VopX effector proteins [40]. Although VopE is not required for infant mouse colonization, a conserved Rho GTPase-activating domain is responsible for an activity that interferes with mitochondrial dynamics and innate immune responses that utilize mitochondria as a signaling platform [18,40,61-63]. VopX is dispensable for colonization in the infant mouse model, but mediates a cell growth defect in *S. cerevisiae* by interacting with components of the cell wall integrity (CWI) MAPK pathway, similar to results observed when VopE is expressed in yeast [64,65]. In place of VopX, some strains encode an effector similar to *Shigella* OspB, which has been shown to modulate the host inflammatory response [66].

The 5' and 3' flanking regions in *V. parahaemolyticus* are more variable and can encode VopO, VopT, VopC, VopA/P and/or VopG, although *vopT* is not present in T3SS2 stains. The unique VopO effector has no known homologues, but is critical for host cell stress fiber formation and epithelial barrier disruption *in vitro* [67]. VopT functions as an ADP-ribosyltransferase that targets the mammalian small G protein, Ras, and plays a role in *in vitro* cytotoxicity of Caco2 and HCT-8 cells [68]. VopA/P (a YopJ homolog, independent from the VopA encoded within the core region) is an acetyltransferase that inactivates MAP Kinase proteins through acetylation [69,70]. Although widely distributed in *V. cholerae* and *V. parahaemolyticus*, VopG (function unknown) is not required for *V. cholerae* infant mouse colonization and combined with the variable location, led to exclusion as a core cluster effector.

The ability of *V. parahaemolyticus* strain RIMD 2210633 (T3SS2 α) to invade HeLa and Caco2 cells as well as HeLa cell invasion by T3SS2 β *V. cholerae* strain 1587 has been attributed to VopC effector protein activity [71-73]. Limited *Vibrio* species invasion was documented more than 30 years ago prior to T3SS identification, and although the T3SS status of all strains in earlier studies is unknown, it is interesting to note that strain 1587 was among the strains analyzed [74-78]. However, current data indicate that the presence of a

T3SS cannot be strictly correlated with an invasive phenotype, and VopC is not required for *V. parahaemolyticus* colonization or fluid accumulation in the infant rabbit model, nor is it present in *V. cholerae* T3SS α clade strains [73]. Furthermore, *in vivo* imaging data from infant rabbit model studies and recently published evidence of intracellular K⁺ levels in target cells serving as a signal to switch secretion from middle (translocator) to late (effector) substrates strongly support the predominantly extracellular nature of the vibrio-host relationship during gastrointestinal infection [49,50,79].

The *V. cholerae* mosaic 3' region (~6.4 kb-17 kb) is comprised of sequences that lie downstream of VopF/N/L. Most *V. cholerae* strains encode four effector proteins in the 3' region: VopG, VopK, VopY, and VopZ (which is not a *V. parahaemolyticus* homolog despite the same name) [40], although VopY is annotated in a limited number of strains. In AM-19226, no 3' encoded effector is required for infant mouse colonization, but in an infant rabbit model of infection, moderate reductions in the incidence and severity of diarrhea as well as a slight decrease in colonization is observed during infection with VopK or VopY deletion strains [18]. In yeast, VopK toxicity is dependent on residues in the C-terminal domain postulated to comprise an MCF1-SHE serine peptidase domain, although peptidase activity was not detected and motif conservation is imprecise [80].

Conclusions

As we continue to recognize and catalog both similarities and differences, we begin to uncover how *Vibrios* have diversified T3SS functions to suit specific roles, adaptations, or environments. Thus, it seems likely that effector proteins present in the conserved core region of all T3SS2 islands dictate common mechanisms employed during infection, such as colonization, whereas the mosaic regions encode unique sets of effector proteins that may dictate specific characteristics of infection. Although T3SS1 has been difficult to definitively associate with disease in non-human pathogens, discovering T3SS1 encoded effector associated phenotypes has provided insight into how *Vibrio* encoded effectors interface with host proteins and pathways. As both *V. cholerae* and *V. parahaemolyticus* are considered to be environmental organisms that can act as human pathogens, we must consider whether the T3SS2 provides beneficial phenotypes in the natural aquatic reservoir. Matz *et al.* demonstrated that T3SS2 promotes *V. parahaemolyticus* survival during co-culture with a marine flagellate, which correlates with flagellate killing, and ciliates and amoeba were also susceptible to T3SS2-mediated killing [81]. Further studies examining the molecular mechanisms of effector protein function will help to elucidate how these proteins collectively or individually contribute to bacterial fitness and survival in the environment. Indeed, many challenges remain: to identify which effectors are both necessary and sufficient for colonization, to identify effectors specific for activity in the human host during disease, to determine whether effectors are required for a particular niche or lifestyle, and to elucidate effector protein functions, be they unique or redundant within a strain or across T3SS clades and species.

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Highlights

- *Vibrio* spp. can encode one or two unique T3SSs
- An environmental role for the T3SS remains largely unknown
- Type 3 Secretion System 2 genomic islands in *Vibrio* species have conserved features
- Effectors encoded in the conserved, core gene cluster are critical for colonization
- Variable sequence flanking regions encoded effectors are clade and species-specific

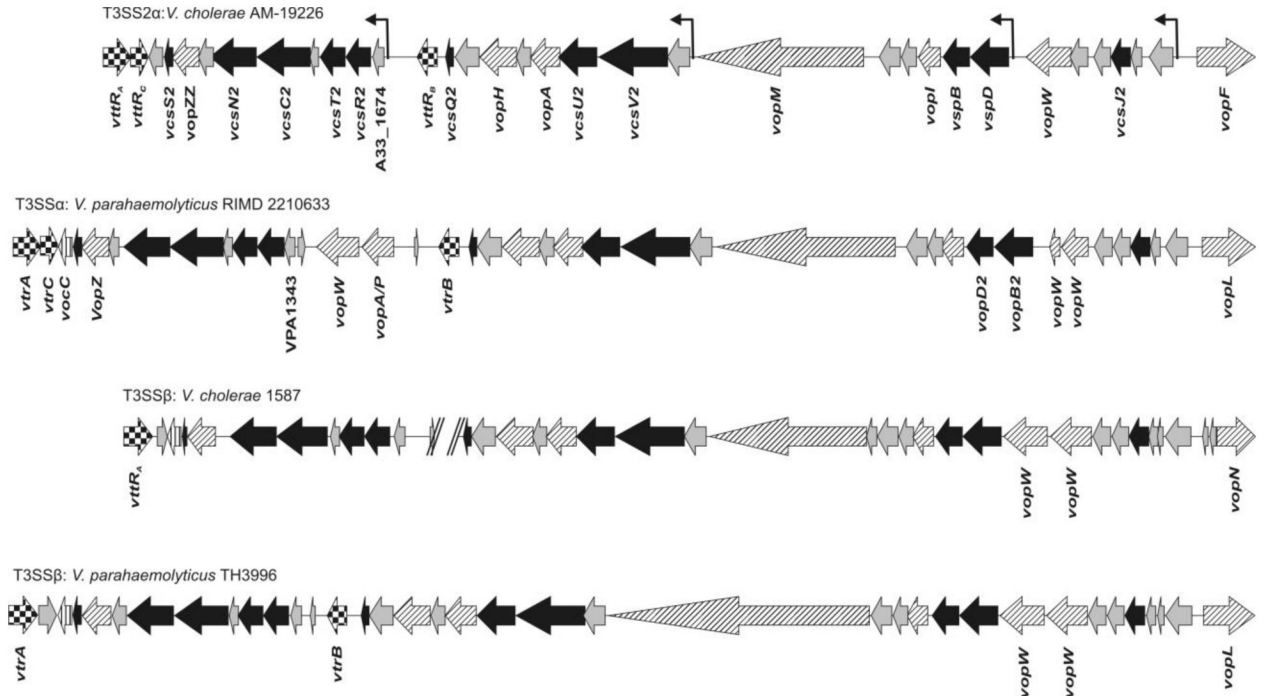


Figure 1. Genetic organization of representative *V. cholerae* and *V. parahaemolyticus* T3SS2 island core regions. T3SS2 α clade islands are depicted for *V. cholerae* strain AM-19226 and *V. parahaemolyticus* strain RIMD 2210633. T3SS2 β clade islands are depicted for *V. cholerae* strain 1587 and *V. parahaemolyticus* strain TH3996. Black arrows designate structural apparatus genes, checkered arrows designate transcriptional regulator genes, diagonally lined arrows designate effector protein genes, vertically lined arrows designate chaperone genes, and gray arrows designate genes predicted to encode hypothetical or conserved hypothetical proteins. In addition to *vopW* position and copy number, differences include a variable length sequence between *vcsR2* and *vtrB*, encoding one of two ToxR-like T3SS transcriptional regulators. *vopW* is denoted as an effector protein gene here, although its role as a structural component has been described and is discussed in the text. Double hatch lines indicate the end of a contig in the NCBI sequence. Bent arrows above the genes indicate putative promoters for the three major operons, named for the genes encoding the 11 identified structural proteins in the core region: 1) *vcsRTCNS2*, 2) *vcsVUQ2*, and 3) *vcsJ2*, *vspD*, and *vspB* [45,82]. Transcriptional reporter fusion data combined with transcriptome analyses suggest the presence of a fourth promoter upstream of *vspD* (downstream of *vopW*). 5' and 3' genes and regulatory genes are labeled in all four strains for positional reference. Otherwise, only genes differing in position among the four strains are labeled.

Table 1.

T3SS Nomenclature. Although a universal nomenclature remains elusive (even between *Vibrio* species), Table 1 provides a reference to itemize T3SS components. Note that *Vibrio* ORFs encoding the needle filament, the needle length control protein, pilotin, and inner rod were not identified by initial sequence annotation and remain unidentified.

Predicted Function	<i>Yersinia</i> spp.	<i>Salmonella</i> SPI-1	<i>V. parahaemolyticus</i> T3SS2	<i>V. cholerae</i> T3SS2
IM ring	LcrD/YscV	InvA	VscV2	VcsV2
IM ring	YscU	SpaS	VscU2	VcsU2
IM ring	YscR	InvL/SpaP	VscR2	VcsR2
IM ring	YscT	InvN/SpaR	VscT2	VcsT2
IM ring	YscS	SpaQ	VscS2	VcsS2
IM ring	YscD	PrgH	-	VopH?
Periplasmic ring	YscJ	PrgK	VscJ2	VcsJ2
Inner Rod	YscI	PrgJ	-	-
ATPase	YscN	InvC/SpaL	VscN2	VcsN2
Cytoplasmic ring	YscQ	InvK/SpaO	VscQ2	VcsQ2
Complex with ATPase	YscL	-	-	-
Secretin (OM ring)	YscC	MxiD	VscC2	VcsC2
Pilotin	YscW	MxiM	-	-
Needle	YscF	PrgI	-	-
Needle length determinant	YscP	InvJ	-	-
Hydrophilic translocator	LcrV	SipD	VopW	VopW
Translocon	YopB	SipB	VopB2	VspD2
Translocon	YopD	SipC	VopD2	VspB2

SPI-1, *Salmonella* pathogenicity island 1; IM, inner membrane OM, outer membrane