Isolation and Host Range of *Vibrio campbellii* Bacteriophages Isolated from Cockles

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ABSTRACT

Vibrio campbellii has been reported to cause luminous vibriosis in cultured shrimp which could lead to dramatic losses in aquaculture. The use of antibiotics for routine controlling of bacterial infections in aquaculture has resulted in the development of antibiotic-resistant bacterial strains. The alternative biocontrol method using lytic bacteriophage has become more interest. The objectives of this work were to isolate bacteriophages from cockle samples and to determine the host range of isolated bacteriophages. In this study, a total of 5 lytic bacteriophages were isolated from cockle samples (n=6) using *V. campbellii* PSU3282 as a host strain. The host range of OKB54 phage extended over 5 of the 6 tested *V. campbellii* strains but not other *Vibrio* spp. A diverging host range including 4 strains of *V. campbellii*, *V. alginolyticus* and *V. parahaemolyticus* was observed in OPB11 phage. None of the phages was effective against *V. cholerae*, *V. harveyi*, and *V. fluvialis*. This study provides the basis for isolation and further studies in of *V. campbellii* bacteriophage for biocontrol in aquaculture.

Keywords: Aqualculture, Bacteriophages, *Vibrio campbellii*

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Introduction

Shrimp aquaculture has expanded rapidly in recent years. Previously, Thailand is one of the world's largest shrimp exporters, exported around 400,000 tons per year (Portley, 2016). However, the shrimp production in Thailand has dropped because of the disease outbreaks that led to the reducing shrimp population which have caused significant economic losses. Vibriosis is a bacterial disease of cultured shrimp caused by *Vibrio* spp. which are normally found in aquaculture environments (Shakibazadeh, *et al.*, 2009). The most frequently causative agents are *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. campbellii*. Recently, *V. campbellii* has been reported as one of a significant bacterial pathogen for luminous vibriosis in cultured shrimp from several countries (Defoirdt, *et al.*, 2007)

In general, prevention and control against shrimp bacterial infections were highly dependent on various antibiotics. This may result in emergence of antibiotic resistance in bacterial populations (Schwarz, *et al.*, 2001). Bacteriophages are bacterial natural viruses that infect their specific bacterial host and are able to inducing bacterial lysis. Lytic phages are able to regulate bacterial populations, thus they are reported to be the potential candidates for control bacterial pathogens (Mahony, *et al.*, 2011). The study on bacteriophage's potential as a biocontrol agent in aquaculture had led to the isolation of novel phage from marine environment (Madhusudana Rao and Lalitha, 2015). Several researches are focused on the isolation of *V. harveyi* or *V. parahaemolyticus*-specific bacteriophages (Vinod, *et al.*, 2006, Jun, *et al.*, 2014) To date, bacteriophage isolated from shrimp environments have proven to be effective against *V. harveyi*, one of the causative agent of luminous vibriosis disease in shrimp (Defoirdt, *et al.*, 2007) . However, there are no previous reports describing the study of bacteriophages from *V. campbellii*.

Objectives of the study

This study aimed to isolate and evaluate host range of *V. campbellii* lytic bacteriophages from cockles (*Anadara granosa*), one of the most important aquaculture species frequently carry *Vibrio* spp. including *V. campbellii* for application in biocontrol for aquaculture.

Methodology

Bacterial strains and growth conditions

All of the bacterial strains used in this study were from the culture collection of the Department of Microbiology, Prince of Songkla University, Thailand. *V. campbelli* PSU3282, a shrimp isolate was used as host for phage isolation. A panel of 6 *V.campbelli* strains (PSU3280, PSU3284, PSU3285, PSU3286, PSU3288, and PSU3292), *V. alginolyticus* PSU6, *V. cholerae* DMST16261, *V. fluvialis* PSU5036, *V. harveyi* PSU15, and *V. parahaemolyticus* PSU578 were used for host range testing. Unless specified, bacteria were cultured on Tryptic Soy agar with 1% Nacl (TSA+1% NaCl) at 35 °C. To prepare broth cultures, Tryptic Soy broth culture (TSB+1% NaCl) was used to subcultured a colony and incubated at 35 °C with shaking (150 rpm, 6 h). Optical density was adjusted to 0.8 at 600 nm, corresponding to 10^9cells/mL.

Treatment of samples and isolation of bacteriophages

Cockle samples used for isolation of bacteriophages were obtained from various fresh markets including Hat Yai Plaza market, Kim Yong market, and Trang Municipal Food market. Twenty grams of each cockle samples were crushed, homogenized, and added to 20 mL of TSB with double concentration. To increase the likelihood of isolating phages, an enrichment protocol was done. Enrichment broths were produced using a combination of 200 µL of broth culture of *V. campbellii* PSU3282 that were grown in TSB at 35°C with shaking (100 rpm, 6 h). After incubation, bacterial-free supernatants were produced by centrifugation (10,000 rpm, 10 min) followed by filtration using 0.22 µm syringe filters.

Bacteriophages against *V. campbellii* PSU3282 were detected by the plaque assay method. Double-layer agar plates were prepared as described previously (Klieve, 2005, Kropinski, *et al.*, 2009). In a 15 mL centrifuge tube, 100 µL of phage samples were mixed with 100 µL of log phase (OD600nm = 0.8) host strain followed by addition of 3 mL of soft agar (TSB+0.7% agar and 1% NaCl), and were poured onto TSA plate. Plates were then incubated at 35°C for 18 h, and then inspected for zones of clearing which indicated plaque formations. A single plaque formed was picked up in SM buffer (5.8 g/L NaCl, 2.0 g/L $MgSO_4$, 50 ml/L 1 M Tris, pH 7.5). In order to isolate bacteriophages, plaque was transferred to 1 mL SM-buffer. Single plaque isolation was repeated three times to insure purity of bacteriophages (Pereira, *et al.*, 2016).

Propagation of bacteriophages

The double-layer agar method was used for propagation of bacteriophages. The plates with lysis plaque was flooded with 3 mL SM buffer and placed at 35°C on a shaker agitator (100 rpm, 6 h). The suspension was then transferred to a centrifuge tube and centrifuged (10,000 rpm, 10 min) at 4°C prior to 0.22 µm syringe filtering. The propagated phages were stored at 4°C and the bacterial residue was excluded by adding 1% chloroform (final volume). Concentration of the bacteriophage was determined as plaque-forming units (pfu) per mL on the host strain used for isolation.

Host range analysis

Bacterial susceptibility to bacteriophage was carried out by spot test. Bacterial lawns were prepared by pouring 3 mL soft agar containing 100 µL broth culture onto TSA plates, and was allowed to solidify at room temperature. Plates were spotted with 3 µL drops of purified phage suspension on triplicate plates. After incubated at 35°C for 18 h, the presence of lysis zone indicate the lytic efficiency of each phage on host.

Results

Isolation of bacteriophages

A total of 6 samples were collected and used for isolation of *V. campbellii* bacteriophages. Five bacteriophages (OPB11, OPB35, OKB54, OKB58, and OTB66) were successfully isolated from enriched samples after three times plague purification (Table 1). Isolated bacteriophages demonstrated various plaque sizes ranged from 0.5 to 1 mm but exhibited the same plaque characteristic (Fig. 1).

Table 1 Overview of isolated bacteriophages.

Origin of cockle samples	Names of isolated bacteriophages	Plaque size (mm)
Hat Yai Plaza market	OPB45	0.75
Thasa-arn market	OPB48	1
Kim Yong market	OKB54	1
	OKB58	1
Trang Municipal Food market	OTB66	0.5

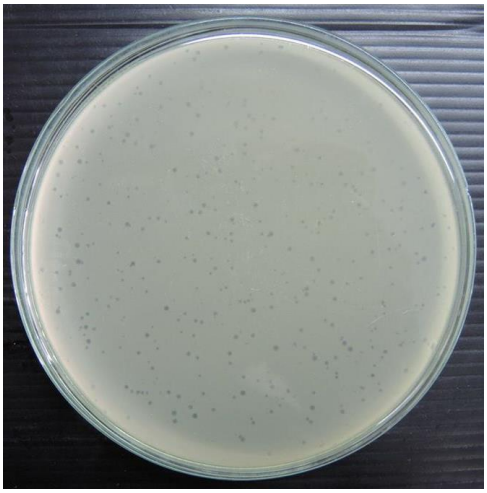

Fig. 1 Example of plaque characteristics of OPB45phage.

Host range analysis

In total the phages were tested on 6 different strains of *V. campbellii* and 5 other *Vibrio* spp. Of the *V. campbellii* strains tested in spot assay, all strains could be lysed by at least one of the phages. *V. campbellii* PSU3286 was sensitive to most of the phages. The widest phage host range against *V. campbellii* was achieved by phage OKB54, which was the most efficient isolate clearing 5 of the 6 tested *V. campbellii* strains but not other *Vibrio* spp. Phage OPB45 had a diverging host range by being most effective in clearing 4 strains of *V. campbellii*, *V. alginolyticus* and *V. parahaemolyticus*. None of the five phages was effective against *V. cholerae*, *V. harveyi*, , and *V. fluvialis* (Table 2).

Table 2 Host range analysis of isolated bacteriophages by spot test.

Bacteria	Reaction of spot test*				
	OPB45	OPB35	OKB54	OKB58	OTB66
V. campbellii					
PSU3280	−	+	−	−	−
PSU3284	+	−	+	−	−
PSU3285	+	+	+	−	−
PSU3286	+	+	+	−	+
PSU3288	+	−	+	+	−
PSU3292	−	−	+	+	−
V. alginolyticus PSU6	+	−	−	+	−
V. cholerae DMST16261	−	−	−	−	−
V. fluvialis PSU5036	−	−	−	−	−
V. harveyi PSU15	−	−	−	−	−
V. parahaemolyticus PSU578	+	−	−	−	−

* +, indicate positive reaction in the spot test; − indicate negative reaction in the spot test.

Discussion and Conclusions

V. campbellii has been known as one of the marine bacteria which causes luminous vibriosis in cultured shrimp (Gomez-Gil, *et al.*, 2004, Defoirdt, *et al.*, 2006). The most widely method used for initially control bacterial disease in aquaculture is the use of antibiotics (Alderman and Hastings, 1998). However, frequently use of these compounds have led to the emergence of antibiotic-resistant bacterial strains and may leave chemical residues in the pond environment and shrimp product (Karunasagar, *et al.*, 2007). Bacteriophages have a potential application as an alternative to antibiotics and will not effect on beneficial bacteria or micro flora.

Bacteriophages are generally isolated from various environmental sources such as sewage, soil, water, ponds or from aquatic animals. Titers of *V. parahaemolyticus* bacteriophages occasionally exceeded 10^6 per g of oyster during the summer months. The high incidence of bacteriophages in oysters indicates that phage replication may be occurring within these animals that are the environments where these host organisms are present in high numbers (Baross, *et al.*, 1978). In this study, 5 phages were isolated from 6 cockle samples. We reported here the isolation of five *V. campbelli* phages with plaques formation approximately 1 mm. The host range of OPB45 and OKB 58 phage was relatively broad, including strain of the closely related species *V. alginolyticus* and *V. parahaemolyticus* (Sawabe, *et al.*, 2007). The reported bacteriophage OKB54 in this study could be useful for high use as a biocontrol agent against *V. campbellii*. Further studies about using cocktails phages and phage characterization are required prior to their application as biocontrol agent in shrimp aquaculture.

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