

REVIEW ARTICLE

Type Six Secretion System (T6SS) in Aquatic Pathogens

Mary Nia M. Santos 

Aquaculture Research and Development Division, National Fisheries Research and Development Institute Corporate 101 Bldg. Mother Ignacia Avenue, South Triangle, Quezon City 1103 Philippines

ABSTRACT

For aquaculture to overcome challenges in diseases, preventive and therapeutic interventions are needed. One solution of great potential is the use of bacterial nanomachines. Protein secretion systems facilitates nutrient acquisition, communication and disease through the delivery of virulence factors. The Type VI secretion system is one of the protein secretion systems that is extremely widespread and targets bacterial and eukaryotic cells for fitness and pathogenicity. The T6SS function can be redirected as target for vaccine development and therapeutics for aquaculture application. Choosing a strain that encodes T6SS and understanding its function and activity are vital to accomplishing this. This review outlines the current knowledge on the function, organization and regulation of T6SS in aquatic pathogens, including important fish and crustacean pathogens *Vibrio*, *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Pseudomonas* and *Francisella*. Overall, to date, the T6SS in *Vibrio* and *Edwardsiella* are the two well studied groups. The review identifies gaps in research and direct future studies for the development of technologies to control diseases caused by pathogens of aquaculturally important species. Future research and development on T6SS can be applied to important, newly emerging and re-emerging bacterial, viral and fungal diseases.

*Corresponding Author: nia.santos@nfrdi.da.gov.ph

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1. INTRODUCTION

Many bacterial pathogens use a variety of protein secretion systems to deliver virulence effectors into hosts. Type III, IV and VI secretion systems are important virulence determinants (Yu and Lai, 2017). The Type VI Secretion System (T6SS) is encoded by about 25 % of Gram-negative bacteria including human, animal, plant pathogens and non-pathogens (Bingle et al., 2008).

In all T6SS identified to date, 13 conserved and essential genes make up a core gene cluster that is common among all species (Boyer et al., 2009). The core components of the machinery is composed of membrane complex (*tssJ*, *tssL*, *tssM*), baseplate (*tssK*, *tssE*, *tssF*, *tssG*, *vgrG*), tube (*hcp*), and contractile sheath (*tssB*, *tssC*) that surround the tube and attaches to the baseplate (Brackmann et al., 2017). ClpV is a disassembly ATPase that provides energy for the machine.

The assembly starts with membrane complex formation to position the baseplate. The baseplate serves as a platform for contractile tail elongation. The tube is built by stacked Hcp rings and tipped with a puncturing VgrG and PAAR encoding protein (Shneider et al., 2013). Sheath contraction propels the VgrG carrying the effectors toward the target. After delivering effectors, the sheath is disassembled and subunits are recycled to build another one (Figure 1). In most of the aquatic pathogen encoding T6SS, the T6SS gene cluster is designated as *tss* or type six secretion (*tssA-tssM*). In *Edwardsiella*, the T6SS is called *Edwardsiella virulence protein* (EVP) gene cluster encoding *evpA-evpO*. While in *Vibrio anguillarum* and *V. crassostreae*, this is called *Vibrio type six secretion* encoding *vtSA-I*. The homologs in T6SS gene clusters discussed in this review are summarized in Table 1.

T6SS confers competitive advantage against bacterial competitors, helps in adapting to stress conditions (Yu et al., 2021) and facilitates metal ion uptake (Lin et al., 2017). There has been a wide

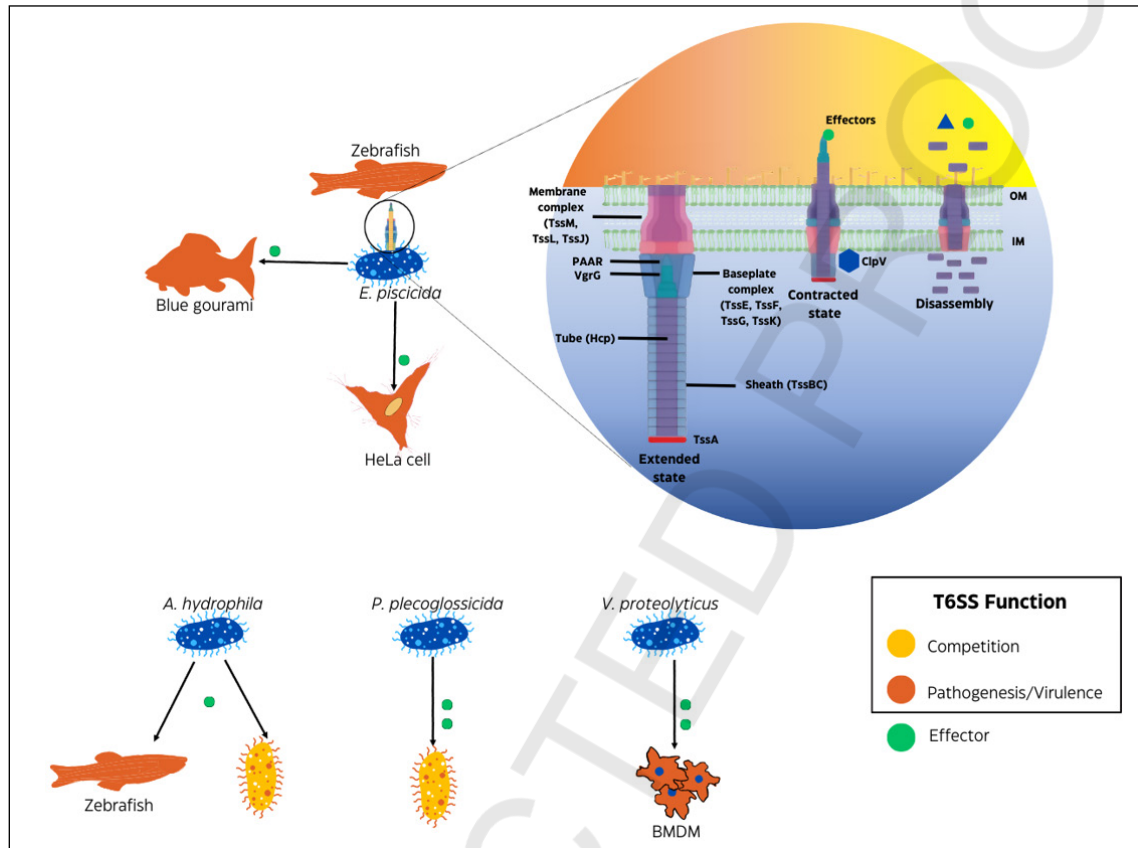


Figure 1. The Type VI Secretion System components, assembly and functions in aquatic pathogens. T6SS encoding aquatic pathogen (blue) delivers effectors (green circle) to other bacterium for inter-bacterial competition (yellow) and eukaryotic cells for pathogenesis (orange) as shown by *in vitro* (i.e. HeLa cells, bone marrow-derived macrophages or BMDM) and *in vivo* fish infection model discussed in this review.

distribution of interbacterial T6SS in *Vibrio* species according to recent studies. In the colonization of squid, the *V. fischeri* T6SS is responsible for controlling competition (Speare et al., 2018). T6SS has also been reported to be involved in pathogenesis (Pukatzki et al., 2006; Wang et al., 2022). One protein that contains the forkhead-associated (FHA) domain, TagH, is involved in regulating the virulence of *V. cholerae* (Wang et al., 2022). The T6SS of coral and shellfish pathogen *Vibrio coralliilyticus*, mediates mortality in brine shrimp *Artemia nauplii* and macrophages (Mass et al., 2024). Additionally, it has been observed that T6SS influences the microbiome makeup of polymicrobial communities (Tang et al., 2022).

To date, there are few studies on the characterization of T6SS in pathogens causing diseases in aquaculture (Table 2). An overview of the current understanding of T6SS encoded in significant aquatic pathogens is discussed in this paper.

In addition, future perspectives are provided on the possible application of T6SS. Since diseases

result in the largest financial losses in aquaculture, new approaches to disease prevention, management, and treatment are urgently needed. The T6SS can be utilized as a delivery platform to target different locations (Allsopp and Bernal, 2023). Using T6SS over other protein secretion systems for delivery has several advantages. First, it does not require a receptor to deliver effectors to the cytoplasm of the target cell. Secondly, in contrast to T3SS, which can only deliver unfolded proteins, T6SS can deliver folded proteins, their native conformation. Thirdly, it is extremely widespread, encoded by one-fourth of Gram-negative bacterial genomes and has a wide array of target cells, targeting both eukaryotic and prokaryotic cells.

2. *Vibrio* sp.

Vibrio is capable of causing diseases in a variety of marine animals including corals, shrimp, mollusks, and fish, both wild and farmed (Rubio et al., 2019).

Table 1. Homologs of T6SS genes and their function described in this review. The T6SS gene cluster is called *Edwardsiella virulent protein* (Evp) in *Edwardsiella* spp; type six secretion (Tss) in *Vibrio parahaemolyticus* and *V. proteolyticus*; and *Vibrio* type six secretion (Vts) in *Vibrio anguillarum* and *V. crassostreae*.

Edwardsiella spp.	<i>V. parahaemolyticus</i> , <i>V. proteolyticus</i>	<i>V. anguillarum</i> , <i>V. crassostreae</i>	Function
EvpA	TssA		assembly chaperone and tube/sheath cap
EvpB	TssB	TssB	contractile sheath
EvpC	Hcp	Hcp	tube
EvpD	N/A		
EvpE	TssC	TssC	contractile sheath
EvpF	N/A		
EvpG	TssE		baseplate
EvpH	TssH	TssH/ClpV	Sheath disassembly
EvpI	VgrG	VgrG	Spike
EvpJ	N/A		
EvpK	N/A		
EvpL	TssJ	VtsF/TssJ	membrane complex
EvpM	TssK	VtsG/TssK	baseplate
EvpN	TssL	VtsH/ IcmH/DotU	membrane complex
EvpO	TssM	VtsI/TssM/IcmF	membrane complex
N/A	TssF	TssF	baseplate
N/A	TssG	TssG	baseplate
N/A	N/A	VtsE	
N/A	N/A	VtsA	not found in other characterized T6SS
N/A	N/A	VtsB	not found in other characterized T6SS
N/A	N/A	VtsC	not found in other characterized T6SS
N/A	N/A	VtsD	not found in other characterized T6SS

Vibrio parahaemolyticus

Initial studies revealed that *Vibrio parahaemolyticus* encodes two T6SS, namely T6SS1 and T6SS2, encoded on chromosome 1 and 2, respectively (Yu et al., 2012). The T6SS2 gene cluster is encoded by all *V. parahaemolyticus* strains, but not the T6SS1 (Jana et al., 2022). A recent pan genome analysis identified two additional clusters with very limited distribution – T6SS3 and T6SS4, which may have been acquired through horizontal gene transfer (Jana et al., 2022). The third T6SS gene cluster was named T6SS3 because of its similarity in genetic structure to *V. proteolyticus* T6SS3 described in Ray et al. (2017). T6SS3 gene cluster homologs are also found in other pathogenic *Vibrio* spp. including *V. proteolyticus*, *V. vulnificus*, *V. splendidus*, *V. crassostreae* and *V. anguillarum* (Cohen et al., 2022). The T6SS4 in some strains are encoded either in a plasmid or chromosome, such as in *V. parahaemolyticus* strain VP157 and strain 160807, respectively, which are both

isolated from white leg shrimp (*Penaeus vannamei*). Yu et al. (2012) found differences in the role of T6SS1 and T6SS2 in cell adhesion. T6SS2 mediates adherence to HeLa cells, whereas T6SS1 is implicated in cell adhesion as evaluated using Caco-2 and HeLa cells. As a result of deleting the entire T6SS gene cluster 1 (*t6ss1*), there was a reduction in bacterial adhesion to the Caco-2 cells. While in HeLa cells, both $\Delta t6ss1$ and $\Delta t6ss2$ showed decreased adhesion to HeLa monolayers. The difference in results may be due to the surface characteristics of the cells such as receptors that interact with T6SS.

Li et al. (2017) demonstrated that the T6SS1 was significantly varied across *V. parahaemolyticus* strains and found two sites—referred to as Sites 1 and 2. In shrimp, *Vibrio parahaemolyticus* is a major cause of acute hepatopancreatic necrosis disease or AHPND (Lai et al., 2015). It was suggested that T6SS is associated with AHPND. A comparative genome sequence analysis demonstrated that the T6SS gene cluster is a distinguishing factor between strains that

Table 2. Function of Type VI Secretion System in aquatic pathogens

Organism	Function	Reference
Vibrio spp.		
<i>V. vulnificus</i>	Antibacterial activity	Hubert and Michell, 2020
<i>V. tasmaniensis</i>	Phagocytosis dependent cytotoxicity	Rubio et al., 2019
<i>V. crassostrea</i>	No reported characterized function yet, only genome analysis	Bruto et al., 2017; Rubio et al., 2019, Cohen et al., 2022
<i>V. parahaemolyticus</i>	Bacterial adhesion, pathogenicity in shrimp; antibacterial activity	Yu et al., 2012; Salomon et al., 2013; Wang et al., 2022
<i>V. alginolyticus</i>	Antibacterial activity; bacterial adhesion, motility and pathogenicity to zebrafish	Yang et al., 2018; Wu et al., 2023
<i>V. anguillarum</i>	Stress response and quorum sensing	Weber et al., 2009
<i>V. harveyi</i>	Not characterized yet, only genome analysis; T6SS component as a putative antigen and fish vaccine	Fu et al., 2021; Hu and Sun, 2011; Sun et al., 2019
<i>V. proteolyticus</i>	Antibacterial activity, toxicity to HeLa cells, actin rearrangements in yeast and macrophage cells; pathogenicity to brine shrimp <i>Artemia salina</i> nauplii	Ray et al., 2017; Cohen et al., 2023; Salomon et al., 2014
Aeromonas spp.		
<i>A. hydrophila</i>	Antibacterial activity, bacterial adhesion, biofilm formation, virulence to zebrafish, channel catfish and grass carp	Wang et al., 2018; Ma et al., 2020; Tekedar et al., 2019
<i>A. veronii</i>	Antibacterial activity, virulence regulation, biofilm formation, bacterial adhesion, motility and pathogenicity to zebrafish and crucian carp	Song et al., 2020; Wang et al., 2023
<i>A. salmonicida</i>	Bacterial adhesion, biofilm formation, extracellular product secretion and pathogenicity to grouper	Cai et al., 2022
Edwardsiella spp.		
<i>E. piscicida</i>	Fish phagocyte regulation, virulence to blue gourami, zebrafish, Japanese flounder and blue gourami; and bacterial translocation/invasion/colonization, calcium flux	Rao et al., 2004; Zheng and Leung, 2007; Hu et al., 2014; Wang et al., 2009; Tan et al., 2019; Zhang et al., 2018; Li et al., 2021; Chen et al., 2017
<i>E. ictaluri</i>	Pathogenicity to catfish fingerlings, bacterial adhesion, colonization, internalization, replication	Rao et al., 2004; Abdelhamed et al., 2018; Kalindamar et al., 2020; Kalindamar et al., 2021; Kalindamar et al., 2023
<i>E. anguillarum</i>	Not characterized yet, only genome analysis	Shao et al., 2015
Pseudomonas spp.		
<i>P. plecoglossicida</i>	Pathogenicity to large yellow croaker, grouper, biofilm formation, motility, bacterial adhesion and replication, antibacterial activity, chemotaxis,	Tao et al., 2020; Huang et al., 2019; Li et al., 2022; Luo et al., 2019; Yang et al., 2023; Wang et al., 2018; Jin et al., 2021
Francisella spp.		
<i>F. noatunensis</i> subsp. <i>orientalis</i>	Pathogenicity to zebrafish	Hansen et al., 2021
Flavobacterium spp.	Not characterized yet, only genome analysis	Tekedar et al., 2017; Kumru et al., 2020

cause AHPND and those that do not (Li et al., 2017). In *V. parahaemolyticus* that does not cause AHPND, the T6SS gene is not present.

In another study, Yang et al. (2019) examined genetic variations among 15 strains of *V. parahaemolyticus* and found that the genetic structure of T6SS1 is genotype-dependent. Wang et al. (2022) demonstrated that T6SS gene clusters in *V. parahaemolyticus* may be horizontally acquired between chromosomes and plasmids. Based on

the comparison of genes from chromosomes and plasmids, it was determined that there are no distinct groups of T6SS core genes.

The T6SS1 of *V. parahaemolyticus* has been characterized as an antibacterial system, found in pathogenic isolates predominantly in AHPND-causing isolates. It has been demonstrated that T6SS1 is active under marine-like conditions, whereas T6SS2 is active under low salt conditions, as demonstrated by the production of Hcp1 and Hcp2 under such

conditions (Salomon et al., 2013). Further, the T6SS-mediated killing is strain specific, targeting *V. cholerae*, *E. coli*, *Yersinia pseudotuberculosis* and *Vibrio natriegens* but not *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. The T6SS gene cluster of *V. parahaemolyticus* VP157, isolated from white leg shrimp (*Penaeus vannamei*), is encoded in a plasmid and the antibacterial activity is T6SS-dependent (Wang et al., 2022). In a co-culture assay, the wildtype exhibited higher antibacterial activity against gram negative bacteria (*V. cholerae* and *E. coli*) and gram-positive bacterium (*Bacillus pumilus*), compared to T6SS deletion mutant strain ($\Delta t6ss$). A mock microbial community comprising seven commonly found bacteria in shrimp ponds was used to test the survival of wildtype and T6SS deficient strains. Results showed that the growth wildtype was significantly higher and it is T6SS dependent, suggesting that T6SS may be able to alter microbiome compositions of shrimp ponds (Wang et al., 2023a). For the regulatory mechanisms of *V. parahaemolyticus*, each T6SS cluster is differentially regulated by quorum sensing. T6SS1 and T6SS2 are negatively and positively regulated by the quorum sensing master regulator OpaR (Salomon et al., 2013).

Vibrio vulnificus

Three biotypes are recognized based on the biochemical traits of *Vibrio vulnificus*. Biotype 2 causes warm-water vibriosis, an acute hemorrhagic septicemia affecting eels and other teleost. Several important aquaculture species are susceptible to *V. vulnificus*, including eels (*Anguilla* sp.), tilapia (*Oreochromis* sp.), grass carp (*Ctenopharyngodon idella*), trout (*Oncorhynchus mykiss*), pompano (*Trachinotus ovatus*) and shrimp (*P. vannamei*) (Amaro et al., 2015; Janampa-Sarmiento et al., 2024). The genome of *V. vulnificus* encodes T6SS1 and T6SS2 each with 13 core genes (Church et al., 2016). Interestingly, only a small number of *V. vulnificus* strains had T6SS1, however all sequenced strains have been shown to have T6SS2.

Functional characterization revealed that *V. vulnificus* T6SS plays an important role in interspecies and intraspecies competition in oyster hosts (Hubert and Michell, 2020). The antibacterial activity is thermoregulated, T6SS1 and T6SS2 are both active at 21 °C, but only T6SS1 is active at 30 °C. When the wildtype *V. vulnificus* 106-2A was used to compete at 30 °C, it reduced the survival of both *V. vulnificus* 99-743

and *Salmonella enterica* serovar Enteritidis CC012 by 1000 fold. There is a T6SS dependency as no reduction was observed with the T6SS deletion mutant ($\Delta hcp1$). On the other hand, a 10-fold reduction in the survival of target *V. vulnificus* 99-743 was observed when cocultured with wildtype strain and no reduction with T6SS2 deletion mutant ($\Delta hcp2$; $\Delta icmF2$) at 21 °C (Hubert and Michell, 2020; Church et al., 2016). The IcmF (intracellular multiplication protein F), initially identified as T4SS component share similarities with TssM (Bonemann et al., 2010; Li et al., 2019). Further, no killing was observed when cocultured with the deletion of both T6SS1 and T6SS2 ($\Delta hcp1\Delta hcp2$). Interestingly, the reduction of killing of *S. enterica* serovar Enteritidis CC012 can only be observed in $\Delta hcp1\Delta hcp2$ and no significance difference can be observed between the wildtype, $\Delta hcp1$ and $\Delta hcp2$ alone. Aside from thermoregulation, T6SS in *V. vulnificus* is negatively regulated by increasing salinity concentrations (Church et al., 2016).

There is no T6SS-dependent anti-eukaryotic activity in *V. vulnificus* when tested against *Dictyostelium discoideum* and *Galleria mellonella* (Hubert and Michell, 2020; Church et al., 2016). Using plaque assay, plaques were observed on T6SS deletion mutants $\Delta hcp1$ and $\Delta hcp2$, indicating predation by *D. discoideum* (Hubert and Michell, 2020). The virulence is not attenuated in $\Delta icmF1$ and $\Delta icmF2$ in *G. mellonella* infection assay (Church et al., 2016).

Vibrio tasmaniensis

After being isolated from diseased oysters, the virulence of *V. tasmaniensis* was confirmed in oyster infection experiments (Gay et al., 2004). The hemocyte intracellular pathogen *V. tasmaniensis* LGP32 encodes the T6SS1 and T6SS2 gene clusters. T6SS1 is encoded in three other virulent *V. tasmaniensis* strains analyzed and absent from avirulent strains (Rubio et al., 2019). All the virulent strains contain an ortholog *evpP*, a T6SS effector in *Edwardsiella tarda*. T6SS is induced upon oyster colonization. As a result of inactivating *vipA* in the T6SS1 gene cluster, oyster experimental infections were rendered non-virulent and no cytotoxicity on oyster hemocytes was observed. VipA is a homolog of TssB, a component of the T6SS contractile sheath. This implies that the cytotoxicity depends on phagocytosis and necessitates the T6SS. In contrast, T6SS2 inactivation showed no effect on cytotoxicity or virulence, indicating that it has no effect on the host-pathogen interaction.

Vibrio crassostreae

The T6SS genes are present in all *V. crassostreae* virulent strains, including J2-9, J5-4, LGP8 and J5-20 (Rubio et al., 2019). In the virulent strain J2-9, T6SS is found on pGV1512, the virulence plasmid for *V. crassostreae*. The T6SS gene cluster consist of *vtsA-vtsI*. The T6SS was induced eight hours after experimental infection of oyster (*Crassostrea gigas*) with J2-9. Further, the mobile genetic elements involved in conjugative transfer were also highly induced. This corroborates with Cohen et al. (2022) wherein the T6SS gene cluster is encoded next to mobile elements including integrase or transposase.

A T6SS deletion was generated in *V. crassostreae* strain J2-9 using allelic exchange and assessed for virulence to specific pathogen free (SPF) oysters. The T6SS deletion did not affect the virulence of *V. crassostreae* after 24 hours post infection (Bruto et al., 2017).

Vibrio alginolyticus

The T6SS expression in *V. alginolyticus* is mediated by quorum sensing (QS) regulators and alternative sigma factor (Sheng et al., 2012). Positive regulation is provided by LuxO, and negative regulation is provided by LuxR and LuxS. Moreover, the enhancer-binding protein VasH and the alternative sigma factor RpoN control the expression of T6SS1. A mutation in RpoN or VasH caused a decrease in the expression of Hcp1, suggesting that those genes are positive regulators of Hcp1. In addition, LuxR, RpoN, and VasH may play a role in regulating the expression of other T6SS genes such as *clpV1*, *icmF1* and *vasA1*, a homolog of *tssF* (Sheng et al., 2012).

T6SS1 and T6SS2 are present in the strain EPGs of *Vibrio alginolyticus* (Sheng et al., 2013). The T6SS2 gene cluster encodes the phosphatase PppA that participates in a complex regulatory network that also involves the T6SS substrate Hcp1, quorum sensing and 30,50-cyclic diguanylic acid (c-di-GMP) (Sheng et al., 2013). Whole genome transcriptome analysis has revealed a number of PppA regulatory targets, including flagellar proteins, exotoxin alkaline serine protease (Asp), quorum sensing regulator LuxR, Hcp, and proteins involved in polysaccharide biosynthesis and transport. PppA negatively regulates the Hcp1 expression and secretion. The deletion of *pppA* could induce the transcription and protein expression of *hcp1*. Upon deletion of *pppA*, c-di-GMP expression increased and LuxR and the exotoxin Asp were decreased. Further, cell aggregation, increased biofilm

formation and reduced swarming were observed in $\Delta pppA$.

The fish-isolated *V. alginolyticus* EPGs has T6SS2-antibacterial activity against *E. coli*, *V. alginolyticus*, and *Edwardsiella* (Yang et al., 2018). The bacterial competition assay against *E. coli* showed that $\Delta hcp1$ had a similar killing as wildtype strain. The $\Delta hcp2$ and $\Delta hcp1\Delta hcp2$ were unable to kill but killing was restored when *hcp2* is complemented in $\Delta hcp1\Delta hcp2$ strain. PpkA2, a serine-threonine kinase PpkA homolog encoded in *V. alginolyticus* T6SS2 modulates antibacterial activity and Hcp2 secretion. The $\Delta pppA2$ was defective in killing *E. coli*. Moreover, the activation of T6SS2 is controlled by PpkA2 phosphorylation and quorum sensing. The phosphoproteomic analysis showed the substrates of the T6SS regulator PpkA2 kinase. T6SS2 function depended on PpkA2 and VtsR (*Vibrio* type six secretion regulator) phosphorylation. It is believed that VtsR phosphorylation controls the expression of the quorum sensing (QS) gene by regulating the expression of LuxR, one of the key regulators of QS. This suggest that PpkA2 phosphorylation cascade regulates T6SS and quorum sensing pathways in concert since LuxR is required for the expression of T6SS2 (Yang et al., 2018).

Wu et al. (2023) showed that the T6SS of *V. alginolyticus* is involved in mediating motility, adhesion, and pathogenicity by impacting its flagellar system. The *V. alginolyticus* HY9901 with $\Delta hcp2$ deletion impaired the swarming motility, reduced adhesion, and attenuated the virulence against zebrafish. Transmission electron microscopy (TEM) revealed abnormal morphology of flagella, the $\Delta hcp2$ had severely hollow-like structure of flagellar filament. Further, the levels of three flagellum FlaA, FlaB, and FlaC protein and assembly-associated proteins (i.e. FliH, FliF and FlgE) were decreased in $\Delta hcp2$. The transcription of the three flagellum genes and sigma factors *rpoN*, *fliA*, and *rpoS* were also reduced in the $\Delta hcp2$ strain.

Vibrio anguillarum

Vibrio anguillarum affects freshwater, brackishwater and marine fish, crustacean and bivalves, resulting in losses to aquaculture industry (Frans et al., 2011). Chemotactic motility and adhesion, hemolysin, metalloproteases, iron absorption system, lipopolysaccharides, outer membrane proteins, quorum sensing, and sigma factors are among the virulence factors that have been found (Guanhua et al., 2018).

On the basis of whole genome sequencing, it has been reported that *V. anguillarum* MVM425 a highly pathogenic strain isolated from moribund turbot in the China, encodes T6SS (Guanhua et al., 2018).

The *V. anguillarum* T6SS modulates stress response and quorum sensing (Weber et al., 2009). *V. anguillarum* strain NB10 serotype O1 isolated from a diseased fish, contains VtsA-H (vibrio type six secretion) proteins. The proteins VtsA-D are not found in other characterized T6SS. The sequence and protein secretion analysis of VtsE-H revealed that these are T6SS components. The VtsE-H are homologs of the forkhead-associated protein, outer membrane lipoprotein TssJ, TssK, IcmH-related protein/DotU and TssM, respectively. DotU is a conserved inner membrane component of T6SS, a T4SS-like component and similar to ImpK/TssL (Filloux et al., 2008). T6SS regulated the expression of metalloprotease EmpA and PrtV. The protease activity decreased in $\Delta vtsB$, $\Delta vtsE-H$ and Δhcp and increased in $\Delta vtsA$, $\Delta vtsC$ and $\Delta vtsD$. This regulation occurs as T6SS positively regulates the expression of RpoS, a stress response regulator and VanT, a quorum sensing master regulator, which then regulates the protease expression. A study of T6SS mutants exposed to hydrogen peroxide, ethanol or low pH was conducted to explore pigment production, *hpd* expression, and survival after exposure to these conditions. The gene *hpd* encodes a 4-hydroxyphenylpyruvate dioxygenase and functions in pigment production. T6SS may control the stress response since stress indicators increased in $\Delta vtsA$, $\Delta vtsC$, and $\Delta vtsD$ and decreased in $\Delta vtsB$, $\Delta vtsE-H$, and Δhcp . In addition, no attenuation of virulence was observed when rainbow trout was infected with *vtsA-H* mutants.

Vibrio harveyi

Vibrio harveyi is an opportunistic pathogen affecting the marine aquaculture industry (Austin and Zhang, 2006). Marine vertebrates and invertebrates affected include gilthead sea bream, sea bass, common dentex, oyster, Senegalese sole, tiger prawn, groupers, flounder, puffer fish and large yellow croaker (Tu et al., 2017; Fu et al., 2021). Virulence mechanisms of *V. harveyi* have been linked to extracellular synthesis of hemolysins, phospholipases, proteases, and cytotoxins (Zhang and Austin, 2000).

In *V. harveyi* QT520, which was isolated from diseased golden pompano grown cages in China, three T6SS gene clusters were identified (Tu et al., 2017). The whole genome sequencing of *V. harveyi* from diseased

fish collected from farms also showed three T6SS gene clusters (Fu et al., 2021). *V. harveyi* strains analyzed by comparative genomic analysis have highly conserved T6SS1 and T6SS2 genes.

Outer membrane proteins have been recognized as potential vaccine candidates. Sun et al. (2019) demonstrated that the *V. harveyi* TssJ is a putative antigen and can be used as vaccine. The sequence analysis, western blot and agglutination assay using the anti-TssJ antibody confirmed that the TssJ in *V. harveyi* is anchored in the outer membrane. Golden pompano was vaccinated using two different types of vaccines: DNA vaccine (pCTssJ) and recombinant subunit vaccine (rTssJ). The use of conserved antigens in a subunit vaccine was shown to be safer compared with the inactivated vaccine because there is no risk of reversing the virulence (Fu et al., 2021). While DNA vaccines has been proven to provide immune protection for fish, safe to use, easy to produce and store (Hu and Sun, 2011). After the challenge with *V. harveyi* QT520, the relative percent survival (RPS) reached 52.39% and 69.11% for rTssJ and pCTssJ, respectively. There was a significant increase in the amount of alkaline phosphatase (AKP), acid phosphatase (ACP), lysozyme, and superoxide dismutase (SOD) activity in fish vaccinated with rTssJ and pCTssJ, providing evidence that rTssJ may enhance the activation of antioxidant enzymes and lysosomes. Three to eight weeks after vaccination, both rTssJ and pCTssJ induced the production of specific serum antibodies, as determined by ELISA. Further, the transcription of immune related genes were upregulated four weeks after vaccination and challenge in rTssJ and pCTssJ with higher folds in pCTssJ (Sun et al., 2019). These findings imply that TssJ might be a useful antigen against *V. harveyi* infections and might be applied in the development of vaccines.

Using the VgrG of *V. harveyi* QT520, the same group created a DNA vaccine and a subunit vaccine which yielded higher RPS, 71.43% and 76.19% for rVgrG and pCNVgrG, respectively (Du et al., 2024). Both vaccinations were successful in eliciting an adaptive immunological response, resulting in increase in serum antibody production against rVgrG. Fish immunized with rVgrG and pCNVgrG also exhibited increased expression of immune genes and enzyme activity.

Vibrio proteolyticus

Vibrio proteolyticus was isolated from diseased Indo-Pacific corals and were shown to be virulent to

fish and *Artemia* (Cervino et al., 2008; Verschuere et al., 2000; Bowden et al., 2018). The genome of *V. proteolyticus* ATCC 15338 (NBRC 13287), isolated from a marine isopod intestine, (*Limnoria tripunctata*) encodes three T6SS gene clusters, T6SS modules and effectors that are not in the main gene clusters. The analysis of *V. proteolyticus* secretome revealed T6SS structural components such as Hcp, VgrG, PAAR-containing proteins and putative effectors with MIX domain, indicating the presence of functional T6SS (Ray et al., 2016). When HeLa cells were infected with *vgrG* deletion mutant ($\Delta vgrG1/2/3$) in each T6SS cluster, the LDH release was the same as with the wild type, indicating that the cytotoxicity of *V. proteolyticus* is not mediated by T6SS. The T6SS3 of *V. proteolyticus* is similar to T6SS3 of *V. parahaemolyticus* (Jana et al., 2022), which was suggested to have anti-eukaryotic activity and induce inflammasome-mediated cell death in macrophages.

Similar to *V. parahaemolyticus*, the *V. proteolyticus* T6SS1 mediates antibacterial activity under warm marine-like conditions, killing activity is prominent at 30°C with 3% NaCl (Ray et al., 2017). T6SS1 delivers polymorphic effectors to both bacterial and eukaryotic targets. The antibacterial action against *E. coli* and *V. parahaemolyticus* was lost in T6SS1 deletion mutant ($\Delta vgrG1$, $\Delta tssG1$), while neither deletion in T6SS2 ($\Delta vgrG2$) nor T6SS3 ($\Delta vgrG3$) showed any effects. The expression and secretion of VgrG1 are positively correlated with the antibacterial activity. The complementation of *tssG1* rescued the antibacterial activity and secretion. Using mass spectrometry, six putative antibacterial effectors and three putative anti-eukaryotic effectors were identified in the T6SS1 gene cluster. One of the effectors encode a MIX domain and cytotoxic necrotizing factor 1 (CNF1) domain which are deamidases that target and activate Rho GTPases. When exogenously produced in yeast and HeLa cells, it causes toxicity in the latter and actin cytoskeleton rearrangements and morphological abnormalities in the former. The phenotype of catalytic site mutant suggests that the observed effects were dependent on a functional CNF1 domain. Moreover, no actin rearrangements were observed in RAW 264.7 cells when infected with $\Delta tssG1$. When *V. proteolyticus* infects eukaryotic cells, the actin rearrangements produced in macrophages but not in HeLa cells appear to indicate that the uptake of T6SS1 by phagocytic cells may be required. The ability of *V. proteolyticus* to cause T6SS1-mediated alterations in macrophages was impaired by the deletion of CNF1-containing effector. The complementation of this effector, but not

its catalytic mutant, rescued the effect to induce actin rearrangements.

Bone marrow-derived macrophages (BMDM) undergo phagocytosis-dependent cellular death as a result of delivery of *V. proteolyticus* anti-eukaryotic T6SS3 effectors (Cohen et al., 2022). The activation of the NLRP3 inflammasome is caused by the effectors *tie1* (T6SS3 inflammasome-inducing effector 1) and *tie2*, which in turn cause the processing and release of caspase-1, IL-1 β , and caspase-1, gasdermin D (GSDMD). The absence of GSDMD activates a compensatory T6SS-induced pathway involving gasdermin E (GSDME) and caspase-3.

In another study, T6SS3 was shown to mediate virulence in the brine shrimp *Artemia salina* nauplii (Cohen et al., 2023). The survival rate of *Artemia* nauplii was significantly lower than that of wildtype and $\Delta vprh$ strains when challenged with *V. proteolyticus* $\Delta vprh/\Delta hns1$, strain with an active T6SS3. VPRH is a pore-forming hemolysin. T6SS is negatively regulated by H-NS (histone-like nucleoid-structuring protein) in *Vibrio*, and deletion attenuates this effect (Salomon et al., 2014). The deletion of T6SS1 ($\Delta tssG1$) and T6SS2 ($\Delta vgrG2$) did not affect the *Artemia* survival, while the T6SS3 deletion ($\Delta tssL3$) resulted in significantly higher survival of *Artemia* compared to $\Delta vprh/\Delta hns1$.

3. *Aeromonas* sp.

An important bacterial disease affecting aquaculture is Motile Aeromonas Septicemia (MAS). It is caused by motile aeromonads: *A. hydrophila*, *A. caviae*, *A. veronii* biovar *sobria* and *A. veronii* (Hanson et al., 2012; Legario et al., 2023). Disease signs include septicemia, inflammation of anus, external and internal hemorrhages, exophthalmia and abdominal swelling, leading to high mortality within a short period of time (Hanson et al., 2012).

Aeromonas hydrophila

As an opportunistic pathogen found in aquatic environments, *Aeromonas hydrophila* affects amphibians, birds, fishes, reptiles, and mammals. The hypervirulent *A. hydrophila* (vAh) causes outbreaks of MAS resulting to high mortality and economic losses in cultured carp including silver carp, bighead carp and common carp; channel catfish, and tilapia (Ma et al., 1998; Hossain et al., 2014; Aboyadak et al., 2015).

The T6SS is encoded in seven fish pathogenic *A. hydrophila* with complete genomes (Jin et al., 2020)

including large yellow croaker, diseased blunt nose sea bream, channel catfish, gold fish and crucian carp.

Comparative genome analysis revealed that T6SS is a key genotype that differentiates the vAh isolates. A virulent *A. hydrophila* GD18 isolated from diseased grass carp encodes a T6SS gene cluster with 25 conserved T6SS genes, including two *VgrG*. Another *hcp* and two *vgrG* are also encoded outside the main T6SS cluster (Li et al., 2021a). A complete set of T6SS gene cluster is found in vAh isolated from carp in China while an incomplete (lack majority of the T6 core genes), encoding only *hcp1*, *tssH*, and *vgrG* were found in isolates from channel catfish in the US (Rasmussen-Ivey et al., 2016; Tekedar et al. 2019).

In particular, *A. hydrophila* NJ-35 with one complete T6SS gene cluster is encoded on a genomic island (Pang et al., 2015). *Aeromonas hydrophila* NJ-35 is associated with high mortality rate in aquaculture. Three Hcp proteins are encoded together with a functional T6SS; one of them belongs to the main cluster of T6SS genes, while the other two belong to the other. In NJ-35, the three Hcp function differently in terms of environmental adaptation and virulence (Wang et al., 2018a). The secretion of *hcp* in wildtype, mutant and complementation strains using western blot revealed that *hcp1* functions in secretion and expression of Hcp proteins. In addition, *hcp1* plays a role in T6SS-mediated antibacterial activity. Growth inhibition in *E. coli* were observed when co-cultured with single and multiple *hcp* deletion mutants ($\Delta hcp1$, $\Delta hcp1/2$, $\Delta hcp1/3$, $\Delta hcp1/2/3$) and restored after complementation of *hcp1*. On the other hand, $\Delta hcp2$ and $\Delta hcp3$ deletion mutants showed the similar growth inhibition to the wild type and $\Delta hcp2/3$ mutant resulted to an enhanced antibacterial activity.

The $\Delta hcp1$ significantly decreased the adhesion to HEp-2 cells (derived from human epidermoid larynx carcinoma) compared to the wildtype. On the other hand, Hcp3 positively affects bacterial adhesion as revealed by an increase in adhesion capability in $\Delta hcp2$ and $\Delta hcp1/2$. The ability of $\Delta hcp1/2$ to form biofilms might indicate that Hcp3 play a role biofilm formation. Whereas, the ability of $\Delta hcp2$ to form biofilm significantly increased indicating that Hcp2 negatively impacts biofilm formation. The virulence of all the *hcp* single, double and triple deletion mutant except $\Delta hcp1/3$ were all higher than the wild-type strain suggesting that Hcp2 is involved in the virulence in zebrafish. Various *hcp* deletion mutants $\Delta hcp2$, $\Delta hcp1/2$, and $\Delta hcp1/2/3$ exhibited a significant increase in median lethal dose (LD50).

A. hydrophila NJ-35 secretes type VI lipase effector (Tle1) that is involved in virulence and biofilm formation. Ma et al. (2020) showed that the deletion of *tle1* ($\Delta tle1$) led to a significant decrease antibacterial competition ability, biofilm formation, and virulence. The $\Delta tle1$ had a reduced killing against *E. coli* and *V. parahaemolyticus* and an 11-fold higher LD50 in intraperitoneally injected zebrafish compared to wild-type strain.

Though having an incomplete T6SS gene cluster, *A. hydrophila* ML09-119, the deletion of *hcp1* and *vgrG1* attenuated the virulence to SPF channel catfish fingerlings (Tekedar et al. 2019). ML09-119 has caused bacterial septicemia outbreaks in channel catfish cultured in the United States. The mortality rate after the immersion challenge was significantly lower in $\Delta hcp1$ and $\Delta vgrG1$ compared with the wildtype. Compared to control, fingerlings surviving both T6SS mutant infections and re-challenge with wildtype have survival rates of 92% and 100%, respectively, whereas control fingerlings only have survival rates of 60%.

The attenuation of virulence in *A. hydrophila* ML09-119 corroborates with *A. hydrophila* NJ-35, but the latter encodes a complete T6SS while ML09-119 only encodes three T6SS core genes. It has been hypothesized that genomic islands associated with the Hcp and VgrG may drive functional diversification by acquiring T6SS effectors and development of evolved Hcp and VgrG proteins (De Maayer et al., 2011). Given that the T6SS in *A. hydrophila* NJ-35 is encoded on a genomic island, the mobilization of genetic islands by Hcp and VgrG may drive genomic variation in *A. hydrophila* T6SS (Tekedar et al. 2019). Future research is needed to elucidate the role of T6SS and the effect of a reduced or rearranged components on the virulence of *A. hydrophila*.

Conditional regulation of T6SS was observed in *A. hydrophila*. Low temperature strongly induced the expression of *hcp*, *tle1* and the transcriptional regulator *vasH*, while Hcp secretion was abrogated at 37 °C. Simulating *in vivo* conditions was achieved using fish serum with the assumption that T6SS expression increases during infection. As grass carp serum conditions were applied, *hcp*, *vasH*, *clpV*, and *dotU* transcriptional levels increased. Further, VasH is required for T6SS function in *A. hydrophila* strain GD18. The *hcp* transcription and expression was abolished in *vasH* deletion, indicating the inactivation of T6SS. With the deletion of *vasH* ($\Delta vasH$), significant decrease in *vgrG* and *tle1* transcription were observed. Importantly, *vasH* and *hcp* mutants abolished the antibacterial activity, suggesting that T6SS contributes

to the antibacterial activity and VasH mediates the antibacterial activity in *A. hydrophila* GD18. Virulence and systemic dissemination are dependent on VasH. The LD50 value of $\Delta vasH$ using a grass carp infection model is 1.19×10^3 compared to 2.73×10^2 in wildtype strain. Moreover, There was a significant reduction in bacterial loads in the spleen, kidney, and liver in $\Delta vasH$.

The mechanism of regulation needs to be determined in future investigations. It is probable that *A. hydrophila* strains may employ different mechanisms to adapt to different environments and hosts. Developing therapeutics, vaccines and antimicrobials against MAS could be made possible by future studies of VasH.

Aeromonas veronii

The spread of *A. veronii* is increasing and poses a serious threat to fish (Xu et al., 2022a, 2022b; Rahman et al., 2002). Disease outbreaks caused by *A. veronii* has been reported in cultured channel catfish in China and tilapia in Saudi Arabia (Liu et al., 2016; Hassan et al., 2017). The symptoms observed are skin ulcers, bleeding from organs and severe ascites (Austin and Austin, 2016).

The virulent *A. veronii* TH0426 isolate from Chinese yellow catfish farms, possesses a full T6SS (Song et al., 2020). A component of the T6SS membrane-bound complex is the inner membrane protein DotU. The *A. veronii* TH0426 *dotU* shares 97% sequence identity with *impK* of *Aeromonas salmonicida*. Higher expression of *dotU* in *A. veronii* TH0426 was observed compared to attenuated strain and non-virulent strain, suggesting its role in virulence regulation. Results showed that *dotU* contributed to biofilm formation and pathogenicity in *A. veronii* TH0426. DotU deletion mutants formed 2 fold more biofilms than wild type, indicating a significant increase in biofilm formation. Moreover, the $\Delta dotU$ LD50 was 50-fold higher than wild-type, suggesting that the deletion of *dotU* resulted to attenuation of pathogenicity. In addition, in comparison to wild type strain, the deletion of *dotU* exhibits a lower adhesion rate to EPC cells.

Wang et al. (2023) showed that T6SS regulates motility in *A. veronii* TH0426. The *hcp* deletion mutant had a reduced motility and loss of polar flagella. It has been shown that flagella influences virulence. Interestingly, the *hcp* deletion (Δhcp) had an increased expression of flagella-related genes, indicating a negative feedback loop may regulate

flagella-related genes in *A. veronii* TH0426. Further work is needed to clarify the mechanism on how *hcp* can regulate motility. Moreover, in comparison with the wildtype strain, the Δhcp strain was significantly less capable of forming biofilms and the killing ability against *E. coli* was reduced significantly and adhesion and invasion of EPC (endothelial progenitor cell) is lower compared with wildtype. Similar result was observed in *A. hydrophila* NJ-35. The inactivation of T6SS (Δhcp) and the consequent loss of polar flagella may reduce the ability of bacterium to adhere. The ability to form biofilm and adhere, and invade may be affected by the regulation of *hcp* via flagellar assembly. Furthermore, a reduction in LD50 was observed for zebrafish and crucian carp when compared with wild type strain and a significant reduction in bacterial load was observed in crucian carp, suggesting that the T6SS or *hcp* gene can affect *A. veronii* pathogenicity by regulating flagella assembly (Wang et al., 2023).

Aeromonas salmonicida

Furunculosis is caused by *Aeromonas salmonicida*, a bacterial septicemia of salmonids (Hiney and Olivier, 1999). It is widely distributed affecting wild and cultured salmon, rainbow trout, Atlantic salmon, black fin reef shark, flounder, and turbot (Cai et al., 2022). In the aquaculture industry, especially in salmon farming, mortality rates can reach 100% within a week after infection.

T3SS, polymeric extracellular substances, siderophores, quorum sensing, and S-layer proteins are associated with *A. salmonicida* pathogenicity (Cai et al., 2022). Virulence factors can also be related to other secretion system.

The *Aeromonas salmonicida* subsp. *salmonicida* A449 encodes 16 T6SS proteins on the chromosome and three T6SS genes on the plasmid (pAsa4) (Reith et al., 2008). However, it is probable that the T6SS in this strain is not functional because of several truncated T6SS genes. There is a possibility that insertion sequence elements assisted in the transfer of T6SS genes to pAsa4. Interestingly, while other species of *Aeromonas* are opportunistic pathogens, even very low levels of *A. salmonicida* infection cause disease in healthy fish (Daly et al., 1996).

A. salmonicida SRW-OG1 was isolated from diseased orange-spotted grouper (Huang et al., 2020). The RNAseq results revealed that the *hcp* expression in this strain was significantly affected by temperature. Therefore, Cai et al. (2022) explored the role of Hcp in virulence by creating a stable silent strain of *hcp*.

The silencing of *hcp* resulted to decreased adhesion, growth, biofilm formation, extracellular product secretion and enzyme activities, including protease, lipase, lecithinase, and caseinase. Importantly, groupers infected with the *hcp*-RNAi strain resulted in significantly delayed death and a lower mortality rate than the wild-type strain. The bacterial colonization in the spleen of infected grouper in *hcp*-RNAi group was significantly lower. Further, a high level of conservation of the *hcp* is found in *A. salmonicida* strains as well as *A. hydrophila* strains, which emphasizes its function in this group of bacteria. The prediction of protein structure of Hcp *A. salmonicida* SRW-OG1 revealed highest similarity with the *Vibrio cholerae* (PDBID: 5mxn1) with 78.49% amino acid sequence homology. In *A. hydrophila* NJ-35, *hcp* also affects biofilm formation, with *hcp3* and *hcp1* causing positive effects and *hcp2* causing negative effects (Wang et al. 2018a).

4. *Edwardsiella* sp.

There are five species of *Edwardsiella*, including three species of fish pathogen (*E. piscicida*, *E. anguillarum*, and *E. ictaluri*), and two non-fish pathogen species (*E. tarda* and *E. hoshinae*) (Bujan et al., 2018). *Edwardsiella piscicida* contains pathogenic *Edwardsiella* isolates previously identified as *E. tarda* on the basis of their phenotypic and genetic characteristics (Abayneh et al., 2013). After the reclassification, *E. tarda* includes human and environmental isolates and do not encode T6SS (Yang et al., 2012; Shao et al., 2015). Freshwater and marine fish are infected by fish pathogens, which have serious consequences for aquaculture worldwide.

Edwardsiella piscicida (old name *E. tarda*)

Aquaculture worldwide encounters a significant problem with Edwardsiellosis caused by *Edwardsiella piscicida*, an intracellular pathogen. In addition to its wide geographic distribution, it has been found in more than 20 fish host species including chinook salmon, eel, channel catfish, mullet, flounder, carp, tilapia, and striped bass (Park et al., 2012; Bujan et al., 2018). The clinical signs associated with Edwardsiellosis include exophthalmia, external and internal hemorrhages, dermal ulcerations, abdominal distension, surface discoloration, and erratic swimming.

Several virulence factors have been implicated in the pathogenesis of *E. piscicida*, including the production of exoenzymes (hemolysin), presence of

T3SS and T6SS, ability to adhere, invade, survive and reproduce in epithelial and phagocytic cells (Leung et al., 2019).

Edwardsiella piscicida is the first *Edwardsiella* to demonstrate critical role for the T6SS (Rao et al., 2004; Zheng and Leung, 2007). The *E. tarda* virulence protein (EVP) gene cluster containing 16 genes (*evpA-evpO*) of strain PPD130/91 encodes T6SS (Zheng and Leung, 2007). Avirulent strains were not hybridized with probes for *evpP*, *evpK*, and *evpO*, indicating that there is a wide distribution and conservation of *evp* genes among virulent strains of *E. tarda*.

In another study, the replication rate in phagocytes, protein secretion, and virulence in blue gourami was lower in *E. tarda* PPD130/91 with deletions of *evpB* and *evpC* (Rao et al., 2004). The partial recovery of observed phenotypes was noted when *evpB* and *evpC* complementation occurred, indicating that these genes may play a role in fish phagocyte replication and could be associated with intramacrophage growth. Further functional analysis to elucidate the mechanism is needed.

The deletion of 14 T6SS genes resulted to attenuation of *E. tarda* virulence with two logs LD50 difference compared to wildtype. The *evpJ* deletion resulted in slight attenuation, but the *evpD* mutant do not differ significantly. The secretion assay demonstrated that 13 proteins, EvpA-EvpC, EvpE-EvpI and EvpK-EvpO are essential for the secretion of EvpC, EvpI and EvpP. The EvpO is homologous to VasK in *V. cholerae* and IcmF1 in *P. aeruginosa* while EvpH is a homolog of ClpV. Three proteins were secreted T6SS proteins, including EvpC (Hcp), EvpI (VgrG) and EvpP. The EvpP deletion mutant did not impact the secretion of EvpC and EvpI. However, the deletion of either *evpC* or *evpI* halted the secretion of EvpP, indicating their essential role in the secretion process. Further, EvpP and EvpC function together in the *E. tarda* cytoplasm, indicating that EvpP may target inflammasome activation in macrophages (Zheng and Leung, 2007; Hu et al., 2014).

On the basis of macrophage-induced gene regulation, two putative T6SS effectors were identified, EseL and EseM (Zhang et al., 2018). EseL and EseM in the T6SS mutant ($\Delta evpAB$) had a reduced translocation into HeLa cells, compared to wildtype *E. piscicida* EIB202. No immunofluorescence was detected in the HA-tagged EseL and EseM in the T6SS mutant, confirming the T6SS-dependent translocation of EseL and EseM.

EsrA-EseB, the ferric uptake regulator (Fur) protein, and the histone-like nucleoid structuring

protein (H-NS) regulate the transcription of *EvpP* (Wang et al., 2009; Chakraborty et al., 2011; Zhang et al., 2014). *EvpP* expression could be downregulated by H-NS, suggesting that it could act as an *evpP* repressor. T6SS expression is regulated by Fur through *EsrC* (*E. tarda* secretion regulator C) in *E. tarda* strain PPD130/91 in response to changes in iron concentration (Chakraborty et al., 2011).

EvpP can be translocated into the cytosol of infected cells (Chen et al., 2017). Also, outer membrane vesicles (OMVs) contain *EvpP* (Park et al., 2011). It was found that *EvpP* was an essential effector in *E. piscicida* virulence (Xiao et al., 2008; Wang et al., 2009). In a mariculture farm in China, *E. tarda* EIB202 was isolated from diseased turbot with high mortality outbreaks due to bacterial septicemia and the role of *EvpP* as virulence determinants was investigated (Xiao et al., 2008). Zebrafish and Japanese flounder lethality was significantly attenuated by *evpP* deletion mutants, the sheep erythrocyte hemolytic activity was reduced, and *evpP* mutants failed to adhere or penetrate the Japanese flounder mucus and decreased serum resistance (Wang et al., 2009; Tan et al., 2019). The complementation of *evpP* restored the phenotypes. Importantly, in the invasion assay, the $\Delta evpP$ displayed deficiency in internalization of epithelial papilloma of carp (EPC) cells, suggesting that *EvpP* is an important component of *E. tarda* invasion mechanisms. *EvpP* has been implicated in several studies related to *E. piscicida* infection (Chen et al., 2017; Tan et al., 2019).

In their study, Chen et al. (2017) explored the mechanism through which the *E. tarda* T6SS interacts with eukaryotic hosts. Inflammasome activation is negatively regulated by *E. tarda* T6SS as low NLRP3 inflammasome activity occurs during infection. The T6SS-deficient mutant, $\Delta evpAB$ significantly enhanced caspase-1 activation, IL-1 β secretion, and cell death in bone marrow-derived macrophages (BMDM) and J774A.1 cells (Chen et al., 2017).

T6SS-dependent transport of *EvpP* into HeLa cells was observed. Immunofluorescence analysis verified the injection of *EvpP* into host cells and *EvpP* positive signals were detected in HeLa cells infected with *E. tarda*. Moreover, *EvpP* was found to localize in the membrane fraction after *E. tarda* infection in HeLa and J774A.1 cells. In this context, *EvpP* can be considered a non-VgrG T6SS effector (Chen et al., 2017).

It is evident that *EvpP* inhibits NLRP3 inflammasome activation by suppressing ASC oligomerization that is JNK-dependent and impair the oligomerization of ASC, an adaptor of

inflammasome. In J774A.1 cells, the deletion of *evpP* ($\Delta evpP$) significantly induced Jnk phosphorylation in comparison to the wildtype. A dose-dependent inhibition of Jnk phosphorylation and inflammasome activation induced by $\Delta evpP$ or $\Delta evpAB$ was observed when *EvpP* is overexpressed in J774A.1 cells. An activated Jnk plays a critical role in NLRP3 inflammasome activation, which is dependent on ASC oligomerization. The number of ASC foci was significantly higher in J774A.1 cells that had been infected with $\Delta evpP$ (Chen et al., 2017).

EvpP inhibits intracellular Ca^{2+} signaling which regulates the NLRP3 inflammasome by inhibiting intracellular Ca^{2+} signaling as measured by a Ca^{2+} sensitive fluorescent probe and time-lapse fluorescence reader. It was found that *evpP* deletion caused J774A.1 cells to have higher intracellular calcium flux (Chen et al., 2017).

In mice, *EvpP*-mediated inhibition of the inflammasome promotes colonization of *E. tarda*. In the $\Delta evpP$, it was observed that Casp⁻¹⁻¹ mice had a significant reduction in IL-1 β levels, indicating that their inflammasome activities were minimized. The $\Delta evpP$ elevated IL-1 β levels significantly higher in wild-type and *Nlrp3*⁻¹⁻¹ mice sera compared to wild-type, suggesting an enhanced NLRP3 inflammasome activation *in vivo* (Chen et al., 2017).

These results corroborates with the results of Tan et al. (2019) using an *in vivo* zebrafish larvae infection model. *EvpP*-mediated manipulation of Jnk-MAPK signaling *in vivo* was found to enhance Jnk activation in larvae infected with *EvpP*. As a result of inhibiting Jnk signaling, *EvpP* decreased chemokine ligand 8 (*cxcl8a*), matrix metalloproteinase 13 (*mmp13*) and interleukin-1 β (IL-1 β). Neutrophil-associated genes were upregulated in response to $\Delta evpP$ infection. When zebrafish larvae were pretreated with Jnk inhibitor SP600125, the $\Delta evpP$ infection-induced recruitment of neutrophils was restored. These results suggest that *EvpP* inhibits the neutrophil recruitment.

The role of inflammasome in regulating neutrophils recruitment was investigated using zebrafish expressing GFP driven by a neutrophil-specific myeloperoxidase (*mpo*) promoter (Tan et al., 2019). A caspase-1 homolog and IL-1 β morpholino were injected into the zebrafish larvae embryo, then infected with selected *E. piscicida* strains. Neutrophil recruitment induced by infection with *E. piscicida* was inhibited by caspases and IL-1 in zebrafish larvae, suggesting an inflammasome-mediated mechanism. Also, higher mortality and bacterial load were observed in caspase or IL-1 β zebrafish larvae. The

caspace- or IL-1 β morpholino knockdown larvae were more susceptible to infection and failed to restrict bacterial colonization in vivo.

A crucial component of *E. piscicida*-macrophage interactions, EvpP is required for macrophage survival and replication (Qin et al., 2020). The $\Delta evpP$ had a reduced replication and survival in macrophages under oxidative and acid stress. Moreover, the $\Delta evpP$ caused macrophages to undergo apoptosis, as demonstrated by increased Annexin V binding and activation of caspase-3 which are involved in apoptosis. EvpP interacts with ribosomal protein S5 (RPS5) as determined by yeast two hybrid screening and co-immunoprecipitation assays (Qin et al., 2020).

An *E. piscicida* EvpQ effector encoded by a mobile genetic element (MGE) was characterized by Li et al (2021b). Through the T6SS, EvpQ is secreted and translocated into host cells. In a blue gourami infection model, *evpQ* deletion attenuated *E. piscicida* PPD130/91 virulence. Fur negatively regulates EvpQ transcription, while EsrC positively regulates it.

Edwardsiella ictaluri

In the catfish aquaculture industry, *E. ictaluri* is the most important endemic infection. It was first isolated from pond cultured channel catfish (Hawke et al, 1981) and causes enteric septicemia.

The *E. ictaluri* T6SS also consist of 16 genes, *evpA-O* (Tekedar et al., 2020). As homologs of *E. ictaluri* Eip20, Eip55, and Eip18, *E. tarda*'s EvpA, EvpB, and EvpC are expressed during an infection and are antigenic for channel catfish (Rao et al., 2004; Moore et al., 2002). The sequence analysis of Eip20, Eip55 and Eip19 showed high identity to the ImpB, ImpC, and ImpF proteins in *Rhizobium leguminosarum* bv. *trifolii*. In *Rhizobium* and *Agrobacterium*, the T6SS is encoded in two different operons (*imp* and *hcp*). Temperature-dependent protein secretion is regulated by the *imp* operon in *Rhizobium leguminosarum* (Bladergroen et al., 2003). The expression of *E. tarda* EvpA and EvpC was temperature dependent, expression was suppressed at 37 °C (Rao et al., 2004). Also, the survival blue gourami is higher when challenged with bacterial cells grown at 37 °C, compared to cells grown at 25 °C.

During infection, *E. ictaluri* EvpB is expressed and is highly similar to *E. tarda* EvpB (Rao et al., 2004). As a result of the deletion of *evpB*, *E. ictaluri* virulence was almost completely lost in catfish fingerlings, and *evpB* was highly internalized in catfish peritoneal macrophages (Abdelhamed et al., 2018).

Apoptosis in anterior kidney macrophages is increased by *E. ictaluri evpP* colonization in channel catfish ovary (CCO) cells (Kalindamar et al., 2020). *E. ictaluri* internalization and adhesion to catfish ovary cells were reduced when *evpP* was deleted. When oxidative stress and limited nutrients are present, *evpP* promotes survival and increases apoptosis and necrosis in catfish anterior kidney macrophages (Kalindamar et al., 2020). Compared with wildtype, the number of live macrophages in $\Delta evpP$ was significantly increased. Interestingly, macrophages exposed to $\Delta evpP$ shows significantly lower necrosis than wildtype. Further, the deletion of T6SS genes $\Delta evpA$, $\Delta evpH$, $\Delta evpM$, $\Delta evpN$ and $\Delta evpO$ resulted to reduced replication inside peritoneal macrophages and attachment to CCO cells (Kalindamar et al., 2023). All the deletion mutants were less virulent than *E. ictaluri* wildtype in catfish fingerlings.

Other T6SS genes, *hcp1* (*evpC*) and *hcp2* were also implicated in virulence through adhesion to epithelial cells, and replication within catfish peritoneal macrophages, demonstrating the role of T6SS in *E. ictaluri* pathogenesis (Kalindamar et al., 2021).

Edwardsiella anguillarum

E. anguillarum ET080813T, isolated from diseased eel contains three T6SS in Chromosome 1, has high level of virulence to fish but this strain has not been extensively studied (Shao et al., 2015). The T6SS2 and T6SS3 have a high level of genetic conservation along with their percent similarity, while the T6SS1 is highly conserved in *E. anguillarum*, *E. piscicida* and *E. ictaluri*.

5. *Pseudomonas* sp.

Pseudomonads are opportunistic pathogens and are an important biological factor in the outbreak of fish disease (Rao et al., 2019). The causative agents of hemorrhagic septicemia in fish include *P. fluorescens*, *P. anguilliseptica*, *P. aeruginosa*, and *P. putida* (Eissa et al., 2010). The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites and exophthalmia (Eissa et al., 2010). *Pseudomonas fluorescens* was first described as a pathogen of mirror and leathern carp, and its symptoms are similar to those of motile aeromonad septicemia. *Pseudomonas anguilliseptica* poses a serious threat to a variety of fish cultured in marine and brackishwater (Mekasha and Linke, 2021). It

causes red spot disease, a major disease of Japanese eels. *Pseudomonas aeruginosa* causes septicemia in freshwater fish, such as catfish (*Clarias gariepinus*) and tilapia (*O. niloticus*), resulting in immense economic losses in the industry (Roberts, 2012). *Pseudomonas putida* has only been isolated from ayu (*Plecoglossus altivelis*) and yellowtail (*Seriola quinqueradiata*) in Japan. Unlike other pathogens in aquaculture, it has only been reported to infect tilapia and rainbow trout (*Oncorhynchus mykiss*) (Altinok et al., 2006; Salama and Gharib, 2009).

To date, *P. plecoglossicida* is the only aquatic pathogen with characterized by T6SS. *P. fluorescens*, *P. aeruginosa*, and *P. putida* T6SS has been characterized in plant and clinical isolates (Decoin et al., 2014; Chen et al., 2015; Bernal et al., 2017).

Pseudomonas plecoglossicida

Pseudomonas plecoglossicida is an important fish pathogen that is causing severe economic losses in aquaculture and currently there is no efficient preventive and control measures (Mao et al., 2024). The pathogen replicates in infected fish macrophages and is a facultative intracellular pathogen (Mao et al., 2013). The first report came from ayu (*Plecoglossus altivelis*) (Nishimori et al. 2000) and subsequent reports came from twenty marine fish species, including large yellow croaker, orange-spotted grouper, yellow drum and rainbow trout (Mao et al. 2013; Huang et al. 2019; Xiang et al. 2020; Akaylı et al. 2011). Signs of disease include hemorrhagic ascites in ayu and white spots covering the liver, spleen, and kidney of affected marine fishes, leading to the term visceral white spot disease (Nishimori et al. 2000; Yuan et al., 2022).

P. plecoglossicida's virulence is influenced by temperature. The bacterium induce natural infection at low (12.0–25.5°C) water temperature (Huang et al. 2018). Aside from temperature, several studies revealed that virulence genes, such as T6SS associated genes regulate the virulence of *P. plecoglossicida* (Tao et al., 2020; Li et al., 2022, Yang et al., 2023; Zhang et al., 2023).

The *P. plecoglossicida* strain XSDHY-P and *P. plecoglossicida* strain NB2011, encodes three T6SS (T6SS-1, T6SS-2, and T6SS-3) gene clusters (Tao et al., 2018; Tao et al., 2020; Jin et al., 2021). The three clusters are all encoded in Chromosome 1 of strain XSDHY-P. Each gene cluster contain all the 13 core gene components. In *P. plecoglossicida*, T6SS1 plays a major role in virulence and anti-eukaryotic functions. The deletion of the entire *t6ss1*, *tssH1*, and *tssD1* gene

reduced *P. plecoglossicida* virulence, and was unable to induce granulomas in large yellow croakers (Tao et al., 2020). Symptoms of granulation in the spleen and total mortality were observed by fish infected with WT, Δ T6SS2 or Δ T6SS3 eight days after infection. There was no evidence that the T6SS3 played a significant role in either virulence or bacterial killing.

In gram-negative bacteria, RpoE acts as a gene regulatory system to cope with stress. The knockdown of *rpoE* in *P. plecoglossicida* significantly reduces biofilm formation, swarming motility, adhesion, and virulence (Huang et al., 2019). In groupers, a mutant *rpoE* induces humoral and cell-mediated immune responses. In *P. plecoglossicida*, RpoE contributes to T6SS-mediated killing. The survival of the target *E. coli* was higher when co-cultured with *P. plecoglossicida* Δ *rpoE*. It suggests that the RIP (regulated intramembrane proteolysis) cascade is crucial for T6SS2 mediated killing activity in *P. plecoglossicida* as revealed by significant increase in *E. coli* survival when co cultured with deletion of RIP genes (Tao et al., 2020).

P. plecoglossicida gains a competitive advantage by using T6SS2 for interbacterial killing. The co-culture of *P. plecoglossicida* XSDHY-P and strains with deletion of T6SS1 (Δ *t6ss-1*) and T6SS 3 (Δ *t6ss-3*) resulted to a significant decrease in the growth of target strains *Photobacterium damsela* subsp. *damsela* 69YT1 and *E. coli* XL10 (Tao et al., 2020). Through bioinformatic analysis, four putative antibacterial T6SS effectors were identified in this strain namely Txe1, Txe2, Txe3, and Txe4 (Li et al., 2022). Among these, three putative effectors encodes DNase domain - Txe2 and Txe4 encodes a Tox-AHH toxin, while Txe3 contains an HNHc endonuclease. Txe1, Txe2, or Txe4 degraded plasmid DNA when expressed in *E. coli*, suggesting that these effectors have a nuclease activity. Interbacterial activity is mediated by only Txe1 and Txe4 with Txe1 being predominant. In Txe1, the PAAR and RhS domains are located at the N-terminus, while the C-terminus has a conserved dipeptide HH motif. Changing the catalytic site of Txe1 by site-directed mutagenesis abolished its nuclease activity and toxicity to *E. coli* suggesting that Txe1 is indeed a nuclease effector.

There have been several studies investigating the function of a single T6SS gene in the pathogenicity of *P. plecoglossicida*. In *P. plecoglossicida* NZBD9, Luo et al. (2019) showed that in groupers infected with *clpV*-RNAi strain, no mortality was observed. The results are supported by gene expression changes of bacterial pathogens in tissue by RNA-seq and verified

by virulence comparison between the *clpV* gene knock down (*clpV*-RNAi strain) and *P. plecoglossicida* wildtype strain.

It has been proposed that VgrG, one of the structural and core components of T6SS, contributes to the pathogenicity of *P. plecoglossicida* (Yang et al., 2023). The *vgrG* deletion mutant ($\Delta vgrG$) in *Acinetobacter baumannii* ATCC 19606 strain resulted in decreased adherence to BEAS-2b human alveolar epithelial cells and impaired lethality in BALB/c mice (Wang et al., 2018b). The deletion of *vgrG* ($\Delta vgrG$) significantly reduced virulence of *P. plecoglossicida*, including chemotaxis, cell adhesion, and biofilm formation (Yang et al., 2023). Transcriptome data analysis supports this finding, indicating lower expression of genes associated with adhesion, chemotaxis, biofilm, T6SS, and T3SS in $\Delta vgrG$ strain. *P. plecoglossicida* virulence may be affected by *vgrG* by inhibiting the secretion of virulence factors and affecting biofilm formation through quorum sensing. Moreover, the LD50 of groupers infected with the $\Delta vgrG$ strain was 50 times higher than wildtype strain, indicating the role of *vgrG* in *P. plecoglossicida* pathogenicity.

The deletion of *tssD* ($\Delta tssD1$) is highly attenuated in large yellow croaker (Tao et al., 2020), therefore Ye et al (2021) constructed a *P. plecoglossicida* strain with deletion of *tssD-1*, and evaluated its efficacy as vaccine in large yellow croaker juveniles. The live attenuated vaccine provided significant relative percentage survival of 86.3% against wildtype *P. plecoglossicida* XSDHY-P eight weeks after primary vaccination. A significant increase in serum IgM specific to *P. plecoglossicida* and immune gene expression was observed after $\Delta tssD-1$ vaccination. In the vaccinated fish injected with a high dose of $\Delta tssD-1$, there were no clinical signs of disease, no bacteria were recovered from the spleen or kidney of surviving fish, and no mortality occurred. The *P. plecoglossicida* $\Delta tssD-1$ showed potential as a live fish vaccine. It is necessary to conduct further studies to assess its efficacy and understand how vaccines induce protection via cell-mediated immunity.

In a recent study, Zhang et al (2023) showed that the RpoE of *P. plecoglossicida* positively controls T6SS expression in a temperature-dependent manner. RIP signal cascades activate RpoE to regulate the expression of genes in response to environmental stimuli. RNA sequencing showed that *rpoE* was significantly expressed at virulent temperature (18 °C) compared to non-virulent temperature (28 °C).

Transcriptome sequencing of *P. plecoglossicida* incubated at virulent (18 °C) and non-virulent (28 °C) temperatures showed that *rpoE* was significantly expressed at 18 °C (Huang et al., 2018). A challenge test in orange spotted grouper showed that *P. plecoglossicida*'s virulence is significantly reduced when *rpoE* is knocked down, suggesting that *rpoE* contributes to its pathogenesis (Huang et al., 2019). The RNA sequencing results revealed that at 18 °C, the transcription of T6SS2 genes were downregulated in *rpoE* deletion mutant.

According to qRT-PCR analysis, T6SS2 gene transcript levels were significantly downregulated in *rpoE* mutant compared to wildtype at 18 °C. RpoE deletion does not induce *hcp2* transcription, which indicates a temperature-dependent regulation of T6SS2 by RpoE. When host cells are exposed to low temperatures, the secretion of T6SS1 is strongly induced.

Interestingly, the expression of *hcp1* was not affected by temperature regulation, however, *rpoE* deletion affects *hcp1* expression and secretion. Thus, it may be possible that RpoE is involved in both T6SS1 and T6SS2 expression with T6SS2 exerting a dominant control.

The possible role of RpoE for infection in eukaryotic cells has been investigated. The replication of T6SS-2 deficient *P. plecoglossicida* strain exhibited reduction in macrophage J774A.1 compared with wildtype (Jin et al., 2021). *Pseudomonas plecoglossicida* infected cells exhibited severe cytoplasmic vacuolation and dying morphology, while $\Delta rpoE$ -infected cells had fewer lesions. Moreover, there was a lower level of intracellular replication of $\Delta T6SS2$ in J774A1 than in wildtype. This results indicate RpoE regulates virulence determinants such as T6SS2 and other pathways associated with intracellular replication of *P. plecoglossicida* in macrophages.

6. *Francisella noatunensis* subsp. *orientalis* (Fno)

Fish mortality rates range from 30-75% in marine and freshwater environments due to *Francisella noatunensis*, an emerging pathogen affecting both wild and farmed species (Hsieh et al., 2006; Olsen et al., 2006; Birkbeck et al., 2007). Two genetic types of *Francisella* spp. cause disease in cold and warm water fish species, respectively: *Francisella noatunensis* subsp. *noatunensis* (Fnn) and *Francisella noatunensis* subsp. *orientalis* (Fno) (Lewis and Soto, 2019; Mikalsen et al., 2009; Kamaishi et al., 2005). Colonization and replication of *F. noatunensis*

occur in phagocytes and endothelial cells (Soto et al., 2017). As a result of the disease, multiple organs, especially the kidney and spleen, experience severe granulomatous inflammation (Birkbeck et al., 2011). Higher mortalities are observed at temperatures below 28 °C (Colquhoun and Duodu, 2011).

The Fno genome contains several virulence determinants, including gene sequences that might encode proteins similar to *F. tularensis* T6SS components including IglA (TssA homolog), IglB (TssB homolog), VgrG, DotU, and PdpB (Clemens et al., 2018). The gene transcription analysis of T6SS homologs in Fno, suggests its role in pathogenicity (Lewis and Soto, 2019). Higher expression of T6SS homologs was observed at lower temperature (< 30 °C) and when exposed to H₂O₂, suggesting a role in oxidative stress tolerance. A better understanding of the pathogenicity mechanism employed by Fno requires further research.

The genome of *Francisella* encodes a Francisella Pathogenicity Island (FPI) which represents a unique T6SS subtype, T6SSⁱⁱ (Nano and Schmerk, 2007; Russell et al., 2014). PdpA encodes the pathogenicity determinant protein A in FPI, which is conserved in *Francisella* and used to secrete effectors by the *Francisella* T6SS (Schmerk et al. 2009; Eshraghi et al., 2016; Hansen et al., 2021).

Using a specific-pathogen-free zebrafish infection challenge through intraperitoneal injection, Hansen et al (2021) demonstrated that *pdpA* deletions in Fno resulted in attenuated virulence, impaired intracellular replication and cytotoxicity and the complementation restored wildtype levels of virulence. No mortality and histopathological changes were observed. Wildtype challenge test revealed significant protection from an acute lethal dose after immunization with Fno Δ *pdpA*. A study in Nile tilapia by de Alexandre Sebastião et al. (2022) evaluated the efficacy of a mutant *pdpA* as a live attenuated vaccination. Immunized tilapia had a 45% relative percent survival, and no clinical signs associated with Francisellosis were observed after the immersion challenge. Comparing the vaccinated and non-vaccinated fish, the vaccinated fish had significantly higher IgM levels. These studies revealed the potential use of attenuated Fno strains as vaccine against Francisellosis in farmed and wild fish.

7. *Flavobacterium* sp.

Worldwide, *Flavobacterium* is an important pathogen of wild and cultured fish (Wahli and

Madsen, 2018). In salmon and channel catfish farming, *F. psychrophilum*, *F. branchiophilum* and *F. columnare* have been reported to cause economic losses. A bacterial cold water disease in freshwater fish, bacterial gill disease, and columnaris disease are caused by *F. psychrophilum*, *F. branchiophilum* and *F. columnare* (Wahli and Madsen, 2018). Other *Flavobacterium* species, including *F. johnsoniae*, *F. succinicans*, *F. hydatis*, *F. chilense*, *F. araucanum*, *F. spartansi*, *F. plurextorum* and *F. tructae* have been associated with diseased fish (Zamora et al., 2012; Loch and Faisal, 2014a; Loch and Faisal, 2014b; Wahli and Madsen, 2018). Despite extensive research, no effective methods has been proposed to reduce its devastating effect.

To date, only comparative gene analysis has been done in *Flavobacterium* fish pathogens. *F. branchiophilum*, *F. araucanum*, *F. chilense*, *F. spartansii*, and *F. tructae* encode a complete T6SS, while *F. columnare*, *F. hydatis*, and *F. plurextorum* encode partial T6SS (Tekedar et al., 2017; Kumru et al., 2020). Interestingly, multiple *tssD* is encoded by *F. columnare*, *F. branchiophilum* and *F. johnsoniae* (Tekedar et al., 2017). Considering *Flavobacterium*'s high prevalence of T6SS, further research is warranted to determine how it impacts pathogenicity and environment adaptation.

8. Conclusion/Future Perspective

By gaining a better understanding of T6SS in aquatic pathogens, we can develop diagnostic tools, more effective therapeutics, and vaccines for mitigating diseases in the aquaculture industry. Interestingly, comparative genome analysis revealed that virulent *A. hydrophila* and AHPND-causing *V. parahaemolyticus* may be distinguished from other strains by the presence of T6SS (Pang et al., 2015; Li et al., 2017). The next step in future studies will be to characterize the functional properties of T6SS in *V. crassostrea*, *E. anguillarum*, and *Flavobacterium* spp, following the genome studies. Most of the T6SS functions in aquatic pathogens described above are antibacterial activity, pathogenicity, regulation of motility (Huang et al., 2019; Wu et al., 2023) and modulation of microbiome composition (Tang et al., 2022) (Table 2). Not many antibacterial and virulence effectors has been characterized (Figure 1).

T6SS demonstrates antibacterial action against a variety of bacteria, primarily gram-negative bacteria and, in the case of *V. parahaemolyticus*, gram-positive bacteria (Wang et al., 2022). *In vivo*

competition assay demonstrated a reduced killing by T6SS deletion mutant against target competitors in oyster and zebrafish host (Hubert and Michell, 2020; Ma et al., 2020). Specific T6SS effectors, such as lipase and nuclease were implicated for the killing activity in *A. hydrophila* and *P. plecoglossicida*, respectively (Ma et al., 2020; Li et al., 2022).

Several *in vitro* studies demonstrated how T6SS plays a role in colonization of host cell and the cytotoxic activity in various cells/cell lines through exogenous expression and deletion mutant. For instance, reduced translocation of *E. piscicida* effectors was observed in T6SS mutant ($\Delta evpAB$) in HeLa cells (Zhang et al., 2018). Whereas, the exogenous expression of *V. proteolyticus* effector induced cytoskeleton rearrangements and morphological abnormalities in yeast as well as toxicity in HeLa cells.

T6SS has been shown to mediate virulence *in vivo* using brine shrimp nauplii, catfish fingerlings, grass carp, crucian carp, orange spotted grouper, Japanese flounder and blue gourami (Table 2). The deletion or inactivation of T6SS gene resulted to attenuation of pathogenicity. Studies demonstrating the histopathology of wild-type vs T6SS mutant may support the possibility that T6SS is directly involved in causing the disease. Identifying the effectors directly involved in pathogenesis will also be of interest.

The regulation of T6SS is mediated by temperature, salinity and quorum sensing. There is still a lack of knowledge about the conditions, cues, and mechanisms that regulate T6SS activity for many of the T6SS studied to date.

Bacterial secretion systems are promising targets to develop anti-virulence drugs, when inactivated it would result to pathogen attenuation or loss of virulence (Baron and Coombes, 2007). It may be possible to develop anti-virulence drugs targeting conserved T6SS components such as extracellular apparatus components. T6SS components in *V. harveyi* and *P. plecoglossicida*, described above, have been employed as an antigen in the production of vaccines (Sun et al., 2019; Ye et al., 2021). Yang et al. (2015) developed a live attenuated vaccine (LAV) for turbot aquaculture using *Edwardsiella tarda* YWZ47 *esrB* mutant with low T6SS and T3SS secretion. The LAV was administered to turbot (*Scophthalmus maximus*) through immersion and subsequently challenged with the wildtype *E. tarda* strain EIB202. The YWZ47 could confer a high protection rate and higher RPS (64.4 %) for vaccinated fish compared to $\Delta esrB$ with only 51.1% RPS. The vaccine can be administered via immersion making it ideal for use in aquaculture. An attenuated

E. ictaluri vaccine was tested by Abdulhamed et al (2018) using a strain with an *evpB* mutation. The vaccine provided protection to catfish fry and fingerlings challenged with *E. ictaluri* wildtype with 80.34% survival. Another study (Kalindamar et al., 2023) showed the efficacy of vaccination using T6SS mutants in channel catfish fingerlings after challenge with *E. ictaluri*. All the T6SS mutants provided better protection than the control, especially $\Delta evpD$, $\Delta evpE$, $\Delta evpG$, $\Delta evpJ$, and $\Delta evpK$. While $\Delta evpA$, $\Delta evpH$, $\Delta evpM$, and $\Delta evpN$ caused less protection.

Several studies have demonstrated that secretion systems can be used for the delivery of recombinant proteins (Ittig et al., 2015; Simon et al., 2015; Walker et al., 2017; Bai et al., 2018). *Yersinia enterocolitica* T3SS substrate YopE allows fast and controlled delivery of bacterial, viral, and human proteins to target cells using the injectisome of extracellular bacteria (Ittig et al., 2015). Through nanobody-fusion proteins, multiple proteins can be simultaneously injected and targeted to different subcellular locations. Proteins can be released from the YopE fragment by T3S-translocated viral proteases or fusion with ubiquitin and cleavage by endogenous ubiquitin proteases after delivery. T3 Pharmaceuticals is currently doing a clinical trial using the system to deliver therapeutic proteins to selectively target cancer cells. Promising results showed immune cell activation and regression of primary tumor and metastasis.

Ting et al. (2020) generated strains expressing surface-displayed antibodies or nanobody which is directed at unique surface antigen of the target cell. The system makes use of the antibacterial activity of the T6SS to eradicate specific bacteria from polymicrobial community. A nanobody recognizing an antigen on the cell surface of aquatic bacterium or virus can be generated to control important diseases in aquaculture. This system can be integrated with the platform developed by Jana et al. (2021) for controlled delivery of effectors to improve the specificity of biocontrol strain to target aquatic bacterial pathogens. Jana et al. (2021) introduced an exogenous antibacterial T6SS from *Vibrio parahaemolyticus* into *Vibrio natriegens* and engineered an on/off switch to activate T6SS in response to an external cue.

Another recent development in the field is engineering the T6SS to deliver an exogenous effector and Cre recombinase, a genetic editing protein into target bacteria (Hersch et al., 2021). The system can be used to manipulate the microbiome or as a next-generation antimicrobial.

One of the most prevalent disease affecting aquaculture is fungal infection. T6SS effectors targeting fungal cells has been reported. The delivery of T6SS antifungal effectors in *Serratia marcescens* leads to fungal cell death (Trunk et al., 2018). Another study described an effector in *Klebsiella pneumoniae* that can target bacteria and yeast (Storey et al., 2020). It is likely that effectors with activity against aquatic fungal pathogens will be identified in the future. This is another direction to harness the T6SS in aquatic animal health management.

The use of T6SS-encoding bacteria as probiotics is possible through symbiotic bacterial strains or engineered strains. We have seen some potential of this T6SS application in the control of plant pathogens (Bernal et al., 2017; Decoin et al., 2014). In addition, T6SS activity has been shown to be affected by host and dietary components. The study by Bachmann et al (2015) demonstrated that microbiota modify bile acids in order to inhibit the production of T6SS in pandemic *V. cholerae*. A future application might be to modulate microbiota behavior using certain dietary components to control pathogenicity. Lastly, this can lead to the development of vaccines targeting secreted virulence factors as prophylactic strategies.

CONFLICTS OF INTEREST

The work was carried out without any financial or commercial ties that might be seen as having a conflict of interest.

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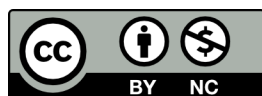
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