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Edwardsiella piscicida: A versatile emerging pathogen of fish

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ABSTRACT

Edwardsiella piscicida is an Enterobacteriaceae that is abundant in water and causes food and waterborne infections in fish, animals, and humans. The bacterium causes Edwardsiellosis in farmed fish and can lead to severe economic losses in aquaculture worldwide. *E. piscicida* is an intracellular pathogen that can also cause systemic infection. Type III and type VI secretion systems are the bacterium's most lethal weapons against host defenses. It also possesses multi-antibiotic resistant genes and is selected and enriched in the environment due to the overuse of antibiotics. Therefore, the bacterium has great potential to contribute to the evolution of the resistome. All these properties have made this bacterium a perfect model to study bacteria virulence mechanisms and the spread of antimicrobial genes in the environment. We summarize recent advance in *E. piscicida* biology and provide insights into future research in virulence mechanisms, vaccine development and novel therapeutics.

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Introduction

Edwardsiella species are abundant in freshwater and marine environments and one particular species, *E. piscicida* (old name as *E. tarda*) is a common and important fish pathogen. *E. piscicida* causes severe infections in a wide variety of marine and freshwater animals, especially in fish, in the USA, Europe, Asia, and around the world [1,2]. Factors that have contributed to the severity of *Edwardsiella* infections in fish include intensive fish farming methods, the overuse of antimicrobial chemicals in aquaculture and agriculture, the bacterium's broad host range and the development of multi-antibiotics resistance. *Edwardsiella* can move through the food chain to infect humans and other farm animals. This emerging pathogen is a member of the Enterobacteriaceae and is capable of transferring multi-antibiotics resistant genes to other enterics and to the resistome in water and soil microbiomes [3]. Currently, approximately 80% of *Edwardsiella* infections in humans result in gastroenteritis in patients with other underlying diseases [4]. Among the *Edwardsiella* species, *E. piscicida* is the most studied and is therefore a useful model organism to study enterics, intracellular pathogens, systemic infections, and crosstalk between multiple secretion systems. Some strains of *Edwardsiella* used in research are given in Table 1. Furthermore, understanding the organism's interactions with the food and human microbiome can further our understanding of the evolution of the resistome in relations to other food and waterborne diseases.

Identification, taxonomy and classification of Edwardsiella *bacteria*

Edwardsiella was described as a new genus in the mid-1960s in isolates recovered from wounds, blood, urine, and feces of infected humans and animals in the USA, Brazil, Ecuador, Israel and Japan [10]. Some of the first reports of *E. tarda* infections in aquaculture were reported in channel catfish in Arkansas, USA [11], but the organism is now recognized as a pathogen of farmed and wild fish worldwide [1]. Recently, the genus *Edwardsiella* was reclassified into five species based on genomic information and phylogenetic analysis. The five species include three fish pathogens (*E. piscicida*, *E. anguillarum*, and *E. ictaluri*) and two non-fish pathogens (*E. tarda* and *E. hoshinae*) [2,12–14]. The three fish pathogens infect a wide variety of marine and freshwater fish globally and are major threats to the aquaculture industry worldwide [1,15].

E. piscicida now includes the fish pathogens under the old species name of *E. tarda* that are isolated from diseased fish and contains one type III and

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Table 1. *Edwardsiella* strains used by researchers in the literature.

Edwardsiella strain	Isolated from; characteristics[a]	Reference
E. piscicida ET883^T (NCIMB 14824^T, CCUG 62,929)	European eel, *Anguilla anguilla*; Greaker, Norway	[12]
E. piscicida EIB202 (CCTCC M208068)	Turbot, *Scophthalmus maximus*; Mariculture farm, Yantai, China; Col^r Cm^r Tc^r	[85]
E. piscicida PPD130/91	Ornamental fish, *Serpae tetra*; PPD, Singapore; Km^s Col^r Amp^s	[22,29]
E. anguillarum ET080813^T	Japanese eel, *Anguilla japonica*; Fujian, China	[13]
E. hoshinae ATCC33379^T	Female puffin, *Fratercula arctica*; France	[5]
E. ictaluri ATCC33202^T	Channel catfish, *Ictalurus punctatus*; Georgia, USA	[6]
E. ictaluri 93–146	Channel catfish, *Ictalurus punctatus*; Baton Rouge, USA	[7]
E. tarda ATCC15947^T	Human feces; Kentucky, USA	[10]
E. tarda TX1*	Japanese flounders, *Paralichthys olivaceus*; Tc^r; Qingdao, China	[8]

[a]Resistant (r) or susceptible (s) to Amp (ampicillin); Col (colistin); Km (kanamycin); Cm (chloramphenicol); Tc (tetracycline).
*This *E. tarda* strain has a T3SS [9] and may belong to *E. piscicida* based on the analysis by Yang et al. [14].

one type VI secretion system (T3SS and T6SS) [12,14]. *E. anguillarum* contains two T3SSs and three T6SSs and is highly virulent to fish although not much work has been done on this organism [13,15–18]. It is interesting to note that isolates of this species have gone by different names; *E. piscicida*-like species, atypical *E. piscicida*, or just *E. tarda* strains [15,18]. However, *E. anguillarum* is taxonomically distinct as described by Shao et al. [13] and Buján et al. [15]. *E. ictaluri* is found in colder climates, harbors one T3SS and one T6SS and is responsible for catfish enteric septicemia (ESC) [14]. *E. tarda* now describes human or environmental isolates that do not contain any T3SS and T6SS [14,19] whereas *E. hoshinae* is a pathogen of reptiles and birds [1]. In the *Edwardsiella* literature prior to 2013, *E. tarda* described both *E. tarda* and *E. piscicida* and this old classification has made it difficult to examine the contribution of *E. piscicida* to human infections. Likewise, it is not clear whether the new organisms now referred to as *E. tarda* isolates play any role in fish infections. Finally, work to understand the evolution of virulence genes, passage of these genes to the resistomes or other bacteria and humans, and adaptation to various environments is required in order to understand the pathogenicity of *Edwardsiella*. Additionally, comparative studies on *Edwardsiella* and other enterics such as pathogenic *Escherichia coli* and *Salmonella* species can shed light on the various virulence mechanisms employed during the infection process.

E. piscicida, a model of the enteric pathogen causing food and waterborne diseases

Isolation of *E. tarda/E. piscicida* from human feces and from infected fish strongly suggest that these bacteria are important enteric zoonotic pathogens [20]. In fact, *Edwardsiella* and many other bacteria such as *Aeromonas, Salmonella, Vibrio* and *Yersinia* species, have been considered as established zoonotic pathogens that affect both humans and animals [20].

Most studies on virulence mechanisms of bacteria human pathogens (such as enterics) use mammalian tissue cultures and mammalian infection models to gain insights into the mechanisms and principles of bacterial pathogenesis. Although studies of these pathogens in humans are crucial, investigations of bacterial pathogens in non-human hosts can help us gather useful information before extending the applications to humans. Many similarities exist between organisms that cause gastroenteritis, such as *E. piscicida*, pathogenic *E. coli*, and *Salmonella* species. Therefore, *E piscicida* is increasingly becoming an attractive model organism for studying enteric bacteria in non-human cells and other hosts [2]. *E. piscicida* infects many fish including blue gourami, turbot, Japanese flounder and zebrafish [1]. Significant differences in LD_{50} values between virulent strains of *E. piscicida* and their T3SS and/or T6SS attenuated mutants have been observed, making *E. piscicida* an attractive model organism for studying food and waterborne pathogens [21,22].

Studying *E. piscicida* infections in non-mammalian host models can provide vital information for the development of appropriate therapeutics such as live vaccines for the aquaculture industry. These models may also illustrate potential treatments for enteric infections in humans and other animals. *E. piscicida* is able to infect mammalian infection models such as mice and mammalian tissue culture cells: HeLa, HEK293A, Hep-2, and J774A.1 [23,24]. Using *E. piscicida* as the model organism to study infection biology, with an emphasis on virulence factors/mechanisms, response to environmental signals, regulation of virulence mechanisms, and interaction between virulence factors and host immune systems, will pave ways to the development of new therapeutics for bacterial diseases in fish. Information gained from such studies has potential for application to the prevention of enteric infections (such as gastroenteritis) in humans as well.

E. piscicida *as a model organism for studying intracellular and systemic infections*

Eukaryotic cells use endomembrane systems such as secretory and endocytic pathways, for vesicular trafficking to distribute and recycle vital macromolecules and to eliminate harmful pathogens [25,26]. The secretory pathway distributes proteins and lipids produced in the endoplasmic reticulum to the plasma membrane, endosomes and lysosomes. In contrast, the endocytic pathway internalizes extracellular materials such as plasma membrane proteins and lipids. Internalized materials are channeled to the early and late endosomes, to lysosomes for degradation, or to the trans-Golgi for recycling [25].

In the tug of war between pathogens and host cells, some bacteria choose an intracellular lifestyle to avoid the hostile external environments and to access the plentiful nutrients and spacious compartments within host cells [27]. These pathogens often acquire new properties to exploit the endomembrane systems for their survival and replication inside host cells. Intracellular pathogens can reside and replicate within a vacuole after internalization. Some pathogens, such as *Salmonella* Typhimurium and *Legionella pneumophila*, choose to modify the vacuole environment and prevent fusion between endosomes and lysosomes to avoid destruction [28]. Others, escape from the vacuoles and replicate inside the host cytoplasm (*Listeria monocytogene*) or persist within the vacuoles and follow the endocytic trafficking (*Helicobacter pylori*) [28].

Our previous studies suggested that the actin cloud or actin condensation were important for the internalization of *E. piscicida* in carp epithelial cells [29]. Sui et al. [30] confirmed the involvement of actin, in addition to microtubules, in *E. piscicida* internalization using clathrin- and caveolin-mediated endocytosis in mouse macrophage RAW264.7 cells. Upon entering the host, *E. piscicida* prefers an intracellular lifestyle in *Edwardsiella*-containing vacuoles (ECVs) in either epithelial [29] or phagocytic cells [31] (Figure 1). The T3SS effector, such as EseJ, empowers the bacteria to thrive intracellularly by disrupting the production of reactive oxygen species (ROS) in J774A.1 cells [32]. The trafficking of *Edwardsiella* in ECVs followed the pathway from early and late endosomes to lysosomes [30]. Additionally, the acidification of the inside of ECVs was required, similar to that reported for *E. ictaluri* by Baumgartner et al. [33]. A second T3SS effector, EseG, appears to be translocated and injected into the membrane fraction of host cells and at the vacuole membrane of ECVs after the internalization of *E. piscicida* in both phagocytic and epithelial cells [34]. EseG may function in microtubule disassembly or remodeling of the endomembrane for the expansion of ECVs [23].

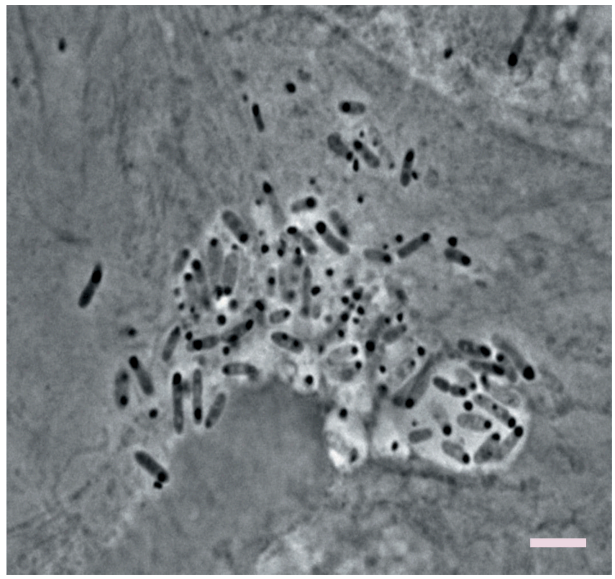

Figure 1. Caco-2 cells showing successful internalization of *E. piscicida* PPD130/91. Caco-2 cells were infected with wild type PPD130/91 for 6 h at 35°C. *Edwardsiella*-containing vacuoles (ECVs) with internalized bacteria are clearly visible under phase microscopy. Scale bar = 10 µm.

Studies on the involvement of *E. piscicida* in systemic infection in animals are limited and the evidence is fragmented. Most evidence comes from histopathological observations of diseased fish, infection kinetics studies in fish, and *in situ* protein-protein interactions. Clinical signs in diseased fish include poor pigmentation, protrusion and opacity of eyes, lesions on the skin, petechial hemorrhage, liquefaction and necrosis of tissues and organs such as kidney, spleen, and liver [1]. Phagocytes loaded with *Edwardsiella* were prominent and accompanied watery and bloody ascites [1]. These clinical signs of *E. piscicida* infection are similar to those observed in fish infected with other fish pathogens such as *Aeromonas hydrophila* and *Vibrio anguillarum* [1]; T3SSs and T6SSs are believed to play key roles in this infection pathway. *E. piscicida* is therefore a good model to study systemic infection in fish and other animals. Novel *in vivo* and molecular based approaches are needed to fully understand this complicated process. Ling et al [35], used GFP tagged *E. piscicida* to infect fish by an immersion method to reveal three virulent entry sites; gills, gastrointestinal tract, and skin. The virulent strain but not the avirulent strain, proliferated in various fish tissue and organs and mortalities were reported within three days-post infection. After bacterial entry, intracellular replication is speculated to be the major event (first inside intestinal cells and then inside phagocytic cells) before progressing to systemic infection as bacteria reach a critical

mass and spread deeper inside the host's body [36]. The hosts eventually die due to multiple organ failure and high bacteria load. The surface proteins of *Edwardsiella* interact with host proteins during local and systemic infection. Protein-protein interactions between outer membrane proteins (OMP) of *E. piscicida* and proteins in the gills are suggested to be responsible for bacterial entry into the fish [37].

So far, no T3SS or T6SS effectors of *E. piscicida* appear to be responsible for systemic infections. However, using time series Tn-seq technology and pattern analysis of conditional essentiality (PACE) algorithm to evaluate the fitness costs of each transposon insertion in the *E. piscicida* genome, the East China University of Science and Technology (ECUST) group in Shanghai was able to systematically assay the virulence factors or determinants *in vivo* in fish [38]. About 417 genes, including nearly all the previously identified or established virulence factors, were validated to be associated with *in vivo* colonization and progression of infection in *E. piscicida* [38]. Advances in these new technologies are unprecedented and show a lot of promise to help us dissect the infection pathway of *E. piscicida* in a systematic manner. Overall, *E. piscicida* appears to use its T3SS and T6SS effectors to sustain an intracellular lifestyle and (possibly) systemic infection in the hosts because T3SS and T6SS mutants of *E. piscicida* have attenuated ability to replicate in phagocytic cells and show reduced bacterial loads during infections [21,39]. One future direction is to elucidate the precise roles played by T3SS and T6SS effectors in the intracellular lifestyle and systemic infection at the molecular level, and to examine separately and collectively their, roles in the infection pathway.

E. piscicida in humans, fish, and environments

E. tarda *in humans*

E. tarda infections in humans are rare and the reported cases have been associated with *Salmonella*-like gastroenteritis [40]. Extra-intestinal manifestation includes biliary tract infection, bacteremia, skin and soft tissue infection, liver abscess, peritonitis, intra-abdominal abscess, tubo-ovarian abscess, and mycotic aneurysm [41,42]. Immunocompromised individuals and persons with other underlying diseases such as hepatobiliary diseases, malignancy, and diabetes are most susceptible to infection [41]. Although T3SS and T6SS are the main virulence mechanism in *E. piscicida* and *E. ictulari*, these virulent mechanisms are lacking in *E. tarda* isolates responsible for human infections [13]. It is not clear which virulence factors/mechanisms *E. tarda* uses to infect human cells. Environmental isolates of *E. tarda* and *E. piscicida* may also be active players in the dissemination of multi-antibiotic resistant bacteria resistome in the aquatic microbiome.

E. tarda/E. piscicida *in aquaculture*

The aquaculture industry has grown rapidly over the past few decades and FAO [43] estimated that the industry contributed about 53% to the total fish consumed in 2016. This rapid growth in the aquaculture industry has raised concerns about the quality and safety of farmed fish [44]. The use of intensive and semi-intensive farming practices greatly increases the risk of bacterial diseases in aquaculture [45]. Although there are many bacteria pathogens of fish, only a few genera are responsible for most of the important economic losses worldwide. Important fish pathogens include species of *Edwardsiella, Aeromonas, Vibrio, Flavobacterium*, and *Streptococcus* [45]. *E. piscicida* causes Edwadsiellosis in many commercially important fish including eels, channel catfish, mullet, chinook salmon, flounder, carp, tilapia, and striped bass [1].

Edwardsiella *species and resistome in microbiome*

Fish reared in aquaculture are often kept under crowded and stressful conditions and require prophylactic and therapeutic use of antimicrobials to control disease outbreaks [44]. The use of antibiotics in aquaculture introduces a selective pressure on the microbial flora in aquatic environments and often promotes antibiotic resistance [46]. Resistant bacteria can thus multiply after the suppression of sensitive bacteria. Antibiotic resistance and virulence genes are often clustered on pathogenic islands located on the chromosome and or plasmid [47]. Thus, antibiotic resistant bacteria such as *E. piscicida* can serve as reservoirs of antimicrobial resistance genes in the environment and can facilitate the transfer of these genes to other bacteria resistome [48,49].

Human activity in the form of wastewater discharge, manure disposal, and aquaculture is the main source of antibiotics in the environment [50]. The appearance of antimicrobial resistant zoonotic pathogens in agricultural environments correlates to the use of antimicrobials in animal husbandry [51]. Antimicrobial resistance genes and antimicrobial-resistant bacteria harboring these genes can also pass from industrially grown animals to human beings and vise versa [51]. The role of aquaculture in the transfer antimicrobial resistance genes from bacteria to bacteria or in the spread of antimicrobial resistant bacteria has not received a lot of attention despite the rapid growth of the aquaculture industry [44].

Additionally, aquaculture can facilitate the transfer of antimicrobial resistance genes from aquatic environments to human pathogens and the human resistome [51,52]. Antibiotic resistance mechanisms in bacteria vary and may include inactivation of drugs via hydrolysis or modification, alteration or bypass of the drug target, changes in the permeability of bacterial cell wall, active efflux of the antibiotic from the microbial cell, and biofilm formation to avoid death [53].

The spread of antibiotic resistance and virulence in bacteria is often mediated by mobile genetic elements. These elements include insertion sequence, transposons, integrons, bacteriophage, genomic pathogenicity island, plasmids or combinations of these elements [54,55]. For example, *Yersinia ruckerii*, the cause of Yersiniosis in fish, shares an antimicrobial resistance plasmid and other antimicrobial resistance genes with *Yersinia pestis*, which is responsible for the plague in humans [56]. A plasmid encoding tetracycline resistance is shared between different *Aeromonas* species and *E. coli* in various environments [57]. The origin of the *qnr* genes involved in plasmid mediated quinolone resistance in clinical and environmental bacterial species belonging to the Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae, Xantho monadaceae, Moraxellaceae, and Shewanellaceae, all have their origin in the waterborne *Shewanella* species [58].

Edwardsiella species in the environment and aquaculture include human pathogens of *E. tarda*, non-T3SS and non-T6SS avirulent *E. piscicida*, and virulent *E. piscicida* that habour T3SS and T6SS. *Edwardsiella* species are under constant selection pressure from all the antibiotics used in aquaculture, farms and human medicine. These organisms are part of the multi-antibiotics resistance bacteria (MARB) found in the environment and can exchange antibiotic resistance genes with other bacteria in the microbiome. For example, the *E. piscicida* plasmid sequence contains six genes known to confer antibiotic resistance to tetracycline (*tet*A and *tet*R), streptomycin (*str*A and *str*B), sulfonamides (*sul*II) and chloramphenicol (*cat*A3) [59,60]. Genetic analysis of the *E. piscicida* plasmid revealed an incomplete set of type IVA secretion (T4AS) genes (VirB2, -B4, -B5, -B6, -B8, -B9, -B10, -B11, -D2, and -D4) [49]. T4AS genes are widespread in nature and promote (conjugative) dissemination of multiple-antibiotic resistance. The presence of plasmid encoded antibiotic resistance genes (tetracycline, streptomycin, sulfonamides, and chloramphenicol) together with T4AS genes, suggests that *E. piscicida* is a good candidate for the dissemination of antibiotic resistance to other bacteria in the aquatic environment and to the human resistome. Thus, *Edwardsiella* species may be involved in the dissemination of multi-antibiotic resistant genes to other bacteria. Therefore, *Edwardsiella* species not only harbour antimicrobial resistance genes but they can also increase the number of MARB in the environment.

Virulence mechanisms and bacterial pathogenesis

Although *Edwardsialla* has been known as a serious pathogen of aquatic animals for a long time, its pathogenicity mechanisms are yet to be fully elucidated. The pathogenesis of *E. piscicida* appears to be multifactorial, and include many virulence factors such as the production of exoenzymes (hemolysin) [1], possession of T3SS and T6SS [21,22], ability to adhere, invade, survive and replicate in both epithelial and phagocytic cells [36]. Additionally, the organisms produces virulence regulators such as EsrA-EsrB, EsrC, PhoP-PhoQ, QseB-QseC and PhoB-PhoR [36] (Figure 2). Some virulent genes of *E. piscicida* are thought to have been acquired by horizontal gene transfer and include the two-component system of EsrA-EsrB that is essential for T3SS and T6SS mediated pathogenesis [61], and the locus enterocyte effacement (LEE) from pathogenic *E. coli* [16]. Among these factors, the T3SS and T6SS are the leading virulence systems that contribute to the success of *E. piscicida* pathogenesis and disruptions in T3SS or T6SS resulted in 1–4 logs attenuation in the LD_{50} values in different fish models [21,22,62].

T3SS and T6SS as the major players in Edwardsiella *pathogenesis*

T3SS is a flagellar-like structure and a contact-dependent device consisting of an injectisome, effectors, regulators, and chaperones [63,64]. Cornelis [65] used phylogenetic analyses to propose seven major families of T3SSs in Gram-negative bacteria. These needle-like nano-machines directly inject protein effectors into eukaryotic host cells using a one-step mechanism through an internal conduit of about 25Å in diameter [63]. Effectors have diverse functions and target specific host proteins to enable the pathogens to take control of the host cells [66]. Effector functions include modifications of cytoskeleton components, ubiquitination and phosphorylation of the host proteins [66]. Concerted actions of these effectors disrupt and/or mimic host cellular processes as well as interfere with the host immunity. Some T3SS effectors of plant and animal pathogens have common properties, suggesting that bacteria have evolved similar functions or inherited these genes through horizontal transfers to hijack host cells and disrupt host defense mechanisms [66]. Seventy percent of the T3SS effectors were estimated belonging to 91 families

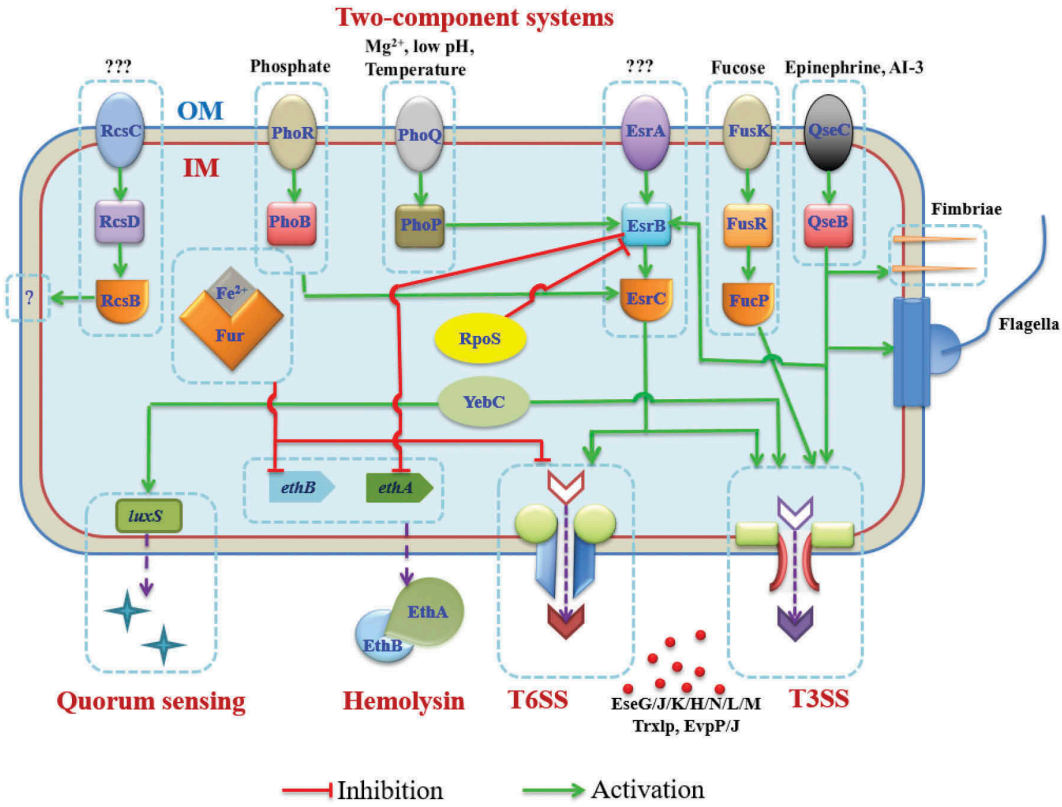

Figure 2. Virulence regulators and crosstalk among the different regulators in *E. piscicida*. Two-component systems and other global regulators control the delivery of the effectors of T3SS and T6SS, and other virulent factors in response to external environmental stimuli. Green lines indicate activation and red lines indicate inhibition.

based on BLASTs results [67]. However, the remaining 30% of effectors appear to be species or strain specific, indicating a specific role in host cell/tissue tropism and host specificity [66,67]. Studies on effectors are still in their infancy and we are only now starting to appreciate their complexity and importance in host-pathogen interactions.

Leung's group was the first to identify a T3SS in *E. piscicida* and found it to be analogous to the *Salmonella* pathogenicity island 2 (SPI-2) T3SS family encoded by *S.* Typhimurium [21]. Using a blue gourami fish model, T3SS deletion mutants were attenuated by 1 log in LD_{50} values and bacteria replication rates inside fish phagocytes were also reduced [21]. The identified T3SS secreted proteins, EseB, EseC and EseD, are homologous to *Salmonella* needle and translocon proteins, suggesting that they may form the extracellular conduit to deliver effectors [21]. EscC was characterized as a chaperone for EseB and EseD [68], while EscA and EseE are chaperones for EseC [69,70]. Similar to the corresponding homologs in *Salmonella*, the EsrA-EsrB two-component system (TCS) regulated the injection apparatus genes of the T3SS [36]. EsrC is an additional regulator (not found in *Salmonella* species) and used to regulate genes encoding the apparatus and effector proteins of T6SS. Therefore, EsrC enables crosstalk between T3SS and T6SS in *E. piscicida* [36]. EseG and EseJ were the first two T3SS effectors studied from Leung's, and researchers at the Institute of Hydrobiology group in Wuhan [23,32]. Using transcriptome analysis, scientists at the ECUST in Shanghai, were able to identify six additional novel T3SS effectors, including EseH, EseK, and Trxl [71,72] (Table 2). Functional studies on T3SS effectors of *Edwardsiella* is the next frontier to unlock key host-pathogen interactions of this important fish pathogen.

T6SSs are also a contact-dependent secretion system reported in about 25% of sequenced genomes of Gram-negative bacteria, especially proteobacteria [63]. A hallmark of T6SSs is the contractile phage-tail-like injection apparatus used to puncture and deliver effectors directly into adjacent host or bacterial cells [74]. The second major component is the membrane complex with some of its inner membrane proteins that have similarities to components of T4SSs [63]. In the tail complex, a spike component called VgrG trimer is responsible for puncturing targeted cells, supported and assisted by the Hcp tube [63,74]. Together, the spike-tube complex or the puncturing device, deliver effectors into target cells.

The majority of T6SS effectors identified so far are related to anti-bacteria action and bacterial fitness or

Table 2. T3SS and T6SS effectors in *E. piscicida* EIB202.

#	Protein name or tag #	T3SS or T6SS effector	Function	Location	Reference
1	ETAE_0866, EseG	T3SS	Interact with α-tubulin and disassemble microtubule	Membrane	[23,34,71,72]
2	ETAE_0888, EseJ	T3SS	Inhibit adhesion and promote intracellular replication, decrease ROS production		[32,71,72]
3	ETAE_1586, EseK	T3SS	Inhibit MAPK activation, promote colonization in zebrafish larvae		[24,71,72]
4	ETAE_1604	T3SS	Hypothetical protein		[72]
5	ETAE_1757, EseH/ EseN*	T3SS	Phosphothreonine lyase, inhibit MAPK signalling pathway	Nucleus	[71,72,84]
6	ETAE_2186, Trxlp	T3SS	Thioredoxin-like, promote inflammasome NLRC4 activation		[72,73]
7	ETAE_2188	T3SS	Hypothetical protein		[72]
8	ETAE_3282	T3SS	Hypothetical protein		[72]
9	ETAE_1303, EseL	T6SS	Major cold shock protein?		[71]
10	ETAE_2316, EseM	T6SS	Major cold shock protein?		[71]
11	ETAE_2428, EvpP	T6SS	Prevent NLRP3 inflammasome activation, promote colonization in mice	Membrane	[71,77]
12	ETAE_2438, EvpJ	T6SS	Hypothetical protein		[72]

*Two names were assigned for the same effector [71,72].

survival in the natural environments. For example, bacteriolytic activity against peptidoglycan in surrounding bacteria allows some bacteria to compete for space in the microbiota [75]. However, there is mounting evidence to suggest other functions such as evading host cell immunity similar to the T3SS anti-host effectors [76]. For example, EvpP of *E. piscicida* was reported to inhibit the Nod-like receptor P3 (NLRP3) inflammasome by supressing Ca^{2+}-dependent c-Jun N-terminal kinase (Jnk) activation in bone marrow-derived macrophages and J774A.1 cells, and to promote bacterial colonization in vivo [77].

Zheng and Leung [22] first discovered a T6SS gene cluster that encodes 16 *E. piscicida* virulent proteins (Evp). Mutants with 14 individual evp gene deletions resulted in virulence attenuation of about 2 logs in LD_{50} values in a fish model. Proteins involved in cross-talk, such as EsrB and EsrC, connected T3SS and T6SS in *E. piscicid*a [36]. Data from Leung's group provided some of the strongest evidence that the T6SS is important for bacterial pathogenesis in host cells. The ECUST group later identified four novel T6SS effectors, namely EvpP, EseL, EseM, and EvpJ [71,77] (Table 2).

T3SS and T6SS effectors identification and characterization

One of the next frontiers of *Edwardsiella* research is to characterize all the novel effectors of T3SS and T6SS and to examine their individual and combined functions in bacterial infection and host defence. *E. piscicida* has one T3SS and one T6SS that together translocate the 12 effectors reported so far (Table 2). Many more effectors are likely to be identified in the future. Some *Edwardsiella* effectors may share commonalities with effectors from other bacteria. Therefore, knowledge attained through *E. piscicida* studies is applicable to the biology of bacterial effectors in general. Although T3SS effectors are common in human and plant pathogens, our understanding of effectors in fish pathogens is limited. Studying *Edwardsiella* effectors will expand our understanding of fish diseases and lead to the development of good control measures. Identified T3SS effectors would form the foundation for the design of vaccines and novel therapeutics. Live *E. piscicida* vaccines have been developed by constructing mutants with deletions in genes that encoded for the translocon and needle proteins (EseB, EseC and EseD) [78], and regulator proteins (EsrB) [62]. Poly-T3SS effector mutants of *E. piscicida* such as those involving the disruption of five and nine effectors were used to produce more effective vaccines [71,72]; T3SSs can also serve as vectors for delivering heterologous antigens into the host cells [79].

The majority of the T3SS and T6SS effectors are not in the main pathogenicity gene clusters of the bacteria, but are scattered throughout the genome as hypothetical proteins among the unknown ORFs. For example, *E. piscicida* strain EIB202 has about 3,700+ genes and 1,000+ (about 30%) of them are hypothetical proteins, some of which may be putative effectors. Novel platform technologies using genome-wide experimental and bioinformatics methods are used to identify potential effectors among the hypothetical proteins before conducting labor intensive confirmation experiments using translocation assays such as TEM-β-lactamase [23] or Cya assay [32]. Several approaches to identify T3SS and T6SS effectors are available. Firstly, a BLAST search on hypothetic proteins in *E. piscicida* can identify sequence similarities with known T3SS and T6SS effectors of other Gram-negative bacteria proteins in the NCBI database [80]. However, this method may not be able to identify novel *Edwardsiella* effectors. Secondly, RNA expression in wild type *E. piscicida* or T3SS/T6SS regulator mutants (such as Δ*esrB*) can be compared under effector inducing versus non-inducing

conditions using RNA sequencing and transcriptome analysis. Using the above methods, two known T3SS effectors and ten novel T3SS and T6SS effectors were identified (Table 2) [71,72]. Interestingly, Zhang et al. [71] also found three effectors that required the outer membrane vesicles (OMV) for translocation but did not require either T3SS or T6SS; the mechanism is unclear or whether these effectors are related to T3SS and/or T6SS.

Future effector prediction approaches include secretome analysis followed by protein identifications using mass spectrometry and machine learning [80]. Machine learning on known *Edwardsiella* effectors can predict *Edwardsiella*-specific translocation signals found in T3SS and T6SS effectors. Previous results from Leung's group suggested that established effector prediction programs such as EffectiveT3 [81] and T3SEpre [82] were not very useful for predicting effectors in *E. piscicida* strain EIB202 (Leung et al., unpublished data). None of the proteins in the top 30 ranked proteins using the above two algorithms were true effectors and only the needle or translocon proteins were identified (EseC, EseB, and EseD; Leung et al., unpublished data). However, it is possible to create a reliable machine learning method that is species or genus specific. Hobbs et al. [83], used 21 attributes to create a reliable machine learning method called, Genome Search for Effectors Tool (GenSET) to predict T3SS effectors. Known effectors and non-effector sequences from one genome were used to train five machine learning algorithms. An averaging algorithm was then applied to predict known and unknown effectors in a testing set. The GenSET program was species-specific, gave better performance, and successfully predicted effectors in four known genomes including *S.* Typhimurium and *E. coli* [83]. A similar approach can be applied to *Edwardsiella* strains to predict T3SS and T6SS effectors in the future.

After the identification of a putative *Edwardsiella* effector and confirmation by a translocation assay, the next challenge is to characterize its function(s). In general, the following methods for effector characterization can be used: (a) Assay for a known *Edwardsiella* key host-alteration phenotype such as its role in intracellular living, decreased rates in adhesion, invasion, or intracellular replication; e.g. EseJ decreases intracellular replication rates in J774A.1 cells [32]. (b) Identify and examine host partner proteins and study their functions in relation to the function of the effector, e.g. EseG interacts with α-tubulin and destabilizes microtubules [23]. (c) Examine known homologs of effectors; e.g. EseH is a phosphothreonine lyase that inhibits the MAPK signalling pathways [84].

E. piscicida *as a model for the study of T3SS and T6SS and their crosstalk*

E. piscicida is an ideal microbe for studies on T3SS and T6SS because of several reasons: (a) Possesses both T3SS and T6SS and has an intracellular lifestyle, a phenotype believed to be T3SS and T6SS dependent [21,22]. (b) Secretes predominantly T3SS and T6SS proteins that are easy to assay in the supernatants of cultured cells [22]. (3) Its effectors are largely unknown. (4) It is a great candidate for studying the roles of effectors in intracellular lifestyle and systemic infection of hosts. (5) It is an excellent model organism to study the role of T3SS and T6SS in other enterics and fish pathogens. Future studies on effectors of *E. piscicida, E. italuri* and *E. anguillarum* will provide a complete picture of effector biology in *Edwardsiella* and other Gram-negative bacteria.

Virulence regulators and crosstalk regulation of T3SS/T6SS

The precise expression and targeting of virulence factors includes a repertoire of T3SS or T6SS effectors, essential for the pathogenesis of *E. piscicida*. Many regulators have been implicated, directly or indirectly, in regulating T3SS/T6SS expression. However, exact regulation mechanisms of many of the regulators are presently unknown. *E. piscicida* strain EIB202 harbors ~33 TCSs as important virulence regulators [85]. These include EsrA-EsrB, PhoQ-PhoP, PhoR-PhoB, QseC-QseB [36], Rcs cascade (RcsC-RcsD-RcsB) [86], and FusK-FusR for fucose signaling [87] (Figure 2). The horizontally acquired EsrA-EsrB controls the expression of ~1,006 genes (27.2% of the genome) through the EsrB binding to the specific 7–4-7 (ATCAGGTgattACCCGAT) motifs in a manner similar to that of SsrB in *Salmonella* in the activated state [72]. The major virulence traits (i.e. T3SS/T6SS and their effectors), as well as genes related to other virulence factors (EthA and siderophore) and metabolic related genes, are controlled by EsrB [72]. The genes encoding EsrA-EsrB are also under the feedback control of EsrB and from PhoP, PhoB, and QseB in response to the Mg^{2+}, iron, phosphate concentrations, pH, as well as temperatures, epinephrine, or quorum sensing signals such as AI-3. Not much is known about the other 30+ TCSs, although the T3SS defective phenotypes are observed in their respective mutants; but their regulatory mechanisms as well as their distinct signal ligands or substrates are still unclear and remain to be elucidated.

Known as a global regulator for stress adaptation, RpoS, was initially showed to be less related to virulence in zebrafish [88]. However, recent investigations indicated that RpoS is involved in virulence regulation by directly repressing *esrB* and other genes' expression by binding to the −6G sites in their respective discriminator sequences in the promoter regions [89]. The regulation of *esrB* links the stress conditions and virulence expression level during the progression of infection in *E. piscicida* and other phylogenetically related pathogens such as *Salmonella* species [89]. Hfq is another global regulator of an mRNA/sRNA chaperon associated with pathogenesis and interacts with ~49 sRNA in *E. piscicida*; there were ~148 sRNAs including 129 novel sRNAs that were associated with regulation of adversity adaptation and pathogenicity [90]. Although the distinct sRNAs and targeted mRNAs remain unknown, preliminary data in *E. pisicicida* suggested that Hfq is involved in T3SS/T6SS expression and *in vivo* pathogenesis (Wang QY et al., unpublished communication). Recently, YebC was shown to regulate quorum sensing and activate T3SS expression by directly binding to the promoter region of the T3SS gene ETAE_0873 involved in bacteria colonization in fish [91]. The ferric uptake regulator (Fur) is a global regulator of iron acquisition, resistance to acids, resistance to oxidative stress, resistance to host serum, hemolysin EthA production, and virulence towards fish [92]. Fur is intertwined with PhoB-PhoR to regulate EsrC expression, and thus to control T3SS/T6SS expression in *E. pisicicida* [36]. Together, these findings demonstrated that T3SS and T6SS expression is under a complex regulatory network that is triggered by distinct environmental cues and may play different roles in the pathogenicity *E. piscicida* in fish hosts. This regulatory network and environmental factors may be highly adapted to *E. piscicida* when compared to the phylogenetically related *Salmonella* or other pathogens, although the above-mentioned regulators are also present in these pathogens.

Other virulence factors or determinants and mechanisms

Other non-T3SS/T6SS virulence factors of *E. piscicida* include chondroitinase (that may relate to cartilage degradation and adhesion), hemolysins (EthA and HlyA), adhesins (AIDA), invasin (Inv1), flagellar structures, and other surface structures or extracellular products that may be involved in the initial steps of infection in hosts [1,15,36,93]. Inside the host, a series of factors such as serum resistance, resistance to oxidative stresses, and replication in macrophages are used to evade adverse host defenses [36,92]. *E. piscicida* circumvents serum attack by preventing, largely, the activation of the complement using the alternative pathway [94]. Sip1, also named as Aur or aureolysin, is a serum-induced zinc metalloprotease implicated in serum resistance and is essential for fish infection [95]. In addition, Sip2 (homologue of HypB, a putative hydrogenase) protects *E. piscicida* in fish serum and significantly increases cellular and tissue infection by allowing the bacteria to cope with acidic stress [96]. Although the direct regulon is unclear, the cyclic AMP receptor protein (CRP) was shown to be essential for flagellar biosynthesis and motility, hemolytic activity, and *in vivo* virulence in *E. piscicida* [97]. Finally, factors such as universal stress proteins [98], TonB [99], *E. tarda* hemolysin activator (Eha) [100], lysozyme inhibitor (MliC) [101], serine protease autotransporter (Tsh) [102], vibrioferrin siderophore [103], HU proteins [104], and the twin-arginine translocation system (Tat) [105], have all been reported to be closely linked to *E. piscicida* virulence.

Conclusion

New and cutting-edge technologies in bacterial functional genomics have been used to investigate the pathogenesis of *E. piscicida*. These include proteomics and genomics studies as well as secretomes, transcriptomes, interactomes, and metabolomics studies. Proteomics and metabolomics studies have enabled researchers to examine protein-protein interactions between *E. piscicida* cells and host tissues, such as fish gills [37] and livers [106]; in antibiotics resistome variations [107] and stress adaptation [108]. RNA-seq, ChIP-seq and Tn-seq are new and powerful technologies that couple defined transposon mutant library with high-throughput sequencing of transposon insertion sites to comprehensively map genetic determinants of bacterial fitness. Yin et al. [89] used Tn-seq technology to systematically identify the regulators of EsrB, the critical virulence regulator in *E. pisicicida*. Time resolved Tn-seq analysis termed PACE is a fascinating technology that is used to study the genes dynamically required for *in vivo* infection [38]. In these studies, PACE facilitated the heuristics identification of targets for live attenuated vaccine development based on fitness curves of the inserted mutants. The above platforms, along with others, will facilitate studies on *Edwardsiella*-host interactions as well as the development of vaccines and novel disease control.

Increased demand for fish and other healthier proteins in our diet has lead to the rapid growth of the aquaculture industry. *Edwardsiella* are abundant in

marine and freshwater habitats globally. *Edwardsiella* infection in aquaculture is a major constraint in fish farming and farmers have resorted to using antibiotics and other antimicrobial agents for prophylactic and therapeutic purposes. The overuse or abuse of antibiotics and chemicals in aquaculture is selecting and enriching for these organisms in the environment. Research on *E. piscicida* has progressed rapidly in the last two decades, from studies on an obscure fish bacterium a few years ago to become an active area of research in China, Asia, and around the world. *Edwardsiella* is an emerging fish pathogen, and *E. piscicida* will increasingly become common as a pathogen of both animals and humans. Thus, *E. piscicida* is a perfect model for elucidating bacteria pathogenesis at the molecular, cellular, and systemic level. *Edwardsiella* species and *E. piciscida* in particular is equipped with a deadly arsenal of virulent mechanisms such as a wide range of antibiotic resistance genes, T3SS, and T6SS. Some of these virulent mechanisms are also present in human pathogens and the knowledge generated from *Edwardsiella* studies will no doubt benefit public health and human medicine. Additionally, *Edwardsiella* species are capable of acquiring antibiotic resistance genes from other bacteria and can transmit these genes to the microbiomes in soil, water, animals and humans. Hence, *Edwardsiella* is not only a fish and human pathogen, but it has potential to spread and transmit, using novel methods, antibiotics- or chemical-resistant genes to other bacteria. Therefore, *Edwardsiella* species may be important players in the resistome and will likely attract a lot of attention in the next decades. New and powerful technologies such as dual RNA-seq, super resolution bio-imaging, all the omics, and structural biology technologies will facilitate studies to elucidate the biology of *Edwardsiella*. These new technologies will also provide new methods to assess the impact of pathogenic microorganisms on the environment, and lead to better diagnostic and control measures of other bacterial diseases of animals and humans.

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