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CHARACTERIZATION OF A
VIRULENCE RELATED HYPOTHETICAL PROTEIN IN
EDWARDSIELLA ICTALURI

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for a degree of
Master of Science

In

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of Pathobiological Sciences

by
Ildiko Katalin Polyak
B.S., Clark University, 2003
December 2007

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Mohandas Ghandi once said, “Whatever you do will be insignificant and it is very important that you do it.” Although simple, his wisdom is very powerful. It has been an influential spark during my graduate studies, speaking to me at every level of my research, lab work, studies, and daily life. Many people have influenced, supported, and advised me in my pursuit of a graduate degree, they deserve not only credit and recognition, but my endless gratitude. I would like to thank my committee members, Dr Thune, Dr Morgan, and Dr Hawke, for all of their advice and guidance in every part of my thesis. Lots of thanks to all of the members of the Thune lab for all of their help and hard work, especially Denise Fernandez, Karen Plant, and Matt Rogge, all who made the challenges of learning and research enjoyable and exciting and were always there to help when I hit a wall. I would like to thank my fan club, my incredibly supportive parents, Steve and Kathy Poyak; my loving brothers and sister-in-law, Levente, Steve, and Lynda; and also all of my friends in Baton Rouge who served as my family away from home, especially Bridget, Danny, Adam, Harley, and the King family. They were always there to hand me a beer and listen to all the trials of graduate school and tribulations of research. Lastly, I would like to thank my advisor again, Dr Thune, for all of his genuine and infectious enthusiasm in and out of the lab. He is an inspiration for all students and was truly the best advisor I could have asked for, even though I didn’t catch any fish in Grand Isle.

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ABSTRACT

Although the biochemical characterization of *E. ictaluri,* the subsequent disease progression of enteric septicemia of catfish (ESC), and the associated pathologic lesions are well characterized, the mechanism of invasion of *E. ictaluri* into a susceptible host is poorly understood. Identification and confirmation of virulence factors and associated genes of *E. ictaluri* is crucial to elucidating the pathogenesis of this important disease. A signature tagged mutagenesis (STM) study conducted by Thune et al. (2006) identified 50 *E. ictaluri* clones with transposon insertions in genes potentially involved in pathogenesis. A specific STM mutant, 233PR, carrying a transposon insertion in a gene encoding a hypothetical adhesin protein, was selected for further characterization. In addition, an isogenic mutant was created by inserting a kanamycin resistance cassette 222 bp downstream from the site of the transposon insertion in 233PR in order to examine the effects of differential protein truncation on function. Bioinformatic analysis of the *E.* ictaluri genome revealed a pathogenicity island encoding genes with similarity to a gene cluster encoding putative adhesin/hemolysin genes in *E. coli* CL3 (Shen et al. 2004). *In vivo* results demonstrated the importance of the putative adhesin's role in *E. ictaluri* pathogenesis and that protein length correlated to the level of attenuation. *In vitro* data did not support a role in adhesion, invasion, or intracellular replication in cell culture. The *E. ictaluri* PAI genes were designated *eacA-H* for Edwardsiella attenuation complex Results demonstrate that EacF, the putative adhesin, is a virulence factor, but further investigation is required to determine its specific role in *E. ictaluri* pathogenesis.

INTRODUCTION AND LITERATURE REVIEW

Aquaculture was first attempted in the United States in the late 20th century, and while Asian countries continued to dominate aquaculture production, the value of U.S. aquaculture production rose over 400% between 1980 and 1998, making it an important part of U.S. agriculture and the U. S. economy. The channel catfish (*Ictalurus punctatus*) industry is the largest sector of the U.S. aquaculture industry, accounting for almost half of all sales at $433.6 million in 2005 (Harvey 2006). Despite increased production, profitability of the catfish industry is declining, with disease related problems constituting the largest single cause of economic losses. Bacterial pathogens cause the most overall disease problems, with Gram-negative bacteria leading the most frequent causes of bacterial disease mortalities in finfish (USDA 2003). Enteric septicemia of catfish (ESC) is the principle disease problem in catfish culture, costing over $10 million in losses annually (Hawke et al. 1998).

Enteric septicemia of catfish was first identified in moribund catfish in Georgia and Alabama in 1976, which led to the discovery of its etiologic agent, *Edwardsiella ictaluri* in 1979 (Hawke 1979; Hawke et al. 1981). Outbreaks of ESC occur primarily during the spring and early fall when the water temperatures are naturally in the range of 22°C - 28°C, a temperature range known as the "ESC window" (MacMillan 1985; Francis-Floyd 1987). Originally, Hawke (1979) suggested that *E. ictaluri* was an obligate pathogen that could only survive in water for eight days, but it has since been shown that it can survive for up to 95 days at 18°C and 25°C when inoculated into sterile pond mud (Plumb and Quinlan 1986). Outbreaks of ESC occur primarily in the southeastern United States, especially Mississippi, Arkansas, Alabama, Louisiana, Georgia, and Florida. The disease occurs less frequently in Virginia, Texas, Idaho, Indiana, Kentucky, California, Arizona, and Maryland (Hawke et al. 1998). Although channel

catfish are the most susceptible to infection, white catfish, brown bullhead, walking catfish, and blue catfish can also be infected by *E. ictaluri*. Experimentally exposed golden shiners, largemouth bass, and bighead carp are all resistant to infection, while tilapia are mildly susceptible (Plumb and Sanchez 1983). However, European catfish (Plumb and Hilge 1987), rainbow trout and Chinook salmon (Baxa and Hedrick 1989) are all susceptible to infection following experimental exposure to ESC. Natural outbreaks have also been reported in non-ictalurid species, including green knife fish (Kent and Lyons 1982), danio (Waltman et al. 1985), rosy barbs (Humphrey et al. 1986), and walking catfish (Kasornchandra et al. 1986).

The biochemical characterization of the causative agent of ESC, *Edwardsiella ictaluri,* was first described by Hawke, et al. (1981), with further studies by Waltman et al. (1986) and Plumb et al. (1989). Briefly, *E. ictaluri* is a Gram negative member of the enterobacteriaceae family and is most similar to *Edwardsiella tarda* and *Yersinia ruckeri. Edwardsiella ictaluri* measures 0.75 x 2.5 µm at 26 C and is weakly motile with peritrichous flagellation. Smooth, circular colonies grow on brain heart infusion (BHI) agar at a slow rate that requires 48 hours at 26 C to produce colonies 2 mm in size. Optimal growth occurs between 25 C – 30 C, which overlaps the catfish disease susceptibility range of 22 C – 28 C (Francis-Floyd 1987). The organism is cytochrome oxidase negative, reduces nitrate to nitrite, does not produce a pigment, is positive for lysine and ornithine decarboxylase, and oxidizes and ferments glucose. It is negative for indole, citrate, protease, esterase, pectinase, chitinase, lipase, alginase, collagenase, hyaluronidase, and many carbohydrates. *Edwardsiella ictaluri* was previously characterized as urease negative, but Booth (2006) recently described an acid activated urease gene that is involved in pathogenesis in the catfish host.

Edwardsiella ictaluri causes a disease that is best described as an acute, rapidly progressive septicemia in exposed or inoculated healthy fish. Environmental factors that are favorable for proliferation of *E. ictaluri* and stressful for the host favor development of ESC in catfish. Transmission can occur by either introduction of an ESC-infected fish into a pond with healthy fish or by stocking healthy fish into a pond of asymptomatic carrier fish. Clinical signs of ESC vary with fish size, stocking density, and water temperature (Hawke 1979; MacMillan 1985). Fish can be seen swimming erratically in tight circles or hanging listlessly in the water column in a head up and tail down position. They normally stop eating shortly after becoming infected (Jarboe et al. 1984; Blazer et al. 1985), eliminating treatment with medicated feed as a potential cure.

The disease process of ESC is well characterized. ESC can manifest as either an acute gastrointestinal septicemia with rapid mortality or as a chronic disease exhibiting a very characteristic "hole-in-the-head" lesion (Shotts and Blazer 1986; Johnson 1989). External lesions include small red and white ulcers covering the skin, petechial hemorrhages around the mouth, base of fins, or ventral and lateral surfaces, pale and swollen gills, exophthalmia, and a very swollen abdomen due to the accumulation of ascitic fluids (Areechon and Plumb 1983; Jarboe et al. 1984; MacMillan 1985; Hawke et al. 1998). This blood tinged or clear yellowish ascitic fluid is a hallmark of acute septicemia that is also caused by other types of bacterial pathogens. The intestine contains clear red fluid and is partially filled with gas. Other internal lesions include petechial hemorrhages in the muscles, intestine, fat, and liver. The liver is friable with characteristic pale foci of tissue necrosis, and there is massive necrosis in the spleen and kidney (Hawke 1979; Areechon and Plumb 1983; Jarboe et al. 1984; Blazer et al. 1985; MacMillan 1985; Miyazaki and Plumb 1985; Waltman et al. 1985).

Although the biochemical characterization of *E. ictaluri,* the subsequent disease progression of ESC, and the associated pathologic lesions are well characterized, the mechanism of invasion into a susceptible host is poorly understood. Studies suggest various possible routes of invasion. Miyazaki and Plumb (1985) proposed that *E. ictaluri* first colonizes the olfactory sac and enters the brain via the olfactory bulb (Miyazaki and Plumb 1985), then disseminates systemically and concludes in fatal septicemia. In another study, fish exposed orally to *E. ictaluri* developed the acute form of ESC and fish exposed via the water, the route that favors colonization of the olfactory sac, developed the chronic form of ESC (Shotts and Blazer 1986). Newton et al. (1989) exposed channel catfish to viable *E. ictaluri* cells by direct immersion, resulting in the development of the acute septicemic form of ESC in 93% of the fish exposed, with development of the chronic form of ESC in the remaining 7% of the fish. Microscopic lesions were present within 2 days of immersion challenge. In other studies, catfish kidney was culture positive for *E. ictaluri* as early as 15 minutes after exposure by gastric lavage, and liver was culture positive by 30 minutes post exposure (Baldwin and Newton 1993).

The rapid spread of *E. ictaluri* from the intestinal lumen to the internal organs suggests circulation through the vasculature as free organisms or within migrating phagocytic leukocytes (Shotts and Blazer 1986; Janda et al. 1991; Baldwin 1992; Reger et al. 1993). Several authors have suggested that *E. ictaluri* is capable of survival and growth within catfish macrophages based on the histological observation of intracellular bacteria that appeared to be in the process of cellular division within the macrophage phagocytic vacuoles, although bacteria were also observed within interstitial spaces (Miyazaki and Plumb 1985; Shotts and Blazer 1986; Baldwin and Newton 1993). It was also demonstrated through histological observation that *E. ictaluri* is generally found in vacuoles of phagocytic leukocytes, and is only occasionally found free in

tissue when associated with degenerating leukocytes (Shotts and Blazer 1986; Baldwin and Newton 1993). Booth et al. (2006) determined that *E. ictaluri* can successfully invade, survive, and replicate in catfish macrophages *in vitro*. Their study used bacteria opsonized with either a normal autologous catfish serum or with heat-inactivated serum to resemble first exposure to a natural infection and successful entry of *E. ictaluri* into the macrophage. Ingestion of non-opsonized bacteria suggest that *E. ictaluri* may have a surface invasion ligand that is recognized by a specific receptor on the macrophage (Booth et al. 2006).

Little work has been conducted to evaluate receptor mediated adherence of *E. ictaluri* to host cells, although mannose sensitive and mannose resistant hemaggultination of non-fish erythrocytes has been demonstrated in some strains (Wong et al. 1989). A cell associated hemolysin that was thought to contribute to reduction of hematocrit, hemoglobin, plasma protein, and plasma glucose associated with ESC infection has been described (Waltman et al. 1986). Another study utilizing a model in which *E. ictaluri* was coincubated with erythrocytes from guinea pigs, sheep, and rabbits reported that *E. ictaluri* does not express any associated hemolysins (Janda et al. 1991). However, since *E. ictaluri* is a host specific fish pathogen, it is possible that it possesses hemolysins that do not lyse mammalian erythrocytes. Williams and Lawrence (2005) identified a two-component hemolysin in *E. ictaluri* that was homologous to a virulence factor in *E. tarda*. However, virulence tests using an isogenic hemolysin mutant strain in channel catfish challenges did not demonstrate significant difference in virulence compared to the wild type (WT) *E. ictaluri* strain (Williams and Lawrence 2005). There are only two virulence factors that have been identified and confirmed in *E. ictaluri* using isogenic mutants: the secreted enzyme chondroitinase (Cooper et al. 1996), which plays a putative role in the formation of the chronic "hole-in-the-head" lesions due to cartilage degradation (Shotts and

Blazer 1986), and the O polysaccharide (OPS) (Lawrence et al. 2001; Lawrence et al. 2003), an important part of the Gram-negative outer membrane that can mediate resistance to complement-mediated killing. Identification and confirmation of more virulence factors and associated genes of *E. ictaluri* is crucial to elucidating the pathogenesis of this important disease.

Signature tagged mutagenesis (STM) is a well accepted method of identifying virulence associated genes that is based on the selection of mutants that have lost their ability to survive in a host, allowing the discovery of virulence genes prior to ascertaining their function (Autret and Charbit 2005). Signature tagged mutagenesis was first developed by David Holden in 1995 as a method that utilized dot-blot hybridization techniques with polymerase chain reaction (PCR) amplified tags (Hensel et al. 1995). Using this method many mutants can be screened at the same time, allowing for rapid analysis of virulence factors in many organisms. Despite a few drawbacks to STM methods, such as signal quality and reproducibility (Autret and Charbit 2005), Holden's STM method has been successfully used to identify virulence factors in pathogens such as *Staphylococcus aureus, Vibrio cholera,* and *Yersinia enterocolitica* (Mei et al. 1997; Chiang and Mekalanos 1998; Darwin and Miller 1999).

More recently, modifications to Holden's method that are simpler and faster have been developed by Lehoux et al. (1999). This optimized method was utilized by Thune et al. (Thune et al. 2007) in studies to identify virulence genes of *E. ictaluri*. Briefly, the optimized STM technique utilizes transposon insertion mutagenesis, where each transposon carries a unique tag that can be identified by PCR analysis. Libraries of bacterial mutants are constructed using pUTmini-Tn*5Km2* plasmids. Pools containing the uniquely tagged mutants are then used to infect the suitable host, channel catfish. Following infection, mutants that invaded and persisted *in vivo* are recovered and screened by PCR. Those strains found to be missing from the host

tissue are assumed to have the transposon insertion in genes required for establishment and persistence of infection. Sequencing the flanking *E. ictaluri* genomic DNA of the insertion cassette identifies the gene of insertion and further sequence analysis provides additional information regarding surrounding genes.

The STM study conducted by Thune et al. (2006) identified 50 *E. ictaluri* clones with transposon insertions in genes potentially involved in pathogenesis. Specifically, STM mutant 233PR, carrying a transposon insertion in a gene encoding a hypothetical virulence protein, was selected for further characterization. The STM attenuation was confirmed with a competitive index (CI) that was determined by challenging the host catfish with equal colony forming units (CFU) of mutant and wild type (WT) bacteria and then dividing the recovery ratio of mutant CFU/WT CFU, by the input ratio of mutant CFU/WT CFU. Values for the CI range from 0 to 1, with values closer to 0 indicating greater attenuation compared to full virulence at values closer to 1. The 233PR CI of 0.00089 indicates that this clone was attenuated and is a good candidate for further analysis (Thune et al. 2007). Based on bioinformatic analysis, the gene mutated in 233PR has similarity to putative adhesin, hemolysin, and hemagglutinin genes of other pathogenic bacteria and has a downstream region that encodes other adhesion related motifs. Elucidation of adherence and invasion mechanisms of *E. ictaluri* could greatly contribute to our understanding of the pathogenesis of ESC; therefore, mutant 233PR was selected as a promising candidate to study.

Adherence of pathogenic bacteria to host cell surfaces and invasion of host tissues are crucial initial steps to colonization and establishment of a bacterial infection (Ofek and Beachey 1980). Adherence allows extracellular pathogens to withstand the mechanical clearing mechanisms of the host and is a necessary step for uptake and invasion by intracellular

pathogens. Adhesins are specialized surface proteins that mediate bacterial adhesion by controlling contact between the bacterium and the host cells (Ofek et al. 2003). Bacterial interaction with host cell receptors function to target a pathogen to a particular niche, activate signaling pathways, establish persistent infection, and induce invasion of the pathogen (Finlay and Cossart 1997). Different bacterial species and strains produce many different adhesin determinants, allowing adherence to a variety of host cells and receptors. Due to the vast arsenal of adhesins in the prokaryotic realm, it is necessary to focus on those most related to the hypothetical protein in *E. ictaluri*, specifically Gram-negative *Enterobacteriaceae* members.

Adhesins are either located on filamentous surface structures called pili (also known as fimbria), or on surface structures not assembled into pili. Pili are adhesive hair-like structures that protrude from the surface of bacteria and function with an adhesin to attach bacteria to a surface. They are composed of a rod anchored to the bacterial outer membrane and a bacterial adherence factor, or adhesin, at the tip, which confers the binding specificity (Pizarro-Cerda and Cossart 2006). The pilin domain, responsible for anchoring the adhesin to the pilus, is encoded in the carboxy terminus of the protein, while the receptor binding domain is encoded in the amino terminus (Choudhury et al. 1999).

Many types of pili have lectins, which are adhesins that show high substrate specificity for carbohydrate (Haslam et al. 1994). The type 1 pilus, the most frequently expressed pilus of enterobacteria, is encoded by the *fim* gene cluster and exported by the chaperone-usher pathway. Receptor specific adhesion is mediated by the fimbrial tip associated lectin-like subunit FimH, which dictates the pilus adhesin (Orndorff and Falkow 1984; Soto and Hultgren 1999; Thomas et al. 2002). The most common enteric bacterial adhesin, FimH, binds specifically to mannose carbohydrates on cell surfaces (Ofek et al. 1982; Karlsson et al. 1992; Ofek and Doyle 1994). It

was traditionally thought that internal bodily fluid flow reduced bacterial adhesion. In this model, bacteria bound to surfaces by receptor ligand bonds that were described as 'slip-bonds' (Dembo et al. 1988; Wang et al. 1995; Dickinson et al. 1997; Shive et al. 1999), whose bond adhesive strength weakened exponentially under force (Bell 1978; Evans 2001). However, recent studies have demonstrated that the binding strength of FimH increases with shear stress (Forero et al. 2006; Nilsson et al. 2006; Anderson et al. 2007) due to the formation of catch bonds that strengthen under force (Thomas et al. 2004; Nilsson et al. 2006; Thomas et al. 2006). In its native state FimH binds with a short-lived, weak bond to mannose presenting surfaces under static and low-flow conditions, but then undergoes a conformational change to long-lived, strong bonds induced by high flow shear stress, termed shear dependent stick and roll adhesion (Thomas et al. 2004; Nilsson et al. 2006).

Adhesins frequently work in concert to promote colonization and invasion under various environmental conditions and bind to numerous specific and non-specific receptors and surfaces, making it very difficult to determine which genes are involved in different stages of attachment and invasion during colonization and infection. Pathogenic bacteria have evolved an incredibly vast and diverse array of adhesion and invasion molecules that enable them to exploit a variety of host-cell surface components. Based on this knowledge, the underlying hypothesis of this study is that the hypothetical gene encoding mutant 233PR has an important role in the pathogenesis of *E. ictaluri* as a possible adhesin.

Using the 233PR strain and a newly created isogenic mutant strain to test the hypothesis, studies were completed to determine the role of the hypothetical adhesin in the pathogenesis of *E. ictaluri* in channel catfish. Mortality and persistence studies, along with a competition challenge, were performed to determine if the WT strain was able to supplement the defect *in*

vivo of the attenuated mutant strain. To assess the effect of disruption of the hypothetical adhesin gene on invasion, intracellular survival, and replication, a gentamicin exclusion assay was performed using channel catfish head kidney derived macrophages (HKDM) and in a channel catfish ovary (CCO) cell line. The following sections provide detailed analysis of the procedures and results that examines the putative adhesin as a virulence factor in the pathogenesis of *E. ictaluri*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli*, used for maintaining the plasmids during mutation of the putative adhesin gene, was grown in Luria-Bertani (LB) broth at 37 C throughout the entire study. *Edwardsiella ictaluri* strains were grown in either brain-heart infusion (BHI) broth or LB broth supplemented with either fish peptone (LB-FP) or mannitol (LB-Man) at 28 C. *E. coli* strain CC118 λ*pir* was used to maintain the delivery plasmids and to isolate plasmid DNA prior to introduction into the conjugation strain, SM10 λ*pir*. Antibiotics, used for the selection of resistant and sensitive bacterial strains, were used in the following concentrations: kanamycin (Km) at 50 μg/ml, colistin (Col) at 10 μg/ml, tetracycline (Tet) at 65 μg/ml, and ampicillin (Amp) at 20 μg/ml. *E. ictaluri* transconjugates that were re-isolated from fish were grown on tripticase-soy agar plates supplemented with 5% de-fibrinated sheep blood (BA).

Specific-Pathogen-Free (SPF) Channel Catfish. All animal use was in accordance with LSU laboratory animal use guidelines and approved animal use protocol. Egg masses were obtained from commercial channel catfish producers with no history of enteric septicemia of channel catfish (ESC) outbreaks, disinfected with 100 mg/L free iodine, and hatched in closed recirculating systems in the LSU SPF laboratory. All experimental fish were reared in the SPF laboratory. Holding systems consisted of four 350-liter fiberglass tanks connected to a 45 liter biological bead filter. Water temperature was maintained at 27±2 C and water quality parameters, consisting of total ammonia nitrogen, total nitrate, pH, hardness, and alkalinity, were measured 3 times per week using a HACH (Loveland, CO) aquaculture kit. Water quality was adjusted as necessary to maintain optimal conditions.

TABLE 1 Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Source
Escherichia coli		
CC118 λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (Am) *recA* λ*pir* phage lysogen	(de Lorenzo and Timmis 1994)
SM10 λ*pir*	Km^r *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* phage lysogen	(de Lorenzo and Timmis 1994)
XL1-Blue MRF'	*-hsdSMR-mrr*) *173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proABblacIgZ.M15* Tn5 (Kan)]	Stratagene, La Jolla, CA
Edwardsiella ictaluri		
93-146	Wild-type *E. ictaluri* isolated in 1993 from moribund channel catfish in a natural outbreak of ESC on a commercial farm	Louisiana Aquatic Diagnostic Laboratory
233PR	Derived from the parental WT strain 93-146, having an insertion of STM tag P/R in an adhesin gene	This study
EacF::Km	Isogenic mutant strain derived from the parental WT strain 93-146, having a pUT-mini-Tn5Km cassette insertion in place of the restriction enzyme *Bsa*BI site located in an adhesin gene	This study
Plasmids		
pBluescript SK-	Phagemid cloning vector	Stratagene, La Jolla, CA
pUT-Km-MCS	pUT-mini-Tn5Km with multiple cloning site containing *Eco*RV, *Xba*I, and *Apa*I restriction sites	(Thune et al. 2007)
pGP704	Ap^r, pBR322 derivative with R6K *ori, mob* RP4, polylinker from M13 tg131	(Miller and Mekalanos 1988)

Bioinformatic Analysis. In order to further characterize the genetic region surrounding the site of insertion of the STM transposon, the Blast Local Alignment Search Tool (BLAST) was used to analyze the *E. ictaluri* genomic database (http://microgen.ouhsc.edu/cgi-bin/blast_form.cgi). Resulting sequence from the data base was analyzed using the Open Reading Frame (ORF) finder tool, with subsequent BLAST analysis of the individual ORFs at the National Center for Biotechnology Information, The LALIGN program at www.ch.embnet.org was used to align and compare individual sequences when required.

Construction of EacF::Km Mutant Strain. The bioinformatic analysis resulted in the identification of substantial sequence surrounding the STM transposon insertion site, which was used to design a strategy for constructing an isogenic mutant of the open reading frame (ORF) encoding the putative adhesin. Briefly, primers were designed that amplified a 1617 bp fragment, 836 bp upstream and 781 bp downstream from the proposed transposon insertion site in 233PR (233PR F TAATCAAGCAGAACACCC and 233PR R ATCACAGAACCACCAATAG). The reaction was performed in a 50 µl volume consisting of 0.5 µg template, 1.0 Unit of PFU DNA polymerase (Stratagene, La Jolla, CA), 1.0 µM for forward and 1.0 µM for reverse primers, 1.0 µM 10 X Pfu PCR buffer (Stratagene, Ind., La Jolla, CA) and 200 µM of each dNTP. PCR was performed in a GeneAmp 9700 thermal cycler (PE-Applied Biosystems, Foster City, CA). Cycling parameters were 94 C for 5 min, followed by 35 cycles of 94 C for 30 s, 55.1 C for 45 s and 72 C for 1 min, followed by 72 C for 5 min. The PCR reaction was purified using Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and the fragment generated was confirmed by DNA sequencing.

For cloning, 1.5 pmol of the PCR product was phosphorylated with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA). A total of 10 µg of the phagemid cloning

vector pBluescript SK- plasmid DNA was digested with *Eco*RV (New England Biolabs, Inc., Beverly, MA), alkaline phosphatase treated using calf intestinal phosphatase (CIP) (New England Biolabs, Inc., Beverly, MA), and phenol extracted. The gene insert was ligated into the pBS SK- vector using T4 DNA ligase (New England Biolabs, Inc., Beverly, MA). The ligation was purified by drop dialysis on a 0.025 µm Millipore nitrocellulose filter (Millipore Corporation, Billerica, MA) over sterile distilled deionized water, electroporated into *E. coli* XL1-Blue MRF' competent cells using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA), allowed to recover in 1 mL LB broth at 37 C, and then spread onto S-Gal-Amp (3,4-cyclohexenoesculetin-b-D-galacto-pyranoside) agar (Sigma Chemicals, St. Louis, MO) plates to differentiate between *lac*+ and *lac*- colonies for selection of transformants. Plasmid DNA was isolated from the selected grey colony using the Qaigen midiprep kit (Qiagen, Valencia, CA), run on a 0.7% agarose gel alongside uncut pBS SK- DNA to ensure that the vector contained the insert at approximately 1.6 kb. The plasmid was then sequenced using *E. coli* specific T3 and T7 primers. Plasmid DNA carrying the putative adhesin insert was linearized with restriction enzyme *Bsa*BI (New England Biolabs, Inc., Beverly, MA), and treated with Antarctic Phosphatase (New England Biolabs, Inc., Beverly, MA) in preparation for insertional mutagenesis using the kanamycin resistance cassette.

The 897 bp mini-Tn5-Km gene was amplified using primers kan 757 (GAAGCCCTGCAAAGTAAA) and kan 1635 (GCTCAGAAGAACTCGTCAA) from pUT-mini-Tn5Km DNA template. The reaction was performed in a 50 µl volume containing 0.5 µg template, 1.0 Unit of Pfu Ultra DNA polymerase (Stratagene, La Jolla, CA), 1.0 µM of each forward and reverse primer, 1.0 µM 10 X Pfu Ultra PCR buffer (Stratagene, Ind., La Jolla, CA), and 200 µM of each dNTP. PCR was performed in a GeneAmp 9700 thermal cycler (PE-

Applied Biosystems, Foster City, CA). Cycling parameters were 94 C for 5 min, followed by 35 cycles of 94 C for 30 s, 54.0 C for 45 s and 72 C for 1 min, followed by 72 C for 5 min. The PCR reaction was purified using Qaigen PCR purification kit (Qiagen, Valencia, CA) and phosphorylated using T4 polynucloetide kinase (New England Biolabs, Inc., Beverly, MA).

The mini-Tn5-Km gene was ligated into the digested plasmid DNA carrying the hypothetical adhesin insert using T4 DNA ligase (New England Biolabs, Inc., Beverly, MA). The ligation was purified by drop dialysis on a 0.025 μm Millipore nitrocellulose filter (Millipore Corporation, Billerica, MA) over sterile distilled deionized water, and then electroporated into *E. coli* XL1-Blue MRF' competent cells using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA). The cells were allowed to recover in 1 mL LB broth at 37°C, and then spread onto LB-km-amp agar plates for selection of transformants carrying the mini-Tn5-Km insert and associated chromosomal DNA flanking the site of insertion. Plasmid DNA was isolated from the selected colonies using the Qaigen midiprep kit (Qiagen, Valencia, CA) and run on a 0.7% agarose gel alongside uncut pBS SK- DNA and uncut pBS with the 233PR insert DNA to ensure that the new construct contained the hypothetical adhesin carrying the mini-Tn5-Km insert.

The suicide vector pGP704 was isolated from CC118 λ*pir* using the Qaigen midiprep kit (Qiagen, Valencia, CA), digested with *Eco*RV (New England Biolabs, Inc., Beverly, MA), and treated with Antarctic Phosphatase (New England Biolabs, Inc., Beverly, MA). A total of 5.0 μg of plasmid DNA containing the hypothetical adhesin carrying the mini-Tn5-Km insert was digested with restriction enzymes *Spe*I and *Xho*I (New England Biolabs, Inc., Beverly, MA), treated with 5.0 units of Klenow fragments (New England Biolabs, Inc., Beverly, MA), and separated by electrophoresis on a 2.0% agarose gel. The band that contained the 2486 bp

eacF::km fragment was excised, gel purified using the Mo-Bio ultra-clean gel spin kit (Mo Bio Laboratories, Inc., Carlsbad, CA), and ligated into the digested pGP704 suicide vector using T4 DNA ligase (New England Biolabs, Inc., Beverly, MA). The ligation was purified by drop dialysis on a 0.025 µm Millipore nitrocellulose filter (Millipore Corporation, Billerica, MA) over sterile distilled deionized water, electroporated into *E. coli* CC118 λ*pir* competent cells using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA), allowed to recover in 1 mL LB broth at 37°C, and then spread onto LB-km-amp agar plates for selection of transformants carrying the *eacf::km* fragment.

The genetic construction of the selected colonies was confirmed by PCR. Each reaction was performed in a 50 µl volume consisting of 3 µL of boil-prepped DNA, 0.5 Unit of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA), 1.0 µM 10 X Applied Biosystem PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 1.0 µM of each forward and reverse primers, and 200 µM of each dNTP. PCR was performed in a GeneAmp 9700 thermal cycler (PE-Applied Biosystems, Foster City, CA). Cycling parameters were 94C for 5 min, followed by 35 cycles of 94 C for 30 s, 55.0 C for 45 s and 72 C for 1 min, followed by 72 C for 5 min. Plasmid DNA from the correct colonies carrying the *eacf::km* fragment was isolated using the Qaigen miniprep kit (Qiagen, Valencia, CA) and directly electroporated into *E. coli* SM10 λ*pir* competent cells, allowed to recover in 1 mL LB broth at 37 C, and then spread onto LB-km-amp agar plates for selection of transformants carrying the *eacf::km* fragment. Plasmid DNA was isolated from select colonies using the Qaigen miniprep kit (Qaigen, Valencia, CA), digested with restriction enzyme *Xba*I (New England Biolabs, Inc., Beverly, MA) and separated by gel electrophoresis on a 0.7% agarose gel to confirm that the selected colonies carried the *eacf::km* fragment.

The mutated gene was then introduced into *E. ictaluri* by conjugation. Briefly, *E. ictaluri* 93-146 was mated with *E. coli* SM10 λ*pir* containing pGP704 *eacf::km* on a Pall 0.4 µM membrane filter (Pall Corporation, Ann Arbor, MI). The filter was incubated on a BA plate and the bacterial cells were recovered in 1 mL LB-FP, spread onto LB-FP-km-col plates, and incubated at 28°C. Putative transconjugates were patched onto LB-FP-km-amp and LB-FP-km-col plates and incubated at 28°C. As *E. ictaluri* is resistant to colistin but sensitive to ampicillin, a single crossover event resulted in colonies that were km and amp resistant, indicating plasmid integration. The desired double crossover resulted in colonies that were km resistant and amp sensitive due to gene replacement by homologous recombination and loss of the suicide plasmid.

Those colonies that grew on the LB-FP km-col plates but not the LB-FP-km-amp plates were further examined by PCR. Each reaction was performed in a 50 µl volume containing of 3 µl of chromosomal DNA, 0.5 Unit of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA), 1.0 µM 10 X Applied Biosystem PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 1.0 µM for each forward and reverse primers, and 200 µM of each dNTP. PCR was performed in a GeneAmp 9700 thermal cycler (PE-Applied Biosystems, Foster City, CA). Cycling parameters were 94 C for 5 min, followed by 35 cycles of 94 C for 30 s, 55.0C for 45 s and 72 C for 1 min, followed by 72 C for 5 min. The PCR product was separated by gel electrophoresis on a 0.7% agarose gel to confirm the presence of the mini-Tn5-Km insert. Chromosomal DNA was isolated according to the method of Ausubel et al. (Ausubel et al. 1994) and stored at 4°C. The isogenic mutant strain was designated EacF::Km (the altered *E. ictaluri* adhesin protein carrying a kanamycin resistance cassette) to differentiate it from mutant strain 233PR from the STM project.

The EacF::Km construct was further confirmed by differential PCR using gene specific primers 233PR F and 233 PR R and mini-Tn5-Km specific primers kan 757 and kan 1635 in various combinations to examine the full length gene including the mini-Tn5-Km insert (233PR F x 233PR R), the mini-Tn5-Km insert (kan 757 x kan 1635), from the beginning of the gene to the end of the mini-Tn5-Km insert (233PR F x kan 1635) and the beginning of the mini-Tn5-Km insert to the end of the gene (kan 757 x 233PR R). Each reaction was performed in a 50 µl volume consisting of 0.5 µg template EacF::Km DNA, 0.5 Unit of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA), 1.0 µM 10 X Applied Biosystem PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 1.0 µM of each forward and reverse primers, and 200 µM of each dNTP. PCR was performed in a GeneAmp 9700 thermal cycler (PE-Applied Biosystems, Foster City, CA). Cycling parameters were 94 C for 5 min, followed by 35 cycles of 94 C for 30 s, primer specific annealing temperature for 45 s and 72 C for 1 min, followed by 72 C for 5 min. The annealing temperatures for primer pairs was 59 C for 233PR F x 233PR R, 60.1 C for kan 757 x kan 1635, 58.5 C for 233PR F x kan 1635, and 55.2 C for kan 757 x 233PR R. The PCR reactions were purified using Qaigen PCR purification kit (Qiagen, Valencia, CA) and sequenced to confirm proper genetic construction of the new mutant strain.

Confirmation of a SingleIinsertion Event in the EacF::Km and 233PR Mutant Strains. It was necessary to confirm that there was only a single transposon insertion in 233PR and also a single Km-resistance cassette insertion in the EacF::Km mutant strains. A single insertion event was confirmed using genomic DNA prepared from the 233PR and EacF::Km mutant strains using the standard method of Ausbel et al. (1994). A total of 10 µg of DNA was digested to completion with *Cla*I, which does not cut the mini-Tn5-Km insert or Km-STM transposon. Digested genomic DNA was separated on a 1.0% agarose gel and transferred to an ECL Hybond

N+nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). A 140 bp Km product was amplified from pUT-mini-Tn5Km using primers KanGeneF (AGAAAGTATCCATCATGGC) and KanGeneR (ATCATCCTGATCGACAAG) and labeled using the ECL nucleic acid labeling system (Amersham Pharmacia Biotech, Piscataway, NJ). The probe was hybridized to each digested genomic DNA on the membrane, and detected using the ECL detection reagents according to the manufacturer's instructions.

Competitive Challenge with WT *E. ictaluri*. Comparative attenuation of the two strains, EacF::Km and 233PR verified that the gene that was successfully knocked-out is a virulence factor of *E. ictaluri*. One tank of 25 fish was inoculated by immersion challenge as described by Thune, et al. (1999). Briefly, experimental SPF fish were transferred to a separate laboratory into 20 L tanks supplied with a continuous flow of de-chlorinated water maintained at 27±1C at a flow rate of 500-600 ml per minute. Fish were stocked at a density of 25 fish per tank and fed commercial catfish feed every day during a four week acclimation. After lowering the tank water level to four liters and turning off water flow, 40 ml (approximately 1 X 10^{8} CFU/ml final concentration) of WT strain 94-146 and 40 ml (approximately 1 X 10^{8} CFU/ml final concentration) of the EacF::Km strain was added to the water and left for one hour, after which the water flow was resumed. Air circulation was constant during the challenge. Liver samples from fish that died were removed, weighed, homogenized and spread on both LB-Man agar and LB-Man-Km agar plates. The plates were incubated at 28 C for 48 hours, after which CFU/gm of tissue for WT and mutant bacterial strains was determined. The competitive index (CI) was determined by dividing the recovery ration of mutant/WT by the input ratio of mutant/WT, with values closer to 0 indicating greater attenuation compared to values closer to 1.

Mortality Assay. Three tanks of 25 fish were inoculated by immersion challenge as described above with 80 ml (1 X 10^8 CFU/ml final concentration) of either WT, 233PR, or EacF::Km *E. ictaluri* strains for one hour and observed for mortality. Inoculation with 80 ml LB broth served as a negative control. Liver samples from all dead fish were streaked on BA plates and incubated at 28 C for 48 hours to confirm the presence of *E. ictaluri*.

Persistence Studies. Three tanks of 25 fish were inoculated by immersion challenge as described above with 80 ml (1 X 10^8 CFU/ml final concentration) of either WT, 233PR, or EacF::Km *E. ictaluri* strains for 15 minutes. One tank inoculated with 80 ml LB-Man broth served as a negative control. One fish was removed and euthanized daily from each of the three tanks per strain for the duration of the study. Head kidney tissue from the fish was removed, weighed, homogenized, spread on LB-Man agar plates, and incubated at 28 C for 48 hours. Recovered CFU/gm of tissue was determined for each strain each day of the study.

Intracellular Survival in Channel Catfish Macrophages. The gentamicin survival assay was performed as described by Booth et al. (2006). This assay enables quantification of internalized bacteria at various time points of post infection, in order to accurately examine intracellular invasion, survival, and replication in the channel catfish head kidney derived macrophage (HKDM). Briefly, channel catfish were anesthetized with tricane methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) and bled from the caudal vein to collect autologous serum. Macrophages were prepared from channel catfish head kidney tissue, suspended in channel catfish macrophage media (CCMM), diluted to 1 X 10^7 cells/ml with CCMM, plated 1 ml/well in a 24 well poly-D-lysine coated cell culture plates (Biocoat Cell Environments, Becton Dickinson Labware, Bedford, MA) and allowed to adhere for 16 hours at 28 C with 5% CO_2 in a CO_2 water jacketed cell culture incubator (Forma Scientific, Inc.,

Marjeta, OH). For microscopic observations, glass cover slips coated with poly-D-lysine (Biocoat Cell Environments, Becton Dickinson Labware, Bedford, MA) were placed in wells prior to the addition of cells.

After 16 hours, the HKDM were washed three times to remove non-adherent cells, and the media was replaced with fresh CCMM, yielding approximately 1×10^5 cells per well. Overnight late-log phase cultures of WT, 233PR and EacF::Km strains were serially diluted to 1×10^6 bacteria/ml and treated with either autologous normal serum (NS) or heat-inactivated autologous serum (HIS) to examine possible surface invasion ligands capable of recognizing specific receptors located on macrophages. Macrophages were inoculated with 1×10^4 CFU per well of NS and HS treated WT, 233PR, or EacF::Km strains at a multiplicity of infection (MOI) of 1:10 (bacteria:macrophage). Once the wells were inoculated with the appropriate strain, one replicate plate was placed on the platform of a rotary shaker (The Belly Dancer, Stoval Life Science, Inc., Greensboro, NC) inside of the cell culture incubator and rotated at a speed of 50 rpm to add movement to the media in the plate in attempts to simulate host internal milieu shear force, while another replicate plate remained static on the shelf of the cell culture incubator during the infection stage. The plates remained in their assigned conditions and were removed only briefly for sampling for the duration of the assay. Coverslips were placed only into static plates. Following a 30 minute incubation, a killing dose of gentamicin at a final concentration of 100 µg/ml was added to each well to kill extracellular bacteria. The cells were allowed to incubate for one hour to ensure that all extracellular bacteria were killed. Cells were then washed once and 1 ml of CCMM containing a static dose of gentamicin at a concentration of 0.35 µg/ml was added to control extracellular growth of released bacteria from lysed cells. At 0, 4, 8, and 12 hour time points (0 hour is equivalent to 90 minutes post infection (PI)) the cells

were lysed with 100 µl of 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) at room temperature for 10 minutes, serially diluted, and spread onto blood agar plates to determine percent invasion, survival, and replication. Coverslips with infected macrophages were removed from wells without lysing, mounted on glass slides with Permount (Fisher Scientific, Fair Lawn, NJ), stained with Hema 3 Stain Kit (Fisher Scientific, Fair Lawn, NJ), and observed with light microscopy. The experiment was repeated ten times with triplicate wells at each time point in each experiment.

Intracellular Survival in Channel Catfish Ovary Cells. Determination of intracellular survival in the channel catfish ovary cell line was performed in a manner similar to the macrophage assay described above. Briefly, viable channel catfish ovary (CCO) cells were diluted to a concentration of 2×10^5 cells/ml with Liebowitz-15 (L-15) supplemented with 10% fetal bovine serum (FBS) and 1 ml/well was added to 24 well Falcon tissue culture plates (Becton Dickinson Labware, Becton Dickinson and Company, Fanklin Lakes, NJ). The plates were wrapped with parafilm to exclude CO_2, and allowed to adhere for 20 hours at 28 C in the cell culture incubator. A 16 hour late log phase culture of WT, 233PR, and EacF::Km *E. ictaluri* strains were diluted to a concentration of approximately 1×10^6 bacteria/ml using sterile Dulbecco's phosphate buffered saline (PBS) (calcium and magnesium free). CCO cells were inoculated with approximately 2×10^4 CFU of either WT, 233PR, or EacF::Km strains at an MOI of 1:10 (bacteria:cells). Once the wells were inoculated with the appropriate strain, one replicate plate was placed on the rotary shaker inside of the cell culture incubator and rotated at a speed of 50 rpm, while another replicate plate remained static on the shelf of the cell culture incubator (28°C). The plates remained at those conditions for the duration of the assay except for brief removal for sampling. Following a 30 minute incubation, a killing dose of gentamicin

at a final concentration of 100 μg/ml was added to each well to kill extracellular bacteria. The cells were allowed to incubate for one hour to ensure that all extracellular bacteria were killed, after which the cells were washed and 1 ml of L-15 with 10% FBS containing a static dose of gentamicin at a concentration of 0.35 μg/ml was added to control extracellular growth of bacteria released from lysed cells. At 0, 4, 8, and 12 hour time points (0 hour is the equivalent to 90 minutes PI) the cells were lysed with 100 μl of 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) at room temperature for 10 minutes, serially diluted, and spread onto blood agar plates to determine percent invasion, survival, and replication. This experiment was repeated ten times with triplicate wells at each time point in each experiment.

Statistical Analysis. The mortality, persistence and competition challenge experimental design was completely randomized with a factorial arrangement of treatments. All data were analyzed by General Linear Methods Procedure as CFU recovered/well following a natural log transformation of the number of CFU recovered/well (SAS Institute, Inc. 2003). When the overall model indicated significance at $p \leq 0.05$, Tukey's test was used for pairwise comparison of main effects, and a least square means procedure was used for pairwise comparison of interaction effects.

RESULTS

Bioinformatics Analysis. Analysis of the sequence encoding the 233PR mutation and surrounding open reading frames (ORF's) using BLAST revealed a hemolysin/adhesin cluster in *E. ictaluri* that is similar to the hemolysin/adhesin cluster (Figure 1 and Table 2) located in a pathogenicity island (PAI) of locus of enterocyte effacement (LEE) –negative strains of *E. coli* CL3, described by Shen et al. (2004). The putative hemolysin/adhesin cluster in *E. ictaluri* encodes 8 open reading frames (ORF's) that have been named EacA-H for Edwardsiella attenuation cluster. The 8 *eac* genes are flanked by two transposases (TnpA) that are homologous to other transposases in *E. ictaluri*. The presence of flanking transposases is a common feature of PAI's and is indicative of horizontal transfer of genetic material (Hacker and Kaper 1999). The overall G/C content of the 8 ORFs is 54%, very similar to the 53% for the *E. ictaluri* genome. The protein encoded by the first ORF downstream from the transposase, EacA, has 64% identity to S1, a homologue to the hemolysin activator HlyB of *E. coli* CL3, and the protein shows 60% identity to the conserved domain of FhaC. HlyB and FhaC are hemolysin secretion/activation proteins that are involved in intracellular trafficking and secretion (Willems et al. 1994). EacB has 57% identity to S2, hemolysin activator HlyC, of *E. coli* CL3, as well as the HlyC conserved domain. HlyC is a hemolysin activating protein that is involved in post-translational modification and protein turnover (Bhakdi et al. 1988). EacC is 51% identical to a putative PagC-like membrane protein of *Y. enterocolitica* and the protein has 50% identity to the conserved domain Ail Lom, a protein that directly promotes invasion (Heffernan et al. 1994). EacD has 50% identity to S3, a putative hemolysin/hemagglutinin in *E. coli* CL3, and shows 41% identity to the conserved hemagglutination activity domain. EacE is a large protein with 48% identity to S4, a putative adhesin/hemagglutinin/hemolysin of *E. coli* CL3, and with a

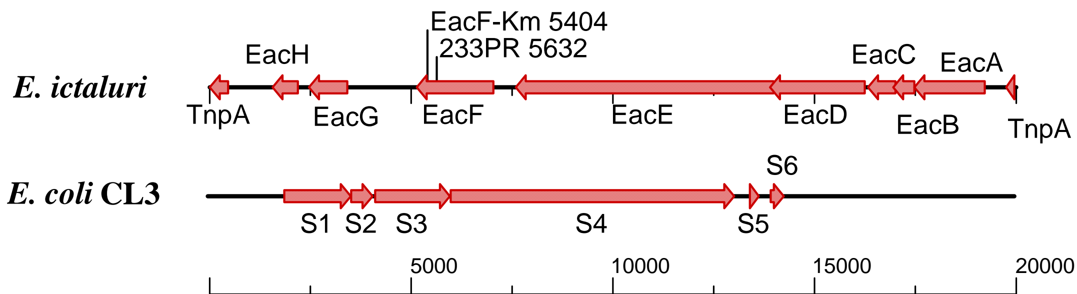


Figure 1 Map of hemolysin/adhesin clusters found in *E. ictaluri* and *E. coli* CL3. Sequencing and subsequent BLAST analysis in the NCBI databases located the insert in the carboxy terminus of a gene with similarity to a putative gene found in verocytotoxin producing *E. coli* CL3 (0113:H21). Analysis of the *E. ictaluri* partially completed genome using the 233PR sequence identified a 19,953 bp fragment of the genome, encoding genes with similarity to the hemolysin/adhesin cluster located in the pathogenicity island (PAI) of the locus of enterocyte effacement (LEE) -negative strain of *E. coli* CL3 (Shen et al. 2004). Table 2 gives full description of those genes located in *E.ictaluri* from the above map. The 233PR transposon insertion mutation and the isogenic EacF::Km mutations are both located in the carboxy-terminus of the *eacF* gene.

Table 2 Amino acid (aa) homologies of the putative open reading frame's (ORF) identified from the hemolysin/adhesin cluster in *E. ictaluri* that aligned to the hemolysin/adhesin cluster in *E. coli* CL3, as described in Figure 1, as well as related proteins from other bacteria.

ORF and Location (bp)	Size (no. of aa)	Conserved Domain	Similar *E. coli* CL3 Protein	Homologous Protein	% Identity/ no. of aa	Accession No. of Homolog
TnpA (19953-19750)	67	None	S6	TnpA, transposase, *E. ictaluri*	69/52	ABD93710
EacA (19217-17475)	580	FhaC	S1	S1, putative hemolysin activator HlyB, *E. coil* CL3	64/546	AAQ19124
				COG2831: Hemolysin activation/ secretion protein, *Y. frederiksenii*	60/566	ZP_00827590
EacB (17475-16948)	175	HlyC	S2	S2, putative hemolysin activator HlyC, *E. coil* CL3	57/169	AAQ19125
EacC (17006-16323)	227	Ail_Lom	None	putative PagC-like membrane protein, *E. coli* EDL933	51/177	NP_289546
				attachment invasion locus protein, *Y. enterocolitica*	32/200	YP_00100609 1
EacD (16244-13893)	783	Haemagg_act	S3	S3, putative hemolsyin/ hemagglutinin, *E. coil* CL3	50/639	AAQ19126
				COG3210: Large exoproteins involved in heme utilization or adhesion, *Y. mollaretii*	41/778	ZP_00824144
EacE (13984-7592)	2130	FhaB	S4	S4, putative adhesin/hemagglutinin/ hemolysin, *E. coil* CL3	48/2140	ABG33928
				YPO2490, putative hemolysin, *Y. pestis* CO92	36/2075	YP_651897
				COG3210: Large exoproteins involved in heme utilization or adhesion, *Y. intermedia*	34/2169	ZP_00833445
				YPO0599, putative hemolysin, *Y. pestis* CO92	33/2187	NP_993634

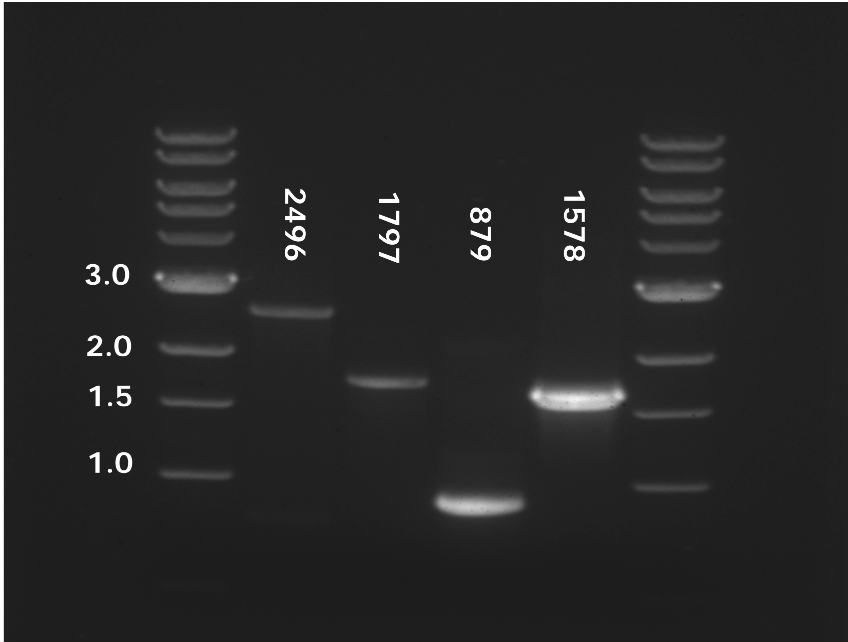
(Table 2 continued)

ORF and Location (bp)	Size (no. of aa)	Conserved Domain	Similar *E. coli* CL3 Protein	Homologous Protein	% Identity/ no. of aa	Accession No. of Homolog
EacF (7035-5134)	633	None	S4	S4, putative adhesin/hemagglutinin/ hemolysin, *E. coil* CL3	70/632	ABG33928
				COG3210: Large exoproteins involved in heme utilization or adhesion, *Y. pestis*	55/381	ZP_01174535
				YPO2490, putative hemolysin, *Y. pestis* CO92	55/381	YP_651897
				YPO0599, putative hemolysin, *Y. pestis* CO92	38/636	NP_993634
EacG (3414-2464)	316	None	S4	S4, putative adhesin/hemagglutinin/ hemolysin, *E. coil* CL3	74/304	ABG33928
				COG3210: Large exoproteins involved in heme utilization or adhesion, *Y. pestis*	55/315	ZP_01174535
				YPO2490, putative hemolysin, *Y. pestis* CO92	55/315	YP_651897
				YPO0599, putative hemolysin, *Y. pestis* CO92	51/316	NP_993634
EacH (2188-1559)	209	None	S4	hypothetical protein YmolA_01003827, *Y. mollaretii*	68/124	ZP_00823794
				S4, putative adhesin/hemagglutinin/ hemolysin, *E. coil* CL3	61/144	ABG33928
				Adhesin HecA, *B. xenovorans* LB400	47/129	YP_555132
TnpA (458-2)	151	Transposase	None	TnpA, transposase, *E. ictaluri*	100/47	ABD93710

conserved domain similar to FhaB, a large exoprotein involved in heme utilization and adhesion during intracellular trafficking and secretion (Willems et al. 1994). EacF, EacG, and EacH also display identities of 70%, 74%, and 68% to S4 of *E. coli* CL3, respectively, but without any conserved domains. Further analysis of the S4 homologues indicates that the first 1086 bp of *eacF* have 94% identity to base pair 633 to 1737 in the 5' end of *eacE* and that the entire *eacG* ORF has 98.6% identity to the same regionsof *eacF* and *eacE.* Both the 233PR and the EacF::Km mutations are located in the carboxy terminus of EacF at 5,632 and 5,404 bp, respectively.

Construction of the EacF::Km Isogenic Mutant. Following ligation of the mini-Tn5 Km gene into the PCR generated insert and eventual conjugation into *E. ictaluri* 93-146 WT strain, the isogenic EacF::Km strain was sequenced for confirmation of the insertion and associated sequence flanking the site of insertion (Figure 2). Amplification with the four primers produced the four PCR fragments and sequencing confirmed the construction. The sequence was also used to identify the gene and surrounding adhesin related genes in the partially completed *E. ictaluri* genome database (http://microgen.ouhsc.edu/cgi-bin/blast_form.cgi) using ORF Finder and the BLAST in the National Center for Biotechnology Information (NCBI). The sequenced EacF::Km also allowed for the comparison of protein length to 233PR, in respect to location of the insert that truncated the functional protein.

Confirmation of a Single Insert. A Southern blot of *ClaI* digested DNA from *E. ictaluri* 233PR and transconjugate EacF::Km mutant strains detected a single band upon hybridization with a Km probe, indicating a single insertion event in these two strains (Figure 3). This verified that the STM and the constructed mutant strains had only a single insertion in the gene.



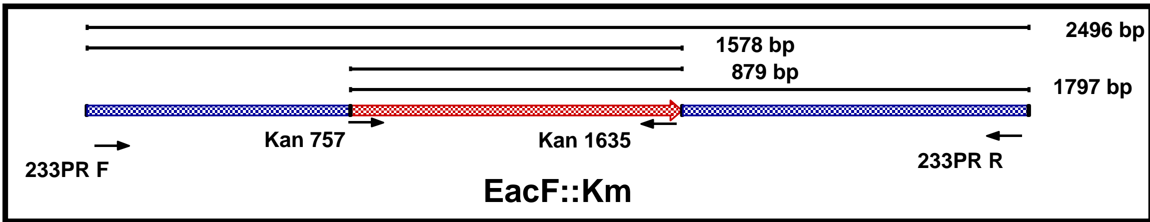


Figure 2 Confirmation of the EacF::Km mutant construction. Far right and left lanes contain 1 Kb markers. The first lane is the entire insertion sequence of the 233PR gene interrupted with the Km^R gene insert (2496 bp). The second lane shows beginning at the mini-Tn5-Km insert through to the end of the 233PR inserted gene (1797 bp). The third lane has only the entire mini-Tn5-Km insert (879 bp). The fourth lane shows the beginning of the 233PR gene stopping at the end of the mini-Tn5-Km insert (1578 bp).

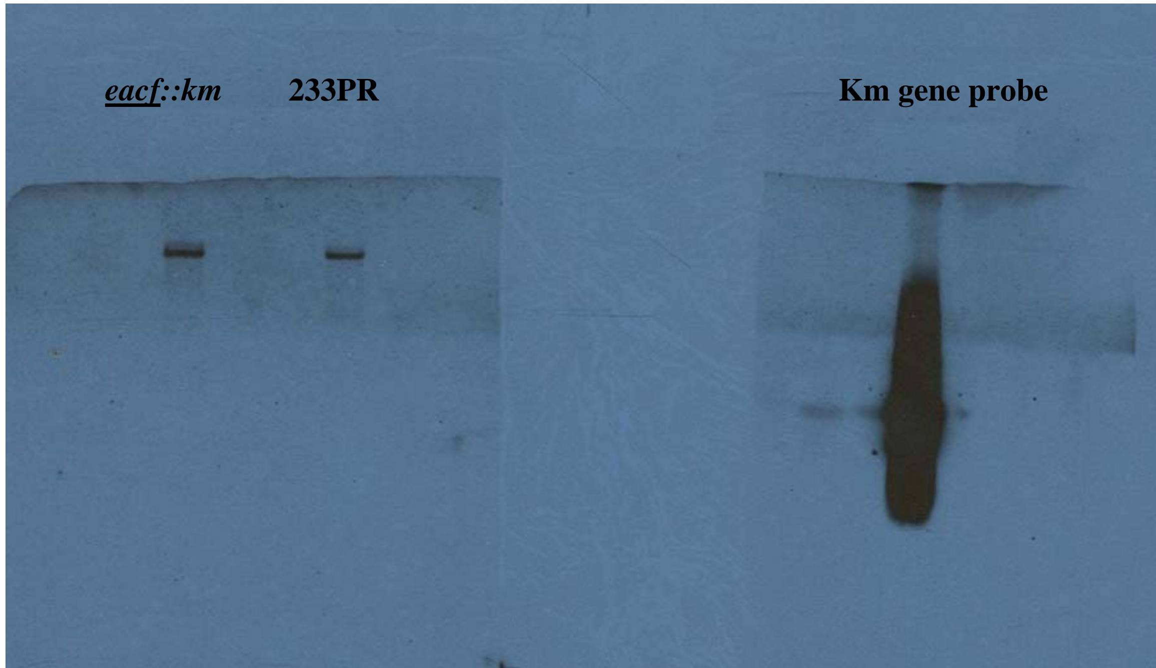


Figure 3 Southern blot confirmation of single insertion events in 233PR mutant and constructed *eacf-km*. *Cla*I digested DNA was hybridized with a labeled Km probe. Generation of a single band in both mutant strains is indicative of a single insertion event.

Competitive Challenge with WT *E. ictaluri*. Previous competitive challenge of the 233PR mutant with WT from the STM study resulted in a CI of 0.00089 for 233PR (Thune et al. 2007). In the current study, challenge with a 50/50 mix of EacF::Km and WT 93-146 strains of *E. ictaluri* resulted in a recovered average of 2.71 X 10+08 (±9.04 X 10+07) CFU per gram of liver WT organisms recovered and 1.43 X 10+08 (±4.27 X10+07) CFU per gram of tissue EacF::Km organisms. The EacF::Km mutant CI of 0.25 defines it as moderately attenuated in relation to the virulent WT strain, and less attenuated than the 233PR mutant strain.

Mortality Studies. No mortalities were observed in fish challenged with the 233PR strain or in the media-only control. Total average mortality for fish challenged with the EacF::Km strain was 39% and the total average mortality for fish challenged with the WT *E. ictaluri* strain was 96% by day 12 (Figure 4). These results support the previous data that the 233PR mutant strain is more attenuated than the EacF::Km mutant strain, and both are moderately attenuated in respect to the WT strain.

Persistence Studies. Persistence results are shown in Figure 5. Although all three strains invaded the catfish host by 24 hours post infection at a statistically similar rate ($P>0.05$), all three strains behaved differently ($P<0.05$) after the first day of sampling. The WT strain persisted and increased in numbers by day 6, but no data is available past day 6 because all fish had died. The 233PR mutant numbers declined until the strain was cleared from head kidney tissue by day 8. The EacF::Km strain persisted at a constant level until day 10, after which numbers declined until the strain was cleared by day 13. Although there was a difference in persistence, both mutant strains were completely cleared from the catfish host while all of the fish infected with WT strain died. The clearance rates of the two mutants is consistent with the mortality data, in

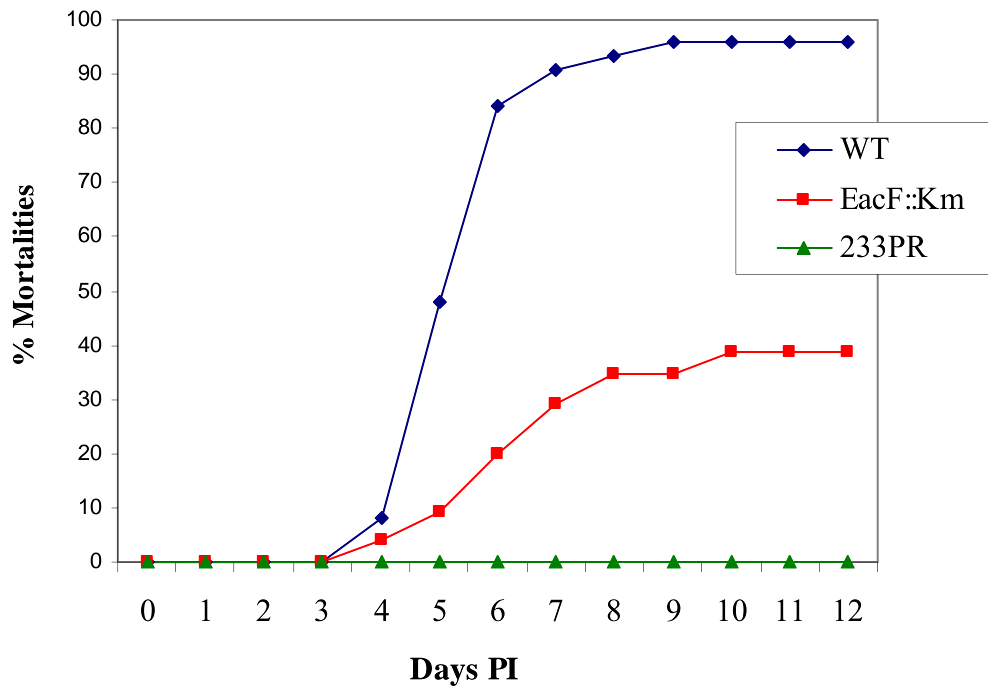


Figure 4 Mortality results for WT, 233PR mutant, and EacF::Km mutant *E. ictaluri* strains in fingerling channel catfish following immersion challenge. Each point is the average mortality in three replicate tanks of 25 fish. There were no mortalities in the control LB broth (not shown on graph). These results support the previous data that the 233PR mutant strain is more attenuated than the EacF::Km mutant strain, and both are moderately attenuated in respect to the WT strain.

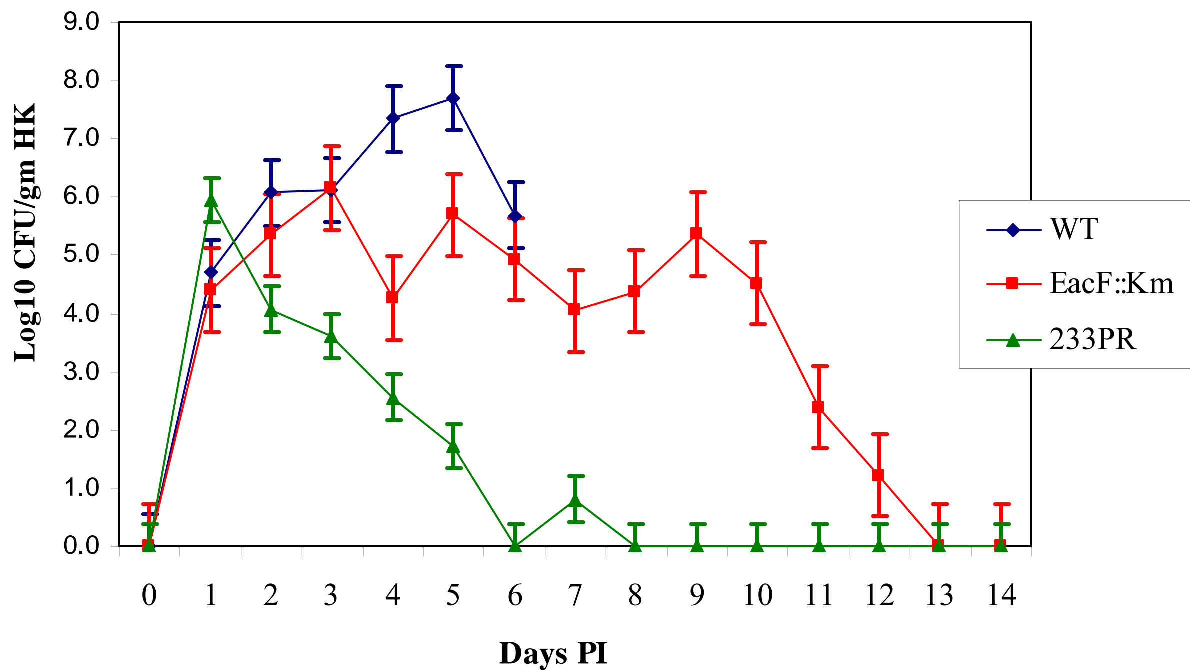


Figure 5
Persistence results for WT, 233PR, and EacF::Km strains in fingerling channel catfish. Each point represents the average of CFU/gm of head kidney from three individual fish from three separate tanks per strain and transformed to LOG_{10} values. Error bars represent the standard error among the three fish tested for each strain that day. All fish from the WT group had died by day 6 so there are no values for any of the days past day 6. There were no bacteria recovered in the control fish treated with LB-Man broth only (not shown in graph).

that EacF::Km, carrying a less truncated EacF protein, maintained higher levels for a longer period of time indicated.

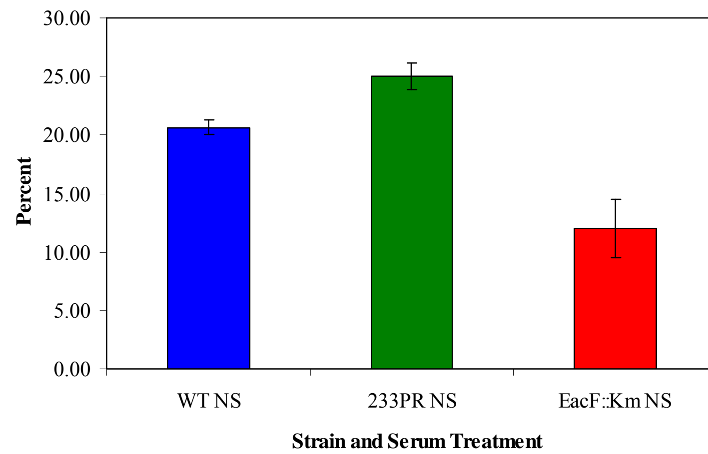
Intracellular Survival in Channel Catfish Macrophages. Survival and replication results of WT, 233PR, and EacF::Km strains of *E. ictaluri* in channel catfish head kidney derived macrophages (HKDM) are presented in Table 3. There was no significant (P>0.05) difference in initial uptake (Figure 6 A and B) or intracellular replication (Figure 7) of either strain of *E. ictaluri* in the HKDM, but there was a significant difference (P<0.05) between serum treatments (Figure 7). Attempts to add shear flow forces did not affect invasion. Light microscopy was used to identify an increase in bacteria in HKDM from 0 hour to 12 hours post infection with EacF::Km mutant strain (Fig 8).

Intracellular Survival in Channel Catfish Ovary Cells. Survival and replication of WT, 233PR, and EacF::Km strains of *E. ictaluri* in channel catfish ovary (CCO) cells is presented in Table 4. There was no significant difference found in invasion data (Figure 9) or growth of any of the strains of *E. ictaluri* in CCO cells (Figure 10). Attempts to add shear flow forces did not affect invasion.

Table 3 Increase in number of colony forming units (CFU) per well over time for *E. ictaluri* WT, 233PR, and EacF::Km strains following infection of channel catfish head kidney derived macrophages (HKDM). Multiplicity of infection (MOI) ratio was one bacteria to ten HKDM. Bacteria were pre-treated with either normal autlogous serum (NS) or heat inactivated autologous serum (HIS) to examine if the putative adhesin was involved in recognition of specific receptors on HKDM. Means with different letters indicate significant differences between times for the same serum treatment in the least squares means procedure ($P<0.05$). Means with different numbers indicate significant differences between the serum treatments at the same time in the least squares means procedure ($P<0.05$). However, there was no significant difference found between the three strains for the same serum treatment in the same times in the least squares means procedure ($P>0.05$). Results from this single experiment are representative of data from four experiments, with triplicate wells for each treatment at each time in each experiment.

Time (Hr)	Strain &Treatment	CFU/well Recovered	Percent Internalized	Fold Increase
0	WT NS	1,070 ± 33.33 [A,1]	20.65 ± 0.65 [1]	
	233PR NS	570 ± 76.38 [A,1]	25.00 ± 2.55 [1]	
	EacF::Km NS	300 ± 104.08 [A,1]	12.00 ± 4.16 [1]	
	WT HIS	43.3E ± 2.40 [A,2]	0.58 ± 0.03 [2]	
	233PR HIS	9.33 ± 3.53 [A,2]	0.20 ± 0.08 [2]	
	EacF::Km HIS	6.00 ± 2.00 [A,2]	0.13 ±0.04 [2]	
4	WT NS	1,050E ± 57.74 [B,1]		0.98 [A]
	233PR NS	667 ± 16.67 [B,1]		1.17 [A]
	EacF::Km NS	400 ± 57.74 [B,1]		1.33 [A]
	WT HIS	40.0 ± 5.00 [B,2]		0.92 [A]
	233PR HIS	20.0 ± 4.41 [B,2]		2.14 [A]
	EacF::Km HIS	20.0 ± 15.00 [B,2]		3.33 [A]
8	WT NS	2,720 ± 120.19 [C,1]		2.55 [B]
	233PR NS	2,120 ± 185.59 [C,1]		3.71 [B]
	EacF::Km NS	820 ± 133.33 [C,1]		2.73 [B]
	WT HIS	107 ± 21.28 [C,2]		2.46 [B]
	233PR HIS	31.7 ± 9.28 [C,2]		3.39 [B]
	EacF::Km HIS	25.0 ± 2.89 [C,2]		4.17 [B]
12	WT NS	5,500 ± 1258.31 [D,1]		5.16 [C]
	233PR NS	5,000 ± 288.68 [D,1]		8.77 [C]
	EacF::Km NS	3,000 ± 288.68 [D,1]		10.00 [C]
	WT HIS	108 ± 10.93 [D,2]		2.48 [C]
	233PR HIS	80.0 ± 16.07 [D,2]		8.57 [C]
	EacF::Km HIS	98.3 ± 4.41 [D,2]		16.39 [C]

A



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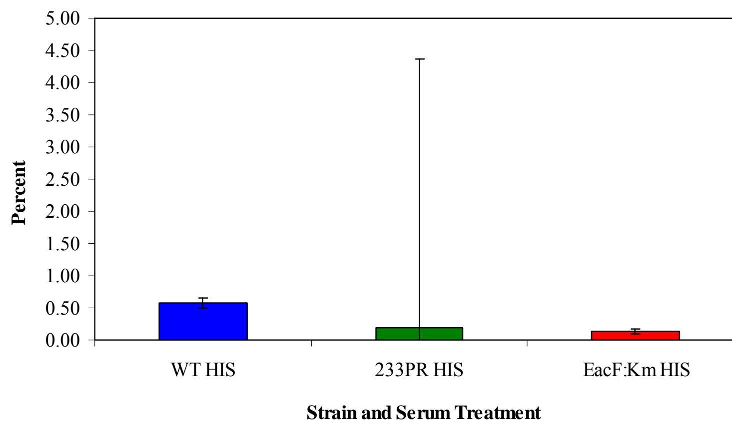

Figure 6 A and B

Percent internalization of WT, mutant 233PR, and mutant EacF::Km *E. ictaluri* strains recovered from channel catfish head kidney derived macrophages (HKDM). **A)** represents the graphical data for opsonized bacteria (treated with normal autologous catfish serum, while **B)** represents graphical data for non-opsonized bacteria (treated with heat inactivated autologus catfish serum). There was no statistical difference found between strains (P>0.05) for either serum treatment. There was a significant difference (P<0.05) in internalization between serum treatments. Results from this single experiment are representative of data from four experiments, with triplicate wells for each treatment at each time in each experiment.

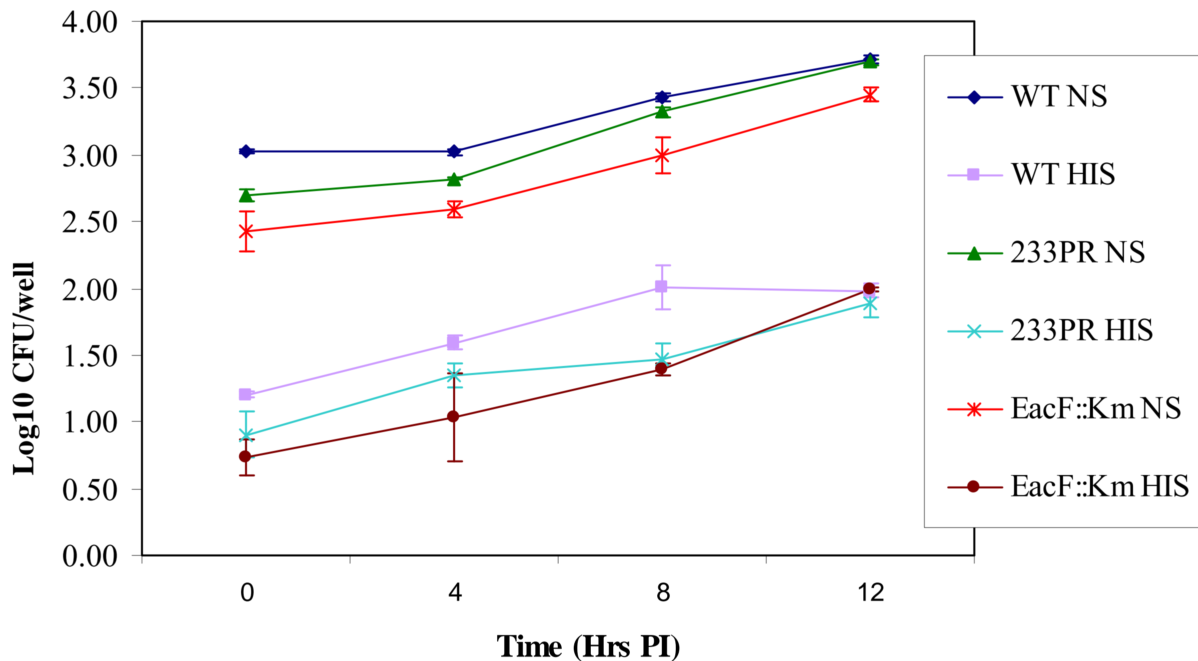

Figure 7
Intracellular survival and replication of WT, 233PR mutant, and EacF::Km mutant strains treated with either normal autologous serum (NS) or heat inactivate autologous serum (HIS) in channel catfish head kidney derived macrophage (HKDM). Each point represents the average CFU/well from triplicate wells transformed to LOG_{10} values. Error bars represent the standard error among the three wells. There is no significant difference between the three NS treated strains, nor is there any significant difference between the three HIS treated strains ($P>0.05$) at any time point. However, there is a significant difference between the NS and the HIS treated strains ($P<0.05$). Results from this single experiment are representative of data from four experiments, with triplicate wells for each treatment at each time in each experiment.

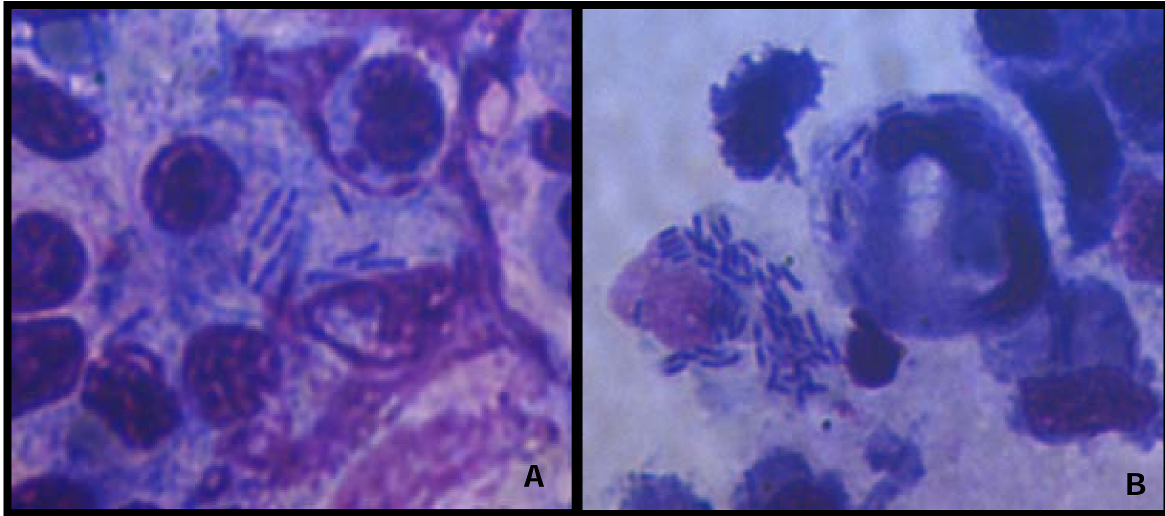

Figure 8 A and B
Light microscopy of *E. ictaluri* EacF::Km growth in channel catfish head kidney derived macrophages (HKDM). Coverslips were stained and observed at 630X. **A)** HKDM after 0 hour incubation (90 minutes post infection). **B)** Macrophages after 12 hour incubation.

Table 4 Increase in number of CFU over time for *E. ictaluri* WT, 233PR mutant, and EacF::Km mutant strains following infection of channel catfish ovary cell line at an MOI rate of one bacteria to ten cells. Means with different letters indicate significant differences between times for infection with the same strain in the least squares means procedure ($P<0.05$). Means with different numbers indicate significant differences between different strains at the same time in the least squares means procedure ($P<0.05$). There is no significant difference between strains, regardless of static or shaken conditions (shaken data not shown). Fold increase is relative to the amount of internalized bacteria. Results from this single experiment are representative of data from four experiments, with triplicate wells for each treatment at each time in each experiment.

Time (hours)	Strain	CFU/well Recovered	Percent Invasion	Fold Increase
0	WT	$41.3 \pm 1.33^{A,1}$	0.016 ± 0.04^{1}	
	233PR	$48.7 \pm 2.40^{A,1}$	0.018 ± 0.03^{1}	
	EacF::Km	$28.7 \pm 2.67^{A,1}$	0.014 ± 0.03^{1}	
4	WT	$143 \pm 12.02^{B,1}$		3.47
	233PR	$70.0 \pm 5.77^{B,1}$		1.44
	EacF::Km	$16.7 \pm 6.67^{B,1}$		0.58
8	WT	$1670 \pm 192.21^{C,1}$		40.32
	233PR	$783 \pm 192.21^{C,1}$		16.10
	EacF::Km	$225 \pm 52.04^{C,1}$		7.85
12	WT	$7170 \pm 927.96^{D,1}$		173.39
	233PR	$2980 \pm 591.84^{D,1}$		61.30
	EacF::Km	$1400 \pm 264.58^{D,1}$		48.84

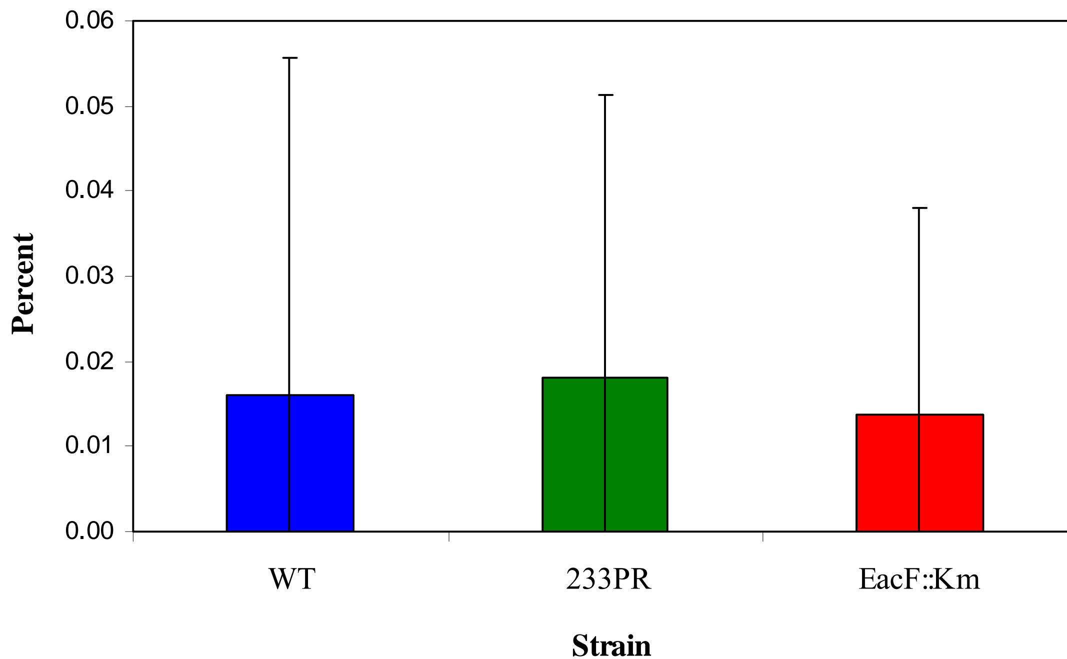

Figure 9
Percent invasion of WT and mutant strains in the channel catfish ovary cell line at time 0 hour (90 min PI). Error bars represent the standard error among the three wells. There was no statistical difference in invasion of the CCO cells by any of the *E. ictaluri* strains (P>0.05). Bacteria invaded the CCO cells consistently at a very low rate (0.01 – 0.02%) in all four experiments. Results from this single experiment are representative of data from four experiments, with triplicate wells for each treatment at each time in each experiment.

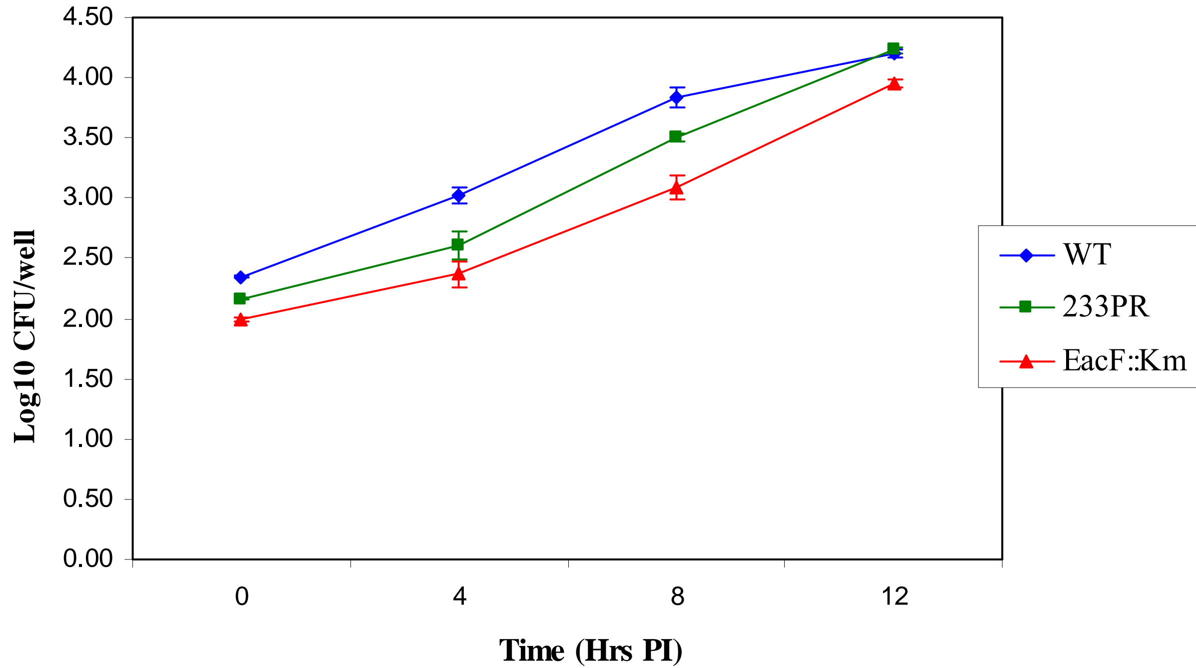

Figure 10
Intracellular survival and replication of WT, mutant 233PR, and mutant EacF::Km strains in channel catfish ovary cells. Each point represents the average CFU/well from triplicate wells transformed to LOG_{10} values. Error bars represent the standard error among the three wells. Results from this single experiment are representative of data from ten experiments, with triplicate wells for each treatment at each time in each experiment. There is no significant difference ($P>0.05$) between the three *E. ictaluri* strains at any point in the experiment regarding invasion, survival, or replication.

DISCUSSION

The mortality, persistence, and competition challenge results suggest that EacF is essential for persistence of *E. ictaluri* in the channel catfish host. The persistence study revealed that both the 233PR and the EacF::Km mutant strains were able to invade host tissue, but were incapable of sustaining an infection, as demonstrated by the complete clearance of bacteria by days 8 and 13 for 233PR and EacF::Km, respectively. Infection with the 233PR or EacF::Km mutant strain resulted in no mortality or 36% mortality, respectively, whereas infection with the WT strain resulted in 98% mortalities over a 16 day period following infection during the mortality study. A competitive index of 0.00089 for 233PR and 0.25 for EacF::Km indicate attenuated mutant strains, although the lower index for 233PR suggests greater attenuation than the EacF::Km strain. Despite *in vivo* results suggestive of attenuated, disruption of *eacF* did not significantly affect intracellular survival or replication in channel catfish head kidney derived macrophages (HKDM) or the channel catfish ovary (CCO) cell line under static conditions during the 12 hour assay. In addition, neither 233PR nor the EacF::Km mutant strain were significantly different from the WT in the rate of phagocytosis or in their ability to invade HKDM or CCO cells. Although the *in vitro* results indicate that *eacF* is not involved in invasion or intracellular replication under the experimental conditions that were used, the *in vivo* results define EacF as a gene involved in *E. ictaluri* pathogenesis. Further analysis of the putative hemolysin/adhesin cluster is required to define its role in the disease process.

The location of the mobile DNA sequences at either end of the *eac* complex suggests the generation of the PAI by horizontal transfer. The similarity of the G+C content is not typical of many pathogenicity islands, where the G+C content is often different than the core genome. This is not the case, however, if the donor of the PAI has a similar or identical G+C content as

the recipient. It is also possible that the acquired DNA assumed properties similar to the rest of the genome during the evolution of the pathogen (Hacker and Kaper 1999).

Figure 11 displays the location of the two mutations located in the carboxy terminus of *eacf*. In 233PR, there are 1,386 bp encoding 46% of the total functional protein, while in EacF::Km there are 1,608 bp of functional protein, with both insertions located towards the carboxy terminus. The extra 222 bp translated into functional protein in EacF::Km appears to reduce the level of attenuation compare to the more truncated 233PR, but at the same time is less virulent than the WT strain. Because the carboxy terminus of many adhesins are the portion of the protein that is involved in attachment to the bacterial surface (Choudhury et al. 1999), truncating the C- terminal domain often affects binding of the protein to the bacterium. In this instance, the slightly shorter 233PR product may encode less of the EacF receptor binding domain. Alternatively, the shorter 233PR protein may be stearically hindered from attachment to the host receptor. Although a differential effect of protein length on pathogenicity was seen in *in vivo* studies, it did not affect results of the *in vitro* assays.

A study that analyzed FimH from commensal *E. coli* found that gentle shaking of guinea pig erythrocytes in the presence of *E. coli* allowed rapid agglutination, indicating that shear flow can allow commensal FimH derivatives to bind erythrocytes (Thomas et al. 2002). This indicates the potential of shear flow analysis in cell and tissue culture, if the special static needs of cell culture methods can be overcome. One last limitation that must be considered when dealing with cell culture assays is that immortalized cells invariably have unstable genotypes and develop genetic mutations that differentiate them from their starting tissue's phenotype (Drexler et al. 1999; Daniele et al. 2002; Ben-Porath and Weinberg 2004). Based on the level of attenuation *in vivo*, *eacF* is involved in *E. ictaluri* pathogenesis, but changes in experimental

ATGCACAGCG ACTATGCCTC GGTGGAGAGC CAGAGCGGCA TCTTTGCCGG
TACCGGTGGC TTTGATATCA CCGTGGGTAA TCATACCCAG CTGGACGGCG
CGGCCATTGC CAGCGCGGCC GGTCAGGAGA GCAACCGCTT AGATAGCGGA
ACCCTGGGCT TTGCCGATAT CGACAACCGC GCTGAGTTCA GGGCGCAGCA
CCAGGGGTTT GGCCTTAGCT CCGGCGGCAG CATTGGCGGC CAGTTCGCGG
GCAATATGGC CAATAGCCTG TTGGCGGGCG CCAATCATCA GGAGCGCGCC
CGCGGCACCA CCCAGTCGGC TATCGCCGAC GGCGCGATCG TCGTGCGCGA
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GGGCGCACCA GCCCCTCACC CCTATCTTCG ACAAGGAGAA AGCGCAGCGG
CGCCTGCAGC AGGCCCGGCT GATCGGTGAG ATTGGCAATC AGGTGGCCGA
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CGGCGGCGGT ACAGGGGCTG GCGGGCGGCA ACCTGCAGGC GGCGCTGGCG
GGCGGCGCGG CGCCGTATAT TAGTGAGATA ATCAAGCAGA GCACCCCGGA
CGGCGCGGGG CGTGTGGCGG CCCATGCGGT GGTAAATGCG GCGCTTGCCG
CAGCACAGGG GAATAACGCC CTTGCCGGAG CCGCGGGTGC GGCCACCGGC
GAAATCGTCG GCATGCTGGC CACGGAGATA TACCAGAAAC CGGTCGCTGA
GCTCAGCGAA AGCGAGAAAC AGACCGCTTC AACACTGGCC ACCGTTGCCG
CCGGACTGGC CAGTGGTCTG GTGGGCGACG CGGGGGCTTC CGCGCTCGCC
GGGGCAGAGT CGGGTAAGAC GACGGTGGAG AATAATTTCC TTGGGACAAA
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AGACGTTAGC GACGGCGAAA GAGTTACTTC AATTAGAAAA CGCAGACAAA
CGGAGTGACA TACTTATCAC GAAGTTTGTG TCAGATCCTA CCCAACTGAA
TAGCGCTGAA CGAAGCGAAT TAGCTGGTTA TTTGCGTATT TACGCAGCAG
AGATGGAGAC GGAATATGGT ACAGGTGTTG CTCAGGAGCT AGTCAATGGA
TTGCTTTCAG GGAGCGACTA TCTAAAACGA GGCCCAG**ACT CTGATGTAAT**
GGCTGAGGCG CAAAATATAA TGAGGGCATG GGTATATCAT AAATCCAATG
CGAGTATTGG TGATGCACCG CTGCTGTTTT CTGGCAGTAT GCTGGGTATT
ACAATAAAAG GAGGTATGGC TGTTAATGCG GCGATAGGCA TAGGTGTCAA
CACTGTGGTT CAATTAAGTG GAGACGATCC TTTCAGTTAT GTGGATGCAA
GTATTGCGGG ATTGATATCA GCCGCGACGA CAGGAAAAGG TTGGCAGGCT
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AGAGGATCCA ACTAATGCAG TGATCGGAGC TGGAATTGGT AGTGTGGGTG
GTAGTATAGG AGGGAAAACT GTTGACTCAT TATCTACAAT AACAAATCAA
GCAGTAAAGG ATGTTATTGG TACAGTTACT GGTTCTACAC TTAATGAAGT
CACAGGAAAG ATAGTGAAAG ATGAATTAGA TGGAACTAAT AACAATGAAT
AG

Figure 11 Location of 233PR and EacF::Km mutations in the *eacf* gene sequence. The bold letters indicate where the STM 233PR transposon insertion alters the gene sequence. The underlined letters indicate where the mini-Tn5-Km resistance cassette truncated the protein in the EacF::Km mutant. The EacF::Km knockout is located 221 nucleotides downstream from the original 233PR transposon insertion.

conditions, using shear forces and/or different cell types cultures may be required. The hemolysin/adhesin cluster located in a PAI of locus of enterocyte effacement (LEE) –negative strain of *E. coli* CL3, described by Shen et al. (2004) represents potential virulence genes. When S1 was used as a marker for the cluster, however, it was not present in strains associated with uremic syndrome or epidemic disease in humans, but was found in 5 of 11 strains that were only isolated from cattle (Shen et al. 2004). The identification of this cluster as a factor in *E. ictaluri* pathogenesis and the negative correlation to human disease suggests a role in animal pathogens. Although the gene encoding the hemolysin/adhesin cluster is only proposed as a potential virulence gene by Shen et al. (2004), the research presented here suggests that the homologous genes in *E. ictaluri* are virulence genes that play a role in pathogenesis.

CONCLUSION

An isogenic *E. ictaluri* EacF::Km mutant was constructed based on a specific attenuated mutant, 233PR, carrying a transposon insertion in a gene encoding a putative adhesin. The EacF::Km was constructed so that the Km-resistance cassette that interrupts the gene's function was located downstream from the transposon insertion found in 233PR for the purpose of examining protein truncation function differences. Analysis of the sequence encoding the 233PR mutation and surrounding open reading frames (ORF's) using BLAST revealed a hemolysin/adhesin cluster in *E. ictaluri* that is similar to the hemolysin/adhesin cluster located in a PAI of LEE–negative strains of *E. coli* CL3, described by Shen et al. (2004). The location of the mobile DNA sequences at either end of the *eac* complex suggests the generation of the PAI by horizontal transfer. The putative hemolysin/adhesin cluster in *E. ictaluri* encodes 8 open reading frames (ORF's) that have been named EacA-H for Edwardsiella attenuation cluster. Both the 233PR and the EacF::Km mutations are located in the carboxy terminus of EacF at 5,632 and 5,404 bp, respectively.

In vivo results were presented that demonstrated the importance of the putative adhesin's role in *E. ictaluri* pathogenesis, but *in vitro* data was unable to support a role in adhesion, invasion, or intracellular replication under the experimental conditions that were used. The 233PR and EacF::Km mutant strains were shown to be attenuated in the natural host, channel catfish, with respect to the WT parental strain. Both mutants were able to invade the host at relatively the same levels as the WT, but were unable to persist in host tissue for more than 8 days for the 233PR strain and 13 days for the EacF::Km strain. Infection with the 233PR strain resulted in no mortalities over a 12 day study, infection with the EacF::Km strain resulted in a total 36% mortality rate, and infection with the WT strain resulted in a total 98% mortality

rate over the same time period. Results from a competitive challenge calculated a competitive index (CI) of 0.25 for EacF::Km and a CI for 233PR from a previous study (Thune et al. 2007) of 0.00089 indicating that the EacF::Km mutant was less attenuated than 233PR. *In vivo* results are all consistent with differential attenuation of 233PR and EacF::Km based on the degree of truncation of EacF. The extra 222 bp translated into functional protein in EacF::Km appears to reduce the level of attenuation compare to the more truncated 233PR, but at the same time it is less virulent than the WT strain. This study has proven that *eacF* is a virulence factor, but further analysis of the putative hemolysin/adhesin cluster is required to define its role in the disease process.

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VITAE

Ildiko Katalin Polyak was born in 1981 as a first generation American. Her loving parents, Istvan and Katalin Poyak, emigrated from Hungary to America in the 1950's, where they could raise a family and provide the opportunities that were not available in Hungary. The youngest of three, Ildiko had two older brothers, Istvan and Levente, who were not only her protectors, but also her best friends.

After completing high school in Connecticut, she attended a small private liberal arts school in Massachusetts, Clark University, where she excelled in the sciences and sports. She graduated in 2003 with a bachelor degree in biology and was also captain of the swim team. During her undergraduate studies, she had a summer internship at a non-profit organization, The Ocean Alliance. It was there that her love for the ocean and everything in it blossomed. It was through her work with Cynde Bierman that she knew that her life's work would forever be entwined with the ocean and all of its environmental obstacles. After she graduated from Clark University, she became an employee at The Ocean Alliance and worked at The Woods Hole Oceanographic Institute studying toxilogical effects on marine mammals. She was then promoted to the position of science manager aboard the research vessel, The Odyssey, where she became part of an effort to sample areas in the Indian Ocean and the Mediterranean Seas for further scientific research. This fueled her desire to know more about all of the destructive issues facing the marine and aquatic systems. This led her to pursue a master's degree at Louisiana State University in 2004 in the research lab of Dr Thune, focusing on bacterial pathogens that affect aquatic and marine organisms. After graduating from LSU in 2007, she will be starting an exciting new job in Washington working with marine and aquatic bacteria in a diagnostic and commercial setting.