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Journal or publication title: Memoirs of Faculty of Fisheries Kagoshima University

Volume: 56
Page range: 55-62
URL: http://hdl.handle.net/10232/4782
Characterization of *Vibrio harveyi* Bacteriophages Isolated from Aquaculture Tanks

Sho Okano, Takeshi Yoshikawa, Armertonnette A. de la Cruz, and Taizo Sakata*

**Key words:** Bacteriophage, *Vibrio harveyi*, sensitivity, one step growth

**Abstract**

Twelve bacteriophage strains of *Vibrio harveyi* were isolated from sea water samples obtained from aquaculture tanks. Two phage strains infected one bacterial strain and the other showed broad host ranges. These bacteriophage strains were divided into five groups based on their host ranges. Four representative strains, ϕH17-5c, ϕH17-7b, ϕH17-8b and ϕH17-9b which infect *Vibrio harveyi* ATCC 14126, were selected and characterized according to various tests such as heat stability, chloroform stability, adsorption rate to *V. harveyi* ATCC 14126, and one-step growth experiment. Phage strains, ϕH17-5c, ϕH17-7b and ϕH17-8b were inactivated completely with heat treatment over 60°C for 10 min. Adsorption rates of these strains to host cells were approximately 70%. On the other hand, ϕH17-9b was inactivated at around 45°C and the adsorption rate is approximately 25%. One-step growth experiments indicated that the latent period and burst size at 25°C of ϕH17-5c, ϕH17-7b, ϕH17-8b and ϕH17-9b strains were 70 min and 5.4, 35 min and 100, 40 min and 170, and 50 min and 5.5, respectively.

*Vibrio harveyi* is a marine luminescent bacterium which causes luminous vibriosis of aquacultured black tiger shrimp (*Penaeus monodon*) and has caused major losses to shrimp farmers in the Philippines and elsewhere.¹-⁴ The symptoms of luminescent vibriosis include loss of appetite, slow growth, high mortality, and luminescence of the bodies of infected shrimps.¹⁻³ At present, clinical therapies against bacteria pathogens have been dependent mainly on antibiotics. But therapeutic uses of various antibiotics have caused the increase of antibiotic-resistant pathogens. Phage therapy is considered to be a potential alternative for preventing bacterial infections.⁵⁻⁶ Bacteriophages infecting fish pathogenic bacteria have been reported by various investigators.⁷⁻¹⁰ Some studies showed that viral particles in the marine waters are generally found at concentrations ranging from 10⁴⁻¹⁰⁷ particles per ml.¹¹ Treatment with bacteriophage was shown to improve survival of shrimp larvae (*P. monodon*) and it was suggested that bacteriophage have a potential for biocontrol of *V. harveyi*.¹² In this paper, the authors reported the isolation and partial characterization of some bacteriophages infecting *V. harveyi* from sea water samples in aquaculture tanks in order to select bacteriophage strains effective for biocontrol of luminous vibriosis.
Materials and Methods

Bacteria and bacteriophage strains

_Vibrio harveyi_ strains ATCC 14126, ATCC 35084 and _V. alginolyticus_ ATCC 17749 were obtained from American Type Culture Collection (ATCC). _Vibrio_ strains including 9M-P5-1, 9M-B9, 9O-S4, 9J-F4, FF-P1, FF-P2, and FF-P4 were isolated from sea water of aquaculture facilities in Japan and the Philippines as described in a previous paper.\(^{13}\)

Sea water samples were obtained from aquaculture tanks in Kagoshima Prefecture and brought to the laboratory for enrichment. Around 100 ml of water sample was added to 100 ml of broth culture of _Vibrio_ sp. strains including _V. harveyi_ ATCC 14126, 9M-P5-1 and FF-P1 and incubated at 25°C for 3 days. After incubation, the phage lysate suspension was centrifuged at 12,000 x g for 15 min to remove the bacterial cells. The supernatant was then filtered through a Millipore filter membrane (pore size; 0.45 µm), and the filtrate was stored at low temperature. The phage lysates obtained were spotted on a lawn of _Vibrio_ spp. on Z-CII double-layered agar plates to examine infection to host strains.

Phylogenetic analysis of 16S rDNA from _Vibrio_ isolates

Chromosomal DNA of _Vibrio_ spp. was extracted as described by Rogers and Bendich.\(^{14}\) Small subunit 16S ribosomal RNA genes (16S rDNA) were amplified by PCR with eubacterial universal forward primer Primer1 (tgt tgg gag agt ttg atc ctg) and _Vibrio harveyi_-specific reverse primer VhSSU.1R (ags gct acc ttg tta yga c). The amplified products were subjected to direct sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and the 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). A phylogenetic tree inferred from the 16S rDNA nucleotide sequences was constructed by the neighbor-joining method\(^{15}\) with a program ClustalW 1.83 (Thompson, _et al._).\(^{16}\) Type strains of the Genus _Vibrio_ species and _Pseudomonas aeruginosa_ as outgroup were included in the phylogenetic tree.

Phage titration

Bacteriophage particles in phage lysate suspensions were counted by titration method.

The phage lysates were diluted and 1.0 ml of each diluted lysate was added to an equal volume of broth culture of a single host strain. An aliquot of 0.2 ml from the phage-host mixtures was added to 3.5 ml of Z-CII soft agar medium and spread over the basal layer of Z-CII agar medium.\(^{13}\) The resulting double layer agar plates were allowed to harden at room temperature. These were then incubated overnight at 25°C. The appearance of plaques and the resulting quantity signifies the infection of one phage to a particular host strain.

Purification of bacteriophages

One plaque from a double layer plate was then aseptically picked up and inoculated into a rotating L-tube containing the host cells in Z-CII liquid medium. Again the phage-host mixture was incubated overnight, centrifuged at 12,000 x g for 20 min and filtered using a 0.20 µm membrane filter. The filtrate was poured into 100 ml of liquid culture of its bacterial host and incubated for 3 days. After incubation the phage-host mixture was centrifuged and filtered. The filtrate was subjected to spot test and titration in order to check morphology and number of plaques.
**Heat sensitivity of bacteriophages**

The heat sensitivity of the bacteriophages was examined by subjecting the phage lysates (10^5 pfu/ml) to various temperatures from room temperature to 100°C for 10 min. Heat-treated phage lysates were then serially diluted and 0.5 ml from each dilution was taken and mixed with an equal volume of host culture. Then, 0.2 ml of the mixture was added into 3.5 ml of Z-CII soft agar. After mixing thoroughly, the soft agar was spread on a basal layer of Z-CII agar plates and incubated overnight at 25°C. The number of phage particles can be determined by the number of plaques left in the plates for samples treated at various temperatures.

**Chloroform sensitivity of bacteriophages**

The phage lysate was diluted to 10^5 pfu/ml. An addition of 0.2 ml chloroform to one of the phage dilution solution was made and incubated at 30°C for 30 min. Just 0.5 ml of the mixture was sampled and subjected to titration. The number of resulting plaques was counted and the survival rate was calculated.

**Adsorption to host cells**

To prepare phage-host mixtures at 0.01 of m.o.i (multiplicity of infection), 4.5 ml the broth culture of host (10^9 cfu/ml) and 0.5 ml of phage lysates (10^8 pfu/ml) were mixed. This phage-host mixture was incubated at 30°C for 3 hr. Aliquots of the mixture were sampled at specific time intervals and filtered using a 0.20 µm membrane filter. The filtrate was subjected to titration and the number of resulting plaques was counted to calculate the adsorption rate.

**One-step growth experiment**

A mixture of 0.9 ml of host culture (10^9 cfu/ml) and 0.1 ml of phage lysates (10^8 pfu/ml) (m.o.i.=0.1) was serially diluted in order to reach a concentration of 10^5 pfu/ml. The total volume of the phage-host mixture was adjusted to 50 ml and incubated at 30°C. An aliquot of 0.5 ml was taken at certain time intervals, serially diluted and portions were added to equal volumes of host culture. A mixture of 0.2 ml of the phage-host suspension and 3.5 ml of Z-CII soft agar was spread over a basal layer of Z-CII agar. The number of plaques formed on the double layered plates incubated overnight at 25°C was counted to determine latent period and burst size.

**Electron microscopy**

The phage lysate and host cell mixture was added with 0.25% glutaraldehyde (final concentration). One drop of the mixture was placed on a carbon coated cupper grid covered with collodion film for 2 min. These specimens were stained with 0.1-0.5% phosphotungstic acid solution for 2 min before the excess solution was removed with a filter paper and observed with a transmission electron microscope (JEM-3010VII, 300 kv, JEOL, Japan).

**Results**

**Phylogenetic analysis of host strains**

A phylogenetic tree inferred from 16S rDNAs of *Vibrio* isolates used in this experiment is shown in Fig. 1. The tree indicated that most of host strains have a very high degree of genetic homology in 16S rDNA sequence and that they belong to the same cluster with *Vibrio harveyi* and only one strain 9M-B9 have high homology with *V. fischeri*.
Isolation of bacteriophages

Twelve bacteriophages infecting *Vibrio harveyi* strains were isolated from 4 sea water samples obtained in aquaculture facilities in Kagoshima Bay, Japan. These bacteriophages were divided into 5 groups based on host range analysis and the representative strains are shown in Table 1 and Fig. 2. Strains $\phi H17-5c$ and $\phi H17-7a$ were found to infect only one host strain, while strain $\phi H17-9b$ infected most of host strains tested as shown in Table 1. A cell form (Fig. 3, A) of host bacterium *V. harveyi* ATCC 14126 and virion morphology (Fig. 3, B and C) of a phage strain, $\phi H17-8b$ are shown in Fig. 3. A phage particle of $\phi H17-8b$ has a head of about 60 nm in diameter and a long tail of about 200 nm in length, suggesting that this phage belongs to siphoviridae.

Heat and chloroform stability of representative strains

Four representative strains, $\phi H17-5c$, $\phi H17-7b$, $\phi H17-8b$ and $\phi H17-9b$ which infect *Vibrio harveyi* ATCC 14126, were selected and characterized by various tests.

As shown in Fig. 4, $\phi H17-5c$, $\phi H17-7b$ and $\phi H17-8b$ were stable to heat treatment under 50°C for 10 min.

<table>
<thead>
<tr>
<th>Table 1. Sampling sources and plaque formation of isolated bacteriophages</th>
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<tbody>
<tr>
<td><strong>Phage strain</strong></td>
</tr>
<tr>
<td>$\phi H17-5c$</td>
</tr>
<tr>
<td>$\phi H17-7b$</td>
</tr>
<tr>
<td>$\phi H17-8b$</td>
</tr>
<tr>
<td>$\phi H17-9b$</td>
</tr>
<tr>
<td>$\phi H17-7a$</td>
</tr>
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</table>
Table 2. Host ranges of bacteriophages infecting *Vibrio* spp.

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Phage strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\phi$H17-5c</td>
</tr>
<tr>
<td><em>V. harveyi</em></td>
<td>+ c*</td>
</tr>
<tr>
<td>ATCC 14126</td>
<td>-</td>
</tr>
<tr>
<td>ATCC 35084</td>
<td>-</td>
</tr>
<tr>
<td>90-S4</td>
<td>-</td>
</tr>
<tr>
<td>9M-P5-1</td>
<td>-</td>
</tr>
<tr>
<td>FF-P1</td>
<td>-</td>
</tr>
<tr>
<td>FF-P2</td>
<td>-</td>
</tr>
<tr>
<td>FF-P3</td>
<td>-</td>
</tr>
<tr>
<td>FF-P4</td>
<td>-</td>
</tr>
<tr>
<td><em>V. alginolyticus</em></td>
<td>ATCC 17749</td>
</tr>
<tr>
<td>PS2-1</td>
<td>-</td>
</tr>
<tr>
<td>MS2-3</td>
<td>-</td>
</tr>
<tr>
<td><em>V. fischeri</em></td>
<td>9M-B9</td>
</tr>
</tbody>
</table>

**+,** sensitive; **−,** not sensitive; **c,** clear plaque; **t,** turbid plaque.

Fig. 3. Electron micrographs of a host cell and bacteriophage particles.

A, *V. harveyi* ATCC 14126 cell and bar of 200 nm; B, $\phi$H17-8b phage particles and bar of 200 nm; C, $\phi$H17-8b phage particle and bar of 50 nm.

Fig. 4. Heat sensitivity of bacteriophages.

Symbols: ◆, $\phi$H17-5c; ■, $\phi$H17-7b; ▲, $\phi$H17-8b; ×, $\phi$H17-9b.

Fig. 5. Adsorption rate of bacteriophages to *V. harveyi* ATCC 14126.

Phage lysate of $\phi$H17-7b was added to an exponential phase-culture of *V. harveyi* ATCC 14126 at a m.o.i. of 0.01.

Symbols: ◆, $\phi$H17-5c; ■, $\phi$H17-7b; ▲, $\phi$H17-8b; ×, $\phi$H17-9b.
Survival rate of bacteriophages after chloroform treatment was 98.1% for \( \phi H17-5c \), 71.4% for \( \phi H17-7b \), 29.2% for \( \phi H17-8b \), and 1.2% for \( \phi H17-9b \) (Table 3).

**One step growth of bacteriophages**

Adsorption rates of bacteriophage particles of the representative strains including \( \phi H17-5c \), \( \phi H17-7b \) and \( \phi H17-8b \) to *V. harveyi* ATCC 14126 cells were approximately 70%. On the other hand, the adsorption rate of \( \phi H17-9b \) was lower than that of others (Fig. 5).

One-step growth experiments determined the latent period, rise period and burst size of the representative strains. Fig. 6 shows one-step growth of strain \( \phi H17-7b \). The latent period, rise period and burst size of strain \( \phi H17-7b \) were 35 min, 45 min and 100 particles per cells. \( \phi H17-5c \) showed 70 min, 20 min and 5.4, \( \phi H17-8b \) did 40 min, 40 min and 170 particles, and \( \phi H17-9b \) did 50 min, 40 min and 5.5 particles (Table 3).

**Discussion**

Luminous vibriosis is known to be caused mainly by *Vibrio harveyi* and bring about major losses to shrimp farmers in Southeast Asia.\(^{1-4}\) *Vibrio harveyi* grows and survives around shrimp culture environments, often acting as an opportunistic pathogen in affected shrimp under stress. Since the discovery of bacteriophages, they have been expected to be used for prevention of various bacterial infections. In this paper, the authors attempted to isolate and select useful bacteriophage strains to suppress luminous vibriosis in shrimp aquaculture.

Twelve bacteriophage strains infecting *Vibrio harveyi* were isolated from sea water of aquaculture tanks in Kagoshima Prefecture, Japan. Two phage strains infected one bacterial strain and the other showed broad host ranges. These bacteriophage strains were divided into five groups based on their host ranges. Among them, \( \phi H17-9b \) shows the broadest host range and is thought to be effective in phage therapy compared with the other strains. On the other hand, strains \( \phi H17-5c \) and \( \phi H17-7a \) have narrow host range and can be used to identify a specific strain of *V. harveyi*.

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**Table 3. Brief characteristics of the representative phage strains**

<table>
<thead>
<tr>
<th>Phage strain</th>
<th>Survival rate (%) in Chloroform</th>
<th>Beginning temperature of inactivation (°C)</th>
<th>One step growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \phi H17-5c )</td>
<td>98.1</td>
<td>60</td>
<td>Latent period (min) 70 Rise period (min) 20 Burst size 5.4</td>
</tr>
<tr>
<td>( \phi H17-7b )</td>
<td>71.4</td>
<td>50</td>
<td>Latent period (min) 35 Rise period (min) 45 Burst size 100</td>
</tr>
<tr>
<td>( \phi H17-8b )</td>
<td>29.2</td>
<td>50</td>
<td>Latent period (min) 40 Rise period (min) 40 Burst size 170</td>
</tr>
<tr>
<td>( \phi H17-9b )</td>
<td>1.2</td>
<td>40</td>
<td>Latent period (min) 50 Rise period (min) 40 Burst size 5.5</td>
</tr>
</tbody>
</table>

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**Fig. 6.** One-step growth curve of phage strain \( \phi H17-7b \). Phage lysate of \( \phi H17-7b \) was added to an exponential phase-culture of *V. harveyi* ATCC 14126 at a m.o.i. of 0.1.
Examination on the influence of temperature and chemicals on bacteriophages is the way of determining the physical and chemical property of the phage particles. Strain $\phi$H17-9b was found to be completely inactivated at 50°C for 10 min and in 20% chloroform for 30 min and to be more sensitive to temperature and chloroform treatment than other strains such as $\phi$H17-5c, $\phi$H17-7b and $\phi$H17-8b. Strain $\phi$H17-5c showed heat resistance at 60°C and survival rate of 98.1% after chloroform treatment.

In one-step growth experiment, Amemura et al. (1991) reported that the latent period and burst size of a bacteriophage strain which infected Edwardsiella tarda were 45 min and 58 particles. Strains $\phi$H17-5c and $\phi$H17-9b had a very small burst size of 5.4 and 5.5, while strains $\phi$H17-7b and $\phi$H17-8b had a large burst size of 100 and 170, respectively, and were considered to be effective in bacteriolysis of V. harveyi ATCC 14126.

Acknowledgments

The authors thank the staff of MBC Aquaculture Farm in Kagoshima Prefecture, Japan for providing sea water samples from shrimp culture tanks, and Mr. Terumi Kakoi for his technical guidance in electron microscopy.

References


