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Comparative Genomic Studies of Catfish and Zebrafish Strains of Edwardsiella ictaluri

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COMPARATIVE GENOMIC STUDIES OF CATFISH AND ZEBRAFISH STRAINS OF *EDWARDSIELLA ICTALURI*

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ABSTRACT

Edwardsiella ictaluri is a gram negative bacterium that is the causative agent of enteric septicemia of catfish. In 2011, this bacterium was identified as the causative agent of massive death in zebrafish populations in U.S. In this project, we found that isolates of *E. ictaluri* from zebrafish comprise a unique strain that differs from the type strain of *E. ictaluri* phenotypically as well as genetically. Also, strains of *E. ictaluri* from zebrafish are non-infectious in channel catfish *Ictalurus punctatus* by immersion.

Here we sequenced two zebrafish strains of *E. ictaluri* and compared the potential virulence genes in these strains with their homologous genes from a typical catfish strain. One of the major differences between the catfish strain and the zebrafish strain was found in the O-antigen biosysnthesis cluster. The catfish strain and the zebrafish strain each contained unique genes in their O-antigen biosynthesis cluster and the entire O-antigen biosynthesis cluster of the zebrafish strain of *E. ictaluri* was nearly identical to that of *E. piscicida* C07-087. The differences in the O antigen were further confirmed by observing the different banding patterns of the purified LPS samples from the catfish and the zebrafish strain of *E. ictaluri*.

Comparative genomic DNA analysis revealed that the major part of the type III secretion system is present and consistent among the zebrafish strains and the catfish strain but single nucleotide polymorphisms (SNPs) were found in certain type III secretion system genes. The type IV secretion system harbored the most variations between the catfish strain and the zebrafish strain. Other potential virulence related

systems, the type VI secretion system and the urease system, are conserved between the catfish and zebrafish strains of *E. ictaluri* with only few SNPs.

In addition, to protect against outbreaks of edwardsiellosis in zebrafish populations, the wild type zebrafish strain of *E. ictaluri* was mutated with the goal of generating attenuated strains that could serve as live attenuated vaccines. Both of our mutants, the *ureG* and *esrC* mutant, were proven to be fully attenuated by immersion in zebrafish. Further study is needed to test their efficacy as live attenuated vaccines.

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

Edwardsiella Genus

The genus *Edwardsiella*, in the family *Enterobacteriaceae*, is named after P.R. Edwards in memory of his contributions to enteric bacteriology [1]. Prior to 2014, there were three known bacterial species in the genus, *Edwardsiella tarda*, *Edwardsiella hoshinae* and *Edwardsiella ictaluri* [2-5]. *Edwardsiella tarda* was the first species validated as a new taxon in the *Edwardsiella* genus by DNA hybridization [6]. In addition, because of the characters of *E. tarda*, the *Edwardsiella* genus was classified as a member of the *Enterobacteriaceae* family [7]. In 1980, another bacterial species, *Edwardsiella hoshinae*, was described and classified in the *Edwardsiella* genus [4]. The *E. hoshinae* strains comprised a new DNA hybridization group and were 37-58% related to *E. tarda* [4]. The other member in this genus *Edwardsiella ictaluri*, known as the causative agent of enteric septicemia of catfish (ESC), was characterized as a new species in 1981 [5]. DNA hybridization studies suggested that *E. ictaluri* is closely related to *E. tarda* and the relative binding ratio can reach to 56-60% at 60 ℃ [5].

Differences in host range have been noted among the different species of *Edwardsiella*. *Edwardsiella tarda* and *E. ictaluri* both can cause disease in various kinds of fish but *E. tarda* can also infect humans, marine mammals and reptiles [8-10]. *Edwardsiella hoshinae* is also found in reptiles and birds [11]. The diversity of *E. tarda* presents both phenotypically and genetically [12, 13]. In addition, *E. tarda* displays serological heterogeneity, and the strains are classified into 61 O antigen groups and 45 H antigen groups based on the international serotyping scheme [14]. Genetically, two genotypes are defined, DNA group I and DNA group II. The DNA group II has higher

similaritity to *E. ictaluri* [15]. Recently, based on further characterization of the original *E. tarda* strains, clusters of *E. tarda* from fish and eel are proposed as two novel species in this genus, *E. piscicida* and *E. anguillarum,* respectively [16, 17]. Although *E. tarda* can be associated with fish kills, *E. piscicida* may be the more frequent reason for disease outbreaks in the U.S. catfish industry [18]. *Edwardsiella piscicida* was first characterized as a new species based on the analysis of European and Asian strains but later, forty-four isolates from diseased catfish in the U.S. were recognized as *E. piscicida* [16, 18]. Abayneh et al. [16] have shown that most of the *E. piscicida* strains studied so far are also highly pathogenic to zebrafish *Danio rerio* by intramuscular injection while the typical *E. tarda* can cause disease in adult zebrafish only by intraperitoneal (ip) injection [19]. Strains of *E. ictaluri* are highly infectious in channel catfish, *Ictalurus punctatus.* The LD_{50} of *E. ictaluri* when injected into channel catfish is appromaximately 10^4 CFU/ml [20]. Biochemically, *E. tarda* and *E. ictaluri* share many characters but unlike *E. tarda*, *E. ictaluri* does not produce indole or H_2S in sulfide indole motility (SIM) medium or H_2S in the triple sugar iron (TSI) slant medium. In addition, *E. tarda* can grow at 42 ℃ and is motile at 37 ℃ while *E. ictaluri* cannot grow well above 30 ℃ and is non motile outside the range of 22-28 ℃ [21]. *Edwardsiella piscicida*, previously classified as *E. tarda*, cannot be distinguished from *E. tarda* by typical biochemical tests. However, many, but not all, *E. piscicida* strains are found to be negative for degradation of β-methyl- D-glucoside, citric acid and L-proline. These characters can potentially differentiate *E. piscicida* from *E. tarda* [16].

Initial isolation and characterization of *E. ictaluri.* *Edwardsiella ictaluri* was first isolated from diseased channel catfish *Ictalurus punctatus* in 1976 [22, 23]. In 1981, it

was identified as the causative agent of ESC and was described as a new bacterial species, most closely related to *E. tarda,* in the family *Enterobacteriaceae* [5]. It is a gram-negative, rod-shaped bacterium with peritrichous flagella and is motile at 25 ℃. *Edwardsiella ictaluri* is described as being negative for indole, urease, H_2S production, citrate utilization, and gas production from glucose [5, 21]. *Edwardsiella ictaluri* is not particularly fastidious, can be isolated on standard bacteriological media such as TSA 5% blood agar plates but is slow growing upon primary isolation. For *in vitro* growth on TSA 5% blood agar plates, it takes approximately 48 hours to form 2 mm diameter colonies at 28 ℃ [23]. The optimum water temperature range in which *E. ictaluri* causes disease is 22-28 ℃ [24].

Outbreaks of enteric septicemia of catfish. From 1981 to 1990, ESC spread throughout the U.S. where catfish farming is practiced, primarily Mississippi, Arkansas, Alabama and Louisiana [25]. During this period it became the most economically important disease in the catfish industry causing millions of dollars of losses yearly. Early on, it was believed to be a disease that only affected ictalurid catfishes but in recent years, the host and geographic ranges of *E. ictaluri* have expanded. Cases have been reported from cultured freshwater catfish *Pangasius hypophthalmus* [26], walking catfish *Clarias batrachus* [27], and yellow catfish *Pelteobagrus fulvidraco* [28] A few non-catfish species such as Nile tilapia *Oreochromis niloticus* [29], green knife fish *Eigemannia virescens* [30], devario *Danio devario* [31], rosy barb *Puntius conchonius* [32], and Ayu *Plecoglossus altevelis* have also been mentioned as possible hosts in the literature.

The homogeneity of *E. ictaluri.* *Edwardsiella ictaluri* has historically been considered a homogeneous species in terms of biochemical phenotype, isozymes, plasmids, serotype

and genotype [21, 33]. There are 2 plasmids that are typically maintained in *E. ictaluri* from catfish, designated as pCL1 and pCL2 by Lobb et al. or pEI1 and pEI2 by Newton et al [23, 34]. DNA sequences of both plasmids have homology to parts of the Type III secretion system which functions to transfer effector proteins to the host cells [35]. However, recent data indicate that plasmids in isolates from fish species other than channel catfish vary [23, 34, 36-38]. Therefore, it is interesting to know whether the differences between isolates from various fish are due to or partially due to the proteins encoded by the plasmids. Serologically, catfish isolates of *E. ictaluri* are believed to be homogenous when analyzed using monoclonal antibody [39] and only the isolate from the green knife fish is serologically different from the catfish isolates. The serological difference is believed to reside in the O polysaccharide antigen [23].

Zebrafish *Danio rerio* and initial isolation of the zebrafish strain of *E. ictaluri.* The zebrafish *Danio rerio* is a tropical freshwater fish. It is a member of the minnow family *Cyprinidae*. The original range of the species is the Himalayan region of India, but the fish now has been introduced to many different parts of the world through the aquarium trade. In recent years it has become an important laboratory fish due to its hardiness and ease of reproduction in captivity. The genome of *Danio rerio* has been sequenced and many transgenic strains are important in human biomedical research. Acute bacterial diseases are not common in cultured populations of zebrafish. Those that occur are usually chronic or asymptomatic infections caused by *Mycobacterium* spp, most often *M. chelonae* [40, 41]. Although species like *M. marinum* and *M. haemophilum* have been associated with outbreaks of morbidity and mortality, these outbreaks are generally protracted and the onset of mortalities is not acute [42, 43]. Acute mortalities in zebrafish

facilities are more likely to be associated with minimal or uncompensated physiologic stress due to poor water quality. Opportunistic pathogens may then be responsible for environmental gill disease or septicemia caused by secondary gram-negative bacterial infections (e.g., *Aeromonas* spp., *Pseudomonas* spp., *Pleisiomonas* spp.).

Acute bacterial diseases were not previously reported from laboratory or pond populations of zebrafish, however in 2011, *E. ictaluri* was determined to be the cause of high mortality rates at zebrafish rearing facilities in 4 different states [38]. From these outbreaks and other strains sent to the Louisiana Aquatic Diagnostic Laboratory (LADL) at Louisiana State University (LSU) for identification, a total of 8 isolates of *E. ictaluri* from zebrafish were collected and archived. The highly infectious nature of *E. ictaluri* in zebrafish emphasizes the utility of quarantine in preventing the introduction and spread of this contagious pathogen into a laboratory with valuable zebrafish colonies [38].

Pathological comparison of edwardsiellosis in channel catfish and zebrafish. Catfish affected by acute ESC develop septicemia and high mortalities over a short period of time [44, 45]. Catfish can develop chronic ESC as well, characterized by meningoencephalitis. Infected fish may swim erratically and exhibit an open ulcer in the top of the head [23]. Clinical signs are minimal in the acute phase of the disease; however, in the sub-acute and chronic phases clinical signs such as hemorrhagic ulcers or petechial hemorrhage in the skin may be obvious. Fish may also hang in the water with head up and tail down or swim lethargically. Other signs may include focal areas of necrosis (white spots) in the liver and swelling of the spleen and head kidney.

Clinical signs in zebrafish usually include hemorrhage in the skin near the eyes, on the ventral surface of the head and abdomen, and at the base of fins. Abdominal swelling

due to ascites is a common clinical sign. Histopathology reveals necrotic foci in the kidney, spleen, liver, hematopoietic tissue, nasal pits and the intestine. Other clinical signs include, pale gills and liver, swollen spleen and occasionally skin ulcers. It is common for zebrafish primarily infected with *E. ictaluri,* to also have secondary infection with *Aeromonas, Plesiomonas* or *Pseudomonas spp*. [38, 46]. These faster growing colonies can overgrow and obscure *E. ictaluri* colonies, complicating the diagnosis.

The pathogenesis of *E. ictaluri* infection in channel catfish. *Edwardsiella ictaluri* is an enteric bacterium, so the intestine is one of the important routes for the bacteria to enter to the host. However, this is not the only route it can gain entrance into the host. The bacterium can potentially invade via the nares and travel to the olfactory organs and brain. The bacterium may also enter the gill or through skin abrasions on the fish [47-49]. The host innate immune response, especially via phagocytes, is designed to exclude these invading bacteria. Detailed mechanisms have been reviewed previously [50, 51]. Briefly phagocytes in the host can successfully engulf and destroy the bacteria since the phagosome that carries the bacteria can fuse with lysosomal elements, which contain bactericidal agents, to form the phagolysosome. A combination of defense mechanisms work in concert in the phagolysosome to kill the engulfed bacteria. First, low pH inhibits bacterial replication, degradative enzymes in the phagolysosome can then lyse the engulfed bacteria and reactive oxygen species decompose the components of the bacteria. However, *E. ictaluri* has evolved multiple mechanisms to escape the phagocyte killing and proliferate in the host cells [52-55]. During *E. ictaluri* infection, depending on the ratio of bacteria and immune cells as well as the status of the host cells, the bacteria may be able to survive and replicate intracellularly, even with the existence of bactericidal

molecules, such as reactive oxygen species and nitrous oxide. The immune cells, specifically macrophages, may become a vehicle for bacterial dissemination in the host [56], in a similar fashion to how macrophages function during *Salmonella* infection [57]. So far, studies indicate that *E. ictaluri* has the ability to invade epithelial cells [58], macrophages [59] and neutrophils [60]. Evidence from multiple references indicate that *E. ictaluri* can gain entry through actin polymerization, disrupting junctions of the host cells and being internalized by receptor-mediated endocytosis [58, 61].

Once *E. ictaluri* enters the host, it can live both intracellularly and extracellularly. Early studies demonstrated the low antibody titers in the host, strong cell mediated immunity with stimulators e.g. lipopolysacchrides (LPS), and bacterial cell replication in head kidney derived macrophages (HKDM) [59, 62, 63]. All these studies indicate that *E. ictaluri* is more favorably adapted to survive the intracellular environment.

For *E. ictaluri*, as well as other members of the *Enterobacteriaceae,* including *Salmonella typhi*, *E. tarda* and *E. piscicida*, the first step in initiation of infection is adhesion and invasion of the host epithelial cells. The surface structures of the bacterial cells contribute the most to this step, through the action of adhesins, flagella, fimbria, pili, LPS and secretion systems. As a result, these virulence factors have become the emphasis of the comparative studies between *E. ictaluri* strains.

Genome-wide Study of *Edwardsiella spp.*

Development of whole genome sequencing techniques. The first gold standard nucleotide sequencing method was developed by in 1975 by Edward Sanger. This sequencing method used four kinds of dideoxynucleoside triphosphate (ddNTP), the analogues of the normal deoxynucleoside triphosphates, to terminate reactions in four

tubes. The sequencing results were then read manually from the various DNA bands after electrophoresis on a denaturing polyacrylamide gel [64]. Sanger sequencing was used for the 13-year-long human genome project [65]. Following this project, the need for a high efficiency and low cost sequencing method was realized. Pyrosequencing, a method based on the detection of pyrophosphate (PPi) during reactions, was developed for confirmatory sequencing and de novo sequencing. This method can detect sequences within real time and is cost-effective [66]. However, the specificity and sensitivity of this sequencing method were a concern [67]. The limits of previous sequencing methods were the driving force behind the development of next-generation sequencing (NGS) techniques. Next generation sequencing methods are good for their high throughput, high efficiency and low cost. There are two widely used platforms, the LifeTechnologies Ion Torrent Personal Genome Machine (PGM) and the Illumina MiSeq. The Illumina Miseq method is based on the detection of fluorescence when a fluorescently labeled nucleotide is added to a growing strand [65, 68]. In contrast, the mechanism behind the Ion Torrent sequencing method is the sensing of hydrogen ions that are released upon incorporation of a nucleotide into a DNA strand [65, 68].

Genomic studies of the *Edwardsiella* genus. With the development of novel sequencing techniques, the genomes of more bacterial strains have been revealed. To date, a total of 13 strains in the *Edwardsiella* genus have been sequenced including seven *E. tarda* strains, two *E. ictaluri* strains, three *E. piscicida* strains and one *Edwardsiella hoshinae* strain. Genome sequencing provides clues for classification of the unknown bacteria and specifies the intra/inter-species taxonomic relationships of the originally defined species. In addition, it can also present hints for virulence factor identification. Comparative

genomic studies have shown that *E. tarda* strains can be grouped into two major genotypes, EdwG I and EdwG II [69]. Now the newly sequenced bacterial strain, which was originally known as *E. tarda* EdwG I from fish, is considered a novel new species and has been named *E. piscicida.* Other researchers, who analyzed the genomes of the EdwG I strains from eel by comparative genomic analysis and phylogenetic comparison found that the bacteria isolated from eel should also be classified as a new species, *Edwardsiella anguillarum* sp. nov [70].

Virulence Factors

Lipopolysaccharides (LPS). As an outer layer of the outer membrane in gram-negative bacteria, lipopolysaccharide (LPS) is a key component in terms of pathogenesis. The LPS molecules can interact with each other and form a barrier to prevent harmful hydrophobic molecules such as antibiotics and complement from disturbing the cell. They also have an effect on the host cell membrane permeability and can even participate in destroying the host cells [71-73]. The LPS molecule has three regions: lipid A, core oligosaccharide and O polysaccharides. The hydrophobic lipid A is embedded in the membrane, and joined to the inner core, leaving the outer core connected to the hydrophilic O-polysaccharides (Figure 1.1). Based on the structural differences, LPS samples are classified into three categories, the smooth, semi-rough and rough LPS [74].

Structurally, the O-polysaccharides display the most inter-species and intra-species variations compared to the lipid A and the core oligosaccharides. The variations in the O-antigen length are associated with the differences in virulence of *Pseudomonas aeruginosa*, *Shigella flexneri* and *Brucella abortus* [75-77]. Research has suggested that in the *Enterobacteriaceae,* the LPS O-antigens tend to present a bimodal length

distribution and the bacteria with short O-antigen chains display a low level of complement resistance and are highly attenuated [78]. Studies of LPS in model Gram-negative organisms *E. coli* and *Salmonella* suggest the essential role of LPS in cell viability, however this is strain-dependent [71].

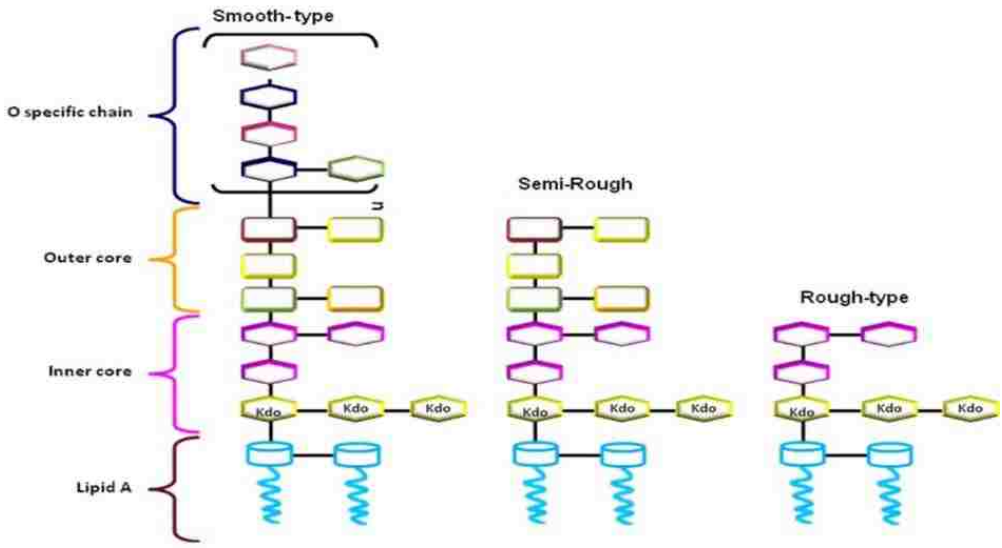

Figure 1.1 The schematic representation of LPS structures. This figure is adapted from Reyes et.al [74]

Secretion systems. In gram-negative bacteria, secretion systems are designed to transport virulence factors, nutrients, extracellular appendages and DNA to maintain the communication between bacterial cells, as well as between bacterial cells and host cells [79, 80]. There are seven types of known secretion systems, including the type I secretion system (T1SS) to the type VI secretion system (T6SS) and the chaperon-usher (CU) system. Among those, the type I, type III, type IV and type VI secretion systems are one-step transport systems, which work independently of the general secretion pathway and twin-arginine translocation pathway, transferring the substrates across the bilayer of the cell membrane directly. This review will focus primarily on T3SS and T4SS.

Type III secretion systems include chaperones, effectors, the injectisome, the translocon, secretion signals and regulators (Figure 1.2). The T3SS forms an injectisome, also known as the type III system apparatus, to transport effector proteins in a one-step way from the cytoplasm of the bacterial cell directly to the host cell. The injectisome structure consists of approximately twenty-five proteins and is relatively conserved among bacterial species. Eight of the twenty-five proteins in the injectisome share high similarity with the flagellar components [81]. Thus, the type III secretion system is divided into flagellar and non-flagellar types [82]. The T3SS we described here is the non-flagellar type that can translocate effectors to the host cells instead of secreting the components of the flagellum.

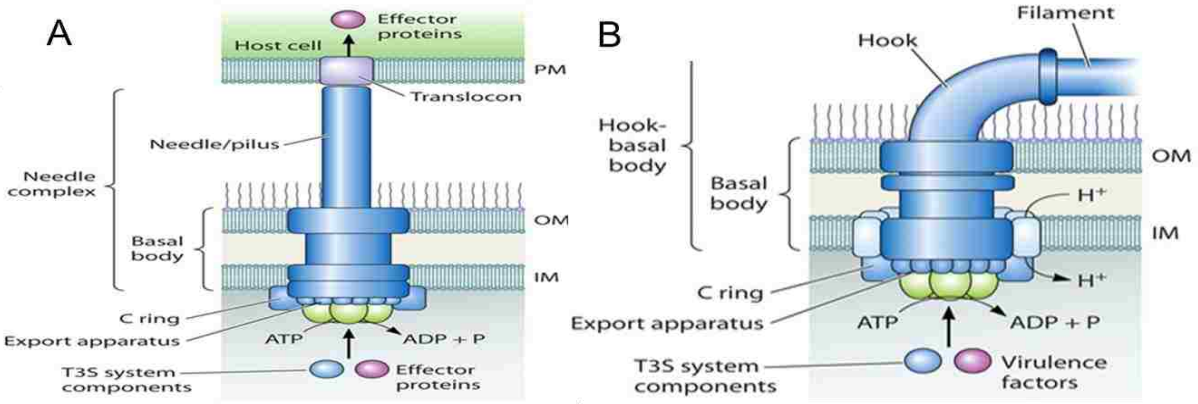

Figure 1.2 Schematic representations of the type III secretion systems. (A) The non-flagellar type of T3SS which transfers effectors to the host cells (B) The flagellar type of T3SS which secretes extracytoplasmic components of the flagellum. This image is adapted from Buttner et al. [83].

Upon assembly completion, most T3SS are in an off mode until bacteria-host contact activates the system to inject effectors into the host. The effectors secreted by T3SS can facilitate bacterial invasion, survival and intracellular replication. Many reviews are available covering the function of T3SS in various bacteria [84-90]. More specifically, injection of effectors into host cells can impair cell signaling and host responses. In *Salmonella,* a closely related bacterium to *E. ictaluri* in the

Enterobacteriaceae family, T3SS effectors can prevent phagosome maturation, inhibit apoptosis and affect host inflammation pathways [91-93]. In addition, T3SS effectors can also subvert cellular trafficking [94-96]. Examples of how T3SS effectors manipulate host cell pathways are described in many enteric pathogenic bacteria, e.g. *E. coli* [97, 98] and *Shigella* [99, 100].

In addition to T3SS, bacteria have also evolved other secretion systems to bring about transport across the membrane barrier. One example of that is the T4SS (Figure 1.3). The T4SS can be categorized into three groups based on their functions, including the contact mediated conjugation system which delivers DNA substrates to bacterial or eukaryotic cells; the effector translocator, which is also contact mediated, transferring protein effectors to eukaryotic cells; and the DNA uptake and release systems to communicate with the extracellular milieu [101]. All of the three types of T4SS are present in gram-negative bacteria, but only the conjugation subfamily is found in gram-positive bacteria [102, 103].

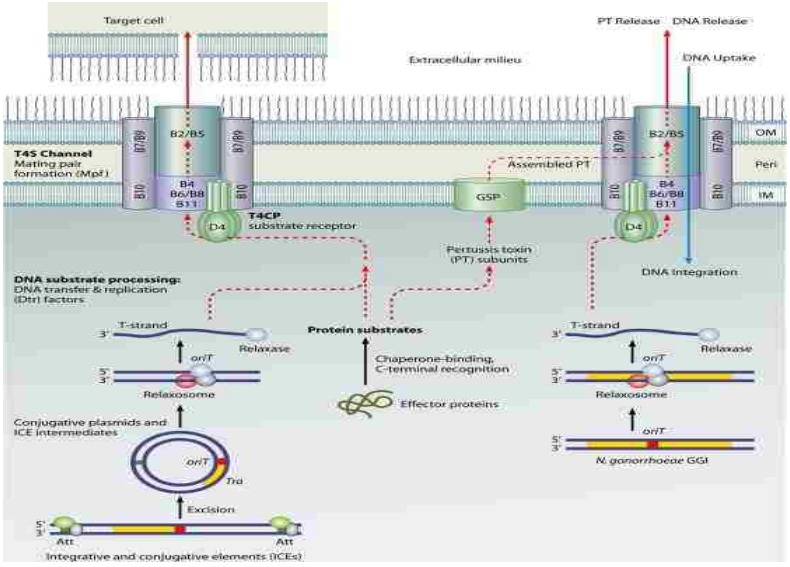

Figure 1.3 The conjugation, effector translocator, and DNA release/uptake subfamilies of T4SS. This figure is adapted from Alvarez-Martinez and Christie [101].

Vaccine Studies in Fish

Vaccines are biological preparations that help build host immunity against certain pathogens. They can stimulate the natural immune processes and will not induce resistance like antibiotics do. Thus, vaccines are considered an effective way of controlling diseases in fish as well as other animals. The first licensed and widely used vaccine in fish was a bacterin made from formalin killed *Yersinia ruckeri* for protection against enteric redmouth disease in trout [104]. This is the first generation of vaccines that used formalin-killed bacteria by immersion to induce humoral immunity. Later generations of vaccines were designed based on the immunogenic proteins or peptide antigens that have protective potential. These vaccines are recombinant, DNA, subunit and vector type vaccines [105]. In the 1990s, more research emphasized the production of modified live vaccines (MLV). Modified live vaccines are made primarily of genetically altered live bacteria cells and these cells can survive in the host but lose their ability to cause overt disease. The attenuated bacterial pathogens comprising the MLV were shown to be efficacious as vaccines for fish [106, 107]. This kind of vaccine does not require an adjuvant to induce the immune response since the attenuated live bacteria have the ability to infect the host, remain viable in the host for several days and induce cellular immunity (CD4 or CD8 T-cell responses) and later humoral responses [107]. These vaccines can be given to fish in multiple ways, including intramuscular (IM) or intraperitoneal (IP) injection, immersion or orally by mixing vaccines with feed [108].

Vaccine studies with *E. ictaluri.* The modified live *E. ictaluri* strain RE-33 was reported to be efficacious as a vaccine against *E. ictaluri* infection in channel catfish in 1999 [109]. This vaccine was developed by using rifampicin to induce a rough phenotype of *E.*

ictaluri and this rough appearance live strain was proved to be LPS O-side chain negative [110]. The efficacy of this live vaccine was tested and relative percent survival ranging from 58.4 to 77.5 [111]. The strain RE-33 was later developed into a commercial product AQUAVAC-ESC™ (Merck) which is a licensed vaccine against enteric septicemia of catfish. Studies of vaccination by immersion exposure on eyed channel catfish (*Ictalurus punctatus*) eggs proved the safety and efficacy of this live *E. ictaluri* vaccine (AQUAVAC-ESC™) [112]. Research conducted at LSU to optimize the efficay of vaccines against ESC focused on the production of live attenuated strains with defined mutations in virulence genes. Initially, a mutated strain of *E. ictaluri* in aromatic metabolism pathway named LSU-E1 and an adenine-auxotrophic strain LSU-E2 were examined and found efficacious as immersion vaccines [113, 114]. Later attenuated strains were identified with a technique known as signature tagged mutagenesis [115]. Work is continuing with type III secretion system mutants in various effector proteins to produce a more efficacious vaccine against ESC [115]. Studies in other labs proved that the novobiocin-resistant *E. ictaluri* AL93-58 and O polysaccharide (OPS) mutant strain 93–146 R6 can be potential vaccine candidates as well [105].

However, all of these vaccines have their own limitations and they are all designed based on attenuation of the catfish strain of *E. ictaluri*. Since the zebrafish strains of *E. ictaluri* can be differentiated from the traditional catfish strain from various aspects including monoclonal antibody recognition [38], and possibly vary in their surface antigens, a newly designed vaccine specific against the zebrafish strain of *E. ictaluri* is needed to provide better protection in zebrafish. This leads to our study which focuses on reproduction of disease through developing laboratory challenge models of *E. ictaluri*

infection in zebrafish, constructing the attenuated strain using a parent strain from zebrafish, to control *E. ictaluri* infections in zebrafish colonies.

Objectives and Hypothesis.

One of our objectives is to find the answer to this question: how the newly identified zebrafish strain of *E. ictaluri* become highly virulent in zebrafish while the typical catfish strain has never been reported as a causative agent in zebrafish in natural outbreaks historically? Therefore, in the following chapters, we mainly focused on the investigation of the differences in the genome by comparing the sequences of the known virulence factors from the catfish and zebrafish strain of *E. ictaluri*. Our hypothesis is that there are differences in the genome between catfish and zebrafish strain of *E. ictaluri* that may contribute to the variations in pathogenesis. Meanwhile, an effective vaccine against edwardsiellosis for zebrafish is needed urgently. Thus, another objective is to attenuate the zebrafish strain for vaccine development purpose. We hypothesized that by mutating *esrC* and *ureG* genes, we can attenuate the zebrafish strain to create potential vaccine candidates.

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CHAPTER II. PLASMID AND WHOLE GENOME SEQUENCING OF TWO ZEBRAFISH STRAINS OF *EDWARDSIELLA ICTALURI* AND COMPARATIVE GENOMIC ANALYSIS WITH A TYPICAL CATFISH STRAIN

Introduction

Zebrafish strains of *Edwardsiella ictaluri*. *Edwardsiella ictaluri*, first isolated from channel catfish *Ictalurus punctatus* in 1976, was described as the causative agent of enteric septicemia of catfish (ESC) [1, 2]. In 2013, the zebrafish *Danio rerio* was reported as a natural host of *Edwardsiella ictaluri* following the description of infections in laboratory populations [3]. Specimens submitted to the Louisiana Aquatic Diagnostic Laboratory (LADL) in 2011 from the Department of Biological Sciences of Louisiana State University (LSU) and the University of Massachusetts at Amherst were found to be infected with *E. ictaluri*. Strains LADL11-100 and LADL 11-194 isolated from zebrafish samples were further identified as the primary cause of high mortality in both facilities and were archived as type strains from zebrafish. Research at Mississippi State University, College of Veterinary Medicine, had previously shown that zebrafish can be used as an experimental model for edwardsiellosis by injection and immersion using a catfish strain, however, a high dose was required to cause mortality by immersion [4]. The strains isolated from zebrafish have been shown to be virulent for zebrafish but not for channel catfish by immersion. This led to our study of examining the differences in potential virulence genes in representative strains from the catfish and zebrafish by whole genome sequencing.

Plasmids. The plasmid is a self-replicating extrachromosomal DNA molecule that can carry important virulence factors in many pathogenic bacterial species [5-9], including *Salmonella spp.* [10], *Edwardsiella tarda* [11] and the catfish strain of *E. ictaluri* [12].

Since the two plasmids in zebrafish strains of *E. ictaluri* are of different sizes than the catfish strain plasmids pEI1 and pEI2, sequencing these plasmids can provide us with additional insight into virulence genes that might vary between the two strains and affect their virulence in different hosts.

Next-generation sequencing (NGS). Next-generation sequencing, the non-Sanger-based sequencing technology, allows large scale DNA templates to be sequenced at the same time. The development of NGS has revolutionized genetic research in the past five years. There are three major platforms released in 2011 including the Ion Torrent ™ Personal Genome Machine (PGM), Pacific Biosciences' RS and the Illumina MiSeq [13]. Of these, the Ion Torrent sequencing platform is known for its efficiency and cost effectiveness. The essential mechanism for ion torrent technology is the use of a semiconductor chip that can translate the chemical signal to digital information. In this process, template DNA is fragmented and then flanked by the Ion Torrent sequencing adaptors. These fragments are then amplified on the beads before being applied to the Ion Torrent chips. When sequencing starts, dNTPs flow through the chips. If a nucleotide incorporates, the chip will sense the proton released and send an electrical signal to the PGM [14].

Single nucleotide polymorphism (SNPs). A single nucleotide polymorphism (SNP) is a DNA sequence variation among individuals. Mapping of SNPs provides fundamental new insights into biology, evolution, pharmacogenomics and diseases in humans and other species [15-18]. In the bacterial genome, a SNP can fall in the coding or non-coding region. It is possible for SNPs that occur in the coding region to have no effect on amino acid sequences due to degeneracy of the genetic code. These are referred to as

synonymous SNPs. Other SNPs that can change the amino acid sequences of the proteins are called non-synonymous SNPs.

Since the zebrafish strains of *E. ictaluri* can be differentiated from the catfish strain of *E. ictaluri* in many aspects, e.g. biochemical and plasmid size profiles, motility, as well as host specificity [3], we hypothesized that there are differences at the DNA level between catfish and zebrafish strains. In this study, both the plasmids and the whole genome of zebrafish strains of *E. ictaluri* LADL11-100 and LADL11-194 were sequenced. All the resulting data were compared with those derived from the catfish strain of *E. ictaluri* available from the National Center for Biotechnology Information (NCBI) website. Lists of SNPs were obtained after comparing the genome sequences of each zebrafish strain to that of the reference catfish strain LADL93-146. Here, we mainly focused on the non-synonymous SNPs in zebrafish strains of *E. ictaluri.* All of these valuable data reveal the differences at the genomic level between catfish and zebrafish strains of *E. ictaluri.*

Materials and Methods

Plasmid sequencing. All strains used in this study are listed in Table 2.1. To prepare for plasmid sequencing, *E. ictaluri* LADL 11-100 was grown in Bacto porcine brain-heart infusion (BHI) broth overnight at 28 ℃.

Plasmids, named pEIZ1 and pEIZ2 from the zebrafish strain of *E. ictaluri*, were isolated and further separated by electrophoresis. Each plasmid was purified by cutting the band from the gel and purifying them using a gel extraction kit (Qiagen). The plasmids were then digested with restriction enzyme BstZ17I and inserted into the plasmid pBluescript SK-. The resulting plasmids, pBSEIZ1 and pBSEIZ2, were

electroporated into *E.coli* XL1 Blue MRF' to sustain the plasmids. Plasmids were isolated and sent to LSU GENELAB, together with appropriate primers, for sequencing. All primers designed for amplifying and sequencing of the plasmids are listed in Table 2.2. Plasmid sequences were compared with the published pEI1 and pEI2 plasmid sequences on the NCBI website (accession numbers AF244083.1 and AF244084.1).

Table 2.1 Bacterial strains and plasmids used in Chapter II.

Bacterial strains or plasmids	Description	Source
Bacterial strains		
E.ictaluri LADL 11-194	Wild type *E.ictaluri* isolated from zebrafish *Danio rerio* in a natural outbreak in 2011 at University of Massachusetts, Amherst	Louisiana Aquatic Diagnostic Laboratory
E.ictaluri LADL 11-100	Wild type *E. ictaluri* isolated from zebrafish *Danio rerio* in a natural outbreak in 2011 at Louisiana State University	Louisiana Aquatic Diagnostic Laboratory
E.coli XL1 Blue MRF'	(*mcrA*)183 (*mcrCB-hsdSMR-mrr*)173 endA1 *supE44 thi-* 1 *recA1 gyrA96 relA1 lac* [F' *proABlacIqZ.*M15 Tn5(Km)]	Stratagene, La Jolla, CA
Plasmids		
pBluescript SK-	Cloning vector	Stratagene, La Jolla, CA

Table 2.2 Oligonucleotide sequences used for amplifying and sequencing of the two plasmids in *E. ictaluri* LADL11-100.

Primer name	Primer type	Sequences
pEIZ1		
pEI1R1	R	CTGACCAGGCAGCTTTATAC
F7	F	CAGAACAGGCGGTATTT
F4	F	CGTCACTGCCTGCGATATAA
R2	R	CGCACCTTGGTAGGTGCTGT
Ra	R	CCACCTCTGACTTGAGCACC
F1	F	GCAATGGCTCCCTAATC
Fa	F	CGCATTGAACATAACATCCG
pEIZ2		
3R	R	GGAATGAGTTTAAGGTAGCT
	F	AGATACGCTCGGAAAG
	F	CAGCAGCGTGGTAAA
	R	AAGAGCGGAGCTATTC
3F	F	GACAGACAGGAAAAGAGGGT

The missing genes from the plasmids were searched against the genome of zebrafish strains of *E. ictaluri* LADL11-100 to check the existence of certain genes in the genome under the RAST/SEED viewer (RAST=Rapid Annotation using Subsytems Technology) [19, 20].

Genome sequencing and polymorphism discovery. *Edwardsiella ictaluri* LADL11-100 and LADL11-194 were grown in BHI broth for 18 hours and the genomic DNA, extracted using High Pure PCR Template Preparation Kit (Roche Applied Science).

Genomic DNA was eluted into the low TE buffer of Ion Torrent ion plus fragment library kit (Life Technologies). Both genomic DNA samples, which were isolated from *E. ictaluri* LADL 11-100 and LADL 11-194, were treated with RNase to remove any RNA contamination before sending to the Division of Biotechnology and Molecular Medicine (BioMMED) at Louisiana State University for sequencing. Both samples were sequenced with the Ion Torrent PGM using a 316D chip. The resulting sequences were aligned to the catfish strain of *E. ictaluri* LADL 93-146 (NCBI accession no. CP001600). The SNPs were detected based on the genome-wide comparison results of *E. ictaluri* LADL11-100, LADL11-194 and LADL93-146.

Genome assembly and annotation. The CLC Genomics Workbench version 7.5 (CLC Bio) was used for de novo assembly. The requirement of a minimum of eleven nucleotides in common at the ends of the reads was chosen when assembling the reads. The fastq and sff files were imported and assembled to the reference genome. The results from both files were merged to one file for each strain. The resulting contigs were sent to Sequencher 5.2.4 (Gene Codes Corporation) to check and further assemble manually. All the genes in the assembled contigs were annotated using the RAST server [21, 22].

SNP analysis and protein structure prediction. All detected SNPs from LADL 11-100 and LADL 11-194 were compared manually to eliminate the unique one in each strain, generating a whole list of SNPs that are shared by both strains. These SNPs were further analyzed by NextGENe software to find those SNPs that are in the coding region as well as non-synonymous SNPs. All predicted genes with SNPs in the zebrafish strain of *E. ictaluri* were categorized into clusters of orthologous groups (COG).

Comparative genome studies. The assembled contigs from the zebrafish strain of *E. ictaluri* LADL11-100, together with the genome sequences from *E. piscicida* (NCBI CP004141) and catfish strain of *E. ictaluri* LADL93-146 (NCBI CP001600) were submitted to a genome alignment package Mauve to view gene arrangements, the similarities of the genes as well as the potential aligned locations for each assembled contig [23]. In addition, contigs from LADL11-100 and LADL 11-194 were sent to CONTIGuator for draft genome generation with the genome sequence of LADL93-146 as a reference [24]. The draft genome from zebrafish strain of *E. ictaluri* LADL11-100 was aligned to catfish strain of *E. ictaluri* LADL93-146 as well as *E. piscicida* C07-087 in a sequence-based manner to show the similarities among these strains. This was done in SEED viewer [21]. Meanwhile, the unique genes in LADL11-100 were extracted when compared to LADL93-146 and these genes were blasted on NCBI website to search for their potential functions.

Results

Plasmid sequence comparison between catfish strain LADL 93-146 and zebrafish strain LADL11-100. The plasmid sequencing results of a zebrafish strain of *E. ictaluri* are compared to the plasmid sequences in the catfish strain on the NCBI website (Figure 2.1). Plasmids are named pEIZ1 and pEIZ2 based on the similarities to the plasmids pEI1 and pEI2 of the catfish strains. The sizes of the plasmids in the zebrafish strain are 3,930 bp (pEIZ1) and 3,363 bp (pEIZ2) while the sizes of the plasmids in catfish strains are 4,807 (pEI1) and 5,643bp (pEI2). These two plasmids have been shown to be consistently seen in zebrafish isolates of *E. ictaluri* [3].

Plasmid pEIZ1 contains the same *eseH, orf4, oriV, orf5* and RNAi sequences as pEI1, but the ISEI1 sequences in pEI1 from 2,479 to 3,337bp are absent in pEIZ1. Plasmid pEIZ2 shares the same *ori2, ori3*, RNAi and *rep* sequences with pEI2 but is missing *eseI*, the potential chaperon for *eseI*, and *ori3* sequence.

Genome sequencing and polymorphism discovery. Sequencing produced an output total of 5,506,555 and 4,634,986 reads for LADL 11-100 and 11-194, respectively, of which, 4,020,880 and 2,894,144 are usable reads with the average read length being 221bp and 217bp. For LADL 11-100, a total of 891 million bases were produced and among those, 808 million bases can be aligned to a typical catfish strain LADL 93-146, indicating 91% bases were aligned. In LADL 11-194 sequencing result, 569 million of 628 million total bases were aligned. The average G+C content of both zebrafish strains is 57.4% that is identical to that of catfish strain LADL 93-146.

Genome assembly and annotation. All the reads from NGS outputs are uploaded into CLC workbench for primary assembly, resulting in 220 contigs for LADL11-100 and 225 contigs for LADL11-194. These assembled contigs are then checked manually for misassembly and other errors in Sequencher, yielded 128 and 130 contigs that are >1,000bp for LADL 11-100 and LADL 11-194, respectively. The maximum length of the contigs from each strain is approximately 160kb. The program RAST predicts a total of 3,613 coding DNA sequences (CDS) and 85 RNAs for LADL 11-100 and 3,638 CDS and 94 RNAs for LADL 11-194. The predicted genes are categorized based on the potential functions of each gene (Figure 2.2).

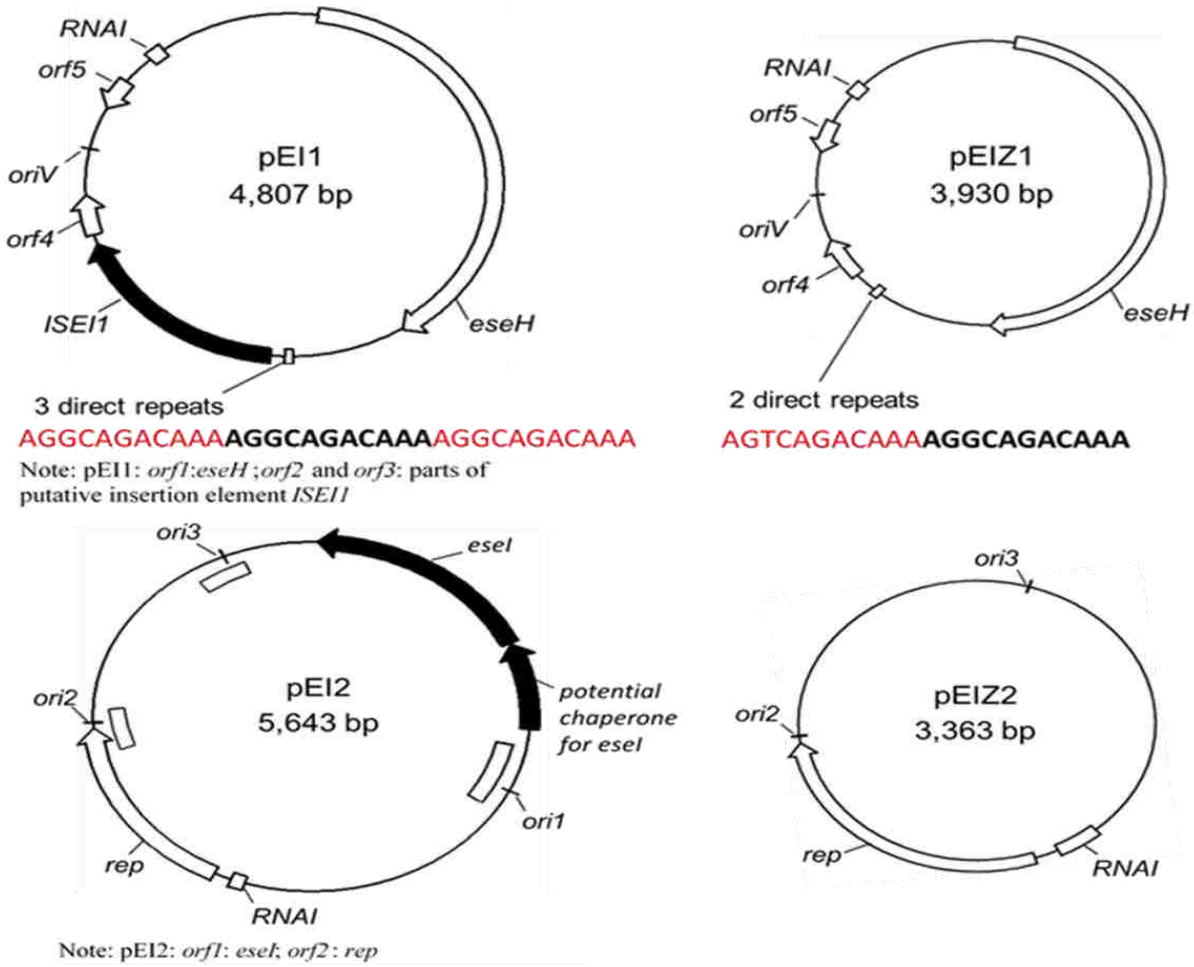

Figure 2.1 Comparison of the plasmids in catfish and zebrafish strains of *E. ictaluri*. The dark region in pEI1 and pEI2 represent the sequences missing in pEIZ1 and pEIZ2. The size of each plasmid, the coding regions and origin of replication on the plasmids are marked.

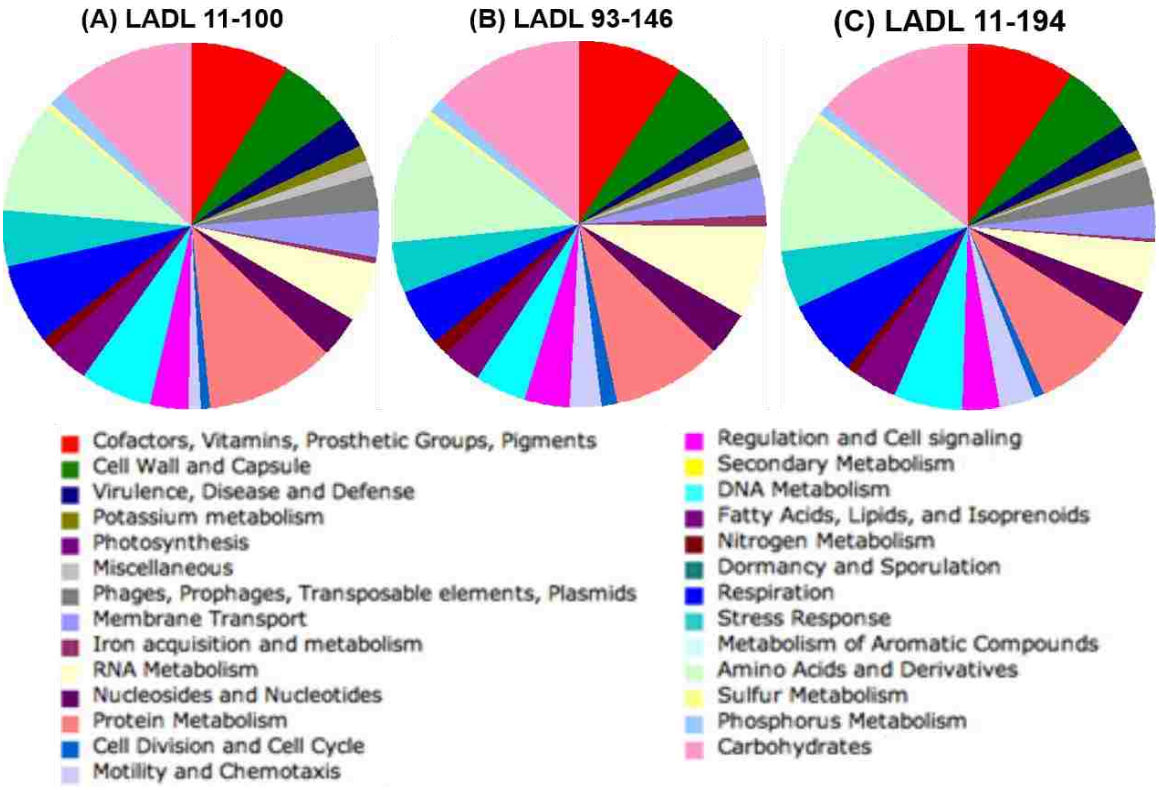

Figure 2.2 The subsystems in zebrafish strain of *E. ictaluri* LADL11-100 (A), LADL11-194 (C) and catfish strain LADL93-146 (B).

SNP analysis. For strains LADL11-100 and LADL11-194, 8,708 and 8,501 SNPs were discovered respectively, using *E. ictaluri* LADL93-146 genome sequence as the reference. The SNPs from LADL11-100 and LADL11-194 were checked manually and 8,287 SNPs are found consistently in zebrafish strains. The SNPs that are not in the coding region or the synonymous ones are removed from the list, resulting in a list with 2,224 non-synonymous SNPs. The proteins that carry these SNPs are categorized into 20 groups. Among these, 7% of the SNPs were located in the genes encoding cell wall, membrane or envelope biogenesis related proteins. Detailed information of orthologous groups is shown in Figure 2.3. All the non-synonymous SNPs from both LADL11-100 and LADL 11-194 are listed in the appendix.

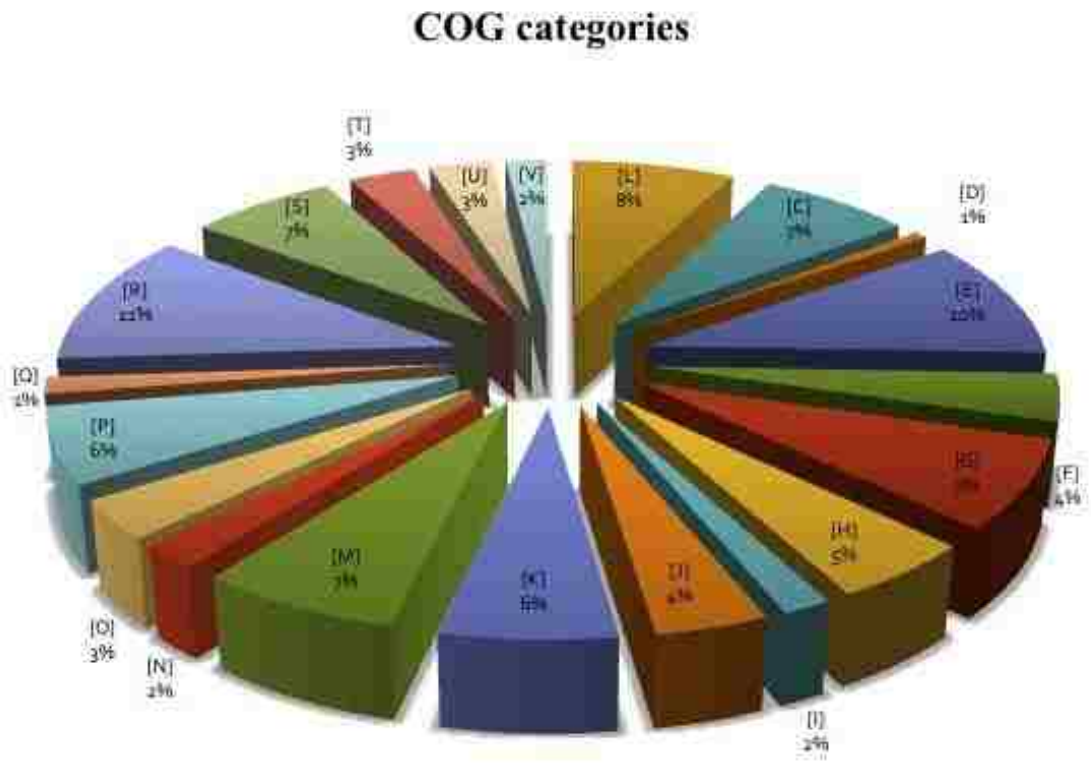

Figure 2.3 Orthologous group categories of proteins that are encoded by the genes carrying SNPs. [J] Translation, ribosomal structure and biogenesis; [A] RNA processing and modification; [K] Transcription; [L] Replication, recombination and repair; [B] Chromatin structure and dynamics; [D] Cell cycle control, cell division, chromosome partitioning; [Y] Nuclear structure; [V] Defense mechanisms; [T] Signal transduction mechanisms; [M] Cell wall/membrane/envelope biogenesis; [N] Cell motility; [Z] Cytoskeleton; [W] Extracellular structures; [U] Intracellular trafficking, secretion, and vesicular transport; [O] Posttranslational modification, protein turnover, chaperones; [C] Energy production and conversion; [G] Carbohydrate transport and metabolism; [E] Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [H] Coenzyme transport and metabolism; [I] Lipid transport and metabolism; [P] Inorganic ion transport and metabolism; [Q] Secondary metabolites biosynthesis, transport and catabolism; [R] General function prediction only; [S] Function unknown.

Comparative genome studies. All the contigs from LADL11-100 submitted to MAUVE are compared to the sequences from *E. ictaluri* LADL 93-146 as well as *E. piscicida* C07-087 to compute the alignment of all the contigs with the similarities of each gene shown as the coverage of each box (Figure 2.4).

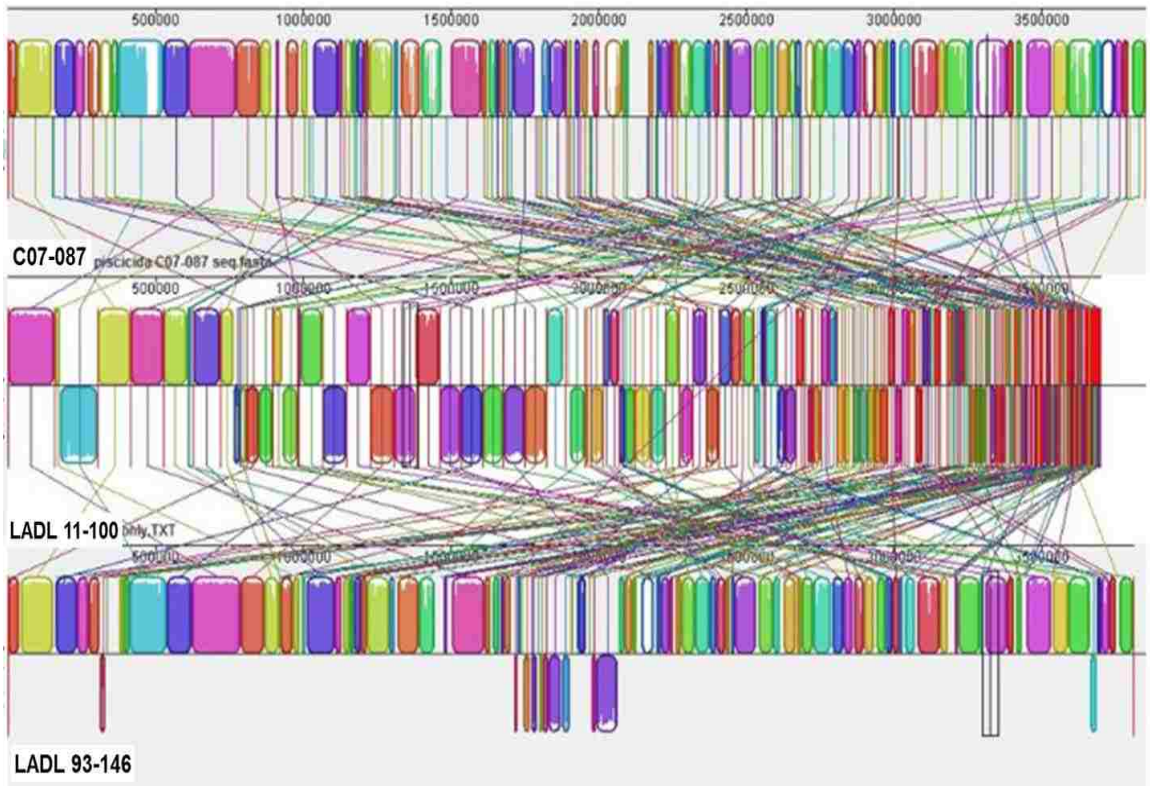

Figure 2.4 The overall alignment of the contigs from zebrafish strain of *E. ictaluri* LADL11-100 (the middle line) to *E. ictaluri* LADL93-146 (the last line) and *E. piscicida* C07-087 (the first line). The full coverage in a box indicated the perfect identity.

MAUVE provides the overall alignment of the genomes, however, it also provides multiple potential locations for the short contigs. Therefore, CONTIGuator was used to align the contigs to catfish strain and more importantly, generate the draft genomes. There were 111 of 128 and 111 of 130 contigs from zebrafish strains LADL11-100 and LADL11-194 aligned to the catfish strain of *E. ictaluri* by CONTIGuator (Figure 2.5). Of the genome from LADL11-100, a total of 3,619,029 bp were aligned to the catfish strain LADL93-146 while 3,634,092 bp from the genome of LADL 11-194 aligned. In both cases, approximately 95% of the catfish strain genome was covered.

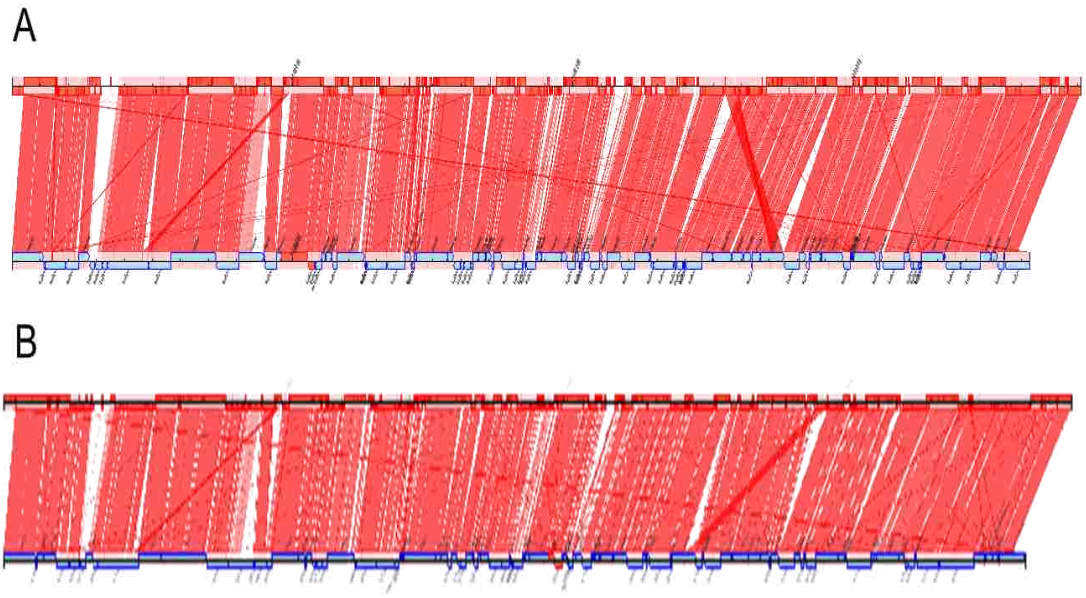

Figure 2.5 Generation of the draft genomes for zebrafish strains LADL11-100 and LADL11-194 from the assembled contigs. (A) Contigs from LADL11-100 align to LADL93-146; (B) Contigs from LADL11-194 align to LADL93-146.

The aligned contigs are assembled as draft genomes in scaffold fasta files which are used to generate the BLAST dot plots against *E. ictaluri* LADL93-146. The draft genome of LADL11-100 is also compared with genome of *E. piscicida* C07-087 for purposes of comparison (Figure 2.6). The plotting of LADL11-100 against LADL93-146 is much closer to a straight line, compared to the irregular broken plot of the genome of *E. piscicida.* It can be concluded that the draft genome of LADL11-100 is more similar to the catfish strain of *E. ictaluri* in terms of gene arrangement than to *E. piscicida*, since 91% of the sequenced bases of the zebrafish strain match the genome of the catfish strain.

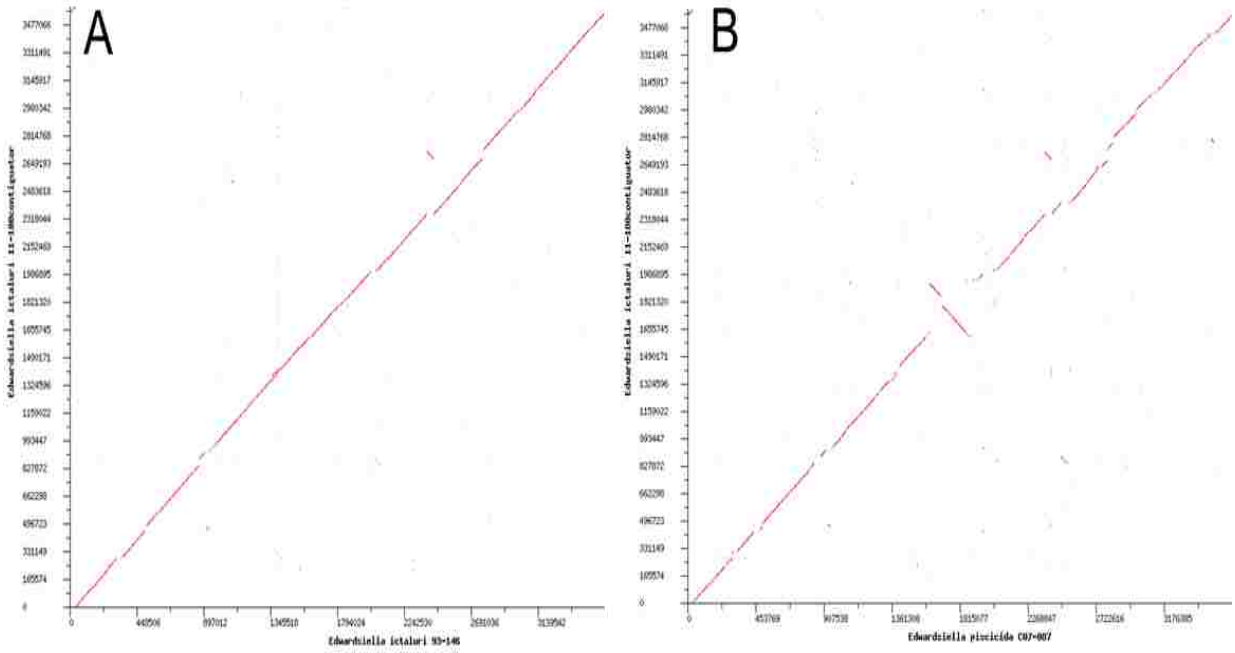

Figure 2.6 The blast dot plots of draft genomes from zebrafish strain of *E. ictaluri* LADL11-100 and the catfish strain of *E. ictaluri* LADL93-146(A) or *E. pisicicida* C07-087(B).

Since there are unaligned contigs from both LADL11-100 and LADL11-194, the contigs from both zebrafish strains are uploaded into RAST for annotation. There are 3,225 and 3,227 putative genes detected from zebrafish strains of LADL11-100 and LADL11-194, respectively. The annotated contigs are studied in SEED viewer to extract the unique genes in LADL11-100 and LADL11-194 and potential functions are assigned to the unique genes (Table 2.3). When comparing the genes between catfish and zebrafish strains of *E. ictaluri*, we find 26 unique putative genes in LADL11-100 including 14 that are phage-related, and 29 unique genes in LADL11-194 including 14 phage-related putative genes.

Table 2.3 Lists of the putative unique genes in zebrafish strain of *E. ictaluri* LADL11-100 (A) and LADL11-194 (B). The "peg" numbers are the catalog number for each gene.

A.

Category	Subcategory	Subsystem	Role	LADL11-100
Amino Acids and Derivatives	Lysine, threonine, methionine, and cysteine	Lysine degradation	L-lysine permease	peg.1042
Cell Wall and Capsule	Gram-Negative cell wall components	LOS core oligosaccharide biosynthesis	Beta-1,3-glucosyltransferase	peg.179
Clustering-based subsystems	no subcategory	CBSS-211586.9.peg.2729	Acyl-CoA thioesterase YciA, involved in membrane biogenesis	peg.1532
Membrane Transport	Cation transporters	Transport of Nickel and Cobalt	Additional component NikL of nickel ECF transporter	peg.2215
Nucleosides and Nucleotides	Purines	GMP synthase	GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)	peg.2678
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage packaging machinery	Phage portal protein	peg.1813, peg.2332
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage packaging machinery	Phage terminase, large subunit	peg.1815, peg.1816, peg.2330
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail fiber proteins	Phage tail fiber assembly protein	peg.2350
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage minor tail protein	peg.2337, peg.2339, peg.2340, peg.2342, peg.2343
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail assembly	peg.2338

Table 2.3 A-continued

Category	Subcategory	Subsystem	Role	LADL11-100
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail assembly protein I	peg.2346
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail length tape-measure protein 1	peg.2341
Regulation and Cell signaling	no subcategory	cAMP signaling in bacteria	Prophage Clp protease-like protein	peg.1810, peg.1811, peg.2333
Respiration	ATP synthases	F0F1-type ATP synthase	ATP synthase F0 sector subunit b (EC 3.6.3.14)	peg.65
Respiration	no subcategory	Formate hydrogenase	Putative formate dehydrogenase oxidoreductase protein	peg.1414, peg.1415
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Copper homeostasis	Multidrug resistance transporter, Bcr/CflA family	peg.1179

B.

Category	Subcategory	Subsystem	Subsystem	LADL11-194
Amino Acids and Derivatives	Lysine, threonine, methionine, and cysteine	Lysine degradation	Lysine degradation	peg.2596
Cell Wall and Capsule	Capsular and extracellular polysacchrides	Sialic Acid Metabolism	Sialic Acid Metabolism	peg.3568
Cell Wall and Capsule	Gram-Negative cell wall components	LOS core oligosaccharide biosynthesis	LOS core oligosaccharide biosynthesis	peg.3553
Clustering-based subsystems	no subcategory	CBSS-211586.9.peg.2729	CBSS-211586.9.peg.2730	peg.1880
Clustering-based subsystems	no subcategory	Primosomal replication protein N clusters with ribosomal proteins	Primosomal replication protein N clusters with ribosomal proteins	peg.1087
Cofactors, Vitamins, Prosthetic Groups, Pigments	Quinone cofactors	Menaquinone and Phylloquinone Biosynthesis	Menaquinone and Phylloquinone Biosynthesis	peg.1961, peg.3347

Table 2.3 B-continued

Category	Subcategory	Subsystem	Role	LADL11-194
Dormancy and Sporulation	no subcategory	Persister Cells	Persister Cells	peg.1124
Membrane Transport	Cation transporters	Transport of Nickel and Cobalt	Transport of Nickel and Cobalt	peg.2945
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage packaging machinery	Phage packaging machinery	peg.869, peg.3124
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail proteins	peg.3113, peg.3114, peg.3116, peg.3117, peg.3119
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail proteins	peg.3118
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail proteins	peg.3110
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage tail proteins	Phage tail proteins	peg.3115
Regulation and Cell signaling	no subcategory	cAMP signaling in bacteria	cAMP signaling in bacteria	peg.867, peg.868, peg.3123
Respiration	ATP synthases	F0F1-type ATP synthase	F0F1-type ATP synthase	peg.20
Respiration	no subcategory	Formate hydrogenase	Formate hydrogenase	peg.1731
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper homeostasis	peg.2118

The annotated draft genomes are also blasted against LADL93-146 as well as *E. piscicida* C07-087 for a sequence identity check (Figure 2.7). The differences in color represent the various similarity levels. For the most part, the outer circle shows dark blue

indicating a high similarity (>99%) between catfish and zebrafish strains of *E. ictaluri* while the inner circle exhibits light green color which means a slightly lower similarity (~95%) between zebrafish strain of *E. ictaluri* and *E. piscicida.*

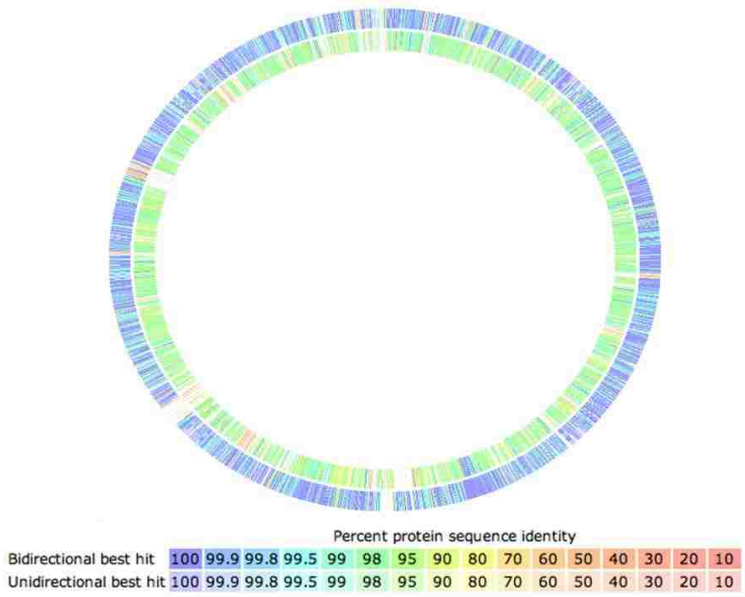

Figure 2.7 The overall protein identities between the draft genome of *E. ictaluri* LADL11-100 and the genome *E. ictaluri* LADL93-146 (outer circle); the identities between LADL11-100 and *E. piscicida* C07-087 (inner circle).

Discussion

The plasmids in enteric bacteria often encode important virulence factors and examples include the plasmids in *Escherichia coli, Shigella spp., Yersinia spp*, and *Salmonella* [25-28]. In previous studies, the plasmids in the catfish strain of *E. ictaluri* pEI1 and pEI2 were sequenced and it was shown that *eseI, eseH* and *escD* genes on those plasmids could affect pathogenesis [29, 30]. In addition, *eseI* and *escD*, encoded in pEI2, are type III secretion system homologous proteins. Another group demonstrated that an *eseI* mutant has decreased ability for adherence and reduced virulence [12]. In this study, the plasmids in the zebrafish strain of *E. ictaluri* were sequenced and the results indicated that *eseI* and *escD* were absent from the plasmids as well as the genome. One possible

explanation for that is genetic redundancy. However, since these genes are important virulence factors, they may contribute or partially contribute to the fact that zebrafish strains are not infectious in channel catfish by immersion (data shown in chapter V). Previous studies indicated that, under low pH or low phosphate condition, *eseI* of the catfish strain of *E. ictaluri* is required to be upregulated to facilitate bacterial survival [31]. Therefore, it will be of interest to study the mechanism of *E. ictaluri* survival inside zebrafish cells. Since *eseI* and *escD* are missing, it is possible that the zebrafish strain has employed other virulence factors to replace them or utilize a completely different way to adhere to the cell and fight against the harsh environment in the host.

In this study, the genomic DNA from zebrafish strains of *E. ictaluri* was also sequenced to generate short reads. These reads are successfully assembled, resulting in 128 contigs for *E. ictaluri* LADL11-100 and 130 contigs for LADL11-194. MAUVE alignment provides the potential locations for each contig in the genome using the genome from catfish strain as a reference. Using the software CONTIGuator, the draft genomes of both zebrafish strains were generated for further analysis.

The overall similarities between the proteins in *E. ictaluri* LADL11-100 and *E. piscicida* C07-087 is relatively high, >=95%. However, even higher similarities are seen to the proteins from *E. ictaluri* LADL93-146, with >=99% similarities to the proteins in the catfish strain. This indicates the close relationships within *E. ictaluri* species, and also between *E. piscicida* and *E. ictaluri.* The close relationship between catfish and zebrafish strains of *E. ictaluri* has also been proved by repetitive sequence mediated PCR (rep-PCR) using different primer sets (personal communication with Dr. Matt Griffin).

Many unique genes are found in zebrafish strains of *E. ictaluri* when compared to a representative catfish strain and 14 unique putative genes in zebrafish strains are phage related. The incorporation of phage functions to drive the diversity of the genome through frequent transfer of phage material by recombination, thus it can possibly affect virulence and transmissibility [32, 33]. BLAST searching of these phage related genes provides us with a hint of the potential source of the phage. It is possible that there are phages carrying virulence factors from *E. piscicida* to the zebrafish strain of *E. ictaluri* and this has resulted in the differences in virulence of *E. ictaluri* strains from zebrafish and catfish.

The category of the genes that have SNPs indicated that 7% of the genes are cell wall, membrane, and envelope biogenesis related. Since these are all potential virulence factors, it would be interesting to zoom in to all of these genes in the future and determine the potential effects of SNPs in these genes.

To summarize, in this study, we sequenced and assembled the short reads of the genomic DNA from the zebrafish strain of *E. ictaluri* to generate contigs and the draft genomes. We also identified the differences at the DNA level and determined the phylogenetic relationship between catfish and zebrafish strains of *E. ictaluri* by comparative genomic analysis. Even though these differences were identified, the overall similarity between the genomes of catfish and zebrafish strains of *E. ictalulri* are relatively high, the existence of the non-synonymous SNPs in many genes are observed and these SNPs can affect the sequences of the protein they encoded. All of these data provide valuable information to direct further study on *E. ictaluri* and illuminate the mechanism of host specificity.

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CHAPTER III. COMPARISON OF THE LIPOPOLYSACCHRIDE O-ANTIGEN BIOSYNTHESIS GENE CLUSTER IN ZEBRAFISH AND CATFISH STRAINS OF *EDWARDSIELLA ICTALURI*.

Introduction

Lipopolysaccharide (LPS) is a component of the gram negative bacterial outer membrane that consists of three unique regions, lipid A, the core oligosaccharides and O side chain polysaccharides. It is the major ligand for host cell recognition by LPS binding protein (LBP), CD14 and the Toll-like receptor 4 (TLR4)–MD-2 complex which leads to inflammation in the host [1-3]. Moreover, LPS can determine the permeability of the bacterial cell and the host cells. In fact, LPS forms a barrier on the bacterial surface to prevent the entry of antibiotics and anti-metabolites into the cell and can also make the host cell membrane more permeable by modulating the expression and localization of host cell surface markers, like toll-like receptor 4 (TLR4) and CD14, to facilitate its survival in harsh environments [4, 5].

The zebrafish strain of *E. ictaluri* can be differentiated from a typical catfish strain by failure of monoclonal antibody recognition of the LPS by Mab Ed9 [6], exhibiting autoagglutination following broth culture, and weak or no motility [7]. Changes in LPS can possibly affect all of the characters above since research has shown that LPS, more specifically O-antigen mutants of the typical catfish strain, can autoagglutinate in broth and have weaker or no motility [8]. Thus, it is necessary to investigate the differences in LPS composition between catfish and zebrafish strains of *E. ictaluri*.

Within the LPS, the O-polysaccharide region varies significantly from one bacterial strain to another while the lipid A and core are relatively conserved [9]. The O-polysaccharide region can have a number of repeating units from zero to above one

hundred in addition to a large repertoire of sugar components and linkage options [10]. The different numbers of O-antigen repeating units and different sugar structures or compositions can influence the virulence of the cells [11-16].

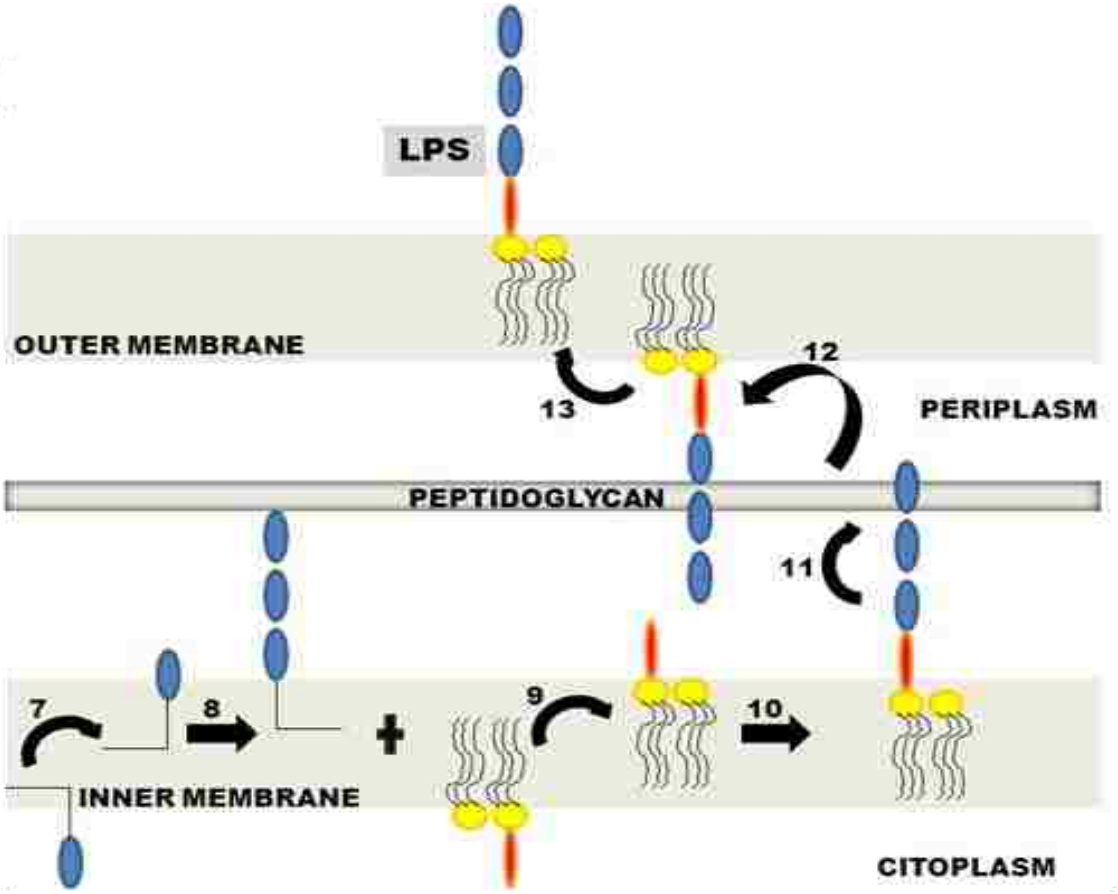

Figure 3.1 The LPS biosynthesis process. The O antigen unit is assembled in the cytoplasm, flipped to the periplasm side of the inner membrane and polymerized in the periplasm. Then the assembled O-antigen is ligated to the lipid A-core complex in the periplasm. This whole molecule is transported to the cell surface. The enzymes needed for these processes are: 7. Wzx; 8. Wzy, Wzz; 9. MsbA; 10. WaaL; 11. LptBCFG; 12. LptA; 13. LptDE. The lipid A appears as yellow circles; red and blue ovals represent the core and O antigen, respectively [17].

In the gram negative bacterial chromosome, genes encoding for O-antigens are in close proximity and form a cluster with lower G+C content than the rest of the genome, possibly because these genes are laterally transferred from other bacteria [18]. The O-antigen cluster usually consists of the following genes: a gene encoding an O-antigen

flippase that flips the synthesized O units from the cytoplasm to the peptidoglycan side of the inner membrane; an O-antigen polymerase gene that determines the length of the O-antigen; and genes that encode many enzymes that work in concert in the sugar synthesis and transfer pathways during LPS biosynthesis [19, 20]. After the O-antigen is polymerized, it is integrated with the core-lipid A complex which is assembled separately and then the whole molecule is transferred from the periplasm to the outer membrane. This synthesis process is shown in Figure 3.1 [17].

The O-antigen biosynthesis cluster in the catfish strain of *E. ictaluri* was previously sequenced and characterized [21]. The genes for core oligosaccharide and lipid A biosynthesis were previously reported in *E. tarda* [22]. Hence, it is interesting to search for the existence of the O-antigen cluster as well as the genes that encode core oligosaccharides and lipid A in the genome of the zebrafish strain of *E. ictaluri*, and to characterize and compare them with the homologous genes in the catfish strain and other closely related bacteria.

Materials and Methods

Genome sequencing, O-antigen biosynthesis cluster detection and analysis. The genome sequencing method was previously described in Chapter II. To determine the existence of an O-antigen biosynthesis cluster in the zebrafish strain, all sequences of the genes that were previously mapped in the catfish strain of *E. ictaluri* O-antigen biosynthesis gene cluster were used as query to search for matching sequences in the zebrafish strain LADL 11-100. The BLAST method was chosen on Rapid Annotation using Subsystem Technology (RAST)/SEED viewer interface for the purpose of searching for related sequences [23, 24]. All matched nucleotide sequences together with

the undetected sequences in intergenic regions were translated and were sent for BLAST at the National Center for Biotechnology Information (NCBI) website for identifying the homologous sequences in other bacterial species based on the similarity of amino acid sequences. To show the phylogenetic relationship of the genes in O-antigen biosynthesis clusters, CLUSTALX (version 1.81) and MEGA 4.0 with the bootstrap test (500 replicates) were used for multi-alignment of protein sequences and to create phylogenetic trees [25, 26].

For detection of the genes encoding core and lipid A, the sequences of *E. tarda* EIB202 (accession number CP001135) were acquired from the NCBI website. In the genome sequences of *E. tarda,* 23 genes and 11 genes related to core and lipid A biosynthesis were found. These sequences were used for BLAST on RAST/SEED viewer interface to search against zebrafish strain LADL 11-100 as well as BLAST at NCBI website to find homologous genes in the catfish strain of *E. ictaluri* LADL 93-146 and *E. piscicida* which is a new member in the *Edwardsiella* genus.

Lipopolysaccharide isolation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining. Lipopolysaccharides from both catfish and zebrafish strains of *E. ictaluri* were extracted using LPS Extraction Kit (iNtRON Biotechnology, Inc. Korea). The resulting LPS samples were then separated on a 15 % SDS gel with a 4% stacking gel following by silver straining using PlusOne Silver Staining Kit (GE. Healthcare, Freiburg, Germany) to observe the LPS profile of both samples.

Lipopolysaccharide composition and structural analysis. Both catfish and zebrafish strains of *E. ictaluri* were grown on trypticase soy agar supplemented with 5% sheep blood (BA, Remel Products, Lenexa, KS) at 28 ℃ for 24 hours and these plates were sent

to the Complex Carbohydrate Research Center (CCRC) (Athens, GA, USA) for LPS extraction and further compositional analysis. Combined gas chromatography/mass spectrometry (GC/MS) was used to analyze glycosyl composition. This technique was performed on the per-O-trimethysilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the LPS sample by acidic methanolysis. The procedure was previously described in detail [27, 28].

Results

LPS O-antigen biosynthesis cluster in the zebrafish strain differs from that of the catfish strain. As shown in Figure 3.2, the structure of O-antigen cluster from zebrafish strain of *E. ictaluri* (LADL 11-100) is virtually identical to that of *E. piscicida,* but quite different from that of the catfish strain of *E. ictaluri* (LADL 93-146). Meanwhile, the identity on the gene level of the two clusters from strain LADL 11-100 and *E. piscicida* C07-087 is 92%.

Further, by comparing all the genes in the O-antigen cluster in catfish and zebrafish strain of *E. ictaluri,* unique genes in each O-antigen cluster are identified. All the proteins that are encoded by the O-antigen biosynthesis cluster from the zebrafish strain of *E. ictaluri,* the potential functions of those proteins, the percentage of similarities to the homologous proteins in the catfish strain and/or their closely related proteins are listed in Table 3.1.

The genes of the O-antigen gene clusters that vary between catfish and zebrafish strains of *E. ictaluri* are divided into three groups: group I includes the genes shared by both zebrafish and catfish strains but with low similarities; group II includes the unique

genes in the zebrafish strain LADL 11-100 and group III includes the unique genes in the catfish strain LADL 93-146.

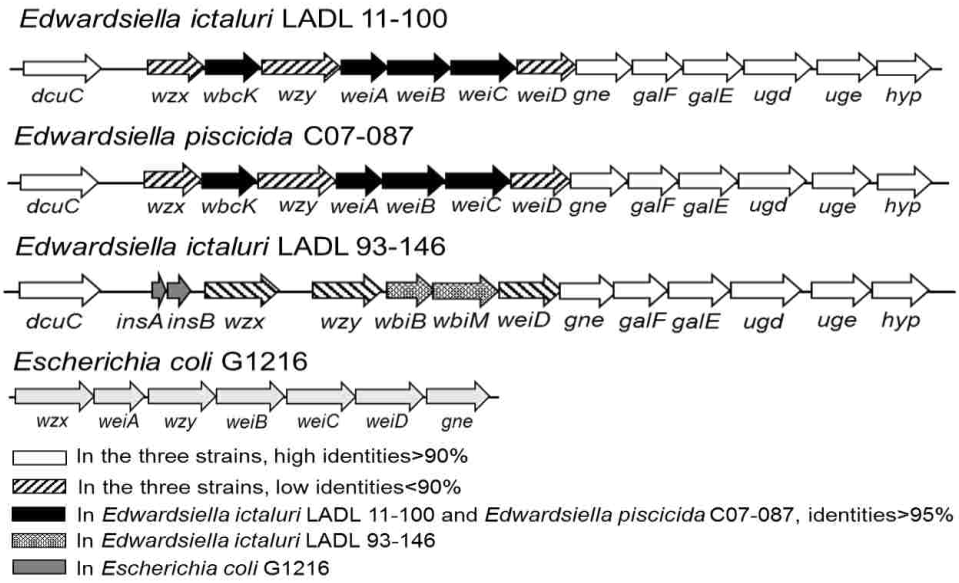

Figure 3.2 Maps of O-antigen biosynthesis gene clusters in *E. ictaluri*, *E. piscicida* and *E. coli*.

Table 3.1 Characteristics of the ORFs in zebrafish strain O-antigen biosynthesis cluster.

Protein	G +C content of gene (%)	No. of residues	Putative function	Related protein(s)		
				Protein (accession no.)	No. of residues	Identity (%)
DcuC	60.3	452	C4-dicarboxylate transporter	DcuC of *Edwardsiella piscicida* C07-087 (AGH73259.1)	452	98
				DcuC of *Edwardsiella ictaluri* LADL93-146 (AAL25626.1)	452	100
Wzx	34.7	324	O unit flippase	Wzx of *Edwardsiella piscicida* C07-087 (AGH73260.1)	324	100
				Wzx of *Edwardsiella ictaluri* LADL93-146 (AAL25627.1)	415	36
				Wzx of *Escherichia coli* G1216 (ADC54955.1)	417	25
WbcK	29.4	317	family 2 glycosyl transferase	WbcK of *Edwardsiella piscicida* C07-087 (AGH73261.1)	317	95
				WbcK of *Edwardsiella tarda* EIB202 (YP_003295248.1)	323	47
				WbcK of *Escherichia coli* (AIG56919.1)	319	28

Table 3.1 - continued

Protein	G +C content of gene (%)	No. of residues	Putative function	Related protein(s)		
				Protein (accession no.)	No. of residues	Identity (%)
Wzy	29.5	453	O antigen polymerase	Wzy of *Edwardsiella piscicida* C07-087 (AGH73262.1)	453	100
				Wzy of *Edwardsiella ictaluri* LADL93-146 (AAL25628.1)	387	26
				Wzy of *Escherichia coli* G1216 (ADC54957.1)	357	26
WeiA	35.7	268	Glycosyltransferases group 2	WeiA of *Edwardsiella piscicida* C07-087 (AGH73263.1)	268	99
				WeiA of *Escherichia coli* G1216 (ADC54956.1)	269	41
				WbcL of *Yersinia enterocolitica* serotype O : 3 (CAA87700)	292	38
WeiB	34.4	359	Glycosyltransferases group 1	WeiB of *Edwardsiella piscicida* C07-087 (AGH73264.1)	359	98
				WeiB of *Escherichia coli* G1216 (ADC54958.1)	360	39
WeiC	34.4	372	Glycosyltransferases group 1	WeiC of *Edwardsiella piscicida* C07-087 (AGH73265.1)	372	100
				WeiC of *Escherichia coli* G1216 (ADC54959.1)	363	43
				WbcN of *Yersinia enterocolitica* serotype O : 3 (CAA87702)	344	33
WeiD (WbiH)	32.2	347	Glycosyltransferases group 1	WeiD of *Edwardsiella piscicida* C07-087 (AGH73266.1)	347	95
				WbeiH of *Edwardsiella ictaluri* LADL93-146 (AAL25632.1)	345	48
				WeiD of *Escherichia coli* G1216 (ADC54960.1)	358	42
				WbcQ of *Yersinia enterocolitica* serotype O : 3 (CAA87705)	349	37
Gne	41	323	UDP-N-acetylglucosamine 4-epimerase	Gne of *Edwardsiella ictaluri* LADL93-146 (AAL25633.1)	323	100
				Gne of *Edwardsiella piscicida* C07-087 (YP_007628627.1)	328	94
				Gne of *Escherichia coli* G1216 (ADC54961.1)	337	26

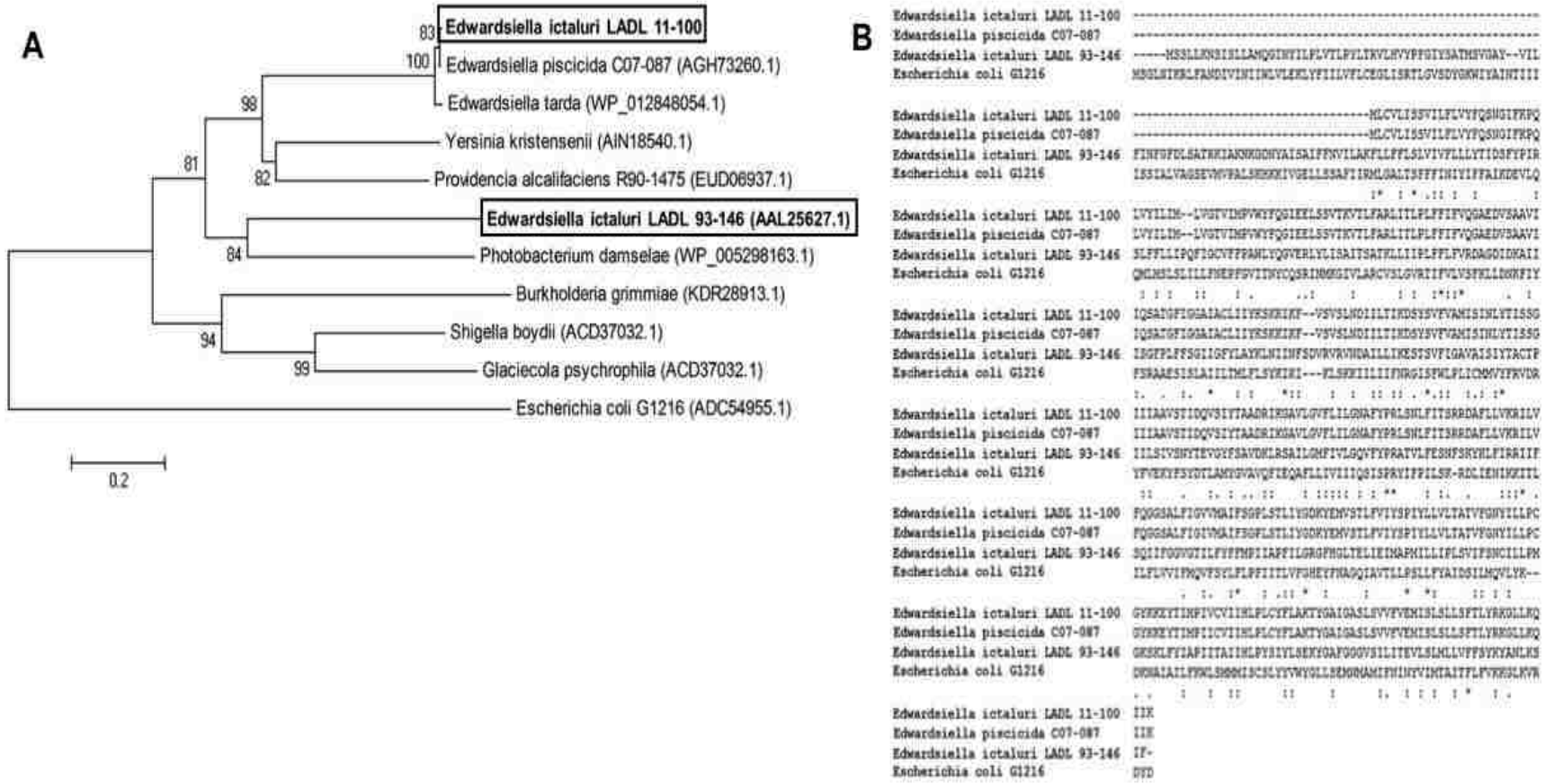

Figure 3.3 Comparison of Wzx protein and its homologous proteins in *E. ictaluri* LADL 11-100, LADL 93-146, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of Wzx proteins in listed bacterial strains. The sequences from 9 closely related bacterial strains are shown in addition to zebrafish strain LADL 11-100 and catfish strain LADL 93-146. B. Multi-alignment of Wzx amino acid sequences with its homologous proteins. The asterisks at the bottom line of the alignment indicate perfect matches among all listed strains; one and two dots represent highly and moderately conserved residues, respectively. Dashes refer to the missing amino acid sequences.

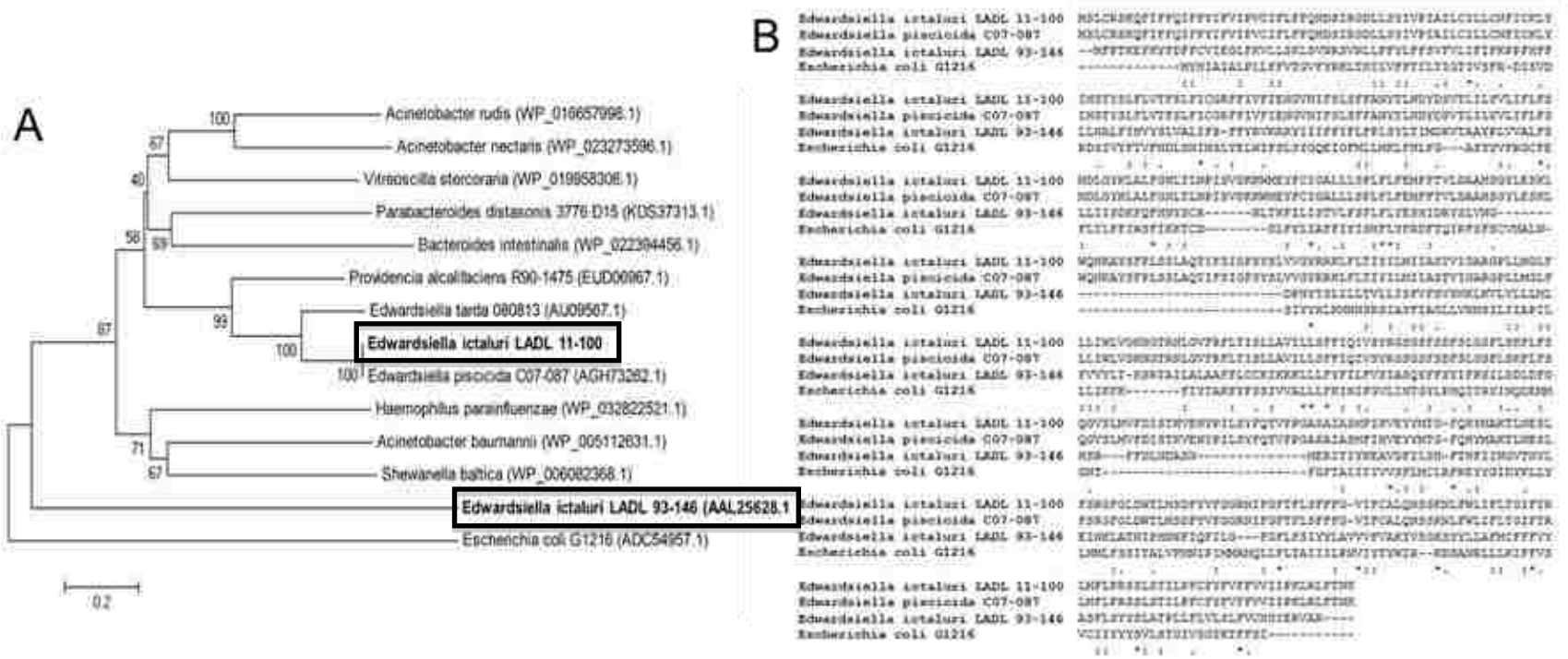

Figure 3.4 Comparison of Wzy and its homologous proteins in *E. ictaluri* LADL 11-100, LADL 93-146, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities among Wzy proteins in listed bacterial strains. The respective proteins from 9 closely related bacterial strains are shown in addition to zebrafish strain LADL 11-100 and catfish strain LADL 93-146. B. Multi-alignment of Wzy amino acid sequences with its homologous proteins. Symbols are described in Figure 3.3.

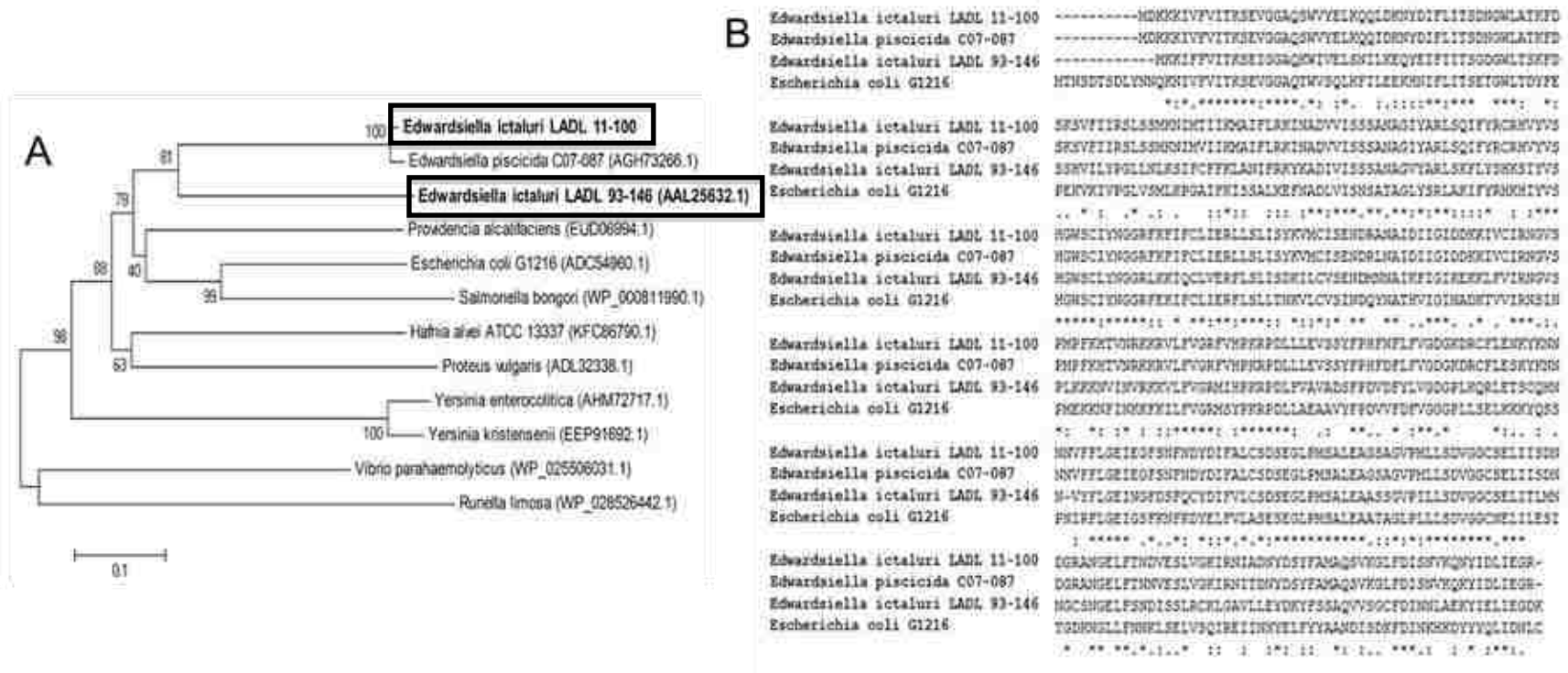

Figure 3.5 Comparison of WeiD and its homologous proteins in *E. ictaluri* LADL 11-100, LADL 93-146, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of WeiD proteins in listed bacterial strains. The respective proteins from 10 closely related bacterial strains in addition to zebrafish strain LADL 11-100 and catfish strain LADL 93-146 are shown. B. Multi-alignment of WeiD amino acid sequences with the homologous proteins. Symbols are described in Figure 3.3

Three genes in group I are *wzx, wzy* and *weiD* which encode a flippase, a polymerase and a glycosyltransferase, respectively. Phylogeny evolution analysis and sequence alignment of the proteins of the O-antigen biosysnthesis cluster from zebrafish and catfish strains are further analyzed to show their respective homology and phylogenetic relationships. The closest neighbors of the Wzx protein encoded by the zebrafish strain are the respective proteins in *E. piscicida*, and the second closest relative is the homologous protein from *E. tarda.* The proteins from the catfish strain are quite different, with 36% amino acid similarities. This phylogenetic relationship also applies to Wzy from the zebrafish strain of *E. ictaluri* which shared 26% of the amino acids with Wzy in the typical catfish strain. See Figure 3.3 and 3.4 for details. WeiD shares 58% of the amino acids with the corresponding protein WbiH in the catfish strain but is 95% identical to the homologous protein in *E. piscicida*. See Figure 3.5 for WeiD protein analysis (Figure 3.5).

Four genes belonging to group II are *wbcK*, *weiA, weiB* and *weiC.* Amino acid sequences of WbcK, WeiA, WeiB and WeiC proteins share no homology with any sequences in the catfish strain. However, they are highly homologous to the proteins in a closely related bacterium, *E. piscicida* C07-087 and have higher similarities to *E. coli* G1216 than to the catfish strain of *E. ictaluri*. The phylogenetic relationships between these proteins and their homologous proteins in closely related bacteria are revealed. The sequences corresponding to each protein are aligned to the closely related protein in the catfish strain of *E. ictaluri, E. piscicida, E. tarda* or *E.coli* and a phylogenetic tree is created to show the evolutional relationships of these O-antigen related proteins among various bacterial strains.

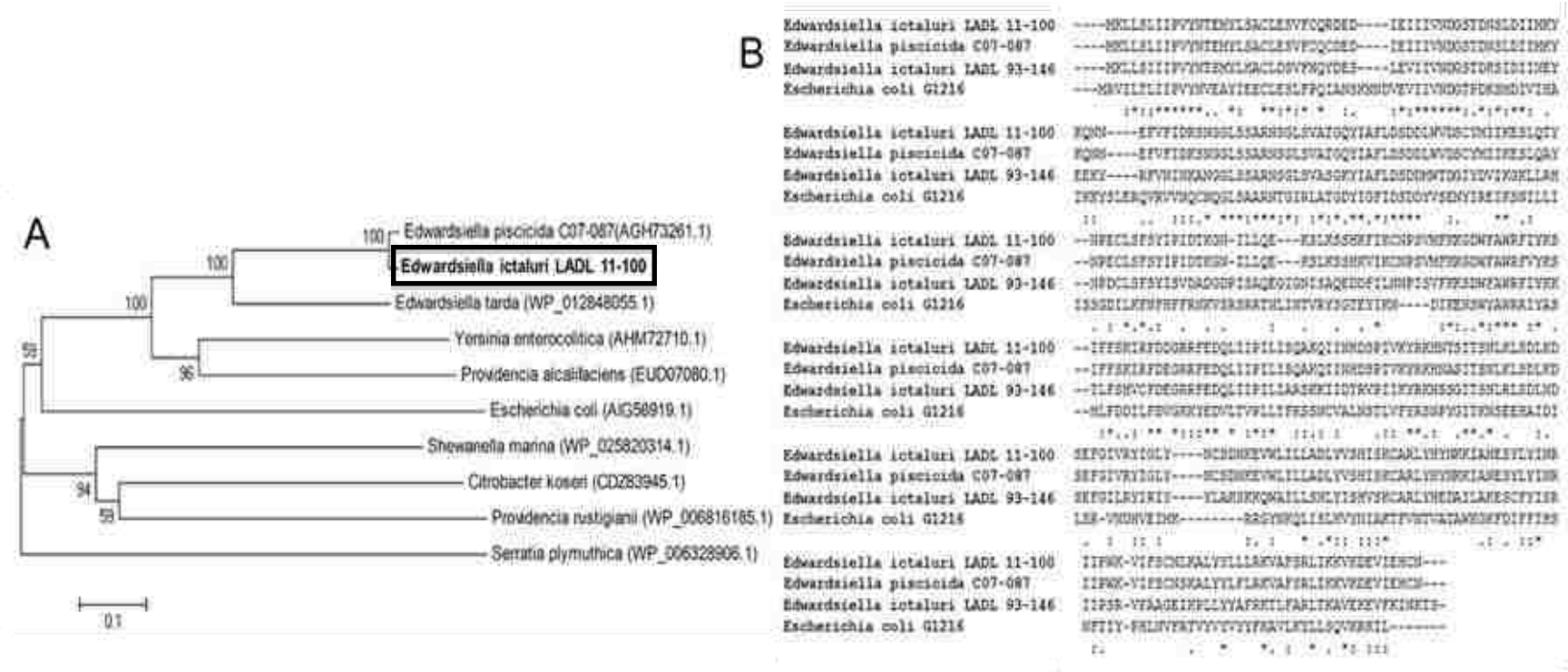

Figure 3.6 Comparison of WbcK and its homologous proteins in *E. ictaluri* LADL 11-100, *E. tarda* EIB 202, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of WbcK proteins in listed bacterial strains. The respective proteins from 9 closely related bacterial strains in addition to zebrafish strain LADL 11-100 are shown. Catfish strain LADL 93-146 does not have a protein that is matching to WbcK. B. Multi-alignment of WbcK amino acid sequences with the homologous proteins. Symbols are described in Figure 3.3.

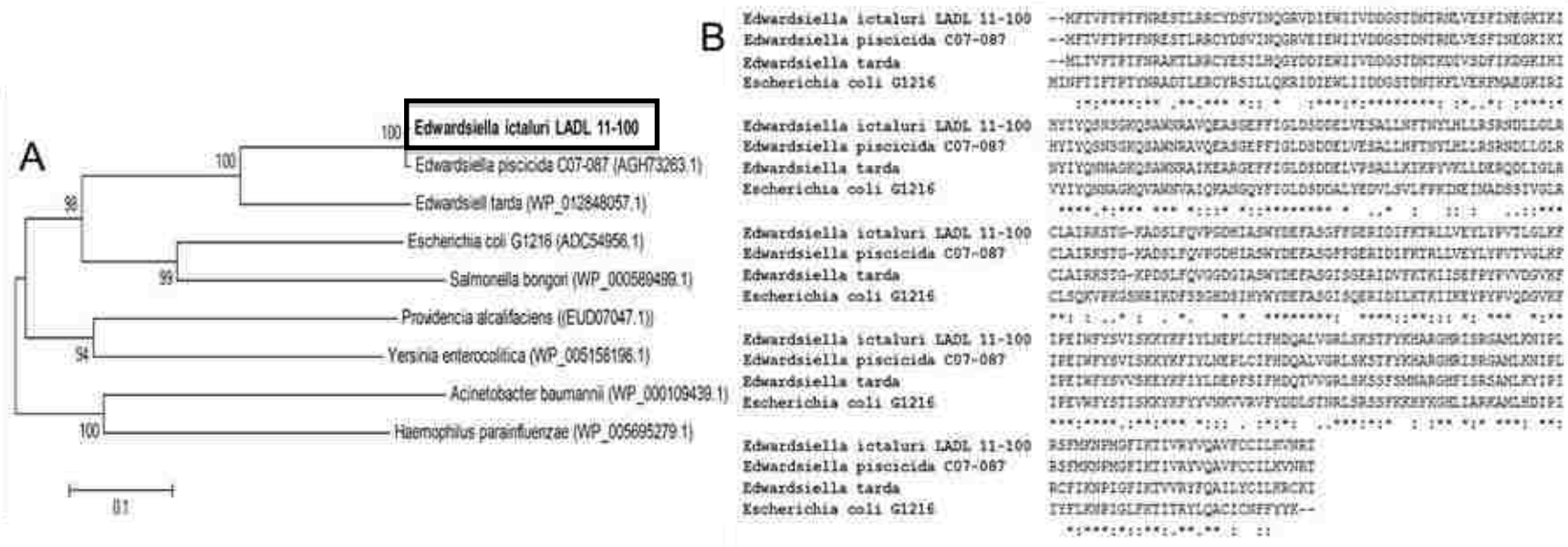

Figure 3.7 Comparison of WeiA protein in *E. ictaluri* LADL 11-100, *E. tarda*, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of WeiA proteins in listed bacterial strains. The respective WeiA proteins from 8 closely related bacterial strains in addition to zebrafish strain LADL 11-100 are shown. Catfish strain LADL 93-146 does not have a protein that matches to WeiA. B. Multi-alignment of WeiA amino acid sequence with the homologous proteins. Symbols are described in Figure 3.3.

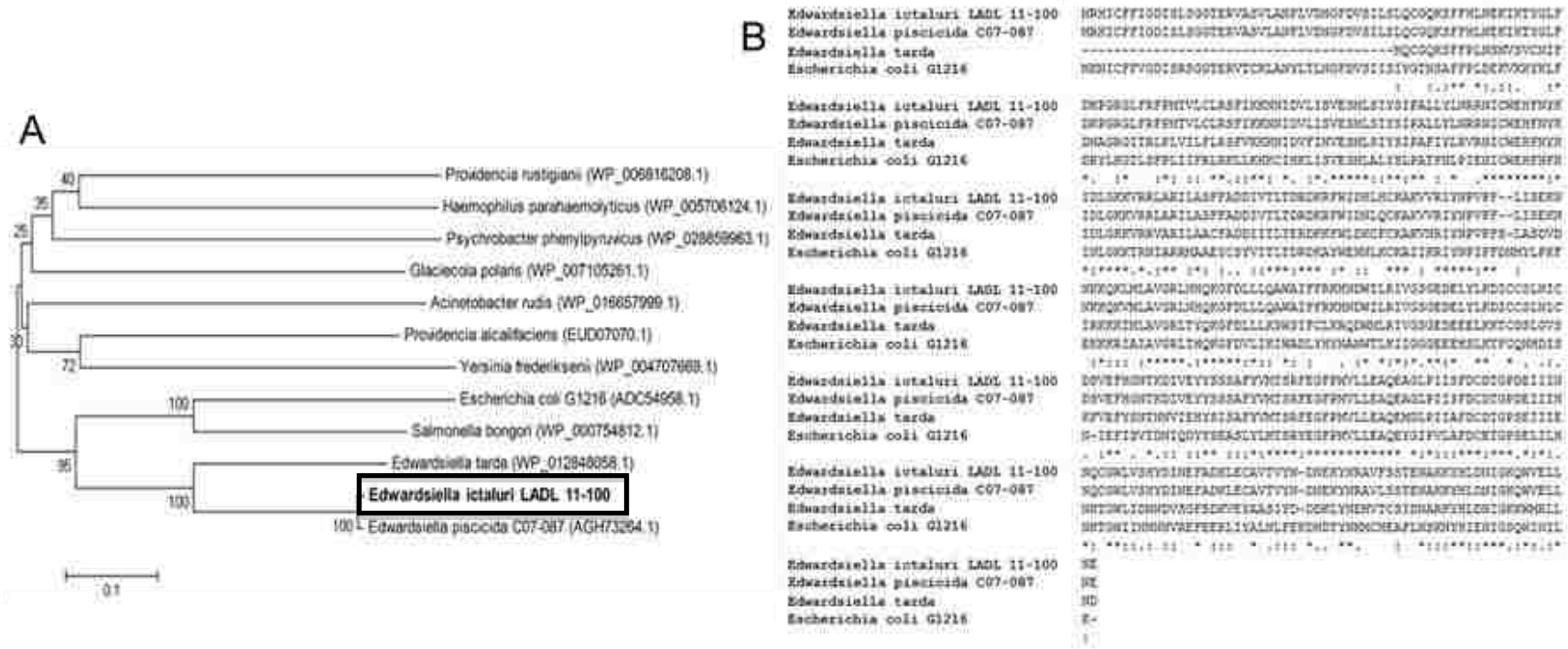

Figure 3.8 Comparison of WeiB and its homologous proteins in *E. ictaluri* LADL 11-100, *E. tarda*, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of WeiB proteins in listed bacterial strains. The WeiB protein sequences from 11 closely related bacterial strains in addition to zebrafish strain LADL 11-100 are shown. Catfish strain LADL 93-146 does not have a protein that matches to WeiB. B. Multi-alignment of WeiB amino acid sequences with its homologous proteins. Symbols are described in Figure 3.3.

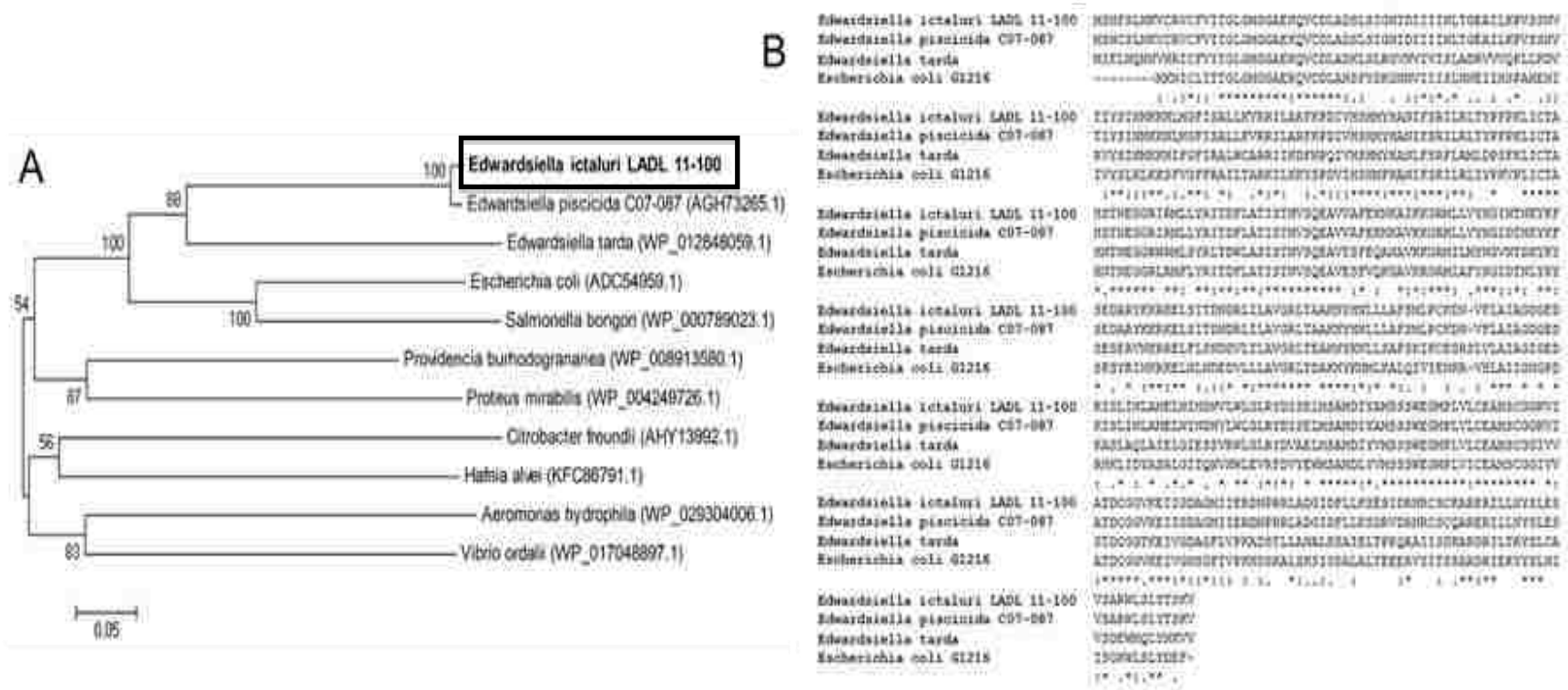

Figure 3.9 Comparison of WeiC protein in *E. ictaluri* LADL 11-100, *E. tarda*, *E. piscicida* C07-087, *E. coli* G1216 and other bacteria. A. Phylogenetic tree based on the similarities of WeiC proteins in listed bacterial strains. The sequences from 10 closely related bacterial strains in addition to zebrafish strain LADL 11-100 are shown. Catfish strain LADL 93-146 does not have a protein that matches to WeiC. B. Multi-alignment of WeiC amino acid sequences with its homologous proteins. Symbols are described in Figure 3.3.

Interestingly, WbcK, WeiA, WeiB and WeiC are all glycosyltransferases. The BLAST of the amino acid sequences of WbcK, WeiA, WeiB and WeiC found no homologous proteins in the catfish strain, however, homologous proteins are found in *E. piscicida*, with higher than 90% similarities. This indicates that the glycosyltransferases that relate to O antigen biosynthesis are quite different between the zebrafish and catfish strains of *E. ictaluri*. Figures 3.6-3.9 for the analysis results of WbcK and WeiABC. The results of multi-alignment of the WbcK, WeiA, WeiB and WeiC amino acid sequences with their homologous proteins are demonstrated in these figures.

The unique sequences that belong to group III are *insA, insB, wbiB* and *wbiM*. The 2 insertion sequences (*insA* and *insB*) on the cluster exist in the catfish strain LADL 93-146 [21], but they are absent from the O-antigen clusters in zebrafish strain LADL 11-100 as well as *E. piscicida* C07-087.The other two genes *wbiB* and *wbiM* encode glycosyltransferases. The proteins that are encoded by the unique genes in the LPS biosynthesis cluster from the catfish strain are listed in Table 3.2.

To investigate the evolutionary relationships of proteins encoded by the O-antigen biosynthesis cluster among various bacterial strains, the unique proteins encoded by the O-antigen cluster in the catfish strain, WbiB and WbiM, were analyzed. Blast and phylogenetic trees both suggest that WbiB and WbiM proteins from the catfish strain of *E. ictaluri* are homologous to proteins in *Salmonella enterica*, which is another enteric bacteria that is closely related to *E. ictaluri*. Detailed results are shown in Figure 3.10 and 3.11.

Table 3.2 Characteristics of the unique ORFs in catfish strain O-antigen biosynthesis cluster.

Protein	G +C (%)	No. of residues	Putative function	Related protein(s)		
				Protein (accession no.)	No. of residues	Identity (%)
WbiB (AAL25629.2)	31.8	261	Glycosyltransferase	lacto-N-neotetraose biosynthesis glycosyltransferase lgtB of *Neisseria meningitides* (WP_002226737.1)	258	20
				beta-1,4 galactosyltransferase of *Pasteurella multocida* (WP_032854432.1)	280	20
WbiM (AAL25631.1)	34.3	366	Glycosyltransferase, group 1	glycosyl transferase [*Vibrio cholerae*] (WP_001931828.1)	362	41
				glycosyl transferase of *Providencia rustigianii* (WP_006816207.1)	366	38

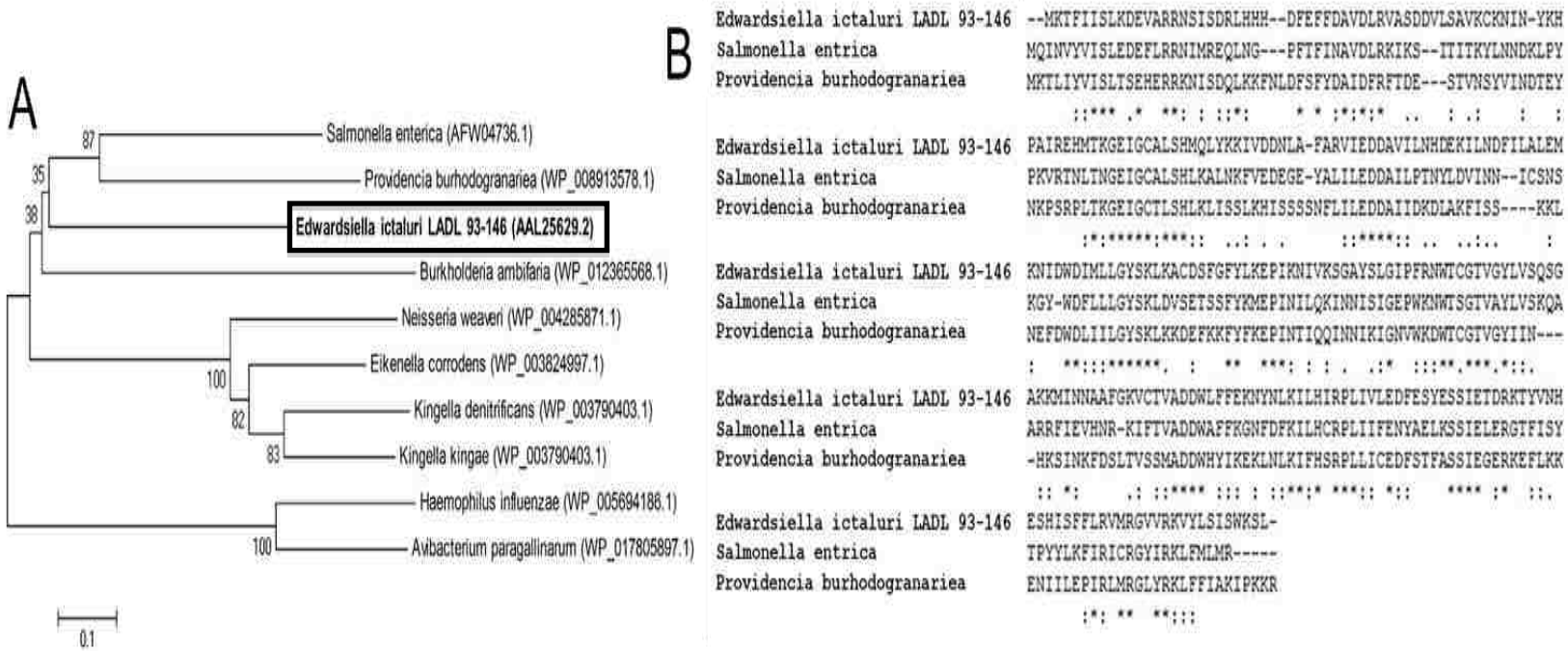

Figure 3.10 Comparison of WbiB protein and its homologous proteins in *E.ictaluri* LADL 93-146, *S. enterica* and other closely related bacteria. A. Phylogenetic tree based on the protein similarities of WbiB in listed bacterial strains. The sequences from 9 closely related bacterial strains in addition to catfish strain LADL 93-146 are shown. B. Multi-alignment of WbiB and its homologous proteins. Symbols are described in Figure 3.3.

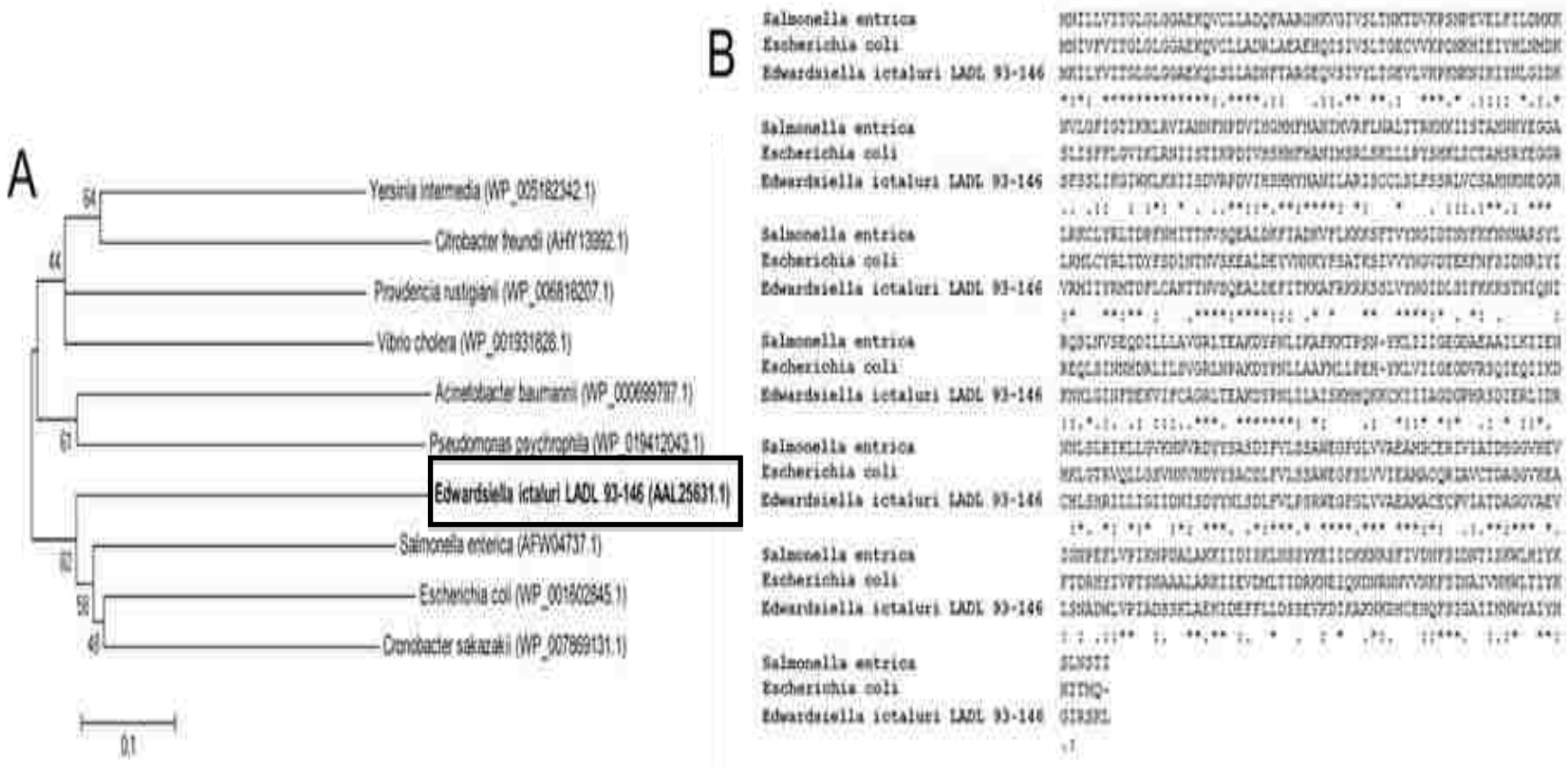

Figure 3.11 Comparison of WbiM protein and its homologous proteins in *E. ictaluri* LADL 93-146, *S. enterica* and other bacteria. A. Phylogenetic tree based on the protein similarities to WbiM in catfish strain of *E. ictaluri*. The sequences from 9 closely related bacterial strains in addition to catfish strain LADL 93-146 are shown. B. Multi-alignment of WbiM in catfish strain of *E. ictaluri* and its homologous proteins. Symbols are described in Figure 3.3.

The core and lipid A related genes are relatively conserved. The genes related to core and lipid A biosynthesis are found in all strains that are investigated, including *E. piscicida*, catfish and zebrafish strains of *E. ictaluri.* The exceptions are the *waaE* and *wabK* genes. These two genes, *waaE* and *wabK*, exist in *E. piscicida* and the catfish strain of *E. ictaluri* but not in the draft genome of the zebrafish strain of *E. ictaluri*. Both of them encode glycosyltransferases and are related to the inner core biosynthesis. The zebrafish and catfish strains of *E. ictaluri* share identities with all the genes in the core and lipid A except *waaE* and *wabK*. Detailed data appear in Table 3.3.

Table 3.3 Putative genes related to core and lipid A biosynthesis.

Core oligosaccharides (from *E. tarda* EIB 202)	Putative Function	*E. ictaluri* LADL11-100 (n.t.)	*E. piscicida* C07-087 (n.t.)	*E. ictaluri* LADL93-146 (n.t.)
Inner core				
waaE (ETAE_0071)	glycosyltransferase involved in cell wall biogenesis	No hits found	771/777 (99%)	732/777 (94%)
waaA (ETAE_00725)	3-deoxy-D-manno-octulosonic-acid transferase	1199/1275 (94%)	1273/1275 (99%)	1197/1275 (94%)
wabH (ETAE_0073)	glycosyltransferase	1017/1095 (92%)	1092/1095 (99%)	1016/1095 (93%)
wabG (ETAE_0074)	glucuronic acid transferase	1063/1128 (94%)	1123/1128 (99%)	1062/1128 (94%)
waaQ (ETAE_0075)	heptosyl III transferase	1005/1077 (93%)	1075/1077 (99%)	1005/1077 (93%)
wabN (ETAE_0076)	deacetylase	901/972 (92%)	970/972 (99%)	902/972 (93%)
wabK (ETAE_0077)	glycosyltransferase	No hits found	1209/1212 (99%)	346/388 (89%)
walR (ETAE_0078)	glycosyltransferase	274/307 (89%)	1105/1107 (99%)	309/355 (87%)
ETAE_0079	glycosyltransferase	1015/1153 (88%)	1155/1158 (99%)	1021/1160 (88%)

Table 3.3 - continued.

Core oligosaccharides (from *E. tarda* EIB 202)	Putative Function	*E. ictaluri* LADL11-100 (n.t.)	*E. piscicida* C07-087 (n.t.)	*E. ictaluri* LADL93-146 (n.t.)
waaL (ETAE_0080)	lipid A core - O-antigen ligase and related enzyme	1024/1134 (90%)	1127/1134 (99%)	1023/1134 (90%)
waaC (ETAE_0081)	ADP-heptose:LPS heptosyltransferase I	909/966 (94%)	964/966 (99%)	911/966 (94%)
rfaF (ETAE_0082)	ADP-heptose:LPS heptosyltransferase II	997/1062 (93%)	1059/1062 (99%)	996/1062 (94%)
rfaD (ETAE_0083)	ADP-L-glycero-D-manno-heptose-6-epimerase	918/965 (95%)	964/965 (99%)	919/965 (95%)
Outer core				
wzzE (ETAE_0102)	lipopolysaccharide biosynthesis protein	979/1038 (94%)	1035/1038 (99%)	978/1038 (94%)
wecB (ETAE_0103)	UDP-N-acetylglucosamine 2-epimerase	1073/1131 (94%)	1127/1131 (99%)	1074/1131 (95%)
wecC (ETAE_0104)	UDP-N-acetyl-D-mannosaminuronate dehydrogenase	1204/1263 (95%)	1260/1263 (99%)	1204/1263 (95%)
rffG (ETAE_0105)	dTDP-D-glucose 4,6-dehydratase	1029/1071 (96%)	1064/1071 (99%)	1024/1071 (96%)
rffH (ETAE_0106)	glucose-1-phosphate thymidylyltransferase	852/882 (96%)	882/882 (100%)	849/882 (96%)
wecD (ETAE_0107)	TDP-D-fucosamine acetyltransferase	642/696 (92%)	690/696 (99%)	641/696 (92%)
wecE (ETAE_0108)	TDP-4-oxo-6-deoxy-D-glucose transaminase	1078/1131 (95%)	1130/1131 (99%)	1076/1131 (95%)
wzxE (ETAE_0109)	membrane protein involved in the export of O-antigen and teichoic acid	1200/1251 (95%)	1245/1251 (99%	1201/1251 (96%)
wecF (ETAE_0110)	4-alpha-L-fucosyltransferase	1027/1077 (95%)	1074/1077 (99%)	1025/1077 (95%)
wzyE (ETAE_0111)	putative enterobacterial common antigen polymerase	1326/1389 (95%)	1378/1389 (99%)	1328/1389 (96%)

Table 3.3 - continued.

Core oligosaccharides (from *E. tarda* EIB 202)	Putative Function	*E. ictaluri* LADL11-100 (n.t.)	*E. piscicida* C07-087 (n.t.)	*E. ictaluri* LADL93-146 (n.t.)
wecG (ETAE_0112)	UDP-N-acetyl-D-mannosaminuronic acid transferase	683/728 (93%)	739/741 (99%)	693/741 (94%)
Lipid A				
htrB (ETAE_0468)	lipid A biosynthesis lauroyl acyltransferase	863/929 (92%)	925/929 (99%)	863/929 (93%)
lpxC (ETAE_0643)	UDP-3-O-acyl N-acetylglucosamine deacetylase	875/916 (95%)	913/918 (99%)	875/916 (96%)
lpxD (ETAE_0747)	DP-3-O-(3-hydroxymyristoyl)-glucosamine N-acyltransferase	943/1023 (92%)	1019/1023 (99%)	947/1023 (93%)
fabZ (ETAE_0748)	(3R)-hydroxymyristoyl-(acyl carrier protein) dehydratase	440/456 (96%)	456/456 (100%)	440/456 (96%)
lpxA (ETAE_0749)	UDP-N-acetylglucosamine acyltransferase	755/789 (95%)	786/789 (99%)	758/789 (96%)
lpxB (ETAE_0750)	lipid-A-disaccharide synthase	1080/1186 (91%)	1178/1185 (99%)	1083/1187 (91%)
msbB (ETAE_1445)	lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase	874/963 (90%)	960/963 (99%)	875/963 (91%)
lpxK (ETAE_2171)	tetraacyldisaccharide 4-kinase	893/992 (90%)	995/996 (99%)	893/996 (90%)
msbA (ETAE_2172)	lipid transporter ATP-binding/permease protein	1641/1755 (93%)	1752/1755 (99%)	1644/1755 (94%)
ETAE_2369	lipopolysaccharide glycosyltransferase	798/893 (89%)	936/945 (99%)	832/936 (89%)
ETAF_2432 (*Edwardsiella tarda* FL6-60)	UDP-2,3-diacylglucosamine hydrolase	714/769 (92%)	780/780 (100%)	724/780 (93%)

LPS composition analysis. LPS banding patterns as well as the LPS sugar composition analysis reveal significant differences in LPS samples between catfish and zebrafish strains of *E. ictaluri*. Purified LPS from the catfish strain of *E. ictaluri*, when analyzed by SDS-PAGE, demonstrates a ladder-like pattern while similar bands between 10 and 37 kD are not visible in the LPS from the zebrafish strain. In fact, only two bands, including one between 10 and 15 kD and the other one between 25 and 37kD, are strongly visible in the gel for the zebrafish strain LPS sample. There is also a diffused band appearing between 75 and 150 kD in the LPS sample from zebrafish strain of *E. ictaluri*. Therefore, the results in Figure 3.12 obviously demonstrate the differences in the LPS between zebrafish and catfish strains of *E. ictaluri*.

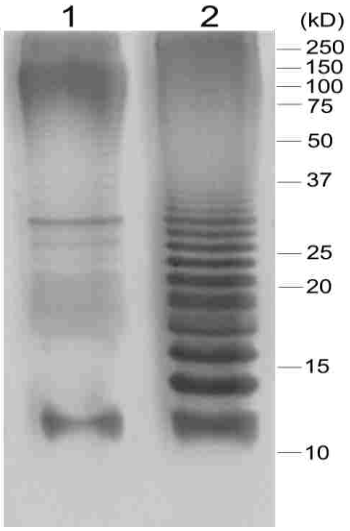

Figure 3.12 LPS samples from zebrafish and catfish strain of *E. ictaluri*. Lane 1 is the LPS sample from zebrafish strain LADL11-100 and lane 2 is the catfish strain LADL 93-146 LPS.

Analysis of the LPS from the two strains by GC/MS are shown in Table 3.4 and 3.5. Both strains have glucose and heptose as their major sugars, however, LPS samples from zebrafish strain contain less glucose and more heptose than the LPS samples from catfish strain. Other sugars that were detected in these 2 samples are ribose, xylose,

galacturonic acid (GalA), mannose, galactose and 2-keto-3-deoxy-octanoate (KDO). Obvious differences were observed in the molar percentages of galactose, glucose and KDO. The KDO makes up 21.3% of total molar percentage in zebrafish strain LPS samples, while in the catfish strain, KDO only comprises 2% of the total LPS samples. Therefore, we can conclude from this analysis that the LPS samples from the catfish strain and the zebrafish strain have the same sugar composition but different sugar percentage ratios.

Table 3.4 Monosaccharide composition of LPS sample from zebrafish strain of *E. ictaluri* LADL 11-100.

Monosaccharide	Weight (μg)	% by mole
Arabinose (Ara)	nd	-
Ribose (Rib)	2.2	1.1
Rhamnose (Rha)	nd	-
Fucose (Fuc)	nd	-
Xylose (Xyl)	0.2	0.1
Glucuronic Acid (GlcA)	nd	-
Galacturonic acid (GalA)	12.8	4.9
Mannose (Man)	1.3	0.5
Galactose (Gal)	9.1	3.8
Glucose (Glc)	40.1	16.6
N-Acetyl Galactosamine (GalNAc)	nd	-
N-Acetyl Glucosamine (GlcNAc)	nd	-
N-Acetyl Mannosamine (ManNAc)	nd	-
Heptose	145.7	51.7
KDO	68.1	21.3
Sum	279.6	100.0
% carbohydrates	0.23	

[1]Data are obtained from 121.80 mg wet bacterial cells; nd = not detected.

Table 3.5 Monosaccharide composition of LPS sample from catfish strain of *E. ictaluri* LADL 93-146.

Monosaccharide	Weight (μg)	% by mole
Arabinose (Ara)	nd	-
Ribose (Rib)	0.3	0.2
Rhamnose (Rha)	nd	-
Fucose (Fuc)	nd	-
Xylose (Xyl)	0.7	0.6
Glucuronic Acid (GlcA)	nd	-
Galacturonic acid (GalA)	5.6	3.4
Mannose (Man)	2.3	1.5
Galactose (Gal)	17.3	11.3
Glucose (Glc)	56.8	37.3
N-Acetyl Galactosamine (GalNAc)	nd	-
N-Acetyl Glucosamine (GlcNAc)	nd	-
N-Acetyl Mannosamine (ManNAc)	nd	-
Heptose	77.8	43.6
KDO	38.6	2.0
Sum	199.4	100.0
% carbohydrates	0.30	

[1]Data are obtained from 65.93 mg wet bacterial cells; nd = not detected.

Discussion

Genome sequencing can provide us with valuable information concerning the existence of certain important virulence factors; the identity of genes that are essential for certain pathways and an understanding of the evolutionary relationship among bacterial species. In this study, all the genes related to LPS biosynthesis are examined in the genome of zebrafish strain LADL 11-100 and compared to those in the catfish strain LADL 93-146 as well as some other closely related bacteria. Genes associated with core and lipid A biosynthesis are found to have high similarities except for two transferases. This result is in accordance with the previous finding that the structures of the core and lipid A of the lipopolysaccharide molecule are highly conserved among bacteria in the

same genera [9]. The O-antigen biosynthesis cluster is also found in the genome of the zebrafish strain *E. ictaluri* LADL11-100. The O-antigen biosynthesis cluster was further characterized and compared with the respective proteins in the catfish strain and other closely related strains. Here we present that O-antigen biosynthesis clusters in zebrafish and catfish strains vary significantly in the structure of the clusters and the identities of protein sequences. The gene arrangement of the cluster in the zebrafish strain of *E.ictaluri* LADL11-100 is identical to that of *E. piscicida* C07-087 and the protein sequences share >90% identity with the homologous proteins in *E. piscicida* C07-087. Analysis of the O-antigen biosynthesis cluster in the catfish strain has shown the existence of two insertional sequences, which can possibly make this cluster different from other O-antigen biosynthesis clusters in the strains of the *Edwardsiella* genus. The differences in the gene cluster structure indicate that horizontal gene transfer, caused by insertional sequences, which are probably the reason for the variation of the O-antigen biosynthesis cluster in the catfish strain. Meanwhile, these variations also lead to the changes of the identities of protein sequences. The amino acid sequences of proteins encoded by this cluster in zebrafish strain LADL11-100 are more closely related to the proteins in *E. piscicida* C07-087 and *E. tarda* strains than to the typical catfish strain of *E. ictaluri* LADL93-146. Meanwhile, according to the result of our phylogeny analysis on WbiB and WbiM (Figure 3.10 and Figure 3.11), these unique proteins in catfish strain of *E. ictaluri* are phylogenetically close to *Salmonella enterica*. It is possible that the O-antigen biosynthesis clusters in *Salmonella enterica* and the *E. ictaluri* catfish strain originated from a similar source by horizontal gene transfer.

Intriguingly, *E. piscicida* is highly virulent in the zebrafish *Danio rerio* by intramuscular injection [29] and whether this bacterium can cause mortality in zebrafish by immersion remains unknown. *E. tarda*, which is phylogenetically more distant from the zebrafish strain of *E. ictaluri* is virulent in zebrafish embryos but not adult fish by immersion [30]. Zebrafish *Danio rerio* are not susceptible to infection by the catfish strain of *E. ictaluri* by immersion unless a high dose of bacteria are applied to the fish. However, even in the case of high dose exposure, the overall mortality of zebrafish is still relatively low [31]. The typical catfish strain differs in its O-antigen biosynthesis cluster when compared to the zebrafish strain of *E. ictaluri*. Whether the age of zebrafish can be a factor that influences *E. ictaluri* infection is not clear, but we predict that the changes in the O-antigen biosynthesis cluster, to a certain extent, contribute to the various levels of infectivity in the host since the O antigen is important for host colonization, invasion and immune evasion and modifications of O antigen can affect the infectivity in specific hosts [32-34]. Our results also show that different genes in the O-antigen biosynthesis cluster can lead to the differences in O antigen profile as we observed from the SDS-PAGE. Taken together, the vital functions of the O-antigen in bacterial infection and the differences in the DNA and protein levels of the O antigen biosynthesis cluster, which are associated with the different banding patterns of LPS samples, reveal the clues for the host specificity.

Within the O-antigen biosynthesis cluster, two of the putative genes that show relatively low similarities between catfish and zebrafish strains are *wzx* and *wzy* which encode a flippase and an O-antigen polymerase, respectively [35]. Studies of the *wzx* gene have shown that mutations in this gene can abolish or delay the expression of

certain bands in the LPS profile [36]. Other studies on LPS biosynthesis have shown that LPS can be synthesized *in vitro* using Wzy and another protein named Wzz which functions as a regulator to guide the synthesis of LPS of specific length. Wzy, as the sole enzyme in the assembly process, works in a distributive manner to add the O subunit to the growing chain [37, 38]. Mutations in the *wzy* gene can change the LPS phenotype completely from smooth to rough and influence serum sensitivity [19]. All of these indicate that the low similarities of Wzx and Wzy can lead to changes in LPS banding profiles and possibly affect the infectivity in the host.

There are an additional four genes on the O-antigen biosynthesis cluster from the zebrafish strain of *E. ictaluri* that do not have corresponding genes on the O-antigen biosynthesis cluster from the catfish strain. These genes encode glycosyltransferases which function to transfer sugars to form an oligosaccharide on a carrier lipid, undecaprenyl phosphate (UndP) before being flipped to the periplasm side of the inner membrane [20]. These transferases tend to bind preferred sugars and are probably related to the differences in sugar compositions of LPS samples from catfish and zebrafish strains of *E. ictaluri* [39, 40]. The changes in these genes explain, to some extent, the significant variation in sugar composition of LPS samples from the catfish and zebrafish strains.

Among all the reported sugars in the LPS samples, the sugar that varies the most in terms of molar percentage is KDO. This eight-carbon sugar is part of the inner core and also the molecule that connects lipid A with the rest of the LPS. In addition to the changes in KDO percentage, a low level of glucose is observed. Glucose only makes up 16.6 % of the total sugars in the LPS of zebrafish strain compared to 37 % in the LPS of

the catfish strain. It's been shown that in *Haemophilus ducreyi*, the virulent and avirulent strains tend to have different ratios of sugar contents in the LPS [41]. Differences in sugar compositions can influence the ability for bacteria to be phagocytosed by the host cells and their efficient intercellular movement in the host. Thus, the differences in sugar composition can affect virulence of bacteria [42, 43].

The different banding patterns of the LPS purified from catfish and zebrafish strains of *E. ictaluri* are probably the result of variations on the O-antigen biosynthesis cluster between the two strains. The ladder-like pattern of LPS from the catfish strain in this study is the same as the results in previous studies [44, 45]. However, the LPS from zebrafish strains contain far less bands. After silver staining, only one low-molecular-mass band, one high-molecular-mass band and one diffused band arranged from 75 kD to 150 kD are obvious. All the other bands in catfish LPS sample are not present in zebrafish LPS sample. It has been shown that all the bands in LPS samples from catfish strains are highly immunogenic and mutations in certain genes that result in O antigen deficient organisms can also fully attenuate the strain [21, 45]. In fact, each band on the SDS-PAGE represents the lipid A- core complex with a specific number of O-antigen repeat units attached [46]. Therefore, the absence of many of the bands in the LPS of the zebrafish strain reveals the structural differences between catfish and zebrafish strain LPS samples. Thus, different banding patterns on SDS-PAGE can explain or partially explain why the zebrafish strain lacks virulence by immersion exposure in the catfish host [44, 47].

Even though the zebrafish strain LPS sample didn't exhibit ladder-like pattern bandings, based on the colony morphology of the zebrafish strain *E. ictaluri* as well as

the observed bands of the LPS samples from zebrafish strain, the LPS samples should still be considered as smooth type LPS since there are indications of the existence of O-antigen. It's possible that many of the components of the O-side chains in the catfish strain LPS are not in the zebrafish LPS molecule at the same level. It's also possible that LPS samples from the zebrafish strain actually contain bands of different sizes than that of the catfish strain and these zebrafish strain specific bands cannot be detected by the silver stain method very well. Other groups have shown that certain O-antigen mutants of catfish strain *E. ictaluri* exhibit autoagglutination and weaker or no motility [8]. Our zebrafish strain of *E. ictaluri* also autoagglutinates and shows weaker motility [7]. Early studies indicate that LPS usually confers the negative charge because of phosphorylation and the changes in the surface charge can possibly lead to autoagglutination [48, 49]. To explain the autoagglutination, we predict that the variations in O-antigen biosynthesis clusters can change the LPS structural composition and also the charge, thus leading to autoagglutination, For many years, it has been known that the catfish strain of *E. ictaluri* is resistant to complement killing by the alternative pathway. One reason for that is the O side chains can help prevent complement, more specifically, C3 from binding to the cell in the alternative pathway [50, 51]. In contrast, reduced LPS exposes the cell surface to complement binding and thus increases membrane disruption and killing [52]. It is possible that the LPS molecule of the zebrafish strain is truncated or reduced in O side chain length, no longer protecting bacteria from being killed by catfish complement system and thus is avirulent in catfish.

To our knowledge, this is the first study that characterized the differences in gene organization and protein profiles derived from LPS gene clusters of bacteria in the genus

Edwardsiella. Here we provide an overview of the differences in the LPS biosynthesis cluster at the DNA and protein level. Lateral gene transfer, acting as an important role in the evolution of bacteria, is apparently responsible for some of the changes in gene arrangement among members of this genus. In the case of *E. ictaluri*, strains pathogenic for catfish, probably obtained alien genes by insertional sequences. Variations in gene expression in different strains of this bacterial species may be associated with the newly observed host specificity of zebrafish and catfish strains of *E. ictaluri.* Finally, this study provides directions for further research aiming to investigate the pathogenesis of bacteria in the *Edwardsiella* genus.

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CHAPTER IV. DIFFERENCES IN OTHER POTENTIAL VIRULENCE FACTORS BETWEEN CATFISH AND ZEBRAFISH STRAINS OF *EDWARDSIELLA ICTALURI*

Introduction

Virulence factors. Bacterial virulence factors refer to the encoded proteins that facilitate bacteria to gain entry into, survive, and proliferate in the host resulting in disease. Important virulence factors usually include the surface structures, e.g. outer membrane proteins, bacterial secretion systems, flagella and fimbriae. Thus, it is interesting to compare the important virulence factors of the catfish strain of *E. ictaluri* with that of the zebrafish strain.

Type III secretion system (T3SS). Type III secretion systems are commonly found in gram-negative bacteria and help inject bacterial proteins, also known as effectors, into the host cells. The T3SS has been reported in *Salmonella spp., Vibrio spp., Pseudomonas spp., Aeromonas spp., Shigella spp.* and enteroinvasive *E. coli* [1-6], In some bacteria, more than one T3SS was found. Examples are *Salmonella typhimurium, Yersinia enterocolitica, Vibrio parahaemolyticus* and *Escherichia coli* [7-10]. The T3SS is an important virulence factor in many pathogenic bacteria because it can inject the effectors into the host cells to facilitate its adhesion, invasion and colonization [11, 12]. Effectors that are secreted by these T3SSs can damage the host cell, make the host environment more favorable for the bacteria and lead to disease progression in the host [12, 13]. The effectors in the catfish strain of *E. ictaluri* have been identified by Thune et, al. [14].

Type IV secretion system (T4SS). Type IV secretion systems (T4SS) are found in both gram positive and gram negative bacteria. Their functions are versatile: some can transfer DNA from one cell to another in a contact-dependent way; some function to uptake and

release DNA to the extracellular milieu; or others can inject effector proteins into the host cells [15, 16]. So far, the most heavily studied T4SS is the vir system in *Agrobacterium tumefaciens* Ti plasmid. Thus far there are no descriptions of T4SS in *E. ictaluri*, and only three of the putative genes can be found on the National Center for Biotechnology Center (NCBI) website.

Other virulence factors, including Type VI secretion system (T6SS) and urease. Another protein transport system in gram negative bacteria is the Type VI secretion system (T6SS). The T6SS mediates contact-dependent competitor killing by introducing effectors to the sister cells [17]. The urease system, encoded in catfish strain of *E. ictaluri*, is an important virulence factor as it is required for bacterial intracellular replication [18].

The newly identified zebrafish strain is different from the typical catfish strain by exhibiting the following characters: weaker motility, different biochemical profiles and the lacking of monoclonal antibody Ed9 recognition of LPS [19]. Our study has also shown that the zebrafish strain cannot cause mortality in channel catfish by immersion. Based on all this information, it is interesting to investigate the differences in the important virulence factors and find the potential reasons for the differences between these two strains on the DNA level.

Materials and Methods

Tools used for preparing the data. The following programs were useful in analysis of genome sequences, BioEdit, NextGENe, virulence factor database (VFDB) [20-22], blast tool on NCBI website, SWISS-MODEL [23, 24],and Protein Homology/analogY Recognition Engine (Phyre) V 2.0 [25].

Virulence factor identification. First, the genomes of the zebrafish strains LADL11-100 and LADL11-194 and the genome of the catfish strain of *E. ictaluri* LADL93-146 were uploaded in VFDB to blast against all known virulence factors. The hits from each strain were compared to the other two strains of *E. ictaluri* manually and the resulting virulence factors were clustered into four groups based on the alignment results.

Second, the well-studied virulence factors in the catfish strain of *E. ictaluri* were blasted in the genome of the zebrafish strain of *E. ictaluri* LADL11-100 one by one. These include the genes involved in T3SS, T6SS and the urease system. Another system that was studied is T4SS. All of the reference sequences and their accession numbers are listed in Table 4.1. The amino acids encoded by the unique genes in zebrafish strain LADL11-100 were blasted against the NCBI database to search for homologous proteins. The phylogenetic trees were created based on the sequences of the genes in the T4SS that differ between the catfish and zebrafish strains of *E. ictaluri*. The related genes in the zebrafish strain of *E. ictaluri* LADL11-194 were also analyzed to confirm the differences between the zebrafish and catfish strain of *E. ictaluri*.

Table 4.1 The reference genes used in this Chapter IV.

Name	Source	Accession number
T3SS genes	*E. ictaluri* 93-146	DQ233733.1
T4SS genes	*E. ictaluri* 93-146	Personal communication with Dr. Ronald Thune
T6SS genes	*E. ictaluri* 93-146	CP001600.2
Urease genes	*E. ictaluri* 93-146	AY607844.2
Fimbrial gene cluster	*E. ictaluri* 93-146	AY626368.2

Third, four of the potential important virulence factors with SNPs from the zebrafish strain of LADL11-100 were sent to Swiss-model software together with their homologous genes from catfish strain of *E. ictaluri* for prediction of their protein structure.

Table 4.2 The virulence factors identified in catfish strain (LADL 93-146) and two zebrafish strains of *E. ictaluri* (LADL11-100 and LADL11-194).

alignment number	Gene symbol and putative function	significant alignments (bits) Value		
		LADL 11-100	LADL 11-194	LADL 93-146
VFG0479	pykF - pyruvate kinase I (formerly F)	456 e-125	456 e-125	456 e-125
VFG2329	fliG - flagellar motor switch protein	365 1e-97	365 1e-97	357 3e-95
VFG2331	fliI - flagellum-specific ATP synthase	327 2e-86	327 2e-86	327 3e-86
VFG1074	orf58 - IS1328 transposase	240 4e-60	262 1e-66	527 e-146
VFG0477	rpoS - sigma S (sigma 38) factor of RNA polymerase	230 4e-57	230 4e-57	230 4e-57
VFG2499	fliI - flagellum-specific ATP synthase	224 3e-55	224 3e-55	222 1e-54
VFG2356	flhA - flagellar biosynthesis protein FlhA	218 2e-53	218 2e-53	226 7e-56
VFG0562	mutS - methyl-directed mismatch repair	202 1e-48	202 1e-48	202 1e-48
VFG0574	mgtB - Mg2+ transport protein	198 2e-47	198 2e-47	214 3e-52
VFG0478	fur - ferric iron uptake regulon transcriptional repressor	182 9e-43	182 9e-43	182 9e-43
VFG0576	ssb - ssDNA-binding protein	167 5e-38	167 5e-38	168 1e-38
VFG1862	katB - catalase-peroxidase	163 8e-37	163 8e-37	163 9e-37
VFG1495	ORF44 - pseudo: with similarity to IS630 transposase	161 3e-36	212 1e-51	220 4e-54
VFG1263	flhA - flagellar biosynthesis protein FlhA	153 8e-34	153 8e-34	145 2e-31
VFG2522	flhA - flagellar biosynthesis protein FlhA	153 8e-34	153 8e-34	145 2e-31
VFG2323	fliC3 - flagellin	151 3e-33	151 3e-33	143 8e-31
VFG2322	YE2522 - thermoregulated motility protein	151 3e-33	151 3e-33	143 8e-31
VFG2321	fliC2 - flagellin	151 3e-33	151 3e-33	143 8e-31
VFG2335	fliM - flagellar motor switch protein FliM	145 2e-31	145 2e-31	145 2e-31
VFG2336	fliN - flagellar motor switch protein FliN	143 8e-31	143 8e-31	143 8e-31
VFG2345	flgH - flagellar L-ring protein precursor	139 1e-29	139 1e-29	139 1e-29
VFG2344	flgI - flagellar P-ring protein precursor	139 1e-29	139 1e-29	149 1e-32
VFG1443	ompA - outer membrane protein A	137 5e-29	137 5e-29	137 5e-29
VFG2338	fliP - flagellar biosynthetic protein	129 1e-26	129 1e-26	129 1e-26
VFG2319	fliA - RNA polymerase sigma 28 (sigma F) factor	127 5e-26	127 5e-26	127 5e-26
VFG0863	astA - heat-stable enterotoxin 1	127 5e-26	127 5e-26	127 5e-26
VFG2346	flgG - flagellar basal-body rod protein	117 4e-23	117 4e-23	117 5e-23
VFG1102	hsdM - DNA methylase	115 2e-22	115 2e-22	100 1e-17
VFG1855	htpB - Hsp60, 60K heat shock protein HtpB	113 7e-22	113 7e-22	113 7e-22
VFG0188	pscR - translocation protein in type III secretion system	111 3e-21	111 3e-21	111 3e-21
VFG1670	orf47 - putative cadaverin decarboxylase	109 1e-20	109 1e-20	109 1e-20
VFG1483	ORF32 - Pseudo [Escherichia coli 536]	107 4e-20	260 5e-66	293 4e-76
VFG2526	cheB - chemotaxis-specific methylesterase	107 4e-20	107 4e-20	107 4e-20
VFG2531	cheA - chemotaxis two-component sensor kinase	107 4e-20	107 4e-20	107 4e-20
VFG0605	SF3705 - putative transposase	98 4e-17	113 7e-22	105 2e-19
VFG0638	SF2974 - orf, partial conserved hypothetical protein	105 2e-19	105 2e-19	105 2e-19
VFG1691	orf68 - truncated InsB protein	103 7e-19	103 7e-19	100 1e-17
VFG0438	spvC - Salmonella plasmid virulence: hydrophilic protein	101 3e-18	101 3e-18	113 7e-22
VFG2525	cheY - chemotaxis protein	100 1e-17	100 1e-17	100 1e-17
VFG0776	orf6.1 - unknown [Escherichia coli E2348/69]	98 4e-17	98 4e-17	90 1e-14
VFG1405	sigA - RNA polymerase sigma factor	96 2e-16	96 2e-16	96 2e-16
VFG1602	orf68 - hypothetical protein [Escherichia coli 536]	96 2e-16	109 1e-20	101 3e-18
VFG1254	fliI - flagellum-specific ATP synthase	94 6e-16	94 6e-16	94 7e-16
VFG2461	bsaQ - Type III secretion system protein	94 6e-16	94 6e-16	88 4e-14
VFG2076	clpV1 - probable ClpA/B-type chaperone	90 1e-14	90 1e-14	90 1e-14
VFG2494	fliP - flagellar biosynthesis protein	90 1e-14	90 1e-14	90 1e-14
VFG1715	c3594 - Putative Transposase	88 4e-14	88 4e-14	88 4e-14
VFG2328	fliF - flagellar M-ring protein	86 2e-13	86 2e-13	86 2e-13
VFG1248	fleQ - transcriptional regulator FleQ	84 6e-13	84 6e-13	84 6e-13
VFG2529	tsr - methyl-accepting chemotaxis protein I	84 6e-13	84 6e-13	84 6e-13
VFG1246	fliC - flagellin type B	82 2e-12	82 2e-12	82 3e-12
VFG1504	z1203 - Z1203 protein [Escherichia coli 536]	80 1e-11	80 1e-11	80 1e-11
VFG2351	flgB - flagellar basal-body rod protein	78 4e-11	78 4e-11	78 4e-11
VFG2348	flgE - flagellar hook protein	78 4e-11	78 4e-11	78 4e-11
VFG1098	hsdR - type I restriction enzyme	76 2e-10	76 2e-10	74 6e-10
VFG2359	flhD - flagellar transcriptional activator	74 6e-10	74 6e-10	74 6e-10
VFG1668	orf45 - putative lysil-tRNA synthetase	74 6e-10	74 6e-10	74 6e-10
VFG2325	fliS - flagellar protein	72 2e-09	72 2e-09	72 2e-09
VFG0519	ssaR - Secretion system apparatus	72 2e-09	72 2e-09	72 2e-09
VFG2480	clpV - Clp-type ATPase chaperone protein	72 2e-09	72 2e-09	72 2e-09
VFG0384	yscV - putative membrane-bound Yop protein	70 9e-09	70 9e-09	66 2e-07
VFG0524	fhlA - formate hydrogen-lyase transcriptional activator	70 9e-09	70 9e-09	70 1e-08
VFG0474	phoQ - sensory kinase protein in two-component regulatory system	70 9e-09	70 9e-09	70 1e-08
VFG2350	flgC - flagellar basal-body rod protein	68 4e-08	68 4e-08	68 4e-08
VFG0475	phoP - response regulator in two-component regulatory system	68 4e-08	68 4e-08	68 4e-08
VFG1242	flgI - flagellar P-ring protein precursor	68 4e-08	68 4e-08	68 4e-08
VFG1867	sodB - superoxide dismutase	68 4e-08	68 4e-08	68 4e-08
VFG2533	motB - flagellar motor protein	68 4e-08	68 4e-08	68 4e-08

Results

The type III secretion system in the zebrafish strain is highly homologous to that of the catfish strain of *E. ictaluri*. The identities of the genes in the T3SS system between catfish and zebrafish strains of *E. ictaluri* range from 86.8% to 99.9%. All of the genes that encode T3SS proteins contain less than 10 SNPs with the exception of *eseD*, *eseC* and *esrA*. Each of these three genes in the zebrafish strain includes more than 20 SNPs. Another two genes that encodes EseB and EseG have 7 and 8 SNPs, respectively. The T3SS in the zebrafish strain of *E. ictaluri* is mapped with the SNPs marked in Figure 4.1.

Table 4.3 The identities between the catfish and zebrafish strain of *E. ictaluri* T3SS.

T3SS Product	Function	% Identity at DNA level to catfish strain *E. ictaluri*	No. of non-synonymous SNPs
EsaC	TTSS oligomeric outer membrane secretin	99.9	2
EseG	TTSS effector protein	95.9	8
EscB	TTSS chaperone	98.8	2
EseD	TTSS translocon protein	91.1	24
EseC	TTSS translocon protein	94.2	24
EscA	TTSS chaperone	97.6	3
EseB	TTSS translocon filament protein	86.8	7
EscC	TTSS translocon filament protein	95.5	2
EsaU	TTSS integral apparatus membrane protein	99.1	6
Slt	soluble lytic murein transglycosylase	94.9	4
EsrA	TTSS regulatory sensor kinase	96.2	25

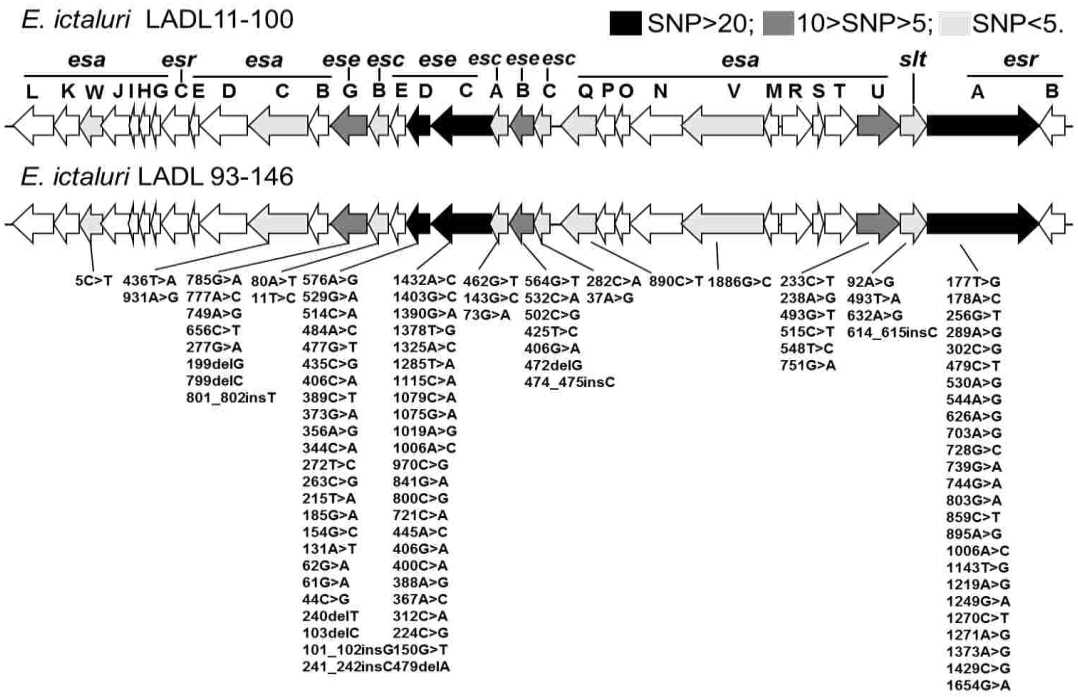


Figure 4.1 The schematic map of the T3SS in zebrafish strain of *E. ictaluri*. The SNPs are labeled with the number indicating the location and in front of the symbol '>' is the DNA sequence in catfish strain and the one after that sign is the DNA in zebrafish strain of *E. ictaluri*.

The putative type IV secretion system varies significantly between catfish and zebrafish strains of *E. ictaluri* in terms of the protein identities. Nineteen putative T4SS genes were checked and 17 of them are found in the putative T4SS gene cluster in the zebrafish strains LADL11-100 and LADL11-194. The putative gene cluster of T4SS in zebrafish strain LADL11-100 is shown in Table 4.4. These genes locate on two contigs of both zebrafish strain draft genomes. See Figure 4.2 for the gene arrangement of the putative T4SS genes in the zebrafish strain LADL11-100 in comparison with the catfish strain putative T4SS gene cluster. Two genes NT01EI_0335 and NT01EI_0338 in the catfish strain are not found in zebrafish strains and two putative genes, labeled as peg 939 and peg 2915, in zebrafish strain are also not found in the catfish strain. The phylogenetic studies of the putative T4SS clusters in the catfish and zebrafish strains indicate the

evolutionary distance between these two strains. Except for NE01EI_0337, all the other genes in the catfish strain putative T4SS cluster are not closely related to the homologous genes in the zebrafish strain of *E. ictaluri* (Figure 4.3 and 4.4). Actually, BLAST results indicates that the corresponding proteins of NT01EI_ 0331 and NT01EI_0332 in the catfish strain, and their homologous proteins in the zebrafish strains are probably proteins that have different functions since they contain different conserved domains (Figure 4.5 and 4.6). The gene NT01EI_0348 and its related gene in the zebrafish strain are unique in *E. ictaluri* with no matching conserved domains from the NCBI website database (Figure 4.7). Zebrafish strains LADL11-100 and LADL11-194 provide identical results in terms of the putative T4SS cluster gene arrangement and similarities to the respective genes in the catfish strain on the DNA level.

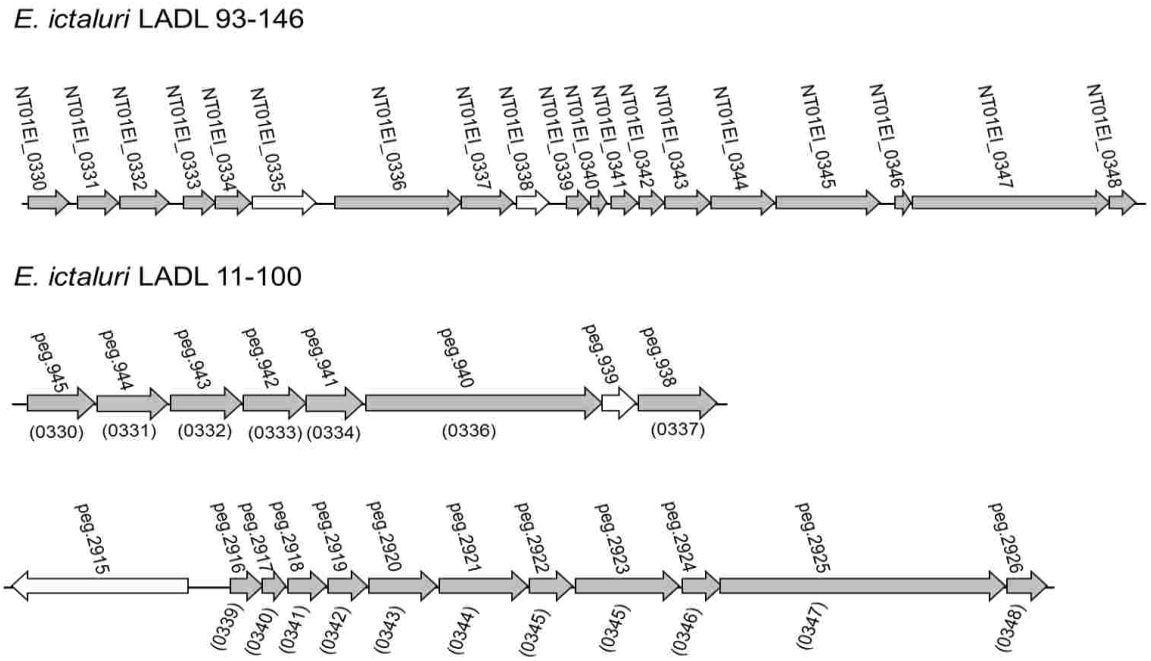


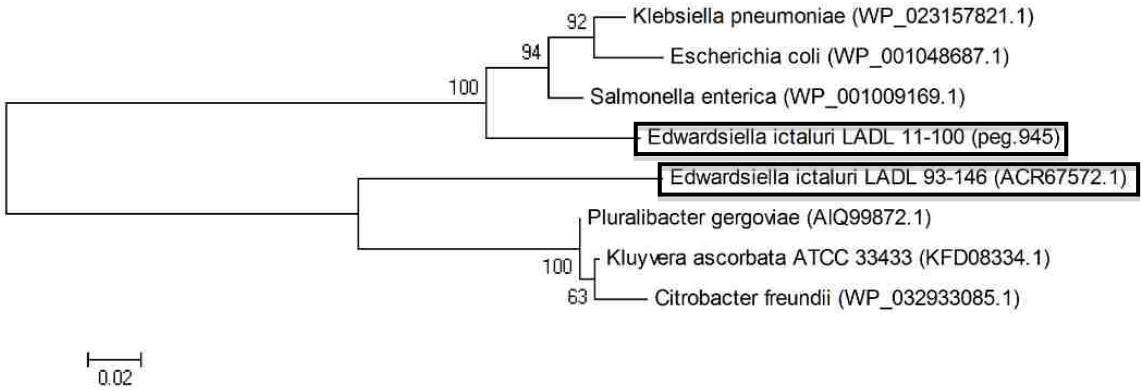
Figure 4.2 The schematic map of the putative T4SS in catfish strain (LADL 93-146) and zebrafish strain (LADL11-100). The genes in grey indicate the genes shared by catfish and zebrafish strains of *E. ictaluri* while the empty arrows indicate the one that is not found in the other strain.

Table 4.4 The putative gene cluster of T4SS in LADL 11-100.

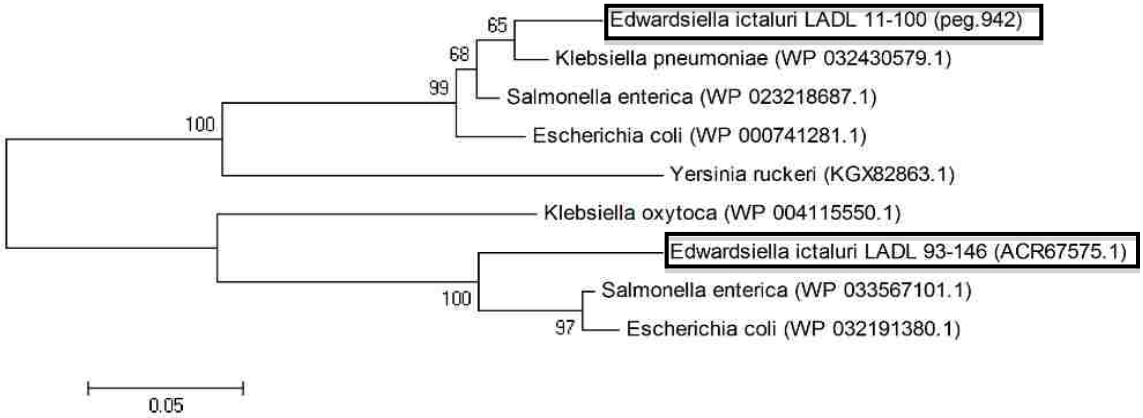
T4SS NT01EI-	Total AA	Putative function	Blast AA in LADL11-100	Location in LADL11-100
0330	198	integrating conjugative element protein PilL	112/175 (64%)	fig|6666666.91609 .peg.945
0331	240	hypothetical protein	36/76 (47%)	fig|6666666.91609 .peg.944
0332	234	conjugal transfer protein	113/209 (54%)	fig|6666666.91609 .peg.943
0333	151	lytic transglycosylase	85/120 (70%)	fig|6666666.91609 .peg.942
0334	170	conjugal transfer protein	72/167 (43%)	fig|6666666.91609 .peg.941
0335	306	hypothetical protein	___	No found
0336	617	hypothetical protein	423/605 (69%)	fig|6666666.91609 .peg.940
0337	251	hypothetical protein	140/229 (61%)	fig|6666666.91609 .peg.938
0338	154	hypothetical protein	___	Not found
0339	113	hypothetical protein	55/94 (58%)	fig|6666666.91609 .peg.2916
0340	80	hypothetical protein	36/78 (46%)	fig|6666666.91609 .peg.2917
0341	128	hypothetical protein	48/93 (51%)	fig|6666666.91609 .peg.2918
0342	118	hypothetical protein	55/107 (51%)	fig|6666666.91609 .peg.2919
0343	229	hypothetical protein	136/219 (62%)	fig|6666666.91609 .peg.2920
0344	309	hypothetical protein	163/294 (55%)	fig|6666666.91609 .peg.2921

Table 4.4 –continued.

T4SS NT01EI-	Total AA	Putative function	Blast AA in LADL 11-100	Location in LADL 11-100
0345	504	hypothetical protein	206/339 (60%); 55/123 (44%)	fig|6666666.91609.peg.2923; fig|6666666.91609.peg.2922
0346	80	hypothetical protein	46/81 (56%)	fig|6666666.91609.peg.2924
0347	954	hypothetical protein	593/950 (62%)	fig|6666666.91609.peg.2925
0348	130	hypothetical protein	55/130 (42%)	fig|6666666.91609.peg.2926

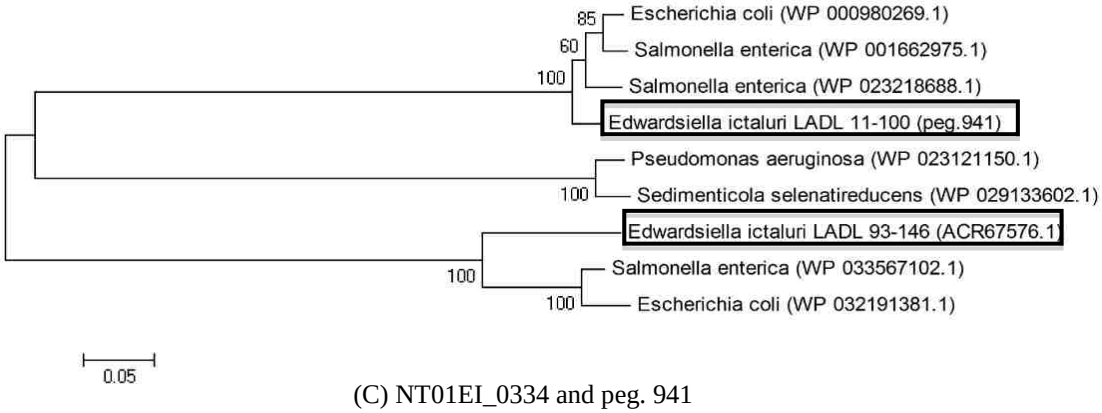


(A) NT01EI_0330 and peg. 945

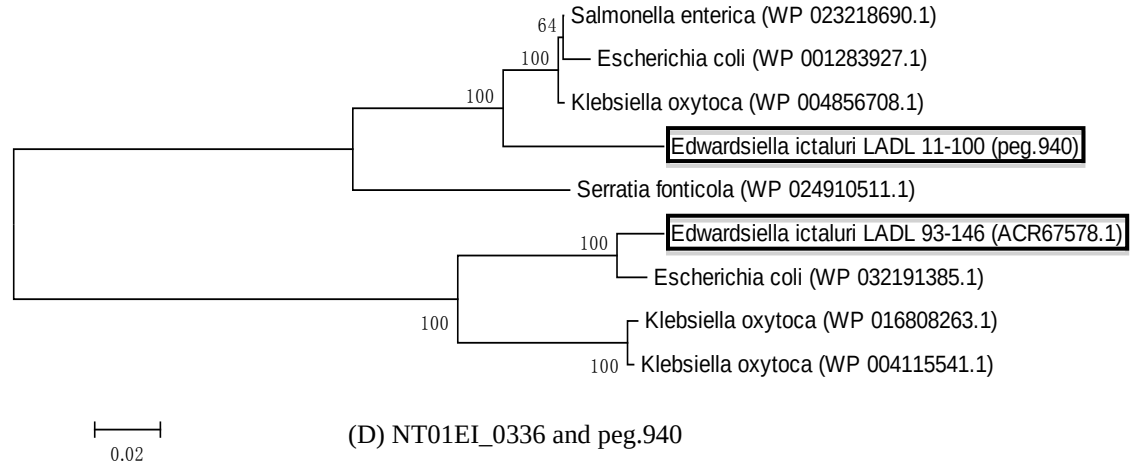


(B) NT01EI_0333 and peg. 942

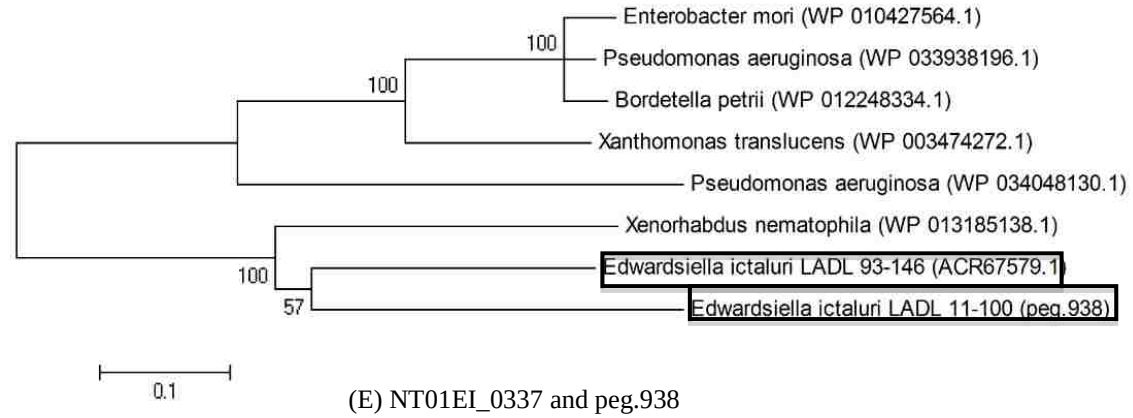
Figure 4.3 The results of phylogenetic analysis on NT01EI_0330, NT01EI_0333, NT01EI_0334, NT01EI_0336 of catfish strain LADL93-146 and their homologous genes in zebrafish strain LADL11-100, cataloged as peg 940, peg 941, peg 942 and peg 945.



(C) NT01EI_0334 and peg. 941

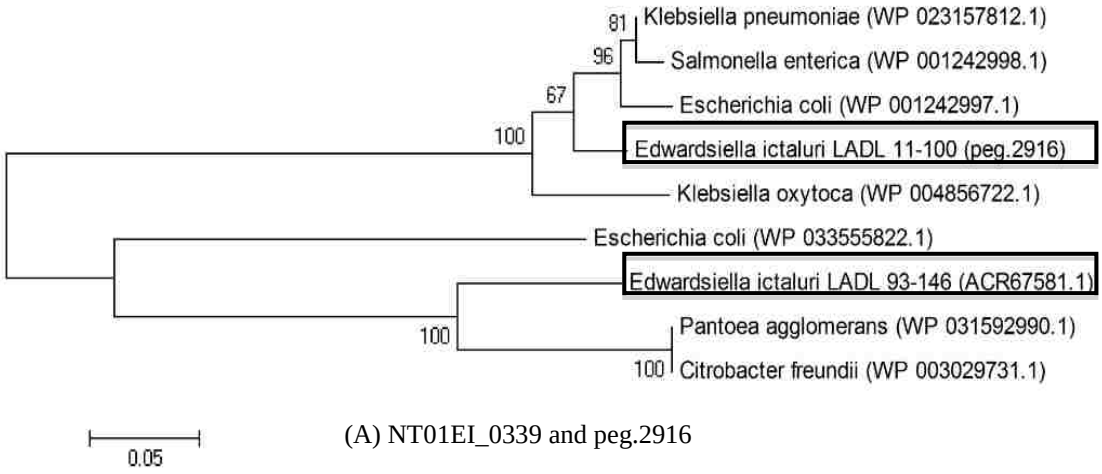


(D) NT01EI_0336 and peg.940

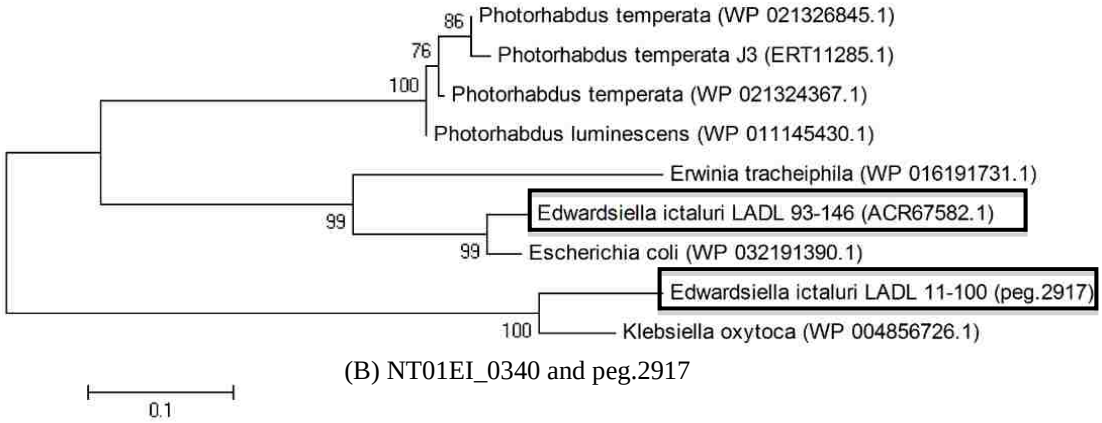


(E) NT01EI_0337 and peg.938

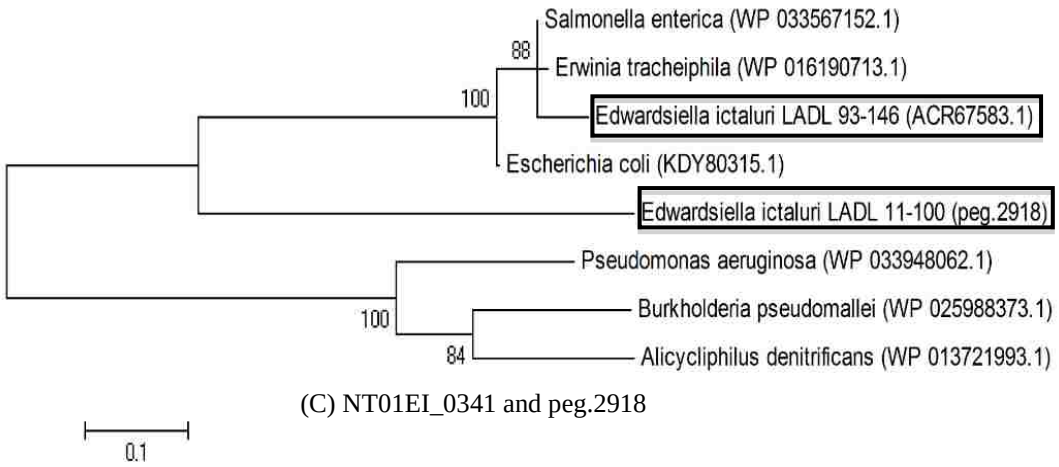
Figure 4.3-continued. The results of phylogenetic analysis on NT01EI_0330, NT01EI_0333, NT01EI_0334, NT01EI_0336 of catfish strain LADL93-146 and their homologous genes in zebrafish strain LADL11-100, cataloged as peg 940, peg 941, peg 942 and peg 945.



(A) NT01EI_0339 and peg.2916

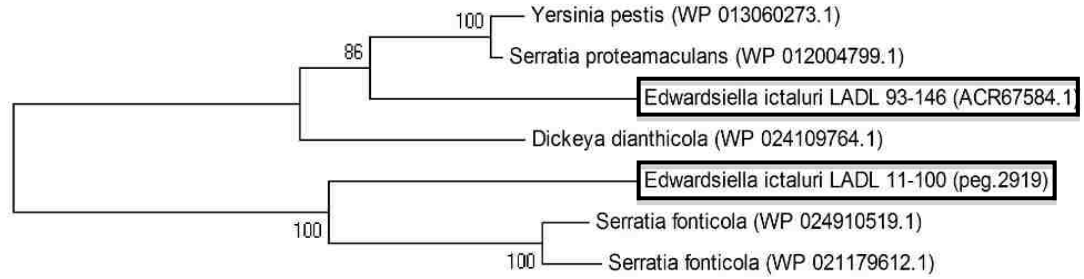


(B) NT01EI_0340 and peg.2917

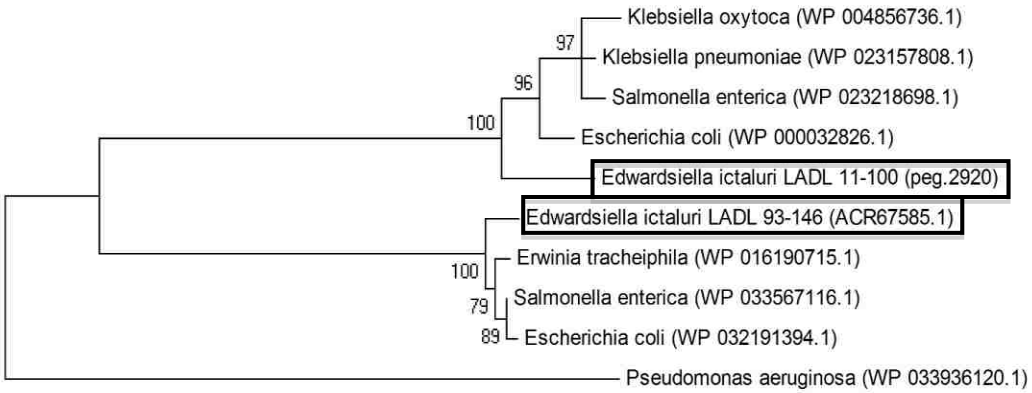


(C) NT01EI_0341 and peg.2918

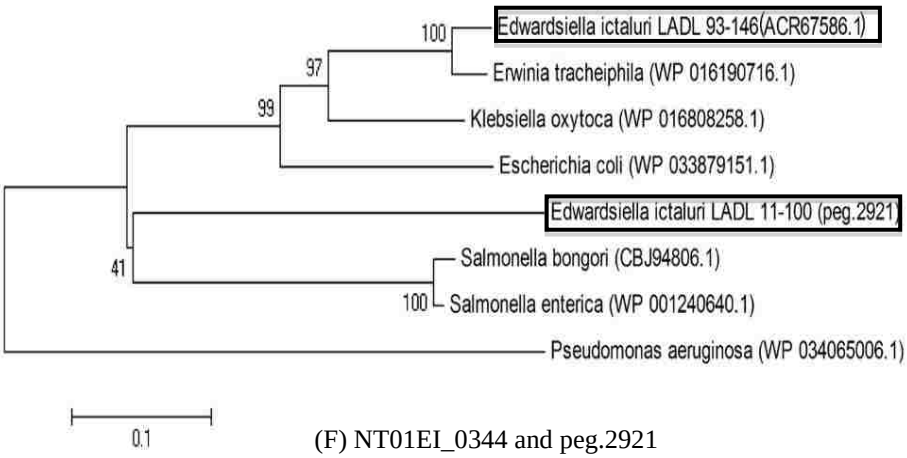
Figure 4.4 The results of phylogenetic analysis on NT01EI_0339 to NT01EI_0347 of catfish strain LADL93-146 and their homologous genes in zebrafish strain LADL11-100, cataloged as peg 2916 to peg 2925.



(D) NT01EI_0342 and peg.2919

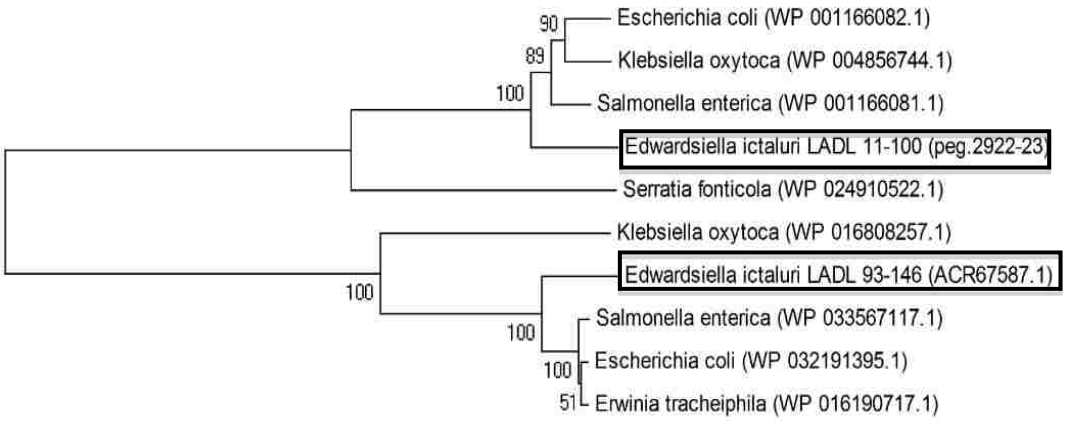


(E) NT01EI_0343 and peg.2920

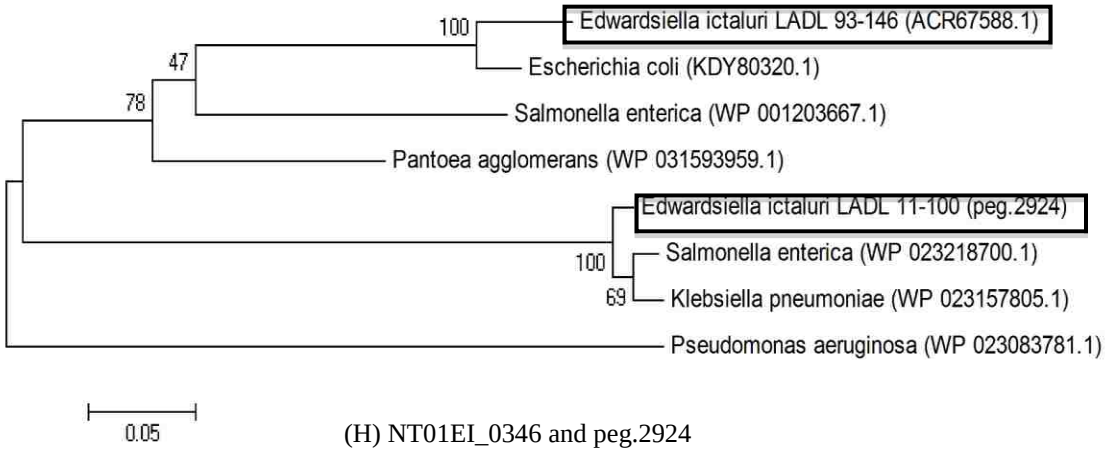


(F) NT01EI_0344 and peg.2921

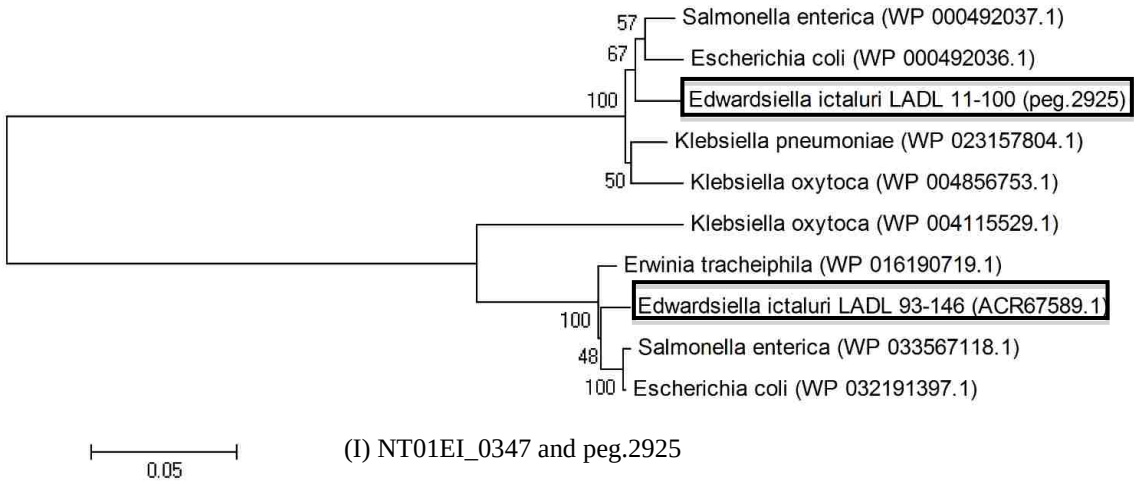
Figure 4.4- continued. The results of phylogenetic analysis on NT01EI_0339 to NT01EI_0347 of catfish strain LADL93-146 and their homologous genes in zebrafish strain LADL11-100, cataloged as peg 2916 to peg 2925.



(G) NT01EI_0345 and peg.2922-23



(H) NT01EI_0346 and peg.2924



(I) NT01EI_0347 and peg.2925

Figure 4.4-continued. The results of phylogenetic analysis on NT01EI_0339 to NT01EI_0347 of catfish strain LADL93-146 and their homologous genes in zebrafish strain LADL11-100, cataloged as peg 2916 to peg 2925.

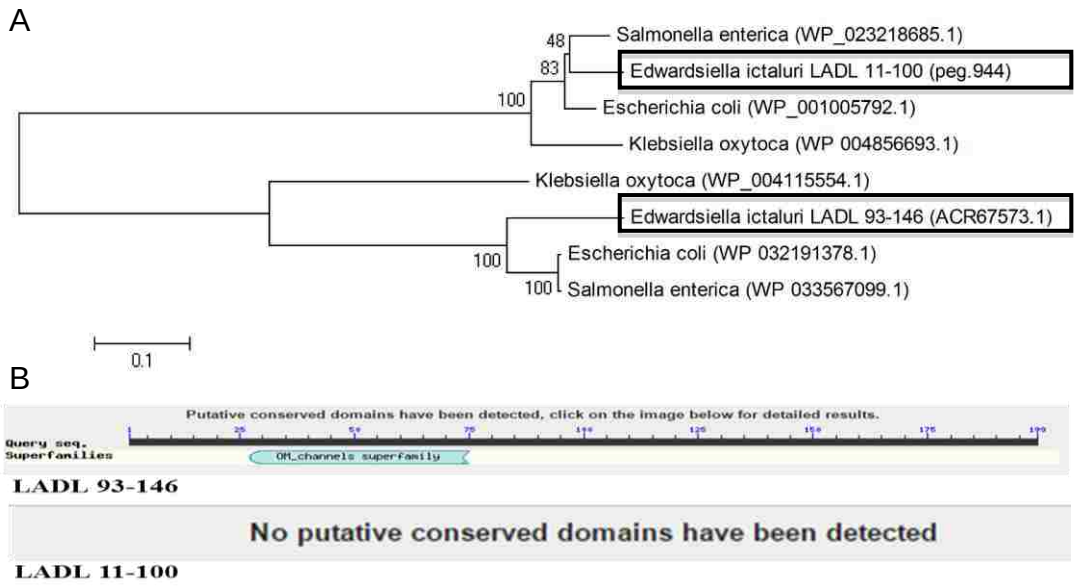


Figure 4.5 The phylogenetic analysis as well as conserved domain detection on protein encoded by NT01EI_0331 of catfish strain LADL 93-146 (A) and its corresponding protein in zebrafish strain LADL11-100 (B).

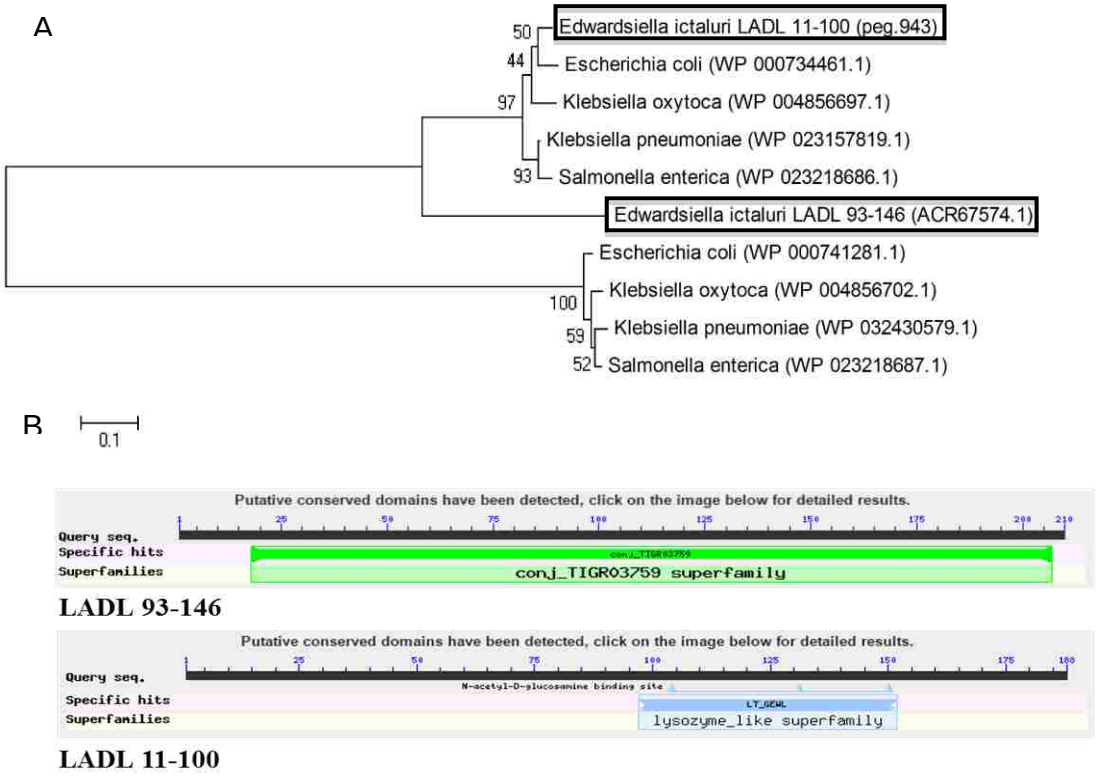


Figure 4.6 The phylogenetic analysis as well as conserved domain detection on protein encoded by NT01EI_0332 of catfish strain LADL 93-146 (A) and its corresponding protein in zebrafish strain LADL11-100 (B).

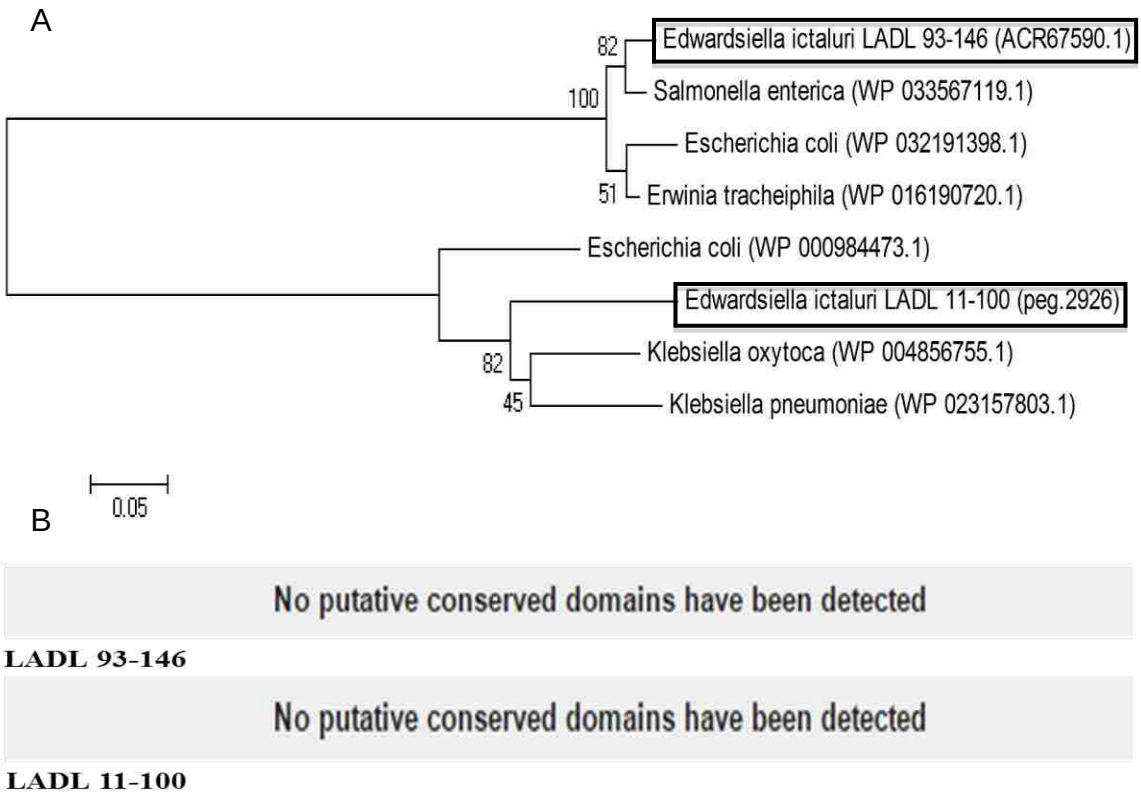


Figure 4.7 The phylogenetic analysis as well as conserved domain detection on protein encoded by NT01EI_0348 of catfish strain LADL 93-146 (A) and its corresponding protein in zebrafish strain LADL11-100 (B).

The T6SS and urease, in zebrafish and catfish strains of *E. ictaluri,* are highly homologous and contain few SNPs. Five genes of the putative T6SS from the catfish strain of *E. ictaluri* are used as templates to find the homologous genes in the zebrafish strain. As shown in Table 4.5, all of the homologous genes in the zebrafish strain contain few SNPs ranging only from one to five. The urease system between catfish and zebrafish strain is more homologous with the overall identity close to 100%. Only one SNP was found in all analyzed nine genes of the urease system between catfish and zebrafish strain of *E. ictaluri* (Table 4.6).

Table 4.5 The putative T6SS in catfish and zebrafish of *E. ictaluri*.

T6SS Product	Putative Function	Locus tag in reference genome	No. of non-synonymous SNPs	Mutation at DNA level	Amino Acid Change
EvpD	hypothetical protein	NT01EI_2740	2	882G>C; 892C>A	294M>I; 298L>I
EvpH	type VI secretion ATPase, ClpV1 family	NT01EI_2744	1	566A>G	189H>R
EvpI	type VI secretion system Vgr family protein	NT01EI_2745	2	236A>G; 748C>G	79Q>R; 250Q>E
EvpO	hypothetical protein	NT01EI_2751	5	861_862 insG; 863delG; 1583A>C; 1710A>G; 3291T>G	FS; FS; 528E>A; 570I>M; 1097H>Q;
Hcp1 family T6SS effector	type VI secretion system effector, Hcp1 family	NT01EI_3420	2	11T>C; 128A>G	4L>P; 43E>G

The SNPs are labeled with the number indicating the location and in front of the symbol '>' is the DNA sequence in catfish strain and the one after that sign is the DNA in zebrafish strain of *E. ictaluri*.

Table 4.6 The putative urease system in catfish and zebrafish of *E. ictaluri*.

Urease PAI Product	Putative function	Locus tag in reference genome	No. of non-synonymous SNPs	Mutation at DNA level	Amino Acid Change
UreA	urea amidohydrolase gamma subunit	NT01EI_2063	0	—	—
UreB	urea amidohydrolase beta subunit	NT01EI_2062	0	—	—

Table 4.6 - continued.

Urease PAI Product	Putative function	Locus tag in reference genome	No. of non-synonymous SNPs	Mutation at DNA level	Amino Acid Change
UreC	urea amidohydrolase alpha subunit	NT01EI_2061	1	967A>G	323I>V
UreE	urease accessory protein E	NT01EI_2060	0	—	—
UreF	urease accessory protein F	NT01EI_2059	0	—	—
UreG	urease accessory protein G	NT01EI_2058	0	—	—
UreD	urease accessory protein D	NT01EI_2057	0	—	—
UreI	urea transporter	NT01EI_2056	0	—	—
AmtB	ammonium transporter	NT01EI_2055	0	—	—

The SNPs are labeled with the number indicating the location and in front of the symbol '>' is the DNA sequence in catfish strain and the one after that sign is the DNA in zebrafish strain of *E. ictaluri*.

Potential structural changes in putative virulence factors due to SNPs. Some of the potentially important virulence factors in the zebrafish strain of *E. ictaluri* contains many SNPs when compare to their homologous genes in the catfish strain. The existence of SNPs can potentially change the structure of these proteins. Examples for that are the regulator in type III secretion system EsrA encoded by the *esrA* gene, the flagellar hook protein FlgE encoded by the *flgE* gene, a fimbrial usher family protein and a permease protein in an ABC transport system (Table 4.7). The amino acid level alignment of the homologous proteins from catfish and zebrafish strain of *E. ictaluri* as well as the predicted structures are shown in Figure 4.8-4.11.

Table 4.7 Examples of potential virulence factors that may have different structures in catfish strain and zebrafish strain of *E. ictaluri*.

Gene product	Chromosome location in LADL 93-146	The size of the gene	No. of non-synonymous SNPs
T3SS regulator EsrA	952369-955104	2736	25
Flagellar hook protein FlgE	1334869-1336143	1275	36
Fimbrial usher protein	1506869- 1509385	2517	8
Permease protein	2059701-2060729	1029	32

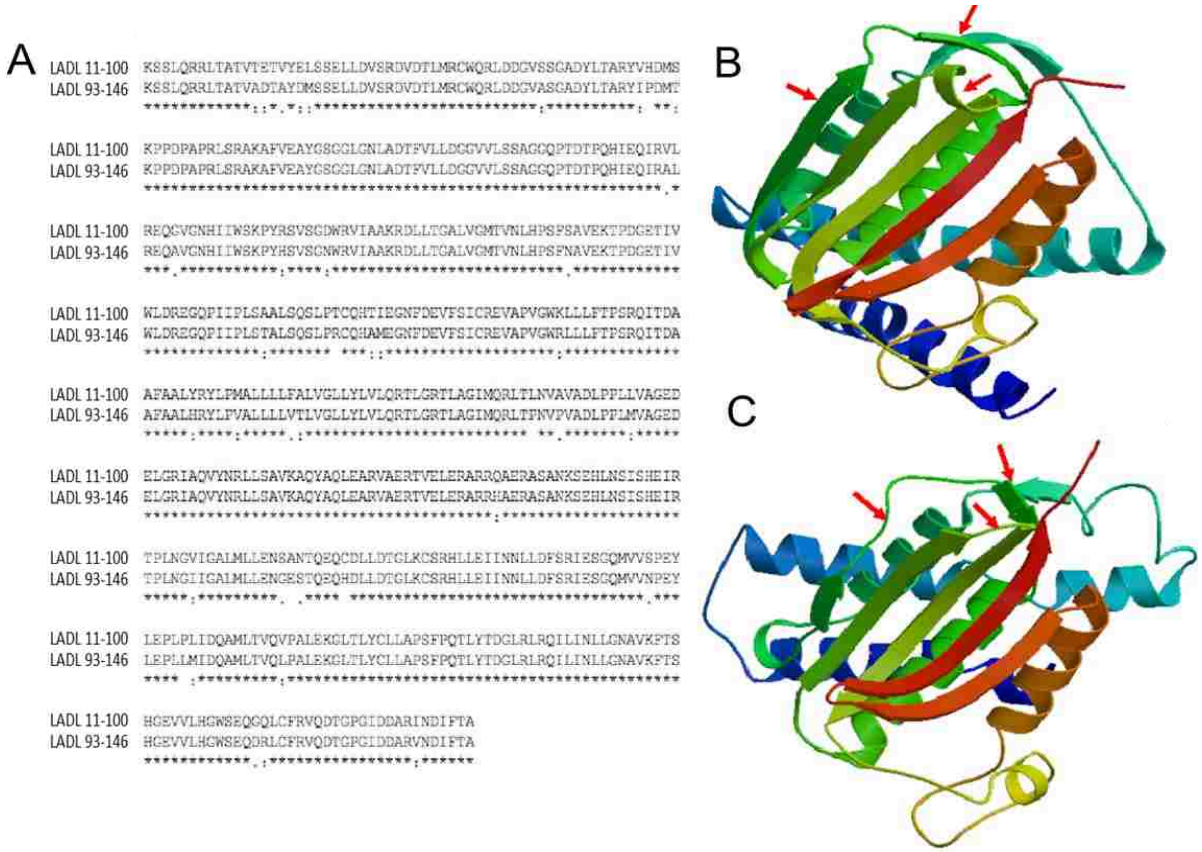


Figure 4.8 The alignment of the amino acids (A) and the structural differences in EsrA between catfish and zebrafish strains of *E. ictaluri*. B: EsrA from catfish strain LADL93-146; C: EsrA from zebrafish strain LADL11-100.

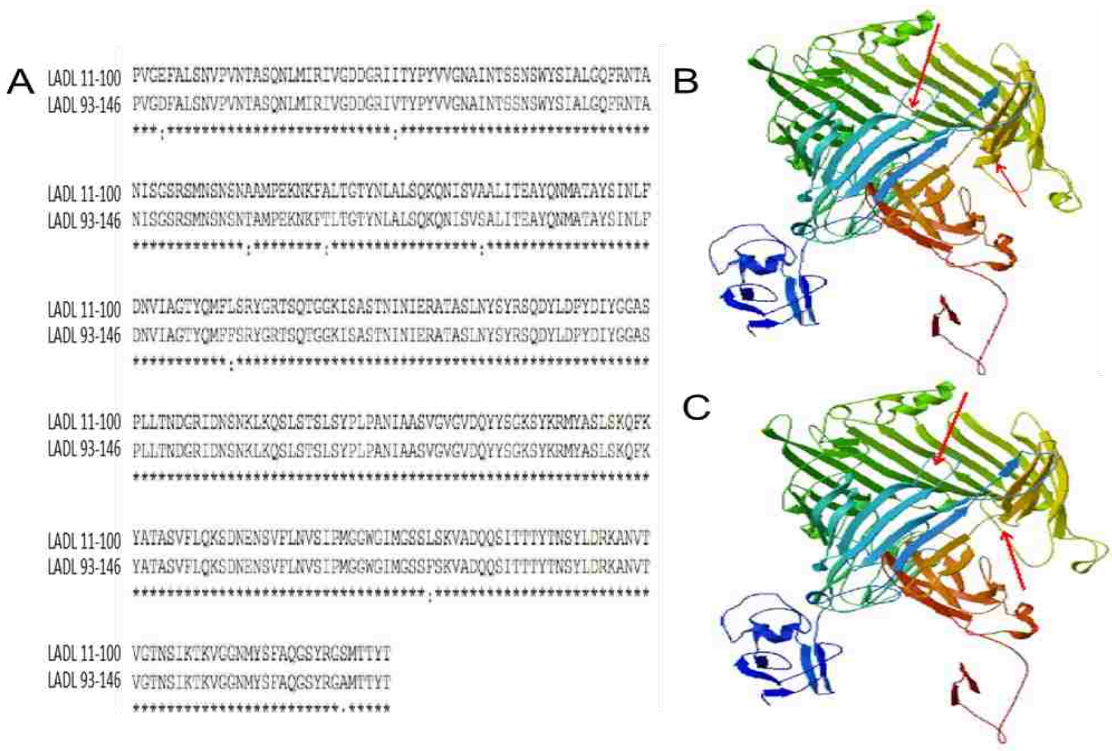


Figure 4.9 The alignment of the amino acids (A) and the structural differences in the fimbrial usher protein between catfish and zebrafish strains of *E. ictaluri*. B: the fimbrial usher protein from catfish strain LADL 93-146; C: the fimbrial usher protein from catfish strain LADL 11-100.

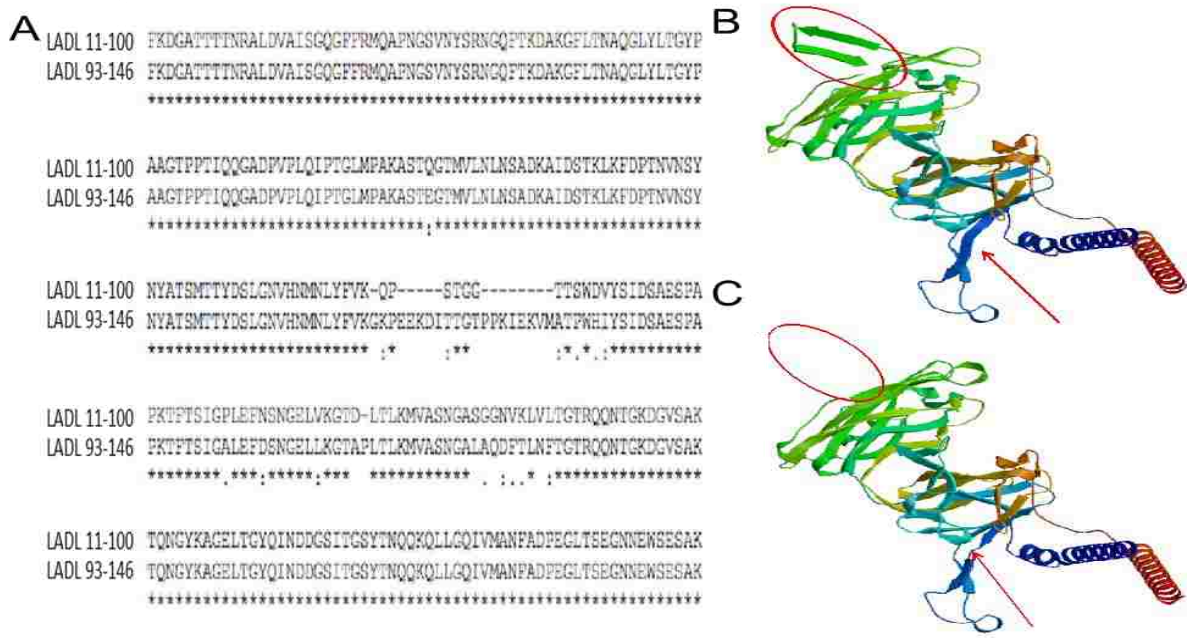


Figure 4.10 The alignment of the amino acids and the structural differences in the FlgE between catfish and zebrafish strains of *E. ictaluri*. A. FlgE from catfish strain LADL 93-146; B. FlgE from zebrafish strain LADL11-100.

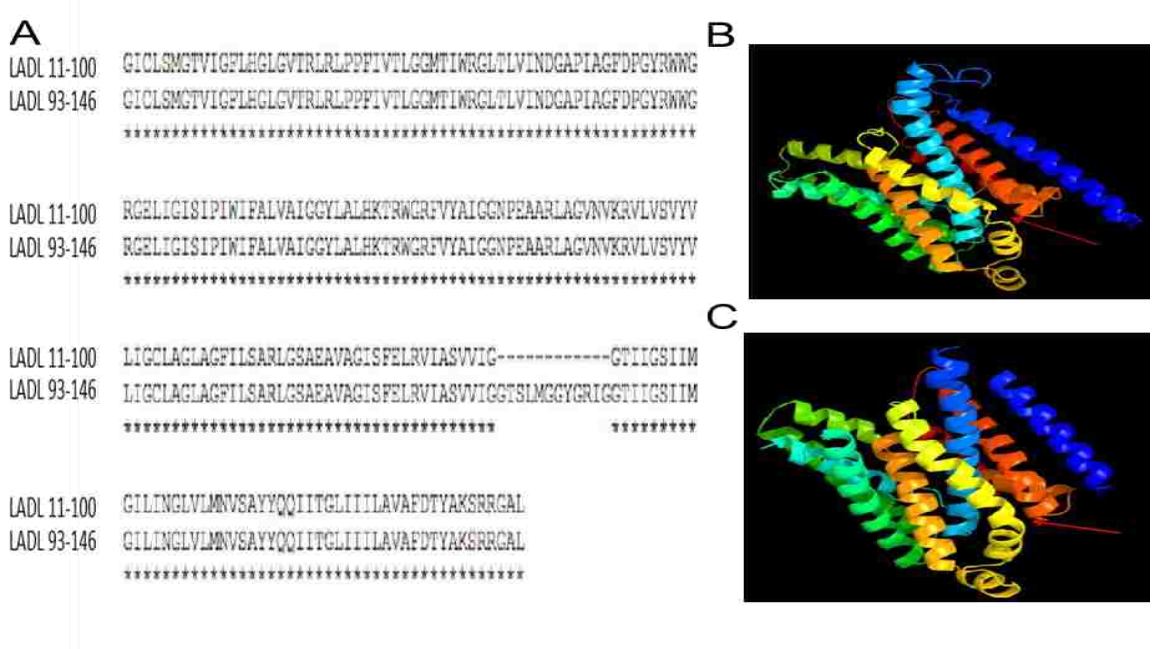


Figure 4.11 The alignment of the amino acids and the structural differences in the permease protein in amino acid or sugar ABC transport system between catfish(A) and zebrafish strains(B) of *E. ictaluri*.

Discussion

Bacteria are equipped with many virulence factors to enhance their pathogenicity. The loss or change of these virulence factors can possibly alter the basic characteristics of these cells, and attenuate them, making them avirulent in certain hosts. Here we present the major differences in the secretion systems, T3SS and T4SS, and the minor variations in the T6SS and the urease system between the catfish and zebrafish strain of *E. ictaluri.*

Since the secretion systems are well-known virulence factors in *E. ictaluri*, especially the type III secretion system, here we compare all the known genes in the T3SS of the catfish and zebrafish strain of *E. ictaluri* [26]. The major structure of the gene cluster in the zebrafish strain is identical to that of catfish strain. The differences between the T3SSs are primarily in the sequences of EsrA and EseCD. EsrA is a regulator of T3SS in the catfish strain of *E. ictaluri* and this protein is also known to

control the expression of a type VI secretion system (T6SS) indicating its cross linked essential functions in terms of pathogenesis [27]. The SNPs in the *esrA* gene can possibly change the structure of this regulator and cause it to lose its function as a regulator or work in a different way. If either of these happens, it will probably cause the alteration of the virulence in the channel catfish and zebrafish.

EseCD, on the other hand, forms the translocon of T3SS together with EseB. This EseBCD complex is later dispersed and secreted. Lacking these proteins can cause the cells to be unable to replicate [28]. In *E. ictaluri*, the secretion of EseCD is increased if the pH is low [26]. When comparing the sequences of EseC and EseD in the catfish and zebrafish strains of *E. ictaluri*, more than twenty SNPs are found in each one of them. These SNPs can potentially make EseCD in the zebrafish strain function differently than their homologous proteins in the catfish strain. Since effectors can have various functions, it is very interesting to further investigate the way EseC and EseD work in the zebrafish strain of *E. ictaluri*. Accordingly, studies in *E. tarda* have shown that EseCD can be related to the autoagglutination property [29]. Thus, it would be very interesting to further investigate whether the SNPs in the *eseCD* genes are the reasons for the character of autoagglutination in the zebrafish strain of *E. ictaluri*.

In addition to the differences in T3SS proteins, we also found more variations between the catfish and zebrafish strain of *E. ictaluri* in the T4SS. The ordering of these proteins in the zebrafish strain is the same as that of the catfish strain except for those unique proteins in each strain. However, most of the proteins in the putative T4SS from catfish strain are found with low identities with their homologous protein in the zebrafish strain. The exact role of T4SS in *E. ictaluri* is not yet clear. Which category this T4SS

belongs to, the conjugation system, the DNA uptake and release system or the effector injection system, remains in question. With the identification of the low overall gene identities and the unique putative T4SS genes in the zebrafish strain, a new world is open to be investigated. If the T4SS works as a conjugation system or the communication system with the surrounding cells, then the differences in these genes can be associated with the variations in the genes that are laterally transferred to the catfish and zebrafish strains of *E. ictaluri*. However, if this is the system that injects effectors into the host, then the differences we found may indicate the variations in the effectors, which can also affect the virulence of the strain due to the functional change of effectors. Further research is needed to confirm the exact function of this T4SS but the differences we found between the strains indicate a potential for significantly different effects of this system with zebrafish and catfish strains of *E. ictaluri*.

Few SNPs are located in the T6SS and the urease system. This possibly emphasizes the essentiality and accuracy of these systems. Studies have shown that T6SS genes are activated when the cell occupies a phagosome-like environment and it is required for virulence. The urease system in the catfish strain of *E. ictaluri* is required for intracellular replication and virulence by altering the pH in the phagolysosome [18, 27, 30].

In this study, many SNPs are observed in the genes that encode potential important virulence factors, including the fimbrial usher protein, the flagellar hook protein and ABC transporter proteins. Fimbriae are essential for bacterial adhesion and invasion to host cells and they have been studied in many bacteria species [31-33]. The structural changes in the fimbriae usher family protein can potential affect the biosynthesis of

fimbriae and thus lead to impaired ability to adhere to and invade host cells. The predicted changes in the structure of FlgE, which is the flagellar hook protein, indicated the potential influence in the motility. This is in accordance with the weaker motility in glucose motility deeps (GMD) [19].

The ABC transporter can be involved in the transportation of surface glycoconjugates [34]. Thus, the differences in the ABC transporter genes can possibly affect the export of certain glycans and may affect the surface structure of the cell. Since the surface structures are very important for host cell recognition, the SNPs in the ABC transporter proteins can be part of the reason for host specificity as well.

To summarize, here we identified the putative T3SS, T4SS, T6SS and the urease system in zebrafish strain of *E. ictaluri*. The ordering of the genes in these systems are basically the same as those in catfish strain. However, relatively low levels of similarity due to a number of SNPs are found in *esrA*, *eseCD* and the T4SS genes. All of these may contribute to the host specificity and virulence of *E. ictaluri*.

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CHAPTER V. ATTENUATION OF THE ZEBRAFISH STRAIN OF *EDWARDSIELLA ICTALURI* BY MUTATING THE *UREG* AND *ESRC* GENES

Introduction

The zebrafish *Danio rerio* is a small tropical freshwater fish in the minnow family (*Cyprinidae*) that has become an important vertebrate used in biomedical research, including studies in genetics, developmental biology, human diseases and pharmacology [1-8]. The characteristics of zebrafish that make them a pre-eminent model for these studies are their small size, regenerative abilities, optical transparency at early stages and high genetic similarity to human beings [6, 8, 9]. Given their key roles in biomedical research as well as broad acceptance in the tropical aquarium pet fish trade, diseases in zebrafish can lead to significant economic losses. Thus, disease control in zebrafish becomes quite important in both highly inbred genetic strains or wild-type pond raised fish for the aquarium trade.

Prior to 2011, acute bacterial disease was not known to cause mortality in laboratory colonies or pond populations of zebrafish. *Edwardsiella ictaluri,* the causative agent of enteric septicemia of catfish (ESC) [10], was believed to be very host specific for catfish species until 2011 when the bacterium was diagnosed as the cause of significant mortality in laboratory populations of zebrafish from four states in the U.S., Louisiana, Massachusetts, Pennsylvania and Florida [11].

Stress is an important factor that can predispose fish to health problems resulting from loss of homeostatic balance with their environment. Under prolonged or repeated stress fish can succumb to opportunistic infections. [12, 13]. Thus, maintaining a stable and favorable environment for fish is essential for preventing disease in fish. What's

more important is avoidance of potential pathogens by the practice of good biosecurity in the aquaculture facility. One of the common sources of pathogenic bacteria is newly introduced fish into a facility. Thus, quarantine should be practiced as a routine measure at facilities to prevent bacteria being introduced into the main facility by new introductions. Another way to prevent disease outbreak is the "eggs only" methods where only the disinfected eggs are brought to the facility. For biosecurity, any zebrafish that is infected with *E. ictaluri* should be euthanized to prevent further outbreaks in the zebrafish populations. In cases where highly valuable populations of zebrafish in research laboratories were affected, antibiotic medicated feeds were used to try to control the disease leading to variable results. In many cases the fish were euthanized and the systems depopulated and disinfected. It has been shown that the zebrafish strain of *E. ictaluri* is susceptible to Romet®, oxytetracycline, florfenicol and enrofloxacin [10]. Further research is needed to find the best way to prevent *E. ictaluri* infection in zebrafish populations. We propose vaccination will be an important method of disease prevention in zebrafish populations in the future.

The zebrafish strain shares many characteristics with the typical catfish strain, including most of the genome sequences (see chapter II), and biochemical phenotype with the exception of being positive in the citrate utilization test. Differences include: a different plasmid profile, lack of LPS recognition by Mab Ed9, weak or lack of motility and autoagglutination in broth [11]. Our thought, however, is that zebrafish strains have surface antigens that are different from the catfish strain and therefore a zebrafish strain must be used as the parent strain in the design of a live attenuated vaccine. Therefore, in order to control edwardsiellosis in zebrafish populations, we explored the development of

live attenuated vaccines that can be applied by immersion. The vaccines were created by mutating genes that are homologous to known virulence factors of the catfish strain in the zebrafish strain of *E. ictalu*ri.

The catfish strain of *E. ictaluri*, like most gram-negative bacteria in the family *Enterobacteriaceae,* encodes a type III secretion system (T3SS) which transfers virulence factors to the host, thus is very important in terms of pathogenesis. Different subsets of proteins work corporately to contribute to the pathogenesis. Some are the secreted proteins, known as effectors and some function as structural proteins to form the translocation apparatus or the pore forming structure to facilitate effectors being injected into the host cells [14, 15]. All of these proteins are controlled by by the regulators which are usually encoded in the same gene cluster. In *E. ictaluri*, specific regulators, named EsrA, EsrB and EsrC, regulate this T3SS. Mutagenesis studies have shown that an *esrC* mutant is able to replicate inside the host cell, but is avirulent, whereas *esrA* and *esrB* mutants cannot replicate inside the macrophage and are also avirulent [16]. In addition, *esrC* also functions to co-regulate another protein EvpC, which is part of the type VI secretion system [16]. Previous studies in channel catfish with the catfish strain combined with the high homology at the genome level with the zebrafish strain provide us with a clue that a mutation in the zebrafish strain *esrC* might be a good vaccine candidate. To test that, we mutated *esrC* gene in zebrafish strain and examined the virulence of the mutant in zebrafish.

Another important system in facultative intracellular bacteria to facilitate bacterial survival in the host is the urease system. Urease is the first enzyme in the world that was crystallized and was characterized as a metalloenzyme that contains nickel [17, 18]. This

enzyme is a known virulence factor in many pathogenic bacteria [19-23] and among those, the urease system in *Helicobacter pylori* has been the most studied. In order to survive in the acidic environment of the phagolysosome in the macrophage, bacteria can import urea into the cell and by the action of the urease enzyme, urea is hydrolyzed to ammonia and carbamic acid. The carbamic acid can spontaneously decompose into carbonic acid and another ammonia molecule [24]. Since ammonia is a basic molecule, this causes an increase the pH of the environment and a gain in bioenergetics for growth. Bacteria like *H. pylori* can activate their urease system to increase the pH in the cytoplasm and buffer the pH in the periplasm, thus survive in the acidic environment [25, 26]. In the catfish strain of *E. ictaluri,* it has been proven that bacteria can alter the pH of the phagolysosome in head kidney derived macrophages by the urease system along with arginine decarboxylase. This modulation of pH is required for intracellular replication of the bacterial cells [27, 28]. Moreover, generating more ammonia can lead to severe cytotoxic effects in the surrounding epithelium cells [29, 30]. Studies have shown that urease in the catfish strain of *E. ictaluri* is required for intracellular replication as well as virulence.

Accessory proteins are required for *in vivo* activation of the urease system [26]. The catfish strain of *E. ictaluri* has a gene cluster that encodes a urease system which includes UreA, UreB, UreC, UreE, UreF, UreG, UreD, and UreI, followed by an ammonium transporter. Of those, UreA, UreB and UreC are primary enzymatic subunits that form the apoenzyme while UreD, UreE, UreF and UreG are the accessary proteins. Mutation in the *ureG* gene of the catfish strain can reduce its virulence significantly in the channel catfish and make it avirulent by immersion challenge [31].

Based on the knowledge of the functions of the virulence factors in the catfish strain of *E. ictaluri*, and the close relationship between the catfish and zebrafish strains, we decided to focus on the *esrC* and *ureG* genes to attenuate the zebrafish strain of *E. ictaluri* for developing potential vaccines to prevent edwardsiellosis in zebrafish.

Materials and Methods

Bacterial strains, plasmids, media and reagents. Bacterial strains and plasmids used in this study are listed in Table 5.1. All *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB plates at 37 ℃. If necessary, antibiotics were added at the following concentrations: ampicillin (Amp) 200 μg ml^{-1}, colistin (Col) at 10 μg ml^{-1}. 5% sucrose was used when needed. The two *E. coli* strains used in this study were CC118 *λpir* for maintaining plasmids and SM10 *λpir* for conjugation. For proper growth, *E. ictaluri* was cultured in Bacto brain-heart infusion (BHI) broth at 28 ℃. Plasmids were isolated from the bacterial cultures using QIA Spin Miniprep Kit (Qiagen Inc., Valencia, CA.) and were further purified by QIAGEN MinElute Gel Extraction Kit (Qiagen Inc., Valencia, CA.).

Table 5.1 Bacterial strains and plasmids used in Chapter V.

Strains or plasmids	Description	Source
Bacterial strains		
E.coli CC118 *λ pir*	Δ(*ara-leu*) *ara*D Δ*lac*X74 *gal*E *gal*K phoA20 thi-1 *rps*E *rpo*B *arg*E (Am) *rec*A1 λpir lysogen	[31]
E. coli SM10 *λ pir*	*thi*1 *thr*1 *leu*B *sup*E44 *ton*A21 *lac*Y1 *rec*A-::RP4-2- Tc::Mu Kmr λ::pir	[32]
Edwardsiella ictaluri LADL 11-100	Zebrafish strain of *E. ictaluri* isolated from a diseased zebrafish from a natural outbreak at LSU in 2011	LSU Louisiana Aquatic Diagnostic Laboratory
Edwardsiella ictaluri LADL 12-140	Zebrafish strain of *E. ictaluri* isolated from a diseased zebrafish from University of Massachusetts Amherst	LSU Louisiana Aquatic Diagnostic Laboratory

Table 5.1-continued.

Strains or plasmids	Description	Source
Δ*esrC*	11-100 with 70-402 bp of *esrC* deleted	This study
Δ*ureG*	11-100 with 153-495 bp of *ureG* deleted	This study
Plasmids		
pRE107	Plasmid suicide vector for allelic exchange, pGP704 derivative	[33]
pRE*::*Δ*esrC*	pRE107 with Δ*esrC* inserted at *Kpn* I and *Xba* I	This study
pRE*::*Δ*ureG*	pRE107 with Δ*ureG* inserted at *Sal* I and *Sac* I	This study
pRE*::ureG*	pRE107 with *ureG* inserted at *Sal* I and *Sac* I	This study
pBBR1-MCS4	Broad-host-range cloning vector	[34]
pBBR*::esrC*	pBBR1-MCS4 with *esrC* inserted at *Xba* I and *Xho* I	This study

Specific pathogen free (SPF) Zebrafish *Danio rerio*. Zebrafish were obtained from sources with no history of *E. ictaluri* outbreaks. After introduction of the fish to the laboratory, a complete necropsy was performed on a sample of fish insure they were *E. ictaluri* negative. For verification purposes, a sample (10 fish) of the SPF fish were challenged with wild type *E. ictaluri* to confirm that they were susceptible to *E. ictaluri* infection. The live fish were separated into groups of 10, then placed in 20 L tanks with free flowing water, while maintained at a constant temperature of 26 ± 2 ℃. All fish were fed at 2% of their body weight daily and acclimated for 2 weeks before starting the experiment.

SPF channel catfish. Channel catfish egg masses were obtained from a facility with no ESC outbreak history. The egg masses were hatched in a closed recirculating aquaculture system at the specific-pathogen-free (SPF) aquatic laboratory at the LSU School of Veterinary Medicine after disinfected with 100 ppm free iodine. Fish were fed at a rate of 2-3% of their body weight twice a week before the challenge and 1% of their body

weight once a day after the challenge. All catfish were between 15-20 g when exposed to *E. ictaluri* LADL11-100.

Generation and verification of *E. ictaluri* 11-100 mutants. Genomic DNA was isolated from the zebrafish strain of *E. ictaluri* using High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN). Primers P1 and P2 were used to amplify the whole *ureG* or *esrC* gene together with upstream and downstream sequences of the respective gene for recombination. The PCR products were sequenced for confirmation before manipulations. Subsequently, P3 and P6 primers were used to amplify upstream sequences for homologous recombination and P4 and P5 primers for downstream sequences. Both PCR products were purified, digested with *Xba* I or *EcoR* I and then ligated together resulting in one DNA piece with the gene of interest deleted. These ligation products were used as templates for PCR to obtain more replicates for further digestion and ligation. The PCR products were digested with restriction enzymes *Sal* I and *Sac* I accordingly, for constructing the *ureG* mutant and *Kpn* I and *Xba* I for constructing the *esrC* mutant. Plasmid pRE107 was extracted from *E. coli* cells and digested with either set of enzymes concurrently. All digested products were purified and the insertional pieces were ligated to previously digested pRE107 to create pRE::Δ*ureG* and pRE::Δ*esrC.* These plasmids were electroporated into *E.coli* CC118 *λ pir* for maintaining the plasmids and the insertional sequences were confirmed by sequencing before transforming these plasmids to *E. coli* SM10 *λ pir* cells for conjugation.

Mid-log phase of *E. coli* SM10 *λ pir* cells carrying pRE::Δ*ureG* or pRE::Δ*esrC* and the zebrafish strain of *E. ictaluri* LADL11-100 were mixed and filtered through a metrical membrane disc filter (Pall corporation, Ann Arbor, MI). After overnight

incubation at 28 ℃, cells were harvested from the filter and were then plated on LB plates with Man, Col and Amp added to stimulate the plasmids entering *E. ictaluri* cells and promotion of a single crossover event. Selected cells were then passed on LB with Col plates and LB with Col and sucrose plates to facilitate the second crossover. Finally, the colonies were selected and tested by PCR and sequencing to confirm the constructs of the mutants.

Table 5.2 Oligonucleotide sequences used for mutagenesis of *ureG* and *esrC* genes in the zebrafish strain of *E. ictaluri*.

Primer	Primer type	Sequences	Source
ureG sF	P1	5'-CGACAGCATCTTATCTTGCCTGAC-3'	This study
ureG sR	P2	5'-CATACCTCTCGGGCTAACTTCCA-3'	
ureG fF	P3	5'-ATATATGTCGACCGAGAGTGCCGATGCGGAAT-3'	
ureG fR	P4	5'-ATATATGAGCTCCCTTAAAGCAGGCGGCGAGT-3'	
ureG mF	P5	5'-ATATATTCTAGATGGAGAGTGACACCAAGGTAG-3'	
ureG mR	P6	5'-ATATATTCTAGACCTGCTTAGCATCTTCAGTGGT-3'	
esrC sF	P1	5'-GCATCAGCCTCACTACGCC-3'	
esrC sR	P2	5'-CCAAAGGCAGCGGGTAT-3'	
esrC fF	P3	5'-TATATAGGTACCCGTCTGCAACGATACGCT-3'	[16]
esrC fR	P4	5'-TATATATCTAGACCATTGTTGATGAGGGCC-3'	
esrC mF	P5	5'-TATATAGAATTCCACTTCAGTCAGTCGCCA-3'	
esrC mR	P6	5'-TATATAGAATTCCAGCCTGAGCATGGTTTC-3'	

Primer type P1 and P2 are designed for verification of constructs. Sequences 'GTCGAC', 'GAGCTC', 'TCTAGA', 'GGTACC' and 'GAATTC' indicate *Sal* I, *Sac* I, *Xba* I, *Kpn* I and *Eco*R I site respectively. Underlined sequences are linkers incorporated into the primers used for cloning.

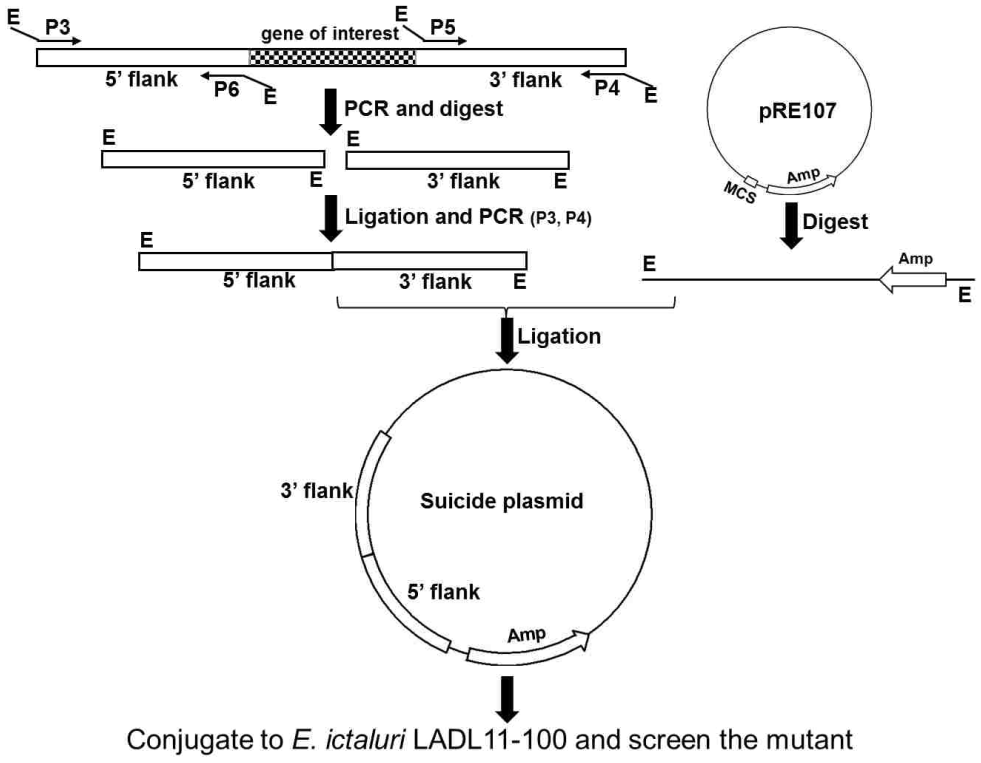

Figure 5.1 Schematic descriptions of the construction of Δ*esrC* and Δ*ureG*.

Complementation of Δ*esrC* and Δ*ureG*. To complement Δ*esrC*, wild type *esrC* gene from the zebrafish strain and 214 bp upstream were amplified to insure the inclusion of the promoter of *esrC*. Primers used for amplification were synthesized based on earlier studies of the catfish strain. Forward primer sequences is 5'-TTTAATTTCTAGAATCGACTGCCTCAATGACGC-3' and reverse primer is 5'-TTTAATTCTCGAGACCGTGACCATGTTTAGGCG-3' [16]. This PCR product, as well as the cloning vector pBBR1-MCS4, were then digested with *Xba* I and *Xho* I before ligation to create pBBR::*esrC*. This plasmid was further transformed into Δ*esrC* by conjugation and the existence of the plasmid confirmed by observing the third plasmid in a gel.

Primers *ureG* fF and *ureG* fR were used to amplify the *ureG* gene. The PCR product was digested with *Sal* I and *Sac* I and inserted into plasmid pRE107 that had been previously digested with the same enzymes. The resulting plasmid, pRE*::ureG*, was used for conjugation and through homologous recombination, Δ*ureG* is reverted back to the wildtype. The final construct was confirmed by PCR and sequencing afterwards. Detailed steps are the same as we described earlier in "Generation and verification of *E. ictaluri* mutants" section.

LD_{50} assays for two zebrafish strains in zebrafish and channel catfish. Zebrafish strain LADL 11-100 and LADL 12-140 were grown in BHI broth for 18 hours at 28 ℃ and cells were enumerated by making serial dilutions of the original cultures and counting colonies on blood agar (BA) plates using the drop plate method. For experimental challenge in catfish, 8ml of original culture was diluted in 72 ml 1*PBS for 8 times to make 9 different dilutions of 80 ml each. The water level in the tank was lowered to 4L and 80ml of broth dilutions were poured into each tank. Catfish were immersed for 2 hours with aeration maintained. Mortalities were recorded daily for 21 days.

For challenge in zebrafish, the same 18-hour *E. ictaluri* culture was serially diluted 1:10 with 900ml tank water in 1.9 L buckets to achieve 9 different dilutions of this culture including the original culture from approximately 1×10^9 CFU/ml to 1×10^1 CFU/ml. Ten zebrafish from each tank were immersed in each bucket. After 2 hours, the water level in the tank was lowered to 4 L and the broth dilutions and zebrafish were poured back to each tank. Mortalities were recorded for 21 days.

LD_{50} assays of Δ*esrC* and Δ*ureG* in zebrafish. Broth cultures were inoculated, incubated for 18 hours at 28 ℃ and a series of 1:10 dilutions of the broth were prepared in 1.9 L buckets. Nine different concentrations of each strain including the undiluted broth, were included in the experimental design. Cells were enumerated by counting colonies on BA by the drop plate method with three replicated drops taken from each dilution. Ten fish were then transferred to each bucket for immersion for 2 hours with aeration maintained throughout the exposure. Following the challenge, fish were transferred to 20 liter tanks with flow through water conditions. Mortalities were recorded daily for 21 days and brain samples were collected from all dead fish to confirm *E. ictaluri* infection.

Experimental challenge with *ureG* or *esrC* mutant, their complemented strain and wild type *E. ictaluri* in zebrafish *Denio rerio*. To assess the attenuated virulence of both *ureG* and *esrC* mutants and to confirm attenuation was the result of the targeted mutations and not accidental mutations in the cell, five strains, including the wild type strain, both mutants, and their complemented strains were used to challenge zebrafish. Cells were grown in BHI broth for 18 hours at 28 ℃. Based on the LD_{50} of the wild type strain, 2 ml of each culture was added to 998 ml tank water for each group of 10 fish. There were 4 replicate tanks for each challenge with each bacterial strain. Mortalities were recorded for 21 days.

Results

Construction of *esrC* and *ureG* mutants. Both mutants, Δ*esrC* and Δ*ureG*, were achieved by allelic exchange. In Δ*esrC*, 333 bp internal sequences out of 693 bp were removed from wildtype *esrC* gene. This deleted region was the same region that was

removed previously in the catfish strain resulting in loss of virulence [16]. Previous studies indicate that the deletion of these DNA sequences can remove amino acid 101-211 of EsrC which covers at least one of the helix-turn-helix domain and most of the other helix-turn-helix domain, so this mutation can significantly reduce the virulence of the catfish *E. ictaluri* strain [16, 36]. In Δ*ureG*, 339 bp out of 630 bp were removed from wild type *ureG* gene. Removal of amino acids 152-264 eliminate part of the potential DNA-binding domain.

The zebrafish strains LADL12-140 and LADL 11-100 are virulent in zebrafish but not in channel catfish by immersion exposure. The number of bacterial cells was enumerated by making a series of dilutions of the BHI broth culture, plating on blood agar plates by the drop plate method, and counting colonies following overnight culture at 28 ℃. The original concentrations of LADL 12-140 and LADL 11-100 in the broth culture were 4.3 x 10^9 CFU/ml and 1.4 x 10^9 CFU/ml, respectively. Death was first observed on the sixth day post challenge. Sixteen days after challenge, 100% mortality was observed from three groups that were treated with highest dose of bacteria. The cumulative mortalities for all groups are shown in figure 5.2. In contrast, there was no death for a 21-day period post challenge in the catfish group even including the group that was challenged with the undiluted overnight bacterial culture (see Figure 5.3).

Zebrafish strain *esrC* and *ureG* mutants are attenuated by immersion exposure in zebrafish. The original concentrations of bacteria in overnight BHI broth cultures were determined to be 1.7 x 10^9 CFU/ml for Δ*esrC* and 3.8 x 10^9 CFU/ml for Δ*ureG.* All tanks were checked daily post challenge for mortalities but no zebrafish from these groups died during the 21-day challenge period. Data are shown in figure 5.4 and 5.5.

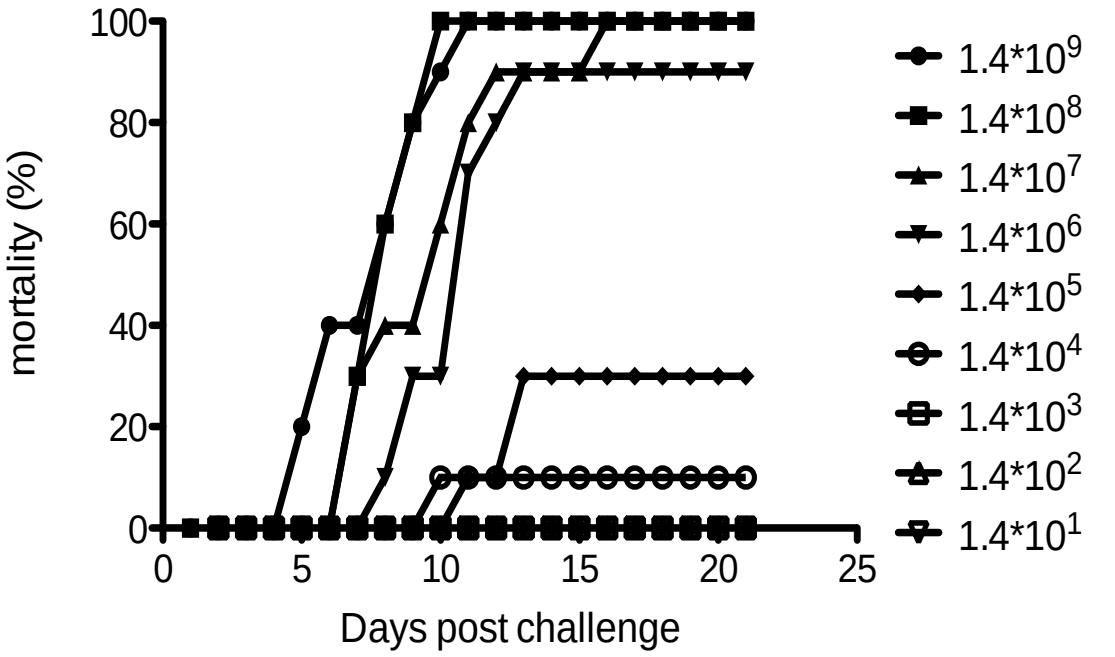

Figure 5.2 Cumulative mortality of zebrafish challenged with wild type zebrafish strain of *E. ictaluri*.

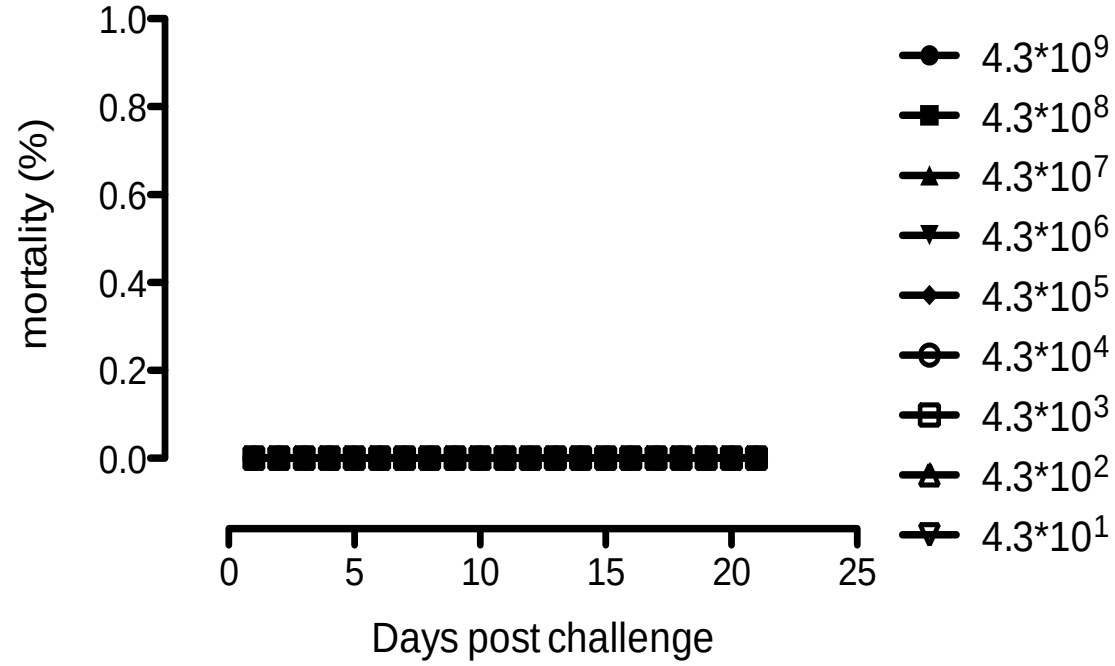

Figure 5.3 Cumulative mortality of channel catfish challenged with wild type zebrafish strain of *E. ictaluri*.

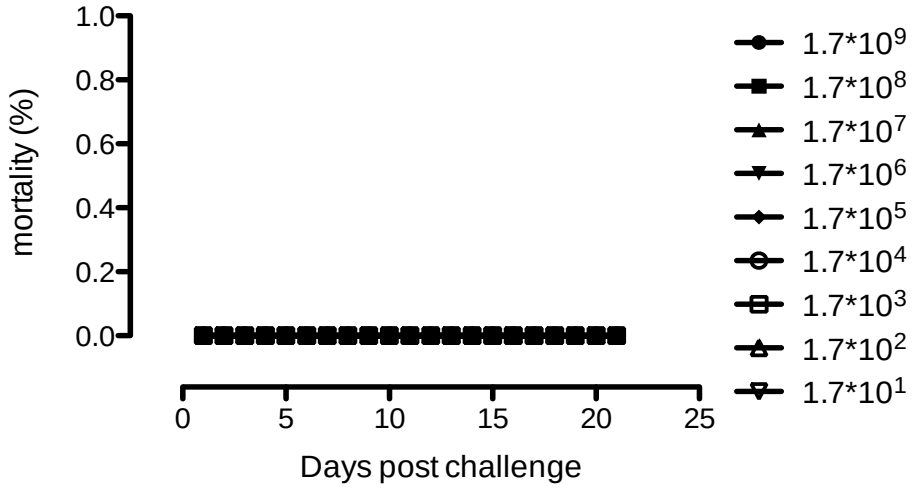

Figure 5.4 Cumulative mortality of zebrafish challenged with Δ*esrC* by immersion.

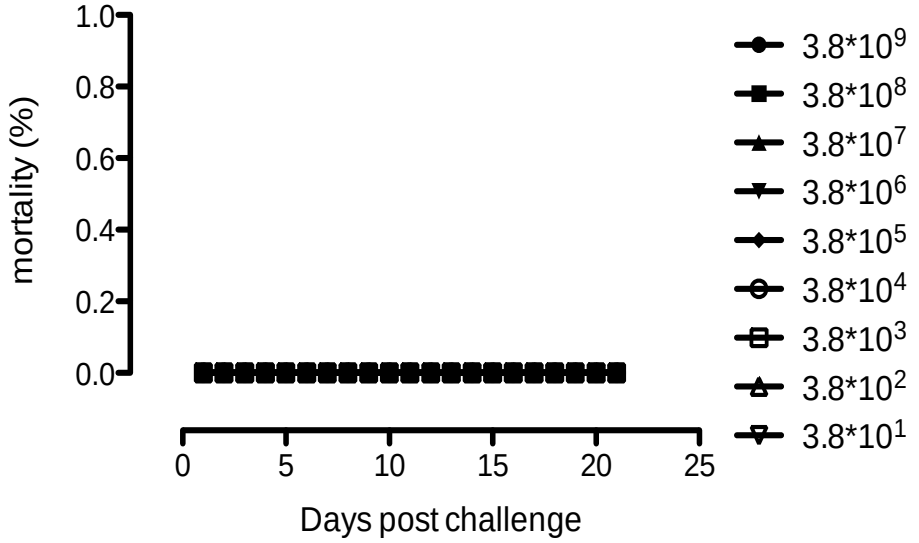

Figure 5.5 Cumulative mortality of zebrafish challenged with Δ*ureG* by immersion.

Attenuation of wild type *E. ictaluri* is due to the mutation in *ureG* or *esrC* gene.

Zebrafish were challenged with the complemented strain of *ureG* and *esrC* mutants. Data are shown in figures 5.6 and 5.7.

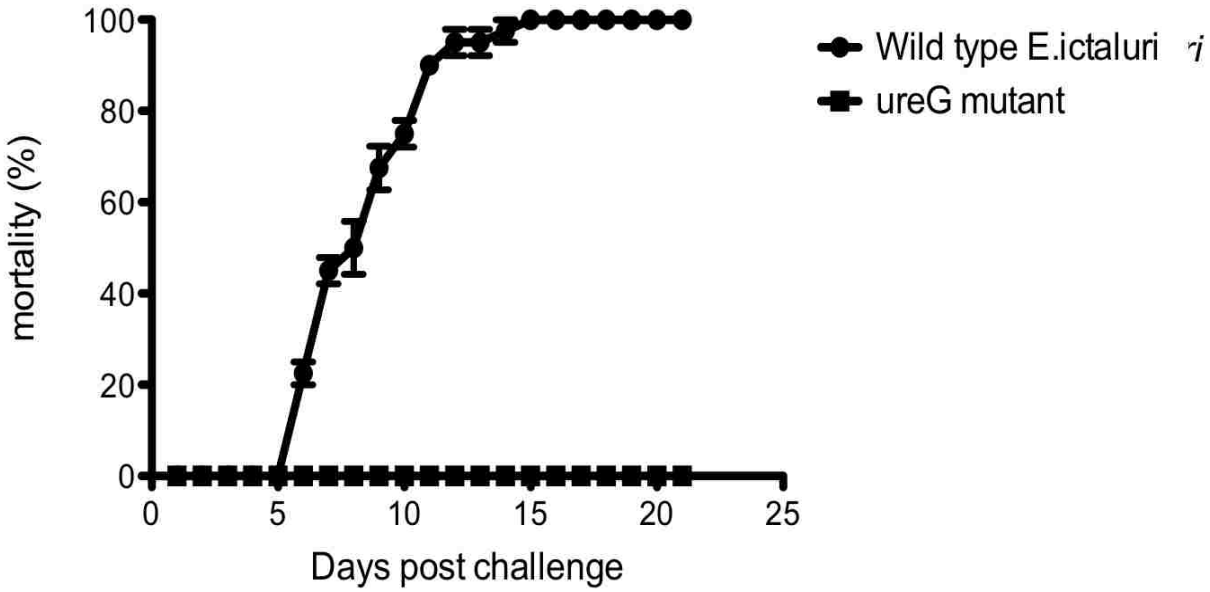

Figure 5.6 Cumulative mortality of zebrafish challenged with Δ*ureG* or wild type *E. ictaluri* by immersion.

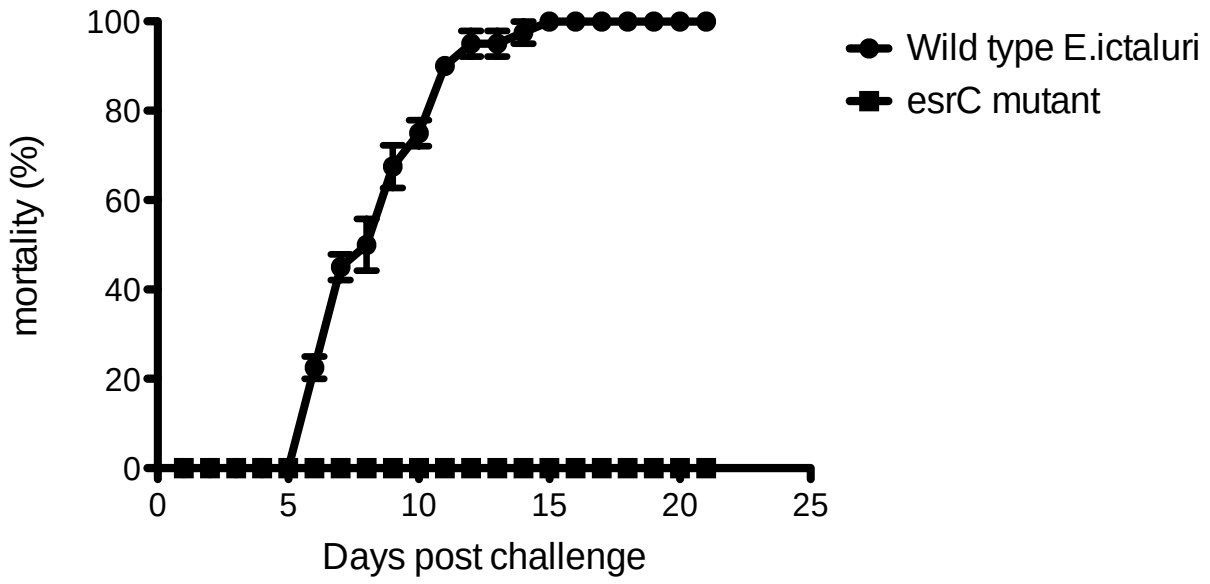

Figure 5.7 Cumulative mortality of zebrafish challenged with Δ*esrC* or wild type *E. ictaluri* by immersion.

Discussion

In 2011, strains of *E. ictaluri* were isolated from laboratory populations of zebrafish and characterized as having unique characteristics allowing them to be

differentiated from catfish strains of the pathogen. Multiple cases were reported, but this specific *E. ictaluri* strain was only isolated from zebrafish [11] and little is known about whether this strain infects zebrafish specifically. Therefore, to test the host specificity of *E. ictaluri* strains, the first step was to challenge channel catfish with the zebrafish strain of *E. ictaluri* to assess relative susceptibility. Research had previously shown that the zebrafish is, susceptible to infection by injection with catfish strain LADL 93-146 but somewhat resistant to immersion challenge with the same strain [37]. In this study, LD_{50} tests are performed to measure the virulence levels and the results indicate that channel catfish juveniles are resistant to this zebrafish strain by immersion. Since immersion more closely mimics the natural infection, we feel this is the proper method to assess host specificity. Catfish can survive even when treated by the highest dose of zebrafish strain of *E. ictaluri*. The specific reason for the host specificity is still unknown but could be related to the variations in the secretion systems or the changes in LPS biosynthesis proteins. It could also be related to minor differences in the genome that encode virulence factors for host adaptation. Thus, genome sequencing was applied and the possible mechanisms were described in earlier chapters.

Previous study of the urease system in *Klebsiella aerogenes* indicated that UreG is essential for the assembly of urease [38]. Studies have shown that the *ureG* gene is required for intracellular replication by neutralizing the pH in the macrophage phagosome and the macrophage arginase is the source of urea [31]. In our study, the zebrafish strain of *E. ictaluri* is successfully mutated in *ureG* gene with amino acid 151 to 264 deleted. The resulting *ureG* mutant is avirulent in zebrafish by immersion. This result is in accordance with previously reported result on catfish strain that *ureG* gene is an

important virulence factor in catfish strain of *E. ictaluri* and mutation in *ureG* gene can attenuate the wildtype strain. This result indicates Δ*ureG* can be a potential good vaccine candidate for preventing *E. ictaluri* infection in zebrafish. In addition, knowing that *ureG* mutant of catfish strain does not replicate intracellularly provides us a hint that Δ*ureG* from the zebrafish strain of *E. ictaluri* can be a safe vaccine candidate for zebrafish [31].

Another important factor in catfish strain is EsrC and a blast search for EsrC sequences returns many AraC family transcriptional regulators which are a common type of regulator in bacteria. Studies have shown that EsrC can regulate type III secretion system (T3SS) and the expression of EvpC, a type VI secretion system protein, in both *E. ictaluri* and *E. tarda* [36, 39]. With the knowledge that T3SS and EvpC are important virulence factors, and EsrC can regulate both T3SS and EvpC to affect virulence, we hypothesized that that mutation in *esrC* can reduce the virulence of our zebrafish strain of *E. ictaluri*. Our study on the LD_{50} of the Δ*esrC* proved this hypothesis since this strain is avirulent by immersion. In the catfish strain, Δ*esrC* is also avirulent and it can actually replicate inside of the host cells [16]. Since our strain has high overall DNA homology to the catfish strain, it is possible that our Δ*esrC* also can replicate in the host. If that is the case, this mutant can be considered as a vaccine candidate that may have a prolonged effect of protection to the host. This would make it superior to the urease mutant.

To develop a vaccine, a balance between safety and efficacy is always important. Further study is needed to test Δ*esrC* and Δ*ureG* for both safety and efficacy as vaccine candidates.

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CHAPTER VI. CONCLUSION

In this study, we have shown that the channel catfish is not susceptible to the zebrafish strain of *E. ictaluri* by immersion challenge. Comparative genomic studies indicate that the identities between the zebrafish and catfish strain genomes, for the most part, are above 95%. Most of the genes in the type III secretion system, type VI secretion system and the urease system in catfish strain are virtually identical to their homologous genes in the zebrafish strain. However, the zebrafish strain contains an O-antigen biosynthesis cluster that differs significantly from that of the catfish strain with unique genes identified. Our observations of different banding patterns for the LPS samples purified from the catfish and zebrafish strains of *E. ictaluri* further support this genomic finding that there are major differences between the catfish and zebrafish strains in the LPS. In addition, the putative type IV secretion system in the catfish and zebrafish strains of *E. ictaluri* share very low similarities.

Vaccination is considered as a potential method for controlling of *E. ictaluri* infection in zebrafish. Based on work with ESC in catfish we believe a live attenuated strain administered by immersion will be an appropriate and effective vaccine. The wildtype zebrafish strain is attenuated by mutating either the *ureG* or *esrC* gene. Challenge results indicate that both of the mutants are fully attenuated and can be potential vaccine candidates.

E. ictaluri is a gram negative bacterium that causes enteric septicemia of catfish (ESC). For years, all of the *E. ictaluri* strains have been considered a homogeneous group [1]. However, in 2011, Hawke et al. [2] isolated *E. ictaluri* from moribund zebrafish *Danio rerio* and found that this strain is different from the typical catfish strain in many

respects, including weaker motility, different plasmid profiles, different API codes and the absence of monoclonal antibody recognition of lipopolysaccharides [2]. Thus it is interesting to investigate the differences between the typical catfish and newly identified zebrafish strain to elucidate the variation in terms of pathogenesis.

In this study, we proved that in addition to the previously described differences between the catfish and the zebrafish strain mentioned above, the *E. ictaluri* strain is also host specific by immersion since the zebrafish strain fails to cause mortalities in channel catfish even when a high dose of bacteria are given to the fish by immersion. This finding led to two research directions. One is the study on the genome level to look for the potential reason(s) for host specificity of *E. ictaluri* and the other direction is to create a potential vaccine to protect zebrafish against this unique zebrafish strain of *E. ictaluri*.

For the genomic level analysis, we mainly focused on the LPS, type III secretion system (T3SS), type IV secretion system (T4SS), type VI secretion system (T6SS) and the urease system. For each of these systems, the overall structure of the gene cluster was checked first to find out whether there are differences in gene arrangement and if there were any unique genes in the catfish and zebrafish strain of *E. ictaluri*. Second, each of these putative clusters in the zebrafish strain was checked further for any differences at the DNA and protein levels. The non-synonymous, single-nucleotide polymorphisms (SNPs) of the genes in these systems were collected from the full SNP list which included the synonymous SNPs as well as the SNPs that are not in the coding regions. These non-synonymous SNPs can change the amino acid sequences of the proteins and potentially affect the structure and the function of the protein. There are many examples that the SNPs in human genome can lead to diseases in humans [3-5].

Lipopolysaccharides are a major ligand for host cell recognition and can bind to host cell Toll-like receptor 4 (TLR4)–MD-2 complex, initiating inflammatory responses in the host [6-8]. Thus the variations in the LPS can possibly cause the bacterial cells to become highly attenuated [9-11]. In this study, we found that the core and the lipid A related genes in the catfish strain LADL93-146 and zebrafish strain, LADL11-100 and LADL11-194, are in the same order and are virtually identical with none or few SNPs. In contrast, the O-antigen biosynthesis cluster varies significantly. The gene arrangements in the catfish and zebrafish strains are different and the identities of the genes in this cluster are low in comparison with the rest of the genome. Interestingly, the O-antigen cluster in the zebrafish strain is virtually identical to that of *E. piscicida* C07-087, with overall similarity >95%. There are three genes, *wzx*, *wzy* and *weiD*, which encode a flippase, a polymerase and a glycosyltransferase, which are found in both the catfish and zebrafish strain O-antigen cluster with low similarities. The identities of these three proteins between the catfish and zebrafish strain of *E. ictaluri* range from 26% to 48%. The catfish strain and the zebrafish strains both have unique genes in their O-antigen clusters and these genes are primarily glycosyltransferase-encoding genes. All these differences in the LPS biosynthesis related genes can possibly alter the structure of the LPS. Our findings that LPS samples from the catfish and zebrafish strains of *E. ictaluri* exhibit different banding patterns on SDS-PAGE further support this theory.

The secretion systems are the systems used by the bacterial cell to communicate with the host cells, the sister cells or the extracellular milieu. The type III secretion system (T3SS) in the catfish strain of *E. ictaluri* has been shown to be an important virulence factor [12-14]. Here we find that the most of the genes in the T3SS between

catfish and zebrafish strains of *E. ictaluri* are highly similar and only the *esrA*, *eseC* and *eseD* genes contain more than 20 SNPs. The putative type IV secretion system, on the other hand, varies significantly between the catfish strain and the zebrafish strain of *E. ictaluri*. Phylogenetic trees of the putative T4SS proteins show that most of the proteins in T4SS of the zebrafish and catfish strain of *E. ictaluri* are not close evolutionally. Whether the T4SS in the catfish strain and zebrafish strain actually have similar or different functions remains in question [15]. There are no significant variations in the type VI secretion system and the urease system between the two strains, only few SNPs were found.

To prevent edwardsiellosis in zebrafish populations, I propose to design a vaccine that can be administered by immersion. Two genes that were chosen to mutate in two different strains of the wild type bacterium LADL11-100 are *esrA* and *ureG*. EsrA is a regulator in the type III secretion system of the catfish strain and it regulates the expression of the T3SS as well as T6SS [13]. Since mutation of *esrA* gene can fully attenuate the catfish strain and *esrA* gene in zebrafish strain is identical to that of the catfish strain, we feel this is a good candidate. The *esrA* gene in the zebrafish strain LADL11-10 is in-frame deleted and our data suggest that the *esrA* mutant of the zebrafish strain is avirulent in zebrafish by immersion. The other mutant constructed in this study is the *ureG* mutant. This gene is part of the urease system that works to increase the pH of the phagosome and facilitates bacterial replication intracellularly [16, 17]. Our study has shown that *ureG* mutant can also attenuate the zebrafish strain in a similar fashion as the *ureG* mutant of the catfish strain did in catfish [17]. Further research is needed to test the

ureG and *esrC* mutants in zebrafish to check for the relative protection when applied as immersion vaccines.

To summarize, the zebrafish strain of *E. ictaluri* is characterized at the genome level. The most differences are found in the lipopolysaccharide O-antigen biosynthesis cluster and these differences on the DNA level is supported by the observation of the different banding pattern of these LPS samples. Second, the important secretion systems are examined and the type IV secretion system differs significantly from the catfish strain to the zebrafish strain. This secretion system can have various functions and the exact function in the catfish and zebrafish strains may vary due to the very low level of similarities. Other systems between the catfish and the zebrafish strain of *E. ictaluri* have very high identities. Certain type III secretion system protein encoding genes are missing on the plasmids as well as in the genome of the zebrafish strain. Meanwhile, three of the T3SS proteins actually contain many SNPs. In addition, we also constructed the *ureG* and *esrC* mutant and both of them are fully attenuated by immersion in zebrafish, indicating their potential function as vaccine candidates against edwardsiellosis in zebrafish populations.

Our study provides clues for illuminating the mechanism of the host specificity of *E. ictaluri*. Future studies can focus on the variations we found in the LPS related genes and genes in type III and type IV secretion systems. Lipopolysaccharide as the outer structure of the bacterial cell can play a vital role in terms of pathogenesis, thus it is very interesting to further examine two LPS samples from the catfish and zebrafish strain of *E. ictaluri*. Structural analysis (chemical analysis) can be done to observe the structures of both LPS samples. Meanwhile, more work is needed to identify the function of the genes

in the O antigen biosynthesis gene cluster that vary between catfish and zebrafish strain of *E. ictaluri*. Combining the results from the chemical and molecular biological analyses will help us clarify the mechanism of the host specificity of *E. ictaluri*.

In addition, we constructed two mutants, the *esrC* and *ureG* mutants, and both mutants proved to be fully attenuated in zebrafish. For the development of the vaccine, the complemented strain of the *esrC* and *ureG* mutants needs to be tested in zebrafish to make sure they regain virulence. Also, a vaccine trial is required to calculate the relative percentage of survival and confirm the protectiveness of these vaccine candidates. Many farms and facilities in U.S. need a vaccine against edwardsiellosis of zebrafish. Once these mutants are proved to be protective for zebrafish, they can be widely used in practice immediately.

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APPENDIX I.
ABBREVIATIONS COMMONLY USED IN THIS DISSERTATION

BLAST – Basic Local Alignment Search Tool

CFU – Colony forming unit

Col – Colistin

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)

LB – Luria Bertani broth

LPS - Lipopolysaccharide

ORF – Open reading frame

PCR – Polymerase chain reaction

pEI1 – *E. ictaluri* plasmid 1

pEI2 – *E. ictaluri* plasmid 2

pEIZ1– zebrafish strain of *E. ictaluri* plasmid 1

pEIZ2– zebrafish strain of *E. ictaluri* plasmid 2

RAST– Rapid Annotation using Subsystems Technology

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP–Single nucleotide polymorphism

SPF – Specific pathogen-free

T3SS – Type III secretion system

T4SS– Type IV secretion system

T6SS – Type VI secretion system

WT – Wild type

APPENDIX II.
THE DRAFT GENOME SEQUENCES OF THE ZEBRAFISH STRAINS OF *EDWARDSIELLA ICTALURI* LADL11-100 AND LADL11-194

See the attached file ("*Edwardsiellaictaluri*LADL11_100andLADL11_194draft genomesequences").

VITA

Rui Wang was born in June, 1985 in Jilin, China. She spent her childhood in a small town in Jilin province with her father Hongtu Wang, her mother Yuqin Li and her younger brother Geng Wang. After high school, she left her hometown and went to Changchun, the capital city of Jilin province, to pursue her bachelor's degree in veterinary medicine at Jilin University. From 2004 to 2009, she completed all the course work and for the first time, stayed in a laboratory for three months to finish a project using molecular biological techniques. There she found her interest in studying microbial pathogenesis. Therefore, after graduation, she decided to continue researching in this area. Luckily, she got an opportunity to keep study at Louisiana State University The School of Veterinary Medical Sciences in the Department of Pathobiological Sciences, so she flied all the way across the Pacific Ocean, from China to U.S.A. In August 2009, she settled down in Baton Rouge, a quiet town perfect for researches and in 2010, she joined in Dr. John Hawke's lab to work on fish pathogens. Under the guidance of Dr. John Hawke, she was able to finish her dissertation research and completed the requirements for the degree of Doctor of Philosophy in February of 2015.