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Characterization of bacteriophage VVP001 and its application for the inhibition of *Vibrio vulnificus* causing seafood-borne diseases

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ABSTRACT

Vibrio vulnificus is a major food-borne pathogen that causes septicemia and cellulitis with a mortality rate of >50%. However, there are no efficient natural food preservatives or biocontrol agents to control *V. vulnificus* in seafood. In this study, we isolated and characterized a novel bacteriophage VVP001. Host range and transmission electron microscopy morphology observations revealed that VVP001 belongs to the family *Siphoviridae* and specifically infects *V. vulnificus*. Phage stability tests showed that VVP001 is stable at a broad temperature range of −20 °C to 65 °C and a pH range from 3 to 11, which are conditions for food applications (processing, distribution, and storage). *In vitro* challenge assays revealed that VVP001 inhibited *V. vulnificus* MO6-24/O (a clinical isolate) growth up to a 3.87 log reduction. In addition, complete genome analysis revealed that the 76 kb VVP001 contains 102 open reading frames with 49.64% G + C content and no gene encoding toxins or other virulence factors, which is essential for food applications. Application of VVP001 to fresh abalone samples contaminated with *V. vulnificus* demonstrated its ability to inhibit *V. vulnificus* growth, and an *in vivo* mouse survival test showed that VVP001 protects mice against high mortality (survival rate >70% at a multiplicity of infection of 1000 for up to 7 days). Therefore, the bacteriophage VVP001 can be used as a good natural food preservative and biocontrol agent for food applications.

1. Introduction

Vibrio vulnificus is a halophilic, motile, vibrio-shaped, gram-negative, pathogenic marine bacterium normally found worldwide in estuarine waters or coastal areas, especially from spring to fall seasons (DePaola et al., 1998; Horseman and Surani, 2011; Strom and Paranjpye, 2000). *V. vulnificus* infection, which causes fatal septicemia, occurs by ingestion of raw or undercooked seafood (especially oysters) or by wound contact with contaminated seawater, with a mortality rate of 50% and 17%, respectively (Horseman and Surani, 2011; Morris, 1988; Phillips and Satchell, 2017). The symptoms of septicemia include fever, chills, nausea, hypotensive septic shock, and the formation of secondary lesions (Kumamoto and Vukich, 1998). Septicemia is lethal to chronic liver disease patients (Haq and Dayal, 2005; Jones and Oliver, 2009). The various virulence factors of *V. vulnificus* are associated with three major elements, the capsular polysaccharide (Wright et al., 1990), RtxA1 toxin (Chung et al., 2010), and iron availability and acquisition systems (Kim et al., 2007; Wright et al., 1981).

In South Korea, the Korea Centers for Disease Control and Prevention reported 325 patients infected by *V. vulnificus*, of which 159 (48.92%) died from 2011 to 2016. In 2017, the United States Centers for Disease Control and Prevention estimated that *V. vulnificus* causes approximately 205 infections every year, and 1 in 7 patients with a *V. vulnificus* wound infection dies. Upon diagnosis, it is recommended that patients be immediately administered antibiotic therapy (Dechet et al., 2008); however, the excessive use of antibiotics has resulted in the emergence of antibiotic-resistant *V. vulnificus* (Han et al., 2007). Some studies have reported that *V. vulnificus* strains are resistant to doxycycline, tetracycline, aminoglycosides, and cephalosporins (Baker-Austin et al., 2009). In addition, the Food and Drug Administration (FDA) and the US

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Department of Agriculture prohibit the application of antibiotics to foods, and chemical food preservatives are not preferred (Donoghue, 2003; Lee et al., 2016). Therefore, alternative biocontrol agents that can replace antibiotics and chemical antimicrobials are needed in order to prevent *V. vulnificus* infection.

Bacteriophages are bacterial viruses that infect and lyse specific host bacteria via lytic and lysogenic cycles (Sulakvelidze et al., 2001). Bacteriophages kill the bacterial host after infection initiation in the lytic cycle, and they continue to survive without cell lysis in the lysogenic pathway (Dalmasso et al., 2014). In addition, bacteriophages infect specific bacteria with recognition-specific host receptors, such as the O-antigen of bacterial lipopolysaccharides, BtuB (vitamin B12 transporter), FhuA (ferrichrome outer membrane transporter), OmpC (outer membrane protein C), and flagella, giving the bacteriophages host specificity (Rakhuba et al., 2010). Because of high host specificity, bacteriophages infect specific bacteria without affecting other bacteria, and no serious side effects have been reported in human applications (Gyles, 2007).

Because of their efficient host lysis activity, high host specificity, safety, and effectiveness in controlling antibiotic-resistant bacteria, bacteriophages can be used to treat bacterial infectious diseases. Since the first attempt by Félix d'Hérelle to administer bacteriophages to treat a 12-year-old boy with severe dysentery (d'Herelle, 1917; Summers, 1999), other studies regarding bacteriophage therapy have been conducted, especially in Eastern Europe and in the former Soviet Union (Sulakvelidze et al., 2001). In addition, many commercial products for bacteriophage treatment of bacterial infections have been developed, such as Phagestaph™ (JSC Biochimpharm, Tbilisi, GA), *Escherichia coli* bacteriophage™ (Microgen, Moscow, Russia), and Complex pyobacteriophage™ (Micogen) (Bai et al., 2016). With the efficacy and safety of bacteriophages, the first multicenter clinical trial France, Switzerland, and Belgium was begun in 2015 to treat wounds with *E. coli* or *Pseudomonas aeruginosa* (Kingwell, 2015; Sansom, 2015).

Because of chemical preservative limitations in foods, especially fresh fruits, vegetables, and ready-to-eat products, bacteriophages can also be used to effectively control bacterial contamination in foods (Kazi and Annapure, 2016). LISTEX™ P100 (Micreos Food Safety, Wageningen, the Netherlands) was given Generally Recognized as Safe status by the FDA in 2006. LISTEX™ P100 decreases *Listeria monocytogenes* in meat and poultry, fish and shellfish, and dairy products by 1.7–3.4 log colony-forming units (CFUs) at the maximum dose (Allende et al., 2016). Because of these characteristics, bacteriophages are considered as a natural food preservative or biocontrol agent for food, in addition to their therapeutic uses.

In 1995, nine bacteriophages (S1, P13, P38, P53, P65, P68, P108, P111, and P147) specific for *V. vulnificus* were first isolated in oysters collected from the Gulf of Mexico (Pelon et al., 1995). Bacteriophage pools were created by mixing the nine bacteriophage stocks and the pools were tested for bactericidal activity in fresh raw oysters by combining a component of oyster extracts that could decrease the *V. vulnificus* load by 4–5 log units (Pelon et al., 2005). In addition, bacteriophages CK-2, 153A-5, and 153A-7 that infect *V. vulnificus* were examined in an iron-dextran-treated mouse model for evaluation of their therapeutic effects. CK-2 (specific for *V. vulnificus* MLT403) and 153A-5 (specific for *V. vulnificus* MO6-24/O and VV1009) were shown to protect infected mice (Cerveny et al., 2002). Although these studies might be important for the application of bacteriophages as food preservatives or biocontrol agents to regulate food-borne pathogens, there are few studies on the isolation, characterization, and application of *V. vulnificus*-targeting bacteriophages.

In this study, we isolated and characterized a novel bacteriophage VVP001 that mainly infects *V. vulnificus* MO6-24/O to understand its phenotypic characteristics. In addition, we sequenced and analyzed the VVP001 genome using bioinformatics tools to predict the safety of this bacteriophage in foods. Also, to evaluate VVP001 effectiveness, we investigated growth inhibition of the *V. vulnificus* MO6-24/O clinical isolate in a fresh abalone sample and an *in vivo* mouse survival test. Our findings are useful for developing a natural food preservative for food applications with high safety to humans.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Table 1 lists all bacterial strains and growth media used in this study. *Vibrio* strains were grown with shaking at 30 °C in Luria–Bertani medium with 2% NaCl supplementation (LBS) (Lee et al., 2014). Other bacterial strains were cultivated with shaking at 37 °C in each optimized medium (Table 1). *Vibrio vulnificus* MO6-24/O, a clinical isolate, was selected as an indicator strain for isolation and characterization of bacteriophages. All medium components were purchased from Difco Laboratories (Detroit, MI, USA).

2.2. Bacteriophage isolation and propagation

Abalone samples were collected from the coastal area of Yeonpo in Tae-An, Korea, and used for isolation of *V. vulnificus*–infecting bacteriophages. Bacteriophage isolation was performed as previously described (Kim and Ryu, 2011) with modifications. Here, the phage isolation process was repeated at least five times for pure isolation of a single phage and this isolation was confirmed by TEM morphological observation.

For bacteriophage propagation, the lysate of a single plaque from a single phage isolation and purification was added to the *V. vulnificus* MO6-24/O host strain culture when the optical density (OD) at a

Table 1
Host range of the bacteriophage VVP001.

Bacterial strain	Plaque formation	Source or reference	Medium
V. vulnificus			
MO6-24/O	+++	Wright et al. (1990)	LBS
FORC_036	++	CCFORC	LBS
FORC_037	–	CCFORC	LBS
FORC_009	–	CCFORC	LBS
FORC_017	–	Chung et al. (2016)	LBS
CMCP6	–	SNUCC	LBS
V. cholerae ATCC 14033	–	ATCC	LBS
V. parahaemolyticus KCTC 2471	–	KCTC	LBS
Gram-negative strains	–		
Escherichia coli	–		
ATCC 43890	–	ATCC	LB
ATCC 43895	–	ATCC	LB
Salmonella Enteritidis ATCC 13076	–	ATCC	LB
Cronobacter sakazakii ATCC 29544	–	ATCC	TSB
Shigella flexneri 2a strain 2457 T	–	IVI	TSB
Shigella boydii ATCC 8700	–	ATCC	TSB
Yersinia enterocolitica ATCC 29544	–	ATCC	TSB
Gram-positive strains	–		
Bacillus cereus ATCC 13061	–	ATCC	BHI
Listeria monocytogenes ATCC 15313	–	ATCC	BHI
Staphylococcus aureus ATCC 12600	–	ATCC	TSB

+++, EOP of 1–0.5; ++, EOP of 0.5–0.2; +, EOP <0.2; -, no susceptibility.
EOP, efficiency of plating; CCFORC, Culture Collection of the Food-borne Pathogen Omics Research Center; SNUC, Seoul National University Culture Collection; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; IVI, International Vaccine Institute; LB, Luria–Bertani medium; LBS, Luria–Bertani medium supplemented with 2% NaCl; TSB, Tryptic Soy Broth medium; BHI, brain–heart infusion medium.

wavelength of 600 nm (OD_{600}) reached a value of 0.5–0.6 and was incubated at 30 °C for 12 h. Cell debris was removed by centrifugation at 6000×*g* for 20 min, and the supernatant was filtered using a 0.45-μm-diameter syringe filter (Pall Corporation, Port Washington, NY, USA). For phage concentration, VVP001 particles were precipitated with polyethylene glycol 6000 (Junsei, Tokyo, Japan), and CsCl density gradient ultracentrifugation (Optima XE; Beckman Coulter, Brea. CA, USA) was performed at 78,500×*g* at 4 °C for 2 h. After extraction of the phage band with a sterilized syringe, the concentrated VVP001 particles were dialyzed in NaCl–magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris–HCl at pH 7.5; Sigma-Aldrich, St. Louis, MO, USA). Finally, purified VVP001 was stored at 4 °C for further experiments.

2.3. Transmission electron microscopy

Observation of purified VVP001 was performed as previously described (Kim and Ryu, 2011). To determine VVP001 morphology, VVP001 (2×10^7 PFU) was negatively stained with 2% aqueous uranyl acetate (pH 4.0) on carbon-coated copper grids and was observed using a Hitachi H-7600 biological transmission electron microscope (Tokyo, Japan) at an accelerating voltage of 80 kV. The morphology of VVP001 was classified and identified on the basis of International Committee on Taxonomy of Viruses guidelines (King et al., 2011).

2.4. Host range analysis

All *Vibrio* strains listed in Table 1 were tested for VVP001 host range determination. After incubation with test bacterial strains (10^8 CFU/mL), 100 μL of each test bacterial culture was added to 6 mL of 0.4% molten soft top agar, and the mixture was overlaid on a 1.8% agar plate. Then, 10 μL of serially diluted VVP001 suspension (10^{10} PFU/mL) was spotted on the overlaid agar plates and incubated at 30 °C. To determine the sensitivity of each *Vibrio* test strain to VVP001, we measured the efficiency of plating by comparing titers between the *Vibrio* test strains and *V. vulnificus* MO6-24/O as a reference strain. All experiments were performed in triplicate.

2.5. In vitro adsorption assay

After 1% sub-inoculation of *V. vulnificus* MO6-24/O, the bacterial strain was cultivated until bacterial cell concentration reached 10^8 CFU/mL, and the culture was diluted 10,000-fold with fresh LBS. Next, VVP001 was added to each diluted culture at a multiplicity of infection (MOI) of 0.01, and the mixed cultures were incubated at 30 °C without shaking. Then, 1 mL of each mixed culture was collected at 5-min intervals and centrifuged at 6000×*g* for 1 min. The supernatants were filtered using 0.22-μm-diameter syringe filters (Pall Co.), and filtrates containing VVP001 particles were used to determine the VVP001 titer using *V. vulnificus* MO6-24/O–overlaid agar plates. All experiments were conducted in triplicate.

2.6. One-step growth curve

One-step growth curve analysis with *V. vulnificus* MO6-24/O was performed as previously described (Lee et al., 2016). VVP001 was added to the bacterial culture (10^8 CFU/mL; MOI = 0.01) and absorbed at room temperature for 20 min. To remove the non-absorbed VVP001, the mixture was harvested by centrifugation at 6000×*g* for 10 min, and the supernatant was discarded. Next, the pellets were resuspended with fresh LBS and incubated with shaking at 30 °C. The samples were collected every 5 min. Subsequently, 1% chloroform (final concentration) was added and the contents vigorously mixed to release intracellular VVP001. These two samples were serially diluted 10-fold, and the suspension was spotted on *V. vulnificus* MO6-24/O–overlaid agar plates to measure VVP001 titration. The eclipse period, latent period, and burst size were determined on the basis of a comparison of the number of plaque-forming units (PFUs) per mL between samples with and without chloroform. All experiments were performed in triplicate and statistically analyzed.

2.7. Bacterial challenge test

V. vulnificus MO6-24/O was cultivated with shaking at 30 °C for 12 h. Subsequently, 1% of the culture was sub-inoculated into 100 mL of fresh LBS. After incubation at 30 °C until OD_{600} reached 1.0, the culture was divided into equal volumes of 50 mL each. To determine bacterial lytic activity of VVP001, we added VVP001 (MOI = 10) to one of the divided cultures, and the two samples were incubated with shaking at 30 °C. The samples were collected at 1 h intervals, serially diluted, and spread on LBS agar plates to determine CFUs per mL using the viable cell count method. All tests were conducted in triplicate.

2.8. Stability test under various stress conditions

To determine VVP001 stability under various temperature conditions, VVP001 at a final concentration of 10^8 PFU/mL was added to SM buffer, and the mixtures were incubated at different temperatures of −20 °C, 4 °C, 20 °C, 30 °C, 37 °C, 40 °C, 50 °C, 60 °C, and 65 °C for 12 h. After incubation, VVP001 titers were measured using a standard plaque assay with *V. vulnificus* MO6-24/O (Payne, 2017). To evaluate VVP001 stability at different pH conditions, pH of the SM buffer was adjusted with 4 N HCl (Daejung Chemical, South Korea) or 2 N NaOH (Duksan Chemical, South Korea) to a pH range of 1–12, and the VVP001 lysate at a final concentration of 10^8 PFU/mL was treated with each SM buffer solution. After incubation at 37 °C for 12 h, VVP001 titration was determined with a standard plaque assay using the same reference strain. All experiments were conducted in triplicate.

2.9. Genome sequencing and bioinformatics analysis

To increase our understanding of VVP001 at the genomic level, we performed genome sequencing and bioinformatics analysis. Genomic DNA of VVP001 was extracted and purified as previously described (Wilcox S, 1996) and then sequenced using the Illumina HiSeq DNA sequencer (Illumina, San Diego, CA, USA) with the HiSeq SBS V4 Library Preparation kit (125-bp paired-end reads; Illumina, USA) by LabGenomics Co. (Seongnam, Korea). Sequence quality was filtered greater than 80% of bases above Q30 at 2 × 125 bp. Qualified sequence reads were *de novo* assembled using CLC Genomics Workbench v10.0.1 (Qiagen, Hilden, Germany) and a contig was obtained, which is the complete genome sequence of VVP001. Prediction of all open reading frames (ORFs) was performed using Glimmer3 (Delcher et al., 2007), FgenesV (Softberry, Inc., Mount Kisco, NY, USA), and GeneMarkS (Besemer et al., 2001), and ribosomal binding sites (RBSs) were predicted using RBSfinder for the confirmation of ORF predictions (J. Craig Venter Institute, Rockville, MD, USA). Annotation and functional analysis were conducted using BLASTP (Altschul et al., 1997) and InterProScan with protein domain databases (Quevillon et al., 2005). The genome map was generated using the GenVision program (DNASTAR, WI, USA). In addition, bacteriophage virulence factor analysis was performed using Virulence Searcher (Underwood et al., 2005). The complete genome sequence was handled and edited using Artemis16 (Carver et al., 2008).

2.10. Food applications

To validate the biocontrol activity of VVP001 for *V. vulnificus* MO6-24/O in the food environment, fresh abalone samples were prepared by removing the flesh from the shells. The host strain (10^4 CFU of *V. vulnificus* MO6-24/O) was spread on the surface of a 5 g abalone flesh sample. After inoculation of the host strain, VVP001 (MOI = 10^5 or 10^6) was added to each abalone fresh sample. The abalone flesh was

incubated at 4 °C for up to 8 h, and samples were collected at 2 h intervals. The collected abalone flesh samples were homogenized with 45 mL of 0.1% sterilized peptone water (pH 7.2) using a BagMixer® 400 CC stomacher (Interscience, St. Nom, France) for 30 s. After stomaching, abalone debris was removed using the stomacher filter bag and the filtrate was transferred to new centrifuge tubes. To separate the host cells from VVP001, the filtrate was centrifuged at 6000×*g* for 10 min and the supernatant containing VVP001 was removed. Then, the pellet containing the host cells was resuspended with 1 mL of 0.1% sterilized peptone water. This separation/washing process was performed three times to remove as many VVP001 phages as possible. Finally, the host cells were resuspended with 1 mL of 0.1% sterilized peptone water and serially diluted via 10-fold dilutions. Each diluted sample was spread on three thiosulfate-citrate-bile salts-sucrose agar plates and incubated at 30 °C for 24 h. After incubation, the colonies were counted for viable cell count. All tests were conducted in triplicate.

2.11. In vivo mouse experiments

To evaluate the protection effects of VVP001 for *V. vulnificus*-infected mouse survival, the *in vivo* mouse survival test using specific-pathogen-free, 6-week-old female CrljOri:CD1 (Institute of Cancer Research [ICR]) outbred mice (CD-1®, Orient Bio Co., Korea) was conducted. Overnight cultures of *V. vulnificus* MO6-24/O were sub-inoculated and incubated with shaking at 30 °C to exponential phase (OD_{600} = 1.0). Next, bacterial cells were harvested and washed twice with phosphate-buffered saline (PBS; pH 8.0), and bacterial pellets were resuspended in PBS. Then, 3×10^6 CFUs of the bacterial suspension in 100 μL of PBS were injected into the intraperitoneal cavities of mice, and 100 μL of purified VVP001 samples (MOIs = 0, 10, 100, and 1000, with each test group of 10 mice) was injected into the other side immediately after bacterial challenge. Uninfected mice with VVP001 (MOI = 1000) were used as controls. The mouse survival rate was recorded for 7 days, and the number of viable cells in blood was counted by sacrificing groups of 3 mice each at 4, 8, and 12 h after bacterial cell and purified VVP001 injection. Each blood sample was serially diluted in PBS, and individual colonies on LBS agar plates supplemented with polymyxin B (final concentration = 10^5 units/L) were counted.

All experiments involving mice were approved by the Institutional Animal Care and Use Committee of Dankook University (Cheonan, Korea; approval no. DKU-19-003) and conducted in accordance with Care and Use of Laboratory Animals guidelines.

2.12. Nucleotide sequence accession number

The complete genome sequence of *V. vulnificus*–infecting VVP001, with its annotation information, was deposited in the National Center for Biotechnology Information database under the GenBank accession no. MG602476.

2.13. Statistical analysis

Statistical significance was assessed by Student's *t*-tests. The IBM SPSS (NY, USA) program was used to perform all statistical tests. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Host range and morphological observations

A novel bacteriophage VVP001 that infects *V. vulnificus* MO6-24/O was isolated from abalone samples collected from the coastal area of Yeonpo, Tae-An, South Korea. The host range test of the isolated VVP001 showed lytic activity against *V. vulnificus* MO6-24/O as a clinical isolate and *V. vulnificus* FORC_036 as an environmental isolate, indicating that VVP001 has a narrow host range with specificity for infection of some *V. vulnificus* strains (Table 1). Subsequent transmission electron microscopy morphology analysis revealed that VVP001 belongs to the family *Siphoviridae*. VVP001 has a noncontractile and flexible tail with tail fibers (Fig. 1) with head length, width, and tail length of VVP001 at 101.25 ± 0.13 nm, 56.25 ± 0.19 nm, and 178.75 ± 0.05 nm, respectively.

3.2. One-step growth curve analysis

The adsorption time of VVP001 was 20 min for *V. vulnificus* MO6-24/O (data not shown), which was used as the basis for one-step growth curve analysis and the monitoring of PFUs. We found that the eclipse and latent periods were both 30 min, and the burst size was 10 PFU per infected cell (Fig. 2). Interestingly, the eclipse and latent periods were the same and relatively short for replication and reconstruction in the host strain when compared to SSP002, suggesting that VVP001 might have a different infection pattern compared with the bacteriophage SSP002, whose eclipse and latent periods are 65 and 100 min, respectively (Lee et al., 2014).

3.3. Bacterial challenge test

To evaluate the inhibition of host bacterial growth by VVP001, we monitored the host strain viable cells after VVP001 infection and found that the number of CFUs showed a 3.87 log CFU/mL reduction at 4 h incubation after VVP001 infection (Fig. 3). However, the host strain growth completely recovered in 12 h, demonstrating the generation of a temporary bacteriophage-insensitive mutant similar to that seen in other phages (Park et al., 2012). The BIM frequency was determined at $1.36 \times 10^{-4} \pm 9.92 \times 10^{-5}$ CFU/mL, according to the BIM frequency calculation method (O'Flynn et al., 2004). Therefore, VVP001 might be useful for initial growth inhibition of *V. vulnificus* strains.

3.4. Phage stability test under various stress conditions

It is important to maintain phage viability for food applications (processing, distribution, and storage) under various conditions. Interestingly, we found little or less loss of VVP001 at a temperature range of

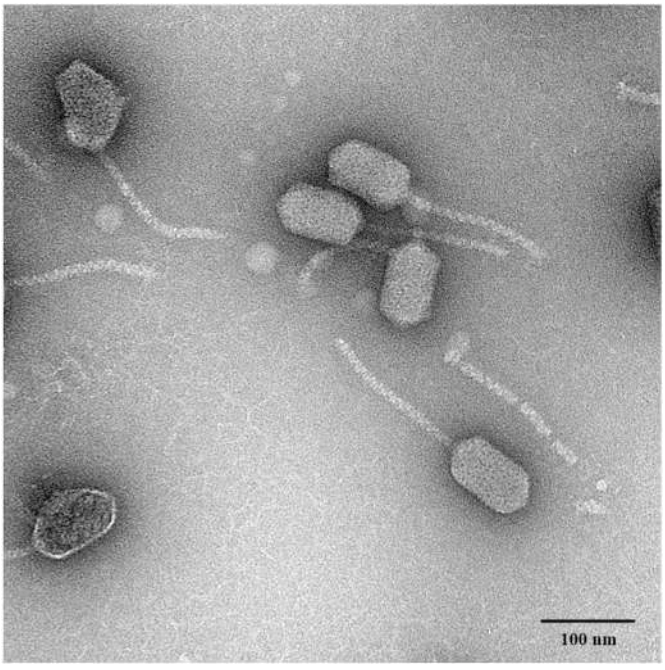


Fig. 1. Transmission electron microscopy morphology of bacteriophage VVP001. Scale bar = 100 nm.

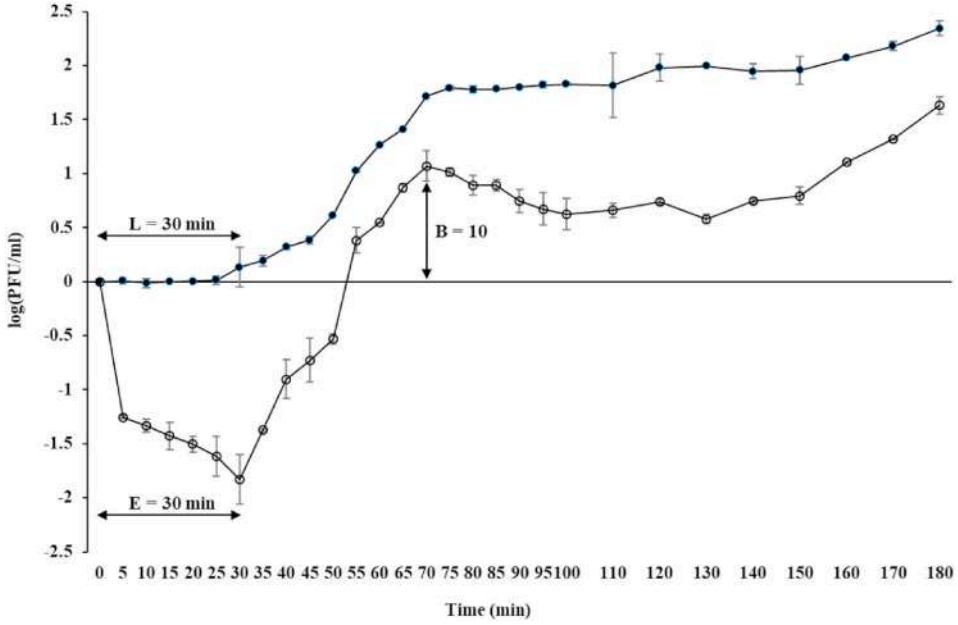


Fig. 2. One-step growth curve analysis of bacteriophage VVP001. Closed circles, chloroform-treated sample; open circles, non-chloroform-treated sample. E, L, and B refer to the eclipse period, latent period, and burst size, respectively. PFU, plaque-forming unit.

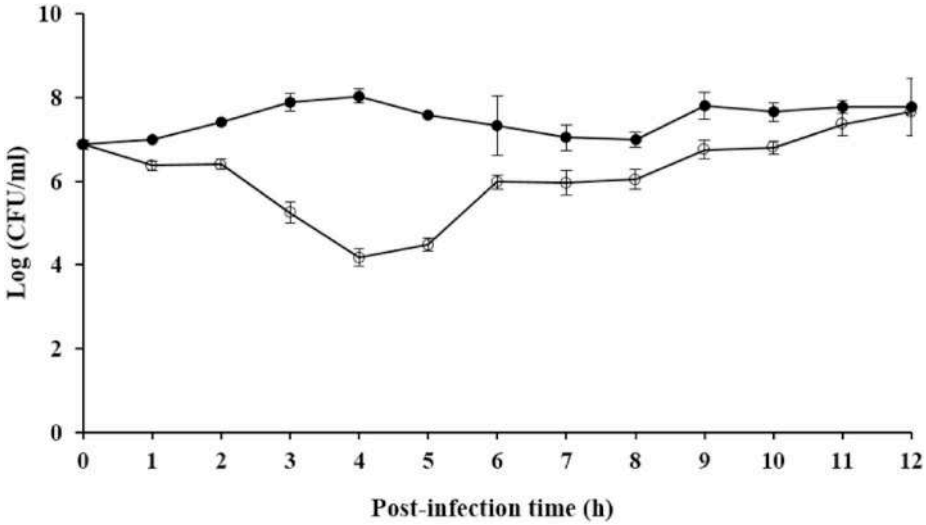


Fig. 3. Bacterial challenge assay of bacteriophage VVP001 with *Vibrio vulnificus* MO6-24/O. Open circles, VVP001-infected samples; closed circles, non-VVP001-infected samples. CFU, colony-forming unit.

−20 °C to 60 °C and a pH range of 3–10, indicating that VVP001 might be suitable as a natural food preservative in various food applications (Fig. 4).

3.5. Genome characterization

To understand the genomic characteristics of VVP001 and to verify its safety for food applications, we determined the complete genome sequence of VVP001. The genome is 76,423 bp DNA length with 49.64% G + C content. Of all the 102 ORFs predicted, 17 (16.67%) were predicted to have specific functions, whereas the others were unknown, probably because of insufficient *Vibrio*-infecting bacteriophage genome information in public genome databases. On the basis of the genome annotation results, we categorized these functional ORFs into six groups: DNA replication/modification (exonuclease, DNA repair exonuclease, RecA protein, DNA polymerase I, DNA polymerase II subunit, DNA polymerase III beta subunit, DNA helicase, and DNA ligase), structure and packaging (structure protein, head morphogenesis domain protein, terminase large subunit, and HNH endonuclease), host lysis (endolysin-like protein), tail (phage tail protein, tail assembly protein, tail assembly structure protein, and tail measure protein), transcription regulation (transcription regulator), and additional function (thymidylate kinase, thymidylate synthase, and adenosine triphosphate-binding domain-containing protein). This showed that VVP001 has all the required core genes for its own replication, reconstruction, and host lysis. Of note, the genome contains no gene encoding toxin or virulence factor, suggesting that VVP001 is safe as a natural food preservative in various food applications (Fig. 5).

3.6. Comparative genome analysis

The complete genome sequence of VVP001 was compared with two

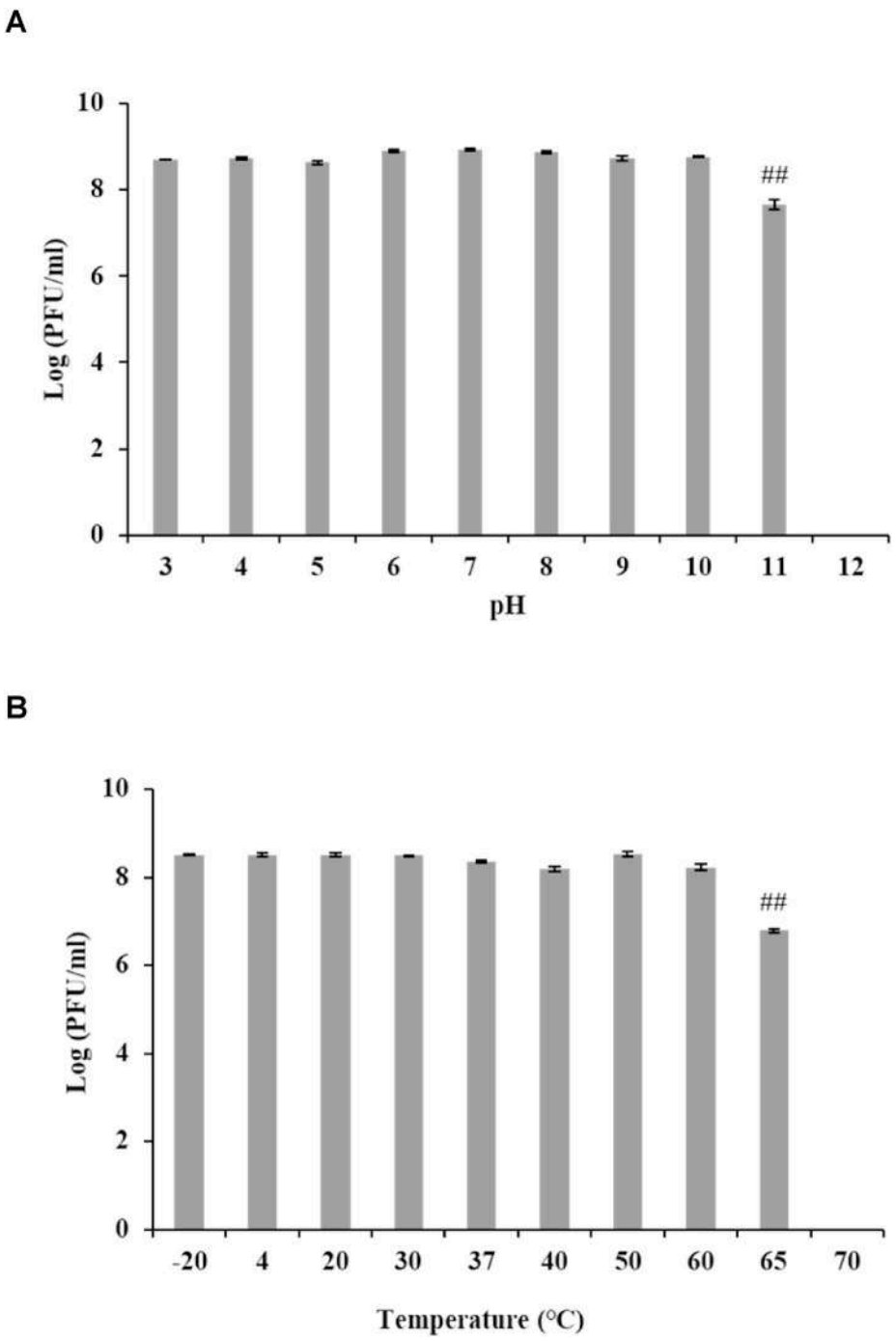


Fig. 4. Stability of bacteriophage VVP001 under various stress conditions with *Vibrio vulnificus* MO6-24/O: (A) pH and (B) temperature stability. PFU, plaque-forming unit. The sharp indicates statistically relevant differences among the different conditions (##, $p < 0.01$).

closely related *Vibrio* bacteriophage genomes, *V. vulnificus*–infecting SSP002 (96.84%) and *V. parahaemolyticus*–infecting vB_VpaS_MAR10 (79.23%) (Lee et al., 2014; Villa et al., 2012) (Table 2). Although most of the core genes were highly homologous among the three genomes, gene clusters encoding tail-related regions were quite different, indicating that tail regions might be associated with host specificity for bacteriophage infection. To further determine the differences in host range between the three phages, we compared amino acid sequences of the tail-related predicted proteins with those of others using the BLASTP program (Table 3).

Tail-related gene clusters in the two *V. vulnificus* phages (VVP001, SSP002) and a *V. parahaemolyticus* phage (vB_VpaS_MAR10) contain 8 genes, encoding tail tape measure protein, tail assembly proteins, phage tail protein, and hypothetical proteins (Fig. 6). Comparative analyses of the amino acid sequences showed two groups (groups I and II), based on amino acid sequence identity. Group I consists of two genes encoding a tail tape measure protein and phage tail protein that are highly homologous, and group II has six genes encoding two tail assembly proteins and four other hypothetical proteins that have low homology. While the function of the proteins in group II are not clearly understood, the phage tail protein-encoding gene in the group I has been suggested to be a determinant for host specificity and host range (Lee et al., 2016; Park et al., 2012). Interestingly, amino acid sequence identity between VVP001_064 and SSP002_049 is 98%, but the identity between

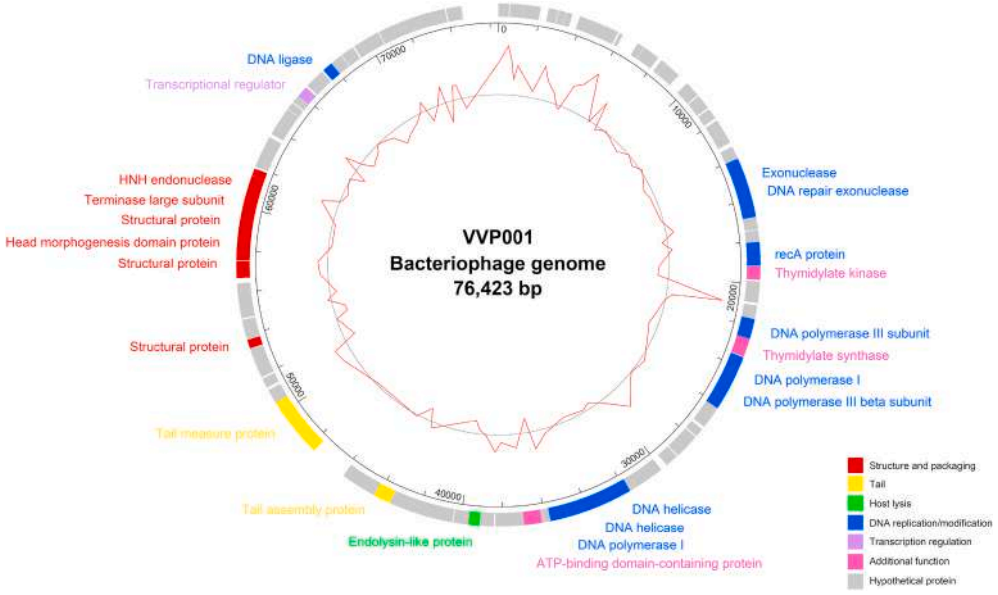


Fig. 5. Genome map of bacteriophage VVP001. Functional categories are indicated using specific colors. ATP, adenosine triphosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
General genome characteristics of the bacteriophage VVP001 and other closely related *Vibrio* phages.

Characteristics	VVP001	SSP002	vB_VpaS_MAR10
Infection host	*V. vulnificus*	*V. vulnificus*	*V. parahaemolyticus*
Morphology (family)	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*
Genome size (bp)	76,423	76,350	78,751
G + C content (%)	49.64	48.78	49.70
Predicted ORFs	102	102	104
Tail structure proteins	4	2	1
Host lysis related proteins	1	1	0
GenBank accession no.	MG602476 (This study)	JQ692107 (Lee et al., 2012)	JX556418 (Villa et al., 2012)
Amino acid sequence identity with VVP001	–	96.84%	79.23%
Amino acid sequence identity with SSP002	96.84%	–	79.09%
Amino acid sequence identity with vB_VpaS_MAR10	79.23%	79.09%	–

ORF, open reading frame.

VVP001_064 and MAR10_050 is only 72%. Previous host range tests revealed that the host ranges of VVP001 and SSP002 are slightly different at the strain level. Phage SSP002 inhibits the two clinical *V. vulnificus* MO6-24/O and CMCP6 strains as well as the environmental strain SS108A3 A (Lee et al., 2014). However, phage VVP001 inhibits only two clinical *V. vulnificus* strains, MO6-24/O and FORC_036, supporting this. However, phage vB_VpaS_MAR10 inhibits only *V. parahaemolyticus*, and not *V. vulnificus*. This different host range of vB_VpaS_MAR10 from those of other *V. vulnificus* phages may be due to relatively low amino acid sequence identity of the phage tail proteins between *V. vulnificus* phages (VVP001 and SSP002) and this phage.

3.7. Food applications

By comparing the number of viable cells between abalone samples intentionally contaminated with *V. vulnificus* MO6-24/O, we determined the potential of VVP001 as a natural food preservative. At 6 h of incubation, the number of CFUs showed a 2.51 log CFU/mL reduction at MOI = 10^6 and a 2.06 log CFU/mL reduction at MOI = 10^5, whereas the *V. vulnificus* MO6-24/O concentration in the control group increased after incubation (Fig. 7). In addition, VVP001 steadily inhibited *V. vulnificus* MO6-24/O up to 8 h, although we observed bacterial strain recovery after inhibition, indicating that VVP001 could be a potential candidate for developing a natural food preservative against *V. vulnificus*

Table 3
Comparative amino acid sequence analysis of functional open reading frames in tail-related regions of the bacteriophage VVP001.

Locus tag	Predicted function	Length[a]	BLASTP best matches	Identity (%)[b]/Coverage (%)	References
VVP001_054	Putative phage tail protein	852	Hypothetical protein SSP002_049 [*Vibrio* phage SSP002]	99%/100%	AFE86376.1
			Hypothetical protein MAR10_049 [*Vibrio* phage vB_VpaS_MAR10]	72%/100%	YP_007111896.1
VVP001_057	Putative tail assembly protein	269	Putative tail assembly protein SSP002_046 [*Vibrio* phage SSP002]	79%/100%	AFE86373.1
			Hypothetical protein MAR10_046 [*Vibrio* phage vB_VpaS_MAR10]	69%/100%	YP_007111893.1
VVP001_058	Tail assembly structure protein	559	Hypothetical protein SSP002_045 [*Vibrio* phage SSP002]	59%/100%	AFE86372.1
			Hypothetical protein MAR10_045 [*Vibrio* phage vB_VpaS_MAR10]	41%/89%	YP_007111892.1
VVP001_061	Putative tail tape measure protein	949	Putative tail tape measure protein SSP002_042 [*Vibrio* phage SSP002]	99%/100%	AFE86369.1
			Tail tape measure protein MAR10_043 [*Vibrio* phage vB_VpaS_MAR10]	84%/100%	YP_007111889.1

[a] Number of amino acids.
[b] Amino acid sequence identity.

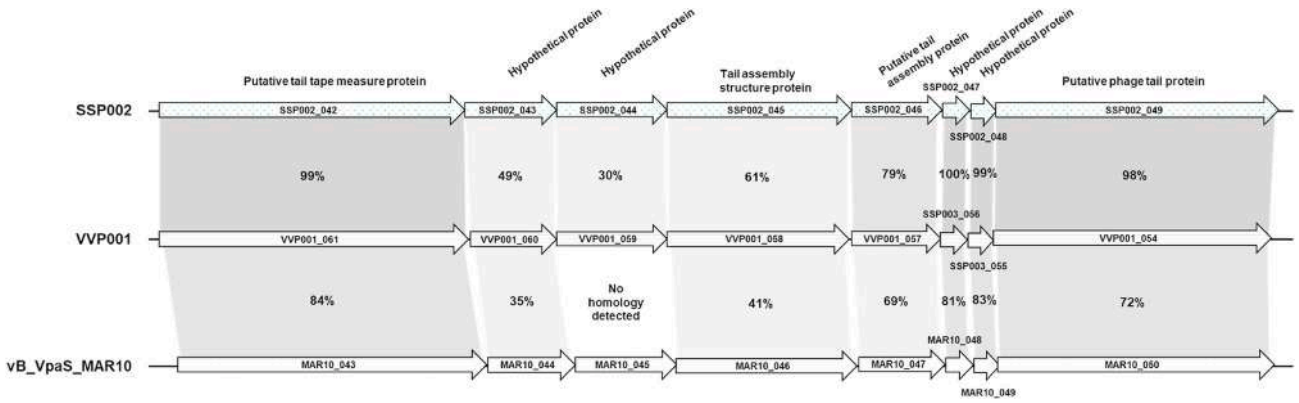


Fig. 6. Comparative amino acid sequence analysis of bacteriophage VVP001 tail regions with two closely related *Vibrio* bacteriophage genomes (SSP002 and vB_VpaS_MAR10). Amino acid sequence identities are indicated as percentages in the shadow box.

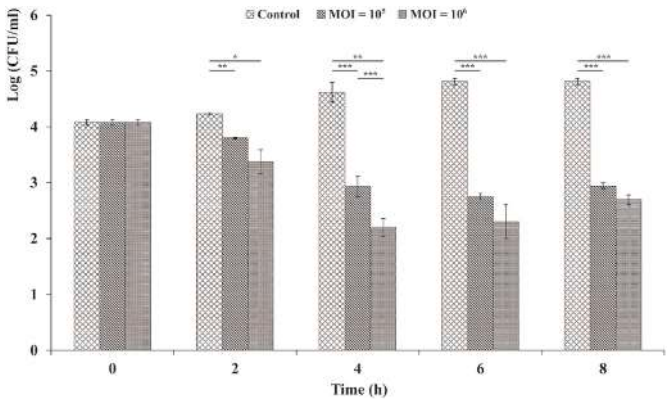


Fig. 7. Food application of bacteriophage VVP001 with *Vibrio vulnificus* MO6-24/O. Closed squares, control sample without VVP001; closed circles, MOI = 10^5; open circles, MOI = 10^6. MOI, multiplicity of infection; CFU, colony-forming unit.

MO6-24/O in raw seafood.

3.8. In vivo mouse experiments

Mouse survival tests with ICR mice after *V. vulnificus* MO6-24/O infection were performed. As shown in Fig. 8A, VVP001-untreated mice (MOI = 0) died within 12 h, indicating that injection with 3×10^6 CFU bacterial cells causes rapid death. However, the survival rate of VVP001-treated mice (MOI = 100 and 1000) was at 50% and 70% after 7 days, respectively, although all VVP001-treated mice (MOI = 10) died within 48 h. These results indicate that VVP001 protects mice against *V. vulnificus* MO6-24/O and that the protection effect increases in a dose-dependent manner. In addition, VVP001-treated mice without *V. vulnificus* MO6-24/O infection as a control group were completely normal, indicating that VVP001 is safe to use, consistent with the lack of virulence genes in the complete genome sequence.

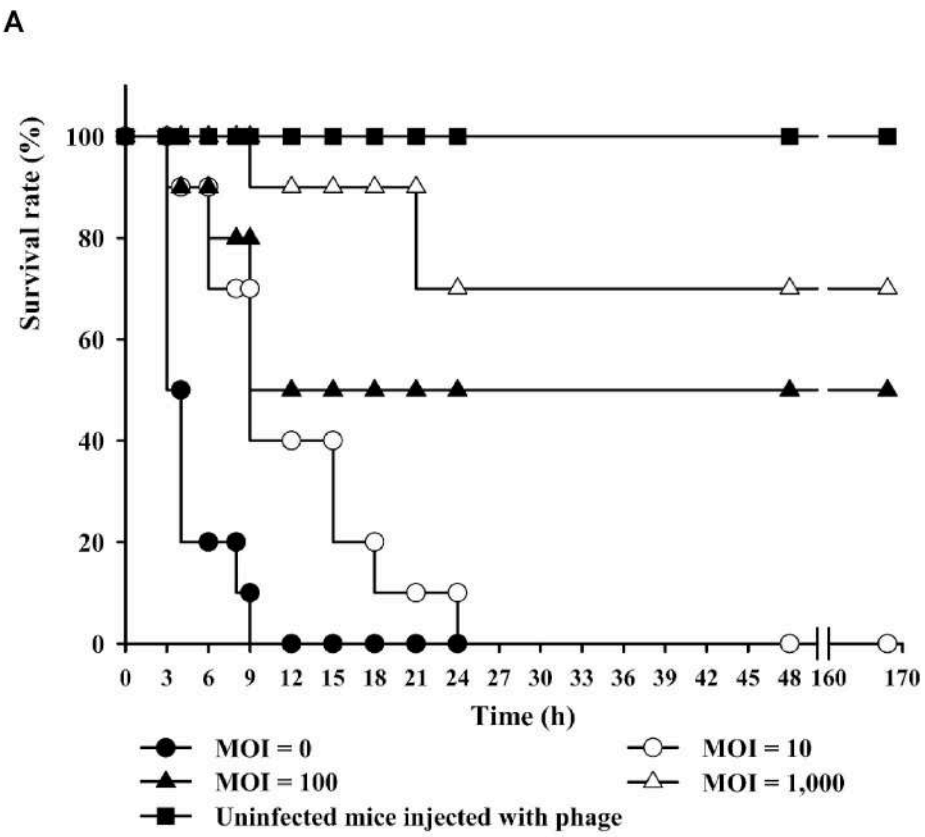
To confirm whether VVP001 administration of phage inhibits bacterial proliferation, we determined the number of viable *V. vulnificus* MO6-24/O cells in the blood of mice. Bacterial cells in the blood of VVP001-untreated mice (MOI = 0) were 4.93 log CFU/mL 4 h after infection and gradually increased at 8 and 12 h to 5.91 and 7.50 log CFU/mL, respectively (Fig. 8B). In VVP001-treated mice (MOI = 10), regardless of *V. vulnificus* MO6-24/O levels, which were not detected after 4 h, the number of CFUs reached 4.52 log CFU/mL at 8 h, indicating that the therapeutic effect of VVP001 slightly improves at low concentrations. However, VVP001-treated mice (MOI = 100 and 1000) showed low *V. vulnificus* MO6-24/O levels at every time point. These results show that VVP001 can effectively protect the host from *V. vulnificus* MO6-24/O infection.

4. Discussion

V. vulnificus is a virulent pathogen causing various diseases in humans, ranging from mild gastroenteritis to severe life-threatening septicemia (DePaola et al., 1998; Horseman and Surani, 2011; Morris, 1988; Strom and Paranjpye, 2000). *V. vulnificus* infection has the highest mortality rate per case among all food-borne infections reported in the United States, so immediate administration of antibiotics to patients is important to relieve symptoms and ultimately decrease the high mortality rate (Hoffmann et al., 2015; Phillips and Satchell, 2017). However, despite continuous antibiotic treatment, *V. vulnificus* is still lethal because the excessive use of antimicrobial agents has led to the emergence of multidrug-resistant *V. vulnificus* strains (Han et al., 2007; Kumamoto and Vukich, 1998). In addition, using antibiotics and chemical antimicrobials in foods is one of the main causes of infection (Donoghue, 2003; Lee et al., 2016). Therefore, alternative biocontrol agents are needed to replace antibiotics and chemical antimicrobials. Although the bacteriophage approach might show promise to control *V. vulnificus* infection, only four *V. vulnificus* bacteriophages have been reported and characterized to date (DePaola et al., 1997, 1998; Villa et al., 2012): SSP002, CK-2, 153A-5, and 153A-7.

In this study, a newly isolated *V. vulnificus* bacteriophage VVP001 was characterized and tested for food applications. Subsequent genome analysis predicted the safety of VVP001 for food applications at the molecular level. Comparative genome sequence analysis of two *V. vulnificus* phages (VVP001 and SSP002) and one *V. parahaemolyticus* phage (vB_VpaS_MAR10) revealed that their tail-related proteins are quite different (Fig. 6). These tail-related proteins have been suggested to be responsible for host recognition and specificity. In particular, four genes encoding a hypothetical protein (VVP001_060) and three tail assembly proteins (VVP001_057–VVP001_059) might be key elements in distinguishing each host range spectrum.

Comparative host range spectrum analysis showed that the host ranges of VVP001, SSP002, and vB_VpaS_MAR10 are also quite different. VVP001 infects only two strains, *V. vulnificus* MO6-24/O (clinical isolate) and *V. vulnificus* FORC_036 (environmental isolate), whereas SSP002 infects three strains, *V. vulnificus* MO6-24/O (clinical isolate), *V. vulnificus* CMCP6 (clinical isolate), and *V. vulnificus* SS108A3 A (environmental isolate) (Lee et al., 2014), indicating that host range spectra of *V. vulnificus* bacteriophages are narrow and host specific at the strain level. However, vB_VpaS_MAR10 infects 13 of the 21 *V. parahaemolyticus* strains, indicating that it has a relatively wide host range spectrum but is host specific at the species level (Villa et al., 2012). Although clear functions of these putative proteins are still unknown, probably because of insufficient information on *Vibrio* bacteriophage



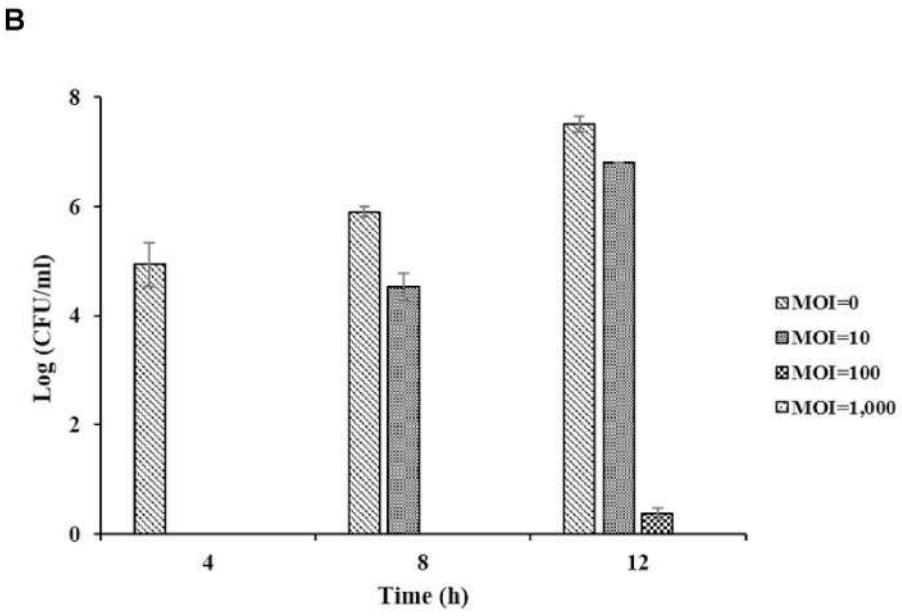


Fig. 8. (A) *In vivo* mouse survival test using ICR mice infected with *Vibrio vulnificus* MO6-24/O and bacteriophage VVP001. Closed circles, infected mice without VVP001 treatment (MOI = 0) as a control group. Open circles, closed squares, and open squares indicate infected mice with VVP001 treatment at MOI = 10, 100, and 1,000, respectively. Closed triangles, uninfected mice with VVP001 treatment. (B) Viable cells of *V. vulnificus* MO6-24/O in the blood of ICR mice. MOI, multiplicity of infection; ICR, Institute of Cancer Research; CFU, colony-forming unit.

genomes in public genome databases, the four genes in the tail-related cluster might cause the difference in host recognition and specificity. In addition, although the host receptor of SSP002 is the host flagellum (Lee et al., 2014), VVP001 does not use the host flagellum as a host receptor (data not shown), indicating that the difference in host receptors between these *V. vulnificus* bacteriophages might contribute to host recognition and specificity. However, to determine their exact functions, further molecular studies are required.

While a few endolysin genes have been detected in *Vibrio* phage genomes, no experimental characterizations of these *Vibrio* phage endolysins were reported. Interestingly, two *V. vulnificus* phages (VVP001 and SSP002) and one *V. parahaemolyticus* phage (vB_VpaS_MAR10) have their own endolysin genes. Comparative sequence analysis of endolysins in *V. vulnificus* bacteriophages revealed that endolysins of VVP001 and SSP002 share 98.40% amino acid sequence identities.

Since 2006, the FDA approved the direct use of bacteriophages for food applications; many commercial products using bacteriophages have been released to control food-borne pathogens (Bai et al., 2016). In this study, we confirmed the lysis activity of VVP001 in fresh abalone

samples for the development of a natural food preservative and its high phage stability under broad temperature and pH conditions. Interestingly, results with *V. vulnificus* MO6-24/O revealed a 2.51 log CFU/mL reduction at MOI = 10^6 and a 2.06 log CFU/mL reduction at MOI = 10^5, indicating that VVP001 has potential as a natural food additive against *V. vulnificus* MO6-24/O in abalone samples (Fig. 7). However, the bacterial challenge assay in the LBS standard condition revealed a 3.87 log CFU/mL reduction at MOI = 10, suggesting that phage inhibition efficiency in LBS standard conditions is much better than that in abalone conditions. The difference of phage inhibition efficiencies between the LBS standard condition and abalone condition may be due to possible hindrance of host-phage contact due to the food matrix or abalone-derived viscous materials.

In vivo mouse experiments with ICR mice infected by *V. vulnificus* MO6-24/O and VVP001 were conducted to evaluate the protection effect of VVP001 for survival of *V. vulnificus*-infected mice. Results of *in vivo* mouse survival tests revealed that VVP001 protects the infected mice against mortality, suggesting that VVP001 might be protection-effective against *V. vulnificus* MO6-24/O infection (Fig. 8). Therefore, phage VVP001 has a protection effect from *V. vulnificus* infection as well as safety for applications. Previous studies investigated *in vivo* bacteriophage therapy against *V. vulnificus* using the isolated bacteriophages CK-2, 153A-5, and 153A-7 (DePaola et al., 1997, 1998). Results showed the possibility of therapeutic effects and that these bacteriophages could protect infected mice (Pelon et al., 2005).

We characterized the novel *V. vulnificus*–infecting bacteriophage VVP001 at the phenotypic and genotypic levels. On the basis of our results, we evaluated and confirmed specific growth inhibition of *V. vulnificus* MO6-24/O by VVP001 infection for further seafood applications. Therefore, VVP001 might be a good alternative approach to efficiently control *V. vulnificus* in seafood as well as develop a novel natural food preservative for seafood processing, distribution, storage, and preservation in the food industry.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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