DISCOVERY, ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES

SPECIFIC FOR *EDWARDSIELLA ICTALURI*

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

John Kiremerwa Walakira

Certificate of Approval:

Mark R. Liles Assistant Professor Biological Sciences	Jeffery S. Terhune, Chair Associate Professor Fisheries and Allied Aquacultures
Covadonga R. Arias Associate Professor Fisheries and Allied Aquacultures	Joe F. Pittman Interim Dean Graduate School

ARDC, KAJJANSI LIBRARY
02 JUL 2008
RECIEVED

Ms WAL
Acc 926
ID 150

DISCOVERY, ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC FOR *EDWARDSIELLA ICTALURI*

John Kiremerwa Walakira

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
May 10, 2008

DISCOVERY, ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC FOR *EDWARDSIELLA ICTALURI*

John Kiremerwa Walakira

Permission is granted to Auburn University to make copies of this thesis at its discretion, upon request of individuals or institutions at their expense. The author reserves all publication rights.

_____
Signature of Author

_____
Date of Graduation

THESIS ABSTRACT

DISCOVERY, ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC FOR *EDWARDSIELLA ICTALURI*

John Kiremerwa Walakira

Master of Science, May 10, 2008
(B.S., Makerere University, 1997)

80 Typed Pages

Directed by Jeffery S. Terhune

Two novel bacteriophages that infect *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), were isolated from aquaculture pond water and from a diseased channel catfish. Both phages (ΦeiDWF and ΦeiAU) produced clear plaques ranging from 0.5 to1 1 mm in diameter, and have an icosahedral head that is approximately 50 nm in diameter with a non-rigid tail structure. The phages are tentatively classified in the family *Siphoviridae*. Phages ΦeiDWF and ΦeiAU are double-stranded DNA viruses with approximate genome sizes of 39 and 45 kb, respectively, with many restriction fragments in common as revealed by multiple restriction digests. Phage infection is calcium-dependent, with an optimal concentration of 500 μM $CaCl_2$ resulting in a increase in plaque forming unit per milliliters (PFU ml^{-1}) of over three orders of magnitude compared to media without supplemental $CaCl_2$. Phage titers were also

highest under the ideal growth conditions for *E. ictaluri*, with an optimal temperature range of 25-30°C. The latent period for phages is approximately 40 min with an estimated burst size of 270 viral particles per bacterial cell. At a multiplicity of infection (MOI) of 0.1 (phage: host), an *E. ictaluri* culture was reduced in colony forming units per milliliters (CFU ml^{-1}) by over six orders of magnitude within a 6 h incubation period. All *E. ictaluri* strains tested were susceptible to phage infection with variable plaquing efficiencies. These phages have not been observed to produce plaques on any other bacterial species. The specificity of these bacteriophages for *E. ictaluri* isolates makes them attractive candidates as diagnostic agents and for bacteriophage therapy of ESC.

ACKNOWLEDGMENTS

The author would like to thank Dr. Jeffery Terhune and Dr. Mark Liles for making it possible to conduct research at Auburn University, and for their valuable lessons and guidance in the development and implementation of this project. He would like also to recognize the contributions made by Dr. J. Plumb and K. Hayden who provided samples collected from Auburn University. Dr. C. Arias and Dr. J.C. Newton for providing *E. ictaluri* isolates. He is grateful for the technical contributions made by members of the Liles and Terhune laboratories, Auburn University.

Style manual or journal used: Journal of Diseases of Aquatic Organisms

Computer software used: Microsoft Word 2003, Microsoft Excel 2003, SAS 9.1.3

TABLE OF CONTENTS

LIST OF TABLES xi

LIST OF FIGURES xii

I. INTRODUCTION 1

II. LITERATURE REVIEW 5

A. Enteric septicemia of catfish disease . . . 5

B. *Edwardsiella ictaluri* 6

C. Pathogenesis 8

D. Epidemiology 9

E. Control of ESC 10

F. Bacteriophages 12

G. Classification of bacteriophages 13

H. Phage replication and growth 15

I. Phage resistance 23

J. Bacteriophage therapy 24

III. MATERIALS AND METHODS 28

A. Bacteria and media 28

B. Discovery, enrichment and isolation of bacteriophages . 29

C. Electron microscopy 30

D. Isolation and restriction bacteriophage nucleic acids . 31

E. Effects of temperature, Ca and Mg on bacteriophage replication 31

F. One-step growth 32

G. *In vitro* challenges. 32

H. Host range determination 33

I. Prophage induction 33

J. Statistical analysis 33

IV. RESULTS 35

A. Isolation of bacteriophages 35

B. Size and morphology of bacteriophages . . . 35

C. Bacteriophage nucleic acid 36

D. Phage production 36

E. Burst size and Latent period 37

F. Kinetics of phage-induced lysis 37

H. Host specificity of phages 38

I. Prophage induction 38

V. DISCUSSION 40

LITERATURE CITED 46

LIST OF TABLES

1. Host range of phage infection tested on *Edwardsiella ictaluri* strains and other bacterial species collected from different locations. The EOP for phage was determined as a ratio of PFU ml^{-1} for each strain relative to that obtained from *E. ictaluri* strain 219 determined after 12 h of incubation at 30°C 59

LIST OF FIGURES

1. Electron micrographs of phages ΦeiAU and ΦeiDWF negatively stained with 2% phosphotungstic acid 60

2. Restriction fragment analysis of phages with *Eco*RI resolved by agarose gel electrophoresis. Arrows show presence of DNA fragments (double bands) unique to phage ΦeiAU. . . 61

3. Effect of temperature on phage titer when infecting log-phase *E. ictaluri* 62

4. Effects of $CaCl_2$ and $MgCl_2$ on titer of phage ΦeiAU when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (± SD) 63

5. Effects of $CaCl_2$ and $MgCl_2$ on titer of phage ΦeiDWF when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (± SD) 64

6. Effects of adding 500 μM $CaCl_2$/$MgCl_2$ and a combination, on titers of phage ΦeiDWF and ΦeiAU when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (+ SE). The asterisks indicate a significant difference from the control (* $P < 0.05$: Dunnett's test). 65

7. One-step-growth-curve showing the latent period (40 min) and the average burst size (270 viral particles per host cell) . . 66

8. Phage-induced lysis of *E. ictaluri* cells grown in BHI broth supplemented with 500 μM $CaCl_2$. Phage ΦeiAU and ΦeiDWF were separately inoculated into log phase cultures of *E. ictaluri* strain 219 while incubating at 30°C. Error bars indicate mean (± SD). 67

9. Effects of inoculating phage ΦeiDWF into *E. ictaluri* strain 219 cultures in log phase (after 6h) and stationary phase (after 19h). Bacterial counts were compared with the control (with out phage). Cultures were supplemented with 500 μM $CaCl_2$ and incubated at 30°C. Error bars indicate mean (+ SD) 68

INTRODUCTION

Edwardsiella ictaluri is the leading fish pathogen affecting farm-raised channel catfish (*Ictalurus punctatus* Rafinesque) in the southeastern states of the United States (Hawke et al. 1981, Hawke et al. 1998, Plumb 1999, Hawke & Khoo 2004). Economic loses due directly to enteric septicemia of catfish (ESC) outbreaks are estimated between $20 and $30 million per year, affecting 78% of all aquaculture farms (Wagner et al. 2002, USDA 2003a, USDA 2003b). The disease primarily affects channel catfish but has also been experimentally reisolated from other species: i.e walking catfish *Clarias batrachus* Linneaus (Thailand), European catfish *Silurus glanis* Linneaus, Chinook salmon *Oncorhynchus tshawytscha* Walbaum and rainbow trout *Oncorhynchus mykiss* Walbaum (Inglis et al. 1993, Plumb 1999). ESC outbreaks are seasonal occurring during late spring and early fall when temperatures range from 22°C to 30°C (Tucker & Robinson 1990, Hawke et al. 1998). However, adverse environmental conditions that exist in an aquaculture system greatly accelerate the severity of ESC causing mortalities of over 50% of cultured fish (Plumb 1999).

Control and preventive measures such as the use of antibiotics and a vaccine (Wise & Johnson 1998, Klesius & Shoemaker 1999, Shoemaker et al.1999, Wise & Terhune 2001) have not been adopted by all catfish producers, limiting their utility in eradicating ESC. Application of medicated feed is an expensive practice, marginally

effective coupled with the antibiotic resistance of *E. ictaluri* to oxytetracycline and ormetoprim–sulphadimethoxine (drugs approved for use on food fish) raises concerns about the long-term efficacy of antibiotic treatment in the commercial production of catfish (Johnson 1991, DePaola et al. 1995, Plumb et al. 1995). Furthermore, consumer preference is increasingly toward farm-raised products utilizing responsible and sustainable management practices, and the decreased use of antibiotics. Similarly, disease outbreaks often occur within vaccinated catfish populations (Thune et al. 1994) and the vaccine has not been universally adopted.

Biological control agents such as bacteriophages may provide an alternative mechanism to control bacterial diseases in both human and veterinary medicine (Barrow & Soothhill 1997, Barrow 2001). The use of phages to treat bacterial infections has a long history in the former Soviet Union, and only recently has Western medicine subjected phage therapy to clinical studies (Sulakvelidze et al. 2001, Levin & Bull 2004). Phage therapy typically involves isolation of diverse bacteriophages specific to a bacterial pathogen that can be used in combination as a bacteriophage "cocktail" (Sulakvelidze et al. 2001). By treating infections with phage, the infected bacterial cells may carry the phage deeper into infected tissue that upon host cell lysis may spread to and infect other bacterial cells. Because a phage can exhibit strong host specificity, express efficient systems for host cell lysis, and spread avidly within an aquatic medium, there has been an increasing interest in their use in the aquaculture industry to control fish pathogens. Studies have demonstrated that *in vitro* and *in vivo* challenges with bacteriophages may reduce mortalities in abalone, loaches, milkfish, brook trout, eastern oysters, yellowtail and Ayu fish (Wu et al. 1981, Wu et al. 1984, Nakai et al. 1999, Tai-

wu 2000, Park et al. 2000, Pelon et al. 2005, Imbeault et al. 2006, Vinod et al. 2006). Recently, the United States Food and Drug Administration (FDA) approved the safe use of a bacteriophage on ready-to-eat meat and poultry products as an additive against *Listeria monocytogenes* (Bren 2007), thereby increasing opportunities for phage application in the food industry.

Two principal challenges in the use of bacteriophages as biological control agents in aquaculture are the selection for bacterial resistance to phage infection and rapid clearance of phage by the fish reticuloendothelial system (Russell et al. 1976, Nakai & Park 2002, Levin & Bull 2004, Dabrowska et al. 2005). Bacterial resistance to phage infection may be lessened as a problem by using phage cocktails that include phages that target diverse host cell receptors. Furthermore, selection for phage-resistance may result in avirulent phenotypes depending upon the mechanism of phage-resistance (i.e. whether the phage receptor is required for bacterial virulence). Such loss of bacterial virulence in a phage-resistant bacterial mutant has been demonstrated previously in a fish pathogen (Park et al. 2000). The problem of reticuloendothelial system clearance of phage within fish may be lessened by selecting for phage variants with reduced clearance rates, via serial passaging of phage within the animal host as has been demonstrated with long-circulating phage variants in a mouse model (Merril et al. 1996). Therefore, the ability to control an aquaculture pathogen through the use of bacteriophage therapy will depend upon several factors, including the route of pathogen infection into an animal host, having multiple phage types that infect diverse genotypes of the bacterial pathogen, the kinetics of phage infection of the bacterial host, burst size of the phage, and whether the phage can enter a lysogenic stage.

While ESC is in some respects an ideal bacterial disease for bacteriophage therapy (i.e. high-density of catfish in aquaculture ponds, fecal-oral route of infection, closed aquatic system), no phage that infects *E. ictaluri* has ever been reported. Clearly not every phage isolated would be an attractive candidate for phage therapy of ESC. Hence, this study focused on isolating bacteriophages with *E. ictaluri* host-specificity, without evidence of lysogeny, and capable of producing large, clear plaques upon pathogenic strains of *E. ictaluri*.

LITERATURE REVIEW

Enteric septicemia of catfish disease

Enteric septicemia of catfish (ESC) is a highly infectious and systemic fish disease affecting farm-raised channel catfish (*Ictalurus punctatus* Rafinesque) in the southeastern states of the United States, and also in Idaho, Indiana, California, New Mexico and Arizona (Hawke 1979, Inglis et al. 1993, Hawke et al. 1998, Plumb 1999, Hawke & Khoo 2004). Economic loses have been estimated to be between $20 and $30 million per year and affecting 78% of commercial farms (Plumb & Vinitnantharat 1993, Wagner et al. 2002, Wise et al. 2002, USDA 2003a, USDA 2003b). The disease primarily affects channel catfish, however, it has been reisolated in experimental infections in other species from different geographical regions: i.e walking catfish *Clarias batrachus* Linneaus (Thailand), European catfish *Silurus glanis* Linneaus, Chinook salmon *Oncorhynchus tshawytscha* Walbaum and rainbow trout *Oncorhynchus mykiss* Walbaum (Inglis et al. 1993, Plumb 1999).

ESC was first reported in 1976 in channel catfish exhibiting a range of clinical signs: behaviorally, a "head-up-tail-down" posture, spiral spinning and anorexia; externally, petechial hemorrhages, pale gills, exophthalmia and "hole-in the head" between the eyes; internally, cloudy or bloody ascites in the visceral cavity, enlargement of the kidney, spleen and often the abdomen, necrosis of the liver, ecchymoses in the

gastrointestinal mucosa, and inflamed adipose and peritoneal tissues (Hawke 1979, Inglis et al. 1993, Plumb 1999).

The disease manifests itself in chronic to acute forms where the latter is more prevalent (Newton et al. 1989, Klesius 1992a). Chronically infected fish develop a "hole-in-the-head" between frontal bones of the skull but with low mortalities, and acute forms are characterized with high mortalities and general septicemia, affecting mostly young-of-the-year fingerlings (Newton et al. 1989, Inglis et al. 1993, Plumb 1999, Wise et al. 2002).

Edwardsiella ictaluri

Edwardsiella ictaluri, the causative agent of ESC, was first reported and described in 1976 at the Southern Cooperative Fish Disease Laboratory of Auburn University in channel catfish (Hawke 1979). It belongs to *Enterobacteriacae* family: Gram-negative, rod-shaped, fermentative bacterium and motile using peritrichous flagella (Hawke 1981, Hawke et al.1998, Plumb 1999). Biochemically, it is cytochrome oxidase negative, citrate negative, phenyl alanine deaminase negative, indole and H_2S negative, ferments and oxidizes glucose releasing gases and is less tolerant to higher levels of 1.5% NaCl (Waltman et al. 1986, Plumb 1999).

However, most of above characteristics are presumptive as they are generally shared among most members of *Enterobacteriacae*, hence conclusive tests or studies should be conducted to identify *E. ictaluri* (Hawke 1979, Waltman et al. 1986, Plumb 1999). Therefore, confirmatory tests include the use of a selective media such as *Edwardsiella ictaluri* medium (EIM) that was formulated by Shotts & Waltman (1990).

In addition, *E. ictaluri* does not produce hydrogen sulphide on triple sugar iron (TSI) and is non-motile at temperatures above 37°C (Plumb 1999). Other commercial identification tests that can be used to identify *E. ictaluri* are Biolog (Biolog Inc., Hayward CA, USA) and API-20 E (bioMérieux® sa, Marcy l'Etiole, France), according to AFS-FHS (American Fishery Society-Fish Health Section, Bethesda, Maryland, 2005). A validated real-time polymerase chain reaction (PCR) assay was developed to detect the presence *E. ictaluri* pathogens in tissue samples of fish thereby quantifying bacterial DNA per cell equivalents (Bilodeau et al. 2003). Recently, an indirect fluorescent antibody (IFA) test has been demonstrated to be an efficient tool for rapid detection of *E. ictaluri* during outbreak episodes (Panangala et al. 2006). A new intervening sequence (IVS) in the 23S rRNA of *E. ictaluri* was also identified and can be utilized for ESC detection (Zhang & Arias 2006).

Phylogenetic analysis based on the 16S-23S rRNA intergenic spacer region (ISR) revealed a more than 96% similarity between *E. ictaluri* and *E. tarda* species (Panangala et al. 2005). This was further confirmed by a study performed by Zhang and Arias (2006) when they compared the two species with the 'core' members of the *Enterobacteriaceae* using a Parsimony tree based on 23S rRNA gene sequences. However, *E. tarda* is more diversified than *E. ictaluri* (Panangala et al. 2005) and both can be distinguished, biochemically; *E. tarda* is H_2S positive and motile above 37°C (Plumb 1999). Additionally, *E. ictaluri* is reported to be a clonal bacterial species compared to the less monomorphic *E. tarda* (Panagala et al. 2006).

Pathogenesis

Edwardsiella ictaluri can infect fish through two routes: (1) free-living bacteria invade the olfactory organ through the nasal cavity then into the brain, and the infection spreads from meninges to the skull and skin, creating the 'hole-in-the head' condition; this is the chronic form (Miyazaki & Plumb 1985, Shotts et al. 1986), and (2) the bacteria can also be ingested and enter the blood system via the intestinal membranes leading to systemic infections in the dermis, thus causing necrosis and depigmentation of the skin: this is the acute form (Shotts et al. 1986, Inglis et al. 1993).

In systematic infections of the catfish the most affected organs are the trunk kidney and spleen that develop necrosis as well as the liver that becomes edematous (Areechon & Plumb 1983, Plumb 1999). Systemic infections are characterized by the necrosis of the hepatocytes and pancreatic cells (Plumb 1999). Externally, a proliferation interlamellar gill tissue occurs but also destruction and depigmentation of the skin due to the accumulation *E. ictaluri* cells in the capillaries was reported (Miyazaki & Plumb 1985, Plumb 1999). For the "hole-in-the-head" condition, *E. ictaluri* attach on to the olfactory epithelium then penetrates the submucosa causing degeneration of sensory cells (cilia and microvilli) and the supporting cells (Plumb 1999). Shotts et al. (1986) demonstrated that when catfish were orally exposed to *E. ictaluri* they developed enteritis, hepatitis, interstitial nephritis and myositis.

The pathogenic mechanism is associated with extracellular products that may cause virulence (Saed 1983, Stanley et al. 1994). The lipopolysaccharide (LPS) is an endotoxin, capable by itself of causing damage in the host and fibrillar processes used for attachment may play a vital role in the pathogenesis of *E. ictaluri* (Lawrence et al. 2001,

2003). Cells of *E. ictaluri* are reported to survive in catfish neutrophils and also able to replicate inside catfish macrophages (Morrison & Plumb 1994, Stanley et al. 1994). Copper et al. (1996) suggested a possible role of chondroitin sulfatase activity in the virulence of *E. ictaluri*. When channel catfish were challenged, intraperitoneally, with *E. ictaluri* transposon mutant MI 15 cells (deficient of chondroitinase activity) and held for two weeks, no mortalities were observed and none showed signs of ESC (Copper et al. 1996). Chondroitinase activity may mediate the degradation of cartilage tissue creating the "hole-in-the-head" condition in chronic infections (Waltman et al. 1986). Thune et al. (2007) identified virulence-related genes at a 26, 135-bp pathogenecity island containing 33 genes of a type III secretion system (TTSS) using a signature-tagged mutagenesis of *E. ictaluri* approach.

Epidemiology

Edwardsiella ictaluri was described as an obligate pathogen (Hawk 1979) but subsequent studies showed that it can survive in ponds sediments for more than 90 d at 25°C, and in water for 15 d at 25°C or below, provided there is less microbial competition (Plumb & Quinlan 1986, Plumb 1999). *Edwardsiella ictaluri* was also detected in the intestines of catfish that were experimentally infected and from natural waters, in cormorants and herons although their viability and survivability has not been demonstrated (Klesius 1992, Mqolomba & Plumb 1992, Plumb 1999). Klesius et al. (2003) isolated the pathogen from natural populations of madtom tadpoles, *Noturus gyrinus* which showed a distinct population of *E. ictaluri*.

ESC outbreaks are frequently experienced in the Southeastern United States of America where channel catfish farming is concentrated (Hawke et al. 1998, Plumb 1999).

Seasonal epizootics normally occur during late spring and early fall when temperatures range from 18°C to 30°C (Tucker & Robinson 1990). Mortalities in catfish fingerlings have been demonstrated to be highest at 25°C, lower at 23°C and 28°C, and none at 17, 21 or 32°C (Francis-Floyd et al. 1987, Mqolomba & Plumb 1992). However, adverse environmental conditions accelerate the severity of ESC disease causing mortalities that vary from less than 10% to over 50% in all aquaculture systems (Plumb 1992, Inglis et al. 1993).

Edwardsiella ictaluri infects both fingerlings and food-size fish reared in ponds, recirculating and cage systems. The mode of transmission is reported to be through: (1) carrier fish populations that seclude the bacteria (2) contact with fish carcasses of ESC disease where large numbers of bacteria are shed (Klesius 1992, Plumb 1992, Inglis et al. 1993), (3) vectors like cormorants and herons (Taylor 1992). Vertical transmission is a possibility but has not yet been demonstrated (Plumb 1992).

Control of ESC

Therapeutic agents are available to fish farmers in an effort to control ESC outbreaks. Currently, antibiotics approved by FDA to treat ESC are Romet 30® (Alpharma, Inc., Animal Health Division, Fort Lee, New Jersey) a 5:1 mixture of sulfadimethoxine and ormetoprim and Florfenicol or Aquaflor ® (Schering-Plough Animal Health Corp., Union, New Jersey, USA) (Tucker & Robinson 1990, Gaikowski et al. 2003). Development of a live attenuated vaccine, *E. ictaluri* RE-33, AQUAVASEC ® (Intervet, Inc., Millsboro, DE, USA) was successful and is licensed for sale (Shoemaker et al. 2002). Other measures include restriction of feed regime (Wise & Johnson 1998)

ARDC, KAJJANSI LIBRARY
02 JUL 2008
RECIEVED

and ensuring good management practices at farm level (Tucker & Robinson 1990). Despite these interventions, the industry continues to suffer economic losses due to ESC mainly because of short-comes encountered in many of these control strategies.

Klesius and Shoemaker (1999) reported that the application of antibiotic medicated feed as an expensive and marginally effective practice in that diseased fish become anorexic and resistance to oxytetracycline and ormetoprim-sulfamethoxine drugs was eminent. Previously, Romet® had palatability problems due to presence of ormetropim (Poe & Wilson 1989) but this has been corrected by adjusting the fish meal composition from 4 to 16 % (Robinson et al. 1990); i.e the use of a new top coating method for applying the drug on feed. Furthermore, Florfenicol has recently gained FDA approval that is shown to be effective in treating ESC and no palatability problems have been reported (Gaunt et al. 2003). Resistance to Romet® has been observed in several strains of *E. ictaluri* and may be plasmid mediated (Starliper et al. 1993). The efficacy of feeding regimes was evaluated under field conditions and it was revealed that the feeding frequency is a primary factor affecting the severity of ESC infections; i.e. survival and weight gain was shown to be high in fish that was completely withheld from feeds or those fed every other day/s (Wise & Johnson 1998). Nevertheless, restricted feeding also results in less production (Wise et al. 2001).

Vaccine applications against ESC show far-reached objectives since most catfish farms have not adopted its usage. Initially, studies conducted on bacterins revealed inconsistent protection against bacterial infections and this is attributed to their inability to induce a cell mediated immune response against intracellular pathogens (Shoemaker & Klesius 1997). Development of a live attenuated vaccine, AQUAVACESC®, provided

the best alternative, however, further investigations on the vaccine show mixed results. Vaccinated fingerlings at a dose of 1.0×10^{7} CFUml^{-1} had significantly low cumulative mortality rates compared to those that were not vaccinated, however, at a lower dose of 1.0×10^{6} CFUml^{-1}, RE-33 provided some protection during laboratory challenges (Wise & Terhune 2001). Carrias (2005) found out that the mean survival of 32 d post-hatch catfish fry nursed in tanks was significantly higher that than the controls compared to the 10 d vaccinates.

Bacteriophages

Bacteriophages (phages) are defined as viruses specific to bacteria with remarkable capabilities to lyse host cells (hence termed "bacteria eaters"), and the lytic agent is transmissible in series from cultures of susceptible bacteria (Twort 1915, d'Herelle 1917, Adam 1959). This definition is further broadened to include prophages; i.e. phage genome is inserted in the linear structure of the bacterial DNA chromosome to form temperate phages and the host cell enters into a state of lysogeny (Adams 1959).

In 1915, Twort described changes in morphology that occurred in the Staphylococci disease but did not pursue this matter further (Adams 1959). Then in 1917, Felix d'Herelle discovered bacteriophages from stool samples of dysentery patients that were inoculated in naive Shiga's bacillus and found out that some samples were clear and sterile (Adams 1959). Subsequently, research on bacteriophages intensified in the 1920s onward, in trying to understand many aspects of virology and its capabilities in therapeutic applications (Adams 1959). However, in the 1930s and early 1940s research into phage therapy declined mainly due to inconsistent therapeutic results as opposed to what was observed from antibiotics: since antibiotics were effective and had a broader

therapeutic spectrum (Summers 2001, Sulakvelidze et al. 2001, Merril et al. 2003, Levin & Bull 2004). However, use of phages to treat bacterial infections has recently gained attention in western medicine mainly due to resistance to antibiotics agents (Sulakvelidze et al. 2001, Levin & Bull 2004).

Classification of bacteriophages

The taxonomy and nomenclature of bacteriophages was recently streamlined under the auspices of the International Committee on Taxonomy of Viruses (ICTV), in liaison with the International Union of Microbiological Societies (IUMS). These entities are responsible for developing, refining, and maintaining a universal virus taxonomy using individual phylogenies or comparative genomics (Nelson 2004, ICTV 2007). A database called *ICTVdb-The Universal Virus Database* has been established with updated versions (ncbi/ICTV 2006).

Historical classification of phages, under ICTV conventions, was based on a hierarchal system invented by Linnaeus in 1758. Viruses that shared common characteristics were grouped together with subgroups that also share a common attribute (Fenner 1976). For instance, *Cardovirales* describes tailed-phage that are subdivided into three families; *Myoviridae* - phage with long, contractile tails; *Siphoviridae*- phage with long, noncontractile tails; *Podoviridae* - phage with short tails. These families are further broken down into genus and subgenus using genome configuration (linear, circular and super coiled), host range and genome size (Nelson 2004). However, this approach has been overwhelmingly criticized because it overlooks genomic and proteomic information. For example, *Salmonella* P22 phage was erroneously grouped in *Podoviridae* for 30

years but recently using molecular techniques showed its relatedness to a long-tailed lambda phage (Botstein & Herskowitz 1974, Nelson 2004). Another problem also cited with current classification system is that the ICTV system is based on vertical transmission of genetic characteristics whereas phage genomes are known to be highly mosaic due to horizontal exchange of material among common genetic pools (Hendrix et al. 1999, Pedulla et al. 2003, Nelson 2004). The current taxonomic model also depends on electron microscopy studies but: (a) electron microscopy underestimates the size, in general, due to unavoidable shrinkage when drying samples (Adams 1959), (b) it is faster and more convenient to obtain genome sequences (Nelson 2004), and (c) sometimes phage exists as lysogenic prophage that do not produce mature virions (Breitbart et al. 2002, Nelson 2004).

The concept of classifying bacteriophages based on genomic data becomes problematic as phages are known to lack ribosomal DNA in addition to the absence of unique conserved genes or protein sequences shared by all phages (Rowher & Edwards, 2002, Nelson 2004). Hence, Nelson (2004) described three new approaches that are proposed by three phage research groups. First, is the construction of a "phage proteome tree" method that analyzes the entire predicted proteome for a given phage using BLASTP or the PROTDIST program, and thereafter a matrix is developed (Rowher & Edwards 2002). The second is using the Linnaean hierarchal approach whereby viruses are divided into "domains" according to genome type (double-stranded DNA, single-stranded DNA, single-stranded RNA and double stranded RNA) and further split into "divisions" that define separate features like tailed phages from filamentous phages (Nelson 2004). The third uses a scheme of classification based on comparative genomics

of a structural gene module adopted from the oldest and most conserved module in dairy phage (Lactococcal phage) that was used to determine graded relatedness between DNA and protein sequences (Proux et al. 2002, Nelson 2004).

Phage replication and growth

According to Adam (1959) bacteriophage may exist in three states: (1) mature phage that exists outside the host cell and is metabolically inert, resembling the spore state of bacteria, (2) vegetative phage that exists inside the host after the adsorption process, (3) prophage or temperate phage were hereditary material is integrated with the host cell leading to lysogeny. Lysogenic bacteria are non-infectious but capable of multiplying without being destroyed, however, any spontaneous change such as exposure to UV radiation may lead to the host destruction (Adams 1959).

Bacteriophage multiplication process is divided into (i) the attachment or adsorption stage where the phage particle attaches to the host cell, (ii) penetration stage of the phage into the bacterium, (iii) biosynthesis or intracellular multiplication of the viral components, (iv) maturation stage, (v) release of phage progeny from the host cell (Adams 1959).

Adsorption or phage attachment to the host is the initial step of the infectious cycle of a bacteriophage. During adsorption, the phage attaches to a complementary receptor site of the host cell membrane, forming weak bonds (Adams, 1959). Adams (1959) categorized phage attachments into: (i) those that adsorb irreversibly, (ii) those that adsorb reversibly, (iii) those that do not adsorb at all. Other phage attachments are specific to the flagella or fimbriae of hosts as demonstrated by a phage that is active on

Caulobacter vibriodes CV6 flagellum (Jollick & Wright 1974).

The adsorption process is specific and attachment rate on viable bacteria was demonstrated to be 2.6 times faster than on heat-killed bacteria; attributed to the destruction of receptor-sites during the heat-kill processes (Adams 1959). A bacterium receptor is reported to capture phage at each collision (Adams 1959, Schwartz 1976). When phage T4 infects *E. coli* B cells the phage tail fibers attach to the outer membrane receptors (e.g. lipopolysaccharide) before injecting its DNA inside the host (Crawford & Goldberg 1980). Using mathematical models, the adsorption capacity is estimated to range from 140 – 300 phage per bacterium, based on experiments conducted using log-phased and heat killed *E. coli* infected with a coliphage and T2 phage, respectively (Delbrück 1940, Adams 1959).

The mechanism of the capture rate of phage was first explained by Delbrück (1940) and Adam (1959) as a reaction rate enhancement by dimensional reduction (RREDR); that is a phage particle encounters a bacterium by diffusion in the three-dimensional (3D) fluid, and it undergoes a "random walk" on the host surface as a two-dimensional (2D) fluid, after it is adsorbed. Numerous studies confirmed a 3D+2D strategy to be the most likely model (Berg & Purcell 1977). Berg and Purcell (1977) further explained that the feasibility of a 2D diffusion strategy requires sufficient adsorption energies to bind the phage but weak enough such that the 2D diffusion is not impeded. It was also suggested that the swimming action of bacteria could be the cause of fast adsorption of phage (Berg & Purcell 1977). However, Moldovan et al. (2007) suggested that phage binding to their hosts, in a defined medium, proceeds in two distinctive steps: a fast and a slow exponential relaxation. This two-step process is

different from the classical two-stage capture model which postulates that binding is membrane-assisted or RRIEDR. Based on observations made on *E. coli* CR 63 that lacks functional receptors, phage-biding abilities failed, however, attained its biding capabilities when a plasmid that expresses LamB (gene coding for the phage λ receptor protein in *E. coli*) was introduced (Moldovan et al. 2007). This two-step process is basic and essential to the receptor-phage interaction and is independent of processes mediated by the bacterial membrane (Moldovan et al. 2007).

In vitro experiments show factors that influence phage adsorption are presence of cations (e.g. Mg and Ca), pH, temperature and the growth medium. Ions have an electrostatic bonding effect in the interactions of phage–bacterium systems (Puck et al. 1951, Moldovan et al. 2007). Moldovan et al. (2007) found out that a concentration of 10^{-2} M Magnesium sulphate buffer (prepared from 10 mM $MgSO_4$, 20 mM Tris buffer, adjusted to pH 7.4) was optimal for the adsorption of λ phage to *E. coli* cells and almost no adsorption of phage occurred at concentration below 10^{-1} M $MgSO_4$ buffer. A rapid decrease was also observed in concentrations greater than 0.1 M $MgSO_4$ buffer due to phage being unstable or high salinities preventing infection of the bacteria (Moldovans et al. 2007). Additionally, when *E. coli* cells are infected with filamentous bacteriophage the addition of $CaCl_2$ increases the concentration of phage particles at the surface of bacteria by creating a shield around negatively charged bacteria and phage particles (Russell et al. 1988). Calcium is reported to alter the structure of the bacterial membrane thereby increasing the accessibility of filamentous phages to a co-receptor complex (Russell et al. 1988). Calcium ions are also reported to be a requirement in stages subsequent to adsorption, for example, in infections of phage PL-1 that is active against *Lactobacillus*

easel ATCC 27029 (Watanabe & Takesue 1972). Some phages require organic compounds as precursors for adsorption, for example tryptophan is shown to activate some variants of coliphage T4 (Adams 1959). The effect of pH on adsorption was also investigated using phage T2 and *E. coli* B; maximum adsorption occurred at pH 6.0 -8.0 (Puck et al. 1951, Adams 1959). Moldovan et al. (2007) also reported a significant increase in adsorption rate by 30 times when the temperature rose from 4 to 40 °C. Furthermore, Delbrück (1940) and Adams (1959) explained that in the adsorption process, the reversible rate (first step process) is independent of temperature whereas the irreversible rate (second step) is temperature-dependent. Carbon sources have also been reported to have an effect on phage adsorption. For instance, Moldovan et al. (2007) observed that adsorption on Ymel cells grown in maltose is stronger for cells grown in glucose because maltose causes cells to produce more receptors.

After adsorption, the phage then injects its genetic material inside the bacterium; therefore the penetration process. Prior to penetration an enzyme, lysozyme is released from the phage's tail to breakdown a portion of the bacterial cell wall (Brown et al. 1972). The tail sheath then contracts, driving through the cell wall and plasma membrane. The nucleic acid is released inside the host, leaving its capsid outside the host (Adams 1959). Penetration in some bacteriophages requires the presence of divalent ions like magnesium or calcium (Watanabe & Takesue 1972). Luria and Steiner (1954) demonstrated that an optimal concentration of 10^{-3} M $CaCl_2$ was crucial for the penetration phase of phage T5 DNA in *E. coli* B cells cultured in nutrient broth. Additionally, magnesium and strontium can be a substitute for calcium ions but with less effect compared to calcium (Luria & Steiner 1954).

Studies have described the mechanism of penetration of phage DNA into the host cell to be an ionic channel-mediated type of transport model (Boulanger & Letellier 1988). Boulanger and Letellier (1988) showed that when T4 phages and phages depleted of DNA(ghost phages) are in infecting *E. coli* B cells, a K^+ efflux induced by the phages competes with influx of H^+, Na^+ or Li^+ for entry through the channels, thereby depolarizing the cell and allowing the passage of DNA (Labedan & Goldberg 1979). Hence, DNA polyanion overcomes the membrane potential existing in non-infected cells. Insertion and conformation of these channels is also temperature dependent. For example, when T4 phage infects *E. coli* B cells, DNA freely diffuses through the channels at temperatures above 29°C but the efficiency of DNA penetration is reduced and conformation changed from 29 to 15°C (Boulanger & Letellier, 1987). Below 14°C, potassium efflux stopped and penetration was severely impaired (Boulanger & Letellier, 1987). The change in conformation is explained by the fact that 29°C is the mid-point that the order-disorder transition of isolated LPS occurs (Van Alphen et al. 1979). Furthermore, Grayson and Molineaux (2007) described a "syringe type model" as insufficient to explain how a phage genome can enter an infected cell because: (1) not all phage genomes contain double-stranded DNA which is the source of pressure in the syringe model, and (2) some phages lack a long tail that can span the cell envelope whereby a syringe model would result in the deposition of phage genome in the extracellular medium or the cell periplasm.

Biosynthesis of viral components commences after the phage DNA enters the host cytoplasm after which synthesis of viral nucleic acids and proteins occurs. This process is also characterized by an eclipse period which is described by the presence viral DNA and

proteins components (Adams 1959). Protein synthesis in the host bacteria ceases when phage induces degradation of host DNA, phage proteins interfere with transcription, and repression of translation occurs (Stone & Burton 1962, Cohen & Ennis 1966, Bautz et al. 1966, Hsu et al. 1967, Kennel 1970, Davis 2004). Bacteriophages utilize the host's nucleotides and enzymes to synthesize copies of its DNA before synthesis of its macromolecules. Thereafter, synthesis of enzymes and capsid proteins are produced from mRNA transcribed from phage DNA. The host's ribosomes, enzymes and amino acids are used for the translation process. However, Champe (1963) observed that little protein synthesized in the eclipse is incorporated in the mature phage particles. These early produced proteins are presumed to be enzymes and are essential for phage multiplication. It is argued that the bacterial host contains information in its genetic material and that phage DNA elicits specific inducers of the new proteins or removes inhibitors against preformed proteins (Champe 1963). These processes are mediated by mRNA polymerase together with other genes.

Thereafter maturation process occurs which is described by a step-by-step assemblage of phage DNA and capsid structure, into complete bacteriophage units (Adams 1959). In the single-stranded DNA (ssDNA) bacteriophage φ X 174, viral particles are produced by packaging the phage ssDNA into a precursor capsid or prohead that is composed of structural proteins [gpF (major capsid protein), gpG (spike protein), gpH (spike tip protein)] and non-strucatural proteins [gp(prohead assembly protein) and gpJ (external scaffold protein)] (Fujisawa et al. 1977, Mukai et al. 1979). The initial assemblage product is the precursor capsid (procapsid) that lacks DNA but contains numerous scaffolding proteins (absent in mature virion) that undergoes transitional

changes to form mature capsid (Casjens & Hendrix 1988). Scaffolding molecules are released from the procapsid via proteolysis (Murialdo & Becker 1978, Casjens & Hendrix 1988). Using phage-encoded packaging proteins and ATP, DNA is packaged into the capsid, and the overlaying layer undergoes conformational changes into a mature structure (Black 1989, Zhang et al. 2000). However, Zhang et al. (2000) suggested that DNA is packaged inside the procapsid in the presence of scaffolding proteins and not in the mature phage. During the late stage of maturation process, encapsulation of double stranded DNA occurs within phage heads, and may involve precursors of phage heads (proheads) and a linear concatemeric DNA substrate (Humphreys & Trautner 1981). It has been observed that in λ phage and T-odd coliphages the concatemers are cut at defined sites that are one genome apart, generating a population of identical unit-length molecules (Rhoades & Rhoades 1972, Humphreys & Trautner 1981). For a capsid to become stable a series of transformations occur. For example, in the maturation of T4 head, the precursor particle (r-particle which is membrane associated) transforms during processing of proteins; i.e. the core (gp22 and internal proteins) and the shell (gp 23, gp 24) the expand with the filling of DNA and finally assemblage into a mature phage (Biljenga et al. 1974). DNA molecules with sizes greater than the genome length are then sequentially packaged depending on the size of the head (Humphreys & Trautner 1981).

Matured phages inside the host cell are released upon lysis of the cell membrane subsequently infect other susceptible host cells (Adams 1959). Young (1992) describes two mechanisms and regulations involved in phage-induced lysis: (1) the normal release of progeny virions from a phage-encoded enzyme which degrades the murein (e-lysozyme or R-transglycosylases) and, (2) the release of progeny without the

participation of phage-enconded enzymes. A gene that codes for amino acid sequence of lysozyme is synthesized once the coat proteins begins to appear, after maturation is complete leading to the rupture of the cell wall. Furthermore, cell lysis may be due to phage-encoded proteins, endolysin, which can be of several unrelated types of enzymes that attack either the glycosidic bonds (lysozyme and transglycosylases) or peptide (amidases) bonds (Young 1992). Gründling et al. (2001) demonstrated that in double-stranded bacteriophages, a phage-encoded membrane protein holin created a lesion in the cell membrane that resulted in production of endolysin attacking the cell and subsequently lysing it. However, in some phages such as ΦX174 and MS2 the role of lysozyme has not been investigated. In phages like λ, T4 and T7 the presence of lysozyme activity is demonstrated to be irrelevant to the actual scheduling of lysis (Young 1992). Other mechanisms may be involved in either degrading the murein or the host enzymes are utilized. Ishiguro and Dai (1990) reported the genetic relationship between the autolysis of *E. coli* cells and phage development exists whereby phage progeny is released upon autolysis of the host cells. Delbrück (1940) describes phage that can lyse bacteria in two ways: lysis from within and lysis from without. Lysis from within occurs when a single phage particle infects a bacterium and multiplies exponentially until a threshold value, releasing the cell contents into solution without deformation of the cell wall. Lysis from without happens when the adsorption of phage particles exceeds the threshold value, thereby releasing cell contents after the cell wall is distended and destroyed.

A one-step-growth-curve demonstrates all stages involved in the multiplication of bacteriophages. From this curve, the latent period (lysis time) and the burst size are

determined (Adams 1959). It has been shown that for any ecological setting there is a positive relationship between latent period and burst size such that an optimal latent period exists leading to high phage fitness (Wang et al. 1996, Wang 2005). It is also reported that a trade-off exists between the latent period and the burst size were latent periods that optimize plaque fecundity are longer than latent periods that optimize plaque size. Additionally, an increase in burst size may contribute to plaque size or larger plaques with larger burst size (Abedon & Culler 2007). Chemicals such as cyanide may interfere with phage growth leading to a prolonged latent period, causing lysis inhibition (Adams 1959).

Phage resistance

Bacteria have evolved resistance to phage infection that may result from *in vitro* or *in vivo* treatments (acquired resistance) or transmitted through various mechanisms (primary resistance). Levin and Bull (2004) explain three mechanisms involved in the emergence of resistance strains. The first being that mutations of single genes that usually result in alteration or loss of the receptor that many phages use for attachment. Hence, phage can not replicate in those bacteria (thus, envelope resistance). The second mechanism described by mucoid colonies that confer bacterial cells to have partial resistance to phage infection. However, phage is capable of adsorbing to and replicate in these bacteria at low rate. The third uses plasmid-encoded restriction endonucleases that exist in bacteria to degrade the genome of infecting phage thereby disrupting the lytic cycle.

Interestingly, phages have also evolved to overcome bacterial resistance by creating an "evolutionary arms race" (i.e. phages fight back due to host-range mutations)

a perspective envisaged to be advantageous over antibiotic therapy (Lenski & Levin 1985, Bohannan & Lenski, 2000, Levin & Bull 2004). Normally, selection will favor mutant phages that have the ability to infect and replicate in these resistant bacteria (Levin & Bull 2004). Therefore, the best strategy might be the application of a phage "cocktail" that would have a broad lytic spectrum and provide an indefinite control of bacterial population (Levin & Bull 2004).

Evolution of phage-resistant bacteria may result in the development of less virulent bacterial strains as receptors used for phage attack might be capsules or virulence factors (Smith et al. 1987, Westwater et al. 2003). Such loss of bacterial virulence in a phage-resistant bacterial mutant has been demonstrated previously in fish pathogens (Park et al. 2000). Basically, resistance reduces the fitness of the bacteria rendering it to compete unfavorably with its phage-sensitive ancestors (Lenski & Levin 1985, Bohannan & Lenski 2000, Levin & Bull 2004).

Bacteriophage therapy

Recently, the emergence of antibiotic resistance against antimicrobial agents has re-ignited the search for phages that have the potential to treat and prevent bacterial infections (Levin & Bull 2004). In response, bacteriophages are continuing to be developed for use in human and veterinary medicine, agriculture and aquaculture (Smith & Huggins 1983, 1987, Merril et al. 1996, Barrow & Soothhill 1997, Barrow 2001, Park & Nakai 2003, Mastuzaki et al. 2005). Many of these achievements are due to improvements made in genetic and biological studies about bacteriophage (Adams 1959, Levin & Bull 2004)

The use of bacteriophage as biocontrol agents in aquaculture is possible. Experiments using phage PPp W- 4 and PPpW-3, isolated and experimentally used to control *Pseudomonas plecoglossicida* infections in fish ayu (*Plecoglossus altivelis* Temminck & Schlegel) showed significantly lower mortalities in phage-treated fish groups compared to those of untreated controls (Park et al. 2000). Imbeault et al. (2006) demonstrated that counts of *Aeromonas salmonicida* (etiological agent of Furunculosis) decreased by six log units in 3 d after the introduction of a phage HER 110, at a multiplicity of infection factor of 1. Bacteriophage of *Vibrio harveyi* was reported and the survival of white shrimp *Penaeus monodon* Fabricius larvae to be 80% versus 25 % observed in the controls (Vinod et al. 2006). Furthermore, *in vitro and in vivo* challenges with bacteriophages isolated from aquatic environments have demonstrated the potential of reducing mortalities, in yellowtail *Seriola quinqueradiata* Temminck & Schlegel and Ayu fish *Plecoglossus altivelis*, abalone *Haliotis discus hannai* Ino, loaches *Misgurnus anguillicaudatus* Cantor, brook trout *Salvelinus fontinalis* Mitchill and eastern oysters *Crassostrea virginica* Gmelin (Wu et al. 1984, Li et al. 1999, Nakai et al. 1999, Pelon et al. 2005, Imbeault et al. 2006). Other virulent bacteriophages that have been isolated and are of great importance to the aquaculture industry include; phage that infect *Chondrococcus columnaris* or *Flavobacterium columnare* (Kingsbury & Ordal 1966); phage PLgY of *Lactococuss garvieae* isolated from diseased yellowtail (Park et al. 1997); phages against *Yersinia ruckeri* (Stevenson & Airdric 1984); and phages Ach1 and Ach2 of *Aeromonas hydophila* have been isolated (Chow & Rouf 1983).

Advantages of phages therapy include: (1) they are specific and therefore cannot infect other microbial cell organisms that are ecologically important. (2) bacteriophage

switch to a dormant state soon after all host cells are destroyed and hence will disperse harmlessly. (3) human patients who are allergic to antibiotics are treated with phages with no side effects and are safe because they do not attack human and animal cells. (4) phages can be administered in various routes. (5) phages produce exponentially, hence, a single dose can be sufficient to treat an infection. (6) when resistant strains of the host develop, the phage has capabilities of mutating in step with the evolving bacteria. (7) production of bacteriophages is simple and inexpensive. (8) bacteriophages are ubiquitous. (Barrow & Soothhill 1997, Barrow 2001, Summers 2001, Sulakvelidze et al. 2001, Merril et al. 2003, Levin & Bull 2004, Mastuzaki et al. 2005).

Nakai & Park (2002) demonstrated the ability of bacteriophages to penetrate the fish body via the skin or gills that were traced in the kidneys after fish were exposed to a bacteriophage solution. It was also reported that phage appeared in the kidneys of fish after an oral administration but without host bacterial cells used as a transport vehicle (Nakai & Park 2002). Nakai and Park (2002) observed that phage particles administered intraperitoneally were recovered in the intestines of fish after 3h of administration and recovery ended after 10h. Furthermore, no antiphage antibodies have been detected in fish after oral treatments and intramuscular injections (Nakai & Park 2002, Dabrowska et al. 2005). However, clearance of bacteriophages in kidneys has been reported were phages are detected in fish kidneys even after a single administration (Russell et al. 1976).

Disadvantages in using bacteriophages include; (1) their specificity implies that the causative bacterial pathogens have to be identified prior to their administration and the lytic spectrum may be limited to one subtype bacterial pathogen. (2) low or no

efficacy have been reported in certain cases but may be attributed to insufficient diagnosis of the disease and phage-dose, together with improper delivery mechanisms, (3) phage administration requires a neutralized environment which is rarely found in the digestive system of animals due to presence of gastric secretions, (4) the lytic life-cycle of bacteriophages described in *in vitro* environments may not be maintained under normal physiological conditions found in the body, and instead may revert to a lysogenic cycle, (5) the existence of bacteriophage as a prophage may lead to propagated resistance of the bacteria towards antibiotics (Russell et al. 1976, Barrow & Soothhill 1997, Barrow 2001, Summers 2001, Sulakvelidze et al. 2001, Nakai & Park 2002, Merril et al. 2003, Levin & Bull 2004, Dabrowska et al. 2005, Mastuzaki et al. 2005).

To date no bacteriophage that infects *E. ictaluri* has ever been reported, therefore, the objectives of this study were to: (1) discover bacteriophages that are specific for *E. ictaluri* from aquaculture ponds, (2) isolate and propagate lytic phages, and (3) characterize all different phages, isolated, through *in vitro* assays. Since not every phage isolated would be an attractive candidate for phage therapy of ESC, this study focused on isolating bacteriophages with *E. ictaluri* host-specificity, without evidence of lysogeny, and capable of producing large, clear plaques upon pathogenic strains of *E. ictaluri*.

MATERIALS AND METHODS

Bacteria and media

Twenty five bacterial isolates from the Southern Cooperative Fish Disease laboratory with the Department of Fisheries and Allied Aquacultures, College of Veterinary Medicine Department of Pathobiology, Auburn University and ATCC collections were used in the study (Table 1). With the exception of *E. ictaluri* strain RE-33, *E. ictaluri* strain R4383, *E. ictaluri* strain C91-162, *Citrobacter freundii* strain ATCC 8090, *Klebsiella pnuemoniae* ATCC 25953, *Proteus mirabilis* and *Salmonella enterica* ATCC 12324 all isolates were obtained from disease cases submitted from farms in various geographical locations. The *E. ictaluri* strain 219 was used for the general characterization of the bacteriophages. The remaining isolates were used to test for host range of the phages.

Flavobacterium columnare isolates were grown on Hsu-shots agar. The remaining bacterial isolates were propagated using brain heart infusion (BHI) broth (Difco, Sparks Md. USA) at 30°C and stored in BHI broth at -80°C in 10% glycerol. Biochemical tests were performed using protocols described by the AFS-FHS (American Fishery Society-Fish Health Section), Bethesda, Maryland, USA. Cryopreserved *E. ictaluri* bacteria were revived on Remel BHI agar (Fisher Scientific, Lenexa, KS, USA).

Discovery, enrichment and isolation of bacteriophages

Water samples (5-L) were collected from commercial catfish ponds that were diagnosed with ESC in West Alabama in fall 2006. Removal of algal cells and debris, and concentration of samples was performed using 30 – 100 kDa Amicon Centricon Plus-70 ultra filtration membranes (Millipore, Billerica, MA, USA) and 5 kDa Amicon centrifugal filters (Millipore, Billerica, MA, USA), respectively, while centrifuging at 3,600 *g* for 15 min. Samples were subsequently purified through 0.22 µm PVDF filters (Millipore, Bedford, MA, USA).

Bacteriophages specific to *E. ictaluri* were enriched with methods described by O'Flynn et al. (2004) with some modifications. Viral sample concentrates (5 ml) were added to a 30 ml log-phase *E. ictaluri* strain 219 culture (3.1×10^7 CFU ml^{-1}) grown overnight at 30°C. Enrichment cultures were incubated overnight at 30°C with shaking (150 rpm). One percent chloroform (Fisher Scientific, Sair Lawn, NJ, USA) was added to 1.5 ml sample and centrifuged at 3,600 *g* for 10 min at 4°C. The supernatant (1ml) was then concentrated down to 100 µl using 5 kDa filters while centrifuging at 3,600 *g* for 10 min. Presence of lytic phages was tested with 5µl of filtrate spotted on a lawn of *E. ictaluri* grown at 30°C on BHI agar. Phages were also isolated in 1985 from primary cultures of *E. ictaluri* obtained from infected channel catfish reared at E.W Shell Fisheries Center Auburn, Alabama, USA. Since initial isolation, the phages remained in storage at 80°C in 1985 (J. Plumb, personal communication). These were also analyzed in this study.

Bacteriophages were triple purified from isolated plaques using the soft agar overlay method (Adam 1959). A mixture of 100 µl of viral concentrate and 200 µl of

log -phase *E. ictaluri* strain 219 were added to 5 ml of molten 0.7% BHI agar (maintained at 35°C) and then poured over BHI agar plates. Plates were incubated overnight at 30°C to allow for plaque formation. Isolated plaques were picked using sterile wooden toothpicks then dropped, separately, into 5 ml log–phase *E. ictaluri* broth cultures and incubated at 30°C with shaking (150 rpm) for 8 h. The purified phages were then stored in SM buffer [100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5)], and 0.002% (w/v) gelatin at 4°C with the addition of 7 % dimethyl sulfur oxide (DMSO) at –80°C.

Phage stocks used in this study were prepared using soft agar overlays as described previously (Su et al. 1998). A confluently lysed plate was flooded with 7ml of SM buffer and incubated at 30°C while shaking at 60 rpm for 4 h. Phage suspension was then centrifuged at 3,600 ***g*** for 10 min. to remove cells and debris, and the supernatant filter-sterilized through 0.22 µm PVDF filters. Plaque assays as described by Adams (1959) were performed to determine the titer of a phage stock. After a 10-fold dilution of the phage stock, 10 µl of each dilution were spotted on a lawn of *E. ictaluri* and then incubated overnight at 30°C to determine the number of plaque forming units (PFU). Samples were stored at 80°C in 7% DMSO for further studies.

Electron microscopy

Five microliters of CsCl-purified phage (10^{12} 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 15 min. and each sample was negatively stained with 2% phosphotungstic acid. Using a Zeiss EM10 transmission electron microscope (Zeiss/LEO, Oberkochen, Germany), the grids were examined at various magnifications to determine the morphology and size of each phage.

Isolation and restriction of bacteriophage nucleic acids

Isolation and restriction of phage nucleic acids was done using methods described by O'Flynn et al. (2004) with modifications. Contaminating host chromosomal DNA was removed from a phage stock (10^{11} PFU ml^{-1}) by adding 250 units/µL of benzonase and incubating overnight at 37°C. Benzonase was inactivated by addition of 10 mM EDTA and heating at 70°C for 10 min. Phage protein coats were degraded using 1 mg ml^{-1} proteinase K and 1% sodium dodecyl sulphate and incubated at 37°C for 2 h. Proteins were removed by phenol-chloroform extraction, and phage DNA was ethanol precipitated and resuspended in 75 µl nuclease free, deionized and distilled water. Bacteriophage DNA was digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, or *Pst*I 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).

Effects of temperature, Ca and Mg on bacteriophage replication

The effects of calcium, magnesium and temperature were examined to determine optimal conditions for the replication of both phages. An overnight bacterial culture was sub-cultured into 50 ml BHI broth prior to adding phage at a multiplicity of infection (MOI) of 0.1 (phage:host). Effects of supplementing $CaCl_2$ and/or $MgCl_2$ to BHI broth on phage production in *E. ictaluri* cultures was examined at eight concentrations ranging from 0 to 1mM. Samples were assayed to determine the PFU ml^{-1} and bacterial culture turbidity (spectrophotometrically determined using optical density, OD_{600}) after 8 h of incubation at 30°C.

To monitor the effect of temperature on phage production, a log-phase *E. ictaluri* strain 219 (10^6 CFU ml^{-1}) culture in BHI broth was infected with about 10^4 PFU ml^{-1} and samples were incubated at different temperatures between 17-30°C for 5 h. Phage lysates were centrifuged at 16,100 ***g*** for 5 min, filter-sterilized through 0.22 µm PVDF filters and then quantified.

One–step growth

A one–step growth experiment was conducted based on Adams (1959) with modifications. Duplicates of ΦeiDWF and ΦeiAU were added to *E. ictaluri* strain 219 broth cultures that contained 1mM potassium cyanide (KCN), at a MOI of 0.1. Cultures were incubated at 30°C for 10 min to allow phage-bacteria adsorption. Cells were pelleted by centrifugation (20,000 ***g***, for 2 min at 4°C), resuspended in fresh BHI broth, diluted 10^5-fold and incubated at 30°C while shaking. Aliquots were removed at 5 min intervals and PFU determined by the soft agar overlay method described above.

In vitro challenges

A time course experiment was used to determine the phage-induced lysis of host cells as described by O'Flynn et al. (2004) with modifications. An overnight culture of *E. ictaluri* strain 219 was inoculated (1% v/v) into BHI broth media with 500 µM $CaCl_2$ then incubated at 30°C while shaking. After 7 h, triplicates of ΦeiDWF and ΦeiAU were separately introduced in log phase *E. ictaluri* strain 219 cultures (~10^6 CFUml^{-1}) at a MOI of 0.1, and none in the control cultures. Samples were drawn every 1 h and plated for CFU counts. Phage was also added stationary phase *E. ictaluri* strain 219 cultures (~10^{10} CFU ml^{-1}) at a MOI of 0.1 and incubated at 30°C.

Host range determination

The host range of both phages was assessed on a range of Gram-negative bacteria (Table 1). Susceptibility of various bacterial isolates was tested using the drop-on-lawn technique (Zimmer et al. 2002). The efficiency of plaquing (EOP) was then determined based on *E. ictaluri* strain 219 as a reference strain. The EOP of a phage on a given strain of *E. ictaluri* was expressed as the ratio of the PFU ml^{-1} of a host strain to that observed on *E. ictaluri* strain 219 cells.

Prophage induction

All isolates of *E. ictaluri* used in the host range study were tested to determine whether lysogenic bacteria existed using a method described by Fortier and Moineau (2007) with modifications. An overnight culture of *E. ictaluri* was subcultured (3% v/v) in fresh BHI broth and incubated at 30°C with shaking until cultures reached OD_{600} of 0.100. To a 5 ml of *E. ictaluri* culture, Mitomycin C was added to a final concentration of 1 μg ml^{-1} and then incubated for 30 min. Cells were pelleted by centrifugation at 3,700 ***g*** for 5 min, resuspended in fresh BHI broth and incubated for 5 h at 30°C while shaking (150 rpm). Samples were then centrifuged at 3,700 ***g*** for 5 min and 10 μl of supernatant spot assayed for presence of phage.

Statistical analysis

Data collected was analyzed using Statistical Analysis System version 9.1.3 software (SAS Institute, Inc., North Carolina, USA). Differences between mean treatments within each phage were assessed using a one-way analysis of variation (ANOVA) at 5% significant level. Comparisons were done using the pair wise

comparisons with Dunnett's test method for comparing treatments to those obtained from the control.

RESULTS

Isolation of bacteriophages

A total of 22 bacteriophages were isolated from plaques that ranged 0.5--11 mm in diameter on *E. ictaluri* strain 219. Sixteen phages were isolated from samples collected from a pond in West Alabama (ΦeiDWF) and six were isolates collected from samples froze in 1985 (ΦeiAU). Phages isolated from West Alabama ponds had plaque size ranging from 0.5 to11 mm in diameter and those isolated from Auburn University ranged from 4 to 7 mm. Both phages produced clear plaques on a lawn of host bacteria. However, for some *E. ictaluri* strains colonies appeared within these lytic zones when the incubation period was prolonged for 48 h but upon restreaking, clear plaques were still produced.

Size and morphology of bacteriophages

Transmission electron microscopy (TEM) revealed similarity in morphology between ΦeiAU and ΦeiDWF (Fig.1). Both have an icosahedral shaped head, 50 nm in diameter, and a non-rigid tail. Tail lengths of ΦeiAU and ΦeiDWF are approximately 100 nm. Based on the morphology and the rules provided by International Committee on Taxonomy of Viruses (ICTV, Bethesda MD, USA) both phages are tentatively placed in the *Siphoviridae* family (Murphy et al. 1995, Nelson 2004).

Bacteriophage nucleic acid

Phage nucleic acids were not digested by exonuclease I indicating that the phages are double-stranded DNA phages. Gels generated from endonucleases *Bam*HI, *Hin*dIII and *Pst*I were inconclusive since no distinct restriction pattern was yielded from both phage DNA. However, restriction analysis of DNA from eight different phage stocks revealed two unique bacteriophages with enzymes *Eco*RI and *Eco*RV. Digestion with *Eco*RI showed many bands in common but phage ΦeiAU had two different bands that are unique (Fig. 2). These two bacteriophages were subsequently designated ΦeiAU and ΦeiDWF; i.e. isolated from Auburn University and Dean Wilsons Farms in West Alabama, respectively. Their genome sizes are approximately 39kb (ΦeiDWF) and 45 kb (ΦeiAU).

Phage production

Infection of *E. ictaluri* by ΦeiAU and ΦeiDWF is enhanced upon both temperature and the presence of calcium and/or magnesium salts. The optimal temperature for growth of *E. ictaluri* (25-30°C) also supports rapid replication of these phages. Over three orders of magnitude decrease were observed in PFU ml^{-1} when the temperature decreased to 20°C. Similarly low phage titers were obtained at higher temperature of 37°C (Fig. 3).

Production of phage in BHI broth is enhanced in the presence of calcium and magnesium ions. However, phage titers increased 2-folds in cultures with $CaCl_2$ compared with those grown in $MgCl_2$ (Fig. 4 and 5). The optimal range observed for calcium and magnesium is 500-750 μM at which high phage titers were attained;

ΦeiDWF (~10^4 PFU ml^{-1} in Mg^{2+} and ~ 10^7 PFU ml^{-1} in Ca^{2+}) and ΦeiAU (~10^5 PFU ml^{-1} in Mg^{2+} and ~ 10^7 PFU ml^{-1} in Ca^{2+}). Also, low numbers of bacterial turbidity were observed at corresponding high phage titers. In the presence of phage ΦeiDWF the bacterial counts were reduced to OD_{600} readings of 0.040, and with phage ΦeiAU turbidity was reduced to 0.200. Low phage titers were attained in the controls but also decreased in number at concentrations of 1mM $MgCl_2$ or $CaCl_2$.

The effects of supplementing $CaCl_2$ and/or $MgCl_2$ (standardized at 500 μM) showed a significant increase ($P < 0.05$: Dunnet's test) in phage titers compared to the controls: observed only in cultures with $CaCl_2$ and a combination of $CaCl_2$ and $MgCl_2$. Higher titers of phage (~1 log PFUml^{-1}) were obtained with phage ΦeiDWF compared to phage ΦeiAU (Fig. 6).

Burst size and latent period

From the one step-growth curve the latent period was estimated to be approximately 40 min and the average burst size to be approximately 270 virions per bacterial cell (Fig.7). Determination of burst size was based on the ratio of mean yield of phage particles liberated to the mean phage particles that infected the bacterial cells. There was no difference in burst size between a one-month old phage stock and a freshly prepared phage stock.

Kinetics of phage-induced lysis

Within 6h of incubation about 10^6 CFU ml^{-1} *E. ictaluri* strain 219 were reduced to below detectable levels (Fig. 8). During this 6h period, bacterial cultures with phage gradually cleared while the controls remained turbid. Clearance and drop in CFU ml^{-1} due

to both phages was attained within the same incubation period. Furthermore, no clearance was observed in bacterial cultures that were in stationary-phase; cultures remained turbid throughout the experiment (Fig. 9).

Host specificity of phages

Both ΦeiAU and ΦeiDWF lysed all *E. ictaluri* strains tested (Table 1). Within this lytic spectrum, clear plaques were produced on all strains except on *E. ictaluri* strain AL93-92 and AL98-25-42A which had a mixture of the opaque and clear plaques. Plaque size ranged from 0.5 to 4 mm. However, pin-point plaques were produced with *E. ictaluri* strains 196, C91-162 and R4383 observed in zones that were spotted with high phage titers (>10^6 PFU/ml).

Variable ranges of EOP (0.4-300%) were observed among *E. ictaluri* strains. Both phages produced high EOP values (>50%) with *E. ictaluri* strains ATCC 33202, 218, S97 773, RE-33, AL93-92 AU98-25-42A and 195 while low values(< 40%) where observed with *E. ictaluri* strains196, C91-162, and R4383. However, in general phage ΦeiAU has a higher efficiency compared to ΦeiDWF but the latter produces high numbers of phage titers when each host is considered. None of the other bacterial species tested were observed to have any evidence of phage plaques.

Prophage induction

The purpose of adding Mitomycin C to the 11 cultures of *E. ictaluri* in log-phase was to exorcise prophages existing in the host cells (Goh et al. 2005). An increase in turbidity was observed in all cultures tested with OD_{600} readings reaching about 0.250 during the 5 h of incubation. No plaques were observed with an all lawns of the host

indicating absence of temperate phage existing in on all *E. ictaluri* isolates used in this study.

DISCUSSION

Bacteriophages specific to *E. ictaluri* were isolated from aquaculture ponds with outbreaks of ESC. This suggests that *E. ictaluri*–specific phage exists in aquatic environments and may contribute to some degree in lessening the severity or persistence of ESC outbreaks. Since *E. ictaluri* is also reported to survive in water and pond bottom sediments for several hours (Inglis et al. 1993, Hawke et al. 1998, Plumb 1999) there is reason to suspect that both *E. ictaluri* and its respective phages may persist in aquaculture ponds. The ubiquity of bacteriophages (d'Herelle 1926, Adams 1959) makes it relatively easy to isolate them from aquatic environments and from diseased fish as previously reported from studies that focused on phages specific to fish pathogens (Park et al. 1997, Nakai et al. 1999, Park et al. 2000, Imbeault et al. 2006, Shivu et al. 2007). Therefore, catfish pond waters and diseased fish are a good source for discovery of phages specific to *E. ictaluri*.

The phages described in this study were isolated from samples collected from different areas but electron microscopy revealed similar morphotypes, classified as *Siphoviridae*. Furthermore, restriction digest using *Eco*RI and *Eco*RV showed similar but unique patterns, suggesting that ΦeiAU and ΦeiDWF may have genetic loci in common. Another *E. ictaluri*-infective phage ΦMSLS-1 was isolated from aquaculture ponds in Mississippi with a history of ESC infection (Dr. T. Welch and Dr. G. Waldbeiser, USDA,

personal communication). A comparison of the *Eco*RV restriction profiles of ΦMSLS-1, ΦeiAU, and ΦeiDWF showed a majority of restriction fragments in common (data not shown). Furthermore, a PCR primer set specific to genes contained within ΦMSLS-1 similarly amplified the target sequences within ΦeiAU and ΦeiDWF (data not shown), providing further evidence of genetic elements in common between these three phages. Preliminary genome sequences from ΦMSLS-1, ΦeiAU, and ΦeiDW also support this conclusion (data not shown).

The primary factors influencing *in vitro* phage infectivity for *E. ictaluri* were temperature (optimal 22-33°C), metal cations (especially calcium) and the host growth stage. Phage reproduction is also dependent on the physiological state of the bacterial host (Adams 1959, Taddei & Paepe 2006, Poranen et al. 2006). Normally, ESC epizootics occur when temperatures range from 18 to 30°C that are characterized by acute infections and high mortalities within catfish fingerlings (Francis-Floyd et al. 1987 Tucker & Robinson 1990, Durborrow et al. 1991, Inglis et al. 1993). Temperature influences the metabolic activities of the host but also accelerates the adsorption rate of phage (Adams 1959, Fujimura & Kaesberg 1962, Moldovan et al. 2007). Moldovan et al. (2007) demonstrated an increase in adsorption rate (~ 30 times) when the temperature rose from 4 to 40°C when λ phage was incubated with bacteria Ymel (derivative of *Escherichia coli* strain K12). The role of Ca^{2+} and Mg^{2+} ions in phage-host interaction may be in the adsorption, penetration processes or in other growth stages of phage (d'Herrelle 1926, Luria & Steiner 1954, Adams 1959, Moldovan et al. 2007). It is also postulated that Ca^{2+} ions may increase the concentration of phage particles at the host surface or alter the structure of a cell surface receptor thereby increasing accessibility to

the receptor molecules or transfer of phage nucleic acids (Watanabe & Takesue 1972, Russell et al. 1988). Interestingly, results show that the optimal Calcium concentration for phage infection (500 μM) is equivalent to 50 ppm Ca^{2+} recommended in commercial catfish ponds (Tucker and Robinson, 1990). Incidentally, pond environments have varying degrees of Ca^{2+} hence phage infectivity in aquaculture ponds might be influenced by hardness of water. Future studies will address the mechanism(s) of metal cation-induced increases in phage titers, and the role of metal cations in phage biological control of ESC in aquaculture ponds.

Both phages were specific to *E. ictaluri* strains. Although *E. tarda* is reported to be closely related to *E. ictaluri* (Zhang & Arias, 2006), it was not susceptible to phages evaluated in this study. Because of their specificity, both phages will have the potential to control *E. ictaluri* infections in fish and may subsequently be utilized as prophylactic and/or treatment agents. Secondly, phage therapy will target only *E. ictaluri* strains and not other beneficial bacterial flora existing in the same environment. Nevertheless, *E. ictaluri* strain RE-33 (a vaccine strain) was observed to be the most susceptible host among the isolates tested. This could be attributed to changes in the receptor site or absence of the O-side chain LPS reported in the strain RE-33 (Klesius & Shoemaker 1999, Arias et al. 2003). But the efficacy of the vaccine may be affected when used in combination with phage therapy. Hence, suitable therapeutic strategies should be formulated and tested if the two agents are to be used against ESC. Additionally, these phages may also be used as diagnostic tools in fish disease laboratories for detection of *E.ictaluri* strains. It is reported that homogeneity exists among *E. ictaluri* strains (Plumb & Vinitnantharat, 1989, Arias et al. 2003, Panangala et al. 2006) which explains the

susceptibility of all *E. ictaluri* strains (tested to date) to phage infection. The relative EOP data reveals significantly better plaquing efficiency for ΦeiAU compared to ΦeiDWF among many of the tested *E. ictaluri* strains. No other bacterial phenotypes are known that correlate with the lower EOP for the three less-susceptible *E. ictaluri* strains. Variation in susceptibility among host strains may be largely due to differences in host receptor sites, modification or loss of receptor molecules, or other host resistant mechanisms such as abortive infection (Zorzopulous et al. 1979, Duckworth et al. 1981). However, compared to chemotherapeutants which have a broad spectrum of different species (Nelson, 2004), an individual phage may not effectively control aquatic pathogens yet a "cocktail" of *E. ictaluri* specific phages may have better efficiency as a biological control strategy (O'Flynn et al. 2004, Skurnik & Strauch 2006, Verner-Jeffreys et al. 2007).

In vitro challenges with phage added to *E. ictaluri* strain 219 in log-phase demonstrates that both phages have a potential control to ESC infections. The fact that these phages are specific to *E. ictaluri* strains and occur naturally in aquaculture ponds justifies their use as biocontrol agents for ESC. Future studies include molecular characterization of phages specific to *E. ictaluri* and evaluating the protective effects of these phages in ESC disease models.

Summary

The discovery of lytic bacteriophages specific to *E. ictaluri* is an important step towards the control of ESC. However, the quest for more phages specific to *E. ictaluri* is vital for effective control of ESC since a few types of phages may not efficiently control

the disease. Hence, a "cocktail of phages" would be administered to target a range of *E. ictaluri* cells, some of which may evolve to be resistant to phage infection. Results from TEM and restriction fragment length polymorphism (RFLP) not only confirmed the existence of these phages (ΦeiAU and ΦeiDWF) in aquaculture ponds but also provided a basis for understanding their nature. Subsequently, using molecular techniques the whole genome sequence of both phages can be determined and evaluated thereby broadening our knowledge about these phages. Furthermore, since these are unique (novel) bacteriophages, their characterization (using molecular approach) will contribute to the evolutionary history information of phages.

The specificity of both phages to *E. ictaluri* isolates is of great advantage for therapeutic purposes and also ecologically important. However, from the host range results the vaccine *E. ictaluri* strain RE-33 is the most sensitive to both phage infections; i.e. EOP for ΦeiAU and ΦeiDWF were150 and 306 %, respectively. This may affect its performance when applied together with these phages therefore control strategies need to be addressed prior to their use.

Phage production is shown to be enhanced by temperature (22-33°C) in presence of 500 mM Mg/Ca. Therefore, in aquarium or pond conditions the performance of these phages might be affected by the existing temperature and the ionic composition of water. Additionally, the optimal 500 mM Ca/Mg is equivalent to 50 ppm which is the recommended hardness for catfish ponds. Beyond these optimal limits low efficacy levels of phage therapy might be attained. This may be lessened by using phage cocktails that include phages that have a higher efficiency beyond the observed limits.

The ability to use these phages as biocontrol agents against ESC will require further studies focusing on their molecular characterization, *in vitro* and *in vivo* challenges in aquarium and pond conditions. Hence, future directions will delve on: (1) isolating additional phages that infect *E. ictaluri* thereby formulating a "phage cocktail", (2) evaluate the protective effects of lytic *E. ictaluri* phages on infected channel catfish fingerlings in aquarium and aquaculture pond systems, (3) evaluate various mechanisms of phage delivery in aquaria experimental infections with *E. ictaluri*, and (4) determine and annotate the whole genome sequence of ΦeiAU and ΦeiDWF.

LITERATURE CITED

Abedon ST, Culler RR (2007) Optimizing bacteriophage plaque fecundity. J Theo Biol 219:582–592.

Adams MH (1959) Bacteriophages. Interscience, New York, NY, USA.

Areechon N, Plumb JA (1983) Pathogenesis of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus*. J World Maricult Soc 14:249–260.

Arias CR, Shoemaker CA, Evans JJ, Klesius PH (2003) Comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin-mutant (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses. J Fish Dis 26:415–421.

Barrow PA, Soothhill JS (1997) Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. Trend Microbiol 5:268–271.

Barrow PA (2001) Review The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. J Chem Technol Biotechnol 76:677–682.

Bautz EK, Kasai T, Reilly E, Bautz FA (1966) Gene-specific mRNA. II Regulation of mRNA sysnthesis in *E. coli* after infection with bacteriophage T4. Proc Natl Acad Sci USA 55:1081–1088.

Berg HC, Purcell EM (1977) Physics of chemoreception. Biophys J 20:193–213.

Bijlenga RKL, Van den Broek R, Kellenberger E (1974) The transformation of T-particles into T4 heads. I. Evidence for the conservative mode of this transformation. J Supramol Struct 2:45–59.

Bilodeau AL, Terhune JS, Waldbiesser GC, Wolters WR, Wise DJ (2003) A real-time PCR assay of the bacterium *Edwardsiella ictaluri* in channel catfish. J Aquat Anim Health 15:80–86.

Black LW (1989) DNA packaging in dsDNA bacteriophages. Annu Rev Microbiol 43:267–92.

Bohannan BJM, Lenski RE (2000) Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecol Lett 3:362–377.

Bostein D, Herskowitz I (1974) Properties of hybrids between *Salmonella* phage P22 and coliphage lambda. Nature 251:585–589.

Boulanger P, Letellier L (1988) Characterization of ion channels involved in the penetration of phage T4 DNA into *Escherichia coli* cells. J Biol Chem 263:9767–9775.

Bren L (2007) U.S Food and Drug Administration. Accessed 4 Feb. www.fda.gov/fdac/.

Breitbart M, Salamon P, Andersen B, Mahaffy JM, Segall AM, Mead D, Azam F, Rohwer F (2002) Genomic analysis of uncultured marine viral communities. Proc Natl Acad Sci USA 99:14250–14255.

Brown DT, Mackenzie JM, Bayer ME (1972) Mode host cell penetration by bacteriophage fx174. J Virol 7:836–846.

Carrias A (2005) Evaluation of the live attenuated vaccine AQUAVACESC and the effects of a primary nursery phase on the production of channel catfish fingerlings in earthen ponds. Master Thesis. Auburn University, Auburn, Alabama, USA.

Casjens S, Hendrix R (1988) Control mechanisms in dsDNA bacteriophage assembly. In the bacteriophages (Calender, Plenum, New York (R ed). 1:15–91.

Champe SP (1963) Bacteriophage reproduction. Annu Rev Microbiol 17:87–110.

Chow MS, Rouf MA (1983) Isolation and partial characterization of two *Aeromonas hydrophila* bacteriophages. Appl Environ Microbiol 45:1670–1676.

Crawford JT, Goldberg EB (1980) The function of tail fibers in triggering baseplate expansion of bacteriophage T4. J Mol Biol 139:679–690.

Cohen PS, Ennis HL (1966) *In vivo* stability of bacteriophage T4 messenger ribonucleic acid. J Bacteriol 92:1345–1350.

Cooper RK, Shotts EB (Jr), Nolan LK (1996) Use of a mini-transposon to study chondroitinase activity with *Edwardsiella ictaluri*. J Aqua Anim Health 8:319–324.

Davis TH (2004) Meselson and Stahl: The art of DNA replication. Proc Natl Acad Sci USA 52:17895–17896.

Dabrowska K, Switala-Jelen K, Opolski A, Weber-Dabrowska B, Gorski A (2005) A Review Bacteriophage penetration in vertebrates. J Appl Microbiol 98:7–13.

DePaola A, Peeler JT, Rodrick GE (1995) Oxytetracycline-medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. Appl Environ Microbiol 61:2335–2340.

d'Herelle FH (1917) Sur un microbe on the invisible antagonite des bacilles dysenteriques. C R Acad Sci 165:373–375.

d'Herelle MD (1926) The Bacteriophage and its behavior. The Williams and Wilkins company, Baltimore, MD, USA.

Delbrück M (1940) The growth of bacteriophage and lysis of the host. J Gen Physiol 23:643–660.

Duckworth DH, Glenn J, McCorquodale DJ (1981) Inhibition of bacteriophage replication by extrachromosal genetic elements. Microbial Rev 45:52–71.

Durborrow RM, Taylor PW, Crosby MD, Santucii TD (1991) Fish mortality in the Mississippi Catfish Farming Industry in 1988: Causes and treatment. J Wildlife Dis 27:144–147.

Fenner F (1976) The classification and nomenclature of viruses. J Gen Virol 31:463–470.

Francis-Floyd H, Beleau MH, Waterstrat P, Bowser PR (1987) Effect of temperature on clinical outcome of infection with *Edwardsiella ictaluri* in channel catfish. J Am Vet Med Assoc 191:1413–1416.

Fortier LC, Moineau S (2007) Morphological and genetic diversity of temperate phages in *Clostridium difficile*. Appl Environ Microbiol 73:7358–7366.

Fujisawa H, Hayashi M (1977) Assembly of bacteriophage X174: identification of a virion capsid precursor and proposal for the functions of bacteriophage gene products during morphogenesis. J Virol 24:303–313.

Fujimura R, Keasberg P (1962) The adsorption of bacteriophage {phi} X174 to its host. Biophys J 2:433–449.

Gaikowski MP, Wolf JC, Endris RG, Gingerich WH (2003) Safety of Aquaflor (Florfenicol, 50% Type A medicated article), administered in feed to channel catfish, *Ictalurus punctatus*. Toxicol Pathol 31:689–697.

Gaunt P, Endris R, Khoo L, Leard AT, Jack S, Santucci T, Katz T, Radecki SV, Simmons R (2003) Preliminary assessment of the tolerance and efficacy of Florfenicol against *Edwardsiella ictaluri* administered feed to channel catfish. J Aquat Anim Health 15:239–247.

Goh S, Riley TV, Chang BJ (2005) Isolation and characterization of temperate bacteriophages of *Clostridium difficile*. Appl Environ Microbiol 71:1079–1083.

Grayson P, Molineaux IJ (2007) Is the phage DNA "injected" into cells- biologists and physicists can agree. Curr Opin Microbiol 10: 401–409.

Gründling A, Manson MD, Young R (2001) Holins kill without warning. Proc Natl Acad Sci USA 98:9348–9352.

Hawke JP (1979) A bacterium associated with disease of pond cultured channel catfish, *Ictalurus punctatus*. J Fish Res Bd Can 36:1508–1512.

Hawke JP, McWhorter AC, Steigerwalt C, Brenner DJ (1981) *Edwardsiella ictaluri* sp. nov, the causative agent of enteric septicemia of catfish. Int J Syst Bact 31: 396–400.

Hawke JP, Durborow RM, Thune RL, Camus AC (1998) Enteric Septicemia of Catfish. SRAC Publication No. 477.

Hawke JP, Khoo LH (2004) Infectious diseases. In: Biology and culture of channel catfish (ed. by CS Tucker & JA Hargreaves) Elsevier, Amsterdam, the Netherlands. pp 387–443.

Hendrix RW, Smith MCM, Burns RN, Ford ME, Hatfull GF (1999) Evolutionary relationships among diverse bacteriophages and prophages: all world's a phage. Proc Natl Acad Sci USA 96:2192–2197.

Hsu W, Foft JW, Weiss SB (1967) Effect of bacteriophage infection on the sulphur-labeling of sRNA. Proc Natl Acad Sci USA 58:2028.

Humphreys GO, Trautner TA (1981) Maturation of bacteriophage SPP1 DNA: limited precision in the sizing of mature bacteriophage genomes. J Virol 37: 832–835.

ICTV (2007) International Committee on Taxonomy of Viruses [online] cited 10th January 2008. http://www.ictvonline.org.

Imbeault S, Parent S, Legace M, Uhland CF, Blais JF (2006) Using bacteriophages to prevent furunculosis caused by *Aeromonas salmonicida* in farmed brook trout. J Aquat Anim Health 18:203–214.

Inglis V, Roberts RJ, Bromage NR (1993) Enteric septicemia of catfish. In: Bacterial Diseases of Fish. Blackwell Science Ltd. Osney Mead, Oxford, London, p67–79.

Ishiguro EE and Dai D (1990) Two new mutant loci (*smh*B and *lyt*D) in *Escherichia coli* which confer temperature -sensitive growth and lysis phenotypes. Can J Microbiol 36:827–833.

Johnson MR (1991) Bacterial resistance to antibiotics: a growing problem in the channel catfish industry. In: Reigh RC(ed) Proceedings of Louisiana Aquaculture Conference. Louisiana State University Agricultural Center, Baton Rouge, Louisiana, p22–23.

Jollick JD, Wright BL (1974) A flagella specific bacteriophage for *Caulobacter*. J Gen Virol 22:197–205.

Kennell D (1970) Inhibition of host protein synthesis during infection of *Escherichia coli* by bacteriophage T4. II Indiction of host messenger ribonucleic acid and its exclusion from polysomes. J Virol 6:208–217.

Kingsbury DT, Ordal EJ (1966) Bacteriophage infecting the Myxobacterium *Chondrococcus columnaris*. J Bact 91:3.

Klesius PH (1992) Carrier state of channel catfish infected with *Edwardsiella ictaluri*. J Aqua Anim Health 4:227–230.

Klesius PH, Shoemaker CA (1999) Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. In: Schultz RD(ed). Advances in Veterinary Medicine 41:523–537.

Klesius P, Lovy J, Evans J, Washuta E, Arias C (2003) Isolation of *Edwardsiella ictaluri* from tadpole Madtom in a southwestern New Jersey River. J Aquat Anim Health 15:295–301.

Labedan B, Goldberg EB (1979) Requirement for membrane potential in injection of phage T4 DNA. Proc Natl Acad Sci USA 76:4669–4673.

Lawrence ML, Banes MM, and Williams (2001) Phenotype and virulence of a transposon-derived lipopolysaccharide O side-chain mutant strain of Edwardsiella ictaluri. J Aquat Anim Health 13:291–299.

Lawrence ML, Banes MM, Azadi P, Reeks BY (2003) The *Edwardsiella ictaluri* O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. Microbiology 149: 1409–1421.

Levin BR, Bull JJ (2004) Population and evolutionary dynamics of phage therapy. Nature Reviews Microbiology 2:166–173.

Lenski RE, Levin BR (1985) Constraints on the coevolution of bacteria and virulent phage: some experiments, and predictions fro natural communities. AM Nat 125:585–602.

Li T, Xiang J, Liu R., Ding M, Shi P, Wang S (1999) Studies on bacteriophage control pustule disease of abalone *Haliotis discus hannai*. Oceanologia et Limnologia Sinica, 30:374-380.

Linneaus C (1758) Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. 10th ed, vol I Stockholm, Sweden.

Luria SE, Steiner DI (1954) The role of calcium in the penetration of bacteriophage T5 into its host. J Bacteriol 67:635–639.

Matsuzaki S, Rashel M, Uchiyama J, Sakurai S, Ujihara T, Kuroda M, Ikeuchi M, Tani T, Fujeida M, Wakiguchi H, Imai S (2005) Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J Infect Chemother 11:211–219.

Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, Adhya S (1996) Long-circulating bacteriophage as antibacterial agents. Microbiol 93:3188-3192.

Merril CR, Scholl D, Adhya SL (2003) The prospect for bacteriophage therapy in western medicine. Nat Rev Drug Discov 2:489–497.

Miyazaki T, Plumb JA (1985) Histopathology of *Edwardsiella ictaluri* in channel catfish, *Ictalurus punctatus* (Rafinesque). J Fish Dis 8:389–392.

Mqolomba T, Plumb JA (1992) Effect of temperature and dissolved oxygen concentration on *Edwardsiella ictaluri* in experimentally infected channel catfish. J Aquat Anim Health 4:215–217.

Moldovan R, Chapman-McQuiston E, Wu XL (2007) On kinetics of phage adsorption. Biophys J 93:303–315.

Morrison EE and Plumb JA (1994) Olfactory organ of channel catfish as a site of experimental *Edwardsiella ictaluri* infection. J Aquat Anim Health 6:101 - 109.

Mukai R, Hamatake RK, and Hayashi M (1979) Isolation and identification of bacteriophage XX174 prohead. Proc Natl Acad Sci USA 76:4877 488.

Murialdo H,Becker A (1978) Head morphogenesis of complex double-stranded deoxyribonucleic acid bacteriophages. Microbiol Rev 42:529- 576.

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA and Summers MD (1995) Virus taxonomy- 6th report of ICTV. Virology Division, International Union of Microbiological Societies, SpringerVerlag, New York, p1 - 586.

Nakai T, Park SC (2002) Bacteriophage therapy of infectious diseases in aquaculture. Res Microbiol 153:13 -18.

Nakai T, Sugimoto R, Park KH, Matsuoka S, Mori K, Nishioka T, Maruyama K (1999) Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. Dis Aquat Org 37:33– 41.

ncbi/ICTV (2006) The Universal Virus Database, 2006 cited 10th January 2008, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB

Nelson D (2004) Phage taxonomy: We agree to disagree. J Bacteriol 186:7029– 7031.

Newton JC, Wolfe LC, Grizzle JM, Plumb JA (1989) The pathology of experimental enteric septicemia in channel catfish, *Ictalurus punctatus* (Rafinesque) following immersion-exposure in *Edwardsiella ictaluri*. J Fish Dis 12:335- 347.

O'Flynn G, Ross RP, Fitzgerald GF, Coffey A (2004) Evaluation of a cocktail of three bacteriophage for biocontrol of *Escherichia coli* 0157: H7. Appl Environ Microbiol 70:3417- 3424.

Panangala VS, Van Santen VL, Shoemaker CR, Klesius PH (2005) Analysis of 16S-23S intergenic spacer regions of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish. J Appl Microbiol 99:657 - 669.

Panangala VS, Shoemaker CA, McNulty ST, Arias CR, Klesius PH (2006) Intra- and interspecific phenotypic characteristics of fish-pathogenic *Edwardsiella ictaluri* and *E. tarda*. Aquaculture Res 37:49- 60.

Park KH, Matsuoka S, Nakai T, Muroga K (1997) A virulent bacteriophage of *Lactococcus garvieae*(formerly *Enterococcus seriolicida*) isolated from yellow tail *Seriola quinqueradiata*. Dis Aquat Org 29:145-149.

Park SC, Shimarura I,Fukunaga M, Mori K, Nakai T (2000) Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. Appl Environ Microbiol 66:1416-1422.

Park SC, Nakai T (2003) Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu (*Plecoglossus altivelis*). Dis Aquat Org 53:33-39.

Pedulla ML, Ford ME, Houtz JM et al.(20 co-authors) (2003) Origins of highly mosaic mycobacteriophage genomes. Cell 113:171-82.

Pelon W, Luftig RB, Johnston KH (2005) *Vibrio vulnificus* load reduction in Oysters after combined exposure to *V. vulnificus*-specific bacteriophage and an oyster extract component. J Food Prot 68:1188–1191.

Plumb JA and Quinlan EE (1986) Survival of *Edwardsiella ictaluri* in pond water and bottom mud. The Progressive Fish-Culturist 48:212–214.

Plumb JA, Vinitnantharat S (1989) Biochemical, Biophysical, and Serological Homogeneity of *Edwardsiella ictaluri*. J Aquat Anim Health 1:51-56.

Plumb JA, Vinitnantharat S (1993) Vaccination of channel catfish, *Ictalurus punctatus* (Rafinesque), by immersion and oral booster against *Edwardsiella ictaluri*. J Fish Dis 16:65-67.

Plumb JA, Sheifinger CC, Shryock TR, Goldsby T (1995) Susceptibility of six bacterial pathogens of channel catfish to six antibiotics. J Aquat Anim Health 7:211-217.

Plumb JA (1999) Catfish bacterial diseases. In: Health maintenance and principle microbial diseases of cultured fishes. Iowa State University Press, Ames, Iowa, p187-194.

Poe WE, Wilson RP (1989) Palatability of diets containing sulfadimethoxisine, ormetoprim, and Romet 30 to channel catfish fingerlings. The Progressive Fish-Culturist 51:226–228.

Poranen MM, Ravantii JJ, Grahn AM, Gupta R, Auvinen P, Bamford DH (2006) Global changes in cellular gene expression during bacteriophage PRD1 infection. J Virol 80:8081-8088.

Proux C, Van Sinderen D, Suarez J, Garcia P, Ladero V, Fitzgerald GF, Desiere F, Brussow H (2002) The dilemma of phage taxonomy illustrated by comparative genomics of Sfi21-like *Siphoviridae* in lactic acid bacteria. J Bacteriol 184:6026-6036.

Puck TT, Garen A, Cline J (1951) The mechanism of virus attachment to host cells: The role of ions in the primary reaction. J Exp Med 93:65-88.

Rhoades M, Rhoades EA (1972) Terminal repetition in the DNA of bacteriophage T5. J Mol Biol 69:187-200.

Robinson EH, Brent JR, Crabtree JT, Tucker CS (1990) Improved palatability of channel catfish feed containing Romet-30. J Aquat Anim Health 2:43-48.

Rowher F, Edwards R (2002) The phage proteomic tree: a genome based taxonomy for phage. J Bacteriol 184:4529-4535.

Russell WJ, Taylor SA, Sigel MM (1976) Clearance of bacteriophage in poikilothermic vertebrates and the effect of temperature. J Reticuloendothelial Soc 19:91.

Russell M, Whirlow H, Sun T, Webster RE (1988) Low-frequency infection of F- bacteria by transducing particles of filamentous bacteriophages. J Bacteriol 170:5312-5316.

Saed M (1983) Chemical characterization of the lipopolysaccharides of *Edwardsiella ictaluri* and the immune response of channel catfish to this function and to whole cell antigen with histopathological comparisons. Ph.D dissertation. Auburn University, USA.

Shoemaker CA, Klesius PH (1997) Protective immunity against enteric septicemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following controlled exposure to *Edwardsiella ictaluri*. J Fish Dis 20:361-368.

Shoemaker CA, Klesius PH, Bricker JM (1999) Efficacy of a modified live *Edwardsiella ictaluri* vaccine in channel catfish as young as seven days post hatch. Aquaculture 176:189–193.

Shoemaker CA, Klesius PH, Evans JJ (2002) *In ovo* methods for utilizing the modified live *Edwardsiella ictaluri* vaccine against enteric septicemia in channel catfish. Aquaculture 203:221–227.

Shotts EB, Blazer VS, Waltman WD (1986) Pathogenesis of experimental *Edwardsiella ictaluri* infections in channel catfish *Ictalurus punctatus*. Can J Fish Aqua Sc 43:36-42.

Shotts EB, Waltman WD (1990) A medium for the selection isolation of *Edwardsiella ictaluri*. J Wildlife Dis 26:214–218.

Shivu MM, Rajeeva BC, Girisha SK, Karunasagar I, Krohne G, Karunasagar I (2007) Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. Environ Microbiol 9:322–331.

Shwartz M (1976) The adsorption of coliphage lamda to its host: effect of variation in the surface density of receptors and in phage–receptor affinity. J Mol Biol 103.

Skurnik M, Strauch E (2006) Review Phage therapy: facts and fiction. Int J Med Microbiol 296:5–14.

Smith HW, Huggins MB (1983) Effectiveness of phages in treating experimental *Escherichia coli* diarrhea in calves, piglets and lambs. J Gen Microbiol 129:2659–2675.

Smith HW, Huggins MB, Shaw KM (1987) The control of experimental *Escherichia coli* diarrhea in calves by means of bacteriophage. J Gen Microbiol 133: 1111–1126.

Stanley LA, Hudson JS, Schwedler TE, Hayasaka SS (1994) Extracellular products associated with virulent and avirulent strains of *Edwardsiella ictaluri* from channel catfish. J Aquat Anim Health 6:36–43.

Starliper CE, Cooper RK, Shotts (Jr) EB, Taylor PW (1993) Plasmid-mediated Romet resistance of *Edwardsiella ictaluri*. J Aquat Anim Health 5:1–8.

Stevenson RMW, Airdrie DW (1984) Isolation of *Yersinia ruckeri* bacteriophages. Appl Environ Microbiol 47:1201–1205.

Stone AB, Burton (1962) Studies on the deoxyribonucleases of bacteriophage–infected *Escherichia coli*. Biochem J 85:600.

Su MT, Venkatesh TV, Bodmer R (1998) Large- and small-scale preparation of bacteriophage λ lysate and DNA. BioTechniques 25:44–45.

Sulakvelidze A, Alavidze Z, Alavidze Z, Morris Jr GJ (2001) Minireview Bacteriophage therapy. Antimicrob Agents Chemother 45:649–659.

Summers WC (2001) Bacteriophage therapy. Annu Rev Microbiol 55:437–451.

Taddei F, Paepe M (2006) Viruses' life history: Towards a mechanistic basis of a trade-off between survival and reproduction among phages. PLoS Biol 4: e273.

Tai-wu L (2000) Studies on phage control of pustule disease in abalone *Haliotis discus hannai*. J Shellfish Res 19:535.

Taylor PW (1992) Fish-eating birds as potential vectors of *Edwardsiella Ictaluri*. J Aquat Anim Health 4:240–243.

Thune RL, Hawke JP, Johnson MC (1994) Studies on vaccination of channel catfish, *Ictalurus punctatus*, against *Edwardsiella Ictaluri*. J Appl Aquaculture 3:11-23.

Thune RL, Fernandez DH, Benoit JL, Kelly-Smith M, Rogge ML, Booth NJ, Landry CA, Bologna RA (2007) Signature-tagged-mutagenesis of *Edwardsiella Ictaluri* identifies virulence related genes, including a Salmonella pathogenicity island 2 class of type III secretion systems. Appl Environ Microbial 73:7934- 7946.

Tucker CS, Robinson EH (1990) Channel catfish farming handbook. Van Nostrand Reinhold, New York, New York.

Twort FW (1915) An investigation on the nature of ultra-microscopic viruses. Lancent ii:1241- 1246.

US Department of Agriculture, USDA (2003a) Part I: Reference of fingerling catfish health and production practices in the United States. National health monitoring system, 2003a, Fort Collins, Colorado, USA.

US Department of Agriculture, USDA (2003b) Part II: Reference of foodsize catfish health and production practices in the United States. National health monitoring system, 2003b, Fort Collins, Colorado, USA.

Van Alphen L, Lugtenberg B, Rietshel ET, Mombers C (1979) Architecture of the outer membrane of *Escherichia coli* K12. Phase transitions of the bacteriophage K3 receptor complex. Eur J Biochem 101:571- 579.

Verner Jefferys DW, Aligoet M, Pond MJ, Virdee HK, Bagwell NJ, Robert EG (2007) Furunculosis in Atlantic salmon (*Salmo salar* L.) is not readily controllable by bacteriophage therapy. Aquaculture 270:475-484.

Vinod MG, Shivu MM, Umesha KR, Rajeeva BC, Krohne G, Karunasagar I, Karunasagar I (2006) Isolation of *Vibrio harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. Aquaculture 255:117–124.

Waltman WD, Shotts EB, Hsu TC (1986) Biochemical characteristics of *Edwardsiella ictaluri*. Appl Environ Microbial 51:101–104.

Wagner BA, Wise DJ, Khoo LH, Terhune JS (2002) The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health 14:263–272.

Wang IN, Dykhuizen DE, Slobodkin LB (1996) The evolution of phage lysis timing. Evol Ecol 10:545–558.

Wang IN (2005) Lysis timing and bacteriophage fitness. Genetics 172:17–26.

Watanabe K, Takesue S (1972) The requirement for Calcium in infection with Lactobacillus phage. J gen Virol 17:19–30.

Westwater C, Kasman LM, Schofield DA, Werner PA, Dolan JW, Schmidt GM, Noris JS (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. Antimicrob Agents Chemother 47:1301–1307.

Wise DJ, Johnson MJ (1998) Effects of feeding frequency and Romet-medicated feed on survival, antibody response, and weight gain of fingerling channel catfish *Ictalurus punctatus* after natural exposure to *Edwardsiella ictaluri*. J World Aquaculture Society 29:169–175.

Wise DJ, Terhune JS (2001) The relationship between vaccine dose and efficacy in channel catfish *Ictalurus punctatus* vaccinated as fry with a live attenuated strain of *Edwardsiella ictaluri* (RE-33) J World Aquaculture Society 32:177–183.

Wise DJ, Khoo LH, Terhune JS (2002) The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health 14:263–272.

Wu J, Lin H, Jan L, Hsu Y, Chang L (1981) Biological control of fish bacterial pathogen, *Aeromonas hydrophila*, by bacteriophage AH 1. Fish Pathol 15:271–276.

Wu J, Chao WJ (1984) The epizootic of milkfish vibriosis and its biological control by bacteriophage AS10. COA Fisheries No. 10 Fish Dis Res 6:34–46.

Young RY (1992) Bacteriophage lysis: mechanism and regulation. Microbial Rev. 56:430--481.

Zhang Z, Greene B, Thuman-Commike PA, Jakana J, Prevelige PE, King J, Chiu W (2000) Visualization of the maturation transition in bacteriophage P22 by electron cryomicroscopy. J Mol Biol 297:615--626.

Zhang Y, Arias CR (2006) Identification and characterization of an intervening sequence within the 23R ribosomal RNA genes of *Edwardsiella ictaluri*. Syst. Appl. Microbiol. 27:573--580.

Zimmer M, Scherer S, Loessner MJ (2002) Genomic analysis of *Clostridium perfringens* bacteriophage φ3626, which integrates into *guaA* and possibly affects sporulation. J Bacteriol.184:4359-4368.

Zorzopulos J, Kozloff LM, Chapman V, Delong S (1979) Bacteriophage T4D receptors and the *Escherichia coli* cell wall structure: Role of spherical particles and protein b of the cell wall in bacteriophage infection. J Bacteriol 137:545 555.

Table 1. Host range of phage infection tested on *Edwardsiella ictaluri* strains and other bacterial species collected from different locations. The EOP for phage was determined as a ratio of PFU ml^{-1} for each strain relative to that obtained from *E. ictaluri* strain 219 determined after 12 h of incubation at 30°C.

Bacteria	EOP[1]		Source[2]
	Φ eiAU	Φ eiDWF	
Edwardsiella ictaluri strains			
ATCC 33202	106	223.1	Catfish, Mississippi
AL93-92	61.1	77.9	Catfish, Alabama
AU98-25-12A	76.4	157.4	Catfish, Alabama
195	51.4	4.7	Catfish, Alabama
196	20.8	2.8	Catfish, Alabama
218	112.5	131.8	Catfish, Mississippi
219	100	100	Catfish, Alabama
S97-773	106.9	66.8	Catfish, Alabama
RE-33	150	306.1	AUFDL
C91-162	18.1	0.4	AUCVM
R4383	37.5	1.4	AUCVM
Aeromonas hydrophila GA-06-05			Catfish, Georgia
Citrobacter freundii ATCC 8090		–	ATCC
Edwardsiella tarda AL 93-38			Catfish, Alabama
Enterobacter aerogenes CDC 659-66		–	ATCC
Flavobacterium columnare ALG 530		–	Catfish, Alabama
Flavobacterium columnare AL-01-35			Tilapia, Alabama
Flavobacterium columnare CR-04-02			Tilapia, Costa Rica
Flavobacterium columnare SC-04-04			Carp, South Carolina
Flavobacterium columnare TN-02-01	–		Catfish, Tennessee
Klebsiella pneumoniae ATCC 25953	–		ATCC
Proteus mirabilis			AUFDL
Salmonella enterica ATCC 12324	–		ATCC
Yersinia ruckeri biotype I MO-06-08	–		Trout, Missouri
Yersinia ruckeri biotype II SC-04-13			Trout, South Carolina

[1] Efficiency of plaquing (EOP) for susceptible host bacteria expressed as percentages (%): –, host was not susceptible to phage infection.

[2] AUCVM, Auburn University College of Veterinary Medicine (Department of Pathobiology); AUFDL, Auburn University Fish Diagnostic Laboratory.

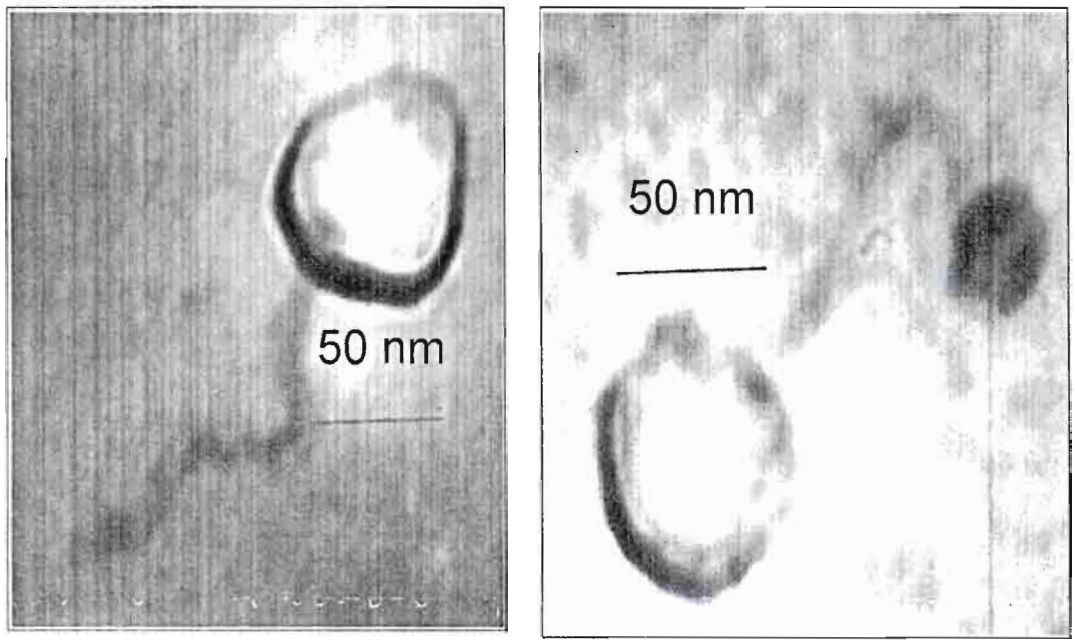

Figure 1. Electron micrographs of phages ΦeiAU and ΦeiDWF negatively stained with 2% phosphotungstic acid.

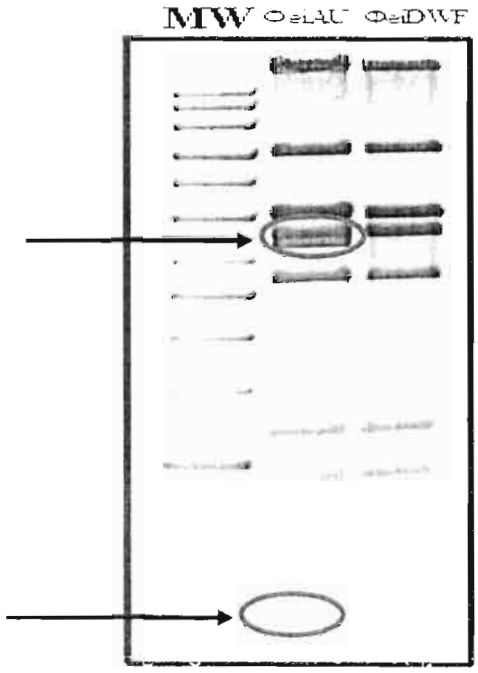

Figure 2. Restriction fragment analysis of phages with *Eco*RI resolved by agarose gel electrophoresis. Arrows show presence of DNA fragments (double bands) unique to phage ΦeiAU.

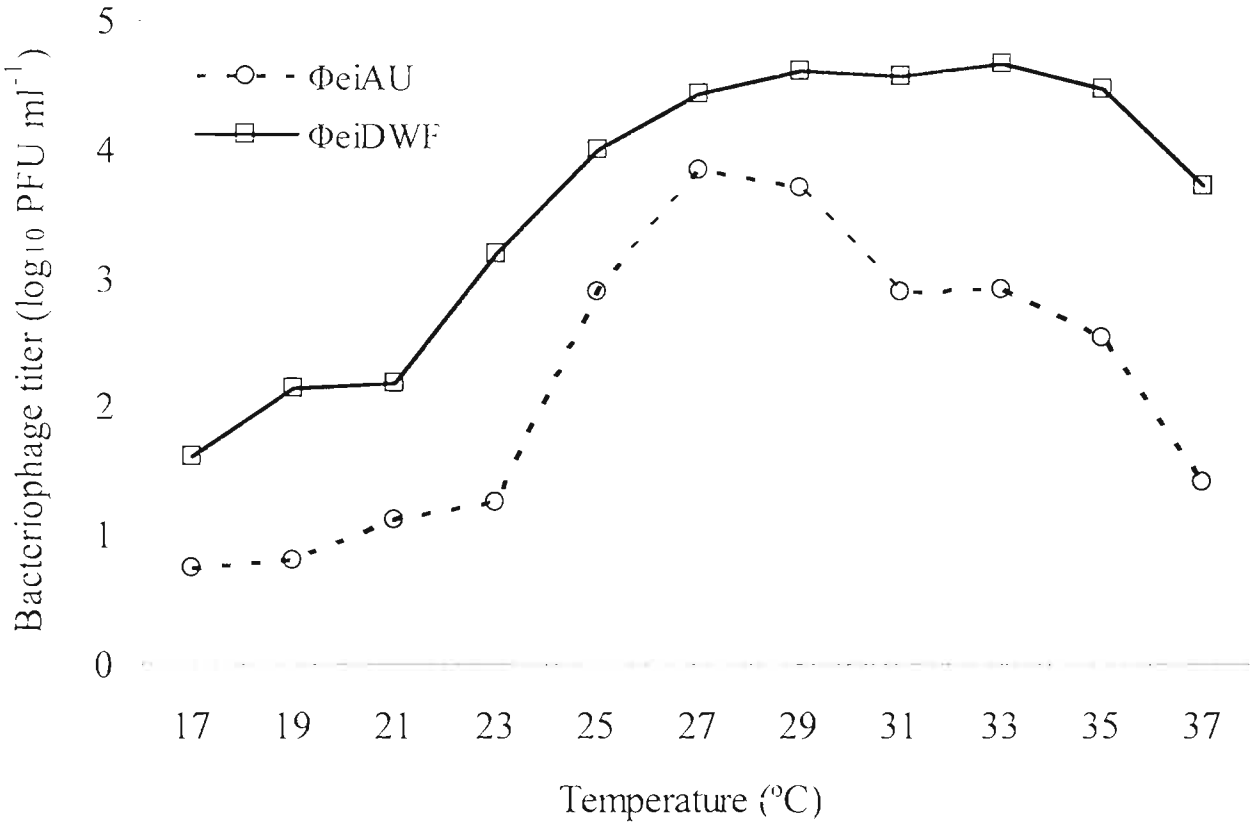

Figure 3. Effect of temperature on phage titer when infecting log-phase *E. ictaluri*.

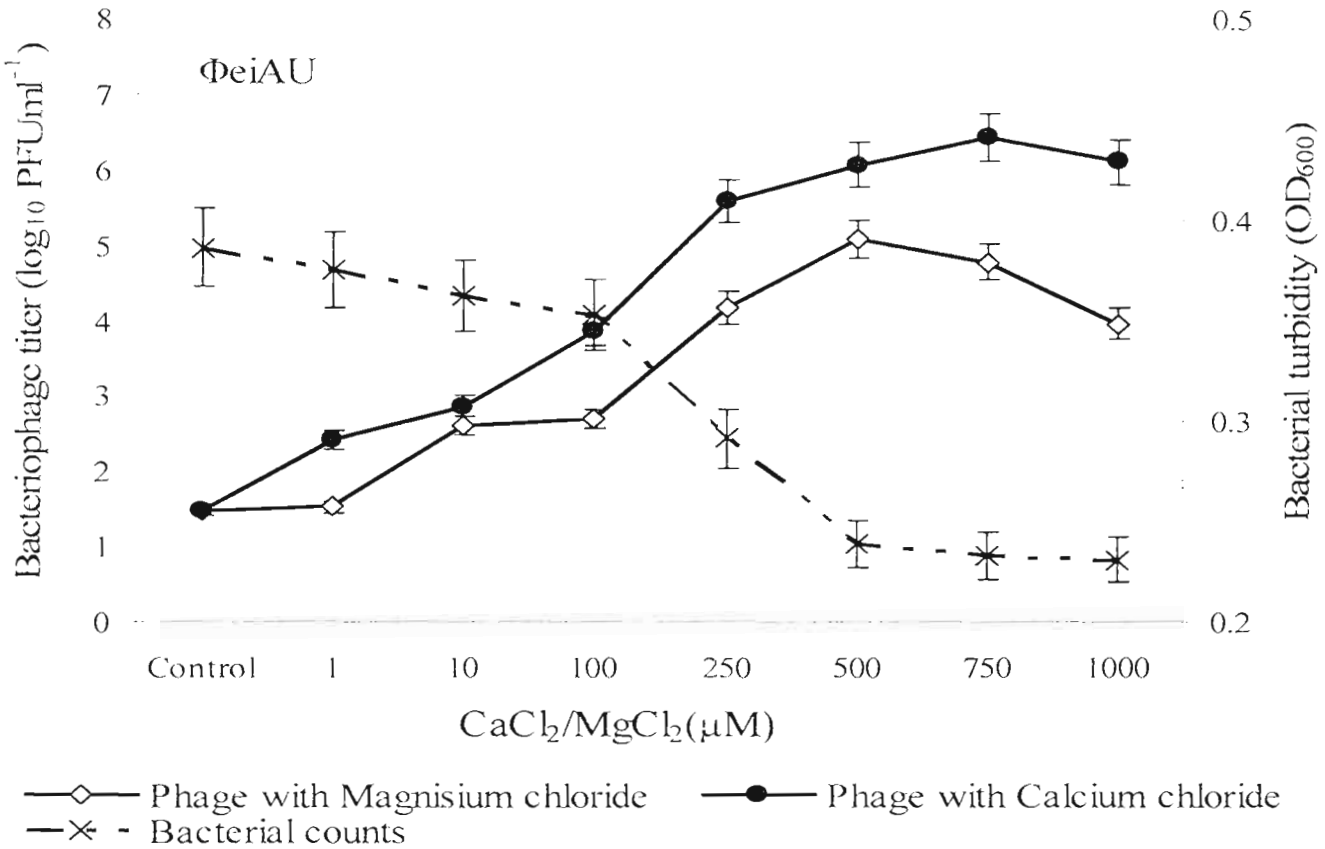

Figure 4. Effects of $CaCl_2$ and $MgCl_2$ on titer of phage ΦeiAU when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (± SD).

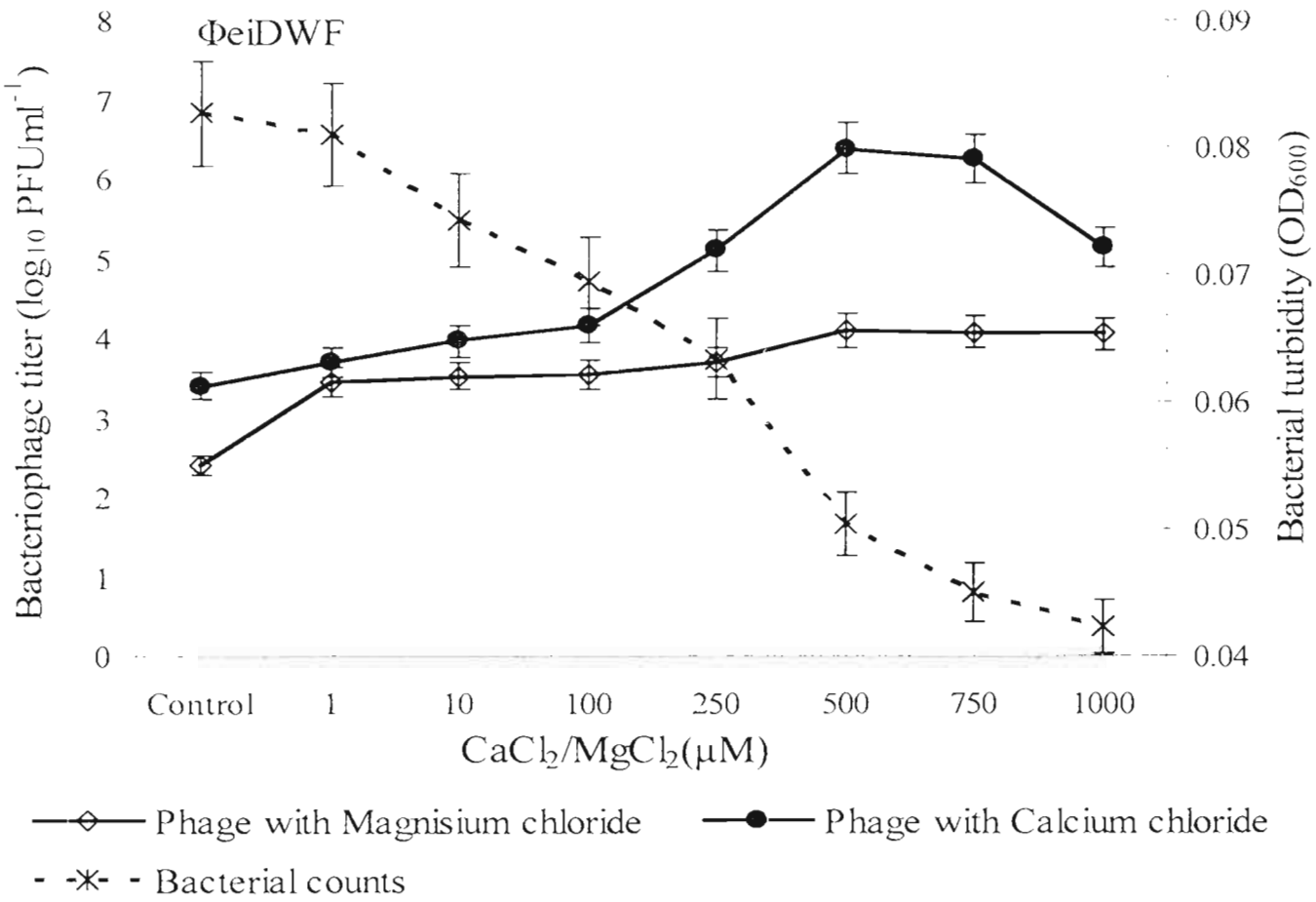

Figure 5. Effects of $CaCl_2$ and $MgCl_2$ on titer of phage ΦeiDWF when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (± SD).

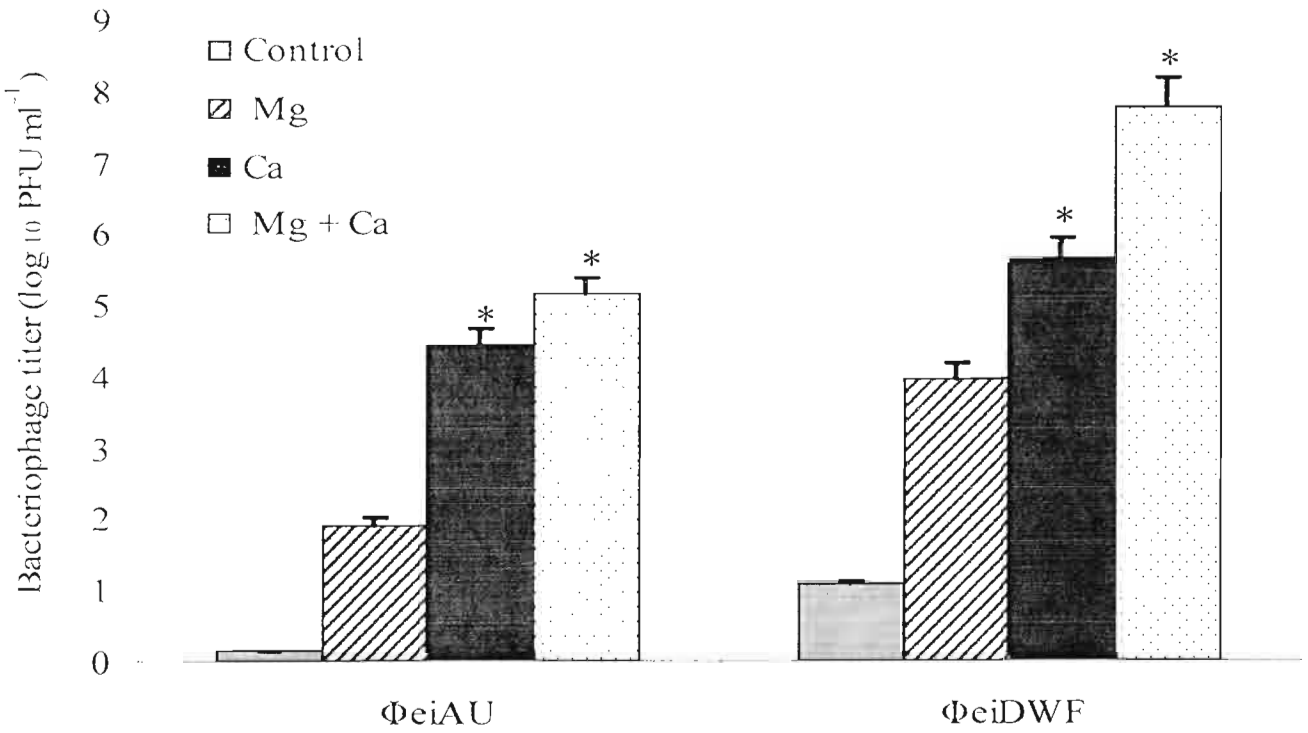

Figure 6. Effects of adding 500 μM $CaCl_2$, $MgCl_2$ and a combination, on titers of phage ΦeiDWF and ΦeiAU when added to cultures of *E. ictaluri* strain 219. Error bars indicate mean (± SE).The asterisks indicate a significant difference from the control (* $P < 0.05$: Dunnett's test).

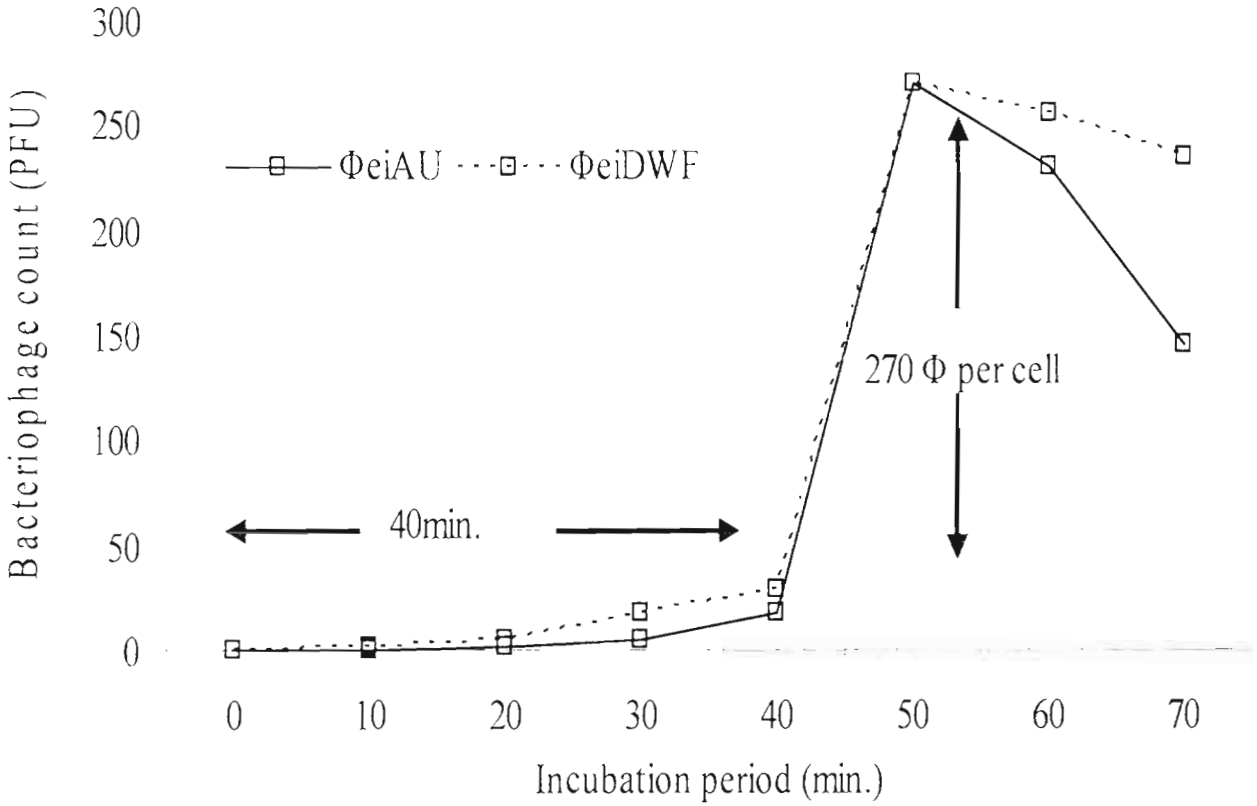

Figure 7. One-step-growth-curve showing the latent period (40 min) and the average burst size (270 viral particles per host cell).

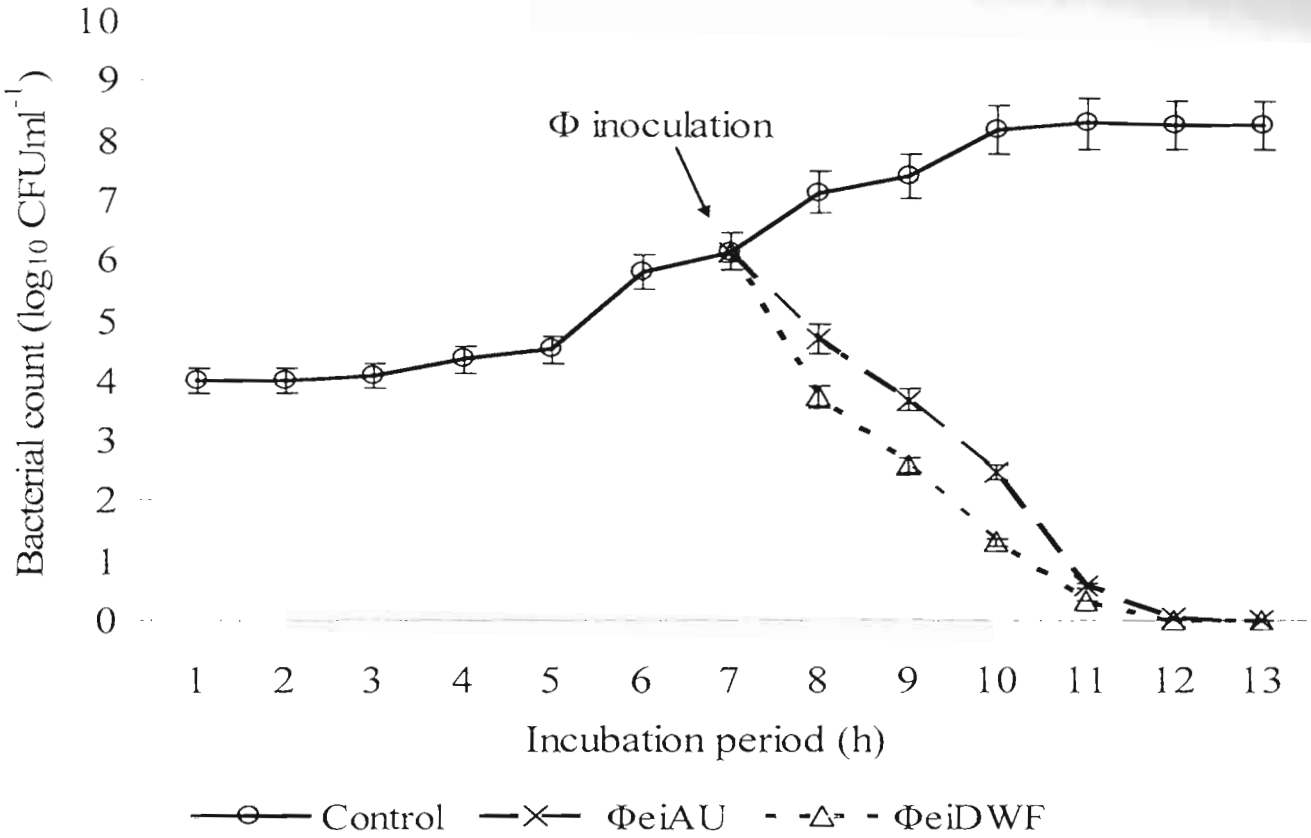

Figure 8. Phage-induced lysis of *E. ictaluri* cells grown in BHI broth supplemented with 500 µM $CaCl_2$. Phage ΦeiAU and ΦeiDWF were separately inoculated in log phase cultures of *E. ictaluri* strain 219 while incubating at 30°C. Error bars indicate mean (± SD).

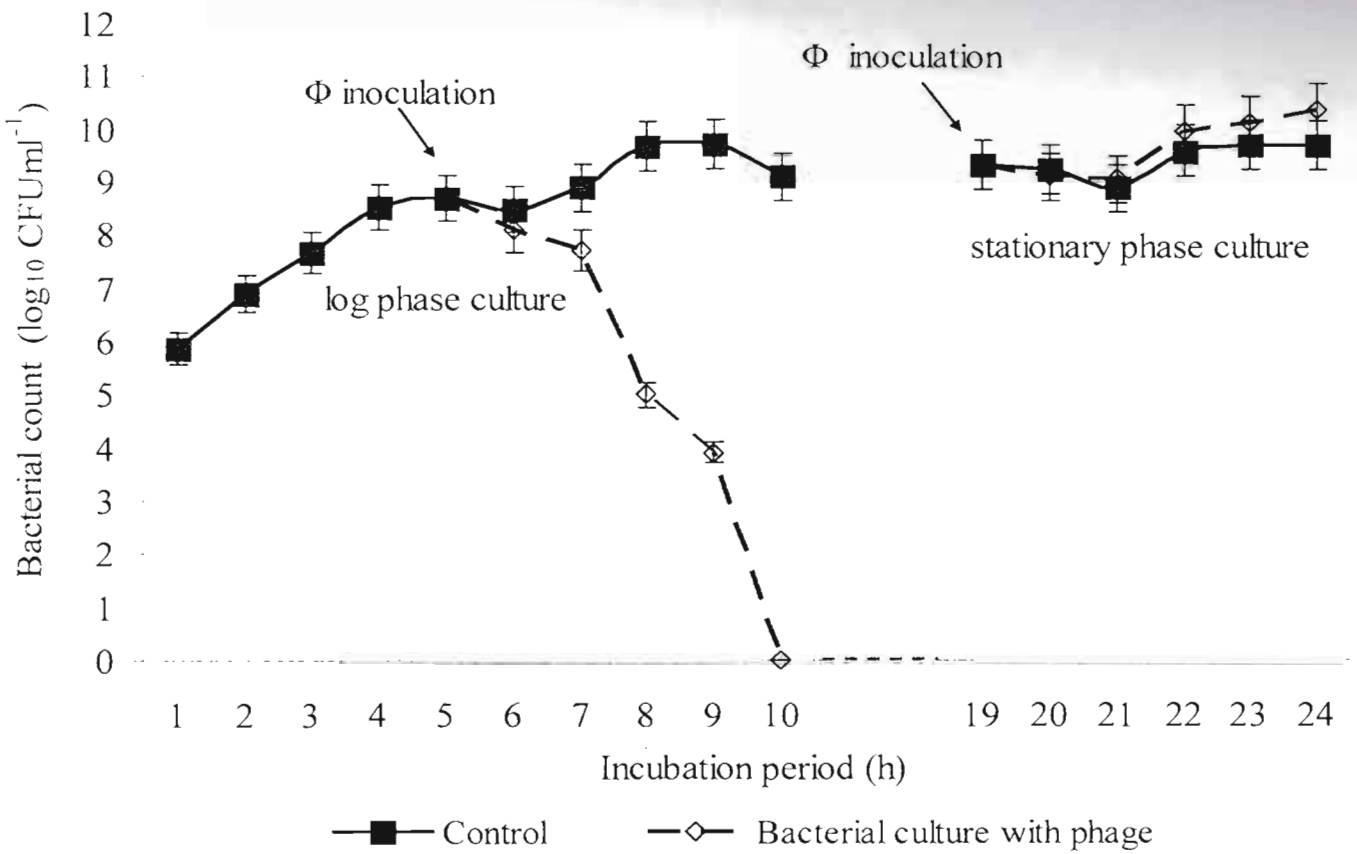

Figure 9. Effects of inoculating phage ΦeiDWF into *E. ictaluri* strain 219 cultures in log phase (after 6h) and stationary phase (after 19h). Bacterial counts are compared with the control (with out phage). Cultures were supplemented with 500 μM $CaCl_2$ and incubated at 30°C. Error bars indicate mean (± SD).