12-8-2017

Understanding molecular mechanisms of host-Edwardsiella ictaluri interaction

Nawar Hadi Al-Janabi

Understanding molecular mechanisms of host-Edwardsiella ictaluri interaction

By

Nawar Al-Janabi

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Sciences
in the Department of Basic Sciences in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2017

Understanding molecular mechanisms of host-Edwardsiella ictaluri interaction

By

Nawar Al-Janabi

Approved:

_____

Attila Karsi
(Major Professor)

_____

Mark L. Lawrence
(Committee Member)

_____

Lesya M. Pinchuk
(Committee Member)

_____

Daniel G. Peterson
(Committee Member)

_____

Larry A. Hanson
(Graduate Coordinator)

_____

Mark L. Lawrence
Associate Dean
College of Veterinary Medicine

Name: Nawar Al-Janabi

Date of Degree: December 8, 2017

Institution: Mississippi State University

Major Field: Veterinary Medical Sciences

Major Professor: Dr. Attila Karsi

Title of Study: Understanding molecular mechanisms of host-Edwardsiella ictaluri interaction

Pages in Study: 118

Candidate for Degree of Doctor of Philosophy

Catfish, the "king" of the U.S. aquaculture, is threatened by a severe, systemic bacterial disease known as enteric septicemia of catfish (ESC). This disease causes high mortality and massive economic losses in cultured channel catfish (*Ictalurus punctatus*) in the United States. *E. ictaluri* penetrates catfish intestinal epithelia quickly and establishes a systemic infection rapidly. However, our knowledge on catfish intestine and *E. ictaluri* interaction is very limited. In Particular, catfish intestinal immune responses and virulence genes needed by *E. ictaluri* to evade host defenses are not well understood. Hence, our long-term goal is to identify the molecular mechanisms of *E. ictaluri*-host interactions. The overall objectives of this study were to understand catfish immune responses to *E. ictaluri* infection and determine essential genes of *E. ictaluri* during the intestinal invasion. To accomplish the overall objectives of this research, intestinal ligated loops were constructed surgically in live catfish and loops were injected with wild-type *E. ictaluri* and two live attenuated *E. ictaluri* vaccine strains developed recently by our research group. We first determined catfish intestinal immune responses against *E. ictaluri* wild-type and live attenuated vaccine strains. Then, we analyzed the global gene

expression patterns of wild-type *E. ictaluri* and vaccine strains during catfish intestinal invasion using high throughput RNA-Seq technology. Results showed a moderate level of neutrophil and B cell infiltration correlated with significantly lower expression of TNF-α, CD4-1, and CD8-α in the vaccine injected intestinal tissue compared to that of wild-type injected intestinal tissue. Further, RNA-Seq data analysis showed the prominent expression of genes related to bacterial secretion systems, ATP production processes, and multidrug resistance (MDR) efflux pumps in wild-type *E. ictaluri*. In contrast, the prominently expressed genes in vaccine strains were related to the phosphotransferase system and sugar metabolism processes. All these data suggest that our live attenuated vaccines are capable of triggering effective immune responses in catfish without causing damage to the host.

DEDICATION

I would like to dedicate this research to my parents Hadi Yaser Al-Janabi and Kareema Fanharawa, my sister Safa, and my brothers Mustafa, Ammar, and Yaser.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major professor Dr. Attila Karsi, who has provided excellent mentorship and support in all aspects of my research. I would also like to give my sincere thanks and appreciation to all my committee members, Dr. Mark Lawrence, Dr. Lesya Pinchuk, and Dr. Daniel Peterson, for their suggestions and encouragements. I would like to thank Dr. Hossam Abdelhamed and Dr. Jingjiun Lu for their support with lab work. I am thankful to Mrs. Michelle Banes and all my lab members, Safak, Ali, Iman, Hasan, and Salih for their advice and company. I would also like to thank Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University for providing me great environment, which is crucial for the successful completion of this dissertation. Finally, I would like to acknowledge the financial support from the Ministry of Higher Education of Iraq. My deepest gratitude goes to my parents Hadi Yaser Al-Janabi and Kareema Fanharawa for their great support and encouragement throughout my doctoral program. Without all of you, completion of this research would have been difficult.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

Edwardsiella ictaluri

Edwardsiella ictaluri is a Gram-negative, rod-shaped, facultative intracellular pathogen. The size of the bacterium is 0.75 x 1.5 – 2.5 μm, and it is slightly motile at 25-30ºC and non-motile at higher temperatures. *E. ictaluri* grows slowly on agar plates, which requires 48 hours at 30°C. It was first reported as a primary channel catfish pathogen in an Alabama farm in 1978 (Hawke, 1979; HAWKE et al., 1981). *E. ictaluri* is the etiological agent of an economically important catfish disease named enteric septicemia of catfish (ESC) (Ainsworth and Chen, 1990; Booth et al., 2009). ESC is characterized by two forms; acute infection represented by hemorrhagic enteritis and septicemia, and chronic infection represented by meningoencephalitis (Newton et al., 1989). The infection is readily spread from fish to fish, and usually occurs during spring and fall when water temperatures are between 22 and 28°C. The *E. ictaluri* genome length is 3.81 base pairs, containing 3,903 genes that encode 3,784 proteins, with an average of 57.4%, GC content (Williams et al., 2012). This bacterium has eight ribosomal operons (Williams et al., 2008), harbor two plasmids known as pEI1 and pEI2 (Lobb and Rhoades, 1987; Newton et al., 1988). This bacterium known to encodes many virulence mechanisms that may be responsible for causing the disease including, type III and type VI secretion systems, flagella, type 1 fimbriae (Williams et al., 2012), lipopolysaccharide

(LPS) (Lawrence et al., 2003; Santander et al., 2013; Weete et al., 1988), hemolysins (Williams and Lawrence, 2005), and outer membrane proteins (OMP) (Newton et al., 1990; Vinitnantharat et al., 1993). Recently, *E. ictaluri* has been reported to cause gastrointestinal septicemia in catfish as the bacterium utilizes catfish intestinal epithelium as a frequent route of entry (Baldwin and Newton, 1993; Santander et al., 2014), and it's capable of surviving in catfish intestinal immune cells including neutrophils (Karsi et al., 2009; Lawrence et al., 2003) and macrophages (Booth et al., 2009). Although *E. ictaluri* can penetrate intestinal membranes rapidly and establish systemic infection, the precise mechanism by which this occurs is underexplored yet.

Innate immune system in catfish

Catfish innate immunity has a complete defense systems. Advanced research in catfish genomic studies revealed the presence of a vast number of innate immune-relevant genes, such as those encoding pattern recognition receptors, antimicrobial peptide, complements, lectins and cytokines (Gao et al., 2012). The innate immune systems are very beneficial for the catfish in providing immediate defense against many bacterial pathogens such as *E. ictaluri*, *Aeromonas hydrophila*, and *Flavobacterium columnare.* In numerous studies, it has been shown that genes encoding innate immune responses in catfish are having variations in their activities and these variations are responsible for the differences in resistance or susceptibility to disease and consequently for the health condition of catfish. (Whyte, 2007). The most important innate immune-related genes in catfish are discussed below.

Pattern Recognition Receptors (PRRs)

Pattern Recognition Receptors (PRRs) are responsible for the recognition of bacterial pathogens through their sensing structures called pathogen-associated molecular patterns (PAMPs), which are present in all microbial species. The PAMPs include bacterial peptidoglycan, LPS in Gram-negative bacteria, lipoteichoic acid in Gram-positive bacteria, double-stranded RNA from viruses, and β-1,3-glucan on fungal cell walls (Medzhitov and Janeway, 2000). Also, PRRs recognize the molecules released from the damaged cells, or damage associated molecular patterns (DAMPs), including oxidized phospholipids and oxidized cholesteryl esters (Jault et al., 2004). Many classes of PRRs have been identified in catfish, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs).

The first class of PRRs is Toll-like receptors (TLRs). There are at least 17 Toll-like receptors have been identified in a variety of fish species, and five functional TLRs have been characterized in catfish (TLR2, TLR3, TLR5, TLR20, and TLR21) (Pridgeon et al., 2010). The first TLRs identified in catfish were the TLR3 and TLR5, in which they were found to be expressed in response to *E. ictaluri* challenge. TLR3 was associated with many immune functions, while TLR5 contributes to the aggregation of macrophages during *E. ictaluri* infection (Bilodeau-Bourgeois et al., 2008; Bilodeau et al., 2006; Bilodeau and Waldbieser, 2005; Peterson et al., 2005). In the catfish intestine, TLR3 plays a significant role in innate immune signaling against *E. ictaluri*. TLR2 can recognize the lipopeptides that are present on the surface of most Gram-negative bacteria

including *E. ictaluri*. The expression pattern of all five TLRs in catfish following an encounter with the pathogens demonstrated their important role in response to the acute infection (Pridgeon et al., 2010).

The other class of PRRs is NOD-Like Receptors (NLRs). Three subfamilies of NLRs have been detected: (1) The NODs (nucleotide-binding oligomerization domain) and IPAF (ICE protease-activating factor) (CARD). (2) The NAIPs (neuronal apoptosis inhibitory proteins) (BIR). (3) The NALPs (NACHT domain-, leucine-rich repeat-, and PYD-containing proteins) (PYD) (Carneiro et al., 2008; Rosenstiel et al., 2008). In catfish, 5 NLRs have been characterized: NOD1, NOD2, NLRC3, NLRC5, and NLRX (Sha et al., 2009). In response to the intracellular pathogen such as *E. ictaluri*, NOD 1 is found to be expressed. Later, nearly 22 NLRs have been identified, and they are co-expressed after microbial infection (Rajendran et al., 2012a).

The last class of PRRs to be discussed here is the Retinoic Acid-Inducible Gene (RIG)-I-Like Receptors (RLRs). RLRs are very important PRRs in recognizing viruses (Takeuchi and Akira, 2008). Generally, RLRs encode three genes; (1) Retinoic acid-inducible gene I (RIG-I), (2) Melanoma differentiation associated antigen 5 (MDA5), and (3) (LGP2) (Rajendran et al., 2012b). In channel catfish, the three RLRs genes have been detected, and their expression significantly increased after bacterial infection, suggesting their important role in anti-bacterial immune responses in addition to their role in antiviral immune responses.

Antimicrobial Peptides (AMPs)

Antimicrobial peptides are an important component of the innate immune system and defense mechanism against pathogens for many organisms including catfish. In catfish, antimicrobial peptides play a critical role in immune response against pathogens, especially against *E. ictaluri*. Several types of antimicrobial peptides have been identified in catfish such as Cysteine-rich AMPs, including Hepcidin and liver-expressed antimicrobial peptide 2 (LEAP-2), NK-lysin, Bactericidal permeability-increasing protein (BPI), Histone-like proteins (HLPs), and hemoglobin-derived AMPs as well as defensins and natural resistance-associated macrophage protein (Nramp)(Bao et al., 2006; Douglas et al., 2003; Zhou et al., 2011).

Complement system

The complement system plays a crucial role in catfish innate immunity and involves in many defenses strategies against pathogens such as phagocytosis, microbial killing, inflammatory reactions, immune complex clearance, and antibody production (Holland and Lambris, 2002). Complement is composed of 30 distinct plasma proteins and membrane-bound proteins. Complement system activated in three ways; (1) the classical pathway, which is mediated by antibodies. C1q is the first sub-component in classical pathway and is responsible for recognizing the antibody in immune complexes, (2) The mannan-binding lectin pathway, which is activated by the host serum lectins when it bind and recognize the carbohydrate motifs on the surface of the microbe and from a complex of mannose-binding lectin-associated serine proteases (MASP), and (3)

The alternative pathway, which is activated by the foreign molecules such as bacterial LPS, is continuously active (Abernathy et al., 2009; Boshra et al., 2006).

All the three pathways converge in the generation of C3 convertase that cleaves C3 into C3a and C3b. C3b binds to hydroxylic group of carbohydrate or proteins of the invading pathogens. The most important function of the complement is the opsonization of the microbes with C3b, so it can be recognized by the complement receptors that are expressed on phagocytes (CR1 and CR2). The other important function is cytolytic activity, where the target-bound C3b forms a protease complex to activate C5 into C5a and C5b and form the membrane attack complex (MAC) by C6-C9. MAC induce the osmotic lysis of the microbes. The C3a, C4a, and C5a are chemotactic for leukocytes, stimulate the release of inflammatory mediators, and enhance the movement of leukocytes and plasma proteins into the tissues. Although complement system role is very important in response to the microbial pathogens, self-destruction could occur resulting in immunological diseases (Mollnes et al., 2002). Complement factor I, and complement membrane attack complex inhibitor CD59 (both as inhibitors in complement system) have been identified in catfish to regulate the activity of complement system (Abernathy et al., 2009; Yeh and Klesius, 2007).

Lectin

Lectins play an important role in fish innate immune response and resistant to pathogens by recognizing the pathogen-carbohydrate chains (Saito et al., 1997; Tasumi et al., 2002). Lectins bind the carbohydrate molecules on the surface of pathogens resulting in clearing the pathogens by phagocytosis and oxidative burst activities (Tateno et al.,

2002; Wolfe et al., 1998). Lectins activate the complement system and form a complex of mannose-binding lectin-associated serine proteases (MASP) as mention above. Many types of lectins have been identified in fish, such as C-type lectins, F-type lectins, intelectins including IntL1, and IntL2 (Vasta et al., 2011).

Cytokines

Cytokines play an important role in catfish immune responses by inducing numerous reactions that enable catfish to respond to the infection. Therefore, they are considered strong markers indicating the activation of inflammatory responses. Many groups of catfish cytokines have been identified including chemokines, interferons (IFNs), interleukins, and Tumor Necrosis Factor (TNF). Chemokines, which are produced in the early stages of infection by the infected tissues. Chemokines play an important role in recruiting the effector immune cells to the site of infection and injury (Zhu et al., 2013). Also, their role in translating the innate immune response into an adaptive immune response (bridge) is critical. Chemokines could be divided into four major subfamilies: CXC, CC, C, and CX3C. However, research in teleost fish prove the existence of many other groups belong to these chemokines subfamilies. Interferons (IFNs) are secreted proteins possess antiviral properties and have the ability in suppressing the tumor and immune modulation (Samuel, 2001). There are three classes of IFNs, the first class is IFNs-I, and consider the first line of defense against viral infection (Robertsen, 2006). The second class is IFN-II, which include IFN-γ1, and IFN-γ2, and they are important in innate and adaptive immune responses, and they are both identified in catfish. The third class is IFNs-III, which also has a role against viral infection (Zou and Secombes, 2011).

Interleukins play a significant role in early inflammatory responses. They are a large group of cytokines, and they were first seen to express by leukocytes. Interleukins are secreted proteins and signaling molecules. In catfish, IL-1β and interleukin-8 are the most studied interleukins and found to be expressed in response to the *E. ictaluri* infections (Chen et al., 2005; Wang et al., 2006). The last group of cytokines is Tumor Necrosis Factor (TNF) which play a key role in innate immune response by regulating cell death and survival (Locksley et al., 2001). In channel catfish, the best identified and characterized member of TNFs is TNFα (Zou et al., 2003). TNFα mRNA in catfish appears to be expressed in response to *E. ictaluri* infection in all catfish tissues.

Transferrin

Transferrin is a blood protein that has high affinity to extracellular free iron in the catfish body. Iron is essential for many metabolic processes for the catfish as well as for the pathogens. The role of transferrin is to prevent the access to iron by pathogens, and at the same time, transferrin can transport the iron to catfish tissues as required. It has been reported that gene encodes transferrin in catfish is expressed in response to *E. ictaluri* infection (Wang et al., 2006).

Mucus secretion

Fish secrete high amount of mucus as a defense mechanism against infection. Mucus contains a variety of antimicrobial peptides, proteases, complement proteins, and lysozyme (Gomez et al., 2013). Very few studies have examined the impact of mucus secretion in catfish immune responses. Catfish secret mucus from the epithelial or goblet cells, which are mainly composed of mucins (high molecular weight glycoproteins)

(Peatman et al., 2015). In catfish, mucins are secreted against many bacterial pathogens including, *Edwardsiella, Aeromonas,* and *Flavobacterium.* Therefore, it has an important role in shedding microbes and maintaining fish health. Recently, transcriptomic analysis has been done to reveal the expression pattern of catfish intestinal immune genes in response to *E. ictaluri* infection, and it was discovered that two mucin genes are differentially expressed (MUC2, and MUC5B) (Li et al., 2012).

Adaptive immune system in catfish

The central cell types of the adaptive immune responses are the lymphocytes, which are responsible for the diversity of antigen recognition and memory. However, the key components of catfish adaptive immune systems are discussed below.

B lymphocytes and Immunoglobulins

The source for B cell populations in fish is the primary lymphoid tissue, the head kidney (HK) or pronephros (the bone marrow equivalent) (Zwollo et al., 2005). A large number of B cell populations are also found in fish spleen (the secondary lymphoid tissue), where B cell activation, plasmablast formation and differentiation into plasma cells occur. Although B cells are differentiated in the spleen, HK harbors more Ig-secreting cells because the plasma cells are migrated from the spleen to the HK after their differentiation. B cells are also present in all fish mucosal tissue (MALT), and there are three classes of immunoglobulins have been identified in teleost fish IgM, IgD, and IgT (Danilova et al., 2005; Hansen et al., 2005; Solem and Stenvik, 2006). IgM is the most abundant antibody in fish systemic circulation. The main producers of IgM are the plasma cells and plasmablasts, which are located in (HK) (Bromage et al., 2004; Zwollo

et al., 2005). This antibody isotype has been used as a marker for protection against diseases caused by extracellular bacteria, diseases caused by viruses, and also can control the intracellular bacteria. The IgT is the predominant antibody in the gut mucosa, playing a crucial role in mucosal immunity of many fish species, and considered as equivalent to IgA antibody. However, It is important to mention that catfish, do not express IgT antibody isotype and fish generally do not express IgA antibody (Rombout et al., 2011). IgT antibody isotype secreting cells and the mechanism by which this antibody is transferred to the mucosal surfaces are unknown. In 2005, a new Ig isotype was described in zebrafish and named as IgZ and later, two IgZ subclasses were described in carp (IgZ1 and IgZ2), and it was stated that IgZ1 is expressed in the systemic organs, while IgZ2 is expressed in mucosal tissues (Schorpp et al., 2006). Further speaking about the IgT and IgM antibodies, research showed that IgT^+ B cells are the preponderant B cell subset in most mucosal tissues, while IgM^+ B cells are the main subset in the spleen and head kidney (Sunyer, 2012).

Furthermore, the last antibody isotype is IgD, which has only been characterized in catfish (Edholm et al., 2011). This antibody isotype function as pattern recognition molecule and it has been reported that catfish contain IgD -armed granulocytes (Chen et al., 2009) and three B cell subsets; IgM^+/IgD^-, IgM^+/IgD^+, and IgM^-/IgD^+ (Edholm et al., 2010).

T lymphocytes

T cell development and maturation in fish occur in the primary lymphoid organ, thymus (Ge and Zhao, 2013; Rezzani et al., 2014). Generally, T cell populations in

teleost fish have similar characteristics to those found in mammals, in which they have two major T cell receptors; (1) The TCRα,β found on the surface of circulating αβ-T cells, and (2) The TCRγ,δ that found more on mucosa-associated γδ-T cells (Salinas, 2015). Also, all T cells markers including, CD4, CD8, CD3, co-stimulatory CD28, and co-inhibitory CTLA-4 surface molecules exist in fish (Castro et al., 2011). According to the functions, T cells could be categorized into two general populations: helper T (Th) cells, and cytotoxic T cells (CTLs). CTLs express CD8 molecules that interact with peptides presented in MHC class I, while helper T cells express CD4 molecules that interact with peptides presented in MHC class II (Nakanishi et al., 2015). Helper T (Th) cells are also could be categorized into Th1, Th2, Th17 and they all exist in fish (Castro et al., 2011). The other helper T cells, such as Th22, Th9, and Tfh as well as T regulatory cells (Tregs) have not been characterized in fish yet. T cells populations are distributed throughout the fish lymphoid tissues, such as thymus, kidney, and spleen. However, T cells populations are more abundant in fish mucosal tissues (MALT) such as, intestine, gill, and skin, which account for 50%–70% of all lymphoid cells. Moreover, it has been shown that T cell populations are constituted around 10%–20% of all lymphoid cells only in GALT (Boardman et al., 2012).

Significance of research and objectives

The aquaculture industry has become the fastest growing agricultural commodities in the United States, with an annual sales value of $1.2 billion (USDA, 2014). The channel catfish is the most important cultured fish species, accounting for $ 381 million in 2017 (USDA, 2017), the majority of which is produced in Mississippi,

contributing more than 50.2% of all catfish consumed in the nation. ESC reduced the production of catfish by about 37% at the cost of $60 million. Disease control is the most prominent challenge catfish growers face, and the antimicrobial treatment is costly and not effective due to reduced feeding of catfish during infection. Use of antimicrobials is also not environment-friendly, causing drug-resistant strains. Although a commercial vaccine is available for controlling of ESC, the disease continues to be the most prevalent bacterial diseases of catfish. Catfish gut is frequently utilized primary sites of invasion by *E. ictaluri*. Following its entry, *E. ictaluri* causes massive intestinal tissue damages and disrupts the intestinal barrier. However, molecular mechanisms by which *E. ictaluri* crosses the intestinal mucosa rapidly are not well characterized. Lack of this knowledge is a critical problem preventing the development of new therapeutic strategies, which is needed to reduce economic losses due to *E. ictaluri* impact. The overall objectives of this study were to understand catfish immune responses to *E. ictaluri* infection and determine essential genes of *E. ictaluri* during the intestinal invasion. Determination of catfish immune responses and identification of differentially expressed genes of *E. ictaluri* should advance our knowledge about pathogen-intestine interactions and may lead to the development of novel treatment strategies. Therefore, the specific objectives of this study were:

1. Determine E. ictaluri pathogenesis and immune responses in catfish small intestine.

The *working hypothesis* for this aim is that catfish intestines will evoke immune responses against *E. ictaluri* to prevent bacterial invasion. We will test this hypothesis by

identifying expression of immune-relevant genes and pathogenesis in catfish intestines using real-time polymerase chain reaction technique (qPCR).

2. Identify differentially expressed E. ictaluri genes during infection of catfish small intestine.

The *working hypothesis* for this aim is that *E. ictaluri* global gene expression profiles during the invasion of catfish small intestine will show important early events in pathogenesis. We will test this hypothesis by injecting *E. ictaluri* into catfish intestinal loops and determining differentially expressed *E. ictaluri* genes by RNA-Seq analysis.

CHAPTER II

THE INTESTINAL IMMUNE RESPONSES OF CHANNEL CATFISH AGAINST LIVE ATTENUATED *EDWARDSIELLA ICTALURI* VACCINES

Abstract

Edwardsiella ictaluri is a facultative intracellular pathogen causing gastrointestinal septicemia in catfish. *E. ictaluri* invades catfish intestine within 15 minutes and establishes a systemic infection. Despite the importance of intestinal route in *E. ictaluri* infections, intestinal immune responses against *E. ictaluri* are mostly unknown. Recently, we developed two live attenuated vaccines protecting catfish against *E. ictaluri* infection. In this work, we report catfish intestinal immune responses against *E. ictaluri* wild-type and attenuated vaccine strains. To achieve our goal, intestinal ligated loops were constructed surgically in live catfish and loops were injected with *E. ictaluri* strains. Histopathological examination showed no significant tissue damage or inflammation in the intestinal tissue exposed to the vaccine strains. In contrast, massive expansion of lamina propria with congested and dilated blood vessels and intestinal inflammation were observed in the intestinal tissue exposed to wild-type *E. ictaluri*. Also, immunohistochemical staining revealed a moderate level of neutrophil and B cell infiltration in the vaccine injected intestinal tissue compared to that of wild-type injected intestinal tissue. We further assessed the expression levels of IL-1β, IL-8, TNF-α, CD4-1, and CD8-α in catfish intestine injected with *E. ictaluri* strains using quantitative real-time

PCR. Vaccine exposed tissue showed significantly lower expression of TNF-α, CD4-1, and CD8-α compared to that of the wild-type exposed tissue. All these data suggest that our live attenuated vaccines are capable of priming immune responses in catfish without causing damage to the host.

Introduction

Catfish, the "king" of the U.S. aquaculture, is threatened by a severe systemic bacterial disease known as enteric septicemia of catfish (ESC). *Edwardsiella ictaluri* is the etiological agent of ESC and continuously causing high mortality and massive economic losses in cultured channel catfish (*Ictalurus punctatus*). *E. ictaluri* utilizes several portals of entry including mucosal epithelia on the gastrointestinal tract, nares, gills, and skin (Griffin and Mitchell, 2007; Karsi et al., 2006; Ourth and Chung, 2004; Plumb and Chappell, 1978). Following its entry, *E. ictaluri* establishes acute and chronic infections in catfish (Skirpstunas and Baldwin, 2002; Thune et al., 2007). However, intestinal epithelium of catfish is a frequently utilized route of entry by *E. ictaluri* (Santander et al., 2014). The intestinal epithelium is well recognized as a selectively permeable barrier regulated by junctional proteins and as a primary route of infection for many enteric pathogens including bacteria, viruses, and parasites (O'Hara and Buret, 2008). Bacterial pathogens disrupt and exploit the intestinal barriers either by stimulating host immune responses, which ultimately compromise junctional integrity or by co-opting the barrier component as receptors to maintain its effect and attachment, through pathogen release of targeted effector molecules (Li et al., 2012). *E. ictaluri* invades catfish intestine within 15 minutes post-infection and crosses the intestinal mucosa,

which consequently resulted in massive damage to the tissue and disruption of the intestinal barriers (Baldwin and Newton, 1993). *E. ictaluri* developed several strategies to infect and colonizes the small intestine of catfish, which ultimately causes disruption of the intestinal tissues and ended up with diarrhea (Guttman and Finlay, 2009). Although the molecular processes and pathways triggered *E. ictaluri* invasion and passage through the intestinal epithelium of catfish have been reported (Jima et al., 2009), the mechanisms underlying intestinal immune responses triggered by *E. ictaluri* invasion in catfish intestine remains unclear.

Vaccination is an effective approach for the protection of catfish industry against *E. ictaluri*. Fortunately, this pathogen is composed of a single serotype and colonizes the internal lymphoid tissues of the catfish after infection. Therefore, it is a suitable pathogen for developing live attenuated vaccines (Bertolini et al., 1990). However, it has been shown that bacteria interact with its host and other bacteria through secreted proteins (Abdallah et al., 2007; Filloux, 2009). Type VI secretion system (T6SS) is a specialized system for directly export proteins from bacteria via a needle-like apparatus to the host cell cytoplasm (Cambronne and Roy, 2006). Approximately, 15 closely linked and correlated genes encoded (T6SS), however, many of these gene's role remain mostly unknown and poorly understood (Hood et al., 2010). Recently, our research group has determined that *evpB* gene in the T6SS operon is required for *E. ictaluri* virulence (Unpublished work). Our finding corroborated an earlier study showing that *evpB* plays a key role in *E. tarda* pathogenesis (Zheng and Leung, 2007). We constructed *Ei*Δ*evpB* strain by in-frame deletion of *evpB* gene and found that *E. ictaluri* is completely

attenuated in catfish fingerlings and fry. Catfish vaccinated with our *Ei*Δ*evpB* strain gained adequate immune protection. Furthermore, our research group reported that genes encode tricarboxylic acid cycle (*sdhCfrdA*) and one-carbon metabolism (*gcvP*) were essential for *E. ictaluri* virulence (Dahal et al., 2014a; Dahal et al., 2013, 2014b). Similarly, we constructed *E. ictaluri* mutant strain carrying triple gene mutations in both TCA and C1 metabolism pathways. We introduced an in-frame deletion of Glycine dehydrogenase (*gcvP),* Succinate dehydrogenase *(sdhC)*, and Fumarate reductase *(frdA)* genes in *E. ictaluri* 93-146 strain and named it as ESC-NDKL1 (*EiΔgcvPΔsdhCΔfrdA*) (unpublished work). Our *EiΔgcvPΔsdhCΔfrdA* mutant strain combined TCA cycle enzymes and C1 metabolism protein attenuate *E. ictaluri* virulence in catfish as well.

In the present study, we investigated catfish intestinal immune responses against *E. ictaluri* wild-type, *Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frd* strains. The relative expression levels of catfish immune-related genes in response to mutant strains were compared to that of wild-type *E. ictaluri*. Our data demonstrate that exposure of catfish intestine to the vaccine strains resulted in immune protective stimulation without harming the host. Our study highlights an appropriate method for evaluating the effectiveness of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains as the potential vaccine candidates and provides sufficient knowledge for understanding catfish immune responses during the intestinal invasion.

Materials and Methods

Bacterial strains, media, reagents, and growth conditions

Edwardsiella ictaluri wild-type and the vaccine strains used in this study are listed in Table 2.1. Bacterial strains were grown on brain heart infusion (BHI) agar and broth (Difco, Sparks, MD) at 30 °C to an optical density at 600 nm (OD_{600}) of 0.8 to 0.9 (~10^8 CFU/ml). When required, the culture media were supplemented with colistin sulfate (Col: 12.5 mg/ml) from (Sigma–Aldrich, Saint Louis, MN). Strains were sedimented by centrifugation (8,000 rpm) for 10 min at room temperature and resuspended in 1X phosphate-buffered saline (PBS) to 10^7 CFU/ml.

Table 2.1 Bacterial strains used in this study

Bacterial strain	**Relevant characteristics**	**References**
E. ictaluri 93–146	Wild-type; $pEI1^+$; $pEI2^+$; Col^r	(Lawrence et al., 1997)
EiΔevpB	93–146 derivative; $pEI1^+$; $pEI2^+$; Col^r; *EiΔevpB*	Unpublished work
EiΔgcvPΔsdhCΔfrdA	93-146 derivative; pEI1+; pEI2+; Col^r; *ΔfrdAΔgcvP ΔsdhC*	Unpublished work

Construction of catfish intestinal loops

All fish experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. Five specific-pathogen-free channel catfish (3 years old; mean weight 2 kg ± 10 g) were obtained from the fish hatchery at the College of Veterinary Medicine (CVM) at Mississippi State University and used in this study. The fish were acclimatized and

fastened in 100-liter tanks at 26 ± 1°C three days before surgery. The fish were anesthetized with buffered tricaine methanesulfonate (pH 7.5). Three anesthesia doses were prepared: a fish surgery dose (100 mg/liter, 20°C), a recovery dose (30 mg/liter, 20°C), and a euthanasia dose (300 mg/ liter, 15°C). The fish were moved from acclimatization tanks to the small aquaria containing surgery anesthesia dose for 10 min to achieve a proper handling, and then the fish were transferred to the surgery table in a supine position and connected to the surgical anesthesia dose (Figure 2.1). The fish body was partially moisturized with water from a sponge, and the recirculating water containing the anesthesia surgery dose flow continuously through the mouth and over the gills (Figure 2.1A). When the fish were completely anesthetized, 3 cm incision near the intestinal site in its body wall was made (Figure 2.1B). The lower part of fish's small intestine was isolated, and four loops of about 3-4 cm each were constructed by double ligation (Figure 2.1C). After the construction of intestinal loops, 1 ml of a 10^7 CFU/ml dose of *E. ictaluri* strain 93-146 was injected into the first intestinal loop. Same infection dose of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA)* strains were injected into the second and third loop respectively. The forth intestinal loop was injected with 1 ml of PBS as a negative control (Figure 2.1D and E). After the injection process, the lower intestine was placed back into the fish body (Figure 2.1F), and the fish wall was sutured closed (Figure 2.1G and H). The fish then were moved to a tank containing a recovery anesthesia dose for 8 hours to allow the intestine to interact and respond to the bacterial strains (Figure 2.1I). After this time, the fish were transferred to aquaria containing a high concentration of buffered tricaine (300 mg/liter).

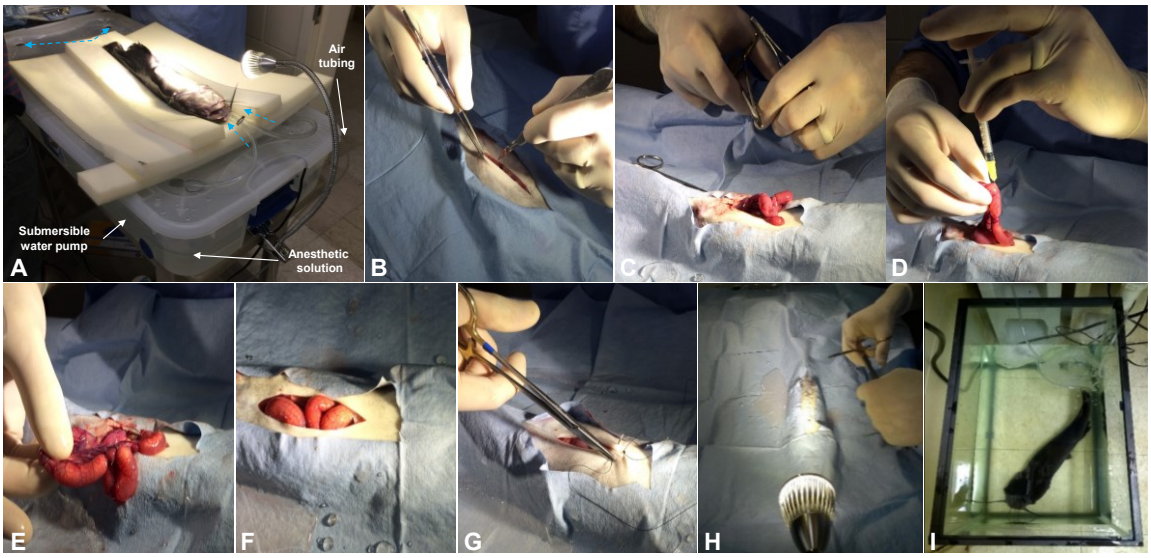


Figure 2.1 Construction of catfish intestinal loops process.

(A) Catfish surgery table. The arrows show the direction of water flow. (B) Incision of the catfish abdomen. (C) Isolation of the small intestine and construction of loops. (D-E) Injection of the intestinal loops. (F) Placement of the intestines into the abdominal cavity. (G-H) Suturing the incision. (I) Recovery.

Test of ligated intestinal loops efficiency

This experiment was undertaken to check for leaks or contamination crossed over our intestinal loops. Similarly, another set of four intestinal ligated loops were constructed in a separate experiment. 1 ml of a 10^7 CFU/ml dose of *E. ictaluri* 93–146 strain carrying pAK*gfplux*1 plasmids with fluorescence excitation was injected into the first and fourth intestinal loops (Karsi and Lawrence, 2007). Second and third loops were injected with 1 ml PBS as a negative control. Bioluminescence imaging technology was performed by using an IVIS 100 Imaging System to assess the bioluminescence signal of bacterial inoculates. We detect the bioluminescence signal as performed by Karsi et al. to visualize the location of the injected bacterial strain within catfish intestinal loops (Karsi et al., 2009).

Determination of mucus secretion

From the euthanized fish, intestinal loops were isolated, and the length of each loop was measured. The intestinal fluid was collected in tubes containing RNA*later* (Ambion, Austin, TX) and recorded with the volume-to-length ratio in milliliters per centimeter for each loop to calculate the mucus secretion.

Determination of pathology in catfish small intestine

Following the collection of intestinal fluid, a piece of intestinal tissue was aseptically cut from each loop and fixed in 15 ml of 10% buffered formalin for 24 hours until use. The fixed tissues were then dehydrated through a graded series of alcohols, then cleared in xylene, and embedded in paraffin wax. A thick section of five microns was cut per slide by microtome and stained with hematoxylin-eosin (H&E) (Leica, Germany). Slides were then examined using OLYMPUS BX60 light microscope (U-T V1X, Japan) with attached INFINITY3 digital camera and software program (Lumenera, Japan).

Immunohistochemistry (IHC)

In a further experiment to detect the presence and distribution of injected *E. ictaluri* strains in the intestinal tissue, and to determine the involvement of neutrophil and B cell in the catfish intestine immune responses to *E. ictaluri* infection, immunohistochemical staining of intestinal tissues paraffin slides were performed as previously described procedure by our lab using different specific primary antibodies (Kordon et al., 2016). The primary antibodies used in this study were prepared from hybridoma cells using mouse antiEd9, anti-neutrophil, and anti-B monoclonal antibody (C.J. Lobb, 1982; N.W. Miller, 1987). Briefly, the slides were deparaffinized in xylene

and rehydrated up to 70% alcohol. The antigen retrieval was examined by incubation of sections in target retrieval solution (Dako, Denmark) for 30 min at 100°C. Following this incubation, slides were incubated in hydrogen peroxide (H_2O_2) for 30 min followed by protein block (Dako, Denmark) for an hour. After this period, sections were incubated with primary antibody diluted at a concentration of 0.2 mg/ml (1:500 from stock solution) for an hour in a humid at room temperature. After the incubation with primary antibody, Slides were then washed with PBS and incubated with secondary antibody (EnVision + Dual link system-HRP, Dako, Carpinteria, Inc, USA) for another hour followed by 10 min incubation in DAKO liquid DAB-HRP + substrate chromogen system. After this, the hematoxylin (Leica, Biosystems Richmand, Inc, USA) was applied on the slides as a contrast and washed with acid ammonia water. Slides were then dehydrated in graded series of alcohols and cleared by xylene. Finally, the slides were analyzed and photographed using OLYMPUS BX60 light microscope (U-T V1X, Japan) with attached INFINITY3 digital camera and software program (Lumenera, Japan).

Total RNA extraction and cDNA synthesis

Intestinal tissues were washed with cold PBS, and a small piece of each loop was immediately transferred into RNase-free tubes containing ten volumes of RNA*later* (Ambion, Austin, TX). Total RNA was isolated from the collected tissues using the FastRNA™ SPIN Kit for Microbes and the FastPrep-24™ Instrument (MP Biomedicals, Santa Ana, CA) following manufacturer's instructions. Catfish genomic DNA was eliminated from the total RNA by using on-column DNase treatment with RNase-Free DNase Set (QIAGEN, Hilden, Germany). The quantity and quality of total RNA were

analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). Tissue RNAs were then converted into cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). The cDNA was synthesized in a final volume of 20 µl reaction containing 5 µg of total RNA, 4 µl of 5X reaction mix, 2 µl of maximum enzyme mix, and to 20 µl of nuclease-free water and incubated at 25°C for 10 min, 50°C for 30 min, and at 85°C for 5 min to terminate the reactions.

Quatitative real-time PCR and data analysis

Catfish-specific immune-related genes with their GenBank accession numbers and primers used in this study are listed in Table 2.2. Expression of the catfish immune genes were detected by real-time PCR using FastStart Universal SYBR Green Master (ROX; Roche, Basal, Switzerland). Primers were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and synthesized commercially (MWG Eurofins Genomics). The primers were designed by spamming the intron sequence by including 16 bases from one exon and 6 bases from another exon to avoid any non-specific amplification or possible genomic DNA contamination in our cDNA samples. Introns/exon junctions for CD4-1 and CD8-α, genes were predicted using the National Center for Biotechnology Information (NCBI) data base. However, due to lack of DNA sequence for IL-1β, IL-8, and TNF genes, primers instead were designed using mRNA sequence with reduced elongation time during the amplification to acquire only the desirable bands. Each qPCR reaction consisted of a total volume of 20 µl containing 10 µl FastStart Universal SYBR Green Master (ROX), 2 µl of cDNA, 0.6 µl primers, and 6.8 µl nuclease free water. qPCRs were performed on the Stratagene Mx3005P™ Real-Time PCR instrument (Agilent Technologies, USA) with programmed

thermal cycling conditions consisting of 45 cycles of 95°C for 10 s, 95°C for 15 s, 57°C for 30 s, and 72°C for 15 s, which reduced to 5 s when required. qPCR analysis was performed in triplicate runs (technical replicates) to confirm expression patterns. The relative expression levels of immune genes were determined by subtracting the cycle threshold (Ct) of the samples by that of the reference gene (18S rRNA) for normalization as per the formula: ΔCt = Ct (sample) – Ct (reference gene). The relative expression levels of immune genes in response to *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains compared to that in *E. ictaluri* wild type was then calculated by the $2^{\Delta\Delta Ct}$ method.

Table 2.2 GenBank accession numbers, and primers used for qPCR in this study

Genes	Accession NO.	Primers	References
18S ribosomal RNA (18S rRNA)	AF021880	F-GAGAAACGGCTACCACATCC R-GATACGCTCATTCCGATTACAG	(Karsi et al., 2004)
Interleukin 1 Beta type a (IL-1β)	DQ157743	F- TGATCCTTTGGCCATGAGCGGC R- AGACATTGAAAAGCTCCTGGTC	This study
Interleukin-8 (IL-8)	AY145142	F- CACCACGATGAAGGCTGCAACTC R- TGTCCTTGGTTTCCTTCTGG	(Santander et al., 2014)
Tumor necrosis factor (TNF-α)	AJ417565	F- GCACAACAAACCAGACGAGA R- TCGTTGTCCTCCAGTTTCAA	This study
CD4-like protein one gene (CD4-1)	DQ435305	F-TCCGCTTCAAAAACAGTGCACA R-GTGGCGGTGTAGACATCATT	This study
CD8 alpha gene (CD8-α)	HQ446239	F-CTACGCGGAGAGACAGTCCCAA R-CTCACAACCCAAAAGCACATC	This study

Statistical analysis

Statistical analysis was performed using SAS 9.4 software (SAS Institute, Inc., Cary, NC). The ANOVA test was used to assess differences among the bacterial strains. $P < 0.05$ was considered statistically significant.

Results

Construction and evaluation of ligated catfish intestinal loops

Our bioluminescence screening procedure showed no leaking or contamination crossed over the intestinal loops as our bacterial inoculates were visualized in the same area of injection within the intestinal loops after 8 hours post-injection with 1 ml of 10^7 CFU/ml of *E. ictaluri* (Figure 2.2). These results suggested that catfish intestinal loops model is an ideal method to study the bacterial virulence genes and corresponding catfish intestine immune responses.

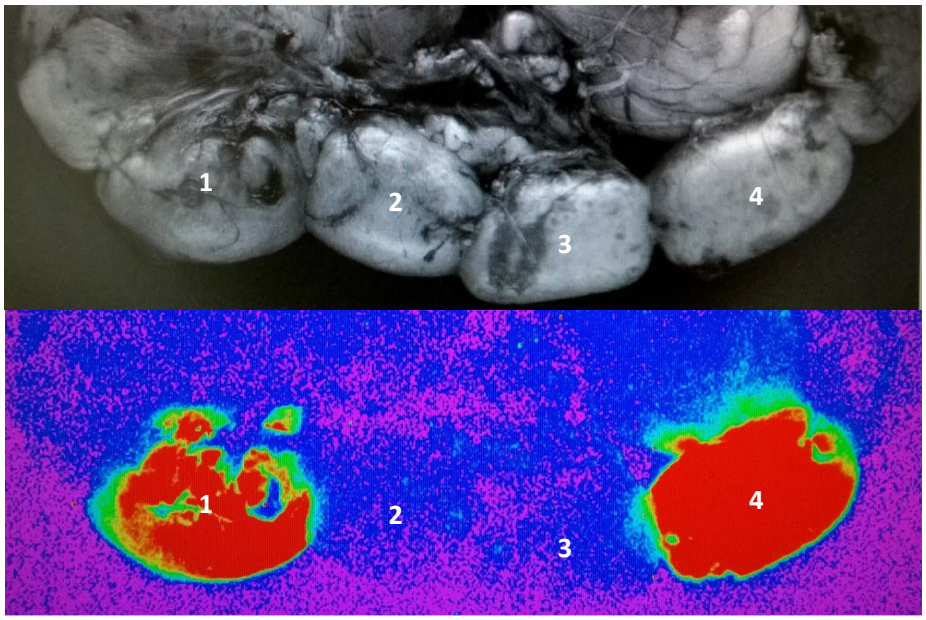


Figure 2.2 Bioluminescence signal from *E. ictaluri* carrying pAK*gfplux*1 (10^7 CFU/ml) injected into the catfish intestinal loops.

Catfish intestinal mucus secretion

Previously, it was shown that pathogenic bacterial infection causes severe mucus secretion in the fish intestinal epithelium, which disrupts intestinal barrier and causes intestinal tissue damage (Gomez et al., 2013). To compare pathogenicity, we measured the amount of mucus secreted under infection with *E. ictaluri* wild-type and vaccine strains. We observed significantly low level of mucus secretion in the catfish intestinal loops injected with *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains compared to the intestinal loops injected with wild-type *E. ictaluri* (Figure 2.3). Furthermore, no significant differences were observed in mucus secretion levels triggered by our vaccine strains compared to the control. In contrast, *E. ictaluri* wild-type triggered significantly high amount of mucus secretion when injected into the catfish intestinal loops.

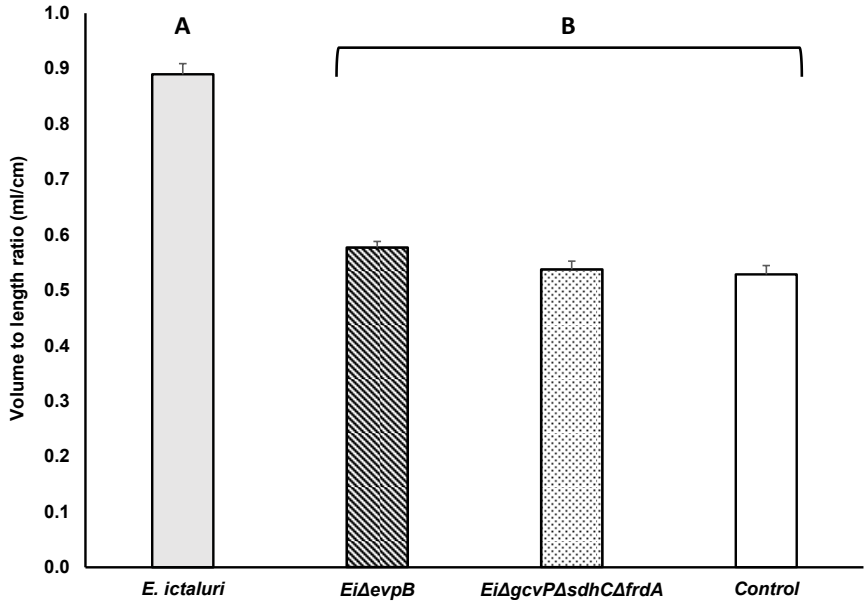


Figure 2.3 Fluid secretion of catfish intestinal loops after 8 hours post-injection with *E. ictaluri* strains (10^7 CFU/ml) and PBS.

Fluid secretion calculated in volume-to-length ratio from 5 fish (biological replicates).

Histopathology of catfish intestine

Histopathological examination of catfish intestinal tissue showed that *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains did not cause any significant tissue damage or inflammation of the intestinal epithelia. In contrast, *E. ictaluri* wild-type causes massive and significant tissue damage correlated with intestinal inflammation (Figure 2.4). We observed that intestinal tissue exposed to *Ei*Δ*evpB* strain appeared to be normal, in which no tissue damage was detected, and the intestinal epithelia were intact compared to the control with mild denudation of the apical villar part (Figure 2.4A and B). Whereas *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain causes mild pathological lesions represented by congestion in the lamina propria, but no inflammation was observed (Figure 2.4C). On the other hand, the wild-type *E. ictaluri* causes a marked loss of apical enterocytes replaced by eosinophilic cellular and karyorrhectic debris (necrosis) of the intestinal tissue (Figure 2.4D). Also, massive expansion of lamina propria with congested and dilated blood vessels admixed with edema was observed in the intestinal tissue injected with *E. ictaluri* wild-type (Figure 2.4E). Furthermore, leukocytic infiltration including macrophages and lymphocytes were detected in the muscular mucosa (Figure 2.4F). This result suggests that both vaccine strains are attenuated and exhibited a low degree of pathogenicity compared to the severe effect of wild-type *E. ictaluri*.

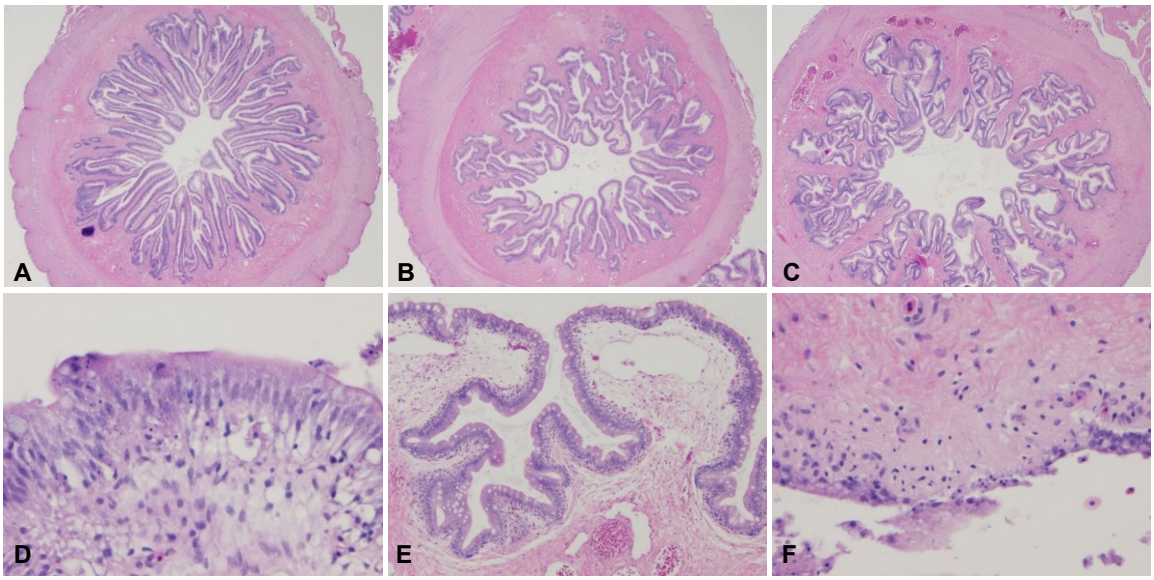


Figure 2.4 Histopathology of catfish intestinal loops after 8 hours post-infection with *E. ictaluri* strains (10^7 CFU/ml) or 1 ml of PBS.

(A) The intestinal tissue of control loop showed the typical architecture of the catfish intestine with normal layers. (B) The intestinal tissue of loop injected with *Ei*Δ*evpB* strain and showed normal intestinal layers with mild denudation of the apical villar part. (C) The intestinal tissue of loop injected with *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain and showed the focal area of sloughed epithelial cells and expanded submucosa with congested blood vessels. (D-F) Intestinal tissue of loop injected with wild-type *E. ictaluri* (D) marked villar tips necrosis with pyknosis and karyotic debris of most enterocytes, (E) massive expansion of lamina propria with severe edema and congestion of blood vessels, and (F) infiltration of muscular mucosa with moderate number of inflammatory cells mainly macrophages and lymphocytes. Magnifications: A, 20X; B, 20X; C, 20X; D, 400X; E, 100X; F, 400X.

Immunohistochemistry (IHC)

Immunohistochemical staining procedure revealed the presence of abundant numbers of *E. ictaluri* strains in the catfish intestinal tissue (Figure 2.5). The intestinal tissue of the control loop appeared very clear without any immune positive stains (Figure 2.5A). The wild-type *E. ictaluri* and *Ei*Δ*evpB* strains were observed in the lumen of catfish intestine (Figure 2.5B and D). While the *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain was present inside the phagocytic cells in the muscularis layers and the lumen of catfish intestine

(Figure 2.5D). This result suggests that the inflammation and tissue damages in the fish intestine were caused by our injected *E. ictaluri* strains. Next, we detect that neutrophils were involved in the immune response of catfish intestine as a defense mechanism against the *E. ictaluri* invasions (Figure 2.6). The intense population of neutrophils infiltration was observed in the intestinal tissue of loop injected with *E. ictaluri* wild-type (Figure 2.6B). In contrast, fewer numbers of neutrophils were stimulated in response to *Ei*Δ*evpB* and

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains (Figure 2.6C and D). Furthermore, a similar finding was seen in the levels of B cell infiltration by our *E. ictaluri* strains in the catfish intestinal tissue (Figure 2.7). In a similar pattern, few numbers of B cells were stimulated in the intestinal tissue injected with our mutant strains (Figure 2.7C and D) while B cell population were more abundant in the intestinal tissue injected with *E. ictaluri* wild-type (Figure 2.7B). B cells have a phagocytic ability, and their numbers induced by the wild-type *E. ictaluri* show that catfish intestines were exposed to the strong antigen.

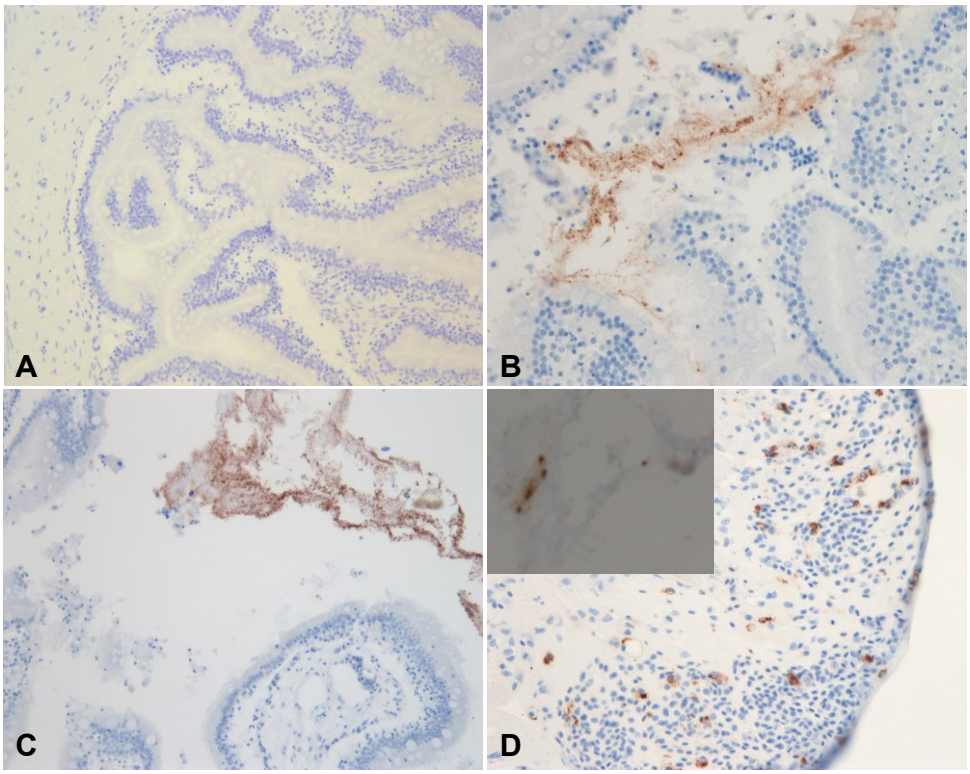


Figure 2.5 Immunohistochemical staining of catfish intestine demonstrating the distribution of *E. ictaluri* strains.

(A) The intestinal tissue of control loop without any positive bacterial staining. (B) The intestinal tissue of loop injected with *E. ictaluri* wild-type showing intense numbers of positive staining in the intestinal lumen. (C) Intense numbers of *Ei*Δ*evpB* positive staining in the intestinal lumen. (D) Intense numbers of *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* positive staining inside the phagocytic cells and blood vessels of muscularis mucosa and in the lumen of catfish intestine. Magnifications: A, 200X; B, 400X; C, 400X; D, 400X.

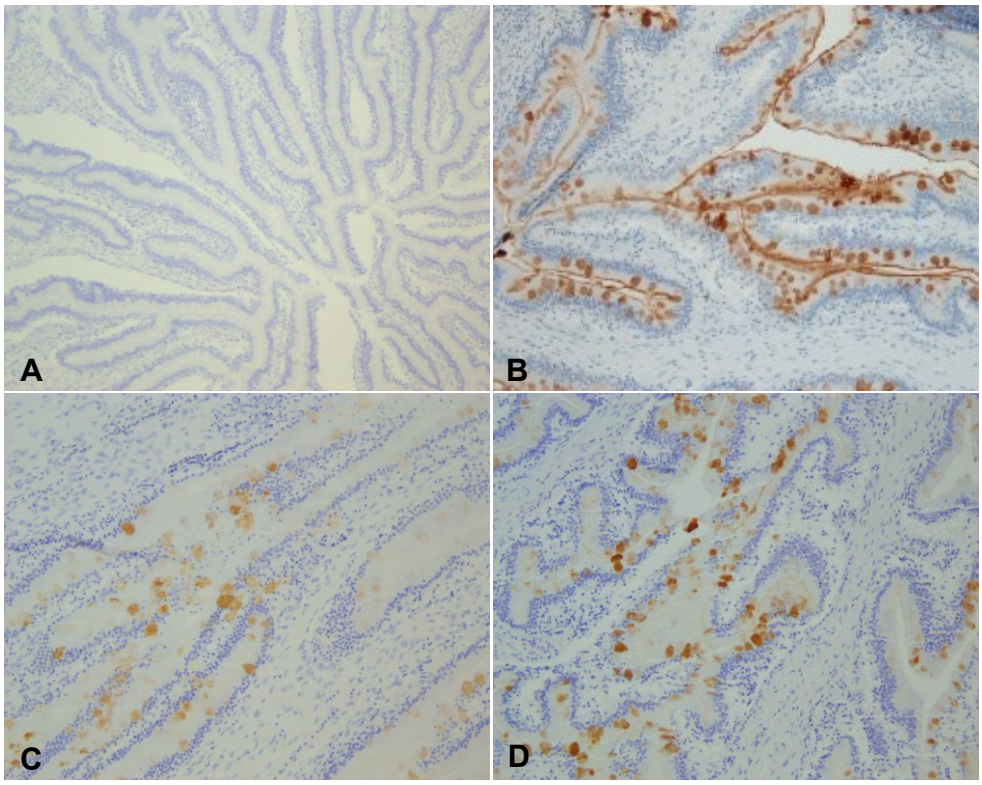


Figure 2.6 Immunohistochemical staining of catfish intestine demonstrating the presence of neutrophilic cells.

(A) The intestinal tissue of control loop without any positive staining. (B) The intestinal tissue of loop injected with *E. ictaluri* wild-type showing intense numbers of positive brown granular neutrophils. (C). The intestinal tissue of loop injected with *Ei*Δ*evpB* showing fewer numbers of positive neutrophilic cells staining. (D) The intestinal tissue of loop injected with *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* showing fewer numbers of neutrophilic cells positive staining. Magnifications: A, 200X; B, 400X; C, 200X; D, 200X.

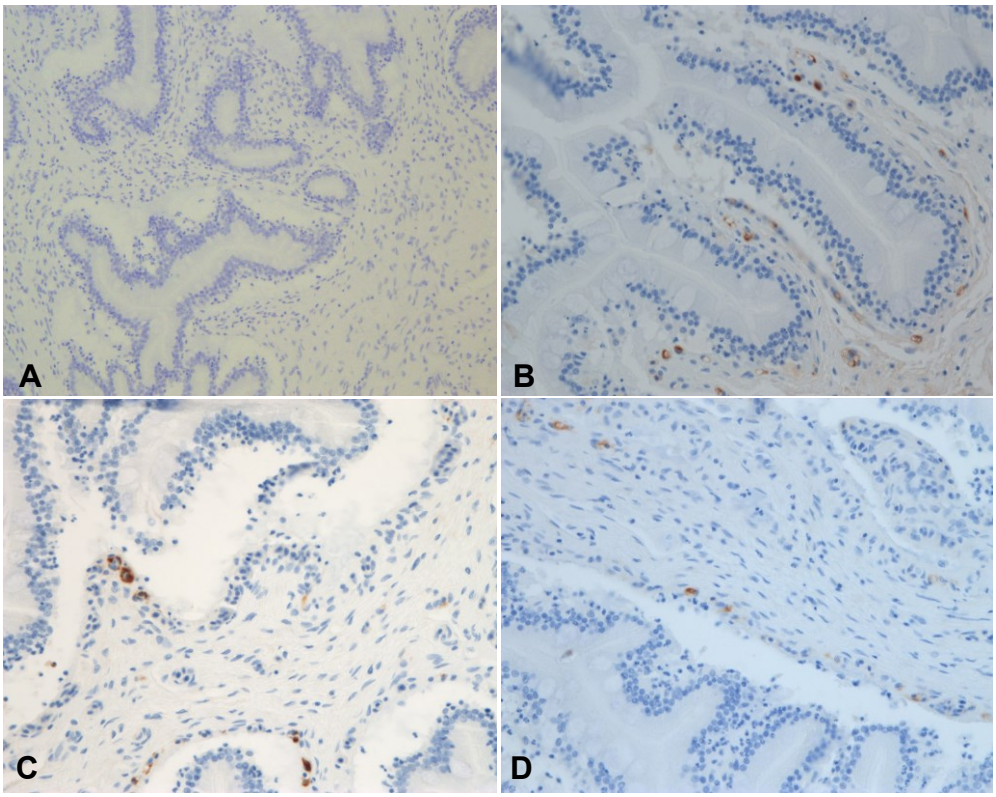


Figure 2.7 Immunohistochemical staining of catfish intestine demonstrating the presence of B cell.

(A) The intestinal tissue of control loop without any positive staining. (B) The intestinal tissue of loop injected with *E. ictaluri* wild type showing clear and moderate numbers of B cell. (C). The intestinal tissue of loop injected with *Ei*Δ*evpB* showing fewer numbers of positive B cell presence than in loop injected with wild-type. (D) The intestinal tissue of loop injected with *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* showing fewer numbers of B cell than *E. ictaluri* wild-type. Magnifications: A, 200X; B, 400X; C, 400X; D, 400X.

Expression pattern of catfish immune-related genes

qPCR was undertaken to assess the expression levels of genes related to proinflammatory responses and T cell responses in the catfish intestine, such as IL-1β, IL-8, TNF-α, CD4-1, and CD8-α. All genes were detected in the catfish intestinal tissue, and they were expressed relatively low in response to the *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains compared to the wild-type *E. ictaluri* (Figure 2.8). Our

expression analysis experiment showed that there was only a numerical decrease in the secretion levels of IL-1β and IL-8 in response to vaccine strains compared to *E. ictaluri* wild-type without statistical differences. However, the secretion level of TNF-α was significantly lower in response to our mutants compared to the *E. ictaluri* wild-type. Similarly, *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains induce the expression levels of CD4-1 and CD8-α significantly lower than wild-type *E. ictaluri*. Interestingly, we observed that CD8-α transcript levels exceeded those of CD4-1 in the catfish intestines. This result suggests that many of CD8-α expressing cells resided in catfish intestine after 8 hours post-infection, while only a few CD4-1 expressing cells and very rare MHCII were localized.

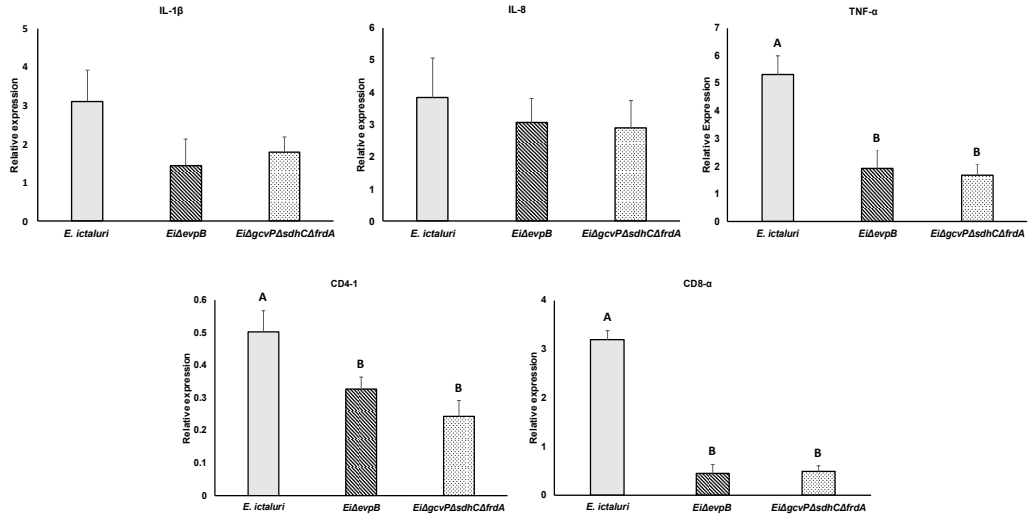


Figure 2.8 Differential expression of immune genes in the catfish small intestines induced by *E. ictaluri* strains.

Catfish intestinal loops were injected with 107 CFU/ml of each strain. qPCR analysis included 5 fish (biological replicates) and repeated in triplicate runs (technical replicates).

Discussion

The objective of this study was to investigate catfish intestinal immune responses during *E. ictaluri* invasions. We constructed catfish intestinal ligated loops to evaluate an efficacy and associated immune responses of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* vaccine candidate strains and compare it to the wild-type *E. ictaluri*. The ligated intestinal loop has been reported previously as an effective method to investigate host-pathogen interactions (Santos et al., 2002; Wallis et al., 1989). We utilized bioluminescence imaging technology to evaluate our loop's efficiency. No leaking or cross-contamination was detected and inoculated bacterial strains were visualized in the same area within the intestinal loops after 8 hours post-injection.

We monitored the histopathological changes in the catfish intestine after the invasion of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains and compared to the effect of *E. ictaluri* wild-type. No inflammation or tissue damage were observed in the small intestine of catfish exposed to the vaccine strains. In contrast, wild-type *E. ictaluri* damaged the intestinal tissue and stimulated inflammatory immune cells to the site of infection. This result in line with a previously reported study showing that *E. ictaluri* wild-type causes massive tissue damages and inflammation to the intestinal epithelia of catfish (Santander et al., 2014). Linked with histopathological changes, both vaccine strains and *E. ictaluri* wild-type were detected by IHC in the fish intestinal tissue. This suggests that our vaccines colonization do not induce strong infiltration of immune cells. In contrast, *E. ictaluri* wild-type induces severe inflammation as showed by loops histopathological examination. This result also confirmed by the level of inflammatory neutrophil and B

cell infiltration in our intestinal tissue. Immunohistochemical staining procedure revealed that our vaccine candidate strains stimulated moderate numbers of neutrophil and B cell in the catfish intestinal tissue, while large number of these cells were stimulated by *E. ictaluri* wild-type as a sign of pathogenic inflammatory response. In catfish intestine, neutrophils are the predominant phagocytic immune cell (Hebert et al., 2002). The phagocytic function of fish neutrophils plays an important role in protection against encountered pathogens (Companjen et al., 2006; Neumann et al., 2001). Additionally, research on a teleost fish show for the first time the phagocytic and intracellular bactericidal ability of fish primary B cell (Li et al., 2006; Sunyer, 2012). However, it is known that the first response of the host after an encounter with a foreign pathogen is the mobilization of the phagocytic immune cells into the sites where the foreign pathogen has invaded (Ainsworth and Chen, 1990). As our results showed, scores of neutrophils and B cells in our catfish intestinal tissue were stimulated in response to the *E. ictaluri* wild-type invasion. It has been reported that overstimulation of host intestinal immune responses leads to disrupt and exploit the intestinal barriers (Li et al., 2012). Therefore, *E. ictaluri* wild-type induces pathogenic inflammatory responses in our intestinal tissue by overestimating catfish intestinal immune cells. On the other hand, vaccine strains stimulated the protective level of immune responses by inducing the presence of moderate numbers of neutrophils and few numbers of B cells in the intestinal tissue. This finding suggests that our mutant strains are attenuated and have immune protection abilities. These observations also put forward that *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains could be potentially an effective commercial vaccine.

To provide more evidence that vaccine candidates have immune protective abilities, we assessed the expression levels of IL-1β, IL-8 and TNF-α pro-inflammatory cytokines in the intestinal tissue exposed to our vaccine candidate strains and compared to the intestinal tissue exposed to *E. ictaluri* wild-type. Proinflammatory cytokines play an important role in innate immunity. In channel catfish, IL-1β, IL-8 and TNF-α like genes have been identified in response to *E. ictaluri* infections (Chen et al., 2005; Wang et al., 2006; Zou et al., 2003). Cytokines secretion followed the bacterial infection are strong markers indicating an activated inflammatory response (Seppola et al., 2008). We observed that invasion of *E. ictaluri* to the catfish intestine lead to induce the secretion of IL-1β, IL-8, and TNF-α cytokines. Interleukins (ILs) and tumor necrosis factors (TNFs) are early expressed cytokines as mechanisms of host defense in the early stage of infection. These cytokines are produced by many cell types mainly blood monocytes and macrophages. Secreted cytokines induce numerous reactions in the organisms, which causes inflammation, this enables the organism to respond to the infection (Huising et al., 2004). Our analysis of catfish intestine TNFα expression levels revealed that the pathogenic inflammatory immune response is stimulated upon invasion of *E. ictaluri* wild-type and not to our mutants, but we could not find any statistical differences in the expression levels of IL-1β, IL-8 between *E. ictaluri* wild-type and our vaccine strains even though there were clear numerical decreases in their secretion levels compared to *E. ictaluri* wild-type, and that maybe due to the statistical variation among the fish.

For further understanding, the catfish intestinal immune responses, the expression levels of CD4-1 and CD8-α genes were also assessed. It has been suggested that many of

fish species have a robust mucosal defense system (Cain et al., 2000; Fletcher and White, 1972; Rombout et al., 1986). Recent research on the intestinal immunology of fish established that intestinal tissue of any fish species is rich in T cell populations (Picchietti et al., 2011a; Rombout et al., 2011). Also, it has been shown that the major leucocyte populations present in the teleost gut are the mucosal T lymphocytes (Abelli et al., 1997; Bernard et al., 2006; Rombout et al., 1998). However, all these observations promote the researchers on the fish mucosal immunity field to claim that T cells are detected very early after bacterial infection in the fish intestine. In mammals, intestinal T cell plays an important regulatory role to maintain the integrity of epithelial tissue by coordinated the innate and adaptive immune responses (Chen et al., 2002). The presence of T cells in the intestinal epithelium help regulate gut metabolism and prevent undesirable immune responses (Fahrer et al., 2001). In channel catfish, T cells have equivalents function and structure to those in mammals (Miller et al., 1986; Wilson et al., 1998; Zhou et al., 2001) and classified by the presence of CD4 or CD8 molecules. Both molecules serve as co-receptors that binding MHC II and MHC I, respectively. CD4 and CD8 molecules play an important role in early T cell activation and signaling by stabilizing the interaction of the TCR-peptide-MHC complex and recruit the co-receptor bound the protein tyrosine kinase $p56^{lck}$ in their cytoplasmic tails to the TCR signaling complex (Swain et al., 2012; Turner et al., 1990). In many fish species including channel catfish, CD4 and CD8 genes have been identified and expressed in many lymphoid tissues (Edholm et al., 2007; Quiniou et al., 2011). However, no report to our knowledge indicates the expression of these genes in the fish intestine next to few hours of bacterial infection. In this study, we observed that the expression levels of CD4-1 and CD8-α were significantly up-regulated in the

catfish intestinal tissue after 8 hours post-injection with *E. ictaluri* wild-type, while vaccine strains exposed tissue showed significantly lower expression of CD4-1 and CD8-α. Interestingly, the transcript levels of CD8-α exceeded those of CD4-1 in catfish intestines. Similar to our finding was seen by analyzing qPCR data that were confirmed by *In situ* hybridization and digoxigenin-labeled RNA probes, in which CD8-α expressing genes were more abundant in the intestinal mucosa of sea bass, while rare CD4 expressing genes were detected (Picchietti et al., 2011b). Also, a similar finding was indicated the predominant occurrence of transcript levels of CD8α than CD4 in teleost fish (Quiniou et al., 2011).

Taken together, catfish intestinal tissue histopathological examinations, IHC staining, and immune genes expression profiles are all provide strong evidence that both of the vaccine candidate strains generated protective immunity in catfish without causing damages after their colonization. In contrast, *E. ictaluri* wild-type launched strong inflammatory response, which ultimately leads to disrupting the epithelial tissue. *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains stimulated catfish immune signaling pathways in protective level. Therefore, they are an essentially commercial vaccine candidates needed to enhance the growth and diversity of our Nation's aquaculture. The evaluation of vaccines in the fish intestine is considered an effective approach due to abundant presence of many immune cells in the fish gut, which make vaccine stability greatly affected by the fish immune relevant gene expression and gut function. In the future, this area of research may be used for monitoring the vaccines efficacy and fish immune responses. Because of the crucial role that intestine organ plays in the fish immune

system, the mechanisms involved in regulating different level of immune stimulation by modified live vaccines need for further investigation to improve the vaccines effectiveness and U.S aquaculture industry.

CHAPTER III

TRANSCRIPTOME ANALYSIS OF EDWARDSIELLA ICTALURI DURING COLONIZATION OF A NATURAL HOST DETERMINED BY RNA-SEQ

Abstract

Edwardsiella ictaluri is Gram-negative bacillus in the family of *Enterobacteriaceae* and a leading cause of an economically important bacterial derived gastrointestinal septicemia of farm-raised channel catfish in the USA. *E. ictaluri* encodes many virulence genes expresses under various stresses conditions indicative of its ability to invade and colonize catfish intestine rapidly. Despite this ability to regulate gene expression, virulence genes during invasion and colonization of catfish intestine remain poorly understood. In this study, we determined gene expression profiles of wild-type *E. ictaluri* during catfish intestinal invasion and compared it to that of *Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains using high throughput RNA-Seq technology. A total of 238 DEGs in wild-type *E. ictaluri*, 190 DEGs in *Ei*Δ*evpB*, and 235 DEGs in *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains were identified with a FDR < 0.05 and fold change > 1.5 or < -1.5. Further, the GO enrichment analysis with a P < 0.5 indicated that the DEGs were enriched to 27, 23, and 22 GO terms in *E. ictaluri, Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* respectively. Also, a KEGG pathway analysis with a P < 0.5 determined that the DEGs of wild-type *E. ictaluri, Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains were involved in 9, 8, and 13 pathways respectively. Moreover, 10 DEGs were chosen to validate the RNA-Seq

data using real-time PCR analysis, and our results showed the consistency of the expression levels between RNA-Seq and qPCR data. Finally, our results provide insights into how *E. ictaluri* can invade and colonize catfish intestine through regulating its virulence gene expressions.

Introduction

Edwardsiella ictaluri is Gram-negative, rod-shaped bacterium, and associated with widespread mortality of farm-raised catfish in the USA. *E. ictaluri* is primary catfish pathogen and can infect catfish of all ages especially during the late spring and fall. This pathogen has many potential routes of entry, but it has recently been confirmed that it mostly gains entry through the intestinal epithelium by crossing the intestinal mucosa rapidly and develop acute and chronic infections in catfish (Li et al., 2012; Santander et al., 2014). The acute infection of *E. ictaluri* causes rapid and high mortalities, while the chronic infection reduces catfish production suggesting that the bacterium may have invasion and survival strategies similar to other members of the *Enterobacteriaceae* family (Baldwin and Newton, 1993). For example, the direct transport in blood and phagocytic cells of the host. This mechanism of survival and replication in catfish phagocytic cells has been reported in many other previous studies conducted on this pathogen (Ainsworth and Chen, 1990; Baldwin and Newton, 1993; Miyazaki and Plumb, 1985; Shotts et al., 1986; Stanley et al., 1994).

Although many virulence factors have been reported in *E. ictaluri* including, type III and type VI secretion systems, flagella, type 1 fimbriae (Williams et al., 2012), lipopolysaccharide (LPS) (Lawrence et al., 2003; Santander et al., 2013; Weete et al.,

1988), hemolysins (Williams and Lawrence, 2005), outer membrane proteins (OMP) (Newton et al., 1990; Vinitnantharat et al., 1993), little is known about the *E. ictaluri* virulence factors that are involved in recognition, attachment, invasion, and colonization of catfish intestine and mucosal membranes. Much research has been conducted for characterizing the pathogenesis of this bacterium, but no study has been done towards understanding the global gene expression profiles during catfish intestinal invasion and colonization. Recently, High throughput RNA-Seq technology has emerged as a robust approach for identifying the global gene expression in many bacterial species (Camejo et al., 2009; Dugar et al., 2013; Mandlik et al., 2011; Sharma et al., 2010). RNA-Seq has many advantages such as the ability to detect and quantify transcripts of all bacterial genomic regions, sensitivity for detection of low abundance transcript, and single nucleotide resolution (Wang et al., 2009b).

The pathogenesis of *E. ictaluri* depends on its ability to regulate its virulence genes to avoid intestinal defenses and causes gastrointestinal septicemia in catfish, therefore, prevention of *E. ictaluri* from establishing an infection in channel catfish should be focused on identifying the genes that are required for invasion and colonization of catfish intestine. Thus, study of the *E. ictaluri* transcriptional profiles is significant for understanding its pathogenic mechanisms. In this study, we report the first comprehensive transcriptome expression analysis of wild-type *E. ictaluri*, after 8 hours post-injection into the catfish intestine and compare it to that of our vaccine candidate *Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains. The outcomes of this study should accelerate

our understanding of the pathogenic mechanisms of *E. ictaluri* and provide sufficient evidence for commercializing our vaccine strains.

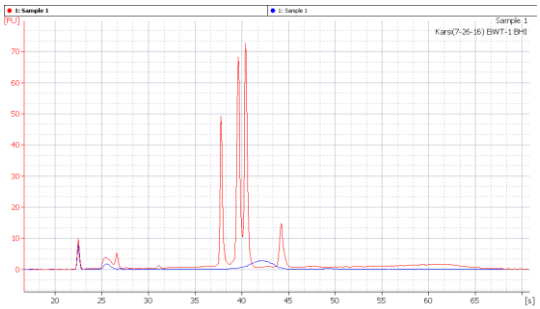
Materials and Methods

RNA isolation

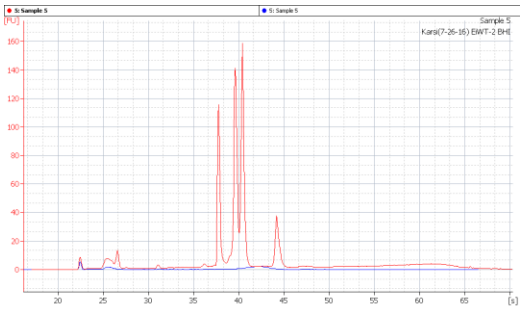
Following 8 hours post-injection of wild-type *E. ictaluri*, *Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains in catfish intestine loops as mentioned in chapter II, bacterial strains were then harvested from each intestinal loop of euthanized fish and immediately transferred into RNase-free tubes containing ten volumes of RNA*later* (Ambion, Austin, TX). Total bacterial RNA was isolated from the bacterial strains using the FastRNA™ SPIN Kit for Microbes and the FastPrep-24™ Instrument (MP Biomedicals, Santa Ana, CA) following manufacturer's instructions. RNA samples for the control were isolated from *E. ictaluri* wild-type grown overnight on brain heart infusion (BHI) agar and broth (Difco, Sparks, MD) at 30 °C to an optical density at 600 nm (OD600). Bacterial genomic DNA was eliminated from the total RNA by using on-column DNase treatment with RNase-Free DNase Set (QIAGEN, Hilden, Germany). The quantity and quality of total RNA were analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The RNA samples were depleted of prokaryotic 16S, 23S and Eukaryotic 18S, 28S rRNA species using combined Bacteria+ Human Ribo-Zero™ rRNA Removal Kits (from Illumina), which performed by LC Sciences LCC, USA. Removal of contaminating rRNA species from each sample was assessed with the Agilent Bioanalyzer RNA 6000 nanochip (Agilent Technologies) as details shown in Figure 3.1, then subjected to cDNA library construction, deep sequencing, and bioinformatic analysis.

Table 3.1 The sequences of primers used in qPCR experiments.

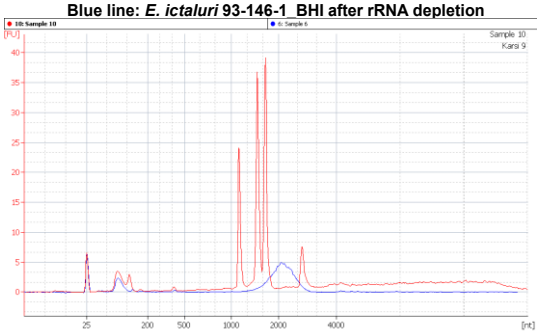
Genes	Locus tag	Forward primers	Reverse primers
EsaJ	NT01EI_0936	AGTTATTTCCCAGCGGACAG	GACGCCATCCATGCTACTAA
EsaH	NT01EI_0938	GCCGTTAACTGCGGGAT	AACAGTAGAATGGCTTCGCA
EsaE	NT01EI_0941	CTTGAAGATTCGCTTCGACAC	CTGACGCTGACGCTCAA
EvpH	NT01EI_2744	GTGGTCTTGCTGGATGAAGTAG	GGTCAGCAGGATAACGGTATTG
EvpB	NT01EI_2738	GCCTGCTGGATAGCATCATT	GACAGAACCTCACTTACCCATTC
EvpA	NT01EI_2737	CGCTTCCCAAGCTGAAAGA	TCAGTGCGGAATGACAGATG
AspA	NT01EI_0377	GTACTGCTGAACGAAGAAGTGA	CTGATAGCCTTCCGGTGTATTC
GlpQ	NT01EI_3469	GAAGCAAATTGCCCGGTATG	TGCGCCTCTTTCACCATATT
PrkA	NT01EI_1637	ATGTTCTCCAACACCGAAGAG	CCATCATACGGTGAACGAAGT
GlgS	NT01EI_1937	GCTGCGTTTAGCACAAATACC	CACCGTCATATTTCCCTGTACC
16S rRNA	NT01EI_R00011	TCAAGTCATCATGGCCCTTAC	CCGGACTACGACGTACTTTATG



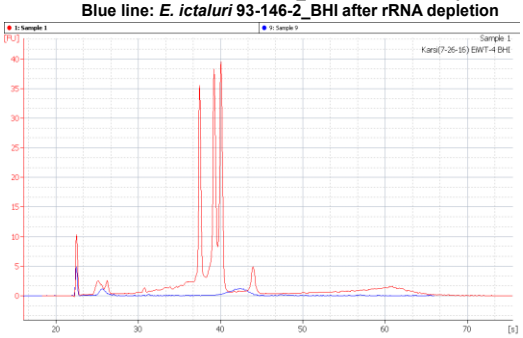
Red line: *E. ictaluri* 93-146-1_BHI before rRNA depletion
Blue line: *E. ictaluri* 93-146-1_BHI after rRNA depletion



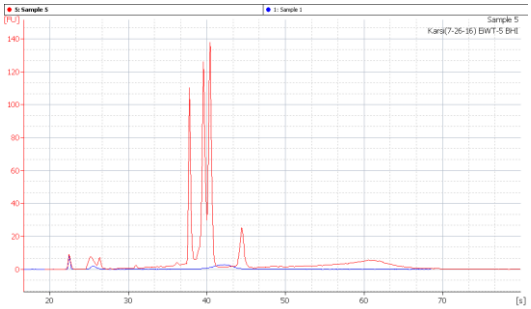
Red line: *E. ictaluri* 93-146-2_BHI before rRNA depletion
Blue line: *E. ictaluri* 93-146-2_BHI after rRNA depletion



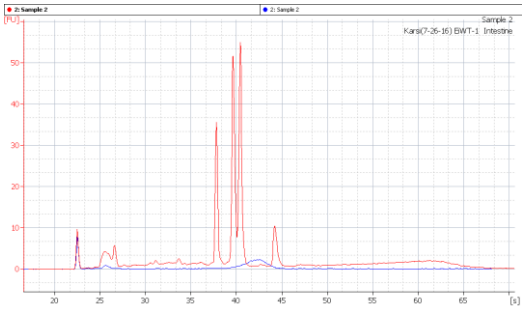
Red line: *E. ictaluri* 93-146-3_BHI before rRNA depletion
Blue line: *E. ictaluri* 93-146-3_BHI after rRNA depletion



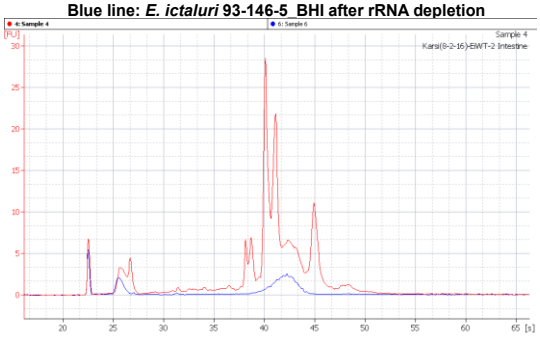
Red line: *E. ictaluri* 93-146-4_BHI before rRNA depletion
Blue line: *E. ictaluri* 93-146-4_BHI after rRNA depletion



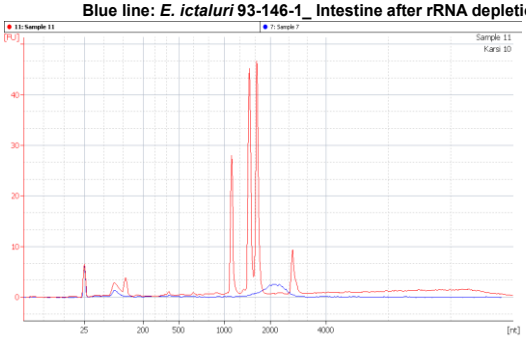
Red line: *E. ictaluri* 93-146-5_BHI before rRNA depletion
Blue line: *E. ictaluri* 93-146-5_BHI after rRNA depletion



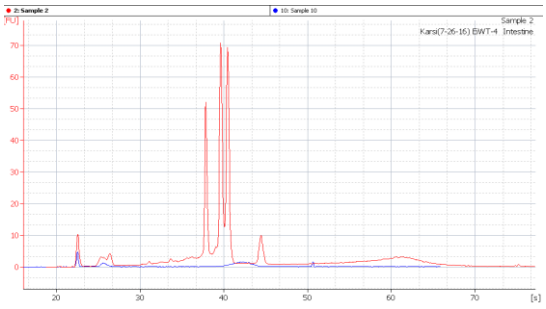
Red line: *E. ictaluri* 93-146-1_ Intestine before rRNA depletion
Blue line: *E. ictaluri* 93-146-1_ Intestine after rRNA depletion



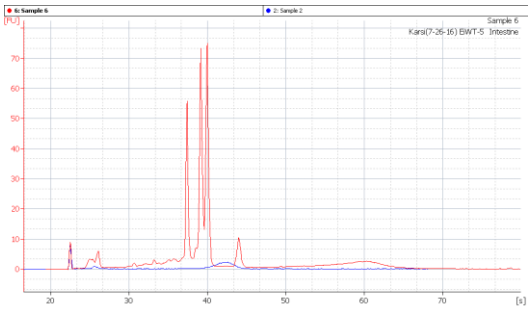
Red line: *E. ictaluri* 93-146-2_ Intestine before rRNA depletion
Blue line: *E. ictaluri* 93-146-2_ Intestine after rRNA depletion



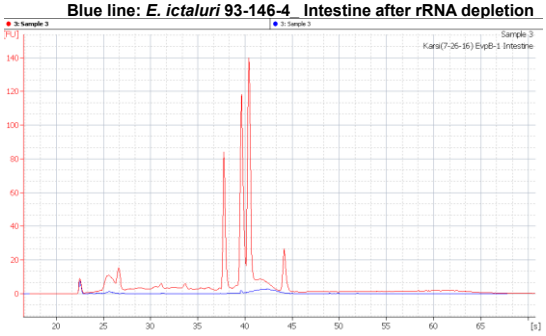
Red line: *E. ictaluri* 93-146-3_ Intestine before rRNA depletion
Blue line: *E. ictaluri* 93-146-3_ Intestine after rRNA depletion



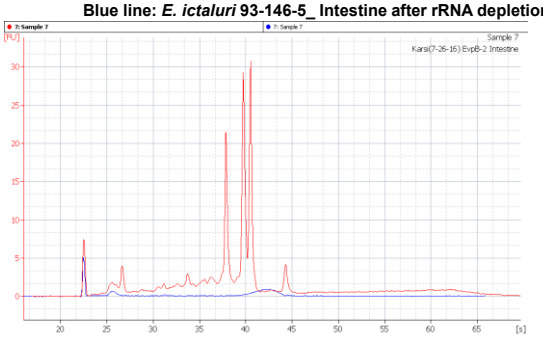
Red line: *E. ictaluri* 93-146-4_ Intestine before rRNA depletion
Blue line: *E. ictaluri* 93-146-4_ Intestine after rRNA depletion



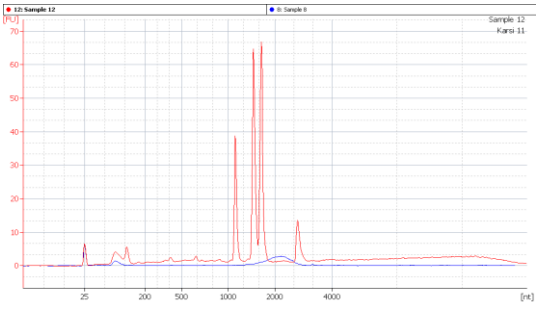
Red line: *E. ictaluri* 93-146-5_ Intestine before rRNA depletion
Blue line: *E. ictaluri* 93-146-5_ Intestine after rRNA depletion



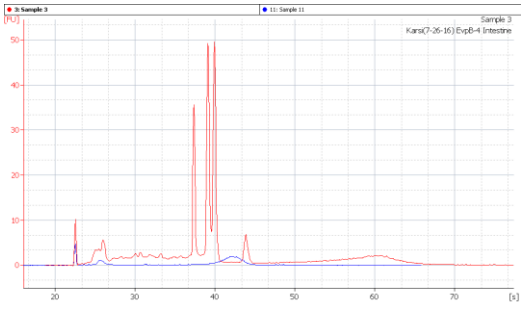
Red line: *Ei*Δ*evpB*-1_ Intestine before rRNA depletion
Blue line: *Ei*Δ*evpB*-1_ Intestine after rRNA depletion



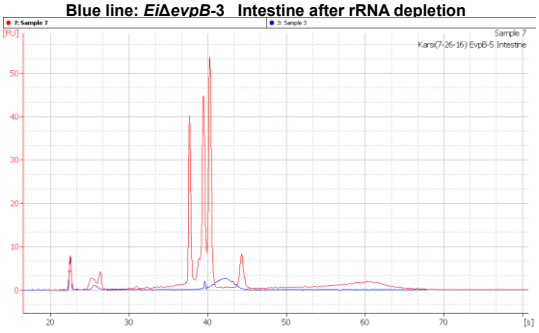
Red line: *Ei*Δ*evpB*-2_ Intestine before rRNA depletion
Blue line: *Ei*Δ*evpB*-2_ Intestine after rRNA depletion



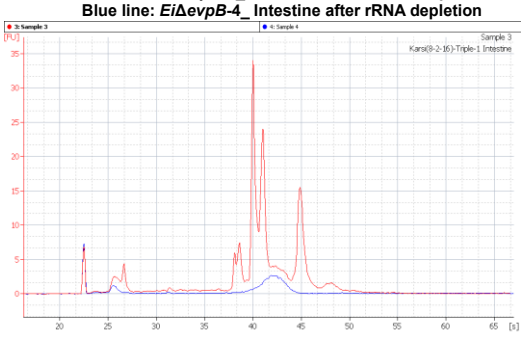
Red line: *Ei*Δ*evpB*-3_ Intestine before rRNA depletion
Blue line: *Ei*Δ*evpB*-3_ Intestine after rRNA depletion



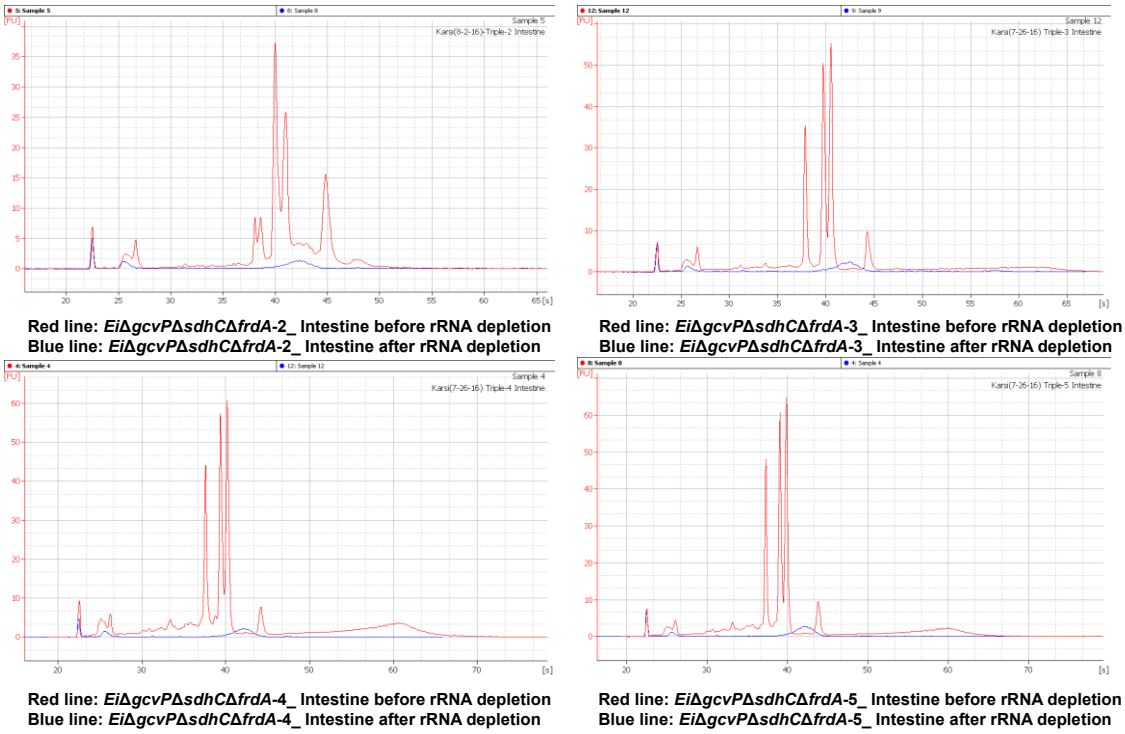
Red line: *Ei*Δ*evpB*-4_ Intestine before rRNA depletion
Blue line: *Ei*Δ*evpB*-4_ Intestine after rRNA depletion



Red line: *Ei*Δ*evpB*-5_ Intestine before rRNA depletion
Blue line: *Ei*Δ*evpB*-5_ Intestine after rRNA depletion



Red line: *EiΔgcvPΔsdhCΔfrdA*-1_ Intestine before rRNA depletior
Blue line: *EiΔgcvPΔsdhCΔfrdA*-1_ Intestine after rRNA depletion



Red line: *EiΔgcvPΔsdhCΔfrdA*-2_ Intestine before rRNA depletion
Blue line: *EiΔgcvPΔsdhCΔfrdA*-2_ Intestine after rRNA depletion

Red line: *EiΔgcvPΔsdhCΔfrdA*-3_ Intestine before rRNA depletion
Blue line: *EiΔgcvPΔsdhCΔfrdA*-3_ Intestine after rRNA depletion

Red line: *EiΔgcvPΔsdhCΔfrdA*-4_ Intestine before rRNA depletion
Blue line: *EiΔgcvPΔsdhCΔfrdA*-4_ Intestine after rRNA depletion

Red line: *EiΔgcvPΔsdhCΔfrdA*-5_ Intestine before rRNA depletion
Blue line: *EiΔgcvPΔsdhCΔfrdA*-5_ Intestine after rRNA depletion

Figure 3.1 Efficiency of rRNA depletion using combined Bacteria + Human Ribo-Zero™ rRNA Removal Kits.

The red line represents the rRNA ratio of *E. ictaluri* strains before depletion with Ribo-Zero kit, and the blue line represents the rRNA ratio of *E. ictaluri* after depletion with Ribo-Zero kit.

RNA sequencing and bioinformatic analysis

Paired-end 100 nt sequencing reads were obtained from Illumina HiSeq 2500 following the protocol of TruSeq Stranded Total RNA Sample Preparation (from Illumina). After trimming the sequencing adaptor from the raw reads, mappable/clean reads were mapped to the *E. ictaluri* genome from https://www.ncbi.nlm.nih.gov/nuccore/NC_012779 using Bowtie2, and the gene abundance was estimated and mapped to different regions of the genome using EDGE Pro v1.3.1 (Magoc et al., 2013). The differential expression analysis was conducted using

EdgeR v3.12.1 (Robinson et al., 2010) and significant genes with false discovery rate (FDR < 0.05) and fold changes (FC > 1.5) were defined as differentially expressed genes (DEGs). The significantly DEGs were then selected for Gene Analogy (GO)/ Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway enrichment analysis. The GO annotation and the (KEGG) Pathway enrichment analysis was conducted using Cytoscape software v3.2.1 (Shannon et al., 2003). Web Gene Ontology Annotation Plot (WEGO) software was used for visualizing, comparing, and plotting GO annotation results.

Quantitative reverse transcriptase polymerase chain reaction (qPCR).

To validate the RNA-Seq data, 10 genes were chosen from all of the DEGs including 6 upregulated genes (*EvpA*, *EvpB*, *EvpH*, *EsaE*, *EsaH*, and *EsaJ*) and 4 downregulated genes (*AspA*, *GlpQ*, *PrkA*, and *GlgS*) for qPCR analysis. Bacterial RNA samples were converted into cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). The cDNA was synthesized in a final volume of 20 µl reaction containing 5 µg of total RNA, 4 µl of 5X reaction mix, 2 µl of maximum enzyme mix, and up to 20 µl of nuclease-free water and incubated at 25°C for 10 min, 50°C for 30 min, and at 85°C for 5 min to terminate the reactions. The primers used for qRT-PCR are listed in Table 3.1, and the expression levels of these selected genes were then determined by qPCR using FastStart Universal SYBR Green Master (ROX; Roche, Basal, Switzerland). Primers were designed using PrimerQuest Tool http://www.idtdna.com/primerquest/home/index and synthesized commercially (MWG Eurofins Genomics). Each qPCR reaction consisted of a total volume of 20 µl containing 10 µl FastStart Universal SYBR Green Master (ROX), 2 µl of cDNA, 0.6 µl primers, and 6.8 µl nuclease free water. qPCRs were performed on the Stratagene Mx3005P™ Real-

Time PCR instrument (Agilent Technologies, USA) with programmed thermal cycling conditions consisting of 45 cycles of 95°C for 10 s, 95°C for 15 s, 57°C for 30 s, and 72°C for 15 s. qPCRs analysis was performed in triplicate runs (technical replicates) to confirm expression patterns. The relative expression levels of our selected genes were determined by subtracting the cycle threshold (Ct) of the samples by that of the reference gene (16S rRNA) for normalization as per the formula: ΔCt = Ct (sample) – Ct (reference gene). The expression levels of our selected genes compared to that of RNAseq data was then calculated by the 2-ΔΔCt method.

Results

Mapping and expression profile analysis of RNA-seq to the *E. ictaluri* 93-146 genome

To determine the gene expression profiles of wild-type *E. ictaluri* 93-146, *Ei*Δ*evpB*, and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains during catfish intestinal invasion, a total of 20 cDNA libraries (five biological replicates of wild-type *E. ictaluri* 93-146 cultures grown in BHI broth used as control, and five biological replicates of each bacterial strains injected in the catfish intestinal loops) were constructed by Illumina sequencing in a single run. The average number of reads obtained per each sample was ~47 million reads with ~71% of reads mapped to the *E. ictaluri* 93-146 genome, and the average of Illumina sequencing coverage was ~648x as shown in Table 3.2.

Table 3.2 Summary of RNA sequencing results of bacterial strains cDNA libraries.

Samples	Raw reads (R1+R2)	Raw reads (R1+R2)		Coverage
		Count	Percentage	
E. ictaluri 93-146-1_BHI	43,919,730	31,060,450	70.72%	603.47
E. ictaluri 93-146-2_BHI	37,843,080	27,052,345	71.49%	562.70
E. ictaluri 93-146-3_ BHI	46,601,756	32,616,326	69.99%	627.34
E. ictaluri 93-146-4_ BHI	59,352,348	41,563,239	70.03%	782.80
E. ictaluri 93-146-5_ BHI	48,236,794	35,202,652	72.98%	679.05
E. ictaluri 93-146-1_ Intestine	44,134,724	34,271,701	77.65%	678.97
E. ictaluri 93-146-2_ Intestine	45,683,010	31,736,549	69.47%	609.03
E. ictaluri 93-146-3_ Intestine	75,702,290	56,959,652	75.24%	1,142.60
E. ictaluri 93-146-4_ Intestine	40,437,438	27,769,545	68.67%	509.13
E. ictaluri 93-146-5_ Intestine	39,823,754	29,325,056	73.64%	579.65
*Ei*Δ*evpB*-1_ Intestine	38,044,716	24,887,125	65.42%	500.68
*Ei*Δ*evpB*-2_ Intestine	58,802,032	43,581,714	74.12%	850.70
*Ei*Δ*evpB*-3_ Intestine	44,883,892	34,385,012	76.61%	692.75
*Ei*Δ*evpB*-4_ Intestine	63,414,018	46,859,895	73.90%	924.02
*Ei*Δ*evpB*-5_ Intestine	34,250,780	23,576,448	68.83%	438.78
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*-1_ Intestine	36,863,108	23,690,876	64.27%	424.53
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*-2_ Intestine	45,586,492	30,120,786	66.07%	548.81
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*-3_ Intestine	39,122,606	25,941,499	66.31%	473.71
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*-4_ Intestine	49,074,294	35,298,697	71.93%	688.25
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*-5_ Intestine	45,711,532	32,799,639	71.75%	639.09

Our results showed that the number of RNA-seq reads were mapped to different regions of *E. ictaluri* genome, in which ~42% of RNA-seq reads were mapped to open reading frames (ORFs), ~3% of reads mapped to Ribosomal RNA (rRNA), ~3% of reads

mapped to non-coding RNA (ncRNAs), ~1% of reads mapped to transfer RNA (tRNA), and ~51% of reads mapped to intergenic regions on the genome (Figure 3.2).

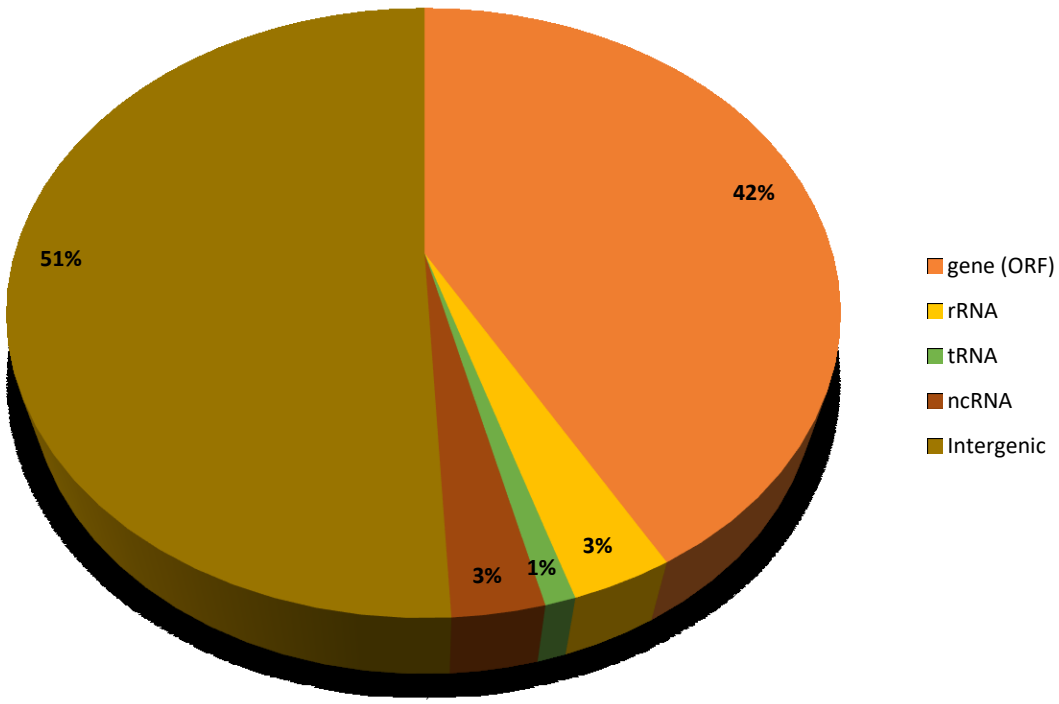


Figure 3.2 RNA-Seq reads distribution that mapped to different locations on the E. ictaluri genome.

Furthermore, the global gene expression profiles in wild-type *E. ictaluri* of intestine samples compared to BHI samples identified 238 genes that were significantly differentially expressed with a FDR > 0.05 and fold change > 1.5 or < -1.5; 191 genes were upregulated and 47 genes were downregulated (Figure 3.3, Appendix A). Comparing wild-type *E. ictaluri* intestine samples to *Ei*Δ*evpB* intestine samples identified 190 genes were significantly differentially expressed (FDR > 0.05, FC > 1.5 or < -1.5); 84 genes were upregulated and 106 genes were downregulated (Figure 3.3, Appendix B). While the comparison between wild-type *E. ictaluri* intestine samples to

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* intestine samples identified 235 genes significantly differentially expressed (FDR > 0.05, FC > 1.5 or < -1.5); 65 genes were upregulated, and 170 genes were downregulated (Figure 3.3, Appendix C). About 50 of the upregulated genes and 83 of the downregulated genes were found to be shared *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains, while no up or downregulated genes were shared in comparison between wild-type *E. ictaluri* to the vaccine strains suggesting that wild-type *E. ictaluri* differ in their action properties than vaccine strains during invasion and colonization of catfish intestine.

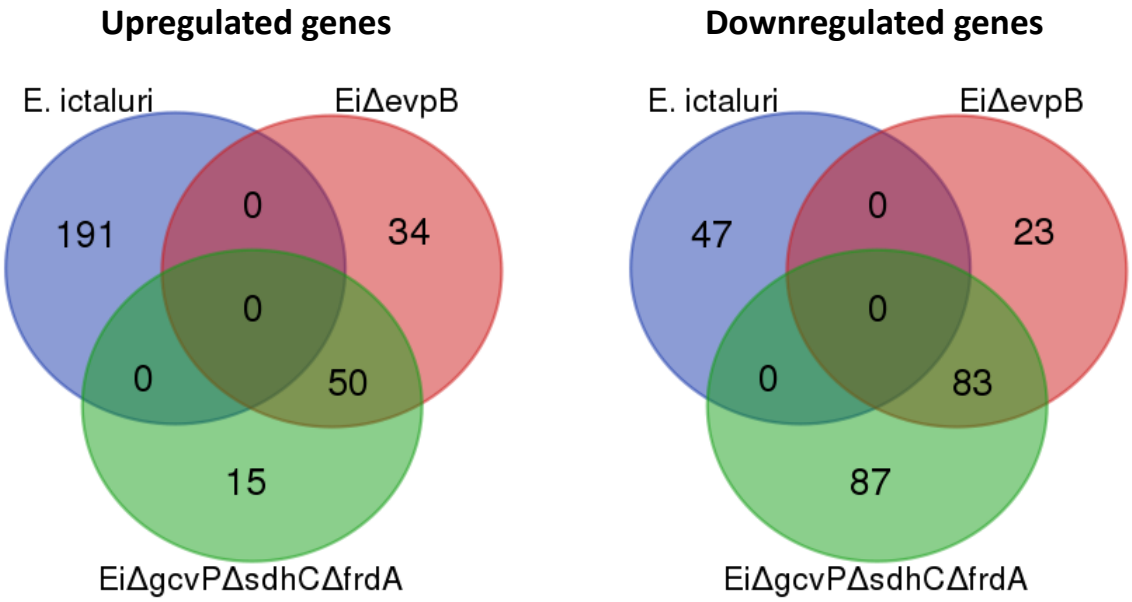


Figure 3.3 Venn diagram of upregulated and downregulated genes in wild-type *E. ictaluri* compared to *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains.

Gene Analogy (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis of differentially expressed genes (DEGs)

The GO annotations and KEGG pathways analysis of DEGs were determined using Cytoscape software v3.2.1 (Shannon et al., 2003). The GO enrichment analysis with a $P < 0.05$ showed that among 238 DEGs of wild-type *E. ictaluri*, 64 (27.9%), 29 (12.1%), and 16 (6.7%) genes were involved in 12 GO terms of biological process, 10 GO terms of molecular function, and 5 GO terms of cellular component categories respectively (Figure 3.4, Appendix D). Also, among 190 DEGs of *Ei*Δ*evpB* strain, 43 (22.6%), 28 (14.7%), and 19 (10%) genes were involved in 14 GO terms of biological process, 6 GO terms of molecular function and 3 GO terms of cellular component categories respectively (Figure 3.5, Appendix E). Finally, among the 235 DEGs of *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain, 42 (17.9%), 32 (13.6%), and 9 (3.8%) genes were involved in 12 GO terms of biological process, 6 GO terms of molecular function and 4 GO terms of cellular component categories respectively (Figure 3.6, Appendix F).

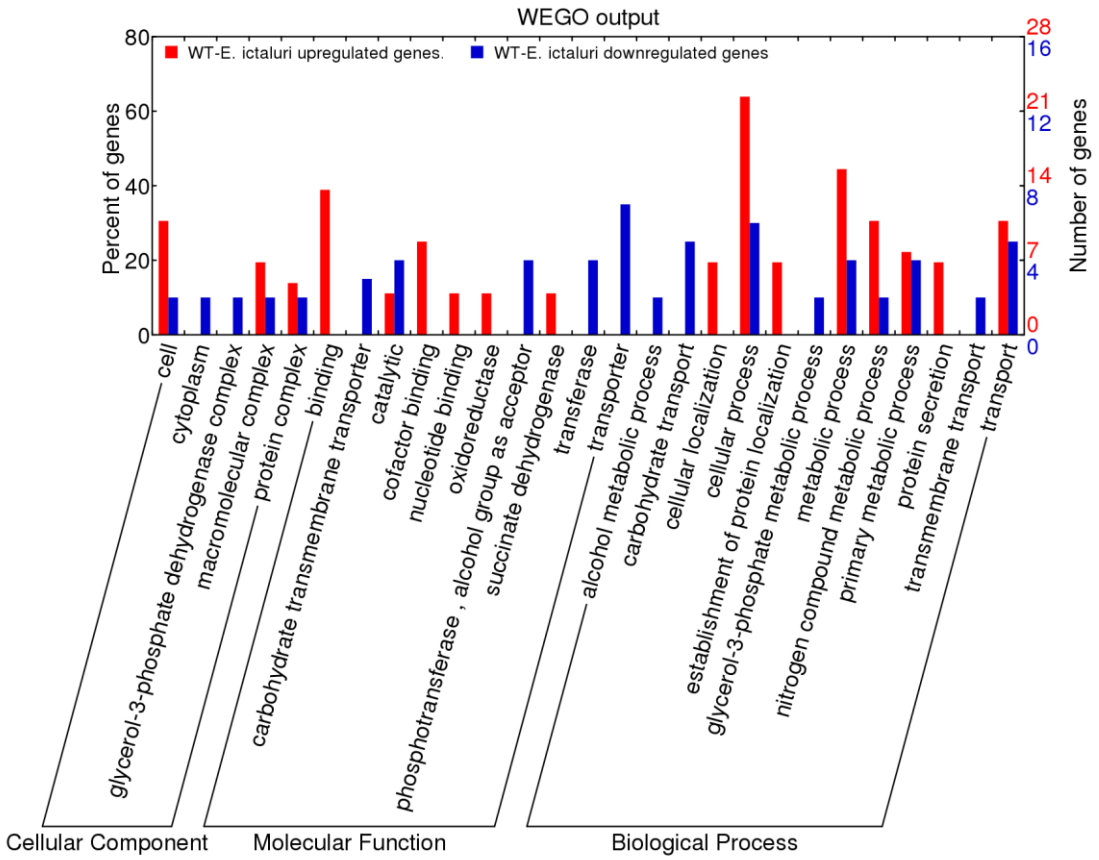


Figure 3.4 GO annotations of differentially expressed genes (DEGs) of wild-type *E. ictaluri* following invasion of catfish intestine.

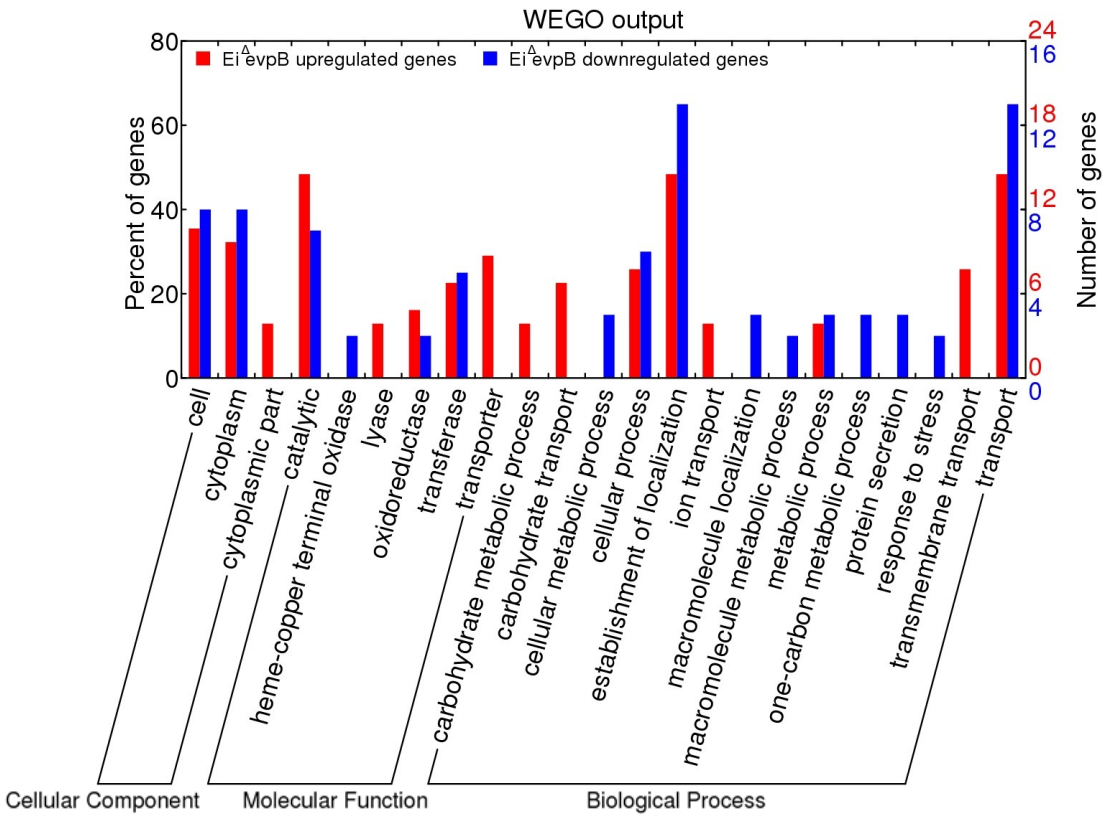


Figure 3.5 GO annotations of differentially expressed genes (DEGs) of *Ei*Δ*evpB* strain following the invasion of catfish intestine.

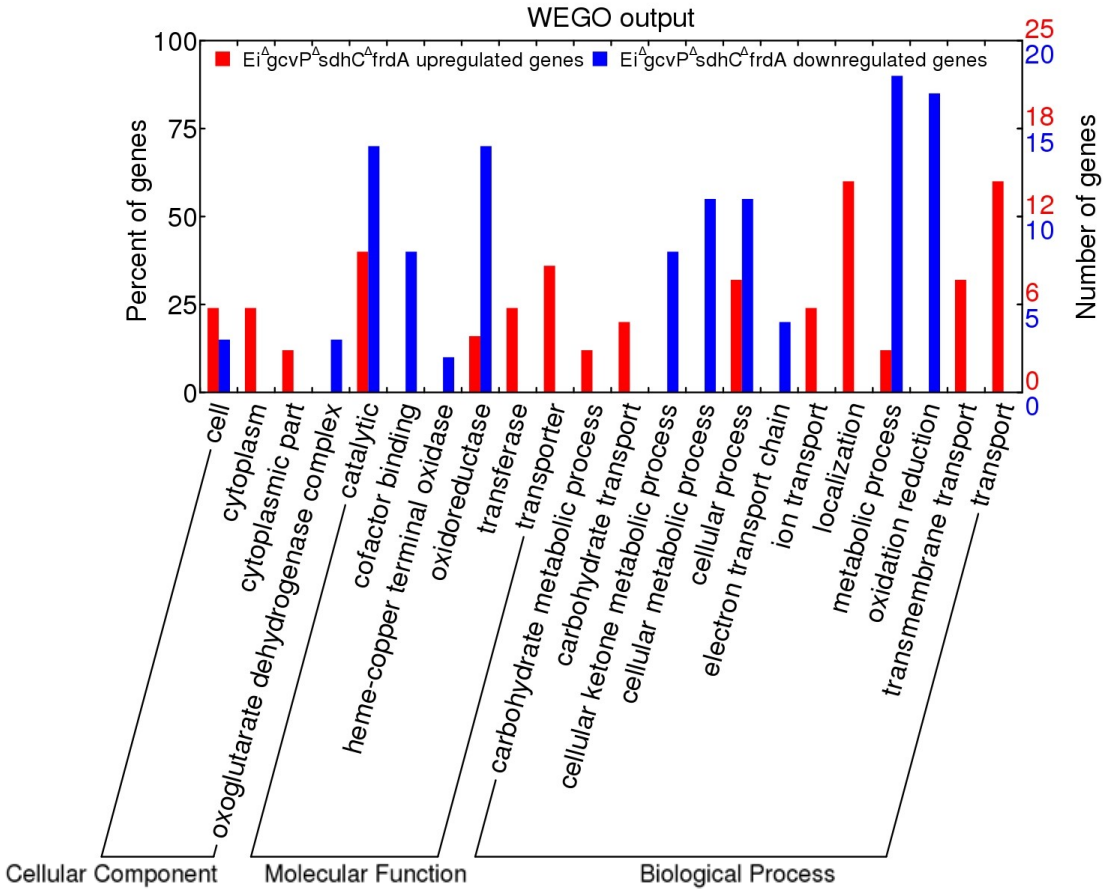


Figure 3.6 GO annotations of differentially expressed genes (DEGs) of *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain following the invasion of catfish intestine.

Furthermore, the significantly enriched KEGG pathways enrichment analysis with a $P < 0.05$ determined that DEGs of wild-type *E. ictaluri* were enriched to 9 KEGG pathways as the details for these pathways are shown in (Figure 3.7, Appendix G). Also, the DEGs of *Ei*Δ*evpB* strain were enriched to 8 KEGG pathways (Figure 3.8, Appendix H). While the DEGs of *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain were enriched in 13 KEGG pathways (Figure 3.9, Appendix I).

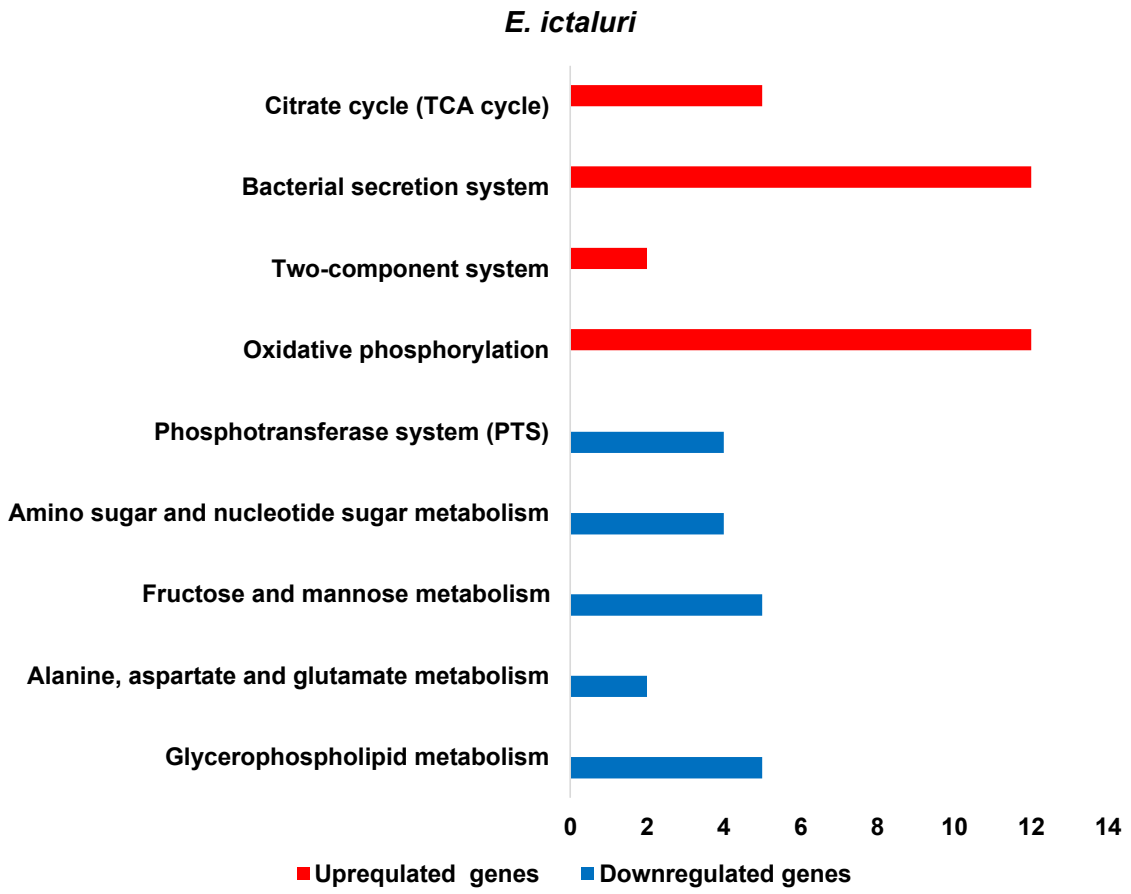


Figure 3.7 KEGG pathway analysis of differentially expressed genes (DEGs) of wild-type *E. ictaluri* following the invasion of catfish intestine.

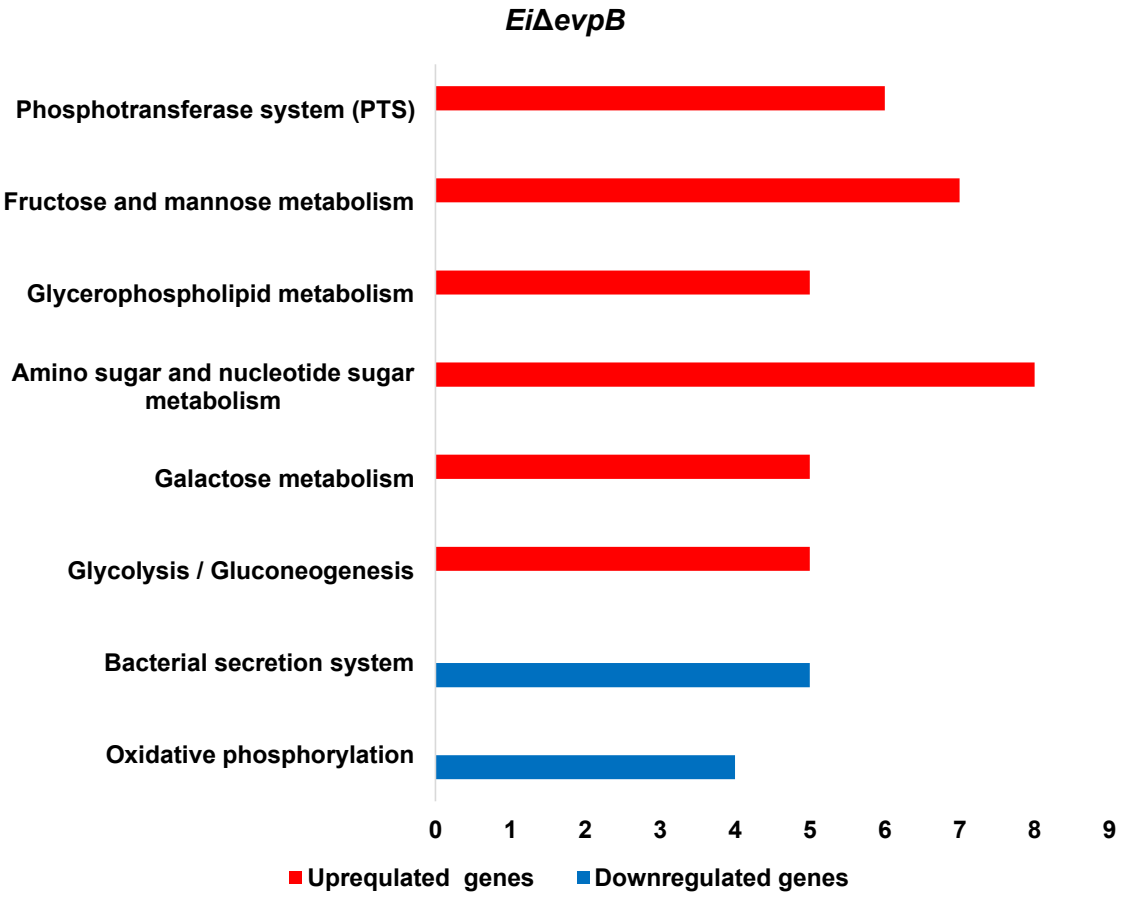


Figure 3.8 KEGG pathway analysis of differentially expressed genes (DEGs) of *Ei*Δ*evpB* strain following the invasion of catfish intestine.

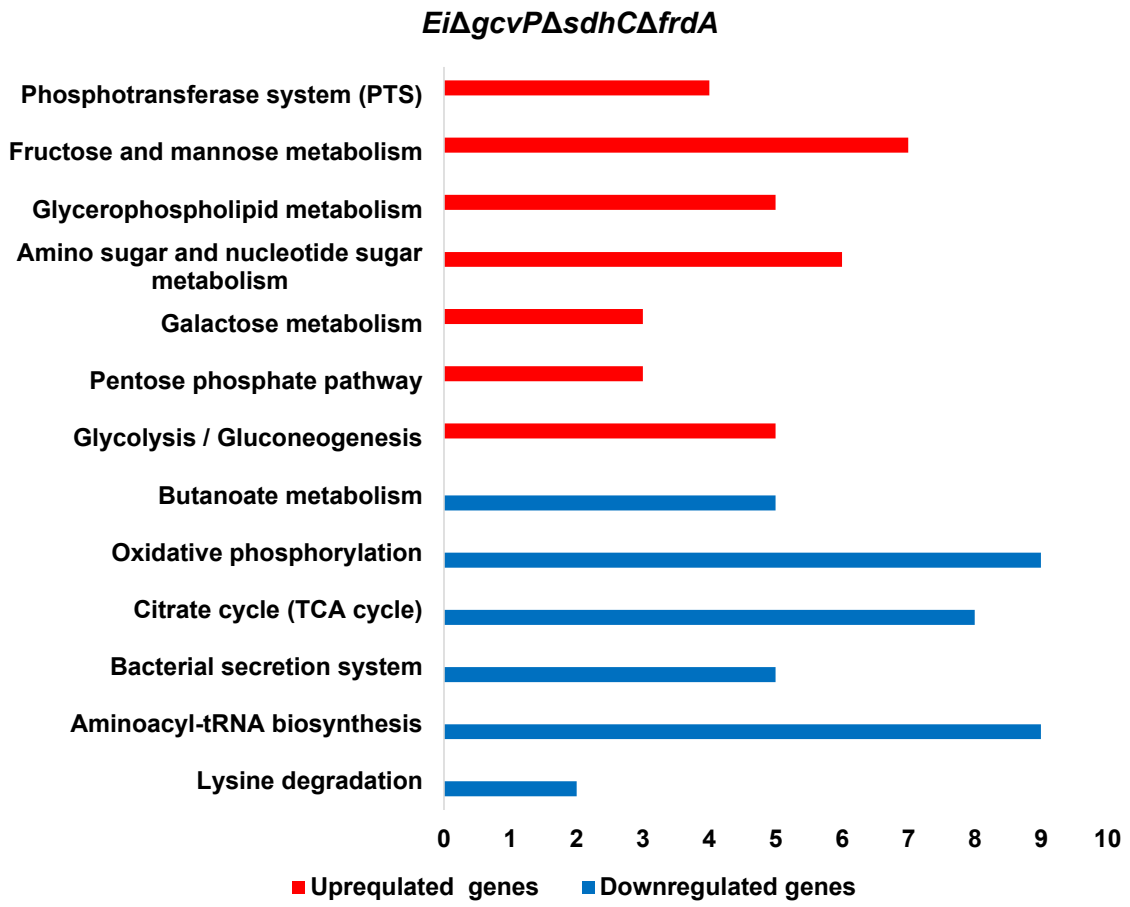


Figure 3.9 KEGG pathway analysis of differentially expressed genes (DEGs) of *EiΔgcvPΔsdhCΔfrdA* strain following the invasion of catfish intestine.

qPCR validation of differentially expressed genes

To verify the reliability of the DEGs results identified by RNA-Seq, we performed qPCR analysis on our selected 10 DEGs, which include 6 upregulated genes (*EvpA*, *EvpB*, *EvpH*, *EsaE*, *EsaH*, and *EsaJ*) and 4 downregulated genes (*AspA*, *GlpQ*, *PrkA*, and *GlgS*). Our results as shown in (Figure 3.10) confirm the consistency of the fold-change values for RNA-Seq data and the values for qPCR analysis in all our selected genes.

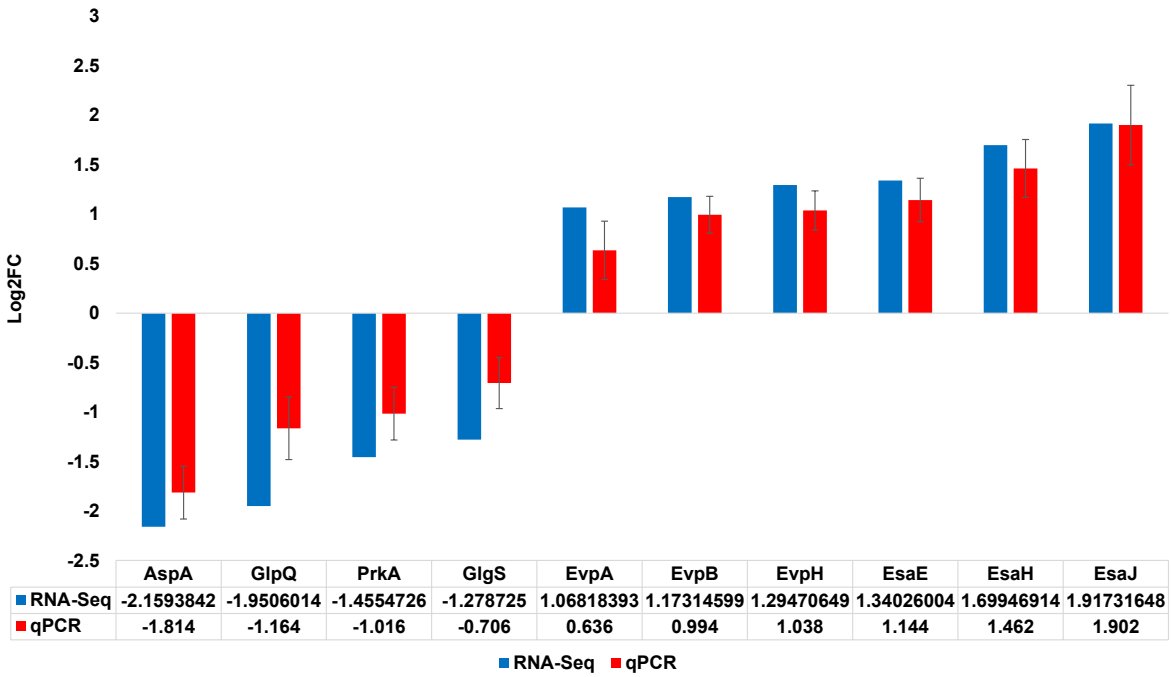


Figure 3.10 Validation of RNA-Seq data by qPCR analysis.

The qPCRs analysis included five biological replicates and repeated in triplicate runs (technical replicates).

Discussion

The purpose of this study was to identify the global gene expression profiles of *E. ictaluri* during catfish intestine invasion and investigate their possible role in pathogenesis. Invasive pathogenic bacteria such as *E. ictaluri* have many virulence mechanisms that are responsible for penetrating the host and evade killing. Therefore, in this study, we focus on *E. ictaluri* virulence mechanisms during catfish intestine adaptation and its infection pathways. We also determined the gene expression profiles of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains during catfish intestinal invasion for their evaluation as safe and essential vaccine candidates for improvement of the US catfish industry. Such a comprehensive transcriptomic study will pave the way to prevent

gastroenteritis in catfish. However, in recent years, transcriptome expression profiles analysis has been proved to be an efficient and advanced approach to capturing the molecular mechanisms of host-pathogen interactions in various studies (Cattoir et al., 2013; Song et al., 2017; Taveirne et al., 2013). Therefore, studying global gene expression profiles of *E. ictaluri* will provide useful information for development of new treatment for bacterial infections and prevent future outbreaks. This work represents, the first RNA-Seq based study to determine the *E. ictaluri* genes that are responsible for invasion and colonization of catfish intestine.

In the previous study, *E. ictaluri* wild-type and vaccine candidate strains showed apparent differences in their virulence in catfish intestine as significant tissue damages and strong immune responses were induced by wild-type *E. ictaluri*, while our vaccine strains were able to stimulate moderate level of immune responses without causing any significant damage to the intestinal tissue (Chapter II). In line with this finding, gene expression profiles in wild-type *E. ictaluri* was also different than that of vaccine candidate strains as shown in Figure 3.3. Surprisingly, no mutual genes were shared between the wild-type *E. ictaluri* and the vaccine candidates whereas some genes were found simultaneously in the vaccine candidate strains. Further analysis of gene expression profiles of wild-type *E. ictaluri* revealed that the upregulated genes were belonging to the bacterial secretion system and ATP production process, indicating their critical role in virulence of *E. ictaluri* during catfish intestinal invasion among other virulence factors. Indeed, our vaccine strains, which have deletion in *evpB* gene of type VI secretion system or deletion in *gcvP, sdhC,* and *frdA* genes of tricarboxylic acid cycle

(TCA), attenuated *E. ictaluri* virulence in catfish in this study (Chapter II) or our previous research, and thus, their gene expression profiles confirmed downregulation of bacterial secretion system and ATP production process pathways.

RNA-Seq data analysis showed the prominent upregulation of gene expressions related to type III secretion system in wild-type *E. ictaluri*, indicating the importance role of this system as a mechanism of virulence utilized to gain entry and replicate inside epithelial cells and gastrointestinal tissue of catfish. Type III secretion system is a complex protein machine and trigger effector protein from bacterial cytoplasm to the host cell cytosol, which contribute to bacterial virulence, intracellular replication, and survival inside the host cell (Dubytska et al., 2016). Twenty three genes encoding type III secretion system were upregulated in wild-type *E. ictaluri* during catfish intestinal invasion including the two-component regulatory system; sensor kinase *EsrA* (1.7 FC); and the response regulator *EsrB* (1.6 FC). It has been previously reported that type III secretion system genes in *E. ictaluri* are regulated through the EsrA/EsrB two-component regulatory system (Rogge and Thune, 2011). These proteins are homologous the component regulatory systems EsrA/EsrB of *E. tarda* (Tan et al., 2005), and SsrA/SsrB of *Salmonella* species (Cirillo et al., 1998), and responsible for their type III secretion system regulation as well. In addition to EsrA/EsrB two-component regulators, type III secretion system in *E. ictaluri* and *E. tarda* encode *EsrC* regulator (Tan et al., 2005; Thune et al., 2007). However, it has been proved that the expression of *EsrC* regulator in *E. tarda* induces the expression of its type VI secretion system genes (Zheng and Leung, 2007; Zheng et al., 2005). In this study, relatively high expression of *EsrC* (3.0 FC) and

eleven genes of type VI secretion system in *E. ictaluri* were observed, suggesting that the regulation of type VI secretion system genes in *E. ictaluri* may also be induced by the expression of *EsrC* regulator. Type VI secretion system is virulence-associated nano-machine and structurally similar to phage tail and has very important role in bacterial replication within the host cytosol and spread to the neighboring cells (Wong et al., 2015).

Both type III and type VI secretion systems play essential roles in bacterial internalization, intracellular replication, and spreading the infection further to adjacent epithelial cells of the host (Okuda et al., 2008; Wang et al., 2009a). In line with our finding, the upregulation of genes encoded type III and type VI secretion systems in wild-type *E. ictaluri* during catfish intestinal invasion suggests their essential role in establishing the typical process of causing gastroenteritis in catfish. The ability to cause gastroenteritis by enteric pathogens is the first step in progression to cause more severe diseases in the host. Bacterial secretion system is an active system in *E. ictaluri* and requires energy to transport the substrate from the bacteria to the host cytosol. Therefore, the upregulation of genes related to ATP production process in wild-type *E. ictaluri* is necessary to provide adequate energy required for utilizing these systems to invade and colonize catfish intestine. To survive in the fish intestinal epithelium, *E. ictaluri* not only need defense mechanism against host immune system but also maintaining its replication. The gene expression profiles of our vaccine candidates commonly indicated that the genes related to bacterial secretion system and ATP production were downregulated. However, the carbohydrate metabolic processes become active. Phosphotransferase

system transports sugars into the bacterial cell, and bacteria utilize various carbon sources to generate energy (Deutscher et al., 2006). The highly-upregulated genes related to the phosphotransferase system and sugar metabolism in the vaccine candidate strains support that the metabolism of the bacteria was more likely toward to the growth and survival rather than invasion of the host. Therefore, our vaccine candidates have strong phenotype to trigger an effective immune response in catfish intestine with less virulence.

Furthermore, wild-type *E. ictaluri* expresses genes that may have an important role in colonization and adaptation in the catfish intestinal environment, such as *YecC, LptB, TcaB, EmrE*, and *MarA*. The proteins encoded by those genes function as efflux pumps, which transports antimicrobial or toxic substances to the outside of the bacterial cell and renders high resistance to antimicrobial compounds (Putman et al., 2000). Bacterial efflux pump system is complex machinery, known as membrane-bound multidrug resistance (MDR) efflux pumps, and plays a role in response to stress, bacterial colonization, virulence and survival in the hosts (Li and Nikaido, 2009; Martinez et al., 2009). The most interesting function of the bacterial efflux pumps is its role in resistance to antimicrobial compounds, such as bile in the intestine (Ruiz et al., 2013). Bile is considered as one of the main antibacterial components that present in the intestine (Santander et al., 2014). It has been shown that deletion of genes encoded efflux pumps system conferred sensitivity to bile and certain antibiotics in Lactobacillus, Campylobacter, and Bifidobacterium species (Gueimonde et al., 2009; Lin et al., 2003; Pfeiler and Klaenhammer, 2009; Ruiz et al., 2013). In general, fish continuously secretes a wide range of antimicrobial substances including bile as an innate defense mechanism

against many pathogenic bacteria (Bly and Clem, 1991; Brogden, 2005). In this study, MDR efflux pumps genes were significantly upregulated in wild-type *E. ictaluri* during catfish intestine invasion include *YecC* (2 FC), *LptB* (1.6 FC), *TcaB* (3.8 FC), *EmrE* (1.7 FC), and *MarA* (1.6 FC). It has been reported that *YecC* and *LptB* belong to ATP-binding cassette transporters which confer multidrug resistance in bacteria by APT hydrolysis (Moussatova et al., 2008). Also, the major facilitator superfamily gene *TcaB* has been proved in many pathogens to mediate antibiotic resistance (Fernandez and Hancock, 2012). *EmrE* is also well known to be involved in resistance to a variety of antimicrobial compounds in many bacterial pathogens such as *Pseudomonas aeruginosa, Escherichia coli, and Bacillus subtilis* (Masaoka et al., 2000; Minato et al., 2008; Putman et al., 2000). Finally, It has been shown that the expression of *MarA* is responsible for the multidrug resistance in *E. coli* (Gambino et al., 1993). Our finding indicates that *E. ictaluri* may utilize the efflux pumps system as virulence mechanism against the antimicrobial molecules secreted by catfish intestine. Interestingly, *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains showed downregulation in expression of the genes encoding efflux pump systems, which is in line with our finding above describing that alteration in carbohydrate metabolism affect the virulence mechanism of *E. ictaluri*. In the future, the role of multidrug efflux pump system in *E. ictaluri* need to be characterized. Further studies on the functions of these genes may provide more insights to understand the pathogenesis of *E. ictaluri* in catfish.

In summary, RNA-Seq data analysis showed clear differences in global gene expression profiles of wild-type *E. ictaluri* and vaccine strains suggesting that wild-type

E. ictaluri strongly differ in their pathogenic properties compared to our vaccine strains. *E. ictaluri* rapidly regulates their gene expressions in response to diverse environmental conditions. This work provides key insights into the genes and functions involved in the adaptation and colonization of the catfish intestinal environment.

CHAPTER IV

CONCLUSION

The channel catfish farming is the most significant contributor to aquacultural production in the United States Catfish growers in the United States produced more than $386 million worth of catfish, of which $179 million (50.2%) was produced in Mississippi (USDA., 2017). Catfish farmers reported that more than 37% at the cost of $60 million annual loss due to enteric septicemia of catfish (ESC) (USDA, 2011). Although antibiotics are used against ESC, and a commercial live attenuated vaccine is available, the disease still a major threat to the catfish industry. Live attenuated vaccines are considered the best prospect for protection against diseases by stimulating host's immune responses. Successful live attenuated vaccines must achieve a precise balance between lack of pathogenicity and provide a protective level of immunity.

Based on previous research, vaccination of catfish fry and fingerlings with *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains provided complete protection against wild-type *E. ictaluri*. The primary aim of this study was to investigate catfish immune responses to *E. ictaluri* and determine essential genes of *E. ictaluri* during the intestinal invasion. The secondary aim of this study was to evaluate the pathogenicity and immunogenicity of two patent live attenuated *E. ictaluri* vaccine strains, *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* in catfish intestine. *Ei*Δ*evpB* was constructed by an in-frame

deletion of the major component of *E. ictaluri* type VI secretion system (*evpB)*. *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* was constructed by an in-frame deletion of the *E. ictaluri* tricarboxylic acid cycle (TCA) and one-carbon (C1) metabolism protein *(gcvP, sdhC, frdA).*

In the present study, intestinal ligated loops were constructed surgically in live catfish and loops were injected with wild-type *E. ictaluri* and the live attenuated vaccine strains (*Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA*). Our findings demonstrated that wild-type *E. ictaluri* and attenuated vaccine strains showed apparent differences in their virulence in catfish intestine. Histopathological examination showed a massive expansion of lamina propria with congested and dilated blood vessels and intestinal inflammation were observed in intestinal tissue injected with *E. ictaluri* wild-type. On the other hand, no significant tissue damage was detected in intestinal tissue exposed to *Ei*Δ*evpB* strain, whereas, *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strain causes mild pathological lesions represented by congestion in the lamina propria, but no inflammation was observed. Also, immunohistochemical staining revealed a moderate level of neutrophil and B cell infiltration in the vaccine injected intestinal tissue compared to that of wild-type injected intestinal tissue. Furthermore, real-time PCR analysis showed that catfish intestinal tissue exposed to the vaccine strains induced significantly lower expression of TNF-α, CD4-1, and CD8-α compared to that of wild-type *E. ictaluri* exposed tissue.

Moreover, the global gene expression profiles of wild-type *E. ictaluri* revealed the prominent expression of genes related to bacterial secretion systems, ATP production processes, and multidrug resistance (MDR) efflux pumps, which are needed for the

invasion and colonization of catfish intestine. In contrast, the global gene expression profiles of vaccine strains showed the prominent expression of genes related to the phosphotransferase system and sugar metabolism processes, which are needed for the bacterial growth and survival in catfish intestine rather than invasion of the host.

All these data are linked to each other and demonstrate that wild-type *E. ictaluri* acts as a potent antigen by over stimulating catfish immune signaling, leading to early detection of B cell and T cell in the catfish intestine, and exhibiting a high degree of pathogenicity. In contrast, live attenuated vaccine strains were able to stimulate protective levels of immune responses in catfish without causing severe damage to the host. Therefore, our study suggests that *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* strains have the potential for use as a live attenuated vaccines for catfish industry. With successful commercialization of *Ei*Δ*evpB* and *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* vaccines, we expected to significantly lower the impact of *E. ictaluri* mortality on fish farms, as well as reduce antibiotics use and more sustainable aquaculture.

REFERENCES

Abdallah, A.M., Gey van Pittius, N.C., Champion, P.A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C.M., Appelmelk, B.J., Bitter, W., 2007. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol 5, 883-891.

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L., Scapigliati, G., 1997. Immunohistochemistry of gut-associated lymphoid tissue of the sea bassDicentrarchus labrax(L.). Fish & Shellfish Immunology 7, 235-245.

Abernathy, J.W., Lu, J., Liu, H., Kucuktas, H., Liu, Z., 2009. Molecular characterization of complement factor I reveals constitutive expression in channel catfish. Fish Shellfish Immunol 27, 529-534.

Ainsworth, A.J., Chen, D.X., 1990. Differences in the phagocytosis of four bacteria by channel catfish neutrophils. Dev Comp Immunol 14, 201-209.

Baldwin, T.J., Newton, J.C., 1993. Pathogenesis of Enteric Septicemia of Channel Catfish, Caused by Edwardsiella ictaluri: Bacteriologic and Light and Electron Microscopic Findings. Journal of Aquatic Animal Health 5, 189-198.

Bao, B., Peatman, E., Xu, P., Li, P., Zeng, H., He, C., Liu, Z., 2006. The catfish liver-expressed antimicrobial peptide 2 (LEAP-2) gene is expressed in a wide range of tissues and developmentally regulated. Mol Immunol 43, 367-377.

Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P., Benmansour, A., 2006. Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. J Immunol 176, 3942-3949.

Bertolini, J.M., Cipriano, R.C., Pyle, S.W., McLaughlin, J.J., 1990. Serological investigation of the fish pathogen Edwardsiella ictaluri, cause of enteric septicemia of catfish. J Wildl Dis 26, 246-252.

Bilodeau-Bourgeois, L., Bosworth, B.G., Peterson, B.C., 2008. Differences in mortality, growth, lysozyme, and toll-like receptor gene expression among genetic groups of catfish exposed to virulent Edwardsiella ictaluri. Fish Shellfish Immunol 24, 82-89.

Bilodeau, A.L., Peterson, B.C., Bosworth, B.G., 2006. Response of toll-like receptors, lysozyme, and IGF-I in back-cross hybrid (F1 male (blue x channel) x female channel) catfish challenged with virulent Edwardsiella ictaluri. Fish Shellfish Immunol 20, 29-39.

Bilodeau, A.L., Waldbieser, G.C., 2005. Activation of TLR3 and TLR5 in channel catfish exposed to virulent Edwardsiella ictaluri. Dev Comp Immunol 29, 713-721.

Bly, J.E., Clem, L.W., 1991. Temperature-mediated processes in teleost immunity: in vitro immunosuppression induced by in vivo low temperature in channel catfish. Vet Immunol Immunopathol 28, 365-377.

Boardman, T., Warner, C., Ramirez-Gomez, F., Matrisciano, J., Bromage, E., 2012. Characterization of an anti-rainbow trout (Oncorhynchus mykiss) CD3epsilon monoclonal antibody. Vet Immunol Immunopathol 145, 511-515.

Booth, N.J., Beekman, J.B., Thune, R.L., 2009. Edwardsiella ictaluri encodes an acid-activated urease that is required for intracellular replication in channel catfish (Ictalurus punctatus) macrophages. Appl Environ Microbiol 75, 6712-6720.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol 20, 239-262.

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3, 238-250.

Bromage, E.S., Kaattari, I.M., Zwollo, P., Kaattari, S.L., 2004. Plasmablast and plasma cell production and distribution in trout immune tissues. J Immunol 173, 7317-7323.

C.J. Lobb, L.W.C., 1982. Fish lymphocytes differ in the expression of surface immunoglobulin. Dev. Comp. Immunol.

Cain, K.D., Jones, D.R., Raison, R.L., 2000. Characterisation of mucosal and systemic immune responses in rainbow trout (Oncorhynchus mykiss) using surface plasmon resonance. Fish & Shellfish Immunology 10, 651-666.

Cambronne, E.D., Roy, C.R., 2006. Recognition and delivery of effector proteins into eukaryotic cells by bacterial secretion systems. Traffic 7, 929-939.

Camejo, A., Buchrieser, C., Couve, E., Carvalho, F., Reis, O., Ferreira, P., Sousa, S., Cossart, P., Cabanes, D., 2009. In vivo transcriptional profiling of Listeria monocytogenes and mutagenesis identify new virulence factors involved in infection. PLoS Pathog 5, e1000449.

Carneiro, L.A., Magalhaes, J.G., Tattoli, I., Philpott, D.J., Travassos, L.H., 2008. Nod-like proteins in inflammation and disease. J Pathol 214, 136-148.

Castro, R., Bernard, D., Lefranc, M.P., Six, A., Benmansour, A., Boudinot, P., 2011. T cell diversity and TcR repertoires in teleost fish. Fish Shellfish Immunol 31, 644-654.

Cattoir, V., Narasimhan, G., Skurnik, D., Aschard, H., Roux, D., Ramphal, R., Jyot, J., Lory, S., 2013. Transcriptional response of mucoid Pseudomonas aeruginosa to human respiratory mucus. MBio 3, e00410-00412.

Chen, K., Xu, W., Wilson, M., He, B., Miller, N.W., Bengten, E., Edholm, E.S., Santini, P.A., Rath, P., Chiu, A., Cattalini, M., Litzman, J., J, B.B., Huang, B., Meini, A., Riesbeck, K., Cunningham-Rundles, C., Plebani, A., Cerutti, A., 2009. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. Nat Immunol 10, 889-898.

Chen, L., He, C., Baoprasertkul, P., Xu, P., Li, P., Serapion, J., Waldbieser, G., Wolters, W., Liu, Z., 2005. Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with Edwardsiella ictaluri. Dev Comp Immunol 29, 135-142.

Chen, Y., Chou, K., Fuchs, E., Havran, W.L., Boismenu, R., 2002. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A 99, 14338-14343.

Cirillo, D.M., Valdivia, R.H., Monack, D.M., Falkow, S., 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol Microbiol 30, 175-188.

Companjen, A.R., Florack, D.E.A., Slootweg, T., Borst, J.W., Rombout, J.H.W.M., 2006. Improved uptake of plant-derived LTB-linked proteins in carp gut and induction of specific humoral immune responses upon infeed delivery. Fish & Shellfish Immunology 21, 251-260.

Dahal, N., Abdelhamed, H., Karsi, A., Lawrence, M.L., 2014a. Tissue persistence and vaccine efficacy of tricarboxylic acid cycle and one-carbon metabolism mutant strains of Edwardsiella ictaluri. Vaccine 32, 3971-3976.

Dahal, N., Abdelhamed, H., Lu, J., Karsi, A., Lawrence, M.L., 2013. Tricarboxylic acid cycle and one-carbon metabolism pathways are important in Edwardsiella ictaluri virulence. PLoS One 8, e65973.

Dahal, N., Abdelhamed, H., Lu, J., Karsi, A., Lawrence, M.L., 2014b. Effect of multiple mutations in tricarboxylic acid cycle and one-carbon metabolism pathways on Edwardsiella ictaluri pathogenesis. Vet Microbiol 169, 107-112.

Danilova, N., Bussmann, J., Jekosch, K., Steiner, L.A., 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat Immunol 6, 295-302.

Deutscher, J., Francke, C., Postma, P.W., 2006. How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. Microbiology and Molecular Biology Reviews 70, 939-1031.

Douglas, S.E., Gallant, J.W., Liebscher, R.S., Dacanay, A., Tsoi, S.C., 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev Comp Immunol 27, 589-601.

Dubytska, L.P., Rogge, M.L., Thune, R.L., 2016. Identification and Characterization of Putative Translocated Effector Proteins of the Edwardsiella ictaluri Type III Secretion System. mSphere 1.

Dugar, G., Herbig, A., Forstner, K.U., Heidrich, N., Reinhardt, R., Nieselt, K., Sharma, C.M., 2013. High-resolution transcriptome maps reveal strain-specific regulatory features of multiple Campylobacter jejuni isolates. PLoS Genet 9, e1003495.

Edholm, E.-S., Bengten, E., Wilson, M., 2011. Insights into the function of IgD. Developmental & Comparative Immunology 35, 1309-1316.

Edholm, E.S., Bengten, E., Stafford, J.L., Sahoo, M., Taylor, E.B., Miller, N.W., Wilson, M., 2010. Identification of two IgD+ B cell populations in channel catfish, Ictalurus punctatus. J Immunol 185, 4082-4094.

Edholm, E.S., Stafford, J.L., Quiniou, S.M., Waldbieser, G., Miller, N.W., Bengten, E., Wilson, M., 2007. Channel catfish, Ictalurus punctatus, CD4-like molecules. Dev Comp Immunol 31, 172-187.

Fahrer, A.M., Konigshofer, Y., Kerr, E.M., Ghandour, G., Mack, D.H., Davis, M.M., Chien, Y.H., 2001. Attributes of gammadelta intraepithelial lymphocytes as suggested by their transcriptional profile. Proc Natl Acad Sci U S A 98, 10261-10266.

Fernandez, L., Hancock, R.E., 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25, 661-681.

Filloux, A., 2009. The type VI secretion system: a tubular story. EMBO J 28, 309-310.

Fletcher, T.C., White, A., 1972. Antibody production in the plaice (Pleuronectes platessa L.) after oral and parenteral immunization with Vibrio anguillarum antigens. Aquaculture 1, 417-428.

Gambino, L., Gracheck, S.J., Miller, P.F., 1993. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in Escherichia coli. Journal of Bacteriology 175, 2888-2894.

Gao, L., He, C., Liu, X., Su, H., Gao, X., Li, Y., Liu, W., 2012. The innate immune-related genes in catfish. Int J Mol Sci 13, 14172-14202.

Ge, Q., Zhao, Y., 2013. Evolution of thymus organogenesis. Dev Comp Immunol 39, 85-90.

Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol 35, 1729-1739.

Griffin, B.R., Mitchell, A.J., 2007. Susceptibility of channel catfish, Ictalurus punctatus (Rafinesque), to Edwardsiella ictaluri challenge following copper sulphate exposure. J Fish Dis 30, 581-585.

Gueimonde, M., Garrigues, C., van Sinderen, D., de los Reyes-Gavilan, C.G., Margolles, A., 2009. Bile-inducible efflux transporter from Bifidobacterium longum NCC2705, conferring bile resistance. Appl Environ Microbiol 75, 3153-3160.

Guttman, J.A., Finlay, B.B., 2009. Tight junctions as targets of infectious agents. Biochim Biophys Acta 1788, 832-841.

Hansen, J.D., Landis, E.D., Phillips, R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc Natl Acad Sci U S A 102, 6919-6924.

Hawke, J.P., 1979. A Bacterium Associated with Disease of Pond Cultured Channel Catfish, Ictalurus punctatus. Journal of the Fisheries Research Board of Canada 36, 1508-1512.

HAWKE, J.P., McWHORTER, A.C., STEIGERWALT, A.G., BRENNER, D.J., 1981. Edwardsiella ictaluri sp. nov., the Causative Agent of Enteric Septicemia of Catfish. International Journal of Systematic and Evolutionary Microbiology 31, 396-400.

Hebert, P., Ainsworth, A.J., Boyd, B., 2002. Histological enzyme and flow cytometric analysis of channel catfish intestinal tract immune cells. Dev Comp Immunol 26, 53-62.

Holland, M.C., Lambris, J.D., 2002. The complement system in teleosts. Fish Shellfish Immunol 12, 399-420.

Hood, R.D., Singh, P., Hsu, F., Guvener, T., Carl, M.A., Trinidad, R.R., Silverman, J.M., Ohlson, B.B., Hicks, K.G., Plemel, R.L., Li, M., Schwarz, S., Wang, W.Y., Merz, A.J., Goodlett, D.R., Mougous, J.D., 2010. A type VI secretion system of Pseudomonas aeruginosa targets a toxin to bacteria. Cell Host Microbe 7, 25-37.

Huising, M.O., Stet, R.J., Savelkoul, H.F., Verburg-van Kemenade, B.M., 2004. The molecular evolution of the interleukin-1 family of cytokines; IL-18 in teleost fish. Dev Comp Immunol 28, 395-413.

Jault, C., Pichon, L., Chluba, J., 2004. Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol Immunol 40, 759-771.

Jima, D.D., Shah, R.N., Orcutt, T.M., Joshi, D., Law, J.M., Litman, G.W., Trede, N.S., Yoder, J.A., 2009. Enhanced transcription of complement and coagulation genes in the absence of adaptive immunity. Mol Immunol 46, 1505-1516.

Karsi, A., Gulsoy, N., Corb, E., Dumpala, P.R., Lawrence, M.L., 2009. High-throughput bioluminescence-based mutant screening strategy for identification of bacterial virulence genes. Appl Environ Microbiol 75, 2166-2175.

Karsi, A., Lawrence, M.L., 2007. Broad host range fluorescence and bioluminescence expression vectors for Gram-negative bacteria. Plasmid 57, 286-295.

Karsi, A., Menanteau-Ledouble, S., Lawrence, M.L., 2006. Development of bioluminescent Edwardsiella ictaluri for noninvasive disease monitoring. FEMS Microbiol Lett 260, 216-223.

Karsi, A., Waldbieser, G.C., Small, B.C., Liu, Z., Wolters, W.R., 2004. Molecular cloning of proopiomelanocortin cDNA and multi-tissue mRNA expression in channel catfish. Gen Comp Endocrinol 137, 312-321.

Kordon, A.O., Scott, M.A., Ibrahim, I., Abdelhamed, H., Ahmed, H., Baumgartner, W., Karsi, A., Pinchuk, L.M., 2016. Identification of Langerhans-like cells in the immunocompetent tissues of channel catfish, Ictalurus punctatus. Fish Shellfish Immunol 58, 253-258.

Lawrence, M.L., Banes, M.M., Azadi, P., Reeks, B.Y., 2003. The Edwardsiella ictaluri O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. Microbiology 149, 1409-1421.

Lawrence, M.L., Cooper, R.K., Thune, R.L., 1997. Attenuation, persistence, and vaccine potential of an Edwardsiella ictaluri purA mutant. Infect Immun 65, 4642-4651.

Li, C., Zhang, Y., Wang, R., Lu, J., Nandi, S., Mohanty, S., Terhune, J., Liu, Z., Peatman, E., 2012. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following Edwardsiella ictaluri infection in channel catfish, Ictalurus punctatus. Fish Shellfish Immunol 32, 816-827.

Li, J., Barreda, D.R., Zhang, Y.A., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat Immunol 7, 1116-1124.

Li, X.Z., Nikaido, H., 2009. Efflux-mediated drug resistance in bacteria: an update. Drugs 69, 1555-1623.

Lin, J., Sahin, O., Michel, L.O., Zhang, Q., 2003. Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of Campylobacter jejuni. Infect Immun 71, 4250-4259.

Lobb, C.J., Rhoades, M., 1987. Rapid plasmid analysis for identification of Edwardsiella ictaluri from infected channel catfish (Ictalurus punctatus). Appl Environ Microbiol 53, 1267-1272.

Locksley, R.M., Killeen, N., Lenardo, M.J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104, 487-501.

Mandlik, A., Livny, J., Robins, W.P., Ritchie, J.M., Mekalanos, J.J., Waldor, M.K., 2011. RNA-Seq-based monitoring of infection-linked changes in Vibrio cholerae gene expression. Cell Host Microbe 10, 165-174.

Martinez, J.L., Sanchez, M.B., Martinez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., Alvarez-Ortega, C., 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev 33, 430-449.

Masaoka, Y., Ueno, Y., Morita, Y., Kuroda, T., Mizushima, T., Tsuchiya, T., 2000. A two-component multidrug efflux pump, EbrAB, in Bacillus subtilis. J Bacteriol 182, 2307-2310.

Medzhitov, R., Janeway, C., Jr., 2000. Innate immune recognition: mechanisms and pathways. Immunol Rev 173, 89-97.

Miller, N.W., Deuter, A., Clem, L.W., 1986. Phylogeny of lymphocyte heterogeneity: the cellular requirements for the mixed leucocyte reaction with channel catfish. Immunology 59, 123-128.

Minato, Y., Shahcheraghi, F., Ogawa, W., Kuroda, T., Tsuchiya, T., 2008. Functional gene cloning and characterization of the SsmE multidrug efflux pump from Serratia marcescens. Biol Pharm Bull 31, 516-519.

Miyazaki, T., Plumb, J.A., 1985. Histopathology of Edwardsiella ictaluri in channel catfish, Ictalurus punctatus (Rafinesque)*. Journal of Fish Diseases 8, 389-392.

Mollnes, T.E., Song, W.C., Lambris, J.D., 2002. Complement in inflammatory tissue damage and disease. Trends Immunol 23, 61-64.

Moussatova, A., Kandt, C., O'Mara, M.L., Tieleman, D.P., 2008. ATP-binding cassette transporters in Escherichia coli. Biochim Biophys Acta 1778, 1757-1771.

N.W. Miller, J.E.B., F. van Ginkel, C.F. Ellsaesser, L.W. Clem, 1987. Phylogeny of lymphocyte heterogeneity: identification and separation of functionally distinct subpopulations of channel catfish lymphocytes with monoclonal antibodies.

Nakanishi, T., Shibasaki, Y., Matsuura, Y., 2015. T Cells in Fish. Biology 4, 640-663.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M., 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Dev Comp Immunol 25, 807-825.

Newton, J.C., Bird, R.C., Blevins, W.T., Wilt, G.R., Wolfe, L.G., 1988. Isolation, characterization, and molecular cloning of cryptic plasmids isolated from Edwardsiella ictaluri. Am J Vet Res 49, 1856-1860.

Newton, J.C., Blevins, W.T., Wilt, G.R., Wolfe, L.G., 1990. Outer membrane protein profiles of Edwardsiella ictaluri from fish. Am J Vet Res 51, 211-215.

Newton, J.C., Wolfe, L.G., Grizzle, J.M., Plumb, J.A., 1989. Pathology of experimental enteric septicaemia in channel catfish, Ictalurus punctatus (Rafinesque), following immersion-exposure to Edwardsiella ictaluri. Journal of Fish Diseases 12, 335-347.

O'Hara, J.R., Buret, A.G., 2008. Mechanisms of intestinal tight junctional disruption during infection. Front Biosci 13, 7008-7021.

Okuda, J., Kiriyama, M., Yamanoi, E., Nakai, T., 2008. The type III secretion system-dependent repression of NF-kappaB activation to the intracellular growth of Edwardsiella tarda in human epithelial cells. FEMS Microbiol Lett 283, 9-14.

Ourth, D.D., Chung, K.T., 2004. Purification of antimicrobial factor from granules of channel catfish peripheral blood leucocytes. Biochem Biophys Res Commun 313, 28-36.

Peatman, E., Lange, M., Zhao, H., Beck, B.H., 2015. Physiology and immunology of mucosal barriers in catfish (Ictalurus spp.). Tissue Barriers 3, e1068907.

Peterson, B.C., Bosworth, B.G., Bilodeau, A.L., 2005. Differential gene expression of IGF-I, IGF-II, and toll-like receptors 3 and 5 during embryogenesis in hybrid (channel x blue) and channel catfish. Comp Biochem Physiol A Mol Integr Physiol 141, 42-47.

Pfeiler, E.A., Klaenhammer, T.R., 2009. Role of transporter proteins in bile tolerance of Lactobacillus acidophilus. Appl Environ Microbiol 75, 6013-6016.

Picchietti, S., Guerra, L., Bertoni, F., Randelli, E., Belardinelli, M.C., Buonocore, F., Fausto, A.M., Rombout, J.H., Scapigliati, G., Abelli, L., 2011a. Intestinal T cells of Dicentrarchus labrax (L.): gene expression and functional studies. Fish Shellfish Immunol 30, 609-617.

Picchietti, S., Guerra, L., Bertoni, F., Randelli, E., Belardinelli, M.C., Buonocore, F., Fausto, A.M., Rombout, J.H., Scapigliati, G., Abelli, L., 2011b. Intestinal T cells of Dicentrarchus labrax (L.): Gene expression and functional studies. Fish & Shellfish Immunology 30, 609-617.

Plumb, J.A., Chappell, J., 1978. Susceptibility of blue catfish to channel catfish virus. Proc Annu Conf Southeast Assoc Fish Wildl Agen 32.

Pridgeon, J.W., Russo, R., Shoemaker, C.A., Klesius, P.H., 2010. Expression profiles of toll-like receptors in anterior kidney of channel catfish, Ictalurus punctatus (Rafinesque), acutely infected by Edwardsiella ictaluri. J Fish Dis 33, 497-505.

Putman, M., van Veen, H.W., Konings, W.N., 2000. Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev 64, 672-693.

Quiniou, S.M.A., Sahoo, M., Edholm, E.-S., Bengten, E., Wilson, M., 2011. Channel catfish CD8α and CD8β co-receptors: Characterization, expression and polymorphism. Fish & Shellfish Immunology 30, 894-901.

Rajendran, K.V., Zhang, J., Liu, S., Kucuktas, H., Wang, X., Liu, H., Sha, Z., Terhune, J., Peatman, E., Liu, Z., 2012a. Pathogen recognition receptors in channel catfish: I. Identification, phylogeny and expression of NOD-like receptors. Dev Comp Immunol 37, 77-86.

Rajendran, K.V., Zhang, J., Liu, S., Peatman, E., Kucuktas, H., Wang, X., Liu, H., Wood, T., Terhune, J., Liu, Z., 2012b. Pathogen recognition receptors in channel catfish: II. Identification, phylogeny and expression of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Dev Comp Immunol 37, 381-389.

Rezzani, R., Nardo, L., Favero, G., Peroni, M., Rodella, L.F., 2014. Thymus and aging: morphological, radiological, and functional overview. Age (Dordr) 36, 313-351.

Robertsen, B., 2006. The interferon system of teleost fish. Fish Shellfish Immunol 20, 172-191.

Rogge, M.L., Thune, R.L., 2011. Regulation of the Edwardsiella ictaluri type III secretion system by pH and phosphate concentration through EsrA, EsrB, and EsrC. Appl Environ Microbiol 77, 4293-4302.

Rombout, J.H., Abelli, L., Picchietti, S., Scapigliati, G., Kiron, V., 2011. Teleost intestinal immunology. Fish Shellfish Immunol 31, 616-626.

Rombout, J.H., Joosten, P.H., Engelsma, M.Y., Vos, A.P., Taverne, N., Taverne-Thiele, J.J., 1998. Indications for a distinct putative T cell population in mucosal tissue of carp (Cyprinus carpio L.). Dev Comp Immunol 22, 63-77.

Rombout, J.W., Blok, L.J., Lamers, C.H., Egberts, E., 1986. Immunization of carp (Cyprinus carpio) with a Vibrio anguillarum bacterin: indications for a common mucosal immune system. Dev Comp Immunol 10, 341-351.

Rosenstiel, P., Jacobs, G., Till, A., Schreiber, S., 2008. NOD-like receptors: ancient sentinels of the innate immune system. Cell Mol Life Sci 65, 1361-1377.

Ruiz, L., Margolles, A., Sanchez, B., 2013. Bile resistance mechanisms in Lactobacillus and Bifidobacterium. Front Microbiol 4, 396.

Saito, T., Hatada, M., Iwanaga, S., Kawabata, S., 1997. A newly identified horseshoe crab lectin with binding specificity to O-antigen of bacterial lipopolysaccharides. J Biol Chem 272, 30703-30708.

Salinas, I., 2015. The Mucosal Immune System of Teleost Fish. Biology (Basel) 4, 525-539.

Samuel, C.E., 2001. Antiviral actions of interferons. Clin Microbiol Rev 14, 778-809, table of contents.

Santander, J., Kilbourne, J., Park, J.Y., Martin, T., Loh, A., Diaz, I., Rojas, R., Segovia, C., DeNardo, D., Curtiss, R., 3rd, 2014. Inflammatory effects of Edwardsiella ictaluri lipopolysaccharide modifications in catfish gut. Infect Immun 82, 3394-3404.

Santander, J., Martin, T., Loh, A., Pohlenz, C., Gatlin, D.M., 3rd, Curtiss, R., 3rd, 2013. Mechanisms of intrinsic resistance to antimicrobial peptides of Edwardsiella ictaluri and its influence on fish gut inflammation and virulence. Microbiology 159, 1471-1486.

Santos, R.L., Zhang, S., Tsolis, R.M., Baumler, A.J., Adams, L.G., 2002. Morphologic and molecular characterization of Salmonella typhimurium infection in neonatal calves. Vet Pathol 39, 200-215.

Schorpp, M., Bialecki, M., Diekhoff, D., Walderich, B., Odenthal, J., Maischein, H.M., Zapata, A.G., Boehm, T., 2006. Conserved functions of Ikaros in vertebrate lymphocyte development: genetic evidence for distinct larval and adult phases of T cell development and two lineages of B cells in zebrafish. J Immunol 177, 2463-2476.

Seppola, M., Larsen, A.N., Steiro, K., Robertsen, B., Jensen, I., 2008. Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). Mol Immunol 45, 887-897.

Sha, Z., Abernathy, J.W., Wang, S., Li, P., Kucuktas, H., Liu, H., Peatman, E., Liu, Z., 2009. NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish. Dev Comp Immunol 33, 991-999.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13, 2498-2504.

Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermuller, J., Reinhardt, R., Stadler, P.F., Vogel, J., 2010. The primary transcriptome of the major human pathogen Helicobacter pylori. Nature 464, 250-255.

Shotts, E.B., Blazer, V.S., Waltman, W.D., 1986. Pathogenesis of Experimental Edwardsiella ictaluri Infections in Channel Catfish (Icta lurus punctatus). Canadian Journal of Fisheries and Aquatic Sciences 43, 36-42.

Skirpstunas, R.T., Baldwin, T.J., 2002. Edwardsiella ictaluri invasion of IEC-6, Henle 407, fathead minnow and channel catfish enteric epithelial cells. Dis Aquat Organ 51, 161-167.

Solem, S.T., Stenvik, J., 2006. Antibody repertoire development in teleosts--a review with emphasis on salmonids and Gadus morhua L. Dev Comp Immunol 30, 57-76.

Song, X., Hu, X., Sun, B., Bo, Y., Wu, K., Xiao, L., Gong, C., 2017. A transcriptome analysis focusing on inflammation-related genes of grass carp intestines following infection with Aeromonas hydrophila. Sci Rep 7, 40777.

Stanley, L.A., Hudson, J.S., Schwedler, T.E., Hayasaka, S.S., 1994. Extracellular Products Associated with Virulent and Avirulent Strains of Edwardsiella ictaluri from Channel Catfish. Journal of Aquatic Animal Health 6, 36-43.

Sunyer, J.O., 2012. Evolutionary and functional relationships of B cells from fish and mammals: insights into their novel roles in phagocytosis and presentation of particulate antigen. Infect Disord Drug Targets 12, 200-212.

Swain, S.L., McKinstry, K.K., Strutt, T.M., 2012. Expanding roles for CD4(+) T cells in immunity to viruses. Nat Rev Immunol 12, 136-148.

Takeuchi, O., Akira, S., 2008. MDA5/RIG-I and virus recognition. Curr Opin Immunol 20, 17-22.

Tan, Y.P., Zheng, J., Tung, S.L., Rosenshine, I., Leung, K.Y., 2005. Role of type III secretion in Edwardsiella tarda virulence. Microbiology 151, 2301-2313.

Tasumi, S., Ohira, T., Kawazoe, I., Suetake, H., Suzuki, Y., Aida, K., 2002. Primary structure and characteristics of a lectin from skin mucus of the Japanese eel Anguilla japonica. J Biol Chem 277, 27305-27311.

Tateno, H., Ogawa, T., Muramoto, K., Kamiya, H., Saneyoshi, M., 2002. Rhamnose-binding lectins from steelhead trout (Oncorhynchus mykiss) eggs recognize bacterial lipopolysaccharides and lipoteichoic acid. Biosci Biotechnol Biochem 66, 604-612.

Taveirne, M.E., Theriot, C.M., Livny, J., DiRita, V.J., 2013. The complete Campylobacter jejuni transcriptome during colonization of a natural host determined by RNAseq. PLoS One 8, e73586.

Thune, R.L., Fernandez, D.H., Benoit, J.L., Kelly-Smith, M., Rogge, M.L., Booth, N.J., Landry, C.A., Bologna, R.A., 2007. Signature-tagged mutagenesis of Edwardsiella ictaluri identifies virulence-related genes, including a salmonella pathogenicity island 2 class of type III secretion systems. Appl Environ Microbiol 73, 7934-7946.

Turner, J.M., Brodsky, M.H., Irving, B.A., Levin, S.D., Perlmutter, R.M., Littman, D.R., 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell 60, 755-765.

USDA, 2011. Catfish production, by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA). 2011.

USDA, 2014 Catfish production, by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA). 2011.

USDA, 2017. Catfish production, by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA).

Vasta, G.R., Nita-Lazar, M., Giomarelli, B., Ahmed, H., Du, S., Cammarata, M., Parrinello, N., Bianchet, M.A., Amzel, L.M., 2011. Structural and functional diversity of the lectin repertoire in teleost fish: relevance to innate and adaptive immunity. Dev Comp Immunol 35, 1388-1399.

Vinitnantharat, S., Plumb, J.A., Brown, A.E., 1993. Isolation and purification of an outer membrane protein of Edwardsiella ictaluri and its antigenicity to channel catfish (Ictalurus punctatus). Fish & Shellfish Immunology 3, 401-409.

Wallis, T.S., Hawker, R.J., Candy, D.C., Qi, G.M., Clarke, G.J., Worton, K.J., Osborne, M.P., Stephen, J., 1989. Quantification of the leucocyte influx into rabbit ileal loops induced by strains of Salmonella typhimurium of different virulence. J Med Microbiol 30, 149-156.

Wang, X., Wang, Q., Xiao, J., Liu, Q., Wu, H., Xu, L., Zhang, Y., 2009a. Edwardsiella tarda T6SS component evpP is regulated by esrB and iron, and plays essential roles in the invasion of fish. Fish Shellfish Immunol 27, 469-477.

Wang, Y., Wang, Q., Baoprasertkul, P., Peatman, E., Liu, Z., 2006. Genomic organization, gene duplication, and expression analysis of interleukin-1beta in channel catfish (Ictalurus punctatus). Mol Immunol 43, 1653-1664.

Wang, Z., Gerstein, M., Snyder, M., 2009b. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10, 57-63.

Weete, J.D., Blevins, W.T., Chitrakorn, S., Saeed, M.O., Plumb, J.A., 1988. Chemical characterization of lipopolysaccharide from Edwardsiella ictaluri, a fish pathogen. Can J Microbiol 34, 1224-1229.

Whyte, S.K., 2007. The innate immune response of finfish--a review of current knowledge. Fish Shellfish Immunol 23, 1127-1151.

Williams, M.L., Gillaspy, A.F., Dyer, D.W., Thune, R.L., Waldbieser, G.C., Schuster, S.C., Gipson, J., Zaitshik, J., Landry, C., Banes, M.M., Lawrence, M.L., 2012. Genome sequence of Edwardsiella ictaluri 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish. J Bacteriol 194, 740-741.

Williams, M.L., Lawrence, M.L., 2005. Identification and characterization of a two-component hemolysin from Edwardsiella ictaluri. Vet Microbiol 108, 281-289.

Williams, M.L., Waldbieser, G.C., Dyer, D.W., Gillaspy, A.F., Lawrence, M.L., 2008. Characterization of the rrn operons in the channel catfish pathogen Edwardsiella ictaluri. J Appl Microbiol 104, 1790-1796.

Wilson, M.R., Zhou, H., Bengten, E., Clem, L.W., Stuge, T.B., Warr, G.W., Miller, N.W., 1998. T-cell receptors in channel catfish: structure and expression of TCR alpha and beta genes. Mol Immunol 35, 545-557.

Wolfe, K.G., Plumb, J.A., Morrison, E.E., 1998. Lectin Binding Characteristics of the Olfactory Mucosa of Channel Catfish: Potential Factors in Attachment of Edwardsiella ictaluri. Journal of Aquatic Animal Health 10, 348-360.

Wong, J., Chen, Y., Gan, Y.H., 2015. Host Cytosolic Glutathione Sensing by a Membrane Histidine Kinase Activates the Type VI Secretion System in an Intracellular Bacterium. Cell Host Microbe 18, 38-48.

Yeh, H.Y., Klesius, P.H., 2007. Molecular cloning and expression of channel catfish, Ictalurus punctatus, complement membrane attack complex inhibitor CD59. Vet Immunol Immunopathol 120, 246-253.

Zheng, J., Leung, K.Y., 2007. Dissection of a type VI secretion system in Edwardsiella tarda. Mol Microbiol 66, 1192-1206.

Zheng, J., Tung, S.L., Leung, K.Y., 2005. Regulation of a type III and a putative secretion system in Edwardsiella tarda by EsrC is under the control of a two-component system, EsrA-EsrB. Infect Immun 73, 4127-4137.

Zhou, H., Stuge, T.B., Miller, N.W., Bengten, E., Naftel, J.P., Bernanke, J.M., Chinchar, V.G., Clem, L.W., Wilson, M., 2001. Heterogeneity of channel catfish CTL with respect to target recognition and cytotoxic mechanisms employed. J Immunol 167, 1325-1332.

Zhou, J.G., Wei, J.G., Xu, D., Cui, H.C., Yan, Y., Ou-Yang, Z.L., Huang, X.H., Huang, Y.H., Qin, Q.W., 2011. Molecular cloning and characterization of two novel hepcidins from orange-spotted grouper, Epinephelus coioides. Fish Shellfish Immunol 30, 559-568.

Zhu, L.Y., Nie, L., Zhu, G., Xiang, L.X., Shao, J.Z., 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Dev Comp Immunol 39, 39-62.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev Comp Immunol 35, 1376-1387.

Zou, J., Secombes, C.J., Long, S., Miller, N., Clem, L.W., Chinchar, V.G., 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). Dev Comp Immunol 27, 845-858.

Zwollo, P., Cole, S., Bromage, E., Kaattari, S., 2005. B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J Immunol 174, 6608-6616.

APPENDIX A

COMPLETE LIST OF DEGs IN WILD-TYPE *E. ICTALURI* DURING INVASION OF CATFISH INTESTINE

Gene	FC	FDR	Product
NT01EI_0936	3.78	0.00	type III secretion apparatus lipoprotein, YscJ/HrcJ family, putative
NT01EI_2114	3.75	0.00	drug resistance transporter, Bcr/CflA subfamily
NT01EI_0935	3.68	0.00	hypothetical protein
NT01EI_2450	3.61	0.00	cold-shock DNA-binding domain protein
NT01EI_1374	3.49	0.00	hypothetical protein
NT01EI_2741	3.26	0.00	type VI secretion system lysozyme-related protein
NT01EI_0938	3.25	0.00	type III secretion system protein, SsaH family
NT01EI_1375	3.24	0.00	hypothetical protein
NT01EI_0934	3.09	0.00	hypothetical protein
NT01EI_2740	3.09	0.00	hypothetical protein
NT01EI_0940	3.06	0.00	transcriptional regulator, AraC family
NT01EI_0946	3.00	0.00	type III secretion system chaperone protein, putative
NT01EI_2448	2.99	0.00	Protein of unknown function (DUF343)
NT01EI_2394	2.97	0.00	AMP-binding enzyme
NT01EI_0709	2.95	0.00	hypothetical protein
NT01EI_2739	2.87	0.01	Protein of unknown function (DUF796)
NT01EI_1519	2.79	0.00	hypothetical protein
NT01EI_2742	2.74	0.00	type VI secretion protein, VC_A0110 family
NT01EI_1373	2.69	0.00	hypothetical protein
NT01EI_2536	2.68	0.00	hypothetical protein
NT01EI_0941	2.53	0.00	type III secretion system protein, YseE family
NT01EI_1633	2.52	0.00	hypothetical protein
NT01EI_3313	2.51	0.00	hypothetical protein
NT01EI_3021	2.51	0.00	Protein of unknown function (DUF465)
NT01EI_2746	2.50	0.00	PAAR motif protein
NT01EI_0945	2.45	0.01	hypothetical protein
NT01EI_2744	2.45	0.00	type VI secretion ATPase, ClpV1 family, putative
NT01EI_2743	2.45	0.00	type VI secretion protein, VC_A0111 family
NT01EI_0939	2.45	0.00	type III secretion apparatus needle protein, putative
NT01EI_1865	2.44	0.00	cold-shock DNA-binding domain protein
NT01EI_0937	2.42	0.00	type III secretion apparatus protein, YscI/HrpB, C-terminal domain protein
NT01EI_3398	2.41	0.00	Cyclopropane-fatty-acyl-phospholipid synthase
NT01EI_0942	2.40	0.00	type III secretion apparatus protein, YscD/HrpQ family, putative
NT01EI_1841	2.39	0.00	periplasmic peptide-binding protein, putative
NT01EI_2447	2.38	0.00	3-deoxy-D-manno-octulosonate cytidylyltransferase, putative
NT01EI_1679	2.37	0.00	YCII-related domain protein
NT01EI_0954	2.37	0.00	hypothetical protein
NT01EI_1245	2.35	0.00	hypothetical protein
NT01EI_2223	2.35	0.00	protoheme IX farnesyltransferase, putative
NT01EI_2224	2.35	0.00	cytochrome o ubiquinol oxidase subunit IV, putative

NT01EI_3900	2.33	0.00	aspartate-ammonia ligase, putative
NT01EI_0955	2.31	0.00	hypothetical protein
NT01EI_1396	2.27	0.00	Protein of unknown function (DUF307)
NT01EI_3539	2.27	0.00	hypothetical protein
NT01EI_2738	2.26	0.01	type VI secretion protein, EvpB/VC_A0108 family
NT01EI_0957	2.25	0.00	type III secretion ATPase FliI/YscN family, putative
NT01EI_1695	2.23	0.00	hypothetical protein
NT01EI_1696	2.20	0.00	amino acid permease family protein
NT01EI_2225	2.19	0.00	cytochrome o ubiquinol oxidase, subunit III, putative
NT01EI_1889	2.18	0.00	50S ribosomal protein L20, putative
NT01EI_2395	2.17	0.00	hypothetical protein
NT01EI_1075	2.17	0.00	putative lipoprotein
NT01EI_0933	2.16	0.00	type III secretion effector delivery regulator, TyeA family
NT01EI_1888	2.14	0.00	50S ribosomal protein L35, putative
NT01EI_1781	2.14	0.00	hypothetical protein
NT01EI_0956	2.13	0.00	hypothetical protein
NT01EI_1520	2.13	0.00	hypothetical protein
NT01EI_1266	2.12	0.00	Na+ dependent nucleoside transporter family protein
NT01EI_2679	2.12	0.00	NADH-quinone oxidoreductase subunit A, putative
NT01EI_2398	2.12	0.00	hypothetical protein
NT01EI_3595	2.10	0.01	30S ribosomal protein S10, putative
NT01EI_2737	2.10	0.02	type VI secretion protein, VC_A0107 family
NT01EI_2776	2.10	0.00	hypothetical protein
NT01EI_2490	2.09	0.00	translation initiation factor IF-1, putative
NT01EI_3491	2.09	0.00	cell shape determining protein, MreB
NT01EI_2898	2.08	0.00	flavodoxin, putative
NT01EI_1799	2.08	0.00	hypothetical protein
NT01EI_1560	2.05	0.00	hypothetical protein
NT01EI_1697	2.04	0.01	bacterial extracellular solute-binding protein, family 3
NT01EI_0943	2.03	0.01	type III secretion outer membrane pore, YscC/HrcC family, putative
NT01EI_2435	2.02	0.00	Bacterial protein of unknown function (DUF882)
NT01EI_3521	2.01	0.00	hypoxanthine oxidase XdhD, putative
NT01EI_2871	2.00	0.00	succinate dehydrogenase, hydrophobic membrane anchor protein, putative
NT01EI_1246	1.99	0.03	cysteine synthase A, putative
NT01EI_1244	1.98	0.00	phosphocarrier, HPr family
NT01EI_2399	1.98	0.00	hypothetical protein
NT01EI_3522	1.98	0.00	selenate reductase, FAD-binding subunit, putative
NT01EI_2745	1.95	0.00	type VI secretion system Vgr family protein
NT01EI_3222	1.95	0.00	16S rRNA processing protein RimM, putative
NT01EI_3935	1.94	0.01	50S ribosomal protein L34, putative
NT01EI_0963	1.93	0.00	type III secretion protein, YscU/HrpY family

NT01EI_3223	1.92	0.01	30S ribosomal protein S16, putative
NT01EI_2400	1.92	0.01	ABC transporter, ATP-binding protein, putative
NT01EI_3538	1.92	0.00	Protein of unknown function (DUF419)
NT01EI_2605	1.92	0.00	hypothetical protein
NT01EI_1959	1.92	0.00	Protein of unknown function (DUF1471)
NT01EI_1521	1.92	0.00	hypothetical protein
NT01EI_3523	1.91	0.01	selenium metabolism protein SsnA, putative
NT01EI_2703	1.90	0.00	phosphoenolpyruvate phosphomutase, putative
NT01EI_2757	1.90	0.00	phosphohistidine phosphatase SixA, putative
NT01EI_0089	1.88	0.01	transcription termination factor Rho, putative
NT01EI_2748	1.87	0.00	type VI secretion lipoprotein, VC_A0113 family
NT01EI_1056	1.86	0.00	riboflavin biosynthesis protein RibD, putative
NT01EI_2692	1.86	0.00	Protein of unknown function, DUF412
NT01EI_0962	1.86	0.00	type III secretion apparatus protein SpaR/YscT/HrcT, putative
NT01EI_2926	1.85	0.00	hypothetical protein
NT01EI_2494	1.85	0.00	Protein of unknown function (DUF535)
NT01EI_3197	1.85	0.00	Transglycosylase, SLT domain protein
NT01EI_3227	1.85	0.01	S-Ribosylhomocysteinase (LuxS)
NT01EI_2855	1.85	0.00	Tol-Pal system protein YbgF
NT01EI_1979	1.84	0.00	hypothetical protein
NT01EI_3520	1.83	0.00	xanthine/uracil permease family protein
NT01EI_3594	1.82	0.04	ribosomal protein L3
NT01EI_2678	1.81	0.00	NADH-quinone oxidoreductase, B subunit, putative
NT01EI_1732	1.81	0.01	transporter, dicarboxylate/amino acid:cation (Na+ or H+) symporter (DAACS) family
NT01EI_2865	1.81	0.02	succinyl-CoA synthetase (ADP forming), alpha subunit, putative
NT01EI_1055	1.80	0.00	transcriptional regulator NrdR, putative
NT01EI_1601	1.79	0.00	ATP-binding protein of zinc transporter, putative
NT01EI_0568	1.79	0.00	hypothetical protein
NT01EI_0958	1.79	0.04	type III secretion protein, HrcV family
NT01EI_3899	1.79	0.00	hypothetical protein
NT01EI_3532	1.78	0.01	diaminopropionate ammonia-lyase, putative
NT01EI_1708	1.78	0.00	hypothetical protein
NT01EI_1405	1.78	0.00	Uncharacterized protein family UPF0005
NT01EI_1753	1.77	0.00	hypothetical protein
NT01EI_2770	1.76	0.01	hypothetical protein
NT01EI_1113	1.76	0.01	hypothetical protein
NT01EI_0034	1.76	0.00	guanylate kinase, putative
NT01EI_3910	1.76	0.01	ATP synthase F1, alpha subunit, putative
NT01EI_1000	1.75	0.00	aspartate racemase, putative
NT01EI_2640	1.75	0.00	molybdenum cofactor biosynthesis protein A, putative
NT01EI_2949	1.75	0.00	twin arginine-targeting protein translocase, TatA/E family

NT01EI_3581	1.74	0.04	ribosomal protein S14p/S29e
NT01EI_1932	1.74	0.00	multidrug resistance protein, SMR family
NT01EI_2656	1.74	0.00	hydrogenase expression/formation protein, putative
NT01EI_3208	1.74	0.00	competence lipoprotein ComL
NT01EI_0552	1.74	0.00	DNA polymerase III, psi subunit, putative
NT01EI_3683	1.73	0.00	hypothetical protein
NT01EI_2869	1.72	0.03	succinate dehydrogenase iron-sulfur subunit, putative
NT01EI_3531	1.72	0.02	M20/DapE family protein YgeY, putative
NT01EI_0966	1.71	0.03	two component sensor/regulator EsrA, putative
NT01EI_0567	1.71	0.00	Nickel-dependent hydrogenases b-type cytochrome subunit
NT01EI_1634	1.70	0.00	hypothetical protein
NT01EI_2870	1.70	0.03	succinate dehydrogenase, flavoprotein subunit, putative
NT01EI_3221	1.69	0.01	tRNA (guanine-N1)-methyltransferase, putative
NT01EI_0569	1.69	0.00	phosphocarrier, HPr family protein
NT01EI_3023	1.69	0.01	CTP synthase, putative
NT01EI_2927	1.69	0.00	magnesium and cobalt efflux protein CorC, putative
NT01EI_0473	1.68	0.04	tetratricopeptide repeat protein
NT01EI_1784	1.68	0.00	hypothetical protein
NT01EI_0571	1.67	0.00	PTS IIA-like nitrogen-regulatory protein PtsN, putative
NT01EI_2172	1.67	0.00	Protein of unknown function (DUF453)
NT01EI_1054	1.67	0.01	hypothetical protein
NT01EI_2771	1.66	0.00	hypothetical protein
NT01EI_2950	1.66	0.00	hypothetical protein
NT01EI_3556	1.66	0.00	yrdC domain protein
NT01EI_0574	1.65	0.03	ABC transporter, ATP-binding protein
NT01EI_2747	1.65	0.02	type VI secretion-associated protein, ImpA family
NT01EI_0007	1.64	0.00	hypothetical protein
NT01EI_3492	1.64	0.00	cyclic diguanylate cyclase/phosphodiesterase domain protein
NT01EI_2872	1.64	0.00	succinate dehydrogenase, cytochrome b556 subunit, putative
NT01EI_0490	1.64	0.00	hypothetical protein
NT01EI_1771	1.64	0.00	hypothetical protein
NT01EI_1642	1.63	0.01	methionine-R-sulfoxide reductase, putative
NT01EI_2620	1.63	0.05	transcriptional regulator, AraC family
NT01EI_1935	1.62	0.00	hypothetical protein
NT01EI_1879	1.62	0.00	hypothetical protein with ferritin-like domain
NT01EI_2616	1.61	0.00	hypothetical protein
NT01EI_3519	1.61	0.02	guanine deaminase, putative
NT01EI_1576	1.61	0.00	D-isomer specific 2-hydroxyacid dehydrogenase family protein, NAD binding domain protein
NT01EI_2462	1.61	0.00	formate/nitrite transporter
NT01EI_3068	1.60	0.00	SsrA-binding protein, putative
NT01EI_2177	1.60	0.00	hypothetical protein

NT01EI_3912	1.60	0.04	ATP synthase F1, beta subunit , putative
NT01EI_3530	1.60	0.01	dihydropyrimidinase, putative
NT01EI_2280	1.59	0.00	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, putative
NT01EI_3841	1.59	0.02	pyridine nucleotide-disulfide oxidoreductase, putative
NT01EI_3112	1.59	0.00	cyanate transporter family protein
NT01EI_0849	1.59	0.01	beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ, putative
NT01EI_1773	1.58	0.00	hypothetical protein
NT01EI_2862	1.58	0.01	cyd operon protein YbgE, putative
NT01EI_1783	1.58	0.00	hypothetical protein
NT01EI_3907	1.58	0.01	ATP synthase F0, C subunit, putative
NT01EI_1749	1.57	0.03	hypothetical protein
NT01EI_0840	1.57	0.00	ribosome recycling factor, putative
NT01EI_0965	1.57	0.05	response regulator receiver domain protein
NT01EI_3467	1.56	0.01	hypothetical protein
NT01EI_2610	1.56	0.00	Protein of unknown function (DUF1414)
NT01EI_2171	1.56	0.01	3-isopropylmalate dehydratase small subunit 1, putative
NT01EI_0728	1.56	0.01	S-adenosyl-methyltransferase MraW, putative
NT01EI_0022	1.56	0.00	hypothetical protein
NT01EI_2756	1.55	0.00	Smr domain protein
NT01EI_1074	1.55	0.00	AmpG-related permease
NT01EI_2283	1.55	0.01	hypothetical protein
NT01EI_1933	1.54	0.00	putative Mg2+ transporter-C (MgtC) family
NT01EI_2353	1.53	0.00	GTP cyclohydrolase II, putative
NT01EI_0964	1.53	0.05	Transglycosylase SLT domain protein
NT01EI_0169	1.53	0.00	transcription termination/antitermination factor NusG, putative
NT01EI_1752	1.52	0.00	hypothetical protein
NT01EI_2442	1.52	0.00	hypothetical protein
NT01EI_0685	1.52	0.00	xanthine dehydrogenase, FAD-binding subunit
NT01EI_3861	-1.52	0.01	hypothetical protein
NT01EI_0688	-1.52	0.04	hypothetical protein
NT01EI_0791	-1.52	0.01	sugar fermentation stimulation protein, putative
NT01EI_3834	-1.56	0.00	outer membrane porin
NT01EI_1570	-1.57	0.04	protein of unknown function (DUF336)
NT01EI_1377	-1.57	0.00	Protein of unknown function (DUF1379)
NT01EI_0899	-1.58	0.01	Amino acid permease
NT01EI_0378	-1.59	0.00	FxsA cytoplasmic membrane protein
NT01EI_3466	-1.61	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit A, putative
NT01EI_0865	-1.65	0.04	hypothetical protein
NT01EI_2118	-1.67	0.01	cation efflux system protein CusF, putative
NT01EI_0216	-1.70	0.00	maltose-binding periplasmic protein precursor, MalE, putative
NT01EI_2311	-1.73	0.01	hypothetical protein

NT01EI_0376	-1.77	0.00	anaerobic C4-dicarboxylate transporter DcuA, putative
NT01EI_2308	-1.78	0.01	hypothetical protein
NT01EI_1636	-1.79	0.03	Protein of unknown function (DUF444)
NT01EI_3465	-1.80	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit B, putative
NT01EI_1720	-1.81	0.01	PTS system, mannose/fructose/sorbose family, IIC component
NT01EI_3848	-1.84	0.00	hypothetical protein
NT01EI_2107	-1.92	0.00	hypothetical protein
NT01EI_0516	-2.00	0.03	Protein unknown function (DUF388)
NT01EI_3789	-2.02	0.03	hypothetical protein
NT01EI_2460	-2.04	0.00	L-asparaginase, type 2, putative
NT01EI_1007	-2.10	0.00	Dihydrodipicolinate synthetase family protein
NT01EI_3468	-2.12	0.00	glycerol-3-phosphate transporter, putative
NT01EI_1393	-2.19	0.01	hypothetical protein
NT01EI_1721	-2.21	0.00	PTS system, mannose/fructose/sorbose family, IIB component
NT01EI_2591	-2.28	0.00	1-phosphofructokinase, putative
NT01EI_3679	-2.30	0.00	FAD dependent oxidoreductase, putative
NT01EI_2999	-2.32	0.00	hypothetical protein
NT01EI_1558	-2.35	0.00	hypothetical protein
NT01EI_1937	-2.43	0.01	Glycogen synthesis protein
NT01EI_0488	-2.63	0.00	hypothetical protein
NT01EI_1719	-2.66	0.00	PTS system, mannose/fructose/sorbose family, IID component
NT01EI_3464	-2.67	0.00	glycerol-3-phosphate dehydrogenase, anaerobic, C subunit
NT01EI_1663	-2.69	0.00	H-NS histone family protein
NT01EI_1637	-2.74	0.00	PrkA serine protein kinase
NT01EI_1892	-2.78	0.00	integration host factor, alpha subunit, putative
NT01EI_2590	-2.97	0.00	PTS system, Fru family, IIBC component
NT01EI_3469	-3.87	0.00	glycerophosphoryl diester phosphodiesterase, putative
NT01EI_3091	-4.04	0.00	Ferritin-like domain protein
NT01EI_0859	-4.09	0.00	Orn/Lys/Arg decarboxylase family protein, putative
NT01EI_2792	-4.28	0.00	hypothetical protein
NT01EI_0900	-4.41	0.00	hypothetical protein
NT01EI_0377	-4.47	0.00	aspartate ammonia-lyase, putative
NT01EI_2159	-5.63	0.00	hypothetical protein
NT01EI_3001	-5.67	0.00	hypothetical protein

APPENDIX B

COMPLETE LIST OF DEGs IN *Ei*Δ*evpB* STRAIN DURING INVASION OF CATFISH INTESTINE

Gene	FC	FDR	Product
NT01EI_3001	8.95	0.00	hypothetical protein
NT01EI_0900	7.25	0.00	hypothetical protein
NT01EI_0377	6.71	0.00	aspartate ammonia-lyase, putative
NT01EI_0859	6.42	0.00	Orn/Lys/Arg decarboxylase family protein, putative
NT01EI_1007	5.59	0.00	Dihydrodipicolinate synthetase family protein
NT01EI_3091	5.15	0.00	Ferritin-like domain protein
NT01EI_3469	4.35	0.00	glycerophosphoryl diester phosphodiesterase, putative
NT01EI_2792	4.28	0.00	hypothetical protein
NT01EI_2159	4.12	0.00	hypothetical protein
NT01EI_2999	3.82	0.00	hypothetical protein
NT01EI_2842	3.81	0.00	galactose-1-phosphate uridylyltransferase, putative
NT01EI_2590	3.78	0.00	PTS system, Fru family, IIBC component
NT01EI_2591	3.31	0.00	1-phosphofructokinase, putative
NT01EI_3464	3.21	0.00	glycerol-3-phosphate dehydrogenase, anaerobic, C subunit
NT01EI_1637	3.07	0.00	PrkA serine protein kinase
NT01EI_0564	3.05	0.01	phosphopentomutase, putative
NT01EI_3377	2.99	0.00	MFS transporter, sugar porter family protein
NT01EI_1719	2.93	0.00	PTS system, mannose/fructose/sorbose family, IID component
NT01EI_1665	2.90	0.00	aldehyde-alcohol dehydrogenase 2, putative
NT01EI_3468	2.90	0.00	glycerol-3-phosphate transporter, putative
NT01EI_1892	2.86	0.00	integration host factor, alpha subunit, putative
NT01EI_3679	2.86	0.00	FAD dependent oxidoreductase, putative
NT01EI_0565	2.86	0.02	purine nucleoside phosphorylase, putative
NT01EI_0133	2.81	0.01	uridine phosphorylase, putative
NT01EI_1663	2.78	0.00	H-NS histone family protein
NT01EI_2460	2.73	0.00	L-asparaginase, type 2, putative
NT01EI_1721	2.60	0.00	PTS system, mannose/fructose/sorbose family, IIB component
NT01EI_2843	2.59	0.02	galactokinase, putative
NT01EI_2841	2.55	0.00	UDP-glucose 4-epimerase, putative
NT01EI_3789	2.52	0.00	hypothetical protein
NT01EI_0516	2.50	0.00	Protein unknown function (DUF388)
NT01EI_3466	2.45	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit A, putative
NT01EI_3833	2.43	0.00	haloacid dehalogenase (HAD) superfamily (subfamily IIIB) phosphatase, putative
NT01EI_0563	2.41	0.04	thymidine phosphorylase, putative
NT01EI_0488	2.34	0.00	hypothetical protein
NT01EI_3465	2.31	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit B, putative
NT01EI_1570	2.31	0.00	protein of unknown function (DUF336)
NT01EI_0234	2.30	0.00	citrate (pro-3S)-lyase ligase, putative
NT01EI_0232	2.28	0.00	citrate (pro-3S)-lyase, beta subunit, putative

NT01EI_3184	2.26	0.03	iron-sulfur cluster assembly transcription factor IscR, putative
NT01EI_3785	2.25	0.00	triose-phosphate isomerase, putative
NT01EI_1558	2.20	0.00	hypothetical protein
NT01EI_1876	2.20	0.00	hypothetical protein
NT01EI_1006	2.20	0.00	hypothetical protein
NT01EI_3832	2.12	0.01	5'-nucleotidase, lipoprotein e(P4) family, putative
NT01EI_1720	2.08	0.00	PTS system, mannose/fructose/sorbose family, IIC component
NT01EI_0376	2.08	0.00	anaerobic C4-dicarboxylate transporter DcuA, putative
NT01EI_1937	2.08	0.01	Glycogen synthesis protein
NT01EI_1641	2.05	0.03	glyceraldehyde-3-phosphate dehydrogenase, type I, putative
NT01EI_1845	2.02	0.02	outer membrane lipoprotein LPP with repeating sequence
NT01EI_1877	2.01	0.01	hypothetical protein
NT01EI_3834	2.01	0.00	outer membrane porin
NT01EI_0231	2.01	0.00	citrate lyase alpha chain
NT01EI_2844	1.96	0.05	galactose mutarotase, putative
NT01EI_3729	1.94	0.03	universal stress protein A, putative
NT01EI_0899	1.94	0.00	Amino acid permease
NT01EI_0987	1.90	0.00	hypothetical protein
NT01EI_1636	1.90	0.01	Protein of unknown function (DUF444)
NT01EI_0216	1.89	0.00	maltose-binding periplasmic protein precursor, MalE, putative
NT01EI_3810	1.88	0.04	exported protein with LTXXQ motif
NT01EI_0233	1.85	0.01	citrate lyase acyl carrier protein, putative
NT01EI_2118	1.85	0.05	cation efflux system protein CusF, putative
NT01EI_2107	1.77	0.03	hypothetical protein
NT01EI_0858	1.73	0.00	amino acid permease family protein
NT01EI_2592	1.72	0.05	multiphosphoryl transfer protein, putative
NT01EI_0378	1.65	0.00	FxsA cytoplasmic membrane protein
NT01EI_0959	1.64	0.05	hypothetical protein
NT01EI_0290	1.64	0.01	basic amino acid/polyamine antiporter (APA) family
NT01EI_0210	1.64	0.00	glucose-6-phosphate isomerase, putative
NT01EI_T00088	1.64	0.02	Sec tRNA
NT01EI_1233	1.61	0.03	malic enzyme, putative
NT01EI_2094	1.60	0.02	glutathione S-transferase
NT01EI_0969	1.59	0.01	hypothetical protein
NT01EI_0329	1.59	0.00	hypothetical protein
NT01EI_1377	1.58	0.01	Protein of unknown function (DUF1379)
NT01EI_2492	1.58	0.04	ATP-dependent Clp protease adaptor protein ClpS
NT01EI_1174	1.57	0.03	Protein of unknown function (DUF1255)
NT01EI_0791	1.57	0.02	sugar fermentation stimulation protein, putative
NT01EI_2815	1.54	0.01	PTS system, mannose/fructose/sorbose family, IIB component
NT01EI_1270	1.53	0.04	hypothetical protein
NT01EI_T00021	1.53	0.05	Leu tRNA

NT01EI_0479	1.52	0.01	hypothetical protein
NT01EI_0988	1.52	0.01	hemolysin activator protein
NT01EI_3376	1.52	0.00	methionine adenosyltransferase, putative
NT01EI_1634	-1.52	0.02	hypothetical protein
NT01EI_1601	-1.52	0.01	ATP-binding protein of zinc transporter, putative
NT01EI_1000	-1.52	0.01	aspartate racemase, putative
NT01EI_2009	-1.53	0.01	addiction module toxin, RelE/StbE family
NT01EI_0162	-1.53	0.01	pantothenate kinase, putative
NT01EI_3492	-1.53	0.04	cyclic diguanylate cyclase/phosphodiesterase domain protein
NT01EI_1835	-1.53	0.04	hypothetical protein
NT01EI_1935	-1.53	0.00	hypothetical protein
NT01EI_1699	-1.54	0.01	transcriptional regulator, AraC family
NT01EI_2086	-1.54	0.00	Electron transport complex, RnfABCDGE type, A subunit , putative
NT01EI_1055	-1.54	0.03	transcriptional regulator NrdR, putative
NT01EI_2945	-1.55	0.00	D-alanyl-D-alanine carboxypeptidase family protein
NT01EI_0034	-1.55	0.04	guanylate kinase, putative
NT01EI_1576	-1.56	0.01	D-isomer specific 2-hydroxyacid dehydrogenase family protein, NAD binding domain protein
NT01EI_2024	-1.57	0.00	hypothetical protein
NT01EI_2523	-1.57	0.00	methylated DNA protein cysteine S-methyltransferase family, putative
NT01EI_2283	-1.57	0.03	hypothetical protein
NT01EI_0957	-1.58	0.05	type III secretion ATPase FliI/YscN family, putative
NT01EI_3491	-1.58	0.05	cell shape determining protein, MreB
NT01EI_2462	-1.58	0.01	formate/nitrite transporter
NT01EI_2748	-1.58	0.01	type VI secretion lipoprotein, VC_A0113 family
NT01EI_1093	-1.59	0.00	6-O-methylguanine DNA methyltransferase, DNA binding domain protein
NT01EI_2196	-1.59	0.00	hypothetical protein
NT01EI_0933	-1.59	0.03	type III secretion effector delivery regulator, TyeA family
NT01EI_1708	-1.60	0.02	hypothetical protein
NT01EI_0955	-1.61	0.00	hypothetical protein
NT01EI_1753	-1.61	0.03	hypothetical protein
NT01EI_1642	-1.61	0.02	methionine-R-sulfoxide reductase, putative
NT01EI_1883	-1.62	0.03	hypothetical protein
NT01EI_3112	-1.62	0.00	cyanate transporter family protein
NT01EI_2656	-1.63	0.01	hydrogenase expression/formation protein, putative
NT01EI_2757	-1.64	0.00	phosphohistidine phosphatase SixA, putative
NT01EI_0954	-1.64	0.00	hypothetical protein
NT01EI_0569	-1.65	0.00	phosphocarrier, HPr family protein
NT01EI_3899	-1.66	0.01	hypothetical protein
NT01EI_1979	-1.67	0.01	hypothetical protein
NT01EI_1056	-1.67	0.00	riboflavin biosynthesis protein RibD, putative

NT01EI_1054	-1.67	0.03	hypothetical protein
NT01EI_1532	-1.68	0.00	hypothetical protein
NT01EI_1783	-1.69	0.00	hypothetical protein
NT01EI_0937	-1.69	0.02	type III secretion apparatus protein, YscI/HrpB, C-terminal domain protein
NT01EI_1244	-1.70	0.04	phosphocarrier, HPr family
NT01EI_2771	-1.71	0.01	hypothetical protein
NT01EI_2926	-1.71	0.00	hypothetical protein
NT01EI_2927	-1.72	0.00	magnesium and cobalt efflux protein CorC, putative
NT01EI_2745	-1.73	0.04	type VI secretion system Vgr family protein
NT01EI_2679	-1.74	0.04	NADH-quinone oxidoreductase subunit A, putative
NT01EI_1959	-1.75	0.00	Protein of unknown function (DUF1471)
NT01EI_2435	-1.76	0.00	Bacterial protein of unknown function (DUF882)
NT01EI_1785	-1.78	0.00	hypothetical protein
NT01EI_1784	-1.78	0.00	hypothetical protein
NT01EI_1560	-1.79	0.00	hypothetical protein
NT01EI_3197	-1.79	0.01	Transglycosylase, SLT domain protein
NT01EI_0941	-1.79	0.00	type III secretion system protein, YseE family
NT01EI_2225	-1.83	0.03	cytochrome o ubiquinol oxidase, subunit III, putative
NT01EI_1750	-1.84	0.00	hypothetical protein
NT01EI_3539	-1.84	0.04	hypothetical protein
NT01EI_2898	-1.89	0.01	flavodoxin, putative
NT01EI_1799	-1.89	0.00	hypothetical protein
NT01EI_1396	-1.90	0.00	Protein of unknown function (DUF307)
NT01EI_1113	-1.90	0.01	hypothetical protein
NT01EI_1075	-1.91	0.00	putative lipoprotein
NT01EI_0568	-1.91	0.00	hypothetical protein
NT01EI_2692	-1.92	0.00	Protein of unknown function, DUF412
NT01EI_2703	-1.92	0.00	phosphoenolpyruvate phosphomutase, putative
NT01EI_3900	-1.93	0.01	aspartate-ammonia ligase, putative
NT01EI_1749	-1.94	0.00	hypothetical protein
NT01EI_2744	-1.94	0.02	type VI secretion ATPase, ClpV1 family, putative
NT01EI_1781	-1.95	0.04	hypothetical protein
NT01EI_1841	-1.95	0.03	periplasmic peptide-binding protein, putative
NT01EI_0942	-1.95	0.00	type III secretion apparatus protein, YscD/HrpQ family, putative
NT01EI_1679	-1.97	0.00	YCII-related domain protein
NT01EI_2395	-2.00	0.00	hypothetical protein
NT01EI_1521	-2.01	0.00	hypothetical protein
NT01EI_1429	-2.04	0.05	cold-shock DNA-binding domain protein
NT01EI_1865	-2.04	0.04	cold-shock DNA-binding domain protein
NT01EI_3313	-2.06	0.02	hypothetical protein
NT01EI_1519	-2.06	0.05	hypothetical protein

NT01EI_1695	-2.10	0.00	hypothetical protein
NT01EI_2223	-2.10	0.00	protoheme IX farnesyltransferase, putative
NT01EI_2743	-2.15	0.00	type VI secretion protein, VC_A0111 family
NT01EI_1696	-2.16	0.00	amino acid permease family protein
NT01EI_2447	-2.19	0.00	3-deoxy-D-manno-octulosonate cytidylyltransferase, putative
NT01EI_2224	-2.22	0.00	cytochrome o ubiquinol oxidase subunit IV, putative
NT01EI_0938	-2.24	0.00	type III secretion system protein, SsaH family
NT01EI_0934	-2.24	0.00	hypothetical protein
NT01EI_3398	-2.24	0.00	Cyclopropane-fatty-acyl-phospholipid synthase
NT01EI_1245	-2.26	0.00	hypothetical protein
NT01EI_3021	-2.31	0.00	Protein of unknown function (DUF465)
NT01EI_2742	-2.34	0.00	type VI secretion protein, VC_A0110 family
NT01EI_2740	-2.47	0.02	hypothetical protein
NT01EI_1520	-2.58	0.00	hypothetical protein
NT01EI_0935	-2.60	0.00	hypothetical protein
NT01EI_2741	-2.62	0.00	type VI secretion system lysozyme-related protein
NT01EI_2399	-2.66	0.00	hypothetical protein
NT01EI_2448	-2.67	0.00	Protein of unknown function (DUF343)
NT01EI_0936	-2.78	0.00	type III secretion apparatus lipoprotein, YscJ/HrcJ family, putative
NT01EI_1374	-2.81	0.00	hypothetical protein
NT01EI_2536	-2.88	0.00	hypothetical protein
NT01EI_2398	-2.96	0.00	hypothetical protein
NT01EI_0709	-3.18	0.00	hypothetical protein
NT01EI_2114	-3.31	0.00	drug resistance transporter, Bcr/CflA subfamily
NT01EI_2450	-3.34	0.00	cold-shock DNA-binding domain protein
NT01EI_2394	-3.39	0.00	AMP-binding enzyme
NT01EI_1375	-3.91	0.00	hypothetical protein
NT01EI_2738	-6.26	0.00	type VI secretion protein, EvpB/VC_A0108 family

APPENDIX C

COMPLETE LIST OF DEGs IN *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* STRAIN DURING INVASION OF CATFISH INTESTINE

Gene	FC	FDR	Product
NT01EI_0859	8.36	0.00	Orn/Lys/Arg decarboxylase family protein, putative
NT01EI_3001	7.02	0.00	hypothetical protein
NT01EI_0900	6.31	0.00	hypothetical protein
NT01EI_0377	5.11	0.00	aspartate ammonia-lyase, putative
NT01EI_3469	4.57	0.00	glycerophosphoryl diester phosphodiesterase, putative
NT01EI_3091	4.57	0.00	Ferritin-like domain protein
NT01EI_3464	3.88	0.00	glycerol-3-phosphate dehydrogenase, anaerobic, C subunit
NT01EI_2590	3.73	0.00	PTS system, Fru family, IIBC component
NT01EI_3679	3.33	0.00	FAD dependent oxidoreductase, putative
NT01EI_2792	3.32	0.00	hypothetical protein
NT01EI_2591	2.91	0.00	1-phosphofructokinase, putative
NT01EI_3466	2.89	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit A, putative
NT01EI_1665	2.87	0.00	aldehyde-alcohol dehydrogenase 2, putative
NT01EI_0564	2.80	0.01	phosphopentomutase, putative
NT01EI_3468	2.79	0.00	glycerol-3-phosphate transporter, putative
NT01EI_3465	2.78	0.00	anaerobic glycerol-3-phosphate dehydrogenase, subunit B, putative
NT01EI_1007	2.67	0.00	Dihydrodipicolinate synthetase family protein
NT01EI_0565	2.59	0.02	purine nucleoside phosphorylase, putative
NT01EI_3377	2.51	0.00	MFS transporter, sugar porter family protein
NT01EI_2999	2.43	0.00	hypothetical protein
NT01EI_0858	2.42	0.00	amino acid permease family protein
NT01EI_3785	2.26	0.00	triose-phosphate isomerase, putative
NT01EI_0563	2.19	0.02	thymidine phosphorylase, putative
NT01EI_2841	2.18	0.00	UDP-glucose 4-epimerase, putative
NT01EI_2460	2.15	0.00	L-asparaginase, type 2, putative
NT01EI_1637	2.13	0.00	PrkA serine protein kinase
NT01EI_3833	2.13	0.00	haloacid dehalogenase (HAD) superfamily (subfamily IIIB) phosphatase, putative
NT01EI_1641	2.11	0.02	glyceraldehyde-3-phosphate dehydrogenase, type I, putative
NT01EI_2842	2.11	0.00	galactose-1-phosphate uridylyltransferase, putative
NT01EI_1663	2.10	0.00	H-NS histone family protein
NT01EI_0488	2.09	0.00	hypothetical protein
NT01EI_2159	2.08	0.00	hypothetical protein
NT01EI_1775	2.06	0.01	hypothetical protein
NT01EI_0376	2.06	0.00	anaerobic C4-dicarboxylate transporter DcuA, putative
NT01EI_1719	2.05	0.00	PTS system, mannose/fructose/sorbose family, IID component
NT01EI_0232	1.96	0.00	citrate (pro-3S)-lyase, beta subunit, putative
NT01EI_3376	1.95	0.00	methionine adenosyltransferase, putative
NT01EI_1138	1.94	0.01	copper-translocating P-type ATPase, putative
NT01EI_0602	1.93	0.00	Arylsulfotransferase (ASST)

NT01EI_1721	1.92	0.01	PTS system, mannose/fructose/sorbose family, IIB component
NT01EI_0899	1.88	0.02	Amino acid permease
NT01EI_2592	1.87	0.03	multiphosphoryl transfer protein, putative
NT01EI_3832	1.87	0.01	5'-nucleotidase, lipoprotein e(P4) family, putative
NT01EI_1892	1.85	0.02	integration host factor, alpha subunit, putative
NT01EI_0601	1.85	0.00	DsbA-like thioredoxin domain containing protein
NT01EI_T00089	1.80	0.01	Trp tRNA
NT01EI_2386	1.77	0.01	fumarate hydratase class I, anaerobic, putative
NT01EI_0216	1.77	0.00	maltose-binding periplasmic protein precursor, MalE, putative
NT01EI_0516	1.76	0.02	Protein unknown function (DUF388)
NT01EI_2873	1.74	0.04	hypothetical protein
NT01EI_R0006	1.71	0.00	5S ribosomal RNA
NT01EI_T00088	1.70	0.00	Sec tRNA
NT01EI_3808	1.69	0.02	6-phosphofructokinase, putative
NT01EI_1558	1.68	0.00	hypothetical protein
NT01EI_0234	1.67	0.04	citrate (pro-3S)-lyase ligase, putative
NT01EI_0479	1.65	0.00	hypothetical protein
NT01EI_0988	1.64	0.00	hemolysin activator protein
NT01EI_3273	1.64	0.00	formate hydrogenlyase, subunit E, putative
NT01EI_3834	1.62	0.00	outer membrane porin
NT01EI_3792	1.60	0.00	glycerol kinase, putative
NT01EI_R0009	1.58	0.00	5S ribosomal RNA
NT01EI_R0001	1.55	0.03	5S ribosomal RNA
NT01EI_R0005	1.55	0.01	5S ribosomal RNA
NT01EI_0210	1.53	0.01	glucose-6-phosphate isomerase, putative
NT01EI_3793	1.53	0.00	MIP family channel protein
NT01EI_0567	-1.52	0.00	Nickel-dependent hydrogenases b-type cytochrome subunit
NT01EI_1063	-1.52	0.04	exodeoxyribonuclease VII, small subunit, putative
NT01EI_2024	-1.52	0.00	hypothetical protein
NT01EI_2802	-1.52	0.00	HNH endonuclease domain protein
NT01EI_3934	-1.52	0.02	ribonuclease P protein component, putative
NT01EI_2485	-1.52	0.01	leucine-responsive regulatory protein, putative
NT01EI_1603	-1.52	0.00	Opacity-associated protein A N-terminal motif protein
NT01EI_0034	-1.53	0.01	guanylate kinase, putative
NT01EI_1626	-1.53	0.00	Uncharacterized protein family (UPF0153)
NT01EI_1701	-1.54	0.00	hypothetical protein
NT01EI_1700	-1.55	0.02	DNA polymerase III, theta subunit
NT01EI_1940	-1.55	0.00	hypothetical protein
NT01EI_0465	-1.55	0.03	hypothetical protein
NT01EI_3334	-1.55	0.01	hypothetical protein
NT01EI_1879	-1.56	0.00	hypothetical protein with ferritin-like domain
NT01EI_1973	-1.56	0.04	hypothetical protein

NT01EI_1832	-1.57	0.00	phage shock protein A, putative
NT01EI_2855	-1.57	0.04	Tol-Pal system protein YbgF
NT01EI_1804	-1.57	0.00	hypothetical protein
NT01EI_2177	-1.57	0.00	hypothetical protein
NT01EI_3227	-1.57	0.01	S-Ribosylhomocysteinase (LuxS)
NT01EI_3222	-1.57	0.04	16S rRNA processing protein RimM, putative
NT01EI_1835	-1.58	0.01	hypothetical protein
NT01EI_0462	-1.58	0.05	preprotein translocase, SecG subunit, putative
NT01EI_1932	-1.58	0.00	multidrug resistance protein, SMR family
NT01EI_2559	-1.58	0.00	hypothetical protein
NT01EI_0192	-1.58	0.01	Protein of unknown function, DUF485
NT01EI_2280	-1.58	0.00	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, putative
NT01EI_2996	-1.59	0.00	antimicrobial peptide resistance and lipid A acylation protein PagP
NT01EI_1628	-1.59	0.00	disulfide bond formation family protein
NT01EI_1737	-1.60	0.04	hypothetical protein
NT01EI_2748	-1.60	0.00	type VI secretion lipoprotein, VC_A0113 family
NT01EI_2610	-1.60	0.00	Protein of unknown function (DUF1414)
NT01EI_1391	-1.60	0.00	hypothetical protein
NT01EI_0022	-1.60	0.00	hypothetical protein
NT01EI_1576	-1.60	0.00	D-isomer specific 2-hydroxyacid dehydrogenase family protein, NAD binding domain protein
NT01EI_3112	-1.61	0.00	cyanate transporter family protein
NT01EI_2757	-1.61	0.01	phosphohistidine phosphatase SixA, putative
NT01EI_1979	-1.61	0.00	hypothetical protein
NT01EI_2537	-1.61	0.00	hypothetical protein
NT01EI_1738	-1.62	0.00	transglycosylase SLT domain protein
NT01EI_1796	-1.62	0.00	ProP effector, putative
NT01EI_T00029	-1.62	0.00	Lys tRNA
NT01EI_1601	-1.62	0.00	ATP-binding protein of zinc transporter, putative
NT01EI_1560	-1.62	0.00	hypothetical protein
NT01EI_1055	-1.62	0.00	transcriptional regulator NrdR, putative
NT01EI_0676	-1.62	0.01	hypothetical protein
NT01EI_1056	-1.62	0.00	riboflavin biosynthesis protein RibD, putative
NT01EI_2679	-1.63	0.03	NADH-quinone oxidoreductase subunit A, putative
NT01EI_2692	-1.63	0.00	Protein of unknown function, DUF412
NT01EI_1799	-1.63	0.01	hypothetical protein
NT01EI_1634	-1.64	0.00	hypothetical protein
NT01EI_0490	-1.64	0.00	hypothetical protein
NT01EI_2950	-1.64	0.00	hypothetical protein
NT01EI_T00080	-1.64	0.01	Ser tRNA
NT01EI_0942	-1.64	0.00	type III secretion apparatus protein, YscD/HrpQ family, putative

NT01EI_1783	-1.66	0.00	hypothetical protein
NT01EI_T0008	-1.66	0.02	Gly tRNA
NT01EI_2417	-1.67	0.00	hypothetical protein
NT01EI_3065	-1.67	0.00	SmpA / OmlA family protein
NT01EI_T00069	-1.67	0.03	Gln tRNA
NT01EI_1244	-1.68	0.01	phosphocarrier, HPr family
NT01EI_3467	-1.68	0.00	hypothetical protein
NT01EI_2926	-1.68	0.00	hypothetical protein
NT01EI_1959	-1.68	0.00	Protein of unknown function (DUF1471)
NT01EI_1753	-1.68	0.00	hypothetical protein
NT01EI_0007	-1.69	0.00	hypothetical protein
NT01EI_2018	-1.70	0.00	hypothetical protein
NT01EI_3491	-1.70	0.00	cell shape determining protein, MreB
NT01EI_1549	-1.70	0.00	iso-IS1 ORF1
NT01EI_0939	-1.71	0.02	type III secretion apparatus needle protein, putative
NT01EI_3208	-1.72	0.00	competence lipoprotein ComL
NT01EI_T00068	-1.72	0.01	Leu tRNA
NT01EI_1054	-1.74	0.00	hypothetical protein
NT01EI_2494	-1.74	0.01	Protein of unknown function (DUF535)
NT01EI_2462	-1.74	0.00	formate/nitrite transporter
NT01EI_1532	-1.74	0.00	hypothetical protein
NT01EI_2867	-1.75	0.03	dihydrolipoyllysine-residue succinyltransferase, E2 component of oxoglutarate dehydrogenase (succinyl-transferring) complex, putative
NT01EI_3538	-1.75	0.00	Protein of unknown function (DUF419)
NT01EI_2435	-1.75	0.00	Bacterial protein of unknown function (DUF882)
NT01EI_2868	-1.76	0.03	oxoglutarate dehydrogenase (succinyl-transferring), E1 component, putative
NT01EI_3520	-1.76	0.01	xanthine/uracil permease family protein
NT01EI_1633	-1.77	0.00	hypothetical protein
NT01EI_1901	-1.77	0.00	NlpC/P60 family protein
NT01EI_2283	-1.77	0.00	hypothetical protein
NT01EI_1405	-1.77	0.00	Uncharacterized protein family UPF0005
NT01EI_2225	-1.77	0.01	cytochrome o ubiquinol oxidase, subunit III, putative
NT01EI_2745	-1.77	0.01	type VI secretion system Vgr family protein
NT01EI_2442	-1.78	0.00	hypothetical protein
NT01EI_1784	-1.80	0.00	hypothetical protein
NT01EI_1075	-1.80	0.00	putative lipoprotein
NT01EI_1266	-1.80	0.01	Na+ dependent nucleoside transporter family protein
NT01EI_T00016	-1.81	0.02	Leu tRNA
NT01EI_2870	-1.81	0.01	succinate dehydrogenase, flavoprotein subunit, putative
NT01EI_3521	-1.82	0.04	hypoxanthine oxidase XdhD, putative
NT01EI_3899	-1.82	0.00	hypothetical protein

NT01EI_2927	-1.82	0.00	magnesium and cobalt efflux protein CorC, putative
NT01EI_1781	-1.83	0.03	hypothetical protein
NT01EI_1679	-1.84	0.01	YCII-related domain protein
NT01EI_1093	-1.84	0.00	6-O-methylguanine DNA methyltransferase, DNA binding domain protein
NT01EI_1732	-1.85	0.00	transporter, dicarboxylate/amino acid:cation (Na+ or H+) symporter (DAACS) family
NT01EI_1889	-1.85	0.01	50S ribosomal protein L20, putative
NT01EI_2656	-1.85	0.00	hydrogenase expression/formation protein, putative
NT01EI_2703	-1.87	0.00	phosphoenolpyruvate phosphomutase, putative
NT01EI_2746	-1.87	0.02	PAAR motif protein
NT01EI_2865	-1.89	0.02	succinyl-CoA synthetase (ADP forming), alpha subunit, putative
NT01EI_0941	-1.89	0.00	type III secretion system protein, YseE family
NT01EI_0934	-1.89	0.01	hypothetical protein
NT01EI_1521	-1.90	0.00	hypothetical protein
NT01EI_3523	-1.90	0.01	selenium metabolism protein SsnA, putative
NT01EI_1517	-1.91	0.00	hypothetical protein
NT01EI_1520	-1.93	0.00	hypothetical protein
NT01EI_2605	-1.95	0.00	hypothetical protein
NT01EI_0938	-1.96	0.00	type III secretion system protein, SsaH family
NT01EI_2744	-1.96	0.01	type VI secretion ATPase, ClpV1 family, putative
NT01EI_1396	-1.97	0.00	Protein of unknown function (DUF307)
NT01EI_1000	-1.98	0.00	aspartate racemase, putative
NT01EI_0946	-1.99	0.03	type III secretion system chaperone protein, putative
NT01EI_2072	-2.00	0.00	hypothetical protein
NT01EI_T0009	-2.01	0.00	Thr tRNA
NT01EI_1113	-2.02	0.00	hypothetical protein
NT01EI_2871	-2.02	0.00	succinate dehydrogenase, hydrophobic membrane anchor protein, putative
NT01EI_2224	-2.03	0.00	cytochrome o ubiquinol oxidase subunit IV, putative
NT01EI_2449	-2.04	0.00	cold-shock DNA-binding domain protein, putative
NT01EI_1519	-2.05	0.00	hypothetical protein
NT01EI_1697	-2.06	0.00	bacterial extracellular solute-binding protein, family 3
NT01EI_1841	-2.06	0.00	periplasmic peptide-binding protein, putative
NT01EI_T00034	-2.06	0.01	Val tRNA
NT01EI_2400	-2.06	0.00	ABC transporter, ATP-binding protein, putative
NT01EI_1749	-2.10	0.00	hypothetical protein
NT01EI_2869	-2.11	0.00	succinate dehydrogenase iron-sulfur subunit, putative
NT01EI_0935	-2.11	0.00	hypothetical protein
NT01EI_3900	-2.12	0.00	aspartate-ammonia ligase, putative
NT01EI_T00067	-2.13	0.00	Met tRNA
NT01EI_3522	-2.14	0.00	selenate reductase, FAD-binding subunit, putative
NT01EI_1696	-2.15	0.00	amino acid permease family protein

NT01EI_2447	-2.16	0.00	3-deoxy-D-manno-octulosonate cytidylyltransferase, putative
NT01EI_2743	-2.19	0.00	type VI secretion protein, VC_A0111 family
NT01EI_0568	-2.19	0.00	hypothetical protein
NT01EI_0936	-2.20	0.00	type III secretion apparatus lipoprotein, YscJ/HrcJ family, putative
NT01EI_2742	-2.21	0.00	type VI secretion protein, VC_A0110 family
NT01EI_1245	-2.21	0.00	hypothetical protein
NT01EI_3398	-2.24	0.00	Cyclopropane-fatty-acyl-phospholipid synthase
NT01EI_3313	-2.25	0.00	hypothetical protein
NT01EI_3539	-2.27	0.00	hypothetical protein
NT01EI_2223	-2.30	0.00	protoheme IX farnesyltransferase, putative
NT01EI_3197	-2.32	0.00	Transglycosylase, SLT domain protein
NT01EI_1373	-2.32	0.00	hypothetical protein
NT01EI_2490	-2.33	0.00	translation initiation factor IF-1, putative
NT01EI_1695	-2.34	0.00	hypothetical protein
NT01EI_0709	-2.37	0.00	hypothetical protein
NT01EI_2398	-2.38	0.00	hypothetical protein
NT01EI_1865	-2.38	0.00	cold-shock DNA-binding domain protein
NT01EI_2399	-2.39	0.00	hypothetical protein
NT01EI_3021	-2.46	0.00	Protein of unknown function (DUF465)
NT01EI_2740	-2.49	0.00	hypothetical protein
NT01EI_2741	-2.64	0.00	type VI secretion system lysozyme-related protein
NT01EI_2395	-2.64	0.00	hypothetical protein
NT01EI_1429	-2.66	0.00	cold-shock DNA-binding domain protein
NT01EI_2536	-2.72	0.00	hypothetical protein
NT01EI_1374	-2.82	0.00	hypothetical protein
NT01EI_2776	-2.93	0.00	hypothetical protein
NT01EI_2114	-3.11	0.00	drug resistance transporter, Bcr/CflA subfamily
NT01EI_2394	-3.14	0.00	AMP-binding enzyme
NT01EI_1375	-3.44	0.00	hypothetical protein
NT01EI_2448	-3.62	0.00	Protein of unknown function (DUF343)
NT01EI_2450	-3.82	0.00	cold-shock DNA-binding domain protein
NT01EI_0392	-9.10	0.00	fumarate reductase, flavoprotein subunit, putative
NT01EI_2872	-31.62	0.00	succinate dehydrogenase, cytochrome b556 subunit, putative
NT01EI_3351	-85.19	0.00	glycine dehydrogenase, putative

APPENDIX D

GENE ONTOLOGY (GO) CLASSIFICATION OF THE DEGS IN WILD-TYPE *E. ICTALURI* DURING INVASION OF CATFISH INTESTINE

E. ictaluri Upregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	No. Genes Found
GO:0006807	nitrogen compound metabolic process	0.01	47
GO:0044765	single-organism transport	0.00	
GO:0045333	cellular respiration	0.00	
GO:0006810	transport	0.00	
GO:0071702	organic substance transport	0.00	
GO:0015031	protein transport	0.00	
GO:0046903	secretion	0.00	
GO:0009306	protein secretion	0.00	
GO:0034641	cellular nitrogen compound metabolic process	0.01	
GO:0046483	heterocycle metabolic process	0.01	
GO:1901360	organic cyclic compound metabolic process	0.01	
GO:0006139	nucleobase-containing compound metabolic process	0.01	

E. ictaluri Downregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	No. Genes Found
GO:0009066	aspartate family amino acid metabolic process	0.01	17
GO:0044699	single-organism process	0.00	
GO:0006072	glycerol-3-phosphate metabolic process	0.00	
GO:0044765	single-organism transport	0.00	
GO:0071702	organic substance transport	0.00	
GO:0008643	carbohydrate transport	0.00	
GO:0034219	carbohydrate transmembrane transport	0.01	
Total No. Genes Found_Ontology Source: GO_BiologicalProcess			64

E. ictaluri Upregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	No. Genes Found
GO:0044424	intracellular part	0.03	14
GO:0043234	protein complex	0.01	
GO:0032991	macromolecular complex	0.01	

E. ictaluri Downregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	No. Genes Found
GO:0009331	glycerol-3-phosphate dehydrogenase complex	0.00	2
Total No. Genes Found_ Ontology Source: GO_CellularComponent			16

E. ictaluri Upregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0048037	cofactor binding	0.04	17

GO:0000104	succinate dehydrogenase activity	0.00	
GO:0050660	flavin adenine dinucleotide binding	0.02	
GO:0003676	nucleic acid binding	0.00	

E. ictaluri Downregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0052591	sn-glycerol-3-phosphate:ubiquinone-8 oxidoreductase activity	0.00	12
GO:0022857	transmembrane transporter activity	0.00	
GO:0015144	carbohydrate transmembrane transporter activity	0.00	
GO:0016773	phosphotransferase activity, alcohol group as acceptor	0.01	
Total No. Genes Found_Ontology Source: GO_MolecularFunction			29

APPENDIX E

GENE ONTOLOGY (GO) CLASSIFICATION OF THE DEGS IN *Ei*Δ*evpB* STRAIN DURING INVASION OF CATFISH INTESTINE

*Ei*Δ*evpB* Upregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	No. Genes Found
GO:0019318	hexose metabolic process	0.00	27
GO:0044281	small molecule metabolic process	0.00	
GO:0006810	transport	0.01	
GO:0044765	single-organism transport	0.00	
GO:0055085	transmembrane transport	0.04	
GO:0071702	organic substance transport	0.00	
GO:0008643	carbohydrate transport	0.00	
GO:0015711	organic anion transport	0.01	
GO:0034219	carbohydrate transmembrane transport	0.00	

*Ei*Δ*evpB* Downregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	
GO:0032259	methylation	0.05	16
GO:0006281	DNA repair	0.05	
GO:0006810	transport	0.05	
GO:0044765	single-organism transport	0.00	
GO:0046903	secretion	0.00	
GO:0009306	protein secretion	0.01	
GO:1902600	hydrogen ion transmembrane transport	0.01	
Total No. Genes Found_Ontology Source: GO_BiologicalProcess			43

*Ei*Δ*evpB* Upregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	No. Genes Found
GO:0005622	intracellular	0.01	11
GO:0005737	cytoplasm	0.00	
GO:0044444	cytoplasmic part	0.00	

*Ei*Δ*evpB* Downregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	
GO:0005737	cytoplasm	0.00	8
GO:0044424	intracellular part	0.01	
Total No. Genes Found_Ontology Source: GO_CellularComponent			19

*Ei*Δ*evpB* Upregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	0.01	21
GO:0052591	sn-glycerol-3-phosphate:ubiquinone-8 oxidoreductase activity	0.00	

GO:0016772	transferase activity, transferring phosphorus-containing groups	0.03	
GO:0022891	substrate-specific transmembrane transporter activity	0.00	
GO:0015144	carbohydrate transmembrane transporter activity	0.00	
GO:0016773	phosphotransferase activity, alcohol group as acceptor	0.03	
GO:0015075	ion transmembrane transporter activity	0.01	
GO:0008514	organic anion transmembrane transporter activity	0.01	

*Ei*Δ*evpB* Downregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0008168	methyltransferase activity	0.05	7
GO:0003908	methylated-DNA-[protein]-cysteine S-methyltransferase activity	0.00	
GO:0008827	cytochrome o ubiquinol oxidase activity	0.00	
GO:0004674	protein serine/threonine kinase activity	0.01	
Total No. Genes Found_Ontology Source: GO_MolecularFunction			28

APPENDIX F

GENE ONTOLOGY (GO) CLASSIFICATION OF THE DEGS IN *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* STRAIN DURING INVASION OF CATFISH INTESTINE

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Upregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	No. Genes Found
GO:0044281	small molecule metabolic process	0.02	24
GO:0019318	hexose metabolic process	0.00	
GO:0044765	single-organism transport	0.00	
GO:0071702	organic substance transport	0.00	
GO:0008643	carbohydrate transport	0.00	
GO:0006810	transport	0.00	
GO:0006811	ion transport	0.04	
GO:0055085	transmembrane transport	0.04	
GO:0015711	organic anion transport	0.03	

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Downregulated Genes Ontology
Ontology Source: GO_BiologicalProcess

GOID	GOTerm	Term PValue	No. Genes Found
GO:0022900	electron transport chain	0.02	18
GO:1902600	hydrogen ion transmembrane transport	0.03	
GO:0055114	oxidation-reduction process	0.00	
GO:0045333	cellular respiration	0.00	
GO:0009060	aerobic respiration	0.00	
GO:0019752	carboxylic acid metabolic process	0.05	
Total No. Genes Found_Ontology Source: GO_BiologicalProcess			42

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Upregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	No. Genes Found
GO:0005737	cytoplasm	0.03	6
GO:0044444	cytoplasmic part	0.00	

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Downregulated Genes Ontology
Ontology Source: GO_CellularComponent

GOID	GOTerm	Term PValue	No. Genes Found
GO:0045252	oxoglutarate dehydrogenase complex	0.04	3
Total Nr. Genes Found_Ontology Source: GO_CellularComponent			9

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Upregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	0.01	17
GO:0052591	sn-glycerol-3-phosphate:ubiquinone-8 oxidoreductase activity	0.00	
GO:0016772	transferase activity, transferring phosphorus-containing groups	0.03	
GO:0022891	substrate-specific transmembrane transporter activity	0.00	

GO:0015144	carbohydrate transmembrane transporter activity	0.00	
GO:0016773	phosphotransferase activity, alcohol group as acceptor	0.03	
GO:0015075	ion transmembrane transporter activity	0.01	
GO:0008514	organic anion transmembrane transporter activity	0.01	

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Downregulated Genes Ontology
Ontology Source: GO_MolecularFunction

GOID	GOTerm	Term PValue	No. Genes Found
GO:0016491	oxidoreductase activity	0.01	15
GO:0008827	cytochrome o ubiquinol oxidase activity	0.00	
GO:0048037	cofactor binding	0.03	
GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	0.00	
GO:0050660	flavin adenine dinucleotide binding	0.01	
GO:0008177	succinate dehydrogenase (ubiquinone) activity	0.00	
Total No. Genes Found_Ontology Source: GO_MolecularFunction			32

APPENDIX G

KEGG PATHWAY ANALYSIS OF THE DEGS IDENTIFIED IN WILD-TYPE *E. ICTALURI* DURING INVASION OF CATFISH INTESTINE

E. ictaluri Upregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00190	Oxidative phosphorylation	0.00	12
eic02020	Two-component system	0.03	2
eic03070	Bacterial secretion system	0.00	12
eic00020	Citrate cycle (TCA cycle)	0.01	5
E. ictaluri Downregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00564	Glycerophospholipid metabolism	0.00	5
eic00250	Alanine, aspartate and glutamate metabolism	0.03	2
eic00051	Fructose and mannose metabolism	0.00	5
eic00520	Amino sugar and nucleotide sugar metabolism	0.00	4
eic02060	Phosphotransferase system (PTS)	0.00	4

APPENDIX H

KEGG PATHWAY ANALYSIS OF THE DEGS IDENTIFIED IN *Ei*Δ*evpB* STRAIN DURING INVASION OF CATFISH INTESTINE

*Ei*Δ*evpB* Upregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00010	Glycolysis / Gluconeogenesis	0.00	5
eic00052	Galactose metabolism	0.00	5
eic00520	Amino sugar and nucleotide sugar metabolism	0.00	8
eic00564	Glycerophospholipid metabolism	0.00	5
eic00051	Fructose and mannose metabolism	0.00	7
eic02060	Phosphotransferase system (PTS)	0.00	6
*Ei*Δ*evpB* Downregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00190	Oxidative phosphorylation	0.01	4
eic03070	Bacterial secretion system	0.00	5

APPENDIX I

KEGG PATHWAY ANALYSIS OF THE DEGS IDENTIFIED IN *Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* STRAIN DURING INVASION OF CATFISH INTESTINE

*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Upregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00010	Glycolysis / Gluconeogenesis	0.00	5
eic00030	Pentose phosphate pathway	0.03	3
eic00052	Galactose metabolism	0.02	3
eic00520	Amino sugar and nucleotide sugar metabolism	0.00	6
eic00564	Glycerophospholipid metabolism	0.00	5
eic00051	Fructose and mannose metabolism	0.00	7
eic02060	Phosphotransferase system (PTS)	0.00	4
*Ei*Δ*gcvP*Δ*sdhC*Δ*frdA* Downregulated KEGG Pathways			
KEGG PathwayID	KEGG Pathway Term	Term PValue	No. Genes
eic00310	Lysine degradation	0.05	2
eic00970	Aminoacyl-tRNA biosynthesis	0.05	9
eic03070	Bacterial secretion system	0.01	5
eic00020	Citrate cycle (TCA cycle)	0.00	8
eic00190	Oxidative phosphorylation	0.00	9
eic00650	Butanoate metabolism	0.00	5