

## Characterization of *Vibrio harveyi* Bacteriophages Isolated from Aquaculture Tanks

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**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,  $\phi$ H17-5c,  $\phi$ H17-7b,  $\phi$ H17-8b and  $\phi$ H17-9b 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,  $\phi$ H17-5c,  $\phi$ H17-7b and  $\phi$ H17-8b 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,  $\phi$ H17-9b 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  $\phi$ H17-5c,  $\phi$ H17-7b,  $\phi$ H17-8b and  $\phi$ H17-9b 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.<sup>1-4)</sup> The symptoms of luminous vibriosis include loss of appetite, slow growth, high mortality, and luminescence of the bodies of infected shrimps.<sup>1-3)</sup> 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.<sup>5,6)</sup>

Bacteriophages infecting fish pathogenic bacteria

have been reported by various investigators.<sup>7-10)</sup> Some studies showed that viral particles in the marine waters are generally found at concentrations ranging from  $10^4$ - $10^7$  particles per ml.<sup>11)</sup> 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*.<sup>12)</sup>

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.

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## 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.<sup>13)</sup>

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.<sup>14)</sup> Small subunit 16S ribosomal RNA genes (16S rDNA) were amplified by PCR with eubacterial universal forward primer Primer1 (ttg ttg 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<sup>15)</sup> with a program ClustalW 1.83 (Thompson, *et al.*).<sup>16)</sup> 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.<sup>13)</sup> 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  $100^\circ\text{C}$  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  $25^\circ\text{C}$ . 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  $30^\circ\text{C}$  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  $30^\circ\text{C}$  for 3 hr. Aliquots of the mixture were sampled at specific time intervals and filtered using a  $0.20\ \mu\text{m}$  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 ( $10^8$  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  $10^3$  pfu/ml. The total volume of the phage-host mixture was adjusted to 50 ml and incubated at  $30^\circ\text{C}$ . 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  $25^\circ\text{C}$  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 copper 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

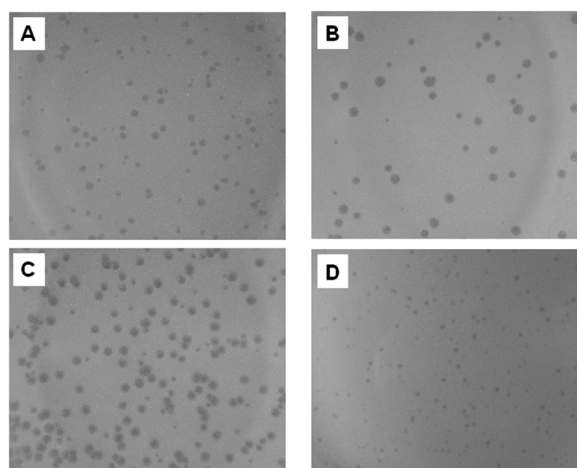
A phylogenetic tree inferred from 16S rDNAs of *Vibrio* isolates used in this experiment is shown in Fig. 1. The tree indicated that most of host strains have a very high degree of genetic homology in 16S rDNA sequence and that they belong to the same cluster with *Vibrio harveyi* and only one strain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



**Fig. 1.** Phylogenetic tree inferred from 16S rDNA sequences of *Vibrio* isolates. The tree was constructed by the neighbor-joining method. A scale bar indicates genetic distance. Numbers at the branching points indicate bootstrap values of 100 replicates.

### 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



**Fig. 2.** Photographs of plaques formed by the representative bacteriophages on the double layer agar plates with a lawn of *V. harveyi* ATCC 14126.

A,  $\phi$ H17-5c; B,  $\phi$ H17-7b; C,  $\phi$ H17-8b; D,  $\phi$ H17-9b.

in Table 2. 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

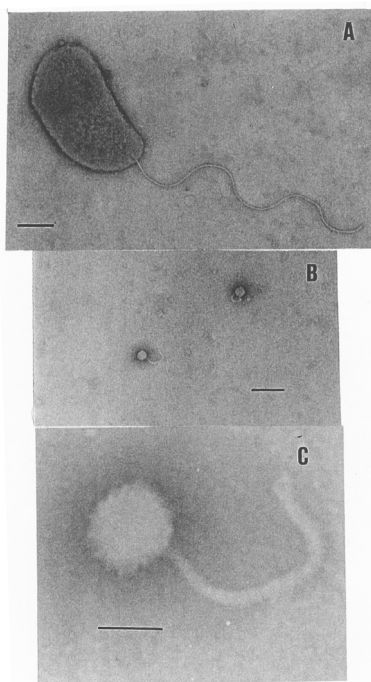
**Table 1.** Sampling sources and plaque formation of isolated bacteriophages

Phage strain	Sampling source	Host strain	Plaque diameter and type
$\phi$ H17-5c	Tiger puffer tank in Kamoike	<i>V. harveyi</i> ATCC 14126	2.3 mm, clear
$\phi$ H17-7b	Tiger puffer tank in Kamoike	"	3.1 mm, clear
$\phi$ H17-8b	Kuruma shrimp tank in Kokubu	"	2.3 mm, clear
$\phi$ H17-9b	Kuruma shrimp tank in Bounotu	"	1.0 mm, clear
$\phi$ H17-7a	Kuruma shrimp tank in Kamoike	<i>V. sp.</i> 9M-P5-1	3.1 mm, clear

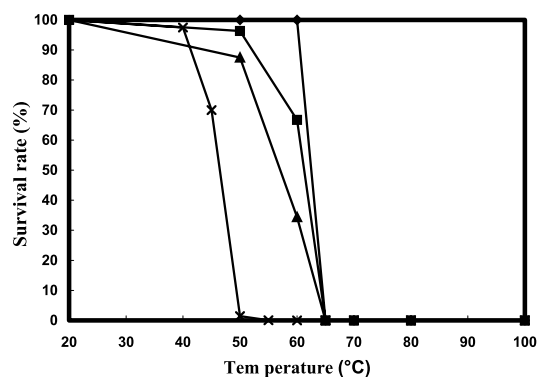
**Table 2.** Host ranges of bacteriophages infecting *Vibrio* spp.

Host strain		Phage strain				
		$\phi$ H17-5c	$\phi$ H17-7a	$\phi$ H17-7b	$\phi$ H17-8b	$\phi$ H17-9b
<i>V. harveyi</i>	ATCC 14126	+ c*	-	+ c	+ c	+ c
	ATCC 35084	-	-	+ c	+ c	-
	9O-S4	-	-	-	+ t	+ c
	9M-P5-1	-	+ c	-	-	+ t
	FF-P1	-	-	-	-	+ c
	FF-P2	-	-	-	-	+ c
	FF-P3	-	-	+ c	+ c	+ t
	FF-P4	-	-	-	-	+ t
<i>V. alginolyticus</i>	ATCC 17749	-	-	-	-	-
	PS2-1	-	-	-	-	+ t
	MS2-3	-	-	-	-	+ t
<i>V. fischeri</i>	9M-B9	-	-	-	-	-

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

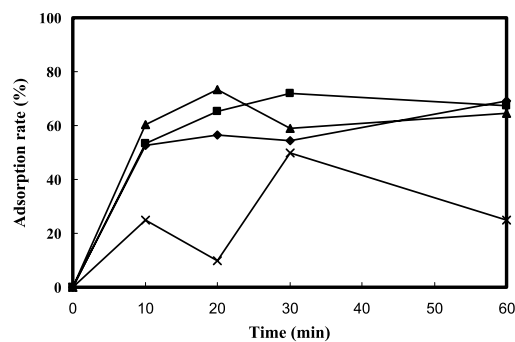


**Fig. 3.** Electron micrographs of a host cell and bacteriophage particles. A, *V. harveyi* ATCC 14126 cell and bar of 200 nm; B,  $\phi$ H17-8b phage particles and bar of 200 nm; C,  $\phi$ H17-8b phage particle and bar of 50 nm.



**Fig. 4.** Heat sensitivity of bacteriophages.

Symbols:  $\blacklozenge$ ,  $\phi$ H17-5c;  $\blacksquare$ ,  $\phi$ H17-7b;  $\blacktriangle$ ,  $\phi$ H17-8b;  $\times$ ,  $\phi$ 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:  $\blacklozenge$ ,  $\phi$ H17-5c;  $\blacksquare$ ,  $\phi$ H17-7b;  $\blacktriangle$ ,  $\phi$ H17-8b;  $\times$ ,  $\phi$ H17-9b.

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

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

and completely inactivated over 65°C. On the other hand,  $\phi$ H17-9b was almost completely inactivated at around 50°C.

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).

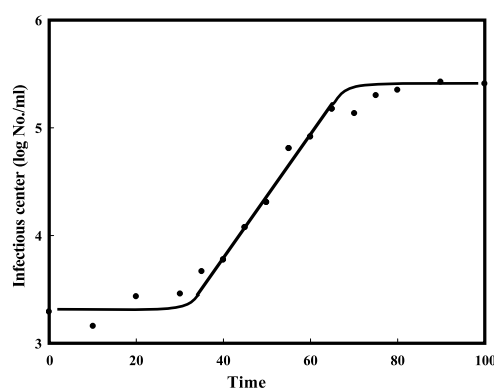
### One step growth of bacteriophages

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.<sup>1-4)</sup> *Vibrio harveyi* grows and survives around shrimp culture environments, often



**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.

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*.

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)<sup>17)</sup> 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.

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