Growth inhibitory effect of bacteriophages isolated from western and southern coastal areas of Korea against *Vibrio parahaemolyticus* in Manila clams

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Received: 7 October 2015 / Accepted: 5 January 2016 / Published online: 2 February 2016 © The Korean Society for Applied Biological Chemistry 2016

**Abstract** *Vibrio parahaemolyticus* is one of the leading causes of seafood-related illnesses in Korea and the whole world. The growth inhibitory effect of *V. parahaemolyticus*-specific phages (Vpp) in clam samples was investigated. Six bacteriophages specific to *V. parahaemolyticus* were isolated by the agar overlay method using sediment and seafood samples from the western and southern coastal areas of Korea. Host range, restriction digestion pattern, and transmission electron microscope images of phage isolates were examined. The most effective phage, Vpp2, at a multiplicity of infection (MOI) of 10 showed 4.2 log CFU/mL reduction in *V. parahaemolyticus* growth at 30°C and 6 log CFU/mL reduction after the incubation at 37°C for 6 h. In a food application study, Vpp2 at an MOI of 100 demonstrated a 2.1 log CFU/mL reduction at 25°C after a 24-h incubation in shell-less Manila clams spiked with *V. parahaemolyticus*. Additional studies are needed to characterize the newly discovered Vpp and their potential applications as biocontrol agents in food.

**Keywords** Biocontrol · Growth inhibition · Manila clam · *Vibrio parahaemolyticus* · *Vibrio parahaemolyticus*-specific phage

**Introduction**

*Vibrio parahaemolyticus* is a halophilic gram-negative bacterium that occurs in sea water and estuaries. It causes gastroenteritis with severe abdominal pain and diarrhea via the ingestion of raw or undercooked seafood and cross contamination from unsanitary cooking environments (Su and Liu 2007; Robert-Pillot et al. 2010). It is also one of the leading causes of seafood-related illnesses in Korea. From July to September, *V. parahaemolyticus* accounted for 14 of 18 total outbreaks (79 %) in 2010, 8 of 9 outbreaks (89 %) in 2011, 9 of 11 outbreaks (82 %) in 2012, 5 of 5 outbreaks (100 %) in 2013, and 7 of 7 outbreaks (100 %) in 2014 (KMFDS 2015); therefore, the species requires additional research and monitoring, particularly during the summer, as does *V. vulnificus*.

Various physical and chemical control techniques effectively inhibit *V. parahaemolyticus* growth; however, they could also cause changes in the physicochemical and sensory properties and the freshness of seafood and seafood products (Lin et al. 2005; Ren and Su 2006; Su and Liu 2007; Chae et al. 2009; Larsen et al. 2013; Rong et al. 2014). Furthermore, as many consumers in Korea and Asia prefer raw or partially cooked seafood, the continual development of novel control methods against *V. parahaemolyticus* contamination is needed.

Lytic bacteriophages are viruses that invade target bacteria, disrupt bacterial metabolism, and cause bacteria to lyse. Bacteriophage and phage-derived antimicrobials have many potential applications in the area of food safety, such as in phage therapy for livestock, biosanitation and biocontrol in the foodstuff industry, biopreservation in storage and marketing, and aquaculture (Hudson et al. 2005; García et al. 2008; Alagappan et al. 2010; Rong et al. 2014).
Bacteriophages have important functions as alternative antibiotic agents against threats of antibiotic-resistant food-borne pathogenic bacteria, despite a number of future challenges, such as host range limitations, better knowledge of phage-host physiology, issues of consumer acceptance, and so on (Hudson et al. 2005; García et al. 2008). The initial approval for the application of phages specific to Listeria monocytogenes for food safety purposes (i.e., LISTEX P100™) has accelerated research on the use of phage against other food pathogens (García et al. 2008). However, reports on food protection using phages and their lytic mechanism against V. parahaemolyticus are rare, except in the area of aquaculture. Applications of bacteriophages to seafood as non-thermal treatments are expected not only to inhibit the growth of Vibrio species, but also to maintain their desirable characteristics. The exploration and selection of appropriate bacteriophages are key factors in the success of phage-mediated control of bacterial infections (Rong et al. 2014).

The purpose of this study was to characterize V. parahaemolyticus-specific phages isolated from sediment and seafood samples and to examine the growth inhibitory effect of these phages against V. parahaemolyticus for use as biocontrol agents in foods.

Materials and methods

Bacterial strains and characterization

All reference strains were donated from the Korea Agricultural Culture Collection, Korea Culture Center of Microorganisms, and Korea Collection for Type Cultures. V. parahaemolyticus reference strains, ATCC 27969 and 17802, and V. parahaemolyticus isolates were grown at 37 °C on Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) supplemented with 1 % NaCl.

Vibrio strains were also isolated from sea water and clam samples in 2013. The isolation of V. parahaemolyticus was performed according to the modified KMFDS Bacteriological Analytical Manual (KMFDS 2013). After screening using thiosulphate-citrate-bile salt sucrose (TCBS) agar, the triple-sugar-iron (TSI) agar test, or CHROMagar Vibrio, the isolates were identified using the VITEK 2 system (bioMérieux, Marcy-l’Étoile, France) according to the manufacturer’s manual. TSA, TSB, TCBS agar, and TSI agar were purchased from BD (Franklin Lakes, NJ, USA), and CHROMagar Vibrio was purchased from CHROMagar (Paris, France). The biochemically identified V. parahaemolyticus isolates were further verified by molecular characterization using PCR to detect the presence of species-specific genes (toxR and th) and virulence genes (tdh and trh) (Tada et al. 1992; Bej et al. 1999; Kim et al. 1999; Yang et al. 2008; Abd-Elghany and Sal-lam 2013). An antibiotic-resistance test against V. parahaemolyticus isolates was performed using the Vitek card AST-N169 (biomérieux) with various antibiotics, such as amoxicillin/clavulanic acid, ampicillin/sulbactam, amikacin, cefalotin, cefazolin, cefotetan, cefoxitin, cefotaxime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, imipenem, nalidixic acid, tetracycline, and trimethoprim/sulfamethoxazole.

Vibrio parahaemolyticus-specific phage preparation

Six V. parahaemolyticus-specific lytic bacteriophages were isolated using the agar overlay method (Baross et al. 1978; Comeau et al. 2005). One hundred microliters of the 24-h enrichment culture (TSB) filtered through a 0.45-μm membrane was mixed with 100 μL of the host strain (reaching an optical density of 1.0 at 600 nm from the overnight culture) and 3 mL of 0.7 % soft TSB. The mixture was then immediately overlaid on the TSB. The plates were incubated at 37 °C overnight, and the number of plaques were counted and expressed as plaque-forming units per milliliter (PFU mL⁻¹). V. parahaemolyticus ATCC 27969 and 17802, and V. parahaemolyticus isolates were used as hosts for the assays. A single plaque was selected and resuspended in SM buffer (5.8 g of NaCl, 2 g of MgSO₄·7H₂O, 50 mL of 1x phosphate buffered saline, pH 7.8 in 1 L), after which it was centrifuged at 10,000 × g for 10 min at 4 °C and filtered through a 0.45-μm membrane to remove intact bacteria and bacterial debris. Three successive single-plaque isolations were performed to obtain the pure phage stock. The stocks were stored at 4 °C with the addition of 1 % chloroform for future studies.

Host range determination of phages

The lytic spectrum of phage based on the extent of host range was tested as follows. One hundred microliters of a phage sample [multiplicity of infection (MOI), 0.001] was added to 100 μL of bacterial overnight culture, and the agar overlay method was used. The presence of plaque plaques as indicators of bacterial lysis was checked after 18–24 h of incubation.

Restriction digestion pattern of the phage genome

The phage DNA was extracted using the Genomic DNA Extraction Kit (Qiagen Tissue and Blood Kits, Chatsworth, CA, USA) according to the manufacturer’s instructions. After DraI (TaKaRa, Shiga, Japan) digestion, the DNA fragments were separated by electrophoresis in a 0.8 % agarose gel in TAE buffer at 80 V for 40 min.
Phage morphology

The phage suspension (10^9 PFU/mL) was centrifuged with a 40 and 10% glycerol gradient at 35,000 rpm for 90 min at 4 °C. After dialysis against SM buffer, phage samples were deposited on formvar-coated copper grids (Ted Pella, Inc., USA) fixed with 2.5% glutaraldehyde for 2 h and washed twice with phosphate buffer (pH 7.4). All phages were examined using a Tecnai F20 Cryo transmission electron microscope operated at 60 kV at a magnification of ×20,000 after they were negatively stained with 2% uranyl acetate.

V. parahaemolyticus growth inhibition by the phage in culture

After 100 mL of TSB with 1% NaCl was inoculated with fresh overnight bacterial culture to reach 1 × 10^4 CFU/mL, 100 µL of purified phage suspension at an MOI of 0.1, 1, and 10 was added to the bacterial culture and incubated with shaking for 12 h at 25, 30, and 37 °C. One milliliter of culture medium was collected every 2 h and used to count the colonies grown on plate count agar with 2% NaCl. The growth inhibitory effect of the phage was measured by a comparison with the CFU of the control group, which was not treated with phage.

V. parahaemolyticus phage application to spiked seafood samples

Live Manila clams were purchased from a local market and transferred to the laboratory immediately. Fresh shell-less Manila clam samples were washed with three volumes of sterile water three times, and 10 g of sample was placed into a sterile stomach plastic bag with membranes. The samples were spiked with 100 µL of diluted overnight host bacterial cells (1 × 10^4 CFU/mL) and then 100 µL of each purified phage suspension at an MOI of 1, 10, or 100 was applied to the contaminated samples, which were incubated for 24 h at 4, 15, and 25 °C. To measure the viable cells in each treatment, an aliquot of 1 mL was collected at 0, 2, 4, 6, and 24 h. After adding 99 mL of alkaline peptone water, serially diluted samples were plated on a TCBS agar plate and incubated for 24 h until the green–blue colonies appeared at 36 °C.

Statistical analysis

Results of the experiment performed in triplicate are presented as mean ± SD. The statistical significance among treatments was analyzed using a Student’s t test. P values of less than 0.05 were considered statistically significant.

Results and discussion

Isolation of Vibrio parahaemolyticus-specific phages

Bacteriophages specific to V. parahaemolyticus were isolated using sediment and seafood samples collected in the western and southern coastal areas from March to September of 2013. Seafood samples, such as oysters and clams, salt-fermented seafood, and other sources were collected from marine environments or from local fish markets. The 6 presumptive phages specific to each V. parahaemolyticus isolate, which were obtained from pure subcultures using three successive single-plaque isolations, were acquired: V. parahaemolyticus phage (Vpp) 1 from an oyster in Gwangyang, Jeonnam, Vpp2 from sediment in Ganghwa, Incheon, Vpp3 from a scallop in Tongyeong, Geongnam, Vpp4 from a warty sea squirt in Tongyeong, Geongnam, Vpp5 from sediment in Dangjin, Chungnam, and Vpp6 from sediment in Siheung, Gyeonggi, Korea.

Characterization of Vibrio parahaemolyticus isolates

Eight V. parahaemolyticus strains were isolated from seawater and clam samples using selective media, TCBS/TSI agar, or CHROMagar Vibrio, and were identified biochemically using the VITEK 2 systems (Table 1). The species-specific genes toxR and tl were detected in all isolates, including two V. parahaemolyticus reference strains (ATCC 27969 and 17802). However, none of the isolates were verified as pathogenic; the virulence genes tdh and trh genes were not detected (Table 1). Only V. parahaemolyticus reference strain ATCC 17802 had the virulent trh gene. These results confirmed the results of previous reports indicating that most V. parahaemolyticus isolates from environmental and seafood samples are tdh- and trh- negative and are not pathogenic; 1–6% of samples

| Vp | toxR | tdh | trh | tl | Ampicillin resistance |
|----|-----|-----|-----|----|----------------------|
| ATCC 27969 | + | - | - | + | + |
| ATCC 17802 | + | - | + | + | + |
| Isolate KF1 | + | - | - | + | + |
| Isolate KF2 | + | - | - | + | + |
| Isolate KF3 | + | - | - | + | + |
| Isolate KF4 | + | - | - | + | + |
| Isolate KF5 | + | - | - | + | + |
| Isolate KF6 | + | - | - | + | + |
| Isolate KF7 | + | - | - | + | + |
| Isolate KF8 | + | - | - | + | + |
contain tdh- and trh-positive isolates (Lee et al. 2008; Rosec et al. 2009). According to the results of an antimicrobial resistance test, V. parahaemolyticus was not resistant against fifteen antibiotics, but was resistant to ampicillin/sulbactam.

**Characterization of Vibrio parahaemolyticus-specific phages**

The lytic spectrum of phages was investigated against 3 Gram-positive and 22 Gram-negative strains (Table 2). Six phages showed no lytic activity against major food-borne pathogens, such as *Escherichia coli* O157:H7, *Salmonella* strains, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. All 6 phages showed lytic activity against *Vibrio* species (i.e., either the reference strains or isolates), and had a narrow lytic spectrum. Only Vpp2 had lytic properties against *V. cholera* and *V. mimicus* as well as *V. parahaemolyticus*.

The restriction analysis of the phage genome using *Dra*I revealed that the pairs Vpp1, Vpp2, Vpp3, Vpp4, Vpp5, and Vpp6 showed similar patterns (Fig. 1). However, the host range analysis of the three phage pairs with similar pattern revealed differences between the two phages in each pair (Table 2). Vpp1 and Vpp2 were isolated from different sources in distant locations; they had similar restriction digestion patterns, but divergent host ranges, similar to the pattern observed for Vpp5 and Vpp6. In an analysis of phage morphology based on transmission electron microscopy (Fig. 2), Vpp2 belonged to *Podoviridae* with short extensions or *Siphoviridae* with tails cut via operational mistakes (Fig. 2(A)). Vpp3 and Vpp 4 belonged to *Siphoviridae*, and were characterized by a flexible tail (Fig. 2(B), (C)). Vpp6 also belonged to *Siphoviridae* and exhibited an elongated head (Fig. 2(D)).

**Table 2** Host range of *Vibrio parahaemolyticus*-specific phages against various food-borne pathogens

| Bacterial strains                  | Lysis by bacteriophages |
|-----------------------------------|-------------------------|
|                                   | Vpp1 | Vpp2 | Vpp3 | Vpp4 | Vpp5 | Vpp6 |
| Gram positive                     |      |      |      |      |      |      |
| *Bacillus cereus*                 | ATCC 14579 | - | - | - | - | - |
| *Listeria monocytogenes*          | KCCM 40307 | - | - | - | - | - |
| *Staphylococcus aureus*           | ATCC 25923 | - | - | - | - | - |
| Gram negative                     |      |      |      |      |      |      |
| *Escherichia coli* K12            | ER2738 | - | - | - | - | - |
| *Escherichia coli* O157:H7        | NCCP 1109-047 | - | - | - | - | - |
| *Salmonella enterica enterica*    | ATCC 13076 | - | - | - | - | - |
| *Salmonella enteritidis*          | KCCM 12021 | - | - | - | - | - |
| *Salmonella typhimurium*          | KCCM 11862 | - | - | - | - | - |
| *Vibrio parahaemolyticus*         | ATCC 27969 | + | - | - | + | - |
| *Vibrio parahaemolyticus*         | ATCC 17802 | + | + | + | + | - |
| *Vibrio vulnificus*               | ATCC 27562 | - | - | - | - | - |
| *Vibrio alginolyticus*            | Isolate | - | - | - | - | - |
| *Vibrio cholera*                  | Isolate | - | + | - | - | - |
| *Vibrio fluvialis*                | Isolate | - | - | - | - | - |
| *Vibrio metchnikovii*             | Isolate | - | - | - | - | - |
| *Vibrio mimicus*                  | Isolate | - | + | - | - | - |
| *Vibrio vulnificus*               | Isolate | - | - | - | - | - |
| *Vibrio parahaemolyticus*         | Isolate (KF1) | - | - | + | - | + |
| *Vibrio parahaemolyticus*         | Isolate (KF2) | - | - | - | - | + |
| *Vibrio parahaemolyticus*         | Isolate (KF3) | - | - | + | + | + |
| *Vibrio parahaemolyticus*         | Isolate (KF4) | + | + | - | - | + |
| *Vibrio parahaemolyticus*         | Isolate (KF5) | + | - | - | + | + |
| *Vibrio parahaemolyticus*         | Isolate (KF6) | + | + | - | - | + |
| *Vibrio parahaemolyticus*         | Isolate (KF7) | + | - | - | + | + |
| *Vibrio parahaemolyticus*         | Isolate (KF8) | - | - | - | - | - |
**Fig. 1** Digestion of bacteriophage DNA using the restriction enzyme *Dra*I. (A) undigested bacteriophage DNA; (B) *Dra*I-digested bacteriophage DNA; lane *M*1, 1 kb DNA marker; lane *M*2, Lambda HindIII/EcoRI DNA marker; lane 1 Vpp1; lane 2 Vpp2; lane 3 Vpp3; lane 4 Vpp4; lane 5 Vpp5; lane 6 Vpp6

**Fig. 2** TEM images of *Vibrio parahaemolyticus* phages. (A) Vpp2; (B) Vpp3; (C) Vpp4; (D) Vpp6

**V. parahaemolyticus** growth inhibition by the phage

*Vibrio parahaemolyticus* phage-mediated growth inhibition was examined in culture medium at two different temperatures, 30 and 37 °C (at an MOI of 0.1–10). In a preliminary study of the six phages, Vpp2 and Vpp4 had the highest lytic activity and were selected for further analyses. Both Vpp2 and Vpp4 at an MOI of 10 exhibited greater inhibition of *V. parahaemolyticus* at 37 than 30 °C (Fig. 3). When Vpp2 was incubated in the presence of its host, Vp-KF4, significant decreases of 6 log CFU/mL (*P* < 0.001) at 37 °C and 4.2 log CFU/mL (*P* < 0.01) at 30 °C were
observed after 6 h. When Vpp4 was incubated in the presence of its host, VP-KF3, a significant decrease of 4 log CFU/mL ($P \leq 0.01$) at 37°C was observed after 4 h. There were no dramatic differences in CFU between the Vpp4-treated group and the control group after 6 h. Vpp2 and Vpp4 each have lytic properties against the pathogenic strain $V. \text{parahaemolyticus}$ ATCC 17802 based on the host range study (Table 2). Using the same host, Vp-KF4, the two-phage mixture (Vpp1 and Vpp2) was not more effective than each single phage with respect to lytic activity in the culture medium (data not shown). Only Vpp2 was used for the food application study described belows because it showed the highest activity among the phages.

When Vpp2 was added to Vp-KF4-spiked clam samples at MOIs of 1, 10, and 100, its growth inhibitory effect was dose-dependent (data not shown). $V. \text{parahaemolyticus}$ growth in clam samples treated with Vpp2 at an MOI of 100 revealed a significant reduction of 2.1 log CFU/mL ($P < 0.01$) at 25°C until 24 h, but there was no effect of Vpp2 treatment on $V. \text{parahaemolyticus}$ growth at 4°C (Fig. 4). The phage had a much lower inhibitory effect on $V. \text{parahaemolyticus}$ growth in clam samples than in culture medium. However, the effect of phage application on clam samples persisted for 24 h, but the effect in culture medium reached a maximum at 6 h and then gradually decreases (Fig. 4). The mode of phage treatment on the surface of food, such as pipetting or spraying, could influence the lytic activity of the phage because complex factors in the food matrix affect the relationship between host and phage. This might explain the differences in the effects of phages on bacterial growth inhibition between culture conditions and food matrices.

The key to successful biocontrol is to isolate promising phages that exhibit high potency and persistent lytic activity. Rong et al. (2014) found that depuration of infected oysters at 16°C with an 0.1 MOI was optimal for reducing $V. \text{parahaemolyticus}$ by 2.35–2.76 log CFU/mL. According to Jun et al. (2014), when a phage was surface-applied to the flesh of oysters after $V. \text{parahaemolyticus}$ inoculation, bacterial growth was inhibited by 6 log CFU/mL for 12 h. Interestingly, this result implied a lack of
phage resistance overtime. This was not consistent with our results, presumably owing to the different phages that were used. Additional research on host–phage relationships is necessary to improve our understanding of phage resistance. Further food application studies should be performed to control Vibrio species in seafood using phage mixtures or phage endolysin to enhance the effectiveness of whole single phages.

Acknowledgments This study was supported by grants from the Korea Food Research Institute and the Ministry of Trade, Industry and Energy, Republic of Korea, and by the Chung-Ang University Excellent Student Scholarship.

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