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ORIGINAL ARTICLE

Identification and characterization of bacteriophages specific to the catfish pathogen, *Edwardsiella ictaluri*

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Abstract

Aims: To identify and characterize bacteriophages specific for *Edwardsiella ictaluri*, the causative agent for enteric septicemia of catfish (ESC).
Methods and Results: Two bacteriophages were isolated that infect *Edw. ictaluri*. They both produce clear plaques, have icosahedral heads with a non-rigid tail, and are tentatively classified as *Siphoviridae*. Phages ΦeiDWF and ΦeiAU are dsDNA viruses with approximate genome sizes of 40 and 45 kb, respectively. The addition of 500 μmol l^{-1} $CaCl_2$ enhanced phage titres. Both phages have a latent period of 40 min and an estimated burst size of 270. Every *Edw. ictaluri* strain tested was susceptible to phage infection with variable plaquing efficiencies and with no evidence of lysogeny, with no plaques detected on other bacterial species.
Conclusions: Two unique bacteriophages were isolated that show host-specificity for *Edw. ictaluri*, have temperature and metal cation-dependent infectivity, and are tentatively placed within the family *Siphoviridae*.
Significance and Impact of the Study: This is the first report of bacteriophages specific to *Edw. ictaluri*, an important fish pathogen affecting farm-raised channel catfish. Initial characterization of these bacteriophages has demonstrated their potential use as biotherapeutic and diagnostic agents associated with ESC.

Introduction

Edwardsiella ictaluri, causative agent of enteric septicemia of catfish (ESC), is one of the leading fish pathogens affecting farm-raised channel catfish (*Ictalurus punctatus* Rafinesque) in the southeastern states of the United States (Hawke *et al.* 1981, 1998; Plumb 1999; Hawke and Khoo 2004). Economic loses due directly to ESC outbreaks are estimated between $20 and $30 million per year, affecting 78% of all aquaculture farms (Wagner *et al.* 2002, USDA 2003a,b). The disease primarily affects channel catfish but has also been experimentally reisolated from other species: walking catfish (*Clarias batrachus* Linnaeus), European catfish (*Silurus glanis* Linnaeus), Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Inglis *et al.* 1993; Plumb 1999). ESC outbreaks are seasonal with occurrences during late spring and early fall when temperatures range from 18°C to 30°C (Tucker and Robinson 1990; Hawke *et al.* 1998). However, adverse environmental conditions that exist in an aquaculture system can greatly accelerate the severity of ESC causing mortalities of over 50% of cultured fish (Plumb 1999).

Control and preventive measures against ESC such as the application of antibiotics and a vaccine are available (Wise and Johnson 1998; Klesius and Shoemaker 1999; Shoemaker *et al.* 1999; Wise and Terhune 2001) but have not been adopted by all catfish producers. Application of medicated feed is an expensive practice and is marginally effective. Antibiotic-resistance of *Edw. ictaluri* to oxytetracycline and ormethoprim–sulphadimethoxine (drugs approved for use in catfish) raises concerns about the long-term efficacy of antibiotic treatment in commercial production (Johnson 1991; DePaola *et al.* 1995; Plumb *et al.* 1995). Similarly, disease outbreaks often occur within vaccinated catfish populations (Thune *et al.* 1994).

Biological control agents such as bacteriophages may provide an alternative mechanism to control bacterial diseases in both human and veterinary medicine (Barrow and Soothhill 1997; Barrow 2001). 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). 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 yellowtail (*Seriola quinqueradiata* Temminck and Schlegel), Ayu fish (*Plecoglossus altivelis* Temminck and Schlegel), abalone (*Haliotis discus hannai* Ino), loaches (*Misgurnus anguillicaudatus* Cantor), brook trout (*Salvelinus fontinalis* Mitchill) and eastern oysters (*Crassostrea virginica* Gmelin) (Wu *et al.* 1981; 1984; Li *et al.* 1999; Nakai *et al.* 1999; Tai-wu 2000; Pelon *et al.* 2005; Imbeault *et al.* 2006).

Two principal challenges in the use of bacteriophages as biological control agents are the selection for bacterial resistance to phage infection, and rapid clearance of phage by the fish reticuloendothelial system (Russell *et al.* 1976; Nakai and Park 2002; Levin and 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 *Edw. ictaluri* 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 genomovars 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 *Edw. 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 *Edw. ictaluri* host-specificity, without evidence of lysogeny, and capable of producing clear plaques upon pathogenic strains of *Edw. ictaluri.*

Method and materials

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 this study (Table 1). With the exception of *Edw. ictaluri* strain RE–33, *Edw. ictaluri* strain R4383, *Edw. 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 *Edw. 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 in Hsu-Shotts medium (Bullock *et al.* 1986) and the remaining bacterial isolates were propagated on brain heart infusion (BHI) media (Difco, Sparks, MD, USA) at 30°C, and stored in their respective broth at –80°C in 10% glycerol. Biochemical tests were performed using protocols described by the AFS–FHS Blue Book (American Fishery Society-Fish Health Section, Bethesda, MD, USA). Various assays (e.g. Gram stain, cytochrome oxidase, indole production, hydrogen sulfide production, and motility) were performed on *Edw. ictaluri* strains grown on Remel BHI agar (Fisher Scientific, Lenexa, KS, USA).

Enrichment and isolation of bacteriophages

Water samples were collected from eight commercial catfish ponds that had recently been diagnosed with ESC (at least 3 l were collected for processing from each pond). Algal cells and debris were pelleted by centrifugation at 3600 ***g*** for 30 min. Following removal of most cells, viruses within the supernatant were concentrated using 30–100 kDa Amicon Centricon Plus-70 ultrafiltration membranes (Millipore, Billerica, MA, USA) while centrifuging at 3600 ***g*** for 15 min. Samples were subsequently sterilized through 0·22 μm PVDF filters (Millipore, Bedford, MA, USA).

Bacteriophages specific to *Edw. ictaluri* were enriched as described by O'Flynn *et al.* (2004) with some modifications. Pond concentrates (~5 ml) were added to 30 ml

Table 1 EOP of ΦeiAU and ΦeiDWF on *Edw. ictaluri* strains and other bacterial species isolated and collected from different locations

Bacteria	EOP*		
	ΦeiAU	ΦeiDWF	Source†
Edwardsiella ictaluri strains			
ATCC 33202	106	223·1	Catfish, Mississippi
AL93-92	61·1	77·9	Catfish, Alabama
Au98-25-42A	76·4	157·4	Catfish, Alabama
195	27·3	33·8	Catfish, Alabama
196‡	10^{-4} to 10^{-7}	10^{-4} to 10^{-7}	Catfish, Alabama
218	112·5	131·8	Catfish, Mississippi
219	100	100	Catfish, Albama
S97 773	106·9	66·8	Catfish, Albama
RE-33	150	306·1	AUFDL
C91-162‡	10^{-4} to 10^{-7}	10^{-4} to 10^{-7}	AUCVM
R4383‡	10^{-4} to 10^{-7}	10^{-4} to 10^{-7}	AUCVM
Aeromonas hydrophilia GA-06-05	–	–	Catfish, Georgia
Citrobacter freundii ATCC 8090	–	–	ATCC
Edwardsiella tarda AL 9338	–	–	Catfish, Albama
Enterobectar aerogenes CDC 65966	–	–	ATCC
Flavobacterium columnare ALG 530	–	–	Catfish, Albama
Flavobacterium columnare AL-04-35	–	–	Tilapia, Albama
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
Protein mirabilis	–	–	AUFDL
Salmonellaenterica ATCC 12324	–	–	ATCC
Yersinia rucleri biotype I MO-06-08	–	–	Trout, Missouri
Yersinia rucleri biotype II SC-04-13	–	–	Trout, South Carolina

*****The EOP for each phage was determined as a ratio of PFU ml^{-1} for each strain relative to that obtained from *Edw. ictaluri* strain 219, determined after 12 h of incubation at 30°C.
†AUCVM, Auburn University College of Veterinary Medicine (Department of Pathobiology); AUFDL, Auburn University Fish Diagnostic Laboratory.
‡Quantification of EOP was difficult in these strains due to a very small plaque size (<1 mm).

log-phase *Edw. ictaluri* strain 219 cultures ($3·1 \times 10^7$ CFU ml^{-1}) and grown overnight at 30°C with shaking (150 rev min^{-1}). One percent chloroform (Fisher Scientific, Sair Lawn, NJ, USA) was added to 1·5 ml of culture and subjected to centrifugation at 3600 ***g*** for 10 min at 4°C. The supernatant (1 ml) was then concentrated down to 100 μl using ultrafiltration filters while centrifuging at 3600 ***g*** for 10 min. The presence of lytic phages was tested by spotting 5 μl of filtrate onto a lawn of *Edw. ictaluri* grown at 30°C on BHI agar.

In addition, samples from diseased catfish reared at E.W Shell Fisheries Center in Auburn, AL, were also analyzed for presence of bacteriophages. Kidney and liver samples were homogenized and spread onto BHI agar for isolation *Edw. ictaluri* and identification of phage plaques. Identified plaques were inoculated into a log-phase culture of *Edw. ictaluri*, and the phage lysate stored at −80°C until further analysis (J. Plumb, personal communication).

Bacteriophages were triple purified from isolated plaques using the soft agar overlay method (Adams 1959). A mixture of 100 μl of viral concentrate and 200 μl of log-phase *Edw. 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 into a 5 ml log-phase *Edw. ictaluri* broth culture and incubated at 30°C with shaking (150 rev min^{-1}) for 8 h. Purified phages were then stored in SM buffer [100 mmol l^{-1} NaCl, 8 mmol l^{-1} $MgSO_4$, 50 mmol l^{-1} Tris–HCl (pH 7·5)], and 0·002% (w/v) gelatin at 4°C with the addition of 7% dimethyl sulfoxide (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 7 ml of SM buffer and incubated at 30°C with shaking at 60 rev min^{-1} for 4 h. Phage suspensions were then centrifuged at 3600 ***g*** for 10 min to remove cells and debris, and the supernatant was filter-sterilized through a 0·22 μm PVDF filter. Plaque assays as described by Adams (1959) were performed to determine the titre of

a phage stock. After a 10-fold dilution of the phage stock, 10 μl of each dilution were spotted on a lawn of *Edw. ictaluri* and then incubated overnight at 30°C to determine the number of plaque forming units (PFU). Stock 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

Contaminating host chromosomal DNA was removed from a phage stock by adding 250 units of Benzonase® (Novagen, Inc., Madison, WI, USA) and incubating overnight at 37°C. Benzonase was inactivated by addition of 10 mmol l^{-1} EDTA and heating at 70°C for 10 min. Phage protein coats were degraded using 1 mg ml^{-1} proteinase K (Novagen, Inc., Madison, WI, USA) and 1% sodium dodecyl sulfate 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 *Eco*RI for at least 3 h at 37°C, and resolved by agarose gel electrophoresis on 1% agarose gels at 70V for 3 h. Gels were stained with ethidium bromide and visualized with an Alpha-Imager® HP gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA).

Effects of temperature, Ca and Mg on bacteriophage replication

The effects of calcium, magnesium and temperature were examined to determine optimal conditions for the infectivity of both phages. To monitor the effect of temperature on phage multiplication, a log-phase *Edw. ictaluri* strain 219 (10^6 CFU ml^{-1}) culture in BHI broth was infected with approximately 10^4 PFU ml^{-1} and samples were incubated at temperatures between 17°C and 37°C for 5 h. Phage lysates were subjected to centrifugation at 16 100 ***g*** for 5 min, filter-sterilized through 0·22 μm PVDF filters and then quantified by spotting serial dilutions onto *Edw. ictaluri* lawns.

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). The effect of $CaCl_2$ and/or $MgCl_2$ (ranging from 0 to 1 mmol l^{-1} added to BHI broth) on phage titres was determined. Samples were assayed to determine the PFU ml^{-1} and the bacterial culture turbidity (OD_{600}) after eight hours of incubation at 30°C. Statistical analysis of the differences between treatment means for each phage was assessed using a one-way analysis of variance (ANOVA) at a 5% significant level.

One-step growth

A one-step growth experiment was conducted based on methods described by Adams (1959) with modifications. Duplicates of ΦeiDWF and ΦeiAU were separately added to *Edw. ictaluri* strain 219 broth cultures with 1 mmol l^{-1} potassium cyanide (KCN), at a MOI of 0·1. Samples were then 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.

Phage lysis of host cells

A time course experiment was used to determine the phage-induced lysis of host cells as described by O'Flynn *et al.* (2004) with slight modifications. An overnight culture of *Edw. ictaluri* strain 219 was inoculated (1% v/v) into BHI broth media with 500 μmol l^{-1} $CaCl_2$ then incubated at 30°C while shaking. After 7 h, triplicate samples of ΦeiDWF and ΦeiAU were separately introduced into log phase *Edw. ictaluri* strain 219 cultures (approx. 10^6 CFU ml^{-1}) at a MOI of 0·1, and none in the control cultures. Samples were drawn every hour and plated for CFU ml^{-1}. Both phages were also added to stationary phase *Edw. ictaluri* strain 219 cultures (approx. 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 using *Edw. ictaluri* strain 219 as a reference strain. The EOP of a phage on a given strain of *Edw. ictaluri* was expressed as the ratio of the

PFU ml^{-1} of a given host strain relative to that observed on *Edw. ictaluri* strain 219.

Prophage induction

All isolates of *Edw. ictaluri* used in the host range study were tested for lysogenic phage using a method described by Fortier and Moineau (2007) with modifications. An overnight culture of *Edw. ictaluri* was sub-cultured (3% v/v) in fresh BHI broth and incubated at 30°C with shaking until cultures reached an OD_{600} of 0·100. To a 5 ml of *Edw. ictaluri* culture, Mitomycin C (Sigma-Aldrich, St Louis, MO, USA) was added to a final concentration of 1 μg ml^{-1} and then incubated for 30 min. Cells were pelleted by centrifugation at 3700 ***g*** for 5 min, resuspended in fresh BHI broth and incubated for 5 h at 30°C with shaking (150 rev min^{-1}). Samples were then centrifuged at 3700 ***g*** for 5 min and 10 μl of supernatant spot assayed for presence of phage against all tested strains.

Results

Isolation of bacteriophages

From aquaculture pond enrichments, one out of eight pond enrichments had evidence of *Edw. ictaluri* phage plaques. Sixteen phages were double purified from samples collected from Dean Wilson Farms in western Alabama, and six phages were double purified from samples obtained from an infected catfish kidney tissue from the E.W Shell Fisheries Center in Auburn, AL. Phages isolated from the aquaculture pond had plaques ranging from 0·5 to 11 mm in size and those isolated from infected catfish kidney tissue ranged from 4 to 7 mm. Both phages produced clear plaques on a lawn of host bacteria. No differences were observed in the restriction fragment profiles between the 16 separate phage isolates from the aquaculture pond, or between the six phage isolates from the catfish kidney tissue (data not shown), and one representative phage was chosen from the aquaculture pond enrichment (ΦeiDWF) and the catfish kidney tissue (ΦeiAU) for further study.

Size and morphology of bacteriophages

Electron microscopy revealed similarity in morphology between ΦeiAU and ΦeiDWF (ΦeiAU shown in Fig. 1). Both have an icosahedral shaped head, 50 nm in diameter, and a non-rigid tail. Tail lengths of ΦeiAU and ΦeiDWF are both 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).

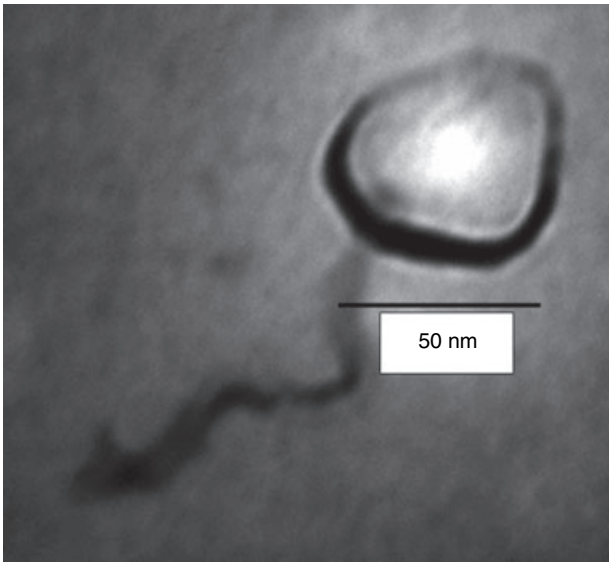

Figure 1 Electron micrograph of phage ΦeiAU, negatively stained with 2% phosphotungstic acid.

Bacteriophage nucleic acid restriction fragment analysis

Phage nucleic acids were not digested by exonuclease I, indicating that the phages are double-stranded DNA phages. Restriction endonuclease digestion of ΦeiAU and ΦeiDWF with *Eco*RI showed many bands in common (Fig. 2); however, phage ΦeiAU had two additional restriction fragments compared to ΦeiDWF (Fig. 2). Their dsDNA genome sizes are approximately 40 kb (ΦeiDWF) and 45 kb (ΦeiAU).

Effects of temperature and metal cations on phage titre

Infection of *Edw. ictaluri* by ΦeiAU and ΦeiDWF is dependent upon temperature and the presence of calcium and magnesium salts. The optimal temperature for growth of *Edw. 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 was lowered to 20°C. Similarly low phage titres were obtained at temperatures higher than of 30°C (data not shown).

Phage titres of both ΦeiAU and ΦeiDWF are increased by the addition of calcium and magnesium salts to BHI broth. The addition of calcium to BHI broth increased phage titres for both ΦeiAU and ΦeiDWF by several orders of magnitude in a dose-dependent manner (Fig. 3). It is important to note that the initial phage

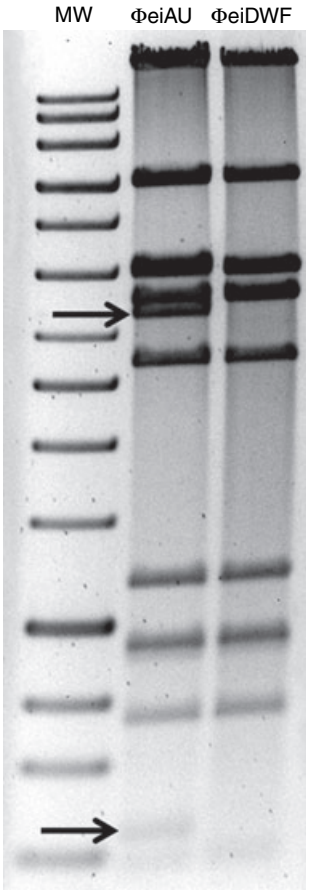

Figure 2 Restriction fragment analysis of phages with *Eco*RI resolved by agarose gel electrophoresis. Arrows show presence of DNA fragments unique to phage ΦeiAU.

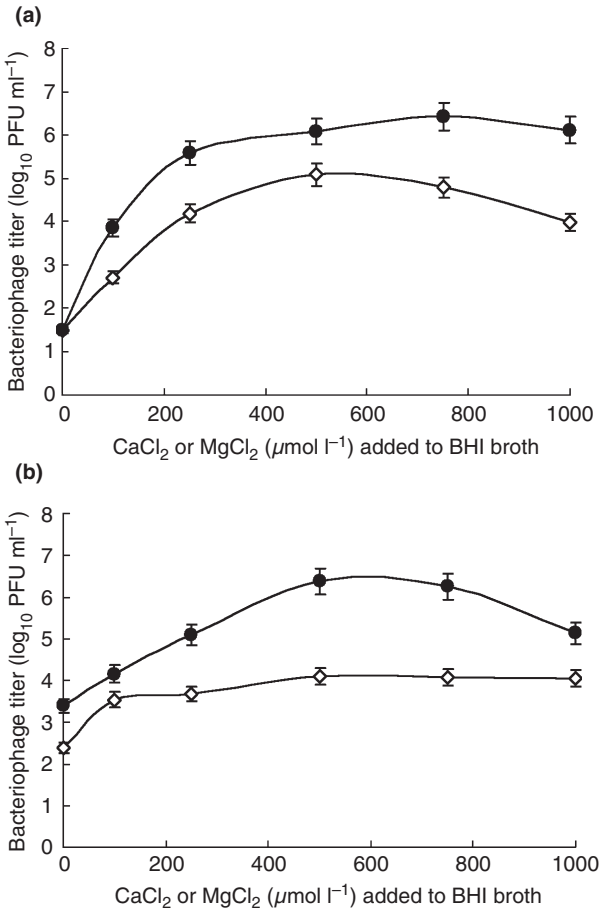

Figure 3 Effects of $CaCl_2$ (●) and $MgCl_2$ (◇) on titre of (a) phage ΦeiAU and (b) ΦeiDWF when added to broth cultures of *Edw. ictaluri* strain 219. Error bars indicate mean (± SD). Bacterial turbidity (X) determined spectrophotometrically at 600 nm.

inoculum in these experiments was approximately identical ($\sim 1 \times 10^4$ PFU ml^{-1}) for ΦeiAU and ΦeiDWF, yet in the absence of supplemental calcium or magnesium the phage titre of ΦeiAU decreased substantially during the 5 h of incubation. The optimal range observed for calcium and magnesium is 500–750 μmol l^{-1} at which a substantial decrease in bacterial turbidity was observed with a corresponding increase in phage titres. The effects of supplementing $CaCl_2$ and $MgCl_2$ (both standardized at 500 μmol l^{-1}) showed a significant increase ($P < 0{\cdot}05$: Dunnet's test) of approximately one to two orders of magnitude relative to the titres obtained with addition of $CaCl_2$ alone for ΦeiAU and ΦeiDWF, respectively (data not shown).

Burst size and latent period

The one-step growth curve was performed for both ΦeiAU and ΦeiDWF, revealing an identical latent period for these bacteriophages of approximately 40 min and with an average burst size estimated to be 270 viral particles (ΦeiAU and ΦeiDWF) per host cell. These calculations were based on the ratio of mean yield of phage particles liberated to the mean phage particles that infected the bacterial cells in the latent period.

Kinetics of phage-induced lysis

Within six hours of incubation of either phage into a log-phase *Edw. ictaluri* strain 219 culture (about 10^6 CFU ml^{-1} at the time of inoculation) the CFU were reduced to below detectable levels (ΦeiDWF shown in Fig. 4). During this 6-h period, bacterial cultures with phage rapidly cleared while the controls remained turbid. The loss of turbidity and drop in CFU ml^{-1} due to both phages was attained within the same incubation period. Furthermore, when ΦeiDWF was inoculated into stationary-phase *Edw. ictaluri* strain 219 cultures, no clearance of the bacterial culture was observed throughout the

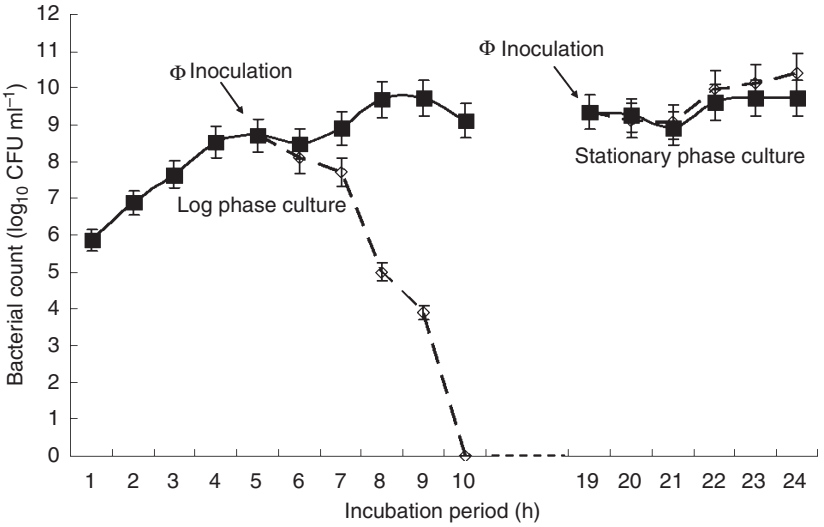

Figure 4 Effects of inoculating phage ΦeiDWF into *Edw. ictaluri* strain 219 cultures in log phase (after 6 h) and stationary phase (after 19 h). Bacterial CFUs in the absence of phage (■) are compared with the cultures inoculated with phage (◇). Cultures were supplemented with 500 μmol l^{-1} $CaCl_2$ and incubated at 30°C. Error bars indicate mean (± SD).

incubation period (Fig. 4). However, when the phage inoculated, stationary phase culture of *Edw. ictaluri* was pelleted by centrifugation and resuspended in fresh medium, the culture turbidity rapidly cleared and the phage titres increased by several orders of magnitude (data not shown).

Host specificity of phages

Both ΦeiAU and ΦeiDWF infected every *Edw. ictaluri* strain that was tested (Table 1). Clear plaques were produced on all strains except on *Edw. ictaluri* strain AL93–92 and AL98-25–42A which had a mixture of opaque and clear plaques. Plaque size ranged from 0·5 to 4 mm. However, small pin-point plaques were produced on *Edw. ictaluri* strains 196, C91–162 and R4383 that appeared only when high phage titres (>10^6 PFU ml^{-1}) were used. Variable ranges in EOP (∼10^{-7} to 300% relative to strain 219) were observed among *Edw. ictaluri* strains. Both phages produced high EOP values (>50% relative to strain 219) with *Edw. ictaluri* strains 218, S97-773, RE-33, AL93-92 AU98-25-42A and 195 while low values (EOP < 10^{-4}) where observed with *Edw. ictaluri* strains 196, C91–162, and R4383. None of the other bacterial species tested were observed to have any evidence of phage plaques including the closely related *Edw. tarda*.

Prophage induction

Mitomycin C was added to cultures of 11 different *Edw. ictaluri* strains in log-phase to induce any prophage(s) existing in the host cells (Goh *et al.* 2005). An increase in turbidity was observed in all cultures tested during the 5 h of incubation. No plaques were observed on any strain of *Edw. ictaluri* indicating the absence of temperate phages in the *Edw. ictaluri* isolates used in this study.

Discussion

Bacteriophages specific to *Edw. ictaluri* were isolated from aquaculture ponds with outbreaks of ESC. This finding suggests that *Edw. Ictaluri*-specific phages exist in aquaculture ponds and may contribute to some degree in lessening the severity or persistence of ESC outbreaks. Since *Edw. 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 *Edw. ictaluri* and its respective phages may persist in aquaculture ponds. This finding is in accordance with the idea that bacteriophages are ubiquitous in the environments inhabited by their respective host(s) (d'Herelle 1926; Adams 1959). Therefore, catfish pond waters and diseased fish are a good source for discovery of phages specific to *Edw. ictaluri*. In addition, the gut microbiota of channel catfish with ESC is an as-yet-unexplored environment in which to identify bacteriophages specific to *Edw. ictaluri*.

The phages described in this study were isolated from samples that differed both temporally and spatially, however electron microscopy revealed similar morphotypes, classified as *Siphoviridae*. Furthermore, restriction digests using *Eco*RI and *Eco*RV showed similar but unique patterns, suggesting that ΦeiAU and ΦeiDWF may have genetic loci in common. Another *Edw. Ictaluri*-infective phage, ΦMSLS-1, has been recently isolated from aquaculture ponds in Mississippi with a history of ESC infection (Dr T. Welch and Dr G. Waldbieser, 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 with only a few unique restriction fragments (data not shown). 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 *Edw. ictaluri* were temperature (optimal 22–33°C), metal cations (especially calcium), and the host growth stage. Phage reproduction is dependent on the physiological state of the bacterial host (Adams 1959; Poranen *et al.* 2006; Taddei and Paepe 2006). Normally, ESC epizootics occur when temperatures range from 22°C to 28°C and are characterized by acute infections and high mortalities within young-of-the-year catfish fingerlings (Francis-Floyd *et al.* 1987; Tucker and 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 and Keasberg 1962; Moldovan *et al.* 2007). Moldovan *et al.* (2007) demonstrated an increase in adsorption rate (approx. 30 times) when the temperature rose from 4°C to 40°C when λ phage was incubated with *E. coli* strain Ymel. 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'Herelle 1926; Luria and 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 and Takesue 1972; Russell *et al.* 1988). The observation that ΦeiAU had a substantial decrease (~1000-fold) in titre after incubation with *Edw. ictaluri* in the absence of supplemental calcium or magnesium, yet could productively infect *Edw. ictaluri* when calcium or magnesium were added to the medium, supports the hypothesis that ΦeiAU (and to a lesser degree, ΦeiDWF) adsorbs to an *Edw. ictaluri* surface receptor that permits productive infection (e.g. phage nucleic acid transfer) in the presence of metal cations. Alternatively, divalent metal cations could be integral to the structural integrity of the bacteriophage(s). Interestingly, results show that the optimal calcium concentration for phage replication (500 μmol l^{-1}) 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 water hardness. Future studies will address the mechanism(s) of metal cation-induced increases in phage titres, and the role of metal cations in phage biological control of ESC in aquaculture ponds.

Both phages are specific to *Edw. ictaluri* strains without generating plaques on any other bacterial species. Although *Edw. tarda* is reported to be closely related to *Edw. ictaluri* (Zhang and Arias 2006), it was not susceptible to phages evaluated in this study. Because of their specificity, both phages will have the potential to help control *Edw. ictaluri* infections in aquaculture raised catfish without infecting beneficial bacteria that could contribute to the biological control of ESC. Interestingly, *Edw. 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 strain RE-33 (Klesius and Shoemaker 1999; Arias *et al.* 2003). Since the efficacy of the vaccine may be affected when both strain RE-33 and bacteriophages are used to control ESC, the vaccine strain should be applied before any bacteriophage application.

Additionally, these phages may also be used as diagnostic tools in fish disease laboratories for detection of *Edw. ictaluri* strains. It is reported that homogeneity exists among *Edw. ictaluri* strains (Plumb and Vinitnantharat 1989; Arias *et al.* 2003; Panangala *et al.* 2006) which explains the susceptibility of all *Edw. ictaluri* strains (tested to date) to phage infection. No other bacterial phenotypes are known that correlate with the lower EOP for the three less phage-susceptible *Edw. 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 (Zorzopulos *et al.* 1979; Duckworth *et al.* 1981). Compared to chemotherapeutants that have a broad spectrum activity on different species (Nelson 2004), an individual phage may not effectively control aquatic pathogens, yet a 'cocktail' of *Edw. ictaluri* specific phages may have better efficiency as a biological control strategy (O'Flynn *et al.* 2004; Skurnik and Strauch 2006; Verner–Jefferys *et al.* 2007). For effective biological control of ESC, additional bacteriophages would need to be identified with good infectivity for *Edw. ictaluri* strains 196, C91-162, and R4383; alternatively, serial passage of ΦeiAU and/or ΦeiDWF in the less-susceptible strains of *Edw. ictaluri* may be an effective means of enhancing the infectivity of these bacteriophages.

In vitro phage infection of *Edw. ictaluri* demonstrates that both phages have the potential to control ESC infections. The observations that these phages are specific to *Edw. ictaluri* strains, occur naturally in aquaculture ponds, and are not lysogenic encourages further work to evaluate their use as biocontrol agents for ESC. Future studies include molecular characterization of phages specific to *Edw. ictaluri* and evaluating the protective effects of these phages in ESC disease challenge models.

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