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The Role of Universal Stress Proteins in Edwardsiella ictaluri Virulence

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The role of universal stress proteins in *Edwardsiella ictaluri* virulence

By

Ali Akgul

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The role of universal stress proteins in *Edwardsiella ictaluri* virulence

By

Ali Akgul

Approved:

_____
Attila Karsi
(Major Professor)

_____
Daniel Peterson
(Minor Professor)

_____
Mark L. Lawrence
(Committee Member)

_____
Justin A. Thornton
(Committee Member)

_____
Larry Hanson
(Graduate Coordinator)

_____
Mark L. Lawrence
Dean
College of Veterinary Medicine

Name: Ali Akgul

Date of Degree: May 5, 2017

Institution: Mississippi State University

Major Field: Veterinary Medical Sciences

Major Professor: Attila Karsi

Title of Study: The role of universal stress proteins in *Edwardsiella ictaluri* virulence

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Candidate for Degree of Doctor of Philosophy

Edwardsiella ictaluri is an intracellular Gram-negative pathogen, causing enteric septicemia of catfish (ESC). Universal stress proteins (USP) are important in bacterial virulence, but the role of USPs in *E. ictaluri* virulence is not explored yet. Our aim was to analyze gene expression of 13 *usp* (*usp01-usp13*) and 7 USP-interacting genes (*groEL*, *groES*, *dnaK*, *dnaJ*, *clpB*, *grpE*, and ppGpp) under low pH, H_2O_2, catfish serum, and in vivo stress conditions, construct USP mutants, and determine mutants' role in *E. ictaluri* virulence. We found that *usp*05, *usp*07 and *usp*13 genes were highly expressed under all stress conditions, while *groEL*, *groES*, *dnaK*, *grpE*, and *clpB* were highly expressed in oxidative stress. Among the 10 *E. ictaluri* USP mutants, *Eiusp05-07-08-09-10, and 13* were significantly attenuated in catfish and highly protective against wild type *E. ictaluri* infections in catfish. *Eiusp*05-07-08-09*, and* 13 were sensitive to oxidative stress, and all mutants were sensitive to pH exposure.

DEDICATION

I am dedicating this research to my wife (Ayfer Akgul) and my two sons (Salih
Yigit Akgul and Melih Said Akgul).

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

The channel catfish industry

Catfish production is an important agricultural activity in the United States. In 2015, Mississippi catfish growers had fish sales more than 178 million pounds of large, medium, and small size catfish accounting for a total sales value of $201 million, up 8% from 2014 (USDA, 2016). However, the channel catfish industry is currently facing severe economic stress from increased feed prices and pressure from foreign competition. *Edwardsiella ictaluri* is a primary pathogen that affects all sizes of catfish, leading to considerable economic losses to catfish producers.

Edwardsiella ictaluri

E. ictaluri is a Gram-negative bacterium that causes enteric septicemia of catfish (ESC), one of the most important diseases in channel catfish (Hawke*, et al.*, 1981). *E. ictaluri* is a facultative intracellular pathogen in the *Enterobacteriaceae*. It was first recognized from an epizootic of fish mortalities in Alabama at Auburn University in 1976, and it is named as *E. ictaluri* (Hawke, *et al*., 1981). The size of *E. ictaluri* is 0.75 x 1.5 – 2.5 μm, and it is slightly motile at 25-30 °C, but it is not motile at higher temperatures. The bacterium grows at 30 °C in 48 hours on agar medium. The genome of *E. ictaluri* 93-146 strain is slightly GC-rich (57.5%) and consists of 3.81 million base

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pairs, containing 3,903 genes that encode 3,784 proteins (Williams, *et al.*, 2003). All *E. ictaluri* isolates harbor two native plasmids of 5.7 kb and 4.9 kb (Fernandez, *et al.*, 2001).

E. ictaluri is indole, lactose, and cytochrome oxidase negative. It ferments glucose and is catalase positive. On Tryptic Soy Infusion (TSI) agar, it produces an alkaline slant and acid without H_2S. It is nonfermentative for other sugars and does not grow on salinity higher than 1.5%. It is a rather homogenous species biophysically, biochemically, and serologically (Fernandez, *et al.*, 2001).

E. ictaluri is assumed to be a facultative intracellular pathogen with the ability to survive inside professional phagocytic cells. In two studies, phagocytosed bacteria appeared to be in binary division (Baldwin, 1993, Newton and Triche, 1993). These studies determined that *E. ictaluri*'s ability to survive in phagocytes might be responsible for the development of a carrier state for ESC (Klesius, 1992), and could be a mechanism for dissemination (Miyazaki, 1985, Shotts, *et al.*, 1986). *E. ictaluri* isolates were investigated and the minimal degree of antigenic variability determined. It showed that most isolates have only a single antigenic serotype of *E. ictaluri*, and it suggested that it can be a good candidate for vaccine development (Bertolini, *et al.*, 1990, Plumb, 1988).

Proposed mechanisms for spreading of pathogen include direct transport in blood or inside phagocytic cells (Booth, 2006, Thune, 1993). Previous research conducted on *E. ictaluri* identified various virulence factors, including flagella (Newton and Triche, 1993), extracellular capsular polysaccharide (Stanley, *et al.*, 1994), lipopolysaccharide (Arias, *et al.*, 2003, Lawrence, *et al.*, 2003, Lawrence, *et al.*, 2001, Newton and Triche, 1993, Weete, *et al.*, 1988, Williams, *et al.*, 2003), outer membrane proteins (Bader, *et al.*, 2004, Newton, *et al.*, 1990, Skirpstunas and Baldwin, 2003, Vinitnantharat, *et al.*, 1993,

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Williams, *et al.*, 2003, Yang*, et al.*, 2016), hemolysins (Williams and Lawrence, 2005) and chondroitinase (Cooper*, et al.,* 1996).

Enteric septicemia of catfish (ESC)

ESC has become a major problem for the catfish industry as it is responsible for annual losses of $60 million dollars to the cultured channel catfish industry in the USA (USDA, 2014). The disease was first identified, and the causative agent determined later in 1976. ESC has been a serious problem affecting the catfish industry since it was first described. *E. ictaluri* affects all sizes and ages of catfish during the late spring or early summer and the fall when water temperatures are between 22-28ºC. Typically, this thermal range is associated with pond temperatures in spring and fall. *E. ictaluri* is host specific to some extent though it was also isolated from other fish species.

The disease occurs in one of the two forms: an acute form and a chronic form (Miyazaki, 1985, Morrison, 1994, Shotts, *et al.*, 1986). In the acute form, farmers experience economic losses due to rapid mortalities because fish start dying 3-14 days post-infection by *E. ictaluri (*Shotts*, et al.,* 1986*)*. In the chronic form, farmers experience economic losses due to decreased production with fish manifesting signs 3-4 weeks after an acute outbreak (Newton *et al.,* 1989). The dead fish in acute ESC show bacterial septicemia, while those that died of the chronic form show "hole in the head" lesions. Behaviorally, sick fish consume less food, swim listlessly, hang vertically at the water surface, or spiral in water. The disease can be horizontally transferred from infected fish or fish that have died from ESC to naïve fish (Shotts, *et al.,* 1986).

Although ESC may occur in healthy fish in nonstressful environmental conditions, stress factors such as handling, close confinement, inappropriate diet, low

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water chlorides, deprived water quality, and water temperature variations lead to increased susceptibility to infection (Chappell, 2008). Fish that survive an outbreak can carry the bacterium in the brain, kidney, and liver for extended periods (up to 200 days). These survivors develop specific immunity that protects them from subsequent infection and disease. Once a fish is infected, it may either:1) become clinically ill and die; 2) become clinically ill, recover and develop protective immunity; 3) become clinically ill, recover and develop protective immunity but become a carrier and 4) become sub-clinically ill (the catfish producer cannot identify the infection) and develop protective immunity (Chappell, 2008).

The only treatment currently available is to feed pellets containing oxytetracycline, sulfadimethoxine, or florfenicol antibiotics. However, one of the earliest clinical signs of ESC is anorexia. Therefore, these antibiotics are only effective in limiting the spread of an outbreak and not in treating the disease because the antibiotics are usually food-fed. Hence, vaccines became an alternative way to reduce or eliminate the impact of *E. ictaluri*.

E. ictaluri is considered as a good candidate for the development of a vaccine (Bertolini, *et al.,* 1990), and killed bacterins have only been efficacious under controlled laboratory conditions when given by injection (Saeed and Plumb, 1986), so this route of delivery is not practical for commercial production. The initial method for controlling ESC is to feed catfish with antibiotic-containing food. However, this practice is expensive and usually ineffective, and continuous administration of antibiotics has led to the development of antibiotic resistance (Tu*, et al.*, 2008). Also, vaccines are important components and a proven effective preventing method for bacterial infections, and they

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are reducing the use of antibiotics in farmed fish (Hastein, *et al.*, 2005, Yang, *et al.*, 2016).

Some *E. ictaluri* mutants that are susceptible to catfish neutrophils (Karsi, *et al.*, 2009), and the neutrophil-susceptible *E. ictaluri* strains were highly attenuated and demonstrated good potential as live attenuated vaccines. In particular, strains with mutations in TCA cycle enzymes and in glycine cleavage system protein generated better protection than the currently available commercial vaccine when juvenile catfish were vaccinated by immersion (Dahal, *et al.*, 2014). Only limited numbers of genes were tried as a vaccine candidate, and still, the mechanism of attenuation for the vaccine candidates has not been fully determined for *E. ictaluri*.

Universal stress proteins (USPs)

Bacteria possess protection mechanisms to overcome environmental stresses such as nutrient starvation, stress and toxic agents (Kim, *et al.*, 2012). One of the stress-responsive genes against various deleterious stresses is the universal stress protein (USP) genes, first reported in *E. coli* (Nystrom and Neidhardt, 1992). They are found in archaea, fungi, flies, and plants (Kvint, *et al.*, 2003). *E. coli* has six *usp* genes divided into four classes based on their amino acid sequence similarities (Kvint, *et al.*, 2003). Gene *uspA*, *uspC*, and *uspD* belong to class I, and *uspF* and *uspG* are members of class II (Fig.2). The *uspE* possesses two domains, E1 and E2, which were previously classified as class III-IV, and *uspB* is in class V (Fig. 2). In *E. coli*, three isoforms of *uspA* have been identified in vivo, and two of them are phosphorylated on serine and threonine residues in response to stasis (Freestone, *et al.*, 1997).The *uspG* is dimeric and possesses autophosphorylation and autoadenylation activities (Weber and Jung, 2006). There have

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been many reports regarding the roles of USPs in defending against diverse stresses in bacteria. *E. coli uspA* mutant caused a survival defect under a variety of growth-arrested conditions, (Nystrom and Neidhardt, 1992, Nystrom and Neidhardt, 1993) (Fig.1.1).

E. coli USP has undefined roles in functions shifting from oxidative stress to adhesion and motility (Nachin*, et al.*, 2005) (Fig.1.2). USP family member USPE is a tandem-type USP that consists of two USP domains. The USPE expression levels of the *E. coli* become elevated in response to oxidative stress and DNA damaging agents, including exposure to mitomycin C, cadmium, and hydrogen peroxide (Fig.1.1). It has been shown that USPA family members are survival factors during cellular growth arrest. The structures and functions of the USPA family members control the growth of *E. coli* in animal hosts (Xu*, et al.*, 2016). However, the role of USP in bacterial virulence is not known in many bacteria, and how USPs function biochemically is also not known.
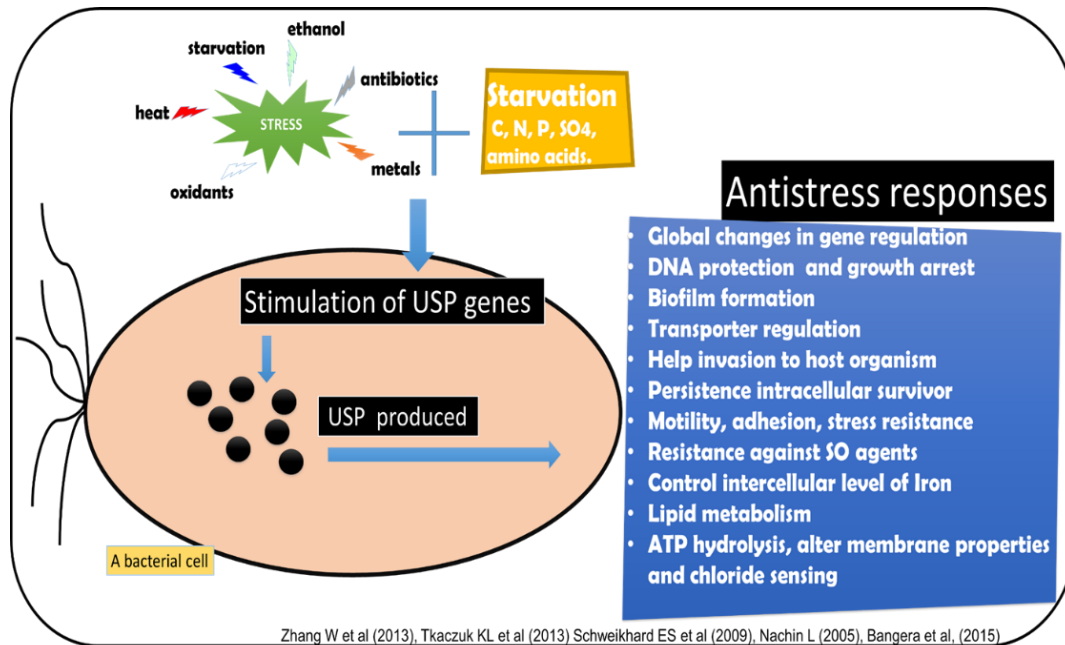
Figure 1.1   This schematic shows a generalized bacterial response to an environmental stress. Adapted from (Zhang*, et al.*, 2013)
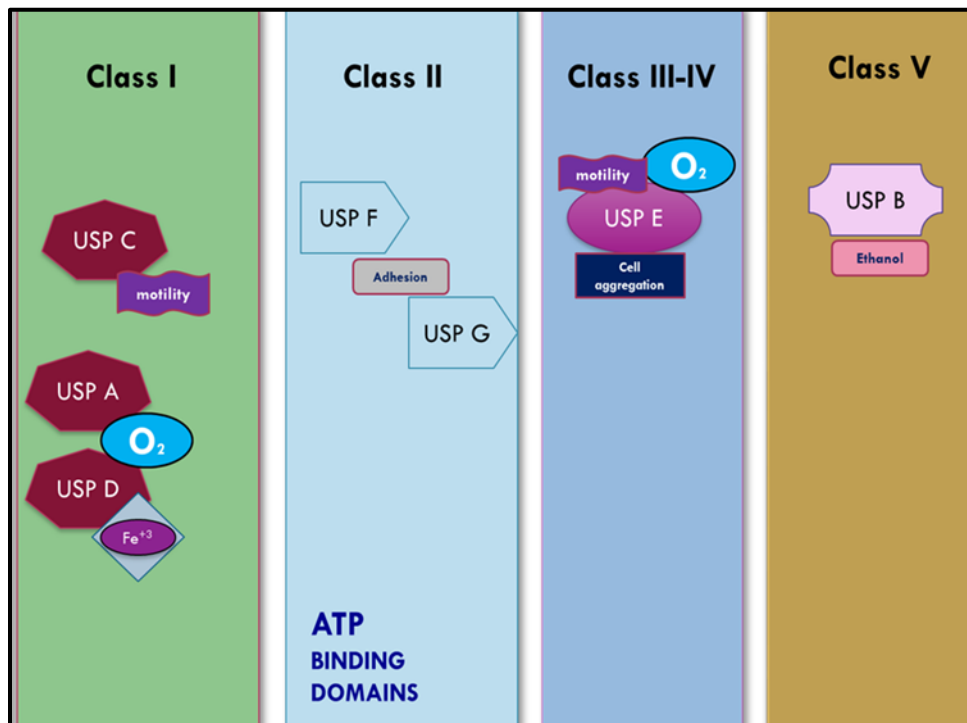
Figure 1.2   USP groups in *E. coli* based on their amino acid sequence similarities. Adapted from (Kvint, *et al.,* 2003).

The *usp* genes are regulated by sigma factors within RNA polymerases, which includes sigma factor σ70 via binding a single promoter region, and they are regulated in a monocistronic fashion (Kvint*, et al.*, 2003). Additionally, *uspA, uspC, uspD, and uspE* are over induced in stationary phase by regulation of RecA, which has a function in repair of DNA by homologous recombination. Thus, the USP genes are assumed to be mediating the protection of DNA (Diez*, et al.*, 2000).

Under stress, USPs are overproduced and through a variety of mechanisms aid the organism in surviving in such uncomfortable conditions (Tkaczuk*, et al.*, 2013). It is also known that USPs are helping pathogens, such as *Salmonella*, *Klebsiella*, or *Mycobacterium*, in the invasion of the host organisms (Hensel, 2009). Involvement of *usp* genes in the persistence and intracellular survival in *Mycobacterium tuberculosis* was reported. (Hingley-Wilson*, et al.*, 2010). Similarly, USPs play a significant role in *Salmonella* growth arrest, stress, and virulence (Liu*, et al.*, 2007). Additionally, *Listeria monocytogenes* intracellular growth adaptation depends on up-regulation in the expression of USPs (Chatterjee*, et al.*, 2006). Two *usp* mutants in *L. monocytogenes* significantly decreased the virulence and survival in macrophages (Seifart Gomes*, et al.*, 2011). Proteomics analysis of *Burkholderia pseudomallei* showed that a down-regulated *usp* is associated with virulence (Al-Maleki*, et al.*, 2014). In a transcriptome study of *Staphylococcus aureus* in lung environment, in vivo expression of *uspA* were increased (Chaffin*, et al.*, 2012). Also, a recent study reported that *uspA* in *Acinetobacter baumannii* is essential for pneumonia and sepsis pathogenesis (Elhosseiny*, et al.*, 2015).

Other USP-related Proteins

Most organisms respond to heat shock by synthesizing a conserved set of proteins, including the DnaK-DnaJ-GrpE and GroEL-GroES complexes (Yura*, et al.*, 1993). These proteins are involved in the maturation of synthesized proteins, and take a

role in the refolding or degradation of proteins (Georgopoulos and Welch, 1993, Hartl, *et al.*, 1994). The heat shock response has been studied well in *Escherichia coli* and *Bacillus subtilis* (Kilstrup, *et al.,* 1997). However, there is no study on heat shock proteins in *E. ictaluri*. Thus, we study six heat shock proteins (GroEL, GroES, DnaK, DnaJ, ClpB, GrpE) and guanosine tetraphosphate (ppGpp), which either regulate or be regulated by USPs.

USPs have a very strong interaction with some other proteins; for example, it was identified that USPA has 83 target proteins are overlap with DnaK, DnaJ, and GroEL, also interactions of 776 ORFs with GroEL and 310 with DnaK (HSP70) in *E. coli* (Arifuzzaman, *et al.*, 2006). GroEL/ES proteins prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions (Begley and Hill, 2015). DnaK/J plays an essential role in DNA replication and participates actively in response to hyperosmotic shock, and DnaK is involved in refolding heat-damaged proteins (Fang, *et al.*, 2016). GroEL was previously determined with various other proteins as a related protein to the bacterial virulence in *Edwardsiella tarda* (Sakai, *et al.*, 2009). Also, it is showed that GroEL was highly expressed in pathogenic strain compared to nonpathogenic strain (Bujan, *et al.*, 2015). Similarly, a selected gene, ClpB controls protein disaggregation in cooperation with the DnaK, DnaJ and GrpE (Motohashi, *et al.*, 1999), and it is sensitive to extreme stresses (Mogk, *et al.*, 2003, Squires, *et al.*, 1991). In *E. tarda*, protein expression of ClpB was higher in virulent strain than non-virulent (Bujan *et al.*, 2015). ClpB is important for recovery of the cell from damage with several rounds of ATP-dependent interactions between DnaJ, DnaK and GrpE are required for fully efficient folding (Fang, *et al.*, 2016).

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The stringent response (SR) manages adaptations to nutritional starvation and various stress conditions (Hauryliuk, *et al.*, 2015, Liu, *et al.*, 2015). SR in bacteria, causing the inhibition of RNA synthesis while they face a shortage of amino acids. RelA/SpoT homology (RSH)-type ppGpp synthetases produces ppGpp and pppGpp by transferring pyrophosphate originating from ATP (Steinchen and Bange, 2016). The ppGpp causes the up-regulation of many other genes involved in stress response such as the genes for amino acid uptake and biosynthesis (Srivatsan and Wang, 2008). Alarmones act by modifying the activity of many cellular targets including DNA replication, transcription, translation, ribosome assembly and metabolism (Dalebroux and Swanson, 2012, Hauryliuk, *et al.,* 2015, Steinchen and Bange, 2016), and it has recently discovered that ppGpp has role in DNA repair mechanism (Kamarthapu, *et al.*, 2016). SR is in the center of bacterial survival and virulence (Ancona, *et al.*, 2015, Chatnaparat, *et al.*, 2015, Dalebroux and Swanson, 2012).

Significance of research and objectives

E. ictaluri establishes systemic infection rapidly in catfish, which requires a quick adaptation to host immune responses. However, mechanisms of *E. ictaluri* stress adaptation are not known. USPs and proteins USPs interact with are expected to take a role in stress adaptation process. Therefore, our aim was to determine the roles of 13 *usp*s (*usp*01 to *usp*13) and 7 stress-related genes (*groEL, groES, dnaK, dnaJ, clpB and grpE, ppGpp*) under various in vivo and in vitro stresses.

This work has two objectives

1: Identify differentially expressed stress related genes in E. ictaluri under

various stressors.

2: Mutate selected E. ictaluri genes and determine virulence in channel catfish.

CHAPTER II

EXPRESSION OF *EDWARDSIELLA ICTALURI* STRESS-RELATED GENES UNDER

VARIOUS STRESSES

Abstract

Edwardsiella ictaluri is a Gram-negative facultative anaerobic rod and the

causative agent of enteric septicemia of catfish (ESC). *E. ictaluri* is one of the most

prevalent pathogens of catfish, causing a significant economic loss in the catfish industry.

E. ictaluri is resistant to complement system in catfish serum and can establish systemic

septicemia rapidly. More studies are needed for understanding the mechanisms of *E.

ictaluri* stress resistance mechanisms. In the present study, we studied *E. ictaluri* stress

responses under in vitro and in vivo stress conditions. In vitro stressors included

hydrogen peroxide, low pH, and catfish serum while in vivo stressor was the catfish

immune system in spleen and head kidney. Among the 13 Universal Stress Proteins

(USP), we found that 3 *usp* genes (*usp05, usp07,* and *usp13*) were highly expressed in all

stress conditions. Also, among the 7 USP-interacting proteins, 5 heat shock proteins

(*groEL, groES, dnaK, grpE, and clpB*) were highly expressed in oxidative stress, and

grpE was also highly expressed in spleen and head kidney environments of *E. ictaluri*

and in low pH condition. Finally, ppGpp was highly expressed in *E. ictaluri* in spleen and

head kidney environments. We are expecting that our results will help a better

understanding the role of USPs and stress response in *E. ictaluri* virulence. Because

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stress proteins are essential for bacterial survival in the catfish host, they may be potential targets for live vaccine development against ESC.

Introduction

E. ictaluri is a Gram-negative bacterium that causes enteric septicemia of catfish (ESC), which is one of the most important diseases causing significant economic loss in farm-raised channel catfish (Shotts, *et al.,* 1986, Thune*, et al.*, 1997)*. E. ictaluri* is a facultative intracellular pathogen like other well-known species in the *Enterobacteriaceae*, and it is capable of surviving inside channel catfish neutrophils and macrophages. Proposed mechanisms of pathogen spread in the host include direct transport in blood or inside phagocytic cells (Booth, 2006, Thune, 1993). Several *E. ictaluri* virulence factors were also determined, which include flagella (Newton and Triche, 1993), extracellular capsular polysaccharide (Stanley, *et al.,* 1994), lipopolysaccharide (Arias*, et al.,* 2003, Lawrence, *et al.,* 2001, Newton and Triche, 1993, Weete, *et al.,* 1988, Williams, *et al.,* 2003), outer membrane proteins (Bader, *et al.,* 2004, Newton, *et al.,* 1990, Skirpstunas and Baldwin, 2003, Vinitnantharat*, et al.,* 1993, Williams, *et al.,* 2003), hemolysins (Williams and Lawrence, 2005) and chondroitinase (Cooper*, et al.,* 1996). Although, mediated feeds with oxytetracycline, sulfadimethoxine, or florfenicol are used in catfish farms to treat ESC, medicated feed is effective for limiting the spread of an outbreak rather that treating the disease because of early onset of anorexia. As an alternative treatment method, live attenuated vaccines could be an effective approach for prevention of ESC in catfish farms.

Pathogenic bacteria can sense and respond to diverse microenvironmental stresses encountered during infection. A detailed understanding of bacterial stress responses

provides understandings of host microenvironments, mechanisms of virulence and potential targets for treatment of infectious diseases (Fang, *et al.,* 2016). A conserved group of stress proteins called universal stress proteins (USPs) are generally between 140-160 amino acids. Archaea, bacteria, plants, and fungi have these domains, but the human does not have USPs. *E. coli* has six *usp* genes, which involves in several functions from oxidative stress to adhesion and motility (Nachin, *et al*., 2005). These genes are regulated by sigma factors within RNA polymerases, which include sigma factor σ70. Also, ppGpp is an important regulator of USPs. (Kvint, *et al*., 2003). Under stress, USPs are overproduced and aid the organism in surviving in stressful conditions (Tkaczuk*, et al.,* 2013). It is known that USPs are important for *Salmonella*, *Klebsiella*, and *Mycobacterium* to invade the host organisms (Hensel, 2009). USP are also involved in the persistence and intracellular survival of *Mycobacterium tuberculosis* (Hingley-Wilson, *et al.,* 2010), growth arrest and virulence in *Salmonella* (Liu, *et al.,* 2007) virulence of *Burkholderia pseudomallei* (Al-Maleki, *et al.,* 2014), intracellular growth adaptation of *Listeria monocytogenes* (Chatterjee*, et al.,* 2006). In *Staphylococcus aureus*, virulence factors were downregulated in vivo while expression of *uspA* increased (Chaffin, *et al.,* 2012). In *Acinetobacter baumannii*, *uspA* is essential for pneumonia and sepsis pathogenesis (Elhosseiny, *et al*., 2015).

USPs can interact with several heat shock proteins and other stress related proteins to help bacterial virulence and stress response. Therefore, we planned to study the expression of 13 *usp* (*usp01* to *usp13*) and 7 stress related genes, which include 6 heat shock proteins (*groEL, groES, dnaK, dnaJ, clpB, and grpE*) and a guanosine pentaphosphate (*ppGpp* synthetase). We expect that expression analysis of selected genes

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will help to better understand stress responses in *E. ictaluri* and some genes might be targeted for genetic mutation to develop live attenuated vaccines.

Materials and Methods

Bacterial growth conditions

E. ictaluri strain 93-146 was grown at 30°C using brain heart infusion (BHI) broth and agar (Difco, Sparks, MD). *E. coli* were cultured at 37°C using Luria-Bertani (LB) broth and agar (Difco). *E. coli* SM10λ*pir* were used for transferring pAK*gfplux*1 into *E. ictaluri*. Ampicillin (100 µg/ml) and colistin (12.5 µg/ml) antibiotics were used when needed.

Acid stress (low pH exposure)

A single *E. ictaluri* wild-type colony was inoculated in 5 ml of BHI broth, followed by 16-18 h incubation at 30ºC with shaking. The next day, 40 ml of BHI broth were inoculated then subculture and grown to an optical density at 600 nm (OD_{600}) of 0.4 before our experiments. Then each culture was divided into four aliquots, 10 ml each and cells were harvested by centrifugation at 6,000 g for 15 min. Furthermore, the supernatant removed and cells were resuspended in a BHI broth that acidified with a 6 N HCL solution (Sigma) to pH 04, and with only BHI broth. These tubes incubated with shaking (180 rpm) at 30 ºC for 30 min., and harvested pellets in 7000 RPM for 15 min, and placed into RNAlater and stored for a week at -20ºC until RNA is isolated.

Oxidative stress

E. ictaluri 93-146 streaked on brain heart infusion (BHI) (Difco) agar plate containing 12.5 µg/ml of colistin (Sigma) and incubated at 30 ºC for 48 h. Small cultures

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prepared by inoculating colonies in 5 ml of BHI broth with 12.5 μg/ml of colistin and incubating for 16-18h at 30 ºC with shaking at 200 rpm. 40 ml of BHI broth were inoculated then subculture and grown to an optical density at 600 nm (OD600) of 0.4 before our experiments. Then each culture was divided into four aliquots, 10 ml each and cells were harvested by centrifugation at 6,000 g for 15 min. Furthermore, the supernatant removed, and cells were resuspended in BHI broth or in 20 ml of different fresh BHI supplemented with 1.5mM (0.05%) H_2O_2. These tubes incubated with shaking (180 rpm) at 30 ºC for 30 min. The bacteria were harvested by centrifuge them at 7000 RPM for 15 min., then bacteria pellets were taken directly into RNA later and stored a week at -20ºC until RNA isolation.

Serum stress

E. ictaluri exposed to naïve catfish serum; normal serum was our treatment and heat-inactivated catfish serum was our control, and each treatment included four biological replicates. *E. ictaluri* cultures washed three times using 1.25 ml of cell wash buffer (10 mM TrisCl and 5 mM magnesium acetate). Normal and heat-inactivated serum (1.25 ml) added onto *E. ictaluri* pellet, mixed, and incubated for 30 min at 30 ºC during incubation; the tubes inverted to mix bacteria and serum thoroughly. After the incubation, serum-bacteria mixture was used for total RNA isolation.

Host stress

Experiments involving live catfish were conducted by the "Institutional Animal Care and Use Committee (IACUC) at Mississippi State University. Approximately eighteen-month-old SPF channel catfish fingerlings (19.728 cm, 18.544 g) were stocked

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into two tanks at a rate of 7 fish/tank. After one week of acclimation, Fish were anesthetized in water containing 100 mg/L MS222 and injected with *E. ictaluri* wild type (approximately 1×10^4 CFU) in 100 µl PBS. Negative control was injected with 100 µl PBS.

Bioluminescent imaging (BLI) was conducted using an IVIS Imaging System to measure some photons emitted by bioluminescent bacteria in fish (Karsi, *et al.,* 2006) (Fig.3). Plasmids pAKgfplux1 was transferred from *E. coli* donor strain SM10 λpir into *E. ictaluri* recipient strain 93-146 by conjugation (Karsi and Lawrence, 2007) (Table 2.2). Catfish were anesthetized in water containing 100 mg/L MS222 and transferred immediately to the photon collection chamber for image capture. Total photon emissions from the whole fish body were collected at an exposure 30 seconds. Following BLI imaging, fish were returned to the well-aerated water for recovery. BLI was conducted at 3, 6, 12, 24 and 30 h postinfection. Bioluminescence was quantified from the fish images using Living Image Software v 4.2 (Caliper Life Sciences., Hopkinton, Massachusetts), and mean photon counts for each treatment were used in the statistical analysis (Fig.2.3, Fig.2.4).

At the end of the 30h exposure, head kidneys and spleens were collected in all fish. The organs immediately dropped to RNA later to stabilize gene expression. Then RNA isolated with a previously described method with an RNeasy mini kit (Qiagen). Bioluminescent pictures of fish were taken.

Total RNA isolation

Total RNA was isolated from four biological replicates by using RNeasy Protect Bacteria Mini Kit (Qiagen). Contaminating bacterial DNA was eliminated by DNase I

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treatment with RNase-Free DNase Set (Qiagen). The quality and concentration of the

isolated total RNA measured by NanoDrop 1000 (Thermo Scientific).

cDNA preparation

First-strand cDNA was produced from 1 µg total RNA using Maxima First Strand

cDNA Synthesis Kit for RT-qPCR (Thermo Scientific).

Gene determination

In this study, we used NCBI to determine 13 USPs in *E. ictaluri,* and from

literature searches, some regulatory proteins for USPs were found. Nucleotide sequences

of the *E. ictaluri* USPs and other stress-related genes were obtained from the *E. ictaluri*

genome (Williams*, et al.*, 2012). It is also used protein-protein interaction network online

software STRING10 (Szklarczyk et al., 2015) to determine USP related genes.

Real-Time qPCR

For gene-expression analysis, quantitative real-time PCR (qRT-PCR) was

performed using an Mx3005P qPCR System (Agilent Technologies, CA, USA) and

DyNAmo SYBR Green qPCR Kit was used (Finnzymes Oy, Espoo, Finland). Each 20 µl

PCR reaction contained 10 ul SYBR Green 2X mix, 0.2 µM each of forward and reverse

primers (Table 2.1), and 1 µl of 100 x diluted cDNA. The PCR was set to initial

denaturation at 95°C for 3 min, 45 cycles of denaturation at 95°C for 15 s, annealing at

60°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 3 min. At

the end of the PCR, a melting curve program from 60°C to 95°C with 0.5°C increase

every 15 s were run. The assessment was done by PCR to determine the suitability of

primers and experimental conditions. We used 16S RNA gene internal control. A sample

from unstressed condition set as a calibrator in each experiment except heat-treated serum was used as a calibrator against normal serum. Relative expression rates were calculated by the threshold cycle changes in sample and calibrator. The ΔΔct method (Livak and Schmittgen 2001) was used to calculate the expression level of related genes. All expression values were normalized against 16S rRNA. Relative expression was determined by the comparative CT method of relative quantification (RQ), calculated with the arithmetic formula $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct$ was calculated by $\Delta\Delta Ct = \Delta Ct$ (stress condition) - ΔCt (non-stress condition), where ΔCt is the normalized signal level in a sample ($\Delta Ct = Ct$ of target gene – Ct of reference gene). The one-way Analysis of Variance (ANOVA) test was used to compare gene expression among conditions by using SAS 9.1.4 (SAS Inc., USA). P value selected for statistical significance was < 0.05.

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Table 2.1   Primers used for expression analysis of 20 genes

Gene Name	Gene ID	Primer	Size	Sequence (5`-3`)	Product (bp)	Locus ID
usp01	-	F	21	TCTACAGGCGTTGGCTGATAC	126	NT01EI_1627
		R	21	ATCAGGATCAGGTCGGCATC		
usp02	uspF	F	20	TGAACACACCCATTTCGAGA	175	NT01EI_1751
		R	18	CCGGTCTATGGCGCAAAG		
usp03	-	F	21	AGGACGCTCATATCCATTTCC	190	NT01EI_1786
		R	21	GAGCCGATGGCAATAGTACAG		
usp04	uspE	F	21	GATATTCTGGCAGCAGAGTGC	134	NT01EI_1962
		R	21	TCTGCAACCTCTTTCAACACC		
usp05	uspA	F	20	GAAAATTGACGGTGATGGCG	203	NT01EI_1981
		R	20	GCTGAGCGTCAATCCATCAA		
usp06	uspA	F	22	CTACCACGATCCACTGAACATC	165	NT01EI_2616
		R	22	CGAATAGCCTCGTTGTAGATCC		
usp07	kdpD	F	21	CGCATGTCCAGAAATTACCAT	147	NT01EI_2891
		R	21	GCCACGGAAAGAAATGAGATA		
usp08	uspA	F	22	ATGTTCTGGTTCTGATTCATGG	150	NT01EI_3729
		R	22	TCAGATTGACATCCAGCATACC		
usp09	uspA	F	21	CTGGTGATGGTCTACCTGCTC	186	NT01EI_3778
		R	21	AATACCGACGATCAGCATCAC		
usp10	-	F	20	AGAATTCCAGGTGTAGCGGT	110	NT01EI_1634
		R	19	GCAGTAACGGCGCTCATAG		
usp11	-	F	21	GTTAATCTGGGCGATATGCAA	195	NT01EI_1803
		R	21	GCTCCAGAAGTCCTGATGATG		

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Table 2.1 (Continued)

usp12	*uspB*	F	20	TACTGACACAGAATGCGTTGG	120	NT01EI_3728
		R	20	TACCAAATGAGTAGGCGGTGA		
usp13	*cpxP*	F	20	AGAGTTTGATCCTGGCTCAG	147	NT01EI_3810
		R	19	GGTTACCTTGTTACGACTT		
clpB	*clpB*	F	21	CCAAGTATTTACCGTCCGCAA	166	NT01EI_3205
		R	21	ACCCTGAGCTGTTTTAATGCG		
dnaJ	*dnaJ*	F	20	CTGAGTGCGATGTCTGCCAC	123	NT01EI_0673
		R	20	AAGGCTGCTGTACGGTGAAG		
dnaK	*dnaK*	F	21	TACAACATGGATCTGACGCTG	186	NT01EI_0672
		R	20	CTGTACGGTGAAGAAGCCCT		
GroEL	*groL*	F	20	GTGGTATCGTGAAAGTGGCG	124	NT01EI_0380
		R	21	AGCTCCATACCGATCTCTTCA		
groES	*groS*	F	20	TTGAGTCCAAGTCTGCTGGC	162	NT01EI_0379
		R	21	ACCATCGTTGAAAATCACGGT		
grpE	*grpE*	F	21	GATGTCGAGAAGGCGCACAAG	158	NT01EI_3062
		R	21	AGCAGGGATTTCAGGGTCAG		
ppGpp	*ppGpp* synthetase	F	20	CGGGTGAAGGATTACATCGC	170	NT01EI_0032
		R	21	GACTCGCCCTCTTTATACGCC		
16sRNA	-	F	20	AGAGTTTGATCCTGGCTCAG	150	NT01EI_0010
		R	19	GGTTACCTTGTTACGACTT		

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Table 2.2   Bacterial strains and plasmids for expression analysis

Strain	Relevant characteristics	References
Edwardsiella ictaluri 93-146	Wild type; pEI1+; pEI2+; Col^r	(Lawrence*, et al.*, 1997)
Escherichia coli SM10λ*pir*	thi; thr; leu; tonA; lacY; supE; recA;::RP4-2-Tc::Mu; Kmr; lpirR6K	(Miller and Mekalanos, 1988)
Plasmids		
	5681bp, EcorI, SacI, T7, PstI, EcoRI, HpaI, AseI, BstBI	(Karsi and Lawrence, 2007)

Results

E. ictaluri USP and stress-related genes

We determined 13 USPs and 7 stress related genes interacting with USPs in the *E. ictaluri* genome (Fig.2.1).

1	USP01	11	USP11
2	USP02	12	USP12
3	USP03	13	USP13
4	USP04	14	ClpB
5	USP05	15	DnaJ
6	USP06	16	DnaK
7	USP07	17	GroEL
8	USP08	18	GroES
9	USP09	19	GrpE
10	USP10	20	ppGpp
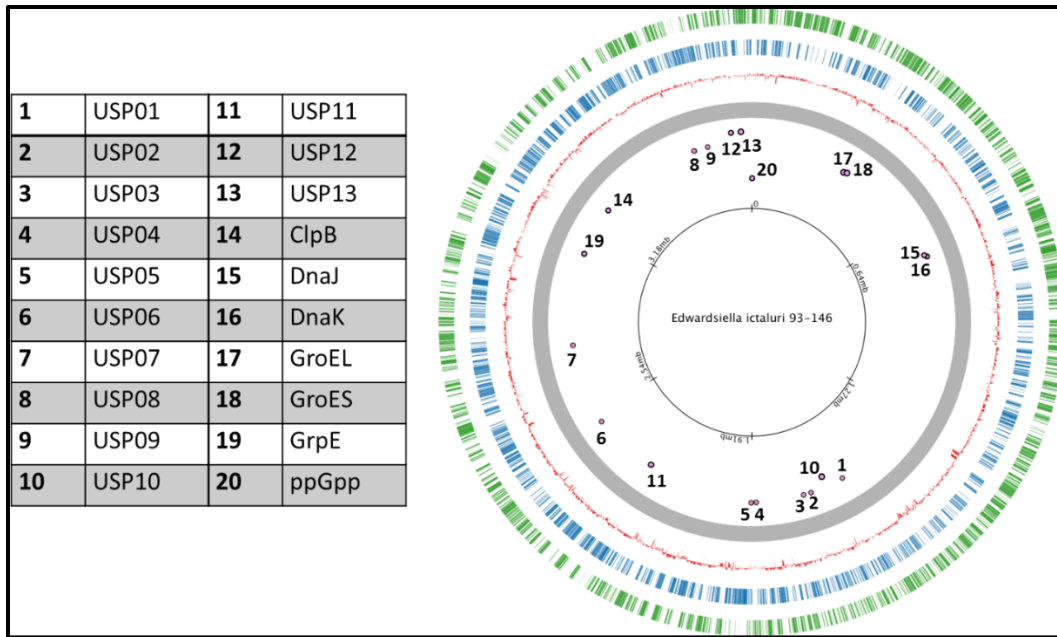
Figure 2.1   Gene's location in *E. ictaluri.*

Protein-protein interaction network indicated that six USPs were in direct relation to six heat shock proteins (GroL, GroS, DnaJ, DnaK, ClpB, and GrpE), which were chosen for expression analysis (Fig.2.2) (Szklarczyk et al., 2015). Additionally, ppGpp synthetase is known as an important regulator for some USPs (Liu*, et al.*, 2007), which was also included in our gene expression analysis.

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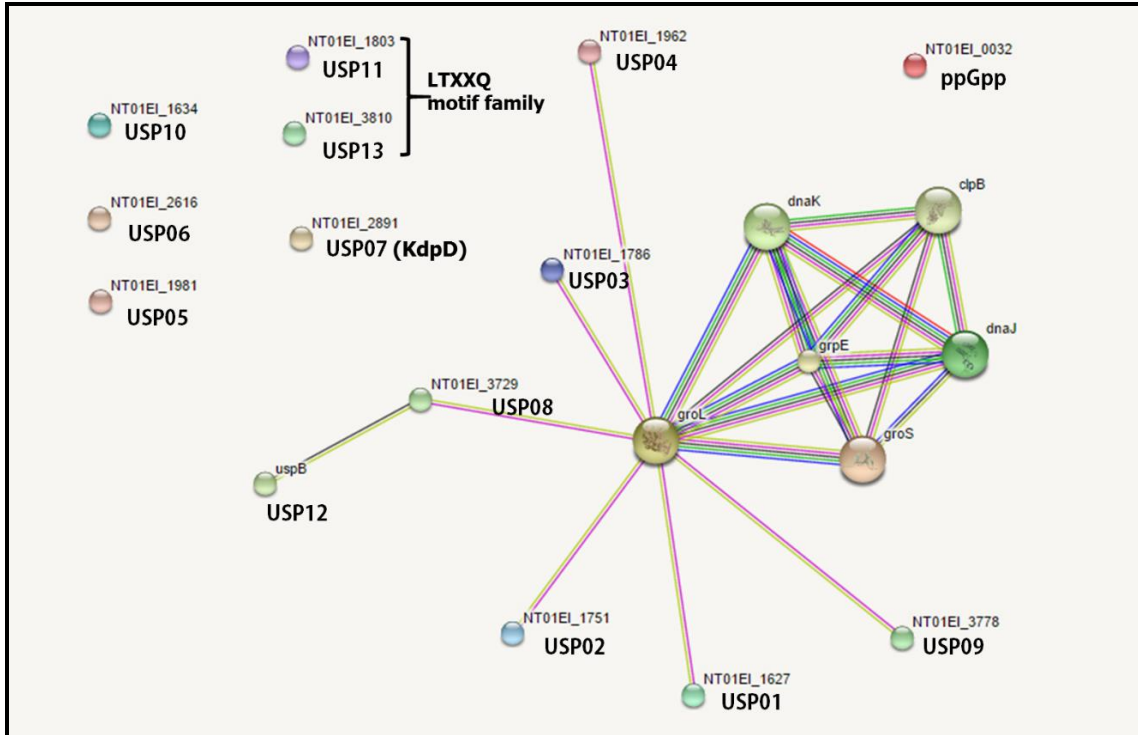
Figure 2.2   Functional protein association network showing the relationship among USPs and heat shock proteins in *E. ictaluri* (Szklarczyk et al., 2015).
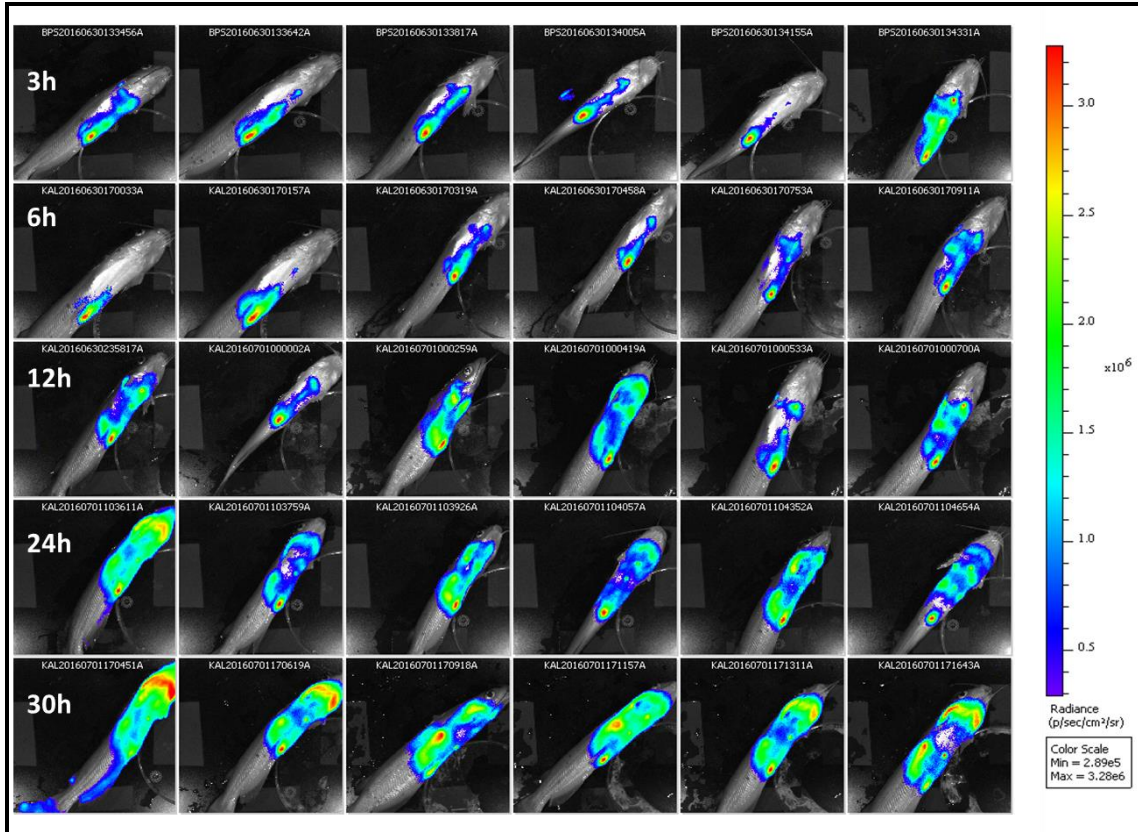
Figure 2.3   Bioluminescent imaging of *E. ictaluri* in live catfish after intraperitoneal injection.

Each line shows time interval for exposures. At the end of 30 hours catfish organs were taken.
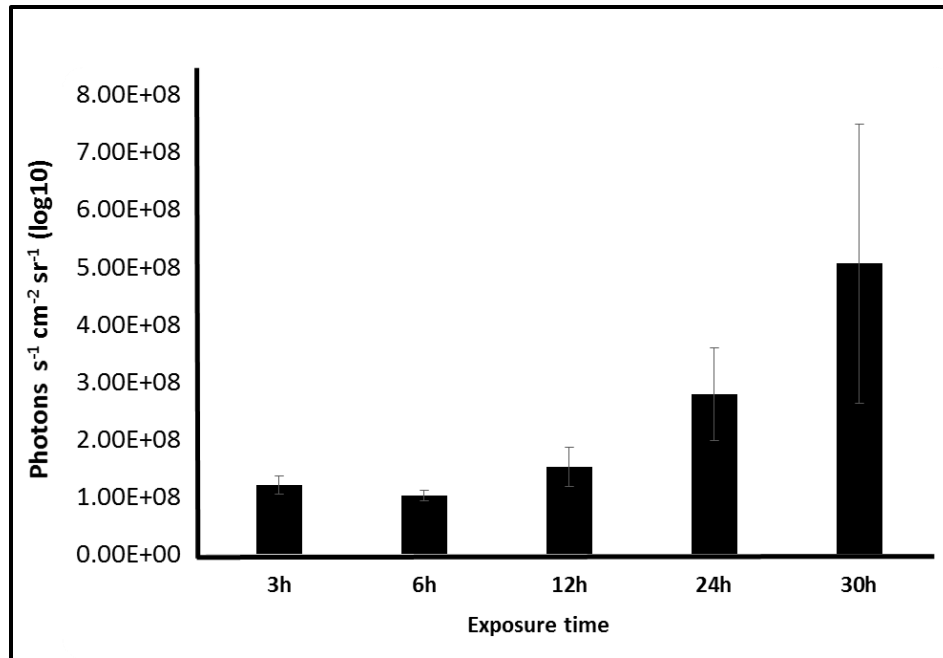
Figure 2.4   Total photon emissions calculated from six catfish at each time point.

Expression analysis of stress-related genes in acid stress

Stress genes were exposed to acidic pH 04, and expression of 20 genes was determined (Fig.2.5). The *usp13* showed the highest expression level with 34 Fold Change (FC) relative to non-stress condition. Additionally, *usp05* and *usp11* expressions were more than 5FC. Finally, *usp08, usp09, usp10, usp12* and *grpE* showed more than 2 FC high expression (Fig.2.5).

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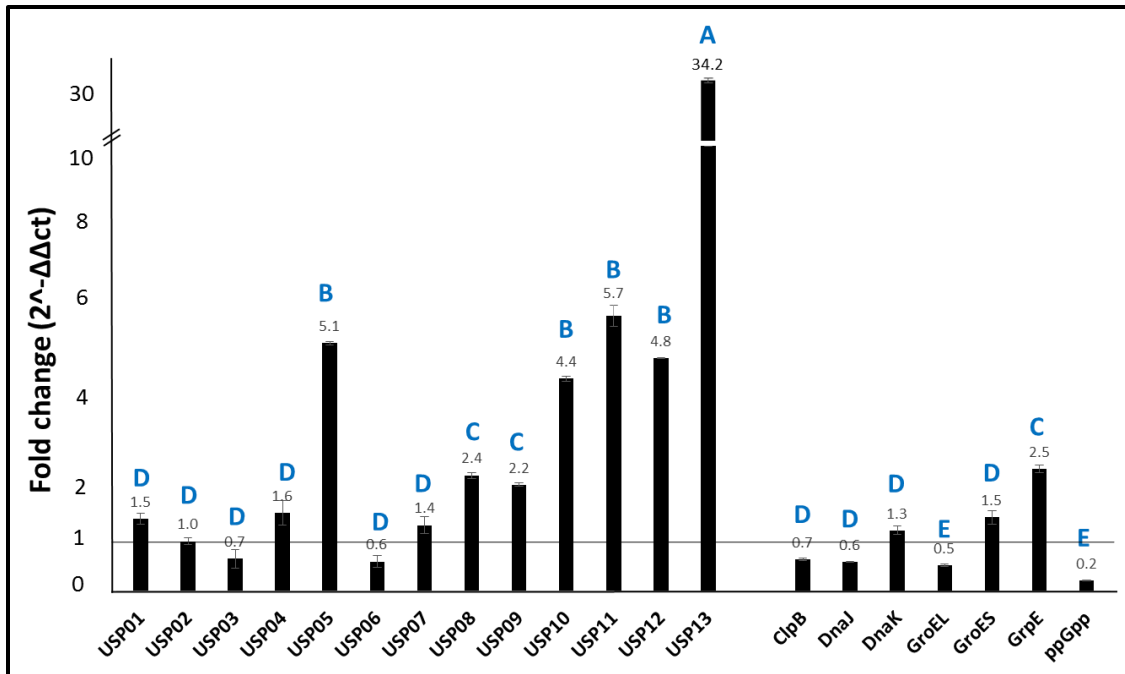
Figure 2.5   Relative expression values of 20 stress genes in *E. ictaluri* exposed to pH 4 stress.

Expression values were normalized by 16S rRNA expression. Letters indicate statistical groupings. Significance level was P < 0.05.Higher than 1FC is upregulated and less than 1FC is down regulated.

Expression analysis of stress-related genes in oxidative stress

Expression of 20 stress genes determined when E. ictaluri exposed to 0.05% H2O2 (Fig.2.6). Gene groES and dnaK showed 18 fold and 11 fold high expression levels, respectively, which was significantly higher than other genes. Also, usp05, groEL, and grpE showed more than 8 fold high expression. The gene clpB had 4.5 fold high gene expression, and finally, usp08, usp09, and usp11 had more than 2 fold high expression (Fig.2.6).

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Figure 2.6   Relative expression values of 20 stress genes in *E. ictaluri* exposed to H_2O_2.

Expression values were normalized by 16S rRNA expression. Letters indicate statistical groupings. Significance level was $P < 0.05$. Higher than 1FC is upregulated and less than 1FC is down regulated.

Expression analysis of stress-related genes in serum

      E. ictaluri exposed to non-treated and treated serum, and the expression of 20 genes was determined (Fig.2.7). Normal serum group used as a stressor and heat treated serum was the control group. The *usp13* showed the highest expression level with 62.2 FC relative to non-stress condition. The *usp11* had 7.7 FC high expression. Furthermore, *usp02, usp03, usp04, usp06, usp08, usp09, usp10* and *usp12* had more than 4 FCs high expression. Finally, *usp05* showed more than 2 FC expression (Fig.2.7).

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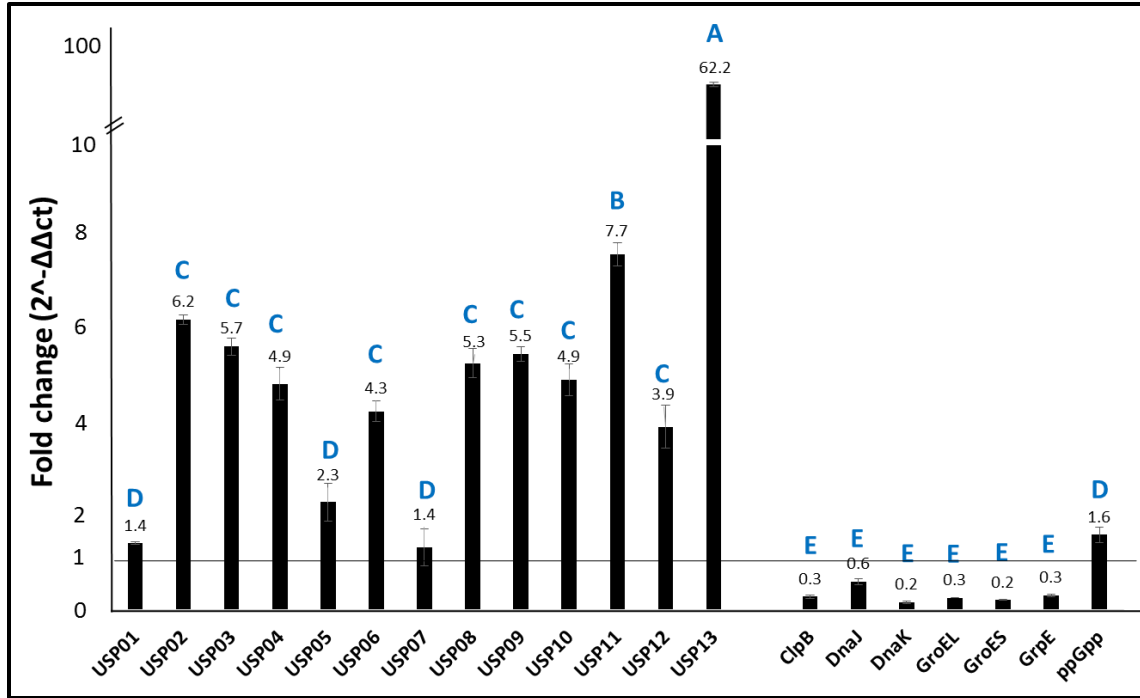
Figure 2.7   Relative expression values of 20 stress genes in *E. ictaluri* exposed to naïve
catfish serum and heat treated serum.

Expression values were normalized by 16S rRNA. Letters indicate statistical groupings.
Significance level was $P < 0.05$. Higher than 1FC is upregulated and less than 1FC is
down regulated.

Expression analysis of stress-related genes in catfish

The expression levels of 20 stress genes were determined in the host at 30h after
injection of wild-type *E. ictaluri*. The expression levels of each *E. ictaluri* gene in spleen
and head kidney were compared to that of *E. ictaluri* grown in BHI broth. The *usp05* and
USP13 showed the highest expression levels in the spleen with 191 and 137 FC. *usp07*
showed 18 FC expression; *usp04, usp06, usp10* and *grpE* expressions were more than 7.8
FC; and *usp08, usp09, usp11*, and *ppGpp* expressions were more than 2 FC (Fig.2.8).
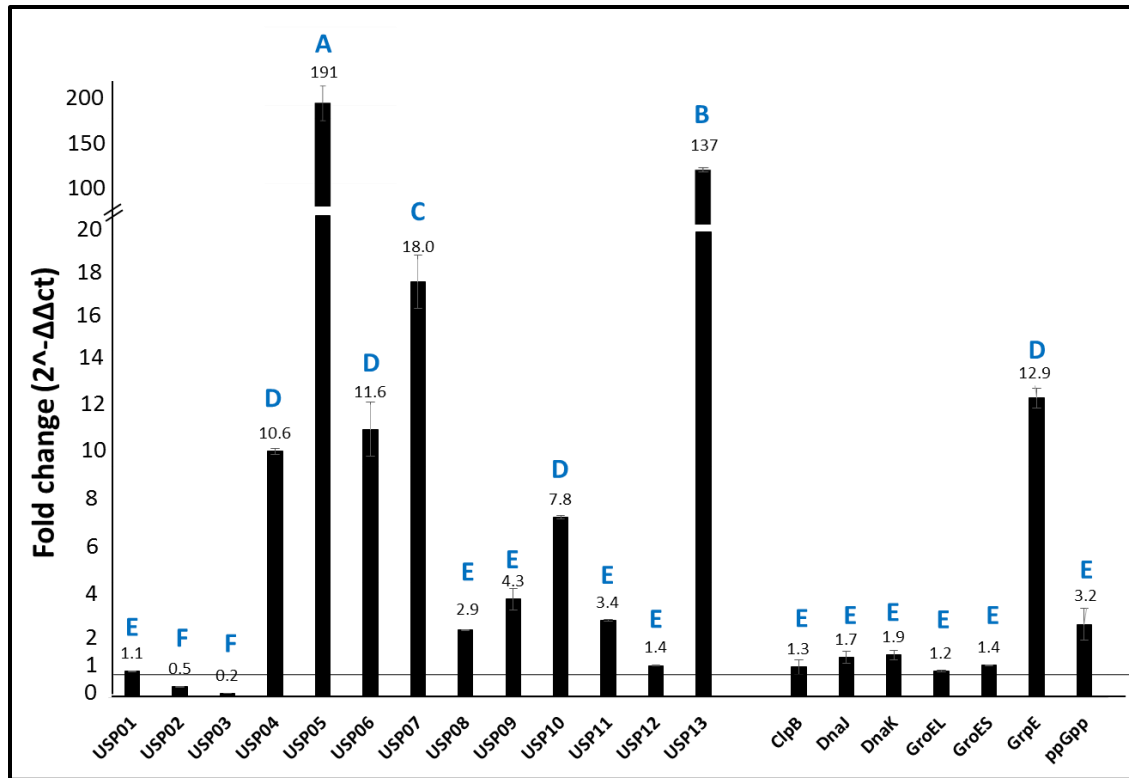
Figure 2.8   Relative expression values of 20 stress related genes in catfish spleen.

Expression values were normalized by 16S rRNA. The expression level with the same letter are not significantly different; and significant difference (P<0.05) showed with an LSD ANOVA. Higher than 1FC is upregulated and less than 1FC is down regulated.

Relative expression of 20 stress genes in infected catfish head kidney is shown in (Fig.2.9). Gene *usp07* showed the highest expression level with 139 FC relative to non-stress condition. The *usp05* and *usp13* showed a significant increase with more than 22 FC. The *usp10* showed more 10 fold higher expression. Also, *usp04, usp06, usp09, grpE* and *ppGpp* showed more than 4 FC. The gene expression of *usp08, usp11, usp12, dnaK* and *GroEL, GroES* were higher than 2 FC.
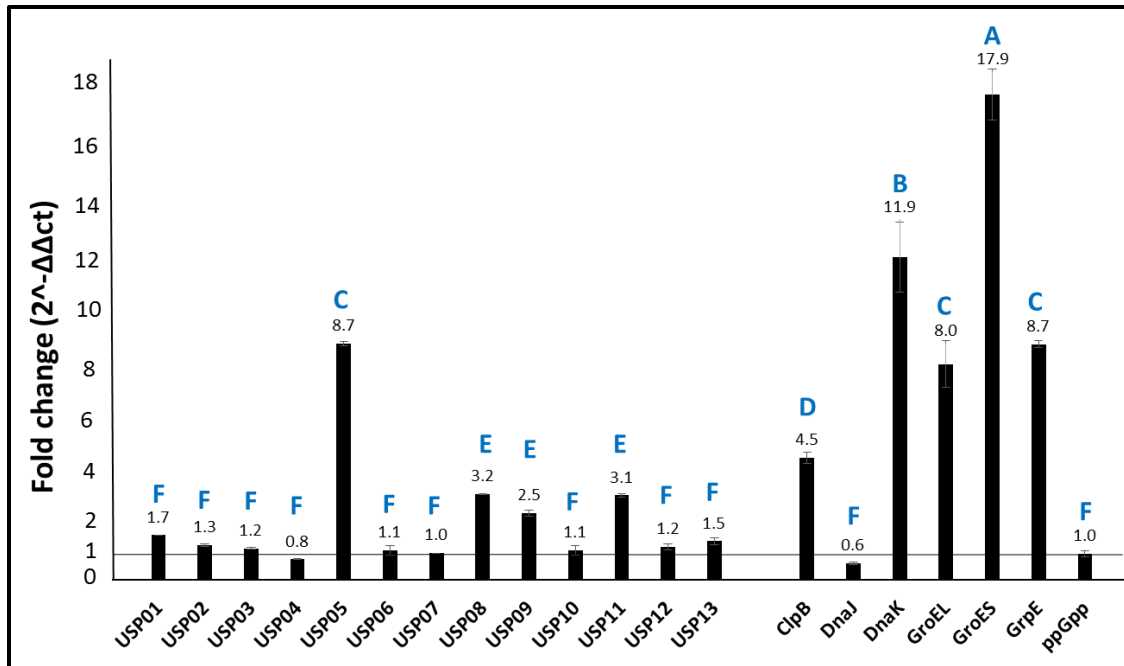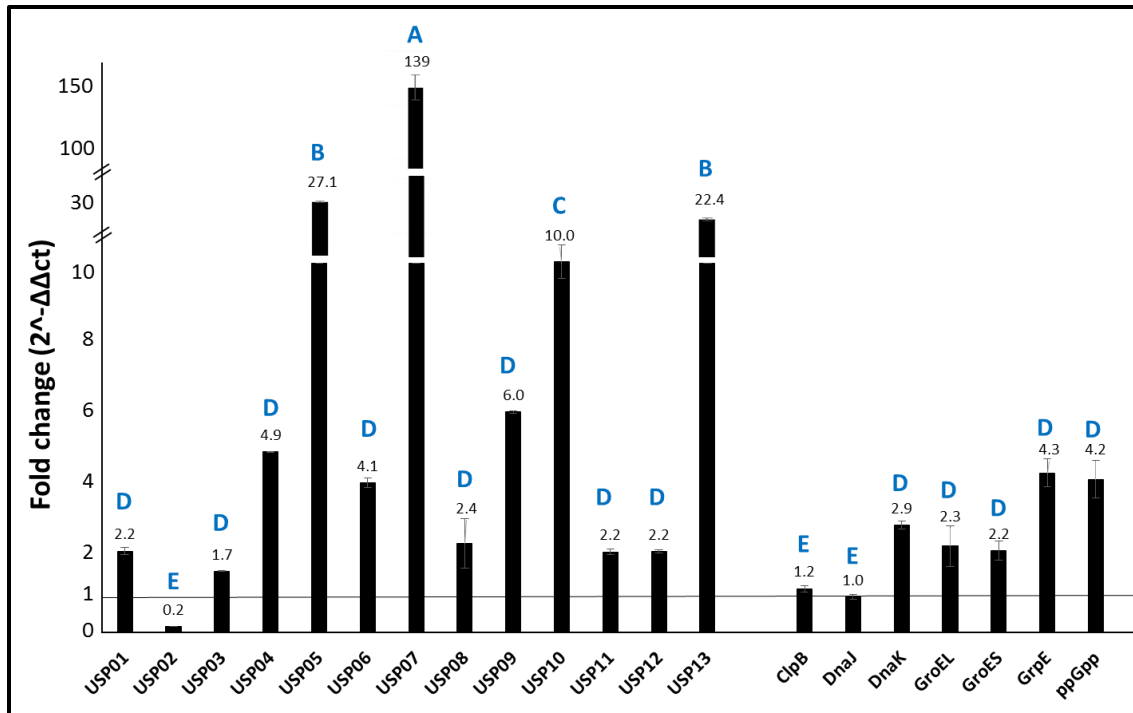
Figure 2.9   Relative expression values of 20 stress genes in infected catfish kidney.

Expression values were normalized by 16S rRNA. The expression level with the same letter are not significantly different; and significant difference (P<0.05) showed with an LSD ANOVA. Higher than 1FC is upregulated and less than 1FC is down regulated.

Gene Expression Analysis (q-RT PCR)					
Gene IDs/Conditions→	In-vitro			In-vivo	
	pH	H2O2	Serum	Spleen	Head Kidney
usp01	1.5	1.7	1.4	1.1	2.2
usp02	1.0	1.3	6.2	0.5	0.2
usp03	0.7	1.2	5.7	0.2	1.7
usp04	1.6	0.8	4.9	10.6	4.9
usp05	5.1	8.7	2.3	191	27.1
usp06	0.6	1.1	4.3	11.6	4.1
usp07	1.4	1.0	1.4	18	139
usp08	2.4	3.2	5.3	2.9	2.4
usp09	2.2	2.5	5.5	4.3	6.0
usp10	4.4	1.1	4.9	7.8	10
usp11	5.7	3.1	7.7	3.4	2.2
usp12	4.8	1.2	3.9	1.4	2.2
usp13	34.2	1.5	62.2	137	22.4
clpB	0.7	4.5	0.3	1.3	1.2
dnaJ	0.6	0.6	0.6	1.7	1.0
dnaK	1.3	11.9	0.2	1.9	2.9
groEL	0.5	8.0	0.3	1.2	2.3
groES	1.5	17.9	0.2	1.4	2.2
grpE	2.5	8.7	0.3	12.9	4.3
ppGpp	0.2	1.0	1.6	3.2	4.2

Figure 2.10   Relative expression values of 20 stress genes in all condition.

Expression values were normalized by 16S rRNA. The expression levels showed with FCs. The FCs values higher than 2 were indicated with bold.

Discussion

Universal stress proteins (USPs) present in archaea, bacteria, plants and fungi, but they are not present in humans (Nachin, *et al.,* 2005). *E. ictaluri* is a Gram-negative bacterium that causes ESC, and expression analysis in USPs and other stress-related genes interacting with USPs, such as heat shock proteins, were not studied before. Therefore, we explored how *E. ictaluri* responds various stressful conditions by studying expression analysis of 13 *usp* genes, 6 heat-shock proteins, and *ppGpp* in different stress conditions. Identification of essential genes providing resilience to *E. ictaluri* under stress may yield new targets for live attenuated vaccine development against ESC.

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The process, known as phagocytosis, involves attaching to the bacterial cell, bringing it into phagosome, then forming a phagolysosome. The lysosome releases acid-activated enzymes and toxic metabolites, the vacuole acidifies to pH 4-5, and the bacterial cell is killed and broken down by the enzymes and toxins. *E. ictaluri* is able to avoid this process and replicate in these cells. The key mechanisms that *E. ictaluri* uses to advance the disease process and avoid the host defense system. Selected stress conditions were phagosome imitated like previous works in *E. ictaluri* (Rogge and Thune, 2011).

The complement system can lyse foreign cells and opsonize foreign organisms and destroy by attracting phagocytes. Fish complement system can inactivate bacterial exotoxins (Yano, 1996). In fish, three pathways of complement activation have been identified which are the classical complement pathway (CCP), alternative complement pathway (ACP), and the lectin complement pathway (LCP) (Holland and Lambris, 2002). First, CCP can be initiated by acute-phase protein such as ligand-bound C-reactive protein, by binding antibody to host cell surface, or by some viruses, bacteria and virus-infected cells (Petersen *et al.,* 2000). Second, ACP is directly activated by viruses, bacteria, fungi, or tumor cells without antibody binding. According to the mechanism mentioned, ACP is antibody independent. Third, LCP can be activated by binding of mannose-binding lectin (MBL), a protein complex, and the serine proteases. The MBL connects proteases 1 and 2 (MASP-1 and MACP-2) to mannans on bacterial cell surface; therefore, the activation of LCP is independent of antibody also (Holland and Lambris, 2002).

E. ictaluri (Ourth and Bachinski, 1987), is resistant to serum. In *E. ictaluri*, sialic acid-induced suppression on the activation of the ACP was thought to be the mechanism

to explain why *E. ictaluri* can survive in normal serum obtained from channel catfish (Ourth and Bachinski, 1987). Moreover, in virulent *E. ictaluri*, `larger amount of surface proteins and polysaccharide capsular material were found more than that in avirulent strains (Stanley *et al.,* 1994) These surface components most probably prevent bactericidal materials in normal serum, such as complement and lysozyme, from contacting with vulnerable sites on the bacteria surface (Evelyn, 1996). In our study, gene expression of USPs in serum were upregulated; on the other hand, gene expression of HSPs were almost down regulated.

We determined that *usp05, usp08, usp09, usp10, usp11, usp12, usp13*, and *grpE* have more than 2-fold high expression levels in acid stress, especially *usp13* showed 34.2 fold increase. Oxidative stress resulted in more than 2-fold high expression in *usp05*, *usp08*, *usp09*, *usp11*, *clpB*, *dnaK*, *groEL*, *groES*, and *grpE*. Especially, *usp05*, *dnaK*, *groEL*, *groES*, and *grpE* expressions were more than 8-fold high. Serum exposure caused high expression levels in the majority of *usp* genes. In particular, usp13 expression was 62.2 fold higher compared to control group. In vivo stress conditions caused more than 2-fold high expression in the majority of the *usp* genes. In spleen, *usp04, usp05, usp06, usp07, usp13*, and *grpE* expression were more than 10 fold higher compared to control. Similarly, in head kidney, *usp05, usp07, usp10*, and *usp13* expressions were more than 10 fold higher compared to control. In vitro condition, *usp05, usp08, usp09*, and *usp11* were always highly expressed, and in vivo condition, *usp04, usp05, usp06, usp07, usp08, usp10, usp13, grpE* and *ppGpp* were highly expressed. These data indicate that in overall, *usp05, usp07, and usp13* may possibly be important for *E. ictaluri* to cope with several difference stressors includes in vivo and in-vitro conditions.

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In a previous two studies in our lab, *usp05* was deleted by transposon insertion and resulting mutant had reduced attenuation in catfish and provided protection to vaccinated catfish against wild-type infection (Kalindamar, 2013). Also, *usp02* and *usp04* showed a lower expression in our microarray analysis study when host encounter (Lu, 2013). Similarly, *usp02* were less expressed in spleen and kidney; however, *usp04* were upregulated in kidney and spleen oppositely to microarray study. The gene *usp05* is known as *uspA*, and it is found that it is an important regulator of survival and virulence in many pathogens (Tkaczuk, *et al.,* 2013). *E. coli uspA* mutant caused a survival defect under a variety of growth-arrested conditions, whereas overexpression induces growth in the growth-arrested state (Tkaczuk, *et al.,* 2013). *E. coli* has six *usp* genes involving in functions ranging from oxidative stress to adhesion and motility (Nachin, *et al.,* 2005). Our data suggests that *usp05* could be an important virulence gene in *E. ictaluri* survival in the host.

USP07 is known as KdpD protein, and the USP domain within KdpD shares similarities to the USP proteins of the USPA subfamily (Heermann*, et al.*, 2009). Biochemical studies revealed that USPC interacts specifically with the USP domain in the stimulus perceiving N-terminal domain of KdpD, and UspC stabilized the KdpD/KdpE and DNA complex to act as a scaffolding protein under salt stress (Moscoso*, et al.*, 2016). KdpD function as a membrane-associated protein kinase that phosphorylates KdpE in response to environmental signals (Heermann, *et al.,* 2009). It has been suggested that binding of other USP proteins might either alter the secondary structure of the protein or perhaps facilitate activation. The ability of KdpD/KdpE to sense and respond to stress may thus be mediated via this USP-binding domain, and the variability

of that domain between species might contribute to differences in bacterial virulence (Freeman *et al.,* 2013). Mutant *kdpD* in *Salmonella typhimurium* is attenuated in animal and macrophage survival experiment. Also it is promoting resistance to osmotic, oxidative and antimicrobial stresses (Alegado*, et al.*, 2011). Furthermore, antimicrobial stress, osmotic stress, and oxidative stress are associated with KdpD/KdpE activity KdpD/KdpE, therefore, appears to be an important adaptive two component system employed during host infection, promoting bacterial virulence and survival through mechanisms both related to and distinct from its conserved role in K+ regulation (Freeman*, et al.*, 2013). In our study especially in the organs (head kidney and spleen) USP07 has a very high expression, and it is important to note that this gene might have involved in host survival of *E. ictaluri.*

USP13 is known as extracytoplasmic adaptor protein CpxP, and Cpx system is comprised of the inner membrane histidine kinase CpxA and the cytoplasmic response regulator CpxR (Debnath*, et al.*, 2013, Vogt and Raivio, 2012). CpxR binding sites are located upstream of the *cpxP* gene, and CpXP is the most highly inducible member of the Cpx regulon, and it has elevated expression in response to both envelope stress and entry into stationary phase growth (DiGiuseppe and Silhavy, 2003, Raivio*, et al.*, 1999). Also, CpxP also functions as an adaptor protein, carrying periplasmic misfolded proteins to the DegP protease for degradation (Buelow and Raivio, 2005, Isaac*, et al.*, 2005). In this process, CpxP is degraded along with its misfolded substrate, suggesting a mechanism by which bacteria can posttranslationally modulate CpxP levels (Debnath, *et al.,* 2013) By varying the amounts of CpxP within the periplasm, bacteria may be able to fine-tune the Cpx stress response, limiting inappropriate activation of CpxA in the absence of envelope

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stress and permitting rapid shutoff of the system once the stress is under control (Buelow and Raivio, 2010, Vogt and Raivio, 2012). Cpx system is important and required for virulence in both Gram-negative and -positive bacteria (Raju, *et al.*, 2012). CpxP also modulates the activity of the Cpx system by dynamic interaction with CpxA in response to specific stresses (Tschauner, *et al.*, 2014). On the other hand, cpxP overexpression results in a reduced Cpx-response (Raivio, *et al.,* 1999), hence interfering with the induction of envelope stress response. Thereby, CpxP inhibits autophosphorylation of reconstituted CpxA (Fleischer, *et al.*, 2007). According to the current model, the inhibitory and supporting functions of CpxP for envelope stress response are linked: In unstressed cells, CpxP associates with CpxA to shut off the Cpx-TCS. Envelope-stress conditions induce the displacement of CpxP from CpxA resulting in Cpx-TCS activation (Tschauner, *et al.,* 2014). In *E. ictaluri*, CpxP showed very high expression in all stress conditions except oxidative stress. CpxP is an important regulator of cell membrane stress in bacteria during host infection, and it might promote the virulence and survival mechanisms related to distinct salt and acidic conditions.

Protein–protein interactions play key roles in protein function and in a large scale protein-protein interaction study in *E. coli* showed that a USPA has 83 target proteins, which mostly overlap with DnaK, DnaJ, and GroEL, also interactions of 776 ORFs with GroEL and 310 with DnaK (HSP70) were identified (Arifuzzaman, *et al.,* 2006). The functional relationship between these chaperone systems and the overlap of DnaK, GroEL; 92 proteins appear to be substrates of both DnaK and GroEL. These proteins may require both systems for folding, degradation, or translocation (Arifuzzaman, *et al.,* 2006).

Functions of GroEL/ES is in protein folding and possibly in intercellular signaling, and these proteins prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions (Begley and Hill, 2015). DnaK/J plays an essential role in DNA replication and participates actively in response to hyperosmotic shock. Also, DnaK also is involved in refolding heat-damaged proteins (Fang, *et al.,* 2016). GroEL was previously determined with various other proteins as a related protein to the bacterial virulence in *E. tarda* (Sakai, *et al.,* 2009). Also, in another proteomics study comparing two strains of *E. tarda* with different degrees of virulence, authors showed that GroEL was highly expressed in pathogenic strain compared to nonpathogenic strain (Bujan, *et al.,* 2015). Our result indicated that expression of *groEL and groES* were high in the oxidative stress, and *dnaK* was highly upregulated. Therefore, because of highly expressed heat shock proteins in oxidative stress, further works need to be done to explain folding and unfolding process in oxidative stress of *E. ictaluri*. ClpB plays an important role in protein control to protein disaggregation in collaboration with the DnaK, DnaJ and GrpE (Motohashi, *et al.,* 1999), and it is sensitive to heat shock and other extreme stresses (Mogk*, et al.,* 2003, Squires, *et al.,* 1991). In *E. tarda*, protein expression of ClpB was higher in virulent strain than non-virulent (Bujan, *et al.,* 2015). In our study, the expression of *clpB* was high in oxidative stress like *dnaK*, *groEL* and *groES*. ClpB is part of a stress-induced system involved in the recovery of the cell from damage in cooperation with DnaK, DnaJ, and GrpE. Several rounds of ATP-dependent interactions between DnaJ, DnaK, and GrpE, are required for fully efficient folding (Fang, *et al.,* 2016). The *grpE* was also shown similar high expression in oxidative stress and in the internal organs. Further, it has a very high

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expression in low pH. Thus, *grpE* may be an important heat shock protein to cope with stress in *E. ictaluri*.

The stringent response (SR) coordinates adaptations to nutritional starvation and various stress conditions (Hauryliuk, *et al.,* 2015, Liu, *et al.,* 2015). SR relies on the 'alarmones' ppGpp and pppGpp alarmone synthetases of the RelA/SpoT homology (RSH)-type produce ppGpp and pppGpp by transferring pyrophosphate originating from ATP (Steinchen and Bange, 2016). The ppGpp acts by modifying the activity of many cellular targets including DNA replication, transcription, translation, ribosome assembly and metabolism (Dalebroux and Swanson, 2012, Hauryliuk, *et al.,* 2015, Steinchen and Bange, 2016). Also, it is recently found that it has a role in DNA repair mechanism (Kamarthapu*, et al.,* 2016). The SR is in the center bacterial survival and virulence (Ancona, *et al.,* 2015, Chatnaparat*, et al.,* 2015, Dalebroux and Swanson, 2012). In our study, we determined that the expression of *ppGpp* was increased in internal organs kidney and spleen. Similarly, serum affects the expression of *ppGpp*. Therefore, we think that ppGpp synthetase could be essential for *E. ictaluri* adaptation in the host. Further mutation study needs to be done in the *ppGpp* synthetases gene to better understand the role of the gene in virulence and the regulation of USPs in *E. ictaluri*.

In this study, we identified that 3 *usp* genes (*usp 05-07-13*) were highly expressed in many stress conditions, and 5 heat shock proteins were highly expressed in oxidative stress (*groEL/ES, dnaK, grpE* and *clpB*), and *grpE* was highly expressed in spleen, kidney, and low pH as well. Finally, *ppGpp* was high expressed in kidney and spleen. As a result, we expect these findings will help us understand the role of stress proteins in *E.*

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ictaluri virulence. Furthermore, essential USPs and stress regulators in *E. ictaluri* can be potential targets for live vaccine development against ESC.

CHAPTER III

CONSTRUCTION AND EVALUATION OF USP MUTANTS IN *E. ICTALURI*

VIRULENCE

Abstract

Edwardsiella ictaluri is an intracellular Gram-negative facultative pathogen causing enteric septicemia of catfish (ESC), which causes large economic losses in the United States catfish industry. More studies are needed to understand the mechanisms of stress response in *E. ictaluri* virulence. Our gene expression study outlined in the previous chapter indicated that several universal stress proteins (USP) were highly express under various stress conditions, which indicate they may be essential in *E. ictaluri* virulence. However, the role of these USPs in *E. ictaluri* virulence in is not explored yet. Therefore, in the present study, we explored the role of USPs in *E. ictaluri* virulence by constructing and evaluating 10 *usp* mutants (*EiΔusp02-10 and EiΔusp13*) in catfish fingerlings. The result indicated that *EiΔusp05*, *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, *EiΔusp10*, and *EiΔusp13* were significantly attenuated (20%, 45%, 20%, 20%, 55%, and 10% mortality) compared to *E. ictaluri* WT (70% mortality). Efficacy test showed that *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, *EiΔusp10*, and *EiΔusp13* were very protective (100% survival), and *EiΔusp05* had a moderate protection (52 % survival) compared to *E. ictaluri* BHI (31 % survival). *EiΔusp05* showed *EiΔusp05*, *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, and *EiΔusp13* were sensitive to oxidative stress. All the *usp* mutants were

sensitive to acidic conditions at pH 04. However, serum exposure did not affect the growth of mutants or WT negatively. We showed that some of USPs in *E. ictaluri* play important roles in resistance to low pH and oxidative stress.

Introduction

Enteric septicemia of catfish (ESC) is one of the most prevalent diseases of cultured channel catfish. ESC is responsible for annual losses of $60 million dollars (USDA, 2014). The most common practice in ESC treatment is mediated feed including oxytetracycline, sulfadimethoxine, or florfenicol. However, one of the earliest clinical signs of ESC is anorexia. Thus, these antimicrobials are only effective in limiting the spread of an outbreak and rather than treating the disease. Also, antibiotic use may lead to the emergence of resistant *E. ictaluri* strains (Tu, *et al.*, 2008). Vaccination could be the preferred method for prevention of ESC.

The killed bacterins have efficacious only under laboratory conditions, and this route of delivery is not practical for commercial production (Saeed and Plumb, 1986). On the other hand, live attenuated vaccines (LAV) can be very protective if they activate the catfish immune system. Several LAV candidates were developed previously such as chondroitinase (Cooper, *et al.*, 1996) and auxotrophic (*aroA* and *purA*) (Lawrence, *et al.*, 1997), but they are not commercially available. Only commercial LAV is Aquavac-ESC (RE-33), which was developed by serial passages of increasing rifampin selection (Klesius and Shoemaker, 1999). The introduction of antibiotic resistance to the environment via vaccines is not a desired feature of LAV. Also, the genetic basis for attenuation in RE-33 is not known completely (Dahal*, et al.*, 2013).

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The universal stress protein domain is a conserved domain of 140-160 amino acids, and they are present in the archaea, bacteria, plants and fungi, but humans (Nachin, *et al*., 2005). USP domain originates from the *uspA* of *E. coli*. The *uspA* mutant caused a survival defect under a variety of growth-arrested conditions, whereas overexpression induces a growth arrested state (Tkaczuk, *et al*., 2013). *E. coli* has six *usp* genes, undefined roles in functions shifting from oxidative stress to adhesion and motility (Nachin, *et al*., 2005). Under stress, USP is overproduced and through a variety of mechanisms aid the organism in surviving in such uncomfortable conditions (Heermann, *et al*., 2009). It is also known that USPs are helping pathogens (Hensel, 2009). USPs affect persistence and survival of *Mycobacterium tuberculosis* (Hingley-Wilson, *et al*., 2010), and cause growth arrest and reduce the virulence in *Salmonella* (Liu, *et al*., 2007) and *Burkholderia pseudomallei* (Al-Maleki, *et al*., 2014). USPs also affect the intracellular growth adaption of *Listeria monocytogenes* (Chatterjee, *et al.,* 2006). Similarly, *Staphylococcus aureus* virulence factors were downregulated in vivo while expression of *uspA* increased (Chaffin, *et al.,* 2012). *Acinetobacter baumannii uspA* is essential in pneumonia and pathogenesis (Elhosseiny, *et al.,* 2015). Based on our previous work and literature, we hypothesize that mutation of *E. ictaluri usp* genes will cause attenuation, and mutant strains can be used as a live attenuated vaccine.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 3.1. *E. ictaluri* 93-146 was grown at 30°C using Brain Heart Infusion (BHI) broth and agar (Difco, Sparks, MD). *E. coli* were cultured at 37°C using Luria-Bertani (LB) broth and

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agar (Difco). *E. coli* CC118λ*pir* was used for cloning and SM10λ*pir,* or BW19851λ*pir* were used for transferring pMEG-375 or pAKgfplux1 into *E. ictaluri*. When required, the following antibiotics and reagents (Sigma-Aldrich, Saint Louis, MN) were added to culture medium at the following concentrations: ampicillin (Amp: 100 μg/ml), colistin (Col: 12.5 μg/ml), sucrose (5%), and mannitol (0.35%).

Construction of USP in-frame deletion Δ*usp* mutants

Overlap extension PCR was used to delete the functional *usp* genes from the *E. ictaluri* genome (Horton*, et al.*, 1990). Four primers were designed for each gene including forward, internal-reverse, internal forward, and reverse primers (Table 3.2). Restriction sites were included in forward and reverse primers. Genomic DNA was isolated from *E. ictaluri* using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and used as template in PCR. The upstream and downstream regions of each gene were amplified, and products were gel-extracted using a QIAquick Gel Extraction Kit (Qiagen). The amplified upstream and downstream fragments were mixed equally, were used as a template in the subsequent overlap extension PCR, to generate the in-frame deletion fragment for each gene. The in-frame deletion fragment was digested with appropriate restriction enzymes (NEB) (Table 3.1 and 3.2), cleaned up, and digested. The suicide plasmid pMEG-375 was purified from an overnight *E. coli* culture by a QIAprep Spin Miniprep Kit (Qiagen) and cut with appropriate restriction enzymes respective to the inserts. The purified PCR product with in-frame deletion was ligated into the pMEG-375 vector using T4 DNA Ligase (NEB) at 16ºC overnight. *E. coli* CC118λ*pir* was transformed by electroporation and plated on BHI agar with Amp. Plasmids were prepared from the colonies and confirmed by size, restriction enzyme digestion, and

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finally by sequencing. The plasmids named as p*Ei∆usp02-10* and p*Ei∆usp13* were transferred into *E. coli* SM10λ*pir* or BW19851λ*pir* by chemical transformation and mobilized into *E. ictaluri* 93-146 by conjugation. First integration was selected by Amp and amp resistant colonies were propagated on BHI agar to allow for the second crossover allelic exchange. After this step, colonies were streaked on BHI plates with 5% sucrose, 0.35% mannitol, and colistin to select for loss of pMEG-375. Potential mutant colonies were tested for ampicillin sensitivity to ensure the loss of the plasmid and confirmed by PCR and sequencing.

Construction of bioluminescent *Ei∆usp* mutants

The pakgfplux1 plasmid was transferred into *Ei∆usp02-10* and *Ei∆usp13* mutants by conjugation as described previously (Karsi and Lawrence, 2007) to construct bioluminescent labeled *Ei∆usp* mutants.

Growth of the *E. ictaluri* ∆*usp* mutants in normal media

Growth kinetics of the *E. ictaluri* ∆*usp* and *E. ictaluri* WT were compared in regular BHI medium. The experiment was conducted using four replicates. The small cultures were grown in a shaking incubator at 30°C for 18 h. The optical densities were measured at 600 nm (OD_{600}), and adjusted volumes were added to 15 ml BHI. Cultures were grown for 24 h and the growth kinetics studied by measuring the bacterial growth at 2, 4, 8, 12 and 20 h.

Survival assay in acidic pH, H₂O₂, and catfish serum

Constructed bioluminescent *Ei∆usp* mutant strains used for survival tests. The ability of *E. ictaluri* USP mutants to survival in the acid stress (pH 5.5), oxidative stress

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3mM (0.1 % of H$_2$O$_2$), and catfish serum were compared to *E. ictaluri* WT. Bacteria were grown overnight, and OD$_{600}$ values were used to adjust culture volumes. From each group, 5 µl of bacteria were inoculated into 195 µl of BHI broth added ampicillin and colistin. All experiment was done in 96 well black plates with four replicates using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT), and the mean photon counts for each treatment following BLI after 3h incubation at 30ºC. Bioluminescence picture of 96 well plate was taken using IVIS (Image Visualization and Infrared Spectroscopy), 100 Series (Caliper Corporation, Hopkinton, Massachusetts). Three independent experiments were done and used for statistical analysis. We calculated photon exposure differences between treatment and non-treatment by using their means, and fold change is calculated by using WT as a control.

Assessment of virulence and efficacy of the *E. ictaluri* Δ*usp* mutants in catfish fingerlings

Specific pathogen free catfish were obtained from the MSU-CVM Hatchery. Virulence and efficacy trial were conducted as reported by our group (Karsi, *et al*., 2009) by following institutional guidelines of Animal Care and Use Committee. The experiment included 10 *E. ictaluri* USP mutants, positive control (*E. ictaluri* WT) and negative control (BHI). Approximately 720 SPF channel catfish fingerlings (13.728 cm, 10.544 g) were stocked into 36 tanks at a rate of 20 fish/tank. Each treatment had three replicate tanks. After one week of acclimation, fish were challenged by immersion with 1.3 x 10^7 CFU/ml water for 1h. Mortalities were recorded daily for 21 days, and the total percent mortalities were calculated for each treatment group. To determine the efficacy of the *E.*

ictaluri USP mutants, the remaining fish in all groups were re-challenged with *E. ictaluri* WT (2.8 x 10^7 CFU/ml water).

Statistical Analysis

Photon counts were transformed by taking the base 10 logarithm to improve normality. Two independent samples t-tests were used for symmetrical variables for comparison between groups (mutant and WT). Relative percent survival (RPS) was calculated according to the formula RPS = [1-(% mortality of vaccinated fish/% mortality of non-vaccinated fish)] x 100 (Amend, 1981) which expresses the proportion of fish saved due to vaccination.

Table 3.1   Bacterial strains and plasmids used for mutant construction.

Strain	Relevant characteristics	References
Edwardsiella ictaluri		
93-146	Wild type; pEI1+; pEI2+; Col^r	(Lawrence, *et al.,* 1997)
EiΔusp02	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp02*	This study
EiΔusp03	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp03*	This study
EiΔusp04	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp04*	This study
EiΔusp05	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp05*	This study
EiΔusp06	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp06*	This study
EiΔusp07	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp07*	This study
EiΔusp08	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp08*	This study
EiΔusp09	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp09*	This study
EiΔusp10	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp10*	This study
EiΔ*usp13*	93-146 derivative; pEI1+; pEI2+; Colr; Δ*usp13*	This study
Escherichia coli		
CC118λ*pir*	D(ara-leu); araD; DlacX74; galE; galK; phoA20; thi-1; rpsE; rpoB; argE(Am); recAl; lpirR6K	(Herrero, *et al.*, 1990)
SM10λ*pir*	thi; thr; leu; tonA; lacY; supE; recA;::RP4-2-Tc::Mu; Kmr; lpirR6K	(Miller and Mekalanos, 1988)
BW19851λ*pir*	RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510	(Metcalf, *et al.*, 1994)

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Table 3.1 (Continued)

Plasmids		
pMEG-375	8142 bp, Amp^r, Cm^r, lacZ, R6K ori, mob incP, sacR sacB	(Dozois*, et al.*, 2003)
pAK*gfplux*1	5681bp, PstI, EcoRI, HpaI, AseI, BstBI	(Karsi and Lawrence, 2007)
p*Ei∆usp02*	9939 bp, *∆usp02*, pMEG-375	This study
p*Ei∆usp03*	9960 bp, *∆usp03*, pMEG-375	This study
p*Ei∆usp04*	10096 bp, *∆usp04*, pMEG-375	This study
p*Ei∆usp05*	10080 bp, *∆usp05*, pMEG-375	This study
p*Ei∆usp06*	10101 bp, *∆usp06*, pMEG-375	This study
p*Ei∆usp07*	10026 bp, *∆usp07*, pMEG-375	This study
p*Ei∆usp08*	10087 bp, *∆usp08*, pMEG-375	This study
p*Ei∆usp09*	9843 bp, *∆usp09*, pMEG-375	This study
p*Ei∆usp10*	9795 bp, *∆usp10*, pMEG-375	This study
p*Ei∆usp13*	9975 bp, *∆usp13*, pMEG-375	This study

Table 3.2   The primers used for *E. ictaluri* USP mutant construction and sequence confirmation.

Genes	Primer ID	Primer Sequence (5`→3`)	Restriction Enzyme
Eiusp02	Ei1751EF01 Ei1751IR01 Ei1751IF01 Ei1751ER01 1751F	cccc**tctaga**AGTGCGGATTGCATTACAAC GAGGTCGATGGAAACCAG ctggtttccatcgacctcGTACTGGTCGTCCGCTGATC cccc**ggatcc**GATTAACAACGGCAAAGTGG ACCTGTGCCATTTCCGCTGCC	*Xba*I *BamH*I

Table 3.2 (Continued)

Eiusp03	Ei1786EF01	cccc**gcggccgc**TTTTCGTCGCGATAGACTTC	*Not*I
	Ei1786IR01	GACGGGAACCAAAATCGTC	
	Ei1786IF01	gacgattttggttcccgtcACCACCAGCGTCTTGGTAGTG	
	Ei1786ER01	cccc**gagctc**CAGCTGCTCCATGAAATTACG	*Sac*I
	1786F	GTATGGCGGTGATAACATCC	
Eiusp04	Ei1962EF01	ccccgcggccgcGGAAAAACGTGTCATTCGTC	*Not*I
	Ei1962IR01	GTTTTGGTTCGGATCGATAG	
	Ei1962IF01	ctatcgatccgaaccaaaacGAAGATGAGCATGATGAC	
	Ei1962ER01	cccc**gcatgc**CATCTCTTTCCTGCTGATGC	*Sph*I
	1962F	TGATTTGTTGCTCGTCGGTA	
Eiusp05	Ei1981EF01	cccc**gcggccgc**GGATCATATAGCCCATGCTG	*Not*I
	Ei1981IR01	GGATCCGGTTTTAAGATCAAG	
	Ei1981IF01	cttgatcttaaaaccggatccGACACCATTAGCATTGATACG	
	Ei1981ER01	cccc**gagctc**GAAATCCTGACAGCCACTTCTG	*Sac*I
	1981F	TTACCATGGCGCATTTAGGC	
Eiusp06	Ei2616EF01	cccc**gcggccgc**ATTGTGACGGAGGAGAGATG	*Not*I
	Ei2616IR01	CAGAACCAGAACATGGTG	
	Ei2616IF01	caccatgttctggttctgGACCTTGAGACCGACGTTCTGG	
	Ei2616ER01	cccc**gagctc**TGGGAAATGGTAAAACATGG	*Sac*I
	2616F	ATATCCGTCGCCGTCATACC	
Eiusp07	Ei2891EF01	cccc**gcggccgc**GCGCTGATCATCGTCTTACTG	*Not*I
	Ei2891IR01	CTGTGCCAGCAGGGTGTC	
	Ei2891IF01	gacaccctgctggcacagGAAACCGATAAGGAGATGACAGAC	
	Ei2891ER01	cccc**gcatgc**GATACAGGAGCAGGGAGTTCTGG	*Sph*I
	2891F	CGTCGAGGCTCTGATTACCA	
Eiusp08	Ei3729EF01	cccc**gcggccgc**TCTCCGACCTGTAACAATCC	*Not*I
	Ei3729IR01	CGGAGAAAGGTCTACAGCAAC	
	Ei3729IF01	gttgctgtagacctttctccgCATATCGACATGCTGATCGTC	
	Ei3729ER01	cccc**gagctc**AGCAGCTTGCCATAGTTCAG	*Sac*I
	3729F	GCGTTTACAACTGACTCCGG	
Eiusp09	Ei3778EF01	cccc**gcggccgc**AATCGGTGTAGAAGGTGTCG	*Not*I
	Ei3778IR01	CTCTTCAATATCGACAGGTAC	
	Ei3778IF01	gtacctgtcgatattgaagagAAGACCAATGTGCTGGTG	
	Ei3778ER01	cccc**gagctc**AGAATCAGGGAGGAGTCCAG	*Sac*I
	3778F	ACAATCTCCGGACTCTGTGG	

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Table 3.2 (Continued)

Eiusp10	Ei1634EF01	at**cccggg**TATTTGCTACCCCTACAGTGCC	*xma*I
	Ei1634IR01	CAGATCGAGGAGTGTACTCAT	
	Ei1634IF01	atgagtacactcctcgatctgGATCAGCCGACACAAAGCCTC	*Sph*I
	Ei1634ER01	at**gcatgc**CGACGGTGTTGGATGAGAGCT	
	1634F	CCACCGAACACACTAGCAATA	
Eiusp13	Ei3810EF01	at**cccggg**AGCATCAGTACCACCATCAG	*Xma*I
	Ei3810IR01	GGTCAGGGTTGCAGTCTTATG	
	Ei3810IF01	cataagactgcaaccctgaccCAGTTAAACGCACGCTATCAG	*Xba*I
	Ei3810ER01	at**tctaga**CGGACAATGCGGATGATCTGA	
	3810F	TCAGCTGTGTGGGTAGACTG	

Note: Bold letters shows restriction enzymes. First four primers used for mutant construction and the last primers in each group used for sequence confirmation

Results

Construction of the *E. ictaluri* USP mutants

We were able to in-frame delete 10 *E. ictaluri* USPs. The size of upstream and the downstream regions and deleted regions of each targeted genes are reported in Table 3.3. All mutants were verified by colony PCR (Fig.3.1) and by sequencing of the mutated fragment.

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Table 3.3   Properties of the *E. ictaluri usp* genes and percentage of deletion

| Gene	Original ORF (bp)	Remaining Upstream ORF (bp)	Remaining Downstream ORF (bp)	Deleted Fragment Size (bp) / (%)	Upper Fragment Size (bp)	Lower Fragment Size (bp)	Total Fragment Size (bp)	Plasmid Size (bp)
usp02	435	15	0	420 / (97)	884	913	1797	9939
usp03	432	8	6	418 / (97)	998	820	1818	9960
usp04	960	28	6	926 / (96)	974	980	1954	10096
usp05	417	24	21	372 / (89)	1025	913	1938	10080
usp06	420	12	14	394 / (94)	952	1007	1959	10101
usp07	2709	30	9	2670 / (99)	877	1007	1884	10026
usp08	438	12	27	399 / (91)	975	970	1945	10087
usp09	429	18	12	399 / (93)	828	873	1701	9843
usp10	258	0	9	249 / (97)	807	846	1653	9795
usp13	480	3	63	415 / (86)	948	885	1833	9975
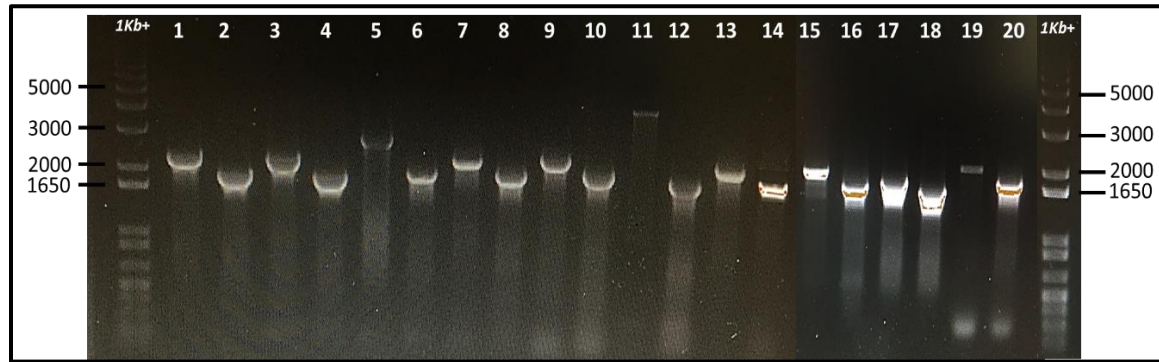
Figure 3.1   The genotypic confirmation of *E. ictaluri* Δusp mutants by in vitro in-frame gene deletion.

Genomic DNAs was amplified from the *E. ictaluri* wild type and mutants using the two outside primers and separated on 1% agarose gel. **1-** *EiΔusp02 (Δusp02)* **2-** *EiΔusp02 (ΔUSP02)* **3-** *EiWT (usp03)* **4-** *EiΔusp03 (Δusp03)* **5-** *EiWT (usp04)* **6-***EiΔusp04 (ΔUSP04)* **7-** *WT (usp05)* **8-***EiΔusp05 (Δusp05)* **9-***Ei WT (usp06)* **10-***EiΔUSP06 (ΔUSP06)* **11-***Ei WT (USP07)* **12-***EiΔusp07 (Δusp07)* **13-***Ei WT (USP08)* **14-***EiΔUSP08 (ΔUSP08)* **15-***Ei WT (USP09)* **16-***EiΔUSP0*9 *(ΔUSP09)* **17-***Ei WT (USP10)* **18-** *EiΔUSP1*0 *(ΔUSP10)* **19-***Ei WT (USP13)* **20-***Ei WT (USP13)*

Growth of the *E. ictaluri usp* mutants

The growth of *E. ictaluri* WT and USP mutants in BHI broth indicated that

EiΔusp02 and *EiΔusp03* have a significantly faster growth than *E. ictaluri* WT (Fig.3.2).

The growth of *E. ictaluri* WT was 23.6% and 17.42% lower than that of *EiΔusp02* and

EiΔusp03, respectively (Fig.3.2).

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Survival assays

When pH 5.5 applied, all *E. ictaluri* USP mutant and WT exhibited reduced growth. Especially, *EiΔusp03* growth rate was 4.1 fold reduced compared to WT (Fig. 3.3). Serum exposure showed that all mutants and WT have increased bioluminescence. All mutants similarly have more bioluminescence than WT, and the difference between WT and Δ*usp* were between 0.7-1.4 fold (Fig. 3.4). We found that hydrogen peroxide affected some mutants significantly. *EiΔusp05* and *EiΔusp08* had 13.6 and 5.3 fold more reduction than WT (Fig. 3.5).
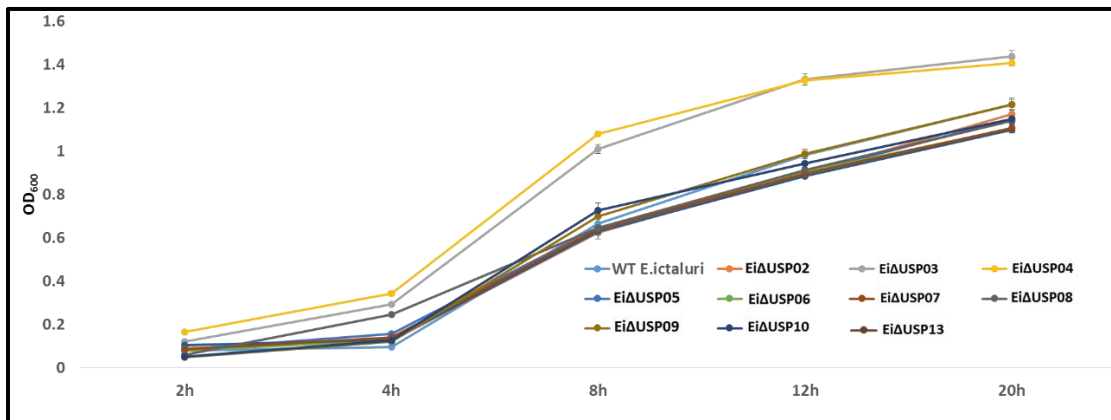
Figure 3.2   Growth curve of *E. ictaluri* Δ*usp* mutants and WT in BHI broth.
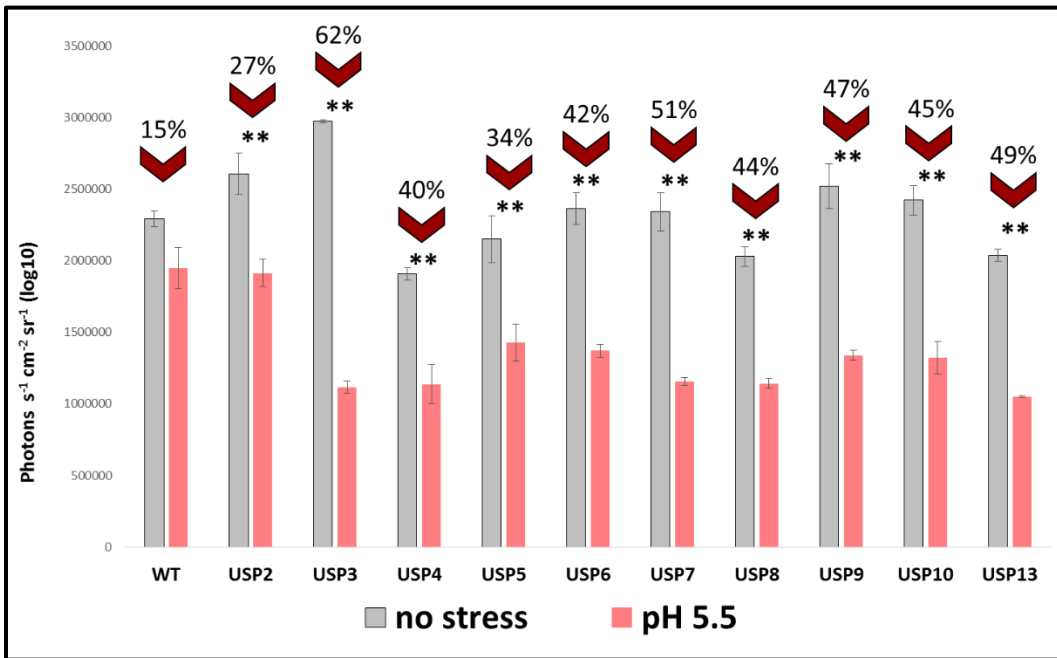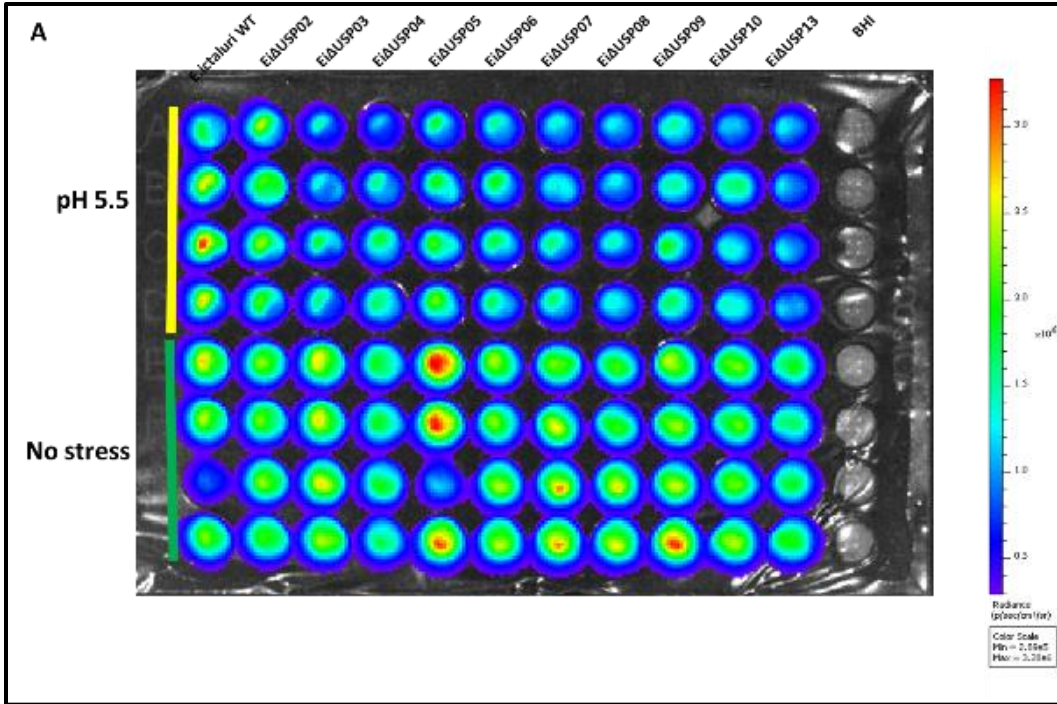
Figure 3.3   Survival assay *E. ictaluri* WT and Δ*usp* mutants in pH 5.5.

A. Each line shows a strain with stressor and non-stress control growth in 96 well plate. Strains start with *E. ictaluri WT*, *EiΔusp02-13* and BHI control. The bars showed percentage of difference between USP mutants and wild-type (WT) in bioluminescence. ****** indicated a significant difference **P < 0.01**; mutant compared with the WT bioluminescence by Student's two-tailed t test.

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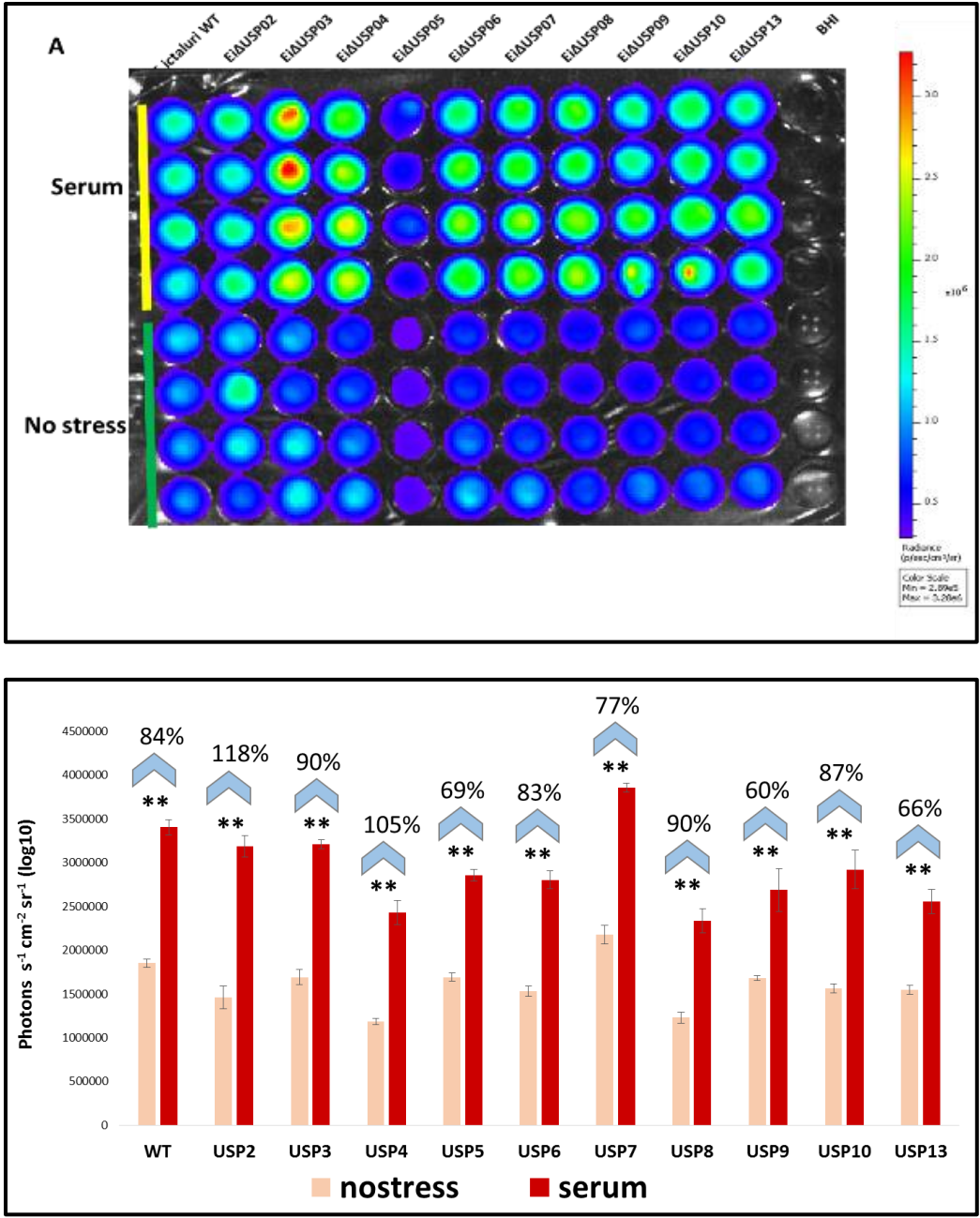
Figure 3.4   Survival assay of *E. ictaluri* WT and Δ*usp* mutants in catfish serum
            exposed.

A. Each line shows a strain with stressor and non-stress control growth in 96 well plate. Strains start with
E. ictaluri WT, *EiΔusp02-13* and end with BHI control. **B.** The bars showed percentage of difference
mutant-wild-type (WT) in bioluminescence.

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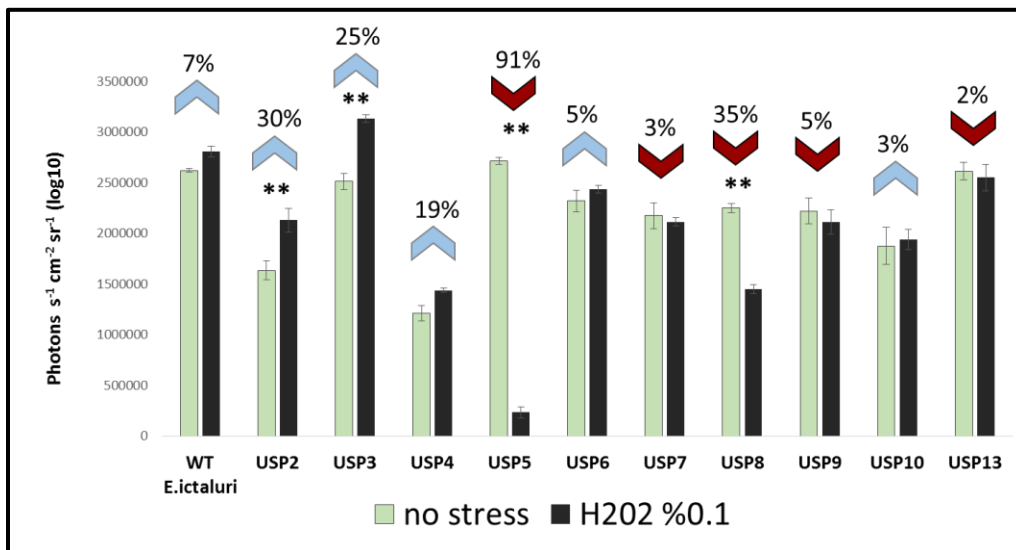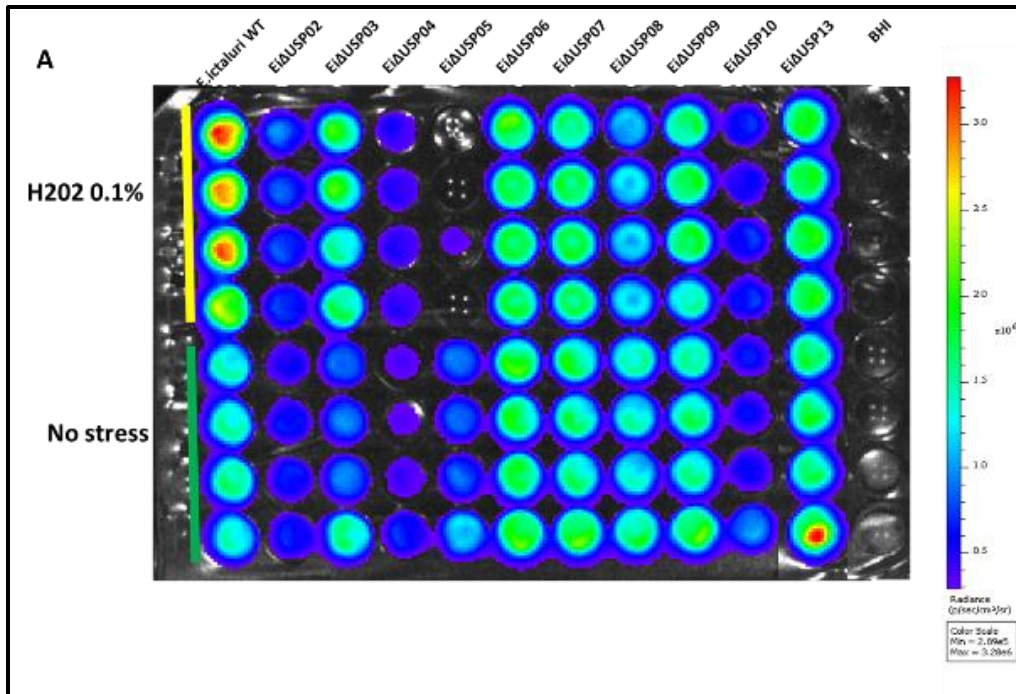
Figure 3.5   Survival assay of *E. ictaluri* WT and Δ*usp* mutants exposed to 0.1% H_2O_2.

A. Each line shows a strain with stressor and non-stress control growth in 96 well plate. Strains start with *E. ictaluri WT*, *EiΔusp02-13* and BHI control. **B.** The bars showed percentage of difference mutant-wild-type (WT) in bioluminescence.

Virulence and efficacy of the *E. ictaluri* Δ*usp* mutants

      *Ei*Δ*usp05, Ei*Δ*usp07*, *Ei*Δ*usp08*, *Ei*Δ*usp09*, *Ei*Δ*usp10*, and *Ei*Δ*usp13* showed

good virulence attenuation (20%, 45%, 20%, 20%, 55% and 10%, respectively) (Fig.3.6).

The protective level of these mutants was high (100% survival), except, *Ei*Δ*usp05* (42%

survival) (Fig.3.7).
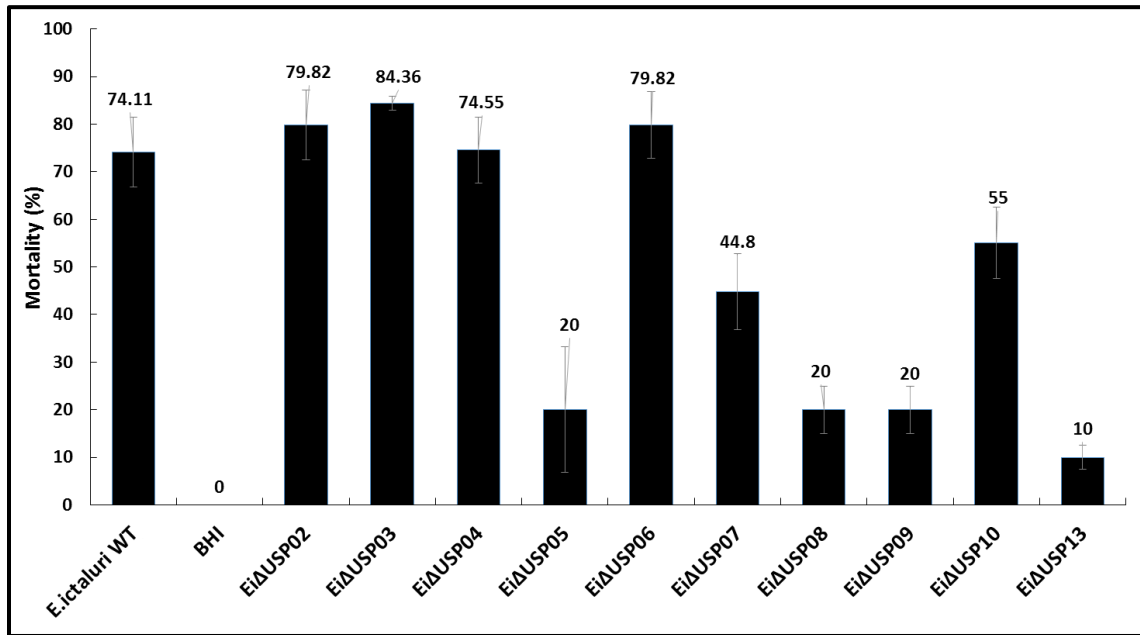
Figure 3.6   Percent mortalities of channel catfish fingerlings challenged with *E. ictaluri* Δ*usp* mutants and *E. ictaluri* WT.
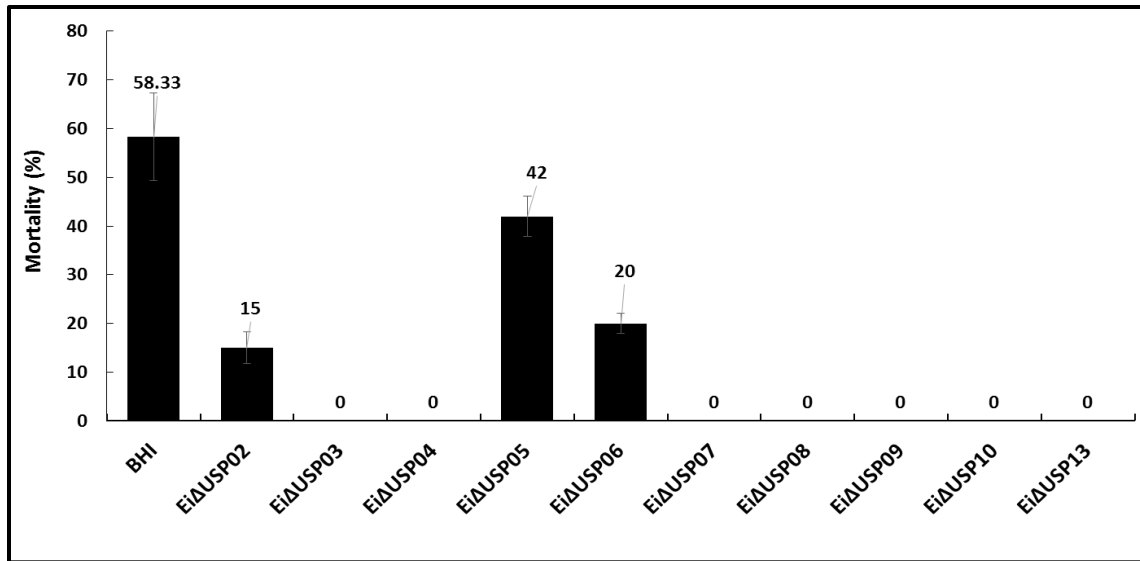
Figure 3.7   Percent mortality of channel catfish fingerlings immunized with the *E. ictaluri* Δ*usp* mutants and re-challenged with *E. ictaluri* WT at 21 d post-vaccination.

Gene ID	q-RT PCR					Mutant ID	Mutant survival			Vaccination	
	pH	H2O2	Serum	Kidney	Spleen		pH	H2O2	Serum	Virulence	Efficacy
usp02	1	1.3	6.2	0.2	0.5	*Ei∆USP02*	1.8	-4.5	1.4	80	15
usp03	0.7	1.2	5.7	1.7	0.2	*Ei∆USP03*	4.2	-3.7	1.1	84	0
usp04	1.6	0.8	4.9	4.9	10.6	*Ei∆USP04*	2.7	-2.8	1.2	75	0
usp05	5.1	8.7	2.3	27.1	191	*Ei∆USP05*	2.2	13.6	0.8	20	42
usp06	0.6	1.1	4.3	4.1	11.6	*Ei∆USP06*	2.8	-0.8	1	80	20
usp07	1.4	1	1.4	139	18	*Ei∆USP07*	3.4	0.4	0.9	44	0
usp08	2.4	3.2	5.3	2.4	2.9	*Ei∆USP08*	2.9	5.3	1.1	20	0
usp09	2.2	2.5	5.5	6	4.3	*Ei∆USP09*	3.1	0.7	0.7	20	0
usp10	4.4	1.1	4.9	10	7.8	*Ei∆USP10*	3	-0.5	1	55	0
usp13	34.2	1.5	62.2	22.4	137	*Ei∆USP13*	3.2	0.4	0.8	10	0

Figure 3.8   Overall summary of 10 *E. ictaluri* Δ*usp* mutants. qRT-PCR values were shown by fold changes; and mutant survival fold changes calculated by mutant/wild-type (WT) reduction in bioluminescence.

Vaccination virulence and efficacy were shown by percentage of mortalities.

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Discussion

We aimed to determine whether the USPs have a role in the virulence of *E. ictaluri* or not. We were able to delete a large portion (86-99%) of the *usp* genes (Table.3.3) by using gene splicing and overlap extension method.

We determined that *EiΔusp02* and *EiΔusp03* have increased more than WT and other mutants in growth curve experiment (Fig.3.2). However, no differences were observed between wild-type and *usp* mutants in the growth of *Listeria monocytogenes* (Seifart Gomes, *et al.,* 2011) and *E.coli* (Nystrom and Neidhardt, 1994) when they cultured in regular media. The group of USPs that was studied in previous experiments was *uspA*. This is the most prevalent studied group of USP in bacteria, and in *E. ictaluri* *EiΔuspA* (*EiΔusp05-06, EiΔusp08-09*) did not show any difference in growth experiment.

We found that *E. ictaluri* can survive and continue growth in up to 3 mM of H_2O_2 and pH 5.5. When the *usp* mutants and WT-exposed to pH, *E. ictaluri* WT growth did not change significantly (Fig.3.3). However, *EiΔusp03* reduced the growth 67% when compared to growth in BHI. Oppositely, when mutants were exposed to serum, they were growing faster than the BHI control (Fig.3.4). Whereas only *EiΔusp05, EiΔusp08,* reduced in growth very differently than WT (Fig.3.5) in oxidative stress, *EiΔusp03-13* were reduced growth more than 2 fold WT (Fig.3.5). This is showing that a diverse response of USPs in *E. ictaluri*.

The effect of USPs in bacterial infection has only been examined for a few bacteria, and their role has not been described (Seifart Gomes, *et al.*, 2011). Deletion of the *uspA* genes resulted in a strain with decreased virulence in *Salmonella, Listeria*, and *Acinetobacter* (Elhosseiny, *et al.,* 2015, Liu, *et al.,* 2007, Seifart Gomes, *et al.,* 2011).

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Also, USPA affects the host invasion and survival in *Salmonella*, *Klebsiella*, or *Mycobacterium* (Hensel, 2009, Hingley-Wilson, *et al.*, 2010). Our virulence assessment demonstrated that *EiΔusp05, EiΔusp07, EiΔusp08, EiΔusp09, EiΔusp10,* and *EiΔusp13* showed a significant attenuation (20%, 42%, 20%, 20%, 55% and 10% mortality, respectively) compared to *E. ictaluri* WT (72% mortality) (Fig. 6). On the other hand, *EiΔusp02, EiΔusp03, EiΔusp06,* and *EiΔusp10* did not show a reduction in virulence. Protection of *EiΔusp03, EiΔusp04, EiΔUSP07, EiΔusp08, EiΔusp09, EiΔusp10, and EiΔusp13* mutants were 100%. However, *EiΔusp02, EiΔusp05,* and *EiΔusp06* showed 85%, 55%, and 80% respectively. We aimed to develop completely attenuated live vaccines in commercial scale catfish vaccination. Therefore, constructing double USP mutants may attenuate *EiΔusp13* strain further. Our study is evidence that USPs are involved in the intracellular pathogenic lifestyle of *E. ictaluri*.

The expression of *E. coli* USPs is controlled by some effector proteins and signaling molecules like SOS repose proteins (Gustavsson*, et al.*, 2002, Kvint, *et al.*, 2003, Persson*, et al.*, 2007). However, mechanisms of USPs in other bacterial species are not known completely. How USPs are protective in the bacteria, and which proteins triggers their induction are not known. We wanted to determine if the any of USPs of *E. ictaluri* used its protective effects in *E. ictaluri* to survive and cause ESC.

Our results indicated that deletion of the *usp* genes did not cause a significant growth defect in *E. ictaluri*, but improved growth of *EiΔusp02* and *EiΔusp03* at all stages of in vitro growth. The growth of the *EiΔusp05, EiΔusp07,* and *EiΔusp09* mutant diminished significantly when exposed to H_2O_2. This finding goes in accordance with those reported for previously studied USPs from various species, which were crucial for

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protecting the cells from the damaging effects of ROS (Elhosseiny, *et al.,* 2015, Liu*, et al.,* 2007, Nachin*, et al.,* 2005, Seifart Gomes*, et al.,* 2011). We also tested their effects on the survival on pH and serum stress, lack of *usp* genes resulted in improved in growth in serum similarly with WT, and oppositely pH exposure reduces the growth of USP mutants. The findings of this study model the *EiΔusp08, EiΔusp09,* and *EiΔusp13* as a unique target to develop a live vaccine against ESC. The field of the molecular pathogenesis of *E. ictaluri* is still potential with very few reports about proven virulence studies. The data presented in this study display that is essential for both stress physiology and pathogenesis.

CHAPTER IV

CONCLUSIONS

The body of work makes clear that there is a need to determine *E. ictaluri* virulence genes required for catfish invasion. Since *usp* genes in many other bacteria are involved in virulence, we believe that USPs in *E. ictaluri* may have a role in virulence in catfish.

We studied *E. ictaluri* in vitro stress responses under low pH, H_2O_2, and catfish serum and in vivo stress responses in catfish spleen and head kidney. Among the 13 Universal Stress Proteins (USP), we found that 3 *usp* genes (*usp05, usp07,* and *usp13*) were highly expressed in all stress conditions. Also, among the 7 USP-interacting proteins, 5 heat shock proteins (*groEL, groES, dnaK, grpE, and clpB*) were highly expressed in oxidative stress, and *grpE* was also highly expressed in *E. ictaluri* in spleen and kidney environments and low pH condition. Finally, *ppGpp* was highly expressed in *E. ictaluri* in kidney and spleen environments.

Our gene expression study indicated that universal stress proteins (USP) are essential in *E. ictaluri* virulence and responded to the stress. Based on USPs response stress, we decided to delete 10 *usp* genes in *E. ictaluri* for further tests. The virulence and efficacy of the constructed mutants were evaluated in catfish fingerling. The result indicated that *EiΔusp05*, *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, *EiΔusp10*, and *EiΔusp13* were significantly attenuated (20%, 45%, 20%, 20%, 55%, 10%, respectively against 70% WT

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mortality), and they are highly protective in catfish. Furthermore, *EiΔusp05*, *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, and *EiΔusp13* were sensitive to oxidative stress. While all the *Δusp* were sensitive to pH exposure; however, the serum did not effect to the growth of mutants or WT negatively. We showed that some of USPs in *E. ictaluri* play important roles in resistance to low pH and oxidative stress.

We are expecting that our results will help a better understanding the role of USPs in *E. ictaluri* virulence. Because stress proteins are essential for bacterial survival in the catfish host, they may be potential targets for live vaccine development against ESC.

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