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Expression of the *Edwardsiella ictaluri* type III secretion system and its relationship to the native plasmids

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EXPRESSION OF THE *EDWARDSIELLA ICTALURI* TYPE III SECRETION SYSTEM AND
ITS RELATIONSHIP TO THE NATIVE PLASMIDS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

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

by

Matthew Rogge

B.S., University of Wisconsin – Stevens Point, 2000

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ABSTRACT

Edwardsiella ictaluri encodes a type III secretion system (T3SS) required for intracellular replication. Analysis of *in vitro* gene expression indicates the T3SS translocon proteins express and secrete in acidic pH. Expression of other T3SS genes, however, requires that phosphate be limited in the culture media, in addition to acidic pH. Responses to environmental stimuli are mediated through the T3SS-encoded regulatory proteins EsrA, EsrB, and EsrC. Mutations in these genes result in differing phenotypes. Mutation of EsrA results in moderately reduced expression of T3SS genes, but translocon protein secretion is retained in the mutant. However, the EsrA mutant is attenuated intracellularly and *in vivo*. Mutation of EsrB results in severely decreased T3SS gene expression and translocon protein secretion, leading to intracellular and *in vivo* attenuation. EsrB is also required for expression of type VI secretion system (T6SS)-related proteins through the modulation of EsrC expression. EsrC mutation has an effect on T3SS gene expression, but less so than EsrB, and does not abolish T3SS translocon secretion. EsrC mutation, however, does inhibit expression of T6SS-related proteins, indicating one of its functions is to coordinate expression of the T6SS with that of the T3SS. The EsrC mutant is not attenuated intracellularly, but does exhibit attenuated virulence *in vivo*. Expression of the T3SS also is dependent on two plasmids carried by strains of *E. ictaluri* virulent for channel catfish. Mutation of both of the plasmids results in severe decreases in T3SS gene expression, resulting in attenuation of *E. ictaluri* intracellularly and *in vivo*. The plasmids do not encode proteins with homology to known regulatory proteins, but may integrate into the genome near putative regulatory genes, thereby modulating their expression. The results presented here demonstrate that *E. ictaluri* responds to conditions mimicking the intracellular environment by expression a T3SS, which is required for intracellular survival. The expression of this T3SS absolutely is dependent on EsrB, and EsrA and EsrC are required for optimal T3SS expression.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

EDWARDSIELLA ICTALURI

Edwardsiella ictaluri is a Gram-negative bacterium of the family Enterobacteriaceae and is the etiological agent of enteric septicemia of catfish (ESC), a destructive disease in channel catfish (*Ictalurus punctatus*) production. *Edwardsiella ictaluri* was isolated, named, and characterized by Hawke et al. (Hawke 1979; Hawke et al. 1981). *Edwardsiella ictaluri* may have been present in the catfish industry as early as 1969 (Mitchell and Goodwin 1999). At temperatures between 22°C and 28°C, channel catfish populations are at risk for infection (Francis-Floyd et al. 1987), correlating to heavy spring and fall outbreaks of ESC.

Edwardsiella ictaluri strains isolated from fish are quite homogeneous. Waltman et al. (Waltman et al. 1986) surveyed 119 isolates and found little biochemical activity or diversity among the strains. Plumb and Vinitnantharat (Plumb and Vinitnantharat 1989) also found few biochemical differences among forty isolates of *E. ictaluri* from areas including the Southeast, Midwest, and Northeast US, as well as Thailand. *Edwardsiella ictaluri* isolates are also serologically homogeneous (Plumb and Vinitnantharat 1989; Bertolini et al. 1990; Newton and Triche 1993). However, Bader et al. (Bader et al. 1998) surveyed 20 isolates of *E. ictaluri* and found four genotypes of *E. ictaluri* not identified by biochemical differences.

Generally, *E. ictaluri* strains carry two conserved plasmids. Isolates from fish species other than channel catfish, however, vary in plasmid profile (Newton et al. 1988; Reid and Boyle 1989; Lobb et al. 1993). Isolates capable of channel catfish pathogenesis carry two conserved plasmids, pEI1 and pEI2 (Newton et al. 1988; Bertolini et al. 1990). Both plasmids are sequenced, and both carry genes encoding proteins homologous to type III secretion system (T3SS) proteins of *Salmonella* and *Shigella* (Fernandez et al. 2001).

Externally, *E. ictaluri* infection results in many clinical signs, including a distended abdomen caused by fluid accumulation and petechial hemorrhaging of the skin near the mouth and at the base of fins. Behavioral changes occur, including reduced feeding and abnormal swimming. Internal examination of diseased fish shows petechial hemorrhaging of the liver, spleen, anterior and posterior kidney, intestine, and muscle. The liver also will exhibit multifocal white spots of necrosis. Chronic infection results in the erosion of the skull, leading to a lesion along the frontal bones of the skull.

The preferred entry of *E. ictaluri* into the channel catfish is unknown. Studies indicate infection occurs following exposure in the olfactory sac via the nerves (Miyazaki and Plumb 1985; Shotts et al. 1986; Morrison and Plumb 1994; Wolfe et al. 1998), through the gut (Shotts et al. 1986; Baldwin and Newton 1993), by intraperitoneal injection (Areechon and Plumb 1983), and immersion (Newton et al. 1989). Baldwin and Newton (Baldwin and Newton 1993) found *E. ictaluri* present in the trunk kidney 15 minutes following intragastric intubation and in phagocytic cells within 24 hours.

The pathology of *E. ictaluri* in channel catfish is well characterized. Following intragastric intubation, *E. ictaluri* is present in the kidneys within 15 minutes and the liver in 30 minutes (Baldwin and Newton 1993). *Edwardsiella ictaluri* is isolated from the liver and kidneys in both natural and experimental infections (Jarboe et al. 1984; Miyazaki and Plumb 1985; Shotts et al. 1986; Newton et al. 1989; Mqolomba and Plumb 1992; Baldwin and Newton 1993). Other tissues affected by *E. ictaluri* infection include the skin, muscle, spleen, intestine, and brain, and can persist in tissues up to 65 days post-infection (Mqolomba and Plumb 1992).

Within 24 hours following gut exposure, enteritis occurs in the intestine characterized by mononuclear cell infiltration (Shotts et al. 1986). Bacteria are present along the brush border at 30 minutes, and dilated and vacuolated cells, possibly macrophages, are present near the

basement membrane (Baldwin and Newton 1993). After an hour post-infection, enterocytes are necrotic. Lesions in the intestine are also reported following natural infections (Jarboe et al. 1984; Shotts et al. 1986) or infection by immersion (Newton et al. 1989).

Infection of the brain by *E. ictaluri* also occurs, resulting in meningoencephalitis. Infection of the channel catfish olfactory sac leads to damage of the epithelium within hours (Morrison and Plumb 1994). Inflammation of the olfactory sacs spreads into the tissues, and nerves are infected by *E. ictaluri*, leading to infection of the brain. While this brain infection occurs in the acute stage of infection, it does not always result in chronic disease, which is characterized by the formation of a hole along the midsagittal plane of the skull.

Inflammation of infected tissues is characterized by the presence of mononuclear cells commonly containing intracellular *E. ictaluri* (Shotts et al. 1986; Baldwin and Newton 1993). The presence of degenerate macrophage-like cells in the intestine following infection, and the presence of *E. ictaluri* in connective tissues only when adjacent to degenerate leukocytes indicates *E. ictaluri* disseminates by infection of macrophages (Baldwin and Newton 1993).

Edwardsiella ictaluri survives within host cells. Shoemaker et al. (Shoemaker et al. 1997) found macrophages from immune channel catfish have the ability to kill *E. ictaluri*; however, macrophages from naive fish have a diminished ability to kill, especially if the bacteria are coated with anti-*E. ictaluri* antibody. Klesius and Sealey (Klesius and Sealey 1996) found extracellular products of *E. ictaluri* attract macrophages to an exoantigen injection site.

Edwardsiella ictaluri also survives in channel catfish neutrophils (Ainsworth and Dexiang 1990; Waterstrat et al. 1991).

Edwardsiella ictaluri infects a number of cultured cell lines of fish and mammals (Skirpstunas and Baldwin 2002). Booth et al. (Booth et al. 2006) developed a gentamicin exclusion assay using primary cultures of channel catfish head kidney-derived macrophages to

study the intracellular replication of wild type and mutant strains of *E. ictaluri*. Wild type *E. ictaluri* exhibits rapid intracellular replication between four and eight hours post-infection. Replication of *E. ictaluri* in channel catfish macrophages is dependent on the activity of an acid-inducible urease system (Booth 2005) and a type III secretion system (T3SS) (Thune et al. 2007).

Channel catfish production is a \$400 million dollar industry (Anonymous 2003b). *Edwardsiella ictaluri* causes mortalities in over 60% of foodsize fish operations (Anonymous 2003b), and over 50% of fingerling operations report losses due to ESC (Anonymous 2003a). In total, ESC is the leading cause of fry and fingerling disease-related mortality. Treatment for ESC outbreaks includes medicated feed containing Terramycin, Romet, or Aquaflor and reducing or eliminating feeding during an outbreak. Medicated feed is the primary treatment at 18% of facilities, whereas 27% of farms use medicated feed in conjunction with other treatments (Anonymous 2003a). Medicated feed is expensive, and it may not be efficacious, as sick catfish typically do not eat. In addition, *E. ictaluri* develops resistance to the antibiotics contained in the feed (Cooper et al. 1993; Starliper et al. 1993; DePaola et al. 1995; Welch et al. 2009). Recently, florfenicol was approved for use in aquaculture. Unfortunately, *E. ictaluri* already can develop resistance to florfenicol (Welch et al. 2009). Despite the availability of commercially produced vaccines, few facilities report using vaccination against *E. ictaluri* infection. Only 11% of fry and fingerling operations (Anonymous 2003a) and 16% of foodsize channel catfish producers (Anonymous 2003b) vaccinate fish against *E. ictaluri*.

Despite the enormous economic impact of this organism, little is known about virulence genes associated with pathogenesis in channel catfish. Cooper et al. (Cooper et al. 1996) found *E. ictaluri* encodes a chondroitin sulfatase gene involved in virulence. Chondroitinase activity is the cause of the erosion of the skull along the sagittal line of the cranium seen in chronic brain infections. However, mutation of chondroitinase prevents both acute and chronic disease in

catfish. Lipopolysaccharide (LPS) is also an important determinant in *E. ictaluri* virulence. An O side-chain mutant of *E. ictaluri* is highly attenuated *in vivo* (Lawrence et al. 2001), with the LPS mutant more susceptible to killing in normal serum. However, the mutant is still able to survive within neutrophils (Lawrence et al. 2003).

Moore and Thune (Moore and Thune 1999; Moore et al. 2002) identified immunogenic proteins of *E. ictaluri*. Those identified include proteins homologous to *Rhizobium leguminosarum* proteins involved in a protein secretion system. This type of secretion system is classified as a type VI secretion system (T6SS). *Edwardsiella tarda* encodes a similar T6SS recently characterized by Zheng and Leung (Zheng and Leung 2007). The *E. tarda* T6SS contains both apparatus and effector genes, and mutagenesis of a majority of these genes results in attenuation *in vivo*. *Edwardsiella ictaluri* encodes a locus containing genes homologous to those in the *E. tarda* T6SS. Each *E. ictaluri* gene has high homology with the *E. tarda* counterpart suggesting homologous function. Zheng and Leung (Zheng and Leung 2007) determined the entire locus is expressed in one transcript and is under control of the T3SS-encoded regulation protein EsrC. Three proteins, EvpC, EvpI, and EvpP, are secreted, but the functions of these proteins in virulence are unknown. EvpC has homology to Hcp (hemolysin co-regulated protein), a common type of T6SS protein that forms a hexamer structure and may serve as the tube which allows translocation of T6SS proteins. EvpI has homology to Vgr (valine-glycine repeats) proteins also common to T6SS. EvpP has no homologs outside *Edwardsiella* in the genetic database. Secretion of EvpP is dependent on both EvpC and EvpI, which are also dependent upon each other for secretion (Zheng and Leung 2007). This dependence suggests EvpC and EvpI function in some capacity within the secretory structure of the T6SS.

Edwardsiella ictaluri is historically characterized a urease-negative bacterium (Hawke et al. 1981; Waltman et al. 1986). However, Booth (Booth 2005) reports a urease system is

involved in pathogenesis of *E. ictaluri*. *Edwardsiella ictaluri* strains carrying mutations in the urease operon are unable to replicate in channel catfish-derived macrophages and are attenuated in channel catfish immersion challenges. Wild type *E. ictaluri* tolerates, but does not replicate in pH levels as low as 2.5. At pH 5.5, however, *E. ictaluri* is able to modulate the pH of its environment to more neutral conditions and flourish. The pH modulation is urea-dependent and lost with mutations in the urease system.

Thune et al. (Thune et al. 2007) conducted a signature-tagged-mutagenesis (STM) project to identify virulence factors involved in the pathogenesis of *E. ictaluri* in channel catfish. Of the 50 virulence-related mutants identified, three are in genes associated with a T3SS. The first mutation is found in *EsaU*, a protein homologous to *SsaU* of the *Salmonella* pathogenicity island 2 (SPI-2) T3SS involved in intracellular survival. The *EsaU* mutant is attenuated *in vivo* and *ex vivo* in head kidney-derived channel catfish macrophages and channel catfish ovary cells. The other two STM T3SS mutations occur in the plasmids of *E. ictaluri*. A *pEI1* mutation is in an open reading frame (ORF) encoding an amino acid sequence similar to *SspH1*, *SspH2*, and *SlrP* of *Salmonella* and *IpaH* of *Yersinia* (Fernandez et al. 2001), all of which are secreted effector proteins of a T3SS. The second plasmid mutation is in *pEI2* upstream of an ORF encoding a sequence similar to *Spa15* of *Shigella* and *InvB* of *Salmonella* (Fernandez et al. 2001), both of which are chaperone proteins of secreted T3SS effector. These findings indicate a T3SS encoded within the *E. ictaluri* genome is important for pathogenesis within channel catfish and, more specifically, involved in *E. ictaluri* replication within channel catfish cells.

The ability of *E. ictaluri* to survive in channel catfish macrophages and other phagocytic cells gives it an advantage against the immune response of channel catfish. The discovery of a T3SS similar to the SPI-2 T3SS that enables *Salmonella* to replicate intracellularly suggests the T3SS of *E. ictaluri* acts to provide a similar benefit intracellularly. The finding that an *E. ictaluri*

T3SS mutant, *esaU*⁻, is unable to replicate within macrophages supports the hypothesis that the *E. ictaluri* T3SS is required for intracellular survival. Further study and characterization of the *E. ictaluri* T3SS will provide insight to an important method of immune avoidance and pathogenesis of *E. ictaluri* and may lead to improved methods of prevention and treatment of ESC.

TYPE III SECRETION SYSTEMS

This review will focus primarily on the SPI-2 T3SS of *Salmonella*, because of the homology to the *Edwardsiella* T3SS. However, the *E. tarda* T3SS was recently characterized and provides insight to the possible function of the *E. ictaluri* T3SS. Many reviews are available covering T3SS translocation, distribution, function, evolution, and other topics of a variety of T3SS in other pathogens (Mecenas and Strauss 1996; Finlay and Falkow 1997; Hueck 1998; Galan and Collmer 1999; Bennett and Hughes 2000; Hensel 2000; Francis et al. 2002; Holden 2002; Page and Parsot 2002; Knodler and Steele-Mortimer 2003; Parsot et al. 2003; Waterman and Holden 2003; Macnab 2004; Saier 2004; Ehrbar and Hardt 2005; Jones 2005; Patel and Galan 2005; Sorg et al. 2005; Troisfontaines and Cornelis 2005; Abrahams and Hensel 2006; Galan and Wolf-Watz 2006; Schlumberger and Hardt 2006; Angot et al. 2007).

T3SS are found only in Gram-negative bacteria and function to translocate effector proteins involved in virulence directly from the bacterial cytoplasm to the host cytoplasm in an energy-dependent manner. T3SS are homologous to flagellar systems. The system includes inner and outer membrane components as well as a periplasm-spanning domain that resembling the flagellar basal body. Furthermore, apparatus proteins of the T3SS share homology with basal body proteins. The translocon is constructed outward from the outer membrane domains in a fashion similar to that of flagellar construction, i.e., by adding protein subunits to the end of a growing chain.

The apparatus proteins of T3SS are rather unremarkable, regardless of the T3SS function; the various functions of T3SS among bacteria result from the effector proteins encoded and translocated by the system. The effector proteins modulate the activities of host cells, generating a pathogenic effect that varies among bacterial species. The homogeneity of the apparatus makes the discovery of a T3SS relatively easy, but identifying genes encoding effector proteins is more difficult due to their generally unconserved nature.

In the case of *Salmonella*, there are two T3SS encoded by separate pathogenicity islands. SPI-1 encodes a T3SS for translocation of proteins that induce bacterial uptake into cells and is required for the invasion of Peyer's patches in the mammalian host gut. SPI-1 effectors induce membrane ruffling of the target cell, leading to bacterial uptake, in addition to modulating the host immune response. The second T3SS, encoded by SPI-2, is required for the intracellular replication of *Salmonella*. Although it is used for intracellular replication, Brown et al. (Brown et al. 2005) report that the SPI-2 system is expressed prior to host cell invasion. The SPI-2 effector proteins act to create a suitable environment for *Salmonella* in many ways: 1) by creating a spacious vacuole referred to as a *Salmonella*-containing vacuole (SCV); 2) by directing intracellular trafficking of cell vesicles to the SCV, providing building blocks for the SCV; 3) by remodeling the actin cytoskeleton; and 4) by modulating or subverting the host immune response. SPI-2 effector proteins of known function are involved in one or more of the above processes. Most of the identified SPI-2 effectors are encoded outside of the pathogenicity island, indicating acquisition by horizontal transfer of genes.

Bacteriophages are associated with the horizontal transfer of some SPI-2 T3SS effector genes. Gifsy phages are active in *Salmonella* and involved in systemic virulence in mice (Figuroa-Bossi et al. 1997; Figuroa-Bossi and Bossi 1999; Figuroa-Bossi et al. 2001). Not all strains of *Salmonella* contain each Gifsy phage, however. SspH1 is an effector protein contained

within the Gifsy-3 phage sequence (Figuroa-Bossi et al. 2001), but Miao et al. (Miao et al. 1999) found SspH1 in only one *Salmonella* serotype. SseI is an SPI-2 effector that is carried by the Gifsy-2 phage (Figuroa-Bossi et al. 2001; Ho et al. 2002). SspH2 is encoded in a region surrounded by possible phage genes, indicating it may be carried by a phage as well (Miao and Miller 2000).

***SALMONELLA* PATHOGENICITY ISLAND 2 T3SS**

The SPI-2 T3SS translocates at least 20 proteins, most of which are involved in the assembly and maintenance of the SCV or intracellular trafficking within the host cell. Many of the effector proteins are encoded outside the pathogenicity island; SseF, SseG, and SpiC are the only known effector proteins encoded within the SPI-2 island. The proteins SseB, SseC, and SseD have the notation of effectors (Sse = *Salmonella* secreted effector), but their function is to generate the translocon apparatus on the surface of the bacterial cell as opposed to a virulence function in the host cell. This apparatus serves as the conduit for transfer of effector proteins from the bacterium to the host cell.

A group of proteins called *Salmonella* translocated effectors (STE) share a common secretion signal in their amino termini (Brumell et al. 2000; Miao and Miller 2000). Within the amino termini is the conserved sequence WEK(I/M)xxFF (hereafter referred to as the WEKI sequence), where x is any amino acid. Deletion of EKIx from the SspH2 sequence inhibits translocation (Miao and Miller 2000). Initially, seven proteins were identified as having the WEKI sequence: SifA, SifB, SseI, SseJ, SspH1, SspH2, and SlrP (Brumell et al. 2000; Miao and Miller 2000). Later, SopD2 was identified as another protein containing the WEKI translocation sequence (Brumell et al. 2003; Brown et al. 2006).

There are many *Salmonella* effector proteins possessing an internal leucine-rich repeat (LRR) region. LRR are involved in protein-protein interactions in eukaryotes (Kobe and Kajava

2001); however, little is known regarding the function of these repeats in *Salmonella* effectors. The LRR of SspH1 mediates the interaction between SspH1 and the host cell nuclear protein PKN1, a serine/threonine kinase involved in NF- κ B activation (Haraga and Miller 2006). The LRR of the *Salmonella* effectors SspH2 and SlrP, however, do not bind PKN1, indicating PKN1 binding is not a function shared by the LRR of *Salmonella* effector proteins.

Translocon Proteins. The translocon of the SPI-2 T3SS consists of the proteins SseB, SseC, and SseD, which are proteins encoded within SPI-2 and secreted by the T3SS. The proteins are not translocated to the host cell, however. Instead, they assemble on the outside of the cell, forming the syringe, or translocon. Mutation of these proteins attenuates *Salmonella* (Hensel et al. 1998), because there is no longer a conduit available for translocation of proteins across the bacterial and host cell membranes. Nikolaus et al. (Nikolaus et al. 2001) found that all three translocon proteins are required for translocation of effectors. SseB, SseC, and SseD are detected on the surface of *Salmonella* (Beuzon et al. 1999; Nikolaus et al. 2001; Chakravorty et al. 2005), and are observed as a polar structure with few copies on the bacterial surface. Klein and Jones (Klein and Jones 2001) determined that both SseC and SseD associate loosely with the membrane and are required for virulence. In the absence of SseB, however, SseC and SseD are secreted, but do not associate with the bacterial surface (Nikolaus et al. 2001). SseE, encoded in the same operon as SseB, SseC, and SseD, is not characterized, but may serve as a chaperone or accessory role (Coombes et al. 2004).

***Salmonella*-Containing Vacuole Biogenesis.** *Salmonella* resides intracellularly within a spacious vacuole. Genesis and maintenance of the SCV are reviewed (Knodler and Steele-Mortimer 2003; Steele-Mortimer 2008). The formation and maintenance of the SCV is dependent upon both the SPI-1 and SPI-2 T3SS. During *Salmonella* infections, the Golgi network surrounds the SCV, and only bacteria in vacuoles associated with the Golgi replicate

(Salcedo and Holden 2003). *Salmonella* inhibits the fusion of lysosomes to the SCV in phagocytic cells (Harrison et al. 2004). In non-phagocytic cells, however, *Salmonella* does not prevent acidification of the SCV or fusion of lysosomes (Drecktrah et al. 2007). Acidification of the SCV within non-phagocytic cells is delayed, however, but not mediated by the T3SS.

SifA is involved in the formation of *Salmonella* induced filaments (Sifs). Stein et al. (Stein et al. 1996) first discovered *sifA* while studying mutations lacking Sif formation. Beuzon et al. (Beuzon et al. 2000) determined SifA is a secreted effector of the T3SS, and mutation of *sifA* results in the inability of *Salmonella* to maintain the SCV. The instability of the SCV results in the inability to replicate within macrophages (Beuzon et al. 2000; Brumell et al. 2001; Salcedo et al. 2001; Ruiz-Albert et al. 2002; Boucrot et al. 2003). Interestingly, *sifA*-deficient strains escape the SCV (Beuzon et al. 2000) and are able to live in the cytosol of epithelial cells, but not macrophages (Beuzon et al. 2002; Brumell et al. 2002b) or dendritic cells (Petrovska et al. 2004). SifA directs Sif formation along microtubules and associates the SCV to Sifs (Brumell et al. 2002a).

Along with SseF and SseG, which are described later, SifA redirects the transport of sphingolipid-containing vesicles normally in the exocytotic pathway to the SCV (Kuhle et al. 2006) and mediates the juxtannuclear position of the SCV by recruiting microtubular motor proteins (Abrahams et al. 2006). Inhibition of host kinesin or dynein prevents Sif formation, indicating these cellular proteins are important for SCV construction and maintenance (Guignot et al. 2004). Dynein localization to the SCV requires Rab7, which recruits Rab7-interacting lysosomal protein (RILP). Normally, RILP associates with the dynein motor complex, and directs vesicles to intracellular areas rich in lysosomes. However, SifA uncouples Rab7 from RILP, inhibiting lysosomes from fusing to the SCV (Harrison et al. 2004). If RILP couples with Rab7, lysosomes fuse to the SCV, and *Salmonella* does not replicate (Marsman et al. 2004). SifB

also associates with the SCV membrane, but moves away from the vacuole along Sifs, an action dependent upon SifA (Freeman et al. 2003).

SseJ is involved in SCV maintenance and trafficking. SseJ localizes to the SCV and moves away from it along Sifs (Freeman et al. 2003). Birmingham et al. (Birmingham et al. 2005) found SseJ negatively affects Sif formation in the first 8-10 hours. Ruiz-Albert et al. (Ruiz-Albert et al. 2002) found SseJ and SifA have complementary activities by observing the loss of SCV membrane in a *sifA* mutant, but not an *sseJ* mutant. In a double mutant, no vacuolar loss was observed. SseJ is homologous to the GDSL family of lipases (Ohlson et al. 2005), and is shown *in vitro* to have deacylase activity, indicating SseJ induces lipid modifications.

PipB and PipB2 also associate with SCV and Sifs (Knodler et al. 2002). PipB and PipB2 are encoded in a pathogenicity island also containing SPI-1 effector genes (Wood et al. 1998; Knodler et al. 2002). The function of PipB is unknown, but mutation of *pipB* does not result in attenuation (Wood et al. 1998; Pfeifer et al. 1999). Knodler et al. (Knodler et al. 2003) found PipB2 localizes to detergent resistant microdomains in host membranes rich in cholesterol. Cholesterol accumulation in the SCV membrane is also T3SS-dependent (Catron et al. 2002). PipB2 localizes to SCV and Sifs near the periphery of host cells (Knodler et al. 2003), and results in extension of Sifs along microtubules, affecting the distribution of late endosomes/lysosomes (LE/Lys) in host cells (Knodler and Steele-Mortimer 2005). This effect is mediated by a pentapeptide repeat, which is not shared with PipB. PipB2 associates with kinesin through the pentapeptide repeat, driving transport along microtubules, and is sufficient for kinesin-1 recruitment to the SCV (Henry et al. 2006). Interestingly, a *sifA/pipB2* double mutant is more virulent than a *sifA* mutant indicating the functions of PipB2 contribute to virulence defects in *sifA* strains (Henry et al. 2006).

SifA and myosin II act in concert against the effects of PipB2 and SseJ, and vice versa, to maintain the perinuclear location of the SVC (Wasylnka et al. 2008). A SPI-1 effector, SopB, activates myosin II following infection with *Salmonella*. The interaction between SifA/myosin and SseJ/PipB2 demonstrates the dynamic and complicated nature of the SPI-2 T3SS in providing a suitable intracellular environment for growth. The involvement of SopB indicates an interaction between the effectors of the SPI-1 and SPI-2 systems for the maintenance of the SCV.

SopD2 associates with the SCV and endosomes (Brumell et al. 2003) via its amino terminus. A reduced capacity for Sif formation is observed in *sopD2* mutants, but only appears relevant in macrophages, because infection with *sopD2* mutants in epithelial cells shows no replication defect (Jiang et al. 2004). Brown et al. (Brown et al. 2006) determined the signal for localization to endocytic vesicles is contained within the STE WEKI sequence. Short peptides derived from SifA, SseJ, and SspH2, proteins also containing the WEKI sequence, localize to vesicles, but the amino acid sequence surrounding the STE sequence also influences localization.

Modulation of Intracellular Trafficking. There are three translocated effector proteins encoded within SPI-2: SseF, SseG, and SpiC. All are involved in modulating intracellular trafficking of the host cell. Deiwick et al. (Deiwick et al. 2006) determined that SseF and SseG interact physically, and are functionally linked. SseF and SseG associate with endosomal membranes and microtubules inside host cells (Kuhle and Hensel 2002; Kuhle et al. 2004) and are required for host endocytic structure alteration (Guy et al. 2000). Kuhle et al. (Kuhle et al. 2006) found SseF and SseG are required for exocytic transport of vesicles to the SCV, perhaps to deliver nutrients and membrane materials. Strains deficient for SseF and SseG do not recruit dynein to the SCV, a protein involved in directing vesicle transport. SseF and SseG are also required for maintenance of the SCV in a juxtannuclear position (Abrahams et al. 2006; Deiwick et al. 2006). SseG contains

a Golgi-targeting domain for localization to the Golgi network. SCV that do not associate with the Golgi do not permit bacterial replication (Salcedo and Holden 2003).

SpiC is reported in the literature as a translocated and non-translocated T3SS protein. Yu et al. (Yu et al. 2002) and Freeman et al. (Freeman et al. 2002) were unable to detect secretion of SpiC from *Salmonella*. The *spiC* mutant also does not secrete other T3SS effector proteins properly, resulting in a virulence defect. The conclusion was that SpiC is either involved with the apparatus or acts as a chaperone. The genes encoded in the same operon as *spiC* are the apparatus genes *ssaCDE*, suggesting SpiC may also be an apparatus protein.

Uchiya et al. (Uchiya et al. 1999), however, found that SpiC interferes with intracellular trafficking in macrophages and detected SpiC in the cytosol of macrophages infected with *Salmonella*, indicating SpiC is translocated. Lee et al. (Lee et al. 2002) used a yeast two hybrid system to demonstrate SpiC associates with a host protein similar to vesicular trafficking proteins. Suppression of this host protein, called TassC, allows *spiC* mutants to survive in macrophages, indicating SpiC is involved in preventing TassC activity. SpiC also associates with Hook3, which also is involved in intracellular trafficking (Shotland et al. 2003). Hook3 is found in infected and non-infected cells, and its expression is associated with disruption of the Golgi and late endosomes and lysosomes (LE/Lys). Shotland et al. agree with Yu et al. (Yu et al. 2002) and Freeman et al. (Freeman et al. 2002) regarding the inability of *spiC* mutants to secrete other effector proteins. The group speculated a similar situation as found in *Shigella* and *Yersinia* whereby secreted effectors are involved in the secretion of other effectors. Yu et al. (Yu et al. 2004) found that SsaM, a SPI-2 T3SS apparatus protein, interacts with SpiC within the bacterial cell, possibly as a mechanism for ordered secretion of translocon and effector proteins.

Remodeling of the Actin Cytoskeleton. Meresse et al. (Meresse et al. 2001) observed SPI-2-dependent accumulation of actin to the SCV, forming a meshwork around the vacuole. *De novo*

actin assembly occurs following *Salmonella* invasion, and treatment of host cells with actin depolymerization agents decreases intracellular *Salmonella* replication, indicating actin modifications are important for *Salmonella* virulence.

SPI-2 translocated proteins are involved in modulating actin polymerization. SspH2 binds filamin through its N-terminus and profilin through its C-terminus (Miao et al. 2003). Filamin crosslinks filamentous actin and profilin modulates the conversion of G-actin to F-actin. The association with profilin, however, is not a direct interaction. *In vitro*, the presence of SspH2 inhibits actin polymerization, but mutations in *sspH2* do not prevent vacuole-associated actin polymerizations. SspH2 shares 97% homology in the first 61 amino acids with SseI, which also associates with filamin. At the C-terminus, SspH2 shares 77% homology to SspH1; however, SspH1 does not associate with profilin as SspH2 does, indicating that high levels of homology between effector protein amino acid sequences are not sufficient to presume homologous function between proteins.

Lawley et al. (Lawley et al. 2006) found SseI is involved in systemic disease. SseI localizes to areas of actin polymerization within the host cell through an interaction of the N-terminus with filamin (Miao et al. 2003). Worley et al. (Worley et al. 2006) found that SseI binds the host protein TRIP6 and promotes *in vitro* motility of phagocytes, leading to systemic spread away from the intestine of the mouse. Cells positive for CD18 carry *Salmonella* from the GI tract through the bloodstream to the spleen and liver, indicating *Salmonella* is disseminated by leukocytes.

SteC is also an effector required for the formation of the F-actin meshwork surrounding the SCV (Poh et al. 2007). SteC has kinase activity dependent upon a conserved lysine residue. Mutation of the Lys residue has no effect on localization of the protein, but the F-actin mesh does not form.

Host Immunity Avoidance. The SPI-2 T3SS has many roles in the subversion of host immune function by *Salmonella*. Type III secretion system activity interferes with the trafficking of oxidase-containing vacuoles to the SCV, inhibiting the oxidative burst resulting from NADPH oxidase (Vazquez-Torres et al. 2000). Gallois et al. (Gallois et al. 2001) found flavocytochrome *b₅₅₈* is excluded or removed from the SCV, preventing construction of the NADPH oxidase complex; mutants deficient in SPI-2 processes are unable to achieve this exclusion. Reactive nitrogen processes also are inhibited during *Salmonella* infection. iNOS is present in vacuoles containing SPI-2-deficient bacteria, but when infected with wild type strains, *Salmonella* inhibits production of reactive nitrogen intermediates (RNI) rather than surviving in the presence of the RNI (Chakravorty et al. 2002; Fang and Vazquez-Torres 2002; Bjur et al. 2006).

Uchiya et al. (Uchiya et al. 2004) determined *Salmonella* upregulates expression of IL-10 in the infected host cell. COX-2 expression is also induced by SPI-2 T3SS, resulting in increased PGE2 and PGI2 production (Uchiya and Nikai 2004), and COX-2 inhibitors abolish growth of wild type *Salmonella* in macrophages. COX-2 activity is increased through ERK1/2 phosphorylation in a SpiC-dependent manner. In addition, the SPI-2 system affects the JAK/STAT pathway, inhibiting cytokine production (Uchiya and Nikai 2005).

The SPI-2 system is involved in decreasing MHC-II activity in dendritic cells (Cheminay et al. 2005). Multiple SPI-2 T3SS effector proteins are involved in the inhibition of antigen presentation (Halici et al. 2008). However, the function of each protein in altering presentation was not evaluated.

SspH1 localizes to the host nucleus and downregulates expression of IL-8 through inhibition of NF- κ B-mediated gene expression (Haraga and Miller 2003). SspH1 binds a serine/threonine kinase protein kinase N1 (PKN1) (Haraga and Miller 2006), a process mediated by the LRR of SspH1. The LRR of SspH2 or SlrP, however, is not sufficient for this interaction.

Constitutive activation of PKN1 results in decreased NF- κ B expression, and using RNAi against PKN1 results in an increase in NF- κ B expression. This suggests SspH1 associates with and activates PKN1, resulting in a decrease in NF- κ B activity.

SseL is produced by bacteria within macrophages and found primarily in the cytoplasm of infected cells (Coombes et al. 2007). SseL is a de-ubiquitinase involved in a delayed cytotoxic effect in macrophages (Rytkonen et al. 2007). A lack of SseL results in the accumulation of ubiquitinated proteins in infected cells. The authors suggest this effect likely affects the signaling cascade involved in cytotoxicity rather than proteasome-dependent degradation of ubiquitinated proteins.

Other Proteins Translocated by the SPI-2 T3SS. Some proteins are secreted by the SPI-2 T3SS, but their function is not known. SlrP is a LRR-containing protein containing the STE WEKI sequence. Mutation of SlrP results in attenuation of *Salmonella* in mice (Tsolis et al. 1999), and SlrP is involved in the inhibition of MHC-II antigen presentation (Halici et al. 2008). GogB has a partial LRR, but lacks a WEKI motif (Coombes et al. 2005a). GogB is secreted by both the SPI-1 and SPI-2 T3SS and localizes to the host cytoplasm. SseK1 and SseK2 are 61% homologous to each other, but differ in their N-termini (Kujat Choy et al. 2004). SseK1 and SseK2 are similar to proteins of *Escherichia coli* involved in attachment and effacement. The first 32 amino acids of SseK1 localize it to the host cytosol. SseK2 is involved in long-term infection of the mouse (Lawley et al. 2006).

***SALMONELLA* PATHOGENICITY ISLAND 2 T3SS REGULATION**

Many *in vitro* conditions induce the SPI-2 T3SS. The SPI-2 T3SS is activated by low magnesium ion concentrations (Garcia Vescovi et al. 1996; Soncini et al. 1996; Beuzon et al. 1999; Deiwick et al. 1999; Hansen-Wester et al. 2002; Hautefort et al. 2003; Norte et al. 2003; Shin and Groisman 2005), low calcium ion concentrations (Deiwick et al. 1999; Garmendia et al.

2003; Linehan et al. 2005), low inorganic phosphate concentrations (Beuzon et al. 1999; Deiwick et al. 1999; Hansen-Wester et al. 2002; Lober et al. 2006), low osmolarity (Lee et al. 2000; Garmendia et al. 2003; Linehan et al. 2005), low pH (Cirillo et al. 1998; Beuzon et al. 1999; Lee et al. 2000; Garmendia et al. 2003; Hautefort et al. 2003; Coombes et al. 2004; Feng et al. 2004; Kim and Falkow 2004; Lober et al. 2006; Walthers et al. 2007), in response to ppGpp levels (Thompson et al. 2006), and in the presence of cation chelators (Kim and Falkow 2003; Kim and Falkow 2004). Miao et al. (Miao et al. 2002) found SPI-2 T3SS genes express in both low and moderate levels of calcium and magnesium and at both neutral and acid pH; however, alkaline pH represses expression. SPI-2 T3SS activity is also positively affected by the presence of natural resistance-associated macrophage protein (Nramp) (Zaharik et al. 2002), providing a potential mechanism of recognition of the intracellular environment.

Expression of SPI-2 T3SS genes is regulated by at least three two-component regulatory systems: SsrAB, PhoPQ, and OmpR/EnvZ. PhoQ, EnvZ, and SsrA are membrane sensor kinase proteins that phosphorylate the response regulators PhoP, OmpR, and SsrB, respectively, which in turn regulate expression of many virulence-associated genes. SsrA and SsrB are encoded within the SPI-2 locus and are required for T3SS expression (Shea et al. 1996; Valdivia and Falkow 1996; Cirillo et al. 1998; Deiwick et al. 1999; Worley et al. 2000; Garmendia et al. 2003; Feng et al. 2004; Deiwick et al. 2006; Dieye et al. 2007; Walthers et al. 2007). SsrB controls regulation of T3SS genes both within and outside the SPI-2 locus (Worley et al. 2000). Interestingly, SsrB is active in the absence of SsrA, indicating SsrB can be phosphorylated by other kinases (Walthers et al. 2007).

PhoP and PhoQ have long been associated with *Salmonella* virulence (Groisman et al. 1989; Miller et al. 1989; Behlau and Miller 1993; Belden and Miller 1994; Garcia Vescovi et al. 1996; Soncini et al. 1996). Both the PhoPQ and OmpR/EnvZ systems are involved in expression

of T3SS genes (Deiwick et al. 1999; Lee et al. 2000; Worley et al. 2000; Feng et al. 2003; Garmendia et al. 2003; Feng et al. 2004; Kim and Falkow 2004; Bijlsma and Groisman 2005; Brown et al. 2005; Merighi et al. 2005; Lober et al. 2006). PhoPQ and OmpR/EnvZ act on *ssrA* and *ssrB* in *Salmonella*, but do not modulate expression of other T3SS genes (Lee et al. 2000; Worley et al. 2000; Feng et al. 2003; Garmendia et al. 2003; Feng et al. 2004; Bijlsma and Groisman 2005). *phoP* expression is greatly affected by pH changes intracellularly (Martin-Orozco et al. 2006), indicating it is a mediator of the SPI-2 T3SS activity in acidic conditions.

SlyA, originally identified as a cytolysin (Libby et al. 1994), is encoded in the *Salmonella* genome and is involved in the regulation of the SPI-2 T3SS regulators SsrAB (Feng et al. 2004; Linehan et al. 2005). SlyA is required for survival inside macrophages (Daniels et al. 1996; Norte et al. 2003) and resistance to oxidative stress within macrophages (Buchmeier et al. 1997). SlyA has redundancy with OmpR, because both are involved in the responses to low osmolarity and calcium concentration (Linehan et al. 2005). Okada et al. (Okada et al. 2007) found SlyA binds directly to the promoter of *ssrA*, and mutation of *slyA* results in loss of SPI-2 T3SS function. Expression of *slyA* may also be modulated by PhoP (Norte et al. 2003); however, Navarre et al. (Navarre et al. 2005) found little influence of PhoP on *slyA*.

In addition to regulating gene expression, environmental conditions regulate secretion of proteins. Acidic conditions are required for secretion, but not expression of SPI-2 translocon proteins (Beuzon et al. 1999; Nikolaus et al. 2001; Hansen-Wester et al. 2002; Coombes et al. 2004; Chakravorty et al. 2005). Beuzon et al. (Beuzon et al. 1999) found SseB accumulates in cells at neutral pH, but upon a shift to a pH less than 5.0, SseB is detected extracellularly within minutes. In the absence of acidic pH, SseB is not degraded intracellularly, but rather accumulates within the cell (Coombes et al. 2004). Coombes et al. (Coombes et al. 2004) hypothesized growth of *Salmonella* to stationary phase induces expression of *sseB*, suggesting relaxed control

of SPI-2 regulation in stationary phase or a separate regulatory mechanism. Acidic pH is also required for localization of the apparatus protein SsaC to the outer membrane and oligomerization of SsaC subunits (Rappl et al. 2003).

SsaL of *Salmonella* is homologous to SepL, a protein of *Es. coli*, and is required for secretion of SPI-2 encoded effector proteins (Coombes et al. 2004; Deng et al. 2004), but is not required for secretion of effector proteins encoded outside of the pathogenicity island. Expression of *ssaL* requires an acidic minimal media and SsrB. A lipoprotein, YfgL, is also required for optimal SPI-2 gene expression (Fardini et al. 2007).

SPI-2 genes are induced intracellularly. Eriksson et al. (Eriksson et al. 2003) used microarray to study intracellular SPI-2 gene expression. Upregulation of regulatory, apparatus, and effector genes is slightly, moderately, or strongly induced, respectively. Based on genes involved in ion transport, the researchers conclude magnesium and phosphate are limiting intracellularly. However, iron, potassium, and amino acids are available to intracellular *Salmonella*.

The route of *Salmonella* entry affects SPI-2 T3SS gene expression. Drecktrah et al. (Drecktrah et al. 2006) found SPI-2 genes are expressed faster following SPI-1 induced uptake than when taken up via phagocytosis. Expression is also greater in non-opsonized than opsonized bacteria, although expression of some SPI-2 genes are not affected. Interestingly, the pH changes of the vacuole are also dependent on opsonization; acidification is more rapid following non-opsonized uptake.

Response Regulator Binding Domains. Feng et al. (Feng et al. 2004) studied the ability of SsrB to bind SPI-2-related promoters. SsrB protects regions of DNA upstream of *ssrA*, *ssrB*, and *sseI* in a DNaseI protection assay, indicating those regions contain SsrB binding sites. The C-terminus of SsrB is sufficient for transcriptional activation of those genes. The SsrB N-terminus

contains a phosphorylation domain that, when phosphorylated, modifies the structure of the C-terminus, resulting in an active state. The amino acid residue subject to phosphorylation is an aspartic acid residue, and substitution of this amino acid with alanine prevents transcription of *sseI*. Walthers et al. (Walthers et al. 2007) found additional SsrB binding sites upstream of the SPI-2 T3SS genes *ssaB*, *sseA*, *ssaG*, and *ssaM*.

Feng et al. (Feng et al. 2003) observed OmpR binding to the *ssrA* promoter intracellularly, but not *in vitro*, suggesting that the signal received by EnvZ is related to the macrophage environment and cannot be replicated *in vitro*. However, Lee et al. (Lee et al. 2000) found that OmpR modulates *ssrA* expression intracellularly and when cultured in broth at pH 4.5. Both of these studies used a different strain of *Salmonella*, however. Feng et al. (Feng et al. 2004) also found an OmpR binding site upstream of *sseI*.

PhoP regulates *ssrB* expression by binding the promoter region and inducing expression (Bijlsma and Groisman 2005). While a binding region is not upstream of *ssrA*, PhoP controls *ssrA* post-transcriptionally by binding a portion of the 5' untranslated region. PhoPQ is a negative regulator of the SPI-1 T3SS required for invasion, indicating that PhoPQ may act to turn off SPI-1 and turn on SPI-2. PhoP activity downregulates HilA, a protein required for SPI-1 T3SS gene expression (Behlau and Miller 1993; Bajaj et al. 1996).

***Salmonella* Pathogenicity Island 2 T3SS Negative Regulators.** Nucleoid binding proteins are negative regulators of the SPI-2 T3SS. YdgT is a protein of *Salmonella* homologous to Hha, which negatively regulates virulence gene expression of *Es. coli* (Fahlen et al. 2000; Fahlen et al. 2001). Mutation of *ydgT* results in *Salmonella* strains overproducing virulence genes, resulting in attenuation (Coombes et al. 2005b). Initially, there is enhanced intracellular growth, but with time the overproduction results in attenuation, suggesting strict control over both up- and downregulation of the SPI-2 T3SS is required for virulence of *Salmonella*. Deletion of *ssrB*

inhibits the early-enhanced virulence of *ydgT* mutants, indicating that loss of *ydgT* does not abolish the requirement for *ssrB*. Hha also is found in *Salmonella* and is responsible for a majority of the repressor activity involved in SPI-2 T3SS expression (Silphaduang et al. 2007). When *ydgT* and *hha* are mutated, SseB is overexpressed in non-inducing conditions, but expression of *hha in trans* lowers expression of SseB to less than that of a wild type phenotype.

Salmonella pathogenicity island 2 T3SS expression is negatively regulated by the nucleoid-associated protein Fis (Lim et al. 2006). The apparatus gene *ssaG* contains four Fis-binding domains in its promoter region. Expression of *ssaG* and survival within macrophages decreases with a *fis* mutation. Expression of *ssrA* is also reduced in a *fis* mutant; Fis interacts with the promoter regions of both *ssaG* and *ssrA* (Kelly et al. 2004). In addition, Fis regulates SPI-1 expression (Wilson et al. 2001). Fis is involved in the upregulation of T3SS genes in relation to relaxed supercoiling of the bacterial genome (Ó Cróinín et al. 2006). Mutations in *fis* result in greater relaxation of the genome, making DNA sequences more accessible to transcriptional factors.

H-NS is an additional nucleoid binding protein that negatively regulates SPI-2 T3SS expression (Lucchini et al. 2006; Navarre et al. 2006; Walthers et al. 2007). H-NS binds curved DNA containing AT-rich regions, potentially silencing foreign gene expression (Lucchini et al. 2006; Navarre et al. 2006). Pathogenicity islands within *Salmonella* likely are acquired by horizontal transmission, based on their higher AT content than the rest of the genome, indicating H-NS would preferentially bind these regions. SsrB, however, relieves repression of H-NS on SPI-2 gene promoters (Walthers et al. 2007). Removal of H-NS results in uncontrolled expression of *Salmonella* pathogenicity islands and reduces bacterial fitness (Lucchini et al. 2006), supporting the findings of Coombes et al. (Coombes et al. 2005b) that overexpression of SPI-2 genes results in attenuated virulence.

SPI-2 T3SS expression is negatively regulated by HilA, a regulator of the SPI-1 invasion-associated T3SS (Thijs et al. 2007). HilA interacts with the promoter of *ssaH* and repress its expression. Other proteins of the SPI-1 T3SS regulatory cascade affect SPI-2 T3SS expression. Dieye et al. (Dieye et al. 2007) found *sirA*, *hilC*, and *invF* repress expression of *sifB*.

TYPE III SECRETION SYSTEM GENE EXPRESSION ANALYSIS USING GFP

Detection of green fluorescent protein (GFP) expression from various promoters of the SPI-2 T3SS by fluorescence-activated cell sorting (FACS) is used to determine *in vitro* conditions sufficient for induction of T3SS promoters. Analysis of expression in media commonly is done by measuring the fluorescence of bacterial-sized particles from cultures (Kim and Falkow 2004; Yu et al. 2004; Linehan et al. 2005). Similar methods are used to measure expression of bacterial cells cultured intracellularly.

Valdivia and Falkow (Valdivia and Falkow 1996) measured fluorescence of bacterial-sized particles from lysed macrophages infected by *Salmonella* strains carrying promoter fusions to *gfp*. This method of detection is used in many studies evaluating promoter fusions to GFP (Lee et al. 2000; Garmendia et al. 2003; Bijlsma and Groisman 2005; Lim et al. 2006). Hautefort et al. (Hautefort et al. 2003), however, measured fluorescence of *Salmonella* carrying GFP-promoter fusions from infected macrophage lysates and labeled the *Salmonella* using indirect fluorescence with a phycoerythrin-conjugated anti-*Salmonella* antibody. Bacteria were analyzed using the phycoerythrin signal rather than by size. The number of total bacteria and the number of bacteria expressing the particular promoter were able to be determined, as opposed to the method described above in which particle size is used. Using the antibody method, the number of bacteria producing GFP can be separated from the population not expression GFP. Hautefort et al. (Hautefort et al. 2003) constructed promoter fusions to GFP and inserted them into the genome as a single copy rather than being expressed from a plasmid. This eliminated problems

with differential copy number of plasmids that lead to differential fluorescence (Hansen-Wester et al. 2002). In addition, Hautefort et al. were able to insert the fusion in a segment of DNA such that the native gene was not disrupted, avoiding loss of virulence phenotypes.

Using GFP expression as a reporter has proven to be ineffective in some instances. Hansen-Wester et al. (Hansen-Wester et al. 2002) found that SPI-2 expression is improperly regulated when genes are expressed from medium copy plasmids with native promoters. A similar problem may arise when analyzing activity of reporter genes fused to promoters and expressed from plasmids. However, Dieye et al. 2007 found *sifA* fusion expression is not detected as a single insertion in the chromosome, but can when expressed from plasmid, suggesting supercoiling or chromatin structure has an effect on expression. Knodler et al. (Knodler et al. 2005) reported that cloning vectors and fluorescent proteins can impair the ability of *Salmonella* to replicate in mammalian cells and in mice.

THE *EDWARDSIELLA TARDA* T3SS

Edwardsiella tarda encodes a T3SS similar to that of the SPI-2 T3SS. An *E. tarda* gene, *esrB*, similar to *ssrB* of *Salmonella*, results in attenuation when mutated (Srinivasa Rao et al. 2003). Secreted proteins EseB, EseC, and EseD of *E. tarda* are homologous to SseB, SseC, and SseD of *Salmonella* and are present in the supernatant of *E. tarda* cultures, indicating *E. tarda* encodes a T3SS similar to the SPI-2 T3SS of *Salmonella* (Srinivasa Rao et al. 2004). The EseBCD proteins form a complex after secretion, supporting the proposal that these proteins serve as the translocon of a T3SS (Zheng et al. 2007). Secretion of EseB and EseD is dependent on a putative chaperone protein, EscC (Zheng et al. 2007), and mutation of *escC* prevents secretion of EseB and EseD. Mutation of *esrB* reduces secretion of the *E. tarda* T3SS proteins EseB, EseC, and EseD, indicating *EsrB* acts as a regulator of the *E. tarda* T3SS.

Edwardsiella tarda replicates within fish phagocytes (Srinivasa Rao et al. 2001; Okuda et al. 2006) similar to intracellular replication of *Salmonella*. Okuda et al. (Okuda et al. 2006) found the T3SS is required for survival and replication of *E. tarda* in murine macrophages, and anti-apoptotic genes are upregulated in a NF- κ B dependent manner by wild type *E. tarda* compared to strains with T3SS mutations. Conversely, Okuda et al. (Okuda et al. 2008) report that wild type *E. tarda* downregulates NF- κ B activity in epithelial cells, and like in macrophages, a T3SS-deficient mutant is unable to replicate in the epithelial cells.

The genome of *E. tarda* has not been sequenced, but the pathogenicity island encoding the *E. tarda* T3SS was sequenced by Tan et al. (Tan et al. 2005), identifying 35 ORFs encoding putative T3SS apparatus, effector, chaperone, and regulatory proteins. Mutation of various genes within the island attenuates *E. tarda* in fish phagocytes, and LD₅₀ values increase at least 10 times that of wild type *E. tarda*. *Edwardsiella tarda* encodes an AraC-type regulator protein, EsrC, which *Salmonella* does not encode in SPI-2 (Tan et al. 2005; Zheng et al. 2005). Mutation of *esrC* increases LD₅₀ values and decreases secreted T3SS proteins. In addition, proteins associated with a type VI secretion system (T6SS) do not secrete in the *esrC* mutant. Expression of *esrC* is dependent upon the EsrA and EsrB two-component system, and expression of some *E. tarda* T3SS genes is EsrC-dependent.

***Edwardsiella tarda* T3SS Regulation.** Expression of the *E. tarda* T3SS does not require acidic media or low phosphate concentrations, in contrast to the SPI-2 T3SS. *Edwardsiella tarda* T3SS promoter activity and protein secretion is achieved by culture in Dulbecco's Modified Eagle Medium at pH 7.0 (Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005). EsrA and EsrB are involved in regulation of the *E. tarda* T3SS (Srinivasa Rao et al. 2003; Tan et al. 2005; Zheng et al. 2005), and mutation of *esrB* decreases expression of putative translocon proteins EseB, EseC, and EseD (Srinivasa Rao et al. 2004). Similar to *Salmonella*, EsrA and EsrB are

required for intracellular replication within macrophages (Srinivasa Rao et al. 2003; Tan et al. 2005). EsrC is also involved in intramacrophage replication; however the effect of *esrC* mutation is less than that of *esrA* or *esrB* (Zheng et al. 2005). Expression of EsrC is dependent upon EsrA and EsrB, and EsrC is also involved in the expression of secreted translocon proteins of the T3SS (Zheng et al. 2005). However, EsrC is not involved in controlling T3SS apparatus gene expression. In addition to regulating *E. tarda* T3SS genes, EsrC regulates expression of T6SS genes (Zheng et al. 2005; Zheng and Leung 2007).

Because the *E. tarda* genome is not sequenced, the presence of genes homologous to SPI-2 regulators, such as OmpR/EnvZ, PhoPQ, or SlyA, is not known. Therefore, association of these proteins with regulation of the *E. tarda* T3SS regulation has not been studied. However, genes involved in phosphate transport (*pstSCABphoU*) are associated with virulence in *E. tarda* (Srinivasa Rao et al. 2003; Srinivasa Rao et al. 2004). The PST operon is involved in phosphate transport, and mutation of PST genes results in decreased T3SS gene expression indicating low intracellular concentrations of inorganic phosphate downregulate the *E. tarda* T3SS. The role of PST in virulence, however, may be a result of reduced fitness due to the bacteria being unable to maintain intracellular phosphate concentrations. Low inorganic phosphate concentrations in culture media induce SPI-2 T3SS gene expression; however, low phosphate media conditions are an extracellular signal as opposed to PST mutants, which would cause a low phosphate signal intracellularly due to the inability to import phosphate.

THE *EDWARDSIELLA ICTALURI* T3SS

Edwardsiella ictaluri encodes a T3SS homologous to the *E. tarda* T3SS and the SPI-2 T3SS (Thune et al. 2007). A list of genes encoded by the *E. ictaluri* T3SS pathogenicity island is provided in Table 1.1 along with their putative functions and homologous genes in *E. tarda* and *Salmonella*. The *E. ictaluri* T3SS is necessary for replication of the bacterium in channel catfish

macrophages and pathogenesis in the channel catfish host. In 2001, T3SS-like genes were sequenced from the plasmids of *E. ictaluri* by Fernandez et al. (Fernandez et al. 2001). However, the sequence of the pathogenicity island was not determined until later (Thune et al. 2007). Within the pathogenicity island, there are genes similar to the SPI-2 T3SS, including regulatory, apparatus, chaperone, and effector genes (Figure 1.1). A schematic describing the putative construction of the *E. ictaluri* T3SS is provided in Figure 1.2.

Edwardsiella ictaluri encodes a protein in the T3SS pathogenicity island that is homologous to the effectors SseF and SseG. However, the sequence in *E. ictaluri* is a hybrid of the two proteins; that is, the predicted amino acid sequence of the single open reading frame has similarities to both SseF and SseG of *Salmonella*. Interestingly, SseF and SseG associate physically and functionally for virulence (Deiwick et al. 2006), suggesting the single protein of *E. ictaluri* may have a similar function as the two *Salmonella* proteins SseF and SseG.

SpiC is a SPI-2 effector protein encoded at the beginning of an operon containing the apparatus genes *ssaCDE*. *Edwardsiella ictaluri* does not have a gene with high homology to *spiC*, but it does encode EsaB, which has minor homology to SpiC and is encoded in a similar position as *spiC*: at the beginning of an operon encoding the apparatus genes *esaCDE* (Figure 1.3). It is unknown if EsaB is an effector protein of the *E. ictaluri* T3SS, but its positioning relative to that of *spiC* in *Salmonella* suggests the possibility.

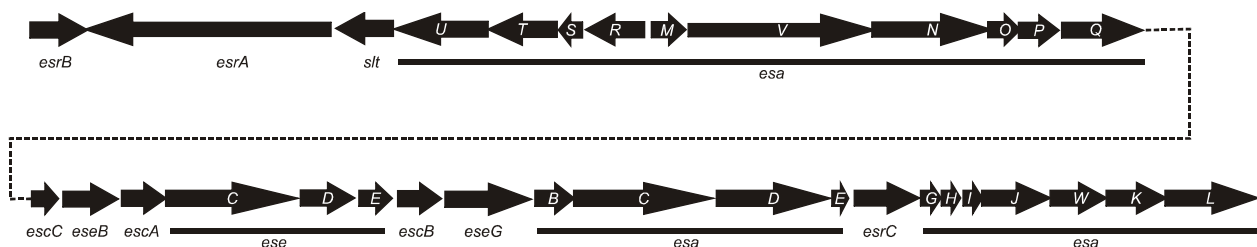


Figure 1.1. Schematic representation of the *Edwardsiella ictaluri* type III secretion system pathogenicity island genetic organization.

Table 1.1. Genes of the *Edwardsiella ictaluri* type III secretion system (T3SS) and homologs in *E. tarda* and *Salmonella* pathogenicity island 2 (SPI-2).

<i>E. ictaluri</i>	<i>E. tarda</i>	SPI-2	Encoded within the <i>E. ictaluri</i> T3SS	Putative function
<i>esaB</i>	<i>esaB</i>	<i>spiC (ssaB)</i>	Yes	Putative translocated effector
<i>esaC</i>	<i>esaC</i>	<i>ssaC</i>	Yes	Apparatus
<i>esaD</i>	<i>esaD</i>	<i>ssaD</i>	Yes	Apparatus
<i>esaE</i>	<i>esaE</i>	<i>ssaE</i>	Yes	Apparatus
<i>esaG</i>	<i>esaG</i>	<i>ssaG</i>	Yes	Apparatus
<i>esaH</i>	<i>esaH</i>	<i>ssaH</i>	Yes	Apparatus
<i>esaI</i>	<i>esaI</i>	<i>ssaI</i>	Yes	Apparatus
<i>esaJ</i>	<i>esaJ</i>	<i>ssaJ</i>	Yes	Apparatus
<i>esaK</i>	<i>esaK</i>	<i>ssaK</i>	Yes	Apparatus
<i>esaL</i>	<i>esaL</i>	<i>ssaL</i>	Yes	Apparatus
<i>esaM</i>	<i>esaM</i>	<i>ssaM</i>	Yes	Apparatus
<i>esaN</i>	<i>esaN</i>	<i>ssaN</i>	Yes	Apparatus
<i>esaO</i>	<i>esaO</i>	<i>ssaO</i>	Yes	Apparatus
<i>esaP</i>	<i>esaP</i>	<i>ssaP</i>	Yes	Apparatus
<i>esaQ</i>	<i>esaQ</i>	<i>ssaQ</i>	Yes	Apparatus
<i>esaR</i>	<i>esaR</i>	<i>ssaR</i>	Yes	Apparatus
<i>esaS</i>	<i>esaS</i>	<i>ssaS</i>	Yes	Apparatus
<i>esaT</i>	<i>esaT</i>	<i>ssaT</i>	Yes	Apparatus
<i>esaU</i>	<i>esaU</i>	<i>ssaU</i>	Yes	Apparatus
<i>esaV</i>	<i>esaV</i>	<i>ssaV</i>	Yes	Apparatus
<i>esaW</i>	<i>esaW</i>	<i>sty1410</i>	Yes	Apparatus
<i>escA</i>	<i>escA</i>	<i>sscA</i>	Yes	Chaperone for translocon proteins
<i>escB</i>	<i>escB</i>	<i>sscB</i>	Yes	Chaperone for EseG
<i>escC</i>	<i>escC</i>	<i>sseA</i>	Yes	Chaperone for translocon proteins
<i>escD</i>	-	-	No; pEI2	Chaperone for EseI

Table 1.1 continued.

<i>E. ictaluri</i>	<i>E. tarda</i>	SPI-2	Encoded within the <i>E. ictaluri</i> T3SS	Putative function
<i>eseB</i>	<i>eseB</i>	<i>sseB</i>	Yes	Translocon protein
<i>eseC</i>	<i>eseC</i>	<i>sseC</i>	Yes	Translocon protein
<i>eseD</i>	<i>eseD</i>	<i>sseD</i>	Yes	Translocon protein
<i>eseE</i>	<i>eseE</i>	<i>sseE</i>	Yes	Possible translocon chaperone
<i>eseG</i>	<i>eseG</i>	<i>sseF</i> and <i>sseG</i>	Yes	Translocated effector
<i>eseH</i>	-	<i>sspH1</i> and <i>sspH2</i>	No; pEI1	Translocated effector; downregulates immune response or inhibits actin polymerization
<i>eseI</i>	-	hypothetical not yet associated with SPI-2	No; pEI2	Translocated effector; downregulates immune response
<i>eseJ</i>	-	<i>sspH1</i> and <i>sspH2</i>	No; genomic	Translocated effector; downregulates immune response or inhibits actin polymerization
<i>eseK</i>	-	<i>sspH1</i> and <i>sspH2</i>	No; genomic	Translocated effector; downregulates immune response or inhibits actin polymerization
<i>eseL</i>	-	<i>sspH1</i> and <i>sspH2</i>	No; genomic	Translocated effector; downregulates immune response or inhibits actin polymerization
<i>eseM</i>	-	<i>sspH1</i> and <i>sspH2</i>	No; genomic	Translocated effector; downregulates immune response or inhibits actin polymerization
<i>esrA</i>	<i>esrA</i>	<i>ssrA</i>	Yes	Membrane sensor kinase
<i>esrB</i>	<i>esrB</i>	<i>ssrB</i>	Yes	Cytosolic response regulator
<i>esrC</i>	<i>esrC</i>	-	Yes	AraC-type transcriptional activator
<i>slt</i>	<i>slt</i>	-	Yes	soluble lytic transglycosylase
<i>orf29/30</i>	<i>orf29</i> and <i>orf30</i>	-	Encoded near the T3SS	Possibly a T3SS effector

Host cell cytoplasm

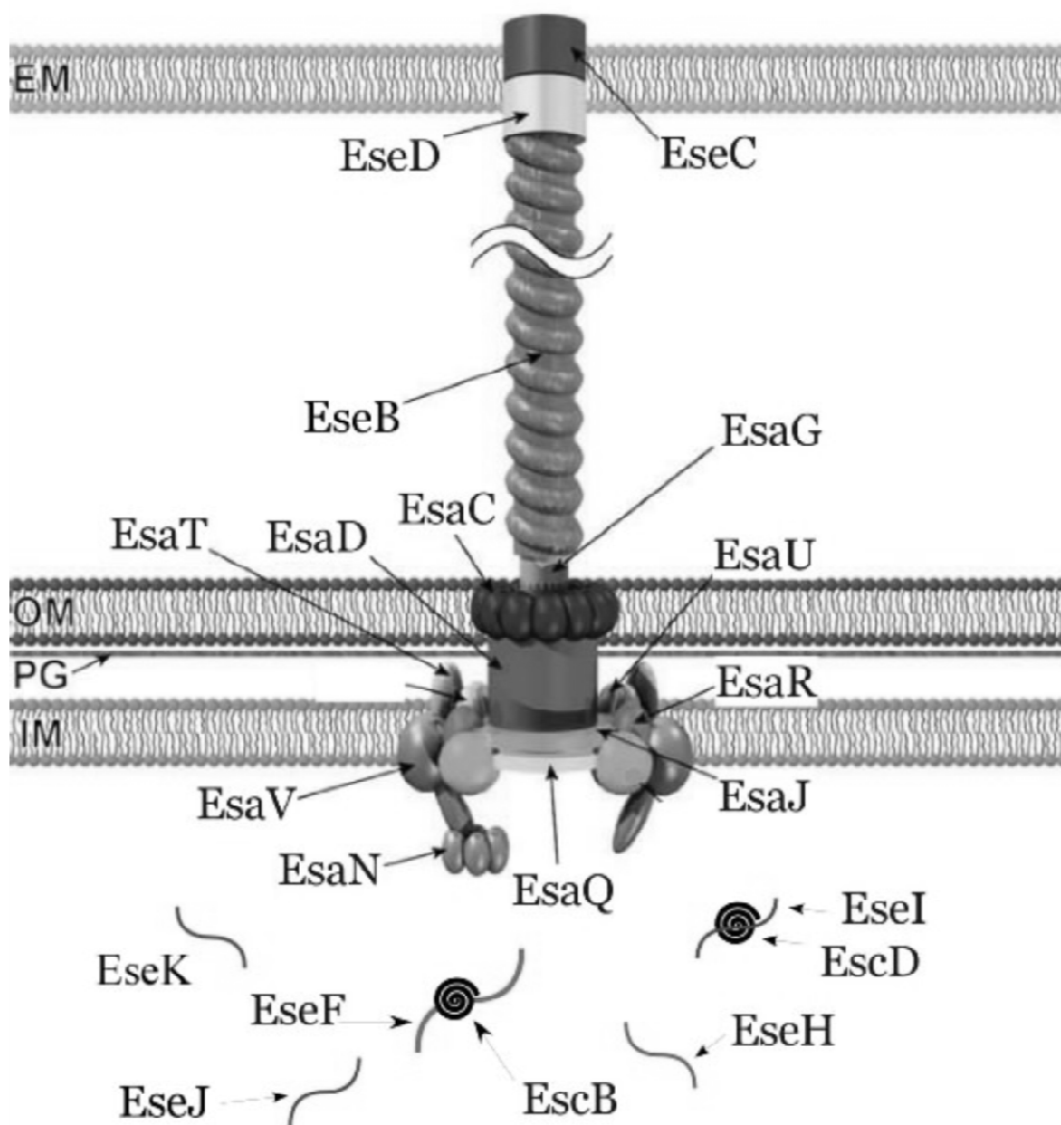


Figure 1.2. Schematic representation of the *Edwardsiella ictaluri* type III secretion system (T3SS). The T3SS translocates proteins from the bacterial cytoplasm to the host cytoplasm. Bacteria-encoded apparatus proteins (Esa) assemble in the inner membrane, periplasmic space, and outer membrane to form a channel for effector proteins (Ese) to pass. Translocon proteins (EseBCD) function as a conduit between the bacterial and host cell membranes to translocate effector proteins. Chaperone proteins (Esc) bind effector proteins to keep them in an inactive state inside the bacteria. The identification of the *E. ictaluri* proteins in the membranes is based on amino acid homology to *Es. coli* T3SS proteins. The image is adapted from Pallen et al. (Pallen et al. 2005).

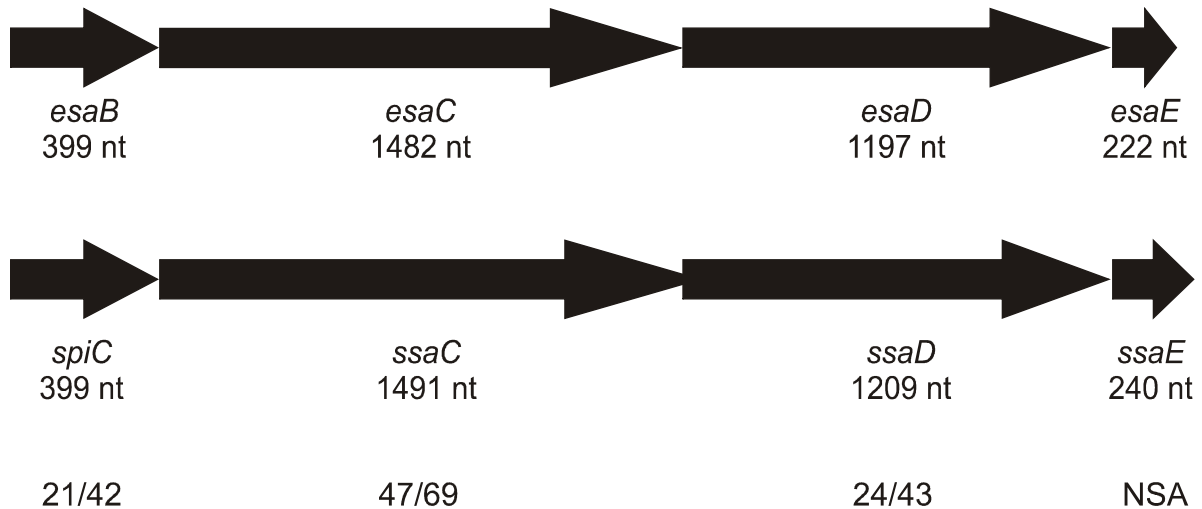


Figure 1.3. Comparison of the *Edwardsiella ictaluri* *esaB* operon to the *spiC* (*ssaB*) operon of *Salmonella*. Although similar in size and organization, predicted amino acid sequences are not greatly homologous. *EsaC/SsaC* and *EsaD/SsaD* are predicted apparatus genes, but *SpiC* is a secreted effector of SPI-2 T3SS, suggesting *EsaB* may have a similar function. Percent identity and similarity between significant amino acid alignments are given below each alignment. NSA indicates no significant alignment between the sequences.

The homology of *E. ictaluri* T3SS proteins to SPI-2 encoded proteins suggests a similar function. *Edwardsiella ictaluri* does not induce filaments as *Salmonella* does, and likewise, no genes are identified that encode proteins with similar amino acid sequences to *Salmonella* proteins involved in Sif formation. *Edwardsiella ictaluri*, however, does encode proteins with homology to *Salmonella* effectors involved in intracellular trafficking (*SseF*, *SseG*, and *SpiC*) and immune modulation (*SspH1*), suggesting the T3SS of *E. ictaluri* provides a similar benefit of modulating trafficking of vesicles.

Many SPI-2 effector proteins are encoded outside of the pathogenicity island. Both plasmids of *E. ictaluri* contain a putative T3SS gene (Fernandez et al. 2001). *pEI1* encodes a gene with a predicted amino acid sequence similar to T3SS secreted proteins *SspH1* and *SspH2* of *Salmonella*, *YopM* of *Yersinia*, and *IpaH* of *Shigella*. The similarities to *YopM* and *IpaH* primarily are in a region encoding leucine rich repeats. However, both the amino and carboxy

termini of SspH1 and SspH2 have homology to the pEI1-encoded protein. pEI2 encodes a small protein with minor homology to chaperone proteins of organisms containing T3SS. Generally, a chaperone protein is a small protein with an acidic isoelectric point (pI) (Page and Parsot 2002). The molecular weight (MW) of the predicted pEI2 protein is 16.5 kDa, and its theoretical pI is 4.78 as predicted by ProtParam (Gasteiger et al. 2005). This dissertation also describes the finding of a potential effector gene three base pairs downstream of the pEI2 chaperone sequence, possibly transcriptionally linked to the chaperone. Furthermore, analysis of the genome reveals at least four other sequences with homology to T3SS secreted effectors of *Salmonella* and *Shigella*. Further analysis of the genome may identify more genes involved in T3SS translocation, which will lead to a better understanding the factors involved in pathogenesis of *E. ictaluri*.

The *E. ictaluri* T3SS pathogenicity island encodes proteins with amino acid sequences similar to SsrA and SsrB of *Salmonella* and EsrA and EsrB of *E. tarda*. SsrA and SsrB are required for pathogenesis of *Salmonella*, and EsrA and EsrB are required for *E. tarda* pathogenesis. Like *E. tarda*, *E. ictaluri* encodes a third regulatory gene, *esrC*. EsrC has homology to AraC-type transcriptional activators, but its function in *E. ictaluri* is unknown. EsrC in *E. tarda* is necessary for virulence, and involved in the regulation of T3SS and non-T3SS virulence factors (Zheng et al. 2005).

Analysis of *E. ictaluri* T3SS gene expression will provide valuable insight into the pathogenic mechanisms of this organism. Type III secretion systems are found throughout Gram-negative bacteria, both in plant and animal pathogens, and in non-pathogenic bacteria as well. The *E. ictaluri* T3SS not only has homology to SPI-2 of *Salmonella*, a pathogen of higher vertebrates, but also to *Ralstonia solaraceum*, a plant pathogen, and to *Chromobacterium violaceum*, an environmental opportunistic human pathogen (Thune et al. 2007). The study of these and other T3SS may result in treatments applicable to a wide variety of T3SS-encoding

organisms. T3SS activity is affected negatively by a variety of chemicals (Muschiol et al. 2006; Hudson et al. 2007; Negrea et al. 2007; Pan et al. 2007). Furthermore, the T3SS has been used as an experimental delivery system for potential vaccines by fusing the translocation signal of T3SS effector proteins to immunogenic proteins of pathogens (Husseiny and Hensel 2005; Panthel et al. 2005; Wilson and Nickerson 2006; Husseiny et al. 2007). The study of the *E. ictaluri* T3SS may also provide insight into the spread and distribution of this SPI-2 type of T3SS among Gram-negative bacteria.

TYPE VI SECRETION SYSTEMS

Edwardsiella ictaluri encodes a T6SS homologous to the *E. tarda* T6SS (Figure 1.4) necessary for virulence (Zheng and Leung 2007). T6SS were recently reviewed by Filloux et al. (Filloux et al. 2008), Bingle et al. (Bingle et al. 2008), and Pukatzki et al. (Pukatzki et al. 2009). A number of plant and animal pathogens including *Salmonella*, *Vibrio*, *Francisella*, *Pseudomonas*, and others encode T6SS. Many T6SS have a role in virulence, particularly with intracellular survival. Interestingly, the *E. tarda* and the *Salmonella* T6SS are both regulated by T3SS-encoded regulatory genes. The *E. tarda* T6SS is positively regulated by EsrC, which is under EsrB control (Zheng et al. 2005; Zheng and Leung 2007), and the *Salmonella* T6SS protein SciS is under negative control by SsrB (Parsons and Heffron 2005). Furthermore, Parsons and Heffron (Parsons and Heffron 2005) report SciS is involved in limiting *Salmonella* replication within macrophages, in opposition of SPI-2 T3SS effects. The limited replication results the ability of *Salmonella* to persist longer intracellularly. Mutation of *sciS* results in hyper-replication intracellularly and an attenuated phenotype because of the unregulated intracellular expression. The T6SS of *E. tarda* is required for virulence *in vivo*, but whether it is involved in intracellular replication is not known.

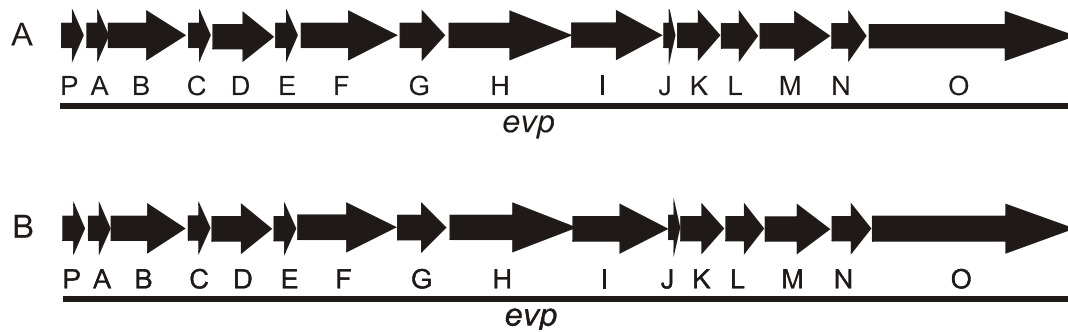


Figure 1.4. Schematic representation of the *Edwardsiella ictaluri* and *E. tarda* type VI secretion system (T6SS) genetic loci. The open reading frames of the T6SS of *E. ictaluri* (A) and *E. tarda* (B) are arranged in the same order between the two species, and the nucleotide sequences across the entire pathogenicity island are 88% identical.

OBJECTIVES AND HYPOTHESES

The goal of this dissertation research was to characterize expression of the *E. ictaluri* T3SS to determine its role in channel catfish pathogenesis. The objectives of this research were threefold: 1) characterize *in vitro* expression of the *E. ictaluri* T3SS; 2) characterize regulatory genes involved in T3SS gene expression *in vitro*; and 3) characterize the role of the *E. ictaluri* plasmids in pathogenesis. The hypotheses for each of the above objectives were 1) *E. ictaluri* T3SS gene expression is upregulated in conditions mimicking those of the intracellular environment of a macrophage, including nutrient limitation and acidic pH; 2) mutation of each T3SS regulatory gene will result in virulence defects both *ex vivo* and *in vivo*; and 3) mutations in T3SS-related genes carried on the plasmids of *E. ictaluri* will result in virulence defects *ex vivo* and *in vivo* due to disruption of the T3SS genes.

The second chapter demonstrates *E. ictaluri* T3SS expression in a number of culture conditions, with strong upregulation of the T3SS when *E. ictaluri* is exposed to acidic environments with low inorganic phosphate concentrations. Acidic pH alone upregulates the translocon operon, and translocon proteins are identified in the supernatants of pH 5.5 cultures. Expression of other T3SS genes in low phosphate, acidic media is confirmed using quantitative

real-time PCR (qPCR) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), with protein spot identification by peptide-mass-fingerprinting matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (PMF MALDI-TOF/TOF MS).

The third chapter describes the mutagenesis of *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and *esrC*. T3SS gene expression was evaluated in each mutant strain using qPCR to determine the effect of each mutation on gene expression. Each mutation causes a decrease in expression of T3SS genes assayed, but mutation of *esrB* has the greatest effect, and expression of *esrC* is dependent on EsrB. Interestingly, even though mutation of *esrC* results in decreased T3SS gene transcription, the mutant is able to replicate equally to the wild type in channel catfish head kidney-derived macrophages (HKDM) up to 10 hrs post-infection. Both *esrA* and *esrB* mutants are significantly attenuated for replication in HKDM. All three of the mutants, however, are attenuated in a channel catfish infection assay.

The fourth chapter describes the characterization of mutants created in the plasmids of *E. ictaluri* by signature tagged mutagenesis (STM) (Thune et al. 2007). One mutant strain carries a mutation in pEI1 at the 3' end of *eseH*. A second STM mutant carries a mutation in pEI2 about 100 nt upstream of *escD*. Upon further analysis, another gene is found three bases downstream of *escD* encoding a protein with homology to proteins from *Pseudovibrio*, *Shigella*, *Vibrio*, *Es. coli*, *Chromobacterium*, and *Salmonella*. Both of the STM plasmid mutants are severely attenuated in macrophages and in three channel catfish challenges evaluating catfish mortality, competition with wild type, and persistence in channel catfish head kidney. However, analysis of T3SS gene expression in the pEI1 and pEI2 mutants demonstrates they are severely deficient in expression of a majority of T3SS genes. Protein analyses corroborate the qPCR findings by showing the proteins EseB and EseD are missing in whole cell lysates of the pEI1 and pEI2 mutants grown in T3SS expression-inducing conditions. The cause of the severe downregulation

is unknown, but may be associated with integration of the plasmid into the genome near putative regulatory genes. Additionally, four putative T3SS effector genes, *eseJ*, *eseK*, *eseL*, and *eseM*, which have homology to *sspH1* and *sspH2* of *Salmonella*, are encoded in the genome. Analyses of the amino acid sequences suggest each is a T3SS translocated effector gene.

The final chapter describes the general conclusions of the studies. The *E. ictaluri* T3SS is upregulated in acidic, low-phosphate media, conditions that mimic the intracellular environment. The expression of the T3SS is dependent upon the T3SS regulatory genes *esrA*, *esrB*, and *esrC*. Mutation of these genes results in attenuation of virulence *in vivo*, and the *esrA* and *esrB* mutants are incapable of replication in HKDM. The *esrC* mutant, however, is able to replicate in channel catfish macrophages for up to 10 hrs post-infection. Finally, the plasmids carried by *E. ictaluri* have an integral, but unknown function in the virulence of *E. ictaluri*. Mutation of each of the plasmids results in complete attenuation *in vivo* and *ex vivo* and results in drastic downregulation of T3SS gene expression, indicating that the plasmids function in T3SS expression.

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CHAPTER 2
IN VITRO* EXPRESSION OF THE *EDWARDSIELLA ICTALURI
TYPE III SECRETION SYSTEM

INTRODUCTION

Edwardsiella ictaluri is a Gram-negative bacterium of the family Enterobacteriaceae and the etiological agent of enteric septicemia of catfish (ESC). *Edwardsiella ictaluri* was isolated, named, and characterized by Hawke et al. (Hawke 1979; Hawke et al. 1981). Enteric septicemia of catfish is the leading cause of disease-related mortality in channel catfish (*Ictalurus punctatus*) production facilities (Anonymous 2003a; Anonymous 2003b). External signs of acute infection include petechial hemorrhaging and lethargy. Internal signs include petechial hemorrhaging and necrotic foci on organs, as well as fluid accumulation (Jarboe et al. 1984; Shotts et al. 1986; Newton et al. 1989; Baldwin and Newton 1993). Chronic infection also occurs, characterized by lesions on the head, causing the formation of a mid-cranial hole. Fish in the chronic stage also exhibit disordered swimming and listlessness because of meningoencephalitis. Despite the enormous economic impact of this organism, little is known about the genetic determinants involved in the pathogenesis in channel catfish.

Type III secretion systems (T3SS) are ubiquitous in Gram-negative bacteria. Type III secretion systems translocate effector proteins from the cytoplasm of bacteria across the bacterial and host cell membranes to the cytoplasm of a host target cell. Many recent reviews are available describing structure, function, and evolution of T3SS (Macnab 2004; Saier 2004; Ehrbar and Hardt 2005; Jones 2005; Patel and Galan 2005; Sorg et al. 2005; Troisfontaines and Cornelis 2005; Abrahams and Hensel 2006; Galan and Wolf-Watz 2006; Schlumberger and Hardt 2006; Angot et al. 2007). Type III secretion system apparatus proteins generally are conserved; the effector proteins vary, however, resulting in the differing functions of T3SS among species. Functions of T3SS include induction of uptake into host cells (*Shigella* and *Salmonella*),

prevention of phagocytosis (*Yersinia*), induced attachment/effacement (*Escherichia*), and induction of intracellular replication (*Salmonella* and *E. tarda*).

A pathogenicity island encoding a T3SS was recently identified and sequenced in *E. ictaluri* (Thune et al. 2007). Mutation of the T3SS apparatus gene *esaU* results in attenuated virulence both in channel catfish macrophages and *in vivo*, indicating the T3SS is required for intracellular replication and virulence. The T3SS is arranged similarly to the *E. tarda* T3SS, which is involved in replication within fish macrophages (Tan et al. 2005; Okuda et al. 2006). The *E. ictaluri* and *E. tarda* T3SS are similar to the *Salmonella* pathogenicity island 2 (SPI-2) T3SS involved in intracellular replication.

An understanding of the conditions required for expression of the *E. ictaluri* T3SS will provide insight as to potential environments within the host that result in expression of the T3SS. Determining how and when the T3SS is expressed will provide valuable information to enable further studies concerning *in vivo* expression of the system and development of preventative measures. In this study, expression of the *E. ictaluri* T3SS was analyzed in a variety of *in vitro* growth conditions. Environmental conditions, particularly low pH and low phosphate, positively influence transcription of *E. ictaluri* T3SS genes. Furthermore, T3SS translocon proteins require acidic pH for secretion into the culture supernatant.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table 2.1. *Edwardsiella ictaluri* strains were grown in Luria Bertani broth (LB) supplemented with either fish peptone (LB-FP) or mannitol salts (LB-Man). For minimal media, *E. ictaluri* defined minimal media MM19 (Collins and Thune 1996) was used. *Edwardsiella ictaluri* was also grown on trypticase soy agar supplemented with 5% sheep blood (BA, Remel Products, Lenexa, KS).

Table 2.1. Bacterial strains and plasmids used in Chapter 2.

Bacterial Strains or Plasmid	Description	Source
<i>Edwardsiella ictaluri</i>		
93-146	Wild type <i>E. ictaluri</i> isolated from a moribund channel catfish from a natural outbreak at a commercial facility in 1993	LSU aquatic disease laboratory
65ST (<i>esaU</i> -)	Derived from parental wild type strain 93-146; carries an insertion of a signature-tagged mutagenesis tag S/T in the <i>esaU</i> gene	(Thune et al. 2007)
<i>eseH-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>eseH</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>esaB-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>esaB</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>esaM-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>esaM</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>esaR-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>esaR</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>escB-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>escB</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>escC-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>escC</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>esrA-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>esrA</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>esrC-gfp</i> +	93-146 carrying a <i>gfp</i> + construct under control of the <i>esrC</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
- <i>promgfp</i> +	93-146 carrying a promoterless <i>gfp</i> + construct between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>kmprom</i>	93-146 carrying a <i>gfp</i> + construct under control of the <i>km</i> promoter between <i>serC</i> and <i>aroA</i> Cm ^r	This work
<i>Escherichia coli</i>		
XL1 Blue MRF ⁺	(<i>mcrA</i>)183 (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proABlacIqZ.M15</i>	Stratagene, La Jolla, CA
SM10λ <i>pir</i>	Tn5(Km)] <i>thi1 thr1 leuB supE44 tonA21 lacY1 recA</i> ::RP4-2-Tc::Mu Km ^r λ::pir	(Simon et al. 1983)
Plasmids		
pBluescript SK-	Cloning vector	Stratagene, La Jolla, CA
pBS:: <i>serCaroA</i>	pBluescript carrying the <i>serC-aroA</i> region of <i>E. ictaluri</i> genomic DNA	This work
pBS:: <i>SΔTn</i>	pBS:: <i>serCaroA</i> with a cryptic transposase removed from between the <i>serC</i> and <i>aroA</i> regions and a <i>BstZ171</i> recognition site inserted	This work
pZep08	<i>gfp</i> + promoter trap vector Km ^r Ap ^r Cm ^r	(Hautefort et al. 2003)
pZep:: <i>eseH</i>	pZep08 carrying the promoter for the <i>E. ictaluri</i> type III secretion system (T3SS) pEII-encoded effector gene <i>eseH</i>	This work
pZep:: <i>esaB</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> (T3SS) apparatus operon (<i>esaBCDE</i>)	This work

Table 2.1 continued.

Bacterial Strains or Plasmid	Description	Source
pZep:: <i>esaM</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS apparatus operon <i>esaM</i> (<i>esaMVNOPQ</i>)	This work
pZep:: <i>esaR</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS apparatus operon <i>esaR</i> (<i>esaRSTU</i>)	This work
pZep:: <i>escB</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS chaperone and effector operon <i>escB</i> (<i>escB, eseG</i>)	This work
pZep:: <i>escC</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS translocon operone (<i>escAC, eseBCDE</i>)	This work
pZep:: <i>esrA</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS regulatory gene <i>esrA</i>	This work
pZep:: <i>esrC</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS regulatory gene <i>esrC</i> and apparatus operon (<i>esrC, esaGHIJWKL</i>)	This work
pZep:: <i>orf29/30</i>	pZep08 carrying the promoter region for the <i>E. ictaluri</i> T3SS associated gene <i>orf29/30</i>	This work
pZep:: <i>-promgfp+</i>	pZep08 carrying a promoterless <i>gfp+</i> construct	This work
pZep:: <i>kmprom</i>	pZep08 carrying the promoter for the Km resistance gene	This work
pBS:: <i>SΔTn::eseHgfp+</i>	pBS:: <i>SΔTn</i> with the <i>eseH</i> promoter fusion to <i>gfp+</i>	This work
pGP704	R6K <i>ori mob</i> Amp ^r ; suicide vector used for allelic exchange	(Miller and Mekalanos 1988)
pGP:: <i>SΔTn::eseHgfp+</i>	pGP704 carrying the the <i>SΔTn::eseHgfp+</i> construct	This work
pGP:: <i>SΔTn::esaBgfp+</i>	pGP704 carrying the the <i>SΔTn::esaBgfp+</i> construct	This work
pGP:: <i>SΔTn::esaMgfp+</i>	pGP704 carrying the the <i>SΔTn::esaMgfp+</i> construct	This work
pGP:: <i>SΔTn::esaRgfp+</i>	pGP704 carrying the the <i>SΔTn::esaRgfp+</i> construct	This work
pGP:: <i>SΔTn::escBgfp+</i>	pGP704 carrying the the <i>SΔTn::escBgfp+</i> construct	This work
pGP:: <i>SΔTn::escCgfp+</i>	pGP704 carrying the the <i>SΔTn::escCgfp+</i> construct	This work
pGP:: <i>SΔTn::esrAgfp+</i>	pGP704 carrying the the <i>SΔTn::esrAgfp+</i> construct	This work
pGP:: <i>SΔTn::esrCgfp+</i>	pGP704 carrying the the <i>SΔTn::esrCgfp+</i> construct	This work
pGP:: <i>SΔTn::orf29/30gfp+</i>	pGP704 carrying the the <i>SΔTn::orf29/30gfp+</i> construct	This work
pGP:: <i>SΔTn::-promgfp+</i>	pGP704 carrying the the <i>SΔTn::-promgfp+</i> construct	This work
pGP:: <i>SΔTn::kmprom</i>	pGP704 carrying the the <i>SΔTn::kmprom</i> construct	This work

Low phosphate MM19 (MM19-P) was formulated similar to MM19, except salts containing phosphate are removed. In order to maintain similar levels of sodium and potassium in the media, an additional 2.5 g sodium chloride (NaCl) and 4.0 g potassium chloride (KCl) were added. Phosphate provides the buffering capacity in MM19, and removal of the phosphate results in pH instability of the media, so 2-(N-Morpholino)ethanesulfonic acid (MES) and 3-(N-Morpholino)propanesulfonic acid (MOPS) were used to buffer pH 5.5 and 7.0 low phosphate media, respectively. Each buffer was added to a final concentration of 80 mM. Minimal phosphate required for growth was maintained by adding 0.5 ml/L of a solution of 125 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 125 mM KH_2PO_4 , resulting in 0.125 mM phosphate in MM19-P.

Edwardsiella ictaluri cultures were grown at 28° C with aeration unless otherwise noted. Antibiotics were added as appropriate in the following concentrations: ampicillin 200 µg/ml (Ap), chloramphenicol 12.5 µg/ml (Cm), colistin 10 µg/ml (Col), and kanamycin 50 µg/ml (Km). *Escherichia coli* strains were cultured in LB broth with appropriate antibiotics at 37° C with aeration.

DNA Manipulation. Genomic DNA was isolated using a protocol previously described by Ausubel et al. (Ausubel et al. 1994). Briefly, cells were collected by centrifugation at 3700 x g for 5 minutes and resuspended in TE. Cells were lysed with 0.5% SDS in the presence of 50 µg/ml RNase A and 100 µg/ml proteinase K. Protein was extracted by using phenol:chloroform:isoamyl alcohol (25:24:1) with a chloroform wash. DNA was ethanol precipitated in the presence of 0.12 M sodium acetate, collected by centrifugation at 3700 x g, and resuspended in water. Plasmid DNA was isolated using the Qiagen Miniprep Kit (Qiagen Inc., Valencia, CA.). Restriction digests were done using enzymes purchased from New England Biolabs (Ipswich, MA), and DNA was purified from restriction digests using the Qiaquick Kit or Minelute Kit (Qiagen).

DNA Sequence Analysis. Percent identity and percent similarity across homologous sequences were determined using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997). Gene and operon predictions were done by using FGENESB to determine putative open reading frames and operons and BPROM to determine putative promoter regions for genes (SoftBerry, Inc., Mount Kisco, NY). In addition, open reading frames were verified using ORF finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>).

T3SS Promoter Fusion to *gfp*⁺. In order to evaluate expression of the T3SS effector protein EseH, the *eseH* promoter (P_{eseH}) was fused to green fluorescent protein (GFP) and inserted into the *E. ictaluri* genome between *serC* and *aroA*. pBS::*serCaroA* was constructed by amplifying a portion of the *E. ictaluri* genome containing *serC*, *aroA*, and the non-coding region between *serC* and *aroA* using primers *serCspeI* and *aroAapaI* (Table 2.2). The resulting PCR product was digested with *SpeI* and *ApaI* (New England Biolabs) following manufacturer's protocol and ligated into the *SpeI* and *ApaI* site of pBluescript resulting in pBS::*serCaroA* (Figure 2.1A).

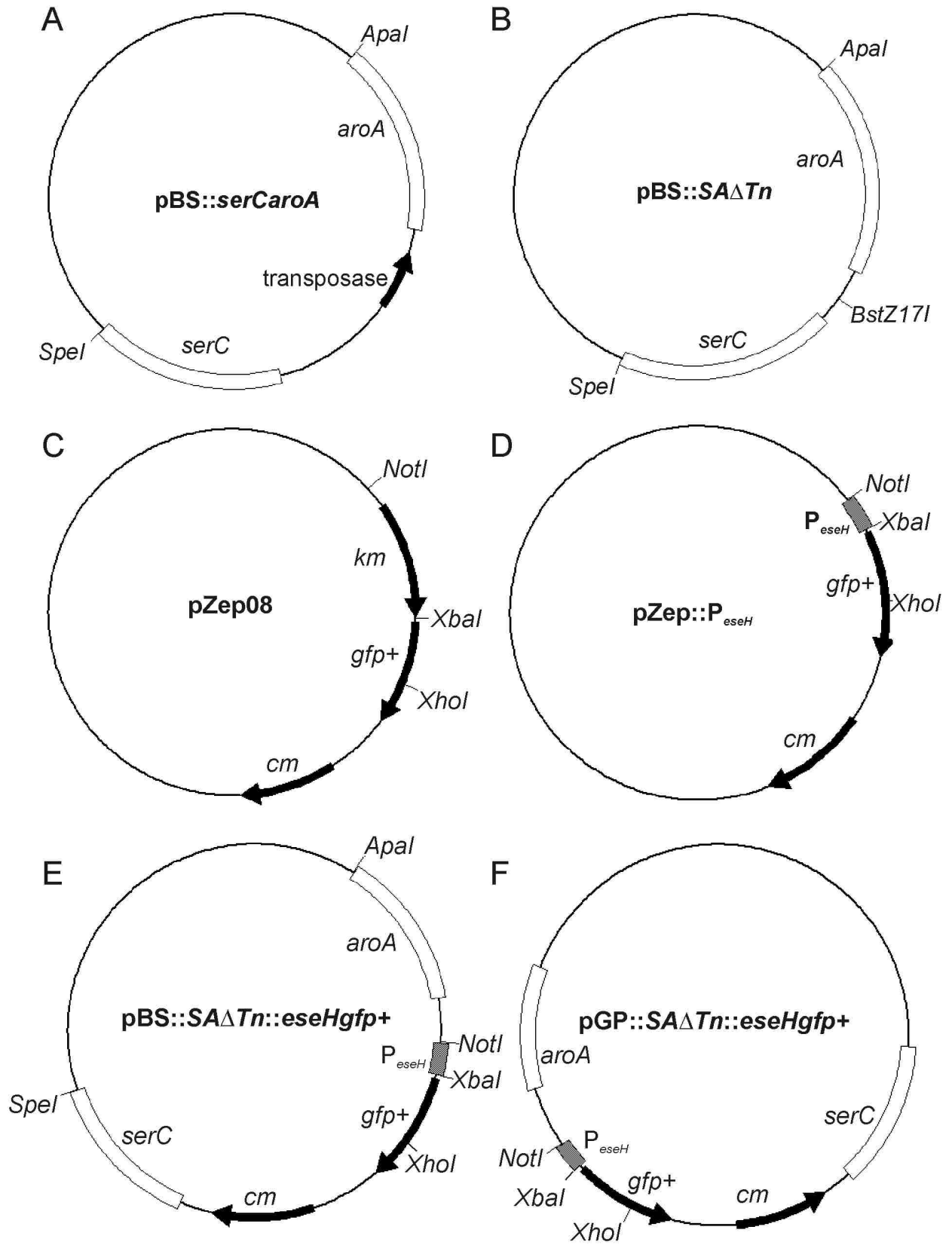
Following construction of pBS::*serCaroA*, inverse PCR was done using primers *serCBstZ17I* and *aroASallI* (Table 2.2). The product of this PCR amplification removed a transposase region, which may have complicated allelic exchange because of multiple homologous regions elsewhere in the genome. The inverse PCR product was gel purified using Qiaquick Kit (Qiagen), and its 3' ends were phosphorylated using polynucleotide kinase (PNK, New England Biolabs) following the manufacturer's protocols. PNK-treated DNA was purified using Qiaquick Kit (Qiagen), and the DNA was self-ligated using T4 DNA Ligase (New England Biolabs) following manufacturer's instructions. The resultant plasmid had 843 nt removed from the *serC/aroA* intergenic region of pBS::*serCaroA* and a *BstZ17I* site inserted into the *serCaroA* intergenic sequence (Figure 2.1B). This plasmid was called pBS::*SΔTn*.

The *eseH* promoter region was amplified using primers containing 5' *NotI* and 3' *XbaI* sites engineered into the primers (Table 2.2). This PCR product was digested with *NotI* and *XbaI* (New England Biolabs), and ligated into the *NotI* and *XbaI* region of pZep08 (Figure 2.1C) (Hautefort et al. 2003), placing it upstream of a promoterless GFP variant *gfp+* and removing the Km cassette. The resultant plasmid was electroporated into *Es. coli* XL1 Blue MRF', and cells were selected for Cm and Ap resistance and Km sensitivity. The resultant plasmid was called pZep::P_{*eseH*} (Figure 2.1D).

Table 2.2. Oligonucleotides used for construction of *Edwardsiella ictaluri* type III secretion system gene promoter fusions to *gfp+*. Underlined portions indicate *NotI* recognition sites added to the primer, incorporating the site into the 5' end of the PCR product. Italicized portions indicate *XbaI* recognition sites added to the primer, incorporating the site into the 3' end of the PCR product.

	Forward primer sequence	Reverse primer sequence
serCspeI	ATTC <u>ACTAGTCGGTAATGGAGATCAGCC</u>	-
aroAapaI	ATATAGGGCCCGCCCTTTGGCAAACAGCG	-
serCBstZ17I	ATAATAGTATACTTCCCACGATGGCCTCTC	-
aroAsaII	ATAATAGTCGACTGTAGAGAAGGCGCTGGT	-
pZep08	TGGGGTAATGACTCTCTAGC	CGTCATTTCTGCCATTCATCC
serCaroA	CGGTAATGGAGATCAGCC	GCCCTTTGGCAAACAGCG
<i>eseH</i>	TTTAAG <u>CGGCCGC</u> ATTACATCTAAGAAA	TTTCACTCTAGATGTGAAATTTTCCATT
<i>escC</i>	TTTAAG <u>CGGCCGC</u> ACTGGAAACGTCGCTC	TTTCACTCTAGAAGGGAAACCTCCTATTGACG
<i>escB</i>	ATTATT <u>GCGGCCGCTGATCGCTCTTATGGGG</u>	ATATATTCTAGAGGGGATCTCCAAAGAGTC
<i>esaB</i>	ATTATT <u>GCGGCCGC</u> ACTCGGCACGGCCATG	TATATATCTAGAGACATTAGGGTAAAGCGA
<i>esaM</i>	ATTATT <u>GCGGCCGC</u> CAGCCCGTAAAGCGCAAG	ATATATTCTAGAGAATATTCTCCGCGATCGT
<i>esaR</i>	TTTAAG <u>CGGCCGC</u> CTCGGGCTGCAATAGCT	TTTCACTCTAGACAGGTATTCACCCTATGCC
<i>esrA</i>	ATATAT <u>GCGGCCGCAACCGCGTGATAATCTG</u>	ATTATTTCTAGAAGGGAACCTCTTATGTG
<i>esrC</i>	ATTAAT <u>GCGGCCGC</u> TCAGGTGATGACCCGATA	ATATATTCTAGAGGTGATGGGCAACGGTTGA
<i>orf29/30</i>	ATTATT <u>GCGGCCGC</u> CAGGTTTCATGATGTCTGTG	ATATATTCTAGAGAAGAGACCTCCCAATCG

Figure 2.1 (Following page). Schematic maps of plasmids used in chapter 2. pBS::*serCaroA* (A) was constructed by amplifying the genomic region of *Edwardsiella ictaluri* containing *serC* and *aroA* and inserting it into the *speI* and *apaI* sites of pBluescript. pBS::*SAΔTn* (B) was generated by inverse PCR of pBS::*serCaroA*, removing the transposase region between *serC* and *aroA*. The restriction site *BstZ17I* was engineered into the PCR primer for a cloning site between *serC* and *aroA*. pZep08 (C) is a promoter trap vector used for fusion of promoter regions of interest to *gfp+* (Hautefort et al. 2003). Insertion of the promoter region is accomplished by digestion of pZep08 with *NotI* and *XbaI*, removing the kanamycin (Km) cassette and replacing it with a promoter of interest. The promoter region of *eseH* was amplified by PCR and inserted into pZep08 at the *NotI* and *XbaI* sites placing it upstream of a promoterless *gfp+* generating pZep::P_{*eseH*} (D). Expression of *gfp+* is dependent upon the promoter activity of the DNA region inserted. The *eseH* promoter fusion to *gfp+* in pZep::P_{*eseH*} was amplified and inserted into the *BstZ17I* site of pBS::*SAΔTn* generating pBS::*SAΔTn*::*eseHgfp+* (E). The region of pBS::*SAΔTn*::*eseHgfp+* containing *serCaroA*::*eseHgfp+* was removed by digestion with *ApaI* and *SpeI*, then blunt ended and inserted into the *EcoRV* site of pGP704 generating pGP::*SAΔTn*::*eseHgfp+* (F). This plasmid was conjugated to *E. ictaluri*, resulting in allelic exchange at the native *serC/aroA* region. Recombination maintained the sequences of *serC* and *aroA*, but removed the intergenic transposon sequence and added *eseH-gfp+* fusion and chloramphenicol (Cm) resistance into the region, as determined by DNA sequencing of the region following allelic exchange.



The promoter-*gfp*⁺ fusion was amplified, including the Cm cassette, using pZep08 forward and reverse primers (Table 2.2). The resultant blunt-end PCR product was purified using Minelute kit (Qiagen), DNA ends were phosphorylated using PNK (New England Biolabs), and the product was ligated into the *Bst*Z17I site of pBS::*SΔTn*. The resultant plasmid was electroporated into *Es. coli* XL1 Blue MRF⁺ and selected for Cm and Ap resistance. Correct insertion was verified using differential PCR with pZep08 and *serCaroA* primers (Table 2.2) and differential restriction digest using *NotI* and *XbaI* (New England Biolabs). The resultant plasmid was called pBS::*SΔTn*::*eseHgfp*⁺ (Figure 2.1E).

The promoter fusion, Cm cassette, and flanking *serC* and *aroA* regions were excised using *ApaI* and *SpeI* (New England Biolabs), and the ends were blunt ended using the Klenow fragment (New England Biolabs). The resultant DNA was PNK treated and ligated into the *EcoRV* site of pGP704 using T4 DNA Ligase (New England Biolabs). The resultant plasmid was electroporated into *Es. coli* SM10 λ *pir* cells. Resultant colonies were selected for Cm and Ap resistance. Correct insertion was verified by differential PCR using pZep08 and *serCaroA* primers, digestion using either *NotI*, *XbaI*, or *HindIII* (New England Biolabs), and DNA sequencing. The resulting plasmid was called pGP::*SΔTn*::*eseHgfp*⁺ (Figure 2.1F).

Insertion of the *eseHgfp*⁺ sequence into the *E. ictaluri* genome between *serC* and *aroA* was accomplished using allelic exchange. *Es. coli* SM10 λ *pir* carrying pGP::*SΔTn*::*eseHgfp*⁺ was used as a donor strain to transfer the plasmid to *E. ictaluri* by conjugation. *E. ictaluri* and *Es. coli* were mixed at a 2:1 ratio (CFU *E. ictaluri* : CFU *Es. coli*) and filtered through a GN-6 25 mm 0.45 μ m Metrical Membrane Filter (Pall Corp., Ann Arbor, MI). Filters with cells were placed cell-side up on BA and incubated for 24 hrs. Following incubation, cells were resuspended in 3 ml media by vortexing vigorously. Cultures were plated on LB-FP

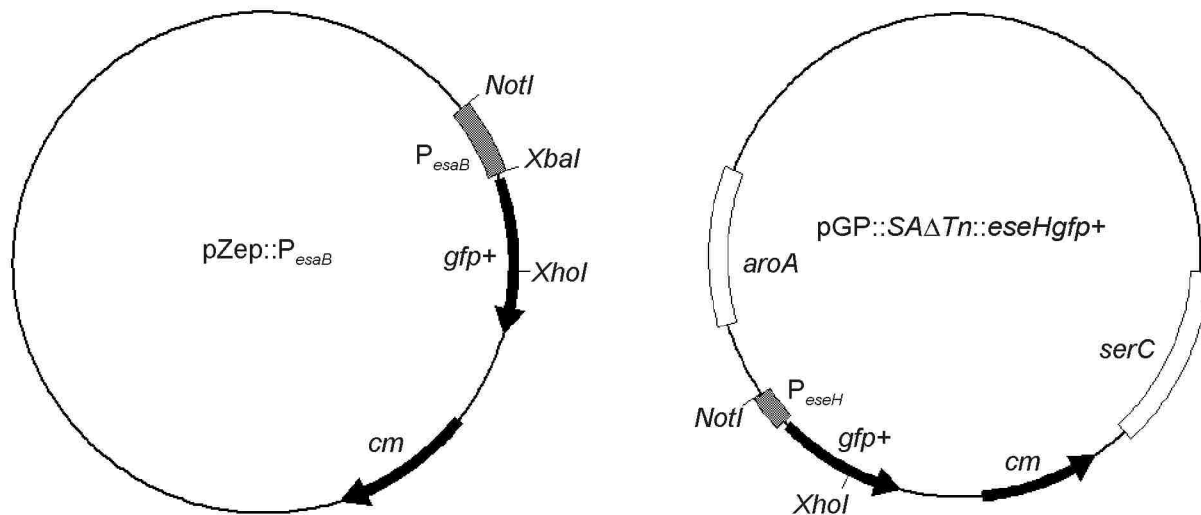
supplemented with Cm and Col. Resultant colonies were replicate plated onto LB-FP with Cm and Col and LB-FP with Cm and Ap. Correct insertion of the *eseHgfp*⁺ construct between *serC* and *aroA* was verified by PCR followed by DNA sequencing of PCR products.

In order to detect expression of other T3SS promoters, the promoter regions of *esaB*, *esaM*, *esaR*, *escB*, *escC*, *esrA*, *esrC*, and *orf29/30* were amplified by PCR using specific primers (Table 2.2) incorporating *NotI* and *XbaI* restriction sites in the 5' and 3' ends, respectively. *esaB*, *esaM*, *esaR*, *escB*, *escC*, and *esrC* are the first genes of putative operons within the island, and each has a predicted promoter region upstream of their respective translational start sites. *esrA* is encoded downstream of *slt*, possibly within the *esaR* operon; however, there is a predicted promoter sequence upstream of *esrA*. *orf29/30* appears to be a single gene located near the 5' end of *esrB* at the outer edge of the pathogenicity island with a promoter region upstream of the predicted translational start site. PCR products were gel purified (Qiagen) and digested with *NotI* and *XbaI* (New England Biolabs). Digested PCR products were purified and ligated to the *NotI* and *XbaI* site of pZep08, similar to the insertion of the *eseH* promoter described above. This resulted in the insertion of each target promoter region upstream of *gfp*⁺.

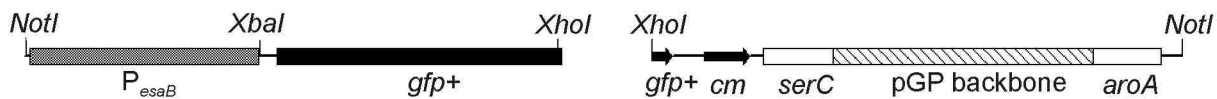
As a positive control for fluorescence, the Km promoter and gene upstream of *gfp*⁺ in pZep08 were left intact, resulting in a transcriptional linkage between Km and *gfp*⁺, allowing expression of *gfp*⁺ from the Km promoter. For a negative control, pZep08 was digested with *eagI*, removing the first 35 nt of *km* and 155 nt upstream of the *km* start. The remaining DNA was ligated at the *eagI* ends, resulting in a promoterless construct.

Resultant pZep plasmids containing T3SS promoter regions fused to *gfp*⁺ and the control constructs were digested with *NotI* and *XhoI* to excise the promoter region and a partial sequence of *gfp*⁺ (Figure 2.2). The excised DNA was inserted into the *NotI/XhoI* site region of pGP::*SAΔTn::eseHgfp*⁺, replacing the *eseH* promoter region with the new promoter region and

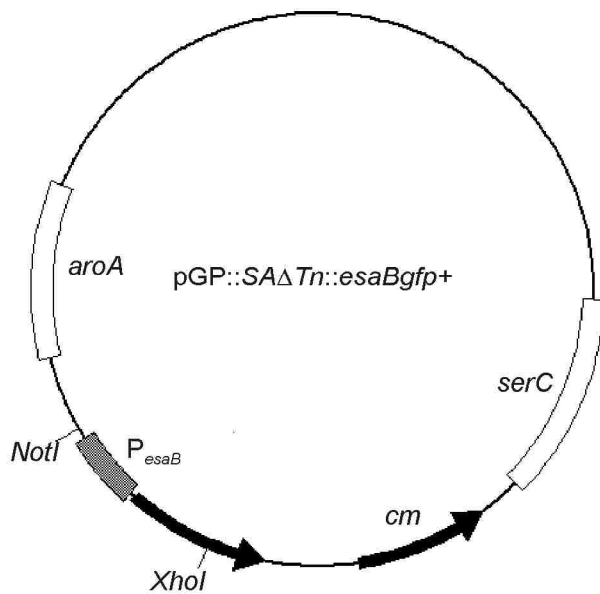
Figure 2.2 (Following page). Construction of *Edwardsiella ictaluri* type III secretion system (T3SS) gene promoter fusions to *gfp*⁺. The *esaB* promoter region was inserted into pZep08 upstream of *gfp*⁺. Resulting plasmids were digested with *NotI* and *XhoI*. The fragment containing the promoter and partial *gfp*⁺ region was inserted into the *NotI/XhoI* site of pGP::*SAΔTn::eseH**gfp*⁺ replacing the *eseH* promoter with the desired *esaB* promoter. This procedure was repeated for all additional T3SS promoter fusions.



Digest each plasmid with *NotI* and *XhoI*



Ligate fragments



generating pGP::*SAΔTn::esaBgfp*⁺. This allowed for rapid construction of promoter fusions in the pGP704 backbone. PCR and DNA sequencing was used to verify the correct sequence of each construct. *Es. coli* strains carrying pGP::*SAΔTn* variants with T3SS promoter fusions to *gfp*⁺ were used as donors for conjugation to wild type (WT) *E. ictaluri* for transfer of the plasmid. Following conjugation, allelic exchange resulted in insertion of the fusions into the genome between *serC* and *aroA*, which was verified by using PCR and DNA sequencing.

Measurement of GFP Activity from Promoter Fusion Strains. Cultures grown to late log phase were pelleted and resuspended in phosphate buffered saline adjusted to the same pH as the culture. Cultures were concentrated to an OD of about 1 – 1.1 units for 200 μl of culture.

Fluorescence (excitation: 491 nm; emission: 512 nm) and OD₆₀₀ of each sample were measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Fluorescence readings were divided by OD to normalize fluorescence readings within each treatment.

RNA Isolation. Total RNA was isolated using Bacteria RNAprotect RNeasy Mini Kit (Qiagen) and subjected to DNase treatment with Baseline Zero DNase (Epicentre Biotechnologies, Madison, WI) to remove contaminating DNA. Following DNase treatment, RNA samples were diluted to 20 ng/μl for RT-PCR and 10 ng/μl for quantitative PCR.

RT-PCR. Reverse transcriptase PCR (RT-PCR) was conducted using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene, La Jolla, CA) using gene specific oligonucleotides to prime the reverse transcriptase at the 3' end of the desired transcripts (Table 2.3). RT-PCR reactions were incubated 1 hr at 50° C, followed by incubation at 70° C to inactivate the reverse transcriptase. Resulting cDNA was used as template for PCR using gene specific primers (Table 2.3) and Phusion High Fidelity DNA Polymerase (New England Biolabs). Absence of contaminating DNA in RNA samples was verified by the absence of PCR products using the DNase-treated RNA as template. PCR was conducted under the following

conditions: denaturation at 98° C for 30 sec, 35 cycles of 98° C for 10 sec, 56° C for 30 sec, and 72° C for 30 sec, and a final extension period of 72° C for 7 min.

For determination of transcriptional linkages, RT-PCR was conducted as described above. To determine linkage of *escD* and *eseI*, a specific primer for *eseI* was used to prime the reverse transcriptase for cDNA amplification. Polymerase chain reactions were done using the *eseI* cDNA as template using primers specific to both *eseI* and *escD* (Table 2.3). Reactions were done using Phusion High Fidelity DNA Polymerase and cycling conditions described above.

Quantitative Real Time PCR. High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) was used to generate cDNA from total RNA. Reaction conditions were 37° C for 60 min to amplify cDNA and 95° C for 5 minutes to inactivate the reverse transcriptase. The resulting cDNA was used as template for relative quantitative real-time PCR (qPCR) reactions using the Power SYBR Green PCR Master Mix (Applied Biosystems) and gene specific primers (Table 2.4). Reaction conditions were 95° C for 10 min, 40 cycles of 95° C for 15 sec followed by 60° C for 1 min. Following amplification, a dissociation curve was run for each sample at 95° C for 15 sec, 60° C for 1 min, slowly to 95° C to measure fluorescence at each step, and 60° C for 15 sec, which ensured amplification of only the single target amplicon. When a target amplicon contained within an operon was amplified, it is assumed that the remaining genes in the operone are also present. Reactions were run using cDNA amplification reactions lacking reverse transcriptase to ensure amplification did not occur from contaminant DNA.

Data were collected using an Applied Biosystems 7500 Fast Real Time PCR System using Sequence Detection Software v1.4 (Applied Biosystems). Relative quantitation was conducted using 16s rRNA as the endogenous gene. Expression values were determined for each condition by comparing target gene ΔC_t values to those of MM19 pH 7.0 cultures (calibrator) using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008).

Table 2.3. Oligonucleotide sequences used for reverse transcriptase PCR (RT-PCR). Reverse transcriptase PCR primers were used for cDNA synthesis from total RNA. Amplification of cDNA was done using 5' and 3' primers.

Gene	RT-PCR primer	5' primer	3' primer
<i>esaM</i>	TTCTCCATCACGCGATCG	GCGGCAAACCGAACTTTG	CCGCTGAAATCCACAGTG
<i>esaR</i>	TGAACGGCATGGAGATCG	CCGGTTTTCCATACAGGT	GATGGCGATAAAGGGCAA
<i>escC</i>	AAAGTCATGCCCCGATAGC	ATGAGCCAACACGTCACT	CTTCAGGGTGGCGTTAAG
<i>escD</i>	CTGTTTCGGTAGCGTGAC	CCCTACAAGAAAACCTTCCTC	GCGTCGTAATAAGTAGTCCA
<i>eseH</i>	GGTATTCACGCTCATAATCC	ACACCCGGCTGGATGTCT	CCTGTCCTCACAGCTTGA
<i>eseI</i>	TCTGGCAAACGACGTAGATT	GCCACACTGGAGCAAATG	CTCGTTGCTTAATGTCTGGG
<i>esrA</i>	TGCTCGCTCTTATTGGCG	GGCATGACGGTAAACCTG	GCGATTATAGACCTGCGC
<i>esrB</i>	CTTGCGCATCAGGTTGAG	GAAGACCATGCCTTGCTG	CGATATCCCGGTTGCGAT
<i>esrC</i>	CTTGCGCACTGACTGAAG	GGATGCCGACGATGAAAC	GCGTTACAGAATCCGGAC
<i>orf29/30</i>	TTCTCGGTCTCCGTCGTC	GCTGTATCTTTGTGCCCG	GTATGTCCCAGCCTATCC

Table 2.4. Oligonucleotide sequences used for quantitative PCR in Chapter 2.

Gene	5' Primer	3' Primer
<i>escB</i>	CTTTACCTTGCGATTTGCCTGCGT	AACAGGCACTCCGCCATATGAAAC
<i>escC</i>	AATGCAAGACCTACAGCAGCGTCA	GCGTTGCGATCTCTTGCTGTAACG
<i>eseH</i>	AAGAGGCTGGATGTCTCTGGTACT	GGTAGCCTTGGCATAGGAGTGTTA
<i>eseI</i>	GCAACCCTGCTTAGCAAGTGGAAA	TCAAAGCCTTCCTGGGCCTAATGGA
<i>esrA</i>	AGAGCGAGCATCTGAACAGCATCA	AGTAAGTCATGCTGCTCCTGCGTA
<i>esrB</i>	CAATGCAGCATGCATCACTGGGAA	TCAGCGATATCCCGGTTGCGATTA
<i>esrC</i>	AAAGTTTGGGATGGCGCCGAA	GAGAAATGGGCGGCGTTACAGAAT
16s	AACGCGAAGAACCTTACCTGGTCT	GCTCGTTGCGGGAATTAACCCAA

Whole Cell Lysate and Supernatant Protein Preparation. Whole cell lysate proteins were prepared similar to Moore and Thune (Moore and Thune 1999). Briefly, cell cultures were pelleted by centrifugation at 3,000 x g for 15 min at 4° C. Supernatant was set aside for extracellular protein isolation (described below), and cells were washed three times with phosphate buffered saline (PBS) pH 7.0 or pH 5.5, depending on the pH of the bacterial culture media. Pellet volume was estimated, and cells were resuspended at a rate of 1 ml of sterile distilled water per 100 µl of cell pellet. Phenylmethylsulphonyl fluoride (PMSF) was added to a final concentration of 1 mM, and the suspension was incubated on ice for 10 min to inhibit protease activity. Cells were dismembranated by sonication at 45% amplitude using a Fisher 500 sonic dismembrator (Fisher Scientific, Pittsburgh, PA). Sonicated suspensions were incubated at 4° C for 1 hr, then centrifuged at 12,000 x g at 4° C for 30 min to remove cellular debris. The supernatant containing the whole cell lysate proteins was collected, and 10% thimerosal was added to a final concentration of 0.01% (Moore and Thune 1999).

Supernatants from above were filtered through a 0.22 µm cellulose acetate low protein-binding filter (Corning Inc., Corning, NY). Trichloroacetic acid (TCA, Ricca Chemical Co., Arlington, TX) was added to a final concentration of 10%, and samples were incubated at 4° C for 16 hrs to precipitate extracellular proteins. Precipitates were pelleted by centrifugation at 24,000 x g for 30 min at 4° C. Pellets were air dried 10 min, resuspended in 1 ml sterile water, and stored at -80° C in 100 µl aliquots. Protein concentrations for both the whole cell lysate and extracellular proteins were estimated using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

2D-PAGE Analysis. Protein samples were purified by using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories) and resuspended in the appropriate volume of rehydration buffer to achieve a concentration of 100 µg of protein per 185 µl of rehydration buffer for whole cell

lysate preps. Because of varying concentrations of protein in supernatants due to differences in protein secretion in acidic and neutral pH, extracellular protein samples (100 μ l) were each cleaned and resuspended in 185 μ l rehydration buffer. Each extracellular protein sample was the equivalent of the amount of protein precipitated from 20 ml of culture. Immobilized pH gradient (IPG) strips (11 cm, pH 4-7) (Bio-Rad Laboratories) were rehydrated using 185 μ l of protein sample in rehydration buffer. Strips were allowed to rehydrate for 24 hrs at room temperature. Isoelectric focusing was done by using a Bio-Rad Protean IEF Cell (Bio-Rad Laboratories). Focusing conditions were maintained by the following program at 20° C: rapid ramping to 250 V for 15 min; slow ramping to 2000 V for 1 hr; hold at 2000 V for 3 hrs; linear step to 5000 V for 1 hr; hold at 5000 V for 3 hrs; rapid ramping to 8000 V for 1 hr; 8000V for 40,000 Vhrs.

Focused IPG strips were equilibrated using ReadyPrep 2-D Starter Kit Equilibration Buffers (Bio-Rad Laboratories), loaded onto a Criterion precast 12.5% gel (Bio-Rad Laboratories), and separated for 55 min at 200 V. Gels were removed and fixed with a solution of 10% methanol and 7% acetic acid for 1 hr. Gels were stained with Sypro Ruby stain (Bio-Rad Laboratories) for at least 3 hrs. Following staining, gels were washed in fixative for 1 hr and rinsed three times with distilled water. Gels were imaged under UV light using a Bio-Rad Gel Doc XR and Quantity One software (Bio-Rad Laboratories). Each protein sample was isolated and analyzed by 2D-PAGE in triplicate to ensure reproducibility.

Protein Digestion and Mass Spectrometry. Nevada Proteomics Center, University of Nevada, Reno analyzed selected proteins by trypsin digestion and MALDI-TOF/TOF analysis. Spots were digested using a previously described protocol with some modifications (Rosenfeld et al. 1992). Samples were washed twice with 25 mM ammonium bicarbonate (ABC) and 100% acetonitrile, reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide and incubated with 75 ng sequencing grade modified porcine trypsin (Promega Corp., Madison, WI)

in 25 mM ABC for 6 hours at 37° C. Samples were spotted onto a MALDI target with ZipTip μ -C18 (Millipore Corp., Billerica, MA). Samples were eluted with 70% acetonitrile, 0.2% formic acid and overlaid with 0.5 μ l 5 mg/ml MALDI matrix (α -Cyano-4 hydroxycinnamic acid 10 mM ammonium phosphate). All mass spectrometric data were collected using an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems), using their 4000 Series Explorer software v. 3.6. The peptide masses were acquired in reflectron positive mode (1-keV accelerating voltage) from a mass range of 700 – 4000 Daltons, 1250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks 842.51 and 2211.10 to within 20 ppm. Any sample failing to internally calibrate was analyzed under default plate calibration conditions of 150 ppm. Raw spectrum filtering/peak detection settings were S/N threshold of 3, and cluster area S/N optimization enabled at S/N threshold 10, baseline subtraction enabled at peak width 50. The twelve most intense ions from the MS analysis, which were not on the exclusion list, were subjected to MS/MS. The MS/MS exclusion list included known trypsin masses: 842.51, 870.54, 1045.56, 1126.56, 1420.72, 1531.84, 1940.94, 2003.07, 2211.10, 2225.12, 2239.14, 2283.18, 2299.18, 2678.38, 2807.31, 2914.51, 3094.62, 3337.76, 3353.75. For MS/MS analysis the mass range was 70 to precursor ion with a precursor window resolution of -1 to +4 Da with an average 2500 laser shots for each spectrum, CID on, metastable suppressor on. Raw spectrum filtering/peak detection settings were S/N threshold of 5, and cluster area S/N optimization enabled at S/N threshold 6, baseline subtraction enabled at peak width 50. The data was then stored in an Oracle database (Oracle database schema v. 3.19.0 Data version 3.90.0).

MALDI Data Analysis. The data was extracted from the Oracle database and a peak list was created by GPS Explorer software v 3.6 (Applied Biosystems) from the raw data generated from the ABI 4700. Analyses were performed as combination MS + MS/MS. MS peak filtering

included mass range 700 – 4000 Da, minimum S/N filter 10. A peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 65. MSMS peak filtering included mass range of 60 Da to 20 Da below each precursor mass. Minimum S/N filter 10, peak density filter of 50 peaks per 200 Da, cluster area filter used with maximum number of peaks 65. The filtered data were searched by Mascot v 1.9.05 (Matrix Science) using NCBI nr database (NCBI 20070908), containing 5,454,477 sequences. Searches were performed without restriction to protein species, M_r , or pI and with variable oxidation of methionine residues and carbamidomethylation of cysteines. Maximum missed cleavage was set to 1 and limited to Trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 20 ppm and ± 0.2 Da, respectively.

Peak lists were also created using ABI's 4000 Series Explorer software v. 3.6 Peaks to MASCOT feature. MS peak filtering included mass range 700 – 4000 Da, minimum S/N filter 0. A peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 200. MSMS peak filtering included mass range of 60 Da to 20 Da below each precursor mass. Minimum S/N filter 0, peak density filter of 50 peaks per 200 Da, cluster area filter used with maximum number of peaks 200. The filtered data were searched by Mascot v 2.1.03 (Matrix Science) using NCBI nr database (NCBI 20070908), containing 5,454,477 sequences. Searches were performed without restriction to protein species, M_r , or pI and with variable oxidation of methionine residues and carbamidomethylation of cysteines. Maximum missed cleavage was set to 1 and limited to Trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 20 ppm and ± 0.2 Da, respectively. These files were analyzed using Proteome Software's Scaffold software.

Statistical Analyses. For measurement of GFP fluorescence, cultures and readings were conducted in triplicate. The mean fluorescence of each strain was calculated with standard error.

Each qPCR reaction was done in triplicate. C_t , ΔC_t , and $\Delta\Delta C_t$ values were calculated using Applied Biosystems Sequence Detection Software v1.4 (Applied Biosystems). $\Delta\Delta C_t$ values for each reaction were converted to fold expression using the equation $2^{-\Delta\Delta C_t}$. Fold expression values were log transformed for homoscedasticity, and mean log fold expression and standard error were calculated.

Statistical differences in treatments were determined by analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis Systems v9.1 (SAS Institute, Cary, NC). Where ANOVA indicated a significant difference, posthoc tests were used to determine pairwise differences. For GFP fluorescence data, Dunnett's one-tailed test was used to determine if fluorescence levels of T3SS promoter fusions were higher than negative controls. For qPCR data, Tukey's HSD was used for pairwise comparisons of T3SS gene expression in different culture conditions. Expression comparisons among different genes were not done. Differences were considered significant where $P \leq 0.05$, except for GFP fluorescence data, which were considered significant if $P \leq 0.005$.

RESULTS

Identification of a Putative T3SS Effector Gene on pEI2. *Edwardsiella ictaluri* carries two plasmids, pEI1 and pEI2, in all strains isolated from channel catfish (Newton et al. 1988; Bertolini et al. 1990). Fernandez et al. (Fernandez et al. 2001) sequenced both plasmids and report a putative T3SS-related gene carried by each. The gene on pEI1, *orf1*, encodes an amino acid sequence with homology to T3SS translocated effector proteins from *Salmonella*, *Shigella*, and *Yersinia*. This gene is named *eseH* for *Edwardsiella* secreted effector H. A pEI2 gene, *orf1*, encodes a protein with homology to a T3SS chaperone protein of both *Salmonella* and *Shigella* and is named *escD* for *Edwardsiella* secretion chaperone D.

Upon further analysis of the pEI2 DNA sequence, three bases downstream from *escD* is an open reading frame encoding a protein with homology to OspB of *Shigella flexneri* and *Vibrio parahaemolyticus* and hypothetical proteins of *Chromobacterium violaceum*, *Es. coli*, and *Salmonella enterica* (Table 2-5). However, the similarity of all proteins is only in the carboxy half of the amino acid sequence, except for the *C. violaceum* protein, which has homology only in the amino terminus. Recently, a sequence was added to the genetic database from *Pseudovibrio spp.* that has homology to *E. ictaluri* protein along the entire length of the amino acid sequence. Of these proteins, only the function of *Shigella* OspB is known (Zurawski et al. 2009). OspB, along with other *Shigella* effector proteins, works to dampen inflammatory cytokine production in host cells. The *E. ictaluri ospB*-like gene is named *eseI*. Figure 2.3G shows the positioning of *eseI* in relation to *escD*.

Table 2.5. Percent identity and similarity of *Edwardsiella ictaluri* EseI to proteins of other pathogens.

Protein	EseI amino acids (comparison amino acids)	Percent Identity	Percent Similarity
<i>Pseudovibrio sp.</i> hypothetical	22 – 331 (25 – 325)	35	53
<i>Chromobacterium violaceum</i> hypothetical	244 – 332 (34 – 123)	53	76
<i>Salmonella enterica</i> subsp. Enterica hypothetical	179 – 302 (148 – 261)	31	57
<i>Vibrio parahaemolyticus</i> putative OspB	154 – 302 (125 – 261)	29	50
<i>Es. coli</i> hypothetical	171 – 301 (129 – 249)	30	52
<i>Shigella flexneri</i> OspB	164 – 330 (125 – 282)	25	44

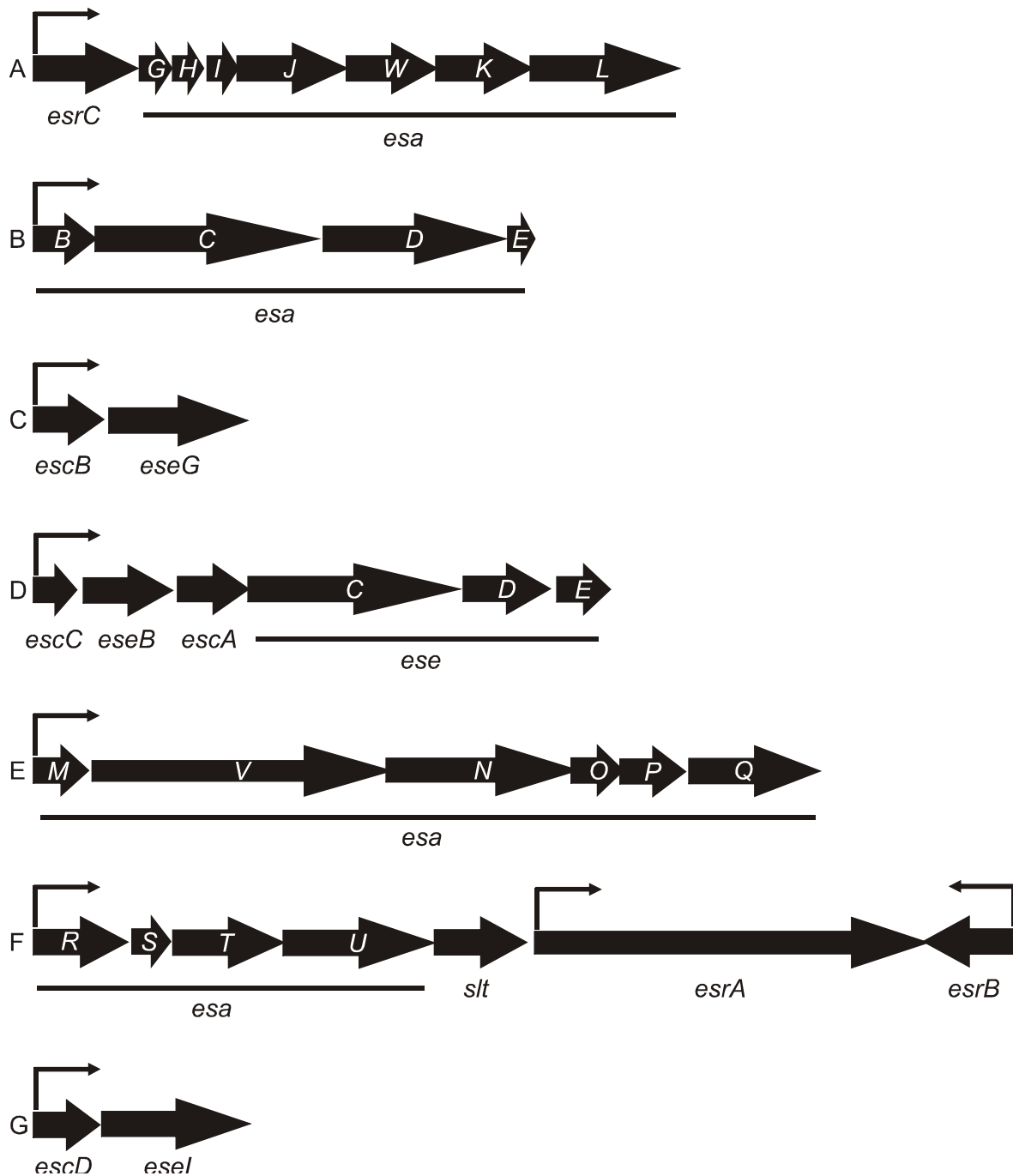


Figure 2.3. Putative operons of the *Edwardsiella ictaluri* type III secretion system (T3SS). The *E. ictaluri* T3SS is encoded within operons in the pathogenicity island. The first gene of each putative operon is used to identify each operon. *esrC* encodes a regulatory protein and seven apparatus proteins (A). *esaB* encodes four apparatus proteins (B). *escB* encodes a chaperone and effector protein (C). *escC* encodes two chaperone proteins and four effector proteins that make up the translocon (D). *esaM* encodes six apparatus proteins (E). *esaR* encodes four apparatus proteins, a murein transglycosylase (*slt*), and possibly the regulatory protein EsrA. However, *esrA* may express independently of *esaR* (F). *esrB* is a single gene added to demonstrate its association with *esrA*. *escD* is carried on pEI2 and encodes a chaperone protein and effector protein (G). Arrows indicate putative promoter regions.

Transcriptional Linkages in pEI2-Encoded T3SS Genes. *eseI* is encoded three bases downstream of the stop codon for *escD*, suggesting transcriptional coupling exists, and FGENESB (Softberry Inc., Mount Kisco, NY) predicts the pEI2-encoded genes *escD* and *eseI* are transcriptionally linked (Figure 2.3G). Reverse transcriptase PCR verifies this linkage. Amplification of cDNA using primers specific for *escD* and *eseI* and a cDNA template generated from an *eseI*-specific RT-PCR reaction indicates *escD* and *eseI* are transcriptionally linked (Figure 2.4). Individual products are amplified from the *eseI*-specific cDNA as well as a larger product when pairing *escD* and *eseI* primers. Amplification of each of these products from a single *eseI*-specific message indicates both *escD* and *eseI* are transcribed on the same message and, therefore, are transcriptionally linked. The transcriptional coupling of these two genes suggests EscD is a chaperone for EseI.

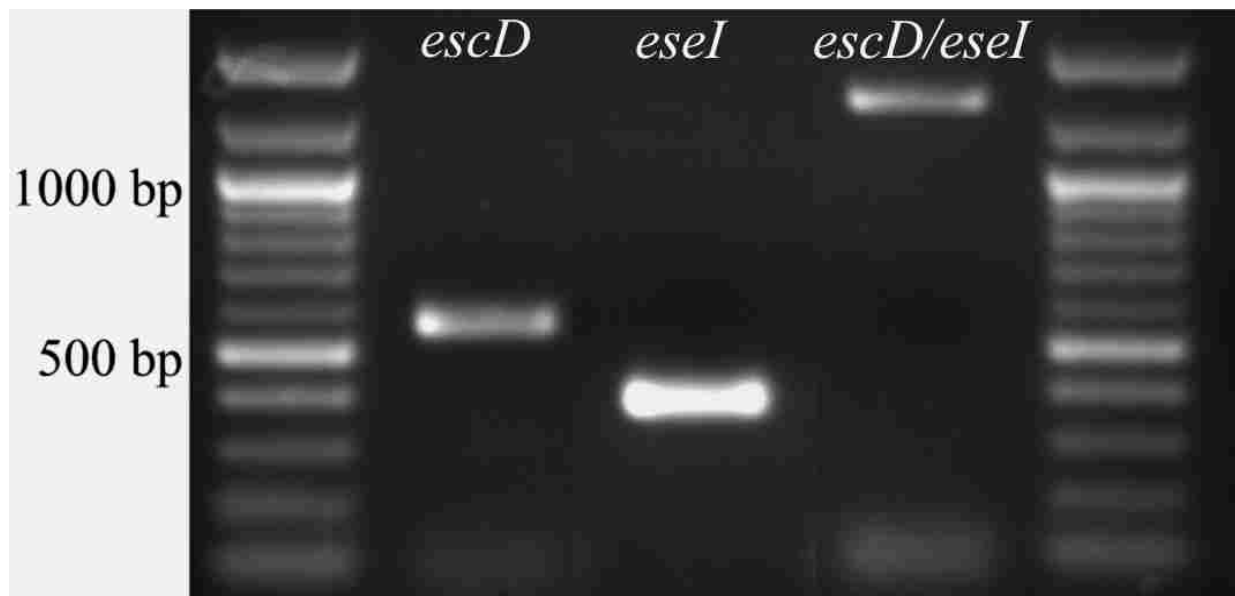


Figure 2.4. Transcriptional linkage of *Edwardsiella ictaluri* *escD* and *eseI*. Reverse transcriptase PCR was conducted to show a transcriptional linkage between *escD* and *eseI*. An *escD/eseI* band was amplified using the 5' primer of *escD* and the 3' primer of *eseI*. Generation of the products from a single cDNA sequence indicates *escD* and *eseI* expression is linked.

T3SS Promoter Regions Induce *gfp*⁺ Expression in Many Conditions. Promoter regions for T3SS-related operons (Figure 2.3) were evaluated for their ability to express *gfp*⁺ in *E. ictaluri*. In addition to the promoter regions indicated in Figure 2.3, the promoter for *orf29/30* encoded at the end of the T3SS pathogenicity island (Thune et al. 2007), and the promoter for the putative T3SS effector protein *eseH* were also fused to *gfp*⁺ to evaluate their expression. Promoter fusions were inserted singly between *serC* and *aroA* to eliminate variation associated with copy number and expression instability associated with plasmids (Hansen-Wester et al. 2002). Hautefort et al. (Hautefort et al. 2003) used a similar method to create promoter fusions and used flow cytometric analyses to measure fluorescence of promoter-*gfp*⁺ fusions in *Salmonella*. In this study, however, mixed results were encountered when using flow cytometric analyses on an inducible *purA* promoter fusion *E. ictaluri* strain in comparison to a non-fluorescing strain (unpublished results). Therefore, fluorescence was measured using an M2 SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Unfortunately, the plate reader also was unable to measure the fluorescence of cultures consistently. Cultures were concentrated 2-10x before fluorescence of a positive control could be detected. Comparing fluorescent values among growth conditions proved difficult because of variations in OD readings among treatments. Therefore, comparisons were done only against control readings within the same culture condition. Because of the issues in measuring fluorescence, differences were not considered significant unless $P \leq 0.005$.

Edwardsiella ictaluri T3SS promoter activity is detected in both neutral and acidic MM19 (Figures 2.5 and 2.6). Culture at pH 7.0 results in significant detection of more promoter fusions, but there are greater levels of fluorescence detected in acidic media with *esaB* and *escC* fusions. Both *esrA* and *esrC* fusions lose significance in acidic media, as does *orf29/30*. Fluorescence trends are similar in MM19-P (Figures 2.7 and 2.8). Many promoter fusions are

detected at pH 7.0, but a greater increase in fluorescence occurs in acidic cultures. Activity of *orf29/30* is not detected in pH 5.5, indicating acidic conditions repress the *orf29/30* promoter. Once again, *esrA* and *esrC* are not detected in acidic pH. Contrary to normal phosphate conditions, *esrC* is not detected in low phosphate pH 7.0, but when cultured in MM19-P pH 5.5, expression of the *esrC* promoter is significantly higher than WT, but not the promoterless *gfp+* strain. Activity of *esaM* is detected in both pH 7.0 and pH 5.5 low phosphate media, but not in either of the regular phosphate pH conditions. Fluorescence of the *eseH* fusion is detected only in MM19-P pH 7.0. Fluorescence of GFP expressed from the *esaR* promoter is not detected in any growth condition.

Other media conditions were also evaluated, including low magnesium at pH 5.5 and 7.0 and MM19 at pH 4.5 (data not shown), and the results are similar to the above: neutral pH results in more promoters being detected, but acidic pH results in greater fluorescence of some T3SS promoter fusions. *orf29/30* consistently fluoresces significantly in all neutral pH conditions assayed, and is insignificant in all acidic conditions assayed. Fluorescence from the *esaR* promoter is not detected in any condition studied. Fluorescence from *esaM*, *eseH*, *esrA* and *esrC* promoters are generally low, even when significantly higher than controls.

Fluorescence measurements of T3SS promoter fusions to *gfp+* suggest an upregulation in acidic conditions for apparatus and translocon operons (*esaB* and *escC*). Low phosphate also appears to induce expression of *esaM*. While two T3SS-related operons are upregulated in acidic media, *orf29/30* has reduced fluorescence in these conditions. However, the *gfp+* fusion data seem unreliable based on the problems encountered measuring fluorescence, and the low levels of fluorescence observed. To verify results from the promoter fusions, RT-PCR was used to evaluate the presence of T3SS transcripts during culture in the conditions used to assay *gfp+* expression from T3SS promoters.

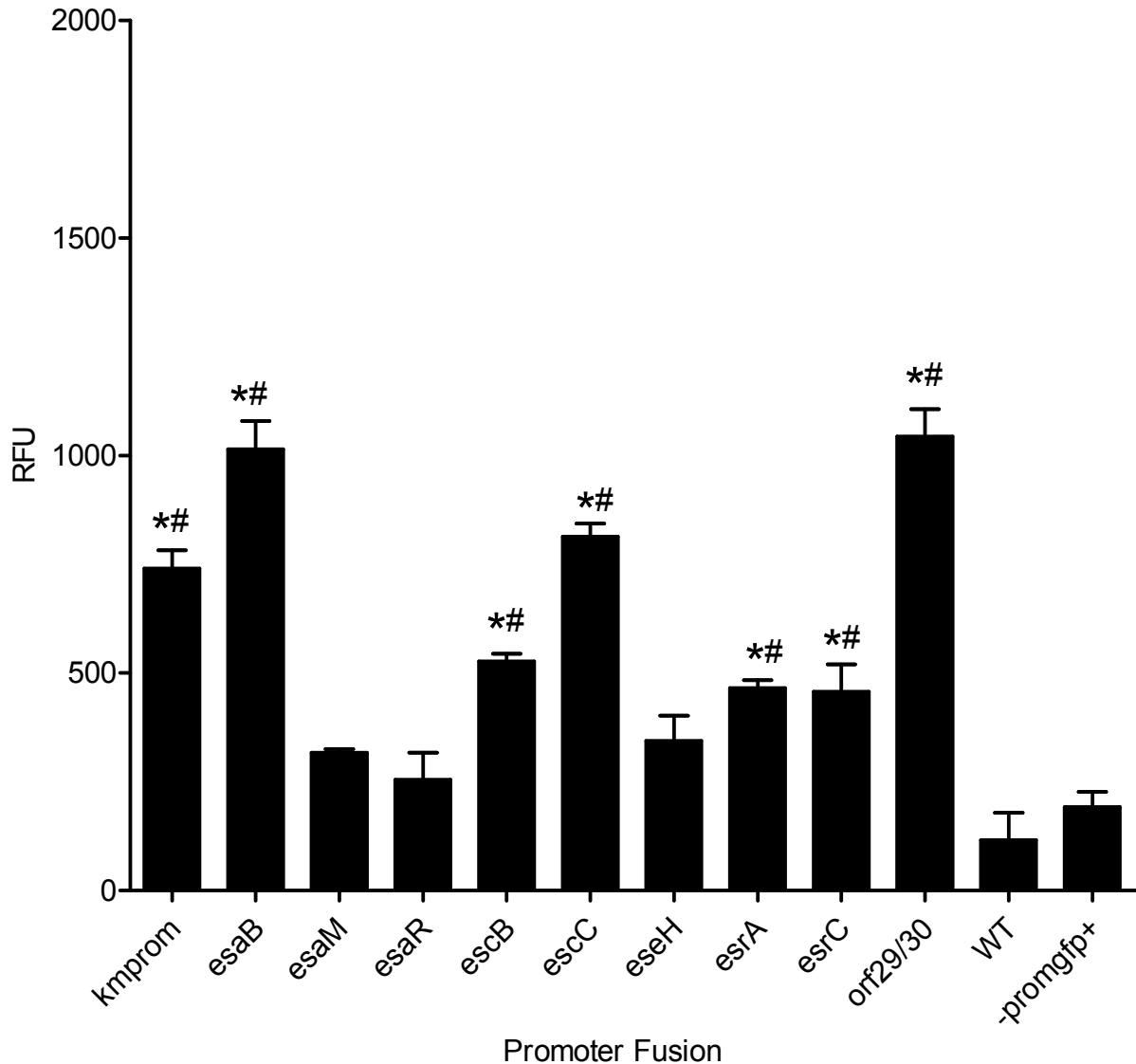


Figure 2.5. *Edwardsiella ictaluri* type III secretion system (T3SS) gene promoter activity in minimal media (MM19) at pH 7.0. *Edwardsiella ictaluri* strains carrying T3SS promoter fusions to *gfp+* were grown to late log phase in MM19 pH 7.0, and fluorescence was measured as relative fluorescence units (RFU). The strain *kmprom* serves as a positive control, and the wild type (WT) and *-promgfp+* strains serve as negative controls. Bars indicate the mean (\pm SEM) of triplicate samples. * indicates a significant difference from WT, and # indicates a significant difference from *-promgfp+* ($P \leq 0.005$).

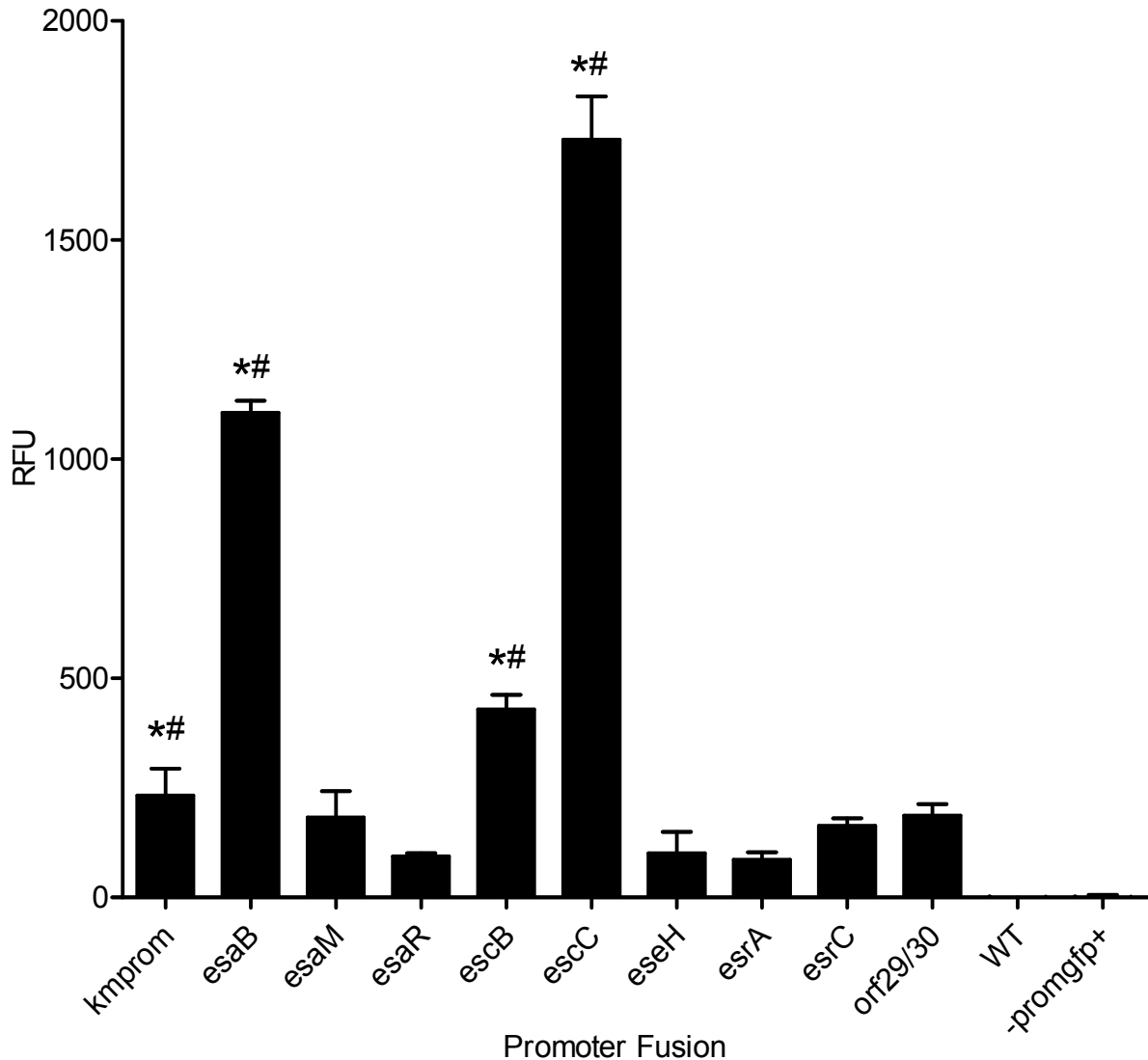


Figure 2.6. *Edwardsiella ictaluri* type III secretion system (T3SS) gene promoter activity in minimal media (MM19) at pH 5.5. *Edwardsiella ictaluri* strains carrying T3SS promoter fusions to *gfp+* were grown to late log phase in MM19 pH 5.5, and fluorescence was measured as relative fluorescence units (RFU). The strain *kmprom* serves as a positive control, and the wild type (WT) and *-promgfp+* strains serve as negative controls. Bars indicate the mean (\pm SEM) of triplicate samples. * indicates a significant difference from WT, and # indicates a significant difference from *-promgfp+* ($P \leq 0.005$).

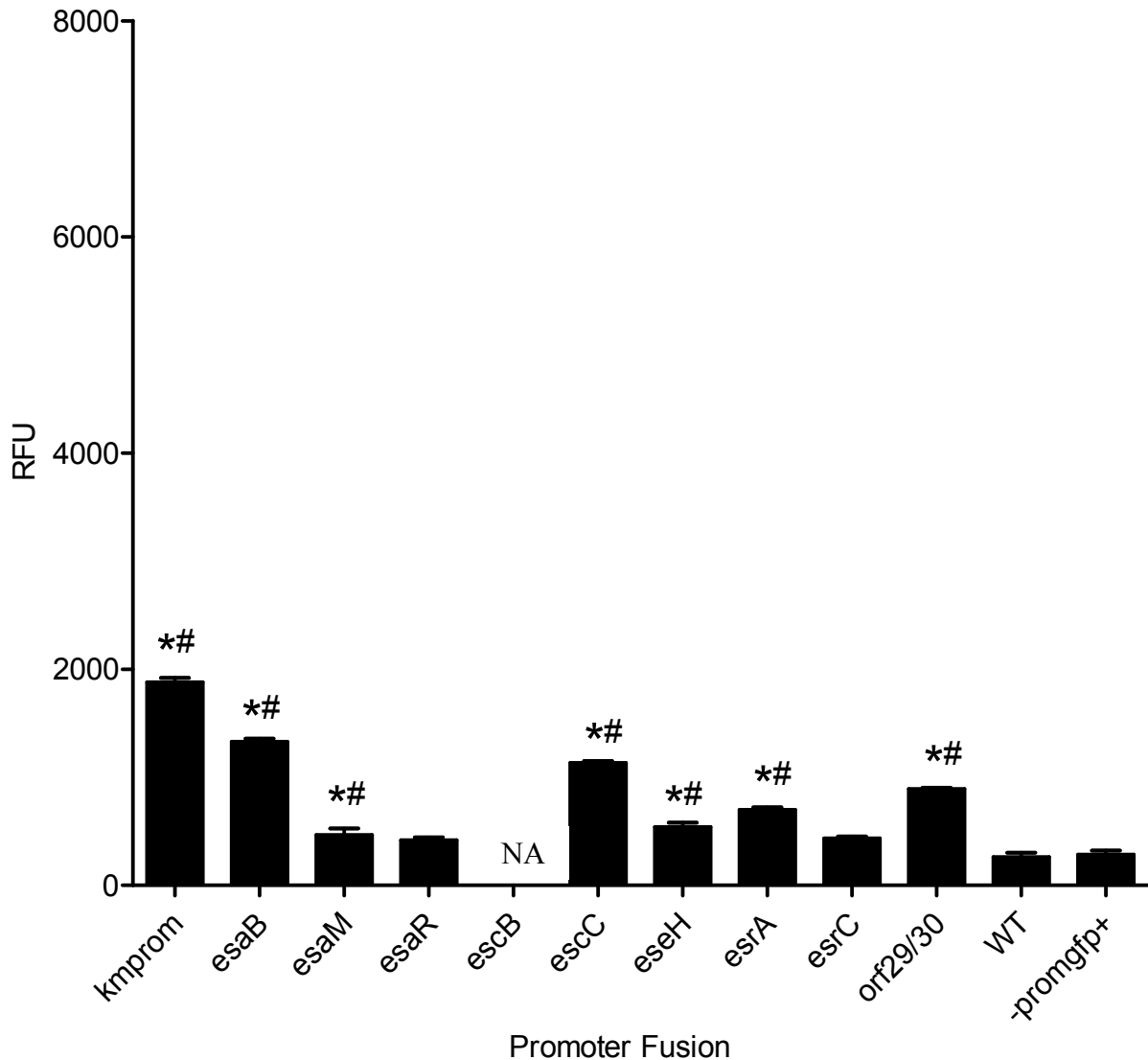


Figure 2.7. *Edwardsiella ictaluri* type III secretion system (T3SS) gene promoter activity in low phosphate minimal media (MM19-P) at pH 7.0. *Edwardsiella ictaluri* strains carrying T3SS promoter fusions to *gfp+* were grown to late log phase in MM19-P pH 7.0, and fluorescence was measured as relative fluorescence units (RFU). The strain *kmprom* serves as a positive control, and the wild type (WT) and *-promgfp+* strains serve as negative controls. Bars indicate the mean (\pm SEM) of triplicate samples. * indicates a significant difference from WT, and # indicates a significant difference from *-promgfp+* ($P \leq 0.005$). NA indicates the promoter fusion fluorescence was not assayed.

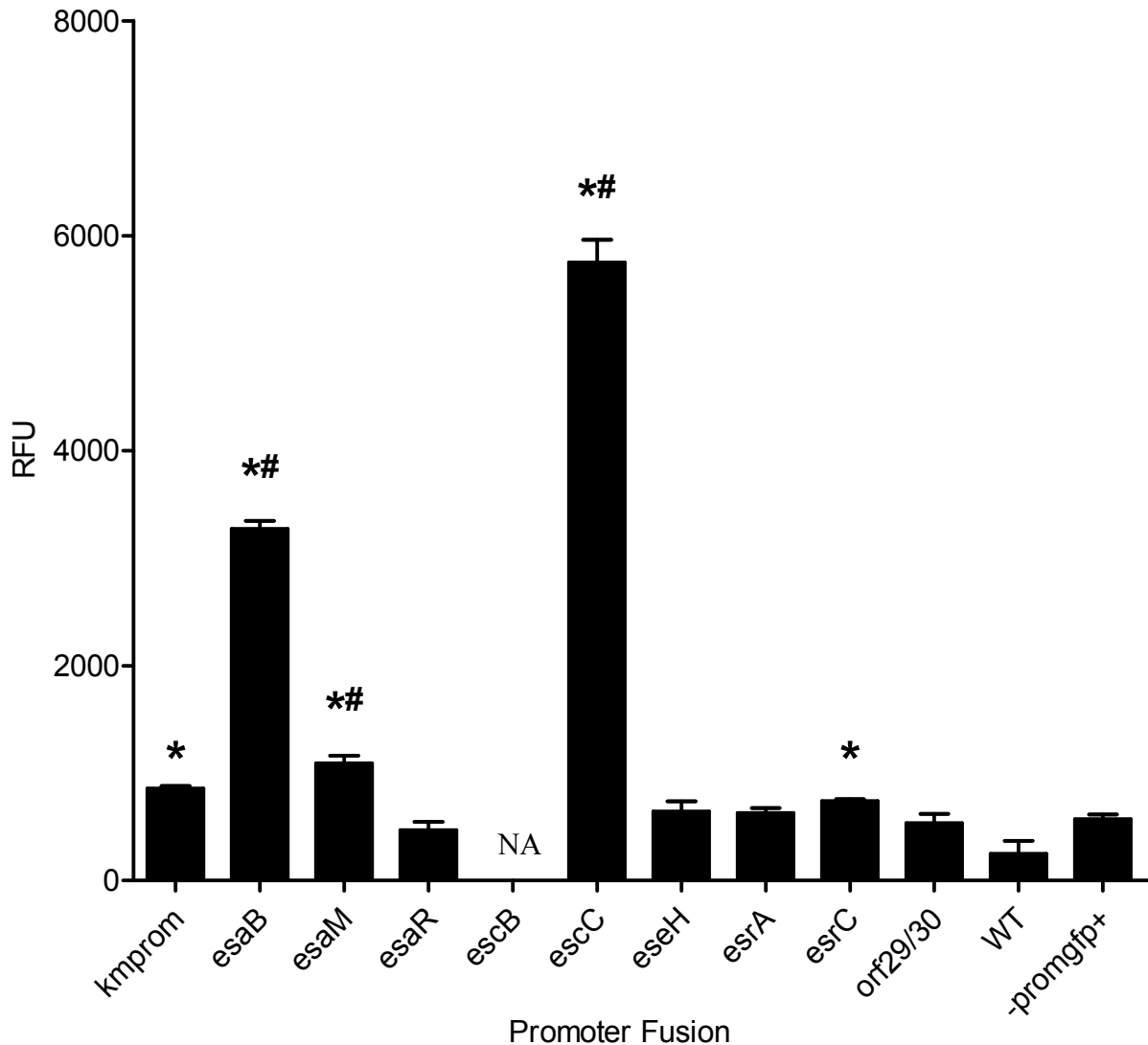


Figure 2.8. *Edwardsiella ictaluri* type III secretion system (T3SS) gene promoter activity in low phosphate minimal media (MM19-P) at pH 5.5. *Edwardsiella ictaluri* strains carrying T3SS promoter fusions to *gfp+* were grown to late log phase in MM19-P pH 5.5, and fluorescence was measured as relative fluorescence units (RFU). The strain *kmprom* serves as a positive control, and the wild type (WT) and *-promgfp+* strains serve as negative controls. Bars indicate the mean (\pm SEM) of triplicate samples. * indicates a significant difference from WT, and # indicates a significant difference from *-promgfp+* ($P \leq 0.005$). NA indicates the promoter fusion fluorescence was not assayed.

The T3SS of *E. ictaluri* Is Expressed in Varying Conditions. The promoter fusions are not definitive in showing expression of promoters in the conditions assayed. Therefore, RT-PCR was used to detect the presence of T3SS gene mRNA in various culture conditions. RT-PCR analyses were conducted on apparatus, translocon, regulatory, and effector genes (Table 2.3).

For pH 5.5 and pH 7.0 cultures with or without phosphate limitations, most transcripts are detected strongly except for *esaR* (Figure 2.9), which also is not detected in the *gfp+* fusions. To determine if growth to late log influenced the presence of T3SS message, RNA isolated from mid-log phase cultures were also analyzed by RT-PCR, and similar results were seen as when grown to late log (data not shown).

Similar to the results from GFP fluorescence, *orf29/30* detection is reduced at pH 5.5 compared to pH 7.0 (Figure 2.9). To verify this, the RT-PCR reactions for *orf29/30* were repeated, and the results demonstrate a visible decrease in PCR product intensity from the pH 5.5 sample relative to pH 7.0 sample (Figure 2.10). This indicates *orf29/30* expression is downregulated in acidic pH in contrast to T3SS genes, which are expressed in both neutral and acidic pH. Therefore, it is unlikely that *orf29/30* is associated with the *E. ictaluri* T3SS based on downregulation in acidic culture conditions.

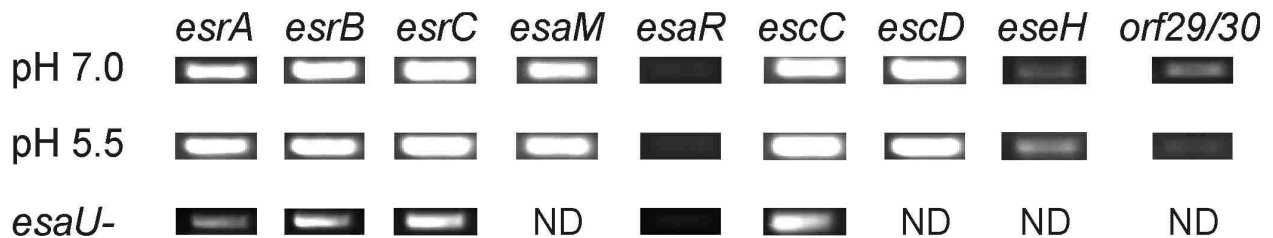


Figure 2.9. Reverse transcription PCR (RT-PCR) results for *Edwardsiella ictaluri* type III secretion system (T3SS) gene expression. Presence of *E. ictaluri* T3SS message in cultures of wild type *E. ictaluri* grown in pH 7.0 or pH 5.5 MM19 and a T3SS-deficient *E. ictaluri* strain, *esaU*-, grown in MM19 pH 5.5 media was determined by RT-PCR. ND indicates genes were not assayed in those conditions.



Figure 2.10. Effect of acidic pH on *Edwardsiella ictaluri orf29/30* expression. Reverse transcription PCR (RT-PCR) was used to detect the presence of *orf29/30* message during culture in pH 7.0 and pH 5.5 MM19. Absence of *orf29/30* message in pH 5.5 indicates *orf29/30* is not expressed in acidic conditions, suggesting its expression pattern differs from that of *E. ictaluri* type III secretion system genes.

Edwardsiella ictaluri has a temperature window of 22 – 28° C in which it causes disease of channel catfish (Francis-Floyd et al. 1987). To determine if temperature has an effect on T3SS expression, RNA was harvested from *E. ictaluri* cultured to late log phase at 18°C and 32°C. When grown in temperatures outside of the ESC temperature window at pH 7.0, message is detected for the same genes as in all other conditions tested (data not shown). *Edwardsiella ictaluri* grown in rich media (LB-mannitol) also has the same message profile as in other conditions (data not shown), indicating rich media does not inhibit T3SS expression.

Thune et al. (Thune et al. 2007) identified a T3SS-deficient mutant, 65ST, using signature tagged mutagenesis. The mutation occurs in the T3SS apparatus gene *esaU* and results in a strongly attenuated virulence phenotype intracellularly and *in vivo*. The mutation will hereafter be referred to as *esaU*-. Reverse transcription PCR was used to determine if the transposon insertion in *esaU* results in any effect on expression of other T3SS genes. Of the genes assayed, none expresses differently than in the WT strain, indicating the transposon insertion does not affect T3SS expression (Figure 2.9).

The results of the RT-PCR studies indicate some level of T3SS expression in all conditions studied. Few differences are observed in PCR product intensity to determine if any conditions result in a higher level of expression. However, *orf29/30* activity is greatly reduced in

acidic conditions, mirroring results from *gfp*⁺ expression analysis. Expression of the T3SS is not temperature dependent, indicating temperatures outside of the “ESC window” do not prohibit T3SS expression. The RT-PCR results offer qualitative data in regards to the presence of message in different conditions, but the data cannot be quantified to determine differences in expression of regulatory, translocon, or effector genes. Upregulation of genes will likely be an indicator of T3SS activity, i.e., secretion of proteins. Therefore, expression of regulatory, translocon, and effector genes were evaluated using qPCR.

The *E. ictaluri* T3SS Is Upregulated in Acidic Phosphate-Limited Conditions. Reverse transcription PCR results indicate T3SS genes are expressed in a number of conditions, and the GFP fluorescence data indicate upregulation of some T3SS genes in acidic conditions. Quantitative PCR was used to measure differences in the expression of T3SS genes in various pH and phosphate concentration conditions. The expression of regulatory, translocon, and effector genes was evaluated. Because *orf29/30* appears to have a different expression pattern than T3SS genes, it was not evaluated further by qPCR. The apparatus genes *esaB* and *esaM* analyzed by *gfp*⁺ fusion and RT-PCR above were also excluded, because expression of the T3SS translocon and effectors are a better indicator of active T3SS protein secretion. Studies conducted on the SPI-2 T3SS demonstrate that expression of T3SS genes is detected in conditions that do not result in active secretion of the T3SS effector proteins, so the system is not truly active in those conditions. The goal for these qPCR reactions was to determine when the *E. ictaluri* T3SS may be upregulated to identify when protein secretion may occur.

Modulation of gene expression was determined by relative quantification using expression in MM19 pH 7.0 as the calibrator sample and *E. ictaluri* 16s rRNA as the endogenous control. When WT *E. ictaluri* is cultured at pH 5.5, there is a significant decrease in expression of the regulatory genes *esrA* and *esrC* (Figure 2.11), but a significant increase in expression of

the *escC* operon (Figure 2.12), which encodes the translocon proteins EseB, EseC, and EseD. These findings support the results observed for *esrA*, *esrC*, and *escC* promoter fusions. This suggests EsrA and EsrC are not necessary for upregulation of the *escC* operon. The expression of the other T3SS-related genes, however, is unchanged in this culture condition, indicating acidic pH is not sufficient for expression of the entire system.

Culture in MM19-P pH 7.0 does not result in an increase of either regulatory or non-regulatory T3SS genes. When cultured in MM19-P at pH 5.5, however, T3SS gene expression increases significantly. *esrC* expression in MM19-P pH 5.5 is significantly higher than in MM19 pH 7, again similar to the results observed in the *esrC* promoter fusion to *gfp* when cultured in MM19-P pH 5.5. The translocon operon again is upregulated in the low phosphate acidic media. The operon encoding *escB* and *eseG* also increases in expression in MM19-P pH 5.5, as does expression of the pEI1-encoded putative effector gene *eseH*. Expression of *eseI* increases in both of the low phosphate conditions, but because of variability in results among triplicate samples, the differences are not significant. These results indicate that phosphate limitation, in addition to acidic pH media, results in an increase in expression of T3SS pathogenicity island-encoded regulatory, translocon, and effector genes, as well as the pEI1-encoded putative effector *eseH*.

Expression of *esrA* and *esrB* generally is similar in all conditions; all log expression values are within 0.5 logs of expression in MM19 pH 7.0. *esrC* appears to be affected more by environmental conditions than *esrA* and *esrB* based on the range of expression values observed among the culture conditions. This suggests EsrC may be a regulator through which T3SS gene expression is modulated in response to environmental conditions.

Results from qPCR indicate expression of the operon encoding the translocon proteins is upregulated in acidic conditions, but both *esrA* and *esrC* are significantly downregulated in

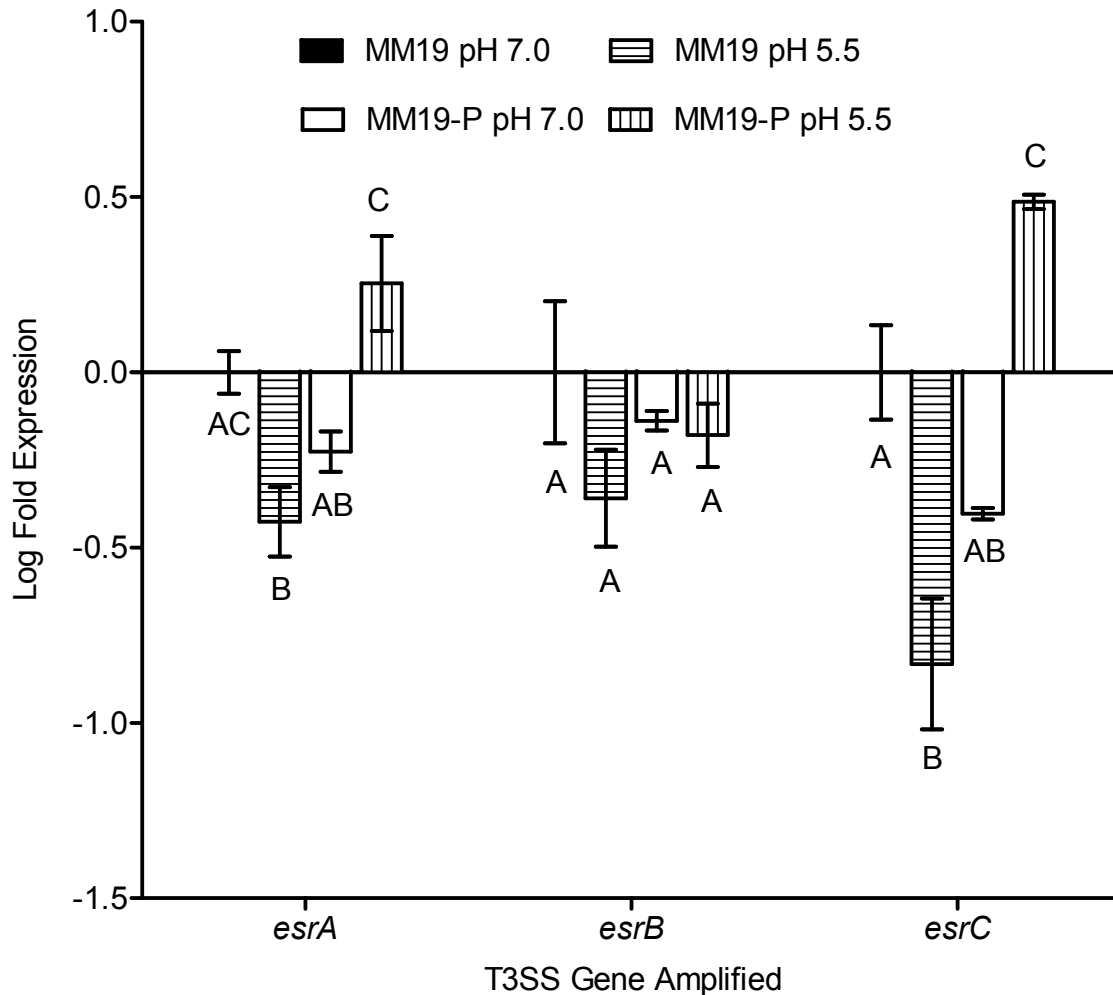


Figure 2.11. Effect of media pH and phosphate concentration on expression of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory genes. Quantitative PCR was used to estimate the expression of *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and the *esrC* operon containing *esrC* and the apparatus genes *esaGHIJWKL* in neutral and acidic MM19 and low phosphate MM19 (MM19-P). Bars represent the mean (\pm SEM) of the log fold expression from triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

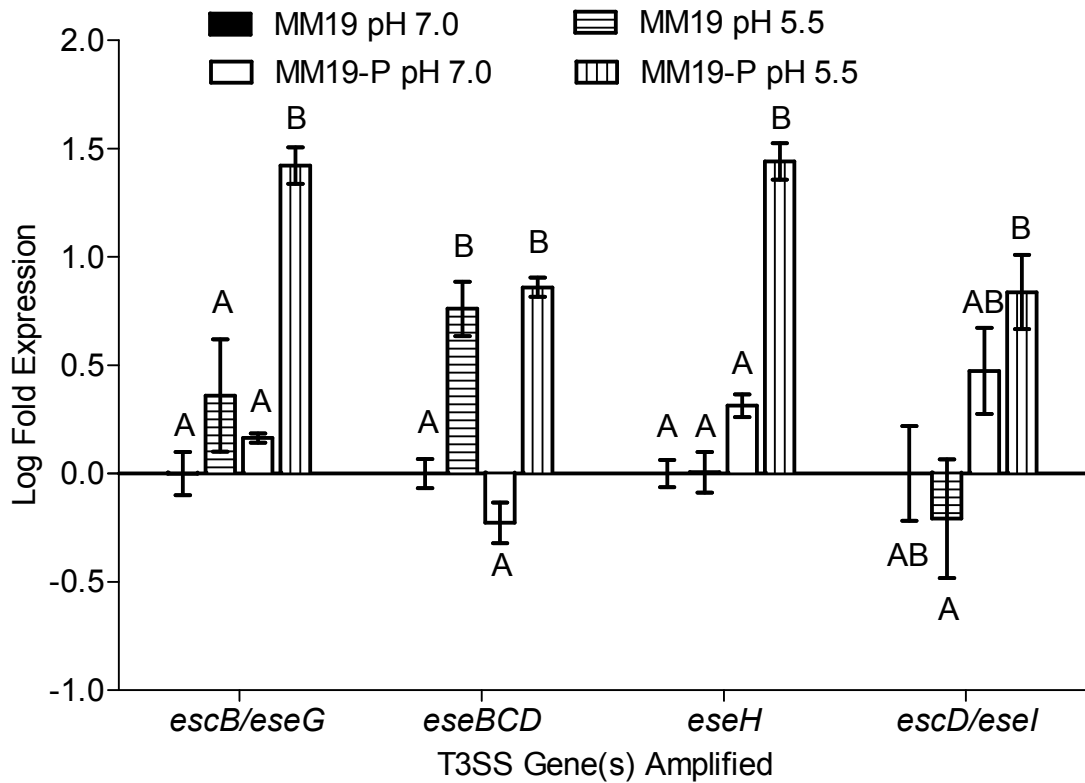


Figure 2.12. Effect of media pH and phosphate concentration on expression of *Edwardsiella ictaluri* type III secretion system (T3SS) non-regulatory genes. Quantitative PCR was used to estimate the expression of the *E. ictaluri* T3SS non-regulatory genes encoded by the *escB/eseG* operon, the *escC* operon containing the translocon genes *eseBCD*, the pEI1-encoded *eseH*, and the pEI2-encoded *escD/eseI* operon in neutral and acidic MM19 and low phosphate MM19 (MM19-P). Bars represent the mean (\pm SEM) of the log fold expression of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

MM19 pH 5.5. This is interesting, because the *esrC* operon also encodes apparatus genes, which would presumably be required for construction of the T3SS apparatus to allow secretion of translocon proteins. The shift in expression of *esrC* in MM19 pH 5.5 and MM19-P pH 7.0 compared to MM19-P pH 5.5 indicates that acidity or phosphate limitation alone has a negative effect on expression, but in an acidic and low phosphate environment, an increase in expression occurs. Both *escB/eseG* and *eseH* are expressed in MM19-P pH 5.5, but show little activity in other conditions. Expression of the pEI2-encoded chaperone *escD* and *eseI* genes is not significantly upregulated above MM19 pH 7.0 expression in the conditions assayed; however, decreased phosphate concentration appeared to have a slightly positive effect at either pH.

The Translocon Proteins Are Secreted to the Culture Supernatant in Acidic Media.

Analyses of whole cell lysate and extracellular proteins were conducted by using 2D-PAGE to determine if the increased transcription of T3SS genes correlates to translation and secretion of T3SS proteins. Whole cell lysate and supernatant proteins were isolated from WT *E. ictaluri* grown to late log phase in MM19 pH 7.0, pH 5.5, and MM19-P pH 5.5. To determine if the secretion of any proteins to the supernatant is T3SS-dependent, the T3SS mutant *esaU*- (Thune et al. 2007) was also evaluated. Protein spots of interest were identified by PMF MALDI-TOF/TOF MS.

Edwardsiella ictaluri whole cell lysate protein gels are uniform among the three culture conditions (Figure 2.13). The 2D gels of the pH 5.5 whole cell lysates were analyzed by PMF MALDI-TOF/TOF MS to confirm protein identities. A majority of the proteins visible on whole cell lysate gels are metabolic and housekeeping genes. However, the T3SS proteins EscA (chaperone), EseB (translocon), and EseD (translocon) are identified. In addition to T3SS proteins, type VI secretion system (T6SS) proteins are also identified. Corresponding T3SS and

T6SS proteins are present, albeit at reduced levels in WT pH 7.0 and *esaU*- whole cell lysate gels, indicating expression of those proteins is not pH or *esaU* dependent.

Extracellular protein samples were loaded onto the IPG strips based on the amount of sample per a given volume of supernatant rather than based on the protein concentration. The reason for this was to compare relative amounts of secretion rather than the proteins present or absent in a particular concentration of protein. That is, for each bacterial culture, precipitated proteins from 200 mls were resuspended in 1 ml of water and divided into 100 μ l aliquots; therefore, a 100 μ l volume of supernatant protein is the equivalent of the secreted protein in 20 mls of culture.

Two-dimensional polyacrylamide gel electrophoresis analysis of the supernatant proteins verifies that T3SS proteins are secreted to the culture supernatant at pH 5.5, but not pH 7.0 (Figure 2.14). Proteins are abundant in the WT pH 5.5 supernatant, but greatly reduced in both the WT pH 7.0 and *esaU*- pH 5.5 supernatants. Protein spot identification by PMF MALDI-TOF/TOF MS demonstrates the T3SS translocon proteins EseB, EseC, and EseD are secreted to the pH 5.5 supernatant. Spots for EseB and EseD are very close together, as demonstrated in the WT pH 5.5 whole cell lysate gel (Figure 2.13B). The concentration of both EseB and EseD in the supernatant causes the spots to focus together, making it difficult to determine the boundaries of each spot. EseB appears to be expressed at higher levels, because vertical and horizontal streaks that originate from the EseB/EseD spot are also identified as EseB. To ensure EseD is present in the supernatant, a section of the upper right edge of the spot was analyzed by PMF MALDI-TOF/TOF MS and verified to contain EseD. Corresponding spots are absent from pH 7.0 supernatants and reduced or absent from pH 5.5 *esaU*- culture supernatants. Protein samples from MM19-P pH 5.5 cultures are similar to pH 5.5 cultures with normal inorganic phosphate concentrations (data not shown). In addition to the identification of T3SS proteins in the

Figure 2.13 (Following page). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of *Edwardsiella ictaluri* whole cell lysate proteins. Whole cell lysate proteins collected from *E. ictaluri* cultured in MM19 at pH 7.0 (A), pH 5.5 (B), and the *esaU*- type III secretion system (T3SS)-deficient mutant at pH 5.5 (C) were separated by 2D-PAGE. Spots corresponding to T3SS proteins EscA, EseB, EseD, and the type VI secretion system proteins EvpA, EvpB, and EvpC are labeled. Gels were run in triplicate, and a representative gel is shown.

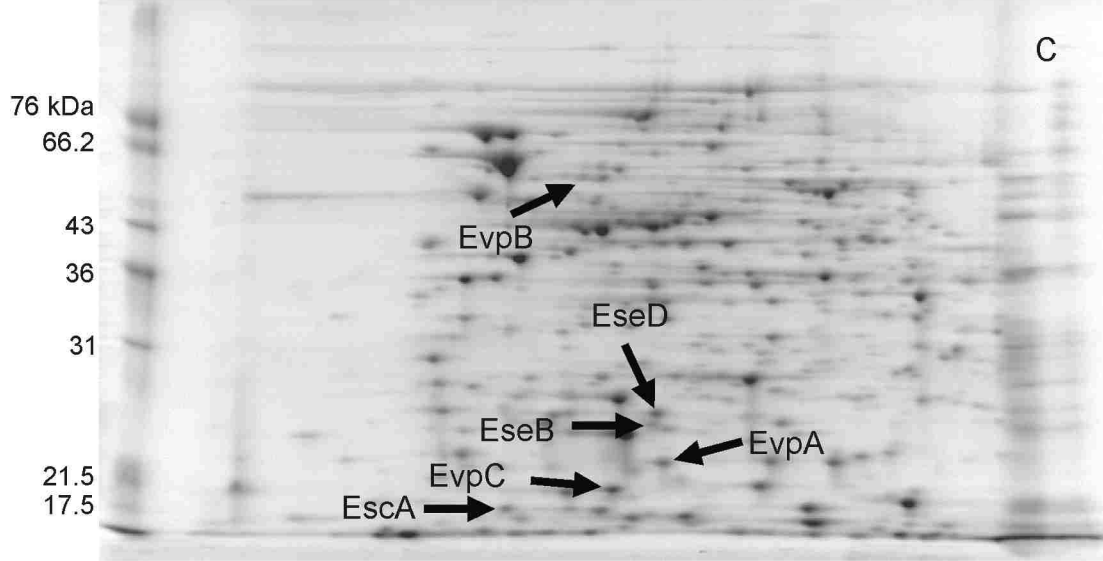
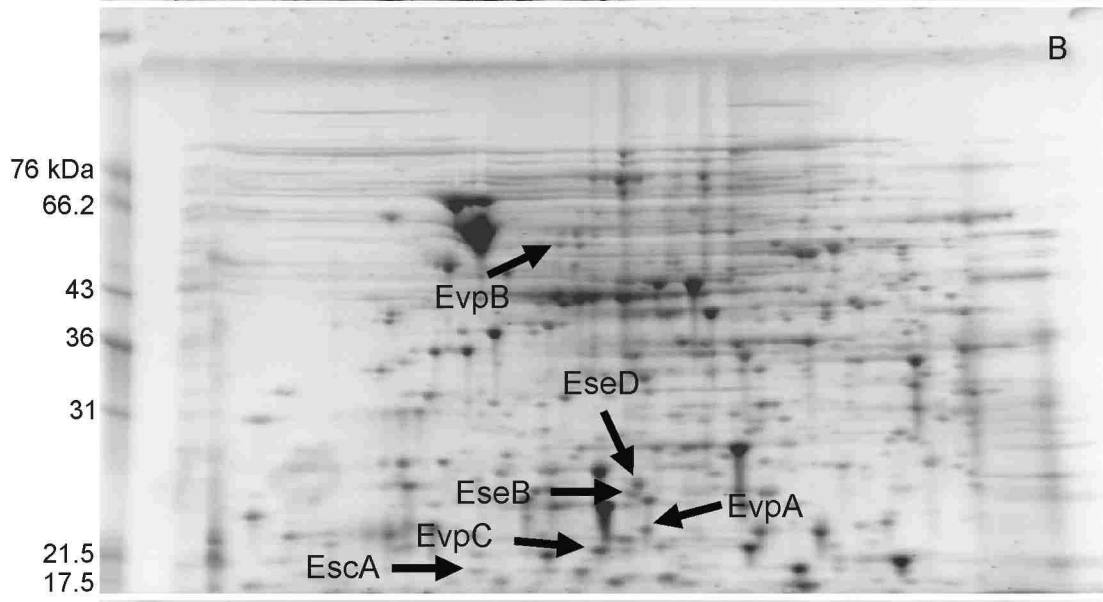
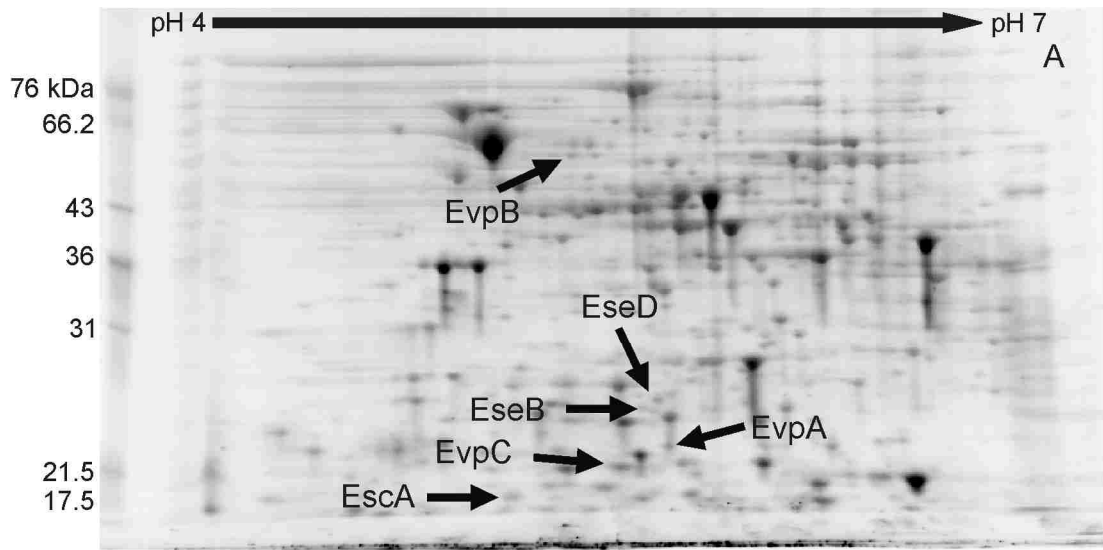
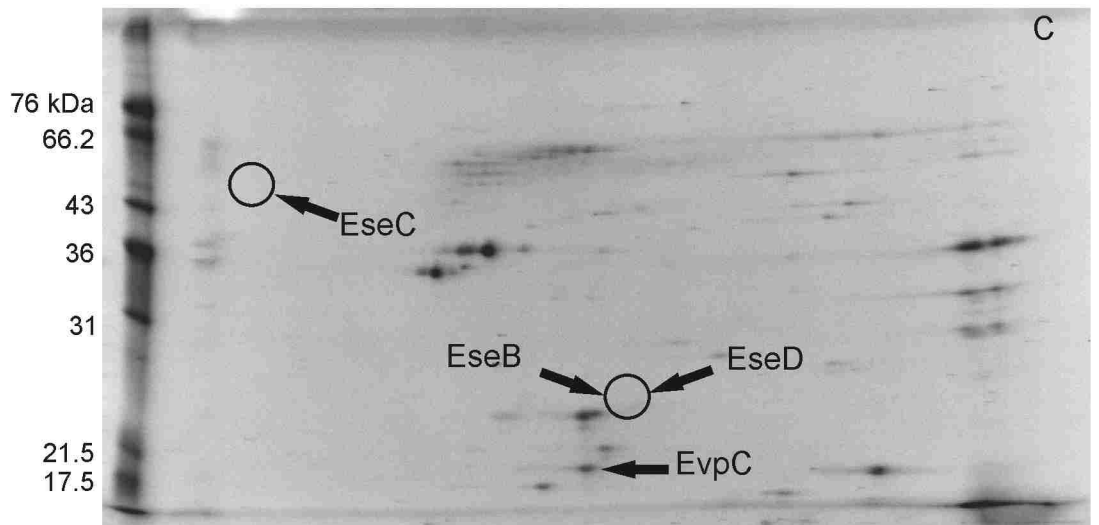
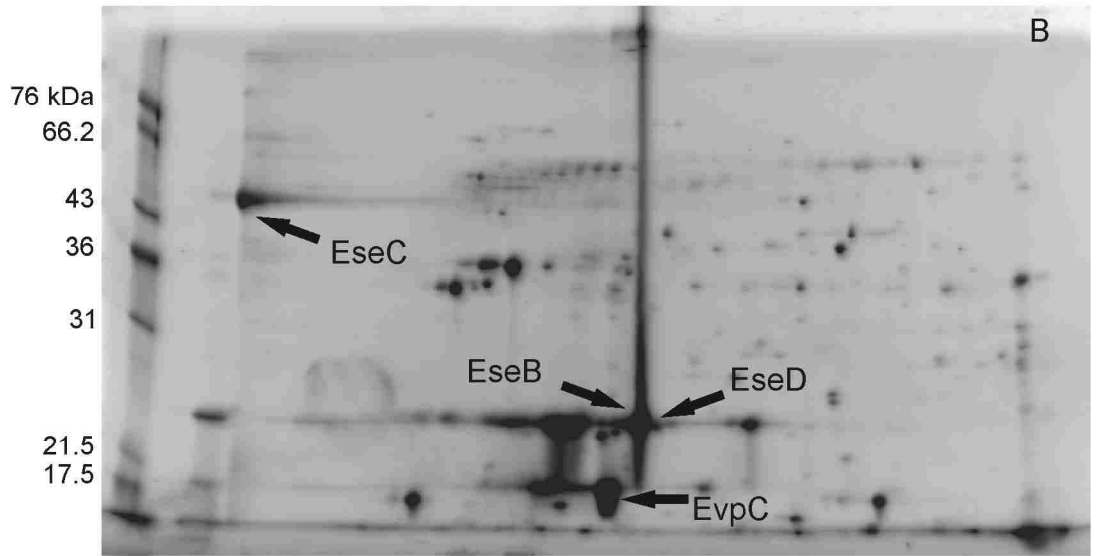
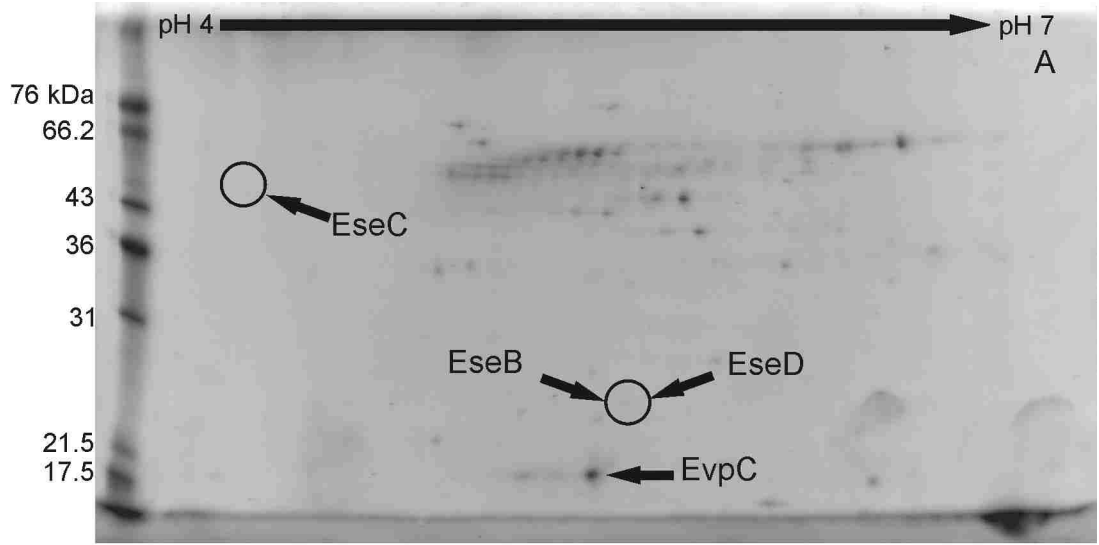


Figure 2.14 (Following page). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of *Edwardsiella ictaluri* extracellular proteins. Extracellular proteins collected from *E. ictaluri* cultured in MM19 at pH 7.0 (A), pH 5.5 (B), and the *esaU*- T3SS-deficient mutant at pH 5.5 (C) were separated by 2D-PAGE. Spots corresponding to the type III secretion system translocon proteins EseB, EseC, EseD, and the type VI secretion system protein EvpC are labeled. Circles indicate regions in gels where a protein spot would occur, but is absent in the culture supernatant. Gels were run in triplicate, and a representative gel is shown.



supernatant, a T6SS protein, EvpC, is identified (Figure 2.14). EvpC is secreted regardless of pH or an intact T3SS. However, EvpC appears to be secreted at lower levels in pH 7 media or in the *esaU* mutant at pH 5.5 than in WT pH 5.5.

The results of the 2D-PAGE analyses of whole cell lysate and extracellular proteins indicate that the translocon proteins EseB, EseC, and EseD are translated at both pH 5.5 and 7.0, and also produced by the *esaU*- strain. However, each protein is secreted only in pH 5.5 media in a T3SS-dependent fashion. In addition to finding secreted T3SS proteins in culture supernatants, a protein homologous to a T6SS secreted protein of *E. tarda* (Zheng et al. 2005) is identified extracellularly. Contrary to T3SS translocon proteins, secretion of EvpC is not abolished in the *esaU*- strain, although it is reduced. EvpC, unlike T3SS translocon proteins, is secreted to the supernatant at pH 7.0. However, it appears EvpC is secreted in greater amounts at pH 5.5. EvpC is found in all whole cell lysate protein preparations as are the T6SS proteins EvpA and EvpB.

The translocon genes of *E. tarda* are secreted in pH 7.0 media (Srinivasa Rao et al. 2004; Zheng et al. 2007). However, the *E. tarda* cultures were grown for 24 hrs in Dulbecco's Modified Eagle Medium (DMEM) without aeration. *Edwardsiella ictaluri*, on the other hand, reaches late log phase after about 16 hours of culture, which is when total RNA was harvested from cells in this study. To determine if growth to stationary affects *E. ictaluri* T3SS expression, *E. ictaluri* was cultured 24 hours in MM19 pH 7.0, and total RNA was harvested for qPCR analysis. Expression of both *esrA* and *esrB* in stationary phase is comparable to expression when cultured to late log phase (Figure 2.15). However, expression of *esrC*, *escB/eseG*, *eseH*, and the translocon operon are significantly decreased (Figure 2.15 and 2.16), whereas expression of *eseI* is unchanged in stationary phase. These results indicate 24-hour culture of *E. ictaluri* to stationary phase in MM19 pH 7.0 does not induce T3SS expression. Because of poor growth of *E. ictaluri* in anaerobic conditions, T3SS gene expression was not evaluated in those conditions.

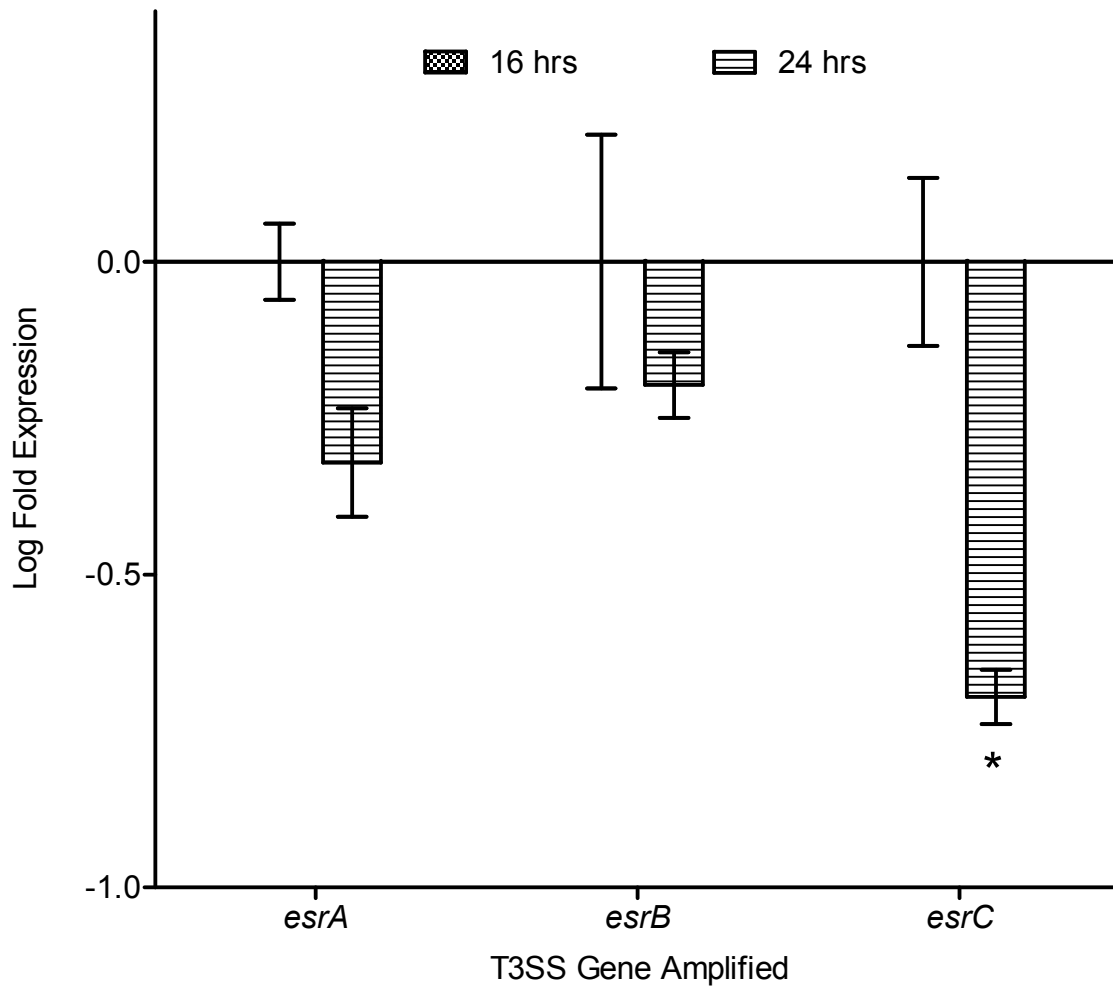


Figure 2.15. Effect of culture to stationary phase on *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory gene expression. Quantitative PCR was used to estimate the expression of *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and the *esrC* operon containing *esrC* and the apparatus genes *esaGHIJWKL* following culture to late log (16 hrs) or stationary phase (24 hrs) in MM19 pH 7.0. Bars represent the mean (\pm SEM) of the log fold expression of triplicate samples. * indicates a significant difference in expression of the target gene between culture conditions ($P \leq 0.05$).

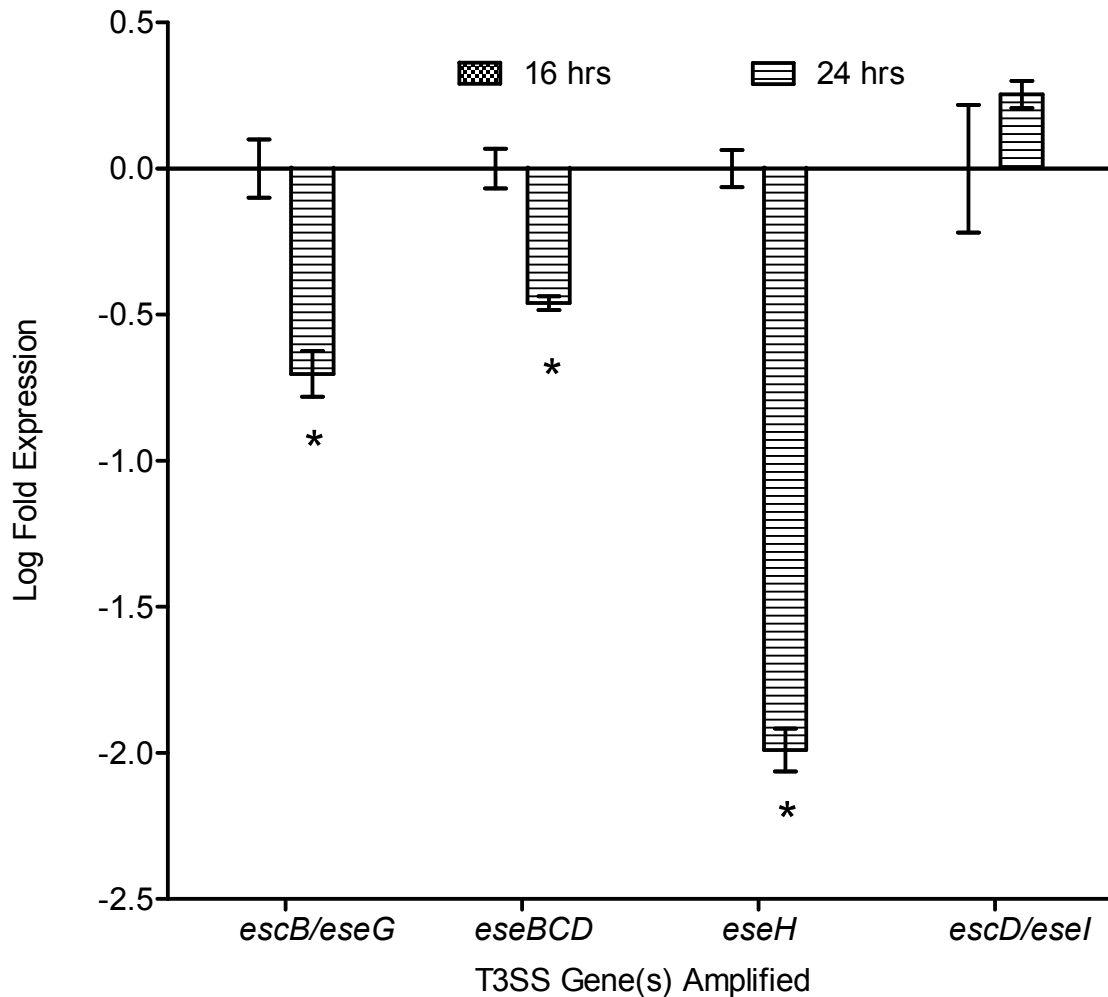


Figure 2.16. Effect of culture to stationary phase on *Edwardsiella ictaluri* type III secretion system (T3SS) non-regulatory gene expression. Quantitative PCR was used to estimate the expression of *E. ictaluri* T3SS non-regulatory genes encoded by the *escB/eseG* operon, the *escC* operon containing the translocon genes *eseBCD*, the pEI1-encoded *eseH*, and the pEI2-encoded *escD/eseI* operon following culture to late log (16 hrs) or stationary phase (24 hrs) in MM19 pH 7.0. Bars represent the mean (\pm SEM) of the log fold expression of triplicate samples. * indicates a significant difference in expression of the target gene between culture conditions ($P \leq 0.05$).

DISCUSSION

The T3SS of *E. ictaluri*, like those of *E.tarda* and *Salmonella*, is involved in intracellular replication (Thune et al. 2007). Little is known about how the T3SS influences pathogenesis of *E. ictaluri* in the channel catfish host, which proteins are secreted, what their function is, or what regulates expression of the T3SS. The SPI-2 T3SS is activated in conditions that mimic the intracellular environment, including low pH and inorganic phosphate. However, the *E. tarda* T3SS is active and able to secrete T3SS proteins in neutral media, but other conditions such as low pH or nutrient limitation have not been evaluated for *E. tarda* T3SS activity. Because of *E. ictaluri*'s dependence on the T3SS for intracellular replication, it was hypothesized that expression of the T3SS would be induced by exposure to an environment containing acidic or low phosphate conditions. Using T3SS promoter fusions to *gfp+*, RT-PCR, and quantitative real-time PCR, *E. ictaluri* T3SS gene expression is detected in neutral media, but expression of the T3SS operons *esrC*, *escC*, *escB*, and the pEI1-encoded *eseH* is significantly upregulated in acidic MM19 and acidic MM19-P. 2D-PAGE analyses support the findings of the T3SS promoter fusions and qPCR data, because *E.ictaluri* T3SS protein secretion to the supernatant is detected in the acidic conditions that upregulate translocon gene expression. T3SS translocon proteins EseB, EseC, and EseD are abundant in the supernatants of *E. ictaluri* acidic media cultures, as is the T6SS protein EvpC.

pEI2 Encodes a Protein Homologous to T3SS Secreted Effectors. Genetic analysis of pEI2 reveals an open reading frame, *eseI*, downstream of *escD* that is transcriptionally linked to *escD*. EseI has homology to proteins among many genera including *Pseudovibrio*, *Vibrio*, *Salmonella*, *Shigella*, *Escherichia*, and *Chromobacterium*. However, only the homolog of *Shigella*, OspB, is characterized. EseI and OspB share homology only in the C-terminus.

OspB is a secreted effector of *Shigella* (Buchrieser et al. 2000; Santapaola et al. 2002; Lucchini et al. 2005; Santapaola et al. 2006) and is localized to the nucleus of the host cell, affecting the innate immune response of the host cell by reducing cytokine production (Zurawski et al. 2009). The sequence similarity between EseI of *E. ictaluri* and OspB of *Shigella* suggests a similar function of the *E. ictaluri* protein; however, the active site of OspB is not reported, so it may not be in the homologous C-terminus. The N-terminus of OspB targets the protein to the nucleus and early endosomes (Zurawski et al. 2009), suggesting either the N-terminus of EseI has a different function or the signal required for nuclear localization is different.

In *Shigella*, Spa15 is a chaperone for OspB (Page et al. 2002). The similarity of EscD to Spa15 (Fernandez et al. 2001) and the proximity of it in relation to *eseI* suggest *escD* and *eseI* are transcriptionally linked, which is verified by RT-PCR analysis. Encoding a chaperone and effector in a single transcriptional unit ensures the effector protein is not expressed without its chaperone.

Besides EseI, *E. ictaluri* encodes a second plasmid-encoded protein, EseH, homologous to T3SS proteins of other organisms involved in the downregulation of the host inflammatory response. EseH has homology to SspH1 from *Salmonella* that downregulates NF- κ B activity in host cells, suggesting that the *E. ictaluri* T3SS also has a downregulating effect on the host immune response. The T3SS of *E. tarda* either up- or downregulates cytokine production depending on what cell type it infects (Okuda et al. 2006; Okuda et al. 2008). *Edwardsiella ictaluri* upregulates host IL-8 expression during infection of channel catfish (Chen et al. 2005); however, no studies are reported evaluating if the IL-8 response is facilitated or limited by the *E. ictaluri* T3SS.

***E. ictaluri* T3SS Gene Promoter Fusions to GFP Produce Variable Results.** The initial plan of this research was to construct *E. ictaluri* strains containing promoter fusions to *gfp*⁺ for two

reasons. The first was to have a rapid method of culturing *E. ictaluri* in varying conditions and assaying the cultures for green fluorescence to determine T3SS-inducing conditions. The second reason was for tracking the expression of T3SS genes intracellularly using fluorescence microscopy. The promoter fusions are inserted into a non-coding region of the *E. ictaluri* genome between *serC* and *aroA* in a similar manner to that used by Hautefort et al. (Hautefort et al. 2003). Single genomic inserts are preferred to expression from a plasmid vector to eliminate possible variability associated with plasmid instability or copy number variations (Hansen-Wester et al. 2002). Hautefort et al. (Hautefort et al. 2003) were able to reliably detect *Salmonella* carrying single inserts of T3SS promoter-*gfp*⁺ fusions using FACS, but attempts using FACS to detect fluorescence of an inducible *E. ictaluri purA* promoter fusion to *gfp* were only moderately successful (unpublished results). No attempts to use FACS with any of the *E. ictaluri* T3SS promoter fusions were made, because all T3SS promoters produce less than 60% the fluorescence of the *purA* promoter (unpublished results).

Fluorescence detection using the spectrophotometer also proved difficult. Because of the low nutrient growth conditions, culture densities were usually low, requiring concentration for detection of fluorescence. In addition, *E. ictaluri* produces autofluorescence levels that confound fluorescence analysis. The low level of fluorescence generated by the promoter fusions also makes them irrelevant for fluorescence microscopy, because they are difficult to visualize unless present in high densities, which likely would not occur in cell culture until perhaps late in the infection process.

The problems encountered with the promoter fusions are not universal. Promoters for the genes *orf29/30*, *esaB*, and *escC* are detectable using the spectrophotometer, and growth in different conditions demonstrates the environmental influence on these promoters. The reason the other promoters are not detected is unknown. Dieye et al. (Dieye et al. 2007) found that *gfp*⁺

fusions to the promoter for the SPI-2 T3SS gene *sifA* cannot be detected when expressed as a single insertion into the genome, and suggested that surrounding DNA structure has an effect on promoter activity. Expression of T3SS genes in *Salmonella* is dependent upon relaxation of DNA (Ó Cróinín et al. 2006), suggesting that the DNA surrounding the fused promoter insertion is not as susceptible to DNA relaxation as the native promoter regions.

The insertion of the promoter fusions between *serC* and *aroA* in the *E. ictaluri* genome could have resulted in a similar problem. In order to insert DNA between *serC* and *aroA*, a transposase sequence with multiple copies in the genome was deleted. Perhaps portions of regulatory regions of the transposase were not removed, allowing them to affect activation of the T3SS promoter fusions. It is possible regulatory regions or termination sequences for *serC* or *aroA* could have affected expression of the promoter fusions. However, any DNA sequence in the area of insertion that could inhibit promoter activity likely would affect expression of all promoter fusions inserted. Another possibility is that not enough of the promoter regulatory region was included for *gfp*⁺ fusions, leading to inefficient regulation of the promoter.

***Edwardsiella ictaluri* T3SS Genes Are Expressed in Many Conditions.** The limited findings from the promoter fusion suggest that culture pH has an effect on the expression of T3SS genes. Fusions using the promoters for *esaB* and *escC* are strongly upregulated in acidic conditions, whereas the promoter for *orf29/30* is consistently active only in neutral media. RT-PCR analyses of total RNA demonstrate many *E. ictaluri* T3SS genes are expressed to some degree in all culture conditions assayed. However, the analysis is not effective in determining up- or downregulation of most T3SS genes. Similar to the promoter fusion results above, however, *orf29/30* expression is noticeably reduced in acidic media compared to neutral media, indicating downregulation of *orf29/30* in acidic conditions. Surprisingly, an operon encoding T3SS apparatus genes (*esaR*) is not detected in any condition. Perhaps the primers used for *esaR* RT-

PCR analysis are not sufficient for efficient amplification, but the same lack of detection is observed for the *esaR* promoter fusion, suggesting low to no expression of this operon in the conditions assayed. Secretion of translocon proteins in acidic media, however, suggests the proteins encoded by the *esaR* operon are expressed and translated, allowing for secretion of the translocon proteins. Furthermore, analysis of secreted protein from the T3SS-deficient *E. ictaluri* strain *esaU*⁻, which has a mutation in the *esaR* operon, indicates expression of that operon is required for secretion of EseB, EseC, and EseD. Therefore, secretion of translocon proteins in acidic media indicates EsaR is present, but expression is undetectable using the *esaR* promoter fusion or RT-PCR.

The *esaR* operon encodes apparatus proteins predicted to be required in low concentration. Operons such as *escC* and *esaB* both are detected by *gfp*⁺ promoter fusion, and they encode proteins that associate into multimers, either within the membrane or outside the membrane. The requirement for multiple copies of proteins for construction of the translocon or membrane-spanning pores suggest higher expression of those genes, which would be detected more easily by promoter fusions or RT-PCR than *esaRSTU*, which encodes proteins all predicted to be monomeric components of the apparatus.

The *gfp*⁺ fusion and RT-PCR results for *orf29/30* suggest *orf29/30* is not associated with the *E. ictaluri* T3SS. GFP fluorescence is reduced to negative-control levels in all acidic media assayed, and RT-PCR results demonstrate a decline of fluorescence intensity in acidic cultures compared to pH-neutral cultures. This is contradictory to the findings by Zheng et al. (Zheng et al. 2005) whom found expression of *E. tarda orf29* and *orf30* is dependent on the T3SS regulatory protein EsrC. While *esrC* expression is significantly downregulated in MM19 pH 5.5, similar to the GFP and RT-PCR results for *orf29/30*, *esrC* is upregulated in MM19-P pH 5.5, a condition in which *orf29/30* promoter activity is not detected.

Orf29 and Orf30 of *E. tarda* are hypothesized to be effector proteins of the T3SS based on the presence of a high number of coiled regions in the predicted secondary structure and a lack of homology to conserved T3SS apparatus proteins (Zheng et al. 2005). While expression of *orf29* and *orf30* of *E. tarda* is dependent upon *EsrC*, expression was only evaluated in neutral media; therefore, the effect of acidic pH on the expression of *orf29* and *orf30* in *E. tarda* is unknown. Perhaps *esrC* has an influence on *orf29/30* expression in *E. ictaluri* cultured in neutral culture conditions, but the regulation is lost in acidic environments.

The DNA region upstream of *orf29/30* of *E. ictaluri* also is different from *E. tarda*. There is a transposase insertion between *esrB* and *orf29/30* in *E. ictaluri* (Thune et al. 2007) that is not present in *E. tarda*, which may affect the expression of *orf29/30* in *E. ictaluri*. Alternatively, *orf29/30* may be involved in other aspects of T3SS not associated with acidic environments. The *gfp+* fusion and RT-PCR analyses indicate that T3SS genes are being transcribed in neutral conditions, albeit at a lower level than in acidic conditions. The T3SS may have a function in neutral conditions in which the other low phosphate or acid induced genes are either not required or not required in great amounts.

Environmental Cues Influence Expression of the *E. ictaluri* T3SS. Quantitative PCR analyses give a better indication of T3SS gene expression in different culture media than either the promoter fusions or RT-PCR. Analyses of apparatus genes were not conducted using quantitative PCR, so further evaluation of the *esaR* operon was not done. qPCR analyses focused on putative effector, translocon, and regulatory genes, because of the assumption that the expression of those genes is more indicative of T3SS secretion activity.

The operon encoding the translocon proteins is the only one found to be upregulated in acidic minimal media with normal phosphate. Upregulation of the translocon operon in acidic media indicates a departure from the observed regulation of the SPI-2 translocon. Many studies

with *Salmonella* conclude that acidic pH induces secretion and construction of the SPI-2 translocon, but that translocon gene expression is not increased by acidic pH (Beuzon et al. 1999; Nikolaus et al. 2001; Hansen-Wester et al. 2002; Coombes et al. 2004; Chakravorty et al. 2005).

Most *E. ictaluri* T3SS gene upregulation occurs in MM19-P pH 5.5. Low phosphate growth conditions are also a strong inducer of SPI-2 T3SS expression (Beuzon et al. 1999; Deiwick et al. 1999; Hansen-Wester et al. 2002; Lober et al. 2006). Lober et al. (Lober et al. 2006) used microarray analysis of intracellular *Salmonella* to determine that the intracellular environment is phosphate-limited based on expression of a number of low phosphate-inducible genes. The upregulation of the *E. ictaluri* T3SS in conditions similar to conditions that induce SPI-2 T3SS is an indication that the T3SS is expressed and active intracellularly. Thune et al. (Thune et al. 2007) found a T3SS-deficient mutant to be highly attenuated for intracellular replication, supporting the findings that the T3SS is induced in conditions that mimic the intracellular environment of a macrophage.

Effect of phosphate starvation on T3SS gene expression is not reported in *E. tarda*. However, *E. tarda* phosphate-specific transport (PST) mutants that are inhibited in the transport of phosphate into bacterial cells exhibit downregulated expression of *esrC* and *esaC* (Srinivasa Rao et al. 2004; Zheng et al. 2005). Mutation of PST, however, likely results in a different signal than low environmental phosphate. The inability to import phosphate may create an internal signal as opposed to the extracellular signal created by decreased phosphate in the media. An internal low phosphate signal may result in a different signal cascade than in response to an externally low phosphate signal, thereby resulting in dissimilar changes in gene expression in the two conditions.

Because of the inability of PST mutants to import phosphate, the decreased T3SS expression may result in a physiological shortage. The PST mutants may lack the phosphate

concentrations necessary to fulfill their physiological phosphate requirements or lack the ability of sensor kinases to phosphorylate response regulators, thereby impeding signal cascades. Because expression of *esaC* and *esrC* is dependent on EsrB (Zheng et al. 2005) and EsrB requires phosphorylation for activation, the inability of PST mutants to import phosphate may result in the inability of EsrB to regulate T3SS gene expression.

Although *E. ictaluri* T3SS genes are upregulated in response to phosphate limited acidic media, detection of gene transcription is not a guarantee of translation. Some T3SS genes of *Yersinia* are thought to have post-transcriptional control over translation (Anderson and Schneewind 1997). The structure of the mRNA protects itself from being translated unless it comes in contact with the T3SS apparatus, at which time the message is translated and the protein translocated. Also, PhoP can post-transcriptionally regulate SsrA expression in *Salmonella* (Bijlsma and Groisman 2005). Whether or not transcriptional upregulation is indicative of T3SS activity, the results of this study indicate *E. ictaluri* T3SS gene expression is upregulated in the presence of conditions likely to be encountered intracellularly: low pH and low phosphate.

Secretion of *E. ictaluri* T3SS Translocon Proteins *In Vitro* Is pH-Dependent. Acidic media induces secretion of the translocon proteins to the culture media, similar to the response in *Salmonella* (Beuzon et al. 1999; Coombes et al. 2004), but different from *E. tarda* (Srinivasa Rao et al. 2003; Srinivasa Rao et al. 2004; Zheng et al. 2007). The correlation of translocon secretion to the outside of the cell in a pH-dependent manner makes sense if the T3SS is involved in intracellular replication as it is in *Salmonella*. It is interesting, however, that translocon secretion occurs for *E. tarda*, but not *E. ictaluri*, in neutral pH conditions. Perhaps the evolutionary paths of each organism have led to differences in regulatory control of their T3SS.

Edwardsiella tarda causes a much different disease in channel catfish, indicating it either lacks some of the virulence mechanisms of *E. ictaluri* or does not regulate them in the same way.

Although expression of the *E. ictaluri* T3SS effector genes *eseG* (*escB* operon), *eseH*, and *eseI* is detected, their respective proteins are not detected in the whole cell lysate or supernatant. The predicted isoelectric points (pI) of of EseH and EseI, 5.54 and 6.32, respectively, both predict the proteins would fall in the range of the IPG strips used for 2D-PAGE analysis (pH 4-7). EseG, however, has a predicted pI of 7.09, and may be only on the boundaries of the gel. Supernatant effector proteins may simply be obscured by other protein spots from the whole cell lysate gels. However, that likely is not the case in the supernatant gels. Possible explanations for effector proteins not being detected in the supernatant are numerous. First, the proteins may not be expressed or secreted at a high enough level for detection. Second, an apparatus or translocon protein may serve as a cap that prevents secretion of effector proteins unless contact is made with a host membrane. Third, presence of T3SS mRNA does not indicate presence of protein. As described above, some T3SS genes of *Yersinia* are regulated post-transcriptionally by the mRNA, so genes are transcribed, but secretion and presumably translation is dependent on relieving mRNA secondary structure (Anderson and Schneewind 1997). RNA signalling for T3SS protein is reviewed by Sorg et al. (Sorg et al. 2005).

Deiwick et al. (Deiwick et al. 2002) studied the SPI-2 proteome and found that many proteins are not produced at a high enough level for detection using silver staining, which has a comparable detection level to the Sypro Ruby stain used in this study for 2D gel visualization. Proteins had to pulse labelled with [³⁵S] methionine/cysteine for visualization of most SPI-2 T3SS-related proteins. These findings, along with observations that the SPI-2 T3SS is expressed in low numbers on the surface of *Salmonella* (Nikolaus et al. 2001; Chakravorty et al. 2005), suggest that production of effectors, and possibly the apparatus and translocon proteins, is low.

Determination of *E. ictaluri* T3SS effector production and secretion may require alternative methods, such as production of antibodies against the native effector proteins or fusing an epitope tag to the effector proteins. Alternatively, if *in vitro* secretion of effector proteins is limited by a cap on the T3SS apparatus, mutation of the cap protein may result in less restrictive protein secretion. Similar to the results described here, the secreted T3SS proteins of *E. tarda* visible on 2D gels were also limited to translocon proteins (Tan et al. 2002; Srinivasa Rao et al. 2004; Zheng et al. 2005).

The SPI-2 T3SS translocates over twenty proteins. Only a few of those proteins, however, are encoded within the T3SS pathogenicity island. The remaining effector proteins are encoded throughout the genome. *Edwardsiella ictaluri* also encodes putative T3SS effectors outside the T3SS pathogenicity island. However, no effector genes outside the *E. tarda* T3SS are identified. Because of the small number of known T3SS effectors in *E. ictaluri* and *E. tarda*, it is difficult to predict functional homologies between the *E. ictaluri* and *E. tarda* T3SS. While the apparatus and regulatory genes are highly homologous, the function of the T3SS in each organism will be defined by the effectors they secrete. Two putative effector proteins outside of the *E. ictaluri* T3SS have been identified, EseH and EseI. Both are homologous to proteins involved in downregulation of the host immune response, but EseH also has homology to actin polymerization-modulating proteins of *Salmonella* (Miao et al. 2003). It is unknown if *E. tarda* encodes homologous proteins.

Both *E. ictaluri* and *E. tarda* modulate expression of host cell cytokines (Chen et al. 2005; Okuda et al. 2006; Okuda et al. 2008). Host cytokine modulation by *E. tarda* is dependent on an intact T3SS; however, cytokine modulation depends on what type of cell the bacterium has infected (Okuda et al. 2006; Okuda et al. 2008). Infection of channel catfish with *E. ictaluri* results in an increase in IL-8 production (Chen et al. 2005). No work, however, is reported that

uses *E. ictaluri* T3SS mutants to determine if the *E. ictaluri* T3SS modulates the increased IL-8 production in the catfish host. Analysis of *E. ictaluri* T3SS mutants on the production of host cytokines may give insight into the comparative functions of the *E. ictaluri* and *E. tarda* secretion systems.

Both *E. ictaluri* and *E. tarda* have homologous T6SS as well, and both secrete EvpC to the supernatant in similar conditions as secretion of the T3SS translocon proteins. This suggests a functional and regulatory link between the T3SS and T6SS. In *E. tarda*, EsrC is verified as being a regulator of the T6SS, demonstrating crosstalk between two types of secretion systems, likely for coordinated expression and secretion. EvpC secretion is not dependent on the T3SS in either organism; however, *E. ictaluri* extracellular protein 2D-PAGE results indicate a lower concentration of EvpC in the supernatants of a T3SS-deficient strain. Perhaps the inability of the T3SS results in the accumulation of T3SS proteins in the cell, resulting in feedback inhibition of the system. The function of the *Edwardsiella* T6SS is unknown, but it is required for *E. tarda* virulence (Zheng and Leung 2007).

Future Work. Future work is needed to determine the role of EsrA and EsrB on *E. ictaluri* T3SS gene expression, and to determine the importance of the *E. ictaluri* AraC-like regulator EsrC. The EsrC homolog of *E. tarda* is important for expression of some *E. tarda* T3SS genes, as well as T6SS-related virulence genes (Tan et al. 2005; Zheng et al. 2005). Expression of EsrC is dependent on the *E. tarda* EsrAB two-component regulatory system, indicating some effects of EsrA and EsrB on T3SS gene expression is through regulation of EsrC. The qPCR results from this study indicate *esrC* expression is upregulated in acidic, low phosphate media, but its relationship to *esrAB* is unknown. Because the activity of *E. tarda* EsrC is only evaluated in neutral pH culture conditions, it is unknown if other culture conditions effect *esrC* expression or activity. The differences seen in expression of T3SS genes between *E. ictaluri* and *E. tarda* may

be indicative that while their T3SS are homologous, they serve different purposes in pathogenesis. Further analysis of both the *E. tarda* and *E. ictaluri* T3SS will be required to determine the degree of homology between secreted effectors in both sequence and function.

Further analysis of the putative T3SS genes encoded by the *E. ictaluri* plasmids also is needed. Thune et al. (Thune et al. 2007) reported the attenuation of *E. ictaluri* carrying Km insertions in the *E. ictaluri* plasmids in or near the coding regions of suspected T3SS-related genes *eseH* and *escD*. Interestingly, both mutants carry the native and mutant copies of the plasmids, suggesting the mutations have a gene copy number effect rather than a knockout effect. Characterization of these mutants and their role in *E. ictaluri* virulence will provide further knowledge concerning the pathogenesis of *E. ictaluri*.

This work is the first to describe expression of the *E. ictaluri* T3SS in response to environmental stimuli. The environmental conditions simulated are those potentially experienced within the intracellular environment of a macrophage phagosome. Further work is needed to measure expression of T3SS genes within the macrophage to verify the *in vitro* responses are analogous to intracellular responses. A T3SS mutant, *esaU*⁻, is completely attenuated for replication within the macrophage (Thune et al. 2007), indicating that a functioning T3SS is required for replication within the phagosome. Study of the T3SS and the factors involved in its expression in relation to pathogenesis within channel catfish will provide valuable insight into an important facet of the virulence mechanisms employed by this pathogen.

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CHAPTER 3

MUTAGENESIS OF THE *EDWARDSIELLA ICTALURI* T3SS-ENCODED REGULATORY GENES

INTRODUCTION

Edwardsiella ictaluri causes enteric septicemia of catfish (ESC) and is found throughout the Southeastern United States, the primary region of channel catfish production (Anonymous 2003a; Anonymous 2003b). *Edwardsiella ictaluri* encodes a *Salmonella* pathogenicity island 2 (SPI-2) class of type III secretion system (T3SS) required for replication within host cells (Thune et al. 2007). Encoded within the *E. ictaluri* T3SS pathogenicity island are the putative regulatory genes *esrA*, *esrB*, and *esrC*. *EsrA*, *EsrB*, and *EsrC* are homologous to *E. tarda* *EsrA*, *EsrB*, and *EsrC*. *EsrA* and *EsrB* of both *E. ictaluri* and *E. tarda* are homologous to *SsrA*-*SsrB* proteins of *Salmonella*, which serve as a two component regulatory system required for expression of the SPI-2 T3SS (Shea et al. 1996; Valdivia and Falkow 1996; Cirillo et al. 1998; Deiwick et al. 1999; Worley et al. 2000; Garmendia et al. 2003; Feng et al. 2004; Deiwick et al. 2006; Dieye et al. 2007; Walthers et al. 2007). *Salmonella*, however, does not have a homolog to *EsrC* in the SPI-2 T3SS. *EsrC* is homologous to *AraC*/*XylS* type transcriptional regulators, which are generally involved in positive regulation of gene transcription (Gallegos et al. 1997).

The T3SS of *E. ictaluri* has strong homology to the T3SS of *E. tarda* (Srinivasa Rao et al. 2003; Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005). Expression of *E. tarda* *esrC* is dependent on the expression of *EsrA* and *EsrB*. Mutation of *esrB* and *esrC* results in decreased expression of the *E. tarda* T3SS translocon proteins *EseB*, *EseC*, and *EseD*, which greatly attenuates *E. tarda* intracellularly and *in vivo* (Srinivasa Rao et al. 2004; Zheng et al. 2005). A mutation in *esrC*, however, maintains some ability to replicate intracellularly (Zheng et al. 2005).

EsrC also regulates expression of the type VI secretion system (T6SS) in *E. tarda*. Expression of T6SS proteins is reduced in an *esrC* mutant, suggesting *EsrC* has a crosstalk

function between secretion systems (Zheng et al. 2005). *Edwardsiella tarda* T6SS proteins have homology to *E. ictaluri* proteins recognized as immunogenic by the channel catfish immune system (Moore and Thune 1999; Moore et al. 2002). *Edwardsiella ictaluri* secretes a T6SS protein, EvpC, in both pH 7.0 and pH 5.5 conditions, whereas the T3SS translocon proteins are secreted only in acidic media, indicating regulation of the two systems is different (Chapter 2).

Expression of the SPI-2 T3SS is greatly dependent on acidic pH and low phosphate *in vitro* (Cirillo et al. 1998; Beuzon et al. 1999; Deiwick et al. 1999; Lee et al. 2000; Hansen-Wester et al. 2002; Garmendia et al. 2003; Hautefort et al. 2003; Coombes et al. 2004; Feng et al. 2004; Kim and Falkow 2004; Lober et al. 2006; Walthers et al. 2007). The effect of environmental conditions on expression of the SPI-2 T3SS is primarily through expression and activation of the SsrAB two-component regulatory system. However, expression of *ssrAB* is affected by two other global two-component regulators PhoPQ and OmpR/EnvZ (Lee et al. 2000; Worley et al. 2000; Feng et al. 2003; Garmendia et al. 2003; Feng et al. 2004; Bijlsma and Groisman 2005) and also SlyA (Okada et al. 2007). The involvement of multiple regulatory systems for expression of the T3SS presents a complex network of signaling and activation.

The *E. ictaluri* T3SS, like the SPI-2 T3SS, is upregulated when cultured in acidic media with limited inorganic phosphate (Chapter 2). Expression of T3SS-related genes is detected at neutral pH and with normal phosphate concentrations, but at a much lower level than when cultured in acidic media with limited inorganic phosphate. Conversely, the *E. tarda* T3SS is active at neutral pH in Dulbecco's Modified Eagle Medium. The differences in conditions required for T3SS induction between *E. tarda* and *E. ictaluri* suggest differences in T3SS expression regulation and potentially different functions of the T3SS effector proteins. The T3SS of both organisms, however, is required for intracellular replication (Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005; Okuda et al. 2006; Thune et al. 2007; Okuda et al. 2008)

In this study, the EsrAB two-component regulatory system of *E. ictaluri* was mutated by gene replacement in both reading frames, and the AraC-type regulator EsrC was mutated by deleting the putative helix-turn-helix DNA binding domains. Quantitative PCR was used to determine the effect of the T3SS regulatory gene mutations on expression of *E. ictaluri* T3SS-related chaperone, effector, and translocon genes. To determine effects on subsequent virulence phenotypes, the mutants were evaluated for their ability to survive in channel catfish macrophages and in channel catfish. The results demonstrate that T3SS-encoded regulators control virulence gene expression inside and outside of the T3SS pathogenicity island. Mutation of all three genes reduces T3SS expression; however, mutation of *esrB* results in greater loss of T3SS gene expression than mutation of *esrA* or *esrC*. Loss of EsrC, however, negatively effects production of the putative T6SS secreted protein EvpC, indicating that EsrC has a role in coordinating expression of *E. ictaluri* virulence genes other than those in the T3SS. Only mutations in *esrA* and *esrB* affect intracellular replication within channel catfish macrophages; however, all T3SS regulatory genes are required for virulence in channel catfish. These studies indicate each *E. ictaluri* regulatory gene is important for infection of channel catfish and suggest differences in the regulation of the T3SS between *E. ictaluri* and *E. tarda*. This study demonstrates the dependence of T3SS expression on EsrB and indicates EsrC has a function in virulence beyond T3SS gene regulation.

MATERIALS AND METHODS

Bacterial Strains and Culture. The bacterial strains and plasmids used in this study are listed in Table 3.1. *Escherichia coli* strains were cultured with aeration in Luria-Bertani (LB) broth supplemented with appropriate antibiotics at 37° C. *Edwardsiella ictaluri* strains were grown in LB supplemented with mannitol salts at 28° C with antibiotics as appropriate. *E. ictaluri* also was cultured in MM19 (Collins and Thune 1996) at pH 5.5 and 7.0 and low phosphate MM19

Table 3.1. Bacterial strains and plasmids used in Chapter 3.

Bacterial Strains or Plasmid	Description	Source
<i>E. ictaluri</i>		
93-146	Wild type <i>E. ictaluri</i> isolated from a moribund channel catfish from a natural outbreak at a commercial facility in 1993	LSU aquatic diagnostic laboratory
65ST (<i>esaU</i> -)	Derived from parental wild type strain 93-146; carries an insertion of an STM tag S/T in <i>esaU</i>	(Thune et al. 2007)
Δ <i>esrA</i> :: <i>km</i>	93-146 with nucleotides 112 – 2610 of <i>esrA</i> deleted, Km ^r	This work
Δ <i>esrB</i> :: <i>km</i>	93-146 with nucleotides 58 – 564 of <i>esrB</i> deleted, Km ^r	This work
Δ <i>esrC</i> :: <i>km</i>	93-146 with nucleotides 70 – 402 of <i>esrC</i> deleted, Km ^r	This work
Δ <i>esrC</i>	93-146 with nucleotides 70 – 402 of <i>esrC</i> deleted	This work
Δ <i>esrA</i> :: <i>km</i> / <i>pesrA</i>	93-146 with nucleotides 112 – 2610 of <i>esrA</i> deleted carrying <i>pesrA</i> , Km ^r Ap ^r	This work
Δ <i>esrB</i> :: <i>km</i> / <i>pesrB</i>	93-146 with nucleotides 58 – 564 of <i>esrB</i> deleted carrying <i>pesrB</i> , Km ^r Ap ^r	This work
Δ <i>esrC</i> / <i>pesrC</i>	93-146 with nucleotides 70 – 402 of <i>esrC</i> deleted carrying <i>pesrC</i> , Ap ^r	This work
WT/ <i>pesrA</i>	93-146 carrying <i>pesrA</i> , Ap ^r	This work
WT/ <i>pesrB</i>	93-146 carrying <i>pesrB</i> , Ap ^r	This work
WT/ <i>pesrC</i>	93-146 carrying <i>pesrC</i> , Ap ^r	This work
<i>Escherichia coli</i>		
XL1 Blue MRF ⁺	(<i>mcrA</i>)183 (<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F ⁺ <i>proABlacIqZ.M15</i> Tn5(Km)]	Stratagene, La Jolla, CA
CC118 λ <i>pir</i>	Δ (<i>ara-leu</i>) <i>araD</i> Δ <i>lacX74 galE galK phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recA1</i> λ <i>pir</i> lysogen	(Herrero et al. 1990)
SM10 λ <i>pir</i>	<i>thi1 thr1 leuB supE44 tonA21 lacY1 recA</i> ::RP4-2-Tc::Mu Km ^r λ :: <i>pir</i>	(Simon et al. 1983)
Plasmids		
pBluescript SK-	Cloning vector	Stratagene, La Jolla, CA
pBS:: Δ <i>esrA</i>	pBluescript carrying <i>esrA</i> with nucleotides 112 – 2610 deleted and replaced with an <i>EcoRI</i> recognition site inserted between <i>KpnI</i> and <i>XbaI</i>	This work
pBS:: Δ <i>esrA</i> :: <i>km</i>	pBluescript carrying <i>esrA</i> with nucleotides 112 – 2610 deleted and <i>km</i> inserted at <i>EcoRI</i> site	This work
pBS:: Δ <i>esrB</i>	pBluescript carrying <i>esrB</i> with nucleotides 58 - 564 deleted and replaced with an <i>EcoRI</i> recognition site inserted between <i>KpnI</i> and <i>XbaI</i>	This work

Table 3.1 continued.

pBS:: <i>ΔesrB::km</i>	pBluescript carrying <i>esrB</i> with nucleotides 58 - 564 deleted and <i>km</i> inserted at <i>EcoRI</i> site	This work
pBS:: <i>ΔesrC</i>	pBluescript carrying <i>esrC</i> with nucleotides 70 - 402 deleted and replaced with an <i>EcoRI</i> recognition site inserted between <i>KpnI</i> and <i>XbaI</i>	This work
pBS:: <i>ΔesrC::km</i>	pBluescript carrying <i>esrC</i> with nucleotides 70 - 402 deleted and <i>km</i> inserted at <i>EcoRI</i> site	This work
pGP704	R6K <i>ori mob Ap^r</i> ; suicide vector used for allelic exchange	(Miller and Mekalanos 1988)
pGP:: <i>ΔesrA::km</i>	pGP704 with <i>ΔesrA::km</i> inserted at <i>KpnI</i> and <i>XbaI</i>	This work
pGP:: <i>ΔesrB::km</i>	pGP704 with <i>ΔesrB::km</i> inserted at <i>KpnI</i> and <i>XbaI</i>	This work
pGP:: <i>ΔesrC::km</i>	pGP704 with <i>ΔesrC::km</i> inserted at <i>KpnI</i> and <i>XbaI</i>	This work
pGP:: <i>ΔesrC</i>	pGP704 with <i>ΔesrC</i> at <i>KpnI</i> and <i>XbaI</i>	This work
pBBR1-MCS4	Broad-host-range cloning vector, <i>Ap^r</i>	(Kovach et al. 1995)
<i>pesrA</i>	pBBR1-MCS4 carrying <i>esrA</i> at <i>XbaI</i> and <i>XhoI</i>	This work
<i>pesrB</i>	pBBR1-MCS4 carrying <i>esrB</i> at <i>XbaI</i> and <i>XhoI</i>	This work
<i>pesrC</i>	pBBR1-MCS4 carrying <i>esrC</i> at <i>XbaI</i> and <i>XhoI</i>	This work

(MM19-P) at pH 5.5. Antibiotics were used in the following concentrations: kanamycin (Km) 50 µg/ml, colistin (Col) 10 µg/ml, and ampicillin (Ap) 200 µg/ml. *Edwardsiella ictaluri* strains isolated from channel catfish or macrophage were cultured on trypticase soy agar plates supplemented with 5% sheep blood (BA, Remel Products, Lenexa, KS).

Specific Pathogen Free Channel Catfish. Channel catfish egg masses obtained from production facilities with no history of *E. ictaluri* outbreaks were disinfected with 100 ppm free iodine and hatched in a closed recirculating aquaculture system at the specific-pathogen-free (SPF) aquatic laboratory at the LSU School of Veterinary Medicine. Commercial catfish diets were used to rear fish at a rate of 2 to 3% body weight per day. Catfish used for immersion infections were between 10 and 20 g at exposure to *E. ictaluri*, and those used for harvesting head kidney-derived macrophages (HKDM) were between 500 and 750 g at the time of harvest.

DNA Protocols. Genomic DNA was isolated similar to the protocol described by Ausubel et al. (Ausubel et al. 1994). Briefly, cells were pelleted by centrifugation at 3700 x g for 5 minutes and resuspended in TE. Cells were lysed with 0.5% SDS in the presence of 50 µg/ml RNase A and 100 µg/ml proteinase K. Phenol:chloroform:isoamyl alcohol (25:24:1) was used to extract protein followed by a chloroform wash. DNA was ethanol precipitated in the presence of 0.12 M sodium acetate. Precipitated DNA was collected by centrifugation at 3700 x g and resuspended in water. Plasmid DNA was isolated using the Qiagen Miniprep Kit (Qiagen Inc., Valencia, CA.). Restriction digests were conducted using enzymes purchased from New England Biolabs (Ipswich, MA), and DNA was purified using the Qiaquick Kit or Minelute Kit (Qiagen).

T3SS Regulatory Gene Mutagenesis. Deletion mutants were constructed using a modified fusion PCR procedure. Polymerase chain reaction was used to amplify 5' and 3' regions of the target genes using gene specific primers (Table 3.2). Rather than using fusion PCR to join the 5' and 3' regions, ligation at restriction sites was used (Figure 3.1). Restriction sites for *KpnI* and *XbaI* were added to P1 and P2 primers, respectively. *EcoRI* sites were added to the P3 and P4 primers. Following amplification using P1 and P2 primers paired with P3 and P4 primers, respectively to amplify the 5' and 3' flank regions, 5' and 3' PCR products were digested and ligated via the *EcoRI* sites. The ligation product was used as template for PCR using primers P1 and P2 (Figure 3.1), resulting in deletion of an internal sequence of the gene of interest. The construct was ligated into pBluescript at *KpnI* and *XbaI* sites, and a Km cassette was inserted into the *EcoRI* deletion site. The construct containing the deletion mutant with the antibiotic marker and flanking regions was excised from pBluescript using *KpnI* and *XbaI* and ligated into pGP704, a suicide vector for *E. ictaluri*. The vector was transferred to *E. ictaluri* via conjugation, and mutagenesis occurred by allelic exchange. Successful recombinants were identified by resistance to Km and AP sensitivity.

Table 3.2. Oligonucleotide sequences used for PCR in the mutagenesis of *Edwardsiella ictaluri* type III secretion system regulatory genes. Bold text indicates a *KpnI* site, underlined is an *EcoRI* site, and italicized is an *XbaI* site.

Primer	Primer Type	Sequence
5' <i>esrA</i> -F	P1	5'-TATATAG GTACCA ACCCTACCCATATTGCC-3'
5' <i>esrA</i> -I	P3	5'-TATATAG <u>AATTC</u> GTTCAGCAGCAGCGTCAC-3'
5' <i>esrB</i> -F	P1	5'-ATATAT GGTACCC GC GGG GACATTATCAGGA-3'
5' <i>esrB</i> -I	P3	5'-TATATAG <u>AATTC</u> ATTACGGATGCCATCGGC-3'
5' <i>esrC</i> -F	P1	5'-TATATAG GTACCC GTCTGCAACGATACGCT-3'
5' <i>esrC</i> -I	P3	5'-TATATAG <u>AATTC</u> CAGCCTGAGCATGGTTTC-3'
3' <i>esrA</i> -F	P2	5'-TATATATCTAGAGCTGCCACTGATTCGGAG-3'
3' <i>esrA</i> -I	P4	5'-TATATAG <u>AATTC</u> GGCCTGACGTTGGTACAT-3'
3' <i>esrB</i> -F	P2	5'-ATATATCTAGATGCGAGAAAAGCGCGATC-3'
3' <i>esrB</i> -I	P4	5'-TATATAG <u>AATTC</u> AACTGATGCGCAAGCTG-3'
3' <i>esrC</i> -F	P2	5'-TATATATCTAGACCATTGTTGATGAGGGCC-3'
3' <i>esrC</i> -I	P4	5'-TATATAG <u>AATTC</u> CACTTCAGTCAGTCGCCA-3'

Because both *esrA* and *esrB* are single genes (Figure 3.2A), the insertional mutations will not cause polar effects on transcriptionally linked downstream genes. However, *esrC* may be transcriptionally linked to downstream apparatus genes (Figure 3.2B). To ensure expression of these genes is not compromised by an antibiotic cassette insertion, *esrC* must be a markerless in-frame deletion. To accomplish this, the process described above was conducted to create a $\Delta esrC::km$ insertion mutant. Following successful recombination and creation of a $\Delta esrC::km$ mutant, a second conjugation was done, this time using a pGP704 vector carrying only the $\Delta esrC$ construct without the Km cassette. Successful recombination was identified by the loss of Km resistance. In-frame mutagenesis was conducted in this manner in order to have a trait (Km resistance) to select against for markerless deletion mutagenesis. PCR and DNA sequencing verified all mutations.

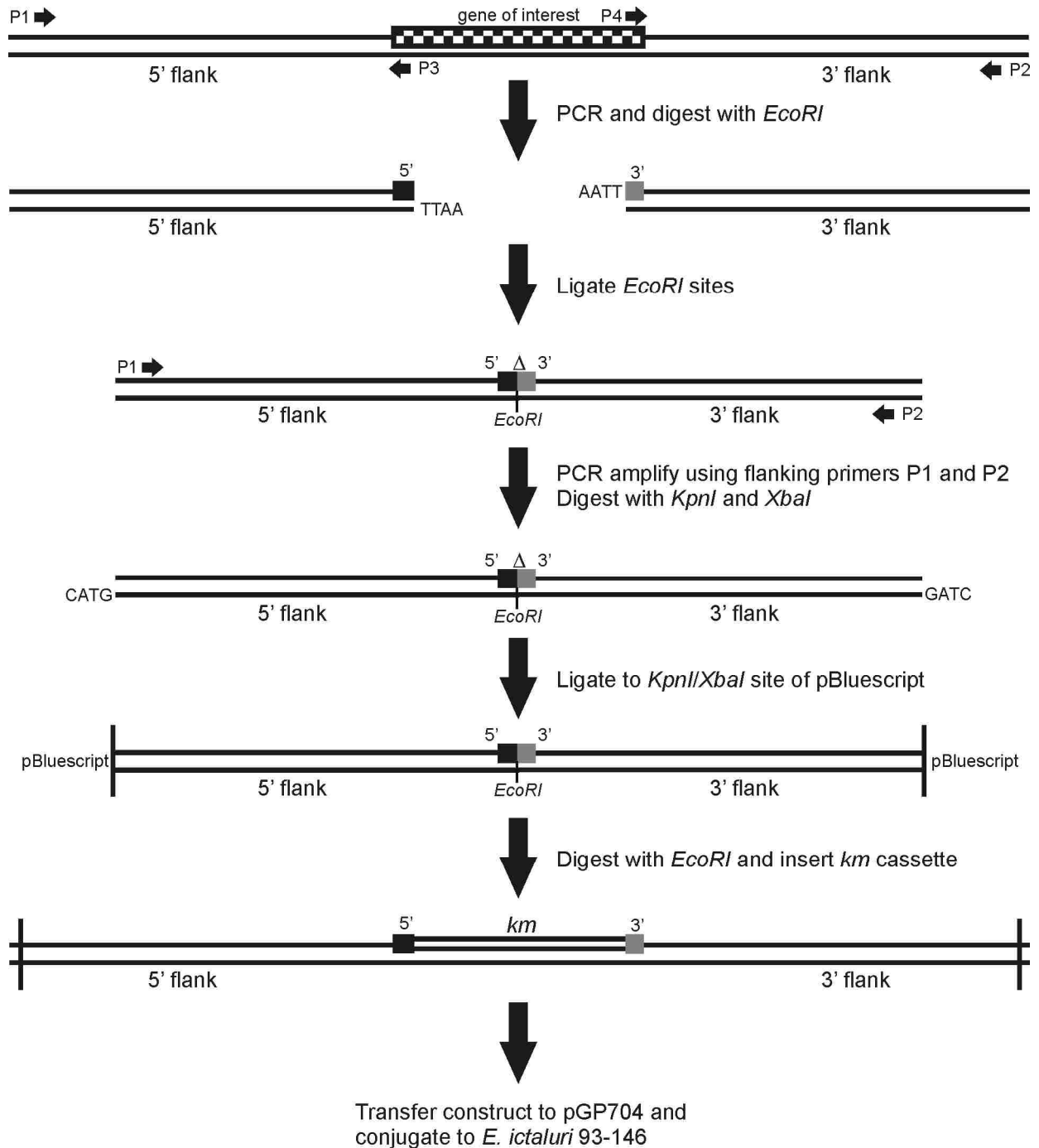


Figure 3.1. Schematic describing construction of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory mutants. The 5' and 3' ends of T3SS regulatory genes were amplified by PCR along with at least 800 nt of DNA flanking the genes. The 5' and 3' fragments were ligated via *EcoRI* ends resulting in the in-frame deletion of an internal coding sequence. Deletion constructs were inserted into pBluescript. A Kanamycin (Km) resistance gene was inserted into the *EcoRI* site, and constructs were inserted into the *E. ictaluri* genome via allelic exchange, resulting in mutation of the regulatory genes at their native locus. For *esrC*, a second allelic exchange was done using the markerless deletion construct to remove the Km cassette, resulting in a markerless in-frame *esrC* mutation.

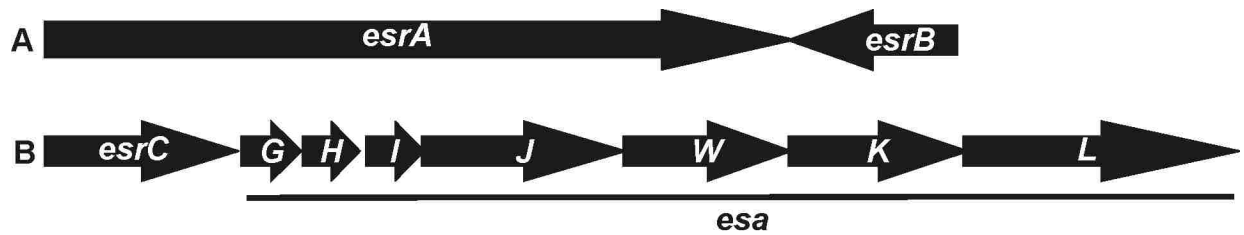


Figure 3.2. Genetic organization of *Edwardsiella ictaluri* type III secretion system regulatory genes. *esrA* and *esrB* are encoded adjacent to each other in the opposite direction (A). *esrC* may be the first gene of an operon encoding *esaGHIJWKL* (B).

Complementation of T3SS Regulatory Mutants. *Edwardsiella ictaluri* genomic DNA was used as template for PCR to amplify the T3SS regulatory genes *esrA*, *esrB*, and *esrC*. Included in the amplified product were at least 250 bases of upstream DNA containing the promoter regions of the genes. Primers for PCR (Table 3.3) were designed to contain the restriction sites for *XbaI* and *XhoI* in their 5' and 3' ends, respectively. Following amplification, PCR products were digested with *XbaI* and *XhoI* (New England Biolabs) and ligated into the *XbaI* and *XhoI* region of pBBR1-MCS4 resulting in *pesrA*, *pesrB*, and *pesrC*. Plasmids were maintained in the *Es. coli* strain CC118 λ *pir*. For conjugation of the complementation plasmids into *E. ictaluri*, *Es. coli* SM10 λ *pir* was used as the donor strain. In addition to conjugating the complementation plasmids to the appropriate *E. ictaluri* mutant strains, each complementation plasmid was conjugated to *E. ictaluri* 93-146 to determine to what extent, if any, the presence of the complementation plasmid has on gene expression and virulence of the wild type (WT) strain.

RNA Isolation. Total RNA was isolated using RNeasy Protect Bacteria RNeasy Mini Kit (Qiagen) and quantified using 260/280 absorbance ratios. Total RNA was treated with Baseline Zero DNase (Epicentre Biotechnologies, Madison, WI) to remove contaminating DNA and adjusted to a concentration of 10 ng/ μ l.

Table 3.3. Oligonucleotides used for construction of complementation plasmids for *esrA*, *esrB*, and *esrC* mutants in *Edwardsiella ictaluri*. Underlined sequences are *XbaI* sites and bolded sequences are *XhoI* sites.

Primer	Sequence
<i>esrA</i> -For	5'-ATTTAATTTCTAG <u>A</u> ATGCAGGTGATGCCGGAAA-3'
<i>esrA</i> -Rev	5'-TTTAATTCT C GAGGCTGGAGGTTTAATCCGCCT-3'
<i>esrB</i> -For	5'-ATTTAATTTCTAG <u>A</u> CGATGCATTCCACAAATCCA-3'
<i>esrB</i> -Rev	5'-ATTTAATTCT C GAGATACGCTAAAGGGGTTGGCC-3'
<i>esrC</i> -For	5'-TTTAATTTCTAG <u>A</u> ATCGACTGCCTCAATGACGC-3'
<i>esrC</i> -Rev	5'-TTTAATTCT C GAGACCGTGACCATGTTTAGGCG-3'

Quantitative PCR. *Edwardsiella ictaluri* WT and mutant strains were cultured to late log phase in MM19-P pH 5.5 to determine the effect of T3SS regulatory gene mutation on T3SS gene expression. Reverse transcriptase PCR (RT-PCR) was conducted by using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qPCR) was conducted using cDNA generated from the RT-PCR reaction as template. Amplification of cDNA was accomplished using T3SS gene specific primers (Table 3.4) and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative quantification was done using 16s rRNA as the endogenous control and expression in MM19 pH 7.0 as the calibrator. Cycling was done as follows: 1x 95° C 10 min followed by 40x 95° C 15 sec and 60° C 1 min. A dissociation curve was done for each reaction to verify that the amplified product was specific. Reactions were run using cDNA amplification reactions lacking reverse transcriptase to ensure amplification did not occur from contaminant DNA.

Whole Cell Lysate and Extracellular Protein Preparation. Whole cell lysate and extracellular protein preparations were done as described in Chapter 2. Briefly, *E. ictaluri* strains were cultured in MM19 pH 5.5 to late log phase. Bacteria were pelleted, and the supernatant was set aside for extracellular protein precipitation. Bacterial pellets were washed in phosphate buffered

Table 3.4. Oligonucleotides used for quantitative PCR in Chapter 3. Putative functions of translational products of the genes in *Edwardsiella ictaluri* are given. Multiple functions indicate more than one type of protein is encoded by the operon.

Gene	Putative Function(s)	5' Primer	3' Primer
<i>escB</i>	chaperone; effector	CTTTACCTTGCGATTTGCCTGCGT	AACAGGCACTCCGCCATATGAAAC
<i>escC</i>	chaperone; translocon	AATGCAAGACCTACAGCAGCGTCA	GCGTTGCGATCTCTTGCTGTAACG
<i>eseH</i>	effector	AAGAGGCTGGATGTCTCTGGTACT	GGTAGCCTTGGCATAGGAGTGTTA
<i>eseI</i>	chaperone; effector	GCAACCCTGCTTAGCAAGTGGAAA	TCAAAGCCTTCCTGGGCCTAATGGA
<i>esrA</i>	regulatory	AGAGCGAGCATCTGAACAGCATCA	AGTAAGTCATGCTGCTCCTGCGTA
<i>esrB</i>	regulatory	CAATGCAGCATGCATCACTGGGAA	TCAGCGATATCCCGGTTGCGATTA
<i>esrC</i>	regulatory; apparatus	AAAGTTTGGGATGGCGCCGAA	GAGAAATGGGCGGCGTTACAGAAT
16s		AACGCGAAGAACCTTACCTGGTCT	GCTCGTTGCGGGAATTAACCCAA

saline pH 5.5 three times before being resuspended in water at a rate of 1 ml of water per 100 μ l of pelleted cell volume. Phenylmethylsulphonyl fluoride (PMSF) was added as a protease inhibitor to a final concentration of 1 mM and incubated on ice for 10 min. Bacterial suspensions were subjected to dismembration by sonication using a Fisher 500 sonic dismembrator at 45% amplitude for 1min per ml of suspension. Samples were pelleted by centrifugation at 12,000 x g for 30 min at 4° C, and supernatants were collected. Thimerosal was added to each sample for a final concentration of 0.01% (Moore and Thune 1999) to prevent microbial contamination. Whole cell lysates were stored at -80° C until two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis.

Supernatants from above were filtered through a 0.22 μ m cellulose acetate low protein-binding filter (Corning Inc., Corning, NY) to remove any remaining bacteria. Trichloroacetic acid (80%) was added to a final concentration of 10% and incubated 16-24 hrs at 4° C. Proteins were pelleted by centrifugation at 24,000 x g for 30 min at 4° C. Pellets were air dried briefly and resuspended in 1 ml water. Samples were vortexed well and stored at -80° C in 100 μ l aliquots.

2D-PAGE. The Bio-Rad Protein Assay (Bio-Rad Laboratories) was used to determine the concentration of protein samples. A similar amount of whole cell lysate protein was analyzed for each *E. ictaluri* strain. However, extracellular proteins were loaded based on the amount of supernatant from which they were precipitated in order to compare relative amounts of protein secreted by each strain.

Whole cell lysate and extracellular protein samples from above were purified by using ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories). Whole cell lysates were resuspended in rehydration buffer at a rate of 100 µg of protein per 185 µl rehydration buffer. Each 100 µl extracellular protein prep was cleaned and resuspended in 185 µl of rehydration buffer. The final concentration of extracellular protein in rehydration buffer varied with the amount of protein secreted by each *E. ictaluri* strain.

Immobilized pH gradient (IPG) strips, pH 4 – 7 (Bio-Rad Laboratories) were rehydrated using 185 µl of each protein prep resuspended in rehydration buffer. Strips were allowed to rehydrate 24 hrs at room temperature. Following rehydration, isoelectric focusing was conducted using a Bio-Rad Protean IEF Cell (Bio-Rad Laboratories). Each protein sample was focused using the following program at 20° C: rapid ramping to 250 V for 15 min; slow ramping to 2000 V for 1 hr; hold at 2000 V for 3 hrs; linear step to 5000 V for 1 hr; hold at 5000 V for 3 hrs; rapid ramping to 8000 V for 1 hr; 8000V for 40,000 Vhrs; rapid ramping to 500 V until strips were removed. Following isoelectric focusing, strips were removed and processed for the second dimension.

Focused IPG strips were prepared for SDS-PAGE by washing with ReadyPrep 2D Starter Kit Equilibration Buffers I and II for 15 min each. Following equilibration, strips were loaded onto Criterion precast 12.5% gels (Bio-Rad Laboratories). Gels were subjected to electrophoresis for 55 min at 200V, then removed and fixed for 1 hr in 10% methanol and 7% glacial acetic acid.

Following fixation, gels were stained with Sypro Ruby stain (Bio-Rad Laboratories) for at least 3 hours, washed in fixative for 1 hr to destain, rinsed three times in water, and imaged under UV light using a Bio-Rad Gel Doc XR and Quantity One software (Bio-Rad Laboratories). Each protein sample was isolated and analyzed by 2D-PAGE in triplicate to ensure reproducibility.

Bacterial Survival and Replication in Channel Catfish Macrophages. Head kidney-derived macrophages (HKDM) were collected from channel catfish and infected with *E. ictaluri* WT and mutant strains as described by Booth et al. (Booth et al. 2006). Briefly, HKDM were harvested and seeded into poly-D-lysine-coated cell culture plates and used in a gentamicin exclusion assay. HKDM were infected with *E. ictaluri* strains at an MOI of 10:1 HKDM:bacteria.

Macrophages were lysed at designated time points using a solution of 1% Triton X-100. Lysates were serially diluted and plated on BA. Colony forming units (CFUs) per well were calculated.

Channel Catfish Infection Challenge. Twenty-liter tanks were stocked with 25 channel catfish each. For immersion, water levels were dropped to 4 L, and bacterial cultures were added for a final concentration of approximately 3×10^{11} CFU/L with aeration maintained. After 1 hour, the water supply was restored. Tanks were observed daily for mortalities. Mortalities due to *E. ictaluri* were confirmed by streaking liver tissue from each mortality onto BA plates and verifying the presence of *E. ictaluri*. Sampling continued until 3 days passed without a mortality.

Statistical Methods. For qPCR samples, cultures were grown in triplicate for RNA samples. Each qPCR reaction was also done in triplicate. C_t , ΔC_t , and $\Delta\Delta C_t$ values were calculated using Applied Biosystems Sequence Detection Software v1.4 (Applied Biosystems). $\Delta\Delta C_t$ values for each reaction were converted to fold expression using the equation $2^{-\Delta\Delta C_t}$. Fold expression was log transformed for homoscedasticity. Mean log fold expression for each strain was calculated with standard error.

For macrophage studies, fold increase was determined by dividing the CFU/well of bacteria at 10 hours by the mean CFU/well at hour 0 and subtracting 1 to adjust for the number of bacteria present at hour 0. Mean fold replication was then calculated with standard error. For mortality challenge, daily mortalities were recorded, and cumulative percent mortality was determined for each tank. Mean cumulative percent mortality was calculated with standard error and reported in graphs. Percent mortality for each tank was arcsine transformed and analyzed as below. Mortality data reported in graphs is non-transformed cumulative percent mortality, but statistical significance in each graph is based on arcsine-transformed data.

Statistical differences were determined by analysis of variance (ANOVA) using the general linear model (Proc GLM) of Statistical Analysis Systems v9.1 (SAS Institute, Cary, NC). Where ANOVA indicated a significant difference, Tukey's HSD posthoc test was used to determine pairwise differences. Differences were considered significant if $P \leq 0.05$.

RESULTS

Construction of Regulatory Gene Mutations. Mutations in the *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and *esrC* were made by allelic exchange. An internal portion of each gene was deleted, and for *esrA* and *esrB*, a Km cassette was inserted into the deleted region, yielding strains $\Delta esrA::km$ and $\Delta esrB::km$. Because *esrA* and *esrB* do not have genes encoded downstream from them on the same strand (Figure 3.2A), the insertion of *km* does not cause polarity issues. However, *esrC* may be the first gene of an operon (Figure 3.2B), and insertion of *km* may cause polar effects on downstream apparatus genes. Therefore, a markerless deletion mutant of *esrC* was created, resulting in strain $\Delta esrC$. In addition, a double mutant, $\Delta esrC/\Delta esrB::km$, was made by creating the $\Delta esrB::km$ mutation in the $\Delta esrC$ strain.

Mutation of *EsrA* results in the removal of amino acids 112 – 2610 from the amino acid sequence. Native *EsrA* contains between two and six transmembrane domains as determined by

using the computer programs DAS (Cserzo et al. 1997), TMpred (Hofmann and Stoffel 1993), TMHMM Server v. 2.0, and Mobyli to predict transmembrane helices. Deletion and subsequent insertion of a Km cassette produces a truncated predicted sequence of 63 amino acids, which results in the deletion of all but one transmembrane domain and no kinase motifs recognizable by Motif Scan (Hulo et al. 2008).

Mutation of *esrB* results in the deletion of amino acids 58 – 564, and with the Km cassette, a predicted amino acid sequence is produced with a length of 51 amino acids, 24 of which are from the inserted Km sequence. The resultant amino acid sequence has no homology to helix-turn-helix (HTH) DNA-binding motifs as determined by three HTH domain prediction programs: GYM 2.0 (Gao et al. 1999; Narasimhan et al. 2002), NPS@ Helix Turn Helix Motif Prediction (Combet et al. 2000), and Motif Scan (Hulo et al. 2008). Motif Scan also does not identify significant matches to any type of amino acid motif contained in the abbreviated *esrB* sequence.

Mutation of *EsrC* results in the removal of amino acids 101 – 211. Using HTH domain predictors, two possible HTH domains are identified in native *EsrC*. The first is between amino acids 139 – 160 or 143 – 164, based on analysis by NPS@ (Combet et al. 2000) and GYM (Gao et al. 1999; Narasimhan et al. 2002), respectively. GYM identifies a second sequence beginning around amino acid 177 that has weak homology to HTH (Gao et al. 1999; Narasimhan et al. 2002). However, using Motif Scan (Hulo et al. 2008), this second sequence is identified as a strong match between amino acids 178 – 222, while the first is identified as a weak match. In either circumstance, the *EsrC* deletion results in the loss of the first HTH sequence and a loss of the majority of the second sequence. Using the above programs to detect motifs in the *EsrC* deletion sequence, no significant HTH sequences are identified.

Deletion of *E. ictaluri* T3SS Regulatory Genes Affects Expression of Genome and Plasmid-Encoded Genes. Quantitative PCR was used to measure the expression of T3SS genes in each of the regulatory mutants. Genes assayed are listed in Table 3.3 and represent regulatory, chaperone, translocon, and effector genes of the T3SS. In the graphs, qPCR data from WT *E. ictaluri* is included as a reference for non-induced expression of T3SS genes. Comparisons in the text to gene expression of mutant strains, however, are generally in relation to the expression of T3SS genes in WT *E. ictaluri* cultured in MM19-P pH 5.5, because regulatory mutants were cultured in acidic, low phosphate media.

Expression of *esrA* is not affected by mutation of either *esrB* or *esrC* (Figure 3.3). Expression of *esrB* similarly is not affected by mutation of either *esrA* or *esrC*. Expression of *esrC*, however, is significantly reduced in both the $\Delta esrA::km$ and $\Delta esrB::km$ strains, indicating *esrC* expression is modulated by the *E. ictaluri* EsrAB two-component regulatory system.

Expression of genomic and plasmid T3SS-related genes, with the exception of *eseI*, is significantly reduced in all of the mutants (Figure 3.4). Although T3SS gene expression is decreased in each mutant, there are differences in the scale of downregulation. Mutation of *esrA* and *esrC* decreases expression of *escB/eseG* approximately one log from WT expression, but mutation of *esrB* results in a near 2.5 log decrease in expression, which is significantly lower than both the *esrA* and *esrC* mutations. Similarly, expression of the *escC* operon is reduced in both the *esrA* and *esrC* mutants, but expression in $\Delta esrB::km$ is significantly lower than in $\Delta esrA::km$ or $\Delta esrC$ and about 2 logs lower than in WT.

T3SS regulators also affect the expression of *eseH*, which is not encoded in the pathogenicity island, but rather on pEI1. Mutation of *esrA* decreases *eseH* expression, but not significantly ($P = 0.0574$). However, mutation of *esrB* and *esrC* have a significant impact on *eseH* expression. Because EsrB is required for expression of *esrC*, and deletion of *esrB*, *esrC*, or

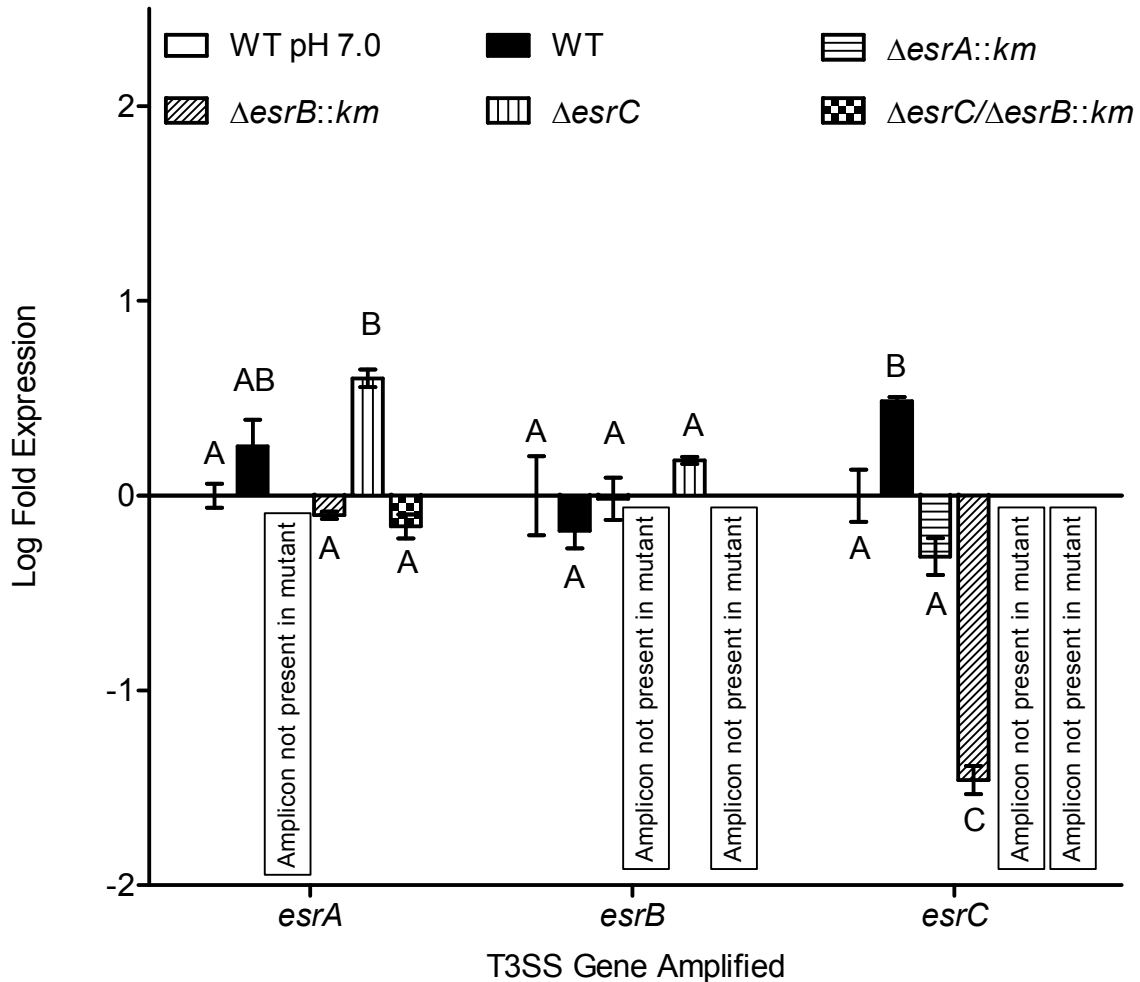


Figure 3.3. Effect of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory gene mutagenesis on expression of T3SS regulatory genes. Quantitative PCR was used to measure expression of the *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and the *esrC* operon containing *esrC* and the apparatus genes *esaGHIJWKL* in wild type (WT) *E. ictaluri* and strains carrying T3SS regulatory gene mutations in *esrA* ($\Delta esrA::km$), *esrB* ($\Delta esrB::km$), *esrC* ($\Delta esrC$), and a *esrB/esrC* double mutant ($\Delta esrC/\Delta esrB::km$). Bacteria were cultured in pH 5.5 low phosphate minimal media (MM19-P). Data for WT cultured in minimal media (MM19) pH 7.0 is presented as a comparison for uninduced T3SS gene expression. Bars indicate the mean (\pm SEM) of the log fold expression of triplicate samples. Values with the same letter within each gene group are not significantly different ($P > 0.05$).

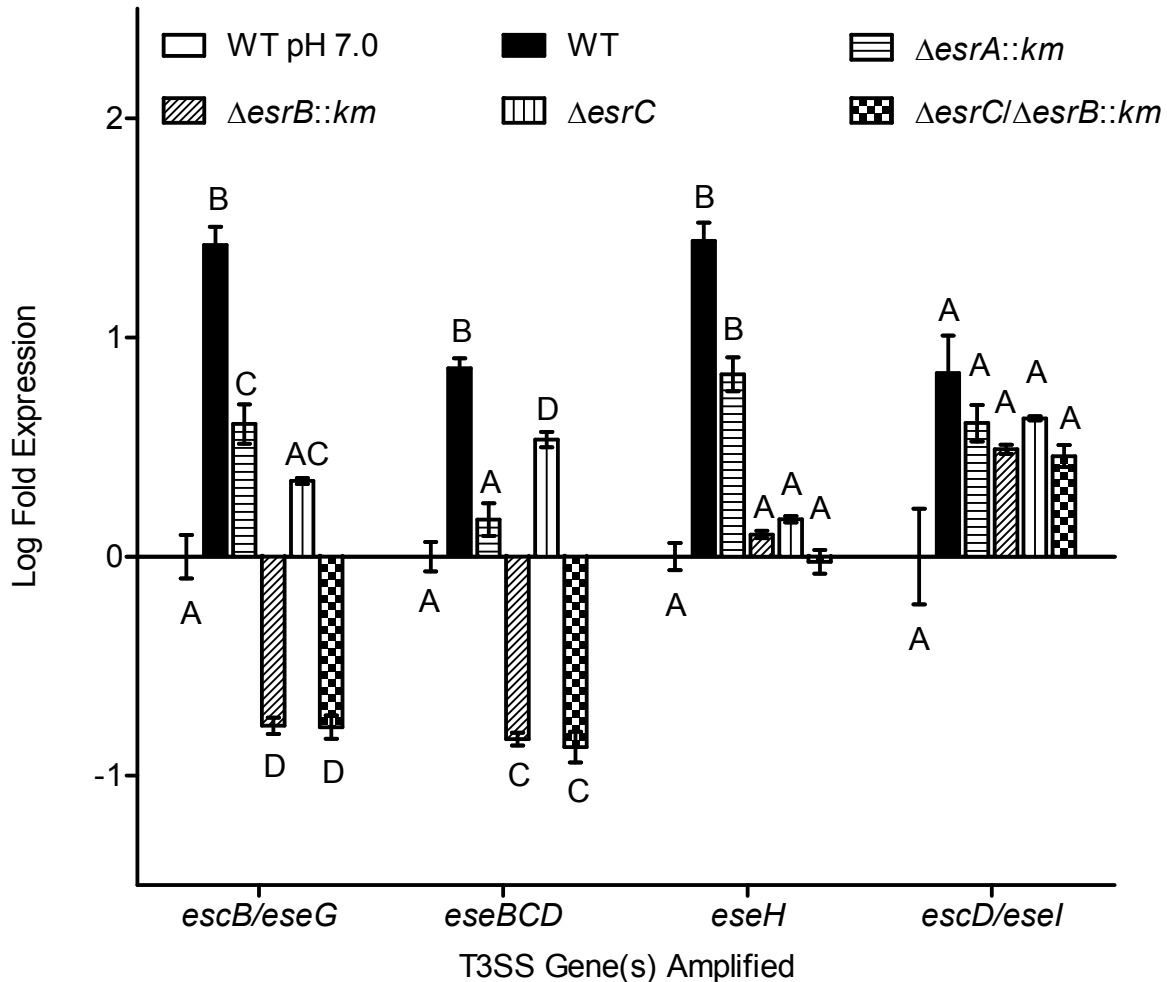


Figure 3.4. Effect of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory gene mutagenesis on expression of T3SS non-regulatory genes. Quantitative PCR was used to measure expression of *E. ictaluri* T3SS non-regulatory genes encoded by the *escB/eseG* operon, the *escC* operon containing the translocon genes *eseBCD*, the pEI1-encoded *eseH*, and the pEI2 encoded *escD/eseI* operon in wild type (WT) *E. ictaluri* and strains carrying T3SS regulatory gene mutations in *esrA* ($\Delta esrA::km$), *esrB* ($\Delta esrB::km$), *esrC* ($\Delta esrC$), and a *esrB/esrC* double mutant ($\Delta esrC/\Delta esrB::km$). Bacteria were cultured in pH 5.5 low phosphate minimal media (MM19-P). Data for WT cultured in minimal media (MM19) pH 7.0 is presented as a comparison for uninduced T3SS gene expression. Bars indicate the mean (\pm SEM) of the log fold expression of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

both results in a similar amount of *eseH* expression, the effect of EsrB on *eseH* may be through regulation of EsrC. None of the mutations has a significant impact on expression of a pEI2-encoded gene, *eseI*. All mean values of *eseI* expression in the mutants were slightly less than WT expression, and similar to each other. The inability of T3SS regulatory mutants to affect *eseI* expression indicates *eseI* expression is not modulated by T3SS regulatory genes.

Double mutation of *esrB* and *esrC* has a similar effect on T3SS gene expression as the single *esrB* mutant, indicating the *esrB* mutation is the dominant phenotype. The dominance of EsrB makes it difficult to determine from this data what the role of EsrC is in regards to control of other genes. EsrB is expressed in Δ *esrC*, and it continues to modulate T3SS gene expression. It is possible EsrC could activate T3SS gene expression in the absence of EsrB, but because EsrB is required for *esrC* expression, an *esrB* mutant strain would need be engineered such that *esrC* is still be expressed to determine the effect of EsrC alone on T3SS gene expression.

These data suggest mutation of *esrB* has a much more profound effect on T3SS gene expression than either *esrA* or *esrC*. T3SS genes are expressed, albeit at reduced levels in *esrA* and *esrC* mutants, indicating expression is influenced, but not dependent on EsrA or EsrC. EsrB and EsrC may act in concert for full activity of the T3SS. The inability of the *esrA* mutant to match the effect of *esrB* on T3SS gene expression suggests EsrB activity is not dependent solely on EsrA for activation, and EsrB can be phosphorylated by other kinases. However, full activity of the T3SS requires EsrA, as evidenced by the significant decreases in T3SS expression in the *esrA* mutant. The finding that T3SS regulators modulate expression of a gene encoded outside of the T3SS, *eseH*, indicates that the effects of the regulators are not limited to the pathogenicity island-encoded genes.

Regulatory Mutants Exhibit Different Protein Secretion Phenotypes. Mutations in T3SS-encoded regulatory genes decrease expression of T3SS translocon genes. However, measurement

of gene activity by qPCR does not provide information on the ability of the regulatory mutants to produce and secrete proteins. Wild type *E. ictaluri* cultured in MM19 pH 5.5 secretes the translocon proteins EseB, EseC, and EseD into the supernatant (Chapter 2). The inability of *E. ictaluri* to produce and secrete these proteins to the cell surface would inhibit the activity of the T3SS. Therefore, the secreted proteins of each T3SS regulatory mutant were analyzed to determine their abilities to secrete translocon proteins.

EseB and EseD are T3SS translocon proteins with molecular weights of 21.6 and 21, respectively, and isoelectric points (pI) of 5.15 and 5.32, respectively. Because of their similarity, they run to similar positions in the gel, making it difficult to determine if both are present. However, both proteins are identified from WT whole cell lysate and extracellular proteins (Chapter 2, Figures 2.13 and 2.14). EseB is abundant in the extracellular protein fraction, as evidenced by the gel streaking that originates from its spot. The abundance of EseB masks the appearance of EseD on extracellular protein 2D gels. For the purposes of these studies, because *eseB*, *eseC*, and *eseD* are encoded by the same operon, expression and secretion of EseD is assumed by the expression and secretion of EseB and EseC, as it is in WT *E. ictaluri*.

Each single mutant has a different protein secretion pattern *in vitro*; however, the $\Delta esrC/\Delta esrB::km$ strain has a phenotype more like $\Delta esrB::km$ than $\Delta esrC$ (Figure 3.5), which is similar to the findings of qPCR. Mutation of *esrA* does not have a noticeable effect on the secretion of EseB, EseC, or EseD into the supernatant (Figure 3.5A) compared to WT (Figure 3.5E). There likely is less protein secreted by the *esrA* mutant, due to the decrease in translocon gene expression shown by qPCR. However, the decrease in expression is not enough to affect translocon secretion.

The *esrB* mutant has severely reduced secretion of EseB, EseC, and EseD into the supernatant (Figure 3.5B). The 2D-PAGE images correlate to the findings of qPCR, which

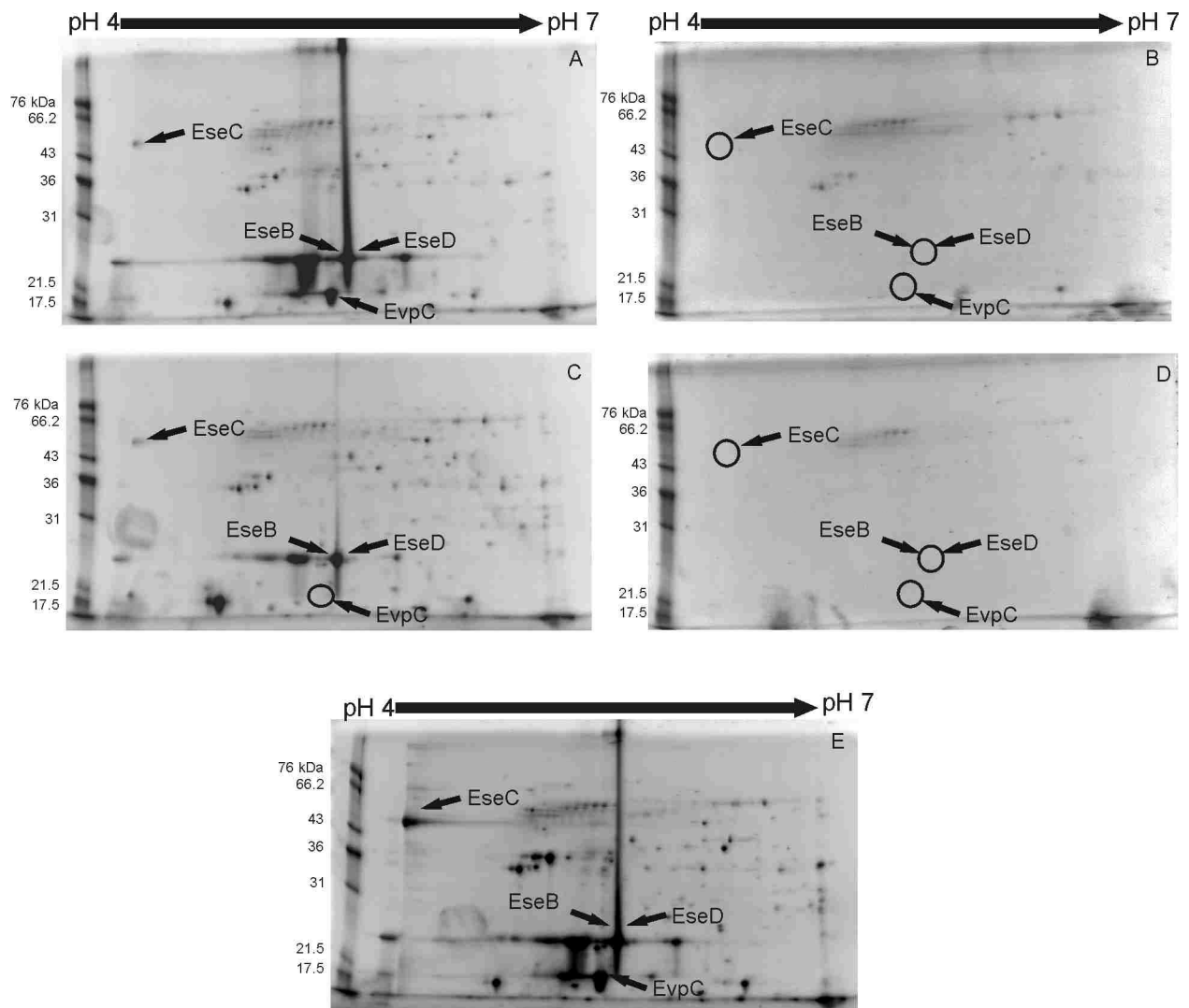


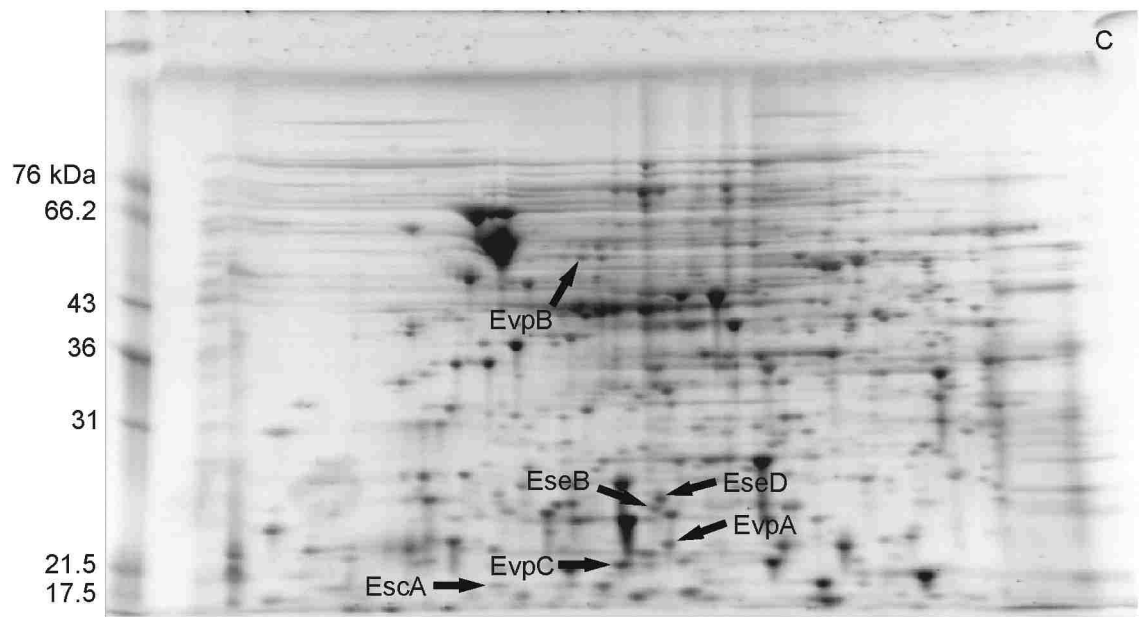
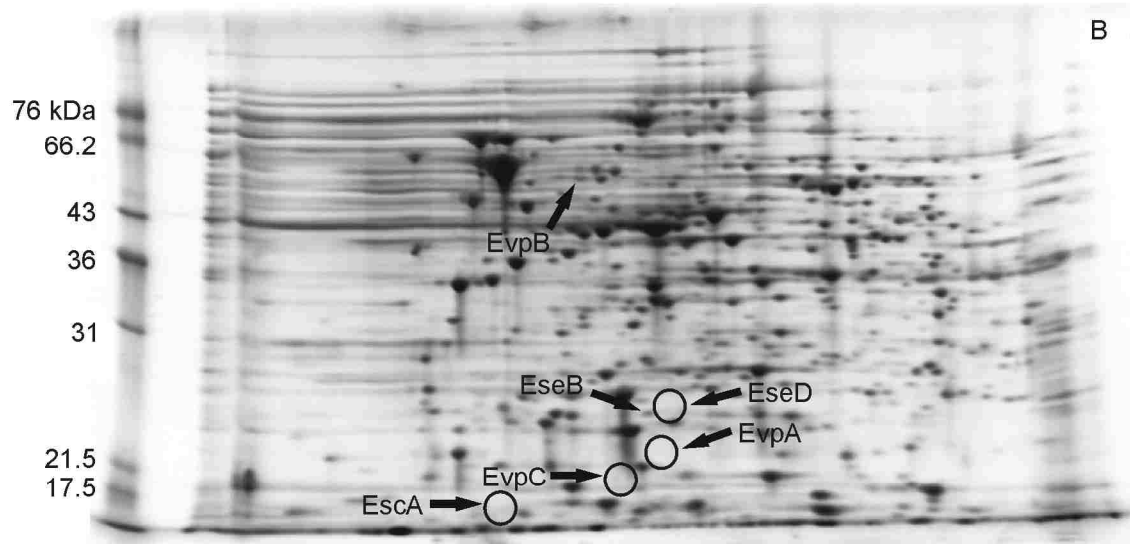
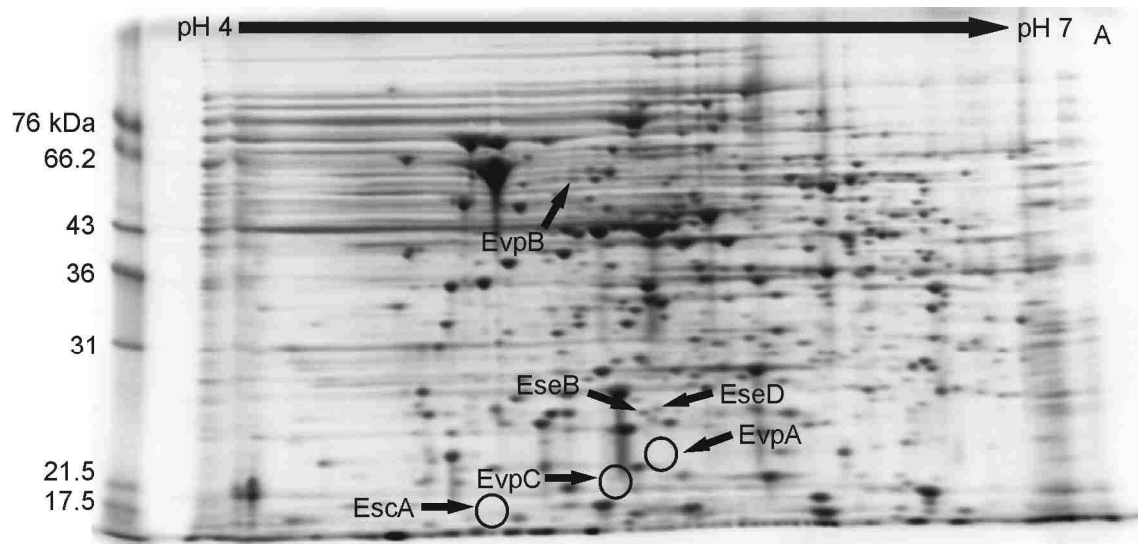
Figure 3.5. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of extracellular protein products of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory gene mutants. Extracellular proteins from T3SS regulatory gene mutants $\Delta esrA::km$ (A), $\Delta esrB::km$ (B), $\Delta esrC$ (C), and $\Delta esrC/\Delta esrB::km$ (D) cultured in pH 5.5 minimal media were collected and separated by 2D-PAGE. Extracellular proteins secreted by wild type *E. ictaluri* cultured in MM19 pH 5.5 are shown (E) for a comparison of normal secretion. Known supernatant proteins are labeled. Circles indicate areas on the gel in which the protein(s) are present when secreted. 2D-PAGE gels were run in triplicate, and a representative gel is shown.

demonstrate that mutation of *esrB* severely decreases translocon gene expression. Also, the protein spot corresponding to a putative T6SS protein, EvpC, is missing. These 2D-PAGE findings also strengthen the notion that EsrB is active in the absence of EsrA, because protein secretion is maintained in the *esrA* mutant. Furthermore, EvpC secretion is also maintained in the *esrA* mutant.

Deletion of *esrC*, like mutation of *esrA*, does not prevent the expression or secretion of translocon proteins (Figure 3.5C). The presence of EseB, EseC, and EseD extracellularly supports the qPCR findings that mutation of *esrC* does not result in a loss of translocon expression. Furthermore, the data demonstrate that EseB, EseC, and EseD are secreted readily in a Δ *esrC* background, indicating EsrC is not required for translocon protein expression. The ability to secrete the translocon proteins also suggests EsrC does not have a significant impact on the expression of apparatus genes that would be required for secretion of the translocon. However, similar to the *esrB* mutant, deletion of *esrC* results in the loss of EvpC secretion. Because EsrB is required for *esrC* expression (Figure 3.3), and EsrC is required for secretion of EvpC, it is likely that EsrC controls expression of *evpC*, and perhaps more T6SS-related gene expression, and the loss of EvpC in Δ *esrB::km* is due to the reduced expression of EsrC as opposed to a direct effect of EsrB on *evpC* expression.

Mutation of *esrB* and *esrC* Decreases Translocon Protein Production. To determine if the loss of protein secretion in Δ *esrB::km* and Δ *esrC* is a result of decreased translational activity, a secretion deficiency, or both, the whole cell lysate proteins from both strains were separated by 2D- PAGE (Figure 3.6). Corresponding spots for the T3SS protein EscA and the T6SS proteins EvpA and EvpC are absent in whole cell lysates from both strains. T3SS proteins EseB and EseD, however, are both present in the *esrB* mutant, indicating some expression of those genes

Figure 3.6 (Following page). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of whole cell lysate proteins of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory gene mutants. Whole cell lysate proteins from *E. ictaluri* T3SS regulatory gene mutants $\Delta esrB::km$ (A) and $\Delta esrC$ (B) cultured in MM19 pH 5.5 were collected and separated by 2D-PAGE. Whole cell lysate protein of the wild type (WT) strain (C) demonstrates the amount of T3SS and T6SS protein found intracellularly when cultured in MM19 pH 5.5. Known whole cell lysate proteins are labeled. Circles indicate areas in which the protein(s) are present in WT whole cell lysates. 2D-PAGE gels were run in triplicate, and a representative gel is shown.



still occurs in the absence of EsrB. The lack of extracellular EseB and EseD, therefore, may be due to suboptimal expression of the translocon, expression defects for T3SS apparatus genes, or a combination of both. EseB is also found in the *esrC* mutant, but EseD is not present. This may indicate EseD is secreted at a similar rate as it is produced, but EseB expresses at a high enough level to accumulate intracellularly as well as being secreted. Although they are secreted in high amounts, both EseB and EseD accumulate intracellularly in WT *E. ictaluri*, as does the T6SS protein EvpC (Figure 3.6C).

The results of the 2D-PAGE analyses of extracellular and intracellular proteins indicate differential importance of the regulators for protein production. Mutation of *esrA* results in near-normal secretion of T3SS translocon proteins and T6SS EvpC. Mutation of *esrB* has a significant effect on the secretion of *E. ictaluri* translocon proteins, correlating to the decreased expression results obtained by qPCR. In addition, EvpC is not secreted to the $\Delta esrB::km$ culture supernatant. Mutation of *esrC* results in a decrease, but not a loss of translocon secretion. Mutation of *esrC*, however, does result in the loss of EvpC secretion. Analyses of whole cell lysates for the *esrB* and *esrC* mutant strains indicate EseB and EseD production occurs in the *esrB* mutant in low levels, suggesting the lack of EseB and EseD in the supernatant is in part possibly due to a T3SS secretion defect.

Replication of *E. ictaluri* T3SS Regulatory Mutants in Channel Catfish Macrophages. Both the *esrA* and *esrB* mutants are attenuated for replication in channel catfish macrophages (Figures 3.7 and 3.8). After 10 hours, each mutant has less than a fold increase while the WT strain has greater than 10 fold replication. However, it is surprising that mutation of *esrA* results in an inability to replicate intracellularly as $\Delta esrA::km$ is able to secrete translocon proteins comparable to WT *E. ictaluri in vitro*, and T3SS gene expression is not greatly reduced. Interestingly, the *esrC* mutant has a similar fold increase as the WT after 10 hours (Figure 3.9),

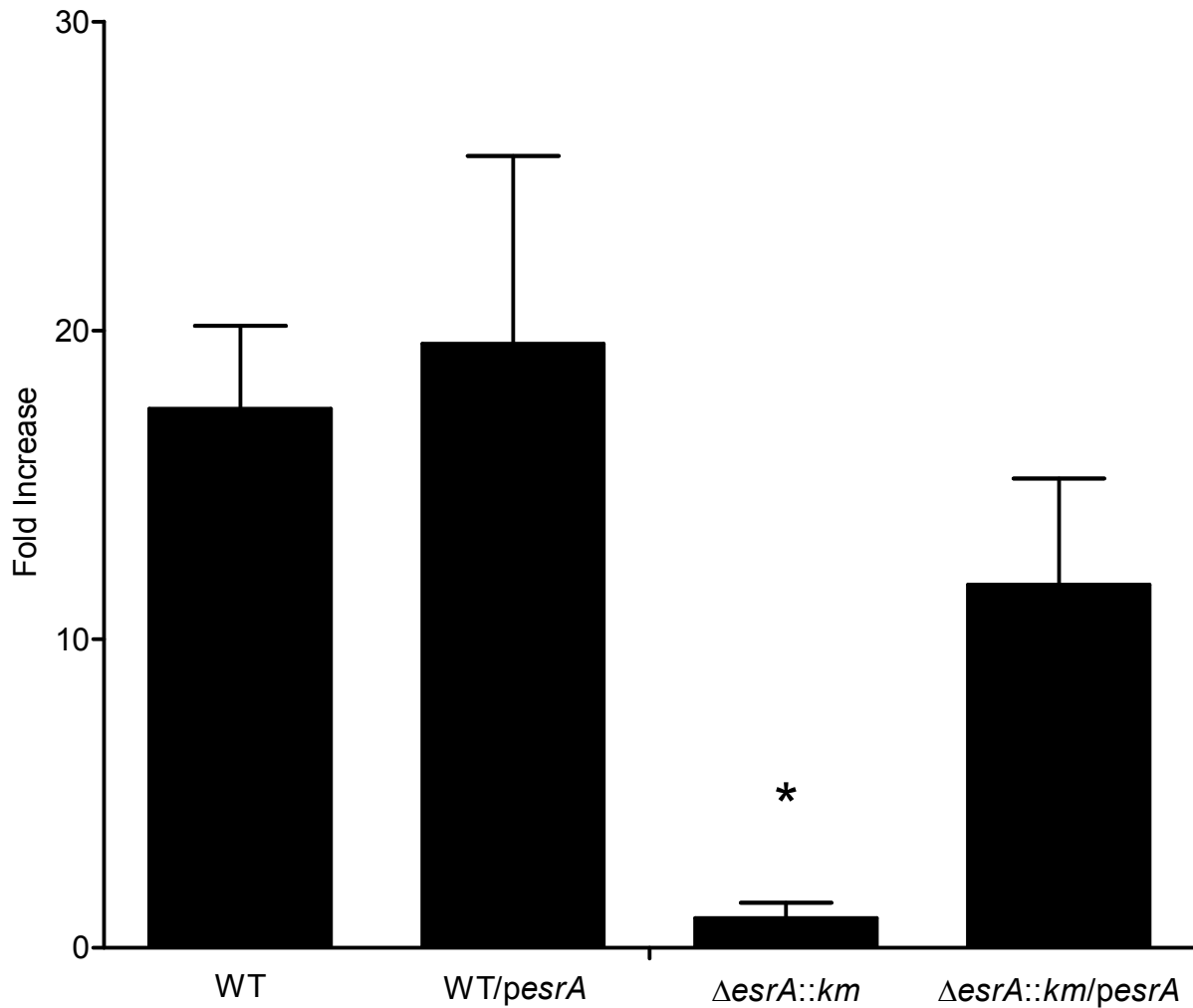


Figure 3.7. Replication of an *Edwardsiella ictaluri* $\Delta esrA::km$ strain in channel catfish macrophages. Head kidney-derived channel catfish macrophages were infected with the following *E. ictaluri* strains: wild type (WT), WT carrying the *esrA* complementation plasmid (WT/pesrA), $\Delta esrA::km$, and $\Delta esrA::km$ carrying the *esrA* complementation plasmid ($\Delta esrA::km/pesrA$). Bars indicate the mean fold replication (\pm SEM) 10 hrs post-infection during triplicate gentamicin exclusion assays. * indicate a significant difference from WT fold increase ($P \leq 0.05$).

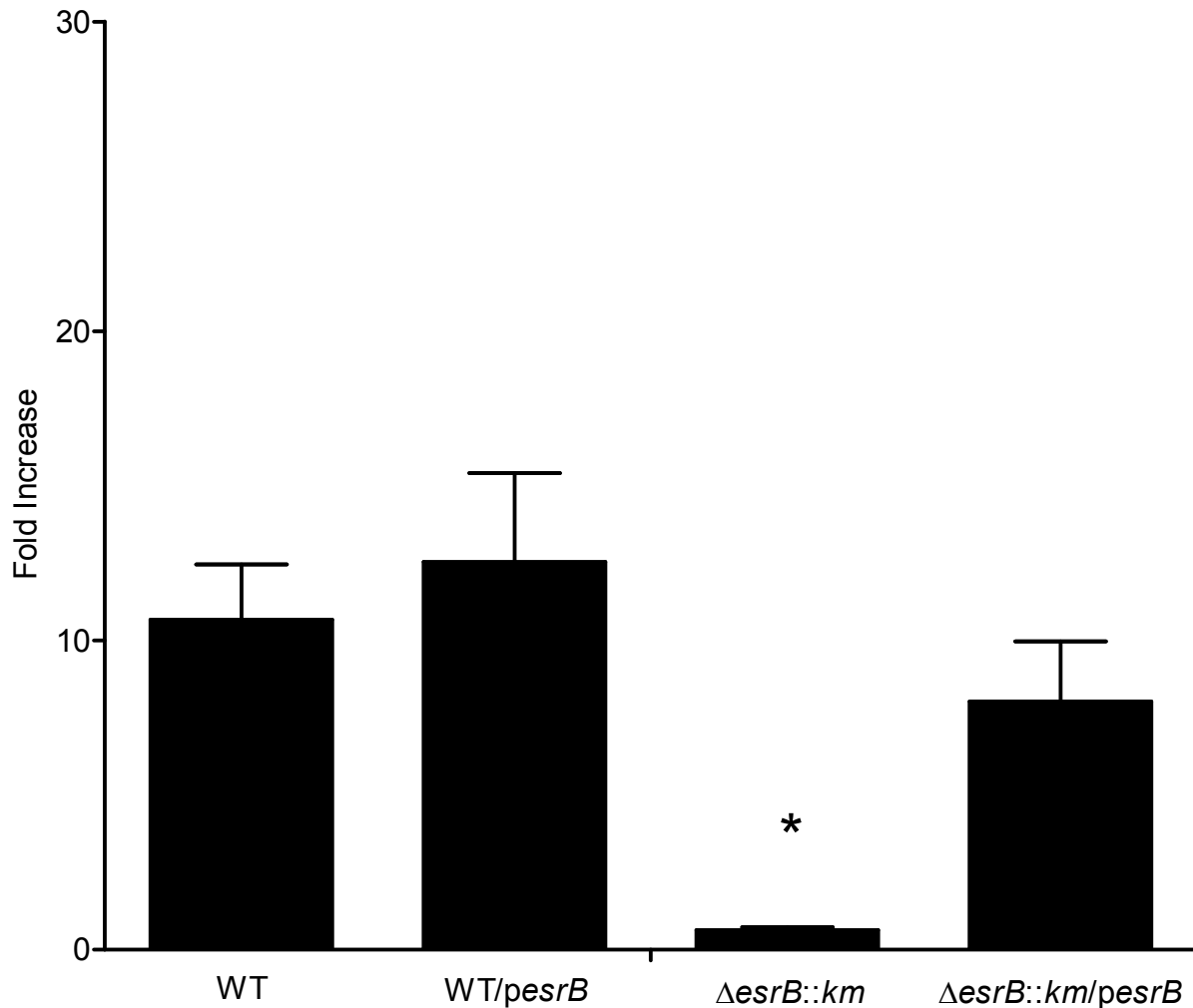


Figure 3.8. Replication of an *Edwardsiella ictaluri* $\Delta esrB::km$ strain in channel catfish macrophages. Head kidney-derived channel catfish macrophages were infected with the following strains of *E. ictaluri*: wild type (WT), WT carrying the *esrB* complementation plasmid (WT/pesrB), $\Delta esrB::km$, and $\Delta esrB::km$ carrying the *esrB* complementation plasmid ($\Delta esrB::km/pesrB$). Bars indicate the mean fold replication (\pm SEM) 10 hrs post-infection from triplicate gentamicin exclusion assays. * indicate a significant difference from WT fold increase ($P \leq 0.05$).

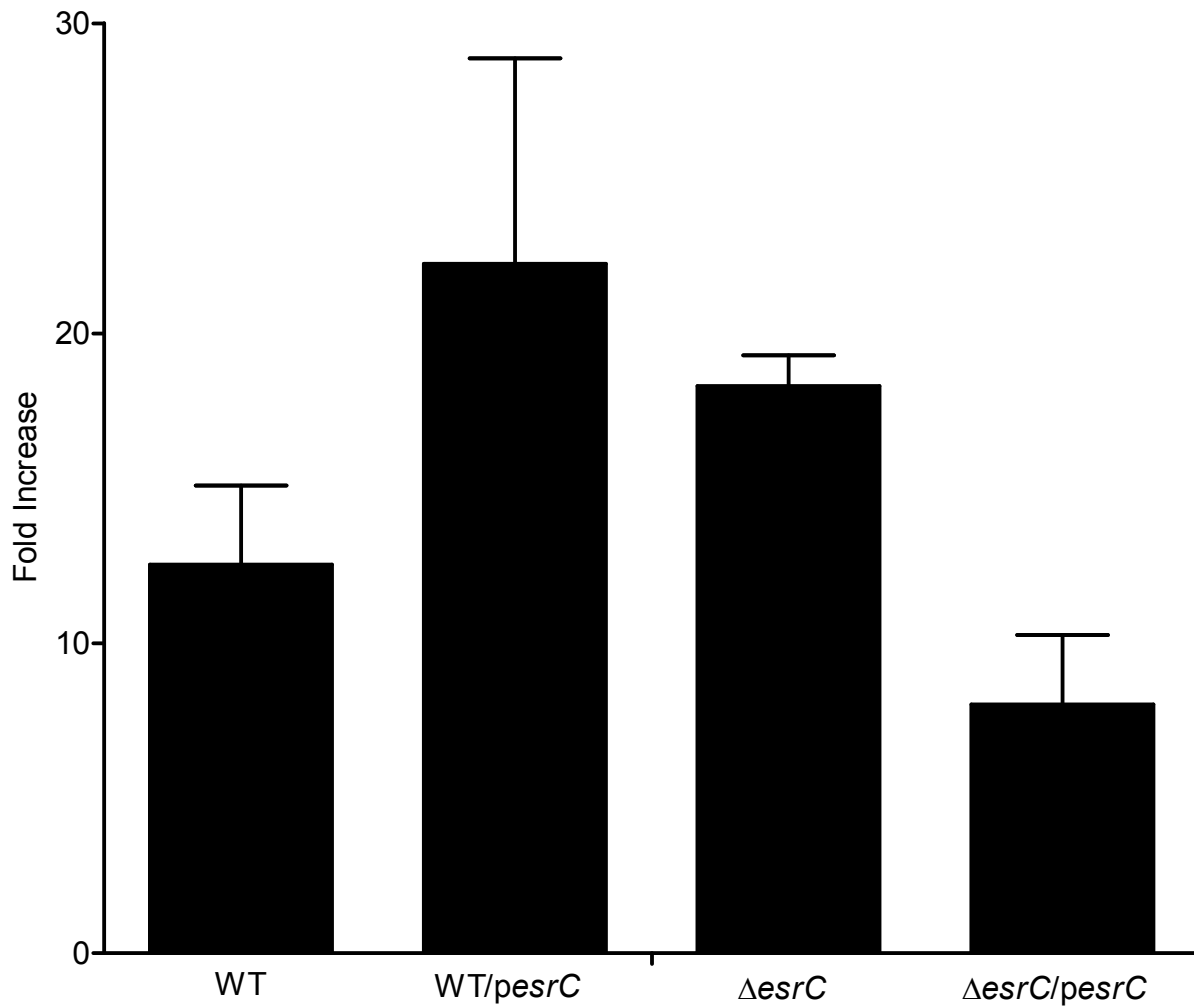


Figure 3.9. Replication of an *Edwardsiella ictaluri* $\Delta esrC$ strain in channel catfish macrophages. Head kidney-derived channel catfish macrophages were infected with the following strains of *E. ictaluri*: wild type (WT), WT carrying the *esrC* complementation plasmid (WT/pesrC), $\Delta esrC$, and $\Delta esrC$ carrying the *esrC* complementation plasmid ($\Delta esrC$ /pesrC) Bars indicate the mean fold replication (\pm SEM) 10 hrs post-infection from triplicate gentamicin exclusion assays.

indicating there is not attenuated intracellular virulence as a result of the mutation. The ability of $\Delta esrC$ to replicate intracellularly comparably to WT suggests EsrC regulation is not required early in the infection process as it pertains to macrophages. However, there may be an effect beyond the initial 10 hrs of host cell infection. The *esrBC* double mutant has a phenotype similar to that of the *esrB* mutant (Figure 3.10).

Plasmids were constructed containing the native *esrA*, *esrB*, and *esrC* open reading frames and promoter regions for complementation of the regulatory gene mutations. Because *E. ictaluri* encodes possible virulence genes on plasmids (Fernandez et al. 2001; Thune et al. 2007), the introduction of complementation plasmids could affect the stability of or gene expression from the native plasmids. Therefore, each complementation plasmid was conjugated into the WT strain, as well as into each appropriate mutant strain.

Each complemented mutant and WT strain was evaluated for replication within channel catfish macrophages. All WT strains carrying a complementation plasmid replicated similarly to the WT strain, indicating the presence of the complementation plasmid has no adverse effect on replication of *E. ictaluri* within macrophages (Figures 3.7 to 3.9). The *esrA* and *esrB* mutant strains are able to replicate in macrophages when *esrA* or *esrB* is supplied *in trans* from the complementation plasmid (Figures 3.7 and 3.8). Complementation of *esrC* in the *esrC*-deficient strain also replicates at a level similar to the WT strain (Figure 3.9) indicating the complementation plasmid does not have a negative effect intracellularly.

The results of the gentamicin exclusion assays in channel catfish macrophages indicate that *esrA* and *esrB* are required for replication of *E. ictaluri* in the early stages of macrophage infection, and their mutation can be complemented by expression of native *esrA* and *esrB* from a plasmid. Mutation of *esrC*, however, has no effect on replication of *E. ictaluri* intracellularly, indicating it is not required for replication within macrophages in the first 10 hours following

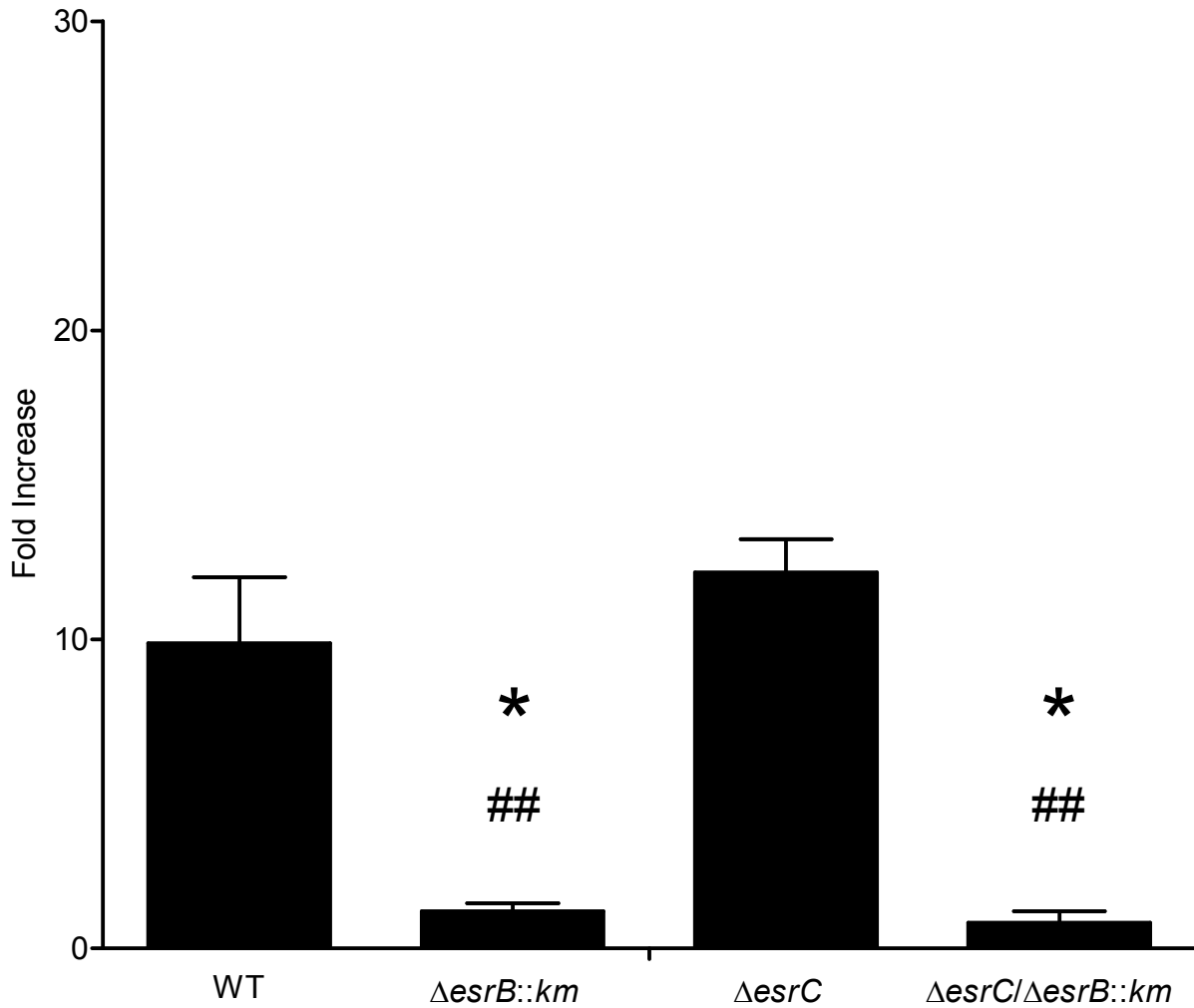


Figure 3.10. Replication of an *Edwardsiella ictaluri* *esrB* and *esrC* double mutant in channel catfish macrophages. Head kidney-derived channel catfish macrophages were infected with the following strains of *E. ictaluri*: wild type (WT), $\Delta esrB::km$, $\Delta esrC$, and the $\Delta esrC/\Delta esrB::km$ double mutant. Bars indicate the mean (\pm SEM) fold replication 10 hrs post-infection from triplicate gentamicin exclusion assays. * indicates a significant difference from WT fold replication ($P \leq 0.05$); ## indicate a significant difference from $\Delta esrC$ fold increase ($P \leq 0.05$).

infection. However, it is possible EsrC is required intracellularly later than 10 hours after infection of macrophages. Furthermore, because EsrC is required for secretion of the T6SS protein EvpC, the ability of the *esrC* mutant to replicate intracellularly suggests the *E. ictaluri* T6SS is not required for the early stages of macrophage infection.

T3SS Mutations Prevent Channel Catfish Mortality Caused by *E. ictaluri*. Channel catfish were infected by immersion with WT *E. ictaluri* and T3SS regulatory gene mutants, both with and without complementation plasmids. WT strains carrying the complementation plasmids were included to determine if the presence of the plasmid or the regulatory gene encoded on the plasmid has an adverse effect on pathogenesis *in vivo*.

Infection with the WT strain results in 70% mortality. Wild type *E. ictaluri* carrying the complementation plasmids for *esrA* and *esrB* produce statistically similar mortality rates (Figures 3.11 and 3.12). However, the presence of the *esrC* complementation plasmid reduces the WT mortality to about 40% (Figure 3.13). Because *pesrA* and *pesrB* do not affect pathogenesis of WT *E. ictaluri* in channel catfish, it is likely the decrease in mortality for WT carrying *pesrC* is due to overexpression of EsrC from the plasmid rather than the presence of the expression plasmid.

All T3SS regulatory mutants are unable to cause mortality in fish (Figures 3.11 to 3.13). The *esrBC* double mutant also causes no mortality (data not shown). Complementation of $\Delta esrA::km$ and $\Delta esrB::km$ with *pesrA* and *pesrB*, respectively, restores the virulence effect of *E. ictaluri* (Figures 3.11 and 3.12). However, $\Delta esrC$ complemented with *pesrC* does not result in restored virulence (Figure 3.13). The complemented *esrC* mutant has only about 10% mortality, which is significantly higher than uncomplemented $\Delta esrC$, but also significantly less than WT or WT carrying *pesrC*.

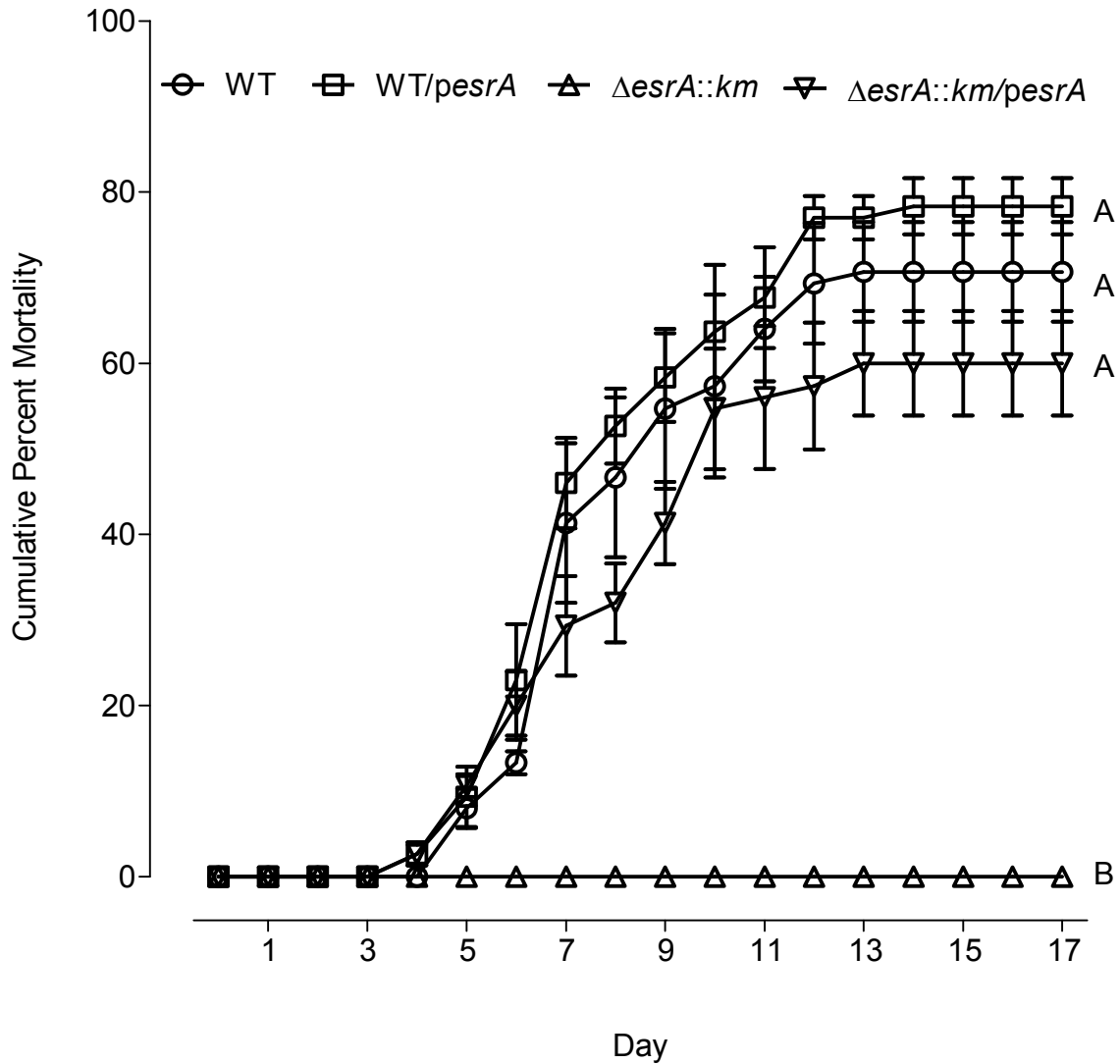


Figure 3.11. Cumulative mortality of channel catfish following infection by $\Delta esrA::km$ *Edwardsiella ictaluri*. Channel catfish were experimentally infected by immersion with wild type *E. ictaluri* (WT), WT carrying the *esrA* complementation plasmid (WT/*pesrA*), $\Delta esrA::km$, and $\Delta esrA::km$ carrying the *esrA* complementation plasmid ($\Delta esrA::km/pesrA$). Bars indicate the mean (\pm SEM) of triplicate challenge tanks. Mortality curves with the same letter indicate no significant difference in cumulative percent mortality ($P > 0.05$).

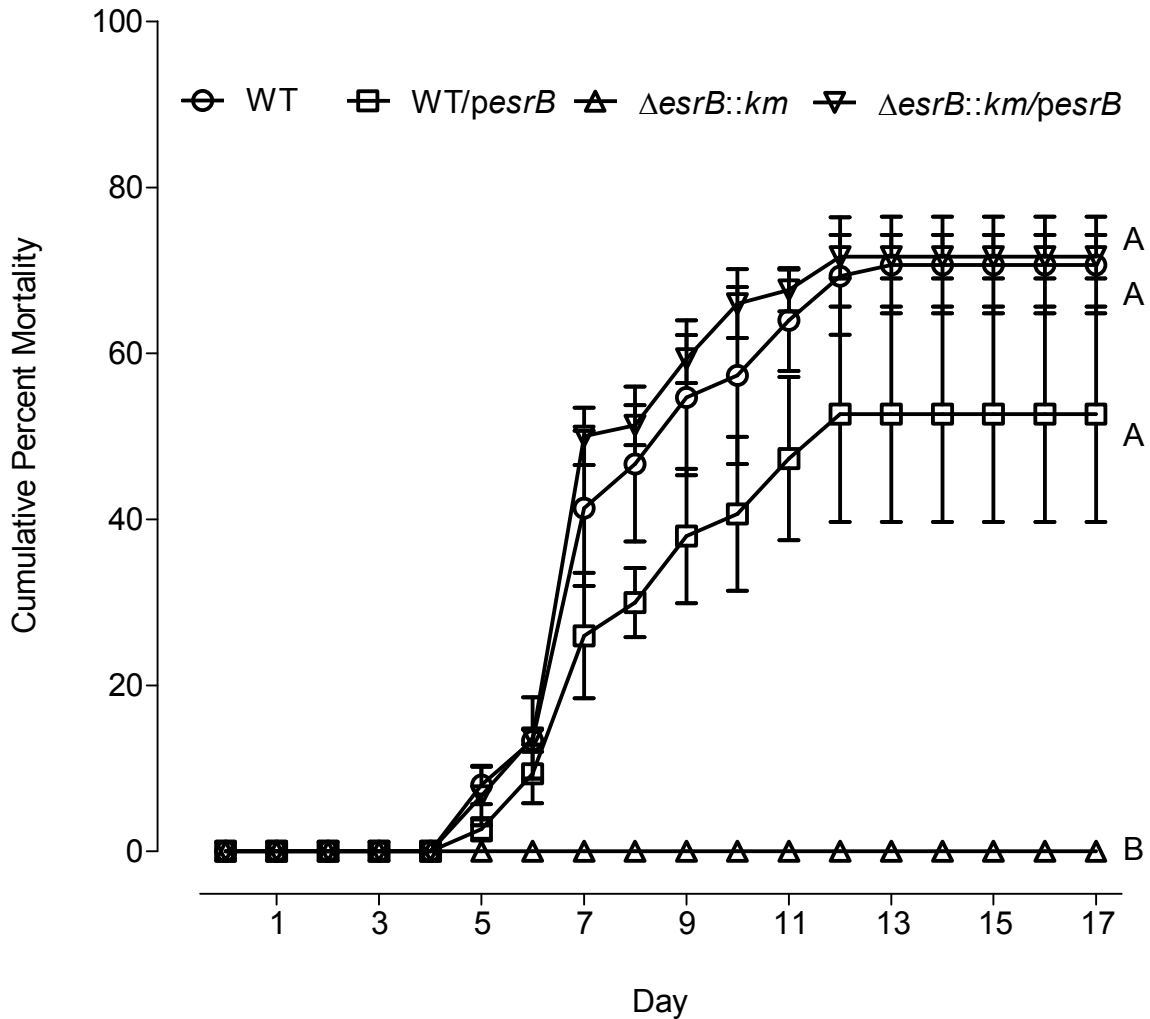


Figure 3.12. Cumulative mortality of channel catfish following infection by $\Delta esrB::km$ *Edwardsiella ictaluri*. Channel catfish were experimentally infected by immersion with wild type *E. ictaluri* (WT), WT carrying the *esrB* complementation plasmid (WT/*pesrB*), $\Delta esrB::km$, and $\Delta esrB::km$ carrying the *esrB* complementation plasmid ($\Delta esrB::km/pesrB$). Bars indicate the mean (\pm SEM) of triplicate challenge tanks. Mortality curves with the same letter indicate no significant difference in cumulative percent mortality ($P > 0.05$).

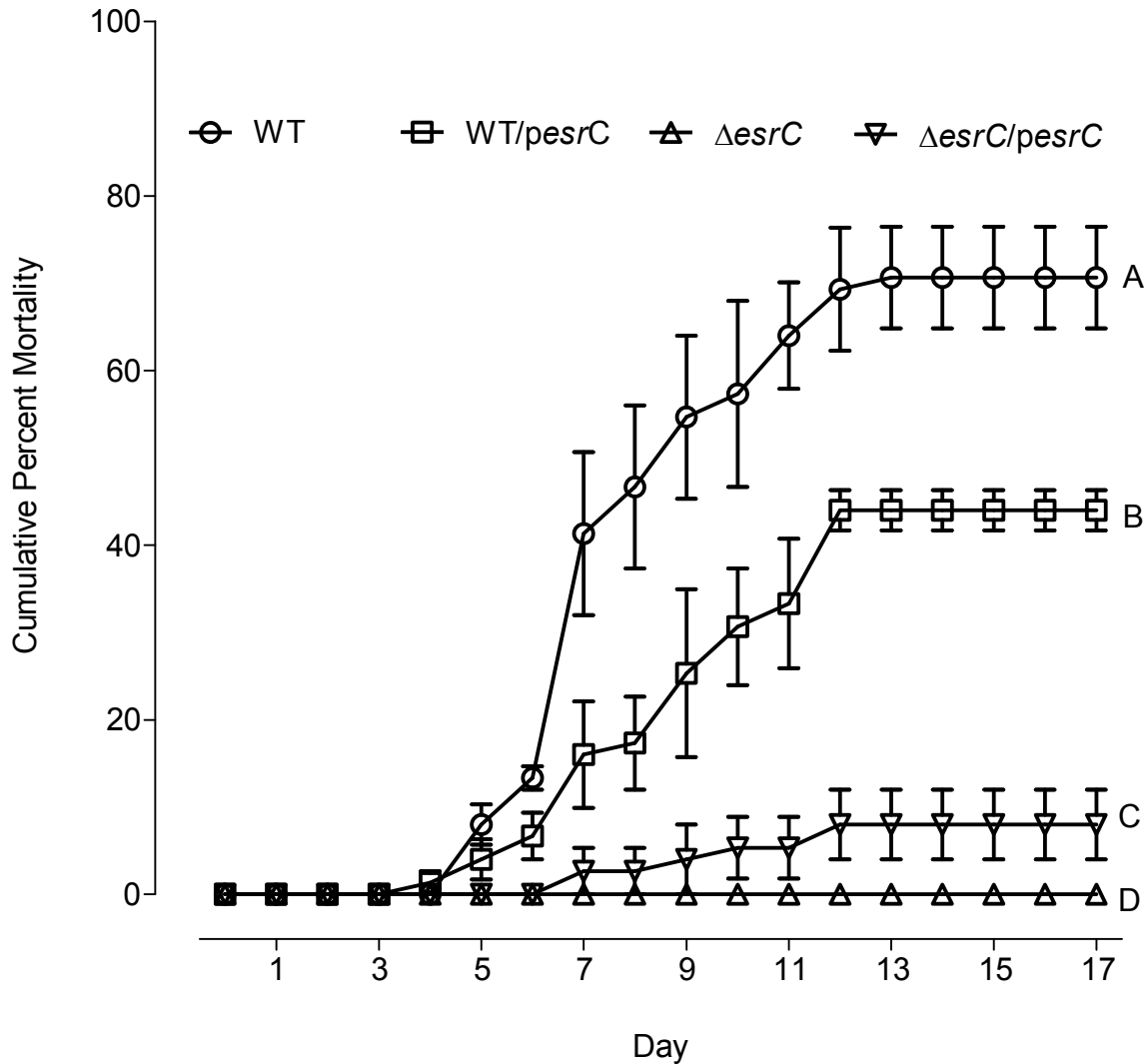


Figure 3.13. Cumulative mortality of channel catfish following infection by $\Delta esrC$ *Edwardsiella ictaluri*. Channel catfish were experimentally infected by immersion with wild type *E. ictaluri* (WT), WT carrying the *esrC* complementation plasmid (WT/*pesrC*), $\Delta esrC$, and $\Delta esrC$ carrying the *esrC* complementation plasmid ($\Delta esrC/pesrC$). Bars indicate the mean (\pm SEM) of triplicate challenge tanks. Mortality curves with the same letter indicate no significant difference in cumulative percent mortality ($P > 0.05$).

The differences between WT and strains carrying *pesrC* are unexpected. Complementation with *pesrC* was expected to restore virulence of the mutant strain and to not affect WT virulence. While *pesrC* slightly increases mortality of the mutant, it greatly decreases mortality caused by the WT strain. This indicates a partial ability of *pesrC* to complement *esrC* mutation *in trans*, but also an overall negative effect of *pesrC* on *E. ictaluri* virulence.

These data show that each regulatory gene is required for virulence *in vivo*. Mutations to *esrA* and *esrB* can be complemented *in trans* using an expression vector carrying *esrA* or *esrB*, respectively. However, deletion of *esrC* is unable to be fully complemented *in trans*. The presence of the *esrC* complementation plasmid also reduces the virulence of the WT strain, which the *esrA* and *esrB* complementation plasmids do not. Because complementation is done using a plasmid, the gene copy number of *esrC* is higher than in WT *E. ictaluri*, perhaps resulting in unfavorable levels of *esrC* expression that affect the signaling cascade of the T3SS and possibly other virulence mechanisms outside the T3SS.

Effect of *esrC* Complementation Plasmid on Expression of T3SS Genes. Because of the differences seen in mortality of channel catfish when challenged with the WT and Δ *esrC* strains carrying *pesrC*, expression of T3SS gene expression was measured by using qPCR in both of the *E. ictaluri* strains carrying the *esrC* complementation plasmid. The WT strain carrying *pesrC* has significantly higher expression of each of the T3SS regulatory genes than the parent WT strain (Figure 3.14), a finding surprising considering that Δ *esrC* has no effect on expression of *esrA* or *esrB*. Δ *esrC/pesrC* also has significantly higher expression of *esrB* than the parent Δ *esrC* strain; however, *esrA* is not significantly higher. Complementation of *esrC* in either the WT or mutant strain increases *esrC* expression higher than that observed in WT *E. ictaluri*. Expression of *esrC* from the plasmid also increases expression of the *escC* operon and *escB/eseG* for both WT and mutant strains (Figure 3.15). However, *pesrC* is not able to restore expression of *eseH* in Δ *esrC*,

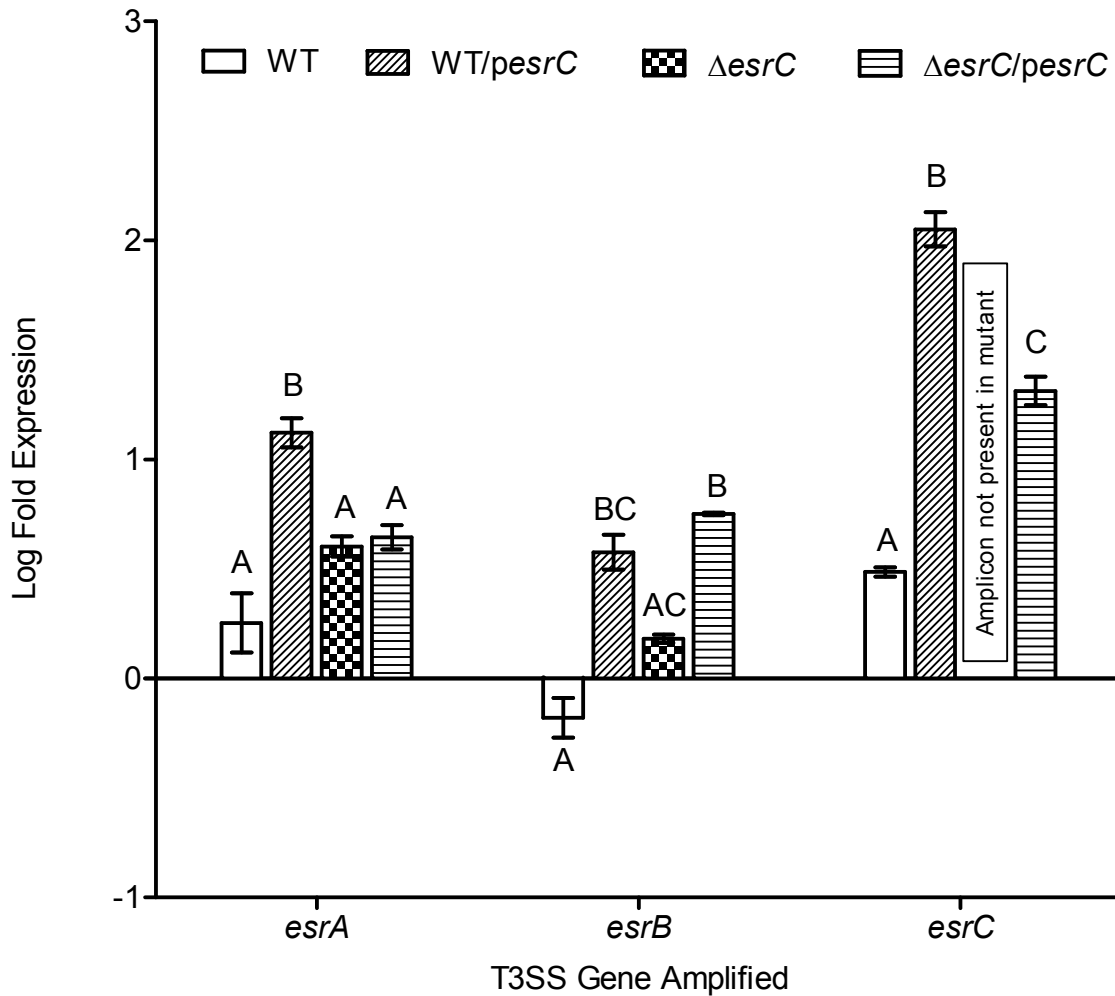


Figure 3.14. Effect of *pesrC* on expression of *Edwardsiella ictaluri* type III secretion system (T3SS) regulatory genes. Quantitative PCR was used to measure expression of *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and the *esrC* operon encoding *esrC* and the apparatus genes *esaGHIJWKL* in wild type (WT) and Δ *esrC* *E. ictaluri* strains carrying an *esrC* complementation plasmid and cultured in T3SS expression-inducing media. Bars indicate the mean (\pm SEM) of the log fold expression of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

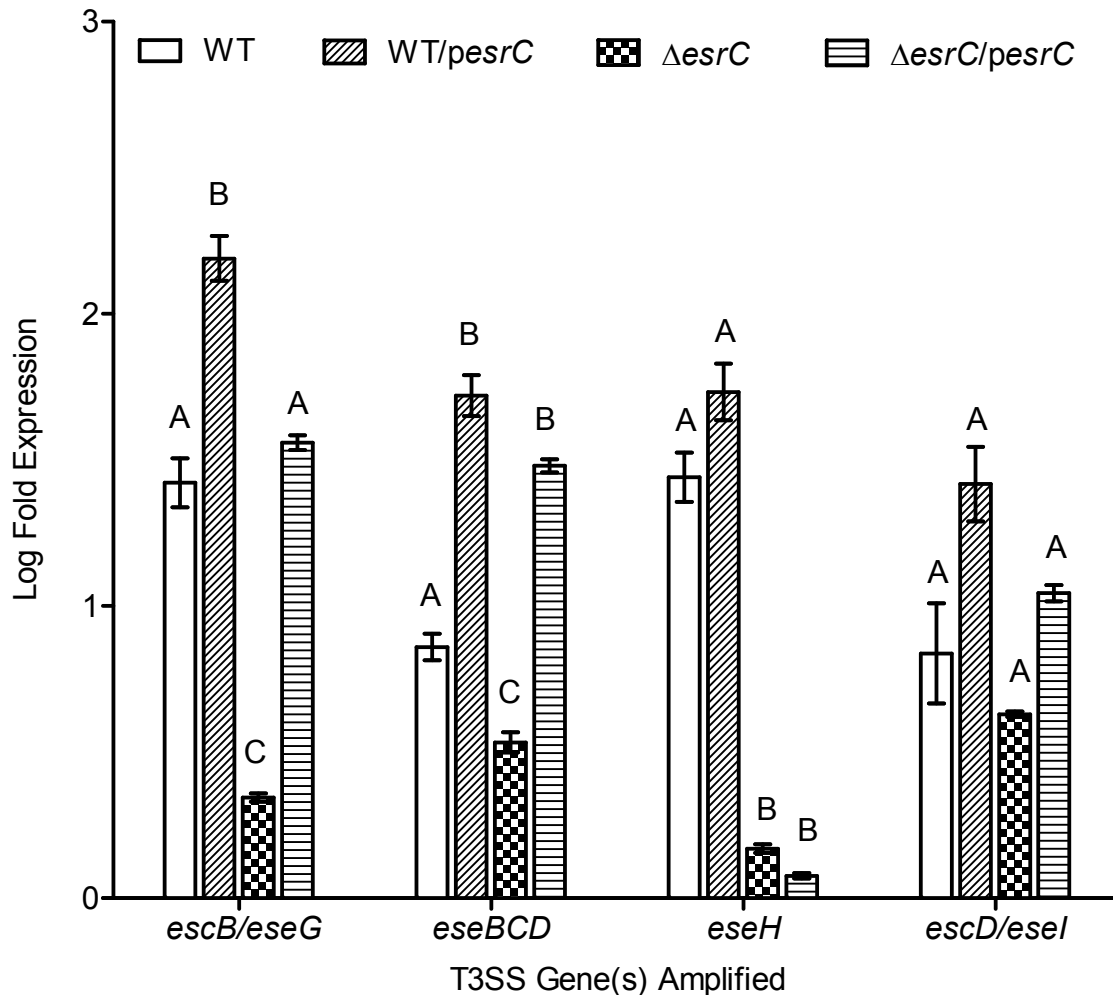


Figure 3.15. Effect of *pesrC* on expression of *Edwardsiella ictaluri* type III secretion system (T3SS) non-regulatory genes. Quantitative PCR was used to measure expression of *E. ictaluri* T3SS non-regulatory genes encoded by the *escB/eseG* operon, the *escC* operon containing the translocon genes *eseBCD*, the pEI1-encoded *eseH*, and the pEI2 encoded *escD/eseI* operon in wild type (WT) and Δ *esrC* *E. ictaluri* strains carrying an *esrC* complementation plasmid and cultured in T3SS expression-inducing media. Bars indicate the mean (\pm SEM) of the log fold expression of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

nor does it result in increased expression of *eseH* in the WT strain. Expression of *eseI* is not increased significantly by *pesrC* in either strain.

The results of these analyses demonstrate that expression of *esrC* from a multicopy plasmid has a strong effect on the expression of many T3SS genes. The extra copies of *esrC* not only result in increased expression of itself, but also the regulatory gene *esrB*, which may have a confounding effect on the expression of *esrC*, because *esrC* is regulated by EsrB.

Overexpression of the regulators leads to the increased expression of other T3SS genes, and likely other genes both inside and outside the T3SS not measured in this study. The increased expression of these genes may be the cause of the differences in virulence noted in the channel catfish immersion challenge for the *E. ictaluri* strains carrying the *esrC* complementation plasmid.

DISCUSSION

Mutations of *E. ictaluri* T3SS regulatory genes result in decreased T3SS gene expression in *E. ictaluri* T3SS-inducing media. However, each mutation has a different gene expression phenotype. Mutation of *esrA* results in reduced T3SS gene expression and protein secretion. The decreased T3SS expression results in the inability to replicate in macrophages and an attenuated virulence in channel catfish. Mutation of *esrB*, however, results in large-scale decreases in T3SS gene expression and protein secretion, and results in loss of EvpC secretion. Like $\Delta esrA::km$, $\Delta esrB::km$ also is unable to replicate in macrophages, and has attenuated virulence in channel catfish. Mutation of *esrC* reduces T3SS gene expression, though not to the degree of *esrB* mutation. $\Delta esrC$ has visibly reduced, but not absent T3SS translocon protein secretion, and like $\Delta esrB::km$, has no detectable EvpC secreted. $\Delta esrC$, surprisingly, is able to replicate intracellularly as well as WT *E. ictaluri*, but is attenuated for virulence *in vivo*.

EsrB Controls the *E. ictaluri* T3SS. Like *Salmonella* and *E. tarda*, the two-component regulatory system encoded within the T3SS pathogenicity island is necessary for optimal expression of the T3SS. As demonstrated by mutation of *esrB*, T3SS activity is arrested at the transcriptional level without EsrB. Mutation of *esrB* results in substantially greater reductions in gene expression and protein production than either $\Delta esrA::km$ or $\Delta esrC$. The importance of EsrB in T3SS expression is mirrored in *E. tarda* (Srinivasa Rao et al. 2003; Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005) and with SsrB of *Salmonella* (Shea et al. 1996; Valdivia and Falkow 1996; Cirillo et al. 1998; Deiwick et al. 1999; Worley et al. 2000; Garmendia et al. 2003; Feng et al. 2004; Deiwick et al. 2006; Dieye et al. 2007; Walthers et al. 2007). Not surprisingly, $\Delta esrB::km$ is avirulent both intracellularly and *in vivo*.

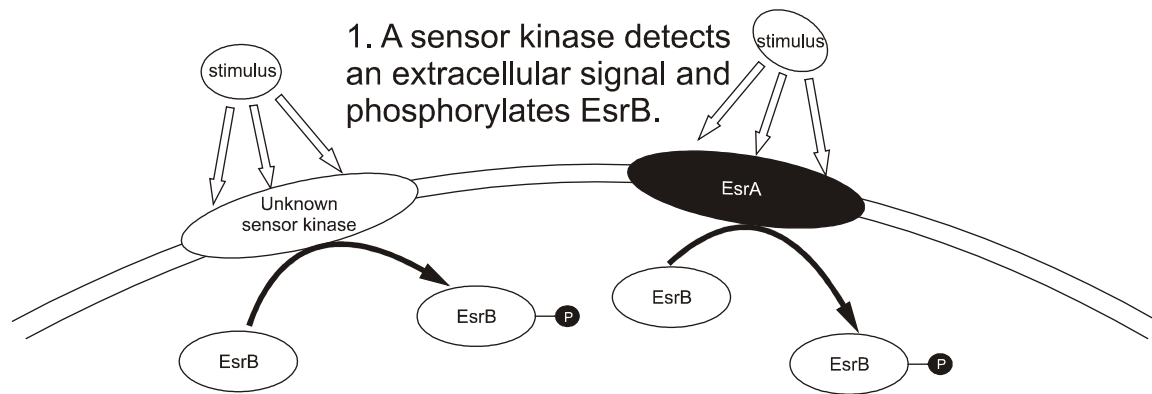
Like SsrB of *Salmonella* (Parsons and Heffron 2005), EsrB also has a role in the expression of the T6SS of *E. ictaluri*. However, this interaction may not be direct in *E. ictaluri*, because EsrC also regulates the T6SS of both *E. ictaluri* and *E. tarda* (Zheng et al. 2005; Zheng and Leung 2007). Because expression of *esrC* is dependent on EsrB, (Figure 3.3) and mutations in both *esrB* and *esrC* result in abolished EvpC expression and secretion (Figures 3.5 and 3.6), the effect of EsrB on the T6SS is likely to be through transcriptional regulation of *esrC*. As opposed to SsrB of *Salmonella*, which has a negative effect on the T6SS, EsrB and EsrC of *Edwardsiella* appear to positively affect expression of the T6SS protein EvpC. The T6SS of *Salmonella*, however, is not active until about 24 hrs post-infection. The analyses done in this study are done after *in vitro* culture of *E. ictaluri* to late log phase, and therefore do not indicate what occurs earlier. The *Edwardsiella* T6SS may be expressed only after a certain amount of T3SS gene expression.

EsrC Serves an Accessory Role for T3SS Gene Expression, but Is Required for T6SS Expression. Mutation of EsrC does not prohibit expression or secretion of T3SS proteins,

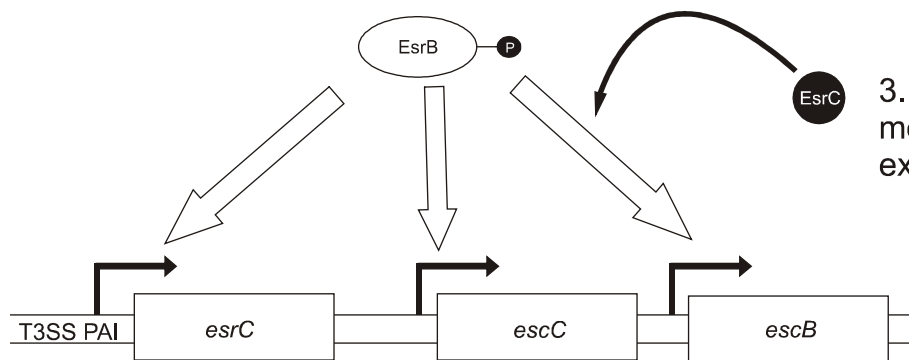
contrary to findings in *E. tarda* (Zheng et al. 2005). However, EsrC does appear to be required for optimal T3SS expression in conjunction with EsrB. Both proteins are required for WT levels of T3SS gene expression, and since EsrB is required for *esrC* expression, mutation of *esrB* results in loss of both proteins. What is unknown, however, is the effect EsrC has on T3SS gene expression in the absence of EsrB. In order to study that scenario, EsrC would need to be expressed independently of EsrB, most likely with an EsrB-independent promoter fused to *esrC*. However, the fact that T3SS protein expression and secretion occurs in the absence of EsrC indicates its presence is not required for T3SS expression. This finding is different than what is reported for *E. tarda*. EsrC of *E. tarda* absolutely is required for expression and secretion of T3SS translocon proteins (Zheng et al. 2005). The results of this study, however, indicate both EsrB and EsrC are required for optimal virulence of *E. ictaluri* in channel catfish.

A model of gene regulation by EsrB and EsrC is given in Figure 3.16. In this model, EsrB is activated by sensor kinases that detect environmental signals. Because T3SS expression occurs in the absence of EsrA, EsrB likely can be activated by other sensor kinases similar to SsrB of *Salmonella* (Walthers et al. 2007). EsrB activates expression of regulatory, translocon, and effector genes encoded within the T3SS pathogenicity island, including *esrC*. Expression of EsrC enhances the transcriptional activity of EsrB and activates virulence genes outside the T3SS, including the T6SS.

EsrC may regulate genes outside the T3SS independently of EsrB activity. Mutation of *esrC* is sufficient to abolish production of the T6SS protein EvpC in this study, and similar results are described for *E. tarda* (Zheng et al. 2005). EsrC and EsrB are also required for expression of Orf29 and Orf30 in *E. tarda*. Both *orf29* and *orf30* are encoded on the outer edge of the T3SS pathogenicity island upstream of *esrB*. However, in *E. ictaluri*, *orf29* and *orf30* are a single large open reading frame and are separated from the T3SS by a transposase insertion not



2. Activated EsrB upregulates the activity of T3SS genes including *esrC*.



3. EsrC enhances EsrB-modulated T3SS gene expression.

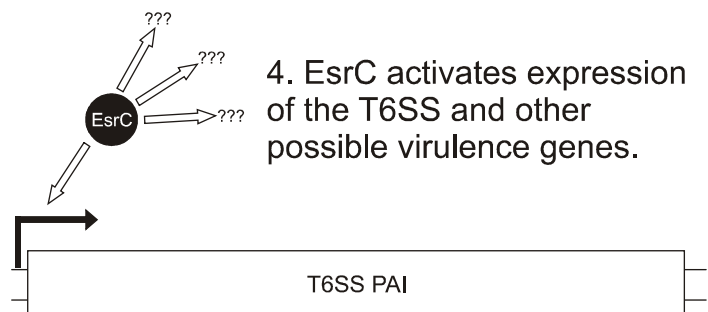


Figure. 3.16. Proposed *Edwardsiella ictaluri* type III secretion system (T3SS) regulation model. Environmental stimuli induce phosphorylation of EsrB by EsrA and possibly other sensor kinases. Activated EsrB upregulates activity of T3SS gene promoter regions in the pathogenicity island (PAI) and increases gene expression. EsrC enhances EsrB activation of T3SS gene expression. EsrC also activates expression of the type VI secretion system (T6SS) pathogenicity island and possibly other virulence genes.

present in *E. tarda*. Zheng et al. (Zheng et al. 2005) hypothesize that Orf29 and Orf30 are T3SS translocated effectors based on their dependence on EsrB and EsrC for expression and the presence of coiled-coil regions in the predicted structure of the proteins. Expression of *E. ictaluri orf29/30* was not evaluated in this study to determine if T3SS regulators are required for *orf29/30* regulation. Transcription of *orf29/30*, however, is reduced in low phosphate, acidic media as shown in Chapter 2, indicating *orf29/30* is downregulated in conditions that increase T3SS gene expression in an EsrB- and EsrC-dependent manner. These results call into question whether or not *orf29/30* is T3SS-related. Further analysis of *orf29/30* is required to determine if it encodes T3SS secreted effectors and its relationship to EsrB and EsrC.

Mutation of *esrB* and *esrC* in this study result in similar expression levels of *eseH*, a putative T3SS effector encoded by pE11, an *E. ictaluri* plasmid. The similar expression levels suggest EsrC regulates *eseH* expression, because expression of *eseH* is decreased in the Δ *esrC* mutant in which EsrB is present. Conversely, the inability of *pesrC* to restore or upregulate *eseH* expression (Figure 3.15) in either the WT or the Δ *esrC* strain suggests EsrC has no regulatory control over *eseH*. Further work is required to determine the effects of EsrB and EsrC on gene expression outside the T3SS pathogenicity island.

The phenotypic differences in *esrC* mutations between *E. ictaluri* and *E. tarda* are significant and more dissimilar than expected. Translocon gene expression is not detected using promoter fusions to *lacZ* in an *E. tarda esrC*-deficient background, and no extracellular translocon proteins are detected (Zheng et al. 2005). Conversely, in an *E. ictaluri esrC* mutant, translocon gene expression is detected by using qPCR and is still significantly higher than in the WT strain cultured in non-inducing media. Furthermore, extracellular translocon proteins are present in high amounts. Whereas the *E. ictaluri esrC* mutant is not attenuated intracellularly, an *E. tarda esrC* mutant is moderately attenuated for intracellular replication. However, both *E.*

ictaluri and *E. tarda* *esrC* mutants are attenuated significantly *in vivo*, indicating both genes ultimately are required for virulence.

There could be a functional difference in the *esrC* mutations made. Zheng et al. (Zheng et al. 2005) deleted amino acids 34 – 200 in *E. tarda* EsrC, while amino acids 101 – 211 of *E. ictaluri* EsrC are deleted in this study. Although no HTH or other regulatory motif is detected in the mutated EsrC sequence of *E. ictaluri*, it is possible the difference results in a protein with partial functionality. However, Zheng et al. (Zheng et al. 2005) made additional deletions in both the N- and C-termini and found similar phenotypes in all mutations, making it unlikely that the differing *esrC* mutant phenotypes between *E. ictaluri* and *E. tarda* are due to differences in construction of the mutation. EsrC of *E. tarda* and *E. ictaluri* shares 98% homology across the entire amino acid sequence, suggesting their active regions would operate similarly in both species, and that mutation would result in similar phenotypes.

It is possible the regulatory control maintained by EsrC is different in the two species. EsrC does have an effect on *E. ictaluri* T3SS gene expression, as demonstrated by the decreased, but not absent expression of translocon genes. *E. tarda* EsrC may have developed stronger control over T3SS gene expression through evolution of stronger EsrC recognition sequences upstream of T3SS genes, providing EsrC greater control over T3SS gene expression. The differences in regulation of the T3SS between *E. ictaluri* and *E. tarda* in response to environmental conditions as described in Chapter 2 could be a function of differential regulation of the T3SS by EsrC.

The differences in T3SS regulation and the effect of EsrC may not be surprising, however. Although both organisms are of the same genus and encode homologous T3SS, their host range and pathogenesis are quite different. *Edwardsiella ictaluri* is generally restricted to the channel catfish, whereas *E. tarda* will infect other fish species in addition to a number of

higher vertebrates. The ability of *E. tarda* to infect higher vertebrates may involve not only different virulence mechanisms, but also different regulation of those mechanisms. The differences observed in the function of EsrC between *E. ictaluri* and *E. tarda* may be part of what determines the respective niches of these two organisms.

The methods used for analyzing T3SS gene expression of *E. tarda* and the methods used in this study may be a partial source of the differences in phenotype between the two species. Studies evaluating *E. tarda* T3SS gene expression have employed T3SS gene promoter fusions to *lacZ* carried by plasmids to study up- and downregulation of genes in WT and mutant strains of *E. tarda* (Tan et al. 2005; Zheng et al. 2005). Hansen-Wester et al. found expression of SPI-2 genes from plasmids resulted in improper regulation of those genes. This study used qPCR to measure the quantity of message produced at a given point in time, relying on native gene expression rather than reporter gene expression. *Edwardsiella ictaluri* T3SS gene promoter fusions to GFP were attempted in Chapter 2, but were limited in their usefulness. However, this study and those evaluating the *E. tarda* T3SS used similar methods of protein production and secretion analysis, and those results demonstrate a difference in the phenotypes of *E. tarda* and *E. ictaluri* in regards to T3SS expression. Therefore, it is difficult to determine if the differences seen in the expression of the *E. ictaluri* and *E. tarda* T3SS are real or possibly due to variations in the methods used to measure expression.

EsrA and EsrB Are Required for Intracellular Survival and Pathogenesis in Channel

Catfish. The reduced intracellular replication of an *esrB* mutant is not surprising, considering the severe effects observed in gene expression and protein secretion. However, it is curious that mutation of *esrA* results in a phenotype similar to *esrB* mutation intracellularly and *in vivo*. Δ *esrA::km* has significantly less T3SS gene expression than WT *E. ictaluri*; however, T3SS genes are still expressed significantly higher than Δ *esrB::km*, and in many cases not much

differently than $\Delta esrC$, which was not attenuated intracellularly. Differences between the *esrA* and *esrC* mutants, however, were observed in translocon expression, suggesting that decreased expression of the translocon in $\Delta esrA::km$ might result in reduced ability to construct the translocon and secrete proteins. However, $\Delta esrA::km$ secreted easily detectable amounts of translocon proteins to the supernatant suggesting they are not limiting (Figure 3.5). It is possible that EsrA is more involved in the expression of T3SS genes not expressed, such as apparatus genes in the *esaB*, *esaM*, or *esaR* operons. Alternatively, EsrA may serve as a sensor kinase for response regulators other than EsrB that have affects on gene expression outside of the T3SS pathogenicity island.

$\Delta esrA::km$ was observed to grow slower than the other mutant and WT strains of *E. ictaluri* used. This growth defect is lost when $\Delta esrA::km$ is complemented with *pesrA*, suggesting *esrA* mutation affects growth of *E. ictaluri*. Because EsrA is a putative membrane protein, the mutation of EsrA may affect membrane dynamics, resulting in reduced growth. However, mutation of *esrA* in *E. tarda* or SsrA in *Salmonella* is not reported to affect bacterial growth.

Another possibility for the severe virulence defect of $\Delta esrA::km$, while still expressing and secreting T3SS proteins, is that efficient pathogenesis requires strict control of gene expression, and any departure from that control will result in abnormal expression and attenuation. Expression of the SPI-2 T3SS is complex, and departures from the ordered expression and secretion of T3SS effectors can have significant impacts (Coombes et al. 2005; Lucchini et al. 2006). The inability of an *esrA* mutant to infect macrophages or fish may be the result of subtle inefficiencies in T3SS gene expression, as opposed to the gross lack of T3SS gene expression as observed in the *esrB* mutant.

EsrC Is Expendable in Early Macrophage Infection. Deletion of *esrC* has no effect on the ability of *E. ictaluri* to replicate within channel catfish macrophages. This assay, however, only measures the replication within the first 10 hrs post-infection. Both the *esrA* and *esrB* mutants, however, exhibit severe decreases in intracellular replication within the 10 hr period. This demonstrates the importance of EsrB for intracellular survival and further obscures the role of EsrC. Because *E. ictaluri* is dependent on the T3SS for intracellular survival (Thune et al. 2007), the early T3SS expression required for intracellular survival must not be dependent on EsrC. This further supports the conclusion that expression of the translocon proteins is not EsrC-dependent, as opposed to requirement of EsrC in *E. tarda* for translocon expression. However, protein expression at later intracellular stages may be EsrC-dependent and may involve the EsrC-regulated T6SS.

As mentioned above, the *Salmonella* T6SS functions to attenuate intracellular growth, an effect that occurs 24 hrs after macrophage infection. Mutation of the T6SS results in hyper-replication intracellularly and hypervirulence in mice (Parsons and Heffron 2005). The function of the *Edwardsiella* T6SS is not known, although Zheng et al. (Zheng and Leung 2007) found that mutagenesis of the *E. tarda* T6SS results in *in vivo* attenuation, suggesting that the T6SS does not have a similar function to the *Salmonella* T6SS. Based on our intracellular replication results for Δ *esrC*, EsrC is not required for the establishment of a hospitable environment intracellularly. Perhaps the T6SS is involved in escaping the macrophage or infecting non-phagocytic cells. The latter may be unlikely, however, because Thune et al. (Thune et al. 2007) found *E. ictaluri* invades non-phagocytic cells poorly.

The lack of *eseH* expression seen in Δ *esrC*, coupled with the observation that Δ *esrC* is not attenuated for growth in macrophage, suggests that activity of EseH is not required for early intracellular replication. EseH has homology to both SspH1 and SspH2 of *Salmonella*, which do

not have homologous functions. SspH1 is involved in *Salmonella*'s resistance to host immunity (Haraga and Miller 2003; Haraga and Miller 2006). SspH2 is involved in actin remodeling (Miao et al. 2003) and is upregulated intracellularly, but SspH1 is not (Miao et al. 1999). Also, *sspH1* is not regulated by EsrB, but *sspH2* is. These comparisons are interesting and suggest that EseH may function more like SspH1, which downregulates host immune responses, because EseH expression is not required intracellularly. Furthermore, the qPCR results for $\Delta esrC$ and $\Delta esrB::km$ suggest the regulator of *eseH* is EsrC, not EsrB.

Mutation of *esrA* and *esrB*, but Not *esrC*, Can Be Complemented *In Trans*. Plasmids carrying copies of the native T3SS regulatory genes restore virulence deficiencies of $\Delta esrA::km$ and $\Delta esrB::km$ in macrophages and *in vivo*. $\Delta esrC$ is not attenuated *ex vivo*, and complementation of $\Delta esrC$ does not adversely affect replication of the WT or mutant strains intracellularly. However, *pesrC* does not complement *esrC* activity in the *esrC* mutant *in vivo*. This is not a result of a negative interaction of the expression plasmid with the native *E. ictaluri* plasmids, because *pesrA* and *pesrB* are both able to restore virulence to *esrA* and *esrB* mutants both intracellularly and *in vivo*.

Complementation was done using the expression vector pBBR1-MCS4, which is shown to have about 30 to 40 copies in *Es. coli* (Antoine and Lochter 1992), indicating a medium copy number. Expressing *esrC* from pBBR1-MCS4, therefore, introduces a significant increase in the gene copy number of *esrC* in *E. ictaluri*. The scope of regulation by EsrC is unknown, and it is possible that excess EsrC expression by the plasmids has effects elsewhere in the genome that affect virulence. Type III secretion system expression is tightly regulated in other organisms and over-expression can result in unfit bacteria (Coombes et al. 2005; Lucchini et al. 2006).

However, it is unclear why *esrB* expressed from a plasmid does not have the same deleterious effect on *in vivo* virulence. EsrB has a much greater impact on T3SS gene expression than EsrC, and because EsrB regulates *esrC*, one might conclude that an increase in *esrB* expression would also lead to an increase in EsrC. However, in that instance, there will still only be one copy of *esrC* for expression, which could limit the amount of *esrC* for transcription.

Conversely, while expression of *esrB* stays constant in various culture media (Chapter 2), *esrC* expression is upregulated in acidic low phosphate media. Perhaps even though *esrB* is expressed from a plasmid for complementation, by virtue of its stable expression, it does not express to a level in which it induces severe overexpression of virulence genes. Because *esrC* responds to environmental cues, expression would be magnified further by expression from each copy of the plasmid. This would result in a much greater amount of EsrC present, and therefore, a greater amount of gene expression activated by EsrC. Complementation of an *E. tarda* *esrC* mutant results in restored phenotypes *in vitro* and *in vivo*.

Although the presence of *pesrC* affects virulence of *E. ictaluri* *in vivo*, it does not inhibit bacterial replication *ex vivo*. This suggests the effects of *pesrC* are not detrimental for replication in the macrophage, and further supports the notion that EsrC regulation is important for pathogenesis only after the first 10 hours of infection. Studies done measuring intracellular replication of *E. tarda* demonstrate moderate differences between WT *E. tarda* and an *esrC* mutant after five hours of growth in the macrophage. However, at five hours WT *E. ictaluri* has low intracellular replication, but between five and ten hours exhibits rapid intracellular replication. This suggests a more prominent role in early intracellular replication for EsrC in *E. tarda* than in *E. ictaluri*.

Overabundant production of EsrC may result in EsrC binding similar, but non-optimal recognition sequences and upregulating genes it normally would not control. Some evidence of

such an action is provided by analysis of the effects of $\Delta esrC$ on *esrA* and *esrB* expression. Mutation of *esrC* does not result in a significant decrease in *esrA* or *esrB* expression indicating EsrC does not regulate *esrA* and *esrB*. However, when expressed from a plasmid, EsrC significantly upregulates both *esrA* and *esrB*. This sort of abnormal regulation could occur with other genes, resulting in abnormal gene expression and reduced fitness of the bacteria.

Overproduction of SPI-2 T3SS genes results in attenuation *in vivo* (Coombes et al. 2005; Lucchini et al. 2006). Overexpression of EsrC may reduce the fitness of *E. ictaluri in vivo*, resulting in reduced fitness and possibly making the complementation plasmid less stable. Instability of the plasmid could result in the complementation plasmid curing from the bacteria. If *pesrC* were unstable and eliminated from the bacterium, the phenotype would revert to that of the parental strain. This would explain the virulence pattern exhibited in the channel catfish challenge by the WT and mutant strains carrying the *esrC* complementation plasmid: complementation results in decreased virulence of the WT and increased virulence of $\Delta esrC$. In retrospect, sampling isolates from channel catfish livers for Ap resistance conferred by the complementation plasmid would determine if the plasmid was present in the bacteria upon death of the fish. Analysis of *pesrC* stability in *E. ictaluri* would be important in determining the reason for the differential virulence effect *in vivo*.

Future Work. Further study of other potential virulence regulators is required. SPI-2 of *Salmonella* is under the regulation of a number of global regulators including PhoPQ, OmpR/EnvZ, and SlyA. Whether or not expression of the T3SS of *E. ictaluri* is under control of similar regulators is unknown. To assist in determining the regulons of both EsrB and EsrC, further analysis is required to identify genetic sequences recognized by EsrB and EsrC. Feng et al. (Feng et al. 2004) used DNaseI protection assays to determine SsrB-binding domains upstream of the SPI-2 T3SS genes *ssrA*, *ssrB*, and *sseI*. Walthers et al. (Walthers et al. 2007)

found additional SsrB-binding domains upstream of *ssaB*, *sseA*, *ssaG*, and *ssaM*. Feng et al. (Feng et al. 2003; Feng et al. 2004) also determined OmpR binding domains occur upstream of SPI-2 T3SS genes *ssrA* and *sseI*. Additionally, PhoP binds the *ssrB* regulatory region and post-transcriptionally controls the expression of *ssrA* (Bijlsma and Groisman 2005). This type of analysis is important to determine which genes EsrB and EsrC regulate. Elucidation of regulatory systems involved in T3SS expression and other virulence mechanisms will provide further insight into the pathogenesis of *E. ictaluri*. Furthermore, regulatory analysis may provide important evolutionary data regarding the association of T3SS regulation to global regulators within bacteria.

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CHAPTER 4

***EDWARDSIELLA ICTALURI* PLASMIDS ARE IMPORTANT FOR PATHOGENESIS IN CHANNEL CATFISH**

INTRODUCTION

All isolates of *Edwardsiella ictaluri* from channel catfish carry two plasmids, one being 5,643 nt and the other 4,807 nt (Lobb and Rhoades 1987; Newton et al. 1988). The larger plasmid is designated pCL1 and the smaller plasmid pCL2 by Lobb and Rhoades (Lobb and Rhoades 1987); Newton et al. (Newton et al. 1988) named them pEI2 and pEI1, respectively. The nomenclature established by Newton et al. (Newton et al. 1988) will be used hereafter. Both plasmids were sequenced by Fernandez *et al.* (Fernandez et al. 2001), and putative virulence-related genes are encoded by each plasmid. No virulence function of the *E. ictaluri* plasmids is reported in the literature.

On pEI1, *eseH* encodes a protein with homology to IpaH of *Shigella flexneri* and SspH1, SspH2, SseI, and SlrP of *Salmonella enterica* serovar Typhimurium. Each of the homologous proteins is a translocated effector of a type III secretion system (T3SS), a common virulence protein secretion system of Gram-negative pathogens. IpaH, secreted by the Mxi-Spa T3SS, is multicopy on the *Shigella* invasion plasmid (Hartman et al. 1990), and is localized to the nucleus of host cells (Toyotome et al. 2001) where it inhibits NF- κ B activation of genes. SspH1 is secreted by both the SPI-1 and SPI-2 T3SS and like IpaH, localizes to the host nucleus, inhibiting NF- κ B gene regulation (Haraga and Miller 2003; Rohde et al. 2007). SlrP is also secreted by both *Salmonella* T3SS (Miao and Miller 2000), but no specific function is attributed to this protein. Both SlrP and SspH2 have a role in the inhibition of MHC-II antigen presentation in dendritic cells, whereas SspH1 and SseI do not (Halici et al. 2008). SspH2 is secreted only by the SPI-2 T3SS (Miao et al. 1999; Miao and Miller 2000). SspH2 associates via its amino terminus with filamin of the host cell, localizing to areas of actin polymerization (Miao et al.

2003). There it associates with profilin, inhibiting it from binding G-actin, thereby inhibiting actin polymerization. SseI is involved in the systemic spread of *Salmonella* by directing the motility of infected cells (Worley et al. 2006). Interestingly, SspH1, SspH2, and SseI are encoded by bacteriophages and are spread horizontally (Miao and Miller 2000), which may explain their homology to proteins in *E. ictaluri*.

On pEI2, *escD* encodes a protein with similarities T3SS chaperones: low molecular weight, acidic isoelectric point (pI), and predicted helical structure (Bennett and Hughes 2000). Immediately downstream of *escD* is *eseI*, a gene encoding a protein with homology to OspB of *Shigella* (Chapter 2). OspB is a T3SS secreted effector of *Shigella* (Buchrieser et al. 2000; Santapaola et al. 2002; Lucchini et al. 2005; Santapaola et al. 2006) that localizes to the nucleus of host cells and affects activity of NF- κ B, leading to decreased cytokine expression (Zurawski et al. 2009).

Recently, two mutations identified in pEI1 and pEI2 resulted in an avirulent phenotype (Thune et al. 2007). A pEI1 mutation in the 3' end of *eseH* is carried by the *E. ictaluri* mutant strain 217UV. Given the homology of EseH to SPI-2 T3SS proteins involved in intracellular replication, this mutation may disrupt a portion of *E. ictaluri*'s ability to reside in host cells, causing attenuation. However, EseH also has homology to SspH1, which is involved in the modulation of the host immune response by *Salmonella*. Therefore, mutation of this *E. ictaluri* gene may affect the ability of *E. ictaluri* to evade immune detection.

The second mutant, strain 166ST, carries a mutation in a region of pEI2 about 125 nt upstream of *escD* and *eseI*, possibly in a regulatory region. EscD and EseI are putative *E. ictaluri* T3SS chaperone and effector proteins, respectively. If the mutation is in a regulatory region of the *escD/eseI* operon, the attenuation may be related to modified expression of *escD* and *eseI* caused by the transposon insertion into the regulatory region.

In this study, the sequences of putative T3SS effector genes were used to survey the *E. ictaluri* genome to identify potential effector genes encoded outside of the pathogenicity island. From this search, four sequences in the genome were found with homology at the nucleotide and amino acid levels to EseH. The ability of the plasmid mutants to infect channel catfish was evaluated. Both mutants have significantly attenuated replication in channel catfish and virulence *in vivo*. Finally, quantitative PCR and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were used to evaluate the expression of T3SS genes in the plasmid mutants. Expression of T3SS regulatory, chaperone, translocon, and effector genes are reduced significantly in both mutants, indicating the avirulent phenotype of the mutants is not related to the sequences mutated within the plasmids. The effect of the plasmid mutations on T3SS gene expression may be due to the plasmid integration into the genome via homologous sequences, thereby affecting gene expression in the vicinity of integration. These are the first studies to evaluate the relationship of *E. ictaluri* plasmids to the pathogenesis of *E. ictaluri* in channel catfish.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Bacterial strains used in this paper are described in Table 4.1. *Edwardsiella ictaluri* was grown in brain-heart infusion (BHI) or Luria-Bertani broth (LB) supplemented with mannitol at 28°C in a Cell-Gro Tissue Culture Rotator (Lab-Line, Melrose, IL). *Edwardsiella ictaluri* was also cultured in *E. ictaluri* defined minimal media (MM19) (Collins and Thune 1996) at pH 7.0 or pH 5.5. A low phosphate media based on the MM19 formula (MM19-P, Chapter 2) at pH 5.5 was also used for culture of *E. ictaluri* strains. Isolates of *E. ictaluri* from channel catfish tissues or channel catfish macrophages were cultured on trypticase soy agar supplemented with 5% sheep blood (BA, Remel Products, Lenexa, KS). Antibiotics were used in the following concentrations when applicable: kanamycin (Km) 50 µg/ml and colistin (Col) 10 µg/ml.

Table 4.1. Bacterial strains used in Chapter 4.

Bacterial Strains or Plasmid	Description	Source
<i>Edwardsiella ictaluri</i> 93-146	Wild type <i>E. ictaluri</i> isolated from a moribund channel catfish from a natural outbreak at a commercial facility in 1993	LSU aquatic diagnostic laboratory
166ST (<i>escD</i> -)	93-146 with a transpositional mutation upstream of <i>escD</i> on pEI2, Km ^r	(Thune et al. 2007)
217UV (<i>eseH</i> -)	93-146 with a transpositional mutation in the 3' end of <i>eseH</i> on pEI1, Km ^r	(Thune et al. 2007)

Specific Pathogen Free Channel Catfish. Egg masses were obtained from Ben Hur Aquaculture Research Facility and disinfected with 100 ppm iodine before being hatched in a closed recirculating aquaculture system at the LSU School of Veterinary Medicine specific pathogen-free (SPF) laboratory. Fish were fed a commercially prepared diet at 2-3% body weight per day. Fish used for immersion challenges were 20 – 40 g; fish used for isolation of head kidney-derived macrophages were 500 – 750 g.

***Edwardsiella ictaluri* Mutant Construction.** *Edwardsiella ictaluri* mutants *eseH*- and *escD*- were constructed by signature-tagged mutagenesis (STM) as described by Thune et al. (Thune et al. 2007). Briefly, *E. ictaluri* was subjected to random transposon insertional mutagenesis. Genomic DNA from strains showing a virulence deficiency were sequenced from the transpositional insertion to determine the insertion site. Of the attenuated strains identified, one mutation was found in both of the *E. ictaluri* plasmids. Mutant 166ST (*escD*-) has a mutation in pEI2 in a region upstream of *escD* and *eseI*. Mutant 217UV (*eseH*-) carries a mutation in pEI1 at the 3' end of *eseH*. Positioning of STM mutations in the plasmids is described in Figure 4.1.

DNA Manipulation. Genomic DNA was isolated using a protocol previously described (Ausubel et al. 1994). Briefly, bacterial cells were pelleted by centrifugation at 12,000 x g for 5 minutes and resuspended in TE. Cells were lysed using 0.5% SDS in the presence of 50 mg/ml RNase A and 100 µg/ml proteinase K. Protein was extracted by using phenol:chloroform:

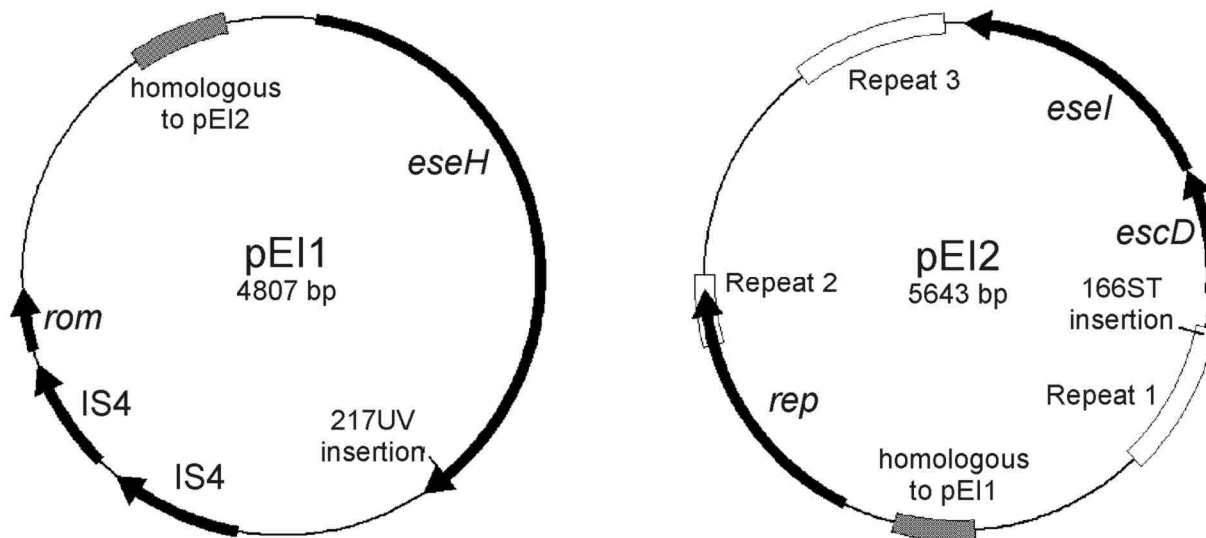


Figure 4.1. Schematic representations of *Edwardsiella ictaluri* plasmids. Shown on the maps are the type III secretion system-related genes of interest (*eseH*, *escD*, *eseI*), genes involved in plasmid replication (*rom*, *rep*), repeat regions within pEI2, insertion sites of signature-tagged mutagenesis mutations (217UV and 166ST), pEI1 IS4-related genes homologous to sites in the genome, and regions of homology between plasmids pEI1 and pEI2.

isoamyl alcohol (25:24:1) with a chloroform wash. DNA was ethanol precipitated in the presence of 0.12 M sodium acetate and resuspended in water. Plasmid DNA was isolated using the Qiagen miniprep kit (Qiagen Inc., Valencia, CA). Restriction digestion was done using enzymes purchased from New England Biolabs, Inc. (Ipswich, MA).

Southern Hybridization. Southern hybridization by was conducted using a probe to the STM-inserted *Km* cassette to determine the presence of a single *km* insert into the mutant *E. ictaluri* genomes. A *km*-specific probe was amplified by PCR using *Taq* DNA polymerase (Applied Biosystems, Foster City, CA) and *km*-specific primers Km757 (5'-GAAGCCCTGCAAAGTAAA-3') and Km1635 (5'-GCTCAGAAGAAGACTCGTCAA-3') using the following amplification cycle: 1x 95° C for 5 min; 35x 95° C for 30 sec, 54° C for 30 sec, 72° C for 1 min; 1x 72° C for 7 min. Products were purified using the Minelute Kit (Qiagen, Inc., Valencia, CA).

Plasmid and genomic DNA from strains wild type 93-146 (WT), *eseH*-, and *escD*- were digested to completion using *BsaI* (New England Biolabs, Inc., Ipswich, MA) and separated on a 1% agarose gel. The DNA was transferred to an ECL Hybond N+ nylon membrane (Amersham Biosciences, Piscataway, NJ) and hybridized to WT, *eseH*-, or *escD*- genomic and plasmid DNA. Hybridization was detected using ECL detection reagents (Amersham Biosciences).

Intracellular Survival in Catfish Macrophages. A gentamicin exclusion assay previously described by Booth et al. (Booth et al. 2006) was used to determine the ability of each mutant to replicate intracellularly. The channel catfish head kidney was removed aseptically, ground through sterile mesh, and diluted with channel catfish macrophage media (CCMM) to yield approximately 10^7 macrophages/ml. Wells of a 24 well plate were seeded with 1 ml of the cell suspension and incubated 16 hrs at 28° C with 5% CO₂.

Macrophages were infected with approximately 1×10^4 colony forming units (CFU) of complement opsonized WT, *escD*-, or *eseH*- *E. ictaluri* for a multiplicity of infection (MOI) of 10 macrophage to one bacterium. At times 0, 5, and 10 hrs, the media was removed, and 100 µl 1% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) was added to wells and incubated at room temperature for 10 min. The lysate was serially diluted, plated on BA, and incubated 48 hrs at 28° C. CFUs were counted, and CFUs/well were determined. Percent uptake was calculated by dividing the mean CFU/well at time 0 by the number of bacteria added to the wells. Fold replication was calculated by dividing CFU/well of bacteria at time 5 or 10 by the mean CFU/well at time 0 and subtracting 1 to adjust for the number of bacteria present at time 0.

Experimental Challenge in Channel Catfish. Fingerling fish were stocked into 20-L tanks at a rate of 25 fish per tank. Fish acclimated 4 weeks prior to challenge and were fed 1.5% body weight per day. Three challenges were conducted to evaluate the ability of *eseH*- and *escD*- to infect channel catfish: mortality, persistence in head kidney, and *in vivo* competition with WT.

For mortality, 12 tanks of 25 fish were used for infection by WT, *escD*-, and *eseH*- with three tanks per treatment. Water in tanks was lowered to 4 L during immersion, and water flow was turned off. Catfish were challenged with approximately 1×10^{11} CFU/L of each *E. ictaluri* strain, and after 1 hr, water flow was restored to the challenge tanks. Fish were monitored daily for mortalities. Upon death, fish were removed, and a sample of liver was taken and streaked onto BA for verification that *E. ictaluri* was present at death.

For persistence challenges, tank water levels were lowered to 4 L, and fish were challenged with approximately 3×10^{11} CFUs/L of each *E. ictaluri* strain. After 15 minutes, water flow was restored. Short- and long-term persistence in the head kidney were monitored. For short-term persistence, samples were taken at hours 0, 0.5, 2, 4, 8, and 12. Long-term persistence was evaluated on days 1 through 7. At each time point, three fish per treatment were sampled. Fish were euthanized in water containing 1 g/L tricaine methanesulfanate (MS-222). Head kidney tissue was removed aseptically, weighed, and homogenized in 500 μ l sterile saline. Homogenates were serially diluted to 10^{-2} and 100 μ l of each dilution were spread onto BA plates. Following incubation for 48 hrs at 28° C, colonies were counted and CFU/g of tissue were calculated.

For the competitive challenges, tank water was lowered to 4 L and fish were challenged with 6×10^{10} CFU/L of both the WT and mutant strains. Following 1 hr, water flow was restored. The first four mortalities were collected, and liver tissue were aseptically collected and homogenized in 0.5 ml sterile saline. The suspension was serially diluted to 10^{-8} , with each dilution done in triplicate. Dilutions were plated onto BHI and BHI supplemented with Km. Following incubation for 48 hrs at 28° C, colony counts were recorded. Only bacteria carrying the Km cassette (mutants) grew on BHI-Km, but all viable bacteria grew on BHI. The difference between the count on BHI and BHI-Km represents the number of WT *E. ictaluri* remaining at

death. From this information, a competitive index was calculated using the equation (Mutant CFU recovered / WT CFU recovered) ÷ (Mutant CFU input / WT CFU input). Values nearer to zero indicate higher attenuation.

RNA Isolation. Total RNA was isolated using the Bacteria RNeasy Protect RNeasy Mini Kit (Qiagen) from *E. ictaluri* strains grown to late log phase in MM19 pH 7.0 and MM19-P pH 5.5. Following purification, total RNA was treated with Baseline Zero Dnase (Epicentre Biotechnologies, Madison, WI) to remove DNA contamination. RNA samples were adjusted to 10 ng/μl.

Quantitative Real-Time PCR. DNase treated RNA (10 ng) was used as template for quantitative PCR (qPCR). The High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used to generate cDNA under the following conditions: 1x 37° C for 60 min followed by 1x 95° C for 5 min to heat inactivate the reverse transcriptase. Quantitative PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems) was used to amplify cDNA using gene specific primers listed in Table 4.2. Primers used correspond to operons and individual genes associated with the *E. ictaluri* T3SS. Polymerase chain reaction was cycled under the following conditions: 1x 95° C for 10 min, 40x 95° C 15 sec and 60° C for 1 min. Following each qPCR reaction, a dissociation curve was used to verify the purity of each target PCR product. Each qPCR reaction also was conducted using cDNA reactions conducted in the absence of reverse transcriptase as template to ensure contaminant DNA was not amplified. Data were collected and analyzed using an Applied Biosystems 7500 Fast Real Time PCR System using Sequence Detection Software v1.4 (Applied Biosystems). Relative quantitation was done using 16s rRNA as the endogenous control gene. Quantitative PCR from WT cultured in MM19 pH 7.0 was used as the calibrator for $\Delta\Delta C_t$ quantification.

Table 4.2. Oligonucleotides used for quantitative PCR in Chapter 4.

Gene	5' Primer	3' Primer
<i>escB</i>	CTTTACCTTGCGATTTGCCTGCGT	AACAGGCACTCCGCCATATGAAAC
<i>escC</i>	AATGCAAGACCTACAGCAGCGTCA	GCGTTGCGATCTCTTGCTGTAACG
<i>eseH</i>	AAGAGGCTGGATGTCTCTGGTACT	GGTAGCCTTGGCATAGGAGTGTTA
<i>eseI</i>	GCAACCCTGCTTAGCAAGTGGAAA	TCAAAGCCTTCCTGGGCCTAATGGA
<i>esrA</i>	AGAGCGAGCATCTGAACAGCATCA	AGTAAGTCATGCTGCTCCTGCGTA
<i>esrB</i>	CAATGCAGCATGCATCACTGGGAA	TCAGCGATATCCCGGTTGCGATTA
<i>esrC</i>	AAAGTTTGGGATGGCGCCGAA	GAGAAATGGGCGGCGTTACAGAAT
16s	AACGCGAAGAACCTTACCTGGTCT	GCTCGTTGCGGGAATTAACCCAA

Whole Cell Lysate Protein Purification. Whole cell lysate proteins were collected from WT, *eseH*⁻, and *escD*⁻ strains of *E. ictaluri* cultured to late log phase in MM19 pH 5.5 similar to the procedure described by Moore and Thune (Moore and Thune 1999). Bacteria were pelleted by centrifugation at 3800 x g for 5 min, and pellets were washed 3x with PBS at the appropriate pH and resuspended in water at a rate of 1 ml of water per 100 µl cell pellet volume.

Phenylmethanesulphonyl fluoride (PMSF) was added to samples for a final concentration of 1 mM. Cell suspensions were sonicated using a Fisher 500 sonic dismembrator (Fisher Scientific, Pittsburgh, PA) at 45% amplitude for 1 min per ml of cell suspension or until suspensions turned from milky to clear. Following sonication, samples were incubated at 4° C for 1 hr. Whole cell lysate samples were pelleted by centrifugation at 12,000 x g for 30 min at 4° C to remove cellular debris, and supernatants were collected. Thimerosal was added to each sample for a final concentration of 0.01% to prevent microbial contamination (Moore and Thune 1999). Whole cell lysate samples were stored at -80° C until two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was conducted.

2D-PAGE Analysis. Whole cell lysate sample concentrations were estimated using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were purified by using the ReadyPrep 2D Cleanup Kit (Bio-Rad Laboratories). Samples were suspended in rehydration buffer at a rate of 100 µg protein per 185 µl rehydration buffer. Protein samples were used to passively rehydrate 11 cm pH 4-7 immobilized pH gradient (IPG) strips (Bio-Rad Laboratories) for 24 hrs at room temperature. Following rehydration, IPG strips were subjected to isoelectric focusing using a Bio-Rad Protean IEF Cell (Bio-Rad Laboratories). Focusing conditions were maintained by the following program at 20° C: rapid ramping to 250 V for 15 min; slow ramping to 2000 V for 1 hr; hold at 2000 V for 3 hrs; linear step to 5000 V for 1 hr; hold at 5000 V for 3 hrs; rapid ramping to 8000 V for 1 hr; 8000V for 40,000 Vhrs; rapid ramping to 500 V until strips were removed.

Immobilized pH gradient strips were prepared for PAGE separation using the 2D Starter Kit Equilibration Buffers I and II (Bio-Rad Laboratories). Equilibrated IPG strips were transferred to wells of a precast 12.5% Criterion SDS-PAGE gel (Bio-Rad Laboratories). Proteins focused by isoelectric point on IPG strips were separated on the basis of molecular weight by electrophoresis at 200 V for 55 min. Following electrophoresis, gels were removed and washed in fixative (10% methanol, 7% glacial acetic acid) for 1 hr. Following fixation, gels were stained with Sypro Ruby protein stain (Bio-Rad Laboratories) per manufacturer's instructions. Gels were washed in fixative an additional hour to destain. Following destaining, gels were washed 3x with distilled water and imaged under UV light using a Bio-Rad Gel Doc XR and Quantity One software (Bio-Rad Laboratories).

Statistical Analyses. For the persistence study, CFU/g data were log transformed, and mean log CFU/g and standard error for each time point were calculated. Daily mortality data are reported as mean cumulative percent mortality (\pm SEM). However, statistical analyses were done using

arcsine transformed cumulative percent mortality. For gentamicin exclusion assays, percent invasion at time 0 (number of bacteria isolated at time 0 / number of bacteria added to wells) and mean percent increase at each time point (CFU at each time / CFUs at time 0) were calculated with standard error. Quantitative PCR data were calculated using the $\Delta\Delta C_t$ method. Values were converted to fold increase log transformed. Means and standard errors of the log-transformed data were calculated.

Analysis of variance (ANOVA) was done for each of the above data sets using the general linear model (Proc GLM) of Statistical Analysis Systems (SAS) v9.1 (SAS Institute, Cary, NC). When ANOVA showed a significant difference, Tukey's HSD was used to adjust the P values for pairwise comparisons. Differences were considered significant if $P \leq 0.05$.

RESULTS

***Edwardsiella ictaluri* Encodes Putative T3SS Effector Genes Outside the T3SS**

Pathogenicity Island. The STM insertion in pEI1 is found in *eseH*, which encodes a protein with homology to SspH1, SspH2, SseI, and SlrP of *Salmonella enterica* serovar Typhimurium and the *Shigella* secreted effector IpaH (Fernandez et al. 2001). Homologies of these proteins are given in Table 4.3. Each of these proteins, including EseH, contains a region of leucine rich repeats (LRR) (Venkatesan et al. 1991; Tsolis et al. 1999; Miao and Miller 2000). Currently, only the function of the LRR of SspH1 is reported (Haraga and Miller 2006). The LRR of SspH1 mediates an interaction with the host cell protein PKN1, resulting in decreased NF- κ B activity. SspH1 and IpaH9.8 ubiquitinate PKN1 in the host nucleus, affecting the function of NF- κ B (Rohde et al. 2007). OspB of *Shigella* is involved in a similar effect of downregulating the host immune response (Zurawski et al. 2009). A gene on pEI2, *eseI*, encodes a protein with homology to OspB. It is interesting that both plasmids encode proteins with homology to translocated effectors involved in the modulation of host immune responses.

SspH1, SspH2, SseI, and SlrP share a conserved translocation sequence in the first 140 amino acids of each protein. Along with SseJ, SopD2, SifA, and SifB, these proteins are called *Salmonella* translocated effectors (STEs) (Brumell et al. 2000; Miao and Miller 2000; Brumell et al. 2003; Brown et al. 2006). The conserved amino sequence is WEK(I/M)xxFF (hereafter referred to as the WEKI sequence), where x is any amino acid.

EseH of *E. ictaluri* has high homology to STE-containing proteins in its first 141 amino acids (Table 4.3), and contains other conserved translocation sequences of STE proteins (Figure 4.2). The presence of conserved *Salmonella* SPI-2 translocation signals in EseH suggests EseH is a translocated effector of the *E. ictaluri* T3SS. SspH2 and SseI are *Salmonella enterica* serovar Typhimurium effector proteins shown to bind filamin in the mammalian host cell via the first 61 amino acids (Miao et al. 2003). EseH of *E. ictaluri* also shares homology with this sequence, having a percent identity and similarity of 66/85 to SspH2 and 65/83 to SseI. SspH1 modulates actin polymerization of the host cell (Miao et al. 2003), and SseI directs infected cell motility, resulting in systemic spread of *E. ictaluri* (Worley et al. 2006). EseH is also similar in the carboxy terminus (amino acids 267 – 619) to SspH1, SspH2, SlrP, and IpaH. The carboxy terminus of SspH2 mediates binding to the host cell protein profilin, resulting in inhibition of actin polymerization within the host cell (Miao et al. 2003).

Table 4.3. Percent identity and similarity of amino acid sequences between EseH of *Edwardsiella ictaluri* and type III secretion system effectors of *Salmonella* and *Shigella*. Homologies were determined using BLAST (Zhang et al. 2000). NSA = no significant alignment

Amino Acids	SspH1	SspH2	SlrP	SseI	IpaH9.8
EseH 1-619 (whole sequence)	54/69	57/72	43/57	57/72	38/55
EseH 1-141 (amino terminus)	40/53	60/75	36/57	57/72	NSA
EseH 267-619 (carboxy terminus)	55/70	58/73	45/62	23/34	40/57

The DNA sequence of *eseH* was used to query the genome of *E. ictaluri* to identify similar genetic sequences. Four open reading frames homologous to *eseH* are found in the genome (Table 4.4). These putative genes are named *eseJ*, *eseK*, *eseL* and *eseM*. These sequences were identified from contigs constructed during the *E. ictaluri* genome-sequencing project. The sequence of *eseK*, however, is not included in the final assembly of the *E. ictaluri* genome. The amino and carboxy termini are all similar in length, but the length of the LRR portions vary. Each protein sequence also has a WEKI sequence (Figure 4.2). The presence of the WEKI sequence in each of the *EseH* homologs suggests each is an *E. ictaluri* T3SS translocated effector protein.

The finding of putative effector genes in both *E. ictaluri* plasmids and in the genome of *E. ictaluri* outside of the T3SS pathogenicity island is consistent with the SPI-2 T3SS-related effectors of *Salmonella*. Three SPI-2 effectors are encoded within the pathogenicity island, but a majority is encoded elsewhere in the genome. The finding of six putative effector genes, *eseH*, *eseI*, *eseJ*, *eseK*, *eseL*, and *eseM*, outside of the *E. ictaluri* pathogenicity island indicates a similar situation, and presents the possibility that other effector genes are encoded in the *E. ictaluri* genome. A number of hypothetical genes were mutated, resulting in attenuation of *E. ictaluri*, in the STM mutagenesis study conducted by Thune et al. (Thune et al. 2007). Although the genes do not match T3SS translocated effectors of other organisms, some may be *Edwardsiella*-specific proteins translocated by the T3SS.

STM Insertions Occur at One Site. Plasmid DNA from WT and mutant strains was isolated, linearized, and separated by agarose gel electrophoresis. As expected, WT *E. ictaluri* 93-146 carries two plasmids, pEI1 and pEI2, which are approximately 4.8 kb and 5.6 kb, respectively (Newton et al. 1988; Bertolini et al. 1990; Fernandez et al. 2001). Both mutant strains, however, carry three plasmids: pEI1, pEI2, and the respective STM mutant plasmid (Figure 4.3A).

A *Salmonella* SspH1 - MFNIRNTQPS
Salmonella SspH2 - MPFHIGSGCL
E. ictaluri EseH - MPLHVGTGHI
E. ictaluri EseJ - MPLYVGTGCF
E. ictaluri EseK - MPLYVGSgyf
E. ictaluri EseL - no match
E. ictaluri EseM - MPLYIGTGCF

B *Salmonella* SspH1 - WEKIQVFF
Salmonella SspH2 - WEKMKEFF
E. ictaluri EseH - RENIQEFF
E. ictaluri EseJ - HEKIQVFF
E. ictaluri EseK - RENVHEYF
E. ictaluri EseL - WEKIKAFF
E. ictaluri EseM - HEKIQAFF

C *Salmonella* SspH1 - EAQQCLAELCH
Salmonella SspH2 - EALECIWTICH
E. ictaluri EseH - EALNCLWEICH
E. ictaluri EseJ - EVLNCLREICY
E. ictaluri EseK - EALTCIQEICH
E. ictaluri EseL - EALSCIREICH
E. ictaluri EseM - EVLNCLREICY

D *Salmonella* SspH1 - SKEILSITLDDAGNY
Salmonella SspH2 - SQEILSVTLDDAGNY
E. ictaluri EseH - GQEMLSVILGDADTY
E. ictaluri EseJ - GQEIVSVTLDDTGTY
E. ictaluri EseK - GQEMLSVILGDDEKY
E. ictaluri EseL - GREILSVTIDAD-TY
E. ictaluri EseM - GREILSVTIDAD-TY

Figure 4.2. Alignment of *Salmonella* translocated effector (STE) conserved amino-terminus domains to *Edwardsiella ictaluri* putative type III secretion system (T3SS) effector proteins. *Edwardsiella ictaluri* putative effector protein sequences encoded outside the T3SS pathogenicity island have strong homology to conserved translocation signals of SspH1 and SspH2 of *Salmonella* (Miao and Miller 2000). The sequences span similar amino acid positions in each protein. The first nine amino acids of SspH1 and SlrP differ from other STE proteins, including SspH2. *Edwardsiella ictaluri* sequences align to the SspH2 sequence MPx(I/V)GxGx(L/F) rather than the SspH1 sequence MFNIxNxQ, where x is any amino acid (A). The WEKI sequence of *Salmonella*, WEK(I/M)xxFF, is required for translocation and is shared among all STE as well as *E. ictaluri* proteins (B). Two other conserved regions were described in the amino termini of STE (C and D) that are also similar in *E. ictaluri* proteins. Shaded amino acids match the *Salmonella* consensus sequences or have a biochemically similar amino acid in that position.

Table 4.4. Percent identity and similarity of leucine rich repeat proteins encoded by *Edwardsiella ictaluri*. Homologies were determined by BLAST (Zhang et al. 2000).

Protein (AA)	EseH (619)	EseJ (789)	EseK (929)	EseL (717)	EseM (792)
EseH (619)		69/76	65/77	62/70	70/79
EseJ (792)	69/76		59/69	72/80	70/79
EseK (929)	65/77	59/69		63/75	57/68
EseL (717)	62/70	72/80	63/75		79/86
EseM (792)	70/79	70/79	57/68	79/86	

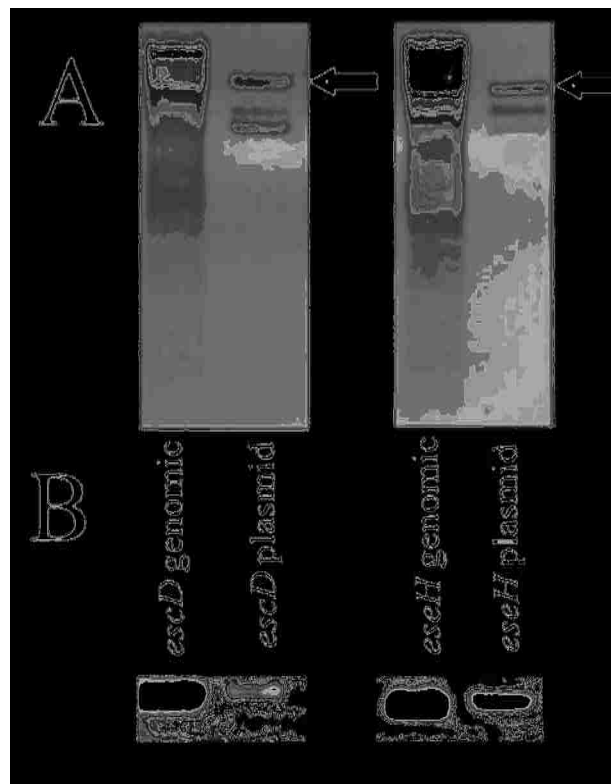


Figure 4.3. Single insertion of the signature-tagged mutagenesis transposon in *Edwardsiella ictaluri* plasmid mutants. Genomic and plasmid DNA digested with *BsaI* was separated on a 1% agarose gel (A). Southern hybridization was conducted using a probe for the inserted kanamycin cassette (B). Arrows indicate the mutant plasmid in each strain.

Southern hybridization verifies that the transpositional insertion site is limited to the plasmids and that a double-insertion event did not occur. For each strain, a single band is detected in the genomic and plasmid DNA preps (Figure 4.3B). The detected band from the genomic DNA is from plasmid DNA collected with the genomic DNA. Both bands are in the same position, indicating that insert occurs once in each strain in the plasmid. The *km* probe does not hybridize to WT genomic or plasmid DNA (data not shown).

Plasmid Mutants Are Attenuated for Replication in Channel Catfish Macrophages. A

gentamicin exclusion assay (Booth et al. 2006) was used to determine the ability of STM *E. ictaluri* plasmid mutant strains to replicate within channel catfish macrophages. Bacterial entry into the macrophages is not significantly different among strains in the gentamicin exclusion assays (Table 4.5), indicating the mutations do not affect the ability of *E. ictaluri* to enter host cells. However, uptake for *E. ictaluri* strains is between 14 and 117% depending on the experiment, indicating a wide range of uptake among macrophages of different experimental fish. The insignificant differences in uptake indicate the mutations do not affect *E. ictaluri*'s ability to infect macrophages.

Table 4.5. Percent uptake of signature-tagged mutagenesis *Edwardsiella ictaluri* plasmid mutants by channel catfish macrophages. Mean percent uptake (\pm SEM) of wild type (WT), *escD*-, and *eseH*- strains of *E. ictaluri* by channel catfish macrophages was calculated from triplicate gentamicin exclusion assays. Values with the same letter in a row are not significantly different ($P > 0.05$).

Experiment	WT	<i>escD</i> -	<i>eseH</i> -
1	88 \pm 17 a	46 \pm 17 a	117 \pm 17 a
2	30 \pm 4 b	28 \pm 4 b	14 \pm 4 b
3	54 \pm 4 cd	30 \pm 4 c	58 \pm 4 d

Replication within macrophages was significantly different between the WT and mutant strains (Figure 4.4). After 10 hours, the mutants replicated less than 1 fold in macrophages, whereas WT increased close to 35 fold. The greatest replication for the WT strain occurs between 5 and 10 hours. The ability of the mutants to survive within macrophages is not compromised, as evidenced by their isolation and culture after 10 hours within macrophages, but their ability to proliferate in or spread among the cells appears lost. Generally, there is low replication in macrophages in the first 5 hours even for WT, indicating the bacteria are not initially able to replicate well within macrophages. This suggests *de novo* synthesis of virulence factors, such as the T3SS, is required for replication.

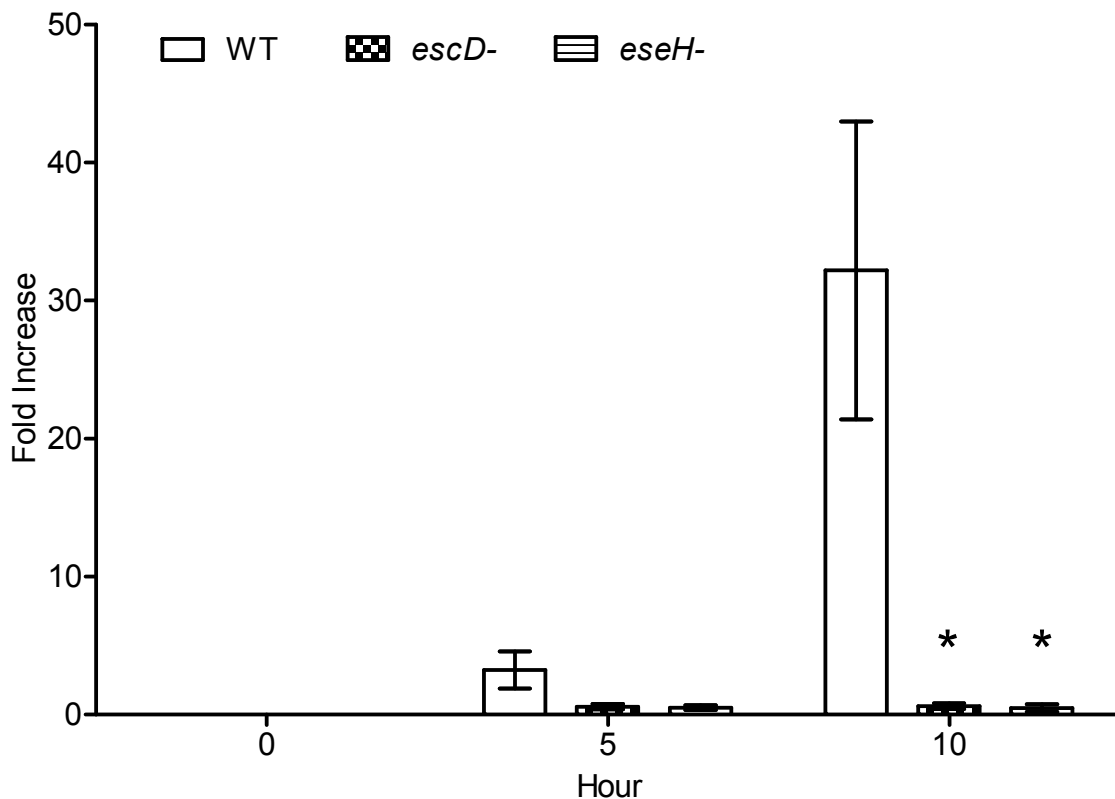


Figure 4.4. Replication of signature-tagged mutagenesis *Edwardsiella ictaluri* plasmid mutants in channel catfish macrophages. Wild type (WT) *E. ictaluri* replicates greater than 30-fold in 10 hrs within macrophages. However, both STM mutant strains *escD*- and *eseH*- replicate poorly in the same timeframe. Bars indicated the mean (\pm SEM) fold increase of triplicate gentamicin exclusion assays. * indicate a significantly lower fold increase than WT at that time ($P \leq 0.05$).

Signature-Tagged Mutagenesis *E. ictaluri* Plasmid Mutants Are Attenuated *In Vivo*.

Specific pathogen-free channel catfish were challenged with WT and STM plasmid mutant strains of *E. ictaluri* to characterize the virulence phenotype of the mutants. Three challenges were done evaluating mortality, persistence within the channel catfish head kidney, and *in vivo* competition with WT *E. ictaluri*.

During the mortality challenge, mortalities began on day 4 post-challenge in fish challenged with the WT strain (Figure 4.5), resulting in cumulative mortality of 77% after 18 days. Infection with either plasmid mutant results in no mortality. These data demonstrate the severe virulence attenuation of the *E. ictaluri* mutant strains. However, the data do not discern if the mutants have lost the ability to infect the host or have lost the ability to replicate *in vivo*.

In order to evaluate the ability of the mutants to infect and replicate within channel catfish, short- and long-term head kidney persistence of the strains *in vivo* was determined. Both mutants are detected in channel catfish head kidney tissue within 30 min of immersion and continue at a relatively constant level for seven days after challenge (Figure 4.6). However, levels of WT bacteria in the head kidney increase significantly over the same time period. Wild type CFU/g values are significantly higher than either the *eseH*- and *escD*- strains starting on day 2 and continuing throughout the remainder of the challenge. These data demonstrate that the mutant strains of *E. ictaluri* infect channel catfish and survive *in vivo* as well as WT *E. ictaluri* for at least 7 days. However, mutant strains are unable to replicate normally *in vivo*, whereas the WT increases up to four logs higher than the initial density.

Competitive indices indicate a significant decrease in virulence for the STM mutants. Neither mutant strain is competitive *in vivo* with the WT strain, because at death, only WT bacteria are isolated from the tissue, resulting in a competitive index of 0 for each mutant. This

data also indicates that defects created by transpositional mutagenesis of the *E. ictaluri* plasmids cannot be complemented by the activity of the WT strain present in the fish.

The combined results of these *in vivo* challenges demonstrate that both plasmid mutants are attenuated for virulence, but can persist in head kidney tissue for at least a week. These data indicate the bacteria can survive within the fish, but the ability of *E. ictaluri* to replicate and spread *in vivo* is reduced. The inability of the mutants to survive in competition with the WT strain demonstrates a significant loss of virulence and suggests that products of the WT strain do not complement the virulence deficiencies of the plasmid mutants.

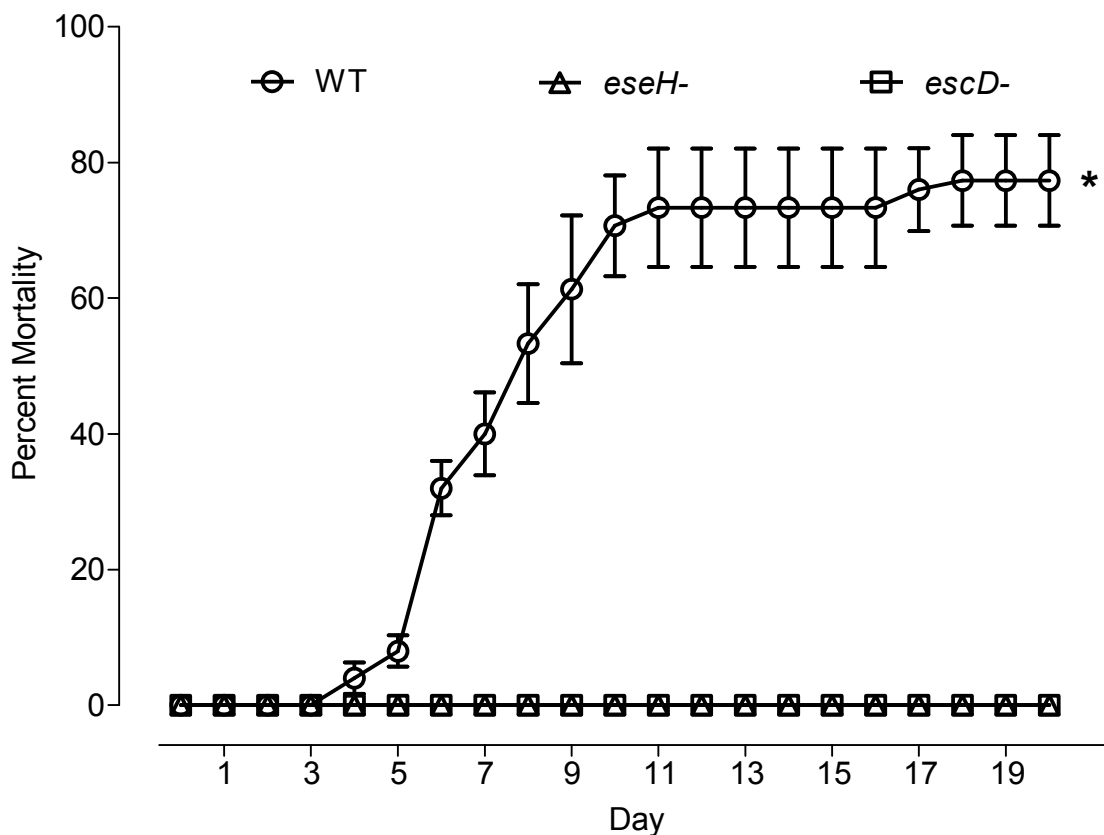


Figure 4.5. Cumulative mortality of channel catfish following infection with signature-tagged mutagenesis (STM) *Edwardsiella ictaluri* plasmid mutants. Wild type (WT) infection results in channel catfish mortality as early as four days post-immersion, resulting in 77% mortality. However, immersion with STM mutant strains *escD*- and *eseH*- resulted in no mortality. Data indicates the mean (\pm SEM) cumulative percent mortality for each *E. ictaluri* strain from triplicate challenge tanks. * indicate significantly higher mortality ($P \leq 0.05$).

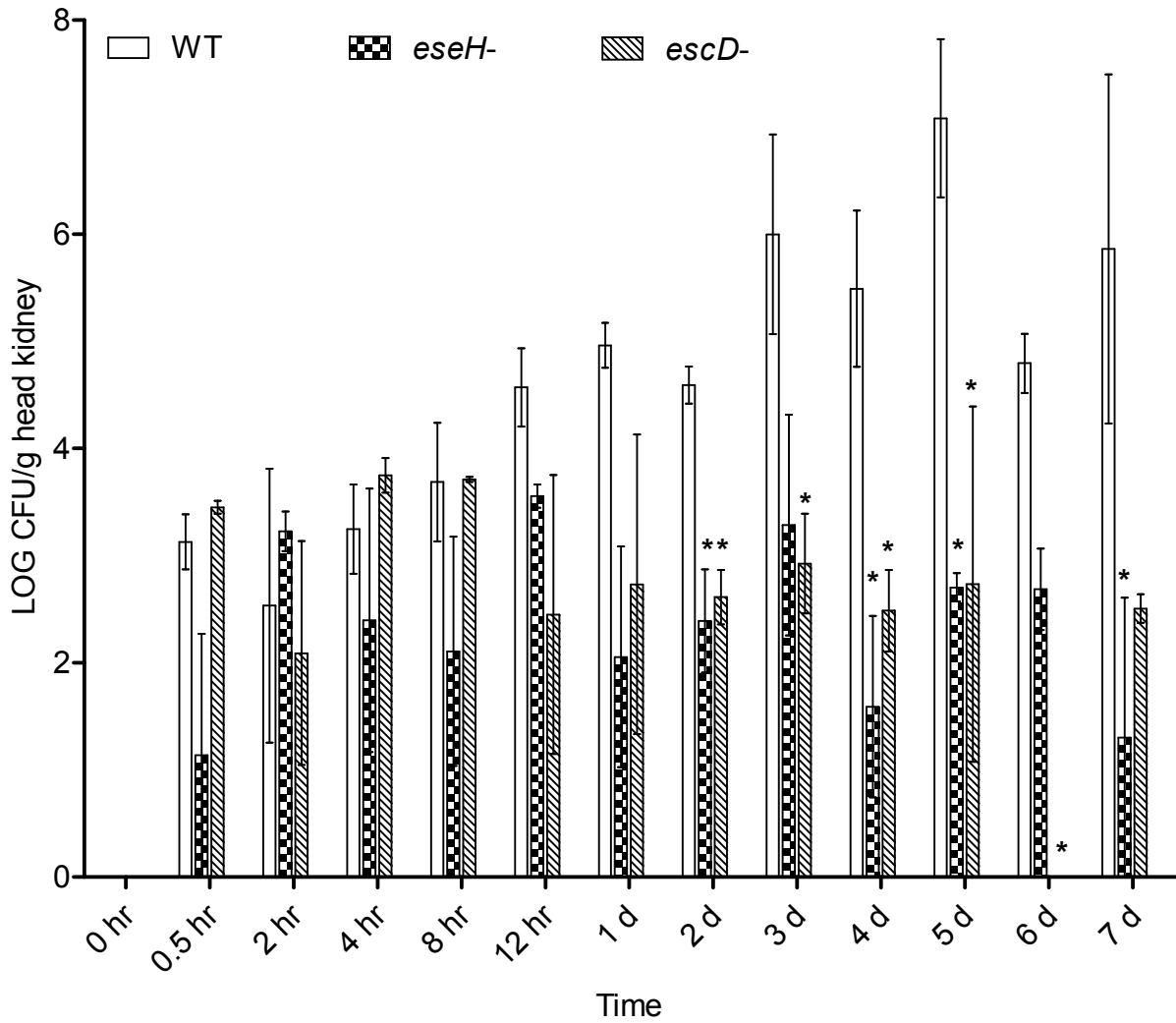


Figure 4.6. Persistence of signature-tagged mutagenesis *Edwardsiella ictaluri* plasmid mutants in channel catfish head kidney tissue. Channel catfish were infected with wild type (WT) and plasmid mutant strains of *E. ictaluri*. Head kidney tissue was sampled at the designated time points and CFU/g of tissue were estimated. Bars indicate mean (\pm SEM) log CFU/g of triplicate samples. * indicate significant differences from WT at that time point ($P \leq 0.05$).

Mutant Plasmids Result in Decreased Expression of T3SS Genes. To determine if the mutations in the *E. ictaluri* plasmids affect expression of other T3SS-related genes, quantitative PCR was conducted on total RNA isolated from *E. ictaluri* cultures grown in MM19-P pH 5.5. Both plasmid mutants exhibit substantial decreases in the expression of T3SS regulatory, chaperone, and effector genes. Expression of *esrB* and *esrC* is not detected in the strains, and

expression of *esrA* is significantly reduced (Figure 4.7). Similarly, *escB/eseG* expression is not detected, and expression of both the translocon genes *eseBCD* and the pEI1-encoded *eseH* is severely reduced (Figure 4.8).

Of the genes assayed, only *eseI* is expressed normally in the two mutants. Other genes, including the T3SS regulatory genes, are significantly reduced compared to expression seen in WT cultured in MM19-P pH 5.5. The significant defect in expression of T3SS genes may be related to the severe decrease in *esrB* expression, which is required for expression and secretion of T3SS proteins (Chapter 2). Mutation of *esrB*, however, does not result in the same degree of *esrA* and *eseH* downregulation as seen in the *eseH*- or *escD*- strains, suggesting that other factors are involved in the loss of T3SS gene expression. These data suggest that the mutations in the *E. ictaluri* plasmids affect T3SS gene expression, likely upstream of EsrAB two-component control. The data also indicate expression of *escD/eseI* is not under similar regulation as the *E. ictaluri* T3SS, because *eseI* is still expressed when other T3SS-related genes are not.

Verification of Reduced T3SS Proteins in Bacterial Whole Cell Lysates. To verify the reduced expression of T3SS genes in the plasmid mutants, whole cell lysate proteins were collected and separated by 2D-PAGE. The T3SS translocon proteins EseB and EseD, as well as the chaperone protein EscA, were all absent from the whole cell lysates of *eseH*- and *escD*- *E. ictaluri* (Figure 4.9A and B). These proteins are readily detected in the whole cell lysate from WT *E. ictaluri* cultured in MM19 pH 5.5 (Figure 4.9C). Furthermore, EseB and EseD are present in *esrB*-deficient *E. ictaluri* whole cell lysates, indicating the T3SS expression defect of the STM plasmid mutants is more severe than observed in an *esrB* mutant. Also missing from the whole cell lysates are the T6SS proteins EvpA and EvpC. Both of these proteins are detectable in the whole cell lysate of the WT strain. The absence of EscA, EseB, and EseD in the STM mutant whole cell lysates verifies the qPCR data showing significant decreases in T3SS expression.

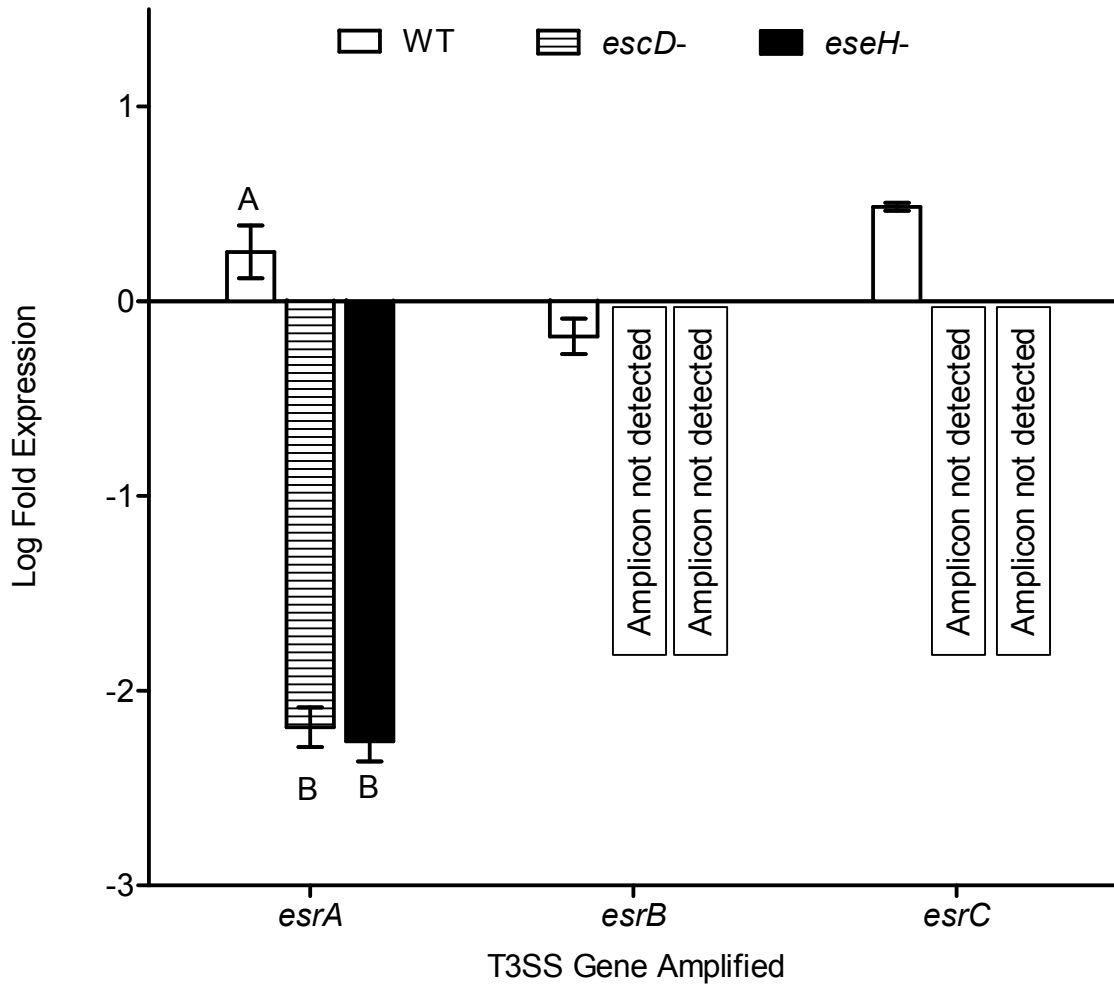


Figure 4.7. Effect of signature-tagged mutagenesis *Edwardsiella ictaluri* plasmid mutation on expression of type III secretion system (T3SS) regulatory genes. Quantitative PCR analyses were used to measure expression of the *E. ictaluri* T3SS regulatory genes *esrA*, *esrB*, and the *esrC* operon containing *esrC* and the apparatus genes *esaGHIJWKL* from wild type (WT), *eseH*⁻, and *escD*⁻ strains cultured in low phosphate minimal media (MM19-P) at pH 5.5. Bars indicate the mean of the log fold expression (\pm SEM) of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

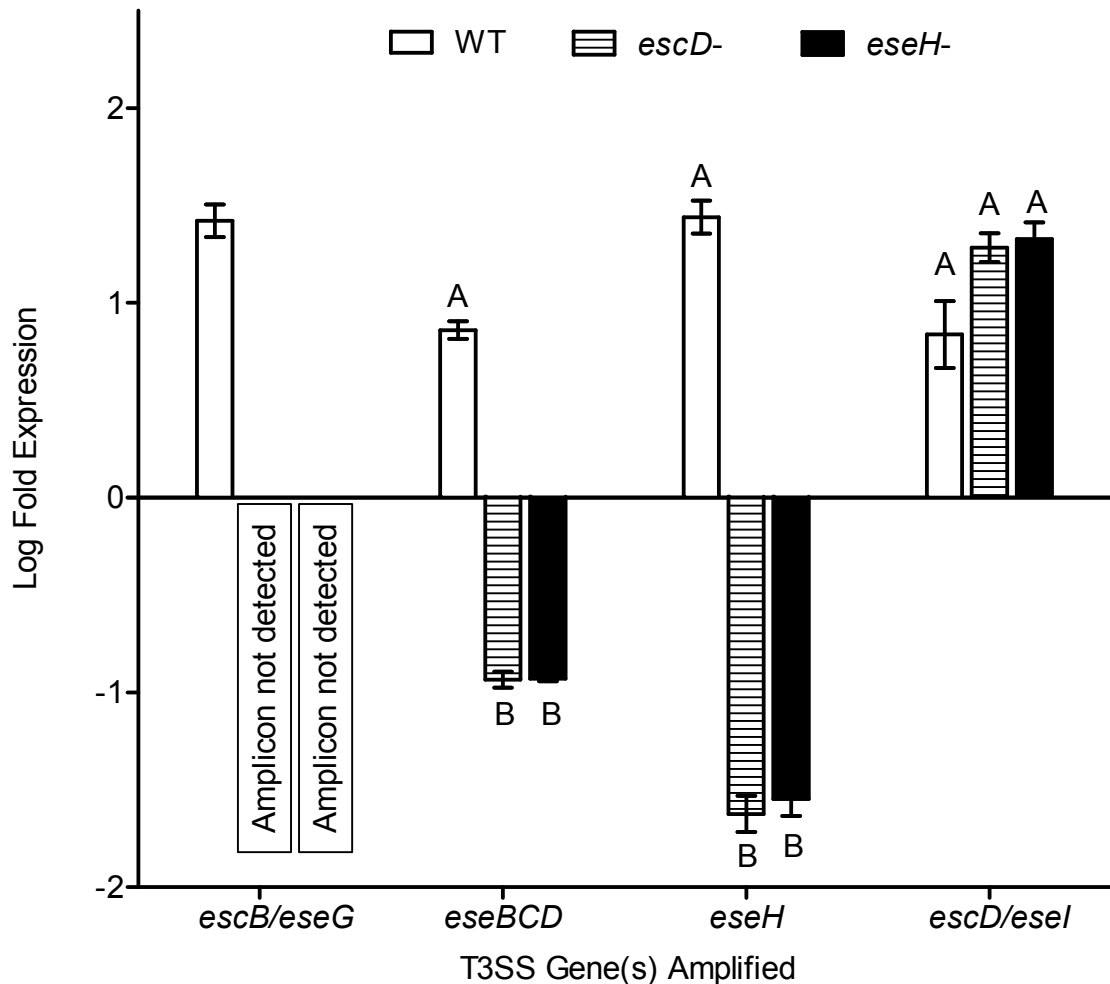
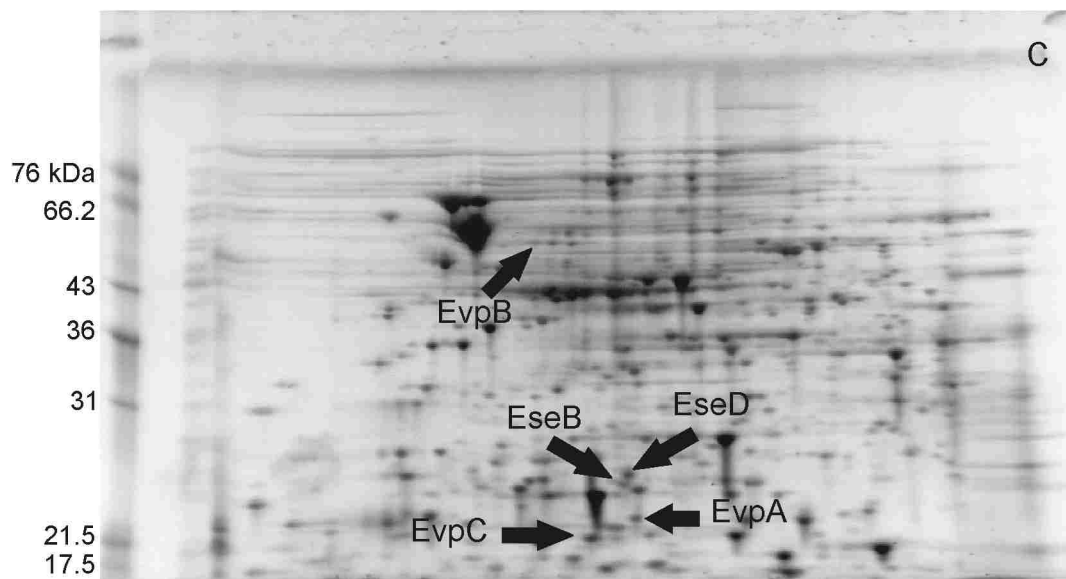
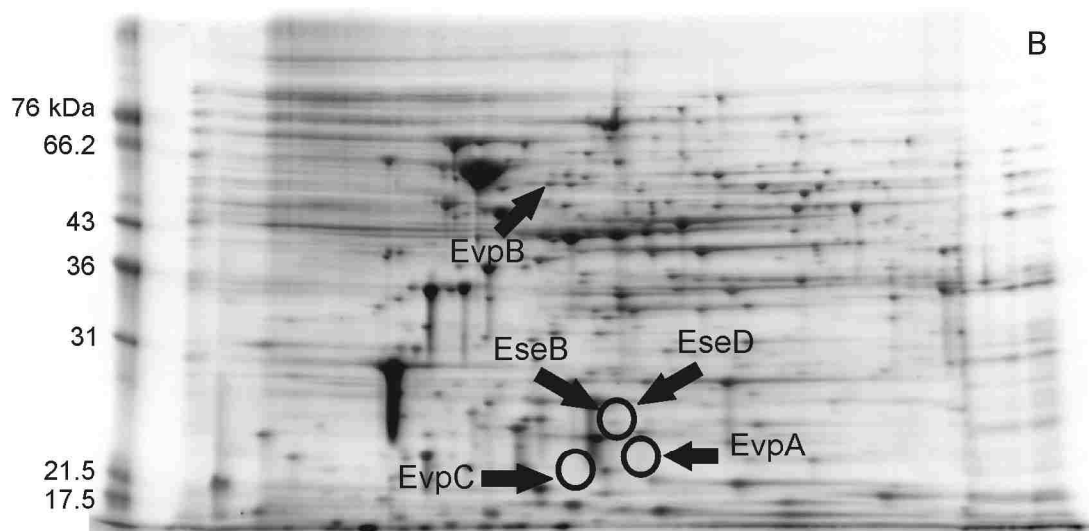
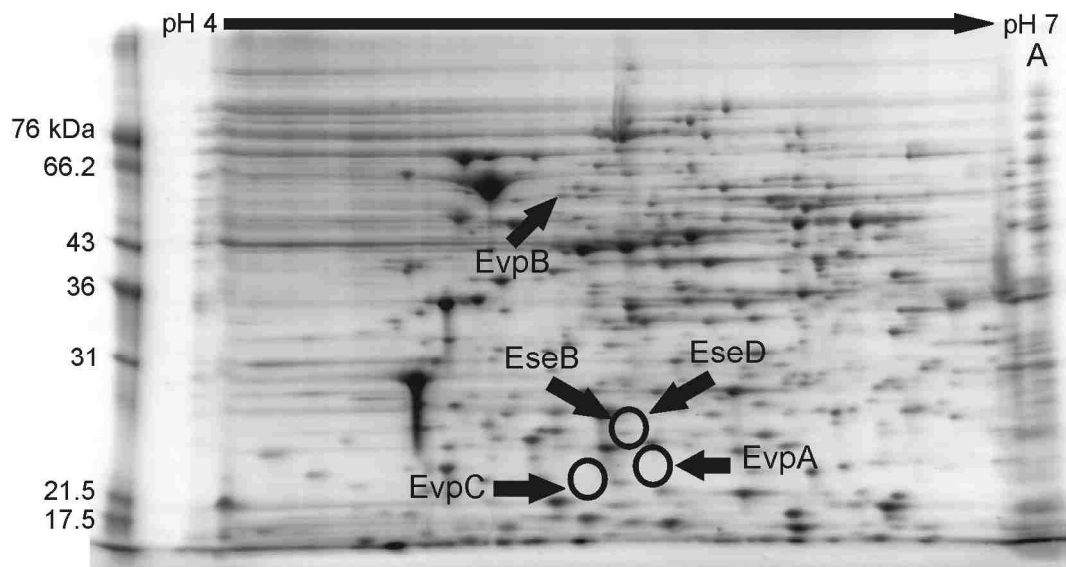


Figure 4.8. Effect of signature tagged mutagenesis *Edwardsiella ictaluri* plasmid mutation on expression of type III secretion system (T3SS) non-regulatory genes. Quantitative PCR analysis was used to measure expression of the *E. ictaluri* T3SS genes encoded by the *escB/eseG* operon, the *escC* operon containing the translocon genes *eseBCD*, the pEI1-encoded *eseH*, and the pEI2-encoded operon containing *escD* and *eseI* from wild type (WT), *eseH*-, and *escD*- strains cultured in low phosphate minimal media (MM19-P) at pH 5.5. Bars indicate the mean of the log fold expression (\pm SEM) of triplicate samples. Values with the same letter within gene group are not significantly different ($P > 0.05$).

Figure 4.9 (Following page). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of signature-tagged mutagenesis *Edwardsiella ictaluri* plasmid mutant whole cell lysate proteins. Whole cell lysate protein from *eseH*- (A), *escD*- (B), and wild type (C) strains of *E. ictaluri* were collected from cultures in MM19 pH 5.5 media and separated by 2D-PAGE. Labels indicate protein spots corresponding to type III secretion system translocon proteins EseB and EseD or type VI secretion system proteins EvpA, EvpB, and EvpC. Circles indicate areas where proteins should appear, but are missing. Gels were run in triplicate, and a representative gel is shown.



Identification of Potential Plasmid Integration Sites in the *E. ictaluri* Genome. The combined results of the qPCR and 2D-PAGE analyses indicate the virulence defects in plasmid mutant strains are not due to the specific insertion sites in the respective plasmids, but rather through some other mechanism. This is surprising, because no sequence in the plasmids encodes a known regulatory protein. Interestingly, the plasmids have regions of homology to each other (Figure 4.1), and pEI1 has IS4-related sequences that are shared throughout the *E. ictaluri* genome, and in some cases, these regions are upstream of hypothetical regulatory genes of unknown function.

Eleven regions in *E. ictaluri* genomic DNA have a 100% match to over 850 nt of the pEI1 IS4 sequence. An additional fourteen regions have a greater than 90% match. These regions of homology provide areas capable of homologous recombination and integration of the plasmid. These regions are located near hypothetical protein kinases, phage genes, transcriptional regulators, transposases, the putative *E. ictaluri* T3SS secreted effector EseJ, heat shock proteins, an ammonia transporter possibly involved with the urease system, and hemagglutinins.

pEI2 has less homology in the genome than pEI1. The greatest homology occurs over a 327 nt sequence in which there is 88% homology to the genome. This region of pEI2 is 890 nt away from the region mutated by signature-tagged mutagenesis. However, pEI1 and pEI2 share 86% homology across a 359 nt sequence in addition to 84% and 76% homology across 122 nt and 139 nt sequences, respectively. These regions of homology may allow for the combination of pEI1 and pEI2, and integration of both plasmids into the genome via the IS4 sequence in pEI1. Furthermore, the pEI1/pEI2 homologous region occurs in the genome near the putative *E. ictaluri* T3SS effector gene *eseL*, and is downstream of the putative effector gene *eseJ*. Although there is no evidence that integration of either plasmid into the genome occurs, there are compelling hypothetical interactions that may occur to modulate expression of virulence genes.

DISCUSSION

A signature-tagged mutagenesis project identified two mutations in the plasmids of *E. ictaluri* resulting in attenuation (Thune et al. 2007). Both mutant strains are unable to replicate within HKDM, and both are strongly attenuated *in vivo*. In addition, the mutations result in severe downregulation of many T3SS-related genes, including the regulatory genes *esrA*, *esrB*, and *esrC*. This effect on the T3SS indicates that the attenuated phenotype of the mutants is not solely due to the mutation of *eseH* or *escD*, but rather due to another mechanism that effects T3SS gene expression.

***Edwardsiella ictaluri* Encodes Putative Effector Proteins Outside of the T3SS Pathogenicity**

Island. *EseH* has homology to many T3SS translocated proteins, with the highest similarity to SspH2 of *Salmonella*. In addition, it shares 85% and 83% similarity to the first 61 amino acids of SspH2 and SseI, respectively, which is the sequence required for these proteins to bind filamin (Miao et al. 2003). Based on these homologies, *EseH* may be an effector involved in survival of *E. ictaluri* within host cells, possibly with a role in modulating cytoskeletal elements during the spacious vacuole formation. *EseH* also has homology to SspH1 encoded by *Salmonella*, which downregulates the host immune response (Haraga and Miller 2003; Haraga and Miller 2006). However, homologous sequence does not guarantee a homologous function. SspH1 and SspH2 have 68% homology (Miao et al. 1999), but do not share a common function. Mutation of *esrC* results in a significant decrease in *eseH* expression (Chapter 3), but the *esrC* mutant was able to replicate as well as WT *E. ictaluri* in HKDM, suggesting *EseH* is not required for early intracellular survival.

Four sequences similar to *eseH* are found in the genome, indicating that, like *Salmonella*, *E. ictaluri* encodes additional T3SS effector genes outside of the pathogenicity island. These four proteins, along with *EseH*, contain LRR regions and homology to conserved sequences of

Salmonella STE proteins (Brumell et al. 2000; Miao and Miller 2000) required for translocation by the T3SS. In addition, the genes encoding EseH, EseJ, EseK, EseL, and EseM in *E. ictaluri* have high DNA and protein sequence homology to each other, with the only significant differences being in the length of the LRR regions.

The fact that *eseH* and *eseI* are encoded on a multicopy plasmid, i.e., a high gene copy number, suggests a need for producing large quantities of this protein during the course of an infection. Interestingly, both plasmids encode proteins with homology to proteins of other pathogens involved in the downregulation of the host immune responses (Haraga and Miller 2003; Haraga and Miller 2006; Zurawski et al. 2009). If the plasmid-encoded proteins have similar functions to SspH1 and/or OspB, perhaps the higher copy number of the plasmid allows for increased production of the proteins, thereby allowing for rapid responses against host immune defenses. However, if EseH has a function more like EseH or EseI, which are involved in host cell actin dynamics (Miao et al. 2003; Worley et al. 2006), the higher copy numbers may allow increased expression for a more rapid modulation of the host cell cytoskeleton.

***Edwardsiella ictaluri* Plasmid Mutants Are Attenuated *Ex Vivo* and *In Vivo*.** Macrophage infections demonstrate the WT strain survives and replicates well during a 10 hr period with the most rapid growth occurring after 5 hours. For both *escD*- and *eseH*-, however, the number of intracellular bacteria increases only slightly, indicating the bacteria are able to survive, but not replicate well within the macrophage. Whether the mutant bacteria are replicating very slowly or are killed at a similar rate as replication is not known.

The results of the immersion challenges indicate each mutant is highly attenuated. Neither of the mutants causes mortality during the course of a three week challenge, while the WT strain causes 77% mortality. The persistence challenge results indicate the mutants colonize the head kidney as well as the WT, because at 30 minutes post-immersion, both mutants and the

WT were found in similar numbers in the head kidney. Over the 7 day experiment, both mutant strains remain relatively stable in numbers, but the WT increases in concentration as time progresses. The competition challenge supports the findings of the mortality and persistence challenges, where neither of the mutant strains are present in liver tissue at the time of death, but large numbers of the WT strain are present. Disappearance of the mutant strains suggests they cannot compete or survive during a co-infection with the WT strain. It also indicates that protein expression of the WT strain does not complement the attenuation of the mutant strains. Based on the results of these experiments, it is apparent that both plasmid mutants are able to survive in the fish for at least a week, but they are unable to grow or spread.

Mutations in the *E. ictaluri* Plasmids Cause a Defect in T3SS Gene Expression. The expectations of the plasmid mutants were that the transpositional inserts cause a regulation defect for expression of *escD/eseI* in *escD*- and a functional defect in EseH in *eseH*-, resulting in attenuation for both mutants. However, qPCR and 2D-PAGE analyses demonstrate that the mutant strains have a severe defect in T3SS gene expression. This is interesting, because the mutated DNA regions in the plasmid do not have any homology to regulatory genes. Yet, the level of downregulation is similar to the severe loss of T3SS gene expression noted in an *esrB* mutant (Chapter 3). However, in the *esrB* mutant, the downregulation of *esrA* and *eseH* is less than that of both *escD*- or *eseH*-. Because expression of these genes is more reduced, it is likely that the effect of the plasmid mutations is on a global regulatory system that controls expression of *esrA* and *esrB*, and possibly other regulatory genes or virulence genes.

The SPI-2 of T3SS of *Salmonella* is in part controlled by regulatory systems outside of SPI-2, including PhoPQ, OmpR-EnvZ, and SlyA (Deiwick et al. 1999; Lee et al. 2000; Worley et al. 2000; Feng et al. 2003; Garmendia et al. 2003; Norte et al. 2003; Feng et al. 2004; Kim and Falkow 2004; Bijlsma and Groisman 2005; Brown et al. 2005; Linehan et al. 2005; Merighi et al.

2005; Lober et al. 2006). Feng et al. (Feng et al. 2003) and Lee et al. (Lee et al. 2000) found OmpR binds DNA upstream of *esrA* and activates expression. PhoP regulates both *ssrA* and *ssrB* at the translational and transcriptional levels, respectively (Bijlsma and Groisman 2005). The role of these regulatory systems on gene expression is unknown in *E. ictaluri*, but the plasmid mutations, which result in abrogated *esrA* and *esrB* expression, may affect these systems.

The *E. ictaluri* plasmids may integrate into the genome and affect expression of genomic genes near the sites of insertion. pEI1 carries genes with homology to the IS4 family of insertion sequences. Although there are regions for potential integration, this phenomenon has not been observed in *E. ictaluri*. Integration and excision may be induced under conditions involved in upregulation of the *E. ictaluri* T3SS. In that scenario, the signature-tagged mutagenesis plasmid mutations may affect either a plasmid's ability to integrate or to affect the function of the plasmid once integrated into the genome. There is much yet to be determined in regards to the roles of the plasmids in virulence, but the homology of the plasmids between each other and the genome suggest a possible role for combination or integration. Conversely, the insertion of the STM tag, which is approximately 2.5 kb, may have affected plasmid supercoiling, allowing these areas of homology to become available for integration. In other words, the mutations may allow the plasmids to integrate or combine when they otherwise would not.

The *E. ictaluri* genome is interspersed with transposase and phage sequences. The activity and functionality of these regions are not known, but the homologous DNA regions repeated throughout the genome make rearrangements possible. How these rearrangements could affect virulence is unknown. The appearance of five homologous genes related to T3SS effector genes also suggests movement and copying of genes. Perhaps the plasmid-encoded *eseH* is the source of those other genomic sequences. If pEI1 has the ability to integrate and excise from the

genome, over time some DNA sequences may have remained in the genome and evolved to the *eseH*-like sequences reported in this study.

Concluding Remarks. The importance of the putative effector genes encoded by pEI1 and pEI2 cannot be determined from this work. The T3SS expression phenotypes indicate the plasmid mutations are not the primary factor in attenuation. The potential for genomic gene expression to be disrupted by mutation in the plasmids makes it difficult to formulate methods to study these plasmid-encoded genes. Furthermore, if the plasmids integrate and affect expression of T3SS genes, it may be difficult to determine all areas of the genome affected by integration. Equally challenging is discerning if a decrease in virulence in a plasmid T3SS gene is a product of an effect on the T3SS, or if there are additional regions of the genome affected, as is the case in the analysis of these STM plasmid mutants.

The study of the STM mutants, however, does give insight into some aspects of EscD and EseI. Expression of *eseI* is the same in the plasmid mutants as observed in the WT strain. T3SS genes, however, were greatly downregulated in the STM mutants. Parallel to that is the effect of *esrA*, *esrB*, and *esrC* deletion on T3SS gene expression. T3SS genes are downregulated in each of those mutants; however, *eseI* maintains a stable expression level. These results indicate EscD and EseI are not T3SS-regulated genes, because mutation of EsrA, EsrB, or EsrC does not affect their transcription. Both SspH1 and SlrP of *Salmonella* are expressed independently of either SsrB or HilA, which regulate the SPI-2 and SPI-1 T3SS, respectively (Miao and Miller 2000). However, both SspH1 and SlrP are secreted by both *Salmonella* T3SS; therefore regulatory dependence of a gene on the T3SS regulators is not a determining factor for secretion by that system.

Further research is needed to determine how mutations in the plasmids can have such a profound effect on the expression of an entire T3SS, particularly when those mutations do not

occur in a gene homologous to known regulators of gene expression. Also, the functions of the plasmid-encoded T3SS proteins of *E. ictaluri* need to be studied to determine their contribution to virulence. Further research to identify other secreted effectors encoded in the genome and determine the functions of the *E. ictaluri* T3SS effectors will be important in developing strategies to combat this devastating disease. In addition, learning about the similarities of effectors of a lower vertebrate pathogen and effectors of higher mammal pathogens may help advance the understanding of the development and distribution of the effector proteins of these ubiquitous secretion systems.

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CHAPTER 5 GENERAL CONCLUSIONS

The *E. ictaluri* T3SS is required for survival within macrophages. Its expression is induced in conditions mimicking the intracellular environment, and mutation of regulatory genes required for expression result in virulence defects intracellularly and *in vivo*. The T3SS genetic structure and sequences are highly homologous to the T3SS of *E. tarda*. However, there are significant differences in the regulation and expression of the respective T3SS. The similarities and differences are summarized in Table 5.1. Although differences appear to exist, both T3SS function to allow intracellular replication, similar to the SPI-2 T3SS. *Edwardsiella ictaluri* shares many characteristics of the SPI-2 T3SS, including expression in response to acidic, low phosphate conditions, homologous T3SS protein sequences, and the encoding of effector genes outside the T3SS pathogenicity island (Table 5.1).

This is the first work to study the expression of the *E. ictaluri* T3SS and the regulatory genes involved in controlling expression of the system. The findings of this study support hypotheses presented. However, the hypothesis that *EsrC* is required for T3SS gene expression is proven incorrect. That hypothesis was based on the findings in *E. tarda* that *EsrC* is required for the expression and secretion of translocon proteins (Zheng et al. 2005). Although mutation of *esrC* in *E. ictaluri* results in a significant decrease in translocon gene expression, it does not prohibit expression, and translocon proteins continue to be secreted from the $\Delta esrC$ strain. The hypothesis that the plasmid mutations result in attenuation due to the inability to produce *EseH* or *EseI* also was incorrect. While both mutations result in significant attenuation, the effect appears to be associated with regulation of the T3SS system upstream of *EsrA/EsrB* regulation rather than being directly attributable to the mutation of *escD/eseI* or *eseH* in the respective *E. ictaluri* mutant strains.

Table 5.1. Comparison of type III secretion system (T3SS) gene organization, expression, and protein function among *Edwardsiella ictaluri*, *E. tarda*, and *Salmonella* pathogenicity island 2.

	<i>Salmonella</i> SPI-2	<i>E. tarda</i>	<i>E. ictaluri</i>
Role of T3SS in virulence	Intracellular replication	Intracellular replication	Intracellular replication
<i>In vitro</i> expression of T3SS genes	Many inducing conditions including low pH or low phosphate; translocon genes not upregulated in acidic	Induced in neutral pH, no nutrient limitation reported	Expression in many conditions, but upregulated in acidic and low phosphate; low phosphate alone does not induce
Secretion of T3SS translocon proteins	Acidic pH required	Neutral pH	Acidic pH required
Relationship between EsrB and EsrC	EsrC homolog not encoded	EsrC dependent on EsrB	EsrC dependent on EsrB
Effect of EsrC on T3SS	EsrC homolog not encoded	Regulates expression of translocon, but not apparatus genes	Partially regulates expression of translocon genes, effect on apparatus genes unknown
Effect of EsrC on the type VI secretion system (T6SS)	EsrC homolog not encoded; T6SS negatively regulated by SsrB	Required for T6SS expression and protein secretion	Required for T6SS expression and protein secretion
Intracellular replication of <i>esrA/ssrA</i> mutant	Attenuated	Attenuated	Attenuated
Intracellular replication of <i>esrB/ssrB</i> mutant	Attenuated	Attenuated	Attenuated
Intracellular replication of <i>esrC</i> mutant	EsrC homolog not encoded	Moderately attenuated	Not attenuated
<i>In vivo</i> virulence of <i>esrA/ssrA</i> mutant	Attenuated	Attenuated	Attenuated
<i>In vivo</i> virulence of <i>esrB/ssrB</i> mutant	Attenuated	Attenuated	Attenuated
<i>In vivo</i> virulence of <i>esrC</i> mutant	EsrC homolog not encoded	Attenuated	Attenuated
Effector genes encoded outside pathogenicity island	Yes	???	Yes
<i>orf29/30</i> association with T3SS	<i>orf29/30</i> homolog not encoded	Possible effector; expressed in same conditions as T3SS; regulated by EsrC	Downregulated in conditions that upregulate the T3SS; relationship to EsrC not determined

***Edwardsiella ictaluri* Encodes Putative T3SS Effector Proteins Outside the Pathogenicity**

Island. The first putative T3SS genes discovered in *E. ictaluri* are carried on the *E. ictaluri* plasmids pEI1 and pEI2 (Fernandez et al. 2001). Thune et al. (Thune et al. 2007) reported that mutations in the T3SS-like pEI1 and pEI2 genes attenuate *E. ictaluri* virulence in channel catfish and also characterized an attenuated strain with a mutation in *esaU*, a putative T3SS apparatus gene. Using the sequence associated with *esaU* to probe the partially completed *E. ictaluri* genome, Thune et al. (Thune et al. 2007) discovered a pathogenicity island encoding a SPI-2 type T3SS. The SPI-2 T3SS secretes effector proteins involved in the intracellular survival and replication of *Salmonella* (Ochman et al. 1996). Thune et al. (Thune et al. 2007) reported a similar role for the T3SS of *E. ictaluri*, as did researchers studying the *E. tarda* T3SS (Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005).

Salmonella pathogenicity island 2 T3SS effector genes are scattered throughout the *Salmonella* genome, but are generally under control of the SPI-2 encoded regulatory protein SsrB. The occurrence of putative T3SS genes on both plasmids (Fernandez et al. 2001) suggests *E. ictaluri* also carries T3SS genes outside its T3SS pathogenicity island. Probing the *E. ictaluri* genome database using the *eseH* DNA sequence uncovered four putative effector genes outside of the T3SS pathogenicity island: *eseJ*, *eseK*, *eseL*, and *eseM*. However, the sequence of *eseK* is omitted from the final genome assembly, calling into question its existence in the genome. Each of these proteins contains a leucine rich repeat (LRR) region of various lengths, and each contains conserved sequences of *Salmonella* T3SS secreted proteins (Brumell et al. 2000; Miao and Miller 2000). These proteins are at least 57% identical and 68% similar to each other, and the homology increases if the LRR regions are excluded from analysis. The *Salmonella* proteins with the greatest homology are SspH1 and SspH2. SspH1 localizes to the host nucleus and modulates NF- κ B activity (Haraga and Miller 2003; Haraga and Miller 2006). SspH2, on the

other hand, associates with host actin polymerization-related proteins filamin and profilin (Miao et al. 2003) and is involved in the inhibition of MHC-II presentation in dendritic cells (Halici et al. 2008).

SspH1 and another LRR protein of *Salmonella*, SlrP, are secreted by both the SPI-1 and SPI-2 T3SS (Miao and Miller 2000). However, SspH2 and other LRR proteins are secreted only by the SPI-2 T3SS. Both SlrP and SspH1 differ from SspH2 and the other LRR proteins in their first nine amino acids. All five of the *E. ictaluri* proteins have sequences matching the conserved sequence of SspH2 rather than SspH1. These and other N-terminal sequence homologies suggest these *E. ictaluri* LRR proteins are translocated by the *E. ictaluri* T3SS.

Two T3SS-related genes, *escD* and *eseI*, are located in an operon on pEI2. EseI has homology to proteins of many pathogens encoding a T3SS, including OspB of *Shigella*. OspB is the only characterized protein with homology to EseI. OspB is secreted by the *Shigella* T3SS (Buchrieser et al. 2000; Santapaola et al. 2002; Lucchini et al. 2005; Santapaola et al. 2006), localizes to the nucleus, and downregulates NF- κ B induced expression of host immune responses (Zurawski et al. 2009). EscD has homology to Spa15 (Fernandez et al. 2001), which is the chaperone of OspB (Page et al. 2002).

Expression of *eseI* is not affected by mutation of any of the three T3SS regulatory genes. Also, culture of WT *E. ictaluri* to stationary phase does not affect *eseI* expression, whereas other T3SS genes are negatively affected. Moreover, in the STM mutants that significantly reduced all T3SS gene expression, *eseI* expression is maintained in comparable levels to the WT strain. However, SlrP and SspH1 of *Salmonella* are not regulated by the SPI-2 T3SS genes, but are secreted by the SPI-2 T3SS (Miao and Miller 2000). Interestingly, *eseI* appears to be upregulated in the presence of acidic pH and low phosphate concentrations similar to the T3SS. This suggests that more than one regulatory protein is responsible for activation of gene expression in response

to those particular environmental conditions. As demonstrated by the mutation of *esrB*, however, this other regulatory gene does not modulate expression of T3SS genes. However, perhaps the regulator of *escD/eseI* is involved in regulation of *esrA* and *esrB*.

No work is reported for gene sequences of putative *E. tarda* T3SS effectors outside the T3SS pathogenicity island. Given the high homology of both the T3SS and T6SS between *E. ictaluri* and *E. tarda*, it is possible that other T3SS effector genes are shared. However, given the host ranges of *E. ictaluri* and *E. tarda*, it would not be surprising that *E. tarda* has a much different arsenal of T3SS effectors than *E. ictaluri*. Further work is needed to determine the proteins secreted by the *E. ictaluri* T3SS and how their function contributes to the pathogenesis of *E. ictaluri* in channel catfish.

Type III Secretion System Gene Expression Is Upregulated in Response to Environmental

Conditions. *Edwardsiella ictaluri* T3SS promoter fusions to *gfp*⁺ and RT-PCR of T3SS

transcripts indicates T3SS genes are expressed in many culture conditions. However, significant upregulation of translocon genes occurs in acidic media. Furthermore, phosphate-limited acidic media upregulates expression of T3SS regulatory, chaperone, and effector genes. The T3SS upregulation in response to acidity and low phosphate is similar to the SPI-2 T3SS (Cirillo et al. 1998; Beuzon et al. 1999; Deiwick et al. 1999; Lee et al. 2000; Hansen-Wester et al. 2002; Garmendia et al. 2003; Hautefort et al. 2003; Coombes et al. 2004; Feng et al. 2004; Kim and Falkow 2004; Lober et al. 2006; Walthers et al. 2007). Acidic pH is required for secretion of the *E. ictaluri* T3SS translocon proteins EseB, EseC, and EseD, analogous to the requirements for SPI-2 T3SS translocon protein secretion and construction (Beuzon et al. 1999; Nikolaus et al. 2001; Hansen-Wester et al. 2002; Coombes et al. 2004; Chakravorty et al. 2005), indicating regulation of the *E. ictaluri* T3SS is similar to SPI-2. However, whereas *E. ictaluri* translocon

gene expression is upregulated in acidic conditions, acidic media does not induce upregulation of SPI-2 translocon genes.

Interestingly, expression of the *E. tarda* T3SS is much different. *Edwardsiella tarda* secretes T3SS translocon protein in neutral media (Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005). While *E. ictaluri* expresses T3SS genes to some extent in neutral pH, no translocon proteins are observed in pH 7.0 culture supernatants. *Edwardsiella tarda* cultures were grown in Dulbecco's Modified Eagle Medium without aeration for 24 hrs. *Edwardsiella ictaluri* does not grow well without aeration, so T3SS gene expression was not evaluated under those conditions. Culture of *E. ictaluri* in MM19 pH 7.0 for 16 hrs approaches late log phase, and culture for 24 hrs enters late stationary phase. Culturing *E. ictaluri* to stationary phase in MM19 pH 7.0 results in significantly less T3SS gene expression than when cultured for 16 hrs, indicating growth to stationary phase does not increase T3SS gene expression. However, protein secretion in stationary phase was not analyzed. While the qPCR results in this study indicate gene expression is not increased in stationary phase, translocon protein secretion may be induced in stationary phase without an increase in translocon gene expression. Coombes et al. (Coombes et al. 2004) also speculated growth to stationary phase may induce SPI-2 T3SS secretion. These results suggest that the *E. ictaluri* and *E. tarda* T3SS are expressed in response to different environmental conditions.

It is interesting that *E. ictaluri* is more similar to *Salmonella* than *E. tarda* in terms of T3SS expression conditions. The homology between the T3SS of *E. ictaluri* and *E. tarda* would suggest expression to be similar to SPI-2 T3SS expression, and the fact that the systems are required for intracellular survival of both species suggests intracellular conditions (acidity, nutrient limitation) induce T3SS expression in both organisms. Perhaps the T3SS of *E. tarda* is more loosely regulated than that of *E. ictaluri*, resulting in expression in more conditions.

However, the expanded host range of *E. tarda*, ranging from fish to higher vertebrates, may require that the *E. tarda* T3SS operates in a different manner than the *E. ictaluri* T3SS.

An important point to make is in regards to the methods used to measure T3SS gene activity between this study and those evaluating *E. tarda* T3SS gene expression. Promoter fusions to GFP, RT-PCR, and qPCR were used in this study to evaluate expression of T3SS genes in different media and different *E. ictaluri* mutants. The promoter fusions and RT-PCR were limited in their ability to measure gene expression, leading to the use of quantitative PCR to measure expression of the native genes from their native loci in the genome and plasmids. Studies measuring *E. tarda* T3SS gene expression, however, used T3SS gene promoter fusions to *lacZ*, and expressed the fusions from a plasmid rather than as single inserts in the genome (Zheng et al. 2005). While reporter genes are widely used and can be accurate for evaluation of gene expression, Hansen-Wester et al. (Hansen-Wester et al. 2002) found that SPI-2 T3SS genes were improperly regulated when expressed from a plasmid. The differences in methodologies used to measure T3SS gene expression may have resulted in some of the phenotypic differences observed. However, both this study and those evaluating the T3SS of *E. tarda* employed 2D-PAGE analyses to study intracellular and secreted proteins, and the results from 2D-PAGE analysis support some of the differences observed between *E. ictaluri* and *E. tarda*.

Furthermore, the growth curves for the two bacteria in the different media used for culture may have resulted in the measurement of T3SS gene expression at different phases in the growth curve. A growth curve was conducted for *E. ictaluri* in each media condition evaluated in this study, and a culture time was chosen based on the amount of time required for *E. ictaluri* to reach late log phase, which correlated to different culture times for different media. Generally, acidic pH took longer to reach late log, presumably from the stressful conditions of acidic pH minimal media. Low phosphate conditions shortened the growth curves, presumably because of

the consumption of the available phosphate. However, by sampling the different media at different time points, every RNA and protein sample were taken at the same phase of bacterial growth to maintain consistency among the samples.

Another important point to make is that these measurements are taken at a single timepoint. This is not reflective of dynamics that may take place over the course of culture. Differential regulation of genes may occur at different timepoints corresponding to a hierarchy of T3SS gene expression. Further analysis of gene expression at different time points and in different media is required for a better understanding of the temporal expression of *E. ictaluri* T3SS genes *in vitro*.

Orf29/30 Expression Differs from T3SS Expression. *orf29* and *orf30* of *E. tarda* are hypothesized to be T3SS-related genes based on their transcriptional dependence on EsrC, proximity to the T3SS, and the finding of coiled-coil regions (Zheng et al. 2005). *Edwardsiella ictaluri* also encodes a region with homology to *orf29* and *orf30*, although the open reading frames are fused into one open reading frame that maintains homologous amino acid sequences to both *orf29* and *orf30* of *E. tarda* (Thune et al. 2007). The combination of *orf29* and *orf30* in *E. ictaluri* suggests these proteins may be physically linked in their function.

Expression of *orf29/30* in *E. ictaluri* does not occur with T3SS gene upregulation. While the T3SS is upregulated in acidic conditions, *orf29/30* expression is not detected in acidic conditions, but is present at neutral pH. This does not demonstrate that Orf29/30 is not secreted by the *E. ictaluri* T3SS, but it does beg the question why a T3SS effector would be downregulated in conditions when the T3SS is upregulated. The dependence of *orf29/30* on EsrC was not studied in this dissertation; however, EsrC expression was also upregulated in acidic, low phosphate media. Further work is required to determine the relationship between *orf29/30* and T3SS regulatory proteins and evaluate the role, if any, of *orf29/30* in the *E. ictaluri* T3SS.

EsrB Is Required for *E. ictaluri* T3SS Gene Expression in Response to Acidic, Low Phosphate Media. Mutation of *esrB* results in reduced expression of translocon and effector genes relative to WT even when cultured in non-inducing conditions. Neither mutation of EsrA nor EsrC has as severe an effect on T3SS expression as EsrB. In comparison, mutation of *esrA* and *esrC* results in decreased expression of T3SS genes, but expression is still higher than that observed in WT cultured in non-inducing media. These results demonstrate an absolute requirement of EsrB for T3SS gene expression.

In the case of both *E. tarda* and *E. ictaluri*, the expression of the T3SS is dependent on expression of EsrB (Srinivasa Rao et al. 2004; Tan et al. 2005; Zheng et al. 2005; Lan et al. 2007). EsrB is homologous to SsrB, which controls the SPI-2 T3SS. Mutation of *esrB* or *ssrB* significantly affects T3SS expression of all three bacteria and attenuates their virulence. However, *Salmonella* does not have a SPI-2 homolog to *esrC*. The presence of the second transcriptional activator suggests regulation of the *Edwardsiella spp.* T3SS varies from that observed in *Salmonella*. Zheng et al. (Zheng et al. 2005) found EsrC to be important for expression of translocon genes, but is not required for apparatus gene expression. Furthermore, EsrC is absolutely required for secretion of *E. tarda* translocon proteins. EsrC of *E. ictaluri* is required for optimum expression of the T3SS, but EsrB has a greater influence on T3SS gene expression. Furthermore, mutation of *esrC* does not abolish secretion of *E. ictaluri* translocon proteins, whereas *esrB* mutation does. Mutation of *esrB* in *E. tarda* has a similar effect on translocon secretion (Tan et al. 2005). Further research is required to determine the genes regulated by EsrB and EsrC to determine what interaction, if any, is required between EsrB and EsrC for optimum expression of the T3SS.

EsrC Serves an Accessory Role for T3SS Gene Regulation in *E. ictaluri*. Mutation of *esrC* in *E. tarda* completely inhibits protein secretion to the *E. tarda* culture supernatant (Zheng et al.

2005) and is similar to the effect observed in an *esrB* mutant (Tan et al. 2005). Mutation of *esrC* in *E. ictaluri*, on the other hand, results in reduced T3SS gene expression, but not to the extent of an *esrB* mutant. Protein secretion, however, is not abolished in Δ *esrC*. The differences observed in the regulatory capabilities of EsrC may be a determinant for the difference in the environmental conditions that induce T3SS expression between *E. ictaluri* and *E. tarda*.

Although EsrC is not critical for expression of T3SS genes, it is required for expression of the putative T6SS protein EvpC. EvpC is also absent in Δ *esrB::km*, but that is likely a result of control of *esrC* expression by EsrB. Both *esrB* and *esrC* mutants exhibit similar EvpC expression and secretion phenotypes, indicating a link between the expression of the two systems.

Specifically, EsrC appears to coordinate expression of the T6SS with expression of the T3SS.

EsrC is equally important for T6SS protein expression in *E. tarda* (Zheng et al. 2005). Further research is required to determine the level of regulatory control EsrC has over T6SS expression.

EsrC Is Not Required for Intracellular Survival of *E. ictaluri*. *Edwardsiella ictaluri* carrying a mutation in *esrC* is able to replicate in channel catfish macrophages as well as the parental WT strain. This indicates the genes regulated by EsrC are not required for intracellular survival. Furthermore, this indicates EsrC does not have a prominent role in T3SS expression, because the *E. ictaluri* T3SS is required for intracellular survival (Thune et al. 2007). The *esrA* and *esrB* mutants present further evidence for the T3SS role in intracellular replication as both are unable to replicate intracellularly.

EsrA and EsrB mutations in *E. tarda* also result in the inability to replicate intracellularly (Tan et al. 2005). Mutation of *esrC*, on the other hand, results in a moderate decrease in intracellular replication (Zheng et al. 2005). Because mutation of *esrC* in *E. ictaluri* does not affect intracellular survival, but moderate attenuation is observed when *esrC* is mutated in *E. tarda*, the importance of EsrC for intracellular survival appears to be different in the two species.

The amino acid sequences of the two proteins, however, are 98% homologous, suggesting the two proteins would act similarly. The differences in T3SS expression, EsrC influence on T3SS expression, and the requirement of EsrC for intracellular survival suggest that although homologous, the T3SS of *E. ictaluri* and *E. tarda* behave in significantly different ways. Further research is required to determine the effect of EsrC on virulence as it relates to T3SS and T6SS expression and intracellular survival.

***Edwardsiella ictaluri* and *E. tarda* Encode Homologous T6SS.** Type VI secretion systems are not well characterized. However, studies indicate that the T6SS is required for intracellular replication for *Francisella tularensis*, *Salmonella enterica*, and *Vibrio cholerae* (Filloux et al. 2008). However, the *Salmonella* T6SS, when expressed, works against the effects of the SPI-2 T3SS and is inhibited by SsrB (Parsons and Heffron 2005). The *Salmonella* T6SS is not expressed until at least 24 hours post-infection, and works in part to attenuate intracellular replication, allowing *Salmonella* to maintain an ordered pathogenesis. It is unknown whether the *E. ictaluri* and *E. tarda* T6SS mediate similar effects. Because *esrC* mutants in *Edwardsiella* do not abolish replication in the macrophage, it is likely that the T6SS is not required for early macrophage growth, suggesting the *Edwardsiella* T6SS may be similar to the T6SS of *Salmonella*. Further analysis of the *Edwardsiella* T6SS will be beneficial in determining the requirement for EsrC regulation and how EsrC regulation relates to EsrB regulation.

The notion that *Edwardsiella* T6SS proteins mediate intracellular growth attenuation is intriguing; however, it is odd that SsrB inhibits the *Salmonella* T6SS, while EsrB upregulates expression of EsrC, a protein positively controlling T6SS expression. EsrB would be upregulating a protein involved in opposing its activity. However, that may also be an important method of feedback inhibition. As more EsrC is accumulated, more T6SS expression will occur,

which would then oppose the activity of the T3SS, thereby preventing possible negative effects of T3SS overexpression.

The findings of *esrC* overexpression in *E. ictaluri*, however, do not support this notion. Expression of *esrC* from an expression vector used for complementation does not significantly decrease macrophage replication, which would be expected if the EsrC-dependent T6SS is involved in intracellular growth attenuation. In fact, overexpression of *esrC* results in an overall increase in nearly all T3SS genes including genes not shown to be affected by deletion of *esrC*.

Mutations in the *E. ictaluri* Plasmids Affect Function of the T3SS. *Edwardsiella ictaluri* carries two plasmids found in all strains isolated from channel catfish (Newton et al. 1988; Bertolini et al. 1990). There are no reports of successful attempts to cure either of the plasmids, indicating both are stable and required for virulence. Each plasmid is small and, other than *eseH* and *escD/eseI*, contains little more than the genes necessary for replication (Fernandez et al. 2001). There are various small unidentified open reading frames on the plasmid, but none that match proteins involved in plasmid addiction.

Thune et al. (Thune et al. 2007) mutated both *E. ictaluri* plasmids by transpositional mutagenesis. Mutations occur in or near the T3SS-related genes on both plasmids, greatly attenuating both. However, the attenuation appears to be unrelated to the T3SS-related genes mutated. Both mutants have significantly reduced T3SS gene expression indicating the mutation of the plasmids has some effect on the global regulation of the T3SS and not the individual components thought to be affected by the transposon insertion into the plasmids. Moreover, the mutant strains carry native copies of the plasmids in addition to the mutant copies, indicating the virulence defects cannot be complemented. This suggests that the copy number of the plasmids must be strictly controlled in order to maintain a virulent phenotype. How the plasmids have such strong control of T3SS gene expression, however, is a difficult phenomenon to explain.

pEI1 has significant homology across at least 800 nt in over 30 regions of the genome. This homology may allow for pEI1 to insert into the genome and somehow modulate virulence genes, although pEI2 has little homology to the genome and likely does not have the ability to integrate like pEI1. However, pEI1 and pEI2 share a homologous sequence, suggesting pEI1 and pEI2 may be able to recombine and insert into the genome via the pEI1 sequence homology. Regions in the genome with homology to the pEI1 800 nt sequence include areas upstream of hypothetical sensor kinase and response regulator genes, phage genes, transposase sequences, and putative T3SS effector genes. The association of the homologous sequences to potential regulatory genes suggests that plasmid integrations can have an important effect on the expression of genes. However, the role of these regulatory genes is currently unknown. Further research is required to determine other regulatory systems encoded by *E. ictaluri* to resolve what role they play in T3SS regulation. However, based on the findings of this study, the *E. ictaluri* plasmids are clearly important to the pathogenesis of *E. ictaluri*, and their role in virulence requires further study.

Hypothesized T3SS Regulatory Cascade. The findings of these studies indicate the *E. ictaluri* T3SS can be induced by environmental conditions. Acidic pH and low phosphate concentration were found to induce T3SS transcription, but other signals are likely to modulate T3SS expression, as well. Furthermore, there may be other signals required for expression and secretion. Effector proteins are not detectable *in vitro*, suggesting their secretion is not induced in the conditions assayed. However, Deiwick et al. (Deiwick et al. 2002) found that many T3SS proteins of *Salmonella* are not produced at high enough levels for analysis by SDS-PAGE. Study of *E. ictaluri* T3SS translocated effectors will likely require the use of protein-specific antibodies or epitope tags fused to the protein sequence to determine signals required for specific effector protein secretion.

EsrA and EsrB are homologous to the SsrAB two-component regulatory system of *Salmonella*, in which SsrA is a membrane-bound sensor kinase that phosphorylates SsrB, a response regulator, once a stimulus is detected. SsrB then induces T3SS promoter activity resulting in transcription of genes within the regulon. Therefore, based on the *Salmonella* SsrAB system, EsrA receives a signal and phosphorylates EsrB, thereby activating it for upregulation of T3SS promoter activity. EsrB activates expression of translocon, chaperone, and effector genes, as well as the AraC-type regulator EsrC. *Salmonella* does not encode an EsrC homolog to base function from, but limited findings in *E. tarda* indicate EsrC can regulate T3SS gene expression as well as modulate expression of non-T3SS genes, such as the T6SS. EsrC of *E. ictaluri* and *E. tarda* appear to have somewhat different effects on gene expression within the T3SS, but both are absolutely required for virulence *in vivo*.

Analysis of EsrB and EsrC transcriptional control in *E. ictaluri* indicates EsrB is required for expression of the T3SS, and EsrC activity enhances EsrB-dependent T3SS gene expression (see Chapter 3, Figure 3.16). EsrC induces expression of the T6SS, which has a yet unknown function. EsrC may also regulate the pEI1-encoded gene *eseH* and other putative T3SS effector genes encoded throughout the *E. ictaluri* genome. The coordinated efforts of EsrB and EsrC, as well as other possible virulence gene regulators, results in an efficient, coordinated response to *in vivo* stimuli. Further work is required to determine other *in vitro* and *in vivo* stimuli required for expression of the *E. ictaluri* T3SS. Analysis of DNA sequences recognized by EsrB and EsrC will provide a tool for identification of genes potentially regulated by these proteins, and perhaps elucidate the roles they have in overall virulence gene expression.

Concluding Remarks. The T3SS of *E. ictaluri* has proven to be a complex system that requires extensive study. The continued study of the *E. ictaluri* T3SS is important for finding treatments for *E. ictaluri* or for manipulating the T3SS to prevent disease. The study and understanding of

T3SS-containing pathogens of animals and humans is advancing quickly. Similar studies in T3SS-containing pathogens of fish need not keep up with human pathogen research, but do need to advance the understanding of T3SS used by pathogens of lower vertebrates. As the knowledge base advances for mammals and humans, and treatments against T3SS are developed, similar methods of treatment may be applicable to fish, providing an understanding of the T3SS in the pathogen of interest is known. Many chemicals have already been shown to inhibit T3SS expression (Muschiol et al. 2006; Hudson et al. 2007; Negrea et al. 2007; Pan et al. 2007).

Edwardsiella ictaluri has plagued the channel catfish industry for over 30 years with no end in sight. An understanding of how this pathogen operates within the host is required before any significant treatment, cure, or preventative can be developed.

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APPENDIX

ABBREVIATIONS COMMONLY USED IN THIS DISSERTATION

2D-PAGE – Two-dimensional polyacrylamide gel electrophoresis

ANOVA – Analysis of variance

Ap – Ampicillin

BA – Blood agar plates

BLAST – Basic Local Alignment Search Tool

CCMM – Channel catfish macrophage media

cDNA – copy DNA

CFU – Colony forming unit

Cm – Chloramphenicol

Col – Colistin

DMEM – Dulbecco's Modified Eagle Medium

Esa – *Edwardsiella* secretion apparatus (T3SS-related)

Esc – *Edwardsiella* secretion chaperone (T3SS-related)

ESC – Enteric septicemia of catfish

Ese – *Edwardsiella* secreted effector (T3SS-related)

Esr – *Edwardsiella* secretion regulator (T3SS-related)

Evp – *Edwardsiella* virulence protein (T6SS-related)

FACS – Fluorescence-activated cell sorting

GFP – Green fluorescent protein

HKDM – Head kidney-derived channel catfish macrophages

HTH – Helix-turn-helix

IEF – Isoelectric focusing

IPG – Immobilize pH gradient

Km – Kanamycin

LB – Luria Bertani broth

LE/Lys – Late endosomes and lysosomes

LRR – Leucine rich repeat

MM19 – *E. ictaluri* defined minimal media

MM19-P – *E. ictaluri* defined minimal media with low phosphate concentration

MOI – Multiplicity of infection

NF- κ B – Nuclear factor-kappa beta

ORF – Open reading frame

PCR – Polymerase chain reaction

pEI1 – *E. ictaluri* plasmid 1

pEI2 – *E. ictaluri* plasmid 2

pI – Isoelectric point

PMF MALDI-TOF/TOF MS – Peptide mass fingerprinting matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

PMSF – Phenylmethanesulphonyl fluoride

qPCR – Quantitative PCR

RT-PCR – Reverse transcriptase PCR

SCV – *Salmonella* containing vacuole

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

Sif – *Salmonella*-induced filament

Slt – Soluble lytic transglycosylase

SPI-1 – *Salmonella* pathogenicity island 1

SPI-2 – *Salmonella* pathogenicity island 2

SPF – Specific pathogen-free

Ssa – *Salmonella* secretion apparatus

Sse – *Salmonella* secreted effector

Ssp – *Salmonella* secreted protein

Ssr – *Salmonella* secretion regulator

STE – *Salmonella* translocated effector containing the WEKI translocation sequence

STM – Signature tagged mutagenesis

T3SS – Type III secretion system

T6SS – Type VI secretion system

TCA – Trichloroacetic acid

WEKI – Conserved amino acid translocation signal shared by a group of *Salmonella* T3SS secreted effectors

WT – Wild type

VITA

Matthew Lee Rogge, born in December, 1976, is the youngest of four children. Originally from Chippewa Falls, Wisconsin, he spent a great deal of his childhood in Malvern, Arkansas. After moving back to Wisconsin the summer between his junior and senior year in high school, Matthew graduated from Chippewa Falls Senior High School and enrolled in the biology program at University of Wisconsin – Stevens Point where he met his wife Rebecca. Matthew graduated in 2000 with a bachelor of science in biology and a minor in chemistry. He and Rebecca left Wisconsin for Ames, Iowa, to pursue their master's degrees at Iowa State University. Matthew joined the Animal Ecology Program, working with Dr. Joseph E. Morris studying the culture of walleye fingerlings in plastic-lined ponds at a state hatchery. Shortly after enrollment, Matthew and Rebecca were engaged. In 2002, Matthew and Rebecca graduated and were soon married. The couple moved to Baton Rouge, Louisiana, where Rebecca accepted a position with Louisiana State University, and Matthew pursued his doctorate in The School of Veterinary Medical Sciences through the Department of Pathobiological Sciences under the guidance of Dr. Ronald Thune. In January of 2007, Matthew and Rebecca welcomed their first child, a daughter named Morgan. In May of 2009, Matthew finished his dissertation research and completed the requirements for the degree of Doctor of Philosophy.