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Characterization of Type VI Secretion System in Edwardsiella Ictaluri

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Characterization of type VI secretion system in *Edwardsiella ictaluri*

By

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Edwardsiella ictaluri causes enteric septicemia of catfish (ESC), which is one of the most important bacterial diseases causing significant economic losses in the US catfish industry. Understanding the virulence mechanisms of *E. ictaluri* plays a vital role to develop preventives, such as vaccines for the disease. Therefore, further research is necessary to discover the new virulence mechanisms of this pathogen. The long-term goal of our group is to determine the mechanism of *E. ictaluri* pathogenesis and to develop effective live attenuated vaccines against ESC. The overall goal of this project is to understand the role of Type 6 secretion system (T6SS) in *E. ictaluri* virulence and determine the safety and efficacy of T6SS mutants in the catfish host. The central hypothesis is that T6SS in *E. ictaluri* provide an ability to invade the host cells and survive inside of the channel catfish neutrophils and macrophages, and mutation of T6SS genes will cause attenuation of the bacterial virulence. The rationale for the proposed research is that characterization of the T6SS in *E. ictaluri* will enlighten its role in *E. ictaluri* virulence, and T6SS genes can be targeted to develop live attenuated vaccines.

In this study, we first constructed mutants of individual T6SS genes and a double mutant. The persistence, virulence, and vaccine efficacy of T6SS mutants were

determined in the catfish fingerlings and fry infection model. The T6SS mutants *Ei*Δ*evpC, Ei*Δ*evpC*Δ*hcp2, Ei*Δ*evpD, Ei*Δ*evpE, Ei*Δ*evpG, Ei*Δ*evpJ,* and *Ei*Δ*evpK* were significantly attenuated and provided better protection against *E. ictaluri 93-146* in channel catfish fingerlings. The role of T6SS mutants in adhesion and invasion of *in vitro* catfish epithelial indicated that *Ei*Δ*evpN, Ei*Δ*evpO,* and *Ei*Δ*evpP* significantly were less adherent and invasive. The survival and replication of T6SS mutants in *in vitro* catfish peritoneal macrophages cell line showed that T6SS mutants could survive up to 6 hours after phagocyted by catfish macrophages. The survival and resistance of T6SS mutants to stress conditions present in macrophages phagosome showed that hydrogen peroxide could limit the growth of T6SS mutants in BHI and minimal medium. *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* exhibited a significant growth decrease.

DEDICATION

I would like to dedicate this research to my parents Pakize Kalindamar and Selahattin Kalindamar, and my brother Donem Kalindamar.

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CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

The catfish industry

Farm-raised channel catfish (*Ictalurus punctatus*) production is one the largest aquaculture commodity in the southeastern U.S., including Mississippi, Alabama, Arkansas, Louisiana, and Texas (USDA 2009). These states are leading producers of farm-raised channel catfish with 94% of total sales ($341 million in 2011) (USDA 2012). Among these states, Mississippi has been the leading state with production acreage of 51,200 and sales of $165 million from 180 catfish operations in 2012 (Mississippi State University 2012). Catfish industry in the southeastern U.S. contributes local economy and creates job opportunities, in particular, in Mississippi Delta region. However, bacterial diseases such as enteric septicemia of catfish (ESC) can cause a significant problem in the production of catfish. *Edwardsiella ictaluri* is well-adapted to catfish and causes ESC (Hawke 1998).

Enteric septicemia of catfish (ESC)

Enteric septicemia of catfish (ESC) causes a severe disease in the U.S. farm-raised channel catfish industry. ESC has been one of the most prevalent disease of channel catfish since it's identification, especially in the southeastern U.S. including Mississippi, Arkansas, Georgia, and Alabama. The impact of ESC in the U.S. catfish

aquaculture was reported to be 50-80 million U.S. dollars (Russo et al., 2009). The occurrence of ESC on channel catfish depends on temperature. *Edwardsiella ictaluri*'s optimal growth temperature is 25-30 ºC. ESC can be observed in channel catfish in the U.S. during the late spring and fall seasons while water temperature is from 18 to 28 ºC. The optimum temperature for the disease is 28 ºC, whereas low mortalities can also occur at 17, 21, and 32 ºC (Francis-Floyd et al., 1987). Moreover, the rapid temperature changes from 11-17 ºC to 20-30 ºC can cause a quick spread of ESC in channel catfish population (Bly et al., 1991).

As an approved treatment method of ESC in the U.S., antibiotics oxytetracycline, sulfadimethoxine, and florfenicol are administered orally medicated as food additives (McGinnis et al., 2003). However, one of the first signs of infection is anorexia. Thus, antibiotics limit the spread of an outbreak, rather than treating the infected fish. The use of live attenuated vaccines has shown that they can provide an alternative for protection with expressing protective antigens without causing infection in the host (Lan et al., 2007). A more efficient way of vaccine delivery is achieved by immersion exposure (Cooper et al., 1996; Lawrence et al., 1997; Klesius et al., 1999; Thune et al., 1999). Live attenuated vaccine candidates developed for *E. ictaluri* includes mutants with a deletion of the virulence-related genes essential for bacterial pathogenesis such as chondroitinase mutant (Cooper et al., 1996) and auxotrophic mutants (aroA and purA) (Lawrence et al., 1997; Thune et al., 1999). There is a commercial live attenuated vaccine (Aquavac-ESC) developed by rifampin resistance.

Edwardsiella ictaluri

Edwardsiella ictaluri is a Gram-negative facultative-anaerobic bacterium that recently classified in *Hafniaceae. E. ictaluri* was first isolated from fish mortalities in 1976 in Alabama (Hawke 1979; Hawke et al., 1981). The optimal growth temperature of *E. ictaluri* is 25° C. The optimal temperature for motility of *E. ictaluri* is 25-30° C. However, higher temperatures above optimal growth temperature can restrict the motility of *E. ictaluri*. *E. ictaluri* is well known as a slow-growing bacterium. The optimal growth of *E. ictaluri* requires 48 hours at 30 ºC on an agar plate. *E. ictaluri* 93-146 strain was the first fully sequenced genome of *E. ictaluri*. The *E. ictaluri* 93-146 genome consists of 3.8123 million nucleotide base pairs with a GC content of 57.4% and 3,201 proteins (Williams et al., 2012). To date, *E. ictaluri* RUSVM-1, *E. ictaluri* ATCC 33202, *E. ictaluri* LADL11-100, and *E. ictaluri* LADL11-194 strains genomes are sequenced, and their genomes are available (Wang et al., 2015; Reichley et al., 2017). *E. ictaluri* primarily infects farm-raised channel catfish but is also able to cause severe infection on other freshwater fish species (Plumb et al., 1983; Soto et al., 2012; Hawke et al., 2013). *E. ictaluri* causing infections on fish were reported from the different regions in U.S., and disease associated *E. ictaluri* strains were isolated from farm-raised channel catfish, tilapia, and zebrafish (Griffin et al., 2016). *E. ictaluri* is not only isolated in U.S. but also reported from across regions of Asia, Central America, and Caribbean (Crumlish et al., 2002; Yuasa et al., 2003; Sakai et al., 2008; Liu et al., 2010; Phillips et al., 2017).

Intracellular survival of *Edwardsiella ictaluri*

E. ictaluri is a facultative intracellular pathogen adapted for surviving inside the channel catfish immune cells such as macrophage and neutrophils (Miyazaki et al., 1985;

Shotts et al., 1986; Baldwin et al., 1993). *E. ictaluri* can invade and proliferate for a long-term survival inside the catfish head kidney-derived macrophages (HKDM) and peritoneal macrophages (Shoemaker et al., 1997; Booth et al., 2006). The short-term intracellular survival has also been reported in the catfish neutrophils (Ainsworth et al., 1990). The intracellular-survival of *E. ictaluri* is virtually unknown in the catfish phagocytic cells. The adaptation of *E. ictaluri* as a facultative intracellular pathogen to the host immune cells is possible with activating the survival and host resistance mechanisms. *E. ictaluri* employs Type III Secretion System (T3SS) is upregulated within catfish macrophages (Rogge et al., 2011)**.** A successful replication of *E. ictaluri* in catfish macrophages depends on the secreted effector proteins of T3SS imported from *E. ictaluri* to the *E. ictaluri* containing vacuole (ECV) in macrophage phagosome to manipulate the pathways responsible for killing bacteria (Zhao et al., 2013; Dubytska et al., 2016). T3SS-dependent secreted effector proteins are responsible for the survival of *E. ictaluri* in HKDM. Any mutation in these proteins can cause lower replication in HKDM (Thune et al., 2007). Recent studies also show that some genes may be crucial for the proliferation of *E. ictaluri* inside the neutrophils including tricarboxylic acid cycle (TCA) enzymes, glycine cleavage system, a sigmaE regulator, the SoxS oxidative response system, and a plasmid-encoded T3SS effector (Karsi et al., 2009).

Type VI secretion system

Type VI secretion system (T6SS) is a contact-dependent secretion system that embedded in the cell membrane of Gram-negative bacteria (Pukatzki et al., 2006). The needle-like structure of T6SS that is structurally homolog to bacteriophage T4 puncture device (Kanamaru 2009). The primary function of T6SS is described in bacterial

pathogenesis and competition (Ho et al., 2014; Zoued et al., 2014). T6SS plays a crucial role in pathogenic bacteria by delivering T6SS-dependent secreted proteins in host. Moreover, T6SS mediates antibacterial determinants for the inter-bacteria and intra-bacteria competition in mixed bacterial populations by secreting lethal toxins. This bacterial antagonism assists to bacterial pathogens to colonize in the host by destroying commensal flora of the host. This is ensured by delivering the effector proteins into the eukaryotic target cells or prokaryotic cells (Hachani et al., 2016).

T6SS operon consists of 13 conserved genes that assemble a functional T6SS. These core genes of T6SS assembly are classified as membrane-associated proteins and T4 phage-related proteins. Two secreted proteins of T6SS are Hcp (Hemolysin co-regulated protein) inner tail tube and VgrG (Valine glycine repeat protein) spike complex proteins. Hcp and VgrG are homologs of the bacteriophage T4 tail tube gp19 and spike proline-alanine-alanine-arginine (PAAR) proteins (Leiman et al., 2009; Shneider et al., 2013). T6SS can be found 25% of Gram-negative bacterial genomes including pathogenic and non-pathogenic bacteria (Shrivastava et al., 2008). Some human and animal pathogenic bacteria have multiple T6SS copies in their genomes such as *Burkholderia pseudomallei*, *Burkholderia thailandensis*, and *Pseudomonas aeruginosa* (Filloux et al., 2008; Schwarz et al., 2010). T6SS is tightly regulated by environmental conditions including temperature, metal concentration (Iron, magnesium, zinc, and phosphate), and pH.

Bacterial secretion systems in *Edwardsiella ictaluri*

Type III secretion system

E. ictaluri may have acquired the vital Type III secretion system (T3SS) horizontally. The importance of T3SS genes of *E. ictaluri* in catfish virulence first revealed in 2007 (Thune et al., 2007). This study showed that T3SS of *E. ictaluri* shares a high functional similarity with the *Salmonella* Pathogenicity Island 2 (SPI-2). T3SS is required for proliferation of *E. ictaluri* within channel catfish head kidney-derived macrophages (HKDM). The activation and expression of T3SS genes require particular conditions in the host environment such as low pH and low organic phosphate (Rogge et al., 2011). In particular, *E. ictaluri* in the vacuoles of HKDM induces T3SS for successful replication (Baumgartner et al., 2014). The adaptation of *E. ictaluri* in these growth-restricted conditions of HKDM achieved by secretion of T3SS dependent secreted effector proteins. Some of the T3SS-dependent secreted effector proteins are located on native plasmids pE1 and pE2 (Zhao et al., 2013). For example, *EseI, EseH,* and *EscD* T3SS effector proteins are encoded on these native plasmids. More T3SS-dependent secreted effector proteins have been recently identified in *E. ictaluri* (Dubytska et al., 2016).

Type VI secretion system

The function of T6SS was first identified in the *Edwardsiella* genus with *Edwardsiella tarda* (Rao et al., 2004; Zheng et al., 2007). This study showed that *evpC*, *evpI*, and *evpP* proteins are T6SS-dependent secreted proteins. Mutation in T6SS genes decreased virulence of *E. tarda*, and T6SS mutants were highly attenuated in the host infection model (except *evpD*. The two-component regulatory (TCs) systems PhoP-PhoQ senses the changes in environmental temperature and Mg^{2+} concentration to regulate T6SS

in *E. tarda* (Chakraborty et al., 2010). The TCs of PhoB-PhoR and ferric uptake regulator (Fur) detect P_i and iron concentration and regulate the expression level of T6SS in *E. tarda* (Chakraborty et al., 2011). Thus, deletion of esrB and PhoP response regulator proteins resulted in complete loss of functional T6SS (Lv et al., 2012). On the other hand, T6SS-dependent secreted protein evpP is also tightly regulated via TCs of esrA-esrB and iron concentration in media (Wang et al., 2009). T6SS-dependent secreted chaperone protein *evpC* contains a disordered region for binding to *evpP* secreted protein (Hu et al., 2014). These two T6SS proteins are secreted to the host environment. *evpP* secreted protein recently identified as an effector protein that can target the pathways to inhibit inflammasome formation in macrophages (Chen et al., 2017).

T6SS proteins of *E. ictaluri* that eip19 (*evpE*), eip18 (*evpC*), eip55 (*evpB*), eip20 (*evpA*) have been first identified in catfish *E. ictaluri* interaction. This study revealed that T6SS proteins were secreted while the invasion of *E. ictaluri* in catfish (Moore et al., 2002). T6SS operon transcriptionally regulated from *evpA* to *evpO* by *esrC* that is an Ara-C type regulatory protein. *esrC* as a part TCs is also controlled by esrA-esrB (TCs), which senses host environment for rapid pH changes and inorganic phosphate (P_i) concentration (Rogge et al., 2011). Conversely, an *evpP* gene located out of T6SS operon is tightly regulated by ferric uptake regulator (*Fur*) protein. Fur regulatory protein can bind to Fur box located the upstream of *evpP* to control the expression level of *evpP* (Santander et al., 2012).

Significance of research and objectives

Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC) and causes significant economic losses in the U.S. aquaculture industry. *E. ictaluri* is a Gram-negative facultative intracellular pathogen that possesses specific virulence

mechanisms involved in the adaptation of bacteria to host and interfere with host immune system to survive inside channel catfish neutrophils and macrophages. In this process, bacterial protein secretion systems play an essential role. *E. ictaluri* tend to quickly regulate the different complex set of pathogenic mechanisms in channel catfish. However, T6SS in *E. ictaluri* is not explored thoroughly.

The *overall goal* of this project is to understand the role of T6SS in *E. ictaluri* virulence and determine the safety and efficacy of T6SS mutants in channel catfish host. The central hypothesis is that T6SS in *E. ictaluri* involves in invasion and survival in the host cells, and mutation of T6SS genes will cause attenuation of the bacterial virulence. The *rationale* for the proposed research is that characterization of the T6SS in *E. ictaluri* will enlighten its role *E. ictaluri* virulence, and T6SS genes can be targeted to develop live attenuated vaccines. The specific objectives of this study were:

1: Construction of Type 6 Secretion System (T6SS) mutants. The *working hypothesis* for this aim is that mutation of T6SS genes in *E. ictaluri* will cause attenuation of the bacterial virulence. To attain this objective, 17 T6SS genes will be in-frame mutated.

2: Determination of invasion and survival of T6SS mutants in catfish cells. The *working hypothesis* for this research objective is that T6SS is an essential secretory machine used by the pathogen to invade and survive in host cells and mutants' invasion and survival is catfish cells will be affected compared to wild-type *E. ictaluri*. To attain this objective, invasion and survival analysis will be conducted using channel catfish ovary cells and peritoneal macrophages.

3: Determination of safety and efficacy of T6SS mutants in channel catfish. The *working hypothesis* for this aim is that in-frame deleted T6SS mutants will attenuate in

catfish, and the attenuated strains can be potential vaccine candidate. To attain this objective, safety and efficacy of the mutants will be characterized in catfish fingerlings and fry.

CHAPTER II

THE EFFECT OF HCP FAMILY PROTEINS OF TYPE VI SECRETION SYSTEM IN *EDWARDSIELLA ICTALURI* VIRULENCE

Abstract

Edwardsiella ictaluri is a facultative intracellular fish pathogen causing enteric septicemia of catfish (ESC), a devastating disease resulting in significant economic losses in the U.S. catfish industry. Bacterial secretion systems are essential components in bacterial virulence, and Type III and Type VI Secretion Systems (T3SS and T6SS) are two of the distinctive machinery in the *E. ictaluri* 93-146 genome. The T3SS assists *E. ictaluri* survival inside catfish macrophages by secreting effector proteins. However, the roles of secreted proteins by T6SS are still unknown in *E. ictaluri*. In this research, we generated *E. ictaluri* hemolysin co-regulated family proteins (Hcp) mutants (*Ei*Δ*evpC*, *Ei*Δ*hcp2*, and *Ei*Δ*evpC*Δ*hcp2*), which are potentially secreted by T6SS. The survivability of *Ei*Δ*hcp2* inside the catfish peritoneal macrophages was significantly less than that of *Ei*Δ*evpC, Ei*Δ*evpC*Δ*hcp2,* and wild-type. The cell attachment of *Ei*Δ*hcp2* and *Ei*Δ*evpC*Δ*hcp2* were significantly decreased compared to wild-type, whereas the cell invasion rates of these mutants were same as that of wild-type. Mutants exposed to catfish serum *in vitro* showed that all mutants were serum resistant. The fish challenges showed that *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* were significantly attenuated and provided excellent protection against wild-type *E. ictaluri* infection in catfish fingerlings.

However, *Ei*Δ*hcp2* caused a high catfish mortality with severe disease signs. Taken together, our data demonstrated that Hcp proteins of T6SS seem to be important in different stages of *E. ictaluri* colonization, adhesion, survival, and virulence. *hcp2* plays a role in adhesion to epithelial cells and survival inside catfish macrophages. However, *evpC* is more critical for *E. ictaluri* virulence in catfish fingerlings and fry.

Introduction

E. ictaluri is the causative agent of enteric septicemia of catfish (ESC) in the southeastern U.S. (Hawke et al., 1981). Although *E. ictaluri* is well-adapted to channel catfish, it can infect other freshwater fish species (Plumb et al., 1983; Soto et al., 2012; Hawke et al., 2013). At the early stages of host invasion, *E. ictaluri* encounters with host immune system (Miyazaki et al., 1985; Shoemaker et al., 1997). However, *E. ictaluri* is capable of surviving and replicating inside the catfish macrophages and neutrophils (Booth et al., 2006). To proliferate successfully inside the host cells, *E. ictaluri* must resist the bacterial killing mechanisms present in the host macrophages and neutrophils such as oxidative and nitrosative stress (Chen et al., 1991; Yeh et al., 2013; Yao et al., 2014).

The survival of *E. ictaluri* highly depends on its resistance to stress factors and modulating the host environment. *E. ictaluri* encodes urease that is activated in acidic macrophage phagosomes to cope with low pH (Booth et al., 2009; Baumgartner et al., 2014). Low pH and low phosphate concentration inside the phagosome can trigger the expression level of genes in Type III and Type VI secretion systems (T3SS and T6SS), which assists *E. ictaluri* survival inside the host immune cells (Rogge et al., 2011). It was shown that the secreted effector proteins via T3SS have an important role in virulence of

E. ictaluri, and mutation of these genes caused decreased intracellular replication inside catfish head kidney-derived macrophages (Zhao et al., 2013; Dubytska et al., 2016).

Hcp family proteins of T6SS plays an important role in different level of bacterial interaction with many eukaryotic hosts. Hcp proteins are particularly involved in adhesion and invasion, intracellular survival of bacteria, bacterial cytotoxicity, and virulence (Hachani et al., 2016). T6SS proteins eip19 (*evpE*), eip18 (*evpC*), eip55 (*evpB*), eip20 (*evpA*) have been first identified with the catfish host-pathogen interaction study in *E. ictaluri* (Moore et al., 2002). The secretion of *evpC* is transcriptionally controlled by two-component system regulatory protein *esrC* in low-pH and phosphate conditions in *E. ictaluri* (Rogge et al., 2011). Fish pathogen *E. tarda* also possesses T6SS that is required for its virulence (Zheng et al., 2007). In *E. tarda*, *evpC* plays a dual role as a chaperone and T6SS-dependent secreted protein (Jobichen et al., 2010), which belongs to hemolysin co-regulated family (Hcp) proteins that bind and guide the T6SS-dependent effector proteins through T6SS needle (Pukatzki et al., 2007). Due to their role as a chaperone protein, *evpC* interacts with the T6SS-dependent effector proteins such as *evpP* in *E. tarda* (Hu et al., 2014). This study recently showed that *evpP* effector protein secreted via *evpC* could target the inflammasome activation in macrophages (Chen et al., 2017).

We found a complete T6SS operon in *E. ictaluri* 93-146 genome, and two of proteins are classified as Hcp family proteins named *evpC* (*Hcp1*) and *hcp2*. Although *evpC* is located inside the T6SS operon, *hcp2* was found outside of the major T6SS operon. Here, we evaluated the role of Hcp family proteins of T6SS in *E. ictaluri*-catfish interaction. Our study reveals the role of Hcp family proteins, *evpC* and *hcp2*, in adhesion and invasion of catfish epithelial cells, survival and replication inside catfish peritoneal

macrophages, adaptation and survival to host stress factors mimicking phagosomal conditions, and virulence and efficacy in the catfish fingerlings and fry.

Material and methods

Bacteria, plasmids, and media

Bacterial strains and plasmids used in this work were listed in Table 1. *E. ictaluri* 93-146 Wild Type (WT) strain was grown at 30ºC in brain-heart infusion (BHI) broth or agar plates. *Escherichia coli* CC118λ*pir* and BW19851 strains were cultured on Luria–Bertani (LB) agar or broth and incubated at 37º C. Antibiotics were added to the culture medium at the following concentrations: ampicillin (100 μg/ml) and colistin (12.5 μg/ml).

Table 1 Bacterial strains and plasmids.

Strain or plasmid	Description	Reference
Edwardsiella ictaluri		
93-146	Wild-type; pEI1; pEI2; Col^r	(Lawrence et al., 1997)
*Ei*Δ*evpC*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpC*	This study
*Ei*Δ*hcp2*	93-146 derivative; pEI1; pEI2; Col^r, Δ*hcp2*	This study
*Ei*Δ*evpC*Δ*hcp2*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpC*Δ*hcp2*	This study
Escherichia coli		
CC118λ*pir*	Δ(*ara-leu*); *araD*; Δ*lacX74*; *galE*; *galK*; *phoA20*; *thi-1*; *rpsE*; *rpoB*; *argE*(*Am*); *recA1*; λ*pirR6K*	(Herrero et al., 1990)
SM10λ*pir*	*thi; thr; leu; tonA; lacY; supE; recA;::RP4-2-Tc::Mu; Kmr; lpirR6K*	(Miller et al., 1988)
BW19851λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510*	(Metcalf et al., 1994)

Table 1 (continued)

DH5α	dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1 (2)	(Taylor et al., 1993)
Plasmids		
pMEG375	8142 bp, Amp^r, Cm^r, lacZ, R6K ori, mob incP, sacR sacB	(Dozois et al., 2003)
pEiΔ*evpC*	9939 bp, pMEG-375, Δ*evpC*	This study
pEiΔ*hcp2*	9939 bp, pMEG-375, Δ*hcp2*	This study
pEiΔ*evpC*Δ*hcp2*	9939 bp, pMEG-375, Δ*evpC*Δ*hcp2*	This study
pAK*gfplux*1	5681bp, PstI, EcoRI, HpaI, AseI, BstBI	(Karsi et al., 2007)

In frame deletion of *evpC* and *hcp2*

The nucleotide sequences of *evpC* (NT01EI_RS11900) and *hcp2* (NT01EI_RS14960) were obtained from the *E. ictaluri* 93-146 genome (GenBank accession: 95 CP001600) (Williams et al., 2012). Overlap extension method was used to generate *evpC* and *hcp2* mutants in *E. ictaluri*. Briefly, external and internal primers were designed to amplify the regions for upstream and downstream of each gene (Table 2). Amplified two fragments were combined through splicing by overlap extension (SOEing) (Horton et al., 1989). The overlap PCR product and the suicide pMEG375 plasmid were digested with the same restriction enzymes, and the mutated insert was ligated into the pMEG375. After electroporation and selection of the correct plasmid in *E. coli* CC118, pMEG375 was transferred to *E. coli* BW19851 by electroporation, which was used to transfer pMEG375 into *E. ictaluri s*train 93-146 by conjugation. Two-step selection was used to obtain in-frame deletion mutants. At the first step, ampicillin resistant *E. ictaluri* colonies were inoculated into BHI broth containing ampicillin and colistin. At the second step, after confirmation of the presence of the deleted gene by colony PCR and agarose

gel electrophoresis, colonies were streaked on the BHI agar containing colistin. These colonies were re-streaked on the BHI agar with 5% sucrose, 0.35% D-mannitol, and colistin. Ampicillin-sensitive colonies with the mutant band were the correct *E. ictaluri* colonies. Deletion of each gene was confirmed by both PCR and sequencing.

Table 2 Primers used for in-frame deletion.

Primers	Sequence (5' to 3')
*Ei*Δ*evpC*EF01	cccctctagaATCGGGGATTATGAGTTCAGC
*Ei*Δ*evpC*IR01	ggaacggtacagggtgacatatAGCGGACCTCTCTTGTGAC
*Ei*Δ*evpC*IF01	ATATGTCACCCTGTACCGTTCC
*Ei*Δ*evpC*ER01	ccccggatccCAGTCCCACCATGATAAAGC
*Ei*Δ*hcp2*EF01	ccctctagaACAGGCCAACAAAATTCTCGC
*Ei*Δ*hcp2*IR01	gtcagaggggtatttgcttcGACTACCGGAGAGCCATTCTC
*Ei*Δ*hcp2IF*01	GAAGCAAATACCCCCTCTGAC
*Ei*Δ*hcp2ER*01	cccgagctcGTGGTGTACCGAGAACCACTG

Construction of bioluminescent strains

The pAK*gfplux*1 plasmid was used to develop bioluminescent *Ei*Δ*evpC, Ei*Δ*hcp2, and Ei*Δ*evpC*Δ*hcp2* strains as described previously (Karsi et al., 2007). Briefly, *E. coli* SM10λ*pir* carrying pAK*gfplux*1 and mutant strains were grown overnight and mixed at the ratio of 1:2 (Donor: recipient). Mixture pellet was inoculated with 0.45 µM filter paper on a BHI agar plate at 30°C for 24 h. Filter paper containing a mixture of bacteria were dissolved in BHI broth with ampicillin and colistin. Serial dilutions were inoculated

on BHI plates containing ampicillin and colistin for selection. Ampicillin resistant mutant colonies carrying pAK*gfplux*1 appeared on the selective plates after 30° C for 24–48 h.

Serum treatment

Bioluminescent *Ei*Δ*evpC, Ei*Δ*hcp2, and Ei*Δ*evpC*Δ*hcp2* strains were exposed to naïve catfish serum. Bioluminescent positive control *E. ictaluri* WT and negative control *E. coli* DH5α were also included in each experiment. Briefly, wells of a 96-well black plate were added 195 µl catfish serum. Then, 5 µl overnight culture was mixed with the serum and inoculated for 4 hours at 30°C. The experiment included four replicates, and bioluminescence was measured by using SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices).

Bioluminescent imaging

Four specific-pathogen-free (SPF) catfish fingerlings obtained from the CVM hatchery (12.72 ± 1.00 cm, 24.95 ± 5.47 g) were stocked into each tank. Three tanks were assigned to *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* (Treatments), and one tank for *E. ictaluri* WT (control). 100 ml of bacterial culture was added directly to each tank (final dose of 5 x 10^7 CFU/ml water). After 1 h, water flow was restored to each tank. Bioluminescent imaging was taken by using IVIS Lumina XRMS in Vivo Imaging System Series III (Waltham, PerkinElmer). 100 mg/L MS222 was used to anesthetize catfish in water. Anesthetized fish were quickly placed into the photon collection chamber to capture images. Exposure time was set for one minute to collect total photon emissions from the whole fish body. Following bioluminescent imaging, fish were placed

in well-aerated water for recovery. Bioluminescent imaging was conducted at 0, 6, 12, and 24 h post-infection, and subsequent daily intervals until 14 days.

Bacterial killing assay

The bacterial killing assay was performed as previously described (Booth et al., 2009; Russo et al., 2009). Briefly, peritoneal macrophages were collected from a year-old channel catfish (250-300 g) injected 1 ml squalene (Sigma). After post-injection, peritoneal macrophages were collected by injection of 10 ml of 1 X cold phosphate-buffered saline (PBS) to the peritoneal cavity of catfish. Harvested cells were washed with PBS three times. Peritoneal macrophages were resuspended in channel catfish macrophage medium (CCMM) including RPMI (RPMI 1640 sans phenol red & L-glutamine, Lonza, Walkersville, MD, USA) containing 1x glutamine substitute (GlutaMAX –I CTS, Invitrogen, Carlsbad, CA, USA), 15 mM HEPES buffer (Invitrogen), in 0.18% sodium bicarbonate solution (Invitrogen), 0.05 mM 2-beta-mercaptoethanol (Sigma, St.Louis, MO, USA), and 5% heat-inactivated (HI) pooled channel catfish serum. Then, peritoneal macrophages were transferred into 96-well black plate. Peritoneal macrophages and bioluminescent mutants or WT *E. ictaluri* were suspended at the 1:1 ratio. Each mutant was placed in a 96-well black plate with four replicates, and one control group without bacteria. A mixture of cells and bacteria was centrifuged at 1500 rpm for 5 mins at 24^{o} C to compact the cells and bacteria. The cell suspension was incubated with CCMM for 1 h at 32^{o} C to allow the invasion of catfish peritoneal macrophages by bioluminescent *E. ictaluri* mutants and WT. Following incubation, CCMM containing 100 µg/ml gentamicin was used to incubate cell suspension for 1 h at 32^{o} C to kill non-phagocyted *E. ictaluri*. Each well was washed by

three times with PBS to remove extracellular bacteria. The suspension was incubated with CCMM containing 10 µg/ml gentamicin for 48 h with 5% CO_2 at 32° C to determine the number of survived bioluminescent bacteria inside the catfish peritoneal macrophages. Statistical analysis was done with the results acquired from Cytation 5 Cell Imaging Multi-mode Reader (BioTek).

Attachment and invasion assays

Attachment and invasion assays were performed using channel catfish ovary (CCO) cell line as previously described (Santander et al., 2013). Briefly, CCO cells were suspended in DMEM medium 1 X (Sigma) with 10% fetal bovine serum and 4mM L-glutamine at a final concentration of $1x10^7$ cells ml^{-1}. Bioluminescent *E. ictaluri* WT and mutants were also adjusted to $1x10^7$ cells ml^{-1}. Bacteria and CCO cells were mixed at the multiplicity of inception (MOI) 1:1 and placed in a 24-well plate with four replicates. One control group without bacteria was also included. CCO and bacteria suspension were incubated 1 h at 28° C for the attachment of *E. ictaluri* WT and mutants to CCO. For invasion, the number of bacteria was determined by incubating medium with 100 µg/ml gentamicin for 1 h to kill the non-invasive *E. ictaluri* WT and mutants. Statistical analysis was done with the results acquired from IVIS Lumina XRMS in Vivo Imaging System Series III**.**

Stress assays

Bioluminescent *Ei*Δ*evpC, Ei*Δ*hcp2, and Ei*Δ*evpC*Δ*hcp2* strains were used for the survival stress tests. The mutants' survival in oxidative stress in hydrogen peroxide (H_2O_2) and nitrosative stress in sodium nitroprusside (SNP) were tested in BHI (rich

medium) and low phosphate minimal medium at pH 5.5 (MM19-P) (Collins et al., 1996). Bacteria were grown overnight, and OD_{600} adjusted to 0.5 for each culture. From each strain, 5 µl of bacteria were inoculated into 195 µl of BHI and MM19-P broth containing 0.75 mM H_2O_2 and 5mM SNP. 96-well black plates were used with three replicates for each mutant and *E. ictaluri* WT as a control. The mean photon counts for each stress treatments were measured after 4, 8, 12, and 24 h incubation at 30°C by using IVIS Lumina XRMS in Vivo Imaging System Series III.

Virulence and efficacy of mutants in catfish fingerlings and fry

All fish experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. Vaccination and efficacy were performed as previously described (Karsi et al., 2009). Briefly, specific-pathogen-free (SPF) channel catfish fingerlings and fry obtained from the MSU-CVM Hatchery. Catfish fingerlings (10.46 ± 0.86 cm, 14.03 ± 3.57 g) were stocked into 15 tanks at a rate of 25 fish/tank. Catfish fry were stocked in 12 tanks at a rate of 50 fish/tank. They were acclimated for one week. Chlorine, dissolved oxygen, and temperature were monitored daily. Treatments were randomly assigned to *Ei*Δ*evpC,* *Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* (vaccination), *E. ictaluri* WT (positive control), and BHI (negative control) groups. Each treatment had three replicates. Immersion vaccination was applied by lowering the water in each tank to 10-L, and by adding 100 ml of bacterial culture (final dose of 2.4 x 10^7 CFU/ml water). After 1 h, water flow (1-l/min) was restored to each tank. Mortalities were recorded daily for a total of 21 days, and the percent mortalities calculated for each group.

To assess the protective capabilities of mutants, all fish that survived the *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* vaccination were re-challenged with *E. ictaluri* WT (2.8 x 10^7 CFU/ml) 21 days post-vaccination as described above. Fish mortalities were recorded daily, and the experiment was terminated when no fish mortalities were observed for three consecutive days.

Statistical analysis

The significance of the differences between means of treatments was established by one-way ANOVA and two-way ANOVA procedures with Tukey's test in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). The level of significance for all tests was set at $p < 0.05$.

Results

Serum treatment

Channel catfish serum was used to evaluate mutants' survival under complement stress. *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* were able to survive after 4 hours of incubation in catfish serum (Figure 1), and there were no differences between the mutants and wild-type. There were significant differences in bioluminescence values at 0 and 4 hours ($p<0.05$), which indicate the wild type and mutants were resistant to complement killing and able to replicate in catfish serum.

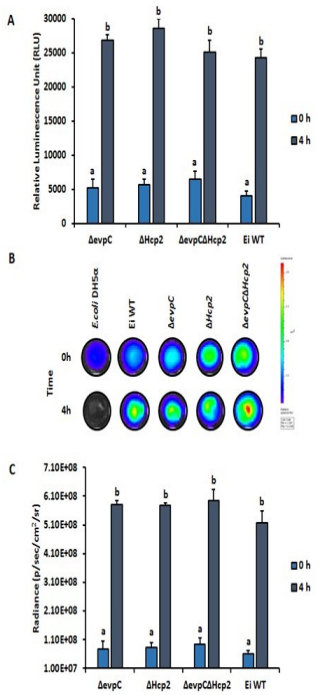

Figure 1 The catfish serum treatment of *Ei*Δ*evpC*, *Ei*Δ*hcp2, Ei*Δ*evpC*Δ*hcp2* and *E. ictaluri* WT.

The data represented the mean of each treatment ±SD. The letters (a and b) show the significant differences between treatments ($p < 0.05$). A. The bar graph indicates the relative luminescence unit (RLU) obtained from the mutant strains and *E. ictaluri* WT treated with the catfish serum. B. The bioluminescent images of mutant strains, *E. ictaluri* WT, and *E. coli* DH5α treated with the catfish serum after 0 and 4 hours. C. Bioluminescence values obtained from mutants and *E. ictaluri* WT.

Bioluminescent imaging

The bioluminescent imaging was used to monitor *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* mutants' persistence in catfish fingerlings. *E. ictaluri* WT was able to kill all catfish fingerlings in 5 days shortly after ESC symptoms observed. Catfish fingerlings exposed to *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* mutants were able to survive for 14 days with clearance of mutants from the catfish fingerlings. However, the immersion challenge of *Ei*Δ*hcp2* showed severe mortality of all catfish fingerlings in 8 days (Figure 2A). The bioluminescent photon counts obtained from the catfish fingerlings showed that the

number of *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* mutants on the catfish fingerlings were peaked the highest point from 12 h to 3 days. On the other hand, the number of *Ei*Δ*hcp2* peaked the highest point from 6 to 7 days (Figure 2B). These findings demonstrate that *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* were attenuated and cleared from catfish fingerlings, while *Ei*Δ*hcp2* was not attenuated.

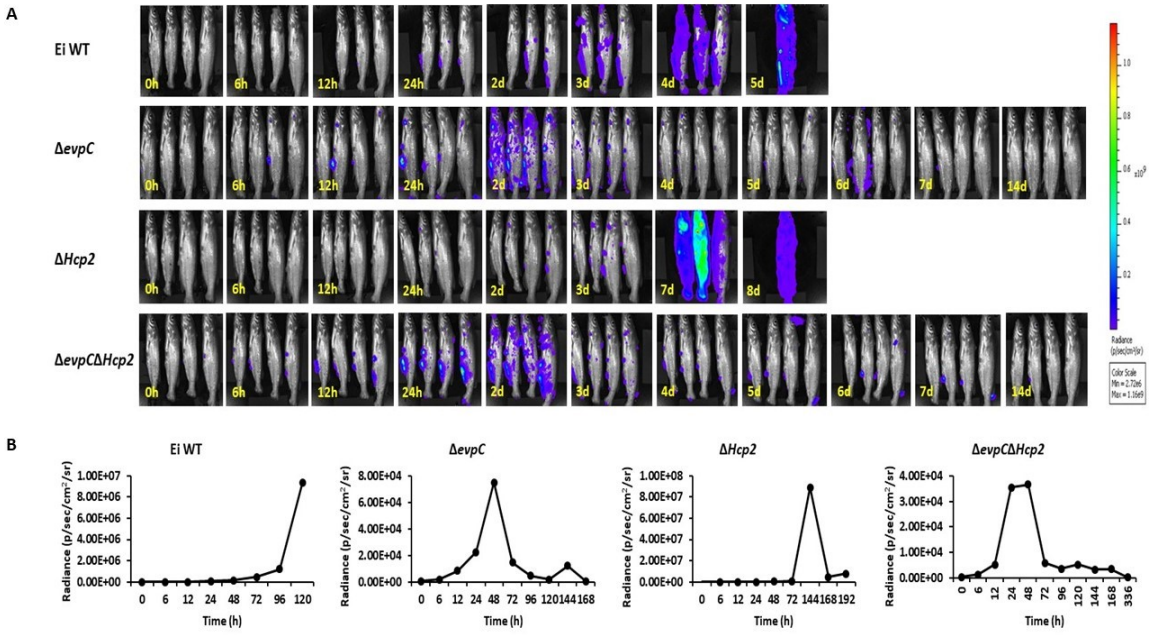

Figure 2 The bioluminescent imaging of catfish fingerlings challenged with *Ei*Δ*evpC*, *Ei*Δ*hcp2*, *Ei*Δ*evpC*Δ*hcp2* and *E. ictaluri* WT.

A. The bioluminescent image was obtained from four fish at 0, 6, 12, 24 h and subsequent daily intervals until 14 days. B. Total photon emissions obtained from the images.

Bacterial killing assay

The phagocytic uptake and survival of *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* inside the catfish peritoneal macrophages were evaluated by the bacterial killing assay. The proliferation of *Ei*Δ*hcp2* inside catfish peritoneal macrophages was significantly less

than *Ei*Δ*evpC* at 6 and 12 h (p<0.05), but was similar to *E. ictaluri* WT and *Ei*Δ*evpC*Δ*hcp2* (Figure 3). The survival of all mutants and *E. ictaluri* WT decreased by time, which was lower at 24 h, and there were no significant differences between the groups. This indicates that *E. ictaluri* WT and mutant strains can replicate inside catfish macrophages up to 6 hours, after which macrophages can prevent bacterial replication and initiate bacterial killing.

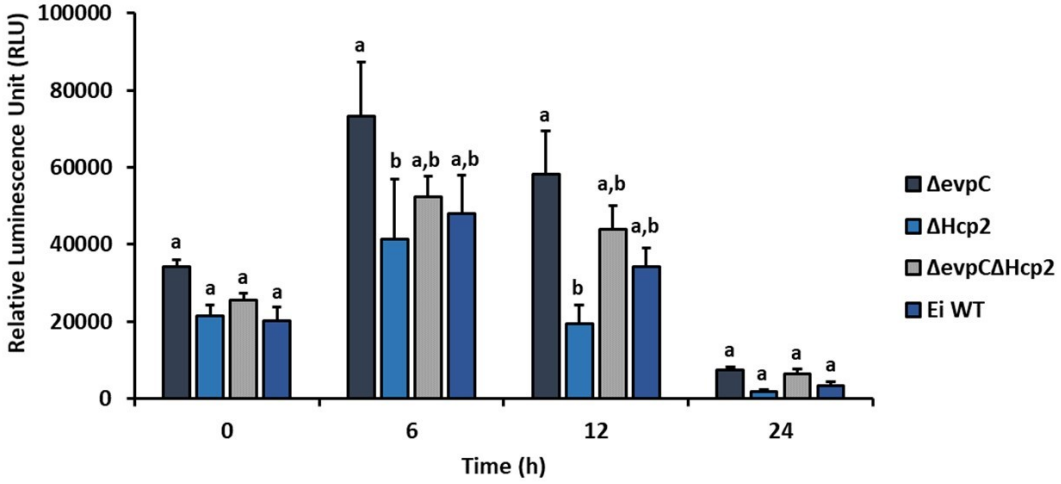

Figure 3 The bacterial killing assay of *Ei*Δ*evpC*, *Ei*Δ*hcp2*, *Ei*Δ*evpC*Δ*hcp2* and *E. ictaluri* WT.

The graph represented the mean bioluminescence of each treatment ±SD. Letters above bars show the significant differences (p < 0.05).

Attachment and invasion

CCO cell line was used to assess the attachment and invasion capability of *Ei*Δ*evpC, Ei*Δ*hcp2,* and *Ei*Δ*evpC*Δ*hcp2* mutants. Although attachment of mutants reduced compared to wild type, there was no significant difference between *Ei*Δ*evpC* and

E. ictaluri WT (p<0.05). However, attachments of *Ei*Δ*hcp2* and *Ei*Δ*evpC*Δ*hcp2* were significantly lower than that of *E. ictaluri* WT (p<0.05) (Figure 4A). The invasion and replication of all mutants were reduced, but there was no significant difference compared to *E. ictaluri* WT (p<0.05) (Figure 4B).

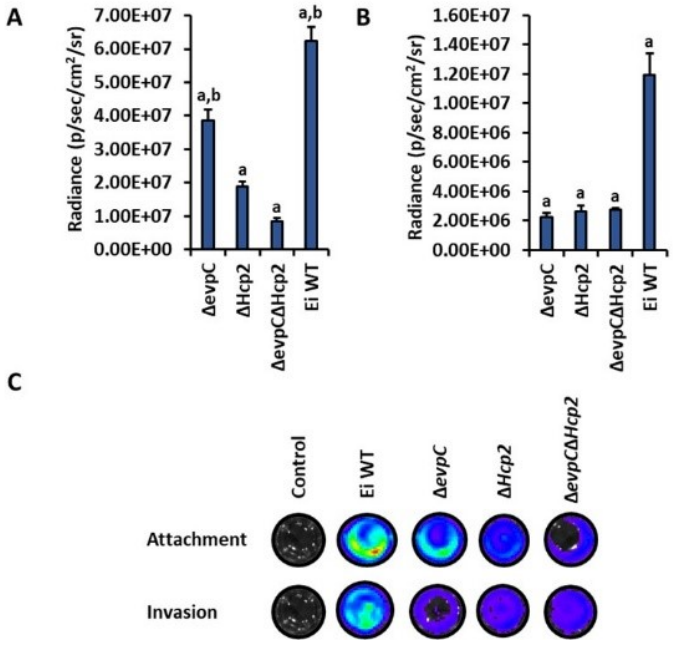

Figure 4 The attachment and invasion of CCO cells by *Ei*Δ*evpC*, *Ei*Δ*hcp2,* *Ei*Δ*evpC*Δ*hcp2* and *E. ictaluri* WT.

A. The mean of photon emission obtained from each well in 24-well plate incubated with mutants, *E. ictaluri* WT, and control (Non-infected) after one-hour incubation. The bar graph indicates the mean of photons emitted from four biological replicates to test the attachment ability of mutant strains and *E. ictaluri* WT. The data represented the mean of each treatment ±SD. Letters above bars show the significant differences between treatments (p < 0.05). B. The mean of photon exposure obtained from the same 24-well plate incubated with mutant strains, *E. ictaluri* WT, and control including gentamycin for an hour after attachment. The bar graph indicates the mean of photons obtained from four biological replicates to test the attachment ability of mutant strains and *E. ictaluri* WT. C. The bioluminescent imaging of CCO treated with mutant strains and *E. ictaluri* WT and control (no bacteria).

Stress assays

The survival and resistance of the mutants to nitrite oxide and hydrogen peroxide were evaluated. The treatment of mutants and *E. ictaluri* WT with SNP and H_2O_2 in BHI and MM19-P had a variation in the growth rate of bacteria, which their resistance were more increased in MM19-P than BHI up to 12 hours (Figure 5 B, D). Due to the low pH (5.5) in MM19-P, the resistance of mutants and *E. ictaluri* WT were enhanced in 0 and 4 hours. The mutants and *E. ictaluri* WT were able to grow exponentially up to 24h in BHI, whereas their survival rate restricted in MM19-P at 24h (Figure 5 A, C). *EiΔevpCΔhcp2* double mutant had more resistant to SNP and H_2O_2 stress in BHI and MM19-P up to 12 hours. *EiΔevpC* and *EiΔhcp2* showed similar growth rate in BHI and MM19-P.

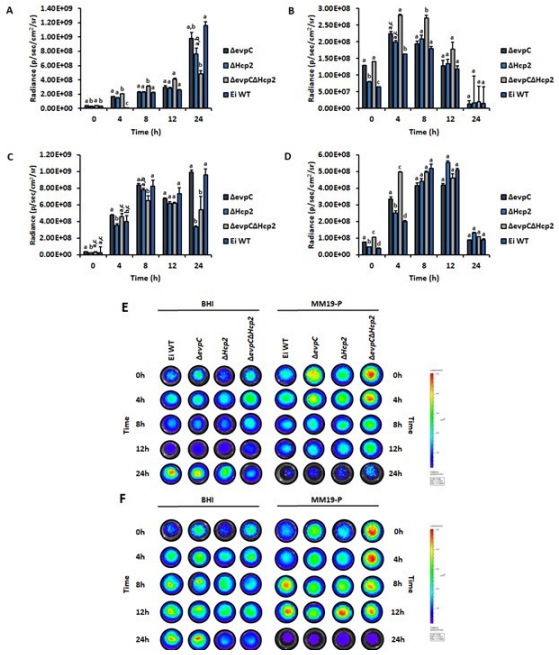

Figure 5 The stress assays of *EiΔevpC*, *EiΔhcp2, EiΔevpCΔhcp2* mutants and *E. ictaluri* WT.

The bar graphs indicate the mean of photons obtained from three biological replicates. The data represented the mean of each treatment ±SD. Letters above bars show the

significant differences between treatments ($p < 0.05$). A, B. Mutants and *E. ictaluri* WT exposed to SNP in BHI and MM19 for 24h. C, D. Mutants and *E. ictaluri* WT exposed to H_2O_2 in BHI and MM19 for 24h. E. The bioluminescent imaging of mutants and *E. ictaluri* WT exposed to SNP in BHI and MM19 for 24h. The picture represents one of three biological replicates. F. The bioluminescent imaging of mutant strains and *E. ictaluri* WT exposed to H_2O_2 in BHI and MM19 for 24h. The picture represents one of three biological replicates.

Assessment of virulence and efficacy

The immersion challenge was used to evaluate mutants' virulence and efficacy in catfish fingerlings and fry. *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* were completely attenuated in catfish fingerlings. Interestingly, *Ei*Δ*hcp2* mutant was caused a severe and quick death (93.94 % mortality) on catfish fingerlings (Figure 6A). The efficacy of *Ei*Δ*evpC* (0 % mortality) and *Ei*Δ*evpC*Δ*hcp2* (0 % mortality) were significantly less than *E. ictaluri* WT (Figure 6B). In catfish fry challenge, virulence of *Ei*Δ*evpC* (18.72 % mortality) and *Ei*Δ*evpC*Δ*hcp2* (35.90 % mortality) decreased significantly comparison to *E. ictaluri* WT (100%) (Figure 6C) ($p<0.05$). These mutants protected fry compared to sham-vaccinated group WT (Figure 6D). Protection of *Ei*Δ*evpC* (33.93 % mortality) was better than that of *Ei*Δ*evpC*Δ*hcp2* (58.42 % mortality).

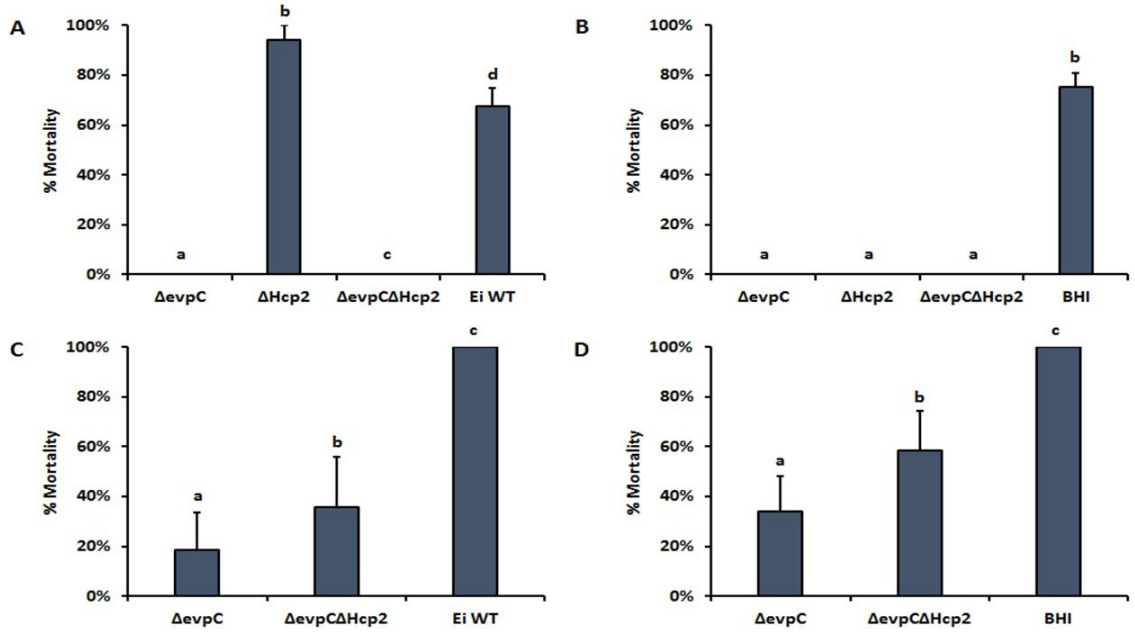

Figure 6 The virulence (A) and efficacy (B) of *Ei*Δ*evpC*, *Ei*Δ*hcp2, Ei*Δ*evpC*Δ*hcp2* mutants and *E. ictaluri* WT in catfish fingerlings.

The virulence (C) and efficacy (D) of *Ei*Δ*evpC, Ei*Δ*evpC*Δ*hcp2* mutants and *E. ictaluri* WT in catfish fry.

Discussion

The goal of this research was to understand potential roles of Hcp proteins in *E. ictaluri* virulence. Adhesion and invasion of catfish epithelial cells, survival and replication inside catfish peritoneal macrophages, adaptation and survival to host stress factors, persistence in catfish fingerlings, and virulence and efficacy in catfish fingerlings and fry were investigated.

According to previous studies, Hcp family proteins are involved in adherence and invasion of the host epithelial tissues. Disruption in *hcp* genes can cause different results in Hcp-mediated cell adhesion and invasion in pathogenic bacteria. Hcp mutants displayed reduced adhesion and invasion of epithelial cells in *Campylobacter jejuni*, *Escherichia coli*, and *Vibrio parahaemolyticus* (Lertpiriyapong et al., 2012; Yu et al.,

2012; Zhou et al., 2012). However, deletion of *hcp* caused increased adhesion and invasion of MODE-K cell line in *Helicobacter hepaticus* (Chow et al., 2010). We founded that there was a difference between *evpC* and *hcp2* in adhesion to CCO cell line. However, their invasion ability for CCO was similar. Mutation in *evpC* did not decrease attachment to CCO. In contrast, the deletion of *hcp2* and a double mutation in *evpC* and *hcp2* decreased adherence of *E. ictaluri* to CCO. These results suggest that the Hcp family proteins may differently regulate in the host environment for adhesion but not for the invasion of CCO. Thus, *hcp2* may play a different role than *evpC* in adhesion of *E. ictaluri* to the catfish epithelial cells.

Hcp family proteins encoded in intracellular pathogens are secreted inside host macrophages and required for intracellular survival in host macrophages (Burtnick et al., 2010; Peng et al., 2016). Lacking a functional *Hcp* reduced survival of *Burkholderia pseudomallei* in macrophages (Burtnick et al., 2011; Hopf et al., 2014). In *E. tarda*, deletion of *evpC* caused lower replication rate in gourami phagocytes (Srinivasa Rao et al., 2004). Similarly, we found that the mutation in *hcp2* displayed a lower replication rate for the intracellular growth of *E. ictaluri* inside the catfish peritoneal macrophages. The numbers of intracellular *Ei*Δ*evpC*, *Ei*Δ*hcp2*, *Ei*Δ*evpC*Δ*hcp2*, and wild-type cells were similar at 0 and 24 h postinfection. At 6 and 12 h postinfection, numbers of intracellular *Ei*Δ*evpC* and wild-type cells were similar, but intracellular replication of *Ei*Δ*hcp2* cells was significantly impaired. This result suggests that *hcp2* may be important for *E. ictaluri* replication within catfish macrophages.

In vivo and *in vitro* infection models indicated that Hcp family proteins are associated with bacterial virulence and host colonization. In *Aeromonas hydrophila, E.*

coli, and *B. pseudomallei Hcp* is required for virulence because Hcp mutants were less virulent compared to wild-type (Suarez et al., 2008; de Pace et al., 2010). Additionally, *evpC* is essential for the virulence of *E. tarda* (Zheng et al., 2007). Here, we showed that the *evpC* and *hcp2* contribute to the pathogenicity of *E. ictaluri* in catfish fingerlings and fry. Vaccination of catfish fingerlings with *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* provided an excellent safety and protection against ESC. However, *Ei*Δ*hcp2* showed a hypervirulent phenotype causing higher mortality with severe symptoms in catfish fingerlings. The mortality rates of *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* in catfish fry challenge indicated that *Ei*Δ*evpC* showed significantly less mortality and better protection than *Ei*Δ*evpC*Δ*hcp2*. The real-time bioluminescent imaging with live catfish fingerlings indicated that all mutants were able to invade and establish an infection on catfish fingerlings. The catfish fingerlings infected with *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* were able to survive up to 14 days post-infection. *Ei*Δ*evpC* and *Ei*Δ*evpC*Δ*hcp2* were able to proliferate inside catfish up to 72 h post-infection. However, *Ei*Δ*hcp2* did not replicate well in catfish up to 72 h post-infection and suddenly caused severe death of catfish. These results demonstrate that *Ei*Δ*evpC* may be developed as a live attenuated vaccine candidate for catfish fingerlings and fry.

T6SS facilities uptake of essential metals under stress conditions by releasing proteinaceous metallophores into the host environment (Chen et al., 2016). The role of T6SS in manganese scavenging under oxidative stress has been recently revealed in *Burkholderia thailandensis* (Si et al., 2017). On the other hand, intracellular compartmentalization of *Salmonella typhimurium* inside macrophages initiates stress conditions including nitrosative and oxidative stress to suppress the replication of

bacteria (Burton et al., 2014). To investigate the survivability of Hcp family proteins of T6SS in *E. ictaluri*, we applied nitrosative and oxidative stress with SNP and H_2O_2 in BHI and MM19-P to imitate macrophage phagosome conditions. Our results indicated that mutants and wild-type were able to survive up to 24h in MM19-P. In BHI, mutants and wild-type can survive, and their number increased under nitrosative and oxidative stress in 24h. Interestingly, the number of *Ei*Δ*evpC*Δ*hcp2* increased in both stress conditions in BHI and MM19-P, but its resistance for H_2O_2 was more than SNP.

In conclusion, the two different Hcp family proteins of T6SS found in the *E. ictaluri* 93-146 genome have diverse functions in *E. ictaluri* pathogenesis. *hcp2* is vital in adherence to epithelial cells and replication within macrophages. However, *evpC* plays a crucial role in *E. ictaluri* virulence in catfish.

CHAPTER III

CHARACTERIZATION OF TYPE VI SECRETION SYSTEM IN *EDWARDSIELLA ICTALURI*

Abstract

Edwardsiella ictaluri is a Gram-negative facultative intracellular fish pathogen causing enteric septicemia of catfish (ESC). Bacterial secretions systems are important in *E. ictaluri* survival in the host environment. Particularly, type III secretion system (T3SS) is required by *E. ictaluri* for survival within catfish macrophages. Although activation of T3SS triggers type VI secretion system (T6SS) expression in the host environment, the role of T6SS has not been well studied in *E. ictaluri.* Here, we report construction and characterization of fourteen T6SS mutants in *E. ictaluri*. Mutants' uptake and survival in peritoneal macrophages, attachment and invasion in epithelial cells, virulence and efficacy in catfish, and resistance to stress were evaluated. *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* showed a reduction in catfish peritoneal macrophages. *Ei*Δ*evpN, Ei*Δ*evpO,* and *Ei*Δ*evpP* exhibited lower attachment to and invasion of channel catfish ovary (CCO) cells, while *Ei*Δ*evpM* showed lower attachment. T6SS mutants were more resistant to stress conditions in BHI than minimal medium. *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* were sensitive to hydrogen peroxide in both BHI and minimal medium. The fish challenges of T6SS mutants showed that *Ei*Δ*evpD, Ei*Δ*evpE,*

*Ei*Δ*evpG, Ei*Δ*evpJ,* and *Ei*Δ*evpK* were significantly attenuated and provided excellent protection against wild-type *E. ictaluri* infection in catfish fingerlings.

Introduction

Edwardsiella ictaluri, a Gram-negative rod-shaped bacterium in *Hafniaceae* was first isolated from channel catfish and has been an important pathogen of cultured fish (Hawke 1979; Hawke et al., 1981). *E. ictaluri* infects channel catfish (*Ictalurus punctatus*) promiscuously, causing acute septicemia or chronic encephalitis (MacMillan 1985; Shotts et al., 1986; Newton et al., 1989). *E. ictaluri* is a facultative intracellular pathogen adapted to survival in catfish immune cells such as macrophage and neutrophils (Miyazaki et al., 1985; Shotts et al., 1986; Baldwin et al., 1993). *E. ictaluri* can invade and proliferate in catfish head kidney-derived and peritoneal macrophages for a long-term survival (Shoemaker et al., 1997; Booth et al., 2006). The short-term intracellular survival of *E. ictaluri* has also been reported in catfish neutrophils (Ainsworth et al., 1990). The intracellular survival and replication of *E. ictaluri* are virtually unknown.

E. ictaluri adapts itself to intracellular stress by using different defense mechanisms. Type III secretion system (T3SS) is tightly regulated and activated inside catfish macrophages (Rogge et al., 2011). The T3SS secreted effector proteins are transported to the macrophage environment to manipulate the host cell pathways (Zhao et al., 2013; Dubytska et al., 2016). Genes that might play a significant role in the survival of *E. ictaluri* inside the neutrophils include tricarboxylic acid cycle (TCA) enzymes, glycine cleavage system, a sigmaE regulator, the SoxS oxidative response system, and a plasmid-encoded T3SS effector (Karsi et al., 2009). Among all of these genes, T3SS

effector proteins could invade catfish macrophages, whereas mutation in these genes was impaired in intracellular replication (Thune et al., 2007).

Type VI secretion system (T6SS) is a supramolecular protein complex located in the cell membrane of Gram-negative bacteria (Pukatzki et al., 2006). T6SS is specialized for active transportation of the effector proteins through a needle-like nanomachine, which is a structural homolog to the puncturing device of bacteriophage T4 (Kanamaru 2009). The thirteen conserved core genes called as core components encode a functional T6SS. In *E. ictaluri* 93-146 genome, *in silico* analysis of the T6SS gene cluster have shown that T6SS is encoded by an operon including 16 genes *evpPABCDEFGHIJKLMNO*, which is tightly regulated by the transcriptional regulatory mechanisms. The core genes involved in T6SS assembly classified as membrane-associated proteins (*evpN*, *evpO*, *evpL*, and *evpM*) and bacteriophage T4 phage-related proteins (*evpK*, *evpA*, *evpB*, *evpC (Hcp1)*, *evpE*, *evpI*, *evpH*, *evpF*, and *evpG*) (Zoued et al., 2014). Functions of *evpP, evpD*, and *hcp2* genes is not known in *E. ictaluri*.

Initially, T6SS proteins of *E. ictaluri* eip19 (*evpE*), eip18 (*evpC*), eip55 (*evpB*), eip20 (*evpA*) were identified during a catfish-pathogen interaction study, showing that these proteins were expressed during *E. ictaluri* infection (Moore et al., 2002). Transcriptional regulation of the T6SS genes from *evpA* to *evpO* is controlled by Ara-C type regulatory protein e*srC*, which is regulated by *EsrA-EsrB* two-component system (TCs), sensing by the environmental pH changes and inorganic phosphate (P_i) concentration (Rogge et al., 2011). Unlikely, the expression of *evpP* gene is regulated by ferric uptake regulator (Fur) protein that binds to Fur box in the upstream promoter of *evpP* (Santander et al., 2012). T6SS provides a dual role in either dealing with survival

inside the host cells or competing for the inter-bacteria and intra-bacteria species. This is ensured by delivering the effector proteins into the eukaryotic target cells or prokaryotic cells (Hachani et al., 2016).

The essential role of T6SS in the *Edwardsiella* genus was first described in the fish pathogen *Edwardsiella tarda* (Rao et al., 2004; Zheng et al., 2007). In this study, *evpC*, *evpI*, and *evpP* proteins identified as secreted proteins, and mutation in T6SS genes caused attenuation in the host except for *evpD*. The possible interaction between the *evpC* secreted protein and a disordered region of *evpP* suggest that *evpP* serves as a secreted effector protein targeting the inflammasome activation in macrophages (Hu et al., 2014; Chen et al., 2017). The activation and repression of T6SS in *E. tarda* are quite similar with *E. ictaluri* T6SS depending on temperature, pH, Mg^{2+}, P_i, and iron concentration (Chakraborty et al., 2010; Chakraborty et al., 2011). Although T6SS has been identified in *E. tarda*, we know little about the functional significance of T6SS and roles of its genes in *E. ictaluri*. Despite this ingrained interaction, there is no unifying answer how *E. ictaluri* employs T6SS for virulence.

This study aimed to understand the role of T6SS in *E. ictaluri* virulence and determine the safety and efficacy of T6SS mutants in the catfish host. Our study reveals the role of individual T6SS proteins in *E. ictaluri* adhesion and invasion to catfish epithelial cells, survival and replication inside macrophages, adaptation and survival to stress, and virulence in the catfish fingerlings and fry.

Material and methods

Bacteria, plasmids, and media

Bacterial strains and the plasmid used in this work were listed in Table 1. *E. ictaluri* 93-146 Wild Type (WT) strain was grown at 30° C in brain-heart infusion (BHI) broth or agar plates. *Escherichia coli* CC118λ*pir* and BW19851 strains were cultured on Luria–Bertani (LB) agar or broth and incubated at 37° C. Antibiotics were added to the culture medium at the following concentrations: ampicillin (100 µg/ml) and colistin (12.5 µg/ml).

Table 3 Bacterial strains and plasmids.

Strain or plasmid	Description	Reference
Edwardsiella ictaluri		
93-146	Wild-type; pEI1; pEI2; Col^r	(Lawrence et al., 1997)
*Ei*Δ*evpA*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpA*	This study
*Ei*Δ*evpB*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpB*	(Karsi et al., 2017)
*Ei*Δ*evpD*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpD*	This study
*Ei*Δ*evpE*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpE*	This study
*Ei*Δ*evpF*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpF*	This study
*Ei*Δ*evpG*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpG*	This study

Table 3 (continued)

*Ei*Δ*evpH*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpH*	This study
*Ei*Δ*evpI*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpI*	This study
*Ei*Δ*evpJ*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpJ*	This study
*Ei*Δ*evpK*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpK*	This study
*Ei*Δ*evpL*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpL*	This study
*Ei*Δ*evpM*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpM*	This study
*Ei*Δ*evpN*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpN*	This study
*Ei*Δ*evpO*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpO*	This study
*Ei*Δ*evpP*	93-146 derivative; pEI1; pEI2; Col^r, Δ*evpP*	This study
Escherichia coli		
CC118λ*pir*	Δ*(ara-leu); araD;* Δ*lacX74; galE; galK; phoA20; thi-1; rpsE; rpoB; argE(Am); recAl;* λ*pirR6K*	(Herrero et al., 1990)
SM10λ*pir*	*thi; thr; leu; tonA; lacY; supE; recA;::RP4-2-Tc::Mu; Kmr;* λ*pirR6K*	(Miller et al., 1988)
BW19851λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510*	(Metcalf et al., 1994)

Table 3 (continued)

DH5α	*dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1 (2)*	(Taylor et al., 1993)

Plasmids		
pMEG375	8142 bp, Ampr, Cmr, lacZ, R6K ori, mob incP, sacR sacB	(Dozois et al., 2003)
pEiΔ*evpA*	9939 bp, pMEG-375, Δ*evpA*	This study
pEiΔ*evpB*	9939 bp, pMEG-375, Δ*evpB*	(Karsi et al., 2017)
pEiΔ*evpD*	9939 bp, pMEG-375, Δ*evpD*	This study
pEiΔ*evpE*	9939 bp, pMEG-375, Δ*evpE*	This study
pEiΔ*evpF*	9939 bp, pMEG-375, Δ*evpF*	This study
pEiΔ*evpG*	9939 bp, pMEG-375, Δ*evpG*	This study
pEiΔ*evpH*	9939 bp, pMEG-375, Δ*evpH*	This study
pEiΔ*evpI*	9939 bp, pMEG-375, Δ*evpI*	This study
pEiΔ*evpJ*	9939 bp, pMEG-375, Δ*evpJ*	This study
pEiΔ*evpK*	9939 bp, pMEG-375, Δ*evpK*	This study
pEiΔ*evpL*	9939 bp, pMEG-375, Δ*evpL*	This study

Table 3 (continued)

pEiΔ*evpM*	9939 bp, pMEG-375, Δ*evpM*	This study
pEiΔ*evpN*	9939 bp, pMEG-375, Δ*evpN*	This study

pEiΔ*evpO*	9939 bp, pMEG-375, Δ*evpO*	This study
pEiΔ*evpP*	9939 bp, pMEG-375, Δ*evpP*	This study
pAK*gfplux1*	5681bp, PstI, EcoRI, HpaI, AseI, BstBI	(Karsi et al., 2007)

In frame deletion of T6SS genes

The nucleotide sequences of *E. ictaluri* T6SS genes were obtained from the *E. ictaluri* 93-146 genome (GenBank accession: 95 CP001600) (Williams et al., 2012). The *evpP* nucleotide sequence was manually identified in the *E. ictaluri* 93-146 genome. Overlap extension PCR method was used to generate T6SS mutants in *E. ictaluri*. Briefly, external and internal primer pairs were designed to amplify the regions for upstream and downstream of each gene (Table 2). Amplified two fragments were combined through splicing by overlap extension (SOEing) (Horton et al., 1989). The overlap PCR product and the pMEG375 suicide plasmid were digested with the same restriction enzymes, and the mutated insert was ligated into the pMEG375. After electroporation and selection of the correct plasmid in CC118, pMEG375 was transferred to *E. coli* BW19851by electroporation, which was used to transfer pMEG375 into *E. ictaluri* 93-146 by conjugation. Two-step selection was used to obtain in-frame deletion mutants. At the first step, ampicillin resistant *E. ictaluri* colonies were inoculated into BHI broth containing ampicillin and colistin. At the second step, after confirmation of the presence of the deleted gene by colony PCR and agarose gel electrophoresis, colonies were streaked on the BHI agar containing colistin. These colonies were re-streaked on the BHI agar with 5% sucrose, 0.35% D-mannitol, and colistin. Ampicillin-sensitive colonies

with the mutant band were the correct *E. ictaluri* colonies. Deletion of each gene was confirmed by both PCR and sequencing.

Table 4 Primers used for in-frame deletion.

Primers	Sequence (5' to 3')
*Ei*Δ*evpA*EF01	ccccgcggccgcATCCCAGTAGGCATATATTGTTG
*Ei*Δ*evpA*IR01	tctgttcgctcattattgctgTCCGTAACATTTCTTACAACACC
*Ei*Δ*evpA*IF01	CAGCAATAATGAGCGAACAGA
*Ei*Δ*evpA*ER01	ccccggatccGGTACCCTTACAGTGGGTCAG
*Ei*Δ*evpB*EF01	aatctagaGGACGACTCACCTCCGTTATC
*Ei*Δ*evpB*IR01	TACGTCACCGGAAACTGTCAC
*Ei*Δ*evpB*IF01	GTGACAGTTTCCGGTGACGTAGATGTCAGC
*Ei*Δ*evpB*ER01	aatctagaGTTGATCGCTGTACCGATGTC
*Ei*Δ*evpD*EF01	cccctctagaGCCCAAGGAATATGACAGTGA
*Ei*Δ*evpD*IR01	gactgccagcgttttcagataCCGCTTGTCATCATCAGTGAG

Table 4 (continued)

*Ei*Δ*evpD*IF01	TATCTGAAAACGCTGGCAGTC

*Ei*Δ*evpD*ER01	ccccgagctcTCCCAGGGTATTCAGATGATG
*Ei*Δ*evpE*EF01	cccctctagaGTTCGATTCAACATCCTTTGG
*Ei*Δ*evpE*IR01	tcacctgtctattcggtacagGGCTCAACTCACGGGATTGTC
*Ei*Δ*evpE*IF01	CTGTACCGAATAGACAGGTGA
*Ei*Δ*evpE*ER01	ccccgagctcTCGGGAATAATTTGGTACTCG
*Ei*Δ*evpF*EF01	cccctctagaGACCCAGCAGATTATCAATGC
*Ei*Δ*evpF*IR01	ctgctgggtgcgtacatgCGCCAGTTCCCGGTTATAGTAG
*Ei*Δ*evpF*IF01	CATGTACGCACCCAGCAG
*Ei*Δ*evpF*ER01	ccccgagctcAAGTCACATCGCGTGGTAGG
*Ei*Δ*evpG*EF01	ccctctagaCCTGACCAAACTGACCGATA
*Ei*Δ*evpG*IR01	ctgtgcacccgatttatcgCATGACAGCACCGCCTTA
*Ei*Δ*evpG*IF01	CGATAAATCGGGTGCACAG
*Ei*Δ*evpG*ER01	cccgagctcGTACCGGCTTCAGCAGATTG
*Ei*Δ*evpH*EF01	cccctctagaTTTTCAGGCCGTCAGGATAC

Table 4 (continued)

*Ei*Δ*evpH*IR01	ccatccttcactcccctctagGCAGGTCTGGTTCATTTTCC

*Ei*Δ*evpH*IF01	CTAGAGGGGAGTGAAGGATGG
*Ei*Δ*evpH*ER01	ccccgagctcATTTAGGCAGGCAGACGAAG
*Ei*Δ*evpI*EF01	cccctctagaGTGCAGACCTGTGTGGATGA
*Ei*Δ*evpI*IR01	cctttgatggaggtcatcacGGTGGATAAGGACAATAGCCG
*Ei*Δ*evpI*IF01	GTGATGACCTCCATCAAAGG
*Ei*Δ*evpI*ER01	ccccgagctcTGATGGCTTGTTGAAGATCG
*Ei*Δ*evpJ*EF01	cccctctagaTACCAGAACCACTTCGTCTGC
*Ei*Δ*evpJ*IR01	ccttaaccgccaatcaacacCCAATACCATACCGCAGAGC
*Ei*Δ*evpJ*IF01	GTGTTGATTGGCGGTTAAGG
*Ei*Δ*evpJ*ER01	ccccgagctcTAACCCAATACACCCTTGAGC
*Ei*Δ*evpK*EF01	cccctctagaGTCTATAACGCCAACCAGACG
*Ei*Δ*evpK*IR01	ttcatccagtctcatattgccACTGATGGGAGCCAGTAAACG
*Ei*Δ*evpK*IF01	GGCAATATGAGACTGGATGAA
*Ei*Δ*evpK*ER01	ccccgagctcTTAGCCCCAGGTAGACATTGA

Table 4 (continued)

*Ei*Δ*evpL*EF01	cccctctagaGTGTTGATTGGCGGTTAAGG

*Ei*Δ*evpL*IR01	tccactgatatcgctcagccgGGTCAGACTGAGCAGCAGG
*Ei*Δ*evpL*IF01	CGGCTGAGCGATATCAGTGGA
*Ei*Δ*evpL*ER01	ccccgagctcCGAATAATCAGATCGCGTACC
*Ei*Δ*evpM*EF01	cccctctagaGTGGAGGGAAAAGGGATGAC
*Ei*Δ*evpM*IR01	tcgaaccagctgaatgcaatCTGTAAAAACATGCCCTGATGC
*Ei*Δ*evpM*IF01	ATTGCATTCAGCTGGTTCGA
*Ei*Δ*evpM*ER01	ccccgagctcTACCGTCATGCATCTCATGG
*Ei*Δ*evpN*EF01	cccctctagaCCGACCGTTCAATGTCTACC
*Ei*Δ*evpN*IR01	cgacatccgcatactcatataaGAGCATGGGCTGAATAAATGC
*Ei*Δ*evpN*IF01	TTATATGAGTATGCGGATGTCG
*Ei*Δ*evpN*ER01	ccccgagctcCGGTAGGGAACATCAATAGGG
*Ei*Δ*evpO*EF01	cccctctagaATTTCCTGATCACCCTTGAGC
*Ei*Δ*evpO*IR01	gcttgtcggttaactgtttggcAATCAGTGCAATCGCTATCC
*Ei*Δ*evpO*IF01	GCCAAACAGTTAACCGACAAGC

Table 4 (continued)

*Ei*Δ*evpO*ER01	ccccgagctcCAGCCGGTACCACAGGATCT

*Ei*Δ*evpP*EF01	ccccgcggccgcTGGTTTTTCTTCACCAGTTGC
*Ei*Δ*evpP*IR01	gcttgtcggttaactgtttggcAATATTACCGACATGGTTCCAG
*Ei*Δ*evpP*IF01	GCCAAACAGTTAACCGACAAGC
*Ei*Δ*evpP*ER01	ccccgagctcCAGCCGGTACCACAGGATCT

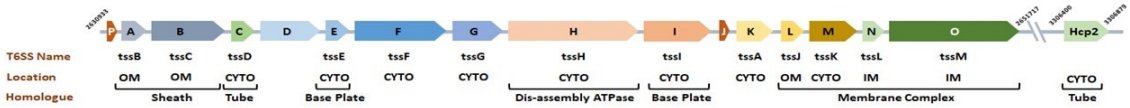

Figure 7 Gene organization of T6SS operon in *E. ictaluri* 93-146 genome.

T6SS genes were represented with the common T6SS name (TSS), proteins location (OM: Outer membrane, CYTO: Cytoplasmic, IM: Inner membrane), and their homolog of bacteriophage T4 (Baseplate, dis-assembly ATPase, sheath, and membrane complex). Gene sizes and distances were represented relatively.

Construction of bioluminescent strains

The pAK*gfplux*1 plasmid was used to develop bioluminescent T6SS mutants as described previously (Karsi et al., 2007). Briefly, *E. coli* SM10λ*pir* carrying pAK*gfplux*1 and T6SS mutant strains grown overnight and mixed at the ratio of 1:2 (Donor:

recipient). Mixture pellet was inoculated with 0.45 µM filter paper on a BHI agar plate at 30° C for 24 h. Filter paper containing a mixture of bacteria were dissolved in BHI broth with ampicillin and colistin. Serial dilutions were inoculated on BHI plates containing ampicillin and colistin for selection. Ampicillin resistant mutant colonies carrying pAK*gfplux*1 appeared on the selective plates after 30° C for 24-48 h.

Serum treatment

Bioluminescent *Ei*Δ*evpP* strain was exposed to naïve catfish serum. Bioluminescent positive control *E. ictaluri* WT and negative control *E. coli* DH5α were also included in each experiment. Briefly, wells of a 96-well black plate were added 195 µl catfish serum. Then, 5 µl of overnight culture was mixed with the serum and inoculated for 4 hours at 30° C. The experiment included four replicates, and bioluminescence was measured by using SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). Bioluminescent imaging was conducted at Cytation 5 Cell Imaging Multi-mode Reader (BioTek). Living Image Software v 4.2 was used to quantify bioluminescence from each well.

Bioluminescent imaging

Four specific-pathogen-free (SPF) channel catfish fingerlings obtained from the CVM hatchery (12.72 ± 1.00 cm, 24.95 ± 5.47 g) were stocked into a tank and acclimated for one week. Two tanks were assigned to bioluminescent *Ei*Δ*evpP* strain (Treatment), and bioluminescent *E. ictaluri* WT (control). The water level in each 50-liter tank was lowered to 10 liters, and 100 ml of bacterial culture was added directly to each tank (Final dose of 5 x 10^7 CFU/ml water). After 1 h, water flow was restored to each tank.

Bioluminescent imaging was taken by using IVIS Lumina XRMS in Vivo Imaging System Series III (Waltham, PerkinElmer). 100 mg/L MS222 was used to anesthetize catfish in water. Anesthetized fish were quickly placed into the photon collection chamber to capture the images. Exposure time was set for one minute to collect total photon emissions from the whole fish body. Following bioluminescent imaging, fish were replaced with the well-aerated fresh water for recovery. Bioluminescent imaging was conducted at 0, 6, 12, and 24 h post-infection, and subsequent daily intervals until 14 days.

Bacterial killing assay

Bacterial killing assay was performed as previously described with minor modifications (Booth et al., 2009; Russo et al., 2009). Briefly, 1 ml squalene (Sigma-Aldrich) was injected to a year-old channel catfish (250-300g) to activate peritoneal macrophages. Peritoneal macrophages were collected after four days. 10 ml of 1X cold phosphate-buffered saline (PBS) was injected into the abdomen of catfish. Harvested cells were washed and centrifuged by three times with PBS. Peritoneal macrophages and *E. ictaluri* WT and T6SS mutants were resuspended in channel catfish macrophage medium (CCMM) including RPMI [(RPMI 1640 sans phenol red & L-glutamine, Lonza, Walkersville, MD, USA) containing 1x glutamine substitute (GlutaMAX –I CTS, Invitrogen, Carlsbad, CA, USA)], 15 mM HEPES buffer (Invitrogen), in 0.18% sodium bicarbonate solution (Invitrogen), 0.05 mM 2-beta-mercaptoethanol (Sigma, St. Louis, MO, USA), and 5% heat-inactivated (HI) pooled channel catfish serum at the ratio 1:1. Macrophage and bacterial cell suspension was centrifuged at 1500 rpm for 5 mins at 24 ^{o}C to contact each other. T6SS mutants were placed into a 96-well black plate with the

four replicates. Control group was placed in 96-well black plate without bacteria suspended with catfish peritoneal macrophages. The cell suspension was incubated within CCMM for 1 h at 32 ºC to allow the invasion of catfish peritoneal macrophages by bioluminescent *E. ictaluri* mutants and WT. 100 µg/ml gentamicin was used to kill extracellular *E. ictaluri*. Each well was washed by three times with PBS to remove non-invasive extracellular bacteria. The suspension was incubated with CCMM containing 10 µg/ml gentamicin for 48 h with 5% CO_2 at 32 ºC to determine the number of survival *E. ictaluri* inside the catfish peritoneal macrophages. Statistical analysis was done with the results acquired from Cytation 5 Cell Imaging Multi-mode Reader (BioTek).

Attachment and invasion assays

Attachment and invasion assays were performed using channel catfish ovary (CCO) cell line as previously described (Santander et al., 2013). Briefly, CCO cells were suspended in DMEM medium 1X (Sigma) with 10% fetal bovine serum and 4mM L-glutamine at the final concentration of $1x10^7$ cells ml^{-1} to a 24-well plate. Bioluminescent *E. ictaluri* WT and mutants were adjusted to of $1x10^7$ cells ml^{-1}. Bacteria and CCO cells were mixed at the multiplicity of inception (MOI) 1:1 and placed in a 24-well plate with four replicates. One control group without bacteria was also included. CCO and bacteria suspension were incubated 1 h at 28º C for the attachment of *E. ictaluri* WT and mutants to CCO. For invasion, the number of bacteria was determined by incubating medium with 100 µg/ml gentamicin for 1 h to kill the non-invasive *E. ictaluri* WT and mutants. Statistical analysis was done with the results acquired from IVIS Lumina XRMS in Vivo Imaging System Series III**.**

Stress assays

Bioluminescent T6SS mutants and *E. ictaluri* WT were used for the survival stress tests. The mutants' survival in oxidative stress in hydrogen peroxide (H_2O_2) and nitrosative stress in sodium nitroprusside (SNP) were tested in BHI (Rich medium) and low phosphate minimal medium (pH 5.5) (MM19-P) (Collins et al., 1996). Bacteria were grown overnight, and OD_{600} adjusted to 1.0 for each overnight grown bacterial culture. From each strain, 5 µl of bacteria were inoculated into 195 µl of BHI and MM19-P broth containing 0.75 mM H_2O_2 and 5mM SNP. 96-well black plates were used with three replicates for each mutant and *E. ictaluri* WT as a control. The mean photon counts for each stress treatments were measured after 4, 8, 12, and 24 h incubation at 30° C by using IVIS Lumina XRMS in Vivo Imaging System Series III.

Assessment of virulence and efficacy of T6SS mutants in catfish fingerlings and fry

All fish experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. Vaccination and efficacy were conducted as described in previously (Karsi et al., 2009). Specific-pathogen-free (SPF) channel catfish fingerlings and fry obtained from the MSU-CVM Hatchery (10.46 ± 0.86 cm, 14.03 ± 3.57 g) were stocked into 50 tanks at a rate of 25 fish/tank and acclimated for one week. Chlorine, dissolved oxygen, and temperature were monitored daily. Tanks were randomly assigned to T6SS mutants (vaccination), *E. ictaluri* WT (positive control), and BHI (negative control) groups, with three tanks per group. Immersion vaccination was applied by lowering the water in each tank to 10-L, and by adding 100 ml of bacterial culture (final dose of 3 x 10^7 CFU/ml water). After 1 h, water flow (1-l/min) was restored to each tank. Mortalities were recorded daily for a total

of 21 days, and the percent mortalities calculated for each group. To assess the protective capabilities of T6SS mutants, all fish that survived the T6SS mutant's vaccination were re-challenged with *E. ictaluri* WT (2.8 x 10^7 CFU/ml) 21 days post-vaccination as described above. Fish mortalities were recorded daily, and the experiment was terminated when no fish mortalities were observed for three consecutive days.

Statistical analysis

The significance of the differences between means of treatments was established by one-way ANOVA and two-way ANOVA procedures with Tukey's test in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). The level of significance for all tests was set at $p < 0.05$.

Results

Serum treatment

Channel catfish serum was used to evaluate *Ei*Δ*evpP* survival in catfish blood and resistance to the complement killing system of catfish serum (Figure 8A). *Ei*Δ*evpP* was able to survive after incubated 4 hours with catfish serum (Figure 8B). There were significant differences between *Ei*Δ*evpP* and wild-type in 0 and 4 hours ($p<0.05$) (Figure 8C). This result indicated that *Ei*Δ*evpP* is capable of surviving in catfish blood and robust to the complement killing activity of catfish serum.

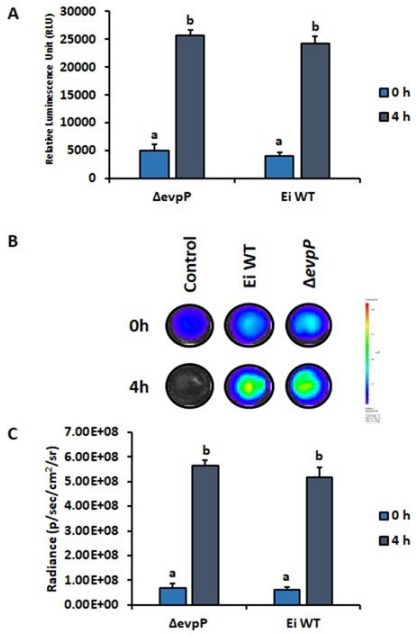

Figure 8 The catfish serum treatment of *Ei*Δ*evpP* and *E. ictaluri* WT.

The data represented the mean of each treatment ±SD. The letters (a and b) show the significant differences between treatments ($p < 0.05$). A. The bar graphs indicated the relative luminescence unit (RLU) obtained from the *Ei*Δ*evpP* and *E. ictaluri* WT treated with the catfish serum. B. The bioluminescent imaging of *Ei*Δ*evpP* and *E. ictaluri* WT treated with the catfish serum after 0 and 4 hours. C. The photon exposure obtained from *Ei*Δ*evpP*, *E. ictaluri* WT, and negative control (*E. coli* DH5α).

Bioluminescent imaging

The real-time bioluminescent imaging was used to track *Ei*Δ*evpP*'s persistence in catfish fingerlings. ESC symptoms appeared 3 days after the challenge of fish with *E. ictaluri* WT and *Ei*Δ*evpP*. Fish mortality occurred on day 5 in fish challenged with *Ei*Δ*evpP* (Figure 9A). Similarly, *E. ictaluri* WT caused mortality on catfish fingerlings in 5 days. The bioluminescence obtained from the catfish fingerlings showed that *Ei*Δ*evpP* and *E. ictaluri* WT were both reached the highest point in 5 days (Figure 9B). These findings demonstrate that there are no major differences in virulence of *Ei*Δ*evpP*

compared to *E. ictaluri* WT. The disease progression on catfish fingerlings was similar for both *Ei*Δ*evpP* and *E. ictaluri* WT.

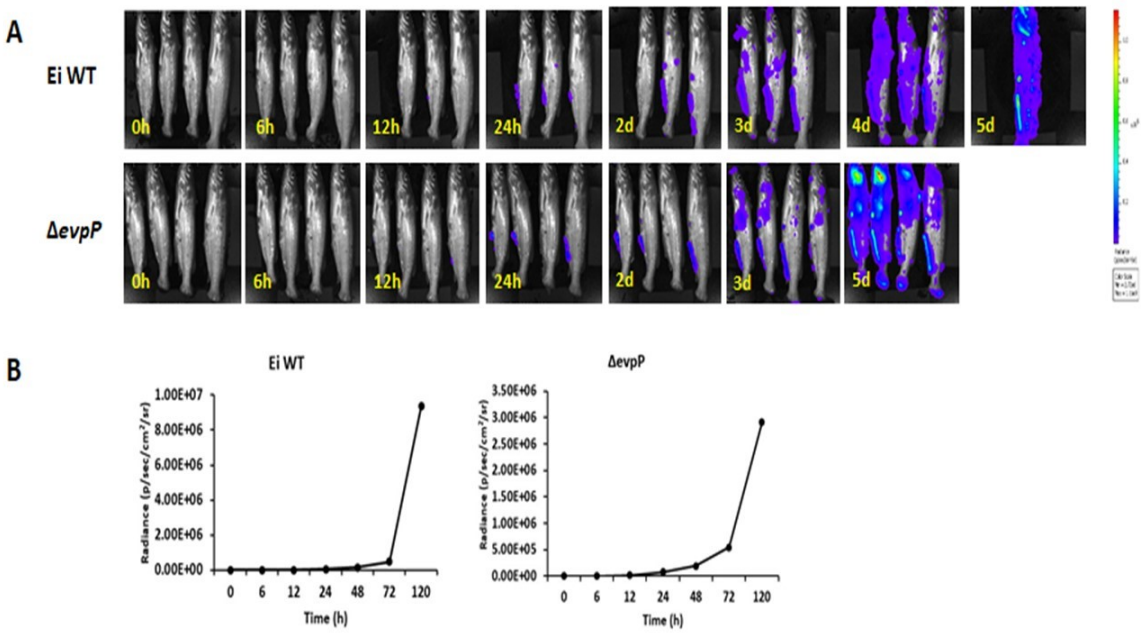

Figure 9 The bioluminescent imaging of catfish fingerlings immersion exposure challenged with *Ei*Δ*evpP* and *E. ictaluri* WT.

A. The bioluminescent image was taken with four fish exposed to *Ei*Δ*evpP* and *E. ictaluri* WT in 0, 6, 12, 24 hours and subsequent daily intervals until 5 days. B. The total photon emissions from four fish exposed with bioluminescent *Ei*Δ*evpP* and *E. ictaluri* WT for 5 days.

Bacterial killing assay

The phagocytic uptake (0 h) and intracellular survival of T6SS mutants and *E. ictaluri* WT within the catfish peritoneal macrophages were evaluated by bacterial killing assay (Figure 10). In phagocytic uptake at 0 h, *Ei*Δ*evpF, Ei*Δ*evpG, Ei*Δ*evpI, Ei*Δ*evpJ,* and *Ei*Δ*evpL* were significantly more internalized than other T6SS mutants and *E. ictaluri* WT. At 6 hour, the number of intracellular *Ei*Δ*evpF*, *Ei*Δ*evpG, Ei*Δ*evpI, Ei*Δ*evpJ,* and *Ei*Δ*evpK* were significantly higher than *E. ictaluri* WT. *Ei*Δ*evpF* and

*Ei*Δ*evpG* replicated significantly more than *Ei*Δ*evpI, Ei*Δ*evpJ,* and *Ei*Δ*evpK*. Interestingly, *Ei*Δ*evpB* and *Ei*Δ*evpO* remained at the lower replication rate than *E. ictaluri* WT at 6 h. *Ei*Δ*evpF, Ei*Δ*evpG,* and *Ei*Δ*evpJ* survived inside the catfish peritoneal macrophages up to 12 h. *Ei*Δ*evpL* also had a higher phagocytic uptake rate, but the number of intracellular *Ei*Δ*evpL* increased up to 6 h. Although *Ei*Δ*evpE* and *Ei*Δ*evpK* had similar phagocytic uptake as *E. ictaluri* WT, the number of intracellular *Ei*Δ*evpE* and *Ei*Δ*evpK* increased up to 12 h. At 24 h, the number of intracellular T6SS mutants and *E. ictaluri* WT were significantly decreased, and there were no significant differences among T6SS mutants and *E. ictaluri* WT. Together, these data indicated that *E. ictaluri* WT could replicate in catfish peritoneal macrophages for a limited time (6 h) and after this, macrophages kill *E. ictaluri* WT gradually. Also, deletion of T6SS genes caused an increased intake of some mutants by macrophages at 6 h, but overall macrophage killing of mutants progressed at a phase similar to WT.

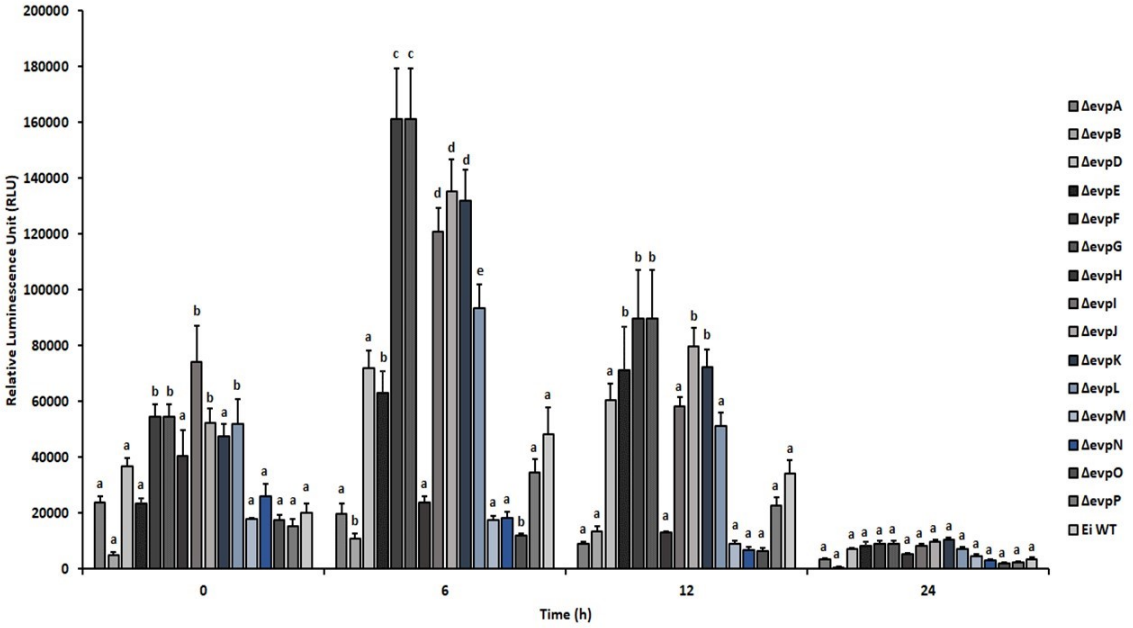

Figure 10 The bacterial killing assay of T6SS mutants and *E. ictaluri* WT.

The mean of photon exposure obtained from each T6SS mutants and *E. ictaluri* WT. The data represented the mean of each treatment ±SD. The letters (a, b, c, and d) show the significant differences between treatments ($p < 0.05$).

Attachment and invasion

The attachment and invasion of T6SS mutants and *E. ictaluri* WT were assessed in CCO cell line. *Ei*Δ*evpF, Ei*Δ*evpG, Ei*Δ*evpK,* and *Ei*Δ*evpL* had significantly increased attachment rate compared to *E. ictaluri* WT (Figure 11A). *Ei*Δ*evpF* and *Ei*Δ*evpG* showed the highest attachment ability compared to other mutants. On the other hand, attachments of *Ei*Δ*evpM, Ei*Δ*evpN, Ei*Δ*evpO,* and *Ei*Δ*evpP* were significantly lower than that of *E. ictaluri* WT. Invasion patterns of mutants have shown a similar pattern to their attachment pattern. *Ei*Δ*evpF, Ei*Δ*evpG,* and *Ei*Δ*evpL* had significantly more invasion rate compared to other mutants and WT (Figure 11B), while invasion capabilities of *Ei*Δ*evpN, Ei*Δ*evpO,* and *Ei*Δ*evpP* were the lowest of all (Figure 11C).

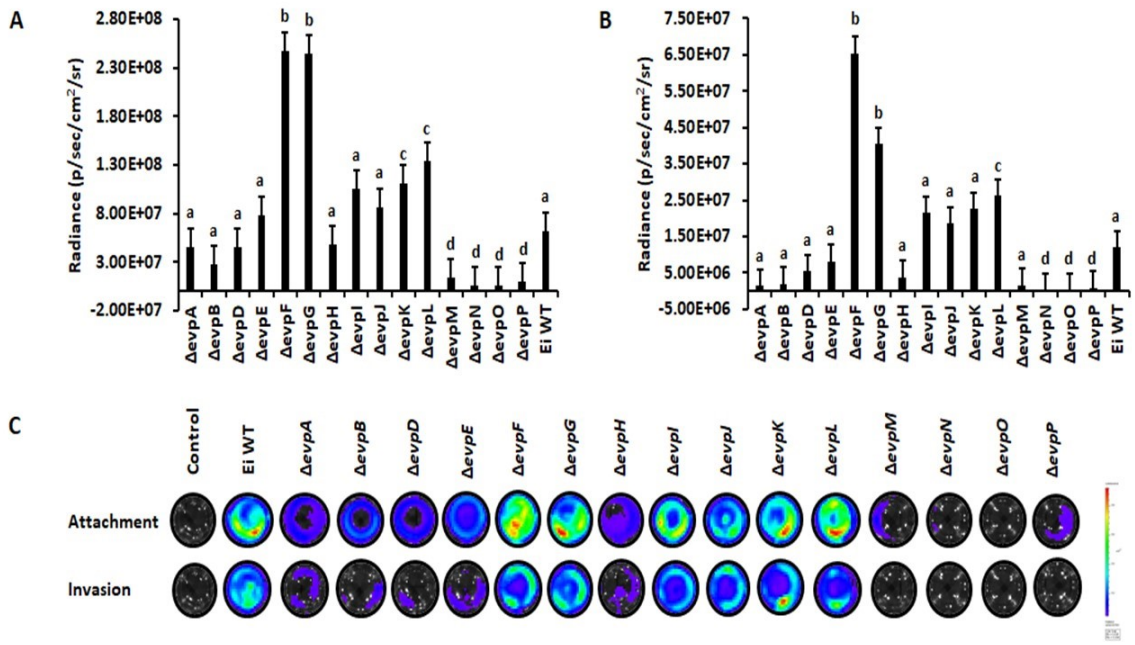

Figure 11 The attachment and invasion of T6SS mutants and *E. ictaluri* WT to CCO.

A. The mean of photon exposure obtained from each well in 24-well plate incubated with T6SS mutant, *E. ictaluri* WT, and control (non-treated) after an hour incubation. The bar graph indicates the mean of photons obtained from four biological replicates to test the attachment ability of T6SS mutants and *E. ictaluri* WT. The data represented the mean of each treatment ±SD. The letters (a, b, c, and d) show the significant differences between treatments ($p < 0.05$). B. The mean of photon exposure obtained from the same 24-well plate incubated with T6SS mutants, *E. ictaluri* WT, and control including gentamycin for an hour after attachment. The bar graph indicates the mean of photons obtained from four biological replicates to test the attachment ability of T6SS mutants and *E. ictaluri* WT. C. The bioluminescent imaging of CCO treated with bioluminescent T6SS mutants and *E. ictaluri* WT and control (non-treated).

Stress assays

Nitrite oxide and hydrogen peroxide were applied to evaluate mutants' abilities to cope with stress. There was a variation in growth of mutants and *E. ictaluri* WT with SNP in BHI and MM19-P (Figure 12A, B). All T6SS mutants were resistant to SNP at 4 h in BHI (Figure 12D). *Ei*Δ*evpF* and *Ei*Δ*evpI* growth gradually increased in the presence of SNP up to at 8 h in BHI (Figure 12E). *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* remained more resistant to SNP than other T6SS mutants at 12 h in BHI (Figure

12F). Although T6SS mutants decreased in growth at 24 h in BHI, *Ei*Δ*evpB* and *Ei*Δ*evpP* mutants were similarly resistant to SNP as *E. ictaluri* WT (Figure 12G). *Ei*Δ*evpG, Ei*Δ*evpI, Ei*Δ*evpJ,* and *Ei*Δ*evpK* showed decreased in growth up to 8 h in MM19-P (Figure 12J). However, *Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* were more resistant to SNP for 12 h in MM19-P (Figure 12K). The growth of *Ei*Δ*evpA* and *Ei*Δ*evpF* after treatment with SNP were similar to *E. ictaluri* WT for 24 h in MM19-P (Figure 12L).

The treatment of T6SS mutants with H_2O_2 caused reduced bacterial growth in both BHI and MM19-P (Figure 13 A, B). *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* were significantly affected by stress for 24 h. *Ei*Δ*evpI, Ei*Δ*evpJ, Ei*Δ*evpK,* and *Ei*Δ*evpL* were resistant to H_2O_2 in BHI up to 4 h (Figure 13D). After this time point, their growth started to decrease. However, *Ei*Δ*evpB* and *Ei*Δ*evpP* were not affected by H_2O_2 in BHI for 24 h. In MM19-P, *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpI, Ei*Δ*evpK, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* were significantly lost their ability to survive with H_2O_2 for 24 h (Figure 13L). *Ei*Δ*evpB, Ei*Δ*evpF, Ei*Δ*evpG, Ei*Δ*evpJ, Ei*Δ*evpL,* and *Ei*Δ*evpP* showed less growth than *E. ictaluri* WT for 12 h (Figure 13K). These results show that T6SS mutants are more sensitive to SNP and H_2O_2 in MM19 due to the low pH (5.5) in MM19-P. The resistance of T6SS mutants to SNP is more than H_2O_2. Hydrogen peroxide can restrict the bacterial growth of T6SS mutants in both MM19-P and BHI. Taken all together, T6SS mutants are sensitive to stress factors that imitate phagosomal killing conditions inside catfish macrophages.

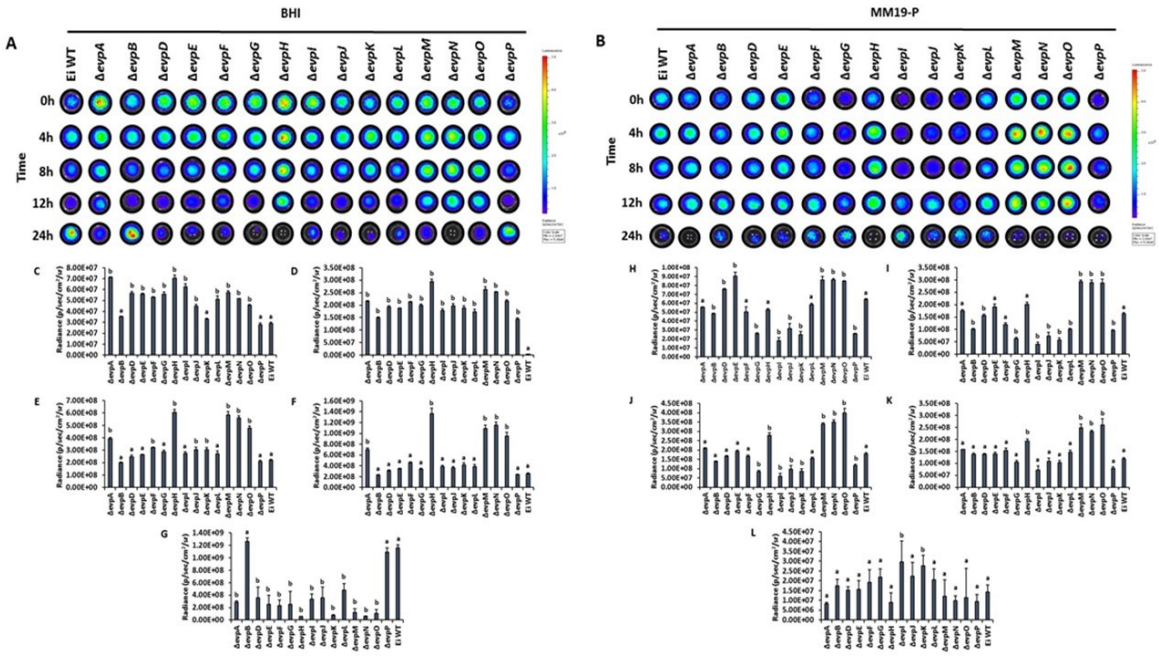

Figure 12 The stress assays of T6SS mutants and *E. ictaluri* WT exposed to 5mM of SNP.

The bar graphs indicate the mean of photons obtained from three biological replicates. The data represented the mean of each treatment ±SD. The letters (a and b) show the significant differences between treatments ($p < 0.05$). A. Bioluminescent image of T6SS mutants and *E. ictaluri* WT exposed to SNP in BHI for 24 h. B. Bioluminescent image of T6SS mutants and *E. ictaluri* WT exposed to SNP in MM19-P for 24 h. C-G. The bar graphs indicated the relative luminescence unit (RLU) obtained from the T6SS mutants and *E. ictaluri* WT treated SNP in BHI for 0, 4, 8, 12, and 24 h. H-L. The bar graphs indicated the relative luminescence unit (RLU) obtained from the T6SS mutants and *E. ictaluri* WT treated SNP in MM19-P for 0, 4, 8, 12, and 24 h.

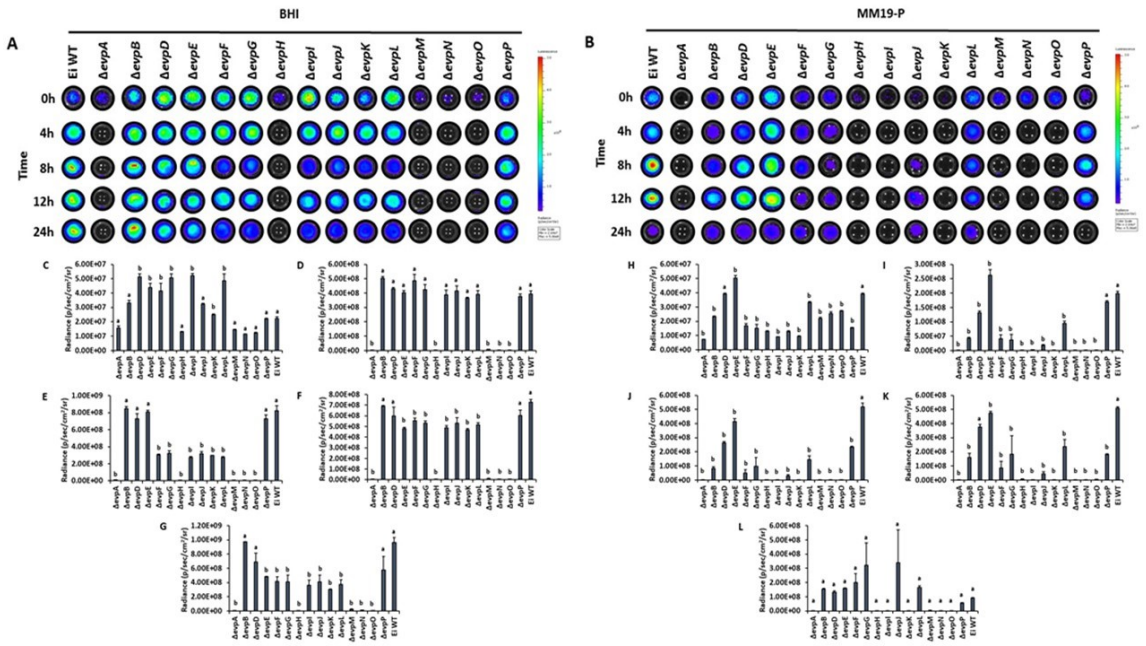

Figure 13 The stress assays of T6SS mutants and *E. ictaluri* WT exposed to 0.75 mM of H_2O_2.

The bar graphs indicate the mean of photons obtained from three biological replicates. The data represented the mean of each treatment ±SD. The letters (a and b) show the significant differences between treatments ($p < 0.05$). A. Bioluminescent image of T6SS mutants and *E. ictaluri* WT exposed to H_2O_2 in BHI for 24 h. B. Bioluminescent image of T6SS mutants and *E. ictaluri* WT exposed to H_2O_2 in MM19-P for 24 h. C-G. The bar graphs indicated the relative luminescence unit (RLU) obtained from the T6SS mutants and *E. ictaluri* WT treated H_2O_2 in BHI for 0, 4, 8, 12, and 24 h. H-L. The bar graphs indicated the relative luminescence unit (RLU) obtained from the T6SS mutants and *E. ictaluri* WT treated SNP in MM19-P for 0, 4, 8, 12, and 24 h.

Assessment of virulence and efficacy

The immersion challenge method was used to evaluate T6SS mutants in catfish fingerlings and fry. *Ei*Δ*evpA, Ei*Δ*evpD, Ei*Δ*evpE, Ei*Δ*evpG, Ei*Δ*evpH, Ei*Δ*evpJ, Ei*Δ*evpK, Ei*Δ*evpM, Ei*Δ*evpN* were significantly less virulent than *E. ictaluri* WT in catfish fingerlings (Figure 14A) ($p<0.05$). *Ei*Δ*evpF* (16.95% mortality), *Ei*Δ*evpI* (13.18% mortality), *Ei*Δ*evpL* (1.75 % mortality), and *Ei*Δ*evpO* (2.38% mortality) had also decreased mortality rate in virulence compared to *E. ictaluri* WT. *Ei*Δ*evpP* (44.73%

mortality) was as virulent as *E. ictaluri* WT. Re-challenged with *E. ictaluri* WT at 21-days post infection showed that all mutants provided better protection compared to sham group (Figure 14B). *Ei*Δ*evpD, Ei*Δ*evpE, Ei*Δ*evpG, Ei*Δ*evpJ,* and *Ei*Δ*evpK* provided better protection against *E. ictaluri* WT. Although there was a significant decrease in virulence, *Ei*Δ*evpA* (37.42% mortality), *Ei*Δ*evpH* (35.80% mortality), *Ei*Δ*evpM* (37.78% mortality), and *Ei*Δ*evpN* (21.80% mortality) caused significantly less protection (Figure 14B). Interestingly, *Ei*Δ*evpP* exhibited low attenuation but high protection rate.

Immersion challenge experiment with 2-weeks old catfish fry showed significantly reduced attenuation of *Ei*Δ*evpD* (19.45% mortality), *Ei*Δ*evpE* (15.93% mortality), *Ei*Δ*evpG* (13.86% mortality), *Ei*Δ*evpJ* (15.43% mortality), and *Ei*Δ*evpK* (22.13% mortality) compared to *E. ictaluri* WT (Figure 14C) ($p<0.05$). *Ei*Δ*evpF* (94.96% mortality), *Ei*Δ*evpI* (82.31% mortality), and *Ei*Δ*evpL* (90.41% mortality) mortality rate were significantly higher than other T6SS mutants. As for the protection of fry by mutant vaccination, all mutants showed better protection compared to sham vaccination, but protective levels of *Ei*Δ*evpD, Ei*Δ*evpF, Ei*Δ*evpG, Ei*Δ*evpI,* and *Ei*Δ*evpJ* were better than that of other T6SS mutants. (Figure 14D) ($p<0.05$).

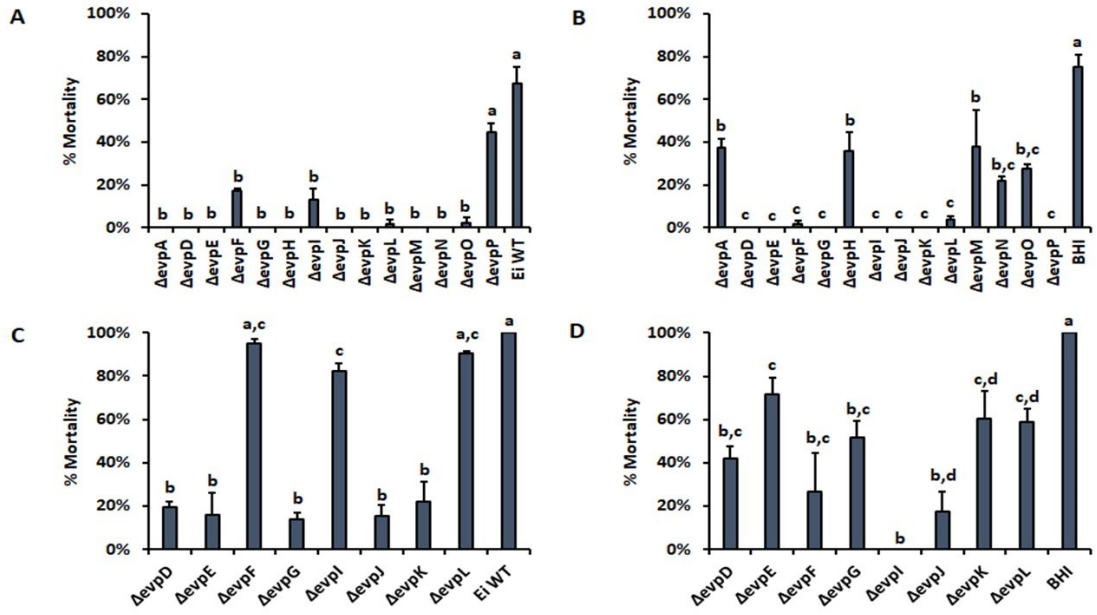

Figure 14 The virulence (A) and efficacy (B) of T6SS mutants and *E. ictaluri* WT in catfish fingerlings.

The virulence (C) and efficacy (D) of T6SS mutants and *E. ictaluri* WT in catfish fry.

Discussion

In this work, we aimed to characterize the T6SS and its role in role in *E. ictaluri* virulence. To accomplish this, T6SS mutants were developed by in-frame deletion, and their adhesion and invasion to catfish CCO cells, survival and proliferation in catfish peritoneal macrophages, adaptation and resistance to the stress, persistence in catfish, and virulence and efficacy in catfish fingerlings and fry were investigated.

E. ictaluri possess a single T6SS operon with all genes from *evpA* to *evpO*. The genes encoded outside of T6SS operon are *evpP* and *hcp2* (Figure 7). Although *evpP* was not annotated in the *E. ictaluri* 93-146 genome, sequence analysis revealed its presence before the *evpA* gene. The complete T6SS operon of *E. ictaluri* contains 20,784 nucleotides, which is relatively smaller than other T6SS founded in pathogenic bacteria.

evpP encoded outside of the T6SS operon in *E. tarda* characterized as a secreted protein via T6SS (Zheng et al., 2007). The secretion of *evpP* highly depends on *evpC*, a chaperone protein assisting to secreted proteins pass through the T6SS tube structure (Hu et al., 2014). The secretion of the *evpP* gene is tightly regulated by iron concentration in media and EsrA-EsrB two-component system in *E. tarda* (Wang et al., 2009). These studies showed that *evpP* is secreted in the host environment. It has been recently identified that *evpP* is an effector protein secreted by T6SS to manipulate inflammasome activation within macrophages in *E. tarda* EIB202 (Chen et al., 2017). Our results showed that *Ei*Δ*evpP* was able to survive inside catfish peritoneal macrophages similar to *E. ictaluri* WT. However, *Ei*Δ*evpP* had significantly less attachment and invasion abilities in CCO cells. Virulence assessment demonstrated that *Ei*Δ*evpP* was not attenuated but provided good protection. Stress assays showed that *Ei*Δ*evpP* was resistant to SNP and H_2O_2 stress in BHI. However, it had decreased growth in MM19-P under both stresses.

Recent studies suggest that T6SS contributes to host cells adherence and invasion. Disruption of *tssM* in which bacteria reduced T6SS-mediated adhesion and invasion of host epithelial cells (de Pace et al., 2011; Lertpiriyapong et al., 2012; Yu et al., 2012). In contrast, mutation of *tssM* caused increased adhesion in *Helicobacter hepaticus* (Chow et al., 2010). *Ei*Δ*evpO* homolog of *tssM* has significantly reduced ability in attachment and invasion for CCO cell lines. *evpP* had less ability to invade host epithelial cells to initiate internalization in *E. tarda* (Wang et al., 2009). Similarly, *Ei*Δ*evpN* and *Ei*Δ*evpP* showed significantly less attachment and invasion of the host epithelial cells. Loss of *vgrG* can also affect the attachment and invasion of bacteria for host epithelial cells (Ma et al.,

2014). *Ei*Δ*evpI*, a homolog of *vgrG*, has no significant difference with *E. ictaluri* WT regarding attachment and invasion for CCO. Remarkably, *Ei*Δ*evpF (tssF), Ei*Δ*evpG (tssG)*, and *Ei*Δ*evpL (tssJ)* are significantly more adhesive and invasive than *E. ictaluri* WT. Although *Ei*Δ*evpK (tssA)* has more attachment ability, *Ei*Δ*evpK* invasion rate is limited.

Surviving inside the host immune cells is important for *E. ictaluri* as a facultative intracellular pathogen. Deletion of T6SS genes such as *tssH* (*clpV*) and *tssM* enhance intracellular replication of *Bordetella* and *Salmonella* (Parsons et al., 2005; Bendor et al., 2015). However, a mutation in *tssE*, *tssM*, and *tssH* can also decrease the intracellular proliferation of intracellular bacteria in macrophages (Burtnick et al., 2010; de Pace et al., 2011; Mulder et al., 2012; Blondel et al., 2013). The uptake of *Ei*Δ*evpF (tssF), Ei*Δ*evpG (tssG), Ei*Δ*evpI (tssI), Ei*Δ*evpJ (tssJ),* and *Ei*Δ*evpL (tssL)* inside peritoneal macrophages were higher than other T6SS mutants. Among these mutants, *Ei*Δ*evpF, Ei*Δ*evpG,* and *Ei*Δ*evpJ* had increased number of intracellular replication in peritoneal macrophages. All T6SS mutants and *E. ictaluri* WT were not able to replicate in peritoneal macrophages. This may be due to the activated state of the catfish peritoneal macrophages. Although deletion of *tssM* and *tssH* resulted in different effect for the fitness of many intracellular bacteria for survival in host immune cells, a mutation in *evpO* (*tssM*) and *evpH* (*tssH*) had only numerical decreased in intracellular replication of *E. ictaluri*. The various effects of T6SS mutants for the intracellular lifestyle of *E. ictaluri* displays that T6SS is critical secretion system for *E. ictaluri* to adapt to catfish peritoneal macrophages.

The role of T6SS in stressful conditions reveal that T6SS may have an important role in the uptake of crucial metals required for surviving in host environment (Chen et

al., 2016). These metals include manganese, which is vital under oxidative stress conditions in *Burkholderia thailandensis* (Si et al., 2017). Once bacteria initiated an internalization in host macrophages, distinct stress and killing mechanisms were activated by host macrophages to limit intracellular replication bacteria (Burton et al., 2014). *E. ictaluri* can resist these stress and killing mechanisms by upregulating expression genes helping phagosomes replication (Baumgartner et al., 2014). T6SS mutants treated with SNP and H_2O_2 showed that the growth of most of the T6SS mutants was limited in BHI and MM19-P with H_2O_2. The growth of *Ei*Δ*evpA, Ei*Δ*evpH, Ei*Δ*evpM, Ei*Δ*evpN,* and *Ei*Δ*evpO* was defective in BHI and MM19-P. This result indicates that T6SS can play an important role surviving in stressful conditions in *E. ictaluri*. Deletion of some T6SS mutants may affect the survivability of *E. ictaluri* in stress conditions that mimic phagosomal conditions of macrophages.

Loss of a functional T6SS can cause reduced virulence and persistence of pathogenic bacteria. *tssM* deletion in *Aeromonas*, *Campylobacter,* and *Acinetobacter* cause attenuation in a mouse model (Suarez et al., 2008; Lertpiriyapong et al., 2012; Repizo et al., 2015). Moreover, *evpH*, *evpI*, *evpC*, and *evpP* in *E. tarda* were found to be important for establishing an infection in blue gourami (Zheng et al., 2007). Our data suggest that deletion of T6SS genes caused attenuation in catfish fingerling and fry. *Ei*Δ*evpP* is the only mutant without reduced attenuation in catfish fingerling. However, the protection level of *Ei*Δ*evpP* was significantly higher. Considering the role of *evpP* as an effector protein in *E. tarda*, *evpP* might have a different role in *E. ictaluri* pathogenesis. In the catfish fry model, T6SS mutants were attenuated, except *Ei*Δ*evpF* and *Ei*Δ*evpL*. Some T6SS mutants protected fry after vaccination. However, the

protection levels were not as good as that of fingerlings, which may be due to the less developed immune system of fry.

In summary, we have shown that T6SS is involved in *E. ictaluri* pathogenesis and contributes attachment and invasion of catfish epithelial cells. T6SS assists *E. ictaluri* for replicating inside catfish peritoneal macrophages, and for resisting to the host stress.

CHAPTER IV

CONCLUSIONS

The overall goal of this project was to understand the role of T6SS in *E. ictaluri* virulence and determine the safety and efficacy of T6SS mutants in channel catfish. *E. ictaluri* has a single T6SS operon with 20,784 nucleotides and genes *evpA* to *evpO*. Also, T6SS releted genes *evpP* and *hcp2* were located outside of the T6SS operon. Results in chapter II showed that *evpC* and *hcp2* had a difference in adhesion to CCO cell line, whereas the invasion ability of *evpC* and *hcp2* in CCO was similar. A double mutation in *evpC* and *hcp2* caused an important effect on adherence to CCO. *hcp2* mutant with a lower replication rate in catfish peritoneal macrophages showed that *hcp2* gene is required for intracellular survival of *E. ictaluri*. The uptake of *evpC*, *hcp2*, the double mutant, and wild-type were similar. This result indicates that *hcp2* may be crucial for the survival of *E. ictaluri* inside the catfish macrophages. The vaccine trials of *hcp* family mutants revealed that the immersion vaccination of catfish fingerlings with *evpC* and double mutant (*evpC*, *hcp2*) provided a better safety and protection against ESC. The catfish fingerlings infected with *evpC* and double mutant survived after fourteen days infection. However, *hcp2* mutant caused a rapid death with severe clinical signs in catfish fingerlings. These results demonstrate that *evpC* mutants might serve as a better vaccine candidate than *hcp2* mutant in catfish fingerlings. Treatment of mutants in stress conditions showed that mutants and wild-type were able to survive in nitrosative and

oxidative stress conditions in BHI. However, the growth of double mutant was increased in BHI and MM19-P. Results in chapter III showed that mutation in *evpP* caused no significant difference in survival inside catfish peritoneal macrophages. However, *evpP* was less adhesive and invasive for catfish epithelial cells. Deletion of *evpP* did not decrease the virulence of *E. ictaluri* significantly, but the protection level of *evpP* mutant was significantly better. *evpP* was resistant to nitrosative and oxidative stress in BHI, not in MM19-P, for both stress factors. *evpN* and *evpP* mutants were significantly less adhesive and invasive in the catfish epithelial cells. Adversely, *evpF*, *evpG*, and *evpL* mutants showed significantly more attachment and invasion compared to wild-type. The internalization rates of *evpF*, *evpG*, *evpI*, *evpJ*, and *evpL* mutants in peritoneal macrophages were significantly higher, and *evpO* and *evpH* mutants were numerically different from wild-type regarding replication inside catfish peritoneal macrophages. The growth of *evpA*, *evpH*, *evpM*, *evpN*, and *evpO* mutants in nitrosative and oxidative stress conditions were significantly limited in both BHI and MM19-P. Deletion of *evpP* caused mortality in catfish fingerling, but protected catfish significantly.

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