Universal Stress Proteins Contribute Edwardsiella ictaluri Virulence in Catfish

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Edwardsiella ictaluri is an intracellular Gram-negative facultative pathogen causing enteric septicemia of catfish (ESC), a common disease resulting in substantial economic losses in the U.S. catfish industry. Previously, we demonstrated that several universal stress proteins (USPs) are highly expressed under in vitro and in vivo stress conditions, indicating their importance for E. ictaluri survival. However, the roles of these USPs in E. ictaluri virulence is not known yet. In this work, 10 usp genes of E. ictaluri were in-frame deleted and characterized in vitro and in vivo. Results show that all USP mutants were sensitive to acidic condition (pH 5.5), and Ei usp05 and Ei usp08 were very sensitive to oxidative stress (0.1% H2O2). Virulence studies indicated that Ei usp05, Ei usp07, Ei usp08, Ei usp09, Ei usp10, and Ei usp13 were attenuated significantly compared to E. ictaluri wild-type (EWT; 20, 45, 20, 20, 55, and 10% vs. 74.1% mortality, respectively). Efficacy experiments showed that vaccination of catfish fingerlings with Ei usp05, Ei usp07, Ei usp08, Ei usp09, Ei usp10, and Ei usp13 provided complete protection against EWT compared to sham-vaccinated fish (0% vs. 58.33% mortality). Our results support that USPs contribute E. ictaluri virulence in catfish.

Keywords: stress, ESC, USP, mutation, vaccine

INTRODUCTION

Enteric septicemia of channel catfish (ESC) is one of the most prevalent diseases of cultured catfish, causing significant losses (USDA, 2014). The most common practice in ESC treatment is use of feed medicated with oxytetracycline, sulfadimethoxine, or florfenicol. However, one of the earliest clinical signs of ESC is reduced appetite. Thus, these antimicrobials are only useful in limiting the spread of an outbreak and rather than treating the disease. Also, medicated feed may lead to the emergence of resistant Edwardsiella ictaluri strains (Tu et al., 2008).

The universal stress proteins (USP) have a conserved domain of 140–160 amino acids, and are present in archaea, bacteria, and plants (Nachin et al., 2005), but not in animals and human (Siegele, 2005). In Escherichia coli usp are involved in various functions from oxidative stress to adhesion and motility (Nachin et al., 2005). Under stress, USPs are overproduced and through a variety of mechanisms aid the survival of organisms in stressful conditions (Heermann et al., 2009b). The uspA mutation caused decreased survival in E. coli (Tkaczuk et al., 2013). It is known that USPs are needed by 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 typhimurium C5 (Liu et al., 2007) and Burkholderia pseudomallei (Al-Maleki et al., 2014). USPs are also necessary for 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).

Although increased expression of several usp genes in *E. ictaluri* under various stresses has been reported (Akgul et al., 2018), the role of USPs in *E. ictaluri* virulence is not known yet. Therefore, in this study, 10 *E. ictaluri* usp genes were studied by introducing in-frame deletions and determining their survival under acidic and oxidative stress conditions. Also, the virulence and protective properties of mutants against ESC infection were tested in catfish fingerlings.

**MATERIALS AND METHODS**

**Animals**

All fish experiments were performed based on a protocol approved by the Mississippi State University Institutional Animal Care and Use Committee (protocol number 15-043). Channel catfish fingerlings were obtained from the fish hatchery at the College of Veterinary Medicine, Mississippi State University, and maintained at 25–28°C during experiments. Tricaine methanesulfonate (MS-222, Western, Chemical, Inc.) was used to sedate (100 mg/ml) or euthanize (400 mg/ml) the catfish.

**Bacterial Strains, Plasmids, and Growth Conditions**

Bacterial strains and plasmids used in this work are listed in Table 1. *E. ictaluri* 93–146 wild-type (WT) was grown at 30°C using Brain Heart Infusion (BHI) broth and agar (Difco, Sparks, MD, United States). *E. coli* strains were cultured at 37°C using Luria-Bertani (LB) broth and agar (Difco). *E. coli* CC118,pir was used for cloning and SM10x.pir or BW19851 were used for transferring pMEG-375 or pAKgfpflux1 into *E. ictaluri*. When required, the following antibiotics and reagents (Sigma-Aldrich, Saint Louis, MN, United States) 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 In-Frame Deletion Mutants**

The nucleotide sequences of 10 *E. ictaluri* usp genes were obtained from the *E. ictaluri* 93–146 genome (GenBank accession: CP001600), and four primers were designed for each gene (Tables 2, 3). Restriction sites were included in forward and reverse primers. Overlap extension PCR was used to delete the functional usp genes from the *E. ictaluri* genome (Horton et al., 1990). Genomic DNA was isolated from *E. ictaluri* using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, United States) and used as template in PCR. The upstream and downstream

| Strain | Relevant characteristics | Reference |
|--------|--------------------------|-----------|
| *Edwardsiella ictaluri* | | |
| 93–146 | Wild type; pEI1−; pEI2−; Colr | Lawrence et al., 1997 |
| EiΔusp02 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp02 | This study |
| EiΔusp03 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp03 | This study |
| EiΔusp04 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp04 | This study |
| EiΔusp05 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp05 | This study |
| EiΔusp06 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp06 | This study |
| EiΔusp07 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp07 | This study |
| EiΔusp08 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp08 | This study |
| EiΔusp09 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp09 | This study |
| EiΔusp10 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp10 | This study |
| EiΔusp13 | 93–146 derivative; pEI1−; pEI2−; Colr; Δusp13 | This study |

| Escherichia coli |
| CC118.pir |
| Djaral-leu; araD; Diax74; gaiE; gaK; phoA20; thi-1; rpsE; rpoB; argE[Am]; recA1; lpirR6K | Herrera et al., 1990 |
| SM10x.pir |
| thi; thr; leu; tonA; lacY; supE; recA::PPr4-2-Tc::Mu; Km+; rpsE; lpirR6K | Miller and Mekalanos, 1988 |
| BW19851.pir |
| RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510 | Metcalf et al., 1994 |

| Plasmids |
| pMEG-375 |
| 8142 bp, AmpR, CmR, LacZ, R6K or, mob incP, sacrif SasB | Dozois et al., 2003 |
| pAKgfpflux1 |
| 5681 bp, PstI, EcoRII, Hpal, Asel, BstBI | Karsi et al., 2006 |
| pEIΔusp02 |
| 9939 bp, Δusp02, pMEG-375 | This study |
| pEIΔusp03 |
| 9860 bp, Δusp03, pMEG-375 | This study |
| pEIΔusp04 |
| 10096 bp, Δusp04, pMEG-375 | This study |
| pEIΔusp05 |
| 10080 bp, Δusp05, pMEG-375 | This study |
| pEIΔusp06 |
| 10101 bp, Δusp06, pMEG-375 | This study |
| pEIΔusp07 |
| 10026 bp, Δusp07, pMEG-375 | This study |
| pEIΔusp08 |
| 10087 bp, Δusp08, pMEG-375 | This study |
| pEIΔusp09 |
| 9843 bp, Δusp09, pMEG-375 | This study |
| pEIΔusp10 |
| 9795 bp, Δusp10, pMEG-375 | This study |
| pEIΔusp13 |
| 9975 bp, Δusp13, pMEG-375 | This study |
### TABLE 2 | The primers used for mutant construction and sequence validation.

| Genes | Primer ID | Primer Sequence (5'-3') | RE |
|-------|-----------|-------------------------|----|
| Eiusp02 | E1751EF01 | A cccccctctagaaagtgcgaggtgctgcttaca | XbaI |
|        | E1751EF01 | B gagaagacggatgagaaacag | |
|        | E1751EF01 | C ctggtcctagctactgatcgtgctgcagctgcttaca | NotI |
|        | E1751EF01 | D cccccggatccgatgggctgcagctgcttaca | SacI |

| Eiusp03 | E1786EF01 | A cccccggccggcttttctgctgactaagctc | NotI |
|         | E1786EF01 | B gacgggaacaaaaattgtgc | |
|         | E1786EF01 | C gacgatgtgctctctgctgctgg | |
|         | E1786EF01 | D cccccgagctcagctcgctcagctgcttgctgcagctgcttaca | SacI |

| Eiusp04 | E1962EF01 | A cccccggccgggtgaaaaccgtccttacgcttc | NotI |
|         | E1962EF01 | B gttggtgctcgtcgtag | |
|         | E1962EF01 | C ctatcgatccgaaccaaaac | |
|         | E1962EF01 | D cccccgcatgcacttttcctcgctgcagctgcttaca | SacI |

| Eiusp05 | E1981EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E1981EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E1981EF01 | C cttgatcttaaaccggatcgcagacattagcattgcttcg | |
|         | E1981EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp06 | E2161EF01 | A cccccggccggcatggtggagggagagatg | NotI |
|         | E2161EF01 | B cggagaaaggtctacagcaac | |
|         | E2161EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E2161EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp07 | E2289EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E2289EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E2289EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E2289EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp08 | E3279EF01 | A cccccggccggctctccgacctgtaacaatcc | NotI |
|         | E3279EF01 | B cggggaagggcctacacgacac | |
|         | E3279EF01 | C gttgctgctgctgctgcagctcagctgctgcttaca | SacI |
|         | E3279EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp09 | E3377EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E3377EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E3377EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E3377EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp10 | E3434EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E3434EF01 | B cggggaagggcctacacgacac | |
|         | E3434EF01 | C gttgctgctgctgctgcagctcagctgctgcttaca | SacI |
|         | E3434EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp11 | E3810EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E3810EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E3810EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E3810EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp12 | E3810EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E3810EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E3810EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E3810EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

| Eiusp13 | E3810EF01 | A cccccggccggctgcatataagcccaagttgcttc | NotI |
|         | E3810EF01 | B ggtcgctgctgcttcagtaagactc | |
|         | E3810EF01 | C gacgattttggttcccgtcaccaccagcgtcttggtagtg | |
|         | E3810EF01 | D cccccgagctcagctcagctgcttacgctcagctgcttaca | SacI |

*Primers A, B, C, and D were used for mutant construction. Bold letters show restriction enzymes added to A and D primers. Underlined letters in primer C indicate reverse complemented primer B sequence. The last primer in each group used for sequence confirmation.*
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 and used as a template in the subsequent overlap extension PCR to generate the in-frame deletion fragment for each gene. The in-frame deletion fragments were digested with appropriate restriction enzymes (NEB) (Table 2) and cleaned up. The suicide plasmid pMEG-375 was purified from an overnight
E. coli culture by a QIAprep Spin Miniprep Kit (Qiagen) and digested with appropriate restriction enzymes respective to the inserts. The in-frame deletion fragments were ligated into the linearized pMEG-375 vector using T4 DNA Ligase (NEB) at 16°C overnight. E. coli CC118:pir was transformed by electroporation and plated on LB agar plus ampicillin. Resulting plasmids were isolated from the colonies and confirmed by size, restriction enzyme digestion, and finally by sequencing. The resulting plasmids named as pEiΔusp02-10 and pEiΔusp13 were transferred into E. coli SM10:pir or BW19851 by chemical transformation and mobilized into E. ictaluri WT by conjugation. First integration was selected by ampicillin, and ampicillin resistant colonies were propagated on BHI agar to allow for the second crossover allelic exchange. After this step, colonies were streaked on counter selective BHI plates with 5% sucrose, 0.35% mannitol, and colistin to allow loss of pMEG-375. Potential mutant colonies were tested for ampicillin sensitivity to ensure the loss of the plasmid, confirmed by PCR, and sequencing.

**Construction of Bioluminescent USP Mutants**

The constructed USP mutants were made bioluminescence using pAKgflux1 plasmid as described previously (Karsi and Lawrence, 2007). Briefly, the overnight culture of both recipient (USP mutants) and donor cells (E. coli SM10:pir carrying pAKgflux1) were mixed at 1:2 ratio (donor : recipient) and centrifuged briefly. Pellet was transferred onto sterile 0.45 μM filter papers placed on a BHI agar and incubated at 30°C for 24 h. Bacteria on the filter paper were collected in BHI broth with ampicillin and colistin and then spread on BHI plates containing ampicillin and colistin. After incubation at 30°C for 24–48 h, ampicillin resistance colonies carrying pAKgflux1 appeared on plates.

**Growth Kinetics of the E. ictaluri USP Mutants in BHI**

Growth kinetics of the ten E. ictaluri USP mutants was compared to E. ictaluri WT in BHI medium as previously described (Abdelhamed et al., 2016). Each bacterial strain had four replicates. Overnight cultures were grown in a shaking incubator at 30°C for 18 h. The optical densities (OD_{600}) were measured, and adjusted volumes were added to 15 ml fresh BHI (1:100 dilution). Cultures were grown for 24 h by sampling and measuring OD_{600} values at 2, 4, 8, 12, and 20 h.

**Survival of E. ictaluri USP Mutants in Low pH Stress**

Survival of bioluminescent USP mutants and EiWT was determined under acidic stress (pH 5.5) as previously described (Seifart Gomes et al., 2011). Bacteria were cultured overnight, and OD_{600} values were used to adjust culture volumes. The experiment was performed in 96 well black plates with four replicates at acidic and neutral pH. For each well, 5 μl of bacteria were inoculated into 195 μl of BHI broth plus ampicillin and colistin. The plates were incubated in Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, United States), and the photon emissions were collected for 3 h at 30°C. Bioluminescence imaging (BLI) of the 96-well plate was taken using IVIS 100 Series (Caliper Corporation, Hopkinton, MA, United States). Three independent experiments were done and used for statistical analysis.
Survival of *E. ictaluri* USP Mutants in Oxidative Stress

The survival of the ten USP mutants in BHI supplemented with 0.1% of H_2O_2 were determined as previously described (Seifart Gomes et al., 2011). The experiment was performed in 96 well plates with four replicates under oxidative stress and normal conditions. The plates were incubated in CyToxion 5 Cell Imaging Multi-Mode Reader, and the photon emissions were collected for 3 h at 30°C.

Virulence and Efficacy of *E. ictaluri* USP Mutants in Catfish Fingerlings

Virulence and vaccine efficacy trials were conducted as reported by our group (Karsi et al., 2009). Approximately 720 channel catfish fingerlings (average: 13.72 cm, 10.544 g) were stocked into 36 tanks at a rate of 20 fish/tank. Tanks were divided into twelve groups with three replicate tanks each group. The experiment included 10 *E. ictaluri* USP mutants, positive control (*Ei*WT), and negative control (BHI exposed). After 1 week of...
acclimation, fish were challenged/vaccinated by immersion with $1.3 \times 10^7$ CFU/ml water for 1 h. Mortalities were recorded daily for 21 days, and the mean percent mortalities were calculated for each treatment group. Protective properties of USP mutants against EiWT infection was determined by challenging vaccinated catfish with EiWT ($2.8 \times 10^7$ CFU/ml water). Fish mortalities were recorded daily, and the percent mortality was calculated for each group.

Statistical Analysis

For the growth kinetic experiment, significant differences between EiWT and USP mutants were determined by Student’s $t$-test. For acid and hydrogen peroxide assays, photon counts were log$_{10}$ transformed $t$-tests were conducted. Percent reduction in bioluminescence was calculated by dividing mean photon emissions of USPs to mean photon emission of EiWT. For fish experiments, percent mortalities were arcsine transformed, and analysis of variance (ANOVA) was carried out using PROC GLM of SAS v9.4 (SAS Institute, Inc., Cary, NC, United States). In virulence/vaccination trial, the percent mortalities of USP mutants were compared to that of EiWT, while in efficacy trail, the comparisons were made to the sham-vaccinated group at the alpha level of 0.05.

**FIGURE 5** The survival assay of E. ictaluri WT and USP mutants exposed to 0.1% H$_2$O$_2$. (A) Each strain had four replicates (column A–D). Strains start with E. ictaluri WT, Ei/usp02-13 and BHI control. (B) The bars show the difference between bioluminescence of USP mutants and WT. *indicates a significant difference between stress and non-stress at $P < 0.01$. 
RESULTS

Construction of the *E. ictaluri* USP Mutants

Thirteen universal stress proteins were identified in the *E. ictaluri* genome (Williams et al., 2012) by sequence similarity (Figure 1). They were scattered through the chromosome, and no operon structure was observed. We were able to delete 10 *E. ictaluri usp* genes in-frame, and mutants were verified by PCR (Figure 2) as well as sequencing. Properties of wild-type and mutated *usp* genes are shown in Table 3. In-frame deletion resulted in removal of a large portion (86–99%) of the wild-type *usp* genes (Table 3).

Growth Kinetics of the *E. ictaluri* USP Mutants in BHI

The growth of *EiWT* and USP mutants in BHI broth indicated that *EiΔusp03* and *EiΔusp04* have a significantly (*p < 0.001*) higher growth rate than *EiWT*. After 20 h incubation, the growth of *EiWT* was 23.6 and 17.42% lower than *EiΔusp03* and *EiΔusp04*, respectively (Figure 3). Whereas, no significant differences were observed in the growth kinetics of *EiWT* and *EiΔusp02*, *EiΔusp05*, *EiΔusp06*, *EiΔusp07*, *EiΔusp08*, *EiΔusp09*, *EiΔusp10*, and *EiΔusp13* strains at all tested time points.

Survival of *E. ictaluri* USP Mutants in Low pH Stress

To evaluate the role of *usp* genes in survival and growth of *E. ictaluri* at low pH, mutants and *EiWT* were exposed to acidic pH (5.5) and neutral pH, and bacterial growth (quantified by bioluminescent signal) were calculated. The growth rate (photon numbers) of the all USP mutants in low pH was significantly lower than that of in neutral pH. In contrast, the growth of *EiWT* at low pH was lower but not significant (Figures 4A,B). The strongest effect of low pH was observed in *EiΔusp03* growth (62% reduction) compared to *EiWT*. The order of susceptibility of USP mutants in low pH as follows: *Δusp03 > Δusp07 > Δusp13 > Δusp09 > Δusp10 > Δusp08 > Δusp06 > Δusp04 > Δusp05 > Δusp02*. The reduced growth of the USP mutants indicates that *usp* genes contribute *E. ictaluri* survival under acidic conditions.

Survival of *E. ictaluri* USP Mutants in Oxidative Stress

Exposure to hydrogen peroxide (0.1% H₂O₂) significantly reduced growth of *EiΔusp03* and *EiΔusp08* compared to no stress group (91 and 35% reduction, respectively), while growth of *EiΔusp02* and *EiΔusp03* increased under oxidative
stress (Figures 5A,B). No differences for EiΔusp04, EiΔusp06, EiΔusp07, EiΔusp09, and EiΔusp10 were observed.

Virulence and Efficacy of E. ictaluri USP Mutants in Catfish Fingerlings

The percent mortalities in catfish challenged with EiΔusp05, EiΔusp07, EiΔusp08, EiΔusp09, EiΔusp10, and EiΔusp13 were significantly lower than that of EiWT (20, 44.8, 20, 20, 55, and 10% vs. 74.1% mortality, respectively) (Figure 6A). In contrast, no significant differences between EiΔusp02, EiΔusp03, EiΔusp04, and EiΔusp06 and EiWT (79.8, 84.4, 74.6, and 79.82% vs. 74.1% mortality, respectively) were observed (Figure 6A). The order of attenuation in the 10 USP mutants are as following: EiΔusp13 > EiΔusp05 > EiΔusp08 > EiΔusp09 > EiΔusp07 > EiΔusp10 > EiΔusp04 > EiΔusp06 > EiΔusp02 > EiΔusp03.

At 3 weeks post-immunization, EiΔusp05, EiΔusp07, EiΔusp08, EiΔusp09, EiΔusp10, and EiΔusp13 provided significant protection against EiWT challenges (no mortalities; p < 0.01) compared to sham-vaccinated fish (58.33% mortality) (Figure 6B). Although immunization with EiΔusp03 and EiΔusp04 protected catfish significantly, they were not safe. EiΔusp05, EiΔusp08, EiΔusp09, and EiΔusp13 were both safe and protective among all USP mutants.

Figure 7 provides overall summary of the results.

DISCUSSION

Several previous studies reported that universal stress proteins (USPs) play a role in different bacteria to respond to different stress conditions, such as heat, substrate starvation, exposure to antimicrobial agents, acidic stress, and oxidative stress (Seifart Gomes et al., 2011). The objective of this study was to determine the role of E. ictaluri usp genes in acidic and oxidative stresses as well as in virulence. Also, mutants’ vaccine potentials were determined.

The uspA gene among usp genes has been studied in different bacterial strains. Deletion of the uspA genes resulted in decreased virulence in Salmonella typhimurium C5, Listeria monocytogenes, and Acinetobacter baumannii (Liu et al., 2007; Seifart Gomes et al., 2011; Elhosseiny et al., 2015). Also, uspA affected the host invasion and survival in Salmonella enterica and Mycobacterium tuberculosis (Hensel, 2009; Hingley-Wilson et al., 2010). In the present study, there were four usp genes (usp05, usp06, usp08, and usp09) with high similarity to uspA. The growth rate of EiΔusp05, EiΔusp06, EiΔusp08, and EiΔusp09 were similar to E. ictaluri WT. However, EiΔusp05 and EiΔusp08 showed reduced growth in oxidative and acidic stresses compared to EiWT. Virulence data showed that EiΔusp05, EiΔusp08, and EiΔusp09 were significantly attenuated compared to E. ictaluri WT. However, EiΔusp06 was not attenuated. These results are consistent with a previous study in L. monocytogenes where not all uspA are involved.

### Table 1

| Mutant ID | Survival (%) | Vaccination (%) |
|-----------|-------------|-----------------|
|           | pH | H2O2 | Virulence | Efficacy |
| EiΔusp02  | ↓27 | ↑30 | 80 | 15 |
| EiΔusp03  | ↓62 | ↑25 | 84 | 0 |
| EiΔusp04  | ↓40 | ↑19 | 75 | 0 |
| EiΔusp05  | ↓34 | ↓91 | 20 | 0 |
| EiΔusp06  | ↓42 | ↑5 | 80 | 20 |
| EiΔusp07  | ↓51 | ↓3 | 44 | 0 |
| EiΔusp08  | ↓44 | ↓35 | 20 | 0 |
| EiΔusp09  | ↓47 | ↓5 | 20 | 0 |
| EiΔusp10  | ↓45 | ↑3 | 55 | 0 |
| EiΔusp13  | ↓49 | ↓2 | 10 | 0 |

- (↓) Reduction or (↑) increase % in bioluminescence
- Mortality shown by % in vaccination and efficacy

![Figure 7](https://example.com/figure7.png)
in reduced virulence (Seifart Gomes et al., 2011). Previously, our group reported that transposon insertion mutants in usp05 reduced E. ictaluri virulence in catfish and provided better protection against ESC (Kalindamar, 2013). Additionally, expression of usp05 were very high in response to host stress or high level of H$_2$O$_2$ in E. ictaluri (Akgul et al., 2018). The usp05 gene (uspA) is an important regulator of survival and virulence in many pathogens (Tkaczuk et al., 2013). In E. coli, uspA mutant caused a survival defect under a variety of growth-arrested conditions, whereas overexpression induced growth in the growth-arrested state. Our data suggest that usp05, usp08, and usp09 are important virulence genes in E. ictaluri.

We demonstrated that EiΔusp03 and EiΔusp04 have a faster growth rate than EiWT and other USP mutants. However, lack of usp genes did not cause growth differences in Listeria monocytogenes (Seifart Gomes et al., 2011), E. coli (Nystrom and Neidhardt, 1993) or other bacteria when cultured in conventional media (Liu et al., 2007; Hingley-Wilson et al., 2010). Indeed, EiΔusp03 and EiΔusp04 did not show any virulence attenuation in E. ictaluri, which was similar to USP mutant RV2623 in Mycobacterium tuberculosis (Hingley-Wilson et al., 2010). This study suggested that usp genes might play a role in latency and persistence of chronic TB infection. We think that usp03 and usp04 are not involved in virulence but may play other roles in stress responses in E. ictaluri.

Edwardsiella ictaluri can survive and continue growth in up to 3 mM of H$_2$O$_2$ and low acidic pH 5.5. When the USP mutants and EiWT exposed to low pH, growth rates did not change significantly. As shown previously, L. monocytogenes ATP Binding USPs exhibited role in the response to acid stress during exponential growth phase (Tremonte et al., 2016).

Our results indicated that E. ictaluri usp07 contributes to virulence of E. ictaluri. Mortality was significantly decreased in the EiΔusp07 mutant compared to EiWT strain. The usp07 is a KdpD protein, and it contains a uspA domain (Heermann et al., 2009a). We included whole KdpD as usp07 because USP domain is located between the N-terminal sensor domain and C-terminal catalytic domain of this Osmo-sensitive K$^+$ channel histidine kinase. Mutant KdpD in Salmonella typhimurium is attenuated in animal infection model and macrophage survival experiments. It also promotes resistance to osmotic, oxidative and antimicrobial stresses (Alegado et al., 2011). KdpD is also involved in oxidative-osmotic stress, response to host, and virulence (Freeman et al., 2013). In our gene expression study after host stress, usp07 showed a very high expression level (Akgul et al., 2018). It is important to note that usp07 involved in E. ictaluri virulence and acid stress response.

The usp13 was described as a universal stress protein and extra cytoplasmic adaptor protein (CpxP) like protein (Williams et al., 2013). The usp13 (CpxP) is placed in the inner membrane with histidine kinase CpxA and CpxR, a response regulator (Vogt and Raivio, 2012; Debnath et al., 2013). 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 (Motohashi et al., 1999; DiGiuseppe and Silhavy, 2003). The CPX system is important and required for virulence in both Gram-negative and -positive bacteria (Raju et al., 2012). Previously, we determined that E. ictaluri, usp13 is highly expressed when exposed low acidic pH (5.5) and the catfish invasion (Akgul et al., 2018). The usp13 (cpxP) is an essential regulator of cell membrane stress in bacteria during host infection. Therefore, it is involved in the virulence of E. ictaluri with a very high reduction in virulence (Figure 6).

The expression of E. coli usp genes is controlled by some effector proteins and signaling molecules, such as SOS repress proteins (Gustavsson et al., 2002; Kvint et al., 2003; Persson et al., 2007). However, mechanisms of USPs in other bacterial species are not known entirely. Overall our results are in line with studies from various species that USPs were crucial for protecting the cells from the damaging effects of reactive oxygen species (ROS) (Nachin et al., 2005; Liu et al., 2007; Seifart Gomes et al., 2011; Elhosseiny et al., 2015; Figure 7).

CONCLUSION

Our lab aims to develop live attenuated vaccines to protect catfish against E. ictaluri infections. Live attenuated bacterial should be both safe and confer full protection against wild-type infections. This study identified that EiΔusp05, EiΔusp08, EiΔusp09, and EiΔusp13 strains have vaccine potential and further efforts, such as constructing double mutants to improve their safety, could be pursued. The data presented in this study display that USPs are essential for both stress physiology and pathogenesis in E. ictaluri.

AUTHOR CONTRIBUTIONS

AK and ML conceived the project and designed the experiments. AA, SN, SK, HT, and HA conducted the experiments. AA wrote the manuscript. SN, SK, HT, HA, ML, and AK reviewed the manuscript.

FUNDING

This project was supported by Agriculture and Food Research Initiative Competitive grant no. 2016-67015-24909 from the USDA National Institute of Food and Agriculture.

ACKNOWLEDGMENTS

We thank the Laboratory Animal Resources and Care at the College of Veterinary Medicine for providing the SPF channel catfish. AA was supported by a fellowship from the Republic of Turkey.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.