Molecular Interactions between phage and the catfish pathogen *Edwardsiella ictaluri* and Comparative Genomics of Epidemic strains of *Aeromonas hydrophila*

by

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Abstract

Edwardsiella ictaluri causes Enteric Septicemia of Catfish (ESC) which is responsible for significant economic loss of the catfish aquaculture industry in the southeastern United States. A better diagnostic tool is required for the identification of *Edw. ictaluri* from ESC-affected catfish. Bacteriophages ΦeiAU and ΦeiDWF are specifically lytic to *Edw. ictaluri* strains and have tremendous potential to be used as diagnostic agents for the identification of *Edw. ictaluri* from ESC-affected catfish. However, bacteriophages ΦeiAU and ΦeiDWF demonstrate varying degree of lytic activity to different *Edw. ictaluri* strains. To identify the basis for variation and understand the mechanisms of phage-host interactions, phage resistant *Edw. ictaluri* mutants were generated by transposon mutagenesis of wild type *Edw. ictaluri* strains. Characterization of phage resistant *Edw. ictaluri* mutants revealed different host factors including integral membrane proteins, molecular chaperon, protein involved in LPS biosynthesis, proteins with proteolytic and regulatory activities, and proteins of unknown functions contribute to phage infection. Since outer membrane porin protein LC (OmpLC) is predictably located on the outermost layer of bacterial cells and deletion of the *ompLC* from *Edw. ictaluri* abolished phage infectivity, we investigated the role of OmpLC in varying degree of phage susceptibility. Phage binding assay with *Edw. ictaluri* and an *ompLC* mutant demonstrated that OmpLC is the receptor for phage attachment and infection to *Edw. ictaluri* strains. The prediction of the three-dimensional structure of the OmpLC protein revealed a typical porin structure with 16 antiparallel β strands and 8 extracellular loops. Site-directed mutagenesis and deletion of loop 8

of the OmpLC protein demonstrated that OmpLC protein is the modulating factor for phage susceptibility nature in different *Edw. ictaluri* strains.

Mutant phages with enhanced lytic activity to phage resistant *Edw. ictaluri* strains were isolated by serial passaging and phage factors involved in broader host range were investigated. Pairwise comparison of the whole genome sequences of wild type and mutant phages with enhanced host lytic activity revealed that mutant phages accumulated several point mutations in six different open reading frames (ORFs). Recombineering experiment carried out with genomic DNA of wild type phage and PCR amplicons of mutated ORFs from mutant phages followed by the generation of recombinant phages demonstrated that phage host specificity protein (HspP) is responsible for the broader host susceptibility to *Edw. ictaluri* strain.

This study highlights the importance of both the OmpLC protein of *Edw. ictaluri* and the ΦeiDWF phage protein HspP in defining the nature of phage susceptibility in different *Edw. ictaluri* strains. Generation of recombinant phages through directed mutagenesis of HspP protein will provide bacteriophages with enhanced infectivity for phage-resistant *Edw. ictaluri* strains and these bacteriophages with broader lytic activity could be used as diagnostic agents to identify *Edw. ictaluri* from ESC-affected catfish.

In addition to ESC, the recent epidemic outbreak of motile *Aeromonas* septicemia (MAS) of catfish caused by a highly virulent and emerging strain of *Aeromonas hydrophila* is a major threat to the catfish industry in the southeastern United States. The lack of complete genome sequence of epidemic *A. hydrophila* hampered efforts to understand the highly virulent nature of this emerging pathogen. To investigate the pathogenic nature of these epidemic strains, a total of 12 *A. hydrophila* isolates including six recent epidemic isolates and six reference isolates were sequenced at >160-fold coverage. Genome-wide comparisons were carried out to identify

epidemic-associated genomic regions exclusively present within the epidemic isolates. Comparative genomics revealed that the epidemic *A. hydrophila* isolates are highly clonal whereas reference isolates are greatly diverse. These epidemic isolates share 54 unique genetic regions comprising 326 kb with 307 predicted genes that putatively encode prophage elements, virulence factors, and gene clusters predicted to be involved in pilus biogenesis and *myo*-inositol utilization, along with many predicted genes of unknown functions. Five different novel O-antigen biosynthesis gene clusters were identified within the genome of sequenced *A. hydrophila* isolates, and all epidemic strains shared a single type of O-antigen biosynthesis gene cluster. A large percentage of the epidemic-associated unique genomic regions were present within genomic islands which was suggestive of possible events of lateral genes transfer for the acquisition of those unique regions. An *in vitro* growth assay demonstrated that epidemic *A. hydrophila* isolates were able to use *myo*-inositol as a sole carbon source and that this phenotypic property could be used to test field isolates for detection of epidemic *A. hydrophila* strains. Our comparative genomic study revealed new insights into the evolutionary changes that have occurred in *A. hydrophila* strains associated with epidemic outbreaks in channel catfish and provides a foundation for studying the specific molecular determinants of virulence in this emerging pathogen.

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Introduction and Literature Review

1.1 Introduction

The catfish aquaculture industry is one of the major aquaculture industries in the US (USDA, 2012). Channel catfish production in the US is more than a $400 million industry (Hanson & Sites, 2012) which contributes significntly in the economy of southeastern United States. US farm-raised catfish was ranked sixth in the 2010 'Top 10' fish and seafood consumption among Americans with an average of about 0.8 pound of catfish consumption per person per year (Hanson & Sites, 2012). The channel catfish production is hampered by several diseases caused by bacteria, viruses, fungi, helminthes and parasitic copepods and other parasites (Stickney, 2012). The US catfish industry is also affected by the increase in feed prices and the import of frozen catfish fillets which now account for 74% of all US sales of frozen catfish fillet product (Hanson & Sites, 2012).

Enteric Septicemia of Catfish (ESC) is caused by *Edwardsiella ictaluri*, the most important pathogen affecting farm-raised channel catfish (Hawke *et al.*, 1981; Hawke *et al.*, 1998). The ESC disease is solely responsible for significant economic losses which are estimated between $30 and $50 million each year (Shoemaker *et al.*, 2009). Though ESC was a disease of major concern in the Unites States, it has been reported in Vietnam and China where the

freshwater catfish and yellow catfish, respectively, were infected with *Edw*. *ictaluri* (Crumlish *et al.*, 2002 and Lie *et al*, 2010).

The current diagnostic protocol commonly used for the detection of *Edw. ictaluri* from catfish afflicted with ESC is time consuming since the confirmatory tests require several days to identify this pathogen (International Office of Epizootics, Aquatic Animal Health Standards, 2009). Early detection of ESC outbreak in catfish farms helps to prevent the loss associated with this disease by treating ESC infected catfish with antibiotics. The delayed diagnosis of the ESC makes the treatment harder since fish progressively reduce feed intake during the infection. Though several numbers of PCR based molecular techniques have been developed for the rapid identification of *Edw. ictaluri* from ESC infected fish, many of the fish diagnostic laboratories are not equipped with necessary devises to carry out those rapid diagnostic tests. Therefore, a rapid alternative diagnosis of ESC is essential to minimize the loss associated with this disease.

Bacteriophages ΦeiAU and ΦeiDWF that are specifically lytic to *Edw. ictaluri* have recently been isolated from aquaculture ponds with history of ESC outbreak (Walakira *et al.*, 2008). These two bacteriophages have enormous potential to be used as a therapeutic to treat ESC infected catfish and can potentially be used for the detection of *Edw. ictaluri* from ESC infected catfish. However, like many other bacterial strains, *Edw. ictaluri* strains are not equally susceptible to their bacteriophages (Walakira *et al.*, 2008). Therefore, it is essential to isolate new *Edw. ictaluri* specific bacteriophages with broader host susceptibility for their therapeutic and diagnostic applications.

The *Edw. ictaluri* host factors required for phage ΦeiAU and ΦeiDWF infection have not yet been identified. The determination of host factors required for phage infection helps to

understand the pathogen better that eventually can help to devise strategies to control the ESC disease. Phage ΦeiAU and ΦeiDWF molecular determinants that modulate the phage susceptibility nature to their host, *Edw. ictaluri*, are yet to be studied. Identification of phage determinants for their infectivity to *Edw. ictaluri* can pave the way to rationally design bacteriophages with broader host specificity that would eventually be able to detect or treat phage resistant *Edw. ictaluri* strains. This dissertation identified the *Edw. ictaluri* determinants required for phage ΦeiAU and ΦeiDWF infection. It also identified phages ΦeiAU and ΦeiDWF molecular determinants required for broader host specificity to different *Edw. ictaluri* strains.

Motile Aeromonas septicemia (MAS) is a systemic disease of fish caused by *Aeromonas* species (Austin & Adams, 1996). The primary species of *Aeromonas* responsible for MAS are *A. hydrophila*, *A. caviae* and *A. sorbia* (Popoff *et al.*, 1981). Different fishes that include but are not limited to channel catfish, minnows and baitfish, carp, gizzard shad, striped bass, largemouth bass, and tilapia are affected by this disease. MAS in channel catfish caused by *A. hydrophila* was not a disease of concern because the catfish aquaculture operations in the southeastern United States have not experienced a major outbreak caused by this pathogen before 2009. In 2009 the catfish farming operations in West Alabama experienced an epidemic outbreak of MAS caused by an *A. hydrophila* strain (Hemstreet, 2010). Since after its inception in 2009, the MAS outbreak caused by *A. hydrophila* is continuing in Alabama and has spread to the neighboring states of Mississippi and Arkansas (Bebak *et al.*, 2011). These epidemic outbreaks were responsible for killing over 7 million pounds of market-size catfish (Hemstreet, 2010). It has been demonstrated that *A. hydrophila* isolates from recent epidemic outbreaks are highly virulent compared to the *A. hydrophila* isolates obtained from non-epidemic outbreak (Pridgeon & Klesius, 2011).

Due to the devastating nature of the epidemic MAS outbreak caused by *A. hydrophila*, it is highly essential to find virulence-associated genes within the genome of *A. hydrophila* strains identified in the epidemic. Until now unique genetic features that contribute to the highly virulent nature of the epidemic *A. hydrophila* in channel catfish have not been identified. Since only one antibiotic, oxytetracycline (Terramycin®), is approved for the treatment of MAS infected fish, the treatment options are limited due the variable antibiotic resistance nature of *A. hydrophila* strains. The development of vaccines by targeted deletion of virulent genes could be an effective way to prevent the outbreaks of MAS in channel catfish. Until now, no vaccines, designed by targeted deletion of virulent genes, were available for the prevention of MAS outbreaks in channel catfish by *A. hydrophila*. Targeted deletion of virulent genes in epidemic *A. hydrophila* is difficult to achieve due to the lack of genome sequences of epidemic *A. hydrophila* strains.

This dissertation described the evolutionary nature of the epidemic *A. hydrophila* isolates involved in epidemic outbreak of MAS in channel catfish using comparative genomics of 12 *A. hydrophila* isolates that includes six epidemic *A. hydrophila* and six reference *A. hydrophila* isolates. It also identified the epidemic-associated unique genetic regions within the genome of epidemic *A. hydrophila* isolates with their possible role in virulence to catfish.

1.2 Objectives of the study

This study intended to determine the molecular determinants of *Edw. ictaluri* host factors required for phages ΦeiAU and ΦeiDWF infection. The degree of susceptibility of these phages to different *Edw. ictaluri* was varying and the underlying mechanisms of this varying degree of phage susceptibility were unknown. Many of the *Edw. ictaluri* strains were naturally resistant to

phage ΦeiAU and ΦeiDWF infection. This study emphasized identification of the *Edw. ictaluri* host mechanisms for resisting the phage infections and to determine the underlying mechanisms of varying degree of phage susceptibility.

In this study, we proposed to develop a recombineering technique, i.e., genetic engineering by homologous recombination, to modify the genome of *Edw. ictaluri*. It was reasoned that applying the recombineering technique would be highly efficient and faster to manipulate the *Edw. ictaluri* genome.

This study aimed to isolate mutant phages with broad host specificity to different *Edw. ictaluri* strains that were previously resistant to *Edw. ictaluri*-specific phages. This study also aimed to identify the molecular determinants of the mutant phages with broad host range specificity to *Edw. ictaluri*. It was rationalized that identification of phage determinants for broader host specificity will pave the way to design recombinant phages to avoid the host imposed phage resistance mechanisms.

This study emphasized comparison of the whole genome sequences of highly virulent epidemic *A. hydrophila* (EAh) isolates with that of reference *A. hydrophila* (RAh) isolates and identification of epidemic-associated genetic elements to reveal mechanisms fostering the hyper-virulent nature of the EAh strains. It was reasoned that the molecular characterization of epidemic strains will provide the framework for the development of vaccines, therapeutics, and rapid diagnostics to facilitate the control of this emerging catfish pathogen.

2. Literature Review

2.1 Channel catfish and Aquaculture

Channel catfish *Ictalurus punctatus* (Rafinesque, 1818) belong to the family *Ictaluridae* of the order Siluriformes and this family of catfish is native to North America. Though there are approximately 44 valid species of the *Ictaluridae* (Page & Lundberg, 2007*)*, only six including blue catfish (*I. urcatus,* LeSueur), white catfish (*I. catus*, Linnaeus), black bullhead (*I. melas,* Rafinesque), brown bullhead (*I. nebulosus*, LeSueur), yellow bullhead (*I. natalis,* LeSueur) and flathead catfish (*Pylodictis olivaris*, Rafinesque) have been cultured or have potential for commercial production (Wellborn, 1988). Though the origin of channel catfish is unknown, it is believed that channel catfish might have originated from Oklahoma stocks (Chapman, 2012). Channel catfish is the most important species of farm-raised aquatic animal commercially culture in the United States. Southeastern United States including Alabama, Arkansas, Louisiana and Mississippi and are the leading commercial catfish producing states.

2.2 Diseases of Channel Catfish

Channel catfish are subjected to a wide variety of diseases caused by virus, bacteria, fungi, helminthes and parasitic copepods (Stickney, 2012). Enteric septicemia of catfish caused by *Edw. ictaluri* is the primary disease in channel catfish (Hawke *et al.*, 1981; Klesius, 1992). Since after its first reporting in 1979 (Hawke *et al.*, 1981), this disease spread throughout the industry and consistently affects the catfish industry (Klesius, 1992). This bacterium is now frequently found in the catfish farming environment (Klesius, 1992) and still remains as an economically significant disease causing agent of farm-raised channel catfish (USDA, 2011). A recent survey demonstrated that many catfish operations with food-sized catfish are experiencing

ESC (USDA, 2011), though it was widely believed and experimentally shown that catfish fingerlings are more susceptible to ESC.

Columnaris, caused by *Flavobacterium columnare*, is the second leading cause of mortality in channel catfish (Durborow *et al.*, 1998). In addition to channel catfish, it also infects many other commercially and ornamentally important fish species (Austin & Austin, 2007; Plumb, 1999). Out of the three existing genomovars of *F. columnare*, genomovar II is highly pathogenic to channel catfish (Arias *et al.*, 2004; Shoemaker *et al.*, 2008). In wild fish populations, genomovar II is almost exclusively found within catfish (Olivares-Fuster *et al.*, 2007). The Columanis disease in catfish might be cutaneous or systemic and can be characterized as an acute to chronic infection of the gills and the integumentary system (Wolke, 1975). A yellowish-brown ulcer in the dorsal fin termed as 'saddleback' is one of the important pathological signs of Columnaris disease in catfish (Arias *et al.*, 2012). Sometimes the systemic infection in catfish could be asymptomatic with little or without any pathological signs (Arias *et al.*, 2012; Hawke & Khoo, 2004).

Motile Aeromonas septicemia (MAS) of channel catfish caused by motile members of *Aeromonas* genus including *A. hydrophila*, *A. sorbia* and *A. caviae* are common in farm-raised channel catfish. *A. hydrophila* is the more frequently isolated species involved in MAS and is more virulent than *A. sorbia* and *A. caviae* (Popoff & VéEron, 1976). A detailed description of MAS caused by *A. hydrophila* is provided in Chapter IV.

Channel catfish viral disease (CCVD) is specific to channel catfish and mostly infects fish fry and fingerlings (Camus, 2004). *Channel catfish virus* (CCV) that causes severe hemorrhagic disease in young channel catfish is a member of the Herpesvirus family 1 and is

cytopathic in nature (Plumb, 1977; Wolf & Darlington, 1971). This viral disease is responsible for 1 to 2% of total disease losses in channel catfish (Camus, 2004).

Saprolegniasis or winter kill, caused by parasitic and opportunistic oomycete pathogen *Saprolegnia* spp., can infect channel catfish eggs, fry fingerlings and adults (Durborow *et al.*, 2003). *Saprolegnia* is ubiquitous in fresh water. *Saprolegnia parasitica* is the primary pathogen of this disease, whereas *S. declina* and *S. saprolytica* cause disease in the presence of pre-existing illness and environmental stress (Durborow *et al.*, 2003). A sudden decrease of water temperature is one of the abiotic factors that make the channel catfish susceptible to *Saprolegnia* spp. due to immuno-suppression of fish associated with cold temperature (Bly *et al.*, 1992). Saprolegniasis disease in catfish is the most common and economically important oomycete disease in catfish (Durborow *et al.*, 2003).

Channel catfish are infected with several protozoan parasites including but not limited to *Ichthyophthirius multifiliis* (Klesius & Rogers, 1995), *Ichthyobodo necator* (Tucker & Robinson, 1990), *Trichodina heterodentata* (Martins *et al.*, 2010), *Tetrahymena* sp. (Hoffman *et al.*, 1975), *Epistylis* sp. (Hubert & Warner, 1975) and *Apisoma* spp. (Tucker & Hargreaves, 2004).

Proliferative gill disease (PGD) of catfish is the fourth most commonly diagnosed disease in the southeastern United States (Mitchell *et al.*, 1998). Several myxosporeans including *Henneguya ictaluri* (Bowser & Conroy, 1985), *Henneguya exilis* Kudo *(Duhamel et al., 1986), Sphaerospora ictaluri (Hedrick et al., 1990)*, and *Aurantiactinomyxon* sp. (Pote & Waterstrat, 1993; Styer *et al.*, 1991) are found to be implicated as etiologic agents for PGD.

Parasitic copepods including *Neoergasilus japonicus* (Hudson & Bowen, 2002), *Ergasilus versicolor* (Roberts, 1969), *Achtheres pimelodi* (Harms, 1960)**,** *Lernaea cyprinacea*

(Goodwin, 1999) were isolated and believed to be responsible for channel catfish infestation. *L. cyprinacea* was involved in an epizootic outbreak in channel catfish in Arkansas in 1998 (Goodwin, 1999).

2.3 Pathogenesis of Enteric Septicemia of Catfish (ESC) caused by *Edw. ictaluri*

Identification of several *Edw. ictaluri* virulence factors required for ESC in catfish has advanced the understanding of the pathogenesis of this bacterium (Cooper *et al.*, 1996; Thune *et al.*, 2007). It has been shown that the routes of entry of *Edw. ictaluri* to the catfish are the nares (nasal opening), gills, olfactory mucosa and gastrointestinal tract (Blazer *et al.*, 1985; Hawke *et al.*, 1998) with no apparent damage to the epithelial cells. It was postulated that the clinical status of ESC depends on the route of entry of *Edw. ictaluri* to catfish (Newton *et al.*, 1989). Bacteria entering through the olfactory sacs develop a chronic form of ESC, whereas an acute form of ESC develops if bacteria enter via the gastrointestinal tract. A study demonstrated that *Edw. ictaluri* invades the catfish gut epithelial cells, and actin polymerization and receptor-mediated endocytosis are involved in the uptake of *Edw. ictaluri* by epithelial cells (Skirpstunas & Baldwin, 2002). This invasion helps *Edw. ictaluri* to cross the intestinal epithelial cell barrier and then may be transported systemically via the bloodstream once engulfed by macrophages (Miyazaki & Plumb, 1985).

Studies demonstrated that *Edw. ictaluri* is capable of entry, survival and replication within head kidney-derived macrophages from channel catfish (Booth *et al.*, 2006). Recently, the survival mechanism of *Edw. ictaluri* within channel catfish macrophages has been revealed (Booth *et al.*, 2009). They demonstrated that *Edw. ictaluri* survives and replicates within the catfish macrophage with the help of an acid-activating urease, an *Edw. ictaluri* virulence factor

required for the establishment of ESC in catfish (Thune *et al.*, 2007). It has been postulated that urease from *Edw. ictaluri* helps this pathogen to survive and replicate within the macrophage by neutralizing the acidic environment within the phagosome (Booth *et al.*, 2009). Like urease, T3SS is a virulence factor of *Edw. ictaluri* and required for the survival and replication of *Edw. ictaluri* in channel catfish macrophages (Rogge & Thune, 2011; Thune *et al.*, 2007). It has been demonstrated that though urease and T3SS are required for the survival and replication of *Edw. ictaluri* within the channel catfish macrophages, they are not required for the entry of this pathogen to macrophages (Booth *et al.*, 2009; Rogge & Thune, 2011).

Though numerous studies have been conducted to determine the role of catfish macrophages in protecting *Edw. ictaluri* from the attack of antibodies and transport of *Edw. ictaluri* to different organs of catfish, the mechanism of colonization of *Edw. ictaluri* into the gut epithelium of catfish has not been previously studied. Successful bacterial colonization of a host requires resisting the physical removal of the pathogen from their host and their subsequent establishment of infection (Ghose, 1996). Different types of fimbriae and adhesins are used by bacteria for colonization to their host (Pizarro-Cerdá & Cossart, 2006). Recently, it has been identified that a putative fimbrial usher membrane protein and a putative adhesin act as virulence factors of *Edw. ictaluri* and required for the establishment of ESC in catfish (Thune *et al.*, 2007). A fimbrial usher membrane protein is required for the biogenesis of fimbriae (Nuccio & Bäumler, 2007). Fimbrial usher membrane protein and adhesins, which were identified as virulence factors in *Edw. ictaluri* (Thune *et al.*, 2007), could be potential colonization factors of *Edw. ictaluri* required for the establishment of ESC in channel catfish.

2.4 *Edw. ictaluri* virulence factors for ESC

Though ESC is an economically important disease affecting the catfish industry, the virulence factors of *Edw. ictaluri* have not been fully characterized (Thune et al., 1993). Lipopolysaccharide (Lawrence *et al.*, 2001), chondroitinase (Cooper *et al.*, 1996) urease (Booth *et al.*, 2009) were found to be potential virulence factors in *Edw. ictaluri*. It was demonstrated that *Edw. ictaluri* strains with a mutation in the chondroitinase gene failed to produce any chronic or acute disease in catfish (Cooper *et al.*, 1996). Chondroitin sulfatase can depolymerize chondroitin sulfate of connective tissue and cartilage and is believed to be responsible for the hole-in-the-head symptom of catfish suffering from chronic ESC (Cooper *et al.*, 1996), wherein the sagittal line of the cranium is eroded followed by the degradation of chondroitin sulfate of connective tissue that finally exposes the brain (Shotts *et al.*, 1986). The lipopolysaccharide O-antigen side chain of *Edw. ictaluri* is an essential virulence factor for ESC in catfish and protects this bacterium from the complement-mediated killing of catfish serum (Lawrence *et al.*, 2001). Signature-tagged mutagenesis of *Edw. ictaluri* followed by the challenge of channel catfish helped to identify a repertoire of virulence factors that are involve in the pathogenesis of *Edw. ictaluri* in channel catfish (Thune *et al.*, 2007). A urease encoded within a pathogenicity island of *Edw. ictaluri* was found to be required for the intracellular replication of *Edw. ictaluri* within the catfish macrophage (Booth *et al.*, 2009). This urease is only activated in acidic environments (Booth *et al.*, 2009) and this was one of the reasons for a negative urease test for *Edw. ictaluri* in which most of the tests were done at neutral pH (Waltman *et al.*, 1986). This urease catalyzes the hydrolysis of urea which is required for the neutralization of the acidic environment of the phagosome. This neutral environment helps *Edw. ictaluri* to survive and replicate within the catfish macrophage. An *Edw. ictaluri* type III secretion system (T3SS), which has a high degree

of similarity to that of the *Salmonella enterica* serovar Typhimurium (Cirillo *et al.*, 1998), is a virulence factor of this pathogen in channel catfish (Thune *et al.*, 2007). This T3SS system is also encoded within an *Edw. ictaluri* pathogenicity island (Thune *et al.*, 2007) and is required for the replication of *Edw. ictaluri* within catfish head kidney-derived macrophages (Rogge & Thune, 2011). It was demonstrated that a putative adhesin of *Edw. ictaluri* acts as a virulence factor in catfish (Thune *et al.*, 2007). Another study confirmed the virulent properties of this adhesin in catfish (Polyak, 2007). However, the mechanism by which this adhesin promotes ESC has not been determined.

2.5 *Aeromonas hydrophila* and its virulence in fish.

A. hydrophila can infect a broad range of hosts that include fish (Thune *et al.*, 1993), amphibians (Rigney *et al.*, 1978) and humans (von Graevenitz & Mensch, 1968). The pathogenesis of *A. hydrophila* is multifactorial since concerted actions of several factors are required to produce disease (Yu *et al.*, 2005). The regulatory protein AhyR, which is a LuxR-type response regulator and plays a role in *A. hydrophila* quorum sensing (Swift *et al.*, 1997), serves as a virulence factor of *A. hydrophila* in fish (Bi *et al.*, 2007). This response regulator protein regulates the expression of several *A. hydrophila* virulence factors that include proteases, amylase, DNase, hemolysin and S layer (Bi *et al.*, 2007).

Fimbriae or pili, one of the most important bacterial surface appendages, are involved in the colonization of a bacterial pathogen (Bieber *et al.*, 1998; Hokama *et al.*, 1990; Salomonsson *et al.*, 2011; Wairuri *et al.*, 2012). Pili of *A. hydrophila* were similarly found to be involved in the colonization of their host (Ho *et al.*, 1990; Hokama *et al.*, 1990). Isolates of *Aeromonas* spp. from gastroenteritis possess two distinct types of type IV pili including a bundle forming pilus

(Bfp) and a Tap pilus (Barnett *et al.*, 1997). Bfp pilus from diarrhea-associated *Aeromonas* isolates acts as an intestinal colonization factor (Kirov *et al.*, 1999). The Tap pilus obtained from fish isolates of *A. hydrophila* did not demonstrate any virulence properties in an animal model (Kirov *et al.*, 2000). Though genetic loci involved in pilus biogenesis were found within the genome of *A. hydrophila* (Seshadri *et al.*, 2006), the direct implications of pili from *A. hydrophila* in their pathogenesis to channel catfish has not been studied. Two different types of types of pili, such as straight and flexible pili, were identified in *A. hydrophila* AH26. The agglutination nature of flexible pilus to human, guinea pig, bovine, and avian erythrocytes were suggestive of *A. hydrophila*'s ability to cause infections both in cold-blooded and warm-blooded vertebrate animals, including humans (Ho *et al.*, 1990).

S-layers, a regularly ordered planar array of proteinaceous subunits found in many Gram-negative and Gram-positive bacteria, may act as a virulence factor (Sabet *et al.*, 2003; Sleytr & Beveridge, 1999). S-layer proteins may contribute to bacterial virulence by protecting bacteria from complement and the attack of phagocytes as a protective barrier (Sleytr & Beveridge, 1999). The presence of S-layer proteins on the surface of virulent strains of *A. hydrophila* isolated from diseased catfish and eels have been reported in several studies (Dooley & Trust, 1988; Esteve *et al.*, 2004; Ford & Thune, 1991). The S-layer proteins of *A. salmonicida* act as a colonization factor to cause disease in fish (Trust, 1986).

The O-antigen of lipopolysaccharide (LPS) acts as an adhesin in *A. hydrophila* and is one of the virulence factors of this pathogen in fish (Canals *et al.*, 2006, 2007; Merino *et al.*, 1996a; Merino *et al.*, 1998; Vilches *et al.*, 2007). This adhesin was found to be a primary colonization factor of *A. hydrophila* in the gut of a germ-free chicken (Merino *et al.*, 1996b). It was

demonstrated that the growth temperature of *A. hydrophila* influences the composition of LPS and hence its virulence in fish (Isberg & Falkow, 1985).

A 43 kDa outer membrane protein in *A. hydrophila* was responsible for invasive properties in fish epithelial cells and is considered a virulence factor of fish pathogen *A. hydrophila* PPD134/91 (Atkinson *et al.*, 1987; Lee *et al.*, 1997). Secretory elastase of *A. hydrophila* has been shown to contribute to virulence in fish (Cascón *et al.*, 2000).

The type III secretion system (TTSS) was found to be responsible for pathogenesis of *A. hydrophila* in fish (Yu *et al.*, 2004). TTSS and Act, a type II-secreted cytotoxic enterotoxin, has effects on the production of lactones that are involved in quorum sensing of *A. hydrophila* (Sha *et al.*, 2005). TTSS of *A. hydrophila* is interconnected with different virulence factors that include lipopolysaccharide and the *ahyIR* quorum sensing system (Vilches *et al.*, 2009).

Hemolysins are also considered to be important virulence factors in bacterial pathogens (Goebel *et al.*, 1988; Maslow *et al.*, 1999; Russo *et al.*, 2005; Wong *et al.*, 1998). It was demonstrated that hemolysin is the principal virulence factor of *A. hydrophila* in fish (Allan & Stevenson, 1981). However, some isolates of *A. hydrophila* with hemolytic activity did not show any virulence in channel catfish (Thune *et al.*, 1986). Both the alpha- and beta-hemolysin are frequently found in *A. hydrophila* species and in most other *Aeromonas* species, including *A. sobria*, *A. caviae*, and *A. trota* (Janda & Abbott, 2010). *A. hydrophila* isolates from clinical and environmental sources that carried both the hemolysin (*hlyA*) and aerolysin (*aerA*) were virulent in a suckling mouse (Heuzenroeder *et al.*, 1999). Though *A. hydrophila* isolates with *hlyA* and *aerA* genes are frequently isolated from fish, the respective contribution of *HlyA* or *AerA* proteins of *A. hydrophila* on virulence in fish has not been determined (Choresca *et al.*, 2010; Xia *et al.*, 2003).

Virulence factors of *A. hydrophila* isolates from human infections include a cytotoxic enterotoxin (Act) (Chopra & Houston, 1999), cyclic-di-guanosine monophosphate (c-di-GMP) (Kozlova *et al.*, 2012), cold shock exoribonuclease (Lawal *et al.*, 2011), DNA adenine methyltransferase (Dam) (Erova *et al.*, 2006), and a type 6 secretion system (T6SS) (Suarez *et al.*, 2008). These virulence factors have not been evaluated for their relative contribution to *A. hydrophila* virulence in fish.

2.6 Role of lateral gene transfer (LGT) in bacterial pathogenicity

Lateral gene transfer (LTG) is the transfer of genetic material between organisms rather than vertical transmission of genetic material to offspring. Lateral gene transfer has profound impact on the ecological and pathological traits of bacterial species (Ochman *et al.*, 2000). Three different methods of lateral gene transfer have been described in bacteria: Transformation is the process by which bacteria uptake naked DNA without the help of any vehicle (Avery *et al.*, 1944). Some bacteria are naturally competent whereas others can uptake DNA after certain inductions (Dubnau, 1999; Solomon & Grossman, 1996). Conjugation is another mode of bacterial genetic exchange wherein donor and recipient cells are physically in contact through conjugative pili and transfer their genetic material (Tatum & Lederberg, 1947). Plasmids are the most frequently transmitted genetic material via conjugation. Recently, integrative and conjugative elements (ICEs) have also been identified in both Gram-positive and Gram-negative bacteria that can transfer by conjugation and integrate into the recipient bacterial chromosome (Wozniak & Waldor, 2010). These self-transmissible genetic elements greatly contribute to bacterial genome evolution (Burrus & Waldor, 2004). In addition to antibiotic resistance genes (Beaber *et al.*, 2002; Waldor *et al.*, 1996), ICEs can mediate the transfer of a very diverse set of functions including virulence factors (Osorio *et al.*, 2008; Seth-Smith *et al.*, 2012), resistance to

heavy metals (Rodríguez-Blanco *et al.*, 2012), aromatic compounds degradation (Ohtsubo *et al.*, 2012; Ravatn *et al.*, 1998), and nitrogen fixation (Sullivan & Ronson, 1998).

Transduction is a process in which genetic material is transferred from one bacterium to another by bacteriophages. Bacteriophages acquire new genetic material from bacteria randomly (generalized transduction) or specific regions adjacent to the phage genome integration sites (specialized transduction). Upon their subsequent infection of another bacterial cell, the new genetic material is introduced after integration of the phage genome into the bacterial genome. Many phages establish lysogeny by integrating their genome into the bacterial genome as a prophage. Prophages act as an agent for lateral gene transfer in many bacteria (Canchaya *et al.*, 2003). Lysogenic conversion of an avirulent bacterium to a virulent state through lysogeny is a common phenomenon in bacterial populations (Brüssow *et al.*, 2004). Lysogenic conversion genes introduced by prophages may provide a selective advantage to the recipient bacterial host (Canchaya *et al.*, 2003). Prophages with virulence factors are found within the genome of *Vibrio cholerae*, *Corynebacterium diphtheriae*, *E. coli*, *Clostridium botulinum*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Salmonella enterica* serovar Typhimurium (Brüssow *et al.*, 2004).

Some natural habitats of microbes are tightly interwoven in such a way that they can easily exchange their genetic materials within bacteria living in the same community (Koonin *et al.*, 2001). For instance, microbial mats (Risatti *et al.*, 1994; Ward *et al.*, 1998), biofilms (Madsen *et al.*, 2012), and the normal microbiota of animals (Tannock, 2000; Vieira de Souza F, 2012) provide good environments for lateral transfer of genes between diverse bacteria. It was demonstrated that a conjugative plasmid can laterally transfer genes within a biofilm (Ghigo, 2001). It was found that demonstrated that *Lactobacillus* spp. can exchange antibiotic resistance

genes between native bacterial strains within the gastrointestinal tract of chickens (Vieira de Souza *et al.*, 2012).

Lateral gene transfer of specific genes can provide a selective advantage to the recipient organism to cope with adverse conditions such as stress, exposure to antibiotics or heavy metals (Ochman *et al.*, 2000), to acquire virulence properties for better infectivity in their host (Blanc-Potard & Lafay, 2003; Davis & Waldor, 2003), or to introduce specific metabolic traits for sustaining new ecological niches (Blanc-Potard & Lafay, 2003; Chan *et al.*, 2011; Ochman *et al.*, 2000). Antibiotic resistance genes can transfer from one bacterial to another by plasmid, transposon or integron. Though it has been widely believed that antibiotic resistance genes are disseminated through mobile genetic elements, recently it has been reported that bacteriophages act as a reservoir of antibiotic resistance genes and can potentially transfer the resistance genes to different bacteria horizontally (Colomer-Lluch *et al.*, 2011). Acquisition of virulence factors through lateral gene transfer is a common phenomenon in bacterial species (Ochman *et al.*, 2000). Virulence plasmids are frequently isolated from pathogenic bacteria that include but are not limited to *Shigella* (Maurelli *et al.*, 1985), *Yersinia* (Portnoy *et al.*, 1981), *Salmonella* (Ahmer *et al.*, 1999), and *Enterococcus* spp. (Stevens *et al.*, 1992). Many of the plasmids containing virulence factors are self-transmissible and transmit their plasmid borne-virulence factors to the recipient cells via conjugation (Isberg & Falkow, 1985; McDaniel & Kaper, 1997).

In addition to virulence plasmids, genomic islands contribute significantly to lateral transfer of genes among different bacterial species (Hacker *et al.*, 1997). Pathogenicity islands contribute significantly to the virulence of many pathogenic bacteria (Hacker & Kaper, 2000) and many of the virulence factors are disproportionately associated with genomic islands (Ho Sui *et al.*, 2009; Lerouge & Vanderleyden, 2002). The lateral transfer of genes among different

bacterial species by genomic islands is revealed by the persistence of same genomic islands among distantly related bacteria separated by millions of years of evolutionary change (Barinaga, 1996). The promiscuous dissemination of genomic islands among bacteria is shown by the presence of common genomic islands in different bacterial species at different chromosomal regions (Hare *et al.*, 1999).

Lateral gene transfer may result in the introduction of new metabolic traits in bacteria that help them to survive in adverse ecological niches (Lawrence, 1999; Ochman *et al.*, 2000). Espinosa-Urgel and Kolter (Espinosa-Urgel & Kolter, 1998) showed that laterally transferred *gapC* gene, which was predicted to encode a glyceraldehyde-3-phosphate dehydrogenase, provided an adaptive role in the survival of *E. coli* in aquatic environments. A laterally transferred *scr* gene of *Salmonella senftenberg that encodes a* phosphotransferase system is found within a conjugative transposon CTnscr94 (Hochhut *et al.*, 1997). This *scr* gene confers the ability to use sucrose as a sole carbon source, thereby broadening the versatility of this bacterium to adapt new ecological niches. The cj0480c–cj0490 genomic island of *Campylobacter jejuni* encodes enzymes required for L-fucose utilization that help this bacterium to grow on this carbon source and provides a competitive advantage to colonize the chicken gut (Stahl et al., 2011).

2.7 Application of phages in bio-control

The concept of phage therapy to treat infectious disease is centuries old. The advent of antibiotics and its application to treat patients with infectious disease successfully has downsized the research and practices of phage therapy. However, the emergence of antibiotic resistance bacterial strains has made the treatment of infectious diseases harder and led to a resurgence of

interest in phage therapy for pathogens that are resistant to multiple antibiotics. Moreover, some infectious diseases are more difficult to treat with antibiotics due to the presence of bacteria in a specialized colonized form, e.g., biofilms. Therefore, the search for alternative anti-infective treatments, with the aid of recent technological advances, has rejuvenated the interest and scientific capability in using phage therapy for treating infectious diseases in animals and plants (Fischetti *et al.*, 2006). Recently, it has been have demonstrated that bacteriophages ϕMR299-2 and ϕNH-4 can effectively eliminate *Pseudomonas aeruginosa* from the murine lung and on cystic fibrosis lung airway cells (Alemayehu *et al.*, 2012). This finding could be an important development for treating cystic fibrosis patients infected with multi-drug resistance *P. aeruginosa* associated with accelerated progression of cystic fibrosis (Lechtzin *et al.*, 2006). It showed that bacteriophage P3-CHA can be used effectively as a prophylactic and therapeutic agent for the prevention and treatment of pulmonary infection cause by *P. aeruginosa* (Morello *et al.*, 2011). Bacteriophage treatments targeting *Burkholderia cenocepacia (Carmody et al., 2010)* and *B. cepacia* (Seed & Dennis, 2009), responsible for acute pulmonary disease, in an infected animal model were highly effective. Over the past three decades, several other animal model experiments were carried out to treat bacterial infections with bacteriophages (O'Flaherty *et al.*, 2009; Pouillot *et al.*, 2012; Smith & Huggins, 1983; Tiwari *et al.*, 2011). The first phase II clinical trial performed under European regulations on bacteriophage treatments to treat chronic otitis due to antibiotic-resistant *P. aeruginosa* with bacteriophage were proved to be safe and effective (Wright *et al.*, 2009). Bacteriophages were shown to effectively control *Listeria monocytogenes* contamination in Ready-To-Eat foods (Guenther *et al.*, 2009; Holck & Berg, 2009).

In addition to bacteriophages, phage endolysins are also promising candidates for the treatment of infectious diseases (Borysowski *et al.*, 2006; Fenton *et al.*, 2010). Most of the endolysins experimentally used to treat bacterial infections were effective against Gram-positive bacteria due to the inherent nature of their cell walls wherein the peptidoglycan layer is the outermost layer (Cheng *et al.*, 2005; Jado *et al.*, 2003; O'Flaherty *et al.*, 2005). The outer membrane envelope of Gram-negative bacteria inhibits the exposure of peptidoglycan layer that may be attacked by phage ensolysins. Without the help of lysis accessory proteins or membrane-disrupting agents, endolysins cannot attack Gram-negative bacteria externally. Recently, an endolysin from T4 phage was engineered with the FyuA-targeting domain derived from pesticin, a soluble protein which also shares a domain similar to T4 lysozyme (Lukacik *et al.*, 2012). This hybrid lysine is able to penetrate the outer membrane barrier of pathogens *Yersinia pestis* and *E. coli* and kill them effectively. Endolysins with broader activity that can kill both Gram-negative and Gram-positive bacteria have also been identified (Yoong *et al.*, 2004).

2.8 Application of bacteriophage in aquacultures

Since cultured fish, like human and other animals, are threatened by microbial pathogens, effective treatment and preventive measures are essential to avoid economic loss. The treatment of fish diseases caused by bacteria is hampered by the emergence of antibiotic-resistance strains (Hemstreet, 2010; Nakai & Park, 2002; Welch *et al.*, 2009). The treatment of *Pseudomonas plecoglossicida* infected Ayu fish with bacteriophages PPpW-3, PPpW-4 proved to be effective in both the experimental aquaria (Nakai & Park, 2002) and commercial ponds (Park and Nakai 2003) to reduce fish mortality in aquaculture settings. Nakai and Park also showed that yellowtail infection with *Lactococcus garvieae* can be treated with feed-supplemented

bacteriophage PLgY (Nakai & Park, 2002). Bacteriophages ΦeiAU, ΦeiDWF and ΦeiAUP10 that are specific to *Edwardsiella ictaluri* have demonstrated limited protection against Enteric Septicemia of Catfish (ESC) caused by *Edw. ictaluri* (Carrias, 2011).

2.9 Application of bacteriophage in diagnostics

The application of bacteriophages as diagnostic agents has advantages over other conventional techniques. The interactions of phages with their bacterial hosts are very specific. The bacteriophage infection of bacterial cells followed by their amplification provides a high analytical sensitivity of the phage based diagnosis tools. The detection of bacterial pathogens using a bacteriophage as a diagnostic tool is cost-effective compared to other conventional diagnostic tools. A simple plaque assay can detect the presence of specific bacterial pathogens by determining the zone of bacterial lysis (Głłośnicka & Dera-Tomaszewska, 1999; Marples & Rosdahl, 1997). A highly sensitive and specific qPCR based technique was developed for the rapid identification of *Yersinia pestis* using a lytic bacteriophage (Sergueev *et al.*, 2010).

Bacteriophages have been engineered with reporter genes for rapid identification of bacterial pathogens in food, water and clinical samples (Smartt *et al.*, 2012). The detection of food-borne pathogens using bacteriophages has advantages over traditional technique since phage-based tests are rapid and only detect viable bacteria responsible for food-borne illness. The *gfp, lux* and *luc* are the most frequently used genes or gene clusters to engineer bacteriophages for rapid and highly sensitive diagnostic applications (Funatsu *et al.*, 2002; Namura *et al.*, 2008; Oda *et al.*, 2004; Piuri *et al.*, 2009; Schofield *et al.*, 2012; Tanji *et al.*, 2004). The *luxAB* tagged bacteriophages were effectively used for the identification of *E. coli*

(Waddell & Poppe, 2000), *Salmonella* (G *et al.*, 2008), *Listeria* (Loessner *et al.*, 1997), *Yersinia* (Schofield *et al.*, 2009), and *Bacillus* (Schofield & Westwater, 2009).

In addition to the identification of bacterial pathogens, engineered reporter phages have also been applied to monitor the drug susceptibility of bacterial pathogens (Banaiee *et al.*, 2001; Jacobs *et al.*, 1993; Jain *et al.*, 2012). For example, the drug susceptibility nature of *Mycobacterium tuberculosis* was determined using luciferase tagged phage (Jacobs *et al.*, 1993).

Several commercial kits, including FASTPlaqueTB kit, FASTPlaque-Response™ kit, and KeyPath™ test use phage-based techniques for the detection of bacterial pathogens. The FASTPlaqueTB kit (Biotec Laboratories, Ipswich, UK) is based on the phage (mycobacteriophage D29) amplification technique for the detection of *Mycobacterium tuberculosis* (Albay *et al.*, 2003). The FASTPlaque-Response™ kit applies the same principle of the FASTPlaqueTB kit with the exception that it uses duplicate samples, one with antibiotic and another without antibiotics, to discriminate between antibiotic-resistant and antibiotic-sensitive *M. tuberculosis* (Mole & Maskell, 2001). The KeyPath™ test designed by MicroPhage (Longmont, Colorado, USA) is based on bacteriophage amplification that is detected by a lateral flow immunoassay with colloidal gold particles conjugated with monoclonal antibodies specific to the assayed bacteriophage (Dreiling *et al.*, 2010). This kit is intended for the detection and differentiation of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) directly present in the blood. Recently, the FDA has approved this detection kit for commercial use in different hospitals and clinical settings (http://microphage.com/news/files/FDA%20Press%20Release.pdf).

2.10 Advantages of using bacteriophages for phage therapy

Phage therapy has several advantages over conventional treatment with antibiotic and other chemotherapy. Moreover, the paucity of novel antibiotics to treat multi-drug resistant bacterial strains makes the treatment of many infectious diseases harder and sometimes impossible to treat them effectively (Stubbings & Labischinski, 2009).

Bacteria that form biofilms are more difficult to kill with antibiotics due to the inherent differences in bacterial physiology and inaccessibility when living within a biofilm (Hoyle & Costerton, 1991; Mah & O'Toole, 2001; Zhang *et al.*, 2011). Bacteriophages can serve as a potential alternative to antibiotics to kill bacteria living within biofilm (Azeredo & Sutherland, 2008; Donlan, 2009). Bacteriophages with the ability to degrade bacterial biofilms have been described recently (Chibeu A *et al.*, 2012; Curtin & Donlan, 2006; Lu & Collins, 2007). Lu and Collins (Lu & Collins, 2007) engineered bacteriophage T7 with dispersin B, an enzyme that can degrade hydrolyze adhesin required for biofilm formation, to kill bacteria by attacking bacteria in biofilms as well as the biofilm matrix.

The application of bacteriophages for phage therapy is cost-effective and much lower than that associated with antibiotics since the production of large-scale preparations of bacteriophages depends on the growth of their bacterial host which is typically very cost-effective to grow (Loc-Carrillo & Abedon, 2011). Recent technological advances also make the production of bulk amounts of pharmaceutical grade phage preparations for therapeutic application economically viable (Kramberger *et al.*, 2010).

Due to the highly specific nature of phage-host interactions, bacteriophages attack and kill specific bacterial strains, and can have a restricted host range limited to specific strains or

can have a very broad host range encompassing closely related bacterial genera without affecting the normal bacterial microbiota (Chibani-Chennoufi *et al.*, 2004; Hyman & Abedon, 2010). Unlike bacteriophages, treatment of infectious diseases with antibiotics can indiscriminately kill the members of the normal microbiota (Jernberg *et al.*, 2010) and cause a disturbance in the ecological balance between the host and the normal microbiota (Sullivan *et al.*, 2001).

The application of a phage as a therapeutic agent has a low environmental impact since phages have a narrow host range (Hyman & Abedon, 2010) and, unlike antibiotics, the compositions of phages themselves do not have any adverse effects on the environment (Abedon & Thomas-Abedon, 2010; Loc-Carrillo & Abedon, 2011). On the other hand antibiotics and other chemical therapeutics exposed to the environment may disturb microbial community structures (Edlund *et al.*, 2012), expand antibiotic resistance by exerting selective pressure on bacteria in the environment (Thiele-Bruhn & Beck, 2005) and finally affect the overall ecological balance of the microbe in the environment (Levy, 1997).

2.11 Disadvantages of using bacteriophage for phage therapy

The application of bacteriophages as therapeutic agents also has some potential disadvantages. The potentially narrow host range of bacteriophages and the generation of phage-resistant bacterial mutants make them limited in their use as therapeutic agents (Hyman & Abedon, 2010). However, a technique has been devised to overcome the narrow host range of bacteriophages (Kelly *et al.*, 2011). In this technique phages with narrow host range are passaged on the phage resistant bacterial strain until the appearance of mutant phages with lytic activities to their hosts.

The administration of bacteriophage through an oral route may inactivate the phage due to the highly acidic conditions of the stomach along with the presence of enzymes and other digestive compounds (Joerger, 2003). Moreover, orally administered bacteriophages have been shown to be ineffective at reaching the targeted affected organs (Oliveira *et al.*, 2009). However, microencapsulation of bacteriophages into chitosan-alginate microspheres can prevent the inactivation of bacteriophages in the gastrointestinal tract while preserving phage bioactivity (Ma *et al.*, 2008).

Bacteriophages introduced into a host for phage therapy have a tendency to be cleared by the host reticuloendothelial system (RES) (Geier *et al.*, 1973). This shortcoming was overcome by serially passaging phage to generate long-circulating bacteriophages (Merril *et al.*, 1996). Point mutations on the phage capsid protein have been attributed for the avoidance of expulsion from the host RES system (Merril *et al.*, 1996; Vitiello *et al.*, 2005). Conjugation of bacteriophages with polyethylene glycol (PEG) also can increase the blood circulation time and reduce T-helper type I immune responses (Kim *et al.*, 2008).

The application of bacteriophage as therapeutic agents may result in the release of endotoxin and superantigens that could induce severe inflammation and septic shock (Munford, 2006). To avoid the release of endotoxin after bacterial lysis, lysis-deficient and/or non-replicating bacteriophages have been designed (Hagens & Blasi, 2003; Hagens *et al.*, 2004; Matsuda *et al.*, 2005). Recent technological advances have helped to obtain highly purified bacteriophages devoid of endotoxin (Boratynski *et al.*, 2004; Merabishvili *et al.*, 2009).

2.12 Phage-host interactions

The interactions between bacteria and bacteriophages are very diverse and complex. Bacteriophages infect bacteria and exploit their cellular machinery to synthesize their macromolecules for propagation. For the initiation of infection, phages interact with bacterial surface molecules that include outer membrane proteins (Morona *et al.*, 1985), lipopolysaccharides (Lindberg, 1973), flagella (Bradley *et al.*, 1984), pili (Faruque *et al.*, 2005) and other bacterial appendages (Lindberg, 1973). After a successful attachment, the interaction between phage tail and bacterial surface appendages trigger the release of phage genomic DNA inside the bacterial cytoplasm. The transport of phage DNA across the cytoplasmic membrane is coordinated with the help of phage (Alcorlo *et al.*, 2007; Perez *et al.*, 2009) and host proteins (García & Molineux, 1996).

The evolutionary clash with phage has forced bacteria to evolve multiple defense mechanisms (Petty *et al.*, 2007). These host defense mechanisms are functional at all stages of the phage infection cycle (Hoskisson & Smith, 2007). Bacterial hosts exploit numerous mechanisms to avoid phage infection (Labrie *et al.*, 2010). The principal mechanisms of phage resistance include adsorption inhibition, interference with phage DNA injection, abortive infection (Abi), restriction-modification (R-M) system and clustered regularly interspaced short palindromic repeats (CRISPRs) that mediate phage resistance (Barrangou *et al.*, 2007; Forde & Fitzgerald, 1999). Despite intensive research, the mechanism of host defenses and phage counter measures are still poorly understood.

The first line of bacterial defense is the inhibition of phage attachment to bacterial surface appendages (Lindberg, 1973). Bacteria can prevent the exposure of phage receptors on the

bacterial cell surface by synthesizing surface structures that block phage receptors (Labrie, Samson et al. 2010). Bacteriophage BF3 that infects *E. coli* encodes a receptor-blocking lipoprotein (Llp_{BF3}) that is synthesized after infection (Mondigler *et al.*, 2006). This lipoprotein blocks the binding of phage BF3 to its receptor BtuB (Mondigler *et al.*, 2006). Protein A of *S. aureus* inhibits the binding of the staphylococcal typing phages by masking the phage receptors, thereby resulting in phage resistance (Nordström & Forsgren, 1974). Likewise, the M protein of *Streptococci* prevents phage A25 infection by masking the phage-binding receptors (Cleary & Johnson, 1977). Changing of the three-dimensional conformation of a bacterial receptor required for phage infection can result in phage resistance. As described in this thesis, bacteriophage ΦeiAU cannot infect *Edw. ictaluri* strain Alg-08-183 with an altered OmpLC due to a point mutation in the loop 8 of OmpLC that is required for phage ΦeiAU infection (Hossain *et al.*, 2012). The production of extracellular matrix on the bacterial surface can inhibit the access of bacteriophages to the bacterial receptor (Labrie *et al.*, 2010). Likewise, the overproduction of alginate in *Azotobacter chroococcum* encapsulates the bacterium that can then resist phage infection (Hammad, 1998).

Restriction modification systems are by far the most characterized and best understood phage defense mechanisms, and function by degrading incoming phage DNA whereas host DNA is protected by methylation (Hoskisson & Smith, 2007). Recently a novel type IV enzyme, GmrSD, has been identified as an evolutionary counter defense for the modification of phage DNA after the emergence of hydroxymethyl cytosine (Bair & Black, 2007). Surprisingly, a new phage counter measure has emerged to avoid the GmrSD mediated exclusion of T4 phage (Bair *et al.*, 2007). This example indicates that the bacteria and virus are engaged in an evolutionary competition whereby each side is constantly changing their strategy to outmaneuver each other.

The Abi, arguably the most efficient mechanism to restrict phage infection, is characterized by a normal start of phage infection followed by an interruption in lytic cycle development, leading to the release of few or no progeny particles and cell death. Most of the Abi systems were identified and characterized in *Lactococcus lactis* and about 20 different Abi systems have been isolated in this species (Chopin *et al.*, 2005). In *Erwinia carotovora*, a novel Abi mechanism based on protein-RNA toxin-antitoxin (TA) pair has been described (Fineran *et al.*, 2009). This phage resistance mechanism unraveled not only a novel TA family but also the novel function of this pair to prevent phage infection in diverse Gram-negative bacteria.

Phage DNA injection inhibition is another strategy used by bacteria to prevent phage infection (Labrie *et al.*, 2010). *Lactococcus lactis* strains MG1363 and 112 can resist phage c2 and 923 infection, respectively, by preventing their genomic DNA entry into the host cells (Rakonjac *et al.*, 2005). Superinfection exclusion system of bacteria provides phage resistance by inhibiting the phage DNA entry into the cytoplasm (Labrie *et al.*, 2010). Recently, it was demonstrated that gp15 encoded within the prophage HK97 prevents phage infection by phages HK97 and HK75 by preventing the phage DNA entry into the host *E. coli* cytoplasm (Cumby *et al.*, 2012).

Chapter II

An Outer Membrane Porin Protein Modulates Phage Susceptibility in *Edwardsiella ictaluri*

1. Abstract

Bacteriophages ΦeiAU and ΦeiDWF are lytic to the catfish pathogen *Edwardsiella ictaluri*. The *Edw. ictaluri* host factors that modulate phage-host interactions have not been described previously. This study identified eleven unique *Edw. ictaluri* host factors essential for phage infection by screening a transposon mutagenized library of two *Edw. ictaluri* strains for phage-resistant mutants. Two mutants were isolated with independent insertions in the *ompLC* gene that encodes a putative outer membrane porin. Phage binding and efficiency of plaquing (EOP) assays with *Edw. ictaluri* EILO, its *ompLC* mutant and a complemented mutant demonstrated that OmpLC serves as a receptor for phage ΦeiAU and ΦeiDWF adsorption. Comparison of translated OmpLCs from 15 different *Edw. ictaluri* strains with varying degrees of phage susceptibility revealed that amino acid variations were clustered on the predicted extracellular loop 8 of OmpLC. Deletion of loop 8 of OmpLC completely abolished phage infectivity in *Edw. ictaluri*. Site-directed mutagenesis and transfer of modified *ompLC* genes to complement the *ompLC* mutants demonstrated that changes in *ompLC* sequences impact the degree of phage susceptibility. Furthermore, *Edw. ictaluri* strain Alg-08-183 was observed to be resistant to ΦeiAU, but phage progeny could be produced if phage DNA was electroporated into this strain. A host-range mutant of ΦeiAU, ΦeiAU-183, was isolated that was capable of

infecting strain Alg-08-183 by using OmpLC as a receptor for adsorption. The results of this study identified *Edw. ictaluri* host factors required for phage infection and indicated that OmpLC is a principal molecular determinant of phage susceptibility in this pathogen.

2. Introduction

Bacteriophages ΦeiAU and ΦeiDWF are lytic to the Gram-negative enteric pathogen *Edw. ictaluri* (Walakira *et al.*, 2008), the causative agent of enteric septicemia (ESC) of catfish (Hawke *et al.*, 1981). These two bacteriophages have similar morphology with icosahedral heads and non-contractile tails and are classified as a member of the *Siphoviridae* (Carrias *et al.*, 2011; Walakira *et al.*, 2008). Our laboratory has previously published a comparative genome analysis of these phages which revealed that the phage ΦeiAU and ΦeiDWF genomes are >95% identical at a nucleotide level with some minor (albeit potentially functionally significant) changes in their predicted tail fiber proteins (Carrias *et al.*, 2011).

The dynamics of phage-host interactions are complex. Bacteriophages rely on bacterial hosts for their propagation, with the nature of the phage-host interaction being dependent upon both the bacterial host and the specific phage (Friedman *et al.*, 1984; Roucourt & Lavigne, 2009). Receptors on the bacterial cell surface are the first site of phage interaction and successful phage adsorption triggers phage genome ejection into the bacterial cytoplasm (Adams, 1959). Upon entry, the phage exploits host intracellular components for their transcription and replication (Guttman *et al.*, 2005). For example, T7 and T3 phages utilize *E. coli* RNA polymerase (Chamberlin *et al.*, 1970; Dharmgrongartama *et al.*, 1973) and thioredoxin (Krüger & Schroeder, 1981; Mark & Richardson, 1976) for the transcription of early genes and replication of phage genomic DNA, respectively. Though the host factors for phage T7 (Krüger & Schroeder, 1981) and phage λ (Friedman *et al.*, 1984) have been studied extensively, recent

genome-wide screening of an *E. coli* K-12 in-frame, single gene knock out library (Baba *et al.*, 2006) provided a more complete analysis of T7 (Qimron *et al.*, 2006) and λ phage (Maynard *et al.*, 2010) dependencies on host factors. In addition to finding specific host gene products directly involved in phage infection and development, genome-wide screening also enables the identification of host factors that may be indirectly involved in phage infection, such as regulatory factors. *Edw. ictaluri* host factors for phage ΦeiAU and ΦeiDWF infection have not been previously investigated. This study will facilitate application of *Edw. ictaluri*-specific phages as a specific diagnostic assay of primary disease isolates from channel catfish and more broadly contribute to our knowledge of phage-host molecular interactions.

The cell surface components of Gram negative bacteria such as outer membrane proteins (Datta *et al.*, 1977), lipopolysaccharides (LPS) (Lindberg, 1973), pili (Chibeu *et al.*, 2009) and flagella (Schade *et al.*, 1967) serve as receptors for phage adsorption and subsequent infection processes. Bacteriophages K2, SSI, T5 and H8 adsorb to OmpA (Datta *et al.*, 1977), OmpC (Beher & Pugsley, 1981), TonA (Menichi & Buu, 1983) and FepA (Rabsch *et al.*, 2007), respectively, for their initiation of infection. In addition to binding to primary protein receptors, some bacteriophages also utilize secondary receptors, mostly LPS, for their adsorption. For instance, bacteriophages K20 (Silverman & Benson, 1987), Ox2 (Sukupolvi, 1984), TuII (Datta *et al.*, 1977), T2 (Lenski, 1984) and T4 (Yu & Mizushima, 1982) can utilize outer membrane porin proteins and lipopolysaccharide (LPS) of *E. coli* as a receptor for phage adsorption and infection. Moreover, bacteriophage Ox2 uses OmpA and OmpC of *E. coli* K-12, though these two proteins are very different with respect to their primary structures and functions, as a receptor for adsorption and infection (Morona & Henning, 1984). The lack of expression or altered structure of those bacterial surface components modulates phage susceptibility of

different bacterial strains to their specific phages. In a previous study, *Edw. ictaluri* strains were observed to have varying degrees of phage susceptibility (Walakira *et al.*, 2008). This study was initiated to determine the molecular determinants of phage infection in *Edw. ictaluri*.

3. Materials and Methods

Bacterial strains and culture conditions. *Edw. ictaluri* strains were grown on Trypticase Soy Broth or Agar (TSB/TSA) at 28°C with aeration. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium or 2×YT (Yeast Extract Tryptone), with aeration. Commercially-prepared electrocompetent *E. coli* strain DH10B (Invitrogen) was routinely used for electroporation of plasmid constructs for cloning purposes. All of the primers used in this study are listed in Table 1. When required, growth medium was supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), colistin (10 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}) and/or $CaCl_2$ (500 μM). For propagation of phage ΦeiAU, ΦeiDWF, or ΦeiAU-183, *Edw. ictaluri* strains EILO, ML-08-116, or Alg-08-183 were routinely used, respectively. Phage lysates were prepared by double layered soft agar overlay (Fortier & Moineau, 2009).

Transposon mutagenesis and screening for phage-resistant mutants. Transposon mutagenesis of *Edw. ictaluri* EILO and ML-08-116 was carried out by filter mating experiments according to the methods described previously (Maurer *et al.*, 2001). Briefly, the donor *E. coli* SM10λpir containing pLOF-Km (Herrero *et al.*, 1990) was grown to an OD_{600} of 1.0. The recipient *Edw. ictaluri* EILO was grown to an OD_{600} of 1.0. Cultures were mixed in a ratio of 4:1 (recipient: donor) in a 15 ml polypropylene conical tube containing 10 mM $MgSO_4$. The mixture was vacuum filtered through 0.45-μm MicroFunnel filter unit (Pall Corporation). This membrane filter was transferred to TSA plates and incubated for 4 hours at 24°C. After 4 hours of incubation, the filter was transferred to LB plates containing 1 mM IPTG and incubated for 16

hours for the induction of transposase. Filters were then suspended in 3 ml 10 mM $MgSO_4$ and vortexed to dislodge cells. Cell suspensions were then mixed with a high titer of relevant phages (~5.0×10^{12} PFU/ml) and incubated for 15 min at room temperature to allow for phage adsorption. Phage-inoculated cells were plated and selected on TSA plates containing kanamycin and colistin for the selection of phage-resistant *Edw. ictaluri* mutants. The phage-resistant mutants were further verified by determining their EOP according to the methods described above.

Identification of transposon insertion sites in phage-resistant mutants. Transposon insertion sites in the chromosome of *Edw. ictaluri* phage-resistant mutants were determined by inverse PCR (Ochman *et al.*, 1988). Genomic DNA was isolated from an overnight culture of each mutant according to the methods described previously (Ausubel *et al.*, 1999), digested with HindIII and self-ligated with T4 DNA ligase. One µl of the ligation mix was used as template for PCR with PyroPhage 3173 DNA polymerase (Lucigen) using transposon-specific primers (Table 1). Amplicons were purified with Promega Wizard SV PCR purification kit. If inverse PCR failed to amplify the transposon-flanking regions, subcloning was performed to identify the transposon insertion sites. One µg of genomic DNA was digested with SphI and ligated into a SphI-digested and dephosphorylated pUC19. The ligation mix was then transformed into *E. coli* strain DH10B and subclones were selected on 2×YT containing kanamycin. Plasmid DNA was purified using the Promega Wizard SV miniprep kit. Inverse PCR amplicons and plasmid inserts from subclones were sequenced with nested transposon-specific primers (Table 1). Sequencing was performed at the Lucigen using an ABI 3730xl sequencer. The transposon insertion sites and genes were compared to genes within the GenBank nr/nt database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTx and BLASTn algorithms.

Complementation of selected phage resistant mutants. To confirm the involvement of *Edw. ictaluri* genes required for phage infection, a complementation study of selected phage-resistant mutants was performed by introducing the wild-type copy of the disrupted gene. Phage-resistant mutants R-165, ML-17, ML-41 and ML-64 were complemented with *ompLC*, hypothetical gene NT01EI_2881, *psoB* gene and porin thermoregulatory gene, *envY*, respectively, from their corresponding wild-type strain. In addition to complementation of the R-165 mutant with *ompLC* from *Edw. ictaluri* EILO, the R-165 mutant was also complemented with *ompLC* from *Edw. ictaluri* Alg-08-183, ML-08-116, ML-08-117 and Alg-08-199. The *ompLC* gene with its predicted promoter region was amplified from *Edw. ictaluri* strains EILO, Alg-08-183, ML-08-116, ML-08-117 and Alg-08-199 using the high-fidelity Takara Ex Taq polymerase and cloned into the pCR2.1 vector (Invitrogen), a plasmid with a pUC19 origin of replication that has a low copy number at reduced growth temperatures (Lin-Chao *et al*., 1992), resulting in pOmpLC-EILO, pOmpLC-183, pOmpLC-116, pOmpLC-117 and pOmpLC-199, respectively. Complementation of the *ML-17* mutant was accomplished by cloning the hypothetical gene NT01EI_2881 from *Edw. ictaluri* ML-08-116 into plasmid pCR2.1 with its native promoter. The *psoB* and *envY* genes from *Edw. ictaluri* ML-08-116 were amplified by PCR with PyroPhage 3137 Polymerase (Lucigen). The PCR-amplified DNA fragments were purified by Wizard SV PCR purification kit, digested with *HindIII*, and ligated with HindIII-digested pUC19. Ligation mixtures were electroporated into *E. coli* strain DH10B. The recombinant clones selected after blue-white screening were confirmed by PCR and sequencing to contain the correct insert sequence. These constructs were then introduced into the appropriate *Edw. ictalur*i mutants by electroporation using previously described procedures (Russo *et al.*, 2009). The phage sensitivity of each complemented mutant was determined as described above

and compared to those of its respective isogenic parental mutant strain containing the empty vector.

Modeling of the OmpLC protein. The three-dimensional structural model of *Edw. ictaluri* OmpLC was predicted by the **SWISS**-MODEL protein structure homology-**modeling** server (Bordoli *et al.*, 2008). The osmoporin OmpK36 of *Klebsiella pneumoniae* was used as a template model due to its high degree of similarity with OmpLC from *Edw. ictaluri* (Dutzler *et al.*, 1999). Amino acids 23 to 360 of *Edw. ictaluri OmpLC* were included in the model. The resulting homology structure generated by SWISS MODEL was visualized and reproduced by PyMOL (version 1.3r) (DeLano, 2004).

Isolation of mutant phage ΦeiAU-183 lytic to *Edw. ictaluri* strain Alg-08-183. Mutant phage ΦeiAU-183 that is lytic to *Edw. ictaluri* strain Alg-08-183 was isolated by serially passaging wild-type phage ΦeiAU on *Edw. ictaluri* strain Alg-08-183, a wild-type strain resistant to the original phage ΦeiAU. *Edw. ictaluri* strain Alg-08-183 was grown from a primary glycerol stock (not serially passaged) and challenged with phage ΦeiAU by double layered soft-agar overlay. Phages were collected from the top agar and subsequently used for challenging another primary culture of *Edw. ictaluri* strain Alg-08-183. The experiment was repeated until visible plagues were observed on the top agar. Once visible plaques were observed, phages were doubly purified and stored in 7.0% dimethyl sulfoxide (DMSO) at -80°C until future use.

Construction and Complementation of *Edw. ictaluri* Alg-08-183 *ompLC*::Tn5 mutant resistant to ΦeiAU-183. The *ompLC* gene in pOmpLC-183 was disrupted *in vitro* using EZ-Tn5 <Tet> insertion kit (Epicentre Biotechnologies) and electroporated into *E. coli* strain DH10B. Colonies isolated from 2×YT plates containing tetracycline, kanamycin and ampicillin

were screened by PCR to find a clone with an appropriate Tn5 insertion within the *ompLC* coding sequence. Transposon insertion sites were mapped by sequencing of the PCR product with the transposon-specific primer Tn5out (Table 1). Extracted plasmid DNA from desired clones was used as a template for the amplification of the Tn5 transposon and its 500 bp flanking *ompLC* sequences with primer $165F_{BglII}$ and $165R_{BglII}$ (Table 1). Amplicons were subjected to gel electrophoresis to separate template plasmid from PCR products and an excised DNA band was purified with the Wizard SV PCR purification kit. BglII-digested amplicons were ligated into a BglII-digested and dephosphorylated suicide vector pGP704 (Miller & Mekalanos, 1988) using T4 DNA ligase and the resulting vector, pGP183, was electroporated into SM10λpir. The pGP183 plasmid in SM10λpir was delivered to *Edw. ictaluri* strain Alg-08-183 by conjugation according to the methods described previously (Maurer *et al.*, 2001). *E ictaluri* strain Alg-08-183 transconjugants resistant to phage ΦeiAU-183, due to the disruption of the *ompLC* gene by allelic exchange, were selected by challenging with a high titer (~5.0×10^{12} PFU/ml) of phage ΦeiAU-183 on 2×YT supplemented with tetracycline and colistin. The site of the *ompLC* gene disruption on the genome of *Edw. ictaluri* Alg-08-183 *ompLC*::Tn5 mutant was verified by PCR followed by sequencing.

Edw. ictaluri strain Alg-08-183 *ompLC*::Tn5 was complemented with *ompLC* from strain Alg-08-183 cloned into plasmid pCR2.1 (pOmpLC-183) with its native promoter. The *Edw. ictaluri* strain Alg-08-183 *ompLC*::Tn5 mutant was also complemented with *ompLC* from strain EILO cloned into plasmid pCR2.1 (pOmpLC-EILO). The EOPs of the complemented mutants were determined with phage ΦeiAU and ΦeiAU-183 according to the methods described above.

Nucleotide sequence accession numbers. The *Edw. ictaluri ompLC* genes reported in this study were sequenced from the wild-type *Edw. ictaluri* strains listed in Table 3 and submitted to GenBank under accession numbers JN604516-JN604530.

4. Results

Transposon mutagenesis and isolation of phage-resistant mutants of *Edw. ictaluri*. Bacterial host factors play significant roles in the growth and propagation of bacteriophages (Friedman *et al.*, 1984; Hashemolhosseini *et al.*, 1994; Qimron *et al.*, 2006). To identify the *Edw. ictaluri* host factors required for phage infection, we screened mini-Tn10 transposon-mutagenized libraries of *Edw. ictaluri* strains EILO (n= approximately 25,000 mutants) and ML-08-116 (n= approximately 30,000 mutants) that are susceptible to infection with both ΦeiAU and ΦeiDWF. The transposon-mutagenized libraries were challenged with a high titer of phages ΦeiAU or ΦeiDWF, respectively, to isolate phage-resistant mutants. A total of 12 mutants were confirmed as unique phage-resistant mutants by a soft agar overlay assay. The growth rate of the mutants was tested and none of the mutants showed an impaired growth rate in TSB as compared to wild-type strains (data not shown).

Identification of genes interrupted in phage-resistant mutants. With either inverse PCR (mutant ML-43, ML-44 and ML-100) or subcloning (rest of the mutants) followed by sequencing with primers specific to the Tn10 transposon, the transposon insertion sites were identified within the genome of phage-resistant mutants. The list of predicted ORFs (open reading frames) that were disrupted by transposon insertion is provided in Table 3.

All of the transposon insertions that resulted in a phage-resistant phenotype were within predicted ORFs of *Edw. ictaluri*. We identified 11 unique genes from 12 phage-resistant mutants. The predicted outer membrane porin protein LC gene, *ompLC*, was interrupted in two phage-

resistant mutants (R-6 and R-165) and analysis of transposon-insertion sites in these two mutants indicated independent insertion events in different sites within the *ompLC* gene. In mutant ML-64, the transposon was inserted within an insertion sequence (IS element) that was adjacent (1695 bp from the ATG start codon) to a putative porin thermoregulatory protein gene*, envY*. Three different hypothetical proteins with unknown functions were disrupted in three separate phage-resistant mutants (ML-17, ML-100 and R-148). The mutant ML-44, containing an insertion within a predicted ATP-dependent RNA helicase gene, *deaD*, was the only mutant that demonstrated a partial phage-resistant phenotype (Table 3).

Complementation of *Edw. ictaluri* phage-resistant mutants. To confirm that the phage-resistant phenotype observed in *Edw. ictaluri* mutants was due to a transposon insertion, selected mutants (R-165, ML-17, ML-41, and ML-64) were complemented with their wild type genes to test for the restoration of a phage-sensitive phenotype. The complementation of other genes identified in this study (e.g. FimD) will be described in future research. Among these mutants the gene with a transposon insertion in mutant R-165 was targeted first for complementation due to the known involvement of outer membrane porins in phage infectivity in other bacterial species. Mutant R-165, in which the outer membrane porin protein gene (*ompLC*) was inactivated, was complemented with the *ompLC* gene from *Edw. ictaluri* strain EILO. The introduction of *ompLC* to mutant R-165 in *trans* fully restored the phage-sensitive phenotype and exhibited an EOP similar to wild type strain EILO (Figure 1). This finding, together with bioinformatic analyses suggesting that OmpLC serves as a putative outer membrane porin protein, led to the hypothesis that OmpLC acts as a receptor for phage ΦeiAU/ ΦeiDWF adsorption and initiation of infection.

Mutant ML-41, in which *psoB* gene was inactivated by transposon insertion, was complemented with the wild-type *psoB* gene. The *psoB* gene is predicted to encode a GDP-fucose synthetase that is involved in lipopolysaccharide (LPS) biogenesis in many Gram-negative bacteria (Barua *et al.*, 2002; Moran *et al.*, 1994; Skurnik & Zhang, 1996). As expected, the introduction of *psoB* in *trans* restored the phage-sensitive phenotype of mutant ML-41 strain (Table 3).

Mutant ML-17 had a transposon insertion within a gene that had a top BLAST hit indicating a hypothetical protein in *Edw. ictaluri* strain 93-146 (NT01EI_2881), with additional BLAST hits (with similarly low E values) indicating that this gene may encode a Tn10 transposase. Since this was an unexpected result associating a putative transposase with phage infectivity, complementation was attempted for mutant ML-17. Initial attempts to PCR amplify this gene were unsuccessful, likely due to the lack of available genome sequences for this strain. A larger PCR amplicon containing downstream sequences was eventually obtained that also included another downstream ORF (accession number YP_002934278.1) with a predicted gene product that has significant sequence similarity (84% similar) to a hydrolase-oxidase encoded by *Pectobacterium wasabiae*. The introduction of this plasmid pML17 into mutant ML-17 and restoration of a phage-sensitive phenotype confirmed the involvement of this genetic region in phage infection (Table 3). Further experiments are necessary to determine any specific role for a putative Tn10 transposase in phage infectivity.

The first attempt at complementation in *trans* was not successful for mutant ML-64, which had a transposon insertion within an ORF designated as an insertion (IS) element. However, inclusion of an open reading frame that encodes a putative porin thermoregulatory protein EnvY, which is downstream of that IS element, in the complementing plasmid restored

the phage-sensitive phenotype of mutant ML-64 (Table 3). This regulatory protein is predicted to have been inactivated due to a polar effect of the transposon insertion. The predicted EnvY protein has a homologue in *E. coli* K-12 that modulates the temperature-dependent expression of several porin proteins, most notably OmpF and OmpC and lambda phage receptor, LamB (Lundrigan & Earhart, 1984). Further experiments are required to clarify the role of *envY* in the putative regulation of expression of the *ompLC* gene in *Edw. ictaluri*.

OmpLC is the receptor for phage ΦeiAU adsorption. To test the hypothesis that OmpLC serves as a receptor for phage ΦeiAU adsorption, phage binding assays were performed with wild type *Edw. ictaluri* strain EILO, its *ompLC* mutant R-165 and its complemented mutant. Phage ΦeiAU was able to bind to *Edw. ictaluri* strain EILO and its complemented *ompLC* mutant with much higher efficiency (adsorption rate >97%) as compared to its *ompLC* mutant R-165 (EILO *ompLC*::Tn10) that exhibited a binding rate less than 4% (Figure 2). Since the preliminary results obtained from EOPs and binding assays of phage ΦeiAU and ΦeiDWF on *Edw. ictaluri* strain EILO were very similar, the plaquing and binding efficiency of phage ΦeiDWF were not examined in further detail.

Results obtained from the phage binding assay were in agreement with the results from EOP assays carried out with strain EILO, *ompLC* mutant R-165 and its complemented mutant R-165 (pOmpLC-EILO) (Figure 1). Strain EILO and its complemented *ompLC* mutant had very similar EOP (~1.0) whereas phage ΦeiAU was not able to produce any plaques on mutant R-165 at a phage titer of $\sim 1.0 \times 10^{12}$. Together these results indicated that OmpLC protein of *Edw. ictaluri* EILO serves as a receptor for phage ΦeiAU adsorption.

OmpLC variability among *Edw. ictaluri* strains mapped to a 3D protein model. *Edw. ictaluri* strains have varying degrees of susceptibility to phage ΦeiAU infection (Walakira *et al.*,

2008). The reduced phage susceptibility among some strains might be due to the lack of a receptor(s), inhibition of phage DNA injection, restriction modification of phage nucleic acids, and/or abortive infection. To investigate the role of OmpLC protein in contributing to the varying degrees of phage susceptibility, the *ompLC* genes from 15 different *Edw. ictaluri* strains were sequenced and variations in their translated protein sequences were identified. Comparison of *ompLC* gene sequences from 15 different *Edw. ictaluri* strains demonstrated that the promoter and upstream regulatory regions are strikingly identical and differences were primarily observed in the 3' coding region (data not shown).

A three-dimensional model of the OmpLC protein structure was obtained by the SWISS-Model using the structure of the OmpK36 porin from *Klebsiella pneumoniae* as a template (Bordoli *et al.*, 2008; Dutzler *et al.*, 1999). The predicted structure of the OmpLC protein demonstrated a typical porin structure with 16 anti-parallel β strands and 8 extracellular loops (Figure 4). The comparison of the amino acid sequences among 15 different *Edw. ictaluri* OmpLC demonstrated that the amino acid variations, if present in OmpLC, are on the surface-exposed loop 8 (amino acid residues 336 to 349) of the predicted OmpLC protein (Figure 3).

The OmpLC loop 8 domain is critical for phage infectivity. The complementation of *ompLC* mutants of *Edw. ictaluri* strains EILO and Alg-08-183 with loop 8-deleted OmpLC, pOmpLC-ΔL8, completely abolished phage ΦeiAU susceptibility (EOP $<1.0\times10^{-11}$) (Table 4), whereas pOmpLC-EILO, a plasmid construct with a wild-type *ompLC*, complemented *ompLC* mutants of *Edw. ictaluri* strains EILO and Alg-08-183 were highly susceptible to phage ΦeiAU infection (Figure 1 and Figure 5). Four *Edw. ictaluri* strains (ML-08-113, ML-08-116, Alg-08-195 and Alg-08-200) with an aspartate to alanine substitution on residue 344 of OmpLC demonstrated reduced phage susceptibility compared to *Edw. ictaluri* strain EILO (Figure 4).

Edw. ictaluri strains ML-08-117 and Alg-08-199, with two amino acids (isoleucine and serine) inserted at residue 346 and a single amino acid (glutamate to aspartate) substitution at residue 339, respectively, were resistant to phage ΦeiAU infection (Figure 4). The importance of the amino acid sequence on loop 8 of OmpLC for determining the degree of phage susceptibility was confirmed by introducing plasmid constructs pOmpLC-116 (*ompLC* which shared identical sequence with *Edw. ictaluri* strains ML-08-116, ML-08-113, Alg-08-195 and Alg-08-200), pOmpLC-117 (*ompLC* from strain ML-08-117) or pOmpLC-199 (*ompLC* from Alg-08-199) to *ompLC* mutant *Edw. ictaluri ompLC*::Tn10 (mutant R-165). These complementations resulted in reduced phage ΦeiAU susceptibility compared to the wild-type strain that correlated with the phage susceptibility of their corresponding strains (Table 4).

The comparison of OmpLC sequences from phage-sensitive *Edw. ictaluri* strain EILO and phage-resistant *Edw. ictaluri* strain Alg-08-183 demonstrated that two amino acid substitutions were present in OmpLC-183, a tyrosine to glutamine substitution on residue 174 located on the predicted transmembrane β7 and a valine to alanine substitution on residue 343 predicted to be located on loop 8 of the C-terminal end of the OmpLC protein (Figure 3). Based on these data it was hypothesized that the single amino acid substitution on residue 343 of OmpLC protein in *Edw. ictaluri* strain Alg-08-183 is localized on a surface-exposed loop and is an important site for phage attachment. To address this hypothesis, the OmpLC of *Edw. ictaluri* EILO was modified by site-directed mutagenesis to introduce the amino acids tyrosine and valine at residues 174 and 343, respectively, in separate plasmid constructs pOmpLC-174 and pOmpLC-343. The *ompLC* gene of *Edw. ictaluri* strain Alg-08-183 was interrupted by a Tn5 transposon, resulting in an Alg-08-183 *ompLC::*Tn5 mutant. The complementation of this *ompLC* mutant with pOmpLC-174 and pOmpLC-343, separately, showed that OmpLC with a

valine substitution at residue 343 was resistant to phage ΦeiAU infection, whereas a glutamine substitution at residue 174 was susceptible to phage infection (Table 4). These results demonstrated that an altered OmpLC with a point mutation predicted to be localized on loop 8 at reside 343, and not at residue 174, results in reduced phage susceptibility.

Transformation of strain Alg-08-183 with ΦeiAU DNA yields phage progeny. The transformation of phage ΦeiAU genomic DNA into an otherwise phage-resistant *Edw. ictaluri* Alg-08-183 resulted in the generation of progeny phages (data not shown). This result ruled out all other potential phage-resistance mechanisms except adsorption/DNA injection-mediated phage resistance. The introduction of *ompLC* from the phage-susceptible *Edw. ictaluri* strain EILO to the completely phage-resistant *Edw. ictaluri* strain Alg-08-183 made this strain susceptible to phage ΦeiAU infection (Figure 1). The EOP of phage ΦeiAU on wild-type *Edw. ictaluri* strain Alg-08-183 complemented with *ompLC* from *Edw. ictaluri* EILO was ~5.82×10^{11} times higher than that of the original strain. This finding indicated that OmpLC is the primary factor contributing to ΦeiAU infectivity in *Edw. ictaluri* strain Alg-08-183.

Passaged ΦeiAU adapts to a unique OmpLC epitope in strain Alg-08-183. A mutant phage ΦeiAU-183 was isolated by passaging phage ΦeiAU in the phage-resistant *Edw. ictaluri* strain Alg-08-183. This mutant phage ΦeiAU-183 was highly lytic to *Edw. ictaluri* Alg-08-183 with the EOP value ~ 0.8 whereas no noticeable plaques were observed on this strain with the wild-type ΦeiAU using a titer of ~1.0×10^{12} PFU/ml (Figure 5). To determine whether OmpLC of *Edw. ictaluri* strain Alg-08-183 (*ompLC*-183) is required for phage ΦeiAU-183 infection, the EOP of phage ΦeiAU-183 was determined against strain Alg-0-183, the *ompLC* mutant of Alg-08-183 and its *ompLC*-183 complemented mutant Alg-08-183 *ompLC*::Tn5 (pOmpLC-183). Interestingly, the *ompLC* mutant of strain Alg-08-183 (*ompLC*::Tn5) was resistant to phage

ΦeiAU-183 infection and complementation of this mutant with the *ompLC* gene from *Edw. ictaluri* strain Alg-08-183 with plasmid pOmpLC-183 restored a phage-sensitive phenotype (Figure 5).

A phage binding assay was conducted with phage ΦeiAU against *Edw. ictaluri* strain EILO, its *ompLC* mutant EILO *ompLC*::Tn10 (R-165) and *Edw. ictaluri* strain Alg-08-183, demonstrated that phage ΦeiAU can bind strongly to strain EILO whereas the adsorption rate was poor with *ompLC* mutant R-165 and *Edw. ictaluri* strain Alg-08-183 (Figure 2a). The binding assay was also conducted with phage ΦeiAU-183 against strain Alg-08-183, its *ompLC* mutant Alg-08-183 *ompLC*::Tn5, complemented mutant Alg-08-183 *ompLC*::Tn5 (pOmpLC-183) and strain EILO, and demonstrated that phage ΦeiAU-183 has a stronger adsorption rate for wild type Alg-08-183 (>80%), complemented mutant Alg-08-183 *ompLC*::Tn5 (pOmpLC-183) (>85%) and EILO (>95%) as compared to its *ompLC* mutant (<3%) (Figure 2b). These findings demonstrated that the passaged phage ΦeiAU-183 can utilize the OmpLC of *Edw. ictaluri* strains Alg-08-183 and EILO for adsorption and subsequent infection. These results also demonstrated that the lack of *ompLC* gene expression is not the reason for phage ΦeiAU resistance to Alg-08-183 as it was observed that mutant phage ΦeiAU-183 was capable of using OmpLC-183 as a means for establishing a productive infection in this strain. The observation that complementation of the Alg-08-183 *ompLC*::Tn5 mutant with *ompLC* from strain EILO resulted in sensitivity to phage ΦeiAU (Figure 5) was in agreement with the previous observation that complementation of wild type Alg-08-183 strain with *ompLC* from EILO resulted in sensitivity to phage ΦeiAU infection (Figure 1).

To further confirm the role of OmpLC as a phage susceptibility determinant, the *ompLC* gene from *Edw. ictaluri* Alg-08-183 (on pOmpLC-183) was introduced into the *ompLC* mutant

of EILO (R-165) and the EOP of phage ΦeiAU was determined. The EOP of the pOmpLC-183 complemented mutant R-165 was about 1.35×10^4 times less than that observed from complementation with pOmpLC-EILO (Figure 1). These data further confirmed that phage ΦeiAU was unable to infect *Edw. ictaluri* Alg-08-183 strain due to its altered OmpLC protein. Taken together, these data demonstrate that OmpLC is a phage susceptibility determinant of phage infection to *Edw. ictaluri*.

5. Discussion

In this study, the *Edw. ictaluri* host factors required for phage ΦeiAU and ΦeiDWF infection were identified in order to further the understanding of phage-host interactions and allow development of a phage-based diagnostic assay for *Edw. ictaluri* primary disease isolates. Transposon-mutagenized libraries of *Edw. ictaluri* strains EILO and ML-08-116 were screened for phage-resistant mutants and 12 unique mutants were isolated that showed complete or partial resistance to phage ΦeiAU and ΦeiDWF infection. Eleven *Edw. ictaluri* genes were identified from those phage-resistant mutants. Host factors identified by this genome-wide screening of *Edw. ictaluri* are predicted to be involved in different stages of phage infection with potential roles from initiation of infection to phage morphogenesis. To the best of our knowledge, this study identified several genes that have never been reported in any bacterial species as an essential host factor for bacteriophage infection.

The deletion of *ompLC* gene, site-directed mutagenesis and complementation assays demonstrated that the putative outer membrane porin OmpLC of *Edw. ictaluri* is important for phage adsorption and serves as a receptor for phage ΦeiAU infection. Many outer membrane proteins of *E. coli* such as OmpC, OmpF, OmpT and PhoE serve as receptors for phages TulB and T4 (Yu & Mizushima, 1982), T2 (Riede *et al.*, 1985), M2 (Hashemolhosseini *et al.*, 1994),

and TC45 (Chai & Foulds, 1978), respectively. The *ompLC* gene is flanked on the chromosome of *Edw. ictaluri* strain 93-146 by genes encoding a hypothetical protein (NT01EI_1358) and a putative asparaginyl-tRNA synthetase. Since neither of these adjacent genes are part of a genetic operon with *ompLC,* this further confirms that the transposon insertion in *ompLC* did not result in any polar effect on adjacent genetic loci. In this study, it was also demonstrated that the putative porin thermoregulatory protein, EnvY, is required for phage infection in *Edw. ictaluri*. In *E. coli*, it was reported that EnvY modulates the temperature-dependent expression of several porin proteins, most notably OmpF, OmpC, and the lambda phage receptor, LamB (Lundrigan & Earhart, 1984). The requirement of EnvY for phage infection suggests that the expression of OmpLC is regulated by this protein and that the inactivation of the *envY* gene results in phage-resistance due to the lack of OmpLC expression.

In our previous study, we observed that *Edw. ictaluri* strains had varying degrees of susceptibility to phage infection (Walakira *et al.*, 2008). *Edw. ictaluri* strains recently obtained from diseased catfish also showed variability in their degree of phage susceptibility (Figure 3). This study showed that OmpLC from different *Edw. ictaluri* strains varied in amino acid sequences, and in seven *Edw. ictaluri* strains (out of 15 tested) the variations in amino acid sequence were predicted to be clustered on the surface-exposed loop 8 of OmpLC. None of the *Edw. ictaluri* strains with amino acids substitutions on loop 8 of their OmpLC protein showed higher phage susceptibility compared to strain EILO that contained a "typical" OmpLC sequence. Furthermore, the deletion of loop 8 from OmpLC completely abolished *Edw. ictaluri* phage susceptibility. These results showed striking similarities with a previous finding that demonstrated that alterations clustered in a small region near the surface-exposed carboxy terminus of Tsx protein resulted in Tsx-specific phage-resistance phenotypes (Schneider *et al.*,

1993). The involvement of other surface-exposed regions in phage attachment is not unexpected as it was demonstrated by mutational analysis of several phage receptors (Cole *et al.*, 1983; Gehring *et al.*, 1987; Heine *et al.*, 1988).

The OmpLC proteins from *Edw. ictaluri* strains EILO and Alg-08-183 with altered phage adsorption phenotypes were studied in detail by phage binding and EOP assays with their corresponding *ompLC* mutants. Several lines of evidence indicate that the OmpLC of *Edw. ictaluri* modulates phage susceptibility in *Edw. ictaluri*. First, the introduction of phage ΦeiAU genomic DNA by electroporation to phage-resistant *Edw. ictaluri* strain Alg-08-183 resulted in progeny phages by avoiding the natural route of phage infection. This transformation experiment demonstrated that phage-resistance in strain Alg-08-183 is due to a phage adsorption/DNA injection deficiency. Secondly, this strain was capable of being infected by introducing the OmpLC from the phage-sensitive strain EILO. This result demonstrated that inefficient phage adsorption due to an altered OmpLC is responsible for the ΦeiAU phage-resistance in Alg-08-183. Third, the introduction of the OmpLC from strain Alg-08-183 into the *ompLC* mutant of strain EILO (mutant R-165) resulted in reduced phage sensitivity, reflecting the poor attachment of ΦeiAU to OmpLC-183. The same consistent patterns were observed when this EILO *ompLC* mutant was complemented with altered *ompLC* from *Edw. ictaluri* strains ML-08-117, ML-08-116 or Alg-08-199 (Table 4). The OmpLC-117 (OmpLC from *Edw. ictaluri* strain ML-08-117) and OmpLC-199 (OmpLC from *Edw. ictaluri* strain Alg-08-199) completely resisted phage infectivity when introduced into the *ompLC* mutant of EILO, as observed in their wild-type strains. The OmpLC-116 complemented *ompLC* mutant of EILO showed approximately the same degrees of phage susceptibility as observed with their wild-type strains ML-08-113, Alg-08-195 and Alg-08-200. However, the phage susceptibility on this OmpLC-116 complemented

EILO mutant was higher than that on wild-type strain ML-08-116 which has identical *ompLC* sequences to *Edw. ictaluri* strains ML-08-113, Alg-08-195 and Alg-08-200. These findings suggest that in addition to OmpLC, other host factors contribute to phage infection in *Edw. ictaluri*. Other studies have shown that the sugar moieties of LPS side chains (Skurnik *et al.*, 1995; Yu & Mizushima, 1982) and the spatial orientation of LPS and outer membrane proteins are important for phage attachment and infection (Beacham & Picken, 1981). Fourth, a phage that was serially passaged and adapted to strain Alg-08-183 (ΦeiAU-183) also utilized OmpLC-183 as a receptor for infection. It was demonstrated that coliphage TuIa, which uses the OmpF protein as a receptor, can acquire mutational changes to exploit OmpC or LamB proteins or both as a substitute receptor (Moreno & Wandersman, 1980). Likewise, host-range mutants of phage Ox2 can exploit two different outer membrane proteins, OmpA and OmpC, of *E. coli* K-12 as a receptor for infection (Morona & Henning, 1984). Phage Ox2 can switch from protein to carbohydrate receptors by altering a tail fiber protein by a single mutation (Drexler *et al.*, 1991). In this study we have demonstrated that OmpLC is a determinant of host specificity and modulates the degree of phage infectivity in *Edw. ictaluri* strains.

In addition to OmpLC, there were other *Edw. ictaluri* host factors that were demonstrated to contribute to phage infection. The *psoB* gene complemented the *Edw. ictaluri psoB*::Tn10 mutant and is predicted to encode a GDP-fucose synthetase that is involved in LPS biogenesis in many Gram-negative bacteria (Barua *et al.*, 2002; Skurnik & Zhang, 1996). This result suggests that LPS might be a potential co-receptor for phage ΦeiAU infection in *Edw. ictaluri*.

The disruption of a putative outer membrane fimbrial usher gene, *fimD*, of *Edw. ictaluri* also resulted in a phage-resistant phenotype. It was reported that an outer membrane

fimbrial usher protein, which is a molecular chaperon, is a potential virulence factor in *Edw. ictaluri* required for catfish infection (Thune *et al.*, 2007). This result is in agreement with previous observations that acquisition of phage-resistance in bacteria may result in a less virulent phenotype (Capparelli *et al.*, 2010; Evans *et al.*, 2010; Santander & Robeson, 2007). The studies of *Staphylococcus aureus* fitness cost associated with phage resistance showed that emerging phage resistant bacteria provide broad immunity against *S. aureus* infection in mice (Capparelli *et al.*, 2010).

Finally, another phage-resistant mutant contained a transposon insertion in a putative ATP-dependent RNA helicase DeaD gene (*deaD*) that resulted in partial resistance to ΦeiAU and ΦeiDWF. The orthologs of this RNA helicase have been studied in *E. coli* and they are involved in the dissociation of RNA duplexes (Bizebard *et al.*, 2004), mRNA processing (Py *et al.*, 1996), and in ribosome biogenesis (Charollais *et al.*, 2004; Charollais *et al.*, 2003). However, an ATP-dependent RNA helicase has never been implicated in phage infection. The lack of DeaD protein might affect the transcription of phage encoded mRNAs and could be responsible for reduced phage susceptibility. Further studies are required to determine the exact nature of DeaD mediated modulation of phage infection in *Edw. ictaluri*.

In conclusion, this study has identified *Edw. ictaluri* host factors required for phage infection. OmpLC has been determined to serve as a receptor for phage infection and variation in its protein sequence modulates the nature of phage infectivity in different *Edw. ictaluri* strains. Knowledge gained from studies of phage-host interactions in *Edw. ictaluri* will further our collective knowledge of the molecular determinants of phage infection, and will also result in improved results for the application of these phages in the diagnosis of ESC.

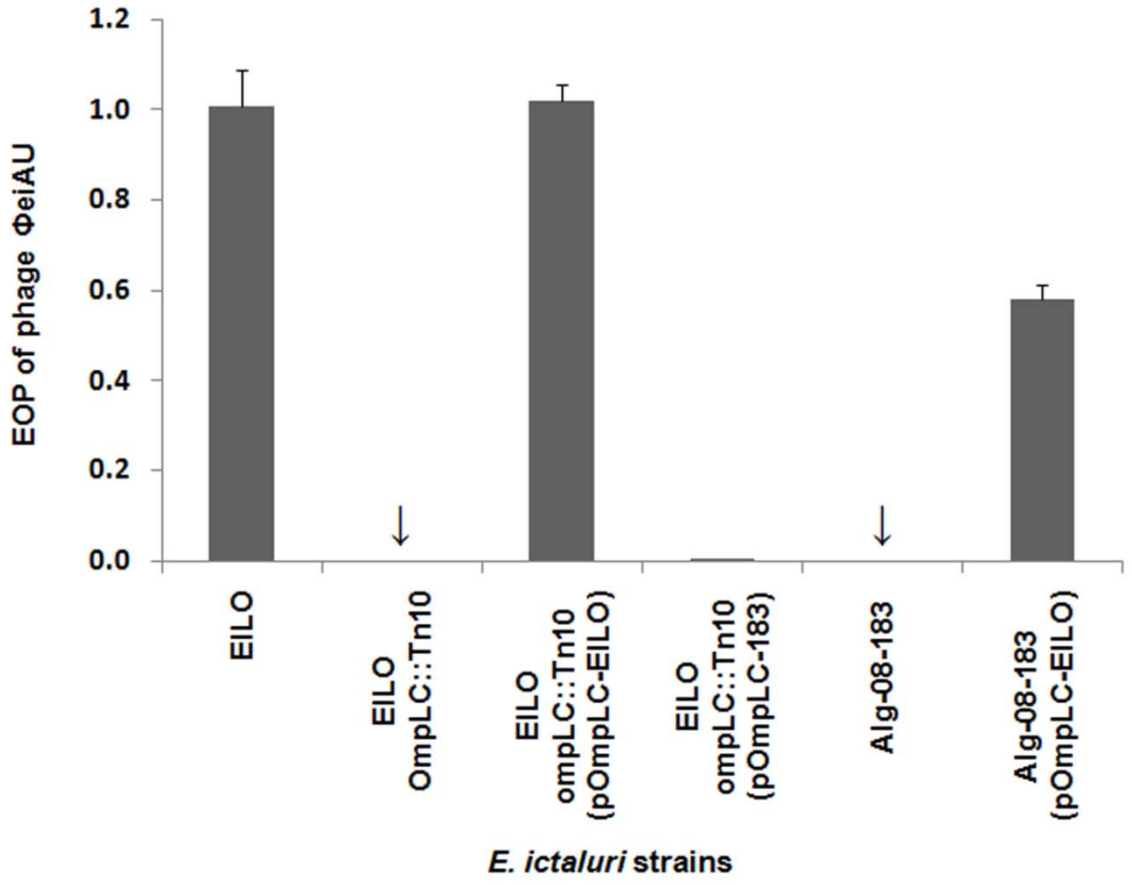


Figure 1. The EOP of phage ΦeiAU on different *Edw. ictaluri* strains. EOPs were calculated by determining the ratio of phage titer on tested strains to the phage titer on phage-sensitive *Edw. ictaluri* strain 219. The means and standard deviations of EOPs were from at least three independent experiments. The designations of the strains are as follows: Strain EILO, *Edw. ictaluri* EILO; EILO *ompLC*::Tn10, *ompLC* mutant of *Edw. ictaluri* EILO; EILO *ompLC*::Tn10 (pOmpLC-EILO), EILO *ompLC*::Tn10 mutant complemented with *ompLC* from *Edw. ictaluri* EILO; EILO *ompLC*::Tn10 (pOmpLC-183), EILO *ompLC*::Tn10 mutant complemented with *ompLC* from *Edw. ictaluri* Alg-08-183; Alg-08-183, wild type *Edw. ictaluri* Alg-08-183; Alg-08-183 (pOmpLC-EILO), wild type *Edw. ictaluri* Alg-08-183 supplemented with *ompLC* from *Edw. ictaluri* EILO. An arrow (↓) indicates that no plaques were observed after applying ~1.0×10^{12} PFU on the tested strains (EOP <1.0×10^{-12}).

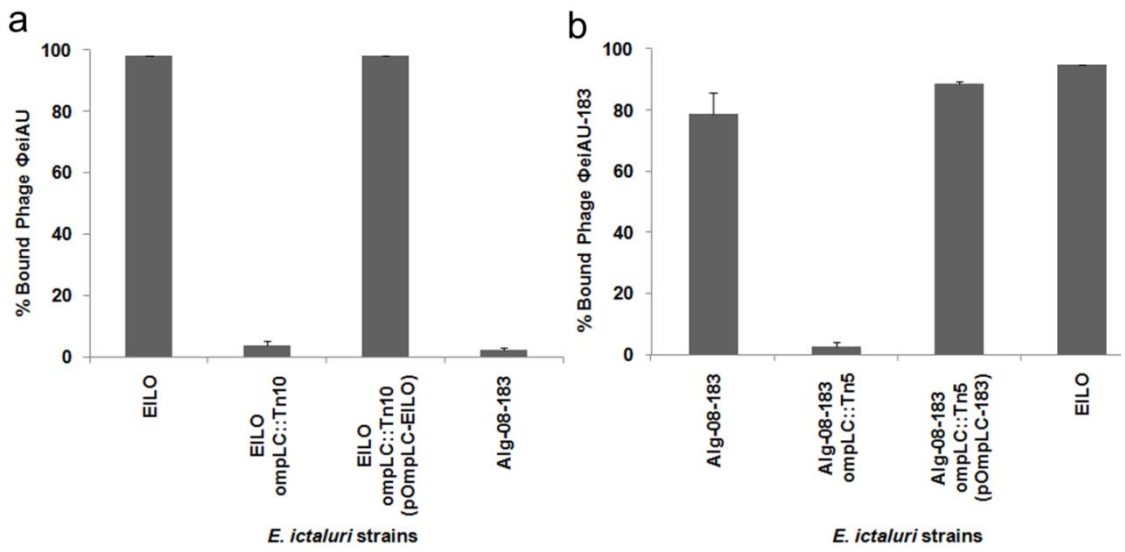


Figure 2. Adsorption of phage ΦeiAU (a) and ΦeiAU-183 (b) to different *Edw. ictaluri* strains. For phage adsorption assays, bacterial cells (~1.0×10^9 CFU/ml) were grown until OD_{600} was ~ 0.975 in TSB supplemented with 500 μM $CaCl_2$. After incubating for 35 min at 28°C with ~1.0×10^7 PFU/ml of phage, bacterial cells with their attached phages were removed by centrifugation in a microcentrifuge and supernatant was tested for its phage titer using *Edw. ictaluri* strain EILO. The percentage of adsorption was calculated as follows: [(phage titer of a control reaction without cells – phage titer of supernatant after cells were removed)/phage titer of a control reaction without cells)] × 100. The designations of the strains are as follows. Strain EILO, *Edw. ictaluri* EILO; EILO *ompLC*::Tn10, *ompLC* mutant of *Edw. ictaluri* EILO; EILO *ompLC*::Tn10 (pOmpLC-EILO), *ompLC* mutant of *Edw. ictaluri* EILO complemented with *ompLC* from *E. ictalrui* EILO; Alg-08-183, wild type *Edw. ictaluri* Alg-08-183; Alg-08-183 *ompLC*::Tn5, *ompLC* mutant of Alg-08-183; Alg-08-183 *ompLC*::Tn5 (pOmpLC-183), Alg-08-183 *ompLC*::Tn5 complemented with *ompLC* from *Edw. ictaluri* Alg-08-183. Strains EILO and EILO *ompLC*::Tn10 (pOmpLC-EILO) are highly susceptible to phage ΦeiAU and strains EILO

ompLC::Tn10 and Alg-08-183 are completely resistant to phage ΦeiAU. Phage ΦeiAU-183 is highly lytic to Alg-08-183, EILO and their complemented *ompLC* mutants.

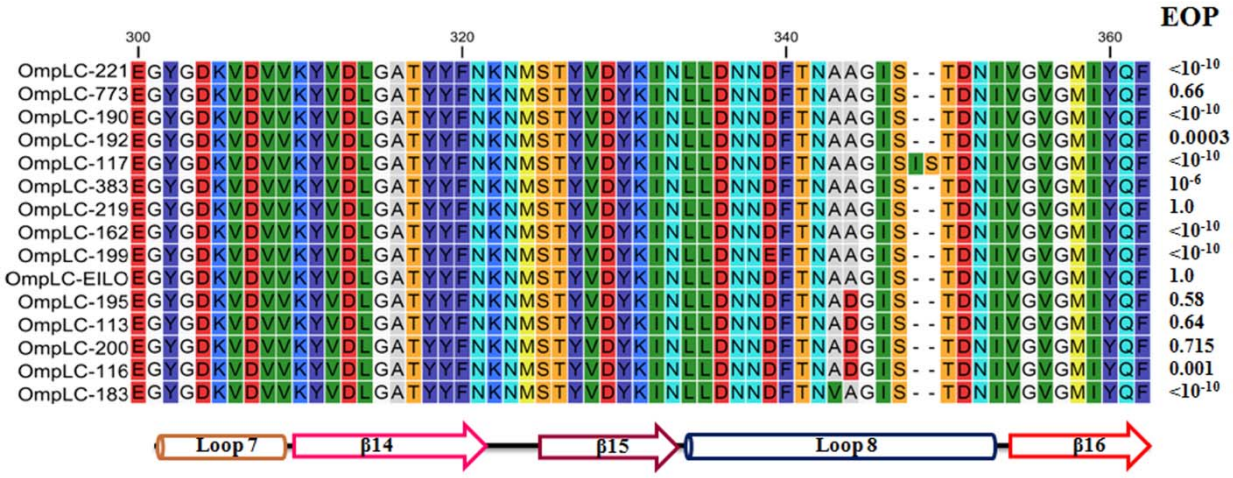


Figure 3. Multiple amino acid sequence alignment of OmpLC protein from 15 different *Edw. ictaluri* strains. The C-terminal region of OmpLC proteins is shown in this alignment (amino acid residues from 300 to 360 for all 15 OmpLC proteins except residues 300 to 362 for OmpLC-117). OmpLC sequences were aligned using CLC Genomics Workbench as described in Material and Methods. Predicted beta strands and loops of OmpLC proteins were indicated by arrows and cylinders, respectively. The variation in the amino acid sequences of OmpLC proteins (OmpLC-195, OmpLC-113, OmpLC-200, OmpLC-116, OmpLC-199, OmpLC-183 and OmpLC-117) were clustered on loop 8 of OmpLC proteins. The sources of aligned OmpLC proteins are as follows. OmpLC-221, *Edw. ictaluri* Alg-08-221; OmpLC-773, *Edw. ictaluri* S97-773; OmpLC-190, *Edw. ictaluri* Alg-08-190; OmpLC-192, *Edw. ictaluri* Alg-08-192; OmpLC-117, *Edw. ictaluri* ML-08-117; OmpLC-383, *Edw. ictaluri* R4383; OmpLC-219, *Edw. ictaluri* 219; OmpLC-162, *Edw. ictaluri* C91-162; OmpLC-199, *Edw. ictaluri* Alg-08-199; OmpLC-EILO, *Edw. ictaluri* EILO; OmpLC-195, *Edw. ictaluri* Alg-08-195; OmpLC-113, *Edw. ictaluri* ML-08-113; OmpLC-200, *Edw. ictaluri* Alg-08-200; OmpLC-116, *Edw. ictaluri* ML-08-116;

OmpLC-183, *Edw. ictaluri* Alg-08-183. The EOPs of the respective wild-type *Edw. ictaluri* strains are indicated on the right side of the OmpLC alignment. EOPs were calculated by determining the ratio of phage titer on tested strains to the phage titer on phage sensitive *Edw. ictaluri* 219.

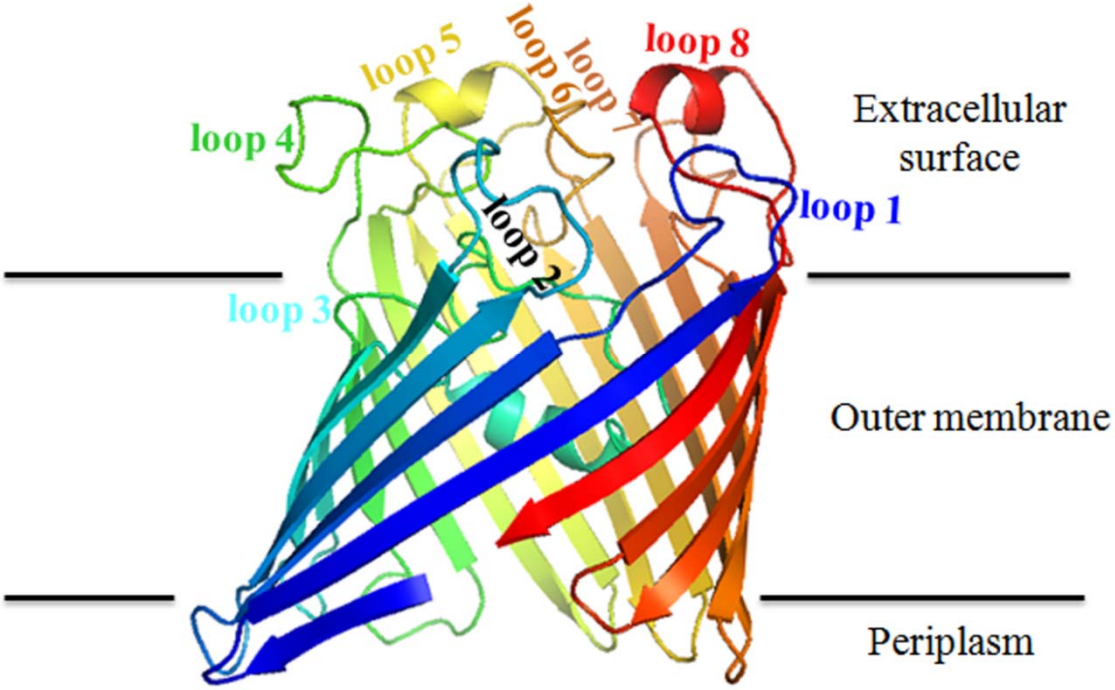


Figure 4. The three-dimensional model of OmpLC from *Edw. ictaluri* strain EILO. This model was determined by SWISS-Model based on the X-ray crystallography structure of *K. pneumoniae* OmpK36 (Dutzler *et al.*, 1999). Surface-exposed loops are labeled loop 1 to loop 8. Alignment of OmpLC proteins from 15 different *Edw. ictaluri* strains indicated that all of the variability in amino acid sequences is present on loop 8 of OmpLC (see Figure 3). The extracellular loops are located on the upper side of the structure and the periplasmic loops are located at the bottom side of the structure. Diagonal strands produced a barrel-like structure with 16 anti-parallel β strands. Loop 3 is inserted within the lumen of the OmpLC porin. Horizontal lines indicate the position of the membrane lipid bilayers.

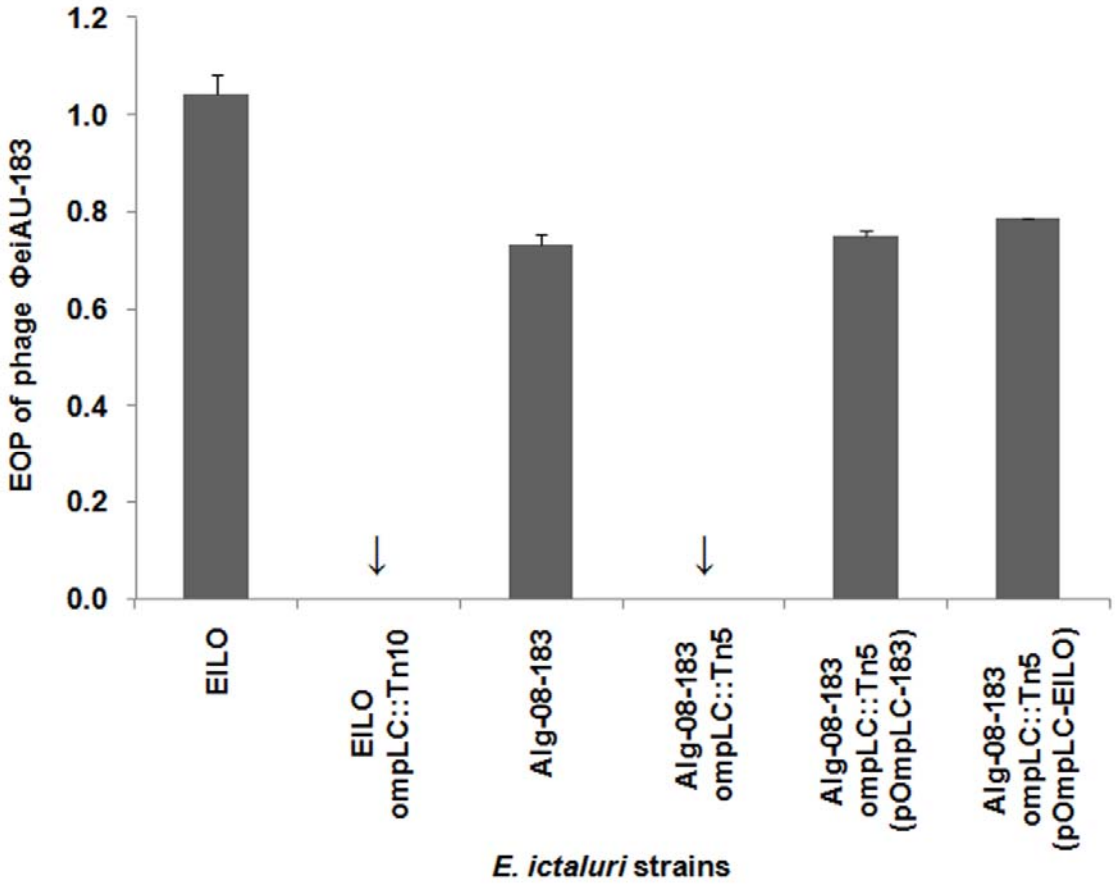


Figure 5. The EOP of phage ΦeiAU-183 on different *Edw. ictaluri* strains. EOPs were calculated by determining the ratio of phage titer on tested strains to the phage titer on phage sensitive *Edw. ictaluri* strain 219. The means and standard deviations of EOPs were from at least three independent experiments. The designations of the strains are as follows: strain EILO, *Edw. ictaluri* EILO; EILO *ompLC*::Tn10, *ompLC* mutant of *Edw. ictaluri* EILO; Alg-08-183, wild type *Edw. ictaluri* Alg-08-183; Alg-08-183 *ompLC*::Tn5, *ompLC* mutant of Alg-08-183; Alg-08-183 *ompLC*::Tn5 (pOmpLC-183), Alg-08-183 *ompLC*::Tn5 complemented with *ompLC* from *Edw. ictaluri* Alg-08-183; Alg-08-183 *ompLC*::Tn5 (pOmpLC-EILO), Alg-08-183 *ompLC*::Tn5 complemented with *ompLC* from *Edw. ictaluri* EILO. Phage ΦeiAU-183 was serially passaged on *Edw. ictaluri* strain Alg-08-183, a previously phage-resistant strain. An arrow (↓) indicates

that no plaques were observed after applying ~1.0×10^{12} PFU on the tested strains (EOP <1.0×10^{-12}).

Table 1. List of oligonucleotides used in this study.

Primer ID	Sequences	Applications
R-165F	5'-TATGCAAGCTTGTAGTTCTTGCTGGTCTC-3'	Cloning and sequencing omp*LC* gene
R-165R	5'-TATGCAAGCTTGTAACGCAACATTCTAAC-3'	Cloning and sequencing *ompLC* gene
ML-17F	5'-TATGCAAGCTTAGGTTACGAGATATATAGGT-3'	Cloning of gene NT01EI_2881 and its downstream ORF
ML-17R	5'-TATGCAAGCTTTAACGACATCAACCTGTA-3'	Cloning of gene NT01EI_2881 and its downstream ORF
ML-41F	5'-TATGCTCTAGAGACATGGCTGAGTATATC-3'	Cloning of *psoB*
ML-41R	5'-TATGCTCTAGAGAGTGAGCCACTAATACA-3'	Cloning of *psoB*
ML-64F	5'-TATGCTCTAGACAATAGAGACGACGATAG-3'	Cloning of IS and *envY*
ML-64R	5'-TATGCTCTAGAACATTGTAGCACTAGACTG-3'	Cloning of IS
ML-64EnvyR	5'-TATGCTCTAGAACATTGTAGCACTAGACTG-3'	Cloning of *envY*
Tn10BF	5'-CTCGTCCAACATCAATAC-3'	Inverse PCR for amplifying Tn10 flanking sequences
Tn10BR	5'-GTTGTAACACTGGAGAG-3'	Inverse PCR for amplifying Tn10 flanking sequences
Tn10EF	5'-TGCAATGTAACATCAGAG-3'	Inverse PCR for amplifying Tn10 flanking sequences
Tn10ER	5'-AGTGATTTTGATGACGAG-3'	Inverse PCR for amplifying Tn10 flanking sequences
Tn10out	5'-ACAAGATGTGTATCCACC-3'	Sequencing of Tn10 flanking sequences
$670F_{seq}$	5'-AGTCGAACTGGTACTGAG-3'	*ompLC* sequencing
$886R_{seq}$	5'-GCTTACTCCAACTCTAAC-3'	*ompLC* sequencing
$1085F_{seq}$	5'-GTAGGTCATGTAGTTATCG-3'	*ompLC* sequencing
$1181R_{seq}$	5'-TAACTACGGTGTGGTCTA-3'	*ompLC* sequencing
$165F_{BglII}$	5'-TTCGATAGATCT GTAGTTCTTGCTGGTCTC-3'	Cloning Tn5 disrupted

		ompLC gene in pGP704
$165R_{BglII}$	5'-ATATGAAGATCTGTAACGCAACATTCTAAC-3'	Cloning Tn5 disrupted *ompLC* gene in pGP704
Tn5out	5'-TTGAGATGTGTATAAGAGAC-3'	Mapping Tn5 insertion on *ompLC* gene
Dnt-SIF	5'-CATACCAACGCCAACGATGTTCAGCAGGTTGATTTTGTAATC-3'	Deletion of loop8 from OmpLC
Dnt-SIR	5'-GATTACAAAATCAACCTGCTGAACATCGTTGGCGTTGGTATG-3'	Deletion of loop 8 from OmpLC
174F	5'-GAACGTTGCCCTGCAGTACTATGGTAAAAACGGTGCCGTAG-3'	Site–directed mutagenesis
174R	5'-CTACGGCACCGTTTTTACCATAGTACTGCAGGGCAACGTTC-3'	Site–directed mutagenesis
343F	5'-AACAACGACTTCACCAATGTAGCCGGTATCAGCACCGAC-3'	Site–directed mutagenesis
343R	5'-GTCGGTGCTGATACCGGCTACATTGGTGAAG-TCGTTGTT-3'	Site–directed mutagenesis

Table 2. Summary of bacterial strains, phages and plasmids used in this study.

Bacterial strains, phages and plasmids	Relevant characteristics	Reference or source
***E. coli* strains**		
SM10λpir	*thi-1thr leu tonA lacY supE recA*::RP4-2-TcT::Mu Km^r *λpir*	Simon *et al*., 1983
CC118λpir	*Δ(ara-leu) araD ΔlacX74 galE galKphoA20 thi-1 rpsE rpoB argE(Am) recA1 λpir*	(Herrero *et al*., 1990)
DH10B	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) *end*A1 *rec*A1 Φ80*dlac*ZΔM15 Δ*lac*X74 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K *rps*L *nup*G λ- *ton*A	Invitrogen
XL10-Gold	TetrΔ (*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* The [F' proAB *lacI*qZΔ*M15* Tn*10* (Tetr) Amy Camr]	Stratagene
***Edw. ictaluri* strains**		
EILO	Wild-type strain isolated from diseased catfish	Walakira *et al*., 2008
ML-08-116	Wild-type strain isolated from diseased catfish	This study
Alg-08-183	Wild-type strain isolated from diseased catfish	This study
Alg-08-221	Wild-type strain isolated from diseased catfish	This study
S97-773	Wild-type strain isolated from diseased catfish	Williams *et al*., 2003
Alg-08-195	Wild-type strain isolated from diseased catfish	This study
Alg-08-190	Wild-type strain isolated from diseased catfish	This study
ML-08-113	Wild-type strain isolated from diseased catfish	This study
Alg-08-200	Wild-type strain isolated from diseased catfish	This study
Alg-08-192	Wild-type strain isolated from diseased catfish	This study
Alg-08-117	Wild-type strain isolated from diseased catfish	This study
R4383	Wild-type strain isolated from diseased catfish	Williams *et al*., 2003
C91-162	Wild-type strain isolated from diseased catfish	Walakira *et al*., 2008
Alg-08-199	Wild-type strain isolated from diseased catfish	This study
219	Wild-type strain isolated from diseased catfish	Walakira *et al*., 2008
Alg-08-183 *ompLC*::Tn5	*ompLC* mutant of *Edw. ictaluri* Alg-08-183	This study
R-6	*ompLC*::Tn10 , phage resistant mutant of EILO	This study
R-11	Phage resistant mutant of EILO	This study
R-137	*fimD*::Tn10, phage resistant mutant of EILO	This study
R-148	Phage resistant mutant of EILO	This study

R-165	*ompLC*::Tn10, phage resistant mutant of EILO	This study
ML-17	Phage resistant mutant of ML-08-116	This study
ML-41	*psoB*::Tn10, phage resistant mutant of ML-08-116	This study
ML-42	*deaD*::Tn10, phage resistant mutant of ML-08-116	This study
ML-44	*dtrA*::Tn10, phage resistant mutant of ML-08-116	This study
ML-64	*envY*::Tn10, phage resistant mutant of ML-08-116	This study
ML-82	*ptrA*::Tn10, phage resistant mutant of ML-08-116	This study
ML-100	*ybaJ*::Tn10, phage resistant mutant of ML-08-116	This study
Phages		
ΦeiAU	Wild type *Edw. ictaluri* specific phage	Walakira *et al*., 2008
ΦeiDWF	Wild type *Edw. ictaluri* specific phage	Walakira *et al*., 2008
ΦeiAU-183	Mutant phage derived from phage ΦeiAU	This study
Plasmids		
pUC19	Cloning vector, Ap^r	
pOmpLC-EILO1	*ompLC* gene from EILO cloned into pUC19	This study
pCR2.1	TOPO-TA cloning vector, Ap^r and Km^r	Invitrogen
pOmpLC-EILO	*ompLC* gene from EILO cloned into pCP2.1	This study
pOmpLC-116	*ompLC* gene from ML-08-116 cloned into pCR2.1	This study
pOmpLC-117	*ompLC* gene from ML-08-117 cloned into pCR2.1	This study
pOmpLC-183	*ompLC* gene from Alg-08-183 cloned into pCR2.1	This study
pOmpLC-199	*ompLC* gene from Alg-08-199 cloned into pCR2.1	This study
pOmpLC-174	Change on residue 174 (Gln→Tyr) of OmpLC in pOmpLC-EILO1	This study
pOmpLC-343	Change on residue 343 (Ala→Val) of OmpLC in pOmpLC-EILO1	This study
pOmpLC-ΔL8	14 amino acids (Loop 8) of OmpLC deleted from pOmpLC-EILO1	This study
pPsoB	*psoB* gene from ML-08-116 cloned into pUC19	This study
pML17	Genes encoding hypothetical proteins NT01EI_2881 and NT01EI_2882 from *Edw. ictaluri* ML-08-116 cloned into pCR2.1	This study
pML64	IS element from ML-08-116 cloned into pUC19	This study
pEnvY	*envY* from ML-08-116 cloned into pUC19	This study
pLOF-Km	*Tnl0*-based delivery plasmid, Km^r and Ap^r	(Herrero *et al.*, 1990)
pGP704	Suicide vector, Ap^r	Miller & Mekalanos, 1988
pGP183	Tn5 disrupted *ompLC* gene cloned into suicide vector pGP704, Tet^r, Km^r and Ap^r	This study

Ap^r, Ampicillin resistance; Km^r, Kanamycin resistance; Tet^r, Tetracycline resistance

Table 3. The identity of transposon-interrupted ORFs in *Edw. ictaluri* phage-resistant mutants

Mutant ID	Efficiency of Plaquing (EOP)*		Phage resistance status	Complemented mutation	% Identity §	Top BLAST hit
	phage ΦeiAU	phage ΦeiDWF				
R-6	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND† (see mutant R-165)	100	Outer membrane porin protein LC (OmpLC)
R-11	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	100	Orn/Lys/Arg decarboxylase family protein
R-137	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	100	Outer membrane fimbrial usher protein (FimD)
R-148	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	95	Hypothetical protein NT01EI_2357
R-165	$<10^{-12}$	$<10^{-12}$	Completely resistant	Complemented	100	Outer membrane porin protein LC (OmpLC)
ML-17	$<10^{-12}$	$<10^{-12}$	Completely resistant	Complemented with additional downstream ORF	100	Hypothetical protein NT01EI_2881 (Transposase)
ML-41	$<10^{-12}$	$<10^{-12}$	Completely resistant	Complemented	100	PsoB
ML-42	$<10^{-5}$	$<10^{-5}$	Partially resistant	ND	93	ATP-dependent RNA helicase (DeaD)
ML-44	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	98	Drug resistance transporter (DtrA)
ML-64	$<10^{-12}$	$<10^{-12}$	Completely resistant	Complemented	100	Porin thermoregulatory protein (EnvY)
ML-82	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	99	Protease 3 (PtrA)
ML-100	$<10^{-12}$	$<10^{-12}$	Completely resistant	ND	100	Hypothetical protein NT01EI_1097 (YbaJ)

*EOPs were calculated by determining the ratio of phage titer on tested strains to the phage titer on phage sensitive *Edw. ictaluri* strain 219.

†Not determined

‡Accession number of all identified proteins except OmpLC corresponds to the orthologs of *Edw. ictaluri* strain 93-146, the only *Edw. ictaluri* strain whose genome sequences are available to the GenBank.

§Percent identity of the identified proteins corresponds to the proteins from the *Edw. ictaluri* strain 93-136.

Table 4. Effect of alterations on loop 8 of OmpLC on the infectivity of phage ΦeiAU against *Edw. ictaluri*

Types of OmpLC*	*Edw. ictaluri* strains	Source of OmpLC	EOP† of ΦeiAU	
			EILO *ompLC:*Tn10 (pOmpLC‡)	Alg-08-183 *ompLC*::Tn5 (pOmpLC‡)
Empty vector	-	-	$<10^{-11}$	$<10^{-11}$
Typical OmpLC	EILO, 219, Alg-08-221, S97-773, Alg-08-190, Alg-08-192, R4383, C91-162	Cloned *ompLC* from *Edw. ictaluri* EILO	0.99±0.08	0.66±0.01
Asp344Gly	ML-08-116, Alg-08-195, ML-08-113, Alg-08-200	Cloned *ompLC* from *Edw. ictaluri* ML-08-116	0.63±0.09	ND§
346→IleSer	ML-08-117	Cloned *ompLC* from *Edw. ictaluri* ML-08-117	$<10^{-11}$	ND
Glu339Phe	Alg-08-199	Cloned *ompLC* from *Edw. ictaluri* Alg-08-199	$<10^{-11}$	ND
Tyr174Gly, Val343Ala	Alg-08-183	Cloned *ompLC* from *Edw. ictaluri* Alg-08-183	$(7.54\pm0.25)\times10^{-5}$	$(1.27\pm0.71)\times10^{-6}$
Tyr174Gly	-	Site directed mutagenesis¥	ND	0.20±0.05
Val343Ala	-	Site directed mutagenesis	ND	$(5.99\pm4.49)\times10^{-5}$
Δ336-349	-	Site directed mutagenesis	$<10^{-11}$	$<10^{-11}$

*Alterations within the mature OmpLC sequence

[†]EOPs were calculated by determining the ratio of phage titer on tested strains to the phage titer on phage sensitive *Edw. ictaluri* strain 219. EOPs are the means and standard deviations from at least three experiments.

[‡]*ompLC* from corresponding *Edw. ictaluri* strains cloned into a vector of pUC19 origin.

[§]Not determined

[¥]Site-directed mutagenesis of selected OmpLC residues was carried out directly on pOmpLC-EILO1, a pUC19 vector in which *ompLC* from *Edw. ictaluri* EILO was cloned.

Chapter III

Highly Efficient Markerless Deletion of *Edwardsiella ictaluri* Genes by Conjugal Transfer of Recombinogenic Plasmid pMJH46

1. Abstract

The genetic modification of *Edwardsiella ictaluri*, causative agent of enteric septicemia of catfish (ESC), by traditional methods is a labor-intensive and time-consuming process. A fast and efficient gene manipulation system in *Edw. ictaluri* has not been developed previously. Herein we describe the development of a PCR-based gene disruption procedure adapted from the λ Red-mediated recombineering technique for efficient deletion of genes from different *Edw. ictaluri* strains. A mobilizable plasmid, pMJH46, was constructed by introducing *oriT* from an IncP plasmid to the temperature-sensitive pKD46 for the introduction of a lambda Red cassette (containing *bet, exo* and *gam* genes necessary for recombineering) to *Edw. ictaluri* by conjugation. To test the feasibility of recombineering-mediated gene manipulation in *Edw. ictaluri, ompLC, dtrA, ptrA* and *eihA* genes from *Edw. ictaluri* strains Alg-08-183 and R4383 were targeted for deletion. Double stranded DNA, carrying the kanamycin resistance gene flanked by 50 bp of DNA sequence homologous to the targeted gene, was obtained by PCR and electroporated into *Edw. ictaluri* expressing the lambda Red recombination apparatus. Mutants

were selected via resistance to kanamycin. All of the targeted genes were successfully deleted by this recombineering technique, and this was confirmed phenotypically and genotypically. Over 70% of the colonies isolated from antibiotic selective plates were validated as the desired mutants. The antibiotic resistance marker integrated into the genome of *Edw. ictaluri* strain by homologous recombination to replace the targeted genes was precisely removed using a Flp recombinase. This new variant of an established method allowed efficient genetic manipulation of *Edw. ictaluri*. Furthermore, the transfer of mobilizable plasmid pMJH46 should also help to manipulate genes from diverse Gram-negative bacteria by λ Red-mediated recombineering.

2. Introduction

Recombineering, genetic engineering based on homologous recombination, is a fast, efficient and reliable technique for the modification of genes in bacteria. This technique was first efficiently implemented for the deletions of genes from *Saccharomyces cerevisiae* (Baudin *et al.*, 1993). Bacteriophage lambda Red-mediated homologous recombination has been exploited for gene modifications in *Escherichia coli* and is now a common practice for gene modifications in different Gram-negative bacteria (Datsenko & Wanner, 2000; Murphy, 1998; Yu *et al.*, 2000). The *E. coli* Rac prophage encodes RecE and RecT, which are functionally analogous to the Exo and Bet proteins encoded by bacteriophage λ (Hall *et al.*, 1993). These λ genes are the most commonly used recombination apparatus (Muyrers *et al.*, 1999; Zhang *et al.*, 1998). In addition to the λ Red and RecET systems, several new recombineering systems have been developed that function in diverse bacterial species (Datta *et al.*, 2008; Swingle *et al.*, 2010a; van Kessel & Hatfull, 2007, 2008). Unlike *recA*-dependent homologous recombination which requires longer regions of sequence homology with the targeted genetic region (Jasin & Schimmel, 1984), the λ Red apparatus can efficiently recombine DNA with homologous regions as short as 30 to 50 bp,

which can easily be incorporated into an oligonucleotide primer in a PCR assay(Datsenko & Wanner, 2000; Yu *et al.*, 2000). The λ Red system consists of three proteins, Exo, Beta, and Gam, that work coordinately to recombine single and double stranded DNA (Datsenko & Wanner, 2000; Murphy, 1998; Yu *et al.*, 2000). Exo has a 5' to 3' double stranded DNA (dsDNA)-dependent exonuclease activity that generates 3' single stranded DNA (ssDNA) overhangs (Cassuto *et al.*, 1971; Little, 1967; Matsuura *et al.*, 2001) that serve as a substrate for the ssDNA-binding protein Beta, which anneals complementary DNA strands for recombination (Court *et al.*, 2002; Karakousis *et al.*, 1998; Muniyappa & Radding, 1986). Gam, an inhibitor of host exonuclease activity due to RecBCD (Poteete *et al.*, 1988), helps to improve the efficiency of λ Red-mediated recombination with linear double-strand DNA. However, Datta et al. (2008) demonstrated that Gam is not absolutely essential for λ Red-mediated recombination. Unlike dsDNA which requires all three proteins for a successful recombination event, ssDNA can recombine with its targeted DNA using Beta protein only (Ellis *et al.*, 2001). However, recent studies have reported that electro-transformed single-stranded DNA oligonucleotides can site-specifically recombine with bacterial chromosomes in the absence of any additional phage-encoded functions (Bryan & Swanson, 2011; Swingle *et al.*, 2010b).

Recombineering is a popular and reliable tool for the modification of bacterial (Datsenko & Wanner, 2000; Yu *et al.*, 2000) and eukaryotic genomes (Copeland *et al.*, 2001). The recombineering technique is widely used for precise deletion (Datsenko & Wanner, 2000), substitution (Datta *et al.*, 2008) or insertion (Rivero-Müller *et al.*, 2007) of genes within genomic DNA. The accuracy and speed of this system helped to construct a set of precisely defined, in-frame single-gene knockout mutants in *E. coli* K-12, the Keio collection (Baba *et al.*, 2006), that provided important information on *E. coli* for systematic studies (Inoue *et al.*, 2007; Maynard *et*

al., 2010; Sharma *et al.*, 2009). This technique has also been extensively used for the epitope tagging of chromosomal (Uzzau *et al.*, 2001) and bacteriophage genes (Marinelli *et al.*, 2008). In addition to chromosome engineering, recombineering has been successfully used for the modification of Bacterial Artificial Chromosome (BAC) (Lee *et al.*, 2001; Muyrers *et al.*, 1999; Yang & Sharan, 2003) and plasmid DNAs (Thomason *et al.*, 2007). Lee et al (Lee *et al.*, 2001) demonstrated that BACs can be modified with recombineering and clones with single based alternations can be identified by a PCR-based screening method called mismatch amplification mutation assay-PCR (MAMA-PCR) (Cha *et al.*, 1992) in the absence of drug selection. Furthermore, a recombineering system has been extensively applied to the modification of phage genomes (Marinelli *et al.*, 2008; Oppenheim *et al.*, 2004; Thomason *et al.*, 2007). One of the biggest advantages of the recombineering method is that modifying DNA can precisely eliminate the antibiotic selection markers for subsequent modification of the targeted DNA (Datsenko & Wanner, 2000; Muyrers *et al.*, 2000; Yu *et al.*, 2000).

The modification of the *Edw. ictaluri* or other bacterial genome using traditional suicide plasmid-based techniques are cumbersome, time consuming and sometimes very difficult to achieve. Therefore, a highly efficient gene modification technique is essential for studying the contributions of specific genetic loci in the *Edw. ictaluri* genome for virulence or phage interactions.

3. Materials and Methods

Bacterial strains and plasmids. The list of bacterial strains used in this study is presented in Table 1. *Edw. ictaluri* strains used on this study were routinely grown on Trypticase Soy Broth (TSB) medium and when required Brain Heart Infusion (BHI) medium supplemented

with 5% Sheep blood. *E. coli* SM10λpir (Simon *et al.*, 1983) was used for the conjugal transfer of mobilizable plasmid to *Edw. ictaluri* strains. *E. coli* BW25141 and BT340 (Datsenko & Wanner, 2000) were received from the Yale University Genetic Stock Center.

Construction of broad host range recombinogenic plasmid pMJH46. The mobilizable plasmid pMJH46 was constructed by introducing a 'mob cassette' that includes *traK*, *traJ* and *oriT* sequences into the pKD46 plasmid (Datsenko & Wanner, 2000) that contains an arabinose-inducible λ-Red cassette (*exo*, *bet* and *gam* genes) required for recombineering. For the introduction of the mob cassette into pKD46, the mob cassette was fused with a chloramphenicol resistance gene, *cat*, by overlap extension PCR according to the method describe by Szewczyk et al (Szewczyk *et al.*, 2007). The mob cassette was amplified by PCR from the bacterial artificial chromosome vector pGNS-BAC (Kakirde *et al.*, 2011) using primers MobF and MobR. Then, a 1.3-kb DNA fragment that contained the *cat* gene was amplified by PCR from the pGNS-BAC with the primers pair CatF and CatR (Table 2). The forward primer CatF and reverse primer MobR specific to the *cat* gene and mob cassette, respectively, were complementary to each other at their 5' ends to assist the fusion of mob cassette and *cat* gene together by overlap extension PCR. The purified fragments of the mob cassette and *cat* gene were fused by PCR using nested primers Mob-intF (forward) and Cat-intR (reverse) to generate a 3.4-kb DNA fragment (*mob-cat*). The amplified *mob-cat* cassette was digested with EcoRV to make it blunt-ended. The pKD46 plasmid was digested with NcoI and blunt-ended using a DNA Terminator kit according to the manufacturer's instructions (Lucigen Corp., Middleton, WI). The EcoRV digested *mob-cat* cassette and blunt-ended pKD46 was ligated with T4 DNA ligase (Promega, WI). The ligation mixture was then electroporated into *E. coli* electrocompetent cells E. cloni 10G (Lucigen). Transformants containing an appropriate plasmid construct were selected on 2×YT medium

supplemented with ampicillin and chloramphenicol after incubation overnight at 30°C. The introduction of the mob-*cat* cassette into pKD46, resulting in pMJH46, was confirmed by PCR and sequencing. Plasmid pMJH46 was extracted using a Wizard SV plasmid purification kit (Promega, WI).

Conjugal transfer of pMJH46 to *Edw. ictaluri* Alg-08-183 and R4383. The mobilizable plasmid pMJH46, bearing the λ-Red cassette, was introduced into *E. coli* SM10λpir by electroporation according to the method described elsewhere (Sambrook *et al.*, 1998). *Edw. ictaluri* clones harboring the pMJH46 plasmid were selected on 2×YT plates supplemented with chloramphenicol after incubation for overnight at 30°C. Plasmid pMJH46 was introduced into *Edw. ictaluri* by filter mating experiments according to the methods described previously (Maurer *et al.*, 2001). Briefly, the donor *E. coli* SM10λpir containing pMJH46 was grown to an optical density at 600 nm (OD_{600}) of 1.0. The recipient *Edw. ictaluri* strains, which are naturally resistant to colistin, were also grown to an OD_{600} of 1.0. Cultures were mixed in a ratio of 4:1 (recipient:donor) in a 15 ml conical tube containing 5-ml of 10 mM $MgSO_4$. The mixture of recipient and donor cells was vacuum filtered through a 0.45µm MicroFunnel filter unit (Pall Corporation, MI). This filter was transferred to BHI Blood agar plate and incubated for 16 hours at 24°C. After 16 hours of incubation, the filter was resuspended in 3 ml of 10 mM $MgSO_4$ and vortexed to dislodge cells. Fifty and 100 µl of resuspended cells were then spread onto the surface of TSA plates containing chloramphenicol and colistin for the selection of *Edw. ictaluri* cells harboring the pMJH46 plasmid and incubated at 28°C for 24 hours. The introduction of pMJH46 to *Edw. ictaluri* transconjugants was confirmed by plasmid profiling and PCR with primers specific to the λ Red cassette.

Preparation of linear DNA fragment for recombineering. All linear double stranded DNA fragments used for recombineering were generated by PCR amplification of Km^R cassette with its flanking FRT sequences using pKD4 as a template (Datsenko & Wanner, 2000), and the appropriate primers listed in Table 2. PCRs using oligonucleotides carrying 50 bp homologous sequences of the targeted genes were performed using a high fidelity Takara Ex Taq Polymerase and buffers accompanied with the PCR kit (Clontech, CA). Three 50 µl volumes of positive PCRs were pooled together and purified by phenol-chloroform extraction followed by ethanol precipitation (Sambrook *et al.*, 1998). Purified PCR products were resuspended in nuclease-free water and used for transformation into electrocompetent *Edw. ictaluri*.

Deletion of *Edw. ictaluri* genes by recombineering. For the preparation of electrocompetent cells, *Edw. ictaluri* strains harboring Red helper plasmid pMJH46 were grown at 28°C for overnight in 2 ml of TSB medium supplemented with Cm. The overnight grown culture was diluted 1:70 in 40 ml of Super Optimal broth (SOB) medium supplemented with Cm and 10 mM of L-arabinose and grown at 28°C with vigorous shaking until the culture reached an OD_{600} of 0.45. Cells were harvested by centrifugation at 4°C using 5000×*g* for 8 min, washed three times with ice-cold 10% glycerol and finally cells were concentrated 200-fold by re-suspending with 100 µl of GYT (10% glycerol, 0.125% yeast extract and 0.25% tryptone) medium. Freshly prepared electrocompetent cells were immediately used for electroporation.

For the transformation of PCR products, 50 µl of cell suspension was mixed with ~5.0 µg DNA, transferred to a pre-chilled electroporation cuvette (0.1-cm), and pulsed at 1.8 kV, 25 µF, and 200 Ω using an Eppendorf Electroporator 2510 (Hamburg, Germany). Electro-transformed *Edw. ictaluri* cells were added to 950 µl of Super Optimal broth with Catabolite repression (SOC) supplemented with 10 mM of L-arabinose and incubated at 28°C with vigorous

shaking for at least 4 hrs. Cells were then spread onto 2×YT agar plates supplemented with kanamycin and incubated for 48 hrs at 28°C to select Km^R resistant transformants.

Verification of correct deletion of targeted genes by PCR and sequencing. PCR was used to check for the correct insertion of the antibiotic cassette into the targeted genes. Two PCRs were performed using primers specific to the antibiotic cassette and flanking sequence of the targeted loci to confirm both of the newly introduced junctions. A third PCR was performed to verify the loss and gain of parental loci and mutant-specific fragments, respectively, using flanking primers. For the preparation of PCR template, a single colony was suspended with 50 µl of water and heated at 99°C for 10 minutes using an Eppendorf Thermocycler. Cell debris was precipitated by a brief spin and 5 µl of supernatant was used for PCR in a 25 µl reaction using EconoTaq Polymerase (Lucigen, WI). The PCR conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 to 3 min, which varied depending on the length of the amplified products. PCR products were purified using a Promega Wizard SV PCR purification kit (Promega, WI) and used for Sanger sequencing at Auburn University's sequencing center.

Curing of pMJH46 from *Edw. ictaluri* mutants. Plasmid pMJH46 which contained the ampicillin and chloramphenicol resistant markers was cured from *Edw. ictaluri* mutants as follows. *Edw. ictaluri* mutants were grown on TSB medium at 28°C until the OD_{600} was 1.0. Cultures were then heat induced by incubating at 43°C for 1 hr with shaking at 250 rpm. After incubation, cultures were serially diluted with sterile water and 50 and 100 µl of aliquots from dilutions 10^{-1}, 10^{-2} and 10^{-3} were spread onto BHI Blood Agar plates and incubated at 28°C for 36 hours. To confirm the loss of the pMJH46 plasmid, colonies grown on BHI Blood Agar plates were replica plated by picking and patching onto BHI Blood Agar plates without any antibiotics

and TSA plates supplemented with 12.5 µg/ml of chloramphenicol. Wild type *Edw. ictaluri* and *Edw. ictaluri* harboring pMJH46 plasmid were plated on the respective plates as negative and positive controls, respectively. Plates were incubated at 28°C for 24-36 hours until single colonies appeared. Colonies grown on BHI Blood Agar plate but not on TSA containing Cm plates were further confirmed by PCR with a pair of primers that anneal outside of the homologous regions.

Flp-mediated excision of the kanamycin resistance gene to generate unmarked mutant with a single FRT scar remaining on the target site. Electrocompetent *Edw. ictaluri* mutants were prepared according to the methods described above. Plasmid pCP20 that contains the Flp recombinase (Cherepanov & Wackernagel, 1995) required for FRT sequence specific recombination was electroporated into *Edw. ictaluri* mutants according to the methods described above. *Edw. ictaluri* mutants harboring pCP20 were selected on 2×YT supplemented with chloramphenicol. *Edw. ictaluri* mutants (pCP20) were grown on TSB medium at 28°C until OD_{600} was at 1.0. Cultures were then transferred to an incubator at 37°C and incubated for 1 hr with shaking at 250 rpm. After incubation, cultures were serially diluted with sterile water and 50 and 100 µl of aliquots from dilutions 10^{-1}, 10^{-2} and 10^{-3} were spread onto the surface of BHI Blood Agar plates and incubated at 28°C for 36 hours. To verify successful FRT recombination and loss of the *kanR* cassette from *Edw. ictaluri* mutants, colonies grown on BHI Blood Agar plates were replica plated by picking and patching onto BHI Blood Agar plates without any antibiotics and TSA plates supplemented with 30 µg/ml of kanamycin. Plates were incubated at 28°C for 24 to 36 hours until single colonies appeared. Colonies grown on BHI Blood Agar plate but not on TSA containing Km plates were further confirmed by PCR with a pair of primers that anneal outside of the homologous regions.

4. Results

Construction of mobilizable λ Red-expressing plasmid. The presence of *Exo*, *Bet* and *Gam* proteins is a prerequisite for the deletion of bacterial genes by recombineering (Datsenko & Wanner, 2000). Though *Edw. ictaluri* is capable of accepting foreign DNA of up to 45 kb by electroporation (Hossain *et al.*, 2012), our several attempts to introduce the recombinogenic plasmid pKD46 (Datsenko & Wanner, 2000) to *Edw. ictaluri* failed. We cloned the λ-Red cassette (*exo*, *bet* and *gam* genes) into pUC19, a low-copy number plasmid at the reduced growth temperature of 30 (Lin-Chao *et al.*, 1992) which is also an optimum growth temperature for *Edw. ictaluri*. However, the introduction of the λ-Red cassette into pUC19 did not help to electroporate the resulting plasmid pUC19-Red into *Edw. ictaluri*. To introduce the recombinogenic plasmid pKD46 to *Edw. ictaluri*, a mobilizable plasmid was constructed by introducing the 'mob cassette' (*oriT* region and *traJ* and *traK*) along with chloramphenicol resistance (*cat*) gene into pKD46, resulting in plasmid pMJH46 (Figure 1, accession no. JQ070344). The *cat* gene introduction broadens the applicability of this plasmid since some *Edw. ictaluri* strains are resistance to ampicillin (Welch *et al.*, 2009) and the pMJH46 plasmid could not be maintained without selection. This conjugally transferable recombinogenic plasmid was introduced into different *Edw. ictaluri* strains by conjugation with *E. coli* SM10λpir bearing plasmid pMJH46.

Deletion of *Edw. ictaluri* genes by recombineering. To determine the feasibility of this recombineering technique in *Edw. ictaluri*, in our first attempt we deleted the *ompLC* gene (required for phage attachment) from *Edw. ictaluri* strain Alg 08-183. The PCR screening of colonies grown on antibiotic selection plates showed that approximately 1 % colonies were true mutants (data not shown). Although plasmid pKD4, the template for the recombineering

substrate, was a suicide vector and PCR products were treated with *DpnI* to eliminate the template plasmid pKD4, hundreds of colonies grew on 2×YT plates supplemented with kanamycin. To avoid the occurrence of background colonies, we used the genomic DNA of *Edw. ictaluri* Alg 08-183 *ompLC::kanR* mutant as a template for the amplification of the kanamycin gene cassette. In our subsequent deletion experiment, we obtained 20 to 25 colonies per experiment on average and ~ 90% of them were true mutants in which the targeted gene was replaced with an antibiotic cassette successfully (Figure 2). We decided to delete three additional genes including *dtrA*, and *ptrA* of *Edw. ictaluri* Alg 08-183 (Hossain *et al.*, 2012) and *eihA* of *Edw. ictaluri* R4383 (Williams & Lawrence, 2005). All four genes that were targeted for deletion from *Edw. ictaluri* strains were successfully deleted by recombineering. PCR amplification followed by sequencing showed that the correct targeted genes had been replaced with a kanamycin resistance gene (*kanR*) marker.

Removal of antibiotic resistance cassette by Flp recombinase. Temperature induction of *Edw. ictaluri* Alg-08-183 *ompLC::kanR, dtrA::kanR and Edw. ictaluri* R4383 *eihA::kanR* mutant at 43°C for 1 hr followed by plating on BHI blood agar plates removed the recombinogenic plasmid pMJH46 (data not shown). We found that rich medium supplemented with 5% Sheep Blood, unlike TSA, supported the growth of the high temperature-induced *Edw. ictaluri* strains. The introduction of plasmid pCP20, that contains the Flp recombinase (Cherepanov & Wackernagel, 1995), followed by their growth at 37°C resulted in the removal of the antibiotic marker from the *Edw. ictaluri ompLC* mutants (Figure 3). The PCR amplification of the targeted genes with their flanking primers confirmed that the frequency of the removal of the antibiotic marker was 100%. The antibiotic resistance markers from *Edw. ictaluri dtrA* and *eihA* mutant were also removed using the Flp recombinase (Figure 4). We found that, in addition

to the removal of the antibiotic resistance marker, the heat induction efficiently cured the plasmid pCP20 from all of the mutant colonies tested and this curing made the mutants ready for the deletion of additional genes.

5. Discussion

Construction of genetically isogenic strains of *Edw. ictaluri* is complicated by poor recombination rates and the lack of generalized transducing phages for *Edw. ictaluri*. Here, we describe a fast, efficient, and highly proficient method for deleting *Edw. ictaluri* genes using a recombineering system that is readily transferrable by conjugation, and demonstrate the applicability of this method by constructing a new plasmid that works for the modification of genes in *Edw. ictaluri*, and possibly in other bacterial species.

The application of recombineering for gene manipulation relies on three different proteins such as Exo, Bet and Gam originally encoded within the lambda prophage. The pKD46 is a low copy-number, temperature-sensitive plasmid that carries three genes (*exo*, *bet* and *gam*) required for recombineering and can be introduced into different Gram-negative bacteria via electroporation. However, multiple attempts to introduce this recombinogenic plasmid to different *Edw. ictaluri* by electroporation failed. Similar difficulties were observed by several other researchers who failed to introduce the pKD46 in *E. coli* by electroporation, demonstrating the need for an alternative route to introduce the recombineering system, i.e., via conjugation. The cloning of the red cassette into the pUC19 vector also did not help to introduce the resulting pUC19-Red plasmid into *Edw. ictaluri* by electroporation. As yet, we do not know the reasons for this failure, although it is likely that the *Edw. ictaluri* strains have active endonucleases that can degrade foreign dsDNA. Finally, the introduction of a mob cassette (*oriT* sequence and *traJ*

and *traK* that help plasmid to conjugally transfer from one bacteria to another) to pKD46 permitted the resulting plasmid pMJH46 to transfer into different *Edw. ictaluri* strains by conjugation.

Although suicide plasmid pKD4 was used as a substrate for the PCR amplification of antibiotic cassette for recombineering substrate, we recovered too many background colonies on the antibiotic selection plates without the replacement of the targeted genes. Even after *DpnI* treatment, the numbers of background colonies were not reduced. Finally, the use of *Edw. ictaluri* Alg-08-183 *ompLC::kanR* as a source of genomic DNA as a template for recombineering substrate reduced the number of background level substantially.

The removal of the recombinogenic plasmid pMJH46 from *Edw. ictaluri* strains subjected to gene modifications was essential since the Flp recombinase is also on plasmid pCP20 and shares the same antibiotic selection marker. The curing of temperature-sensitive plasmid pMJH46 required heat induction at 43°C for 1 hr before plating on solid medium to isolate colonies that were cured of the plasmid. We found in this study that heat-induced *Edw. ictaluri* mutants do not grow on their normal growth medium since all *Edw. ictaluri* strains are mesophilic bacteria that grow optimally at or around 28°C. Finally, it was found that BHI media supplemented with 5% sheep blood supported the growth of the heat-induced *Edw. ictaluri* mutants and had lost the temperature-sensitive recombinogenic plasmid pMJH46.

The construction of *Edw. ictaluri* mutants with multiple gene knock-outs also requires the removal of the previously introduced antibiotic marker due to the limited number of antibiotic resistance markers that can be used in *Edw. ictaluri* strains. In this study, we have found that the

Flp recombinase can be exploited to efficiently remove the antibiotic resistance cassette, thereby allowing construction of defined, markerless mutations in *Edw. ictaluri*.

We have described a highly efficient and rapid procedure for the generation of markerless mutants in *Edw. ictaluri* by recombineering. The newly constructed conjugally transferable recombinogenic plasmid pMJH46 can presumably be used to delete genes from other Gram-negative bacteria. Since recombineering is a common practice for epitope tagging of bacterial proteins (Uzzau *et al.*, 2001) this newly devised recombineering procedure could be used to tag *Edw. ictaluri* proteins very efficiency and in a very short amount of time.

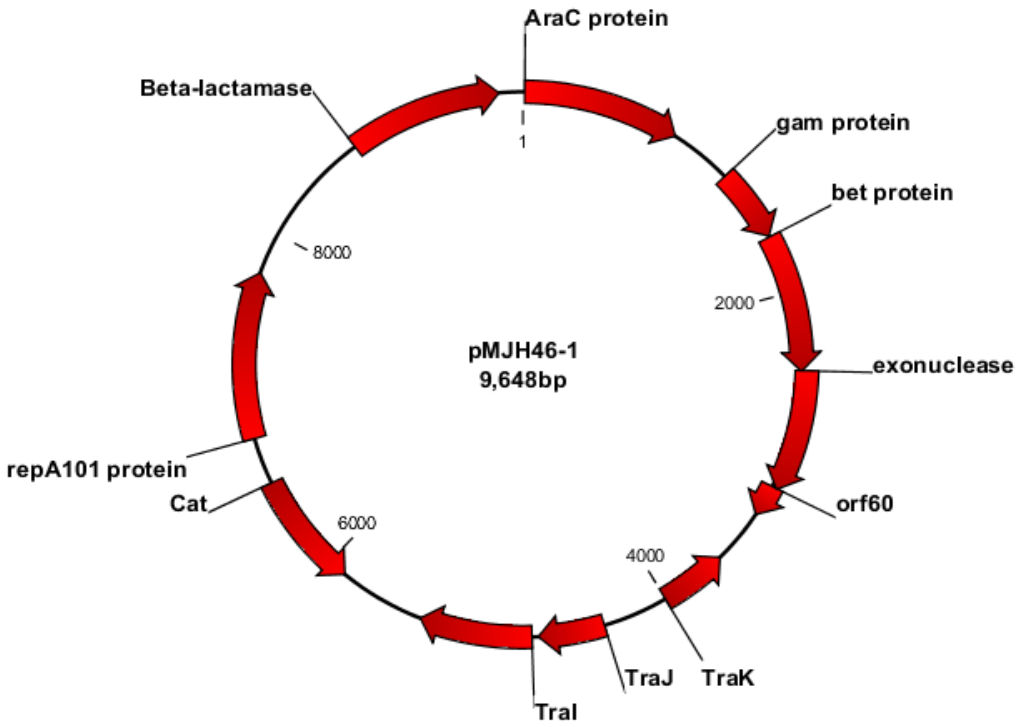


Figure 1. Schematic map of conjugally transferable recombinogenic plasmid pMJH46 (GenBank accession number JQ070344). This mobilizable plasmid was constructed by introducing an IncP origin of conjugal transfer (*oriT*) and chloramphenicol resistance gene (*cat*) at a unique NcoI restriction site of recombinogenic plasmid pKD46 (Datsenko & Wanner, 2000). Plasmid

pMJH46 encodes the λ- Red cassette (*exo*, *bet* and *gam*) that is required for RecA-independent homologous recombination of 50- to 60- bp homology extensions to the targeted genes. The plasmid map was generated by CLC Genomics Workbench (version 4.7.1).

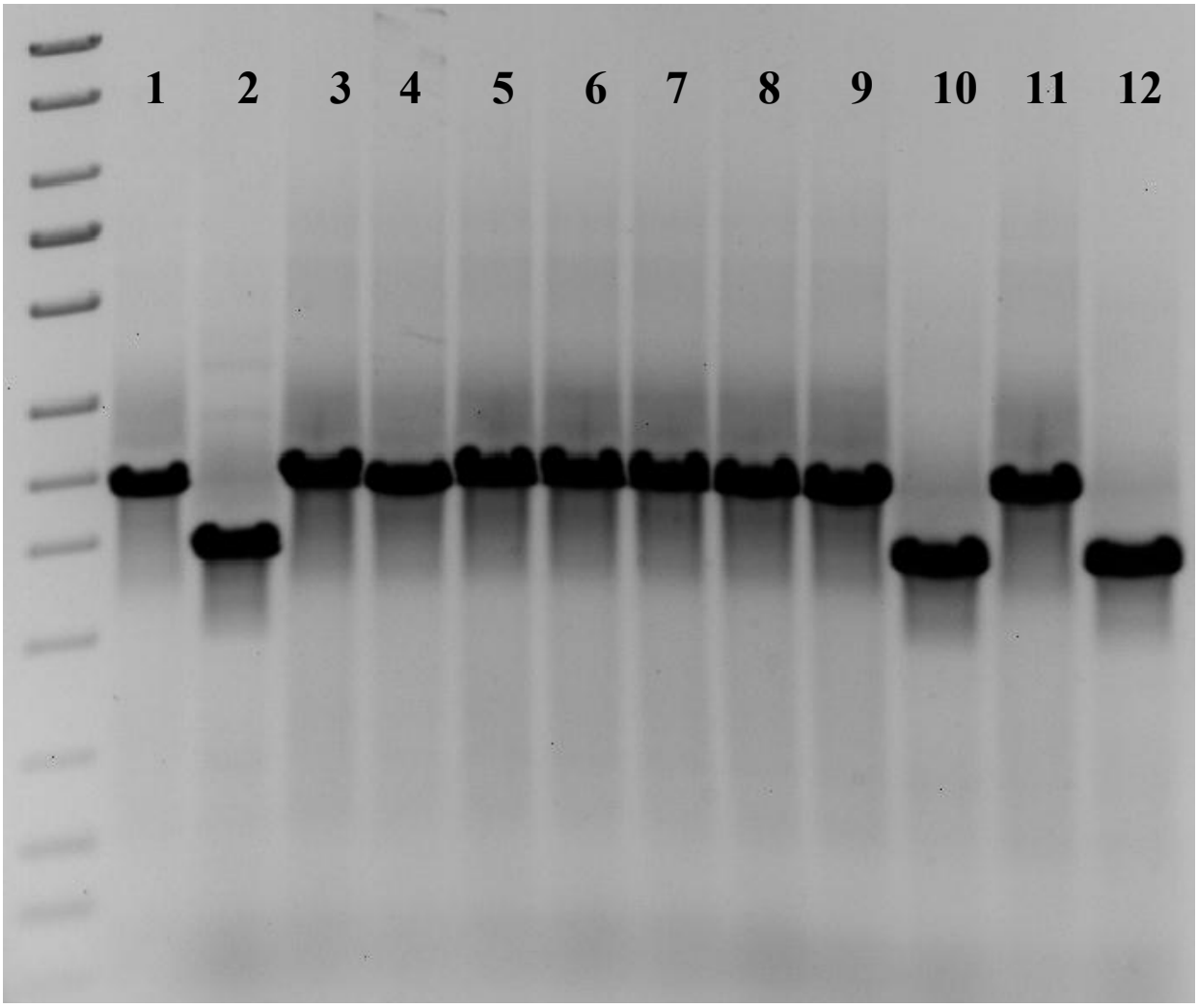


Figure 2. Deletion of *ompLC* gene from *Edw. ictaluri* by recombineering. A number of colonies gown on 2×YT plates supplemented with kanamycin were selected for PCR for the screening of *ompLC* gene deleted mutants. On average 90% of colonies were found to be deleted for *ompLC* gene by recombineering. Lane 1, 3-9 and 11 represents the PCR product of *ompLC* gene

disrupted with kanR gene (*ompLC::kanR*) and lane 2, 10 and 12 represents the PCR product of wild type *ompLC* gene of *Edw. ictaluri* strain Alg-08-183.

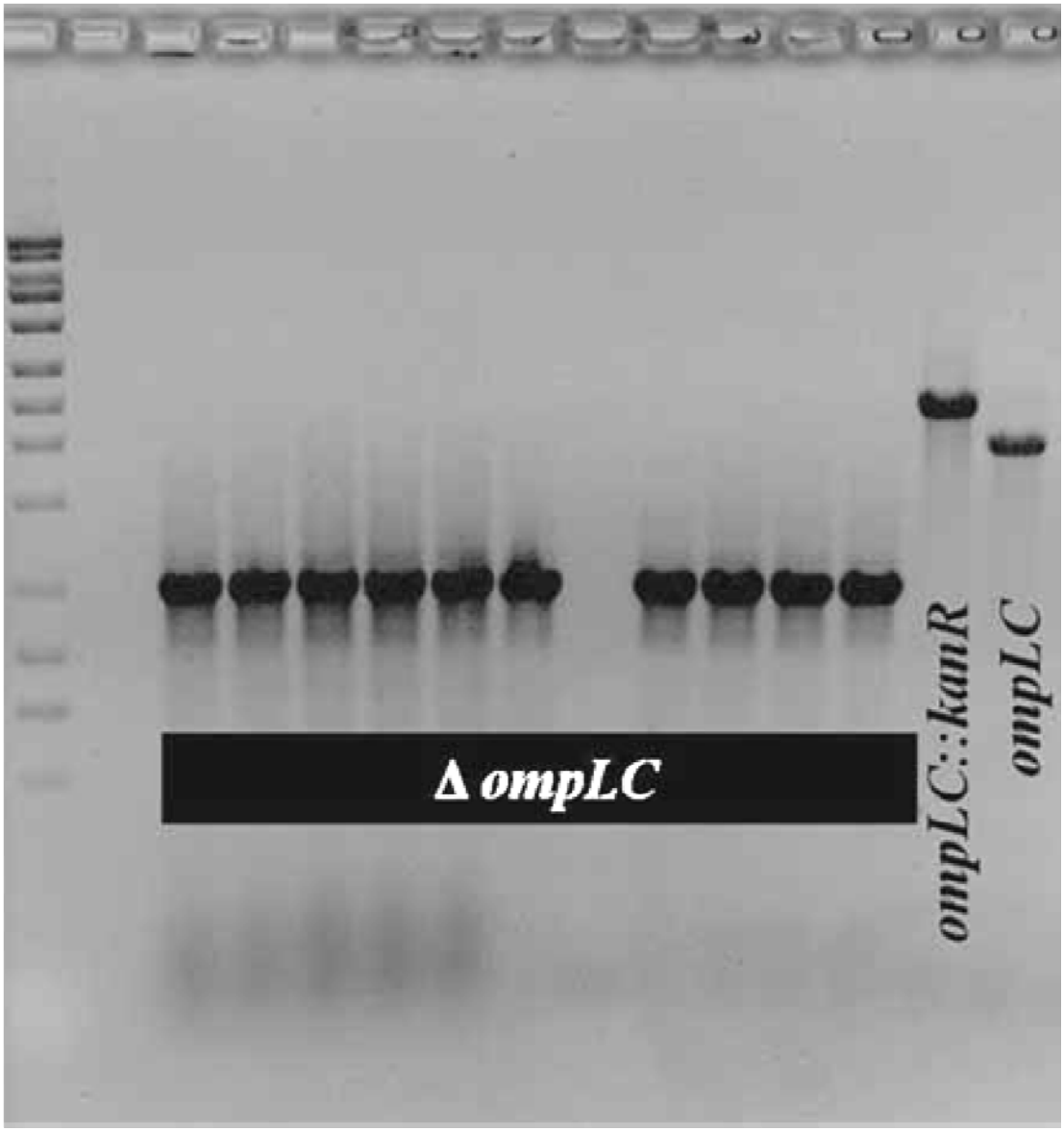


Figure 3. Removal of antibiotic resistance marker by Flp recombinase. PCR screening of *Edw. ictaluri* mutant colonies plated after temperature induction showed that all the mutants tested lost the kanamycin resistance marker. The absence of the mutant strains' growth on chloramphenicol also demonstrated that the pCP20 plasmid was cured after heat induction.

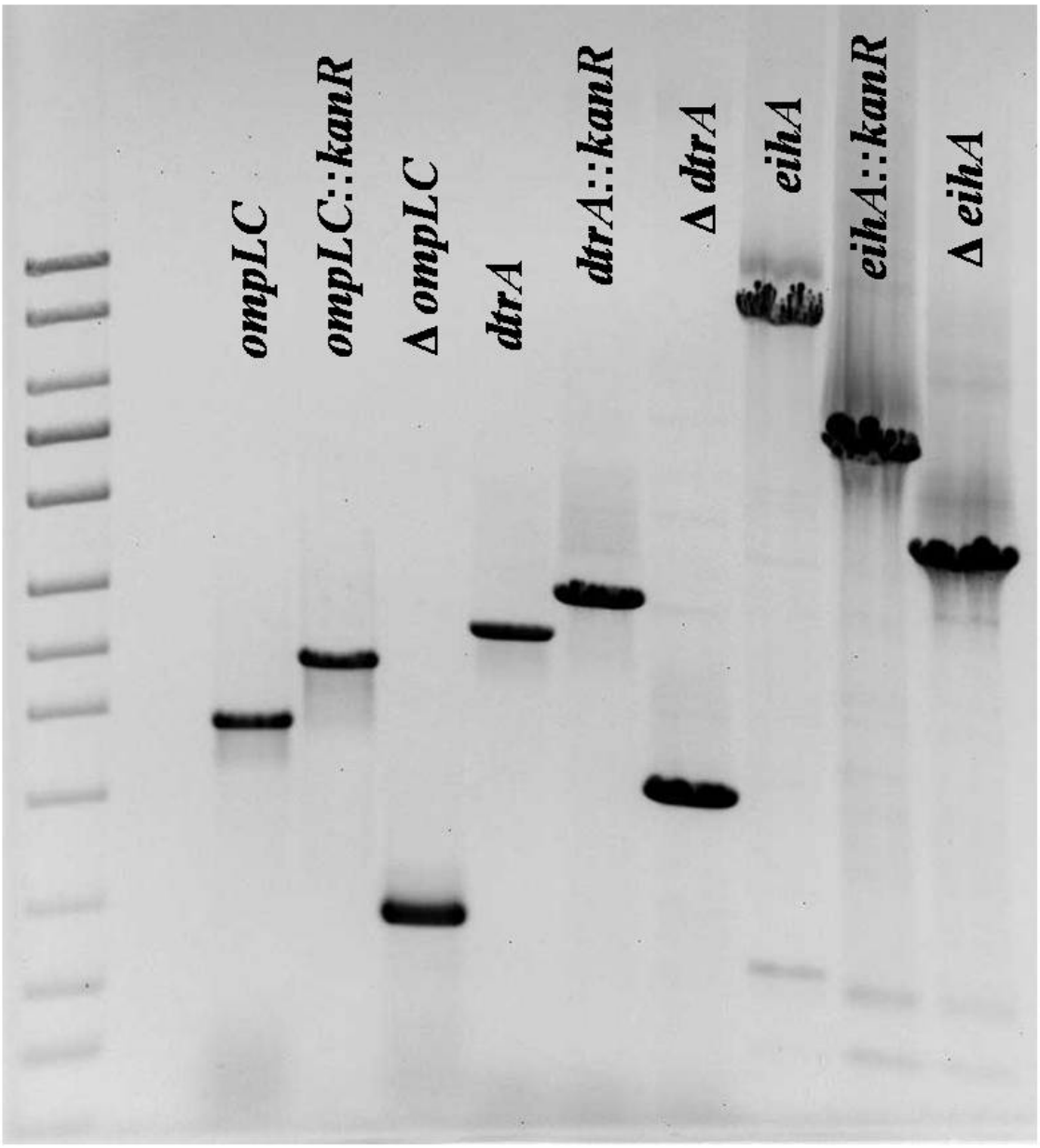


Figure 4. The modification of three different genes from *Edw. ictaluri*. The *ompLC* and *drtA* genes from *Edw. ictaluri* Alg-08-183 and *eihA* from *Edw. ictaluri* R4383 were replaced with *kanR* by recombineering. The *kanR* gene was removed from each of the mutant with Flp recombinase encoded from plasmid pCP20.

Table 1. The list of bacterial strains and plasmid used in this study.

Bacterial strains or plasmid	Features	References
E. coli		
SM10λpir	*thi-1thr leutonAlacYsupE recA*::RP4-2-TcT::Mu Km^r *λpir*	(Simon *et al.*, 1983)
BW25113/pKD46	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, pKD46	(Datsenko & Wanner, 2000)
BT340	F-, *Δ(argF-lac)169*, φ80d*lacZ58*(M15), *glnV44*(AS), λ^-, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *spoT1*, *thiE1*, *hsdR17*, pCP20	(Datsenko & Wanner, 2000)
BW25141/pKD4	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *Δ(phoB-phoR)580*, λ^-, *galU95*, *ΔuidA3::pir*$^+$, *recA1*, *endA9*(del-ins)*::FRT*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, pKD4	(Datsenko & Wanner, 2000)
Edw. ictaluri		
Alg-08-183	Phage ΦeiAU-183 resistant *Edw. ictaluri* strain	(Hossain *et al.*, 2012)
R4383	Highly hemolytic *Edw. ictaluri* strain	(Williams & Lawrence, 2005)
Alg-08-183 *ompLC::kanR*	Replacement of hemolysin *ompLC* gene with *kanR* gene	This study
Alg-08-183 *drtA::kanR*	Replacement of hemolysin *dtrA* gene with *kanR* gene	This Study
Alg-08-183 *ptrA::kanR*	Replacement of hemolysin *ptrA* gene with *kanR* gene	This Study
R4383 *eihA::kanR*	Replacement of hemolysin *eihA* gene with *kanR* gene	This Study
R4383 Δ*eihA*	In-frame deletion of hemolysin gene *eihA*	This study
Plasmids		
pKD46	Temperature-sensitive recombinogenic plasmid	(Datsenko & Wanner, 2000)
pKD4	Template for recombineering substrate	(Datsenko & Wanner, 2000)
pMJH46	Conjugally transferrable recombinogenic plasmid	This study
pCP20	Temperature-sensitive Flp recombinase plasmid	(Cherepanov & Wackernagel, 1995)
pGNS-BAC	Conjugally transferable BAC vector	(Kakirde *et al.*, 2011)

Table 2. List of primers used in this study.

Name of primers	Sequence
pKD4-ompLCf	5’-AACTGGTAGATCATACCAACGCCAACGATGTTGTCGGTGCTGATACCGGCGTGTAGGCTGGAGCTGCTTC-3'
pKD4-ompLCr	5’-GTTCAAAAAATTCCCGATGGAATCAAATTAGGCAGTGGCAGGTGTCAAAACATATGAATATCCTCCTTAGT-3'
ML44-RedF	5’-ATGCTTACAACAAAAAATATGCCAGCCAATGCTGGGCTGGCAGCGTTTTCTGGTGTAGGCTGGAGCTGCTTC-3'
ML44-RedR	5’-TTAGCAAGGGGGAAGATGCTCTGGTGGTGATGGTCTGTTTTTCTGATGATAGCATATGAATATCCTCCTTAGT-3'
Hemo-redF	5'-TTCCTTTTAACTCTGCTTTGGCGCCCATGGGCGCTGATATGAGGCAATCTCTGTGTAGGCTGGAGCTGCTTC-3'
Hemo-redR	5'-ACGGCGGCCCGCAGGCCGCCGTTGAGGATGGATAACGTCGCCACTATCCGGTCATATGAATATCCTCCTTAGT-3'
ML82-RedF	5'-GTGATACGTAGACAAGGTGCGACCATCGTACTGTGGATATTACTGCTGTTTTGTGTAGGCTGGAGCTGCTTC-3'
ML82-RedR	5'-TCAGCGGGTCACCTCTATCTTCAGCGTCTTTTGCAGCGCACTGACGTCCGGGTCATATGAATATCCTCCTTAGT-3'
RedtrackF	5'-GATGTCTATCTGTTCAGCTC-3'
RedtrackR	5'-GTACGCAATACCAATAGTG-3'
RE33-165F	5'-TATGCAAGCTTGTAGTTCTTGCTGGTCTC-3'
RE33-165R	5'-TATGCAAGCTTGTAACGCAACATTCTAAC-3'
k1	5’-CAGTCATAGCCGAATAGCCT-3'
k2	5’-CGGTGCCCTGAATGAACTGC-3'
kt	5’-CGGCCACAGTCGATGAATCC-3'
MobF	5'-ATGCAGATATCGGATCCTTTTTGTCCG-3'
MobR	5'-ACGCAGCAGTCAGTCACGATACAGCCGACCAGGCT-3'
CatF	5'-TATCGTGACTGACTGCTGCGTGTAGACTTCCGTTGAACT-3'
CatR	5'-ATGCAGATATCGCCTAATGAGTGAGCTAA-3'
MobicatF	5'-AGAGTGCTGACAGATGAG-3'
MobicatR	5'-ACGCAGCAGTCAGTCACGATAATGATGTGGTCTGTCCT-3'
MCF-int	5’- CATGCGATATCACCGCTAACCTGTCTT-3’
CatR-int	5'- CATGCGATATCTAATGAATCGGCCAAC-3'

Chapter IV

Identification of broad host specificity determinant of *Edwardsiella ictaluri* specific bacteriophages

1. Abstract:

Edwardsiella ictaluri strains responsible for enteric septicemia in catfish (ESC) are not equally susceptible to bacteriophage ΦeiAU and ΦeiDWF. In our previous study, we have established that the outer membrane porin protein LC (OmpLC) of *Edw. ictaluri* is a primary determinant for varying degree of phage susceptibility (Hossain *et al.*, 2012). Some *Edw. ictaluri* strains, such as Alg-08-183 and C91-162, are highly resistant to phage infection. However, by serially passaging phages in these strains, we isolated mutant phages, designated ΦeiDWF-183 and ΦC91-162 from these host strains. In this study, the genome of phages ΦeiDWF-183 and ΦC91-162 were sequenced using next generation sequencing in order to identify the molecular determinants in these phages that enabled broader host range infectivity. The comparative genomics of mutant and wild type phages demonstrated that mutant phages ΦeiDWF-183 and ΦC91-162 have experienced mutations in seven out of 56 predicted proteins. Genes that encode phage tape measure protein and phage host specificity protein (encoded by *hspP*) experienced 14 and 9 mutations, respectively. Using recombineering experiment with a mutated *hspP* and wild type phage genomic DNA followed by the generation of recombinant phages with broader host

specificity demonstrated that the C-terminal end of the phage host specificity protein (HspP) is responsible for the broader host specificity of phage ΦeiDWF-183. The information gained from this study will help to rationally design bacteriophages for better infectivity to phage-resistant *Edw. ictaluri* strains, and more generally to better understand the nature of phage-host interactions at a molecular level.

2. Introduction

Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC) which is a primary disease that affects farm-raised channel catfish (Hawke *et al.*, 1981; Hawke *et al.*, 1998). Catfish producers in the Southeastern United States incur a significant annual economic loss which is estimated between 30 and 50 million dollars per annum (Shoemaker *et al.*, 2009). A recent survey conducted by USDA's National Animal Health Monitoring System (NAHMS) within the catfish producers in the Southeastern United States showed that about 37% of food-size catfish producers have experienced economic loss due to ESC (USDA, 2011). Though channel catfish (*Ictalurus punctatus*) is the primary target of natural infection with *Edw. ictaluri*, the natural infection of freshwater catfish (*Pangasius hypophthalmus*) in Vietnam (Crumlish *et al.*, 2002) and yellow catfish (*Pelteobagrus fulvidraco) in China (Liu et al., 2010)* caused by *Edw. ictaluri* has been reported recently. The rapid diagnosis of ESC is essential for the treatment of ESC infected catfish (Gaunt *et al.*, 2006). However, the current culture-based methods for the detection of ESC are time-consuming, taking several days to get confirmatory results (International Office of Epizootics. Aquatic Animal Health Standards, 2009). Although several number of PCR-based detection techniques have been developed to detect *Edw. ictaluri* rapidly (Sakai *et al.*, 2009; Williams & Lawrence, 2010; Yeh *et al.*, 2005), the diagnostic approaches are not cost effective and not available in many fish diagnostic laboratory. It is highly

essential to devise a rapid, cost-effective and specific diagnostic procedure for the detection of ESC.

Bacteriophages are efficient tools for the rapid identification (McNerney *et al.*, 2004; Sergueev *et al.*, 2010) and typing (Chakrabarti *et al.*, 2000; Rabsch, 2007) of bacterial pathogens. We identified bacteriophage ΦeiAU and ΦeiDWF that are specific to *Edw. ictaluri* (Walakira *et al.*, 2008) and these phages could potentially be used for the rapid diagnosis of ESC. However, our host range analysis of the two phages to different *Edw. ictaluri* strains demonstrated that all *Edw. ictaluri* strains are not equally susceptible to phage ΦeiAU and ΦeiDWF infection (Hossain *et al.*, 2012). The outer membrane porin protein LC (OmpLC) of *Edw. ictaluri*, receptor for phage adsorption and infection, modulates phage ΦeiAU and ΦeiDWF susceptibility in different *Edw. ictaluri* strains (Hossain *et al.*, 2012). We isolated a mutant phage ΦeiDWF-183 of ΦeiDWF origin which acquired the ability to infect phage ΦeiDWF and ΦeiAU-resistant *Edw. ictaluri* strain Alg-08-183 by overcoming the host-imposed phage resistance due to an altered *Edw. ictaluri* OmpLC protein. The molecular determinants of phage ΦeiDWF-183 which support broader host specificity to *Edw. ictaluri* strains have not been identified. Moreover, many of the *Edw. ictaluri* strains are still resistant to our existing phage stocks.

Tailed phages recognize their host receptors that include lipopolysaccharide, outer membrane porin protein, flagella, and pili with their tail fiber protein (Werts *et al.*, 1994; Yu & Mizushima, 1982). The C-terminal portion of the phage tail fiber proteins is responsible for host receptor recognition (Garcia-Doval & van Raaij, 2012; Montag *et al.*, 1990; Wang *et al.*, 2000) (Wang *et al.*, 2000). The C-terminus of the tail fiber protein contains variable regions (Duplessis & Moineau, 2001) and is highly prone to change for binding adaptability to modified bacterial receptors (Meyer *et al.*, 2012), whereas the N-terminus of receptor-binding proteins are mostly

conserved to maintain the stable protein-protein interactions with other phage proteins (Tétart *et al.*, 1998). Lambda phage extend their host range by changing the C-terminal portions of their tail fiber protein J (Werts *et al.*, 1994) that specifically adsorbs with LamB protein of *E. coli* K-12 for the initiation of infection (Wang *et al.*, 2000). Recently, it was demonstrated that λ phage switches from its natural receptor LamB to a new receptor OmpF by changing the C-terminal region of receptor binding protein J (Meyer *et al.*, 2012). The host range specificity of T4 phage is determined by a C-terminal variable region of receptor recognizing protein 37 and changing amino acid sequences on the variable region that enables a switch from an outer membrane protein OmpC to lipopolysaccharide as a receptor (Montag *et al.*, 1990). In addition to point mutations, phages can also extend their host range by swapping the variable protein domain (Tétart *et al.*, 1998) or by inverting the DNA sequences of the receptor binding proteins (van de Putte *et al.*, 1980). Recently, it was demonstrated that a tail fiber protein of phage T4 can be engineered to cross the species boundary for the detecting, infecting and killing of Gram-negative bacteria distantly related to *E. coli* (Pouillot *et al.*, 2010). The characterization of phage anti-receptor proteins will help to identify different regions of this protein that are involved in broader host range and finally would help to modify the regions by directed mutagenesis for further extending their host range.

3. Materials and Methods

Bacterial strains, phages and plasmids. Bacterial strains, phages and plasmids used in this study are listed in Table 1. *Edw. ictaluri* strains were routinely grown on Trypticase Soy Broth (TSB) at 30°C with aeration. Bacteriophages were propagated using a soft agar overlay technique using *Edw. ictaluri* strains susceptible to phage infection. An *E. coli* strain containing recombinogenic plasmid pKD46 was grown on 2× YT medium at 30°C with aeration. If needed,

growth medium was supplemented with arabinose (10mM), ampicillin (100 µg/ml), kanamycin (50 µg/ml), and/or colistin (20 µg/ml).

Passaging of bacteriophages for isolation of mutants with broad host specificity. Wild type phage ΦeiDWF, which shares an identical host specificity to *Edw. ictaluri* strains with phage ΦeiAU and is the most recent phage isolate (Walakira *et al.*, 2008), was passaged on phage ΦeiDWF-resistant *Edw. ictaluri* strains C91-162, R4383, S89-9, Alg-08-183, ML08-117, Alg-08-192, Alg-08-199, Alg-08-196 and Alg-08-221 according to the methods described previously (Hossain *et al.*, 2012).

Sequencing and assembly of phage ΦeiDWF-183 and ΦC91-162 genome. To determine the nucleotide polymorphisms responsible for broad host range-specificity of phage ΦeiDWF-183, the genome sequence of this phage was determined using an Illumina MiSeq. Phage ΦeiDWF-183 genomic DNA was extracted according to the methods described elsewhere (Hossain *et al.*, 2012). A phage ΦeiDWF-183 genomic DNA library was prepared using a Nextera sample preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions with minor modifications. Briefly, a tagmentation reaction was assembled by adding 11 µl of phage ΦeiDWF-183 genomic DNA (total 50 ng), 25 µl of 2× TD buffer, 5 µl of TDE1 enzyme and 9.0 µl of nuclease free water to make the reaction volume up to 50 µl. The tagmentation reaction was carried out at 55°C for 5 minutes using a Thermalcycler with a heated lid. Tagmented DNA was purified using the Zymo DNA purification system (Zymo Research, Irvine, CA) according to manufacturer's instructions. Purified tagmented DNA was eluted with 25 µl of resuspension buffer. A 5-cycle PCR was carried out to amplify and enrich the tagmented DNA library. The PCR reaction included purified, tagmented DNA, index 1, index 2, PCR master mix and a PCR primer cocktail in a 50 µl reaction volume. The amplified and enriched

phage genomic DNA library was purified and size selected using an E.Z.N.A.® Size Select-IT Kit (Omega Bio-tek, Norcross, GA) according to manufacturer's instructions. DNA fragments between 150 bp to 700 bp were selected for sequencing using an Illumina MiSeq. Paired-end sequences obtained from the Illumina MiSeq were trimmed and assembled using the CLC Genomics Workbench under default parameters. Phage ΦC91-162 genomic DNA was sequenced using a 454 sequencer according to the method described previously (Hossain *et al.*, 2012).

Comparison of phage ΦeiC91-162 and phage ΦeiDWF-183 genomes with that of the wild type phage ΦeiDWF. The predicted ORFs of phage ΦeiC91-162 and phage ΦeiDWF-183 genome were defined using GeneMarkS gene prediction tool (Borodovsky & Lomsadze, 2002). Each of the ORFs within the genome of phage ΦeiDWF-183 was compared with those of the phage ΦeiDWF using CLC Genomics Workbench. A local database was created in the CLC Genomics Workbench using the complete ORFs collection of phage ΦeiDWF. All of the ORFs from phage ΦC91-162 and ΦeiDWF-183 were compared by BLAST against the predicted ORFs of phage ΦeiDWF using the BLASTp algorithm to determine the variations within the ORFs of phage ΦeiDWF-183 that resulted in broad host specificity of this phage. Each of the ΦeiDWF-183 ORFs with point mutations as compared to that of phage ΦeiDWF was examined further to determine their potential role(s) in broad host range specificity.

Identification of phage ΦeiDWF-183 proteins involved in broad host specificity. The *hspP* of phage ΦeiDWF-183 showed variations with that of phage ΦeiAU and ΦeiDWF and was PCR amplified and cloned into the TOPO-TA cloning vector pCR2.1 using primers listed in Table 2. Plasmid constructs were introduced into *Edw. ictaluri* 219 or EILO separately by electroporation according to the methods described in elsewhere (Hossain *et al.*, 2012). *Edw. ictaluri* strain 219 harboring a plasmid containing the *hspP* gene was infected with wild-type

phage using a soft agar overlay to obtain confluent lysis of the bacterial cells after overnight incubation. Plates were soaked with 1.0 ml of SM buffer and phages were collected by scrapping the top agar in a 15 ml polypropylene conical tube. Cellular debris was removed by centrifugation at 3600 ×g for 20 minutes and phages were purified by 0.2 µm filter. Purified phages were used to challenge phage ΦeiAU and ΦeiDWF-resistant *Edw. ictaluri* strain Alg-08-183 using a soft agar overlay (Hossain *et al.*, 2012). Phages from individual plaques were doubly purified and targeted regions believed to be involved in enhancing infectivity to *Edw. ictaluri* Alg-08-183 were amplified by PCR and subjected to sequencing.

A region of HspP from phage ΦeiDWF-183 involved in broad host specificity was identified by recombineering. To determine the regions of HspP of phage ΦeiDWF-183 involved in broad range host specificity, a method of bacteriophage recombineering of electroporated DNA (BRED) (Marinelli *et al.*, 2008) was modified. Instead of native host *Edw. ictaluri*, *E. coli* was used as a heterogeneous host for recombineering of wild-type phage genomic DNA. Recombineering plasmid pKD46 (Datsenko & Wanner, 2000) was introduced into *E. cloni* 10G (Lucigen, WI) using electroporation. This *E. coli* strain can support the growth of phage ΦeiAU, ΦeiDWF, or ΦeiDWF-183 after introducing phage DNA by electroporation, even though these phages cannot infect it naturally. A high fidelity PCR product was generated using TaKaRa Ex Taq Polymerase, primer MaF and BabaR, and 50 ng of phage ΦeiDWF-183 genomic DNA as a template by targeting the mutated region of phage ΦeiDWF-183 *hspP* gene. This PCR product, which was 157 bp in length included 50 and 50 bp of homologous sequence upstream and downstream of the mutated regions, respectively. The PCR product was treated with the DpnI restriction enzyme to get rid of the phage ΦeiDWF-183 genomic DNA template. Four separate electroporation reactions were carried out using electrocompetent *E. coli* cells

harboring plasmid pKD46 and grown in the presence of 10 mM arabinose. Two hundred ng of PCR product was mixed with 120 ng of wild-type phage genomic DNA and electroporated into *E. coli* (pKD46). In another experiment, two hundred ng of PCR product was electroporated into *E. coli* (pKD46) to confirm the absence of phage ΦeiDWF-183 genomic DNA template after DpnI digestion. Additionally, 120 ng of wild-type phage and ΦeiDWF-183 genomic DNAs were electroporated separately into *E. coli* (pKD46). After 1 hour of recovery at 30°C, phages were prepared by chloroform lysis followed by centrifugation at 15,000 ×g for 2 minutes. Titer of the progeny phages was determined by soft agar overlay using *Edw. ictaluri* strain 219 and Alg-08-183 separately.

4. Results

Isolation of mutant phages with higher infectivity to phage-resistant *Edw. ictaluri* strains. Since *Edw. ictaluri* strains exhibit varying degree of phage susceptibility ranging from highly susceptible to completely resistant to their phages (Hossain *et al.*, 2012), it is highly essential to expand the host range of *Edw. ictaluri*-specific phages for their application in diagnostics and/or therapy. A total of nine highly resistant *Edw. ictaluri* strains that included *Edw. ictaluri* strains C91-162, R4383, S89-9, Alg-08-183, ML08-117, Alg-08-192, Alg-08-199, Alg-08-196 and Alg-08-221 were challenged with phage ΦeiDWF to obtain mutant phages with higher infectivity. Six mutant phages were isolated that were able to kill the phage ΦeiDWF-resistant *Edw. ictaluri* strains C91-162, R4383, S89-9, Alg-08-183, Alg-08-192 and Alg-08-221. Mutant phages obtained by challenging on *Edw. ictaluri* strains Alg-08-199 and Alg-08-196 were not able to produce noticeable plaques on these strains but were able to infect and produce noticeable plaques on *Edw. ictaluri* strains Alg-08192 and Alg-08-221 (data not shown). The

comparison of the efficiency of plaquing (EOP) of the mutant phages with that of the wild type phage showed that the EOP of mutant phages had been increased greatly (Figure 1).

Sequencing of mutant phages ΦeiDWF-183 and phage ΦC91-162. To determine the phage molecular determinants for broad host specificity, we sequenced the genomes of the mutant phages ΦeiDWF-183 and ΦC91-162 that are lytic to phage ΦeiAU and ΦeiDWF-resistant *Edw. ictaluri* strains Alg-08-183 and C91-162, respectively, at greater than 116 and 15× coverage, respectively. The *de novo* assembly of the sequences produced a single large contig for each of the phage genomes. The sizes of these contigs were very close to the genome of the wild type phage ΦeiDWF (Walakira *et al.*, 2008). The genome size of phages ΦeiDWF-183 and ΦC91-162 were 43,017 and 42,923 bp, respectively. The comparison of phage ΦeiDWF-183 and ΦC91-162 genome sequences with that of the wild type phage ΦeiDWF demonstrated that each of the phage genomes encode 56 predicted open reading frames (ORFs). Pairwise comparison of the predicted ORFs from mutant and wild type phages showed that there were no deletions or insertions within the genomes of phages ΦeiDWF-183 and ΦC91-162. A total of 37 amino acids substitutions were observed within the proteome of each of the phage genomes. Mutations within the predicted ORFs of phage ΦeiDWF-183 and ΦC91-162 are documented in Table 1 and 2, respectively. Though phage ΦC91-162 and ΦeiDWF-183 were isolated by passaging on two different *Edw. ictaluri* strains, it was observed that both phages had 33 identical amino acid mutations. There were two mutations in both of the phage proteomes that were located in independent sites. Phage ΦeiDWF-183 had 9 mutations within the phage host specificity proteins HspP. Among the 9 amino acid substitutions, 7 of these were located in the C-terminal end of the protein. It was demonstrated that the C-terminus of phage host specificity protein J of λ phage experienced most of the mutations that conferred broader host specificity (Meyer *et al.*, 2012).

The role of HspP of phage ΦeiDWF-183 in extended host range. To determine the role of the mutated HspP protein of phage ΦeiDWF-183 for infectivity to *Edw. ictaluri* strain Alg-08-183, the complete ORF of the *hspP* gene of phage ΦeiDWF-183 was cloned and introduced into *Edw. ictaluri* strain EILO. The EILO strain bearing the phage ΦeiDWF-183 gene was infected with wild type phage. Recombinant phages generated after allelic exchange of mutant *hspP* gene with the wild type *hspP* gene of phage ΦeiAU through homologous recombination were selected on wild-type phage-resistant but ΦeiDWF-183-sensitive *Edw. ictaluri* strain Alg-08-183. Three recombinant phages were isolated and sequencing of the *hspP* gene from these phages showed that they shared sequence similarity with that of phage ΦeiDWF-183 (Figure 2). This result confirmed that the HspP protein in phage ΦeiDWF-183 is responsible for its broader host range.

The C-terminal region of HspP in phage ΦeiDWF-183 is involved in extended host range. The analysis of HspP in recombinant phages obtained by allelic exchange demonstrated that most of the amino acid substitutions were clustered within the C-terminus of HspP. To determine the role of these C-terminal amino acid substitutions, a recombineering system was used in which a heterogeneous host *E. coli* with a recombinogenic plasmid was used to modify electroporated phage genomic DNA. The co-electroporation of wild-type phage genomic DNA and a PCR product that included the 3′ end of the *hspP* gene of phage ΦeiDWF-183 into the recombinogenic *E. coli* produced 1.0×10^3 recombinant phages/μg of phage genomic DNA infective to wild-type phage-resistant *Edw. ictaluri* strain Alg-08-183 (Figure 4). In contrast, the electroporation of recombinogenic *E. coli* with an equal amount of wild-type phage genomic DNA did not produce any recombinant phages infective to wild-type phage-resistant *Edw. ictaluri* strain Alg-08-183 (Figure 4). These results suggest that the 3’ end of the PCR product of

the *hspP* gene of phage ΦeiDWF-183 had integrated into the genome of the wild-type phage and produced recombinant phages similar to phage ΦeiDWF-183. The sequencing of the C-terminus of the *hspP* gene of the doubly purified recombinant phages obtained by recombineering determined that the PCR products had integrated into the genome of the wild-type phage, resulting in recombinant phages infective to *Edw. ictaluri* strain Alg-08-183 (Figure 3). These findings confirmed the role of the C-terminus of HspP in phage ΦeiDWF-183 for the infectivity of *Edw. ictaluri* strain Alg-08-183.

5. Discussion

This study isolated six different mutant phages that are now able to infect a large number of previously phage-resistant *Edw. ictaluri* strains. The repertoire of mutant phages that have been isolated in this study is now being routinely used for the detection of primary isolates of *Edw. ictaluri* strain obtained from diseased fish at the West Alabama Fish Farming Center (William Hemstreet, director). This study demonstrated that a large number of mutant phages with broad lytic activity could be isolated in a short period of time.

The comparative genomics of phage ΦeiDWF-183 and ΦC91-162 genomes with that of the wild-type phage ΦeiDWF demonstrated that a large number of phage proteins (seven ORFs out of 56 total ORFs) have experienced mutation(s) after passaging on a phage-resistant *Edw. ictaluri* strain. This finding is in contrast to the nature of the mutant λ phages with extended host range in which only the phage tail fiber protein J experienced several mutations on the way to their recognition of the new receptor OmpF (Meyer *et al.*, 2012). The large number of mutations suggests that the *Edw. ictaluri*-specific phages might need to overcome several lines of host

defenses to gain their lytic capacity against *Edw. ictaluri* primary isolates obtained from diseased catfish.

In this study we determined that the C-terminus of the phage host specificity protein (HspP) in phage ΦeiDWF is responsible for broad host infectivity to *Edw. ictaluri*. The C-terminal portion of the phage tail fiber proteins of λ, T4 and T7 are involved in the recognition of host receptors required to initiate infection. Previously, we have demonstrated that the outer membrane porin protein LC (OmpLC) of *Edw. ictaluri* is the receptor for phage ΦeiAU and ΦeiDWF, with mutations in OmpLC severely affecting the binding as well as infection capacity of this phage in *Edw. ictaluri* strains (Hossain *et al.*, 2012). Mutant phage ΦeiDWF-183 is known to infect *Edw. ictaluri* Alg-08-183 strain that has an altered OmpLC (Hossain *et al.*, 2012). The involvement of the C-terminus of the phage host specificity protein in increasing the host range suggests that phage ΦeiDWF evolved to overcome the phage resistance in the *Edw. ictaluri* strain due to altered OmpLC receptor by changing its tail fiber protein. The C-terminus of the phage host specificity protein could be targeted for rational design of phages with enhanced attachment to diverse bacterial hosts.

This study described a novel experimental technique to study the phage determinants for broader host specificity. A heterogeneous *E. coli* host, instead of the native host *Edw. ictaluri*, bearing a recombinogenic plasmid was used to modify wild type bacteriophage by recombineering for the production of recombinant phages. This technique amplified the mutated segment of the phage genome from mutant phages obtained by passaging on wild type, phage-resistant *Edw. ictaluri* strains. The co-electroporation of mutated PCR products and genomic DNA from wild type phages into the recombinogenic *E. coli* resulted in recombinant phages with lytic capability as mutant phages from which the PCR products were obtained. A technique has

recently been described that called Bacteriophage Recombineering of Electroporated DNA (BRED), in which a highly efficient recombineering system for the mutagenesis of phage DNA is utilized directly on electroporated phage DNA (Marinelli *et al.*, 2008). In this system the native bacterial host *Mycobacterium smegmatis* cells are used for the mutagenesis of phage using recombineering. Though *Edw. ictaluri* strains are capable of receiving plasmids or phage genomic DNA through electroporation, the transformation efficiencies are much lower than that of *E. coli* strains (Russo *et al.*, 2009). Since we have found that strains of *E. coli* support the growth of progeny phages after electroporation of wild-type phage genomic DNA but cannot be infected naturally, we used the heterogeneous host *E. coli* for the mutagenesis of electroporated phage ΦeiAU genomic DNA by recombineering.

The passaging experiment carried out in this study failed to obtain mutant phages that were lytic to *Edw. ictaluri* strains ML08-117, Alg-08-196 or Alg-08-199. The loop8 of OmpLC of *Edw. ictaluri* ML08-117 strain contained a two amino acid insertion that is believed to be altered in the surface-exposed structure (Hossain *et al.*, 2012). An altered phage host specificity protein found in this study is responsible for broad host specificity. In many bacteriophages, the phage host specificity protein binds to their host receptor(s) and initiates the infection process. *Edw. ictaluri*-specific bacteriophages can exploit the OmpLC protein for phage infection and this OmpLC modulates phage susceptibility by changing its surface-exposed loop 8 (Hossain *et al.*, 2012). The failure of passaging experiments to obtain mutant phages lytic to *Edw. ictaluri* ML08-117 might be attributed to the altered OmpLC of this strain. However, random mutagenesis of the phage tail fiber protein followed by their incorporation into phages through recombineering could provide recombineering phages that can be lytic to ML08-117 and other primary disease isolates.

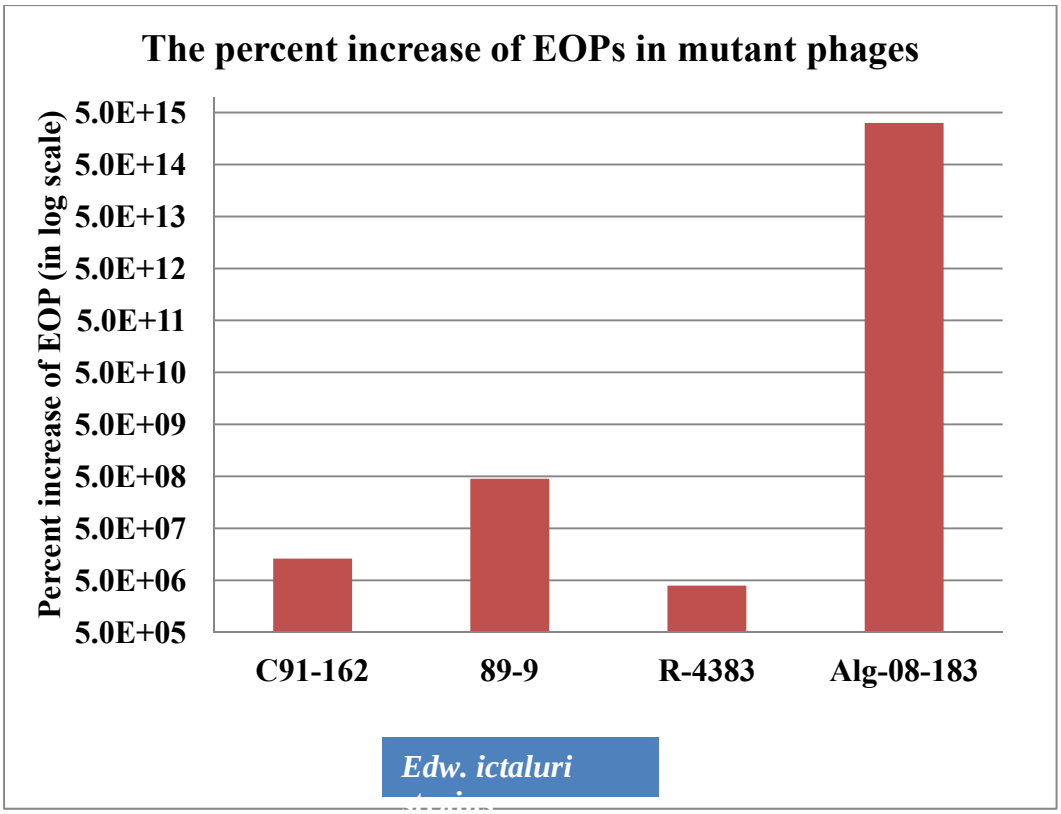


Figure 1. The percent increase in the efficiency of plaquing (EOP) of different mutant phages to different *Edw. ictaluri* strains that were previously resistant to wild type phages ΦeiAU and ΦeiDWF.

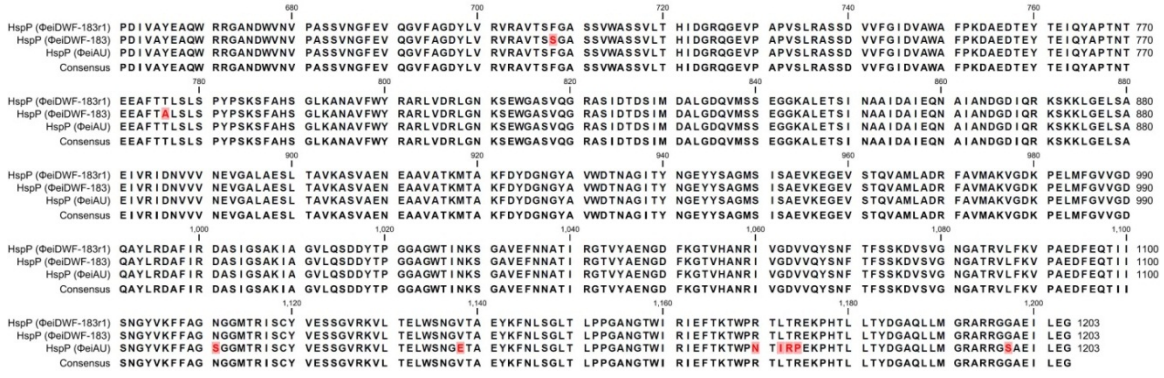


Figure 2: The alignment of amino acids sequences of HspP of phage ΦeiDWF-183r, a recombinant phage obtained by homologous recombination, wild type phage and mutant phage ΦeiDWF-183.

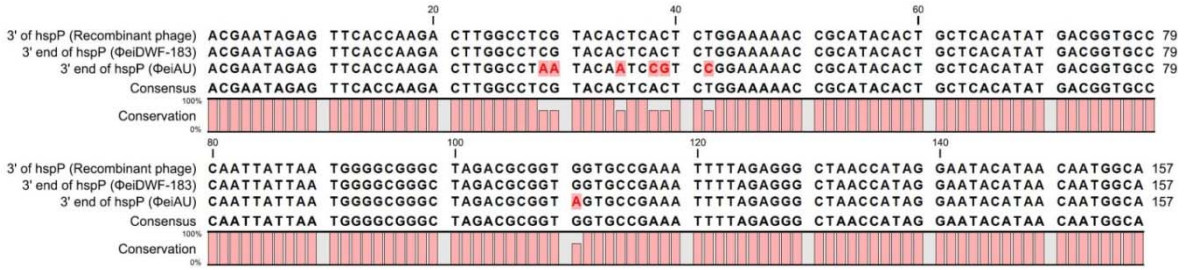


Figure 3. The alignment of C-terminal ends of the *hspP* gene from wild-type phage, ΦeiDWF-183 and recombinant phages lytic to *Edw. ictaluri* Alg-08-183 obtained by recombineering of PCR product and the wild-type phage genome.

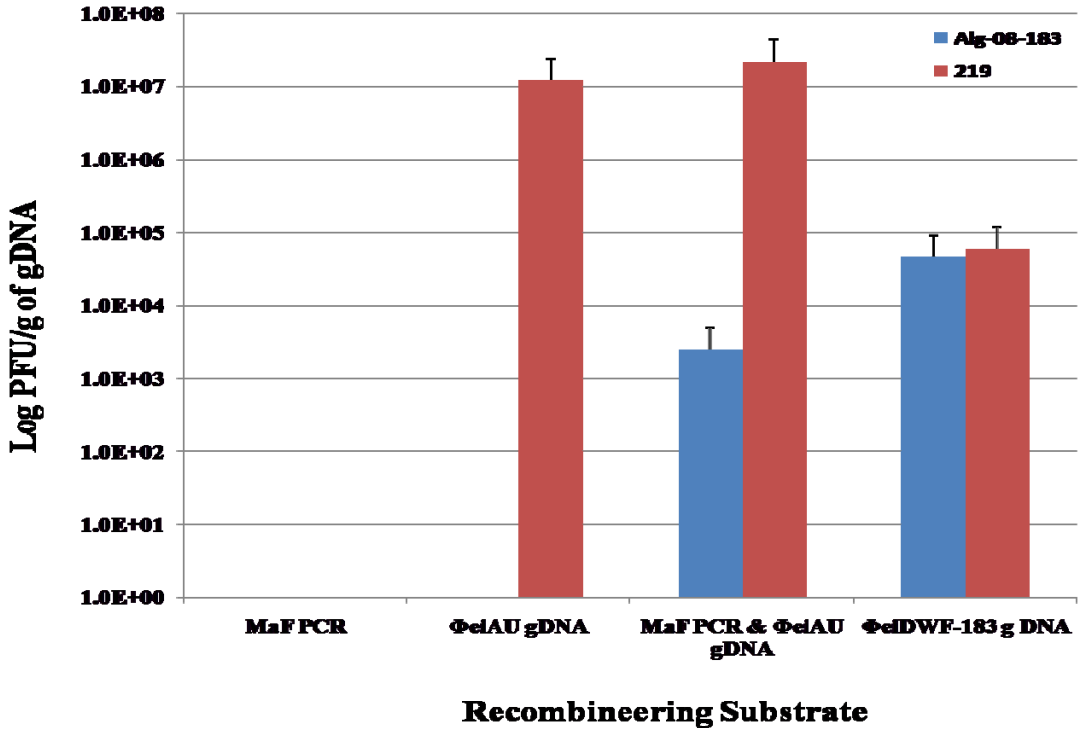


Figure 4: The frequency of recombinant phages obtained by a recombineering approach. In each experiment, where appropriate, 200 ng of PCR product was electroporated with 120 ng of phage genomic DNA.

Table 1. Proteins of phage ΦeiDWF that have been altered in mutant phage ΦeiDWF-183 after passaging on phage-resistant *Edw. ictaluri* Alg-08-183.

ORFs of ΦeiDWF-183	% Identity to phage ΦeiDWF ORFs	ΦeiDWF ORFs	Phage Proteins	Amino acid substitutions within the predicted ORFs
1	98	HQ824622	putative bacteriophage tail tape measure protein	D434N, I440T, S443A, L445Q, E669D, A680I, D682E, K683Q, N684D, K691R, E698D, M701T, A704S, D705A
6	99	HQ824617	phage host specificity protein	F708S, T776A, S1111N, E1138V, N1170R, I1172L, R1173T, P1174L, S1197G
16	99	HQ824608	unknown	D53Y
17	99	HQ824607	methyltransferase	A33S
19	95	HQ824606	gp31	T3S, I24L, S41T, Q45L, Y54F, P81Y, A86S, V108A,
21	97	HQ824550	unknown	K62E, A65G, G106E
40	99	-		X32R

Table 2: Proteins of phage ΦeiDWF that have been altered in mutant phage ΦC91-162 generated after passaging on phage-resistant *Edw. ictaluri* C91-162.

ORFs of ΦC91-162	% Identity to phage ΦeiDWF ORFs	ΦeiDWF ORFs	Phage Proteins	Amino acid substitutions within the predicted ORFs
14	99	DWF_45		X32R
36	98	HQ824622	putative bacteriophage tail tape measure protein	S172F, Y391N, D434N, I440T, S443A, L445Q, E669D, A680I, D682E, K683Q, N684D, K691R, E698D, M701T, A704S, D705A
41	99	HQ824617	phage host specificity protein	F708S, T776A, S1111K, E1138V, N1170H, R1173T, P1174Q,
50	99	HQ824608	unknown	D53Y
51	99	HQ824607	methyltransferase	A33S
53	95	HQ824606	gp31	T3S, I24L, S41T, Q45L, Y54F, P81Y, A86S, and V108A
55	97	HQ824550	unknown	K62E, A65G and G106E

Chapter V

Implication of lateral genetic transfer in the emergence of *Aeromonas hydrophila* isolates of epidemic outbreaks in channel catfish

1. Abstract

To investigate the molecular basis of the emergence of *Aeromonas hydrophila* responsible for an epidemic outbreak of motile Aeromonas septicemia (MAS) of catfish in the Southeastern United States, we sequenced 12 *A. hydrophila* isolates that included six reference and six recent epidemic isolates. Comparative genomics revealed that recent epidemic *A. hydrophila* isolates are highly clonal, whereas reference isolates are greatly diverse. We identified 54 epidemic-associated unique genetic regions with 307 predicted genes that are present in epidemic isolates but absent from reference isolates and 35% of the unique regions are located within the genomic islands, suggesting their acquisition through lateral gene transfer. The epidemic-associated regions encode prophage elements, pathogenicity islands, metabolic islands, fitness islands and genes of unknown functions, and 28 of the genes encoded in these regions were predicted as virulence factors. We found two pilus biogenesis gene clusters encoded within predicted pathogenicity islands. A functional metabolic island, that encodes a complete pathway for *myo*-inositol catabolism, was evident by the ability of epidemic *A. hydrophila* isolates to use *myo*-inositol as a sole carbon source. Testing of *A. hydrophila* field isolates found a consistent correlation between *myo*-inositol utilization and the presence of an epidemic-specific genetic

marker. All epidemic isolates and one reference isolates shared a novel O-antigen cluster. Altogether we identified five different O-antigen biosynthesis gene clusters within the 12 sequenced *A. hydrophila* genomes. Our study reveals new insights into the evolutionary changes that have resulted in the emergence of recent epidemic *A. hydrophila* strains.

2. Introduction

A. hydrophila is the causative agent of motile Aeromonas septicemia (MAS) of catfish (Thune *et al.*, 1993). MAS was not a disease of major concern for the catfish industry in the Southeastern United States until 2009 (Bebak *et al.*, 2011) when several commercial catfish operations in western Alabama experienced outbreaks of MAS resulting in industry-wide losses of food-sized catfish totaling over 8 million pounds (Hemstreet, 2010). Since 2009 this MAS epidemic has spread within the Southeastern United States and cases have now been identified in Mississippi and Arkansas (Bebak *et al.*, 2011). Experimental disease challenges have demonstrated that epidemic *A. hydrophila* (EAh) isolates obtained from recent epidemic outbreaks in catfish are highly virulent to channel catfish compared to reference isolates of *A. hydrophila* (RAh) obtained from diseased fish during previous non-epidemic outbreaks (Pridgeon & Klesius, 2011).

Virulence factors of *A. hydrophila* including pili (Pepe *et al.*, 1996), hemolysin (Thune *et al.*, 1982), serine protease (Rodriguez LA *et al.*, 1992 ; Thune *et al.*, 1982), metalloprotease (Yu *et al.*, 2005), cytotoxic enterotoxin (Li *et al.*, 2011; Santos *et al.*, 1988), S-layer (Dooley & Trust, 1988), and the type III secretion system (Yu *et al.*, 2005) have been shown to be important for fish disease. Virulence factors of *A. hydrophila* are multifactorial and the concerted actions of several factors are required to cause disease in fish (Santos *et al.*, 1988; Yu *et al.*, 2005). Though a large number of *A. hydrophila* virulence factors involved in different fish diseases have been

reported, most of their mechanisms of pathogenesis have yet to be studied, and no studies have evaluated the specific virulence factors within EAh strains. Recently, a PCR-based subtractive genomic hybridization approach was used to identify epidemic-associated genes within EAh isolates (Pridgeon *et al.*, 2011). However, this study did not provide comprehensive information on the genomic regions and functions associated with EAh strains.

The emergence of infectious agents is frequently driven by the plasticity of bacterial genomes due to the loss and acquisition of foreign genetic elements (Rasko *et al.*, 2011). Lateral gene transfer (LGT) by means of prophages (Brüssow *et al.*, 2004; Pullinger *et al.*, 2004), integrating conjugative elements (Burrus *et al.*, 2002) and plasmids (Le Roux *et al.*, 2011) play significant roles in bacterial virulence. Prophages are well known for their ability to induce lysogenic conversion by introducing virulence genes (Pullinger *et al.*, 2004) and changing the genome architecture by introducing genetic elements that increase fitness (Brüssow *et al.*, 2004). The rapid onset of the recent epidemic in catfish is suggestive of an emerging strain of *A. hydrophila* that has acquired new genetic elements via LGT.

Until now none of the *A. hydrophila* strains obtained from fish have been subjected to whole genome sequencing. The complete genome sequence of *A. hydrophila* ATCC 7966 (obtained from a milk sample) has been determined, yet the nature of the pathogenicity of this strain has not been published in fish (Seshadri *et al.*, 2006). Phylogenetic analysis and virulence studies have demonstrated differences between the EAh and RAh strains, with the EAh strains being at least 200 times more virulent than a RAh isolate obtained from a diseased catfish in 1998 (Pridgeon & Klesius, 2011). This study was initiated to compare the genomes of highly virulent EAh isolates with that of RAh isolates and identify epidemic-associated genetic elements to reveal mechanisms fostering the hyper-virulence of these EAh strains. The molecular

characterization of epidemic strains will provide the framework for the development of vaccines, therapeutics, and rapid diagnostics to facilitate the control of this emerging catfish pathogen.

In this study we have sequenced the genome of 12 *A. hydrophila* isolates including 6 epidemic and 6 reference isolates using next-generation sequencing technology. Comparative analysis of these *A. hydrophila* genomes demonstrated that recent epidemic isolates are clonal and carry a large number of epidemic-associated unique genetic regions missing in reference isolates. This study provides detailed insight into the molecular evolutionary changes that have occurred in *A. hydrophila* epidemic isolates and suggests that the recent outbreaks of MAS in catfish are the result of the emergence of a pathogenic strain through the acquisition of novel genetic elements via LGT.

3. Materials and Methods

Bacterial isolates. Based on the biochemical and molecular phylogenetic data, a collection of 12 *A. hydrophila* isolates ((n=6 epidemic *A. hydrophila* (EAh); n=6 historical "reference" *A. hydrophila* isolates (RAh)) were selected for sequencing (Table 1).

Phylogenetic analysis. Evolutionary relationships of 107 *A. hydrophila gyrB* gene sequences were determined by the construction of a phylogenetic tree using MEGA5 (Tamura *et al.*, 2011). The *gyrB* gene sequence of *A. hydrophila* subsp. *decolorationis* was included as an outgroup for rooting the phylogenetic tree. The evolutionary history was inferred using the Maximum Parsimony method (Eck & Dayhoff, 1996). Results from a maximum parsimony analysis of all 107 strain *gyrB* sequences were used to remove clades that were more distantly related to the EAh strains, while retaining all RAh strains, and 37 strains were re-analyzed by maximum parsimony analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All

positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 929 positions in the final dataset. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). The *gyrB* gene sequences were deposited within the GenBank nr/nt database under the accession numbers JX275833 to JX275847.

Sequencing, assembly and annotation. Barcoded Illumina libraries were prepared from each strain using a Nextera DNA Sample Prep Kit (Epicentre, Madison, WI), equal amounts of library products were pooled, and paired sequences were obtained from an Illumina GAIIx sequencer using 150bp read lengths (Illumina, Inc., San Diego, CA). Sequences from each library were deconvoluted and assembled *de novo* using ABySS v1.2.6 [59] on the Amazon Elastic Compute Cloud. A minimum of 10 paired reads was required to join unitigs into contigs. Multiple assemblies were produced per isolate using varied kmer settings, and 200 bp or larger contigs from the most contiguous assembly was further analyzed. In addition to Illumina sequencing, the EAh type strain ML09-119 was subjected to 454 pyrosequencing. A bar-coded phage DNA sublibrary was prepared at the Lucigen Corporation (Middleton, WI) and sequencing was conducted at Engencore (Univ. of South Carolina). The methods for the determination of prophage sequences are described in the SI methods. The reads from Illumina and 454 were *de novo* assembled using CLCBio Genomics Workbench (version 4.9). Gene prediction and annotation were carried out using GeneMark (Lukashin & Borodovsky, 1998) and the RAST annotation server (Aziz *et al.*, 2008), respectively.

Identification of unique regions within the genome of ML09-119. *In silico* genomes for each of the reference isolates including AL06-01, AL06-06, AL097-91, GA97-22, MN98-04 and TN97-08 were constructed by force-joining each of their respective contigs. Each of the genomes were greater than 4.8 Mbp which was presumed as a near complete genome since the size of the only sequenced *A. hydrophila* ATCC 7966 is about 4.7 Mbp (Seshadri *et al.*, 2006). The *in silico* genome of AL06-06 was used as a scaffold to assemble trimmed, paired-end sequence reads of epidemic isolate ML09-119 using CLC Bio Genomic Workbench (v 4.9). The parameters that were used for each reference mapping was as follows: mismatch cost =2, insertion cost =3, deletion cost =3, length fraction =0.5 and similarity =0.9. The un-mapped (paired) reads of ML09-119 sequences from this reference mapping were then reference mapped against the force-joined contigs of AL97-91, and this process was repeated with the RAh strains AL06-01, GA97-22, MN98-04, TN97-08, and ATCC 7966. The un-mapped ML09-119 sequence reads that did not match with any of the six RAh strains or ATCC 7966 strain were considered as ML09-119-associated sequences that were uniquely present in strain ML09-119. To identify the distribution of those un-mapped reads in the genome of ML09-119, we conducted reference mapping of the un-mapped sequence reads to the *de novo* assembled genome of ML09-119 which was about 5.0 Mbp. The regions of the ML09-119 genome that were aligned with ML09-119 un-mapped reads were considered as ML09-119-associated unique regions. Those ML09-119-specific sequences were extracted manually for further analysis. Since later analyses determined that all of the genomic regions that were ML09-119-specific were also present within each of the other EAh strains, these regions are subsequently referred to as EAh-associated genomic regions.

Defining the Pan and Core genome. Conserved gene families within the genome of *A. hydrophila* isolates were identified according to the methods described as (Friis *et al.*, 2010) that used '50/50' rule for defining conserved protein families (Tettelin *et al.*, 2005). According to this rule two sequences are considered as a member of the single family if alignment between two proteins is 50% in a single span and contained at least 50% identities. The conserved gene families in the collection of *A. hydrophila* genomes were found by using the BLASTp algorithm for all of the proteins of each proteome against all the proteins of the query proteome using the microbial pan-genomics tool (Snipen & Ussery, 2010) kindly provided by David W. Ussery, at The Technical University of Denmark, Lyngby, Denmark.

BLAST Matrix. The BLAST matrix algorithm was used for the pairwise comparison of the proteomes of each of the 12 *A. hydrophila* isolates to another according to the methods described elsewhere (Friis *et al.*, 2010). This algorithm determines the percent similarities between two isolates by measuring the ratio of the number of conserved gene families shared between isolates to the total number of gene families in the isolates. The distribution of the conserved gene families within the genome of 12 *A. hydrophila* isolates was presented in a triangle-shaped matrix.

Prediction of Genomic Islands. The epidemic *A. hydrophila* strain ML09-119 genome sequences were subjected to genomic island prediction using IslandViewer (Langille *et al.*, 2008), a computational tool that integrates three different genomic island prediction methods including IslandPick, IslandPath-DIMOB, and SIGI-HMM (Langille *et al.*, 2008). The concatenated contigs of ML09-119 strain consisted of ~ 5.0 Mbp sequences that were converted to GenBank format using the Sequin program (version 11.9). The GenBank formatted sequence file was uploaded to the IslandViewer web based tools for scanning of the ML09-119 genome

for the presence of genomic Islands. IslandViewer used three different *Aeromonas* species such as *A. hydrophila strain* ATCC 7966*, A. salmonicida* subsp. salmonicida A449 and *A. caviae* Ae398 for the comparison of query sequences of ML09-119 provided for GIs prediction. To identify the epidemic-associated unique GIs, the nucleotide sequences of all the GIs in the ML09-119 strain predicted with IslandViewer tools were forced joined and used as a reference sequence to conduct a reference mapping against trimmed pair-end reads of all five RAh strains. The regions of concatenated GI sequence that did not map with the sequence reads of RAh strains were considered as EAh-associated unique GIs.

Prediction of virulence factors in the epidemic-associated unique genomic regions. Virulence factors were predicted within the unique EAh-associated genome sequences using the Virulence Factors Database (VFDB) (Chen *et al.*, 2005) which contains 2,353 proteins from different pathogenic bacteria as of March 2012. All of the proteins from the VFDB were retrieved and a local database was created in the CLC Bio Genomic Workbench. Predicted proteins encoded by genes within the unique regions were subjected to BLASTp analysis against the virulence factors database using CLC Bio Genomics Workbench to identify the occurrence of virulence factors associated with epidemic strains. An E-value threshold of 10^{-10} was selected to exclude proteins of distant homologs.

Electron Microscopy. Concentrated phage particles obtained from a mitomycin C-treated *A. hydrophila* ML09-119 culture were negatively stain with 2% phosphotungstic acid (pH 6.5) after placing on 300 mesh formvar- and carbon-coated copper grids (Electron Microscopy Services, PA) for 15 minutes. The grids were examined at different magnifications to determine the size and morphology of phages using a Zeiss EM10 Transmission Electron Microscope (Zeiss, Germany).

454 pyrosequencing of induced prophage genome. Phage genomic DNA was extracted from concentrated phage lysates obtained from mitomycin C-treated *A. hydrophila* ML09-119 strain as previously described (Hossain *et al.*, 2012). A bar-coded phage DNA sublibrary was prepared at the Lucigen Corporation (Middleton, WI) compatible with 454 titanium chemistry. A 1/8 plate sequencing run of the 454 pyrosequencer was conducted at Engencore (Univ. of South Carolina) that yielded 25,873,898 bp from 96,898 reads (267 bp average length) from the phage DNA library.

Screening of *A. hydrophila* strains for EAh-associated genes by PCR. A PCR assay was used to test for the presence of EAh-associated genes within the genome of *A. hydrophila* cultured isolates. An EAh-associated gene whose presence was confirmed on the complete genome sequence of all six EAh strains, and had no significant BLAST hit against the GenBank nr/nt database was used for PCR screening of *A. hydrophila* cultured isolates obtained from diseased catfish, pond sediments, and fish samples taken from a processing plant. Two oligonucleotides EAhL (CTA TTA CTG CCC CCT CGT TC) and EAhR (ATT GAG CGG TAT GCT GTC G) used for PCR were developed using the Primer-BLAST program at GenBank. Genomic DNA was extracted from *A. hydrophila* isolates according to the methods described by (Wilson, 2001). One hundred ng of genomic DNA per 25 µl PCR reaction was used as a template for PCR amplification of EAh-associated genes using the following thermal cycling parameters: 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Type strain *A. hydrophila* ATCC 7966 was used as a negative control whose genome sequence does not possess any epidemic-associated genes (Seshadri *et al.*, 2006).

Evaluating the growth of *A. hydrophila* using *myo*-inositol as a sole carbon source. An isolated colony from a pure culture of an *A. hydrophila* isolate was used to inoculate a 2 ml Tryptic Soy Broth (TSB) culture that was grown at 28°C overnight with shaking at 200 rpm. The cell suspension was pelleted by centrifugation at 10,000 ×g for 10 min and then the cells were washed twice with 1x PBS buffer and resuspended in M9 medium supplemented with 5.5 mM of *myo*-inositol (M9I) to an OD_{600} of 0.5. The cell suspension was then serially diluted 1:100 in M9I and 100 µl of the 1:100 diluted cell suspension was used to inoculate 1.9 ml of M9I. Bacterial cultures were then grown for 72 hours and the OD_{600} was recorded at 24, 48, and 72 hours for determining the ability of each strain to use *myo*-inositol as a sole carbon source.

4. Results

Phylogenetic analysis of *A. hydrophila* isolates. Phylogenetic analysis based on 16S rRNA gene sequences of epidemic *A. hydrophila* isolates demonstrated they are 100% identical to previously reported *A. hydrophila* strains (data not shown). Phylogenetic analysis based on *gyrB* gene sequences of representative *A. hydrophila* isolates demonstrated sufficient resolution to separate EAh and RAh isolates. A maximum parsimony (MP) tree generated from the alignment of EAh and RAh *gyrB* nucleotide sequences revealed that all of the EAh strains consistently grouped together as a single clade with strong bootstrap support (Figure 1). Although the EAh isolates formed a coherent clade based on *gyrB* gene sequences, there was not sufficient phylogenetically informative nucleotide positions within the *gyrB* gene sequence to develop an EAh-specific primer set. Interestingly, the *A. hydrophila* strain ZC1 obtained from a diseased grass carp in China (Guo-Cheng *et al.*, 2009) clustered with EAh isolates.

***A. hydrophila* genome sequencing, assembly and annotation.** Summary statistics for each of the *A. hydrophila* genome sequences and their assemblies are provided in Table 1. The

average number of contigs obtained per genome was 114. After trimming, the quality Illumina sequence reads totaled 9510.8 Mb, with an average coverage of >160-fold per genome. 454 pyrosequencing of an induced prophage from strain ML09-119 (see below) provided a total of 96,598 high-quality sequences with an average read length of 268 bp. The *de novo* combined assembly of sequence reads generated by Illumina and 454 provided a total of 42 contigs with an average size of 119.2 kb. The average genome size of the EAh and RAh isolates were 5.0 Mb and 4.8 Mb, respectively. The %G+C content of the 12 strains ranged from 60.5% to 61.5% (Table 1) which was consistent with the previously reported %G+C content of 61.5 % for *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006). NCBI accession numbers for each *A. hydrophila* genome sequence are provided in Table 1.

Identification of unique genomic regions associated with EAh isolates. The EAh strain ML09-119, originally cultured from the kidney tissue of a diseased channel catfish establishing characteristic MAS symptoms, has been identified as an *A. hydrophila* strain by biochemistry and 16S rRNA gene sequencing and is highly virulent to channel catfish by intraperitoneal injection, was used as a type strain for all further analysis in this study. We found that the strain ML09-119 genome contains 54 unique regions (data not shown) that are missing in all six RAh isolates (AL06-01, AL06-06, AL97-91, GA97-22, MN98-04 and TN97-08) and *A. hydrophila* ATCC 7966. These 54 unique regions are also present in all five of the other sequenced EAh isolates (AL09-79, AL10-121, ML09-121, ML09-122, and PB10-118). These epidemic–associated unique regions contain 325,813 bp, accounting for 6.5% of the ML09-119 genome. A total of 307 ORFs are encoded by these unique regions (data not shown). Region C2R1 is the smallest region with one predicted ORF, whereas C15R7 is the largest region (33,402 bp) predicted to encode 36 different proteins. More than 252,453 bp of these unique

sequences are part of 16 predicted genomic islands (GIs; see below for detailed description of these GIs). About 46% (141 out of 307) of the EAh-associated genes are predicted to encode proteins with unknown functions (data not shown). The average %G+C content of these unique regions is 46.8%, whereas the %G+C content of the ML09-119 genome is 60.9%. The %G+C content bias of the EAh-associated regions supports the hypothesis that novel genomic segments were acquired through LGT.

Determining the Pan and Core-genome of *A. hydrophila*. A total of 7,394 pan-gene families comprising full sets of non-orthologous genes families were found within the genome of the 12 *A. hydrophila* isolates. We found 3,326 conserved core gene families present within these 12 *A. hydrophila* genomes. Based on these estimates the number of pan-genes is approximately twice the number of core-genes in *A. hydrophila*. Considering the 4,766 average number of gene families present in each of the *A. hydrophila* isolates sequenced in this study, it was observed that 70% of the predicted genes were core genes that are shared among all of the *A. hydrophila* isolates. From a plot of pan- and core-genomes it was observed that the number of genes in the pan-genomes reached its maximum among the EAh strains (Figure 3). There was a negligible increase in the number of new gene families among the EAh genomes, supporting the conclusion that the six epidemic *A. hydrophila* genomes are highly similar.

Pairwise proteome comparison of *A. hydrophila* genomes. A pairwise BLAST Matrix was generated to determine the similarity in each of the conserved protein families present within the *A. hydrophila* genomes. The proteome comparison revealed that the average protein family similarity between any two *A. hydrophila* genomes ranges from 63-99% while the intra-proteome homology among protein families within each isolates is less than 5.6% (Figure 2). The pairwise comparison of proteomes showed that the six EAh strains share a very high degree

of homology (>99%) (Figure 2, highlighted with triangle). In contrast, the pairwise proteome comparison between the EAh and RAh strains and among RAh strains including *A. hydrophila* ATCC 7966 revealed a range from 65% to74% homology. These results demonstrated that EAh strains are genomically distinct from RAh strains and that there is a highly coherent EAh genome. The BLAST matrix results also indicated that RAh proteomes are diverse, with an average 73.2% sequence identity. One exception was the 95% sequence identity between the AL97-91 and MN98-04 proteomes. Since these two strains had been isolated from Tilapia this may reflect their isolation from a common host fish. These results suggest there was significant diversity among RAh strains sampled in this study, especially in contrast to EAh strain genomic homogeneity.

EAh strains contain a *myo*-inositol utilization pathway. The comparative analysis of *A. hydrophila* genomes revealed that a 17.5 kb genomic region predicted to be involved in *myo*-inositol catabolism is present in all EAh isolates and is part of the epidemic-associated unique regions in EAh isolates (data not shown). Consistent with this finding, it was observed that all EAh isolates were able to grow in *myo*-inositol as a sole carbon source. Neither the six RAh strains nor the ATCC 7966 reference proteome (Seshadri *et al.*, 2006) was predicted to contain genetic regions involved in *myo*-inositol catabolism. These findings were supported by the inability of any RAh isolate to grow in *myo*-inositol as a sole carbon source. The 17.5 kb *myo*-inositol catabolism cluster (*iol*) contains 11 ORFs that are predicted to be involved in *myo*-inositol transport and catabolism. In EAh strains, as in *Bacillus subtilis* (Yoshida *et al.*, 1994), *myo*-inositol catabolism and transport genes are clustered together within a single region whereas in some bacteria, including *Corynebacterium glutamicum* (Krings *et al.*, 2006) and *Caulobacter crescentus* (Boutte *et al.*, 2008), these genes are split into two or more clusters and dispersed

across the chromosome. The comparison of the *iol* cluster of EAh isolates with that of *Bacillus subtilis* (Yoshida *et al.*, 2008; Yoshida *et al.*, 1997) and *Klebsiella (Aerobacter) aerogenes* (Anderson & Magasanik, 1971) revealed that the EAh *myo*-inositol catabolism pathway cluster encodes all of the enzymes necessary for *myo*-inositol utilization with the exception of 2-deoxy-5-keto-D-gluconic acid 6-phosphate aldolase, which is required for the degradation of *myo*-inositol to acetyl-CoA (Figure 4). However, a search in the ML09-119 genome did reveal a gene predicted to encode a homolog of 2-deoxy-5-keto-D-gluconic acid 6-phosphate aldolase, with 98% similarity to its nearest BLASTx hit within the genome of *A. hydrophila* ATCC 7966.

The low %G+C content of the region encoding the *iol* cluster was 56.2% compared to the average 60.9 %G+C content of the entire genome and the presence of transposase flanking the *iol* cluster (Figure 4) suggest that the genetic region encoding the *myo*-inositol catabolism genes has been introduced into the EAh genome via a LGT event(s).

Novel O-antigen biosynthesis gene cluster in EAh isolates. The significant role of O-antigen, the most variable surface structure in terms of its composition, in bacterial virulence (Lerouge I, 2002) prompted us to analyze the O-antigen biosynthesis gene cluster of the *A. hydrophila* isolates. We found that all of the EAh isolates and one RAh isolate TN97-08 shared a 26.5 kb novel O-antigen biosynthesis gene cluster predicted to encode 25 different ORFs (Table 2, 3 and Figure 6). Though the proteomic comparison of the TN97-08 and EAh isolates showed about 73% similarities (Figure 2), the sharing of an entire O-antigen biosynthesis cluster suggests possible LGT events. None of the O-antigen biosynthesis gene clusters of RAh isolates, except for strain TN98-04, shared homology with that of the EAh isolates and five of the RAh isolates possess four unique O-antigen biosynthesis clusters (Figure 6). Among the six RAh isolates, only AL97-91 and MN98-04 isolates shared homology in their O-antigen cluster (Figure 6), which is

in agreement with their overall proteomic homology (Figure 2). The O-antigen biosynthesis cluster of 12 sequenced *A. hydrophila* isolates are quite different than the previously published *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006), PPD134/91 (Zhang *et al.*, 2002), JCM3980 (Zhang *et al.*, 2002) and AH-3 (Jimenez *et al.*, 2008) O-antigen biosynthesis clusters (Figure 6). A detailed description of the novel EAh O-antigen biosynthesis cluster and a comparative analysis of RAh O-antigen biosynthesis clusters are provided in SI Results. All together we identified five unique O-antigen biosynthesis clusters among the 12 sequenced *A. hydrophila* strains (Figure 5) and this increases the number of known types of O-antigen biosynthesis clusters in *A. hydrophila* to a total of eight (Figure 6). The diversity of O-antigen biosynthesis clusters in *A. hydrophila* isolates also suggests the possible contribution of LGT events.

Novel O-antigen cluster in EAh isolates. The analysis of gene content within the EAh O-antigen cluster suggests that EAh strains express a previously uncharacterized O-antigen among *A. hydrophila* strains. The comparison of the EAh O-antigen biosynthesis cluster with that of strains PPD134/91 and AH-3 revealed that the 2.6 kb EAh O-antigen gene cluster is larger than the 19 kb clusters found in both PPD134/91 and AH-3. The analysis of the EAh O-antigen gene cluster revealed 25 ORFs and the annotation of each ORF demonstrated that this O-antigen gene cluster contains predicted gene products required for the synthesis of activated nucleotide sugars, transport of those sugars to the growing O-antigen chain, and the processing of the O-antigen (Table 2). The EAh O-antigen biosynthesis cluster contains genes putatively required for the synthesis of nucleotide activated sugars D-rhamnose, D-mannose, GDP-L-Fucose and 3-acetamido-3, 6-dideoxy-d-galactose (D-Fuc*p*3NAc). The *rmlA*, *rmlB*, *rmlC* and *rmlD* genes are usually clustered together (Samuel & Reeves, 2003) and products of those genes are required for the synthesis of dTDP-rhamnose. Each of the EAh and RAh strains possess

rmlA, rmlB, rmlC and rmlD genes in their O-antigen gene cluster. Though each of the unique O-antigen cluster types described in this study contain genes required for D-rhamnose biosynthesis, the homology and organization of those genes varied substantially (Table 2-7). Those genes are also present within the antigen clusters of *A. hydrophila* ATCC 7966, PPD134/91, JCM3980 and AH-3. These collective observations indicate that D-rhamnose is the most common sugar component present within the O-antigen of *A. hydrophila*. The presence of all five of the genes required for D-Fuc*p*3NAc synthesis in the EAh O-antigen cluster suggests that this sugar is also a major component of the EAh O-antigen. In contrast, the O-antigen clusters of all RAh and previously sequenced *A. hydrophila* strains do not contain the *fdtA*, *fdtB* or *fdtC* genes required for D-Fucp3NAc synthesis.

The EAh O-antigen gene cluster was predicted to contain five different genes, namely *gmd, fcl, gmm, manC,* and *manB* required for the synthesis of GDP-mannose and GDP-L-fucose from fructose-6-phosphate (Table 2). ManA, ManB, and ManC are required for the synthesis of GDP-mannose from fructose-6-phosphate. Typically, the genes *manB* and *manC* are located within the O-antigen cluster, whereas *manA* is found outside the O-antigen cluster elsewhere within the genome (Guo *et al.*, 2005; Jimenez *et al.*, 2008; Zhang *et al.*, 2002) and this was also observed for the EAh O-antigen cluster. The genes *gmd* and *fcl* encode GDP-mannose 4, 6-dehydratase and GDP-L-fucose synthetase, respectively, which synthesize GDP-L-fucose using GDP-mannose as a precursor. None of the previously sequenced O-antigen biosynthesis gene clusters of *A. hydrophila* contained *gmd* or *fcl* genes. The *fdtA*, *fdtC* and *fdtB* genes are predicted to encode the enzymes dTDP-6-deoxy-3,4-keto-hexulose isomerase (FdtA), dTDP-D-Fucp3N acetyltransferase (FdtC), and aminotransferase (FdtB), respectively, required for the synthesis of the dTDP-sugar 3-acetamido-3,6-dideoxy-D-galactose (dTDP-D-Fucp3NAc) (Pfoestl *et al.*,

2003), an activated nucleotide sugar that could be incorporated into the EAh O-antigen. In addition to these enzymes, D-glucose-1-phosphate thymidyltransferase (RmlA) and dTDP-D-glucose-4,6-dehydratase (RmlB) encoded by the *rmlA* and *rmlB* genes, respectively, are predicted within the EAh O-antigen cluster and are required for the biosynthesis of the nucleotide sugar dTDP-D-Fucp3NAc (Pfoestl *et al.*, 2003).

The EAh O-antigen cluster contained five different glycosyltransferase genes and one acetyltranferase gene (Table 2). A series of glycosyltransferases work consecutively to assemble the nucleotide sugar repeat on the membrane lipid undecaprenol pyrophosphate (Und-PP). The EAh O-antigen gene clusters were predicted to contain the *wecA* gene that encodes a undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase required for the transfer of the GlcNAc-1-phosphate moiety from UDP-GlcNAc onto the carrier lipid undecaprenyl phosphate. The single polysaccharide repeat bound to Und-PP is flipped to the periplasmic side which is catalyzed by O-antigen flippase (Raetz & Whitfield, 2002) and polymerized by the Wzy-dependent pathway (Whitfield, 2006). The EAh strains were also found to possess an O-antigen flippase (*wzxB*) and O-antigen polymerase (*wzyE*) within their O-antigen gene cluster. These findings suggest the presence of smooth LPS on each of the EAh strains.

Comparative analysis of O-antigen biosynthesis gene cluster of RAh strains. The predicted O-antigen clusters in the RAh strains AL06-01, AL06-06 and GA97-22 are unique. The O-antigen biosynthesis gene cluster of AL06-01, AL06-06 and GA97-22 are 29 kb, 18.86 kb and 23 kb in length that encode 25, 17 and 21 ORFs, respectively. The gene content and organization of O-antigen clusters of AL06-01, AL06-06 and GA97-22 varied substantially with those of the other EAh and RAh strains used in this study (Tables 2-7 and Figure 5). The AL06-01 strain contains all 4 genes (*rmlA*, *rmlB*, *rmlC* and *rmlD*) required for the biosynthesis of

dTDP-rhamnose from glucose-1-phosphate. It also contains gene that encode UDP-glucose 6-dehydrogenase required for the biosynthesis of UDP-D-glucuronic acid from UDP-glucose. These findings suggest the presence of D-rhamnose and D-glucuronic acid on the O-antigen of strain AL06-01. The O-antigen cluster of RAh strain GA97-22, like the EAh strains, contains all five genes required for the synthesis of D-mannose and D-L-fucose (Table 7). In contrast, the unique O-antigen cluster of RAh strains AL06-01 and AL06-06 do not contain any genes required for the synthesis of D-mannose or D-L-fucose (Table 4 and 5). Instead, the AL06-06 O-antigen cluster is predicted to encode UDP-N-acetyl-D-galactosamine dehydrogenase and an epimerase/dehydratase family WbfY-like protein. Those two enzymes are required for the biosynthesis of UDP-GalNAcA which is a common O-antigen sugar for many Gram-negative bacteria.

The O-antigen gene cluster of AL06-01 and AL06-06 contain six and four different glycosyltransferase genes, respectively, that are required for the assembly of nucleotide sugar repeat on the membrane lipid undecaprenol pyrophosphate (Und-PP) (Table 4 and 5). On the other hand, GA97-22 contains five different glycosyltransferase genes and one acetyltranferase gene (Table 7). AL06-06 and GA97-22 O-antigen clusters, unlike the EAh O-antigen cluster, were predicted to contain the *wbtB* gene that encodes undecaprenyl-phosphate alpha-N-acetylgalactosaminyl 1-phosphate transferase required for the transfer of the GalNAc-1-phosphate moiety from UDP-GalNAc onto the carrier lipid undecaprenyl phosphate (Figure 5). In contrast, the O-antigen cluster of AL06-01, like the EAh O-antigen cluster, contains the *wecA* gene that encodes undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase required for the transfer of the GlcNAc-1-phosphate moiety from UDP-GlcNAc onto the carrier lipid undecaprenyl phosphate (Figure 5).

A genome-wide comparison of the RAh isolates AL97-91 and MN98-04 using BLAST matrix showed that they are highly similar (>94%) in terms of their conserved gene families (Figure 2). Both the isolates were predicted to contain an O-antigen biosynthesis gene cluster that was highly similar to each other in terms of gene content, relative organization of the genes and the percent identity of their predicted gene products (Table 6 and Figure 5). The O-antigen biosynthesis gene clusters of RAh isolates AL97-91 and MN98-04 contain 15 ORFs predicted to be involved in O-antigen biosynthesis, including gene products predicted to be required for the biosynthesis of dTDP-rhamnose and D-glucose (Table 6). They contain six different glycosyltransferase genes and one acetyl transferase gene in their O-antigen clusters (Table 6). Additionally, they contain the *wecA* gene required for the transfer of the GlcNAc-1-phosphate moiety from UDP-GlcNAc onto the carrier lipid undecaprenyl phosphate. The absence of an O-antigen polymerase gene within those clusters suggests these two isolates may produce a semi-rough O-antigen. The analysis of the O-antigen biosynthesis gene cluster of strains AL97-91 and MN98-04 demonstrated that these two strains contain two additional cluster of genes required for S-layer protein synthesis and type II secretion in their O-antigen clusters (Table 6 and Figure 5). These results suggest the O-antigen of these two strains anchor the S-layers and most probably the S-layers of *A. hydrophila* isolates AL97-91 and MN98-04 are secreted by a type II secretion system, unlike in *Caulobacter crescentus* that secretes S-layer proteins via a type I secretion system (Awram & Smit, 1998).

Epidemic-associated genomic islands (GIs). Since genomic islands contribute to lateral gene transfer and bacterial evolution (Juhas *et al.*, 2009) we analyzed the epidemic *A. hydrophila* isolates for the presence of genomic islands. We identified 16 GIs, ranging from 8 kb to 30 kb and comprised of 252.45 kb that encodes 255 ORFs (data not shown), within the genomes of

EAh isolates (Figure 7). Nine of the GIs were considered as epidemic-associated GIs since they were absence from the RAh isolates (Table 10). The GI 2 region contains a cluster of genes involved in *myo*-inositol catabolism. GI 3, the largest among the nine epidemic-associated unique GIs with 25 ORFs, includes genes coding hypothetical proteins, proteins involved in thiamine and cobyric acid biosynthesis and RNA metabolism. GI 12 includes a type I restriction modification system, DNA helicase, DNA repair protein, anticodon nuclease, as well as transposases (T7 like) and regulatory proteins along with hypothetical proteins of unknown function (data not shown). This GI is predicted to be generated in EAh isolates after T7 transposition since a GI with these fitness-enhancing features is generated after integrating at an *attTn7* site within a bacterial genome (Sambrook & Russell, 2001). GI 13 encodes a CS5 pilus biogenesis cluster (Table 8) which is similar to that of the enterotoxigenic *E. coli* O115:H40 (Duthy *et al.*, 1999). GI 16 of EAh isolates is also predicted to contain a cluster of genes required for pilus biogenesis.

Virulence factors in epidemic-associated unique regions. The highly virulent nature of the recent epidemic *A. hydrophila* isolates (Pridgeon & Klesius, 2011) and the presence of most of the predicted virulence factors within genomic islands (Ho Sui *et al.*, 2009) prompted us to search for virulence factors within the epidemic-associated genomic regions of epidemic *A. hydrophila*. We found 28 predicted virulence factors within the epidemic-associated unique regions of EAh isolates (Table 8). The average percent identity of those proteins to their homologous virulence factors was 35%. We found that 42% (12 out of 28) of the virulence factors were located within the GIs, which is in agreement with the occurrence of virulence factors within genomic islands (Ho Sui *et al.*, 2009). Genes predicted to encode a fimbrial major subunit, fimbrial usher, and fimbrial chaperon were found within GI 13 and GI 16, respectively.

Two putative TonB-dependent receptor coding genes were identified within the epidemic-associated unique regions of EAh isolates, with one showing 41% homology to a TonB-dependent receptor in *Neisseria meningitidis* MC58 (serogroup B) and the other with 26% homology to a yersiniabactin receptor protein of *Yersinia pestis* CO92 (Table 9). Three genes (*iolG*, *rbsA* and *iolA*) located within the *myo*-inositol utilization cluster (Figure 4), which is also part of GI 2, were predicted to encode virulence-related proteins (Table 9). In GI 12, we found *hsdR* and *hsdM* of a putative type I restriction modification system that share 25% and 24% identity to their homologs in *Vibrio cholerae* N1696, respectively (Table 9). Guanylate cyclases, involved in bacterial cell division, motility, biofilm formation and pathogenesis (Wolfe *et al.*, 2010), were predicted within the epidemic-associated unique regions C32R2 and C27R1. Putative virulence factors found within the epidemic-associated unique regions could potentially contribute to enhance pathogenicity of EAh strains.

Predicted prophages within the genome of *A. hydrophila* isolates. Prophages contribute significantly to the evolution of their bacterial hosts (Casjens, 2003).We predicted 5 prophages (AH1, AH2, AH3, AH4 and AH5) within the genome of EAh isolates (Table 11). While prophage AH1 was present in all EAh and RAh isolates, prophages AH2, AH3 and AH5 were present only in EAh isolates (Table 11). Prophage AH2 shares significant homology with phiO18P of *A. media* (Beilstein & Dreiseikelmann, 2008) and phiO18P-like prophages of *A. caviae* Ae398 (Beatson *et al.*, 2011) and *A. salmonicida* subsp. *salmonicida* A449 (Zhou *et al.*, 2011). AH2 was found to be integrated into the tRNA-Leu gene at an *attL* site on the EAh genome.

Prophage AH4 (36 kb in size with 40 ORFs), found within the genome of all EAh isolates and RAh isolates AL97-91, TN97-08 and MN98-04, shows significant homology to the

Mu-like prophage D108 of *Escherichia* coli origin (Hull *et al.*, 1978). None of the ORFs from this prophage has any known affiliation with any previously described *A. hydrophila* phage. The M and S subunits of a type I restriction modification system and secretion activator proteins encoded in this AH4 prophage could be potential virulence factors in *A. hydrophila*. Induction of EAh isolate ML09-119 followed by sequencing of phage DNA and mapping of the phage sequences against the ML09-119 genome suggests that AH4 is the only inducible prophage in EAh isolates (Figure 8). Induced phage particles were visualized with electron microscopy revealing a phage morphology with an icosahedral head and a contractile tail. These results strongly suggest that *A. hydrophila* ML09-119, and presumably all other EAh strains, contain a lysogenic phage. Prophage AH5, found within the EAh isolates but absent from RAh isolates, shows the highest number of protein similarities to phiKO2 of *Klebsiella oxytoca* (Casjens *et al.*, 2004). AH5 is 29.2 kb and encodes 33 predicted ORFs. The %G+C content of this prophage is 52.38% which is much lower than the average %G+C content (61%) of *A. hydrophila* isolates. AH5 is predicted to encode a N^6-methyltransferase and this could potentially contribute to *A. hydrophila* virulence in catfish due to the virulence properties of this protein in many bacteria including *A. hydrophila* (Erova *et al.*, 2006; Kim *et al.*, 2008).

Establishment of genotypic and phenotypic tests to identify epidemic strains. The clonal nature of sequenced EAh isolates prompted us to develop an EAh-specific PCR and to compare results from the PCR-based detection of EAh strains with the ability of each strain to use *myo*-inositol as a sole carbon source. We designed primers by targeting the epidemic-associated region C13R2 (region 2 of contig 13 of *A. hydrophila* ML09-119) present in all sequenced EAh isolates and which does not have any significant BLAST hit to any entry in the GenBank nr/nt database. A multiplex PCR was carried out with the *gyrB* gene as an internal

positive control and the EAh-specific C13R2 specific primers to screen 68 *A. hydrophila* isolates obtained from catfish, with the majority of these being presumably EAh isolates from catfish that had evidence of MAS. We also used as negative controls the sequenced RAh isolates along with other presumably RAh isolates from pond sediment or fish cloaca with no evidence of MAS. Among 68 *A. hydrophila* isolates tested, 47 isolates (69%) were positive for the C13R2-specific PCR and all were positive for *gyrB* PCR.

The presence of genetic loci involved in *myo*-inositol catabolism in all sequenced EAh isolates prompted us to determine the ability of *A. hydrophila* isolates to use *myo*-inositol as a sole carbon source. We tested the same 68 *A. hydrophila* isolates (evaluated above for C13R2 region-specific PCR) for their growth in M9 minimal medium containing *myo*-inositol. Of the 68 isolates, the same 47 isolates (69%) that were positive by C13R2-specific PCR were also capable of using *myo*-inositol as a sole carbon source. Taken together, these results demonstrated that 100% of the isolates showed a strong correlation between the growth on *myo*-inositol and the presence of epidemic-associated genes in *A. hydrophila* isolates.

5. Discussion

This study provided valuable insights into the evolutionary changes within the genomes of highly virulent *A. hydrophila* isolates responsible for an epidemic of disease in catfish. Comparative genomics of 12 *A. hydrophila* isolates demonstrated that recent epidemic isolates are highly clonal while a great deal of diversity was observed among *A. hydrophila* isolates obtained from diseased fish prior to any epidemic outbreak. Recent EAh isolates have considerable genomic differences with RAh strains that may contribute to their emergence as highly pathogenic strains in aquaculture farmed catfish.

The recent epidemic outbreak of MAS caused by highly virulent *A. hydrophila* is unique since the catfish farming operations in the southeastern United States have not experienced a large-scale outbreak of MAS (Hemstreet, 2010). Moreover, experimental disease challenges in aquaria models indicate that the *A. hydrophila* isolates responsible for recent epidemic outbreaks are highly virulent as compared to the RAh isolates that were historically regarded as an opportunistic bacterial pathogen isolated from stressed fish (Pridgeon & Klesius, 2011). Since after introduction into catfish farming in western Alabama in 2009, this unprecedented epidemic has expanded its geographic territory and caused frequent outbreaks in the summer months, resulting in millions of pounds of losses in Alabama, Mississippi and Arkansas.

Epidemic isolates used in our study were obtained as pure cultures from tissues (kidney or brain) taken from diseased catfish in ponds experiencing an outbreak of MAS. Our comparative genomics data distinguishes these contemporary epidemic isolates (EAh strains) from reference isolates (RAh strains) based on the presence of specific genetic polymorphisms. Our findings suggest that those epidemic-associated genetic markers have been acquired during the course of evolution of highly virulent strains. Our comparative genomic analysis demonstrated that all of the EAh isolates contain a large number of unique regions within their genomes that are completely absent in the genomes of RAh isolates. A total of 54 regions comprising 325,813 bp were identified as epidemic-associated unique regions in EAh isolates. These EAh-associated genomic regions all together encode 307 ORFs that are predicted to be involved in different functions. A large fraction of these EAh-associated genomic regions (252 kb out of 326 kb in total) are contained within genomic islands, suggesting their possible acquisition through lateral transfer. A total of 28 predicted genes that had significant similarity with proteins in a virulence factor database were predicted within these EAh-associated regions.

Further experiments will be required to determine the specific contribution of each genetic locus to EAh virulence in catfish.

Two different pilus biogenesis gene clusters were identified within two different genomic islands, each of which was within an EAh-associated region. One pilus biogenesis cluster located within the GI 13 was predicted to encode all six proteins required for CS5 pilus biogenesis, which has been extensively studied in enterotoxigenic *E. coli* (Duthy *et al.*, 2002; Duthy *et al.*, 1999). The comparison of EAh-associated genetic loci with the virulence factor database from VFDB predicted that the CS5 major pilin subunit encoded from *hsfA* is a putative virulence factor. This predicted virulence gene, discovered within a genomic island with a %G+C content of 35, suggests that GI 13 might be a pathogenicity island that has been acquired by lateral transfer.

A cluster of genes (*iol*) involved in *myo*-inositol catabolism and transport were consistently present in all sequenced EAh isolates, and were not identified in any other *A. hydrophila* genome. This is the first identification of an *iol* cluster, which encodes all of the proteins required for the transport and catabolic degradation of *myo*-inositol to acetyl-CoA, within any *Aeromonas* species. The existence of a functional *myo*-inositol catabolism pathway in epidemic-associated *A. hydrophila* isolates was formally demonstrated by the ability of these strains to grow in a minimal medium in the presence of *myo*-inositol as a sole carbon source. Burtle and Lovell (1989) suggested that the liver and intestine of catfish (*Ictalurus punctutus*) synthesize *myo*-inositol *de novo* and hence dietary supplementation is unnecessary. These *myo*-inositol pathways, like L-fucose utilization pathways in *Campylobacter jejuni* (Stahl *et al.*, 2011), could provide a competitive advantage for *A. hydrophila* strains expressing an *iol* cluster to grow in liver, intestine or other fish tissue and could be responsible for enhanced virulence in

catfish. Alternatively, the abundance of *myo*-inositol in soil and pond sediments could facilitate growth and survival of *A. hydrophila* isolates, providing a competitive advantage over other bacterial taxa that lack the capacity to utilize *myo*-inositol.

This study, by sequencing a large number of epidemic and reference *A. hydrophila* isolates followed by the identification of epidemic-associated genomic regions, has provided valuable tools for studying the molecular epidemiology of the ongoing MAS epidemic in the southeastern United States. In this study we found a strong correlation between presumptive EAh isolates with the presence of specific genomic regions and the ability to use *myo*-inositol as their sole carbon source. In the future, epidemic outbreaks of MAS could be investigated using a genotypic and/or phenotypic assay based on the presence of epidemic-associated genetic loci in epidemic isolates and/or the ability to use *myo*-inositol as a sole carbon source. In addition to routine diagnostics, these assays will also help to track the geographic distribution of the current epidemic *A. hydrophila* strains affecting catfish farming.

In this study another objective was to attempt to ascertain the origin of this highly virulent genotype. The comparative genomic analysis of EAh and RAh strains identified, in addition to epidemic-associated genomic regions, several other genomic regions that could help trace the emergence of this virulent *A. hydrophila* strain. The lateral transfer of an O-antigen biosynthesis gene cluster is widely considered as a prominent way to generate novel serotypes with highly virulent attributes (Mooi & Bik, 1997). In this comparative genomics study, we have identified five different novel O-antigen biosynthesis gene clusters among 12 different isolates. These novel O-antigen clusters varied substantially based on their nucleotide sequences, gene content and their relative genetic organization within the clusters. The EAh-type O-antigen cluster, which is present in all sequenced epidemic isolates and one reference isolate TN97-08, is

predicted to include the five sugars D-rhamnose, D-mannose, D-Fucose, and 3-acetamido-3, 6-dideoxy-d-galactose (D-Fuc*p*3NAc). An O-antigen with amino-sugar D-Fuc*p*3NAc is reported, in this study, for the first time in any *Aeromonas* species. Although the genome-wide pairwise comparison between the conserved gene families of ML09-119 and TN97-08 demonstrated that they are less than 75% homologous, it is intriguing to discover that the 26.5 kb O-antigen gene cluster of strain TN97-08, obtained in 1998 from a diseased fish in Tennessee, was 100% identical to that of the recent epidemic *A. hydrophila* isolates. In addition to the O-antigen cluster, prophage AH4 present in EAh isolates also shared significant homology with the genome of TN97-08. The sharing of an identical O-antigen cluster and highly similar prophage suggests that EAh strains and TN97-08 might have evolved from a common ancestor and diversified via the successive acquisition of genes via LGT. Future experiments will be required to determine whether the novel O-antigen cluster present in epidemic isolates provides any direct role in the virulence of EAh strains in catfish.

The expansion of MAS in catfish aquaculture caused by highly pathogenic *A. hydrophila* is threatening the catfish farming industry in the southeastern United States. Currently, there is no effective vaccine or therapeutic agent demonstrated to be effective for the prevention and/or control of MAS in catfish aquaculture ponds. It is our goal to use the molecular insights gained from this comparative genomic analysis for design of improved diagnostic and therapeutic approaches for control of epidemic MAS caused by *A. hydrophila*.

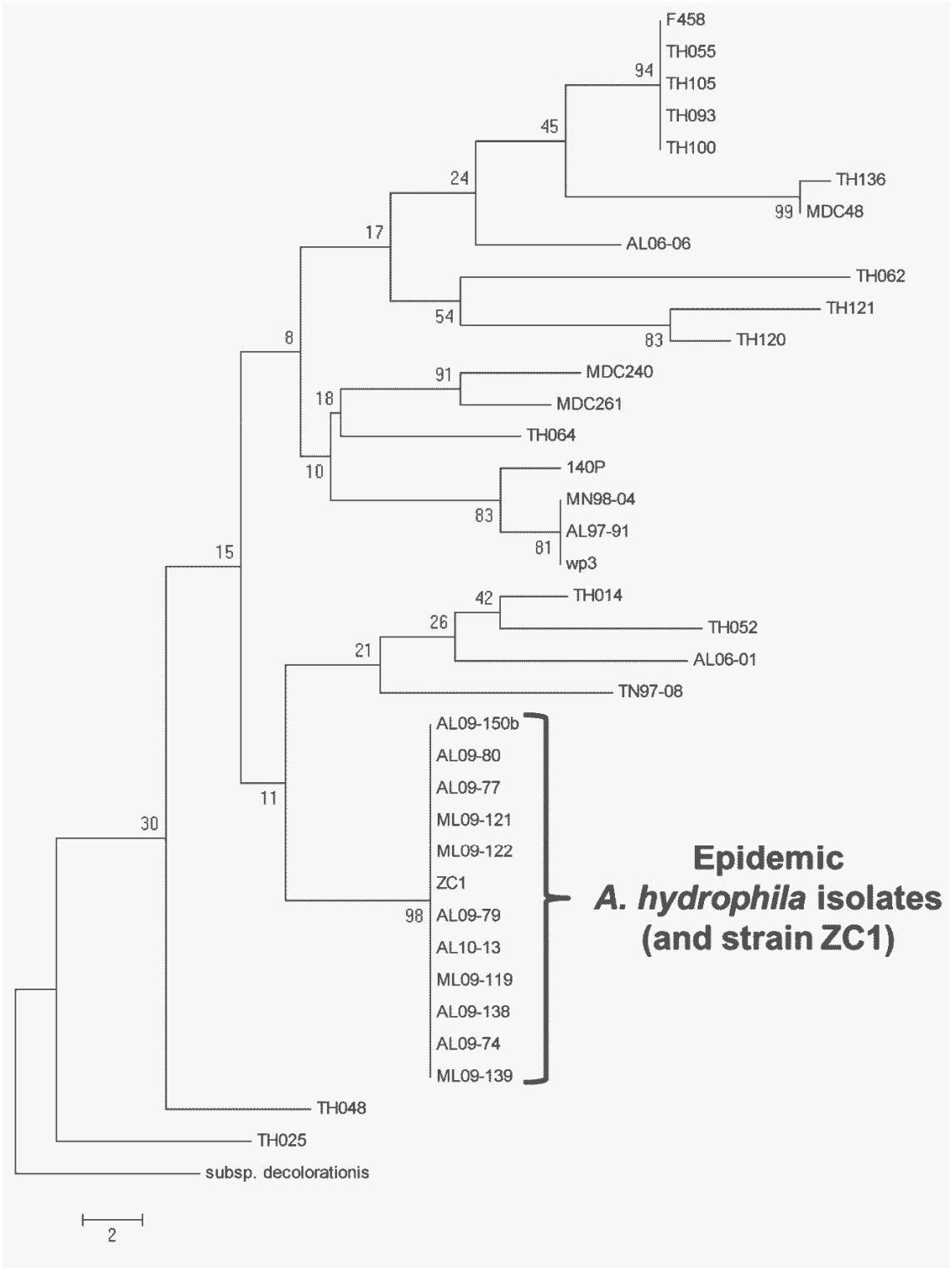


Figure 1. Evolutionary relationships of 37 *A. hydrophila* taxa based on *gyrB* gene sequences (out of a larger dataset of 107 *A. hydrophila gyrB* sequences). The evolutionary history was inferred

using the Maximum Parsimony method. Tree #1 out of 67 most parsimonious trees (length = 218) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence.

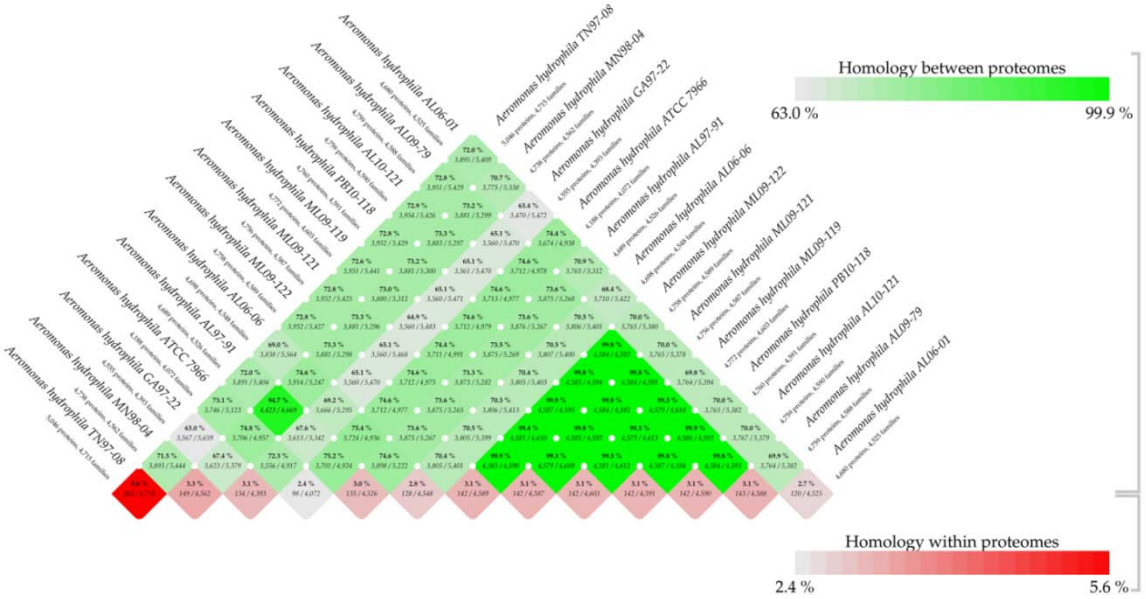


Figure 2. BLAST MATRIX of 12 different *A. hydrophila* isolates. The proteomes of each of the *A. hydrophila* strains were compared using all-against-all BLASTp according to the methods described previously (Friis *et al.*, 2010). This matrix showed the output from pairwise comparison of conserved protein families of each of the isolates to each other. The green color represents the % homology between proteomes and the red color represents % homology within proteomes. This matrix showed that all the epidemic *A. hydrophila* isolates are similar to each other but substantially different to reference isolates. All of the reference isolates, except for MN98-04 and AL97-91 that were highly similar to each other, possessed substantial amount of diversity in their protein families.

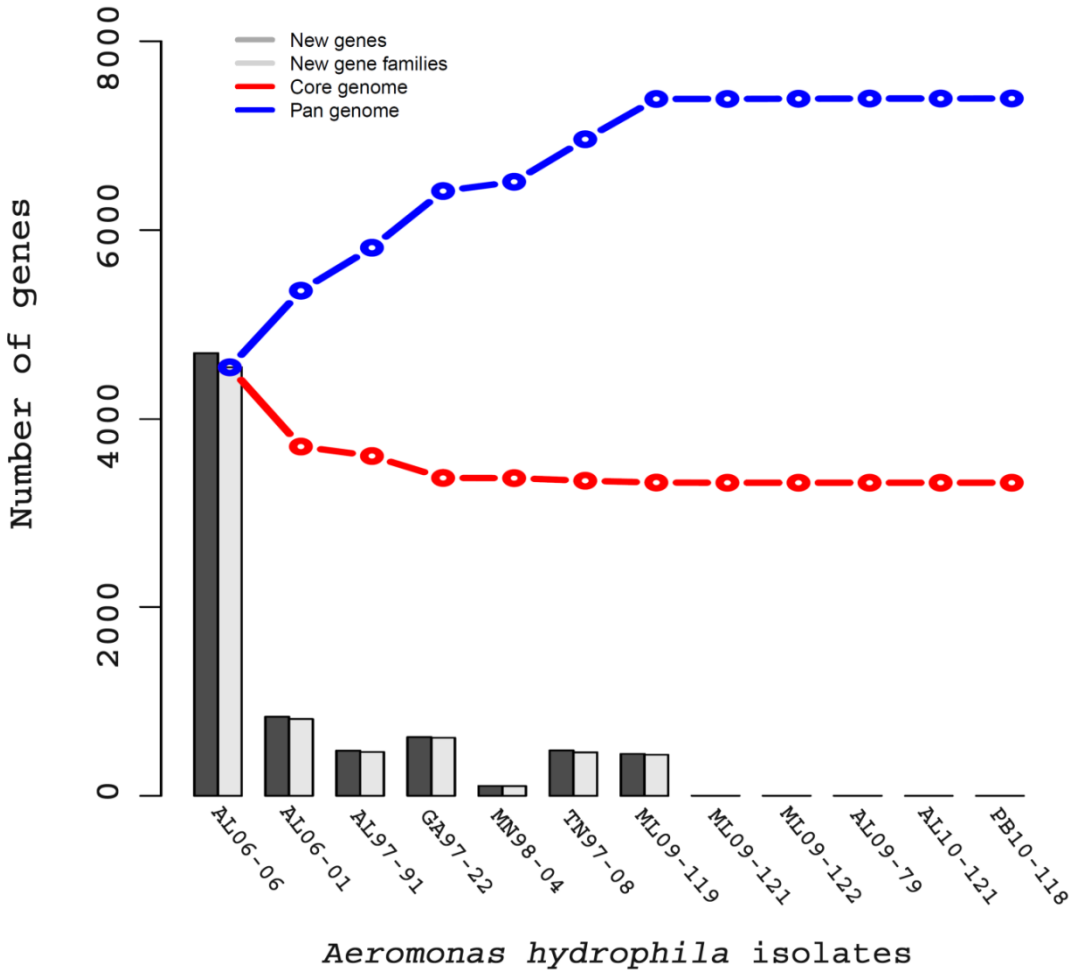
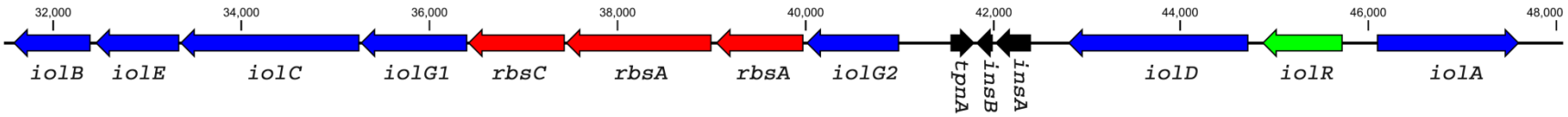


Figure 3. Pan and core-genome plot of 12 different *A. hydrophila* isolates. The red and blue lines indicated the number of genes within the core and pan-genomes, respectively. The *A. hydrophila* core genome contained 3354 core genes whereas the *A. hydrophila* pan-genome contained 7290 genes. Note that the addition of other EAh strains after *A. hydrophila* ML09-119 did not significantly increase the number of new gene families, which was in agreement with the highly clonal nature of EAh strains.



myo-inositol catabolism myo-inositol transport regulation of *iol* gene transcription Transposase

ORF ID	Nucleotide positions*	Putative functions	Gene	Bacteria (Nearest neighbor)	% Identity/ % Similarity	E-value	Accession Number
ORF1	31577..32398	5-deoxy-glucuronate isomerase	*iolB*	*Dickeya dadantii* Ech586	73/83	1E-143	YP_003332313
ORF2	32450..33340	Myo-inosose-2 dehydratase	*iolE*	*Enterobacter cloacae subsp. cloacae* ATCC 13047	97/98	0	YP_003614284
ORF3	33352..35256	5-dehydro-2-deoxygluconokinase	*iolC*	*Enterobacter cloacae subsp. cloacae* ATCC 13047	99/99	0	YP_003614285
ORF4	35269..36402	Myo-inositol 2-dehydrogenase	*iolG1*	*Enterobacter cloacae subsp. cloacae* ATCC 13047	99/99	0	YP_003614286
ORF5	36411..37439	Ribose transport system permease	*rbsC*	*Enterobacter cloacae subsp. cloacae* ATCC 13047	99/99	0	YP_003614287
ORF6	37451..38998	Ribose transport system ATP-binding protein	*rbsA*	*Enterobacter mori* LMG 25706	98/99	0	ZP_09038867
ORF7	39047..39976	Ribose ABC transporter	*rbsB*	*Enterobacter cloacae subsp. cloacae* ATCC 13047	98/99	0	YP_003614289
ORF8	40008..40994	Myo-inositol 2-dehydrogenase	*iolG2*	*Enterobacter mori* LMG 25706	89/92	0	ZP_09038869
ORF9	41539..41793	Transposase IS3	*tpnA*	*Aeromonas caviae* Ae398	74/79	1.1E-21	ZP_08522329
ORF10	41813..41989	Integrase catalytic subunit	*insB*	*Aeromonas salmonicida* subsp. *salmonicida* 01-B526	83/86	1.4E-10	EHI50204
ORF11	42017..42394	Integrase catalytic region	*insA*	*Aeromonas caviae* Ae398	91/94	4.2E-59	ZP_08522330
ORF12	42791..44725	3D-(3, 5/4)-trideoxycyclohexane-1,2-dione hydrolase	*iolD*	*Photorhabdus luminescens* subsp. *laumondii* TTO1	74/84	0	NP_929083
ORF13	44874..45728	Transcriptional regulator	*iolR*	*Providencia stuartii* ATCC 25827	66/81	7E-122	ZP_02961459
ORF14	46092..47603	Aldehyde dehydrogenase	*iolA*	*Photobacterium profundum* SS9	78/89	0	YP_132151

* The number corresponds to the nucleotide positions in the contig 15 of *A. hydrophila* strain ML09-119.

Figure 4. Genetic elements involved in *myo-inositol* utilization in EAh strains. The schematic organization depicts the cluster of genes involved in *myo*-inositol utilization in epidemic *A. hydrophila* ML09-119. The presence of a functional *myo*-inositol utilization pathway in EAh strains was confirmed by their ability to grow on *myo*-inositol as a sole carbon source.

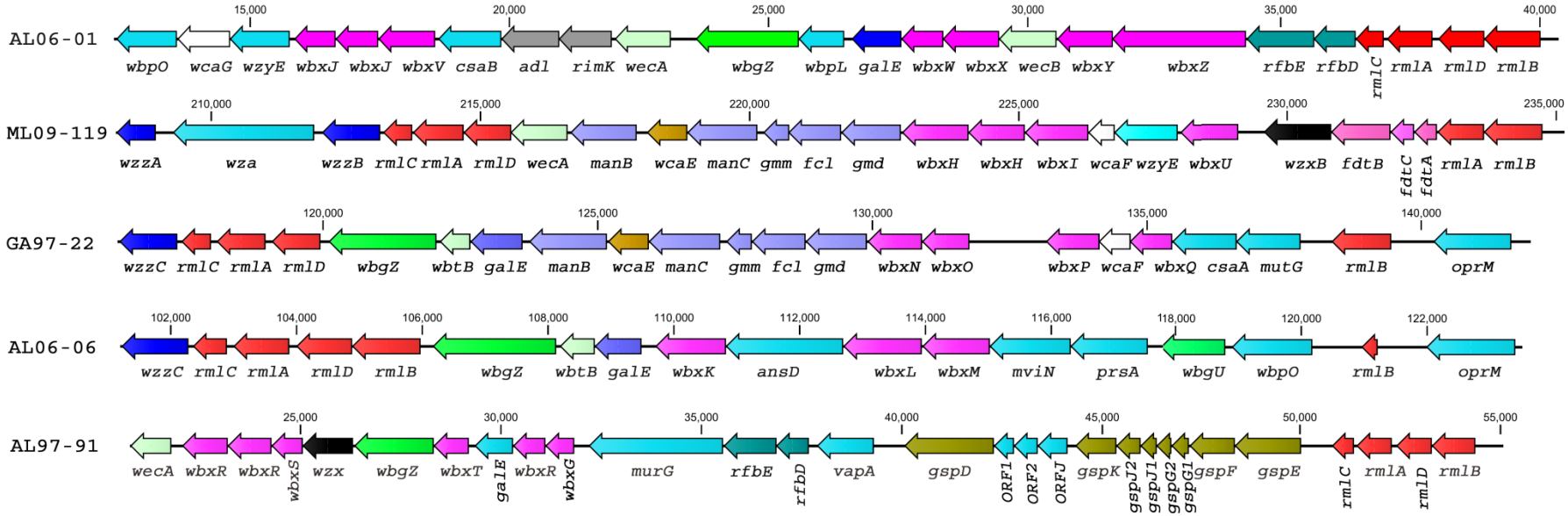

AL06-01
15,000
20,000
25,000
30,000
35,000
40,000
wbpO wcaG wzyE wbxJ wbxJ wbxV csaB adl rimK wecA wbgZ wbpL galE wbxW wbxX wecB wbxY wbxZ rfbE rfbD rmlC rmlA rmlD rmlB
ML09-119
210,000
215,000
220,000
225,000
230,000
235,000
wzzA wza wzzB rmlC rmlA rmlD wecA manB wcaE manC gmm fcl gmd wbxH wbxH wbxI wcaF wzyE wbxU wzxB fdtB fdtC fdtA rmlA rmlB
GA97-22
120,000
125,000
130,000
135,000
140,000
wzzC rmlC rmlA rmlD wbgZ wbtB galE manB wcaE manC gmm fcl gmd wbxN wbxO wbxP wcaF wbxQ csaA mutG rmlB oprM
AL06-06
102,000
104,000
106,000
108,000
110,000
112,000
114,000
116,000
118,000
120,000
122,000
wzzC rmlC rmlA rmlD rmlB wbgZ wbtB galE wbxK ansD wbxL wbxM mviN prsA wbgU wbpO rmlB oprM
AL97-91
25,000
30,000
35,000
40,000
45,000
50,000
55,000
wecA wbxR wbxR wbxS wzx wbgZ wbxT galE wbxR wbxG murG rfbE rfbD vapA gspD ORF1 ORF2 ORFJ gspK gspJ2 gspJ1 gspG2 gspG1 gspF gspE rmlC rmlA rmlD rmlB

Figure 5. Schematic organization of the five different types of O-antigen biosynthesis gene cluster present within the genome of the 12 *A. hydrophila* isolates sequenced in this study. All of the genes on the cluster are transcribed in the same direction. All EAh strains along with RAh strain TN97-08 shared the ML09-119-type O-antigen biosynthesis gene cluster. This cluster encodes proteins predicted to be involved in the biosynthesis of the nucleotide sugars D-rhamnose, D-mannose, D-Fucose, and 3-acetamido-3, 6-dideoxy-d-galactose (D-Fuc*p*3NAc). The AL97-91-type cluster (that was also shared with MN98-04) encodes genes predicted to be required for S-layer biosynthesis and transport in addition to O-antigen biosynthesis. Genes that encode conserved proteins with similar functions are marked with the same color. The number displayed next to the maps indicates the nucleotide positions on the respective contig from each strain. The designation of each of the genes presented on the schematic map of the AL06-01, ML09-119, AL06-06, AL97-91 and GA97-22 O-antigen clusters are found in the supplemental material Table 4, Table 2, Table 5, Table 6 and Table 7, respectively.

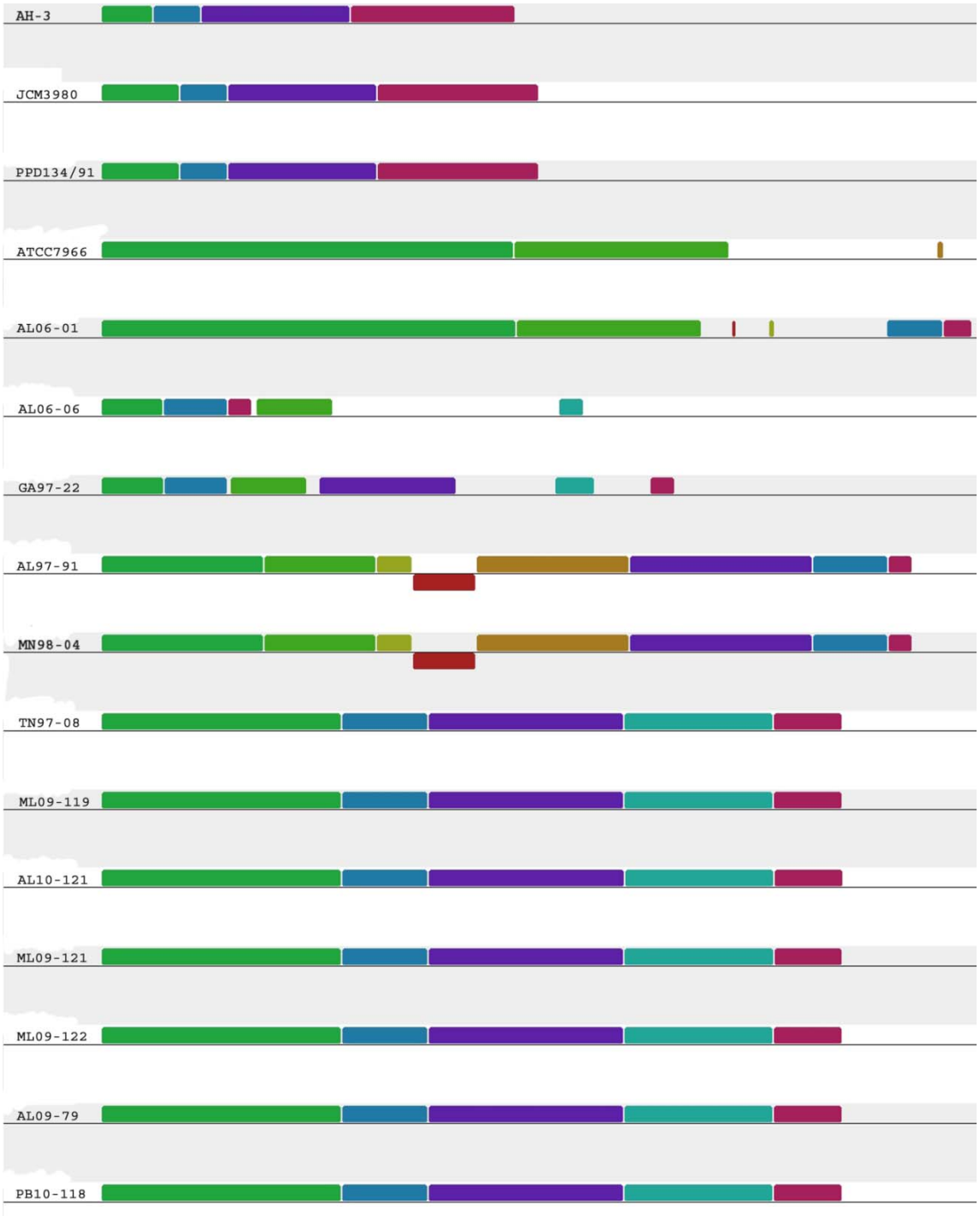


Figure 6. Mauve Alignment of the O-antigen cluster from 16 different *A. hydrophila* isolates. Segments with a similar color indicate homologous regions. The alignment is on scale based on the size of the O-antigen cluster of *A. hydrophila* ATCC 7966 which is 44 kb in length. All of the

EAh strains including RAh strain TN97-08 shared the ML09-119-type O-antigen biosynthesis gene cluster.

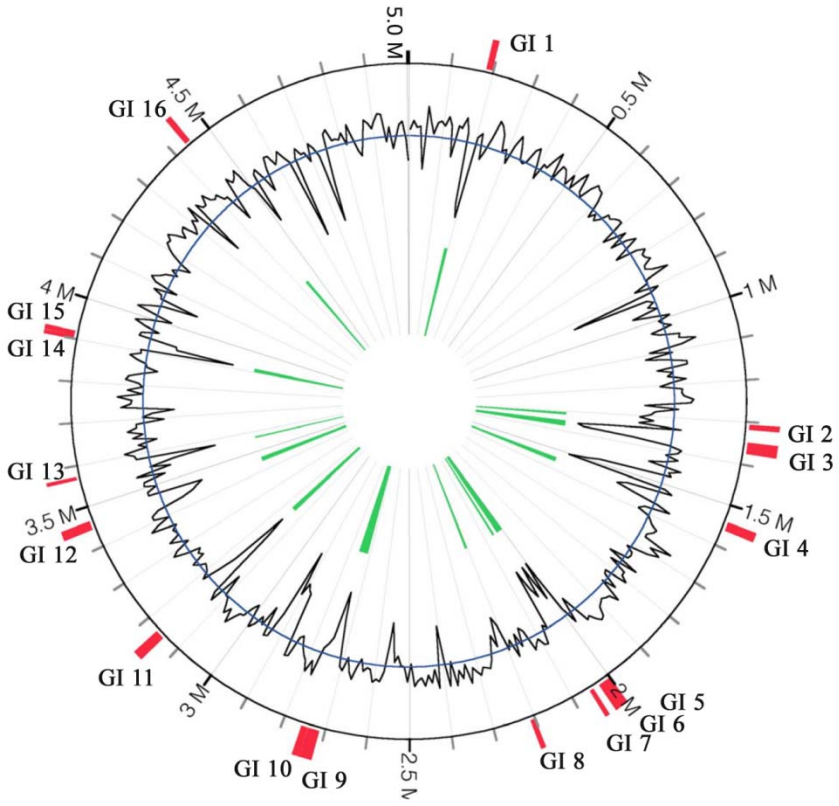


Figure 7. Predicted genomic islands (GIs) within the genome of *A. hydrophila* ML09-119. GIs were predicted using the IslandViewer tool (Langille *et al.*, 2008). The black line indicates the %G+C content. All of the predicted GIs showed a %G+C content bias much lower than the average %G+C content of *A. hydrophila* (61.0%).

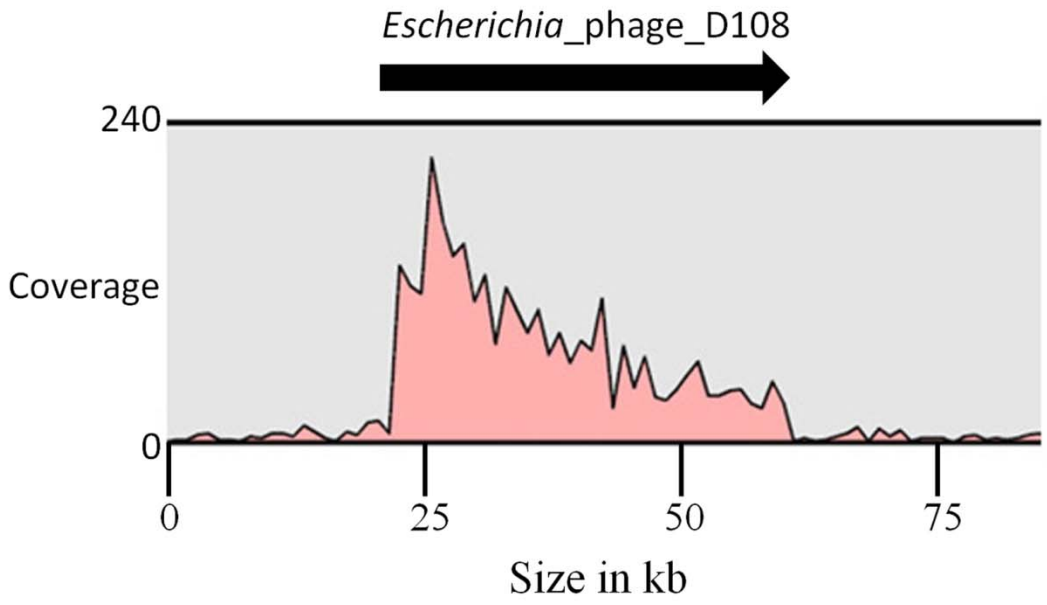


Figure 8. Identification of induced prophage after mitomycin C treatment. Induced phage DNAs were subjected to 454 pyrosequencing and were reference mapped against 42 contigs of the *A. hydrophila* ML09-119 genome using CLC Bio Genomics Workbench. Prophage sequence reads were aligned with only one region within the ML09-119 genome designated as AH4 which has similarity to prophage Escherichia_phage_D108.

Table 1. Summary of draft genome sequences from 12 different *A. hydrophila* isolates obtained from diseased fish.

Strains	Source of isolates	Year of isolation	Type[a]	Contigs >200bp	Total bp in Assembly Mbp	GenBank Accession no.
AL97-91	Diseased Tilapia	1997	RAh	111	4.8	SRX157795
GA97-22	Rainbow Trout	1997	RAh	160	4.75	SRX157828
TN97-08	Diseased Blue Gill	1997	RAh	94	5.2	SRX157873
MN98-04	Diseased Tilapia	1998	RAh	98	4.86	SRX157796
AL06-01	Diseased catfish	2006	RAh	122	4.75	SRX157912
AL06-06	Diseased Goldfish	2006	RAh	133	4.84	SRX157794
AL09-79	Diseased Catfish	2009	EAh	91	4.97	SRX157791
ML09-119	Diseased Catfish	2009	EAh	100	5.0	SRX157759
ML09-121	Diseased Catfish	2009	EAh	93	5.0	SRX157784

ML09-122	Diseased Catfish	2009	EAh	156	4.97	SRX157790
AL10-121	Diseased Catfish	2010	EAh	98	5.0	SRX157792
PB10-118	Diseased catfish	2010	EAh	100	5.06	SRX157793

Table 2. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of EAh isolate ML09-119.

ORF ID	Nucleotide positions in contig 15	Predicted Function	Gene	Top BLASTx hit	% Identity
ORFu	207127..207798	lipoprotein YmcC	*ymcC*	*Edwardsiella tarda* ATCC 23685	43
ORF1	208240..208968	O-antigen length determinant protein	*wzzA*	*Ferrimonas balearica* DSM 9799	59
ORF2	209286..211910	periplasmic protein involved in polysaccharide export	*wza*	*Marinomonas sp.* MWYL1	45
ORF3	212056..213138	O-antigen chain length determinant protein	*wzzB*	*Aeromonas veronii* B565	80
ORF4	213193..213732	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Halomonas sp.* TD01	58
ORF5	213746..214687	glucose-1-phosphate thymidylyltransferase	*rmlA*	*Lutiella nitroferrum* 2002	61
ORF6	214687..215571	rmlD gene product	*rmlD*	*gamma proteobacterium* HdN1	54
ORF7	215558..216619	undecaprenyl-phosphate N-acetylglucosaminy	*wecA*	*Photobacterium damselae subsp. damselae* CIP 102761	57

		l 1-phosphate transferase			
ORF8	216670..217902	phosphomannomutase	*manB*	*Vibrio nigripulchritudo* ATCC 27043	69
ORF9	218091..218843	Colanic acid biosynthesis glycosyl transferase	*wcaE*	*Shigella dysenteriae* 1012	67
ORF10	218843..220144	mannose-1-phosphate guanylyltransferase	*manC*	*Photobacterium profundum* 3TCK	71
ORF11	220254..220736	GDP-mannose mannosyl hydrolase	*gmm*	*Tolumonas auensis* DSM 9187	63
ORF12	220720..221700	GDP-fucose synthetase	*fcl*	*Yersinia pestis* KIM 10	84
ORF13	221704..222810	GDP-mannose 4,6-dehydratase	*gmd*	*Vibrio angustum* S14	86
ORF14	222831..224066	group 1 glycosyl transferase	*wbxH*	*Pectobacterium carotovorum subsp. carotovorum* WPP14	49
ORF15	224063..225112	group 1 glycosyl transferase	*wbxH*	*Pectobacterium carotovorum subsp. carotovorum* PC1	52
ORF16	225112..226293	glycosyl transferase group 1	*wbxI*	*Methylobacter tundripaludum* SV96	52
ORF17	226290..226781	acetyltransferase	*wcaF*	*Methylobacter tundripaludum* SV96	59
ORF18	226774..227952	O-antigen polymerase	*wzyE*	*Bacteroides sp.* 2_1_7	29
ORF19	228003..229094	group 1 glycosyl transferase protein	*wbxU*	*Dysgonomonas gadei* ATCC BAA-286	42
ORF20	229570..230820	O-antigen flippase	*wzxB*	*Shewanella baltica* OS625	81
ORF21	230817..231920	aminotransferase	*fdtB*	*Shewanella baltica* OS195	81
ORF22	231922..232359	dTDP-D-Fucp3N acetyltransferase	*fdtC*	*Shewanella baltica* OS195	85
ORF23	232361..232783	dTDP-6-deoxy-3,4-keto-hexulose isomerase	*fdtA*	*Shewanella putrefaciens* 200	72
ORF24	232794..233660	glucose-1-phosphate thymidylyltransferase	*rmlA*	*Shewanella putrefaciens* 200	79

ORF25	233657..234745	dTDP-glucose-4-6-dehydratase	*rmlB*	*Aeromonas hydrophila*	93
ORFd	235360..238509	AcrB protein	*acrB*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99

Table 3. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of RAh isolate TN97-08.

ORF ID	Nucleotide positions in Contig 3483_64512	Predicted Function	Gene	Top BLASTx hit	% Identity
ORF1	5776..6504	O-antigen length determinant protein	*wzz,*	*Ferrimonas balearica* DSM 9799	59
ORF2	6822..9446	periplasmic protein involved in polysaccharide export	*otnA*	*Marinomonas sp.* MWYL1	45
ORF3	9592..10674	O-antigen chain length determinant protein	*wzz*	*Aeromonas veronii* B565	80
ORF4	10729..11268	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Halomonas sp.* TD01	58
ORF5	11282..12223	glucose-1-phosphate thymidylyltransferase	*rmlA*	*Lutiella nitroferrum* 2002	61
ORF6	12223..13107	rmlD gene product	*rmlD*	*gamma proteobacterium HdN1*	54
ORF7	13094..14155	undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase	*wecA*	*Photobacterium damselae subsp. damselae* CIP 102761	57
ORF8	14206..15438	phosphomannomutase	*manB*	*Vibrio nigripulchritudo* ATCC 27043	69
ORF9	15627..16379	Colanic acid biosynthesis glycosyl transferase	*wcaE*	*Shigella dysenteriae* 1012	67

ORF10	16379..17680	mannose-1-phosphate guanylyltransferase	*manC*	*Photobacterium profundum* 3TCK	71
ORF11	17790..18272	GDP-mannose mannosyl hydrolase	*gmm*	*Tolumonas auensis* DSM 9187	63
ORF12	18256..19236	GDP-fucose synthetase	*fcl*	*Yersinia pestis* KIM 10	84
ORF13	19240..20346	GDP-mannose 4,6-dehydratase	*gmd*	*Vibrio angustum* S14	86
ORF14	20367..21602	group 1 glycosyl transferase	*wbxH*	*Pectobacterium carotovorum subsp. carotovorum* WPP14	49
ORF15	21599..22648	group 1 glycosyl transferase	*wbxH*	*Pectobacterium carotovorum subsp. carotovorum* PC1	52
ORF16	22648..23829	glycosyl transferase group 1	*wbxI*	*Methylobacter tundripaludum* SV96	52
ORF17	23826..24317	acetyltransferase	*wcaF*	*Methylobacter tundripaludum* SV96	59
ORF18	24310..25488	O-antigen polymerase	*wzyE*	*Bacteroides sp.* 2_1_7	29
ORF19	25539..26630	group 1 glycosyl transferase protein	*wdaN*	*Dysgonomonas gadei* ATCC BAA-286	42
ORF20	27106..28356	O-antigen flippase	*wzxB*	*Shewanella baltica* OS625	81
ORF21	28353..29456	aminotransferase	*fdtB*	*Shewanella baltica* OS195	81
ORF22	29458..29895	dTDP-D-Fucp3N acetyltransferase	*fdtC*	*Shewanella baltica* OS195	85
ORF23	29897..30319	dTDP-6-deoxy-3,4-keto-hexulose isomerase	*fdtA*	*Shewanella putrefaciens* 200	72
ORF24	30330..31196	glucose-1-phosphate thymidylyltransferase	*rmlA*	*Shewanella putrefaciens* 200	79
ORF25	31193..32281	dTDP-glucose-4-6-dehydratase	*rmlB*	*Aeromonas hydrophila*	93

Table 4. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of RAh isolate AL06-01.

ORF ID	Nucleotide positions in contig 46	Predicted function	Gene	Top BLASTx hit	% Identity
ORFu	10305..10733	low molecular weight protein-tyrosine-phosphatase ptp	*ppt*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99
ORF1	10969..12084	capsular polysaccharide transport protein	*wza*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99
ORF2	12420..13586	UDP-glucose 6-dehydrogenase	*wbpO*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99
ORF3	13599..14612	nucleotide sugar epimerase	*wcaG*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99
ORF4	14609..15763	O-antigen polymerase	*wzyE*	*Vibrio alginolyticus 40B*	32
ORF5	15853..16647	glycosyl transferase family protein	*wbxJ*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	86
ORF6	16649..17470	glycosyl transferase family protein	*wbxJ*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	98
ORF7	17474..18568	glycoside hydrolase family protein	*wbxV*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	99
ORF8	18631..19845	polysaccharide biosynthesis protein	*csaB*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	100
ORF9	19847..20962	NAD(P) transhydrogenase subunit alpha	*adl*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	100

ORF10	20955..21977	hypothetical protein	*rimK*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	100
ORF11	22036..23115	UDP-phosphate alpha-N-acetylglucosaminyl 1-phosphatetransferase	*wecA*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	100
ORF12	23600..25585	protein WbgZ	*wbgZ*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	98
ORF13	25582..26460	glycoside hydrolase family protein	*wbpI*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	92
ORF14	26607..27563	UDP-glucose 4-epimerase	*galE*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	91
ORF15	27563..28372	glycosyl transferase family protein	*wbx W*	*Aeromonas salmonicida* subsp. *salmonicida* A449	65
ORF16	28363..29448	glycosyltransferase	*wbxX*	*Enterobacter aerogenes* KCTC 2190	53
ORF17	29445..30554	UDP-N-acetylglucosamine 2-epimerase	*wecB*	*Aeromonas salmonicida* subsp. *salmonicida* A449	80
ORF18	30566..31642	group 1 glycosyl transferase	*wbxY*	*Enterobacter aerogenes* KCTC 2190	43
ORF19	31629..34331	family 2 glycosyl transferase	*wbxZ*	*Enterobacter aerogenes* KCTC 2190	47
ORF20	34344..35648	ABC transporter ATP-binding protein	*rfbE*	*Enterobacter aerogenes* KCTC 2190	64
ORF21	35638..36447	ABC-2 type transporter	*rfbD*	*Pseudomonas chlororaphis O6*	60
ORF22	36449..36988	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Aeromonas caviae Ae398*	78
ORF23	37051..37929	glucose-1-phosphate thymidylyltransferas	*rmlA*	*Aeromonas veronii B565*	92

		e 1			
ORF24	38041..38928	dTDP-4-dehydrorhamnose reductase	*rmlD*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	95
ORF25	38928..40013	putative dTDP-glucose-4-6-dehydratase	*rmlB*	*Aeromonas hydrophila*	96
ORFd	40701..42110	outer membrane protein OprM	*oprM*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	97

Table 5. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of EAh isolate AL06-06.

ORF ID	Nucleotide positions in contig 9046_152365	Predicted function	Gene	Top BLASTx hit	% Identity
ORFu	99408..101126	lipid A core - O-antigen ligase	*waaL*	*Aeromonas hydrophila subsp. hydrophila* ATCC 7966	97
ORF1	101221..102282	O-antigen chain length determinant	*wzzC*	*Aeromonas caviae* Ae398	69
ORF2	102355..102894	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Halomonas sp.* TD01	57
ORF3	102999..103886	glucose-1-phosphate thymidylyltransferase 1	*rmlA*	*Aeromonas veronii* B565	92
ORF4	103998..104885	dTDP-4-dehydrorhamnose reductase	*rmlD*	*Aeromonas hydrophila subsp. hydrophila* ATCC 7966	97
ORF5	104885..105973	dTDP-glucose-4, 6-dehydratase	*rmlB*	*Aeromonas hydrophila*	98
ORF6	106168..108129	epimerase/dehydratase family WbfY-like protein	*wbgZ*	*Aeromonas caviae* Ae398	96
ORF7	108188..108742	lipid carrier : UDP-N-acetylgalactosaminyl transferase	*wbtB*	*Aeromonas veronii* B565	96
ORF8	108745..109485	UDP-glucose 4-	*galE*	*Vibrio metschnikovii*	77

		epimerase		CIP 69.14	
ORF9	109708..110829	glycosyl transferase, group 1 family protein	*wbxK*	*Shewanella oneidensis* MR-1	59
ORF10	110826..112691	asparagine synthetase, glutamine-hydrolyzing	*asnD*	*Shewanella oneidensis* MR-1	74
ORF11	112697..113941	glycosyl transferase, group 1	*wbxL*	*Vibrio ichthyoenteri* ATCC 700023	46
ORF12	113954..115024	group 1 glycosyl transferase	*wbxM*	*Achromobacter piechaudii* ATCC 43553	35
ORF13	115021..116310	virulence factor MVIN family protein	*mviN*	*Burkholderia ubonensis* Bu	39
ORF14	116307..117536	ribose-phosphate pyrophosphokinase	*prsA*	*Candidatus Sulciamuelleri* CARI	36
ORF15	117765..118793	UDP-GlcNAc 4-epimerase	*wbgU*	*Shewanella oneidensis* MR-1	81
ORF16	118896..120176	UDP-glucose dehydrogenase/UDP-N-acetyl-D-galactosamine dehydrogenase	*wbpO*	*Vibrio vulnificus* MO6-24/O	88
ORF17	120958..121215	dTDP-D-glucose-4,6-dehydratase	*rmlB*	*Aeromonas hydrophila*	89
ORFd	121989..123404	outer membrane protein OprM	*oprM*	*Aeromonas hydrophila*	99

Table 6. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of RAh isolate AL97-91.

ORF ID	Nucleotide positions in contig 4239_186716	Predicted function	Gene	Top BLASTx hit	% Identity
ORFu	19045..20706	lipid A core - O-antigen ligase	*waaL*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	97
ORF1	20759..21784	UDP-glucose lipid carrier transferase	*wecA*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	73
ORF2	22052..23188	glycosyltransferase	*wbxR*	*Aeromonas salmonicida* subsp. *salmonicida* A449	75
ORF3	23185..24279	glycosyltransferase, group 2 family protein	*wbxR*	*Aeromonas salmonicida* subsp. *salmonicida* A449	70
ORF4	24281..25060	glycosyltransferase, group 2 family protein	*wbxS*	*Escherichia coli*	48
ORF5	25057..26313	integral membrane protein AefA/O-antigen flippase	*wzx*	*Salmonella bongori* NCTC 12419	70
ORF6	26344..28326	epimerase/dehydratase family WbfY-like protein	*wbgZ*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	94
ORF7	28323..29201	glycosyl transferase, group 4 family protein	*wbxT*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	91
ORF8	29348..30304	UDP-glucose 4-epimerase	*galE*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	92
ORF9	30304..31110	Glycosyltransferase, family 2	*wbxR*	*Aeromonas salmonicida* subsp. *salmonicida* A449	65
ORF10	31112..31831	acyltransferase family protein	*wbxG*	*Flavobacteria bacterium* BAL38	30
ORF11	32191..35541	putative N-acetyl	*murG*	*Serratia odorifera*	50

		glucosaminyl transferase		4Rx13	
ORF12	35559..36875	transporter	*rfbE*	*Serratia odorifera* 4Rx13	73
ORF13	36865..37683	ABC-2 type transporter	*rfbD*	*Thermosinus carboxydivorans* Nor1	65
ORF14	37885..39303	surface layer protein	*vapA*	*Aeromonas hydrophila*	100
ORF15	40063..42294	S-protein secretion D	*gspD*	*Aeromonas hydrophila*	100
ORF16	42298..42798	ORF2	ORF2	*Aeromonas hydrophila*	100
ORF17	42795..43391	ORF1	ORF1	*Aeromonas hydrophila*	100
ORF18	43388..44134	ORFJ, partial	ORFJ	*Aeromonas hydrophila*	99
ORF19	44328..45350	general secretion pathway protein K	*gspK*	*Pseudomonas stutzeri* DSM 4166	49
ORF20	45350..45955	type II secretion system protein	*gspJ2*	*Azotobacter vinelandii* DJ	48
ORF21	45952..46356	type II secretion system protein	*gspJ1*	*Pseudomonas stutzeri* DSM 4166	58
ORF22	46350..46721	type II secretion system protein	*gspG2*	*Pseudomonas stutzeri* DSM 4166	48
ORF23	46724..47158	General secretion pathway protein G	*gspG1*	*Pseudomonas stutzeri* DSM 4166	84
ORF24	47177..48376	type II secretion system protein	*gspF*	*Azotobacter vinelandii* DJ	60
ORF25	48376..50031	type II secretion system protein E	*gspE*	*Azotobacter vinelandii* DJ	76
ORF26	50799..51347	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Escherichia sp.* TW09308	81
ORF27	51412..52290	glucose-1-phosphate thymidylyltransferase	*rmlA*	*Aeromonas salmonicida* subsp. *salmonicida* A449	94
ORF28	52403..53290	dTDP-4-dehydrorhamnose reductase	*rmlD*	*[Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	98
ORF29	53290..54375	dTDP-glucose 4,6-dehydratase	*rmlB*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	97

Table 7. Summary of ORFs encoded by the O-antigen biosynthesis gene cluster of RAh isolate GA97-22.

ORF ID	Nucleotide positions in contig 5016_170856	Predicted function	Gene	Top BLASTx hit	% Identity
ORFu	114463..116178	lipid A core - O-antigen ligase	*waaL*	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966	91
ORF1	116294..117349	O-antigen chain length determinant	*wzzC*	*Aeromonas caviae* Ae398	68
ORF2	117422..117961	dTDP-4-dehydrorhamnose 3,5-epimerase	*rmlC*	*Halomonas sp.* TD01	55
ORF3	118066..118953	glucose-1-phosphate thymidylyltransferase 1	*rmlA*	*Aeromonas veronii* B565	91
ORF4	119066..119953	dTDP-4-dehydrorhamnose reductase	*rmlD*	*Aeromonas caviae* Ae398	91
ORF5	120103..122064	epimerase/dehydratase family WbfY-like protein	*wbgZ*	*Aeromonas caviae* Ae398	92
ORF6	122125..122682	lipid carrier : UDP-N-acetylgalactosaminyltransferase	*wbtB*	*Vibrio metschnikovii* CIP 69.14	84
ORF7	122685..123632	UDP-galactose 4-epimerase	*galE*	*Shewanella oneidensis* MR-1	54
ORF8	123752..125167	phosphomannomutase	*manB*	*Vibrio sp.* EJY3	66
ORF9	125186..125929	Colanic acid biosynthesis glycosyl transferase	*wcaE*	*Shigella dysenteriae* CDC 74-1112	56
ORF10	125929..127230	mannose-1-phosphate guanylyltransferase	*manC*	*Photobacterium profundum* 3TCK	70
ORF11	127340..127804	GDP-mannose mannosylhydrolase	*gmm*	*Vibrio angustum* S14	63
ORF12	127806..128783	GDP-fucose synthetase	*fcl*	*Yersinia pestis* KIM10+	80
ORF13	128787..129899	GDP-D-mannose dehydratase	*gmd*	*Vibrio angustum* S14	82
ORF14	129913..130902	glycosyltransferase, group 1 family	*wbxN*	*Salmonella enterica*	49

ORF15	130903..131766	glycosyl transferase, group 2 family protein	*wbxO*	*Clostridium perfringens* SM101	35
ORF16	133160..134134	group 1 glycosyl transferase	*wbxP*	*Escherichia coli* O104:H4 str. C227-11	36
ORF17	134119..134700	acetyltransferase	*wcaF*	*Actinobacillus pleuropneumoniae* serovar 2 str. 4226	40
ORF18	134685..135470	glycosyl transferase, group 2 family	*wbxQ*	uncultured *delta proteobacterium*	29
ORF19	135472..136644	Polysaccharide biosynthesis protein	*csaA*	*Bacillus cereus* MM3	32
ORF20	136634..137806	MutG family lantibiotic protection ATP binding cassette transporter permease subunit	*mutG*	*Haemophilus parainfluenzae* ATCC 33392	29
ORF21	138371..139456	dTDP-glucose-4, 6-dehydratase	*rmlB*	*Aeromonas hydrophila*	96
ORFd	140225..141643	outer membrane protein OprM	*oprM*	*Aeromonas salmonicida* subsp. *salmonicida* A449	94

Table 8. Summary of ORFs encoded within GI 13 of EAh isolate ML09-119 involved in CS5 pilus biogenesis.

ORF ID	Nucleotide positions in contig 27	Putative function	Gene	Top BLASTx hit	% Identity
ORF1	106..456	Extracellular solute-binding protein family 3	-	*Paenibacillus lactis* 154	48
ORF2	909..1490	CS5 fimbrial major pilin subunit	*hsfA*	*Escherichia coli*	38
ORF3	1555..2247	25.9 kDa protein in CS5 3' region precursor	*hsfB*	*Edwardsiella ictaluri* 93-146	36
ORF4	2303..3643	P pilus assembly protein porin PapC-like protein	*hsfC*	*Edwardsiella ictaluri* 93-146	29
ORF5	3945..4679	P pilus assembly protein porin PapC-like protein	*hsfC*	*Edwardsiella ictaluri* 93-146	28
ORF6	4669..5244	Hypothetical protein	*hsfE*	*Escherichia coli*	26
ORF7	5205..6023	CS5 fimbrial minor pilin subunit	*hsfD*	*Escherichia coli*	35
ORF8	6369..7127	EAL domain protein	-	*Vibrio parahaemolyticus* AN-5034	45
ORF9	7258..8034	Alpha/beta hydrolase, putative	-	*Vibrio cholerae* MZO-3	49

Table 9. Predicted genes that have homology to putative virulence factors and are present within EAh-associated genomic regions

Unique region ID	VFDB ID	GIs	Gene	Putative functions	Organisms	% Identity
C8R1	VFG1693	GI 1	*int*	Prophage P4 integrase	*Escherichia coli* CFT073	33
C10R1	VFG0893	-	*papA_2*	PapA protein	*Escherichia coli* CFT073	33
C10R1	VFG0075	-	*InlA*	Internalin A	*Listeria monocytogenes* (serovar 1/2a) EGD-e	30
C13R2	VFG0783	-	*intL*	Putative integrase for prophage 933L and the LEE pathogenicity island	*Escherichia coli* O157:H7 EDL933	27
C15R4	VFG0038	GI 2	*bplA*	probable oxidoreductase	*Bordetella pertussis* Tohama I	28
C15R4	VFG0344	GI 2	*hitC*	iron(III) ABC transporter, ATP	*Haemophilus influenzae* Rd	33
C15R5	VFG0082	-	*aldA*	aldehyde dehydrogenase	*Vibrio cholerae* N16961	31
C15R6	VFG0598	-	*intC*	Sai integrase	*Shigella flexneri* (serotype 2a) 301	47
C18R3	VFG0672	GI 6	*int*	integrase	*Shigella flexneri* (serotype 2a)	66
C20R4	VFG1124	-	*VC1791*	conserved hypothetical protein	*Vibrio cholerae* N16961	41
C20R7	VFG0925	-	*fepC*	Ferric enterobactin transport ATP	*Escherichia coli* CFT073	33
C20R7	VFG0922	-	*chuU*	Putative permease of iron compound ABC transport system	*Escherichia coli* CFT073	39
C20R8	VFG0358	-	*fyuA/psn*	yersiniabactin receptor protein	*Yersinia pestis* CO92	26
C20R8	VFG0167	-	*pchR*	transcriptional regulator PchR	*Pseudomonas aeruginosa* PAO1	41

C26R1	VFG1102	GI 12	*hsdM*	DNA methylase HsdM	*Vibrio cholerae* N16961	25
C26R1	VFG1098	GI 12	*hsdR*	type I restriction enzyme HsdR	*Vibrio cholerae* N16961	24
C27R1	VFG1433	GI 13	*csvA*	CS7 fimbria major subunit CsvA precursor	*Escherichia coli*	39
C27R1	VFG0584	GI 13	*yjcC*	putative diguanylate cyclase	*Salmonella enterica* (serovar *typhimurium*) LT2	29
C32R1	VFG1584	GI 14	*orf50*	hypothetical protein	*Escherichia coli* 536	26
C32R1	VFG1584	GI 14	*orf50*	hypothetical protein	*Escherichia coli* 536	26
C32R2	VFG1888	-	*letS*	sensory box histidine kinase	*Legionella pneumophila* Philadelphia 1	39
C32R2	VFG0584	-	*yjcC*	putative diguanylate cyclase	*Salmonella enterica* (serovar typhimurium) LT2	32
C36R3	VFG1092	GI 16	*int3*	integrase, phage family	*Vibrio cholerae* N16961	26
C36R3	VFG1443	GI 16	*ompA*	outer membrane protein A	*Escherichia coli*	38
C36R3	VFG1548	GI 16	*prfC*	PrfC protein	*Escherichia coli* 536	39
C36R3	VFG1547	GI 16	*prfD*	PrfD protein	*Escherichia coli* 536	51
C39R1	VFG0266	-	*hmbR*	hemoglobin receptor	*Neisseria meningitidis* MC58 (serogroup B)	31
C39R1	VFG0266	-	*hmbR*	hemoglobin receptor	*Neisseria meningitidis* MC58 (serogroup B)	36

Table 10. The distribution of 16 different EAh-specific genomic islands in different *A. hydrophila* isolates used in this study.

GI #	Nucleotide positions	Size (kb)	Number of ORFs	Contig ID in ML09-119	%G+C	*Aeromonas hydrophila* isolates	Epidemic-associated GIs
GI 1	8728..22581	13,853	12	8	44.0	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 2	30195..41895	11,700	13	15	57.8	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 3	71487..98770	27,283	28	15	41.3	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 4	263720..284778	21,058	32	15	54.0	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, AL97-91, MN98-04, TN97-08, AL06-01	-
GI 5	76843..95300	18,457	22	18	51.5	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, AL97-91, MN98-04, TN97-08, AL06-01	-
GI 6	95365..107861	12,496	12	18	48.1	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 7	123898..133969	10,071	12	18	44.5	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, TN97-08, AL06-01	-
GI 8	277559..28	11,108	13	18	54.2	ML09-119, ML09-	-

	8667					122, ML09-121, AL09-79, AL10-121, PB10-118, AL97-91, MN98-04	
GI 9	91274..118831	27,557	36	20	51.1	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, TN97-08	-
GI 10	119893..134877	14,984	20	20	57.4	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, TN97-08	-
GI 11	21645..43624	21,979	33	24	42.3	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 12	25686..46472	20,787	19	26	49.9	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 13	1..8099	8,098	12	27	35.2	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 14	51060..60399	9,339	12	32	63.6	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique
GI 15	60579..71065	10,486	13	32	43.6	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118, TN97-08,	-
GI 16	351791..364972	13,182	18	36	56.4	ML09-119, ML09-122, ML09-121, AL09-79, AL10-121, PB10-118	Unique

Table 11. Distribution of five prophages in different *A. hydrophila* isolates used in this study.

A.hydrophila isolates	AH1 (Contig 13)	AH2 (Contig 13)	AH3 (Contig 15)	AH4 (Contig 18)	AH5 (Contig 20)
GA97-22	Present	Absent	Absent	Absent	Absent
AL06-06	Present	Absent (partial present)	Absent	Absent	Absent
AL06-01	Present	Absent	Absent	Absent	Absent
AL97-91	Present	Absent (partial present)	Absent (partial present)	Present (Some ORFs were missing)	Absent
TN97-08	Present	Present (Many of the ORFs are missing)	Absent (partial present)	present	Absent[a]
MN98-94	Present	Absent	Absent (Only one ORF was present)	Present	absent
ML09-119	Present	Present	Present	Present	Present
ML09-121	Present	Present	Present	Present	Present
ML09-122	Present	Present	Present	Present	Present
AL09-79	Present	Present	Present	Present	Present
AL10-121	Present	Present	Present	Present	Present
PB10-118	Present	Present	Present	Present	Present

[a] The reference mapping of TN97-08 against the prophage AH5 found that some reads from TN97-8 matched with this prophage genome but none of them encoded complete ORFs.

Chapter VI

Conclusions

This study identified 11 unique genes of *Edw. ictaluri* strains that are required for phage ΦeiAU and ΦeiDWF infection. Complementation of phage resistant mutants confirmed the role of the outer membrane porin protein LC (OmpLC), porin thermoregulatory protein, EnvY, PsoB and a hypothetical protein in phage ΦeiAU infectivity to *Edw. ictaluri* strains.

The outer membrane porin protein LC (OmpLC) was identified as a receptor on *Edw. ictaluri* strains required for phage binding and their subsequent infection. The sequencing and comparison of the *ompLC* gene from 15 different *Edw. ictaluri* strains demonstrated that the promoter and upstream regulatory regions are identical and differences were clustered primarily on the 3′ of the coding region.

The three-dimensional model of the OmpLC protein structure determined by SWISS-Model predicted that OmpLC protein is a typical porin structure with 16 anti-parallel β strands and 8 extracellular loops. The amino acid sequence variations found within the OmpLCs from different *Edw. ictaluri* strains were clustered on the loop 8 of this protein.

The deletion and site-directed mutagenesis of the loop 8 of the OmpLC protein from *Edw. ictaluri* strains demonstrated that loop 8 is critical for phage infection to *Edw. ictaluri* strains. The complementation of *Edw. ictaluri ompLC* mutant with different variant of *ompLC* demonstrated that OmpLC protein modulates phage susceptibility in *Edw. ictaluri*.

This study isolated six different mutant phages with enhanced lytic activity to previously ΦeiAU and ΦeiDWF-resistant *Edw. ictaluri* strains C91-162, R4383, S89-9, Alg-08-183, Alg-08-192 and Alg-08-221. The comparison of the efficiency of plaquing (EOP) of the mutant phages with that of the wild type phage showed that mutant phages had expanded their host specificity greatly. The complete genomes of the mutant phages ΦeiDWF-183 and ΦC91-162 that are lytic to phage ΦeiAU and ΦeiDWF-resistant *Edw. ictaluri* strains Alg-08-183 and C91-162, respectively, were sequenced that helped to determine the phage determinant for broad host specificity.

The pairwise comparison of the predicted ORFs from mutants and wild type phages showed that there were no deletions or insertions within the genomes of phages ΦeiDWF-183 and ΦC91-162 and a total of 37 amino acids substitutions were found within the proteome of the mutant phages. The changes observed within the proteomes of phage ΦC91-162 and ΦeiDWF-183 were similar, except two independent mutations, though each of the mutant phages was obtained by passaging on two different *Edw. ictaluri* strains. A large number of mutations were found within the phage host specificity protein HspP.

This study determined that HspP protein in phage ΦeiDWF-183 is responsible for its broader host range specificity to *Edw. ictaluri* strains. Using a novel recombineering experiment, this study also confirmed that the C-terminal end of the HspP protein in phage ΦeiDWF-183 is responsible for the broader infectivity to *Edw. ictaluri* strains.

A conjugally transferrable recombinogenic plasmid pMJH46 (accession no. JQ070344) was constructed by introducing the mobilization cassette (mob) to temperature-sensitive pKD46 that contains lambda Red cassette (*bet, exo* and *gam* genes) required for recombineering. The recombinogenic plasmid pMJH46 was successfully introduced into different *Edw. ictaluri* strains and a total of 4 different genes that includes *ompLC, dtrA, ptrA* and *eihA* were deleted efficiently from different *Edw. ictaluri* strains. After deletion of targeted genes from *Edw. ictaluri*, the recombinogenic plasmid was successfully cured from *Edw. ictaluri* mutants. The antibiotic resistance gene from the *Edw. ictaluri* mutants were removed by Flp-mediated excision of the antibiotic resistance gene to generate unmarked mutant with a single FRT scar remaining on the target site.

A total of 12 *A. hydrophila* isolates including six recent epidemic isolates and six reference isolates were sequenced at > 160-fold coverage and genome-wide comparisons were carried out to identify epidemic-associated genomic regions. The comparative genomic analysis revealed that recent epidemic *A. hydrophila* isolates are highly clonal whereas reference isolates are greatly diverse.

Phylogenetic analysis based on *gyrB* gene sequences of representative *A. hydrophila* isolates demonstrated better resolution to separate EAh and RAh isolates and it was found that all EAh isolates clustered as a single clade. The *A. hydrophila* strain ZC1 obtained from a diseased grass carp in China clustered with EAh isolates and this finding provided a solid ground to study the origin of the epidemic *A. hydrophila* isolates.

The genome of epidemic *A. hydrophila* isolates contain 54 unique regions that are missing in all six RAh isolates and *A. hydrophila* ATCC 7966. The epidemic–associated unique

regions totaled 325,813 bp which accounts for 6.5% of the epidemic *A. hydrophila* genome. A total of 307 ORFs are encoded by these unique regions encode prophage elements, pathogenicity islands, metabolic islands, fitness islands and genes of unknown functions and 28 of the genes encoded in these regions were predicted as virulence factors.

A metabolic island (17.5 kb genomic region contains 11 ORFs) that encodes a complete pathway for *myo*-inositol catabolism present in all epidemic *A. hydrophila* isolates sequenced in this study and the functionality of this island was evident by the ability of epidemic *A. hydrophila* isolates to use *myo*-inositol as a sole carbon source. Neither the six RAh strains nor the ATCC 7966 reference proteome was predicted to contain genetic regions involved in *myo*-inositol catabolism. The lack of myo-inositol utilization cluster in the sequenced genome of RAh isolates was supported by their inability to grow in *myo*-inositol as a sole carbon source. The screening for the presence of epidemic-associated genomic regions in the genomes of *A. hydrophila* field isolates and their ability to grow in myo-inositol as a sole carbon source demonstrated that 100% of the isolates showed a strong correlation between the growth on *myo*-inositol and the presence of epidemic-associated genes in *A. hydrophila* isolates.

The pairwise comparison of proteomes showed that the six EAh strains share a very high degree of homology in their proteomes. In contrast, the pairwise proteome comparison between the EAh and RAh strains and among RAh strains including *A. hydrophila* ATCC 7966 revealed that RAh strains are diverse and varied considerably with the proteomes of EAh isolates. The results of this demonstrated that EAh strains are genomically distinct from RAh strains and that there is a highly coherent EAh genome. These results suggest there was significant diversity among RAh strains sampled in this study, especially in contrast to EAh strain genomic homogeneity.

This study identified a total of 16 genomic islands (GIs), ranging from 8 kb to 30 kb, within the genome of epidemic *A. hydrophila* isolates and 9 of the genomic islands were exclusively present within the genome of epidemic *A. hydrophila* isolates. GIs found within the genome of the EAh isolates were predicted to encode pathogenicity factors, fitness factors, proteins involved in myo-inositol utilization pathways and proteins of unknown functions.

The comparison of the O-antigen cluster from EAh and RAh isolates sequenced in this study demonstrated that the EAh isolates share a single type of O-antigen cluster and all together this study identified five unique O-antigen biosynthesis clusters among the 12 sequenced *A. hydrophila* strains. The gene contents and the relative organization of the O-antigen biosynthesis gene cluster from EAh strain showed that the O-antigen biosynthesis gene cluster of this type has not been reported previously. The O-antigen biosynthesis cluster of 12 sequenced *A. hydrophila* isolates are quite different than that of the previously published *A. hydrophila* ATCC 7966, PPD134/91, JCM3980 and AH-3 O-antigen biosynthesis clusters.

This study identified five different prophage regions within the genome of EAh strains and determined their distribution in different *A. hydrophila* isolates. None of the RAh isolates contains all the prophage regions predicted within the genome of EAh isolates. The prophage AH4 with significant homology to the *Escherichia* phage D108 was the only inducible prophage found within the genome of EAh isolates.

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