

Characterization of four lytic transducing bacteriophages of luminescent *Vibrio harveyi* isolated from shrimp (*Penaeus monodon*) hatcheries

Sanjeevi Thiyagarajan, Bagthasingh Chrisolite, Shankar. V. Alavandi, Modem Poornima, Natarajan Kalaimani & T. Chinnappan Santiago

Central Institute of Brackishwater Aquaculture, Indian Council of Agricultural Research, Raja Annamalai Puram, Chennai, India

Correspondence: Modem Poornima, Central Institute of Brackishwater Aquaculture, Indian Council of Agricultural Research, 75, Santhome High Road, Raja Annamalai Puram, Chennai, India. Tel.: 91 044 24618817; fax: 91 044 24610311; e-mail: poornima@ciba.res.in

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Introduction

Vibrio harveyi, a gram-negative marine bacterium, has been described as a significant pathogen of marine vertebrates and invertebrates (Austin & Zhang, 2006). *V. harveyi* causes luminescent bacterial disease (LBD) in larval shrimp, resulting in considerable economic loss to shrimp hatcheries world over (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). Pathogenicity mechanism of *V. harveyi* has been attributed to various virulence factors such as production of proteases (Liu & Lee, 1999), siderophores (Owens *et al.*, 1996), and hemolysin (Zhang *et al.*, 2001). Besides these virulence factors, the association of a *V. harveyi* myovirus-like (VHML) bacteriophage is reported to impart virulence to *V. harveyi* (Austin *et al.*, 2003). Munro *et al.* (2003) also demonstrated that naïve strains of *V. harveyi* could be converted into virulent strains by infecting them with bacteriophage VHML.

Abstract

Four lytic bacteriophages designated as ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 were isolated from commercial shrimp hatcheries, possessing broad spectrum of infectivity against luminescent *Vibrio harveyi* isolates, considering their potential as biocontrol agent of luminescent bacterial disease in shrimp hatcheries, and were characterized by electron microscopy, genomic analysis, restriction enzyme analysis (REA), and pulsed-field gel electrophoresis (PFGE). Three phages ϕ Vh1, ϕ Vh2, and ϕ Vh4 had an icosahedral head of 60–115 nm size with a long, noncontractile tail of $130\text{--}329 \times 1\text{--}17$ nm, belonged to the family *Siphoviridae*. ϕ Vh3 had an icosahedral head (72 ± 5 nm) with a short tail (27×12 nm) and belonged to *Podoviridae*. REA with DraI and PFGE of genomic DNA digested with ScaI and XbaI and cluster analysis of their banding patterns indicated that ϕ Vh3 was distinct from the other three siphophages. PFGE-based genome mean size of the four bacteriophages ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 was estimated to be about 85, 58, 64, and 107 kb, respectively. These phages had the property of generalized transduction as demonstrated by transduction with plasmid pHSG 396 with frequencies ranging from 4.1×10^{-7} to 2×10^{-9} per plaque-forming unit, suggesting a potential ecological role in gene transfer among aquatic vibrios.

It was almost three decades ago that the first description of bacteriophages infecting luminescent bacteria was reported (Keynan *et al.*, 1974). After a long gap of 25 years, bacteriophage-mediated toxicity of *V. harveyi* in *Penaeus monodon* by the transfer of a gene controlling toxin production was reported (Ruangpan *et al.*, 1999), followed by the description of VHML associated with toxin-producing strains (Oakey & Owens, 2000; Oakey *et al.*, 2002). There are also some reports on the isolation and characterization of lytic bacteriophages of *V. harveyi* from coastal ecosystem and shrimp culture ponds (Shivu *et al.*, 2007). A lytic bacteriophage was evaluated as a biocontrol agent of *V. harveyi* and was reported to provide encouraging results (Vinod *et al.*, 2006; Karunasagar *et al.*, 2007). In our earlier work, we reported isolation of bacteriophages of *V. harveyi* from shrimp hatchery (Chrisolite *et al.*, 2008). Here, we present our work on the characterization of four selected

bacteriophages with broad spectrum of infectivity against luminescent *V. harveyi* isolates, considering their potential as biocontrol agent of LBD in shrimp hatcheries. In addition, the possibility of these lytic phages in the process of transduction was also examined as a parameter of risk assessment in view of the increased number of proposals of vibriophages being proposed as biocontrol agents in mariculture.

Materials and methods

Bacteria and phages

Vibrio harveyi cultures (Vh01, Vh51, Vh102, and Vh105) used as bacterial hosts for the phages in this study were isolated from shrimp hatcheries (Chrisolite *et al.*, 2008). Bacteria were grown at 30 °C in peptone yeast extract sea salt (PYSS) broth (Oakey & Owens, 2000) containing 5 g L⁻¹ peptone and 3 g L⁻¹ yeast extract dissolved in Macleod's artificial sea salt water (HiMedia, Mumbai, India). Phages designated as φVh1, φVh2, φVh3, and φVh4, selected from a pool of 76 phages isolated from shrimp hatcheries (Chrisolite *et al.*, 2008), were used in this study.

Preparation of phage lysates

Phage lysates were prepared (Carlson, 2005) and concentrated by ultracentrifugation at 200 000 g for 2 h at 4 °C, using SW-41 swinging-bucket rotor (Beckman, Palo Alto, CA). Phage pellet was resuspended in sterile phage buffer and treated with DNase I (1 µg mL⁻¹) and RNase A (100 µg mL⁻¹) (Genei, Bangalore, India) to degrade the nucleic acid residues of host bacteria. Phage concentrate was further purified by cesium chloride (SRL, Mumbai, India) gradient ultra-centrifugation at 490 000 g for 18 h at 20 °C. The band containing phage particles was drawn from the centrifuge tube using sterile needle and dialyzed against phage buffer overnight at 4 °C and stored at 4 °C for subsequent studies. Titer of the purified phage suspension was determined by agar overlay method (Carlson, 2005).

Host range

Purified phages (with a titer of about 10⁸ PFU mL⁻¹) were tested by spot assay (Carlson, 2005) to test the spectrum of bactericidal activity against 125 isolates of *V. harveyi* and an isolate each of *Vibrio* species such as *Vibrio logei*, *Vibrio fischeri*, *Vibrio splendidus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio cholerae* (Non-O1), *Vibrio fluvialis*, *Vibrio mimicus*, *Vibrio ordalii*, *Vibrio vulnificus*, and *Vibrio metschnikovii*.

Electron microscopy

A 10-µL suspension of purified phages was placed on 200 mesh carbon-coated copper grids and stained with potassium 2% phosphotungstate (pH-7.2) for 20 s. Excess stain was removed immediately by placing the grids on blotting paper. The grids were examined in a Tecnai G2 Spirit Bio-Twin Transmission Electron Microscope (Eindhoven, The Netherlands).

Extraction and analysis of phage nucleic acid

Total nucleic acid of the bacteriophages was extracted using the protocol described earlier (Santos, 1991) with some modifications, dried, and resuspended in 50 µL of sterile Milli-Q water. The phage nucleic acid was treated with DNase I, RNase A (Genei, Bangalore, India), and S1 nuclease (New England Biolabs, MA) according to the manufacturer's instructions to confirm the nature of the nucleic acid of the bacteriophages (Sambrook *et al.*, 1989).

Restriction enzyme analysis of phage DNA

The genomic DNA of the bacteriophages was subjected to restriction enzyme analysis (REA) with AluI, BamHI, BglII, DraI, EcoRI, EcoRV, HindIII, HaeII, KpnI, NcoI, NotI, PstI, SmaI, and XbaI. The reactions were performed as per the manufacturer's instructions (New England Biolabs, UK). DNA fragments were electrophoresed on 1% agarose gel at 5 V cm⁻¹, in 1× TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0). φ Lambda HindIII digest was used as DNA molecular weight marker. Gels were stained with ethidium bromide and photographed.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of bacteriophage DNA was carried out using the protocols described earlier (Carlson, 2005). Agarose plugs were prepared by mixing 1 mL of bacteriophage concentrate with 1 mL of 1.6% PFGE-grade agarose (Bio-Rad, Hercules, CA) and allowed to solidify at room temperature. These agarose plugs were incubated in EDTA sarcosine proteinase K (ESP) (0.5 M EDTA, pH 9, 1% *N*-laurylsarcosine, and mg mL⁻¹ proteinase K) overnight at 50 °C to digest the bacteriophage coat proteins and then washed thrice with TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). The plugs were then treated with RNase A (µg mL⁻¹) by incubating at 37 °C for 30 min.

Restriction digestion was performed with various enzymes such as AluI, BamHI, BglII, DraI, HindIII, HaeII, KpnI, NcoI, NotI, PstI, XbaI, and ScaI following manufacturer's instructions. The plugs were cut to 2-mm slices,

placed in 1× restriction buffer, and incubated for 10 min in a 37 °C water bath. The buffer was removed, and fresh restriction buffer containing 10 U of enzyme was added and incubated at 37 °C in water bath for 4 h. PFGE was carried out in 1% agarose gels in a BioRad CHEF DR-III PFGE system (Bio-Rad), at 120° angle and 6 V cm⁻¹, using ramped pulse times from 1 to 12 s for 6 h in 0.5× TBE (45 mM Tris, 45 mM borate, 1 mM EDTA pH 8.0) at 14 °C. Low-range PFGE marker was used as molecular weight size standard. Genome size was estimated by adding the length of each DNA fragment in the PFGE profile of ScaI and XbaI separately.

Phylogenetic analysis

The REA and PFGE patterns were captured using the Quantity one electrophoresis analysis system (Bio-Rad). Gel images were digitally normalized to a single DNA marker to reduce gel-to-gel restriction pattern variability, and cluster analysis was carried out using MOLECULAR ANALYST software – Fingerprinting II (Version 3.0; Bio-Rad) by unweighted pair group method with arithmetic mean.

Transduction

Ability of the phages to transduce genetic elements was demonstrated by the transduction of the plasmid pHSG396 (Takara Bio Inc., Shiga, Japan), which possesses two selective phenotypic markers, β-galactosidase and chloramphenicol resistance. An isolate of *V. harveyi* (Vh57) susceptible to all four phages was transformed by CaCl₂ treatment (Sambrook *et al.*, 1989). Transformants carrying the plasmid were grown in 10 mL PYSS broth supplemented with 50 μg mL⁻¹ chloramphenicol at 30 °C. This broth was suitably diluted with sterile PYSS to obtain 10⁸ cells and mixed with four bacteriophage suspensions

at a multiplicity of infection of one in separate tubes and incubated for 15 min at 30 °C. This suspension was mixed with 3.0 mL of molten PYSS soft agar (0.75% agar) held at 45 °C and overlaid on PYSS agar. After incubation at 30 °C for 24 h, the resultant plaques were picked for the preparation of transduced purified phage lysates.

Vibrio harveyi recipient cultures grown to OD_{600nm} = 0.6 (≅ 3 × 10⁸ mL⁻¹; BioRad SmartSpec 3000) in PYSS broth were separately mixed with the above transduced phage lysate at the MOI of one and incubated at 30 °C for 30 min. To prevent re-infection, 100 μL of 1 M sodium citrate was added, and the suspension was centrifuged at 10 000 g for 10 min at 4 °C and washed twice with sterile PBS. The cells were inoculated into 1.0 mL of PYSS broth supplemented with chloramphenicol (50 μg mL⁻¹) and incubated at 30 °C for 1.5 h with shaking. Transductants were serially diluted and enumerated by spread plate technique onto PYSS agar supplemented with chloramphenicol (50 μg mL⁻¹), with Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and isopropyl β-D thiogalactoside (IPTG) (Sambrook & Russel, 2001).

Results

Characteristics of bacteriophages

The four phages produced different plaque morphology on their respective hosts (Table 1). Transmission electron micrograph revealed that all the phages (Fig. 1) had tails and thus belonged to the order Caudovirales (Ackermann, 1999). Phages φVh1, φVh2, and φVh4 had icosahedral head of diameters ranging from 60 to 115 nm with a long, rigid noncontractile tail 130–329 × 12–17 nm size (Fig. 1, Table 1) and were assigned to the family *Siphoviridae*, whereas φVh3 had an icosahedral head (72 ± 5 nm) with

Table 1. Characteristics of *Vibrio harveyi* bacteriophages recovered from shrimp hatchery

Phage	Host	Plaque morphology	Morphology measurements (nm)			Genome size (kb)	Efficiency of transduction* (PFU ⁻¹)
			Head diameter (SD)	Tail length	Tail width		
φVh1	Vh01	Pinpoint, clear centered, and irregular margin	80 (±7)	170	15 (±1)	85	4.7 × 10 ⁻⁹
φVh2	Vh51	0.5–2 mm, clear with irregular margin	60 (±12)	130	14 (±1)	57	2 × 10 ⁻⁹
φVh3	Vh102	1–2 mm, clear centered, and irregular margin	72 (±5)	27	12 (±1)	64	2.3 × 10 ⁻⁹
φVh4	Vh105	Pinpoint, clear centered with turbid halo and irregular margin	115 (±4)	329	17 (±3)	107	4.1 × 10 ⁻⁷

*Plasmid phenotype: Chr^r, LacZ⁺; donor strain: Vh 57.

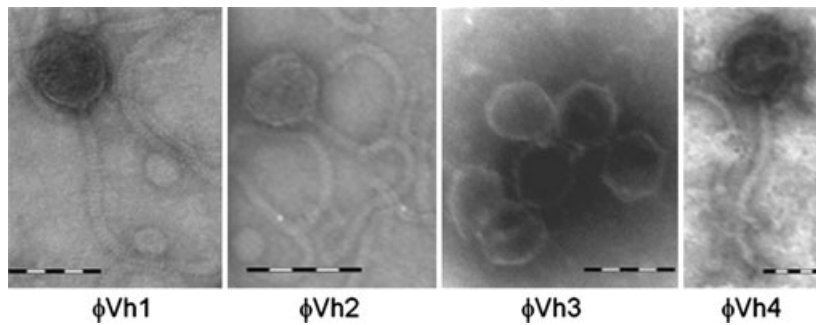


Fig. 1. Electron micrographs of phages ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 of *Vibrio harveyi*.

a short tail (27×12 nm) and was assigned to the family *Podoviridae* (Ackermann, 2005).

Host range

Of a total of 125 isolates tested, it was found that 98%, 78%, 84%, and 96% of *V. harveyi* isolates were susceptible to ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4, respectively. In addition to being able to infect *V. harveyi*, ϕ Vh1, ϕ Vh2, and ϕ Vh3 could also infect other vibrio species such as *V. parahaemolyticus*, *V. alginolyticus*, and *V. logei*, while ϕ Vh4 was found to be specific to *V. harveyi*.

Analysis of nucleic acid and REA

The nucleic acid of all four phages could be completely digested on treatment with DNase I but not with RNase A and S1 nuclease, confirming that the genetic material of the bacteriophages was double-stranded DNA. The enzymes XbaI, DraI, and HindIII were able to splice the phage genomic DNA resulting in 5–12 fragments of various lengths ranging from 818 to 56 818 bp (Fig. 2). The REA patterns of four phages with DraI, HindIII, and XbaI showed different banding patterns, indicating that these phages were distinct from each other. The genomes of all phages were resistant to EcoRI and EcoRV except ϕ Vh4. BamHI, BglII, HaeII, KpnI, NcoI, NotI, PstI, and SmaI did not digest any of the four bacteriophage DNA preparations.

Pulsed-field gel electrophoresis

Among the 12 restriction enzymes used, only XbaI and ScaI produced distinct PFGE profiles. Although the genomic DNA of the four phages had restriction sites for DraI and HindIII, their fragments could not be resolved in PFGE, which showed only streak. PFGE of ScaI produced 13, 12, 13, and 12 fragments of ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 DNA, respectively. The size of DNA fragments ranged from 1030 to 19 937, 1000 to 11 247, 521 to 21 735, and 380 to 31 103 bp in the four phages, ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4, respectively. XbaI produced more number of fragments (13, 12, 16, and 18) ranging from 492 to 28 279, 1034 to 11 254, 458 to 11 331, and 224 to 39 618 bp of the four phages, respectively (Fig. 3).

The genome size based on PFGE profiles generated with ScaI and XbaI showed little variation (0.8–3.3 kb) with the two enzymes, and the genome size of each phage was calculated as an average of the two profiles. The estimated genome size of the four phages was 85, 58, 64, and 107 kb corresponding to ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4, respectively.

Phylogenetic analysis

The phylogenetic analysis of phages based on DraI REA pattern showed distinct nature of phage ϕ Vh3, which separated from the cluster of the other three siphoviruses

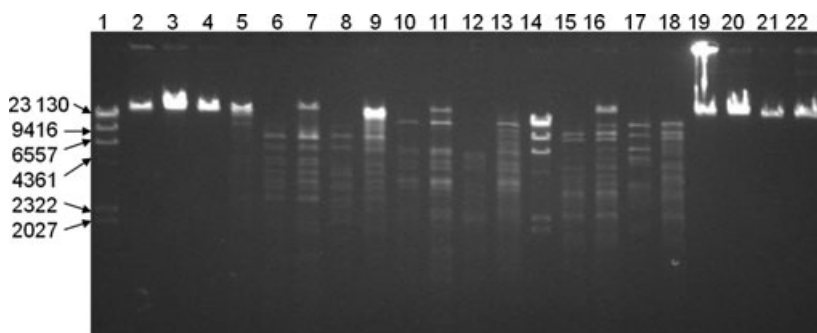


Fig. 2. Restriction enzyme digestion pattern of bacteriophages ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 of *Vibrio harveyi*: lane 1 and 14: λ DNA Hind III digest marker; lanes 2–5: EcoRI digest; lane 6–9: XbaI digest; lanes 10–13: DraI digest; lanes 15–18: Hind III digest; lanes 19–22: PstI digest of the ϕ Vh1– ϕ Vh4 genomic DNA, respectively.

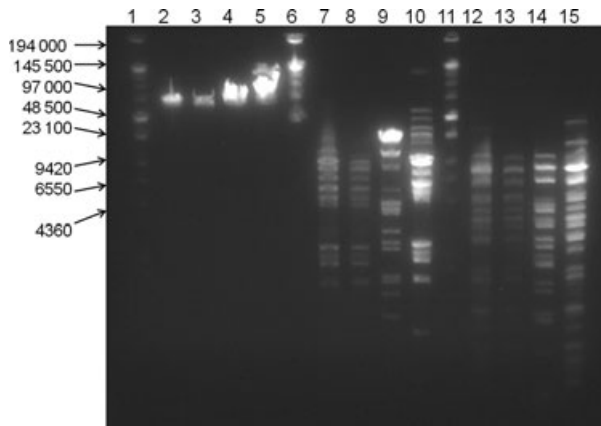


Fig. 3. PFGE of bacteriophages of *Vibrio harveyi*: lane 1, 6, and 11: MW marker; lanes 2–5: uncut genomic DNA of ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4; lanes 7–10: genomic DNA of ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 cut with *ScaI*; lanes 11–15: genomic DNA of ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4 cut with *XbaI*.

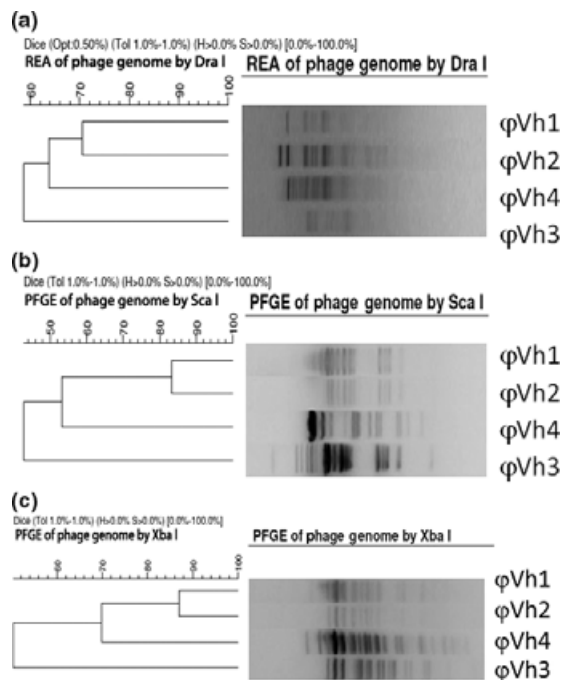


Fig. 4. Phylogenetic analysis of bacteriophages of *Vibrio harveyi* based on *DraI* restriction digest of genomic DNA (a) and PFGE of *ScaI* and *XbaI* digest of genomic DNA (b and c).

at 63% hierarchical level (Fig. 4a). Similarly, the phylogenetic analysis based on PFGE upon restriction with *ScaI* and *XbaI* revealed that the phage ϕ Vh3 was distinct and did not cluster with other three siphoviruses as observed in the cluster analysis of *DraI* REA (Fig. 4b and c). Among the three siphoviruses, phage ϕ Vh4 was distinct

from the other two phages, which branched separately at 56% and 70% hierarchical level in the *ScaI* and *XbaI* PFGE dendrograms, respectively. Phages ϕ Vh1 and ϕ Vh2 showed clustering at 83% and 86% hierarchical level with *ScaI* and *XbaI*, respectively, suggesting their similarity.

Transduction

Vibrio harveyi Vh57 susceptible to all the four phages was successfully transformed with the plasmid DNA (pHSG396). The transformants harboring the plasmid produced blue colonies on PYSS agar supplemented with chloramphenicol, Xgal, and IPTG. The transductants obtained after infection of plasmid transformed donor strain with the four phages grew on PYSS medium supplemented with chloramphenicol producing blue colonies as they acquired the plasmid PHSG 396 DNA. The frequency of transduction of four phages ranged from 4.1×10^{-7} to 2×10^{-9} PFU⁻¹ (Table 1).

Discussion

So far, 227 tailed phages infecting *Vibrio* spp. have been described, among which 67 belonged to the family *Siphoviridae* (Ackermann, 2007). In this study, three phages (ϕ Vh1, ϕ Vh2, and ϕ Vh4) with a long noncontractile tail (130–329 nm long) and an isometric head (approximately 60–115 nm in diameter) belonged to the family *Siphoviridae* and resemble the phages described earlier (Pasharawipas *et al.*, 2005; Vinod *et al.*, 2006; Shivu *et al.*, 2007). One phage, ϕ Vh3, belonged to the family *Podoviridae* according to criteria of head, tail, and genetic material (Ackermann, 2001). According to Ackermann, capsid and tail size of tailed phages range from 30 to 160 and 10 to 800 nm, respectively (Ackermann, 2005). Reports on the isolation of bacteriophages belonging to the family *Podoviridae* from the aquaculture ecosystems are scanty. A member of this group infecting *V. vulnificus* isolated from Gulf Coast oysters has been earlier described (DePaola *et al.*, 1998).

The genome size of the bacteriophages (ϕ Vh1, ϕ Vh2, ϕ Vh3, and ϕ Vh4) based on PFGE was minimal (0.8–3.2 kb) and was estimated to be 85, 58, 64, and 107 kb, respectively, by PFGE. The genome size of the members of the family *Siphoviridae* is reported to range from 14.5 kb in *Lactococcus* prophage bIL311 to 134.4 kb in *Bacillus* phage SPBc2 (<http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10699>). The genome size of the VHS1 *Siphoviridae* phage of *V. harveyi* described earlier was approximately 80 kb (Pasharawipas *et al.*, 2005), and six of them described by Shivu and others had genome sizes ranging from 44 to 94 kb as determined by REA (Shivu *et al.*, 2007). The phylogenetic analysis showed that the four bacteriophages were distinct

from one another as revealed by cluster analysis. The clustering pattern based on both REA and PFGE showed distinct genetic nature of ϕ Vh3.

A marine phage capable of specifically transducing the tryptophan region was described almost three and a half decades back (Keynan *et al.*, 1974). In the present study, all the four bacteriophages were capable of transducing the plasmid DNA between *V. harveyi* with a transduction frequency ranging from 4.1×10^{-7} to 2×10^{-9} PFU⁻¹. A similar efficiency was reported with indigenous marine phage host isolates in an earlier report (Jiang & Paul, 1998). It has been demonstrated that the vibriophages in the coastal environment transfer genes from O1 El Tor strain to non-O1/O139 through transduction, suggesting the process as one of the mechanisms of pathogenicity evolution among environmental *V. cholerae* (Choi *et al.*, 2010). Possibilities of genetic interaction among the bacteriophage genomes and chromosomal and plasmid-borne DNA of vibrios such as *Vibrio parahaemolyticus* strains and of genetic transmission among strains through filamentous phages have been suggested (Chang *et al.*, 1998). The use of a wide variety of antibiotics in aquaculture has resulted in the emergence of antibiotic-resistant bacteria in aquaculture environments (Cabello, 2006). The abundant occurrence of bacteria along with their bacteriophages in seawater and aquatic sediments is known to facilitate such a transfer (Fuhrman, 1999).

In conclusion, results from this study provide description of three bacteriophages of the family *Siphoviridae* and one of the family *Podoviridae*. Literature search shows that the latter group of bacteriophages has not been reported from the shrimp aquaculture ecosystem so far. The significance of the present study is that these bacteriophages were able to bring about generalized transduction and can transfer genetic elements such as antibiotic resistance or pathogenicity traits among *V. harveyi* and possibly in other vibrio species in the brackishwater aquaculture ecosystem.

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