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ORIGINAL ARTICLE

Isolation and characterization of a novel lytic cold-active bacteriophage VNPH-1 from the Napahai wetland in China

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Abstract Wetlands have unique characteristics and play a number of roles in the environment, principally in water purification, flood control, and the maintenance of shoreline stability. In this work, a lytic cold-active bacteriophage designated VNPH-1 was isolated from the Napahai wetland in China together with *Aeromonas sobria* NPH-1 cells, and a preliminary characterization of this bacteriophage was carried out. Electron microscopy revealed that VNPH-1 had an icosahedral head (116.7 nm) and a contractile tail (10 nm in width, 166.7 nm in length). Bacteriophage VNPH-1 was classified as *Myoviridae* and had an approximate genome size of 110 to 120 kb. One-step growth curve revealed that the latent and burst periods were 20 and 10 min, respectively, with an average burst size of 80 bacteriophage particles per infected cell. The pH and thermal stability of bacteriophage VNPH-1 were also investigated. The maximum stability of the bacteriophage was observed at an optimal pH of 9.0, and the phage was comparatively stable at pH 5.0-10.0. The specificity of this bacteriophage for its host makes it an attractive candidate for phage therapy of *A. sobria* infections. As VNPH-1 is a cold-active bacteriophage with a low production temperature, it would be worthwhile to characterize it further and to deepen knowledge of its interaction with *A. sobria* in future studies.

Keywords Cold-active phage · *Aeromonas sobria* · *Myoviridae* · Characterization

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Introduction

A large part of the earth's surface is occupied by low-temperature environments, including the Antarctic, alpine regions, and oceans. Even under such harsh conditions, many cold-adapted microorganisms can grow at 15 °C or lower. Viruses are the most abundant lifeforms on Earth (Breitbart and Rohwer 2005). They are reservoirs of genetic diversity and play important roles in regulating the structure of microbial communities and global biogeochemical cycles (Suttle 2005; Danovaro et al. 2008; Rohwer and Thurber 2009). Bacteriophages (phages), the viruses that infect and proliferate in bacteria, play a significant role in lake and ocean ecosystems (Danovaro et al. 2008; Sawstrom et al. 2008a, b). Despite the prevalence on Earth of environments≤4 °C and the high abundance of phages (an estimated 10^{31} phage particles) in them, little is known about the characteristics of those phages, as only approximately 300 phages have been characterized (Casjens 2008). Cold-active phages were capable of infection and replication at temperatures≤4 °C (Rex et al. 2006).

Phages are classified into 13 families by the International Committee on Taxonomy of Viruses (ICTV) according to their morphology and the type of nucleic acid they contain. These families are: *Podoviridae*, *Siphoviridae*, *Myoviridae*, *Tectiviridae*, *Corticoviridae*, *Lipothrixviridae*, *Rudiviridae*, *Fuselloviridae*, *Guttaviridae*, *Inoviridae*, *Microviridae*, *Cystoviridae*, and *Leviviridae*. All of them have a DNA genome, except for *Microviridae* and *Cystoviridae* that are RNA phages.

Phages replicate via either a lytic cycle alone or a combination of lytic and lysogenic cycles. Bacterial cells are lysed after replication of the virion in the lytic cycle. In contrast, the lysogenic cycle does not result in immediate lysing of the host cell. Lytic phages are able to kill their bacterial hosts without harming their human or animal hosts and are thus more suitable for phage therapy.

Powerful antibiotics have been used to treat infections caused by bacteria; however, with the emergence of multi-drug-resistant bacteria, phage therapy has been the subject of renewed interest (Hermoso et al. 2007). Phages have characteristically high specificity for their bacterial hosts (Li et al. 2012), targeting only one or a few strains of bacteria, while antibiotics have broader effects, killing both harmful and useful bacteria. The specificity exhibited by phages could reduce the chance that useful bacteria would be killed when fighting an infection, and may result in no side effects. Phage therapy research is again attracting attention; until recently, it was considered an alternative method of fighting bacterial infection (Imbeault et al. 2006).

Aeromonas is a Gram-negative, facultative anaerobic, rod-shaped bacterium. It is ubiquitous in fresh and brackish water, food, and soil. Most *Aeromonas* species have been associated with human diseases; among the most common responsible pathogens are *A. hydrophila*, *A. caviae*, and *A. veronii* biovar *sobria. A. sobria* is the primary microflora found in fish. Among approximately 5,600 known viruses, 33 *Myoviridae*, seven *Siphoviridae*, three *Podoviridae*, and 43 tailed bacteriophages have been observed to infect *Aeromonas* (Ackermann 2007). Although some phages that infect *Aeromonas* have been isolated, very few cold-active phages that infect *A. sobria* have been isolated and characterized in detail. Thus, screening to identify phages that infect *A. sobria* would facilitate the potential use of phage therapy to treat infections with this species of bacteria.

Wetlands are unique ecological systems that play a number of roles in the environment, principally in water purification, flood control, and the maintenance of shoreline stability. Because of the high numbers of species, high productivity, and rich biodiversity present in wetlands, those ecosystems are becoming a focus of interest. Napahai is a degraded plateau wetland in northwestern Yunnan, China. The average temperature is 4-8 °C. In this study, a new cold-active bacteriophage VNPH-1 specific for the *A. sobria* NPH-1 strain was isolated from the Napahai wetland, and the biological features of this novel bacteriophage were characterized.

Materials and methods

Sampling and bacteriophage isolation

Culture medium: The PYGV medium used in this study was prepared as follows (Atlas 2004): tryptone 10 g/L, yeast extract 5 g/L, Hutner's basal salts solution 20 mL/L, 25 % D-glucose 10 mL/L, 2×vitamin solution 5 mL/L. Hutner's basal salts solution: $MgSO_4 \cdot 7H_2O$ 29.7 g/L, nitrilotriacetic acid 10 g/L, $CaCl_2 \cdot 2H_2O$ 3.35 g/L, $FeSO_4 \cdot 7H_2O$ 99 mg/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 9.25 mg/L, Metals 44 solution 50 mL/L. Metals 44 solution: $ZnSO_4 \cdot 7H_2O$ 10.95 g/L, $FeSO_4 \cdot 7H_2O$ 5 g/L, sodium EDTA 2.5 g/L, $MnSO_4 \cdot H_2O$ 1.54 g/L, $CuSO_4 \cdot 5H_2O$ 392 mg/L, $Co(NO_3)_2 \cdot 6H_2O$ 248 mg/L, $Na_2B_4O_7 \cdot 10H_2O$ 177 mg/L. 2×Vitamin solution: pyridoxine HCl 20 mg/L, *p*-aminobenzoic acid 10 mg/L, calcium DL-pantothenate 10 mg/L, nicotinamide 10 mg/L, riboflavin 10 mg/L, thiamine HCl 10 mg/L, biotin 4 mg/L, folic acid 4 mg/L, vitamin B_{12} 0.2 mg/L. The pH value was adjusted to 6.0. To prepare the medium in solid or semi-solid form, 15 g/L agar or 4 g/L agarose were added to the medium, respectively.

Bacterial strain: Water samples were obtained from the Napahai wetland (E99°37′28″, N27°53′34″, altitude 3, 265 m, 7 °C, and pH 6.5) located in Yunnan province, China. Samples were spread on PYGV solid plates and incubated 2-3 days at 15 °C. Colonies were then picked and purified by streaking on fresh plates. The molecular identification of isolates was confirmed by 16S rRNA gene sequencing. The following PCR primers were used: 27 F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′) (Liu et al. 2006; Lin et al. 2011). A 1.5-kb PCR product was subcloned into a pMD-18 T vector (Takara, Dalian) and sequenced by Beijing Sangon Biological Engineering Technology and Services in China.

Phage isolation: The NPH-1 bacterial strain was used as the host to isolate, propagate, and characterize phages. A water sample (50 mL) collected from the Napahai wetland was pre-incubated with NPH-1 host cell, which was precultured in PYGV broth medium to logarithmic phase growth (5 mL, OD_{600}=0.4-0.5), to enrich the phages. The culture was centrifuged (at 13,000×*g* for 5 min at 4 °C; Beckman Avanti J-25, CA, USA), and the supernatant was filtered through 0.22 μm filters (Millipore Corp., Bedford, MA, USA) after incubation at 15 °C for 10 d.

Phages were isolated by successive single-plaque isolation using the double-layer agar method (Adams 1959). Bacteriophage stocks (100 μL) were mixed with NPH-1 host cell culture (200 μL, OD_{600}=0.4-0.5). After incubation at 15 °C for 10 min, the mixture was added to 4 mL of the semi-solid medium (0.4 % agarose in PYGV liquid medium), mixed gently, and poured onto the PYGV solid plate (Shen et al. 2012a, b). The double-layer plates were incubated right side up at 15 °C overnight to enumerate plaques.

Purification of phage particles

Phages were purified by successive single-plaque isolations (Xiang et al. 2005). Cultures containing 3×10^9 PFU/mL phage particles were centrifuged (at 12,000×*g* for 30 min at 4 °C; Beckman Avanti J-25, CA, USA) to remove cells. DNase I and RNase A (Sigma-Aldrich, St. Louis, MO, USA) were added to the supernatant to a final concentration of 1 μg/mL. The mixture was incubated at 37 °C for 30 min. To precipitate phage particles, PEG 8000 and NaCl were added into the supernatant to concentrations of 10 % (w/v)

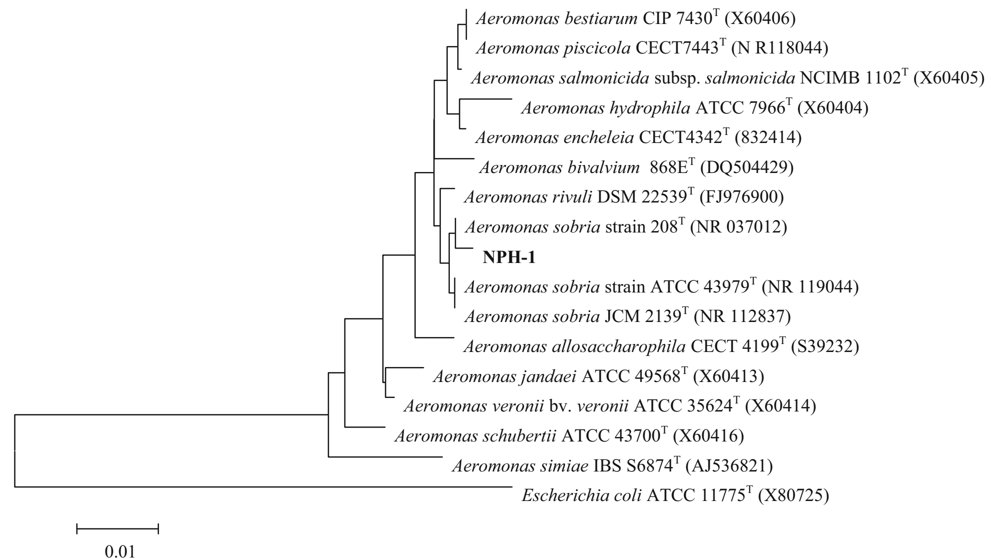

Fig. 1 Phylogenetic analysis of NPH-1 strain based on 16S rRNA gene sequences available from NCBI GenBank database

and 1 M, respectively. After incubating on ice for 24 h, phage particles were pelleted by centrifugation (at 11,000×*g* for 15 min at 4 °C; Beckman Avanti J-25, CA, USA) and then resuspended in SM buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 10 mM $MgSO_4$, 0.01 % gelatin); chloroform was then added to the phage preparations, and the samples were centrifuged (at 11,000×*g* for 15 min at 4 °C; Beckman Avanti J-25, CA, USA). To purify the phage particles further, solid CsCl was added to the supernatant to a concentration of 0.45 g/mL and the samples were ultracentrifuged (at 280,000×*g* for 24 h at 4 °C; SW41 rotor, Beckman LE-80 K, CA, USA). The phage band was collected and dialyzed against SM buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$) at 4 °C. The phage particles were detected using the double-layer agar technique.

Electron microscopy

Phage morphology was examined by TEM of negatively stained preparations. A drop of phage containing approximately 1×10^{10} PFU/mL was applied to the surface of a formvar-coated grid (200 mesh copper grids), negatively stained with 2 % (w/v) uranyl acetate and then examined in a JEM-1230 transmission electron microscope operated at 80 KV (JEOL, Tokyo, Japan).

Adsorption experiments

Phage adsorption experiments were carried out as described (Haq et al. 2012). NPH-1 host cells of (3×10^8 CFU/mL) in PYGV with and without Ca^{2+} were infected with phages at an MOI of 10 and incubated at 15 °C. Aliquots of 100 μL were taken at 0, 10, 20, 30, 40, and 50 min after infection, diluted in 0.9 mL cold PYGV, and centrifuged (at 12,000×*g* for 5 min). The supernatants containing the unadsorbed phages were then titrated using the double-layer agar method.

Characterization of cold-active phage

Determination of the optimal multiplicity of infection (MOI)

The multiplicity of infection was defined as the ratio of virus particles to potential host cells (Lu et al. 2003). NPH-1 host cells of (3×10^8 CFU/mL) were infected with VNPH-1 at six different ratios (0.001, 0.01, 0.1, 1, 10, and 100 PFU/CFU). After the cells were incubated with VNPH-1 for 3.5 h at 28 °C, the phage lysate was centrifuged. The supernatant was then

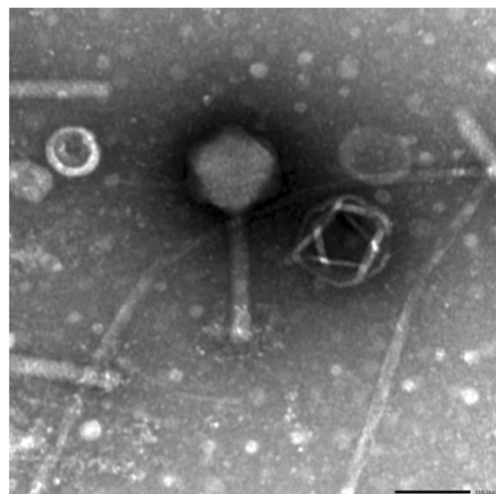

Fig. 2 Transmission electron micrograph of phage VNPH-1. Bar represents 100 nm

Table 1 The optimal multiplicity of infection of VNPH-1

MOI	Phage titer (PFU/mL)	Host cell (CFU/mL)	Phage titer after 3.5 h (PFU/mL)
0.001	1×10^4	1×10^7	1.7×10^4
0.01	1×10^5	1×10^7	7.1×10^4
0.1	1×10^6	1×10^7	1.2×10^6
1	1×10^7	1×10^7	3.4×10^7
10	1×10^8	1×10^7	7.0×10^8
100	1×10^9	1×10^7	8.5×10^7

filtered using a 0.22 μm filter and assayed to determine the phage titer.

One-step growth curve

The phage sample was mixed with 1 mL of a fresh NPH-1 host cell sample (3×10^9 CFU/mL) to obtain an MOI of 10, and the cells were allowed to adsorb the phage for 1 min at 28 °C (Haq et al. 2012). The mixture was centrifuged (at 13, 000× g for 30 s), and the pellets were suspended in 5 mL fresh medium. The cell suspension was then shaken at 28 °C. Phage titer in the culture was measured by the double-layer agar method at 5-min intervals. Plates were incubated at 28 °C overnight to allow the detection of plaques. Assays were carried out in triplicate.

Host range analysis

The host range of the phage was examined using eight bacterial strains (Haq et al. 2012). These strains included *Escherichia coli* ATCC33305, *Acinetobacter* sp. ATCC 9957, *Pseudomonas putida* S-12, *Bacillus cereus* S-8, *Arthrobacter citreus* SW-9, *Flavobacterium* sp. SW-7, *Zoogloea* sp. W-7, and *Stenotrophomonas* sp. SW-10. The latter six strains were isolated from the Napahai wetland. The susceptibility of the bacterial strains to the bacteriophage was tested using the double-layer agar method. Tests were carried out in triplicate.

Thermolability of cold-active phage

To examine the thermolability of the cold-active phage, phage stocks (3×10^9 PFU/mL) were incubated at 40, 50, 60, or 70 °C for 1 h. The rate of survival of each treated phage sample was determined using the double-layer agar technique at 10-min intervals.

pH sensitivity of phage particles

In order to know the pH steady, 10 μL phage stocks (3×10^9 PFU/mL) were added into 0.99 mL PYGV broth medium and incubated for 1 h at room temperature (Haq et al. 2012; Chaudhry et al. 2012). The pH of mixture was adjusted from 3 to 11 with different buffers, including 50 mM citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 9.0), and carbonate buffer (pH 10.0-11.0), respectively. The rates of survival of the phage samples were determined using the double-layer agar technique.

Sensitivity to organic solvent and detergents

To study the sensitivity of the phage to organic solvents and detergents, phage particles were exposed to 1, 2, or 4 % (w/v) chloroform and Triton X-100 (0.3 % w/v) for 10 min at room temperature (Merino et al. 1990; Kim et al. 2012a, b). The number of surviving phages in the supernatant was determined using the double-layer agar technique. Plates were incubated at 15 °C overnight to allow the examination of plaque formation.

Extraction and restriction endonuclease digestion of phage DNA

EDTA, SDS, and proteinase K were added to the CsCl-purified phage suspension to final concentrations of 20 mM, 10 %, and 50 μg/mL, respectively. After incubation at 56 °C for 3 h, the mixture was extracted with phenol/chloroform and ethanol precipitated. The air-dried DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0). The

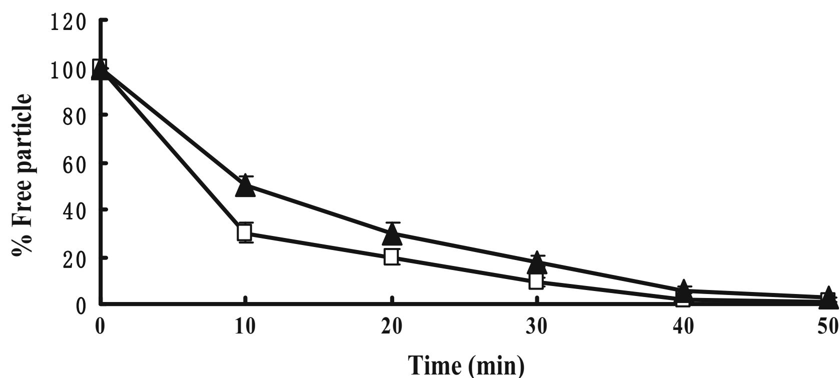

Fig. 3 The adsorption experiences of phage VNPH-1 on cells of NPH-1, in PYGV broth medium with (□) and without (▲) Ca^{2+}. The values are the mean of three determinations

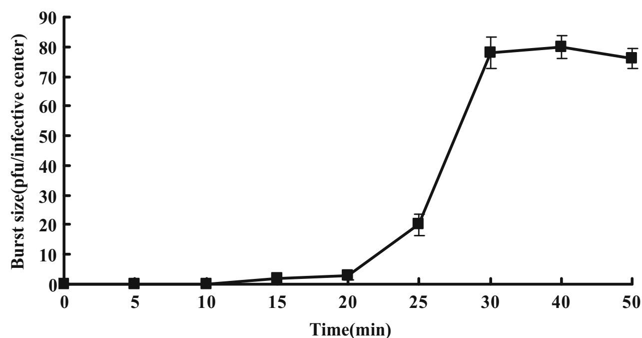

Fig. 4 One-step growth curve of VNPH-1; the values are the mean of three determinations

DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII, and *Xba*I (TaKaRa, Dalian, China).

Protein analysis

CsCl-purified phage particles were mixed with the loading buffer, heated in boiling water for 5 min, and then subjected to SDS-PAGE (12 %). The protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Results

Isolation and morphology

Approximately 170 bacterial strains were isolated from the Napahai wetland. The NPH-1 strain was chosen for further study. Based on the morphology and physiology of NPH-1 and on a phylogenetic analysis of the 16S rRNA gene sequence (Fig. 1), the isolate NPH-1 was identified as a strain of *Aeromonas sobria* and was named *A. sobria* NPH-1. The NPH-1 strain exhibited 99 % similarity to *A. sobria* 208^T (NR_037012). It was found to be a facultative anaerobic, Gram-negative, rod-shaped, non-spore-forming, and psychrophilic bacterium that grows at 4-37 °C, with optimal growth at 10-28 °C.

A lytic phage infecting *A. sobria* NPH-1 was isolated and named VNPH-1. Clear plaques of 1 mm were obtained after incubation of phage at 4 °C for 48 h on a double-layer agar plate. It was, therefore, recognized as a typical cold-active phage. TEM analysis showed that VNPH-1 has an icosahedral head 116.7 nm in diameter and a tail that is 166.7 nm in length and approximately 10 nm in width (Fig. 2), a morphology characteristic of member of the family *Myoviridae*.

Phage production

Temperature range for plaque formation: Following infection with VNPH-1, *A. sobria* NPH-1 was able to produce progeny phage particles when incubated at temperatures ranging from 4 to 28 °C, with optimal phage production at 15-28 °C.

Adsorption experiments: The adsorption rates with or without Ca^{2+} of VNPH-1 to *A. sobria* NPH-1 cells are shown in Fig. 3. A slight influence of Ca^{2+} was observed. The adsorption rates with or without Ca^{2+} reached almost 99 % after 40 min and 50 min, respectively.

MOI: The optimal MOI of VNPH-1 was determined to be 10 (Table 1).

One-step growth curve revealed that the latent and burst periods were 20 and 10 min, respectively, with an average burst size of 80 bacteriophage particles per infected cell (Fig. 4). The latent period was similar to that observed for *Aeromonas punctata* bacteriophage IHQ1 (Haq et al. 2012).

Host range analysis: The effects of phage VNPH-1 on non-host bacteria were tested to determine the host range of VNPH-1. Eight bacterial strains, viz., *Escherichia coli* ATCC33305, *Acinetobacter* sp. ATCC 9957, *Pseudomonas putida* S-12, *Bacillus cereus* S-8, *Arthrobacter citreus* SW-9, *Flavobacterium* sp. SW-7, *Zoogloea* sp. W-7, and *Stenotrophomonas* sp. SW-10 were tested with the VNPH-1 phage. None of these strains was found to be susceptible to the phage. This result indicates that the VNPH-1 phage has a narrow host range.

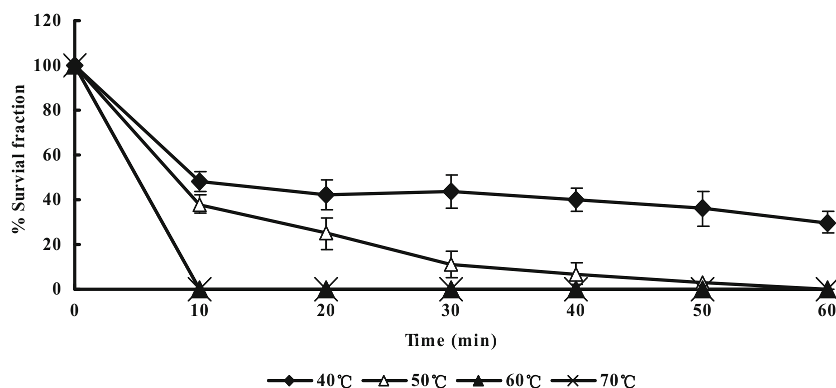

Fig. 5 Thermostability of VNPH-1, the values are the mean of three determinations

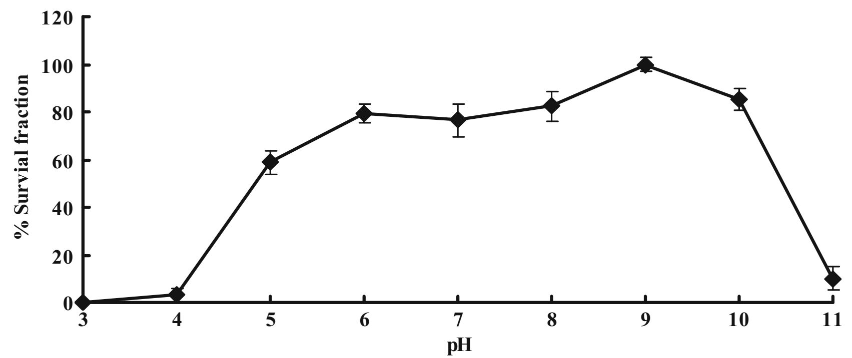

Fig. 6 pH sensitivity of VNPH-1, the values are the mean of three determinations

Thermolability and pH sensitivity

The thermolability profile was the most salient physical feature of cold-active VNPH-1 phage (Fig. 5). The ability of VNPH-1 to infect the host fell rapidly when the temperature exceeded 50 °C. On the other hand, VNPH-1 was shown to be stable between pH 5.0 and pH 10.0, the optimal pH was determined to be 9.0 (Fig. 6).

Sensitivity to chloroform and detergents

VNPH-1 was shown to be sensitive to chloroform and Triton X-100. Plaques could not be observed with 4 % chloroform, while plaque rates of 22, 52.26 and 34.73 % were observed with 2 % chloroform, 1 % chloroform, and 0.3 % Triton X-100, respectively. These results suggest that the capsid of VNPH-1 contains lipids.

Analysis of phage DNA and proteins

VNPH-1 DNA was isolated and digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Xba*I (Fig. 7). The patterns of digestion show that VNPH-1 contained double-stranded DNA with an estimated size of 110-120 kb. Purified phage particles were subjected to analysis by SDS-PAGE (Fig. 8). At least 12 distinct protein bands, with molecular masses ranging from 30 to 120 kDa, were visualized after the gels were stained with Coomassie Brilliant Blue. The most abundant bands in the gel were proteins ranging from 70 to 100 kDa, and most likely represented the major coat protein of VNPH-1.

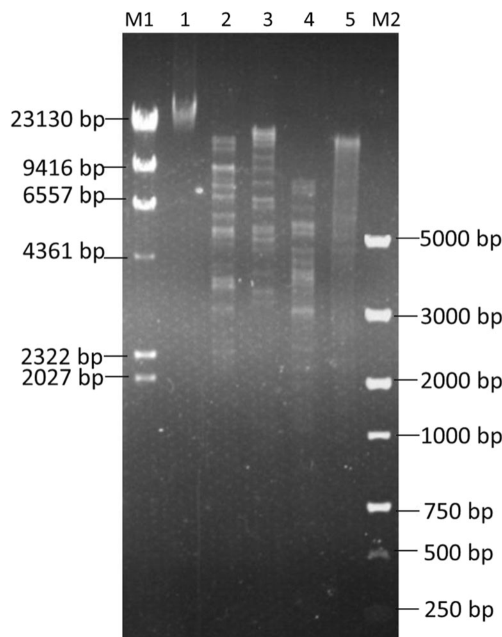

Fig. 7 Restriction endonuclease digestion patterns of VNPH-1. M1, λ/*Hin*dIII DNA markers; *1*, Purified VNPH-1 DNA; *2*, VNPH-1 DNA digested with *Eco*RI; *3*, VNPH-1 DNA digested with *Bam*HI; *4*, VNPH-1 DNA digested with *Hin*dIII; *5*, VNPH-1 DNA digested with *Xba*I; M2, DL5000 DNA markers

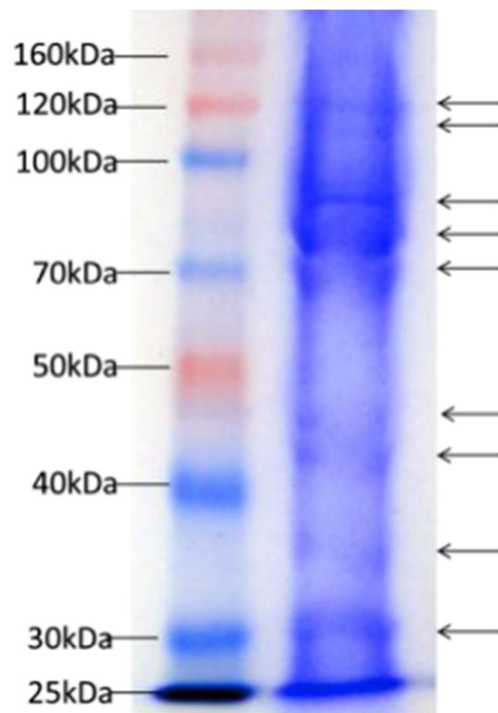

Fig. 8 SDS-PAGE of purified VNPH-1. Lane *1*, protein marker. Lane *2*, purified VNPH-1

Discussion

Cold-active phages have been isolated from low-temperature environments, including marine environments, sewage, food, sea ice, and glaciers (Sawstrom et al. 2008a, b; Anesio et al. 2009a, b; Tamames et al. 2009; Rohwer and Thurber 2009; Sime-Ngando and Colombet 2009; Anesio and Bellas 2011). Morphological examinations have shown that these phages include members of the families *Podoviridae*, *Siphoviridae,* and *Myoviridae* and had genomes consisting of double-stranded DNA. To the best of our knowledge, this is the first study of a virulent phage capable of infecting *A. sobria* to be isolated from a water sample from the Napahai wetland.

The morphology of VNPH-1 was similar to that of most other *Aeromonas* phages that belonged to *Myoviridae* (Haq et al. 2012; Kim et al. 2012a, b). VNPH-1 possessed an icosahedral head and a tail, but it was different from IHQ1 (Haq et al. 2012). VNPH-1 had a burst size of 80 PFU/cell, which is much higher than that observed with the Aeh1 phage (17 PFU/cell) (Chow and Rouf 1983) and smaller than that observed with the Aeh2 (92 PFU/cell) (Chow and Rouf 1983), PAS-1 (116 PFU/cell) (Kim et al. 2012a, b), and IHQ1 (600 PFU/cell) (Haq et al. 2012) phages. IHQ1 had a slightly larger head (128 nm in diameter) and a shorter tail (108 nm) than VNPH-1 (116.7 and 166.7 nm, respectively). The IHQ1 and VNPH-1 phages had similar latent periods (24 min and 20 min, respectively), which were shorter than those of Aeh1 and Aeh2 (39 and 52 min, respectively).

The genomes of *Aeromonas* bacteriophages in the *Podoviridae* (Kim et al. 2012a, b) and *Myoviridae* families have been sequenced (Beilstein and Dreiseikelmann 2008; Shen et al. 2012a). Genome analysis indicated that the VNPH-1 phage had an approximate genome size of 110-120 kb while IHQ1 had a genome size of 25-28 kb (Haq et al. 2012). The adsorption rate of the VNPH-1 phage to the host bacterium was slightly enhanced with Ca^{2+}, whereas the adsorption rate for IHQ1 was significantly enhanced with Ca^{2+}. The optimal pH for VNPH-1 was 9.0 while that for IHQ1 was 7.0 (Haq et al. 2012).

Thermostability was the most salient physical feature of the cold-active VNPH-1 phage. The infectivity gradually decreased when the phage was placed at 40 °C for 10 min, and beyond 60 °C, the VNPH-1 phage became inactive completely; the IHQ1 phage, in contrast, exhibits less thermal stability (Haq et al. 2012).

Aeromonas sobria is widely distribute and has been recognized as a common microbial flora of aquatic ecosystems, as well as opportunistic pathogens for fish, such as epizootic ulcerative syndrome which caused massive death of fish and economic loss. Bacteriophages could decrease the opportunities of bacterial infections and may be an ecological approach to solve the problem. Some research showed that bacteriophages possess the ability to control the pathogens and reduce fish mortality rates in low-temperature aquaculture (Castillo et al. 2014; Madsen et al. 2013; Castillo et al. 2012). For example, *Flavobacterium psychrophilum* is a fish pathogen that causes cold water disease, and it was verified that the lytic bacteriophage infecting *F. psychrophilum* could control this pathogen in artificial laboratory conditions (Castillo et al. 2012). Cold-active VNPH-1 was lytic only against *A. sobria* NPH-1, so it showed high specificity for host cells. The specificity of this bacteriophage for its host makes it an attractive candidate for phage therapy of fish infections caused by *A. sobria* at low temperature.

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Ethical standards The authors declare that all the experiments were conducted according to the current laws of the country in which they were performed.

Conflict of interest The authors have no substantial financial or commercial conflicts of interest with the current work or its publication.

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