

Evaluation of Biological Agents for Controlling Enteric Septicemia of Catfish

by

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Abstract

Enteric septicemia of catfish (ESC), caused by the bacterium *Edwardsiella ictaluri*, is considered the most important bacterial disease of cultured channel catfish *Ictalurus punctatus* and is estimated to cost the industry \$40 to \$60 million yearly in economic losses. Control of ESC has been elusive using available treatment agents, and additional options are required for its control. The use of biological control strategies such as lytic bacteriophages and probiotic bacteria (e.g. *Bacillus* spp.) represent two potential alternative options for use to combat ESC.

A new lytic *E. ictaluri*-specific bacteriophage was isolated from a catfish culture pond during an ongoing *E. ictaluri* infection. The bacteriophage (eiPF) showed specificity to *E. ictaluri*. Electron microscopy revealed that eiPF has an icosahedral head approximately 50 nm, and a non rigid tail. Restriction digestion of the nucleic acids of eiPF and of previously isolated eiAU and eiDWF showed similar restriction fragments among the three bacteriophages. Characterization of eiAU, a candidate for bacteriophage therapy, demonstrated that the bacteriophage is stable in various biological and physico-chemical conditions including pond water, and in catfish fingerling tissues. Bacteriophages administered alone by intra-gastric gavage were not detected in blood circulation and internal organs of catfish. However, when administered in conjunction with *E. ictaluri*, bacteriophages were recovered in blood circulation and in trunk kidney samples collected. Serial passaging eiAU did not confer enhanced capacity on the

passaged variant (eiAU P10) to remain in higher numbers in the gastro-intestinal tract of fish compared to its wild-type parent.

The safety of three previously isolated bacteriophages for therapeutic use was confirmed by sequencing and analyzing their genomes. The genomes for bacteriophages eiAU, eiDWF, and eiMSLS reveal considerable conservation of genomic architecture and sequence identity, even with considerable temporal and spatial divergence in their isolation. The genomic analysis of these bacteriophages revealed no sequence homology to known toxin genes, virulence factors, or genes required for lysogeny, supporting a conclusion that these are virulent bacteriophages, and may be safe for use in bacteriophage therapy without the potential for lysogenic conversion of *E. ictaluri* to a more virulent phenotype.

A one-time administration of bacteriophage, and administration of three different bacteriophage types (eiAU, eiDWF, and eiAU P10) for 15 days, demonstrated limited protection against *E. ictaluri* infection. Protection was observed when bacteriophage was administered for 27 consecutive days, starting 3 days pre-challenge. Additionally, protection was also observed when bacteriophage was administered at a high dose (10^{10} PFU/gram of feed), but not when administered at lower dosages (10^7 and 10^4 PFU/gram of feed). During the challenge studies no bacteriophage-resistant *E. ictaluri* isolates were recovered from fish and no adverse effect were grossly observed in fish that received bacteriophage-only administration.

A preliminary study that was undertaken to isolate and select the best *Bacillus* spp. exclusively for use in biological control of ESC resulted in the isolation of one Gram-positive, spore-forming isolate that produced zones of inhibition on three *E.*

ictaluri isolates tested. Analysis of the 16S ribosomal RNA gene sequence indicates that the isolate (AB01) belongs to the genus *Bacillus*; however, further analysis is needed to conclusively identify the species. A challenge experiment demonstrated the capacity of a mixture of twelve *Bacillus* spp. obtained from a large collection (N=160) to provide protection against artificially induced *E. ictaluri* infection in aquaria. Data from that experiment along with data from the challenge study that evaluated the individual capabilities of seven *Bacillus* spp. to provide protection against *E. ictaluri* will be included as part of an ongoing process to select the best performing *Bacillus* spp. for use in biological control of enteric septicemia of catfish.

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CHAPTER I

Introduction and Literature Review

1. Introduction

The commercial catfish industry is the largest aquaculture industry in the United States of America with annual sales to processing plants of approximately \$389 million (USDA, 2009), and representing 46 % of the value of aquaculture production in the USA (Tucker and Hargreaves, 2004). The industry integrates several other businesses such as feed mills, processing plants, restaurants, among others to generate an economic impact of billions of dollars to the four major producers (Mississippi, Arkansas, Alabama and Louisiana) of channel catfish (Tucker and Hargreaves, 2004).

The catfish industry is striving to remain profitable amidst numerous problems that threaten its sustainability and growth. Issues such as escalating fuel and feed prices, low market prices for catfish, and competition from imported fish are some of the culprits that are putting negative pressure on profitability of the industry. Additionally, catfish farmers continue to incur huge losses due to diseases which reduce the margins of profitability on an individual farm basis.

Enteric septicemia of catfish (ESC) is considered the most important bacterial disease of cultured catfish and is estimated to cost the industry up to \$60 million yearly

(Shoemaker et al., 2002; Shoemaker et al., 2009). Control of ESC has been elusive using available treatment agents as is evident by the many operations (52.9 of catfish fingerling operations and 60.6 % of food-fish operations) experiencing losses due to the disease in a given year (Wagner et al., 2003). The long term economic benefits of an available vaccine for use against ESC is still in question, and the use of available antibiotics is limited by cost, regulations, and increasing evidence of bacterial strains resistant to antibiotics used in aquaculture (Adams et al., 1998; Akashi and Aoki, 1986; Alcaide et al., 2005; Clark et al., 1991; DePaola et al., 1995; Karunasagar et al., 1994; Starliper et al., 1993; Stock and Wiedemann, 2001; Waltman and Shotts, 1986; Welch et al., 2008).

Widespread resistance to antibiotics used in human medicine as well as in agriculture raises concerns about the long term dependency on antibiotics to combat bacterial pathogens. Additionally, consumer concerns about food safety and environmental protection has sparked interest in developing alternatives to antibiotics that are safe, efficacious, and environmentally–friendly. Biological control using lytic bacteriophages (or phages) and using antagonistic bacteria such as *Bacillus* spp. possessing inhibitory activity against bacterial pathogens has gained increased interest for use to control bacterial infections in several areas of agriculture, and may be a source for additional agents to combat diseases in aquaculture.

Lytic phages are viruses that infect and kill bacteria and are generally species-specific (Monk et al., 2010), hence, they pose minimal threat to beneficial bacterial flora (Balogh et al., 2010; Greer, 2005) present in the intestinal tract of organisms such as catfish or in their culture pond environment, and more importantly may represent minimal risk to human consumers. Additionally, phages are natural components of the

environment, are relatively easy and inexpensive to propagate (Greer, 2005), and can be administered through feed. Probiotic or beneficial bacteria (i.e. *Bacillus* spp.) may work in one of several different modes of actions to benefit a fish, including the direct antagonism of bacterial pathogens, stimulating the immune system, and by non-specific competitive exclusion (Korsten and De Jager, 1995; Marco et al., 2006). Although, therapy using phages and *Bacillus* spp., as biological control agents, have long been postulated as potential options for combating bacterial infections, the reality is that there are several potential limitations to the success of such therapies. Additionally, there is a lack of understanding of the interactions of all components (i.e. phage-host-pathogen-fish-environment) that may affect the capacity of these biological agents to control disease in a natural disease outbreak scenario, hence the need for laboratory trials to further understand their therapeutic capacities and limitations.

The main goal of this work was to investigate the therapeutic potential of phages to control ESC. This dissertation describes components relating to the isolation, characterization, and improvement of bacteriophages, sequencing and genome analysis of bacteriophages, and evaluation of bacteriophages to control experimental ESC infections. During work with phage we realized the need and importance of isolating and selecting additional biological control agents for use in therapy plans for the control of ESC. Hence, this work also included isolation of *Bacillus* spp. from fish and selection of *Bacillus* spp. for biological control based on protective effects against *E. ictaluri* infections.

Specifically, the objectives of this study were: 1) to isolate and characterize lytic *E. ictaluri*-specific bacteriophages, 2) to evaluate, the *in vitro* and *in vivo* survival of

three *E. ictaluri* phages (eiAU, eiDWF, and eiAUP10), 3) to use a method of ‘serial passaging’ of phage within catfish to develop a phage variant with enhanced capacity to survive conditions in the gastro-intestinal tract of catfish fingerlings, 4) to analyze the genomes of three *E. ictaluri*-specific phages (eiAU, eiDWF, and eiMSLS) to examine their potential for lysogeny, to ensure they do not harbor virulence or toxin genes and to better understand the genetic basis of their host specificity, 5) to evaluate the protective effects of three phages (eiAU, eiDWF, and eiAU P10) against artificially induced *E. ictaluri* infections in channel catfish fingerlings, and 6) to isolate and select *Bacillus* spp. as candidates for biological control of *E. ictaluri* infections in catfish.

2. Literature review

2.1. The catfish industry

Culture of channel catfish *Ictalurus punctatus* (Rafinesque) began expanding on a large scale commercial level in the Southeastern US in the 1960s (Parker, 1989), and experienced a huge growth in the 1980s, particularly in Mississippi (Mott and Brunson, 1997). Presently, the commercial catfish industry is the largest in the US aquaculture sector representing 46 % of its total value (USDA, 2006).

The consumption of fish in the US is projected to increase and the per capita consumption of catfish (0.42 Kg) is the tenth highest of all consumed fish and sea food products (Muhammad and Jones, 2009) suggesting a strong market for catfish.

Nonetheless, the catfish total production acreage has declined during this decade as has the total weight of catfish processed at processing plants (Muhammad and Jones, 2009). Major issues driving this pattern include the increasing cost of catfish farm operation

(escalating feed and fuel prices), competition from fish imports, lower prices for catfish at the processing plants, and a recession in the US economy (Umali-Maceina, 2007). In addition to these external forces, farm profits are hugely impacted by factors such as management capabilities of individual farmers, stocking densities, feed management, and losses due to diseases.

Production and management schemes used for culturing catfish as well as other aquaculture organisms are designed to produce high numbers of market size fish in the least time possible and in the smallest water area/volume possible in an economically sustainable method. In food fish production, catfish fingerlings (10-50 g) are stocked at high stocking densities (8,500 to 10,000 fish/hectare) and fed high feeding rates (114-136 Kg/ha/day) (Chapman, 2000) leading to stressful conditions in culture environments that have high propensity for the outbreak of diseases. Additionally, production systems (multiple-batch) often used for culturing catfish, commonly practice limited to no water exchanges for extended periods of time and grow catfish of multiple year classes simultaneously in the same pond (Engle and Valderrama, 2001). These situations have been postulated as major determinants of diseases in the catfish industry because pathogens such as *E. ictaluri* may survive in pond sediment for an extended period (Plumb and Quinlan, 1986), furthermore; previously infected fish may serve as carriers of disease and infect newly introduced fish (Wagner et al., 2002).

Considering that the catfish industry provides up to 14,000 on-farm jobs paying an estimated \$168.7 million worth of salaries to workers in the states of Mississippi, Alabama, Arkansas, and Louisiana which are the major producers of catfish (USDA, 2006), it is not only essential for the industry to have better technology, but also essential

for the industry to become more efficient at managing losses from diseases as a means of remaining profitable.

2.2. *Enteric septicemia of catfish*

Enteric septicemia of catfish, a highly fatal systemic disease, is considered the most important disease of cultured channel catfish (Klesius, 1992; Noga, 2000). ESC affects catfish of all ages but predominantly affects young-of-the-year fingerlings (Francis-Floyd et al., 1987). The disease was first reported from pond-raised channel catfish in 1976 (Hawke, 1979), and can be found in most channel catfish culture environments (Hawke et al., 1998). ESC is a seasonal disease, with outbreaks commonly occurring during spring and fall when water temperatures range from 20 °C to 28 °C (Hawke et al., 1998). Although the disease is primarily a disease of channel catfish, its causative agent, *Ewardsiella ictaluri*, has been isolated from species such as walking catfish *Clarias batrachus*, European catfish *Silurus glanis*, Chinook salmon *Oncorhynchus tshawytscha*, and rainbow trout *Oncorhynchus mykiss* (Plumb, 1999). Most recently *E. ictaluri* has been confirmed as the etiological agent of bacillary necrosis of pangasius a newly recognized disease of cultured *Pangasius* spp. from the Mekong Delta of Vietnam (Crumlish et al., 2002; Crumlish et al., 2010), and from yellow catfish *P. fulvidraco* farms in China, indicating that the pathogen may have world economic importance.

In the field *E. ictaluri* infected fish may become listless, may swim erratically, and may hang in the water column with their head up and tail down. Fish tend to go off feed once infected, posing major problems for therapy efforts that involve administering

treatment through feed (Blazer et al., 1985). However, this problem may be circumvented by diagnosing the disease in time to administer treatment before fish become anorexic.

Edwardsiella ictaluri, the causative agent of ESC, is a small (0.75 x 1.5-2.5 µm) Gram-negative, facultative anaerobic rod belonging to the Enterobacteriaceae family (Hawke et al., 1998). The bacterium possesses peritrichous flagella, and fimbriae has been observed by electron microscopy (Menanteau-Ledouble, 2009; Newton and Triche, 1993). Biochemically, *E. ictaluri* is negative for cytochrome oxidase, indole, H₂S, citrate, phenyl alanine deaminase, and positive for methyl red, nitrate reductase, lysine decarboxylase, ornithine decarboxylase, and catalase (Hawke et al., 1981a; Waltman et al., 1986). It is also capable of fermenting and oxidizing glucose releasing gases (Plumb, 1999). *Edwardsiella ictaluri* is closely related to *Edwardsiella tarda*, sharing 96 % identity based on 16S-23S rRNA intergenic spacer region (Panangala et al., 2005b). Additionally, it exhibits high homogeneity based on phenotypic (outer membrane protein profiles, antigenic determinants, and biochemical properties) and genetic characteristics (Panangala et al., 2005a).

Pathogenesis studies have shown that *E. ictaluri* can enter through the gastrointestinal tract, the nares (nasal openings), and possibly through the gills (Hawke et al., 1998). Two clinical forms of ESC occur in channel catfish: an acute form resulting in septicemia characterized by hemorrhage and necrosis of several organs, and a chronic form resulting in chronic encephalitis (Johnson, 1989). A latent state of the disease is also presumed, whereby, infected fish become carriers of the pathogen. It is postulated that when *E. ictaluri* enters the fish through the gut the acute form of ESC develops, and

when it enters through the olfactory sac the chronic form of ESC develops (Newton et al., 1989).

Investigation into the pathogenesis of ESC has revealed that *E. ictaluri* entering through the gut can cross the intestinal mucosa, enter the bloodstream, and migrate to the kidneys within 15 minutes (Baldwin and Newton, 1993). The progression and the mechanisms used by the bacteria for invading catfish has been thoroughly investigated, and is believed to be similar to the strategy used by other enterobacteriaceae such as *Salmonella* and *Yersenia* spp. (Baldwin and Newton, 1993). Studies in which catfish received *E. ictaluri* by intra-gastric administration suggest that *E. ictaluri* penetrates the intestinal epithelium of catfish and is uptaken by macrophages present in the underlying epithelium (Booth et al., 2006; Miyazaki and Plumb, 1985; Thune et al., 1993). No histological damage of epithelial cell is observed suggesting that *E. ictaluri* crosses the epithelial lining by using the normal cell transport system (Baldwin and Newton, 1993). This is reinforced by the observation of a fast passage of the bacteria through the epithelial lining suggesting there is not enough time for *E. ictaluri* to synthesize proteins for this function, and hence must be using the normal cell transport mechanism (Thune et al., 1993). Once inside macrophages *E. ictaluri* can survive, replicate and spread systematically through the blood to other tissues (Booth et al., 2006). Studies in which various cultured epithelial cells and freshly harvested intestinal cells of catfish were used as a model of *E. ictaluri* invasion of catfish cells showed that *E. ictaluri* is internalized through epithelial-lined membranes. It seems *E. ictaluri* utilizes surface exposed ligands recognized by host receptors to aid this internalization (Booth et al., 2006). The spread of *E. ictaluri* to the tissues often result in sepsis and death.

Gross signs associated with ESC were first described by Hawke (1979), signs observed in the acute form of ESC include petechial hemorrhages under their heads, around the mouth, and on the ventral or belly region. Multifocal, hemorrhagic cutaneous lesions (~ 2 mm in diameter) that progress to depigmented ulcers also occur. Internally, the liver and other organs exhibit hemorrhages and necrotic foci (Areechon and Plumb, 1983). In some instances, accumulation of ascitic fluid in the body cavity and enlargement of the spleen have been observed (Hawke, 1979; Jarboe et al., 1984). Histological examination reveals a systemic infection of all organs and skeletal muscles, with the most severe changes being diffuse interstitial necrosis of the anterior and posterior kidney, and focal necrosis in the liver and spleen are also generally seen (Blazer et al., 1985). In some cases gill inflammation, exophthalmia, and anemia may be observed

In the chronic state, studies suggest that *E. ictaluri* enters the brain via the olfactory bulb, having colonized the olfactory sac, causing ulceration of the cranial vault (Miyazaki and Plumb, 1985; Morrison and Plumb, 1994). From within the brain the infection then spreads systemically along the olfactory nerves causing a generalized septicemia infection. Along the way it generates granulomatous inflammation. The encephalitis leads to abnormal swimming behaviors such as swimming in tight circles chasing their tails (Hawke et al., 1998). In late stages of chronic infection swelling of the dorsum of the head occurs as the inflammatory process causes the connective tissue to erode (Morrison and Plumb, 1994). This swelling causes an ulcer that exposes the brain resulting in what is known as the 'hole in the head' (Shotts et al., 1986). In most cases death eventually ensues, often due to secondary opportunistic bacteria.

The virulence factors associated with the pathogenesis of *E. ictaluri* are not fully understood (Thune et al., 1993). It is postulated that capsular polysaccharides, hemolysins, lipopolysaccharide, and several enzymes that degrade chondroitin sulfate may be involved (Cooper et al., 1996; Lawrence et al., 2001; Lawrence et al., 2003; Williams and Lawrence, 2005a). It has been suggested that similar to other pathogenic bacteria, *E. ictaluri* may produce a thick, mucous-like capsular polysaccharide, which cloaks antigenic proteins in the bacterial surface helping the bacteria evade host immune response (Lawrence et al., 2003). Membrane associated hemolysins have also been detected in *E. ictaluri*; however, a recent study suggests that although hemolysins may work in conjunction with other virulence factors they are not essential for virulence in catfish (Williams and Lawrence, 2005b). The lipopolysaccharide (LPS) is considered a major virulence factor in *E. ictaluri* (Lawrence et al., 2003). Bacterial strains containing LPS seem to be more resistant to complement-mediated lysis or phagocytosis than are strains which lack OPS (Merino et al., 1996). Additionally, strains that possess CMP-NANA (cytidine 5 monophospho-N-acetyl neuraminic acid) in their LPS are often more resistant to bacterial killing by the alternative complement pathway. Chondroitinase has been associated with virulence of *E. ictaluri*, and is believed to degrade chondroitin, a component of extracellular matrix and of cartilage, suggesting it may mediate development of chronic “hole-in-the-head” lesion (Cooper et al., 1996).

Outbreaks of ESC tend to occur within the temperature range, from 20 to 28°C, making spring and fall the most common periods for outbreaks in regions where channel catfish are normally cultured (Hawke et al., 1998). However, low-level mortality due to ESC has been observed in carrier populations outside of this temperature range. Other

environmental factors such as poor water quality, high stocking density, poor nutrition, handling, and other stressors predispose the host to ESC (Wise et al., 1993).

Edwardsiella ictaluri is endemic but it is considered an obligate pathogen as it requires a proper host for overall survival (Plumb and Quinlan, 1986). The pathogen can remain viable in pond sediment for up to ninety five days at cool temperatures (Hawke et al., 1998; Plumb and Quinlan, 1986) hence, under optimal conditions it can infect catfish of all ages (Francis-Floyd et al., 1987).

Horizontal transmission of ESC may occur through the shedding of bacteria into the aquatic medium by infected fish, and by cannibalism of infected fish (Hawke et al., 1998; Tucker and Robinson, 1990). It is also postulated that birds, and contaminated equipments used at infected ponds, may also play a role in transmitting the disease from pond to pond or farm to farm (Hawke et al., 1998). In multiple batch production systems previously infected fish may serve as carriers of disease and infect newly introduced fish (Wagner et al., 2002).

Over the course of catfish farming history, apart from the use of chemotherapeutants and a vaccine, various other methods to control ESC have been proposed. These methods include the use of immunostimulants, use of genetically selected fish for resistance, use of specific pathogen free fish, and the practice of restricting feed during an ESC outbreak (Bilodeau-Bourgeois et al., 2007; Dunham et al., 2002; Hawke et al., 1998). Of these methods, restricting feeding has been widely practiced at the commercial farm levels; however, the effectiveness of the practice in controlling the disease is unclear (Lim and Klesius, 2003; Wise and Johnson, 1998). A drawback to this approach is that restricting feed may result in smaller fish at the end of

the production cycle which leads to lower prices at marketing. The use of antibiotics and vaccination are the two major options for controlling the disease at the present moment. In the US, few options are available for controlling fish diseases in general. There are only five drugs of which 3 are antibiotics that have been approved by the U.S. Food and Drug Administration (FDA) for use in aquaculture (Benbrook, 2002). In the catfish industry, only Romet[®] (ormethoprim plus sulfadimethoxine) and the recently approved Aquaflor[®] (florfenicol) are approved by the Federal Drug Administration (FDA) to specifically treat ESC. Aquaflor[®]; however, requires veterinary feed directive from a licensed veterinarian prior to its use by catfish farmers. Although this process may help regulate the use of antibiotics it also poses logistic problems and may also delay treatment. The cost of developing and approving antibiotics, the public concerns of its misuse, coupled with the emergence of resistant strains of bacteria from its systematic use, poses problems for the approval process of additional antibiotics by the FDA (Patrie-Hanson and Jerald Ainsworth, 1999b). In 1994 the American Society of Microbiologists (ASM) antibiotic resistance task force reported that aquaculture represents “one of the biggest concerns” with regards to antibiotic use because aquaculture production is growing rapidly, and disease prevention and treatment practices are presently not standardized or regulated (Benbrook, 2002).

In 2001-2002 the live-attenuated vaccine AQUAVAC-ESC[®] was licensed, and marketed by Intervet, Inc., for use against ESC (Shoemaker et al., 2002). Laboratory studies have demonstrated the safety and efficacy of AQUAVAC-ESC[®] for use in reducing mortality in channel catfish fry and fingerlings (Lawrence and Banes, 2005; Shoemaker et al., 2002; 2007; Wise, 2006). Field studies have reported larger

fingerlings, and improved feed conversion, leading to improved return due to the use of the vaccine; however, data specifically showing similar mortality reduction due to the use of the vaccine as seen in laboratory studies are inconclusive in the field studies (Carrias et al., 2008; Intervet Inc., 2003; McNeely, 2006; Shoemaker et al., 2009; Wise, 2006).

2.3. Biological control

There have been many changes and adaptations to the definition of biological control over the years. One of these (Wilson, 1997) simply defines biological control as: “The control of disease with a natural biological process or the product of a natural process”. In aquaculture, bacteriophages (or phages) and bacteria possessing inhibitory activity against other micro-organisms have received increased consideration for use as bio-control agents to control diseases caused by bacterial pathogens. Although vaccines and antibiotics have improved the wellbeing, and has reduced disease related losses in numerous cultured aquatic organisms, they may not be cost-effective for all aquaculture animals, and may not be available for all pathogens and all hosts (e.g. invertebrates) (Pillay and Kutty, 2005). According to the Food and Agriculture Organization of the United Nations (FAO), the indiscriminate use of antibiotics, particularly in countries with insufficient regulations and limited enforcement for controlling antimicrobial agent use, has contributed to antibiotic resistance problems that may pose potential public health hazards (Hernandez, 2005). These problems have led to an interest in using biological control agents to control diseases in aquaculture. However, the realization of biocontrol strategies to control diseases in aquaculture will only be possible if agents used are safe, effective and economically viable. Hence, consideration of biocontrol therapy for

treating diseases in aquaculture should include as the primary criteria: 1) the efficacy of the biological agent in controlling the target disease(s), 2) the safety to the aquatic host, the environment, and to human consumers, 3) the cost and ease in producing large scale quantities of the biocontrol agent, 4) the mode of action, 5) the stability and shelf life capacity, 6) the route and cost of administration, and 7) the regulatory and approval process. Antimicrobial agents can be either broad-spectrum, which can inhibit the growth or kill a wide range of pathogens; or narrow-spectrum, which are more specific for treatment of certain genera or species (Schwarz *et al.*, 2001). Phage (narrow-spectrum antimicrobial) and bacteria (generally broad-spectrum antimicrobial), are primary examples of biological control agents with these contrasting killing spectrum capabilities. Given the increased bacterial resistance to antibiotics and the interest in consumers for ecologically or organically grown food products the use of biocontrol agents may find increasing use in aquaculture.

2.3.1. Biological control using Bacteriophages

Phages are viruses that infect bacteria and are among the most common entities on earth (Ackermann and DuBow, 1987). Studies on their biology, ecology, and genetics has provided better understanding on how they may interact with their host and their environment, in turn providing a bases for better understanding on how they may work in nature or in phage therapy to reduce bacterial populations.

Phage infection of bacterial cells is highly regulated, and the efficiency and timing of infection may be affected by several factors, one of the major ones being the metabolic state of the host (Kutter and Sulakvelidze, 2005). The first step in phage

infection involves the adsorption to bacterial surface receptors (i.e. lipopolysaccharides) using specialized structures such as fibers or spikes (Goodridge, 2004). Many phage types require cofactors such as divalent cations (Ca^{2+} and Mg^{2+}) for attachment to bacterial surfaces (Kutter and Sulakvelidze, 2005). After attachment, phages transfer their DNA into the cytoplasm of the host bacteria, using mechanisms that are often specific for each phage (Goodridge, 2004). In general the phage tail tip poses enzymatic activity capable of penetrating the peptidoglycan layer of bacterial cells (Kutter and Sulakvelidze, 2005).

Once their DNA has been injected into the bacterium's cell, phages may undergo one of two life cycles, lytic or lysogenic. Temperate phages may undergo a lysogenic life cycle in which they incorporate their DNA into the bacterium's chromosome and become a noninfectious prophage (Barksdale and Arden, 1974). In this life cycle, the phage does not shut down the host's protein synthesis machinery and instead inserts itself into the host's chromosome (Barksdale and Arden, 1974). Expression of the phage genes that control phage replication is blocked by repressor proteins, allowing the phage DNA to replicate as part of the bacterium's DNA (Barksdale and Arden, 1974). The DNA of phage may remain integrated on the host's chromosome until adverse conditions results in their emergence from the host cell. In contrast, phages that utilize a lytic cycle protect themselves by circularizing their DNA to avoid host cell restriction enzymes, or by inhibiting host nucleases. Host RNA polymerase recognizes phage promoters present in the phage DNA giving way to the transcription of phage early genes. The early genes are involved in phage DNA synthesis and in shutting host nucleic acid synthesis. This is achieved by inactivating host proteases, blocking restriction enzymes, terminating

various host biosynthesis, and destroying some host proteins. The middle genes are transcribed next, and their products synthesize the new phage DNA. Then the late genes are transcribed producing products that encode components of the phage particle (structural proteins) and proteins necessary for phage lysis. The final stage of the lytic life cycle is lysis of the host cell (Kutter and Sulakvelidze, 2005). Lysis of host cell is well described in tailed phages, which use an endolysin which degrades cell wall peptidoglycan and a holin which permeabilizes the cell membrane (Lehman et al., 2009).

The concentration of viruses present in various aquatic mediums can range from 10^4 viral particles ml^{-1} and 10^8 viral particles ml^{-1} making them the most abundant entities in water environments (Abedon, 2004). A huge portion of these viruses are believed to be bacteriophages (Abedon, 2004; Fuhrman, 1999). It is postulated that viruses, including phages, influence the cycling of nutrients, the quantity and diversity of host community, and the transfer of genetic material between hosts in the aquatic environment (Heldal and Bratbak, 1991). There is a prevailing view that viruses kill approximately 10-40 % of the standing stock of bacteria in marine ecosystems (Suttle, 1994; 2005). It could therefore be hypothesized that phages may also play a role in balancing bacterial levels in other ecosystems such as in aquaculture environments. This assumption becomes more plausible when one considers that phages are commonly isolated from aquaculture environments where nutrients are abundant and microorganisms flourish (Nakai et al., 1999; Park et al., 2000; Vinod et al., 2006; Walakira et al., 2008).

Phages have been isolated from both fish and their culture environment (Walakira et al., 2008), suggesting that these phages are capable of surviving both the aquatic medium and also inside fish. One of the challenges for phage is locating host

bacteria in the environment before succumbing to harsh environmental conditions (Gill and Abedon, 2003). In soil environments, diffusion of phage through the soil medium may be a problem for phage host encounter (Goodridge, 2004) because they cannot easily diffuse through soil matrix. In fish culture, this may not be as problematic in therapy efforts in which phages are added to the water; however, it may pose a problem for any phage therapy efforts in which phage is administered to the fish. In this scenario phage must be able to survive the adverse conditions inside the fish's gastro-intestinal tract, capable of diffusing to infect its host, and cross intestinal barrier to target pathogens in systemic infections.

Prior to using phages as therapeutic agents a thorough sequence analysis of their genomes is important. Although *in vitro* studies help in selecting lytic phages based on their lytic capabilities, sequence data is needed to verify that they are truly lytic and that they do not possess genes required for integration into host DNA (Carlton, 1999; Sillankorva et al., 2010). Additionally, sequence data may provide insight into understanding how phages differ in their capacity to infect their host, as it is clear that varying degrees of infectivity exists among phage types that may kill a common bacterial species (Jensen et al., 1998; Pantucek et al., 1998; Walakira et al., 2008). Apart from answering these types of questions, the growing information on the genome sequences of phages now allows better comparative genomic analysis and comprehensive taxonomy (Rohwer and Edwards, 2002).

A major challenge to comparative genomic analysis and taxonomy of phages is understanding the high incidence of horizontal exchanges with yet the observation of conserved gene organization among many phages (Brüssow and Desiere, 2001; Brüssow

and Hendrix, 2002; Casjens, 2003; Kwan et al., 2005). In light of recent advances in sequencing technology many new complete genome sequences of phages continuously become available to the research community shedding light on their mosaic nature, a product of horizontal gene transfer (Pedulla et al., 2003). At the same time, it is being realized that synteny exists in genes encoding virion proteins among phages whose genome sequence has been completed. There are suggestions that a strong conservation of gene organization implies low loads of horizontal transfers (Hardies et al., 2007). Obligatory lytic phages have been shown to be less prone to horizontal gene transfer and hence to reorganization of their genome than are temperate phages (Chibani-Chennoufi et al., 2004; Petrov et al., 2006). Recently, researchers have used conservation of gene order in conjunction with other comparative methods as a tool to hypothesize functional assignments for genes that have diverged beyond easy recognition (Hardies et al., 2007). It is agreed, however, that virus evolution does not follow a hierarchical system to classify plants and animals (Ackermann, 2006).

Valuable information may be extracted from the genome sequence of organisms. For example, gene cassettes and gene organization may be used to derive information about the lytic capacity of phages (Hendrix et al., 1999). In general, the genomes of lytic phages that have been sequenced typically show two enzymes, an endolysin which degrades cell wall peptidoglycan and a holin which permeabilizes the cell membrane (Bukovska et al., 2006). *Escherichia coli* phage λ exhibits a typical organization of the genes involved in lysis of host cells; which shows the presence of three lysis genes, gene *S* encoding phage holin, gene *R* encoding phage lysin (transglycosylase) and gene *Rz*. Genes *S* and *R* are necessary for lysis, while the function of the protein *Rz* is not fully

understood but it seems to be required for lysis only in a medium containing excess of divalent cations (Vasala, 1998). Likewise, the genomes of lysogenic phages typically contain genes needed for integration into the host cell. These genes includes genes encoding for proteins such as integrase/recombination associated enzymes, repressor proteins, and antirepressor proteins (Oakey et al., 2002).

The use of bacteriophages to control bacterial diseases like most therapy strategies is also faced with difficulties and concerns. However, potential solutions have been proposed for most of these potential problems.

The specificity of phages is a dual-edged sword, on one side providing an advantage in targeting specific bacterial pathogens, while also in some cases requiring testing of the infecting strain for phage sensitivity prior to treatment, and not covering the diversity of pathogenic bacterial strains that may be encountered in aquaculture (Levin and Bull, 2004). Additionally, companies may see it as too risky to invest in phage therapy since their return on their investment may be limited to a narrow range of pathogenic bacteria species. These limitations may be minimized by using rapid methods for bacterial identification, by using ‘multivalent’ phages that can attach to multiple receptors, or by using phage “cocktails” that collectively possess a broader host-range (Levin and Bull, 2004; Sagor et al.).

Bacterial resistance to phages is one of the most significant potential limitations for phage therapy. There are multiple mechanisms by which bacteria may become resistant to phage, including the mutation of single genes resulting in an alteration or loss of a receptor leading to decreased (or absent) phage adsorption, and restriction modification immunity in which bacteria or plasmids they carry encode restriction

endonucleases that can degrade phage genomes (Levin and Bull, 2004). On a positive note, phage-resistant bacteria mutants may be susceptible to phage infection by other phages possessing a similar target range (Sagor et al., 2005). In contrast, resistance development to multiple antibiotics within an antibiotic class and for many bacterial species and not just for the target bacteria may occur (Salyers and Amabile-Cuevas, 1997). However, as opposed to antibiotics phages do have the capacity to co-evolve, counteracting the mutations of bacteria (Carlton, 1999). Additionally, selecting new phages to target phage-resistant bacteria mutants can be accomplished within days or weeks (Sulakvelidze et al., 2001) as opposed to developing new antibiotics for antibiotic resistant bacteria which may take years (Chopra et al., 1997; Silver and Bostian, 1993). It has been proposed that similarly to the multi-drug treatment strategy used against tuberculosis and HIV the use of a phage “cocktail” to target several receptors on bacterial cells can be used to reduce the incidence of bacterial resistance (Leverentz et al., 2003; Levin and Bull, 2004). Additionally, it has been reported that phage-resistant bacterial strains isolated in several studies, (Loc Carrillo et al., 2005; Park et al., 2000) have reduced virulence or are completely avirulent, and in one case it was reported that phage-resistant *Salmonella enterica* isolates resulted in avirulent vaccines that provided efficacious protection from *S. enterica* infections (Capparelli et al., 2009).

Because of the self-replicating nature of phages, phages may only need to be applied once which may result in a cheaper dose application cost compared to chemotherapeutants (Sulakvelidze et al., 2001). While there is potential for only a single administration to treat an infection, for this to occur the target bacterial population should be sufficiently dense, and physiologically and genetically susceptible to phage infection

(Levin and Bull, 2004). Additionally, self-amplification will not occur if bacterial densities are too low for the phage to replicate faster than they are lost, or if the phage does not propagate in the bacteria (Levin and Bull, 2004).

It is well recognized that some phages (lysogenic phages) have the capacity to carry virulence factors or toxic genes (Brussow et al., 2004; McGrath et al., 2004; Mesyanzhinov et al., 2002) which they may transfer to their bacterial host (i.e., lysogenic conversion). This problem may be avoided by selecting virulent phages that lack the capacity for lysogenic conversion, via establishing the phage-host life cycle and sequencing the genome of phages that are candidates for therapy (Carlton, 1999).

Another possible limitation of phage therapy is the rapid elimination of phages by the reticuloendothelial system (Geier et al., 1973; Merrill et al., 1996). If phage does not rapidly find a host in which to multiply they will rapidly be eliminated before they reach their target, resulting in reduced effectiveness of phage therapy efforts, especially in systemic applications. To mediate this problem it may be possible to serially passage phage to select phage variants that are better at evading the reticuloendothelial system (Merrill et al., 1996).

Anti-phage antibodies have been reported in phage therapy trials in humans (Kucharewicz-Krukowska and Slopek, 1987) and animals (Smith et al., 1987b) and is considered a possible challenge to the success of phage therapies. It has been proposed that treating with a higher dose of phage may compensate for the portion of bacteria that may be eliminated by the antibody response (Carlton, 1999). Recent studies showed low immunogenicity of phage in mice (Capparelli et al., 2007) and in ayu fish (Park and Nakai, 2003) suggesting that antibody production may be minimal in some animals. In

catfish, studies have shown that antibodies against *E. ictaluri* are detected 2 weeks post-exposure with *E. ictaluri* (Patrie-Hanson and Jerald Ainsworth, 1999a) hence allowing some time for control of the pathogen prior to any interference by the fish immune system.

Apart from the specific problems that can potentially affect phage therapy there are other problems that are common to both antibiotic and phage therapy. For success, both antibiotics and phages have to be maintained in sufficient concentrations and densities, both agents must be able to reach the sites of infection, and both must have access to bacteria when they are susceptible (Levin and Bull, 2004). Even if all of these practical issues associated with phage therapy are addressed, realization of phage therapy faces other hurdles regarding approval for use by regulatory agencies. It is a general consensus that using phage “cocktails” will make phage therapy more effective; however, to secure regulatory approval in the US, the US Food and Drug Administration (FDA) requires proven efficacy and safety at both individual and collective level, a process that is very expensive and may impede investment. Additionally, the pharmacokinetics of self-replicating phages is different than that of antibiotics and demonstrating an effective dose for regulatory approval purposes may prove complicated. The treatment dose depends on density-dependant thresholds, and the ability to predict those thresholds may be difficult but are necessary for phage therapy to be practical (Payne and Jansen, 2000). It has been proposed that instead of having to proof efficacy and safety for phage cocktails, the broadening of a single phage type that can infect the majority or all of its targeting pathogenic strains may be an effective strategy (Thiel, 2004).

Phage therapy has garnered interest because of the need for alternative therapies, and due to some of the unique attributes of phage that could provide a comparative advantage over chemotherapy. Phage therapy has been shown to be more effective than antibiotics in controlling deep tissue infections, and also useful in infections where bacteria are resistant to available antibiotics (Kochetkova et al., 1989; Meladze et al., 1982; Sakandelidze, 1991). Additionally, their specificity, their capacity to evolve to counter act buildup of resistance, their self-replicating nature, their abundance and natural presence in nature are qualities that make phage therapy a viable option for therapeutic use (Sulakvelidze et al., 2001).

Phages were independently discovered by Felix d'Herelle and Frederick Twort in 1917 and 1915, respectively (Carlton, 1999). Immediately after discovery their potential for controlling bacterial infections was recognized, and between 1920 to 1940 d'Herelle performed work using phages to treat cholera and other enteric diseases (Monk et al., 2010). After the discovery of penicillin in 1928 and due to the inconsistent therapeutic results in early trials, interest in the use of phages as therapeutic agents to control bacterial infections was lost in the Western Hemisphere; however, countries in the former Soviet bloc continued clinical use of bacteriophages (Kutter, 2005; Levin and Bull, 2004; Monk et al., 2010).

In areas of agriculture other than aquaculture, phage therapy has been evaluated in both animals and plants. In plants, most notable are a series of promising studies (Balogh et al., 2008; Balogh et al., 2003; Civerolo and Kiel, 1969; Flaherty et al., 2000; Flaherty et al., 2001; Saccardi et al., 1993) evaluating the use of phages to control *Xanthomonas* spp. causing bacterial spot or bacterial blight in tomatoes, peach, geranium,

and citrus. The positive results obtained in some of these studies led to the development and commercialization of the phage based product AgriPhage™ <http://www.phage.com> in the USA (Monk et al., 2010). Studies on the use of AgriPhage™ have reported variable results, reporting that foliar application of the commercial product reduced severity of bacterial canker of greenhouse tomatoes on two of three years studied (Ingram, 2009). Other important studies reporting promising results include phage therapy for the control of bacterial blotch of mushroom caused by *Pseudomonas* spp. (Munsch and Olivier, 1995), fire blight, bacterial soft rot caused by *Erwinia* spp. (Ravensdale et al., 2007), bacterial wilt of tobacco caused by *Ralstonia solanacearum* (Tanaka et al., 1990), and potato scab caused by *Streptomyces* spp. (McKenna et al., 2001).

Several promising results have been reported on the use of phages to provide protection against bacterial infections to farm animals used for human consumption. Some examples of successful result of phage therapy include control of diarrhea in calves caused by *E. coli* (Smith et al., 1987a), septicemia and meningitis-like infection in chickens and calves caused by *E. coli* (Barrow et al., 1998), *Salmonella typhimurium* infection in chicken (Berchieri, 1991a), and colibacillosis in poultry caused by *E. coli* (Huff et al., 2004; 2005).

In the area of food processing, phage use is more advanced. P100 used in ready-to-eat food products to kill *Listeria monocytogenes* the causative agent of Listeriosis an often fatal food poisoning condition was extensively evaluated (Carlton et al., 2005), leading to the development and commercialization of LISTEX™ P100 <http://www.ebifoodsafety.com>. LISTEX™ P100 has been approved by the USDA for

Listeria monocytogenes control on both raw and ready-to-eat food products. In fish products LISTEX™ P100 has proved effective in reducing *Listeria monocytogenes* in catfish fillet (Soni et al., 2010). Additionally, three other products, BioTector for use against *Salmonella* in poultry <http://www.cj.co.kr/>, EcoShield™ for targeting *E. coli* 0157:H7 in food and food processing facilities <http://www.intralytix.com>, and ListShield™ for use against *Listeria monocytogenes* contamination in foods and food processing facilities <http://www.intralytix.com> are commercially available.

It is important to recognize that there are also numerous reports on the failure of phage therapy in agriculture, failure has been reported in the use of phages to control beef spoilage caused by *Pseudomonas* spp. (Greer and Dilts, 1990), in reducing nalidixic acid-resistant *Salmonella enteric serovar enteritidis* strain in the ceaca of young chicken (Sklar and Joerger, 2001), in reducing *Yersenia enterocolitica* 0:3 strain that cause infections in pigs (evaluated in mice model) (Skurnik and Strauch, 2006), and others.

In aquaculture, studies on phage therapy include a study of the control of *Edwardsiella tarda* (Wu and Chao, 1982), *Pseudomonas plecoglossicida* (Park and Nakai, 2003; Park et al., 2000), *Vibrio harveyi* (Karunasagar et al., 2007; Karunasagar et al., 2005; Shivu et al., 2007; Vinod et al., 2006), *Streptococcus iniae* (Matuoka et al., 2007), and *Aeromonas salmonicida* (Imbeault et al., 2006; Verner-Jeffreys et al., 2007). Additionally, phages have been isolated against *Flavobacterium psychrophilum* (Kim et al., 2010; Stenholm et al., 2008) and a brief report was published on their therapeutic potential to protect ayu from *F. psychrophilum* infection (Nakai et al., 2010). These studies are reviewed in detail next.

The first reported investigation of phage as biocontrol agents in aquaculture were phages isolated against *E. tarda* and tested for control of edwardsiellosis in loaches *Misgurnus anguillicaudatus* (Wu and Chao, 1982). The effect of phage-infected *E. tarda* on loach mortality was investigated. When loaches were exposed to water containing phage-infected *E. tarda* two minutes post-mixing, no difference in mortality (100%) was observed after 48 h compared to a control group in which loaches were inoculated with bacteria only. Loaches that were exposed to water after two hours post-mixing resulted in 5 % survival for four days, and loaches that were exposed after 8 h post-mixing resulted in 90 % survival.

Lytic phages specific to *Lactococcus garvieae* were isolated in Japan from Yellow tail *Seriola quinqueradiata* culture environments (Park et al., 1997; Park et al., 1998). The capacity of a *L. garvieae* phage (PLgY-16) to control experimentally induced *L. garvieae* infection in yellowtail was evaluated (Nakai et al., 1999). A single administration of phage by i.p. injection resulted in higher survival of fish groups receiving phage (90%) compared to the control group that did not receive phage (45%). The i.p administration of phage at different times (0, 1, and 24 hours) post-bacterial challenge (also by i.p. administration) resulted in higher survival rates for fish that received phage treatment at the earlier time. Survival percentages over ten days was 100 %, 80 %, 50 %, and 10 %, for fish groups that received phage at 0, 1, 24 h post-challenge, and 10 % for the control group receiving no phage, respectively. A delay in mortality was observed in fish groups that received phage compared to control groups that did not receive phage. All fish groups receiving phage had statistically significantly higher survival than the control group. In another experiment, fish that received phage through

oral administration of phage-impregnated feed and were challenged 30 minutes later by anal intubation had survival rates of 90 %, 80 %, and 35 % for fish groups that received phage, phage and *L. garvieae* together, and no phage control, respectively.

Isolation of lytic *Pseudomonas plecoglossicida* phages PPpW-3 (Myoviridae) and PPpW-4 (Podoviridae) from diseased ayu *Plecoglossus altivelis* and from rearing pond water was reported (Park *et al.*, 2000). There are two published reports regarding the evaluation of phages PPW-3 and PPpW-4 as therapeutic agents against *P. plecoglossicida* infection. In the first study (Park *et al.*, 2000), fish that received oral administration of phage-impregnated feed 15 min post-challenge with oral administration of bacteria-impregnated feed resulted in significantly lower mortality (23%) compared to the control (65%) that did not receive phage treatment. In another experiment where smaller fish were used, mortality from fish that were challenged by oral administration of bacteria-impregnated feed and received phage 1 h post-challenge resulted in 78 % lower mortality than its control, and fish that received phage 24 h post-challenge resulted in 68 % lower mortality than control fish. Additionally, in both experiments there was a delay in mortality in the groups that received phage. In the second study, the therapeutic effects of these phages were evaluated for both oral and water borne infection models, as well as in the field in a commercial pond (Park and Nakai, 2003). When fish groups received a single oral administration of phage-impregnated feed immediately after challenge with oral administration of *P. plecoglossicida* also through feed, mortalities of 93%, 53%, 40%, and 20%, were observed for fish groups that did not receive phage (control), that received phage PPpW-3, that received phage PPpW-4, and that received a phage mixture (PPpW-3 and PPpW-4), respectively. In a water borne infection model, fish that were

injected intramuscularly with *P. plecoglossicida* served as a source of infection for groups that received phage-impregnated feed at 24 h and 72 h post-injection. In two trials, mortality in the treated group was 63% and 73% lower than in the controls that did not receive phages. In the field trial, three administrations of feed containing a phage mixture (PPpW-3 and PPpW-4) were given through feed to fish in a pond with an ongoing *P. plecoglossicida* infection. A reduction in bacterial levels and an increase in phage levels were observed in dead fish, live fish, and pond water after phage administration. The authors use this observation in conjunction with the observation that mortality in fish consistently decreased by 5% daily after phage administration to suggest protective effects. However, because no control pond was available it is difficult to determine if the 5% drop in daily mortality was a consequence of phage administration. Further complicating the results was the observation of mixed infection with *F. psychrophilum* during the trial.

Three studies have been reported on the use of bacteriophages to control *Vibrio harveyi* infections in shrimp *Penaeus monodon*. The first reported use of phage to control *V. harveyi* infections used phages isolated from water from shrimp hatcheries or shrimp grow-out ponds (Karunasagar *et al.*, 2005). In a laboratory microcosm two applications of phage at 24 h intervals was able to reduce *V. harveyi* levels by 3 logs and resulted in 80 % shrimp survival compared to 10% survival in the control. In a 'field trial' phages were tested for biocontrol at a hatchery experiencing mortality due to *V. harveyi*. A reduction of *V. harveyi* counts from 10^6 CFU/ml to 10^3 CFU/ml was observed by 48 h and *V. harveyi* was undetectable after 72 h. The survival of larvae was 78 % in the tank that received phage but no survival is reported on the control tank although one

was included. In the second study, a *V. harveyi*-specific phage belonging to the Siphoviridae family of phages was isolated from shrimp farm water in India and was evaluated for its potential to reduce mortality due to *V. harveyi* (Vinod *et al.*, 2006). Similar to the first study, the capacity of phage to reduce *V. harveyi* in a water sample demonstrated that two administrations of phage, at time 0 and 24 h post-bacterial inoculation, resulted in a three log reduction in *V. harveyi* counts (10^6 CFU/ml to 10^3 CFU/ml) and 80% shrimp survival, compared to 25% shrimp survival in the control which also had a one log increase in *V. harveyi* counts. In a hatchery trial, triplicate tanks each containing 500 L of sea water and stocked with 35,000 nauplii of *P. monodon* were used to evaluate the impact of phage addition on shrimp survival. Daily phage administration for 17 days resulted in 86% larvae survival and no *V. harveyi* counts were detected during the trial, compared to an antibiotic treatment (kanamycin at 10 ppm and oxytetracycline at 5 ppm) which resulted in 40% larvae survival and *V. harveyi* counts of 10^5 CFU/ml, and a control treatment that received neither phage or antibiotics and resulted in larvae survival of 17% with *V. harveyi* counts of 10^6 CFU/ml at the end of the study. In the third study, four additional phages lytic to *V. harveyi* were isolated from oyster tissue and from hatchery water and tested for their therapeutic potential in shrimp (Karunasagar *et al.*, 2007). In a hatchery exhibiting mortality due to *V. harveyi*, two phage types were inoculated for four consecutive days into duplicate 10 ton larval tanks each containing 0.5 million *P. monodon* shrimp post-larvae, and another two tanks received antibiotic (oxytetracycline at 5 mg/L and kanamycin at 10 mg/L). The shrimp that received phage had an 87% mean percent survival, whereas shrimp that received antibiotics had a 67% mean percent survival. However, in the hatchery trial only two

replicate tanks were used for each treatment and a control tank receiving no treatment was not included, precluding any statistical analysis of these results.

A bacteriophage (HER 110) also known as bacteriophage 65, which was isolated in the La Petite Mouge River in France in 1971 (Popoff, 1971), was evaluated for its biocontrol potential in brook trout yearlings (Imbeault et al., 2006). A challenge with an *Aeromonas salmonicida* culture (10^8 CFU/ml) was added to aquaria (n=6) and the remaining aquaria (n=6) received sterile culture media. A high titer of phage (10^9 - 10^{10} PFU/ml) was added to some aquaria, and the fish receiving phage treatment (or no bacteria) survived and initially showed no signs of infection. Fish that were exposed to bacteria and did not receive phage rapidly became sick, and by day 45 all were dead. In contrast, the group that received phage remained in good health for a longer period and it was not until day 35 that they were seriously affected by furunculosis, and only 10% of the fish died at the end of the study. In a second study, three previously isolated *A. salmonicida* lytic phages (Rodgers et al., 1981) were evaluated for their capacity to control furunculosis in Atlantic salmon (*Salmo salar* L.) caused by *A. salmonicida* subsp. *salmonicida* (Verner-Jeffreys et al., 2007). This study included two experiments that evaluated the efficacy of phage to control furunculosis. In one experiment, when a combination of three phages (O, R, and B) were administered through i.p. injection to Atlantic salmon at two different time points post-challenge (0 h, 24 h) with *A. salmonicida* (also by i.p. injection) a delay in onset of mortality and a delay in the time to reach 60% mortality was observed in the group that received phage at time zero but not at 24 h post-challenge. However, at the end of the study no significant difference in mortality was observed among the three treatments. In another study a cohabitation

method in which infected fish were used as a source of infection was used to evaluate the efficacy of a daily bath, daily oral administration, or single injection of phages (O, R, and B) on the control of furunculosis. In addition, administration of oxolinic acid for 10 days was also included as a treatment. Administration of phage and oxolinic acid was done four days post-injection of the challenge source fish in the header tank. No protection was provided by the control and the two treatments that received phage, while protection was provided by the group that received oxolinic acid treatment.

Streptococcus iniae lytic bacteriophages were evaluated for their potential to control hemolytic streptococcosis in Japanese flounder *Paralichthys olivaceus* (Matsuoka et al., 2007). The study included four trials in which fish were were injected i.p. with a mixture of phages one hour post injection i.p. with *S. iniae*. In all four trial, the administration of phages resulted in lower mortality compared to controls that did not receive phage.

The isolation of lytic *F. psychrophilum*-specific phages from Danish rainbow trout farms was reported in 2008 (Stenholm et al., 2008). The only report on the use of phages to control *F. psychrophilum* infection was reported in a review on the use of phages in aquaculture (Nakai et al., 2010). They reported that in both the injection and immersion challenge the phage-treated group had significantly lower mortality compared to the control.

In addition to their use as therapeutic agents to control bacterial infections and to their molecular biology application, phages are used in the food industry as antimicrobials to control food poisoning agents, and biopreservatives, and as possible diagnostic tools for detecting bacteria (Atterbury et al., 2003; Carlton et al., 2005; Greer,

2005; Mole and Maskell, 2001). A relatively new area of bacteriophage therapy that has recently gained interest is the use of lysin or endolysin which are enzymes produced by bacteriophages to break down peptidoglycan in bacterial cell walls. Extensive work has been done in this area and successful trials have been reported on the use of lytic enzymes to control bacterial infections such as *Streptococcus pyogenes* (Fischetti et al., 2006; Nelson et al., 2001).

2.3.2. *Biological control using Bacillus spp.*

Antagonistic bacteria, such as those belonging to the genus *Bacillus*, have gained increased consideration for use in biological control of diseases in aquaculture (Newaj Fyzul et al., 2007; Queiroz and Boyd, 1998; Rengpipat et al., 1998). The most important advantage of bacterial biocontrol agents over antibiotics is that multiple mechanisms are potentially involved in the biocontrol process, making it difficult for the pathogens to evolve all of the necessary resistant genes together (Moriarty, 1998). For example, the ability of some bacteria to adhere to intestinal mucus may block the intestinal infection route common to many pathogens (Evelyn, 1996), and some bacteria may stimulate the innate immunity of fish (Brunt and Austin, 2005).

In many cases, *in vitro* antagonism against target pathogens has been used as the primary criterion for screening of candidate antagonistic bacteria (Jack et al., 1996; Robertson et al., 2000). Antagonism may be mediated not only by antibiotics, but also by many other inhibitory substances, for example organic acids, hydrogen peroxide (Gatesoupe, 1999; Ring and Gatesoupe, 1998), and siderophores (Gram and Melchiorson, 1996). The inhibition due to such compounds is highly dependent on the experimental

conditions, which are potentially different *in vitro* and *in vivo*. Therefore, the expression of antagonism *in vitro* is not a sufficient criterion to select candidate biocontrol agents (Riquelme *et al.*, 1997), nor is sufficient the absence of antagonism to rule the strains out (Rico-Mora *et al.*, 1998). Other potentially beneficial properties should also be considered during screening of candidate bacteria.

To date, antagonistic bacteria have shown potential in controlling various infectious diseases in aquaculture, including furunculosis in rainbow trout and Atlantic salmon caused by *A. salmonicida* (Irianto and Austin, 2002; Nikoskelainen *et al.*, 2001; Smith and Davey, 1993), saprolegniosis in eel *Anguilla australis* Richardson caused by *Saprolegnia parasitica* (Lategan *et al.*, 2004), edwardsiellosis in cultured European eel, *Anguilla anguilla* L. caused by *Edwardsiella tarda* (Chang, 2002), lactococcosis and streptococcosis in rainbow trout caused by *Lactococcus garvieae* and *Streptococcus iniae* respectively (Brunt and Austin, 2005) and vibriosis in rainbow trout, turbot larvae, prawn and Atlantic cod fry (Gatesoupe, 1994; 1997; Gildberg and Mikkelsen, 1998; Gram *et al.*, 1999; Moriarty, 1998). Most antagonistic bacteria that have been evaluated for use in the control of diseases are strains of *Pseudomonas* spp., *Alteromonas* spp., *Vibrio* spp., or lactic acid bacteria, in some cases, strains of *Bacillus* spp., *Enterococcus* spp, have also been used (Chang, 2002; Moriarty, 1998; Rengpipat *et al.*, 1998).

The antimicrobial capabilities of *Bacillus* spp., along with their capacity to form spores makes them good candidates for use as therapy agents because spores are resistant to environmental conditions and possess long lasting shelf life in storage (Wang *et al.*, 2008). *Bacillus* spp. has been evaluated for their capacity to reduce mortalities due to several diseases, with variable results. These studies are discussed in detail next.

Bacillus strain IP5832 spores were introduced into the culture medium of rotifers, which were fed to turbot larvae (Gatesoupe, 1991). Survival of turbot larvae fed with the spore-fed rotifers was significantly higher than that of the control group (31 and 10%, respectively) ten days after challenge with an opportunistic *Vibrio* sp. pathogen.

European eels *Anguilla anguilla* L. were fed feed amended with *Bacillus toyoi* for two weeks and then challenged with *E. tarda* 981210L1 by anal injection. Two weeks after challenge, no significant difference was observed between the survival rates of the *B. toyoi* and control groups.

Queiroz applied a commercial product Biostart containing various *Bacillus* spp. strains to channel catfish pond three times a week and demonstrated that survival and net production of fish treated with *Bacillus* spp. was significantly greater than the control (Queiroz and Boyd, 1998). However, during the feeding regime, some fish in all ponds were infected by proliferative gill disease and enteric septicemia of catfish. Therefore, the higher survival of fish in ponds treated with *Bacillus* spp. may or may not suggest the possible protective effect against ESC specifically.

The use of *Bacillus* strain S11 for prevention of disease due to *Vibrio harveyi* when administered in enriched *Artemia* to larvae of *Penaeus monodon* in laboratory aquaria was investigated (Rengpipat *et al.*, 1998; Rengpipat *et al.*, 2003). The *P. monodon* larvae fed the *Bacillus*-fortified *Artemia* had significantly shorter development times and fewer disease problems than did larvae reared without the *Bacillus* strain. After being fed for 100 days with the *Bacillus* strain S11-supplemented feed, *P. monodon* were challenged with *V. harveyi* by immersion. Ten days later all of the groups treated

with *Bacillus* strain S11 showed 100% survival whereas the control group had only 26% survival.

The value of adding selected strains of *Bacillus* to control disease due to *Vibrio* by comparing farms in Indonesia using the same water sources, which contain luminous *Vibrio* strains was evaluated (Moriarty, 1998). The farms that did not use the *Bacillus* cultures experienced almost complete failure in all ponds, with luminescent *Vibrio* disease killing the shrimp before 80 days of culture was reached, whereas the addition of several *Bacillus* cultures in penaeid culture ponds allowed the culture of the shrimps for over 160 days without significant mortality.

In aquaculture farmed fish, the skin, gill, and intestinal microbiota can all contribute to disease prevention, with complex interactions occurring between the host, its associated microorganisms and the surrounding environment that can contribute to biocontrol efficacy. Ultimately, success will depend on the ability of these bacteria to establish within the host environment and to express inhibitory activity during the interactions with their hosts and the target pathogens (Teplitski *et al.*, 2009). The colonization potential is another important factor that may affect therapy success of antagonistic bacteria. The process of colonization is characterized by attraction of bacteria to the mucosal surface, followed by association within the mucous gel or attachment to epithelial cells (Balcázar *et al.*, 2006). Adhesion and colonization of the mucosal surfaces are possible protective mechanisms against pathogens through competition for binding sites and nutrients (Westerdahl *et al.*, 1991), or immune modulation (Salminen *et al.*, 1998). In some cases, the bacteria do not truly colonize the

gastrointestinal tract but rather achieve a sustained transient state (Fuller, 1992; Irianto and Austin, 2002).

CHAPTER II

Isolation, characterization and *in vivo* serial passaging of bacteriophage specific to the catfish pathogen *Edwardsiella ictaluri*

I. Abstract

A new lytic *Edwardsiella ictaluri*-specific bacteriophage was isolated from pond water that was collected at a commercial catfish farm in western Alabama during an ongoing *Edwardsiella ictaluri* infection. The phage, given the name eiPF, showed specificity to *E. ictaluri* and did not form plaques on other fish bacterial pathogens including the closely related *Edwardsiella tarda*. Electron microscopy revealed that eiPF has an icosahedral head approximately 50 nm, and a non rigid tail. Restriction digestion of the nucleic acids of eiPF and of previously isolated eiAU and eiDWF revealed highly similar restriction fragments among the three phages.

In pond water, phage eiAU which is a candidate for phage therapy was capable of reducing *E. ictaluri* titers in both soft (34 ppm as CaCO₃) water and in moderately hard water (137 ppm as CaCO₃). In an *in vivo* analysis on fate and persistence, the administration of phage to catfish fingerlings by intra-gastric gavage resulted in no recovery of phage in blood or internal organs; however, when phage and *E. ictaluri* were administered simultaneously, phages were recovered in blood and in trunk kidney

samples. Phages administered by intra-gastric gavage were recovered from stomach plus intestine samples up to 48h post-administration at titers 1000x lower than its initial concentration.

A method of serial passaging of phage eiAU did not confer enhanced capacity on the passaged variant (eiAU P10) to remain in higher numbers in the gastro-intestinal tract of fish compared to its parent phage.

2. Introduction

The alarming rate at which important human and agricultural bacterial pathogens are becoming resistant to existing chemotherapeutic agents warrants an examination of the use of biological control agents, including the use of bacteriophage (phage) for therapy as an option to control bacterial infections. In aquaculture, use of phages as biological agents to control diseases is undergoing a renewed interest (Imbeault et al., 2006; Nakai et al., 1999; Park and Nakai, 2003; Park et al., 2000; Verner-Jeffreys et al., 2007; Vinod et al., 2006). Although phage therapy has over ninety years of history, particularly in the former Soviet Union, in general, the amount of data on the efficacy and safety of phage therapy in humans, animals, and plants, are limited (Projan, 2004). This is attributed in part due to the lack of uncontrolled early experiments and the lack of understanding of the biology, ecology, and genetics of the phages (Housby and Mann, 2009; Sulakvelidze and Morris, 2001). With new technology, particularly in molecular biology came a better understanding of these potential problems and with it better methodologies for understanding and possibly solving these problems. Detailed explanation of the potential problems associated with phage therapy and how to

potentially mediate them are discussed in various review articles (Carlton, 1999; Matsuzaki et al., 2005; Morrison and Rainnie, 2004; Skurnik and Strauch, 2006).

The specificity of bacteriophages can be both beneficial and detrimental in terms of their therapeutic use. For example, their specificity allows them to be selective enough to target single bacterial species or even strains within a given species (Gill and Abedon, 2003) while posing minimal risk to beneficial bacterial flora (Balogh et al., 2010; Greer, 2005) present in the intestinal tract of phage-therapy targeted organisms and in their culture environments. Their narrow host-range, however, may pose limitations for phage therapy purposes because it requires testing of the infecting strain for phage sensitivity prior to treatment (Levin and Bull, 2004). Additionally, the specificity of phages along with bacterial resistance to phage itself, may render a specific phage type being used in a phage therapy ineffective (Levin and Bull, 2004). This limitation may possibly be minimized by using ‘multivalent’ phages that can attach to multiple receptors or by using phage cocktails that collectively possess broad host-ranges (Levin and Bull, 2004; Sagor et al., 2005). However, this process may require the isolation of several phage types or the isolation of a single phage type that meets the requirements above.

The success of phage therapy for the control of diseases in fish such as enteric septicemia of catfish caused by *Edwardsiella ictaluri* will depend in part on an understanding of phage biology and on the interactions of phages with host *E. ictaluri* within the context of fish and its culture environment. This requires studies on the various components of this complex model, including studies on the dynamics of phage-*E. ictaluri* relationship in pond water, capacity of phage to survive inside catfish, and the fate and persistence of phage inside catfish. Additionally, upon identifying phages for

use in therapy for the control of bacterial diseases, a convenient method is required for long-term storage of these phages without appreciable loss in titers (Keogh and Pettingill, 1966).

The rapid elimination of phages by the organism targeted for phage therapy may also pose problems for phage therapy. If phages do not rapidly find a host, they may be degraded by gastric pH or eliminated by the reticuloendothelial system (Geier et al., 1973; Merrill et al., 1996) before they reach their target, resulting in reduced effectiveness of phage therapy efforts, especially in systemic applications. To overcome this problem a method to serial passage to select phage variants that are better at evading the reticuloendothelial system has been proposed (Merrill et al., 1996).

In response to the problems associated with phage therapy mentioned above, the following were the objectives of this study: 1) to isolate and rapidly differentiate new variants from previously isolated *E. ictaluri* phages using electron microscopy and nucleic acid restriction fragment analysis, 2) to continue physical characterization of previously isolated phages which are candidates for phage therapy, and 3) to conduct serial passaging of the previously isolated phage eiAU within catfish. This study will provide insight on the readiness of isolating *E. ictaluri* phages, on the stability of these phages in physico-chemical and biological conditions, and on the potential of improving characteristics of available candidates for phage therapy.

3. Materials and methods

3.1. Isolation and characterization of new E. ictaluri phages

3.1.1. Bacteria and media

Sixteen bacterial isolates were obtained from the Southern Cooperative Fish Disease laboratory (Department of Fisheries and Allied Aquacultures), College of Veterinary Medicine Department of Pathobiology, Auburn University, and from the American Type Culture Collection (ATCC, Manassas, VA, USA) (Table 1).

Edwardsiella ictaluri isolates obtained from the Southern Cooperative Fish Disease Laboratory were isolated from disease cases submitted from commercial farms in various geographical locations. *Edwardsiella ictaluri* strains C 91-162 and R-4383 which have shown lower efficiency of plaquing towards previously isolated *E. ictaluri* phages eiAU and eiDWF (Walakira et al., 2008) and strain 219, a strain used to previously isolate *E. ictaluri* phages (Walakira et al., 2008) were used for initial isolating and screening of phage isolates. The remaining isolates were used to determine the host range of the phages.

3.1.2. Isolation of bacteriophages specific to Edwardsiella ictaluri

In the fall of 2008, water samples (~ 5 L) were collected from commercial channel catfish ponds located in Central West Alabama, where active ESC outbursts were occurring or had recently occurred. Water samples were collected from various locations and strata within a pond. Algal cells and other debris from water samples were removed by centrifugation at 3,600 x g for 25 min. A stirred cell apparatus powered by nitrogen gas pressure was used to filter and concentrate water samples (2 L) over two super imposed layers (30 kDa and 5 kDa) PVDF (low protein binding) membrane (Millipore, Billerica, MA, USA). Concentrated water samples (5 ml) were filter-purified through 0.22 µm PVDF membrane.

Filtered water samples were used to enrich log-phase *E. ictaluri* cultures according to methods previously described (O'Flynn et al., 2004). Briefly, filtered water sample concentrates (1.5 ml) were added to 30 ml log-phase *E. ictaluri* (5×10^8 CFU/ml) and incubated overnight at 30 °C while shaking at 150 rpm. Overnight enriched cultures were treated with one percent chloroform (Fisher Scientific, Sair Lawn, NJ, USA) and centrifuged at 3,600 x g for 10 min at 4 °C. The supernatant was concentrated down to 250 µl using 5 kDa filters while centrifuging at 3,600 x g. The presence of lytic phages was tested by spotting 10 µl of filtrate on a lawn of log-phase *E. ictaluri* and incubating at 30 °C overnight.

Plaques obtained were purified using the soft agar overlay method (Adams, 1959). Briefly, a single plaque was removed from *E. ictaluri* lawns and used to inoculate 5 ml log-phase *E. ictaluri* broth cultures, and then incubated at 30 °C with shaking (150 rpm) for 8 h. This process was repeated three times. The triply purified phages were then stored in SM buffer [100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCL (pH 7.5)], and 0.002 % (w/v) gelatin at 4 °C with the addition of 7 % dimethyl sulfer oxide (DMSO) at -80 °C.

3.1.3. Host range determination

The host range of both phages was assessed on a range of Gram-negative bacteria. Susceptibility of these bacteria to phage lysis was tested using the drop-on-lawn technique (Zimmer et al., 2002). Briefly, two hundred microliters of each bacterial isolate tested was mixed with molten soft-agar and poured onto a BHI agar plate. After a

10-fold dilution of the phage stock, 10 µl of dilution 10^{-5} were spotted on the lawn of bacteria and then incubated overnight at 30°C to observe the lytic activity.

3.1.4. Electron microscopy

Five microliters of purified phage (10^{10} PFU ml⁻¹) were applied to 300 mesh formvar and carbon-coated copper grids (Electron Microscopy Services, Hatfield, PA, USA). Excess liquid was removed after 10 min and samples were negatively stained with 2 % phosphotungstic acid for 2 min. Grids were allowed to dry for 5 min before being examined at various magnifications using a Philips 301 transmission electron microscope (FEI Co., Hillsboro, OR, USA).

3.1.5. Isolation and restriction of bacteriophage nucleic acid

Phage was propagated on *E. ictaluri* strain 219 using a standard soft agar overlay method (Adams, 1959). The solution (100 ml) containing phage particles was treated for 10 min with 1% (v/v) chloroform to lyse bacterial cells, subjected to centrifugation at 3,600 x g for 25 min, and then filtered through a 0.22 µm filter to remove cell debris. Phages were purified over a cesium chloride gradient and concentrated by precipitation with polyethylene glycol 8000 (Sambrook and Russell, 2001). Phage DNA was extracted using modifications to a previously described method (Sambrook and Russell, 2001). Briefly, concentrated phage particles were resuspended in 200 µl SM buffer. Free nucleic acids from lysed bacterial host cells were degraded with 250 units of benzonase endonuclease for 2 h at 37 °C, after which the benzonase was inactivated by the addition of 10 mM EDTA and heating at 70 °C for 10 min. The phage protein coats were

degraded using proteinase K (1 mg/ml) and SDS (1 %). A phenol-chloroform extraction was performed, and phage DNA was precipitated with ethanol. The washed DNA pellet was resuspended in T₁₀E₁ buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at -20°C. Phage DNA was digested with *Eco*RI for at least 3 h at 37 °C, and resolved by agarose gel electrophoresis on 1 % agarose gels at 70V for 3 h. Gels were stained with ethidium bromide and visualized with an Alpha imager[®] HP gel documentation system (Alpha Innotech corporation, San Leandro, CA, USA).

3.2. Characterization of previously isolated phages that are candidates for phage therapy

3.2.1. Phage and Bacteria

Two previously isolated bacteriophages (eiAU and eiDWF) (Walakira et al., 2008) that are candidates for phage therapy were used to evaluate stability and dynamics of these phages in various physiochemical and biological conditions. High titer phage stocks were propagated on *E. ictaluri* cultures using the double agar method (Adams, 1959). Briefly, 5 ml of semi-solid (0.7 % agar) tryptic soy broth (TSB) or brian hearth infusion (BHI, Difco Laboratories, Detroit, Michigan) was inoculated with 100 µl triple-purified phage stock plus 200 µl of culture of *E. ictaluri* grown overnight, poured onto agar plates, and incubated for at least 15 hr at 30 °C. Phages were recovered from media plates by pouring SM buffer (100 mM NaCl, 8 mM MgSO₄•7H₂O, 50 mM Tris-Cl [1 M, pH 7.5]) over agar plates, incubating for an additional 3-6 h, and then collecting the SMB-phage suspension. Phage suspension was treated with chloroform at a rate of 1:100 (chloroform to phage suspension), and then centrifuged at 3600 x *g* for 25 min. After centrifugation, the supernatant containing phages was filtered through a 0.2 µM filter and

then concentrated by precipitation with polyethylene glycol 8000. Phage stocks were stored at 4 °C for later use. *Edwardsiella ictaluri* isolate 219 and S97-773 used in these experiments were obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University.

3.2.2. Preparation of phage-amended feed

Commercially available slow-sinking pelleted fish feed (2 mm, 40 % protein, Zeigler, Gardners, PA) was frozen and then lyophilized using a VirTis Unitrap 10-100 manifold-style freeze-dryer (VirTis, Gardiner, NY). Lyophilized feed was spray-coated with a phage solution at a rate of 8 % of the dry/lypholyzed weight of the feed. Feed was immediately coated a second time with menhaden fish oil (5 %) at a rate of 4 % of the dry feed weight. The concentration of phage in feed was determined by homogenizing eight, 0.1 g replicate samples of feed each in 900 µL of 10 % beef extract, serially diluting (10-fold), and spotting on a lawn of *E. ictaluri* S97-773.

3.2.3. Dynamics of Phage and *E. ictaluri* in Pond Water

The dynamics of phage-*E. ictaluri* interactions in aquaculture pond water of different levels of hardness were evaluated. Water samples (3 L) were collected from various catfish research and production ponds at the E. W. Shell Fisheries Research Center (Auburn, AL) and from commercial catfish farms in central western Alabama. Water samples were pooled into two groups based on their hardness levels, resulting in a pooled sample of moderately high (137 ppm as CaCO₃) water hardness and another of low water hardness (34 ppm as CaCO₃). Collected water samples were centrifuged at

3600 x g for 20 min and the supernatant was filtered through a 0.2 µM filters. Log phase *E. ictaluri* isolate S97-773 was centrifuged twice and resuspended in the respective water to remove media nutrients. Bacteria and bacteriophage eiAU were added to water samples, targeting a final concentration of 1 x 10⁸ CFU/ml and 1 x10⁵ PFU/ml in soft water and 1 x 10⁹ CFU/ml and 1 x 10⁶ PFU/ml in hard water, respectively. Four replicate water samples from each of the hardness levels were prepared. Additionally, one water sample for each condition was prepared in which either phage only or bacteria only were added to the water as controls. All water samples were incubated at 26 °C for the duration of the experiment. At each time point one ml of water sample was drawn from each of the four replicates of each water condition at times (0 h, 3 h, 9 h, 24 h, and 48 h) and centrifuged at 14,000 rpm for 10 min. The supernatant containing the phage portion was removed and the bacterial pellet was re-suspended in 1 ml water of their respective condition. Ten fold serial dilutions of the phage and bacterial solutions were done and phage and bacterial numbers were quantified.

Pearson correlation coefficients for the relationship between bacterial titer drop and phage titer increase overtime were calculated separately for each water condition. Analysis was done using MINITAB 15 for windows (MINITAB Inc., Pennsylvania, USA).

3.2.4. Fate and persistence of phage inside catfish

The fate and persistence of phage inside channel catfish fingerlings (10-14 cm) was investigated. Phage alone or a mixture of phage and *E. ictaluri* was administered by intra-gastric gavage. The intestines, blood, trunk kidney, and spleens of fish were

processed and analyzed for the presence of phage. Briefly, blood was drawn from the caudal vein of fish directly into sodium heparinized tubes to prevent clotting and then centrifuged at 5,000 x g for 5 min. The supernatant containing blood plasma was removed from blood samples, serially diluted in SM buffer, and subjected to a spot assay for phage quantification. Immediately after removing blood, fish were sacrificed by prolonged exposure to 300 ppm of the anesthetic MS-222 (tricane methanesulfonate). The intestines, trunk kidney, and spleen were aseptically removed, weighed, and homogenized in 0.9 mL of 10 % beef extract using a Pellet Pestle[®] Mortar (Kontes, Vineland, New Jersey USA), and then centrifuged at 10,000 x g for 10 min. The supernatant was removed and filtered through a 0.22 μ M filter, and subjected to a spot assay for phage quantification. Three experiments were carried out separately in this study.

In experiment one, 40 catfish fingerlings were equally distributed into three, 60-L aquarium. Fish in each aquarium were administered 200 μ L of phage eiAU (6×10^8 PFU/ml) by intra-gastric gavage. At 0.5 h, 6 h, 12 h, 24 h, and 48 h post-phage administration, a total of eight fish were randomly removed from the three aquaria, blood was drawn, and then the fish were euthanized with MS-222 and necropsied. Whole intestines were removed from each fish, weighed, and processed along with plasma samples for phage quantification as described above.

In experiment two, 15 channel catfish fingerlings each received by intra-gastric gavage 400 μ L of an equal mixture of phage eiAU P10 (6.3×10^8 PFU/ml) and *E. ictaluri* (7.8×10^8 CFU/ml) and then placed in one aquaria containing 15 L of water. At time 0.5 h, 4 h, and 8 h post-gavage five fish were randomly removed and processed for phage

detection in blood and internal organs. Additionally, two fish received phage only and two fish received bacteria only and were processed as above as negative controls. These fish were kept in separate aquaria.

In experiment three, 15 channel catfish fingerlings were placed in one aquaria containing 15 L of water. Fish were fed phage-amended feed containing 5×10^6 PFU/gram of feed. At times 24 h, 48 h, and 72 h post phage administration the whole intestines were removed from five fish and processed as described previously for phage detection. Additionally, two fish received phage only and two fish received bacteria only and were processed as above as negative controls. These fish were kept in separate aquaria.

A General Linear Model (GLM) with repeated measures ANOVA (Wallace and Green, 2002) tested the effect of time on mean phage titers present in tissue sampled after phage administration. Tukey's pairwise comparisons were conducted between time points sampled, $p < 0.05$. Analysis was done using MINITAB 15 for Windows (MINITAB Inc., Pennsylvania, USA).

3.3. Serial passage of phages in catfish

Serial passaging and re-isolation of phages through the fish was performed to better understand if phages could survive passage through the stomach and into the intestinal tract and whether phage strain improvements could be accomplished. For each passage, channel catfish fingerlings ($n=7$; 10-14 cm) were acclimated in one 60 L aquaria containing 15 L of well water for 5 days. Water temperatures maintained at 26 °C. No feed was given to the fish 3 days before the beginning of the experiment.

Based on preliminary experiments to determine a range of PFU's of phage to pass through the fish and general time points in which to sample the fish after phage exposure, 0.2 ml of bacteriophage solution ($\sim 5 \times 10^8$ PFU/ml) was administered by intra-gastric gavage. Five fish were randomly chosen, euthanized by prolonged exposure to 300 ppm MS-222 the whole intestines were aseptically removed and processed for the quantification of phages present in whole intestines of fish after 24 h. Intestines were weighed, and 0.1g of the organ was homogenized manually for 1 min using a mini tissue grinder (Daigger & company, Inc., Vernon Hills, Illinois, USA) in 900 μ L of beef extract. Homogenized samples were kept on ice until all samples had been processed. Once all sampling was completed, each homogenized sample was mixed by vortexing and then centrifuged at 10,000 x g for 10 min using an Eppendorf 5424 centrifuge. The supernatant from each centrifuged sample was filtered through 0.22 μ m filter, transferred into sterile micro centrifuge tubes and serially diluted (10-fold) six times. Dilutions were spotted onto an overlay of *E. ictaluri* isolate S97-773 for phage detection and quantification. This protocol was performed for a total of ten times, each time isolating a single plaque from a re-isolated phage population, propagating in a culture of *E. ictaluri*, and then readministering the purified phages to the fish. After the final passage, one phage plaque was chosen for phage propagation, double purified, and stored in SM buffer (10 % DMSO) at -80 °C. This phage variant was given the name eiAU P10.

A study was performed to compare parent phage eiAU against its passaged variant, eiAU P10, for their capacity to survive within the catfish gastro-intestinal tract, as determined by phage titers recovered from the intestine of fish at different time

post-administration of phage. Eighty four channel catfish fingerlings (10-14 cm) were equally stocked into six, 60-L aquaria containing 50 L of well water and acclimated to 26 °C. Fish in three randomly chosen aquaria were administered 200 ul of either phage eiAU or phage eiAU P10 by intra-gastric gavage. The target dose of phage administration was 1×10^8 PFU/ml. Eight fish were randomly removed from each treatment as a whole, beginning at 0.5 h, 5 h, 10 h, 20 h, and 40 h after phage administration. Blood was collected from the caudal vein directly into sodium heparinized tubes and then fish were euthanized as above and necropsied. Whole intestine and trunk kidneys were removed from each fish, weighed, suspended in 10 % beef extract, homogenized manually using a micro homogenizer and then centrifuged at 10,000 g for 10 min. The blood was centrifuged at 5,000 x g for 5 min and blood plasma and supernatant from homogenized organs (intestine and trunk kidney) were removed, filtered through a 0.22µm filter, serially diluted in SM buffer, and subjected to a spot assay for phage quantification. Phage quantification was performed on *E. ictaluri* strain S97-773.

Mean phage titers were compared between treatments (eiAU and eiAU P10) using a repeated-measures ANOVA (SAS proc GLM) (Wallace and Green, 2002). The model included time, treatment, and their interaction as main effects. Significant differences were considered at $p < 0.05$. Statistical analyses were carried out using SAS (version 9.1.3, SAS Institute, Cary, North Carolina).

3.4. Long-term storage of phages

The long term stability of phages (eiAU and eiDWF) that are candidates for phage therapy were evaluated in different storage media and at different temperatures. Phages were propagated on BHI using a standard soft agar overlay method (Adams, 1959), concentrated by centrifugation, and resuspended in either BHI or SM buffer containing either glycerol (7 %) or DMSO (7 %). Triplicate samples of each condition were stored at 4 °C and at – 80 °C and quantified at 6 or 10 months post storage.

Mean initial and mean final titers of phage in each storage condition were compared using a t-test. Significant differences were considered at $p < 0.05$. Statistical analyses were carried out using SAS (version 9.1.3, SAS Institute, Cary, North Carolina).

4. Results and discussion

4.1. Characterization of previously isolated phages that are candidates for phage therapy

4.1.1. Isolation of bacteriophages and host specificity

Phages were recovered from two out of six commercial catfish ponds and plaques were only observed on *E. ictaluri* strain 219. Although multiple plaques were observed that differed in size and clarity, six plaques were randomly chosen, triple purified, and propagated. Plaque size after propagation ranged from 8 mm to 10 mm for all six isolates. The host specificity of these six phage isolates are presented in Table 1.

Phages in this study were recovered from two different ponds on two different but adjacent farms, with one pond experiencing an ongoing ESC infection and the other with an ESC outbreak having occurred two weeks prior to sampling. Previously recovered *E.*

ictaluri phages has also been isolated from ponds with ongoing *E. ictaluri* infections (Walakira et al., 2008). The routine isolation of phages from aquaculture environments (Imbeault et al., 2006; Karunasagar et al., 2005; Park et al., 2000; Vinod et al., 2006) may suggest that phages may be actively propagating within their bacterial host(s) in that environment and that they may be playing a role in modulating the population of its host. There is a prevailing view that viruses kill approximately 10-40 % of the standing stock of bacteria in marine ecosystems (Suttle 1994, Suttle 2005). Hence, it has been postulated that viruses (including phages), influence the cycling of organic matter and the quantity and diversity of bacterial communities in aquatic environments (Heldal and Bratbak, 1991).

Counterintuitively, the isolation of phages during the height of an epizootic may suggest that even if there is some influence on the levels of bacterial population in a particular pond, the level of control is not high enough to prevent disease outbreak in that particular pond. It is plausible that phages may play a role in the differences in severity or lack of disease in ponds especially when one considers that phages are obligate parasites requiring their host to proliferate (Day, 2004). However, the impact of phage on the presence or severity of disease is difficult to assess in commercial aquaculture settings because disease severity from pond to pond is dependent on many factors such as the health of the fish, environmental conditions in the pond, and the virulence of the pathogen.

During initial isolation, plaques were observed only on *E. ictaluri* strains 219; however, when high titer phage solutions were prepared and spotted on those strains cell lysis was observed from phage isolate eiPF on both strains C91-162 and R-4383 (Table

1). In a previous study two isolated *E. ictaluri* phages also showed the presence of small pin-point plaques on strains C 91-162 and R-4383 (Walakira et al., 2008). The inability to readily screen for wild-type phages that have broader host-range capabilities to lyse targeted bacteria that are not effectively lysed by previously isolated phages may suggest that a proposed means to mediate problems of phage resistance by continuously isolating phages from the environment may not be practical in the case of *E. ictaluri* phages. However, *in vitro* serial passaging of phage on less susceptible bacterial isolates was shown to increase the capacity of wild type phage eiAU to to lyse C91-162 and R-4383 in our laboratory. This strategy is also part of a long term plan used by OmnyLytix to mediate problems of phage resistance that may arise from the use of their phage-based product AgriPhage™ (<http://www.phage.com>). Based on lysis capacity ϕ eiPF (or eiPF) was chosen for further characterization using electron microscopy and nucleotide restriction analysis.

4.1.2. Morphology and restriction of bacteriophage nucleic acid

Electron microscopy revealed that phage eiPF has an icosahedral shaped head, approximately 50 nm and a non-rigid tail (Figure 1). Based on the morphology and the rules provided by the International Committee on Taxonomy of Viruses (ICTV, Bethesda MD, USA) the phage is tentatively placed in the Siphoviridae family. Restriction digestion of eiPF with *EcoRI* showed that it shares identical restriction fragments with phage eiAU, and is highly similar to the restriction fragments of phage eiDWF (Figure 2) which was also observed by Walakira et al (2008). Based on restriction fragments and

data on the genome size of eiAU (Carrias et al., 2010) the genome size of eiPF is estimated to be approximately 43 kb.

The morphology and genome size of eiPF is highly similar to eiAU. Current data suggests that *E. ictaluri* phages are morphologically and genetically similar, at least those from the geographical regions sampled. The genomes of phage eiAU, eiDWF, and eiMSLS, which were isolated in 1985 in Auburn Alabama, in 2006 in western Alabama, and in 2004 in Washington County Mississippi, respectively, are greater than 95 % identical at the genome level (Carrias et al., 2010). Although phage diversity is common in other bacterial species (Buchen-Osmond, 2003; Pedulla et al., 2003), a low phage diversity for *E. ictaluri* (Panangala et al., 2005a; Panangala et al., 2005b) may reflect the lack of significant genetic heterogeneity among *E. ictaluri* phages and their host. Additional sampling for *E. ictaluri* phages would be necessary to determine the genomic diversity of *E. ictaluri* phages. However, considering that viruses can influence the diversity of prokaryotes (Weinbauer and Rassoulzadegan, 2004) and that *E. ictaluri* is a highly homogeneous species based on phenotypic (outer membrane protein profiles, antigenic determinants, and biochemical properties) and genetic characteristics (Panangala et al., 2005), suggest that phages for *E. ictaluri* may not be as genetically diverse as phages in other bacterial species. For purposes of phage therapy the homogeneity of *E. ictaluri* and the seemingly low diversity of *E. ictaluri* phages would suggest that only a few phage types may be required to cover the diversity of *E. ictaluri* present in catfish culture environments. Tests are being performed at a fish disease diagnostic laboratory on the susceptibility to phage lysis of *E. ictaluri* primary isolates

obtained from diseased catfish. The data collected may provide additional insight into the diversity of phage phenotypes among *E. ictaluri* populations in the environment.

4.2. Characterization of previously isolated phages that are candidates for phage therapy

4.2.1. Dynamics of Phage and E. ictaluri in pond water

A significant negative correlation ($R = -0.843$, $p = 0.049$) and ($R = -0.818$, $p < 0.001$) was observed between phage titers and bacterial titers overtime for soft water/low hardness and hard water/high hardness respectively. In soft water, approximately 10x decrease was observed in bacterial titers after 9 h post-inoculation of phage and bacteria, and over a 100x decrease in bacterial titers was observed after 48 h (Figure 3A). While bacterial titers decreased, phage titers in soft water increased by 10x after 9 h, and 1000x after 48 h (Figure 3A). In hard water, bacterial titers had a 1000x drop after 24 h (Figure 3B). At the same time, phage titers increased by approximately 10x after 9 h and 100x after 24 h (Figure 3B) in hard water. During the same experiments minimal drop in bacterial titers was observed at 48 h post-inoculation in both soft and hard water when only bacteria were added to the water (data not shown). Similarly no increase in phage titers was observed when phage alone was added to the water. The bacterial population in both soft and hard water conditions was not reduced to undetectable levels (Figure 3A and Figure 3B).

In both water conditions the negative correlation of phage titers and bacterial titers recovered indicates that phages were able to infect and reduce bacteria in both water

conditions. The rate of reduction of bacterial titers could not be compared between water conditions; however, a similar pattern of decrease in phage titer over time was observed in water of both low (34 ppm as CaCO_3) and moderately high (137 ppm as CaCO_3) levels of hardness, suggesting that the levels of calcium and magnesium cations in waters of disparate hardness (expressed as CaCO_3) commonly found in catfish production ponds in different regions of the catfish industry (Hariyadi et al., 1994) does not have an impact on the capacity of phage to infect and kill *E. ictaluri*. This observation does not coincide with observations that higher levels of calcium and magnesium cations result in increased lyses of bacterial host cells by their phages (Lu and Breidt, 2003; Walakira et al., 2008). This difference may be explained in part by the percent composition of calcium and magnesium available in the chemical compound used in these experiments. For example, other studies used compounds such as calcium chloride as a source of calcium (Capra, 2006; Lu and Breidt, 2003; Walakira et al., 2008); however, in this experiment water hardness measurements were used as an estimation of the different levels of calcium and magnesium cations that may be present in the two different water samples collected under commercial conditions. Water hardness may be partially composed of compounds other than calcium carbonate such as bicarbonates and sulfates (Tucker and Robinson, 1990), hence the calcium and magnesium portion may not be very different in soft and hard water in some locations. Also, differences in the physiological state of the bacteria in pond water and in a complex medium may impact the results. Regardless, the objective for following phage-bacterial dynamics was to determine if these conditions (hard and soft water) that are prevalent in commercial catfish farms affect capacity of phage to reduce bacterial numbers. The data suggests that it does not.

Even though the phage used in this study (eiAU as an example) was capable of reducing *E. ictaluri* cell numbers in pond water, bacteria were not reduced to undetectable levels. Similar results have been obtained in other studies of fish-bacterial dynamics in culture water (Park et al., 2000), and in a study evaluating the capacity of phage to control the growth of *Erwinia amylovora* in liquid culture which showed that although phage prevented the growth of bacteria it was not able to reduce phage numbers in water, and that phage was able to more effectively lyse its host when in its early growth stage (Schnabel et al., 1998). The inability of phage to eliminate all bacteria present may be explained in part by the different physiological state of bacteria present in laboratory culture in a nutrient rich culture medium (Miller and Day, 2008). Evidence suggests that non-replicating population of bacteria are physiologically insusceptible to most bacteriophages (Adams, 1959; Bull et al., 2002; Stent and Dohm, 1963) or to antibiotics (Tuomanen, 1986). This has been previously observed with *E. ictaluri* that showed to be non-susceptible to phage lysis in log phase but susceptible to phage lysis when in exponential growth (Walakira et al., 2008). However, the inability of phage to completely eliminate host bacterial population in water may be more critical for therapy plans that involve adding phage directly into water as a means of reducing concentrations of host bacteria present in culture environments. However, the main route of *E. ictaluri* infection is through the oral route (Klesius 1994); hence, it is more important for *E. ictaluri* phages to be efficient at targeting bacteria at sites of entrance such as the gastrointestinal tract and at sites of bacterial concentration and proliferation such as trunk kidney, liver, and spleen (Baldwin and Newton, 1993) of catfish.

4.2.2. Fate and persistence of phage inside catfish

In experiment one, a significant effect ($p < 0.001$) of time on mean phage titers recovered post-phage administration was observed. Mean phage titers recovered from the intestine samples of catfish post-phage administration are presented in Figure 4. Mean titers recovered after six hours-post phage administration was 10x lower ($p = 0.0016$), than the initial titer. A 100x decrease ($p = 0.008$) was observed after 12 h, and over a 1000x decrease ($p = 0.007$, $p = 0.007$) was observed after 24 h and 48 h post-phage administration respectively (Figure 4). No phage was detected in the blood or internal organs (trunk kidney, liver and spleen) of catfish. However, in experiment two, when phage and *E. ictaluri* were simultaneously administered to fish, phage was recovered from the blood, and trunk kidney samples, but not from spleen and liver samples of fish at 4 h and 8 h post-phage administration. Phage was not recovered in any of the organs at time zero/0.5 h post-phage administration. Phage titers were not quantified in experiment two. In experiment three, when phage was administered through phage-amended feed, mean phage titers recovered at 24 h and at 48 h from the whole intestines of fish were 3.5×10^4 PFU/gram of fish tissue and 2×10^2 PFU/gram of fish tissue respectively. No phage was recovered at the 72 h sampling time.

High phage titers that were placed directly into the gastro-intestinal tract of catfish through gavage steadily and rapidly declined overtime post-phage administration. Although the titer (5.25×10^3 PFU/gram of fish tissue) remaining in the intestines of fish at 48 h post-phage administration may seem high, it represents a 1000x decrease of the initial titer administered. When phage was administered through phage-amended feed the small (2×10^2 PFU/gram of fish tissue) numbers of phage recovered after 48 h post-administration in the intestines of fish was more pronounced. Similar results were

observed in other studies (Nakai et al., 1999; Park et al., 2000; Verner-Jeffreys et al., 2007), suggesting that although phage can survive adverse conditions such as low pH commonly present in the gastro-intestinal tract of various fish species that have been tested, they rapidly decline in titers. Observations that phage titers present in the stomach plus intestine of fish are greatly reduced after 48 h may have significant implications on the amount/dose and frequency of phage administration needed for effective therapy results. This was observed in a study in which the control of bacterial spot in geranium was achieved by the daily application of phage but not by bi-weekly applications (Flaherty et al., 2001). Therefore, for *E. ictaluri* phages to have better probabilities of reducing *E. ictaluri* inside catfish it may be necessary to administer phage daily or twice a day to ensure that sufficient numbers of phage are present in the gastro-intestinal tract of fish to target bacteria at any given time. This proposition is practical but more costly than feeding phage-amended feed every two or every three days.

When phage alone was administered to catfish by intra-gastric gavage, no phage was recovered from blood or any internal organs sampled. However, when phage was administered in conjunction with *E. ictaluri*, phages were isolated from blood and trunk kidney, but not from the spleen and liver. The recovery of phages in blood circulation and trunk kidney only when phage was administered in conjunction with *E. ictaluri* suggests that phages were transported from the intestine into blood circulation by the host bacteria. This phenomena has been observed previously in phage therapy studies against the intracellular bacterial pathogen *Mycobacterium avium* (Broxmeyer et al., 2002) and has been described as a mechanism analogous to the Trojan horse concept (Skurnik and Strauch, 2006). Similarly, the original eiAU phage isolate was obtained from kidney

samples from fish with an active *E. ictaluri* infection (Walakira et al., 2008) and therefore it is plausible that a phage infected bacterial cell was able to enter the blood stream before being lysed and transported the phage to internal organs. The inability of phages to cross the intestinal epithelium and become systemic may have significant implications for the efficacy of phage therapy efforts against *E. ictaluri* because *E. ictaluri* causes systemic infections and the liver, spleen, and trunk kidney are sites of propagation and infection for the pathogen (Baldwin and Newton, 1993). Furthermore, although the oral route is postulated as the major route of *E. ictaluri* infection, other routes of entrance for the pathogen such as the gills and the nasal cavity are believed possible (Menanteau-Ledouble, 2009; Nusbaum and Morrison, 1996), resulting in a portion of bacteria being inaccessible to phage due to their inability to become systemic. Pathogenesis studies indicate that once *E. ictaluri* crosses the intestinal epithelium they quickly enter macrophages (Booth et al., 2006). Macrophages have been observed to be more concentrated in the kidneys (Baldwin and Newton, 1993) which may in part explain why phages (carried by phage-infected bacteria) were only recovered there and not in the spleen and liver.

4.3. Serial passage and comparison of passaged phage versus wild type parent

Mean phage titers recovered at various time points from fish groups that received either phage eiAU or its serially passaged variant eiAU P10 are presented in Figure 5. No significant effect of phage passaging ($p = 0.052$, $R\text{-Sq}=77.8\%$) was observed on mean phage titers over a 40 h time period. No significant interaction was observed between time points sampled and phage treatment administered to fish ($p = 0.884$).

The lack of any significant difference observed in the mean phage titers for the passaged phage vs. wild-type phage suggests that passaging in the gastro-intestinal tract may not confer an enhanced capacity for passaged phage as a population to remain in the gastro-intestinal tract in higher numbers than its parent phage. It may be possible that the gastric conditions within the catfish GI tract do not inactivate phage at a rapid rate, and that the reduction in phage titer within the GI tract over time is simply a function of the rapid passage of materials through the GI tract. However, in this study fish were not fed. Additionally, the spot assay performed determines only the phage titers present and not the difference in capacity of the two phages to infect bacterial hosts inside gastro-intestinal tract conditions. Ultimately the desired effect of phage passaging is a phage variant that has enhanced capabilities of infecting and killing their host in adverse conditions such as those encountered in the gastro-intestinal tract of fish. Therefore, a challenge study comparing the two phages ability to infect *E. ictaluri* within the catfish GI tract may indicate differences in these two phages' relative ability to infect their host under *in vivo* conditions.

4.4. Long-term storage of phages

The stability of phages eiAU and eiDWF are presented in Table 2. Phages were highly stable when stored at both 4 °C and at -80 °C, both in BHI and SM buffer containing a final concentration of either 7 % glycerol or 7 % DMSO (Table 2). No significant (P-value > 0.05) reduction in phage numbers from initial to final enumeration were observed in any of the conditions tested. No comparisons were made between phages or among storage conditions (i.e. 4 °C vs -80 °C or BHI vs. SM buffer).

Minimal drop in phage titers stored over long periods of time has observed previously in *Aeromonas salmonicida*-phages stored at -20 or -70 °C (Verner-Jeffreys et al., 2007). The capability of *E. ictaluri* bacteriophages to remain viable for a long time is important for maintaining stocks for future uses. Furthermore, the long term stability of a phage at 4 °C is important because it means that cryopreservation in a -80 °C freezer is not required for long-term storage of these phages.

5. Conclusions

The host range, host specificity, morphology, and DNA restriction fragment analysis of a lytic *E. ictaluri* phage isolated in this study indicated that the phage is very similar to previously isolated *E. ictaluri* phages (Walakira et al., 2008). Although *E. ictaluri* phages available to date were isolated in different times, one of them in 1985 (eiAU), and in different regions of catfish production in the southeastern United States, they share greater than 95 % of their genome sequence (Carrias et al., 2010). This information may suggest that *E. ictaluri* phages are not highly genetically diverse.

Phage eiAU demonstrated the capacity of *E. ictaluri*-specific phages to infect and reduce *E. ictaluri* in pond water of low hardness levels and in moderately high hardness levels. Inside channel catfish, phages were capable of surviving conditions in the gastro-intestinal tract, and were recovered from intestines up to 48h post-administration at titers 1000x lower than its initial concentration and up to 72 h in very low numbers.

Although phage eiAU (a candidate for phage therapy) and its passaged variant were not detected in blood circulation and internal organs when administered without *E.*

ictaluri they were detected in blood circulation and in trunk kidneys when they were administered in conjunction with host *E. ictaluri*. This suggests that phage-infected *E. ictaluri* cells may still carry phages into systemic sites of infection. The passaging of phage in the gastro-intestinal tract did not confer increased survival of these phage variants within the gastro-intestinal tract of channel catfish fingerlings; however, further testing in a disease challenge experiment is needed to determine if the serially passaged phage has an enhanced efficiency of attachment and/or killing of its host bacteria under *in vivo* conditions.

Table 1. Specificity of phage infection on *Edwardsiella ictaluri* strains and other bacterial species isolated and collected from different locations. If “+” there is lysis and if “-” there is no lysis.

Bacteria	Lysis						Source ¹
	φA	φB	φC	φD	φE	φF	
<i>Edwardsiella ictaluri</i> strains:							
195	+	-	+	-	+	+	catfish, Alabama
196	-	+	-	+	-	+	catfish, Alabama
219	+	+	+	+	+	+	catfish, Alabama
218	+	+	+	+	+	+	catfish, Mississippi
S97-773	+	+	+	+	+	+	catfish, Alabama
C91-162	-	-	-	-	-	+	AUCVM
R4383	-	-	-	-	-	-	AUCVM
Non- <i>Edwardsiella ictaluri</i> strains:							
<i>Yersenia ruckeri</i> biotype 1 M0-06-06	-	-	-	-	-	-	Trout, Missouri
<i>Yersenia ruckeri</i> biotype 11 SC-04-13	-	-	-	-	-	-	Trout, SC
<i>Aeromonas hydrophila</i> GA-06-05	-	-	-	-	-	-	Catfish, Georgia
<i>Proteus mirabilis</i>	-	-	-	-	-	-	AUFDL
<i>Edwardsiella tarda</i> AL-93-98	-	-	-	-	-	-	Catfish, Alabama
<i>Salmonella enterica</i> ATCC12324	-	-	-	-	-	-	ATCC
<i>Citrobacter freundii</i> ATCC 8090	-	-	-	-	-	-	ATCC
<i>Klebsiella pneumoniae</i> ATCC25943	-	-	-	-	-	-	ATCC

¹Source indicates from what fish species and location the isolate was obtained. AUCVM, Auburn University College of Veterinary Medicine (Department of Pathobiology); AUFDL, Auburn University Fish Diagnostic Laboratory.

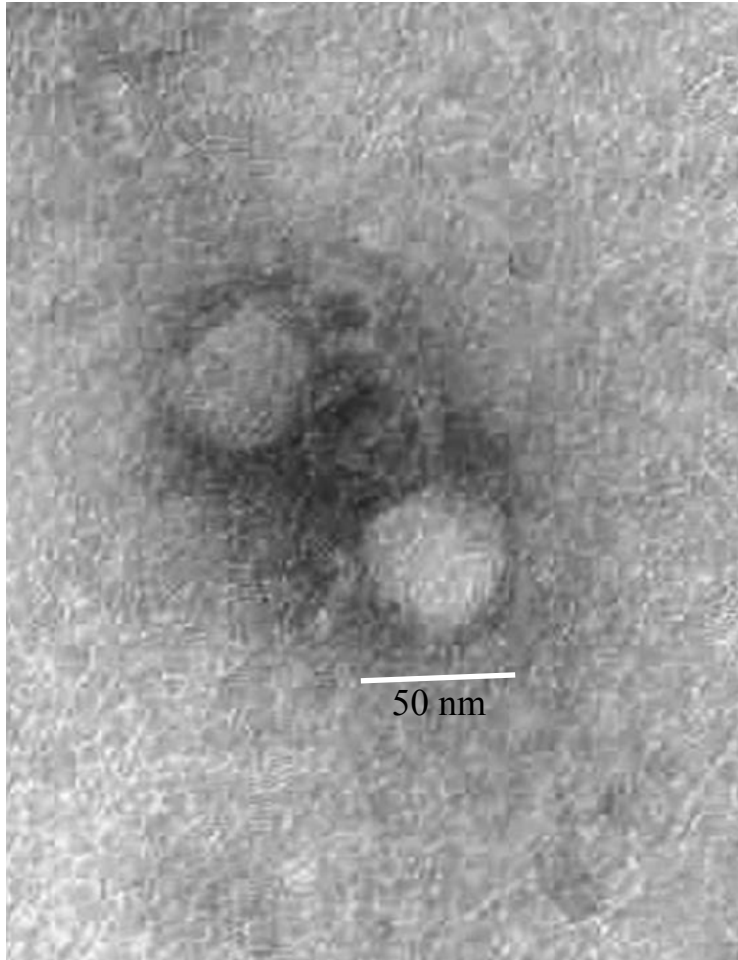


Figure 1. Electron micrograph of phage Φ eiPF negatively stained with 2% phosphotungstic acid.

MW Φ eiPF Φ eiDWF Φ eiAU

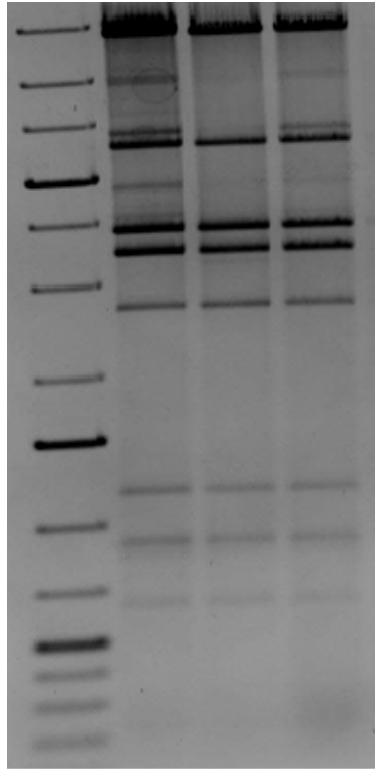


Figure 2. Restriction fragment analysis of phages eiPF, eiDWF, and eiAU with *EcoRI* resolved by agarose gel electrophores. Molecular weight of ladder = 1 Kb.

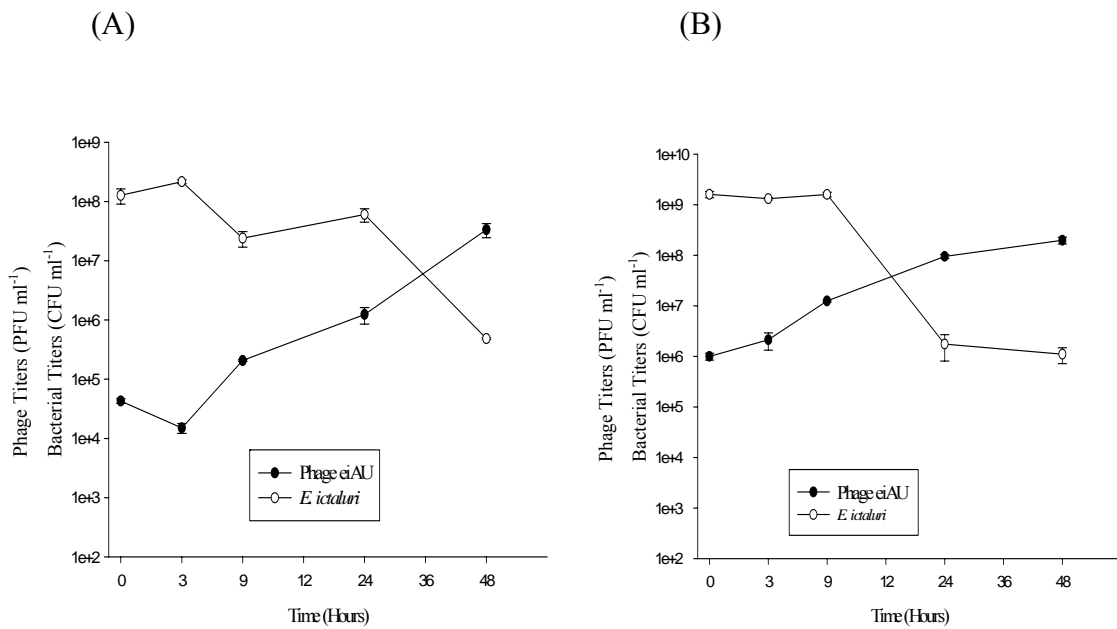


Figure 3. Dynamics of phage eiAU and *E. ictaluri* in water of low hardness level (A) and of high hardness level (B). Mean \pm SD of four replicate samples was plotted at each time point for both phage and bacterial titers.

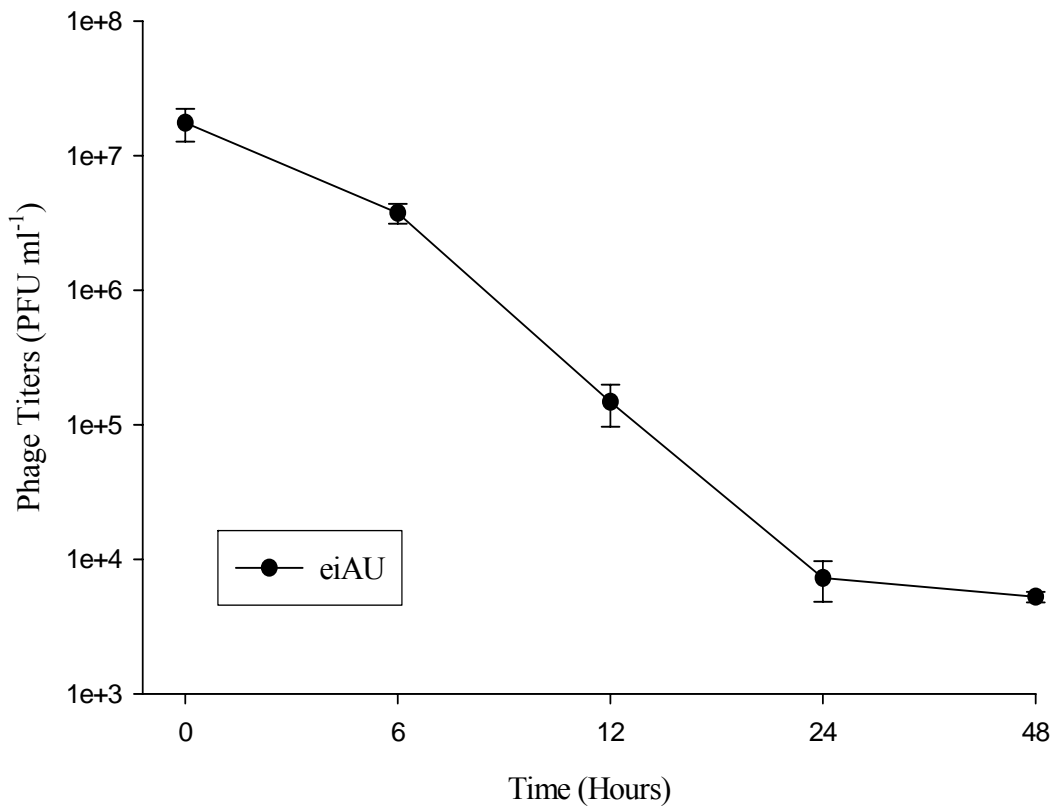


Figure 4. Titers of phage present in the intestines of catfish after phage administration by intra-gastric gavage. Each time point plotted is the mean \pm SD of the titers present in eight replicate fish sampled. A significant ($p < 0.001$) effect of time on mean phage titers recovered overtime was observed.

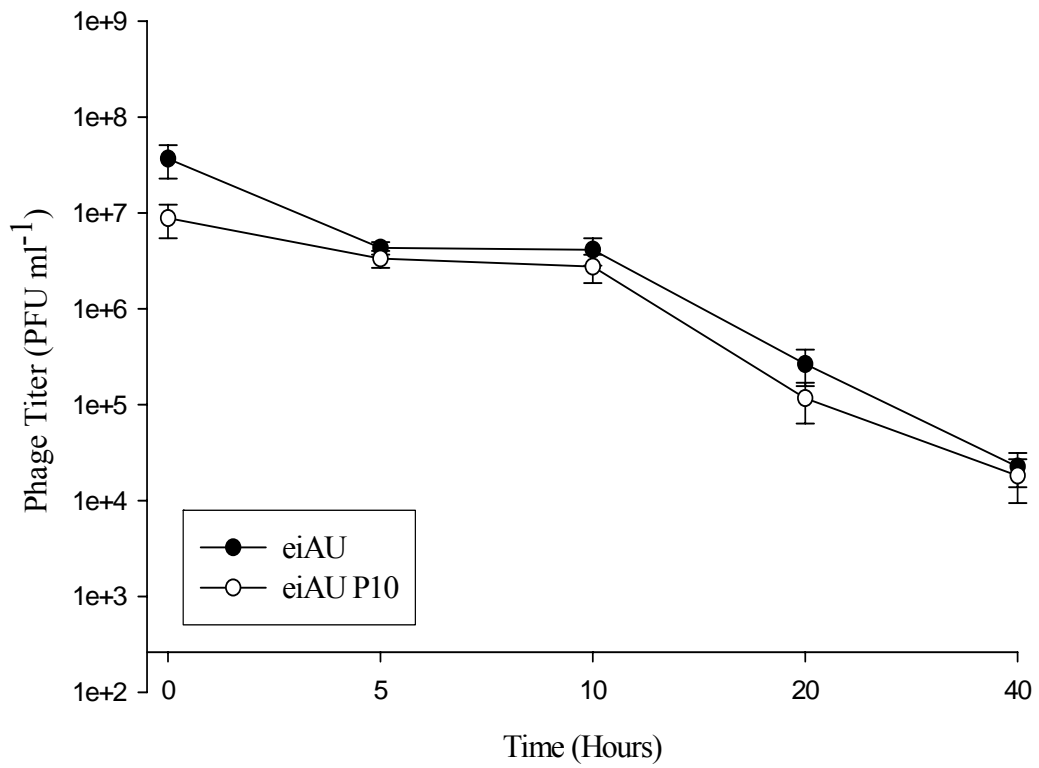


Figure 5. Phage titers of phage eiAU and its variant eiAU P10 recovered from the intestine of channel catfish fingerlings. No significant effect of phage passaging ($p = 0.052$) was observed.

Table 2. Long term storage of phages (eiAU and eiDWF) preserved in different agents, and in different storage temperatures.

Phage	Storage Condition	Temp (⁰ C)	Initial (PFU/mL)	Final (PFU/mL)	Time elapsed
eiAU	SMB (7 % DMSO)	4	2.00E+04	2.00E+04	6 months
eiAU	SMB (7% gly)	-80	1.00E+08	9.00E+07	10 months
euAU	BHI	4	5.00E+06	4.00E+06	6 month
eiDWF	SMB (7 % DMSO)	4	4.00+07	2.00+07	6 months
eiDWF	SMB (7 % gly)	-80	1.00E+08	1.00E+08	10 months
eiDWF	BHI	4	8.00E+04	8.00E+04	6 month

SMB is a sodium-magnesium based buffer, BHI is an acronym for Brain heart infusion.

CHAPTER III

Comparative genomic analysis of bacteriophages specific to the channel catfish pathogen *Edwardsiella ictaluri*

1. Abstract

One of the obstacles to successful use of phage therapy is the capacity of some temperate phages to transduce virulence factors to its bacterial host. This problem may be solved by selecting bacteriophages as therapeutic agents that are well characterized at a genomic level, with no potential for inducing lysogenic conversion.

The genomes of three *Edwardsiella ictaluri*-specific bacteriophages isolated from geographically distant aquaculture ponds, at different times, were sequenced and analyzed. The genomes for phages eiAU, eiDWF, and eiMSLS are 42.80 kbp, 42.12 kbp, and 42.69 kbp, respectively, and are greater than 95 % identical to each other at the nucleotide level. Nucleotide differences were mostly observed in non-coding regions and in structural proteins, with significant variability in the sequences of putative tail fiber proteins. These *E. ictaluri*-specific phage genomes reveal considerable conservation of genomic architecture and sequence identity, even with considerable temporal and spatial divergence in their isolation. The genomic analysis of these phages supports the

conclusion that these are virulent phages, lacking the capacity for lysogeny or expression of virulence genes. This study contributes to our knowledge of phage genomic diversity and facilitates studies on the diagnostic and therapeutic applications of these phages.

2. Introduction

Here we report the complete nucleotide sequence and annotation of the genomes of three bacteriophages specific to the gram negative bacterial pathogen *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC). ESC is a primary cause of mortality in catfish farms with annual direct losses in the range of \$40-60 million dollars in the U.S. (Shoemaker et al., 2009). Economic losses coupled with limited available treatment options for controlling ESC, and concerns regarding the development of resistance to antibiotics used in aquaculture warranted efforts to identify biological control agents that are antagonistic to *E. ictaluri* (e.g., bacteriophage). In addition, the multiple days necessary to obtain a diagnostic result for *E. ictaluri* via biochemical tests was a motivation to identify phage that could serve as specific, rapid, and inexpensive typing agents for ESC disease isolates.

The idea of using phage as antimicrobial agents to treat bacterial infections in agriculture or aquaculture is not a new proposition (Brussow, 2005); however, there is now a better understanding of phage biology and genetics, and with it a better understanding of their potential and their limitations as biological control agents (Skurnik and Strauch, 2006). One of the most serious obstacles to successful use of phage therapy is the capacity of some temperate phages to transduce virulence factors (i.e., lysogenic conversion) to its bacterial host. This problem may be solved by selecting bacteriophages

as therapeutic agents that are well characterized at a genomic level, with no potential for inducing lysogenic conversion (Brussow, 2005; Carlton, 1999; Skurnik and Strauch, 2006).

Two unique *E. ictaluri*-specific phages ϕ eiAU (eiAU) and ϕ eiDWF (eiDWF) were isolated from aquaculture ponds with a history of ESC (Walakira et al., 2008). Phage eiAU was isolated in 1985 at Auburn University and phage eiDWF was recently isolated in 2006 in western Alabama. An additional *E. ictaluri*-specific bacteriophage ϕ eiMSLS (eiMSLS) was isolated directly from culture water from a commercial catfish aquaculture pond in Washington County, MS in 2004 (Timothy Welch, USDA National Center for Cool and Cold Water Aquaculture, WV personal communication). The isolation of each of these bacteriophages was accomplished by concentrating viruses from pond water samples by ultrafiltration and enriching for *E. ictaluri*-specific bacteriophages via enrichment in log-phase bacterial broth cultures. These three bacteriophages were classified initially within the family *Siphoviridae* due to their long, non-contractile tails, but their phylogenetic affiliation could not be assessed in the absence of phage genome sequence analysis (Chibani-Chennoufi et al., 2004; Lima-Mendez et al., 2008; Rohwer and Edwards, 2002). To date no other bacteriophage morphotypes have been observed to infect *E. ictaluri* from pond water enrichment experiments. A genomic analysis of these three phages was initiated to examine the potential of these three bacteriophages for lysogeny, to ensure they did not harbor virulence or toxin genes and to better understand the genetic basis of their host specificity (Walakira et al., 2008). This study represents the first genomic analysis of

bacteriophages specific to *Edwardsiella ictaluri*, and will expand scientific understanding of phage biology, and genomic information (Serwer et al., 2007).

3. Materials and methods

3.1. Bacteriophages and bacterial strains

Phages eiAU and eiDWF used in the study were originally isolated and characterized at Auburn University (Walakira et al., 2008). Phage MSLS was isolated from an aquaculture pond water sample on a lawn of *E. ictaluri* strain I49 (Thad Cochran National Warmwater Aquaculture Center, Aquatic Diagnostic Lab), and clear plaques were doubly purified on an *E. ictaluri* host. Host bacterial isolate *E. ictaluri* strain 219 was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. *E. ictaluri* strains were grown on Brain Heart Infusion (BHI) medium and cryopreserved in BHI containing 10% glycerol at -80°C. In each experiment bacterial strains were grown from the original glycerol stock to maintain low passage number, virulent *E. ictaluri* cultures.

3.2. Isolation of phage DNA

Phages eiAU, eiDWF, and eiMSLS were propagated on *E. ictaluri* strain 219 using a standard soft agar overlay method (Adams, 1959). Phages were harvested by flooding plates with 5 mL SM buffer (100 mM NaCl, 8 mM MgSO₄•7H₂O, 50 mM Tris-Cl (1 M, pH 7.5), and 0.002% (w/v) of 2 % Gelatin), incubating at 30°C while shaking for 6 h, and then collecting the buffer-phage solution. Collected phage suspensions were treated for 10 min with 1% (v/v) chloroform to lyse bacterial cells, subjected to

centrifugation at 3,600 x g for 25 min, and then filtered through a 0.22 µm filter to remove cell debris. Phage solutions were purified over a cesium chloride gradient and concentrated by precipitation with polyethylene glycol 8000. Concentrated phage particles were resuspended in 200 µl SM buffer. Free nucleic acids from lysed bacterial host cells were degraded with 250 units of benzonase endonuclease for 2 h at 37°C, after which the benzonase was inhibited by the addition of 10 mM EDTA. The phage protein coats were degraded using proteinase K (1 mg/ml) and SDS (1 %). A phenol-chloroform extraction was performed, and DNA was precipitated with ethanol. The washed DNA pellet was resuspended in T₁₀E₁ buffer (10mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at -20°C.

3.3. Shotgun library construction and sequencing

Shotgun subclone libraries were constructed at Lucigen Corporation (Middleton, WI) as previously described (Schoenfeld et al., 2008). Briefly, phage genomic DNA was randomly sheared using a Hydroshear instrument (Digilab Genomic Solutions, Ann Arbor, MI) and DNA fragments from 1 to 3 kb in size were extracted from an agarose gel. Phage DNA fragments were blunt-end repaired, ligated to asymmetric adapters, amplified using a proof reading polymerase and ligated into the pSMART[®] GC cloning vector following manufacturer recommendations. The ligation was transfected into electrocompetent *E. coli* cells. *Escherichia coli* transformants were robotically picked into Luria-Bertani (LB) broth containing 30 µg per ml kanamycin and 10 % (w/v) glycerol in a 96-well format using a QPix2 colony picking system (Genitex Limited, Hampshire, UK). Colony PCR was performed on a representative number of clones

(n=10) to assess insert size and the percentage of subclones containing an insert. Plasmid DNA was isolated using standard alkaline-SDS lysis and ethanol precipitation. Alternately, the insert was amplified from the *E. coli* clone glycerol stock using a pSMART vector-specific primer set, with 30 cycles of amplification (95°C denaturation, 50°C annealing, and 72°C extension). The resultant PCR products were treated with exonuclease I and Shrimp Alkaline Phosphatase to remove oligonucleotides. Sanger sequencing from both ends of the insert was obtained using ABI PRISM BigDye™ 3.1 Terminators chemistry (Applied Biosystems, Foster City, CA), and sequencing products were resolved on an ABI 3130XL capillary electrophoresis instrument.

3.4. Contig assembly and primer walking

Raw sequence data from eiMSLS was re-assembled using LaserGene software (DNASTAR Inc., Madison, WI). The eiMSLS sequence was used as a reference for alignment of eiAU and eiDWF sequences. For the latter two genomes, raw sequence data was trimmed for quality and vector sequence was removed using Sequencher™ software (Gene Codes Corporation, Ann Arbor, MI). Contigs were re-assembled using CromasPro v.1.42 (Technelysium Pty, Tewantin, Australia) using 70 % sequence match, and a minimum of 30 bp overlap. Contigs were manually edited to remove nucleotide gaps and mis-called bases. Closure of each respective phage genome was completed by primer walking using either the isolate phage DNA or amplified products as the sequencing template. Each phage was determined to have a circular genome by PCR amplification using primers directed out from the ends of the single large contig comprising the respective phage genome.

3.5. Genome sequence analysis

Open reading frames were identified using a GeneMark heuristic approach for gene prediction in prokaryotes, which is specifically designed for small virus, plasmid, or phage genomes less than 50 kb in size (Watanabe et al., 2007). Additionally, GLIMMER 3.02, and NCBI's ORF Finder (Khardori, 2006) were utilized to corroborate the predicted ORFs obtained from GenMark analysis. The % GC content of phages was calculated using geecee (Wagenaar et al., 2006). The tRNAscan-SE v.1.21 program was used to search for tRNA genes (Lowe and Eddy, 1997); (Fairbrother and Nadeau, 2006) Gene function was predicted by comparing each phage ORF sequence against the GenBank nr/nt sequence database using the BLASTp and BLASTn (Altschul et al., 1990) search algorithms. Iterative PSI-BLAST analysis was used to increase sensitivity of detecting homologous genes for ORFs resulting in hits with low E-values. Searches for secondary structures (profiles, patterns, blocks, motifs, and protein families) were performed using a web server (Oh et al., 2007). Frameshifts were detected using FrameD (Belley et al., 2006). The amino acid identity (AAI) of predicted protein sequences was determined by pairwise BLASTp analysis of each set of phage homologs. Dotplots were generated using the DOTMATCHER tool from EMBOSS (Ian Longden, Sanger Institute, Cambridge, UK). Pairwise global alignment and graphical representation of phage genomes was performed using the CGView server using tBLASTx with an E-value cutoff of 0.001 (Stothard and Wishart, 2005). Genome sequences were annotated using the Artemis software package (The Institute for Genomic Research), and all sequences were deposited in the GenBank database (accession # HQ824548-HQ824705) using Sequin.

3.6. Phylogenetic analysis

The predicted amino acid sequences for phage terminase large subunit and DNA polymerase were used to conduct a phylogenetic analysis of these *E. ictaluri* bacteriophages. The amino acid sequence for each predicted protein was aligned with a collection of homologous sequences using the program ClustalW2 (Larkin et al., 2007). ClustalW2 multiple alignments were exported to Mega4 (Tamura et al., 2007) and a maximum parsimony analysis was used to construct a phylogenetic tree, with bootstrap support (n=1000 replicates).

4. Results and Discussion

4.1. Genome characteristics

Total sequence coverage for the eiMSLS assembly was 9.8X, while coverage for the eiAU and eiDWF assemblies exceeded 30X. The genomes of phages eiAU, eiDWF, and eiMSLS are 42.80 kbp, 42.12 kbp, and 42.69 kbp, respectively. The % GC content is 55.37 %, 55.54 %, and 55.77 % for phage eiAU, eiDWF, and eiMSLS, respectively, and is similar to the 57% GC content of host *E. ictaluri* genome reference strain (GenBank accession NC 012779). No tRNA genes were detected in the genome of any of the three phages. This is unlike several members of the *Siphoviridae* family that carry tRNA genes (Kropinski and Sibbald, 1999).

4.2. Open Reading Frame (ORF) analysis

A total of 54 ORFs were predicted for phage eiAU (Table 1), while 52 ORFs were predicted for eiDWF and 52 ORFs for eiMSLS. Based on sequence similarity (E value < 0.001), 40 out of 54 (74%), 37 out of 52 (71%) and 36 out of 52 (69%) of the ORFs for phages eiAU, eiDWF, and eiMSLS, respectively, share significant sequence similarity to known protein sequences contained in the GenBank nr/nt database (Table 1). Of the ORFs with significant sequence similarity to sequences in GenBank, putative functions could only be assigned to 21 out of 40 (53%), 21 out of 37 (57%) and 20 out of 36 (56%) for phages eiAU, eiDWF, and eiMSLS, respectively. Positions, sizes, sequence homologies and putative functions for each predicted ORF are presented in Table 1.

The genome of phage eiAU contains several overlapping predicted ORFs, which can be an indication of translational coupling or programmed translational frameshifts (Bukovska et al., 2006). Twelve possible sequence frameshifts were predicted in the eiAU genome sequence. Interestingly, one of these frameshifts is conserved in tail assembly genes of dsDNA phages (Xu et al., 2004). In dsDNA phage genomes the order of the tail genes is highly conserved, most notably the major tail protein is always encoded upstream of the gene encoding the tape measure protein (Xu et al., 2004). Between these two genes, two overlapping ORFs are commonly found that have a translational frameshift (Levin et al., 1993). A similar organization of tail genes is observed in phage eiAU, in which two ORFs (22 and 23) lie between the putative phage tape tail measure protein gene (ORF21) and the major tail protein (ORF24) (Table 1). Similarly, phage eiAU contains a frameshift in the two overlapping ORFs between the phage tail measure and the major tail protein. In other phages both of these proteins are

required for tail assembly even though they are not part of the mature tail structure (Xu et al., 2004).

4.3. Overall Genome Organization and Comparison

A schematic representation of one of these phages (eiAU) shows that ORFs in these three phages are organized into two groups; early genes (DNA replication) that are encoded on one strand and the late genes (head, tail, and lysis) that are encoded on the complementary strand (Figure 1). Whole genome comparisons revealed that phages eiAU, eiDWF, and eiMSLS have conserved synteny (Figure 1 and Figure 2). The overall genetic organization of the eiAU, eiDWF, and eiMSLS genomes, typically consisting of “DNA packaging-head-tail-tail fiber-lysis/lysogeny-DNA replication-transcriptional regulation” modules is shared by many phage within the *Siphoviridae* family (Brüssow and Desiere, 2001).

Multiple sequence alignment analysis revealed that the eiAU, eiDWF, and eiMSLS genomes are > 95 % identical at the nucleotide level (Figure 2). Similarly, a high degree of sequence similarity has been observed in the genomes of recently sequence bacteriophages that infect *Campylobacter* (Timms et al., 2010), *Eschericia coli* (Denou et al., 2009), and also many *Mycobacterium* spp. (Hatfull et al., 2010). The high similarity of some phage genomes that infect a single host species suggests that certain phage lineages may be stable over time and over distant geographic areas (Timms et al., 2010). This observation may likely be clarified once additional genome sequences of phages infecting a common host such as *E. ictaluri* become available.

4.4. Comparison of head morphogenesis and structural proteins

Genome sequencing of tailed phages and prophages has revealed a common genetic organization of the genes encoding head morphogenesis and head structural proteins. These gene systems are typically organized as follows: 'terminase – portal – protease – scaffold – major head shell (coat) protein – head/tail-joining proteins – tail shaft protein – tape measure protein – tail tip/base plate proteins – tail fiber' (listed in the order of transcription) (Casjens, 2003). Phages eiAU, eiDWF, and eiMSLS follow a similar organization of genes encoding head morphogenesis and structural proteins, although the direction is reversed in relation to their order of transcription (Figure 1 and Table 1).

The module containing head morphogenesis and tail structure proteins in phage eiAU is the largest module, and is predicted to contain 22 ORFs (ORF14-ORF35). The consecutive ORFs 14 to 32 have significant sequence similarity with phage head morphogenesis and structural proteins, with putative function in tail assembly (ORFs 14, 17, and 18), tail fiber protein (ORF 15), phage host specificity (ORF 16), minor tail proteins (ORFs 19-21), major tail proteins (ORFs 24 and 25), major capsid proteins (ORF 29), structural proteins (ORFs 27, 30 and 33), and a phage head morphogenesis protein (ORF32) (Table 1). ORFs 28, 26, 23, and 22 could not be linked to a putative function based on BLAST search or any other similarity searches. However, all of these ORFs with the exception of ORF28 have sequence similarity to proteins identified within other phage genomes (Table 1). The protein products of ORF34 and ORF35 may encode large and small terminase subunits, respectively. ORF34 is predicted to encode the terminase large subunit. The top BLAST hit for ORF35 is the protein Gp1 encoded by *Sodalis*

phage SO-1; however, it is possible that ORF 35 encodes a small terminase subunit as there is limited sequence similarity to a putative terminase small subunit from *Listonella* phage phiHSIC. This indicates that these *E. ictaluri* phages, similarly to most dsDNA viruses, use a DNA packaging motor consisting of two nonstructural proteins (the large and small terminase subunits) encoded by adjacent genes (Lehman et al., 2009). Most known terminase enzymes have a small subunit that specifically binds the viral DNA and the large subunit with endonuclease activity for DNA cleavage and an ATPase activity that powers DNA packaging (Duffy and Feiss, 2002; Maluf et al., 2005).

No hit for a portal protein or for a protease was obtained either by BLAST or by HmmPfam searches. ORF33 is the most likely candidate for a portal protein based on the observation that the portal protein is generally located immediately downstream of the terminase gene (Bukovska et al., 2006).

4.5. Lytic Cassette

The lytic cassette of phage eiAU is predicted to be encoded by ORFs 36-39. ORF36 encodes a predicted endolysin, and a putative holin protein is encoded by ORF39. All dsDNA phages studied to date use two enzymes to lyse their host, an endolysin which degrades cell wall peptidoglycan and a holin which permeabilizes the cell membrane (Lehman et al., 2009). These two proteins work in conjunction to destroy the cell wall of bacteria and subsequently lyse the cell (Wang et al., 2000). These components of a host lysis cassette are each present in the genome of phages eiAU, eiDWF, and eiMSLS including a putative Rz lysis accessory protein encoded by ORF38 (Table 1.). The RZ protein is predicted to be a type II integral membrane protein and its function, although

not fully understood, may be required for host cell lysis only in a medium containing an excess of divalent cations (Summer et al., 2007). Phage endolysins have been linked to five enzymatic activities, including an N-acetyl muramidase or “true lysosyme”, the lytic transglycosylases, the N-acetylmuramoyl-L-alanine amidases, the endo- β -N-acetylglucosaminidases, and the endopeptidases (Borysowski et al., 2006). Secondary structure analysis predicts that the endolysin of eiAU is a member of the N-acetylmuramoyl-L-alanine amidases class of endolysins.

4.6. DNA replication proteins

ORFs with significant sequence similarity to proteins involved in DNA replication were identified in all three *E. ictaluri*-specific phage genomes. ORF44 is predicted to encode a phage replicative helicase/primase. Several phages use separate primase and helicase proteins while others use a multifunctional protein (primase/helicase) possessing both activities (Bukovska et al., 2006). The helicase/primase protein works in DNA replication by unwinding double stranded DNA into single stranded DNA (Egelman et al., 1995). No predicted function could be assigned to ORFs45 and 46. Also, no predicted function could be assigned to ORF47; however, a search for secondary structures within the predicted ORF47 amino acid sequence detected a helix-hairpin-helix DNA binding motif. Additionally, no putative function could be assigned to ORF48, ORF49, or ORF50. ORF51 had as one of its top BLAST hits an isoprenylcysteine carboxyl methyltransferase known to function in methylating isoprenylated amino acids (Clarke, 1993). ORF52 is predicted to encode a protein similar to gp41 of *Sodalis* phage SO-1, but no putative function could be assigned. ORF53 is predicted to encode DNA

polymerase I. Secondary structure analysis suggested that the DNA polymerase encoded by ORF53 contains a domain that is responsible for the 3'-5' exonuclease proof-reading activity of *E. coli* DNA polymerase I and other enzymes, and catalyses the hydrolysis of unpaired or mismatched nucleotides. The protein encoded by ORF54 is predicted to have a VUR-NUC domain, which are associated with members of the PD-(D/E) XK nuclease superfamily such as type III restriction modification enzymes. ORF2 is predicted to encode a DNA repair ATPase. A search for secondary structures within the ORF2 predicted amino acid sequence revealed a HNH endonuclease. No putative function could be assigned to ORF3. ORF4 is predicted to encode a helicase protein belonging to the SNF2 family, commonly found in proteins involved in a variety of processes including transcription regulation, DNA repair, DNA recombination, and chromatin unwinding (Eisen et al., 1995). ORF6 is predicted to encode a phage methyltransferase. Secondary structure analysis revealed that the methyltransferase predicted to be encoded by ORF6 is a C-5 cytosine-specific DNA methylase which in bacteria is a component of restriction-modification systems. Also, Mg⁺ and ATP binding sites were detected in the predicted protein product of ORF6. ORF7 is predicted to encode a DNA N-6-adenine-methyltransferase within a family of methyltransferase found in bacteria and phage that has site specific DNA methyltransferase activity (Schneider-Scherzer et al., 1990).

No ORF encoding an RNA polymerase was detected in any of the phages suggesting that these phages rely on the host RNA polymerase to transcribe their genes. This is further corroborated by the observation that no phage-encoded transcription factor was detected in the genome of these phages.

4.7. Comparison of ORFs among phages eiAU, eiDWF, and eiMSLS

The three phage genomes revealed extensive homology and limited variability in their gene sequence (Figure 2). The percent identity and percent similarity of each ORF within the three phage genomes (data not shown) revealed that differences exist mainly in predicted ORFs that have no significant sequence similarity to sequences in GenBank database and also to ORFs encoding structural proteins (primarily the tail fiber genes). ORF14 (117 AA) is predicted to encode a phage tail fiber assembly protein/tail assembly chaperone, and in eiAU and eiDWF it is 100 % identical, yet it is not present in eiMSLS. ORF15 (335 AA) is predicted to encode a tail fiber protein and is present in all three phages, with 100 % identity in eiAU and eiDWF, however, it only has 58 % identity to its counterpart in eiMSLS. ORF21 (900 AA) is predicted to encode a phage tail tape measure protein and is present in all three phages at approximately 95 % identity at the amino acid level. ORF23 (118 AA) is predicted to encode a protein homologous to gp15 [*Sodalis* phage SO-1] which is a structural protein that plays a role in cell membrane penetration. This ORF is present in all three phages with 83 % identity at the amino acid level. ORF24 (200 AA) is predicted to encode a major tail protein and is present in all three phages, with 100 % identity between eiDWF and eiMSLS, and with only 90 % identity between those two phage and the ORF counterpart in eiAU. Sequence differences in these structural proteins may help explain the differences observed in the efficiency of these phages to form plaques on various *E. ictaluri* strains (Walakira et al., 2008). Most of the structural proteins described above are expected to be involved in phage infectivity such as adsorption of the phage to the bacterial cell (ORFs 14 and 15), phage tail length (ORF21), and cell membrane penetration (ORF23).

Differences were also observed in the ORFs encoding the putative methyltransferases. In phage eiAU, ORF6 and ORF7 are predicted to encode a phage methyltransferase and a DNA N-6-adenine-methyltransferase respectively, while in phage eiDWF and eiMSLS only one larger ORF encoding a phage methyltransferase was predicted. Similarly, two methyltransferases are present in the genomes of one of two highly similar *Campylobacter* phages (Timms et al., 2010). The authors suggest that the two methyltransferases may enable the phage to avoid DNA restriction in some strains through DNA methylation. This may help explain the differences observed in host range for the *Campylobacter* phages (Timms et al., 2010) as well as differences observed in host specificity of the *E. ictaluri* phages (Walakira et al., 2008). Hence, these methyltransferases may likely be involved in DNA methylation as a means of avoiding the restriction endonuclease (s) of *E. ictaluri*.

4.8. Classification of phages eiAU, eiDWF, and eiMSLS

The majority of the top BLAST hits for these phage genomes are to proteins belonging to lytic phages, including *Yersinia* phage PY100, *Salmonella* phage c341, and *Enterobacteria* phage HK97 (Table 1.). All of the components of a phage lysis cassette (endolysin, holin, and a lysis accessory protein) were detected in these phages and no sequence similarity to lysogenic phages or to any component that is associated with lysogeny such as integrase/recombination associated enzymes, repressor proteins, and anti-repressor proteins (Oakey et al., 2002) were detected. These data along with results documenting the lytic capabilities of these phages (Walakira et al., 2008) all indicate that these phages lack mechanisms for integration into the DNA of their host and that they are

virulent phages without the capacity for lysogeny. Additionally, none of the predicted proteins have similarities to known bacterial pathogenicity factors. These observations indicate that these phages lack any lysogenic or bacterial virulence-inducing capacity that would preclude their potential use as therapeutic agents.

Taxonomic classification of these *E. ictaluri*-specific phages must rely upon a synthesis of morphological and genomic information, considering that phage evolution has been profoundly directed by lateral gene transfer (Nelson, 2004), and that a rational hierarchical system of phage classification should be based on the degree of DNA and protein sequence identity for multiple genetic loci (Proux et al., 2002). Gene modules that have been proposed for using as basis of a phage taxonomy system include the DNA packaging-head gene cluster, the structural gene architecture, and phage tail genes (excluding the tail fiber genes) (Brüssow and Desiere, 2001).

A comparison of phage eiAU to *Enterobacteria* phage SSL-2009a was conducted due to the large number of significant BLAST hits between ORFs in the *E. ictaluri* phage genomes and those respective ORFs within the genome of phage SSL-2009a, which are on average 34.1 % identical at the nucleotide level. A comparative genomic analysis between the genome of phage eiAU and that of phage SSL-2009a revealed that genome regions encoding many putative structural and replication proteins are shared by both phages (Figure 2 and Figure 3). The predicted gene products with sequence similarity between the eiAU and SSL-2009a phage genomes include the putative minor tail proteins/tail tape measure, major tail proteins, major capsid proteins, head morphogenesis, phage terminase small subunit, and the phage terminase large subunit. Interestingly, other structural proteins including the host specificity proteins, the

tail assembly proteins, and particularly the tail fiber/baseplate protein which has been recommended for exclusion in any sequence based phage taxonomy scheme (Proux et al., 2002) are not shared between the two genomes.

4.9. Phylogeny based on multiple genetic loci

The genetic conservation observed in the structural proteins between phage eiAU and *Enterobacteria* phage SSL-2009a led us to further investigate the relatedness of these *E. ictaluri* phages and other enterobacteria phage, based on specific phage genetic loci. The amino acid sequences of one of the conserved structural proteins (large terminase subunit) as well as one of the non structural proteins (DNA polymerase I) were chosen for phylogenetic analysis. The large terminase subunit which is a structural protein is along with the portal protein considered the most universally conserved gene sequence in phages (Casjens, 2003), hence they are good options to aid in phage classification. Phylogenetic analysis based on the large terminase subunit amino acid sequence (Figure 4) and the DNA polymerase I amino acid sequence (Figure 5) of eiAU reveal that phages eiAU, eiDWS, and eiMSLS were most similar to phage that infect other enterobacteria (*Enterobacteria* phage SSL-2009a) and *Sodalis glossinidius* (*Sodalis* phage SO-1). These two phages are dsDNA viruses belonging to the Caudovirales order, one being a *Siphoviridae* (*Sodalis* phage SO-1) (NCBI accession # [NC_013600](#)) and the other an unclassified member of the Caudovirales (*Enterobacteria* phage SSL-2009a) (NCBI accession # [NC_012223](#)). The overall genomic organization of the three new phages is shared by many members of the *Siphoviridae* family of phages sequenced to

date (Brüssow and Desiere, 2001), and is supported by the previously described morphology of these phages (Walakira et al., 2008).

5. Conclusion

This is the first genomic analysis of bacteriophages that infect the bacterial pathogen *E. ictaluri*. Phylogenetic analysis of multiple phage gene products suggests that these phages are similar to those that infect other *Enterobacteria* hosts. The bioinformatic analysis of the genomes of these three *E. ictaluri*-specific bacteriophages corroborate previously published data that indicates that these bacteriophages are lytic, and lack any mechanism for lysogenic conversion of their host. Additionally, none of the predicted proteins have similarities to known bacterial pathogenicity factors or to toxin genes. Even though these three bacteriophages were isolated in different geographic locations within the natural range of catfish over twenty years apart, they are remarkably similar to each other at a genomic level. This genomic analysis suggests that these phages are members of a lineage that is highly stable over time and geographic regions. The information obtained from the analyses of these bacteriophage genomes will facilitate their diagnostic and therapeutic applications.

Table 1. Predicted ORFs for ϕ eiAU, ϕ eiDWF, and ϕ eiMSLS, and the most similar BLAST hits for each of the phage ORFs.

ϕ eiAU						Accession #	Best match E value/ % aa identity	Presence in	
ORF/ Strand	Position Start Stop	Size bp aa	Putative function [Nearest neighbor]					ϕ eiDWF	ϕ MSLS
1/-	220 459	240 79	None					[+]	[+]
2/+	458 925	468 155	DNA Repair ATPase [<i>Salmonella</i> phage]			YP_003090241.1	1E-49/67	[+]	[+]
3/+	922 1260	339 112	None					[+]	[+]
4/+	1319 2668	1350 449	helicase [<i>Enterobacteria</i> phage]			YP_002720041.1	0.0/70	[+]	[+]
5/+	3035 3211	177 58	None					[-]	[-]
6/+	3239 4126	888 295	phage methyltransferase [<i>Edwardsiella tarda</i>]			ZP_06713110.1	4E-98/69	[+]	[+]
7/+	4126 4836	711 236	N-6-adenine-methyltransferase [<i>Escherichia coli</i>]			YP_003041971.1	3e-18/45	[-]	[-]
8/+	5164 5526	363 120	None					[+]	[+]
9/+	5523 5804	282 93	None					[+]	[+]
10/-	6073 5816	258 85	None					[+]	[-]
11/-	6581 6060	522 173	hypothetical protein [Phage PY100]			CAJ28429.1	8E-20/38	[+]	[+]
12/-	6869 6603	267 88	None					[+]	[+]
13/-	7721 7020	702 233	hypothetical protein [Phage PY100]			CAJ28427.1	2E-09/36	[+]	[+]

14/-	8175 7822	354	117	phage tail assembly chaperone gp38 [<i>Enterobacter</i> sp.]	YP_001178193.1	9e-11/53	[+]	[-]
15/-	9179 8172	1008	335	tail fiber protein [<i>Enterobacteria</i> phage]	NP_037718.1	2e-10/38	[+]	[+]
16/-	12809 9198	3612	1203	phage host specificity protein [<i>Yersinia kristensenii</i>]	ZP_04623740.1	0.0/42	[+]	[+]
17/-	13333 12809	524	174	phage tail assembly protein [<i>Yersinia enterocolitica</i> phage]	YP_001006526.1	8E-50/59	[+]	[+]
18/-	14112 13393	720	239	phage minor tail protein [<i>Enterobacteria</i> phage]	YP_002720062.1	4E-56/48	[+]	[+]
19/-	14887 14117	771	256	phage minor tail protein L [<i>Yersinia pseudotuberculosis</i>]	YP_001721823.1	5E-66/51	[+]	[+]
20/-	15228 14884	345	114	phage minor tail protein M [<i>Enterobacteria</i> phage phi80]	CBH95068.1	1E-12/39	[+]	[+]
21/-	17990 15288	2703	900	phage tail tape measure protein [<i>Enterobacteria</i> phage]	YP_002720065.1	8E-126/38	[+]	[+]
22/-	19188 18862	327	108	gp16 [<i>Sodalis</i> phage SO-1]	YP_003344951.1	5E-20/48	[+]	[+]
23/-	19523 19167	357	118	gp15 [<i>Sodalis</i> phage SO-1]	YP_003344950.1	9E-16/38	[+]	[+]
24/-	20305 19703	603	200	putative major tail protein [<i>Enterobacteria</i> phage]	YP_002720068.1	2E-54/58	[+]	[+]
25/-	20766 20338	429	142	gp13 [<i>Sodalis</i> phage SO-1]	YP_003344948.1	1E-08/38	[+]	[+]
26/-	21395 20763	633	210	gp12 [<i>Sodalis</i> phage SO-1]	YP_003344947.1	6E-53/55	[+]	[+]
27/-	21748 21392	357	118	phage structural protein [<i>Enterobacteria</i> phage]	YP_002720071.1	9E-23/48	[+]	[+]
28/-	21884 21729	156	51	None			[+]	[+]
29/-	22387 21887	501	166	hypothetical protein EpSSL_gp33 [<i>Enterobacteria</i> phage]	YP_002720072.1	2E-22/43	[+]	[+]
30/-	23550 22450	1065	353	phage structural protein [<i>Enterobacteria</i> phage]	YP_002720073.1	1E-65/59	[+]	[+]

31/-	24306	23638	669	222	hypothetical protein EpSSL_gp36 [<i>Enterobacteria</i> phage]	YP_002720075.1	1E-39/50	[+]	[+]
32/-	25520	24393	1128	375	phage head morphogenesis protein [<i>Enterobacteria</i> phage]	YP_002720086.1	2E-123/58	[+]	[+]
33/-	26964	25504	1461	486	phage structural protein [<i>Enterobacteria</i> phage]	YP_002720085.1	1E-153/57	[+]	[+]
34/-	28358	26976	1383	460	phage terminase large subunit [<i>Enterobacteria</i> phage]	YP_002720084.1	4E-162/64	[+]	[+]
35/-	28855	28358	498	165	gp1 [<i>Sodalis</i> phage]	YP_003344936.1	2E-24/48	[+]	[+]
36/-	29356	29090	267	88	endolysin [<i>Yersenia</i> Phage PY100]	CAJ28446.1	7E-14/48	[+]	[+]
37/-	29775	29500	276	91	prophage Lp2 protein 33 [<i>Streptococcus pneumoniae</i>]	ZP_01821446.1	2E-09/45	[+]	[+]
38/-	30311	29826	486	161	putative lysis accessory protein [<i>Escherichia</i> phage]	YP_512284.1	1E-10/39	[+]	[+]
39/-	30559	30308	381	127	Putative holin [<i>Burkholderia multivorans</i> CGD1]	ZP_03586913.1	5E-05/30	[+]	[+]
40/-	30996	30775	222	73	None			[+]	[+]
41/-	31670	31026	645	214	None			[+]	[+]
42/-	32769	32128	642	213	Conserved phage protein [<i>Enterobacteria</i> phage]	ADE87955.1	2E-27/37	[+]	[+]
43/-	33112	32882	231	76	None			[+]	[+]
44/-	35397	33988	1410	469	phage replicative helicase/primease [<i>Enterobacteria</i> phage]	YP_002720055.1	7E-114/58	[+]	[+]
45/+	35764	36093	330	109	None			[+]	[+]
46/+	36115	36282	168	55	None			[+]	[+]
47/-	36455	36339	117	38	None			[+]	[+]

48/+	36834	37277	444	147	gp46 [<i>Sodalis</i> phage]	YP_003344981.1	5E-04/36	[-]	[+]
49/+	37326	37862	537	178	gp27 [<i>Sodalis</i> phage]	YP_003344962.1	5E-04/40	[+]	[+]
50/+	37865	38098	234	77	None			[-]	[+]
51/+	38101	39360	1194	396	gp43 [<i>Sodalis</i> phage]	YP_003344978.1	9E-49/50	[+]	[+]
52/+	39455	40192	738	245	gp41 [<i>Sodalis</i> phage]	YP_003344976.1	4E-56/64	[+]	[+]
53/+	40252	42459	2208	735	DNA polymerase I [<i>Enterobacteria</i> phage]	YP_002720046.1	0.0/64	[+]	[+]
54/+	42470	42748	279	92	gp36 [<i>Sodalis</i> phage SO-1]	YP_003344971.1	1E-22/60	[+]	[+]

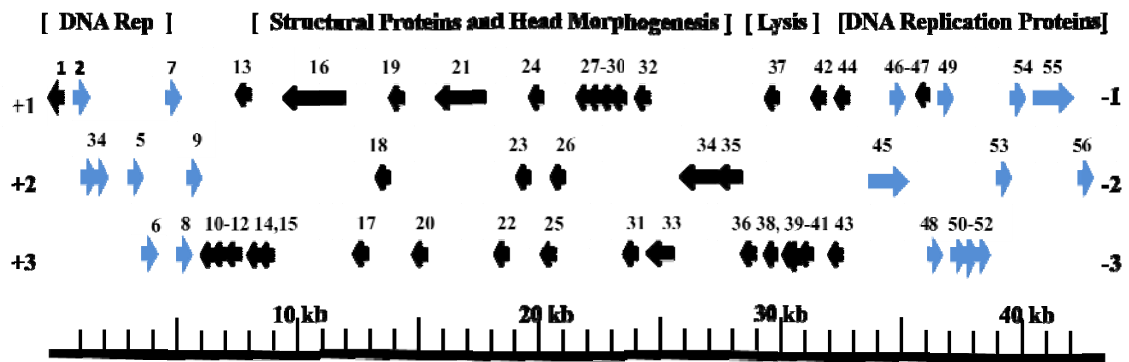


Figure 1. Schematic representation of the genome sequence of bacteriophage eiAU showing its overall genomic organization. The ORFs are numbered consecutively (see Table 1) and are represented by arrows based on the direction of transcription. The numbers +1, +2, +3 represent corresponding reading frames.

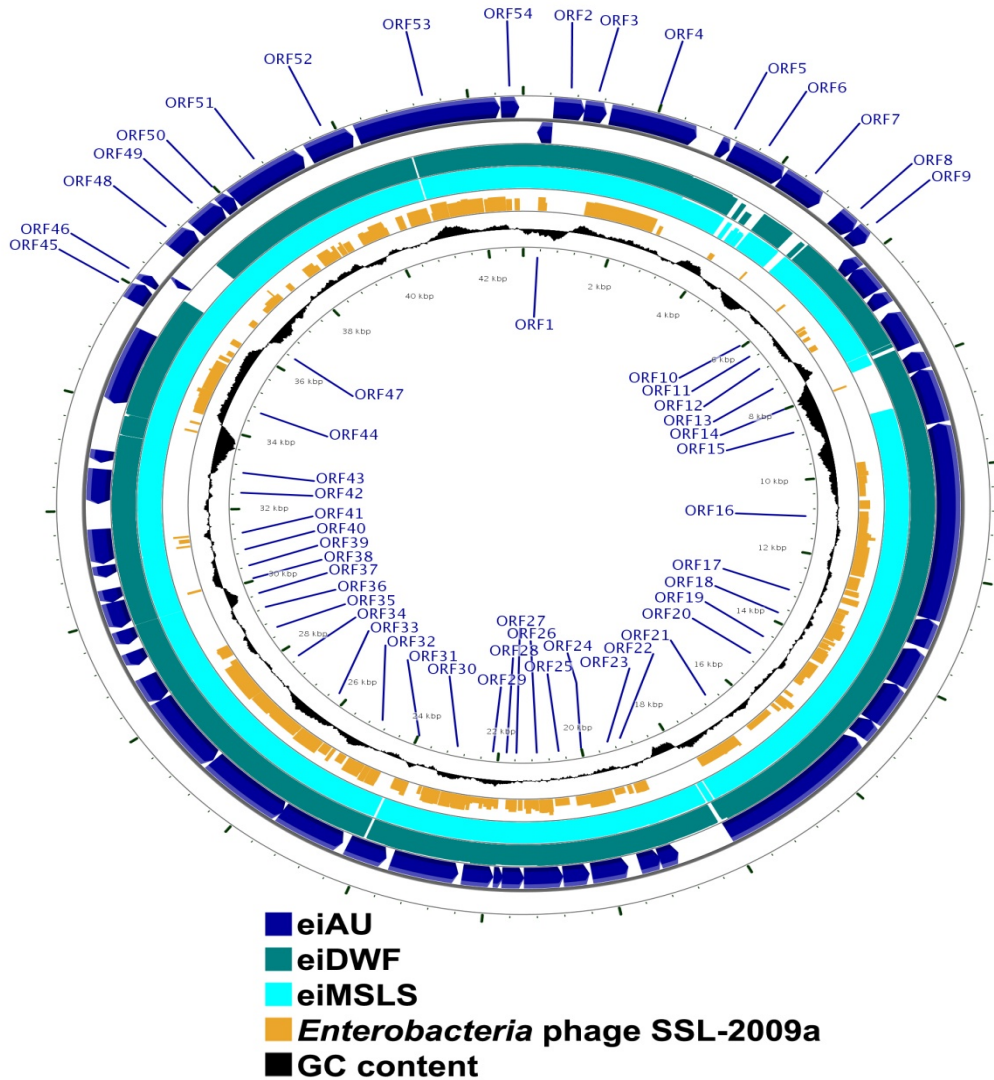


Figure 2. Circular representation depicting the genomic organization of eiAU (two outermost circles, dark blue, showing each predicted ORF and its direction of transcription) and a tBLASTx comparison with the genomes of eiDWF (third circle from outside, green), eiMSLS (fourth circle from outside, light blue), and *Enterobacteria* phage SSL-2009a (fifth circle from outside, orange). The degree of sequence similarity to eiAU is proportional to the height of the bars in each frame. The %G+C content of eiAU is also depicted (sixth circle from outside, black). This map was created using the CGView server (Grant and Stothard, 2008).

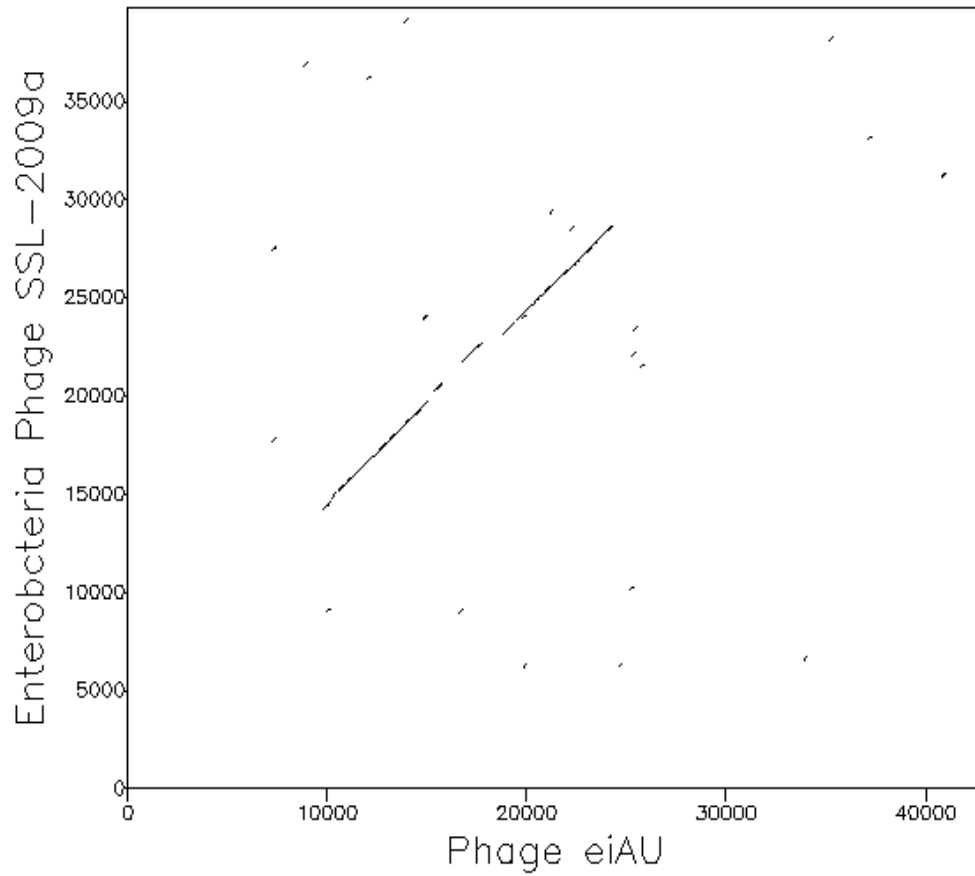


Figure 3. DotPlot matrix comparing the relatedness of the genome sequence of phages eiAU and Enterobacteria phage SSL-2009a. Windows size of 150 and threshold value of 50 were used in Dotmatcher program.

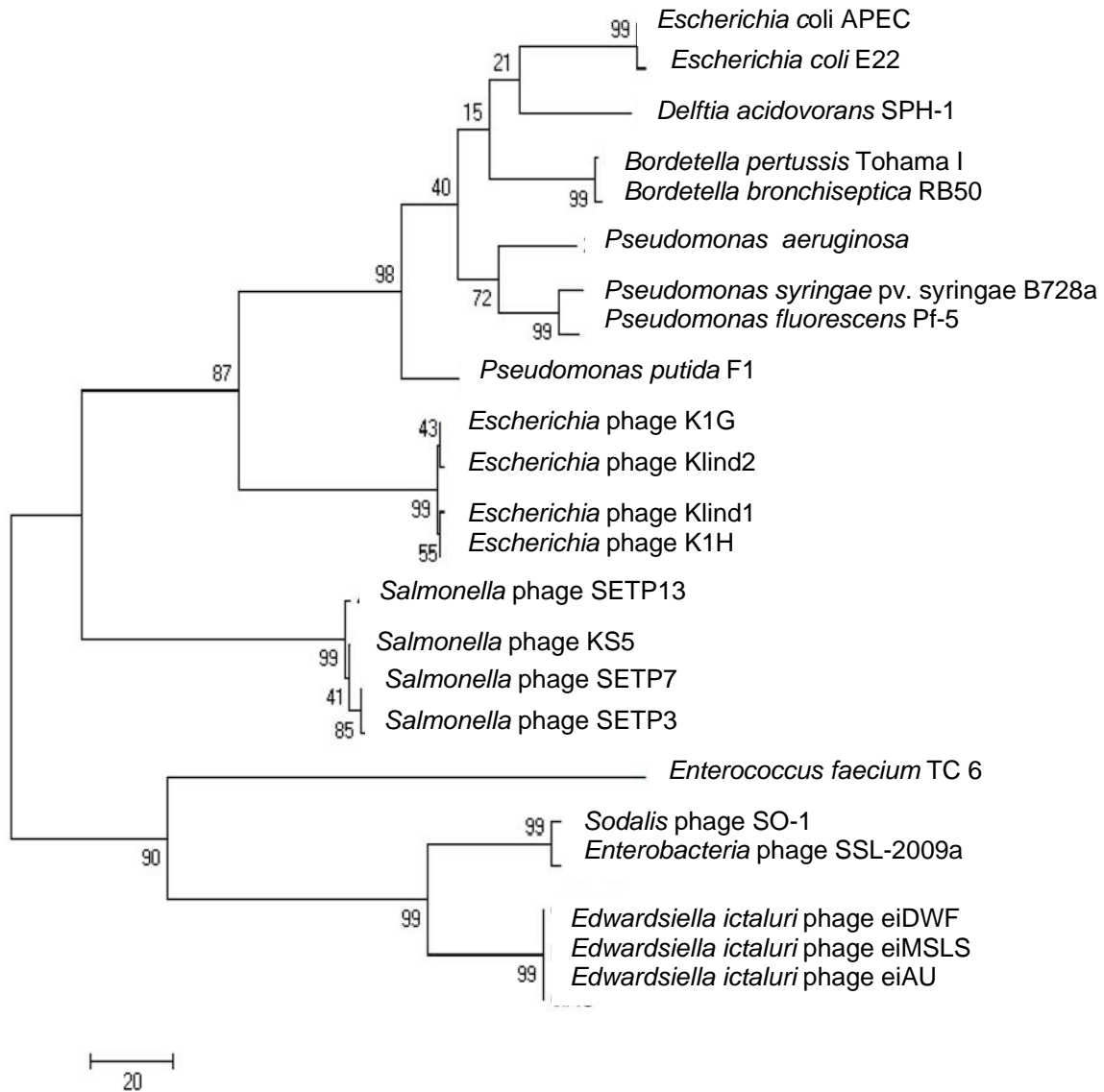


Figure 4. Rooted maximum parsimony tree based on the aligned amino acid sequences of the large terminase subunit gene of phage eiAU and 25 other large terminase genes from diverse phage genomes. The numbers at the nodes represent bootstrap values based on 1,000 resamplings.

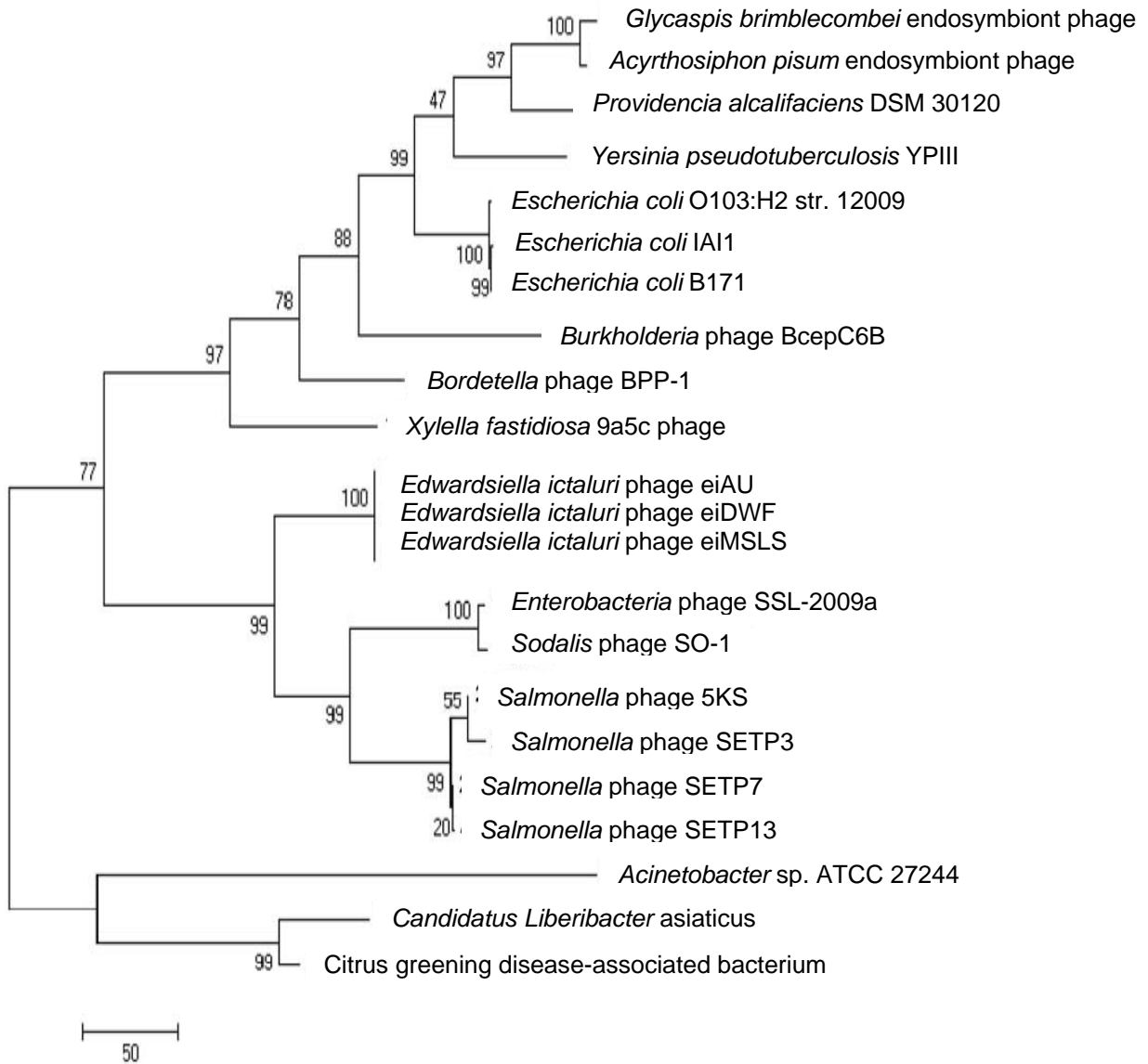


Figure 5. Rooted maximum parsimony tree based on the aligned amino acid sequences of the DNA polymerase subunit gene of phage eiAU and 33 other DNA Polymerases from diverse phage genomes. The numbers at the nodes represent bootstrap values based on 1,000 resamplings.

CHAPTER IV

Application of bacteriophage for biocontrol of enteric septicemia of catfish

1. Abstract

Three lytic *Edwardsiella ictaluri*-specific bacteriophages (eiAU, eiDWF, and eiAU P10) were evaluated for their stability in phage-amended feed, and for their efficacy to protect channel catfish *Ictalurus punctatus* fingerlings from *E. ictaluri* infections. In bacteriophage-amended feed, bacteriophage eiAU titers remained stable exhibiting no significant drop in feed stored at 4 °C and 24 °C for 21 days. However, titers in feed stored at 37 °C remained stable for 8 d and then significantly declined. A one-time administration of bacteriophage, and administration of three different bacteriophage types (eiAU, eiDWF, and eiAU P10) for 15 days, demonstrated limited protection against *E. ictaluri* infection. Protection was observed when bacteriophage was administered for 27 consecutive days, starting 3 days pre-challenge. Additionally, protection was also observed when bacteriophage was administered at a high dose (10^{10} PFU/gram of feed), but not when administered at lower dosages (10^7 and 10^4 PFU/gram of feed). During the challenge studies no bacteriophage-resistant *E. ictaluri* isolates were recovered and no adverse effect was grossly observed in fish that received

bacteriophage-only administration.

We conclude that although the genetic homogeneity of *E. ictaluri* makes it a good candidate for bacteriophage therapy, the inability of bacteriophages used in this study to cross into blood circulation as previously observed, in conjunction with the capacity of *E. ictaluri* to rapidly penetrate, cross the epithelium barrier of catfish, and become intracellular, limits the potential for bacteriophage-bacterial interaction resulting in ineffective use of a bacteriophage therapy approach for the control of *E. ictaluri*.

2. Introduction

Enteric septicemia of catfish (ESC) caused by the pathogen *Edwardsiella ictaluri* causes yearly losses of \$40-60 million dollars to the commercial catfish industry in the US (Shoemaker et al., 2009). In addition, an ESC outbreak may increase production cost due to poor feed efficiency, harvest delays, and from mortality due to secondary infections from opportunistic pathogens (Wagner et al., 2002). Currently, treatment of ESC relies on the use of two approved antibiotics, Aquaflor® (florfenicol) and Romet® (ormetoprim plus sulfadimethoxine). Data from diagnostic services for the years 1999 to 2007 (USDA, 2009) indicate that the number of farms experiencing ESC remained relatively unchanged, supporting other evidence (Storey 2005) that additional options for the control of ESC are required. Development and commercialization of additional antibiotics may be an option; however, the widespread discovery of antibiotic resistant bacteria in aquaculture environments (DePaola et al., 1995) (Geneva 2006) raises concerns about its long term dependency for use in aquaculture. Additionally, economic costs to drug companies to develop and gain governmental approval for use in fish used for human consumption may be prohibitive.

Lytic phages or viruses that infect and kill bacteria may be an attractive option to control diseases in catfish aquaculture caused by bacterial pathogens. They are highly specific, often restricted to particular strains within a single bacterial species (Monk et al., 2010) thereby posing minimal threat to beneficial indigenous flora in animals (Balogh et al., 2010; Greer, 2005) and human consumers. Additionally, phages are naturally present in the environment (Goyal et al., 1987), and phage preparations are relatively easy and inexpensive to prepare (Greer, 2005).

Phage therapy studies in human medicine and in other areas of agriculture have reported inconsistent and variable results. In aquaculture, several studies have recently evaluated the efficacy of phages to control diseases in fish and other aquatic organisms of economic importance. Protective effects of phage therapy has been demonstrated in the control of *Lactococcus garvieae* in yellowtail *Seriola quinqueradiata* (Nakai et al., 1999), *Pseudomonas plecoglossicida* in ayu *Plecoglossus altivelis* (Park and Nakai, 2003; Park et al., 2000), *Aeromonas salmonicida* in brook trout *Salvelinus fontinalis* (Imbeault et al., 2006), and *Vibrio harveyi* in *Penaeus monodon* shrimp larvae (Vinod et al., 2006). However, phages did not prove effective in reducing mortalities in Atlantic salmon *Salmo salar* L. caused by *Aeromonas salmonicida* subsp. *salmonicida* 78027 (Verner-Jeffreys et al., 2007).

The variable results obtained from phage therapy studies may be attributed to issues such as lack of understanding of phage-host interaction, virulence and pathogenicity of the specific bacteria targeted, and inability of phage to reach bacteria at the sites of infection inside the model organism (O'Flynn et al., 2004). Additionally, the potential for development of phage resistance, the capacity of some phages to transduce

undesirable characteristics to its host (e.g. toxins, virulence factors, etc.), the typically narrow host range of phages, rapid clearance by the reticulo-endothelial system, potential for antibody production against phage particles, and environmental effects can be barriers to success in phage therapy to control bacterial diseases (Balogh et al., 2010). These issues must be taken into consideration when evaluating the use of phages to control diseases in the model of infection used and for the phage-host-environment complex specific for the particular bacteria targeted.

The objective of this study was to evaluate the use of lytic *E. ictaluri*-specific phages to control experimentally induced ESC in channel catfish *Ictalurus punctatus* in aquaria. This is the first study in the US to evaluate the use of bacteriophages to control a major disease in aquaculture.

3. Materials and methods

3.1. Phage and Bacteria

Two previously characterized bacteriophages (eiAU and eiDWF) (Walakira et al., 2008) and one variant (eiAU P10), selected by passaging eiAU in catfish fingerlings, were used in this study. High titer phage stocks were propagated on *E. ictaluri* cultures using the double agar method (Adams, 1959). Briefly, 5 ml of semi-solid (0.7 %) tryptic soy broth (TSB) or brain heart infusion (BHI, Difco Laboratories, Detroit, Michigan) was inoculated with 100 µl triple-purified phage stock plus 200 µl of culture of *E. ictaluri* grown overnight, poured onto agar plates, and incubated for at least 15 hr at 30 °C. Phages were recovered from media plates by pouring SM buffer (100 mM NaCl, 8 mM MgSO₄•7H₂O, 50 mM Tris-Cl [1 M, pH 7.5]) over agar plates, incubating for an

additional 3-6 h, and then collecting the SMB-phage suspension. Phage suspension was treated with chloroform at a rate of 1:100 (chloroform to phage suspension), and then centrifuged at 3600 x g for 25 min. Phages used in challenge studies were not treated with chloroform. After centrifugation, the supernatant containing phages was filtered through a 0.2 µM filter and then concentrated by precipitation with polyethylene glycol 8000. Phage stocks were stored at 4 °C for later use.

Edwardsiella ictaluri isolate 219 and S97-773 were obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. Both isolates 219 and S97-773 were used in phage propagation, but only isolate S97-773 was used in fish challenges. Isolate S97-773 was passed through catfish prior to challenge experiments to confirm virulence.

Bacteria cultures for challenge were prepared by inoculating 5 ml BHI with 200 µl of a frozen stock (−80 °C) of *E. ictaluri* culture. The 5 ml culture was incubated for 24 h at 30 °C while shaking at 150 rpm, and then used to inoculate 100 ml of fresh BHI. The second inoculated culture was then incubated for an additional 24 h at 30 °C while shaking at 150 rpm. Prior to use for challenge, the bacterial culture was centrifuged at 3600 x g for 30 min, re-suspended in 100 ml of fresh BHI, and allowed to grow an additional 3 h. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

3.2. Preparation of phage-amended feed

Commercially available slow-sinking pelleted fish feed (2 mm, 40 % protein, Zeigler, Gardners, PA) was frozen and then lyophilized using a VirTis Unitrap 10-100

manifold-style freeze-dryer (VirTis, Gardiner, NY). Lyophilized feed was spray-coated with a phage solution at a rate of 8 % of the dry/lypholyzed weight of the feed. Feed was immediately coated a second time with menhaden fish oil (5 %) at a rate of 4 % of the dry feed weight. Enough feed was prepared to feed fish for the duration of each treatment period and was stored at 4 °C. The concentration of phage in feed was determined by homogenizing eight, 0.1 g replicate samples of feed each in 900 µL of 10 % beef extract, serially diluting (10-fold), and spotting on a lawn of *E. ictaluri* S97-773. Phage titers on feed pellets were quantified twice during the duration of each challenge trial.

3.3. Stability of phage in phage-amended feed

The stability of phage eiAU on sprayed-coated feed pellets was investigated after storage at different temperatures for 21 days. Feed (100 g) was supplemented with 1.5×10^8 PFU/ml of eiAU as described above. The feed was distributed equally into four replicate sterile 50 ml polypropylene plastic tubes for each temperature held and stored in one of three temperature conditions: 4 °C, 24 °C (room temperature), and 37 °C. At the time of storage, four, 0.1 g feed samples were randomly removed aseptically from each temperature condition, weighted, and individually re-suspended in 900 µl of 10 % beef extract. The feed samples were left undisturbed for 30 min, and then disaggregated/grounded for 1 minute using a Pellet Pestle[®] Mortar (Kontes, Vineland, New Jersey USA). Feed samples were centrifuged at 10,000 x g for 10 minutes. After centrifugation, the supernatant from each centrifuged feed sample was removed and placed into a new, 2 ml micro centrifuge tube. Ten-fold serial dilutions were made of

each supernatant and 10 µl of each dilution were spotted onto a lawn of *E. ictaluri* S97-773 for phage quantification. Four feed samples from each storage condition were removed at 24 h intervals for 21 days, and processed as above.

The capacity of phage-amended feed pellets to retain phage once it was placed inside water was also evaluated. Phage (eiAU P10) amended feed pellets were coated with phage 1×10^7 PFU/ml) as described previously. Twenty, 0.1 g phage-coated feed samples were each individually weighed and transferred into one of 20, 100 ml disposable plastic containers. Each container contained 10 ml of filtered (0.22 µm) pond water. Immediately after placing the feed into the water each container was vortexed and then left undisturbed at room temperature (24 °C). At 0, 15, 30, 45, and 60 min post-addition of feed into the water four feed samples were chosen at random for phage quantification. The water from each chosen sample was decanted, and the feed pellets removed aseptically. Pellets from each sample were placed into a 2 ml micro centrifuge tube containing 450 µl of 10 % beef extract, homogenized for 1 minute using a Pellet Pestle[®] Mortar (Kontes, Vineland, New Jersey USA) and then an additional 450 µL of beef extract was added to each tube. Homogenized samples were kept at room temperature until all samples had been processed for all time points. Each homogenized sample was mixed by vortexing and then centrifuged at 10,000 x g for 10 min using an Eppendorf 5424 centrifuge (Eppendorf, Hamburg, Germany). The supernatant from each centrifuged sample was transferred into sterile micro centrifuge tubes and serially diluted (10-fold) six times. Dilutions were spotted onto an overlay of *E. ictaluri* isolate S97-773 for phage quantification.

3.4. Disease challenge protocol

For challenge, *E. ictaluri* was grown in BHI culture media as described above and added directly into each aquarium. Prior to adding bacteria the water flow into each aquarium was restricted for one hour, after one hour exposure in the aquarium to the bacteria, the water flow was resumed. Water volume in each aquarium (15 L) was standardized in order to provide equal bacterial concentrations during challenge.

3.5. Fish and husbandry activities

Channel catfish fingerlings used in this study were grown from larvae housed at indoor facilities either at the College of Veterinary Medicine or at the E.W. Shell Fisheries Research Station, Auburn University, AL. No known exposure to *E. ictaluri* had been documented in these fish populations and fish were considered specific pathogen free. Prior to challenge, fish were placed into 60 L aquarium containing 15 L of well water and acclimated to experimental conditions. Continuous flow-thru water supply (0.3 l/min) maintained at 26 °C and continuous aeration was provided to each aquarium individually. Industrial grade salt was injected into the supply water in order to increase chloride levels and water hardness of the incoming water to a minimum of 0.4 ppt. Fish were fed once a day at approximately 2 % body weight for the duration of all challenge trials. Un-eaten feed and waste material were siphoned out of each aquarium as needed. Each treatment had its own set of equipment such as nets and siphoning hose and disinfected after every use to avoid cross contamination.

During the challenge experiments, fish were observed daily for behavioral changes and for gross signs of disease. Moribund and dead fish were removed twice

daily. Moribund/freshly dead fish (n=15) were necropsied in each challenge experiment and samples from trunk kidney, liver and spleen were streaked on BHI agar plates for bacterial isolation. Bacteria that were isolated from these tissues were identified biochemically (Hawke et al., 1981b) as well as tested for their susceptibility to phage lysis. At the end of each challenge experiment all surviving fish were counted, euthanized with 300 ppm MS-222, and disposed. The intestines, trunk kidney, liver, and spleen from necropsied fish were removed and placed in 10 % beef extract containing 10 % glycerol and stored at -80⁰C for later analysis for the presence phage.

3.6. Challenge experiments

In the first challenge, an experiment was performed to test the effects of a one-time application of phage eiAU to control induced ESC in catfish fingerlings. Ten, channel catfish fingerlings (10-14 cm) were placed into each of 10, 60 L aquaria containing 15 L of filtered well water. In Treatment 1, all fish in each of five aquaria individually received intra-gastric gavage, simultaneous but separate administrations of 200 µl each of *E. ictaluri* isolate S97-73 (8×10^4 CFU/ml) and of a solution containing phage eiAU (7.5×10^4 PFU/ml). A one to one ratio of phage to bacteria was targeted. In Treatment 2 (control), fish in five replicate aquaria received 200 µl PBS and 200 µl of bacteria as described above. The experiment was terminated 21 days post-challenge.

The second challenge experiment was performed to evaluate the efficacy of multiple administrations of phage eiAU P10 through feed to control induced ESC in channel catfish fingerlings. Three hundred channel catfish fingerlings

(10-14 cm) were divided equally into 10, 60 L aquaria (n=30 fish/aquaria) each containing approximately 15 L well water. The study consisted of two treatments, a phage treatment plus a control, each composed of five replicate aquaria. Fish in treatment 1 (control) received oil-coated feed daily for 27 days beginning 3 days prior to challenging fish with *E. ictaluri*. Fish in treatment 2 received phage-amended feed that contained approximately 3×10^6 PFU/gram of feed for 27 days beginning 3 days prior to challenging fish. Additionally, fish in a single negative control aquarium were fed phage-amended feed for 27 days however were not challenged to observe for adverse reactions to phage administration. Feeding on the day of challenge occurred 1 h prior to challenge. Challenge dose was calculated to be 2.7×10^6 CFU/ml of *E. ictaluri* in aquarium water. The experiment was terminated after mortalities had ceased for two days in both treatments.

The third challenge experiment was performed to evaluate the efficacy of three different phages (eiAU, eiDWF, and eiAU P10) to control artificially induced ESC in channel catfish fingerlings. Four hundred channel catfish fingerlings (10-14 cm) were equally divided at 20 fish/tank into 20, 60 L aquaria containing 15 L well water. The study consisted of three treatments plus a control, with each treatment composed of five replicate aquaria. Fish in treatment 1 (control) received oil-coated feed only. Fish in treatment 2 received phage eiAU-amended feed, containing 3.9×10^7 PFU/gram of feed, fish in treatment 3 received phage eiDWF-amended feed, containing 2.3×10^7 PFU/gram of feed, and fish in treatment 4 received phage eiAU P10-amended feed, containing 1.4×10^7 PFU/gram of feed. All groups received experimental feed for 15 days, starting 3 days pre-challenge. Additionally, fish (n=20) in a single aquarium were exposed to challenge

conditions described above minus bacteria as a negative control, and were fed phage-amended feed for 15 d. Feeding on the day of challenge occurred 1 h prior to challenge. After 15 d of phage administration, fish in all treatments were fed regular feed for the remainder of the study. Challenge dose was calculated to be 4.7×10^5 CFU/ml of *E. ictaluri* in aquarium water. The experiment was terminated 20 days post-challenge.

The fourth challenge experiment was performed to evaluate the efficacy of different phage dosages to control artificially induced ESC in channel catfish fingerlings. Four hundred channel catfish fingerlings (10-14 cm) were equally divided at 20 fish/tank into 20, 60 L aquaria containing 15 L well water. The study contained four treatments, each composed of five replicate aquaria. Fish in treatment one (control) received oil-coated feed only. Fish in the remainder of the treatments used phage eiAU P10 amended feed. Fish in treatment two received feed containing approximately 6.9×10^{10} PFU/gram of feed, fish in treatment three received feed containing approximately 6.9×10^7 PFU/gram of feed, and fish in treatment four received feed containing approximately 6.9×10^4 PFU/gram of feed. All groups received experimental feed for 15 days, starting 3 days prior to challenging fish with bacteria. Fish in a single negative control aquarium were exposed to challenge conditions described above minus bacteria, and were fed phage-amended feed at a dose of 10^7 PFU/gram of feed for 15 d. After 15 d, fish in all treatments were fed regular catfish fingerling feed for the remainder of the study. Feeding on the day of challenge occurred 1 h prior to challenge. Challenge dose was calculated to be 5.7×10^6 CFU/ml of *E. ictaluri* in aquarium water. The experiment was terminated 28 days post-challenge.

3.7. Experimental design and statistical analysis

A General Linear Model (GLM) with repeated measures ANOVA (Wallace and Green, 2002) tested the effect of storage temperature on phage titers over time. For each storage temperature, multiple comparisons of mean phage titers recovered at each time point sampled were performed using Tukey's multiple range test, $p < 0.05$. The same analysis was done to test the effect of time on phage titers recovered from phage-amended feed following placement of feed in water. Analysis was done using MINITAB 15 for Windows (MINITAB Inc., Pennsylvania, USA). The final mean percent mortality in the first and second challenge experiments were compared using a one-way ANOVA with Tukey's student test. For the third and fourth challenge experiments, due to aquaria configuration in the challenge laboratory, a blocking effect was incorporated in experimental design to better control for variability between aquaria. The mixed procedure (Wolfinger et al., 1991) with Duncan's multiple range test was used to determine significant differences in final mean percent mortality among treatment groups, in the third and fourth challenge experiments. For each challenge experiments, the survival curves were plotted for each treatment and compared with a logrank test (JM Bland 2004). Statistical analyses for the challenge experiments were carried out using SAS (version 9.1.3, SAS Institute, Cary, North Carolina). Significance between treatments in all experiments was set at $p\text{-value} \leq 0.05$. Relative percent survival (RPS) was calculated according to Amend (Amend, 1981) when significance between treatments was observed.

4. Results and Discussion

4.1. Stability of phage in phage-amended feed

A significant effect of temperature ($p = 0.004$) was observed on phage titer stability over time. Mean phage titers in phage-amended feed stored at 4 °C and 24 °C remained stable, with no significant decrease in phage titers ($p = 0.778$) and ($p = 0.491$), respectively, over a 21 day storage period, while titers in feed stored at 37 °C remained stable for six days ($p = 0.612$) and then significantly decreased at day eight ($p = 0.0017$) and thereafter (Figure 1). A loss of over 100x in phage titer was observed after 21 d of storage at 37 °C.

Mean phage titers retained by phage-amended feed following placement of the feed in pond water are presented in Figure 2. A significant effect of time ($p = 0.001$) on mean phage titers recovered from phage-amended feed was observed. After 15 min no significant decrease in phage titers was observed ($p = 0.999$); however, a significant decrease was observed ($p = 0.042$, $p = 0.048$, and $p = 0.024$) at times 30 min, 45 min, and 60 min respectively. Mean phage titers in phage-amended feed placed in pond water decreased from 4.25×10^6 PFU/ml to 9.25×10^5 PFU/ml representing a 78 % decrease over a 60 min time period (Figure 2).

The observation that phage (eiAU) titers in phage-amended feed remained stable at 4 °C and 24 °C for 21 days indicates that it would be possible to prepare phage-amended feed and store it for extended periods of time in appropriate temperature storage conditions. However, results also suggest that phage titers will drastically drop if phage-amended feed is stored in a storage unit with no temperature control where temperatures

may reach or surpass 37 °C, which often happens on commercial farms (Tucker and Robinson, 1990). Similar results have been previously reported in which no detectable drop in titers were observed in bacteriophage-amended feed stored at 4 °C after 4 weeks (Verner-Jeffreys et al., 2007).

Phage titers in phage-amended feed placed inside water remained unchanged for up to 15 min suggesting that a crude method of coating feed with phage and then coating a second time with menhaden fish oil provides ample time for catfish to feed before phage particles disintegrate from the feed. When feeding catfish on commercial facilities, the goal of producers is to feed only enough feed that fish will eat in 10-15 min (Robinson et al., 2007) for optimal economic returns. The method of incorporating phage into feed used in this study may be appropriate for laboratory experiments; however, a more elaborate method may be needed for industry wide applications of bacteriophages when used under other conditions or for other fish.

4.2. First challenge experiment: A one-time administration of phage-amended feed to control ESC

The daily cumulative percent mortality of fish for each treatment in the first challenge experiment is presented in Figure 3. Fish mortalities due to ESC were first observed and diagnosed four days post-challenge in the control group. A two day delay in the onset of mortalities was observed in the treatment that received a one-time administration of phage immediately after challenge with *E. ictaluri*. One half (47 %) of the final percent mortality for the control treatment was reached seven days post-challenge, while for the treatment that received phage, one half (44 %) of the final

mortality was reached eleven days post-challenge. The delay in onset of mortality and the time to reach 50 % mortality of fish groups that received phage treatment compared to the control were significantly different ($p = 0.045$, and $p = 0.031$, respectively). However, at the end of the experiment the final mean percent mortality \pm SD ($n=5$) of the control (94 ± 9 %) compared to the treatment that received phage (88 ± 13 %) is not significantly different ($p = 0.3139$).

A similar pattern of mortality curve with delays in the onset of mortality and at the time that half of the total mortality was reached was observed in studies evaluating the use of phages to protect Atlantic salmon *Salmo salar* L from Furunculosis caused by *Aeromonas salmonicida* subsp. *salmonicida* 78027 (Verner-Jeffreys et al., 2007). Additionally, in that same study, similar to the results of this experiment, the mean final mortality of the control compared to the phage treated group was not significantly different. The authors postulate that this result may be due to the initial inactivation of a portion of the original inoculum caused by the mixing of phage and bacteria in the intraperitoneal cavity, which would have lowered the infective dose thereby increasing the lag period needed for the infection to be established. This may be a plausible explanation for the delay in mortality observed in this study considering that in this experiment both phages and bacteria were administered by intra-gastric gavage simultaneously, providing an optimal situation for a synchronized phage-bacterial interaction in the gastro-intestinal tract of catfish.

In this experiment a single administration of phage did not result in significant difference in mortality at the end of the study which is contrary to other studies (Nakai et al., 1999; Park and Nakai, 2003; Park et al., 2000). Interestingly, the disease outbreak in

those studies reporting protective effects from a single phage administration is short (acute infection) as compared to other studies (Verner-Jeffreys et al., 2007) and to this one in which the infection of *E. ictaluri* is longer (2-3 weeks), possibly resulting in more opportunities for multiple re-infection or infection of those fish within a tank that were protected initially but may get infected later in the epizootic (Wise et al., 1997).

Although phages have self replicating capabilities and hence multiple administrations of phage may not be required to control bacterial disease in some cases (Barrow and Soothill, 1997), for this to occur the target bacterial population should be accessible and sufficiently dense, and physiologically and genetically susceptible to phage infection (Levin and Bull, 2004). Additionally, self-amplification will not occur if bacterial densities are too low for the phage to replicate faster than they are lost (Levin and Bull, 2004). This was noted in a study where the presence *Erwinia amylovora* phages in high numbers was dependant on the presence of their host (Schnabel et al., 1998). As previously observed in studies of the pathogenesis of *E. ictaluri* in catfish, the rapid movement of *E. ictaluri* from the intestines into blood circulation and their uptake by macrophages once in blood circulation (Booth et al., 2006; Newton et al., 1989) suggests that *E. ictaluri* phages have limited time and opportunity to target *E. ictaluri* before they become inaccessible to phage infection due to their intra-cellular nature. This may further be complicated by results on the fate and persistence of *E. ictaluri* phages in the gastrointestinal tract of catfish (Chapter II) that indicates that phage numbers drop by 100x after 24 h and that these phages by themselves are not capable of crossing from the intestine into blood circulation. This would suggest that the portion of uninfected bacteria that

enters through the GI tract and become systemic within the host fish may become unavailable to phage infection.

4.3. Second challenge experiment: Twenty seven days administration of phage to control ESC

The daily cumulative percent mortality of fish for each treatment in this experiment is presented in Figure 4. Fish mortalities were first observed and diagnosed in the control treatment at 10 d post-challenge. In the treatment that received phage, one fish died 7 d post-challenge, and heavy mortality was first observed eleven days post-challenge. One half (15.7 %) of the final percent mortality for both the control treatment and the phage treatment (10%) was reached simultaneously at 14 d post-challenge. No significant delay in onset of mortality ($p = 0.344$) and on the time to reach 50 % mortality ($p = 0.568$) of fish groups that received phage treatment compared to the control were observed. The final mean percent mortality (\pm SD) ($n=5$) for the control group (29.33 ± 7.23 %) and for the treatment that received multiple administration of phage (20.67 ± 4.94 %) is significantly different ($p = 0.0127$). No mortality occurred in the negative control tank that was not challenged but received phage and no gross indication to suggest detrimental effect of phage administration was observed in fish from this group. *Edwardsiella ictaluri* was diagnosed as the causal agent of mortality in all fish sampled ($n=15$), and phage was recovered from the intestines of five of the fifteen fish sampled. Phage was not recovered from internal organs of any of the fish sampled. All *E. ictaluri* isolates recovered from fish sampled were susceptible to phage lysis.

Contrary to results from the first challenge experiment in which a delay in the onset and in the time to reach 50 % mortality was observed in fish that were administered a one-time administration of phage, no delay in the onset of mortality was observed in this experiment. Similarly, Verner-Jeffreys *et al.*, (2007) observed a delay in onset of mortality and at the time to reach 60 % mortality in fish groups that were challenged by i.p. injection and then received i.p. injections either at 1h or at 24 h post infection; however, they did not observe these delays when fish were challenged by an indirect cohabitation model and phage was administered by various methods (i.p., immersion, feed). This difference may be explained in part by the difference in infection dynamics that occurs with the different methods of infection used.

4.4. Third challenge experiment: Evaluation of various E. ictaluri-specific phages to control ESC

The daily cumulative percent mortality of fish for each treatment in this experiment is presented in Figure 5. Fish mortalities were first observed and diagnosed in all treatments 4 d post-challenge. Final mean mortalities (\pm SD; n=5) were 48 ± 27 %, 44 ± 31 %, 38 ± 11 %, and 42 ± 13 % for the control, eiAU, eiAU P10, and eiDWF phage treatments, respectively. No treatment effect ($p = 0.9385$) was observed in this experiment.

Contrary to results from the second challenge experiment, no significant difference was observed in final mean mortality of treatment that received phage eiAU compared to the control ($p = 0.9235$). This may be explained in part by the difference in the days that fish received phage-amended feed. In the second challenge experiment, fish

were administered phage eiAU for 27 days as opposed to 15 days in this experiment. Phages eiAU and eiDWF are not too dissimilar in their efficiency of forming plaques in *E. ictaluri* S97-773 (Walakira et al., 2008) and are both wild type, which may in part explain why no significant difference ($p = 0.7448$) in the final mean mortality percent of fish that received eiAU compared to fish that received eiDWF was observed. Additionally, no significant difference ($p = 0.6747$) was observed in the final mean percent mortality of fish that received phage eiAU compared to fish that received eiAU P10. Phage eiAU P10 is a variant of eiAU that was obtained by serial passage within fish to enhance the capacity of eiAU to survive conditions in the gastro-intestinal tract of catfish. However, as previously discussed, once *E. ictaluri* enters the gastro-intestinal route it rapidly penetrates the intestinal epithelium and moves into blood circulation, therefore the enhanced capacity to survive for a longer time in the gastro-intestinal tract may not necessarily translate to an increase in the number of phages within a population that comes into contact with bacteria to attach and infect *E. ictaluri*, because the time for phage-bacteria interaction is still relatively short.

All confirmed *E. ictaluri* isolates recovered from fish during this challenge experiment were susceptible to all three bacteriophages (eiAU, eiDWF, and eiAU P10) available. Studies have reported the isolation of phage resistant variants from fish sampled during an ongoing challenge experiment (Verner-Jeffreys et al., 2007); however, others have only observed development of resistance when induced *in vitro* (Imbeault et al., 2006) but not during an ongoing challenge (Park et al., 2000). Although the development of resistant strains of bacteria to phages used was not observed in this study, the data on aquaculture studies above as well as in other studies evaluating the use

of bacteriophages in other animal (Capparelli et al., 2009; Loc Carrillo et al., 2005) confirm that resistance development to phages is very common and may pose a significant challenge to success in phage therapy (Cairns et al., 2009; Kysela and Turner, 2007; Lederberg, 1996; Levin and Bull, 2004). It has been proposed that resistance may be mediated by the use of phage cocktails (Leverentz et al., 2003; Levin and Bull, 2004), additionally, it has been reported that phage resistant strains isolated in several of these studies have reduced or completely loses it virulence to cause disease (Loc Carrillo et al., 2005; Park et al., 2000). In one case it has been reported that phage-resistant *Salmonella enterica* isolates resulted in good vaccines providing protection from *S. enterica* infections (Capparelli et al., 2009). An additional point to note with regards to bacteriophage resistance is that eiAU originally isolated in 1985 still remains highly lytic and is capable of lysing the majority of *E. ictaluri* strains that have been tested to date (Walakira et al., 2008).

4.5. Fourth challenge experiment: Evaluation of phage dosage to control ESC

The evaluation of different phage doses was examined and the daily cumulative percent mortality for each treatment is presented in Figure 6. Fish mortalities were first observed and diagnosed in the control treatment 8 d post-challenge, and in all other treatments 9 d post-challenge. Final mean mortalities (\pm SD ; n=5) were 76 ± 22 %, 71 ± 22 %, 67 ± 23 %, and 64 ± 29 % for fish groups that received the control treatment (no phage), 6.9×10^4 PFU/gram of feed, 6.9×10^7 PFU/gram of feed, and 6.9×10^{10} PFU/gram of feed respectively. Compared to the control, no significant differences $p = 0.5209$ and $p = 0.2551$ were observed for the group that received a phage dose of $6.9 \times$

10^4 PFU/gram of feed and 6.9×10^7 PFU/gram of feed, respectively. A significantly lower mortality ($p = 0.0212$) compared to the control was only observed in the group that received the highest phage dose (6.9×10^{10} PFU/gram of feed). Also, no significant difference ($p > 0.05$) was observed among the groups that received any of the phage treatments.

Contrary to other studies in which the effects of increasing the phage dose on the mean mortality of animal subjects are well defined (Berchieri, 1991b), in this study the effects of incrementing phage doses were not clearly observed. These results are counterintuitive to the idea that the higher the dose of phage present in the feed, the more phage the fish can consume and hence, the more phage available in the gastro-intestinal tract of fish to infect and kill bacteria. However, models of pharmacokinetics and pharmacodynamics assume that success of phage therapy does not only depend on the amount of phage present, but may depend on various factors, including the rate of phage adsorption, the burst size of phage, the latent period, the rate at which bacteria is killed and eliminated from sites of infection, the rate at which bacteria replicate, and the density of phage and bacteria (Levin and Bull, 2004). In this model factors such as the observation that some fish within groups went off feed and some had reduced feeding during the disease outbreak, which is a behavioral characteristic associate with ESC, may affect the dose of phage that each fish consumes within an aquarium. This would in part affect the density of phage within a fish possibly affecting phage therapy success. Furthermore, the pharmacokinetics of self-replicating phages is different than that of antibiotics and demonstrating an effective dose may prove complicated. Treatment dose depends on density dependant thresholds, and the ability to predict those thresholds may

be difficult (Payne and Jansen, 2000). This means that the dose rate may be more important at some points more than others during an infection.

5. Conclusions

The titers of phage eiAU remained stable, in oil-coated phage-amended feed stored at 4 °C and 24 °C for 21 days; however, phage titers in feed stored at 37 °C remained stable for 6 d and then drastically declined. When phage-amended feed were placed into pond water and vortexed to simulate feeding activity, phage titers in the feed remained unchanged for the first 15 min, indicating that the phage coating method employed allows retention of phage in feed with ample time for target doses be consumed by fish for experimental purposes. Protective effects of *E. ictaluri* specific phages against *E. ictaluri* infections in channel catfish fingerlings was evaluated using a method of oral administration of phages through phage-amended feed and a bath/oral infection model of infection. A single simultaneous but separate administration of phage by intragastric gavage did not provide protection against *E. ictaluri* infection. The use of three different phage types (eiAU, eiDWF, and eiAU P10) separately administered for 15 d to fish in three different groups did not provide protection. In an experiment testing three different phage doses administered to fish groups (10^{10} , 10^7 , and 10^4 PFU/gram of feed), only the highest dose provided protection against *E. ictaluri* infection. Protection against *E. ictaluri* infection was also demonstrated when phage was administered for 27 consecutive days, beginning 3 days pre-challenge. These results suggest that dose and frequency of phage administration are important for phage control of ESC. Although using a high phage dose and administering phage daily for 27 d lowered mortality of fish

compared to the control, the high dose used and the long duration of phage administration may not be cost effective for use in catfish culture. During the challenge experiments no adverse effect to phage was observed in control fish that received phage only administration. Although in this study tests were not performed to detect neutralizing antibodies, other studies evaluating phage therapy of bacterial infections in fish (Nakai et al., 1999; Park and Nakai, 2003) and in chicken (Berchieri, 1991b) have reported that none were detected.

Administration of phage for 27 days provided protection, but may be considered un-practical and economically challenging in industry standards. Furthermore, 8.7 % better survival is far lower than survivals reported from the use of Aquaflor® or Romet to treat ESC. Although limited protection is demonstrated in this study, other studies (Imbeault et al., 2006; Nakai et al., 1999; Park and Nakai, 2003; Vinod et al., 2006) have demonstrated much more promising results with regards to phage therapy success to control bacterial infections in fish. We conclude that although the genetic homogeneity of *E. ictaluri* and *in vitro* studies in pure broth culture suggest that this pathogen is a good candidate for phage therapy, the observed inability of these phages to cross into blood circulation, in conjunction with the capacity of *E. ictaluri* to rapidly penetrate, cross the epithelium barrier of catfish, and become intracellular, limits the potential for phage-bacterial interaction resulting in ineffective use of a phage therapy approach for the control of *E. ictaluri* and other pathogens with similar mode of infection and pathogenesis.

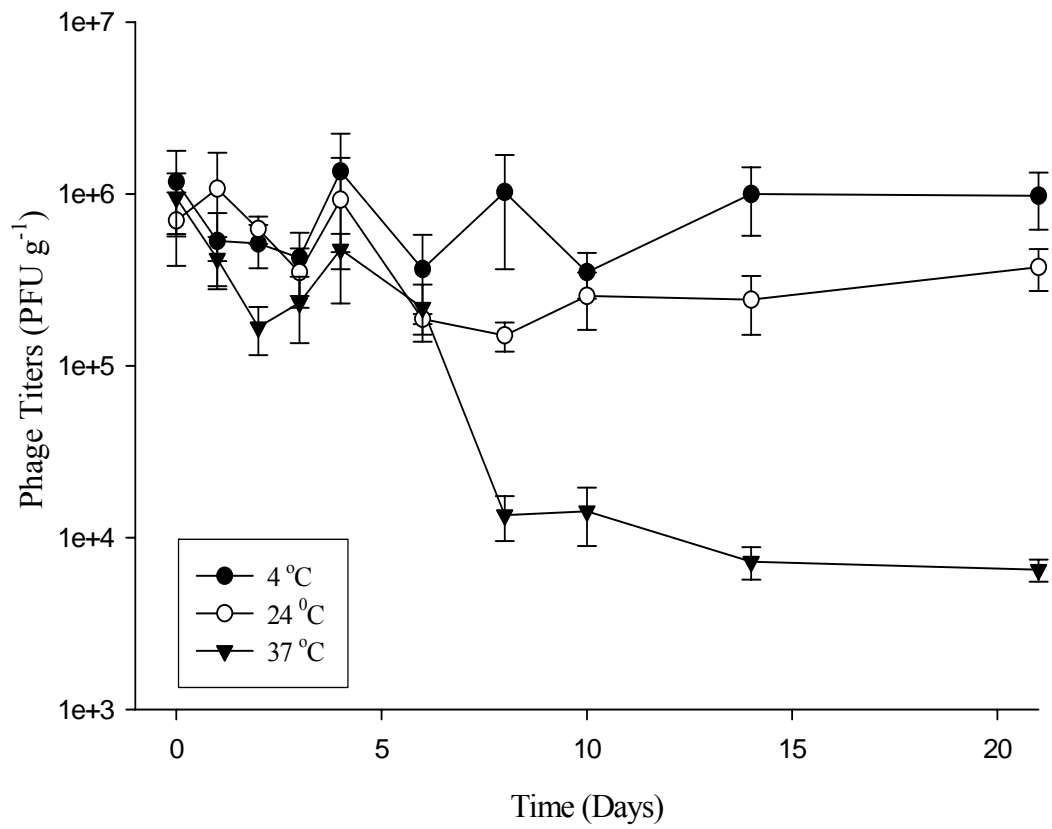


Figure 1. Phage survival in phage-amended feed stored at three different temperatures. Each point plotted is mean \pm SD of four replicate samples.

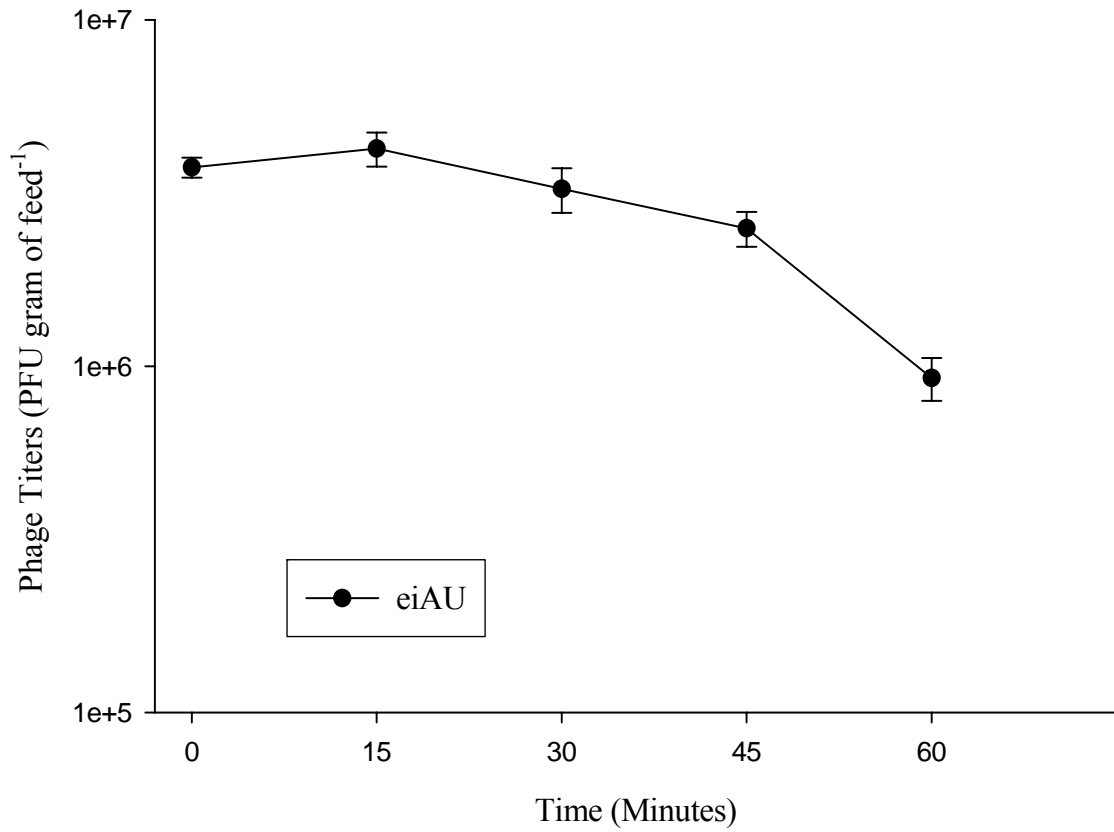


Figure 2. Mean \pm SD phage titers recovered from phage-amended feed following placement of the feed in pond water.

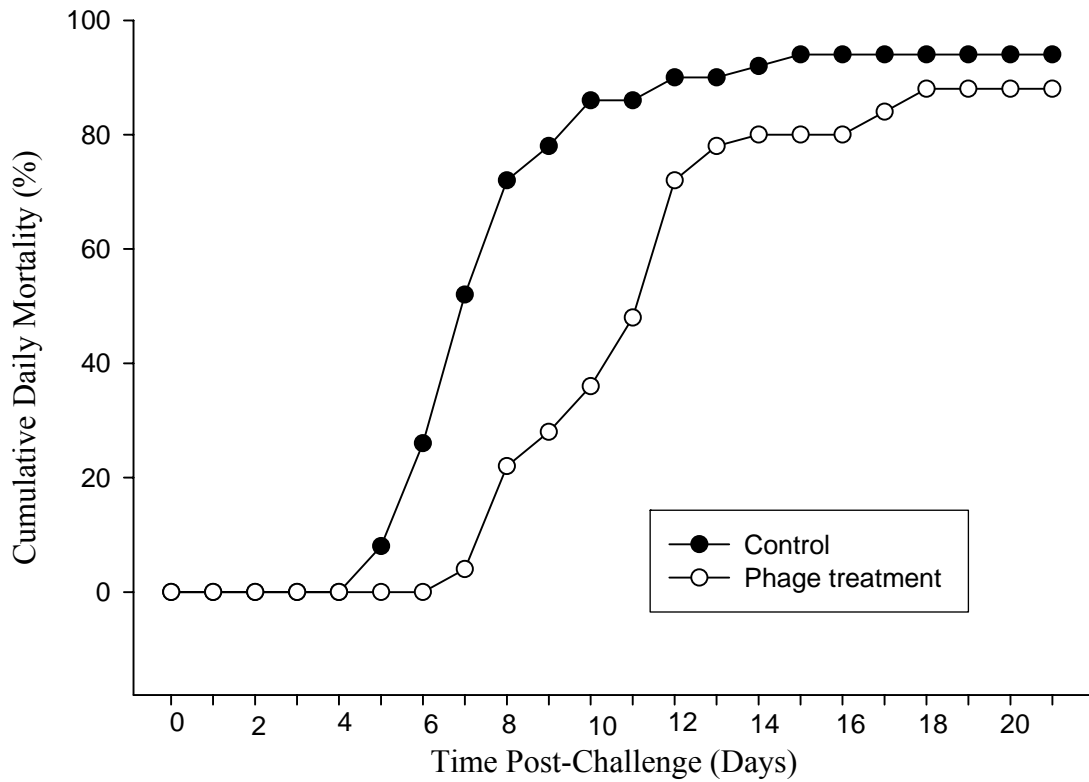


Figure 3. Cumulative mean daily mortality of fish that received a one-time treatment of Phage eiAU via intra-gastric gavage and then challenged with *E. ictaluri*. No significant difference ($p = 0.3139$) in mortality was observed between the two treatments.

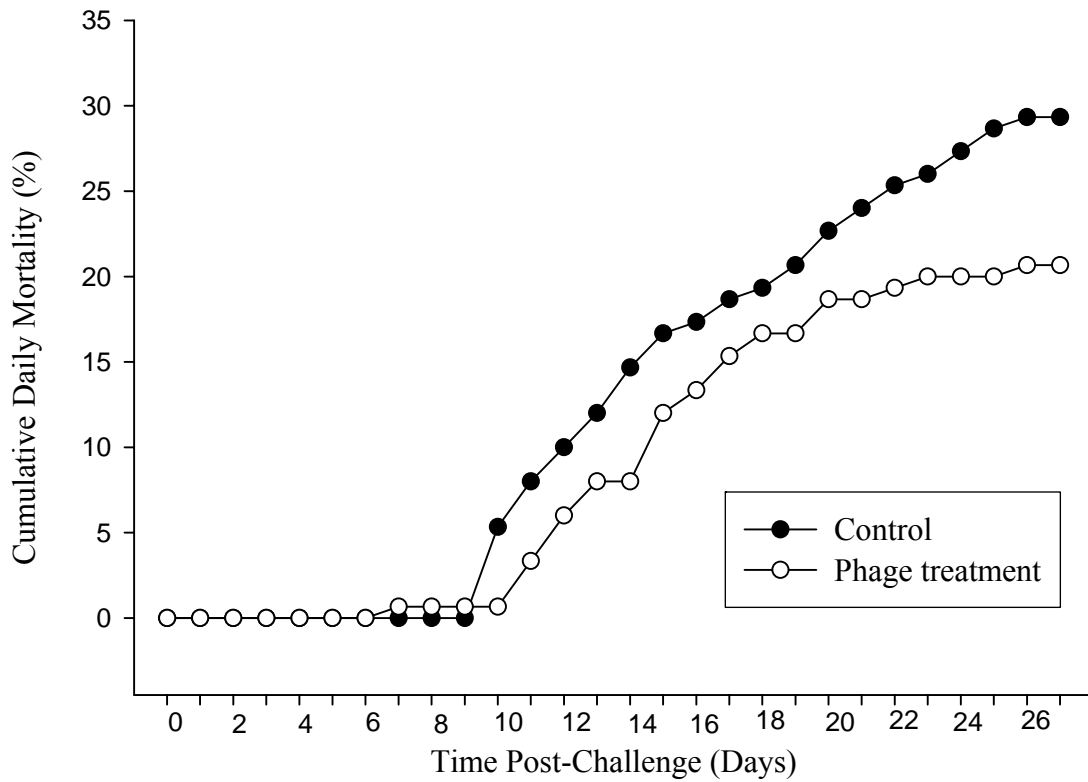


Figure 4. Cumulative mean daily mortality of fish that received a 27-day administration of phage eiAU P10 via feed and were challenged with *E. ictaluri*. Significant difference ($p = 0.0127$) was observed in mean fish mortality percentage between treatments.

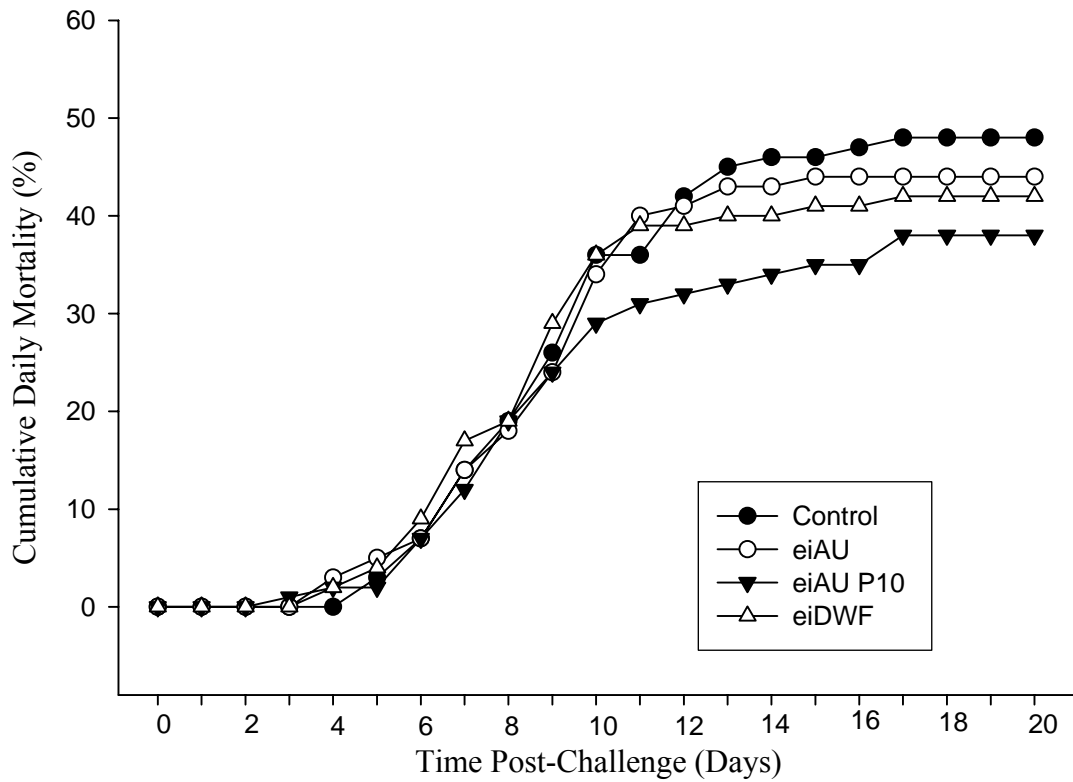


Figure 5. Cumulative mean daily mortality of fish that received a 15 day administration Of three different phage types via feed beginning 3 days prior to being challenged with *E. ictaluri*. No significant difference ($p = 0.9385$) in mean mortality was observed between the phage strains.

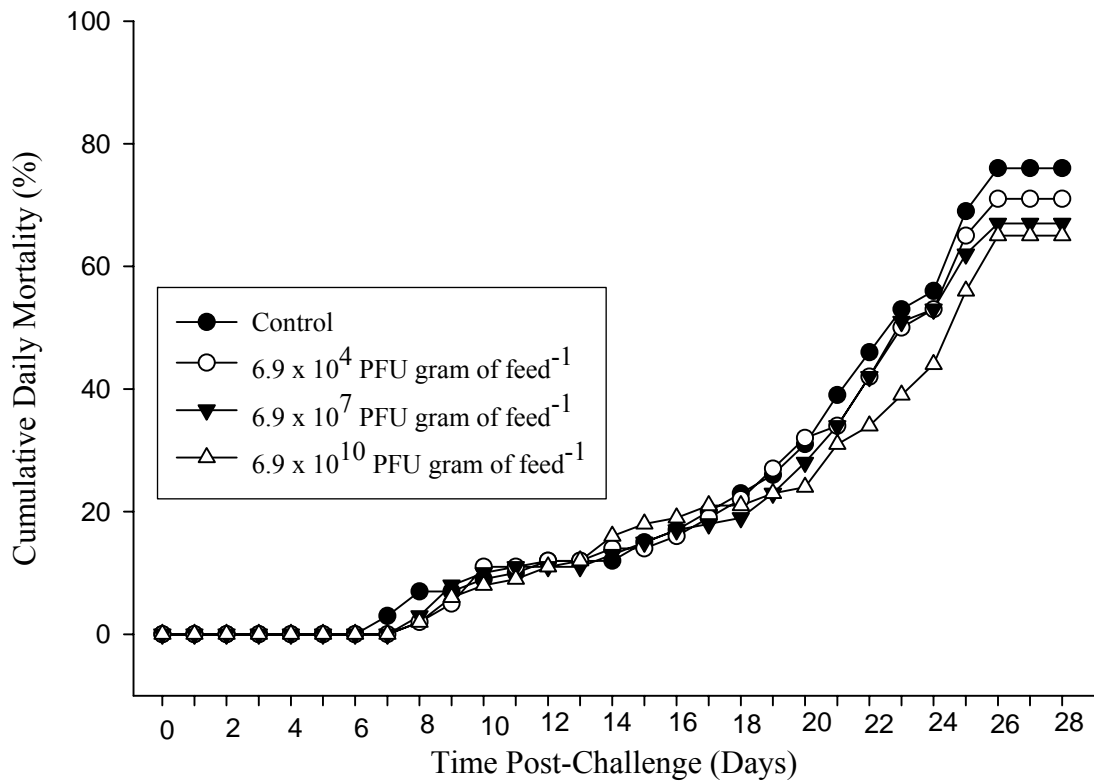


Figure 6. Cumulative mean daily mortality of fish that received a 15 day administration of three different dosages of phage eiAU P10 via feed and challenged with *E. ictaluri*. Only the highest phage dose compared to the control was significantly different ($p = 0.0212$).

CHAPTER V

Isolation and selection of *Bacillus* strains for biological control of *Edwardsiella ictaluri* infections

1. Abstract

Several bacterial species belonging to the genus *Bacillus* have been evaluated for their respective ability to biologically control bacterial diseases in aquaculture. However, the failure to carefully select candidates for biological control of diseases has contributed to failure of therapeutic effects of some of these biological control agents. This study was undertaken to isolate and to select the best *Bacillus* spp. exclusively for use in biological control of enteric septicemia of catfish.

One isolate, given the designation strain AB01 was isolated from the gastro-intestinal tract of channel catfish fingerlings. The isolate is Gram-positive, spore-forming, and produced zones of inhibition on three *E. ictaluri* isolates tested. Analysis of the 16S ribosomal RNA gene sequence indicates that strain AB01 belongs to the genus *Bacillus*, and is a member of the *B. subtilis*/*B. amyloliquefaciens* group. Further analysis will be needed to conclusively identify the species.

Challenge experiment demonstrated the capacity of a mixture of twelve *Bacillus* spp. obtained from a large collection (N=160) to provide protection against artificially

induced *Edwardsiella ictaluri* infection in aquaria. Data from that experiment along with data from another challenge study that evaluated the individual capabilities of seven *Bacillus* spp. to provide protection against *Edwardsiella ictaluri* will be included as part of an ongoing process to select the best performing *Bacillus* spp. for use in biological control of enteric septicemia of catfish.

2. Introduction

Bacteria of the genus *Bacillus* are spore forming, Gram-positive bacteria that have gained consideration for use in controlling diseases in aquaculture. Their antimicrobial capabilities, along with their capacity to form spores makes them good candidates for use as therapeutic agents because their endospores are resistant to environmental conditions and possess a long shelf life in storage (Wang et al., 2008).

In aquaculture, *Bacillus* spp. have been evaluated as part of probiotic formulations administered to aquatic organisms, and the stimulation of the immune response and improvement in health of these organisms have been reported by their use (Gatesoupe, 1999; Gullian et al., 2004; Rengpipat et al., 1998; Salinas et al., 2005). However, only a few studies (Newaj Fyzul et al., 2007; Queiroz and Boyd, 1998; Rengpipat et al., 1998) have reported data specifically on the efficacy of *Bacillus* spp. to provide protection against artificially-induced or naturally-caused bacterial infections.

The selection of *Bacillus* spp. for use as therapeutic agents is the first step in developing bacterial biological control of diseases. In selecting the best strains to use, it should be considered that these natural antagonistic bacteria may provide protection to fish in several ways, including by the competitive inhibition of undesired bacteria (i.e.

pathogens), enhancement of fish immunity, or by the microbial enhancement of the culture environment (Newaj Fyzul et al., 2007). These bacteria may achieve these effects by producing several different inhibitory compounds such as bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, and formation of ammonia and diacetyl (Verschuere et al., 2000). Although bacteria used in biological control strategies have been isolated from various sources such as from commercial probiotic products for humans and livestock use (Nikoskelainen et al., 2001), from the host's intestine and skin mucus (Westerdahl et al., 1991), and from the habitat of aquaculture animals (Rengpipat et al., 1998), in general it is recommended to isolate *Bacillus* spp. from the host in the study and to verify its safety or security for use (i.e. that they are non-pathogenic) (Gómez R. et al., 2007). Additionally, the *in vitro* antagonism against target pathogens may be employed as a primary criterion for screening of candidate biocontrol bacteria (Jack et al., 1996; Olsson et al., 1992; Robertson et al., 2000).

In an effort to select candidates for use as biological agents specifically to control enteric septicemia of catfish (ESC) caused by the pathogen *E. ictaluri*, a study was undertaken to isolate bacteria from the intestinal tract of catfish that possesses inhibitory activity against the pathogen. Additionally, two challenge trials were done in which the mean percent survival of fish at the end of the trial was used as an indicator for selecting the best performing candidates from a collection of soil-derived *Bacillus* species.

3. Materials and methods

3.1. Bacterial strains and preparation of bacterial cultures used in fish challenge

Edwardsiella ictaluri strain S97-773 was used for the primary screening for antibiosis of *Bacillus*-like isolates obtained from fish and for ESC challenge experiments since this strain is highly pathogenic for channel catfish and has previously been used in challenge studies at the fish health disease lab (E. W. Shell Research Center, Auburn University). Bacterial cultures for challenge were prepared by inoculating 5 ml tryptic soy broth with 200 µl of a -80 °C (~ 10⁹ CFU/ml) frozen culture stock and incubating for 24 h at 30 °C while shaking. After 24-h incubation the 5 mL culture was used to inoculate 100 mL of tryptic soy broth, which was also incubated for twenty four hours. After over-night incubation the 100 mL bacterial culture was centrifuged at 3600 x g for 30 min, re-suspended in 100 mL of fresh TSA, and allowed to grow an additional 3 h prior to challenge. Bacterial culture was quantified to verify challenge dose. Target challenge dosage was ~10⁶ CFU/ml aquarium water.

The collection of soil-derived *Bacillus* strains (n= 7) was previously selected based on antibiosis against *E. ictaluri* and other fish pathogens (Chao et al., manuscript in preparation) and are part of a large collection (N=160) provided by the laboratory of Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University).

3.2. Isolation of Bacillus spp. strains from the intestine of channel catfish and evaluation of antimicrobial activity

Catfish fingerlings (10 to 14 cm) were humanely euthanized by administration of an overdose of MS-222 and their intestine plus stomach were aseptically removed in their entirety. Approximately 1.0 g was homogenized in 9.0 ml of sterile saline (0.9 % w/v). Ten-fold serial dilutions were prepared to 10⁻⁶ in fresh saline, and 0.1 ml was spread over

the surface of triplicate plates of tryptone soy agar (TSA) with incubation at 28°C for 48 h (Irianto and Austin, 2002). Colonies exhibiting *Bacillus*-like morphology (Holt et al., 1994) were picked, purified by streaking for isolated colonies on fresh media, and examined for inhibition against the growth of *E. ictaluri* using a modification of a double-layer soft agar method previously described (Jack et al., 1996). Briefly, for soft agar overlays, the bacterial isolates obtained from fish were grown in 5 ml of tryptone soy broth (TSB) for 24 h at 30°C. Then, a volume of 5 µl was spotted onto triplicate plates of TSA, and incubated for a further 24 h. Soft agar (0.7 % w/v agar) prepared with TSB was melted and cooled to 37°C and seeded with an inoculum of log-phase *E. ictaluri* strain S97-773. The bacterial cell suspension in soft agar was immediately poured over the TSA plates and incubated for 24 h at 30°C. After incubation, the presence of zones of clearing in the growth of the lawn of *E. ictaluri* were recorded (in mm) as evidence of growth inhibition. Cultures that were regarded as inhibitory to *E. ictaluri* were subjected to further testing with other pathogenic *E. ictaluri* strains isolated from diseased catfish.

3.3. 16 S Ribosomal RNA sequencing

Genomic DNA of *Bacillus*-like bacteria was extracted from 500 ml cultures grown in TSB using the Promega genomic DNA isolation kit (Madison, WI) according to manufacturer's directions. These cultures were characterized by Gram staining and 16S rRNA gene sequencing using the 'universal' primer set 27F and 1492R (Weisburg et al., 1991). A consensus 16S rRNA gene sequence was produced using Chromas Pro

(Technelysium Pty Ltd., Queensland, Australia) and each sequence was compared to the GenBank nr database by BLASTn.

3.4. Preparation of *Bacillus* spores and spore-amended feed

Bacillus spores were prepared using slight modifications to a method previously described (Kenney and Couch, 1981). Briefly, *Bacillus* strains were grown in TSB at 30°C overnight. Then the broth was spread on spore preparation agar (peptone 3.3 g/l, beef extract powder 1.0 g/l, NaCl 5.0 g/l, K₂HPO₄ 2.0 g/l, KCl 1.0 g/l, MgSO₄ · 7H₂O 0.25 g/l, MnSO₄ 0.01 g/l, lactose 5 g/l, agar 15 g/l) using a sterile cotton swab and incubated at 28°C for 5 to 7 days. To collect the spores, 5 ml of sterile distilled water was added to the plate and the spores were suspended in water using an inoculation loop. The spore suspension was then incubated at 85°C for 15 min to kill the vegetative cells. The concentration of the spore suspension was determined by serial dilution and spreading onto TSA. The final concentration of the spore suspension was manipulated with sterile water to 10⁹ CFU/ml.

To prepare spore-amended feed, finfish slow-sinking starter pellets (2 mm, 40 % protein, Zeigler, Gardners, PA) was spray-coated with a solution containing spores (10⁹ CFU/ml) from one of seven strains of *Bacillus spp.*, at a rate of 8 % of the dry/lyophilized weight of the feed. The feed in all batches was immediately coated a second time with 5 % menhaden fish oil at a rate of 3 % of the dry feed weight. Prepared feed stocks were stored at 4°C until used.

3.5. ESC challenge studies

Channel catfish fingerlings, 3 to 4 inch total length, were divided 20 fish per tank in 60 L aquaria containing approximately 15 L filtered well water. Two challenge experiments were conducted. In experiment one, two treatment groups each composed of four replicate aquaria were included. In one of the experimental treatment groups, fish were administered feed supplemented with a mixture of twelve *Bacillus* strains and in the other treatment group (control) fish were fed oil-coated feed. In experiment two, eight treatment groups each composed of four replicate aquaria were included. In each of the seven experimental treatment groups, fish were administered feed supplemented with spores from one of seven *Bacillus* strains (AP193, AP254, AP143, AP76, AP77, AP79, and AB01). A control treatment was included in which fish received oil-coated feed only, and were challenged.

Prior to challenge, fish were acclimated to conditions in the aquaria, and fish were fed for five consecutive days. For ESC challenge, *E. ictaluri* was added directly into each aquarium to a final target dosage of approximately 5×10^6 CFU/ml aquarium water. Prior to adding bacteria the water flow into each aquarium was restricted, and then resumed one hour after bacteria was added to each aquarium. For each experiment, fish in one single positive control aquarium received sterile SM buffer (100 mM NaCl, 8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM Tris-HCl, 0.002% (w/v) Gelatin, pH 7.5) only, and were exposed to challenge conditions described above except that they did not receive bacteria.

3.6. Daily husbandry activities during challenge

During the duration of the challenge trial continuous flow-thru water supply maintained at 25 ± 2 °C and aeration was provided to each aquarium individually. Rock salt (NaCl_2) and CaCl_2 were added daily to increase chloride levels and water hardness of the incoming water. Water quality parameters (pH, hardness, alkalinity, and chloride levels) were monitored weekly.

In experiment one, fish were fed their respective feed treatments three days prior to challenge, and then for 15 additional days. In experiment two fish were fed their respective feed treatments five days prior to challenge and for an additional 15 d pre-challenge. Un-eaten feed and waste material were siphoned out of each aquarium as needed. All equipments used during the experiment were routinely disinfected using an iodine solution bath. Upon outbreak of ESC, fish were observed for behavioral response and for gross signs of disease. Moribund/freshly dead fish were removed from aquaria, necropsied, and samples from trunk kidney, liver and spleen were streaked on TSA for bacterial isolation. Bacteria that were isolated from these tissues were identified biochemically. Dead fish were removed twice a day once in the morning and once in the evening and recorded.

At the end of the experiment all surviving fish were documented, euthanized by prolonged exposure to 300 ppm MS-222, and properly disposed.

3.7. Experimental design and statistical analysis

For challenge studies, a completely randomized design was used in experiment one, and a block design was used in the experiment two because aquaria were distributed

in three different systems with expected variability. A one way ANOVA with Duncan's multiple range tests was used to determine significant differences in mean percent mortality between treatment groups in experiment one. No statistical analysis was possible in experiment two due to the loss of control aquaria. For experiment one, significant differences were considered at $p < 0.05$. Statistical analysis was carried out using SAS (version 9.1.3, SAS Institute, Cary, North Carolina).

4. Results and Discussion

4.1. Characterization of Bacillus isolates

Gram-positive, endospore forming, bacterial isolates exhibiting similar morphology were recovered from catfish intestine samples. Colonies from these isolates were able to form similar size (~ 5 mm) inhibition zones on lawn overlays of *E. ictaluri* strains S97-773, C91-162 and R4383. Colonies tested exhibited *Bacillus*-like morphology as described by Bergey's manual of determinative bacteriology (Holt et al., 1994). One of these isolates, given the name AB01, was chosen for further studies.

The capacity of AB01 to produce inhibitory activity against *E. ictaluri* strains S97-773, C91-162, and R4383 that are highly pathogenic for catfish is a first step in selecting bacteria for use in biocontrol of *E. ictaluri*. Additionally, its capacity to form spores is a desired characteristic for biological control purposes (Wang et al., 2008) as it suggests that AB01 may be able to withstand adverse environmental conditions and may be highly stable when stored for extended periods of time.

4.2. 16 S Ribosomal RNA sequencing

The highest BLASTn hits to sequences in the GenBank nr/nt database of the consensus 16S ribosomal RNA gene sequence of ABO1 were to 16S ribosomal RNA genes of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. The e-value was zero for the top BLASTn hits.

The three *Bacillus* species cannot be conclusively defined based on 16S rRNA gene sequence data, which would require using other methods such as DNA-DNA hybridization or whole genome sequencing for identification of the species with which strain ABO1 affiliates. Although *Bacillus* spp., including *B. subtilis*, *B. cereus*, *B. circulans*, *B. polymyxa*, *B. megaterium* and *B. licheniformis*, and *B. pumilus* have been used as probiotics in aquaculture (Aly et al., 2008; Balcázar et al., 2006; Oggioni et al., 2003), it is important to identify the species prior to use in therapy as some *Bacillus* species such as *Bacillus cereus* may include strains that have been known to be unsafe to humans as they produce toxins (Rowan et al., 2003; Rowan et al., 2001; Sorokulova, 2008).

4.3. ESC challenge studies

Mortality of channel catfish fingerlings at the end of challenge studies are presented in (Table 1 and Table 2). In experiment one, mean mortality percentage for a fish group that received a mixture of twelve *Bacillus* species (17.3 %) was significantly lower (P value < 0.5) compared to control fish (29.3 %) that received standard oil-coated catfish fingerling feed (Table 1). In experiment two, mortality of fish groups that received feed amended with one of seven *Bacillus* spp. ranged from 7.5 % to 36.3 %

(Table 2). No comparison was done against the control treatment due to mortality of all fish in three of its five replicates caused by water heater failures. However, comparison among the *Bacillus* treatments showed that administration of AP193 resulted in significantly lower ($p = 0.006$) mean fish percent mortality compared to the other *Bacillus* strains.

Results from experiment one demonstrated that feeding catfish fingerlings for 18 d starting three days prior to challenge, with feed amended with a mixture of twelve *Bacillus* spp., provided protection against artificial *E. ictaluri* infection in aquaria. Although a previous study reported improved survival and yield of catfish due to the use of a commercial probiotic product containing a mixture of *Bacillus* spp. (*B. subtilis*, *B. megaterium*, and *B. polymyxa*, *B. licheniformis*), the effects of the probiotic on reduction of mortality specifically due to ESC was not possible due to a mixed infection with proliferative gill disease during the study (Queiroz and Boyd, 1998). Furthermore, that study required the administration of the probiotic to pond water three times a week for five months to achieve the effects obtained. The data obtained in experiment one was the basis for making a decision to continue work to select the best *Bacillus* isolates from a large collection (N=160) for use in biological control of ESC. In addition to data from this challenge experiment, data obtained from other experiments including experiments on the stability of *Bacillus* spp. within catfish, and *in vitro* antibiosis against *E. ictaluri* and other fish and shrimp pathogens have been used to select the best performing *Bacillus* spp. for use in biological control of ESC and other fish diseases (Chao et al., manuscript in preparation). This selection process led to a final collection of nine *Bacillus* spp. which include the seven species used in challenge study number two.

Although the comparison to a control of the levels of protection (i.e. percent mortality) provided by individual *Bacillus* spp. at the end of experiment two was not possible, the results in this experiment may be used as a tool to further narrow down the best performing two or three *Bacillus* strains from a larger collection of *Bacillus* strains. The data indicates that AP193 is the best performing isolate. However, an ongoing experiment that includes proper controls also includes the seven *Bacillus* strains included in experiment two, and the results of that experiment will be used to validate the results of experiment two.

5. Conclusions

This study reports the isolation of a Gram-positive, *Bacillus*-like bacteria isolated from the gastro-intestinal tract of channel catfish fingerlings. The colony morphology, ability to form spores, production of inhibitory compound (s), and analysis of the 16S ribosomal RNA gene sequence indicate that the bacteria belongs to the genus *Bacillus*. Further analysis is needed to conclusively identify the species.

The administration of feed amended with twelve *Bacillus* spp. to channel catfish fingerlings demonstrated the capability of these *Bacillus* spp. as a group to provide protection against artificially induced *E. ictaluri* infections in aquaria. The evaluation of the capacity of individual *Bacillus* spp. to provide protection against artificially induced *E. ictaluri* infection was inconclusive, but has suggested protective effects in each study. However, data on challenge study using individual *Bacillus* spp. may be used to select the best candidates for use in biological control of enteric septicemia of catfish.

Table 1. Mortality of channel catfish fingerlings that were fed for 5 d either with feed amended with a mixture of *Bacillus* spp. (N=12 isolates) or with standard catfish feed, and then challenged with *E. ictaluri*. Values are mean of five replicates \pm SD. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column.

Treatment	Mean mortality (%)
Control	29.3 \pm 7.2 ^a
<i>Bacillus</i> cocktail	17.3 \pm 1.5 ^b

Table 2. Mortality of channel catfish fingerlings that were fed for 5 d either with standard catfish feed or feed that was amended with one of seven *Bacillus* strains, and the challenged with *E. ictaluri*. Values are mean of four replicates \pm SD. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column.

Treatment	Mean mortality (%)
AB01	23.8 \pm 15.4 ^b
AP76	22.5 \pm 15 ^b
AP77	15 \pm 16.8 ^b
AP79	15 \pm 17.8 ^b
AP143	36.3 \pm 29.5 ^b
AP193	7.5 \pm 2.9 ^a
AP254	18.8 \pm 15.5 ^b

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