

Full Length Research Paper

Isolation and partial characterization of a bacteriophage infecting the shrimp pathogen *Vibrio harveyi*

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A bacteriophage infecting *Vibrio harveyi* was isolated from shrimp pond water, it was designated PW2. It infected all strains of *V. harveyi* but not other bacteria used in this study. The phage adsorption rate increased rapidly in the first 15 min of infection to 80% and continued to increase to 90% within 30 min of infection. The stability of phage PW2 was dependent on temperature and pH. It was inactivated by heating at 90°C for 30 min and by treating at pH 2, 3, 11 and 12. From its one step growth curve, latent and burst periods were 30 and 120 min, respectively with a burst size of about 78 plaque-forming unit (pfu) per infected center. As analyzed by transmission electron microscopy, phage PW2 had an icosahedral head (50 ± 3.8 nm in diameter) with a noncontractile tail of 136 ± 6.2 nm long and 11 ± 0.5 nm wide and belongs to the *Siphoviridae* family. Six structural proteins (75, 60, 35, 30, 20 and 15 kDa) were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis, its genome was found to be double stranded DNA with an approximate size of 46 kb.

Key words: Bacteriophage, shrimp, *Vibrio harveyi*.

INTRODUCTION

Vibrio harveyi is a gram-negative bioluminescent bacterium with curved rod shape. It is motile via a single polar flagellum. The bacterium has been recognized as a cause of luminous vibriosis in cultivated black tiger shrimp (*Penaeus monodon*) and has resulted in major losses to shrimp farmers in Thailand and elsewhere (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994; Manefield et al., 2000). The symptoms of luminous vibriosis include loss of appetite, slow growth, high mortality and luminescence of the bodies of infected shrimps (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994; Karunasagar et al., 1994). Currently, antibiotics have been widely used in shrimp aquaculture to control bacterial infections (Baticados and Paclibare, 1992). However, the use of antibiotics has recently become a major public concern because their use can lead to the development of drug resistant bacteria, thereby reducing drug efficacy. Moreover, the accumulation of antibiotics

both in the environment and in shrimp tissues can be potentially risky to consumers and the environment (Alderman and Hastings 1998). Such adverse effects have prompted scientists to search for alternatives to replace antibiotics in controlling diseases in shrimp farms. One of the potential alternatives is bacteriophage based approach.

Bacteriophages or phages are viruses that specifically infect and lyse bacteria. A method using phages for the treatment of bacterial infectious disease is called bacteriophage therapy or phage therapy. Recently, phage therapy has gained an increasing attention because it has many advantages over chemotherapy. Phages have high specificity for their target bacteria, indicating that they do not harm the normal intestinal microflora. Phages are effective against multidrug resistant pathogenic bacteria because the mechanisms by which they induce bacteriolysis differ completely from those antibiotics. Moreover, phages have self limitation, meaning that the number of phages remain in very low level after killing the target bacteria (Nakai and Park, 2002).

Successful uses of phage therapy to control bacterial infectious diseases have been reported since 1980s. Phages were used for both treatment and prophylaxis of

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Escherichia coli infections in mice and farmed animals including calves, piglets and lambs (Smith and Huggins, 1982; Smith and Huggins, 1983; Smith et al., 1987). Polish and Soviet groups reported a series of successful clinical usages of phages for drug resistant bacterial infections in human (Slopek et al., 1987; Alisky et al., 1998). Much of the recent research has focused on using phages to control diseases caused by a variety of human pathogenic bacteria including *Salmonella* (Goode et al., 2003), *Listeria* (Leverentz et al., 2004) and *Campylobacter* (Atterbury et al., 2003) species. However, in addition to the current attempts to apply phages in the control of human pathogens, aquatic animal pathogens have also been investigated as a target for phage therapy. A number of phages have been isolated for potential use in phage therapy against important aquatic animal pathogens such as *Aeromonas salmonicida* in brook trout (*Oncorhynchus fontinalis*) (Imbeault et al., 2006), *Vibrio harveyi* in shrimp (*Penaeus monodon*) (Karunasagar et al., 1994; Vinod et al., 2006; Shivu et al., 2007), *Pseudomonas plecoglossicida* in ayu (*Plecoglossus altivelis*) (Park et al., 2000; Nakai and Park, 2002) and *Lactococcus garvieae* in yellowtail (*Seriola quinqueradiata*) (Nakai et al., 1999).

The application of phage to control a certain bacterial pathogen is complicated by the high degrees of phenotypic and genotypic diversity within populations of both phages and bacteria. Consequently, newly isolated strains of *V. harveyi* may be more or less susceptible or even resistant to previously discovered *V. harveyi* phages. Therefore, there is still a need to find a phage specific for an individual newly emerged *V. harveyi* strain. The purpose of this study was to isolate and characterize a new lytic phage that infect *V. harveyi* and to investigate its lytic property toward its host bacterium under controlled conditions in the laboratory. The study thus provides useful information for further use of the phage in controlling *V. harveyi* infection in shrimp.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strain used for phage isolation was *V. harveyi* CS101. It was isolated from diseased shrimp obtained from a shrimp pond in Songkhla Province, Thailand in May 2007. The bacteria used in phage host range study are present in Table 1. Growth conditions of the bacteria are also included in Table 1. The bacterial stock cultures were stored as frozen cultures at -80°C in appropriate broth containing 20% glycerol (v/v).

Isolation of phage

Phage was isolated from shrimp pond water obtained from five farms in Songkhla Province, Thailand. 50 ml of pond water was filtered through a 0.45 m-pore-size membrane filter (Pall Life Sciences, MI, USA) and mixed with 50 ml of double strength BHI broth supplemented with 1% NaCl (BHI-N broth) containing the log phase cells of *V. harveyi* CS101 (with the cell concentration of

approximately 10^8 cfu/ml). After 48 h of growth at 25°C, the culture was centrifuged and filtered through a 0.45 m-pore-size membrane filter. The presence of lytic phage in the filtrate was examined by using the double layer method with some modifications (Paterson et al., 1969). 100 l of the filtrate was mixed with 400 l of log phase culture of *V. harveyi* CS101 and incubated at 25°C for 30 min. The mixture was added into a 4.5 ml of molten BHI-N top agar (0.5% agar) which was already cooled down to 50°C, mixed gently and poured into a BHI -N agar plate. The plate was left to stand at room temperature for 30 min to allow the top agar to solidify. The presence of lytic phage in the form of plaques was detected after incubation of the plate at 25°C for 48 h.

Purification of phage

A single plaque was picked with a sterile glass Pasteur pipette and put into a log phase culture of *V. harveyi*. After incubated at 25°C for 48 h, the phage-host mixture was centrifuged at 12,000 x g for 10 min and filtered through a 0.45 m-pore-size membrane filter. The filtrate was subjected to the double layer method as mentioned above. Three repeated rounds of single plaque isolation and re-inoculation were performed. The phage was eluted from the final resulting plate by adding 5 ml of SM buffer (50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM MgSO₄, 0.01% gelatin) on top of the plate and incubated at room temperature for 4 h with shaking. The phage containing buffer retrieved from the plate was centrifuged at 12,000 x g for 10 min and filtered through a 0.45 m-pore-size membrane filter. The resulting filtrate was called phage suspension.

Concentration of phage suspension

When needed, phage suspension was concentrated using the method described by Su et al. (1998). The double layer method was performed as mentioned earlier to obtain confluent lysis on the plate. The phage was eluted with 5 ml of SM buffer at room temperature for 4 h with shaking. The buffer was transferred to 50 ml tube and the bacterial debris was pelleted by centrifuged at 12,000 x g for 10 min. The supernatant was filtered through a 0.45 m-pore-size membrane filter. The filtrate was added with 2 M ZnCl₂ at a ratio of 1:50 (v/v) and incubated at room temperature for 30 min. The phage was pelleted by centrifugation at 15,000 x g for 20 min. The pelleted was resuspended in approximately 1:25 of the original volume in SM buffer.

Determination of phage titer

The phage containing solution was serially diluted in SM buffer. Each dilution was subjected to plaque assay using the double layer method as mentioned earlier. Plaques were counted in the plate containing 50 - 300 plaques and expressed as plaque forming unit per milliliter (pfu/ml).

Examination of phage host range

Bacteria listed in Table 1 were used as indicators for the examination of phage host range by a modified version of the spot test method (Chopin et al., 1976). 100 ml of a log phase culture of each tested bacteria were added into a 4.9 ml of 50°C molten soft agar (0.5% agar), mixed gently and poured into an agar plate. After solidification, 5 l of the phage suspension was spotted on the lawn of bacteria. After the plate was left to stand for 30 min at room temperature, it was incubated at an appropriate temperature for 48 h before checking the presence of a clear zone in the plate which indicated the ability of phage in infecting the tested bacteria.

Table 1. Bacteria and growth conditions used in this study.

Bacterial strains	Growth conditions (°C)	Lysis ^a
<i>V. harveyi</i> CSC101	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 14126	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 15702	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 25919	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 33842	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 43515	BHI-N agar and broth, 25	+/C
<i>V. harveyi</i> ATCC 700104	BHI-N agar and broth, 25	+/C
<i>Vibrio alginolyticus</i> ATCC 14582	BHI-N agar and broth, 25	-
<i>Vibrio alginus</i> ATCC 14390	BHI-N agar and broth, 25	-
<i>Vibrio campbelli</i> ATCC 25920	BHI-N agar and broth, 25	-
<i>Vibrio cholerae</i> ATCC 11558	BHI-N agar and broth, 37	-
<i>Vibrio fischeri</i> ATCC 14546	BHI-N agar and broth, 25	-
<i>Vibrio logei</i> ATCC 15382	BHI-N agar and broth, 18	-
<i>Vibrio marinovulgaris</i> ATCC 14394	BHI-N agar and broth, 26	-
<i>Vibrio natriegens</i> ATCC 14048	BHI-N agar and broth, 25	-
<i>Vibrio nereis</i> ATCC 25917	BHI-N agar and broth, 25	-
<i>Vibrio parahaemolyticus</i> ATCC 17802	BHI-N agar and broth, 37	-
<i>Vibrio salmonicida</i> ATCC 43839	BHI-N agar and broth, 15	-
<i>Vibrio splendidus</i> ATCC 25914	BHI-N agar and broth, 25	-
<i>Vibrio tubiashii</i> ATCC 19105	BHI-N agar and broth, 25	-
<i>Aeromonas hydrophila</i> ATCC 7966	BHI agar and broth, 2	-
<i>Campylobacter fetus</i> ATCC 19438	BHI agar and broth, 37	-
<i>Campylobacter jejuni</i> ATCC 700819	BHI agar and broth, 37	-
<i>Escherichia coli</i> ATCC 15922	Nutrient agar and broth, 37	-
<i>Lactococcus garvieae</i> ATCC 49156	MRS agar and broth, 37	-
<i>Listonella anguillarum</i> ATCC 43310	Marine agar and broth, 20	-
<i>Moritella marina</i> ATCC 15381	Marine agar and broth, 18	-
<i>Photobacterium damsela</i> ATCC 33538	Marine agar and broth, 25	-
<i>Proteus vulgaris</i> ATCC 27968	Nutrient agar and broth, 37	-
<i>Pseudomonas anguilliseptica</i> ATCC 33660	BHI agar and broth, 20	-
<i>Pseudomonas fluorescens</i> ATCC 6972	Nutrient agar and broth, 37	-
<i>Salmonella enterica</i> ATCC 33059	Nutrient agar and broth, 37	-
<i>Streptococcus iniae</i> ATCC 29177	MRS agar and broth, 37	-
<i>Streptococcus phoecae</i> ATCC 51973	MRS agar and broth, 37	-

^a + = lysis; - = no lysis; C = clear plaque, BHI, brain Heart Infusion; BHI-N, Brain Heart Infusion supplemented with 1% NaCl; MRS, de Man Rogosa and Sharpe.

Thermal sensitivity test

900 l of sterile deionized water was preheated to a desirable temperature, ranging from 50 to 90°C. 100 l of phage solution (approximately 10⁷ pfu/ml) was added to the preheated water. After heating at the assigned temperatures for 30 min, the solutions were placed in an ice-warm bath. Surviving phage titer was assayed by the double layer method.

pH sensitivity test

The phage (at the final concentration of 10⁶ pfu/ml) was incubated overnight at 25°C in phosphate buffered saline (135 mM NaCl, 1.3 mM KCl, 0.5 mM KH₂PO₄, 3.2 mM Na₂HPO₄, pH 7.4) adjusted in

steps of 1 pH unit from pH 2 to 12 using HCl or NaCl as required. Upon re-adjustment to pH 7, the double layer method was performed to determine phage titer.

Phage adsorption study

Phage adsorption was studied using method described by Sechaurd et al. (1989) modified as follows: log phase growing *V. harveyi* culture in BHI -N broth was centrifuged and the cells were resuspended in BHI-N to a final concentration of 10⁸ cfu/ml. Phage was added at a multiplicity of infection (MOI) of 0.01, and the mixture was incubated at 25°C. Samples were collected every minute during a total period of 15 min. The samples were centrifuged at 12,000 x g for 10 min to sediment the phage-

adsorbed cells. Then, the titers of unadsorbed free phages in the supernatant were determined as indicated above and the results were expressed as percentages of the initial phage counts.

One step growth curve

One-step growth curve was performed as described by Pajunen et al. (2000) with some modifications. Briefly, 10 ml of log phase culture of *V. harveyi* was harvested by centrifugation at 12,000 x g for 10 min and resuspended in 5 ml of fresh BHI-N broth in order to obtain a final concentration of 10^8 cfu/ml. To this suspension, 5 ml of phage suspension was added in order to have a MOI of 0.01 and the phage was allowed to adsorb for 30 min at 25°C. The mixture was then centrifuged at 12,000 x g for 10 min and the pellet was resuspended in 10 ml of fresh BHI-N broth. Samples were taken every 5 min over a period of 3 h and immediately tittered by the method described earlier. Latent period, burst time and burst size were calculated from the one-step growth curve.

Electron microscopy

Phage suspension with a titer of 10^8 pfu/ml was mixed with one-tenth volume of 2.5% buffered glutaraldehyde (50 mM cacodylate pH 7.2, 50 mM KCl, 2.5 mM $MgCl_2$) for 5 min. 25 μ l of fixed phage was added to the surface of a pioloform coated grid and left for 2 min. The phage was negatively stained with 0.5% uranyl acetate for 2 min and then inspected with a Philips EM 300 electron microscope (Philips, Eindhoven, the Netherlands) operated at 60 kV.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970). Briefly 24 l of phage suspension was mixed with 8 l of 4 x Laemmli buffer (200 mM tris pH 6.8, 400 mM 1, 4-dithiothriitol, 20% - mercaptoethanol, 8% SDS, 40% glycerol, 0.1% bromophenol blue) and boiled for 10 min. The mixture then subjected to electrophoresis on a 4 - 12% Bis-Tris polyacrylamide gel at 200 V with a constant current of 100 mA for 45 min. The protein bands were stained with Coomassie blue G-250 (Sigma-Aldrich, MO, USA), followed by destaining with a destain solution (50% methanol and 10% acetic acid).

Phage genome isolation and digestion analyses

Phage genome was isolated using a PureLink Viral RNA/DNA Mini Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The genome was digested with the following enzymes: RNase A, S1 nuclease, *Pst*I, and *Bam*HI (all from Promega, WI, USA) according to the instructions provided by the manufacturer. Uncut and digested nucleic acid was analyzed by agarose gel electrophoresis on 0.8% (w/v) agarose gel in Tris-acetate buffer at 100 V and visualized on Hoefer MacroVue UVis-20 transilluminator (Amersham Bioscience, NJ, USA) after stained with ethidium bromide solution (0.5 μ g/ml, Sigma-Aldrich, MO, USA).

RESULTS

Isolation of phage

By the double layer method using *V. harveyi* CS101 as a

host organism, a phage was isolated from a shrimp pond water sample. The phage produced clear plaques on the lawn of the host, indicating that it was a lytic or virulent phage. The plaques were small with an average diameter of 14 mm. The isolated phage was designated PW2.

Host range determination

Of all 34 strains of bacteria used to determine the host range of phage PW2 by using the spot test method, only *V. harveyi* strains were susceptible to the phage as shown in Table 1. The other bacterial strains used in this study were insensitive to the phage. These results suggested that the phage had a broad host range with specificity to *V. harveyi*.

Heat and pH stability

Phage PW2 was stable upon heat treatment at 50°C for 30 min. The temperature dependent decrease of phage titer was observed when temperature used for the heat treatment was 60°C and above. The phage titer was reduced by 50, 70, and 90% at 60, 70 and 80°C respectively. No phage was detected at 90°C (Figure 1).

Phage PW2 maintained its infectivity when incubated overnight at 25°C in a pH range between 4 and 10. However, phage could not be detected at pH 2, 3 and 11 (Figure 2).

Phage adsorption

Phage PW2 had 2 adsorption phases: a very rapid adsorption to its host cells during the first 15 min is followed by a slower rate of adsorption after 15 min. The number of free (unadsorbed) phage particles was approximately 20% and below 10% within 15 and 30 min after infection, respectively (Figure 3).

One-step growth curve

Multiplication parameters of the lytic cycle of phage PW2 were determined from the one-step growth curve (Figure 4). The latent and burst periods were 30 and 120 min, respectively and the burst size was estimated at approximately 78 pfu per infected cell.

Morphology of phage

Morphological characterization of phage PW2 using transmission electron microscope showed that it had an icosahedral head (50 ± 3.8 nm in diameter) with a non contractile tail of 136 ± 6.2 nm long and 11 ± 0.5 nm

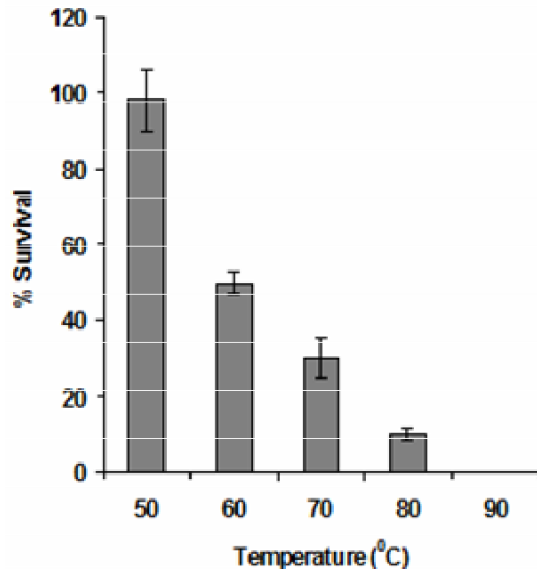


Figure 1. Stability of phage PW2 treated with different temperature for 30 min. Values are the means of 3 determinations.

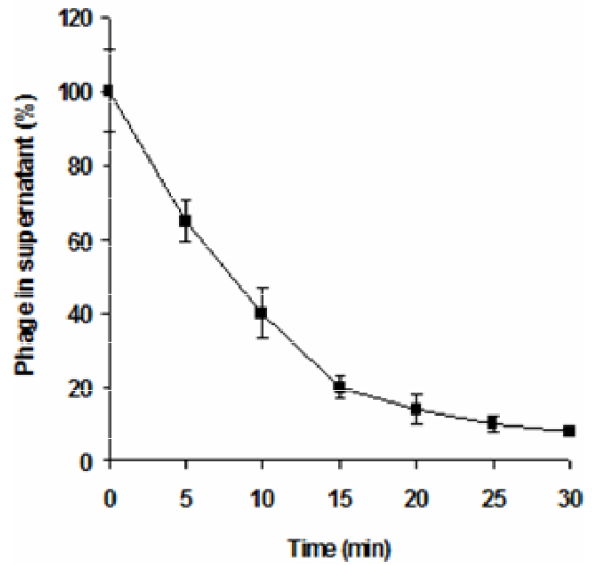


Figure 3. Adsorption of phage PW2 on cells of *V. harveyi* CS101. Values are the means of 3 determinations.

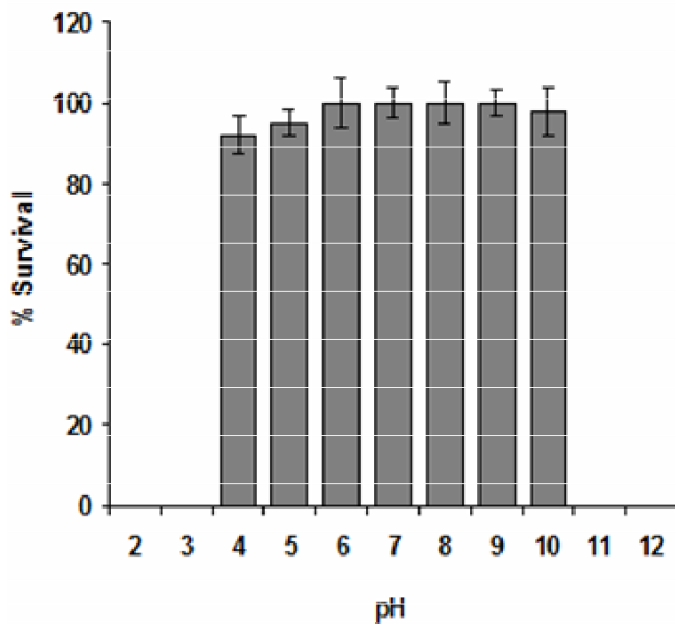


Figure 2. Stability of phage PW2 treated with different pH overnight at 25°C. Values are the means of 3 determinations.

wide. No collar or tail appendages was observed (Figure 5). All values were determined as means \pm SD from 5 measurements.

Phage structural proteins

Protein composition of phage PW2 was analyzed by

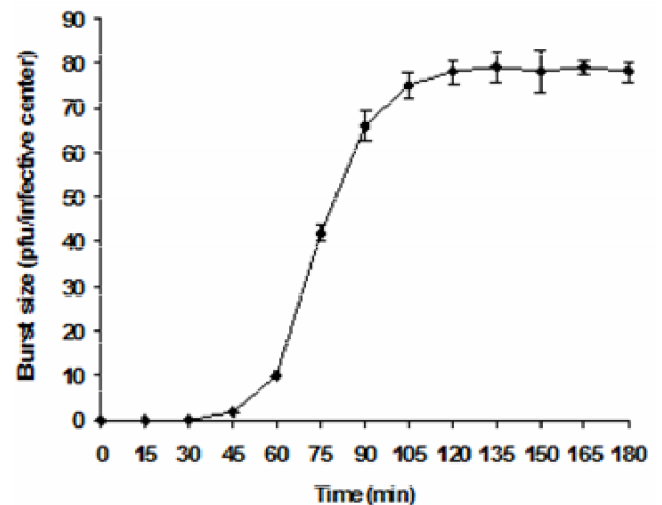


Figure 4. One step growth curve of phage PW2 on *V. harveyi* CS101. Values are the means of 3 determinations.

SDS-PAGE. A total of 6 protein bands were clearly seen in the polyacrylamide gel. Their estimated molecular weights were 75, 60, 35, 30, 20 and 15 kDa (Figure 6).

Phage genome

Nucleic acid of phage PW2 was isolated and subjected to enzymatic digestion analysis. It was not digested with RNase A and S1 nuclease (data not shown). However, it was digested with both *Pst*I and *Bam*HI. The genome size estimated from *Pst*I and *Bam*HI digested fragments was approximately 46 kb (Figure 7).

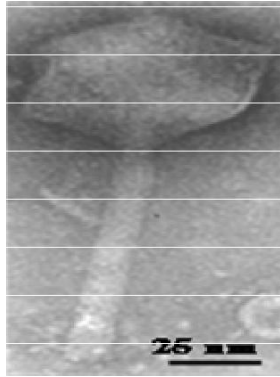


Figure 5. Scanning electron micrograph of phage PW2.

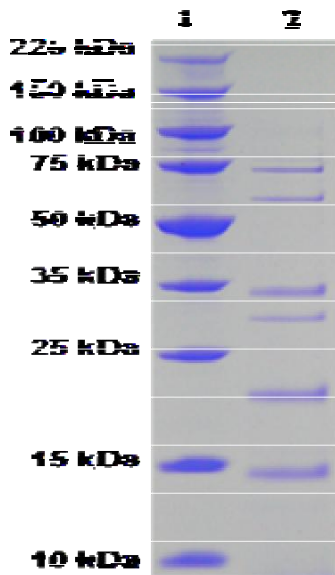


Figure 6. SDS-PAGE analysis of phage PW2 structural proteins. Lane 1, broad range protein molecular weight markers (Promega, WI, USA); Lane 2, phage PW2 proteins.

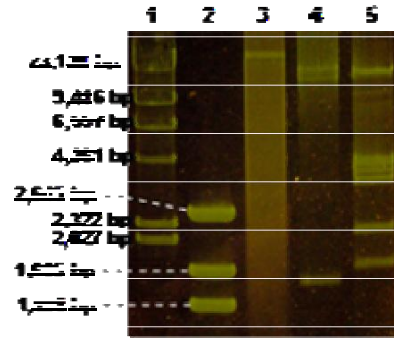


Figure 7. Restriction analysis of phage PW2 DNA. Lane 1, *Hind*III digested lambda DNA (Promega, WI, USA); Lane 2, pGem marker (Promega, WI, USA); Lane 3, uncut PW2 DNA; Lane 4, PW2 DNA cut with *Pst*I, Lane 5; PW2 DNA cut with *Bam*HI.

phage so attractive is their highly discriminatory nature. Most of the known phages interact only with a specific set of bacteria that express specific binding sites. This narrow host range is also a significant challenge for phage therapy. Consequently, there is no known phage that is lytic for all strains of *V. harveyi*. This high specificity of phage-host relationship leads to a need for phages to inhibit newly isolated *V. harveyi*. Phages are generally isolated from environments that are habitats for the respective host bacteria (Nakai and Park, 2002). Since *V. harveyi* used as a main host in phage screening isolated from shrimp farm environment, shrimp pond water would be ideal for isolation of *V. harveyi* phages. For searching phages to use in phage therapy, phages with broad host range are more preferable than those with narrow host range because they can inhibit several strains of bacterial pathogens (Nakai and Park, 2002). In this study, phage PW2 isolated from shrimp pond water had a broad host range infecting all of the tested strains of *V. harveyi*. This characteristic of the phage makes it useful for use individually or in combination with other *V. harveyi* phages as phage cocktails to control *V. harveyi* infection.

As a tailed virus of bacteria, PW2 phage fell into the Order Caudovirales that contains three families of tailed viruses that infect Bacteria and Archaea (van Regenmortel et al., 2000). Possession of an icosahedral head and a long, non contractile tail would tentatively place it in the family Siphoviridae (van Regenmortel et al., 2000). The genome of PW2 phage was found to be double stranded DNA because it was digested with restriction endonucleases but not with RNase A and S1 nuclease. The size of genome estimated by *Pst*I and *Bam*HI digestion analysis was approximately 46 kb. This was close to the genome size of most siphophages ranging from 40 to 50 kb. From previous studies, most of the *V. harveyi* phages were found to be siphophages with double stranded DNA (Pasharawipas et al., 2005; Vinod

DISCUSSION

Bacterial diseases are a major problem affecting shrimp farming and most of the mass mortalities reported in shrimp are associated with luminous disease caused by *V. harveyi* (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994; Karunasagar et al., 1994). Till recently, antibiotics were widely used in shrimp aquaculture to control bacterial infections. However, emergence of antibiotic resistant pathogens and awareness of adverse effects of antibiotics has led to a need for alternatives to antibiotics in shrimp aquaculture.

Phage therapy is one of the promising approaches to control bacterial infections. One feature that makes

et al., 2006; Karunasagar et al., 2007). However, *V. harveyi* phages in other families such as Myoviridae and Podoviridae were also reported (Oakey and Owens, 2000; Oakey et al., 2002; Busico-Salcedo, 2004; Shivu et al., 2007). Several studies documented that thermal and pH stability of phages varied depending on strains of phage. Therefore, it is of interest to investigate the stability of phage PW2 in a wide range of temperatures and pH. This study showed that the phage was tolerant to relatively high temperature ranging from 50 to 80°C and a broad range of pH. These characteristics may be useful for the application of the phage in different environments.

In conclusion, we have isolated and partially characterized phage PW2, a phage infecting *V. harveyi* CS101. The results from this study provide preliminary data useful for designing a rational phage control strategy for *V. harveyi* infection in shrimp aquaculture. However, further characterization of phage PW2 is underway in our laboratory to obtain more information for development of the most effective approach for controlling *V. harveyi* infection.

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